dGH™ in-Site Hybridization Protocol
Standard Hoechst Method for dGH Analysis

Introduction

This protocol is designed for use with dGH™ in-Site™ probes produced by KromaTiD. dGH™ probes may be freely combined according to the guidelines provided in this document.

Please contact techsupport@kromatid.com for more information and guidance or kromatid.com/contact-us/ to request a product quote.

» **NOTE:** Cell samples to be processed using this protocol must be prepared using the **dGH Cell Prep Protocol** and slides should be prepared using KromaTiD's **Metaphase Slide Preparation Protocol** or equivalent. Both protocols can be found at kromatid.com.

» **NOTE:** Please see Appendix C for guidance on choosing probes and fluorophore conjugates compatible for use in this assay.

Reagents & Materials Provided with a dGH™ in-Site Probe Purchase.

- dGH™ in-Site Probes (10 Assay kit), see options below or contact sales@kromatid.com for additional options.
  - Targeted Genomic Probes
    - TRAC–TexRed (DGHP-001-B)
    - B2M–6-FAM (DGHP-002-C)
    - PDCD1–ATTO550 (DGHP-003-A)
    - CIITA–ATTO550 (DGHP-004-A)
    - CD19 Endogenous –ATTO643 (DGHP-005-D)
    - Custom in-Site Gene Probe (DGH-007)
  - Subcentromere Probes (DGH-CEP-0001-A to DGH-CEP-0048-D)
  - Subtelomere Probes (DGH-TEL-0001-A to DGH-TEL-0047-D)
- dGH™ Standard Hybridization Buffer (10 Assay Tube, DGH-051)

Reagents Required

- De-ionized water (DI H_2O)
- Nuclease-free water (Invitrogen™ Ambion Cat# AM9938 or equivalent)
- PN buffer (see Appendix A)
- 1X Phosphate Buffered Saline (PBS) (GrowCells Cat# MRGF-6230 or equivalent)
- Molecular grade ethanol (made in 75%, 85%, and 100% dilutions)
- 2X Saline Sodium Citrate (SSC) buffer (pH 7.0) (see Appendix A for 20x SSC formulation)
- Exonuclease III (100,000 U/mL; New England BioLabs Cat# M0206L or equivalent)
- 10X NEB Buffer 1 (New England BioLabs Cat# BL001S; comes with purchase of Exonuclease III from New England BioLabs)
- Hoechst 33258 (Invitrogen Cat# H3569)
- DAPI (VECTASHIELD Antifade Mounting Medium with DAPI; Vector Labs Cat# H-1200)
- RNase A Solution (17500 units; QIAGEN Cat# 19101); optional, see Appendix B

Equipment and Supplies

- Coverslips (22 x 50 mm No1.5; VWR Cat# 48393-195)
- UV crosslinker, 365 nm (Boekel™ 115V UV Crosslinker, Part No. 234100)
- Thermocycler (BioRad T100 Thermal Cycler or equivalent)
- Slide hybridization system (Cytobrite or equivalent)
- Humidity-controlled incubator (37ºC)
- Hybridization humidity chamber (see Appendix A)
- Coplin jars (glass and opaque with screw caps)
- Water bath (37ºC & 42ºC)
- Vortex mixer
- Benchtop microcentrifuge
- Micropipettes with sterile tips (2 µL – 1000 µL range)
- Microcentrifuge tubes (sterile, DNase/RNase free)
- Calibrated thermometer
- Rubber cement (Elmer's E904 or equivalent)
- Wypall wipes (or equivalent lint-free lab wipe)
- Liquid waste collection – 500 mL beaker or other
- Nail polish (clear and fast drying)

Preparation

- Samples for this assay must be prepared according to KromaTiD's **dGH™ Cell Prep Protocol** followed by KromaTiD's **Metaphase Slide Preparation Protocol** or equivalent.
- Place the ethanol solution series in a -20ºC freezer prior to beginning.
- Prepare the humidity chamber and store it at 37ºC prior to beginning.

» **NOTE:** *For samples found to have high background fluorescence see RNase A Slide Treatment Protocol in Appendix B.*

Slide Pre-Treatment

1. Place the slides into 50 mL of PN Buffer at room temperature (RT) in a glass Coplin jar for 5 minutes.
2. Transfer the slides into 50 mL of 1X PBS at RT in a glass Coplin jar for 3 minutes.
3. Retrieve the ethanol series Coplin jars from the -20ºC freezer.
4. Dehydrate the slides:
   a. Place the slides in the 75% ethanol solution for 2 minutes.
   b. Transfer the slides to the 85% ethanol solution for 2 minutes, wiping the back of the slides between transfers.
   c. Transfer the slides to the 100% ethanol solution for 2 minutes, wiping the back of the slides between transfers.
   d. Allow the slides to air dry completely.

» **NOTE:** ***The remainder of this assay <u>must</u> be performed in dim lighting.***

5. In an opaque Coplin jar, prepare 0.5 µg/mL Hoechst 33258 staining solution as follows:

   » **NOTE** *Make dilution immediately before use.*

Solution	Volume
2X SSC	50 mL
Hoechst (stock 500 µg/mL)	50 µL

6. Place the slides in the Coplin jar with the diluted Hoechst solution for 15 minutes.
7. Immerse the slides in DI H_2O for 1 minute.
8. Immediately lay slides on a flat surface and apply 250 µL of 2X SSC to each. Do not allow slides to dry before applying the 2X SSC.
9. Place a coverslip over each slide and transfer them into the UV crosslinker.
10. Expose the slides to long wave UV light (~365 nm) for 35 minutes.
11. Retrieve the slides and submerge them in DI H_2O to remove the coverslips, then air dry the slides.

   » **NOTE:** *Slides can sit in the dark overnight before proceeding to the next step.*

12. Thaw 10X NEB Buffer I at RT, vortex for 30 seconds, spin down in benchtop microcentrifuge, then keep on ice.
    a. Set up the following 50 µL reaction mixture for each slide:

Solution	Volume
Nuclease-free water	44 µL
10X NEB Buffer I	5 µL
Exonuclease III	1 µL

13. Vortex the reaction for 30 seconds and spin down in benchtop microcentrifuge.
14. Apply the 50 µL Exonuclease III reaction mixture to each slide and add a coverslip.
    a. Performing this step on a slide warmer set to 25°C may guard against ambient temperature variability.
15. Incubate at RT for 35 minutes.
16. Remove the coverslips by immersion in DI H_2O and air-dry the slides completely.

   » **NOTE:** *Slides can sit in the dark overnight before proceeding to the next step.*

Hybridization Procedure

17. Place probes and dGH Standard Hybridization Buffer on ice until completely thawed, then vortex for 30 seconds, spin down in benchtop microcentrifuge and place on ice.
18. Prepare the hybridization mixture immediately before use, as follows.

   » **NOTE:** These are the volumes needed per slide. For example, if hybridizing three slides, the amount of hybridization mixture should total 90 µL, rather than 30 µL. In addition, to account for the possibility of

imperfect pipetting, the values below may each be multiplied by 1.2 to build-in a 20% error margin. Do not store excess hybridization mixture.

Number of Probes Being Hybridized	dGH Standard Hybridization Buffer (µL)	Total Probe Solution Volume (1.00 µL Per Probe Added)	Nuclease-Free Water (µL)	Total Volume (µL)
1	25.3	1.0	3.7	30
2	25.3	2.0	2.7	30
3	25.3	3.0	1.7	30
4	25.3	4.0	0.7	30
5+	25.3	SpeedVac*	0	30

*SpeedVac: Hybridizations involving greater than four probes will exceed the probe solution volume limit of 4.7 µL. Users may carry out such a hybridization by combining the total volume of probe solution needed and dehydrating (**without heat**) the solution using a SpeedVac. The mixture can then be rehydrated with nuclease-free water to the volume corresponding to the total number of slides to be run.

19. Vortex the hybridization mixture for 30 seconds and spin down in a benchtop microcentrifuge.
20. Denature the hybridization mixture at 75°C for 5 minutes in a thermocycler.
21. Immediately place the hybridization mixture on ice.
22. Within 10 minutes after denaturation, apply the hybridization mixture to the slides and coverslip them, taking care to avoid trapping air bubbles.
    a. Performing this step on a slide warmer set to 25°C may guard against ambient temperature variability.
23. Apply rubber cement around the edges of the coverslip.

» **NOTE:** *Any unsealed areas along the perimeter of the coverslip may cause the hybridization mixture to dry out and the assay to fail.*

24. Denature slides for 3 minutes at 68°C on a Cytobrite® system or equivalent.
25. Incubate slides for 12 - 20 hours in a humidity chamber placed inside a 37°C incubator.

Post-Hybridization Slide Treatment

26. Place 50 mL of PN Buffer in a Coplin jar and 50 mL 2X SSC in another Coplin jar. Leave both jars at RT.
27. Allow a vial of DAPI to come to RT on the bench.
28. Place 50 mL of 2X SSC in a Coplin jar and 200 mL of 2X SSC in a bottle, then place them both in a water bath set to 42°C.
29. Set a 500 mL beaker on some paper towels on the bench.
30. Confirm the 2X SSC has reached 42°C using a calibrated thermometer.
31. Bring the humidity chamber to the bench.

32. Remove one slide at a time from the humidity chamber, gently roll off the rubber cement seal over the coverslip and submerge each slide in the 2X SSC at RT to let the coverslip slide off.
33. Once the coverslips are all off, transfer the slides to the Coplin jar with 2X SSC in the water bath.
34. After 5 minutes, retrieve the Coplin jar from the water bath and decant the solution into waste collection container while keeping the slides in place with a gloved hand.
35. Refill the Coplin jar containing the slides with the warm 2X SSC in the bottle. Return the Coplin jar and bottle to the water bath for another 5 minutes.
36. Repeat steps 34 and 35 an additional 3 times for a total of 5 washes.
37. Transfer slides to the Coplin jar containing PN Buffer at RT for 5 minutes.
38. During this five-minute incubation, vortex the DAPI vial and then spin it down in benchtop microcentrifuge.
39. Remove slides from PN Buffer and prop them up to allow excess buffer to drain off.
40. Before the slides dry, lay them flat on a paper towel and pipette 18 µL of DAPI onto the middle of each slide.
41. Coverslip each slide taking care to avoid trapping air bubbles, then allow the slides to rest for at least 15 minutes.
42. Seal all edges of the coverslips with clear nail polish (or equivalent).
43. If slides will not be imaged within two hours, place them in a slide folder or slide box and store at -20ºC.

Appendix A: Reagents

20x Saline Sodium Citrate (Dilute to 2x prior to use in this protocol.)

Reagent	Amount	Supplier
Sodium Chloride	175.3 g	VWR, catalog #: BDH9286-500G
Sodium Citrate	88.2 g	VWR, catalog #: BDH9288-2.5KG
DI H_2O	QS up to 1 L	
pH to 7.0		

1x PN Buffer

Reagent	Amount	Supplier
Na_2HPO_4	14.2 g	Millipore Sigma, catalog #: 795410-100G
*0.1M NaH_2PO_4	50 mL	Millipore Sigma, catalog #: 567545-500GM
Triton™ X-100 (proteomics grade)	1 mL	VWR, catalog #: 97063-864
DI H_2O	QS up to 1 L	

*12 g NaH_2PO_4 in 1 L of DI H_2O

Hybridization Humidity Chamber

KromaTiD uses a 96-well plastic freezer box with the outside wells filled with DI H2O as a humidified chamber. Slides are placed flat and face-up in the chamber before sealing the box with parafilm and transferring it into a humidity-controlled 37ºC incubator.

Appendix B: RNase A Slide Treatment Protocol

» **NOTE** *This optional step can be performed if a sample produces high background.*

1. Combine 0.25 mL of RNase A (100 mg/mL) and 50 mL of sterile 1X PBS in a Coplin jar with a screw lid and place the jar in a 37°C water bath to warm.

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

2. Use a calibrated thermometer to confirm the RNase A solution in the Coplin jar has reached 37ºC.
3. Once the solution has reached 37°C, place the slides into the Coplin jar and cap the jar with the screw lid.
4. After one hour, transfer the slides from the RNase A solution to a Coplin jar filled with DI H_2O for 1 minute.
5. Remove the slides from the DI H_2O and prop the slides up to air dry.
6. Proceed to Step 1 of this Protocol, the PN buffer incubation step.

» **NOTE:** *Once dry, the slides can sit overnight before proceeding to the PN buffer step.*

Appendix C: Fluorophore Compatibility

Fluorophore	Excitation/Emission (nm)
DAPI	376/460
6-FAM	490 / 525
ATTO 550	555 / 576
Texas Red	595 / 620
ATTO 643	643 / 669

Notes

1. Probes labeled with Aqua/ATTO 425 are not compatible with this protocol.
2. KromaTiD recommends that small target probes (<5kb in size) included in the assay be conjugated to ATTO 643.

Reference Documents – Found at www.kromatid.com

1. Microscope Specifications Datasheet
2. dGH in-Site Assay Hardware Requirements
3. Application Note: The Next Generation of Metaphase FISH Techniques; The Why and How of directional Genomic Hybridization (dGH™)

Customer Notification

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