GE Healthcare

illustra TempliPhi Sequence Resolver Kit

Product Booklet

Codes: 28-9035-29 (20 reactions)
28-9035-30 (50 reactions)
28-9035-31 (200 reactions)

Page finder

1. Legal

The **illustra™ TempliPhi™ Sequence Resolver Kit** has been designed, developed, and sold **for research purposes only.** It is suitable **for *in vitro* use only.** No claim or representation is intended for its use to identify any specific organism or for clinical use (diagnostic, prognostic, therapeutic, or blood banking).

It is the responsibility of the user to verify the use of **illustra TempliPhi Sequence Resolver Kit** for a specific application as the performance characteristic of this kit has not been verified to any specific organism.

For use only as licensed by Qiagen GmbH. and GE Healthcare. The Phi 29 DNA polymerase may not be re-sold or used except in conjunction with the other components of this kit. See US patent numbers 5,854,033, 6,124,120, 6,143,495, 6,323,009, 5,001,050, 5,198,543, 5,576,204, and equivalent patents and patent applications in other countries.

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2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only*. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin and eyes. In the case of contact with skin or eyes, wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store the kit at -70°C.
The enzyme mix must be stored at -70°C; all other components may be stored at -20°C.
Thaw components on ice and maintain at 0°C to 4°C during handling.

2.3. Expiry

For expiry date please refer to outer packaging label.

3. Components

3.1. Kit components

Pack size Cat. No.	200 reactions 28-9035-31	50 reactions 28-9035-30	20 reactions 28-9035-29
Sample Buffer (White cap)	1 × 900 µl	1 × 225 µl	1 × 90 µl
Reaction Buffer (Blue cap)	1 × 900 µl	1 × 225 µl	1 × 90 µl
Enzyme Mix (Yellow cap)	1 × 100 µl	1 × 25 µl	1 × 10 µl

3.2. Materials to be supplied by user

Liquid-handling supplies—Vials, pipettes, microcentrifuge, and vacuum centrifuge. Perform all amplification reactions in plastic microcentrifuge tubes (typically 0.5 ml), or in 96-well or 384-well plates suitable for sealing and incubating at 10°C.

Incubator — For incubations at 10°C.

4. Description

4.1. Introduction

The **illustra™ TempliPhi™ Sequence Resolver Kit** is designed to prepare DNA templates containing regions of secondary structures for sequencing. The kit incorporates a novel technology that produces DNA that does not form secondary structures as strongly as DNA prepared by traditional methods. This makes it possible to generate high quality sequence from templates containing repetitive or GC rich regions, particularly for templates that usually generate sequence stops (Figure 1).

The kit is optimized for use with any circular sequencing templates such as plasmids, M13, fosmids and BAC's. It is not intended for use with linear templates. The starting material for amplification can be purified DNA, bacterial colonies, phage plaques, bacterial cultures, glycerol stocks or amplification products produced with the illustra TempliPhi DNA Amplification kits. The sensitivity of the amplification method is such that the TempliPhi Sequence Resolver Kit can even be used when no visible signs of template DNA remain. When a DNA sample has been exhausted the 'empty' well can be rinsed with a small amount of water and then used in an amplification reaction.

Amplifications are carried out at 10°C for 18 hours, generating approximately 1 µg of DNA per 10 µl reaction. The amplified DNA can be used directly in cycle sequencing reactions without any further purification. The amplification product is double-stranded DNA and can be sequenced with forward and reverse primers and is compatible with any sequencing chemistry including DYEnamic™ ET terminator and the ABI™ Prism® BigDye™ Terminator Cycle Sequencing Kits.

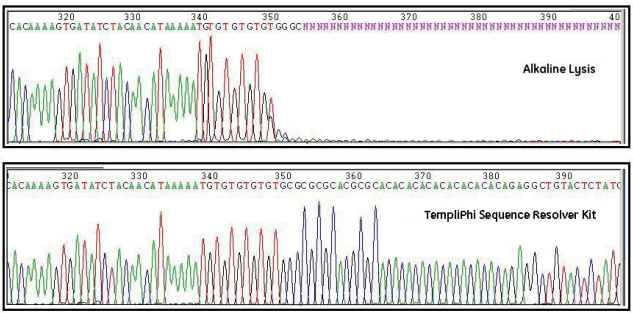

Fig 1. Sequencing data for a difficult to sequence plasmid DNA template. Sequencing of DNA prepared by alkaline lysis resulted in a sequencing stop, probably due to secondary structure formation (top panel). By first amplifying the plasmid template with the TempliPhi Sequence Resolver Kit, high quality sequence was generated through the repeat region (bottom panel). Sequencing was performed with the DYEnamic ET terminator Cycle Sequencing Kit. Data viewed with Chromas © v 2.22.

4.2. The basic principle

The kit consists of three components, sample buffer, reaction buffer, and enzyme mix. The protocol is quick and requires only 20 minutes of hands-on time to prepare a 96 well plate. Briefly, 1 µl of a DNA template to be amplified is added to 4 µl of sample buffer and heated to 95°C for 1 minute to denature the DNA. The sample is cooled, mixed with 4.5 µl of reaction buffer and 0.5 µl enzyme mix and incubated at 10°C overnight (18 hours). On completion of the incubation, the Phi29 DNA polymerase is heat-inactivated during a 10 minutes incubation at 65°C. The tube should now contain amplified DNA that can be used directly in sequencing reactions. The expected yield is approximately 1 µg (Figure 2).

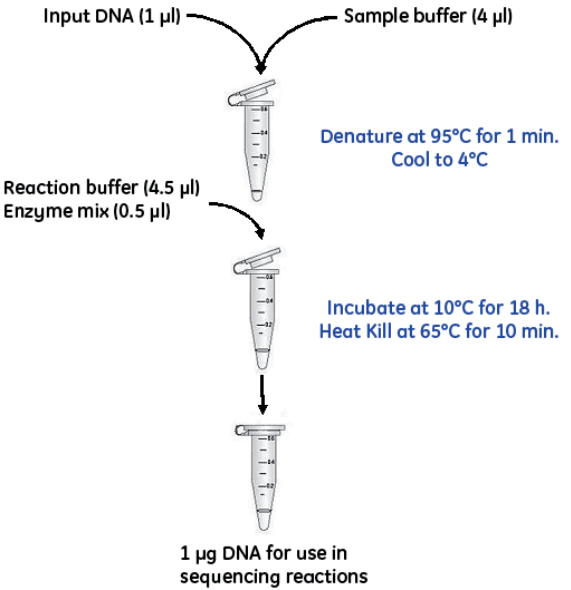

Fig 2. Schematic of TempliPhi Sequence Resolver Kit protocol.

5. Protocol

The following protocol is for M13 or plasmid templates. Please see protocol 5.3 for guidelines on amplification and sequencing of large DNA templates such as fosmids and BACs.

5.1. General Amplification Protocol

1. Heat denaturation of template in sample buffer
Mix 1* µl (1–2 ng) of template DNA with 4* µl of sample buffer.
Heat to 95°C for 1 min.
Cool to 4°C on ice.

* The amount of DNA and sample buffer to use per amplification reaction is dependent on the type of starting material. Please refer to Appendix 1 for recommendations for different starting material types.

2. Preparation of amplification reaction
Defrost reaction buffer and enzyme mix on ice just prior to use. Mix reagents thoroughly by vortexing briefly at medium speed.

For each amplification reaction, combine 4.5 µl of reaction buffer with 0.5 µl of enzyme mix on ice.
Immediately add this to the cooled sample from step 1.

Note: Reaction buffer and enzyme mix should be combined only in sufficient quantities and immediately prior to addition to the sample. Prepare and keep mixture on ice. Do not allow the mixture to warm above 4°C prior to amplification. Discard any unused portion.

3. Incubation
Incubate the sample at 10°C for 18 hours. A thermal cycler or water bath may be used.

Note: Incubation at a temperature other than 10°C, or for less than 18 hours will compromise the quality and quantity of the amplified DNA.

4. Heat inactivation of Phi29 DNA polymerase
Heat the sample to 65°C for 10 min and then cool to 4°C.

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

5. Storage of the amplified material
Store amplification reactions at -20°C or -80°C.

Note: Storage of amplified DNA at -20°C or -80°C is recommended, although short term (few days) storage at 4°C is possible. For long term (many months) storage of amplified DNA we recommend adding 40 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.5) to each sample prior to storage. Stability of amplified DNA is equivalent to template DNA prepared by traditional methods.

5.2. Sequencing Reactions

5.2.1 Preparation of sequencing reactions
Assemble each sequencing reaction as follows. These volumes apply for DYEnamic ET Terminator and BigDye Terminator v3.1 and v1.1 Cycle Sequencing Kits.

96 well

Amplification Product	2 µl (200 ng)
Sequencing Premix	8 µl
Primer (5 pmol/µl)	1 µl
Water	9 µl
Total volume	20 µl

384 well

Amplification Product	1 µl (100 ng)
Sequencing Premix	4 µl
Primer (5 pmol/µl)	0.5 µl
Water	4.5 µl
Total volume	10 µl

After dispensing the reagents, seal the plates and mix thoroughly by gentle vortexing. Centrifuge briefly to bring contents to the bottom of the wells.

Note: The most consistent results will be achieved when sequencing reagent premix is used at full strength. Dilution of sequencing reagent premix will result in a decrease in the quality of sequence data.

Note: 200–500 ng of amplified plasmid DNA and 5 pmol of primer are recommended for routine sequencing. The volume of primer added to each reaction will depend on the concentration. Dilute the primer in deionized water or buffer containing no more than 0.1 mM EDTA. Do not use buffers containing > 0.1 mM EDTA since they may reduce the effective metal cofactor concentration in the reactions.

5.2.2 Thermocycling and Post-Reaction Cleanup

Use manufacturer's recommended cycling conditions and post-reaction cleanup protocols.

5.3. Amplification and Sequencing of Large Constructs

Large templates such as BACs and fosmids are often difficult to sequence due to the large amount of template DNA required for high quality sequence data. Insufficient template DNA in the sequencing reaction can produce low signal strengths that cause poor base-calling and short reads. Also, due to the large size of fosmids and BACs it is difficult to lyse bacterial cells without also releasing chromosomal DNA.

The following should be considered when optimizing amplification and sequencing reactions:

- The TempliPhi Sequence Resolver Kit will amplify any contaminating DNA, including chromosomal DNA, present in the starting material. For the highest success rate amplify purified BAC

or fosmid DNA, as colonies and cultures will contain higher levels of chromosomal DNA.

- Optimal sequencing results are obtained with 0.1–0.2 pmol of DNA template per sequencing reaction (20 µl volume). For a 40 kb fosmid this translates into 4 µg of DNA. As it is not generally feasible to add this amount of DNA to a sequencing reaction, a minimum of 1–2 µg is recommended.
- Increasing sequencing reaction volume and the cycle number can overcome low templates amounts and improve signal strengths.
- Dilution of the sequencing premix will compromise sequencing success rate and data quality.

5.3.1 Amplification Reaction

Note: As increased amounts of DNA are required to successfully sequence large DNA templates, the amplification reactions should be scaled to produce the required level of amplification product. All components of the reaction should be scaled proportionally including template DNA. A 10 µl reaction will produce approximately 1 µg of product DNA. As the TempliPhi Sequence Resolver Kit will amplify any DNA present in the sample, including chromosomal DNA, it is necessary to account for the contaminating DNA when calculating the amount of amplification product required. A purified DNA sample prepared by alkaline lysis will typically contain 10–20% *E.coli* chromosomal DNA.

1. Mix 2 µl of purified DNA with 8 µl of sample buffer.
2. Heat to 95°C for 3 min. Cool to 4°C on ice.
3. For each amplification reaction, combine 9 µl of reaction buffer with 1 µl of enzyme mix on ice. Immediately add this to the cooled sample from step 2.
4. Incubate sample at 10°C for 18 h. A thermal cycler or water bath may be used.
5. Heat the sample to 65°C for 10 min. Cool to 4°C.

6. Store samples at -20°C.

5.3.2 Preparation of large construct sequencing reactions

Assemble each sequencing reaction as follows for sequencing with the DYEnamic ET terminator Cycle Sequencing Kit.

Amplification Product	10 µl
Sequencing Premix	8 µl
Primer (5 pmol/µl)	1 µl
Water	1 µl
Total volume	20 µl

After dispensing all reagents seal the plate and vortex gently. Centrifuge briefly to bring contents to the bottom of the wells.

Place the plate into the thermal cycler and run the following program for **60 cycles**. The increased number of cycles is required to generate sufficient labeled sequencing fragments during cycle sequencing.

95°C for 20 sec

50°C for 20 sec

60°C for 60 sec

Extension time at 60°C can be increased to 2 min to increase signal.

After cycling is complete, briefly centrifuge the plate to collect the reaction mixture at the bottom of the wells.

5.3.3 Post reaction cleanup

Add 2 µl of 7.4 M ammonium acetate to each well

Add 60 µl of 95% ethanol to each reaction and mix by pipetting. Do not vortex.

Note: The final ethanol concentration must be 70%. Final ethanol concentrations of < 65% result in weak signals, while concentrations 75% results in dye blobs.

Centrifuge plate at room temperature or 4°C at 2 500 × g or greater for 30 min.

Discard the supernatant. Remove any remaining liquid by a brief inverted spin (1 min at 300 × g).

Wash the DNA pellets by adding 100 µl of 70% ethanol to each well. Centrifuge for 5 min at 2 500 × g or greater.

Discard the supernatant. Remove any remaining liquid by a brief inverted spin (1 min at 300 × g).

Air-dry or vacuum dry the pellets for 2–5 min. Do not overdry.

6. Appendices

6.1. Template DNA Considerations

The starting material for amplification with the TempliPhi Sequence Resolver Kit may be purified DNA, colonies, phage plaques, cultures, glycerol stocks or amplification products produced with the TempliPhi DNA Sequencing Template Amplification Kits. The amplification method is susceptible to inhibition by large amounts of DNA and components in culture growths and glycerol stocks. Only small amounts of DNA are required for amplification. Please follow the recommendations below based on your starting material to obtain the highest level of success.

1. TempliPhi Amplification Kit or TempliPhi Sequence Resolver Kit products

Add 1 µl of amplification product to 4 µl of sample buffer.

2. Purified plasmid or M13 DNA

Add 1–2 ng in approximately 1 µl to 4 µl of sample buffer.
For templates where the purified DNA sample has been exhausted, wash the 'empty' well with a small amount (5–10 µl) of water. Add 1 µl of sample to 4 µl of sample buffer.

3. Plasmid or M13 from a colony or plaque

Gently touch the colony or plaque with a pin or toothpick and transfer to 5 µl of sample buffer.

Alternately, transfer the entire colony or plaque to a tube containing 10 µl TE (10 mM Tris, 1 mM EDTA, pH 8.5) or water, vortex, and add 1 µl to 4 µl of sample buffer.

4. Liquid bacterial culture

Add 0.5 µl of saturated overnight culture directly to 4.5 µl of sample buffer.

5. M13 in liquid culture

Add 0.5 µl of phage supernatant directly to 4.5 µl of sample buffer

6. Glycerol Stocks
Dilute 1 µl of glycerol stock in 10 µl of TE of water. Add 1 µl of diluted glycerol stock to 4 µl of sample buffer.

6.2. Product Specifications

Typically, a 10 µl TempliPhi Sequence Resolver Kit reaction will yield 1 µg of high molecular weight DNA that appears as a smear when visualized with agarose gel (Figure 3). Cutting the amplified DNA with restriction endonucleases typically results in incomplete digestion (Figure 4). TempliPhi Sequence Resolver Kit improves sequencing success rates and readlengths on many difficult DNA templates compared to other finishing methods such as sequencing reaction additives (Table 1).

1. Product size characterization

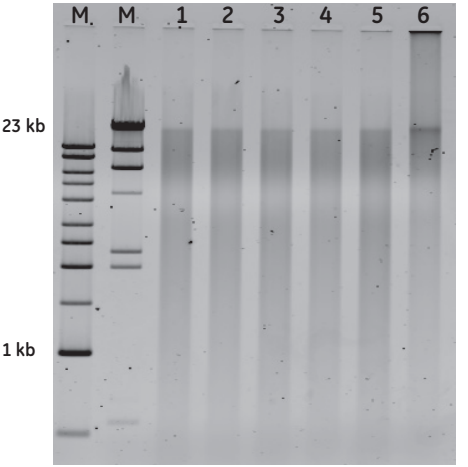

Fig 3. Products of TempliPhi Sequence Resolver Kit amplification reactions are high molecular weight. Lanes 1–6: Amplification products (1 µl) were analyzed on a 0.8% agarose gel.

Products should appear as a DNA smear. Some material may remain in the well.

2. Downstream uses of the amplified product

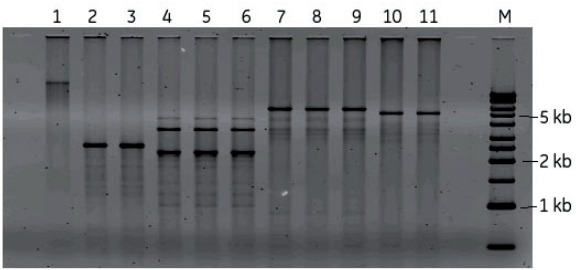

Fig 4. Restriction digestion of TempliPhi Sequence Resolver Kit amplification products. Amplification products (1 µl) and purified pUC18 DNA were digested with Eco RI and analyzed on a 0.8% agarose gel stained with GelStar. Lane 1: undigested pUC18, lane 2–3: digested pUC18, lanes 4–11: digested amplified products.

Motif	TempliPhi Sequence Resolver Kit	TempliPhi 100 Kit	DMSO (5%)	SequenceRx Enhancer Solution A	Betaine (1M)
GA dinuc repeat	731	251	590	307	293
GC rich	716	403	588	612	532
Poly G	480	378	379	410	413
TG dinuc repeat	829	374	379	388	386
CT dinuc repeat	792	190	82	164	427
Poly C	270	223	227	228	235
CA dinuc repeat/ poly C	533	439	424	456	453
TC	829	40	7	31	47
TC at end of sequence	746	353	473	460	515
Inverted repeat	565	199	204	191	201
GC rich	550	291	489	350	366
Inverted repeat	689	481	588	702	764

Table 1. Phred20 readlengths from DYEnamic ET Terminator sequencing reactions with the TempliPhi Sequence Resolver amplified product. When compared side-by-side with other finishing methods, the **TempliPhi Sequence Resolver Kit** resolved most of the difficult regions and yielded the longest readlengths (highlighted).

6.3. Troubleshooting guide

Problem: Reduced yield/ no amplification product

Possible cause	Suggestions
Too much culture or starting material was added to the amplification reaction	• Components of saturated culture media; agar and large amounts of input cellular material are inhibitory to the amplification reaction. . • If inhibition of the amplification reaction still occurs prepare a dilution series of starting material.
Enzyme inactivated by incorrect storage or usage.	• Store enzyme mix at -70°C and ensure that it freezes completely. • Do not freeze-thaw enzyme mix more than 5 times. • Ensure that enzyme mix is thoroughly mixed prior to use. • The premix of enzyme mix and reaction buffer can become inactivated if not kept cold and used promptly.
Insufficient or degraded starting DNA.	• Nicked DNA is a poor amplification substrate. • Use a fresh sample of purified DNA.

Problem: No sequencing result

Possible cause	Suggestions
No amplification product	• See problem "No amplification product".
Contaminating input DNA	• The TempliPhi Sequence Resolver Kit is very sensitive and minute amounts of any input DNA will be amplified. • When amplifying DNA from colonies or cultures do not exceed 3 min denaturation time to prevent release of chromosomal DNA. • For old purified DNA samples, prepare a fresh sample of DNA. • Transfer colonies or plaques using sterile techniques.
Volume reduction and sequencing premix dilution.	• Excessive dilution of the sequencing premix could result in an imbalance of essential components in the sequencing reaction. Follow manufacturer's recommended protocols. • Ensure all sequencing reaction components (DNA, primer, premix) are in the correct proportions.

Problem: Poor sequencing result

Problem	Possible cause and suggestions
Incorrect amount of amplified product used in sequencing reaction.	• Incorrect amount may be the result of inconsistent amplification from sample to sample or high levels of contaminating DNA present in the starting material. • Quantify yield of amplification products by Quant-iT™ Picogreen® dsDNA Assay Kit to ensure sample to sample consistency. If yields are inconsistent use less starting material per amplification reaction. • Use purified DNA as starting material to reduce contaminating levels of chromosomal DNA.
Contaminating input DNA.	• The TempliPhi Sequence Resolver Kit is very sensitive and minute amounts of any input DNA will be amplified. • When amplifying DNA from colonies or cultures do not exceed 3 minute denaturation time to prevent release of chromosomal DNA. • For old purified DNA samples, prepare a fresh sample of DNA. • Transfer colonies or plaques using sterile techniques.

Problem: Poor sequencing result

Problem	Possible cause and suggestions
Volume reduction and sequencing premix dilution.	• Excessive dilution of the sequencing premix could result in an imbalance of essential components in the sequencing reaction. Follow manufacturer's recommended protocols. • Ensure all sequencing reaction components (DNA, primer, premix) are in the correct proportions.

Problem: Difficult sequence region not resolved by TempliPhi Sequence Resolver Kit

Problem	Possible cause and suggestions
AT rich repeat	• Lower success rate is observed with very AT rich template. Attempt other methods to resolve sequence.
Complex GC repeat resulting in very stable secondary structure	• The TempliPhi Sequence Resolver Kit will not resolve every GC rich problem region. • Addition of chemicals that improve DNA denaturation such as betaine and DMSO to the sequencing reaction may help to resolve the sequence.
Polynucleotide repeat	• Very large (> 20 nucleotides) are difficult to sequence and slippage is often observed following the repeat. This is a sequencing related issue not a problem with the amplification reaction.

6.4. Related products available from GE Healthcare

Application	Product	Product code	Pack sizes
Sequencing Products	DYEnamic ET Terminator Cycle Sequencing Kit	US81050	100 reactions
	illustra AutoSeq™G-50	27-5340-01	50 columns
	illustra TempliPhi 100 Amplification Kit	25-6400-10	100 reactions
	illustra TempliPhi 500 Amplification Kit	25-6400-50	500 reactions
	illustra TempliPhi HT DNA Amplification Kit	25-6400-20	10 000 reactions
	illustra TempliPhi Large Construct Kit	25-6400-80	1000 reactions
PCR Products	illustra Hot Start Master Mix	25-1500-01	100 reactions
	illustra Hot Start Mix RTG	28-9006-46	100 reactions
	FideliTaq™ PCR Master Mix (2×)	E71182	100 reactions
	FideliTaq PCR Master Mix Plus	E71183	100 reactions
	ExoSAP-IT™	US78200	100 reactions
DNA Purification Products	illustra MicroSpin™ G-50 Columns	27-5330-01	50 columns

7.0. References:

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