Development of a Sensitive Reverse Transcriptase PCR Assay, RT-PCR, Utilizing Rapid Cycle Times

Sally S. Tan and John H. Weis

Division of Cell Biology and Immunology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132

We describe a procedure to analyze rare gene transcripts quantitatively using a reverse transcriptase-polymerase chain (RT-PCR) reaction. RNA purified from cells and tissues is reverse-transcribed using random hexamer primers and is amplified using very short cycle times. The products are trace-labeled with [³²P]dCTP, which allows for the quantitation of the products by gel electrophoresis and excision of bands. The quantity of the PCR product is directly proportional to the quantity of cDNA added and is reproducible from a single cDNA preparation or from samples derived from separate preparations. Because cDNA synthesis is primed with random oligonucleotides, the same cDNA sample preparation can be examined for many different gene products.

The analysis of transcripts that are in low abundance in the cell requires sensitive procedures. These have included Northern blots utilizing poly(A)-selected mRNA,⁽¹⁾ S1/RNase nuclease protection using DNA or RNA substrates,^(2,3) and, more recently, reverse transcriptase-polymerase chain reaction (RT-PCR).⁽⁴⁻⁸⁾ The key for any of these protocols is that they accurately detect the transcript in question in such a way that transcript levels from different sources can be compared.

Most experiments attempting transcript quantitation have used Northern blot analysis in which equal amounts of RNA are loaded per lane. After transfer of the RNA to a nylon membrane, it can then be probed successively with different gene sequences for transcript comparison. This approach, although accurate, is nonetheless time consuming and requires a significant quantity of cells or tissue if poly(A) mRNA selection is required.

Compared to the products of other genes, the transcripts from the mouse complement receptor Cr2 gene fall within the low-abundance category. Two approximately equal molar mature Cr2 transcripts (3 and 5 kb) are produced via alternative splicing by most, if not all, cells expressing Cr2.⁽⁹⁻¹²⁾ The tissue with the greatest abundance of Cr2 transcripts is the mouse spleen. Detecting Cr2 transcripts in the splenic RNA routinely requires a 2-day exposure of a Northern blot of 1 µg of poly(A) mRNA (approximately equivalent to 25 µg of total splenic RNA).^(9,10) We wanted to develop a protocol that would allow us to detect and distinguish the two different forms

of Cr2 transcripts from quantities of RNA less than 25 µg.

Several groups have described the use of quantitative RT-PCR for measuring transcript levels.⁽⁵⁻⁸⁾ These protocols utilize either murine or avian-derived reverse transcriptase and the antisense oligonucleotide from the gene of choice as the polymerase primer. The reactions are then directly amplified using standard PCR protocols and the products are analyzed. These protocols have definitely demonstrated that RT-PCR can be quantitative when the only limiting element within the reaction is the cDNA and the number of cycles is kept to a minimum. Most of the transcripts analyzed in these articles (HPRT, α-globin, *mdr-1*, etc.), however, do not fall within the low-abundance or rare transcript category.

We attempted to utilize these quantitative RT-PCR protocols for the analysis of splenic Cr2 transcripts and were generally unsuccessful. Our problems included wide day-to-day variance with the same cDNA substrate, high lane background, and a general lack of sensitivity. To increase our sensitivity, remove the variability in these experiments, and generate more specific products, we have extensively modified the standard RT-PCR quantitative protocols. With these modifications, we can routinely and reproducibly quantitate low-abundance transcripts including the Cr2 gene alternatively spliced mRNA. The most dramatic modification within this protocol includes the use of a thermocycler, which allows for a total PCR cycle of 24 sec duration (1 sec denaturation, 1 sec anneal, and 4 sec elongation).⁽¹³⁾ Thus, we will refer to the pro-

tol described in this report as reverse transcriptase-rapid polymerase chain reaction (RT-RPCR).

METHODS AND MATERIALS

Reverse Transcription

RNA was obtained using the method of Chirgwin et al.⁽¹⁴⁾ RNA concentration was determined using a spectrophotometer prior to reverse transcription. Reverse transcription was carried out using 5 µg of total RNA in a 50-µl reaction mixture containing 1× RT buffer (GIBCO-BRL, Gaithersburg, MD), 10 mM DTT (GIBCO-BRL), 0.125 mM each of the four deoxyribonucleotide triphosphates, 0.5 µg of random primers (New England Biolabs, Beverly, MA), and 400 units of Moloney murine leukemia virus (Mo.-MLV) reverse transcriptase (GIBCO-BRL). After 60 min incubation at 37°C, 2 µg of DNase-free RNase was added and incubated for 5 min at 37°C. Reaction volume was adjusted to 270 µl with 0.4 M NaCl, followed by a phenol/chloroform, chloroform extraction, and precipitated with ethanol at -20°C overnight. The cDNA concentration was measured using a spectrophotometer.

cDNA Amplification

PCR was performed with adaptations from C. Wittwer's procedure for rapid cycling with the air thermocycler.⁽¹³⁾ Optimal cDNA concentration and number of cycles for amplification of Cr2 transcripts were determined by a titration from 1–500 ng of cDNA and from 18–39 cycles. Optimal parameters were determined to be 200 ng of cDNA for 20 cycles except where noted. Each 10-µl reaction contained 200 ng of cDNA, 70 pmoles each primer (primers ranged from 20 to 21 nucleotides), 1× reaction buffer [10× stock consisted of 500 mM Tris (pH 8.3), 30 mM MgCl₂, 200 mM KCl, and 5 mg/ml BSA), 0.8 mM dNTPs, 2.5 µCi [³²P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA), and 0.72 units of AmpliTaq DNA polymerase (5 U/µl, Cetus, diluted 1:7]. For consistency between samples, a master mix of 1× reaction buffer, dNTPs, [³²P]dCTP, and AmpliTaq was prepared and aliquoted to separate tubes containing each set of primers (see Table 1:Cr2,43,45,48; β-actin, 61,62). These mixtures were then aliquoted to the cDNA samples. Each 10-µl

reaction was added to glass microcapillary tubes (#1705, Idaho Technology, Idaho Falls, ID), the ends sealed, and the tubes inserted into the 1605 Air Thermocycler (Idaho Technology). PCR conditions were: denaturation, 94°C for 1 sec; annealing, 59°C for 1 sec; and elongation, 72°C for 4 sec. The total time elapsed for a single PCR cycle was 24 sec.

Analysis of PCR Products

Following PCR, the ends of the microcapillary tubes were scored and samples were removed with a Captrol microaspirator. All 10 µl of sample was added to an equal volume to stop solution, heated to 95°C for 5 min, and 5 µl was electrophoresed in a 6% acrylamide gel.⁽¹⁵⁾ Radiographic bands were detected by autoradiographic exposure for variable times at -70°C and cut from the gel for quantitation by liquid scintillation. Sizes of bands were estimated by the migration of ³²P-end-labeled *MspI* digest of pBR322.

RESULTS

Development of the RT-RPCR Protocol

Most quantitative PCR protocols rely upon a single reaction vial containing total or poly(A) mRNA, reverse transcriptase, DNA polymerase, the PCR primers, trace levels of a radionucleotide to be incorporated within the amplified product, and the usual buffers to support both the RT and PCR reaction.^(5–8) These reaction mixes are then incubated at 37°C for a set time period (15–30 min) and followed immediately by PCR amplification. The products from such an amplification are analyzed by agarose or polyacrylamide electrophoresis.

Two major drawbacks are evident within such an RT-PCR assay. First, the PCR primers are used for cDNA production. Because the RT incubation is done at 37°C (or with avian RT at 43°C), utilizing the PCR primers increases the probability of synthesizing nonspecific cDNAs via inappropriate oligo binding to related sequences. These cDNAs can then be inappropriately amplified during the PCR stage. The second major drawback is that if the level of one transcript is to be compared to that of a second or third, such reactions must be set up in parallel and be assumed to be

equivalent not only in cDNA production but in PCR amplification as well.

To devise a quantitative PCR protocol, it is essential that the only limiting element in the reaction be the quantity of cDNA and that if a number of different primers are to be used, the cDNA in each reaction be equivalent.^(5–8) Our approach to such a protocol is detailed in Materials and Methods, but the following points can be made. We synthesized total cDNA from purified total RNA using random primers to ensure that some cDNAs will not be underrepresented due to primer bias. Also, because we use total RNA for cDNA synthesis instead of poly(A) mRNA, there is no bias for smaller mRNA species versus larger forms. In quantitating the PCR products, each reaction must be limited only in the quantity of cDNA and have saturating quantities of dNTPs, primers, and enzyme. In conventional heating block thermocyclers, because of their relatively long amplification cycle time (4–7 min), we found that the enzyme was largely denatured within the first dozen or so cycles.⁽¹⁶⁾ Although the enzyme may be active enough for the detection of abundant mRNA species, our initial results with Cr2 primers to detect the relatively rare Cr2 mRNA forms were mixed and disappointing. However, by using an air cycler machine that allowed for a single cycle of 24 sec⁽¹³⁾ (denature by heating to 94°C, immediately drop to 59°C for oligo annealing, immediately climb to 72°C for an extension time of 4 sec and back up to 94°C) the PCR amplification could be completed before the enzyme activity decayed.

RT-RPCR Strategy for cDNA Amplification of Alternatively Spliced Cr2 Transcripts and Detection of the Products from Splenic cDNA.

To detect Cr2 transcripts using the RT-RPCR protocol, three oligonucleotides were derived from coding sequences (Fig. 1A; Table 1). Oligo 43 was derived from the signal sequence and is present in both the 3- and 5-kb Cr2 transcripts. Oligo 48 is found only in the 5-kb transcript and with oligo 43 produces a product of 123 bp. Oligo 45 is found within short consensus repeat #7 of the Cr2 gene and produces a product of 162 bp with oligo 43. The sequence of oligo 45 is also present within the 5-kb transcript

FIGURE 1 PCR strategy for cDNA amplification of alternatively spliced Cr2 transcripts using oligos 43, 45, and 48. (A) Positions of oligos 43 and 48 for amplification of the Cr2-190 encoding mRNA and oligos 43 and 45 for the amplification of the Cr2-145 encoding mRNA. PCR products are 123 bp and 162 bp, respectively. (B) Specificity of the Cr2 PCR oligos was evaluated by an amplification of oligos, either alone or in sets with Cr2 cDNAs.⁽¹⁰⁾ Lanes 1–7 utilized the 4-1 Cr2 cDNA specific for the Cr2-145 protein and lanes 8–14 utilized the 31-1 Cr2 cDNA specific for the Cr2-190 protein. PCR oligos used were: (lanes 1 and 8) oligo 43 alone; (lanes 2 and 9) oligo 45 alone; (lanes 3 and 10) oligo 48 alone; (lanes 4 and 11) oligos 45/48; (lanes 5 and 12) oligos 43/45; (lanes 6 and 13) oligos 43/48; (lanes 7 and 14) oligos 43/45/48. Product is only evident in lanes 5, 7, 13 and 14.

TABLE 1 PCR Primers for Cr2 and β -Actin Gene Sequences

Primer	Sequence	Description
43	5'-ATGGGATCCTTGGGTTTCGCTC-3'	Cr2 5' signal sequence primer
45	5'-GCTAGGTGAACAAGTGACCT-3'	3' turnaround of Cr2-145 (SCR #7)
48	5'-CTCAGATTATCACTCACAAT-3'	3' turnaround of Cr2-190 (SCR #1)
61	5'-GGGTCAGAAGGACTCCTATG-3'	5' β -actin sequence
62	5'-GTAACAATGCCATGTTCAAT-3'	3' β -actin sequence

but is far enough from the oligo 48 site that only the 43/48 product is produced.

The specificity and product size of the PCR products generated by oligos 43, 48, and 45 are shown in Figure 1B. When the 4-1 cDNA specific for the 3-kb Cr2 transcript was used with these primers, only the 43/45 combination produced the expected product of 162-bp (Fig. 1B, lanes 5 and 7). Additionally, when the 31-1 cDNA specific for the 5-kb Cr2 transcript was used, the expected product of 123-bp was generated from oligos 43/48 (lanes 13 and 14).

When the oligo set described above was used to prime mouse spleen cDNA, PCR products specific for the 5-kb and 3-kb mRNA were observed (Fig. 2). The sizes of the different products were exactly those seen in the plasmid DNA from Figure 1. [When RNA alone, the negative control, was used in this assay (lane 5), no products were present.]

Optimizing RT-RPCR: Cycle Number and Quantity of cDNA

For such a PCR protocol to be quantita-

FIGURE 2 Cr2 gene products from splenic cDNA. Amplification of 100 ng of splenic cDNA for 24 cycles with Cr2 oligos set 43, 45, and 48. (Lane 1) 43/45/48; (lane 2) 45/48; (lane 3) 43/48; (lane 4) 43/45; (lane 5) 43/45/48 using spleen RNA as negative control. The Cr2 products of 145,000 M_r and 190,000 M_r are identified.

tive, it is important that with increasing cycles the amount of product continues to increase. As shown in Figure 3, Cr2-specific products were just visible at 18 cycles and continued to accumulate throughout the duration of the experiment (up to 39 cycles). We have not seen any increase in PCR product if the cycle times were lengthened at any of the three time points (denaturation, annealing, or elongation) (data not shown). Note that with the increase of cycles that the fidelity of the product diminished. The products in this experiment were quantified by excising the gel fragments and determining the cpm (data not shown). Starting from cycle 18 to 24, the products produced were linear on a logarithmic scale and had begun to reach saturation by 27 cycles (see below).

Because these PCR reactions are designed to be quantitative, the amount of cDNA added should directly control the amount of product. As shown in Figure 4, with increasing quantities of spleen cDNA ranging from 1 ng per reaction to 500 ng there is direct correlation of prod-

FIGURE 3 Increasing cycles increases Cr2 gene PCR products. Amplification of 100 ng of splenic cDNA with increasing PCR cycles with Cr2 oligos 43/45/48. (Lane 1) 18 cycles; (lane 2) 21 cycles; (lane 3) 24 cycles; (lane 4) 27 cycles; (lane 5) 30 cycles; (lane 6) 33 cycles; (lane 7) 36 cycles; (lane 8) 39 cycles.

FIGURE 4 Cr2 gene PCR products increase with increasing cDNA. Amplification of varying quantities of splenic cDNA with Cr2 oligos 43/45/48. All reactions were done for 24 cycles. (Lane 1) 1 ng of cDNA; (lane 2) 10 ng of cDNA; (lane 3) 50 ng of cDNA; (lane 4) 100 ng of cDNA; (lane 5) 500 ng of cDNA.

uct increase with input cDNA. This indicated that, for the range of cycles examined, the amount of Cr2 product generated from the PCR step was directly dependent upon the quantity of cDNA added.

The two previous experiments were merged into one by analyzing the quantity of product produced using the Cr2 3-kb specific primers for varying cycle numbers and with varying quantities of cDNA. As shown in Figure 5, either 50, 250, or 500 ng of splenic cDNA was used for the amplification steps over a range of cycles. This experiment indicated that for the first 10 cycles monitored (15–25) the quantity of product was linear. Interestingly, although all three sets of reactions reached saturation from cycle 30 on, the final amount of product generated was dependent upon the initial quantity of cDNA added.

Competition Between Oligonucleotide Sets During RT-PCR

One advantage of generating the cDNA

FIGURE 5 Quantitation of PCR products generated from increasing amounts of splenic cDNA. Splenic cDNA was analyzed with the Cr2 3-kb specific oligonucleotides for varying cycles (15, 18, 21, 24, 27, 30, 33, and 36 cycles). Samples amplified contained either 50 ng ($\square$), 250 ng ($\triangle$), or 500 ng ($\circ$) of splenic cDNA. Eight identical samples were prepared with the same quantity of cDNA and were sequentially removed from the cyclor every third cycle. After electrophoresis and autoradiography, bands were excised from the gel and quantitated by liquid scintillation. Negative control values of amplification of RNA were subtracted as background.

with random primers is the ability to use a number of different primers with a single cDNA preparation. In one cDNA reaction using 5 μ g of total RNA, we usually obtain 7–10 μ g of cDNA. Since each reaction employs only 100–200 ng of cDNA, this would allow from 50 to 100 different PCR reactions with different gene-specific primer combinations to be performed on the same sample. An example of this approach is shown in Figure 6. Primers were developed for the mouse β -actin transcripts (Table 1) and used alone or with the CR2 primers for 18 or 24 cycles. When the β -actin and Cr2 primers were added to one reaction (lanes 3 and 5), all of the expected products were apparent although with some diminution of each of the species compared to when added alone (lanes 1, 2, 4, and 6). Thus, when β -actin primers are used to help quantitate the cDNA products, each primer set is analyzed in its own PCR tube. Since the quantity of β -actin mRNA is much higher than Cr2 mRNA in splenic RNA, a cycle titration with splenic cDNA was also performed with the β -actin oligos (Fig. 7). The quantity of the β -actin product was also proportional to the number of cycles. However, the actin product was seen with as few as nine cycles (lane 2).

FIGURE 6 Effect of amplifying two different gene products in one reaction. PCR analysis of 100 ng of splenic cDNA with Cr2 (43/45/48) and β -actin oligos. Lanes 1–3 were done for 18 cycles. (Lane 1) Cr2 oligos alone; (lane 2) β -actin oligos alone; (lane 3) Cr2 + β -actin oligos. Lanes 4–6 were done for 24 cycles. (Lane 4) Cr2 oligos alone; (lane 5) Cr2 + β -actin oligos; (lane 6) β -actin oligos alone.

Reproducibility of the RT-RPCR Protocol

The last parameter in the development of RT-RPCR assay was to determine its reproducibility. That is, for any set of samples that have identical quantities of Cr2 mRNA, the PCR products should reflect this. Thus, if the RNAs from the spleen and liver of three different mice were obtained and analyzed, they should have comparable quantities of Cr2 and β -actin transcripts. As shown in Figure 8A, when splenic cDNA from three different mice was analyzed with the Cr2 oligos (lanes 1–3), each PCR product was equivalent. This was also evident when the same samples were examined with β -actin primers (lanes 7–9). In the liver samples examined, only slight levels of Cr2 transcripts were evident but equal amounts of β -actin products were seen.

The second test for reproducibility was that for any single cDNA preparation it should be uniform for the percentages of various gene sequences. That is, if a

FIGURE 7 β -Actin RT-PCR products increase with cycle number. Amplification of 100 ng of splenic cDNA with increasing PCR cycles with β -actin oligos. (Lanes 1–9) from 6 to 33 cycles with increments of 3.

FIGURE 8 Reproducibility of PCR amplification for quantitation of products. (A) PCR analysis for 24 cycles with Cr2 oligos (lanes 1–6) and β -actin oligos (lanes 7–12) with 100 ng of cDNA generated from three different spleens (lanes 1–3 and 7–9) and livers (lanes 4–6 and 10–12). (B) PCR analysis for 24 cycles with Cr2 oligos (lanes 1–3) and β -actin oligos (lanes 4–6) with 100 ng aliquots from the same splenic cDNA sample.

single cDNA preparation was used for three different Cr2 PCR assays and three different β -actin reactions, the type and quantity of the products should be the same for each oligo set. As shown in Figure 8B, when such an experiment was performed, the quantities of the PCR products were indeed comparable. Thus, this assay was reproducible both for mRNA/cDNA content and reaction products.

DISCUSSION

In this report we have detailed a modification of the now standard RT-PCR protocol.⁽⁵⁻⁸⁾ We have varied our RT-PCR from the previously published methods to increase the sensitivity of the reactions and to optimize the PCR product. The RT-PCR protocol is as follows:

1. Total RNA is reverse-transcribed using the recombinant Moloney viral enzyme and using random hexamers as primers. This step ensures that all RNAs are represented equally in the cDNA pool. The utility of such primers to synthesize cDNA has been amply demonstrated in the production of cDNA libraries where uniform cDNA production is essential. In this reaction, 5 μ g of total RNA is used producing from 5 to 10 μ g of RNA-free cDNA.
2. A total of 100–200 ng of cDNA is amplified using saturating quantities of primers, dNTPs, buffer, and enzyme. Trace [³²P]dCTP is added to label the product.
3. To ensure that the enzyme remains active throughout the entire amplification process (i.e., the quantity of enzyme remains saturating), a very rapid PCR cycle is used. Contrary to the accepted norm, the enzyme is fully capable of producing PCR products of 50–500 bp within the described 24-sec cycle.
4. The PCR products are visualized by acrylamide electrophoresis. After autoradiography, the bands can be excised from the gel. Alternatively the gel can be directly scanned and the bands quantitated by a phospho-imager.

The RT-PCR protocol is very reproducible from one day to the next either using the same cDNA preparation or ana-

lyzing cDNA samples prepared from identical sources. The sensitivity of the protocol is such that the isolation of poly(A) mRNA is not necessary. The amount of product is directly proportional to the quantity of cDNA added into the reaction, as long as only the cDNA is limiting.⁽¹⁶⁾ Thus, for the early cycle numbers the assay is quantitative.

To compare the relative levels of different transcripts within a single cDNA preparation, it is important to know approximately how many transcripts from a single gene are represented in the cDNA sample compared to a control transcript such as β -actin. Thus, for any cDNA preparation made from mouse spleen or lymph node RNA, we know that actin transcripts can be detected quantitatively from 11 to 16 cycles, and the Cr2 transcripts from 18 to 22 cycles per 200 ng of input cDNA. Thus, any increase or decrease of β -actin or Cr2 transcripts in other cDNA samples can be easily detected within the ranges of cycles described.

Although this report details the analysis of β -actin and Cr2 transcripts, we have used this protocol to measure quantitatively transcripts from the murine β -7, β -1, α -4 integrins, Crry, Fc ϵ 1 (α , β , and γ subunits), Oct-1, and Oct-2 genes.¹⁷⁻¹⁹ For most tissues, the range of quantitative amplification for these gene transcripts is between 18 and 24 cycles using 200 ng of cDNA. The entire protocol can be accomplished easily in 1 day. The usual exposure time of the labeled gel is 12 hr. For the majority of our analyses, we design the oligonucleotide sets to produce PCR products ranging from 80 to 200 bp, and to contain approximately the same number of C residues. Practically speaking, this means that approximately the same exposure time will allow for the detection of the range of products produced from different cDNA samples and gene-specific oligonucleotide sets.

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