PCR Primer

A LABORATORY MANUAL

SECOND EDITION

EDITED BY

Carl W. Dieffenbach
Gabriela S. Dveksler

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This book is dedicated to
Sara,
Rebecca,
Ethan,
and Ari

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WWW RESOURCES

http://www.ncbi.nlm.nih.gov/entrez/query.fcbi?db=PubMed NCBI, National Library of Medicine, PubMed database
http://www.ncbi.nlm.nih.gov/entrez/query.fcbi?db=snp NCBI, National Library of Medicine, Single Nucleotide Polymorphism (SNP) database
www.gelcompany.com The Gel Company, San Francisco, California.

5

19 Reverse Transcriptase In Situ PCR

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Since the discovery of the structure of DNA in 1953, a great deal of effort has been devoted to detecting specific DNA target sequences. The first commonly used technique, filter (or dot-blot) hybridization, offered excellent sensitivity, being able to detect 1 copy per 100 cells. The later introduction of Southern blot hybridization further enhanced detection methods. However, the destructive nature of nucleic acid extraction, upon which these techniques rely, precluded localization of the target to its specific cell of origin. In many instances, this information is critical. For example, although PCR can be used routinely to detect HIV-1 DNA in the lymph nodes of seropositive, asymptomatic patients, it cannot be used to identify which of the many different cell types that reside in this site are reservoirs of the virus, prior to the development of AIDS.

During the last 15 years, direct cellular localization of a DNA or RNA target has been achieved routinely using in situ hybridization. Over the past 10 years, there have been dramatic improvements in the sensitivity of this technique, especially in the use of nonradioactive probes (Crum et al. 1988; Nuovo 1989, 1997; Nuovo and Richart 1989). However, despite these improvements, the usefulness of the technique has been limited by its relatively high detection threshold (~10 copies per cell; Nuovo 1993, 1994a). Although this is not a problem when detecting, for example, productive viral DNA infections, where a given cell may contain thousands of copies, there are common problems, such as the detection of latent viral infection or point mutations, which cannot be solved using in situ hybridization. Detection of low-copy events, of course, has become routine over the last 13 years with the widespread use of PCR. With the hot-start method, PCR can be used to detect one copy in a background of 1 μg of total cellular DNA (Erlich et al. 1991; Nuovo et al. 1991; Chou et al. 1992). However, PCR cannot be used to achieve sub-tissue localization of target sequences.

The last 10 years have seen the development of in situ PCR, a technology that combines the high sensitivity of PCR with the cell localization of in situ hybridization. This chapter presents protocols for in situ detection of PCR-amplified DNA and cDNA, and discusses some of its applications, especially as it relates to HIV-1 and AIDS.

PROTOCOL 1 REVERSE TRANSCRIPTASE IN SITU PCR

This section provides an abbreviated protocol for doing in situ PCR, specifically reverse transcriptase (RT) in situ PCR. Detailed information on the various steps is provided in the remainder of this chapter.

MATERIALS

CAUTION
See Appendix for appropriate handling of materials marked with <!>.

BUFFERS AND SOLUTIONS
- Nuclear Fast Red<!>
- Formalin (v/v), 10%<!> buffered (Polyscientific)
- Detection solution (0.1 M Tris-HCl<!>, pH 9.5, 0.1 M NaCl)
- DEPC<!>-treated H_2O
- Phosphate-buffered saline (PBS)(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4<!>)
- 2 mM KH_2PO_4<!>, pH 7.0, for use with cell cultures
- Xylene<!> (for use with tissue samples)
- Ethanol<!>
- Bovine serum albumin (BSA), 2% (w/v)
- SSC, 20x (3 M NaCl, 0.3 M sodium citrate, pH 7.0)
- NBT/BCIP chromogen<!> (Enzo Clinical Labs)

ENZYMES AND ENZYME BUFFERS
- Pepsin<!> (2 mg/ml) (Enzo Diagnostics)
  - Prepare by adding 20 mg of pepsin, 9.5 ml of H_2O, and 0.5 ml of 2 N HC1<!>.
- RNase-free DNase (Boehringer Mannheim)
- *Taq* DNA polymerase Gold (Applied Biosystems, Foster City, CA)
- RNase inhibitor (Applied Biosystems)
- DNase buffer, 10x
  - Prepare by adding 35 µl of sodium acetate (3 M, pH 3), 5 µl of $MgSO_4$ (1 M), and 60 µl of DEPC H_2O.

NUCLEIC ACIDS AND OLIGONUCLEOTIDES
- Digoxigenin dUTP<!> (Boehringer Mannheim), 1 mM

ANTIBODIES
- Antidigoxigenin conjugate (Boehringer Mannheim)
- Appropriate PCR primers

SPECIAL EQUIPMENT
- Silane-coated slides (Ventana)
- In situ PCR cycler (Applied Biosystems)
- Polypropylene sheet, to be used as coverslips (sold as autoclavable bags)
- Incubators, preset to 37°C, 60°C
- Humidity chamber (lidded box lined by water-saturated paper towel)

ADDITIONAL ITEMS
- RT-PCR kit (Applied Biosystems)
  - This kit includes the buffers, nucleotides, RNase inhibitor, manganese solution, rTth DNA polymerase, and control primers needed for RT-PCR.
- Reagents and equipment for paraffin embedding and sectioning (for use with tissue samples)
- Permount<!>

CELLS AND TISSUES
- Appropriate tissue samples or cell culture
  - Many reagents, including the protease, buffers, washes, chromogen, probes, and counterstains, are part of comprehensive in situ hybridization kits. These are now marketed by several biotechnology companies.

METHOD

Preparation of Tissue Sections and Cell Samples

1. Fix the tissue samples in 10% buffered formalin, preferably from 8 to 15 hours, and then embed in paraffin.
2. For cell cultures, wash the cells once with PBS added directly to the culture plate. Add 10% buffered formalin, let stand overnight, and then scrape the cells off the plate with a rubber policeman. Wash the cells twice in diethylpyrocarbonate (DEPC)-treated H_2O, and centrifuge at 2000 rpm for 3 minutes in a microcentrifuge. Resuspend the cells in 5 ml of DEPC-treated H_2O, and spot 50 µl (the ideal cell density is 2500–7500 cells) onto a slide, air-dry, and store at room temperature.
3. Place several (at least 3) 4-µm paraffin tissue sections or three cell suspensions on silane-coated slides.
   - The silane coating is essential for cell adherence during the procedure.
4. Remove the paraffin from the tissue samples by placing the slides in fresh xylene for 5 minutes and then in 100% ethanol for 5 minutes; air-dry.

Protease Digestion

5. Bathe slides in pepsin solution for the desired incubation time at 37°C.
   - See Tables 1 and 2 for protease incubation times. Proteases other than pepsin may be used for this digestion. For more information, see Protease Digestion, p. 257.
6. Inactivate the protease by washing the slide in DEPC-treated H_2O for 1 minute and then in 100% ethanol for 1 minute; air-dry.
   - This simple wash step is sufficient to remove/inactivate pepsin; heat-inactivation is not required.

DNase Digestion (for RT In Situ PCR)

> It is important to DNase-digest two of the three tissue sections/cell suspensions. The in situ PCR *positive control* is the section that is not treated with DNase. An intense signal in at least 50% of cells demonstrates that protease digestion time is optimal and that the other reagents are working properly; the signal represents DNA repair, genomic amplification, and mispriming. The in situ PCR *negative control* is the cells/tissue section that is DNase-digested and then incubated with irrelevant primers (i.e., primers that correspond to a target that could not be present in the tissue). The absence of signal demonstrates that the DNase digestion has rendered the native DNA template unavailable for DNA synthesis. The *test* is the section digested by DNase that is then treated with RT and PCR using the target-specific primers.

7. To two of the three sections add 1 µl of 10x DNase buffer, 1 µl of RNase-free DNase (10 units/µl), and 8 µl of DEPC-treated H_2O. Alternatively, RT/PCR buffer can be used in place of 10x DNase buffer.

8. Cover the solution with a polypropylene coverslip that is cut to the size of the sample to prevent drying. Place the slides in a humidity chamber at 37°C.
9. After overnight digestion, remove the coverslip, wash for 1 minute in DEPC H_2O, then 100% ethanol, and air-dry.

RT Step

10. In a sterile microcentrifuge tube, assemble the following RT-PCR reagents:

EZ buffer (Applied Biosystems)	10 µl
each of dATP, dCTP, dGTP, dTTP (stock of each nucleotide at 10 mM)	1.6 µl
Bovine serum albumin solution, 20% (w/v)	1.6 µl
DEPC-treated H_2O	15.1 µl
$MnCl_2$ (or Mn acetate) solution, 10 mM	12.4 µl
Sense primer (20 µM)	2.0 µl
Antisense primers (20 µM), 2.0 µl	
RNase inhibitor, 0.6 µl of digoxigenin dUTP (stock solution 1 mM; Applied Biosystems)	0.5 µl
rTth	2.0 µl

11. Add RT-PCR solution to one of the two sections treated with DNase.
12. To prevent drying, anchor the polypropylene coverslip with one small drop of nail polish and place the slide in an aluminum "boat" and cover with sterile mineral oil. Incubate on the block of the thermal cycler at 60°C for 30 minutes.

    The Amplicover/Ampliclip method of Applied Biosystems can be used as an alternative to mineral oil.

PCR Step

> For PCR in situ hybridization (for DNA targets), the hot-start manuever must be used. This can be achieved by withholding the DNA polymerase until the thermal cycler block reaches 60°C, but it is easier to add all the reagents at room temperature, including the *Taq* polymerase Gold. This polymerase contains an antibody that inhibits nonspecific polymerase activity. At the appropriate temperature, the anti-*Taq* antibody is eliminated and the polymerase is activated. The higher temperatures, in turn, prevent the nonspecific side reactions.

13. In a sterile microcentrifuge tube on ice, assemble the PCR reagents in the following order:

Taq polymerase buffer (Applied Biosystems)	5 µl
dNTP solution (each nucleotide at 10 mM stock)	9.0 µl
$MgCl_2$, 25 mM	8.0 µl
Bovine serum albumin solution, 2% (w/v)	1.6 µl
H_2O	22.4 µl
Sense primer, 20 µM	2.0 µl
Antisense primer, 20 µM	2.0 µl
Taq polymerase Gold (10 units/µl)	2.0 µl

14. Apply the PCR mix to each sample and perform the following thermal cycle series:

Cycle number	Denaturation	Annealing/Extension
1	3 min at 94°C*	
20**	1 min at 94°C	1.5 min at 60°C

*This initial denaturation step is used for RT in situ PCR and PCR in situ hybridization.

**If the newly formed cDNA is to be detected using a probe after RT and PCR rather than by direct labeling, increase the number of cycles to 30.

15. Remove the coverslip and nail polish from the slide. Wash for 5 minutes in xylene and 5 minutes in 100% ethanol. Allow the slide to air-dry.

Detection Step

16. Wash the slides for 15 minutes in a 0.2x SSC solution containing 0.2% (w/v) BSA at 60°C (for RT in situ PCR).

    For PCR in situ hybridization using full-length genomic probes of at least 80 bp in size, use the wash described above. For hybridization using oligoprobes of <45 bp, use 1x SSC containing 2% (w/v) BSA at 45°C for 10 minutes.

17. Remove the excess wash solution. To each slide, add 100 µl of a 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl solution containing the antidigoxigenin antibody (1:150 dilution) (Boehringer Mannheim). Incubate at 37°C for 30 minutes.
18. Wash the slides for 1 minute in detection solution and then incubate for 5–30 minutes at 37°C in detection solution containing NBT/BCIP chromogen, as specified in supplier's instructions (Enzo Clinical Labs). Shorter incubations can be used for direct incorporation of label into amplified DNA; longer incubations are required when using probes. Check the slides under a microscope and stop the reaction when the signal is strong.

    For PCR in situ hybridization, the probe–amplicon complex should be hybridized for 15 hours; digoxigenin-labeled probes are recommended for this purpose. It is important to use the longest probes possible (optimally 80–100 bp). Primer oligomerization does not appear to occur with PCR in situ hybridization, so full-length probes that include the region of the primers can be used. It is also important to label the probes by random priming (Nuovo 1997). If shorter probes are used, they must be at least 45 bp in size and labeled with the 3′ end-tailing method (Nuovo 1997).

19. Wash the slides in water for 1 minute, counterstain with nuclear fast red for 5 minutes, wash in water for 1 minute, then in 100% ethanol for 1 minute, and then in xylene for 1 minute. Mount coverslips using Permount. View under a microscope.

▶ TROUBLESHOOTING FOR RT IN SITU PCR

- If the positive control has >50% positive cells, and the negative control shows no positive cells, then the RT/DNase-treated section should show target-specific cDNA amplification.
- If the negative control shows positive cells, increase the protease digestion time. Insufficient protease digestion is the most common cause of problems with RT in situ PCR and is easily recognized using the two in situ PCR controls outlined above.
- If neither the negative nor the positive control shows positive cells, check the tissue morphology. If it is poorly preserved, reduce the protease digestion time. If morphology is well preserved, increase the protease digestion time.

DISCUSSION

Basics of In Situ PCR

Tissue Preparation

The correct choice of fixative is critical for success with in situ PCR. There are several fixatives that can be used for in situ hybridization, but their suitability for use in PCR is vari-

able. In situ PCR can be performed on unfixed, frozen tissue or in tissue fixed in acetone, ethanol, or buffered formalin. Fixatives that include a heavy metal or picric acid cause rapid and extensive degradation of DNA and are incompatible with PCR (Greer et al. 1991). Fixatives such as acetone and ethanol function by denaturing proteins, but the most commonly used fixative in anatomic pathology is 10% buffered formalin, which cross-links proteins and nucleic acids. Tissues fixed for more than 8 hours in solutions that contain either a heavy metal or picric acid are not suitable for in situ hybridization, although shorter-term fixation can yield intense signals (Nuovo and Silverstein 1988).

Table 1 shows the results of a study comparing the utility of different fixatives for in situ PCR. The experiments used a cell line containing a known target sequence (*bcl-2* DNA), and fixatives that allowed a 100% detection rate were seen (Nuovo et al. 1993). Note that under the conditions defined in Table 1, only buffered formalin fixation, followed by protease digestion, gave a 100% detection rate of the target sequence. It is perhaps surprising that acetone or ethanol fixation did not produce 100% detection rates, given that they permit successful solution-phase PCR. Further investigation showed that the poor detection rate for acetone- or ethanol-fixed tissues was due not to inhibition of amplification, but to the migration of the PCR product out of the cell and into the amplification buffer. No such migration was detected in formalin-fixed samples. This does not mean that acetone- or ethanol-fixed cells should not be used for in situ PCR; however, the results obtained with these fixatives are less reliable than those obtained using formalin or its relatives (e.g., glutaraldehyde and paraformaldehyde). Formalin fixation appears to create a migration barrier that, under certain specified conditions, strongly limits the movement of the PCR product from its site of synthesis in the cell. It is reasonable to assume that this relates to the protein–DNA latticework that occurs as a secondary result of formalin fixation.

Because of the highly toxic nature of formalin, there has been a concerted effort to find alternatives to this fixative. Fixatives that do not contain formalin include Histochoice and Streck's solution. These chemicals can be used for in situ PCR with cell suspensions and tissue sections without the need for a protease digestion step. However, in our experience, these fixatives are less reliable than formalin-based reagents, with respect to both detection rate and precision of subcellular localization (Nuovo 1994a). Note that the use of formalin-containing fixatives can be problematic in combination with paraffin embedding (for more details, see Nonspecific Pathways during In Situ PCR, p. 258).

TABLE 1. Effect of fixation chemistry and duration on the detection of amplified *bcl-2* DNA in peripheral blood mononuclear cells

NO PROTEASE DIGESTION (% positive cells)					
Fixation time		formalin	acetone	95% ethanol	Bouin's
5 min		5	2	14	0
15 hr		0	15	31	0
39 hr		not done	0	9	not done
PROTEASE (pepsin) DIGESTION (% positive cells)					
Fixation time	protease conditions	formalin	acetone	95% ethanol	Bouin's
5 min	2 mg/ml 12 min	0	0	0	0
5 min	20 μg/ml 1 min	1	0	0	—
5 min	20 μg/ml 2 min	35	0	0	—
5 min	20 μg/ml 5 min	0	0	0	—
15 hr	2 mg/ml 12 min	100	0	0	0

Protease Digestion

As shown by the data in Table 1, 100% detection of the target sequence was achieved only after prolonged formalin fixation *and* protease digestion. An expanded study, including tissues fixed in buffered formalin for up to 1 week, demonstrated that the signal strength in formalin-fixed tissues is strongly dependent on the duration of both formalin fixation and protease digestion (Nuovo et al. 1994a). These data are presented in Table 2. Note that the study used in situ PCR and not PCR in situ hybridization. For PCR in situ hybridization, using tissues fixed for 4 hours to several days, the optimal protease digestion time is from 20 to 30 minutes (Nuovo 1994a).

Several proteases have been extensively tested for in situ hybridization. These include proteinase K, pepsin, trypsin, trypsinogen, and pronase. All of these proteases are compatible with in situ amplification of DNA and cDNA, but trypsin and pepsin are preferred. These proteases can be inactivated simply by increasing the pH of the reaction to pH 7.0, and they do not have a tendency to over-digest, as does proteinase K. Overdigestion is indicated by the loss of distinct morphology and concomitant absence of signal (Fig. 1).

Inadequate protease digestion is the most common cause of unsuccessful in situ PCR. For both in situ PCR and RT-in situ PCR, the optimal length of protease treatment is dependent on the duration of formalin fixation. Optimal treatment is indicated by distinct morphology, concomitant with an intense signal in more than 50% of cells (3+). If the signal is less than 3+, the protease digestion times should be increased until 3+ is obtained. This important principle is illustrated in Figure 2. Note that the times listed in Table 1 were optimized for skin tissue. The times will vary depending on the tissue type used. Tissues such as lymph nodes, spleen, and kidney typically are more sensitive to protease digestion, as compared to brain, skeletal muscle, and skin. The use of longer formalin fixation times tends to allow a fixed protease digestion time to be used irrespective of cell type.

Amplifying Solution

For those doing PCR in situ hybridization (i.e., analyzing for a DNA target), it is important to note that the optimal $MgCl_2$ concentration is 4.5 mM, as illustrated in Figure 3, irrespective of the primer or target sequences. This relatively high and constant concentration of magnesium may be due to its partial sequestration on the glass slide and cellular proteins (Nuovo et al. 1993; Nuovo 1994a).

TABLE 2. Effect of protease digestion time on the primer-independent signal during in situ PCR as a function of the time of fixation in 10% buffered formalin

	Protease[a] digestion time (min)								
Fixation time	0	5	10	15	30	45	60	75	90
4 hr	0	1+	3+	2+	overdigested[b]				
6 hr	0	0	1+	3+	2+	overdigested[b]			
8 hr	0	0	0	0	1+	3+	—	—	—
15 hr	0	0	0	0	0	1+	1+	2+	3+
48 hr	0	0	0	0	0	0	1+	2+	3+
1 week	0	0	0	0	0	0	1+	1+	2+/3+[c]

The signal was scored as follows: 0, 1+ (<25% of cells positive), 2+ (25–50% cells positive), and 3+ (>50% of cells positive). Signal measurements were made without knowledge of the reaction conditions.

[a]Protease is pepsin (2 mg/ml) at room temperature.

[b]Overdigested refers to loss of tissue morphology with a concomitant loss of the in situ PCR signal.

[c]The 2+ signal was with pepsin; the 3+ signal with proteinase K digestion (1 mg/ml).

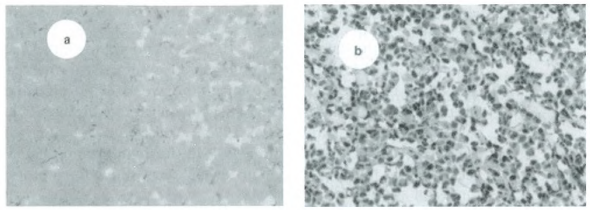

FIGURE 1. Overdigestion of tissue with protease. This tonsillar tissue was fixed in buffered formalin for 4 hours. No signal is evident with in situ PCR using *bcl-2* primers if the protease digestion time (pepsin 2 mg/ml) was 30 minutes (*a*); note the poor morphologic detail. If the digestion time was decreased to 10 minutes, an intense signal and good morphologic detail are evident (*b*).

The composition of the buffer (PCR buffer II, GeneAmp kit, Perkin-Elmer) and the concentration of the nucleotides (200 μM) and primers (1 μM) for in situ PCR are equivalent to those used for solution-phase PCR. The concentration of the DNA (*Taq*) polymerase (Perkin-Elmer or equivalent), however, should be increased to 15 units per 50 μl. This large amount of enzyme is thought to be necessary because the protein binds nonspecifically to the glass slide and plastic coverslip. If BSA is added to the reaction (final concentration 0.015%), the binding is inhibited, and strong signals can be obtained using only 1.5 units of enzyme (Nuovo et al. 1993; Nuovo 1994a). Similarly, higher concentrations of rTth must be used for RT in situ PCR as compared to solution-phase RT PCR.

Nonspecific Pathways during In Situ PCR

Studies have shown that primer-independent DNA synthesis occurs if paraffin-embedded fixed tissues are used for in situ PCR. The nonspecific signal results from the initiation of DNA synthesis at nicks in the DNA, formed during the 65°C paraffin-embedding process. Because of this background, target-specific direct incorporation of the reporter nucleotide into the amplicon is not possible for formalin-fixed, paraffin-embedded tissues for DNA targets.

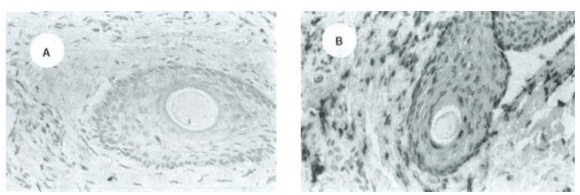

FIGURE 2. Relationship of protease digestion and formalin fixation times for successful in situ PCR. This skin biopsy was fixed in buffered formalin for 8 hours. No signal is evident with in situ PCR if the pepsin digestion time was 15 minutes (*A*). An intense signal is evident if the digestion time is increased to 45 minutes (*B*).

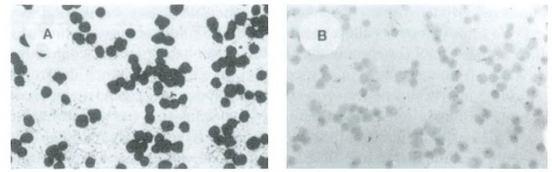

FIGURE 3. Importance of $MgCl_2$ concentration for successful in situ PCR. At an $MgCl_2$ concentration of 4.5 mM all peripheral blood mononuclear cells fixed for 15 hours in buffered formalin and digested have an intense signal using in situ PCR and *bcl-2* primers (*A*). No signal is evident if $MgCl_2$ is omitted (*B*). Similar poor results were obtained with magnesium concentrations of 1.5, 6.0, and 9.0 mM.

In Situ PCR

In situ PCR differs from PCR in situ hybridization (see Fig. 4) in the inclusion of a reporter molecule in the amplification step and, of course, the omission of the in situ hybridization

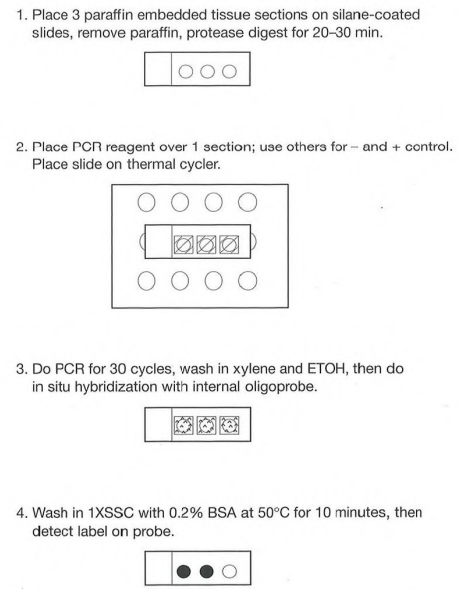

FIGURE 4. Graphic representation of the PCR in situ hybridization protocol.

step. A variety of reporter molecules may be used. Digoxigenin- and biotin-labeled nucleotides allow rapid and simple colorimetric detection of the amplified product by the use of the appropriate alkaline phosphatase-labeled conjugated antibodies (antidigoxigenin and streptavidin, respectively). In situ PCR is much quicker and simpler than PCR in situ hybridization. However, in our experience, it can be employed for DNA targets only when using frozen fixed tissues or cytospins that have not been heated. The power and widespread utility of in situ PCR are much more evident for RNA, where pretreatment overnight in a RNase-free DNase solution allows target-specific incorporation of the reporter molecule in the cDNA synthesized after the RT step.

RT In Situ PCR

The two steps of RT in situ PCR that differ from in situ PCR are the overnight digestion in RNase-free DNase that is performed after the protease digestion, and the RT step, prior to in situ PCR. After optimal protease digestion, a minimum of 7 hours of DNase treatment is needed to eradicate completely the nonspecific signal (Nuovo 1994a; Nuovo and Forde 1995).

An essential point for successful RT in situ PCR is evident from Tables 1 and 2. Specifically, there is a strong relationship between the protease digestion and formalin fixation times. Adequate protease digestion is defined as an intense signal in >50% of the cells and no signal after DNase digestion. It is important to realize that, with too little protease digestion, one usually sees a signal with DNase digestion that is stronger than that for the section not pretreated with DNase. Assuming that the nonspecific signal represents repair of DNA gaps, this DNase enhancement with suboptimal protease may represent the enlargement of these putative gaps under conditions where the DNase cannot completely destroy the DNA template. For RT in situ PCR, it is essential that the positive control (no DNase) and negative control (DNase, no RT) are performed on the same slide as the RT test. A successful run is defined by a strong signal in the positive control and no signal in the negative control (Fig. 5); note that ^{3}H is the reporter molecule. If a signal is seen with the negative control, the most likely reason is inadequate protease times. Another possible problem is no

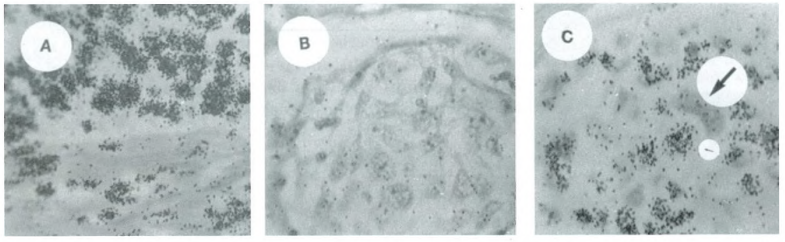

FIGURE 5. The importance of the positive and negative controls with RT in situ PCR. This cervical cancer was analyzed for MMP-92 expression using RT in situ PCR and direct incorporation of [^{3}H]dCTP. Note the intense nuclear signal with the positive control (no DNase, *A*) and the absence of signal with the negative control (DNase, no RT, *B*). These reactions were done on the same slide as the test and demonstrated that the protease and DNase digestions were adequate. On the same glass slide, the RT in situ PCR for MMP-9 was done. Many cells contained this mRNA; note the cytoplasmic localization of the signal (*C; small arrow*, cytoplasm; *large arrow*, nucleus).

signal with the positive control and poor morphology. This signifies overdigestion with protease and should prompt retesting at lower protease digestion times (Fig. 1).

The final important point about RT in situ PCR concerns the localization of the signal. The signal should localize to a different subcellular compartment(s) for the RNA and for the DNA in the positive control. For the DNA positive control, the signal should be pan-nuclear, as illustrated in Figure 6. Note the dramatically different patterns evident with various RNAs, suggesting different pathways from the nucleus to the cytoplasm.

UTILITY OF PCR IN SITU HYBRIDIZATION AND RT IN SITU PCR—PATHOGENESIS OF AIDS

It can be argued that the development of the methodology of in situ amplification of DNA and cDNA was due to AIDS. More specifically, the fact that HIV-1 DNA can integrate as one copy and remain transcriptionally silent (or nearly so) was the impetus to develop technologies that were capable of the routine in situ detection of one target copy. RT in situ PCR and PCR in situ hybridization have revealed the following about HIV-1-associated disease:

There is a massive, covert infection, primarily in the lymph nodes, by HIV-1 long before a person develops AIDS. On average, in the sites of infection, 30% of the CD4 cells are latently infected by the virus. The realization of this essential fact paved the way for aggressive anti-retroviral therapy, long before symptoms of AIDS were evident (Bagasra et al. 1992; Embretson et al. 1993; Nuovo et al. 1994c).

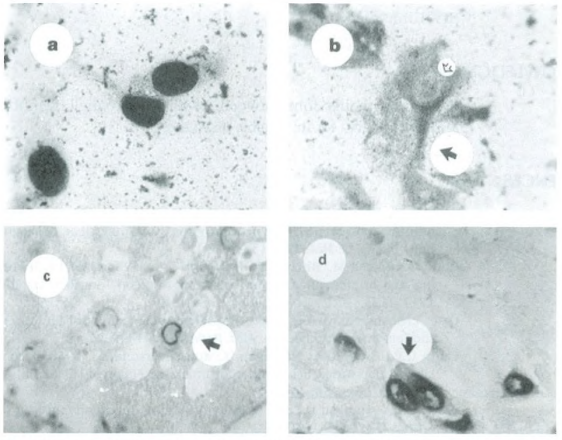

FIGURE 6. Different localizations of the signal with RT in situ PCR. The signal with the positive control, representing DNA repair, mispriming, and genomic DNA synthesis should localize to the nucleus, as is evident with this HT1080 cell line (*a*). The RNA-based signal should not be pan-nuclear. Variable patterns are seen. For example, a cytoplasmic signal is evident for MMP-92 in this cell line (*b; open arrow*, negative nucleus; *large arrow*, cytoplasmic signal). The signal for PCR-amplified hepatitis C RNA localizes to the junction of the nucleus and cytoplasm in the hepatocyte (*c*). A perinuclear and cytoplasmic signal is seen for the β chain of fibrinogen mRNA in trophoblasts (*d; arrow*, cytoplasm).

AIDS dementia is marked by productive HIV-1 infection of astrocytes, microglial cells, and neurons in the brain with concomitant up-regulation of a variety of cytokines in the area of HIV-1 infection but in the neighboring, noninfected cells (Nuovo and Alfieri 1996).

AIDS myopathy and cardiomyopathy involves (primarily) the recruitment of HIV-1-infected macrophages in the skeletal muscle and heart, respectively (Seidman et al. 1994; Cioc and Nuovo 2002).

SUMMARY

Many groups have published data generated by in situ detection of PCR-amplified DNA and cDNA. As with standard in situ hybridization or PCR, a number of variables can affect the results of in situ PCR. These include the type of fixative and duration of fixation, the duration of protease digestion, the composition of the amplifying solution, the size of the probe, and the stringency of the wash conditions. Investigators new to the field of in situ PCR should first try direct incorporation of the reporter molecule into paraffin-embedded tissue sections. Although nonspecific DNA synthesis occurs under these conditions, the experiment can be used to demonstrate successful DNA synthesis inside the nucleus and to demonstrate the importance of optimizing protease digestion for successful RT in situ PCR. In situ detection of PCR-amplified DNA and cDNA will have a strong impact on diverse fields of research, such as oncogenesis, embryology, RNA-trafficking, and detection of viral diseases. It has already significantly advanced our understanding of the pathogenesis of HIV-1 infection.

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