The Basics of RT-PCR

Some Practical Considerations

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1. Introduction

The basic reverse-transcriptase-polymerase chain reaction (RT-PCR) technique is used routinely and widely in most biomedical research laboratories. The fundamental considerations for such basics as primer design, good laboratory set up and practice, and techniques for performing RT-PCR have been fully examined elsewhere (1,2). Thus, I will include only observations from my own experience in this chapter. Although the purpose of this chapter is to help the reader in setting up their own PCR assays, and to provide tips on effective PCR laboratory set up, it should be noted that each individual chapter in this volume contains complete experimental detail on the protocols described.

2. RNA ISOLATION: “Home-Made” Preps vs Commercial Kits

In-house techniques for RNA isolation have been widely employed. These involve homogenizing tissue specimens in a guanidine thiocyanate lysis buffer, followed by phenol extraction and alcohol precipitation (for example, see Chapter 7) (3). A number of kits for RNA extraction are now commercially available; these usually obviate the need for phenol extraction, instead involving spun-column chromatography or magnetic bead separation to purify the RNA. As such, these methods are considerably more rapid and convenient than the in-house methods. However, it should be noted that most techniques for quick RNA isolation, whether in-house or using commercially available kits, result in some contamination with genomic DNA. The best way to purify RNA with-
out DNA contamination is probably by ultracentrifugation of RNA lysates through cesium chloride density gradients (4), a technique that is obviously not suited to routine requirements for RT-PCR. The simplest solution to this problem is the use of intron-spanning primers for the RT-PCR, so that PCR products derived from the mRNA are easily distinguishable from the larger, intron-containing products derived from contaminating genomic DNA. If the target region of mRNA to be amplified does not contain introns, DNA should be eliminated from the RNA, using RNase-free DNase prior to performing RT-PCR. It is usually advisable to repeat the RNA extraction to remove the DNase, which could otherwise hinder cDNA synthesis.

3. Primers for cDNA Synthesis: Oligo-dT, Random Hexamers, or Gene-Specific

An additional factor for consideration in RT-PCR is the choice of primer to synthesize the cDNA. Although oligo (dT) primers may seem to be the best choice, since they will specifically prime from the poly A tail of mRNA, it should be noted that most mammalian mRNAs contain extensive non translated regions (usually from one to several kb) downstream from their coding regions. If PCR primers are located in the coding region, particularly near the 5' end of the mRNA, then the use of oligo (dT) primers necessitates that full-length, or nearly full-length cDNA copies are synthesized from the 3' terminus of the mRNA. Because of secondary structures in mRNA, synthesis of such long cDNAs is not always effective. However, random hexanucleotide primers prime randomly from sites throughout the mRNA, making it more likely that 5' sequences will be represented in the resultant cDNA. Alternatively, a gene-specific primer can be synthesized which will prime only from the target mRNA, so that the cDNA is enriched for the cDNA of interest prior to PCR. This may be particularly useful when the target mRNA is at a low copy-number. Although the anti-sense PCR primer can also be used for cDNA priming, a better approach is to synthesize a separate primer immediately downstream from the anti-sense PCR primer site. The advantage of this method is that the PCR primers will be “nested” relative to the cDNA primer; thus, any nonspecifically primed cDNA should not be amplified during PCR. Because cDNA synthesis is usually performed at relatively low temperatures (e.g., 37°C or 42°C), the specific primer sequence should be shorter than a typical PCR primer, so that its annealing temperature is not considerably higher than the cDNA synthesis temperature; this minimizes non specific priming. For most applications, random hexamers work well, and offer the particular advantage that a single cDNA prep. can be used for PCR detection of many different cDNAs.
4. Primer Design

As previously mentioned, the best strategy for RT-PCR is to select primers that span intron(s), so that mRNA-specific PCR products are obtained. For this, either a complete or partial genomic sequence is required, which indicates the position of at least one intron in the gene. Performing an alignment between the genomic and cDNA sequences may be helpful for this purpose. Even if the genomic sequence is not available, any reasonably sized target region within the mRNA (300–400 bp) has a reasonable probability of spanning an intron. This can be tested empirically by performing PCR with the primers using genomic DNA as a template, to determine whether the product is larger than that obtained from cDNA. (Note: since introns can be large, frequently > 1 kb, intron-spanning primers frequently do not amplify the larger genomic sequence efficiently, and no product is obtained from genomic DNA).

In the early days of PCR, primers were often selected on a fairly random basis (the “let’s stick two forks in the sequence” approach). Primers selected in this way often worked very well. More recently, computer programs have become available that are designed to select the most efficient primers within a given sequence. These programs incorporate a number of theoretical considerations that determine the optimal primer pairs, including:

1. Minimal self-complementarity between both primers in the pair, or within each individual primer; self-complementarity (leading to dimers or hairpin loops) can yield a high level of primer artifacts during PCR.

2. The primers should have a matched annealing temperature. If the optimal annealing temperature for primer 1 is substantially lower than that of primer 2, PCR may need to be performed at an annealing temperature well below the optimal annealing temperature for primer 2; this could allow mis-priming by primer 2.

3. The primers should have a good stability of binding to their target sequences; also, in theory, there should be a gradient of increasing binding strength from the 3’ to the 5’ end. Even transitory binding of the 3’ end of a primer to a non identical target sequence can lead to non-specific priming. By keeping the strength of binding at the 3’ end low (i.e., a relatively [AT]-rich sequence), it is believed to bind only to a perfectly matched target sequence, and the stronger affinity of the 5’ end (i.e., a relatively[GC]-rich sequence) will clamp the primer in place.

4. There should be a good difference between the calculated annealing temperature of the primers and the annealing temperature of the PCR product; this favors binding of the primers to their target sequences over re-annealing of the PCR product itself.

The program will usually select and rank multiple potential primer pairs. Select the best option that spans an intron in the genomic sequence.
A useful analysis to perform at this point is a homology search for the selected primer sequences against a sequence database (e.g., perform a BLAST search of the Entrez Nucleotide database, which incorporates sequences from the GenBank database; this service is available on the Entrez-PubMed website at: http://www.ncbi.nlm.nih.gov/PubMed). This will identify any homologous sequence that the primers may also amplify (such as a closely related member of a gene family). Some homology with other genes is acceptable, particularly if the region of homology does not involve the 3' end of the primer; a perfect match at this end can lead to nonspecific priming, whereas even a single nucleotide difference at the 3' end will render it unlikely to prime the nonidentical target. Unless both primers show strong homology to the related target, nonspecific priming may not be a problem. Another advantage of the homology search is that it cross-checks the primer sequences against multiple versions of the target sequence. For example, databases frequently contain several cDNA and/or genomic sequences for a particular gene, which are independently submitted by different laboratories. Since occasional single-nucleotide discrepancies have been known to occur between different published versions of the same gene sequence, the homology search will establish whether the primer matches all known versions of the target sequence.

One potential problem to consider when selecting primers is the occurrence of processed pseudogenes for the gene of interest. Certain genes have been shown to have additional, redundant copies in the genome that are similar to the mRNA sequence (i.e., minus intron sequences) (β-actin is a notable example, which is often used as a control for equivalence of RT-PCR efficiency between samples). These sequences may represent evolutionary relics of mRNA sequences incorporated into the genome at some point via viral RT activity. If the target is known to have a processed pseudogene, even if intron-spanning primers are designed, they will yield a product from the pseudogene in contaminating genomic DNA identical in size to the mRNA-specific product. If the pseudogene sequence is known, it may be possible to design primers that do not amplify the pseudogene. Alternatively, genomic DNA must be eliminated from the RNA preps.

5. PCR Reaction Conditions

Once the optimal primers have been rationally selected, usually very little optimization of the PCR itself is necessary. In my experience, most primer pairs that are selected using software such as the Lasergene Primerselect program (DNASTAR Inc., Madison, WI) work well with a standard set of PCR conditions. We routinely use PCR primers at a final concentration of 0.1 μM each, dNTPs at 50 μM, MgCl₂ at 1.5 mM, and 1.0 U of Taq DNA polymerase
per 50 µl reaction. These are all somewhat minimal concentrations, at the lower ends of the potential concentration ranges. This is preferable because excessive concentrations of primers, dNTPs or the DNA polymerase are more likely to promote mis-priming, leading to nonspecific PCR products. Better specificity is obtained with moderate reagent concentrations. We commonly use the following program of thermal cycling: denaturation at 96°C for 15 s; annealing at 55°C for 30 s, and extension at 72°C for 3 min. Although the calculated annealing temperature of the selected primers may be somewhat greater or less than 55°C, in our studies this annealing temperature has worked well for a long list of different primer pairs. We always perform “hot start,” by heating the reaction at 80°C for about 1 min before adding the polymerase. This prevents nonspecific priming from occurring during reaction setup, which can occur if all the reaction components were mixed at room temperature—i.e., well below the optimal annealing temperature of the primers. Although heat-stable DNA polymerases usually only reach optimal activity at 72°C, any nonspecific products that are generated at the lower temperature would be efficiently amplified during the subsequent PCR, leading to substantial background. An alternative method for performing hot-start allows the complete PCR setup to be performed at the bench, at room temperature. Wax beads are available that can be inserted in the reaction tube before pipeting in the polymerase, so that the polymerase is physically separated from the rest of the reaction components. When the tube reaches high temperature in the thermocycler, the wax melts, allowing mixing of the polymerase with the rest of the reaction components. Other methods for performing hot-start involve the use of anti-Taq neutralizing antibodies, which prevent activity of the polymerase until antibody binding is lost at higher temperatures. Alternatively, a chemically modified Taq can be used, which only becomes active at high temperatures. We typically perform 30–40 cycles, depending on the relative abundance of the mRNA transcript being detected. PCR product specificity should ideally be confirmed, either by restriction mapping or by DNA sequence analysis. If the PCR product is to be cloned, a proofreading thermostable DNA polymerase (e.g., Pwo, Pfu, Vent, or Ultima) should be used in the PCR to minimize misincorporation errors.

6. Contamination: Detection, Precautions, and Remedies

The source of contamination in PCR is almost invariably amplicons from a previous run. Because amplicons are present in enormous quantity at the end of the PCR, it is easy for the DNA to become aerosolized and spread as contamination throughout the laboratory environment. Although the contamination of reagent stocks leads to false-positives in all reactions, including negative controls, environmental contamination is usually random, affecting only some
tubes in the same run. This type of contamination is thus insidious, because the negative control tubes are often “clean;” therefore, unexpected positive results can be caused simply by contamination. If a contamination problem is suspected, it is sometimes helpful to perform multiple negative-control PCRs, so that at least some tubes should pick up the sporadic contamination. Strictly, every tube in a PCR run should have an individual negative control, to increase the ability to detect random contamination.

Proper handling of the PCR product is the key to avoiding contamination problems. Separate laboratory areas (preferably separate rooms) should be allocated for setup of the PCR and analysis of the PCR products. Traffic between these areas should be minimized, particularly from the analysis area back to the setup area. Separate pipettors, pipet tips, and reaction tubes should be kept in both areas, and materials such as notebooks and markers should not be moved between both areas. A separate lab coat should be worn in each area. It is essential to allocate a separate pipettor for handling of the finished PCR product only (such as loading gels); also, a separate pipetor should be kept next to the thermal cycler to be used for adding the DNA polymerase to the PCR during hot-start, and for no other purpose. As already mentioned, sporadic contamination is usually the result of distribution of aerosolized amplicons in the environment. Therefore, care must be taken after the PCR tube is opened at the end of the run and the products are pipetted out for analysis. Pipet tips and tubes used for handling the PCR products should be carefully disposed, in a closed container; tubes that contained PCR products should be re-capped before disposing. Also, electrophoresis buffer and gel-staining solutions should be carefully disposed as soon as possible after they have been used, since they represent large volumes containing PCR products leached from the gel.

Despite the best efforts at prevention, most researchers encounter PCR contamination at some point, particularly when multiple runs are performed over a long period of time using the same primers. When contamination occurs, a number of steps can remedy the situation. Since contamination is usually environmental, one simple solution that can work is to temporarily move the PCR setup area to a different area of the laboratory—ideally a different room—where there is less likelihood of contamination with PCR amplicons. The move should involve the use of fresh batches of reagents and different pipettors and tips. The use of filtered, “aerosol-resistant” pipet tips during PCR setup may reduce the risk of contamination. In time, the original PCR setup area should be rid of the contamination; scrubbing the bench and surfaces (e.g., using 10% household bleach) may speed up the decontamination process. If contamination is widespread, and other measures fail to clear the contamination, a quick solution is to make a different set of primers for the target, which will not
amplify the contaminant amplicon. However, the cause of the contamination should be investigated so that the problem doesn’t arise again. When repetitive PCRs using the same primer pair are envisaged, it may be worth considering additional precautions to prevent contamination. Although it adds to the expense of the PCR, the use of uracil-N-glycosylase (UNG) is a reliable way to minimize the risk of contamination (5). If dUTP is substituted for dTTP in the PCR, the dU-containing amplicons are destroyed by treatment with UNG. Thus, UNG treatment of the PCR reaction prior to beginning thermal cycling will eliminate any contaminant, dU-containing amplicons from previous runs, without affecting the template DNA/cDNA, or the primers. The UNG enzyme is denatured during the initial denaturation step in the PCR, so that newly synthesized PCR products are not degraded. A cheaper alternative to the use of UNG is to ultraviolet-(UV)-irradiate the PCR reaction tube prior to addition of the template DNA. For example, treatment of the reaction tube with UV light of 254 nm wavelength and intensity of 30,000 µJ/cm² for 2 × 2 min can destroy any contaminating, double-stranded DNA. However, unlike UNG, which destroys contaminant amplicons in the reaction tube immediately prior to beginning the PCR, there is a pipetting step after the UV treatment (adding the DNA template) which allows a window for the introduction of contamination. Of course, if hot-start is performed, the risk of contamination during the addition of the DNA polymerase is still present.

7. Conclusion

A wide range of variations on the basic RT-PCR method have been employed in various situations. As such, RT-PCR is a fairly robust technique, tolerating a wide range in the different reaction conditions. Hopefully, the parameters discussed in this chapter will help in the design and setup of RT-PCR reactions, and will inform good PCR laboratory practice.

References
