dGH™ Assay Cell Preparation Protocol

Purpose

This protocol provides guidance on cell culture and preparation prior to use in KromaTiD directional Genomic Hybridization (dGH™) assays.

Reagents & Materials Provided

- KromaTiD dGH™ Cell Prep Additive – KromaTiD Cat. No. DGH-0004, -0005, or -0006

Reagents Not Provided

- Demecolcine solution, 10 µg/mL (pseudonym Colcemid™) - KromaTiD Cat. No. COL-001, -002, -003; or equivalent.
- 75 mM potassium chloride (KCl) (Fisher Cat. No. 10575090 or equivalent).
- Methanol - molecular grade (Fisher Cat. No. A412-4 or equivalent)
- Glacial acetic acid (Fisher Cat. No. A38C-212 or equivalent)
- 70% ethanol – molecular grade (VWR Cat. No. EM-4450S or equivalent)
- Decon BDD Bacdown Detergent Disinfectant (Fisher Cat. No. 04-355-13 or equivalent)
- Growth media specific for the cell line in culture

Equipment and Supplies

- Certified biosafety cabinet or equivalent
- Appropriate culture vessels
- Serological pipettor & pipets
- Parafilm
- Laboratory benchtop centrifuge and appropriate swing bucket rotor
- 50mL conical tubes
- 15 mL *polystyrene* conical tubes (VWR Cat. No. 21008-197 or equivalent)

» **WARNING:** DO NOT USE POLYPROPYLENE TUBES. *Polypropylene tubes are **NOT** resistant to the fixative and will degrade and/or leak, compromising the sample.*

Handling & Storage of KromaTiD dGH Cell Prep Additive

- Store dGH Cell Prep Additive at 4ºC upon receiving.
- dGH Cell Prep Additive can be stored up to expiry date at 4ºC.

Important Notes Before Beginning

Purpose of dGH™ Cell Prep Additive

Cells for analysis in dGH assays undergo a specialized preparation method to enable the stripping of daughter strands from the metaphase chromosomes prior to dGH probe hybridization. This requires incorporation of the dGH™ Cell Prep Additive while cells are actively dividing and in S-phase.

Choosing dGH™ Cell Prep Additive timepoints

Incubating cultures with dGH Cell Prep Additive at three different timepoints helps ensure capture of sufficient correctly prepared metaphase cells for analysis. Determining the doubling time of your cell line or primary cell culture is essential for choosing the three timepoints to use.

The example timeline in Figure 1 is based on a cell line with a 24-hour doubling time. KromaTiD has found this timing generally works well for all white blood cell types, blood products and immortalized blood cell lines as well as non-cancerous fibroblast and epithelial cell lines. Cancer cell lines and other significantly modified cultures may need alternative planning. Please contact **techsupport@kromatid.com** if you have questions about how your cell timing could impact experimental planning.

Cell Number Requirements

To obtain sufficient metaphase chromosome spreads for dGH analysis, perform the experiments at a sufficient scale to yield a minimum of 1 million cells per timepoint sample at harvest.

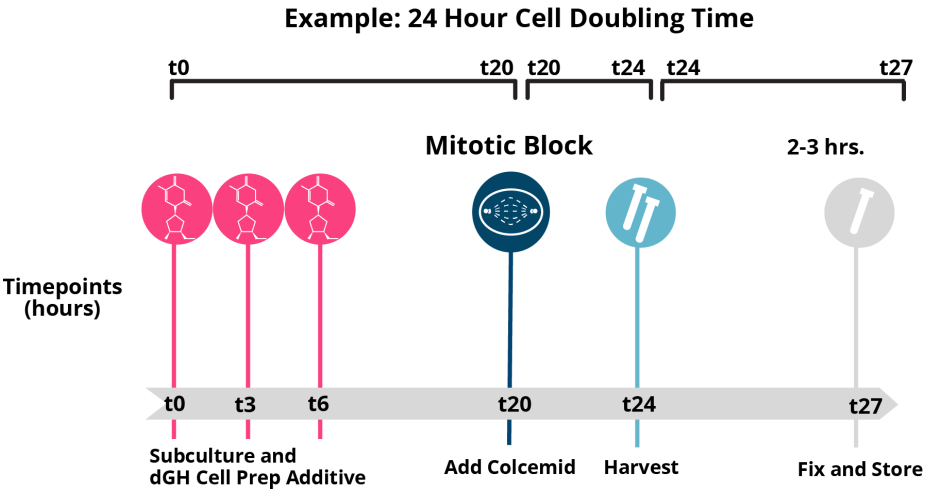

Figure 1: Example workflow for culturing cells with a 24-hour doubling time.

Sample Experimental Plan (Multiple Samples)

11/14 - 11/20

Samples:

- = S1
- = S2
- = S3
- = S4
- = S5

Harvest Timepoints:

T1: 23 hours post analog addition
T2: 27 hours post analog addition

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Nov. 14	15	16	17	18	19	20
					8a: S1, T1 colcemid	
					8a: S2, T1 colcemid	
					8a: S3, T1 colcemid	
					8a: S4, T1 colcemid	
					8a: S5, T1 colcemid	
					9a: S1, T2 colcemid	
					9a: S2, T2 colcemid	
					9a: S3, T2 colcemid	
					9a: S4, T2 colcemid	
					9a: S5, T2 colcemid	
				10a: S1, T2 analog		
				10a: S2, T2 analog		
				10a: S3, T2 analog		
				10a: S4, T2 analog		
				10a: S5, T2 analog		
					12p: S1, T1 harvest	
					12p: S2, T1 harvest	
					12p: S3, T1 harvest	
					12p: S4, T1 harvest	
					12p: S5, T1 harvest	
				1p, S1, T1 analog	1p: S1, T2 harvest	
				1p: S2, T1 analog	1p: S2, T2 harvest	
				1p: S3, T1 analog	1p: S3, T2 harvest	
				1p: S4, T1 analog	1p: S4, T2 harvest	
				1p: S5, T1 analog	1p: S5, T2 harvest	

Figure 2: Example Experimental Plan (Multiple Samples). KromaTiD recommends processing cohorts of no more than 7 samples in parallel. Experiments with more than 7 samples should be planned with staggered cohorts, and separate harvest times.

Cell Culture Protocol for KromaTiD dGH Assays

24 Hours Before Harvest

1. Ensure that the total culture volume is sufficient to yield at least 1 million cells per timepoint at harvest. The cells can be cultured in flasks or multi-well plates and scaled to yield >1 million cells at harvest.
2. Adding fresh media with dGH Cell Prep Additive to cell cultures
   a. Suspension cell cultures
      i. Pellet cells at appropriate centrifuge speed at room temperature in conical tubes.
      ii. Aspirate supernatant and resuspend cell pellet in fresh media, at a density that promotes culture expansion.
      iii. Add 1µL KromaTiD dGH Cell Prep Additive per 1mL of culture volume.
      iv. Return the culture to the incubator.
   b. Adherent cell cultures
      i. When the culture is 40–50% confluent, aspirate media and add fresh media.
      ii. If the cells are more than 60% confluent, consider replating them to allow room for culture expansion.
      iii. Add KromaTiD dGH Cell Prep Additive to the culture, using 1µL of Additive per 1mL of culture volume.
      iv. Return the cultures to incubator.

Day of Harvest

1. Warm 75 mM potassium chloride (KCl) solution to 37°C in water bath and prepare fixative (3:1 Methanol: Glacial Acetic Acid).

   Note: *Fixative needs to be prepared for use within 24 hours and should always be prepared fresh each day of harvest.*
2. Directly add 10 µL of 10 µg/mL demecolcine solution (Colcemid™) per mL of media to each culture vessel, for a final concentration of 0.1 µg/mL.

   Note: *Add demecolcine directly to existing culture media. Do not replace current with fresh media before adding demecolcine.*
3. Return culture to incubator for 4 hours.
4. At the end of the incubation period, transfer the cells from their culture vessels to 15 mL polystyrene conical tubes.
   a. Detach adherent cells thoroughly from vessels before transferring.

Note: *Retain media supernatant prior to detachment (trypsinizing/ scraping) and use it to deactivate trypsin and/or rinse and collect the cells from the vessel prior to transferring to the 15 ml conical tube.*

b. For suspension cells, transfer directly. Multiple wells of sample (single timepoint) can be combined at this step. If media volume exceeds 15 mL, split into multiple 15 mL conical tubes, or use a 50 mL conical tube for this step and transfer volume to 15 mL polystyrene conical tube after step 7.

5. Centrifuge the samples at 180 g for 10 minutes at room temperature. If your centrifuge cannot be programmed using g, and you do not know the radius of your rotor, spin at 1000 RPM. A setting of 1000 rpm using a standard hanging-bucket style rotor centrifuge large enough to accommodate 15-50 mL conical tubes will achieve a sufficiently gentle g-force.

   Note*: Avoid centrifugation at higher g's. Cell pellets will over-clump during high-speed spins and cannot be disrupted sufficiently to provide exposure to the hypotonic in the following step. Please check the rotor size and calculate the RPM required to achieve 180g. G-force formula: RCF (g force) = relative centrifugal force, r = rotational radius (cm) g Force (RCF) = (RPM)2 × 1.118 × 10-5 × r. RPM Formula: RPM = √[RCF/(r × 1.118)] × 1 × 105.*

   Note*: If your specific culture calls for a gentler, lower g-force spin than the one specified in this protocol, consult KromaTiD to determine appropriate modifications, and plan for a longer centrifugation time to accommodate the lower g-force.*

6. Carefully aspirate supernatant, leaving 0.5 mL to 1 mL of media behind with the cell pellet.
7. Thoroughly resuspend cell pellets by vigorously flicking each conical tube, or by pipetting up and down.

   ⚠ Be sure that all cell clumps have been thoroughly dispersed.

   ⚠ If a 50mL conical was required for steps 4–7, transfer the entire resuspended pellet to a 15mL polystyrene conical.

8. Add 10 mL of 37ºC 75 mM KCl hypotonic solution, cap the tubes and gently invert several times.
9. Incubate for 30 minutes at 37ºC, inverting every 10 minutes.
10. Add 1.5 mL of freshly prepared fixative to each 15 mL polystyrene conical tube. Cap the tubes and invert gently several times.
11. Centrifuge the samples at 180 g for 10 minutes at room temperature.

    ⚠ *This step involves centrifugation of swollen, sensitive cells. Centrifugation at higher g's may result in cell damage.*

12. Carefully aspirate supernatant, leaving 0.5 mL to 1 mL of media behind with the cell pellet.
13. Thoroughly resuspend cell pellets by finger flicking the tube. CAUTION: Do not use a pipette to resuspend as the cells are fragile. Be sure that all cell clumps have been dispersed.
14. While vortexing at low-medium speed, add 2 mL fixative **dropwise** with gentle mixing to minimize clumping, followed by an additional 3 mL of fixative.
15. Leave the fixative suspension at room temperature for 20 minutes.

16. Centrifuge the samples at 180 g for 10 minutes at room temperature.

    Note: *At this point, sample tubes can be held at -20°C until ready to perform the remainder of the fixative washes.*

17. Carefully aspirate supernatant, leaving 0.5 mL of media behind with the cell pellet.

    Note: *Some T cells, T cell lines and CAR-T cells may have a translucent or transparent cell pellet that is difficult to visualize after fixation. Take care not to aspirate the cell pellet while removing the supernatant after each fixative wash.*

18. Resuspend the cell pellet by flicking gently.
19. Add 5 mL of fixative to the cell pellets, cap the tubes and gently invert several times.
20. Centrifuge at 180 g for 10 minutes at room temperature.
21. Repeat steps 17–20 twice more for a total of 3 washes in fixative.
22. At this point, the samples can be stored at -20°C until ready to proceed to the **Metaphase Slide Preparation Protocol.**

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