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# THE FOUNDATION OF SUCCESSFUL RT *IN SITU* PCR

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## 1. ABSTRACT

RT *in situ* PCR allows for the routine and rapid detection of low copy viral and human RNAs. Success with RT *in situ* PCR is best accomplished with formalin fixed, paraffin embedded material, which allows the study of archival material. The key variable for RT *in situ* PCR is protease digestion. The optimal digestion time, which is determined by testing a variety of protease digestion times, is defined by an intense signal in the nuclei of most cells irrespective of the primers used, and a loss of this signal with overnight digestion in DNase. This permits the target specific direct incorporation of the labeled nucleotide into the amplified cDNA. A lack of signal with the negative control (DNase, no RT) and an intense nuclear signal in most cells with the positive control (no DNase) is prerequisite for success with RT *in situ* PCR. The localization of the signal (cytoplasmic for human mRNAs and restricted to certain cell types) is another important indicator of successful RT *in situ* PCR. The one step rTth system allows for the reproducible amplification and detection of low copy RNA targets within a few hours. Matrix metalloprotease (MMPs) and their inhibitors (TIMPs) in cervical cancer are used as a model system for RT *in situ* PCR. Analysis of MMP and TIMP expression in cervical cancer demonstrates the following: 1) the signal localizes to the cytoplasm of invasive cancer cells and the surrounding stromal cells; 2) no signal is evident in the adjacent carcinoma *in situ* cells (non invasive component) or the normal epithelium.Cervical cancers of poor prognosis showed a marked increase in the percentage of cells expressing MMP versus TIMP as compared to microinvasive cervical cancer, which has an excellent prognosis.

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## 2. INTRODUCTORY STATEMENT

The field involving the *in situ* detection of PCR-amplified DNA and cDNA has advanced considerably in the last 5 years. A consequence of this rapid movement has been a proliferation of laboratory protocols and procedures and their accompanying “camps” of supporters. These differing approaches have led to many ideas that, over time and in the light of new data, have, in my opinion, been shown to be incorrect. For example, one can use full length probes for PCR *in situ* hybridization, with their much greater signal to background ratio, as they do not *per se* lead to background due to the apparent lack of detectable primer oligomerization inside the cell (1). Also, a high stringency wash after RT *in situ* PCR can eliminate the background that may result from nonspecific binding of labeled primer oligomers that form in the amplifying solution during the procedure (1). There are several other misconceptions and either incorrect or partly correct statements regarding *in situ* PCR that may be hindering the advancement of the field. In particular:

• RT *in situ* PCR (that is, direct incorporation of the reporter nucleotide) invariably leads to false positive results;
• migration of the amplicon during RT *in situ* PCR may lead to false positive results when the amplicon sticks to a cell that does not have the target (2-16). Included in the reference listing (2-16) are the protocols of many different laboratories who have reported data using *in situ* PCR. This list is presented as alternative techniques for doing *in situ* PCR as well as discussions of potential problems with the technique.

In understanding the keys to successful RT *in situ* PCR, it is important to appreciate some differences between doing solution phase PCR in a 50 µl volume in a GeneAmp tube and PCR in a cell of 5 microns with less than 1,000th of the volume. First, there is the marked difference in the surface to volume ratio. In a 0.5 ml tube, the ratio of the surface area to volume is about 1:2. With RT *in situ* PCR, assuming that one uses a 10 mm coverslip and a volume of 10 µl, the surface to volume ratio is over 20 times greater. This will alter the cycling parameters one uses for RT *in situ* PCR, especially the time and temperature of denaturation (17). The second difference is that the amplifying solution in solution phase PCR is mostly water, and the test sample provides relatively scanty amounts of DNA and even smaller amounts of proteins. With RT *in situ* PCR, the nucleus of a cell provides a relatively dense matrix of DNA, RNA and proteins which, if fixed in formalin, will be extensively cross linked to form a complex, 3-dimensional labyrinth. The cytoplasm of the cell also consists of a complex 3-dimensional matrix that is composed of its "skeleton", made primarily of the intermediate filaments, and a wide variety of other proteins and RNA. The relatively dense and complex protein-nucleic acid matrix found in the cell cytoplasm can be exploited to serve as an "anchor" for the amplicon to prevent its migration out of the cell. The relatively concentrated collection of proteins and nucleic acids in a fixed cell are the basis of some fundamental differences in the various DNA synthesis pathways that can be operative during *in situ* PCR versus solution phase PCR.

The first part of this review will focus on the key variables for successful RT *in situ* PCR. These key steps include sample fixation, protease digestion and DNase digestion. This section will be followed by a protocol for RT *in situ* PCR using the one step rTth system. Next detection of matrix metalloprotease mRNAs in cervical cancer cells will be used as a model of the RT *in situ* PCR technique, focusing on the interpretation of the positive and negative controls and the importance of the specific localization of the signal, that is, nuclear versus cytoplasmic.

Before beginning the review, the following terms should be defined:

• *in situ* PCR - direct incorporation of the reporter nucleotide into the PCR product as it is being synthesized within the cell.
• RT *in situ* PCR - detection of PCR-amplified cDNA via direct incorporation of the reporter nucleotide.
• PCR *in situ* hybridization - detection of the PCR product with a labeled probe after it is synthesized within the cell using a hybridization step.

## 3. THE KEY PREPARATORY STEPS

### 3.1. Fixative.

Pathologists use a limited number of fixatives. By far the most common solution used to preserve tissue and cell samples is 10% buffered formalin. Formalin cross-links the amino groups of proteins and nucleic acids to each other, and thus renders degradative enzymes inoperative (18). Other members of this family include paraformaldehyde and glutaraldehyde. One can visualize how this cross linking process creates an intricate, complicated 3-dimensional "roadway" from the nuclear matrix to the cytoskeleton and endoplasmic reticulum of the cell (1,19,20). This structure certainly could form a physical barrier to molecules that otherwise might diffuse readily through the nucleus and cytoplasm (1,19,20). It may also create a "charge" or "ionic" barrier by rigidly fixing the positively and negatively charged side chains of amino acids in space inside the cell (1,20).

Rarely, pathologists add other ingredients to formalin based fixatives. One such additive is picric acid. This acid is thought to improve preservation of nuclear detail, and thus is favored by some as a fixative for lymphoid lesions or testicular biopsies. An example of a picric acid containing fixative is Bouin's solution. A heavy metal, such as mercury or zinc is sometimes added to formalin and is thought by some to improve the cytologic detail of the nucleus. Zenker's solution is an example of this type of preparation.

After fixation the tissue is embedded in paraffin at 65°C for 4 hours. For the non-Pathologist, the paraffin embedded tissue block hardens when brought to room temperature and 4 µM sections can be prepared by the use of an instrument called a microtome. This obligatory heating step has important implications for *in situ* PCR, as it induces single stranded DNA gaps which lead to primer independent direct incorporation of the reporter nucleotide during *in situ* PCR (1,21). This DNA

"repair" pathway, which can be eliminated by DNase digestion, precludes target specific direct incorporation of the labeled nucleotide for DNA targets, but not for RNA targets (1,21).

Other less commonly used fixatives in the pathology laboratory include ethanol and acetone, which render degradative proteins inactive by denaturation. These are used mostly in immunohistochemistry, to preserve the antigenicity of certain epitopes.

Solution phase PCR and standard *in situ* hybridization can be done using unfixed material or samples fixed in formalin, ethanol, or acetone. Picric acid or heavy metal containing solutions should not be used as the tissue fixative for standard PCR or *in situ* hybridization as prolonged (>8 hour) fixation of the samples is associated with extensive degradation of the DNA, and a weak or no amplification or hybridization signal (18,22). One would then anticipate that either unfixed tissues or samples fixed in either a cross-linking fixative such as formalin, or a denaturing fixative such as ethanol or acetone could be used for RT*in situ* PCR. However, optimal RT *in situ* PCR , in my experience, requires formalin fixed material that has been adequately proteased (1,21). This is also true for *in situ* PCR for DNA targets (1,21). When peripheral blood leukocytes, each of which contain two copies of the bcl-2 gene, were fixed in ethanol or acetone, only 0 to 34% of the cells were found to be positive by *in situ* PCR using bcl-2 specific primers. Detection of the target in every cell was possible only if the cells were fixed in 10% buffered formalin and digested with protease prior to *in situ* PCR (1,20).

One may question why ethanol, acetone, or no fixation are suitable for PCR and *in situ* hybridization but not *in situ* PCR. Clearly, PCR and *in situ* PCR both involve the synthesis of DNA whereas *in situ* hybridization and *in situ* PCR both necessitate the complexing of a probe/primer with its target; in both instances similar sized molecules (Taq polymerase has equivalent dimensions with a 100 base pair labeled probe) must transverse a complex, 3-dimensional matrix of proteins and nucleic acids. The major difference between *in situ* PCR and the other two methodologies relates to the compartmentalization of the final product. With *in situ* hybridization, the probe-target complex is fixed to the nuclear matrix. The amplicon will readily diffuse during solution phase PCR to equivalent concentrations throughout the amplifying solution. *In situ* PCR is unique in that the target is fixed to the nucleus (or cytoplasm for RNA) but the amplicon can either remain at its site of origin, or diffuse throughout the entire cell or into the amplifying solution, depending on how the cells were fixed prior to *in situ* PCR.

It is a simple matter to do *in situ* PCR, save the amplifying solution, and determine under what conditions the amplicon remains restricted to the cell. We have performed such experiments (1,20). After *in situ* PCR, the only condition which permitted detection of the target in the cell and no detectable amplicon in the amplifying solution was fixation of the cells with a cross linker, such as 10% buffered formalin, followed by optimal protease digestion. Importantly, the amplicon was readily detected in the overlying solution after *in situ* PCR if the cells were fixed in acetone or ethanol (1,20).

It follows that the cross linking of proteins and nucleic acids by formalin fixation must be creating an "amplicon migration barrier". What is the nature of this barrier? Is it a physical barrier related to pore size, or is it biochemical in the sense of an ionic charge barricade? Obviously, at this stage all one can do is to speculate based on relatively little information.

If this putative barrier was based on the pore size of the cytoplasmic and nuclear membranes, then it should apply equally well for *in situ* hybridization and *in situ* PCR. There is no doubt that DNA sequences can be "stuck" inside a cell during *in situ* hybridization, perhaps by being trapped in these pores. This can be demonstrated by doing *in situ* hybridization with a 100mer probe using a tissue that does not have the corresponding target. If the probe concentration is too high or the post hybridization wash is not stringent enough, a nonspecific signal will be evident (Figure 1). The nonspecific signal, referred to as background, may be seen either in the nucleus or, more commonly, in the cytoplasm. Is background related to the size of the probe? In my experience, for probes that range from 20 to 300 base pairs, background is not related to the size of the probe (1). This would suggest that pore size may not be the most important variable for understanding this "amplicon migration barrier". It should be stressed that background is easily removed by performing a high stringency wash (Figure 1) which suggests that the movement of these DNA segments from 20 to 300 base pairs is not being restricted by pore size, but rather by biochemical forces such as hydrogen bonding and ionic charges. Such biochemical forces can be influenced during the post hybridization wash by conditions such as temperature, salt concentration, and formamide concentration (1,18). However, it has been well documented that probe size is an important variable for successful *in situ* hybridization. This was demonstrated in 1985 by Moench et al. using probes that varied from 70 to 780 base pairs in size (19).

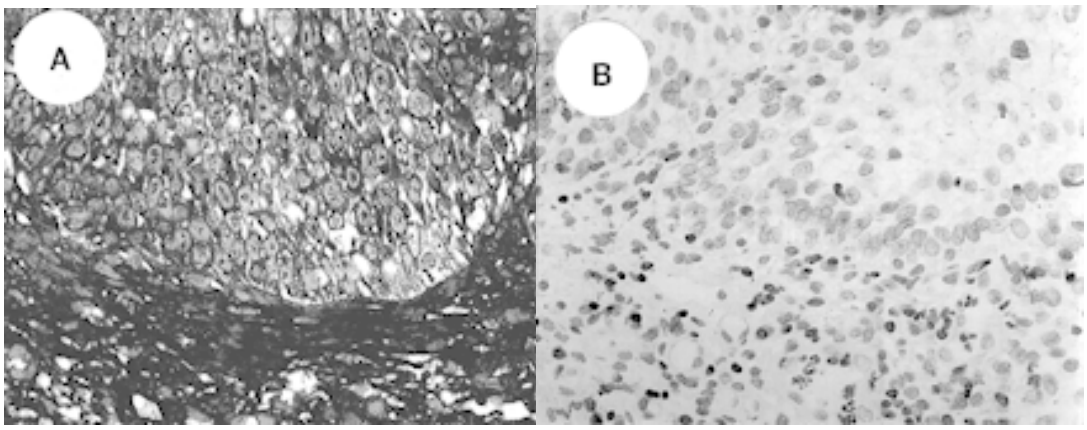


**Figure 1**. Background versus signal with *in situ* PCR. In either *in situ* hybridization or *in situ* PCR, background will result if the labeled DNA sequence binds to cellular proteins or nucleic acids. Background often localizes to the cytoplasm of cells that are known to not contain the target. Background was evident in this HPV negative vulvar biopsy using HPV specific primers, if the post *in situ* PCR high stringency wash was omitted (A). This background was eliminated if a wash of 10 minutes at 60°C in 15 mM salt was done (B).

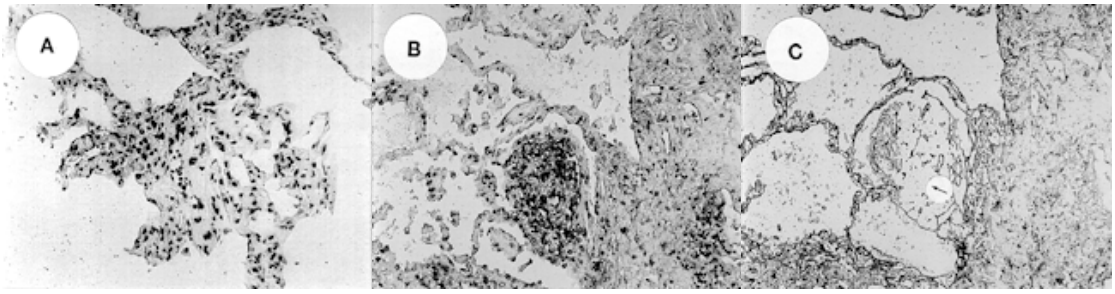


**Figure 2.** Formalin versus ethanol fixation with *in situ* hybridization. Formalin fixed tissue requires protease digestion for an optimal signal with *in situ* hybridization; in this instance, the repetitive alu probe was employed (A). If the same biopsy was fixed in ethanol, a signal is evident without protease digestion (B). If protease digestion is used, the signal was lost and the morphology destroyed (C).

Another observation that suggests that pore size may be part, but not the complete explanation for the "amplicon migration barrier" with formalin fixed material is that, to obtain a signal with *in situ* hybridization, one does not need to protease digest samples fixed with one of the denaturing fixatives. However, especially after prolonged fixation, protease digestion will augment the signal for *in situ* hybridization if the sample has been fixed in formalin (1). Indeed, as evident in Figure 2, protease digestion destroys morphology in ethanol fixed tissue long before this would be evident if the same tissue was fixed in formalin. One may speculate that the cross linking fixatives creates pores or channels that could physically inhibit movement of the probe/primer to its target. Of course, there are other possible explanations for the need for a protease step for *in situ* hybridization with formalin fixed samples. The protein-DNA cross-links may need to be removed for the probe to access the target (1,18).

Pore size, may, therefore, explain part of the putative "amplicon migration barrier" induced bycross linking fixatives with *in situ* PCR but probably is not the most important variable. It is possible that the biochemical correlates of a 3-dimensional matrix of rigid, cross linked proteins and nucleic acids may be a key factor (1). One can liken formalin fixation of a cell to creating a rigid, uniform scaffold where the positively and negatively charged side chains of amino acids would be spaced regularly and be poorly mobile. This can be contrasted with the "floating islands" of denatured proteins that are not as intimately associated with DNA and RNA in cells fixed in acetone or ethanol. In the latter situation, the charged side chains of the amino acids would not be rigidly and uniformly spaced nor in close, formal approximentation with the nucleic acids (Figures 3 and 4). As evident from these figures, two conditions would be required for minimal migration of the amplicon: the presence of a fixed nuclear or

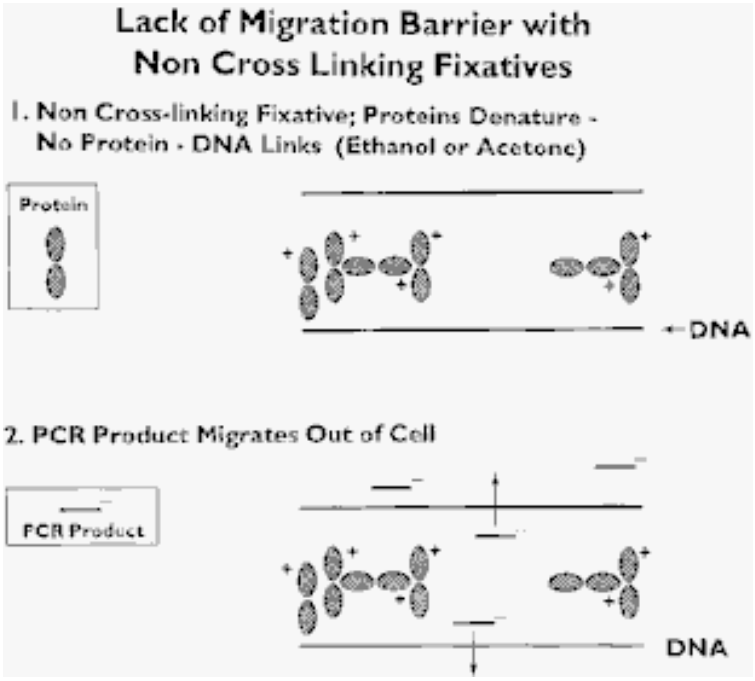


**Figure 3**. Migration of the amplicon during *in situ* PCR: ethanol or acetone fixation. This figure attempts to explain the loss of the amplicon into the amplifying solution and decreased detection rate with *in situ* PCR if a denaturing fixative is used. It is speculated that ethanol or acetone fixatives remove the "ionic barrier" of positive charged protein side chains by denaturing the proteins.

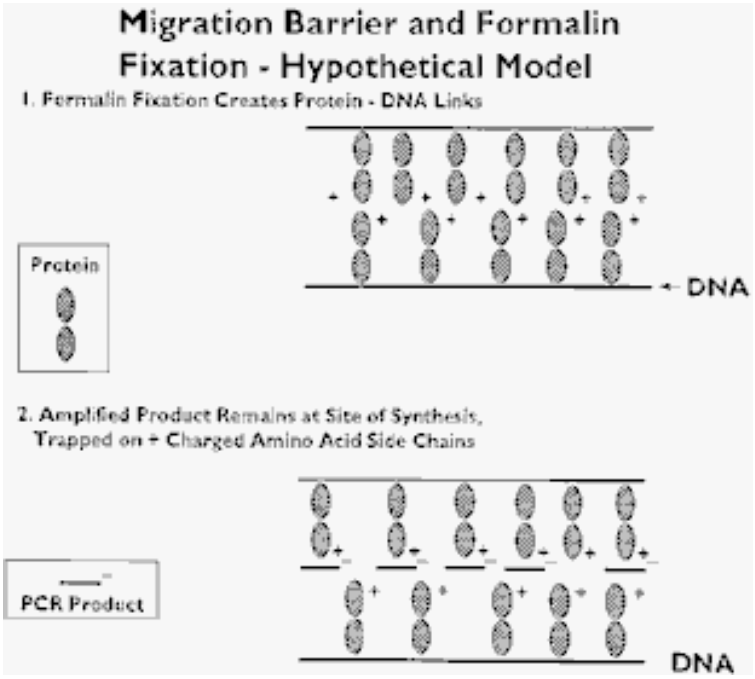


**Figure 4**. Migration of the amplicon during *in situ* PCR: formalin fixation. This figure attempts to explain the apparent lack of migration of the amplicon into the amplifying solution and 100% detection rate with *in situ* PCR if formalin fixation is used. It is speculated that cross-links between proteins and nucleic acids create a regular arrangement of positively charged amino acid side chains that limit migration of the amplicon, assuming optimal protease digestion.

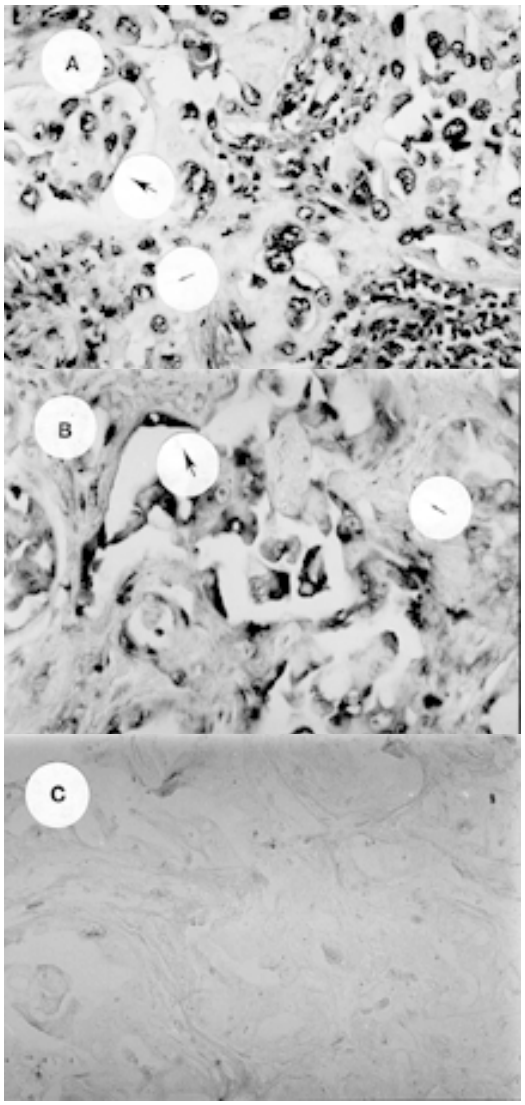


**Figure 5.** Correlation of mRNA expression and histologic features of tissues. The tissue is from a breast cancer. Note the intense nuclear signal in the different cell types (large arrow, carcinoma cells, small arrow, stromal cells) in the positive control where there is no DNase digestion (A). After DNase digestion, and RT *in situ* PCR for MAP kinase mRNA, only the cancer cells show a signal (B). Also note the cytoplasmic localization of the signal; the nuclei are negative. The signal was lost if hepatitis C primers were used in place of the MaP kinase primers (C).

cytoplasmic target and a network of positively charged amino acid side chains to inhibit amplicon migration (1,20). Acetone or ethanol fixed material would have a fixed target but, according to this model, would lack the latter feature. It is obvious that this is a simplistic model that needs further and rigorous testing.

Whatever the correct model, it must be stressed that, under optimal formalin fixation and protease digestion, there is minimal migration of the amplicon from its site of origin. As seen in Figure 5, this can be seen as the sharp cytoplasmic signal of mRNA with RT *in situ* PCR versus the nuclear based signal in the positive control, in which DNA is being synthesized from a nuclear based genomic template. Consistent with a large body of data on RNA trafficking via the nuclear matrix, we have noted different nuclear pathways of premRNAs using RT *in situ* PCR (1,23). The sharp demarcation between the nuclear (for DNA) and cytoplasmic signal (for RNA) also illustrates the marked inhibition of migration of the amplicon under proper conditions of protease digestion and post PCR high stringency washes. This is further illustrated when doing *in situ* PCR for viruses that show marked cellular tropisms. For example, parvovirus infects only red blood cell precursors, which can be easily recognized on cytologic grounds. The signal after RT *in situ* PCR for parvoviral RNA in infected tissues is only evident in these cells under proper conditions, with no evident “back diffusion” to the neighboring white blood cell precursors (1, 15).

Another interesting observation with regards the "amplicon migration barrier" is that, with intentional over digestion with protease, one can make a nuclear based signal move to the cytoplasm with *in situ* PCR in formalin fixed samples (Figure 6). Whether this reflects enlargement of cellular pores to sizes greater than critical migration thresholds or the loss of the protein-DNA tight cross linked network is unclear. The importance of the information available when interpreting *in situ* PCR with formalin fixed cells and protease digestion from the cellular distribution of the signal cannot be overstated. A cytoplasmic localization for a nuclear based target is due to over digestion by the protease. A nuclear based signal for RT *in situ* PCR and the test means under digestion in protease, as will be discussed in more detail. A cytoplasmic signal for RT *in situ* PCR and a nuclear signal for the positive control means adequate protease digestion and the target specific localization of the mRNA of interest.

### 3.1.1. Protease digestion

The amount of time the sample is exposed to protease digestion is arguably the most important variable in RT *in situ* PCR. The complex interconnecting protein-nucleic acid latticework that is created with formalin fixation may be, as just discussed, an essential element for successful *in situ* PCR, both in terms of detection of the amplicon and preventing its migration from inside the cell.

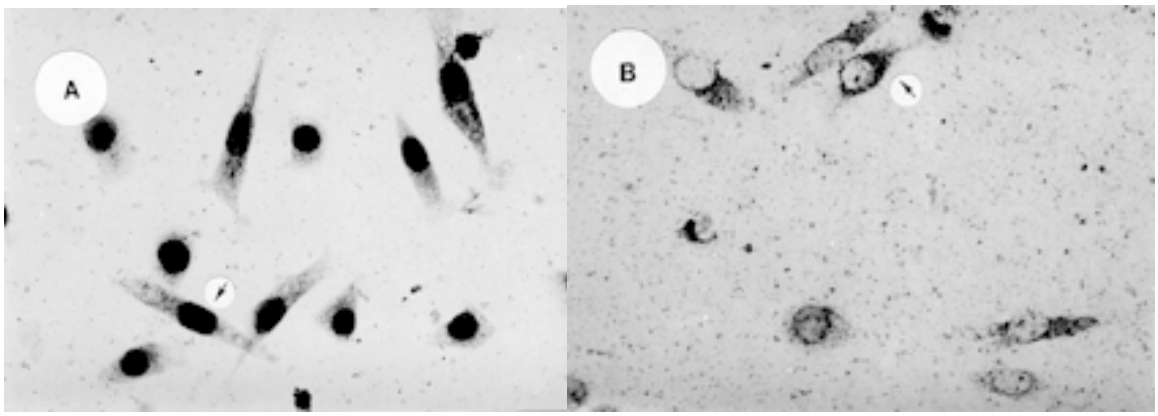


**Figure 6.** Migration of the amplicon during PCR *in situ* hybridization. After optimal protease digestion (30 minutes), a strong nuclear signal is evident in SiHa cells, which contain 1 copy of HPV 16, using PCR *in situ* hybridization for HPV DNA (A). The signal migrates to the cytoplasm if the protease digestion time is increased to 60 minutes (B). The signal is lost, as is cell morphology, if the protease digestion time was increased to 90 minutes (not shown).

However, the use of this fixative necessitates a protease digestion step. An advantage of the denaturing fixatives is that they do not require the based signal during the PCR step.digestion step. Perhaps due to a relatively high probability of migration of the amplicon out of the cell, at this stage in the development of the procedure these fixatives do not appear to allow for detection of the amplicon in all cells that contain the target (1,20,21).

The function of the protease in preparation for standard *in situ* hybridization and PCR *in situ* hybridization is to allow for entry of the probe/primer/Taq polymerase to the target sequence. Although this procedure also applies to RT *in situ* PCR, protease digestion has a further extraordinary function of rendering the entire genomic DNA of the cell non-amplifyable by exposing the DNA to subsequent digestion by DNase. Insufficient protease digestion may result in many persistent DNA-protein cross-links and the DNase may not be able to adequately degrade all the cellular DNA. The result can be the development of a non-specific DNA-repair

**3.1.2 Choice of protease**

The three most commonly used proteases in diagnostic pathology are proteinase K, pepsin, and trypsin. These and other proteases will allow for successful *in situ* PCR. It is best to choose one of these proteases and use it exclusively in order to become familiar with its particular nuances. The actual procedure for preparing these protease solutions follows:

| **Pepsin (or trypsin)** | **Proteinase K** |
|---|---|
| 9.5 ml DEPC water | 10 ml DEPC water |
| 0.5 ml 2N HCl | 10 mg proteinase K |
| 20 mg pepsin. | |

Both of these solutions can be frozen in 1 ml aliquots. When frozen, proteinase K solution will maintain activity for many months, or when stored at 4°C. The pepsin (or trypsin) should be used immediately or frozen and thawed within 1 week. When thawed, the pepsin solution should be stored on ice until ready to be used and then warmed to either room temperature or 37°C and used immediately. DEPC water, which is RNase free, is used for RNA work, although the protease would probably degrade any RNase present in the solution.

**3.1.3 Definition of optimal protease digestion**

The most important point regarding protease digestion is the following:

Optimal protease digestion time typically is very different for RT *in situ* PCR as compared to PCR *in situ* hybridization for the same tissue.

Specifically, for PCR *in situ* hybridization, 30 minutes of digestion with pepsin solution is adequate for most formalin fixed cell and tissue preparations, regardless of length of fixation. However, for most tissues, especially those fixed for $\geq$8 hours, 30 minutes of pepsin digestion would be suboptimal in RT *in situ* PCR. This difference presumably is due to the fact that RT *in situ* PCR requires complete degradation of the genomic DNA template by DNase which, in turn demands extensive

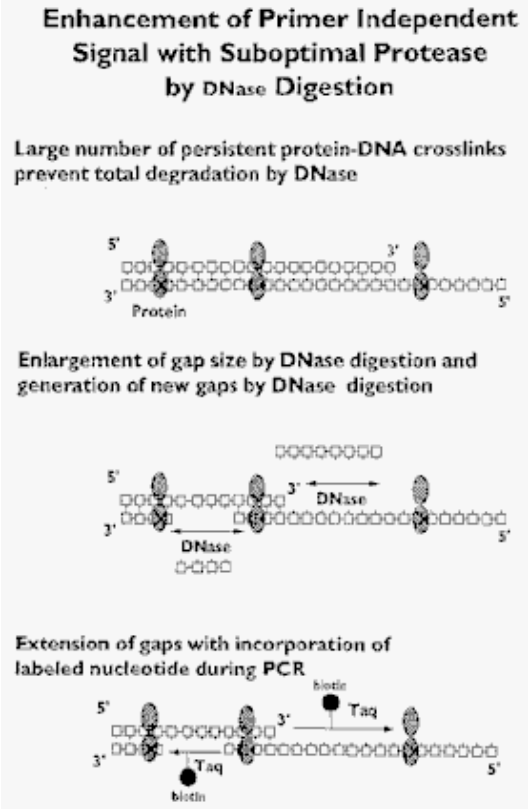


**Figure 7.** Hypothetical model for the enhancement of the signal in the negative control with inadequate protease digestion. It is postulated that DNase digestion after suboptimal protease digestion may enhance the signal by creating new gaps and/or increasing the size of the pre-existent single stranded gaps that may be repaired by Taq polymerase during PCR.

disruption of protein-DNA cross-links. Longer fixation times lead to more extensive protein-DNA cross-links and reflect the strong correlation between protease digestion and formalin fixation times for successful RT *in situ* PCR.

Optimal protease digestion for RT *in situ* PCR (Figure 5) is defined as that protease digestion time which yields the following using direct incorporation of the reporter nucleotide:

No DNase digestion: Intense signal
After DNase digestion: No signal

Sub optimal protease digestion for RT *in situ* PCR is defined as that protease digestion time which yields the following using direct incorporation of the reporter nucleotide:

No DNase digestion: No to moderate signal
After DNase digestion: Moderate to strong signal

The intense nuclear signal for the positive control (no DNase) with optimal protease digestion represents the following pathways:

a) target specific DNA synthesis (assuming that one includes primers that correspond to a genomic DNA sequence);

b) mis-priming (assuming that all reagents are added at room temperature and that primers are included);

c) DNA repair or primer independent DNA synthesis (assuming that the tissue has been heated, as is obligatory for paraffin embedded tissues).

A final and important point concerns the observation that sub optimal protease digestion allows for a signal with the negative control that is usually stronger than that for the corresponding positive control (1,20,21). A possible explanation for this is given in Figure 7. With sub optimal protease digestion, sufficient protein-DNA cross-links exist that interfere with complete DNase digestion of the genomic DNA. However, DNase can enlarge the putative gaps that are the foundation of the primer independent signal (1,20,21). The presence of a relatively strong nuclear based signal with the negative control and test (DNase and RT) in RT *in situ* PCR tells us to repeat the experiment with increased protease digestion time.

**3.1.4 Over-digestion with protease**

The definition of over-digestion with a protease is a test result exhibiting:

1) poorly visualized nuclei and cytoplasm;

2) conspicuous basement membranes;

3) either loss of signal or a weak, cytoplasmic signal in the positive control (*i.e.*, for a DNA based signal) (Figures 2 and 6).

Proteinase K is much more likely than pepsin or trypsin to lead to over digestion of tissues. In RT *in situ* PCR in about 30% of tests, proteinase K (1 mg/ml) results in over-digestion of tissues compared with 5% using pepsin (Nuovo GJ, unpublished observations). For this reason, pepsin or trypsin is preferable.

In RT *in situ* PCR, protease can easily be inactivated by simply washing it off the glass slide. A one minute wash in DEPC water followed by a one minute wash in 100% ethanol will suffice. Do not heat inactivate the protease, as is done for solution phase PCR. Dry heat inactivation of the protease using the tissue on the glass slide will markedly

diminish any hybridization signal, either with standard *in situ* hybridization or *in situ* PCR (1).

**3.1.5 DNase digestion and RT *in situ* PCR**

DNase digestion is best accomplished by overnight incubation at 37°C using the following recipe:
2 µl 10x PCR buffer (Perkin Elmer, Norwalk, CT)
2 µl of DNase (10U/µl, Boehringer Mannheim, Indianapolis, IN)
16 µl of DEPC water per tissue section.

Although adequate DNase digestion can be achieved after 8 hours, a longer digestion time, specifically overnight, is recommended (1).

Inadequate DNase digestion, as defined by the persistence of a signal with the negative control (DNase, no RT or RT with nonspecific primers) is most likely due to inadequate protease digestion. Increasing either the DNase digestion time and/or the concentration of the DNase usually will not rectify this problem (Nuovo GJ, unpublished observations). Under these conditions, increasing the protease digestion time will remove all nonspecific DNA synthesis with the negative control (1,20,21).

**3.1.6 Direct incorporation of the reporter nucleotide**

This section concerns an essential aspect of RT *in situ* PCR: direct incorporation of the reporter nucleotide. It should be stressed that direct incorporation of the tagged nucleotide can be nonspecific if mis-priming, primer oligomerization, or DNA repair are operative inside the cell. Optimal protease digestion and the subsequent DNase digestion will eliminate these nonspecific pathways. The result is that target specific direct incorporation of the reporter nucleotide into the PCR amplified cDNA can be achieved. However, direct incorporation of the reporter nucleotide is not possible in paraffin embedded tissues for DNA targets because DNA repair is invariably operative due to the heating of the tissue during tissue processing. In such cases, a formal hybridization step is needed after the PCR (1,20,21).

We have shown (Nuovo, GJ unpublished observations) that about 8,000 digoxigenin reporter nucleotides are needed inside a cell for a signal to be evident. Similarly, our calculations has suggested that, with the positive control of RT *in situ* PCR, over 100,000 reporter nucleotides may be incorporated into the nucleus, primarily via the DNA repair pathway. This correlates with the intense signal evident with the positive control (Figure 5).

We undertook a series of experiments to determine if a signal could be generated using labeled primers. We used HPV 16 primers that had one biotin or digoxigenin per 20mer. The samples were either SiHa cells (1 HPV 16 copy) or paraffin embedded cervical SILs that contained about 100 copies of HPV 16 per infected superficial cell. No signal was evident with the SiHa cells and direct incorporation of the labeled primer using hot start *in situ* PCR. A weak signal was evident in the cervical SILs that was stronger with the hot start maneuver. We obtained similar results with paraffin embedded placenta tissues and primers specific for the bcl-2 gene, present as two copies per cell. The signal was never as strong as with direct incorporation of the labeled nucleotide. Under these conditions, it was calculated that about 5,000 reporter nucleotides were present per nucleus (1).

To try to circumvent this problem of sensitivity with labeled primers, we obtained HPV 16 primers that contained THREE biotin moieties per primer; such primers are expensive to obtain and must be over 40 bp long. We re-did the experiments with the SiHa cells and the cervical SILs. A signal was seen in only about 10% of the SiHa cells and the signal with the cervical SILs was still not nearly as strong as with either standard *in situ* hybridization or PCR *in situ* hybridization. It is evident that, at this stage, labeled nucleotides are preferable to labeled primers for RT *in situ* PCR. Further, for DNA targets, PCR *in situ* hybridization is preferable to *in situ* PCR with labeled primers. However, it is important to remember that, by using frozen, fixed tissue and hot start, one CAN achieve intense target specific incorporation of the reporter nucleotide for DNA targets (1,20,21). This is of no help with paraffin embedded tissues where DNA repair would preclude using reporter nucleotides.

## 4. THE ONE STEP RT *IN SITU* PCR PROTOCOL

This next section will detail the protocols used for detection of PCR amplified cDNA and DNA.

**RT *in situ* PCR** (17)

1) Place three 4µ tissue sections or three cells suspensions on a silane coated slide (ONCOR, Gaithersburg, MD).
2) Remove paraffin (tissue sections) with 5 min xylene wash, 5 min 100% ethanol wash.
3) Digest with pepsin (2 mg/ml) for optimal time (usually 30-90 min).
4) Incubate two of three sections with 10-20U DNase overnight at 37°C.
5) Wash for 1 min with DEPC water, then 100% ETOH, and air dry.
6) Prepare the following solution from the EZ rTth kit (Perkin Elmer):
10 µl EZ buffer(EZ rTth RNA PCR kit)

1.6 µl each of dATP, dCTP, dGTP, dTTP(each 10 mM stock)
1.6 µl of 2% bovine serum albumin
1.0 µl of Rnasin (2 U/µl)
3.0 µl of primers 1 and 2 (20 µM stock)[1]
0.6 µl digoxigenin dUTP (1 mM stock)
14.6 µl DEPC water
12.4 µl 10 mM $MnCl_2$
2.0 µl rTth (2.5 U/µl)

[1] for the negative control, use nonspecific primers or omit the primers

7) Apply 10-40 µl of above solution to each section, cover with amplicover (Perkin Elmer 1000 cycler) or polypropylene coverslip and anchor with nail polish and mineral oil overlay
8) Incubate at 65°C for 30 min
9) Denature at 94°C for 3 min
10) Cycle at 60°C for 1.5 min, 94°C for 45 sec; do 20 cycles
11) Wash slides in 0.1XSSC/0.2% BSA at 60°C for 15 min
12) Detect digoxigenin as per protocol of Boehringer Mannheim, which follows:

Antidigoxigenin-alkaline phosphatase conjugate at 1:150 in 0.1M TRIS pH 7.5 and 0.1M NaCl for 30 min at 37°C, followed by 10-15 min at 37°C in NBT/BCIP solution = 50 µl of substrate 1 and 50 µl of substrate 2 (Digene Diagnostics, Gaithersburg, MD) in 15 ml of 0.1M TRIS, pH 9.5 and 0.1M NaCl) counterstain for 1-5 min in nuclear fast red.

## 5. APPLICATIONS OF RT *IN SITU* PCR

Assuming adequate protease and DNase digestion, target specific direct incorporation of the tagged nucleotide is routinely achieved with RT *in situ* PCR. To illustrate some of the key points for successful RT *in situ* PCR already discussed, the expression of matrix metalloprotease (MMP) and their inhibitors (tissue inhibitor of matrix metalloprotease -TIMP) in cancers will be used as a model system.

### 5.1. MMP and TIMP expression in cervical cancer

Studies of tumor invasion and metastases have focused on the degradation of the extracellular matrix (ECM) and the endothelial cell basement membrane. Enzymes that have been implicated in the degradation of these compartments include the MMP family. Two of these enzymes, designated MMP-2 and MMP-9, are potent gelatinases and their activity, in conjunction with their inhibitors, TIMP-1 and -2, have been correlated with the processes of tumor cell invasion and metastasis (24,25). The importance of the balance in production of MMPs and TIMPs in tumor cell invasion and metastasis has been suggested by several studies. The inhibition of tumor cell invasion and metastasis in animal models has been demonstrated using *in vivo* injections of TIMP (24,25). The inactivation of TIMP by transfection of mouse 3T3 cells with antisense DNA converted the phenotype of these cells from noninvasive to tumorigenic and metastatic in nude mice (24,25).

MMP and TIMP expression in cervical cancers serves as a good model to illustrate the major points discussed in the first section of this manuscript. The foundation for determining if the signal seen with RT *in situ* PCR is specific is based in the positive and negative controls, which should be done on the same slide, if possible, with the test. The intense, nuclear signal in all cell types with the positive control (no DNase) and the loss of this signal with DNase digestion is the primary way to determine if the key variables, especially protease time, have been optimized (Figure 5). Unless the variables are optimized, one will not get a target specific signal with RT *in situ* PCR. However, there are two other important indicators of specificity with RT *in situ* PCR that require some expertise in histologic analysis to appreciate: the restriction of the signal to certain cell types and the subcellular localization of the signal.

We used the RT *in situ* PCR technique to correlate the presence MMP-9 and MMP-2 and of TIMP-1 and TIMP-2 mRNAs with prognosis in 23 cases of cervical carcinoma (26). It is important to stress that most of these tissues showed, in addition to foci of invasive cancer, areas of noninvasive carcinoma *in situ* and normal epithelium. PCR-amplified MMP and TIMP cDNAs were restricted to the invasive cancer cells and the surrounding stromal cells. Thus, the adjacent carcinoma *in situ* and normal epithelial areas served as important “in-built” negative controls (26). One should be skeptical when obtaining a signal in many different cell types when doing RT *in situ* PCR. Also, as would be expected for human mRNAs (see Figure 5), the signal localizes to the cytoplasm, whereas the genomic based signal with the positive control is seen in the nucleus. A nuclear based signal after RT *in situ* PCR most likely is nonspecific and reflects inadequate protease digestion (1,26).

The ratios of the percentage of cancer and stromal cells expressing MMP-9 and MMP-2 to those expressing TIMP-1 and TIMP-2 were approximently one in those cancers with a good prognosis. This MMP to TIMP ratio in the cancer and stromal cells with a poor prognosis was significantly increased to 5.4 and 3.4 ($p<0.0001$), respectively, reflecting a marked reduction in the percentage of cells expressing TIMP in cancers with a poor prognosis. Expression of human papillomavirus open reading frames E6 and E7, which are important in cell

transformation and immortalization (1) were equivalent in cervical cancers of good and poor prognosis (26).

We then studied the *in vitro* behavior of the Caski and HeLa cells in more detail. The matrigel system was used to study the invasiveness of these cancer cells. We used the RT *in situ* PCR technique to directly compare the expression of cells invading the matrigel with the non-invasive cells. There was a significant increase in the percentage of HeLa cells invading the matrigel expressing MMP-9 (33%) and MMP-2 (48%) when compared to the non-invasive cells (11% and 12%, respectively); there was no change in the percentage of cells expressing either TIMP, HPV E6 or E7 RNA. These simple experiments illustrate an important advantage of RT *in situ* PCR as compared to solution phase PCR; the simple and rapid quantification of the percentage of a given cell type expressing a transcript of interest.

These data derived from RT *in situ* PCR suggest that the balance of MMP-9 and -2 to TIMP-1 and -2 expression, but not HPV expression, is an essential factor in determining the invasiveness of cervical cancer. More specifically, the data suggest a multi-step process in the evolution of cervical cancer. Early invasion in cervical cancer probably requires activation of MMP-9 and MMP-2 expression. This is counterbalanced by an equivalent expression of TIMP-1 and TIMP-2. The controlled production of these two MMPs and two TIMPs may explain, in part, why such superficial cervical cancers, called microinvasive carcinomas, have a metastatic rate of less than 1% and the women have a survival rate of near 100%. More extensive invasion in clinical samples is strongly associated with a decreased in the percentage of cells expressing TIMP. The next step towards increased invasiveness requires the ability to enter microvessels. The data from the clinical samples, including direct detection of MMP and TIMP expression in tumors cells that had invaded the microvasculature, strongly suggests that, for this to occur, there must be an increased percentage of cells expressing MMP-9 and MMP-2 with a marked reduction in the expression of TIMP-1 and TIMP-2.

## 6. CONCLUDING REMARKS

The foundation of successful RT *in situ* PCR is the use of formalin fixed, paraffin embedded samples which have been digested for the optimal time in a protease. The optimal time, which is determined by testing a variety of protease digestion times, is defined by an intense signal in the nuclei of most cells irrespective of the primers used, and a loss of this signal with an overnight digestion in DNase. This permits the target specific direct incorporation of the labeled nucleotide into the amplified cDNA. A lack of signal with the negative control (DNase, no RT) and an intense nuclear signal in most cells with the positive control (no DNase) is prerequisite for success with RT *in situ* PCR. The localization of the signal (cytoplasmic for human mRNAs and restricted to certain cell types) is another important indicator of successful RT *in situ* PCR. The one step rTth system allows for the reproducible amplification and detection of low copy RNA targets within a few hours.

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