Short reports

Novel method for detection of small amounts of RNA based on the semi-nested polymerase chain reaction

S Sanders, G A Thomas

Abstract

A technique for the amplification of very small quantities of cDNA based on a semi-nested polymerase chain reaction system has been developed. The technique uses an additional blocking oligonucleotide "primer" in the second round nest which is complementary to one of the first round primers. This prevents amplification of first round product or of any other unwanted products dependent on that primer, but allows amplification of second round product, using first round product as the template. The second round primers are added through the oil layer, eliminating the possibility of introducing first round product aerosol into the atmosphere, as would be the case in a fully nested system. Contamination is therefore minimised, while amplification of the desired product is maximised.

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Since the initial development of the polymerase chain reaction (PCR) technique, a number of advances and improvements have been made. These include using nested or semi-nested systems to detect very small quantities of nucleic acid. We have developed the semi-nested approach further to provide a system that reduces the chance of contamination and reduces unwanted bands, but has sufficient sensitivity to allow for the amplification of small amounts of template. We tested this approach on two systems: tissue microdissected from histochemically stained sections, and paraffin wax embedded tissue.

A successful PCR system allows for the efficient amplification of the desired product while avoiding contamination and the production of non-specific amplicons. Using a fully nested system (fig 1A) can greatly increase sensitivity but also increases the chance of contamination, partly due to its sensitivity and partly because of the additional manipulation required be-

tween rounds. Fully nested reactions require a small aliquot of the first round to be removed and used in a second, separate reaction. This transfer is thought to be the main culprit creating environmental contamination. Semi-nested systems (fig 1B), although having the drawback of reduced sensitivity, have the advantage of avoiding the additional manipulation, therefore reducing the chance of environmental contamination. However, because the primers that are responsible for amplification of the larger, unwanted, first round product are still present, continued amplification of this species occurs, the out-nested primer from the first round competing for template with the in-nested primer of the second round.

Various methods have been used to reduce these unwanted products, including "touch-down" PCR,¹ "hot start" PCR,^{2,3} and adding additional reagents.⁴

We considered that the introduction of an additional blocking oligonucleotide, added at the same time as the semi-nested primer, would avoid the problems associated with semi-nested systems (fig 1C). This prevents further amplification of any product associated with this first round primer, and removes competition between first and second round templates for the components of the reaction mixture. With unwanted reactions blocked, the only product produced in the semi-nest should be the single, specific product of the semi-nested primer and the remaining, unblocked first round primer (fig 1C).

Using this system, we demonstrated production of only the desired product. The technique was highly sensitive, detecting small amounts of material, extracted by three different methods. Two methods were applied to extracts from fresh liver tissue and from histochemically stained frozen tissue which had been fixed in paraformaldehyde as part of the histochemical protocol. The third was applied to extracts from paraffin wax embedded archival material. All products were checked by direct sequencing to ensure that desired sequences had been amplified.

Department of
Histopathology,
University of
Cambridge, Level 5,
Laboratory Block,
Addenbrooke's
Hospital, Hills Road,
Cambridge CB2 2QQ
S Sanders
G A Thomas

Correspondence to:
Dr Thomas.

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Figure 1 (A) Nested PCR. Second round uses separate reaction with new, unique set of in-nested primers (white triangles). Smaller second round product obtained. (B) Semi-nested PCR. Second round involves addition of single in-nested primer (white triangle). At least two products can be obtained. (C) Semi-nested PCR with blocking oligonucleotide. Second round involves addition of single in-nested primer (white triangle) plus a primer complementary to one of the first round primers (grey triangle).

Methods

FRESH TISSUE EXTRACTIONS

RNA was extracted from frozen mouse liver tissue using a standard RNA extraction protocol⁵ and stored at -20°C for subsequent reverse transcriptase PCR (RT-PCR).

RNA was also extracted from a section of murine ear epidermis containing approximately 40 cells. The $6\text{ }\mu\text{m}$ section had been histochemically stained with a technique that involves a 15 minute post-reaction fixation step using paraformaldehyde. RNA extraction was performed using the commercially available reagent, TriZOL (Life Technologies, Paisley, Scotland). The fixed tissue was first digested using proteinase K treatment for one hour at 50°C . The proteinase K (Sigma, Poole, Dorset) was used at a final concentration of $100\text{ }\mu\text{g/ml}$ in PK buffer (10 mM Tris-HCl, pH 7.8; 5 mM EDTA, pH 8.0; 0.5% sodium dodecyl sulphate). A reverse transcriptase kit

Figure 2 Semi-nested PCR. The method was first tested on high copy number "control" cDNA prepared from BALB/c mouse liver. The PCR was carried out with (lane 2) or without (lane 1) the blocking oligonucleotide in the second round. Note that the spurious bands present in lane 1 were removed, and the intensity of the correct band was increased (lane 2) when the blocking oligonucleotide was used. RNA was extracted from microdissected murine ear epidermis and subjected to RT-PCR. The cDNA was amplified with (lane 4) or without (lane 3) blocking oligonucleotide in the second round. The initially stronger unwanted first round band (lane 3) was converted to the correct size product when blocking oligonucleotide was added to the reaction. Lanes 5 and 6 were the "No DNA" controls. M, phi-X marker (200 ng).

(SUPER RT, HT Biotechnology Ltd, Cambridge) was then used for both extracts. The reaction was performed in a total volume of $25\text{ }\mu\text{l}$ containing $1\times$ reaction buffer (50 mM Tris-HCl; 50 mM potassium chloride; 4 mM dithiothreitol; 10 mM magnesium chloride), 1 mM deoxynucleotides (dNTPs), RNasin, approximately 1000 pmol random hexamer (Pharmacia Biotech, St Albans), 20 U SUPER RT enzyme, and mRNA template.

The target sequence used was that of glucose-6-phosphate dehydrogenase (G6PD), a low copy number mRNA of the order of one copy per cell in the rat liver.⁶ The cDNA product was placed in a first round PCR reaction with $0.2\text{ }\mu\text{M}$ each of primers A₁ (TATCCTAC-CATCTGGTGGCT) and C₄ (TCGGAAC-TGCAGTCTCACTT) which flank exons 4 to 9 (fig 2), $1\times$ Taq buffer (Promega; 50 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100), 0.2 mM dNTPs, and $0.5\text{ }\mu\text{l}$ Taq DNA polymerase (Promega, Southampton, 5 U/ μl). For each PCR a "no DNA" control was also included. All samples were overlaid with sterile oil (Sigma).

A "false" hot start at 80°C for about 30 seconds was used, followed by 15 cycles of three steps: denaturation for 30 seconds at 95°C , annealing at 56°C for 60 seconds, and extension at 72°C for two minutes.

After the 15 cycles of the first round the samples were placed on ice and $2\text{ }\mu\text{l}$ of a premix containing the following were added through the oil: $1\text{ }\mu\text{l}$ in-nested primer A₂ (CTTTGAC-CTTCTCATCACGG) at a concentration of $300\text{ ng}/\mu\text{l}$, $0.5\text{ }\mu\text{l}$ oligonucleotide F₁ ($120\text{ ng}/\mu\text{l}$; complementary to C₄), and 2.5 U Taq DNA polymerase. A further 35 cycles were then run. A final step of five minutes at 72°C was used to

Figure 3 Polyacrylamide gel of PCR products using primers specific for the *ret* gene. Lane 1, single round of 35 cycles; lane 2, semi-nested system without blocking oligonucleotide; lane 3, semi-nested system with blocking oligonucleotide. Addition of the blocking oligonucleotide pushed the reaction from the production of two weak bands to a single, strong band. The "No DNA" control showed no product (not shown). M, phi-X marker (200 ng).

allow for completion of polymerisation on partial products. PCRs were performed on various dilutions of the cDNA to provide an estimate of sensitivity of the technique. The highest dilution from which reliable amplification was obtained was 1:3, representing G6PD mRNA from about 15 cells.

PARAFFIN WAX EMBEDDED MATERIAL

Single 5 µm tissue sections mounted on glass slides were put through three five-minute xylene and three five-minute ethanol washes, followed by at least five minutes in DEPC-treated water. Entire sections were scraped off and placed in PK buffer. Proteinase K was added to a final concentration of 100 µg/ml, and the samples were incubated at 50°C for three hours. Extraction was then carried out as described for histochemically treated and fixed tissue.

All subsequent steps were as described except: (1) primers for a different gene were used. In this case, first round primers (R_1 , GACCTGGAGAAGATGATGGT and R_2 , AAGAGGAGAGACTACTTGGG (fig 3)) spanning a 127 base pair region around the exon 18/19 junction of the *ret* gene were used. The in-nested primer (R_3 , AAGAGGAGAGACTACTTGGG) was designed across the junction to prevent the amplification of genomic DNA in the unlikely event that DNA contamination was present, and an oligonucleotide complementary to R_1 ($\alpha-R_1$) was also added in the nest. In this case, the 3' primer was biotinylated in preparation for sequencing. This should make no difference to the ability of the primer to work in the PCR reaction. (2) Of the RNA extracted, either the entire amount, or half of the total was used in the RT reaction. (3) Of the cDNA produced, not more than 1 µl was used in the PCR. Using more template caused the reaction to become overloaded, and no distinction between a PCR with and without the blocking oligonucleotide could be made.

GELS

All products were run on 10% or 12% polyacrylamide gels in 1 × Tris buffered EDTA. From each sample an aliquot of 4 µl was loaded onto the gel, with 1 µl loading buffer (0.25% wt/vol xylene cyanol, 0.25% wt/vol bromophenol blue, 40% wt/vol sucrose). Samples were run for between three and a half and four hours at 120 V. Gels were stained for 5 to 10 minutes in a 50 µg/ml solution of ethidium bromide to visualise the bands. Gels were then photographed using a thermal imager and an ultraviolet light box.

Results

The presence of the additional blocking oligonucleotide in the second round successfully cleared spurious bands from the "control" cDNA sample (fig 2). In the case of the fixed

tissue sample, it served to force a reaction favouring the unwanted first round product towards one favouring the desired second round product (fig 2). In the paraffin wax embedded sample, absence of the blocking oligonucleotide gave two weak bands at each of the expected positions (127 base pairs and 106 base pairs) (fig 3). Addition of the blocking oligonucleotide yielded a single, strong band at the lower position (106 base pairs). A single round of 35 cycles gave no product (fig 3, lane 1). This band has been sequenced in five cases (different samples) and has been shown to be the predicted sequence (data not shown).

Discussion

The advantages of the semi-nested PCR are twofold. First, by removing one of the first round primers from the second round reaction, spurious bands were eradicated and the correct size product was obtained. Second, by running a short first round, and by adding second round reagents directly, all the first round products were available as templates for the second round. This made for a more efficient reaction, and as no first round product was removed, no contamination of the environment occurred. The system also made the PCR reaction very sensitive in that it could amplify very low levels of template, a great advantage when paraffin wax embedded archival material is used.

We conclude that this is a worthwhile advance and provides an easy and efficient means to amplify low copy number target sequence without having to deal with spurious bands or with unwanted contamination. This is especially relevant when an increased number of cycles is used to amplify extremely small amounts of target (50 cycles in the second round have been used with the same results, data not shown). This technique has been used effectively to amplify very small amounts of cDNA from either fresh or paraffin-embedded material. The products are entirely suitable for direct sequencing.

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