User Manual

QUICK-Clone™ cDNA User Manual

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Table of Contents

I. Introduction

Cloning of cDNA by PCR is a valuable tool for isolating individual cDNAs. Amplification of cDNA by PCR can often circumvent labor-intensive cDNA library construction and screening procedures. PCR cloning makes use of known regions of sequence from previously characterized cDNAs or genes to design PCR primers, either unique or degenerate, which are then used to amplify new cDNA segments. PCR-amplified cDNA fragments, generated using degenerate primers based on amino acid sequence data, can also be used to make hybridization probes to screen conventional cDNA or genomic libraries.

At Clontech, we have found that amplification of specific gene transcripts from purified double-stranded cDNA yields fewer non-specific amplification products than when performing PCR on single-stranded cDNA. For this reason, we have developed **QUICK-Clone™ cDNA**, a highly purified form of double-stranded cDNA ready for PCR amplification. Our cDNA is synthesized from our Premium Poly A^+ RNA using an oligo(dT) primer and purified to remove interfering RNA and genomic DNA.

Human Universal QUICK-Clone cDNA II (Cat.No. 637260) is an optimized mixture of >30 QUICK-Clone cDNAs from normal human tissues. (The exact number may vary based on tissue availability. Please see the Certificate of Analysis for a list of tissues.) It has been specially formulated for the amplification of full-length cDNAs that represent the majority of human genes.

II. Applications Discussion

This section discusses the amplification of QUICK-Clone cDNA and describes several useful post-amplification options.

A. Primer Design

Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. *Always check and recheck your primer design before constructing or ordering primers.* Visit **http://frodo.wi.mit.edu/** on the web for helpful guidelines on primer design.

Length and G-C content: In general, primers should have a T_m of approximately 70°C to achieve optimal results in a two-step cycling program with a 68°C annealing/extension step. Therefore, whenever possible, primers should be *at least* 22 nucleotides (nt) long (25–30-mers are preferred) and should have a G-C content of 45–60%. Furthermore, the 3'-terminal ends of each primer should not be complementary to each other and should possess a low G-C content.

B. Setting up the PCR Reaction

1. cDNA

The quantity of cDNA template to use in the reaction is related to: (a) the abundance of the target mRNA relative to the entire mRNA population used to synthesize the cDNA, and (b) the length of the cDNA required for the 5' primer to recognize its corresponding template. For mRNAs of rare abundance, a larger amount of cDNA template is required. More cDNA may also be required to amplify long cDNA segments (e.g., greater than 2 kb).

2. Primer concentration

For unique sequence primers of lengths between 25–30 nucleotides, we use a final primer concentration of between 0.2 and 0.5 µM. The optimum amount of primers in the PCR reaction depends on their molar concentrations and not simply their masses. If the primers have similar lengths and G-C content, optimal amplification will occur when the molar concentrations of both primers are equal. Unfortunately, there are no general rules for determining the optimal concentration for degenerate primers, although concentrations are generally higher than when unique sequence primers are used.

II. Applications Discussion *continued*

3. Choice of thermostable polymerase

For high-fidelity and efficient amplification of long gene segments (>1 kb), we recommend our Advantage 2 Polymerase Mix (Cat. Nos. 639201 & 639202) and an automatic hot start that reduces nonspecific products. For high yields of products <2 kb, we recommend Clontech's TITANIUM *Taq* DNA Polymerase (Cat. Nos. 639208 & 639209). This enzyme is easy to use and does not require Mg^{2+} optimization. TITANIUM *Taq* contains our TaqStart Antibody (Cat. No. 639250) which allows hot starts for increased specificity and yield in every PCR experiment. See the January 2001 and July 2006 issues of *Clontechniques* for details.

4. Amplification of G-C rich regions

In some instances, amplification of a region with a high G-C content is difficult or impossible using standard PCR. For these instances, we recommend our Advantage-GC Kits and Polymerase Mixes. These products are optimized to amplify cDNA or genomic sequences with a G-C content as high as 90%. Both the kits and the polymerase mixes contain GC-Melt, a component that destabilizes the DNA structure while also making A-T and G-C base pairs equally stable ("isostabilization"; Advantage-GC cDNA PCR Kit (Cat. Nos. 639116 & 639115) Advantage-GC cDNA Polymerase Mix (Cat No. 639112), and Advantage-GC LA Genomic Polymerase Mix (Cat. No. 639153).

C. Example of a control cDNA Amplification Reaction using Human β-actin cDNA

1. cDNA fragment size: 1.1 kb. Distance of 5' primer template from the 3' end of the cDNA: 1.8 kb.
2. Primers: Unique sequence primers (β-Actin Control Amplimers, (Cat. No. 639001). 5' primer (21-mer), 52 % G-C; 3' primer (33-mer), 61% G-C. Final concentration: 0.2 µM each.
3. Human Universal QUICK-Clone cDNA: 500 pg.
4. Cycle parameters:

   20–25 cycles

   Denature at 94°C for 45 sec.

   Anneal at 60°C for 45 sec.

   Extend at 72°C for 2.5 min.

   1 cycle

   Final extension at 72°C for 10 min

II. Applications Discussion *continued*

D. Post-Amplification Procedures

1. Removal of reaction components

We recommend removal of reaction components such as nucleotides and primers by exclusion chromatography. This procedure can be done conveniently using our CHROMA SPIN Columns with gel matrices stored in TE Buffer. CHROMA SPIN Columns are available in sizes that remove nucleic acids smaller than 100 or 400 base pairs (CHROMA SPIN +TE-100 Columns, (Cat. Nos. 636072, 636073) CHROMA SPIN + TE-400, (Cat. No. 636076). The PCR product or reaction mixture (40–45 µl) can be loaded directly onto the pre-spun column with no prior manipulations.

2. Cloning amplified cDNA

Several procedures exist for subcloning PCR products. We recommend In-Fusion™ 2.0 PCR Cloning Kits (Cat. Nos. 639609, 639607 & 639608). Cloning using the In-Fusion 2.0 method does not require PCR product purification and requires simple extensions to your PCR primers. In-Fusion is the easy, precise solution for making expression clones.

E. Troubleshooting

For researchers without extensive experience with PCR, we strongly recommend the use of β-actin or G3PDH control primers as a positive control in your first few experiments. We offer several amplimer sets specific for human β-actin (Cat No. 639001), mouse ß-actin (Cat. No. 639008), and human G3PDH (Cat. No. 639005).

II. Applications Discussion *continued*

1. Perfect sequence primers

When mixing perfect sequence primers, *inefficient or failed amplification* can result from using old reagents, such as old dNTPs or stale reaction buffers. The incidence of this problem can be reduced by storing aliquots of reagents at –20°C, and by periodically discarding working stocks. In rare instances, the DNA polymerase may be defective. Ensure that no reagents have been omitted from the reaction.

2. Degenerate primers

A number of factors may cause partial or complete failure in experiments using degenerate primers. Determining whether the failure of degenerate primer experiments are due to causes associated with perfect sequence experiments, or to causes associated with the degenerate primers, is of utmost importance. To make this determination, you must perform PCR experiments with perfect sequence control primers such as β-actin. If these control experiments are successful, the most likely reasons for amplification failures is that at least one of the degenerate primers contains too many mismatches to allow stable annealing. Lowering the annealing temperature stepwise by 2–5°C at a time may help. However, you will probably have to construct new primers.

In some cases, too many PCR products are observed. This result is usually due to the presence of unwanted, stable primer templates. Increasing the annealing temperature and/or decreasing the primer concentrations may resolve this problem. If you still obtain unwanted products, we suggest that you utilize a hot start PCR. For this purpose, we offer TaqStart Antibody that can be added to *Taq* polymerase. This antibody is also included in our TITANIUM *Taq* and Advantage 2 Kits. If problems persist, we recommend that you construct alternate primers or try nested primers to increase specificity. However, please note that in some cases, additional bands may indicate the presence of alternately spliced transcripts (Nakabeppu & Nathans, 1991).

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