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IN SITU HYBRIDIZATION, *IN SITU* TRANSCRIPTION, AND *IN SITU* POLYMERASE CHAIN REACTION

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Abstract

In situ hybridization, *in situ* transcription, and *in situ* polymerase chain reaction (PCR) are techniques used to detect DNA and RNA sequences within a cell or tissue structure. These three *in situ* methodologies employ the principles of recombinant DNA to form double-stranded hybrids of DNA-DNA, DNA-RNA, or RNA-RNA. The essence of *in situ* hybridization (ISH) is the hybridization of a labeled probe to a complementary target sequence, whereas *in situ* transcription (IST) is the synthesis of complementary DNA incorporating a label directly on the target DNA or RNA within a cell or tissue. In the case of *in situ* PCR (ISPCR), it is the repeated *in situ* duplication of both the sense and antisense strands of DNA to increase the number of copies of the target sequence. ISH, IST, and ISPCR each have their advantages and disadvantages. The purpose of this chapter is to address *in situ* considerations requried of these techniques, emphasizing tissue fixation, pre-hybridization steps, DNA probes, RNA probes, oligoprobes, and probe labeling. Five successfully used protocols are presented as examples. Any given nucleotide target sequence may have its own unique set of optimum conditions, thus requiring some adjustment in the hands of the user.

Key Words: *In situ* hybridization, *in situ* transcription, *in situ* polymerase chain reaction, molecular morphology, cytochemistry.

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Introduction

In situ hybridization, *in situ* transcription, and *in situ* polymerase chain reaction (PCR) are techniques used to detect DNA and RNA sequences in chromosomes, cells or intact tissue sections. These three *in situ* methodologies employ the principles of recombinant DNA (rtDNA) as they are broadly defined [49], and rely on the powerful and widely used technique of nucleic acid hybridization which exploits the ability of complementary sequences in single-stranded DNAs and RNAs to pair with each other to form double-stranded hybrids of DNA-DNA, DNA-RNA, or RNA-RNA. Man-made sequences of nucleotide that specifically bond or hybridize to a target sequence in an A-T (or U) and C-G complementary fashion are called DNA or RNA probes. In the *in situ* hybridization technique, the probes are labeled with a radioisotope or chemically tagged for the detection and localization of the hybridized probes. The hybridized probes are then detected by either a direct or indirect method. In the *in situ* transcription technique, an unlabeled probe is used as a primer and labeled nucleotides are incorporated into the cDNA that is synthesized as the primer is extended on the target template. In the case of *in situ* PCR, the target sequences are bracketed by two primers and are amplified by making a large number of copies, and then the copies are detected or directly visualized.

The essence of *in situ* techniques

The essence of *in situ* hybridization (ISH) is the hybridization of a labeled probe to a complementary target sequence. In its simplest form, ISH is performed by separating the strands of double-stranded nucleic acids by denaturing, hybridizing a labeled probe to its complementary DNA or RNA in tissue sections or individual cells, washing away the unhybridized probe, and detecting the label on the bound probe. Protocols will be presented that will take advantage of simplicity and directness of ISH. The advantages and disadvantages of ISH and the need for controls will be discussed.

The essence of *in situ* transcription (IST) is the synthesis of complementary DNA (cDNA) within a cell. In its simplest form, IST is performed by annealing a specific unlabeled primer to its complementary mRNA in a tissue section or individual cell, washing away the unhybridized primer, and synthesizing cDNA in the presence of reverse transcriptase and deoxynucleotide triphosphates (dNTPs), some of which are labeled. Protocols will be presented that take advantage of the simplicity of IST, the use of Digoxigenin (DIG)-labeled dUTP and an immunochemical bridge. The advantages and disadvantages of IST and Immunogold Silver Staining (IGSS) will be discussed.

The essence of *in situ* PCR (ISPCR) is repeated *in situ* transcriptions of both the sense and antisense strands of DNA to increase the number of copies of the target sequence. In its simplest form, ISPCR is performed by denaturing double-stranded nucleic acids to single-strands, two primers bracketing a sequence of interest are hybridized to their targets, the sequence amplified with PCR, and the amplicons detected. The advantages of ISPCR and the need for controls will be discussed. For more detailed discussion of these techniques, readers are referred to the monographs and reviews published on the topics [14, 16, 58, 63, 75].

Historical background

ISH, IST, and ISPCR, like other scientific tools and the new knowledge they provide, were preceded by other key discoveries and innovations. From our perspective, there were about a dozen or so historic milestones spanning approximately two decades which made molecular biology possible and the application of ISH, IST, and ISPCR to tissues and cells *in situ*, practical. The 1969 work of Gall and Pardue [24], demonstrating the formation and detection of RNA-DNA hybrid molecules *in situ*, were proof-of-principle findings that made the ISH technique possible. In 1970, the discovery of RNA-dependent DNA polymerase (or reverse transcriptase) by two independent groups, Baltimore [5] and Temin and Mizutani [88], and the 1971 work of Kleppe *et al.* [51] demonstrating the replication of short synthetic DNA segments catalyzed by DNA polymerases, demonstrated the possibility of IST. But it was not until Langer *et al.* [59] enzymatically synthesized Biotin-labeled polynucleotides, followed by the work of Saiki *et al.* in 1985 [82] that achieved *in vitro* primer-mediated amplification of genomic DNA, that practical IST was made possible. The IST technique was first described and applied to tissue sections by Tecott *et al.* in 1988 [87] and was confirmed and applied to cells *in vitro* by Longley *et al.* in 1989 [67]. The field picked up steam with three landmark discoveries: i.e., the 1973 work of Cohen *et al.* [17] demonstrating the cloning DNA fragments into plasmid vectors, the 1975 work of Kohler and Milstein [57] demonstrating the development and *in vitro* production of monoclonal antibodies, and the 1976 work of Chien *et al.* [15] discovering thermostable DNA polymerase. This was later followed in 1987 by Kogan *et al.* [56] who introduced the use of thermostable DNA polymerase and made possible the practical use of the polymerase chain reaction (PCR). The introduction of liquid phase PCR and its automation dramatically improved the rate of progress for molecular biology [77]. The aforementioned milestones were essential advances for the advent of ISH, IST, and ISPCR, and were indirectly necessary

for the proof-of-principles of these *in situ* techniques.

Scope of Chapter

Many names have been given to these techniques encompassing a wide range of protocols, but the principles remain the same. In this chapter, we give a concise and comprehensive review of the principles and procedures of these techniques and their major variations. As examples, certain protocols that have been tested by the authors are included at the end of this chapter.

In situ Conditions

General considerations

In vivo nucleotide sequences anneal and separate with a high degree of precision. *In vitro* their behavior also follows certain general rules. The factors that affect their behavior the most include temperature, salt concentration, length of the probes, percentage of C-G content, pH and concentrations of certain chemicals such as formamide. Changing the temperature or pH are the most commonly used methods to manipulate the annealing and denaturation of DNA or RNA [16].

Fixation

The most commonly used fixatives for *in situ* hybridization have been aldehyde-based fixatives such as 10% formalin or 4% paraformaldehyde [14]. The aldehyde fixatives cross link proteins, or proteins and nucleotides, and effectively preserve the nucleotide sequence. Fixatives with picric acid such as Bouin's fixative or heavy metals such as Zenker's solution should be avoided as damage of target nucleotide sequences may occur [27]. Fixation from three hours to overnight appears to be an acceptable range depending on the size and nature of the tissue and the strength of the fixation. Frozen sections can also be used.

Pre-hybridization steps

The aldehyde-fixed samples need to be digested to expose the targets, yet this digestion should be optimized to also preserve the morphology and retain the target sequences after hybridization. Proteinase K, trypsin, pronase and other proteases have all been used successfully. The digested sections should be washed thoroughly to remove or inactivate all the enzymes. A pre-hybridization step is recommended which should eliminate or reduce the background nonspecific reactions. This usually consists of a cocktail of the hybridization solution without the specific probe to exhaust possible nonspecific bonding sites prior to incubation of the specific probes. Additional pretreatment using levamisol, normal rabbit serum, etc. can also be performed to prevent the subsequent detection step causing any background. The samples are then equilibrated in a solution that is close to the hybridization condition [37] and proceed with the hybridization steps. We have also found that it may be beneficial to let the samples dry after the equilibrate washing and applying the hybridization cocktail directly on the dry samples. When nonspecific background staining is controlled, this usually gives stronger signals, presumably because of a better penetration of the probes to the tissue sample and a more controlled concentration of the salts in the immediate environment of the hybridization reaction.

DNA probes

The key in probe design and selection is that the probe sequence has to be specific to the target and not complementary to any other sequence. Double-stranded DNA probes were the initial approach developed for *in situ* hybridization because of their relative stability. They can, however, be destroyed by DNase which requires magnesium to function and can be inhibited by EDTA [80]. They are usually labeled by nick translation or random primer extension. These probes can be as long as a few thousand nucleotides or as short as 1 or 2 dozen nucleotides [80]. Double-stranded probes must be denatured before hybridization, which is generally achieved by increasing the temperature. The drawback of double-stranded probes is that the two strands tend to re-anneal to themselves during hybridization, thereby decreasing the detecting efficiency. Single-stranded DNA probes can also be produced by using the 13M cloning vectors [42, 72] and PCR [68], overcoming the disadvantage of probe re-annealing. Thus, they are more efficient at hybridizing to their targets than double-stranded probes [78].

RNA probes

A riboprobe is a single-stranded RNA probe produced by cloning man-made vectors containing promoters and an insert of cDNA fragment of interest at a known orientation [47, 71, 85]. The recombinant plasmid vectors can be grown and amplified in appropriate bacterial hosts, whereby the inserted cDNA grows in quantity. The inserted sequences are then transcribed with RNA polymerase into RNA probes. Radioisotope or other labeling molecules can be incorporated during transcription. By reversing the orientation of the inserted cDNA, both anti-sense and sense RNA probes can be produced. A "sense" probe refers to a sequence equivalent to the target mRNA and an "antisense" probe is complementary to the sequence and is used to hybridize with the target. Riboprobes are superior to DNA probes in that they are single-stranded and do not reanneal to themselves, resulting in a more efficient target detection [3, 18, 64, 80]. When hybridized to mRNA, it is more stable than DNA-RNA hybridization and can stand stringent hybridization conditions and washing [44]. Following hybridization, it is possible to

destroy the unhybridized single-stranded probes with RNase, further reducing the background. A large amount of the same kind of probe can be generated. However, the preparation of riboprobes requires the availability of the appropriate cDNA, considerable experience with molecular biological skills to prepare and propagate the plasmids and transcripts, and an RNase-free environment for probe preparation and handling. It is more specific than the shorter oligoprobes. A good riboprobe can bind its complementary mRNA more tightly than the shorter oligoprobes allowing for more stringent washing, which can lead to lower backgrounds and a more specific detection of the target.

Oligoprobes

Oligoprobes (or oligoprimers) are small single-stranded probes that possess many advantages [43]. The simplicity in manufacturing those probes make them an easy choice for *in situ* hybridization. If the target sequence is known, oligoprobes can be synthesized with a DNA synthesizer or ordered from a molecular biologic reagent company at a reasonable cost. The length of an oligoprobe should be about 14-50 bases [80]. The sequence has to be unique to the target of interest so that unwanted hybridization will be minimized. The percent of G and C content is also important as this will affect the thermostability of the hybrid or the background labeling. Optimal conditions for individual probes and targets should be established empirically, particularly for oligoprobes, as the window for optimal hybridization is narrower than for longer probes.

Probe labeling

A variety of molecules have been used to label probes. Radioisotope labeling remains the most sensitive approach. ^{35}S and ^{33}P have been a good compromise for probe labeling as they do not require too long an exposure time for autoradiography and give relatively high resolution. These labeling methods are widely used because of their sensitivity and reliability [47, 64].

Non-radioisotope labeling has become increasingly popular and, with refinement of the technique, more sensitive [29, 58, 76]. These methods avoid the hazardous radioisotope handling and disposal, shorten the experimental protocol, and yield high resolution results that can be combined with other techniques such as immunohistochemistry. It is also suitable for *in situ* hybridization at the electron microscopic level when used in combination with immunogold labels. Commonly used labeling molecules include biotin, digoxigenin, alkaline phosphatase, hapten and fluorescein. Biotin enjoys the popularity because of the availability of a wide variety of detection kits. Digoxigenin is a plant molecule that can be recognized by specific antibodies, providing added specificity and sensitivity due to reduced nonspecific binding, and is preferred by many investigators. Fluorescein can yield very high signal-to-noise ratios and detecting sensitivities, and therefore has been widely used in chromosomal labeling, and often goes by the acronym FISH (Fluorescence *In Situ* Hybridization) [48, 69, 93].

Optimizing *in situ* Conditions

The search for optimal condition for hybridization is one of the key issues for successful experiments. It is important to determine the melting temperature at which 50% of the double-stranded sequences are denatured. Equations are available for different factors that govern the relationship between the two complementary sequences in the denaturing and reannealing processes. Generally for ideal hybridization, the reannealing temperature should be set to 20-25°C lower than the melting temperature to generate a stable hybridization product. To ensure specificity for shorter probes, the hybridization temperature may be adjusted at 12-17°C below the melting point. The optimal hybridization temperature should be established empirically in addition to being predicted from the formulas. A temperature range of 37-42°C is a good starting point for the hybridization step. This is derived from inclusion of 50% formamide in the hybridization solution which competes with bases for the formation of hydrogen bonds and thus lowers the temperature requirement. For a DNA sequence with 50% C-G contents in 0.4 M sodium chloride containing 50% formamide, the melting temperature is about 54.5°C, and the hybridization temperature is adjusted to 37-42°C. The concentration of a probe is also important. It is usually used at around 300 ng/ml with a range of 200-1500 ng/ml depending on the size of the probes. The hybridization kinetic is also affected by salt concentrations. At concentrations below 1.5 M, the higher the salt concentration, the higher the rate of hybridization. Polymers of high molecular weight, such as dextran sulfate and polyethylene glycol, are often added at a concentration of about 10% (v/w). Those molecules can create a networking phenomenon, taking up sufficient space in the solution to artificially increase the probe concentration and thus the hybridization rate by about tenfold without creating any noticeable side effects **[1,2]**. The washing solution should be designed to contain the appropriate salt content that will wash away unbound probes and probes in less stable, mismatched hybrids, and leave the perfectly-matched hybrids intact. To set up an *in situ* hybridization protocol for a new target, the best approach is to follow previously established procedures and then adjust the various conditions, one at a time, starting from the probe design, hybridization solution and incubation

temperature. Changing probe design is often the most effective measure to make *in situ* hybridization work.

In situ Hybridization

Technically *in situ* hybridization is a well established method. The protocols are fairly straight forward. When the right probes are selected and the target sequences are preserved and accessible, the properly executed hybridization procedure should lead to clear and well defined signals with low background. The window of opportunity for achieving optimal reaction is fairly broad. Certain small mishaps in performing the technique would not lead to unusable results.

Advantages of ISH

One of the advantages of *in situ* hybridization, in comparison to *in situ* transcription and *in situ* PCR, is that the probes are hybridized to the target DNA or RNA sequences directly without target manipulation or amplification. What are amplified in the *in situ* hybridization protocols are the labeling molecules not the targets themselves. Therefore, there is little problem of product diffusion or amplicon back diffusion, sometimes a formidable problem for *in situ* PCR. In addition, when the signal amplification steps are constant, the amount of reporting signals are approximately proportional to that of the target, thus allowing semiquantitative assessment of the target DNA or RNA. When evaluated jointly with the results obtained with immunocytochemistry, this semiquantitative information can lead to meaningful interpretations of the cellular components under study. For example, they may indicate the balance or imbalance of the expression of a particular gene and the production of the coded protein. When the distributions and relative quantities of the gene expression and its protein are different, certain cellular events could be indicated [29, 86, 88].

Disadvantages of ISH

The disadvantage of *in situ* hybridization is that it is not as sensitive as *in situ* PCR, or *in situ* transcription. Nevertheless, with the newly reported, more powerful signal enhancement techniques such as the nanogold-silver immunostaining and computer enhanced fluorescence *in situ* hybridization, the detecting sensitivity of *in situ* hybridization has been improved markedly [35, 69, 78].

In situ Transcription

IST is initiated by the hybridization of a primer (usually a specific oligonucleotide) to target mRNA in a tissue section or cell preparation on a glass slide as is done in conventional ISH [18, 38, 50, 62, 63, 65, 66]. Transcription of the target mRNA is achieved by adding reverse transcriptase and labeled nucleotides which allow the synthesis of a labeled complementary DNA (cDNA). Since the synthesized cDNA remains associated with its mRNA template as a cDNA-mRNA hybrid, anatomical distribution and cellular localization are preserved, thus, it is one of the main advantages of IST.

In the original works [67, 87], radio labels were used and the localization of the labeled cDNA was detected by autoradiography. Many non-radioactive detection systems have been developed for ISH and can be applied to IST, i.e., fluorochrome-labeled dNTPs in fluorescence methods [6, 7, 52, 65, 66, 89, 93], biotin-labeled [26, 39, 50, 53, 55, 59, 65, 73, 74] or digoxigenin-labeled [19, 20, 36, 39, 79, 91, 92] dNTPs in immunochemical detection methods.

One of the first applications of the IST technique was to the localization of proopinmelanocortin (POMC) mRNA on fresh-frozen paraformaldehyde-fixed sections of rat pituitary using reverse transcriptase and radio-labeled ^{3}H-dCTP in the cDNA elongation process followed by autoradiography [87]. In another early study, IST was applied to localized alpha-2 domain of CD1a mRNA using an oligonucleotide primer specific to the target mRNA, incorporating ^{35}S-dCTP into cDNA followed by autoradiography [67]. IST has also been used to investigate the temporal expression of mRNAs in developing embryos [22, 23]. One of the most intriguing uses of IST was in the analysis of gene expression in live cells where mRNA in single live neurons *in vitro* was injected with primer, dNTPs, and reverse transcriptase via whole cell patch electrode. The contents of the cell was then harvested by suction back into the electrode, the electrode incubated *in vitro* where the cDNA is first synthesized by IST followed by replication of cDNA to many copies of amplified RNA (aRNA) [90]. The aRNA was assessed by Southern and Northern blot analysis. More recently IST has been used to localize γ-GTP mRNA in paraffin section from rat kidney [91] and from cell cultures of rat brain microvessels [92].

One technique closely related to IST for mRNA is termed, PRimed *in situ* labeling (PRINS), which was first used by a Danish group to localize chromosomal DNA [52-55], and was later confirmed by a group in Scotland [26, 73]. For clarity, the PRINS technique applied to DNA is referred to as PRINS-DNA. The PRINS-DNA procedure uses unlabeled DNA probes or oligonucleotides as the primer, and DNA polymerase (Klenow or *Taq*-I) and biotin or digoxigenin labeled dNTPs to synthesize labeled DNA *in situ*. The site of synthesis was detected by immunocytochemistry using fluorochromes as reporter molecules. A variation of PRINS-DNA can be used in the detection of mRNA [54,

74], and when applied to mRNA it is referred to as PRINS-mRNA. PRINS-mRNA is virtually the same as IST in that it uses an unlabeled primer complementary to a specific mRNA sequence, and reverse transcriptase and labeled dNTPs to synthesize a labeled cDNA. Thus all comments made about IST should also apply to PRINS-mRNA.

Advantages of IST

Since chain elongation is independent of the length of the primer, oligonucleotide primers induce as much (if not more) labeling of the target mRNA than a much longer pre-labeled probe, thus increasing the sensitivity of the method. Another advantage of IST is its application to the detection of minor sequence variations in RNA by the proper selection of the sequence; thus, the position of the primer can control whether or not there will be chain elongation. The application of IST to the detection of minor sequence variations should be superior to ISH in that it is well known that the last few nucleotides of the primer are crucial to the initiation of chain elongation [10]. A significant technical advantage of the method is that the probe (primer) is unlabeled and labeling occurs only secondarily to specific hybridization, and the unincorporated labeled nucleotide can be washed away easier, which results in a lower background. In addition, fewer procedural steps allow for a shorter cumulative incubation time resulting in less degradation in the tissue morphology which often accompanies ISH or ISPCR. Another advantage of the milder conditions of IST is that it allows for the detected mRNA in cell suspension intended for flow cytometry without clumping and disintegration of cells [4].

Disadvantages of IST

The main disadvantage of IST is that it requires a high copy number of target mRNA (or DNA). If the copy number is low, ISH may be the technique of choice. If the copy number is very low, then ISPCR is the technique of choice, and has been shown to be able to detect a single copy. See ISPCR below.

In situ PCR

In situ PCR combines the DNA amplifying power of liquid phase PCR with the localizing capability of *in situ* hybridization [28, 63]. First, it amplifies minute quantities of DNA or RNA fragments to millions or billions of identical copies at the site of the original template and then detects or visualizes the amplified signal *in situ*. This technique fills a technical gap and allows detection of low copy numbers of nucleotide sequences against the background of tissue structure, even detecting a single copy of DNA or RNA per cell [78]. ISPCR is particularly important in detecting latent virus infections and studying the pathogenesis of many viral and oncogenic diseases [28, 78].

In situ PCR can be performed in several different ways. It can directly incorporate labeling molecules into amplified products by using labeled primers or labeled free nucleotides in PCR. This way, all amplified sequences have the labeling molecule built into them for subsequent detection. The amplified sequence can also be detected by employing *in situ* hybridization with a labeled probe complementary to the amplified target. RNA can be reverse transcribed into cDNA and then amplified and detected. Several *in situ* PCR machine models are available on the market. Each has its advantages and limitations.

It is well recognized that the key steps in *in situ* PCR include tissue preparation, pretreatment, primer and probe design, washing and detection. However, the prevention of amplified product diffusion is perhaps the most important consideration in designing any *in situ* PCR protocol. This technique is relatively new and technically challenging. False positivity and negativity can occur easily and should be carefully checked with a number of control experiments, including positive and negative tissue samples, and omission of primer, probe or other key components, one at a time, in the amplification or detection mixtures. The ISPCR results should be compared with results obtained by liquid phase PCR using the same primers and probes to detect the same sequences on DNA or RNA extracts from the same tissue samples.

Advantages of ISPCR

The most obvious advantage of *in situ* PCR is, of course, its very high detecting sensitivity while retaining tissue morphology so that minute quantities of DNA or RNA can be visualized and correlated to the surrounding morphology. It was reported that it can detect down to a single copy of a DNA or RNA sequence in intact cells or on tissue sections. This makes it a very valuable tool for many purposes, especially the detection of early or latent viral infections or early genetic changes in oncogenesis. All these can be achieved with commonly used enzyme labeling methods and viewed with a transmitting light microscope. The detecting sensitivity of conventional *in situ* hybridization is not entirely clear, but is believed to need at least 20 copies for detecting and thus leaves a technical gap where *in situ* PCR finds most of its applications. A second advantage is, in theory, *in situ* PCR can be combined with other methods to demonstrate two targets simultaneously on the same tissue preparation. It can also be performed at the electron microscopic level, although the preliminary reports in this regard (mostly in abstract forms) showed very poor ultrastructure preservation. A third advantage,

again in theory, is that direct *in situ* PCR can detect DNA or RNA targets without knowing the entire sequence by using a pair of primers flanking the two ends of the target. Overall, *in situ* PCR has a high detecting sensitivity that has made *in situ* PCR so popular.

Disadvantages of ISPCR

In comparison with *in situ* hybridization and other highly sensitive methods, *in situ* PCR has a number of disadvantages. First, it is technically challenging to set up reliable *in situ* PCR and, once set up, the technique is often not very stable. This is due primarily to the large number of critical steps in the protocol. These include adequate enzyme digestion, efficient amplification, prevention of amplicon diffusion, and reduction of background. It often takes longer and needs more controls to establish reliable *in situ* PCR protocols. The second disadvantage is that the tissue morphology is less than ideal. Because of digestion and, in particular, the harsh treatment by the thermal cycling, the morphology of the samples, although still recognizable, is often distorted and damaged. The third disadvantage is that the protocol of *in situ* PCR requires specially designed machines which makes it more expensive and less convenient to perform. Fourth, because of the very high detecting sensitivity, the less than desirable morphology and the potential problems associated with amplicon diffusion and inadequate digestion or washing, the results could be difficult to interpret. Extensive controls are sometimes called for and this might make the experiment lengthy and less manageable. Strict internal and external controls are needed for *in situ* PCR; however, optimal control kits are not currently available for this technique.

It should be mentioned that although *in situ* PCR is a very powerful and attractive technique, it is not the only one for detecting low copy number of nucleotide fragments. Other procedures such as FISH, nanogold-silver method, 3-SR technique, radioisotope labeled *in situ* hybridization, and autoradiography, etc., can also detect those sequences without some of the drawbacks of *in situ* PCR. Each of the methods has it strengths and weaknesses and should be considered before committing a given *in situ* PCR protocol.

Controls

As for immunohistochemistry, it is extremely important to perform adequate controls to verify the specificity of *in situ* hybridization [14, 16, 58, 63, 75]. For single-stranded probes, a common approach is to use a sense instead of antisense probe as a negative control and follow the exact same protocol. However, it has been reported [44], although rarely noted in the literature, that a small number of antisense sequences may be produced by the target cells, possibly by a mechanism of transcription regulation. Therefore, a sense probe may result in a reduced but still specific and positive labeling. We have observed this phenomenon and learned to interpret the sense probe results with caution [29]. Using an unrelated probe of the same length and C + G content is the best way to get around this problem. Positive controls may include a tissue preparation with a known quantity and distribution of the target sequence. Negative controls may include a piece of tissue that is known not to contain the target sequence. This can be created artificially by treating the tissue sections with RNase or DNase to destroy the target sequences. However, this needs to be performed carefully and followed by extensive washing. Any trace residue of the enzyme that finds its way into the real experiments may destroy the target or probe. Additional controls may include the omission of each of the key elements in the incubation cocktails. This is often effective in checking the ingredients that cause the false positivity or give high background. It is always advisable to perform Southern or Northern blot analysis on DNA or RNA extracts of the same target tissue side-by-side with *in situ* hybridization to verify the presence and quantity of the target sequences. The specificity of the visualization methods also needs to be checked including replacement of each of the key elements, particularly the primary antibody or the first linking reagent to the probe. Only after the key controls give the expected results can the observations with *in situ* hybridization be validated.

It should be noted that new techniques are emerging that can detect low copy numbers of DNA and RNA without going through the elaborate *in situ* amplification steps. The reporting signals can be amplified instead of, or in addition to, the amplification of the target sequences themselves.

EM *in situ* hybridization

In situ hybridization has been applied at the electron microscopical levels using electron-opaque labels [25]. The attempts of applying *in situ* PCR at the EM level have not been very successful because of the deterioration of the ultrastructure caused by the many PCR cycles, although signals have been reported to be visualized under the electron microscope.

Both preembedding and postembedding *in situ* hybridization can be performed on electron microscopic grids. The procedure is similar to that for the light microscope except that the protocols are adjusted to the EM conditions with much gentler digestion and washing. Colloidal gold remains to be the best labeling method at

the electron microscope level. A double labeling with 2 differently sized gold particles can be performed on the same grid to demonstrate the RNA or DNA and its corresponding protein simultaneously, greatly facilitating the morphological elucidation of the subcellular regulatory mechanism of a particular gene and its product [25].

Technically, EM *in situ* hybridization is quite challenging. The tissue samples are very delicate and the optimal balance of the many treatments, washings and reactions need to be established empirically for each target sequence in its host tissue. For detailed protocols, readers are referred to a monograph on this topic edited by Morel [75].

Protocols

Recommended protocols of *in situ* hybridization

The following protocols are selected from many published procedures. They have been tested in our own laboratories and found to be reliable and reproducible. The first is for RNA-RNA detection using riboprobe to detect mRNA. It has been successfully used to detect c-myc, N-myc and L-myc gene expression in small cell lung cancers [31] and atrial natriuretic peptide gene expression in the heart [30, 32]. The second protocol uses oligoprobe that was labeled with FITC and detected with a specific antibody to FITC. We used this protocol to detect peptide mRNA including ANP, neuropeptide Y (NPY), insulin and glucagon. These protocols can be adapted to different purposes. They should be adjusted individually with particular probes, targets and tissues. The protocols rarely give optimal results by just copying and switching from one probe or target to another. Controls should always be performed with the experiments to assist in trouble-shooting and result interpretation. There is inconsistency in tissue preparation, digestion, pretreatment, and detection among the protocols. This was due to the fact that they were developed in different laboratories by different individuals. They have all worked well for their particular applications at the time of the studies.

Common materials and equipment. The equipment needed are incubation oven, adjustable precision pipettes, glassware, glass slides, coverslips, etc. The reagents and solutions needed are xylene, alcohol, dextran sulfate, saline sodium citrate (SSC) (20x, 5x, 2x, 1x), EDTA (1 mM), Tris-buffered saline (TBS) (50 mM Tris/HCl, 150 mM NaCl pH 7.6), alkaline phosphate substrate buffer (100 mM Tris/HCl, 50 mM $MgCl_2$, 100 mM NaCl pH 9.0), formamide ($HCONH_2$), Diethyl Pyrocarbonate (DEPC; Sigma, St. Louis, MO).

Protocol #1: *in situ* hybridization using biotin-labeled riboprobes on tissue sections.

1. Prepare tissue sections by dewaxing, rehydration, washing in DEPC-treated water, etc.

2. Digest tissue with Proteinase K, 5-30 μg/ml at 37°C for 10-30 min in humid chamber.

3. Wash in DEPC-treated water for 3x2 min.

4. Deactivate Proteinase K at 70-75°C for 1 min (optional) wash in double distilled water 5 mins.

5. Prepare prehybridization solution (for 1 ml):

50% Dextran sulfate	250 μl
20xSSC	149 μl
EDTA (1 mM)	120 μl
Herring Sperm DNA	33 μl
DEPC-treated water	448 μl

6. Apply prehybridization solution to tissue section and incubate at 42°C for 30 min.

7. Prepare hybridization solution with biotinylated probe.

Hybridization solution (H.S.) (for 1 ml):

50% Dextran sulfate	250 μl
EDTA (1 mM)	120 μl
20xSSC	100 μl
Formamide	450 μl
Herring Sperm DNA	33 μl
DEPC-treated water	47 μl

Mix well and add probe. Probe final concentration: 0.2 - 1.5 ng/μl.

8. Apply hybridization solution.

9. Incubate 3 hours to overnight at 42°C in humid chamber.

10. Wash in 5x SSC for 5 min.

11. Wash in 2x SSC for 5 min.

12. Proceed to detection with a biotin detection kit with sufficient background blockage.

13. Wash in distilled water (2x5 min).

14. Counterstain and mount.

Notes to Protocol #1

1. Radioisotope or digoxigenin-labeled in situ hybridization can be more sensitive than biotin-labeled in situ hybridization.

2. The probe in the hybridization solution (step 8) can be heated to 95°C for 5 mins before being applied to the tissue section to eliminate any self-annealing or secondary structure formation of the probes.

3. Up to step 10, the procedure should be performed in a RNase-free manner.

4. The hybridization mixture can be applied on dehydrated dry sections or on wet sections. If the latter, there should be as little liquid remaining on the slides as possible to avoid diluting the concentrations of the ingredients in the hybridization solution.

5. For hybridization solution of less than 40 μl per tissue section, a coverslip can be applied and sealed at the hybridization step (step 9) to prevent evaporation.

6. RNase may be used in post hybridization treatment to remove single-stranded probes, thereby reducing

background. This can be applied at 100 μg/ml RNase A and 1 unit/μl RNase T_1 for 30 mins at 37°C.

7. Counterstain should not mask or overshadow the specific labeling.

Protocol #2: *in situ* hybridization using FITC-Labeled oligoprobes on tissue sections.

1. Prepare tissue section by dewaxing, rehydration, washing in DEPC-treated water, etc.

2. Digest tissue sections with Proteinase K at 5-30 μg/ml in 0.05 M Tris/HCl buffer pH 7.6 made with DEPC-treated water, and incubate for 10-30 min at 37°C.

3. Immerse in DEPC-treated, double-distilled water for 3 x 2 min.

4. Dehydrate in increasing grades of ethanol.

5. Air dry for 2 min.

6. Prepare hybridization solution with FITC-labeled probe.

Hybridization solution

30% Formamide
10% Dextran Sulfate
0.6 M NaCl
Mix well and add probe. Probe concentration: 0.2-1.5 ng/μl, optimized for probe and tissue sample selected.

7. Apply hybridization solution and coverslip.

8. Incubate 3 hours at 42°C in humid chamber.

9. Wash slides in TBS containing 0.1% Triton-X-100 for 3 x 3 min. Allow coverslips to drain off in the washing solution - Do not touch coverslips to remove. Dipping may be required to remove any remaining coverslips.

10. Place slides on incubation tray and cover sections with 100 μl of TBS, containing 3% bovine serum albumin, 0.1% Triton-X-100, 20% normal rabbit serum. Incubate for 10 min.

11. Tip off the blocking solution and add rabbit Fab anti FITC conjugated to alkaline phosphatase diluted 1:100 - 1:200 in TBS containing 3% bovine serum albumin and 0.1% Triton-X-100. Incubate for 30 min - 3 hours at 20°C.

12. Wash slides in TBS for 2 x 3 min.

13. Wash slides in alkaline phosphatase substrate buffer pH 9.0 for 5 min.

14. Place slides in humid chamber and demonstrate alkaline phosphatase activity by covering sections with the following solution (for 200 μl):

80 μl 5-Bromo-4-chloro-3-indolylphosphate (BCIP) - 50 mg/ml in dimethyl formamide
80 μl Nitro blue tetrazolium (NBT) - 75 mg/ml in 70% dimethyl formamide
10 μl 1 M levamisole
10 ml alkaline phosphatase substrate buffer

Color development may take from 1 hour to overnight.

15. Wash in running water for 5 min.

16. Counterstain and coverslip.

Notes to Protocol #2

1. In step #2, the enzyme activity can be stopped by optionally heating to 70°C for 1 min. Generelly the activity is stopped by dilution in the subsequent washings.

2. Up to step 9, the procedure should be performed in a RNase-free manner.

3. All the other notes described in Protocol #1 also apply to Protocol #2.

Recommended protocols of *in situ* transcription

Animals perfuse-fixed with 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1M PBS (phosphae buffered saline), and the tissue embedded in paraffin by standard procedures. Under RNAse free conditions, 25-nt oligonucleotide probes complementary to target mRNA were hybridized 5 μm tissue sections, overnight. The primed mRNA was then transcribed *in situ* by incubation with a mixture of nucleotide precursors containing DIG-labeled dUTP, and AMV (Avian Myeloblastosis Virus) reverse transcriptase. After washing the *in situ* transcribed sections, the incorporated DIG was bridged with sheep anti-DIG IgG, and detected with 10nm gold conjugated rabbit anti-sheep IgG followed by silver enhancement. Controls consisted of the omission of the oligonucleotide probes, or the substitution of unrelated 25-nt oligonucleotides in the hybridization step.

IST has been applied to tissue, perfuse-fixed, paraffin-embedded, and handled to permit IST, immunocytochemistry, and enzymecytochemistry on sections all from the same paraffin block. This has been illustrated in detail by De Bault and Wang [19] using γ-Glutamyl Transpeptidase (γ-GTP) in rat kidney to demonstrate the localization of γ-GTP enzyme activity, γ-GTP protein, and γ-GTP mRNA.

Common materials and equipment: The equipment needed is: incubation oven, hot plate, ice bucket, adjustable precision pipettes, glassware, glass slides, coverslips, etc. The reagents and solutions needed are xylene, alcohol, sucrose, bovine serum albumin (BSA), dithiotheitol (DTT), gum arabic, triton X-100, $MgCl_2$, KCl, citric acid, sodium citrate, SSC (20x, 5x, 2x, 1x), EDTA (1 mM), TBS (50 mM Tris/HCl, 150 mM NaCl pH 7.6), RNase inhibitor, Formamide ($HCONH_2$), DEPC, silver lactate or silver acetate, ice, etc.

Protocol #3: *In situ* transcription using unlabeled oligoprimers and DIG-labeled dUTP on tissue sections.

1. Tissue was fixed in 3% paraformaldehyde + 0.5% glutaraldehyde + 2.5% sucrose in 0.1 M PBS, pH

7.4, followed by standard paraffin embedding.

2. Tissue sections were dewaxed, rehydrated in a graded ethanol series, and washed with 2xSSC (2xSSC solution consists of 0.15 M NaCl, 0.015 M sodium citrate, and adjusted to pH 7.0 with 1N HCl). DEPC-treated water was used to make all solutions and buffers.

3. Each slide is incubated with 20 μl of ISH mixture containing oligonucleotide primer(s) (covered with a glass coverslip) at 42°C overnight, followed by 30 min incubation at room temperature. The final mixture contained:

50% Formamide
4xSSC
0.02% bovine serum albumin (BSA)
5 mM DTT
0.6U RNase Inhibitor/μl
10μM Oligonucleotide (see notes #5 and #6 below for ISH solution)

4. Wash 2 times with 2xSSC and 2 times with 0.5xSSC, 15 min each at room temperature.

5. Hold slides in 0.5xSSC for 2 hr before proceeding with IST.

6. Each slide was incubated with 15 μl of IST mixture containing DIG-labeled dUTP (covered with a glass coverslip) at 37°C for 60 min followed by 45°C for 10 min. The final mixture contained:

50 mM Tris-HCl, pH 7.4
6 mM $MgCl_2$
40 mM KCl
5 mM DTT
0.02% BSA
0.1 mM DIG-DNA Labeling Mixture
0.6U RNase Inhibitor/μl
1U AMV Reverse Transcriptase/μl (see note #7 below for IST Solution

7. Wash 2 times with 2xSSC 30 min each at room temperature.

8. Wash 2 times with 0.05xSSC 1 hr each at 35°C.

9. Wash 3 times with 0.05 M TBS pH 7.4 containing 0.25% Triton X-100 10 min each at room temperature.

10. Slides were pre-incubated in 1% BSA in 0.05 M TBS for 15 min at room temperature.

11. Slides were incubated with 50 μl of 1° antibody mixture at +4°C overnight, followed by 1 hour incubation at room temperature. The final mixture contained Sheep Anti-Digoxigenin antibody diluted 1:50 in 1% BSA in 0.05 M TBS.

12. Wash 3 times with 0.05 M TBS 10 min each followed by wash with 0.02 M TBS pH 8.2 containing 0.25% Triton X-100 for 10 min at room temperature.

13. Incubate in 1% BSA in 0.02 M TBS pH 8.2 for 15 min at room temperature.

14. Incubate with 50 μl of 2° antibody mixture for 1 hr at room temperature. The final mixture contained 10nm gold conjugated Rabbit Anti-Sheep IgG diluted 1:10 in 1% BSA in 0.02 M TBS.

15. Wash once with 1% BSA in 0.02 M TBS, pH 8.2 for 10 min, followed by 3 washes of 0.05 M TBS 10 min each.

16. Wash with distilled water.

17. Incubate in Silver Enhancement mixture for about 20 min at 22°C. The final mixture contained [34, 40]:

5.5 mM silver lactate or silver acetate
77 mM hydroquinon
120 mM citric acid
80 mM sodium citrate
10% gum arabic (see note #10 below for Silver Enhancement Solution)

18. Wash with distilled water 5 times 2 min each.

19. Counter stain lightly with hematoxylin and eosin (H&E) (optional).

20. Mount in Permount® and coverslip.

Notes to Protocol #3:

1. All reagents used and steps in the perfusion and fixation procedure were performed at +4°C, unless otherwise stated [45, 61].

2. In the standard paraffin embedding, infiltration was by machine processing and included 2 changes of 95% Ethanol for 20 min each, 3 changes of 100% ethanol for 15, 20, and 30 min respectively, 2 changes of Xylene for 20 and 30 min respectively, all at 40°C, and 2 changes of paraffin for 45 min each at 57°C. The tissue is embedded in flat molds [70, 83]. Completion of the entire embedding process on the same day that the perfusion fixation is performed is important in maintaining maximum antigenicity and mRNA reactivity.

3. 5 μm paraffin sections were floated on a 0.1% DEPC-treated water bath at 42°C, and picked up on silanized slides [81]. It is ***important*** *to perform the in situ transcription and immunogold-silver staining or other detecting procedures immediately after cutting; cut sections stored for days or weeks lose reactivity and background often increases.*

4. All in situ reagents were prepared on ice and used at the indicated temperatures [46, 74, 87].

5. Oligonucleotides were synthesized on an Applied Biosystems, Inc. (Forest City, CA) Model 380B or 394A according to the β-cyanoethyl phosphoramidite chemistry method [12]. The newly synthesized oligo-nucleotides were purified by reverse phase high pressure liquid chromatography on a 4.6 x 250 mm C18 column (Rainin Instrument Co., Woburn, MA) The column was equilibrated with 0.02 M triethylammonium acetate, pH 7.0; and the elution was accomplished by a linear grad-ient of 5% to 30% acetonitrile in 12 min. This typically yields 100 pM/μl of 25-nt oligonucleotides [19].

6. Preparation of in situ Hybridization (ISH) Solutions for step #3: A minimum of 20 µl of final ISH working solution containing the oligoprimers is needed for each slide. The following stock and final working solutions are recommended:

Solution #1

Formulation	*Volumes*	*Final Concentration*
Deionized Formamide	*500 µl*	*50% Formamide*
20xSSC	*200 µl*	*4xSSC*
10mg/10 ml BSA	*200 µl*	*200µg BSA/ml*
38.5mg/5 ml DTT	*100 µl*	*5 mM DTT*
Total	*1 ml*	

Solution #2

Take 160 µl of solution #1 and add 2.5 µl of 40U/µl RNase Inhibitor (Boehringer Biochemica, Mannheim, FRG) to give a total of 100U in 162.5 µl. (Enough for 8 slides) [8-11].

Solution #3

Final working solutions: Makes 44 µl to 46 µl of ISH mixture containing Oligonucleotide. Enough for 2 slides when used at 20 µl/slide. Adjust volume for additional slides.

7. Preparation of in situ Transcription (IST) Solution: Prepare solution with cold reagents and hold on ice until used. 15 µl of final working IST solution is needed for each slide.

Solution #1

Formulation	*Volumes*	*Final Concentration*
0.05M Tris-HCl pH 6.5	*40 ml*	
$MgCl_2.6H_2O$ (MW:203.3)	*48 mg*	*6 mM*
KCl (MW:74.55)	*120 mg*	*40 mM*
DTT (MW:154.3)	*31 mg*	*5 mM*
BSA	*8 mg*	*200 µg/ml*
Total	*40 ml*	

***Solution #2:** Final Working Solution:*

Solution #1	*140.0 µl*	
BM Digoxigenin-DNA Labeling Mix (10X)*	*15.0 µl*	*0.1mM/base*
BM RNase Inhibitor (40U/µl)	*2.5 µl*	*100U/163.5µl*
BM AMV Reverse Transcriptase (24U/µl)	*6.0 µl*	*150U/163.5µl*
Total	*163.5 µl*	

** = Boehringer Mannheim Biochemica [13, 21, 33, 41, 84, 94].*

8. In negative control slides the procedure was modified by: a) omitting the ISH Oligonucleotide step #3, b) omitting the DIG-DNA Labeling Mixture from IST step #6, c) omitting the AMV Reverse Transcriptase from IST step #6, or d) a combination of these omissions.

9. In the immunocytochemical detection of Digoxigenin all immunoreagents were diluted with 1% BSA in 0.05 M Tris buffer saline (TBS) pH 7.4 [60, 87, 95].

10. Preparation of Silver Enhancement Solutions: In the silver enhancement step, a 100 ml final working solution is prepared as follows:

Formulation	*Volumes*	*Final Concentration*
Solution #1:		
Citric Acid, monohydrate	*2.55 g*	*120 mM*
Sodium Citrate, dihydrate	*2.35 g*	*80 mM*
Distilled H_2O	*50 ml*	
Add 50% gum arabic (in H_2O)	*20 ml*	*10%*
Subtotal	*70 ml*	
Solution #2		
Hydroquinon	*0.85 g*	*77 mM*
Distilled H_2O	*15 ml*	
Subtotal	*15 ml*	
Solution #3		
Silver Lactate	*0.11 g*	*5.5 mM*
Distilled H_2O	*15 ml*	
Subtotal	*15 ml*	

Solution #4

Final Working Solution: First mix solutions #1 and #2, just before use add solution #3. The final working solution should be kept in the dark as much as possible, i.e., wrap working solution container and staining jars with aluminum foil.

Recommended protocols of *in situ* PCR

The published protocols for *in situ* PCR and *in situ* RT PCR are very different. Here we presented the protocols for detecting HIV RNA and DNA as an example to illustrate the techniques [96]. HIV ISPCR

and HIV RT-ISPCR protocols take an average of three full working days and follow the same guidelines of other ISPCR methods. The same kits are used as for conventional PCR and RT-PCR. The technician's schedule must be planned in advance. An ISPCR laboratory should have a full-time and motivated staff.

Common materials and equipment. The equipment and reagents needed are identical to those for *in situ* hybridization plus reagents, enzymes and probes specified in the protocols. An *in situ* PCR machine is also required. For our purposes, we employed the *in situ* PCR Machine (PTC-100-16MS) for MJ Research Inc. (Watertown, MA). Other *in situ* PCR machines designed for slides can also be used.

Protocol #4: *In situ* polymerase chain reaction using HIV biotinylated probe on tissue sections.

1. Deparaffined tissue sections or cytological samples are digested with 30 μg/mL of proteinase K solution, inside a humid chamber at 37°C for 15 to 30 min.

2. Wash sections in double-distilled (dd) H_2O for 10 min twice.

3. Place slides on a block at 70-75°C for 1 min to inactivate the enzyme, followed by washing.

4. Prehybridize the sections inside a humid chamber at 42°C for 30 min using 50 μL of a solution composed of 12% dextran sulfate , 2x SSC, 0.12 mM EDTA and 0.33 mg/mL salmon sperm DNA.

5. Remove the excess prehybridization solution from each section and heat each of them to 75°C in a slide thermocycler (MJ Research).

6. Slowly add 50μL of PCR mixture to each section.

PCR Mixture:

	Volume	Final Concentration
ddH_2O	33.75 μL	
10x PCR Buffer II	5 μL	1x
25 mM $MgCl_2$	5 μL	2.5 mM
10 mM each dNTP	1 μL	200 μM each
25 mM each primer (SK38/39 or SK102/432)	1 μL	0.5 mM each
5 U/mL *Taq* DNA polymerase	0.25 μL	1.25 U/50 μL

7. Cover each section with a glass coverslip one at a time and completely seal the edges with an appropriate amount of transparent nail polish. Keep all the slides at 75°C until the PCR mixture has been added to the last one.

8. Start the thermocycling. The temperature parameters for ISPCR are 95°C 1.5 min (initial denaturing), 30 cycles of 95°C (denaturing) 30 s, 55°C (annealing) 45 s, and 72°C (extension) 30 s. This is followed by 72°C (final extension) 1.5 min, and storage at 4°C (soaking).

9. The nail polish is softened with acetone and the cover slips are carefully removed with a surgical blade. Immediately, the sections are washed in 5x SSC, 2x SSC and PBS for 5 min each.

10. Bake the sections in an oven at 60°C for 20 min.

11. One hundred microliters of hybridization solution are added to each section. Heat to 95°C, 5 min for denaturing. Avoid evaporation of the solution. Hybridize overnight in a humid chamber at 45°C.

Hybridization Solution
50% formamide
25% dextran sulfate
2x SSC
0.33 mg/mL salmon sperm DNA
HIV-1 biotinylated probe (SK19 or SK102 for SK38/39 and SK145/431 amplifications, respectively) at 250 to 400 pg/mL concentration.

12. Wash the sections with 5x SSC, 2x SSC and PBS for 5 min each.

13. Detection is performed using a kit for biotinylated probes (K600: DAKO, Carpenteria, CA) based on the linkage of streptavidin and biotinylated alkaline-phosphatase coupled to NBT/BCIP calorimetric reaction (blue color).

14. Once the detection is completed, wash the section in PBS for 5 min. If desired, slides may be slightly counter stained with Pyronin-Y, Nuclear Fast Red or Fast Green. These dyes should be dissolved in 2x SSC or PBS and not in ddH_2O.

15. Dry sections at 50°C in an oven and cover using permanent mounting media.

Notes to protocol # 4.

1. In step #1, the digestion time and temperature vary according to type of specimen and must be empirically verified.

2. In step # 7, avoid excessive nail polish; otherwise, slides will not fit into the compartments of the thermocycler. Use the pipet tip to adjust the cover slips. Bubbles trapped beneath the coverslip will usually come out by themselves during heating. Therefore, do not try to remove them by pressing the coverslip.

3. In step # 13, this reaction is carried out in darkness and monitored at about 15-min intervals under a light microscope, usually for no more than 1 h.

Protocol #5. Reverse transcriptase-*in situ* PCR on tissue sections. Method A. Reverse transcriptase-driven RT-ISPCR.

1. Deparaffined tissue sections or cytological specimens are digested with 30 μg/mL proteinase K solution inside a humid chamber at 30°C for 15 to 30 min. Digestion process may be varied (see above).

2. Sections are washed twice in ddH_2O for 10 min and heated to 70-75°C for 1 min to inactivate the enzyme.

3. Sections are pretreated with RNase-free DNase. Ten to twenty U/section incubating at 37°C for a minimum of 4 h. Overnight incubation is strongly advised to completely destroy the DNA.

4. The sections are extensively washed with several changes of ddH_2O for 20 min.

5. Ten microliters of RT mixture (GeneAmp RNA PCR Kit) are added to each section and incubated inside a humid chamber at room temperature for 15 min.

RT Mixture:

	Volume	Final Concentration
$MgCl_2$	2.0 μL	5 mM
10x Buffer II	1.0 μL	1x
ddH_2O	1.5 μL	-
dNTPs each	1.0 μL	1 mM
RNase inhibitor	0.5 μL	1 U/10 μL
Reverse transcriptase	0.5 μL	2.5 U/10 μL
Random hexamers	0.5 μL	2.5 μM
Total volume	10.0 μL	

6. Sections are incubated in a humid chamber at 42°C for 20 min.

7. Twenty microliters of prehybridization solution (same as used for HIV ISPCR) are added to each section.

8. Slides are placed in the slide thermocycler (MJ Research) set with one cycle above 95°C for 3 min (to inactivate the reverse transcriptase) and 5°C for 5 min.

9. Sections are incubated with the residual prehybridization solution in a humid chamber at 42°C for 20 min.

10. PCR mixture is added to the sections, 40 μL/section.

PCR Mixture

	Volume	Final Concentration
$MgCl_2$	2.0 μL	2 mM
10x PCR buffer	4.0 μL	1x
ddH_2O	37.75 μL	-
Taq DNA polymerase	0.25 μL	1.25 U/50 μL
Primer SK38	0.5 μL	0.25 μM
Primer SK39	0.5 μL	0.25 μM
Total volume	40 μL	

11. The amplification is performed in the slide thermocycler using the same parameters as for HIV ISPCR (see above).

12. Hybridization and detection are also performed as described in HIV ISPCR.

Method B. r*Tth* DNA polymerase-driven RT-ISPCR

1. Deparaffined tissue sections or cytological specimens are digested with 30μg/mL proteinase K solution inside a humid chamber at 37°C for 15 or 30 min.

2. Sections are washed twice in ddH_2O for 10 min and heated to 70-75°C for 1 min to inactivate enzyme.

3. RNase-free DNase pretreatment is performed as described above.

4. Sections are extensively washed in ddH_2O for 20 min.

5. Twenty microliters of RT mixture (Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit; Perkin-Elmer) are added to each section and incubated inside a humid chamber at 70°C for 25 min.

RT Mixture

	Volume	Final Concentration
ddH_2O	11.5 μL	-
10x RT Buffer	2.0 μL	1x
$MnCl_2$	2.0 μL	1 mM
dNTPs each	0.4 μL	200 μM
r*Tth* DNA polymerase	2.0 μL	5 U/20 μL
"Downstream" primer SK39	1.0 μL	0.75 μM
Total volume	20.0 μL	

6. Next the humid chambers containing the slides are placed in a refrigerator at 4°C to stop the reaction.

7. Ten microliters of 12% dextran sulfate solution containing 1 mg of glycogen are added to each section.

8. Eighty microliters of PCR mixture are added to the sections.

PCR Mixture

	Volume	Final Concentration

ddH_2O	61.0 μL	-
10x Chelating buffer	8.0 μL	0.8x
$MgCl_2$	10.0 μL	2.5 mM
"Upstream" primer SK38	1.0 μL	0.25 μM
Total volume	80.0 μL	

9. Amplification is performed in the slide thermocycler (MJ Research) using the same parameters as for HIV ISPCR (see above).

10. Hybridization and detection are also performed as described for HIV ISPCR.

Notes to Protocol #5.

1. Radioisotope or digoxigenin-labeled in situ hybridization could be more sensitive than biotin-labeled in situ hybridization.

2. The probe in the hybridization solution (step 8) can be heated to 95°C for 5 min before being applied to the tissue section to eliminate any self-annealing or secondary-structure formation by the probes.

3. Up to step 10, procedure should be performed in an RNase-free manner.

4. The hybridization mixture can be applied to dehydrated dry sections or to wet sections. If the latter, there should be as little liquid remaining on the slides as possible to avoid disbursing concentrations of the ingredients in the hybridization solution.

5. For a hybridization solution of less than 40 μl per tissue section, a coverslip can be applied and sealed at the hybridization step (step 9) to prevent evaporation.

6. RNase may be used in posthybridization treatment to remove single-stranded probes, thereby reducing the background. The RNase can be applied at 100 μg of RNase A per ml and 1 U of RNase T_1 per μl for 30 min at 37°C.

7. Counterstain should not mask or overshadow the specific labeling.

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Discussion with Reviewers

Reviewer I: Among different fixatives which one would you prefer for expression analyses of a particular gene in the tissue sections?
Authors: 10% formalin or 4% paraformaldehyde in 0.01 M PBS adjusted to pH 7.4.

Reviewer I: Would it be possible to amplify a particular genetic element from a tissue section which has been fixed in paraformaldehyde or formalin for longer period of time like 5-10 years?
Authors: It is possible to amplify targets in formalin or paraformaldehyde fixed archive tissue samples. One example was reported by Isaacson *et al.* (1994) who examined polio, measles, influenza and HTLV-1 in archival brain tissue samples with *in situ* RT-PCR and detected their RNA sequences in individual neurons, glia and vascular endothelial cells. Some of the paraffin tissue blocks were over 25 years old. However, it should be pointed out that the degree of success with archival material is dependent on the completeness of the fixation and the nature of the target sequence.

Reviewer I: You mentioned that the concentration of probes in the range of 200-1500 ng/ml depended on the size of the probe. How does the size of the probe relate to concentration, is it empirical or certain defined rules that govern the concentration and size of the probe?
Authors: The concentration in this case was given in ng/ml. At a given concentration, the larger the probe, the fewer the number probes available per ml of solution. Thus, the concentration should be increased proportionally with the increase in probe length to ensure that a sufficient number of probes are available for hybridization.

Reviewer II: The authors state that single stranded (ss) DNA probes are superior to double stranded (ds) DNA probes because they do not self-anneal. The hybridization signal's intensity ultimately reflects the number of reporter nucleotides on the probe-target complex. For a target of 8,000 base pairs (a small virus), a ds probe can easily be made that covers this entire region; this is much more difficult with the ss DNA probe. In my experience ds DNA probes usually give the best results; this is most notable with the ultimate small single stranded DNA probe, i.e, the oligoprobe. Do the authors have any direct experience showing that a ss DNA probe is superior to a ds DNA probe and, if so, would this be expected for large targets (>1000b)?
Authors: This depends on the size of the probe and the target and how the probes are labeled. When the single stranded and double stranded probes are the same size and identically labeled, single stranded probes are more efficient as they do not normally anneal to themselves. In the case of a large target where large single stranded probes are difficult to make, double stranded probes will be superior. On the other hand, longer probes will find less targets than shorter ones, thereby having decreased detecting sensitivity but giving a more specific signal.

Reviewer II: In the section on trouble-shooting for *in situ* hybridization, the authors state that probe design is a very important factor. In my experience, nick translation and random primer usually give good results for probe synthesis for any DNA template of >500 bp. Define probe design and explain its role in troubleshooting. In the case of no signal with *in situ* hybridization, I would recommend that the investigator use his/her labeling system and an alu DNA probe tem-plate, as the repetitive alu sequence is present as thousands of copies in mammalian cells. Do the authors agree with this strategy or do they recommend some other method to deter-mine the cause of a lack of signal with *in situ* hybridization that involves changing probe design?
Authors: Probe design in this context refers to the selection of a particular fragment of the sequence complementary to the target and the length of this sequence, rather than the way the probes are labeled. In our experience, when experimenting with a new target, repeated negative signals, even after modifying the hybridization conditions, call for a new probe to a different portion of the target. Certain probes work well and others do not, i.e., either too faint a signal or too much a background, even if, in theory, the new probe should work equally well. This may have to do with the degree of the uniqueness of the probe sequence and the way the target sequences are folded and embedded in the tissue sample, presenting different availability of the target sequences to the particular probes selected. The suggested alu DNA probe templet approach is a good method to check the labeling efficiency, the general hybridization conditions and target (alu sequences) availability, but give little indication to the suitability of the particular probe that gives weak or no signal. In addition to employing the alu sequence, shifting the target sequence or using a collection of different sequences to different or overlapping portions of the target should be tested. By changing the probe design, i.e., to use a different probe sequence, we have solved the problems of false negative hybridization in a number cases while other modifications produced little success.

M. Malecki: Do you perform liquid phase PCR as a

way to optimize annealing temperature for a specific target?

Reviewer I: Besides the critical parameters like Proteinase K, probes, and primers, do you think optimization of annealing temperature is also critical, and if it is so, how can one proceed with optimization of the system?

Authors: Optimization of annealing temperature is also very important, of course. This can be established by keeping the other factors constant and testing a number of temperature settings. It can also be established by liquid phase PCR with the same target and primers. Nevertheless, the temperature should not vary too much from target to target.

Reviewer II: Background signals with *in situ* hybridization are due to nonspecific sticking of the labeled probe to cellular proteins and/or nucleic acids. That is, the probe, which presumably enters all cells, will diffuse out unless it finds its complementary target or non-specifically sticks to cellular components. From a practical viewpoint, I would argue that the most likely causes of background are: a) posthybridization wash not stringent enough, where temperature is a key factor; b) probe concentration too high. The questions are: 1) why do the protocols not include a temperature for the post hybridization wash. 2) Most protocols do not include a prehybridization wash, which, in my experience, does not affect background. Have the authors done comparative studies of background with and without prehybridization?

Authors: Background signals with *in situ* hybridization are caused by nonspecific or less specific sticking of the labeled probes to cellular proteins and/or nucleic acids or may be trapped by cytoskeleton structures that in theory can be removed by critical washing. Temperature, stringency of the washing solution, washing duration and vigilance are all factors affecting the efficiency of probe removal from the tissue sample. Temperature is one of the factors that affect the outcome significantly. We usually raise the temperature up to 50°C if the initial washing does not remove most of the background staining. The optimal condition should be established empirically for each case, therefore no particular temperature is recommended. We have performed comparisons between protocols with and without prehybridization washes and found that prehybridization washing reduced background staining is some cases, presumably by blocking potential nonspecific bonding sites in the tissue sample. We also know that this prehybridization washing does not affect the efficacy of amplification nor does it increase background staining. We routinely perform prehybridization washing for both *in situ* hybridization and *in situ* PCR.

Reviewer II: With IST, one is making labeled cDNA inside the cell. Do the authors know of instances where this labeled product diffused out of the cell and into another cell type known to not have the target?

Authors: We know of no published case where the labeled cDNA product of IST diffused into another cell type not containing the target sequence. In IST, the cDNA is literally synthesized on the target sequence creating a double stranded nucleic acid duplex with little or no mismatched NTP pairs. Such double stranded duplexes are very stable and require strong denaturing conditions to separate the new cDNA from its complementary strand, i.e., high melting temperatures well in excess of the 45°C used in the last 10 min of the transcription step, high stringency conditions in excess of 2xSSC at room temperature plus 0.05xSSC at 35°C.

Reviewer II: The authors state that extensive washing of the protease and DNAse is needed with *in situ* PCR. However, in my experience, a 1 minute wash in water and a 1 minute wash in 100% ethanol is enough to eliminate these enzymes. Have the authors demonstrated any change in the *in situ* PCR signal relative to the length of wash after one or both of these enzymes?

Authors: You may be right, but we prefer to be on the safe side. When there are so many steps that can go wrong we want to be sure that the experiments are not ruined by easily avoidable mishaps at the beginning of the protocol. We also know that extensive washing does no harm to the targets and subsequent reactions.

Reviewer II: The authors claim that one can make millions of copies of the amplicon in the cell during *in situ* PCR. However, most investigators report a 200+ fold increase in copy number, and not a million fold increase. It would, on a theoretical basis, seem unlikely that one could amplify one million copies in a space of 10μ, given the extremely high concomitant amplicon concentration which is one of the limits on the extent of amplification in a 100μl reaction volume. What is the basis for the authors claim for such a marked increase in copy number during *in situ* PCR?

Authors: It is true that PCR on tissue sections or intact cells is not as efficient as liquid phase PCR due to the limited accessibility of the target and the interference of fixed tissue structures as well as the smaller amount of solution (we add about 50-100 μl of PCR solution to the slide depending on the size of the sample). But once a sequence is amplified, more than a few hundred copies will result within the next ten cycles. Nobody knows, even roughly, how many copies of amplicons there are after amplification *in situ*. It is difficult, if not impossible, to measure. By theory, the amplicons are free floating and they diffuse out freely following each cycle

of amplification, therefore leaving rooms for new amplicons to form. We believe that the estimated 200 plus copies of the amplicons by others are not grounded. One thing appears to be true, i.e, there are not too many copies left *in situ* by the time the signals are examined under the microscope. Judging by the intensity and the size of the reporting signal, there should not be more than a few hundred copies of the ampli-cons remaining in their original location. Nevertheless, this does not mean that only those amplicons are produced by the PCR, as most of them have diffused or been washed away. There is no reasonable mechanism to keep the amplicons remaining *in situ*, except the possible long amplicon theory proposed by one of the authors (JG) and some possible trapping and network-forming phenomenon to hold the amplicons on site. We speculate that most amplicons diffuse to the supernatant easily.

Reviewer II: The authors state that back diffusion is a formidable problem with *in situ* PCR. Back diffusion would presumably be due to the large amount of labeled amplicon in the overlying solution sticking to cellular proteins and/or nucleic acids. This is equivalent to every *in situ* hybridization reaction, where one has a large amount of labeled DNA that can stick non-specifically to cellular components. This can be removed by a high stringent wash, owing to the weak force of these bonds versus the much stronger hydrogen bonds with 100% homology between probe and target. Explain why back diffusion (background) is any more of an issue with *in situ* PCR versus *in situ* hybridization.

Authors: The answer to this question is related to the issue discussed in a previous question ("In the section on troubleshooting ..."). In the situation of *in situ* PCR, the concentrations of the amplicons are much higher than that used for *in situ* hybridization, and increasingly so until a plateau is reached. This large amount of amplicons will diffuse to other parts of the tissue sample and may stick to them, semi-specifically or non-specifically by bonding to proteins, or simply be trapped and tangled at nonspecific sites. In addition, the variations in sizes of the amplicons may "stick" to non-specific sites more easily than the more uniformly sized probes in the solution of *in situ* hybridization. More-over, the tissue samples for *in situ* PCR tend to be more harshly treated than those used in straight *in situ* hybridization. For these reasons, the so-called back diffusion may be more readily seen during *in situ* PCR than during *in situ* hybridization. It should also be noted that back diffusion does not occur only with labeled amplicons, but unlabeled amplicons produced in the so-called indirect *in situ* PCR protocol can also cause nonspecific sticking and subsequently be picked up by hybridization step used in the detection process resulting in nonspecific staining.

Reviewer I: Between the two *in situ* PCR strate-gies, i.e., direct incorporation of labeled molecules into amplified products or indirect labeling like addition of labeled probe, which one is better and why? Would you please comment on this?

Authors: We prefer the indirect methods, as do most investigators. It has an added step to check the specificity. Since some nonspecific sequences are amplified in PCR, they will not be picked up by the specific probes in the hybridization step used in the detection system. It should also be pointed out that in the indirect method, nonspecific DNA repair, etc. will not affect the final results. This has been discussed extensively in a number of publications.

Reviewer II: An important measure of specificity with ISH, IST, or *in situ* PCR is afforded by knowing which cell type likely contains the target of interest. There is often striking localization of the target to specific cell types. An example would be parvoviral infections where the target (the nucleated red blood cell) is easily identified on morphologic grounds. According to published reports, with *in situ* hybridization and reverse transcriptase (RT) *in situ* PCR, the signal only localizes to the target cell, assuming a high stringent wash. If the wash is not stringent enough, then other cell types show signals with both methods. Explain why background should be any more of a problem with *in situ* PCR versus *in situ* hybridization, where an excess of probe far greater than can be synthesized during the cycling process is present, and why it is not evident in many published reports of viral infection and *in situ* PCR where the target cell is known?

Authors: This question has been partially answered in a previous question ("The authors state that back diffusion ...."). To start with, *in situ* PCR is usually employed only after the conventional *in situ* hybridization failed to detect any signal convin-cingly. The extremely high detecting sensitivity of *in situ* PCR leads the investigators to a new territory where more marginal positivity may be present and positive signals may show up in unexpected cell types. When those occur, extensive controls are called for and the new results may still not be black and white. These all make the interpretation of the *in situ* PCR and, in particular, RT *in situ* PCR results more difficult. For target mRNA, it is often a matter of difference in quantity among different cell types rather than a yes or no answer. We speculate that when the target locations are known, the authors are more confident to publish their results and the articles are more likely to be accepted by journal referees. The many, less than clear cut observations, even though they may be closer to the truth, were buried in the lab's notebooks and data bases.

M. Malecki: Would you be able to estimate the distances between the target sequences and the reporter molecules in ISH, IST, and ISPCR?
Reviewer III: Could you analyze differences in the distribution of reporter molecules around the target sequence labeled by IST and ISPCR as compared to ISH?
Authors: The distances between target sequences and reporter molecules in different methods depend on many factors, including the size of the probes, the size of the target, the type of tissue, the labeling methods, the signal detecting methods, the visualization approach, etc. It all depends on how much the targets have been amplified and retained and what projection range the reporter signal build-up has achieved by the particular detecting method. It can range from the immediate proximity as in IST, to a signal that can engulf the entire cell by one target sequence in IS PCR. In general, *in situ* hybridization gives more localized signals than the other two techniques.

Reviewer I: In the HIV ISPCR protocol, prehybridization is recommended before doing actual ISPCR. Does it provide certain (extra) advantage(s) for *in situ* PCR amplification?
Authors: We routinely perform prehybridization for *in situ* hybridization and find this step reducing background labeling. For indirect *in situ* PCR, prehybridization is also necessary. We perform this step before the PCR cycles rather than afterwards, i.e., directly before the subsequent *in situ* hybridization. This way, we avoided the prehybridization step between the PCR cycles and the hybridization step and believe that this is beneficial for preventing diffusion of or washing away the amplicons from their original sites.

Reviewer I: In the HIV ISPCR protocol, in step #9 acetone is recommended for softening of nail polish. Most people use absolute ethanol for this purpose. Do you think acetone is better over ethanol?
Authors: It is our preference. Both will work. We have not made a systematic comparison.

Additional Reference

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