Abstract

The polymerase chain reaction (PCR) has revolutionized the manner in which molecular biologists are able to examine nucleic acids by offering an extremely sensitive mechanism for amplifying specific target sequences. The combination of increased sensitivity owing to the amplification process and increased specificity owing to primer sequences with subsequent analyses by DNA sequencing, restriction enzyme digestion, or probe hybridization has provided the most powerful molecular tool thus far for both the research and the clinical laboratory (1,2).

1 Introduction

The polymerase chain reaction (PCR) has revolutionized the manner in which molecular biologists are able to examine nucleic acids by offering an extremely sensitive mechanism for amplifying specific target sequences. The combination of increased sensitivity owing to the amplification process and increased specificity owing to primer sequences with subsequent analyses by DNA sequencing, restriction enzyme digestion, or probe hybridization has provided the most powerful molecular tool thus far for both the research and the clinical laboratory (1,2).

The majority of molecular analyses focus on the isolation and purification of nucleic acids from the parent tissues, excluding the possibility of associating the presence of genetic sequences with a specific cell type or region of tissue. In situ hybridization (ISH) protocols have been developed for the detection of nucleic acid sequences within whole cells and/or tissue sections utilizing probe technology. A major disadvantage of ISH is that oftentimes the target of interest may be a single-copy gene or low-copy viral sequence whose detection is below the limit of ISH sensitivity. An extremely powerful technique results from the combination of both in situ PCR (ISP) and ISH. The coupling of these two techniques combines one methodology with extreme sensitivity and specificity (PCR) with the ability to determine cellular localization (ISH) (3–7). Single-copy DNA target sequences that were previously too scarce to detect by ISH can now be amplified so that they are more easily detected. As expected, numerous investigators have described protocols for utilization of these two techniques in the detection of infectious
organisms in tissue samples (3–13), and nucleic acid sequences in cell cytospin preparations (10) and formalin-fixed paraffin-embedded tissues (11–15).

To date, the majority of ISP applications are confined to the detection of foreign nucleic acid sequences with respect to foreign organisms of infectious origins. Although publications do exist that describe the use of ISP for detection of genetic alterations, this application has not yet been fully exploited. Meanwhile, in situ RT-PCR has been well described by Nuovo (5–7) and others. This technology at first may seem limited by the fact that the source of target nucleic acid sequences is whole tissues. However, the applications at this level of cellular architecture are limited only by one’s imagination. As the number of gene therapy protocols continues to increase, so too will the need for determining not only which cells have received the newly introduced gene, but also which of these cells is expressing this gene. This type of information can be provided accurately only by ISP technologies. As our understanding of some infectious diseases has increased owing to information obtained by ISP, gene therapy trials will also benefit from our ability to localize a new gene and/or gene product within specific cell types.

Over the past four years, more than 300 manuscripts and at least two books have been devoted to the development and application of ISP for both research and clinical investigations. Four major steps are involved in the ISP process:

1. 1.
   Tissue preparation;

2. 2.
   Cell permeabilization;

3. 3.
   In situ amplification; and

4. 4.
   Detection.
Like many, if not all other procedures, each one of these steps must be optimized for the application being pursued. Although tedious and time-consuming, initial optimization can save a great amount of time, effort, and frustration.

2 Materials

2.1 Tissues

1. Formalin-fixed paraffin-embedded tissue: This protocol is designed for routinely fixed (10% buffered formalin) tissues from the Anatomic Pathology Laboratory.

2. Tissue sections: routine, 5–6 µm thickness.

3. Organosilane-coated glass slides (Fisher Scientific, Pittsburgh, PA)

2.2 Cell Permeabilization

1. Xylene (Brand New Labs, New Haven, CT).

2. Proteinase K (Promega, Madison, WI).

2.3 LISA
Deoxynucleoside triphosphates (Perkin Elmer, Foster City, CA): Each deoxynucleoside triphosphate (dNTP) is provided separately at a concentration of 10 mM. A working solution of all four dNTPs is made up to a concentration of 1.25 mM each.

2. AmpliTaq DNA polymerase (Perkin Elmer).

3. 10X Taq buffer (Perkin Elmer): 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 0.01% w/v gelatin.

4. 25 mM MgCl₂ (Perkin Elmer).

5. Forward and reverse oligonucleotide primer.

6. Deionized water.

7. Digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN; Genius 1 Kit).
Tissue-culture cloning rings (Bellco Glass Inc., Vineland, NJ) (0.8-cm outer diameter, 0.8-cm inner diameter, 1.0-cm height).


10. Mineral oil (Sigma Chemical, St. Louis, MO).

11. Thermal cycler (Conditions are given for the COY TempCycler II, Grass Lake, MI).

2.4 Detection

1. Acetone (Sigma).

2. Wash buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) (Boehringer Mannheim).

3. Blocking buffer (0.5% blocking reagent in wash buffer 1) (Boehringer Mannheim).
Humidified chamber

5. 5.

Antidigoxigenin antibody (alkaline phosphatase-conjugated antidigoxigenin, (Boehringer Mannheim).

6. 6.

Wash buffer 2: 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5 (Boehringer Mannheim).

7. 7.

Chromogen: nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim).

8. 8.

Stop buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

9. 9.

Eosin stain (Sigma).

3 Methods

3.1 Tissue Preparation

Paraffin-embedded tissue sections fixed in 10% buffered formalin are prepared by the histology laboratory using routine protocol (see Note 1). Sections of 6-μm thickness should be placed onto organosilane-coated glass slides.
3.2 Cell Permeabilization

1. Deparaffinize tissue sections, and rehydrate by sequentially placing the slides into Coplin jars containing the following solutions for the given time: xylene, 10 min; xylene, 10 min; 100% ethanol, 5 min; 100% ethanol, 5 min; 95% ethanol, 2 min; 70% ethanol, 2 min; 50% ethanol, 2 min; and phosphate-buffered saline, pH 7.6, 5 min.

2. Treat tissues with proteinase K (1 µg/mL) for 10 min in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA at 37°C (see Note 2).

3. Dehydrate tissues in a series of graded alcohols (i.e., 70, 95, and 100% for 2 min each).

3.3 LISA

1. Place tissue sections, prepared as described in Section 3.2., onto the slide thermocycler (Tempycler II) with heating blocks designed to accommodate glass microscopic slides (Fig. 1A,B).

2. Heat slides for 1 min at 94°C to inactivate the proteinase K prior to the addition of glass tissue-culture cloning rings. Place these selectively on top of the tissue section at room temperature (see Note 3).

3. 
Seal the bottom of the ring to the tissue section with clear nail polish (approx 50 µL), forming an amplification vessel for the LISA reaction. The nail polish should be allowed to air-dry before proceeding (see Note 4) (Fig. 2).

4. The amplification reaction consists of a total volume of 25 µL/cloning ring and includes 75 ng of both the forward and reverse primer, 0.5 U AmpliTag polymerase in a reaction solution containing per liter: 200 mM of each deoxynucleotide triphosphate, 1.5 mM MgCl₂, 67 mM Tris-HCl, pH 8.8, 10 mM 2-mercapto-ethanol, 16.6 mM ammonium sulfate, 6.7 mM EDTA, and the addition of 10 mM digoxigenin-11-dUTP (see Note 5). The reaction mixture is added to the center of the cloning ring and mineral oil is overlaid to prevent evaporation (see Notes 6 and 7).

5. Place the slides onto the slide thermocycler and denature DNA in situ at 94°C for 2 min prior to amplification (see Note 8).

6. LISA is accomplished by using 20 cycles consisting of a 1-min primer annealing step (55°C), 1.5-min extension step (72°C), and a 1-min denaturation step (94°C).
The COY TempCycler II (A) was one of the first specialized instruments for ISP applications. The specially designed heating block (B) can accommodate up to four glass slides.

Fig. 2.

Application of cloning rings to tissue section on glass slide. The ability to place more than one ring on each section allows for the simultaneous performance of multiple reactions.

3.4 Detection

1. 1.

Detect amplified products containing incorporated digoxigenin-11-dUTP using a modification of the protocol supplied with the Genius 1 kit.

2. 2.

Remove each reaction mixture by pipeting, and rinse the wells with xylene. All solutions and reactions should be at room temperature.

3. 3.

Remove the cloning rings from the glass slides by soaking in acetone for 1–2 min (see Note 9).

4. 4.
Wash the slides three times with approx 500 µL buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), and then incubate for 30 min with 500 µL buffer 2 (0.5% blocking reagent in buffer 1) in a humidified chamber (see Note 10).

5. Rinse the slides with 500 µL buffer 1, and incubate for 1 h with a 1:100 dilution of antibody (alkaline phosphatase-conjugated anti-digoxigenin) in a humidified chamber.

6. Excess antibody is removed by three washes in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) before the addition of the chromogen (nitroblue tetrazolium chloride and 5-bromo-4-chloro3-indolyl phosphate).

7. Monitor development of the detection reaction for optimal staining (approx 10–25 min), and stop by rinsing three times in buffer 4 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (see Note 11).

8. Dehydrate the tissues in a series of graded alcohols and counterstain with eosin before coverslipping for microscopy.

4 Notes

1. Traditionally 10% buffered formalin has been the fixative of choice. However, others have used frozen tissues as well as alcohol or heat-fixed tissues with limited success. Unbuffered formalin and fixatives containing picric acid or heavy metals are not recommended.
2. Protease digestion is necessary to permeate tissue samples. Fixation crosslinks proteins and nucleotides, thus hindering reagent penetration and target sequence availability. The concentration of enzyme, duration of digestion, and temperature are all critical. Excessive digestion will destroy tissue morphology, whereas under digestion will result in poor permeability, decreased amplification, and increased background.

3. It is imperative that the protease be inactivated at this step before proceeding.

4. Clear nail polish is relatively inexpensive. Colored nail polishes of greater or lesser value are not suitable for LISA because of leaching out of color during high-temperature cycling.

5. PCR reaction mixtures can first be optimized by traditional tube amplification reactions. Some reports suggest using higher concentrations of magnesium and polymerase for ISP.

6. AmpliWax beads may also be used in place of mineral oil for “hot-start” PCR protocols. LISA lends itself quite well to such modifications in PCR procedures.

7. Proper control reactions are essential to all ISP protocols. These should consist of at least the following reactions: omit primers, omit polymerase, alternate primer set for other target sequence, and tissue without target sequence present.
8. Other thermal cyclers are also available specific for ISP from MJ Research, Inc. (Watertown, MA), Hybaid Ltd. (Teddington, England), and Perkin Elmer (Foster City, CA). Conditions will need to be optimized for the respective instrument used. ISP can also be performed on traditional thermal cyclers by covering the heating block with aluminum foil.

9. Cloning rings do not need to be removed at this point. They can be left in place throughout the detection process. However, doing so results in loss of tissue outside the ring acting as an internal negative control for the detection process.

10. Humidified chambers are easily constructed out of plastic containers with lids. Tape damp gauze bandage pads to the inner portion of the lid after slides are placed into the container. Cover with lid to keep slides from drying out.

11. Detection is dependent on the amount of amplification and size of the target. This step must be monitored closely so that overstaining does not occur.

References


10. 10.


