6

Long Distance Reverse-Transcription PCR

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1. Introduction

Polymerase chain reaction (PCR) has been applied to the amplification of long DNA fragments from a variety of sources, including genomic, mitochondrial, and viral DNAs *(1–5)*. We have adapted the concept of long PCR technology to reverse-transcription (RT) PCR *(6)*. Here, we describe the parameters critical in producing RT-PCR products of up to 20 kbp. The nature of RT-PCR requires the synthesis of a cDNA by RT prior to its amplification in the PCR reaction. Thus, we focus on the three steps of RT-PCR: the preparation and requirements of the RNA template, the reverse transcription reaction, and the amplification of the cDNA by PCR.

To carry out these studies, we used the genomic RNA of the human coronavirus HCoV 229E as template *(7)*. The HCoV 229E genomic RNA has a length of ca. 27,000 nucleotides and the homogeneity of the RNA can be readily assessed by electrophoresis and hybridization analysis *(8)*. HCoV 229E genomic RNA has two major advantages for the studies reported here. First, as a viral RNA, it is relatively abundant in the infected cell. Second, coronaviruses are positive strand RNA viruses and the genomic RNA has a 3' polyadenylate tract that can be used for affinity chromatography *(9)*.

In **Subheading 3.1.**, we describe a simple and fast technique to purify poly(A)-containing RNA from tissue culture cells. In fact, we believe that the integrity and purity of the RNA template is the most critical parameter when the RT-PCR amplification of sequences more than 5 kb in length is desired *(6)* (*see* **Note 4**, **Fig. 1**). Depending on the source of the RNA template, a method of preparation should be chosen that minimizes degradation of the RNA. In our hands oligo (dT)-based affinity chromatography with magnetic beads has proven to be reliable for the isolation of poly(A)-RNA that can be used to produce cDNA of more than 20 kb by RT.

The conditions of the reverse transcription reaction strongly influence the outcome of the subsequent PCR. Reverse transcription reactions have been performed using the RNase H-deficient reverse transcriptase, SuperScript II and the cDNA has been used for PCR amplification without further purification. In general, an RT-primer should
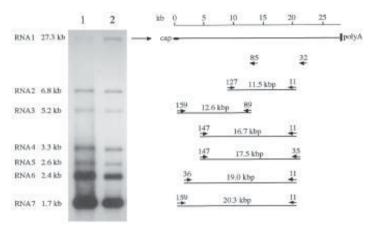
Fig. 1. Northern hybridization analysis of HCoV 229E poly(A)-containing RNA isolated from infected MRC-5 cells. The material shown in lane 1 was prepared using poly(U)-Sepharose *(6)*. The material shown in lane 2 was prepared using oligo(dT)$_{25}$ magnetic beads as described in **Subheading 3.1.** The poly(A)-containing RNAs were separated by gel electrophoresis and the viral mRNAs were hybridized to a ^{32}P-(5'end)-labeled oligonucleotide (5'AGAAACTTCTCACGCACTGG 3'). Also shown is the relationship of the HCoV 229E genomic RNA template, oligonucleotide primers and RT-PCR products. The oligonucleotides are indicated as arrows with their orientation and position relative to the HCoV 229E genomic RNA. The expected sizes of the RT-PCR products are indicated.

be used that is highly specific and great care should be taken to adjust the optimal concentration of the RT-primer in the reverse transcription reaction. In our experience, the major problem that arises is "less stringent" priming during the RT reaction. The fortuitous cDNAs that are synthesized and the small amounts of RT-primer that are carried over into the PCR are responsible for most of the background amplification products observed *(6)*.

Finally, the optimal conditions, regarding the amount of cDNA template, the choice of PCR primers and the cycle profile of the PCR, have to be determined. In this respect, the considerations that apply to the PCR amplification of dsDNA templates are equally applicable to amplification of cDNA produced by reverse transcription.

2. Materials

1. Phosphate-buffered saline (PBS).
2. Lysis buffer: 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM KCl, 1% Nonidet P-40 (NP40).
3. Oligo(dT)$_{25}$-Dynabeads (Dynal) (3.3 × 10^8 beads/mL).
4. Dynal Magnetic Particle Concentrator (Dynal).

5. 2X binding buffer: 20 mM Tris-HCl, pH 7.5, 1 M LiCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 1% sodium dodecylsulfate (SDS).
6. Wash buffer: 10 mM Tris-HCl, pH 7.5, 150 mM LiCl, 1 mM EDTA, 1 mM EDTA pH 7.5.
7. SuperScript II reverse transcriptase (Life Technologies).
8. 5X first-strand buffer (Life Technologies).
9. 10 mM dNTPs (10 mM of each dNTP).
10. 0.1 M dithiothreitol (DTT).
11. RNasin (50 U/μL) (Pharmacia).
12. Thin-wall PCR tubes.
13. Elongase Enzyme Mix (Life Technologies).
14. PCR buffer B (Life Technologies).

3. Methods

3.1. The RNA Template: Preparation of Poly(A)-Containing RNA Using Oligo(dT)$_{25}$-Dynabeads

The protocol below describes the isolation of poly(A)-containing RNA from a confluent layer of adherent cells grown in a 175 cm^2 tissue culture flask (*see* **Note 1**).

1. Wash 200–500 μL oligo(dT)$_{25}$-Dynabeads twice with 2X binding buffer using an appropriate Dynal Magnetic Particle Concentrator (*see* **Note 2**) and resuspend the beads in 1.5 mL 2X binding buffer.
2. Wash the cells twice with ice-cold PBS and then scrape in 10 mL ice-cold PBS.
3. Pellet the cells at 1000g for 2 min at 4°C.
4. Resuspend the cell pellet in 1.5 mL ice-cold lysis buffer and incubate for 30 s on ice.
5. Centrifuge the cell lysate at 1500g for 1 min at 4°C to remove nuclei.
6. Mix the supernatant with oligo(dT)$_{25}$-Dynabeads resuspended in 1.5 mL 2X binding buffer (**Step 1**) and incubate for 5 min at 23°C. Gently mix the sample every 1–2 min.
7. Wash the oligo(dT)$_{25}$ magnetic beads twice with wash buffer using an appropriate Dynal Magnetic Particle Concentrator (*see* **Note 3**).
8. Completely remove the wash buffer and add 50–100 μL 2 mM EDTA, pH 7.5.
9. Transfer the solution into a 1.5-mL Eppendorf tube and incubate for 2 min at 65°C to elute the bound poly(A)-RNA.
10. Take the supernatant containing the poly(A)-RNA, add 5 μL RNasin and store at –70°C in aliquots (*see* **Note 4**).
11. Regenerate the oligo(dT)$_{25}$-Dynabeads according to the manufacturer's instructions (optional; *see* **Note 5**)

3.2. The RT Reaction

1. Add the following components to a volume of 19 μL (*see* **Note 6**):
   RNase-free water
   4 μL 5X first strand buffer;
   2 μL 10 mM dNTPs (10 mM of each dNTP);
   2 μL 0.1 M DTT;
   0.5 μL RNasin (50 U/μL);
   5–15 pmol reverse transcription primer (*see* **Note 7**);
   10–500 ng poly(A)-RNA (0.5–3 μL of poly(A)-containing RNA prepared as described earlier).

2. Incubate for 2 min at 42°C (*see* **Note 8**).
3. Add 1 μL (200 U) SuperScript II reverse transcriptase.
4. Incubate for 60–90 min at 42°C (*see* **Note 9**).
5. Incubate for 2 min at 94°C and chill on ice (*see* **Note 10**). Store at –20°C until use.

3.3. The PCR Reaction

To amplify long DNA fragments from cDNA templates the basic principles of long PCR technology are applicable (*see* **Note 11**). The protocol below describes a typical reaction using the Elongase Enzyme Mix (Life Technologies). In addition, we provide a list of PCR cycle profiles (**Fig. 2**) and corresponding oligonucleotide primers (**Fig. 1**, **Table 1**) that have been used to produce RT-PCR products of 11.5–20.3 kbp in length.

1. Set up the PCR reaction mix in thin-wall PCR tubes on ice [50 μL volume; final concentrations: 60 mM Tris-SO$_4$ (pH 9.1), 18 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 0.2 mM dNTPs, 0.2–0.4 μM PCR primer]:
   10 μL buffer B (Life Technologies) (*see* **Note 12**);
   1 μL 10 mM dNTPs (10 mM of each dNTP);
   1–2 μL forward PCR primer (10–40 pmol);
   1–2 μL reverse PCR primer (10–40 pmol);
   0.2–3 μL RT-reaction;
   1 μL Elongase Enzyme Mix (1 U/μL);
   water to a final volume of 50 μL;
2. Place PCR tubes in an appropriate PCR cycler at 94°C (*see* **Note 13**).
   Cycle conditions:
   1 min 94°C, followed by 30 cycles of 20 s denaturation at 94°C, 20 s annealing at 50°C and elongation for 1 min/kb expected product length at 68°C. Increase the elongation time during the last 18 cycles by 15–30 s in each successive cycle. Incubate additional 10 min at 72°C and terminate the reaction by decreasing the temperature to 4°C.

4. Notes

1. The number of cells will be about 10^7–10^8 depending on the tissue culture cell line used. We recommend preparing the RNA from a confluent cell layer using at least one 175 cm^2 tissue culture flask. This ensures that you will get enough poly(A)-containing RNA to perform several RT reactions using the same RNA preparation.
2. There are different Magnetic Particle Concentrators recommended, depending on the size of the tubes. To lyse the cells and bind the RNA to the oligo(dT)$_{25}$ magnetic beads, we use 15 mL tubes. Before we elute the poly(A)-containing RNA, we transfer the oligo(dT)$_{25}$ magnetic beads into a 1.5-mL Eppendorf tube.
3. If the oligo(dT)$_{25}$ magnetic beads appear to clump and do not resuspend well, you have cellular DNA in your sample. However, you can proceed without affecting the RNA quality or yield, if you perform additional washing steps *(2–4)* until the oligo(dT)$_{25}$ magnetic beads can be resuspended easily.
4. We strongly recommend analyzing the RNA template integrity before cDNA synthesis. We routinely perform a northern hybridization analysis. In **Fig. 1** poly(A)-containing RNA prepared from HCoV 229E infected MRC-5 cells using two different methods are shown. In both RNA preparations, it is possible to identify the HCoV 229E genomic RNA (27.3 kb) and the six subgenomic mRNAs (1.7 kb-6.8 kb) that are characteristic of
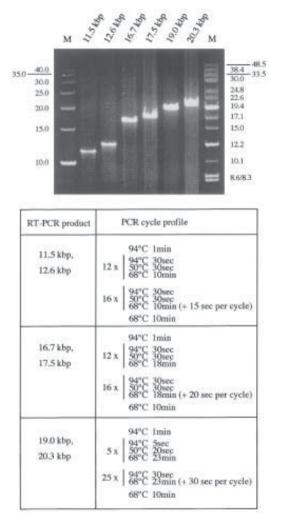
Fig. 2. Pulse-field gel electrophoresis of HCoV 229E RT-PCR products. Five microliters of each PCR reaction were separated by PFGE together with a 5-kbp DNA ladder and a high-molecular weight DNA marker (Life Technologies). Also shown are the cycle profiles that have been used to produce RT-PCR products ranging from 11.5–20.3 kbp in length.

Table 1
Oligonucleotide Primers

Oligonu-cleotide	Sequence (5' to 3')[a]	Position[b]	Orientation[c]	Application
85	ACACACGGTGTATGTCCTCATT	12979–13000	−	RT
32	TATAGGCATTGCGCAACCACCGG	21747–21769	−	RT
127	cgatcgcggccgctggccgaataggccatgGCTGATTACCGTTGCGCTTGT	9071–9091	+	PCR
11	gagaggatccGCAAAACAAAACATTTTATTTAGTTGAGAC	20554–20582	−	PCR
159	cgatcgcggccgctggccgaataggccATGGCCTGCAACCGTGTGACACT	293–315	+	PCR
89	TCATGGTGTATTTAGTAAGAT	12830–12850	−	PCR
147	AAACCAGTCTGCTCATCA	3860–3877	+	PCR
35	tgggacgTCAAAGGACAACTGGTCACATCTCAG	21353–21378	−	PCR
36	TTGGTCTGTTGGTGATTGGACCGGT	1048–1072	+	PCR

[a]The nucleotides corresponding to HCoV 229E sequences are shown in capitals. The nucleotides shown in small case were added for cloning purposes.
[b]The position refers to the nucleotide sequence of HCoV 229E genomic RNA.
[c]Oligonucleotides with mRNA orientation are designated as +.

coronavirus infection. The hybridization analysis indicates that the material prepared using the oligo(dT)$_{25}$ magnetic beads (lane 2) is less degraded than the material prepared using poly(U)-Sepharose (lane 1). RT-PCR amplifications of more than 5 kbp were only possible when poly(A)-containing RNA shown in lane 2 was used as the template for reverse transcription.

5. The oligo(dT)$_{25}$ magnetic beads can be regenerated according to the manufacturer's instructions (Dynal). However, during the repeated washing steps, you will loose about 20% of the magnetic beads.

6. Add the reagents in the order listed in **Subheading 3.2.** To melt RNA structures before the reverse transcription, some protocols recommend to heat the RNA for 10 min at 70°C. However, in most cases this is not necessary.

7. Because the RT is performed at 42°C, cDNAs can be generated during the RT reaction by "less stringent" priming events. This is the reason for most of the background PCR products observed in our system. Therefore, it is absolutely necessary to use a highly specific RT-primer and to adjust the optimal primer concentration in the RT-reaction (*6*). Furthermore, we recommend the use of different primers for the RT and PCR reactions.

8. Before adding the SuperScript II enzyme, the RNA template and the RT-primer should be at 42°C to minimize unspecific binding.

9. We recommend incubation for 90 min when cDNA synthesis of more than 10 kb is desired. Increasing the incubation temperature above 42°C was not beneficial in our hands.

10. Some protocols for the amplification of long mRNAs have required digestion of the RNA with RNase H after cDNA synthesis (*11*). This step did not seem to be necessary for amplification with HCoV 229E genomic RNA. This may be owing to the fact that in our protocols the HCoV 229E RNA was relatively abundant. Experiments with dystrophin mRNA required treatment with RNase H prior to amplification (*6*).

11. The PCR cycle conditions have to be optimized according to the amount of template, the PCR primers and the cycle profile. We recommend that these parameters should be optimized with RT-PCRs of expected product sizes below 5 kbp before trying to synthesize longer RT-PCR products.

12. This will result in a Mg^{2+} concentration of 2 m*M*.

13. Optional: a "real" hot start can be performed by adding the enzyme mix after the PCR sample has reached 94°C.

Acknowledgments

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