Fluorescence *in situ* Hybridization Protocol
for use with
KromaTiD's Human Whole Chromosome Pinpoint FISH™ Paint Probes

Reagents & Materials Provided
- FISH Hybridization Buffer (provided by KromaTiD)
- Probes/Paints (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
- De-ionized water (DI H_2O)
- 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 -20ºC upon receiving.
- Probes/Paints are to be protected from light and dust until ready to begin.

Whole Chromosome Pinpoint FISH™ Paints Protocol

Slide Preparation with Cell Samples

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.

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 37ºC water bath for 1 hour.
4. Transfer the slide to DI H_2O in a Coplin jar for 1 minute. Air dry the slide completely before proceeding.

Slide Pre-Treatment

Note: *Ethanol solutions should be kept at -20ºC 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 H_2O and place in the 37ºC incubator to equilibrate prior to beginning.*

6. Prepare the hybridization mixture for one full slide (30µL).

Solution	Volume
FISH Hybridization Buffer	18 µL
Probe	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 3 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 on 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 75ºC for 5 minutes.
11. Transfer the slide to a pre-warmed humidified chamber. Place the humidified chamber with 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-wash 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 42ºC with a calibrated thermometer. Wash the slide 5 times in 2X SSC at 42ºC 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 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.*
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.

Appendix A: Reagents

20x Saline Sodium Citrate

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

2x Saline Sodium Citrate (SSC)

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

1x PN Buffer

Reagent	Amount
Na_2HPO_4	14.2g
*0.1M NaH_2PO_4	50mL
Triton X-100	1mL
DI H_2O	up to 1L

*12g NaH_2PO_4 in 1L of DI H_2O

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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