RT-PCR Protocol for High-Fidelity Cloning Applications

Protocol

Introduction

It is essential to select reverse transcription and PCR kits capable of high-fidelity replication when performing RT-PCR for applications such as cloning. The iScript™ Select cDNA Synthesis Kit is a highly sensitive reverse transcription kit. The powerful reverse transcriptase and proprietary buffer achieve a high rate of fidelity during cDNA synthesis. The iProof HF Master Mix uses iProof High-Fidelity DNA Polymerase, a unique *Pyrococcus*-like proofreading enzyme fused to the Sso7d dsDNA-binding protein to create a thermostable fusion polymerase that accurately amplifies long products from a variety of DNA templates. iProof DNA Polymerase is 52-fold more accurate than *Taq* polymerase.

Using these two kits in conjunction helps ensure that accurate amplicons are generated for cloning. The protocol below details how to use these kits for RT-PCR when cloning and offers a high-level overview of steps integral to the entire cloning process.

For additional information on specific Bio-Rad products referenced in this protocol, including storage information, see individual product instruction manuals.

- iScript Select cDNA Synthesis Kit Instruction Manual
- iProof High-Fidelity Master Mix Manual

Procedure

1. Reverse Transcription

Before beginning, determine the appropriate RT priming strategy (either oligo(dT) or gene-specific primers) for your cloning target insert.

- Oligo(dT) and gene-specific primers are ideal priming strategies for cloning and are most likely to generate full-length cDNA
  - Oligo(dT) will complement RNA with a 3' poly(A) tail
  - Gene-specific primers can be used for transcripts without 3' polyadenylation
- Random primers can improve yield and coverage throughout the RNA transcript, but they may result in shorter cDNA fragments and are not recommended for cloning

1.1. Thaw all components except iScript Reverse Transcriptase. Mix thoroughly and briefly centrifuge to collect contents at the bottom of the tube before using. Place components on ice.

1.2. (Optional) For difficult targets, such as those high in GC content or with a very long transcript, preincubation of RNA at 65°C for 5 min followed by incubation on ice can help denature RNA secondary structure and improve the yield of full-length cDNA. Preincubate the RNA in diluted TE buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) prior to combining with reaction components.

1.3. On ice, add the following components to a 0.2 ml PCR tube or each well of a 96-well PCR reaction plate (Tables 1 and 2). **Note:** For difficult targets, doubling the volume of iScript Reverse Transcriptase may improve the yield of full-length cDNA.

Table 1. Oligo(dT) primers.

Component	Volume per Reaction, µl
Nuclease-free water	Variable
5x iScript Select Reaction Mix	4
Oligo(dT)$_{20}$ primer	2
RNA sample (1 ng to 1 µg total RNA)*	Variable
iScript Reverse Transcriptase	1
Total volume	**20**

Table 2. Gene-specific primers.

Component	Volume per Reaction, µl
Nuclease-free water	Variable
5x iScript Select Reaction Mix	4
Gene-specific primer (2–10 pmol) (100–500 nM in 20 µl final volume)	Variable
GSP enhancer solution	2
RNA sample (1 ng to 1 µg total RNA)*	Variable
iScript Reverse Transcriptase	1
Total	**20**

* In general, the RT-PCR yield of low-abundance transcripts can be increased by using higher inputs of template RNA.

Scale all components proportionally according to sample number and reaction volumes.

1.4. Mix gently and incubate for 10–60 min at 42°C. Incubation time can be further extended up to 120 min at 42°C to synthesize longer cDNAs for cloning purposes.

Note: For shorter length targets (1–3 kb), incubation time can be as little as 10 minutes. For longer (up to 10 kb) or more difficult targets, incubation for at least 60 minutes is recommended.

1.5. Incubate at 85°C for 5 min to heat-inactivate the reverse transcriptase.

1.6. Store cDNA product at –20 to 4°C.

Note: The resulting cDNA product can be used directly for PCR amplification using the iProof HF Master Mix. Typically, the volume of cDNA synthesis reaction used is one-tenth the volume of the PCR reaction.

2. PCR

iProof HF Master Mix is recommended for cloning for its convenience, speed, and accuracy.

Primer design for amplification of the target insert should take into account whether a blunt or sticky end cloning strategy will be employed.

In the case of blunt end cloning, primers should include a 5' phosphorylation modification to allow for direct ligation of the PCR product to the vector. Alternatively, the blunt-ended PCR product may be treated with a kinase if PCR primers are not 5' phosphorylated.

In the case of sticky end cloning, the PCR product will still be blunt-ended but is followed by a restriction enzyme digestion to create sticky ends complementary to the vector. The recognition sequence of the chosen restriction enzyme(s) can be added to PCR forward and reverse primer sequences. Additional nucleotides flanking the recognition sites may be added to improve digestion efficiency. Confirm that the target insert sequence does not contain any additional restriction sites for the restriction enzyme used to create sticky ends on the PCR product and vector.

2.1. Thaw 2x iProof HF Master Mix. Mix thoroughly and briefly centrifuge to collect contents at the bottom of the tube before using. Place on ice.

2.2. On ice, prepare reaction mix using PCR tubes according to the recommendations in Table 3.

Table 3. Reaction setup.

Component	Volume per Reaction, µl	Final Concentration
2x iProof HF Master Mix	25	1x
Forward and reverse primers	Variable	0.5 µM each primer*
cDNA template	Variable	100 pg–100 ng
Nuclease-free water	Variable	—
DMSO** (optional)	1.5	3%
Total	**50**	**—**

* Recommended final primer concentration is 0.5 µM, but it can range between 0.2 and 1.0 µM if needed.

** DMSO can be added for GC-rich amplicons. See **Notes about Reaction Components** for more details.

Scale all components proportionally according to sample number and reaction volumes.

2.3. Seal tubes and vortex for 30 sec or more to ensure thorough mixing of the reaction components. Spin the tubes to remove any air bubbles and collect the reaction mixture in the vessel bottom.

2.4. Program the PCR cycling protocol on the PCR instrument according to Table 4.

Table 4. Thermal cycling protocol.

Step	Temperature, °C	Time	Cycle
Initial denaturation	98	30 sec–3 min	1
Denaturation	98	5–10 sec	25–35
Annealing	45–72	10–30 sec	
Extension	72	15–30 sec/kb	
Final extension	72	5–10 min	1

Notes about Cycling Conditions

- **Denaturation** — Template denaturation should be performed at 98°C. The high thermostability of iProof DNA Polymerase allows denaturation temperatures greater than 98°C to be used if needed. A 30 sec initial denaturation time is recommended, but this can be extended to 3 min for difficult DNA templates. Subsequent denaturation should be performed for 5–10 sec at 98°C.
- **Annealing** — When using iProof HF Master Mix, a general rule is to anneal primers (>20 nt) for 10–30 sec at 3°C above the primer with the lowest melting temperature (T_m). Primer T_m should be calculated using the nearest neighbor method as results can vary significantly, depending on the method used. For primers ≤20 nt, use an annealing temperature equal to the primer with the lowest T_m.
- **Extension** — Template extension temperature must be 72°C and extension time depends on amplicon length (15–30 sec/kb) and complexity. Do not exceed 1 min per kb for amplicons that are >5 kb.

Notes about Reaction Components

- **DMSO** — Adding 3% DMSO aids in template denaturation for GC-rich templates. Further optimization of DMSO concentration should be made in 2% increments. If a high DMSO concentration is used, the annealing temperatures should be lowered by 5.5–6.0°C.

3. Insert Product, Transform and Grow Cells

3.1. Linearize the desired vector by restriction enzyme digestion. The linearized vector will have blunt or sticky ends depending on the restriction enzymes chosen. In vectors with multiple cloning sites, avoid choosing restriction enzyme digest sites that may disrupt a selectable marker, such as antibiotic resistance.

Note: Including a phosphatase in the digestion reaction for 5' dephosphorylation can help prevent self-ligation of the vector. Directional cloning using two different restriction enzymes to create noncomplementary ends can also prevent self-ligation of the vector. Refer to manufacturer's instructions for optimal digestion conditions.

3.2. Gel purify the target insert and linearized vector.

- Electrophoresis should be run long enough that both the insert and vector consist of single bright bands that can be isolated to remove unwanted fragments.
- If necessary, a scaled-up PCR reaction of the target insert may be performed to increase yield. Refer to the manufacturer's instructions for gel purification.

3.3. Ligate the fragments with DNA ligase. Extending the reaction time overnight may improve low efficiency ligations. Refer to the manufacturer's instructions on blunt or sticky end ligation.

3.4. Transform competent cells using the plasmid DNA. Grow cells overnight on an agar media plate. Use the appropriate selective conditions for the chosen vector. For example, add the appropriate antibiotic to the media if antibiotic resistance was used as the selectable marker. Refer to manufacturer's instructions for transformation of chemically or electrocompetent cells.

3.5. Pick and isolate a few colonies to grow in liquid media overnight at small scale. Again, use the appropriate selective conditions for the chosen vector.

3.6. Perform small-scale plasmid purifications of each clone. Refer to the manufacturer's instructions for purification.

3.7. Perform PCR using the purified plasmid as the template and primers for the target insert to confirm a positive clone. The same cycling conditions for amplification of the target insert from the cDNA template may be used. Alternatively, the cloned fragment can be confirmed by digestion of the plasmid with the appropriate restriction enzymes and followed by agarose gel electrophoresis. Sequencing of positive clones can be used to provide additional confirmation.

Visit bio-rad.com/fidelity for more information.

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