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IN SITU PCR. OVERVIEW OF PROCEDURES AND APPLICATIONS

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1. ABSTRACT

The evaluation of gene expression in the context of cellular morphology is essential to the full understanding of cell biology. A variety of methods for detection of nucleic acids are currently available. Solution PCR requires disruption of the sample and detection of the amplified material by electrophoresis in agarose gels. *In situ* Hybridization methods, on the other hand, permit morphological correlation and provide a high sensitivity that is sufficient for many applications. In some instances, however, the amount of target in the sample is below the limit of detection of this technique. *In situ* PCR allows the detection of minimal amounts of nucleic acids with exquisite sensitivity and

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specificity, while the integrity of the cells and the morphology of tissues remains preserved. This technique, although not exempt from difficulties, is undergoing methodological simplifications that will make it suitable for an increasing number of basic science and clinical applications. The following is a review of the principles, methods and applications of *In situ* PCR.

2. INTRODUCTION

The last decade has witnessed a continuous revolution in molecular biology methods and the parallel development of a competitive biotechnology industry that has made available high quality reagents and sophisticated equipments at a progressively lower cost. This has allowed the routine use, by many laboratories, of state-of-the-art procedures that would be otherwise restricted to highly specialized basic science research groups. The adaptation of some of these procedures, to the detection of nucleic acids in cells and tissues, converts genetic information into a visual signal that can be evaluated *In situ* while preserving cellular integrity and tissue morphology (1-16). This signal can then be evaluated in the context of a homogeneous or heterogeneous cell population, a given cellular compartment, or a particular pathological tissue change. *In situ* PCR is also known as PCR *In situ* (1), PCR ISH (2), *In-cell* PCR (3), and PCR-driven ISH (4), and for those modifications intended to amplify mRNA, RT *In situ* PCR (5) or *In situ* cDNA PCR (6) (appendix 1 and figure 1).

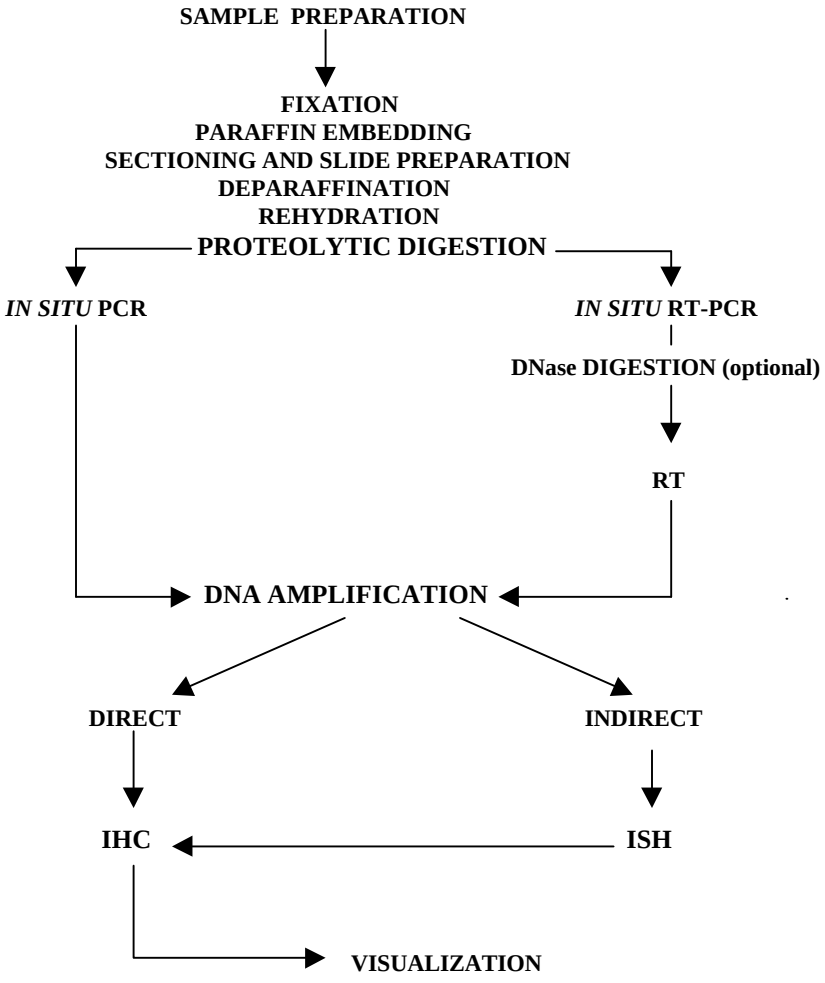

Figure 1. *In situ* PCR protocols for tissue sections

Abbreviations: PCR = Polymerase Chain Reaction, RT = Reverse Transcription, ISH = *In situ* Hybridization, IHC = Immunohistochemistry

3. GENERAL CONSIDERATIONS

3.1 Equipment

Thermocyclers are designed to reach and maintain the necessary temperatures in a reproducible manner over specified periods of time and predetermined numbers of cycles. Some of these instruments have independent "slide" blocks and "tube" blocks, connected to a common programmable controlling unit, providing the opportunity to run different protocols simultaneously and to correlate solution-PCR with *In situ* PCR results. The commercially available thermocyclers, use different mechanisms to prevent evaporation and increase efficiency and consistency of results. In the Omnislide Temperature Cycling System, Hybaid (Teddington, Middlesex, UK, http://www.hybaid.co.uk/), slides are placed in a sealed humid chamber. In the PTC-100-12MS Programmable Thermal Controller (MJ Research, Inc., Watertown, MA, USA, http://www.mjr.com/), the reagents are added directly to the amplification mixture to avoid evaporation. With the Gene-Amp *In situ* PCR System 1000 (Perkin-Elmer Corp., Norwalk, CT, USA, http://www.perkin-elmer.com/), up to 3 samples/slide can be tested by clamping slides individually using water-tight seals. Several cheaper ovens, in which *In situ* PCR can be performed, are also available. The BioOven from Biotherm Corp. (Fairfax, VA, USA), ensures temperature control via a sensor placed under the coverslip or glass slide that sends information to the control unit. Those that already have a conventional solution PCR thermocycler can also perform *In situ* PCR experiments by placing slides in a "tube" block. In these systems, however, heat transfer to the tissues is less reliable.

3.2 Technical aspects

The same precautions that apply to solution PCR procedures, should be exercised in *In situ* PCR experiments. Due to the extreme amplification capability of the *In situ* PCR, any small amount of contaminant DNA in the sample can be inadvertently amplified. During specimen handling, areas where amplified DNA (in particular previously amplified DNA) can be found should be avoided. Devices (positive displacement pipets, pipet tips containing sterile filters, tubes, etc.) have to be specifically allocated to *In situ* PCR experiments and frequent changes of gloves have to become routine. When working with tissue sections, appropriately coated slides (i.e., poly-L-lysine, Aminosilane) should be used to avoid the detachment of sections during the proteolytic digestion step (16-17). To avoid evaporation, a variety of methods have been attempted. The section is overlaid with the PCR mixture and a coverslip is placed over the section. The coverslip may be sealed with nail polish or rubber cement making sure not to introduce bubbles. After amplification, the seal is peeled-off with forceps and the coverslip removed in 0.1X SSC. Alternatively, specially designed cones (1, 8) are applied to the slide sealing the area of interest. The amplification solution is added to the cones making sure that it diffuses evenly over the tissue section without creating air bubbles. If the operator does not have previous experience with the procedure, it is advisable to attempt several dry runs to get acquainted with the peculiarities of the technique (slide manipulation, microscope checks, rubber cement and nail polish application and removal, transfers in and out of the thermocycler, etc.).

3.3 Types of samples (appendix 2)

In situ PCR has been successfully performed on a variety of samples: cell smears, imprints, cell blocks, cytospins, metaphase chromosomes, frozen sections and sections of tissues embedded in plastic or paraffin. Haase *et al* (14) performed *In situ* PCR on fixed cells suspended in the PCR reaction mixture within a microcentrifuge tube. In this procedure, after amplification, cells are recovered by cytocentrifugation onto glass slides (3, 4, 8, 12, 14). An advantage of this method is that cells may be lysed after amplification and the lysate analyzed by gel electrophoresis and Southern blot hybridization (14, 15). Good results can also be obtained in cytospins and cell blocks prepared following standard protocols. When tissues are used, 4-6 micron thick sections are prepared from paraffin or frozen tissue blocks. The morphological changes induced by freezing, and the increase rate of diffusion of the amplified product, make frozen sections less desirable for *In situ* PCR purposes. Although thicker sections may compromise evaluation of morphology and increase noise to signal ratio, it is believed that thick paraffin sections contain more starting nucleic acid material and allow a better yield of amplified signal. Deparaffinization is performed on sections cut from paraffin blocks, according to standard procedures by removing paraffin in xylene and rehydration in decreasing concentrations of ethanol.

3.4 Fixation (appendix 2)

The goal of fixation is to maintain optimal tissue morphology while preserving the integrity of the nucleic acids. It is generally agreed that aldehyde-based fixatives, such as 10% neutral buffered formalin, are superior for preservation of morphology and for minimizing the diffusion of signal (17-20). This is fortunate since the vast majority of archival material in pathology departments has been fixed in 10% neutral buffered-formalin. Nevertheless, not all routine procedures for fixation of specimens are done under ideal standard conditions and, for most protocols, a variety of proteolytic digestion conditions has to be tested. This is due, in part, to cross-linkages between proteins, or between histones and DNA, which are difficult to remove and may impair the efficiency of PCR. Furthermore, aldehyde-based fixatives may induce single-stranded breaks that, when repaired by the DNA polymerase, result in false positivity. Fixatives containing picric acid (Bouin's solution) or mercury (Zenker's solution) seem to degrade nucleic acids and generally are not recommended (17-22). Tissues, preferably less than 0.5 cm in thickness, are fixed at room temperature in 10 % Neutral Buffered Formalin. Most cell suspensions and cytology preparations are routinely fixed in 95 % alcohol for 1 hour either at 4^{o} C or at room temperature. They can be washed in PBS and kept at 4^{o} C for short-term storage or dehydrated and placed at -70^{o} C for long-term storage. Paraformaldehyde also provides excellent results, being the fixative of choice in many laboratories (21, 22). It can be used at concentrations ranging from 1 % to 4 % during a period of 4 to 24 hours, preferably at 4^{o} C. Fixation has to be initiated as early as possible to reduce the chance of

nucleic acid degradation.

3.5 Proteolytic Digestion (appendix 3)

Since fixatives cross-link proteins and induce other changes that diminish the accessibility of reagents to the target, pre-treatment with proteases is necessary, particularly for paraffin-embedded, formalin-fixed tissues (17-19, 21-23). Stringent control over protease digestion is required to maintain preservation of morphology and to reduce noise to signal ratio. Therefore, a balance has to be achieved to avoid false negativity, due to poor penetration of reagents in underdigested samples, and destruction of the tissue architecture due to over-digestion by protease. It is considered that the length of proteolytic digestion correlates with the duration of fixation (8, 19). As a guideline, 10 min of digestion is necessary if tissue was fixed for 4 hours in 10 % neutral buffered formalin. On the other hand, 90 min of protease digestion may be necessary if tissue was fixed for 24 h. Bagasra *et al*. (15), have reported that the presence of "peppery dots", observed on the membrane of digested cells, can be used as evidence of appropriate digestion for *In situ* PCR. In all cases, immediately after digestion, the enzyme should be inactivated.

3.6 Additional pre-treatments (appendix 4)

After proteolytic digestion, additional pretreatment steps may be necessary. When labeled nucleotides are used, reduction of the static charge of tissue sections can minimize nonspecific binding. This can be accomplished by washing the slide for five minutes in a solution of 0.1M Triethanolamine and 0.25 % acetic anhydride. If peroxidase is going to be used in the detection step, endogenous peroxidase should be quenched by any of the routinely used methods. In *In situ* RT-PCR it may be necessary to pre-digest the sample with RNase-free DNase, to destroy endogenous DNA.

4. POLYMERASE CHAIN REACTION (PCR)

Prior to attempting *In situ* PCR protocols, it is important to have an appropriate level of knowledge and expertise in solution PCR methodologies. This is due to the fact that the conceptual background is the same for both techniques. It is also highly desirable to test reagents and conditions by PCR in solution (*in vitro*) as part of the necessary preliminary control process. It is of no use to spend time and resources optimizing *In situ* PCR if one has not confirmed that primers and amplification conditions work as expected, preferably on the nucleic acids extracted from the sample.

4.1 Polymerases (appendix 5)

A variety of polymerases with different properties are commercially available. Ideally, the selected enzyme should work effectively at a given temperature and retain its enzymatic activity at temperatures higher than 95^{o} C . It is important to follow the conditions suggested by the supplier, since, depending on the vendor, the same enzyme may have different assay requirements. In general, 0.5-2.5 units of DNA *Taq* polymerase in a 100 microliter reaction is appropriate (21-24). However, based on the type of targets or primers, the concentration of the enzyme may vary. It is recommended to test concentrations ranging from 0.5 to 5 units/100 microliter.

4.2 Deoxynucleotide triphosphates (dNTPs) (appendix 5)

Equivalent concentrations of all four dNTPs in the range of 20-200 microM are recommended (25-30). Mispriming may be minimized, and sensitivity maximized, by reducing the concentration of the dNTPs (26-30). Typically, in a 100 microliter reaction, a dNTP concentration of 20 microM, will allow the synthesis of 2.6 micrograms of DNA or 10 pmol of a 400 bp sequence (25-27).

4.3 Buffer conditions (appendix 5)

It is important to include the appropriate metallic ion in the amplification buffer and to use the enzyme at optimal pH. In general, as compared with solution PCR, increased concentrations of DNA polymerase and Mg^{2+} ions are required. This is probably related to the sequestration of reagents on slides or due to the presence of inhibitors in the tissue sample (24). The concentration of ions is one of the critical factors in PCR experiments and it has to be optimized to the particular assay conditions. $MgCl_2$ is commonly used at a concentration of approximately 2-5mM.

4.4 Primers

Primers are usually designed to be 18-28 nucleotides in length, with a balanced G/C and A/T ratio, no complementarity between their 3' ends (to avoid primer-dimer formation) and with a low probability of internal secondary structure (23-26). Concentrations between 0.1 and 1.5 microM are generally optimal. The melting temperature (T_m) of oligonucleotides ranging in length from 14-70 bases can be calculated acording to the following formula: T_m in ^{o}C = 2^{o}C (#A+#T) + 4^{o}C (#G+#C) (24).

4.5 Cycling Profile (appendix 5)

For optimal binding of the primers to the DNA, separation (denaturation) of the two strands has to be complete. Incomplete "denaturation" is a common cause of PCR failure (25-30). Typically, denaturation is achieved at 95^{o} C for 30 seconds or at 97^{o} C for 15 seconds, although higher temperatures may be necessary for G+C rich targets. Higher temperatures and longer times may lead to the loss of enzyme activity since the half-life of *Taq* polymerase is approximately 5 minutes at 97^{o} C, and 40 minutes at 95^{o} C (24, 28-30). Annealing temperature and annealing time depend upon the base composition and length and concentration of the primers (26-30). It is generally agreed that an annealing temperature of 5^{o} C below the true T_m of the amplification primers is appropriate and temperatures in the range of 55^{o} C to 72^{o} C yield the best results. The primer extension step (elongation) is usually performed at 72^{o} C for 20 seconds to one minute. Using typical concentrations of primers (0.2 microM), annealing will require a few seconds. However, a longer extension time may be required at the beginning of the cycling when the substrate concentration is low or at later cycles when substrate concentration exceeds the enzyme concentration. Some investigators report that longer extension times (e.g., 2 min) are necessary (30) while others omit the extension step since annealing seems to be the

rate limiting factor (2, 31-33). The reaction is maintained at 72° C for 5-15 minutes to allow completion of partial extension and annealing (25-29). It is believed, however, that *In situ* DNA amplification occurs at a low efficiency. It is generally estimated that in 30 cycles, even under the best conditions, amplification of DNA does not exceed 50-100 fold, in suspended cells, and it may be even lower on tissue sections and cytospins (20-23). The reasons are not clearly understood although cross-linking of histones to DNA, single-stranded DNA breaks, and sequestration of DNA polymerases and other reagents on the surface of slide, have all been proposed as potential causes (24). When all parameters are optimized, the number of cycles will primarily depend upon the starting concentration of DNA (26-30). It has been estimated that if the starting number of target molecules is 300,000, then 25-30 cycles of amplification would be appropriate. On the other hand, if only 50 target molecules are present, 40-45 amplification cycles may be necessary (30-32). Some investigators, however, reported poor results, with more than 20 cycles, in *In situ* PCR experiments (4).

5. *IN SITU* PCR PROTOCOLS

In "direct" methods, labeled nucleotides (i.e., digoxigenin-11-dUTP, fluorescein-dUTP) are incorporated during the amplification step and then amplified product are detected by immunohistochemistry. In "indirect" methods, the amplified products are detected by *In situ* Hybridization (ISH) using a specific labeled probe.

5.1 *In situ* RT-PCR (appendix 6)

Since mRNA cannot serve as a template in PCR, a reverse transcription step is introduced to convert mRNA to cDNA (1-10). The combination of these two techniques is referred to as RT-PCR. RT-PCR may be used for the detection of mRNAs that are present at less than 10 copies/cell (1-12). The enzyme that converts RNA into c-DNA is "Reverse Transcriptase". Three Reverse Transcriptases commercially available are: the mesophilic viral reverse transcriptases from Avian Myeloblastosis Virus (*AMV*) and Moloney Murine Leukemia Virus (*M-muLV*), and *rTth*, a heat-stable DNA polymerase from *Thermus thermophilus*, that can be used to perform reverse transcription and PCR in a single step. The *AMV* and *M-muLV* RTs can reverse transcribe mRNAs up to 10 kb; *rTth* synthesizes cDNAs in the range of 1-2 kb. The most important factors to consider in the selection of the enzyme are: a.) RNAse H activity that degrades RNA in an RNA:cDNA hybrid, b.) temperature (*M-muLV* reaches maximum activity at 37° C and *AMV* at 42° C); *rTth* reverse transcribes mRNA at 60-70° C and amplifies cDNA at 60-94° C), and c.) divalent ion requirements (*rTth* requires manganese). Three types of primers can be used in reverse transcription reactions: a.) Oligo(dT)12-18 (it binds to the endogenous poly (A)+ tail of mRNA), b.) random hexanucleotides (these bind at any complementary sequence throughout the length of mRNA), c.) specific oligonucleotides (based on a specific mRNA sequence).

5.2 Direct *In situ* PCR (appendix 7)

In direct *In situ* PCR, the label, digoxigenin or biotin, is incorporated, into the amplified product, during the PCR step (32-36). Digoxigenin is present in *Digitalis* plants and therefore is not detectable in any other biological material. High affinity, unconjugated, anti-digoxigenin antibodies and anti-digoxigenin antibodies conjugated to alkaline phosphatase, peroxidase, fluorescein or rhodamine, are readily available (37). Biotin is a member of the vitamin B complex and can be detected with an anti-biotin antibodies (37). The final result is the enzymatic localization of a chromogen at the site of DNA amplification. Due to its simplicity, direct *In situ* PCR represents a logical procedure of choice. However, in the experience of many investigators (5, 8, 24, 35), direct detection procedures tend to yield false-positive results (35-36) that may be due to either nonspecific incorporation of labeled nucleotides into fragmented endogenous DNA undergoing "repair" by the DNA polymerase (DNA-repair artifact), or nonspecific priming by cDNA or DNA fragments (endogenous priming) (35-36). The false positive signal is typically present in the nucleus, particularly in apoptotic and senescent cells where DNA fragmentation occurs (26, 29-31). This endogenous DNA amplification is difficult to avoid, even by DNAse pretreatment (5, 35, 36). Several alternatives to reduce the artifact have been attempted with only partial success. These include the use of exonuclease-free DNA polymerase (5, 36-38), repair of DNA nicks by treatment with T4 ligase (39-44), or initial thermal cycling using unlabeled nucleotides (44, 48).

5.3 Indirect *in situ* PCR (appendix 8-9)

The indirect method, although more cumbersome, provides an extra level of specificity since the probe is designed to be complementary to an internal sequence within the amplified product (39, 48, 49). Double-stranded DNA, single-stranded DNA, oligonucleotides 20-30 bases long, and single-stranded RNA have all been successfully used as probes for the detection of amplified products (30). Hybridization of probes to the amplified product follows the general rules described for *In situ* Hybridization (ISH) procedures (50). The kinetics of the reaction are influenced by: a.) accessibility of the target (in *In situ* PCR methods, the target is readily accessible because hybridization takes place after DNA amplification), b.) concentration of the probe, and c.) stringency of hybridization. A series of parameters influence the stability of the hybrids: a.) T_m (formamide decreases the T_m allowing use of lower temperatures to achieve a higher stringency), b.) base composition (the greater the G+C content the higher the T_m), c.) sequence identity between the probe and the target, and d.) composition of hybridization/washing solution (higher concentrations of monovalent cations increase the stability of hybrids). The final sensitivity depends on the method of probe labelling and detection. Labelling of the probe can be done with radioactive labels ([^{3}H], [^{35}S], [^{32}P], [^{33}P]. Factors to be considered are cost, instability, and biohazard potential of the radioisotope

used. Alternatively, the probe can be labeled with non-radioactive haptens, such as biotin, digoxigenin or FITC. The efficiency of the non-radioactive detection methods has been recently improved using immunogold silver detection methods (38, 51).

6. CONTROLS

In *In situ* PCR protocols, it is imperative to control the numerous steps involved, not only to interpret a given signal in the proper context of sensitivity and specificity, but also to identify and correct some of the many potential pitfalls that are likely to occur (appendix 6).

6.1 Causes of false negativity.

Poor thermal conduction by the slide or the block, uneven convection, adsorption of reagents to the glass, presence of DNA polymerase inhibitors, evaporation, excessive washing, and leakage of reagents, can all account for false negativity or inconsistency in the results (43-48). Control for some of these steps should be done during the optimization of the technique. Since most *In situ* PCR protocols are designed to detect nucleic acids within cells placed on slides, the starting material may be present in low amounts (43-45) and uncontrolled fixation of archival material may have further reduced the nucleic acid content, compromising the sensitivity of the procedure. DNA extracted from paraffin blocks is often shorter than 400 bp and mRNA fragments, in paraffin blocks, are usually smaller than 200 bp (1-10, 43-45). Although, some authors have been able to obtain full length DNA from paraffin-embedded material (2, 12-14), other investigators have succeed with *In situ* PCR only when using multiple primers designed to produce short PCR products and relatively long DNA probes or cocktails of oligonucleotide probes (8, 43-45). To ensure that a negative result is not due to poor penetration of reagents and accessibility of the target, it is advisable to include a control sample in which a sequence, such as actin, is amplified.

6.2 Causes of False Positivity.

Specificity can be tested by recovering the product from the slide and analyzing it by gel electrophoresis. In indirect methods, the ISH step introduces an extra level of specificity, determined by the probe. Furthermore, another slide hybridized with an irrelevant probe should give no signal. "Nested" PCR can increase the specificity of PCR by using a second set of primers to amplify sequences within the first amplicon (46-48). Furthermore, testing, in the absence of PCR components (enzyme, primers or probe), should be done to make sure that the detection system does not render a non-specific signal. In experiments with virally-infected cells, it is advisable to mix infected and non-infected cells, at different ratios, and to correlate the results of the test with the expected sensitivity.

6.3 DNA-repair.

Non-specific incorporation of labeled nucleotides may occur as a consequence of "repair" of DNA breaks by DNA polymerase (44). Excessive protease digestion (removal of histones), insufficient DNAse treatment (DNase may break DNA into oligonucleotides), poor fixation, suboptimal processing of the sample, apoptosis, or previous irradiation of the specimen can all induce DNA breaks. The slide, in which the DNA polymerase is omitted, however, should not show this artifact. This type of false positivity is extremely difficult to eradicate but it may be partially reduced by using, exonuclease-free, DNA polymerase or by performing several amplification cycles in the absence of the labeled nucleotide (44, 45).

6.4 Mispriming.

The frequent amplification of "non-desired" sequences seems to be dependent upon factors such as specificity of primers, pH and ion concentration in the PCR mixture, and annealing temperature (1-8). Primers should be designed to detect short template sequences avoiding homology to non-desired sequences and between themselves. Since misprimimg occurs at lower melting temperatures, withholding the *Taq* polymerase, until 55^{o} C is reached reduces non-specific amplification without compromising the specific primer-target annealing. This "hot-start" procedure has been successfully applied to *In situ* PCR (46-48). An alternative method has been developed which takes advantage of the affinity interaction of *Taq* and Anti-*Taq* mAb (46). This interaction initially blocks the activity of the enzyme but the complex dissociates, at higher temperatures, freeing the enzyme at the appropriate time for specific amplification. In the hands of some researchers, this method has provided results similar to hot-start technique with less manipulation of the sample (40-42). Other “cold-start” procedures, using *E. coli* single-strand-DNA-binding protein SSB, or T4 gene protein, at ambient temperatures, have been devised (1, 5, 8, 40, 42, 48, 50-53).

6.5 Endogenous priming.

This artifact, thought to occur due to priming of endogenous DNA and cDNA fragments, is difficult to eradicate and therefore the use of specific primers is imperative (1, 8).

6.6 Diffusion of the PCR products.

Excessive protease digestion (loss of cellular boundaries) and excessive number of amplification cycles may contribute to the diffusion of the amplified product (8). If a product diffuses out of its original location, it may be preferentially amplified, producing a signal in a different cellular compartment or even in an extracellular location with the possibility of "labeling" adjacent negative cells (8, 12, 21). Proper processing of the sample, use of aldehyde-based fixatives, low number of cycles, generation of longer amplicons or more “bulky” products (21, 41), or the use of direct methods (incorporation of digoxigenin) may all reduce this type of false positivity (1, 4, 12, 48).

7. APPLICATIONS

Despite technical difficulties, *In situ* PCR protocols are being utilized in an increasing number of applications. The technique has particular potential in areas such as

embryogenesis, organogenesis, infectious diseases, genetics, immunology, neoplasia or pathology. New genes are continuously being added to the list of target sequences, detected by *In situ* PCR, that includes infectious agents such as human immunodeficiency virus (HIV-1), simian immunodeficiency virus (SIV), human papilloma virus (HPV), hepatitis B virus (HBV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpes virus-6 (HHV-6) and herpes simplex virus (HSV), and molecules such as p53, surfactant protein A, estrogen receptor, growth factors, growth factor receptors, transferrin, and adrenomedullin (88-110). Almost every tissue has been successfully tested (3, 48-57).

One of the areas where *In situ* PCR methodology has proven its value is the detection of viral sequences that are present in vanishingly small numbers (48-57). This is particularly true for slowly progressing viral diseases. Among these are the group of lentiviruses of which HIV is a member where *In situ* PCR has provided a ten-fold increase in sensitivity over the conventional techniques (51-53). Furthermore, the Creutzfeld-Jacob virus of Progressive Multifocal Leukoencephalopathy has been demonstrated by *In situ* PCR in an archival material from 1958, confirming previous solution PCR data and demonstrating that DNA sequences are preserved for long periods of time in routinely processed tissues (57). The increased sensitivity of *In situ* PCR offers the opportunity for viral latency studies, overcoming the need to activate the viruses *in vitro* prior to detection. For example, Herpes Simplex Virus was detected in the trigeminal ganglia of latently infected mice. *In situ* PCR revealed three times more infected neurons than it was shown previously (56).

A fast, alternative method to *In situ* Hybridization, Primed *In situ* DNA Synthesis (PRINS) (60, 61) involves annealing of oligonucleotides or short DNA fragments to denatured complementary nucleic acid sequences (i.e., metapahase or interphase chromosomes) on slides. A thermostable DNA polymerase incorporates nonradioactive labeled nucleotides that are detected by immunofluorescence. A variety of suitable primers for chromosomal sequences are available. A further increase in sensitivity has been achieved with Cycling PRINS. To detect low copy or unique sequences, the PRINS technique can be modified by subjecting the chromosomal preparation to amplification cycles that incorporate biotin or digoxigenin-labeled nucleotides. Detection can be accomplished by immunofluorescence with suitable antibodies.

A related technique, Self-Sustained Sequence Replication-Based Amplification (3SR) (63, 64) is a reiterated cycling of reverse transcription and amplification reactions, catalyzed by AMV-RT and T7 RNA polymerase, intended to replicate a RNA target via RNA/DNA hybrids and double-stranded cDNA intermediates. The cDNA copy of the original mRNA incorporates a T7 RNA polymerase promoter that is used as template for a 10 million fold amplification within 2 h. The destruction of RNA in RNA/DNA hybrids by E. coli RNAseH eliminates the need for denaturation steps.

Obstacles to the wide application of *In situ* PCR have been low amplification efficiency, poor reproducibility, and difficulty in quantitation (21). Improvements in the technology, however, are likely to reduce these drawbacks. Newer reported systems, such as the nanogold-silver detection (51) or the catalyzed reporter deposition methods (37) may reduce background. Another innovative procedure takes advantage of a fluorochrome-labeled oligonucleotide probe, specific for a region of the amplified DNA between the PCR primer sequences, that is designed to emit fluorescence signal only after hybridizing to the appropriate template (4,21).

It is expected that *In situ* PCR protocols will be further simplified in the near future and that this methodology and its variants, despite limitations, will find a deserved place in the repertoire of methods available to the researchers.

8. ACKNOWLEDGEMENT

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10. APPENDICES

Appendix 1 – General information

A typical *In situ* PCR protocol for the detection of DNA (i.e., of viral origin), in tissue sections, will start with the collection and preparation of the sample. This requires optimal fixation of the tissue, followed by tissue embedding, generally in paraffin. The tissue is then sectioned and placed onto a slide. Standard deparaffination protocols are followed by enzymatic digestion to allow penetration of reagents. The tissue is covered with a PCR solution containing specific primers, DNA polymerase, and a mixture of nucleotides in an appropriate buffer. Amplification of the desired nucleic acid sequences is achieved using a cycling profile determined to be optimal for the particular conditions of the experiment. Detection of the amplified material can be done indirectly, by *In situ* Hybridization, using a labeled specific probe or, directly, by incorporating a labeled nucleotide during the amplification step. The label generally used is digoxigenin which can be detected by an immunoenzymatic method. A colored product is deposited at the site of the amplified nucleic acid sequences. If the sequence to be detected is mRNA, prior to the *In situ* PCR, the tissue section may be exposed to DNase, to destroy the endogenous DNA. In a reverse transcription step, mRNA is converted into c-DNA that is further amplified and detected as described.

Appendix 2 - Sample preparation

Cells

1. Wash cells three times x 5 minutes in PBS at 4^{o} C
2. Resuspend cells in PBS at the desired concentration (i.e., 2 million cells/ml)
3. Add 50 microliters onto a slide and air dry or prepare cytospin
4. Fix in 95 % ethanol, 4 % paraformaldehyde or 10 % neutral buffered formalin x 2-15 hours
5. If slides are to be stored, air dry after dehydration and rehydrate prior to use
6. Prior to proceeding with the protocol, wash in PBS and water

Frozen sections

1. Place sections, 5-10 microns in thickness, onto appropriately coated slides
2. Wash twice with PBS at 4^{o} C
3. Fix in 4% paraformaldehyde or 10 % neutral buffered formalin x 2-18 hours
4. Dehydrate through ethanol, air dry, and store at 4^{o} C
5. Rehydrate prior to use

Paraffin-embedded tissue sections

1. Prepare 4-5 microns tissue sections onto coated glass slides
2. Adhere section to slide by heating on a 60^{o} C hot plate for 12 hours
3. Deparaffinize and rehydrate following standard protocols
4. Incubate slides in 0.02M HCl for 10 minutes
5. Wash slides twice in PBS for 5 minutes each
6. Inmerse slides in 0.01 % (v/v) Triton-X 100 in PBS for 2 minutes
7. Wash slides twice in PBS for 5 minutes each
8. Proceed with proteolytic digestion (appendix 3)

Appendix 3 - Proteolytic digestion

Materials - Proteolytic solutions

Proteinase K: 10 to 20 micrograms/milliliter in 0.1M Tris-HCl pH 7.5, 5mM EDTA x 20-30 minutes at 37^{o} C(aliquots can be stored at -20^{o} C). Stop activity with a solution of 20 % acetic acid at 4^{o} C x 10 seconds or with 0.1 M Glycine in PBS x 5 min.

Trypsin: 0.1 % solution in 0.4 % calcium chloride x 30 minutes at room temperature. Inactivate in 0.1 M Tris at pH 7.4.

Pepsin: 0.4 % in 0.04M hydrochloric acid x 30 minutes at room temperature.

Method

1. Incubate sections in buffer at 37° C
2. Add proteolytic solution, coverslip and digest
3. Remove coverslip and inactivate enzyme
4. Wash in PBS and water
5. Dehydrate to 100 % ethanol
6. Proceed with protocol or store in 100 % ethanol at 4° C until use

Appendix 4 – Additional pretreatments

Materials

DNAse I (Life Technologies, Gaithersburg, MD) 0.1 U/microliter in 20 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, and 100 micrograms/milliliter BSA w/v. Incubate overnight at room temperature, in a humidified chamber. Stop reaction with 20 mM EDTA for 10 min at 65° C plus three 10 minutes washes in 50 mM Tris-HCl, pH 7.5.

RNAse-free DNAse (Boehringer Mannheim, Indianapolis, IN) 1 U/microliter in a solution containing 40 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, and 2 mM $CaCl_2$. Incubate overnight at 37° C in a humidified chamber. Rinse slides for 1 min in DEPC-treated water, wash x 1 min in 100 % ethanol and air dried.

Method

1. Incubate sections in DNase solution (i.e.,100 microliters of solution containing 8U RNase-free DNase/75U RNasin at 37° C for 30 min).
2. Inactivate enzyme, wash slides and proceed with protocol

Appendix 5 - RT-PCR

Materials

Components of Reverse Transcription solution

Reagent	Final Concentration
RT Buffer	10 mM Tris, 50 mM KCl (when AMV reverse transcriptase is used) 50 mM Tris-HCl pH 8.3, 40 mM KCl (when M-MLV reverse transcriptase is used) 20 mM Tris-HCl pH 8.4, 50 mM KCl (when Superscript II reverse transcriptase is used)
$MgCl_2$	2.5 mM - 6 mM
dNTP Mix	1 mM (each)
Primer (select one)	
Oligo-p(dT)15	80 nanograms/microliter
Random p(dN)6	160 nanograms/microliter
Sequence-specific	1.0 miligrams/microliter
RNAse inhibitor	0.5 units/microliter
Reverse Transcriptase	1 unit/microliter

Components of the amplification solution in single-step RT-PCR

Reagent	Final Concentration
Buffer	50 mM Bicine, pH 8.2; 115 mM KOAc; 8 % (v/v) glycerol (*) 50 mM Tricine-KOH, pH 8.3; 100 microM (**)
$Mn(OAc)_2$	55 mM (*), 10mM (**)
rTth enzyme	1 unit/10 microliters (*) (**)
dNTPs	200 microM (each)
Dig11-dUTP	100 microM
Primer #1	1.25 microM
Primer #2	1.25 microM
RNAse Inhibitor	25U/10 microliter

(*) Perkin Elmer, Norwalk, CT (http://www.perkin-elmer.com/),
(**) Boehringer Mannheim, Indianapolis, IN (http://www.biochem.boehringer-mannheim.com/)

Method

1. Add 100 microliter reaction mixture containing appropriate number of units of reverse transcriptase in 1X RT buffer and incubate at an appropriate temperature (i.e. 37-42^{o} C) for 30 minutes to 1 hour
2. Wash with 1X SSC
3. Inactivate room temperature by heating to 95^{o} C for 5 minutes
4. Wash five times with 1X SSC
5. Add 100 microliters of PCR amplification solution
6. Cover the slide with a glass coverslip and seal it with nail polish to prevent evaporation and reagent loss
7. Allow nail polish to harden for 5 minutes
8. Proceed with amplification per protocol (specific for the experiment)
9. Remove coverslip with a scalpel and scratch out remaining nail polish with a razor blade
10. Wash slides in 0.1M Tris-HCl, pH 7.4 twice for 5 minutes each
11. Dip slides in 100 % ethanol for 10 minutes to immobilize amplified products
12. Rehydrate in 80 % ethanol and wash in 0.1M Tris-HCl, pH 7.4, twice for 5 minutes
13. Proceed with detection

Appendix 6 - Polymerase chain reaction

Materials

Brief description of the DNA polymerases commonly used in PCR Protocols

Enzyme	Origin	pH	Temperature	Ion concentration	dNTP concentration
Taq (*) Polymerase 1.0-5 U/assay	Thermus Aquaticus	8.9	75^{o} C	$MgCl_2$ 1.5 mM	200 microM
Pwo (*) Polymerase 1.0-3 U/assay	Pyrococcus woesei	8.9	75^{o} C (**)	$MgSO_4$ 2 mM	200 microM
RTth Polymerase 1.0-5 U/assay	Thermus Thermophilus	8.9	70^{o} C (***)	(***)	200 microM

Abbreviations: **dNTP**: Deoxynucleotide triphosphate, **U**: units of enzymatic activity, **C**: Degrees in Celsius,
rTth: recombinant *Tth*, (*) *Taq* and *Pwo* Polymerases have been combined (Expand Long Template PCR system) to amplify up to 40 kb on lambda DNA and 27 kb on human genomic DNA. (**) Greater thermal stability (half-life >2hours at 100^{o} C) than *Taq* Polymerase (>5 min at 100^{o} C). *Pwo* is 10 times more accurate than *Taq* Polymerase due to an inherent 3'-5' exonuclease proofreading activity. (***) *rTth* has polymerase activity in the presence of magnesium ($Mg Cl_2$,1.5 mM) and intrinsic reverse transcriptase activity in the presence of manganese ion ($Mn(OAc)_2$, 2.5 mM).

Method

1. Remove slides from ethanol and rehydrate in water
2. Place approximately 30-50 microliters of amplification cocktail onto the slide
3. Cover slide with a glass coverslip and seal with nail polish to prevent evaporation and reagent loss.
4. Allow nail polish to harden for 5 minutes
5. Proceed with amplification protocol (specific for the experiment)

Typical amplification profile

Step	Temperature and Time	Number of cycles
Denaturation	94^{o}C x 5 minutes	1
Annealing + extension (according to Tm)	90 seconds	1
Denaturation and Annealing + extension (according to Tm)	94^{o}C x 20-40 seconds 90 seconds	30 cycles

Appendix 7 - Direct *In situ* PCR

Materials.

Components of the amplification solution in Didirect *In situ* PCR

Reagent	Final concentration
Forward (5') Primer	1.25 microM
Reverse (3') Primer	1.25 microM
dNTPs	200 microM each
Tris-HCl, pH 8.3	10 mM
KCl	50 mM
$MgCl_2$	2.5 mM
Labeled nucleotide (Digoxigenin-11-dUTP or Bio-14-dATP)	100 microM
Taq DNA polymerase	2 U/microliter
Gelatin	0.001 %

Components of GeneAmp *In situ* PCR Core Kit (Perkin Elmer)

Reagent	Final concentration
10X PCR Buffer	1X
dATP	200 microM
dCTP	200 microM
dGTP	200 microM
dTTP	200 microM
Primer 1	0.2 – 1.0 microM
Primer 2	0.2 – 1.0 microM
$MgCl_2$	2 – 4.5 microM
AmpliTaq	10 U
Biotin-11-dUTP	2.5microM
H2O	as needed

Method

1. Wash briefly in 2XSSC at 40^{o} C x 10 minutes
2. Wash in PBS x 2 minutes and then water
3. Dip slides in 100 % ethanol for 10 minutes to immobilize amplified products
4. Rehydrate in 80 % ethanol and wash in 0.1M Tris-HCl, pH 7.4, twice for 5 minutes each

Sections recommended as controls and for optimization of direct i*n situ* RT-PCR

Slide No.	PK	DNAse	RT	Primers	Taq Polymerase	Detection Reagents	Signal Specific
1	+	+ (*)	-	+	+	+	No
2	+	+	+	-	+	+	No
3	+	-	-	-	+	+	No
4	+	+	+	+	-	+	No
5	+	+	+	+	+	+	Yes (test)
6	+	+	+	+	+	+	Yes (+ control)
7	+	+	+	+	+	+	No (- control)
8	+	+	+	+ (**)	+	+	Yes

Abbreviations: PK = Protease, RT = Reverse Transcription, PCR = Polymerase Chain Reaction, + = The reagent is added, - = The reagent is omitted, (*) Optional, depending on the type of target, (**) Specific primers for the house-keeping gene.

Appendix 8 - Indirect *in situ* PCR Protocol

Materials

Components of the amplification solution in Indirect *In Situ* PCR

Reagent	Final concentration
Forward (5') Primer	1.25 microM
Reverse (3') Primer	1.25 microM
Unlabeled dNTPs	200 microM each
Tris-HCl, pH 8.3	10 mM
KCl	50 mM
$MgCl_2$	2.5 microM
Taq DNA polymerase	0.2 U/microliter
Gelatin	0.001 %

Components of GeneAmp *In Situ* PCR Core Kit (Perkin Elmer)

Reagent	Final concentration
10X PCR Buffer	1X
dATP	200 microM
dCTP	200 microM
dGTP	200 microM
dTTP	200 microM
Primer 1	0.2–1.0 microM
Primer 2	0.2-1.0 microM
$MgCl_2$	2– 4.5microM
AmpliTaq	10 U
Biotin-11-dUTP	2.5microM
H2O	as needed

(*) = 500 mM KCl + 100 mM Tris-HCl pH 8.3

Method

1. Remove coverslip with a scalpel and scratch out remaining nail polish with a razor blade
2. Wash slides in 2X SSC at 37^{o} C for 5 minutes
3. Fix sections in 2 % paraformaldehyde for 10 minutes
4. Washed in PBS twice for 2 minutes each
5. Dehydrate in 100 % ethanol for 10 minutes and air dry
6. Add 20-30 microliter of hybridization solution containing 2X SSC, 50 % deionized formamide, 10 % dextran sulfate, 250 micrograms/milliliter salmon sperm DNA, and 5 nanograms of probe/slide
7. Apply coverslip
8. Denature target and probe DNA simultaneously in a water bath at 96^{o} C for 5 minutes
9. Hybridize 8 hours to overnight in a humidified chamber at 42^{o} C
10. Displace coverslip by dipping slide in 4X SSC at room temperature for 1-2 minutes
11. Wash at high stringency in 2X SSC at room temperature and then in 2 X SSC, 0.5X SSC and 0.1X SSC baths at 42^{o} C

Appendix 9 - Detection methods

Method

1. Rinse slides and place them in PBS x 5 minutes
2. For Biotin, add 100 microliters of the appropriate dilution of alkaline phosphatase-conjugated avidin or Streptavidin-Biotin complex
3. For Digoxigenin, add recommended dilution of Fab'-antidigoxigenin-alkaline-phosphatase
4. Rinse in 1X PBS twice for 5 minutes each
5. Apply appropriate substrate following vendor instructions
6. Stop reaction by transfering slides to a jar containing deionized water twice for 5 minutes each
7. Counterstain with modified Mayer's Hematoxylin x 30 seconds
8. Wash slides in running water x 10 minutes
9. Apply mounting media and coverslip.