A simple and quick method for successful sequencing of difficult genomic regions

Key words: *difficult template • GC region • DNA sequencing • plasmid • fosmid • BAC • dinucleotide repeat • polynucleotide repeat*

Abstract

The illustra™ TempliPhi™ Sequence Resolver Kit produces exceptional sequencing results from difficult templates such as those with high GC content and secondary structures. The kit resolves most of the sequencing stops or gaps that routinely elude other commonly used finishing methods. The use of this kit eliminates the need to use dGTP chemistry, which can affect the accuracy of a sequence due to the appearance of sequence compression. In addition, it eliminates the need for additives in the sequencing reaction. The illustra TempliPhi Sequence Resolver Kit provides a convenient, time-saving, and economical solution to the problem of sequencing difficult templates.

Introduction

Despite significant improvements in sequencing chemistry and the availability of a wide array of finishing tools such as sequencing additives, many DNA templates remain difficult to sequence (1-5). These difficult templates often cause sequence gaps in assemblies, and closing these gaps is often arduous and costly. Unlike most currently available solutions that target the sequencing step, we have developed an effective tool that targets the DNA preparation stage.

This application note describes the use of the illustra TempliPhi Sequence Resolver Kit for the sequencing of difficult templates. This optimized kit utilizes the highly processive, strand-displacing Phi29 DNA polymerase and modified nucleotides to amplify circular templates for subsequent sequencing. The kit can be used with both small (plasmids and M13) and large [fosmids and bacterial artificial chromosomes (BACs)] circular templates. The starting template for the reaction can be purified DNA, glycerol stock, liquid culture or colonies. The reaction takes less than 20 min to prepare and after an overnight incubation at 10°C, it is ready to be used directly in sequencing applications with the sequencing chemistry of choice. A 10 µl reaction typically yields 1 µg of DNA in a standard overnight reaction. The performance of the kit with a variety of difficult motifs is shown in Table 1.

Table 1. Evaluation of the illustra TempliPhi Sequence Resolver Kit with various sequence motifs.

Difficult template type	Kit performance
Large (> 20 bp) polynucleotide repeat Poly G Poly C Poly A Poly T	 + + - -
Secondary structure resulting in a sequencing stop	++
GC-rich templates	++
AT-rich templates	-
Dinucleotide repeats AC/CA AG/GA AT/TA CG/GC CT/TC GT/TG	 ++ ++ - ++ ++ ++

(++) denotes the kit usually resolves these types of sequencing problems.
(+) denotes the kit sometimes resolves these types of sequencing problems.
(-) denotes the kit usually does not resolve these types of sequencing problems.

Materials

Products used

illustra TempliPhi Sequence Resolver Kit	28-9035-31
DYEnamic™ ET Terminator Cycle Sequencing Kit	US81060
illustra TempliPhi 100 Amplification Kit	25-6400-10

Other Materials

ABI PRISM™ dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems)	4390229
SequenceRx Enhancer Solution A (Invitrogen™)	12238-010
Betaine Solution (Sigma)	B0300
Dimethyl Sulfoxide (DMSO) (Sigma)	D9170
BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)	4336917
Quant-IT™Picogreen™ dsDNA Assay Kit (Molecular Probes)	P7589
3730xl DNA Analyzer (Applied Biosystems)	

Methods

1.Amplification of DNA templates

a. Glycerol stocks (1 µl each) of several plasmid templates containing difficult regions were amplified with both the TempliPhi 100 Amplification and the illustra TempliPhi Sequence Resolver Kits according to the manufacturer's protocols. Amplification reactions were performed overnight (18 h) in a thermocycling instrument. For the fosmid templates, the volume of each component was doubled to increase the yield of amplified DNA.

2.Sequencing of amplified templates

a. Sequencing reactions containing 400 ng of template generated with each of the kits above and 5 pM of -40 Universal primer (5'-GTTTTCCCAGTCACGACGTTGTA-3') were performed with either the DYEnamic ET Terminator Cycle Sequencing Kit or BigDye v3.1 Cycle Sequencing Kit according to their manufacturers' protocols. Additional sequencing reactions were performed with 400 ng of the illustra TempliPhi 100 Amplification Kit products with different additives: 5% DMSO, 1 M betaine and 1× SequenceRx Enhancer Solution A.

b. For the fosmid templates, sequencing reactions containing 15 µl (approximately 1.5 µg) of amplified DNA and 5 pM primer (24 µl reaction volume) were performed with the DYEnamic ET Terminator Cycle Sequencing Kit. Slightly modified cycle sequencing reaction conditions of 60 cycles at 95°C for 20 s, 50°C for 20 s, 60°C for 2 min were performed to increase the overall signal.

3.Sample precipitation

a. Each sequencing reaction was precipitated with ethanol according to their manufacturers' instructions and redissolved with 10 µl of MegaBACE™ Loading Solution. All reactions were analyzed on an ABI 3730xl DNA Analyzer.

Results and discussion

Phred 20 read lengths

In general, sequencing templates generated with the illustra TempliPhi Sequence Resolver Kit yielded longer read lengths than either standard TempliPhi amplification or the application of additives (table 2). The illustra TempliPhi Sequence Resolver Kit is particularly effective on repeat sequences and sequencing stops.

Table 2. Phred 20 read lengths (in base pairs) were tabulated for all the samples. The highlighted samples are those that yielded the longest read length with the DYEnamic ET Terminator Cycle Sequencing Kit for each of the templates.

Motif	illustra TempliPhi Sequence Resolver Kit	illustra TempliPhi 100 Kit	DMSO (5%)	SequenceRx Enhancer 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 the end of sequence	746	353	473	460	515
Inverted repeat	565	199	204	191	201
GC rich	550	291	489	350	377
Inverted repeat	689	481	588	702	764

Plasmid templates

A comparative performance evaluation showed that the illustra TempliPhi Sequence Resolver Kit was particularly effective in making difficult templates such as CT-repeat (Fig 1), hairpin (Fig 2), and inverted-repeat (Fig 3) regions amenable to successful and high-quality sequencing.

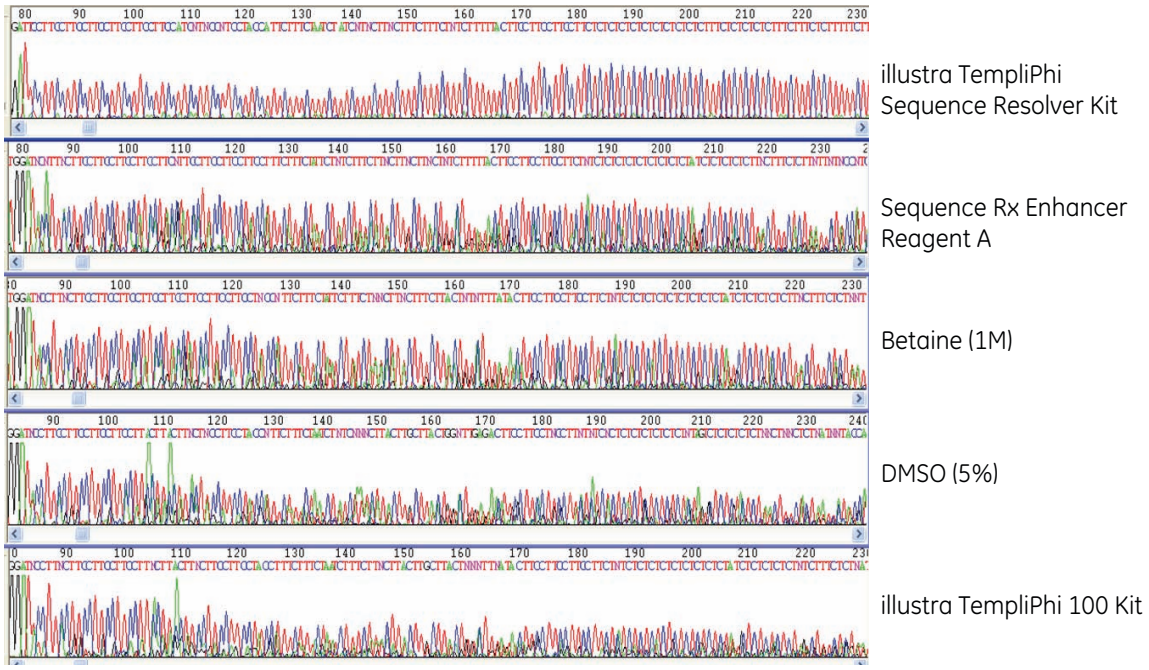

Fig 1. Electropherogram of a CT-repeat region of plasmid DNA. Additive-treated templates produced noisier sequences with Phred 20 read lengths ranging from 40 to 450 bp whereas the sequence result from the samples generated with the illustra TempliPhi Sequence Resolver Kit yielded the longest Phred 20 read length of 829 bp.

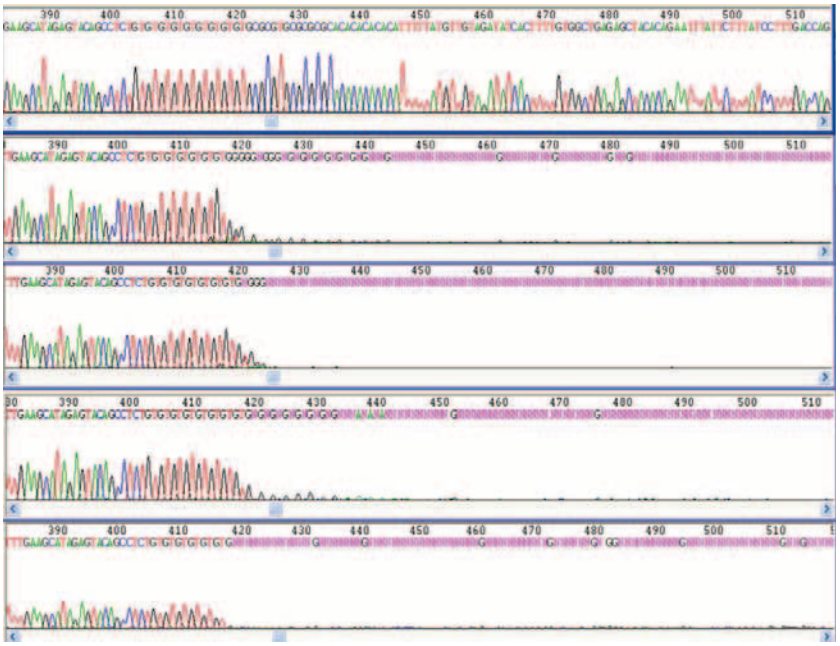

Fig 2. Electropherogram of a hairpin region of plasmid DNA. Apart from the template generated with the illustra TempliPhi Sequence Resolver Kit, the sequencing reaction failed with all the other templates because the enzyme could not get through the hairpin structure.

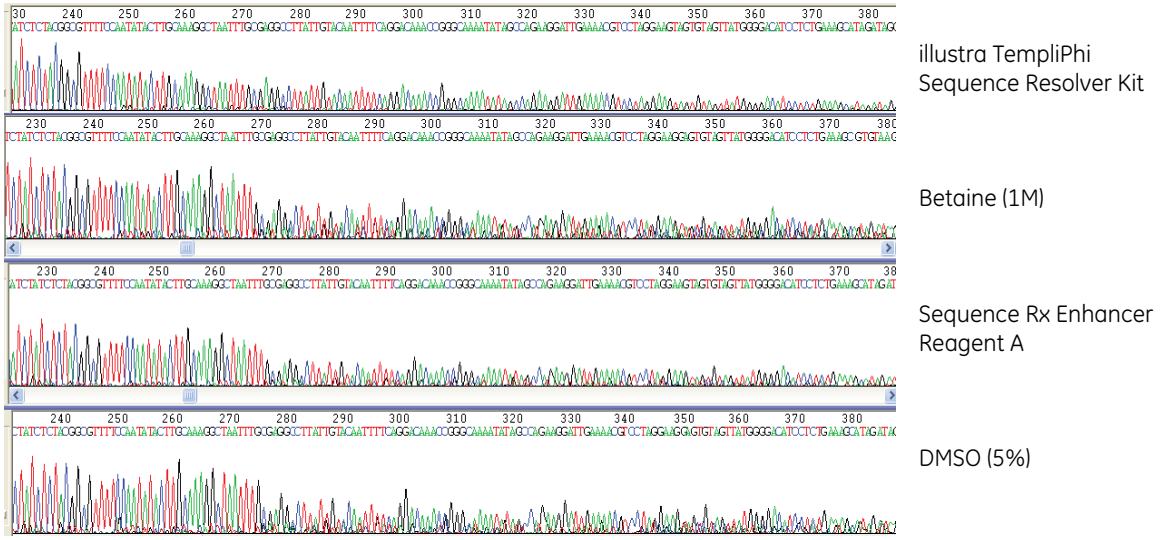

Fig 3. Electropherogram of an inverted repeat region of plasmid DNA. For all the additive-treated templates, the sequence becomes noisy and signal intensity decreases after the AATTT repeat—producing an average Phred 20 read length of 350 bp. In contrast, the template from the illustra TempliPhi Sequence Resolver Kit produced a continuous sequence with a Phred 20 read length of 552 bp.

ABI PRISM dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit results

Unlike dGTP chemistry, the illustra TempliPhi Sequence Resolver Kit was used to successfully sequence a GC-rich region (Fig 4).

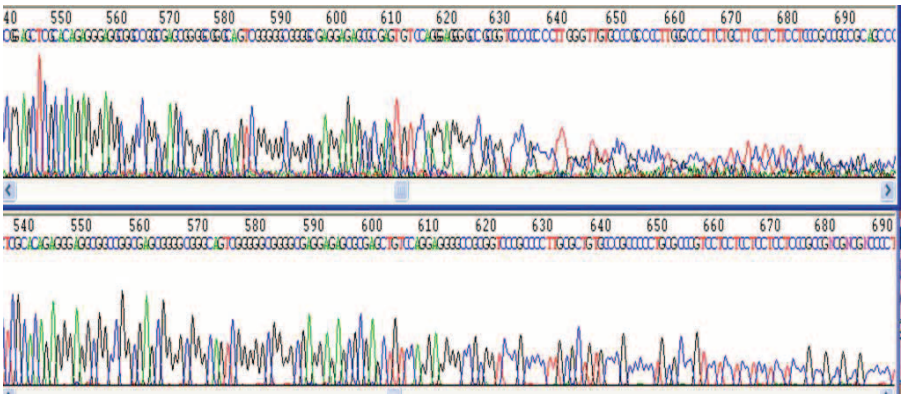

Fig 4. Electropherogram of a GC-region region of plasmid DNA. A GC-rich region toward the end of the sequence showed poor resolution when it was sequenced with dGTP chemistry—resulting in base miscalling. In addition, the dGTP chemistry produced sequencing compressions. All these limitations are absent from the sequence produced with the illustra TempliPhi Sequence Resolver Kit.

Fosmid sequencing

The illustra TempliPhi Sequence Resolver Kit was used to prepare fosmid templates that contained the following motifs: TC-rich (Fig 5), GA-repeat (Fig 6), and inverted repeat (Fig 7).

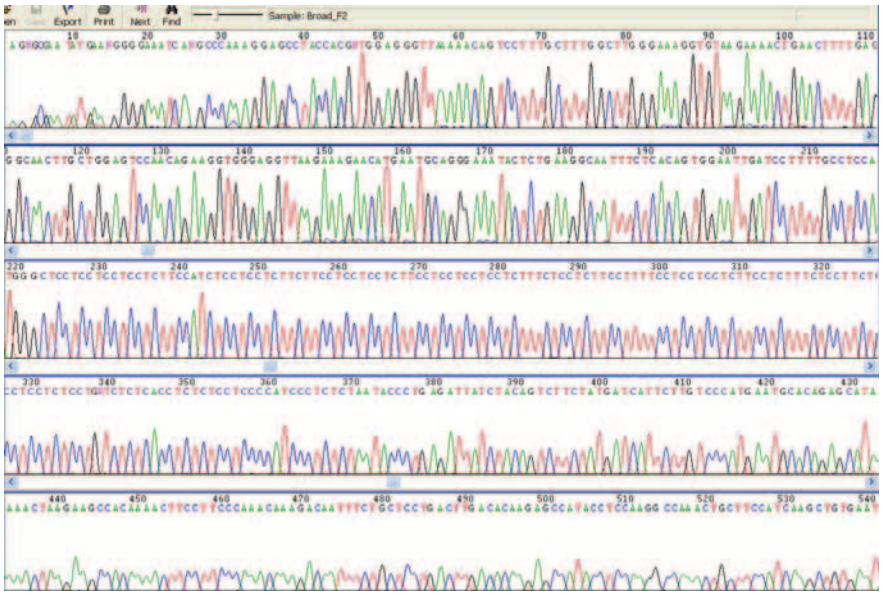

Fig 5. Electropherogram of a TC-rich region of a fosmid template after amplification with the illustra TempliPhi Sequence Resolver Kit shows that there is no need to use additives to successfully resolve such a difficult region.

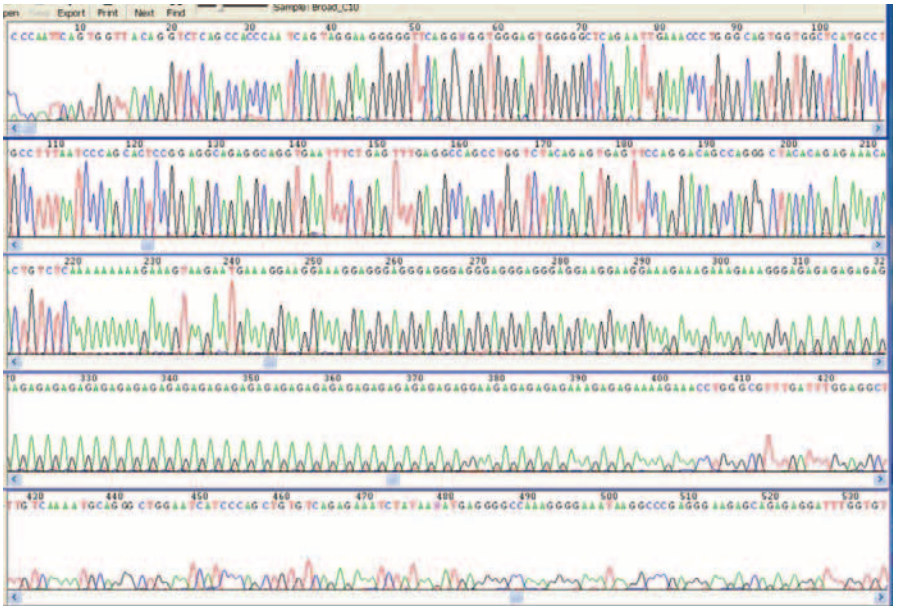

Fig 6. Electropherogram depicting the resolution of GA-repeat region of a fosmid template after amplification with the illustra TempliPhi Sequence Resolver Kit.

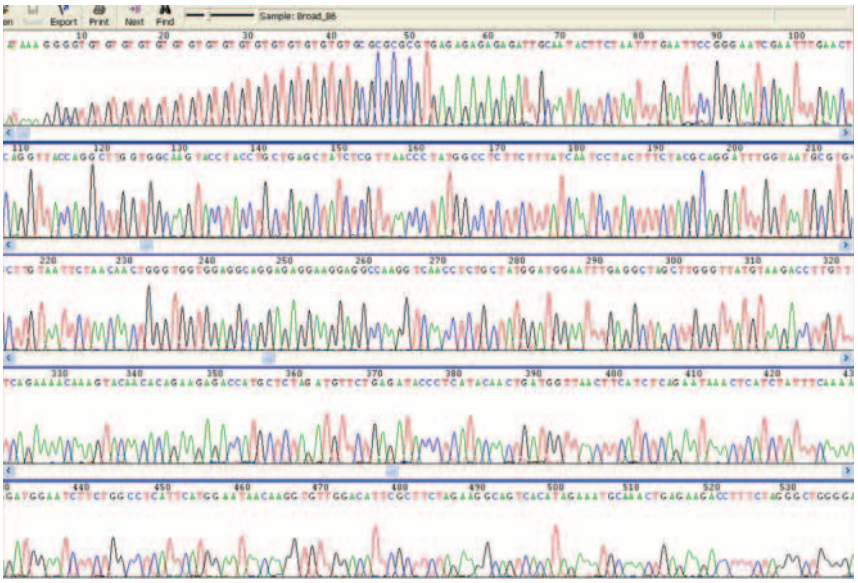

Fig 7. Electropherogram showing the successful resolution of an inverted-repeat region in a fosmid template after amplification with the illustra TempliPhi Sequence Resolver Kit.

Conclusions

illustra TempliPhi Sequence Resolver Kit offers a novel way to resolve difficult templates. The DNA prepared with the kit does not form stable secondary structures and this allows sequence determination of difficult templates. The amplified DNA is compatible with all sequencing chemistries and can be used in conjunction with sequencing reaction additives for extremely stable secondary structures. DNA can be amplified from purified DNA, colonies, plaques, glycerol stocks, or cultures; for both large and small sequencing templates.

The illustra TempliPhi Sequence Resolver Kit offers a quick, robust, and economical alternative to sequencing difficult templates. A single reaction produces sufficient DNA yields for sequencing without the need to purify or quantitate the yield first. It is compatible with both DYEnamic ET Terminator Cycle Sequencing and BigDye v3.1 Terminator Cycle Sequencing kits. The longest Phred 20 read lengths were obtained from templates that were amplified with the illustra TempliPhi Sequence Resolver Kit. The kit also eliminates the presence of sequence compressions commonly seen with dGTP chemistry, which can cause inaccurate basecalling. The illustra TempliPhi Sequence Resolver Kit can be used to amplify fosmid and BAC templates (data not shown) for downstream sequencing with slight modifications to the protocol.

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