Fast and Efficient cDNA Synthesis and PCR Amplification for Molecular Cloning

Keywords: Cloning, Recombinant DNA, qScript® Ultra Flex Kit, repliQa HiFi ToughMix®, sparQ PureMag Beads

ABSTRACT

Molecular cloning is an indispensable technique in any modern laboratory and represents a great technical and economic value to molecular biology research. Generating high quality dsDNA from RNA or DNA templates is the first and one of the most critical steps of cloning. In this application note, we detail the use of qScript Ultra Flex Kit and repliQa HiFi ToughMix to generate insert DNA for cloning in a fast and efficient way.

INTRODUCTION

Molecular cloning has driven numerous advancements throughout the life sciences over the last four decades. It has become an essential and powerful tool of modern scientific research and medicine for multiple applications that heavily depend on recombinant DNA technology.

Molecular cloning refers to the process in which one or more DNA fragments are isolated and inserted into a vector plasmid for replication using bacteria or eukaryotic cells as hosts. The recombinant DNA from the resulting population can be isolated or it can be used to generate recombinant protein after inducing the promoter region for protein synthesis. This method allows for the study of gene function and the massive production of recombinant proteins used for vaccines, antigens, gene probes, cytokines or others.

Over the years, several cloning techniques have been developed to create recombinant DNA, offering options from which researchers are able to choose the most fitting cloning technique based on speed, cost, number of inserts, difficulty, and cloning efficiency. Traditionally, molecular cloning has depended on restriction enzymes and ligases to cut a vector and an insert at specific locations so a DNA ligase can join them together to create a recombinant DNA[1,2]. However, this method can be time consuming, sequence-dependent and leaves behind a short scar in the DNA sequence. As a result, other more efficient and flexible methods were developed including SLIC (Sequence and Ligase Independent Cloning), Gibson Assembly® (Telesis Bio Inc), GeneArt® Seamless Cloning (Life Technologies), Gateway® Cloning (Invitrogen) and TEDA Cloning which rely on the homology of the assembly fragments[3–5].

Regardless of the technique, all of them share the same preliminary steps: the synthesis of the first-strand cDNA when starting from RNA material and the PCR amplification of specific DNA/cDNA targets that later get inserted into a vector. In this application note, we use the qScript Ultra Flex Kit and repliQa HiFi ToughMix to generate DNA fragments for molecular cloning. The qScript Ultra Flex Kit demonstrated rapid and efficient first-strand cDNA synthesis while providing high yields of full-length cDNA products. Likewise, repliQa HiFi ToughMix amplified DNA fragments with high fidelity with an extension time as fast as 1–10 kb/sec, enabling 3x faster PCR reactions. Finally, we used sparQ PureMag Beads to efficiently remove primers, primer dimers, unincorporated nucleotides, salts and any other impurities to improve the downstream cloning performance.

METHODS

Samples

Universal Human Reference RNA (Agilent, #740000), rat brain RNA (Takara, 636653), and human coronavirus OC43 RNA (ATCC, VR-1558D™) were used as the template for cDNA synthesis with a final input of 0.5-1 µg, 50-200 ng, and 1 µl (from ATCC stock) respectively per reaction. DNA from *Escherichia coli* strain K12 (ATCC, 10798) and human genomic DNA (Roche, 11691112001) were used as the template for the PCR amplifications with a final input of 20 ng per reaction.

qScript Ultra Flex Kit

1. Gene-specific reverse primers were designed for the first-strand cDNA synthesis from human, rat, and human coronavirus OC43 genomes. The full-length cDNA products were between 10 to 18 kb (Table 1).

Species	Target	Primer	Sequence 5'–3'	Amplicon size (kb)
Human	SYNE1	SYNE1 Rev	GAGGGCTTTCGCCAAGATCAAGG	12.5
Rat	Dynein	Dynein Rev	AGGTGGCGGCTCAAACACAAAG	17.7
Virus	HCoV-OC43	12174 Rev	GAGAGCCAAATCAGCCATACGCTCCA	11.9
		21745 Rev	AATTTAATCTGAAAACCATCACCCCAA	10.9
		30683 Rev	TGGTAACTTAACATGCTGGCTCTTCCC	10.9

Table 1 Gene specific primers used for the first-strand cDNA synthesis using qScript Ultra Flex Kit.

2. The qScript Ultra Flex master mix was prepared on ice according to Table 2.

Component	Volume for 1 sample (µl)
Nuclease-free water	13-x
Template RNA	x
10 µM Gene-specific primer mix	1
10x GSP Enhancer	2
5x qScript Ultra Reaction Mix	4
Final volume	20

Table 2 Reaction setup for the first-strand cDNA synthesis using qScript Ultra Flex Kit.

3. The first-strand cDNA reaction was run following the cycling program detailed in Table 3.

Step	Temperature	Incubation time cDNA target <20 kb	Incubation time cDNA target >20 kb
1	55°C	10 min	30 min
2	85°C	5 min	5 min

Table 3 Cycling program for first-strand cDNA synthesis using qScript Ultra Flex Kit.

4. After the completion of cDNA synthesis, 4 µl of the reaction was taken for PCR amplification.

repliQa HiFi ToughMix

5. Primer sets were picked to amplify specific regions from human, rat, human coronavirus OC43 and *E. coli* genomes. These region-specific targets were between 208 bp to 17.7 kb (Table 4).

Species	Target	Primer	Sequence 5'–3'	Amplicon size
E. coli	Cyclic di-GMP regulator gene	Fw	CCAGGCAAAGAGTTTATGTTGA	212 bp
		Rv	GCTATTTCCTGCCGATAAGAGA	
	Peptidoglycan-associated lipoprotein (PAL)	Fw	GGCAATTGCGGCATGTTCTTCC	257 bp
		Rv	CCGCGTGACCTTCTACGGTGAC	
	ygjG	Fw	TTGGATCCGATTAATTTGATTTAGATCGCA	1.8 kb
		Rv	TTCTGCAGCCTGCGGGCGTACGCGTCG	
Human	Mevalonate Kinase (MVK)	Fw	TGGGCATAGGACCTTGGCCT	208 bp
		Rv	GAGGAGTGTGATGCCACAGCCAC	
	ABCB11	Fw	CTGCTTCCTACAGATATGGAGGTTAC	997 bp
		Rv	TCAACTGATGGGGGATCCAGTG	
Rat	Dynein	Fw	GCGGCGCTGGAGGAGAA	12.5 kb
		Rv	AGGTGGCGGCTCAAACACAAAG	
Virus	HCoV-OC43	Fw	CCCGCCCATAGGTCACAATGTCGAAG	11.9 kb
		Rv	ATTAGCAGAACCACTAAAACGCGCCTC	
		Fw	ACAGTGACCCTGCAAAATTCTCGTACGC	10.9 kb
		Rv	CACTGCCACCCAGAGCTAACTTGTCTCG	
		Fw	AATTATTTGCCAAGCGCAGTGTTCGAC	10.9 kb
		Rv	CCATTCTGATAGAGAGTGCCTTATCTCGACT	

Table 4 Gene specific primer sets for PCR amplification with repliQa HiFi ToughMix.

6. The repliQa HiFi master mix was prepared on ice according to Table 5.

Component	Volume for 1 sample (µl)
repliQa HiFi ToughMix (2X)	10
10 µM Primer mix	1
Nuclease-free water	9-x
Template DNA/cDNA*	x
Final volume	20

Table 5 Reaction setup for PCR amplification using repliQa HiFi ToughMix.

* The DNA/cDNA input quantities used are mentioned in the section Samples above.

7. The PCR was run according to the cycling programs detailed in Table 6.

2-Step cycling	Temperature	Incubation time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Extend	68°C	1–10 sec/kb*	
Hold	4°C	Hold	1

3-Step cycling	Temperature	Incubation time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	35
Anneal	T_m °C**	5 sec	
Extend	68°C	1–10 sec/kb*	
Hold	4°C	Hold	1

Table 6 2-Step and 3-Step cycling programs for PCR amplification using repliQa HiFi ToughMix. 2-Step cycling was used for primer pairs with a T_m >63°C. Otherwise, a 3-Step cycling program was used.

* The extension time is based on the fragment size: 1 sec for <1 kb; 5 sec/kb for 1–10 kb; 10 sec/kb for >10 kb.
** The annealing temperature used was the same as the T_m calculated using Primer3Plus[6].

PCR Cleanup

8. PCR products were then purified using sparQ PureMag Beads and eluted in 20 µl elution buffer (10 mM Tris-HCl, pH 8.0).

Size Verification

9. To verify amplification of the Dynein and SYNE1 targets, 1 µl PCR products were loaded on a 0.5% agarose-TAE and run at 150 V for 1 hour.

10. For the remaining targets, 1 µl of the DNA was run on a D5000 or Genomic DNA ScreenTape on the 4200 TapeStation® System (Agilent).

RESULTS

Molecular cloning is an essential technique for most modern biomedical research studies and translational applications; therefore, it is important to generate high quality DNA fragments for cloning using a fast and efficient process. In this application note, we chose multiple targets from four different species that were previously used in molecular cloning research and generated DNA fragments with qScript Ultra Flex and repliQa HiFi ToughMix.[7–11]

Workflow Starting from DNA

The first two examples describe the amplification of DNA fragments from human and *E. coli* DNA using repliQa HiFi ToughMix master mix, gene specific primers sets, and sparQ PureMag Beads for purification of the cloning fragments (Figure 1).

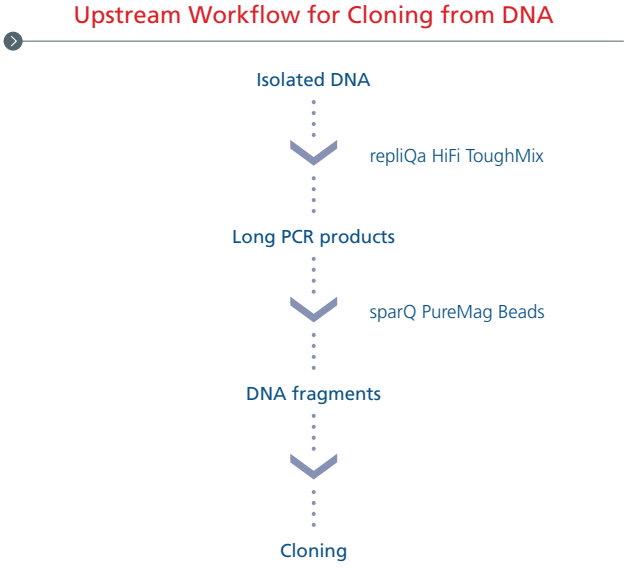

Figure 1 Cloning workflow starting from DNA inputs using repliQa HiFi ToughMix and sparQ PureMag Beads.

Cloning *E. coli* genes

We generated three DNA fragments (212 bp, 257 bp and 1.8 kb) from *E. coli* using repliQa HiFi ToughMix with an extension time of 1 sec for targets < 1 kb and 5 sec for > 1 kb target (Figure 2).

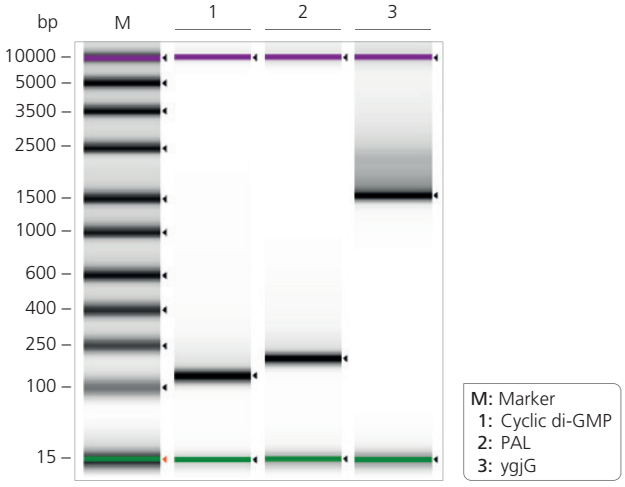

Figure 2 TapeStation analysis of the DNA fragments. **1** Cyclic di-GMP (212 bp), **2** PAL (257 bp) and **3** ygjG (1.8 kb) from *E. coli* genome amplified with repliQa HiFi ToughMix.

Cloning human genes

repliQa HiFi ToughMix was used to generate DNA fragments of 208 bp and 997 bp from the Human Mevalonate Kinase and ABCB11 genes and visualized by TapeStation electrophoresis (Figure 3).

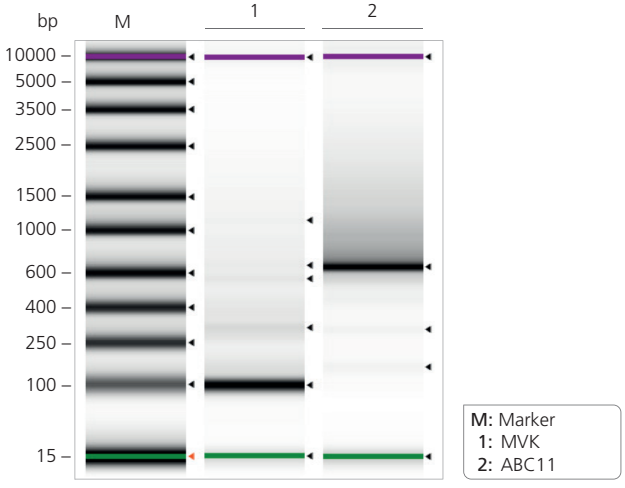

Figure 3 TapeStation analysis of the human targets genes. **1** MVK (208 bp) and **2** ABC11 (997 bp) amplified with repliQa HiFi ToughMix.

Workflow Starting from RNA

In the next examples, we describe the workflow to generate DNA fragments for cloning from human, rat and viral RNA. We used qScript Ultra Flex Kit for the first strand cDNA synthesis, repliQa HiFi ToughMix for PCR amplification and sparQ PureMag Beads for purification of the cloning fragments (Figure 4).

This upstream workflow before cloning is fast, efficient and generates DNA fragments suitable for cloning purpose regardless of the cloning technique.

Figure 4 Cloning workflow starting from RNA inputs using qScript Ultra Flex Kit, repliQa HiFi ToughMix and sparQ PureMag Beads.

Cloning Rat Dynein Gene

Different input amounts (50 ng, 100 ng, and 200 ng) of rat brain RNA were used for the first strand cDNA synthesis with qScript Ultra Flex Kit. To improve the cDNA priming efficiency, GSP enhancer and gene-specific reverse primers were used to target the cytoskeletal motor protein Dynein. Later, repliQa HiFi ToughMix amplified clean DNA fragments of 12.5 kb as seen in the agarose gel (Figure 5).

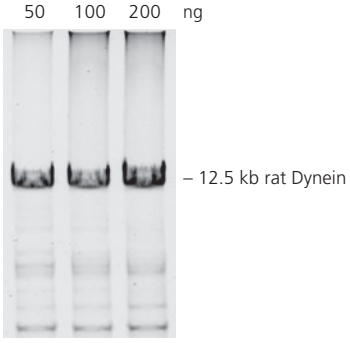

Figure 5 Rat Dynein DNA fragments from different concentrations of rat brain RNA input using qScript Ultra Flex Kit and repliQa HiFi ToughMix.

Cloning human genes

In this next example, different inputs of Universal Human Reference RNA (0.5 µg, 0.75 µg, and 1 µg) were used as starting material. During the first strand cDNA synthesis using qScript Ultra Flex Kit, GSP enhancer and gene-specific reverse primers targeting SYNE1 were used to facilitate the reverse transcription. Then repliQa HiFi ToughMix was used for PCR amplification generating DNA fragments of 17.7 kb as seen in the agarose gel (Figure 6).

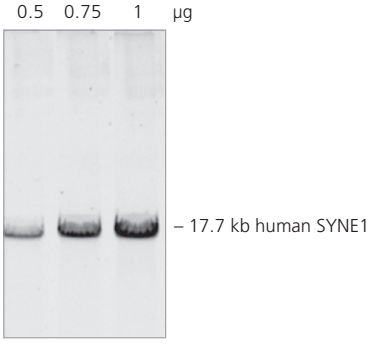

Figure 6 Gel electrophoresis of the human SYNE1 gene using qScript Ultra Flex Kit and repliQa HiFi ToughMix.

Human coronavirus OC43

Later, the human coronavirus OC43 genome was amplified using qScript Ultra Flex Kit and repliQa HiFi ToughMix. To target the whole genome of approximately 30 kb, the sequence was divided into three regions of similar sizes (11.9 kb, 10.9 kb and 10.9 kb) and three gene-specific reverse primers were used with qScript Ultra Flex Kit for the first-strand cDNA synthesis. Then, repliQa HiFi ToughMix was used for the PCR amplification step. TapeStation electrophoresis confirmed the sizes of the DNA fragments at 11.9 kb, 10.9 kb and 10.9 kb (Figure 7).

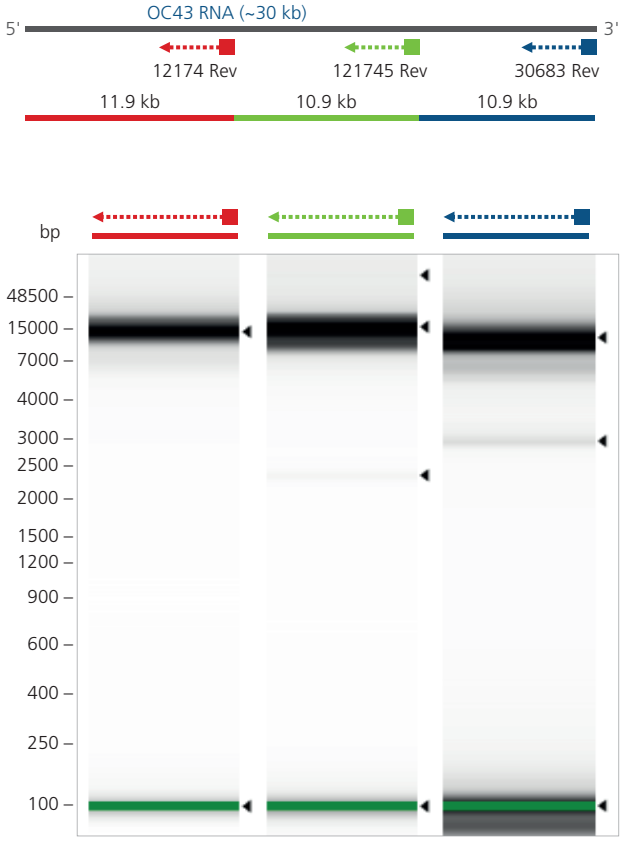

Figure 7 TapeStation analysis of the human coronavirus OC43 DNA fragments using qScript Ultra Flex Kit and repliQa HiFi ToughMix.

Finally, after PCR amplification, all the DNA fragments were purified with sparQ PureMag Beads to remove primers, primer dimers, unincorporated nucleotides, salts and any other impurities before downstream cloning workflows.

CONCLUSIONS

Molecular cloning is an essential technique that has multiple approaches but all of them share the same needs: the synthesis of first-strand cDNA when starting from RNA material and the PCR amplification of specific DNA/cDNA targets. In this application note, we described a fast and efficient upstream cloning workflow using qScript Ultra Flex Kit, repliQa HiFi ToughMix and sparQ PureMag Beads.

We showed that the combination of these three products generate high quality DNA fragments for any cloning approach from a variety of sample input sources and amounts in a fast and efficient process.

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