Diagnostic Molecular Pathology 12(1): 1–13, 2003

Recent Developments in Signal Amplification Methods for In Situ Hybridization

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In situ hybridization (ISH) allows for the histologic and cytologic localization of DNA and RNA targets. However, the application of ISH techniques can be limited by their inability to detect targets with low copies of DNA and RNA. During the last few years, several strategies have been developed to improve the sensitivity of ISH by amplification of either target nucleic acid sequences prior to ISH or signal detection after the hybridization is completed. Current approaches involving target amplification (in situ PCR, primed labeling, self-sustained sequence replication), signal amplification (tyramide signal amplification, branched DNA amplification), and probe amplification (padlock probes and rolling circle amplification) are reviewed with emphasis on their applications to bright field microscopy. More recent developments such as molecular beacons and in situ strand displacement amplification continue to increase the sensitivity of in situ hybridization methods. Application of some of these techniques has extended the utility of ISH in diagnostic pathology and in research because of the ability to detect targets with low copy numbers of DNA and RNA.
Key Words: In situ hybridization—Signal amplification—Tyramine—In situ PCR.

Diagn Mol Pathol 12(1): 1–13, 2003.

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In situ hybridization (ISH) has proven to be a very important molecular tool in research and diagnosis and has greatly advanced the study of gene structure and expression at the level of individual cells in complex tissues. ISH has contributed substantially to the diagnosis and understanding of viral and neoplastic diseases and has provided invaluable insights into hormone regulation, storage, and secretion (1–9). In addition to morphologic identification of gene targets within cells, ISH also allows for quantification of observations, for example with respect to tumor burden or viral load. However, its applicability can be limited by its relatively low sensitivity, especially with nonradioactive reporters (7–12).

Since the introduction of ISH by Pardue and Gall in 1969, there have been many advances in ISH methodology (1). Although the use of radioisotopic labels has long served as a gold standard for ISH, the use of nonisotopic probes has eliminated some of the problems inherent with radioactive ISH, such as long turn-around times, risk from exposure to radioactivity, and radioactive waste disposal. Moreover, the sensitivity of ISH has been greatly improved by the application of PCR-based target amplification and catalyzed signal amplification (CSA or CARD) (8–29). During the last few years, several strategies have been developed to improve the threshold levels for ISH detection by amplification of either target nucleic acid sequences prior to ISH or the detection signals after the hybridization is completed. The ultimate mRNA detection limit is more difficult to determine but may reach the level of rare mRNA molecules in the most optimal test systems (8–10,30,31). For routine detection of nucleic acid sequences in tissue sections, however, these sensitivities may not be reached due to factors such as accessibility and loss of target nucleic acids. As a consequence, ISH detection limits on tissue sections are rather in the range of 40 KB of target DNA and 10 to 20 copies of mRNA or viral DNA per cell. Several strategies have been developed to amplify the nucleic acid targets (target amplification) or immunocytochemical detection signals (signal enhancement) in situ hybridization (Table 1). In addition, increased absolute amounts of

TABLE 1. *Recent approaches used to increase the sensitivity of ISH*

	Advantages	Disadvantages
Target sequence amplification		
In situ polymerase chain reaction (in situ PCR)	Very sensitive	Difficulty to quantify
In situ reverse transcriptase PCR (RT-PCR)	Very sensitive	Difficulty to quantify
In situ self-sustained sequence replication (IS-3SR)	Sensitive	Not used on paraffin
Primed in situ labeling (PRINS)	Relatively simple	False positive
In situ transcription		
Signal amplification		
Catalyzed reporter deposition (CARD)	Rapid, sensitive	Endogenous biotin
Enzyme polymer system		
Branched DNA methods	Quantitative, biotin not used	Lower sensitivity
Probe amplification		
Padlock probes and rolling circle amplification	Sensitive	Not quantitative

hybridized probes have been used successfully to improve the ISH detection sensitivity, e.g., cocktails of oligonucleotides or multiple cRNA probes.

In this overview, we review the principles, applications, and limitations of in situ polymerase chain reaction (24–35), in situ self-sustained sequence replication (3SR) (12,36–38), primed in situ labeling (PRINS) (39–42), and in situ transcription as examples of target amplification methods and catalyzed reporter deposition (8–28) using biotinylated tyramine as an approach to signal amplification during ISH.

IN SITU HYBRIDIZATION

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 labeled nucleic acid strand (terms as probe) anneals to complementary sequences in fixed tissue or cells. The hybrid is formed not only of the two strands of DNA but also of RNA-DNA and RNA-RNA combinations. By labeling one of these two strands, with a reporter molecule annealing can be detected by a variety of methods. The basic requirement of a probe is specificity of the sequence of interest and labeling to allow appropriate detection. Preservation of sufficient morphologic detail in the tissue is needed for identification of the location of the labeled probe. The annealing and separation of the two hybridized 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 (Tm) of hybrids is the point at which 50% of the double-stranded nucleic acid chains are separated. The optimal temperature for hybridization is 15° to 25°C below the Tm. There are various formulas to calculate Tm, depending on probe length and type of hybrids. For DNA-DNA hybrid with probes longer than 22 bases, the following formula can be used: $Tm = 81.5 + 16.5 \log (Na) + 0.41 (\%GC) - 0.62 (\%\ \text{formamide}) - 500/\text{length}$ of base pairs of probe. RNA-RNA hybrids are generally 10° to 15°C more stable than DNA-DNA or DNA-RNA hybrids, and therefore require more stringent conditions for hybridization and posthybridization washing (43–46).

A wide variety of probes can be used for ISH and the appropriate type of probe is determined to a large extent by the application. For DNA detection (for example, DNA viruses, chromosome-specific probes, or locus specific probes), double-stranded DNA probes are the most commonly encountered. For RNA detection, RNA probes (riboprobes) are often used because RNA-RNA hybrids are more stable than DNA-RNA hybrids; riboprobe vectors can be used to generate both sense and antisense probes to allow control of hybridization; and unbound probe can be digested using RNAse A, which does not digest double stranded RNA. Alternatively, oligonucleotides, either singly or as a cocktail, can be used, particularly for the detection of high abundance RNA such as hormone mRNAs. These have the advantage that they can be custom synthesized and have high specificity. This decreased sensitivity with oligonucleotide probes can be overcome in part by using a cocktail of multiple oligoprobes that are complementary to different regions of the target molecules. Careful selection of oligonucleotide probes with low or no homology with other nucleotide sequences is most important to ascertain the specificity of ISH. The use of oligonucleotide probes does not require expertise with many molecular biology techniques such as gene cloning or plasmid preparation (30,47–49).

Labeling of probes is most commonly performed using enzymatic methods (47–49). DNA probes are conveniently labeled by either nick translation or randomly primed extension, both of which introduce labeled nucleotides such as digoxigenin-11-dUTP into the probe

molecules. RNA probes are generally labeled by in vitro transcription whereby the probe sequence is cloned into a vector containing RNA polymerase promoter sites, and probe molecules generated using phage RNA polymerase mediated incorporation of labeled nucleotides such as digoxigenin-11-UTP. A variety of polymerases can be used, including T3, T7, and SP6. Oligonucleotides can be labeled during synthesis, when 5 prime-labeled nucleotides can be incorporated directly into the molecule. Alternatively, enzymatic procedures can be used for either 5-prime or 3-prime labeling. One advantage of 3-prime end labeling is that multiple probe labels can be introduced to form a "tail," thus increasing the sensitivity of detection. Finally, probe labeling can be carried out using polymerase chain reaction (PCR)-based methods, with either direct incorporation of labeled nucleotides or by using labeled primers. Details of the practical details of probe labeling can be found elsewhere (8,49).

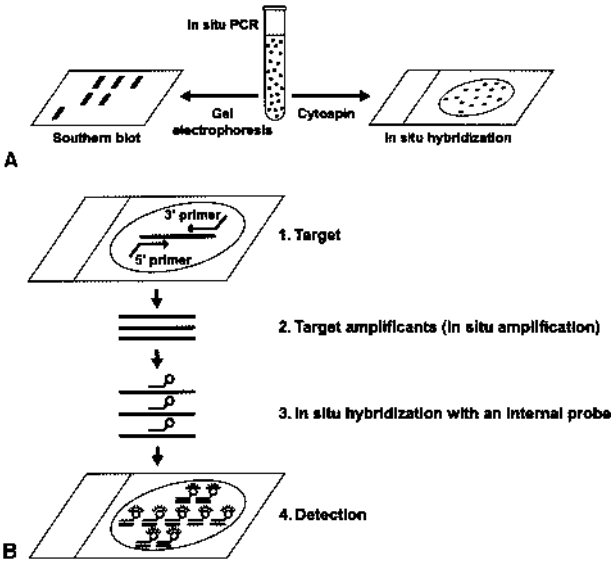

FIG. 1. (A) In situ amplification of cells in liquid phase. The PCR products can be detected by Southern blot or by in situ hybridization. **(B)** Flow diagram showing indirect in situ polymerase chain reaction (PCR). Amplification of the target is usually in the range of 10- to 50-fold.

INCREASING SENSITIVITY BEFORE ISH USING TARGET AMPLIFICATION METHODS

In situ PCR

PCR with ISH (in situ PCR) can amplify specific DNA or RNA sequences inside single cells or tissue sections and increases the copy numbers to levels readily detectable by conventional ISH methods. 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 its inception in 1990, in situ PCR has undergone rapid development. Several in situ PCR protocols with varying modification have been published (24–32). Initially, fixed cells suspended in the PCR reaction mixture were thermal cycled in micro-Eppendorf tubes using conventional block cycler. After PCR, the cells are cytocentrifuged onto glass slides followed by visualization of intracellular PCR products by ISH or immunohistochemistry. In situ PCR of cells or tissue sections on glass slides is performed by two approaches: direct and indirect (Fig. 1). The cells or tissues are overlaid with the PCR mixture under a coverslip, which is then sealed with nail polish, rubber cement or mineral oil to prevent evaporation of the reaction mixture or by using specially designed reaction chambers which are clipped onto the glass slide. In the direct approach a labeled nucleotide is incorporated into the PCR products, whereas in the indirect method, which is more specific and the preferred method. ISH is performed after in situ amplification using labeled oligonucleotide or other probes.

These techniques have been successfully performed on intact cells in suspension, smear or cytospin preparation, metaphase chromosomes, and frozen and paraffin sections. After proper fixation and permeabilization, 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, and Taq DNA polymerase. Generally, 10 to 30 PCR cycles are adequate for in situ amplification. The oligonucleotide primers (sense and antisense) are usually 18 to 24 bases long and the amplified fragments 100 to 800 bp. 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 usually performed before PCR. The amplified signal of indirect in situ PCR can be detected by ISH using an oligonucleotide probe, which recognizes sequences internal to the amplification primers.

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 are many concerns about the specificity of direct in situ PCR. Nonspecific incorporation of labeled nucleotides and misprimings or DNA repair artifacts may occur, resulting in false-positive results. Many different controls are needed to allow adequate interpretation of the results. However, the strategies to optimize reproducibility include controlled approaches to fixation and digestion as well as the use of specially designed equipment for in situ PCR. Inappropriate conditions can lead to false-negative and -positive results.

In situ PCR has been mainly applied to detect DNA sequences that are not easily detected by conventional

ISH, which include human single-copy gene, rearranged cellular genes, and chromosomal translocations, and to map low-copy number genomic sequences in metaphase chromosomes. The use 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. Application of the in situ RT-PCR to detect gene expression is still limited primarily to cell preparation and frozen section (24–32). Relatively few successful applications in paraffin sections with adequate rigorous controls have been reported (50–52). Successful amplification of mRNA by in situ RT-PCR includes hormone, receptor, and oncogenes (Fig. 2A and B) (24–29). A one-step RT in situ PCR procedure that compartmentalizes these sequential steps within a single applications methodology using the enzyme rTth has been recently applied to detect and localize mRNA transcripts for Fas ligand within the immune-privileged placental environment and to provide verification of immunohistochemical localization of gene product (33).

In theory, in situ PCR techniques are straightforward and should be reproducible like conventional PCR. In practice, however, it is associated with many problems, such as low amplification efficiency and poor reproducibility.

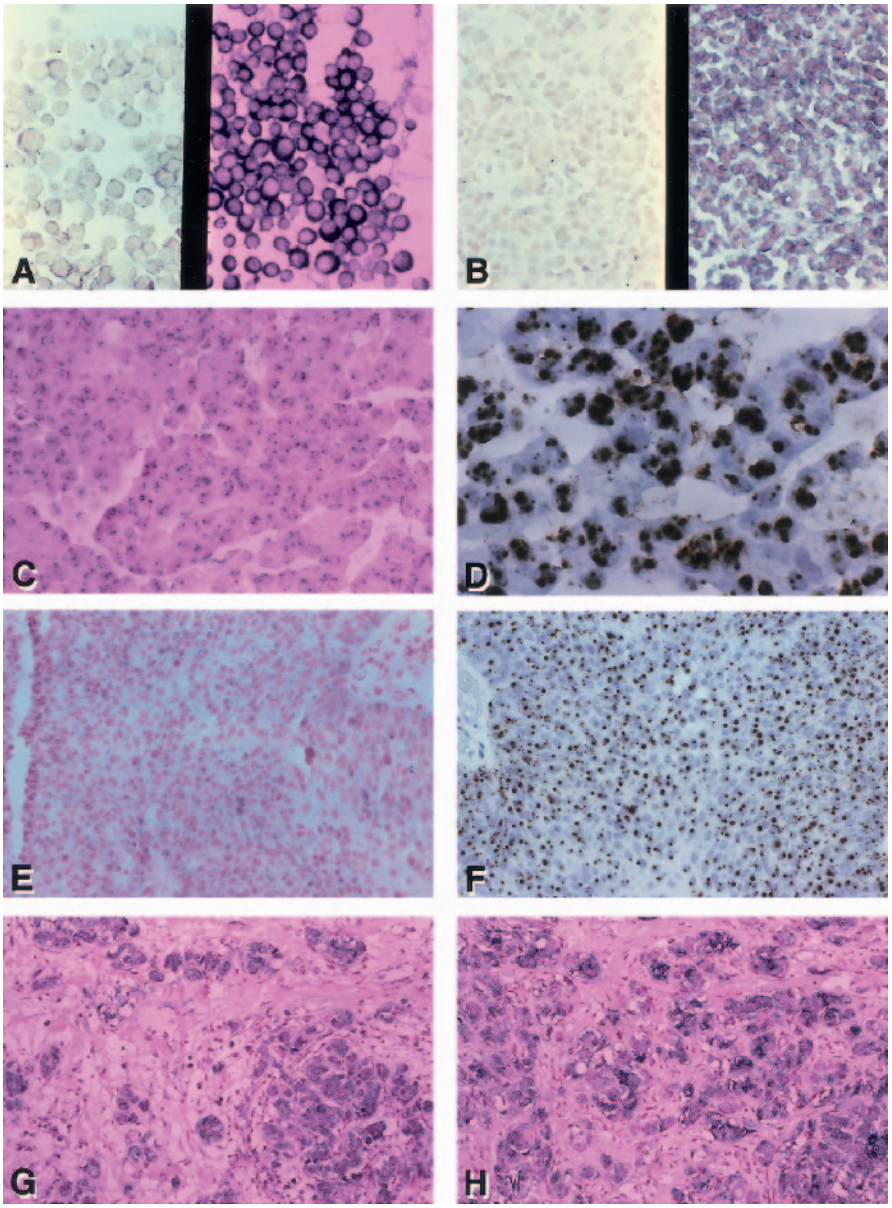

FIG. 2. A: In situ PCR showing prolactin RNA expression in GH3 cells hybridized with prolactin primers. The figure on the left shows in situ hybridization without PCR, and the cells on the right shows the detection signal after indirect in situ PCR with a digoxigenin-labeled probe. **B:** In situ PCR of pituitary tumor using frozen tissue sections. The specimen on the left shows detection of gonadotropin-hormone releasing hormone receptors by regular in situ hybridization. The figure on the right shows the increased signal intensity detected by using in situ PCR with the indirect method and a digoxigenin labeled probe. **C:** In situ hybridization using biotinylated HPV16 and 18 cDNA probes with detection by biotin/alkaline phosphatase and development with NBT/BCIP in Caski cells. **D:** CARD ISH staining (brown) using the same HPV 16 and 18 probes diluted five-fold with Caski cells. There is marked amplification of the hybridization signal with the CARD technique. **E:** In situ hybridization for HPV in a cervical biopsy using 16 and 18 probes with biotin/alkaline phosphatase and development with NBT/BCIP. **F:** The same tissue hybridized by CARD ISH using the same HPV 16 and 18 probe shows that the signal is markedly amplified compared with regular in situ hybridization. **G:** Detection of calcitonin mRNA by in situ hybridization with a digoxigenin labeled riboprobe. A moderately strong in situ hybridization signal is detected. **H:** CARD-ISH with the same calcitonin riboprobe detected calcitonin gene product in the medullary thyroid carcinoma. There is an increase in the hybridization signal.

These difficulties are caused by a number of events resulting from PCR amplification in situ, such as the diffusion of PCR products, which are bound up with the denaturation steps, from the site of synthesis inside and/or outside the cells, followed by the possible extracellular generation of amplificants. In addition, the final results of direct in situ PCR can be influenced by incorporation of labeled nucleotides into nonspecific PCR products and the generation of nonspecific PCR products resulting from mispriming, fragmented DNA undergoing "repair" by DNA polymerase, "repair" artifacts, or priming of nonspecific DNA or cDNA fragments, "endogenous priming," artifacts. These artifacts can also be observed in apoptotic cells or samples that have been pretreated with DNAse before in situ RT-PCR for mRNA detection. It is prudent to use many different controls to allow adequate interpretation of in situ PCR results and to use indirect in situ PCR to increase the specificity of the amplified nucleic acids. In situ PCR is considered a rather cumbersome ISH method, in which sample pretreatment consists of fixation and protease digestion in combination with heating (thermal cycling) during nucleic acid amplification by PCR. Moreover, the increase in detection sensitivity compared with conventional ISH is rather limited, even after optimization.

In situ self-sustained sequence replication (IS-3SR)

The 3SR reaction has a number of theoretical advantages as an alternative method for RNA amplification in comparison to RT-PCR. In situ self-sustained sequence replication (IS-3SR) is based on the use of primers with attached RNA polymerase initiation sites and the combination of three different enzymes in the same reaction mixture (DNA polymerase, RNAse H, and RNA polymerase), resulting in accumulation of target mRNA through the combination of RT, DNA synthesis, and in vitro transcription. Due to the high efficiency of RNA polymerase, initial mRNA target sequences can be multiplied by way of cDNA intermediates with great efficiency and be visualized by ISH with a labeled probe. However, the IS-3SR assay shows significantly less amplification efficiency than in situ PCR when performed in situ. So far, IS-3SR has only successfully been applied to experimental cytocentrifuge preparations and not to archival tissue sections. Amplification of long RNA segments is much less efficient. This may be a result of the phenomenon of "mispriming," meaning that the RNA polymerase has a tendency to fall off its template before the entire sequence has been transcribed. Alternatively, the action of RNase H may partially digest the products of 3SR amplification as well. When a shorter sequence is amplified, the effect of mispriming and product digestion are minimized. Nonspecific amplification by 3SR means that IS-3SR is only reliable as an indirect technique, that is 3SR amplification must be followed by a specific ISH with a probe internal to the 3SR primers. There have been some efforts to modify 3SR for use in an in situ system (IS-3SR) (12,36–38).

IS-3SR is a simple and inexpensive technique, which has a number of advantages in comparison to PCR-based procedures. A number of factors must be taken into account, in particular the fact that since 3SR amplification is quite nonspecific, it is suitable only as an indirect method and must be carefully evaluated with the use of well-designed controls. IS-3SR has the potential to provide information about the location of gene expression in the early phases of viral infection or carcinogenesis, which is not easily obtained by any other method. The amplification of target nucleic acids before hybridization is one of the most powerful approaches for the detection of low-copy number RNA and DNA. IS-3SR can significantly amplify the amount of intracellular RNA with the measles virus study as a model. Such a level of amplification could raise the amount of single copy RNA to the level of detection by conventional in situ hybridization. Although careful controls to ensure its specificity must be carried out, IS-3SR has several advantages, including ease of use, preserved cell morphology, and specificity for RNA amplification. The suitability of IS-3SR as an adjunctive tool in routine pathology as well as in research has to be further explored.

Primed in situ labeling (PRINS)

Primed in situ labeling (PRINS) is a rapid, one-step target amplification technique based on the Taq DNA polymerase-mediated incorporation of labeled nucleotides into newly synthesized DNA by a single primer elongation. PRINS technique has several advantages over conventionally applied fluorescence in situ hybridization (FISH). PRINS is easy to perform, fast, less expensive, and applicable without experience in cloning techniques. PRINS method has been refined to allow the identification of translocations and staining of human telomeres and multiple chromosomes in the same specimen. To further increase the sensitivity of PRINS, cycling PRINS or repeated PRINS protocol has been established (39–42).

However, the detection of single-copy genes by PRINS to paraffin-embedded tissue sections is still in the experimental stage. Potential pitfalls of the PRINS method include false-positive results and background staining due to mispriming and artifacts related to the incorporation of labeled nucleotides into damaged DNA, similar drawbacks to those encountered in the direct in situ PCR method, especially when applied to tissue sections from paraffin-embedded specimens. In the cycling PRINS procedure, which is actually not very different

from in situ PCR, similar potential problems are encountered as already mentioned for experiments with direct in situ PCR-based target sequence amplification. Until recently, PRINS was efficient only on targets containing repetitive sequences. Despite this limitation, PRINS proved useful because (1) it generates highly specific labeling, (2) it can be performed rapidly and easily, and (3) it can be combined with techniques such as FISH and immunofluorescence. The original PRINS targets were repetitive sequences such as those found in telomeres and centromeres.

The PRINS method, which has long been overshadowed by the initial achievements and publicity of in situ PCR, is now gaining more and more attention and has emerged as a robust and highly promising alternative method to in situ PCR and FISH. Its potential clinical diagnostic applications are plentiful, and the speed and simplicity of the PRINS procedures appear to make it an ideal adjunctive tool in clinical pathology for the analysis of structural and numerical chromosomal aberrations in cells obtained from blood and other body fluids, effusions, or touch preparations. Its use for mRNA detection and application on tissue sections from paraffin-embedded specimens, however, is currently limited and substantial improvements of protocols are required to render it more reliable and acceptable.

In situ transcription

In situ transcription is a method for the visualization of mRNA by means of synthesis of labeled complementary cDNA within a cell using an mRNA-complementary primer, reverse transcription, and labeled nucleotides (53–55). It is very similar to the PRINS method for mRNA detection. However, in the latter approach, the subsequently synthesized DNA but not the cDNA is labeled, as in the PRINS technique for genomic DNA detection. Theoretically, the production of a long, labeled cDNA product for each copy of mRNA should provide an improved sensitivity in comparison to conventional ISH. Moreover, the selection of appropriate primers could even allow the identification of minor sequence variations in a particular mRNA species. However, similarly to the PRINS reaction, potential mispriming or nonspecific incorporation of labeled nucleotides may yield nonspecific results or increased background staining, although probably at a significantly lower level than in techniques applying DNA polymerase enzymes. In situ transcription has so far not found broad acceptance in the field of in situ detection of mRNA.

SIGNAL AMPLIFICATOIN AFTER ISH

Catalyzed reporter deposition signal amplification

Several investigators have used catalyzed reporter deposition technique (CARD) with biotinylated tyramine for ISH amplification in cell preparations and tissue sections (16–23). This novel signal amplification technique is based on the deposition of activated biotinylated tyramine onto electron-rich moieties such as tyrosine, phenylalanine, or tryptophan at or near the site of horseradish peroxidase. The binding of tyramide to proteins is believed to be due to the production of free oxygen radicals by the horseradish peroxidase. The biotin sites on the bound tyramide act as further binding sites, e.g., streptavidin-biotin complexes or enzyme- and fluorochrome-labeled streptavidin. In this way, a lot of extra hapten molecules can be introduced at the hybridization site in situ (Fig. 3). CARD allows a 500- to 1000-fold increase in sensitivity of ISH signals when compared with conventional avidin-biotin complex procedures without production of increased background (Fig. 2C–H). ISH sensitivity could be improved to enable the detection

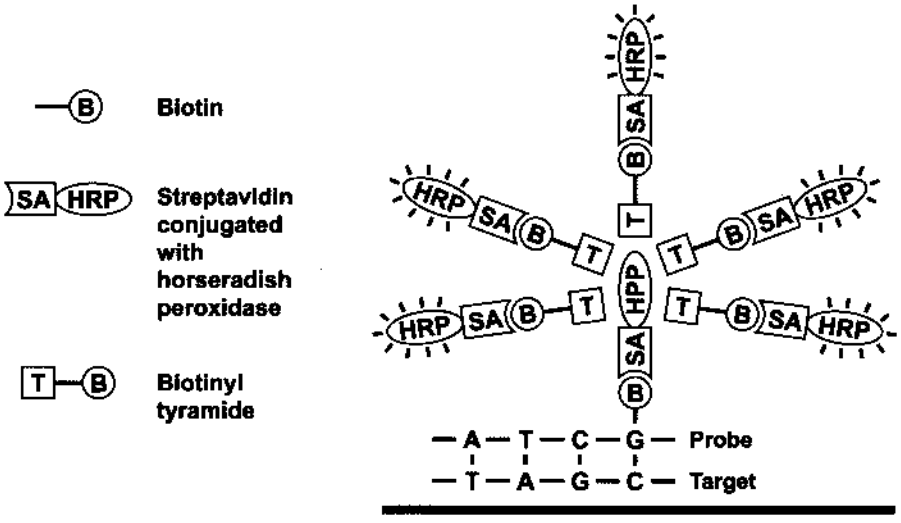

FIG. 3. Schematic of the CARD-ISH method. Hybridized biotinylated probes are detected with primary streptavidin conjugated with horseradish peroxidase (SA/HRP) and reacted with hydrogen peroxide and biotinyl tyramide. Newly deposited biotin molecules are then detected with an application of secondary SA/HRP. Peroxidase is finally reacted with hydrogen peroxide and 3,3′-diaminobenzidene to form an insoluble brown precipitate.

of (1) repetitive and single-copy (up to the level of 1–5 KB) DNA sequences in cell preparations, (2) up to three different DNA sequences (repetitive as well as single-copy) simultaneously in cell preparations, (3) low- and single-copy human papillomaviruses and other microorganisms in cell and tissue preparations (8–21), and (4) rRNA and mRNA ranging from high to low abundance in cell and tissue preparations. Visualization of deposited tyramides can be performed either directly after the CARD reaction with fluorescence microscopy, if fluorochrome-labeled tyramides are used, or indirectly with either fluorescence or bright field microscopy, if biotin, digoxigenin, or di- or trinitrophenyl are used as haptens, which can act as further binding sites for antihapten antibodies or streptavidin conjugates. In addition, fluorescein and rhodamine can be used as haptens because specific antibodies against these fluorochromes are commercially available from several companies (18–22).

The main advantage of using CARD signal amplification for ISH procedures is that it is performed after probe hybridization and stringent washings, so that the specificity of the probe hybridization is not compromised. Therefore, in principle, proper conditions for sample fixation and pretreatment, including endogenous peroxidase inactivation, as well as hybridization in situ, do not need to be modified compared with conventional ISH procedures. Only if nonspecific probe binding is suspected on the basis of control experiments should the probe concentration be further modified (decreased) for optimal results, e.g., in cases where complex DNA probes are used. For instances with chromosome painting, YAC, P1, or cosmid probes containe repetitive elements that need to be blocked by competitor DNA such as Cot-I DNA when an optimal balance is needed between probe and competitor DNA (13,14,19,20). A number of different combinations of probe detection (one to three detection conjugate layers) and CARD signal amplification systems (utilizing different tyramides, amplification buffers, reaction times, and temperatures) have been applied and that optimization of each detection system has been necessary to obtain a high signal-to-noise ratio. Because signal amplification, both specific and nonspecific, are greatly amplified with CARD, it is essential that nonspecific probe binding and detection be avoided or kept at a minimum to apply this procedure successfully.

CARD has also been used for diagnostic nonradioactive oligonucleotide ISH procedure with digoxigenin-labeled probes to increase the sensitivity and to shorten the overall turnaround time of assays (8–10,21–23). This approach allows the detection of peptide hormone mRNA in tissue sections from routinely fixed, paraffin-embedded surgical samples within a single working day and makes the assay suitable for routine diagnostic purposes. Biotinylated tyramides are relatively easy and inexpensive to synthesize. However, biotin is associated with large numbers of endogenous streptavidin binding sites, such as in liver or kidney, and high background staining may be encountered. It is therefore desirable to be able to rely on differently labeled tyramides, e.g., with digoxigenin, di- or trinitriphenyl, or fluorochromes. Moreover, because the tyramide deposition reaction proceeds very quickly, minor differences in amplification reaction time may lead to variations in the final signal intensities. Nevertheless, an amplification factor in the range of five- to ten-fold, or possibly higher, together with preservation of distinct localization of ISH signals, seems to be a realistic goal for both DNA and mRNA ISH.

The practical limitations of CARD are due to a number of factors. Because of high sensitivity, CARD has potential to amplify nonspecific background signal, which may result in an unfavorable signal/noise ratio. Endogenous peroxidases in human tissue are potent enough to catalyze the CARD reaction. To avoid this unwanted reaction, endogenous peroxidase must be blocked or quenched, and appropriate positive and negative controls should be used. CARD ISH uses biotinylated probes, biotinyl tyramide, and streptavidin-conjugated horseradish peroxidase.

Peptide nucleic acid (PNA) probes combined with CARD detection has recently introduced (56). PNA consists of a peptide backbone composed of N-(2 aminoethyl) glycine units to which nucleobases are attached by carbonyl methylene linkers, bringing new possibilities for the in situ hybridization. PNA is an uncharged molecule, which reduces hybridization time and background significantly. The combination of PNA and CARD technology greatly simplifies the in situ hybridization component of the reaction.

CARD signal amplification using labeled tyramides has significantly influenced the ISH methods performed on different biologic specimens because it is an easy, rapid, highly sensitive, and efficient procedure. As a consequence, sensitive ISH procedures incorporating CARD signal amplification is an attractive option for low-copy nucleic acid and antigen detection in situ.

Branched DNA in situ hybridization

Branched DNA (bDNA) technology has been used recently to measure nucleic acids in serum, plasma, or cell lysates and to adapt bDNA technology to an ISH for detection of DNA or RNA. The bDNA ISH method to improve nucleic acid detection increases the signal rather than amplifying the target or probe sequence and uses a series of nonisotopic oligonucleotide probes, hybridized sequentially, to generate chromogenic and fluorescent signals (57–61). Either mRNA or DNA can be detected with the same set of probes. After pretreatment, cells are

incubated with a prehybridization solution, hybridized with target probes, and then with a series of oligonucleotide probes for signal amplification. These include preamplifier, amplifier, and alkaline phosphatase (AP)-conjugated label probes (Fig. 4). To reduce potential nonspecific hybridization, nonnatural nucleotides 5-methyl-2′-deoxyisocytidine (isoC) and 2′-deoxyisoguanosine (isoG) are included in the target, preamplifier, amplifier, and AP-conjugated label probes. Although nonnatural nucleotides have recently been incorporated into bDNA technology, specific and sensitive detection of target nucleic acid sequences is routinely achieved using probes without nonnatural nucleotides. Alkaline phosphatase–labeled probes catalyze the reaction of NBT-BCIP substrate. The advantages of this method are its specificity, while amplification of the signal rather than the target or probe makes bDNA amplification a quantitative procedure. The original amount of the target remains unmodified, unlike the situation with other amplification methods. The disadvantages of bDNA are sensitivity as compared with target and probe amplification methods (58,60).

A couple of features distinguish the bDNA ISH method from CARD ISH. One significant difference is in the probe design. The smaller probes generated for bDNA ISH are easier to synthesize and can detect smaller target sequences, thereby providing greater flexibility than longer probes. In principle, adjusting the number of target probes used can control the degree of signal amplification. Another feature of bDNA is that bDNA ISH does not use an avidin-biotin signal amplification system and hence is not affected by binding of avidin-conjugated reporter molecules to endogenous biotin. By providing greater flexibility in probe design and avoiding potential interference by endogenous biotin, the bDNA ISH method represents a significant advance in low-copy nucleic acid detection.

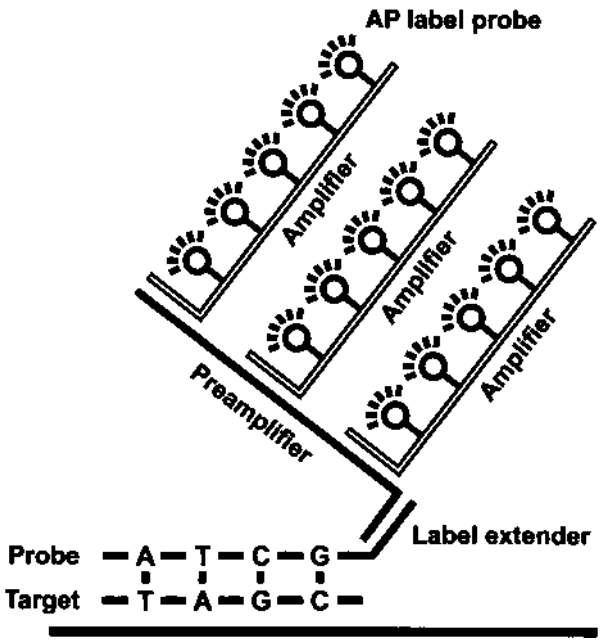

FIG. 4. Basic components of the bDNA ISH method. These include a preamplifier, an amplifier, and alkaline phosphate (AP) conjugated labeled probes. Specific and sensitive detection of target nucleic acid is achieved by amplification.

Compared to PCR-based methods, the bDNA signal amplification system offers a number of advantages for ISH. Because bDNA ISH is based on the sequential hybridization of synthetic DNA probes, it does not require any DNA or RNA polymerase activity and hence is not affected by the presence of polymerase inhibitors in tissue specimens. The potential for diffusion of amplification products away from the target site, which is a concern with PCR-based ISH method, is not a significant concern with bDNA ISH. Because the AP-conjugated reporter molecule that provides the signal is tethered to the spatially fixed nucleic acid target in the bDNA ISH method, the AP–Fast Red (Fast red, #597, Dako, Carpinteria, CA) reaction product is retained at the target site. Moreover, the bDNA ISH method does not require repeated cycling through elevated temperatures. Because repeated incubation at high temperatures can readily damage cell morphology, avoiding high-temperature incubations is important for applications in which preservation of intricate cell morphology is important. Based on bDNA signal amplification technology, the bDNA ISH method is highly sensitive (can detect one or two copies of DNA target per cell), specific, and provides subcellular localization of the target sequence. By modifying a few steps in the bDNA ISH procedure, detection of mRNA or bDNA targets can be achieved using the same set of bDNA oligonucleotide probes. The bDNA ISH method is nonisotopic, rapid, can be adapted to generate chromogenic and/or fluorescent signals, and should be amenable to automation and quantification. Given its ease of use and reliability, the bDNA ISH method is an attractive alternative for sensitive detection of nucleic acid sequences in a well-preserved morphologic context.

bDNA ISH provides a rapid, sensitive, and reproducible means for detection of specific DNA and mRNA sequences in various cell types (58). The sensitivity of the bDNA ISH method is sufficient to detect relatively low-abundance targets, as few as one or two copies of HPV-16 DNA in SiHa cells. The bDNA ISH method agrees with the studies that have detected HPV-16 DNA in SiHa cells using CARD. The two methods have similar sensitivities for detecting HPV-16 DNA in SiHa cells (57,58).

The bDNA ISH method is highly specific and provides subcellular localization of the target sequence. In mixed cell population experiments, signals were detected only in the appropriate cell types, indicating that there

was no diffusion of AP–Fast Red reaction product from one cell type to another. Moreover, signals obtained with bDNA ISH are retained within the cellular compartment in which the target sequence is localized. Subcellular localization experiments with HPV-18-infected HeLa cells showed that viral mRNA was predominantly detected in the cytoplasm, whereas viral DNA was detected in cell nuclei (58).

PROBE AMPLIFICATION

Padlock probes and rolling circle amplification (RCA)

Padlock probes were developed in the early 1990s for the detection and characterization of single-copy genes in genomic DNA samples such as analysis of alleles and point mutations (62–69). Padlock probes are composed of a single-stranded linear oligonucleotide of about 90 base pairs (bp) that hybridize to targets of 30 bases. The 30-base target-binding region of the probe is split into two 15-base segments placed in opposite orientation at each end of the linear probe so that a circle must be formed for hybridization to occur (62,63,66). At 10 bases per helical turn, the hybridized probe wraps around its target three times, and the remaining 70 bases form an unhybridized single-stranded loop. Posthybridization DNA ligation connects the two ends of the probe in the middle of the 30-base binding region. The unbound 70-base loop facilitates probe circularization and permits 20 bases to serve as a primer recognition site for DNA polymerase to replicate the circle.

RCA generates a localized signal via an isothermal amplification of an oligonucleotide circle, which can be performed using padlock probes as templates. The polymerase progresses continuously around the loop until the 100 bases have been replicated hundreds or thousands of times. Incorporating a labeled nucleotide during the RCA reaction produces sufficient signal for easy visualization of the target. RCA technology is a robust and simple procedure that can provide a universal platform for the localization of a wide variety of molecules as a function of either antigenicity or nucleic acid sequence. RCA has been adapted to in situ hybridization and immunohistochemistry to increase sensitivity. In a recent report (67,68) (Fig. 5) after hybridization and stringency wash, the bound immunoconjugates were detected by incubation with a circle homologous to the RCA primer diluted to 200 nmol/L in 100 mmol/L of potassium glutamate. After incubation for 30 minutes at 37°C, slides were rinsed with 100 mmol/L of potassium glutamate. RCA reactions were performed for 45 minutes using Φ29 DNA polymerase at 31°C. The products of these RCA reactions were then visualized by incubation at 37°C for 30 minutes with the appropriate oligonucleotide decorator diluted to 20 nmol/L in 2× standard saline citrate/0.1% Triton X-100.

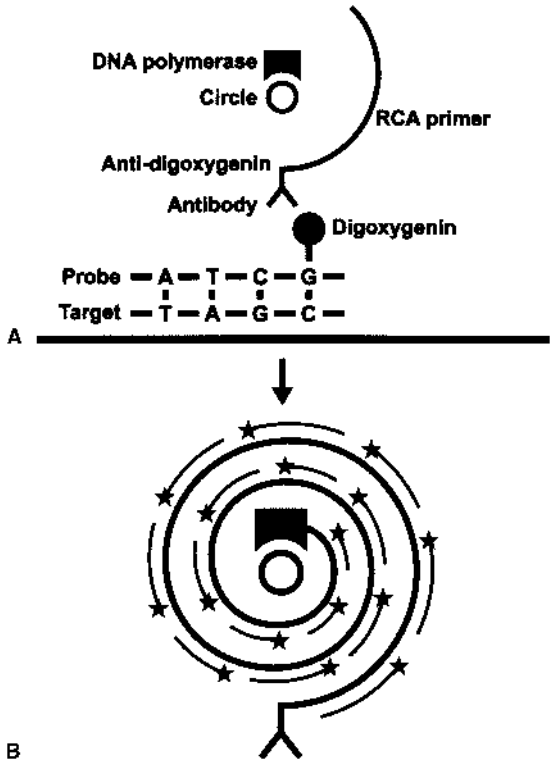

FIG. 5. In situ hybridization with immuno-RCA signal amplification system using digoxigenin-labeled probes. **A:** An anti-digoxigenin antibody harboring a covalently bound oligonucleotide primer binds to a digoxigenin-labeled DNA probe; a circular oligonucleotide then binds to the primer, RCA is initiated by DNA polymerase, and the amplified DNA product is labeled by hybridization with dye-labeled oligonucleotides **(B)**.

RCA is an isothermal nucleic-acid amplification method, but it differs from the polymerase chain reaction and other nucleic-acid amplification schemes in several respects. A linear mode of RCA can generate 10^5-fold signal amplification during a brief enzymatic reaction that has been demonstrated in microarray assays (63–67). The most important feature of linear RCA is that the product of amplification remains tethered to the target molecule. In conjunction with the isothermal nature of the RCA reaction and the capability to localize multiple markers simultaneously, RCA seems well suited to cell- and tissue-based assays where it is critical to maintain morphologic information. RCA amplification permits the localization of signals, representing single molecules with specific genetic or biochemical features. Localized signal amplification by means of RCA can recognize nucleic acid targets through hybridization of nucleic acid probes. Increased sensitivity without compromising cellular and tissue morphology has been demonstrated for applications in immunohistochemistry and ISH. One ad-

vantage of RCA is a significant increase in the signal that is achieved through a single round of enzymatic amplification of a nucleic acid substrate, leading to improvements in relative discrimination as well as absolute amounts of measurable signal. This is in contrast to biotinyl tyramide, which requires a multilayered amplification process.

Immuno-RCA derives its specificity from an antibody-antigen interaction and its signal amplification from nucleic acid hybridization and synthesis. In contrast polymerase chain reaction or other nucleic acid–based amplification approaches involve multiple rounds of amplification that can magnify background as well as specific signal. As a result of the RCA product being tethered to the conjugate, the signal is localized. This localized signal provides RCA with a significant advantage over biotinyl tyramide and PCR. Moreover, because the amplification is relatively simple, there are no additional steps leading to increase background staining. It should be possible to obtain reasonable sensitivity in a short time with greater ease than with conventional techniques or signal amplification techniques that involves serial incubations with detection reagents. The reagents used for immuno-RCA are relatively universal. The same circles, conjugates, and DNA polymerase can be used for cells and tissues fixed in different ways and probed with a range of antibodies. The use of additional pairs of circles and conjugates can permit the simultaneous detection of multiple targets. The detection of single nucleotide polymorphisms in at least one locus has been accomplished using RCA in conjunction with padlock probes on nuclear halo cytologic preparations.

The immuno-RCA protocols have been extended to hapten-labeled nucleic acid probes for nuclear DNA sequences or cytoplasmic mRNA molecules. The use of RCA to detect mRNA in cytologic preparations is sufficiently quantitative to enable transcriptionally mediated dose-response curves to be generated. RCA in situ is useful for discriminating alleles, determining gene copy number, and quantifying gene expression in single cells. The sensitivity, specificity, and speed of RCA may also allow its use for clinical purposes such as prenatal diagnosis and pathologic characterization of tumors. The exquisite sensitivity of in situ RCA may add an entirely new dimension to the fields of genomics, pathology, and cytogenetics.

CONCLUSIONS AND FUTURE PROSPECTS

The relative insensitivity of ISH has been cited as an important limiting factor in many applications, especially in diagnostic pathology. Improving the sensitivity of ISH has been a goal of many investigators in this field. There are two general approaches to achieve this: amplification of target sequences and signal enhancement. Currently, nucleic acid amplification has become both a necessity and a routine procedure in many aspects of molecular biology (70–73). Although extremely versatile and sensitive, in situ PCR has limitations, with poor preservation of morphology of some biologic structures and unreliable as a quantitative method. Clinical application of in situ PCR must await the resolution of some of the current limitations (24–26). Isothermal methods such as IS-3SR and RCA are extremely sensitive qualitative methods (36–38). However, like PCR, target and probe amplification procedures are not appropriate for precise quantification of the target nucleic acid. In contrast, amplifying the signal rather than the target or probes is suitable for

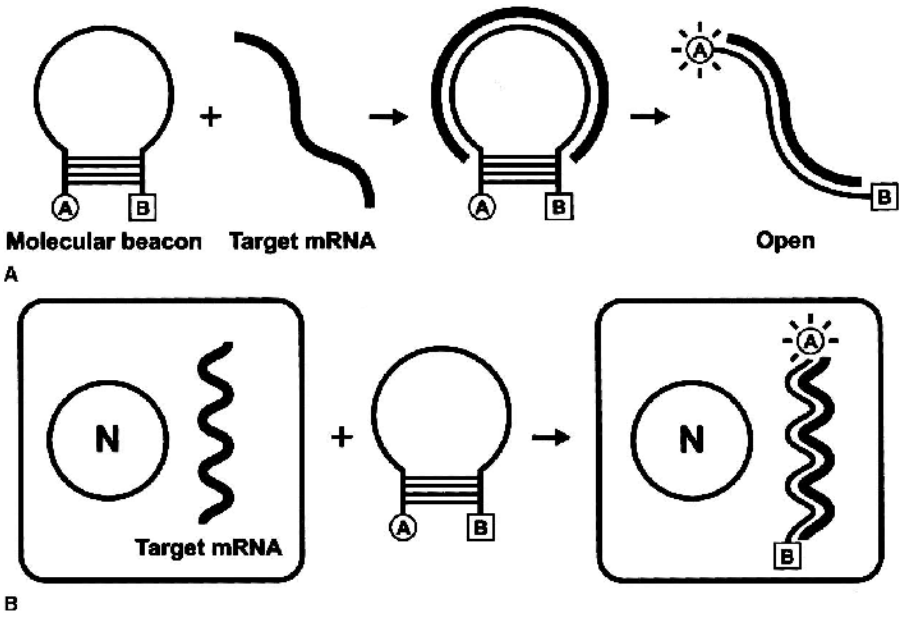

FIG. 6. Schematic diagram of in situ hybridization using molecular beacons. **A:** The molecular beacons are dual labeled oligonucleotides with a hairpin structure. One end is for the fluophor, and the other end is the quencher. When the molecular beacon hybridizes to the target mRNA the molecular beacon unwinds. This increases the fluorescent signal, which can be readily detected. **B:** Molecular beacons can be delivered into cells and they can then hybridize with target mRNA, which results in a fluorescent signal released after hybridization.

quantitative interpretations, as in bDNA ISH, which addresses both the detection and quantification of the target, providing valuable information in the molecular diagnosis of pathogens. CARD with a combination of enzyme/substrate is able to generate and to deposit large numbers of molecules at the detection site. Therefore, ISH results in considerable improvement in sensitivity. In addition, brighter fluorescent polystyrene microspheres and enzyme-labeled fluorescence signal amplification technology are being developed, which could prove useful in the future for the detection (74,75).

The most promising methods for target sequence amplification prior to ISH include in situ PCR, PRINS, and the catalyzed reporter deposition for signal amplification of visualization procedures. The detection of intracellular mRNA or viral RNAs as well as endogenous and foreign DNAs by these methods has many potential applications when the level of gene expression or the number of DNA copies is below that detectable by conventional ISH. Conventional 3SR protocols, which thus far have not found broad acceptance in diagnostic laboratories, along with IS-3SR may be replaced by the newer alternative strategies, which are becoming available for the intracellular analysis of nucleic acids. Signal amplification methods by catalyzed reporter deposition using biotinylated tyramine hold enormous potential to significantly influence future directions in the development of ISH. The combined efforts to target sequence and signal amplification, in particular, are the key strategy for the future to make ISH an easy to perform, fast, highly sensitive, and accurate method and therefore suitable for diagnostic laboratories. An intriguing modification of indirect in situ PCR, involves an adaptation of the TaqMan approach for solution-phase PCR (25,26).

In situ signal amplification approaches have been explored extensively during the past few years, and the CARD signal amplification method appears to be one of the most promising approaches (71). Trends in recent developments suggest that it should possible to use different chromogens for various amplified sequences within the same cell.

More sensitive techniques for bright-field detection of hybridization products are being continuously developed. One new approach has been the use of molecular beacons (MB) (Fig. 6). MB is a new class of nucleic acid probes that become fluorescent when they bind to a complementary sequence. Because of the sequence specificity of nucleic acid hybridization and the marked sensitivity, MB can be used to detect low-copy number of DNA or RNA molecules (76–78). In situ strand displacement amplification has recently been described as an improved technique for the detection of low-copy nucleic acids. This technique was reported to be as sensitive as in situ PCR (79). A bright-field assay for assessment of Her-2/neu gene amplification using GoldEnhance gold-based automatography (Nano probes, Inc., Yaphank, NY), CARD, and a biotinylated-labeled probe that approached the sensitivity of the FISH technique is also a promising development (80). These new developments indicate that the development of highly sensitive and specific probes, and amplification systems for ISH will continue unabated into the near future. □

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