

Use of Gene-Specific Primer Cocktails for First-Strand cDNA Synthesis With a Reverse Transcriptase Kit

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The use of reverse transcriptase (RT), which synthesizes a DNA copy from an RNA template, has allowed researchers to monitor gene expression by coupling reverse transcription of cellular mRNA to conventional polymerase chain reaction (PCR). Typically, cDNA is primed from total RNA using either a poly-T primer that is directed against the poly-A tail of mature mRNAs or random hexanucleotides. If the sequence of a particular gene of interest is known, a gene-specific primer can be used to copy and amplify transcripts of a single gene. This application note describes the use of a cocktail of gene-specific primers to prime first-strand cDNA synthesis with the Eppendorf® cMaster™ RT kit (Brinkmann Instruments, Westbury, NY). Reverse transcription followed by conventional PCR results in the specific and robust amplification of a small group of genes.

Materials and methods

RNA isolation

Whole rat brain was homogenized in 1 mL TRIzol (Life Technologies, Rockville, MD) per 50 mg tissue, and RNA was isolated using the standard TRIzol protocol. Briefly, 0.2 mL of chloroform was added per milliliter of TRIzol; the contents were then mixed, incubated, and centrifuged. The aqueous layer was removed, and RNA was precipitated with isopropanol, washed with 70% ethanol, dried, and resuspended in nuclease-free water. RNA was quantitated using a spectrophotometer.

First-strand cDNA synthesis

Two micrograms of rat brain RNA was used as a template for each RT reaction. First-strand cDNAs were primed using either oligo-(dT)₁₂₋₁₈ (0.5 µg, Stratagene, La Jolla, CA), random hexamers (50 ng, Stratagene), or a cocktail of gene-specific primers (2 pmol/primer, 10 primers total). A two-step RT-PCR protocol was used. In the first step, MasterMix 1, consisting of deoxyribonucleotide triphosphates (dNTPs; 10 mM each, Brinkmann, Westbury, NY), antisense primers (Sigma-Genosys, Haverhill, U.K.), template RNA, and nuclease-free water (up to 10 µL) was incubated at 65 °C for 5 min and then chilled on ice. MasterMix 2, consisting of 4 µL RT_{plus} buffer with 25 mM Mg²⁺, 1 µL cMaster RT enzyme (Brinkmann), 0.5 µL Prime RNase inhibitor solution (Brinkmann), and nuclease-free water (up to 10 µL), was then added to MasterMix 1. The contents were mixed, centrifuged, and incubated at 42 °C for 60 min. The reaction was terminated by incubation at 85 °C for 5 min and samples were placed on ice.

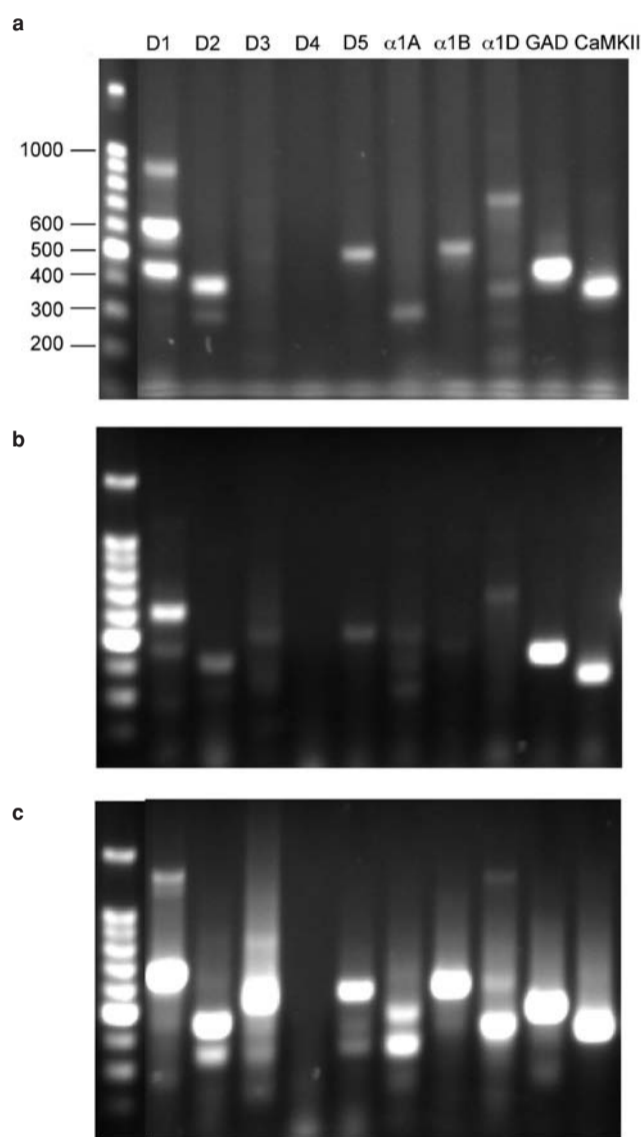


Figure 1 Comparison of RT-PCR products for 10 genes using oligo-(dT), random hexamer, or a cocktail of gene-specific primers. Two micrograms of rat brain RNA was used as a template for RT-PCR. First-strand cDNA synthesis was primed using a) oligo-(dT), b) random hexamer, or c) a cocktail of gene-specific antisense primers. As a template for PCR, 0.5 µL of each RT reaction was used for each of the following genes: D1, D2, D3, D4, and D5 dopamine receptors; α1A, α1B, and α1C adrenergic receptors; GAD; and CaMKII.

Conventional PCR

As a template for conventional PCR, 0.5 µL of the RT reaction was used in a Gradient 40 Robocycler™ (Stratagene). Each PCR reaction contained 10.4 µL Tris (10 mM, pH 8.4), 2 µL 10× PCR buffer (Promega, Madison, WI), 2 µL MgCl₂ (25 mM, Promega), 2 µL dNTPs (2.5 mM each, Promega), 0.4 µL bovine serum albumin (BSA) (1 mg/mL), 0.5 µL of

the RT reaction, and 0.2 µL Taq Polymerase (Promega). The cycling program used to amplify the cDNAs was 95 °C for 45 sec (1 cycle), 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min (40 cycles), and 72 °C for 10 min. Ten microliters of each PCR reaction was run on a 1.5% PCR Plus agarose gel (EM Science, Gibbstown, NJ), stained with ethidium bromide, and photographed using an Eagle Eye II imaging system (Stratagene). Pictures of the gels were digitized and edited using Photoshop® (Adobe Systems, San Jose, CA) and PowerPoint® (Microsoft Corp., Redmond, WA).

Two-step PCR

In the first step, 0.5 µL of RT reaction was used as a template for 20 cycles of PCR using the parameters described above. A cocktail of sense and antisense primers (10 pmol each) for each of the 10 genes was used to prime the PCR reaction. In the second step, 1 µL of the first step reaction was used as a template for 40 cycles of PCR using the parameters described above. A single pair of gene-specific primers was used in each reaction tube to amplify genes in the second PCR step.

Primers

The following primers were used in this study: D1 dopamine receptor (sense 5'-GAC AAC TGT GAC ACA AGG TTG AGC-3', antisense 5'-AAT ACA GTC CTT GGA GAT GGA GCC-3', expected product 609 bp), D2 dopamine receptor (sense 5'-GCA GTC GAG CTT TCA GAG CC-3', antisense 5'-TCT GCG GCT CAT CGT CTT AAG-3', expected product 404 and 317 bp), D3 dopamine receptor (sense 5'-CAT CCC ATT CGG CAG TTT TCA A-3', antisense 5'-TGG GTG TCT CAA GGC AGT GTC T-3', expected product 201 bp), D4 dopamine receptor (5'-TCA TGC TAC TGC TTT ACT GGG CCA-3', antisense 5'-TCT GAG AGA GGT CTG ACT CTG GTC-3', expected product 223 bp), D5 dopamine receptor (sense 5'-AGT CGT GGA GCC TAT GAA CCT GAC-3', antisense 5'-GCG TCG TTG GAG AGA TTT GAG ACA-3', expected product 517 bp), α1A adrenergic receptor (sense 5'-GGA GTC AGC AGT GCC AAG AAT AAG-3', antisense 5'-AGA CTG CCT TCT GCG AAG ACA CTG-3', expected product 313 bp), α1B adrenergic receptor (sense 5'-CAA TGA TGA CAA AGA ATG TGG-3', antisense 5'-CCA AGG ATA CGC ATG AAG GC-3' or 5'-GCC AAC ATA AGA TGA ACA TTC-3' "nested," expected product 524 bp or 355 bp), α1D adrenergic receptor (sense 5'-ACC CTC GAG GCA GGC ATC AAG AG-3', antisense 5'-CTT GAA CTC GGG ACT GGA GC-3', expected product 371

bp—note: a mistake in primer design was made, where the first “C” in the sense primer should be a “G”; however, the primer functioned well despite the incorrect base at this position), glutamic acid decarboxylase (GAD) (sense 5'-GTA TGA CGT CTC CTA CGA TAC AGG-3', antisense 5'-AGG AAA TCG ATG TCA GAC TGG GTG-3', expected product 426 bp), and calcium/calmodulin-dependent protein kinase II (CaMKII) (sense 5'-ACA AGA AGA ATG ATG GCG TGA AGG-3', antisense 5'-CCA GGT ACT GAG TGA TGC GGA TGT-3', expected product 354 bp). The primers for the dopamine receptors, GAD, and CaMKII were described previously.^{1,2} The genes for α 1-adrenoreceptors (α 1ARs) contain a large (~16 kb) intron between the sixth and seventh transmembrane domains.³ The primers used in this study flank the intron; therefore, α 1AR message is the only possible template for PCR product obtained from these primers using the conditions described above. As a control for DNA contamination of RNA samples, RT reactions were run in parallel in which the reverse transcriptase enzyme was left out. PCR reactions using the “no RT” reaction as a template typically resulted in either very faint or no product (data not shown).

Results

Using 2 μ g of total rat brain RNA, first-strand cDNA synthesis was primed with either oligo-(dT), random hexamer, or a cocktail of 10 gene-specific antisense primers. Using 0.5 μ L of each resulting cDNA reaction as a template, PCR was used to amplify the 10 gene transcripts of interest. PCR products of the expected sizes were obtained with all three cDNA templates, with the exception of the D3 and D4 dopamine receptor genes (Figure 1). A product using D3 primers was observed, but it was of incorrect size, and therefore may not correspond to the D3 gene (Figure 1c). Strikingly, the amount of PCR product for each set of primers differed significantly between the cDNA templates. In nearly every case, a more robust PCR product was observed with the cDNA primed with the gene-specific cocktail. This was especially true for some of the less abundant messages, such as the α 1ARs (compare Figure 1c to a and b).

To determine the sensitivity of the cocktail primed RT-PCR reaction, the RT product was diluted by a factor of 10, 100, and 1000, and PCR was performed using primers to either GAD or the α 1BAR. Detectable GAD product was obtained with all three dilutions, while α 1BAR product was detected with the 1:10 and 1:100, but not the 1:1000 dilution (Figure 2). One microliter of the 1:1000 α 1BAR PCR product was then used as a template for a PCR reaction using the same “sense” α 1BAR primer and a nested antisense α 1BAR primer. A robust product of appropriate size was generated from this reaction.

To determine whether two-step PCR reactions following RT synthesis could be generally useful for amplifying very rare messages, 0.5 μ L of either the oligo-(dT) or cocktail RT reaction was used as a template for 20 cycles of PCR with sense and antisense primers from all 10 genes in one tube (10 pmol each primer, 50 μ L total reaction volume). One microliter of the resulting product was then used as a template for a second round of PCR, with each tube containing a single pair of gene-specific primers. In this case, no nested primers were used. This strategy resulted in detectable amplification of all genes, with the exception of D4 (Figure 3). D3 of the correct size was only detected with the cocktail RT template. Similar to Figure 1, use of the cocktail RT

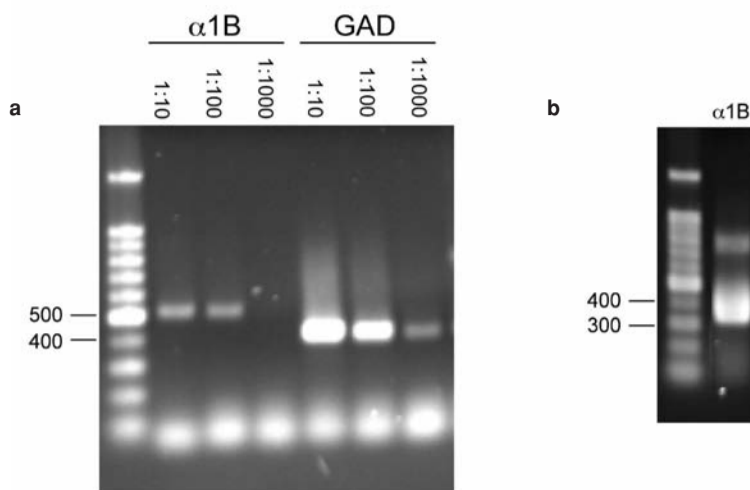


Figure 2 Sensitivity of cocktail-primed RT-PCR. a) Cocktail RT product was diluted 1:10, 1:100, and 1:1000, and was then used as a template for PCR for GAD and the α 1BAR. b) The PCR product from the α 1BAR reaction in (a) was used as a template for a second round of PCR using the same sense primer and a nested antisense primer.

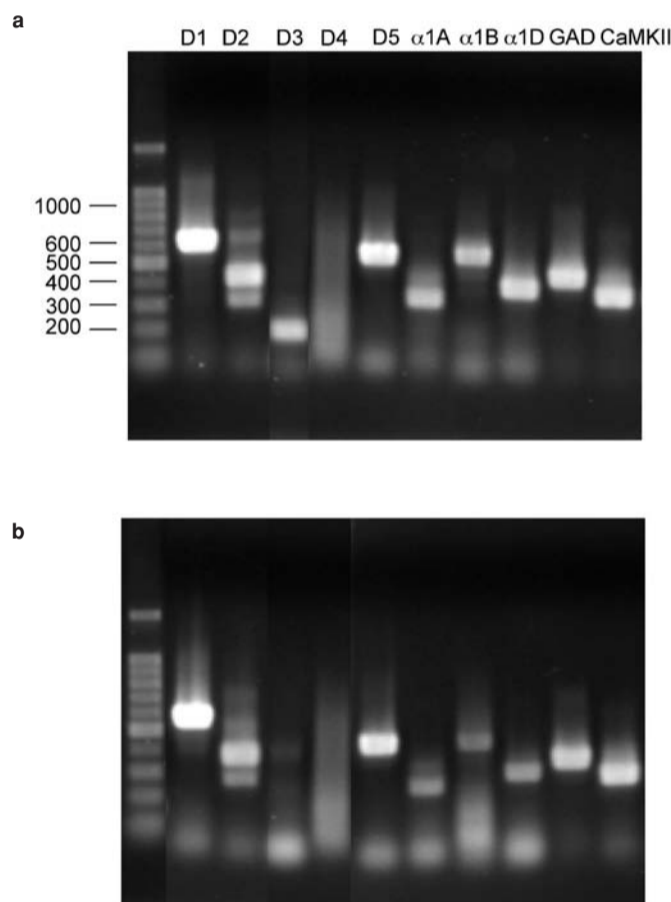


Figure 3 Comparison of two-step RT-PCR products for 10 genes using oligo-(dT) or a cocktail of gene-specific primers. A cocktail of gene-specific antisense primers (a) or oligo-(dT) (b) was used to prime first-strand cDNA synthesis; 0.5 μ L of each RT reaction was used as a template for 20 cycles of PCR containing all 10 pairs of gene-specific primers in a single reaction. One microliter of the product from that reaction was used as a template for a second round of PCR with each reaction containing a single pair of gene-specific primers.

template resulted in more robust PCR products, especially for the α 1ARs.

Discussion

There are three ways to prime first-strand cDNA synthesis, each with a unique set of advantages and disadvantages. Oligo-(dT) priming is useful because it can utilize any transcript with a poly-A tail as a template; therefore, the cDNAs one generates are typically repre-

sentative of the entire pool of mRNAs. One limitation associated with this technique is that because reverse transcription starts at the 3' end of the mRNA, 5' ends of transcripts are underrepresented. In addition, rare transcripts may be difficult to detect because the proportion of rare to common transcripts is conserved. The lack of 5' transcript ends can be addressed by using random hexamers to prime first-strand cDNA synthesis, because priming takes place wherever the hexamers can anneal and is not limited to the 3' end of the transcript. However, this technique will rarely generate full-length transcripts because the chances are slim that priming will begin at the very 3' end of the transcript. Lastly, a gene-specific primer can be used to prime first-strand cDNA synthesis. The 5' or 3' bias is up to the experimenter because the primer can be designed to anneal anywhere in the transcript.

In addition, rare transcripts can be specifically targeted by using a primer that is specific to the gene of interest. The primary caveat associated with this technique is that only one gene can be examined at a time. This study reports that by using a cocktail of multiple gene-specific primers in combination with a highly sensitive reverse transcription system, such as the Eppendorf cMaster RT, robust RT-PCR products can be generated, even from relatively rare transcripts. This technique is useful for situations in which a handful of genes are examined, especially for multiple rare transcripts.

The authors examined the expression of 10 genes in the rat brain, of which eight were amplified robustly. In theory, there is no limit to the number of primers one could use in the cocktail, but more than 20 or so probably would not be practical in terms of cost and effort. The cocktail RT reaction can be diluted at least 1000-fold and still generate PCR product from common transcripts; rare transcripts can be detected after a 100-fold dilution. It has also been shown that two-step PCR, either with or without nested primers, can further aid in amplifying rare transcripts. The cocktail technique is at least as good and probably better than oligo-(dT) priming at generating full-length transcripts, although this parameter was not examined in the present study.

References

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