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In Situ Hybridization: Methods and Applications

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In situ hybridization (ISH) combines molecular biological techniques with histological and cytological analysis of gene expression. RNA and DNA can be readily localized in specific cells with this method. ISH has been useful as a research tool, and recent studies have used this technique in the diagnostic pathology laboratory and in microbiology for the tissue localization in infectious agents.

Other recent developments in the applications of ISH involve in situ polymerase chain reaction (PCR) and in situ reverse transcription (RT)-PCR, which can be used to detect very low levels of nucleic acids in tissues by taking advantage of the powerful amplification capacity of PCR. In situ PCR will contribute significantly to progress in this field because of the marked increase in the sensitivity of this method. J. Clin. Lab. Anal. 11:2–9. © 1997 Wiley-Liss, Inc.

Key words: in situ hybridization; polymerase chain reaction; in situ polymerase chain reaction

INTRODUCTION

Recent developments in molecular biology are rapidly expanding many technological advances used to study disease processes. In situ hybridization (ISH) represents a unique technique in which molecular biological and histochemical techniques are combined to study gene expression in tissue sections and cytological preparations. The method involves a hybridization reaction between a labeled nucleotide probe and complementary target RNA or DNA sequences. Those hybrids can be detected either by autoradiographic emulsion for radioactively labeled probes or by histochemical chromogen development for nonisotopically labeled probes. ISH localizes gene sequences in situ and visualizes the product of gene expression while preserving cell integrity within the heterogeneous tissue, permitting anatomically meaningful interpretations.

ISH analysis of nucleic acids was first described in 1969 (1). Since then many variations of ISH methods have been developed (2–5). During the past decade, ISH has undergone many refinements and has become an important tool in basic scientific research and clinical diagnosis. Between January 1994 and July 1995, more than 4,000 articles using the technique were published. These rapid advances have made ISH analysis feasible in the clinic laboratory as a molecular diagnostic tool (6–8).

This review surveys the basic principles and approaches of ISH technique, the use of ISH in the study of specific diseases, and some new advances of ISH as an experimental tool, especially in situ polymerase chain reaction (PCR) techniques.

IN SITU HYBRIDIZATION METHODS

Principles

DNA is a double-stranded nucleic acid chain consisting of two complementary nucleic acid strands made up of four basic deoxynucleotides linked to one another by phosphodiester linkage. Each of the four nucleotides has a base, including adenine (A), cytosine (C), guanine (G), and thymidine (T). The two strands are oppositely bound with A bound to T with two hydrogen bonds and C bound to G with three hydrogen bonds. RNA is a single-strand nucleotide with the base uridine (U) substituted for T. ISH is the process by which the specific annealing of a labeled nucleic acid probe to complementary sequences in fixed tissue or cells is followed by visualization of the hybridized probe. One can have DNA–DNA, DNA–RNA, RNA–DNA, and RNA–RNA ISH, depending on various types of probes and targets concerned. The annealing and separation of the two hybrided strands depend on various factors, including temperature, salt concentrations, pH, the nature of the probes and target molecules, and the composition of the hybridization and washing solution. The "melting" temperature (T_m) of hybrids is the point at which 50% of the double-stranded nucleic acid chains are separated. The optimal temperature for hybridization is 15–25°C below the Tm. There are various formulas to calculate T_m, depend-

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ing on probe length and type of hybrids (4). For DNA–DNA hybrid with probes longer than 22 bases, the following formula can be used:

$$T_m = 81.5 + 16.5 \log (Na) + 0.41 (\%GC) - 0.62 (\%\ formamide) - 500/length\ of\ base\ pairs\ of\ probe$$

RNA–RNA hybrids are generally 10–15°C more stable than DNA–DNA or DNA–RNA, and therefore require more stringent conditions for hybridization and post-hybridization washing.

Probes

A number of different types of probes are available for ISH, including cDNA, cRNA, and synthetic oligonucleotide probes. Complementary double-stranded DNA probes generally are cleaved by restriction enzymes from the cloned DNA plasmid. Optimal probe sizes for tissue penetration is probably 200–500 base pairs (bp). These probes should be denatured by heating to 95°C before hybridization. Single-stranded DNA probes can be prepared by PCR with single antisense primer extension and Taq polymerase, which allows great flexibility in the choice of probe sequences by using appropriate primers (9). cDNA probes with nonisotopic reporters labeled by nick-translation or random primer methods are most commonly used ones in the detection of an infectious virus.

Complementary RNA probes or riboprobes are prepared by transcription in vitro using the cDNA sequences as a template. The cDNA insert is subcloned into a transcription vector and flanked by two different RNA polymerase (e.g., T7, Sp6) initiation sites, thus enabling either sense-strand or antisense-strand to be synthesized. The sense RNA probe is usually performed as a negative control. Treatment with ribonuclease A (RNAse) after hybridization with riboprobes will reduce nonspecific background signal, since the enzyme digests single-stranded, but not double-stranded, RNA hybrids. cRNA probes are most sensitive since they can be labeled to high specific activity and used for detection of low copy numbers of gene expressions. One of the disadvantages of riboprobes is the instability of the labeled cRNA. There is usually a detectable degradation of the probes after 1 week (3).

Oligonucleotide probes consisting of a single-stranded DNA molecule ranging in size from 20 to 50 bases can be generated with an automated DNA synthesizer. The oligo-probes probably penetrate cells more readily and can produce excellent hybridization signals. With the increasing numbers of cloned genes, oligo-probes can be generated from cDNA maps in the literature or gene bank and synthesized rapidly and inexpensively. Labeling oligo-probes for ISH is commonly done with "tail" labeling with relatively few labeled nucleotides incorporated into each probe, and they are relatively less sensitive than longer cDNA or cRNA probes (3,10). So the oligo-probes are generally considered the most suitable for detection of relatively abundant gene expression such as hormone mRNAs (11–13). This disadvantage can be overcome by using a "cocktail" of multiple oligo-probes that are complementary to different regions of the target molecules (7,14). Careful selection of oligo-probes with low or no homology with other nucleotide sequences is most important to ascertain the specificity of ISH (15). The use of oligo-probes does not require expertise with many molecular biology techniques such as gene cloning or plasmid preparation.

Labeling and Signal Detection

Two major methods for labeling and detection are used: (a) radioisotope labeling, such as ^{3}H, ^{35}S, ^{125}I, ^{32}P, and recently ^{33}P, which is detected with x-ray film and/or by emulsion autoradiography; and (b) nonisotope labeling, such as biotin, digoxigenin, fluorescein, alkaline phosphatase, or bromodeoxyuridine (BrdU), which is visualized by histochemistry or immunohistochemistry (IHC) detection systems.

Radioisotope labeling is the more traditional way to perform ISH. ^{35}S-labeled probes are most commonly used, representing the most sensitive and rapid results (typical exposures of about 1 week) compared to other isotopic labeling (3). The half-life of ^{35}S is 87 days, and labeled probes should be used within 1 month or so. Generally, radioactive probes are more sensitive, and the experimental results can be readily quantitated or semi-quantitated by densitometry counting on film or by silver grain counting. Disadvantages of isotopic probes include biohazards and short half-life, as well as the fact that they are time-consuming.

Nonisotopic ISH methods are being used with increasing frequency. The advantages of oligonucleotide probes include greater stability of labeled probes, rapid results, and better resolution. These practical advantages make oligonucleotide probes very useful in the clinical laboratory. Biotin and digoxigenin are the most commonly used reporters at present because they are readily detectable in tissues. Biotinylated probes were first used in the early 1980s, and have been widely used for detection of viral or relative abundant mRNA at both the light (LM) and electron microscopic (EM) levels. The hybridization signals with biotinylated probes can be detected by addition of avidin or streptavidin conjugated to alkaline phosphatase (AP), horseradish peroxidase (HPO), fluorochromes, or colloidal gold particles. Digoxigenin-labeled probes were recently introduced and are rapidly becoming more widely used compared to biotinylated probes with higher sensitivity and less background staining (10). Because digoxigenin is not present in mammalian cells, this is a particular advantage when studying tissues such as liver or kidney, which may contain endogenous biotin, but not digoxigenin. The signals can be visualized by using an antidigoxigenin antibody fragment conjugated to AP or HPO with respective substrates that yield insoluble-colored products. Nonisotopic probes are generally considered to be less sensitive than the correspond-

ing radioactive probes, and the hybridization results are difficulties to quantify.

Tissue Preparations

Tissue processing including storage and fixation should be optimized to detect intracellular nucleic acids. ISH has been applied to cell specimens (smears, cytospins, or cell pallets) and tissue sections (frozen, paraffin, semithin, and ultrathin plastic section). Nucleic acids are better preserved in frozen tissues than in paraffin-embedded tissues (3). Intact cells are ideal for ISH, as they have less damaged nucleotide sequences. Both fresh frozen tissues (stored at -70°C) and formalin-fixed archival tissues can be used for ISH after storage for several years (3). Ideal fixation for ISH should preserve both RNA/DNA and tissue morphology. Cross-linked fixatives such as paraformaldehyde, formalin, and glutaraldehyde are most commonly used. Generally, fixation 20 min to 1 hr is sufficient for frozen section or single-cell preparation. To ensure adherence of tissue sections to the glass slides, the slides should be pretreated with a suitable coating solution, such as 3-aminopropyltrimethoxysilane or poly-L-lysine.

Pretreatment of Tissue

A series of pretreatment steps before hybridization increases the efficiency of hybridization and reduce nonspecific background staining. Some steps are routinely used in most ISH protocols.

Proteases treatment (e.g., proteinase K) is considered as one of the most important steps to increase the accessibility of the target nucleic acid, especially for nonisotopic probes with paraffin sections. The concentrations of proteinase K (1–50 μg/ml) and the length of treatment (5–30 min) depend on tissue type and length of fixation. Prolonged incubation results in overdigestion resulting in loss of signal and morphologic integrity. A brief post-fixation in paraformaldehyde is commonly recommended.

Acetylation of sections using 0.25% acetic anhydride/0.1 M triethanolamine can reduce charged probes binding to tissues. Some investigators have noted that acetic anhydride prevents the nonspecific binding of unrelated digoxigenin-labeled probes to neuroendocrine cells (13). Adding fresh dithiothreitol (DTT) in the hybridization solution containing ^{35}S-labeled probes can protect the sulfur from oxidation. The background artifacts with ^{35}S-labeled probes can be dramatically reduced by increasing the DTT concentration (16). Incubation of section with prehybridization buffer, normally containing 50% formamide, is generally considered useful for maintenance of the hybridization stringency, even though some investigators prefer to perform ISH in a formamide-free condition (17,18). The presence of endogenous biotin or AP should be anticipated in some tissues when using the nonisotopic probes. Endogenous AP can be inhibited by treatment of sections with 0.2 N HCl and levamisole and endogenous biotin by biotin-blocking agents.

Control for ISH

ISH is essentially a histochemical method, raising the fundamental histochemical concerns of specificity controls of the signals. A variety of controls can be used: (a) pretreatment of tissues with RNAse or DNAse, depending on the target being tested; (b) omission of the specific probes in hybridization reaction; (c) using an unrelated or sense probe; (d) competitive studies with unlabeled probes before adding labeled probes for hybridization; (e) Northern or Southern hybridization to characterize the nuclei acid species of the hybrids; and (f) combining ISH with immunostaining to localize the translated protein product in the same cells.

It is important to assess the integrity of target RNA during ISH. Loss of RNA or failure of probes to detect target RNA may result in false-negative results, particularly for retrospective studies of paraffin-embedded tissues. To evaluate preservation of mRNA, control probes that are used include β-actin (19); poly(dT) (8), and more recently ribosomal RNA (20).

APPLICATION OF IN SITU HYBRIDIZATION

ISH methods have found many applications in clinical research and diagnostic pathology.

Infectious Diseases

Many DNA probes are available for the identification of foreign genes, including bacteria, fungi, and viruses in tissue sections. Detection of those infectious agents with sensitive non-isotopic ISH methods provides the valuable information about the etiology of the infectious disease, since many of the infectious agents can be readily visualized by ISH methods. Detection of human immunodeficiency virus (HIV), cytomegalovirus (CMV), human papillomavirus (HPV), herpes simplex virus (HSV), hepatitis B virus (HBV), Epstein-Barr virus (EBV), and JC viruses are just some of the applications for ISH methods. More than 50 types of HPV and probes specific for the various types are available to assess the infection by particular HPV types associated with neoplastic development (21). Detection of HBV DNA by ISH is superior to IHC detection for HBsAg with a higher degree of sensitivity and more reliability (22). ISH with nonisotopic-labeled oligonucleotide or riboprobes to detect EBV encoded small RNAs (EBERs) has been used extensively in archival surgical and autopsy specimens recently (23,24). EBV infections are found to be associated with a broad range of diseases, including Burkitt's lymphoma, Hodgkin's disease, post-organ transplantation, lymphoproliferative disorders, nasopharyngeal carcinoma, and gastric carcinoma (24,25). Certain tumors, such as lymphoepithelium, are frequently associated with EBV and may be of value in confirming the histologic

diagnosis. Ribosomal RNA (rRNA) is another target that can be used to characterize infectious agents for ISH. Montone and Litzky (26) recently reported a rapid ISH procedure that could specifically detect *Aspergillus* infection on paraffin section by using biotinylated oligo-probe for species specific regions of rRNA sequences.

Cytogenetics

ISH has now developed into a practical technique in cytogenetics and molecular genetics. Fluorescence in situ hybridization (FISH) is most commonly used in this field (4,27). Probes are directly fluorescein labeled or labeled with biotin or digoxigenin, followed by a second signal detection coupled with fluorochrome. The primary applications of FISH in cytogenetic analysis include chromosomal gene mapping, characterization of genetic aberrations, identification of genetic abnormalities associated with genetic disease or neoplasmic disorders, and detection of viral genomes in interphase nuclei or metaphase chromosomes. Synchronization of cell cultures by BrdU or thymidine is used to increase the frequency of chromosomes at the different stages of condensation (metaphase, prometaphase, or prophase) for ISH analysis (4).

The hybridization signals (fluorescent spots) can be visualized by fluorescent microscopy. FISH using probes with nonisotopic labeling has successfully localized single copy gene sequences (4,27).

Gene Expression

ISH methods are powerful tools for the analysis of gene expression and regulation in normal and pathologic tissues (6,27–30). A major advantage of ISH is its ability to localize mRNA at the cellular level in heterogeneous tissues, thus expanding the results of other molecular techniques such as Northern blot hybridization for specific gene analysis. ISH methods have been of particular values for the study of mRNA encoding oncogenes, growth factors and their receptors, hormones and hormone receptors, cytokines, structural proteins, enzymes, collagenase, and others.

There are many practical applications of ISH methods in tumor pathology. The correlation of oncogene expression with prognosis is being investigated in neuroblastomas and epithelial neoplasms such as colon, lung, prostate, and breast carcinomas. Detection of genes encoding cell structural proteins, including tumor-associated markers, represent potential areas of application of ISH methods in pathologic diagnosis. For example, nonisotopic ISH methods for localization of immunoglobulin light chain in mRNAs in hyperplastic and neoplastic lymphoproliferative disorders (14), albumin mRNA to distinguish between hepatocellular and metastatic carcinomas in the liver, chromogranin/secretogranin mRNAs in the classification of neuroendocrine tumors (7) are used in some diagnostic pathology laboratories (Figs. 1–4). ISH methods used to identify cells or tumors on the basis of their specific mRNA content are different from IHC, which is dependent on the protein content of cells. Thus, ISH identifies the gene products from de novo synthesis, rather than nonspecific uptake of proteins by cells, which may result in false-positive immunostaining results. ISH analysis has also been extensively used in studies of endocrine tumors. For example, a significant number of small cell lung carcinomas with few secretory granules are commonly negative for chromogranin proteins, but the mRNAs may be detected by ISH methods (7,12). Studies of gene expression in endocrine tumors, including chromogranin A, thyroglobulin, estrogen receptor proteins, parathyroid hormones, and calcitonin gene-related peptide by ISH, have contributed to our understanding of the biology and pathophysiology of various endocrine disorders (26,28).

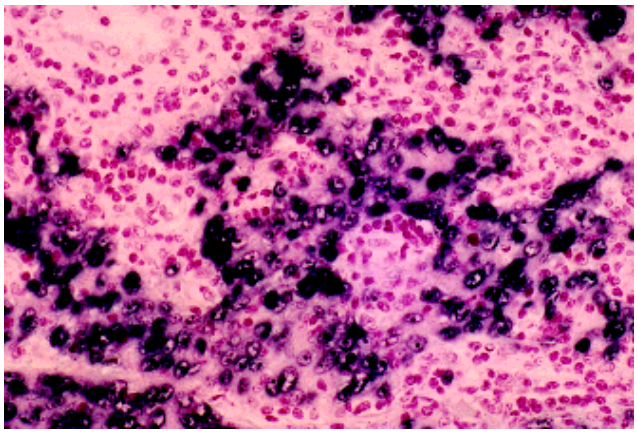

Fig. 1. Detection of Epstein-Barr virus by in situ hybridization with a fluorescein-labeled EBER (Epstein-Barr early RNA) probe cocktail. ×300.

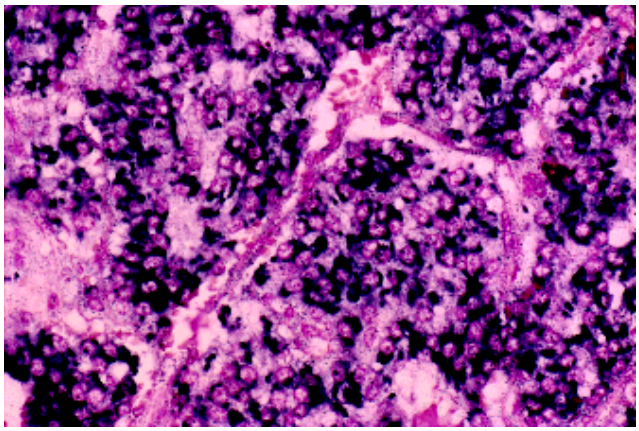

Fig. 2. Localization of chromogranin A + B in a neuroendocrine tumor by in situ hybridization with a digoxigenin-labeled probe cocktail. ×300.

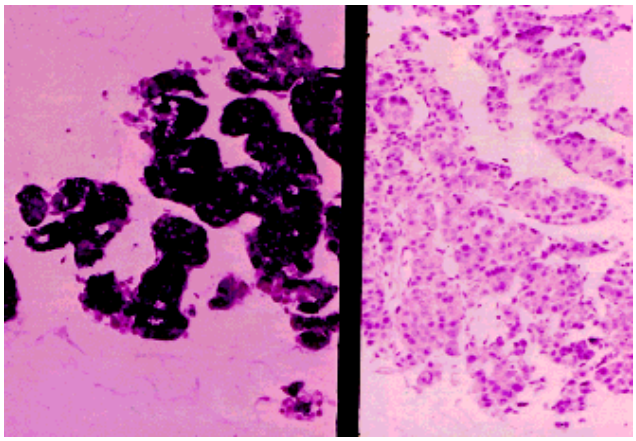

Fig. 3. In situ hybridization localizing albumin mRNA in a hepatocellular carcinoma with a digoxigenin-labeled oligonucleotide probe cocktail *(left)*. Pretreatment with ribonuclease A before hybridization *(right)* eliminates the positive hybridization signal. ×200.

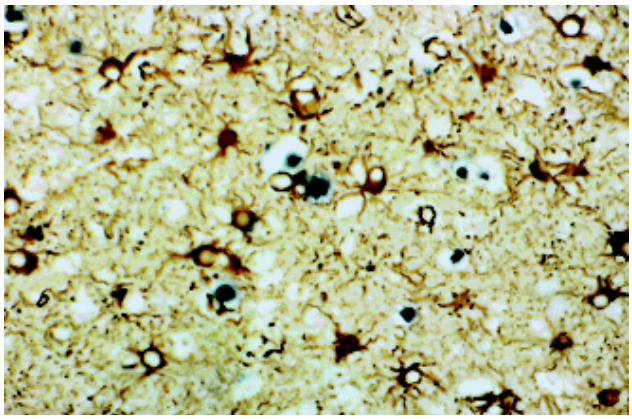

Fig. 4. Detection of JC virus in a brain biopsy of a patient with progressive multifocal leukoencephalopathy by in situ hybridization with a digoxigen-labeled cDNA probe (blue). Double staining with glial fibrillary acidic protein illustrates the glial cells (brown). ×150.

RECENT ADVANCES IN ISH

A variety of new ISH techniques have been developed over the past few years. These advances can be divided two categories: (a) combination use of various ISH approaches for simultaneous detection of multiple target nucleic acids at the LM and EM levels; and (b) increasing the sensitivity of ISH signals by amplification of detecting systems (31) or by amplification of the DNA or RNA targets with in situ polymerase chain reaction (in situ PCR).

Double ISH Localization

Using double-labeling ISH methods involves the simultaneous detection of multiple target nucleic acids such as two mRNAs, DNA, and mRNA, or nucleic acids and proteins if combination ISH with IHC techniques (2,32–36). In performing double ISH, the two probes may be hybridized simultaneously, and the signals are detected either simultaneously or sequentially. For combined ISH/IHC, ISH is generally performed before IHC because it reduces the chances of RNAse contamination. A variety of combination of ISH methods have been reported, mainly including the following.

Combination of nonradioactive and radioactive ISH methods which are mainly used for detection of two mRNAs in the same tissue sections (16,33,34). After histochemical detection for nonisotopic signals, the slides are subject to an autoradiographic approach for radioactive signals. Some investigators have reported that ILford emuslion, rather than Kodak NTB_2 emulsion, can reduce silver grain background by chemographic artifact when simultaneous uses of digoxigenin and ^{35}S-labeled oligonucleotide probes for double ISH detection are performed (35).

Several investigators have successfully applied the techniques of using a combination of two non-isotopic labeled probes, mainly biotin and digoxigenin, conjugated to different enzymes (AP or HPO) or fluorescence followed by respective detective systems to a simultaneous localization of multiple mRNA and genomic DNA at same tissue sections, even same cells (2,27,36). Using probes labeled with different fluorochrome molecules and with different excitation and emission characteristics allows simultaneous analysis of different probe signals on chromosomal and cytogenetic preparations (4,27). An alteration is combination of nonisotopic ISH with FISH, in which the two signals can be visualized by using bright-field and dark-field microscopy (36).

Electron microscopic ISH is mainly used for resolving problems requiring the high precision correlation of molecular function and ultrastructural morphology. Nonradioactive ISH approaches with colloidal gold are the most commonly used for pre- and/or post-embedding EM techniques (37–39). Colloidal gold with different, nonoverlapping particle sizes (6–30 nm) greatly facilitates multiple labeling for ISH analysis at the EM level. Bienz and Egger (37) recently reported the simultaneous localization of P_1 and P_2 virus genome regions in poliovirus infected cells by double EM ISH, in which digoxigenin labeled P_1 probe and biotin labeled P_2 probe were detected through simultaneous direct immunodetection with 10 nM gold-labeled antidigoxigenin and 6 nM gold-labeled antibiotin antibodies.

In Situ PCR

In situ PCR is a relatively new molecular technique that combines the high sensitivity of PCR with the anatomical localization by ISH (40–42). PCR is an extremely sensitive technique which can amplify low copy DNA sequences to high levels but cannot correlate the results with morphological features. ISH localizes gene sequences at the cellular level, but it generally requires at least 20 copies of the mRNA of interest in a cell to make the signals visible. A combination of

PCR with ISH can amplify specific DNA or RNA sequences inside single cells and increase the copy numbers to levels readily detectable by ISH methods. Some terms used in the literature to describe the technique include "in situ PCR," "PCR in situ hybridization," and "in-cell PCR" (43). The term "in situ RT-PCR" is used for the process of detecting mRNA by in situ PCR, in which mRNA is reverse transcribed to single strands of DNA followed by PCR amplification. Since first reported in 1990 (44), in situ PCR technology has undergone rapid development and over 400 articles describing various forms of in situ PCR have been published in peer-reviewed journals.

Several in situ PCR protocols with varying modification have been published. There are two main approaches: direct and indirect. In the direct approach a labeled nucleotide is corporated into the PCR products (45), whereas in the indirect method, ISH can be performed after in situ amplification using labeled oligonucleotide probe. These techniques have been successfully performed on intact cells in suspension, smear or cytospin preparation, metaphase chromosomes, and frozen and paraffin sections (40–53).

For tissue pretreatment, the use of 4% paraformaldehyde and 10% buffered formalin fixation for 1–6 hr is most widely reported. Paraformaldehyde has been shown to be the best all around fixation. One of the most important steps is to digest the tissue sample with a protease such as proteinase K, allowing reagent penetration and unveiling of the target sequence. The strength of digestion (concentration, duration, and temperature) should correlate with the length of fixation and tissue types. Too little or excessive digestion may result in unsatisfactory amplification, leading to false negative, high background or morphology distortion (40). After proper fixation and digestion, the cells with semipermeable membranes permit the PCR reagents to enter the cells more readily and to retard the diffusion of the amplified products.

PCR amplification is carried out in the presence of primers, nucleotides, Taq DNA polymerase, etc. Generally, 20–30 PCR cycles are adequate for in situ amplification. The oligonucleotide primers (sense and antisense) are usually 18–24 bases long, and the amplified fragments 100–800 bp. It is important to design the primers with unique sequences. Ideally, primers that bridge introns to distinguish template source should be used. The multiple primer pairs used in some approaches may generate longer and/or overlapping products, and therefore less diffusible (47). If direct in situ PCR is performed, one of four deoxynucleotides should be labeled (e.g., digoxigenin-dUTP) at a certain ratio.

For in situ RT-PCR, the reverse transcription step is performed before PCR. There are three types of primers for RT, including antisense, oligo-dT, and random primers (46). Antisense primers are commonly used. However, random primers may be more efficient for tissue sections in which damaged nucleic acids with broken fragments may be more easily bound by random primers. After amplification, a postfixation step with 4% paraformaldehyde effectively immobilize the amplified products.

Nonradioactive detection is generally employed for both direct and indirect in situ PCR products. The amplified signal of indirect in situ PCR can be detected by ISH using a oligonucleotide probe which recognizes sequences internal to the amplification primers. The design of specific probes is very important because PCR may amplify more than one sequence and a specific probe will hybridize only the correct one. The specificity of probe may be provided by conventional PCR followed by southern blot hybridization. For direct in situ PCR, the amplified product can be detected directly by IHC or enzyme/chromogen reaction with no need for subsequent ISH detection. However, despite its speed and relative simplicity, there has been concern about the specificity of the reaction (47). Nonspecific incorporation of labeled nucleotides into damaged DNA and misprimings or DNA repair artifacts may occur, resulting in false positive results, especially on tissue section (42).

The number of controls needed to interpret the results depends on the approaches of in situ PCR employed: (a) omission of certain key reagents such as primers, RT enzymes, Taq-polymerases, and probe, to check the specificity of each step; (b) pretreatment of tissues with DNAse or RNAse to destroy the targets before being subjected to in situ PCR; (c) artifactual mixtures of cells to be tested with another cell type known not to contain target sequences at different ratios, providing a control for cell preparations (47–50); (d) gel electrophoresis and Southern blot hybridization of amplified products from supernatants or tissue extracts after in situ PCR (46,47,50); and (e) use of samples that do not express the gene of interest in each experiment. The false-negative or false-positive results may be caused by mispriming, DNA-repair artifacts, RNAse contamination, inappropriate fixation or proteinase digestion, insufficient DNAse digestion, and amplified product diffusion (42,46,47). It is not possible to interpret in situ PCR results without adequate controls.

Over the past few years, in situ PCR has been mainly applied to detect DNA sequences that are not easily detected by conventional ISH. The employment of in situ PCR to detect low copy number of viral genes, especially HIV and hepatitis C, has led to significant discoveries about viral infectious diseases (2,41,48). Ribbo et al. recently reported that surfactant protein A mRNA analysis by in situ PCR can help define the origin of a primary lung adenocarcinoma (49). Application of the in situ RT-PCR to detect gene expression is still limited primarily to cell preparation and frozen section (Figs. 5, 6). Only very few applications in paraffin sections have been reported (46). Successful amplification of mRNA by in situ RT-PCR includes hormone, receptor, oncogenes, etc. (46,50–52). By direct comparison of in situ RT-PCR with radioactive or nonradioactive conventional ISH for mRNA analysis there are 2- to 20-fold increases in hybridization signals (50,51). In situ RT-PCR has enormous future potential application, since

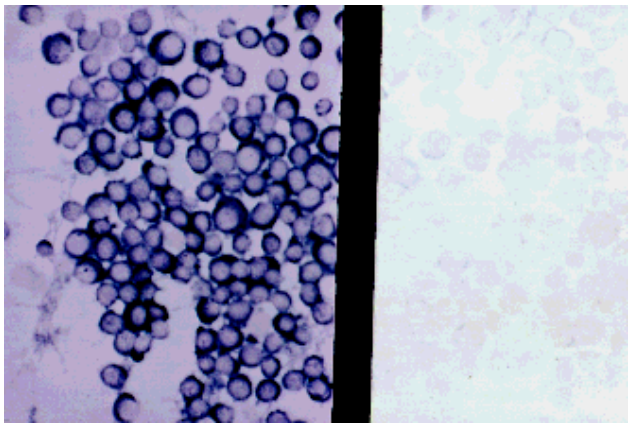

Fig. 5. Comparison of in situ RT-PCR *(left)* with in situ hybridization *(right)* to localize prolactin mRNA in cultured GH_3 pituitary tumor cells. There is greater than a 10-fold increase in the detection of PRL mRNA by in situ RT-PCR. ×200.

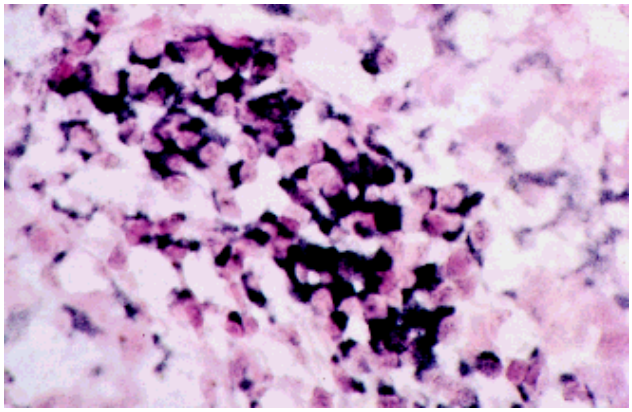

Fig. 6. Localization of epidermal growth factor receptor mRNA in frozen section by in situ RT-PCR in a poorly differentiated squamous cell carcinoma of the lung. The blue cytoplasmic staining indicates a positive reaction product. ×250.

many quiescent or developing genes are known to produce small amounts of mRNA and their roles have not been elucidated. Application of in situ PCR in EM and combination of in situ PCR with IHC were also described (50,53).

In situ PCR is rapidly a developing molecular technique and promises to be a powerful technique to detect low copy numbers of specific genes in the near future.

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