Label IT® Fluorescence *In Situ* Hybridization (FISH) Kits

Product Name	Quantity*	Product No.
Label IT® FISH Cy®3 Labeling Kit	Full Size	MIR 6510
	Trial Size	MIR 6520
Label IT® FISH TM-Rhodamine Kit	Full Size	MIR 6512
	Trial Size	MIR 6522
Label IT® FISH Fluorescein Kit	Full Size	MIR 6513
	Trial Size	MIR 6523
Label IT® FISH Biotin Kit	Full Size	MIR 6514
	Trial Size	MIR 6524

*Each full size kit contains sufficient reagents to label 10 µg of DNA and complete 40 PAINT hybridizations or 200 centromeric hybridizations. Each trial size kit contains sufficient reagents to label 2 µg of DNA and complete 8 PAINT hybridizations or 40 centromeric hybridizations. Please note that the *Label* IT®FISH Biotin Kit does not contain biotin detection reagents.

1.0 INTENDED USE

The *Label* IT® Fluorescence *In Situ* Hybridization (FISH) Kit is optimized for the preparation and hybridization of fluorescently (Cy®3, TM-Rhodamine, Fluorescein) labeled DNA probes to targeted interphase nuclei/metaphase chromosome spreads. In addition, multi-color FISH analysis is possible using spectrally distinct fluorophores. The use of *Label* IT® FISH Biotin probes and subsequent detection using any of a variety of streptavidin and anti-biotin antibody fluorophore conjugates can also expand the sophistication of FISH experiments.

2.0 DESCRIPTION

2.1 General Information

Fluorescence *In Situ* Hybridization (FISH) uses fluorescent DNA probes to vividly paint genes or chromosomes, revealing the localization of specific nucleic acid sequences. This valuable technique has proved useful for gene mapping and the identification of chromosomal abnormalities. FISH entails the preparation of short sequences of fluorescently-labeled DNA (probes) which are complimentary to the target DNA sequences. Metaphase chromosome spreads are prepared, RNase treated, dehydrated and denatured according to the procedure provided. The slides are then hybridized with the fluorescently labeled DNA probe for 16-24 hours. Post hybridization, the slides are washed, counterstained with a 4',6-diamidino-2-phenylidole (DAPI) antifade mixture, and analyzed using fluorescent microscopy.

The optimized *Label* IT® FISH Kits utilize Mirus Bio's *Label* IT® technology to prepare superior labeled probes, by design, and hybridize them to targeted interphase nuclei/metaphase chromosome spreads. The *Label* IT® nucleic acid labeling technology represents a new class of reagents that efficiently and reproducibly attach marker molecules to nucleic acids in a simple, one-step chemical reaction. With the *Label* IT® Reagents, one can covalently attach a variety of fluorescent and non-fluorescent tags to any nucleic acid species within minutes. Traditional nonradioactive labeling methods (nick translation and random priming) are enzyme-mediated and therefore inherently difficult to control. *Label* IT® reactions require a single reagent, and the density and scale of the reaction can be easily controlled. The DNA remains intact after the labeling reaction, which allows quantitative recovery of the labeled product. The *Label* IT® FISH Kits are supplied with labeling reagents, hybridization buffer, and an optimized protocol, which can be used to generate fluorescently labeled probes for a variety of FISH applications, including:

Alpha-satellite centromeric probes—generated from repetitive sequences found at the centromeres of specific chromosomes. Researchers use this technique to enumerate specific chromosomes.

Whole chromosome PAINT probes (Protocol Aberration Identification and Nomenclature Terminology)—collections of small probe sequences that hybridize to different regions along the length of the same chromosome. Researchers use this technique for examining chromosomal abnormalities.

Fluorescent images of PAINT and centromeric analyses using the *Label* IT® FISH Kits are available at www.mirusbio.com/products_FISH.php.

Table 1. Excitation and emission wavelengths of DNA labeled with the *Label* IT® FISH Reagents

Labeling Reagent	Excitation Wavelength (nm)	Emission Wavelength (nm)
Cy™3	549	570
Tetramethyl-rhodamine	559	576
Fluorescein	495	518

2.2 Kit Components

Each full size kit contains sufficient reagents to label 10 µg of DNA and complete 40 PAINT hybridizations or 200 centromeric hybridizations. Each trial size kit contains sufficient reagents to label 2 µg of DNA and complete 8 PAINT hybridizations or 40 centromeric hybridizations. Each FISH Kit includes the appropriate Cy™3, TM-Rhodamine, fluorescein or biotin labeling reagent, labeling buffers and hybridization buffer. Please note that the *Label* IT® FISH Biotin Kit does not contain biotin detection reagents.

Kit Component	Full Size Kit	Trial Size Kit	Reagent Cap Colors
Label IT® FISH Reagent (dried pellet)	Sufficient reagent to label 10 µg DNA	Sufficient reagent to label 2 µg DNA	Varies with reagent
Reconstitution Solution	100 µl	100 µl	Clear cap
10X Labeling Buffer A	100 µl	100 µl	Lilac cap insert
Denaturation Reagent D1	150 µl	150 µl	Blue cap insert
Neutralization Reagent N1	200 µl	200 µl	Clear-no cap insert
FISH Hybridization Buffer	3 ml (2 x 1.5 ml)	0.6 ml	Amber cap

NOTE: Extra volume of the Reconstitution Solution and reagents are supplied to allow for differences in pipetting devices.

2.3 Storage and Stability

Store the *Label* IT® FISH Reagent at -20°C in both its dried pellet and reconstituted form. Store all other supplied reagents at -20°C. The
Label IT® FISH Reagent is stable for 6 months after reconstitution. Unreconstituted *Label* IT® Reagent and all other reagents are stable for up to 1 year from the date of purchase.

2.4 Required Equipment and Reagents (not provided)

Reagents

Biotin detection reagents (if necessary)
DNase-free RNase A (1 mg/ml)
Ultrapure deionized formamide (>99.5% purity)
20X SSC buffer (pH 7, 0.22 µm filtered)
Tween 20
Molecular biology grade water (DNase/RNase free)
5M NaCl
Ethanol (100%), room temperature
1M HCl (molecular biology grade)
Sheared salmon sperm DNA
Species-specific Cot-1 DNA
Nail polish or rubber cement
Antifade mounting medium with 4',6-diamidino-2-Phenylindole (DAPI stain) (e.g. Vectashield containing 1.5 mg/ml DAPI from Vector Labs, Cat#H-1200)
Antifade mounting medium for diluting above stain (Vector Labs, Cat#H-1000)

Equipment

0.2 µm filter units (SFCA, Nalgene)	22 x 50 mm glass coverslips
Coplin jars	22 x 22 mm glass coverslips
pH strips (4.5-10 pH or similar testing range)	Fluorescent microscope equipped with appropriate filters (see Table 1)
Slide warmer	Hybridization chambers (e.g. Corning CMT Cat#2551)
Forceps	Humid chamber

3 temperature controlled water baths (shaking waterbaths are recommended)

2.5 Required Solutions (not provided)

RNase digestion solution: 100 µg/ml RNase A in 2X SSC (600 µl)

1. Make fresh prior to use (approximately 150 µl/slide).
2. To process 4 slides: Mix 60 µl 1 mg/ml RNase A, 60 µl 20X SSC, and 480 µl molecular biology grade water in a microcentrifuge tube.
3. Pipet to mix. Store on ice and use immediately.
4. Discard after each use.

Denaturing solution: 70% formamide in 2X SSC at 72°C (pH 7-7.5) (70 ml; ~70 ml needed for each coplin jar)

1. Mix 49 ml deionized formamide, 7 ml of 20X SSC, and 1 ml molecular biology grade water.
2. Adjust pH to 7-7.5 with 1M HCl.
3. Bring volume to 70 ml with molecular biology grade water. Mix well.
4. Ensure pH is between 7 and 7.5 prior to each use. Prepare fresh if pH is not between 7 and 7.5.
5. Prewarm to 72°C (30-60 minutes) prior to each use.
6. Solution can be used up to 1 week. Store in a sealed coplin jar at 4°C.

Formamide wash solution: 50% Formamide in 2X SSC at 45°C (pH 7-7.5) (210 ml; 70 ml needed for each jar)

1. Mix 105 ml deionized formamide, 21 ml 20X SSC, and 83 ml molecular biology grade water.
2. Adjust pH to 7-7.5 with a few drops of 1M HCl.
3. Bring volume to 210 ml with molecular biology grade water. Mix well.
4. Ensure pH is between 7 and 7.5 prior to each use. Prepare fresh if pH is not between 7 and 7.5.
5. Prewarm to 45°C (30-60 minutes) prior to each use.
6. Solution can be used up to 1 week. Store in a sealed coplin jar at 4°C.

20X SSC stock (1 L)

1. Combine 175 g of NaCl, 88.2 g trisodium citrate dehydrate, and 800 ml molecular biology grade water. Mix well.
2. Adjust pH to 7 with 1M HCl.
3. Bring volume to 1 liter with molecular biology grade water. Mix well.
4. Filter with 0.22 µm SFCA filter unit.
5. Store at room temperature for up to 6 months.

2X SSC at room temperature and 45°C (285 ml)

1. Mix 28.5 ml of 20X SSC with 256.5 ml of molecular biology grade water. Mix well.
2. Three 70 ml coplin jars of 2X SSC will be warmed for post-hybridization washes and the fourth 70 ml coplin jar should be kept at room temperature. The excess 2X SSC will be used to make 0.1X SSC.
3. Discard after each use.

0.1X SSC at 45°C (210 ml)

1. Mix 10.5 ml of 2X SSC with 199.5 ml molecular biology grade water. Store at room temperature until ready to use.
2. Warm to desired temperature in 70 ml coplin jars.
3. Discard after each use.

4X SSC/0.1% Tween 20 (70 ml)

1. Make fresh prior to each use.
2. Mix 14 ml of 20X SSC with 56 ml of molecular biology grade water.
3. Add 70 µl of Tween 20.
4. Mix well and store at room temperature until ready to use. Discard after use.

Antifade DAPI Counterstain:

1. Prepare antifade mounting medium with approximately 500 ng/ml DAPI counterstain. Make a fresh dilution of mounting medium when ready to mount hybridized slides.
2. Use the Vectashield products to process 4 slides with 2 areas per slide (8 areas total) and 15 µl mounting medium per area. Make a 3-fold dilution by adding 40 µl DAPI containing mounting medium into 80 µl of mounting medium. If the DAPI is not diluted, signal intensity may be too bright to allow for optimal FISH localization.

NOTE: DAPI is a possible mutagen based on positive genotoxic effects. AVOID inhalation, ingestion, or contact with skin.
1,4-phenylenediamine (the antifade reagent) is a known dermal sensitizer and a possible respiratory sensitizer. Handle with caution.

Ethanol Solutions:

Prepare ethanol solutions (70%, 85%) by diluting with molecular biology grade water. 100% ethanol will also be needed in this procedure. Store ethanol dilutions (~70 ml per coplin jar) up to 1 week at room temperature. If dilutions are not sealed properly in storage bottles or they become diluted due to excess use, discard properly and make fresh solutions.

3.0 PROCEDURE

NOTE: Ideal probe fragments range in size from 100 to 1000 base pairs, depending on target and specimen preparation. General references using PCR methods to generate probes are provided in Section 5, references 1-3. The *Label* IT® FISH Kits are optimized for use with metaphase spreads; see Section 5, references 4 and 5 for slide preparation of metaphase spreads.

3.1 Probe Labeling Reaction

1. Warm the tube containing the *Label* IT® FISH Reagent to room temperature and quick spin to collect the pellet. If using the full size kit, add 10 µl of Reconstitution Solution to the pellet in the tube. If using the trial size kit, add 8 µl of Reconstitution Solution to the pellet in the tube. To ensure complete reconstitution of the pellet, mix well by gentle pipetting, and perform a quick spin.
2. Prepare the labeling reaction according to the example shown below. Use water that is both DNase- and RNase-free (molecular biology-grade quality). Add the *Label* IT® FISH Reagent last.
   NOTE: Use only purified probe DNA (A_{260}/A_{280} ratio between 1.8 and 2.2) in the labeling reactions.

   Labeling Reaction Examples (labeling 2 µg DNA):

Reaction Component	Full Size Label IT® FISH Kit	Trial Size Label IT® FISH Kit
Molecular biology grade H_2O	32 µl	26 µl
10X Mirus Labeling Buffer A	4 µl	4 µl
1 mg/ml DNA (probe) solution	2 µl	2 µl
Label IT® FISH Reagent	2 µl	8 µl
Total reaction volume	40 µl	40 µl

   NOTE: This recommended labeling ratio and nucleic acid concentration will result in labeling efficiencies that are appropriate for most direct FISH analyses. The labeling reaction may be scaled up or down, depending on the amount of DNA to be labeled. However, the amount of *Label* IT® Reagent should never constitute more than 20% of the total reaction volume. Store unused reconstituted *Label* IT® Reagent tightly capped at -20°C.
3. Quick spin the tube containing the labeling reaction. Incubate reaction at 37°C for 1 hour.

NOTE: When performing the labeling reaction, a quick spin should be performed after 30 and 60 minutes of incubation. This will minimize the effect of evaporation and keep the concentration of the reaction components at the appropriate levels.

4. Add 60 µl molecular biology grade water to bring volume to 100 µl.
5. Remove unreacted labeling reagent from the labeled DNA by ethanol precipitation:
   a. Add 0.1 volume of 5 M sodium chloride and 2 volumes of ice cold 100% ethanol to the completed reaction. Mix and place in a -20°C (or colder) freezer for at least 30 minutes.
   b. Centrifuge at full speed in a cooled (4°C) microcentrifuge for 10 minutes to pellet the labeled DNA. Aspirate the ethanol, being careful not to disturb the pellet.
   NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that you will know where the pellet forms. Small DNA quantities can be invisible to the naked eye.
   c. Gently wash the pellet once with 70% ethanol (400-500 µl; room temperature). After an additional centrifugation (4°C) at full speed for 10 minutes, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become extremely difficult to resuspend. Resuspend the labeled probe in 10 µl 1X Labeling Buffer A or buffer of choice.
6. Quantify resuspended DNA using a spectrophotometer and store protected from light at -20°C, or colder.

3.2 Slide Pretreatment of Metaphase Spreads

See Section 2.5 for preparation of reagents.

NOTE: Warm slides to room temperature prior to pretreatments. Use fresh slides if possible, as slides 6 months or older may not hybridize as well. It is recommended to process only 4 slides per coplin jar in order to maintain proper temperatures.

1. Incubate prepared metaphase spread slides with approximately 150 µl per slide of RNase A digestion solution (100 µg/ml) under a 22 x 50 mm coverslip for 1 hour at 37°C in a humid chamber. A simple humid chamber can be fashioned using a sealed plastic storage container lined with water-soaked paper towels and a dry platform (i.e. microcentrifuge rack) to support slides.
2. Wash slides in 2X SSC until coverslips slide off; slight agitation may be necessary.
3. Dehydrate slides in an ethanol series:
   a. 2 minutes in 70% room temperature ethanol
   b. 2 minutes in 85% room temperature ethanol
   c. 2 minutes in 100% room temperature ethanol
4. Air dry slides. At this point, if necessary, slides can be stored at room temperature overnight. However, once the slides are denatured, they must be used the same day.

3.3 Suggestions for the Preparation of *Label* IT® Labeled Probe With Unlabeled Competitor/Blocking DNA

NOTE: Labeled probes have been quantitatively prepared in Section 3.1. The recommended starting conditions below are for the concentration of labeled probe and competitor DNA. Optimal conditions may need to be tested empirically.

1. a. PAINT probes: combine 150 ng of *Label* IT® labeled probe with 7.5 µg species-specific Cot-1 DNA and 11 µg sheared salmon sperm DNA. Vortex and quick spin.
   b. Alpha-satellite centromeric probes: combine 30 ng of *Label* IT® labeled probe with 1.2 µg species-specific Cot-1 DNA and 18 µg sheared salmon sperm DNA. Vortex and quick spin.
   NOTE: Bring volume to at least 10 µl with molecular biology grade water to ensure accurate denaturing of probe and competitor DNA. Vortex and quick spin.
2. Add 0.1 volume (use probe mixture volume to calculate) Denaturation Reagent D1 to the probe mixture.
3. Mix well and incubate for 5 minutes at room temperature.
4. Add 0.1 volume (use probe mixture volume to calculate) Neutralization Buffer N1 to the denatured probe mixture.
5. Mix well and incubate on ice for at least 5 minutes.
6. Bring volume to 100 µl with molecular biology grade water. Store on ice.
7. Ethanol precipitate labeled probe from Section 3.3 (see Section 3.1 step 5 for details).
8. Complete all steps of Section 3.4 (denaturation of slides) before preparing probe for hybridization.

NOTE: Pellet will be resuspended in the provided hybridization solution (see Section 3.5).

3.4. Denaturing and Dehydrating Metaphase Chromosome Spreads

1. Prepare denaturing solution (see Section 2.5) in a coplin jar and warm to 72°C.
   NOTE: It is very important that the temperature of the denaturing solution in the coplin jar does not fall below 70°C because probe access during hybridization may be impaired. Therefore, only process 4 slides per coplin jar and prewarm the slides to approximately 42°C on a slide warmer before denaturation step. Coplin jars tend to be 3°C lower than the water bath temperature, and each slide will cause a 1°C temperature drop, therefore set temperature of water bath accordingly.
   NOTE: It is important that the pH of the formamide solution is kept between 7 and 7.5, therefore, discard and make fresh solution if it is not in this range.
2. Denature slides for 2 to 4 minutes in denaturing solution.
   NOTE: Depending on target cell type and preparation method, optimal denaturing time will need to be empirically determined. If the denaturing time is too long, chromosomes will appear puffy and distorted. If the denaturing time is too short, probe will appear as a haze over cellular spreads. In general, older slides will take longer to denature.
3. Immerse the slides in the following ethanol series:
   a. 2 minutes in 70% room temperature ethanol
   b. 2 minutes in 85% room temperature ethanol
   c. 2 minutes in 100% room temperature ethanol
4. Tap excess ethanol off of slides and air dry.
   NOTE: Prop slides upside down and at an angle to aid in ethanol removal and uniform drying.

3.5 Hybridization

1. Warm hybridization buffer to room temperature. Remove a 15 µl aliquot of hybridization buffer per 22 x 22 mm coverslip area, warm to 37°C, and resuspend labeled probe pellet. Avoid heating entire supplied hybridization buffer tube to 37°C, as this may alter the pH of subsequent hybridizations.
2. Vortex probe mixtures and incubate probe in hybridization buffer for 10 minutes at room temperature to aid in dissolving the DNA pellet.
3. Incubate probe mixture at 75°C for 10 minutes.
4. Prewarm denatured/dehydrated slides 1 to 2 minutes on a slide warmer to approximately 42°C.
   NOTE: If using 2 hybridization areas per slide, separate probe areas with a streak of nail polish or pap pen to keep probe solutions from mixing.
6. Pipet probe mixture onto slides. Alternatively, pipet probe mixture onto coverslip, invert, and gently place over slide target area.
7. Carefully cover with a 22 x 22 mm glass coverslip and eliminate any visible air bubbles by very gently pressing coverslip to glass slide.
   NOTE: The presence of air bubbles will impede access of probe to the target cell spreads and may yield areas with no signal.
9. Seal in humidified hybridization chamber and incubate at 42°C overnight (16-24 hours) protected from light.

3.6 Post Hybridization Treatment- Staining, Viewing and Preservation of Hybridized Slides

3.6.1 Post Hybridization Washes

1. Prepare antifade DAPI counterstain (see Section 2.5).
   NOTE: Do not let mounting solution come to room temperature before removing aliquot; keep at 4°C until ready to use. DAPI staining intensity will correspond inversely to the degree of chromosomal denaturation.
2. Gently remove nail polish from slide with forceps, and remove coverslips with gentle agitation in a 70 ml coplin jar filled with 2X SSC.
   NOTE: Do not allow slides to dry out at this step and between successive post hybridization washes or chromosomes will appear morphologically deformed with a high fluorescent background.
3. Stringency washes: (wash slides in 50 ml coplin jars at recommended temperatures in a shaking water bath)

   PAINTs 1. Prewarm formamide/SSC solutions to 45°C in separate coplin jars. Prewarm 2X SSC to 45°C in 3 separate coplin jars (30 minutes).
   2. Wash 3 times (5 minutes in each jar) in 50% Formamide wash solution at 45°C (dispose of formamide wash solution properly in sink with excess water flush).

3. Wash 3 times (5 minutes in each jar) in 2X SSC at 45°C.
4. Wash slide in 4X SSC/0.1% Tween 20 for 1 minute.
5. Wash slide in molecular biology water for 1 minute.

NOTE: If detecting biotin, see Section 3.6.2. If detecting fluorescent probes, see Section 3.6.3.

Alpha-satellites

1. Prewarm formamide/SSC solutions to 45°C in 3 separate coplin jars. Prewarm 0.1X SSC to 45°C in 3 separate coplin jars (30-60 minutes).
2. Wash 3 times (5 minutes in each jar) in 50% Formamide wash solution at 45°C (dispose of formamide wash solution properly in sink with excess water flush).
3. Wash 3 times (5 minutes in each jar) in 0.1X SSC at 45°C.
4. Wash slide in 4X SSC/0.1% Tween 20 for 1 minute.
5. Wash slide in molecular biology water for 1 minute.

NOTE: If detecting biotin, see Section 3.6.2. If detecting fluorescent probes, see Section 3.6.3.

3.6.2 Biotin Detection

NOTE: PBS is used to pre-incubate slides because it is the buffer recommended with the streptavidin-fluorescent conjugate. If using a different buffer for the biotin detection reagent, use that buffer instead of PBS throughout detection procedure.

1. Rinse slide in PBS or suitable detection buffer for 1 minute.
2. Quick spin biotin detection reagent stock prior to making dilution to ensure accurate concentration and to avoid any particulates. Dilute biotin detection reagent stock per manufacturer's instructions.
3. Incubate the slide with approximately 200 µl of biotin detection reagent solution at room temperature for 1 hour in humid chamber.
4. Wash 3 times (5 minutes each) in PBS at room temperature, replacing with fresh PBS between each wash.
5. Complete Section 3.6.3.

3.6.3 Counterstaining and Mounting Slides

NOTE: Store hybridized (not yet mounted) slides at -20°C. Store mounted slides at 4°C. Store slides in a slide box, protected from light. Avoid frequent exposure to light as fluorescent signals will fade with time and exposure. View slides with a fluorescent microscope and scan using the appropriate filter for specific signal. Best images are acquired within one to two days and up to one week of mounting.

1. For every 22 x 22 mm coverslip area, dispense approximately 15 µl of diluted antifade DAPI counterstain (for preparation, see Section 2.5) and cover with glass coverslip.
2. Push gently on coverslip with lab tissue to remove all air bubbles and excess mounting solution as these may inhibit proper viewing under the microscope (excess mounting solution results in chromosomes appearing out of focus and probe signal may not be observable).
3. Seal edges of coverslip with nail polish or rubber cement.
4. View slides on fluorescent microscope using appropriate filters (see Table1).

4.0 TROUBLESHOOTING

Suboptimal Signal

- Improper storage of reagents
  Store both reconstituted and unreconsistuted *Label* IT® FISH Reagents tightly capped at -20°C. Protect from exposure to light and moisture.
- Poor quality DNA
  Use purified DNA (A_{260}/A_{280} between 1.8 and 2.2) that is free from proteins, carbohydrates, etc. Avoid nucleic acid degradation by using DNase-free handling procedures and plasticware.
- Incorrect concentration of starting DNA
  Perform spectrophotometer analysis to confirm DNA concentration.
- Labeling reaction was not scaled properly
  Perform reaction in minimal volume, keeping the amount of *Label* IT® FISH Reagent less than 20% of the total reaction volume and the Labeling Buffer A at 1X final concentration in the reaction. See Section 3.1.

- Low labeling ratio
  Increase the labeling ratio (volume of *Label* IT® FISH Reagent to weight of DNA) or increase the incubate time of the reaction to 2 hours.
  NOTE: The relative density of fluorescent labels on purified, labeled DNA can be assessed by:
  1. Agarose gel electrophoresis without ethidium bromide. The DNA may appear faint under UV illumination because the transilluminator emits at approximately 300 nm, which is not optimal for the fluorescent labels (see Table 1).
  2. Spectrophotometric absorbance at λ_{max}.
  3. Fluorescent microscopy. Spot dilutions of labeled DNA onto a glass slide and view with fluorescent microscope.
- Target slide was not denatured
  See Section 3.4 for proper slide denaturation steps
- Suboptimal amount of probe was used
  Requantify stock of labeled probe, or add more probe to hybridization buffer.
- Probe was not denatured
  See Section 3.3 for proper probe denaturation steps.
- Probe was not added to hybridization buffer
  See Section 3.5 for proper hybridization steps.
- Poor probe design
  Check probe sequence, clone identity, PCR products, etc.
- Wash solutions were made incorrectly
  See Section 2.5 for proper temperature, pH, and components.
- Excessive competitor or blocking DNA was used
  Lower concentration of competitor or blocking DNA in hybridization buffer.
- Hybridization washes were too stringent
  Raise salt concentration in post hybridization washes, or lower temperature or number of post hybridization washes.
- Incorrect pH of solutions
  Confirm pH of formamide solutions.
- Low target accessibility
  Protease treatment (after RNase A digestion and prior to denaturation) can serve to increase target accessibility by removing the protein that surrounds the target DNA. Prepare stock solutions of Pepsin or Proteinase K, aliquot, store at -20°C and avoid freeze/thaws to ensure enzyme stability. Each of these solutions should be prepared fresh and discarded after each use. A 10% Pepsin stock (w/v) can be made with molecular-biology grade water or 50% glycerol. A 0.2 mg/ml Proteinase K stock solution can be made with PBS and 0.05% SSC. Optimal incubation times and concentration of protease must be empirically determined for specific fixed-slide target preparations and labeled probe combination. A reasonable starting point for protease digestion is approximately:
  Pepsin treatment: Prewarm 0.01 M HCl to 37°C (approximately 30 minutes) in a coplin jar. Add Pepsin to make a final concentration of 50 µg/ml immediately before slide addition, and incubate for 10-15 minutes.
  Proteinase K treatment: Prewarm PBS solution with 10% SDS to 37°C (approximately 30 minutes) in a coplin jar. Add Proteinase K to make a final concentration of 100 ng/ml, immediately before slide addition, and incubate for 10-15 minutes.
- Coverslips have trapped air bubbles in DAPI mounting medium
  Press gently with lab tissue to remove air bubbles.
- Trouble detecting fluorescent signal
  Use proper filter sets for microscopic detection. See Table 1.

High Background Signal

- Probe sample was not clean
  Use purified DNA (A_{260}/A_{280} between 1.8 and 2.2) that is free from proteins, carbohydrates, etc. Avoid nucleic acid degradation by using DNase-free handling procedures and plasticware.
- Excess probe was used
  Decrease the probe concentration used for hybridization. See Section 3.5.
- Washes were not stringent enough
  Increase number, temperature and/or length of post hybridization washes. Increase formamide concentration in post hybridization washes, or decrease salt concentration in post hybridization washes.
- Suboptimal concentration of competitor DNA was used
  Increase concentration of competitor DNA in hybridization buffer.
- Cellular debris present
  Remake target cell spreads. Treat with Pepsin or Proteinase K. See "Low target accessibility" in troubleshooting section.
- Cross-hybridization
  HCL treatment: precise action of the acid is unknown; however, the extraction of proteins and partial hydrolysis of target sequences may contribute to increase signal-to-noise ratio. After RNase A digestion, and before denaturation, incubate slides in 0.2 N HCl (37°C) for 20 minutes.

Unfocused Chromosomes/ Poor Chromosome Morphology

- Excess Mounting Solution
  Remount slides or remove excess anti-fade solution by gently pressing on edges of coverslip with a tissue prior to sealing.
- Target slide was denatured for too long
  Reduce slide denaturation time to 1-2 minutes.
- Formamide concentration was too high
  Lower formamide concentration in post hybridization wash solutions by 5% increments.
- Slides dried out during initial preparation or between post-hybridization washes
  Keep hybridization chamber humid, and do not let slides air dry until indicated in protocol.

Unexpected Signal

- Cross hybridization between chromosomes
  See Section 5, reference #3.
- Probe concentration too high
  Lower probe concentration in hybridization buffer. See Section 3.5.
- Poor probe design
  Check probe sequence, clone identity, PCR products, etc.
- Slides were denatured for too long
  Shorten denaturation time. See Section 3.4, step 2.

For specific questions or concerns, please contact Mirus BioTechnical Support at 888.530.0801 or techsupport@mirusbio.com
For a list of citations using Mirus Bio products, please visit the Technical Resources section of our website. (www.mirusbio.com)

5.0 REFERENCES

NOTE: Human chromosome similarities which may result in cross hybridization using PAINT probes and alpha-satellite centromeric probes can be found between (13 and 21), (1, 5, 16 and 19), (2, 18, and 20), (4 and 9) and (14 and 22). See Reference 1 below.

1. Ward, David, C., *et al*. 2001. "Small marker chromosome identification in metaphase and interphase using centromeric multiplex FISH." http://info.med.yale.edu/genetics/ward/tani/CMFISH.html. Laboratory Investigation 18(4): 475.
2. PAINT probes: Liu, Siciliano, Seong, Craig, Zhao, Jong, Siciliano. 1993. Dual *Alu* Polymerase Chain Reaction Primers and Conditions for Isolation of Human Chromosome Painting Probes From Hybrid Cells. *Cancer, Genetics, Cytogenetics* 65: 93.
3. Alpha-satellite centromeric probes: Dunham, Lengauer, Cremer, Featherstone. 1992. Rapid generation of chromosome-specific alphoid DNA probes using the polymerase chain reaction. *Human Genetics* 88: 457.

NOTE: Generally, amniocyte, chronic villi, peripheral blood lymphocyte (PBL) WBC, or fibroblast cultures are established and slides are prepared according to standard cytogenetic methods:

4. Knight, Flint. 2002. Multi-Telomere FISH. Molecular Cytogenetics: Protocols and Applications. 204: 164.
5. www.slh.wisc.edu/cytogenetics/protocols/cultureharvest/cultharv.html