

Technical reports

A novel, rapid in cell RNA amplification technique for the detection of low copy mRNA transcripts

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Abstract

Growing interest now focuses on improvements of in situ polymerase chain reaction (PCR) technology for the detection of DNA and RNA cellular sequences. In this study, reverse transcription PCR in situ hybridisation (RT PCR-ISH) was developed and used to determine gene expression of pyruvate dehydrogenase in a cell model system, using human peripheral blood lymphocytes (PBLs). The success of in cell RNA amplification depends on the type of cell/tissue fixation, cell permeabilisation, and the efficiency of reverse transcription and cDNA amplification. This paper presents new approaches to overcome the critical aspects of fixation, permeabilisation, and reverse transcription when performing in cell RNA amplification. A novel fixative, "Permeafix", possessing fixative and permeabilisation properties, was used for cell fixation procedures. "Permeafix" obviated the need for pre-amplification proteolysis, facilitating entry of PCR reagents to target sequences within the cell. In addition, a simple one step RNA in cell amplification protocol using recombinant *Thermus thermophilus* (rTth) DNA polymerase, which reverse transcribes mRNA efficiently to cDNA and then catalyses cDNA amplification, was used. The value of a semi-junctional primer system for in cell gene expression studies, without the need to perform DNase digestion, is demonstrated.

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Keywords: "Permeafix"; reverse transcription polymerase chain reaction in situ hybridisation; recombinant *Thermus thermophilus* DNA polymerase; pyruvate dehydrogenase

In situ reverse transcription polymerase chain reaction (RT PCR) technology is most useful when in situ hybridisation (ISH) fails to detect low copy RNA transcripts within the cell. In cell RNA amplification provides a method for the localisation of cytoplasmic message, allowing evaluation of gene expression in different cells and tissues.

The mRNA of pyruvate dehydrogenase, involved in the glycolytic pathway, was tran-

scribed in our slide based non-isotopic in situ reverse transcription assay. Pyruvate dehydrogenase is a single copy gene, with no pseudogenes, and is expressed constitutively at low concentrations within the cell.¹

Before in situ amplification is carried out, the morphology of the cell must be fixed rigidly to create a microenvironment that facilitates entry of all PCR components and minimises leakage of the PCR product. The combination of fixation and subsequent cell permeabilisation is the biggest problem encountered when performing in cell DNA and RNA amplification assays.² Most in cell amplification assays use formaldehyde/paraformaldehyde fixation and proteinase K or pepsin digestion, with or without mild acid hydrolysis.

In this paper, we introduce a fixative that also acts as a non-crosslinking permeability agent ("Permeafix"; Ortho Diagnostics, Raritan, USA), eliminating the need to perform pre-amplification permeabilisation. This reduces amplicon diffusion out of the cell, during and after amplification, and thus eliminates false positive signal generation. Recent studies have demonstrated the suitability of "Permeafix" for use with flow cytometry.³⁻⁶

Several protocols for in cell RNA amplification have been published.⁷⁻¹⁴ Initially, antisense oligonucleotide primers are annealed to mRNA and then reverse transcribed by avian myeloblastosis virus (AMV) or Moloney mouse leukaemia virus (MMLV) to generate cDNA which is amplified by Taq DNA polymerase. Alternatively, a one step procedure using recombinant *Thermus thermophilus* (rTth) DNA polymerase and a novel bicine buffer system can be used to carry out in cell RNA amplification. rTth possesses reverse transcriptase and DNA polymerase activity¹⁵ and has the advantage of performing reverse transcription at higher temperatures than AMV or MMLV, thereby minimising the effect of RNA secondary structure, which can be overwhelming in the fixed cell. The use of a single bicine buffer system greatly facilitates the performance of in cell RNA amplification without the need to change buffer systems during amplification or use chelating agents.

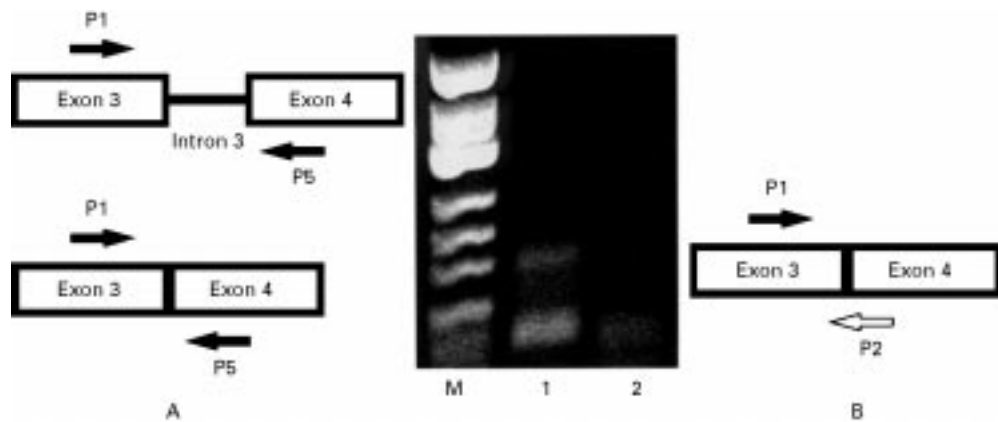


Figure 1 Comparison of different primer sets (A and B) using rTth DNA polymerase in a solution phase pyruvate dehydrogenase RT PCR assay. (A) Set A primers are an exonic, intron spanning primer pair amplifying contaminating DNA and reverse transcribed cDNA. (B) Set B primers are a semi-junctional primer pair generating a single mRNA generated amplicon in RT PCR. Also shown (middle) is a 2% agarose gel showing the single band generated by the junctional primers (P1 and P5; lane 2) and the two bands generated by the exonic primer pair (P1 and P5; lane 1).

Materials and methods

ISOLATION OF PERIPHERAL BLOOD LYMPHOCYTES
Human peripheral blood lymphocytes (PBLs) were isolated from fresh EDTA treated blood by Histopaque 1.077 (Sigma Diagnostics, St Louis, USA) discontinuous density gradient separation. Cells were washed twice in RPMI medium (Gibco BRL Life Technologies, Gaithersburg, USA), containing 10% foetal calf serum (FCS), and were pelleted on to silane coated microscope slides (Perkin Elmer, Foster City, USA) by cytospin centrifugation at 550 22.3 ×g for five minutes at ambient temperature (Shandon Cytospin 2; Shandon, Pittsburgh, USA).

PRETREATMENT

Cells attached to the glass slides were incubated in "Permeafix" solution for one hour, washed twice in phosphate buffered saline (PBS) for five minutes, air dried, and frozen at -80°C. After thawing for 15 minutes at room temperature, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide solution in 0.1% sodium azide for 15 minutes, followed by two washes in DEPC (0.1% solution diethylpyrocarbonate; Sigma Diagnostics) treated water.

PRIMER DESIGN

Three sets of oligonucleotide primers were synthesised for pyruvate dehydrogenase (TIB MolBiol, Berlin, Germany) (fig 1).

Set A primers:

P1: 5' GGT ATG GAT GAG GAG CTG GA 3'

P5: 5' CTT CCA CAG CCC TCG ACT AA 3'

Set B primers:

P1: 5' GGT ATG GAT GAG GAG CTG GA 3'

P2: 5' CAG CCC TCG ACT AAC CTT GT 3'

Set C primers:

P3: 5' TGC TTG GAG AAG AAG TTG CC 3'

P4: 5' ATC TGG GGG TAA CAG TGA CC 3'

Set A primers are located in exon 3 and 4 of the pyruvate dehydrogenase gene and were generated to perform a control solution phase PCR. This is a conventional intron spanning primer approach, which yields products from DNA and mRNA.

Set B primers represent a semi-junction system. Primer P2 is complementary to the exon sequences in exon 3 and 4, adjacent to the intervening intron 3 segment. This system prevents annealing of primers to genomic DNA sequences in the cell and only allows amplification of cDNA sequences, reverse transcribed from RNA. The upstream primer P1 is located in exon 3. The junctional primer P2 also acts as a specific downstream primer for cDNA synthesis.

Set C primers were designed exclusively to generate a digoxigenin labelled PCR amplicon for use as a probe for in situ hybridisation following amplification.

SOLUTION PHASE RT PCR

A qualitative assessment of the rTth DNA polymerase and primer systems was performed using solution phase RT PCR. Total human RNA isolated from fresh EDTA treated blood (Qiagen RNAeasy blood kit; Qiagen Inc, Santa Clarita, USA) was reverse transcribed and cDNA was amplified using the Perkin Elmer Gene Amp 9700 thermocycling system with the following reaction mixture: 0.4 μM primers P1 and P2 or P1 and P5, 400 μM dNTPs (Boehringer Mannheim, Mannheim, Germany), 2.5 mM Mn(OAc)₂ (Perkin Elmer), 1× EZ buffer (bicine buffer system; Perkin Elmer), 5 U rTth DNA polymerase (Perkin Elmer). After 30 minutes of reverse transcription at 58°C and an initial denaturation step at 94°C for five minutes, 30 cycles of: denaturation at 94°C for 15 seconds, annealing at 58°C for 15 seconds, and elongation at 72°C for 15 seconds were performed. Amplicons were analysed on an ethidium bromide stained 2% agarose gel.

To make the dsDNA hybridisation probe for in situ hybridisation following in cell amplification, human genomic DNA was amplified in a solution phase PCR using primers P3 and P4 (0.4 μM), 100 ng human male DNA, 10× PCR

buffer II (Perkin Elmer), 200 μ M dNTPs (Boehringer Mannheim), 1.5 mM $MgCl_2$, and 1 U Amplitaq polymerase (Perkin Elmer). Amplification was carried out initially at 94°C for two minutes and then 94°C for 15 seconds, 58°C for 15 seconds, and 72°C for 15 seconds for 35 cycles. Afterwards, the PCR product was reamplified in the presence of the same reagents, replacing the dNTPs with a 10 \times digoxigenin labelling mixture (Boehringer Mannheim).

RT PCR IN SITU HYBRIDISATION

Reverse transcription and subsequent PCR amplification were carried out using the Gene Amp in situ PCR 1000 system (Perkin Elmer). The following cocktail for one step RT PCR was used: 1 μ M of primers P1 and P2, 1 \times EZ buffer (Perkin Elmer), 400 μ M dNTPs (Boehringer Mannheim), 5 mM $Mn(OAc)_2$ (Perkin Elmer), 10 U rTth DNA polymerase (Perkin Elmer), and DEPC treated water to a final volume of 50 μ l. The PCR mixture was placed on top of the fixed PBLs, covered with silicon rubber Ampli cover discs (Perkin Elmer), and sealed with Ampli cover clips (Perkin Elmer). The following thermal cycling parameters were applied: 58°C for 30 minutes (reverse transcription) and an initial denaturation step of 94°C for five minutes, followed by 94°C for 45 seconds and 58°C for two minutes for 30 cycles. Control samples excluded primers in the PCR mixture. Following amplification, the slides were disassembled and fixed in 100% ethanol. Hybridisation was carried out in a moist Terasaki microtitre plate (Nalge Nunc International, Rochester, USA) in a hybridisation oven. The PCR generated double stranded probe was added to the hybridisation cocktail which comprised: 2 \times saline sodium citrate (SSC), 10% formamide, 5% dextran sulphate, and 2 ng/ μ l probe. Slides were covered with coverslips, denatured at 94°C for 15 minutes, and hybridized at 37°C for eight hours.

DNase digestion was carried out as follows: 10–40 μ l of solution containing 1 μ l 10 \times digestion buffer (35 μ l 3 M sodium acetate, 5 μ l 1 M $MgSO_4$, and 60 μ l dH₂O) 1 μ l 10 U/ml RNase free DNase, and 8 μ l H₂O was placed on top of each section. The sections were covered with autoclaved coverslips and placed in a humidity chamber at 37°C. After two hours, fresh solution was added and left overnight. The procedure was repeated in the morning and sections were left for a further two hours. The coverslips were removed and the slides were

washed for one minute in distilled H₂O and 100% ethanol and allowed to air dry.

RNase digestion was performed as follows: RNase A was dissolved in 2 \times SSC (in DEPC water) to a concentration of 10 mg/ml, boiled for 10 minutes, and diluted further to 100 μ g/ml in 2 \times SSC. RNase T1 was added to a final concentration of 100 U/ml and sections/cells were incubated for 30 minutes at 37°C. After digestion, sections were washed in DEPC water and air dried at 70°C.

DETECTION

Slides were washed initially in 2 \times SSC at 37°C for 15 minutes, followed by a final wash with normal blocking serum (1/100 dilution in 10 mM sodium phosphate; Vectastain Universal Elite ABC kit; Vector, Burlingame, USA) for 10 minutes. Monoclonal mouse antidigoxigenin (1/100 dilution in 10 mM sodium phosphate; Boehringer Mannheim) was used for three step detection, followed by biotinylated universal antibody (1/50 dilution in 10 mM sodium phosphate; ABC kit) for five minutes, and then the previously mixed avidin–peroxidase complex (1/100 dilution in 10 mM sodium phosphate; ABC kit) for 10 minutes. The signal was developed with aminoethylcarbazole (Vector), which produces a red signal. Cells were counterstained with haematoxylin and mounted with glycerol jelly.

Results

Our experiments showed that “Permeafix” is a suitable permeability and fixative agent for in cell RNA amplification (fig 2). The fixed and permeabilised cells remained intact during the temperature cycling PCR process and yielded adequate cell morphology following amplification. Optimal cytoplasmic staining was achieved with 60 minutes “Permeafix” fixation. Longer fixation times (up to 120 minutes) did not compromise amplification or morphology. Clearly, the detergent component of “Permeafix” has sufficient “cell lysis” activity to allow access of large molecules, such as Taq DNA polymerase (100 Å diameter) into the cell. Unlike the proteases used with formaldehyde fixed cells, when using “Permeafix”, it is not necessary to determine empirically the most suitable concentration or duration of pre-treatment.

In our study, we carried out amplification of pyruvate dehydrogenase RNA using a specially designed junctional primer pair (P1 and P2). In solution phase PCR, these primers generate a single band, as shown in the 2% agarose gel (fig 1). In contrast, an exonic primer pair (P1 and P5) flanking intron 3 of the pyruvate dehydrogenase gene generated two bands (fig 1); the upper band is amplification of contaminating genomic DNA, the smaller band is RNA molecules. This phenomenon was not seen when the semi-junctional priming approach was used. Therefore, in cell DNase digestion is not needed when using the semi-junctional primer approach. In general, in cell amplification experiments are easy to perform using this system, once solution phase conditions have been established.

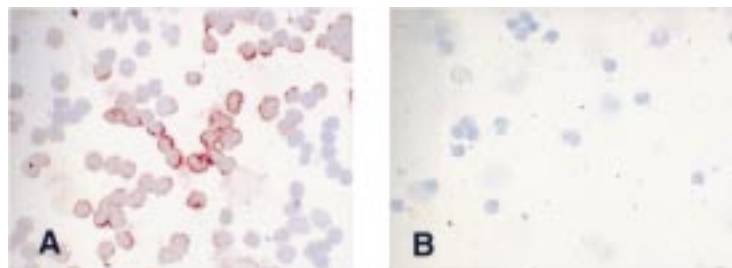


Figure 2 (A) Pyruvate dehydrogenase mRNA in cell amplification using a semi-junctional primer pair model in peripheral blood lymphocytes. (B) In the negative control for RT-PCR-ISH the primers were omitted.

Figure 2 shows that ~ 50–70% of PBLs are strongly positive and, therefore, contain pyruvate dehydrogenase amplicons. Because these cells represent a biological system, expression of pyruvate dehydrogenase mRNA might differ from cell to cell. Although the permeabilisation process and RT PCR are standardised, one cannot exclude incomplete efficiency for both reactions. Using standard non-isotopic in situ hybridisation, pyruvate dehydrogenase RNA sequences could not be detected in the cytoplasm of PBLs. Importantly, no diffusion phenomena occurred in these cytospin preparations, largely because they are whole cell preparations that have not undergone excessive cell lysis by harsh proteolytic regimens, such as proteinase K. Excluding primers from the PCR mix gave no signals, thereby eliminating non-specific priming phenomena that might occur as a result of nicked single stranded DNA fragments acting as primers within the cell. DNase did not abolish the signal, but the specific signal disappeared on RNase digestion.

Discussion

We have shown that non-isotopic RT PCR-ISH is a powerful tool for the detection of human mRNAs where in situ hybridisation fails because of low detection sensitivity. In particular, the use of both “Permeafix” for cell pretreatment and rTth DNA polymerase for amplification, and a semi-junctional priming strategy, greatly reduces the time required to perform in cell RNA amplification. Our protocol can be performed in less than one working day. In addition, it eliminates the need to carry out extensive proteolytic digestion titrations which, by necessity, need to be performed when using formaldehyde fixed tissue.

In earlier work, cells have been fixed with neutral buffered formaldehyde or paraformaldehyde^{7–9 12 16} and digested with proteases.^{7 8 12 15 16} Crosslinking fixatives, such as formalin or paraformaldehyde, allow PCR reagents access to the target sequence only after considerable incubation with a suitable protease. Simple precipitating reagents, like ethanol and acetic acid (and probably also “Permeafix”), with little crosslinking ability, allow PCR mixture penetration with no pretreatment. Indeed, cells are probably “opened up” during the thermocycling process, again facilitating entry of PCR reagents. Importantly, in our study, morphology was well preserved so that it was easy to locate the signal in the cytoplasm of the cell. No migration of PCR amplicon out of PBLs could be seen, or back diffusion into other cells, even though our amplicon was only 92 base pairs in size, which is considered small for in cell amplification.

rTth DNA polymerase was an effective enzyme for use in our RT PCR-ISH assays because it acts both as an RNA dependent reverse transcriptase and a DNA dependent thermostable polymerase. Unlike other protocols for performing reverse transcription and subsequent cDNA amplification, neither the addition of another enzyme nor an alteration in the buffer system is required in this single enzyme assay. The conversion of the standard

Tris-HCl PCR buffer to bicine (a reagent capable of buffering metal and hydrogen ion concentrations) allows the enzyme to be used for copying both RNA and DNA.¹⁷ Another advantage is that this highly stable rTth DNA polymerase enables reverse transcription to be carried out at high temperatures and in the presence of Mn²⁺ ions,¹⁸ conditions unsuitable for standard mesophilic reverse transcription enzymes. Our reverse transcription reaction was carried out at 58°C, which ensures high specificity and reduced background.

The exclusiveness of the cytoplasmic signals derived from the semi-junctional primer system (fig 2) do not necessitate an RNase free DNase digestion step to avoid genomic DNA amplification. Adequate DNase digestion is difficult to perform with most protocols, requiring elaborate three stage digestions, which compromise cell morphology.

In conclusion, this new in cell RNA amplification procedure is a rapid, inexpensive, and robust method for detecting rare and abundant transcripts directly within the cell, and it allows sensitive and specific gene expression studies at the individual cell level.

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