Protocols for the *in situ* PCR-amplification and detection of mRNA and DNA sequences

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In this protocol we describe the *in situ* PCR method for the amplification of both DNA and mRNA targets [*in situ* reverse transcriptase-PCR (RT-PCR)], from frozen or paraffin-fixed tissue sections, cell culture or other single-cell suspensions. Detection of amplicons can be achieved by the hybridization and detection of labeled probes. The protocol includes the following steps: (i) tissue preparation, (ii) *in situ* PCR (or *in situ* RT-PCR), (iii) probe hybridization, (iv) signal detection. The technique has high sensitivity (geometrically PCR-amplifying 150–350 bp fragments of a gene of interest *in situ*) and specificity (derived from *in situ* hybridization with specific fluorescent or biotinylated probes for the target genes). The ability to identify individual cells, expressing or carrying specific genes of interest in a latent form in a tissue section under the microscope provides a visual account of silent genes, and allows the determination of various aspects of normal *versus* pathological conditions, or latent *versus* active viral replication. An average of 48 h is required to carry out the technique.

INTRODUCTION

The ability to identify individual cells expressing or carrying specific genes of interest in a latent form in a tissue section, under the microscope, provides a great advantage over the solution-based PCR methods for the amplification of defined gene sequences[1] in determining the various aspects of normal, as opposed to pathological, conditions. One of the major drawbacks of a solution-based PCR is that the procedure does not allow the direct association of amplified signals of a specific gene segment with the histological cell type or the cellular source[1–23]. *In situ* PCR, however, utilizes the well-recognized and highly developed techniques of PCR or reverse transcriptase-PCR (RT-PCR) so that a gene of interest can be amplified *in situ* in a fixed cell. In this case, the cell mimics a microvessel where amplification can take place and the amplicons can be detected inside various subcompartments of a cellular structure. The most crucial step is to fix the cells in such a fashion that allows the amplification steps to take place without destruction or distortion of the subcellular compartments[7,8,11,13], which requires thorough optimization and familiarity with molecular histology methods[12,13].

Theoretically, there are unlimited numbers of applications for this protocol. For example, the amplification of a fragment of mRNA or DNA, either in the cytoplasm or in the nucleus of a cell by *in situ* RT-PCR, can assist in the determination of the number of cells infected with a specific virus[11], or the number of cells with active viral gene expression compared to latently infected cells (when DNA and mRNA specific primers are applied)[13,21,22]. The percentage of cells with tumor genes[23,24] before and after therapy[23–28] could also potentially be determined by *in situ* PCR; and by utilizing specific primers for genes that are expressed by certain histological cell types, one can potentially determine the origin of metastatic tumors by performing *in situ* RT-PCR[28,29]. It is also possible to determine the subcellular localization of a gene or virus inside the cytoplasmic or nuclear compartments[2–7,12,14,18,24,28,30–32]. In addition, one of the more interesting applications of *in situ* PCR is in the rapid identification of bacterial species in the natural environment[29,33,34].

Our laboratories have been utilizing *in situ* PCR techniques for almost two decades, and we have developed precise, sensitive protocols for both RNA and DNA proven to be reproducible in multiple double-blinded studies[2–7,10,17–18,21]. In the case of viral infection, or to determine the differential expression of a known gene, one can use this method for the amplification of both DNA and mRNA sequences either separately or simultaneously, utilizing primers that anneal to the intron/exon (for DNA or unspliced mRNA) or the splicing junctions of the exons (for mRNA), respectively[8,10,13,16,17,20,21,23,27]. Using multiple, differently colored, labeled probes with different excitation and emission ranges, one can also detect various signals in a single cell[14,21]. Currently, there are >30 different kinds of colored probes commercially available, and one can choose them according to the filter range one has in the laboratory. In addition, one can perform immunohistochemistry to detect RNA and DNA amplification at a single-cell level (triple-labeling[10,22,31]). If one desires to carry out immunolabeling, then it is recommended to treat the tissues in antigen retrieval buffer (following the manufacturer's recommendation) and then label the tissues/cells with primary antibody and then with secondary antibody. The labeling of surface antigen(s) can be carried out by standard immunohistochemistry methods.

In the following pages, we present a detailed protocol currently being utilized in our laboratory. The full procedure is illustrated in **Figures 1** and **2**. One of the limitations of this protocol is the need for a multidisciplinary team or group of individuals having expertise in molecular biology, immunohistochemistry, histology and pathology. In addition, utmost care must be taken to preserve the morphology of the tissues as well as the nucleic acid that one wants to amplify *in situ* (see EXPERIMENTAL DESIGN for further details).

Experimental design

Slide choice and preparation. Before one can perform *in situ* PCRs using the protocols described below, utmost care should be taken to preserve the DNA and mRNA molecules inside the cellular

structures. Therefore, we recommend using + slides or Superfrost glass slides, if possible. These can be purchased from any reliable source and should be autoclaved to sterilize them before applying cells or tissue sections to the slides. However, such slides have a limited shelf-life and may not be available to all researchers; therefore, we also provide an inexpensive method in this protocol for researchers to prepare their own silanized 3-aminopropyl-triethoxysalane (AES) glass slides for use in *in situ* PCR. Treatment of glass slides with AES imparts a strong, persistent positive electrostatic charge, which causes the cells or tissues to adhere strongly to the slides throughout the PCR hybridization procedure.

Tissue preparation. Most of our experience is in the amplification of DNA/RNA from mammalian cells, thus the protocols presented here will need further optimization for different types of cells. The best type of tissue to use for *in situ* PCR is paraffinized tissue fixed in buffered formalin. This is a routine tissue preparation method that preserves the morphology of the tissues. However, cells cultured on glass slides, peripheral blood mononuclear cells (PBMCs), cytopreps or cytospins of cell cultures, cervical lavage, buccal cells or other cells on glass slides, as well as fresh frozen tissues, can all be prepared in an appropriate fashion for *in situ* analyses. Of course, all unfixed cells need to be fixed before *in situ* amplification.

It is possible to use frozen sections for *in situ* PCR-amplification (see **Box 1**); however, the morphology of the tissue following the amplification process is generally not as good as with paraffin-fixed sections. Cryogenic freezing of the tissue, combined with the lack of paraffin substrate during slicing, compromises the integrity of the tissue. Usually thicker slices must be made, and the tissue can 'chatter' in the microtome. Indeed definitive pathological diagnoses are made from paraffin-fixed sections, and this rule-of-thumb seems to extend to the amplification procedure as well. The exception to the rule exists when one wishes to use immunohistochemical techniques to detect additional signals in the cells. Some of these techniques require frozen sections; and in such circumstances, use of frozen sections is appropriate. Frozen sections should, however, only be analyzed by *in situ* PCR if the tissue is fixed in formalin after the section is made. Alternatively, newer fixatives—such as 'Permiofix' from Ortho Diagnostics—can be used to preserve cell surface antigens in frozen tissue.

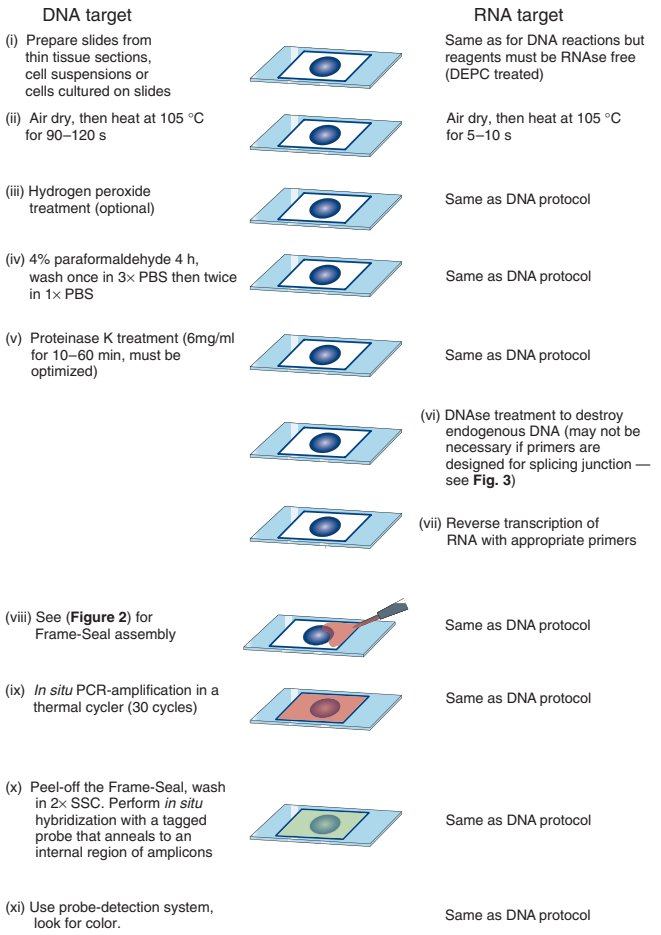

Figure 1 | An overview of the whole procedure. This illustration summarizes all the steps that are required to carry out the *in situ* PCR on slides. This includes: (i) preparation of slides from cell or tissue samples; (ii) air-drying and heat treating the slides; (iii) removal of endogenous signals [e.g., using hydrogen peroxide (H_2O_2)]; (iv) fixation of cells/tissue; (v) proteinase K treatment, (vi) DNAse treatment before reverse transcriptase-PCR (RT-PCR); (vii) RT reaction for the detection of RNA targets (one- or two-step as described in PROCEDURE); (viii) applying Frame-Seal to get the slides ready for *in situ* PCR amplification, (ix) PCR-amplification of the desired mRNA or DNA fragments; (x) *in situ* hybridization of labeled probe; (xi) probe detection.

Proteinase K digestion. The pretreatment of tissues or cells with proteinase K is one of the integral steps of the *in situ* PCR procedure. Generally, cells are reasonably permeable to small nucleic acids (e.g., probes and primers); however, polymerase enzyme is a different story. Therefore, it is necessary to treat the cells with a specific concentration of proteinase K for a short, optimized period. This creates a controlled digestion of proteins in the cell pores that allows the appropriate penetration of the PCR cocktail. The time and temperature of the incubation with proteinase K should be optimized carefully for each cell line or tissue-section type (see **Box 2**). Too little digestion and the cytoplasmic and nuclear membranes will not be sufficiently and equally permeable to primers and enzyme in all cell types, thus amplification will be inconsistent (or nonexistent). Too much digestion will cause the membranes to lose their integrity and to leak amplicons, thus making the surrounding cells falsely positive, or exhibit high background or poor morphology. Often with excessive digestion, many cells will show peri-cytoplasmic staining, which represents leaked signal contaminating cells where no positive signal actually exists. Attention to detail with the proteinase K digestion can often mean the difference between success and failure in an experiment, and this digestion should be practiced on extra sections by anyone attempting to conduct this protocol for the first time. In our laboratory, typically, lymphocytes will require 5–10 min at 25 °C (room temperature), neuronal tissue will require 12–18 min at room temperature, and almost all paraffin-fixed tissue will require between 15 and 30 min at room temperature (these times can be accelerated by using higher temperatures of incubation, up to 55 °C). However, the periods can vary widely and one has to optimize the conditions by carrying out careful reactions with control cells.

Primer design. PCR primers are synthetic oligonucleotides, typically between 18 and 22 bases in length. It is our experience that primers in this range work effectively. The overall GC-content

of the primer should be between 45 and 50%, and it is desirable to have one or two G's and C's at the 3′ (downstream) end of the primer to facilitate annealing. The two primers should be designed so that they have approximately the same annealing temperature. They should also be designed so that they do not form intra- or interstrand base pairs, which may result in hairpin formation, and one should ensure that primers do not anneal to multiple regions of the target. Furthermore, primers should never be complementary to one another, particularly around their 3′-ends. If they are complementary in this region, they often anneal to each other and thus form 'primer dimers', which is always undesirable because it consumes the primers and greatly lowers the efficiency of amplification. Primers should be designed according to standard principles, and computer-aided design programs http://www.bioinformatics.vg/biolinks/bioinformatics/PCR%2520and%2520Primer%2520Design.shtml combined with the data resources offered by GenBank (http://www.ncbi.nlm.nih.gov/entrez/) can often lead to superior primers.

RT-PCR. *In situ* PCR is largely used for the detection of a particular gene expression; thus *in situ* RT-PCR is usually the method of choice. Therefore, the desired mRNA or mRNAs will require conversion to cDNA. The two most commonly used methods are a random primer-based RT reaction and a specific primer-based method. In the former, almost all the mRNAs are converted into cDNA *in situ* using sets of very short oligos of random sequence, generally 'hexamers' only six bases long. They anneal to complementary strands of mRNA, and the RT enzyme extends them. A specific primer pair is then used to amplify the desired fragments of gene or genes. In the latter method, the RT reaction is carried out using a specific primer to reverse-transcribe only the gene of interest from the mRNA, which is subsequently PCR-amplified using the pair of primers designed to amplify the target. In all RT reactions, it is desirable to reverse-transcribe only relatively small fragments of mRNA ($<$1,500 bp). Larger fragments may not completely reverse-transcribe due to the presence of secondary structures. Furthermore, the RT enzymes—AMVRT and MuLVRT, at least—are not very efficient in transcribing large mRNA fragments.

Primers for RT-PCR should be designed so that they do not contain secondary structures, and are not complementary to RNA

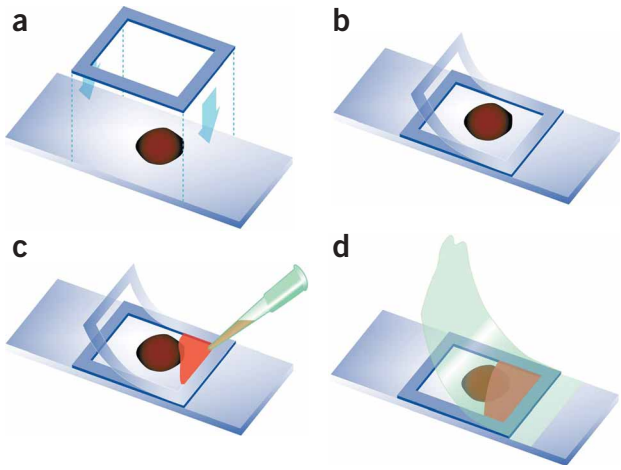

Figure 2 | An overview of Frame-Seal incubation chambers. (**a**) Carefully remove the sticky tape from one of the sides of the square frame. (**b**) Firmly attach the square frame around the areas of the tissues or cell suspension. (**c**) Add 50 μl of PCR-cocktail in the square area and then remove the upper sticky tape (**d**) Carefully seal the slide with the plastic sealer.

BOX 1 | FREEZING TISSUE FOR *IN SITU* PCR ANALYSIS

This procedure can be performed using options A (OCT and liquid nitrogen), B (liquid nitrogen only) or C (OCT and dry ice), as follows:

(A) Freezing tissue using OCT and liquid nitrogen

(i) Cut a 1 × 1 cm^2 piece of styrofoam from a sheet ~3-mm thick.

(ii) Then cut a slice of tissue about the same size as the styrofoam square, but somewhat thicker (up to 1-cm thick).

(iii) Pour ~2 ml of Tissue-Tek OCT (an embedding media for frozen tissue specimens made by Miles Laboratory of Elkhart IN) onto the styrofoam substrate.

(iv) Lay the tissue onto the Styrofoam substrate.

(v) Pour another 2 ml of OCT on top of the tissue, so that the tissue is covered with OCT.

(vi) Fashion an immersion tool from wire or a coat hanger so that the styrofoam/tissue sample can rest on a loop of wire, with a wire handle that allows immersion.

(vii) Slowly lower the apparatus and sample into the liquid nitrogen, and the tissue should freeze in $<$30 s.

(viii) Load the tissue into a cryocassette for cutting or store at −70 °C.

(B) Freezing tissue using liquid nitrogen only

(i) Place the tissue into a small ziplock bag designed for immersion into liquid nitrogen—these bags are common items in pathology laboratories.

(ii) Immerse the tissue in liquid nitrogen.

(iii) Remove the tissue from the bag and load into a cryocassette for cutting or store at −70 °C.

(C) Freezing tissue using OCT and dry ice

(i) If liquid nitrogen is not available, prepare tissue with styrofoam and OCT (as in option A(i–iv) and wrap in aluminum foil.

(ii) Place on dry ice for 10–15 min.

(iii) Store at −70 °C.

▲ CRITICAL STEP Some ice crystallization in the tissue may occur with this method.

▲ CRITICAL STEP Under no circumstance attempt to freeze the tissue by merely placing it into a −70 °C freezer. This will result in an abundance of ice crystals, and the sections will not be suitable for *in situ* procedures.

BOX 2 | OPTIMIZATION OF PROTEINASE K DIGESTION PARAMETERS

1. Prepare four or five extra slides of the specific tissue in question.
▲ CRITICAL STEP It is especially helpful if the slides are successive sections or very similarly prepared, for the morphology of the various slides must be closely compared later on.
2. Prepare an equal number of serial dilutions of proteinase K solution, over the range of 1–6 μg ml^{-1}, for example.
3. Decide upon a standard time and temperature for digestion, for example, 15 min at 37 °C.
4. Treat the slides in the serial solutions for this standard period.
5. Stop the digestion by heating slides on a block for 2 min at 95 °C.
6. Counterstain the slides with hematoxylin or other appropriate stain.
7. Observe slides under a high-power light microscope and look for any morphological change in the cells.
8. Choose the highest concentration of proteinase K that did not result in significant change as the optimized concentration.
9. If all the slides showed change, repeat the process with lower concentrations of proteinase K or shorter incubations.
10. If none of the slides showed change, increase concentrations or incubation times and repeat the procedure until proper conditions are found.
11. Once the optimized digestion is determined, use these conditions to process all slides of that particular tissue, fixation method and thickness of section. Any change in the latter three parameters necessitates re-optimization of the digestion procedure.

that will form secondary structures. One can design primers that anneal only to targeted mRNA sequences by designing the primers to span introns in the genomic DNA. The primers will only then adhere to the mRNA templates and the cDNA copies of mRNA, and not to any genomic DNA copy of the same gene. This concept is illustrated in **Figure 3**. Design of such primers requires prior understanding of the gene of interest, and there are several software programs available that assist in designing such primers.

If one combines these special primers with a special polymerase enzyme that has both RT and DNA polymerase activity, such as the r*Tth* enzyme, then one can amplify mRNA sequences directly without going through any specific RT step (see **Box 3**). This simplifies the whole PCR procedure by eliminating the need for a DNase treatment as well as a buffer change between the RT and polymerase enzyme steps. Better yet, this procedure allows for the amplification of multiple mRNAs or both mRNAs and genomic DNAs simultaneously—because there is no need to destroy all the endogenous DNA: primers for each nucleotide-type can also be included without interfering with the activity of the other. However, one must know a considerable amount about the sequence of the gene-in-question to design these RNA-specific primers.

Length of desired amplicon. Recent publications, using new combinations of polymerase enzymes and buffers, which allow efficient amplification of much larger fragments of DNA or cDNA, have shown that the amplification of genes up to 50 kb is possible[35]. We see no reason to prevent these new techniques from being adapted to *in situ* PCR-amplification. However, this 'long PCR' approach is not frequently used for *in situ* work because the primary purpose of the amplification in most circumstances is to *detect* specific genes, not clone them. For most *in situ* PCR work, relatively short amplicons are used. Our laboratory has had great success routinely amplifying gene fragments in the 150–350 bp range; the amplicons should not be so long as to lower the efficiency of the amplification in the difficult environment of a cellular matrix.

Probe labeling and detection systems. Probes can be labeled indirectly using either biotin or digoxigenin (dig), or directly with a fluorescent label, to allow their subsequent detection by microscopy. Biotin-labeled probes can be detected using enzyme-based color detection systems such as peroxidase or alkaline-phosphatase, using a streptavidin–enzyme conjugate that binds to the biotin label on the probe. Dig-labeled probes can be recognized using an anti-dig-enzyme conjugate for colorimetric detection in a similar fashion.

Optimization of reactions. Before one attempts to conduct *in situ* PCRs on tissues or cells, we strongly recommend that all investigators first optimize the PCRs in solution in order to ensure primers, probes and the incubation temperatures all work effectively before adding the further complication of cellular matrices. Investigators universally report that amplification reactions are more problematic in tissues and cells, while solution-based reactions are relatively easy and one can have success rather quickly—often on the first try. Then the reactions can be quickly optimized to improve the results, and optimized parameters almost always transfer more successfully to the *in situ* protocols. Solution-based reactions can also serve as controls for *in situ* PCR.

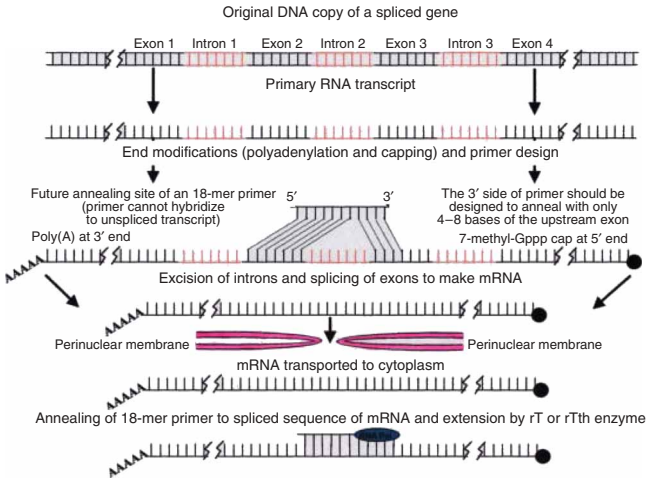

Figure 3 | Most eukaryotic genes are divided into coding segments (exons) and noncoding regions (introns). In order for an mRNA to be functional, all the exons are spliced together and all the segments that contain introns are removed. This characteristic can be utilized in the design of primers to amplify mRNA signals without interference from the DNA genetic code or vice versa. One can simply design primers so that their sequences flank splicing junctions where two exons are joined together so that the complementary primers would bind mRNA only.

BOX 3 | ONE-STEP REVERSE TRANSCRIPTASE-AMPLIFICATION

It is possible to manufacture cDNA that is subsequently amplified by DNA polymerase in a single step, using any of the suitably thermostable reverse transcriptase (RT)/DNA polymerases (that have both RT and DNA polymerase capacity), like *rTth*. In this case, we recommend that you follow the manufacturer's protocol to prepare the amplification mix and PCR amplify the target DNA, using the following general protocol:

1. Prepare one-step RT-PCR master mix, using a kit of your choice, following the manufacturer's instructions.
2. Add 50 μλ master mix to each well of the sample slide and seal slides (see **Fig. 1**).
3. This reaction requires a slightly variant thermal cycling profile. Our laboratory uses the following amplification protocol:

42 °C 45 min
92 °C 3 min
42 °C 15 min 92 °C 3 min
42 °C 15 min
Then, 29 cycles of the following profile:
93 °C 1 min
53 °C 1 min
72 °C 1 min
Final extension 72 °C 10 min

■ **PAUSE POINT** Reactions can be stored at 4 °C.

Validation and controls. It is appropriate to run two or three sets of experiments in multiwelled slides simultaneously, to validate amplification and confirm the subsequent hybridization/detection steps. In all amplification procedures, we use one slide as a control for nonspecific binding of the probe by hybridizing the amplified cells with an unrelated probe. We also use HLA-DQα, or β-actin probes and primers with human PBMCs as positive controls, to check various parameters of our system.

MATERIALS

REAGENTS

- Paraformaldehyde (PFA; ultra pure; Merck, cat. no. 4005) (see REAGENT SETUP)
- 1× PBS (CLP Molecular Reagents, cat. no. 5114) (see REAGENT SETUP)
- Hydrogen peroxide (H_2O_2; Sigma, cat. no.341-500)
- PBS (VWR, Inc., cat. no. EM6508) (see REAGENT SETUP)
- Proteinase K (10 mg powder; Sigma, cat. no. P 6556) (see REAGENT SETUP)
- 2× SSC (CLP Molecular Reagents, cat. no.5115 or Ambion, cat. no. 9763) (see REAGENT SETUP)
- Streptavidin peroxidase (StreptAB Complex/HRP; Dako, cat. no. K0377)
- Color solution: AEC (3-amino-9-ethyl-carbazole; Sigma, cat. no. A6926) or AEC+ (Dako, cat. no. K3461) (see REAGENT SETUP)
- 50 mM acetate buffer pH 5.0 (Fisher Scientific, cat. no. NC9232841) (see REAGENT SETUP)
- *In situ* hybridization buffer (Hyb-buffer)—EKONO hybridization buffer (RPI Corp, cat. no. 248800 or VWR cat. no.82021-3380) (see REAGENT SETUP)
- Streptavidin peroxidase powder (Sigma, cat. no. A-5754) (see REAGENT SETUP)
- *N,N*-dimethyl formamide (Fisher, cat. no. BP277-100)
- Na_2HPO_4 (VWR, cat. no. JT3818-1)
- Sodium citrate (Fisher, cat. no. 5476-3)
- Glacial acetic acid (Fisher, cat. no. A35-500)
- Sodium acetate trihydrate (Fisher, cat. no. NC9232841)
- 10 U μl^{-1} RQ1 DNase (Boehringer, cat. no.776785) (see REAGENT SETUP)
- Formamide (Fisher, cat. no. BP277-100)
- Salmon sperm DNA (ssDNA, 10 mg ml^{-1}; Sigma, cat. no. 50125193)
- 20× SSC (VWR, cat. no. EM8310)
- 50× Denhardt's solution (VWR, cat. no. EM3610)
- Diethyl pyrocarbonate (DEPC)-treated water (Sigma, cat. no. D-5758)
- Phytohemagglutinin
- Xylene
- Ethanol
- Water-based medium (e.g., CrystalMount or GelMount)
- Organic solvent-based medium (Permount; Fisher Scientific)
- 2% AES solution (see REAGENT SETUP)
- Amplification solution (see REAGENT SETUP)
- RT reaction solution (see REAGENT SETUP)
- Nitro-blue-tetrazolium (NBT) (see REAGENT SETUP)
- Blocking solution (see REAGENT SETUP)
- Conjugate dilution buffer (see REAGENT SETUP)
- Buffer A (see REAGENT SETUP)
- Alkaline substrate buffer (see REAGENT SETUP)
- 4-Bromo-5-chloro-3-indolylphosphate (BCIP) (see REAGENT SETUP)

EQUIPMENT

- Thermocycler (e.g., MJ Research PTC-100-16MS or DNA-Engine Twin-Tower 16 × 2; Bio-Rad, cat. no. PTC-0200G) (see EQUIPMENT SETUP) ▲ **CRITICAL** Our laboratory has been using these models of dedicated slide thermocyclers that are specifically designed to hold 16 or 32 slides quite successfully for the past decade or so. The DNA-Engine Twin-Tower has incorporated humidification chambers as well as temperature gradient to optimize the annealing temperatures for PCR. Gradient thermocyclers are especially useful in the optimization of annealing step, reverse transcription and hybridization steps. However, various types of thermocycler will work in this application, and we understand that other laboratories have used stirred-air, oven-type thermocyclers quite successfully.
- Glass slides ▲ **CRITICAL** One should always use glass slides.
- Frame-seal incubation chambers (Bio-Rad Laboratories or Life Science Research Group) ▲ **CRITICAL** Special slides that have Teflon coatings that form individual 'wells' could alternatively be used, and can be purchased from the same source.
- Coplin Jars and Glass Staining Dishes
- Multiplex chamber slides (BD Falcon, cat. no. 354108 or 354118)

REAGENT SETUP

2% AES solution 5 ml AES, 250 ml acetone (prepare fresh and use within 30 min).

2% PFA Take 12 g PFA and add to 600 ml 1× PBS. Heat at 65 °C for 10 min. When the solution starts to clear add four drops of 10 N NaOH and stir. Adjust to neutral pH and cool to room temperature. Filter on Whatman's no. 1. The solution should be used within 6 months after preparation. Can be stored at room temperature.

10× PBS stock solution pH 7.2–7.4 Dissolve 20.5 g $NaH_2PO_4 \cdot H_2O$ and 179.9 g $Na_2HPO_4 \cdot 7H_2O$ (or 95.5 g Na_2HPO_4) in ~4 l of double-distilled water. Adjust to the required pH (7.2–7.4). Add 701.3 g NaCl and make up to a total volume of 8 l. This solution is good for 1 year. ▲ **CRITICAL** Alternatively, pre-made powder can be used from different sources.

1× PBS Dilute the stock 10× PBS at 1:10 ratio (i.e., 100 ml 10× PBS and 900 ml of water for 1 l). Final concentration of buffer should be 0.01 M phosphate and 0.15 M NaCl. This solution should be used within a month.

0.3% H_2O_2 in PBS Dilute stock 30% H_2O_2 at a 1:100 ratio in 1× PBS for a final concentration of 0.3% H_2O_2. This should be used within few hours.

Proteinase K Dissolve powder in water to obtain 1 mg ml^{-1} concentration. Aliquot into 1 ml tubes and store at −20 °C for up to 6 months. For working solution, dilute 1 ml of stock (1 mg ml^{-1}) into 150 ml of 1× PBS. This solution should be used within hours.

20× SSC Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of water. Adjust the pH to 7.0 with a few drops of 10 N solution of NaOH. Adjust the volume to 1 l with water. Sterilize by autoclaving. This solution is good for a year.

2× SSC Dilute 100 ml of 20× SSC and 900 ml of water. This should be used within 6 months.

Streptavidin peroxidase Dissolve powder in PBS to make a stock of 1 mg ml^{-1}. Just before use, dilute stock solution in sterile PBS at a 1:30 ratio. Should be used within 30 min after preparation. ▲ CRITICAL Alternatively, this can be purchased from Dako. Store it in a cold and lightproof area.

AEC solution Dissolve one AEC 20-mg tablet in 2.5 ml of *N,N*-dimethyl formamide. Store at 4 °C in the dark. This can be stored for 6 months. ▲ CRITICAL AEC+ can alternatively be purchased in ready-to-use form from Dako.

Working color solution Mix 5 ml 50 mM acetate buffer, pH (5.8), 250 µl AEC solution and 25 µl 30% H_2O_2. Prepare fresh before each use, keeping solution in the dark. Use within 30 min after preparation.

50 mM acetate buffer pH 5.0 Add 74 ml of 0.2 N acetic acid (11.55 ml glacial acid per l) and 176 ml of 0.2 M sodium acetate (27.2 g sodium acetate trihydrate in 1 l) to 1 l of deionized water and mix. Can be stored for 6 months in refrigerator.

DNase solution 40 mM Tris–HCl, pH 7.4, 6 mM magnesium chloride ($MgCl_2$), 2 mM $CaCl_2$ and 1 U μl^{-1} final volume of DNase. Use within 60 min after preparation. ▲ CRITICAL Use RNase-free DNase, such as 10 U μl^{-1} RQ1 DNase from Boehringer or any other reliable source (reviewed in ref. 1).

Amplification solution 1.25 µM of each primer, 200 µM dATP, dCTP, dGTP, dTTP, 10 mM Tris–HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% stabilizer (including BSA and gelatin) and 2.5 U of DNA polymerase. ▲ CRITICAL DNA polymerase can be purchased from any certified company. Make sure that the appropriate units of DNA polymerase are used.

RT reaction solution Using AMVRT enzyme as an example:

10× Reaction buffer[a]	2.0 µl
10 mM dATP	2.0 µl
10 mM dCTP	2.0 µl
10 mM dGTP	2.0 µl
10 mM dTTP	2.0 µl
RNasin at 40 U μl^{-1} (Promega)	0.5 µl
20 µM downstream primer	1.0 µl
AMVRT 20 U μl^{-1}	0.5 µl
DEPC-water	8.0 µl
Total volume	20.0 µl

[a]10× Reaction buffer comprises 100 mM Tris pH 8.3, 500 mM KCl, 15 mM $MgCl_2$.

Hybridization solution Contains 20–50 pg μl^{-1} of the appropriate probe, 50% deionized formamide, 2× SSC buffer, 10× Denhardt's solution, 0.1% sonicated ssDNA and 0.1% SDS. The following is a convenient recipe (see refs. 1–3):

Probe	2 µl
Deionized formamide	50 µl
20× SSC	10 µl
50× Denhardt's solution	20 µl
10 mg ml^{-1} ssDNA	10 µl
10% SDS	01 µl
H_2O	7 µl
Total volume	100 µl

▲ CRITICAL The ssDNA should be denatured at 94 °C for 10 min before it is added to the Hyb-buffer. ▲ CRITICAL Alternatively, EKONO Hyb-buffer can be used.

Blocking solution To 50 mg ml^{-1} BSA (protein) in 100 mM Tris–HCl (pH 7.8), add 150 mM NaCl and 0.2 mg ml^{-1} sodium azide.

Conjugate dilution buffer Mix 100 mM Tris–HCl, with 150 mM $MgCl_2$, 10 mg ml^{-1} BSA and 0.2 mg ml^{-1} sodium azide.

Buffer A Mix equal volumes of 100 mM Tris–HCl (pH 7.5) and 150 mM NaCl.

Alkaline substrate buffer Mix equal volumes of 100 mM Tris–HCl (pH 9.5), 150 mM NaCl and 50 mM $MgCl_2$.

NBT 75 mg ml^{-1} NBT in 70% (vol/vol) dimethylformamide, freshly prepared.

BCIP 50 mg ml^{-1} in 100% dimethylformamide, freshly prepared.

EQUIPMENT SETUP

Thermocycler setup We suggest that you follow the manufacturer's instructions on the use of your own thermocycler, bearing in mind the following points:

Glass does not easily make good thermal contact with the surface on which it rests. Therefore, a weight to press down the slides and/or a thin layer of mineral oil to fill in the interstices will help thermal conduction. If using mineral oil, make certain that the oil is well smeared over the glass surface so that the slide is not merely floating on air bubbles beneath it.

The top surfaces of slides lose heat quite rapidly through radiation and convection; therefore, use a thermocycler that envelopes the slide in an enclosed chamber (as in some dedicated instruments), or insulate the tops of the slides in some manner. Insulation is particularly critical when using a weight on top of the slides, for the weight can serve as an unwanted heat sink if it is in direct contact with the slides.

Good thermal uniformity is imperative for good results—poor uniformity or irregular thermal change can result in cracked slides, uneven amplification or completely failed reactions. If adapting a thermocycler that normally holds plastic tubes, use a layer of aluminum foil to spread out the heat.

PROCEDURE

AES silanization of glass slides ● TIMING ~24 h

1| Prepare 2% AES solution just before use (see REAGENTS SETUP).

2| Put 2% AES solution into a Coplin jar or glass staining dish and dip glass slides in solution for 60 s.
▲ CRITICAL STEP 250 ml of AES solution is sufficient to treat 200 glass slides.

3| Dip slides five times into a different vessel filled with 1,000 ml of distilled water.

4| Repeat Step 3 three more times, changing the water each time.

5| Air-dry slides in a laminar-flow hood from a few hours to overnight, then store slides in sealed container at room temperature.
■ PAUSE POINT Try to use slides within 15 d of silanation

Preparation of slides from tissue/cell samples for *in situ* PCR analysis

6| Slides can be prepared from the following types of tissue/cell samples for analysis by *in situ* PCR using the different options, as indicated: cell suspensions (option A); cell cultures on slides (option B); paraffin-fixed tissue (option C); frozen tissue (option D); and archived tissue samples (option E).

(A) Preparation of slides from cell suspensions for *in situ* PCR analysis ● **TIMING** ~**2 h**

(i) Divide cell suspensions into two portions. One portion should be used for the preparation of slides and other portion should be saved for confirmation of the results by a different method (i.e., immunohistochemistry or solution-based RT-PCR).

! **CAUTION** The human peripheral blood used in this procedure may be infectious or hazardous to the investigator. Use universal precautions to work with any human fluids or tissues. Proper handling and decontamination and disposal of waste material must be emphasized.

▲ **CRITICAL STEP** Sterile technique must be practiced at all times during the culturing and pre-fixation parts of this protocol. Sterile technique is especially important in handling cell cultures, both to protect the investigator and to avoid introducing microbial contamination of the cell culture system. Such contamination is often the cause of test failure.

(ii) Use two culture tubes each filled with 5 ml of RPMI media and add 0.5 ml of well-mixed whole blood to each tube using a pipette.

▲ **CRITICAL STEP** Use three culture tubes for blood from newborns.

(iii) Rinse pipette with a small volume of RPMI media (1–1.5 ml) three to four times to expel all of the whole blood into the culture tube.

(iv) With a 1-ml syringe, slowly add 0.1 ml phytohemagglutinin (PhA-C) to each tube.

(v) Gently vortex or invert tubes to ensure complete mixing.

(vi) Place tubes in a tray with a slight slope upward toward cap (about an 18° angle). Loosen cap to allow CO_2 penetration.

(vii) Incubate for 66 h at 37 °C with 4.5% CO_2 and 90% humidity (as in a water-jacketed incubator).

■ **PAUSE POINT** The protocol can be paused whilst this incubation takes place.

(viii) Isolate cells on a Ficoll-Hystopaque density gradient method[36].

(ix) Wash cells twice with 10 ml 1× PBS in 15-ml tube by centrifugation at 400*g* for 10 min.

(x) Resuspend cells in 1× PBS at 5×10^5 cells ml^{-1}.

(xi) Add 10 µl of cell suspension to each well of a slide (from Step 5) using a P20 micropipette.

(xii) Air-dry slide in a laminar-flow hood for 1 h

(B) Culture of adherent cells on a slide for *in situ* PCR analysis ● **TIMING** **Overnight or longer depending on the cell growth rate and the intial number of cells seeded**

(i) To culture cells on single-chamber or multiplex-chamber slides (prepared as detailed in Steps 1–5), add 0.5 ml of cells (~$1–5 \times 10^{-3}$ cells per well) to an eight-chamber slide, or 2–3 ml cells for the single-chamber slide. The density of the cells should be adjusted according to the size of the cells.

(ii) Culture overnight in a sterile humidified box.

▲ **CRITICAL STEP** Most cells will grow well at 37 °C at 5% CO_2, but one must use the appropriate media to culture each cell type one is working with and must follow the proper cell culture temperature, pH and growth condition requirements for the cells one is working with. Contrary to common practice, not all cells grow optimally at 37 °C. Any tissue culture chamber designed for cell culture can be used. The time required depends on the cell type, for example, HeLa cells will attach within 2–3 h on the glass surface and form a monolayer within 24 h. On the other hand, the majority of the primary cultures will take several days to attach and may form a monolayer after a week or so.

■ **PAUSE POINT** The protocol can be paused overnight or longer while cells are cultured.

(iii) Wash the cells by gently dipping slides in 1× PBS.

(C) Preparation of paraffin-fixed tissue slides for *in situ* PCR analysis ● **TIMING** **2 h**

(i) To remove the paraffin from the tissue, firstly incubate the slides in an oven at 80 °C for 1 h to melt the paraffin.

(ii) Dip the slides in xylene (EM grade, benzene-free) solution for 5 min, then in 100% ethanol (EM grade) for 5 min, 90% ethanol (EM grade) for 5 min, 80% ethanol (EM grade) for 5 min, 70% (EM grade) ethanol for 5 min, 50% (EM grade) ethanol for 5 min and then in H_2O for 5 min.

(iii) Air-dry the slides for 1 h.

(D) Preparation of slides from frozen tissue for *in situ* PCR analysis ● **TIMING** **1 h**

(i) Freeze tissue as described in **Box 1**.

(ii) Section the tissue with a cryostat.

! **CAUTION** All fresh frozen tissues are considered biohazardous, and precautions should be taken to avoid cutting one's fingers and hands.

▲ **CRITICAL STEP** Use proper sectioning procedure. This procedure takes a considerable degree of experience.

▲ **CRITICAL STEP** It is necessary to use as thin a section as possible, down to 6 µl in thickness. Difficulties may be experienced in slicing sections thinly if the tissue is insufficiently frozen. This is remedied by the use of pathologist's freezing spray—merely blast the central area with a few quick bursts of spray, wait a few moments and proceed. If the

tissue will not slice at all, it could be that the tissue is too solidly frozen. To remedy this problem, allow the tissue to equilibrate overnight at −30 °C while mounted on the disk in the cryostat.

(iii) Apply sliced tissue to slide (from Step 5).

(E) Preparation of archival tissue slides for *in situ* PCR analysis ● TIMING **Overnight**

(i) Remove the coverslip and mount by soaking in 100% methanol (or carefully in acetone if methanol does not pry the coverslips open).

(ii) Deparaffinize as described in Steps 6C(i–iii).

▲ CRITICAL STEP Since archival tissue samples are already on a slide, one does not have a choice about the area of the slide covered by the section or what type of surface preparation to use.

Heat treatment ● TIMING **30 min**

7| Place the slides with adhered tissue/cells on a heat-block at 105 °C for 5–120 s to stabilize the cells or tissues.

▲ CRITICAL STEP Some RNA signals may not be very stable at high temperatures. Therefore, we use shorter incubation times (5–10 s) with RNA targets, and longer times (90–120 s) with DNA signals. The preservation of intact mRNA is of primary importance, and the success of first-strand cDNA synthesis depends upon the integrity of the mRNA of interest. One may need to experiment with different periods in order to find the best heat treatment for the specific tissue and target.

■ PAUSE POINT At this point, slides with adhered tissue/cells can be stored at −70 °C until use.

Note: A small piece of sterile gauze or tissue paper soaked with sterile water can be placed inside the storage box with the slides, and the box should be sealed. This prevents the drying of the slides after long-term storage (over 2 months or longer).

8| Optional. If peroxidase-based color development is to be used, then slides should be further treated by adding 0.3% solution of H_2O_2 in 1× PBS, in a Coplin jar and incubating overnight—either at 37 °C or at room temperature—then washing once with 1× PBS.

▲ CRITICAL STEP In order to be certain that color development after the following procedure is the result of amplification and proper *in situ* hybridization, slides must be treated with 0.3% H_2O_2 to destroy the endogenous peroxidases. This treatment time can be increased for the tissue that generally carried more abundant peroxidase activity (i.e., pancreas, liver and small intestine)

▲ CRITICAL STEP Endogenous phosphatases should be inactivated by heat treatment (Step 7).

Fixation ● TIMING **5 h**

9| Place the slides in a solution of 4% PFA in 1× PBS (pH 7.4, <1 month old) for 4 h at room temperature. The fixation step is carried out by submerging the slide in a specially designed glass slide jar (Coplin jar) that can accommodate either 10 slides or 100 slides at the same time. For the smaller jar, one can fill it up with ~50 ml of liquid and the larger one with ~250 ml. The fixative should be used only once and then discarded, and the jar should be filled with a fresh solution for the next batch of slides. One can double the numbers of slides that can be fixed by loading the slides in the jar in a zig-zag manner.

▲ CRITICAL STEP Use of the recommended Coplin jars or staining dishes facilitates this step.

Washes ● TIMING **1 h**

10| All the slides fixed in 4% PFA must be washed immediately after the completion of fixation in 3× PBS for 10 min, agitating periodically with an up-and-down motion by submerging in a Coplin jar (100 ml volume).

11| Wash the slides with 1× PBS for 10 min, agitating periodically with an up-and-down motion.

12| Repeat Step 11 twice with fresh 1× PBS.

13| Wash the slides in deionized distilled water for 1 min.

14| Air-dry the slides under the laminar-flow hood.

Proteinase K treatment ● TIMING **1 h**

15| Proteinase K digestion can be carried out using previously optimized digestion parameters (as described in **Box 2**), or as follows: treat samples with 6 μg ml^{-1} proteinase K in PBS for 5–60 min at room temperature or at 55 °C (see Experimental design for further details).

16| After 5 min, look at the cells under the microscope at 400× magnification.

17| If the majority of the cells of interest exhibit uniform-appearing, small, round 'bubbles' or 'blebs' or 'peppery dots' on the cytoplasmic membrane, then stop the treatment immediately with the next step. Otherwise, continue treatment for another 5 min and re-examine.

18| After proper digestion, heat slides on a block at 95 °C for 2 min to inactive the proteinase K.

19| Rinse slides in 1× PBS for 10 s.

20| Rinse slides in distilled water for 10 s.

21| Air-dry slides.

***In situ* PCR-amplification of DNA and RNA targets**

22| This step can be carried out using either option A to amplify DNA targets by *in situ* PCR or option B to amplify RNA (or in reality cDNA) targets by *in situ* RT-PCR.

(A) *In situ* PCR-amplification of DNA targets using frame-seal incubation chambers ● TIMING **~5 h**

(i) Add 10–15 µl of amplification solution (see REAGENT SETUP) onto each well with a P20 micropipette so that the whole surface of the well is covered with the solution.
▲ CRITICAL STEP If using a single-well slide for a tissue section, add 12–20 µl of the solution to the well. In all cases, be careful—do not touch the surface of the slide with the tip of the pipette.

(ii) Seal the slides (see **Fig. 2**) by placing the plastic coverslip on top of the slide; or if using tissue sections, use a second slide instead of a coverslip. Carefully seal the edge of the coverslip to the slide.
▲ CRITICAL STEP In the case of archival tissue slides (see Step 6E), not all of the tissue will be subject to the amplification–hybridization procedure, but usually there is a sufficient area in the well region to get adequate results using the Frame Chamber (see **Fig. 2**).

(iii) Place on a heat block for 90 s at 92 °C, to further cure the adhesive.

(iv) Place slides in an appropriate thermal cycler.

(v) Run 30 cycles of the following amplification protocol:
94 °C 2–3 min
45–55 °C 1–2 min
72 °C 1–5 min (depending on the size of amplicons)

(B) *In situ* RT-PCR to amplify RNA targets ● TIMING **6 h**

(i) *DNase treatment*. Add 10 µl of DNase solution (see REAGENT SETUP) to each well.
▲ CRITICAL STEP All reagents for *in situ* RT-PCR should be prepared with RNase-free water (i.e., DEPC-treated water). In addition, the silanized glass slides and all glassware should be RNase-free, which we insure by baking the glassware overnight in an oven at 250–300 °C before use in the RT procedure.

(ii) Incubate the slides overnight at 37 °C in a humidified chamber.
▲ CRITICAL STEP When using liver tissue, this incubation should be extended an additional 18–24 h.
■ PAUSE POINT The protocol can be paused while the slides are incubated overnight.

(iii) Rinse the slides with a similar solution to the DNAse solution, but prepared without the DNase enzyme.

(iv) Wash the slides twice with DEPC-treated water.

(v) *RT reaction*. Add 10 µl of RT reaction solution (see REAGENT SETUP) to each well, containing either primers specific for the target (at final concentration of 200 nM) or random nanomers (at 50 µM concentration) if multiple mRNA targets are to be amplified simultaneously. Carefully place the coverslip on top of the slide.
▲ CRITICAL STEP It is advisable to use a premade RT reaction solution that contains all the components necessary to carry out the reaction, and one only has to add the mRNA template and primers at the end.
▲ CRITICAL STEP The RT-PCR cocktail must be preserved from degradation and should be kept on ice to minimize the formation of nonspecific first-strand products.

(vi) Incubate at 42 °C or the temperature recommended by the manufacturers for 1 h in a humidified atmosphere.
▲ CRITICAL STEP Some RT enzymes work best at 72 °C and or other temperatures, and one should check this before setting the cycles in the thermocycler.

(vii) Incubate slides at 92 °C for 2 min.

(viii) Remove coverslip and wash twice with distilled water.

(ix) Follow Step 22, option A for the amplification procedure, which is the same for both DNA- and RNA-based protocols. Alternatively, the *in situ* PCR-amplification of RNA targets can be achieved by one-step RT-amplification, as described in **Box 3**.

23| Open the slides by carefully prying off the plastic coverslip.

24| Optional. To recover the supernatant for further analyzsis of PCR products or for re-amplification, the supernatant can simply be collected by pipetting. Ideally, one wishes to recover as much of the supernatant as possible, which would typically comprise 10–15 µl of supernatant.

25| Place the opened slides on a heat-block at 92 °C for 1 min—this treatment helps immobilize the intracellular signals.

26| Wash slides with 2× SSC at room temperature for 5 min, two times.

Probe hybridization ● TIMING **12–24 h**

27| Prepare hybridization solution (see REAGENTS SETUP)

▲ CRITICAL STEP Alternatively, there are many companies who market premade Hyb-buffers. Most of them contain a large percentage of dextran sulfate that is originally designed for Southern blots or Northern blots hybridizations. Therefore, it would be wise to first inquire about the contents of the Hyb-buffer before purchasing. However, there are buffers that contain similar ingredients, which we described earlier, and they would be appropriate to use.

28| 2% BSA can be added if one is observing nonspecific binding; add 10 µl of 20% BSA solution and reduce the amount of water.

29| Add 10 µl of hybridization mixture to each well and add coverslips.

30| Heat slides on a block at 95 °C for 5 min.

31| Incubate slides at 48 °C for 2–4 h in a humidified atmosphere.

▲ CRITICAL STEP For optimal results, hybridization can be carried overnight.

Post-hybridization colorimetric detection of probes

32| Post-hybridization colorimetric detection of probes can be achieved using the following options: option A for peroxidase-based colorimetric detection of a biotinylated probe; option B for alkaline phosphatase-based colorimetric detection of a biotinylated probe; option C for colorimetric detection of a dig-labeled probe; option D for detection of fluorescently labeled probes.

(A) Post-hybridization peroxidase-based colorimetric detection of a biotinylated probe ● TIMING **2 h**

(i) Wash slides in 1× PBS twice for 5 min each time.
(ii) Add 10 µl of streptavidin-peroxidase complex (100 µg ml^{-1} in PBS, pH 7.2). Gently apply the coverslips.
(iii) Incubate slides at 37 °C for 1 h.
(iv) Remove coverslip, wash slides with 1× PBS twice for 5 min each time.
(v) Add to each well 100 µl of AEC in the presence of 0.03% H_2O_2 in 50 mM acetate buffer (pH 5.0).
(vi) Incubate slides at 37 °C for 10 min to develop the color—this step should be carried out in the dark. After this period, observe slides under a microscope. If color is not strong, develop for another 10 min.
(vii) Rinse slides with tap water and allow drying.
(viii) Add 1 drop of 50% glycerol in PBS and apply the coverslips.
(ix) Analyze with optical microscope—positive cells will be stained a brownish-red.

(B) Post-hybridization alkaline phosphatase-based colorimetric detection of a biotinylated probe ● TIMING **~3 h**

(i) After hybridization, remove coverslip, wash the slides with two soakings in 2× SSC at room temperature for 15 min.
(ii) Cover each well with 100 µl of blocking solution (see REAGENT SETUP), place the slides flat in a humidified chamber at room temperature for 15 min.
(iii) Prepare a working conjugate solution by mixing 10 µl of streptavidin-alkaline phosphatase conjugate (40 µg ml^{-1} stock) with 90 µl of conjugate dilution buffer (see REAGENT SETUP) for each well.
(iv) Remove the blocking solution from each slide by touching a paper towel to the edge of the slide.
(v) Cover each well with 100 µl of freshly prepared working conjugate solution (see REAGENT SETUP) and incubate in the humid chamber at room temperature for 15 min. Do not allow the tissue to dry out after adding the conjugate.
(vi) Wash slides by soaking in buffer A (see REAGENT SETUP) for 15 min at room temperature two times.
(vii) Wash slides once in alkaline substrate buffer (see REAGENT SETUP) at room temperature for 5 min.
(viii) Prewarm 50 ml of alkaline-substrate buffer to 37 °C in a Coplin jar. Just before adding the slides, add 200 µl NBT and 166 µl of BCIP (see REAGENT SETUP). Mix well.
(ix) Incubate slides in the NBT/BCIP solution at 37 °C until the desired level of signal is achieved (usually from 10 min to 2 h). Check the dark brown color development periodically by removing a slide from the NBT/BCIP solution. Be careful not to allow the tissue to dry out.
(x) Stop the color development by rinsing the slides in several changes of deionized water.
(xi) Analyze with optical microscope—positive cells will be stained a dark brown coloration.

(C) Post-hybridization colorimetric detection of dig-labeled probes ● TIMING **2 h 30 min**

(i) Use anti-dig-peroxidase solution (Sigma, 1:250 dilution in PBS), add 20 ml or in case of a large tissue section 40 ml, enough to completely cover the tissue section.

(ii) Incubate for 2 h at 37 °C.
(iii) Wash three times with PBS.
(iv) Develop color with AEC, as described in Steps 32A(v–ix).

(D) Post-hybridization detection of fluorescently labeled probes ● TIMING 20 min

(i) Wash the slides with PBS three times, 5 min each.
(ii) Mount the slides with 50% glycerol/PBS with antifade reagent.
(iii) Observe under UV microscope with appropriate filter range.

Counterstaining and mounting ● TIMING 5–60 min (depending upon the counterstain)

33| Optional: An appropriate counterstain can be utilized for the cells that are not carrying the amplified signals. This is a very critical step and one has to work out the fine detail of the counterstains before actually applying on the slides that were used for the earlier protocols.

▲ **CRITICAL STEP** In order to use the counterstain, one has to keep the following basic rules in mind: (i) Do not use the counterstain that has the same color as the end products after hybridization will provide (e.g., biotin–strepavidin–peroxidase gives reddish color; therefore, a red counterstain will nullify the result, and (ii) the timing of counterstain for each particular tissues needs to be worked out in the same kind of tissues treated the same fashion, ahead of time. Unstained tissue that has not gone through the same heating and chemical treatments may not behave the same way. Therefore, it is good to have few slides that are run with the experimental slides and then counterstained for the staining-time optimization before the experimental slides are counterstained.

34| To mount slides, rinse in several exchanges of a buffer.

35| Dehydrate the sections through graded ethanol series (50%, 70%, 90%, 100% vol/vol for 1 min each).

36| For permanent mounting, a water-based medium such as CrystalMount or GelMount or an organic solvent-based medium such as Permount (Fisher Scientific) can be used.

37| Apply one drop of mounting medium per each 22 mm to coverslip area.

38| The slides may be viewed immediately, if you are careful not to disrupt the coverslip. The mounting medium will dry after sitting overnight at room temperature.

? TROUBLESHOOTING

● TIMING

Steps 1–5, AES silanization: 10 min procedure plus 24 h incubation
Step 6, preparation of slides from tissue/cell samples: 2 h to a week
Step 7, heat treatment: 30 min
Step 8, removal of endogenous peroxidases/phosphatases: ~30 min
Step 9, fixation: 5 h
Steps 10–14, washes: 1 h
Steps 15–21, proteinase K treatment: 1 h
Steps 22–26, *In situ* PCR amplification of DNA and RNA targets
(A) and (B): ~5 and 6 h, respectively
Steps 27–31, probe hybridization: overnight
Step 32, post-hybridization colorimetric detection of probes
(A) Post-hybridization peroxidase-based color development: 2 h
(B) Post-hybridization alkaline-phosphatase-based color development: 3 h
(C) Post-hybridization detection of digoxinin-labeled probe: 2 h 30 min
(D) Post-hybridization detection of fluorescent probes: 20 min
Steps 33–38, counterstaining and mounting: up to 1 h

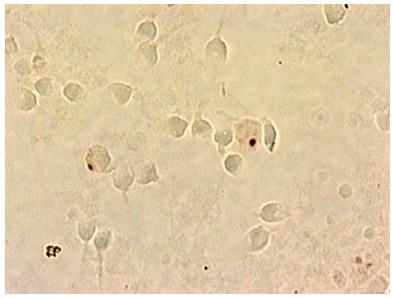

Figure 4 | Detection of human herpesvirus type 8 (HHV8) DNA in the sperms of a HIV-1 infected man by *in situ* PCR. HHV8 conserved sequences were amplified by *in situ* PCR and labeled with a biotinylated probe[16,32]. In this figure, one can easily observe the HHV8 virions in the head of the sperm, represented by red signals following peroxidase-based colorimetric probe detection.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Inconsistent (or nonexistent) *in situ* reverse transcriptase-PCR (RT-PCR) amplification	Improper RNA preservation	All reagents for RT *in situ* PCR should be prepared with RNase-free water [i.e., Diethyl pyrocarbonate (DEPC)-treated water] In addition, the silanized glass slides and all glassware should be RNase-free, which we insure by baking the glassware overnight in an oven at 250–300 °C before use in the RT procedure
	RT-PCR cocktail degradation	Work on ice to minimize the formation of nonspecific first-strand products
	Not enough template	Either true negative or lost mRNA; repeat experiment with new sample
	Primer annealing problems	Check primers for quality and appropriate design (see EXPERIMENTAL DESIGN for further details). Use software to check for secondary structure. For high G/C content templates, increase denaturation temperature to 98 °C. Incubate slides at 96 °C before cycling
	Amplicon size too large	Design the amplicon to be $<$350 bp
	Thermal cycler problems	Make sure that the heating is efficient and reaching the slides. Use a thermometer at the peak 92 °C stage and insert the probe of the thermometer to verify heat
	Too little digestion with proteinase K	In our laboratory, proper digestion parameters vary considerably with tissue type. Typically, lymphocytes will require 5–10 min at 25 °C or room temperature, CNS tissue will require 12–18 min at room temperature, and paraffin-fixed tissue will require between 15 and 30 min at room temperature (these times can be accelerated by using higher temperatures of incubation, up to 55 °C). However, the periods can vary widely, and one has to optimize the conditions by carrying out careful reactions with control cells
Poor morphology	Excessive digestion	In our laboratory, proper digestion parameters vary considerably with tissue type. Typically, lymphocytes will require 5–10 min at 25 °C or room temperature, CNS tissue will require 12–18 min at room temperature, and paraffin-fixed tissue will require between 15 and 30 min at room temperature (these times can be accelerated by using higher temperatures of incubation, up to 55 °C). However, the periods can vary widely and one has to optimize the conditions by carrying out careful reactions with control cells
High background False positive cells Signal leakage Membranes lost integrity		
Nonspecific amplicons	Annealing temperature variations	Optimize the annealing temperatures in solution-based reactions first Use 'touchdown' protocol where the annealing temperature is initially set rather high, but it ratchets down by $\sim$0.5 °C with each subsequent annealing step for the first 10–20 cycles. The idea here is to first create a number of high-fidelity amplicons, which get geometrically amplified in the subsequent cycles—in other words, one increases the signal-to-noise ratio for better results, even though the final annealing temperature might be substantially below the optimum
Poor morphology	Freezing technique	Under no circumstance attempt to freeze the tissue by merely placing it into a $-$70 °C freezer. This will result in an abundance of ice crystals, and the sections will not be suitable for *in situ* procedures It is necessary to use as thin a section as possible, down to 6 μl in thickness. Difficulties may be experienced in slicing sections thinly if the tissue is insufficiently frozen. This is remedied by the use of pathologist's freezing spray—merely blast the central area with a few quick bursts of spray, wait a few moments and proceed. If the tissue will not slice at all, it could be that the tissue is too solidly frozen. To remedy this problem, allow the tissue to equilibrate overnight at $-$70 °C while mounted on the disk in the cryostat

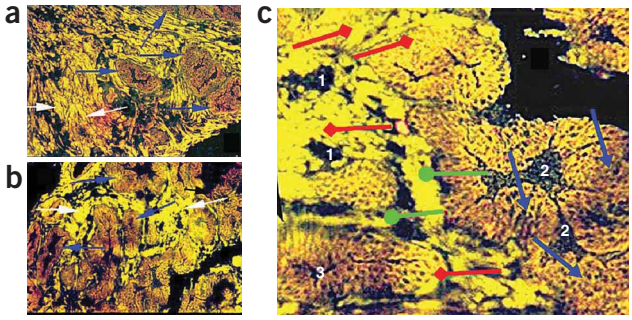

Figure 5 | *In situ* reverse transcriptase-PCR (RT-PCR) detection of hZIP1 (human zinc transporter 1) mRNA in normal and malignant prostate sections. After *in situ* RT-PCR amplification, the amplicons were hybrizided with fluorescein isothiocyanate (FITC)-labeled probe that gives a green color in the positive areas and reddish color in the negative areas. (**a**, **b**) Sections from two prostate cancer subjects at low magnification. White arrows point to acini with normal glandular epithelium that exhibit hZIP1 mRNA. Blue arrows point to adenocarcinomatous glands in which hZIP1 expression is not demonstrable or downregulated. (**c**) A higher magnification view of a section from a cancer patient to show more detail. Red arrows point to acini with normal glandular epithelium, which exhibits hZIP1 expression. These normal acini marked '1' show uniform hZIP1 mRNA expression in the glandular epithelium. Blue arrows point to malignant glands. The malignant epithelial cells exhibit significant downregulation of detectable hZIP1 mRNA in the glandular epithelium. Advanced adenocarcinomatous glands are marked as '2' and show uniform absence of hZIP1 mRNA, while developing early-stage adenocarcinomatous glands, marked '3', show a progression of normal ZIP1 expressing cells and malignant cells that have lost the expression of ZIP1. Green arrows point to stromal (fibromuscular) tissue with no hZIP1 expression.

ANTICIPATED RESULTS

One can anticipate exciting results after a successful *in situ* PCR experiment. However, the first and foremost thing to is to check the controls. If the negative controls give a positive signal then the positive control needs to be compared side by side with the negative control to determine the background activity. If the positive signals in the positive controls are several-fold stronger than the negative control then the weak signals should be considered background signals. Many cell types carry a strong peroxidase activity, and proper precautions should be taken to inactivate the endogenous chemical or enzymatic activities. There are numerous protocols are available to inactivate endogenous activity of a particular specimen[31]. The commercial post-hybridization kits also generally supply the reagents to inactivate the endogenous agents for optimal detection of a signal.

Figure 4 illustrates the use of *in situ* PCR to verify the presence infective viral DNA. In this figure, one can easily observe the human herpes virus type 8 (HHV-8) virions in the head of the sperm, represented by red signals. Of course, we have negative cell lines without any signals and chronically infected cell lines with HHV-8 to serve as positive controls (data not shown, see refs. 16,32). Most of the times, however, the results obtained from an *in situ* RT-PCR may require a series of interpretations and may not be matter of a simple positive or negative signals. **Figure 5** illustrates this point. Here we are attempting to correlate a complex inter-relation between the amount of zinc in the prostate tissues from individuals who have malignant foci and the degree of expression of zinc transporter 1 (ZIP1) with the degree of malignancy (normal, to premalignant or malignant). These two examples represent the two extreme ranges of interpretations that one has to become familiar with when carrying out *in situ* PCR.

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