**UW Medicine - Pathology**

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BrdU Label of Cultures for Replication Analysis (X Inactivation Studies)

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PURPOSE

This technique allows for the differentiation of the two X chromosomes in a female. The inactive X, which is late-replicating, will incorporate BrdU (bromodeoxyuridine) late in S phase. The differential BrdU incorporation can be revealed by several methods including Hoechst 33258 staining, Giemsa protocol, or BrdU antibody. This technique is informative when there is a cytogenetic or FISH detectable difference between X chromosomes. Preferred method is BrdU antibody for staining.

This staining technique is designed to visualize chromosomal regions that incorporated BrdU such as late-replicating X chromosome in female cells, particularly for individuals with X-rearrangements or X;autosome translocations, and in studies of SCE, for the study of chromosome breakage syndromes.

PROCEDURE

### Specimen Requirements

5ml peripheral blood in Sodium Heparin tube (room temperature). Set up blood as per Peripheral Blood Cultures with the additional cultures given by the table below. Accession specimen in GCS as a PB; log into PB book. A normal female control (accessioned as an RE case) should be run in conjunction with patient specimen.

### Material and Equipment

* + - 1. Dry 5% CO2 incubator.
      2. Laminar flow hood [Biosafety Hood (BioGard)]
      3. Centrifuge
      4. 15 ml centrifuge tubes.

### Reagents and Solutions

* + - 1. RPMI 1640 with L-glutamine (Sigma Cat. #R8758): add the following per 100 ml of media: 20 ml fetal calf serum 0.1 ml penicillin-streptomycin.
      2. Colcemid 10 µg/ml (Gibco Cat. #0395).
      3. PHA (Murex Cat. #HA-15). Once in solution good for 1 mo per PHA info sheet.
      4. 0.075 M KCl hypotonic.
      5. 3:1 methanol:glacial acetic acid (FIX).
      6. Fluorodeoxyuridine ‑‑ FUdR (Sigma Cat. #F3503).
      7. Uridine (Sigma Cat. #U-37505
      8. Bromodeoxyuridine ‑‑ BrdU (Sigma Cat. #B5002).
      9. FUdR stock solution (3 x 10-4 M).
         1. FUdR 1 mg
         2. Distilled H2O 10 ml
         3. Store frozen in dark vials
      10. Uridine stock solution (4 x 10-3 M).
          1. Uridine 1 mg
          2. Distilled H2O 1 ml
          3. Store frozen.
      11. BrdU solution
          1. BrdU 3 mg (10-2 M)
          2. FUdR stock solution 0.1 ml (3 x 10-5 M)
          3. Uridine stock solution 0.2 ml (8 x 10-4 M)
          4. Distilled H2O 0.7 ml
          5. Store frozen in dark vial. Solution good for 2 wk.

### Procedure

***Note****:* No mouth pipetting. Adhere to sterile techniques. Use tissue culture hood [Biosafety Hood (BioGard)]. Use gloves.

1. Set up four cultures for proband and one culture for control as usual. Proband: 2 RPMI cultures for replication study and 2 RPMI cultures for routine analysis. Control: 1 RPMI culture for replication study.

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| **X-Inactivation/BrdU Study** | |  | Add: |
| A—72 hr | 9 ml RPMI + 1 ml blood | T25 flask | Nothing |
| B—72 hr (Thy) | 9 ml RPMI + 1 ml blood | T25 flask | 0.3 ml of 1% thymidine after 48 hr |
| C—72 hr (BrdU) | 9 ml RPMI + 1 ml blood | T25 flask | 0.1 ml BrdU on morning of harvest |
| D—72 hr (BrdU) | 9 ml RPMI + 1 ml blood | T25 flask | 0.1 ml BrdU on morning of harvest |
| **Control (normal female)** | |  |  |
| A—72 hr | 9 ml RPMI + 1 ml blood | T25 flask | 0.1 ml BrdU on morning of harvest |

1. After 72 hr, harvest routine cultures as per Peripheral Blood Cultures.
2. After 72 hr, add 0.05 ml BrdU solution to each 5 ml culture for replication study.
3. Incubate culture in dark for 5-6 hr. (Wrap black paper or aluminum foil around culture tube.)
4. Add 0.05 ml colcemid for 30 min.
5. Harvest cultures like routine Peripheral Blood Cultures. Use Hanabi p-II harvester.
6. Prepare slides.
7. Slides can be stained with Hoechst, Giemsa**,** or BrdU antibody. The preferred procedure is BrdU (IX). The X chromosome will appear differentially stained and banding will be evident along the chromosomes.

### Staining

### Method 1: Fluorescence Plus Giemsa (Fpg) Staining

### Material and Equipment

* + - 1. 4" x 4" square Petri dishes.
      2. Coverslips (24 x 50 mm).
      3. Aluminum foil.
      4. Slide warmer at 60-65°C.
      5. Fluorescence microscope (excitation 350 nm, emission 450 nm).

### Reagents and Solutions

* 1. Phosphate Buffered Solution (PBS), pH 7.0 (Gibco Cat #14190-136).
  2. Hoechst 33258 (Sigma B33258).
  3. ***Note***: This is a mutagen, wear gloves.
  4. Distilled H2O.
  5. 2X SSC
  6. Wright stain (Sigma - W3000).
  7. Prepare 50 µg/ml (H2O) Hoechst 33258 stock solution. Keep refrigerated or frozen and wrapped in foil. Expires in 1 yr.
  8. Prepare 0.5 µg/ml Hoechst 33258 by diluting the stock solution in fresh PBS (1:100). Expires 1 month after preparation. Store in refrigerator.
  9. PBS-buffered Hoechst 33258 (1 volume of PBS to 1 volume of the 50 µg/ml Hoechst 33258 solution). Store in refrigerator wrapped in foil.

### Procedure

Prepare slides. Stain slides by the following FPG procedure. Do not bake the slides

* + - 1. Soak 1-2 day old unstained slides for 5 min in 50ml PBS in a coplin jar (1).
      2. Stain for 10 min in 0.5 µg/ml Hoechst 33258 in a coplin jar (2).
      3. Drain slides and place them in 4" x 4" square dishes, using moist filter paper and plastic slide racks for supports.
      4. Cover each slide with 3-4 drops of PBS-buffered Hoechst. Put a 24 x 50-mm coverslip on each slide.
      5. Cover the square dishes and expose them to cool white (20 watt) fluorescent light approximately 4" from the surface of the dishes for 6 hr or overnight.
      6. Rinse the coverslips off the slides under warm running tap H2O. Do not try to pry the coverslips off.
      7. Place the slides in pre-warmed (60-65°C) 2X SSC for 30-45 minutes. Slightly longer treatment may be required; adjust time, if necessary. Agitate the slides periodically.
      8. Rinse the slides well in running distilled H2O.
      9. Stain in Wright stain for 4 minutes and score.
      10. Examine and capture metapases under bright field in computer.
      11. Alternate fluorescence banding method: After step 2, the slide can be rinsed 5 min in PBS, pH 7.0, mounted in McIlvaine, pH 7.5 (chapter 3) and examined under fluorescence microscope (excitation 350 nm, emission 450 nm).

### Method 2: Anti-Brdu Staining (Preferred Method)

### Material and Equipment

* 1. Fluorescence microscope: (fluorescein set up of filters)

### Reagents and Solutions

1. Antibody for BrdU (Monoclonal Sigma B-2531)
2. Antimouse antibody FITC (Sigma #F5262).
3. KCM/5% BSA buffer
   1. 120 mM KCl
   2. 20 mM NaCl
   3. 10 mM Tris-HCl pH 8.0
   4. 0.5 mM Na2·EDTA
   5. 0.1% Triton-X
   6. Bovine Serum Albumin (BSA) (from lypholized powder, freeze dried, not acetylated, SIGMA Cat# B6917).

**Dissolve the following in 400 ml DD-H2O:** 4.47 g KCl, 0.58 g NaCl, 0.09 g Na2·EDTA, 5 ml 1M Tris, 500 µl Triton X-100 and 12.5 ml [2mg/ml] BSA(25 mg). Add H2O to a final volume of 500 ml. Ensure the pH is 8.0. Store at 4°C with a shelf life of 1 year.

### Procedure

***Note:*** Omit steps 1-3 if slide has been hybridized to maintain FISH probe signals.

1. Bake the slides for 20 min at 95oC.
2. Denature the slides by immersing in 0.05 N NaOH (in a Coplin jar) for 1 sec (just one dip).
3. Immediately dip into a Coplin jar of distilled H2O.
4. Wash in KCM/BSA for 5 min at room temperature.
5. Dry the sample area a little bit (don’t let dry out completely).
6. Flood slide with ~50µl Anti-BrdU antibody (diluted 1:80 in KCM/BSA) cover with 24 x 60 mm coverslip (or plastic coverslip) and incubate at room temperature for 30 min.
7. Wash in KCM/BSA at room temperature 2 min X 2.
8. Incubate the slide with ~50µl FITC conjugated anti-mouse IgG (diluted 1:80 in KCM/BSA) as in step 6, above.
9. Wash in KCM/BSA at room temperature 1 min.
10. Dry and mount in dilute DAPI-antifade (DAPI III-Vysis) as in FISH procedure.

### Analysis

Screen 20 cells and identify both X chromosomes. Record the allocyclic (late replicating) chromosome: pale using Hoechst or Geimsa staining, bright using BrdU antibody staining (FITC-anti BrdU). Photograph 5 sample cells. Capture 15-20 pictures as per requirement and use same metaphases for follow up FISH if order received.

The late replicating chromosome will be seen in a fraction of the total cells. Look for cells that exhibit minimal BrdU incorporation. With anti BrdU staining, they appear duller than the average cell.

REFERENCES

1. Verma RS and Babu A, Human Chromosomes: Manual of Basic Techniques. Pergamon Press, Inc., NY, pp. 131-133, 1989.
2. Benn PA et al., Human Cytogenetics. A Practical Approach. Vol. I., Chapter 4, eds. Rooney DE, Czepulkowski BH, IRL Press, 1992.

Written By: Director Approval:

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Cytogenetics Supervisor