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PREPARATION OF SAMPLES FOR POLYMERASE CHAIN REACTION *IN SITU*

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Abstract

The purpose of this paper is to describe the key variables in sample and reagent preparation needed for successful polymerase chain reaction (PCR) *in situ*. Tissue or cell preparations should be fixed in a cross linking fixative, such as 10% buffered formalin, preferably from 15 to 48 hours. Tissues should be embedded in paraffin; cell preparations can be fixed when near confluence, then physically removed and processed. When possible three samples (4 μM tissue sections or 1-5000 cells) should be placed on silane coated glass slides. Digestion in pepsin (2 mg/ml) for 30 min is adequate for DNA detection by PCR *in situ* hybridization whereas optimal protease digestion time is variable and related to formalin fixation time for reverse transcriptase (RT) *in situ* PCR. RT *in situ* PCR requires an overnight digestion with DNase. The amplifying solution should contain 4.5 mM $MgCl_2$, 0.05% bovine serum albumin, and, for RNA analysis, the reporter nucleotide. A false positive signal would be evident with incorporation of the reporter nucleotide for DNA targets due to DNA repair; this can be avoided with frozen, fixed tissues and the hot start maneuver. Otherwise, one needs to use a labeled probe and a hybridization step to detect amplified DNA targets in paraffin embedded tissues.

Key Words: polymerase chain reaction *in situ*, *in situ* hybridization, formalin, fixative, hot start.

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Introduction

The purpose of this paper is to provide a concise, simple reference for sample and reagent preparation for those investigators who wish to do PCR *in situ*. This discussion will presuppose that the reader has minimal experience with cell and tissue preparation in a pathology laboratory. The basis of much of the information presented in this manuscript can be found in several publications [4, 6, 7, 8, 9, 10, 11]. Readers who wish a more in-depth discussion of the different variables important for PCR *in situ* are referred to a textbook [2]. Finally, a video presentation is available for those wishing to see more directly some of the technical, hands-on features of doing PCR *in situ* [3].

The key variables for PCR *in situ*

Introductory statements. From the standpoint of the technique itself and reproducibility of the data PCR *in situ*, in my opinion, is no more difficult than solution phase PCR or *in situ* hybridization. However, it does have a large disadvantage when compared to either of these other two techniques. Many people who do solution phase PCR usually have much expertise in molecular analyses but, often, do not have much experience working with intact tissue sections. Similarly, those who do *in situ* hybridization often have a strong background in histology but not as extensive an experience with other molecular-based methodologies. To achieve the maximum advantages with PCR *in situ*, one needs to have some knowledge of tissue preparation and histologic interpretation as well as basic concepts of molecular analyses in general and PCR in particular.

Definition of terms. There are many different approaches to amplifying DNA or cDNA inside an intact cell. One fundamental difference is whether one detects the amplified DNA or cDNA by incorporating a labeled reporter nucleotide into the amplicon as it is being synthesized or by use of a labeled probe. The following terms relate to this important point:

Polymerase chain reaction (PCR) *in situ*: A general term for amplifying DNA or cDNA inside an intact cell.

PCR *in situ* hybridization: Detection of the amplicon by doing a hybridization step after PCR using a labeled probe. This is best suited to detection of DNA

targets in paraffin embedded tissue.

In situ PCR: Detection of the PCR product via a labeled nucleotide (or, less commonly, a labeled primer) that is incorporated into the amplicon during DNA synthesis. This can not be done for a specific DNA target in paraffin embedded tissues due to the ubiquitous presence of primer independent DNA synthesis in such material. *In situ* PCR is the basis, however, for RNA detection.

Reverse transcriptase (RT) *in situ* PCR: Detection of a specific cDNA target via a labeled nucleotide that is incorporated into the amplicon during amplification. A key step with RT *in situ* PCR is DNase digestion, which, after optimal protease digestion, eliminates the various DNA synthesis pathways which may be operative during *in situ* PCR.

Primer independent DNA synthesis: DNA synthesis operative during *in situ* PCR in cells or tissue that have been exposed to dry heat (e.g., 65°C for 15 min) prior to PCR. The DNA synthesis is due to repair of single stranded gaps. This DNA repair pathway is always operative in paraffin embedded tissues due to the obligatory heating step [2, 10].

Sample Preparation

The glass slide

Cell and tissue adherence is an important issue with PCR *in situ* as the repeated cyclings at elevated temperatures would tend to separate the sample from the slide. Fortunately, one can obtain near 100% adherence of the tissue or cell sample using silane coated slides. Organosilane (Sigma, St. Louis, MO), can be purchased separately and diluted to 2% in acetone for those wishing to pretreat the slides themselves. I recommend that one purchases slides already pretreated in silane, which are readily available and inexpensive from many commercial sources (e.g., ONCOR, Gaithersburg, MD or Enzo Diagnostics, Farmingdale, NY). In my experience, silane coated slides may be used years after they are prepared with no loss of tissue adherence. Other pretreatment regimes to improve tissue adherence are available (e.g., glue, SOBO, L-lysine). However, in my experience, these do not work as well as silane.

The fixative

Fixatives are used in the pathology lab to render native degradative enzymes inoperative and to harden tissues such that they can be cut into thin (1-6 μm sections) for histologic analysis. There are two classes of fixatives which comprise nearly all such material used in the surgical pathology laboratory - cross linking fixatives and denaturing fixatives. The most common cross linking fixative formulation is 10% buffered formalin. Over 95% of surgical specimens in most surgical pathology laboratories will be fixed in buffered formalin. Other cross linking fixatives include glutaraldehyde, routinely used for electron microscopy, and paraformaldehyde. Acetone and 95% ethanol are examples of denaturing type fixatives. They are rarely used in diagnostic pathology, although ethanol is by far the most common fixative used in diagnostic cytopathology. Another category of fixatives that one may encounter in diagnostic pathology include formalin based fixatives to which has been added either picric acid (Bouin's solution) or a heavy metal such as mercury (Zenker's solution).

The optimal fixative for PCR *in situ* is 10% buffered formalin. Most surgical specimens will be fixed from 8 to 24 hours. If one can control the fixation time, it is recommended to use 24-48 hours as this will allow for a broader range of optimal protease digestion times for RT *in situ* PCR, which will be discussed shortly. Do not use fixatives which contain picric acid or a heavy metal, as they cause extensive degradation of the DNA and preclude successful amplification [2, 5, 10]. One can obtain a signal with PCR *in situ* using ethanol or acetone fixation. Such fixatives have the advantage that a protease digestion step is not needed, as compared to the same tissue type fixed in formalin [9]. However, in my experience, cell localization, critical for interpretation with RT *in situ* PCR, is not as distinct with the denaturing fixatives. Further, the detection rate is less than 100% with *in situ* PCR and denaturing fixatives when analyzing for a target that is known to be present in all cell types. This should be contrasted with formalin fixed material where, after optimal protease digestion, the detection rate will be 100%. Under these conditions, if one analyzes the over lying amplifying solution, the amplicon will not be detectable as compared to ethanol or acetone fixed material, where the amplified product is readily detectable in the overlying solution [9].

Unfixed, cryostat sections are sometimes used in immunohistochemistry because certain epitopes may be destroyed after formalin fixation. If one wishes to use such sections with PCR *in situ*, it is recommended that the slides be fixed overnight in 10% buffered formalin prior to amplification. Indeed, one can do target specific direct incorporation hot start *in situ* PCR for a DNA target using such material. This is because such material lacks the primer independent DNA synthesis pathway as it was never exposed to dry heat [2, 10].

Cells versus tissue sections

Tissue sections should be processed in the routine manner of surgical pathology laboratories: 3-4 four μm sections placed on a given silane coated slide. The multiple sections on one slide allows for direct compari-

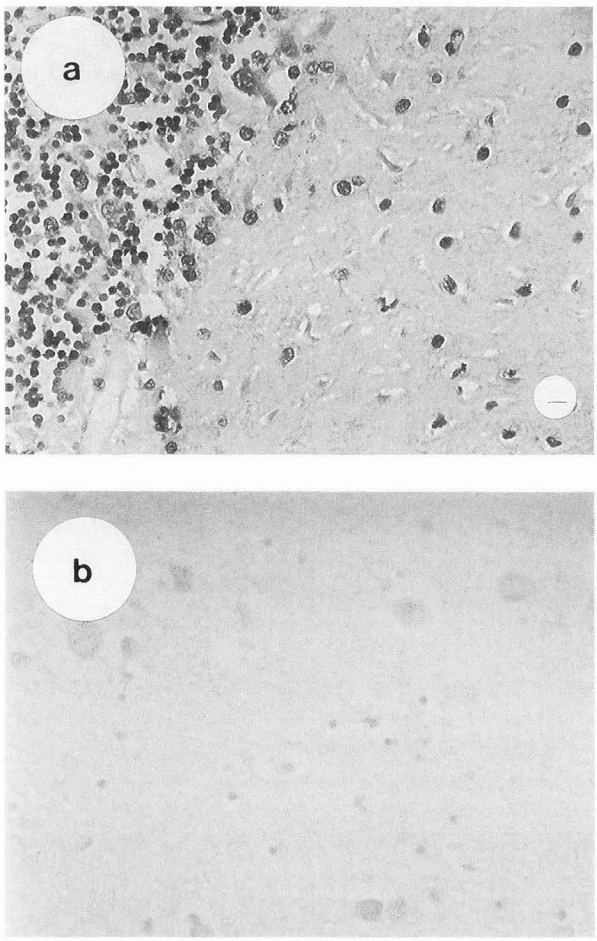

Figure 1. Determination of the optimal protease digestion time. This brain tissue was digested with pepsin (2 mg/ml) for 30, 60, and 90 min. At 90 min, an intense signal was evident in most cell types (**a**). This non specific signal was eliminated by overnight digestion in DNase (**b**), demonstrating that 90 min is the optimal protease digestion time for doing RT *in situ* PCR in this tissue section (see Fig. 4). The bar in each figure represents 10 μm.

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son in the same cells of the critical positive and negative controls.

One has several options when working with cell preparations. If grown in a tissue culture plate, one can added sterile, silane coated slides (or coverslips) and have the cells grow on them directly. Alternatively, one can grow the cells to near confluence, remove the growth media, and then add an ample amount of 10% buffered formalin for 2-3 days. The cells can then be scraped off the plate, the solution placed in a conical tube, and the cells washed twice in diethylpyrocarbonate (DEPC) (RNase free) treated water. The cells can then

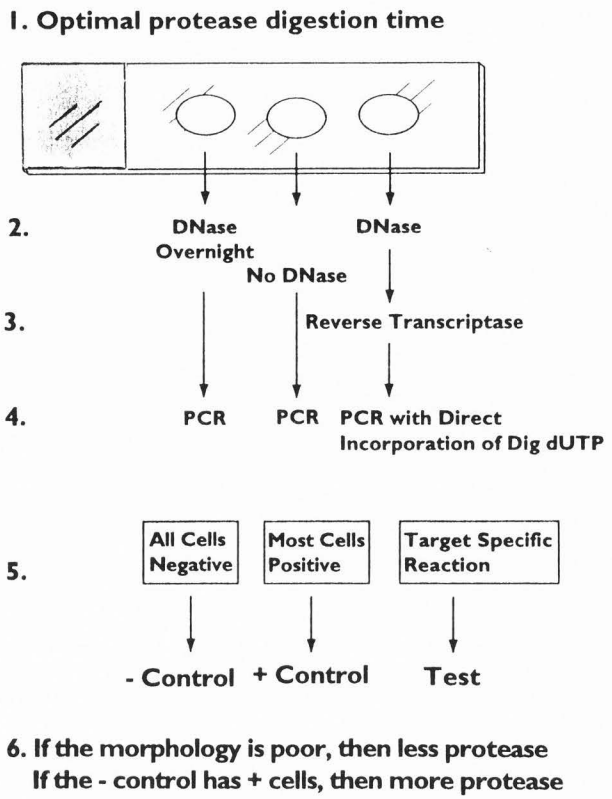

Figure 2. RT *in situ* PCR protocol. After determining the optimal protease digestion time, RT *in situ* PCR can be done by performing the positive control (no DNase), negative control (DNase, no RT or RT with irrelevant primers) and the test (DNase and RT) on the same glass slide.

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be resuspended to a concentration of about 5000 cells per 50 μl, and three 50 μl "spots" can be placed on the silane slide and allowed to dry. For those doing RNA analysis, I recommend exposing the cells to dry heat at 60°C for 15 min to induce DNA repair; this allows for a stronger positive control, as discussed below. For those doing DNA analysis, do **not** expose the cells to dry heat if you wish to do target specific *in situ* PCR.

Protease digestion

If ones uses formalin fixed material, as recommended, then protease digestion will be needed to obtain success with PCR *in situ* [2, 9, 10, 11]. There are a variety of proteases that are routinely used in the diagnostic pathology laboratory. These include pepsin, trypsin, proteinase K, and pronase. It is recommended that one choose one of these proteases and use it exclusively to become familiar with its nuances.

I prefer pepsin (or trypsin) over proteinase K as it can be inactivated by increasing the pH to 8.3 and a

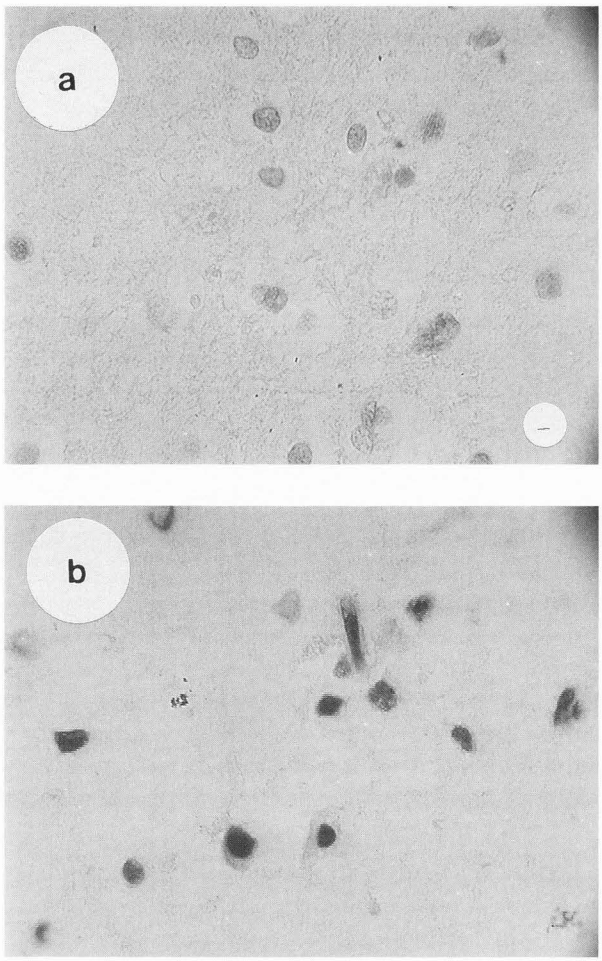

Figure 3. Persistence of non specific signal with DNase digestion after suboptimal protease digestion. This is the same brain tissue illustrated in Fig. 1. A signal is not evident with the no DNase positive control (**a**) if the protease digestion time is suboptimal, e.g., 30 min. However, note that a signal is evident under these conditions with the negative control (DNase, no RT) (**b**). The nuclear based signal in most cell types demonstrates that the signal is non specific.

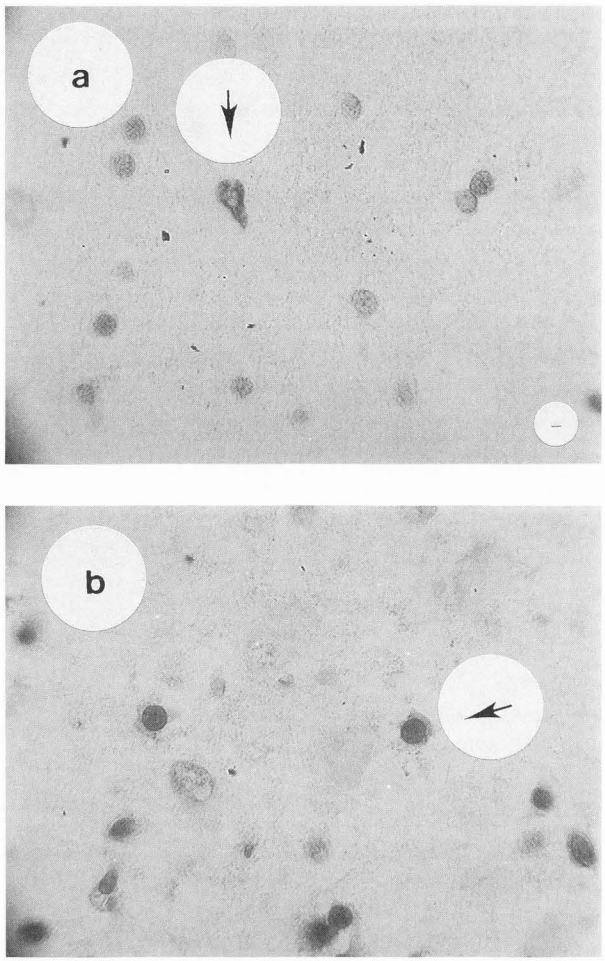

Figure 4. Specific localization of the signal with RT *in situ* PCR. This brain tissue is the same illustrated in Fig. 1. After optimal protease digestion, note that the signal for tumor necrosis factor mRNA localizes to the cytoplasm of a few cells (**a**). This is to be contrasted with the nuclear based signal for HIV-1 RNA (**b**) from this case of a person with severe AIDS dementia.

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simple rinse. Also, pepsin is less likely to over digest the sample, which precludes successful PCR *in situ* (unpublished observations). If one does use proteinase K, do not dry heat inactivate as this will render the DNA unsuitable for subsequent hybridization [2].

To prepare the protease solution, take 20 mg of pepsin and add 9.5 ml DEPC water and 0.5 ml of 2 N HCl. The pepsin should dissolve immediately. Store 1 ml aliquots at -20°C for up to one week. Thaw at either room temperature or 37°C and use immediately after thawing.

The optimal protease digestion time for *in situ* PCR and PCR *in situ* hybridization is very similar for a wide range of tissues whether they have been fixed for a short period of time (e.g., 8 hours) or a long period of time (e.g., 1 week). In most of these instances, 20-30 min of digestion with pepsin at room temperature will allow for successful *in situ* PCR or PCR *in situ* hybridization [2]. It is worthwhile noting that 20-30 min of pepsin digestion is adequate for over 95% of routinely processed surgical pathology specimens analyzed by standard *in situ* hybridization [2]. To remove the protease, simply wash the solution off the glass slide using sterile water, wash in sterile water for another minute, then wash in 100% ethanol for 15 seconds, and allow to air dry.

The optimal protease digestion time is much less

straightforward for RT *in situ* PCR. The likely explanation relates to the need for DNase digestion with RT *in situ* PCR. That is, the function of the protease with RT *in situ* PCR is not only to permit entry of the DNA polymerase but also to render the entire genomic DNA susceptible to DNase digestion! Longer formalin fixation times likely increase the number of protein-DNA cross links which must be removed for DNase to degrade the DNA. This may explain the strong relationship between formalin fixation and protease digestion time for successful RT *in situ* PCR [2, 10].

To determine the optimal protease digestion time for a given sample for RT *in situ* PCR, it is recommended that one place 3 sections on a given silane coated glass slide and digest each a different time with pepsin. For routinely processed surgical pathology material, it is recommended that one start with 20 min, 40 min, and 60 min of pepsin digestion. Then, do *in situ* PCR. For paraffin embedded tissues, it will not matter which primer pair one uses as the primer independent DNA synthesis pathway will be operative. Whichever protease digestion times gives an intense signal in at least 50% of the cells will be the optimal for that particular specimen [2]. To corroborate that this is the optimal protease digestion time, one should digest 2 of the samples for that time, and digest one overnight in DNase (described shortly). The definitive proof of optimal protease digestion time is an intense signal in most cells that is completely eliminated by DNase digestion (Fig. 1). This simple statement is the foundation of RT *in situ* PCR (Fig. 2). The elimination of the genomic based DNA synthesis pathways by DNase digestion after optimal protease digestion will allow for target specific direct incorporation of the reporter nucleotide into the cDNA of interest. It cannot be stressed enough that suboptimal protease digestion will NOT permit for cDNA target specific incorporation during RT *in situ* PCR due to persistence of the genomic, nuclear based DNA synthesis pathways (Fig. 3) [2, 10]. To remove the protease for RT *in situ* PCR, simply wash the solution off the glass slide using DEPC water, wash in DEPC water for another minute, then wash in 100% ethanol for 15 sec, and allow to air dry. I do not follow routine RNase precautions until the protease digestion step, and then use gloves and RNase free materials until the RT step is completed.

DNase digestion

After optimal protease digestion, the genomic DNA can be degraded such that it can no longer serve as a template for DNA synthesis during PCR by DNase digestion. Although 7 hours of DNase digestion can accomplish this task, it is recommended that one do this step overnight [2]. The DNase solution can be prepared by diluting both the PCR buffer II (Perkin Elmer, Norwalk, CT) and the RNase free DNase (Boehringer Mannheim, Indianapolis, IN) 1:10 with DEPC water. To remove the DNase for RT *in situ* PCR, wash the solution off the glass slide using DEPC water, wash in DEPC water for another minute, then wash in 100% ethanol for 15 sec, and allow to air dry.

Reagent conditions

When preparing the amplifying solution for PCR *in situ* hybridization, one can use the same formulation of buffer, nucleotides, and primers as for solution phase PCR. However, one must adjust the concentration of $MgCl_2$ and taq polymerase for optimal PCR *in situ*; further, it may be advantageous to include bovine serum albumin (BSA) in the amplifying solution to block adsorption of the taq polymerase to the glass, thus raising the effective concentration of the enzyme [9]. Optimal magnesium concentration for PCR *in situ* is 4.5 mM. This increased concentration relative to solution phase PCR applies to a wide variety of primer pairs and targets [2, 9]. The optimal concentration of the taq polymerase is 25 U/50 μl of amplifying solution. One can use 5 U/50 μl of the taq polymerase if BSA is added to the amplifying solution [2, 9]. The recipe that is recommended for PCR *in situ* hybridization follows [2]:
5 μl of GeneAmp buffer (the reagents are from the PCR *in situ* kit from Perkin Elmer)
9 μl of $MgCl_2$ (25 mM stock)
8 μl dNTP (final concentration 200 μM)
1.5 μl 2% BSA
3 μl of primer pair (20 μM stock)
22.5 sterile water
1 μl Taq polymerase (hot start, see below)

The recipe that is recommended for RT *in situ* PCR (from the EZ rTth kit from Perkin Elmer) follows [2, 4]:
10 μl EZ buffer
1.6 μl each of dATP, dCTP, dGTP, dTTP (final concentration 200 μM)
1.6 μl of 2% bovine serum albumin
1.0 μl of RNasin
3.0 μl of primers 1 and 2 (20 μM stock) (for the negative control, use nonspecific primers or omit the primers)
0.6 μl digoxigenin dUTP (1 mM stock)
14.6 μl DEPC water
12.4 μl 10 mM $MnCl_2$
2.0 μl rTth

Hot start maneuver

There are several DNA synthesis pathways which may be operative during PCR, either in solution phase or *in situ*. Of course, the pathway specific for the DNA or cDNA target of interest is the one we wish to be

operative. However, the primers may initiate DNA synthesis after annealing to non target DNA (mis priming) or to themselves (primer oligomerization). In either instance, a large amount of DNA may be synthesized which may interfere with target specific amplification. The other DNA synthesis pathway which may be operative during PCR *in situ* is primer independent and is induced by dry heating of the cells (7), which initiates the formation of single stranded DNA gaps. These gaps can serve as "surrogate" primers and initiate DNA synthesis during *in situ* PCR in any paraffin embedded tissue since such specimens are routinely heated during processing.

If one inhibits both mis priming and primer oligomerization during *in situ* PCR, target specific DNA synthesis is enhanced, even if the primer independent pathway is still operative [2, 6, 7]. Inhibition of the two primer dependent nonspecific DNA synthesis pathways is readily accomplished by realizing that their melting temperatures (Tms) will be much lower than for primer-target annealing, due to their lesser homology. That is, if one withholds either the taq polymerase or some essential reagent (such as magnesium) from the amplifying solution until the temperature of the reaction denatures the primer-non target and primer-primer hybrids, without denaturing the primer-target hybrids, then these primer dependent non specific DNA pathways can be blocked. This can be achieved by withholding the taq polymerase until 55°C; hence, the term hot start PCR [1, 2, 6, 7].

The hot start maneuver is needed for routine detection of 1 DNA target per cell using PCR *in situ* hybridization [2, 6, 7]. It is not needed for RT *in situ* PCR, as DNase digestion eliminates all the genomic-based DNA synthesis pathways.

One can either manually withhold the taq polymerase until the temperature of the cycler reaches 55°C, or add some chemical which inhibits primer annealing to non target DNA and other primers. The latter can be achieved with single stranded binding proteins [2, 7, 9] or antibodies against taq polymerase (unpublished observations). The antitaq polymerase antibodies prevents polymerase activity at room temperature; at >55°C, the antibodies are denatured and the taq polymerase can then function.

Cycling parameters

The recommended cycling parameters for PCR *in situ* hybridization and RT *in situ* PCR (using the one step rTth) are listed below. A key point to remember is that one is more likely to inactivate the taq polymerase with PCR *in situ* relative to solution phase PCR due to the much greater surface to volume ratio. Thus, the denaturing times and temperatures should be kept as short as possible.

The cycling parameters for RT *in situ* PCR are [2, 4]:

Incubate at 65°C for 30 min; denature at 94°C for 3 min; cycle at 60°C for 1.5 min, 94°C for 45 sec; do 20 cycles.

The cycling parameters for PCR *in situ* hybridization are [2]:

Incubate at 55°C for manual hot start; add taq polymerase, denature at 94°C for 3 min; cycle at 60°C for 1.5 min, 94°C for 45 sec; do 35 cycles, then do hybridization step.

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Discussion with Reviewers

M. Malecki: Have you noticed any differences in the distribution of the reporter molecules around the target sequence labeled by *in situ* PCR and PCR-*in situ* hybridization?
Author: No. With adequate protease digestion, DNA based signals are evident in the nucleus. RNA based signals vary from nuclear (HIV-1), subnuclear (perinucleolar or nucleolar for premRNAs), nuclear membrane (hepatitis C), and cytoplasmic (tumor necrosis factor (TNF) mRNA). However, with overprotease digestion, these various signals tend to become cytoplasmic. A signal on the cytoplasmic membrane usually reflects background; i.e., sticking of labeled primer oligomers from the amplifying solution onto the cell surface due to an inadequate post PCR wash.

Reviewer I: Would you be able to evaluate the ranges of distances between the target sequences and the reporter molecules in *in situ* PCR and PCR-*in situ* hybridization depending on amplification protocols?
Author: The reporter nucleotide is directly incorporated into the target sequence during *in situ* PCR; about 1 labeled nucleotide will be added for every 20 nucleotides of the amplicon. For PCR-*in situ* hybridization, the label is included on the probe. If the probe is made by nick translation or random priming, then the ratio of label to unlabeled nucleotides will be about the same as for *in situ* PCR. If terminal transferase is used, then from 1 to 5 labeled nucleotides will be present on the probe.

Reviewer II: In sample preparation sometimes epithelial cells like human endothelial cells need specific treatment like coating of the surface with different attachment factor. Do you think that silane coating will interfere with attachment factor if one is growing these cells on silane coated slides?
Author: No. My collaborators and I have obtained good results growing cells on silane coated slides.

Reviewer II: If a choice of fixatives is available which one would you recommend for tissue sections and which for established cell lines?
Author: I would strongly recommend 10% buffered formalin, 2-3 days, for each. This will allow one a broader optimal protease window for RT *in situ* PCR.

Reviewer II: In proteinase K digestion some researchers have mentioned appearance of small dots in the membrane peripheral area, have you experienced the same in your proteinase K digestion strategies?
Author: I do not rely on dots, presumably representing early breakdown of the cell membrane from over protease digestion. Rather, I rely on the primer independent signal to show me what is the optimal protease for RT *in situ* PCR. This is less important for PCR *in situ* hybridization, where 15-30 min of digestion with pepsin at 2 mg/ml will be adequate for most samples. I describe this in detail in reference [2]. Briefly, one can digest paraffin embedded (i.e., tissues exposed to heat) tissues for different times with a given protease, do *in situ* PCR or even isothermal DNA synthesis for 15-30 min, and determine optimal protease based on the strength of the signal.

Reviewer II: In definition of terms you mention *in situ* PCR as detection of the PCR product via a labeled nucleotide and less commonly by a labeled primer. Do you think that labeled nucleotide strategy is advantageous over a labeled probe?
Author: For RNA analysis - yes, for DNA analysis on paraffin embedded tissues, definitely no. The advantage is the time saved and the simplicity of not having to do a hybridization step. The DNase digestion after optimal protease digestion allows on to do target specific incorporation into the cDNA with RT *in situ* PCR. However, for DNA targets, the primer independent signal invariably present in paraffin embedded tissues necessitates detection by a probe step.

Reviewer II: In reagent conditions, you recommend the same formulation of buffer, nucleotides and primers as for solution phase PCR which is quite comprehensible, as far as concentration of these reagents is concerned you only take into consideration the $MgCl_2$ concentration and taq polymerase. What about nucleotides and primer concentration as we know that in the cellular milieu the conditions are not the same as in a tube. Would you recommend higher concentration of primers and probes for *in situ* PCR?
Author: We have varied the concentration of the primers up to 9 μM (about 10 times greater than the protocol we recommend) with no enhancement of the signal [8]. Indeed, the increased risk of primer oligomerization and, potentially, mispriming may be disadvantageous. We have not varied the nucleotide concentration. With regards to the probe, when using an oligoprobe and PCR-*in situ* hybridization, the concentration is a key factor in background and signal.