POLYMERASE CHAIN REACTION

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Abstrak

Real-time PCR, disingkat RT PCR, adalah alat yang umum untuk mendeteksi dan menghitung ekspresi profil dari gen-gen tertentu. PCR biasanya digunakan untuk menggandakan lokasi spesifik dari suatu rantai DNA (DNA tujuan). Terdapat tiga teknik blotting yang berbeda seperti: Southern blot, northern blot, dan western blot. Southern blot untuk mendeteksi DNA, northern blot untuk mendeteksi mRNA (messenger ribonucleic acid) dan western blot untuk mendeteksi protein.

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Abstract

Real-time PCR, hereafter abbreviated RT PCR, is becoming a common tool for detecting and quantifying expression profiles of selected genes. PCR is used to amplify a specific region of a DNA strand (the DNA target). There are three different blotting techniques: Southern blot, northern blot and western blot. The Southern blot detects DNA, the northern blot detects mRNA (messenger ribonucleic acid) and the western blot detects proteins.

Introduction

The invention of polymerase chain reaction (PCR) by Kary Mullis in 1984 was considered as a revolution in science. Real-time PCR, hereafter abbreviated RT PCR, is becoming a common tool for detecting and quantifying expression profiles of selected genes. The technology to detect PCR products in real-time, i.e., during the reaction, has been available for the past 10 years, but has seen a dramatic increase in use over the past 2 years. A search using the key word real-time and PCR yielded 7 publications in 1995, 357 in 2000, and 2291 and 4398 publications in 2003 and 2005, respectively. At the time of this writing, there were 3316 publications in 2006. The overwhelming majority of the current publications in the field of the genomics have been dealing with the various aspects of the application of methods in medicine, with the search for new techniques providing higher preciosity rates and with the elucidation of the principal biochemical and biophysical processes underlying the phenotypic expression of cell regulation. Series of RT PCR machines have also been developed for routine analysis (Deepak, 2007).

PCR is used to amplify a specific region of a DNA strand (the DNA target). Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size (Cheng, 1994).

A basic PCR set up requires several components and reagents. These components include:

- DNA template that contains the DNA region (target) to be amplified.
- Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target.

- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C.
- Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesizes a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
- Monovalent cation potassium ions (Joseph, 2001).

Procedure

The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers.

Initialization step: This step consists of heating the reaction to a temperature of 94-96°C (or 98°C if extremely thermostable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

- Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

- Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

- Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

- Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended (Chien, 1976).

The way to detect PCR results

There are three different blotting techniques: Southern blot, northern blot and western blot. The Southern blot detects DNA, the northern blot detects mRNA (messenger ribonucleic acid) and the western blot detects proteins.

The blotting techniques are often used to detect genetic abnormalities (when blotted for DNA), as well as certain infectious diseases (when blotted for protein).

a.The Southern Blot

The Southern blot is used to detect and identify certain DNA sequences in a sample of bodily fluid. It uses single-stranded DNAs to search out their complementary strands.

When a Southern blot is performed on DNA, the first step is to incubate the DNA with restriction enzymes. Restriction enzymes cut DNA at known sequences, and produces DNA fragments of a certain length. Once the DNA is cut into pieces, scientists conduct electrophoresis to separate them by size.

Then, since the DNA in the gel is double-stranded, it is separated into single strands. The single-stranded DNAs are then transferred to a special piece of paper since a labelled probe cannot reach them inside the gel (too thick). The transfer is exact and does not disturb the location and grouping of the DNAs from gel to paper. This is called "blotting."

The location is important because scientists know what a certain DNA sequence profile should look like. Since there are usually many fragments on a blot, labelled probes are used to flag the different pieces of DNA. If the specific labelled probe meets a complementary pair on the paper blot, it will bond to it. Later, a method called autoradiography is used to read the location of the attachment. Since the identity of the labelled probe is already known, the identity of the DNA from the sample will also be known.

b.The Northern Blot

The only difference between a Southern blot and a northern blot is that the northern blot uses mRNA from samples instead of DNA. So instead of applying gel electrophoresis to DNA, northern blot applies it to mRNA, and since mRNA is naturally single-stranded, there is no need to separate the strands as in a Southern blot.

c.The Western Blot

The western blot is used to detect different proteins. Gel electrophoresis is applied to the specific proteins. This separates them by size and just like in the other blots, the result is blotted onto a special paper. The rest of the steps follow the method for an ELISA (Brown, 2001).

PCR sample in pathology

There are three PCR sample in pathology:

1.Fresh tissue

Rearrangement of the BCL-2 gene is the molecular consequence of the t(14;18) chromosomal translocation, which is found in approximately 60-90% of follicular lymphomas. To investigate the ability of the polymerase chain reaction (PCR) to detect this rearrangement in fixed-tissue samples, we studied 48 cases of follicular lymphoma using DNA extracted from paired samples of fresh-frozen tissue and formalin-fixed, paraffin-embedded tissue. A standard phenol-chloroform DNA extraction method was used for both types of tissue. Rearrangements of the major breakpoint region (MBR) and minor cluster sequence (MCS) were examined. Three segments of the human beta-globin gene were also amplified to estimate the degree of DNA degradation in the fixed-tissue samples. PCR of fresh-tissue (intact) DNA revealed

amplifiable products in 29 of the 48 follicular lymphomas (60%), whereas the fixed-tissue (degraded) DNA studies were positive in 24 (50%). MBR products were detected in 24 fresh-tissue samples, and varied from 80 bp to > 1.5 kb. Twenty of these cases yielded MBR products in the corresponding fixed-tissue DNA, ranging from 80 to 276 bp. Five fresh-tissue and four fixed-tissue samples produced MCS segments that ranged from 340 bp to 1.2 kb. Four of the five samples with no detectable MBR or MCS translocations using degraded DNA had products greater than 1.0 kb in the fresh-tissue studies. A 175-bp segment of the beta-globin gene was amplified in all 29 fixed-tissue samples; a 324 bp fragment was produced in 20 samples (69%), and a 676 bp segment was detected in 13 (45%) (Liu, 1993).

2. Paraffin block

One of the most active research areas in molecular pathology is retrospective studies on archival tissue samples. However, isolating high-quality genomic DNA from formalin-fixed, paraffin-embedded tissue can be difficult because only minimal amounts of intact DNA may be present in the sample.[1] Because of this, analysis of the recovered DNA is generally limited to PCR, and amplification of small target sequences (300 bp or less) is most successful.

The MasterPure™ Complete DNA and RNA Purification Kit was designed to isolate DNA and RNA from a variety of sources, including samples containing small amounts of nucleic acid. Here, we provide a protocol for isolating PCR-ready DNA from paraffin-embedded tissue using the MasterPure Complete Kit. We isolate genomic DNA from a biopsy specimen and show that the DNA is suitable as a template for PCR by amplifying a region of the Factor V gene.

Methods and Results

DNA isolation from breast cancer tissue paraffin sections

The protocol for treatment of formalin-fixed, paraffin-embedded tissue samples prior to purification using the MasterPure Complete DNA and RNA Purification Kit is summarized in Table 1. The isolation of genomic DNA from a breast cancer tissue section was performed following these guidelines. Specifically, DNA was isolated from 0.02 g of 35 µm thick paraffin-embedded samples. (Thin paraffin sections allow the best recovery and quickest extraction times). Five milliliters of xylene were added to the tissue and the sample was incubated for 10 minutes to extract the paraffin. The xylene was poured off and the extraction was repeated. Five milliliters of 100% ethanol were then added and the sample was incubated for 10 minutes. The ethanol was decanted and the ethanol extraction was repeated. The last traces of ethanol were removed by aspiration and the tissue was resuspended in 300 µl of Tissue and Cell Lysis Buffer 1 containing 1 µl of 50 mg/ml Proteinase K. The sample was incubated at 37°C for 30 minutes. The sample was then treated with 150 µl of Protein Precipitation Reagent, mixed by vortexing, and centrifuged for 10 minutes in a microcentrifuge. The DNA-containing supernatant was transferred to a clean microcentrifuge tube and 500 µl of isopropanol were added. The tube was inverted 30 times and then centrifuged for 10 minutes at 4°C in a microcentrifuge. The nucleic acid pellet was then washed twice with 70% ethanol and resuspended in 50 µl of TE buffer.

Table 1. Protocol for the Extraction of DNA from Paraffin-Embedded Tissue.

1. Weigh out 0.01-0.05 g of a 35 µm thick paraffin section.
2. Add 1-5 ml of xylene or Hemo-D (Fisher Scientific) to the paraffin section and

incubate for 10 minutes at room temperature to extract the paraffin. Pour off the xylene or Hemo-D.

3. Repeat step 2.
4. Add 1-5 ml of 100% ethanol and incubate for 10 minutes. Pour off the ethanol.
5. Repeat step 4.
6. Remove the last traces of ethanol by aspiration.
7. Continue with the MasterPure Complete protocol[2] for nucleic acid recovery. (Note: the standard protocol requires only a 15-minute incubation of the sample with Tissue and Cell Lysis Buffer, whereas a 30-minute to 18-hour incubation is required for paraffin-embedded tissue samples (Masterpure).

Factor V amplification using the isolated DNA

One microliter of the human genomic DNA sample (2% of the total isolated) was used to amplify a 267 bp region of the Factor V gene. The sequences of the primers used were: 5'-TGTTATCACTGGTGCTAA-3' and 5'-TGCCCAAGTGCTTAACAAGACCA-3'. The 50 µl reaction contained 1X MasterAmp™ PCR Optimization Kit PreMix B (1X PCR buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP) (Epicentre), 50 pmoles of each Factor V primer, 1 25 units of MasterAmp AmpliTherm™ DNA Polymerase (Epicentre), and 1 µl of the genomic DNA template. Forty cycles of amplification were performed with the following profile: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. Five microliters of the sample were separated on a 2% agarose gel and the gel was stained with ethidium bromide. Figure 1 shows that the 267 bp fragment was easily amplified (Shimizu, 1995).

3.Microdissected tissue section

Tissue sections need to be reviewed and annotated prior to microdissection to histologically identify the desired cells for microdissection. Therefore, an evaluation of the tissue samples by a pathologist or a scientist trained in histologic cell identification of frozen tissues is needed before, during and after microdissection. Also of importance is the orientation of the specimen in the tissue block. This is particularly important so that the cells of interest are adequately represented on the slide. Pathology slide review includes the evaluation of the tissue integrity, histopathology, determination of the adequacy of the sample for microdissection based on the amount of the target cell population, and annotation of the target cells on the slide. The pathologist also can give advice on the staining procedure that will help to better identify the cells of interest under the microscope during dissection.

- The goals of histopathology consideration before proceeding with tissue microdissection are: 1) to evaluate the total amount of tissue and the amount of the target cells in the tissue section present in the block, 2) to study the histopathology of the tissue specimen and identify the target cells, and 3) to plan the microdissection for each specimen, taking in account the heterogeneity of tissue samples.

- Always make a regular H&E slide for histopathologic analysis before proceeding with tissue microdissection. Traditional H&E staining (using longer times in each solution than H&E for LCM) and cover slipping of sections 1, 5, and 10 for histopathologic analysis are recommended prior to beginning the study. These sections will serve as a permanent record of tissue specimen status and show histologic changes that occur in the deeper sections.
- Always label the slides with the tissue block identification label, the number of the recut (e.g. 1, 2, 3, etc.), and the date the section was cut from the tissue block.

- If you are unsure of the tissue histology represented in the traditional H&Es, consult with a pathologist to review the slides to accomplish the general goals listed above.
- Keep the traditional H&Es used for histopathologic assessment with you when performing the tissue microdissection. These slides will help in identifying the cells of interest in the tissue section being used for microdissection. Both slides can be compared side by side.
- Only dissect cells that can be clearly identified. If there is any doubt, do not dissect it without consulting with a pathologist (Heidi, 2009).

PCR-in situ for Histopatology

The use of in situ RT-PCR to examine gene expression in disease tissues has certain advantages over more established hybridisation, PCR amplification or antibody-based techniques. As with immunohistochemistry, detection of gene expression is at the level of individual cells, but whereas polyclonal antibody production by immunisation may take 4 months or longer, and require extensive optimisation, it is relatively easy to characterise and optimise oligonucleotide primers which have considerably less chemical complexity and therefore, inherently more predictable properties. Moreover, while cross-reactivity is a frequent problem when selecting antibodies for protein detection, it is a simple matter to select PCR primers that are specific to a single member of a gene family, or even a particular splice variant of that gene (Heid, 1996).

We have successfully applied *in situ* RT-PCR to 1 mm paraffin-embedded tissue section arrays in order to determine which cells within a cancer are responsible for gene over-expression observed in RNA extracts. A number of technical manipulations were incorporated into the in situ protocol to ensure specificity and fidelity, and these transferred readily to the micro-array format. To our knowledge, this is the first time this procedure has been applied simultaneously to multiple samples in a microarray format.

A DNAse digestion step is commonly used in RT-PCR amplification in order to reduce the risk of spurious amplification of genomic DNA. This can also be carried out on tissue sections but the extensive incubation time required (up to 16 hr) means that considerable tissue autolysis occurs, damaging tissue structure and making post-PCR identification of cells difficult. In our protocol, the DNAse digestion step was omitted so as to better preserve tissue structure. Modifications to experimental design were employed to prevent amplification of genomic DNA. Although other approaches have been taken to obviate nuclease pre-treatment, we employed more conventional means. Firstly, primers were designed to amplify across two different exons, so that the amplified fragment from reverse transcribed, fully spliced mRNA would be small (300 bp), whilst the distance between the same primer sites in genomic DNA was over 3500 bp. Secondly, the number of PCR cycles and the duration of the polymerisation step were minimised so that any priming from genomic DNA would fail to achieve chain-reaction amplification. These strategies had a number of other beneficial effects. the PCR cycle number was kept with the linear range of amplification established by real-time quantitative RT-PCR, giving a more quantitative representation of the mRNA remaining in each cell, and avoiding significant synthesis of non-specific artifacts. Diffusion of reaction products away from the site of synthesis, another problem associated with *in situ* PCR, was reduced by this rapid procedure and exposure of the tissue sections to destructive conditions was also minimised, with the result that post-amplification staining revealed a high degree of preservation of tissue architecture and cellular features.

A consequence of using low PCR cycle numbers is that the degree of

amplification will be limited, with implications for detection of the PCR product. Standard peroxidase-linked antibody detection is insufficiently sensitive. Chemiluminescent or fluorescent detection reagents could be used instead to amplify the signal, but these would require specialised image detection systems and would rapidly diffuse away from the point of detection. Immunogold labelling followed by silver nucleation produced solid particles visible by light microscopy at magnifications suitable for visualising tissue and cellular features. This enabled simultaneous imaging of PCR products and hematoxylin-stained tissue details. The silver particles were bound to PCR products via anti-digoxygenin antibodies, and proved resistant to diffusion, remaining in the same cellular localisation as the original mRNA.

A persistent problem with the *in situ* PCR procedure has been inconsistency of results. Dedicated instrumentation has been designed with the aim of controlling conditions on a microscope slide, and some machines accommodate four or more slides to increase throughput and lower experimental variability. However, variation in the quality of paraffin-embedded tissue sections, and the number of steps involved in *in situ* PCR and the time taken to acquire data on significant numbers of samples affect the reproducibility of the technique. We found that a single, standard *in situ* PCR coverslip covered up to seventy 1 mm sections on a Clinomics tissue microarray, enabling simultaneous amplification of reverse transcribed RNA in each section under selected conditions. Although small tissue sections are more likely to become dislodged during the process of de-waxing and amplification, the use of poly-L-lysine coated slides decreased these losses and the cancer tissues examined were intrinsically more adherent due to their high cellularity. Thus a significant number of tissue samples could be analysed per single experiment. This approach substantially addresses the problem of slide-to-slide variability by subjecting large numbers of samples to identical experimental conditions. In addition, our technical modifications minimised tissue damage during preparation and amplification, preserving useful information on cellular morphology (Staecker, 1994).

REVERSE TRANSCRIPTASE (RT)-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of polymerase chain reaction (PCR), a laboratory technique commonly used in molecular biology to generate many copies of a DNA sequence, a process termed "amplification". In RT-PCR, however, an RNA strand is first reverse transcribed into its DNA complement (*complementary DNA*, or *cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR. Reverse transcription PCR is not to be confused with real-time polymerase chain reaction (Q-PCR/qRT-PCR), which is also sometimes (incorrectly) abbreviated as RT-PCR. RT-PCR utilizes a pair of primers, which are complementary to a defined sequence on each of the two strands of the cDNA. These primers are then extended by a DNA polymerase and a copy of the strand is made after each cycle, leading to logarithmic amplification.

RT-PCR includes three major steps. The first step is the reverse transcription (RT) where RNA is reverse transcribed to cDNA using a reverse transcriptase and primers. This step is very important in order to allow the performance of PCR since DNA polymerase can act only on DNA templates. The RT step can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used (Bustin, 2000).

The next step involves the denaturation of the dsDNA at 95°C, so that the two strands separate and the primers can bind

again at lower temperatures and begin a new chain reaction. Then, the temperature is decreased until it reaches the annealing temperature which can vary depending on the set of primers used, their concentration, the probe and its concentration (if used), and the cations concentration. The main consideration, of course, when choosing the optimal annealing temperature is the melting temperature (Tm) of the primers and probes (if used). The annealing temperature chosen for a PCR depends directly on length and composition of the primers. This is the result of the difference of hydrogen bonds between A-T (2 bonds) and G-C (3 bonds). An annealing temperature about 5 degrees below the lowest Tm of the pair of primers is usually used.

The final step of PCR amplification is the DNA extension from the primers which is done by the thermostable Taq DNA polymerase usually at 72°C, which is the optimal temperature for the polymerase to work. The length of the incubation at each temperature, the temperature alterations and the number of cycles are controlled by a programmable thermal cycler. The analysis of the PCR products depends on the type of PCR applied. If a conventional PCR is used, the PCR product is detected using agarose gel electrophoresis and ethidium bromide (or other nucleic acid staining).

Conventional RT-PCR is a time-consuming technique with important limitations when compared to real time PCR techniques. This, combined with the fact that ethidium bromide has low sensitivity, yields results that are not always reliable. Moreover, there is an increased cross-contamination risk of the samples since detection of the PCR product requires the post-amplification processing of the samples. Furthermore, the specificity of the assay is mainly determined by the primers, which can give false-positive results. However, the most important issue concerning conventional RT-PCR is the fact that it is a semi or even a low quantitative technique, where the amplicon can be visualised only after the amplification ends.

Real time RT-PCR provides a method where the amplicons can be visualised as the amplification progresses using a fluorescent reporter molecule. There are three major kinds of fluorescent reporters used in real time RT-PCR, general non specific DNA Binding Dyes such as SYBR Green I, TaqMan Probes and Molecular Beacons (including Scorpions).

The real time PCR thermal cycler has a fluorescence detection threshold, below which it cannot discriminate the difference between amplification generated signal and background noise. On the other hand, the fluorescence increases as the amplification progresses and the instrument performs data acquisition during the annealing step of each cycle. The number of amplicons will reach the detection baseline after a specific cycle, which depends on the initial concentration of the target DNA sequence. The cycle at which the instrument can discriminate the amplification generated fluorescence from the background noise is called the threshold cycle (Ct). The higher the initial DNA concentration, the lower its Ct will be (Innis, 1990).

QUANTITATIVE (QT)-PCR

Q-PCR used to measure the quantity of a PCR product (commonly in real-time). It quantitatively measures starting amounts of DNA, cDNA or RNA. Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample. Quantitative real-time PCR has a very high degree of precision. QRT-PCR methods use fluorescent dyes, such as Sybr Green, EvaGreen or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time. It is also sometimes abbreviated to RT-PCR (Real Time PCR) or RQ-PCR. QRT-PCR or RTQ-PCR are more appropriate contractions, since RT-

PCR commonly refers to reverse transcription PCR (see below), often used in conjunction with Q-PCR (Heid, 1996).

Conclusion

RT PCR is becoming a common tool for detecting and quantifying expression profiles of desired genes. The review itself indicates that the technology to detect PCR products in real-time, i.e., during the reaction, has seen a dramatic leap in use and application over the past couple of years. The RT PCR allows quantitative genotyping and detection of single nucleotide polymorphisms and allelic discrimination as well as genetic variation. Application of RT PCR combined with other molecular techniques made possible the monitoring of both therapeutic intervention and individual responses to drugs. RT PCR is a valuable methodic tool in clarifying such problems. The needs in clinical application of molecular methods initiated important developments in diagnostics stimulating progress in other branches of science. The introduction of these new methods in other fields of human practices induced rapid expansion of molecular approaches.

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