Exclusive Amplification of cDNA Template (EXACT) RT-PCR to Avoid Amplifying Contaminating Genomic Pseudogenes

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ABSTRACT

Genomic DNA contamination within RNA samples has important implications for RT-PCR, particularly if there is a pseudogene related to the gene under investigation, because amplification from pseudogenes and reverse-transcribed cDNA can be very difficult to distinguish. Methods to remove DNA contamination cannot guarantee the absolute absence of DNA from the sample without a loss of RNA quantity or quality, which can be crucial for small amounts of RNA or for the investigation of transcripts with a low level of expression. Here, we describe a general technique for RT-PCR that applies a sequence to the 5’ tail of reverse-transcribed cDNA that is not present in genomic DNA and uses this for annealing the reverse PCR primer to exclude genomic DNA amplification in unmodified RNA samples.

INTRODUCTION

Most techniques for RNA isolation cannot exclude trace amounts of genomic DNA contamination. While this may not be visualized on an agarose gel, contamination comprising as little as 0.1% of the RNA sample can be sufficiently amplified by PCR to be visualized with ethidium bromide staining (2). This has important implications for studies of gene expression using RT-PCR, particularly if there are genomic pseudogenes related to the sequence under investigation or if the gene has one exon.

Pseudogenes are common, nonfunctional sequences with close similarities to the genes from which they are derived. Those that have arisen by DNA duplication retain the intron-exon arrangement of the productive gene (8) so that amplification of these sequences can be distinguished from cDNA by size, using primers designed to cross intron boundaries (11). This solution is not applicable to intronless “processed pseudogenes” that have arisen by retrotransposition. These pseudogenes lack introns and have a 3’ polyadenylation tract, so they are extremely similar to cDNA derived from the exons of transcribed genes (8). Processed pseudogenes are more common than duplicated pseudogenes; in the complete sequence of chromosome 22, pseudogenes accounted for 19% of the coding sequence, of which 82% were the processed variety (3).

RT-PCR is now frequently used to assess gene expression for its sensitivity in detecting low-abundance mRNA transcripts (5). Co-amplification of processed pseudogenes can lead to false-positive results in RT-PCR studies by generating products that are virtually identical to the reverse-transcribed cDNA target (2). β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes that are often used as controls in RT-PCR studies also have several pseudogenes (8) with up to 96% sequence homology to their mRNAs (10).

Amplification of these sequences can still give rise to a positive β-actin or GAPDH signal, even in specimens in which the RNA has degraded or reverse transcription has failed. This can lead to a false-negative result in which the subsequent failure to detect other genes in that sample would lead to the erroneous conclusion that these genes are not expressed (1).

Pseudogenes are simple to detect by the identification of a positive result in an RT-negative control, but there is no easy solution to prevent their amplification. Methods to remove contaminating genomic DNA can be time consuming and may affect the quality of the RNA sample (6). This is of particular importance for small quantities of tissue or RNA, or in the analysis of genes expressed at low levels.

Although primers cannot be chosen from separate exons to distinguish cDNA from processed pseudogenes, they can be designed to minimize annealing to the pseudogene sequence to prevent its amplification by carefully optimizing the PCR conditions (10). This technique relies on a detailed knowledge of the sequence of the relevant pseudogenes. If that information is not already available, then this approach becomes more complex. Tschentscher et al. (11) cloned and sequenced CK18 pseudogenes from genomic DNA, screened them for sequence differences resulting from substitutions and mutations occurring in the pseudogenes, and then designed primers to permit specific amplification of CK18 cDNA. This method is time consuming, requires precise optimization of the PCR, and, in some cases, the high sequence homology for pseudogenes to their parent gene may not allow specific primers to be sufficiently developed (9).

Recently, Folz et al. (5) described an approach that does not rely on DNase treatment to circumvent genomic DNA amplification. Their technique, called poly(A) cDNA-specific (PACS) RT-PCR, takes advantage of the mRNA’s poly(A) tract to prevent amplification from the genomic sequence from which it is derived. An anchored oligo(dT) primer acts as the antisense primer for PCR after reverse transcription has been performed in the normal fashion. The annealing temperature for PCR is then optimized to ensure that the oligo(dT) primer would amplify cDNA but could not hybridize to genomic DNA (5).

Unfortunately, one of the defining characteristics of processed pseudogenes is the presence of a 3’ polyadenylation tract (8), so the use of this technique may not prevent pseudogene amplification. The method described here also exploits the poly(A) tract on the mRNA, but, instead of anchoring the reverse PCR primer, a unique sequence that is not present in genomic DNA is incorporated at the 5’ end of the cDNA during reverse transcription, as used in standard 3’ rapid amplification of cDNA (RACE) protocols for characterizing cDNA (Clontech Laboratories, Palo Alto, CA, USA). The unique 5’ sequence is then used as the site for reverse primer annealing for PCR, which can therefore only occur from cDNA—hence the name, exclusive amplification of cDNA template (EXACT) RT-PCR. The amplification from contaminating genomic DNA is excluded with any PCR conditions so that optimization can be directed solely to ensure the
amplification of full-length cDNA. Furthermore, because the primer does not require sequence-specific bases at its 3' end, it may be used as a general technique for any RT-PCR without additional modification.

MATERIALS AND METHODS

Cell Culture

The prostate cancer cell lines DU-145 (ATCC no. HTB-81), PC-3 (ATCC no. CRL-1435), LNCaP (ATCC no. CRL-1740), two other prostate cell lines, PNT2 (ECACC no. 95012613) and PNT1A (ECACC no. 95012614), and the breast cancer cell line T47D (ATCC no. HTB-133), were studied using the EXACT RT-PCR method. These cell lines were cultured either in DMEM or RPMI 1640 with 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin antibiotics (all from Invitrogen, Carlsbad, CA, USA).

RNA Extraction and RT-PCR

Total RNA was extracted using TRIZOL® reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using the modified oligo(dT) primer (Clontech Laboratories) (Table 1) and AMV reverse transcriptase enzyme using a cDNA Cycle Kit® (Invitrogen). The cDNA strand produced was then used immediately as the template for the first round of PCR.

PCR Conditions

All of the following PCRs were performed in 50-μL reactions on a PTC-225 thermal cycler (MJ Research, Waltham, MA, USA). One microliter of cDNA template was amplified using 2 U Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA) with the PCR buffer supplied with the enzyme (final concentration 10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 2 mM Mg²⁺, 1 mM mixed dNTPs, and the appropriate concentration of primers, as detailed in Table 1.

An initial denaturation step at 94°C for 2 min was followed by 35 cycles of annealing at 60°C for 50 s, elongation

Table 1. Primer Sequences and Concentrations Used in RT-PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’→3’)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>AAGCAGTGGTAACAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTVN^</td>
<td>500 nM</td>
</tr>
<tr>
<td>β-Actin Forward (10)</td>
<td>CCTGCCTTTTGGCCGATCC</td>
<td>1 μM</td>
</tr>
<tr>
<td>β-Actin Reverse (10)</td>
<td>GGATCTTCATGAGTGATGACTGTC</td>
<td>1 μM</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>ACTGGCGTCTTCACCACCATTG</td>
<td>1 μM</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>GTCATGGATGACCTTGCGGACAG</td>
<td>1 μM</td>
</tr>
<tr>
<td>Nested GAPDH Forward</td>
<td>AGAAGGCTGGGGGCTATTGG</td>
<td>1 μM</td>
</tr>
<tr>
<td>5’ Unique Long (Clontech Laboratories)</td>
<td>CTAATACGACTCATAAGGCA-GAGCAGTGTAACAACCGCAGAGT</td>
<td>20 nM</td>
</tr>
<tr>
<td>5’ Unique Short (Clontech Laboratories)</td>
<td>CTAATACGACTCATAAGGACGAG</td>
<td>100 nM</td>
</tr>
<tr>
<td>5’ Unique Nested (Clontech Laboratories)</td>
<td>AAGCAGTGGTAACAACCGCAGAGT</td>
<td>1 μM</td>
</tr>
</tbody>
</table>

^V = A, C, or G and N = A, C, G, or T

Figure 1. Diagrammatic representation of EXACT RT-PCR. This shows how the 5’ unique sequence is incorporated onto reverse-transcribed mRNA and then acts as the site for the reverse primer, annealing after the first cycle with the gene-specific primer. The generic reverse primer contains a long and short primer for “Step Out” PCR as shown. The use of an internal gene-specific primer for performing nested PCR is also represented.
at 72°C for 1 min, and denaturing at 94°C for 30 s. The PCR regime was concluded with a 4-min extension period at 72°C.

β-actin and GAPDH PCR was carried out with gene-specific forward and reverse primers to determine the success of reverse transcription and to assess the pseudogene amplification from genomic DNA contamination respectively. GAPDH was also amplified using the gene-specific forward primer and a mixture of the primers designed to anneal to the unique sequence at the 5’ end of the cDNA product. One microliter of the above reaction was subjected to nested PCR with a second gene-specific GAPDH forward primer and a unique reverse primer, both internal to those used in the first round (Figure 1).

Southern hybridization with a radiolabeled GAPDH probe was performed to verify the specificity of the EXACT-primed GAPDH PCR and to confirm the absence of product in the reverse transcriptase-negative control lanes.

The human endogenous retrovirus (HERV-K) was amplified using primers and PCR conditions as described by Etkind et al. (4). The Southern blot of this reaction and the EXACT RT-PCR products were both hybridized with a HERV-K-specific probe (see Figure 3).

RESULTS AND DISCUSSION

The β-actin gene-specific primers amplify a 626-bp product. The absence of product in the RT-negative lanes for β-actin illustrates how carefully designed primers can avoid pseudogene amplification and demonstrates the success of reverse transcription (Figure 2a). GAPDH gene-specific primers amplify a 204-bp product in the RT-negative control lanes of each sample that is indistinguishable from the 204-bp product obtained by reverse transcription. This is derived from a processed pseudogene and demonstrates the presence of genomic DNA contamination in each of the samples (Figure 2b).

GAPDH amplification using the unique 5′ primer sequence in place of the gene-specific reverse primer produced faint bands in the RT-positive lanes only (data not shown). Southern blotting and hybridizing with a specific GAPDH probe (Figure 2c) or performing nested PCR with the internal GAPDH primer and the nested RACE primer (Figure 2d) allow these bands to be clearly visualized. These are sensitive methods that show the absence of product from the RT-negative lanes, confirming that these controls are true negatives, and therefore the product in the reverse-transcribed lane must have come from successful RNA reverse transcription. This technique is valuable because it avoids the need to remove contaminating DNA from the RNA sample. DNA removal must be complete to be effective because even a single remaining DNA copy could affect RT-PCR, particularly for low-abundance messages. In practice, this can be difficult to achieve, and the lack of agreement in the literature over the best technique for DNA exclusion bears testament to this (2,6,9). Furthermore, methods to eradicate DNA contamination can lead to a loss of RNA quantity or quality (7). Although the integrity of the RNA can be checked by the amplification of housekeeping genes, these highly abundant sequences are somewhat insensitive to mRNA loss, which may cause genes expressed at low copy numbers to be falsely interpreted as negative (1,6).

The EXACT technique prevents pseudogene amplification through the use of the reverse PCR primer that recognizes the unique 5′ end of the newly synthesized cDNA, a sequence that is...
not present in genomic DNA. This allows the existing gene-specific forward primer to be used without modification and still ensures the amplification of reverse-transcribed products only.

We have extended the use of this technique to investigate the expression of HERVs in tissue samples and in these cell lines. HERV sequences are expressed at low levels but have a number of copies distributed throughout genomic DNA, which can affect the results of RT-PCR. We have found that a published PCR protocol for HERV-K amplification with gene-specific forward and reverse primers produces indistinguishable HERV-K products in both the RT-positive and RT-negative lanes caused by HERV-K sequences in contaminating genomic DNA. (b) HERV-K EXACT RT-PCR. The forward primer is used without modification for the first-round reaction, and the reverse gene-specific primer is converted to a forward orientation for the second-round reaction. The amplification of HERV-K is now confined to the RT-positive lanes only, including some smaller splice variants below the 1.5-kb expected product.

CONCLUSION

The EXACT technique is a sensitive and specific modification of RT-PCR that excludes the amplification from genomic DNA, without requiring the knowledge of the sequence, or even the presence, of processed pseudogenes. This is achieved without the need for processing the RNA sample to eliminate contaminating genomic DNA or adding steps to a standard RT-PCR protocol. Minimizing the amount of processing reduces the likelihood of external contamination and ensures the optimum quantity and quality of RNA in the sample. This is particularly advantageous for low-abundance transcripts, for which any RNA losses can be crucial.
INTRODUCTION

Our previous studies have shown that hPRL demonstrates a stimulatory effect on human breast cancer cell proliferation (1). We have also reported that an hPRL mutant with a single amino acid substitution mutation at position 129 (hPRL-G129R) acts as an hPRL receptor antagonist on human breast cancer cells (1). Further investigation of hPRL-G129R demonstrated that its inhibitory effects on breast cancer cells are through the induction of apoptosis (1). However, the exact mechanism of hPRL-G129R-induced apoptosis is still awaiting further investigation.

To study the physiological mechanisms of different cell types and of cells under different conditions, PCR subtraction hybridization has been used widely over the years (4–6). This technique gives a representation of differentially expressed genes from one group of cells as compared with another. The theory behind the technique is very simple. It first uses mRNA from two populations of cells and converts them into cDNA. The cDNA from cells that contain differentially expressed genes is referred to as the “tester”, and the reference cDNA is referred to as the “driver”. Both tester and driver cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The tester cDNAs are first digested using a 4 base-cutter restriction enzyme to create shorter blunt-ended molecules. The ends of the tester cDNAs are modified by ligating adaptors that will serve as PCR primers. The tester cDNAs are then amplified and sequencing individual cDNAs is a tedious and lengthy process. In this report, an attempt has been made to combine the use of PCR select cDNA subtraction hybridization and cDNA microarrays to identify differentially expressed genes using a nonradioactive chemiluminescent detection method. mRNA from human prolactin (hPRL) or human prolactin antagonist (hPRL-G129R) treated and non-treated breast cancer cells was isolated, and cDNAs were synthesized and used for the PCR subtraction to enrich the differentially expressed genes in the treated cells. The PCR-amplified and subtraced cDNA pools were purified and labeled using the digoxigenin method. Labeled cDNAs were hybridized to a human apoptosis cDNA microarray membrane and identified by chemiluminescence. The results suggest that the strategy of combining all three methods will allow for a more efficient, nonradioactive way of identifying differentially expressed genes in target cells.

REFERENCES


Combination of PCR Subtraction and cDNA Microarray for Differential Gene Expression Profiling

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ABSTRACT

PCR subtraction hybridization has been used effectively to enrich and single out differentially expressed genes. However, identification of these genes by means of cloning and sequencing individual cDNAs is a tedious and lengthy process. In this report, an attempt has been made to combine the use of PCR select cDNA subtraction hybridization and cDNA microarrays to identify differentially expressed genes using a nonradioactive chemiluminescent detection method. mRNA from human prolactin (hPRL) or human prolactin antagonist (hPRL-G129R) treated and non-treated breast cancer cells was isolated, and cDNAs were synthesized and used for the PCR subtraction to enrich the differentially expressed genes in the treated cells. The PCR-amplified and subtraced cDNA pools were purified and labeled using the digoxigenin method. Labeled cDNAs were hybridized to a human apoptosis cDNA microarray membrane and identified by chemiluminescence. The results suggest that the strategy of combining all three methods will allow for a more efficient, nonradioactive way of identifying differentially expressed genes in target cells.

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