EXHIBIT D

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AMPLIFICATION OF RNA

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Sensitive methods for the detection and analysis of RNA molecules are an important aspect of most cell/molecular biology studies. Methods commonly in use include *in situ* hybridization, Northern gels, dot or slot blots, S-1 nuclease assays, and RNase A protection studies. Detailed descriptions of these techniques can be found in several laboratory manuals (Davis *et al*. 1986; Ausubel *et al*. 1987; Berger and Kimmel 1987). The most sensitive of these methods is *in situ* hybridization, in which 10 to 100 molecules can be detected in a single cell. However, the *in situ* hybridization method can be technically difficult and does not lend itself to the processing of a large number of samples. With other techniques, the level of detection is about 0.1 to 1.0 pg of the target sequence. For an average-sized mRNA this translates to 10^5 to 10^6 target sequence molecules, and for most practical purposes the detection limit has been reached.

Adaptations of PCR technology have provided a breakthrough in the area. Seeburg and co-workers were the first to describe (P. Seeburg *et al*. 1986, UCLA Symposium, unpublished) the use of PCR to amplify mRNA sequences from cDNA. The first published

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description of the technique was in 1987 (Veres *et al.* 1987), when it was used to study point mutations in the mouse ornithine transcarbamylase gene by using subclones derived from an amplified segment of the mRNA. This simple modification of the PCR method has not only increased the sensitivity of detection by several orders of magnitude (with the exception of *in situ* hybridization) but has also made possible the sequence analysis of RNA starting with extremely small amounts of template. In this chapter we describe how RNA sequences are amplified by the use of combined complementary DNA (cDNA) and PCR methodologies.

Protocols

Use autoclaved tubes and solutions wherever possible and wear gloves to minimize nuclease contamination from fingers.

Reagents and Supplies

10× PCR buffer: 500 m*M* KCl, 200 m*M* Tris–HCl (pH 8.4) at 23°C, 25 m*M* $MgCl_2$, and 1 mg/ml of nuclease-free bovine serum albumin.

Deoxynucleotide triphosphates: Neutralized 100 m*M* solutions from Pharmacia. The dNTPs are combined to make a stock solution (10 m*M* each dNTP) by using 10 m*M* Tris–HCl (pH 7.5) as diluent.

RNasin: RNase inhibitor from Promega Corporation at 20 to 40 units/μl.

Random hexamer oligonucleotides: 100 pmol/μl solution in TE [10 m*M* Tris–HCl, 1 m*M* EDTA (pH 8.0)]. Hexamers can be purchased from Pharmacia.

PCR primers: 18 to 22 bases in length, 10 to 100 pmol/μl in TE.

Reverse transcriptase: Moloney murine leukemia virus (MoMuLV) from Bethesda Research Laboratories (BRL) at 200 units/μl. Other reverse transcriptases can be used. See "Discussion and Helpful Hints."

Taq polymerase: 5 units/μl from Perkin-Elmer Cetus.

Light white mineral oil: Sigma

Chloroform: Any reagent grade saturated with TE.

NuSieve and ME agarose: Obtained from FMC Corporation.

TEA electrophoresis buffer: 40 m*M* Tris–HCl, 1 m*M* EDTA, and 5 m*M* sodium acetate (pH 7.5).

DNA markers: 123-bp or 1-kb ladder from BRL.

Microfuge tubes: Use only tubes specified for use in the Thermal Cycler. This is an important point, because ill-fitting tubes will result in inconsistent and inefficient amplifications.

Reverse Transcriptase Reaction

Assemble the following reagents in a final volume of 20 μl 1× PCR buffer: 1 m*M* each dNTP, 1 unit/μl of RNasin, 100 pmol random hexamer, 1 to 5 μl of RNA sample (amount will be variable), and 200 units of MoMuLV reverse transcriptase. Incubate 10 minutes at 23°C, and then 30 to 60 minutes at 42°C. Heat the reaction at 95°C for 5 to 10 minutes in a water bath and then quick-chill on ice. The heat treatment denatures the RNA–cDNA hybrid and inactivates the reverse transcriptase. It may be helpful to heat treat the RNA sample at 90°C for 5 minutes and quick chill on ice before addition to the reaction; presumably this breaks up aggregates and some secondary structures that may inhibit the cDNA priming step.

PCR Amplification

To the heat-treated reverse transcriptase reaction add 80 μl of 1× PCR buffer containing 10 to 50 pmol each of upstream and downstream primer and 1 to 2 units of *Taq* polymerase. To prevent evaporation of liquid during thermal cycling, layer 100 μl of mineral oil on top of the PCR solution. The number of PCR cycles required depends on the abundance of the target. Usually, somewhere between 20 and 50 cycles is used, but the optimal number should be determined in each case. A thermal cycle profile that works well for amplification of a target of more than 500 base pairs is (1) denaturing for 30 seconds at 95°C, (2) cooling over 1 minute to 55°C, (3) annealing primers for 30 seconds at 55°C, (4) heating over 30 seconds to 72°C, (5) extending the primers for 30 seconds at 72°C, (6) heating over 1 minute to 95°C, and so on. Variations in this thermal cycle profile will work just as well, but one should be careful about the

times allotted for heating and cooling. It is essential that enough time be allowed for the solutions in the tubes to equilibrate to the correct temperatures; otherwise, the amplification efficiency may be very low.

Analysis of Amplification Products

After amplification, the mineral oil is removed by extraction with 200 to 300 μl of TE-saturated chloroform. The upper (aqueous) phase is saved, and 5 to 10 μl is used for analysis in a 3% NuSieve–1% ME agarose composite gel made in TEA buffer. An 8 or 10% polyacrylamide gel in TBE buffer is also suitable. Use the 123-bp or 1-kb ladder as a convenient marker for size estimates of the products. Stain the gel with ethidium bromide and photograph. Other analytical methods such as Southern gels or dot/slot blots can be done at this point. Detailed descriptions of these standard methodologies can be found in Davis *et al.* (1986), Ausubel *et al.* (1987), and Berger and Kimmel (1987). Further manipulations of the PCR product such as subcloning (Chapter 11) and direct sequencing (Chapter 24) are discussed elsewhere in this book and will not be described here.

Discussion and Helpful Hints

The same buffer is used for both the reverse transcription reaction and the PCR. We have found that the use of PCR buffer throughout does not seem to negatively affect the efficiency of amplification. Although this has simplified the protocol somewhat, one should be cautious if trying to make long cDNA products. We have not rigorously tested the PCR buffer for this purpose.

First-strand cDNA synthesis may be accomplished by extension with random hexamers, the downstream primer, or oligo(dT). In many cases, it does not seem to matter which priming method is used. With the downstream primer, we have successfully used from 5 to 100 pmol in the cDNA reaction. If oligo(dT) is used, 0.1 to 0.2 μg works well. Figure 1 shows the results of an experiment where

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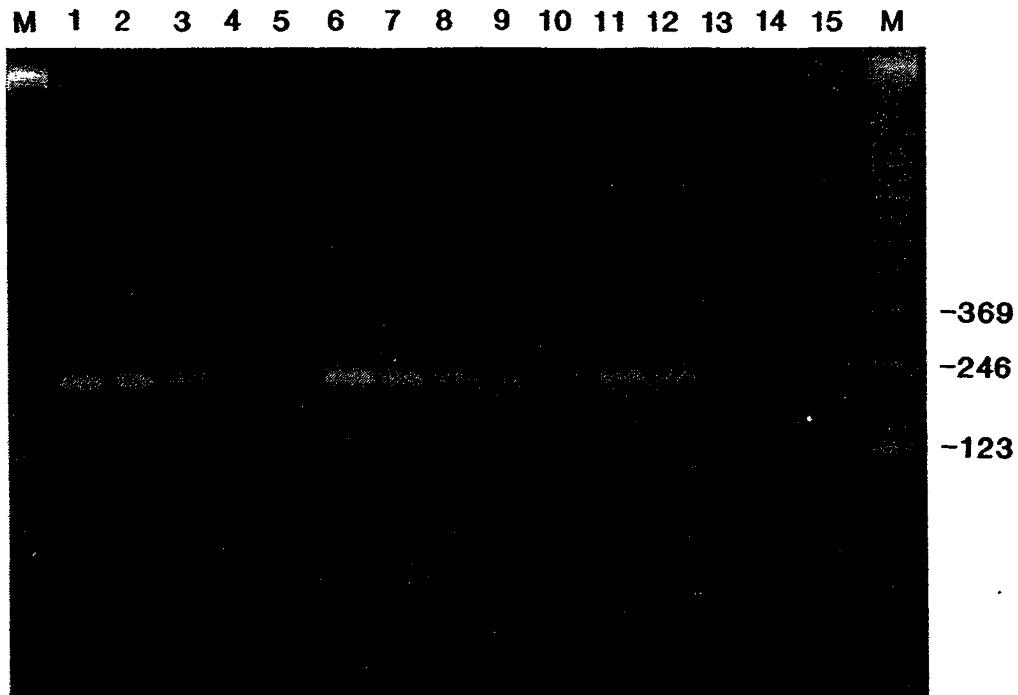

Figure 1 Comparison of random hexamers, downstream primer, and oligo(dT) in the cDNA PCRs. Samples in lanes 1–5 were amplified starting with random hexamers, lanes 6–10 with downstream primer, and lanes 11–15 with oligo (dT). Lanes 1–5 represent amplified cytoplasmic MoMuLV RNA from 50,000, 5000, 500, 50, and 0 cells, respectively. Lanes 6–10 and 11–15 represent the same dilution series but using the different first-strand primers.

the same RNA target (*Pol* gene of MoMuLV) was amplified after reverse transcription with random hexamers (lanes 1–5), downstream primer (lanes 6–10), or oligo(dT) (lanes 11–15). Lanes 1 through 5 represent amplifications starting with viral RNA from 50,000, 5000, 500, 50, and 0 cells, respectively. Lanes 6–10 and lanes 11–15 contain correspondingly the same amounts of starting RNA. As shown, the random hexamer reactions were the most efficient, containing the highest amount of final amplified product. In general, we and others (Veres *et al.* 1987; Noonan and Roninson 1988) have found that the random hexamer approach is the most consistent and results in the highest amplification of target sequence.

The source and type of reverse transcriptase do not seem to be of critical importance. We have used MuLV and AMV enzymes from BRL and Boehringer Mannheim Biochemicals with good results, so enzymes from any reputable source should do as well. For our own research, we have used only the *Taq* polymerase from Perkin-Elmer Cetus, so we cannot comment on the performance of other thermostable polymerases.

It may be useful to titrate the primer oligonucleotides in the PCR to find the smallest amount that can be used to give a "good" amplified product. We have found that a large excess of primer usually gives more extraneous amplified products, which hinders subsequent analysis. As little as 5 pmol of primer pairs has been used to give a very clean amplification. However, it is best to optimize the amount for each sequence you wish to amplify.

The concentration of each dNTP in the PCR should not exceed 0.2 m*M*. This is the reason for the fivefold dilution of the reverse transcriptase reaction; 1 m*M* of each dNTP is lowered to 0.2 m*M*. As little as 0.02 m*M* can work in PCR, but this should be checked for each case. Triphosphate concentrations higher than 0.2 m*M* increase the misincorporation rate or mutation frequency for *Taq* polymerase. This point is crucial if one wishes to subclone a cDNA sequence.

The magnesium concentration is also critical, so care should be taken that the addition of reagents does not lower the magnesium molarity; i.e., some nucleic acid buffers contain 1 m*M* EDTA, and this can chelate out much of the magnesium. In general, try to keep the free magnesium concentration at about 2 m*M*.

Use the smallest number of PCR cycles that gives you the "cleanest" result. More cycles often just give you more nonspecific amplification products. Also, with a large number of amplification cycles, you may run into contamination problems. You may start picking up false positives because of the presence of extremely small amounts of unwanted target sequences.

We have not discussed methods for RNA extraction because detailed descriptions of the latest protocols are described in several laboratory manuals (Davis *et al.* 1986; Ausubel *et al.* 1987; Berger and Kimmel 1987). Purified RNA usually gives the best results, but if the highest sensitivity is not required, protease- or diethylpyrocarbonate-treated samples will work in this system (see Chapters 18, 19, and 20).

Usually, 1 μg of cytoplasmic RNA is sufficient for amplification of rare mRNA sequences (1 to 10 copies per cell). Since a "typical" mammalian cell may contain ~10 pg RNA per cell cytoplasm, 1 μg represents the RNA from about 100,000 cells. Thus the number of target sequences in 1 μg is probably greater than 100,000 and should be easily amplifiable. Therefore, the isolation of poly(A)$^+$ RNA is not required in cases where one is analyzing a homogeneous cell population. In fact, the time-consuming step of mRNA purification should rarely, if ever, be necessary, since one can detect specific mRNA se-

quences from the equivalent of 1 to 1000 cells (Kawasaki *et al.* 1987; Rappolee *et al.* 1988).

Choose your PCR primers to be about 18 to 22 bases in length and not too high or low in G+C content. If you are studying eukaryotic mRNAs, try to use primers derived from separate exons; this will inhibit amplification of any contaminating genomic DNA sequences. When the genomic structure is not known, use primers separated by 300 to 400 bases in the 5′ portion of the coding region. Exons larger than 300 bases in this area are fairly rare in vertebrates (Hawkins 1988), so the primers will have a good chance of residing in separate exons. If the gene in question has no introns, or you are studying bacterial mRNA, RNA viruses, RNA transcripts from viral integrates, etc., a thorough DNase treatment of the RNA will probably be necessary to obtain meaningful PCR results. It requires only a minuscule amount of contaminating genomic DNA to give a false-positive signal in this type of assay.

In summary, the RNA-PCR technique is a powerful new method for the analysis of RNA transcripts. Experiments that were previously extremely difficult or even impossible, such as mRNA quantitation from single cells, can now be easily performed by this modified PCR procedure.

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