



Instructions For Use

Fluorescence *in situ* Hybridization Protocol using KromaTiD's Pinpoint FISH™ Probes

Reagents & Materials Provided

- FISH Hybridization Buffer (provided by KromaTiD)
- Probe Solution (provided by KromaTiD)

Reagents Required

- De-ionized water (DI H₂O)
- Nuclease-free water
- Molecular Grade Ethanol (made in 75%, 85%, and 100% dilutions)
- PN Buffer (see Appendix A)
- 2X Saline Sodium Citrate (SSC) Buffer (pH7.0) (see Appendix A)
- Antifade Mounting Medium with DAPI
- 1X Phosphate Buffered Saline (PBS)
- RNase A Solution (0.05mg/mL)

Equipment and Supplies

- Glass slides (25x75x1mm, positively charged)
- Coverslips (22x50mm)
- 50mL Coplin jars (acetal or glass)
- Water bath (37°C & 42°C)
- Slide hybridization system (Cytobrite or equivalent hot plate)
- Incubator (37°C)
- Humidified chamber (see Appendix A)
- Calibrated thermometer
- Rubber Cement
- Vortex mixer
- Benchtop microcentrifuge
- Micropipette and sterile tips (2-200µL range)
- Microcentrifuge tubes (sterile, DNase/RNase free)
- Forceps

Fluorophore Information

Fluorophore	Excitation/Emission (nm)
Atto425 / Aqua	436 / 485
6-FAM / Spectrum Green	490 / 525
Atto550 / Spectrum Orange	555 / 576
TexRed	595 / 620
Atto643 / 647 / Cy5	643 / 669

Handling/Storage Information

- Store all probes and hybridization buffers at -20°C upon receiving.
- Probes are to be protected from light until ready to begin.

Pinpoint FISH™ Protocol**Slide Preparation for Pinpoint FISH™**

1. Samples for Pinpoint FISH™ hybridization should be prepared using standard metaphase spread preparation techniques on glass slides specified for cytogenetic applications (positively charged).
 - a. Submerging new slides in 100% ethanol may help remove any residue that could be present from the slide manufacturing process.
 - b. For good metaphase spreads, drying should take place in a humidity-controlled chamber to optimize chromosome spreading.
 - c. Freshly dropped slides may be artificially aged for 10 minutes in a 90°C oven.

RNase A Treatment (Optional Step)

Note: This step is performed as deemed necessary to reduce background. If the assay or sample is known to produce high background, this treatment may reduce that effect.

2. Prepare RNase A solution to a final concentration of 0.5mg/mL using sterile 1X PBS in a Coplin jar.

Solution	Volume
RNase A (100mg/mL)	0.25mL
Sterile 1X PBS	50mL

3. Place the slide in the RNase A solution and then place the Coplin jar in 37°C water bath for 1 hour.
4. Transfer the slide to DI H₂O in a Coplin jar for 1 minute. Air dry the slide completely before proceeding.

Slide Pre-Treatment

Note: Ethanol solutions should be kept at -20°C until use.

5. Dehydrate the slides:
 - a. Place the slide in the 75% ethanol solution for 2 minutes.
 - b. Transfer the slide to the 85% ethanol solution for 2 minutes, wiping the back of the slide between transfers.
 - c. Transfer the slide to the 100% ethanol solution for 2 minutes, wiping the back of the slide between transfers.
 - d. Allow the slide to air dry completely.

Note: 100% ethanol should dry completely within approximately 1-2 minutes. If water droplets are still present after 1-2 minutes, replace the 100% ethanol and repeat Step 5c.

Hybridization Procedure

Note: Prepare the hybridization humidity chamber with DI H₂O and place in the 37°C incubator to equilibrate prior to beginning.

6. Prepare the hybridization mixture for one full slide (30µL), or in a 3:2 ratio of FISH Hybridization Buffer to probe solution if a smaller volume of hybridization mix is desired.

Solution	Volume
FISH Hybridization Buffer	18 µL
Probe Solution	12 µL

Note: If combining more than one KromaTiD Pinpoint FISH probe in a single assay, then combine 12uL of each probe into a single tube and proceed to dehydrate the probes. Resuspend the dried probe mixture in 12uL of nuclease-free H₂O.

- a. Vortex the mixture three times for 5 seconds each.
- b. Centrifuge for 10 seconds.
7. Apply hybridization mix to the slide using a micropipette. Use care not to introduce air bubbles.
8. Apply a coverslip.
 - a. If a bubble is present underneath the coverslip, it can be forced out with gentle and even pressure to the coverslip.

Note: Pressing on a coverslip can physically damage cells if not done with great care.

9. Seal the edges of the coverslip with rubber cement. Be sure all edges are sealed to prevent the hybridization mixture from drying out.

Note: Ensure that no rubber cement gets underneath the slide. It can obstruct slide contact with the heat block in the denature step that follows.

10. Denature the slide using a hybridization system (Cytobrite or equivalent hot plate) by placing the slide directly onto the heat source at 75°C for 5 minutes.
11. After the program ends, transfer the slide to a pre-warmed humidified chamber. Place the humidified chamber containing the hybridization slides into the 37°C incubator for a minimum of 4 hours.

Note: An overnight hybridization can be performed in a humidified chamber for up to 20 hours at 37°C.

Postwash Slide Treatment

1. After the hybridization incubation is complete, remove the rubber cement and coverslip with care before moving on to the wash process.
2. Ensure that the internal temperature of the 2X SSC has reached 42°C with a calibrated thermometer. Wash the slide 5 times in 2X SSC at 42°C for 5 minutes each.
3. Without allowing the slides to dry, transfer to a fresh 50mL of PN Buffer at RT for 1-2 minutes.
4. After 1-2 minutes, remove the slides from PN Buffer and air-dry in the dark.

Note: Wipe the excess solution from the back of the slide using a lint-free towel or equivalent to help reduce background during imaging. Also, propping slides label-end down will prevent residues such as ink or adhesive potentially running down onto the sample from the label area.

5. Add 18µL of Antifade mounting medium with DAPI. Apply a coverslip with care to avoid trapping air bubbles.
6. Allow the DAPI stain to react for at least 15 minutes prior to imaging.

Customer Notification

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Appendix A: Reagents**20x Saline Sodium Citrate (SSC)**

Reagent	Amount
Sodium Chloride	175.3g
Sodium Citrate	88.2g
DI H ₂ O	up to 1L
pH to 7.0	

2x Saline Sodium Citrate (SSC)

Reagent	Amount
20x SSC	100mL
DI H ₂ O	up to 1L
pH to 7.0	

1x PN Buffer

Reagent	Amount
Na ₂ HPO ₄	14.2g
*0.1M Na ₂ HPO ₄	50mL
Triton X-100	1mL
DI H ₂ O	up to 1L

*12g Na₂HPO₄ in 1L of DI H₂O

Humidified Chamber

KromaTiD uses 96-well plastic freezer box with the outside wells filled with DI H₂O as the humidified chamber. Slides are placed flat and upright in the chamber before sealing the box with parafilm and placing the entire chamber into a humidified-controlled 37°C incubator.