In Situ Polymerase Chain Reaction

AND RELATED TECHNOLOGY

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In Situ PCR and Related Technology

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Preface

Ever since the introduction of the polymerase chain reaction (PCR) in 1986, morphologists, whose interests lie in the analysis of intact tissue structures, have been attempting to adapt this technique to intact cells or tissue sections to detect low copy numbers of DNA or RNA *in situ* while preserving tissue morphology. The significance of this objective is obvious. A technique finally materialized in 1990 when Dr. Ashley T. Haase and coworkers published results that used multiple primers with complementary tails in intact cells. Since then, a number of laboratories have successfully developed their own versions of the technique. *In situ* PCR is now a well-recognized method that permits the detection of minute quantities of DNA or RNA in intact cells or tissue sections. As a result, morphological analysis of those target nucleotide sequences becomes possible. As anticipated, this advancement has led to significant improvement in our understanding of many normal and abnormal conditions, and its impact is becoming more evident as time passes.

In situ PCR has the characteristics of a new landmark in morphologic technology—it is scientifically fascinating and technically challenging. In essence, it is a combination of *in situ* hybridization and conventional PCR. The wealth of literature, experience and protocols for the two latter techniques can be applied to *in situ* PCR. *In situ* PCR also has its own unique aspects that were not addressed by the other two techniques. For example, *in situ* PCR requires that the target nucleotide be available for amplification without interruption, yet the templates have to be effectively anchored *in situ* by fixation and tissue processing. These can only be achieved by optimal fixation, tissue processing and enzyme digestion. Another critical step is the inhibition or reduction of amplified signal diffusion. In theory, it seems that there is no reason for the amplified products to remain *in situ* rather than to float in the supernatant of the reaction mixture. The fact is that PCR products, or at least some of them, do remain at the site of origin under certain conditions. While the subsequent detection or visualization of the amplified signals should not be an obstacle, the elimination of unspecific amplification binding or distinction of specific and unspecific reactions could be a formidable task. More controls are also needed for *in situ* PCR than for the other techniques. It is the search for these and other empirical conditions that makes the setting up of an *in situ* PCR protocol challenging. Those technical steps are so demanding that only a dozen or so laboratories have reported success in performing *in situ* PCR. This technique is at an exciting stage of development, and more simplified, straightforward versions and widespread applications are bound to appear. This book is intended to accelerate this evolution.

Thus far, applications of this technique have already broken ground in a number of disciplines, particularly in viral infections, tumor diagnosis, AIDS research and gene therapy. For those who want to detect minute quantities of DNA or RNA against a background of tissue structure, *in situ* PCR may be the only technique

available. A number of other techniques, such as fluorescence *in situ* hybridization and some enzyme-driven reactions have similar promise; however, they all have inherent limitations. *In situ* PCR is branching into a number of variations, each with its particular attractive features. Some can be combined with immunohistochemistry, image analysis, confocal microscopy or electron microscopy, thereby broadening the horizon for morphologists and many other professionals.

These chapters were written by experts in the field of *in situ* PCR and related techniques. The authors have had considerable experience in establishing and performing the procedures. Their experience at the bench is particularly valuable for those who intend to perform these techniques. This book is organized to give theoretical consideration, historical review and practical, step-by-step guidance in the protocols for performing *in situ* PCR and its variations. It is hoped that with the help of this book, readers will gain a balanced view of this emerging technology.

I am indebted to the contributing authors who have shared their expertise and time to make this book possible. I am also grateful to Ms. Christine McAndrews for her excellent copyediting work and to Dr. Tak-Shun Choi for his invaluable input. It is our hope that this volume will make a valuable contribution to the literature and to those who intend to use these techniques in their research or practice.

Jiang Gu
August 14, 1995

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The cover photo shows a double staining of human condyloma with in situ *PCR for human papillomavirus (HPV) as detected with the indirect IGSS method with silver acetate autometallography (black; infected nuclei are labeled) and immunohistochemistry for cytokeratins (S-ABC method using DAB as the chromogen; brown; epithelial cells are labeled). The tissue was fixed in formalin, embedded in paraffin and cut into 7-μm-thick sections. The preparation was lightly counterstained with hematoxylin. Control sections were not stained for HPV when* in situ *hybridization alone was used without prior* in situ *PCR amplification, or when primer or polymerase was omitted.*

Contributed by Gerhard W. Hacker (Salzburg, Austria), Ingeborg Zehbe (Uppsala, Sweden) and Cornelia Hauser-Kronberger (Salzburg, Austria).

In Situ PCR—An Overview

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SUMMARY

In situ *polymerase chain reaction (PCR) is a morphological technique used to detect minute quantities of DNA or RNA in tissue sections or intact cells to unveil their distribution. It is derived from a combination of the conventional PCR widely employed by molecular biologists and* in situ *hybridization commonly used by morphologists. The former is capable of amplifying minute quantities of DNA or RNA in test tubes to billions of identical copies for analysis, but it does not allow the correlation of amplified signals with tissue structure. The latter can visualize DNA or RNA sequences in tissue samples and correlate them with tissue structure, but it has limited detecting sensitivity and requires relatively large amounts of DNA or RNA for detection. A combination of the two enables one to pinpoint up to a single copy of DNA or RNA of interest in tissue sections or intact cells and allows subsequent identification of cellular structures. This capability gives new insight into the cellular distribution of low copy numbers of nucleic acid sequences in many situations, such as viral infections, gene mutations, gene alterations, chromosomal translocation, gene therapy and low level gene expression. It is opening up a number of new territories for exploration and is expected to have a significant impact on basic and clinical research and diagnosis. This chapter gives a comprehensive account of the theoretical and practical background of this technology, presents its major variations and considers the commonly used protocols step-by-step. Its current and future applications in research and diagnosis are also discussed.*

INTRODUCTION

Since first reported in 1990 (26), *in situ* PCR technology has undergone rapid development. Many modifications and variations have been made and a number of applications have been reported. Owing to its unprecedented detecting sensitivity, some applications have led to important discoveries in several disciplines. In common with any new technology at its incipient stage, controversy about its reproducibility and significance of results is unavoidable. For the most part, these discussions are healthy and serve to propel the improvement of the method. The technology is still very young, and the procedures are quite complicated and technically demanding. Continuing evolution of the whole technique or parts thereof is inevitable. Its impact on a number of fields, such as viral infection, gene mutation, genetic disorders, gene therapy and low level gene expression, is expected to be significant.

Conventional PCR

In order to understand *in situ* PCR, one must understand conventional PCR. Since its invention by Mullis et al. in 1986 (42), PCR has had a profound impact on

molecular biology. It has provided an extremely sensitive and relatively straightforward means to amplify very small amounts of DNA or RNA—down to a single copy of a gene, to milligram amounts of the same sequences consisting of millions or billions of identical copies for detection, sequencing, cloning, diagnosis, etc. PCR was popularized by the introductions of automatic thermocyclers and thermostable DNA polymerase—*Taq* DNA polymerase. Before its inception, DNA amplification could only be achieved by time-consuming cloning that took several days. PCR can be set up and completed in a matter of hours. The importance of PCR is exemplified by the rapid increase in the number of publications relating to PCR from three in 1986 to more than 1700 in 1990. In 1993, Mullis shared a Nobel prize for this discovery. The principle of PCR is illustrated in Figure 1 (C–H).

DNA is a double-stranded nucleic acid chain consisting of two complementary nucleic acid strands made up by four basic nucleotides—dATP (A), dCTP (C), dGTP (G) and dTTP (T). The four nucleotides are linked to one another by phosphodiester linkage. The two strands are bound together by hydrogen bonds in a complementary fashion with A bound to T with two hydrogen bonds and C bound to G with three hydrogen bonds. The annealing and separation of the two strands depend on a number of factors, particularly temperature, salt concentration, pH, nucleotide composition and length of the sequence concerned. The denaturation or "melting" temperature (T_m) is the point at which 50% of the double-stranded DNA is separated. It can be calculated according to the following formula (6,7,9):

$$T_m = -16.6 \log (Na) + 0.41 (\%GC) + 81.5°C - 0.61 (\% \text{ formamide}) - (500/\# \text{ base pairs in DNA/DNA hybrid})$$

where T_m = melting temperature, (Na) = cationic concentration and %CG = percentage of CG nucleotide. Formamide concentration is irrelevant to PCR but is important in hybridization and washing. The optimal annealing temperature is 25°C below T_m. For example, for a pair of nucleotide strands containing 50% of G and C, in a PCR buffer at 50 mM KCl, T_m will be about 80.4°C, and the best annealing temperature will be 55.4°C. This formula is more applicable to long strands of DNA rather than to the short primers. However, it demonstrates the relationship among the different factors in PCR reaction.

DNA polymerase is an enzyme that can make a complementary strand of a single-stranded DNA. The heat-resistant enzyme, *Taq* DNA polymerase can synthesize DNA at a theoretical rate of about 150 nucleotides per second per enzyme molecule at around 75°–80°C, although the polymerization step in most PCR protocols is set at around 72°–75°C (13,18). It is important to note that *Taq* DNA polymerase has a built-in error rate of about 0.1%–0.3% (60). Other DNA polymerases, such as *Vent* and *Pfu*, may have proofreading ability but amplify at a lower efficiency (41). The polymerization reaction takes place in the presence of DNA polymerase, magnesium and the four free nucleotides. Single-stranded specific nucleic acid sequences, named primers, usually 18–28 nucleic acids in length, serve to initiate specific binding sites for the making of complementary strands. The free nucleotides are added one-by-one by the DNA polymerase, according to the complementary template to one end of the annealing primer. The newly synthesized strand elongates from the 5′ to 3′ direction. For PCR, a pair of such primers is used, each

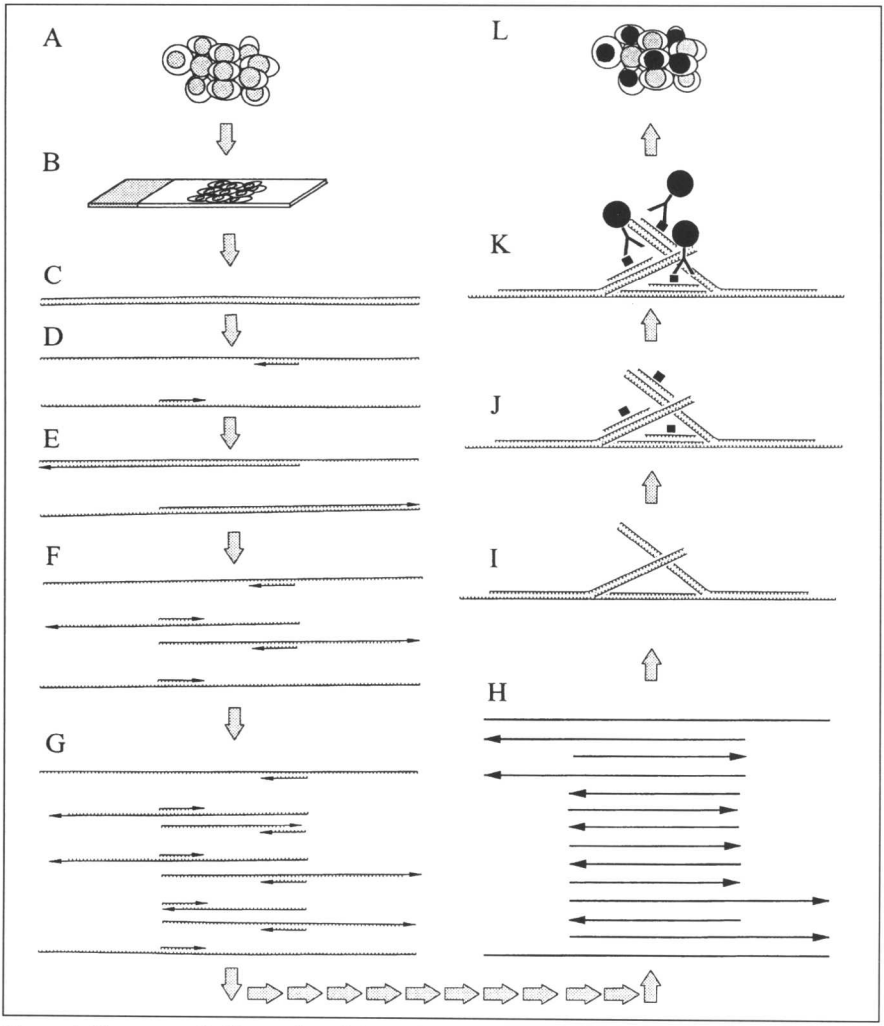

Figure 1. Diagrammatic illustration of the major steps of indirect *in situ* PCR. A) The samples can be individual cells in suspension or solid tissue samples. B) In most cases, the samples can be made into cytospin, smear or tissue sections. Chromosomal preparations may also be used. For cell suspension, *in situ* PCR may be performed in test tubes and then spun down on slides for detection. C) DNA in the nuclei is double stranded before denaturation. D) Above 90°C, DNA is denatured and becomes single-stranded DNAs that are bound by a pair of primers at specific sites. E) The primers serve as the starting sites for initiation of the polymeration reaction and form two new complementary chains of nucleotides. These two chains extend beyond the boundary defined by the primer on the opposite strand of the DNA template. F) The second cycle repeats the first cycle and uses the original DNA as well as the two newly synthesized strands as templates for making new complementary strands. G) The third cycle repeats the process to make more products. H) The reaction repeats itself in a thermocycler. By the end of n cycles, the sequence encased by the 5′ of the two primers (short products) becomes 2^{n-1} copies and the long products become $2 \times n$ copies. I) A possible mechanism for the amplified products to stay *in situ* is that the long products serve to anchor some of the short products as well as to form networks among themselves at the sites of the original templates. J) The amplified sequences and the original sequences are detected by specific probes which are labeled by, for example, digoxigenin. K) Digoxigenin is recognized by specific antibodies and visualized by a variety of markers. L) The cells that contain the very small amounts of target DNAs are thereby amplified and detected on the background of cellular morphology.

complementary to a specific sequence on one strand of DNA. The two primers are usually 100–1000 bases apart. This duplication reaction occurs at an optimal temperature range depending on the reaction condition. Beyond this range, the reaction still takes place but at lower rates (13). PCR is performed in a thermocycler that can be programmed to increase and decrease temperature from about 0°C to 100°C at about 1°C or more per second and maintain at a certain temperature for defined durations. The PCR typically consists of three different temperature gradients. First, at about 94°C, the double-stranded DNA are denatured and become single-stranded. At about 45°–65°C, the primers anneal to the specific complementary sequences of the denatured single-stranded DNA. At about 72°C, the DNA polymerase will add nucleotides onto the complementary strands and complete the extension. After each cycle, the sequence between the 5′ of the two primers doubles. The two newly synthesized strands serve as additional templates for subsequent PCR reactions. The sequence encased by the two primers grows in number exponentially thereafter following each cycle. Theoretically, after 30 cycles, a single copy of DNA sequence will become 10^9 identical molecules. For one target of 250 bp fragments, for example, this will represent approximately 0.3 ng of DNA, easily detectable by Southern blot analysis (13). The above reaction can also be used to amplify single-stranded RNA by first reversely transcribing the RNA into cDNA with a reverse transcriptase followed by the same PCR (30). Once PCR is completed, the end product can be detected by electrophoresis. The bands can be identified by size or by hybridization with labeled nucleotide probes that recognize a specific sequence of interest. A variety of labeling techniques ranging from radioisotopes to proteins can be used to label the probes (12,27). A number of controls are needed. These include the use of negative or positive samples, omission of primers, pretreatment of the samples with DNase or RNase, etc. (13). They all serve to ensure the specificity and the sensitivity of the amplification. Despite its extremely high sensitivity, conventional PCR cannot tell in which cells a particular DNA or RNA sequence is found.

In Situ Hybridization

In situ hybridization entails a hybridization reaction between a labeled nucleotide probe and a complementary strand of target DNA or RNA in tissue sections or intact cells. The hybridization reaction follows the same principle of nucleic acid chain annealing and disassociating as for PCR. Depending on what labeling methods are used, there are several means to visualize the reaction. The principle of *in situ* hybridization is illustrated in Figure 1 (J and K).

During the past decade, *in situ* hybridization has gone through many refinements and has become one of the most important tools in detecting DNA and RNA sequences in tissue samples. In 1992 and 1993, for example, more than 3000 articles utilizing *in situ* hybridization were published. The appropriate conditions for various factors in *in situ* hybridization have been established. These factors include tissue fixation, pretreatment, prehybridization, temperature, incubation duration, salt concentration, formamide concentration, probe length, concentration and composition, washing conditions and detecting systems (66).

Many variations of *in situ* hybridization have been developed. The probe can be either DNA or RNA. Oligoprobes may consist of 17–75 nucleotides, and ribroprobes may be composed of hundreds or thousands of nucleotides. The probe may be labeled with biotin, digoxigenin, fluorescence or radioisotopes. There are DNA-DNA, DNA-RNA, RNA-RNA and RNA-DNA *in situ* hybridizations depicting the various types of probes and targets concerned. An inherent limitation to this technique is that it requires at least 20 copies of identical DNA or RNA sequence in a single cell for detection (44). For this reason, most *in situ* hybridizations have been employed to study mRNA, which is usually present in much higher copy numbers per cell than DNA. In many instances, this leaves a lot to be desired as low copy numbers of DNA or RNA are important for many normal and pathologic conditions. Latent viral infections, for example, often have only a few copies of viral genomes per cell. Gene mutations, chromosomal translocation, gene therapy and early pathological changes in DNA and RNA may all involve fewer copies of nucleotide sequences than those detectable by conventional *in situ* hybridization. Despite their importance, the cellular distribution of these small quantities of nucleotide sequences remained invisible until *in situ* PCR is deployed.

PRINCIPLES AND STEP-BY-STEP CONSIDERATION OF *IN SITU* PCR

Ever since the development of liquid-phase PCR, there have been many attempts to perform *in situ* PCR. Haase and associates were the first to report a successful experiment in 1990 (26). They experimented with fixed intact cells in suspension and successfully performed PCR using the cell nuclear membrane as a sack to retain the amplificants. Enzyme digestion was used to permeate the membranes allowing primers, polymerases and free nucleotides to enter into the nucleus. Then, they managed to enlarge the size of the amplified signals by using multiple primers with complementary tails. The amplified and self-nested products were too large to leak out of the nucleus. The amplification was performed in test tubes and the cells were then spun down on glass slide for detection. Since then, many modifications and advances have been made. Now, more than a dozen laboratories have reported successful applications of *in situ* PCR. In the first issue of *Cell Vision*, many articles relating to this technique were published—most of them using protocols with varying modifications (4,8,11,29,38,61,68,70,71,74–80). Overall, this technique is still in its developmental stages, undergoing multiple phases of advancement. For the most part, many groups concur with the basic principles and the key steps in the procedures. The basic principle and practical considerations for *in situ* PCR are discussed below. The actual protocols can be found in later chapters of this book and in a number of articles of *Cell Vision*.

There are already a number of variations of *in situ* PCR and, undoubtedly, there will be many more. However, the basic steps can be illustrated in Figure 1. A successful *in situ* PCR requires that each step be performed properly and that specificity be verified with appropriate controls. In order to do so, one needs to understand the theoretical background behind each step so that optimal procedures can be established and adapted to suit specific needs.

Tissue Fixation

In situ PCR can be performed on intact cells in suspension, cells in smear, cells in cytospin, metaphase chromosomes, frozen sections and paraffin sections (1,10,15,22,29,34,39,74–80). Ideal fixations for *in situ* PCR should preserve both DNA or RNA and tissue morphology. If immunocytochemistry is to be performed in conjunction with *in situ* PCR on the tissue sample, antigenicity must also be preserved. Fortunately, formalin and paraformaldehyde, the most commonly used fixatives for histopathology and immunocytochemistry, meet these criteria. Fresh tissue is preferred but formalin-fixed archival tissue samples can also be used. Whole cells with intact membranes are ideal as they should have less damaged nucleotide sequences, and their nuclear or cell membrane serves as a natural boundary to retain the amplified products (26,32,44,57). *In situ* PCR on tissue sections is less efficient, but it acquires a higher significance as most pathological tissue samples are fixed in formalin and kept in paraffin blocks. It demands more technical refinement in conducting the procedure. For either paraffin or frozen tissue sections, thicker sections may yield better results. As the target DNA or RNA are molecules of long strands and somewhat randomly distributed in the nuclei or the cytoplasm, a thicker section will contain more target DNA or RNA sequences in their entirety. The depth of the section with more membrane structure and the cross-linked proteins might also serve to trap the amplified products from diffusion. Depending on the availability of the target DNA or RNA, thinner sections (< 7 μm) may also be used, which generally give lower background and better morphology. Immediate fixation in 10% buffered formalin, pH 7.0, for 4 to 6 h at 4°C is an acceptable fixation method. Formalin causes cross-linking between proteins and nucleotides, which may serve to retain the amplified sequences and keep them from being washed away. Unbuffered formalin is not recommended as it has been shown to reduce reaction intensity of conventional *in situ* hybridization (43,54). Alcohol fixation or unfixed tissue samples have also been experimented, but with limited success (10,58). Fixatives with picric acid such as Bouin's fixative or heavy metals such as mercury in Zinker's solution are not recommended as they degrade DNA or RNA after a few hours of fixation (23,24). To test the suitability of a fixative for *in situ* PCR, it is advisable to dissect a portion of the fixed tissue sample and extract DNA or RNA from it. The availability of total DNA or RNA and of the nucleic acid sequence of interest in the fixed tissue sample can be evaluated using conventional PCR, electrophoresis and blotting assays.

Tissue Pretreatment

For *in situ* PCR, it is almost always necessary to digest the tissue sample with a protease, such as proteinase K, trypsin or pepsinogen. It permeates the tissue sample, allowing reagent penetration and unveiling the target sequence for amplification. The concentration of the enzyme and the duration and temperature of the treatment are important. Excessive digestion distorts the tissue morphology and increas diffusion of PCR products through disrupted membrances of intact cells (34,39,44). If under-digested, the tissue will have poor permeability and extensive cross-

linking between proteins and nucleotides, which interfere with PCR (44). Both may lead to false negative results. The strength of digestion should also correlate with the length of fixation. The more extensively the tissue sample is fixed, the heavier the digestion should be. After digestion, the enzyme should be completely inactivated by heating or removed by washing. Minute remnants of the enzyme may destroy the DNA polymerase, which is essential for PCR amplification.

Primer Design

Primers are usually 18–28 nucleotides long and encase a fragment of around 100–1000 bp. For *in situ* PCR, shorter template sequences are preferred. This is especially true for archival tissue samples in paraffin sections as considerable degradation of DNA and RNA have taken place. DNA fragments extracted from paraffin blocks are rarely longer than 400 bp, and RNAs are not more than 200 bases (63). Long sequence amplifications are more prone to nonspecific reactions caused by mispriming on the original DNA (13). Once a wrong sequence has been copied, it will be continuously amplified by the remaining cycles of PCR. It is important to design a pair of primers that has little or no homology to any other sequences in the tissue. They should also have no homology to each other or within themselves. Computer programs are available to verify the degree of homology for each designed fragment. Nonspecific or false positivity generated by PCR is, to a large extent, attributable to mispriming of the primers (12). Generally, one pair of primers will be sufficient. To increase specificity of the amplification, multiple pairs may be used (10,14–16,26,33,39,49,64). A second pair of primers embedded within the sequence amplified by the first pair can be employed. This reaction is called "nested PCR." It allows further amplification of the signal already amplified by the first pair to increase specificity and sensitivity. Multiple pairs of primers, distinct from each other and against different portions of the same gene sequences, may also be used to increase the specificity of the detection.

PCR

PCR performed in tissue sections or intact cells follows the same principles as conventional PCR. One important difference between *in situ* PCR and liquid-phase PCR is that DNA or RNA strands in fixed cells or tissue sections are immobilized. Not all the target sequences are available for complementary bonding and, more likely than not, the entirety of the target sequence has been damaged or truncated during tissue processing and sectioning. Twenty-five to forty cycles are recommended, although the *in situ* amplification reaction may have reached its plateau in less than 20 cycles (1,34,39,52,58,62). *In situ* PCR can be performed on a conventional PCR thermocycler by using aluminum foil paper to make a flat plate out of the platform. The temperature is effectively transmitted through the glass slide to the tissue sample and reaction solution. Mineral oil may be used to facilitate heat conduction. However, conventional PCR machines may only hold about four slides for each run, which is not nearly enough for the many controls necessary for each experiment. Thermocyclers specifically designed for *in situ* PCR have been

available. They can hold from 12 to 20 slides per round and are much more efficient for carrying out experiments. Typically, the duration of each temperature step for *in situ* PCR is slightly longer than that of conventional PCR to overcome the disadvantages inherent in *in situ* PCR and to ensure that sufficient amplification takes place (44). Denaturing at 94°C for 1 min, annealing at 55°C for 30 s and extension at 75°C for 1 min are generally adequate. The *Taq* DNA Polymerase has a half life of about 20 min at 96°C (59). Prolonged total time of heating will diminish its activity. The concentrations of primers, DNA polymerase and magnesium may also be higher than those used in conventional PCR (32,39,49). For tissue sections, cytospins and smears, the amplification is performed on glass slides. Generally, it takes place under a coverslip that is sealed with nail polish or a Pap pen (Newcomer Supply, Oak Park, IL, USA) (69,78).

It is commonly believed that *in situ* PCR is not as efficient as conventional PCR. It was estimated that liquid-phase PCR can amplify a single copy of DNA to billions of identical copies while *in situ* PCR amplifies to much fewer copies (44). This estimation was performed by measuring the end products of the liquid supernatant or homogenate of the tissue samples at the end of *in situ* PCR thermocycling (67). The concentrations of amplified products, however, was calculated against the total tissue volume or liquid volume which, by any measure, is not a fair denominator. The true concentrations of the end products should be calculated against the value of the individual cells that harbor the products. For conventional PCR, as the cycle number increases, amplification efficiency is affected by substrate saturation and exhaustion of reactive solution. At a certain point of amplification, the exponential growth plateaus (13). This effect is influenced by template input and initial amplification efficiency. Over how many cycles this phenomenon occurs for any unknown concentration of template is not completely predictable (36). For *in situ* PCR, it is presumed that this effect comes much earlier and is mainly attributed to the local concentration of the amplificants. This local concentration for *in situ* PCR is likely to be as high as the total concentration of liquid-phase PCR. Detecting sensitivity generally refers to the minimum copy numbers of a target detectable by the method. If both *in situ* PCR and liquid-phase PCR detect down to a single copy of DNA or RNA, they are of equal detecting sensitivity.

A "hot start" technique that increases the specificity of *in situ* PCR has been developed (44,47,49). Initial annealing between the primers and the target sequence determines the amplification specificity. "Hot start" entails the initiation of the primer-target annealing step at a higher temperature, which significantly reduces the possibility of mispriming, and thereby improves the specificity of subsequent PCR (17). There are a number of variations in performing the "hot start." For conventional PCR, a special wax called AmpliWax (Perkin-Elmer, Norwalk, CT, USA) can be applied to separate the reaction mixture and the polymerase. It serves as a barrier between the reagents but will melt at temperatures above 80°C. The DNA polymerase will then be mixed with the reaction mixture as the wax melts, ensuring that PCR starts at a high temperature, a condition strongly unfavorable to nonspecific annealing. For *in situ* PCR, "hot start" requires adding the DNA polymerase after the slides have been heated to about 94°C for a few minutes. Now there is

specific monoclonal antibody available that specifically reacts to *Taq* DNA Polymerase and effectively blocks the enzymatic activity up to 70°C. This inhibition is completely reversed upon the first denaturation step in thermal cycling when the antibody is inactivated by heating (Clontech Laboratories, Palo Alto, CA, USA). It has been confirmed that "hot start" is crucial for a highly specific and efficient *in situ* PCR. Once the PCR starts with specific initial annealing and amplification, the rest of the duplication cycles will usually yield products of high fidelity.

A very important step for a successful *in situ* PCR is product retention. For intact cells, the amplified products may be withheld by cellular and nuclear membranes (26,57). For tissue sections, however, it is not entirely clear why the large number of amplified sequences stay *in situ*, nor is it known how to make them stay at the site of the original template. It is reasonable to believe that most of the amplified sequences have diffused away following established rules of physics. The well-defined distribution of the reaction, as visualized by the detection system, would strongly indicate that certain mechanisms exist which trap the amplified sequences in place. This may, in fact, be the case. During PCR, while the sequences encased by the two primers increase exponentially with each cycle, there are also "long products" that increase in number arithmetically. These long products are generated by duplication of the original template, and their chains extend beyond the boundary of the primer on the opposite strand. Two more such byproducts are produced by each PCR cycle. After 30 cycles, at least 60 such long sequences are generated for each copy of target DNA. They should be able to bind to the intrinsic templates that are partially embedded within the tissue section and also to the short sequences and to each other as they share complementary sequences. The long products could act as anchors, effectively retaining some of the amplified products. Judging from the appearance of the final reaction in the *in situ* PCR preparation, it is possible that those sequences retained by the long products are the major sources of signals that are not diffused or washed away during the procedure. Sixty or more identical target copies retained by the long products should be easily detectable by subsequent *in situ* hybridization and give a well-defined signal made visible by the detecting system. The long products, that are mostly neglected and have little importance in conventional PCR, may be the most important products of *in situ* PCR. This hypothesis is illustrated in Figure 1 (I).

Other measures have been taken to increase the chance of product retention. Thicker sections with deeper spatial structure may serve to trap the amplified sequences in place. Sealed coverslips or plastic bags have been employed to prevent evaporation and also to retain the products *in situ*. Post-PCR drying at about 60°C and fixation in 4% paraformaldehyde are also necessary to effectively immobilize the amplified products (10,15,26,78). It is possible that these measures do not work by themselves but only facilitate the bonding of the long products to the amplified signals described above.

Following PCR, the samples should be washed gently with solutions of appropriate stringency. Many investigators have employed different strategies in this washing step. Excessive washing may remove the amplified products from the initial site. However, without washing, high background or diffused signals may be

seen. The washing stringency can be designed according to the T_m formula described previously. It is sometimes a compromise between the amounts of desired specific signal retention and that of background nonspecific staining.

In Situ Hybridization Detection

The amplified signals are detected by *in situ* hybridization. The initial step normally starts with pre-hybridization, which entails incubating the sections with all the chemical ingredients in the hybridization solution except for the specific probe. Those ingredients will saturate the potential nonspecific bonding sites on the tissue sample to increase the specificity of the detection (66). A pre-hybridization step can be performed before PCR (78,80). This modification is commendable as it will reduce the steps and number of washings after PCR and serve to better preserve the amplificants. An enzyme digestion routinely used in conventional *in situ* hybridization is not necessary for PCR product detection. The *in situ* hybridization can be carried out in a number of ways (27). The most straightforward is to employ an oligoprobe that is labeled with biotin or digoxigenin and hybridized under appropriate conditions with formamide at a temperature of 37°–54°C from 3 h to overnight. Generally, a 3-h incubation at 42°C is sufficient. Longer probes, such as riboprobes, with sequences extending beyond the fragment that is amplified, may be employed. Longer probes increase the detecting specificity but decrease the sensitivity (66). Multiple probes can also be used to ensure that different fragments of the amplified signal correlate well with the sequence of interest. The design of a specific probe is very important as PCR may amplify more than one sequence and a specific *in situ* hybridization will pick up only the correct one (13). Different labeling methods are more a matter of preference than a limiting factor. Generally, radioisotope labeling gives the highest detecting sensitivity but it is compromised by time, hazardous chemicals and reduced resolution (27,28,66). Fluorescent labeling is also very sensitive. However, its signal tends to fade and the background structure is not clearly visible. Protein labeling with enzyme detection is at present the method of choice (27,28). Biotin labeling can be easily detected by the streptavidin-biotin complex that can be coupled to a number of enzymes, such as alkaline phosphatase or horseradish peroxidase. Digoxigenin labeling can be recognized by a specific antibody that will have added specificity.

The detection procedure of hybridization signal is very similar to immunocytochemistry. It may have many variations ranging from direct visualization to immunogold-silver autometallography and display a variety of colors, intensity and textures (27). The sections of tissue can then be counterstained and examined.

Controls

The entire *in situ* PCR procedure is fairly long and technically demanding. Each inappropriate step may create false positive or negative and should be properly controlled. In theory, there are more than 20 controls that should be run with each experiment so that the negative or positive results can be ascertained (13,32,44). If any of the controls give unexpected results, one should be able to explain and define

the causes of the problem and interpret the results accordingly. However, in reality, the following controls should be routinely performed as the first line checklist to ensure the specificity of the reactions. Usually, unrelated primers or omission of the primers should be used as controls. This should give a negative or much weakened signal. The use of unrelated probes or omission of the probes should be the best control for the *in situ* hybridization step. It is important to have good positive and negative samples to run in each experiment to make sure that all the ingredients and steps are properly applied. A negative control, for example, could be a tissue pretreated with DNase or RNase to destroy the targets before being subjected to the *in situ* PCR experiment. When intact cells in suspension are used, artificial mixtures at different ratios with another cell type known not to contain the target sequence provide good controls (1,32,44). The cell preparation may be smeared, cytospun or grown on slides if they attach. They also may be collected into pellets by centrifugation, embedded or frozen and cut, and then serve as controls for tissue sections. One good working model is SiHa cells, a cell line derived from human cervical cancer cells. It is known to contain a single copy of human papilloma virus (HPV) type 16 per cell (44,52,71). Other cell lines such as HeLa cells, known to contain about 25 copies of HPV type 18 per cell, and Caski cells, known to contain about 600 copies of HPV 16 per cell, are also good controls for technical setup. DNA or RNA extracted from the same tissue sample should be assayed with conventional PCR to verify the presence or absence of the sequences of interest. If conventional PCR results do not correlate with those of *in situ* PCR, a check on the fixation or enzyme digestion may be in order. The washing stringency may be adjusted to facilitate a better retention of the amplified signal or a reduction in background staining. The amplification solution should also be examined after the PCR by recovering a small amount of the reagents from the top of slides and running it through liquid-phase PCR or directly through Southern or Northern blotting to check for the presence of the amplified sequence of interest (67). The results normally provide information that will clarify at which steps the procedure went wrong and thereby help to troubleshoot the problem. If there is too much background or nonspecific reaction, adjustments may be made to reduce the PCR cycles, redesign the primers, modify the concentrations, change the annealing temperature, perform the "hot start" carefully, block nonspecific reactions in the detecting steps, increase the washing stringency, etc. It also should be kept in mind that as the detecting sensitivity of *in situ* PCR surpasses other detecting systems, unexpected positivity may reflect the true presence of the sequence in a particular tissue structure. This is true for HIV and a number of other viruses that were not known to be present in a variety of cell types. Their latent phase of infection is now gradually being unveiled by *in situ* PCR (1,3,5,15,16,31,53,57,74–80).

Most of the problems and pitfalls encountered in conventional PCR and *in situ* hybridization also need to be dealt with in *in situ* PCR. The extensively published protocols and modifications on optimizing and troubleshooting conventional PCR are often applicable to *in situ* PCR. PCR product contamination, a nightmare for conventional PCR, is also a concern for *in situ* PCR. A dedicated working area that is nucleic acid-, RNase- and DNase-free is highly recommended. Nevertheless,