# SUNSHINE® CEP X/Y DNA FISH PROBE

REF D-0001-100

## KEY TO SYMBOLS USED

| | | | |
|---|---|---|---|
| | Legal Manufacturer | | Danger |
| REF | Reference Number | | Temperature limitation |
| Σ | Contains sufficient for <n> tests | | Used by |
| | Consult instructions for use | | Biological Risks |

## INTENDED USE

The Sunshine® CEP X SpectrumOrange/Y SpectrumGreen DNA Probe is intended to detect alpha satellite sequences in the centromere region of chromosome X and satellite III DNA at the Yq12 region of chromosome Y in conjunction with routine diagnostic cytogenetic testing. It is indicated for use as an adjunct to standard cytogenetic analysis for identifying and enumerating chromosomes X and Y via FISH in interphase nuclei and metaphase spreads obtained from standard cytogenetic procedures.

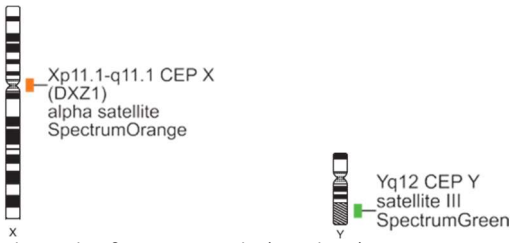
## PRINCIPLES OF THE PROCEDURE

In situ hybridization is a technique that allows the visualization of specific nucleic acid sequences within a cellular preparation. Specifically, DNA FISH involves the precise annealing of a single- stranded fluorescently labeled DNA probe to complementary target sequences. The hybridization of the probe with the cellular DNA site is visible by direct detection using fluorescence microscopy.

Tissue consisting of interphase nuclei or metaphase spreads is attached to glass slides using standard cytogenetic protocols. The resulting specimen DNA is denatured to its single stranded form and then allowed to hybridize with the CEP X/Y DNA probe. Following hybridization, the excess and unbound probe is removed by a series of washes and the chromosomes and nuclei are counterstained with the DNA specific stain DAPI (4,6 diamidino-2-phenylindole) that fluoresces blue. Hybridization of the CEP X/Y DNA probe is viewed using a fluorescence microscope equipped with appropriate excitation and emission filters allowing visualization of the intense orange fluorescent signal concentrated at the centromere of chromosome X, the intense green fluorescent signal concentrated at the Yq12 region of chromosome Y and the blue counterstained chromosomes and nuclei. The enumeration of chromosomes X and Y is conducted by microscopic examination of interphase and/or metaphase nuclei. The fluorescently stained centromeres of chromosome X and satellite III DNA of chromosome Y stand out brightly against the general blue fluorescence of the nuclear DNA provided by the DAPI counterstain. Relative to standard cytogenetic methods, the CEP procedure provides a higher percentage of interpretable nuclei per slide and enables visual enumeration of chromosomes X and Y within the nuclei.

The assay results are reported as the presence of nuclei with an XX, XY or other formula. Each orange fluorescent signal corresponds to the centromere of a chromosome X; each green fluorescent signal corresponds to the satellite III DNA of chromosome Y.

Chromosome enumeration probes consist of chromosome specific tandem-repeat DNA sequences (alpha satellite, satellite II, or satellite III DNA). Sunshine® probes are directly labeled with a fluorophore for detection. Unlabeled blocking DNA is mixed with the DNA sequences to suppress the more highly repetitive sequences that are common to many different chromosomes. To minimize cross-hybridization to other similar repeat sequences on other chromosomes the DNA sequences are hybridized under conditions of high stringency.




The Sunshine® CEP X DNA probe (DXZ1 locus) is a SpectrumOrange directly labeled fluorescent DNA probe specific for the AT rich alpha satellite DNA sequence at the centromeric region of chromosome X (Xp11.1- Xq11.1). The CEP Y DNA probe (DYZ1 locus) is a SpectrumGreen directly labeled fluorescent DNA probe specific for the satellite III DNA at the Yq12 region of chromosome Y. This assay is designed for the detection and quantification of chromosomes X and Y in both interphase nuclei and metaphase spreads by FISH.

## REAGENTS AND INSTRUMENTS

### Materials Provided

This kit contains 1 reagent in quantity sufficient to process approximately 10 assays. An assay is defined as one 20 mm × 20 mm target area.

**Warning & Precautions:** Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions and slides containing fluorophores in reduced light.

### Materials Required but Not Provided

**NOTE:** Where storage conditions are not listed in this insert store reagent per vendor recommendations.

**Laboratory Reagents**

- 20X SSC
- Concentrated (12N) HCl
- 1N NaOH
- Pepsin solution
- Purified water (distilled or deionized)
- DAPI/Antifade
- PBS
- Tween-20
- Formaldehyde 37%
- Ethanol (100%)

**Laboratory Equipment**

- Fluorescence microscope equipped with recommended filters
- Precleaned microscope slides
- Slide warmer (78°C)
- Microliter pipettor (1 to 10 µL)
- pH meter and pH paper
- Calibrated thermometer
- Water bath (73 ± 1°C)
- Air incubator (37-42°C)
- Timer
- Vortex mixer
- Microcentrifuge
- Magnetic stirrer
- Diamond-tipped
- Humidified
- Forceps
- Coplin jars
- Graduated

**Microscope Equipment and Filters**

Microscope: An epi-illumination fluorescence microscope is required for viewing the hybridization results. The microscope should be checked to confirm it is operating properly to ensure optimum viewing of FISH assay specimens. A microscope used with general DNA stains such as DAPI, propidium iodide, and quinacrine may not function adequately for FISH assays. Routine microscope cleaning and periodic "tune-ups" by the manufacturer's technical representative, especially alignment of the lamp, if required, are advisable.

Excitation Light Source: A 100-watt mercury lamp or other lamp with similar intensity and spectral output is the recommended excitation source. The manufacturer's technical representative should be consulted to assure that the fluorescence illumination system is appropriate for viewing FISH assay specimens. Record the number of hours that the bulb has been used and replace the bulb before it exceeds the rated time. Ensure that the lamp is properly aligned, if required.

Objectives: Use oil immersion fluorescence objectives with numeric apertures ≥ 0.75 when using a microscope with a 100-watt mercury lamp or other lamp with similar intensity and spectral output. A 25X or 40X objective, in conjunction with 10X eyepieces, is suitable for scanning the specimen to select regions for enumeration. For enumeration of FISH signals, satisfactory results can be obtained with a 40X, 63X or 100X oil immersion achromat type objective.

Immersion Oil: The immersion oil used with immersion objectives should be one formulated for low autofluorescence and specifically for use in fluorescence microscopy.

Filters: Multi-bandpass fluorescence microscope filter sets optimized for use with the CEP DNA probe kits are available for most microscope models. A triple bandpass DAPI/Green/ Orange filter set is recommended for the CEP X/Y kit. This filter configuration allows the simultaneous excitation and emission of the SpectrumOrange, SpectrumGreen and DAPI fluorophores. The CEP X/Y probe hybridization to its 2 target chromosomes is marked by orange and green fluorescence. All of the other DNA will fluoresce blue with the DAPI stain.

## WARNINGS AND PRECAUTIONS

1. Because it is often impossible to know which might be infectious, all human specimens should be treated with universal precautions.
2. Hybridization conditions may be adversely affected by the use of reagents other than those recommended by manufacturer.
3. Failure to follow all procedures for slide denaturation, hybridization and signal enumeration may cause unacceptable or erroneous results.

4. Fluorophores are readily photobleached by exposure to light. To limit this degradation, handle all solutions containing fluorophores in reduced light. This includes all steps involved in handling the hybridized slide. Carry out all steps which do not require light for manipulation (incubation periods, washes, etc.) in subdued lighting to avoid direct light projecting onto the fluorophore.
5. CEP X/Y DNA Probe contains formamide, a teratogen. Avoid contact with skin and mucous membranes. Refer to MSDS for more information.
6. The use of a calibrated thermometer is strongly recommended for measuring temperatures of solutions, waterbaths, and incubators as these temperatures are critical for optimum product performance.
7. All hazardous materials should be disposed of according to your institution's guidelines for hazardous disposal.

**CEP X SpectrumOrange/Y SpectrumGreen DNA Probe**

**CAUTION:** This preparation contains human sourced and/ or potentially infectious components. No known test method can offer complete assurance that products derived from human sources or inactivated microorganisms will not transmit infection. These reagents and human specimens should be handled as if infectious using safe laboratory procedures, such as those outlined in Biosafety in Microbiological and Biomedical Laboratories, OSHA Standards on Bloodborne Pathogens, CLSI Document M29-A3, and other appropriate biosafety practices. Therefore all human sourced materials should be considered infectious.
These precautions include, but are not limited to, the following:

- Wear gloves when handling specimens or reagents.
- Do not pipette by mouth.
- Do not eat, drink, smoke, apply cosmetics, or handle contact lenses in areas where these materials are handled.
- Clean and disinfect spills of specimens by including the use of a tuberculocidal disinfectant such as 1.0% sodium hypochlorite or other suitable disinfectant.
- Decontaminate and dispose of all potentially infectious materials in accordance with local, state, and federal regulations.

**Danger**

**Hazard-determining components of labeling:** Formamide

| | |
|---|---|
| H360 | May damage fertility or the unborn child. |
| P201 | Obtain special instructions before use. |
| P202 | Do not handle until all safety precautions have been read and understood. |
| P281 | Use personal protective equipment as required. |
| P308+P313 | IF exposed or concerned: Get medical advice/attention. |
| P405 | Store locked up. |
| P501 | This material and its container must be disposed of in a safe way. |

## STORAGE AND HANDLING

Store the unopened CEP X/Y DNA probe at – 20°C protected from light and humidity. Expiration date is indicated on the tube label. This storage condition applies to both opened and unopened tubes.

## ASSAY PROCEDURE: FISH PROCEDURE SUMMARY

The following procedure has been validated for performance on cultured peripheral blood lymphocytes and is used to determine the probe quality.
The user is responsible for validating the procedure for their specific application.

**Preparing the Reagents**

**NOTE:** Where indicated, measure the pH of these solutions at ambient temperature. Use a pH meter with a glass electrode unless otherwise noted.

Pepsin Solution

Add 35 mg Pepsin to 70 mL of 0.01 N HCl and keep at 37 °C. use within 1 week.

Phosphate Buffered Saline (PBS)

Add 10.0 g NaCl, 0.25 g KCl, 1.3 g Na2HPO4, 0.25 g KH2PO4 in 1000 mL of distilled water and mix well.

Fixative Solution

Add 1 mL of 37% formaldehyde and 0.18 g of MgCl2 to 39 mL PBS Store at 4°C and use within 1 month.

20X SSC solution

Mix thoroughly 87.66 g NaCl and 38.70 g in 400 mL purified $H_2O$. Measure pH and adjust to pH 7.0 with HCl/NaOH. Add purified $H_2O$ to bring final volume to 500 mL. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

2X SSC wash solution

Mix thoroughly 100 mL 20X SSC (pH 5.3) with 850 mL purified $H_2O$. Measure pH and adjust to pH 7.0±0.2 with NaOH. Add purified $H_2O$ to bring final volume to 1 liter. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

0.4X SSC wash solution

Mix thoroughly 20 mL 20X SSC (pH 7.0) with 950 mL purified $H_2O$. Measure pH and adjust pH to 7.0-7.5. Add purified $H_2O$ to bring to final volume of the solution to 1 liter. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

2X SSC/0.05% Tween-20 wash solution

Mix thoroughly 100 mL 20X SSC (pH 5.3) with 850 mL purified $H_2O$. Add 1 mL Tween-20 and mix thoroughly until Tween-20 is completely dissolved. Measure pH and adjust to pH 7.0±0.2 with NaOH. Add purified $H_2O$ to bring final volume to 1 liter. Store at ambient temperature. Discard stock solution after 6 months, or sooner if solution appears cloudy or contaminated.

Ethanol Solutions (70%, 85%, 100%)

Prepare v/v dilutions of 100% ethanol with purified $H_2O$ to make stock solutions of 75% and 85% ethanol. Between uses, store covered at ambient temperature. Discard stock solutions after 6 months.
For working solutions, pour 70 mL 100% EtOH into one of three jars; 70 mL 85% EtOH into another, and 70 mL 70% EtOH into the last. Use at ambient temperature. Discard after 7 days or if excessive dilution or evaporation has occurred.

**Procedural Notes:** Prior to use, thaw reagents at ambient temperature, vortex, then centrifuge tube 2-3 seconds using a standard bench-top microcentrifuge. Measure the temperatures of the solutions inside the Coplin jar; use of a calibrated thermometer is required.

**Preparing the Specimen Target**

1. Mark hybridization areas with a diamond tipped scribe on the underside of the specimen slide prepared by standard cytogenetic procedures.
2. Place slides in pepsin at 37°C for 5-10 minutes.
3. Place slides in fixative solution at 4°C for 5 minutes.
4. Rinse slides in PBS at room temperature for 5 minutes.
5. Dehydrate slides for 1 minute in 70% EtOH, followed by 1 minute in 85% EtOH, and 1 minute in 100% EtOH.

**NOTE:** Keep the slides in 100% EtOH until you are ready to dry all slides and apply the probe.
**NOTE:** Immerse no more than four slides in the Coplin jar simultaneously.

**Applying and Hybridizing the Probe to the Specimen Target**

Prepare a humidified box by placing a paper towel moistened with water on the side of an airtight container. Place in 37-42°C incubator.

1. Remove the slides from 100% EtOH and dry slides by touching the bottom edge of the slides to a blotter and wiping the underside of the slides dry with a paper towel.
2. Vortex and then centrifuge probe for 1-3 seconds. Apply 10 µL of probe to one target area and immediately apply a 20×20 $mm^2$ coverslip. Repeat for additional target areas.
3. Seal coverslip with rubber cement if hybridization time will be greater than 2 hours.
4. Co-denature sample and probe simultaneously by placing the slide on a hotplate at 75°C for 5 minutes.
5. Place slides in a prewarmed humidified box and place box in a 37-42°C incubator. Hybridize for 60 minutes to overnight.

**Washing the Slide**

Prepare the post-hybridization wash solutions:
Pour 70 mL of 0.4X SSC into a Coplin jar. Place jar in a 73±1°C water bath at least 30 minutes prior to use. Use 1 day, then discard.
Pour 70 mL of 2X SSC/0.05% Tween-20 into a Coplin jar. Use at ambient temperature. Use 1 day, then discard.

**NOTE:** To maintain the proper temperature in 0.4X SSC, wash four slides simultaneously. If you have less than four slides, add blank slides that are at ambient temperature to bring the total to four. Start timing when the fourth slide is immersed.

**NOTE:** If more than 4 slides have been hybridized, they must be washed in more than 1 batch. The temperature of the wash solution must return to 73±1°C before washing each batch.

1. Remove coverslip from one slide and immediately immerse the slide in the 0.4X SSC. Agitate slides for 1–3 seconds. Remove slides after 2 minutes.

**NOTE:** Ensure the temperature of the wash solution is 73±1°C before washing another four slides.

2. Immerse slides in 2X SSC/0.05% Tween-20. Agitate slides for 1–3 seconds. Remove slides after 5 seconds to 1 minute.
3. Immerse slides in deionized water. Agitate slides for 1–3 seconds. Remove immediately.
4. Air dry slide in darkness

**Visualizing the Hybridization**

1. Apply 10 µL DAPI/Antifade counterstain to the target area of slide and apply coverslip.

View hybridized slides using a suitable filter set on an optimally performing fluorescence microscope. The optical filter sets will visualize the fluorophores used in the hybridization and should be selected according to the following spectral data.

**Fluorochrome Spectral Data**

| Label | Absorption | Emission |
|---|---|---|
| Green | 496 nm | 524 nm |
| Orange | 554 nm | 586 nm |

**Enumerating Signals**

Assessing Slide Adequacy

Evaluate slide adequacy using the following criteria:

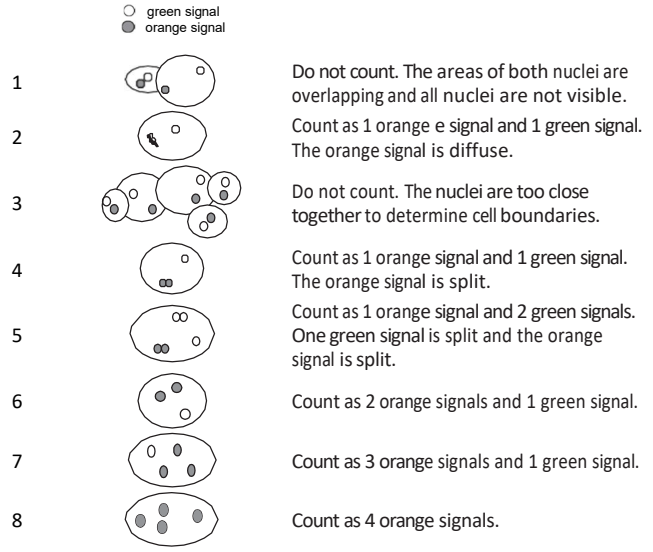
- Probe Signal Intensity: The signal should be bright, distinct, and easily evaluable. Signals should be in either bright, compact, oval shapes or stringy, diffuse, oval shapes.
- Background: The background should appear dark or black and free of fluorescence particles or haziness.
- Cross-hybridization/Target Specificity: The probe should hybridize and illuminate only the target (centromere of chromosome X or Yq12 region of chromosome Y). Metaphase spreads should be evaluated to identify any cross-hybridization to non-target sequences. At least 98% of cells should show 1 or more signals for acceptable hybridization (see guidelines for signal enumeration below).

If any of the above features are unsatisfactory, consult the following Troubleshooting Guide, and process a fresh slide.

Selection of optimum viewing area and evaluable nuclei

Use a 25X objective to scan the hybridized area and examine the specimen distribution. Select an area where the specimen is distributed sparsely, few interphase nuclei or metaphase spreads are overlapping, and several interphase nuclei or metaphase spreads can be scanned within a viewing field. Avoid areas where the distribution of cells is dense, cells are overlapped, or the nuclear border of individual nuclei is unidentifiable. Avoid areas which contain clumps of cells. Enumerate only those cells with discrete signals.

**Figure 1.** Image Examples and Signal Counting Guidelines




Enumeration scan

Using a 40X or 63X objective, begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals in each evaluable metaphase spread or within the nuclear boundary of each evaluable interphase cell. Areas on the slide with a high cell density should be randomly skipped in order to scan the entire target area.

Interphase Enumeration

Enumerate the fluorescent signals in each evaluable interphase nucleus using a 40X or 63X objective. Follow the signal counting guidelines in Figure 1. Objectives with higher magnification (eg, 63X or 100X) should be used to verify or resolve questions about split or diffused signals.

- Two signals that are in close proximity and approximately the same sizes but not connected by a visible link are counted as 2 signals.
- Count a diffuse signal as one signal if diffusion of the signal is contiguous and within an acceptable boundary.
- Two small signals connected by a visible link are counted as 1 signal.
- Enumerate the number of nuclei with 0, 1, 2, 3, 4, or > 4 signals (for both X and Y signals) and record the counts in a 2-way table. Count nuclei with 1 or more FISH signals of either color. If the accuracy of enumeration is in doubt, repeat the enumeration in another area of the slide.
- Do not enumerate nuclei with uncertain signals.

Metaphase Enumeration

- The metaphase spread should have chromosomes that are well separated from each other but are clearly from the same cell. Select a minimum of 20 good quality, complete metaphase spreads with well defined, non-overlapping chromosomes for chromosome enumeration and analysis.
- The CEP X/Y DNA Probe signal will be visible as a distinct fluorescent signal located near the centromere region of the X chromosome and the Yq12 region of chromosome Y. The CEP signal may appear split (2 smaller signals in close proximity) if the chromatids are separated. Chromatid separation occurs when the cell is in the later stages of mitosis (between metaphase and anaphase). The split signal found on each of the 2 chromatids should be counted as one signal. Follow general magnification and scanning guidelines as indicated above in the "Interphase Enumeration".
- In addition to enumerating the X/Y signals, metaphase cells should be assessed to verify locus specificity of the probes and to assure that there are no cross-hybridizing sequences at alternate chromosomal locations.

**Slide Storage**

Store hybridized slides, with coverslip and counterstain, at -20°C in the dark. Hybridized FISH slides can be analyzed for at least 12 months if stored in this way.

**Shipping**

Sunshine DNA probes are shipped at -20°C in darkness.

**Additional Procedural Recommendations**

- The use of a calibrated thermometer is strongly recommended for measuring temperatures of solutions, water baths, and incubators, as these temperatures are critical for optimum product performance.
- Carefully check the temperature of preheated solutions.
- Carefully check the pH value of all solutions. It must be in the range of 7.0 - 7.5 at room temperature.
- The wash concentrations (stringency), pH and temperature are important, as low stringency can result in non-specific binding of the probe and too high stringency can result in a lack of signal.
- Before opening: Spin briefly to collect probe mix at the bottom of the tube.

**TROUBLESHOUTING GUIDE**

**Cross Hybridization**

Repeat on a new specimen using one of the following:

- Increase the temperature of 0.4X SSC by 2°C. As needed, continue to increase the temperature until the cross hybridization is minimal.
- Decrease the denaturation temperature by 2°C. Make sure incubation is at 42°C.

**Probe Appears Dim**

Repeat on a new specimen using one of the following:

- Increase the hybridization time.
- Increase the denaturation temperature. As needed, continue to increase the temperature until the signal becomes acceptable.
- Increase the denaturation time.
- Pretreat this specimen. Call Technical Service for assistance. Wash the slide using 0.4X SSC at 70-73°C.
- Ensure filters are correct and not damaged.

**Diffuse Signal (speckling)**

Repeat on a new specimen using one of the following:

- Decrease the denaturation temperature 2°C. Decrease the denaturation time.

**NOTE:** As needed, reduce the denaturation temperature or time until metaphase morphology has improved and signal intensity is still acceptable.

- Wash the slide using 0.4X SSC at 73–76°C.

**Poor Metaphase Morphology**

Repeat on a new specimen using one of the following:

- Decrease the denaturation temperature 2°C. Decrease the denaturation time.

**NOTE**: As needed, reduce the denaturation temperature or time until metaphase morphology has improved and signal intensity becomes acceptable.

- Age the slides:

1. Prepare a solution of 2X SSC.
2. Immerse the slide in 2X SSC for 2 minutes at 73°C.
3. Immerse the slide several times in purified water.
4. Dehydrate slides through a series of 1 minute EtOH rinses (70%, 85%, 100%).
5. Air dry the slides and continue with hybridization.

When performing FISH on interphase cells, samples containing DNA that is damaged, degraded or destroyed by extreme conditions or treatments such as acid, strong alkali, and heat will not produce FISH results. Icteric, hemolyzed or lipemic specimens may prevent proper culture for standard cytogenetic analysis, but will still provide an acceptable FISH result. Clotted or frozen specimens may lead to poor FISH results, but unclotting or thawing the specimens can achieve successful FISH results. When viewing FISH results, ensure that your microscope is properly aligned and functioning optimally.

The following text lists some examples of variable results that you may encounter using the CEP probes. Probable causes and suggestions to improve performance are included.

**Distorted Chromosome Morphology**

probable causes are as follow:

1. Specimen slides dried too quickly during preparation
   a. Increase temperature of water bath (increases humidity) used when dropping slides.
   b. Decrease the temperature of the slide warmer during sample preparation.
   c. Increase drying time to at least overnight at ambient temperature, and then age slides at least 24 hours at ambient temperature. Do not bake slides at high temperature.

2. Specimen over-denatured
   a. Ensure the denaturation solution was made correctly.
   b. Ensure temperature of denaturation solution is 73±1°C prior to immersing the slide; decrease the temperature to 72°C.
   c. Decrease the time the slide is immersed in the denaturation solution by 1–3 minutes.
3. Specimen slides too fresh prior to denaturation
   a. Age slides at least 24 hours at ambient temperature or artificially age the specimen in 2X SSC at 73°C for 2 minutes.
4. Specimen slides not thoroughly dry prior to immersion in denaturation solution
   a. Warm specimen slides to 45–50°C prior to denaturation or dehydrate slides through a series of 1-minute EtOH rinses (70%, 85%, 100%).

**High Slide Background**

probable causes are as follow:

1. Glass slides not sufficiently cleaned prior to sample preparation
   a. Immerse glass slides in EtOH and wipe dry using lint-free paper prior to dropping slides.
2. Cellular debris in sample preparation.
   a. Wash cell pellet with fresh fixative three times and repeat the slide dropping procedure.
3. Metaphase spreads were aged by baking or contain cytoplasm.
   a. Increase time the slide is immersed in the denaturation solution up to 10 minutes.
4. Slide inadequately washed following hybridization.
   a. Ensure the wash solutions were made correctly. Ensure pH and temperature of wash solutions are correct. Remove coverslip. Repeat the wash procedure. Increase the time that the slide is immersed in the 73°C 0.4X SSC wash solution up to 4 minutes.
5. Wash solutions used too long or stored improperly.
   a. Discard all wash solutions after 1 day.
6. Viewed hybridization using long bandpass filters.
   a. Switch to filters with narrow bandwidths or to multi-bandpass filters to reduce background light.

**Weak or No Signal**

probable causes are as follow:

1. Specimen slide not adequately denatured.
   a. Ensure temperature of denaturation solution in the Coplin jar is 73±1°C prior to immersing the slide.
   b. Increase temperature of denaturation solution to 74°C. Increase the time the slide is immersed in the denaturation solution by 2-4 minutes.
2. Specimen slides improperly aged after dropping specimen.
   a. Age for 24 hours at ambient temperature or artificially age the specimen in 2X SSC at 73°C for 2 minutes, before performing FISH on them.
3. Specimen slides not thoroughly dry prior to immersion in denaturation solution.
   a. Warm specimen slides to 45-50°C prior to denaturation or dehydrate slides through a series of 1-minute EtOH rinses (70%, 85%, 100%).
4. Probe mix dried too much on the specimen slide.
   a. Immediately place the coverslip over the target area after applying probe mix. When washing slides after the hybridization, remove the coverslip from one slide at a time and immediately immerse the slide into the wash solution before removing the coverslip from the next slide.
5. Air bubbles were trapped under the coverslip during hybridization
   a. Apply coverslip by first touching the surface of the probe. Place the slide with the coverslip down on a blotter and very gently press out visible bubbles. Seal the coverslip well with rubber cement, leaving no gaps.
6. Hybridization conditions inappropriate
   a. Ensure that the correct time and temperatures for the hybridization were followed.
   b. Ensure that the temperature of the incubator is 42°C.
   c. Increase the hybridization time.
7. Wash conditions or solutions incorrect
   a. Ensure that the wash solutions were made correctly.
   b. Ensure the temperatures of the wash solutions are at the stated temperatures.
   c. Ensure that the thermometers and pH meters used are calibrated properly.
   d. Remove coverslips before immersing slides in wash solution.
8. Probes or specimen slides stored improperly
   a. Store probe at –20°C in the dark.
   b. Store non-hybridized slides desiccated at -20°C for an extended period or at ambient temperature for short periods.
   c. Store hybridized slides at -20°C in the dark for up to 6 months.
9. Wrong counterstain used or counterstain is too bright
   a. Remove coverslip. Immerse slides for 5 minutes in 2X SSC/0.05% Tween-20 at ambient temperature; dehydrate slide through a series of 1-minute EtOH rinses (70%, 85%, 100%). Air dry and reapply a different counterstain.
10. Viewed hybridization using inappropriate filter set
    a. Multi-bandpass filter sets provide less light than single bandpass filter sets, so probe signals may appear fainter when viewed through the multi-bandpass sets.
    b. Use correct filter for viewing the probe fluorophore.
11. Microscope configuration or objectives not adequate for viewing FISH results, or microscope filters are damaged
    a. Contact your microscope manufacturer.

**Low Signal Specificity**

probable causes are as follow:

1. Inappropriate hybridization conditions.
   a. Ensure temperature of incubator is 37-42°C.
2. Wash temperature too low.
   a. Maintain the wash temperature of the wash solutions by placing no more than four slides in one Coplin jar at a time and ensuringthat the temperature of the wash solution is correct before washing another set of slides.
3. Wash solution stringency too low.
   a. Ensure the wash solutions were made correctly.

**NOTE:** The lower the concentration of salt (SSC), the higher the concentration of Tween-20, the more stringent the wash.

**Bright or Weak Counterstain**

probable causes are as follow:

1. Counterstain appears weak:
   a. specimen slides not dehydrated prior to applying counterstain or oil droplets in counterstain.
2. Counterstain too old or exposed to light for extended period
   a. Ensure the counterstain is not used past the expiration date. DAPI turns dark as it degrades.
   b. Store counterstain at -20°C in the dark.

**TECHNICAL ASSISTANCE**

For technical assistance, call Niagene Biotech Technical Services at (21)88880115.

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