Chapter 12
PNA Fluorescent *In Situ* Hybridization for Rapid Microbiology and Cytogenetic Analysis

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1. Introduction

Hybridization-based assays for the detection of nucleic acids including *in situ* hybridization are increasingly being utilized in a wide variety of disciplines such as cytogenetics, microbiology, and histology. Generally *in situ* hybridization assays utilize either cloned genomic probes for the detection of DNA sequences or oligonucleotide probes for the detection of DNA or RNA sequences. Alternately, peptide nucleic acid (PNA) probes are increasingly being utilized in a variety of *in situ* hybridization assays (***1,2***). The neutral backbone of the PNA molecule allows for the PNA probes to bind to DNA or RNA under low ionic-strength conditions that will either disfavor reannealing of complimentary genomic sequences or are denaturing for RNA secondary structure but are favorable for PNA/DNA or PNA/RNA hybridization (***3,4***). For *in situ* hybridization assays, these unique properties of PNA probes offer significant advantages that allow for the development of fast, simple, and robust assays.

This chapter outlines two protocols that have exploited the unique physic-chemical properties of the PNA molecule in the development of *in situ* hybridization assays for the targeting of chromosome specific human alpha satellite sequence and for the definitive identification of microorganisms by targeting species-specific rRNA sequences.

1.1. Culture Identification by PNA FISH

Fluorescence *in situ* hybridization using PNA probes (PNA-FISH) is a new molecular method for definitive identification of microorganisms using fluorescent-labeled PNA probes targeting species-specific rRNA sequences (***5-8***). Due to the high cellular abundance of rRNA, individual cells can be detected directly by fluorescence microscopy (***9***) and thus identified directly in primary cultures, thereby eliminating the requirement for subculture to obtain pure isolates.

rRNA sequence analysis today is a well-established method for phylogenetic analysis of microorganisms (***10***) and has further enabled design of specific probes against the rRNA of most microorganisms of clinical, industrial, and environmental interest. As a result, molecular diagnostic methods using rRNA sequences are rapidly replacing the classic phenotype-identification methods based on biochemical analysis and morphological characteristics.

The noncharged backbone of PNA allows hybridization to be performed under low-salt conditions that are denaturing for rRNA secondary structure but favorable

for PNA-RNA hybridization (***11***). This enables use of PNA probes targeting rRNA sequences in highly structured regions where many species-specific target sequences are found. Furthermore, the relative hydrophobic character of PNA makes PNA probes able to easily diffuse through the hydrophobic cell wall of fixed bacteria and yeasts. *See* **Fig. 1.**

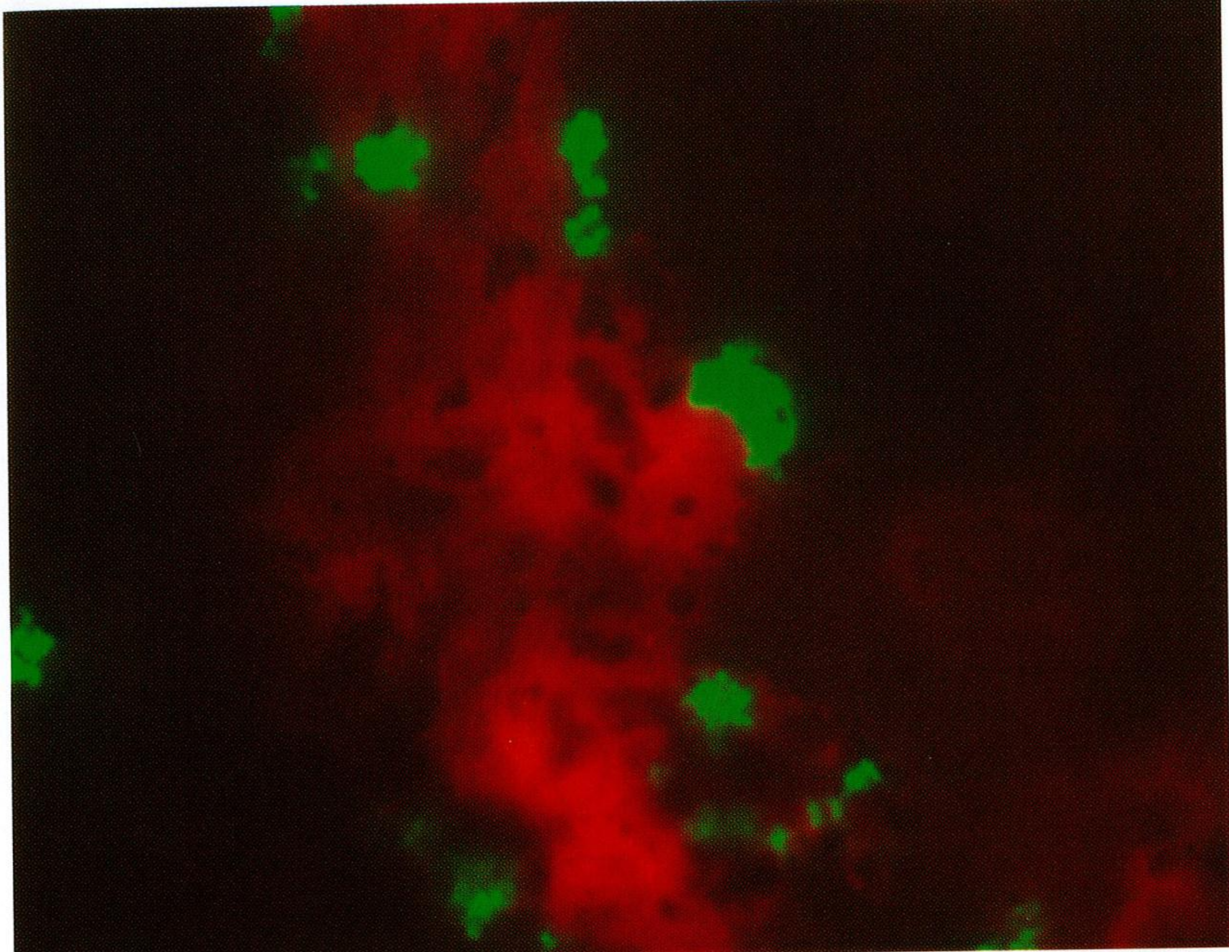

Fig. 1. Identification of *Staphylococcus aureus* from blood culture by PNA-FISH. Identification is based on bright fluorescent cells and thus combines the specificity provided by molecular technologies with the typical cell morphology.

1.2. Molecular Cytogenetic Analysis Using PNA FISH

FISH has become firmly established in cytogenetics, with the vast majority of FISH probes in use being derived from cloned genomic probes. Recently, the utility in FISH assays of directly labeled PNA probes that have been designed from human satellite or telomeric repeat sequence has been demonstrated by a number of investigators (***13-16***). As mentioned previously, PNA probes can bind to DNA under low ionic-strength conditions that disfavor reannealing of complimentary genomic strands. This advantage is particularly important for *in situ* hybridization experiments that target repetitive sequences, because both the length and the repetitive nature of the target sequences will effectively favor renaturation over hybridization with labeled probes (***13***).

These properties of PNA have been exploited in the following protocol; the sensitivity of which when used with a mixture of short synthetic PNA probes is comparable to large cloned probes. *See* **Fig. 2**.

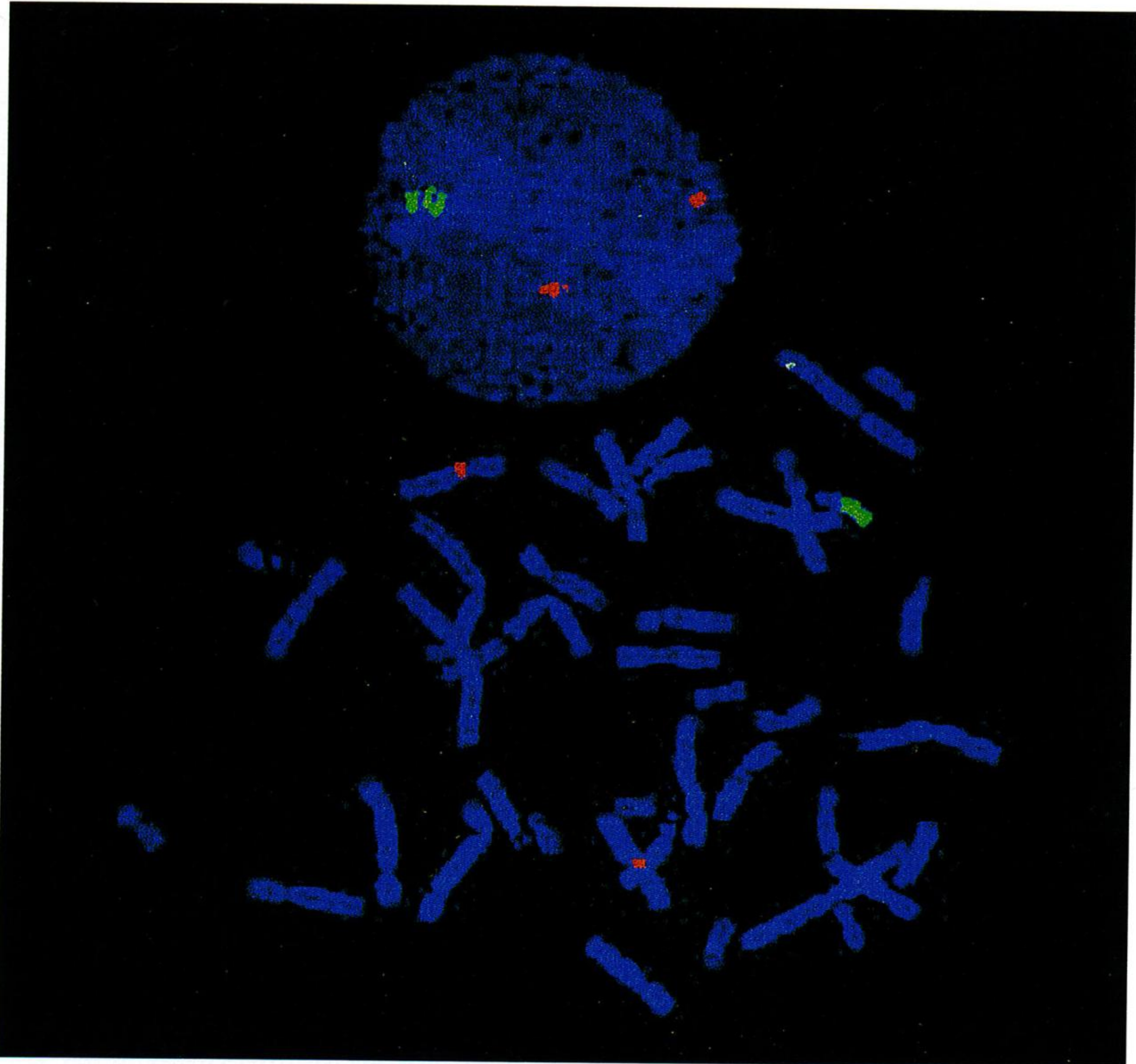

Fig. 2. Detection and enumeration of the X and Y chromosomes in human cells with sex chromosomes abnormalities. A mixture of Cy3 and fluorescein labeled PNA probes specific for the X centromere and Yq12 region, respectively, were used.

2. Materials

2.1. Culture Identification by PNA-FISH

1. Filter-sterilized PBS. It is recommended to add 1% Triton X-100 for culture media containing material, such as charcoal, to prevent nonspecific binding of PNA.

2. Fluorescein-labeled PNA probe targeting rRNA: Typically, PNA probe sequences of approx 15 bases with T_m values in the range of 60-70°C are optimal. Fluorescein-labeled PNA probes targeting *Escherichia coli* 16S rRNA, *Pseudomonas aeruginosa* 16S rRNA, *Salmonella enterica* 23S rRNA, *Staphylococcus aureus* 16S rRNA, *Dekkera bruxellensis* 26S rRNA as well as universal probes targeting eubacterium 16S rRNA and eukarya 18S rRNA are available from Boston Probes (Bedford, MA), whereas custom-made PNA probes can be obtained from Applied Biosystems (Foster City, CA). It is recommended to re-suspend and store PNA probes in 50% aqueous N,N-dimethyl formamide (DMF) at -18°C prior to use.
3. Hybridization Buffer: 10% (w/v) dextran sulfate, 10 m*M* NaCl, 30% (v/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone, 0.2% (w/v) ficoll, 5 m*M* Na_2EDTA, 0.1% (v/v) Triton X-100, 50 m*M* Tris-HCl, pH 7.5. Store at 2-8°C. (*see* **Note 1**).
4. Wash Solution: 25 m*M* Tris-HCl or CAPSO, pH 10.0, 137 m*M* NaCl, 3 m*M* KCl. Store at 2-8°C, preferably as a 60X stock solution.
5. IMAGEN Mounting Fluid (DAKO, Ely, UK). Store at 2-8°C. Alternatively, VectaShield Mounting Medium (Vector Laboratories, CA) may be used, although it tends to give a more reddish smear background (*see* **Note 2**).
6. Ethanol (Histology Grade).
7. Milli Q or deionized water.
8. Teflon-coated microscope slides with 14-mm diameter wells for FISH (ClearCell, Erie Scientific, Portsmouth, NH) (*see* **Note 3**).
9. Coverslips with a 0.15 mm thickness (No. 1).
10. Incubator or hybridization chamber at 55 ± 1°C.
11. Water bath (55 ± 1°C). The temperature of the water bath should be checked using a thermometer in the water.
12. Coplin jar for slides
13. Fluorescence microscope equipped with a 60x or 100x oil objective, a 100 W mercury lamp, and a FITC/Texas Red dual band filter. The use of a 50 W lamp reduces the fluorescent signal. The FITC/Texas Red double filter provides excellent discrimination between autofluorescence and specific signal, because autofluorescence will appear reddish whereas cells detected by the fluorescein-labeled PNA probe will be bright green. In particular clusters of cells and yeast cells tend to autofluoresce and therefore make it difficult to distinguish between positive and negative results using a standard FITC-filter (*see* **Note 4**).
14. Immersion oil. The immersion oil used must comply with the microscope objective and be nonfluorescent.

2.2. Molecular Cytogenetic Analysis Using PNA-FISH

2.2.1. *Reagents*

1. Labeled PNA Probed: Typically, PNA probe sequences of approx 18-20 bases with T_m values in the range of 65-75°C are optimal. Chromosome specific centromere and pan telomere probes are available from Boston Probes, custom-made PNA probes can be obtained from Applied Biosystems. It is recommended

to store the PNA probes in 50% aqueous DMF at -18°C prior to use (1) (*see* **Note 5**).
2. Hybridization buffer: 20 m*M* Tris-HCl, pH 7.5, 70% formamide (Gibco-BRL), 1X Denhardt's solution (Sigma), 10 m*M* NaCl, 100 mg/mL tRNA (Sigma), 100 µg/mL salmon sperm DNA (Sigma), pH 7.0-7.5.
3. Metaphase chromosome and Interphase cell suspension.
4. Ethanol (histology grade).
5. Mill-Q H_2O.
6. Carnoy's Fixative (3:1 methanol/acetic Acid): 3 parts methanol (J.T. Baker) to 1 part glacial acetic acid (J.T. Baker).
7. 20X SSC 1% Tween 20: 3 *M* NaCl, 0.3 *M* Sodium Citrate, 1% (v/v) Tween 20, pH 7.0-7.5. 2X SSC 0.1% Tween 20. Prepare 50 mL for each experiment (sufficient for 5 slides) by making a 1 in 10 dilution of the 20X SSC 1% Tween 20.
8. 10X PBS 1% Tween 20: 27 m*M* KCl, 15 m*M* KH_2PO_4, 1.4 *M* NaCl, 8 m*M* Na_2HPO_4, 1% (v/v) Tween 20, pH 7.5. 1X PBS 0.1% Tween 20. Prepare 50 mL for each experiment (sufficient for 5 slides) by making a 1 in 10 dilution of the 10X PBS. 1% Tween Wash Solution.
9. Mounting medium: Vector Shield (Vector Laboratories) containing 100 ng/mL of DAPI.

2.2.2. Equipment

1. Suitably equipped epifluorescence microscope with appropriate filters (*see* **Note 6**).
2. Microscope slides: Gold Seal precleaned slides, dipped in 100% ethanol, and polished with a lint free cloth (*see* **Note 7**).
3. Cover slips (22 x 22 mm): 0.15 mm thickness Corning brand No. 1.
4. Pipettes (10 µL and 20 µL).
5. Sterile pipet tips.
6. Graduated cylinders.
7. Water bath (55-60°C).
8. Incubator or heating block (70-80°C).
9. Humidified chamber at room temperature.
10. Forceps.
11. Coplin jars (50 mL).

3. Methods

Before starting the assay procedure, prepare working-strength wash solution and start to preheat (*see* **Note 8**).

3.1. Culture Identification by PNA-FISH

3.1.1. Preparation of Smears

1. Place one drop of phosphate-buffered saline (PBS) in a well on a microscope slide.
2. Transfer a small drop (10-50 µL) of re-suspended culture or a small part of a colony to the PBS and mix gently to emulsify.

3. Allow the smears to air-dry.
4. Fix the smears by passing the slide through the blue cone of a Bunsen burner three to four times, by heating for 20 min at 60°C, or by methanol for 5 min (**12**).
5. Immerse the slide in 80% ethanol for 5-10 min. This step primarily serves to disinfect the smear, but may also supplement fixation of the cells.
6. Air-dry until the smears are dry (approx 10 min). The smears may be stored for up to 14 d 2-8°C prior to hybridization, preferably placed in a sealed foil bag with a desiccant.

3.1.2. Hybridization

1. Dilute the fluorescein-labeled PNA probe in Hybridization Buffer. Typically, a concentration in the range of 100-500 n*M* is optimal, but it is recommended to carefully titrate individual probes using a panel of respective species. The diluted PNA probe in the Hybridization Buffer should be stored at 2-8°C and is stable for up to 1 yr.
2. Add one drop of PNA probe in Hybridization Buffer to the well on the microscope slide with the smear.
3. Add coverslip and try to avoid air bubbles trapped by coverships, as this will reduce the hybridization efficiency.
4. Incubate for 90 m in at 55°C.

3.1.3. Stringent Wash

1. Immerse slide in preheated Wash Solution at 55°C in a Coplin jar placed in a water bath at 55°C (*see* **Note 8**).
2. Carefully remove the coverslip.
3. Incubate for 30 min. at 55°C.

3.1.4. Mounting

1. Add one drop of Mounting Medium to the smear.
2. Add coverslip and try to avoid air bubbles as that may interfere with microscopic examination.

3.1.5. Microscopial Examination

1. Examine slides using a Fluorescence microscope. The cell smear appears in general brown/reddish. Positive identification is observed as bright green fluorescent cells and further supported by cell morphology. The intensity may vary between individual cells due differences in the amount of rRNA and/or permeability of the cell wall. The slides are stable when stored in the dark for at least 14 d.

3.2. Molecular Cytogenetic Analysis Using PNA-FISH

3.2.1. Slide Preparation

1. Spot the cell suspension onto a cleaned microscope slide using your current laboratory procedure. Evaluate the metaphase cells using phase-contrast

microscopy. Using a 20X phase objective, the metaphase chromosomes should appear gray/black and free of cytoplasm. If there is excessive cytoplasm present it is not advisable to proceed with the hybridization (*see* **Note 9**).

2. After preparing the slides, for optimal chromosome morphology and staining intensity, the slides should be aged for at least 4 h at room temperature (preferably overnight) before proceeding with the hybridization steps. Alternatively, slides can be stored at -20°C; slides are typically stable for at least 6 mo when stored correctly (*see* **Note 10**).

3.2.2. Hybridization

1. If the slides have been stored in the freezer, allow the slides to warm to room temperature before use.
2. Prepare the PBS wash solution by adding 1X PBS, Tween Wash Solution to a Coplin jar and warm to 57 ± 1°C.
3. Dilute the labeled PNA probe in Hybridization Buffer. Typically, a concentration in the range of 4-100 n*M* is optimal. The diluted PNA probe in Hybridization Buffer should be stored at 2-8°C and is stable for up to 1 yr.
4. Apply a 10 µL aliquot of probe solution to the target area of the slide. Immediately place a 22 x 22 mm glass coverslip over the probe solution and allow the solution to spread evenly under the coverslip. Try to avoid air bubbles because they may interfere with hybridization.
5. Please the slides in an incubator preheated to 70 ± 1°C for 5-8 min (*see* **Note 11**).
6. Prepare a humidified hybridization chamber by placing a damp paper towel in a container at room temperature and cover the chamber with a lid. Remove the slide from the incubator or heating block and place the slide into the humidified hybridization chamber. Reseal the container.
7. Incubate at room temperature for 30 min.

3.2.3. Posthybridization Washes

1. Immerse the slide(s) in 1X PBS, 0.1% Tween Wash Solution at room temperature to remove the coverslip.
2. Transfer the slide(s) to the prewarmed 1X PBS, 0.1% Tween Wash Solution and wash the slide at 57 ± 1°C for 20 min.
3. Rinse the slide(s) in 2X SSC 0.1% Tween wash solution for 1 min.
4. Drain the excess fluid from the slide and apply 20 µL of Mounting Media containing DAPI counterstain to the target area and place a 22 x 22 mm glass coverslip. Try to avoid air bubbles because they may interfere with visualization.
5. Place a tissue over the slide and gently apply pressure with your fingers to squeeze out the excess mounting media.
6. Store the slide in the dark for 15-30 min to allow the DAPI to stain the cells before viewing under the microscope.
7. The slides may be viewed immediately or stored at 4°C or -20°C in the dark. Slides are typically stable for at least 6 mo when stored correctly.

3.2.4. Microscopy Examination

The DAPI counterstain is used as a general DNA stain to aid in the location of metaphase spreads and interphase cells. The fluorescent signals are viewed using an epifluorescence microscope equipped with appropriate excitation and emission filters. In metaphase spreads, the centromere of the chromosome of interest will fluoresce brightly (the color of which will depend on the fluorochrome(s) used). In interphase cells the centromeric signals will be present as bright fluorescent spots. The hybridization efficiency of the PNA probes should be greater than 90% for metaphase and interphase cells.

4. Notes

1. The Hybridization Buffer contains 30% formamide. There is a possible risk of harm to unborn child. It is irritating to eyes, respiratory system, and skin. Wear suitable protective clothing and gloves.
2. Care should be taken when using the Mounting Medium because it may cause skin irritation. Skin should be flushed with water if contact occurs.
3. The use of other slides should be carefully evaluated in respect to the thickness of the Teflon-coating, cell-adhesive characteristics, and background spots due to unspecific binding of PNA probes.
4. It is important that the microscope is functioning properly. Make sure that the microscope bulb is correctly adjusted and is not aged above its specified lifetime.
5. PNA probes are compatible with a wide range of reporter molecules including fluorochromes for the direct detection of the PNA probe such as fluorescein, rhodamine, and the alexa and cyanine series of dyes. For indirect detection of PNA probes, alkaline phosphatase, peroxidases, biotin, DNP, and digoxigenin labels have been utilized.
6. The epifluorescent microscope should be equipped with at least a 10x plan Fluorite objective for locating cells of interest and a 60x and/or a 100x oil plan fluorite NA 1.3-1.4 objectives for observing the cells at high magnification. Below are examples of filters from Omega and Chroma suitable for three of the common fluorochromes used *in situ* hybridization experiments (*see* **Table 1**).
7. The use of clean slides cannot be overemphasized. There are a number of protocols for cleaning microscope slides, the simplest of which is to soak the slides in ethanol and wipe clean with a lint free cloth as described earlier. Other approaches include soaking in detergent, followed by rinsing in deionized water and either kept in distilled water until use of dipped in ethanol and rapidly air-dried.
8. It is important that the temperature of the Wash Solution has reached a temperature of 55°C prior to immersion of the slides.
9. The temperature and humidity of the environment in which the slides are made should be optimized to 20-23°C and 50-60%, respectively. If excessive cytoplasm is present, prepare another slide as follows: spot the cell suspension onto a cleaned microscope slide, as the spot takes on a grainy appearance add 1-2 drops of fixative (3:1 methanol:acetic acid). Allow the slides to dry and re-evaluate.

10. If the slides are to be subjected to a hybridization experiment immediately after preparation, then the following steps may be helpful in maintaining chromosome morphology and signal intensity:
    a. Prepare the pretreatment solution by adding 50 mL of 2X SSC wash solution to a Coplin jar and warm to 55 ± 1°C.
    b. Add the slides to the prewarmed 2X SSX wash solution for 5 min.
    c. Dehydrate the slides for 1 min in each of the cold ethanol (70, 85, 100%) series. Air-dry.
    d. Proceed with the hybridization steps.
11. If you are using a programmable heating block then the block should be programmed to gradually increase (90 s) the temperature to 75°C, hold the temperature for 90 s at 75°C and then ramp down the temperature to room temperature (90 s). If the heating block is humidified, the slides may be left on the heating block for the 30-min hybridization step at room temperature before proceeding with the posthybridization wash steps.

Table 1
Examples of Filters for Common Fluorochromes

Part No.	Ex	Em
Omega XF05/Chroma 31000 (DAPI filter set)	365	400
Omega XF22/Chroma 41001 (FITC filter set)	485	530
Omega XF34/Chroma 41007 (Cy3 filter set)	535	590

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