dGH™ in-Site CAR-T Kit 1 Protocol
Standard Hoechst Method for dGH Analysis

dGH in-Site Hybridization Protocol v0.9 - EARLY ACCESS

Introduction

This protocol is designed for use with the dGH™ in-Site™ CAR-T Kit 1 (Cat. No. DGH-045) which contains probes for human TRAC and B2M loci. Additional dGH probes can be added to the kit. Please contact techsupport@kromatid.com for more information and guidance.

» **NOTE:** Cell samples to be analyzed with this kit must be prepared using the dGH Cell Prep Protocol and slides should be prepared using KromaTiD's Metaphase Slide Preparation Protocol or equivalent.

Reagents & Materials Provided With dGH in-Site CAR-T Kit 1 Cat# DGH-045

- dGH™ Standard Hybridization Buffer (10 Assay Tube CAT# DGH-051)
- dGH™ Probe TRAC (14q11.2) - TexRed (10 Assay Tube CAT# DGHP-001-B)
- dGH™ Probe B2M (15q21.1) – 6-FAM/Spectrum Green (10 Assay Tube CAT# DGHP-002-C)

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)
- 10X NEB Buffer 1 (New England BioLabs Cat# BL001S or equivalent)
- Exonuclease III (100,000 U/mL; New England BioLabs Cat# M0206L or equivalent)
- Hoechst 33258 (Invitrogen Cat# H3569)
- DAPI (VECTASHIELD Antifade Mounting Medium with DAPI; Vector Labs Cat# H-1200)

Equipment and Supplies

- Coverslips (22 x 50 mm No1.5; VWR Cat# 48393-195)
- UV crosslinker, 365 nm (Spectrolinker XL1500 or equivalent)
- Thermocycler (BioRad T100 Thermal Cycler or equivalent)
- Slide hybridization system (Cytobrite or equivalent)
- Humidity-controlled incubator (37°C)
- Hybridization humidity chamber (see Appendix A)
- Coplin jars (glass and opaque with screw caps)
- Water bath (37°C & 42°C)
- Vortex mixer
- Benchtop microcentrifuge
- Micropipettes with sterile tips (2 µL – 1000 µL range)
- Microcentrifuge tubes (sterile, DNase/RNase free)

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- Calibrated thermometer
- Rubber cement (Elmer's E904 or equivalent)
- Wypall wipes (or equivalent lint-free lab wipe)
- 500 mL Beaker
- 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 known 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 must 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. Without allowing the slides to dry, lay them on a flat surface and apply 250 µL of 2X SSC to each.
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.

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» **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 from the Kit components provided.
    a. The total volume of the final hybridization mixture should be 30 µL for each slide required. The table below shows the component volumes needed for 1 to 8 slides.
    b. The hybridization mixture should be prepared immediately before use. Any remaining hybridization mixture should be discarded and not stored as a mixture.

Number of Slides	dGH Standard Hybridization Buffer (µL)	TRAC Probe (µL)	B2M Probe (µL)	Total Volume (µL)
1	25.3	2.35	2.35	30
2	50.6	4.70	4.70	60
3	75.9	7.05	7.05	90
4	101.2	9.40	9.40	120
5	126.5	11.75	11.75	150
6	151.8	14.10	14.10	180
7	177.1	16.45	16.45	210
8	202.4	18.80	18.80	240

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19. Vortex the hybridization mixture for 30 seconds and spin down in a benchtop microcentrifuge.
20. Denature the hybridization mixture at 75ºC 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 the 500 mL beaker 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.

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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.

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Appendix A: Reagents

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

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

1x PN Buffer

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

*12 g Na_2HPO_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 step can be performed if a sample is known to produce 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.*

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