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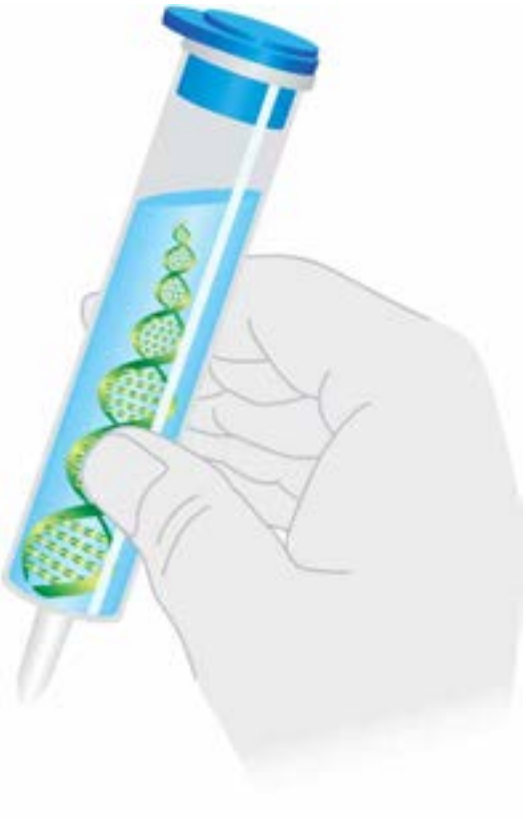

Nucleic Acid Sample Preparation for Downstream Analyses

Principles and Methods

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Nucleic Acid Sample Preparation for Downstream Analyses

Principles and Methods

Contents

Introduction

The changing face of the 'omics era is setting new challenges for today's researchers. New tools have accelerated the volume of knowledge to help us understand the fundamentals of biological systems, while quality publications now demand extensive information covering molecular identity, *in vivo* function studies, disease manifestation, and potential therapeutic opportunities. Little wonder then that the research community has embraced the development of commercial kits to reduce or eliminate mundane procedures. Quality-controlled products can now be applied to many of the laboratory basics, enabling investigators to work in a more cost-effective and time-efficient manner. Whether you are interested in isolating one group of target molecules—DNA, RNA, protein—or all three, the first part of the process is sample preparation.

Most people are familiar with the old adage "garbage in - garbage out." As most of us will have realized at some time, sample preparation is perhaps one of the most crucial aspects of any study. Contemporary methods typically involve several steps in which each subsequent step is dependent on the performance and quality of the preceding step. For this reason, many vendors are quite specific about the types of samples that can be prepared using a specific kit and the appropriate protocol. Poor sample preparation can lead to suboptimal results in downstream applications, and it is for this reason that many optimized versions of kits have emerged to cope with variation in sample source, be it blood, plant tissue, fungi, or bacteria. The goal of this handbook is to provide useful information to help make this very important starting point as prescriptive and efficient as possible. The handbook has several guiding principles:

- Preparing DNA and RNA samples that meet your needs is just as critical as the analysis step.
- Obtaining good yields, quality, and purity and increasing the reproducibility at the preparation stage are keys to achieving your desired results.
- Genomics research workflows involve combinations of biomolecules and are becoming more complex.
- Identifying the right technique or product for each stage of the research process makes it easier to obtain your desired results.

Outline

This handbook provides guidance, hints, and tips and is intended for novice and experienced researchers to aid in "getting it right from the start" when preparing nucleic acids for downstream applications. It contains a "before you begin" chapter, an overview of nucleic acid sample preparation, and eight chapters on nucleic acid sample preparation. There is also a chapter for those interested in purifying genomic DNA, total RNA, and total denatured protein from a single undivided sample.

Sample collection is addressed in a chapter that precedes those for specific subcategories of nucleic acids, with a focus on preserving the integrity of the sample to ensure quality and relevance. For example, a number of scientific studies will use RNA to look at temporal expression profiles. Therefore, several precautions are necessary to prevent degradation of the RNA and to standardize the way in which samples are collected. Similarly, genomic DNA can be used for a number of different applications, some of which are more dependent on molecular size than others. Finally, the requirement for long-term storage is also addressed to introduce the demands of field biology and the requirements for archiving precious samples.

Common acronyms and abbreviations

A_{280}	absorbance at specified wavelength (in this example, 280 nanometers)
aCGH	array comparative genomic hybridization (sometimes referred to as CGH)
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
BAC	bacterial artificial chromosome
CCC	covalently closed circular
cDNA	complementary DNA
CHEF	contour-clamped homogeneous electric field
CsCl	cesium chloride
CSF	cerebrospinal fluid
CsTFA	cesium trifluoroacetate
C_q	quantification cycle, previously known as threshold cycle (C_t)
CTAB	cetyltrimethylammonium bromide
ddNTP	dideoxy nucleotide triphosphate
DIGE	differential gel electrophoresis (sometimes referred to as 2-D DIGE)
DNase	deoxyribonuclease
dNTP	deoxy nucleotide triphosphate
DEPC	diethylpyrocarbonate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EndA+	endonuclease I-positive
FFPE	formalin-fixed, paraffin-embedded
Gua-HCl	guanidine-HCl
GTC	guanidinium thiocyanate
HLA	human leukocyte antigen
KDS	potassium dodecyl sulfate
LB	Luria broth or Luria Bertani broth
LC-MS	liquid chromatography-mass spectrometry
LiCl	lithium chloride
LIMS	Laboratory Information Management System
LOH	loss of heterozygosity
LPS	lipopolysaccharide
miRNA	micro RNA
M-MuLV	Moloney Murine Leukemia Virus
MS	mass spectrometry
OC	open circular
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
qPCR	quantitative real-time PCR
r	recombinant, as in rGST, or ribosomal, as in rRNA
RAPD	random amplified polymorphic DNA

RBC	red blood cells
RCA	rolling circle amplification
RFLP	restriction fragment length polymorphism
RFU	relative fluorescence units
RIN	RNA integrity number
RNAi	interference RNA
RNase	ribonuclease
RSP	robotic sample processor
RT-PCR	reverse transcriptase-polymerase chain reaction
RT-qPCR	reverse transcriptase-quantitative polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
STR	short tandem repeat
TB	Terrific broth
TE	Tris-EDTA buffer (usually 10 mM Tris-HCl, 1 mM EDTA)
TE^{-1}	Tris-EDTA buffer containing 0.1 mM EDTA
u	units (e.g., of an enzyme)
UV	ultraviolet
WBC	white blood cells
WGA	whole genome amplification
YAC	yeast artificial chromosome

Symbols


this symbol indicates general advice to improve procedures or recommend action under specific situations.


this symbol denotes mandatory advice and gives a warning when special care should be taken.

highlights chemicals, buffers and equipment.

outline of experimental protocol.

Chapter 1
Overview of nucleic acid sample preparation

Definition of nucleic acid sample preparation

This handbook provides information about nucleic acid sample preparation. Our working definition of nucleic acid sample preparation is "any operation that is carried out prior to any major manipulation step in a given workflow, starting with a biological sample and ending in nucleic acid analysis." By "major manipulation step" we mean, for example, microarray analysis, DNA sequencing, or transfection. In contrast, a simple preparative step using a spin column is not considered a major manipulation, and thus by our definition is included as part of sample preparation. This handbook focuses on small-scale samples that can be prepared in a microcentrifuge tube, in a mini column, or in a multiwell plate to yield nanogram to microgram quantities of nucleic acid for analysis.

Along with our working definition of sample preparation, we are basing the discussion in the following chapters on the assumption that your goal is to purify a particular group or subgroup of nucleic acids (e.g., genomic DNA, total RNA, etc.) to a sufficient purity and quality and in enough quantity to allow completion of your selected type of analysis. Chapter 2 discusses some key considerations prior to performing sample preparation. Chapter 3 addresses the global issue of sample collection and archiving. Chapters 4, 5, and 6 address preparation of genomic DNA, plasmid DNA, and RNA, respectively. Although plasmids are not the only cloning vector used today, plasmids are a "workhorse" for DNA cloning and subsequent applications; therefore, plasmid DNA preparation is included in this handbook. The next three chapters deal with sample preparation steps that apply to more than one type of nucleic acid. Amplification (Chapter 7) can be used to prepare nucleic acids for further analysis, that is, whole genome analyses or differential expression. Chapter 8 addresses nucleic acid cleanup prior to final analysis, and Chapter 9 provides a discussion relating to increasing nucleic acid sample throughput. The handbook finishes with Chapter 10, which discusses the isolation of genomic DNA, total RNA, and total denatured protein from a single undivided sample and addresses considerations specific to isolating multiple molecules.

Why is nucleic acid sample preparation so important?

Sample preparation is often critical to successful analysis of nucleic acids. Protection from nucleases is one of the most important considerations during nucleic acid sample preparation. This is particularly true when preparing RNA for analysis. For example, if RNA is not protected from degradation during sample disruption, one or more rare transcript may be lost and may fail to be identified in subsequent analyses. In another example, a degraded RNA molecule may fail to bind to a complementary nucleic acid sequence on a microarray because the area of complementarity has been lost. **In these two examples, you may never know that sample preparation was inadequate**. Poor sample preparation can also cause visible problems during analysis. These may be the result of nucleic acid degradation or the presence of contaminants. Examples of visible problems that may be attributed to poor sample preparation are extra products generated during PCR analysis, reduced activity of nucleic acid restriction and modifying enzymes, short read lengths during sequencing, and so on. One of the most noticeable visible problems in genomic DNA preparation is apoptotic nuclease fragmentation, which causes characteristic nucleosomal laddering of DNA when DNA is electrophoresed on agarose gels.

Analysis and *in vitro* manipulation of nucleic acid polymers is typically preceded by a preparative step, the objective of which is to make the polymer accessible to manipulating agents

and sufficiently free from undesired contaminants. The need for accessibility is obvious—a manipulating agent, such as a nucleic acid modifying enzyme, can only interact directly with a target molecule in immediate proximity to it. If this direct interaction is blocked by a cell wall, cell or nuclear membrane, or viral protein coat, or if the nucleic acid is covered with protein on its surface, then the enzyme obviously cannot perform its function. Clearly, these blocking obstacles must be removed or made penetrable for the manipulating agent to do its job.

The goal of nucleic acid preparation is to gain accessibility to nucleic acids in as close to natural form as possible and to remove sufficient quantities of undesired contaminants. The choice of a particular method should be evaluated not only in terms of purification performance but also with respect to time, effort, and monetary expense. Issues to consider include whether the nucleic acid is to be used for one or several downstream applications, and the purity and quality requirements for the most stringent of those applications. In addition to the qualities of the end product, the expense of time and labor should be considered. A clinical laboratory typically has very high time demands, so speed and simplicity are of prime importance. A publicly funded laboratory may place higher value on reagent expense because salaries are budgeted differently from reagent expenses. An industrial laboratory may place higher value on personnel expenses. If a particular procedure is to be used very often, it may even prove worthwhile to automate the operation using a robotic workstation. See Chapter 9 for information on increasing the throughput of sample preparation.

Driving forces behind nucleic acid sample preparation

The completion of the Human Genome Project and other genome sequencing projects, advances in bioinformatics, and the availability of specialized software applications have influenced biomolecular research in a number of ways. Current trends in nucleic acid sample preparation include increased throughput and speed of nucleic acid preparation, smaller sample sizes, and more complex sample types (1).

Increased throughput may be accomplished by automating sample preparation steps or by using a multiwell plate system (e.g., illustra™ RNAspin 96 RNA Isolation Kit from GE Healthcare). Multiwell plates can be used in combination with new single-use centrifuge or vacuum purification methods that are designed to improve sample workflow and extraction times. Increased speed typically goes along with increased throughput.

Blood spots, buccal swabs, FFPE histological sections (for Phi29-mediated WGA [2]), and samples collected by laser capture microdissection (3) are all examples of limited or precious samples. More complex samples for analysis include, for example, biological fluids that are screened for viral nucleic acids (e.g., 4) and feces and hair shafts from which nucleic acids are isolated (e.g., from the South China tiger, 5). In addition, the past decade has brought about a new field of scientific investigation, that of RNAi (6, 7). This relatively recent technique represents an exciting opportunity for nucleic acid sample preparation.

Another important area is the heterologous expression of proteins. The initial step involves introducing recombinant nucleic acid (generally in the form of plasmid or other circular DNA) into the host organism. This requires procedures, such as transfection, in which plasmid DNA is prepared in smaller amounts for the screening step but expanded to relatively large amounts when the final protein expression stage is performed.

The range of downstream applications for nucleic acids has grown with a change to the key applications that have resulted from technology advances. Many applications are now PCR-based, and multiplexing to identify more than one target per reaction is frequently performed (e.g., 8). For many applications, a fluorescent label is incorporated during PCR or in a separate reaction to aid in detection and analysis. Many of these applications are based on real-time or quantitative PCR (see 9). Increasingly, sequencing applications are based on fluorescence and analyzed following capillary electrophoresis or hybridization. Massively parallel sequencing

(10) is an evolving technique for ultra-high throughput genomics. Microarray technologies use fluorescent labeling and hybridization for high-throughput genotyping (11), comparative genome analyses (12), and expression screening (13). Additional applications for nucleic acids relate to functional genomics, gene therapy (e.g., 14), DNA vaccines (e.g., 15), and screening of biological and environmental samples to identify DNA (i.e., forensics; 16). As functional genomics increases in popularity, so does the desire to isolate multiple molecules (e.g., genomic DNA, RNA, and protein) from a single sample and correlate the presence and levels of gene products. The construction of expression plasmids is also one of the key applications in studying the structure/function of the gene products/proteins.

Challenges of nucleic acid sample preparation

Working with RNA provides very different challenges from working with DNA. DNA is much more stable and can be extracted from most sources, whereas the demands on the processes and source material when working with RNA are much higher because of the speed with which RNA is being degraded. The major challenge whenever isolating, manipulating, or analyzing RNA is the presence of RNases. These are extremely stable and very active nucleases capable of degrading RNA. For example, bovine pancreatic ribonuclease A is one of the hardiest enzymes in common usage; one isolation method boils a cellular extract until all proteins other than RNase A are denatured. Therefore, an RNase-free work area is essential when isolating RNA.

Some of the major challenges for nucleic acid sample preparation are listed below:

- Extremely small sample sizes (sometimes just a few cells)
- Presence of contaminants that may interfere with analysis
- Degradation that starts as soon as the sample is collected; particularly relevant when preparing RNA
- Samples that are difficult to disrupt
- Isolation of more than one molecule (e.g., genomic DNA, RNA, protein) from a single sample
- Detection of low-abundant RNA transcripts
- Detection of viral nucleic acids in biological fluids
- Isolation of small (< 200 nt) RNA

Working with nucleic acids is an enormously powerful tool with the ability to provide insight into a large number of biological processes that are key in a wide variety of areas, all the way from basic and applied research to diagnostics. In many areas, the application of molecular biology techniques has facilitated a quantum leap toward understanding biological systems. For example, systems biology is an interdisciplinary field that focuses on the systematic study of complex interactions in a biological system. At its core is the ability to generate, integrate, and analyze complex data from multiple experimental sources using interdisciplinary tools and technologies such as transcriptomics (gene expression measurements), proteomics (complete cellular protein expression pattern, including phosphoproteomics and glycoproteomics), and metabolomics (identification of cellular metabolites). These investigations are frequently combined with large-scale perturbation methods, including RNAi, expression of mutant genes, and chemical approaches using small molecule inhibitor libraries. For examples, see 17 and 18. Therefore, the ultimate goal of studying the biology of an entire cellular system represents a significant driving force for the development of effective and efficient nucleic acid and protein sample preparation reagents and techniques.

This handbook provides advice and guidance on the myriad of nucleic acid extraction reagents and techniques plus tips to overcome many of the challenges of isolating nucleic acids in sufficient quantity, quality, and purity for optimal results in downstream applications.

References

1. Frost and Sullivan Research Service. European advances in nucleic acid purification and amplification technologies (30 June 2005).
2. Huang, J. *et al*. Whole genome amplification for array comparative genomic hybridization using DNA extracted from formalin-fixed, paraffin-embedded histological sections. *J. Mol. Diagn.* **11**, 109–116, (2009).
3. Emmert-Buck, M. R. *et al*. Laser capture microdissection. *Science* **8**, 998–1001 (1996).
4. Cheng, X. *et al*. Micro- and nanotechnology for viral detection. *Anal. Bioanal. Chem.* **393**, 487–501 (2008).
5. Zhang, W. *et al*. A new method for DNA extraction from feces and hair shafts of the South China tiger (*Panthera tigris amoyensis*). *Zoo Biol.* **28**, 49–58 (2009).
6. Elbashir, S. M. *et al*. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498 (2001).
7. Fire, A. Z. Gene silencing by double-stranded RNA. *Cell Death Differ.* **14**, 1998–2012 (2007).
8. De Lellis, L. *et al*. Methods for routine diagnosis of genomic rearrangements: multiplex PCR-based methods and future perspectives. *Expert Rev. Mol. Diagn.* **8**, 41–52 (2008).
9. Watson, D. E. *et al*. TaqMan applications in genetic and molecular toxicology. *Int. J. Toxicol.* **24**, 139–145 (2005).
10. Rogers, Y. and Venter, J. C. Genomics: massively parallel sequencing. *Nature* **437**, 326–327 (2005).
11. Appleby, N. *et. al*. New technologies for ultra-high throughput genotyping in plants. *Methods Mol. Biol.* **513**, 19–39 (2009).
12. Thomson, N. R. *et al*. Comparative genome analyses of the pathogenic Yersiniae based on the genome sequence of *Yersinia enterocolitica* strain 8081. *Adv. Exp. Med. Biol.* **603**, 2–16 (2007).
13. Kulesh, D. A. *et al*. Identification of interferon-modulated proliferation-related cDNA sequences. *Proc. Natl. Acad. Sci. USA* **84**, 8453–8457 (1987).
14. Bagley, J. *et al*. Gene therapy in type 1 diabetes. *Crit. Rev. Immunol.* **28**, 301–324 (2008).
15. Kutzler, M. A. *et al*. DNA vaccines: ready for prime time? *Nat. Rev. Genet*. **9**, 776–788 (2008).
16. Keim, P. *et al*. Microbial forensics: DNA fingerprinting of *Bacillus anthracis* (anthrax). *Anal. Chem.* **80**, 4791–4799 (2008).
17. Bauer, M. *et al*. Reverse genetics for proteomics: from proteomic discovery to scientific content. *J. Neural Transm.* **113**, 1033–1040 (2006).
18. Walduck, A. *et al*. Proteomic and gene profiling approaches to study host responses to bacterial infection. *Curr. Opin. Microbiol.* **7**, 33–38 (2004).

Chapter 2
Key considerations before you begin

Discussion in this chapter focuses on initial, general considerations for sample preparation of nucleic acids, with more detailed, target-specific information presented in later chapters. Because of the numerous end uses of a given target molecule or population of molecules, some of which have fairly specific requirements for the state of the sample, a key focus of the following chapters is to provide information that will help in preparing a target molecule ready for its downstream analysis.

Experimental parameters

The first step in obtaining the best possible analytical results is to define the parameters for sample preparation based on a holistic view of the complete workflow and with the overall purpose in mind. Having the answers to the following interrelated questions will help in designing and optimizing the number and type of sample preparation unit operations to include:

What is the purpose of the intended study?

Which biomolecular class(es) is of interest?

What sample source will be used and what are its characteristics?

Which analytical technique(s) will be used?

What are the capabilities of the analytical technique, and what are the criteria for optimal overall detectability?

- Range of total biomolecular amount?
- Volume range?
- Limit of detection of individual biomolecules?
- Complexity tolerance, for global analysis (i.e., approximate resolving power)?
- Level of purity for analysis of a single biomolecular species?
- Contaminant tolerance?
- How important is overall biomolecular integrity?

Additional considerations include the number of samples to be processed simultaneously (i.e., parallel processing), the amount of initial sample available, required speed, and acceptable cost. The relationships among optimal detectability, yield, and reproducibility are complex and need to be considered as well.

Sample collection (acquisition)

Because the quality of biomolecules begins to decrease at the sample collection stage, we recommend using fresh material whenever possible. When this is not possible, it is important that samples such as tissues and cultured cells are flash frozen in liquid nitrogen immediately and stored at -80°C in a stabilizing agent. When RNA will be isolated, we recommend wearing gloves and preparing the laboratory environment to minimize the introduction of exogenous RNases to the sample. Never allow tissues to thaw before lysis, and disrupt samples in liquid nitrogen, if possible. For DNA isolation, even though DNA is relatively robust, the quality of the downstream analysis will benefit from careful handling of the sample at the time of collection. If room temperature archiving of samples is desired, we recommend using Whatman™ FTA™ technology for sample collection.

Explanation of cell disruption and sample homogenization

If the sample does not consist of separate cells, it will probably need to be physically disrupted using liquid homogenization or another method. This is necessary to provide access to cell walls and cell membranes for cell lysis. For example, solid tissues will usually require physical disruption before cells can be lysed, whereas cultured cells may be lysed directly.

Although animal, plant, and bacterial cells display numerous differences, they all possess a common feature—the cell membrane. The cell membrane surrounds and contains the cellular cytoplasm and functions as a selectively permeable barrier to molecules in the cell's extracellular environment. In addition to the cell membrane, plant and bacterial cells have rigid cell walls that are exterior to their membranes; their chemical nature and arrangement often present challenges to their disruption or removal. For example, the multiple layers of cellulose that comprise a plant cell's wall render it very resilient and refractive to many disruption methods.

With some understanding of the basic differences in cells, the researcher then must devise a protocol that will efficiently disrupt the cell's external barriers—i.e., membranes or walls—while preserving the integrity of the cell's chemical components. **This preservation is critical for molecules that will be studied in downstream applications**. Animal cells are generally easy to disrupt because they lack cell walls. However, the variation in specific cell membrane composition and distribution of molecules either comprising or associated with the membrane can make the process a bit more complex. Because of their unusual resilience, cell walls are generally not as easily disrupted as are cell membranes. Lysis protocols usually involve either physical or enzymatic techniques. Cells with cell walls can be lysed gently following enzymatic removal of the cell wall. This must be done with an enzyme specific for the type of cell to be lysed (e.g., lysozyme for bacterial cells and lyticase for yeast cells).

The ultimate goal of any cell disruption scheme is preservation of the cell's chemical components in as natural a state as possible. By understanding the biology and chemistry of their outer layers, through which cells interact with their environment and protect their internal organelles and molecules, you can choose methods that are most appropriate for yielding those molecules in which you are interested.

Overview of techniques for cell disruption and sample homogenization

Incomplete cell lysis and sample homogenization will not only lead to significantly reduced yield but can also increase the risk of downstream problems, such as clogging of a purification column or inhibition of enzymatic reactions such as PCR. The choice of disruption method depends primarily on whether the sample is from cultured cells, solid tissue, or other biological material and whether the analysis is targeting all of a particular biomolecule (e.g., total DNA or RNA in a cell) or only a component from a particular subcellular fraction (e.g., nuclei or mitochondria). Nucleases and proteases may be liberated and/or activated upon cell disruption. Because the action of these enzymes could extensively complicate (or prevent) the eventual analysis of the target molecule, the sample should be protected from the action of such enzymes during cell disruption and subsequent purification. In particular, inhibition of RNases and proteases is critical to obtaining high-quality RNA and protein, respectively, for analysis. See to Chapter 6 for details on inhibiting RNases.

Numerous methods are available for disrupting cells and homogenizing samples. Table 2.1 summarizes the most popular methods for nucleic acids and indicates for which sample type and target biomolecule the method is appropriate. In general, gentle disruption methods are employed when the sample of interest consists of easily lysed cells (such as tissue culture cells, blood cells, and some microorganisms). Gentle disruption methods can also be employed when only one particular subcellular fraction is to be analyzed. For example, conditions can be chosen in which only cytoplasmic proteins are released, or intact mitochondria or other organelles are

recovered by differential centrifugation. Sometimes gentle disruption techniques are combined (e.g., osmotic lysis following enzymatic treatment; freeze-thaw in the presence of detergent). Moderate disruption methods are employed when cells are less easily disrupted (e.g., cells in solid tissues or cells with tough cell walls). These methods use mechanical or manual methods to physically disrupt tissue. Vigorous disruption methods (e.g., ultrasonication) will result in complete disruption of the cells, but care must be taken to avoid heating or foaming during these procedures. For this reason, vigorous disruption methods have been excluded from Table 2.1.

Table 2.1. Overview of methods for disrupting cells and homogenizing samples for nucleic acid isolation.

Method	Principle	Type of starting material	Used for which biomolecules?	Advantages (+)/ Disadvantages (-)
Gentle				
Osmotic shock lysis	Change from high to low osmotic medium disrupts membranes	Gram-negative bacteria, erythrocytes, and cultured cells	Used primarily for protein isolation. Used for nucleic acid isolation from whole blood to lyse RBC.	+ Simple, inexpensive - Only useful for disruption of cells with less robust walls (e.g., animal cells) - May give low yield
Lysis with chaotropic salts (usually GTC)	Chaotropic agents disrupt the structure of the cell membrane by creating a less hydrophilic environment and weakening hydrophobic interactions	All sample types, but may not fully lyse some Gram-positive bacteria	Used primarily for nucleic acid isolation. Can also be used to isolate nucleic acids and proteins from the same sample.	+ Does a good job of denaturing nucleases and proteases + Can be used to isolate nucleic acids and protein from the same sample + Amenable to increased throughput when used in a mini column or multiwell plate format
Enzymatic digestion	Enzyme digests cell wall, and contents are released by osmotic disruption	Bacteria, yeast, plant tissue, fungal cells	Used for nucleic acid and protein isolation. Often used in combination with other techniques, e.g., freeze/thawing or osmotic shock	+ Gentle + Yields large membrane fragments - Slow - May give low yield
Detergent lysis	Detergents solubilize cellular membranes, lysing cells and liberating their contents	Tissue culture cells	Used for nucleic acid and protein isolation.	
Moderate to vigorous				
Dounce (manual) and/or Potter-Elvehjem (mechanical) homogenization	Cells are forced through a narrow gap with a clearance; cell membrane is disrupted by liquid shear forces	Soft animal tissues and cultured cells	Used for nucleic acid and protein isolation.	+ Excellent for small volumes and cultured cells

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Method	Principle	Type of starting material	Used for which biomolecules?	Advantages (+)/ Disadvantages (-)
Mechanical homogenization (e.g. Polytron™, Waring™ blender, or rotor-stator homogenizer)	Rotating blades break open cells. With the Polytron and rotor-stator homogenizer, the sample is drawn into a shaft with rotating blades	Most plant and animal tissues, blood, cultured cells, bacteria, yeast	Used for nucleic acid and protein isolation.	
Manual grinding with mortar and pestle	Cell walls disrupted by mechanical force	For nucleic acid isolation, samples are often ground in liquid N_2	Solid tissues. Common method to disrupt plant cells.	

Sample sources

Somatic (body) fluids

Whole blood, serum, plasma, CSF, ascites, semen, saliva, amniotic fluid, and lymph are all examples of somatic fluids.

Lysis buffer is typically added directly to cell-free somatic fluids. For whole blood samples, lysis is often a two-step process. First, RBC are selectively lysed and WBC collected. The WBC are resuspended in lysis buffer. Small blood volumes (up to 200 µl) can sometimes be lysed directly (i.e., RBC and WBC are lysed simultaneously).

Viruses

Viral nucleic acids may be obtained from a variety of virus-infected samples but are frequently prepared from cell-free somatic (body) fluids. Lysis buffer with guanidinium is typically added directly to cell-free fluids; Proteinase K may also be required to lyse some viruses. Infected tissues must first be disrupted before isolating viral nucleic acids. Viral nucleic acids may also be obtained from samples archived on Whatman FTA cards or from buccal swabs.

Cultured mammalian cells

Cultured mammalian cells are normally easy to disrupt. Those grown in suspension are typically harvested by centrifugation, washed, and resuspended in an appropriate lysis buffer containing relevant denaturants, inhibitors, stabilizers, and so on. Vortexing will generally complete lysis. Adherent cells can be lysed directly on the culture plate, by adding lysis buffer and scraping the cells followed by transfer of the lysate to a tube and vortexing it to complete the disruption of the cells. Alternatively, adherent cells can be scraped into a tube containing the lysis buffer, mixed, and vortexed.

Animal tissue

Most animal tissue can be processed fresh. The complete disruption and homogenization of animal tissue is critical for good yields and representation of target molecules. Some tissues present special challenges, depending on the biomolecule being sought (i.e., DNA, RNA, or protein). For example, skeletal and cardiac muscle tissue, as well as kidney tissue, are especially difficult to disrupt. Liver and spleen are very active organs transcriptionally, so tissue samples derived from these organs have a very high protein and RNA content and thus, depending on the target molecule, may benefit from inclusion of appropriate inhibitors. Depending on the disruption method, the viscosity of the lysed sample may need to be reduced further for optimal results. Animal tissue that has been frozen after collection will generally benefit from disruption by different methods than those used for fresh tissue. For example, grinding in liquid nitrogen

with a mortar and pestle is effective for most frozen tissue. In general, grinding should be followed by thorough homogenization in an appropriate lysis buffer.

Insects

Treat insects and insect cells as you would animal tissue and mammalian cells, respectively (see above).

Plant tissue

Like animal tissues, plant tissues most often are broken up mechanically. Some soft, fresh plant tissues can be disrupted by homogenization in lysis buffer alone, whereas others need more vigorous treatment (e.g., freezing and grinding in liquid nitrogen or milling). Plants contain polysaccharides, polyphenols, and other molecules that may copurify with the target molecule and inhibit downstream applications. Therefore, additional steps may be helpful to absorb these plant-based contaminants prior to further purification steps.

Yeast and fungi

The cell walls of yeasts make them very difficult to disrupt. They are typically incubated in lyticase, zymolase, glucalase, or some combination thereof, to digest or at least weaken their robust cell walls, followed by vortexing. With some species of yeast, mechanical disruption using a bead mill is effective. To disrupt filamentous fungi, grinding in a mortar in the presence of liquid nitrogen can be effective, followed by sonication in lysis buffer.

Bacteria

Because of the diversity among bacteria, it is difficult to generalize on the effectiveness of a particular extraction method. The volume scale of the extraction has implications as well, because options for extracting small volumes may not be feasible for larger volumes. However, a few generalizations may be made: Because Gram-positive bacteria have a much thicker peptidoglycan layer than do Gram-negative bacteria, they must often be pretreated with lysozyme (or another enzyme; see Table 2.1) to break open the cell wall. Incubation time required with lysozyme will vary between species. Bead milling will lyse most Gram-positive and Gram-negative bacteria, including mycobacteria. Small-volume cultures of some Gram-negative bacteria can be lysed by sonication alone, in an appropriate lysis buffer; however, most Gram-positive bacteria will require more vigorous methods.

Formalin-fixed, paraffin-embedded (FFPE) tissue

For nucleic acid isolation, paraffin-embedded tissue may be deparaffinized using an organic solvent such as xylene. For some PCR applications that do not require intact nucleic acids, FFPE tissue may be used directly. However, using FFPE tissue often poses the risk of poorer results, as the nucleic acid will be fragmented.

Quality and purity considerations

Quality refers the extent to which the isolated biomolecule represents the molecule *in vivo*. RNA degradation is a key concern when isolating RNA. Therefore, inactivation of RNases is of paramount importance in any sample preparation scheme for RNA isolation. Along with intactness of a biomolecule, three-dimensional structure and retained activity are important. For example, nicked (i.e., open circle or OC) and denatured plasmid DNA are distinct from the desired covalently closed circular (i.e., CCC) form.

The quality of isolated biomolecules may be measured by different means than those used to measure purity (see below for a discussion of purity). For example, RIN is a quality measurement for RNA, with 1 corresponding to the poorest quality and 10 to the highest quality (1). DNA sequence quality, which may be a reflection of DNA quality, is often measured using Phred scoring (2, 3).

In sample preparation, the concept of purity varies depending on the type of starting sample, the biomolecule to be isolated, and the requirements of the intended analyses and downstream applications. For nucleic acids, the A_{260}/A_{280} ratio is very important. Because there is not one universally accepted definition of purity, it is probably most correct to talk about removal of contaminants in the context of nucleic acid sample preparation.

Many biological analyses are sensitive to contaminants. To obtain the best possible analytical results, after cell disruption and before the sample is subjected to further preparation or analysis, interfering compounds such as salts, polysaccharides, and non target biomolecules will often need to be removed. Table 2.2 includes a list of common contaminants and options for dealing with them. The table presents contaminants for both nucleic acids and proteins, because in some instances the goal is to prepare more than one biomolecule from the same sample.

Table 2.2. Contaminants that may affect downstream analyses of biomolecules.

Type of contaminant	Relevant to	Source	Reason for removal	Removal techniques
Salts, residual buffers, and other charged small molecules	Both nucleic acid and protein preparations	Carried over from sample preparation	Salts disturb some downstream analyses. See Chapters 4 through 8.	For nucleic acids: Precipitation or washing with alcohol. See Chapters 4 through 8.
Polysaccharides	Both nucleic acid and protein preparations	Starting sample, from plants and certain Gram-negative bacteria	Polysaccharides can clog gel pores. Some polysaccharides contain negative charges and can complex with proteins by electrostatic interactions. Lipopolysaccharides (LPS), often called endotoxins, are found in the outer membrane of various Gram-negative bacteria. LPS can be toxic to tissue culture cells and can elicit endotoxic shock in therapeutic applications.	Ultracentrifugation will remove high-molecular-weight polysaccharides.
Phenolic compounds	For both nucleic acid and protein preparations	Starting sample, particularly plant tissues	Phenolic compounds can modify proteins through an enzyme-catalyzed oxidative reaction (4, 5).	Prevent phenolic oxidation by employing reductants during tissue extraction (e.g. DTT, β-mercaptoethanol, sulfite, ascorbate). Rapidly separate proteins from phenolic compounds by precipitation techniques. Remove phenolic compounds by adsorption.
Insoluble material	For both nucleic acid and protein preparations	Starting sample, particularly tissue	Insoluble material in the sample can clog chromatographic media and gel pores.	Samples may need to be clarified by centrifugation or filtration prior to the next step.

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Type of contaminant	Relevant to	Source	Reason for removal	Removal techniques
Proteins	For nucleic acid sample preparation	Starting sample	The proteins that cause the most concern in nucleic acid sample preparation are nucleases, particularly RNases when RNA is the target biomolecule.	If not fresh, samples should remain frozen during homogenization. Nucleases should be chemically inactivated during lysis. Care should be taken not to introduce exogenous nucleases.
DNA and RNA	For nucleic acid and protein sample preparation	Starting sample	Examples of contaminating nucleic acids are genomic DNA in plasmid DNA and RNA preparations and RNA in DNA preparations.	For nucleic acids: Treat an RNA sample with DNase and a DNA sample with RNase.

General guidelines for cell disruption and sample homogenization

Use procedures that are as gentle as possible; cell or tissue disruption that is too vigorous may denature or shear the target molecule or lead to the excessive release of detrimental enzymes (i.e., proteases and nucleases).

Extraction should be performed quickly, at subambient temperatures and in the presence of a suitable buffer to maintain pH and ionic strength and stabilize the sample. Prechill equipment and keep samples on ice at all times.

Include additives when appropriate to inhibit nucleases or proteases (or other protein modifying enzymes) or to eliminate contaminating biomolecules.

Laboratory practices for nucleic acid sample preparation

Gloves and safety goggles may be required for sample preparation methods that require the use of certain chemicals and equipment. Consult the product information to determine if gloves and safety goggles are required to protect you during your sample preparation scheme.

Consider whether cross-contamination is a concern and take appropriate precautions if necessary. For example, PCR is susceptible to contamination from the products of previous PCR amplifications. For this reason, aerosol pipette tips are often used, and reactions are typically set up in a different location than the one used during PCR product analysis.

DNA can be kept at room temperature for several hours or at 4°C for several days in the presence of EDTA to inhibit the activity of trace amounts of DNase. DNA should be frozen at -20°C or -80°C for long-term storage. Alternatively, biological samples can be collected onto Whatman FTA cards for archiving. Whatman FTA technology is based on a cellulose matrix that contains chemicals to ensure preservation of nucleic acids and inactivate bacteria and viruses, if present.

RNA is very sensitive to trace contaminations of RNases, often found on general labware, fingers and dust. It is necessary to create an RNase-free working environment. It is important to wear gloves at all times during the preparation and change them frequently. We recommend using sterile, disposable polypropylene tubes, which should be kept closed whenever possible. Glassware should be oven-baked for at least 2 h at 250°C before use. Laboratory surfaces should be wiped down with alcohol. To preserve stability, isolated RNA should be kept on ice during use or frozen at -80°C for long-term storage.

For storage of all biomolecules, a manual-defrost freezer is recommended. Store in aliquots to prevent repeated freeze-thaw cycles.

References

1. Schroeder, A. *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurement. *BMC Mol. Biol.* **7**, 3 (2006).
2. Ewing, B. *et al.* Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**, 175–185 (1998).
3. Ewing, B. and Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**, 186–194 (1998).
4. Granier, F. Extraction of plant proteins for two-dimensional electrophoresis. *Electrophoresis* **9**, 712–718 (1988).
5. Flengsrud, R. and Kobro, G. A method for two-dimensional electrophoresis of proteins from green plant tissues. *Anal. Biochem.* **177**, 33–36 (1989).

Chapter 3
Sample collection, transport, archiving, and purification of nucleic acids

Nucleic acid sample preparation begins with the process of sample collection. If samples are not collected and handled properly, it may be impossible to obtain high-quality nucleic acid *regardless* of the method used for DNA preparation. Therefore, sample collection is critical to obtaining optimal results in downstream applications for nucleic acids. Because the path from sample collection to nucleic acid purification may involve sample transportation and archiving, we recommend that you give upfront consideration to sample collection, transport, archiving, and purification of nucleic acids as a workflow (see Fig 3.1) rather than as isolated processes. Special consideration should be given to whether you want to archive samples, that is, whether you want to have access to them years later. Room temperature sample archiving is valuable because it eliminates the need for freezer and refrigerator storage, thus reducing the impact on the environment. In addition, it provides ambient temperature sample libraries that are easily accessible.

A number of factors will influence the choice of a sample collection and biomolecule purification method. The following list includes some of the major considerations:

- Do you want to archive samples and for how long?
- From what sources (e.g., human blood, plant tissue) will you collect samples?
- How many samples will you collect in one day? How many each year? What is the sample size?
- Will sample collection take place outside the laboratory? If so, are facilities available for refrigeration/freezing?
- Is the sample considered a biohazard? Will the sample be transported off-site?
- Which molecule(s) (e.g., DNA, RNA, protein) will be isolated from the samples?
- What is the intended downstream application for the molecule of interest?

In some cases, nucleic acid purification will be performed immediately after sample collection, but this is not the norm. Most samples are stored over the short or long term before they are processed. Regardless of the method for sample collection, it is critical to handle biological samples with care. See Chapter 2 for details on handling biological samples.

Nucleic acid quantity, purity, and quality considerations

It is important to select a sample collection/nucleic acid purification system that will provide nucleic acid of sufficient yield, purity, and quality for subsequent use.

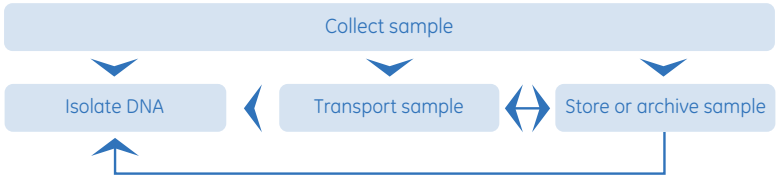

Fig 3.1. Simple workflow for sample collection, transport, archiving, and DNA purification.

The choices for sample collection and nucleic acid purification methods are influenced by the amount of nucleic acid required for the desired experiment or technique. For some downstream applications, microgram quantities of nucleic acids are required; this requirement will affect the method of sample collection. When large quantities of nucleic acids are required, you can collect sufficient fresh or fresh-frozen tissue or cells to directly isolate the desired amount of nucleic acid. If nucleic acids will not be isolated immediately or if you have very limited starting material, you can collect samples via a micro collection method on a paper matrix; a technique such as WGA or total RNA amplification can be used to generate enough nucleic acid for use. See Chapter 7 for information on WGA using Phi29 DNA polymerase.

Many downstream applications for biological molecules are sensitive to contaminants in the starting sample. For example, major contaminants in blood samples include heme and heparin (an anticoagulant that may be used during blood drawing). Additional contaminants (e.g., organic solvents, salts, alcohol) may be introduced with some methods of sample preparation. Contaminant removal should be considered when selecting a sample collection/nucleic acid purification system.

Sample integrity is very important for any nucleic acid analysis. The amplification of long DNA fragments is directly dependent on whether the starting template is intact or degraded. Although methods to repair damaged DNA are available, the goal should always be to isolate intact DNA to obtain the best possible results from subsequent analyses. Sample collection methods that also prevent sample degradation during sample archiving are critical to today's molecular biological analyses.

The remainder of this chapter will discuss sample collection, transport, archiving, and purification of DNA. It will focus on blood samples but will briefly mention several other sample types.

Options for sample collection, transport, archiving, and DNA purification

After sample collection, the other steps do not follow in a specific order. For example, DNA can be prepared from fresh sample, or the sample can first be transported off-site then stored (short term) or archived (long term). A simple workflow is shown in Figure 3.1.

A number of options are available for sample collection, transport, archiving, and DNA purification. These can be categorized into those that are amenable to room temperature archiving and transport, and those that are not. In general, untreated biological samples are not stable at room temperature.

Options that do not allow room temperature archiving and transport

Biological samples collected directly into a tube, multiwell plate, or other vessel are typically not stable at room temperature. To maintain nucleic acid integrity, samples should be processed immediately, stored over the short term (with or without an added chemical stabilizer) at a temperature appropriate for the sample type, or collected onto a matrix suitable for room temperature archiving. For short-term storage, conditions vary by sample type. For example, blood samples can be stored at 4°C for up to 48 h before isolating DNA, while tissue samples and pelleted cells may be stored at -80°C for several weeks or months. Cells may be frozen directly, or a stabilizing agent (see below) may be added. Most tissues should be "flash frozen" in liquid nitrogen for best results. Biological samples that require refrigeration or freezing also require wet or dry ice, respectively, for shipping. In addition, human samples must be labeled as potential biohazards. Shipping of these samples to less accessible regions may be limited to certain days of the week, and the shipper must be willing to deal with biohazardous materials.

Chemical stabilizers may be used to stabilize DNA, RNA, and/or protein. These chemicals allow samples to be stored for short periods (several days or weeks) at room temperature or for longer periods (12+ mo) at -20°C or -70°C. Chemical stabilizers require an additional kit or method to isolate nucleic acids.

Options that allow room temperature archiving and transport

A straightforward way to prepare a sample for room temperature archiving and transport is to collect the sample directly onto a paper matrix. The options for paper matrices include a variety of untreated matrices (e.g., Guthrie or Whatman 903 cards) and chemically treated matrices (i.e., Whatman FTA technology). When nucleic acids will be isolated, we recommend using chemically treated matrices designed to stabilize nucleic acids and denature proteins. Additional details on Whatman FTA technology are provided later in this chapter.

Other products are available for room temperature archiving. However, these products do not stabilize the nucleic acids in the samples themselves; they stabilize previously purified nucleic acids.

See Table 3.1 for a summary of the different options for sample collection, transport, archiving, and DNA purification, including advantages and disadvantages.

Table 3.1. Advantages and disadvantages of different methods for sample collection, transport, archiving, and DNA purification.

Collection	Transport	Can sample be archived?	Additional DNA purification method required?	(+) Advantages/ (-) Disadvantages
Use fresh sample	Dry ice for tissues and cells; wet ice for blood Human samples must be treated as biohazardous	No. Sample is used up.	Yes	+ Does not require a refrigerator or freezer - Sample must be used right away - Can't return to sample for additional analysis because sample was not stored
Use frozen tissue/cells or use blood stored at 4°C	Dry ice for tissues and cells; wet ice for blood Human samples must be treated as biohazardous	No. Short-term storage only in freezer or refrigerator, depending on sample type	Yes	+ Allows user to return to sample for additional analysis - May require use of liquid nitrogen to freeze tissues - Does not allow for sample archiving
Add chemical stabilizers to the sample	Room temperature, wet ice, or dry ice (depends on how long sample is expected to be stable at that temperature)	Yes, in a freezer	Yes	+ Inactivates nucleases (and proteases, depending on the chemical) + Samples can be stored for several days or weeks at room temperature - Requires a freezer for long-term storage - Tissue must be cut to be less than 0.5 cm in at least one dimension - Requires an additional DNA purification method
Isolate nucleic acids, then stabilize with a commercially available product for this purpose	Room temperature	Yes, at room temperature	Yes. DNA must be prepared before using these archiving methods	+ Allows room temperature archiving + Available as single tubes or as 96-well plates

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Collection	Transport	Can sample be archived?	Additional DNA purification method required?	(+) Advantages/ (-) Disadvantages
Spot sample onto untreated matrix (e.g., Guthrie or 903 card)	Refrigerated to preserve integrity of nucleic acids and proteins Human samples must be treated as biohazardous	Yes, at -20°C	Yes	+ Less expensive than chemically treated paper - Nucleic acids must be purified before using product - Nucleic acids not stabilized at room temperature for extended periods of time - Nucleic acids can be stored at room temperature, but the integrity of the nucleic acids is unknown; amplification of long PCR fragments (> 2 kbp) may not be possible
Apply sample onto chemically treated matrix (i.e., Whatman FTA technology)	Room temperature	Yes, at room temperature	No. Requires only FTA Purification Reagent (FTA) or water (FTA Elute) for DNA purification	+ Complete system for sample collection, transport, archiving, and DNA purification + Allows room temperature archiving—more than 17 yr (and counting) real-time stability with human blood on FTA + Allows for noninvasive sample collection (i.e., buccal swabs) + Quick and easy DNA purification + Can be used with a variety of sample types from tissue to plant material to cultured cells and bacterial cultures

Overview of Whatman FTA technology

Whatman FTA technology is a patented process that incorporates chemically coated matrices to collect, transport, archive and isolate nucleic acids in a single device. The technology, which consists of two distinct chemistries for FTA and FTA Elute, has the ability to lyse cells on contact, denature proteins, and protect DNA from degradation caused by environmental challenges and microbial attack. FTA contains chemical denaturants and a free radical scavenger, while FTA Elute contains a chaotropic salt. The difference in the chemical coatings is what allows the DNA to be eluted from FTA Elute into a solution phase, while purified DNA remains bound to FTA. Purified genomic DNA from FTA and FTA Elute is suitable for use in PCR, STR, SNP genotyping, allelic discrimination genotyping, and RFLP analyses. DNA from FTA is also suitable for AFLP; DNA from FTA Elute is also suitable for use in TaqMan™ assays. FTA and FTA Elute are compared in Table 3.2.

Samples are collected onto FTA or FTA Elute cards in a number of ways (see below), and cards are dried. Discs of FTA and FTA Elute are removed from sample areas using a coring device, such as a Harris Micro Punch or Uni-Core. These coring devices come in various sizes (i.e., 1.2 mm, 2.0 mm, and 3.0 mm); the choice of size depends on both the downstream application and the initial sample type. For applications that require DNA in solution, multiple discs can be treated at once. See Chapter 9 for information on semi-automation and full automation of card punching.

Both FTA and FTA Elute are supplied as white cards and as indicating cards. As shown in Figure 3.2, Indicating cards come with a dye that changes from colored to white when a clear colorless sample such as bacteria, cultured cells, saliva, urine, or buccal cell smears is applied to the cards.

Fig 3.2. Indicating FTA Elute. Colorless sample has been applied to the upper left portion of the card.

Sample application

Samples can be applied to FTA and FTA Elute cards in a variety of manners (see Fig 3.3). The simplest method is used to apply liquid samples such as blood, cultured cells, and bacteria. The samples can be pipetted onto the card or, in the case of a finger stick, blood can be dropped onto the card (1). The circles on the different cards can accommodate samples from 40 to 125 µl; refer to the GE Healthcare Web site for product selection. Buccal samples are collected from the inside of the cheek with a swab or foam head such as that found on the EasiCollect buccal cell collection device. Cells scraped and collected onto the foam head are then pressed onto the FTA cards to transfer an even coating of cells onto the card surface. Samples such as plant leaf can be gently crushed onto the cards using a pestle, tack hammer, or round-bottom plastic test tube (2). Insects can be dissected and homogenized, then applied to the FTA card (3) or crushed directly on the card (4). Tissue can be scraped to dislodge cells (5) or pressed onto the FTA card (6). Cards must be dried completely before DNA purification.

Fig 3.3. Options for sample application onto Whatman FTA or FTA Elute cards.

FTA and FTA Elute are compared in Table 3.2.

Table 3.2. Comparison of FTA and FTA Elute.

Characteristic	FTA Indicating FTA	FTA Elute Indicating FTA Elute
Bactericidal Gram -/+	Yes	Yes
Viricidal	Yes	Yes
Fungicidal	Yes	Yes
Blood—shelf life	17.5 yr and counting	12.5 yr and counting
Buccal cells—shelf life	8 yr and counting	3 yr and counting
BACs—shelf life	4 yr and counting	Not applicable
Water elution	No	Yes

Genomic DNA purification from sample applied to FTA cards

Materials

FTA Card (Micro, Mini, or Classic Card); Indicating card for clear colorless samples

Harris Micro Punch or disposable Uni-Core Punch (1.2, 2.0, or 3.0 mm coring device, depending on sample and downstream application)

FTA Purification Reagent

TE^{-1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0)*

* *Note that this buffer contains 0.1 mM EDTA, not 1 mM EDTA.*

Advance preparation

None

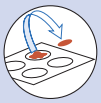
Protocol

1. Collect sample and dry

Apply liquid sample (125 µl per 25 mm circle), crushed tissue, or plant leaf to the FTA card and air dry for 1 h. Cells are lysed, proteins are denatured, pathogens are inactivated, and DNA is released to entangle in the fibers of the matrix.

Card must be completely dry before proceeding to punching and purification.

2. Punch



Cut a disc using a Harris Micro Punch or Uni-Core device and place in a PCR tube.*

3. Rinse the punch with FTA Purification Reagent

Wash with 200 µl of FTA Purification Reagent for 5 min at room temperature; perform three washes. Cell debris and PCR inhibitors are washed away.

4. Rinse the punch with TE^{-1} buffer

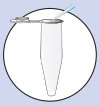
Wash with 200 µl of TE^{-1} buffer for 5 min at room temperature; perform two washes. FTA Purification reagent, which itself is a potent PCR inhibitor, is washed away.

TE with reduced EDTA content is used because EDTA may interfere with PCR.

5. Dry

Air dry the punch for 1 h at room temperature or for 15 min at 50°C.

6. Perform PCR analysis



Add PCR Master Mix and perform PCR according to the protocol chosen.

* *Guidelines: 1.2 mm punch for blood samples and samples with high DNA content; 2.0 mm for buccal cells, plant cells, and bacteria containing plasmid DNA; 3.0 mm to prepare DNA from FTA Cards using illustra tissue & cells genomicPrep Mini Spin Kit.*

Genomic DNA purification from sample on FTA Elute

Materials

FTA Elute card or Indicating FTA Elute card

Harris Micro Punch or Uni-Core Punch, 3.0 mm punch tool

Heating block or thermal cycler calibrated to 95°C

Advance preparation

None

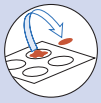
Protocol

1. Collect sample and dry

Apply up to 40 µl of liquid sample or crush leaf tissue onto FTA Elute card. Air dry for 3 h at room temperature or 15 to 20 min at 80°C. The cells are lysed when they contact the chemical coating on the card. Proteins become irreversibly immobilized, and DNA interacts reversibly with the fibers of the matrix.

Card must be completely dry before proceeding to punching and purification.

2. Punch



Use a Harris punch tool to remove a 3.0 mm disc from the dried sample. Place into a 1.5 ml microcentrifuge tube.

3. Rinse the punch

Add 500 µl of sterile water and vortex 5 times. FTA Elute chemicals and cellular debris are washed from the disc; proteins remain bound to the disc.

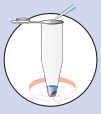
4. Remove water

Centrifuge briefly and remove rinse water. Use a pipette tip to transfer the washed disc to a clean 0.5 ml microcentrifuge tube.

5. Elute purified DNA

Add 30 µl of sterile distilled water and incubate in a calibrated heating block or thermal cycler at 95°C for 30 min. After incubating the disc, vortex for 1 min by pulsing the tube 60 times to dislodge the DNA from the matrix. During heating, DNA is denatured and dissociates from the fibers of the FTA Elute card. Proteins and other PCR inhibitors remain bound to the matrix.

6. Centrifuge



Centrifuge the tube to recover the condensation from the top of the tube and to pellet the disc. Withdraw the disc from the solution and store eluted DNA.

7. Perform PCR analysis

Use approximately 2.5 µl of eluted DNA in a 25 µl PCR. Store the remainder of the DNA at -20°C in aliquots.

It is important to quantitate the amount of purified DNA by either real-time PCR or by fluorescent methods such as OliGreen™. The DNA from FTA Elute is too dilute to be measured with spectrophotometric methods even on a micro-spectrophotometer. The yield and purity will be poor if measured by this method, but be assured that the DNA is of sufficient purity for PCR and other downstream applications.

Comparison of treated versus untreated matrices for DNA stabilization

DNA stored on an FTA-coated matrix versus an uncoated matrix such as 903 Specimen Collection Paper was challenged with UV radiation to mimic accelerated aging radiation. In the experiment shown in Figure 3.4, DNA collected on FTA or 903 was either exposed to UV radiation (red tracings) or kept in the dark at ambient temperature (blue tracings; these represent the starting amount of DNA) as a control. The DNA was then quantitated using a real-time PCR assay to determine the extent of damage to the DNA. The data show that FTA stabilizes the DNA and prevents damage caused by UV radiation.

In Figure 3.4.A, a shift to the right of the blue tracings indicates a loss of DNA by damage. The average C_q shift for the FTA cards is 1.4, indicating a 2.7-fold loss in DNA integrity. In contrast, the average C_q shift for DNA from untreated filter paper (Fig 3.4.B) is approximately 9.7, which represents an 860-fold loss of DNA integrity.

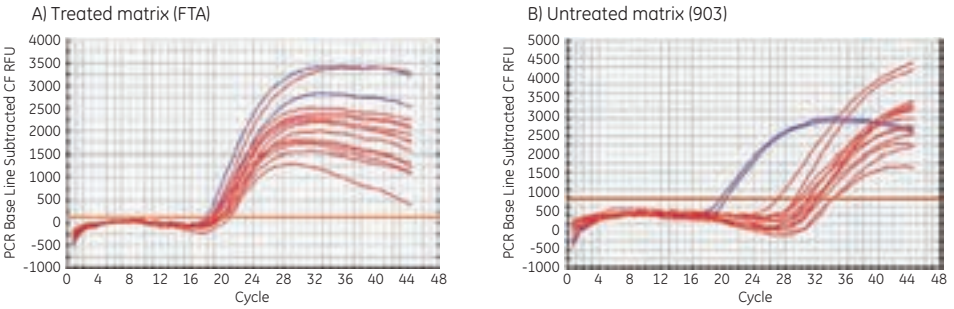


Fig 3.4. Real-time PCR results of DNA from treated **(A)** or untreated **(B)** matrices subjected to UV treatment. Blue lines represent controls that have not been treated with UV radiation; red lines represent results from individual punched discs.

The real-time PCR data show that FTA clearly has a protective effect on DNA under a variety of circumstances and is therefore superior to an untreated matrix for DNA stabilization.

DNA archiving on FTA

The following example shows the power of archiving DNA on FTA cards. Punches (1.2 mm) were manually taken from FTA blood samples archived for 17.5 yr at room temperature and from fresh FTA blood samples. Punches were washed according to standard protocols. Figure 3.5.A represents fresh blood applied to FTA cards, which were then dried and prepared for STR analysis using the PowerPlex™ 16 System. A normal profile is seen, with all the homozygous and heterozygous peaks well represented. The scale of the RFUs is given for reference; the scale should be taken into consideration when comparing samples. In Figure 3.5.B, blood stored for 17.5 yr on an FTA card was analyzed with the same STR kit; the RFU scale is given for reference.

The 17.5 yr old (17.5 y/o) profile shows balanced peaks, no stutter, no peak drop out, and no signs of DNA degradation. Note that the largest peaks, Penta E (blue channel) and Penta D (green channel), are intact. If the sample was degraded, these peaks would drop out or would be greatly reduced.

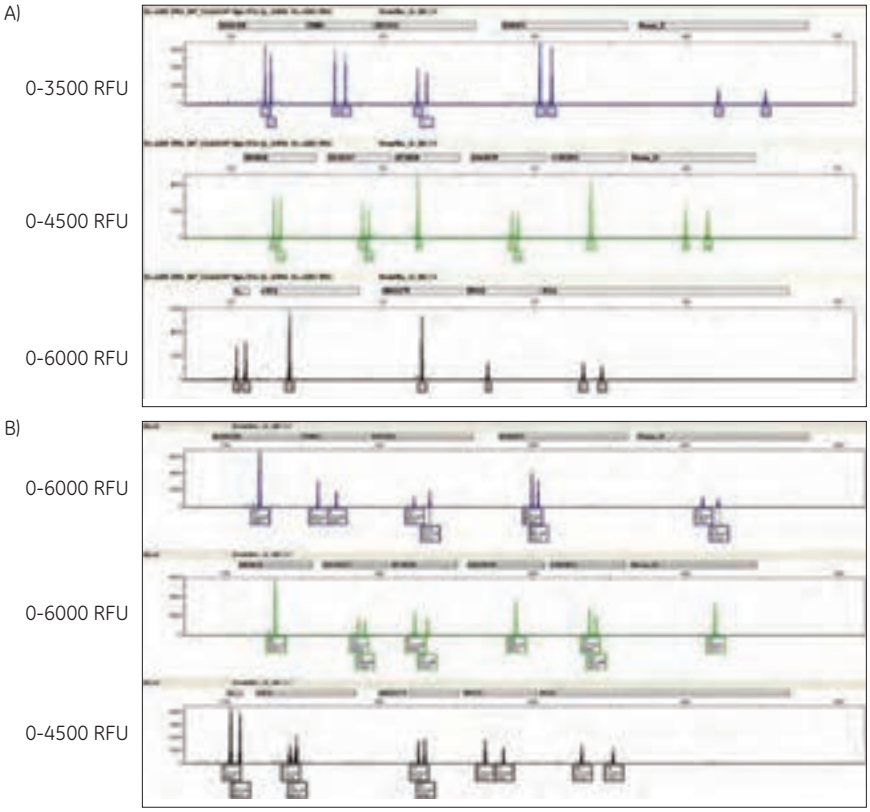


Fig 3.5. STR analysis using the Promega PowerPlex 16 System with DNA from fresh FTA blood **(A)** or 17.5 year-old (17.5 y/o) blood **(B)** on FTA. Data courtesy of Dr. Arthur Eisenberg and Mr. Xavier Aranda, DNA Identification Lab, University of North Texas Health Science Center.

See Chapter 4 for data for Scorpions™ ARMS™ analysis using genomic DNA from FTA Elute.

References

1. Stienstra, Y. *et al.* Susceptibility to Buruli ulcer is associated with the SLC11A1 (NRAMP1) D543N polymorphism. *Genes Immun.* **7**, 185–189 (2006).
2. Ndunguru, J. *et al.* Application of FTA technology for sampling, recovery and molecular characterization of viral pathogens and virus-derived transgenes from plant tissue. *Virol. J.* **2**, 45 (2005).
3. Adams, E. R. *et al.* Trypanosome identification of wild tsetse populations in Tanzania using generic primers to amplify the ribosomal RNA ITS-1 region. *Acta Trop.* **100**, 103–109 (2006).
4. Harvey, M. L. An alternative for the extraction and storage of DNA from Insects in forensic entomology. *J. Forensic Sci.* **50**, 627–629 (2005).
5. Dobbs, L. J. *et al.* Use of FTA gene guard filter paper for the storage and transportation of tumor cells for molecular testing. *Arch. Path. Lab. Med.* **126**, 56–63 (2002).
6. Moscoso, H. *et al.* Molecular analysis of infectious bursal disease virus from bursal tissues collected on FTA filter paper. *Avian Dis.* **50**, 391–396 (2006).

Chapter 4
Genomic DNA preparation

Genomic DNA is responsible for passing on heritable characteristics and as such is used as the primary source for studying genetic similarities and differences between individuals. Within the nucleus of eukaryotic cells, billions of base pairs are wound around histone proteins to condense the DNA into chromatin. Several layers of complexity are exhibited in controlling how and when genes are expressed. These include tertiary structure, DNA methylation, sequence variation, and segmentation. Genomic DNA preparation typically involves the lysis of cell membranes, removal of histone proteins, and separation of DNA from other biomolecules such as RNA and lipids. In plants, yeast, and bacteria, additional preparative methods are often required to disrupt a thick cell wall composed of polysaccharide or peptidoglycan. This chapter discusses the extraction of genomic DNA from a variety of sources, as well as sample preparation considerations for common downstream applications.

The required purity, quality, and quantity of genomic DNA should be considered when selecting a preparation method. The purity and quality of extracted genomic DNA can be assessed by several methods including spectrophotometry, in which the A_{260}/A_{280} may be used to monitor protein contamination. It is generally accepted that good-quality genomic DNA has an A_{260}/A_{280} ratio of 1.8. RNA also absorbs at 260 nm, but it is often removed by an RNase treatment step during genomic DNA preparation. If the A_{260}/A_{280} is close to 1.8, spectrophotometric quantitation of DNA may be performed. Spectrophotometry and other methods of DNA quantitation are discussed in Appendix 4. The quality of genomic DNA may be assessed using agarose gel electrophoresis. Standard electrophoresis will not resolve DNA larger than about 50 kb; if an assessment of length is required, genomic DNA can be electrophoresed using PFGE, which may reveal megabase-sized fragments.

Different applications for genomic DNA have different tolerances to contaminants. Choosing a method for genomic DNA preparation should be based on the requirements for the intended application(s), as well as on the available sample size, preciousness of the sample, desired throughput, and other factors. See Chapter 2 for further information.

Table 4.1 provides a summary of possible contaminants in genomic DNA preparations and applications in which those contaminants may interfere.

Table 4.1. Summary of possible contaminants in genomic DNA preparations and applications in which those contaminants may interfere.

Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
RNA	Sample; some tissues are more transcriptionally active than others, for example liver and kidney	DNA quantitation by spectrophotometry	May give artificially high estimate of DNA concentration
		Cycle sequencing	May result in poor amplification/ low signal
DNases	Sample; some tissues contain high levels of DNase, for example pancreas, thymus, spleen, and lymphoid tissue	All	May degrade genomic DNA
Proteins	Sample	Electrophoretic analysis	Interfere with mobility
		Enzymatic reactions	Interfere with enzyme kinetics
Polysaccharides	Particularly from plant samples, yeast, and fungi	Restriction digestion and amplification	Inhibit restriction enzymes and polymerases
			May reduce overall yield of genomic DNA
Residual salts	May be from sample or introduced during genomic DNA preparation	Action of restriction and modifying enzymes	Inhibit enzyme activity (e.g., restriction enzymes HindIII and SacI)
Organic solvents, for example, alcohols and phenol/chloroform	May be introduced during genomic DNA preparation	Residual organic solvent contaminants will interfere with the majority of routine molecular biology techniques	Inhibit enzyme activity
Contaminating DNA from the environment, particularly DNA from previously run PCR amplifications	Poor technique or hygiene	Amplification reactions	May give false positives in presence/absence studies and genotyping
Pigmented DNA	Sample, particularly blood	Poor amplification/ low signal in cycle sequencing, for example	May give incorrect A_{260}/A_{280} measurement
			Inhibits restriction enzymes and polymerases

Techniques for genomic DNA preparation

Few entirely new methods for genomic DNA preparation have evolved in recent years. "Homemade" methods are typically solution-based and may use organic solvents such as phenol. Reagent kits typically include all required buffers and use a medium for selective binding of genomic DNA. Two purification options predominate in reagent kits: silica and anion exchange media. Protocols are either centrifugation (spin)-based or vacuum-based. Silica is typically provided in a membrane or other solid phase such as magnetic beads, both of which are amenable to automation using liquid handling robotics. Reagent kits may be designed for

a number of sample types, or they may be sample-specific (e.g., specific for blood or plants). Another option for preparing genomic DNA uses Whatman FTA technology. Genomic DNA may be accessed while bound to the card for FTA or released with water and heat for FTA Elute. See Chapter 3 for additional information on Whatman FTA technology.

A simple flowchart for the preparation of genomic DNA is shown in Figure 4.1.

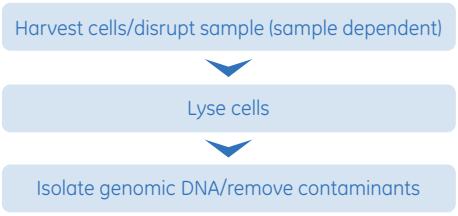


Fig 4.1. Flowchart showing steps in preparing genomic DNA for analysis.

Cell lysis is a critical step in genomic DNA preparation. For hard tissues, it is necessary to first process the sample into small enough pieces so the lysis reagents can access the cells. See Chapter 1 for details on sample disruption. Other samples (plants, fungi, yeast, Gram-positive bacteria) may require a specific enzymatic or organic step to break open the cell wall. Incomplete cell lysis will lead to a decreased yield of genomic DNA and may increase potential contaminants such as polysaccharides and proteins. During lysis, it is also important to inactivate nucleases that may degrade genomic DNA. Proteases and RNases are frequently added to genomic DNA isolation procedures to degrade proteins and RNA, respectively. This decreases the "load" of potential contaminants in the preparation and also decreases sample viscosity. The DNA is subsequently eluted in a high-salt buffer.

Note that RNases and proteases should not be used if the goal is to also isolate RNA or protein, respectively, from the sample.

Filter capture or chromatographic purification

Filter capture or chromatographic purification can circumvent the use of organic extraction for most samples. Genomic DNA binds to silica (in membrane, bead, or other form) in a denaturing, chaotropic environment. After silica-bound genomic DNA is washed with a high-salt buffer containing ethanol, the DNA is eluted in a low-ionic-strength buffer, ready for use. A popular choice for chromatography is ion exchange chromatography, which allows binding of negatively charged DNA.

After elution in a high-salt buffer, genomic DNA must be desalted or precipitated. Other choices for chromatography include hydrophobic interaction chromatography, but this is not typically used on a small scale.

Amplification using Phi29 DNA polymerase

Genomic DNA from limited samples, such as buccal swabs and FTA/Guthrie card blood, can be amplified using Phi29 DNA polymerase. In this isothermal technique, random hexamers prime denatured DNA to form complementary strands. These are displaced and become templates for further rounds of amplification (1). This method prepares microgram quantities of genomic DNA from nanogram input. It is amenable to automation and does not require specific primers or a thermal cycler. See Chapter 7 for further details on amplification using Phi29 DNA polymerase and Chapter 9 for information on increasing throughput of the procedure.

Classical and solution-based methods

A classical method for isolation of high-molecular-weight genomic DNA involves capture of DNA directly in agarose. However, this "in-gel" method requires electrophoresis and at least a crude preparation of genomic DNA. Isolation of genomic DNA for restriction analysis or library construction may require fragment sizes on the order of several hundred kilobases. Protocols for extracting large genomic DNA involve tissue homogenization or lysis of cells with detergent or by osmotic shock. Yeasts, plant cells, Gram-positive bacteria, and tissues high in carbohydrates typically require additional organic or enzymatic steps. Solution-based methods usually use differential precipitation or phenol extraction. In differential precipitation, cells are lysed in the presence of a particular salt, and proteins and other contaminants are selectively precipitated. The precipitate is pelleted during centrifugation, leaving genomic DNA in solution. However, phenol extraction segregates contaminants (typically proteins) by partitioning a lower organic phase from an upper aqueous DNA-containing phase. Phenol extraction is frequently followed by DNA precipitation with alcohol to remove residual phenol. While simple and effective, the use of phenol is recognized as being particularly hazardous, and many laboratories have been active in its prohibition. Safer, alternative methods have been developed that exploit the use of phase separation to accelerate and improve the purification of genomic DNA. The range of Nucleon™ products from GE Healthcare produces a semi-solid stratum between aqueous DNA and the contaminant organic phase, making genomic DNA preparation much easier and safer.

A summary of methods for genomic DNA preparation and their advantages and disadvantages is provided in Table 4.2. Consult references 2 and 3 for further information on some of these methods.

Table 4.2. Advantages and disadvantages of different techniques for genomic DNA preparation.

Technique	Principle	Advantages(+)/ Disadvantages (-)
Silica	This method frequently utilizes chaotropes and proteolytic digestion in combination with silica purification. Genomic DNA binds to silica in a denaturing, high-salt environment. Silica-bound DNA is washed and partially dried. Genomic DNA is eluted in a low-ionic-strength buffer.	+ Eluted DNA is ready to use + Amenable to high throughput when in column or magnetic bead form - Poor scalability–not much beyond ~100 µg - DNA may bind irreversibly if the medium is underloaded or overdried
Anion exchange chromatography	This method frequently utilizes detergent and enzymatic digestion in combination with anion exchange purification. Negatively charged genomic DNA binds to the anion exchanger in a high-salt environment. Genomic DNA is eluted with a higher-salt buffer then desalted or precipitated.	+ Scalable - DNA is eluted in high salt so must be desalted or precipitated
Whatman FTA technology	Sample is spotted onto a chemically treated matrix, which is dried. For FTA Elute, DNA is eluted with addition of water and 95°C incubation; protein and other potential contaminants remain bound to the matrix. For FTA, contaminants are washed away, while purified genomic DNA remains bound to the matrix.	+ Technology allows for long-term ambient temperature archiving of samples + DNA can be used in PCR or isothermal amplification - Spotted samples must have been prepared and thoroughly dried before use

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Technique	Principle	Advantages(+)/ Disadvantages (-)
Amplification using Phi29 DNA polymerase	Phi29 DNA polymerase uses random hexamers to prime denatured DNA to form complementary strands. These are displaced and become templates for further rounds of amplification.	+ ng input of genomic DNA can generate µg output + Can be used in conjunction with Whatman FTA Elute + Most applications do not require high purity of input DNA + Amenable to automation + Does not require custom primers or a thermal cycler
Differential precipitation (e.g., using CTAB, potassium acetate, etc.)	Cells are lysed using enzymatic and/or chemical means. Cellular components are separated based on solubility differences.	+ Inexpensive + Does not use phenol/chloroform - Slow
Methods using phenol and chaotropes	Chaotropic salts lyse cells and denature proteins. Phenol solubilizes and extracts proteins and lipids into an organic phase. DNA in the aqueous phase is precipitated using ethanol and salt.	+ Inexpensive - Requires handling and disposal of caustic/toxic organic solvents - Not amenable to high throughput
In-gel	A crude preparation of genomic DNA can be separated by PFGE. Longer genomic DNA, which migrates the least, can be selectively excised and extracted.	+ High-molecular-weight DNA is obtained - Requires electrophoresis prior to DNA isolation, time-consuming - Not easily adaptable to high throughput

General considerations for genomic DNA preparation

Considerations independent of technique

External factors such as sample type, handling, and storage may affect the quality and yield of isolated genomic DNA. In general, use samples immediately or freeze them, utilize precautions against introducing exogenous DNases, and disrupt your sample using a method appropriate for your sample type. Many contemporary applications such as PCR, SNP detection, sequencing, WGA, and fingerprinting utilize genomic DNA in the size range up to 50 kb. Most "homemade" and commercially available genomic DNA purification protocols generate DNA in this size range. Applications that require high-molecular-weight genomic DNA (e.g., preparation of large insert genomic libraries, requiring > 100 kb inserts), are almost exclusively the domain of "in gel" extraction techniques and PFGE, and are not served well by methods that use silica or anion exchange media. See Chapter 2 for more information on handling of genomic DNA.

It may be tempting to use a larger starting sample size than is recommended for your preparation method. Do NOT overload the purification system with too much starting material or, for solid-phase purification using silica or anion exchange chromatography, too much crude lysate. Overloading of a purification system can lead to decreased yield of genomic DNA and/or to increased levels of contaminants. Please follow the provided instructions for your preparation method. Considering that many analysis methods involve some type of amplification, obtaining sufficient output yield is usually not a limitation.

Following purification, DNA quantity, quality, and purity can be estimated using a number of methods including spectrophotometry, gel quantitation, or DNA-binding dyes. Assessment of quantity and quality is highly recommended. See Appendices 3 and 4 for details.

See Chapter 1 for details on handling and storing genomic DNA.

Although ethanol precipitation is frequently used to concentrate plasmid DNA, precipitation of genomic DNA is best avoided. Ethanol-precipitated genomic DNA may be difficult to redissolve.

If it is necessary to concentrate or desalt purified genomic DNA, isopropanol precipitation may be used (see Appendix 4). Alternatively, a desalting column may be used if DNA concentration is not required.

Often the viscosity of genomic DNA makes it difficult to use standard pipettes and tips successfully. Positive-displacement pipettes such as Gilson Microman™ have been developed to work with highly viscous solutions including genomic DNA.

Considerations when using silica products

Underloading or overloading silica columns can have a number of consequences. For example, if the column is underloaded, the recovery will be compromised, yielding genomic DNA that will typically be at a much lower concentration than expected. This may prevent the direct use of the eluted DNA in an application, and the DNA may need to be concentrated prior to use. Conversely, column overloading can significantly impact the quality of the isolated genomic DNA. Overloading typically causes an increase in processing time and carryover of contaminating RNA and protein through inefficient washing. While the DNA yield appears to have increased based on a high A_{260} value, it is often at the expense of functionality in downstream applications. With overloaded columns, A_{260}/A_{280} measurements typically show a value that is well below the ideal quality metric of 1.8.

Silica-based protocols for genomic DNA purification typically include washing with a buffer that contains ethanol.

Ethanol should be thoroughly removed prior to DNA elution because it can interfere with the use of purified genomic DNA in some applications. For example, residual ethanol can cause samples to "float" out of wells when loading a gel for electrophoresis and can inhibit some enzymatic reactions.

When using silica in a membrane-based column format: Carefully discard the flowthrough and the collection tube. If any of the ethanolic wash solution comes into contact with the bottom of the column, discard the flowthrough and recentrifuge for an additional 1 min. In addition, be sure to thoroughly air dry the sample, but do not overdry as DNA may irreversibly bind to the silica matrix.

Considerations when using anion exchange products

DNA is eluted from anion exchange medium in high salt; this is not compatible with most applications. Purified genomic DNA will need to be desalted using a desalting column or desalted and concentrated using isopropanol. See Appendix 4 for details on isopropanol precipitation.

Overloading or underloading anion exchange systems can lead to the same problems mentioned above for silica-based products.

Sample-specific considerations for genomic DNA preparation

See Chapter 2 for details on sample disruption methods.

Do not use RNase or proteases if RNA or protein, respectively, will also be isolated from the sample.

Animal tissue

The complete disruption and homogenization of animal tissue is critical. See Chapter 1 for further details on disruption and homogenization options. Liver and spleen are very active organs transcriptionally, so tissue samples derived from these organs have a very high RNA content. Therefore, these samples should be treated with RNase prior to column purification. Ensure that animal tissue is fresh or adequately frozen to ensure optimal quality. To process fixed samples, see the section on FFPE tissue later in this chapter.

Blood and blood fractions

Sample considerations

Genomic DNA can be isolated from whole blood, buffy coat, or bone marrow, with only WBC containing genomic DNA. Note that the number of WBC may increase in individuals with certain infections or diseases, so care should be taken not to overload the purification system. In addition, buffy coat and bone marrow have a higher nucleated cell count than whole blood has. Unique differences between species exist in the aggregation tendency of blood, and this can affect the efficient lysis and extraction of WBC. Both yield and purity of the end product will be determined by the total viable WBC count in the blood samples. If heparin is used as an anticoagulant, it should be thoroughly removed because it may inhibit some PCR amplifications (4). If blood is not mixed sufficiently with anticoagulant during blood drawing, it is possible for micro-clots to form; these may interfere with the speed of sample processing and quality of isolated DNA.

Although used often, frozen blood tends to be more viscous, and as a consequence can produce DNA of lower yield and quality as judged by spectrophotometry.

Hemoglobin removal

Extraction of genomic DNA from blood is challenging with regard to removal of hemoglobin. Any remnant coloration in DNA samples tends to indicate incomplete purification. The consequences of colored samples can range from interference with determination of DNA concentration by A_{260} to complete inhibition of enzymatic reactions. In practice, the starting volume of blood used for DNA preparation determines the choice of approach to limit the impact of trace hemoglobin. Small volumes of blood can be processed using a one-step total lysis procedure, while larger starting volumes rely on preferential lysis of heme-containing erythrocytes and washing of pelleted white cells.

Cultured mammalian cells

Cultured mammalian cells are simple to process for genomic DNA extraction. As with any other sample, the cells should be fresh.

To obtain good-quality genomic DNA, ensure that the extraction system is not overloaded and that cell counts are accurately measured.

Bacteria

Gram-positive bacteria have a thicker peptidoglycan layer than do Gram-negative bacteria. Therefore, it is necessary to pretreat Gram-positive bacteria with lysozyme to break open the cell wall. Incubation time required with lysozyme may vary between species.

Plant tissue and fungi

The complete disruption and homogenization of plant tissue is critical. See Chapter 2 for further details on sample homogenization. Leaves, stems, and pollen can be the starting material for plants, while both hyphae and spores can be used for fungi. Accordingly, there are a number of pretreatments including enzymatic treatment and physical disruption to maximize genomic DNA recovery. The cell wall in plants is made up of glycans and cellulose; that of fungi contains glycans and chitins. Enzymes such as lyticase and zymolyase are often used to improve DNA recovery and purity.

Insects

Total disruption using a hand-held homogenizer and standard lysis methods are suitable for most insects. Following lysis, centrifugation should be performed to remove particulate matter prior to loading onto columns. Care should be taken to consider the size of the insect and the potential for overloading columns, which may result in slow processing and poor quality of DNA if the capacity is exceeded.

Yeast

Treat with zymolase or lyticase and pellet spheroplasts prior to purification. Refer to instructions provided with products for this purpose or to reference 2 for further information.

Formalin-fixed, paraffin-embedded (FFPE) tissue

Tissue biopsies and cells can be prepared and maintained in paraffin wax blocks for archival purposes. Most protocols for genomic DNA isolation from these samples require that paraffin be removed prior to purification. Paraffin and water are immiscible, making aqueous extraction and enzymatic treatment inefficient. Two approaches can be taken, in which either xylene is used to dissolve the paraffin wax or heat is used to melt the wax, followed by recovery of cellular debris for standard genomic DNA extraction.

It is also possible to use WGA, via either PCR or strand displacement amplification (e.g., using Phi29 DNA polymerase) to circumvent the requirement for paraffin removal..

FFPE archive samples are often degraded or the genomic DNA fixed to proteins or lipids; both of these scenarios will limit their usefulness and the quality of the analysis.

Scarce or precious samples

DNA from FTA or Guthrie card dried blood spots, buccal swabs, and other scarce or precious samples can be amplified by boiling samples in water followed by standard PCR or WGA. The use of Phi29 DNA polymerase for sample preparation is described in Chapter 7. See Chapter 3 for isolation of genomic DNA from FTA and FTA Elute cards.

Genomic DNA purification using illustra genomicPrep Mini Spin Kits

illustra genomicPrep Mini Spin Kits from GE Healthcare are designed for the rapid extraction and purification of genomic DNA. The protocols have been designed to minimize shearing, resulting in high-quality genomic DNA that is compatible with most molecular biology techniques, including cloning, restriction enzyme digestion, PCR amplification, and genotyping applications.

The developed method uses detergents and a chaotropic agent to extract DNA, denature protein components, and promote the selective binding of DNA to the silica membrane contained in an illustra mini column (5, 6). Proteinase K is the protease of choice to digest protein from samples, because it is active even when enzyme inhibitors such as EDTA and detergents are present in samples. Denatured contaminants are easily removed by subsequent washing of the silica membrane with an ethanolic buffer. The purified genomic DNA is eluted in a low-ionic-strength

buffer at a concentration suitable for most downstream molecular biology applications. Purity is high—A_{260}/A_{280} readings are usually 1.7 to 1.8.

illustra tissue & cells genomicPrep Mini Spin Kit has been optimized to extract DNA from several tissue types such as liver, kidney, and mouse tails. Typical yields are 0.5 to 1.5 µg of genomic DNA per mg of tissue. Between 5 and 50 mg of tissue can be used per miniprep. Purified genomic DNA can be obtained in about 90 min.

illustra blood genomicPrep Mini Spin Kit is designed for use with whole blood, buffy coat, bone marrow, and nucleated RBC and can process in the range of 50 to 1000 µl of whole blood. Purified genomic DNA yields are typically between 4 and 6 µg from 200 µl of whole blood. Purified genomic DNA can be obtained in as little as 20 min.

illustra bacteria genomicPrep Mini Spin Kit has been optimized to extract genomic DNA from Gram-negative and Gram-positive bacteria with yields ranging from 4 to 12 µg of genomic DNA per purification. Bacterial numbers ranging from 1 to 4×10^9 cells in 1 ml can be used for each purification. Purified genomic DNA can be obtained in as little as 40 min.

A general protocol for genomic DNA purification using illustra genomicPrep Mini Spin Kits is provided below.

Materials

illustra mini columns, buffers, lyophilized Proteinase K, and collection tubes are provided with each kit

Water bath or heating block for 70°C incubation

RNase A (optional): 20 mg/ml

Dulbecco's PBS solution is required for tissue homogenization or cell resuspension and for blood samples < 200 µl

Homogenizer for animal tissue, for example, hand-held motor-driven homogenizer (Kimble, part no. 749540 or equivalent) with probe (part no. 749520-0090).

For blood samples > 300 µl

RBC lysis buffer is required. See Advance Preparation, below.

For Gram-positive bacteria only

Lysozyme buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.1 M NaCl, 5% Triton™ X-100)

Lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0)

Advance preparation

Dissolve Proteinase K in water.

Add ethanol to wash buffer.

Heat elution buffer.

For tissue samples: homogenize thoroughly.

RBC lysis buffer

This buffer is needed only for processing blood and/or blood fractions ranging from 300 to 1000 µl using the two-stage lysis method as described in the protocol for this volume range. See product instruction for Blood Mini Spin Kit for details.

10 mM $KHCO_3$

155 mM NH_4Cl

0.1 mM EDTA, pH 8

Filter sterilize using a 0.2 µm filter. The volume of RBC Lysis buffer required per purification is three times the volume of blood to be processed.

PBS

Dissolve the following in 800 ml of distilled H_2O:

8 g of NaCl

0.2 g of KCl

1.44 g of Na_2HPO_4

0.24 g of KH_2PO_4

Adjust pH to 7.4.

Adjust volume to 1 liter with additional distilled H_2O.

Sterilize by autoclaving.

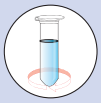
Alternatively, PBS can be purchased as a ready-made solution or as tablets for adding to water prior to autoclaving.

Protocol

Note: Columns and buffers are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure you use the correct columns and buffers for your purification.

1. Prepare sample

a. Homogenize animal tissue
Homogenize 5 to 50 mg of tissue in a small volume of PBS.



b. Resuspend mammalian cells
Resuspend cells in a small volume of PBS.

c. Harvest bacterial culture
Pellet bacterial cells (1 ml) and remove culture medium.

d. Prepare blood
For 50 to 300 µl of blood
For blood samples < 200 µl, dilute up to 200 µl with PBS. Proceed to step 2, lysis.

For 300 to 1000 µl of blood: We recommend a two-stage lysis of blood and its fractions. The first stage involves selective lysis of RBC, while WBC are pelleted and resuspended to 200 µl. In the second stage, nucleated cells are lysed to release DNA that is then purified from the silica membrane column. See product instructions for the Blood Mini Spin Kit for details.

2. Lyse cells

a. Animal tissue, cultured cells, and 50 to 300 µl of blood
Add detergent-containing lysis buffer and Proteinase K. Chaotropic salts in the lysis buffer break open the cells, and Proteinase K digests proteins. Incubate briefly at room temperature (for blood) or for 1 h with heat (for tissue and cells).

b. Gram-negative bacteria
Add two lysis buffers and Proteinase K in the correct sequence. Lysis buffers break open the cells via detergents and osmotic shock, and Proteinase K digests proteins.

c. Gram-positive bacteria
Add lysozyme buffer and lysozyme. After incubation, add Proteinase K.

3. Remove RNA (optional)

Add RNase and incubate at room temperature.

RNase treatment is strongly recommended for cultured mammalian cells as most laboratory-grown cells contain large quantities of RNA.

4. Bind genomic DNA

The chaotropic salt in the lysis buffer promotes selective binding of genomic DNA to the silica membrane. Denatured proteins are collected in the flowthrough following centrifugation.

Do not overload the column. The maximum volume that can be loaded is 720 µl.

5. Wash and dry

Wash the mini column with lysis buffer, followed by wash buffer to remove chaotropic salts and other contaminants.

6. Elute purified genomic DNA

Elute genomic DNA in a low-ionic-strength buffer containing EDTA or simply water if downstream applications are sensitive to EDTA.

For short-term storage, place genomic DNA at 4°C. For long-term storage, aliquot sample and store at -20°C. Do not subject samples to repeated freeze-thaw cycles.

Genomic DNA purification using illustra genomicPrep Midi Flow Kits

illustra genomicPrep Midi Flow Kits from GE Healthcare are designed for high-yield extraction and purification of genomic DNA from animal tissue and cultured mammalian cell lines (tissue & cells Kit) and blood/blood fractions (blood Kit). The entire procedure, including lysis, purification, and desalting, can be completed using a gravity-based or a spin-based method. Highly pure genomic DNA can be obtained with the spin protocol in as little as 3 h for the tissue & cells Kit and as little as 2 h with the blood Kit. Purified genomic DNA is of high molecular weight, quality, and purity, and is therefore compatible with most molecular biology techniques such as PCR amplification, restriction enzyme digestion, and DNA sequencing.

The kits utilize a simple and efficient process, employing a simple lysis procedure (7), purification using anion exchange column chromatography with an illustra Fast Flow Genomic column, and a trouble-free desalting process using an illustra NAP™-25 Desalting column. illustra Fast Flow Genomic columns, used for the purification step, contain an anion exchange chromatography medium with excellent flow characteristics, exceptional chemical and pH stability, and a high capacity for biomolecules. Genomic DNA, but not potential impurities, binds to the anion exchange medium at high salt concentrations. The DNA is subsequently eluted from the column in a buffer containing higher salt concentrations. illustra Fast Flow Genomic columns are disposable, single-use, and robust for either gravity flow or centrifugation protocols. They are supplied pre-equilibrated and have excellent flow characteristics, resulting in reduced hands-on and total process time. The buffer used for pre-equilibration has been optimized to prevent the majority of impurities from binding to the medium, leading to higher purity and yield of genomic DNA. A desalting step based on illustra NAP-25 columns is provided in the kit to avoid the need for isopropanol precipitation.

illustra cells & tissue genomicPrep Midi Flow Kit has been optimized for the purification of genomic DNA from up to 200 mg of animal tissue or up to 2×10^7 cultured mammalian cells. The typical yield is 0.75 to 1.5 µg of DNA per milligram of tissue.

illustra blood genomicPrep Midi Flow Kit is designed for the purification of genomic DNA from 1 to 8 ml of blood. The typical yield from 5 ml of human blood is 80 to 125 µg.

A general protocol for genomic DNA purification using illustra genomicPrep Midi Flow Kits follows.

Materials

illustra purification columns and desalting columns, buffers, lyophilized Proteinase K, and adapters (for centrifugation) are provided with each kit

Optional for gravity protocol: LabMate™ buffer reservoir – (GE Healthcare, product code 18-3216-03)

Required for the spin protocol: Centrifuge equipped with a swing-out rotor and buckets for 50 ml centrifuge tubes

Optional for final concentration step: Isopropanol

For tissues and cells

Dulbecco's PBS solution is required for tissue homogenization or cell resuspension

Water bath or heating block for 60°C incubation

RNase A: 20 mg/ml

For isolating genomic DNA from high densities of cells: Steriflip™ 0.2 µm filter (Millipore, SCGP 00525) or equivalent (requires a vacuum source)

Recommended homogenizer for genomic DNA purification from animal tissues: Hand-held motor-driven homogenizer (Kimble, part no. 749540 or equivalent) with probe (part no. 749520-0090)

Advance preparation

Dissolve Proteinase K in water.

Use water to dilute the elution buffer provided for the desalting step.

For tissue samples: Homogenize thoroughly.

For blood: Prechill the lysis buffers and the DNase-free water.

Prepare PBS: See the recipe in Advance Preparation for Mini Spin Kits, above.

Protocol

Note: Columns and Buffers are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure you use the correct columns and buffers for your purification.

Note: This procedure may be performed by gravity flow or centrifugation. The gravity flow procedure will take slightly longer but will increase the yield.

1. Prepare sample

a. Homogenize animal tissue
Homogenize 100 to 200 mg of tissue in PBS.

b. Resuspend mammalian cells
Rinse up to 2 × 10^7 cells with PBS, then resuspend in TE buffer (provided).

c. Isolate white blood cells
Add red blood cell lysis buffer and incubate briefly at room temperature. Centrifuge to pellet the WBC. RBC are selectively lysed by osmotic pressure.

2. Lyse cells/prepare illustra Fast Flow Genomic column

a. Lyse WBC

Add white blood cell lysis buffer and Proteinase K to lyse the cells and digest proteins. Incubate briefly at room temperature. During the incubation, equilibrate the anion exchange column. WBC are lysed by detergent.

b. Lyse tissue cells or cultured cells

Add lysis buffer and Proteinase K. Lysis buffer breaks open the cells, and Proteinase K digests proteins. Incubate for 1 to 1.5 h with heat. Add RNase A and incubate briefly. Dilute tissue samples with high-salt buffer. High-density cell culture samples will require filtration. During the RNase step, equilibrate the anion exchange column. Cells are lysed by detergent.

3. Purify genomic DNA

Add sample to anion exchange column. Genomic DNA will bind to the anion exchange medium under high-salt conditions, while other contaminants will not. Add additional loading buffer to complete sample loading and to wash away contaminants.

4. Elute genomic DNA/prepare illustra NAP-25 Desalting column

Add high-ionic-strength buffer to elute genomic DNA from the anion exchange medium. Equilibrate the desalting column.

Column equilibration can be conveniently accomplished using LabMate PD-10 buffer reservoir. If the buffer reservoir is maintained only for equilibration of the desalting column, the LabMate accessory is reusable.

It is critical to equilibrate the NAP-25 column because UV-absorbing stabilizers are used in column packing.

5. Desalt genomic DNA

Load eluted genomic DNA onto the prepared desalting column.

6. Elute genomic DNA ready for use

Genomic DNA is eluted in a low-ionic-strength buffer. If highly concentrated DNA is required, an optional isopropanol precipitation may be performed. See Appendix 4.

For short-term storage, place genomic DNA at 4°C. For long-term storage, aliquot sample and store at -20°C. Do not subject samples to repeated freeze-thaw cycles.

Genomic DNA isolation using illustra Nucleon Genomic DNA Extraction Kits

illustra Nucleon DNA extraction kits from GE Healthcare were developed around the proprietary Nucleon resins that bind proteins and polysaccharides, forming a semi-solid barrier during partitioning. High-quality, high-molecular-weight genomic DNA is obtained from the upper aqueous phase. Nucleon kits have been developed for hard tissue and paraffin-embedded tissue, blood, and plant tissue. Refer to product instructions for details.

Downstream applications

The following table summarizes the general quantity and purity considerations for several common applications for genomic DNA.

Table 4.3. Summary of downstream applications for genomic DNA and substances that may interfere.

Application	Quantity required	Potential interfering contaminants
Restriction enzyme digestion	0.5 to 10 µg	Salts, for salt-sensitive enzymes Organic solvents RNA (if gel analysis is performed); use RNase
Analyses using modifying enzymes (e.g., ligation and cloning)	10 µg	Salts Organic solvents EDTA for some modifying enzymes RNA (if gel analysis is performed); use RNase
Cycle sequencing	< 1 µg	Salts Organic solvents EDTA Nucleases
PCR amplification	5 to 50 ng for human genomic DNA	EDTA; $MgCl_2$ concentration can be increased proportionally to compensate Organic solvents Ionic detergents (e.g., SDS and Sarkosyl) Heparin from heparinized blood samples Hemoglobin
Amplification-based SNP genotyping	10 to 50 ng	Same as for PCR amplification, above
Array-based genotyping	0.5 to 1 µg	Same as for PCR amplification, above

Restriction enzyme digestion

Digestion of genomic DNA with restriction enzymes is often performed to prepare the DNA for subsequent manipulation. Between 0.5 and 10 µg of DNA is typically used in a restriction enzyme digest. Genomic DNA may be digested with a rare cutter (e.g., NotI) to generate large fragments, with a common cutter to generate small fragments, or with specific combinations of restriction enzymes.

Salts, organic solvents, and RNA can interfere with restriction enzyme digestion or analysis of digested fragments.

Cloning

The classical technique for cloning of genomic DNA fragments into a plasmid or other vector involves digestion with one or more restriction enzymes followed by enzymatic ligation of the fragments to the linearized vector. Digestion with rare cutters (e.g., NotI) produces large fragments for cloning into YACs and BACs. Digestion with frequent cutters produces smaller fragments that can be cloned into plasmids. When the goal is to prepare random DNA fragments, this is referred to as "shotgun" cloning. For looking at repeats and expansions in genomic DNA, it might be necessary to generate large insert libraries for efficient sequence assembly. The use of silica and anion exchange media for genomic DNA preparation typically does not produce fragments > 50 kb. Therefore, careful consideration is needed to design procedures that may include isolation of digested genomic DNA from gel plugs and size fractionation using PFGE or Contour-clamped homogeneous electric field (CHEF) systems.

Cloning may also be performed using PCR-based techniques. These usually use PCR to amplify genomic DNA fragments prior to ligation to recipient vectors, in which specific point mutations can be introduced to alter function.

Cloned products are typically used for sequencing, hybridization mapping, site-directed mutagenesis, and transcriptional and translational control of gene expression. Other uses are overexpression of specific proteins (with or without tags), as well as transfection experiments to study the structure and function of known proteins.

Sequencing

After cloning and propagation of clones in an appropriate host, DNA may be sequenced. In general, sequencing reactions are very robust and rarely fail to generate at least some data. While there are a number of issues related to DNA sequence composition that may affect base calling, complete failure of reactions is rare.

DNA quality can have an impact on overall performance. Often, simply overloading cycle sequencing reactions with template DNA can have a negative impact. Therefore, we recommend that you accurately determine the concentration and mass of DNA prior to sequencing.

Typically, if sequencing results are poor but genomic DNA amplification has been successful as judged by gel visualization, the consensus is that contamination and inhibition from the genomic DNA are not contributing to the poor results; poor results may be attributable to primers or other reagents used in the sequencing reactions.

Typical sequencing results using illustra triplePrep Kit, which can isolate genomic DNA, total RNA, and total denatured protein from a single undivided sample, or DNeasy™ Kit are shown in Figure 4.2. High Phred 20 scores indicate the high quality of genomic DNA for applications such as PCR and sequencing. See Chapter 10 for more information on triplePrep Kit.

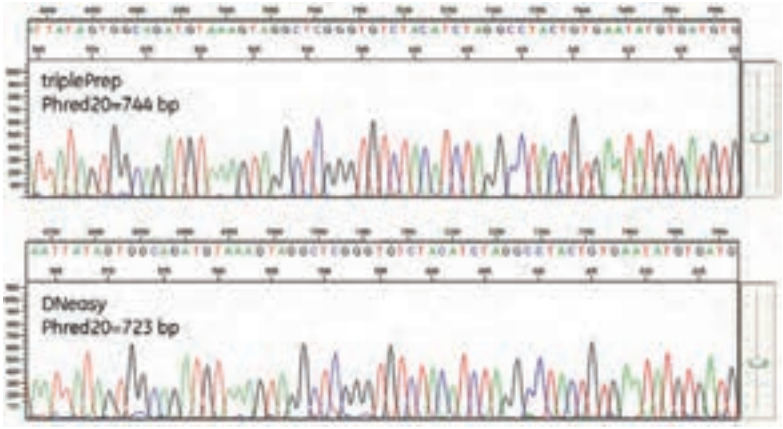


Fig 4.2. Sequencing of a PCR product produced from genomic DNA isolated using illustra triplePrep Kit or DNeasy.

PCR amplification

PCR is a very powerful technique that can be used to prepare fragments of genomic DNA for subsequent applications including sequencing, cloning, and genotyping. If subsequent analysis is to be automated, fluorescent dyes are typically included during PCR. Typical amounts of human genomic DNA used in PCR reactions range from 5 to 50 ng. Of interest to oncologists is the surveillance of tissues and cells for genes associated with cancer that may have been amplified or deleted during the development of a tumor. Methods have been developed that

allow unbiased identification of genomic copy changes by a balanced-PCR process that takes into account PCR saturation and impurity differences in samples when comparing diseased versus normal cells (8). The single-tube procedure used by Wang *et al.* allows application of balanced-PCR to genomic DNA obtained from low numbers of cells with relevance to array-CGH and real-time PCR quantitation. The same procedure can be used for modestly degraded DNA obtained from paraffin-embedded tissue. See earlier in this chapter under FFPE tissue.

Although only small amounts of genomic DNA are necessary for PCR amplification reactions, contaminants can inhibit PCR reactions. These contaminants include hemoglobin or heparin in genomic DNA samples from blood, as well as trace detergents introduced during sample preparation. Poor DNA quality can lead to poor results in PCR.

To minimize DNA degradation from endogenous nucleases, samples should be collected and stored correctly. See Chapter 2 for details. In addition, care should be taken to avoid overloading purification systems, as discussed earlier in this chapter.

In the experiment shown in Figure 4.3, 11 kb amplicons were successfully amplified, and no inhibitory effects were seen with genomic DNA purified using either genomicPrep or QIAamp™. The results demonstrate that the large size of genomic DNA isolated using illustra bacteria genomicPrep Mini Spin Kit makes it suitable for long PCR.

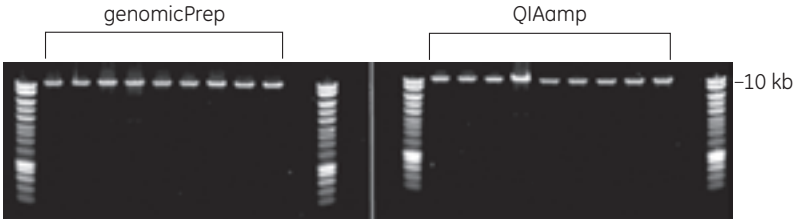


Fig 4.3. Amplification of an 11 kb amplicon from purified bacterial genomic DNA. Five microliters of the eluted genomic DNA solution, corresponding to 50 to 150 ng of the genomic DNA, was used per reaction. Following PCR, equal volumes from each reaction were resolved on a 0.8% agarose gel stained with ethidium bromide. M = 1 kb molecular weight marker.

In another experiment, several genomic DNA samples isolated from human whole blood with Genomic-tip 100/G or illustra blood genomicPrep Midi Flow Kit were evaluated in real-time PCR experiments (Fig 4.4). All samples displayed very similar amplification curves, demonstrating the reproducible quality of the isolated genomic DNA and its compatibility for use in real-time PCR applications.

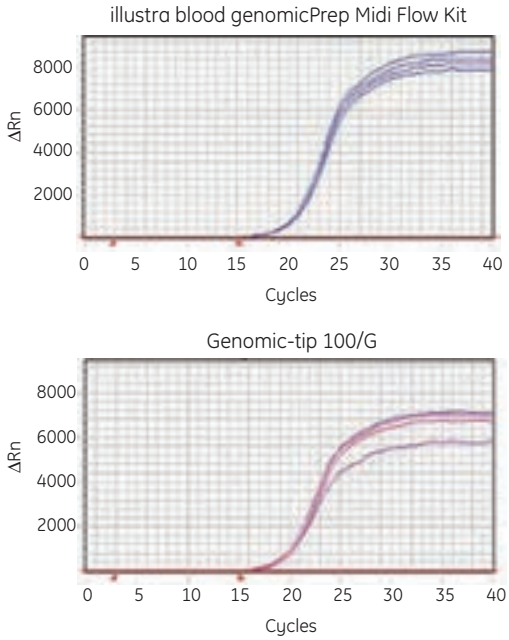


Fig 4.4. Real-time PCR amplification from genomic DNA extracted from 5 ml human whole blood using illustra genomicPrep Blood Midi Flow Kit and Genomic-tip 100/G from Qiagen. Genomic DNA was isolated according to manufacturers' instructions. One hundred ng of genomic DNA was used as template. Chromosome 2 forward and reverse primers were used.

Genotyping

The term genotyping refers to sets of tests that reveal genetic identity and how some traits are inherited from parents. Changes in genomic DNA can correlate with lost or reduced protein function, leading to a measurable change in a cellular biomarker or phenotype. Powerful genotyping tests can be used in forensic identity, disease gene mapping, pharmacogenomics, evolution, and the study of population dynamics. Genetic variation can exist as single-base changes, deletions, insertions, repeated elements, rearrangements, and other sequence modifications such as methylation.

Genotyping has evolved from the simplest tests where DNA fingerprinting was carried out on gels to study RFLP to the more contemporary approaches that use highly parallelized methods and automated detection systems. While probe sets can be developed to reveal a single-base change in a single gene, investigators are frequently interested in revealing many SNP variations across a single genome. Single nucleotide variation has been characterized for many genes and loci within the human genome, accounting for more than 1.5 million SNPs. The International HapMap Project is creating a more detailed, global view of SNPs and their grouping into haplotypes. As methods have developed, it has become possible to do genome-wide association studies to reveal individual SNPs and haplotypes that are correlated with disease susceptibility. Ultimately, mutations can be checked for authenticity by resequencing regions of genomic DNA samples.

Genomic DNA sample preparation is key to achieving optimal results in different genotyping analyses. Individual genotyping tests based on amplicon probes might consume 10 to 50 ng of genomic DNA and require sets of sequence-specific primers and probes. Chips for aCGH and SNP are available that cover hundreds of thousands of allelic variants. Each chip costs ~USD1000 and can report global changes in DNA copy number and sequence-specific variation yet consumes no more than 0.5 to 1 µg of genomic DNA. When very limited amounts of DNA are available it is possible to use WGA to provide sufficient starting material.

Quality factors that can affect the ability to use genomic DNA for genotyping include the degradation state of samples, purity level, and whether samples are contaminated with exogenous DNA.

Elimination of cross-contamination of amplified samples is very important to avoid misinterpretation and the creation of false positives. A number of articles have been published that describe isolation tactics and workflow to minimize cross contamination (9).

An example of genotyping results is shown below. Blood samples from 10 individuals were collected onto Whatman FTA Elute matrix and processed for genotyping analysis of the UGT2B15*2 gene. The samples were run in sets of four replicates and plotted as a function of the fluorescence of the two dyes HEX and FAM. Figure 4.5 shows a clear distinction between heterozygous (wt/mut) and homozygous (mut/mut or wt/wt) samples. These types of data demonstrate that FTA Elute yields a very clean DNA template that is reproducible and excellent for use with the highly sensitive Scorpions ARMS technology.

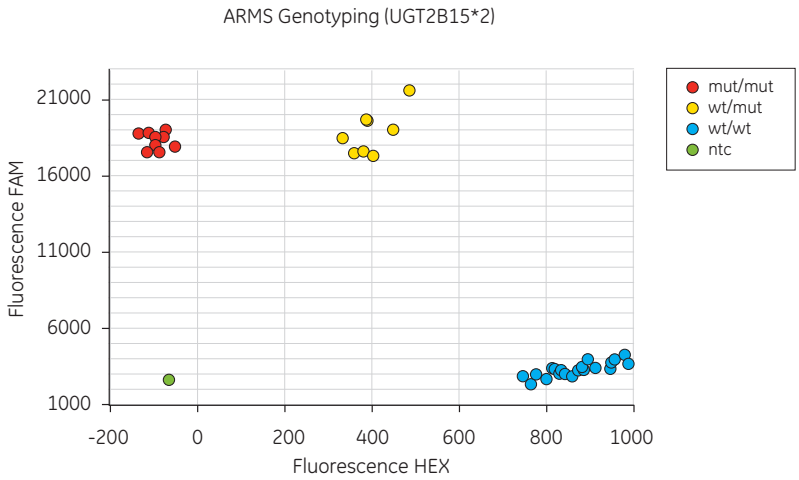


Fig 4.5. Genotyping was performed on four replicates each of 10 different DNA samples purified on FTA Elute. This assay was performed on a Stratagene Mx3000P™ Real-time PCR machine. Scorpions ARMS assays use the specificity of ARMS combined with the signaling of Scorpions probes to generate data. Fluorescence readings were taken pre- and post-PCR. NTC refers to no template control.

See Chapter 3 for additional information on Whatman FTA Elute.

Troubleshooting

Some of the more common problems that can occur during genomic DNA preparation are described in Table 4.4. Refer to the instructions provided with your genomic DNA preparation product for more detailed troubleshooting information.

Table 4.4. Possible causes and solutions for problems that may occur during genomic DNA preparation.

Problem	Possible cause	Solution
Quality of genomic DNA is poor (genomic DNA is not high molecular weight or looks degraded)	Starting sample was not stored properly.	Nucleases may have degraded genomic DNA. Follow recommendations for storage and handling of your sample type.
	Starting sample was fixed with formalin or another fixative (e.g., FFPE tissue).	Genomic DNA may be fragmented in the sample. It is not possible to obtain high-molecular-weight DNA from this sample type.
	Purified genomic DNA was not stored properly.	Follow recommended storage conditions to minimize degradation by nucleases. Do not store DNA in water for extended periods.
DNA yield is low	*If the yield is low and purity/quality is good:*	
	Starting sample size was insufficient.	Limited samples such as buccal swabs, FTA/Guthrie card blood, etc. may require amplification of genomic DNA using Phi29 DNA polymerase.
	If yield and purity/quality are low:	
	Starting sample was not stored properly.	Nucleases may have degraded genomic DNA. Follow recommendations for storage and handling of your sample type.
	Cells were not lysed thoroughly.	Follow homogenization/lysis recommendations for your sample type.
A_{260}/A_{280} of genomic DNA sample is < 1.7 or > 1.9	*A_{260}/A_{280} < 1.7:* Proteins or chaotropes may be present.	Process starting sample according to recommended instructions to ensure thorough removal of proteins. Do NOT overload the purification system. To remove protein contaminants, treat the sample with a protease or perform a phenol extraction + ethanol precipitation. Ensure wash steps are followed closely to eliminate carryover of contaminants into your final sample.
	A_{260}/A_{280} > 1.9: RNA may be present.	Follow recommendations for processing different sample types. Some sample types contain high levels of RNA. RNA may not interfere with some applications.
DNA does not work well in downstream application	Genomic DNA quality is poor.	See troubleshooting tips above for poor-quality genomic DNA.
	If DNA quality is good:	
	Contaminants (lipids, salts, etc.) may be present in the sample.	Do not overload the purification system. This may result in poor yield and/or decreased purity of genomic DNA. If organic solvents such as phenol were used, residual solvent may be present in the sample. Precipitate the DNA with salt and alcohol. See Appendix 4.
	For silica purification using ethanolic wash buffer:	
	Residual ethanol remains in the sample.	Be sure to thoroughly air dry the sample prior to elution.

References

1. Detter, J. C. *et al.* Isothermal strand-displacement amplification applications for high-throughput genomics. *Genomics* **80**, 691–698 (2002).
2. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A laboratory manual,* Cold Spring Harbor Laboratory, 1st and 2nd eds. (2001).
3. Gerstein, A. S., ed., *Molecular Biology Problem Solver: A Laboratory Guide,* Wiley-Liss, Inc. (2001).
4. Beutler, E. *et al.* Interference of heparin with the polymerase chain reaction. *BioTechniques* **9**, 166 (1990).
5. Marko, M. A. *et al.* A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. *Anal. Biochem.* **121**, 382–387 (1982).
6. Melzak, K. A. *et al.* Driving forces for DNA adsorption to silica in perchlorate solutions. *J. Coll. Interf. Sci.* **181**, 635-644 (1996).
7. Aljanabi, S. M. and Martinez, I. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. *Nucl. Acids Res.* **25**, 4692–4693 (1997).
8. Wang, G. *et al.,* Balanced-PCR amplification allows unbiased identification of genomic copy changes in minute cell and tissue samples. *Nucleic Acid Res.* **32**, e76 (2004).
9. Mifflin, T. E. Setting up a PCR Laboratory, in *PCR Primer: A Laboratory Manual,* 2nd edition (Dieffenbach, C. W. and Dveksler, G. S., eds.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 5–14 (2003).

Chapter 5
Plasmid DNA preparation

Plasmid DNA preparation is part of the workflow for cloning genomic DNA or cDNA fragments. Molecular cloning in bacteria is used to produce large amounts of a DNA fragment. Bacterial cloning exploits the rapid growth of bacterial cells and the ability to clone foreign DNA into self-replicating DNA molecules. In bacterial systems, DNA fragments may be cloned into plasmids, vectors from M13 or lambda phage, cosmids, or BACs. Eukaryotic vectors such as YACs and baculovirus vectors are used to clone larger fragments of DNA and to obtain recombinant proteins with post-translational modifications. The remainder of this chapter will discuss preparation of plasmid DNA.

Quantity, purity, and quality of extracted plasmid DNA

The required quantity, purity, and quality of plasmid DNA should be considered when selecting a preparation method. The purity and quality of extracted plasmid DNA can be assessed by several methods including spectrophotometry, in which the A_{260}/A_{280}, A_{260}/A_{230}, and A_{340} measurements are used to monitor protein/RNA, salt, and particulate contamination, respectively. RNA contamination is less of an issue for plasmid DNA purified using commercially available kits, as these routinely contain RNase. The percentage of supercoiled plasmid DNA present can also be determined; this can be used as a broad indication of the amount of damage incurred by the DNA during the extraction procedures. This determination is especially important for plasmid DNA that is to be used in transfection experiments. In general, a higher percentage of supercoiled plasmid DNA is generated using midi- and maxi-scale kits, as these typically involve more gentle protocols and procedures, such as gravity flow, for loading plasmid DNA on the purification medium.

Some molecular biological applications may tolerate a high degree of contaminants. For example, PCR is routinely used during the cloning process to screen bacterial colonies for the desired recombination event. Single bacterial colonies are used as the template source in the PCR reaction without prior isolation or purification of plasmid DNA. However, other techniques such as mammalian cell transfection require plasmid DNA of higher purity, as even small quantities of endotoxin (LPS derived from the bacterial outer membrane) may have an adverse effect on mammalian cell viability. Restriction enzyme digestion is typically fairly tolerant of impurities; however, some restriction enzymes (e.g., HindIII and SacI) are particularly sensitive to contaminating salt that may be introduced during purification from either residual growth medium or carry over of purification buffer.

Table 5.1. Summary of possible contaminants in plasmid preparations and applications in which those contaminants may interfere.

Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
Bacterial genomic DNA	Host bacteria	PCR	Primers may anneal to genomic DNA; this may cause extra bands in PCR Contamination with genomic DNA may require the use of more stringently designed primers and optimal PCR conditions
RNA	Host bacteria	Action of restriction and DNA modifying enzymes Transcription	May interfere with gel analysis of fragments
Nucleases	Host bacteria expressing endonuclease A (EndA$^+$)	Action of restriction and DNA modifying enzymes Transcription	Nucleases degrade plasmid DNA
Carbohydrates, particularly endotoxins	Host bacteria (EndA$^+$ host strains)	Action of restriction and DNA modifying enzymes Transcription	Endotoxin is cytotoxic to mammalian cells
Residual salts	May be introduced during plasmid preparation	Action of restriction and DNA modifying enzymes Transfection	Inhibit enzyme activity (e.g., restriction enzymes HindIII and SacI)
Organic solvents such as alcohols and phenol/ chloroform	May be introduced during plasmid preparation	Residual organic solvent contaminants will interfere with the majority of routine molecular biology techniques	Inhibit enzyme activity

Techniques for plasmid DNA preparation

When choosing a method for purifying plasmid DNA from bacteria, the intended downstream application should be considered. DNA yield, quality, and purity are important factors for consideration. Plasmid DNA isolation kits are available from numerous suppliers. These offer a diverse range of kits based on differences in scale, purity, quality, and methods of extraction. A simple flowchart for the preparation of plasmid DNA is shown in Figure 5.1. Harvesting of bacterial cells is typically done via centrifugation. Pelleted cells are lysed, then plasmid DNA is preferentially bound to specific purification materials, for example, anion exchange or silica resins or membranes. Subsequent washing steps remove contaminants such as carbohydrates and proteins. Finally, purified plasmid DNA is eluted from the membrane or medium.

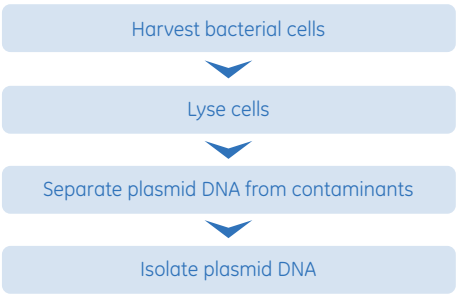


Fig 5.1. Steps in preparing plasmid DNA for downstream applications.

Bacterial cells are typically harvested by centrifugation. Lysis of the host bacteria has in the past been achieved by boiling (1) or exposure to alkali (2–4). Alkaline lysis methods typically use three solutions and centrifugation. In the basic procedure, bacterial cells are harvested by centrifugation and resuspended in a buffered glucose solution. An alkaline solution containing SDS is added. SDS helps to solubilize the cell membrane, and DNA and proteins are denatured in the alkaline environment. An acidic salt solution (typically containing potassium acetate) neutralizes the pH, and the DNA selectively renatures. The small circular plasmid DNA reanneals, but genomic DNA is too large to reanneal correctly. Potassium dodecyl sulfate (KDS), denatured genomic DNA, proteins, and salts, form a white precipitate, which is removed by centrifugation.

Following cell lysis, a simple isopropanol or ethanol DNA precipitation yields a crude plasmid preparation that is of sufficient purity for many enzymatic treatments such as digestion with restriction enzymes. Further purification classically entails RNase treatment, phenol/chloroform extraction, and additional rounds of precipitation for a small-scale preparation, or CsCl ultracentrifugation for a larger scale. Cationic salts such as CTAB form insoluble complexes with nucleic acids and can be used to precipitate plasmids. PEG can also be used to generate clean plasmid DNA. Gel filtration chromatography offers yet another alternative for purifying plasmid DNA and is especially useful for large-scale purification of plasmid DNA in conjunction with CsCl ultracentrifugation.

Purification using anion exchange or silica is a quicker alternative to lengthy precipitations and typically generates plasmid DNA of high purity. Many commercially available plasmid DNA purification kits are based on anion exchange or silica in a membrane or bead format. In anion exchange chromatography, DNA binds to the column matrix in high-salt buffer and is subsequently eluted with a higher-salt buffer. Purified DNA is typically of high purity and quality. The advantage of this technology is that few proteins bind to anion exchange media at high ionic strength, so protein removal is relatively complete. The disadvantage to ion exchange is that salt concentrations of up to 2 M are required to elute the DNA, which must then be desalted. Silica purification can also be utilized to isolate plasmid DNA but at marginally lower quality and purity. This technique offers advantages to anion exchange because the sequence of buffer additions is reversed. High-ionic-strength buffer binds the plasmid to the matrix, and low-ionic-strength buffer releases it. Because the sample is eluted in low-ionic-strength buffer, the isolated plasmid DNA can often be used directly in subsequent applications.

Mini-scale (-prep) plasmid DNA isolation kits are designed for the rapid purification of plasmid DNA from 1.5 to 3.0 ml culture volumes of transformed strains of the Gram-negative bacteria *E. coli*. Generally, the extractions are simple, quick, and allow the processing of multiple samples. The plasmid yield from a 1.5 ml culture of a freshly grown *E. coli* strain containing a high-copy-number plasmid (>300 copies per cell) and grown to an A_{600} of ~2.5 is typically up to 10 µg. The purity and quality of plasmid DNA is compatible with many common molecular biology techniques, including cloning, enzymatic modification (i.e., using restriction enzymes, nucleases, kinases, methylases, etc.), PCR amplification, DNA sequencing (both manual and automated),

and others. In addition, several mini-scale kits allow the isolation of plasmid DNA that is of sufficient quality and purity to facilitate the transfection of robust mammalian cell lines, such as HEK293, at acceptable efficiencies.

When plasmid DNA is required either in greater quantities or at higher levels of purity or quality, extractions are normally performed using midi- or maxi-scale purification kits. Commercially available midi- and maxi-scale extraction kits are designed for the purification of plasmid DNA from 25 to 150 ml *E. coli* cultures, facilitating the extraction of 100 to 250 µg of plasmid DNA depending on strain and plasmid copy number. The plasmid DNA extracted by these kits is typically of sufficient purity and quality for the transfection of the majority of cell lines, including primary cells.

The purification principles for mini- and midi-/maxi-scale protocols are comparable in terms of workflow but are achieved by fundamentally different chemistries. Typically, mini-scale extractions are based on a modified alkaline lysis procedure in combination with silica-based membranes. Genomic DNA, KDS, and denatured cellular debris are removed by centrifugation. Chaotropic salts are included not only to denature protein components but also to promote the selective binding of plasmid DNA to the silica membrane. The denatured components are easily removed by subsequent washing, and purified plasmid DNA is eluted in a low-ionic-strength buffer. Traditionally, this procedure is performed using a small-scale laboratory microcentrifuge at 16 000 × g, and the entire purification process is completed in approximately 10 min.

Midi-/maxi-scale plasmid DNA purifications are also generally based on an alkaline lysis procedure, but chaotropic salts are not included and the DNA purification is achieved using anion exchange-based media or membranes. Plasmid DNA binds in high-salt conditions, and impurities such as RNA, cellular debris, and protein are removed through successive high-salt washes. The purified plasmid is eluted from the anion exchange material at a higher salt concentration. The plasmid DNA is then concentrated and desalted using isopropanol precipitation and a 70% ethanol wash. Because plasmid DNA binds at a high salt concentration, binding of potential impurities is minimized. The midi-/maxi-scale purification procedures are traditionally based on the gravity-assisted flow of buffers through columns. The gravity flow system represents a gentler purification procedure compared with those using centrifuge-based systems.

The different techniques for plasmid DNA preparation are summarized in Table 5.2. See reference 5 for more information on techniques for plasmid DNA preparation.

General considerations for plasmid DNA preparation

The yield and purity of isolated plasmid DNA will be influenced not only by the method of isolation, that is, whether a commercially available purification kit is used, but also by a number of external factors, for example, culture cell density, duration of growth, medium used, the type of plasmid (high or low copy number), the size of the insert, and the host strain used.

Factors affecting plasmid DNA yield and purity

Cell density

Excessively high *E. coli* cell density is the most important factor resulting in the poor yield and purity of plasmid DNA. Cultures grown to an extremely high density (A_{600} > 5) can overload both the mini- (1 to 3 ml) and midi-scale (~50 ml) purification systems. If high cell densities are obtained, you should process smaller culture volumes to ensure no deleterious effect on plasmid recovery and purity. The A_{600} of an overnight culture of the *E. coli* strain TOP10 (transformed with a high-copy-number plasmid, >300 copies/cell) and grown in LB medium is approximately 2.5. Note: LB is a nutritionally complex medium, primarily used for the growth of bacteria. Adjust salt levels as appropriate for the bacterial strain, culture conditions, and salt sensitivity of the antibiotic used.

Table 5.2. Advantages and disadvantages of different techniques for plasmid DNA preparation.[1]

Technique	How it works	Advantages (+)/ Disadvantages (-)
Methods based on alcohol precipitation (may include phenol/chloroform extraction, RNase treatment)	When cations (e.g., Na^+ or K^+) are present at a sufficient concentration to counteract the repulsion caused by the negative charges of the DNA backbone, DNA becomes insoluble in aqueous solutions and is precipitated. DNA is recovered by centrifugation.	+ Inexpensive - Plasmid DNA may be of low purity, particularly if phenol/ chloroform extraction and RNase treatment is not included.
Methods using CsCl	In the CsCl method, supercoiled plasmid DNA is separated from linearized and "open circle" plasmid DNA due to different buoyant densities. Ethidium bromide intercalates into plasmid DNA, allowing visualization.	+ Extremely high purity + Good for scale-up - Low throughput - Requires access to ultracentrifuge and overnight centrifugation
Silica purification; usually in membrane or bead format	Plasmid DNA binds to silica in the presence of high concentration of chaotropes; DNA is eluted at low ionic strength.	+ DNA is eluted in low-ionic-strength buffer, ready for use + Amenable to increased throughput - May not remove sufficient endotoxins to obtain good transfection efficiencies with sensitive cell lines
Anion exchange chromatography; membrane or medium format	Negatively charged plasmid DNA binds to positively charged medium. DNA is eluted in high-ionic-strength buffer as anions displace bound plasmid DNA.	+ Few proteins bind, so protein removal is relatively complete + Effective removal of endotoxins - DNA is eluted in high salt and must be desalted before most applications
Gel filtration	Supercoiled plasmid DNA can be separated from contaminants by size selection.	+ Good for large scale - Low throughput
Amplification using Phi29 DNA polymerase (see Chapter 7)	Phi29 DNA polymerase can be used to increase the amount of plasmid DNA. This is achieved by RCA using nonspecific primers.	+ Good when limited starting template material is available + Amenable to automation - Poor-quality input plasmid DNA may give poor-quality output

[1] *Most techniques use a variation of alkaline lysis to lyse bacterial cells.*

Growth conditions

Specific factors that affect culture growth, and ultimately the density of the culture, are listed below.

Inoculation

To inoculate growth medium for 1 to 3 ml cultures, use a fresh single transformed *E. coli* colony from an agar plate containing the appropriate antibiotics. For midi-prep scale, a small-scale starter culture (1 to 3 ml) is normally initiated as described above. After several hours of growth, this is used to inoculate a larger culture volume (> 50 ml).

Culture medium

When incubated for an equivalent period of time, cultures grown in enriched media such as 2× YT and TB tend to give cell densities that are significantly higher than those achieved with LB medium; therefore, the use of such enriched media when growing cultures for purifying plasmid DNA must be carefully considered in terms of cell density.

Aeration

Cultures should be well aerated during growth. When growing cultures in a 30 ml universal container, no more than 3 ml of medium should be used. Aeration will be poor if cultures are grown in multiwell plates or in 1.5 or 2.0 ml microcentrifuge tubes. Poor aeration will lead to poor culture growth, and subsequently to low yields of plasmid DNA. The problem of aeration is not as significant an issue when isolating plasmid DNA from midi-scale cultures, as these tend to be grown in large-volume flasks. However, a good "rule of thumb" is that the surface of the culture medium should be located near the widest part of the culture vessel to ensure that the maximum air to surface ratio is obtained.

Plasmid copy number

For a given length of incubation and a given culture medium, low-copy-number plasmids will give lower yields than high-copy-number plasmids.

Size of insert

In general, the larger the size of the insert, the lower the yield of plasmid DNA from a given culture medium.

Host strain

Strains that grow poorly or contain large amounts of nucleases or carbohydrates should be avoided. HB101 and its derivatives express endonuclease A (EndA$^+$), which if not inactivated can digest plasmid DNA. These strains may also release carbohydrates that can inhibit restriction digests (5). *E. coli* strains DH5α and TOP10 are EndA$^-$ and therefore do not express an active nuclease. These strains facilitate the extraction of high-quality plasmid DNA.

Length of incubation

For cultures grown in an enriched medium (e.g., 2× YT or TB), the length of the incubation time should not exceed 12 h. Cultures in LB medium should be grown for at least 9 h to obtain sufficient cell mass for processing. Cultures (in any medium) should not be grown for more than 16 h, due to increased rates of cell death, which will affect the yield and quality of extracted plasmid DNA.

Tips for plasmid DNA preparation

Culture preparation

To obtain good aeration, use a sterile tube or flask with a volume of at least four times the volume of the culture. Choose a suitable tube or flask that facilitates the generation of the largest culture medium to air surface interface.

If purifying a high-molecular-weight or low-copy-number plasmid, process twice the recommended culture volume.

Do not process more than the recommended culture volume when the culture was grown in enriched media or when a high-copy-number plasmid was used. If using enriched media, measure the culture growth (A_{600}) and dilute to ≤ 2.5. This is especially important to prevent the blockage of purification columns used in centrifuged-based plasmid miniprep extractions (1.5 to 3.0 ml culture volumes).

To thaw frozen, pelleted cells: Defrost thoroughly at room temperature for 5 to 15 min prior to use. Do not thaw for more than 15 min, as endogenous enzyme activity can affect plasmid yield and purity.

Alkaline lysis procedures

Resuspension: Cell resuspension can be achieved by gentle vortexing or by pipetting up and down. For mini-scale extractions, cells pelleted in microcentrifuge tubes can be resuspended by simply running the tube along the holes of an empty pipette tip box.

Note: Incomplete cell resuspension will result in reduced plasmid DNA recovery.

Cell lysis: Vigorous mixing will shear genomic DNA. These fragments will be copurified with the plasmid DNA. Do not vortex. Do not allow the lysis reaction to exceed 5 min. Alkaline lysis buffers contain NaOH, which will denature the plasmid DNA on prolonged incubation.

Neutralization: Do not shake or mix vigorously because genomic DNA will be sheared and may co-isolate with plasmid DNA; mix by gentle inversion.

Clarification: If supernatant is not clear after neutralization and centrifugation, transfer to a fresh tube and recentrifuge. Using a pipette, immediately transfer the clarified lysate to a fresh tube without disturbing the pellet. The small flecks of flocculent (precipitated KDS and denatured cellular debris) that remain on the surface of the clarified lysate should stick to the pipette.

Mini-scale purification using silica

Following neutralization and centrifugation, use a pipette to transfer the supernatant to the purification column containing a silica membrane. Use a pipette if possible or decant directly. It is important to avoid transferring any cellular debris to the column as this will affect the purity of the isolated plasmid DNA.

Elution: When eluting plasmid DNA from the column, be sure that all residual wash buffer has been removed. The presence of contaminating ethanol in the eluted plasmid DNA may affect downstream applications; therefore, care must be taken to ensure its complete removal.

Purification using anion exchange medium

Overdrying the plasmid DNA pellet (generated after isopropanol precipitation) will make the DNA difficult to dissolve. If the pellet was overdried, heat the TE buffer to 60°C prior to addition, or add the buffer and gently roller mix overnight.

Preparation of plasmid DNA for* in vitro *transcription

If RNase is used during preparation of plasmid DNA, remove it completely by phenol/chloroform extraction or Proteinase K digestion. This is necessary because residual RNase will degrade RNA generated during *in vitro* transcription.

Plasmid DNA purification using illustra plasmidPrep Kits

illustra plasmidPrep Kits from GE Healthcare have been optimized to rapidly isolate plasmid DNA of sufficient quantity, purity, and quality for most common molecular biology applications. Although the purification principles and formats differ for illustra plasmidPrep Mini Spin and Midi Flow Kits (see Table 5.2), both sets of kits produce high-quality DNA. The Midi Flow Kit is recommended for preparing transfection-quality plasmid DNA (see later in this chapter). The purification principles and specifications for the kits are summarized in Table 5.3. Spectrophotometric measurements of plasmid DNA are provided in Table 5.4.

Table 5.3. Purification principles and specifications associated with illustra plasmidPrep Mini Spin Kit and illustra plasmidPrep Midi Flow Kit.

	Mini Spin	Midi Flow
Lysis principle	Modified alkaline lysis plus chaotropic salt	Modified alkaline lysis
Purification principle	Silica-based	Anion exchange chromatography
Purification format	Centrifugation	Gravity
Column capacity	~20 µg	~250 µg
Sample/culture size[1]	1–3 ml	25–150 ml
Plasmid size	<35 kb	<35 kb
Typical yield	9–20 µg	100–250 µg

[1] *Sample size is dependent on growth medium used, conditions, strain of* E. coli, *and the copy number and size of the plasmid.*

Table 5.4. Spectrophotometric measurements used as an indicator of plasmid DNA purity following purification with illustra plasmidPrep Mini Spin Kit and illustra plasmidPrep Midi Flow Kit.

Spectrophotometric analyses	Mini Spin	Midi Flow
A_{260}/A_{280}	1.8–2.0	1.8–2.0
A_{260}/A_{230}	1.6-2.0	2.0–2.5

Spectrophotometric analyses are routinely used as quality indicators. The A_{260}/A_{280} value measures the ratio of nucleic acid to protein, and the A_{260}/A_{230} value measures the ratio of nucleic acid to salt contamination. Good-quality plasmid DNA samples possess A_{260}/A_{280} and A_{260}/A_{230} values in the range of 1.8 to 2.0 and >1.6, respectively. Plasmid DNA generated using the Midi Flow system is generally of better quality than that isolated using the Mini Spin format.

Plasmid DNA purification using illustra plasmidPrep Mini Spin Kit

illustra plasmidPrep Mini Spin Kit utilizes a simple plasmid DNA purification protocol, employing a modified alkaline cell lysis procedure and a novel silica-based membrane. It is designed for the extraction and purification of general molecular biology grade plasmid DNA from *E. coli*. A modified alkaline lysis procedure is followed by clarification through centrifugation to remove precipitated genomic DNA, KDS, and other contaminants. No organic solvents are used; instead, chaotropic salts are included to denature protein components and promote the selective binding of plasmid DNA to the novel silica membrane (6, 7). Denatured contaminants are easily removed by subsequent centrifugation and washing steps. The purified plasmid DNA is eluted in a low-ionic-strength buffer, at a plasmid concentration suitable for most molecular biological applications. The steps for plasmid DNA purification using illustra plasmidPrep Mini Spin Kit are summarized below.

Materials

illustra mini columns, buffers, and collection tubes are provided with the kit

Absolute ethanol

Advance preparation

Warm lysis buffer type 8 to dissolve precipitate, if present. Store at room temperature.

Prior to first use, add absolute ethanol to the bottle containing wash buffer (check product instructions for volume). Mix by inversion, record completion on the label, and store upright and airtight.

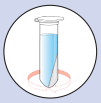
Protocol

Note: Columns and buffers are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure that you use the correct columns and buffers for your purification.

1. Harvest bacterial culture

Harvest bacteria by centrifugation and remove spent medium.

2. Lyse cells



Resuspend cells in an isotonic solution containing RNase. Add alkaline lysis buffer to denature genomic DNA and proteins. Neutralize the pH of the lysate with an acetate-buffered solution containing a chaotropic salt. Centrifuge the sample to pellet cellular debris, including genomic DNA, proteins, and lipids.

Cell resuspension can be achieved by vortexing, pipetting up/down, or by scraping the base of the microcentrifuge tube across the surface of an empty pipette tip box. Incomplete cell resuspension will result in reduced plasmid DNA recovery.

Vigorous mixing will shear genomic DNA, resulting in contamination. Do not allow the cell lysis reaction to exceed 5 min. Solution II contains NaOH, which will denature the plasmid DNA on prolonged incubation.

3. Bind plasmid DNA

Apply the cleared cellular lysate to an illustra plasmid mini column. Plasmid DNA binds to the silica membrane in the presence of chaotrope.

4. Wash (strain dependent)

This step is recommended for EndA$^+$ *E. coli* strains such as HB101. It removes residual nuclease activity and carbohydrates.

5. Wash and dry

Add buffer containing salt and ethanol to remove residual salts and other contaminants. Silica-bound plasmid DNA is dried to remove residual ethanol.

After centrifugation, if any of the wash buffer comes into contact with the bottom of the column, discard the flowthrough and recentrifuge for 30 s. The presence of contaminating ethanol in the eluted plasmid DNA may affect downstream applications. Therefore, care must be taken to ensure its complete removal.

6. Elute purified plasmid DNA

Elute plasmid DNA in a low-ionic-strength buffer containing EDTA.

Plasmid DNA purification using illustra plasmidPrep Midi Flow Kit

illustra plasmidPrep Midi Flow Kit is designed for the extraction and purification of transfection-grade plasmid DNA from *E. coli*. A modified alkaline lysis procedure is followed by clarification through centrifugation to remove precipitated genomic DNA, KDS, and other contaminants. Anion exchange purification is facilitated by plasmid DNA binding in high-salt conditions to illustra Fast Flow plasmid columns. Impurities such as RNA, cell wall debris, and protein are removed through successive high-salt washes. Purified plasmid DNA is eluted from the column at a higher salt concentration. The plasmid DNA is then concentrated and desalted using isopropanol precipitation and a 70% ethanol wash. The steps for plasmid DNA purification using illustra plasmidPrep Midi Flow Kit are summarized below.

Materials

illustra Fast Flow plasmid columns, buffers, and adapters are provided with the kit

Isopropanol (isopropyl alcohol)

Polypropylene or polycarbonate tubes (for lysis and clarification steps)

Polypropylene or polyallomer[1] tubes (for purification and concentration/desalting steps)

[1] *Use of polycarbonate centrifuge tubes is not recommended as they are not resistant to the alcohol used in subsequent steps.*

Advance preparation

Warm lysis buffer type 12 to dissolve precipitate, if present. Store at room temperature. Chill the other lysis buffers to 4°C before use.

Protocol

Note: Columns and buffers are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure that you use the correct columns and buffers for your purification.

1. Harvest bacterial culture

Harvest bacteria by centrifugation and remove spent medium.

2. Lyse cells

Resuspend cells in an isotonic solution containing RNase. Add alkaline lysis buffer to denature genomic DNA and proteins. Neutralize the pH of the lysate with an acetate/buffered solution.

The bacteria should be completely resuspended by pipetting up and down to remove any clumps.

Do not vortex, as this will result in shearing of genomic DNA. The lysate will appear viscous. Do not incubate for more than 5 min because this can result in denaturation damage to supercoiled plasmid.

3. Clarify the lysate

Centrifuge at high speed to pellet denatured genomic DNA and protein, as well as KDS. Plasmid DNA remains in solution. During this centrifugation step, equilibrate the illustra Fast Flow plasmid column.

Centrifugation should be performed in nonglass tubes (e.g., polypropylene).

4. Bind plasmid DNA

Apply the cleared cellular lysate to the column. Plasmid DNA binds to the anion exchange medium in the presence of high salt. Wash to remove contaminants.

The first wash removes most contaminants in the majority of plasmid DNA preparations. A second wash is essential when using large culture volumes and bacterial strains producing large amounts of carbohydrates.

5. Elute purified plasmid DNA and concentrate DNA

Elute plasmid DNA with a high-ionic-strength buffer. Add isopropanol to precipitate DNA.

The protocol can be stopped at this point and eluate stored at 4°C overnight, or at -20°C for longer periods.

6. Desalt plasmid DNA

Centrifuge to pellet DNA and wash with 70% ethanol. Air-dry and resuspend in TE buffer.

Downstream applications

The purity of plasmid DNA in terms of protein, salt, particulates, RNA, and endotoxin contamination, and quality measures such as the percentage of supercoiled plasmid DNA, will all affect downstream functional performance. In general, plasmid DNA extracted using anion exchange-based systems is of higher quality and purity compared with that isolated using silica membrane-based systems. Table 5.5 provides a summary of downstream applications for plasmid DNA and substances that may interfere in each.

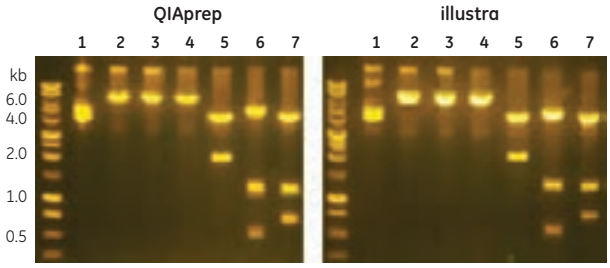
Table 5.5. Summary of downstream applications and contaminants that may interfere.

Application	Quantity of plasmid DNA required	Potential interfering contaminants
Analyses using restriction endonucleases	0.5–1 µg per restriction enzyme	Salts Organic solvents RNA (if gel analysis is performed)
Modifying enzymes, including methylases, phosphatases, kinases, and ligases	0.5–1 µg	Salts Organic solvents EDTA for some modifying enzymes RNA (if gel analysis is performed)
DNA sequencing	1–2 µg for standard sequencing; 200 ng for cycle sequencing	Salts Organic solvents EDTA Nucleases
PCR amplification	0.05–10 ng	Genomic DNA Salts EDTA
Transfection	For CHO cells: 100 ng per ~1×10^4 cells/well in 96-well plate 400 ng per ~4×10^4 cells/well in 24-well plate 1.9 µg per ~1.9×10^5 cells/well in 6-well plate	Carbohydrates, particularly endotoxins Salts Organic solvents Nucleases

Restriction endonucleases

Enzymatic processing of purified plasmid DNA is one of the most common downstream applications. Restriction endonucleases are used to prepare DNA fragments for subcloning experiments (including the screening for the correct recombination event). Several restriction endonucleases are particularly sensitive to elevated salt levels (e.g., HindIII and SacI); digestion with these salt-sensitive enzymes can be used as a very broad indicator of contamination levels. Organic solvents affect enzyme activity, and RNA can interfere with visualizing digestion products on an agarose gel.

Plasmid DNA isolated with either QIAprep™ or illustra Mini Spin Kit was digested to completion in all the restriction digests performed in this study (Fig 5.2). In addition, digests involving low concentrations of the restriction enzyme HindIII (1 unit at 37°C for 1 h) were performed. HindIII activity is diminished in the presence of elevated salt concentrations; therefore, HindIII digestion can be utilized to indicate the presence of prohibitively high salt contamination in the purified plasmid DNA. We observed that plasmid DNA samples isolated with both kits were digested to completion with HindIII (data not shown), suggesting that both illustra and QIAprep Kits produced plasmid DNA samples with negligible salt content.



1. Undigested plasmid DNA
2. NheI (6313 bp)
3. SacI (6313 bp)
4. BamHI (6313 bp)
5. HindIII (4273 and 2040 bp)
6. PsiI (4497, 1252, and 564 bp)
7. HindIII/NheI (4273, 1278, and 762 bp)

Fig 5.2. Restriction enzyme digestion of plasmid DNA samples (400 ng, 5 units, 37°C for 1 h).

Modifying enzymes

Plasmid DNA can also be manipulated by a large and diverse range of modifying enzymes. In traditional ligation and cloning, the endonuclease digestion of plasmid DNA is commonly used to facilitate the subcloning of a DNA fragment from one plasmid into an alternative. Site-specific endonucleases are used to "cut" at enzyme recognition sites flanking the DNA sequence of interest. The digestion products are subjected to agarose gel electrophoresis, and the desired fragments are purified using kits such as illustra PCR and Gel Band Purification Kit from GE Healthcare (see Chapter 8). This process is applied to both the DNA fragment of interest and the alternative plasmid. The latter may also be subjected to treatment with Alkaline Phosphatase, derived from sources such as calf intestines. This enzyme removes terminal 3'-phosphate groups, preventing the religation of DNA molecules possessing compatible termini in the presence of DNA ligase. Thus, this modifying enzyme facilitates the ligation of the DNA fragment of interest into the new or alternative plasmid.

As with DNA endonucleases, many modifying enzymes, such as phosphatases and ligases, are also extremely sensitive to contamination, especially elevated salt and protein levels. Therefore, both the quality and purity of the extracted plasmid DNA are important. When choosing a kit to perform plasmid DNA extraction, take into consideration the quality of plasmid DNA produced and the required sensitivity of downstream applications. Commercially available kits such as those of the illustra range are designed to purify plasmid DNA that can be routinely used in the majority of molecular biology applications.

DNA sequencing

Options for sequencing of cloned DNA include standard enzymatic sequencing (e.g., using T7 DNA polymerase and ddNTP "chain terminators") based on the Sanger method (8) and cycle sequencing. Cycle sequencing is similar to PCR in that it utilizes a thermostable DNA polymerase to generate new DNA. However, in cycle sequencing amplification is linear rather than exponential. Both options for sequencing are usually performed with fluorescent nucleotides so the DNA fragments can be analyzed automatically after gel electrophoresis. However, manual sequencing using radiolabeled nucleotides is still an option.

DNA sequencing can be used to indicate the quality of the extracted plasmid DNA. Quality is determined by automated DNA sequencing and Phred analyses. The Phred program is designed to assign nucleotide bases to DNA sequence traces. Phred reads DNA sequence chromatograms and analyzes the peaks to identify bases. It also assigns quality or Phred scores to each base. After identifying bases, Phred examines the peaks around each base to assign a quality score. Quality scores range from 4 to 60, with higher values corresponding to higher quality. These scores are linked to error probabilities. For example, a Phred score of 40 corresponds to a 1 in 10 000 chance that the base is called incorrectly, that is, 99.99% accuracy.

In general, good-quality plasmid DNA with high purity is required for manual sequencing. This is related to the amount of plasmid DNA required for the sequencing reaction. In comparison, cycle sequencing is more tolerant of contamination since contaminants are effectively diluted in the reaction mix.

Sequencing reactions (see Fig 5.3) were performed on plasmid DNA isolated using two commercially available mini-scale purification kits (illustra and QIAprep™) according to the manufacturers' instructions. DNA sequencing was performed on an ABI™ 3100 automated DNA sequencer using the BigDye™ Terminator v3.1 Cycle Sequencing Kit. Marginally higher Phred20 (99% accuracy) scores are generated when plasmid DNA is extracted using the midi-scale kits (data not shown). This is indicative of the higher quality plasmid DNA associated with the midi-scale anion exchange-based kits.

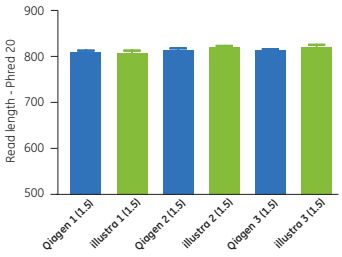


Fig 5.3. Mean autosequence Phred20 quality measurements (see above) for high-copy-number plasmid DNA samples prepared using QIAprep Spin Miniprep Kit (Qiagen) and illustra plasmidPrep Mini Spin Kits. The numbers 1, 2, and 3 refer to the culture from which the plasmid DNA was isolated. Processed culture volume (in ml) is shown in parentheses.

PCR amplification

Purified plasmid DNA is commonly used as a template in PCR for the amplification of specific cloned DNA sequences, for example, to modify the 5′ and 3′ ends of a cloned gene of interest with new restriction sites to facilitate subsequent subcloning experiments. PCR is also used to screen for the correct recombination event in subcloning experiments. However, in this context transformed *E. coli* cells are generally used as the template, indicating the relatively robust nature of PCR (and *Taq* DNA polymerase) in the presence of contaminants for routine amplifications.

A wide variety of DNA polymerases derived from thermophilic organisms are commercially available, including rTaq (GE Healthcare), PfuTurbo™ Hotstart DNA Polymerase and Vent™ DNA Polymerase. All add dideoxy nucleotides to the 3′-OH end of a primer in a template-directed reaction. Pfu and Vent DNA polymerases possess a 3′ to 5′ exonuclease proofreading activity, which reduces the number of misincorporated nucleotides in an extending primer. The error rates for the nonproofreading *Taq* DNA polymerase and Vent DNA polymerase are 285×10^{-6} and 57×10^{-6}, respectively (New England Biolabs).

Due to the amplification associated with PCR, small amounts of plasmid DNA can be used as a template. Therefore, contaminants are essentially diluted away. Even so, routine PCR is a relatively robust process and intact bacterial cells can be successfully used directly in a PCR reaction with little impact on amplification efficiency.

Plasmid DNA purified using illustra plasmidPrep Mini Spin Kit was used as the template in a PCR reaction to amplify a 1.2 kb product with the thermostable DNA polymerases described above. The results are shown in Figure 5.4. All polymerases successfully amplified the product. Marginally higher efficiency was observed using the nonproofreading enzyme rTaq.

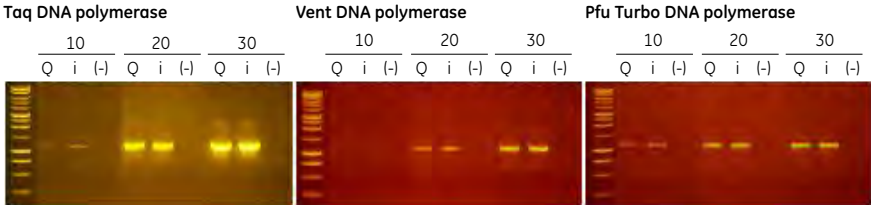


Fig 5.4. End-point PCR using several thermostable polymerases. Lanes Q and i represent amplification products derived from plasmid DNA samples extracted using QIAprep Spin Miniprep Kit (Qiagen) or illustra plasmidPrep Mini Spin Kit, respectively. (-) represents no template control reactions. The numbers 10, 20, and 30 indicate the number of thermal cycles performed. Aliquots (5 µl) of each reaction were loaded on a 1% (w/v) agarose gel.

Rolling circle amplification

In nature, the replication of circular DNA molecules, such as plasmids, occurs via a rolling circle mechanism. In the laboratory, linear rolling circle amplification (RCA) is the extension of an oligonucleotide primer annealed to a circular template DNA. Random hexamer primers are used to perform RCA of both strands of the plasmid, resulting in a cascade of strand displacement reactions producing an exponential amplification. See Chapter 7 for details on using Phi29 DNA Polymerase for RCA of plasmid DNA.

The application of RCA was demonstrated on plasmid DNA extracted using illustra plasmidPrep Mini Spin Kit. The RCA-amplified DNA generated was of sufficient purity and quality to successfully transfect a mammalian cell line, allowing the performance of a cell-based assay (data not shown).

Transfection

Purified plasmid DNA is routinely used for the transfection of mammalian or other eukaryotic cells. This process is very susceptible to contaminants (e.g., salt and endotoxin levels); therefore, the plasmid DNA must be of high purity and quality to ensure high transfection efficiencies and low toxicity. High-quality plasmid DNA is routinely isolated using many of the commercially available midi-/maxi-scale kits that are based on anion exchange, including illustra plasmidPrep Midi Flow Kit. The high-purity and -quality plasmid DNA associated with midi-scale kits is especially important for transfecting more sensitive cells such as primary cell lines and those that are highly sensitive to endotoxin levels. Certain robust cell lines (e.g., HEK293) can be transfected to relatively high efficiencies using plasmid DNA that is purified using silica-based mini-scale systems, including illustra plasmidPrep Mini Spin Kit. Mini-scale silica-based kits generally facilitate the purification of plasmid DNA that is of marginally lower quality in terms of salt and endotoxin levels compared with that extracted using anion exchange-based systems.

Plasmid DNA isolated using Qiagen™ Plasmid Midi and illustra plasmidPrep Midi Flow Kits was used to transfect the cell lines COS7, HeLa, and SH-SY5Y. The first two cell lines are representative of those that are relatively tolerant of contamination levels, while the latter SH-SY5Y cells are sensitive to elevated endotoxin levels. The results of these experiments are shown in Figure 5.5.

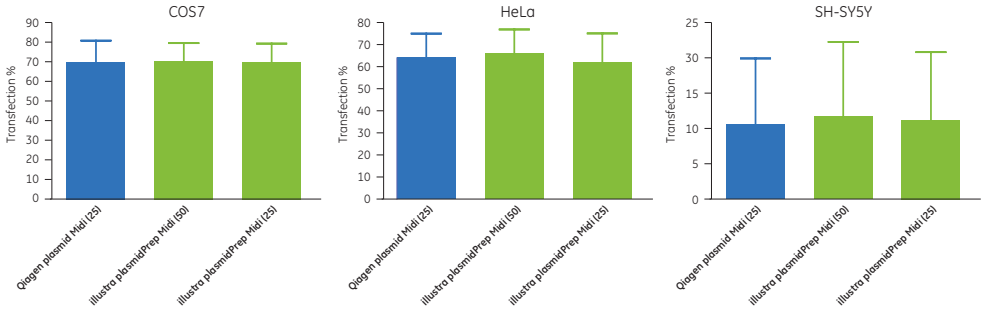


Fig 5.5. Transfection efficiencies of plasmid DNA generated using illustra PlasmidPrep Midi Flow Kit and Qiagen Plasmid Midi Kit. The numbers 25 and 50 indicate the culture volume (ml) from which plasmid DNA was extracted.

For COS7 and HeLa cell lines, both of the anion exchange-based midi-scale kits generated plasmid DNA of sufficient quality and purity to transfect the cells at comparable efficiencies (i.e., >70%). The endotoxin-sensitive SH-SY5Y cell line was transfected at comparable but lower efficiencies (i.e., ~10%), with plasmid DNA generated from both manufacturers' kits.

Plasmid DNA isolated with illustra plasmidPrep Mini Spin and Midi Flow Kits was used to transfect the robust HEK293 cells. These cells are more amenable to transfection compared with the more sensitive cells described above. The highest transfection efficiency (~70%) was observed from plasmid DNA samples purified with illustra plasmidPrep Midi Flow Kit (50% efficiency was observed when using samples obtained from illustra plasmidPrep Mini Spin Kit). illustra Midi Flow Kit generates samples of high purity and quality that possess not only significantly reduced endotoxin levels, but also lower impurities such as proteins/salts/particulates compared with samples from illustra Mini Spin Kit.

To summarize, plasmid DNA purified using silica-based systems can be used to transfect certain robust cell lines. The advantage of using mini-scale extractions is that it facilitates the isolation and transfection of multiple samples relatively quickly and easily. Multiple plasmid DNA extractions based on a 96-well plate format that consists of silica membranes are commercially available and are routinely performed.

Troubleshooting

Table 5.6. Possible causes and solutions for problems that may occur during plasmid DNA preparation[1].

Problem	Possible cause	Solution
Yield of plasmid DNA is low	The bacterial culture was not fresh.	A culture should be processed in a timely manner after it has reached the required cell density. Alternatively, bacterial pellets can be stored at -20°C prior to plasmid DNA extraction with no significant effect on purity or quality.
	The plasmid has a low copy number.	Process a larger volume of culture or grow in a richer medium. Alternatively, clone DNA sequence of interest into a plasmid possessing a higher copy number, for example, pCORON1000 (~300–500 copies per cell).
	The purification system was overloaded.	Measure the A_{600} of the culture before processing to ensure that the purification column is not overloaded with bacterial debris (e.g., when using illustra plasmidPrep Mini Spin system. If the A_{600} is > 5, reduce the volume of culture processed).
	Cells were not adequately resuspended.	Cell resuspension can be achieved by either vortexing, pipetting up/down, or running the microcentrifuge tube containing the bacterial cells along the lid of an empty pipette tip box.
	Cells were not adequately lysed.	Mix gently but completely when alkaline lysis solution is added.
	EndA$^+$ strains (e.g., HB101) possess nucleases and excessive carbohydrates.	When using silica-based purification formats, apply an additional wash to the plasmid purification column (after plasmid DNA binding) using high-salt buffer. This additional step removes residual nucleases and excessive carbohydrates, thereby increasing the yield and quality of the plasmid DNA. Alternatively, use an EndA$^-$ strain, such as TOP10 or DH5α.
Plasmid DNA appears as a smear when viewed on an agarose gel	EndA$^+$ strains (e.g., HB101) possess nucleases.	When using silica-based purification formats, apply an additional wash to the plasmid purification column (after plasmid DNA binding) using high-salt buffer. This additional step removes the residual nuclease activity, thereby increasing yield and quality of the plasmid DNA. Alternatively, use an EndA$^-$ strain such as TOP10 or DH5α.
Plasmid DNA is contaminated with genomic DNA	Alkaline lysis: The sample was mixed too vigorously after adding lysis buffers.	Mix gently by inverting the sample 10–15 times after adding either of the solutions. Vigorous mixing may cause shearing of genomic DNA, thereby facilitating its co-purification with plasmid DNA.
Plasmid DNA is contaminated with RNA	Sample was not treated with RNase.	RNase (100 µg/ml) is included in the lysis buffer of illustra plasmidPrep Mini Spin Kit. When using other methods, add an RNase step if it is not already included.

[1] These troubleshooting tips are applicable to the majority of commercially available plasmid DNA purification kits.

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Problem	Possible cause	Solution
Plasmid DNA does not cut to completion at 37°C	Plasmid DNA was irreversibly denatured and therefore will not cut.	Mix gently by inverting the bacterial sample 10–15 times after adding the alkaline lysis solution. Avoid prolonged exposure (> 5 min) or the NaOH in the lysis solution will irreversibly denature plasmid DNA.
	Silica-based purification: All traces of ethanolic wash buffer were not removed.	The presence of residual ethanol may affect downstream applications and therefore it must be carefully removed, for example, after washing and drying the purification column by centrifugation. If any ethanolic wash buffer comes into contact with the purification column after centrifugation, discard the flowthrough and recentrifuge.

References

1. Holmes, D. S. and Quigley, M. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114,** 193–197 (1981).
2. Birnboim, H. C. and Doly, J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7,** 1513–1523 (1979).
3. Ish-Horowicz, D. and Burke, J. F. Rapid and efficient cosmid cloning. *Nucl. Acids Res.* **9**, 2989–2998 (1981).
4. Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., *Molecular Cloning: A laboratory manual,* Cold Spring Harbor Laboratory, 3rd ed. (2001).
5. Ausubel, F. M., *et al.,* ed., *Current Protocols in Molecular Biology,* John Wiley and Sons, Inc. (2002).
6. Vogelstein, B. and Gillespie, D. Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. USA.* **76**, 615–619 (1979).
7. Marko, M. A., *et al.* A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. *Anal. Biochem.* **121**, 382–387 (1982).
8. Sanger, F. and Coulson A. R. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **94**, 441–448 (1975).

Chapter 6
RNA preparation

Cellular RNA includes ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), mitochondrial RNA (mtRNA), and a range of smaller, micro RNA molecules (miRNA). In the past decade, the study of miRNA has become prevalent. Interference RNA (RNAi) is a naturally occurring system that allows researchers to silence the expression of a gene of interest. This chapter discusses the preparation of total RNA and mRNA. The preparation of smaller RNAs involved in RNAi is frequently accomplished using a specifically designed, commercially available kit and is beyond the scope of this chapter.

The trend in RNA purification is toward purifying total RNA rather than mRNA. This is due in part to the increased sensitivity, over the past decade, of downstream applications, as well as to improvements in workflows for total RNA purification. Total RNA from eukaryotic sources includes ribosomal RNA, polyadenylated (poly $[A]^+$) mRNA, tRNA, and other smaller RNAs. The majority of cellular RNA (about 80%) is ribosomal; mRNA represents just 1% to 5% of cellular RNA. mRNA species are not equally represented in a cell. Some species are highly represented, and others have as few as five copies per cell. Total RNA can be used in most applications, particularly if oligo(dT) primers or other molecules are used to select poly$(A)^+$ mRNA. mRNA may be preferred in some procedures, such as cDNA synthesis. For applications that require labeled mRNA, protocols are available for direct labeling. If total RNA does not give acceptable results in a given application, it may be further purified using oligo(dT) affinity chromatography to isolate mRNA.

The required purity, quality, and quantity of RNA should be considered when selecting a preparation method. The purity and quality of extracted RNA can be assessed by several methods including spectrophotometry, in which the A_{260}/A_{280} ratio may be used to monitor protein contamination. High purity RNA has an A_{260}/A_{280} of 1.9 to 2.1. Because DNA and RNA both absorb at 260 nm, the presence of genomic DNA is typically assessed by formaldehyde agarose gel electrophoresis or by PCR to detect a "housekeeping" gene. Genomic DNA is typically removed during the RNA isolation procedure by DNase treatment or extraction with acid phenol/chloroform. Another non-spectrophotometric method for assessing RNA quality and integrity is analysis using an Agilent Bioanalyzer to determine the RNA integrity number (RIN; 1). Using this method, RNA with an RIN greater than 7 would be suitable for most downstream applications, including microarray analysis. See Appendix 4 for a discussion of RIN.

Molecular biological applications vary in their requirements for RNA quality and purity. For example, Northern blot analysis is fairly tolerant of contaminants but is not as tolerant to partially degraded RNA. However, techniques such as RT-PCR and microarrays require high-purity RNA, as even small quantities of genomic DNA can bind primers, leading to "false-positive" results. Because a number of enzymatic reactions are involved in RT-PCR, it is highly sensitive to other contaminants such as phenol, salts, and ethanol. For microarray analysis, contaminants may interfere with enzymatic labeling reactions or increase background signals.

Table 6.1. Summary of possible contaminants in RNA preparations and applications in which those contaminants may interfere.

Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
RNases	All sample types; prevalence varies by sample type	All applications that require intact RNA, including Northern blots/slot blots	May degrade RNA
Genomic DNA	All sample types	RT-PCR	Primers/probes may anneal to genomic DNA
		Microarrays	Intronic genomic DNA will react as mRNA with the primers/probes and give extra signals
		Nuclease protection assays	Antisense riboprobes are capable of annealing to single-stranded DNA, giving false positives or confounding protection patterns
Polysaccharides	Particularly in plant tissue, animal liver and muscle, and bacteria	RT-PCR	May interfere with extraction, reducing RNA yield and purity
			Exact mechanism for RT-PCR inhibition is not known
EDTA	May be introduced when resuspending an RNA pellet	RT-PCR	Chelates Mg^{2+} required for PCR
		Microarrays	Inhibits cDNA synthesis
Residual salts	May be from sample or introduced during RNA preparation	RT-PCR	Inhibit activity of reverse transcriptase
		Microarrays	Inhibit cDNA synthesis
		in vitro translation	Exact mechanism unknown
Organic solvents such as alcohols and phenol/ chloroform	May be introduced during RNA preparation	A majority of routine molecular biology techniques	Inhibit enzyme activity

Techniques for RNA preparation

The isolation of RNA from tissues and cells is a prerequisite for many procedures, including RT-PCR, real-time or RT-qPCR, microarrays, cDNA synthesis, Northern blot hybridization, nuclease-protection mapping, *in vitro* translation, and other techniques. The goal of RNA purification is to produce highly enriched RNA that is undegraded and free from DNA and proteins. When purifying RNA, it is important to protect RNA from degradation by RNases, which are prevalent both in biological samples and in the laboratory environment. Many methods have been developed to circumvent this problem and to achieve the goal of good-quality RNA.

A simple flowchart for the preparation of total RNA and mRNA is shown in Figure 6.1.

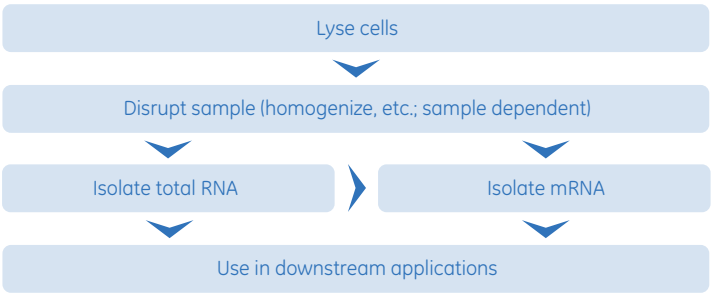


Fig 6.1. Steps in preparing total RNA and mRNA for analysis.

Most of the current methods for isolating total RNA are solution- or silica-based. These methods generally include a chaotrope, denaturant, or other chemical to inactivate RNases. "Homemade" methods are typically solution-based and may use organic solvents such as phenol. Reagent kits typically include all required buffers and are either solution-based and use an organic solvent or are silica-based. Because silica will bind both DNA and RNA, a DNase treatment is typically required in silica-based kits. Silica may be supplied in prefilled columns or in another format such as magnetic beads. Membrane-based silica and magnetic bead formats are amenable to automation using liquid handling robotics. Reagent kits may be useful for a number of sample types, or they may be sample-specific (e.g., for blood or plants). mRNA isolation is typically achieved using affinity chromatography with oligo(dT)-cellulose or other oligo(dT)-derived media. mRNA may be isolated directly from the sample or from total RNA. Some of the most common methods for RNA preparation are discussed in the pages that follow.

When preparing total RNA, guanidinium salts (usually GTC) are a popular choice for lysing cells because they inhibit RNase activity and promote binding of nucleic acids to silica. Some solution-based methods include phenol in conjunction with GTC to further inhibit RNases. Other total RNA preparation methods are described below.

Techniques for preparation of total RNA

Guanidinium + phenol/chloroform + alcohol precipitation

RNases can be inhibited by a variety of reagents including noncompetitive RNase inhibitors (2) or very strong denaturants, such as the chaotropes Gua-HCl or GTC (3), which are often coupled with hot phenol/phenol-chloroform extractions (4, 5). This latter modification is a popular method for making cDNA libraries and for use with differential display technologies. The use of a GTC extraction buffer followed by recovery of RNA using direct precipitation with 4 M LiCl has been demonstrated (6). In this method, purified RNA is obtained after a second precipitation in 3 M LiCl, followed by a phenol/chloroform extraction and ethanol precipitation. In another GTC-based method, phenol and chloroform are added immediately after homogenizing samples in a GTC buffer. RNA partitions to the aqueous phase, whereas DNA and proteins separate to the interphase and organic phase. RNA is then recovered by multiple alcohol precipitations of the aqueous phase.

Guanidinium + density gradient centrifugation

The inclusion of the chaotropic salt in the primary extraction buffer affords a high degree of protection from RNase activity. The effectiveness of GTC for both deproteinizing and protecting RNA has been documented (7). Because both the anion and cation are strong chaotropes, GTC is an excellent denaturant of proteins, including RNases. In addition, GTC aids in cell lysis. Further purification of extracted RNA by equilibrium centrifugation in CsCl results in pelleting of the RNA at the bottom of the centrifuge tube. Although these methods accomplish, to varying degrees, the primary goal of producing highly pure RNA, they also have drawbacks. For example, methods involving CsCl equilibrium centrifugation require an ultracentrifuge and long

centrifugation times (typically 12 to 16 h), while those that use organic solvents, such as phenol and chloroform, are potentially toxic.

Tissues known to contain extremely high RNase activity (e.g., pancreas) have yielded undegraded RNA using GTC followed by ultracentrifugation through a CsCl cushion (7). CsTFA may be substituted for CsCl. The disruptive and protective properties of GTC with selective precipitation and isopycnic centrifugation using LiCl and CsTFA have also been exploited. In addition, the literature describes modifications for the isolation of RNA from notoriously "difficult" samples. For example, Birnboim (8) recommends the use of a mixture of SDS and urea when extracting RNA from cultured cells because leukocyte RNases appear to be more effectively inhibited by these reagents. Plant tissue often contains high levels of soluble starch, so a filtration step and a wash with 3M sodium acetate (pH 5.2) are incorporated to remove polysaccharides (9).

Silica-based methods

Nucleic acids have been shown to bind to silica in the presence of GTC (10). This property can be exploited to purify nucleic acids quickly and without the use of organic solvents such as phenol and chloroform. Because both DNA and RNA bind to silica in the presence of chaotropes, DNase treatment is typically performed to eliminate genomic DNA from total RNA preparations. After washing and drying of the silica, total RNA is eluted in a low-ionic-strength buffer or RNase-free water. The latter is frequently used because it is least likely to interfere with downstream applications.

Charge-based method

This method uses the charge on a solid surface (magnetic beads) for nucleic acid capture. At lower pH (< 6.5), the surface chemistry holds a positive charge, attracting DNA and RNA. DNase I is used to remove bound DNA. At higher pH (> 8.5), the surface chemistry is neutral, thereby releasing any attraction to bound RNA.

Sequence-specific capture

Nucleic acid capture uses a biotinylated probe and streptavidin-coated beads to enrich low-abundance nucleic acids from clinical samples. This method requires a biotinylated PCR product or biotinylated probe. This is an enrichment method, not a complete RNA preparation method. Therefore, it is not included in Table 6.2.

Techniques for preparation of mRNA

In some cases, purification of total or compartmental RNA is an intermediate step in the isolation of mRNA. In others, mRNA is isolated directly from tissue or cells. Traditionally, mRNA has been separated on oligo(dT)-cellulose affinity columns, and this remains the method of choice. Recent improvements, however, have included formatting changes to utilize centrifugation during equilibration, washing, and elution steps. The use of spin columns containing oligo(dT)-cellulose has dramatically reduced the time commitment and improved the efficiency of poly(A)$^+$ RNA isolations over use of gravity-driven columns containing the same medium. When high levels of RNase are not present, affinity capture of poly(A)$^+$ RNA from guanidinium salt solutions using biotinylated oligo(dT) and streptavidin-coated latex or silica beads can decrease preparative time.

A summary of different techniques for RNA preparation are provided in Table 6.2. Consult references 11–13 for details on different RNA preparation techniques.

Table 6.2. Advantages and disadvantages of different techniques for RNA preparation.

RNA preparation category	Technique	How it works	Advantages (+)/ Disadvantages (-)
Total RNA preparation	Guanidinium + phenol/ chloroform + alcohol precipitation	Chaotrope helps lyse cells and inactivates RNases. Proteins are denatured and removed in the organic phase during phenol/chloroform extraction. RNA is precipitated with alcohol and salt.	+ Inexpensive + Methods that use chaotrope containing phenol provide enhanced protection against RNases - Need to use and dispose of caustic/toxic chemicals - Cumbersome
	Guanidinium + density gradient centrifugation	Chaotrope helps lyse cells and inactivates RNases. RNA either bands in CsCl or CsTFA or is pelleted, depending on gradient used. Proteins and DNA are physically separated from RNA.	+ High-purity RNA + Does not require organic extraction or alcohol precipitation + CsTFA is a strong RNase inhibitor - Requires access to ultracentrifuge and overnight centrifugation
	Silica purification in the presence of chaotrope, usually GTC (most commercial kits include an optional DNase step)	Chaotrope helps lyse cells and inactivates RNases. RNA and DNA bind to silica in the presence of chaotrope. (DNA is digested with DNase.) Contaminants are washed away and RNA is eluted at low ionic strength.	+ Fast + Does not require use of organic solvents or alcohol precipitation - May require DNase treatment
	Charge-based purification	This separation technology binds nucleic acids to magnetic beads at low pH and elutes nucleic acids by raising the pH.	+ Fast + Does not require use of organic solvents or alcohol precipitation + Amenable to automation - May require DNase treatment - Only works on cells
mRNA purification	oligo(dT) coupled to cellulose or other matrix; may use paramagnetic particles (PMPs)	mRNAs bind via their poly(A) tails to oligo(dT) in high-salt conditions. Medium is washed with high salt, then low salt, and mRNA is eluted at low ionic strength.	+ RNA is highly enriched for mRNA + Can be used directly from cells/tissues without intermediate preparation of total RNA - Will not work with prokaryotic mRNAs, which are not polyadenylated - May selectively enrich for mRNAs with shorter poly(A) tracts

General considerations for RNA preparation

Considerations independent of technique

A number of parameters should be considered before selecting a method for RNA preparation. See Chapter 2 for a discussion of these. Protection of the sample from both endogenous and exogenous RNases is critical to the success of any method for preparing RNA. Before selecting a method, you must decide if total RNA is sufficient for your application or if mRNA is required. In addition to quantity, purity, and quality considerations, it is important to consider the desired size of the isolated RNA. For example, many methods are not designed to isolate small RNA molecules, such as miRNA. For gene expression studies, it is important to stabilize samples quickly to maintain the cellular mRNA population as it was before samples were harvested. Also, it is important to isolate poorly expressed ("rare") mRNAs, as well as more abundant messages. Finally, gene expression analyses are typically sensitive to the presence of genomic DNA, so this contaminant should be thoroughly removed for these applications.

Before beginning any method for preparing RNA, care must be taken to make the laboratory as free of RNases as possible. Treatment using DEPC (see Appendix 4) will inactivate RNases in solutions. DEPC can be added directly to water and most other solutions, which are then autoclaved to remove residual DEPC. However, DEPC cannot be added directly to buffers containing a primary amine (e.g., Tris, Hepes), because this will effectively decrease the DEPC available to inactivate RNases. For these buffers, chemicals should be dissolved in DEPC-treated water or in commercially available "RNase-free" water. Glassware should be baked rather than autoclaved because autoclaving does not inactivate RNases. Using disposable plasticware instead of glass can reduce the risk of introducing RNases. One of the biggest carriers of exogenous RNA is human skin. Therefore, gloves should be worn at all times when preparing or working with RNA, and these should be changed frequently. In addition, commercially available products for eliminating RNases can be sprayed on gloves or counter tops. Many RNA procedures end with RNA in RNase-free water. Particular care should be taken at this stage because the sample is not protected by chaotropes or other chemicals that inactivate RNases. RNA can be stored at -20°C for the short term and at -80°C for longer term. Ideally, RNA should be stored in aliquots to avoid repeated freeze-thaw cycles.

Starting samples will contain RNases in varying amounts. RNA is not protected against RNase digestion until the sample material is flash frozen or disrupted in the presence of RNase-inhibiting or -denaturing agents. Therefore, it is important that samples be flash frozen in liquid N_2 immediately and stored at -80°C, stored in a stabilizing agent, or processed as soon as possible. Cutting hard tissue samples into tiny pieces will increase the contact area when using stabilizing agents. If tissue is frozen, most procedures recommend that the sample not be allowed to thaw prior to addition of the extraction buffer, which contains chaotropes or other RNase-inhibiting chemicals.

We recommend that you check the quantity, purity, and quality of isolated RNA prior to using it in downstream applications. A number of options are available, including spectrophotometry, quantitation using an RNA-binding dye, and visualization following denaturing electrophoresis. See Appendix 4 for details. When genomic DNA contamination is a concern, genomic DNA may be detected by performing PCR for a "housekeeping" gene. When RNA quality is important, analysis using an Agilent Bioanalyzer may be warranted.

If RNA is of insufficient concentration or volume for use, the sample may be precipitated. See Appendix 4 for details. A number of inert coprecipitants may be used to increase RNA yield, particularly with very dilute solutions. DNase-treated glycogen or linear acrylamide are good options if DNA contamination is a concern.

Considerations when using silica products

When using silica products, it is important to know the RNA binding capacity of the column or other solid phase, as well as the RNA content in the source sample, to ensure that you don't overload the system. Overloading often leads to a decrease in yield, purity, and quality. A highly viscous sample usually indicates the presence of a large amount of genomic DNA. Reduce the sample input to prevent clogging the column.

Silica-based protocols for RNA purification typically include washing with a buffer that contains ethanol. Ethanol should be thoroughly removed prior to RNA elution because it can interfere with the use of RNA in some applications. For example, residual ethanol can inhibit some enzymatic reactions.

When using silica in a membrane-based column format, carefully discard column flowthrough and the collection tube. If any of the ethanolic wash solution comes into contact with the bottom of the column, discard the flowthrough and recentrifuge. In addition, be sure to thoroughly air dry the sample but do not overdry.

Considerations when using oligo(dT) chromatography media

Do not overload the medium. Applying more than the maximum amounts of cells or tissue to oligo(dT) cellulose media will result in a loss of purity as reflected by a decrease in the A_{260}/A_{280} ratio. When overloaded, the medium will appear highly viscous and mucus-like when the sample is mixed in the column.

Do not use this method with bacteria because their mRNAs are not polyadenylated.

Multi-prep methodologies

It is possible to isolate more than one type of biomolecule from the same sample. For example, total RNA and protein may both be isolated. Other variations are also possible. The primary methods for such "multi-prep" isolations are solution-based (with phenol) or silica-based. See Chapter 10 for a discussion of multi-prep methodologies, including illustra triplePrep Kit from GE Healthcare.

If you want to isolate more than one biomolecule, choose a protocol that is designed for this purpose. For example, if you want to isolate DNA or protein, do not add DNase or proteases, respectively.

Sample-specific considerations for RNA preparation

See Chapter 2 for details on lysing and disrupting different sample types.

Cultured animal cells are typically harvested by centrifugation and lysed with an extraction buffer containing GTC or other chemical to inhibit RNases. β-mercaptoethanol is often added to denature RNases by reducing the disulfide bonds that are necessary to maintain RNase activity. β-mercaptoethanol may also help to release RNA when it is bound to proteins. Cell pellets can be frozen before RNA isolation.

Solid animal tissues must be broken up physically as well as lysed. Depending on the disruption method (see Chapter 2), the viscosity of the extracted sample may need to be reduced further for optimal results. It is essential for efficient RNA preparation that all the RNA contained in the sample is released from the cells and that the viscosity of the sample is reduced by homogenization. See Chapter 2 for details on methods to disrupt and homogenize animal tissue. After cell lysis, the sample may be used immediately, frozen, or stabilized using a stabilization reagent. Cell lysates may be homogenized using filters designed for this purpose or by passing the lysate at least five times through a 0.9 mm (20 ga) syringe needle. Thawing of undisrupted animal tissue should be done in the presence of chaotrope, denaturant, or other chemical to inactivate RNases.

Like animal tissues, plant tissues must be broken up physically. See Chapter 2 for details on methods to physically disrupt these tissues. Plants contain polysaccharides, polyphenols, and other molecules that may copurify with RNA and inhibit downstream applications. Additional purification steps, such as the addition of polyvinylpyrrolidone (PVP), may be helpful to absorb these plant-based contaminants.

Bacteria and yeasts are typically incubated in lysozyme or lyticase/zymolase solutions, respectively. By this treatment, the robust cell walls of these organisms are digested or at least weakened, which is essential for effective cell lysis by extraction buffer. For microorganisms with extremely resistant cell walls, such as some Gram-positive bacteria, it may be necessary to optimize the conditions of treatment with lytic enzymes or the cultivation conditions. After lysis, homogenization is achieved by the use of a commercially available homogenizing filter unit or the syringe-needle method. Bacterial mRNAs do not have a poly(A) tail, which means that they cannot be captured by oligo(dT) affinity chromatography media. Additionally, with bacteria random hexamers must be used instead of oligo(dT) primers in downstream applications.

Blood contains high levels of proteins and RNases. In addition, it contains a number of inhibitors to PCR, such as heme. Anticoagulants in blood samples (i.e., heparin and EDTA) can also interfere with downstream applications. In addition to these factors, the volume of whole blood poses challenges to many RNA preparation procedures. When processing whole blood, chaotropes or other chemicals may become too dilute to effectively inhibit RNases. Some procedures selectively lyse RBC; WBC are then collected by centrifugation. RNA is typically protected from RNases during this step.

Cell-free somatic (body) fluids such as serum may contain viral RNA. Virus particles are typically dilute in these samples and may need to be concentrated prior to RNA isolation.

Total RNA preparation using illustra RNAspin RNA Isolation Kits

illustra RNAspin RNA Isolation Kits from GE Healthcare are designed to isolate total RNA from cells, tissue, and bacteria. Note that these kits are not suitable for use with whole blood. The kits can also be used for RNA cleanup (see product instructions). One of the most important aspects in the process of isolating RNA is to prevent the degradation of the RNA during the procedure. With the illustra RNAspin method, cells are lysed by incubation in a buffer containing large amounts of chaotropic ions. This lysis buffer immediately inactivates RNases, which are present in virtually all biological materials, and creates appropriate binding conditions that favor adsorption of RNA to the silica membrane. Contaminating DNA, which is also bound to the silica membrane, is removed by the direct application of a DNase I solution to the silica membrane (RNase-free DNase I is supplied with the kit). Simple washing steps with two different buffers remove salts, metabolites, and macromolecular cellular components. Pure total RNA is eluted under conditions of low ionic strength, using RNase-free water (supplied). illustra RNAspin Kits allow for the isolation of pure RNA with an A_{260}/A_{280} ratio generally exceeding 1.9 (measured in 0.1 M Tris-HCl buffer, pH 7.6). Even biological samples that are sometimes difficult to process will yield high-quality RNA. These include mouse tissue (e.g., liver, brain) and different tumor cell lines. The isolated RNA is ready to use in downstream applications such as RT-qPCR, primer extension, RNase protection assays, cDNA synthesis, and microarray analysis.

Illustra RNAspin RNA Isolation Kits are available in three formats. Examples of input sample material are provided in Table 6.3. Note that the RNAspin 96 Kit can be used with vacuum or centrifugation and that the protocol can be automated. See Chapter 9 for details.

Table 6.3. Sample amounts and typical yields for the three illustra RNAspin RNA Isolation Kits.

Sample type	Format	Sample amount	Yield
Cultured animal cells	Mini	Up to 5 x 10^6 cells	Up to 70 µg
	Midi	Up to 4 x 10^7 cells	Up to 620 µg
	96	Up to 2 x 10^6 cells (vacuum) or 1 x 10^7 cells (centrifuge)	Up to 20 µg (vacuum) or 100 µg (centrifuge)
Animal tissue	Mini	Up to 30 mg	Up to 70 µg
	Midi	Up to 200 mg	Tissue dependent; up to 320 µg for mouse liver
	96	10–30 mg (vacuum) or up to 30 mg (centrifuge)	Up to 40 µg (vacuum) or 100 µg (centrifuge)
Bacteria	Mini	Up to 10^9 cells	*E. coli;* 10^9 cells (with 1 mg/ml lysozyme); RNA yield: approx. 19–25 µg total RNA
	Midi	Up to 5 x 10^9 cells	*E. coli* (with lysozyme): up to 105 µg
Yeast	Mini	Up to 5 x 10^7 cells	Data not available
	Midi	Up to 3 x 10^9 cells	Data not available

Total RNA purification using illustra RNAspin Mini and Midi RNA Isolation Kits

The following protocol summarizes use of the illustra RNAspin Mini and Midi RNA Isolation Kits. Most of the materials and preparations are the same for the RNAspin 96 Kit, but the RNAspin 96 Kit includes one additional buffer for dilution of lysate and for washing. In addition, collection trays are provided with this latter kit. See Chapter 9 for the protocol for RNAspin 96.

Materials

Kits provide illustra RNAspin columns, filter units, buffers, lyophilized DNase I, DNase reaction buffer, RNase-free water, and collection tubes

Gloves

RNase-free tubes and other plasticware

β-mercaptoethanol

For bacteria: lysozyme (0.2 mg/ml final concentration for Gram-negative cells; 2 mg/ml final for Gram-positive cells) in TE buffer, pH 8

For yeast: zymolase/lyticase in sorbitol/EDTA buffer (1 M sorbitol, 100 mM EDTA)

Optional for all samples: 0.9 mm needle (20-gauge) and syringe

Advance preparation

Dissolve DNase I in RNase-free water.

Avoid vigorous mixing of the DNase I enzyme because it is sensitive to mechanical agitation and will be inactivated.

Add ethanol to buffer RA3 concentrate.

Protocol

1. Disrupt sample and lyse cells

a. Disrupt tissue or do other sample pretreatment if needed.

Disrupt animal tissue using liquid nitrogen and a mortar and pestle or other suitable method. Immediately proceed to Step 2 to lyse cells.

Harvest cultured cells by centrifugation. Immediately proceed to Step 2 to lyse cells.

Pretreat harvested bacterial cells with lysozyme.

Pretreat harvested, cultured yeast cells with lyticase or zymolase. Centrifuge and isolate spheroplasts.

b. Lyse cells.

Add lysis buffer RA1 and β-mercaptoethanol. Vortex vigorously. The chaotropic salts in the lysis buffer break open the cells and inhibit RNases. β-mercaptoethanol helps to inhibit RNases and break up RNA-protein complexes.

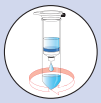
2. Filter lysate

Reduce viscosity and clear the lysate by centrifugation through an RNAspin filter unit.

3. Adjust RNA binding conditions

Add 70% ethanol to the cleared lysate and apply to silica column.

4. Bind RNA



The chaotropic salt in the lysis buffer plus the ethanol promote binding of RNA > 200 nucleotides long to the silica membrane. Denatured proteins are collected in the flowthrough following centrifugation.

After the addition of ethanol, a stringy precipitate may become visible. This will not affect RNA isolation. Be sure to load all the precipitate onto the column as described in step 5.

5. Desalt the membrane

Add desalting buffer to wash away salts.

Maximal loading capacity of RNAspin Mini column is 750 µl. Repeat the procedure if larger volumes are to be processed.

6. Add DNase I

Add RNase-free DNase to digest membrane-bound DNA. Incubate briefly at room temperature.

7. Wash and dry

Wash the column to inactivate DNase, then wash with a low-salt buffer containing ethanol to remove residual salts and other contaminants. Dry the membrane.

8. Elute purified total RNA

Elute purified total RNA using RNase-free water. Immediately place on ice or freeze.

mRNA purification using illustra QuickPrep and QuickPrep Micro *mRNA Purification Kits*

QuickPrep™ mRNA Purification Kit is designed for the rapid isolation of mRNA from eukaryotic cells or tissues without the need for intermediate purification of total RNA. The kit is well suited for the isolation of mRNA from cells or tissues that are available in limited quantities because it eliminates losses associated with intermediate purification of total RNA. Using QuickPrep *Micro* Kit, we have isolated mRNA from up to 25 µl of blood, 10^7 cells, and 100 mg of animal tissue; using QuickPrep Kit, we have isolated mRNA from up to 5×10^7 cells and up to 500 mg of tissue. After one round of oligo(dT)-cellulose affinity chromatography, mRNA represents > 90% of the nucleic acid (starting with 10^7 or fewer cells). A second round of chromatography can be performed with QuickPrep Kit to further enrich the sample for mRNA. Note that because bacterial mRNAs are not polyadenylated, these kits are not suitable for mRNA isolation from bacteria.

QuickPrep and QuickPrep *Micro* mRNA Purification Kits combine the disruptive and protective properties of GTC with the speed and selectivity of oligo(dT)-cellulose chromatography in a spin-column format pioneered by GE Healthcare. The protocol is briefly described below.

Initially, the tissue is extracted by homogenization in a buffered solution containing a high concentration of GTC. This ensures the rapid inactivation of endogenous RNases and the dissociation of cellular components from the mRNA. The extract is then diluted with elution buffer to optimally reduce the GTC concentration to allow efficient hydrogen bonding between poly(A) tracts on mRNA molecules and the oligo(dT) attached to cellulose, but high enough to maintain inhibition of RNases. As an added benefit, a number of proteins precipitate, allowing their easy removal by centrifugation. After a brief second homogenization, the extract is clarified by centrifugation, the supernatant is poured into an oligo(dT)-cellulose spin column, and the polyadenylated fraction is allowed to bind over a short period of time with gentle mixing. The column is subjected to a low-speed centrifugation, and the liquid containing the unbound material is decanted. The medium is washed sequentially with high-salt and then low-salt buffer. Finally, the sample is eluted from the medium with prewarmed elution buffer. A protocol for use of QuickPrep Kits is provided below.

Procedure for mRNA purification using illustra QuickPrep mRNA Isolation Kits

Materials

Buffers, glycogen, and potassium acetate solutions are provided with the kits. Bulk oligo(dT)-cellulose and empty MicroSpin™ columns are provided with QuickPrep *Micro* Kit; oligo(dT)-cellulose spin columns are provided with QuickPrep Kit.

RNase-free or DEPC-treated water

TE buffer (pH 7.4), prepared with RNase-free or DEPC-treated water

Gloves

RNase-free tubes and other plasticware

65°C heating block or water bath

For tissue: Mechanical or manual tissue homogenizer

For QuickPrep mRNA Purification Kit: A centrifuge with a swinging-bucket rotor and appropriate buckets capable of reaching 4000 to 4500 × g is required, as well as 15 ml centrifuge tubes.

Advance preparation

Just before use, heat extraction buffer to 37°C to dissolve crystals. Cool to room temperature.

Protocol

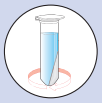
1. Disrupt sample and lyse cells

a. Homogenize animal tissue in extraction buffer containing chaotropic salts. For cultured cells, add extraction buffer to flask or to harvested cells. Blood samples (~25 µl) can be used directly with QuickPrep *Micro* Kit; there is no need to pellet them prior to extraction.

b. Dilute the homogenate with elution buffer. Store remaining elution buffer at 65°C.

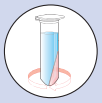
The GTC concentration is lowered to allow binding of mRNA to oligo(dT)-cellulose while inhibiting RNases. The dilution causes a number of proteins to precipitate, giving an initial purification. Centrifuge briefly.

2. Prepare oligo(dT)-cellulose



For QuickPrep *Micro* Kit: Resuspend bulk slurry and dispense into a micro-centrifuge tube. Centrifuge briefly and remove the supernatant. For QuickPrep Kit: Resuspend medium inside the spin column. Centrifuge to remove liquid.

3. Bind mRNA



Add supernatant from Step 1b to prepared medium. Invert to mix. Centrifuge and remove supernatant. Poly(A)$^+$ mRNA will bind to the dT oligos on the cellulose medium. Wash the medium.

4. Transfer medium and wash

Transfer medium to a Microspin column (*Micro* Kit only), and wash with low-salt buffer.

5. Elute purified mRNA

Elute with elution buffer heated to 65°C. Purified mRNA is eluted from oligo(dT)-cellulose when salt is removed.

Optional for QuickPrep Kit: Perform another round of oligo(dT)-cellulose chromatography to further enrich the RNA sample for mRNA.

Optional: Precipitate RNA with glycogen to increase concentration. See Appendix 4 for details.

Downstream applications

The purity of total RNA—in terms of the presence of genomic DNA, salts, organic solvents, and other contaminants—will affect downstream functional performance. It is impossible to specify the extent of the effect of contaminants for all RNA applications, and therefore we recommend obtaining RNA in as pure a form as possible, within the constraints of a given experiment. In general, salt and organic solvents should be eliminated regardless of the downstream application. It is generally true that mRNA isolated by oligo(dT) affinity chromatography will contain the fewest contaminants. However, total RNA isolation is simpler, and RNA recovery will be greater. Table 6.4 provides a summary of downstream applications and substances that may interfere in each.

Table 6.4. Summary of downstream applications for RNA and substances that may interfere.

Application	Quantity of total RNA required	Potential interfering contaminants
RT-PCR and RT-qPCR amplification	< 1 µg	Genomic DNA Organic solvents Salts EDTA
Expression microarrays	2–5 µg	Genomic DNA Organic solvents Salts EDTA
cDNA synthesis (not PCR-based)	< 2 µg	Salts
Northern blots/slot blots	5–20 µg	No specific inhibitors; however, this method is dependent on intact RNA
Nuclease protection assays (RNase or S1)	5–20 µg	Genomic DNA Salts
in vitro translation	2–5 µg	Salts Organic solvents Polysaccharides

RT-PCR and RT-qPCR

RT-PCR is frequently used to analyze mRNA expression levels. When reagents are consumed during amplification, the amount of product "plateaus." Because of this effect, RT-PCR may not be the most effective tool for absolute quantitation of input mRNA. Real-time RT-PCR (also known as RT-qPCR) allows detection of product generated at each cycle of amplification. Current chemistries such as TaqMan, Scorpions, and SYBR™ Green utilize fluorescence to detect PCR products. RT-PCR is fairly tolerant of poor RNA quality; however, contaminants remaining after RNA purification might affect the reaction efficiency. The biggest issue is contamination with genomic DNA, which may lead to false-positive signals. Genomic DNA can be eliminated by DNase treatment or by using at least one primer that spans an exon junction (introns are spliced out during mRNA maturation) so that genomic DNA will not be amplified.

Figure 6.2 shows a typical RT-qPCR amplification plot for total RNA isolated from different rat tissues using illustra triplePrep Kit. See Chapter 10 for details on triplePrep Kit.

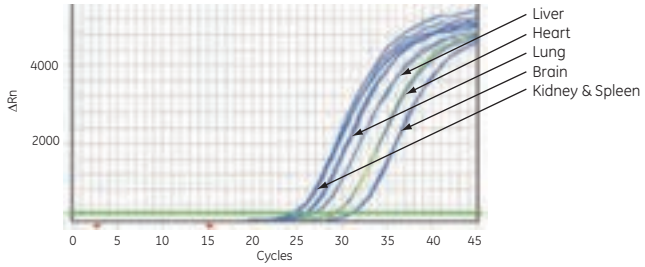


Fig 6.2. RT-qPCR amplification of the c-Fos gene from total RNA isolated from different rat tissues using illustra triplePrep Kit.

Expression microarrays

An expression microarray is a tool for analyzing levels of gene expression. In a typical experiment, many different DNA sequences are "spotted" onto a membrane chip or glass slide. mRNA is typically converted to first-strand cDNA (cRNA), which is labeled using a detectable molecule such as the fluorescent dyes Cy™3 and Cy5. Binding of cRNA molecules to specific DNA sequences is indicated by the presence of the detectable dye.

RNA quality is critical to expression microarrays. We recommend checking RNA quality using an Agilent Bioanalzyer. The RIN value should be at least 7 or higher, and the 28s:18s ratio should be between 1 and 3. UV measurement may be used to analyze RNA purity. The A_{260}/A_{280} ratio is used to indicate protein or DNA contamination. DNA will bind the probes, causing false-positive signals. The A_{260}/A_{230} ratio is used to check for residual salt, which will interfere with labeling efficiency.

Figure 6.3 shows results from a differential expression analysis of hybridized cRNA, demonstrating high concordance between two kidney:liver expression ratios, independently derived from four randomly chosen bioarrays. The n = 19 428 number in Figure 6.4 represents the number of concordantly "good" probes used in the correlation calculation.

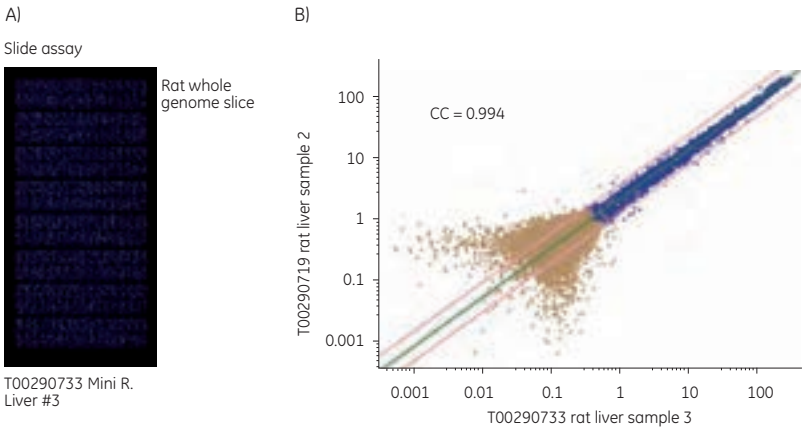


Fig 6.3. Microarray evaluation of total RNA prepared using RNAspin Mini Kit. Labeled cRNA was run on CodeLink™ Rat Whole Genome Bioarrays. **(A)** The low level of background signal is indicated by the entire hybridized array image shown. **(B)** The normalized signal intensity correlation between replicate independent cRNA samples was high (Pearson correlation coefficient of 0.994).

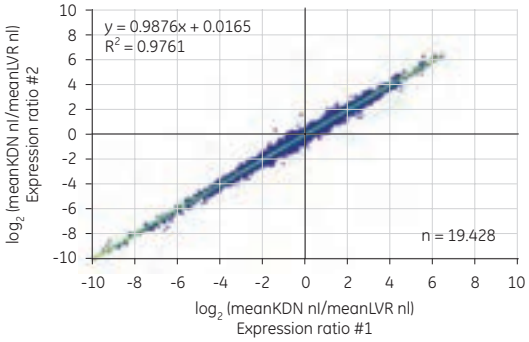


Fig 6.4 . Differential expression analysis of the hybridized cRNA targets using CodeLink Rat Whole Genome Bioarrays.

cDNA synthesis

Classically, cDNA synthesis has been accomplished using reverse transcriptase and oligo(dT) primers, random hexamers, or specific primers to generate cRNA. Second-strand DNA synthesis has been accomplished using a specific RNase and DNA polymerase. Double-stranded DNA may have been further manipulated to prepare it for ligation into a cloning vector to generate a cDNA library. Current methods for cDNA synthesis still rely on reverse transcription but are typically PCR-based (i.e., use RT-PCR). cDNA prepared via RT-PCR can be used for expression studies, library construction, or other applications. The quality requirements for cDNA synthesis are similar to those for expression microarrays. See that section for details.

Northern blots and slot blots

Northern blot analysis remains a mainstay of gene expression analysis. In traditional Northern blots, total RNA is electrophoresed under denaturing conditions. It is then transferred to a membrane for hybridization with a radiolabeled or nonisotopically labeled probe. Northern analysis can be used to compare the relative intensities of bands from different samples on the same membrane. It can also be used to determine the approximate length of hybridized transcripts. Slot and dot blots are similar to Northern blots but eliminate the electrophoresis. Therefore, these techniques are not useful for analyzing transcript length. Northern blots and slot blots are not as sensitive to contaminants as are other methods. However, degraded RNA transcripts will not work well in these applications.

RNase and S1 nuclease protection assays

Nuclease protection assays may be used to quantitate individual mRNAs within a complex mixture of total RNA. In the basic procedure, a radioactive or nonradioactive labeled anti-sense probe is added to a solution of total RNA. After hybridization, any remaining RNA or probe is digested with a nuclease specific for single-stranded RNA. Nuclease protection assays are quicker than Northern blots and are considered to be more quantitative. In addition to detecting and quantitating mRNA, nuclease protection assays may also be used for mapping splice junctions. Genomic DNA and salts are the major contaminants for this application.

in vitro translation

in vitro translation is performed in cell-free extracts, where mRNAs are translated into proteins. Salts, organic solvents, and polysaccharides are the major contaminants for this application.

Troubleshooting

It is good practice to check RNA quality, purity, and yield before using prepared RNA in a downstream application. Troubleshooting downstream applications may require that you go back to the prepared RNA sample. First, check RNA quality and purity using RIN, the 28s:18s ratio, and the A_{260}/A_{280} ratio, as discussed above. If the RNA quality is poor, this is probably the cause of poor results in downstream applications. In this case, either RNA was degraded before the sample was processed or RNA was degraded during preparation. The RNA preparation should be repeated with fresh samples and with more care to prevent RNase contamination during the procedure. If the quality of RNA is good, check the yield of RNA. If the RNA quality is good but the yield is lower than expected, this might be due to incomplete cell lysis, poor binding, or poor elution if using silica purification. The RNA preparation should be repeated with more care to make sure the sample is completely disrupted and the cells completely lysed. If the RNA yield is lower than expected, RNA may need to be concentrated, or more RNA may need to be prepared (e.g., increase sample input). If quality, purity, and yield of RNA are good, the problem may be with the downstream application. See Table 6.5 for more specific troubleshooting information.

Table 6.5. Possible causes and solutions for problems that may occur during RNA preparation.

Problem	Possible cause	Solution
RNA is degraded/is of low quality/purity (e.g., low RIN value, low 28s:18s ratio, or smearing on gel, low A_{260}/A_{280} ratio [< 1.9])	Starting sample was not stored/handled properly.	Endogenous RNases may have degraded the RNA. Follow recommendations for storage and handling of the sample. Quickly add sufficient chaotrope or denaturant to inactivate RNases in sample. Do not let sample thaw if it is already frozen.
	RNases were introduced during/after sample processing.	Make sure to wear gloves at all times and to change them frequently. Follow other laboratory procedures for treating glassware, plasticware, and solutions that will come into contact with RNA.
	Purified total RNA was not stored properly.	Follow recommended storage conditions to minimize degradation. Store RNA at -80°C, especially for extended periods.
Total RNA contains genomic DNA	DNase treatment was not used during preparation or was insufficient.	Treat with DNase. Be sure to completely remove DNase after treatment. For silica purification, if an on-column DNase digestion will be done, washing steps will remove the DNase. Alternatively, perform a phenol/chloroform extraction, precipitate the RNA, and resuspend in RNase-free water (see Appendix 4).
	Purification system was overloaded.	Follow sample size recommendations for your purification system.
Yield of total RNA is low	*If the yield is low but quality is good:*	
	The starting material has low RNA content or the wrong starting material was used (i.e., used some connective tissue instead of the target organ).	Use the correct starting material or more starting material. Do not overload the purification system.
	For silica methods: Elution was not effective.	Apply at least 50 µl of water to the center of the column.
	If both yield and quality are low:	
	Starting sample was not stored/handled properly.	Endogenous RNases may have degraded the RNA. Follow recommendations for storage and handling of the sample. Quickly add sufficient chaotrope or denaturant to inactivate RNases in sample.
	RNases were introduced during/after sample processing.	Make sure to wear gloves at all times and to change them frequently. Follow stringent laboratory procedures for treating glassware, plasticware, and solutions that will come into contact with RNA.
	Sample was not disrupted/ homogenized thoroughly.	Follow disruption/homogenization recommendations for your sample type.
Total RNA performs suboptimally in downstream application	*If RNA quality is poor:*	See troubleshooting tips for poor-quality RNA.
	If RNA quality is good:	
	Contaminants may still be present in the sample.	Do not overload the purification system. This may result in poor yield and/or decreased purity of total RNA.
		If organic solvents were used, residual solvent may be present in the sample. Precipitate the RNA with salt and alcohol. For low concentrations of RNA, add an inert coprecipitant (e.g., glycogen). See Appendix 4.
	For silica purification using ethanolic wash buffer: residual ethanol remains in the sample.	Be sure to thoroughly air dry (e.g., extra spin) the sample prior to elution.
	Genomic DNA is present.	See troubleshooting tips above.

Continues on following page

Continued from previous page

Problem	Possible cause	Solution
For mRNA: Sample contains a significant amount of rRNA	Sample was not disrupted/lysed thoroughly.	Follow disruption/lysis recommendations for your sample type.
	oligo(dT) purification system was overloaded.	Follow sample size recommendations for your purification system. Sample may require a second round of oligo(dT) chromatography.

References

1. Schroeder, A. *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* **7**, 3 (2006).
2. Blackburn, P. Ribonuclease inhibitor from human placenta: rapid purification and assay. *J. Biol. Chem.* **254**, 12484–12487 (1979).
3. Cox, R. A. The use of guanidinium chloride in the isolation of nucleic acids. *Methods Enzymol.* **12B**, 120–129 (1968).
4. Feramisco, J. R. *et al., in Molecular Cloning: A Laboratory Manual,* Maniatis, T. *et al.,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 194 (1982).
5. Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).
6. Cathala G. *et al.* A method for isolation of intact, translationally active ribonucleic acid. *DNA.* **2**, 329–335 (1983).
7. Chirgwin, J. M., *et al.* Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. CsTFA: a centrifugation medium for nucleic acid isolation and purification. *Biochemistry* **18**, 5294–5299 (1979).
8. Birnboim, H. C. Extraction of high molecular weight RNA and DNA from cultured mammalian cells. *Methods Enzymol.* **216**, 154–160 (1992).
9. Logemann, J., *et al.* Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* **163**, 16 (1987).
10. Boom, R. *et al.* Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503 (1990).
11. Ausubel, F. M. *et al., ed. Short protocols in molecular biology: a compendium of current protocols in molecular biology* (5th ed), John Wiley and Sons, Inc. Indianapolis, IN (2002).
12. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A laboratory manual*, Cold Spring Harbor Laboratory, 1st and 2nd eds. (2001).
13. Gerstein, A. S., ed., *Molecular Biology Problem Solver: A Laboratory Guide*, Wiley-Liss, Inc. (2001).

Chapter 7
DNA preparation by amplification

In molecular biology applications, DNA amplification can take many forms, including the ubiquitous PCR, as well as isothermal amplification using Phi29 DNA polymerase. The choice of amplification method depends on a number of factors, for example, the availability of sequence-specific primers, the intended use of the amplified product, and the quantity and quality of source material. When sequence information is available, primer-specific DNA amplification (e.g., PCR) can produce a subpopulation of DNA from the original sample. When cDNA is the objective, RNA is the starting material, so a reverse transcription reaction can be coupled with PCR (RT-PCR). Isothermal amplification (e.g., using Phi29 DNA polymerase) is an option when the amount of input material is small and inadequate sequence information is available to prepare sequence-specific primers.

Amplified genomic DNA and plasmid DNA can be substituted for the original unamplified DNA in most applications. For example, amplified plasmid and other circular DNA are common templates for DNA sequencing reactions. With any amplification method, quality of the output DNA correlates with the quality of the input DNA/RNA.

As is true with other molecular biology applications, DNA amplification is influenced by sample preparation. In some cases, low-quality template can be used. However, in more stringent applications such as long PCR, single-cell or low-copy-number genetic analysis, and rare allele detection, higher-quality RNA or DNA is required. Poor sample preparation can decrease the purity of DNA/RNA and may prevent accurate quantitation by spectrophotometry. Poor quality of DNA/RNA effectively decreases the concentration of usable templates, resulting in a decrease in the signal in PCR experiments and an increase in false-negative results, as well as other problems associated with incorrectly titered template.

Nucleic acid amplification using PCR and RT-PCR

Isothermal amplification is discussed later in this chapter.

PCR is the most commonly used method for amplifying nucleic acid samples. It has widespread utility in both scientific research and applications such as forensics and clinical work. The number of specialized PCR methods continues to grow, and a detailed discussion of all of these is outside the scope of this handbook. Refer to references 1 and 2 for more information. The most common PCR methods are end-point PCR and real-time or quantitative PCR (qPCR). Either of these can be combined with a reverse transcriptase step (RT-PCR and RT-qPCR, respectively) to enable amplification of RNA.

Sample preparation considerations for DNA amplification using PCR and RT-PCR

The yield, fidelity, and quality of any amplification reaction can be influenced by a number of variables, one of which is sample preparation. DNA and RNA for PCR and RT-PCR, respectively, can come from a range of sources, including cell cultures, blood, bacterial cultures, and plant and animal tissues. Potential contaminants to PCR may be sample specific or common to most sample types. Some contaminants may be introduced during sample preparation. A list of some common potential contaminants and their effects are summarized in Table 7.1.

Table 7.1. Summary of possible contaminants that may interfere with DNA amplification.

Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
Genomic DNA	Sample	RT-PCR	Primers may anneal to genomic DNA; this may cause extra bands in PCR. Contamination with genomic DNA may require the use of more stringently designed primers and optimal PCR conditions.
PCR product from a previous PCR amplification	Pipettors or lab environment if care is not taken to reduce cross-contamination	qPCR/PCR	Will support amplicon production in the absence of template
Nucleases	Sample or may be introduced exogenously	DNase activity inhibits all applications; RNases inhibit RT-PCR	Nucleases degrade template DNA or RNA.
Alcohols	May be introduced during sample preparation	All	Inhibit enzyme activity
Organic solvents such as phenol/chloroform	May be introduced during sample preparation	Residual organic solvent contaminants will interfere with the majority of routine molecular biology techniques	Inhibit enzyme activity
Polysaccharides	Plant tissue	All	Inhibit enzyme activity
Heme	Blood	All	Inhibit enzyme activity
Heparin	Preserved blood	All	Inhibit enzyme activity
EDTA	May be introduced during sample preparation	PCR, qPCR, RT-PCR, RT-qPCR	EDTA present in the RNA solution will chelate Mg^{2+} required for PCR and may lead to suboptimal results; can add additional Mg^{2+} to reaction to compensate.

Overview of Ready-To-Go™ bead technology for PCR and RT-PCR

illustra Ready-To-Go PCR beads from GE Healthcare are premixed, predispensed, single-dose reactions for performing PCR amplification. Each ambient-temperature-stable bead provides the convenience of a single-dose reaction that only requires the addition of PCR primers, template, and water. Ready-To-Go PCR Beads are available in several formulations and formats, including standard PCR, "hot start" PCR, RT-PCR, and RAPD analysis. All bead types use high-purity reagents, such as recombinant Taq DNA polymerase, ensuring the lowest possible levels of contaminating DNA in each bead. The bead format significantly reduces the number of pipetting steps, thereby decreasing handling errors, pipetting errors, and contamination of subsequent reactions, all of which increases overall reproducibility. Functional testing of each lot of Ready-To-Go Beads ensures that each lot will deliver similar results. PuReTaq™ Ready-To-Go PCR Beads and Ready-To-Go RT-PCR Beads are provided in 0.5 ml tubes, 0.2 ml tubes, or in a 96-well format. Custom sizes and formulations of Ready-To-Go beads are also available.

DNA amplification using illustra PuReTaq Ready-To-Go PCR Beads

PuReTaq Ready-To-Go PCR Beads from GE Healthcare contain all the necessary reagents (except primer, template, and water) for 25 µl PCR amplifications. The beads are available predispensed into 0.2 ml thin-walled multiwell plates (sufficient for 96 reactions), 0.2 ml thin-walled tubes, or 0.5 ml PCR tubes.

PuReTaq PCR Beads have passed rigorous quality tests to ensure the lowest possible levels of contaminating prokaryotic and eukaryotic nucleic acids.

A protocol for use of Ready-To-Go PuReTaq PCR Beads is provided below.

When performing PCR, exercise extreme care to prevent contamination by nucleic acids. Always use sterile filter pipette tips and microcentrifuge tubes, and avoid carry-over contamination of stock solutions. See Chapter 2 for precautions against introducing DNA contaminants.

For most standard, three-step PCR reactions, 35 cycles results in a 10^5- to 10^9-fold amplification of the target sequence. The yield of PCR product may be increased by increasing the number of cycles to 45. However, an increased number of cycles may also produce spurious bands and increased background. The duration of each step when using a "rapid cycler" such as a PerkinElmer™ 9600 thermal cycler (or equivalent) should be approximately half the time as when using a PerkinElmer 480 thermal cycler (or equivalent).

Materials

Each PuReTaq bead contains stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of PuReTaq DNA polymerase, and reaction buffer. When a bead is reconstituted to a 25 µl final volume, the concentration of each dNTP is 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, and 1.5 mM $MgCl_2$.

Template-specific primers

Thermal cycler

Mineral oil (if required for your thermal cycler)

Optional: 10 mM $MgCl_2$ (if desired to increase final Mg^{2+} concentration above 1.5 mM)

Advance preparation

Prepare the PCR beads as follows: Remove the desired quantity of tubes from the foil pouch. Examine these tubes to verify that a bead is visible at the bottom of each tube. If necessary, gently tap the tube against a hard surface to force each bead to the bottom of the tube. Place the tubes into a container that allows easy access during your experiment.

Protocol

1. Add template DNA, primers, and water to bead

For each reaction, add template DNA, template-specific primers, and water to a PCR bead. Optional: Add additional $MgCl_2$ (see Table 7.2, below).

2. Snap caps onto tubes. Mix.

Flick the tube, vortex gently, and centrifuge briefly.

3. Place tubes on ice or in cold block

4. Perform thermal cycling

Add mineral oil if needed for your thermal cycler and replace caps. Perform thermal cycling according to the instructions for your thermal cycler.

The optimal cycling profile for a given PCR system and thermal cycler will vary and must be determined empirically. Cycle number can range from 20 to 40 depending on the desired yield of product. Thermal cycling results and product yield can vary with cycle conditions and thermal cycler used. Read the instructions provided with your thermal cycler and optimize reaction conditions accordingly.

When each PCR bead is rehydrated in a reaction volume of 25 µl, the mixture will contain 1.5 mM $MgCl_2$. If a higher concentration of Mg^{2+} is desired, Table 7.2 can be used to determine the volume of a sterile 10 mM $MgCl_2$ solution that should be added to increase the Mg^{2+} concentration of the reaction. If $MgCl_2$ is added to the reaction, decrease the amount of water added to the reaction to maintain a final reaction volume of 25 µl.

Table 7.2. Adjusting the reaction concentration of Mg^{2+} in PuReTaq PCR Bead reactions.

Final [$MgCl_2$]	Volume of 10 mM $MgCl_2$ to add
2.0 mM	1.25 µl
2.5 mM	2.50 µl
3.0 mM	3.75 µl
3.5 mM	5.00 µl
4.0 mM	6.25 µl
4.5 mM	7.50 µl
5.0 mM	8.75 µl

Application data using illustra PuReTaq Ready-To-Go PCR Beads are shown later in this chapter.

Amplification using illustra Ready-To-Go RT-PCR Beads

When RNA is the starting material, PCR products can be generated through a cDNA intermediate via RT-PCR. illustra Ready-To-Go RT-PCR Beads provide the reagents for RT-PCR in a convenient ambient-temperature-stable bead. Each bead is optimized to allow the first-strand cDNA

synthesis and PCR to proceed sequentially as a single-tube, single-step reaction. Simply add RNA, first-strand primer, and PCR primers to the reaction, incubate at 42°C for 15 min, and cycle. Even cDNA made from relatively rare transcripts can be successfully amplified using this technique. RT-PCR can also be performed in two steps if desired.

Each illustra Ready-To-Go RT-PCR Bead contains M-MuLV reverse transcriptase, and RNAguard™ ribonuclease inhibitor, in addition to all the reagents required for PCR. The only reagents that must be added to the reaction are template RNA, a first-strand primer, PCR primers, and water. The first-strand cDNA synthesis reaction can utilize different types of primers: oligo(dT), a random primer such as $pd(N)_6$, or a custom (gene-specific) primer complementary to a specific mRNA sequence. PCR may be primed with two gene-specific primers, or with $pd(T)_{12–18}$ and a single gene-specific primer.

A protocol for using illustra Ready-To-Go RT-PCR Beads is provided below.

When performing PCR, exercise extreme care to prevent contamination by nucleic acids. Always use sterile filter pipette tips and microcentrifuge tubes, and avoid carry-over contamination of stock solutions. See Chapter 2 for precautions against introducing DNA contaminants and RNases.

For most standard, three-step PCR reactions, 35 cycles results in a 10^5- to 10^9-fold amplification of the target sequence. The yield of PCR product may be increased by increasing the number of cycles to 45. However, an increased number of cycles may also produce spurious bands and increased background. The duration of each step when using a "rapid cycler" such as a PerkinElmer 9600 thermal cycler (or equivalent) should be approximately half the time as when using a PerkinElmer 480 thermal cycler (or equivalent).

Materials

RT-PCR Beads, control mix beads, and lyophilized $pd(N)_6$ and $pd(T)_{12–18}$ are provided with the product. In addition, extra bubble caps are provided with the 0.2 ml product format. When brought to a final volume of 50 µl, each reaction will contain ~2.0 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 60 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, M-MuLV Reverse Transcriptase (FPLC*pure*™), RNAguard Ribonuclease Inhibitor (porcine), and stabilizers, including RNase/DNase-Free BSA.

Template-specific primers

DEPC-treated water (see Appendix 4 for preparation)

Thermal cycler

Heating block or water bath at 42°C

Mineral oil (if required for your thermal cycler)

Optional: 25 mM $MgCl_2$ (if desired to increase final concentration above 1.5 mM)

Advance preparation

To use all 96 tubes at once for the plate format*:

Remove the plate from the pouch. Check that the bead in each tube is visible at the bottom of the tube. If necessary, gently tap the plate against a hard surface to force each bead to the bottom of the tube. Place the plate, paper side up, on the bench top or other hard surface. Carefully pull off the paper and foil simultaneously and discard both.

* *Fewer than 96 tubes may be used. See product instructions.*

*One-Step RT-PCR Protocol**

1. Place tubes or multiwell plate containing RT-PCR beads on ice

If desired, one or more tubes may be used for a negative control to test for DNA contamination.

2. Determine volume of RNA, primers, and water to add

Determine the total volume of RNA and primers that will be used in an RT-PCR Bead reaction. If additional Mg^{2+} is required, determine the volume to add (see Table 7.3). Calculate the amount of DEPC-treated water that will give a final volume of 50 µl.

If using the two-step protocol, leave the PCR primers out of the reaction. The combined total volume of the two PCR primers should not exceed 10 µl.

3. Add RNase-free water. Incubate briefly on ice.

4. Flick tube or pipette to mix

5. Optional: Prepare negative control reaction

6. Add template and primers individually

Add these to each tube.

7. Prepare positive control

Add a control mix bead to one of the tubes.

8. Add mineral oil if required. Close caps.

9. Incubate briefly at 42°C

Incubate for 15 to 30 min to generate cDNA.

10. Incubate briefly at 95°C

This will inactivate the reverse transcriptase and completely denature the template.

If using the two-step protocol, add the PCR primers after the heat denaturing of the reverse transcriptase and proceed with the cycling step (Step 11).

11. Perform thermal cycling

Cycle 20 to 45 times depending on the abundance of the target and your specific thermal cycler.

* *RT-PCR can also be performed in two steps; see product instructions for further detail. The one-step protocol is most convenient because primers for first-strand cDNA synthesis and PCR are simultaneously added with the template to an RT-PCR Bead. Most RNA templates will amplify using the one-step protocol. The two-step protocol for RT-PCR is slightly less convenient because only the first-strand cDNA synthesis primers are added with the RNA template, so each tube must be opened prior to PCR amplification to add the gene-specific PCR primers. In certain cases, the two-step protocol will produce higher yields and greater specificity than the one-step protocol. For example, if nonspecific priming is a problem or if an extremely low amount of template RNA is applied, the two-step protocol may generate better results.*

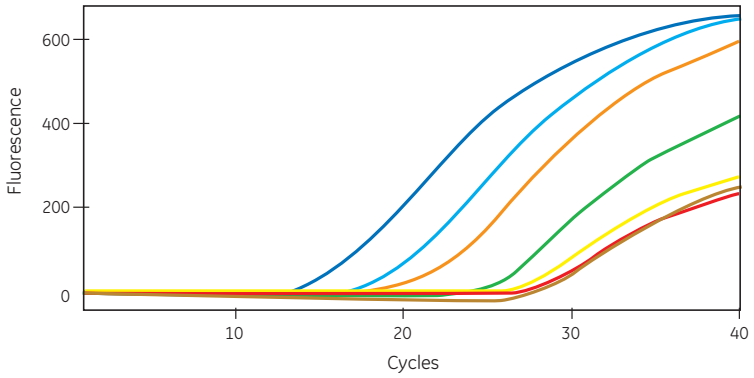
Use the information in Table 7.3 to determine the volume of a sterile 25 mM $MgCl_2$ solution that should be added to increase the Mg^{2+} concentration of the reaction. If extra $MgCl_2$ is added, decrease the amount of water added to the reaction so that the final volume equals 50 µl.

Table 7.3. Adjusting the reaction concentration of Mg^{2+} in RT-PCR bead reactions.

Final [$MgCl_2$]	Volume of 25 mM $MgCl_2$
2.0 mM	1.0 µl
2.5 mM	2.0 µl
3.0 mM	3.0 µl
3.5 mM	4.0 µl
4.0 mM	5.0 µl

Amplification results using illustra Ready-To-Go PCR and RT-PCR Beads

To assess the performance of PuReTaq Ready-To-Go PCR Beads, real-time PCR was run using TaqMan probes and *E. coli* DNA. As shown in Figure 7.1A, the beads produced robust amplification curves and low background fluorescence. In addition, as shown in Figure 7.1B, template titrations were linear after optimizing Mg^{2+} and cycling conditions; no other additions to the PuReTaq bead were necessary to optimize these representative reactions. The results shown here were obtained by running reactions on the Smart Cycler™, but comparable performance was observed when tests were completed on the ABI PRISM™ 7700 and 7900 Sequence Detection Systems or on the Rotor-Gene™ 3000 (data not shown). The beads used in these assays were stored at room temperature according to the manufacturer's instructions, and no dropoff in performance was observed when the same tests, under the same conditions, were run over one year later.



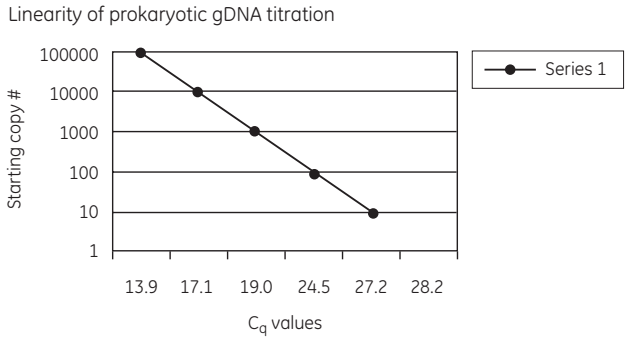
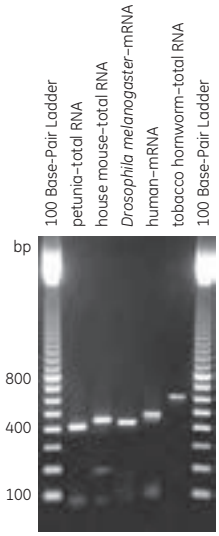


Fig 7.1. (A) Real-time PCR amplification curves obtained for prokaryotic template and no template controls. Template titrations each contain one PuReTaq Ready-To-Go PCR Bead; 10^5 to 10 copies of genomic DNA from E. coli DH1λ; 20 pmol of 16s rRNA primers (forward and reverse); 5 pmol of 16s rRNA FAM-TAMRA probe; 6.1 mM $MgCl_2$; and sterile H_2O. Reactions were amplified as follows: 95°C for 300 s, then 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 40 cycles. **(B)** TaqMan assay reproducibility. Real-time PCR data highlighting reproducibility for 11 replicates of 10^5 to 10 copies of E. coli (DH1λ) genomic DNA.

illustra Ready-To-Go RT-PCR Beads can be used with either total RNA or mRNA isolated using various methods (Fig 7.2).



RNA amount from organism	Target gene
20 ng of total RNA from petunia	light harvesting complex I (3)
2 ng of total RNA from house mouse *(Mus musculus)*	mitotic checkpoint regulator (4)
250 ng of mRNA from *Drosophila*	female sterile homeotic system (5)
100 pg of mRNA from human	*p53* (6)
20 ng of total RNA from tobacco hornworm *(Manduca sexta)*	hemolymph juvenile hormone binding protein (7)

Fig 7.2. RT-PCR detection of specific mRNAs from a range of sources. Source organisms and RNAs are indicated above the lanes. The target genes for amplification and the amounts of RNA used are indicated in the table that accompanies the figure. Consult references 3–7 for further information on the different source materials.

Troubleshooting PCR and RT-PCR amplification

Many of the common problems with PCR and RT-PCR are identified following agarose gel electrophoresis of the reaction products. These include the absence of the expected amplification product, the presence of nonspecific products, excessive smearing, and the presence of a "primer dimer" band. A summary of the possible causes and solutions for these problems is provided in Table 7.4.

Table 7.4. Possible causes and solutions for problems with PCR and RT-PCR amplification.

Problem	Possible cause	Solution
No bands on gel	The thermal cycler was not functioning properly.	If a positive control was not prepared, consider testing reagents in a control reaction with template and primers previously shown to function properly.
	Insufficient template or too much template was added.	Titrate the amount of template required for your system.
	For DNA: Template quality was poor or contains inhibitors.	Impure DNA can fail to amplify properly. Use freshly prepared DNA or isolate template by another method.
	Primer concentration was too low or was unbalanced.	Make sure primer concentration is within recommended range and that concentration of both PCR primers is the same.
	Nucleases were introduced.	Prepare new template. Be sure to follow precautions against introducing nucleases. This is particularly important when the starting material is RNA.
	For RNA: Template is in buffer containing EDTA.	EDTA present in the RNA solution will chelate Mg^{2+} required for the RT-PCR and may lead to suboptimal results. To compensate, add additional Mg^{2+} when assembling the reactions. Refer to product instructions for details.
	Reagents were not thawed properly prior to use.	Thaw and thoroughly mix all reagents before setting up reactions.
	Primer annealing temperature was too high.	Decrease annealing temperature in 2°C increments.
	Denaturing temperature was suboptimal.	Try increasing the temperature or duration time of the initial denaturation step. This is especially useful for GC rich templates.
	Extension time was too short.	Increase extension times. This can be especially important for long PCR.
	Thermal cycler was not at correct temperature.	Repair/calibrate the thermal cycler or use a different machine.
Extra, nonspecific bands on gel	Primers hybridized to a secondary site on the template.	Design new primers that are less specific for the secondary site. Increase the annealing temperature by increments of 2°C to 5°C.
	DNA contamination was introduced in primers or buffers.	Run a negative control reaction (no template). Prepare fresh materials if contamination is detected.
	For RNA: Template is contaminated with DNA.	Perform a DNase step and completely inactivate/remove DNase. Alternatively, prepare RNA using a different method.
	Too much template was added.	Titrate the amount of template for your system.
	Primer concentration was too high.	Titrate the amount of primers for your system.
	Thermal cycler was not at correct temperature.	Repair/calibrate the thermal cycler or use a different machine.

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Problem	Possible cause	Solution
Excessive smearing of amplified DNA after gel analysis	Too much template was added.	Titrate the amount of template for your system.
	Too many cycles were performed.	Reduce the number of cycles below 35.
	Thermal cycling conditions were not optimal for your thermal cycler.	Optimize conditions based on manufacturer's recommendations.
	The annealing temperature was too low.	When using Ready-To-Go Beads, re-optimization of the annealing temperature might be required. Increase the annealing temperature by increments of 2°C to 5°C.
	Primer concentration was too high.	Titrate the amount of primers for your system.
	Extension time was too long.	Decrease extension time in small increments.
	For DNA: Template quality was low.	Impure DNA can fail to amplify properly. Use freshly prepared DNA or isolate template by another method.
	Too much DNA was loaded on gel.	Re-run with less sample.
Primer dimers visible after gel analysis	Too much primer was added/ primer concentration too high.	Titrate the amount of primers for your system.
	Primers possess complementary overlapping sequence.	Design primers to avoid self-complementary internal sequences.
		Increase annealing temperature. Use illustra Hot Start Mix PCR products.

Isothermal DNA amplification

Commercial kits for isothermal amplification are available from several suppliers. Their utility for different types of template may vary. Although isothermal amplification procedures that utilize Phi29 DNA polymerase are generally quite resistant to inhibition by mild contamination with salts, culture media, and cell debris, results may be affected by poor quality template as discussed later in this paragraph. Therefore, DNA should be handled with care during sample preparation. If the template is a circular DNA strand, and RCA is being used, DNA nicks and strand breaks can significantly reduce amplification potential. The likelihood of nicking and strand breaks in circular templates increases as the size of the molecule increases. Because preparation of large intact circular molecules (e.g., BACs) can be quite difficult, care should be taken to avoid rapid pipetting and vortexing. If samples are linear DNA, and multiple displacement amplification is being used, the reaction relies on hybridization of multiple primers to each DNA strand for maximal amplification. Samples containing nicks and breaks can fail to amplify well, and amplification products may contain a number of rearranged products. This may have deleterious effects on downstream applications (e.g., DNA cloning or sequencing). GE Healthcare provides optimized reagents and protocols for difficult templates such as BACs (illustra TempliPhi™ Sequence Resolver Kit) and larger templates (illustra TempliPhi Large Construct Kit).

DNA amplification using TempliPhi and GenomiPhi DNA Amplification Kits

Circular DNA amplification using TempliPhi

illustra TempliPhi DNA Amplification Kits from GE Healthcare are novel products developed to exponentially amplify single- or double-stranded circular DNA templates by RCA, shown schematically in Figure 7.3 (8, 9). Amplified products can be used in downstream applications

such as sequencing and molecular cloning. TempliPhi DNA Amplification Kit provides RCA reagents optimized for circular DNA amplification. The reaction utilizes modified random primers to efficiently prime synthesis by the extremely processive, high-fidelity Phi29 DNA polymerase in a single-step isothermal reaction.

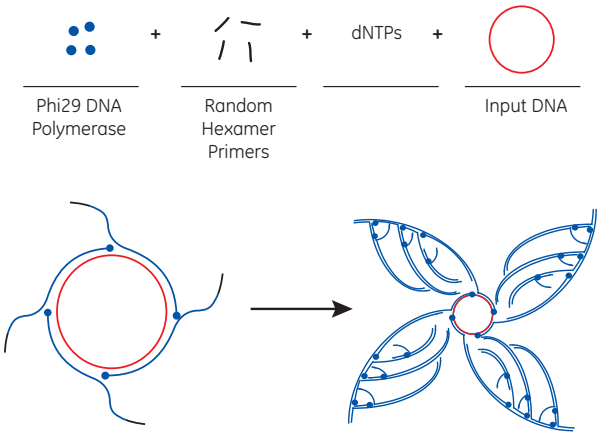


Fig 7.3. Schematic showing the TempliPhi method of RCA.

The starting material for TempliPhi amplification can be a variety of sources—a small number of bacterial cells containing a plasmid, an isolated plasmid, intact M13 phage, or any circular DNA (including ligation reactions) are efficiently amplified. Bacterial colonies can be picked from agar plates, added to a sample buffer, and heated to release the plasmid DNA from the cells. The polymerase is added directly to this material to initiate the amplification reaction. Alternatively, sub-microliter quantities of a saturated bacterial culture or a glycerol stock can be used. Depending on the source of starting material, amplification is completed in 4 to 18 hours at 30°C with no need for thermal cycling. The product of the TempliPhi reaction is high-molecular-weight, double-stranded, tandem-repeated copies of the circular template. Note that when M13 clones are the source material, the TempliPhi product is double-stranded DNA that can be sequenced with both forward and reverse primers. DNA amplified by the TempliPhi method can be used directly in many subsequent applications including cycle sequencing reactions, restriction enzyme analysis, DNA cloning procedures, and *in vitro* transcription reactions in place of supercoiled plasmid DNA. illustra TempliPhi DNA Amplification Kits are used to prepare DNA directly from circular DNA. illustra TempliPhi Large Construct DNA Amplification Kit is designed for large DNA vector (e.g., fosmid and BAC) preparation. illustra TemliPhi HT DNA Amplification Kit is designed for high-throughput use (over 1000 preps/wk). illustra TempliPhi Sequence Resolver Kit is designed to produce a different type of DNA that sequences more easily, solving the most common sequencing problems, and implements a simplified protocol with fewer steps and no overnight culturing.

A protocol for DNA amplification using TempliPhi Amplification Kits follows.

Materials

Sample buffer, reaction buffer, enzyme mix, and positive control DNA are provided with the product

Thermal cycler or water baths for incubations at 30°C, 65°C, and 95°C

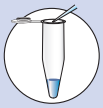
For single- or low-copy number BACs and fosmids: alkaline lysis reagents (see product instructions)

Advance preparation

Thaw sample buffer and reaction buffer on ice.

*Protocol**

1. Mix sample buffer with template DNA†



2. Denature sample at 95°C and cool

This step lyses bacterial cells or phage particles sufficiently to release the circular template into the liquid.

Avoid heating at higher temperatures or for longer than 3 min, which can release bacterial DNA that competes with the desired template during amplification.

3. Prepare enzyme master mix

Prepare the master mix for all the amplification reactions.

4. Transfer to cooled sample

Transfer enzyme master mix to the cooled sample.

5. Incubate at 30°C

DNA is amplified during this step. The time will vary (4 to 18 h) with the amount of input DNA and desired amount of output DNA.

6. Heat-inactivate enzyme

Inactivate the enzyme by incubating briefly at 65°C. Cool to 4°C.

Inactivating the DNA polymerase activity prevents potential interference with subsequent cycle sequencing reactions. Inactivating the proofreading exonuclease activity prevents degradation of template DNA during storage. The heat inactivation step can be eliminated if the amplified DNA sample is used immediately.

* *The protocol can be automated; see Chapter 9.*

† *Alkaline lysis is recommended to denature cultures containing low-copy-number BACs and fosmids to reduce DNA fragmentation caused by thermal degradation; this is unnecessary for cultures containing plasmids or M13 phage.*

DNA amplification results with illustra TempliPhi DNA Amplification Kits

As discussed earlier in this chapter, TempliPhi amplified DNA can be used in the same applications as unamplified DNA. Plasmid, M13, and other circular DNAs are typically used as templates for sequencing (see Chapter 5). TempliPhi amplified plasmid DNA is of high quality and generates accurate sequencing results in automated cycle sequencing (Fig 7.4.).

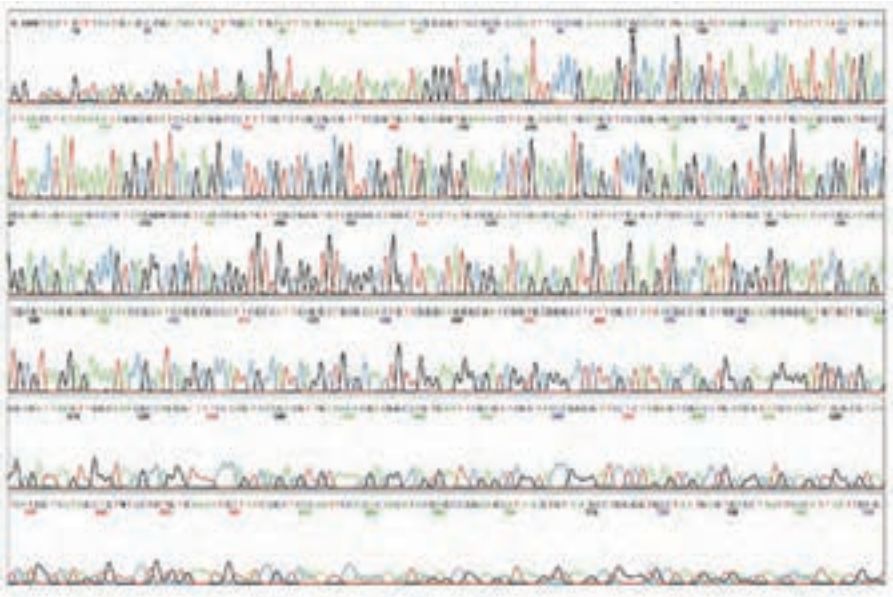


Fig 7.4. Sequence data generated from plasmid DNA amplified with illustra TempliPhi 100 Amplification Kit. Amplified DNA was subsequently sequenced using DYEnamic™ ET Terminator Cycle Sequencing Kit and analyzed on an ABI PRISM 3100 Genetic Analyzer.

Whole genome amplification (WGA) using GenomiPhi

The GenomiPhi™ method of WGA from GE Healthcare uses Phi29 DNA polymerase, a highly processive enzyme with excellent strand-displacement activity, in combination with random-sequence hexamer primers (random hexamers) to amplify DNA in an isothermal process—a thermal cycler is not required. First, random hexamers anneal to multiple sites on the denatured linear DNA template. Next, Phi29 DNA polymerase initiates replication at these sites simultaneously. As synthesis proceeds, strand displacement of upstream replicated DNA generates new single-stranded DNA. Subsequent priming and strand displacement replication of this DNA produces large quantities of several-kilobase-long, double-stranded DNA suitable for genetic analyses that require high-molecular-weight product (Fig 7.5). Microgram quantities of high-molecular-weight DNA can be generated from as little as 10 ng of genomic DNA using this simple and robust procedure. DNA replication is extremely accurate due to the proofreading 3′–5′ exonuclease activity inherent in Phi29 DNA polymerase (10, 11). Note that the GenomiPhi method does not preserve epigenetic methylation patterns.

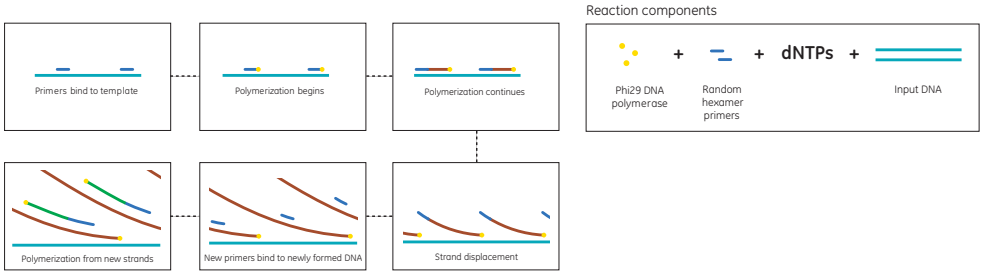


Fig 7.5. Schematic showing the GenomiPhi method of genomic DNA amplification for WGA.

A protocol for DNA amplification using GenomiPhi Amplification Kits is provided below.

Materials

Sample buffer, reaction buffer, enzyme mix, and positive control DNA are provided with the product

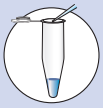
Thermal cycler or water baths for incubations at 30°C, 65°C, and 95°C

Advance preparation

Thaw sample buffer and reaction buffer on ice.

*Protocol**

1. Mix sample buffer with template DNA



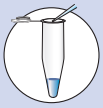
2. Denature sample at 95°C and cool†

Heating the DNA for longer than 3 min or at higher temperatures can damage the DNA.

3. Prepare enzyme master mix

Prepare the master mix for all the amplification reactions.

4. Transfer to cooled sample



Transfer enzyme master mix to the cooled sample.

5. Incubate at 30°C

DNA is amplified during this step. The time will vary (1.5 to 18 h) with the amount of input DNA and desired amount of output DNA.

6. Heat-inactivate enzyme

Inactivate the enzyme by incubating briefly at 65°C. Cool to 4°C.

Heating is required to inactivate the exonuclease activity of the DNA polymerase which may otherwise begin to degrade the amplification product.

* *The protocol can be automated; see Chapter 9.*

† *As an alternative, genomic DNA template may be chemically denatured. See product instructions for details.*

DNA amplification results with illustra GenomiPhi DNA Amplification Kits

As discussed earlier in this chapter, amplified genomic DNA can be used in the same applications as unamplified DNA. For WGA, common applications include library construction, forensic analysis (e.g., DNA fingerprinting), genotyping (e.g., SNP analysis), PCR analysis, DNA cloning, CGH, whole genome DNA sequencing, HLA typing, and loss of heterozygosity (LOH).

Figure 7.6 shows that microgram quantities of genomic DNA can be amplified from small amounts of input DNA using GenomiPhi V2 kit.

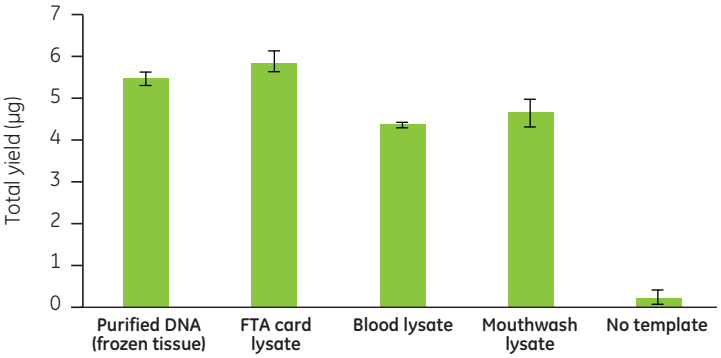


Fig 7.6. Genomic DNA preparation using illustra GenomiPhi V2 kit from various source materials.

Figure 7.7 shows that amplified DNA performs similarly to unamplified genomic DNA in SNP analysis.

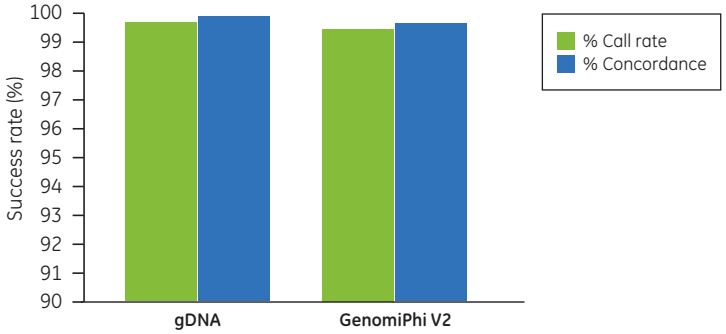


Fig 7.7. SNP analysis of amplified products using Affymetrix GeneChip™ 10K human genome chip. Individual human genomic DNA (gDNA) obtained from Coriell was amplified with illustra GenomiPhi V2 kit and subjected to analysis on Affymetrix 10K SNP chip (green bar = % call rate; blue bar = % concordance).

References

1. Bartlett, J. and Stirling, D. eds. *PCR Protocols (Methods in Molecular Biology).* Humana Press, Inc., New Jersey (2003).
2. Innis, M. *et al. PCR Applications: Protocols for Functional Genomics*. Academic Press (1999).
3. Stayton M. M. *et al.* Characterization of a full-length petunia cDNA clone encoding a polypeptide of the light-harvesting complex associated with photosystem I. *Plant Mol. Biol.* **10**, 127–137 (1987).
4. Starborg M. *et al.* A novel murine gene encoding a 216-kDa protein is related to a mitotic checkpoint regulator previously identified in *Aspergillus nidulans*. *J. Biol. Chem.* **269**, 24133–24137 (1994).
5. Haynes, S. R. *et al.* The Drosophila fsh locus, a maternal effect homeotic gene, encodes apparent membrane proteins. *Dev. Biol.* **134**, 246–257 (1989).
6. Lamb, P. and Crawford, L. Characterization of the human p53 gene. *Mol. and Cell. Biol.* **6**, 1379–1386 (1986).
7. Goodman, W. G. and Chang, E.S. in *Comprehensive Insect Physiology and Pharmacology* (Kerkut, G. A. and Gilbert, L. I., eds.) pp 491, Pergamon Press, Oxford, England (1985).
8. Dean, F. *et al.* Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Research* **11**, 1095–1099 (2001).
9. Lizardi, P. *et al.* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* **19**, 225–232 (1998).
10. Estaban, J. A. *et al.* Fidelity of phi 29 DNA polymerase. Comparison between protein-primed initiation and DNA polymerization. *J. Biol. Chem.* **268**, 2719–2726 (1993).
11. Nelson, J. R. *et al.* TempliPhi, phi29 DNA polymerase based rolling circle amplification of templates for DNA sequencing. *BioTechniques* **32**, S44–S47 (2002).

Chapter 8
Nucleic acid cleanup

Nucleic acid cleanup encompasses the removal of salts, nucleotides, primers, and other contaminants from nucleic acid samples. Typically, this step follows an enzymatic reaction (e.g., PCR, restriction digest, DNA modification, or labeling) but can also be performed after agarose gel electrophoresis of the reaction products. The options for cleanup depend on the sample type, contaminants to be removed, and subsequent application of the nucleic acid of interest. Purification of nucleic acids may be desirable to maximize the quality of the results and the likelihood of success in the intended downstream application. This chapter focuses on the major categories for DNA cleanup and major applications of the cleaned up unlabeled or labeled DNA. Some of the major downstream applications following DNA cleanup are PCR, hybridization (e.g., blot or microarray), automated sequencing/electrophoresis, ligation, and cloning.

Table 8.1. Summary of possible contaminants in DNA reactions and applications in which those contaminants may interfere.

Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
Salts/ions	Reaction mixture or may be introduced during cleanup (e.g., with chaotrope used in silica binding or alcohol precipitation)	Any reaction that utilizes an enzyme, (e.g., PCR, sequencing, restriction digestion, and DNA modification reactions, including ligation and labeling)	Excess salt may inhibit enzyme activity.
Enzymes	Reaction mixture or may be introduced during cleanup (e.g., nucleases and phosphatases)	Ligation and cloning	Contaminating enzymes could modify the wrong DNA fragment.
Nucleotides (labeled or unlabeled)	Reaction mixture	Automated sequencing and hybridization, including microarrays	Unincorporated labeled nucleotides may cause high background in autosequencing and hybridization.
		Determination of DNA yield by UV spectrophotometry	Presence of nucleotides will contribute to a high A_{260} reading. Operator might confuse presence of nucleotides for high DNA yield and use insufficient DNA quantities in downstream applications. Also may affect efficiency of sequencing or labeling reactions by competing with ddNTPs.
Primers (labeled or unlabeled)	Reaction mixture	PCR, sequencing, labeling	Wrong DNA sequence could be amplified, sequenced, or labeled.
"Primer dimers"	Reaction mixture	Ligation	Primer dimers could be ligated instead of desired DNA fragment.
		PCR	Inhibits PCR amplification because primers are not available to start the reaction

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Possible contaminant	Source	Application(s) in which contaminant may interfere	How it may interfere
Organic solvents (e.g., phenol)	May be introduced during cleanup	Residual organic solvent contaminants will interfere with the majority of routine molecular biology techniques	Inhibits enzyme activity
Alcohol (e.g., ethanol, isopropanol)	May be introduced during cleanup (e.g., with silica binding or alcohol precipitation)	Residual alcohol will interfere with the majority of routine molecular biology techniques May also lead to reduced yield from cleanup step	Inhibits enzyme activity
Contaminants from agarose gel	May be introduced when DNA is electrophoresed	Contaminants can interfere with the majority of routine molecular biology techniques May also lead to reduced yield from cleanup step	Inhibits enzyme activity

Techniques for DNA cleanup

Cleanup of PCR products and restricted DNAs

After PCR amplification, the desired PCR product is contaminated with remaining single-stranded oligonucleotide primers, short (< 50 bp) double-stranded DNA ("primer dimers"), unincorporated dNTPs, thermostable polymerase, buffer salts, and possibly other reaction components (e.g., mineral oil). These contaminants are potential inhibitors of subsequent downstream applications, especially cloning or sequencing of the PCR product. They may also produce arbitrary results or cause high background levels. Potential inhibitors may also be present in a reaction mixture following DNA modification or restriction enzyme digestion. PCR and other reaction mixtures are typically cleaned up directly. However, when a particular PCR product or restricted DNA is required, agarose gel electrophoresis may be performed to allow isolation of the band of interest.

The classical method for purifying and/or concentrating DNA is by alcohol precipitation (1), which is frequently preceded by phenol/chloroform extraction (2, 3). However, silica adsorption and gel filtration are two popular techniques that are currently used for cleanup of reaction mixtures containing PCR fragments or restricted DNAs.

Silica-based DNA cleanup products bind DNA in the presence of high concentrations of chaotropic salts and certain pH conditions, while contaminants such as primers, dNTPs, enzyme, and buffer salts are washed away. Silica adsorption is the most highly recommended method for DNA cleanup from PCR amplifications, agarose gel slices, and enzymatic reactions because it removes enzyme from the reaction mixture and can also be used to concentrate DNA. In addition, silica may be used to purify fluorescent-labeled DNA (e.g., Cy3- or Cy5-labeled cDNA). Silica is typically provided as a solid phase such as a membrane or filter, both of which are amenable to increased sample throughput.

Gel filtration products such as Sephadex™ or Sephacryl™ in a microcentrifuge-based column format can be used to purify PCR products or DNA of interest directly from reaction mixture contaminants in approximately 5 min. The contaminants, which are smaller than the DNA product, are retained by the gel matrix while the DNA product is not. Sephadex G-25 and G-50 are typically used to remove unincorporated nucleotides and primers from reaction mixtures. Note that enzyme is not removed using the gel filtration method, which is discussed in more detail later in this chapter. Considerations for gel filtration in a spin format are discussed later in this chapter.

An alternative option for PCR cleanup prior to sequencing is enzymatic treatment. ExoProStar™ from GE Healthcare uses a mixture of two enzymes to degrade unincorporated nucleotides and primers. Figure 8.1 provides an overview of the ExoProStar method. Additional details on ExoProStar are provided in Chapter 9.

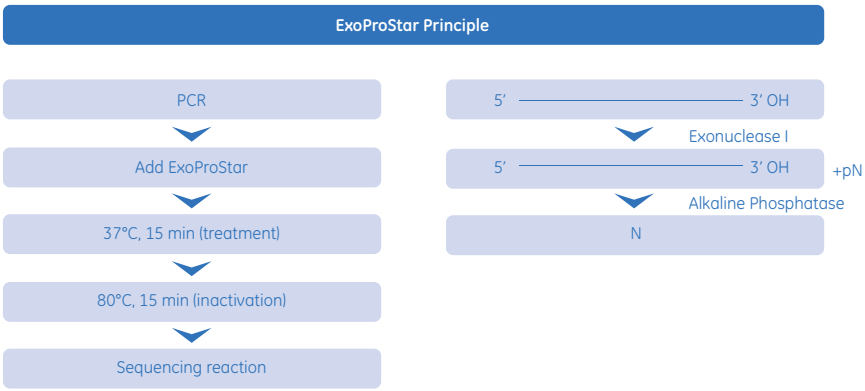

Fig 8.1. ExoProStar enzymatic method for PCR product cleanup. N = nucleotides.

Cleanup of DNA fragments from agarose gels

For some applications, it may be critical for the fragment of interest to be free of competing, contaminating fragments. In such cases, removal of competing fragments can be accomplished by agarose (or polyacrylamide) gel electrophoresis (2). A gel slice containing the fragment of interest can be excised and the fragment extracted using a variety of methods.

Electrophoresis is not only a method for analysis of nucleic acids; it is also a valuable preparative technique. The various electrophoretic separation techniques will not be considered here. However, it is important to note that DNA fragments can be recovered from agarose gels by capturing the fragment on DEAE membrane or by phenol extraction of DNA in low-melting-temperature agarose gels. Recovery of DNA from agarose by any of these methods is time consuming, although fragment yields and quality are high. A more expedient alternative is to excise a gel slice containing the fragment of interest from the gel and dissolve the agarose in a solubilizer solution or with a commercially available enzyme such as agarase. The DNA fragment can then be captured on silica and physically separated from the dissolved agarose.

Cleanup of labeled DNA prior to hybridization

Nucleic acid probes labeled with radioactive or nonradioactive reporter molecules are used in many phases of molecular biology. DNA is generally labeled using techniques such as random priming (also known as oligolabeling), direct labeling, nick translation, or 5′ phosphorylation. Labeling typically involves incorporation or attachment of radioactive, chemiluminescent, or fluorescent labels. For example, cDNA targets used for microarray hybridization are often prepared using random or oligo(dT) primers (or a mixture of both), and Cy3 or Cy5 fluorescently labeled nucleotides. For optimal results in hybridization applications, labeled DNA should be purified from unincorporated labeled nucleotides before use.

Cleanup of automated sequencing reactions prior to electrophoresis

Residual dye-labeled dideoxynucleotide terminators (ddNTPs) can interfere with automated sequencing results and should therefore be removed prior to electrophoresis. Any excess free dye terminators remaining in the reactions will be observed as dye blobs, usually present at the beginning of the sequence. These terminator artifacts may cause base-calling errors. Excess unincorporated labeled ddNTPs can also cause high background fluorescence in automated sequencing gels.

For optimal sequencing results, remaining labeled ddNTPs should be removed prior to electrophoresis. This can easily be accomplished using gel filtration in a spin-column format; however, other technologies may be used.

Table 8.2 provides a summary of techniques for cleanup of unlabeled and labeled DNA.

Table 8.2. Summary of techniques for DNA cleanup.

Technique	Uses	How it Works	Advantages (+)/ Disadvantages (-)
Silica adsorption	Cleanup of PCR products and other DNA fragments from solution or agarose gel Buffer exchange Can be used to concentrate DNA Can also be used for cleanup of labeled DNA (e.g., Cy3- or Cy5-labeled cDNA) prior to hybridization	PCR products and restriction fragments bind to silica in the presence of high concentrations of chaotropic salts. Contaminants such as dNTPs, buffer salts, enzyme, primers, and "primer dimers" < 50 bp are washed away.	+ Can be used to clean up and/or concentrate PCR products from solution or agarose gel + Can use elution volumes as low as 10 µl + Product is eluted in low-ionic-strength buffer and is therefore ready for use in many downstream applications + Membrane-based 96-well formats are amenable to high throughput + Removes enzyme + Removes DNA fragments smaller than 50 bp
Gel filtration	Desalting Removal of unincorporated nucleotides from end-labeled oligonucleotides (G-25) and purification of labeled DNA from unincorporated labeled nucleotides (G-50)	Small molecules are retained in the pores of the matrix. DNA fragments longer than 10 bp (Sephadex G-25) or 20 bp (Sephadex G-50) are eluted.	+ Quick and simple when using spin format + Good purity + Amenable to high throughput, especially when dispensed in a 96-well plate + Sephadex G-50 removes all dNTPs, ddNTPs, and primers that are less than 20 bases in size + Sephadex G-25 removes all dNTPs, ddNTPs, and primers that are less than 10 bases in size - Does not remove enzyme
Enzymatic (i.e., ExoProStar from GE Healthcare)	Cleanup of PCR products prior to sequencing or SNP	Uses a mixture of two enzymes to degrade unincorporated nucleotides and primers	+ Quick and simple + DNA recovery is near 100% + Amenable to high throughput + Any size reaction + Any plate format + No centrifugation or filtration + Fast 30 minute protocol + No additional equipment required + No issues with elution volume because elution is not necessary - Limited to cleanup for sequencing and SNP analysis

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Technique	Uses	How it Works	Advantages (+)/ Disadvantages (-)
Nonsilica microparticles	Cleanup of PCR products and automated sequencing reactions	Magnetic bead-based technology	+ No centrifugation or filtration + Suitable for a wide range of downstream applications + Can be used manually or with automation + Scalable + For sequencing applications, allows dilution of BigDye™ Terminator mix 1/32 - Lengthy protocol compared with ExoProStar - Available in pack sizes for 96 or 384 samples; technology compatible only with certain plate types - Larger reaction volumes require transfer to larger volume plate - Requires magnetic plates for manual or automated use. Some liquid handling systems cannot add and remove magnetic plate, so procedure may not be fully automatable - Elution volume limited by automation—need at least 40 µl for optimal elution
Ultrafiltration	Cleanup of PCR products Dye terminator removal	Small contaminants (e.g., dNTPs, primer, primer dimers, salts) readily pass through the membrane. PCR products remain at the membrane surface and are recovered with water or low-ionic-strength buffer.	+ Amenable to high throughput + No chaotrope or alcohol used + Scalable from 20 to 300 µl reaction volume – Recovery volume > 25 µl so cannot be used to concentrate DNA
Phenol/ chloroform extraction and ethanol precipitation	Cleanup of labeled or unlabeled DNA	Phenol denatures proteins, which are segregated into the lower organic phase after phenol/ chloroform extraction. Salts are removed during ethanol precipitation.	+ Inexpensive + Removes the majority of enzyme - Slow - Requires handling and disposal of hazardous organic chemicals

General guidelines for DNA cleanup

When purifying DNA from either PCR mixtures or restriction enzyme digestions, we recommend running an analytical gel prior to purification to check for a single band representing the DNA species of interest. If multiple bands are present, we recommend performing a preparative gel and excising the band of interest. Silica-based purification is the method of choice for purifying PCR products and other DNAs from reactions or from agarose slices.

Newly synthesized oligonucleotides and labeled DNA are generally cleaned up via gel filtration. We recommend Sephadex G-25 to clean up newly synthesized oligonucleotides > 10 bases and Sephadex G-50 to remove dye terminators prior to electrophoresis for automated sequencing. To clean up other labeled DNA, we generally recommend a Sephadex G-50 product; however, GE Healthcare provides a specific silica-based product for cleanup of Cy3- or Cy5-labeled cDNA (see later in this chapter).

DNA cleanup using silica

Guidelines for silica-based methods

Silica-based protocols for DNA cleanup typically include washing with a buffer that contains ethanol.

Ethanol should be thoroughly removed prior to DNA elution because it can cause interference in many downstream applications. For example, residual ethanol can cause samples to "float" out of wells when loading a gel for electrophoresis and can inhibit some enzymatic reactions.

When using silica in a membrane-based column format: Carefully discard flowthrough and the collection tube. If any of the ethanolic wash solution comes into contact with the bottom of the column, discard the flowthrough and recentrifuge for an additional 1 min.

DNA cleanup using illustra GFX Kits

GE Healthcare provides three silica-based kits for DNA cleanup. illustra GFX™ PCR and Gel Band Purification Kit purifies PCR products and other DNAs from solution or gel slices. This product is discussed in more detail below. illustra GFX 96 PCR Purification Kit purifies PCR products from solution in a 96-well format, allowing for increased throughput. See Chapter 9 for details on the GFX 96 PCR Kit. illustra CyScribe™ GFX Purification Kit is designed for cleanup of Cy3- or Cy5-dye-labeled cDNA prior to microarray or other applications.

illustra GFX PCR DNA and Gel Band Purification Kit is designed for the purification and concentration of DNA from PCR mixtures, restriction enzyme digests, solutions, and agarose gel bands. DNA ranging in size from 50 bp up to 10 kbp can be purified from solution volumes of up to 100 µl and from gel slices of up to 900 mg. No modifications are required for purification of DNA from gels run in borate-based buffers (e. g., TBE).

The developed method uses a chaotropic agent to extract DNA from solution and/or to dissolve agarose and to denature proteins (4, 5). DNA binds selectively to the silica membrane contained in an illustra GFX MicroSpin column. The matrix-bound DNA is washed with an ethanolic buffer to remove salts and other contaminants, and the purified DNA is eluted in a low-ionic-strength buffer.

Product specifications are provided in Table 8.3, and the protocol that follows.

Table 8.3. Product specifications for illustra GFX PCR DNA and Gel Band Purification Kit.

Sample type	PCR mixtures, enzymatic reactions, DNA solutions, agarose gel slices
Sample size range	50 bp–10 kbp
Input volume	100 µl solution or up to 900 mg agarose
Elution volume	10–50 µl
Major subsequent applications	Further PCR amplification, sequencing, labeling, restriction enzyme digestion, ligation, cloning
Yield obtained when purifying a 910 bp fragment at a starting concentration of 8.4 ng/µl	PCR, 10 µl elution volume: 65% PCR, 50 µl elution volume: 82% 300 mg agarose, 10 µl elution volume: 57% 300 mg agarose, 50 µl elution volume: 91%

When purifing a PCR fragment less than 50 bp (but greater than 10 bp) in length, use an illustra MicroSpin G-25 Column.

This kit cannot be used for the purification of RNA.

If handling large numbers of samples in solution, 100 bp–10 kbp in length, use illustra GFX 96 PCR Purification Kit. See Chapter 9.

Procedure for purifying and concentrating DNA using illustra GFX PCR DNA and Gel Band Purification Kit

Materials

Buffers and illustra GFX MicroSpin Columns are included with the product

Heating block or water bath set at 60°C (for agarose gel slices only)

Advance preparation

Prior to first use, add absolute ethanol to the bottle containing wash buffer type 1. See product instructions for volume. Mix by inversion. Indicate on the label that this step has been completed. Store upright and airtight.

General protocol

Note: Buffers and columns are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure you use the correct buffers and columns for your purification.

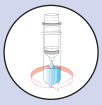
1. Prepare sample for binding

a. From solution: Add Capture Buffer to prepare the sample for binding. Capture Buffer contains chaotropic salts that denature proteins (including enzymes) and promote adsorption of DNA to the silica matrix.

If the sample contains DNA greater than 5 kb, do not vortex, as this will cause shearing of the DNA.

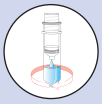
b. From agarose: Add Capture Buffer to dissolve agarose and prepare the sample for binding. Incubate/mix at 60°C until agarose dissolves.

2. Bind DNA



Add sample from step 1 to the GFX column. Centrifuge. DNA greater than 50 bp in length binds to the silica membrane in the presence of chaotropic salts.

3. Wash and dry



Wash the matrix-bound DNA with an ethanolic buffer to remove salts and other contaminants.

Note: the membrane should be free of liquid following the centrifugation step to ensure that ethanol is not eluted into the purified DNA.

Wash and dry steps can be repeated when purity is paramount, for example if the sample is to be used in a blunt-ended ligation.

4. Elute purified and concentrated DNA

Elute DNA from the membrane using 10 to 50 µl of a low-ionic-strength elution buffer. Incubate briefly at room temperature and centrifuge to elute purified DNA. In the absence of chaotropic salts, DNA desorbs from the membrane.

DNA cleanup using gel filtration

Guidelines for gel filtration in a spin format

See Figure 8.2 and accompanying text for an overview of gel filtration principles. Molecules larger than the largest pores in Sephadex or Sephacryl are excluded from the matrix and elute first. Intermediate size molecules penetrate the matrix to varying extents, depending on their size and the medium used. Penetration of the matrix retards progress through the column; very small molecules elute last. The volume required to elute these small molecules is dependent on the volume available both inside and outside the pores, that is, the bed volume.

Gel filtration media do not exhibit a fixed exclusion limit when used in a spin-column format. Exclusion limits of gel filtration media are only meaningful in continuous flow processes where the molecules being purified have sufficient time to reach equilibrium between the time spent in the gel filtration medium and the time spent in the eluent stream. In spin-column chromatography, the observed exclusion properties that allow the product to pass through the gel while the smaller impurities are retained depends on experimental factors, such as the medium used, sample volume, product size, and the g forces used in the purification process.

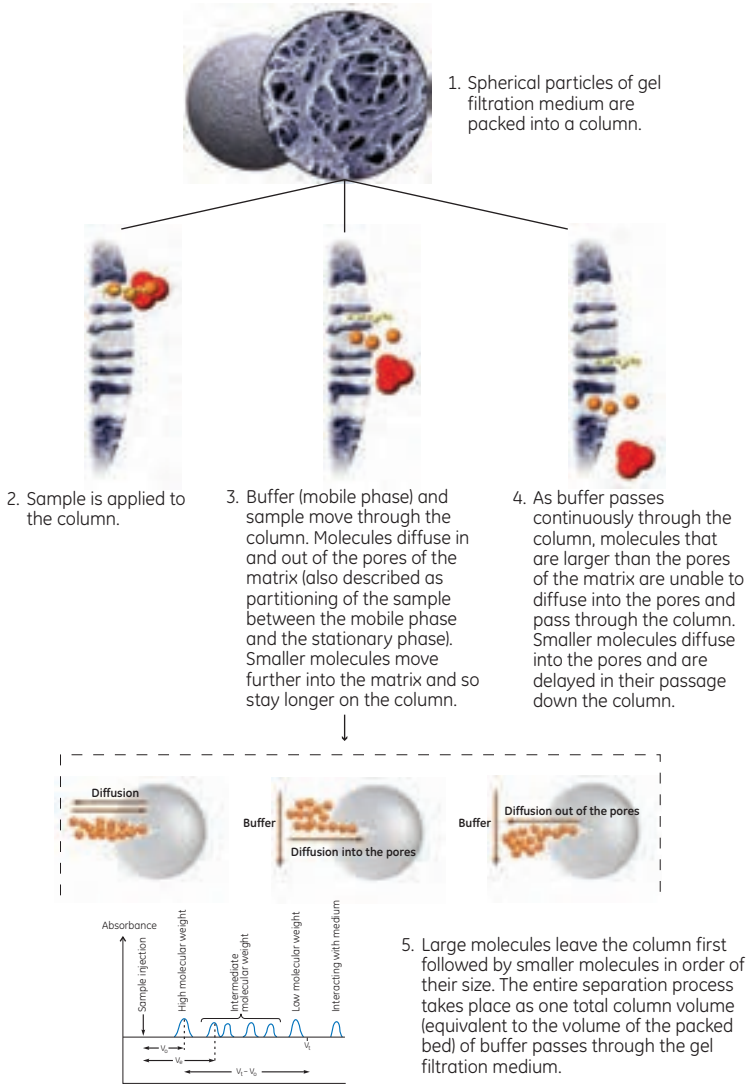


Fig 8.2. Process of gel filtration.

Use columns immediately after preparation to avoid drying out of the medium. If the column medium appears dry, displaced, or cracked after the first spin, this is usually indicative of overcentrifugation (too fast or too long). Rehydrate the column with 250 µl of nuclease-free sterile distilled water, vortex and recentrifuge, checking the settings. Spin speed can be reduced by 20% if necessary.

The medium will have come away from the column slightly to form a pillar. It is essential that the sample being purified is applied slowly to the center of the column bed and is not allowed to run down the sides of the column bed. Avoid touching the column bed with the pipette tip.

GE Healthcare provides a range of gel filtration columns for DNA cleanup. See Appendix 2 to identify the product recommended for your application. Refer to the GE Healthcare Web site (www.gelifesciences.com) for additional information about gel filtration products for DNA cleanup.

Dye terminator removal using illustra AutoSeq G-50 columns

illustra AutoSeq G-50 columns, which contain Sephadex medium DNA grade F, remove dye terminators from sequencing reactions using gel filtration. AutoSeq G-50 columns are configured for the rapid purification (< 5 min) of dye terminator labeled sequencing reactions. The GE Healthcare DYEnamic ET terminator cycle sequencing kit can be used to prepare sequencing reactions for analysis on GE Healthcare MegaBACE™ sequence analyzers. Alternatively, the ABI BigDye v3.1 cycle sequencing kit (catalog number 4337454) can be used to prepare reactions for analysis by all common ABI sequence analyzers (e.g. 377, 3730XL).

A protocol for dye terminator removal using AutoSeq G-50 is provided below.

Materials

AutoSeq G-50 columns are provided with the product

Advance preparation

None

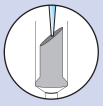
Protocol

1. Prepare column

Resuspend the medium. Remove excess storage buffer by centrifuging briefly.

Use columns immediately after preparation to avoid drying out of the medium.

2. Apply sample



Be sure to apply sample slowly, directly to the center of the column bed.

3. Elute purified DNA

Centrifuge briefly. Unincorporated labeled nucleotides will be retained within the pores of the medium; DNA fragments will flow through.

Downstream applications

Some of the major downstream applications of DNA purified from reaction mixtures or gels include ligation and cloning of PCR products and DNA fragments, use of labeled DNA in hybridization experiments (e.g., microarray analysis), and electrophoresis of dye-labeled DNA from automated sequencing reactions. Optimal results in these applications are obtained when the DNA of interest is purified away from contaminants. See Table 8.1 for details.

Ligation and cloning of purified PCR products

In the following example, ligation and cloning experiments were performed with DNA purified from PCR mixtures and agarose gel bands using GFX PCR DNA and Gel Band Purification Kit compared with four different Qiagen Kits: QIAquick™ PCR Purification Kit, MinElute™ PCR Purification Kit, QIAquick Gel Extraction Kit, and MinElute Gel Extraction Kit. Results are shown in Figure 8.3. In the cloning system that was used, white colonies represent clones that contain successfully ligated DNA and blue colonies represent background clones. Statistical analysis using a one-way ANOVA showed no significant difference in cloning efficacy between the kits ($p > 0.05$) for both PCR amplifications and agarose gel band extractions.

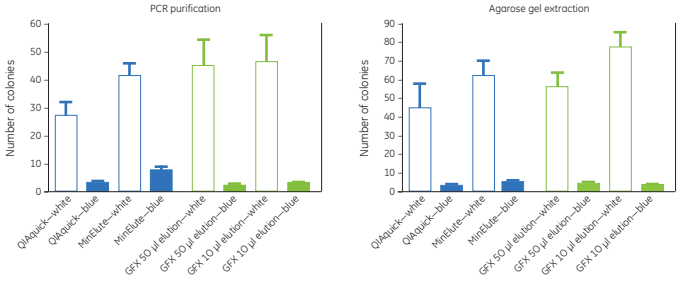


Fig 8.3. Results of ligation and cloning experiments using DNA purified from PCR mixtures and agarose gel bands using GFX PCR DNA and Gel Band Purification Kit compared with four different Qiagen kits.

Cleanup of cDNA labeled with CyDye™

One form of microarray technology anneals labeled cDNA (the probe) to glass- or silicon-immobilized DNA fragments (the target). Purity of dye-labeled cDNA probes has been shown to be a critical factor for the success of these hybridizations (6). Impurities in the dye-labeled samples can obscure spectrophotometric measurements and introduce errors in signal quantitation of array elements.

A microarray slide with high background can reduce accuracy of gene expression ratios and cause a decrease in detection limits. Therefore, it is imperative for labeled cDNA probes to be as free as possible from other labeling reactants.

illustra CyScribe GFX Purification Kit uses a simple, three-step process to purify cDNA probes labeled with either Cy3- or Cy5-dNTPs. The kit uses a glass fiber matrix to bind labeled probes in the presence of a chaotropic salt. Contaminants are removed by washing with an ethanolic buffer. Purified probe is eluted using a low-ionic-strength buffer. Probes purified using this method deliver bright signals, high sensitivity, and low background.

In the following microarray experiment, a human pre-arrayed slide was hybridized with Cy3- and Cy5-labeled cDNA probes made from total RNA. Probes were purified with illustra CyScribe GFX Purification Kit. The results in Figure 8.4 show low background levels, indicating effective removal of unincorporated dye.

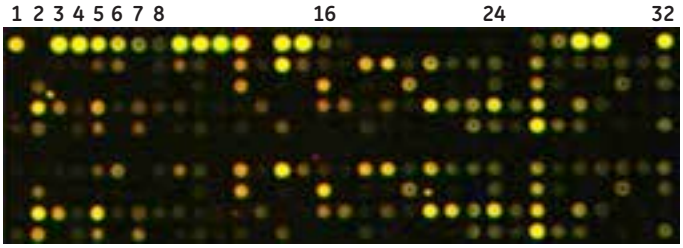


Fig 8.4. Human pre-arrayed slide hybridized with Cy3- and Cy5-labeled cDNA probes made from total RNA. Probes were purified with CyScribe GFX Purification Kit. Universal ScoreCard™ controls were spotted in the first row and consisted of column 1, positive control; column 2, negative control; columns 3 and 4, high-copy-number controls; columns 5 and 6, medium-copy-number controls; columns 7 and 8, low-copy-number controls (17 and 6 copies, respectively); columns 9–12, ratio controls; columns 13–17, housekeeping genes; columns 30 and 31, negative hybridization controls.

Cleanup of dye terminators from sequencing reactions

Phred quality (q) values express the probability of correctly calling a base-peak in a sequencing electropherogram. They are based on key parameters such as resolution, spectral cross-talk, and uniformity of peak spacing (7–9). The q value is logarithmically proportional to the probability of correctly calling the identified peak (i.e., a Phred value of 20 is an accuracy of 99%, Phred30 is 99.9% accurate). The minimal acceptable value for sequencing is a Phred value of 20. For this study, we defined sequencing read length as the total number of bases with a Phred value of 20 or greater (q ≥20) using a 10-base moving window average. Sequencing reactions were purified using Qiagen DyeEx™ 2.0 Spin Kit and illustra AutoSeq G-50 Dye Terminator Removal Kit. We checked for data accuracy at the beginning of the sequence by examining electropherograms for the absence of dye blobs and noting the read length at which the Phred score reached ≥ 20. Dye blobs were absent from all the samples tested, and there was no significant difference in the read length at which the Phred score reached 20 or more ($p = 0.6579$) between illustra AutoSeq G-50 and DyeEx 2.0 columns. A typical electropherogram image depicting the quality and accuracy of data generated with illustra AutoSeq G-50 Kit is shown in Figure 8.5.

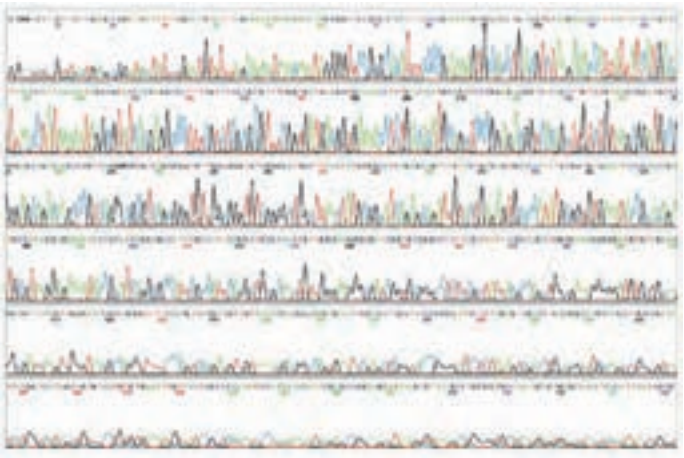


Fig 8.5. Typical electropherogram of sequencing reactions purified using illustra AutoSeq G-50.

References

1. Zeugin, J. A. and Hartley, J. L. Ethanol precipitation of DNA. *Focus* **7** (4): 1–2 (1985).
2. Ausubel, F. M., *et al.,* ed., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc. (2002).
3. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A laboratory manual,* Cold Spring Harbor Laboratory, 1st and 2nd eds. (2001).
4. Vogelstein, B. and Gillespie, D. Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci. U S A* **76**, 615–619 (1979).
5. Marko, M. A. *et al.* A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. *Anal. Biochem.* **121**, 382–387 (1982).
6. Hedge, P. *et al.* A concise guide to cDNA microarray analysis. *BioTechniques* **29**, 548–562 (2000).
7. Ewing, B. *et al.* Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**, 175–185 (1998).
8. Ewing, B. and Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**, 186–194 (1998).
9. Paegel, B. M. *et al.* High throughput DNA sequencing with a microfabricated 96-lane capillary array electrophoresis bioprocessor. *Proc. Natl. Acad. Sci. U S A* **99**, 574–579 (2002).

Chapter 9
Increasing throughput of nucleic acid sample preparation

Sample throughput refers to the number of samples that are processed. Sample throughput can apply to the number of samples processed simultaneously or to the number processed per day, week, or longer. The term *parallel processing* is often used when referring to samples processed simultaneously. Sample throughput needs vary widely, as do the format, scale, and equipment options available for increasing sample throughput.

Several examples may help to distinguish some of the different sample throughput needs for nucleic acid sample preparation. Researcher 1 wants to prepare RNA from 96 samples at a time but does this only occasionally. Researcher 2 wants to remove excess fluorescent label from 12 cDNA labeling reactions at a time. He plans to repeat this process many times per month but does not anticipate processing more than 12 samples simultaneously. And Researcher 3 wants to automate nucleic acid sample preparation from sample collection to analysis of PCR products. She will prepare a large number of samples where yield and high reproducibility are critical.

These three researchers have different needs for nucleic acid sample preparation throughput. Researcher 1 is interested in parallel processing of 96 samples. A product in a 96-well format would be a good choice for this researcher, who may consider a 96-well plate using either magnetic bead or silica membrane technology. The parallel processing need for Researcher 2 is for 12 samples. He may benefit from a product in a mini spin-column format. In his case he could either handle the sample preparation work manually by establishing a standard operating procedure and following it every time, or he could use a robot to handle this repetitive work routinely. For Researcher 3, automated systems for nucleic acid preparation and other processes (e.g., amplification and detection) would be a must-have. Her parallel processing needs are not discussed, but we can assume that she will process at least 96 samples at a time, using as few steps as possible to lose as little as possible of her precious sample.

Many commercially available products for nucleic acid sample preparation are based on silica or another solid phase. Silica-based products usually involve a membrane in a mini spin column or multiwell plate. Magnetic bead products typically use paramagnetic particles (PMPs) as the binding matrix for nucleic acids. Solid-phase product formats, as well as solution-based protocols with few pipetting steps (e.g., Phi29-based DNA amplification), are generally good candidates for increased throughput. Some product manufacturers offer the option of removing strips from a multiwell plate to allow processing of fewer than 96 samples. Solution-based protocols may include reagents predispensed in multiwell plates, or they may offer the user a choice of using multiwell plates, tubes, or other vessels.

Choices for mini column-based products are numerous. Most use centrifugation for sample processing. However, some manufacturers offer automated systems for use with their columns. These systems are often referred to as “closed” systems or systems with closed architecture because they are designed specifically for use with columns from that manufacturer.

The different product formats influence the options for processing equipment. Mini spin columns typically use a microcentrifuge for processing. Multiwell plates may be processed using a centrifuge with a multiwell plate adapter. Plates from some manufacturers may be amenable to processing with an automated handling workstation (robotics), and some may allow use of a vacuum manifold. Magnetic bead products are processed with a benchtop magnetic separator. Many magnetic bead products are amenable to automation.

Automated systems for nucleic acid sample preparation range from simple, closed systems that are coupled with reagents from a specific manufacturer to large, open systems that are amenable for use with more than one sample preparation procedure (1, 2). Closed systems are typically designed for use with mini columns or multiwell plates; some systems use prepacked reagents to increase reproducibility and decrease time for reagent preparation. Open systems are not exclusive to a particular manufacturer. However, these systems are not compatible with all commercially available products for nucleic acid sample preparation. Contact the system manufacturer for more information. Basic open systems handle only nucleic acid sample processing, while integrated systems are coupled with equipment for amplification or other techniques. In general, systems with higher throughput and more integration are larger and more expensive than systems with lower throughput.

Let's return to Researchers 1, 2, and 3. What are their equipment options for sample preparation? Researcher 1 may use a centrifuge, vacuum manifold, or robotics to prepare total RNA in a 96-well plate. Because this researcher processes 96 samples only occasionally, there may not be any interest in investing in an automated liquid handling system. Researcher 2 may use mini columns in a microcentrifuge to process 12 samples at a time. He may also consider using a specific vacuum system provided by the same manufacturer that sells the purification kits or may choose robotics, because he will process 12 samples many times per month. Researcher 3 will want to use an automated liquid handling system for sample preparation. Considerations for choosing a sample preparation system are listed in the following pages.

Considerations for increasing throughput for nucleic acid sample preparation

Several factors should be considered when discussing how to increase sample throughput. Some of these were alluded to in the previous examples.

- For which process or processes do you want to increase throughput?

  For example, do you want to increase throughput of a total RNA preparation, cleanup of dye-labeled DNA, or of multiple steps in an analytical workflow (e.g., for analysis of PCR products)?
- Do you already have an automated liquid handling system? If so, which systems for nucleic acid preparation are compatible with it?
- How many samples will you process in parallel? What is your expected daily, monthly, and yearly sample throughput?
- What is the sample volume to be processed?
- What are your requirements for experimental reproducibility and variation between replicates?
- What is your budget for initial expenditure of equipment for increasing sample throughput?
- How much space can you dedicate to the equipment?
- How much maintenance and training is required?
- How important is it to have a system with low "hands on" time for reactions? Is it important that the system is truly "walk away" (sample in—sample out)?

GE Healthcare provides a number of nucleic acid sample preparation products that are amenable to increased throughput. These are highlighted in the following pages.

Increasing the throughput of FTA card punching

Whatman FTA technology is a patented process that incorporates chemically coated matrices to collect, store, and process nucleic acids in a single device. Samples are collected onto FTA or FTA Elute cards in a number of ways, and cards are dried. Discs of FTA and FTA Elute may be manually removed from sample areas using a coring device such as a Harris Micro Punch or Uni-Core. Alternatively, a semi-automated or fully automated punching system may be used for

disc punching (see below). After punching, the discs are processed to purify DNA, which remains bound to the matrix (FTA) or is released into solution (FTA Elute). See Chapter 3 for details on Whatman FTA technology and manual punching of sample discs.

Two systems are available to increase the throughput of disc punching. These are the BSD600-Duet (semi-automated) and BSD1000-GenePunch (fully automated), both from BSD Robotics (Fig 9.1).

BSD600-Duet is a benchtop system that automates the processes of punch acquisition and delivery to a multiwell plate. LIMS software and barcode scanning capabilities ensure accurate sample tracking. Accompanying software also allows users to customize punching patterns and to include standards/controls. BSD1000-GenePunch is a benchtop system that fully automates punch acquisition and delivery. It accepts hotels (loaded with FTA cards) and delivers up to six plates with punches accurately placed in appropriate wells.

Fig 9.1. BSD600-Duet semi-automated (left) and BSD1000-GenePunch fully automated (right) punching systems.

Parallel processing of up to 96 total RNA samples

illustra RNAspin 96 RNA Isolation Kit from GE Healthcare is designed for fast small-scale preparation of total RNA from tissues or cells in a 96-well format using suitable vacuum manifolds or suitable centrifuges. This kit can also be used in a fully automated flow with vacuum on common laboratory workstations (see protocol for automated workstations on the following pages). illustra RNAspin 96 Kit can be used to process up to 96 samples simultaneously in less than 70 min. Actual automated processing time depends on the configuration of the workstation used. The kit provides reagents and consumables for the purification of up to 100 µg of highly pure total RNA per sample, suitable for direct use in downstream applications like RT-qPCR, primer extension, RNase protection assays, cDNA synthesis, and microarray analysis.

Typical amounts of starting material and anticipated yields for RNAspin 96 Kit are shown in Table 9.1. Note that the yield of total RNA is highly dependent on the starting material and on complete sample disruption and cell lysis, so actual results may vary. For more information on sample disruption and cell lysis, see Chapter 2. illustra RNAspin Kits are also available in Mini and Midi formats; see Chapter 6 for details. A general protocol for isolation of total RNA using RNAspin 96 Kit follows.

Table 9.1. Typical yields of total RNA using illustra RNAspin 96 Kit with the vacuum or centrifuge method.

	Vacuum	Centrifuge
Maximum number of cells to be used	2×10^6	1×10^7
Yield up to	20 µg	100 µg
Maximum amount of tissue to be used	10-30 mg	30 mg
Yield up to	40 µg	100 µg

Procedure for isolating total RNA using illustra RNAspin 96 RNA Isolation Kit

RNA isolation using RNAspin 96 Kit can be performed at room temperature. However, the eluate should be treated with care because RNA is very sensitive to trace contamination of RNases, often found on general labware, fingers, and dust. To preserve stability, keep the isolated total RNA frozen at –20°C for short-term or –80°C for long-term storage.

Materials

Buffers, lyophilized DNase I, DNase reaction buffer, RNase-free water, RNAspin RNA Binding Plate, Wash Plate, and Vacuum Elution Plate are provided with the product

Gloves

RNase-free plasticware

β-mercaptoethanol

Standard 1 ml polypropylene 96-well round-well block, such as those from Macherey-Nagel (Cat No. 740671, pack of 20) or Corning (Cat. No. 3959, pack of 5)

RNAspin 96 Filter Plate (Cat. No. 25-0500-88; recommended if processing tissue or large cell numbers)

An adapter such as Macherey-Nagel Frame (Cat. No. 740680) is required for positioning of the Wash Plate with certain manifolds, e.g., QIAvac™.

*Advance preparation**

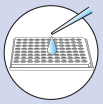
Dissolve DNase I in RNase-free water.

Add ethanol to buffer RA3 and RA4 concentrates. See product instructions for volumes.

* *RNA is not protected against digestion until the sample material is flash frozen or disrupted in the presence of RNase inhibiting or denaturing agents. See Chapter 6 for details.*

*General Protocol**

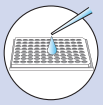
1. Disrupt tissue or harvest cells



Disrupt tissue using a suitable method or harvest cultured cells by centrifugation. Immediately proceed to step 2 to lyse cells.

For tissue: Depending on the type of sample, up to 30 mg can be processed. If the lysate is too viscous, add 300 µl of buffer RA1 and a corresponding amount of buffer RA4. For higher throughput in the 96-well format, add lysis buffer RA1 to frozen or stabilized tissue collected in a round or square well block or tube strips, and immediately disrupt tissue with an appropriate homogenizer.

2. Lyse cells



Add lysis buffer RA1 and β-mercaptoethanol. Vortex vigorously. Chaotropic salts in the lysis buffer break open the cells and denature proteins, and β-mercaptoethanol inhibits RNases.

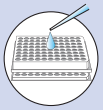
3. Clarify cell lysate (if needed)

For tissue samples, reduce viscosity and clear the lysate by centrifugation through an RNAspin RNA Filter Plate.

4. Prepare vacuum manifold

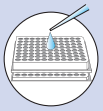
Prepare vacuum manifold if using the vacuum protocol.

5. Bind RNA



Add buffer RA4 to prepare the lysate for binding, and apply cleared lysate to silica membrane. The salts and solvents in the lysis buffer promote binding of RNA to the silica membrane. Denatured proteins are collected in the flowthrough following centrifugation.

6. Add DNase I

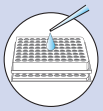


Wash to remove salts and add RNase-free DNase to digest membrane-bound DNA. Incubate briefly at room temperature.

Do not touch the silica membrane with the pipette tips.

Be sure that all the DNase reaction mixture gets into contact with the silica membrane and that the membrane is completely wetted.

7. Wash and dry



Wash the column to remove DNase, then wash with low-salt buffer and ethanol to remove residual chaotropic salts and other contaminants.

The ethanol in buffer RA4 inhibits downstream enzymatic reactions and must be completely removed before DNA elution.

8. Elute purified total RNA

Elute purified total RNA using RNase-free water. Immediately place on ice or freeze.

* *This product can be used with suitable centrifuges or suitable vacuum manifolds. See product instructions for details. If using a common laboratory automation workstation, see the following protocol.*

Standard protocol for automated purification of total RNA using common laboratory automation workstations*

1. **Prepare robotic workstation**
   Place plastic equipment (e.g., plates and the assembled vacuum manifold) at the locations as specified for your workstation.
2. **Prepare buffers**
   Add sufficient buffer to the reservoirs or place the buffer bottles at the corresponding positions on the robot worktable. Calculate the needed buffer volumes plus 10% overage, and fill the reservoirs.
3. **Disrupt tissue or harvest cells**
   Disrupt tissue using a suitable method, or harvest cultured cells by centrifugation. Proceed immediately to the next step.
4. **Incorporate samples into automated workflow**
   Place multiwell plate with samples at the appropriate position of the robotic workstation.
5. **Run automation program**
   Select method for total RNA purification and start the run. Use disposable filter tips for the transfer of sample to the RNAspin RNA Binding Plate. All other steps can be processed with needles.

   Adjust vacuum times and strength, if necessary. Make sure that the solution of DNase I is pipetted into the middle of each well.
6. **Elute purified total RNA**
   Stop the protocol after the final washing step with RA4. Centrifuge to remove residual ethanol. Elute with at least 50 µl of RNase-free water.

* *For a more specific protocol, refer to the manufacturer of your automation system.*

Downstream applications

Total RNA was analyzed following isolation using either illustra RNAspin 96 RNA Isolation Kit using the centrifuge method (Fig 9.2) or Biomek™ FX automated workstation (Fig 9.3).

GAPDH gene expression is often used as a positive control in gene expression profiling. Therefore, GAPDH is expected to be expressed at a detectable level in many cell types, including cultured HeLa cells. The known pattern of GAPDH gene expression in cultured HeLa cells is shown for 48 separate, 96-well culture samples (Fig 9.2). These data highlight the reproducibility aspect of RNAspin 96 Kit and also show that the kit can be used to isolate sufficient, high-quality total RNA for Northern blot analysis.

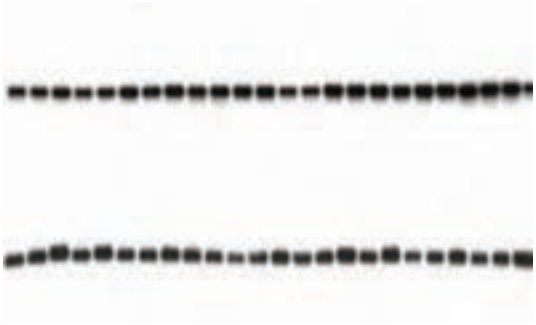


Fig 9.2. Northern blot analysis of the GAPDH transcript in 48 separate HeLa cell cultures. Fifteen µl of the RNA eluate from each HeLa cell culture in a 96-well plate was run per lane.

In another experiment, 30 wells containing 10 mg of mouse liver tissue each were processed for RNA isolation using illustra RNAspin 96 RNA Isolation Kit on a Biomek FX vacuum automation system. The RNA was eluted with 80 µl of RNase-free water, and 1 µl of this was used for RT-qPCR. cDNA was synthesized using GAPDH primers and SYBR Green. The level of GAPDH transcript was measured with the LightCycler™ detection system according to the manufacturer's protocol (Fig 9.3). C_q are shown for all samples. The mean quantitation cycle was 22.04 ± 0.21 with a coefficient of variation (CV) of 0.95%.

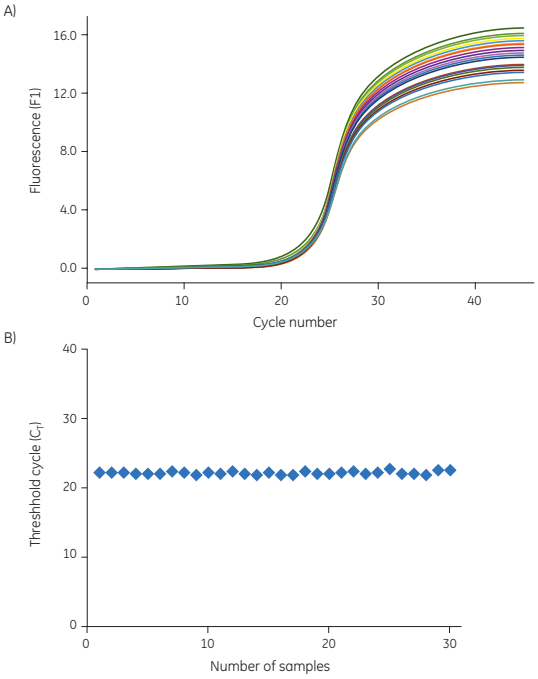


Fig 9.3. Total RNA from 10 mg of liver tissue was prepared using illustra RNAspin 96 RNA Isolation Kit on a Biomek FX. One µl out of the 80 µl eluate from each sample was used for LightCycler detection (GAPDH primer, SYBR Green).

Parallel purification of up to 96 PCR samples

illustra GFX 96 PCR Purification Kit from GE Healthcare is designed for the purification and concentration of DNA from PCR mixtures, restriction enzyme digests, and solutions. DNA ranging in size from 100 bp up to 10 kbp can be purified from solution volumes of up to 300 µl. illustra GFX 96 PCR Purification Kit utilizes glass fiber matrix technology in a 96-well format. DNA fragments are captured by the silica matrix in the presence of a chaotropic salt, and contaminants are removed by washing the matrix with a buffered ethanol solution. Purified DNA is recovered by elution in a small volume of water or in a buffer of low ionic strength. The purified DNA is ready for use in a variety of applications, including fluorescent sequencing, labeling, hybridization, ligation, and transformation. illustra GFX 96 PCR Purification Kit provides Binding, Wash, and Collection plates and all required buffers in one box. The standard configuration of the 96-well plates is compatible with most 96-well plate-adapted benchtop centrifuges and the NucleoVac™ 96 Vacuum Manifold from Macherey-Nagel. There are no hazardous organic extractions, and ethanol precipitation is not required to isolate purified DNA. The kit allows 96 PCR products to be purified in as little as 15 min.

Table 9.2 shows typical percentage yields obtained when purifying a 910 bp fragment.

Table 9.2. Percentage yield of a 910 bp product obtained with illustra GFX 96 PCR Purification Kit.

Protocol	Elution volume (µl)	Yield (%)
Centrifugation	10	55
	50	95
	50	90
Vacuum	50	59
	100	78
Vacuum plus optional additional Wash and dry step	50	51

Procedure for purifying and concentrating DNA using illustra GFX 96 PCR Purification Kit

A general protocol for purification and concentration of DNA from PCR mixtures, restriction enzyme digests, and solutions using illustra GFX 96 PCR Purification Kit is provided below. The kit can be used with suitable centrifuges, vacuum manifolds, or automated robotic workstations.

Materials

Buffers and GFX Binding, Wash, and Collection plates are included with the product

Benchtop microcentrifuge suitable for centrifugation of 96-well plates OR NucleoVac 96 Vacuum Manifold, available from Macherey-Nagel (product code 740681)

Reagent reservoir (optional for multichannel pipetting)

Advance preparation

Prior to first use, add absolute ethanol to the bottle containing wash buffer type 1. See product instructions for volume. Mix by inversion. Indicate on the label that this step has been completed. Store upright and airtight.

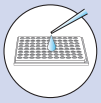
Prepare the vacuum source

GFX 96 PCR Purification Kit can be used with Macherey-Nagel NucleoVac 96 vacuum manifold. To process fewer than 96 samples, use a rubber pad or self-adhering polyethylene foil to cover up any unused wells of the GFX 96 Binding Plate and ensure a vacuum is maintained. Establish a reliable vacuum source for the manifold. A vacuum pump, house vacuum, or water aspirator may be used. We recommend a vacuum of 380 to 630 mbar or 15 to 25 mm Hg. The use of a vacuum regulator is recommended. Alternatively, adjust the vacuum so that during purification the sample flows through the column at a rate of 1 to 2 drops per second. Vacuum times suggested in the product protocol might have to be increased for complete filtration if large sample volumes are used.

General protocol*

Note: Buffers are NOT transferable between GE Healthcare kits in the illustra product range. Please ensure that you use the correct buffers for your purification.

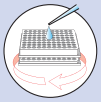
1. Prepare sample for binding



Add capture buffer to prepare the sample for binding.

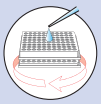
Sample volume may be 20 to 300 µl.

2. Bind DNA



Add sample from step 1 to the GFX Binding Plate. Centrifuge or apply vacuum. DNA binds to the silica membrane in the presence of chaotropic salts. Primers and nucleotides are too small to bind effectively. Enzymes are denatured and will not bind to the column.

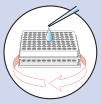
3. Wash and dry



Add wash buffer to membrane-bound DNA. Centrifuge or apply vacuum. The wash buffer contains salts and ethanol to remove unbound contaminants and dry the membrane.

Wash and dry steps may be repeated when purity is paramount, for example if the sample is to be used in a blunt-ended ligation.

4. Elute purified and concentrated DNA



Add low-ionic-strength elution buffer to the membrane. Incubate briefly at room temperature. Centrifuge or apply vacuum. Membrane-bound DNA is eluted from the membrane when chaotropic salts are removed.

When 50 µl volume is used for elution, an average volume of 45 to 48 µl will be recovered.

* *This product can be used with suitable centrifuges or vacuum manifolds. Refer to GFX 96 PCR Purification Kit product instructions for specific centrifuge and vacuum protocols. For details on using this product with automated workstations, refer to the manufacturer of your system.*

Downstream applications

DNA was purified from PCR solutions with GFX 96 PCR Purification and QIAQuick 96 PCR Purification Kits. The ability of the purified DNA to undergo successful ligation reactions into a pUC18-based vector was tested with PCR Cloning plus™ Kit (Fig 9.4.). The PCR product was amplified from an ampicillin-resistant plasmid, but kanamycin was used as the selectable marker during the cloning step to eliminate the risk of plasmid carryover. Recombinant clones were selected using blue/white colony selection. Both kits gave good numbers of colonies. No evidence was seen of primer concatamers being cloned; the negative control PCR mixture gave no colonies. A one-way ANOVA gave p values of 0.316 between operators and 0.415 between kits (CI, 95%). This confirms that there was no statistical difference between operators or kits for the cloning test performed.

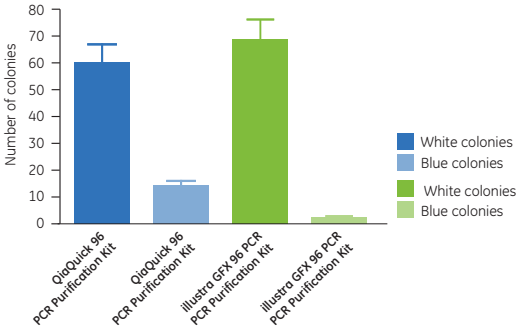


Fig 9.4. Ligation and cloning results of PCR products purified using QiaQuick or illustra GFX 96 Purification Kits.

PCR sample cleanup using illustra ExoProStar™

illustra ExoProStar is designed for fast and efficient purification of PCR products for downstream applications such as sequencing and genotyping (e.g., SNP analysis). ExoProStar consists of two hydrolytic enzymes, Exonuclease I and Alkaline Phosphatase (AP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. illustra ExoProStar offers a 30–min PCR cleanup protocol in either a convenient one or two tube, one-step format*. illustra ExoProStar eliminates the need for spin column purifications, sedimentations, filtrations, beads and/or magnetic separations (3). Both Exonuclease I and AP are active in commonly used PCR buffers, eliminating the need for a buffer exchange step. Recovery for both short (< 100 bp) and long (> 20 kbp) PCR products is 100%. The procedure is scalable and easy to automate with the user determining whether to use 0.5 ml tubes, 0.2 ml tubes, or multiwell plates.

** The one tube format is not available in certain territories.*

Procedure for purifying PCR products using illustra ExoProStar

Materials

Enzyme mix is provided with the product. No other reagents are required.

Thermal cycler or heating block for 37°C and 80°C incubations

Advance preparation

None

Protocol

1. Add enzyme mix

Add enzyme mix to sample. Incubate for 15 min at 37°C. Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced during PCR. AP removes the remaining unincorporated dNTPs from the PCR mixture.

2. Heat-inactivate enzymes

Incubate for 15 min at 80°C to inactivate the enzymes. The heat-inactivated enzymes do not interfere with downstream applications.

Isothermal DNA amplification using Phi29 DNA polymerase

GenomiPhi and TempliPhi DNA Amplification Kits from GE Healthcare use a Phi29 DNA polymerase–based, isothermal DNA amplification method with random hexamer primers to amplify genomic DNA and circular DNA, respectively. This method is a simple, reliable alternative to traditional DNA isolation procedures. The process is amenable to automation, and the method produces DNA ready for direct use in downstream analyses such as genotyping and sequencing. Genomic DNA is amplified in a linear process; plasmid and other circular DNAs are amplified in a process called rolling circle amplification (RCA; 4, 5).

See Chapter 7 for an overview of the GenomiPhi and TempliPhi methods.

Phi29 DNA polymerase-based DNA amplification using an automated robotic workstation

Because the amplification protocols for both GenomiPhi and TempliPhi are simple, automation is straightforward. The Genesis Freedom EVO™ series of Robotic Sample Processors (RSPs) from Tecan are well suited for automating DNA amplification reactions and downstream applications. The arm of the Tecan liquid handler is equipped with four or eight tips and can perform all required liquid handling steps with high sensitivity and accuracy. The amplification reaction stops when the nucleotides in the reaction are exhausted, and DNA yields are equivalent across all samples (Fig 9.5). Note that allthough the figure shows results from an 18 h DNA amplification, the current protocol for GenomiPhi has been optimized for shorter incubation times. Disposable tips eliminate cross-contamination concerns. With an eight-channel liquid handling arm, multiple 96-well plates of TempliPhi or GenomiPhi DNA amplification reactions can be prepared in half the time of manual pipetting. Whatever the post-amplification application, the open platform can be adapted. The Tecan Gemini software system allows easy protocol setup and operation of the workstation.

For details on using GenomiPhi or TempliPhi with your automated liquid handling workstation, contact the manufacturer of your system.

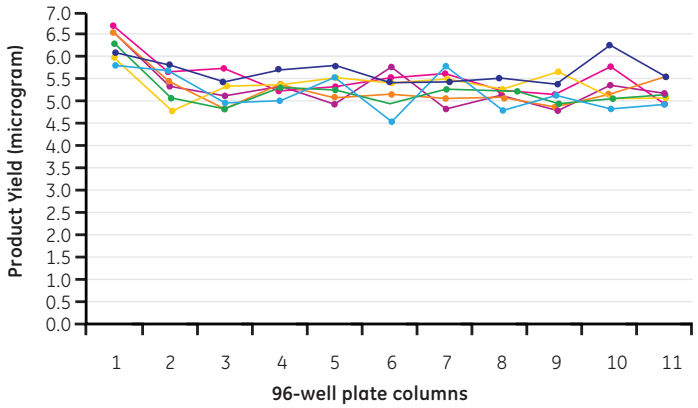


Fig 9.5. DNA yield using GenomiPhi DNA Amplification Kit (18 h incubation) and Genesis RSP workstation.

References

1. Check, W. For nucleic acid extraction, choices galore. *CAP Today*. (October, 2008). [Online.] http://www.cap.org/. Accessed 14 Jan 2009.
2. Bird, C. Nucleic acid purification market expands: potential use in downstream applications will likely contribute to higher growth in future. *Genet*. *Engineer. Biotech News* **28**, (Sep 1, 2008).
3. Dugan, K. A. *et al.* An improved method for post-PCR purification for mtDNA sequence analysis. *J. Forensic Sci.* **47**, 811–818 (2002).
4. Dean, F. *et al.,* Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Research* **11**, 1095–1099 (2001).
5. Lizardi, P. *et al.,* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat*. *Genet*. **19**, 225–232 (1998).

Chapter 10
Preparation of DNA, RNA, and protein from an undivided sample

Until recently, researchers in fields such as functional genomics, molecular genetics, systems biology, and biomarker studies used three separate protocols or kits to isolate DNA, RNA, and proteins for use in downstream applications. However, the use of divided samples can potentially skew results due to heterogeneity between different aliquots of cell and tissue samples. The demand for good correlations between transcript (gene) expression, protein expression, copy number variation, and SNP detection has resulted in the development of methods for isolating genomic DNA, total RNA, and total protein from the same sample to enable direct correlation of DNA, RNA, and total protein levels. Preparation of all three biomolecules from a single sample ensures that results are not an artifact of experimental design but are actually characteristic of the sample.

GE Healthcare provides a method for preparing genomic DNA, total RNA, and total denatured protein from an undivided sample, using illustra triplePrep Kit, which isolates these three categories of molecules sequentially. The chaotropic lysis buffer immediately inactivates DNases, RNases, and proteases in biological materials to minimize molecular degradation, and creates appropriate binding conditions that favor adsorption of DNA to the silica membrane. RNA is adsorbed to a second silica membrane, and in a subsequent step, total denatured protein is precipitated. Further details are provided later in this chapter.

Several factors should be considered before deciding whether to use a multi-molecule isolation method and if so, which method to use. Questions to answer include:

- Which molecules do you want to isolate?
- How much starting material is available?
- Will the quality and purity of the molecules be comparable to that obtained when using methods dedicated to isolating a single molecule?
- Will the yield of the molecules be comparable to that obtained when using methods dedicated to isolating a single molecule?
- How convenient is the multi-molecule isolation method compared with a single method?
- How cost-effective is the multi-molecule isolation method compared with a single method?
- If isolating protein, does it need to be native, or can it be denatured?

In general, the same sample preparation considerations for isolating DNA, RNA, or protein separately also hold true when isolating multiple molecules from the same sample. For example, sample disruption and cell lysis are critical to any nucleic acid or protein sample preparation experiment. When using a multiple molecule isolation method, consideration should be given to minimizing genomic DNA shearing, RNA degradation, and protein degradation. See Chapter 2 for further discussion on these topics. A number of methods that describe the extraction of DNA, RNA, and protein are described elsewhere (1–3).

General considerations for isolating multiple molecules using illustra triplePrep Kit

See Chapter 2 for information on cell disruption and sample homogenization. If samples are not thoroughly disrupted, cells will not be lysed efficiently.

Freshly collected whole blood can be used for the preparation of DNA, RNA, and total protein, when lymphocytes are isolated from freshly drawn whole blood and are used as a source material.

When processing a type of tissue or cell for the first time, start with 5 mg of tissue or 1 million cells. Optimize the sample amount depending on initial results.

The molecules (particularly RNA) within tissue and cells are vulnerable to degradation by both endogenous enzymes and enzymes found in the general laboratory environment. The triplePrep lysis buffer inactivates DNases, RNases, and proteases. See Chapter 2 for tips on handling samples and Chapter 6 for specific details on minimizing introduction of RNases into a sample.

Use fresh biological material whenever possible. Where this is not possible, it is important that fresh samples are flash frozen in liquid nitrogen immediately and stored at -80°C. Alternatively, they can be stored in the presence of a stabilizing agent, although this may not give optimal yields when working with tissue. Storage conditions vary with this latter alternative, based on the type of agent.

Never allow frozen tissues to thaw before addition of lysis buffer type 15. Disrupt samples in liquid nitrogen, if possible.

Loading the system with sample containing more nucleic acids than the column's capacity will not increase yield and sometimes may even lower the quality and purity of eluted nucleic acids. The triplePrep columns have a capacity of 20 µg of genomic DNA (first column) and 60 µg of total RNA (second column).

If you have a large amount of tissue or number of cells, split the samples and process as several samples for better quality and yield of nucleic acids.

Because protein is precipitated without binding to any columns, the yield of protein with triplePrep is relatively linear to sample input amount (i.e., If 100 µg of protein can be isolated from 1 mg of a certain tissue type, then 2000 µg of protein is expected from 20 mg of the same tissue type).

Protein resuspended in 2-D DIGE buffer can be used for SDS-PAGE by mixing with 1 volume of 20% SDS. Add an appropriate sample loading buffer (i.e., 1 volume of 2× Laemmli buffer) and incubate at 70°C for 10 min before loading on the gel.

Note that 2-D DIGE buffer is not compatible with BCA or Bradford assays. We recommend using the 2-D Quant Kit (supplied by GE Healthcare) to determine protein concentration when protein is resuspended in 2-D DIGE buffer. Alternatively, 7 M urea can be used for protein resuspension and subsequently diluted to less than 3 M to be compatible with BCA or Bradford assays.

Overview of illustra triplePrep Kit and expected performance

illustra triplePrep Kit from GE Healthcare is designed for the rapid extraction of genomic DNA, total RNA, and total denatured protein from a single undivided sample starting from as little as 1 mg of animal tissue, 0.3 million cultured mammalian cells, or 10^7 freshly prepared lymphocytes isolated on Ficoll-Paque™ PLUS (GE Healthcare). The protocols for extraction from both tissues and cell lines utilize the same buffers. Although the protocols are rapid, they have been designed to minimize shearing of genomic DNA, maintain the integrity of the RNA, and maximize proteome representation. The entire procedure can be completed in as little as 45 min to yield genomic DNA, total RNA, and denatured total protein that are compatible with most molecular biology applications. These applications are summarized in Table 10.1. Note that the isolated protein is suitable for applications that use denatured protein.

Table 10.1. Summary of applications for molecules isolated using triplePrep.

Molecule	Downstream applications
Genomic DNA	Restriction enzyme digestion, PCR, sequencing, aCGH
Total RNA	RT-PCR, cDNA synthesis, expression array
Total protein	SDS-PAGE, Western blotting, 2-D DIGE, LC-MS

DNA, RNA, and protein have been successfully isolated from a range of tissue types such as liver, kidney, spleen, brain, lung, and intestine, and the cell lines HeLa, NIH-3T3, CHO-K1, and HEK-293. Tissue amounts ranging from 1 to 20 mg can be used for each preparation. Cell amounts ranging from 0.3 to 5 million cells can be used for each preparation. Approximately 10^7 lymphocytes isolated on Ficoll-Paque PLUS are required for nucleic acid and protein preparation.

Although illustra triplePrep Kit is designed to isolate DNA, RNA, and protein, users may select to only isolate two out of the three molecules by following the appropriate protocol. As shown in the workflow (Fig 10.1), the DNA purification step does not affect RNA and protein isolation; the RNA purification step does not affect protein isolation.

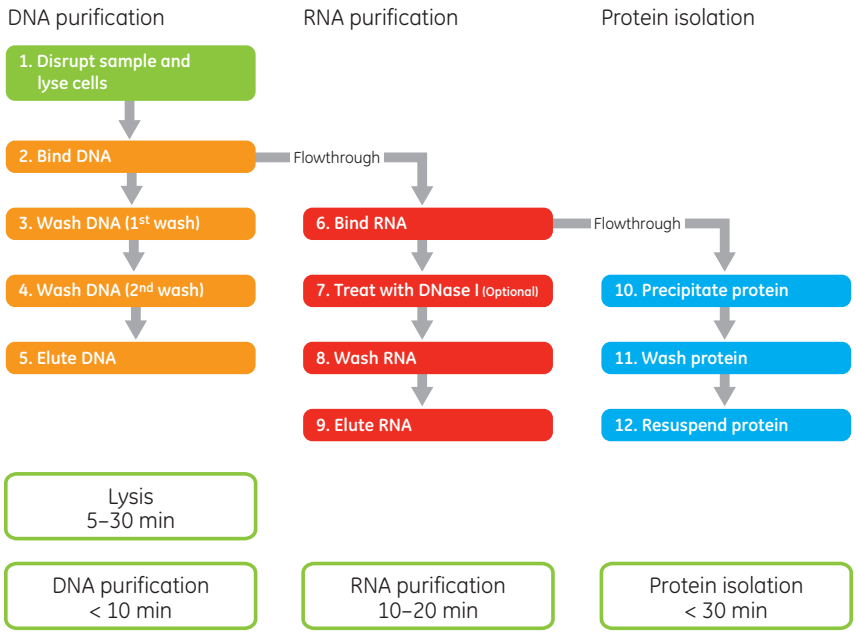


Fig 10.1. illustra triplePrep Kit workflow.

RNA in the flowthrough is stable for about 30 min at room temperature or 60 min on ice depending on sample type. To prevent potential RNA degradation, users may purify RNA immediately at this point by following step 6 before returning to step 3 for DNA purification. DNA bound to the mini column is stable for at least 30 min at room temperature. When DNase treatment is not needed, experienced users may also simultaneously purify DNA and RNA to save time and ensure RNA quality.

illustra triplePrep Kit conveniently provides a flexible lysis buffer for the long-term storage of samples, avoiding the need for costly stabilization reagents. Following sample disruption, samples can be stored in lysis buffer type 15 at -80°C for 6 mo or up to 4 h at room temperature.

The following tables provide information on expected performance (Table 10.2) and typical outputs and yields (Table 10.3) for illustra triplePrep Kit.

Table 10.2. Expected performance of illustra triplePrep Kit.

Sample Type	Rat Liver	HeLa Cells
Sample input amount	1–20 mg	0.3–5 million
Genomic DNA yield	1–3 µg (per mg tissue)	4–12 µg (per million cells)
Total RNA yield	3–7 µg (per mg tissue)	10–15 µg (per million cells)
Protein yield	80–160 µg (per mg tissue)	100–200 µg (per million cells)
Genomic DNA purity (A_{260}/A_{280})	≥1.7	
Genomic DNA size	≥15 kb	
Total RNA purity (A_{260}/A_{280})	≥1.9	
Total RNA quality (RIN)	8.0–10	
Total RNA quality 28s:18s	1.5–2.5	
Time/preparation (excluding lysis)	45–60 min	

Table 10.3. Typical yields and purities of genomic DNA, total RNA, and total protein from different tissues and cells.

	Cell	HeLa	NIH-3T3	CHO-K1	HEK-293
	Cell input amount (million)	1	1	1	1
DNA	Yield (µg)	8.5	9.5	4.4	9.1
	Purity (A_{260}/A_{280})	1.9	1.9	1.9	1.9
RNA	Yield (µg)	13.7	7.9	6.6	10.1
	Purity (A_{260}/A_{280})	2.1	2.1	2.0	2.0
Protein	Yield (µg)	139	127	77	139

	Tissue	Liver	Brain	Heart	Kidney	Lung	Spleen
	Tissue input amount (mg)	10	10	10	10	10	10
	Disruption difficulty	Easy	Easy	Hard	Med	Med	Med
DNA	Yield (µg)	14.6	6.8	9.8	17.0	15.5	18.8
	Purity (A_{260}/A_{280})	1.9	1.8	1.9	1.9	1.8	1.8
RNA	Yield (µg)	44	5.5	2.9	4.4	8.8	18.9
	Purity (A_{260}/A_{280})	2.1	2.0	1.9	1.9	2.0	2.1
	Need DNase	Yes	No	No	Yes	Yes	Yes
Protein	Yield (µg)	1460	746	923	897	592	510

* *Yield and purity of genomic DNA, total RNA, and total protein may vary depending on the user and the nature and condition of input sample.*

Procedure for isolating genomic DNA, total RNA, and total protein using illustra triplePrep Kit

Figure 10.1 shows a flowchart for use of illustra triplePrep Kit. Genomic DNA, total RNA, and denatured protein can be isolated in 12 steps.

Materials

Buffers, lyophilized DNase I, illustra mini columns (2 types), and collection tubes are provided with the product

β-mercaptoethanol

Nuclease-free water, molecular biology grade

Absolute acetone

2-D DIGE buffer* (for easy protein resuspension): see Advance preparation for recipe or purchase one of the 2-D Protein Extraction Buffers from GE Healthcare. Buffer V (28-9435-27) is closest to the recipe provided below.

Optional: PBS if processing fresh cultured cell lines

Optional: Liquid nitrogen

Optional: Protein concentration determination reagents (e.g. 2-D Quant Kit, supplied by GE Healthcare)

1.5 ml DNase- and RNase-free microcentrifuge tubes

DNase- and RNase-free pipette tips

For tissue samples: Rotor-stator homogenizer or mortar and pestle

Optional: Nuclease-free 20-gauge needle and syringe (1 ml) if using mortar and pestle or for cell lysate homogenization

* *Protein resuspended in 2-D DIGE buffer can be used for SDS-PAGE or Western blotting by mixing with 1 volume of 20% SDS. Add an appropriate sample loading buffer (i.e., 1 volume of 2× Laemmli buffer) and incubate at 70°C for 10 min before gel loading.*

Advance preparation

For each sample being processed, aliquot lysis buffer type 15 to a fresh DNase- and RNase-free tube and add 3.5 µl of β-mercaptoethanol.

Add absolute ethanol to wash buffer type 6 before use. Mix by inversion and indicate on the label that this step has been completed.

Add RNase-free water to the DNase vial. Aliquot and store at -20°C.

Prepare 2D-DIGE buffer as follows if you have not purchased it.

	100 ml final vol	**Final concentration**
Distilled water	40 ml	
Urea	42.0 g	7 M
Thiourea	15.2 g	2 M
Tris-HCl	0.36 g	30 mM
CHAPS (3-3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate)	4.0 g	4%

Mix well (do not heat over 37°C).

Adjust final pH to 8.5 (8.0 to 9.0) and adjust to final volume (100 ml) with distilled water.

Dispense into 5 ml aliquots and store at -20°C with a 3 mo expiration date (if using for 2-D DIGE study).

Protocol

Note: GE Healthcare supplies a wide range of buffer types across the illustra nucleic acid and Trap protein purification range. The composition of each buffer has been optimized for each application and may vary between kits. Please ensure you use the correct type of buffers and columns for your purification

1. Disrupt sample and lyse cells

Add lysis buffer with β-mercaptoethanol to the sample. Homogenize if necessary for your sample type. See product instructions for details. The high concentration of chaotropic salts lyses cells and inactivates nucleases and proteases. β-mercaptoethanol inactivates nucleases and proteases and dissociates nucleic acids from proteins.

Note: For the isolation of DNA, RNA, or total protein from whole blood, users should prepare lymphocytes on Ficoll-Paque PLUS. At least 20 ml of whole blood should be processed. Following lymphocyte isolation, cells should be counted. At least 10^7 cells should be used for isolating genomic DNA, total RNA, and protein with illustra triplePrep Kit. Following isolation, lymphocytes should be processed as described in step 1 above.

If using cells, the cell pellet can be stored at -80°C for up to 12 mo.

Homogenized sample in lysis buffer type 15 containing β-mercaptoethanol may be stored at -80°C for up to 6 mo.

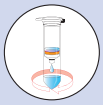
2. Bind DNA

Add homogenized lysate to a DNA mini column. Spin briefly in a microcentrifuge. The buffer conditions have been optimized to promote the selective binding of DNA (but not RNA) to the silica membrane of the DNA mini column.

Collect and save the flowthrough, which contains RNA and protein.

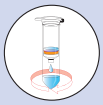
Optional: Purify RNA immediately—go to step 6. If DNase treatment is not needed, DNA and RNA can be purified simultaneously.

3. Wash DNA (1st wash)



Add lysis buffer (without β-mercaptoethanol) to column. Spin briefly. Lysis buffer removes contaminants from membrane-bound DNA.

4. Wash DNA (2nd wash)



Add wash buffer to the column. Spin briefly. The ethanolic wash buffer removes residual salts and dries the silica membrane at the same time.

5. Elute DNA

Add elution buffer type 5. Spin briefly. DNA is eluted in a low-ionic-strength buffer containing EDTA.

Proceed immediately to step 6 if RNA has not yet been isolated. Then proceed to step 7 if DNase treatment is required (e.g., if the crude lysate contains more than 20 µg of DNA).

6. Bind RNA

Add acetone to the column flowthrough from step 2. Apply to an RNA mini column. Spin briefly. Acetone promotes the binding of total RNA to the silica membrane of the RNA mini column in the presence of chaotropic salt in the lysis buffer.

Collect and save the column flowthrough, which contains protein. Flowthrough containing total protein can be stored at -80°C for up to 6 mo.

7. Treat with DNase I (optional)

Add DNase to the column in step 5. Incubate for 10 min at room temperature.

On-column DNase digestion removes residual genomic DNA bound to the silica membrane.

8. Wash RNA

Add wash buffer to the RNA mini column. Spin briefly. The ethanolic wash buffer removes residual salts and dries the silica membrane at the same time.

9. Elute RNA

Apply elution buffer type 9 to the RNA mini column. Spin briefly. RNA is eluted in RNase-free water.

Follow precautions against introducing RNases into the purified RNA sample.

Store purified RNA at -80°C. Purified RNA is stable for up to 12 mo.

10. Precipitate protein

Add protein precipitation buffer to the column flowthrough from step 6. Vortex vigorously and incubate for 5 min at room temperature. Spin for 10 min and remove supernatant. Proteins are precipitated by an acid- and salt-based method.

11. Wash protein

Add water to the protein pellet. Pipette up and down. Spin briefly. Remove the supernatant. Water solubilizes and removes acid and salt from the protein pellet.

12. Resuspend protein

Add 2-D DIGE buffer. Incubate for 5 min at room temperature.

Urea and detergent in 2-D DIGE buffer ensure complete protein pellet solubilization. Note that the protein will be in a denatured form. (Protein is denatured during lysis.)

Downstream applications

The following data show the high yield, purity, and quality of molecules prepared using illustra triplePrep Kit. Sample preparation considerations for different analytical techniques for genomic DNA and total RNA are provided in Chapters 4 and 6, respectively.

Genomic DNA

Genomic DNA was isolated from 10 mg of rat liver tissue using illustra triplePrep Kit or DNeasy™ Kit. Yield, purity, and quality were compared for both samples (Fig 10.2). Genomic DNA was used to amplify a 1.5 kb fragment by PCR. The resulting fragment was then sequenced to compare performance in downstream applications. Results showed higher than average yields with illustra triplePrep Kit (15.4 µg ± 0.60) compared with DNeasy Blood and Tissue Kit (9.8 µg, ± 2.10) using the same amount of input sample. Genomic DNA isolated from both kits produced similar purities as determined by A_{260}/A_{280} optical density ratios (1.90 ± 0.02). The sequenced PCR fragment generated from illustra triplePrep Kit showed higher Phred20 scores (744 bases) compared with that from DNeasy Blood and Tissue Kit (723 bases), indicating the suitability of genomic DNA in downstream applications such as PCR and sequencing. Additional data using genomic DNA isolated with triplePrep Kit are provided in Chapter 4.

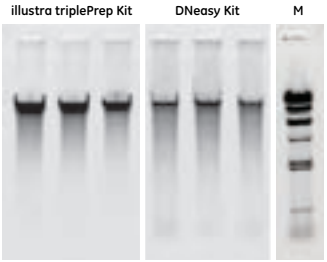


Fig 10.2. 2% of isolated genomic DNA from 10 mg of rat liver analyzed on an 0.8% agarose gel. M = molecular weight ladder.

Total RNA

Total RNA was isolated from 10 mg of rat liver tissue using illustra triplePrep Kit or RNeasy™ Mini Kit. Yield, purity, and quality were compared for both samples (Fig 10.3). Both kits yielded high-quality total RNA (Fig 10.3A).

Total RNA samples were used to amplify mRNA expressed at high-, medium-, and low levels (i.e., 18S ribosomal RNA, cytochrome P450, and c-fos, respectively). Expression levels were monitored by RT-qPCR. Similar slopes and C_q values were obtained in all three cases, indicating similar performance of the kits in downstream applications (Fig 10.3B). Total RNA isolated from both kits produced similar quantities and purities as determined by A_{260}/A_{280} ratios (data not shown).

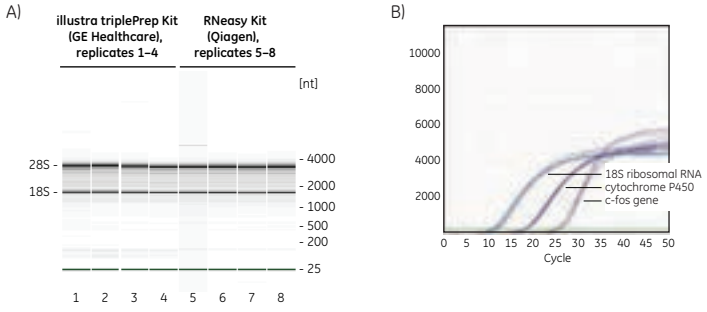


Fig 10.3. Total RNA isolation from 10 mg of rat liver tissue using illustra triplePrep Kit and RNeasy Mini Kits. **(A)** Analysis using Agilent Bioanalyzer. **(B)** RT-qPCR results for amplified 18S ribosomal RNA, cytochrome P450, and c-fos genes. These are expressed at high, medium, and low levels, respectively.

Total protein

Total protein was isolated from the second flowthrough following genomic DNA and total RNA extraction. The total protein yield was similar for both illustra triplePrep Kit and a 2D-DIGE reference method (Fig 10.4A). Western blotting with specific antibodies was used to detect β-actin or GAPDH proteins and showed comparable high quality (Fig 10.4B). Quality was also assessed by separating the peptides by nanoRPC on Ettan™ MDLC coupled to a Finnigan™ LTQ Linear Ion Trap mass spectrometer. The LC-MS data were evaluated using DeCyder™ MS Differential Analysis Software. Protein samples from illustra triplePrep Kit extraction were also analyzed by LC-MS for peptide profiling and showed an average representation of 3115 ± 176 peptides (Fig 10.4C).

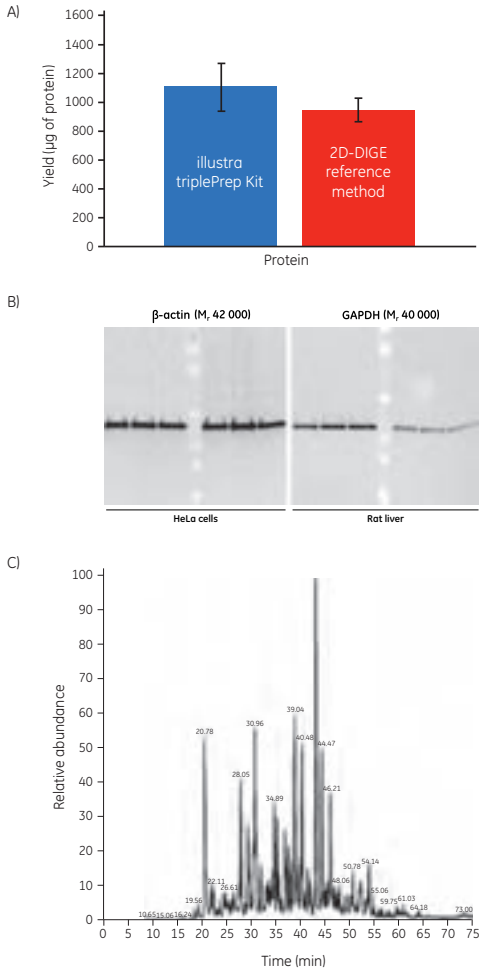


Fig 10.4. (A) Protein isolated from illustra triplePrep Kit and a 2-D DIGE reference method produced similar yields. **(B)** Western blotting analysis of proteins. **(C)** Base peak ion chromatogram of LC-MS.

2-D DIGE results are shown in Figure 10.5. In this experiment, triplePrep Kit and AllPrep DNA/RNA/Protein Mini Kit (Qiagen) were compared. Ten mg of rat liver was used as source material for each kit, and recommended protocols were followed for each method. More spots are detected by the triplePrep method (green spots) than with the competitor's method (red spots).

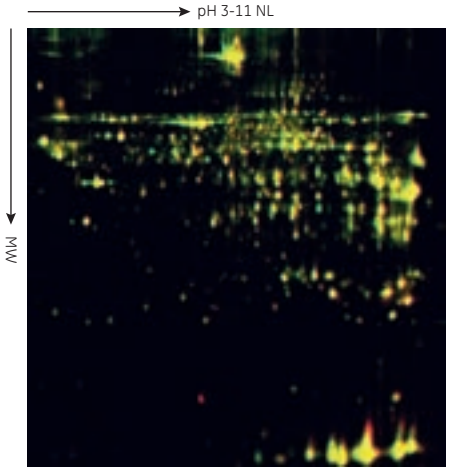


Fig 10.5. 2D-DIGE results from protein isolated from 10 mg rat liver using illustra triplePrep Kit or AllPrep DNA/RNA/Protein Mini Kit (Qiagen). Yellow spot overlay indicates proteins isolated with both methods, green spots indicate spots detected by the triplePrep method, and red spots indicate those detected by the AllPrep DNA/RNA/Protein Mini Kit method. Isoelectric focusing was used for the first-dimension separation step; SDS-PAGE was used for the second-dimension step.
NL = non-linear; MW = molecular weight.

Troubleshooting

Note that this is an abbreviated troubleshooting table. See triplePrep product instructions for a complete troubleshooting guide.

Table 10.4. Possible causes and solutions for problems that may occur when using illustra triplePrep Kit.

Problem	Possible cause	Solution
Low yield of genomic DNA	Homogenization of tissue incomplete	Use of rotor-stator homogenizer is recommended. For difficult to disrupt tissues such as mouse or rat tails, crushing the animal tissue in liquid nitrogen is recommended prior to proceeding with DNA extraction.
	Tissue sample old or subjected to repeat freeze/thaw cycles	For best results, use fresh tissue samples.
Poor quality of isolated genomic DNA	Too much tissue or too many cells used per sample	Ensure correct amount is used.
RNA is degraded/no RNA obtained	RNase contamination	Create an RNase-free working environment. Wear gloves during all steps of the procedure. Change gloves frequently. Use RNase-free, disposable polypropylene tubes. During nucleic acid extraction, keep the tubes closed whenever possible. Glassware should be oven-baked for at least 2 h at 250°C before use.
Poor RNA quality or yield	Reagents not prepared, stored, or applied properly	See product instructions for specifics.
	Suboptimal elution	Be sure that all the elution buffer type 9 gets into contact with the silica membrane. No drops should stick to the walls of the columns. The membrane has to be wetted completely.
		Ionic strength and pH influence A_{260} absorbance as well as ratio A_{260}/A_{280}; thus, for absorbance measurement, use 10 mM Tris-HCl pH 7 as the diluent and when setting the baseline.
Suboptimal performance of RNA in downstream experiments	Carry-over of ethanol or salt	See product instructions for specifics.
Protein pellet can not be resuspended	Wrong protein resuspension buffer used	2-D DIGE buffer is the best-tested buffer to solubilize precipitated protein. Follow the protocol—break the protein pellet with a pipette tip during water wash for effective removal of salts.

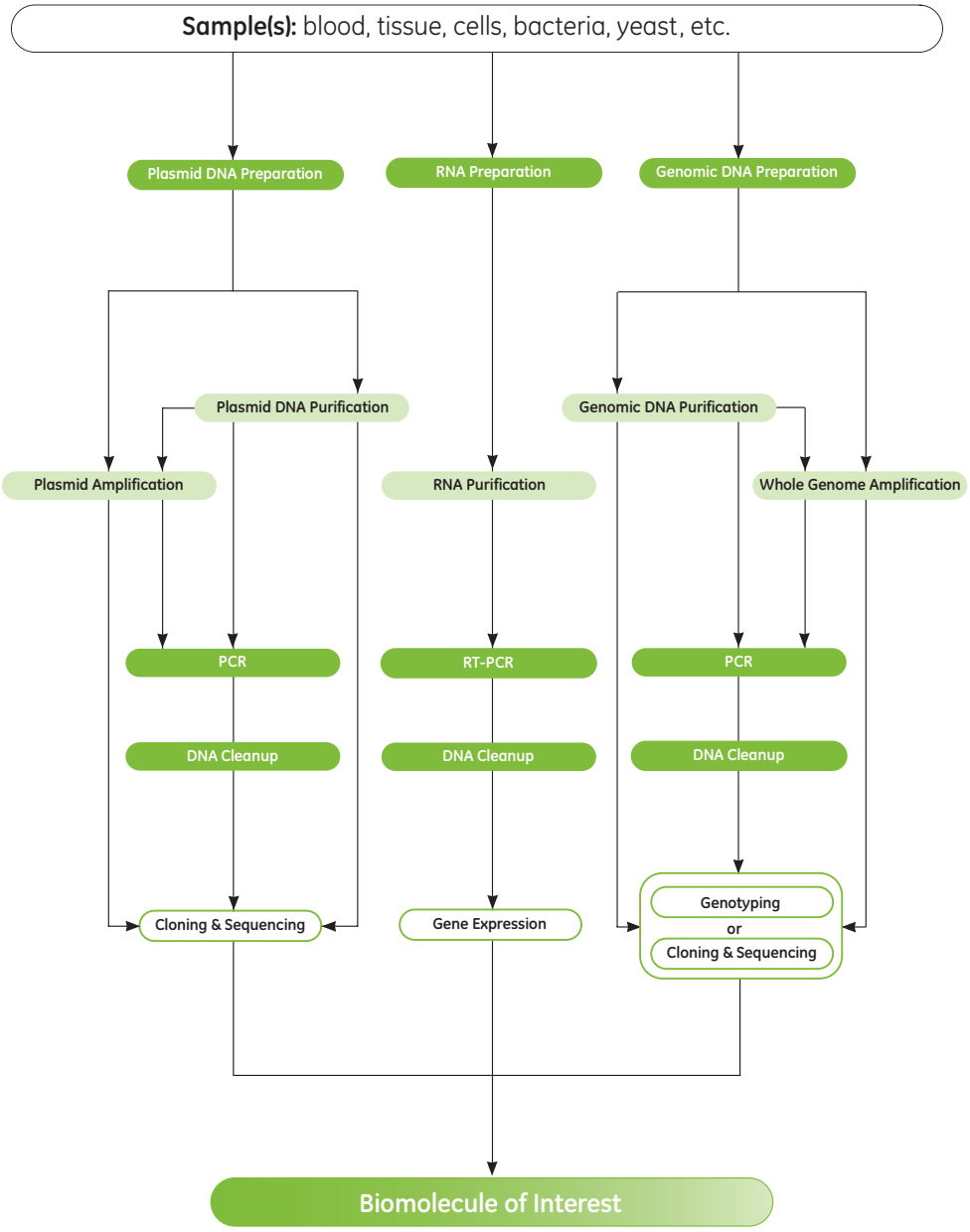
References

1. Coombs, L. M. *et al.,* Simultaneous isolation of DNA, RNA, and antigenic protein exhibiting kinase activity from small tumor samples using guanidine isothiocyanate. *Anal. Biochem.* **188**, 338–343 (1990).
2. Chomczynski, P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques* **15**, 532–534 (1993).
3. Tolosa, J. M. *et al.,* Research Report: Column-based method to simultaneously extract DNA, RNA, and proteins from the same sample. *BioTechniques* **43**, 799–804 (2007).

Appendices

1. Nucleic acid workflow

Genome and Gene Analysis



Sample collection, transport, archiving, and purification	FTA	FTA Elute	Indicating FTA	Indicating FTA Elute
Plant leaf press	●	●		
Plant homogenate	●	●		
Blood	●	●		
Buffy coat	●	●		
Cultured cells			●	●
Urine			●	●
Cerebrospinal fluid (CSF)			●	●
Saliva/buccal cells			●	●
Semen			●	●
Insects	●	●		
Tissue homogenate	●	●		
Tissue press	●	●		
Fungi			●	●
Mold			●	●
Bacteria			●	●
BAC vectors			●	
Plasmid clones			●	

* *Code numbers are for Classic Cards, 100 card pack size with 4 sample areas/card. Other pack sizes and formats are available. See Ordering information.*

See page 161 for Ordering information

Genomic DNA preparation

Sample type	Starting quantity (DNA yield)	tissue & cells Mini Spin	tissue & cells Midi Flow	blood Mini Spin	blood Midi Flow	bacteria Mini Spin	Nucleon BACC1	Nucleon BACC2 & BACC3	Nucleon HT	PhytoPure	GenomiPhi V2	GenomiPhi HY DNA
Animal tissue	> 300 cells (4 to 7 µg DNA yield)										●	
	> 300 cells (40 to 50 µg DNA yield)											●
	5 to 50 mg	●										
	Up to 200 mg		●									
Paraffin-embedded tissue or difficult samples	Up to 25 mg								●			
	20 to 30 µm thick paraffin section								●			
Cultured cells	> 300 cells (4 to 7 µg DNA yield)										●	
	> 300 cells (40 to 50 µg DNA yield)											●
	Up to 5 × 10^5 cells	●										
	1 to 3 × 10^5 cells						●					
	Up to 2 × 10^7 cells		●									
	3 × 10^6 to 1 × 10^7 cells							●				
Blood	5 to 10 µl (4 to 7 µg DNA yield)										●	
	5 to 10 µl (40 to 50 µg DNA yield)											●
	50 to 300 µl			●								
	1 ml						●					
	1 to 8 ml				●							
	10 ml							●				
Buffy coat	5 to 10 µl (4 to 7 µg DNA yield)										●	
	5 to 10 µl (40 to 50 µg DNA yield)											●
	50 to 300 µl			●								
Nucleated red blood cells	10 µl (4 to 7 µg DNA yield)										●	
	10 µl (40 to 50 µg DNA yield)											●
	10 µl			●								
	25 to 200 µl				●							
Bone marrow (suspended cells)	5 to 10 µl (4 to 7 µg DNA yield)										●	
	5 to 10 µl (40 to 50 µg DNA yield)											●
	200 µl			●								
Bacteria (Gram - and +)	> 300 cells (4 to 7 µg DNA yield)										●	
	> 300 cells (40 to 50 µg DNA yield)											●
	Up to 4.0 × 10^9 cells					●						
Plants	1 cm leaf (4 to 7 µg DNA yield)										●	
	1 cm leaf (40 to 50 µg DNA yield)											●
	1 seed (4 to 7 µg DNA yield)										●	
	1 seed (40 to 50 µg DNA yield)											●
	Up to 1 g									●		
FTA paper/Guthrie card	3 × 3 mm piece (4 to 7 µg DNA yield)										●	
	3 × 3 mm piece (40 to 50 µg DNA yield)											●
Buccal swab	Single swab			●								
	Single swab (4 to 7 µg DNA yield)										●	
	Single swab (40 to 50 µg DNA yield)											●
Purified genomic DNA	> 10 ng (4 to 7 µg DNA yield)										●	
	> 10 ng (40 to 50 µg DNA yield)											●
Downstream applications	Genotyping	●	●	●	●	●	●	●	●	●	●	●
	Cloning & sequencing	●	●	●	●	●	●	●	●	●	●	●

See page 162 and 163 for Ordering information

Plasmid DNA preparation

Sample type	Starting quantity	plasmidPrep Mini Spin	plasmidPrep Midi Flow	TempliPhi DNA Sequencing Template Preparation*	TempliPhi 100/500*	TempliPhi Large Construct*	TempliPhi Sequence Resolver*
Bacterial culture	1 µl (high-throughput)			●			
	1 µl (low- to medium-throughput)				●		
	1 to 3 ml	●					
	25 to 500 ml		●				
Bacterial colony	< 1 colony (high-throughput)			●			
	1 colony (low- to medium-throughput)				●		
Bacteria glycerol stock	< 1 µl (high-throughput)			●			
	< 1 µl (low- to medium-throughput)				●		
Purified DNA (small vectors)	> 1 ng (high-throughput)			●			
	> 1 ng (low- to medium-throughput)				●		
Purified BAC/fosmid DNA	> 1 ng					●	
Difficult templates (GC rich, secondary structures)	0.1 to 1 ng						●
M13 phage plaque	1 plaque			●	●		
M13 phage glycerol stock	< 1 µl			●	●		
Downstream applications	Cloning	●	●				
	Sequencing	●	●	●	●	●	●

** Can be used directly for PCR and subcloning. Addtional steps will be needed for transformation or transfection.*

See pages* 162 and 163 for Ordering information

RNA preparation

Sample type and format	Starting quantity	RNAspin Mini	RNAspin Midi	RNAspin 96	QuickPrep mRNA	QuickPrep *Micro* mRNA Purification	mRNA Purification
Total RNA							
Cultured cells and tissue	Up to 200 mg tissue or 5×10^7 cells		●				
Tube format	10 to 30 mg or up to 2×10^6 cells	●					
96-well plate vacuum	10 to 30 mg or up to 2×10^6 cells			●			
96-well plate centrifuge	30 mg or up to 1×10^7 cells			●			
mRNA purification							
Eukaryotic cells or tissue	1 to 1×10^7 cells or 100 mg tissue					●	
Eukaryotic cells or tissue	1 to 5×10^7 cells or 0.5 g tissue				●		
Eukaryotic total RNA	Total RNA or 25 mg to 1 g tissue						●
Downstream applications	Gene expression	●	●	●	●	●	●

See page 162 for Ordering information

PCR and RT-PCR

Amplification method	Starting quantity	Hot Start Mix RTG*	Hot Start Master Mix	PuReTaq RTG* PCR Beads	RTG* RT-PCR Beads	RT-PCR Master Mix	Taq DNA Polymerase (Cloned)
PCR	Basic PCR (amplicon up to 3 kb)	●	●	●			●
	Hot start PCR (amplicon up to 3 kb)	●	●				
	Long-range PCR (amplicon up to 20 kb)						
RT-PCR	Basic RT-PCR (amplicon up to 3 kb)				●	●	
	Long-range RT-PCR (amplicon up to 6 kb)						
Downstream applications	Genotyping	●	●	●	●	●	
	Cloning & sequencing	●	●	●	●	●	
	Gene expression	●	●	●	●	●	

* *RTG is an abbreviation of Ready-To-Go, a single-dose room temperature-stable bead format that contains all PCR or RT-PCR components. You just need to add DNA template, primer, and water for a reaction.*

See page 162 for Ordering information

DNA cleanup

Sample type	Starting quantity	ExoProStar	GFX PCR DNA and Gel Band	GFX 96 PCR	MicroSpin G-25	AutoSeq G-50	ProbeQuant™	NICK Columns	NAP-5 Columns	NAP-10 Columns	NAP-25 Columns	CyScribe GFX	MicroSpin G-50	MicroSpin S-200	MicroSpin S-300	MicroSpin S-400
PCR products	Any size, for sequencing only	●														
	50 bp to 10 kbp, few samples		●													
	100 bp to 10 kbp, many samples			●												
	10 to 50 µl				●											
Agarose gel slices or enzyme removal	50 kbp to 10 bp		●													
Sequencing reactions	12 to 25 µl					●										
Labeled DNA fragments	> 20 bases						●									
	> 10 bases				●											
	100 µg sample in 100 µl or no microcentrifuge							●								
Oligonucleotides	100 to 150 µl				●											
	0.1 to 0.5 ml								●							
	0.5 to 1 ml									●						
	1 to 2.5 ml										●					
cDNA	CyDye labeled probes											●				
A range of sample types	25 to 50 µl for labeled DNA												●			
PCR, sequencing and labeling reactions	10 to 100 µl for buffer exchange or desalting												●			
A range of volumes and sample sizes	PCR (25 to 50 µl) or labeling reaction (25 to 50 µl) > 100 bp													●		
	PCR (25 to 50 µl) or labeling reaction (50 to 75 µl) or fragment > 200 bp														●	
	PCR (50 to 100 µl) or labeling reaction (75 to 100 µl) or remove primers > 24 bases (DNA to be purified should be > 400 bp)															●
Downstream applications	Cloning & sequencing	●	●	●	●	●			●	●	●		●	●	●	●
	Gene expression		●	●	●		●	●	●	●	●	●	●	●	●	●
	Genotyping		●	●	●								●	●	●	●

See page 164 and 165 for Ordering information

Increasing throughput

Nucleic acid type	Sample type	Starting quantity (DNA yield)	RNAspin 96	GFX 96 PCR	ExoProStar	TempliPhi 100/500	GenomiPhi V2	GenomiPhi HY
Total RNA	Cultured cells and tissue, 96-well plate vacuum	10 to 30 mg or up to 2 × 10^6 cells	●					
	Cultured cells and tissue, 96-well plate centrifuge	30 mg or up to 1 × 10^7 cells	●					
PCR product (cleanup)	PCR mixture	Any size product, for sequencing only			●			
		100 bp to 10 kbp	●					
Plasmid DNA	Bacterial culture	1 µl				●		
	Bacterial colony	1 colony				●		
	Bacteria or M13 phage glycerol stock	< 1 µl				●		
	M13 phage plaque	1 plaque				●		
	Purified DNA (small vectors, BAC, or fosmid)	> 1 ng				●		
Genomic DNA	Animal tissue, cultured cells, or bacteria (Gram – or +)	> 300 cells (4 to 7 µg DNA yield)					●	
		> 300 cells (40 to 50 µg DNA yield)						●
	Blood, bone marrow (suspended cells), or buffy coat	5 to 10 µl (4 to 7 µg DNA yield)					●	
		5 to 10 µl (40 to 50 µg DNA yield)						●
	Nucleated red blood cells	10 µl (4 to 7 µg DNA yield)					●	
		10 µl (40 to 50 µg DNA yield)						●
	Plant	1 cm leaf or 1 seed (4 to 7 µg DNA yield)					●	
		1 cm leaf or 1 seed (40 to 50 µg DNA yield)						●
	FTA matrix/Guthrie card	3 × 3 mm piece (4 to 7 µg DNA yield)					●	
		3 × 3 mm piece (40 to 50 µg DNA yield)						●
	Buccal swab	Single swab (4 to 7 µg DNA yield)					●	
		Single swab (40 to 50 µg DNA yield)						●
	Purified genomic DNA	>10 ng (4 to 7 µg DNA yield)					●	
		>10 ng (40 to 50 µg DNA yield)						●

See page 163 and 165 for Ordering information

DNA, RNA, and protein preparation from an undivided sample

Sample type	Starting quantity	tripleprep Kit
Cultured cells	0.3–5 × 10^7 cells	•
Animal tissue	1–20 mg tissue	•
Purified lymphocytes	At least 1 × 10^7 cells	•

Downstream applications	Cloning & sequencing	•
	Gene expression	•
	Genotyping	•
	Protein analysis (1D, 2D, LCMS)	•

See page 165 for Ordering information

3. Nucleic acid conversion data

Common abbreviations

bp	base pair(s)
dNTP	2'-deoxynucleoside 5'-triphosphate
ddNTP	2', 3'-dideoxynucleoside 5'-triphosphate
ds	double-stranded (e.g., double-stranded DNA)
kb	kilobase; 1000 bases (or base pairs)
kDa	kilodalton
M	molarity; number of moles per liter of solution
Mb	megabase; 10^6 bases (or base pairs)
m	molecular mass (g/mol), 1/12 the mass of ^{12}C
M_r	relative molecular mass (molecular weight), ratio between the mass of 1 mole of a substance and 1 mole of ^{12}C. M_r is dimensionless.
mol	mole(s)
NTP	ribonucleoside 5'-triphosphate
ss	single-stranded (e.g., single-stranded DNA)

Codon dictionary

5'-OH Terminal base	Middle base U	C	A	G	3'-OH Terminal base
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Tyr	Asn	Ser	U
	Ile	Tyr	Asn	Ser	C
	Ile	Tyr	Lys	Arg	A
	Met	Tyr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val*	Ala	Glu	Gly	G

* *Codes for Met if in the initiator position.*

Metric conversions

1 kg (kilogram)	$= 10^3$ g
1 g (gram)	= 1 g
1 mg (milligram)	$= 10^{-3}$ g
1 µg (microgram)	$= 10^{-6}$ g
1 ng (nanogram)	$= 10^{-9}$ g
1 pg (picogram)	$= 10^{-12}$ g
1 fg (femtogram)	$= 10^{-15}$ g

Molar masses of nucleic acids

Average molar mass of a deoxynucleotide base	= 324.5 g/mol
Average molar mass of a deoxynucleotide base pair	= 649 g/mol
Average molar mass of a ribonucleotide base	= 340.5 g/mol
1 kb of dsDNA (sodium salt)	= 6.5×10^5 g/mol
1 kb of ssDNA (sodium salt)	= 3.3×10^5 g/mol
1 kb of ssRNA (sodium salt)	= 3.4×10^5 g/mol
1×10^6 g/mol of dsDNA (sodium salt)	= 1.54 kb
λ DNA	= 3.1×10^7 g/mol (1)
pBR322 DNA	= 2.8×10^6 g/mol (1)
ϕX-174 DNA	= 3.6×10^6 g/mol (1)
E. coli DNA	= 3.1×10^9 g/mol (1)

Mass-to-mole conversions

1 µg/ml of DNA	= 3.08 µM phosphate
1 µg/ml of a 1 kb DNA fragment	= 3.08 nM 5′-ends
1 µg of a 1 kb DNA fragment	= 1.5 pmol = 9.1×10^{11} molecules
1 µg of a 1 kb DNA fragment	= 3.0 pmol 5′-ends
1 pmol of a 1 kb DNA fragment	= 0.65 µg
1 µg of pUC18/19 DNA (2686 bp)	= 0.57 pmol = 3.4×10^{11} molecules
1 pmol of pUC 18/19 DNA	= 1.77 µg
1 µg of pBR322 DNA (4361 bp)	= 0.35 pmol = 2.1×10^{11} molecules
1 µg of linear pBR322 DNA	= 0.70 pmol of 5′-ends
1 pmol of pBR322 DNA	= 2.83 µg
1 pmol of 5′-ends of linear pBR322	= 1.4 µg
1 µg of M13mp18/19 DNA (7249 bp)	= 0.21 pmol = 1.3×10^{11} molecules
1 pmol of M13mp18/19 DNA	= 4.78 µg
1 µg of λ DNA (48 502 bp)	= 0.033 pmol = 1.8×10^{10} molecules
1 pmol of λ DNA	= 32.01 µg

Determining the molar mass and moles of ends of a double-stranded DNA fragment

Molar mass of a dsDNA fragment	= (# of bp) × (649 g/mol/bp)
Moles of ends of linear DNA	= 2 × (g of DNA)/(# of bp) × (649 g/mol/bp)
Moles of ends generated by a restriction digest:	
for linear DNA	= (# of cuts) × (moles of DNA) × 2 (ends per cut) + 2 (ends of linear DNA) × (moles of DNA)
for circular DNA	= (# of cuts) × (moles of DNA) × 2 (ends per cut)

4. Methodology

a. Options for analysis of DNA quantity, purity, and quality

Spectrophotometric measurement

Spectrophotometry can be used to estimate DNA or RNA concentration and to analyze the purity of the preparation. Typical wavelengths for measurement are 260 nm and 280 nm. In addition, measurements at 230 nm and 340 nm can provide further information (see later for more detail)

A_{260} readings should be between 0.1 and 1.0 to ensure significance (see "Purity analysis", below). Conversion factors for double-stranded DNA, RNA, and single-stranded DNA are for readings taken at neutral pH (i.e., sample diluted in low-salt, neutral pH buffer). For RNA samples that

will be recovered from the cuvette, use RNase-free cuvettes or treat quartz cuvettes to remove RNases. Dilute RNA with buffer that is RNase-free.

Quantitation

Analyzing the UV absorption of a nucleic acid solution at 260 nm provides a simple estimation of the concentration because purines and pyrimidines in nucleic acids show absorption maxima around 260 nm (247–272 nm). Using a 1 cm light path, the extinction coefficient for nucleotides at 260 nm is 20. Based on this extinction coefficient, a 50 µg/ml solution of double-stranded DNA, a 40 µg/ml solution of RNA, and a 33 µg/ml solution (lower for oligonucleotides; see below) of single-stranded DNA all exhibit an absorbance of 1.0 when analyzed in a quartz cuvette in low salt and at neutral pH. Using this property, an estimation of the concentration of the double-stranded DNA, RNA, or single-stranded DNA can be made by inserting these constants (50, 40, and 33, respectively) into the following formula:

Nucleic acid concentration (µg/ml) = A_{260} × dilution factor × constant

For example, for a double-stranded DNA solution diluted 40× for measurement, with an A_{260} reading of 0.20, the estimated concentration of the undiluted sample would be 0.20 × 40 × 50 = 0.4 mg/ml or 400 µg/ml.

The estimated concentration of the DNA or RNA preparation can be multiplied by the total sample volume to give the total quantity of DNA/RNA. For example, for an RNA solution of 200 µg/ml in a volume of 100 µl, the total quantity of RNA would be 20 µg.

Note: For oligonucleotides, an A_{260} of 1.0 represents anywhere from 20 to 33 µg/ml, with the actual conversion factor dependent on the length and base sequence of the oligonucleotide. For a more accurate approximation, consult reference 2.

Purity analysis

Nucleic acids extracted from cells normally require purification to remove protein impurities. The A_{260}/A_{280} ratio gives an indication of protein contamination; however this measurement is only an indication and not a definitive assessment. DNA and RNA preparations exhibiting an A_{260}/A_{280} ratio of 1.7 to 1.9 and ≥ 2.0, respectively, are indicative of samples possessing good purity. Deviations from these values may indicate the presence of potential impurities; however, care must be taken when interpreting spectrophotometric data, and a more suitable indication of purity and quality should also be considered based upon the successful use of the nucleic acid in downstream applications (e.g., restriction enzyme digestion, etc.).

In the absorbance spectrum for nucleic acids, the absorbance reading at 260 nm is located near the top of a broad peak, whereas the 280 nm reading typically occurs in a steeply sloping region; therefore, small changes in wavelength at 280 nm will result in large changes in absorbance. Consequently, small variations in wavelength accuracy have a much larger effect at 280 nm than at 260 nm. It follows that the A_{260}/A_{280} ratio is susceptible to this effect, and users are warned that spectrophotometers of different designs may give slightly different ratios.

In practice, the concentration of the nucleic acid sample can also affect the A_{260}/A_{280} ratio. This is especially evident when a sample exhibits readings that approach the spectrophotometer's minimum or maximum detection limits. For example, if a solution is too dilute, the 280 nm background reading shows a greater proportional interference and thereby has a disproportionate effect on the final result. Therefore, it is advisable to ensure that the A_{260} value is > 0.1 but < 1.0 for accurate measurements.

Absorbance values at 230 nm can indicate the presence of additional impurities. This wavelength is near the absorbance maximum of peptide bonds and common buffers such as Tris, EDTA, or chaotropic salts. When measuring RNA samples, the A_{260}/A_{230} ratio should be > 2.0; a ratio lower than this is generally indicative of contamination with GTC, a reagent commonly used in nucleic acid purification. This reagent absorbs over the 230 to 260 nm wavelength range; therefore, a wavelength scan can be particularly useful when assessing the purity of nucleic acid samples.

A correction protocol is often used to compensate for the effects of high background absorbance levels. This is performed at a wavelength distant from those associated with the absorbance of nucleic acids and proteins. The procedure can adjust for the effects of turbidity, particulates, and high-absorbance buffers. NanoVue spectrophotometer from GE Healthcare possesses a background correction facility that is set at 320 nm. This default correction is automatically applied when measuring the concentration of all nucleic acids. The use of the background correction facility is recommended since spectrophotometric data can be grossly distorted by contaminating particles. This is especially true when analyzing dilute samples.

Agarose gel electrophoresis

The benefit of subjecting nucleic acid samples to gel electrophoresis (in combination with ethidium bromide staining) is that in addition to facilitating the isolation of specific-sized fragments for downstream applications such as cloning, it can also convey information about sample quality, purity, and concentration (quantity).

Quantitation

Electrophoresis can be used to estimate the amount of nucleic acid in a sample by the direct visual comparison to molecular weight markers of known concentration. Experienced molecular biologists routinely make such comparisons; for less experienced investigators there are a number of gel documentation systems available with software that facilitates a quantitative assessment. These assessments are based on comparing the fluorescent intensities of samples with controls or molecular weight markers of known concentrations.

Purity analysis

Electrophoresis can be used to estimate the purity of DNA or RNA preparations. When analyzing genomic DNA or plasmid DNA, the retention of DNA in the wells of electrophoresis gels and excessive DNA smearing often indicates residual protein contamination and nuclease activity, respectively. During genomic or plasmid DNA extractions in the absence of RNase, RNA will remain intact, and the rRNA bands will be visible. The presence of RNA will contribute to an erroneously high A_{260} absorbance determination, which would result in an over-representation of the concentration of the desired DNA product. During the preparation of total RNA from mammalian cells, two major bands are typically observed after electrophoresis with ethidium bromide. These correlate with the 28S and 18S rRNA, while the majority of mRNA is visualized as a faint smear underlying these two bands. So, for example, enrichment of mRNA using oligo(dT) cellulose would cause a significant reduction in the amount of rRNA present in these samples.

Quality analysis

Electrophoresis of DNA or RNA can provide a general idea regarding the intactness of the nucleic acid of interest. For example, electrophoresis of undigested plasmid DNA provides an opportunity to gauge the performance of the extraction method. In general, the more vigorous the extraction procedure is, the greater the exposure of the plasmid DNA to potential damage. Damage can be assessed by visualization of the amount of intact superhelical plasmid DNA present after purification. Any reduction is indicative of damage. Superhelical plasmid DNA typically runs ahead of covalently closed circular (CCC). In stringent applications such as mammalian cell transfection, extraction methods that facilitate the purification of predominantly superhelical plasmid DNA are generally considered to be more desirable. The size range of genomic DNA up to about 20 kb can be estimated using agarose gel electrophoresis versus a large molecular weight marker. PFGE can be performed to better visualize genomic DNA in excess of 20 kb. RNA integrity can be crudely visualized by comparing the ratio of the 28S and 18S rRNA bands (28S:18S of 2 generally indicates good quality RNA), and analyzing the agarose gel for excessive smearing below the 18S band is indicative of RNA degradation. More accurate quality analysis of RNA can be obtained using the RNA integrity number (RIN; see below).

The suppliers of electrophoresis equipment provide detailed information, references, and protocols with their products to address many variants in gel construction and buffer compositions for resolving nucleic acids of different types and sizes. These should be consulted for further details.

RNA integrity number

RNA quality analysis

The RNA integrity number (RIN) is generated by an algorithm designed to estimate the integrity of total RNA. Using RIN, sample integrity is no longer determined by the simple ratio of the ribosomal 28S and 18S bands, but by the entire electrophoretic trace of the RNA sample. This includes the presence or absence of degradation products. In this way, an automatic interpretation and assessment of an RNA sample is generated. The assigned RIN is independent of sample concentration, instrument, and analyst, and therefore represents an unbiased measurement standard for RNA integrity.

RIN values also allow researchers to compare RNA samples, for example, before and after prolonged storage. In addition, RIN ensures repeatability of experiments. For example, if a given RIN value is suitable for a specific experiment, then in general other samples possessing the same value should also be suitable for similar experiments. In another instance, a sample with, for example, a RIN of 5 might not work for microarray experiments but may be appropriate for RT-PCR experiments. The RNA Integrity Database is a repository of user-submitted total RNA traces and is designed to determine the typical total RNA profile for different tissue types, as well as the effects of using different RNA extraction methods and kits. Consult references 3 and 4 for more details.

Phred measurement

DNA quality

Phred quality (q) values express the probability of correctly calling a base-peak in a sequencing electropherogram. They are based on key parameters such as resolution, spectral cross talk, and uniformity of peak spacing. The q value is logarithmically proportional to the probability of correctly calling the identified peak (i.e., a Phred value of 20 is an accuracy of 99%, while Phred30 is 99.9% accurate). The minimal acceptable value for sequencing is a Phred value of 20 (5, 6). Phred values can be used to estimate the quality of any sequenced DNA.

Fluorescent stains

Quantitation

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm. The major disadvantages of this method are the relative contribution of nucleotides, single-stranded nucleic acids, and proteins to the signal, the interference caused by contaminants commonly found in nucleic acid preparations, the inability to distinguish between DNA and RNA, and the relative insensitivity of the assay (an A_{260} of 0.1 corresponds to a 5 µg/ml double-stranded DNA solution).

A variety of fluorescent stains are available that circumvent many of these problems. These dyes can be used for both the visualization and quantitation of nucleic acid. These range from the ethidium bromide commonly used for the visualization of nucleic acids during agarose gel electrophoresis to the cyanine dye SYBR™ Green. The latter is routinely used in real-time qPCR to detect the accumulation of PCR products.

Ethidium bromide is a DNA intercalating agent used as a fluorescent nucleic acid stain. When exposed to ultraviolet light, it fluoresces with an orange color, intensifying almost 20-fold after binding to DNA. It is commonly used to detect nucleic acids during gel electrophoresis (e.g., double-stranded DNA derived from PCR amplifications, restriction digests, etc.). It can also be used to detect single-stranded RNA because this molecule usually folds back onto itself, providing local base-pairing for the dye to intercalate. Caution should be used with ethidium

bromide because it may be a strong mutagen. It is also widely assumed to be a carcinogen or teratogen, although this has never been carefully tested.

Hoechst (bis-benzimide) dyes are part of a family of sensitive fluorescent stains used for labeling DNA (especially the nuclei) in fluorescence microscopy and for fluorescent-activated cell sorting. Hoechst 33258 is routinely used to quantitate DNA in solution. The dye is essentially selective for double-stranded DNA and does not exhibit any significant interference in fluorescent emission in the presence of proteins or other contaminants. It facilitates the detection and quantitation of DNA concentrations as low as 5 ng/ml.

SYBR Green is generally used for post-electrophoresis staining of double-stranded DNA in agarose or polyacrylamide gels, as well as in real-time qPCR to detect the accumulation of PCR products. The detection limit is ~60 pg per band with 300 nm transillumination (20 pg at 254 nm). SYBR Green can also be used to detect single-stranded DNA and RNA in denaturing agarose/formaldehyde and polyacrylamide/urea gels, although at a reduced sensitivity. The stain is able to detect as little as 1 to 2 ng of a synthetic 24-mer oligonucleotide on a 5% polyacrylamide gel. This sensitivity is 25 to 50 times greater than can be achieved with ethidium bromide. Due to its exceptional sensitivity, SYBR Green is routinely used in applications when only a limited amount of DNA is available.

SYBR Green is also routinely used in real-time qPCR to detect the accumulation of double-stranded PCR products formed during progressive PCR thermal cycles. When SYBR Green is added to a sample, it immediately binds to all double-stranded DNA present. During PCR, the thermostable DNA polymerase amplifies the target sequence to create PCR products. The SYBR Green then binds to each new copy of double-stranded DNA. As PCR progresses, more PCR products are generated. Since the dye binds to all double-stranded DNA, the result is an increase in fluorescence intensity proportionate to the amount of PCR product produced.

The main advantages of using SYBR Green for quantitative, real-time PCR is that it can be used to monitor the amplification of any double-stranded DNA without the use of a specific probe, thus reducing assay setup and running costs. However the nonspecific nature of SYBR green also represents a major disadvantage; that is, because the dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences and may generate false-positive signals.

PicoGreen™ is an alternative ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA in molecular biological procedures such as DNA amplification, cDNA synthesis for library production, and DNA fragment purification for subcloning. The PicoGreen reagent exhibits an emission maximum at 530 nm when bound specifically to double-stranded DNA (unbound PicoGreen reagent exhibits minimal fluorescence in solution). The detection range of PicoGreen when bound to double-stranded DNA is 1 to 1000 ng/ml.

RiboGreen™ is a sensitive fluorescent nucleic acid stain for determining the RNA concentration in solutions. The RiboGreen reagent exhibits minimal fluorescence when free in solution. Upon binding RNA, the fluorescence increases more than 1000-fold. However, a disadvantage of the RiboGreen reagent is that it also binds DNA. RNA-DNA mixed samples require pretreatment with DNase to generate an accurate RNA selective assay. The RiboGreen RNA assay is ~200-fold more sensitive than ethidium bromide-based assays and ~1000-fold more sensitive than absorbance measurements at 260 nm.

Regardless of the dye used, the quantitation of a DNA solution is achieved by the direct comparison against a standard curve of control samples of known concentrations. A stock solution of standard double-stranded DNA (e.g., derived from calf thymus or salmon sperm) resuspended in TE buffer is routinely used to generate the standard curve. However, to serve as an effective control it may be preferable to prepare the standard curve with a nucleic acid most similar to the type being assayed (genomic DNA, plasmid DNA etc.). The control should also be treated in a similar way as the experimental samples.

b. Concentration of nucleic acids by precipitation

Concentration of DNA by isopropanol precipitation

If the purified genomic or plasmid DNA is too dilute for the selected downstream application, DNA may be concentrated by isopropanol precipitation.

1. Add 0.7 volumes of room temperature isopropanol to the purified sample.
2. Vortex, then spin for 15 min at 5000 × g at 4°C.
3. Remove the supernatant by decanting, taking care not to disturb the pellet.
4. Add 2 ml of 70% ethanol that has been pre-chilled to 4°C. Vortex briefly and spin for 10 min at 5000 × g at 4°C.
5. Carefully remove the supernatant without disturbing the pellet. Air dry for 5–10 min. Do not overdry the pellet as this will make the DNA difficult to redissolve.
6. Resuspend the DNA in the desired volume of a suitable buffer (e.g., TE pH 8.0 or 10 mM Tris-HCl pH 8.5). To ensure the pellet is completely dissolved, incubate at 55°C for 1 h.

Concentration of mRNA by precipitation

(Adapted from the protocol in QuickPrep mRNA Kit.)

Glycogen solution: 5–10 mg/ml glycogen in RNase-free or DEPC-treated water*

Potassium acetate: 2.5 M potassium acetate (pH 5.0) solution (prepared using RNase-free or DEPC-treated water)

DEPC-treated water: Prepare a 0.1% (v/v) solution of Diethyl Pyrocarbonate in distilled water, shake vigorously, and allow to stand overnight at room temperature. Autoclave the solution on the following day with the cap loosened. Commercially available RNase-free water may be used instead of DEPC-treated water in this protocol.

** 0.1% DEPC-treated water.*

1. Add 75 µl (1/10 volume) of potassium acetate solution and 10 µl of glycogen solution to 0.75 ml of mRNA in RNase-free water. Add 1.5 ml of chilled 95% ethanol (2 to 2.5 volumes) and incubate the sample at -20ºC for a minimum of 15 min. The amount of glycogen should remain constant regardless of sample volume.
2. Collect the mRNA by centrifuging at max. speed in a microcentrifuge at 4ºC for 10 min. If the RNA will not be used immediately, store it in this precipitated state (in ethanol) at -80ºC.
3. Decant the supernatant and invert the tube over a clean paper towel. Gently tap the tube on the towel to facilitate the removal of excess liquid. Wash the pellet carefully by pipetting 1 ml of prechilled 80% ethanol into the tube and gently invert several times. Centrifuge at full speed at 4ºC for 10 min. Decant ethanol and allow to air dry. When all traces of ethanol are gone, redissolve the precipitated RNA in an appropriate volume of RNase-free water. To determine the appropriate resuspension volume, consider the RNA concentration desired, the concentration before precipitation and the volume of the sample subjected to precipitation. However, the percentage of the RNA recovered after precipitation will depend on the total amount present. With 10 µg of RNA, for example, approximately 70% will be recovered. You may therefore wish to redissolve the pellet in a volume 25 to 50% smaller than would be required if all of the RNA were recovered.

c. Estimation of cell density for cultured mammalian cells

Cell numbers should be determined using an automated cell counter (e.g., NucleoCounter™). Alternatively, individual cells should be counted under a microscope using a standard hemocytometer (Hausser Scientific/VWR, #1483). Below is a basic protocol for determining cell numbers using a hemocytometer.

1. Clean a hemocytometer and the short coverslip thoroughly and wipe clean with ethanol.
2. If working with adherent cells, trypsinize the cells and wash once with phosphate-buffered saline (PBS). If working with cells in suspension, pellet the cells at 5000 rpm for 1 min.
3. Resuspend cells in an appropriate volume of PBS to yield roughly 1×10^6 cell/ml. For example, mammalian cells grown inside a 25 cm^2 flask to confluence yield approximately 2.5×10^6 cells per flask (Visit Corning web site at http://catalog2.corning.com/Lifesciences/). If a 25 cm^2 flask was used add 2.5 ml of PBS. Make sure cells are completely resuspended without any visible clumps.
4. Add 10 µl of resuspended cells separately to two chambers of a hemocytometer (under a small coverslip), making sure the solution spreads completely under the coverslip by capillary action.
5. Place the hemocytometer under a light microscope, focus on the cells using lowest magnification and begin counting cells only at the four corner squares and the middle square in both chambers of the hemocytometer grid (7). Count all cells except those touching the middle lines at the bottom and right. Aim to have 50 to 100 cells per square-grid. If the cell count is >150/grid, dilute the cells, clean the hemocytometer, and re-count the cells.

d. RPM calculation from RCF

The appropriate centrifugation speed for a specific rotor can be calculated from the following formula:

$RPM = 1000 \times \sqrt{(RCF/1.12r)}$

Where RCF = relative centrifugal force; r = radius in mm measured from the center of the spindle to the bottom of the rotor bucket; and RPM = revolutions per minute.

For example, if an RCF of 735 × g is required using a rotor with a radius of 73 mm, the corresponding RPM would be 3000.

References

1. Ausubel, F. M. *et al.* (eds.), *Current Protocols in Molecular Biology*, Wiley and Sons, Inc., New York (1989).
2. Borer, P. N. in *The Handbook of Biochemistry and Molecular Biology*, 3rd edition (Fasman, G. D. ed.) CRC Press, Ohio, p. 589 (1975).
3. Schroeder, A. *et al.* The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol. Biol.* **7**, 3 (2006).
4. Imbeaud, S. *et al.* Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucl. Acids Res.* **33**, e56 (2005).
5. Ewing, B. *et al.* Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* **8**, 175–185 (1998).
6. Ewing, B. and Green, P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* **8**, 186–194 (1998).
7. Sambrook, J. and Russell, D. W. Preparation and analysis of eukaryotic genomic DNA, in *Molecular Cloning, A Laboratory Manual*, pp. 6.1–6.64 (2001).

Product index

Related literature

	Code No.
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illustra GFX PCR DNA and Gel Band Purification Kit: sNAPpier isolation and concentration of DNA fragments	28-9176-72
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TempliPhi DNA amplification methods	63-0046-15

Data files and application notes	
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GFX PCR DNA and Gel Band Purification Kit	18-1126-81
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illustra blood genomicPrep Midi Flow Kit	28-9162-81
illustra blood genomicPrep Mini Spin Kit	28-9090-83
illustra NICK and ProbeQuant G-50 Micro Columns	28-9175-27
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illustra plasmidPrep Mini Spin Kit	28-9075-92
illustra QuickPrep mRNA Purification Kit	18-1131-98
illustra tissue & cells genomicPrep Midi Flow Kit	28-9162-80
illustra tissue & cells genomicPrep Mini Spin Kit	28-9162-79
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Ordering information

Product	Quantity	Code No.
Sample collection, transport, archiving, and purification of nucleic acids		
FTA		
FTA Kit[1]	25 cards	WB120067
FTA Plant Kit[2]	20 cards	WB120068
FTA Starter Pack	N/A	WB120061
FTA Classic Card	25 cards, 4 sample areas/card 100 cards, 4 sample areas/card	WB120305 WB120205
Indicating FTA Classic Card	25 cards, 4 sample areas/card 100 cards, 4 sample areas/card	WB120306 WB120206
FTA Mini Card	25 cards, 2 sample areas/card 100 cards, 2 sample areas/card	WB120355 WB120055
Indicating FTA Mini Card	25 cards, 2 sample areas/card 100 cards, 2 sample areas/card	WB120356 WB120056
FTA Micro Card	25 cards, 1 sample area/card 100 cards, 1 sample area/card	WB120310 WB120210
Indicating FTA Micro Card	25 cards, 1 sample area/card 100 cards, 1 sample area/card	WB120311 WB120211
FTA Gene Card	25 cards, 3 sample areas/card 100 cards, 3 sample areas/card	WB120308 WB120208
PlantSaver FTA Card	25 cards, 4 sample areas/card 100 cards, 4 sample areas/card	WB120365 WB120065
FTA Card/Pouch/Desiccant	1000 cards	WB120217
FTA Elute		
FTA Elute Micro Card	25 cards, 4 sample areas/card 100 cards, 4 sample areas/card	WB120401 WB120410
Indicating FTA Elute Micro Card	25 cards, 1 sample area/card 100 cards, 1 sample area/card	WB120412 WB120411
FTA Elute Sample Pack[3]	N/A	WB120461
FTA reagents and accessories		
FTA Purification Reagent	500 ml	WB120204
FTA Gene Card Tray	20	WB100030
Sterile Foam Tipped Applicators	100	WB100032
Harris Micro Punch 1.2 mm (with cutting mat)	1	WB100005
Replacement Tip 1.2 mm	1	WB100006
Harris Micro Punch 2.0 mm (with cutting mat)	1	WB100007
Harris Micro Punch 3.0 mm (with cutting mat)	1	WB100038
Replacement Tip 2.0 mm	1	WB100008
Replacement Cutting Mat	1	WB100020
Multi-Barrier Pouch, Small (8 × 7 cm)	100	WB100036
Multi-Barrier Pouch, Large (9 × 15 cm)	100	WB100037
Desiccant Packets (1 g)	1000	WB100003

[1] *Includes: 25 FTA Micro Cards, 2 × 25 mL FTA purification reagent, 2 Uni-Core Punches with cutting mat, instructions.*

[2] *Includes: 20 FTA PlantSaver cards, 2.0 mm Uni-Core Punch and cutting mat, 2 × 25 ml FTA purification reagent, 1 pair of nitrile gloves with 1 cutting mat and round bottom test tube for sample application, instructions.*

[3] *Includes: 5 FTA Elute Micro Cards, 3.0 mm Uni-Core Punch, instructions.*

Harris Micro Punch 1.2 mm, Replacement Plunger	1	WB100025
Harris Micro Punch 2.0 mm, Replacement Plunger	1	WB100026
Harris Uni-Core Punch 1.2 mm	4	WB100028
Harris Uni-Core Punch 2.0 mm	4	WB100029
Harris Uni-Core Punch 3.0 mm	4	WB100039
Harris Uni-Core Punch 6.0 mm	4	WB100040
Sterile Omni Swab	100	WB100035

Genomic DNA preparation

illustra tissue & cells genomicPrep Mini Spin Kit	50 preps 250 preps	28-9042-75 28-9042-76
illustra tissue & cells genomicPrep Midi Flow Kit	25 preps	28-9042-73
illustra blood genomicPrep Mini Spin Kit	50 preps 250 preps	28-9042-64 28-9042-65
illustra blood genomicPrep Midi Flow Kit	25 preps 100 preps	28-9042-61 28-9042-62
illustra bacteria genomicPrep Mini Spin Kit	50 preps 250 preps	28-9042-58 28-9042-59
Nucleon BACC1	1 kit	RPN8501
Nucleon BACC2	1 kit	RPN8502
Nucleon BACC3	1 kit	RPN8512
Nucleon PhytoPure	50 preps × 0.1 g 50 preps × 1.0 g	RPN8510 RPN8511
Nucleon HT (hard tissue)	1 kit	RPN8509

Plasmid DNA preparation

illustra plasmidPrep Mini Spin Kit	50 preps 250 preps	28-9042-69 28-9042-70
illustra plasmidPrep Midi Flow Kit	25 preps 100 preps	28-9042-67 28-9042-68
Yeast Plasmid Isolation Kit	50 preps	US79220-50RXNS

RNA preparation

illustra RNAspin Mini Kit	20 preps 50 preps 250 preps	25-0500-70 25-0500-71 25-0500-72
illustra RNAspin Midi Kit	20 preps	25-0500-73
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illustra mRNA Purification Kit	2 purifications 4 purifications	27-9258-01 27-9258-02

DNA preparation by amplification

Phi29 DNA polymerase-based

illustra GenomiPhi V2 DNA Amplification Kit	25 reactions	25-6600-30

	100 reactions	25-6600-31
	500 reactions	25-6600-32
illustra GenomiPhi HY DNA Amplification Kit	25 reactions	25-6600-22
	100 reactions	25-6600-20
	1000 reactions	25-6600-25
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	50 reactions	28-9035-30
	200 reactions	28-9035-31
illustra TempliPhi Large Construct Kit	1000 reactions	25-6400-80

PCR-based

illustra Hot Start Mix RTG (0.5 ml tubes)	100 reactions	28-9006-46
illustra Hot Start Mix RTG (0.2 ml tubes)	96 reactions (12 × 8 strip wells)	28-9006-53
	480 reactions (12 × 8 strip wells × 5)	28-9006-54
illustra Hot Start Master Mix	100 reactions	25-1500-01
illustra PuReTaq Ready-To-Go PCR Beads (0.2 ml tubes/plate)	96 reaction plate	27-9557-01
	5 × 96 reaction plate	27-9557-02
illustra PuReTaq Ready-To-Go PCR Beads (0.5 ml tubes)	100 reactions	27-9558-01
illustra PuReTaq Ready-To-Go PCR Beads (0.2 ml hinged tubes with caps)	96 reactions	27-9559-01
illustra Ready-To-Go RAPD Analysis Beads	100 reactions	27-9500-01
illustra Ready-To-Go RAPD Analysis Kit	100 reactions and 6 primers	27-9502-01
illustra Ready-To-Go RT-PCR Beads (0.5 ml tubes)	100 reactions	27-9266-01
illustra Ready-To-Go RT-PCR Beads (0.2 ml tubes)	96 reactions	27-9267-01
illustra Ready-To-Go RT-PCR Beads (0.2 ml hinged tubes with caps)	96 reactions	27-9259-01
RT-PCR Master Mix (2×)	100 reactions	E78370
Taq DNA Polymerase (cloned)	250 units	27-0798-04
	4 × 250 units	27-0798-05
	10 × 250 units	27-0798-06

Nucleotides

illustra dATP		
2′-Deoxyadenosine 5′-Triphosphate Solution), 100 mM	25 µmol	28-4065-01
	100 µmol	28-4065-02
	500 µmol	28-4065-03
illustra dCTP		
2′-Deoxycytidine 5′-Triphosphate Solution, 100 mM	25 µmol	28-4065-11
	100 µmol	28-4065-12
	500 µmol	28-4065-13
illustra dGTP		
2′-Deoxyguanosine 5′-Triphosphate Solution, 100 mM	25 µmol	28-4065-21
	100 µmol	28-4065-22
	500 µmol	28-4065-23

illustra dTTP

2'-Deoxythymidine 5'-Triphosphate Solution, 100 mM	25 µmol	28-4065-31
	100 µmol	28-4065-32
	500 µmol	28-4065-33

illustra dUTP

2'-Deoxyuridine 5'-Triphosphate Solution, 100 mM	25 µmol	28-4065-41
	100 µmol	28-4065-42

illustra dNTP Sets

illustra dNTP Set (100 mM each A,C,G,T)	4 × 25 µmol	28-4065-51
	4 × 100 µmol	28-4065-52
	4 × 500 µmol	28-4065-53
illustra DNA Polymerization Mix dNTP Set (20 mM each A,C,G,T)	10 µmol	28-4065-57
	40 µmol (4 × 10 µmol)	28-4065-58
illustra PCR Nucleotide Mix dNTP Set (25 mM each A,C,G,T)	500 µl	28-4065-60
illustra PCR Nucleotide Mix dNTP Set (2 mM each A,C,G,T)	1 ml	28-4065-62
illustra PCR Nucleotide Mix dNTP Set (10 mM each A,C,G,T)	500 µl	28-4065-64

Nucleic acid cleanup

Silica

illustra GFX PCR DNA and Gel Band Purification Kit	100 purifications	28-9034-70
	250 purifications	28-9034-71
illustra GFX 96 PCR Purification Kit	10 × 96 well plates	28-9034-45
illustra CyScribe GFX Purification Kit	25 purifications	27-9606-01
	50 purifications	27-9606-02

Gel filtration

illustra MicroSpin G-25 Columns	50	27-5325-01
illustra AutoSeq G-50	50 columns	27-5340-01
	250 columns	27-5340-02
	1000 columns	27-5340-03
illustra MicroSpin G-50 Columns	50	27-5330-01
	250	27-5330-02
illustra ProbeQuant G-50 Micro Columns	50	28-9034-08
illustra MicroSpin S-300 HR Columns	50	27-5130-01
illustra MicroSpin S-200 HR Columns	50	27-5120-01
illustra MicroSpin S-400 HR Columns	50	27-5140-01
illustra NAP-5 Columns	20	17-0853-01
	50	17-0853-02
illustra NAP-10 Columns	20	17-0854-01
	50	17-0854-02
illustra NAP-25 Columns	20	17-0852-01
	50	17-0852-02
illustra NICK Columns	20	17-0855-01
	50	17-0855-02

Enzyme-based PCR product cleanup

illustra ExoProStar	20 reactions	US78220
	100 reactions	US78210
	500 reactions	US78211
	2000 reactions	US78212
	5000 reactions	US78225
illustra ExoProStar 1-Step	20 reactions	US77701

	100 reactions	US77702
	500 reactions	US77705
	2000 reactions	US77720
	5000 reactions	US77750

Increasing throughput of nucleic acid sample preparation

illustra GenomiPhi V2 DNA Amplification Kit	25 reactions	25-6600-30
	100 reactions	25-6600-31
	500 reactions	25-6600-32
illustra GenomiPhi HY DNA Amplification Kit	25 reactions	25-6600-22
	100 reactions	25-6600-20
	1000 reactions	25-6600-25
illustra TempliPhi DNA Sequencing Template Preparation Kit	2000 reactions	28-9642-86
illustra TempliPhi 100 Amplification Kit	100 reactions	25-6400-10
illustra TempliPhi 500 Amplification Kit	500 reactions	25-6400-50
illustra TempliPhi Sequence Resolver Kit	20 reactions	28-9035-29
	50 reactions	28-9035-30
illustra TempliPhi Sequence Resolver Kit	200 reactions	28-9035-31
illustra TempliPhi Large Construct Kit	1000 reactions	25-6400-80
illustra RNAspin 96 Kit	4 × 96 preps	25-0500-75
	24 × 96 preps	25-0500-76
illustra GFX 96 PCR Purification Kit	10 × 96 well plates	28-9034-45
illustra ExoProStar	20 reactions	US78220
	100 reactions	US78210
	500 reactions	US78211
	2000 reactions	US78212
	5000 reactions	US78225
illustra ExoProStar 1-Step	20 reactions	US77701
	100 reactions	US77702
	500 reactions	US77705
	2000 reactions	US77720
	5000 reactions	US77750

Preparation of DNA, RNA, and protein from an undivided sample

illustra triplePrep Kit	50 preps	28-9425-44

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