Fluorescence *in situ* Hybridization Protocol 60ºC
for use with KromaTiD Pinpoint FISH™ Probes

Reagents & Materials Provided
- FISH Hybridization Buffer (provided by KromaTiD)
- Probe Solution (provided by KromaTiD)

Samples
- This protocol requires use of cell samples that have been fixed in 3:1 MeOH: Glacial acetic acid (Carnoy's fixative), prepared on cytogenetic-quality microscope slides (See Appendix A).

Reagents Required
- 20X Saline Sodium Citrate (SSC) Buffer (pH7.0) (see Appendix A)
- 2X SSC Buffer (pH7.0) (see Appendix A)
- 0.4X SSC / 0.05% Tween-20 (see Appendix A)
- Antifade mounting medium with DAPI

Equipment and Supplies
- Glass slides (25x75x1mm, positively charged)
- Coverslips (22x50mm)
- 50mL Coplin jars (acetal or glass)
- Water bath (60ºC)
- Slide hybridization system (Cytobrite or equivalent hot plate)
- Incubator (37ºC)
- 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 -20ºC 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 90ºC oven.

Hybridization Procedure

Note: *Prepare the hybridization humidity chamber with DI H_2O and place in the 37ºC incubator to equilibrate prior to beginning.*

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

    Note: *Keep probe products on ice and away from full light if not in use, preferably in the dark.*

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 H_2O.*

    a. Vortex the mixture three times for 5 seconds each.
    b. Centrifuge for 10 seconds.
3. Apply hybridization mix to the slide using a micropipette. Use care not to introduce air bubbles.
4. 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.*
5. 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.*
6. Denature the slide using a hybridization system (Cytobrite or equivalent hot plate) by placing the slide directly onto the heat source at 75ºC for 5 minutes.
7. After the program ends, transfer the slide to a pre-warmed humidified chamber. Place the humidified chamber containing the hybridization slides into the 37ºC incubator for a minimum of 4 hours.

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

Post-Hybridization Slide Treatment

1. Prepare wash solution 1: place 50mL of 0.4X SSC/ 0.05% Tween-20 solution in a Coplin jar and pre-heat in a water bath set to 60ºC.
2. Prepare wash solution 2: place 50mL of 2X SSC solution in a Coplin jar and leave at room temperature.
3. Gently roll off the dry rubber cement seal and remove coverslip. The coverslip can be removed by submersion in a 2X SSC Coplin jar at room temperature, or the coverslip can be removed gently by hand.
4. Ensure the temperature of the 0.4X SSC/ 0.05% Tween-20 (wash solution 1) inside the Coplin is at 60ºC. Add the slides and wait 1 minute.
5. Move the slides to wash solution 2 (2X SSC at RT).
6. After 1-2 minutes, remove the slides from wash solution 2 and airdry in the dark.

   Note: *Wipe the excess solution from the back of the slide using a lint-free towel or equivalent will help to reduce background during imaging. Also, propping slides up on their sides or label-end down will prevent residues such as ink or adhesive potentially running down onto the sample from the label area.*
7. Add 18µL of Antifade mounting medium with DAPI. Apply a coverslip with care to avoid trapping air bubbles.
8. Allow the DAPI stain to react for at least 15 minutes prior to imaging.

Appendix A: Reagents

20x Saline Sodium Citrate (SSC)

Reagent	Amount
Sodium Chloride	175.3g
Sodium Citrate	88.2g
DI H_2O	QS to 1L
pH to 7.0	

2x Saline Sodium Citrate (SSC)

Reagent	Amount
20x SSC	100mL
DI H_2O	QS to 1L
pH to 7.0	

0.4x SSC / 0.05% Tween-20

Reagent	Amount
20X SSC (pH 7.0)	20mL
Tween-20	500µL
DI H_2O	QS to 1L

Cytogenetic-Quality Microscope Slides

SciGene CytoDrop® Slides Cat# 2060-01-01 or equivalent

Humidified Chamber

KromaTiD uses 96-well plastic freezer box with the outside wells filled with DI H_2O 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.

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