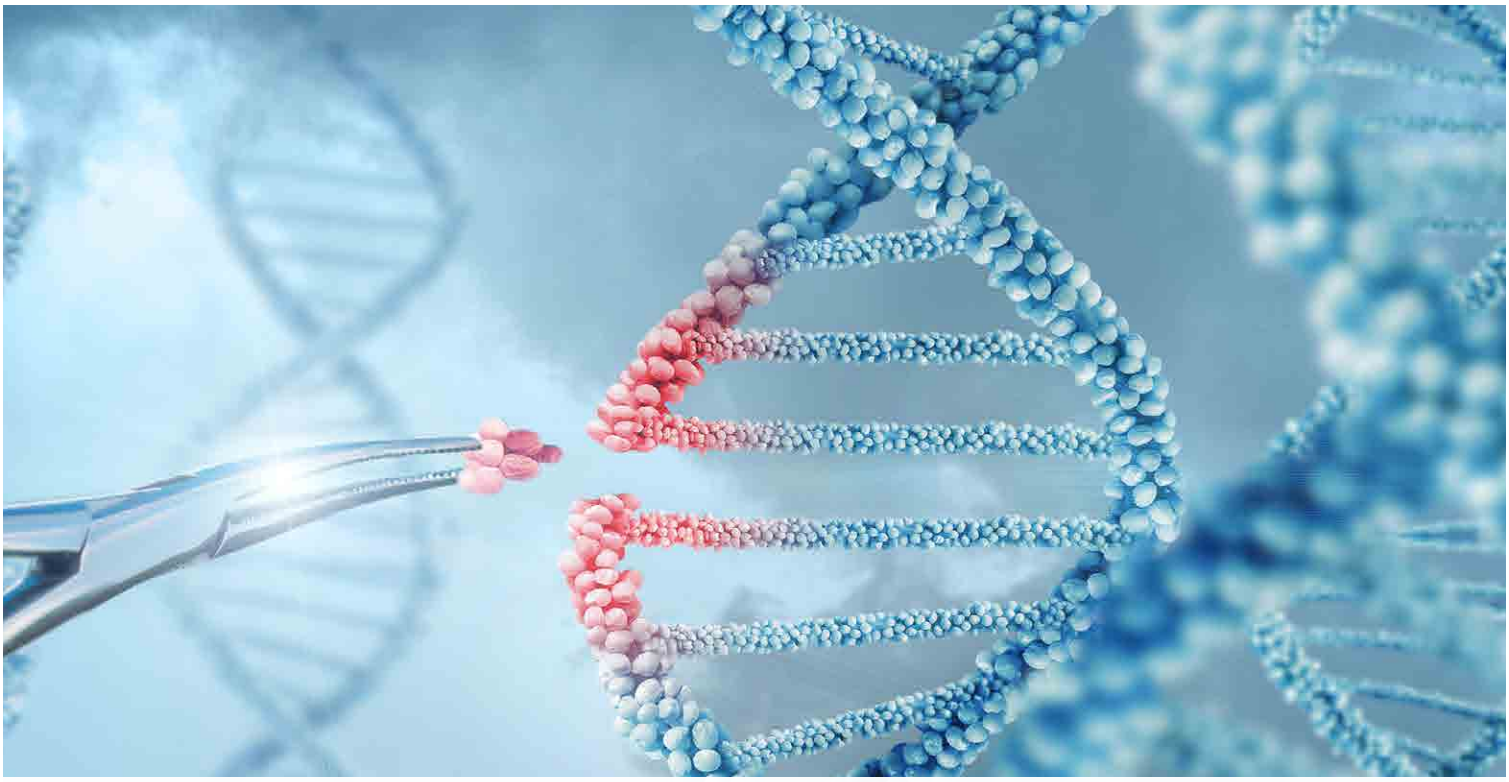

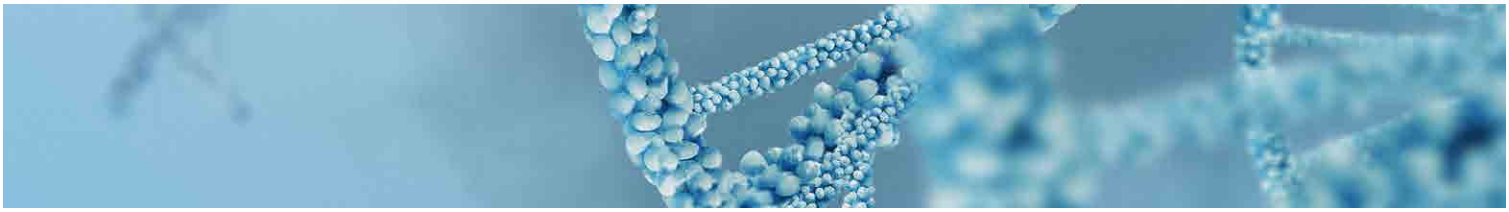

Focus: Gene Editing

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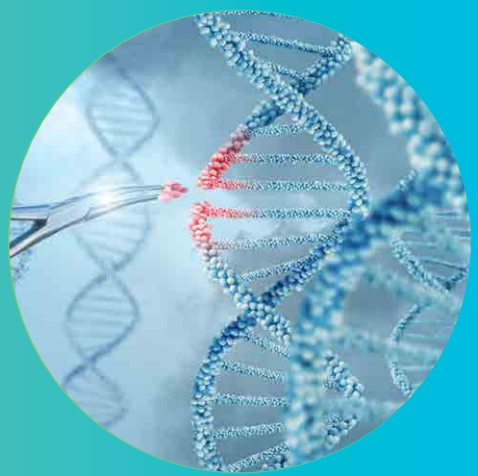
2020



Using aptamers to control enzyme activities

Hot Start *Taq* and beyond

By Nicole M. Nichols, Ph.D. and Nathan A. Tanner, Ph.D., New England Biolabs, Inc.

As molecular biology and diagnostic applications have become more demanding and sensitive, the ability to control enzymatic activities, and therefore reaction specificity, has naturally followed. Biotechnological innovations have led to several "hot start" approaches for the control of DNA polymerases, enabling the spread of PCR from the lab to the clinic with precise enzyme activation. Though powerful, these traditional methods typically require long activation times and very high temperature incubations that are incompatible with essential enzymes like reverse transcriptases. The aptamer-based hot start approach involves the selection of specific, modified aptamers for control of any enzymatic target of interest, even those that can't tolerate the extreme temperatures of PCR. NEB's selection of aptamers targeted to RTs and isothermal polymerases has enabled the first "warm start" enzymes that can be used in RT-qPCR and isothermal amplification. Control of enzyme activities remains a critical feature for demanding nucleic acid amplification methods, and the use of aptamer technology brings the benefits of hot start/warm start enzymes to the next generation of rapid, sensitive, and even isothermal molecular tests.

INTRODUCTION

The polymerase chain reaction (PCR) is a widely used technique, and the foundation of numerous diagnostic applications that seek to detect minute amounts of DNA via exponential amplification. Successful PCR requires a number of components, including a DNA polymerase capable of tolerating high temperature incubations (94°C or higher) that occur during a typical thermal cycling protocol. *Taq* DNA Polymerase, originally isolated from *Thermus aquaticus*, is most commonly used in PCR assays[1]. In the early stages of PCR development, it became clear that reaction specificity impacted experimental success[2]. Like many non-proofreading Family A DNA polymerases, *Taq* Polymerase possesses the ability to add bases onto the end of ssDNA in a non-template-dependent manner (i.e., terminal transferase activity). This activity is present, even at room temperature, and can result in the addition of non-specific bases onto the ends of DNA primers in the reaction, enabling them to bind to undesired locations on the DNA and reducing overall reaction specificity. In contrast, at higher temperatures, non-specific binding is reduced as annealing becomes more stringent. As PCR applications became more complex and demanding, preventing this low temperature activity became critically important to increasing reaction specificity, and numerous techniques and methods have been employed to achieve this protection.

INCREASING REACTION SPECIFICITY

Early methods focused on the exclusion of key reaction components to mitigate undesired activity at low temperature. As the temperature was increased, any missing components (e.g., polymerase, cofactor) could be spiked into the mixture, triggering the reaction under a more restrictive,

high temperature condition – a so-called "hot start" reaction. This method worked, but required the user to open reaction tubes and make very small volume additions, a labor-intensive and contamination-prone process. Subsequent methods aimed to improve upon this approach included sequestering reaction components with wax layers or beads[3]. This was also effective, but was not widely adopted by the research community.

The next generation of hot start methods, still in use today, didn't involve physical removal or exclusion, but instead focused on covalent and non-covalent enzyme interactions or modifications to block activity. The most common of these methods involved the development of an antibody specific to *Taq* Polymerase, which renders *Taq* inactive at room temperature, but denatures and dissociates from the enzyme after the initial, high-temperature denaturation step[4,5]. Antibody-based Hot Start *Taq* (Ab-*Taq*) addressed many of the concerns with prior approaches, and soon became a popular option for anyone looking to increase reaction specificity. However, original Ab-*Taq* offerings utilized animal-derived antibodies (increasing the possibility of reaction contamination) and were limited in commercial practice to those with a license; it was not surprising that other solutions continued to appear in the marketplace. The most successful of these alternatives was the use of chemicals to reversibly modify amino acid side chains of the polymerase[6]. Initial work in this area yielded effective results, but came at a cost: the enzyme could clearly be inactivated but required long activation times at very high temperatures to reactivate, and even then, only a fraction of the initial protein activity could be restored. Advances in this approach, namely changes to the covalent side chain modifications, have resulted in improved versions of chemically based Hot Start *Taq* (Chem-*Taq*), with shorter activation times (4 minutes instead of 10–15 minutes). The increased potential of inducing DNA damage and the persistent need to add significant amounts of protein may have kept this approach from becoming more widely employed.

DEVELOPMENT OF APTAMER-BASED APPROACH DELIVERS ADDITIONAL IMPROVEMENTS

A number of years ago, NEB investigated the use of aptamers to impart hot start activation of our enzymes. Generally, aptamers are engineered oligonucleotides that bind to a specific target molecule through non-covalent interactions. SOMAmers, the aptamer-based technology developed by SomaLogic that we have further adapted for use in our products, include specific nucleobase modifications that can improve inhibition profiles and/or reduce unintended side effects[7,8]. As with previously designed *Taq* inhibitors, the *Taq* aptamer evolved and engineered by NEB also inhibited polymerase activity at room temperature. This function can be monitored by a variety of assays that include read-outs not just for polymerase activity, but also for reaction specificity. For example, one assay includes an excess of off-target DNA in the reaction and results in the production of multiple amplicons in the presence of non-Hot Start *Taq*, but only a single, target product in the presence of the aptamer-based Hot Start *Taq* (NEB-HS). Another assay employed at NEB includes two primers that contain three complementary bases at the 3´ end of each primer[9]. These "poor" primers can serve as a substrate for *Taq*, and even in the presence of additional input DNA, can generate a clear primer-dimer product in the presence of *Taq* alone. However, in the presence of NEB-HS *Taq*, only the desired target specific product is observed (Figure 1, page 4). Although the main function of an aptamer-based inhibitor is similar to other hot start mechanisms, there are some key differences between NEB-HS *Taq* and either Chem-*Taq* or Ab-*Taq*. First, unlike other methods, the aptamer-based inhibition/activation process is fully reversible. At the end of thermal cycling, when the temperature of the reaction is decreased, the aptamer rebinds to *Taq* Polymerase, inhibiting any further activity in the sample. This has proven to be important in workflows where undesired polymerase activity after reaction completion can disturb baseline readings of negative samples, such as in workflows that include a significant delay between interrogation of the first and the last samples of a set

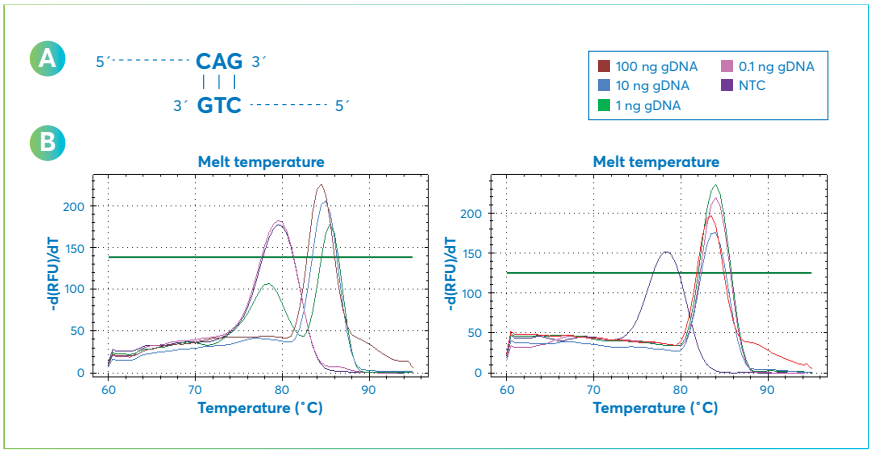


FIGURE 1: Monitoring hot start effectiveness via reaction specificity A. To assess reaction specificity, primers that create a stable, primer-dimer product via 3 complementary bases at their 3´ ends were used in PCR with *Taq* or Hot Start *Taq*. B. Post reaction melt-temp analysis demonstrates the presence of the non specific product only in the absence of template (NTC) for the Hot Start *Taq* reactions (purple curve, right) but reaction specificity is observed at all other inputs. However, in the absence of a hot start mechanism (left), primer-dimer product can be observed with two lower input concentrations as well as in the NTC sample. Reactions (25 µl) contained 0.2 µM primers.

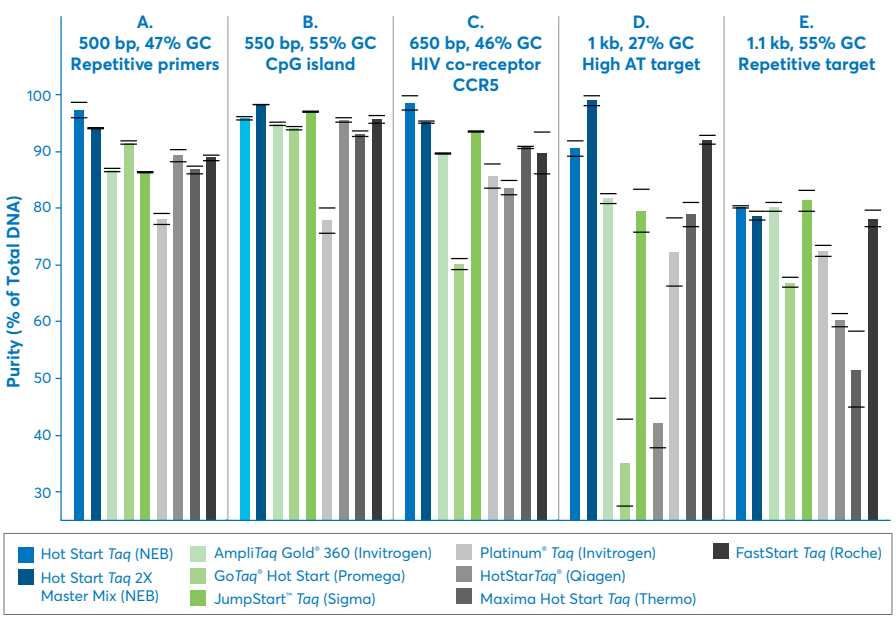


FIGURE 2: Target specificity of commercially available *Taq* products. Endpoint PCR analysis of various Hot Start *Taq* polymerases on human genomic DNA amplicons. Triplicate reactions were conducted according to manufacturer's recommendations in the buffer supplied with each enzyme. Input template concentration (20 ng), number of PCR cycles (30) and source of dNTPs (NEB) were held constant for all reactions. Products were quantitated by microfluidic LabChip® analysis and specificity of the final product was calculated as % purity of the expected amplicon.

TABLE 1

DNA Polymerase	Hot Start Method	Activation Time*
AmpliTaq Gold® 360 (Applied Biosystems)	Chem	10 min.
Platinum® *Taq* (Invitrogen)	Ab	30 sec.
Hot Start *Taq* (NEB)	Aptamer	None
GoTaq® Hot (Promega)	Ab 2	min.
HotStarTaq® (Qiagen)	Chem	15 min.
FastStart *Taq* (Roche)	Chem	4 min.
JumpStart™ *Taq* (Sigma)	Ab 1	min.
Maxima Hot Start *Taq* (Thermo Fisher Scientific)	Chem	4 min.

* May include initial denaturation step, if not specified by manufacturer.

(e.g., strip-tubes, 96-well plates, or droplet digital PCR). The second major difference lies in the release of inhibition. Whereas Ab-*Taq* and Chem-*Taq* are only activated once the reaction temperature is raised to 94–95°C, the aptamer in NEB-HS *Taq* dissociates from the polymerase at much lower temperatures (Tm = approximately 45°C), eliminating the need for a specific high temperature activation step, and enabling faster protocols (Table 1). Furthermore, reaction specificity is not impaired (Figure 2). The benefits of an aptamer-based hot start approach can be seen in the numerous NEB products that contain NEB-HS *Taq,* from the flexible OneTaq family of routine PCR products, to the recently released Luna products that support best-in-class qPCR and RT-qPCR performance.

EXTENDING APTAMER UTILITY TO OTHER APPLICATIONS

Dissociation at lower temperatures has enabled the use of aptamers for a broader range of polymerases and enzymes, including those that cannot tolerate the high temperatures employed in PCR. For example, aptamers have been particularly useful for isothermal amplification applications, where mesophilic and moderately thermophilic enzymes that catalyze these reactions cannot survive an initial high-temperature denaturation step (and instead use a strong strand displacement activity to separate the DNA duplex). In addition to developing aptamers for an enhanced version of Bst DNA Polymerase (WarmStart® Bst 2.0 DNA Polymerase) to increase specificity in these types of workflows, in 2014 NEB launched the first warm start reverse transcriptase, WarmStart RTx Reverse Transcriptase, specifically for RT-LAMP. Similar to the nonspecific primer extension described above, enzymes utilized in isothermal applications can also give rise to undesired products that affect reaction performance. Reaction conditions, such as very high primer and Mg2+ concentrations, as well as lower optimal temperatures for the enzymes, make isothermal methods especially prone to effects from any off-target activity that occurs during reaction set up. As an example, Figure 3 shows a LAMP reaction where room-temperature incubation results in a significant increase in reaction time (from ~6 minutes to nearly 30 minutes); by utilizing the dual-warm start formulation (WarmStart RTx plus WarmStart Bst 2.0), the time increase is prevented and consistent reaction performance is maintained. Critically, this protection requires the activities of both enzymes to be controlled. Having observed the utility of a warm start RT in RT-LAMP applications, NEB next focused its efforts on the development and launch of Luna® WarmStart Reverse Transcriptase, to support RT-qPCR applications. As with our previous aptamer development for the DNA dependent DNA polymerases, these warm start RNA-dependent DNA polymerases (i.e., reverse transcriptases) are inhibited at room temperature, but the aptamer is still released as the temperature is increased, enabling full activity despite the use of moderate reaction temperatures (50–60°C). As with RT-LAMP, it has been through the use of these aptamers that we have been able to identify and prevent RT-mediated non-specific amplification that can occur in certain settings, such as challenging assays or workflows that include a delay between

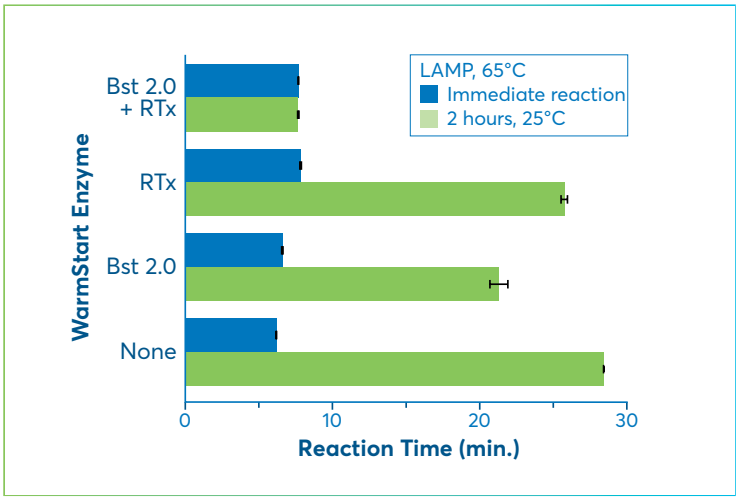


FIGURE 3: RT-LAMP specificity. Duplicate RT-LAMP reactions were prepared on ice and either immediately placed at 65°C with real-time fluorescence detection (blue), or incubated for 2 hours at 25°C before heating to 65°C (green). A 2-enzyme combination of Bst 2.0 and RTx was used for the reactions, with neither, one, or both enzymes used with their specific WarmStart aptamer as indicated on the y-axis. With 0 or 1 of the enzymes in WarmStart form, a dramatic increase in LAMP detection time resulted from the 2 hr room-temperature incubation due to nonspecific activity one or both polymerases. With both WarmStart aptamers used, no change in LAMP time was seen, indicating efficient protection from nonspecific activity by the dual-WarmStart format.

TABLE 2: Taq/Q5 polymerase properties

	TAQ DNA Polymerase	Q5 High-Fidelity DNA Polymerase
Polymerase family	A	B
3´→5´ exonuclease*	No	Yes
5´→3´ flap exonuclease**	Yes	No
5´→3´ polymerase (room temperature)	Yes	No
Aptamer function	Inhibit polymerase activity at room temperature	Inhibit 3´→5´ exonuclease activity at room temperature
Fidelity (relative to Taq)***	1	280

reaction set up and intended initiation. A one-step RT-qPCR example is shown in Figure 4, where a number of reactions were set up simultaneously and half were immediately transferred to a thermocycler and half were left at room temperature for 24 hours. No non-specific amplification can be detected in any of the samples that contained the Luna WarmStart RT. In contrast, clear evidence of non-specific amplification was detected when a more typical RT-qPCR reagent (containing only a Hot Start *Taq*) was used (Figure 4). In addition to polymerase activity, aptamers can also be selected to inhibit or moderate other enzymatic activities. For example, the hot start aptamers that were developed for use with Q5® High Fidelity DNA Polymerase were not created to inhibit the polymerase activity, but instead to inhibit its exonuclease activity. Q5 is an engineered polymerase that most closely resembles an archaeal Family B polymerase, and as such it possesses little-to-no detectable 5´→3´ polymerase activity at room temperature. However, Q5 does have a robust 3´→5´ exonuclease (proofreading) activity that enables high-fidelity replication, and this activity remains even at room temperature. This difference in activity temperature profiles compared to *Taq* (which possesses measureable polymerase activity at room temperature but has no 3´→5´ exonuclease activity at any temperature) can lead to alternate modes of non-specific amplification, thus requiring different solutions (Table 2, above). Whereas the mechanism of non-specific amplification by *Taq* is generally via non-templated addition at the 3´ end of the primers, reducing the probability of a Watson-Crick base pair at the desired annealing site, non-specific amplification with a Family B polymerase that possesses proofreading activity appears to occur via exonucleolytic primer degradation at the 3´ end, again reducing specificity at the desired annealing site and increasing the probability of off-target amplification.

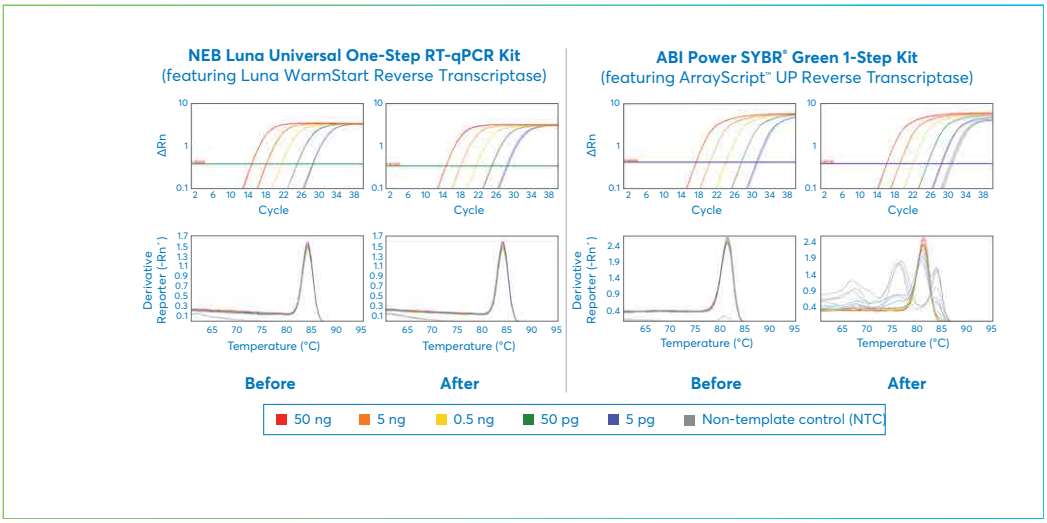


FIGURE 4: WarmStart Luna RT prevents spurious amplification resulting from room-temperature pre-incubation. RT-qPCR targeting human ribosomal protein L32 was performed before and after a 24-hour incubation at room temperature, with triplicate reactions for a 5-log range of input human (Jurkat) total RNA and a non-template control. The Luna Universal One-Step RT-qPCR Kit featuring Luna WarmStart Reverse Transcriptase exhibited robust performance and no detectible non-template amplification, either with or without a 24-hour 25°C pre-incubation, while the ABI 1-Step Kit, featuring a non-WarmStart reverse transcriptase, exhibited significant non-template amplification.

CONCLUSION

NEB's selection of aptamers enables the ability to inhibit activity at room temperature and offers unique features that other hot start approaches cannot. Aptamer-based inhibition is reversible, allowing for an additional level of reaction specificity as the reaction temperature is decreased. Aptamers dissociate rapidly at lower temperatures than traditional hot start methods, eliminating the need for specific activation steps. Additionally, this lower release temperature and the ability to tune release during the aptamer selection process has enabled the creation of "WarmStart" enzymes to bring the benefits of specificity and consistent reaction performance to enzymes outside of typical PCR workflows, such as reverse transcriptases and enzymes used in isothermal amplification methods. Modulating enzyme activity to reduce unwanted side activities remains an important consideration in numerous assays and technologies, and NEB continues to apply its expertise in aptamer chemistry to develop warm and hot start forms of any enzyme where such control is beneficial. But as researchers and developers continue to increase the complexity of these workflows, it is likely that no single hot start technology will serve all needs. As such, NEB continues to evaluate various methods to ensure that our products enable new and existing applications. With a full understanding of each method's benefits and limitations, we aim to provide comprehensive hot start reagents for a wide variety of demanding biotechnology applications.

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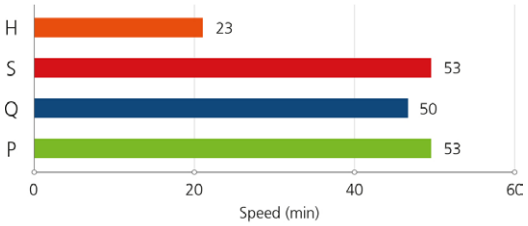

H: repliQa HiFi ToughMix
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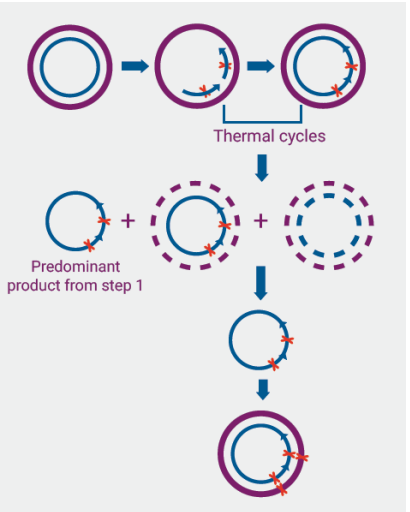

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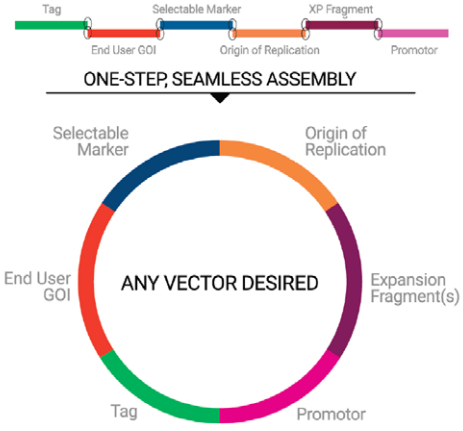

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Simplified DNA extraction to accelerate gene editing and genotyping detection workflows

By Dietrich Koester, Product Manager -Extraction, LGC, Biosearch Technologies, Berlin, Germany

Gene-editing techniques have attracted considerable attention in recent years due to their therapeutic potential and application in diverse areas of research, such as disease modeling, drug discovery, and agrigenomics. A fundamental requirement common to all gene-editing methods is validation of the results. The QuickExtract™ DNA Extraction Solution has emerged as a popular reagent to extract genomic DNA for PCR-based validation, due to its speed, convenience, and ease of automation. It has also been used in PCR-based genotyping with a broad range of sample types.

INTRODUCTION

When isolating nucleic acids for downstream applications, researchers generally choose between two types of methods: purification, which is a multi-step, longer procedure that provides high-quality DNA or RNA; and extraction, which is a rapid procedure designed specifically for subsequent PCR or RT-PCR. The QuickExtract family of products provides a simple, rapid method for the extraction of PCR-ready DNA from a variety of sources. For most sample types, the procedure can be completed in as little as 8 minutes. The QuickExtract products are easy to use, with a single-tube protocol that does not require the use of spin columns, which can cause considerable sample loss.

The QuickExtract workflow avoids the use of toxic chemicals and is easily adapted for multi-well plates with robotic automation systems. Because of the speed of the QuickExtract method, it is ideal for applications where the results from PCR amplification are required to proceed with further experiments, such as screening and genotyping assays.

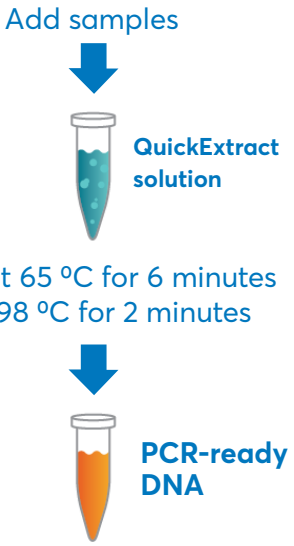

FIGURE 1. **The QuickExtract™.** DNA Extraction Solution isolates PCR-ready DNA in 8 minutes or less.

QuickExtract DNA Extraction Solution is suitable for a broad range of sample types, from hair follicles to cultured cells. Due to the more challenging nature of some sample types, additional QuickExtract kits were developed to handle these difficult samples. The QuickExtract FFPE DNA Kit eliminates the need for tedious extraction procedures that use organic solvents for formalin-fixed, paraffin-embedded (FFPE) samples. The QuickExtract Plant DNA Extraction Solution enables rapid extraction of plant genomic DNA from leaf tissues for both endpoint and real-time PCR. This white paper provides a brief overview of published applications that represent the diverse uses for the QuickExtract DNA Extraction Solution.

CRISPR-CAS GENE EDITING

The ability to make precise, targeted changes to genes within living organisms has undergone a revolution in recent years, largely thanks to the rapid development of CRISPR-Cas technology. This technology has its origins in a bacterial immune defense mechanism that uses clustered regularly interspaced short palindromic repeats (CRISPR) together with CRISPR-associated (Cas) nucleases to recognize and destroy foreign DNA from invading viruses. Several types of CRISPR-Cas systems have been identified, of which the CRISPR-Cas9 system is the most commonly used.

Typically, foreign DNA is fragmented within bacteria and incorporated into CRISPR sequences. When the CRISPR locus is transcribed, the transcripts are processed into smaller CRISPR RNAs (crRNAs). In conjunction with bacterial trans-activating CRISPR RNAs (tracrRNA), these crRNAs can target and cleave complementary sequences in bacteriophage DNA during future infections.

Genome-editing techniques take advantage of the relatively small number of components in the CRISPR-Cas9 system. The sequences of the crRNA and tracrRNA are often combined into a single guide RNA (sgRNA). Sequences coding for sgRNA and Cas9 can be incorporated into a single vector that is used to transfect or transduce the host cell. Alternatively, purified Cas9 and transcribed guide RNA can be complexed and delivered to the host cell as a ribonucleoprotein (RNP). Once inside the cell, the guide will target Cas9 activity to a specific locus. Cas9 activity results in a double-stranded break (DSB), which will be repaired by the error-prone nonhomologous end-joining (NHEJ) pathway. This type of repair often produces insertion and/or deletion (indel) mutations at the target site. If the sgRNA and Cas9 sequences are delivered to the cell with a "repair template," then the cell uses an alternative repair mechanism called homology-directed repair (HDR). A repair template contains a DNA sequence to be inserted so that a locus can be modified in a specific manner.
The modified target sequences are then validated, typically by PCR-based screening or next-generation sequencing (NGS).

SCREENING FOR MUTATIONS

An important part of any gene-editing method is the screening of mutated and wild-type cells to confirm the success and efficiency of the procedure. Several assays have been developed for this purpose. Of these, enzyme mismatch cleavage (EMC) assays have been widely used in CRISPR-Cas-based methods. The first EMC assays used the bacteriophage resolvase enzymes T4 endonuclease VII[1] and T7 endonuclease I[2]. Subsequently, plant-based S1 nucleases were introduced, and the technology is currently commercialized in the line of Surveyor mutation-detection assays from Integrated DNA Technologies (IDT).

In a typical EMC assay, genomic DNA is isolated from a population of cells, containing wild-type and mutant alleles. The targeted region is amplified by PCR and then denatured and reannealed. This process results in mismatched duplexes at the targeted sites, due to cross-annealing of the wild-type and mutated sequences. Digestion of the amplicons with T7 endonuclease I, which selectively cleaves distorted duplex DNA, is followed by analysis of the DNA fragments by polyacrylamide gel electrophoresis.

The use of QuickExtract DNA Extraction Solution to prepare genomic DNA for an EMC assay was originally reported to screen for mutations introduced by zinc-finger nucleases (ZFNs)[3]. The assay is sensitive down to ~1% gene modification in a population, and the authors cite its speed, low cost, and convenience as advantages over other mutation-detection methods for ZFN-based gene editing.

Subsequently, the laboratory of Dr. Feng Zhang at the Massachusetts Institute of Technology developed a standard EMC protocol for mutation detection following CRISPR-Cas9 gene editing, using genomic DNA isolated with QuickExtract DNA

Extraction Solution[4]. The speed and simplicity of the QuickExtract workflow contribute to this specific, scalable, and cost-efficient assay for genome editing in mammalian cells.

GENOME-SCALE KNOCKOUT

Joung et al.[5] described a system for performing pooled genome-scale knockout or transcriptional activation screening using CRISPR-Cas9 in a variety of human and mouse cell lines. They constructed plasmids containing variable sgRNAs that, when pooled, make up an sgRNA library for genome-scale targeting. The system format can either encode the sgRNA, Cas9, and other necessary components on the same plasmid or on separate plasmids. The sgRNA libraries were packaged into lentivirus and transduced into the cell lines being studied. After applying selective pressure to the cells, the resulting population was screened by NGS for changes in sgRNA distribution and, from the results, a number of candidate target genes were selected for validation. Following cloning and lentiviral packaging of validation sgRNAs targeting the candidate genes of interest, these select sgRNAs were transduced into cells. Once the cells reached confluency, genomic DNA was isolated using QuickExtract DNA Extraction Solution in 96-well plates, and the regions containing the sgRNA sequences were amplified by a two-step PCR for initial validation by targeted NGS on an Illumina® platform. The researchers report that although their method is designed for loss-of-function and gain-of-function screening, it can be used for other types of screening as well.

IMPROVED GENE-EDITING EFFICIENCY

Research using human pluripotent stem cells (hPSCs) has enabled an array of novel technologies in the study of human development, as well as disease progression and therapeutic intervention. The ability of CRISPR-Cas9 technology to introduce specific, targeted mutations in hPSCs has added a new dimension to this rapidly expanding field of research. Xie et al.[6] describe a novel, episomal vector-based CRISPR-Cas9 system (epiCRISPR) for the generation of knockout mutations in hPSCs. The vector includes components from Epstein-Barr virus that allow it to replicate once per cell division in eukaryotes, allowing persistent expression of Cas9 and sgRNAs. Further, the transfected cells can be enriched by puromycin selection. The epiCRISPR system was tested in separate experiments for gene knockout and genomic deletions, using custom-designed sgRNA panels. After transfection of hPSCs and puromycin selection, genomic DNA was isolated using the QuickExtract DNA Extraction Solution. The target region was amplified by PCR, purified, and analyzed by restriction fragment length polymorphism (RFLP). Off-target effects were also examined using NGS. The authors reported high efficiency of gene knockouts and up to 100% efficiency for indel generation with the epiCRIPSR system, with no off-target effects.

ALLELE EXCHANGE

While the majority of CRISPR-Cas9 methods are used for gene knock-out or knock-in experiments, the exchange of an entire allele is an attractive prospect for potential therapeutic applications. Kelton et al.[7] provide a proof-of-concept method to reprogram the polymorphic major histocompatibility complex (MHC) locus in murine-derived antigen-presenting cell lines, using a CRISPR-Cas9 system. The authors used Cas9 with sgRNAs that introduced DSBs flanking the MHC-I allele (~3.4 kb), enabling its replacement with an MHC donor cassette, which was introduced as a double-stranded DNA template. To confirm insertion of MHC donor DNA, candidate cells were treated with QuickExtract DNA Extraction Solution, and the genomic DNA was amplified using primers covering the putative cleavage sites at the MHC locus for analysis. The authors conclude that their approach demonstrates the feasibility of replacing large MHC alleles at the native locus, suggesting its future utility for correcting MHC mismatches in allogenic cellular transplantations.

TALENs

Concern about the off-target effects of CRISPR-derived gene-editing methods has spurred renewed interest in TALENs, which typically offer high targeting specificity. Wang et al.[8] optimized a TALEN design and tested their method in human cultured cell lines, as well as human embryonic stem cells (hESCs). The method combined an optimized TALEN with a single-strand oligodeoxynucleotide (ssODN) for high-efficiency gene editing. The cutting efficiency of the optimized TALEN was validated in HEK293T cells after transfection with TALEN plasmid by treating the cells with QuickExtract DNA Extraction Solution and analyzing the percentage of gene modification using a Surveyor nuclease assay. Subsequent experiments established human tumor cell lines and hESCs with homozygous deletions of two microRNA genes: miR-21 and miR-9-2. The authors suggest that the versatility of the TALEN-ssODN method could contribute to its utility in therapeutic applications.

GENOTYPING

The speed of the QuickExtract DNA workflow has made it popular for PCR-based genotyping in a variety of organisms, from bivalves to mice.

MUSSELS

Ferguson et al.[9] characterized population structure, dispersal potential, and reproductive strategies in the freshwater mussel Lampsilis cardium from three watershed sites in Ohio, USA. Genomic DNA was extracted from adult mussels using the PureGene kit (Gentra) and from ethanol-preserved glochidia (mussel larvae) using QuickExtract DNA Extraction Solution. The QuickExtract procedure was modified to extend the time for both heating steps: 30 minutes at 65°C followed by 7 minutes at 98°C. The extracted DNA was used in PCR with primers for 12 microsatellite loci to assess local population structure relative to within-population patterns of relatedness and parentage. The study concluded that long-distance fertilization observed in this mussel species may have implications for improved recovery of imperiled freshwater mussel populations.

MICE

The G protein-coupled trace amine-associated receptor 1 (TAAR1) is stimulated by neurotransmitters and metabolites, and the receptor has multiple physiological roles, including predisposition to drug abuse and related effects. Shi et al.[10] studied the functional effects of single-nucleotide polymorphisms (SNPs) in the TAAR1 gene in mice. Genomic DNA from several mouse strains was extracted from ear or tail tissue using QuickExtract DNA Extraction Solution and amplified by PCR with primers flanking the SNP-containing regions. The amplicons were purified and sequenced. The results showed that TAAR1 mutants resulted in expression of functional, subfunctional, and nonfunctional receptors, with widely differing responses to methamphetamine. The authors suggest that SNPs in TAAR1 could provide a useful screening tool for determining predisposition to a variety of human diseases, as well as for individualizing treatments using TAAR1-specific therapies.

TRANSGENIC MICE

Tumor-specific antigens (TSAs) constitute attractive therapeutic targets in a wide range of cancers. Unlike TSAs, tumor-associated antigens (TAAs) are expressed at varying levels in normal cells as well as tumors, making therapeutic strategies more challenging. Yong et al.[11] studied the TAA human epidermal growth factor 2 (Her2), which is expressed in normal breast tissue but upregulated in 15%-30% of breast cancers. They used a transgenic mouse model to examine therapies that specifically targeted Her2 antigen in tumor cells. While heterozygous Her2+/– mice appear to develop in a similar manner to wild-type mice (Her2–/–), it has proven difficult to generate homozygous Her2+/+ mice, potentially due to embryonic lethality. Genomic DNA was extracted from tail snips of Her2+/+, Her2+/– and Her2–/– mice using QuickExtract DNA Extraction Solution, followed by PCR to examine the integration site of the Her2 transgene. Subsequent whole-genome sequencing confirmed that the integration site of the Her2 transgene was indeed responsible for embryonic lethality, due to its insertion into the gene encoding precocious dissociation of sisters (Pds5b), which is implicated in embryonic development.

CONCLUSION

The speed and convenience of the QuickExtract methods make them ideal for PCR validation during intermediate steps in gene-editing and genotyping workflows. The QuickExtract procedure allows for a large number of samples to be screened in a rapid, automation-friendly process. The utility of the QuickExtract DNA Extraction Solution in applications requiring PCR-based screening has been recognized by its incorporation into standard CRISPR-Cas9 gene-editing protocols provided by IDT (Alt-R™ Genome Editing Detection Kit, Protocol CRS-10056-PR) and New England Biolabs (EnGen® Mutation Detection Kit, Technical Manual E3321).

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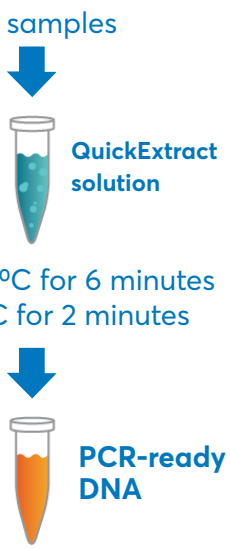

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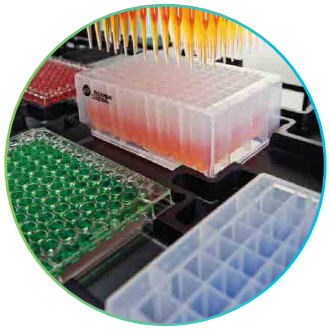

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Nanosep®	10K	Blue	Omega	–	**29300-620**
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Description	MWCO	Color	Membrane material	Pore size	Cat. no.
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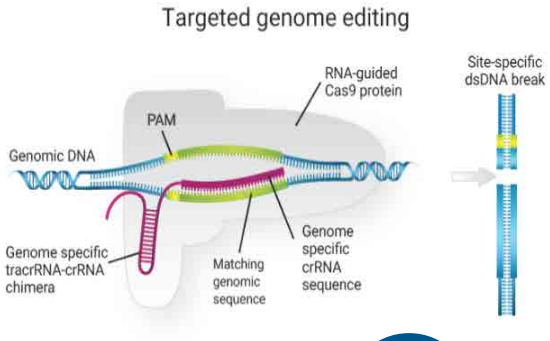

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