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21 Molecular pathology

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Introduction

Molecular pathology seeks to apply gene expression against morphology and use gene expression analysis to validate large numbers of targets. A glossary of common definitions and terminology can be found at the end of this chapter.

Molecular pathology techniques have been used in the clinical laboratory to aid in the diagnosis and monitoring of treatment regimens of many infectious diseases such as HIV, hepatitis B, and tuberculosis (Netterwald 2006). These tests are usually performed on serological or other body fluids, such as sputum and seminal fluid. Currently the most well-known and most advertised molecular testing is for human papillomavirus (HPV) and human epidermal growth factor receptor 2 (HER2).

Clinical and research laboratories may use additional molecular pathology techniques, such as blotting methods which are used to study extracted ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Blotting methods consist of extracting DNA and/or RNA from homogenized tissues and then analyzing them using dot, Southern and Northern blotting filter hybridization methods (Sambrook et al. 1989). Blotting techniques such as these are powerful tools for the qualitative analysis of extracted nucleic acid from fresh or frozen cells and frozen tissues.

The polymerase chain reaction (PCR) is included in molecular pathology methods. PCR is a common method of creating copies of specific fragments of DNA. It rapidly amplifies a single DNA molecule into many billions of copies. In one application of the technology, small samples of DNA, such as those found in a strand of hair at a crime scene, can produce sufficient material to carry out forensic tests. PCR may also be used in addition to *in situ* hybridization (ISH) to study a specific genome of a tissue (Innis et al. 1990).

All of this leads to the role the histology laboratory plays in molecular pathology. In the histology laboratory the main method used in molecular pathology is ISH. John et al. (1969) and Gall and Pardue (1969) described the technique of ISH almost simultaneously.

ISH is a method of localizing and detecting specific mRNA sequences in preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest.

The method consists of denaturing (breaking apart) DNA and RNA strands using heat. A probe (a labeled complementary single strand) is incorporated with the DNA/RNA strands of interest. The strands will anneal with complementary nucleotides bonding back together with their homologous partners when cooled (Fig. 21.1). Some will anneal with the original complementary strands, but some will also anneal or hybridize with the probe. As probes increase in length, they become more specific. The chances of a probe finding a homologous sequence other than the target sequence decreases as the number of nucleotides in the probe increases. A longer probe can hybridize less specifically than shorter probes. Optimal probe size for ISH is small fragments of about 200–300 nucleotides. However, probes may be as small as 20–40 base pairs (bp) or as large as 1000 bp.

The detection of specific nucleic acid sequences (RNA, viral DNA or chromosomal DNA) in cells,

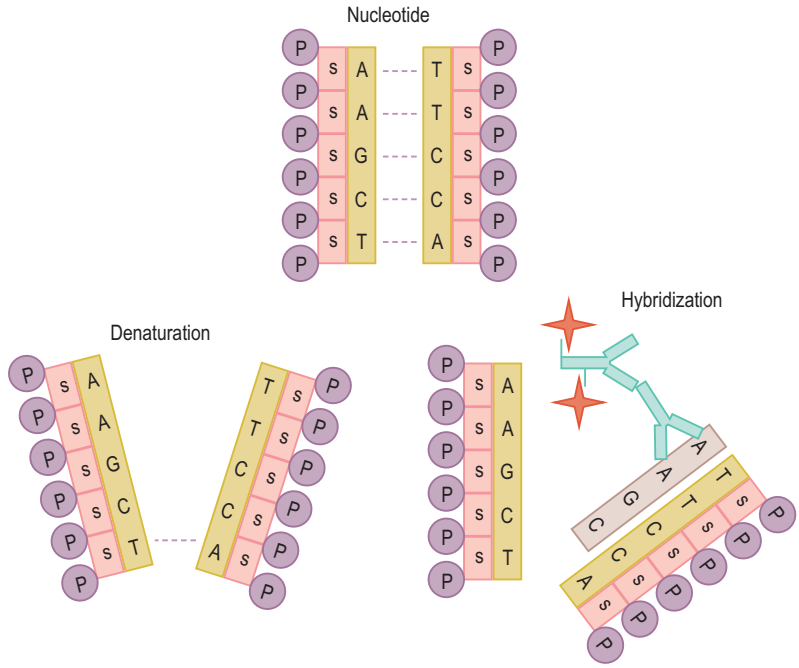


Figure 21.1 The genetic information for humans is encoded in billions of nucleotides (the building blocks of the DNA code) arranged in a double-helix molecule. Nucleotides consist of a base, a sugar (S), and a phosphate (P). The DNA code is written in an alphabet that uses four letters to represent each of the bases: (A) = adenine, (T) = thymine, (C) = cytosine, (G) = guanine. These bases will form pairs. (A) will only pair with (T). (G) will only pair with (C). Therefore, double-stranded DNA consists of two strands of homologous nucleotides. The genetic code in DNA is in triplets such as ATG. The base sequence of that triplet in the partner strand is therefore TAC.

tissues or whole organisms by ISH has numerous applications in biology, clinical and anatomical pathology, as well as in research.

ISH methods may employ radiolabeled probes that are visualized on a photographic film or photographic emulsion. However, most of these probes do not work well on routinely fixed, processed tissues and require the use of frozen sections. They may need 20–50 days of exposure before the results are visible. The development of non-radiolabeled probes that perform well on routine surgical and autopsy specimens has extended the field of anatomic pathology.

Detection of mRNA by using ISH is particularly useful if the protein product is quickly degraded or rapidly transported out of the target cell.

In ISH detection, immunohistochemistry (IHC)-like methods may be incorporated to detect the labeled (biotin, digoxigenin (DIG)) probe. So, the question arises, why not just do IHC? After all IHC is well-established, reliable, and less time consuming than ISH. IHC has been employed in the clinical and research arenas for several decades and has become a routine procedure in the histology laboratory. Furthermore, IHC has provided diagnostic procedures and a close look at the proteins within and on the cell membranes. The advantages of ISH over IHC include:

- High degree of specificity.
- DNA and mRNA are not as sensitive to formalin fixatives.
- Probe-target hybrid is stronger than antibody-antigen complex.
- Provides an alternative means of detection when reliable antibodies are not available.
- Provides a diagnosis at the molecular level.

It is important to understand the 'how and why' of the different stages in the ISH process in order for the testing to result in a functional outcome. This

revised chapter continues to focus on the 'how and why' of ISH, and includes a review of automated ISH versus the manual processes (Sterchi 2008). It also includes a revised comprehensive review of fluorescence *in situ* hybridization (FISH) staining procedures and analysis.

Applications

There are many modifications of ISH methods that relate to application needs. Although the demonstration of DNA and RNA sequences by ISH is a valuable research tool, according to Warford and Lauder (1991) and Mitchell et al. (1992), it is also used diagnostically in:

- detection of abnormal genes
- identification of viral infection
- tumor phenotyping

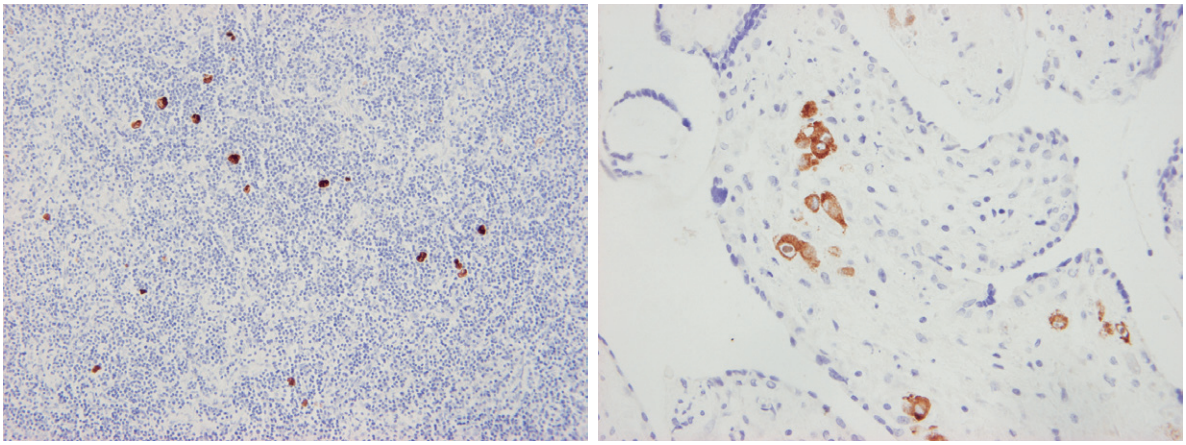
ISH comes in many forms and methods, and over the past 10 to 20 years the methodology has expanded significantly. At one time only FISH was the 'standard' method for ISH. Now there are methods that allow visualization of the stain using a bright field microscope, reducing the need for a fluorescence microscope. However, FISH still has an advantage over chromogenic methods for labeling specific nucleic acid sequences in cells and tissues. It is a 'direct' technique, so it is faster and in some cases it does not require IHC-like detection. These probes employ fluorescent (fluorescein) tags that glow under ultraviolet light to detect the hybridization. FISH allows the use of multiple probes on the same tissue that may spatially or spectrally overlap. The literature suggests that it is possible to distinguish at least four or five different fluorescent signals in a single sample (Haugland & Spence 2005), whereas chromagens are often limited to one or two color options per slide. FISH, also known as molecular cytogenetics, has enabled a huge advance in the diagnostic and prognostic capability of the clinical cytogenetic laboratory. FISH can also vividly paint chromosomes or portions of chromosomes with fluorescent molecules (thus, the term 'chromosome painting').

ISH can provide cytological information on the location and alteration of genomic sequences in chromosomes. Traditionally, the technique has been applied to metaphase chromosome spreads (Davis et al. 1984; Lux et al. 1990), but it has been shown to be applicable to interphase nuclei (Hopman et al. 1988; Poddighe et al. 1992). Routine paraffin wax preparations of tissues can be used and 'interphase cytogenetics', as the method is termed, can provide direct information on chromosomal abnormalities in unselected tumor cell populations.

Viral identification can be undertaken using a variety of methods, of which only immunohistochemistry and ISH provide simultaneous morphological information. The sensitivity of immunohistochemistry for the visualization of viral antigens, and ISH for the demonstration of cytomegalovirus, correlate well (Van den Berg et al. 1989). Most viral ISH methods use probes for DNA. Others, such as in the demonstration of the Epstein-Barr virus (Fig. 21.2), the detection of a virally encoded RNA transcript, provide results that are more sensitive than the use of antibodies and may even approach that of the PCR (Pringle et al. 1992).

The light chain portion kappa and lambda mRNA may be detected in normal and neoplastic B cells in human lymphoid tissue. Restriction of either kappa or lambda mRNA denotes monoclonality of lymphoid neoplasms and is useful in distinguishing between neoplastic and reactive lymphoid proliferations. Due to the destruction of RNAases by formalin fixation, kappa and lambda sequences are conserved in routine surgical tissues. (See Data Sheet Kappa and Lambda Probe ISH Kit, Novacastra.) (Fig. 21.3.)

Chromogenic *in situ* hybridization (CISH) is a method 'that enables the detection of gene expression in the nucleus using a conventional histochemical reaction' (White 2005); it is used for the detection of abnormal genes and to identify a gene therapy treatment direction. CISH can be used as an alternative in screening archived breast cancer tissue samples for HER2/neu (type 1 growth factor receptor gene) (Madrid & Lo 2004). Automated CISH techniques were used for detecting light chain expression in paraffin sections on plasma cell dyscrasias and B-cell non-Hodgkin lymphomas



a b

Figure 21.2 Example of automatic chromogenic *in situ* hybridization (CISH) staining. (a) Epstein-Barr virus-encoded RNA (EBER) and (b) cytomegalovirus (CMV). (Photographs courtesy of Leica Microsystems, Inc.)

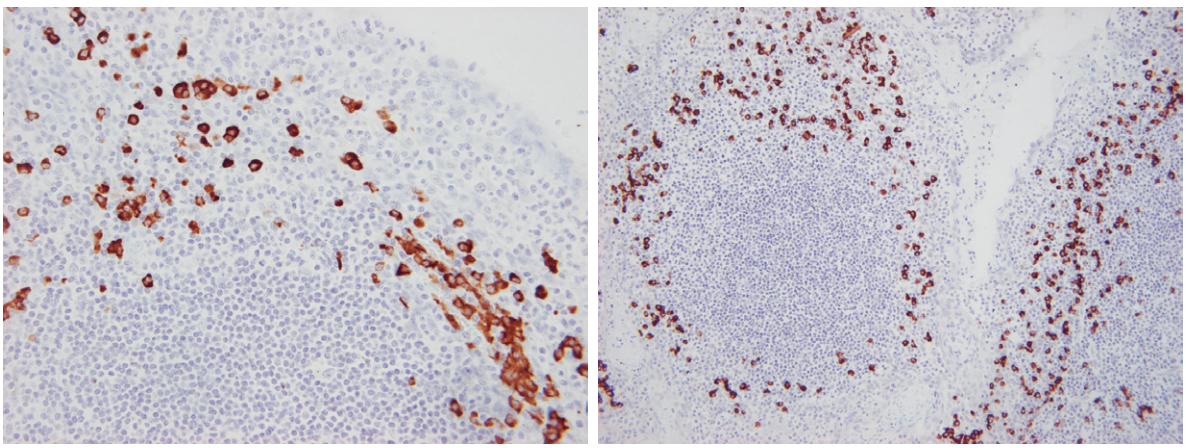


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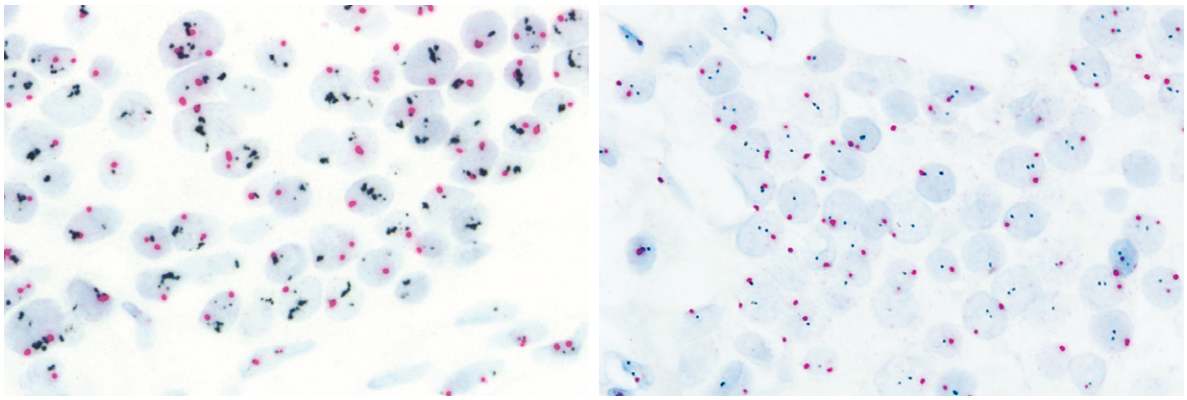
Figure 21.3 Example of automatic chromogenic *in situ* hybridization (CISH) staining (a) kappa (b) lambda. (Photographs courtesy of Leica Microsystems, Inc.)

'appeared superior to IHC' in that the ISH resulted with no background staining (Beck et al. 2003).

Silver precipitation *in situ* hybridization (SISH) is an emerging ISH method that works well with formalin fixed paraffin embedded (FFPE) tissues. It is also similar to FISH performance in detecting the location of genomic targets using probes. The major advantage of CISH and SISH is the possibility of long term storage of the stained slides. The chromagens or silver signals do not quench over time, unlike FISH signals (Fig. 21.4).

In situ zymography (ISZ) is a method that uses specific protease substrates to detect and localize protease activities in tissue sections. In the regulation of biological processes, proteases modulate several cellular functions. Several molecular techniques identify and characterize proteases in cells and tissue, such as a Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) but ISZ works as well. One of its drawbacks is that unfixed fresh frozen tissues must be used. In contrast, its advantages are that it costs less than



a b

Figure 21.4 Example of automatic silver (silver deposition technology) *in situ* hybridization (SISH) staining. (a) HER2 and Chr17. (b) HER2 and Chr17. (Photographs courtesy of Ventana Medical Systems, Inc.)

conventional ISH methods, there are two approaches (one uses a photographic emulsion, the other uses a fluorescent-labeled substrate) and it is applicable to almost any protease (Yan & Blomme 2003).

Immunolabeling electron microscopy (IEM) in combination with ISH has been used in detecting severe acute respiratory syndrome (SARS). Viral immunogold labeling and ultrastructural ISH were used to analyze the morphogenesis of this recently emergent virus. A negative-sense riboprobe was used for the ultrastructural ISH (Goldsmith et al. 2004).

Polymerase chain reaction-ISH (PISH) is another form of ISH. Viral RNA is detected by RT-PCR, using formalin-fixed paraffin-embedded tissue (FFPE). PISH results have been compared to IHC on staining for Newcastle disease in veterinary medicine. Newcastle disease is an avian viral infection that has a potential for rapid spread and may cause serious economic impact and international trade restrictions in the poultry industry (Wakamatsu et al. 2005). PISH is also used in the detection of human papillomavirus in uterine cervical neoplasia (Xiao et al. 2001).

The identification of mRNA sequences by *in situ* hybridization may be the technique of choice for the rapid and sensitive identification of viral infection. Another advantage of *in situ* hybridization for viral detection is that some viral coat antigens are not expressed at certain stages of the viral replication cycle, thus negating the use of immunohistochemical methods.

ISH methods have been developed over the years so that most FFPE tissues, including decalcified tissues, can be used (Janneke et al. 1999).

Another area in which *in situ* hybridization and immunochemistry can be viewed as complementary techniques is in the phenotyping of tumors. Many monoclonal and polyclonal antibodies are available for phenotyping and these may be employed in sensitive and rapid techniques. When problems arise in the interpretation of immunohistochemical results, mRNA phenotyping by *in situ* hybridization can be helpful (Pringle et al. 1990, 1993; Kendall et al. 1991; Ruprai et al. 1991).

Common reagents

Listed here are names of reagents that are used in ISH techniques. The formulae for preparing these reagents are located in Appendix VIII. The majority of these reagents can be purchased pre-mixed or in a kit for easy mixing. Keep in mind that different reagents may be suggested with some ISH methods and automatic IHC equipment often provide prepackage reagents as ready to use (RTU) with their equipment. Purchasing the reagents pre-mixed or

in kits is convenient and safer, and it provides some reassurance that they are mixed according to manufacturer's specifications and guaranteed by the vendor. This may cut down the possibility of human error.

1. Diethylpyrocarbonate (DEPC) treated water
2. 2% aminoalkylsilane (positively charged slides)
   These slides may be purchased pre-coated
   Make sure they are RNA/DNA free
3. Proteinase K
   Aliquot and freeze below –20°C
4. Hyaluronidase
5. 0.1 M triethanolamine (TEA), freshly made
6. 1 M Tris (this is to make buffers that vary in pH: buffer #1, pH 7.5; buffer #2, pH 9.5)
7. 1 M magnesium chloride
8. 5 M sodium chloride
9. Maleic acid buffer a washing buffer
10. 20× Saline sodium citrate (SSC) buffer (this is also used to make 2× SCC and 1× SCC buffers)
11. Denhart's solution
    (may cause increase in background)
12. Prehybridization solution
13. Hybridization solution
14. Detection method reagents:
    a. Streptavidin-alkaline phosphatase
    b. Anti-digoxigenin
    c. Horseradish peroxidase (HRP)
15. Colorimetric detection reagents:
    a. 5-bromo-4-chloro-3-indolyl phosphate (BCIP) nitro-blue tetrazolium salt (NBT)
    b. 3-amino-9-ethylcarbazole (AEC)
    c. Diaminobenzidine (DAB)

Probes and their choice

Probe choice is based on the type of sequence you are trying to detect. The technologist needs to optimize the conditions used as much as possible. The strength of the bonds between the probe and the target plays an important role. The strength decreases in the order RNA-RNA to DNA-RNA. Various hybridization conditions such as concentration of formamide, salt concentration, hybridization temperature, and pH influence this stability.

A probe is a labeled fragment of DNA or RNA used to find its complementary sequence or locate a particular clone. The choice of probes will depend on availability, sensitivity, and resolution required. The sensitivity of the probe will also depend on the degree of substitution and the size of the labeled fragments. Degree of substitution refers to the original nucleotide substituted by the labeled analogues. The sensitivity of detection correlates with the amount of label substituted. In general, probes with 25–32% substitution yield the highest sensitivity. There are several different types of probe. Each has unique characteristics that must be considered for each application.

Probes for DNA:

- Double-stranded DNA
- Single-stranded DNA
- Oligodeoxyribonucleotides

Probe for RNA:

- Single-stranded complementary RNA, a riboprobe

Probe type and means of synthesis

There are essentially four types of probe that can be used in performing *in situ* hybridization. Oligonucleotide probes are usually 20–50 bases in length. They are produced synthetically by an automated chemical synthesis employing a specific DNA nucleotide sequence (of your choice). These probes are resistant to RNases and are small, thus allowing easy penetration into the cells or tissue of interest. However, the small size has a disadvantage in that it covers fewer targets. The label should be positioned at the 3′ or the 5′ end. To increase sensitivity one can use a mixture of oligonucleotides that are complementary to different regions of the target molecule. Oligonucleotide protocols can be standardized for many different probes regardless of the target genes being sought. Another advantage of oligonucleotide probes is that they are single

stranded, therefore excluding the possibility of renaturation.

Single-stranded DNA probes cover a much larger size range (200–500 bp) than oligonucleotide probes. They can be prepared by a primer extension on single-stranded templates by RT-PCR of RNA, or by an amplified primer extension of a PCR-generated fragment in the presence of a single antisense primer, or by the chemical synthesis of oligonucleotides. PCR-based methods are much easier and probes can be synthesized from small amounts of starting material. Moreover, PCR allows great flexibility in the choice of probe sequences by the use of appropriate primers.

Double-stranded DNA probes can be prepared by nick-translation, random primer, or PCR in the presence of a labeled nucleotide, and denatured prior to hybridization in order for one strand to hybridize with the mRNA of interest. They can also be produced by the inclusion of the sequence of interest in bacteria, which is replicated, lysed, and then the DNA is extracted and purified. The sequence of interest is removed with restriction enzymes. Random priming and PCR give the highest specific activities. These probes are less sensitive than single-stranded probes, since the two strands have a tendency to rehybridize to each other, thus reducing the concentration of probe available for hybridization to the target. Nevertheless, the sensitivity obtained with double-stranded probes is sufficient for many purposes, although they are not widely used today.

RNA probes (cRNA probes or riboprobes) are thermostable and are resistant to digestion by RNases. These probes are single stranded and are the most widely used in ISH. RNA probes are generated by *in vitro* transcription from a linearized template using a promoter for RNA polymerase that must be available on the vector DNA containing the template (SP6, T7, or T3). RNA polymerase is used to synthesize RNA complementary to the DNA substrate. Most commonly, the probe sequence is cloned into a plasmid vector so that it is flanked by two different RNA polymerase initiation sites enabling either sense-strand (control) or antisense (probe) RNA to be synthesized. The plasmid is linearized with a restriction enzyme so that plasmid sequences are not transcribed, since these may cause high backgrounds. Single-stranded probes provide advantages over double-stranded probes such as:

- The probe does not self-anneal in solution, so the probe is not exhausted.
- Large probe chains are not formed in solution; thus, probe penetration is not affected.

If high sensitivity is required, single-stranded probes should be used (Table 21.1).

Probe preparation and labeling

To visualize where the probe has bound within your tissue section or within your cells, you must attach a detectable label to your probe before hybridization. Two major choices must be made for the preparation of a probe:

- What type of nucleic acid is to be used (DNA or RNA, single or double stranded)?
- What type of label is to be incorporated into the probe?

A vital consideration is the length of the probe, and the means by which this is controlled depends on the type and the method of synthesis. There are two methods of probe labeling. They are:

- Direct: the reporter molecules (enzyme, radioisotope or fluorescent marker) are directly attached to the DNA or RNA.
- Indirect: a hapten (biotin, digoxigenin, or fluorescein) is attached to the probe and detected by a labeled binding protein (typically an antibody).

Methods for incorporating labels into DNA are nick translation and random primer methods.

Oligonucleotide probe labeling

5′-end labeling

The 5′ end of DNA or RNA undergoes direct phosphorylation of the free 5′-terminal OH groups. The free 5′-OH substrates can be labeled using T4 polynucleotide kinase. This method is usually used

Table 21.1 Probe types

Probe	Labeling	Advantages	Disadvantages
dsDNA	Random primers	Easy to use Subcloning unnecessary Choice of labeling methods High specific activity Possibility of signal amplification (networking) Readily available	Re-annealing during hybridization (decreased probe availability) Probe denaturation required, increasing probe length and decreasing tissue penetration Hybrids less stable than RNA probes
ssDNA	Primer extension	No probe denaturation needed No re-annealing during hybridization (single strand) More sensitive Stable	Technically complex Subcloning required Hybrids less stable than RNA probes Template binding
ssRNA	DNA polymerase transcription	High specific activity No probe denaturation needed No re-annealing Unhybridized probe enzymatically destroyed, sparing hybrid	Subcloning needed Less tissue penetration RNase labile May have higher levels of non-specific binding to tissue components, thus increasing the chance of higher background and lower penetration of the probe into the tissue
Oligo	5′ end 3′ end 3′ tailing	No cloning or molecular biology expertise required Stable Good tissue penetration (small size) Constructed according to recipe from amino acid data No self-hybridization Limited labeling methods Short oligonucleotide sequences can be directly manufactured Can use multiple probes, no competition between probes	Limited labeling methods Lower specific activity, so less sensitive Dependent on published sequences Less stable hybrids

for radiolabeling. Non-radiolabels use a covalent linker.

3′-end labeling

Terminal dexoxynucleotidyl transferase (TdT) is used to add a labeled residue to the 3′ end of a synthetic oligonucleotide that is approximately 14–100 nucleotides in length. These probes provide excellent specificity but only moderate sensitivity. See the oligonucleotide 3′-end labeling procedure on page 555 of sixth edition.

3′ tailing

A tail containing labeled nucleotides is added to the free 3′ end of double- or single-stranded DNA using TdT. These probes are more sensitive than the 3′-end

labeled versions, but can produce more non-specific background. Oligonucleotide tailing kits are commercially available.

It should be noted that the use of commercially available labeling kits can greatly assist in making methods simpler to undertake while providing results of an assured standard.

Purification of labeled probes

There are several methods that can be used to test the purification. If the probe is homemade or purchased it must be tested for its sensitivity to the selected target. Here is a list of methods that can be used, but it is advisable to follow the manufacturers' recommendation on their use:

- Sephadex G-50 column
- Sephadex G-50 chromatography
- Selective precipitation

Estimating the labeling efficiency and testing the probe

It is always good practice to estimate the yield of labeled nucleic acids. This confirms a successful labeling reaction before performing the staining.

Before using a labeled probe, it is useful to prepare and demonstrate test strips to gauge the degree of label incorporation. This may be done by using the normal detection procedure for the ISH method on dots of labeled nucleic acid sequence and a labeled control applied at matching descending concentrations on a positively charged nylon membrane. Some technicians will prepare several test strips from one labeled sequence to compare the sensitivity of different detection systems. The nylon membrane is subjected to an immunological detection which can be either a colorimetric or chemiluminescent method depending on the protocol used. Direct comparison of the signal intensities of sample and control allows estimation of labeling yield. Kits for this technique are commercially available in which the labeled control already exists on a test strip. A much quicker method for estimating the yield of labeled nucleic acids is to use a bioanalyzer, since this can give quantitative results in as little as 30 minutes.

A method for estimating labeling efficiency of the nucleic acid using a dilution series followed by a spot test is described below.

Preparation of the dilution series

1. Dilute the labeling probe using dilution buffer to a starting concentration of 2.5 pmol/µl.
2. Make a dilution series of purified probe in Eppendorf tubes to give nucleic acid concentrations of 300 pg/µl, 100 pg/µl, 30 pg/µl, 10 pg/µl, 3 pg/µl, and one tube containing diluent only. Ensure that all tube volumes are equal. Repeat the same dilution series with your control or used pre-labeled (with control) test strips.
3. Apply 1 µl drops from each tube onto the nylon membrane (Roche). The control dilutions should be lined up with the test sample dilution concentration. For an example of placing spots, see Figure 21.5.
4. Label the position of each application with a pencil on the side of the strip (not on the strip).
5. Fix the nucleic acid to the membrane by either baking the membrane for 30 minutes at 120°C or using a UV light.
6. Wash the membrane briefly in washing buffer.
7. Immerse in blocking solution for 10 minutes.
8. Incubate with reagents used in ISH detection technique.

Note: Dilute reagents in blocking solution and use this solution for washing.

9. Detect enzyme using the same solutions and procedure as for ISH method.
10. Rinse in double-distilled water and blot dry.

Commercially made probes

Custom designed, pre-made cloned DNA and oligonucleotide sequences and labeling kits are commercially available. Their use can make methods simpler

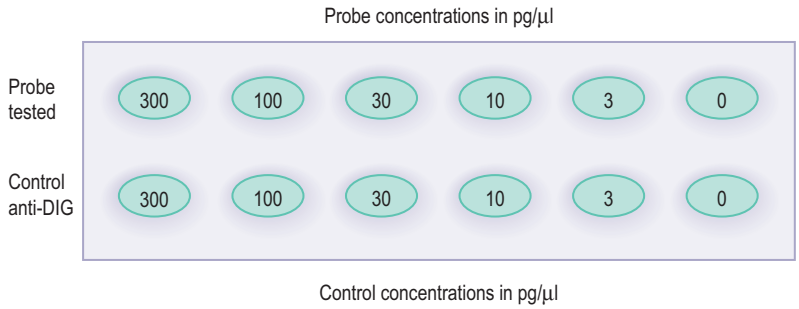


Figure 21.5 Diagram of 'dot test' for estimating the labeling efficiency and testing the probe.

to undertake while providing results of an assured standard. Depending on the type of laboratory you have and how many ISH requests you receive, premixed reagents and pre-labeled probes can be cost-effective. The kits, reagents, or ordering the labeled probes can cut down on precious technologist time, and they come with instructions – a benefit for the novice technologist. However, this does not mean that the theory behind ISH can be ignored. The technologist must understand ISH to order labeled probes and the ISH kits.

Probe concentration

For DNA probes the concentration of the probe will be ~0.5–2 µg/ml. Oligonucleotide probes can be used with, or without, acetylation. Probes without acetylation pretreatment of the sample will have a concentration of ~50–200 ng/ml and may provide more intense results with minimal background. For probes with acetylation pretreatment, a higher concentration of oligonucleotide probe may be used without incurring non-specific background staining.

Length of probe

As mentioned previously, one must consider the length of the probe. Longer probes give weaker signals and they penetrate less effectively into the cross-linked (fixed) tissue. The extent of weaker signals and penetration depends also on the nature of the tissue, the choice of fixative and whether a pretreatment has been carried out.

The length of probe can be controlled in either the synthesis reaction or the subsequent partial cleavage. In nick-translated probes, the DNA length is determined by the amount of DNase in the reaction, whereas in random priming the length is determined by concentration of the primer. Long RNA probes may show poor tissue penetration, whereas chemical shortening (hydrolysis) enhances tissue penetration, and may also increases the likelihood of non-selective binding to other non-targeted gene sequences.

Once a probe is prepared, its size should be checked. If the probe is too small, it may yield low signals with high background. It is necessary to know whether a reduction in probe size (length) will improve both the signal for the tissue and the preparation method used.

Detection

The choice of detection system will be principally determined by the probe label used and secondly by the ISH procedure type. One must consider the sensitivity and resolution required.

Colorimetric detection substrate systems include horseradish peroxidase with either 3-amino-9-ethylcarbazole (AEC) or 3,3′-diaminobenzidine tetra-hydrochloride (DAB) substrates. AEC forms a red-brown product which is alcohol soluble; therefore aqueous mounting media are required. Methyl

green/blue has been the most often used counterstain in earlier publications but is losing popularity. DAB forms a permanent, insoluble, brown product that is compatible with solvent-based mounting media. Alkaline phosphatase systems can use 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) or fast red. BCIP/NBT forms a purple/blue alcohol-insoluble stain. Eosin is a compatible counterstain if a nuclear target is expected, or nuclear fast red if the target is cytoplasmic. Fast red forms an intense red product which is alcohol soluble and an aqueous mounting media is required. Methyl green or blue is compatible if a nuclear target is expected, or light hematoxylin if the target is cytoplasmic.

Detection methods can be either direct or indirect. Incorporation of a stable hapten to a probe is the cornerstone of non-radiographic detection. Hybridized probes can be detected by enzymatic reactions that produce a colored precipitate at the site of hybridization. The most commonly used enzymes for this application are alkaline phosphatase (AP) and horseradish peroxidase (HRP). Although these enzymes can be conjugated directly to nucleic acid probes, such enzyme-coupled probes are often inappropriate for ISH to tissue preparations because probe penetration is hampered by the presence of the conjugated enzyme. Therefore, indirect methods are preferred (Knoll & Lichter 1995).

Biotin, fluorochromes (fluorescein), and digoxigenin (DIG) are the most common labels used. Probes that are labeled with these reporter molecules are usually detected by an AP or HRP conjugate to avidin (for biotin) or antibodies (for DIG). Fluorescein and DIG have an advantage over biotin in that they produce lower levels of background signals in tissues that contain high amounts of endogenous biotin.

Direct detection of a fluorescent label is often employed for the demonstration of multiple chromosome targets (Nederlof et al. 1989), but when single target sequences are to be identified, indirect methods may be used.

To reduce non-specific staining (particularly of collagen) by indirect detection reagents it is advisable to pre-incubate preparations in a Tris (Triton X-100 or Tween) buffer solution containing bovine serum albumin. It may also prove beneficial to use this solution as the diluent for the primary detection reagent. However, at this step it is more important to use a Fab fragment of an antibody as this may reduce background staining.

Indirect detection procedures offer increased sensitivity. The selection of the enzyme and substrate (De Jong et al. 1985) should be included in weighing the benefits of different detection systems. A substrate system that employs conjugated antibodies such as anti-DIG or anti-FITC that are conjugated with AP together with the application of a colorimetric BCIP/NBT that can be cycled to produce an insoluble blue/black precipitate over a period of 24 hours is recommended. Another substrate system one could use for a more intense fluorescent signal is a fluorescent 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate (HNPP) with fast red TR.

The main advantages of this procedure are low levels of non-specific staining, simplicity, and the use of an enzyme substrate system that can produce an insoluble blue/black precipitate.

Many commercially available probes for ISH are labeled with biotin. When used in combination with streptavidin detection systems, high sensitivity can be achieved. A disadvantage of this combination is having a widespread endogenous tissue distribution. Substantial quantities of endogenous biotin are, for example, present in the liver and kidney (Wood & Warnke 1981), as well as in other tissues, such as pituitary, submandibular gland, thyroid, and parathyroid. Furthermore, proliferating cells may often produce enough biotin to make the discrimination between true and false-positive results difficult. However, methods of blocking endogenous biotin have greatly improved and work well to prevent false positives.

Digoxigenin (Herrington et al. 1989) in combination with a Fab fragment-enzyme conjugate detection system currently provides results of equal or superior sensitivity to biotin, with extremely low non-specific background staining. Another label that may be used in conjunction with a single-step detection method is fluorescein. Using this label it is possible to undertake rapid ISH methods in which

target sequences of moderate to high copy number can be demonstrated in a working day.

Sample preparation

Fixation

Fixation is an initial step in specimen preparation or can be an intermediate step in a protocol, as in methods using cryostat sections. The duration, type, and temperature of fixation may also differ according to preparation. Together, these factors will have an effect, not only on the preservation of the tissue but also on the retention of nucleic acid and the resistance of DNA and RNA to nuclease digestion. The choice of fixative will have an influence on the conservation of nucleic acids and their availability for hybridization. Specimens that are immersion-fixed prior to paraffin embedding appear to be unaffected by 'normal' contamination levels of nucleases, thus indicating that only the hybridization solutions need to be scrupulously free of the enzymes.

The functional groups involved in base pairing are protected in the double-helix structure of duplex DNA. RNA is fairly unreactive to cross-linking agents.

Methanol/acetic acid fixation is recommended for metaphase chromosome spreads. Cryostat sections may be fixed with 4% formaldehyde (~30 minutes), Bouin's fixative, or paraformaldehyde vapor fixation. This fixation also helps to secure the tissue to the slide.

Proteins surround DNA and RNA target sequences and the extensive cross-linking of these proteins may mask the target nucleic acid. Therefore, 'permeabilization' procedures may be required.

After the tissue is removed from the patient or animal, it must be fixed to prevent autolysis, inhibit bacterial/fungal growth, and make it resistant to damage from subsequent processing. There are two main groups of fixatives, coagulant and non-coagulant, classified by their reaction with soluble proteins. Ethanol and mercuric chloride are coagulant fixatives. They are not the preferred fixative for use with ISH, since ethanol dehydrates, coagulates, and precipitates cellular proteins, nucleic acids, and carbohydrates. Covalent bonding does not occur with ethanol fixatives and the tissue components, so mRNA is not anchored within the tissue and is likely to be lost during post-fixation processing procedures. For ISH, non-coagulant, cross-linking aldehydes (formaldehyde, paraformaldehyde, and glutaraldehyde) are recommended.

Tissues fixed for ISH should retain mRNA within the tissue but not raise background. Both background and signal are generally higher on perfused-fixed paraffin tissue sections than on frozen sections. The signal-to-noise (S/N) ratio on perfused-fixed tissue sections is better.

Most commonly, tissue specimens are routinely fixed in 10% buffered formalin, processed overnight in an automatic tissue processor, and embedded in paraffin wax. Fixation time of 8–12 hours is optimal. Keep in mind that the longer the fixation, the more rigorous the enzyme digestion is required to optimize the signal. Alcohol-fixed tissues should be post-fixed with an aldehyde fixative to prevent the diffusion of mRNA (if you are looking at RNA).

Slide/section preparation

Sections are cut at 4–6 μm on an alcohol-cleaned microtome using positively charged or hand-coated slides. Sections are drained well and then air-dried at room temperature. After deparaffinization, slides are placed in an alcohol-cleaned staining container of DEPC water. The staining container is then placed in the heated water bath at 23–37°C and held until the start of ISH. Gloves must be worn to prevent contamination, and all utensils, such as brushes and forceps, should be cleaned with alcohol and kept within the cleaned area designated for ISH.

Proteolytic digestion

The use of formaldehyde-based fixatives prior to paraffin embedding of specimens will mask nucleic acid sequences. Digestion is a important step when performing ISH. Digestion improves probe penetration by increasing cell permeability with minimal

tissue degradation. Although the nucleic acid is not directly affected by proteolytic digestion, it is important to control this step carefully. Under-digestion will result in insufficient exposure of the nucleic acid, while over-digestion can sufficiently weaken the protein structure surrounding the sequence, bringing about its loss into subsequent solutions. mRNA sequences tend to be loosely associated with proteins, while DNA targets are intimately associated with histone and other nuclear proteins. Due to these differences, the concentration of proteolytic enzyme required to unmask mRNA will be less than that necessary to expose DNA.

The selection of the proteolytic enzyme is important. This should be of molecular biology grade to ensure the absence of nuclease activity. Proteinase K and pepsin are two enzymes commonly used for digestion. Proteinase K has an advantage over other proteolytic enzymes because during incubation it digests any nucleases that might be present. However, higher concentrations of the enzyme may be required, depending on the tissue fixation time.

Nuclease digestion is used as a negative control. Treatment of same tissue/patient tissue sections with RNase A will demonstrate that ISH signals are due to RNA hybridization.

Proteoglycan digestion is required for bone and cartilage. Kidney and brain may also need proteoglycan digestion, if the signal is weak. Post-fixation in 4% paraformaldehyde is necessary after digestion to prevent tissue loss. In addition, post-fixation after digestion (for all digestions) prevents leaching and will inhibit RNase activity.

When ISH methods are used to demonstrate mRNA sequences, non-specific attachment of digoxigenin and fluorescein-labeled oligonucleotides to epithelial tissues can create non-specific results. To minimize this interaction, preparations can be acetylated after proteolytic digestion and before post-fixation. Acetylation decreases non-specific binding of the probe to the tissue. Positive charges on the tissue are neutralized by reducing electrostatic binding of the probe.

Prehybridization is intended to reduce non-specific binding. Sites in the tissue become saturated with the components of the prehybridization solution preventing non-specific binding. The purpose of the prehybridization solution is to equilibrate the specimen with the hybridization solution prior to the addition of the probe, and to allow anionic macromolecules to block sites of potential non-specific probe interaction. The prehybridization solution contains all the ingredients of the hybridization mixture except the probe. Non-complementary sequences such as bovine serum albumin (BSA at 1 mg/ml), Denhardt solution (Ficoll, BSA, and polyvinylpyrrolidone all at 0.02%), and tRNA are used to reduce non-specific binding. Most data indicate that blocking is required, but a separate prehybridization step may not be necessary. In some cases, adequate blocking may be accomplished during the hybridization step. Electrostatic binding of probes to the tissue and slides can be neutralized by treatment in TEA buffer containing 0.1 M triethanolamine.

Hybridization

Hybridization occurs after denaturization, during cooling, in the presence of a complementary probe, and permits hydrogen bonding of the two strands of nucleic acids. The probe must form stable hydrogen bonds with the target with minimal hybridization with non-target sequences. The probe and target sequences must be single stranded. Simultaneously heating the probe and target to high temperatures may increase the consistency and sensitivity of detection. This can only be met if care is taken to precisely control this step of the ISH procedure. Control is achieved through balancing the various components of the hybridization solution and hybridizing at an optimal temperature for the correct length of time.

If a DNA probe is employed or a DNA target demonstrated, then it is essential that these are rendered single stranded. This is achieved by using dry heat with the hybridization solution placed over the specimen, which is then covered with a coverslip, plastic sheet, or cap. The denaturation will differ according to the percentage of guanine-cytosine

base pairs within the target sequence of interest. When there is a high percentage of guanine-cytosine base pairs present, the third hydrogen bond associated with the base pair will occur at a higher melting temperature (T_m) than in the sequences in which adenosine and thymidine pairing predominates. Overheating to temperatures greater than 100°C at this stage may compromise specimen preservation.

At the molecular level, hybridization involves an initial nucleation reaction between a few bases, followed by hydrogen bonding of the remaining sequences. The control of temperature during hybridization is crucial, as variations will influence the specificity (stringency) of annealing. RNA and DNA hybrids are formed optimally at about 25°C, below their T_m, but when lower temperatures are used some partial homologous annealing may occur. Although this situation should be avoided, it can be usefully employed to screen for sequences with partial homology (e.g. human papillomavirus subtypes). By incorporating formamide, a helix-destabilizing reagent, the annealing may be maintained using lower temperatures (e.g. at 37°C, at which the tissue preservation is not affected).

Stringency can also be altered by adjusting the availability of monovalent cations in the hybridization solution. These cations are usually supplied by sodium chloride and they regulate the degree of natural electrostatic repulsion between the probe and target sequences. When used at high concentration their effect is to produce conditions of low stringency, while at low concentrations only sequences with complete homology can hybridize.

Anionic macromolecules are often included in the hybridization solution to reduce non-specific interactions of the probe. Sonicated and denatured salmon sperm DNA can shield non-homologous nucleic acid sequences from the probe and reduce the opportunity for cellular electrostatic interactions. Dextran sulfate will also reduce the possibility of cellular electrostatic interactions and locally concentrate the probe, enhancing the rate of hybridization. Particular attention should be taken to ensure that hybridization solutions are prepared using reagents free from nuclease contamination.

The rate of annealing will be influenced by time and temperature as well as by the composition of the hybridization solution as discussed above. Due to steric constraints, ISH proceeds at a slower rate than in blotting methods. However, high probe concentrations can be used to compensate for this factor. Hybridization times of 1–2 hours for biotinylated and fluorescein-labeled probes are often effective. However, with digoxigenin-labeled probes, overnight hybridization may be required to provide high sensitivity.

Post-hybridization washes are used to adjust the stringency of hybridization. The sections must be rinsed with solutions that contain high concentrations of salt to remove the unbound probe. Subsequent washing with solutions containing decreasing salt concentrations and increasing temperature reduces mismatching of base pairs. Longer probes and those with higher G + C content are more stable. Increases in temperature and formamide concentration are the destabilizing factors. By reducing the concentration of formamide in the hybridization solution, while maintaining a constant temperature, the annealing conditions will become less stringent, thereby increasing the sensitivity of mRNA detection when using fluorescein-labeled oligonucleotide probes.

Controls

It is essential to include controls to verify ISH results. A positive and no-hybridization control should always be included when undertaking an ISH procedure. The positive control should contain the target sequence being demonstrated and be prepared the same as the test samples. It should receive the same solutions and go through the same procedural steps as the test samples. This will provide a gauge for the overall performance of the technique. If the same positive control is used from run to run, it will help validate the ISH staining reproducibility.

Other controls may be incorporated to test the results' validity. The number and type of controls to be incorporated into the technique is determined at the discretion of the laboratory's personnel and standard operating procedures. As mentioned under the

digestion section earlier in this chapter, nuclease digestion can be used as a negative control.

Equipment and reagent preparation

DNA and RNA can be degraded by nuclease activity. Indeed, high concentrations of DNase and RNase are useful in confirming the nucleic acid type specificity of hybridization. However, at much lower concentrations, nucleases present on the skin may contaminate solutions used in hybridization methods sufficiently to degrade the quality of naked DNA and RNA. For this reason, in addition to the wearing of gloves, elaborate precautions are often taken to ensure the absence of nucleases from solutions used in hybridization techniques.

Treatment of solutions and glassware to destroy nuclease activity

DNase is destroyed by autoclaving. RNase is resistant to heat inactivation and therefore other procedures should be used as described below.

Preparation of DEPC-treated water

Add diethylpyrocarbonate (DEPC) (Sigma D5758) to pure water to a final concentration of 0.1%. This should be done in a fume hood. Shake well to dissolve and allow it to stand overnight. Autoclave the solution and container the following day.

Preparation of DEPC-treated solutions

Prepare solutions, then add DEPC to 0.1%. Shake and leave overnight, then autoclave.

Note

Adding full-strength DEPC directly to a buffer may alter the buffering properties. Solutions that require a buffer of that type should be made up in RNase-free glassware using pre-mixed autoclaved DEPC-treated water.

Preparation of glassware

Treat glassware at 200°C overnight or, if delicate:

1. Wash in a mild/low suds soap or RNase Away solution.
2. Rinse in double-distilled nuclease-free water until detergent is removed.
3. Soak in 3% aqueous hydrogen peroxide, 10 minutes.
4. Rinse in DEPC-treated water.
5. Dry and protect from dust.

Most laboratories purchase DEPC-treated water and use sterile plastic disposable containers in place of the glassware. The information in this chapter is for those laboratories that prefer to prepare their own DEPC water and use glass bottles for buffer storage.

Universal ISH method (no frills or specialized equipment)

Day 1

1. Deparaffinize slides completely. Three changes of xylene and/or substitute for 4–8 minutes each.

Note

Incomplete deparaffinization may cause a weak reaction.

2. Dehydrate through two changes 100% (ethanol) EtOH, 1 change 95% EtOH for 3 minutes each. Rinse in DEPC-treated water or use slides already in warmed DEPC-treated water. Rinse in warmed (23–37°C) Tris/saline buffer #1, pH 7.5, and drain.
3. De-proteinize sections in freshly prepared proteinase K solution at 23–37°C, in a moist chamber for 15 minutes.
4. Rinse in Tris/saline buffer #1 at room temperature for 5 minutes. If necessary, digest proteoglycans and/or acetylation before going to next step.
5. Dehydrate slides through one change of 95% EtOH and two changes 100% EtOH for 2 minutes each. Air-dry for 5 minutes. This step is omitted if prehybridizing (next step).
6. Apply the prehybridization solution by putting 1–2 drops (60–100 µl) on the sections. Incubate in a moist chamber at room temperature for 1 hour. Blot off all excess prehybridization solution before adding the probe.

7. Apply hybridization fluid (probe) and cover with a heat-resistant film (microwave wrap, coverslips, or chambers). The probe is tailed with either biotin-dUTP or digoxigenin-dUTP.
8. Initiate hybridization by denaturing slides for 5–10 minutes at 92–100°C (try not to exceed 100°C). Use a preheated 'metal' tray to set slides on for optimal denaturization. Cool slides to 37–42°C and incubate in humidity chamber for 18–24 hours. Agitation may enhance reaction. Since this method is not using a commercial kit, the staining continues on day 2.

Day 2

9. Rinse slides twice in 2× SSC and twice in 1× SSC for 5 minutes each at 37°C.
10. Apply a 5% blocking solution at 37°C for 10 minutes.
11. Rinse in buffer #1 for 2 minutes, followed by a 10-minute rinse in buffer #2.
12. Add 1–2 drops of detection reagent to each slide (streptavidin-AP or anti-digoxigenin-AP). Incubate in a humidity chamber for 20–30 minutes at 37°C. During the incubation period, prepare the substrate and warm to 37°C.
13. Rinse slides in three changes of 1× SSC for 5 minutes each.
14. Incubate in substrate for 30–60 minutes at 37°C.
15. Stop reaction by washing in buffer at 37°C for 2 minutes. Rinse in two changes of distilled water (DW) for 2 minutes each.
16. Rinse in two changes of DW for 2 minutes each.
17. Counterstain; this is dependent on the chromogen selected. For BCIP/NBT use nuclear fast red, eosin, or methyl green. For DAB use hematoxylin or methyl green. Go on to step 18 if using BCIP/NBT or DAB. For AEC use methyl green and coverslip out of distilled water using an aqueous mounting medium. Do not go through alcohols or clearing.
18. Dehydrate in increasing concentrations of alcohol.
19. Clear using xylene and coverslip in permanent mounting resin (Doran & Sterchi 2000).

Automation

There are several versions of automation for comparing with the universal manual ISH method. Automation types can be placed in the following categories:

1. Fully automated: this is where the cut section/slide comes out of the oven and straight into the stainer. The slide is deparaffinized, digested, prehybridized, hybridized, and detected all in one piece of equipment. This type of equipment can be expensive, but is necessary for large volumes of ISH.
2. Mainly automated:
   a. Requires that the histologist must remove the paraffin before placing it on the stainer.
   b. As above, but the digestion must also be performed before placing the slide on the stainer.

All these options are time-efficient and cost-effective, since they use less reagents than manual staining. Often the hybridization time is less than 2 hours.

3. Semi-automated: usually the manual paraffin removal, digestion, prehybridization and hybridization are performed either manually or using a hybridizer or a thermocyler before finishing the detection part of the staining. Commercial stand-alone hybridizers perform prehybridization and hybridization automatically. Some do not require removal of the slides for the washes and the temperatures and times are programmed into the equipment. The fully programmable ones for prehybridizing, hybridizing and washes are great for laboratories that have IHC stainers only.

Any equipment that will cut down on the hands-on time and provide more consistent and reliable results is advantageous.

Troubleshooting

Tissue sections fall off

- adhesive absent/insufficient poly-L-lysine or amino-alkylsilane (AS) on slide
- insufficient adhesion time/temperature

Table 21.2 Suggested digestion times adjusted to fixation time

Fixation time (hours)	Enzyme	Digestion time (minutes)
4	Pepsin, trypsin, or proteinase K	10
15	Pepsin, trypsin, or proteinase K	90
24	Pepsin, trypsin, or proteinase K	120

- over-digestion (too long/too concentrated)
- overzealous coverslip removal (use pliable wrap, AS slips, or well covers)
- denaturation too long or temperature too high (93–98°C is ideal)
- excessive slide agitation

Weak staining (tissue preparation)

- slides incompletely deparaffinized (add an additional xylene/substitute step to insure that all the paraffin has been removed)
- slides not dehydrated or drained prior to addition of probe (water in tissue will dilute probe)
- over-fixation (increase digestion time)
- insufficient digestion (increase time, concentration, or type of digesting reagent) (see Table 21.2)

Weak staining (hybridization/detection)

- probe insufficiently biotinylated
- probe concentration too dilute (hybridize longer)
- probe or target DNA insufficiently denatured (increase time of denaturation, check the temperature of the hot start)
- incomplete hybridization (prehybridize, increase hybridization time, lower temperature if too high, or check stringency)
- buffer wrong pH (should be alkaline, pH 9.5)
- reagents too cold (warm reagents to 23°C)

High background

- skipped blocking step
- probe too concentrated
- slides dried out during incubations
- washes omitted or shortened
- detergent wash buffer not used after label incubation
- incubated substrate too long

'Negative' positive control

- wrong probe
- reagents bad (improper storage)
- digestion absent (enzymes are unstable)
- denaturation absent (increase temperature)
- omitted step in protocol
- mixed detection reagents improperly

Inconsistent staining (stringency conditions)

Stringency conditions occur if a related but non-homologous probe binds to the target:

- non-homologous sequences hybridize/some mismatch (low stringency – high salt concentration, low temperature, low formamide concentration)
- complete binding only if homologous (high stringency – low salt concentration, high temperature, high formamide concentration)

Digestion

If there is absent or a weak signal, digestion needs to be increased by 10 minutes.

A quick test for sufficient digestion is to place the slide under the microscope; using a 40× objective one cell is observed. When 20 dots can be counted in the nucleus, digestion is complete.

Oligonucleotide 3′-end labeling with DIG-ddUTP

Terminal transferase is used to add a single modified dideoxyuridine triphosphate (DIG-ddUTP, biotin-ddUTP, or fluorescein-ddUTP) to the 3′ ends of an oligonucleotide. In this method, DIG-ddUTP will be used. The method described here is from Roche Applied Science Laboratory; it has been modified from the Boehringer Mannheim procedure. The reagents in this procedure may be purchased in a kit (Roche).

Contents of labeling reagents: the amounts to prepare listed here are the amounts supplied in the kit. The kit will accommodate 25 labeling reactions. If you require other amounts, just adjust your volume calculations for less or more.

1. Reaction buffer, pH 6.6 (this is a 5× concentrated solution)

1 M potassium cacodylate
0.125 M Tris-HCl
1.25 mg/ml bovine serum albumin
Prepare 50–100 µl

2. $CoCl_2$ solution

25 mM cobalt chloride
Prepare 50–100 µl

3. DIG-ddUTP solution

1 mM digoxigenin-11-ddUTP in double distilled water
Prepare 25 µl

4a. Terminal transferase 1 (newer method)

25 µl terminal transferase, in the following:
60 mM potassium phosphate (pH 7.2 at 4°C)
150 mM potassium chloride
1 mM 2-mercaptoethanol
0.5% Triton X-100
50% glycerol
Prepare a solution with a concentration of 400 units/µl

4b. Terminal transferase 2 (older method)

1 µl (50 units) terminal transferase, in the following:
200 mM potassium cacodylate
200 mM potassium chloride
1 mM EDTA
0.2 mg/ml bovine serum albumin
50% glycerol
Add enough double-distilled water to make a final volume of 20 µl

5. 0.2 M EDTA (pH 8.0) made in double distilled water

Procedure

1. Dissolve 100 pmol of the purified oligonucleotide in 10 µl of sterile double distilled water.
2. Add the following to a sterile microcentrifuge tube on ice:
   4 µl of 5′ concentrated reaction buffer
   4 µl of 25 mM cobalt chloride ($CoCl_2$)
   1 µl DIG-ddUTP solution. For this labeling we will use DIG-ddUTP.
   1 µl of terminal transferase (400 units/µl)
3. Mix and centrifuge briefly.
4. Incubate at 37°C for 15 minutes, then place on ice.
5. Stop the reaction by adding 2 µl of 0.2 M EDTA (pH 8.0)

Genetic testing: fluorescence *in situ* hybridization (FISH)

The current methods for genetic testing of hereditary and oncological human disease are vast and continuously evolving, as faster, more reliable, and cheaper techniques are developed. One such method of genetic testing is a specialized form of ISH: fluorescence *in situ* hybridization (FISH), also known as molecular cytogenetics. FISH utilization has enabled a huge advance in the diagnostic and prognostic capability of clinical and research laboratories for both constitutional and acquired disorders. We now concentrate on this one technique of genetic testing.

Methodology

The basic steps in a FISH procedure include the fixation of the DNA, as either metaphase chromosomes or interphase nuclei, on a slide; the DNA is then denatured *in situ*, so that it becomes single stranded. This target DNA is then hybridized to specific DNA probe sequences, which are labeled with fluorochromes to allow for their detection. The labeled

probe is added in excess, so probe binding to target DNA occurs. Fluorescence microscopy then allows the visualization of the probe on the target material; analysis of the probe signals includes observation of gain of signals, loss of signals, positioning of signals, or fusion of signals.

Probes

Most probes used in the clinical laboratory in the United States are commercially available and tend to fall into three categories:

1. Repetitive sequences (such as the centromeres or alpha-satellite regions of chromosomes).
2. Whole chromosome sequences (including the short arm, centromere, and long arm of the chromosome).
3. Unique sequences (ranging in size from < 1 kb to >1 Mb of DNA).

With the availability of data from the Human Genome Project (www.genome.ucsc.edu), virtually any sequence of DNA may be used as a FISH probe for the study of specific regions of the chromosome. Several laboratories utilize the Human Genome Project to create 'homebrew' probes, such as bacterial artificial chromosomes.

Labeling

Commercially available probes are usually directly labeled, such that the fluorochrome is directly attached to the probe nucleotides. This technique involves no other detection of the probe before analysis. Probes may also be indirectly labeled, via incorporation of a hapten (such as biotin or digoxigenin) into the DNA via nick translation, for example. The probes are then detected using a fluorescently labeled antibody (such as strepavidin and anti-digoxigenin). Currently, directly labeled probes may be labeled in green (such as SpectrumGreen™ or fluorescein), red (SpectrumOrange™ or Texas Red), blue (SpectrumAqua™), or gold (SpectrumGold™). The ability of a fused green and red FISH signal to be seen as yellow under a fluorescent microscope is helpful in several hematological FISH studies, as detailed below.

Tissue types

FISH can be applied to a variety of clinical specimens, providing there is DNA in the sample. Cultured cells, such as amniocytes, chorionic villi, lymphocytes, bone marrow aspirates, or from solid tumors, will generally yield metaphase spreads. Metaphase spreads are used routinely in the clinical cytogenetics laboratory and are stained with a variety of special stains to allow interpretation of chromosomal regions and rearrangements; these spreads may also be used for FISH analysis. Analysis of FISH on metaphase spreads allows the exact position of the target signals to be determined, as well as whether they are in their normal location or not. One major advantage of FISH is that it does not require cultured cells or metaphase spreads, and can therefore be applied to interphase or non-dividing cells, including uncultured amniocytes (used for rapid prenatal diagnosis), peripheral blood smears (used in immediate newborn blood analysis), or bone marrow aspirate smears. In addition, FISH may be performed on paraffin block sections, disaggregated cells from paraffin blocks, touch preparations from lymph nodes or solid tumors. Therefore, FISH may be used when metaphase chromosomes are unavailable, such as from archival material or when using samples of poor quality.

Clinical applications of FISH

In the clinical laboratory, FISH is used for both congenital and acquired chromosomal analyses. Standard banding techniques (G-banding) allow for the detection of chromosomal rearrangements of approximately 3–5 Mb. FISH is a necessary adjunct in delineating rearrangements, such as subtle deletions or duplications, which are beyond the level of resolution of standard G-banding. FISH is also used to identify the many recurring translocations observed in oncology specimens, both at the initial diagnosis stage and as a monitor of residual disease.

Prenatal chromosome studies

One of the major advantages of FISH is the ability to detect numerical abnormalities (aneuploidy) in uncultured cells from amniotic fluid or chorionic villi. The turnaround time is generally 24 to 48 hours. In high-risk pregnancies, including those associated with advanced maternal age (older than 35 years), abnormal ultrasound findings, or abnormal maternal screening results, FISH is used as an adjunct to standard cytogenetic analysis to provide aneuploidy screening for chromosomes 13, 18, 21, as well as the X and Y chromosomes. Aneuploidy of these chromosomes accounts for the most common abnormalities detected prenatally (Fig. 21.6). FISH technology on prenatal samples has been found to be effective, sensitive, and specific (Tepperberg et al. 2001).

Microdeletion and microduplication syndromes

Microdeletion or contiguous gene syndromes are caused by a deletion of genetic material, which results in the loss of several genes from one chromosomal region. Generally, these deletions are <2 Mb in size. There are several, clinically recognized, microdeletion syndromes (Table 21.3), for which commercial FISH probes are available (Fig. 21.7). Microduplication syndromes are caused by a gain of genetic material, often in the same regions of the chromosome in which microdeletions are observed. The same FISH probes used for microdeletion analyses may be used for microduplication analyses; the major difference is that interphase nuclei must be scored as well as metaphase cells.

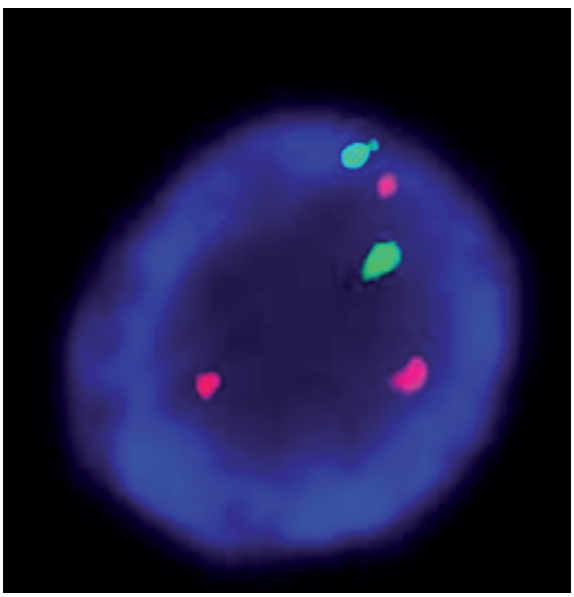


Figure 21.6 Representative FISH image of the AneuVysion™ (Abbott Molecular Inc., Des Plaines, IL) probe set with probes for chromosome 13 (labeled in SpectrumGreen™) and chromosome 21 (SpectrumOrange™) on an interphase cell from an uncultured amniocyte sample. Three red signals are seen for chromosome 21, indicating that this fetus has Down's syndrome.

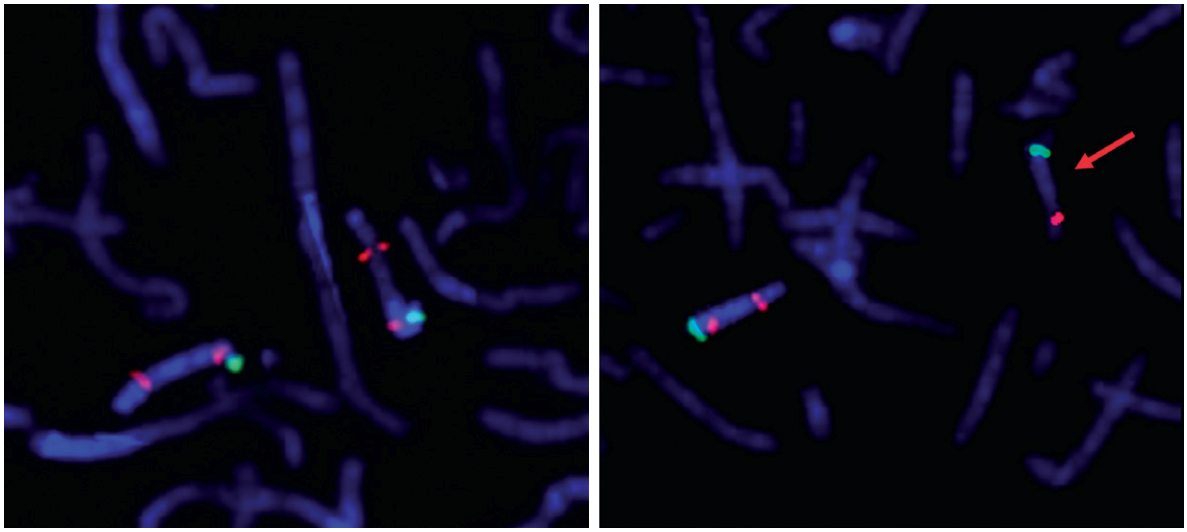
Acquired abnormalities

FISH probes have been developed for the majority of recurrent chromosomal aberrations found in hematological malignancies (Table 21.4). One of the commercial suppliers of hematological FISH probes is Abbott Molecular Inc., an Abbott Laboratories Company (Des Plaines, IL). Their hematological FISH probes are currently divided into four types:

1. Dual-color/single-fusion probes
With the dual-color, single-fusion probes, the DNA probe hybridization targets are located on one side of each of the two genetic breakpoints in the specific translocation (for example, chromosomes 9 and 22 in the case of the Philadelphia chromosome associated with chronic myelogenous leukemia or acute lymphoblastic leukemia).

2. Extra-signal probes
The extra-signal (ES) probes are designed to reduce the frequency of normal cells with an abnormal FISH signal pattern due to random co-localization of probe signals in the nucleus. In this type of probe set, one larger probe (labeled in one color) spans one breakpoint in the specific translocation, while the other probe (labeled in another color) flanks the breakpoint of the other gene involved in the translocation.

3. Dual-color/break-apart probes
Dual-color, break-apart probes are used when a specific gene may have several different chromosomal partners, for example the MLL gene, rearrangements of which are seen in both acute myelogenous leukemia and acute lymphocytic leukemia. These dual-color, break-apart probes are designed so that



a b

Figure 21.7 Fluorescent *in situ* hybridization (FISH) images of the SNRPN probe set used in the diagnosis of Prader-Willi and Angelman syndromes, with SNRPN at 15q11.2 and PML at 15q22 labeled in SpectrumOrange™, and D15Z1 at 15p11.2 labeled in SpectrumGreen™. The D15Z1 and the PML probes are used as internal controls in this probe set. (a) A partial metaphase spread from a peripheral blood specimen. This is the normal signal pattern, with two green signals and four red signals, indicating there is no deletion of any of these probes. (b) A partial metaphase spread from a peripheral blood specimen, showing a deletion of one SNRPN locus at 15q11.2 (red arrow), indicating that this patient has the clinical diagnosis of either Prader-Willi syndrome or Angelman syndrome.

Table 21.3 Common constitutional microdeletion syndromes

Syndrome	Chromosomal region	Critical gene(s)
1p36 deletion syndrome	1p36.3	Unknown
Angelman	15q11.2	UBE3A
Kallmann	Xp22.31	KAL1
Miller-Dieker	17p13.3	LIS1
Prader-Willi	15q11.2	SNRPN
Smith-Magenis	17p11.2	RAI1
Sotos	5q35	NSD1
Steroid sulfatase deficiency	Xp22.31	STS
Velocardiofacial/DiGeorge	22q11.2	TUPLE1 (HIRA)
Williams	7q11.2	ELN

the DNA sequence on either side of the breakpoint in a specific gene is labeled in two colors; when the gene is disrupted due to a translocation, the probe is seen as two separate colors (red and green), rather than as one fused signal pattern (yellow).

4. Dual-color/dual-fusion probes (Fig. 21.8)
This probe set is designed to reduce the number of normal nuclei showing an abnormal signal pattern due to random co-localization; large probes (in different colors) span both breakpoints involved in the

Table 21.4 Common commercially available FISH probes for hematological diseases

Chromosomal aberration	Gene(s)	Associated disease[a]
t(1;19)(q23;p13.3)	PBX1/TCF3	ALL
t(2;5)(p23;q25)	ALK	NHL
3q27	BCL6	CLL, NHL
del(4)(q12q12)	FIP1L1-PDGFRA	HES
t(4;14)(p16;q32.3)	FGFR3/IgH	MM
Trisomy 4	4cen[b]	ALL
-5/del(5q)	EGR1	AML, MDS
6q23	MYB	CLL
-7/del(7q)	D7S486[c]	AML, MDS
t(8;14)(q24;q32)	MYC/IgH	ALL, NHL, MM
t(8;21)(q22;q22)	ETO/AML1	AML
Trisomy 8	8cen[b]	AML,CML,MDS, NHL
9p21 rearrangements	P16 (CDKN2A)	ALL
t(9;22)(q34;q11.2)	ABL1/BCR	CML, ALL, AML
Trisomy 10	10cen[b]	ALL
del(11)(q22.3)	ATM	CLL
t(11;14)(q13;q32)	CCND1/IgH	NHL, MM
t(11;18)(q21;q21.1)	API2/MALT1	NHL
11q23 rearrangements	MLL	ALL, AML
t(12;21)(p13;q22)	TEL/AML1	ALL
12p13 rearrangements	ETV6	ALL, AML
Trisomy 12	12cen[b]	CLL
del(13)(q14.2)	RB1	MDS/MPD
del(13)(q14.3)	D13S319[c]	CLL, NHL, MM
t(14;16)(q32;q23)	IgH/MAF	MM
t(14;18)(q32;q21)	IgH/BCL2	NHL
t(14;18)(q32;q21.1)	IgH/MALT1	NHL
14q11 rearrangements	TCR$\alpha\delta$	ALL
14q32 rearrangements	IgH	NHL, MM
t(15;17)(q22;q21.1)	PML/RARA	APL
inv(16)(p13q22) or t(16;16)(p13;q22)	CBFβ	AML
del(17)(p13.1)	TP53	CLL, MM, NHL
Trisomy 17	17cen[b]	ALL
del(20)(q11q13)	D20S108[c]	MDS

[a]Abbreviations: ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; APL = acute promyelogenous leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; HES = hypereosinophilic syndrome; MDS/MPD = myelodysplastic syndrome/myeloproliferative disorder; MM = multiple myeloma (plasma cell myeloma); NHL = non-Hodgkin's lymphoma (including follicular lymphoma, Burkitt's lymphoma, anaplastic large cell lymphoma, diffuse large cell lymphoma, mantle cell lymphoma and mucosa-associated lymphoid tissue (MALT) lymphoma).

[b]cen = centromere of chromosome (not a gene).

[c]An STS (sequence-tagged site) marker (not a gene).

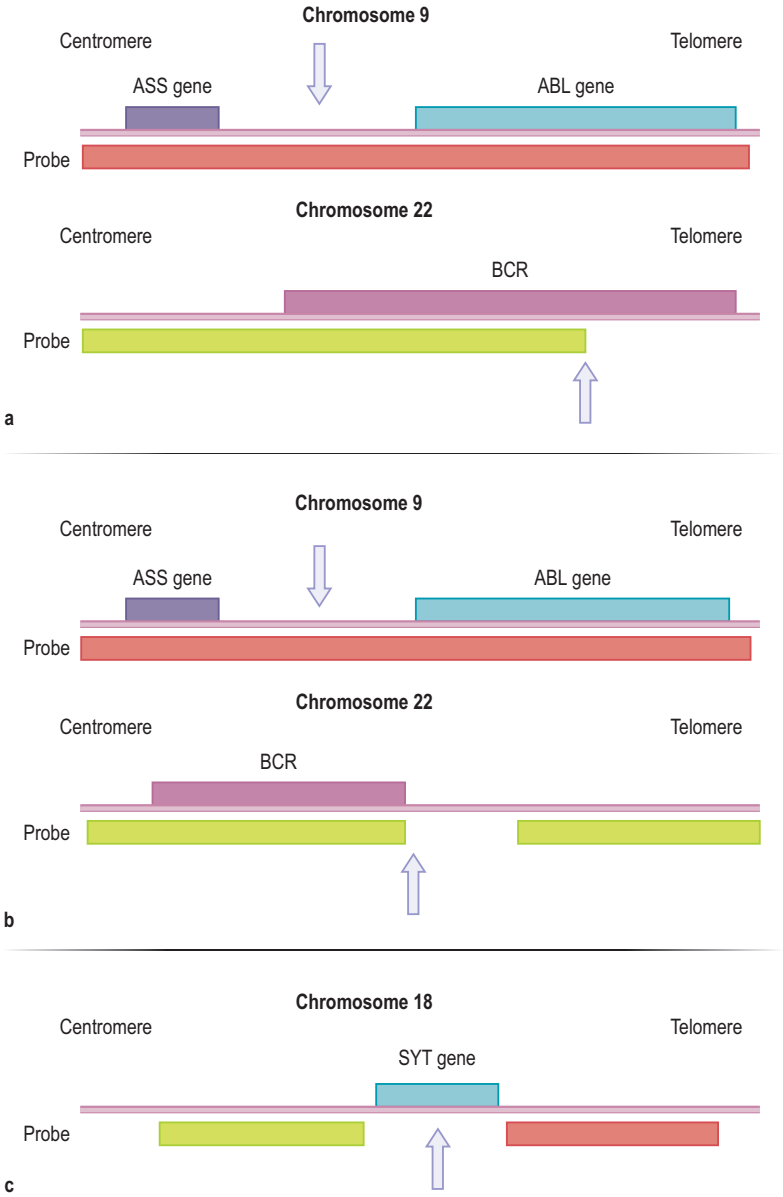
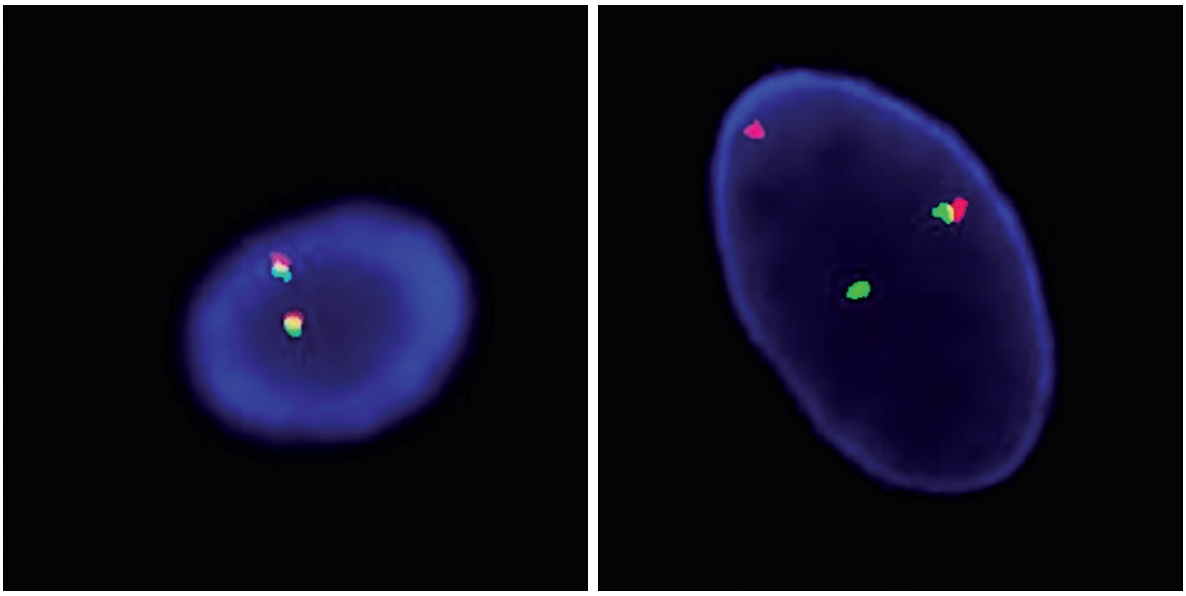


Figure 21.8 (a) Diagram illustrating the design of a dual-color, extra-signal probe set. This probe set is used to identify the Philadelphia chromosome, which results from the translocation between chromosomes 9 (labeled with SpectrumOrange™) and 22 (SpectrumGreen™) in chronic myelogenous leukemia (CML) and some cases of acute lymphocytic leukemia (ALL). The arrows indicate the breakpoints on the chromosomes. The extra signal is due to the presence of the ASS gene, also labeled in SpectrumOrange™, which remains on the derivative chromosome 9, following the translocation. (b) Diagram illustrating the design of the dual-color, dual-fusion signal probe set. This probe set is also used to identify the translocation between chromosomes 9 and 22 seen in CML and some cases of ALL. The arrows indicate the breakpoints on the chromosomes. The two fusion signals arise due to the fusion of part of the red signal on chromosome 9 with part of the green signal on chromosome 22, and vice versa. (c) Diagram illustrating the design of a dual-color, break-apart probe. This probe is used to identify translocations involving the SYT gene on chromosome 18 seen a majority of synovial sarcomas. The probe is labeled in both SpectrumGreen™ and SpectrumOrange™, forming a yellow or fused signal in a normal cell; a translocation in an abnormal cell will disrupt this fused yellow signal, creating a separate red and green signal. (Diagrams adapted from product information with permission from Abbott Molecular Inc.)



a b

Figure 21.9 Representative FISH images of the SYT dual-color, break-apart probe on interphase cells from a bone marrow sample. The SYT gene is located on chromosome 18 and translocations involving this region are seen in the majority of synovial sarcoma patients. (a) Two yellow or fused signals (both SpectrumOrange™ and SpectrumGreen™ present) are seen, which is the normal signal pattern. (b) One fused yellow signal is seen, representing the normal chromosome 18, as well as a separate red and green signal, indicating that a translocation has occurred. The SYT gene is translocated to the X chromosome and forms an abnormal fusion protein with either the SSX1 or SSX2 gene. This analysis was performed in an interphase cell and, therefore, the specific translocation partner of the SYT gene cannot be determined.

rearrangement. In a truly abnormal cell, two fusion signals are generally seen, representing the specific chromosomal translocation, as well as a red and a green signal, representing the normal and uninvolved chromosomes.

Solid tumors

Solid tumors are often difficult to grow in culture, and metaphase spreads can be hard to obtain and/or analyze. FISH is useful in detecting specific rearrangements in interphase cells of solid tumors that have diagnostic and prognostic implications. Some soft tissue masses, such as synovial sarcoma, may be difficult to diagnose by morphology alone. A break-apart, dual-color FISH probe has been designed which is telomeric and centromeric to the SYT gene. In a normal cell, two yellow or fused signals are seen, while in an abnormal cell, one yellow, one red, and one green signal are seen, representing the disruption and translocation of the SYT gene (Fig. 21.9). Such translocations bring the SYT gene on chromosome 18 into contact with either the SSX*1* or SSX2 gene on the X chromosome (Geurts van Kessel et al. 1997). Chromosomal rearrangements involving the DDIT*3* (formerly CHOP) gene on chromosome 12 are common in myxoid/round cell liposarcomas (Aman et al. 1992). This FISH probe, also a break-apart probe, will detect a translocation involving the DDIT*3* gene, but not the specific chromosomal translocation partner. Another break-apart probe involves the FOXO*1* (formerly FKHR) gene on chromosome 13, which, when fused with the PAX*3* gene on chromosome 2 or the PAX*7* gene on chromosome 1, is associated with alveolar rhabdomyosarcoma (Mehra et al. 2008).

Neuroblastoma

Amplification and overexpression of the MYCN oncogene on chromosome 2 is seen in childhood neuroblastoma and is associated with rapid tumor progression and a poor prognosis (Ambros et al. 2009). The MYCN FISH probe is used to detect extra copies or amplification of the gene. Analysis is usually performed on interphase cells, from bone marrow biopsies, fresh or snap-frozen tumor, or paraffin-embedded tumor samples. MYCN amplification is considered to be present when there is more than a 4-fold increase in the MYCN signal number compared to the reference probe signal (generally the centromere of chromosome 2); for example, if there are two signals seen for the centromere of chromosome 2, there must be at least 9 signals present for the MYCN probe for the result to be deemed amplified. Amplification may be seen as either double minutes (dmin) or homogeneously staining regions (hsr) (Storlazzi et al. 2010).

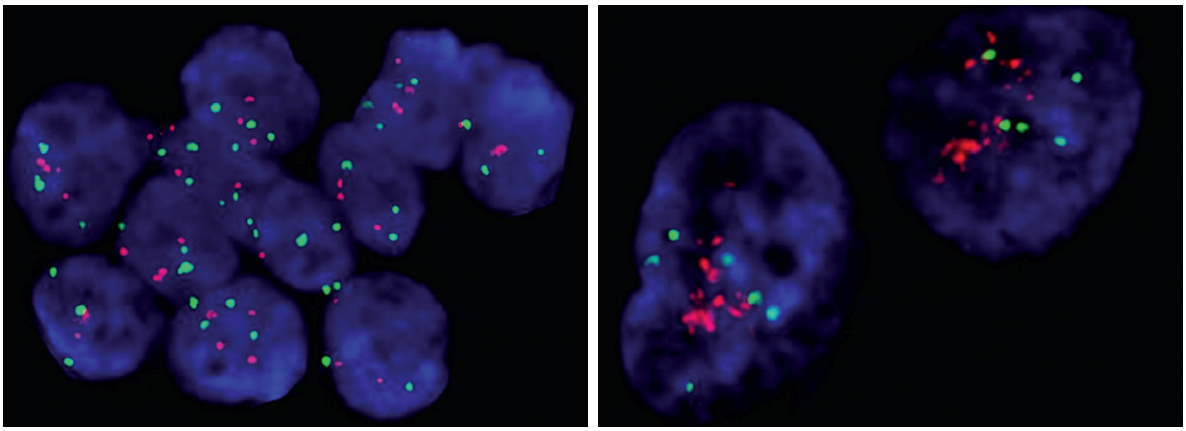
Breast cancer

Breast cancer is the second most common cause of cancer death among women in the United States (American Cancer Society, 2010). A great deal of money and research has been targeted toward the improvement of early detection and effective therapies. This area of genetic testing has arisen because of the advances made in the field of pharmacogenomics, in which therapeutic drug development is dependent upon genetic variations in an individual. One of the major advantages of FISH is the ability to study paraffin-embedded tissue, permitting the analysis of both fresh and archival samples. This has been extremely helpful in the analysis of breast cancer tissue specimens. The HER2 (ERBB2) gene on chromosome 17 has been shown to be overexpressed or amplified in approximately 25% of breast cancers (Kallioniemi et al. 1992). Amplification of the HER2 gene and/or overexpression of its protein product is associated with poor prognosis, an increased risk of recurrence, and a shortened survival time (Press et al. 1997). There are two types of test commonly used to determine HER2 status: immunohistochemistry, which measures the level of expression of the gene; and FISH, which measures the number of copies of the gene. Assessment of HER2 status (Pegram and Slamon, 2000) is useful for determining chemotherapy responsiveness and selection for targeted monoclonal antibody therapy (Herceptin® (trastuzumab), Genentech, Inc., South San Francisco, CA). FISH (for example, the PathVysion™ DNA probe kit from Abbott Molecular Inc.) has been approved by the United States Food and Drug Administration (FDA) as the most sensitive and specific methodology for HER2 detection. The highest Herceptin® response is seen in FISH-positive breast cancer patients. Therefore, knowledge of the status of HER2 amplification is vital to treatment strategies for some breast cancer patients.

The PathVysion™ FISH procedure for the detection of HER2 amplification involves in brief, 4 μm sections of paraffin-embedded breast cancer tumor samples being prepared. The tumor areas are scored by a pathologist, and FISH analysis with a probe set consisting of the HER2 gene in combination with the alpha-satellite probe for the centromere of chromosome 17 is performed. The number of HER2 and chromosome 17 signals is scored, the ratio of the two is determined, and a ratio of greater than 2.2 is taken to indicate amplification of the HER2 gene (Fig. 21.10). These results are then interpreted in conjunction with clinical and pathological findings to determine the best treatment option for patients with stage II, node-positive breast cancer.

Bladder cancer

Bladder cancers are among the most frequent of adult cancers, expected to account for approximately 14,680 deaths (men and women) in the USA in 2010 (American Cancer Society, 2010). Chromosomal aberrations, such as aneuploidy for chromosomes 3, 7, 9, and 17, have been found to be associated with histological progression in bladder cancer (Nemoto et al. 1995). An FDA-approved FISH test has been developed by Abbott Molecular Inc., the UroVysion™ assay, results from which should be used in conjunction with current standard diagnostic procedures for bladder cancer. The assay consists of a panel of probes for the centromeric regions of



a b

Figure 21.10 Representative FISH images of the PathVysion™ (Abbott Molecular Inc., Des Plaines, IL) probe set with probes for the centromere of chromosome 17 (labeled in SpectrumGreen™) and the HER2 probe (labeled in SpectrumOrange™) on a paraffin-embedded sample of two patients with breast cancer. (a) The signal ratio of the two probes was determined to be 1.13 in this sample and, therefore, no amplification of the HER2 gene was seen. (b) The signal ratio of the two probes was determined to be 5.48 in this sample and, therefore, amplification of the HER2 gene was determined to have occurred.

chromosomes 3, 7, and 17, as well as a probe for a region on the short arm of chromosome 9 (9p21). Gain of chromosomes 3, 7, and 17, and/or homozygous loss of chromosome 9p21 are both associated with recurrence of bladder cancer (Fig. 21.11); the UroVysion™ assay may also be used as an aid for the initial diagnosis of bladder cancer in patients with hematuria. This FISH assay is performed on voided urine and is capable of detecting approximately 95% of recurrent transitional cell carcinomas (Wolff 2007).

General FISH procedure

Sample requirements

1. Fixed metaphase or interphase chromosomes on a microscope slide or coverslip are required. One should not bake the slides (as is performed for slides that will be G-banded for routine chromosome analysis). Aging of slides is essential to obtaining good FISH signals, as it hardens the DNA, removes water, and may increase signal intensity. If slides are made and used in a FISH analysis the same day, then artificial aging must be performed, by placing slides for a minimum of 2 hours in a 37°C oven. Alternatively, if the slides have been stored at room temperature for less than 3 weeks, then the slides may be aged in 2× SSC at 73°C for 2 minutes or at 37°C for 60 minutes (followed by dehydration in an ethanol series).

2. For analysis with metaphase FISH probes, at least 15 metaphases per 22 × 22 mm area are required for a complete analysis.

Solutions

20× SSC

Sodium chloride	175.32 g
Sodium citrate	88.20 g
Double distilled (dd) H_2O	800 ml

pH to 7.0 with 1 M HCl. Bring to 1 liter with ddH_2O. Filter through a 0.4 μm filtration unit. Store at room temperature. Expiration: 6 months.

2× SSC

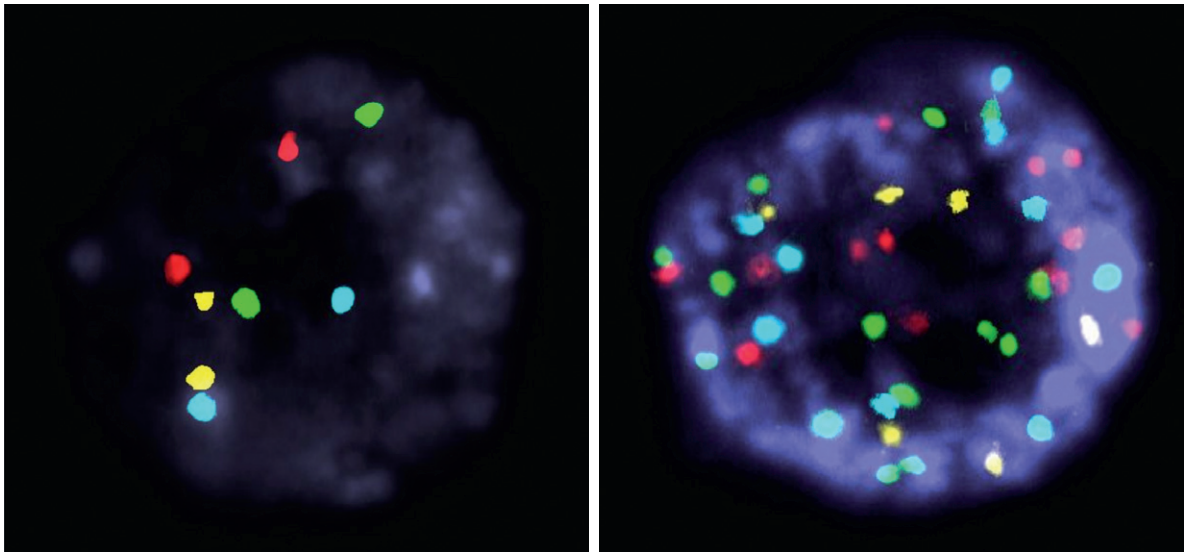
20× SSC	50 ml
ddH_2O	450 ml

Store at room temperature. Expiration: 6 months.

Ethanol series

	70%	80%	95%	100%
Ethanol (ml)	350	400	475	500
ddH_2O (ml)	150	100	25	0

Store at room temperature.



a b

Figure 21.11 Representative FISH images of the UroVysion™ (Abbott Molecular Inc., Des Plaines, IL) probe set with probes for the centromeres of chromosome 3 (labeled in SpectrumRed™), chromosome 7 (SpectrumGreen™), and chromosome 17 (SpectrumAqua™). The chromosome 9p21 probe is labeled in SpectrumGold™. (a) Two signals with each of the probes can be seen in this cell from a urine sample of a patient previously diagnosed with bladder cancer; this is the normal signal pattern. (b) This FISH image is from an abnormal cell found in a urine sample of a patient previously diagnosed with bladder cancer. The extra copies of chromosomes 3, 7, and 17 indicate aneuploidy of these chromosomes and perhaps a recurrence of the bladder cancer.

Denaturation solution (70% formamide/ 2× SSC)

Formamide	35 ml
20× SSC	5 ml
ddH_2O	10 ml

Bring pH to 7.0 with 1 M HCl. Store at 4°C. Expiration: 1 week.

Post-wash solution (2× SSC/0.1% Nonidet P-40 (NP-40))

20× SSC	100 ml
ddH_2O	899 ml
NP-40	1 ml

Bring pH to 7.0 ± 0.2 with 1 M NaOH. Mix well. Store at room temperature. Discard used solution at the end of each day. Expiration: 6 months.

Post-wash solution (0.4× SSC/0.3% NP-40)

20× SSC	20 ml
ddH_2O	977 ml
NP-40	3 ml

Bring pH to 7.5 ± 0.2 with 1 M NaOH. Mix well. Store at room temperature. Discard used solution at the end of each day. Expiration: 6 months.

DNA counterstain

Propidium iodide and DAPI (4′,6-diamidino-2-phenylindole) are fluorescent dyes used to counterstain DNA. Propidium iodide dyes the chromosomes red/orange, while DAPI stains chromosomes blue. Different concentrations of DAPI are available. Abbott Molecular Inc. provides both DAPI I and DAPI II. DAPI I is recommended when a more intense counterstain is required. It provides a distinct banding pattern. DAPI II provides a weaker counterstain, which is useful when viewing smaller probes (unique sequences and centromeric regions).

FISH set-up

This set-up procedure is the basic procedure for use with either commercially available probes or home brew probes. The denaturation times and temperatures may need to be adjusted depending on the

specific probe used (whole chromosome paint versus centromere-specific probes, for example) and the tissue type (peripheral blood specimen slide versus amniocyte coverslip). Different commercially available probes are provided either pre-denatured and in solution with the appropriate hybridization buffer, or require to be prepared with the appropriate hybridization buffer and distilled water (step 5) before denaturation. The two alternatives to denaturation of the slides/coverslips and probes are listed in step 7 (denaturation solution) and step 12 (all-in-one co-denaturation and hybridization system). The stringency of the post-wash solutions is important to remove non-specifically bound probe. The stringency of the post-wash solutions may be altered by changing the salt concentration, the temperature, or the time in each solution. Some specimen types require pretreatment before denaturation, which removes cytoplasmic proteins of the cell membrane, allowing greater accessibility of the DNA. Pretreatment is generally performed using fresh pepsin in an acid solution.

Day one

1. Place denaturation solution in water bath at 73°C (if following step 7).
2. Examine slide or coverslip to determine optimal target area.
3. Treat slide(s) in 2× SSC at 37°C for at least 30 minutes.
4. Dehydrate the slide(s) in a cold ethanol series (70%, 85%, and 100%) for 2 minutes each.

   Allow slides to dry. Store slide(s) in covered slide box until ready to denature.
5. Pre-warm probe to room temperature for about 5 minutes. If probe does not need to be denatured, aliquot 10 µl for each 22 x 22 mm target area; if probe needs to be denatured, aliquot 7 µl hybridization buffer, 2 µl ddH_2O, and 1 µl probe into a microcentrifuge tube. Keep probe in darkness as much as possible. Return probe to freezer as soon as possible.
6. Vortex probe briefly and centrifuge for 2–3 seconds.
7. Denature the slide(s) for exactly 2 minutes in the pre-warmed denaturant at 73°C.

Note

A maximum of three slides should be denatured at one time to maintain the correct denaturation temperature. In addition, denaturation temperatures and times may vary depending on tissue type and type of probe.

8. Dehydrate the slide(s) in a cold ethanol series (70%, 80%, and 100%) for 2 minutes each.
9. Wipe the back of the slide(s) and place on a 37°C slide warmer to dry completely. Leave slide(s) on slide warmer until ready to apply probe mixture.
10. Denature aliquoted probe mix for 5 minutes in a 73°C water bath. Vortex probe briefly and centrifuge for 2–3 seconds.
11. Apply 10 µl probe mix to target area and cover with a 22 × 22 mm glass coverslip. Seal with rubber cement.
12. If not denaturing probes and slides separately, probes and slides can be co-denatured on, for example, the ThermoBrite™ Denaturation and Hybridization system from Abbott Molecular Inc. for 2 minutes at 73°C.
13. Following either form of denaturation: incubate slide(s) at 37°C overnight in a humidified chamber (place moist sponge or paper towels in an airtight, opaque container).

Note

Slide(s) may be left in the ThermoBrite™ instrument for hybridization at 37°C for 4–16 hours. A minimum of 4 hours of hybridization is recommended for any probe.

Day two

14. Warm glass Coplin of 0.4× SSC/0.3% NP-40 to 73 ± 1°C. Do not wash more than three slides at a time, to ensure the correct wash temperature is maintained.
15. Remove coverslip and rubber cement from hybridized slide(s). Keep slides covered as much as possible and away from the light.
16. Wash slide(s) in 0.4× SSC/0.3% NP-40 at 72°C for 2 minutes. Agitate slide(s) for 1–3 seconds.
17. Wash slide(s) in 2× SSC/0.1% NP-40 at room temperature for 1 minute. Agitate slide(s) for 1–3 seconds.
18. Allow the slide(s) to dry while protected from the light.
19. Apply 2 µl of DAPI I or DAPI II to slide(s) and cover with appropriately sized glass coverslip.

Note: DAPI is a carcinogen

For batching FISH samples, or running a FISH procedure with many steps and solution changes, automated FISH processing may be advantageous. One such pre-programmed automated system is supplied by Abbott Molecular Inc., the VP 2000™ Processor, which may be used for a variety of functions, such as deparaffinization and pretreatment of FISH samples, histology/cytology staining, special stains of chromosomes, and routine slide washing.

Specific FISH procedure: HER2 FISH (PathVysion™)

Sample requirements

Formalin-fixed, paraffin-embedded breast cancer tissue specimens should be cut into approximately 4 µm sections. Slides are then floated in a protein-free water bath at 40°C. Each section is mounted on the positive side of an organosilane-coated slide in order to minimize the loss of tissue during processing. The slides are air dried. A hematoxylin and eosin (H&E) slide, scored by a pathologist, should accompany each specimen, with the areas of tumor to be scored clearly delineated. Control slides (one negative and one positive slide) must be run at the same time as the clinical, patient slides in each specimen processing run, to ensure accuracy of signal analysis and to monitor assay performance. If the FISH assay on the control slides does not work, then the patient analysis cannot be reported. In addition, control slides must be run with each new lot of the PathVysion™ probe kit. A more detailed reference of the procedure may be obtained from the PathVysion™ HER2 DNA Probe Kit product data sheet (Abbott Molecular Inc.).

Solutions needed for the VP 2000™ Processor

70%, 85%, 95% ethanol

Posthybridization wash buffer. Note: only one wash buffer used in this procedure.

2x SSC/0.3% NP-40

Protease I reagent

25 mg pepsin/lyophilized per 50 ml 0.01 M HCl. Make fresh.

Pretreatment reagent (1 M sodium thiocyanate)

Keep covered at ambient temperature. Expiration: 6 months.

0.2 M HCl

Keep covered at ambient temperature. Expiration: 6 months.

10% buffered formalin

PathVysion™ HER2 DNA probe kit *(Abbott Molecular Inc.)*

DAPI I

Day one

Bake slide(s) overnight at 56°C (hot plate or oven).

Day two

Slide pretreatment (as run on the VP 2000™ Processor)

1. Deparaffinization in Hemo-De (non-toxic solvent similar to xylene) for 5 minutes at ambient temperature.
2. Repeat twice.
3. 95% ethanol for 1 minute at ambient temperature.
4. Repeat.
5. 0.2 M HCl for 20 minutes at ambient temperature.
6. Rinse in water for 3 minutes at ambient temperature.
7. Pretreatment reagent for 30 minutes at 80°C.
8. Rinse in water for 3 minutes at ambient temperature.
9. Protease treatment for 10 minutes at 37°C.
10. Rinse in water for 3 minutes at ambient temperature.
11. Fixation in 10% buffered formalin for 10 minutes at ambient temperature.
12. Rinse in water for 3 minutes at ambient temperature.
13. Dehydrate for 1 minute each in ethanol series (70%, 85%, and 95% ethanol).
14. Air dry on drying station for 3 minutes at 25°C.
15. Proceed to FISH procedure.

Notes

1. Before each run on the VP 2000™ Processor, each basin should be filled with 470 ml of the appropriate reagent. After approximately 15 runs, all solutions should be discarded and the basins refilled with fresh reagents.
2. The fixative step 11 helps to reduce tissue loss during denaturation.

FISH procedure

See general FISH procedure described previously. It is recommended that denaturation solution (step 7) is used to denature the slide(s). Denature slide(s) at 72 ± 1°C for 5 minutes.

Day three

1. Wash slide(s) in 2× SSC/0.3% NP-40 at 73 ± 1°C for 2 minutes.
2. Air dry slides in the dark.
3. Apply 20 μl DAPI I counterstain and cover with a glass coverslip.

Signal analysis

Using a 40× objective, scan several areas of tumor cells within the area corresponding to the H&E area, as designated by the pathologist. Select an area of good nuclei distribution. Using a 100× objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluable interphase cell. Do not score nuclei with no signals or signals of only one color.

1. Thirty interphase cells are analyzed by one technologist and the results are confirmed by a second technologist. Note: A minimum of 20 interphase cells must be analyzed.
2. Results are recorded as the number of signals for the HER2 probe and the number of signals for the CEP (chromosome enumeration probe) 17 probe (the centromere of chromosome 17).
3. The ratio of the average copy number of HER2 to CEP 17 signals is calculated.
4. If the ratio of HER2 to CEP 17 signals is borderline (between 1.8 and 2.2), then a second technologist will count an additional 30 interphase cells and a new ratio is calculated.
5. A schematic drawing of the section of the tissue analyzed should be made to indicate the area analyzed by each technologist.

Interpretation of results

1. The ratio of the average copy number of HER2 to CEP 17 signals is calculated. If the number is less than 1.8, then the results are reported as not amplified.
2. If the ratio is ≥2.2, the results are reported as amplified.
3. If the ratio is between 1.8 and 2.2, the results are reported as equivocal.

Troubleshooting

One of the most common problems encountered with paraffin-embedded FISH analysis is under- or over-digestion of the tissue sample. If the tissue looks under-digested (too much cytoplasm, weak or no signals), then the incubation time in the pretreatment solution should be increased to 15–60 minutes. Over-digested tissue appears faded, with a loss of cell borders. A repeat sample must be run, with a decrease in the pretreatment time to 15–25 minutes and a decrease in the protease solution to 5–7 minutes. In addition, the denaturation time and temperature may be adjusted to increase the accessibility of the sample DNA to the probe. Formalin fixation, tissue size, and sample quality may all also affect the FISH results.

General scoring analysis criteria for FISH

The scoring analysis criteria described here are based on the minimum requirements of the College of American Pathologists (CAP) guidelines, as well as recommendations by the American College of Medical Genetics (ACMG).

Metaphase scoring criteria

General information

1. Only complete metaphases (i.e. with 46 chromosomes) should be scored. An exception may be made when the number of complete metaphases is low and all the chromosomes of interest are present in one field of view.
2. Analysis of 10 metaphase cells on all clinical samples is required for a complete study. If necessary, additional metaphase cells may be analyzed.
3. Record coordinates and results of at least two representative metaphase spreads analyzed on analysis sheet. At least two images should be captured if the analysis is abnormal; at least one image should be captured if the analysis is normal.

Table 21.5 Troubleshooting FISH

Problem	Possible cause	Possible solution
Slide background	Inadequate post-hybridization wash	Ensure correct wash solution and temperature. Re-wash slide
	Inadequate cleaning of glass slides	Clean slides in ethanol and wipe dry with lint-free paper
Weak or no signal	Specimen inadequately denatured	Ensure correct denaturation solution and temperature. Increase denaturation time
	Specimen slides not aged	Age slides for 24 hours at ambient temperature before use
	Probe not added	Allow probe to thaw completely before use. Vortex probe. Pipette slowly
	Probe inadequately denatured	Ensure correct temperature of water bath
	Counterstain is too bright	Remove coverslip. Re-wash in 2x SSC/ 0.1% NP-40 at ambient temperature. Dehydrate slides; reapply counterstain
	Probes improperly stored	Store probes at –20°C (in dark)
Distorted chromosome morphology	Specimen over-denatured	Ensure correct denaturation solution and temperature. Repeat on new specimen with reduced time of denaturation

4. In cases suspected of mosaicism (more than one cell line present), additional metaphase cells should be counted.

Interphase scoring criteria

General information

1. At least 200 individual interphase cells should be analyzed. Only cells that are in a monolayer with discrete borders are countable. Overlapping cells should not be counted. Not all cells are appropriate for analysis and may be skipped.
2. Signals should be discrete (not diffuse). Signals should be counted as two signals if they are greater than one signal-width apart. Signals that are closer than one signal-width apart should be counted as one signal.
3. Signals should be counted as yellow (fusion of orange and green) if they are on top of each other. A green signal and an orange signal with discrete borders are considered separate (i.e. not a fusion (yellow) signal).

Troubleshooting FISH

There are various problems that may be encountered when setting up a FISH experiment, ranging from over-denaturation of the DNA, to weak or no FISH signals. Table 21.5 highlights some of the more common problems and their solutions; however, the list is by no means exhaustive. Commercially available probes are supplied with package inserts which generally cover a wide array of possible problems with FISH set-up and analysis.

Validation of FISH probes in the clinical laboratory

The majority of probes and materials used for clinical FISH studies in the United States are considered to be analyte-specific reagents (ASRs). They are

exempt from the Food and Drug Administration (FDA) regulations (probes that are FDA-approved include PathVysion™ and UroVysion™). Therefore, these probes must be independently validated by each clinical laboratory. The College of American Pathologists (CAP) and the Standards and Guidelines for Clinical Genetics Laboratories issued by the ACMG state that each probe must be validated for sensitivity (the expected signal pattern is observed) and specificity (the probe is localized to the correct chromosome and chromosomal region). If the probe is to be used on interphase cells, a reportable reference range for that probe must be established. Reportable reference ranges are generally established from a database of cytogenetically characterized cases, so that the percentage of cells exhibiting an 'abnormal' signal pattern by random chance can be determined. Thus, a normal cut-off for each probe can be established. Biannual or continual evaluation of the performance characteristics of each probe in clinical use is also required by CAP.

FISH nomenclature

The ISCN 2009, an International System for Human Cytogenetic Nomenclature, addresses FISH nomenclature. The appropriate FISH nomenclature should accompany each FISH report on a patient, indicating whether the FISH test was performed on metaphase (ish) or interphase (nuc ish) cells, the probe name, the number of signals observed, and for metaphase analyses, the chromosomal location. For example, a result indicating a deletion of the SNRPN gene (see Fig. 21.6) is written as: ish del(15)(q11.2q11.2)(SNPRN-), indicating that one chromosome 15 did not have the SNRPN signal, confirming a diagnosis of either Angelman or Prader-Willi syndrome. A normal HER2 result would be written as: nuc ish(D17Z1,HER2)x2[X], with [X] representing the number of interphase cells analyzed; the result indicates that two signals for both the centromere of chromosome 17 and the HER2 gene were seen, and there was no amplification of the HER2 gene. An abnormal HER2 result may be written as: nuc ish(D17Z1x2),amp(HER2)[20/30], indicating that there were two signals seen for the control probe and amplification was observed for the HER2 probe in 20 out of 30 of the interphase cells counted.

Summary

This section describes only one specialized area of genetic testing: the use of FISH in clinical laboratories. Advances in genetic testing seem to occur on an almost daily basis, including new FISH probes, pharmacogenetics, targeted therapies, and Next Gen sequencing, including exome sequencing. The increasing usage of other technologies such as microarrays (SNP, oligonucleotide, or expression arrays) in clinical laboratories will certainly reduce the utilization of FISH in the future, but it will still remain a valid and user-friendly way of answering many diagnostic and prognostic questions.

Glossary and definitions of the terminology used in this chapter and in ISH techniques

Amplification

Production of additional copies of a gene sequences. This multiplication of the sequence makes it easier to identify.

Anneal

To join two strands of complementary nucleic acids.

Antisense RNA

An RNA which is complementary to mRNA.

Base

A building block of the nucleotide. Four different bases for DNA: (A) – adenine, (T) – thymine, (C) – cytosine, (G) – guanine. In RNA, the thymine is replaced by (U) – uracil.

Base pair (bp)

Two bases which are complementary to each other and joined by hydrogen bonds.

cDNA

A complementary sequence of DNA that has been synthesized from an initial template of RNA by the enzyme reverse transcriptase.

Codon

A set of three consecutive nucleotides in DNA or mRNA that directs the incorporation of an amino acid during protein synthesis or signals the start or stop of translation. Multiple codons will code for the same amino acid.

Complementary sequence

Nucleic acid sequence of bases that can form a double-stranded structure by matching base pairs. For example, the complementary sequence to C-A-T-G (where each letter stands for one of the bases in DNA) is G-T-A-C.

Denature

To dissociate a double-stranded region of nucleic acid into the homologous single strands by breaking the hydrogen bonds. This is usually achieved by heating.

DNA

Deoxyribonucleic acid is the genetic material in chromosomes.

DNA polymerase

An enzyme that synthesizes new DNA using a parental DNA strand as the template.

Downstream

A region extending from the 3′ end of the gene.

Endonuclease

Any enzyme that starts to degrade DNA or RNA within the nucleic acid sequence. These enzymes are used as molecular scalpels to cut DNA or RNA at precise points.

Gene

A DNA segment that codes for a polypeptide.

Gene cloning

The selection, application, and production of an individual nucleotide sequence.

Gene library

A collection of nucleotide sequences that have been artificially inserted into microorganisms or viruses and propagated.

Genome

The total amount of DNA present in a cell.

Hybridization

The action whereby two complementary single-stranded pieces of nucleic acids are joined to form a double-stranded segment.

In situ

In the normal location. An *in situ* tumor is one that is confined to its site of origin and has not invaded neighboring tissue or gone elsewhere in the body.

In situ hybridization

A technique that identifies and quantifies nucleic acid sequences within cells.

Kilobase

A measure of length of nucleic acids. One kilobase (kb) equals 1000 nucleotides of single-stranded nucleic acid; kbp refers to kilobase pairs of double-stranded DNA.

Melting temperature

The temperature at which the hydrogen bonds between complementary nucleotides will break, causing the dissociation of double-stranded nucleic acid. It is dependent on the G + C content of the DNA.

Missense mutation

A single base substitution in DNA that changes a codon for one amino acid into a codon for a different amino acid.

mRNA

Messenger RNA carries the message of the DNA to the cytoplasm of the cell where protein is made. Single-stranded RNA synthesized from a DNA template during transcription binds to ribosomes and directs protein synthesis.

Mutation

A change in the sequence of nucleotides in DNA.

Nucleotide

The unit of DNA or RNA that consists of a phosphate group, a sugar and a base.

Oligonucleotide

A short piece of nucleic acid that can be used as a hybridization probe.

Probe

A single-stranded piece of labeled DNA or RNA that will bind to a complementary sequence (target).

Random priming

A method for labeling double-stranded DNA to produce a probe.

Recombinant DNA

The production of a single piece of DNA from two different sources.

Replication

The process by which an exact copy of parental DNA or RNA is made with the parental molecule serving as a template.

Reverse transcriptase

An enzyme that will synthesize a complementary sequence of DNA from an RNA template.

RNA

Ribonucleic acid. A nucleic acid that plays an important role in protein synthesis and other cell activities.

RNA polymerase

An enzyme that catalyzes the synthesis of RNA from a DNA template, using nucleotide triphosphates as substrates.

RNases

Ubiquitous RNA degrading enzymes.

rRNA

Ribosomal RNA is a component of ribosomes and functions as a non-specific site for making polypeptides.

Sense strand

The DNA strand that RNA polymerase copies to produce mRNA, rRNA, or tRNA.

Stringency

Conditions employed in hybridization reactions used to control the specificity of probe binding. The highest stringency conditions ensure the probe will bind only to completely complementary sequences. Lower stringency conditions will allow binding to sequences with some mismatching.

Template

A strand of DNA or RNA that specifies the base sequence of a newly synthesized complementary strand of DNA or RNA.

Transcription

The process by which a DNA sequence is copied into a complementary RNA sequence.

tRNA

Transfer RNA is a short-chain type of RNA present in cells that transfers specific amino acids in the formation of proteins.

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