RUO
RESEARCH USE ONLY

Instructions For Use

Cell Culture Protocol for use in KromaTiD's Directional Genome Hybridization™ (dGH™) Assay

Reagents & Materials Provided

- KromaTiD dGH Media Additive
- Return shipping materials included (shipping box & internal insulated box)
    - 2mL screw cap cryovials
    - Return shipping label

Reagents Not Provided

- Colcemid (KaryoMax, Gibco Cat#15210-040 or equivalent)
- 75mM Potassium Chloride (KCl) (Fisher Cat# 10575090 or equivalent; must be less than 7 days old if solution is made with dry KCl and molecular grade H_2O)
- Methanol - molecular grade (Fisher Cat# A412-4 or equivalent)
- Glacial Acetic Acid (Fisher Cat# A38C-212 or equivalent)
- 70% Ethanol – molecular grade (VWR Cat#EM-4450S or equivalent)
- Decon BDD Bacdown Detergent Disinfectant (Fisher Cat# 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
- 50mL conical tubes
- ⚠ 15mL *polystyrene* conical tubes (VWR Cat# 21008-197 or equivalent)

Note: *Polypropylene tubes are not resistant to the fixative and will leak. The degradation of plastic can interfere with sample quality as well.*

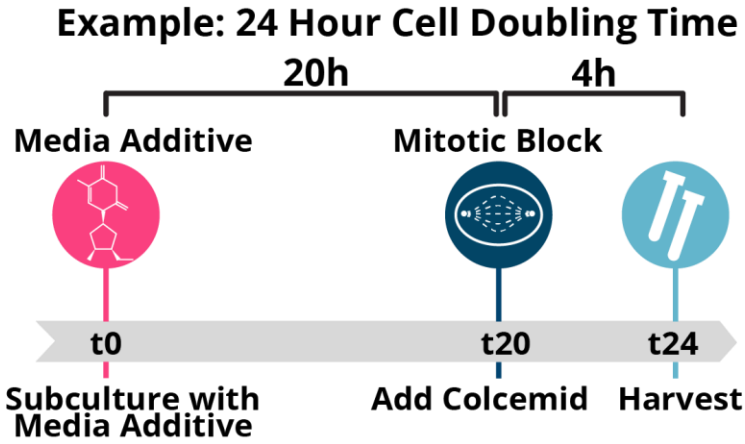
Cell Culture Protocol for use in KromaTiD's dGH Assay

Preparation of Cells

- Prepare at least 1 million cells per sample to be tested.
- Understanding your cell lines doubling time is crucial for choosing three timepoints for dGH media additive inclusion (see note & graphic below). KromaTiD employs cell counting to get accurate doubling times for various cell lines.
- Make sure cell culture has fresh media before media additive inclusion.
    a. Suspension cells should be spun down and resuspended in fresh media.
    b. As for adherent cells, remove old media and add fresh media.

Handling/ Storage of Media Additive

- Store the vial of media additive at -20ºC upon receiving.
- Media additive can be stored for up to 9 months at -20ºC.
- ⚠ Once thawed, do not re-freeze.
- Media additive can be stored for up to 30 days at 4ºC.



Note: *KromaTiD is happy to review and provide guidance on an experimental plan appropriate for your specific cell culture. It is best practice to bracket the cell doubling time with media additive by having three timepoints per sample. This helps to ensure the capture of sufficient metaphase arrested cells. For example, if the cell cycle time is 24 hours, media additive would be included into three separate flasks at three different time points to have 21-, 24- and 27-hour incubation and a single harvest time point.*

Use of Media Additive and Harvest

Note: *37ºC pre-warmed 75mM KCl is required for Step 9.*

1. Add 1µL of the KromaTiD dGH Media Additive per mL of total culture media.
2. Incubate with normal cell culture conditions (temperature, %CO2, and humidity) for specified incubation times, as according to note above.
3. Add colcemid at a final concentration of 0.1µg/mL by adding 10µL of colcemid per mL of culture media and incubate for 4 hours.
4. At the end of the 4 hour colcemid blocking period, transfer the growth medium to a 15mL polystyrene conical, depending on culture volume.
    a. Dissociate adherent cells and transfer the cells to a polystyrene conical.
    b. For suspension cells, transfer the cells to a polystyrene conical.

Note: *If your culture volume is larger than 15mL, then initially transfer to a 50mL conical or other appropriately sized vessel for centrifugation before moving to step 5. Important: before adding fixative ensure the cell pellet has been transferred to a polystyrene conical tube per step 8.*

5. Centrifuge the samples at room temperature at 1000rpm for 5 minutes.
6. Aspirate the supernatant with care to leave 0.5mL to 1mL of media in the tubes with the cell pellet.
7. Thoroughly suspend cell pellets by vigorously flicking the conicals to resuspend the cell pellet.
8. Transfer the entire resuspended cell pellet volume to 15mL polystyrene conicals.

9. Add 6mL of 37ºC 75mM KCl hypotonic solution and incubate for 30 minutes at 37ºC, inverting every five minutes.
10. Add 1.5mL of freshly prepared 3:1 methanol to glacial acetic acid fixative to each 15mL polystyrene conical tube and invert gently several times.
11. Centrifuge the samples at 1000rpm for 10 minutes at room temperature. The cell pellets should be approximately 2-3X larger in size due to the swelling of cells in hypotonic solution.
12. Aspirate the supernatant with care to leave 0.5mL to 1mL of supernatant in the tubes with the cell pellet.
13. Resuspend the cell pellet by gently flicking the conicals.
14. While vortexing at low-medium speeds, add 5mL of 3:1 methanol to glacial acetic acid fixative.
    a. Slowly add the first 2mL dropwise to minimize clumping.
15. Fix at room temperature for 20 minutes.
    a. At this point you may place the tubes at -20ºC until ready to perform the remainder of the fixative washes.
16. Centrifuge the samples at 1000rpm for 10 minutes at room temperature.

Note: *Some cell types (especially iPSCs and T-Cells) will have a translucent cell pellet that is very difficult to visualize after exposure to fixative. In step 17, take care not to aspirate the cell pellet while removing the supernatant after each fixative wash.*

17. Aspirate the fixative supernatant with care to leave 0.5mL in the tubes with the cell pellet.
18. Resuspend the cell pellet by gently flicking the conicals.
19. Add 5mL of the 3:1 methanol to glacial acetic acid to the cell pellets.
20. Centrifuge at 1000rpm for 10 minutes at room temperature.
21. Repeat steps 17 – 20.
22. Repeat steps 17 – 20 again.

Note: *If shipping samples back to KromaTiD for dGH analysis, please follow the instructions below. If not, please follow the dGH Assay Protocol that can be provided at request.*

23. Discard the supernatant after centrifuging, leaving behind 1.0mL to cover the pellets. Gently flick the cell pellets into solution thoroughly and transfer the full volume to individual 2mL screw cap cryovials. Use 0.5mL of fresh fixative to rinse out the 15mL conical tubes to collect any cells on the walls of the tubes and transfer to the cryovial for a total volume of 1.5-1.7mL. Please ensure all tube labels are legible.
24. Please complete KromaTiD's Sample Submission Form (available at request) and submit it to **samples@kromatid.com**. Include any requisition information on this form.
25. Package the cell pellets for return to KromaTiD, see Shipping Instructions below.

Shipping Instructions

Make sure all vials are tightly screwed shut and the top sealed with parafilm before packaging for shipment. Place the sealed 2mL cyrovials in an insulated box with a frozen gel pack (not included). Attach the return shipping label included with the original packaging.