**UW Medicine - Pathology**

400-02-01-08

BrdU Label of Cultures for Sister Chromatid Exchange / Bloom Syndrome

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PURPOSE

This technique allows for the observation of sister chromatid exchanges (SCE). This test is indicated for patients suspected of having Bloom syndrome. In addition, SCE can be used to monitor certain types of DNA damage after exposure to mutagens or carcinogens. This latter type of test is not performed in our lab at present.

PROCEDURE

### Specimen Requirements (See Peripheral Blood Cultures procedure, SOP number 400-02-01-03)

A normal control sample is required in case of Bloom syndrome testing.

### Material and Equipment

* + - 1. Dry 5% CO2 incubator.
			2. Laminar flow hood [Biosafety Hood (BioGard)]
			3. Centrifuge.
			4. 15 ml and 50 ml centrifuge tubes (Falcon Cat. #352095 and 352070, respectively).
			5. T25 flasks (Corning Cat. #430168).
			6. Sterile pipettes.

### 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 (Life Technologies Cat. #10378)]. Final concentration 10 units/ml pen; 10 µg/ml strep. Stock good for one year stored at -20°C.
2. Colcemid 10 µg/ml (GIBCO 15210-040).
3. 0.075 M KCl hypotonic. Store at room temperature one mo.
4. 3:1 methanol:glacial acetic acid ‑ made fresh and kept at room temperature.
5. **10-2 M bromodeoxyuridine (BrdU)** (Sigma Cat. #B5002) stock solution. Weight 0.031 g BrdU: dilute in 10 ml dH2O. Keep frozen 1 year (-6°C), 2 mo refrigerated.
6. **Phytohemagglutinin** (PHA) (Murex Cat. #HA-15) (only for fresh blood, important to reduce toxicity of BrdU).
7. 10-2 M deoxycytidine (Sigma Cat. #D0883).
8. **Bromodeoxyuridine (Sigma Cat. #B5002):** Prepare a 10-2 M solution of BrdU in distilled water (0.03071 g/10 mL). Sterilize by filtration, aliquot, and freeze. Just before use, dilute 1:4 with Hank's balanced salt solution (HBSS) to obtain a 2.5 mM solution.
9. **Fluorodeoxyuridine (Sigma - F0503) and uridine (U3750):** Prepare a 10-3 M solution of fluorodeoxyuridine in distilled water (0.00246 g/10 mL) and a 10-2 M solution of uridine in distilled water (0.02442 g/10 mL). Add 1.0 ml of "F" stock and 1.5 mL of "U" stock to 47.5 mL of HBSS to get a working solution that is 2 x 10-5 M "F" and 3 x 10-4 M "U." Sterilize all three solutions by filtration, aliquot, and freeze.
10. **Deoxycytidine (Sigma D3897)**: Prepare a 10-1 M solution of deoxycytidine in distilled water (0.2272 g/10 mL). Sterilize by filtration, aliquot, and freeze. Just before use, dilute 1:10 in HBSS to obtain a 10-2 M working solution.
11. **Thymidine (Sigma T1895)**: Prepare a 10-2 M solution of thymidine in distilled water (0.02422 g/10 mL). Sterilize by filtration, aliquot, and freeze. Prepare a working solution by adding 1 mL of thymidine stock solution to 49 mL of HBSS.
12. 2x Saline sodium citrate (2x SSC):
	* 17.53 g sodium chloride
	* 8.82 g sodium citrate
	* Bring volume to 1 L with distilled water; adjust pH to 7.0.
		1. **Hoechst33258 stain**: Wear gloves; Hoechst induces SCEs and may be harmful. Dissolve 5 mg Hoechst stain (Sigma cat. #B-2883) in 100 mL distilled water (50 µg/mL). Prepare fresh at least monthly. Prepare stock solution in distilled water; refrigerate in a foil-covered bottle. Prepare working solution by diluting in PBS immediately before use. (Hoechst will precipitate if stored in PBS).
		2. **McIlvaine's buffer**, pH 7.5 (44):
			+ Solution A: 0.1 M citric acid (19.2 g: q.s. to 1 L with distilled water)
			+ Solution B: 0.2 M Na2HPO4 (28.4 g: q.s. to 1 L with distilled water)
		3. For pH 7.5, mix 8 mL of solution A with 92 mL of solution B. Check the pH and adjust if necessary.
		4. **Wright Stain** (Sigma - W3000).

### Procedure

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

**Method I: T-pulse for sister chromatid exchange analysis on blood-Replication analysis:**

1. Set up 4 cultures as usual (see Peripheral Blood Cultures): 2 RPMI cultures for SCE study and 2 RPMI cultures for routine analysis.
	1. Add to the SCE culture: BrdU (Sigma Cat. #B5002): final concentration 10-4 M (stock 10-2 M in H20) and deoxycytidine (Sigma Cat. #D0883): final concentration 10-4 M (stock 10-2 M in H2O).
	2. At about 40 hr in culture, transfer SCE cultures to a sterile centrifuge tube. Spin culture tubes 5 min at 1200 rpm and aspirate supernatant.
	3. Add 2 ml fresh medium (no BrdU; no dC) prewarmed at 37°C. Resuspend the pellet by flicking the tube. Spin as before.
	4. Remove supernatant. Add 10 ml of fresh medium containing thymidine [10-2 M final concentration (Sigma) stock 10-3 M in H20]. Return cultures to incubator for 6 hr.
	5. Add colcemid as usual (see Peripheral Blood Cultures).
2. Harvest routine cultures as per Peripheral Blood Cultures. If possible, use HANAB P-II harvester with other routine harvesting.

**Method II: Lymphocyte culture initiation for terminal thymidine pulse (3rd Ed, AGT Manual)-Sister Chromotid Study:**

1. Set up lymphocyte cultures. Each culture should receive the following:
	* 5 mL RPMI medium complete
	* 200 µL of working BrdU solution
	* 100 µL of working F/U solution
	* 50 µL of working deoxycytidine solution
	* 0.3-0.4 mL whole blood or buffy coat
	* Cover the tubes with foil; BrdU-substituted chromosomes are subject to breakage if exposed to certain wavelengths of light. Incandescent light and GE golden yellow fluorescent light are acceptable, but white fluorescent light and sunlight are not. Cap tubes tightly; invert to mix. Incubate cultures on a slant rack at 37ºC.
		1. At 9:00 a.m. on the 2nd day of culture (~ 40 hrs after culture initiation), resuspend the cells by gentle inversion of the tubes, then centrifuge them for 5 mins at 1000 rpm.
		2. Remove the BrdU-containing medium by aspiration. Resuspend the cells by tapping the bottom of the tube.
		3. Add 3-5 mL of fresh, prewarmed (37ºC) medium MEM to wash the cell pellet. Centrifuge as before.
		4. Remove the wash medium by aspiration. Add 5 mL of prewarmed supplement medium and 0.2 mL of working thymidine solution to each tube; mix thoroughly. Return the foil-covered tubes to the incubator.
		5. Approximately 6 hrs later, add 25 µL of Colcemid® to each tube.
		6. Harvest 30-60 mins later for a 6.5- to 7-hour T pulse. (The time can be varied for studies of replication kinetics.)
		7. Stain slides by the 33258 Hoechst procedure or the FPG procedure.

**SCE Culture Procedure**

Set up lymphocyte cultures as for the terminal thymidine pulse procedure, step 1. Do not change the medium. Harvest 68-72 hours later in harvestor.

**Fluorescence plus Giemsa (FPG) staining**

1. Soak slides for 5 mins in PBS.
2. Stain for 10 mins in 0.5 µg/mL Hoechst 33258. (Dilute the 50 µg/mL stock 1:100 in frest PBS.)
3. Drain slides, and place them in square culture dishes, using moist filter paper and plastic slide racks for supports.
4. Cover each slide with 3-4 drops of PBS-buffered Hoechst (1 part of 50 µg/mL stock to 1 part PBS.) Place a 24 mm x 50 mm glass coverslip on each slide.
5. Cover the dishes, and expose them to a cool white fluorescent light approximately 4 in from the surface of the dishes for overnight.
6. Rinse the coverslips off the slides with distilled water.
7. Place the slides in pre-warmed (60-65ºC) 2x SSC for 15 mins. (Slightly longer treatment may be required; adjust time if necessary.) Agitate the slides periodically.
8. Rinse the slides well with running distilled water.
9. Stain in Wright stain for 4-7 minutes.
10. Examine and photograph as for other brightfield techniques.

**T-pulse for sister chromatid exchange on fibroblasts**

1. Cultures
	1. Add: BrdU final concentration 10-4 M (stock 10-2 M) and deoxycytidine final concentration 10-4 M to a culture 24 hr after it was transferred. Let the culture grow 10-12 hr (11 pm to 9 am).
	2. Rinse the flasks with fresh medium.
	3. Add fresh medium with thymidine (final concentration 10-5 M (stock 10-3 M).
2. Harvest as usual after 7 hr (including 1 hr of colcemid).

**Staining and scoring**

Different staining protocols can be used to reveal BrdU incorporation: X, Hoechst staining; XIX, Giemsa staining, and XX, BrdU antibody staining. At present the preferred method is XIX**.** The number of SCE should be scored by noting alternative pattern of differential staining intensity along the chromatids in 20-100 cells. A control sample (normal in case of testing for Bloom syndrome) should be included. Usually the number of SCE is increased 5- to 10-fold or more in Bloom syndrome samples compared to control cultures.

***Note:*** All vessels that come in contact with specimens or cultures from specimens are biohazardous and should be discarded in appropriate containers following the guidelines for disposal**.**

REFERENCES

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Written By: Director Approval:

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 Cytogenetics Supervisor