# PREPARATION OF MOUSE BONE MARROW PRIMARY CULTURES FOR SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATION STUDIES

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SUMMARY: A procedure for preparing and culturing mouse bone marrow cells for cytogenetic studies is described. Animals are killed by cervical dislocation, the bone marrow is flushed from femora and tibia with Ham's F12 medium into centrifuge tubes. Bone marrow cells are collected by low-speed centrifugation (285 × *g*). One million cells are suspended in a 30-ml Falcon flask with complete medium containing 3.45 ml Ham's F12 medium, 1 ml fetal bovine serum, 0.05 ml penicillin-streptomycin, and 0.5 ml whole uterus extract from pregnant mice. For sister chromatid exchange analysis, 20 μ*M* of 5-bromo-2′-deoxyuridine is also added to the culture medium for 24 to 28 h. The cell cycle is approximately 12 to 14 h, in culture. This culture system can also be used for chromosomal aberration studies.

*Key words:* mouse bone marrow cells; primary culture; genotoxicity; chromosomal aberration; sister chromatid exchange; cytogenetic analyses.

## I. INTRODUCTION

Most genotoxicity studies in rodents have been done by in vivo exposure of animals to the genotoxic compounds. In humans this has been done by in vitro culturing of blood or bone marrow or both from the exposed subjects followed by analysis of chromosomal damage. To extrapolate results from animals to humans it is essential to use the same tissue for both in vivo and in vitro conditions. Bone marrow is one of the tissues used in both animals (in vivo) and humans (in vitro) for chromosome analysis. Although several simple, repeatable procedures have been developed for culturing of human bone marrow to analyze chromosomes (1,2), such procedures are still lacking in the mouse system.

The chromosomal aberration and sister chromatid exchange (SCE) assays have been widely used in recent years to monitor chemical insults to exposed individuals in the environment (3–5). SCEs represent reciprocal exchanges between sister chromatids, which can be visualized in metaphase chromosomes using 5-bromo-2′-deoxyuridine (BrdU) and special staining procedures. SCE has been shown to be an extremely sensitive indicator for the genotoxicity of potential chemical carcinogens both in in vivo and in vitro test systems (4–6). It may be observed that in such studies a bulk of the time is spent not in setting up and harvesting cultures but in the analysis of cells. Obviously, the best way to reduce time spent in analysis is to obtain more and better chromosome spreads.

This report describes a reproducible mouse bone marrow culture system for SCE and chromosomal aberration analyses. The embryo/uterus growth factor reported by Bradley et al. (7) was used to stimulate bone marrow cells; however, the extract preparation has been simplified by eliminating several purification steps. This culture system can be used for in vivo and partial in vivo (exposing animals to chemicals and then culturing cells in vitro) comparison studies and can also be used for in vitro genotoxicity assays.

## II. MATERIALS

A. Equipment

Balance, top loading, Metler, Model PC440,No. 01-913-351, Fisher[1]

Centrifuges

IEC table top, Model HN-SII, No. 05-111[1]

Ultra-centrifuge, IEC/B-60[1]

Homogenizer, tissue, Model 300[1]

Hood, biological safety cabinet 800 series, Bellco[2]

Incubator, Napco Bench Model, No. 11-685-55[1]

Microscopes

AO Biostar, inverted, biological, American Scientific[3]

Light, AO Microstar, General Lab[3]

Pipet-aid, filler and dispenser, No. 13-681-15[1]

Refrigerator, Laboratory Model 812, No. R4040-1[3]

Slide warmer, Lab Line Model 26020, No. M6410[3]

Water bath, general purpose, Model 181, No. 15-474-10[1]

B. Plastics and glassware

Plastics
- Centrifuge tubes
  - Falcon 2095, conical graduated, 15 ml, screw cap, No. 2610-L35, Thomas Scientific[4]
  - Falcon 2098, conical graduated, 50 ml, screw cap, No. 2610-L54[4]
  - Round bottom, polycarbonate, screw cap, Oak Ridge-Type IEC, No. C4049-30[3]
- Filters, membrane
  - Nalgene 120-0020, 0.20 μm, No. 09-740[1]
  - Nalgene 245-0045, 0.45 μm, No. 09-740-5[1]
- Flasks, tissue culture, Falcon 3013, 25 $cm^2$, screw cap, No. 9381-K35[4]
- Pipettes
  - Stripettes, cotton plugged, individually wrapped, 10 ml, No. 4101, Costar[5]
  - Serological, 5 ml, 5 in 1/10 ml, with plug, No. 7529, Falcon[6]
- Vials, Fisher brand, polyethylene, No. 03-338-1C[1]

Glassware
- Beakers, 400 ml Pyrex, No. 1000[3]
- Bottles, 100 and 500 ml, screw cap, GIBCO[7]
- Couplin staining jars, No. S7655-1[3]
- Cylinders, graduated, 50 and 500 ml Pyrex, No. 3025; 100 ml Kimax, No. 20030[3]
- Flasks, filtering, Pyrex, Corning 5320
  - 250 ml, No. 4947-G17[4]
  - 500 ml, No. 4947-G23[4]
- Microslides, frosted, No. 12-552[1]
- Cover glasses, Gold seal rectangular, No. 12-518-105A[1]
- Pasteur pipettes, Fisher brand, No. 13-678-208[1]

C. Media and solutions

- 5-Bromo-2′-deoxyuridine, No. B5002, Sigma[8]
- Colchicine, No. C9754[8]
- Crystal violet dye, No. C3886[8]
- Fetal bovine serum, No. 220-6300[7]
- Ham's F12 with glutamine, No. 12-423-54, Flow Laboratories[9]
- Hanks' balanced salt solution, No. 310-4020[7]
- Penicillin-streptomycin, No. 16-700-46[9]
- Absolute methanol (reagent grade), No. M3641[8]
- Glacial acetic acid (reagent grade), No. A6283[8]
- Giemsa stain (stock), No. 3250-4[3]
- Hoeschst 33258 dye, No. 382061, Calbiochem[10]
- Potassium chloride, KCl, No. P4504[8]
- Potassium phosphate, monobasic, $KH_2PO_4$, No. P5379[8]
- Sodium phosphate, dibasic, $Na_2HPO_4$, No. S0876[8]
- Phosphate buffered saline, No. 18-600-54[9]
- Xylene, No. X516-9[3]

D. Miscellaneous

- Ethyl alcohol 70%, No. 3791-1GL[3]
- Gauze pads, 4 × 4 in., 12-ply, sterile, No. 8519, Johnson [11]
- Gloves
  - Surgical, sterile, No. 30-6180-1, Parke Davis[12]
  - Latex, nonsterile disposable, No. 46-320, Edmont[13]
- Hemocytometer, bright line, No. 5972-H15[4]
- Rubber bulbs, No. 1951-B10[4]
- Test tube rack, 50-ml capacity, S-shaped metal, No. 14-754-25[1]
- Sterile distilled water
- Ice bucket, PVC, No. 6106-A02[3]
- Tissue grinder, sieve type, No. 3425-B05[3]
- Permount, No. So-P-15[1]
- Microslide holder, No. 6707-G20[3]
- Surgical instruments
  - Scissors, diamond-edge operating, No. SU-1757, Mueller[14]
  - Forceps, Halsted, curved, length 5 in., No. SU-2707[14]
  - Forceps, 4½ in., 2 × 3 teeth, No. SU-2340[14]
  - Hemostatic forceps, microline, No. SU-10495[14]
- Timer, 2-h interval, GE, NO. 9371-J70[4]
- Lamp, laboratory, No. 36554-004, VWR[15]
- Black light bulb (lamp tube, long wave UV 360 nm) No. 36557-002[15]
- Silicone rubber adhesive, No. 04-769-5[1]
- Syringe, lock tip, 3 cc, No. S9520-3[3]
- Needle, 25G × 5/8 in., No. S9549-25D[3]

E. Animals

- 17-d-old pregnant $CD_1$ mice, Charles River[16]
- 5 to 8 wk-old male $CD_1$ mice weighing 20 to 30 g[16]

## III. PROCEDURE

A. Prepare the whole uteri extract

1. Use sterile procedures during uterus extract preparation.
2. Kill 10 to 12, 17-d-old pregnant mice by cervical dislocation.
3. Sterilize the abdomen area of animals with 70% ethyl alcohol.
4. Make incision at abdomen and remove whole uterus containing embryos, using surgical instruments.
5. Rinse uterus in phosphate buffered saline and put all uteri in a preweighed, sterile, graduated beaker.
6. Weigh the beaker with uteri and note down wet weight of the uteri.
7. Homogenize the uteri in a homogenizer.
8. Filter the homogenate through a tissue filter.
9. Wash the tissue residue using sterile distilled water (the volume of distilled water should be about 3 times the volume of wet uteri).
10. Centrifuge the filtrate in an ultracentrifuge at 10000 × *g* for 20 min.

11. Decant the supernatant into a glass container and discard the pellet.
12. Heat-inactivate the supernatant in a water bath for 10 min at 60° C.
13. Some proteins denature and the supernatant turns to dark gray precipitate.
14. Cool the supernatant to 10° C using ice in an ice container.
15. Centrifuge this suspension at 10 000 x *g* for 20 min.
16. Decant the supernatant into a sterile container and discard the pellet.
17. Filter the supernatant through a 0.20-μm membrane filter.
18. Dispense the filtrate into 5-ml plastic vials.
19. Freeze these vials at −20° C until needed.
20. Use 10% (vol/vol) of the extract in the medium for cell stimulation.

B. Prepare the stock solutions

1. 5-Bromo-2′-deoxyuridine (BrdU)
   a. Dissolve 10 mg of BrdU in 50 ml sterile distilled water (0.20 μM).
   b. Sterilize by using 0.20-μm membrane filter.
   c. Dispense into 5-ml plastic vials.
   d. Keep these in a suitable container and cover with aluminum foil.
   e. Freeze at −20° C.
2. Colchicine solution
   a. Dissolve 13.2 mg of commercial colchicine powder in 10 ml sterile distilled water.
   b. Keep the stock solution in a refrigerator.
   c. Use 0.05 ml of this stock solution per 5 ml culture medium (33 μ*M*).
3. Fixative
   Mix 3 parts of absolute methanol and 1 part of glacial acetic acid just before use.
4. Giemsa stain
   Dissolve 2.5 ml of commercial Giemsa stock solution in 47.5 ml of Sorenson's buffer (5%) just before use.
5. Hoechst 33258 dye
   a. Dissolve 5 mg of Hoechst 33258 dye in 100 ml distilled water.
   b. Cover the bottle with aluminum foil.
   c. Keep stock solution in a refrigerator.
6. Hypotonic solution (0.075 *M* KCl)
   a. Dissolve 2.8 g of potassium chloride in 500 ml distilled water.
   b. Store at room temperature.
   c. Before use, keep in the incubator (37°C).
7. Sorenson's buffer (pH 6.8)
   a. Dissolve 2.27 g of potassium phosphate (monobasic) and 2.37 g of sodium phosphate (dibasic) in 500 ml distilled water.

C. Prepare the complete culture medium

1. To 69 ml Ham's F12 medium add 20 ml fetal bovine serum (20%), 1 ml penicillin-streptomycin, and 10 ml whole uterus extract.
2. Add 5 ml of this complete medium into a 30-ml Falcon flask (25 $cm^2$) to culture approximately one million bone marrow cells.

D. Prepare the bone marrow cells

1. Expose 5 to 8-wk-old $CD_1$ male mice to the test chemical/control compound for desired length of time.
2. Kill animals by cervical dislocation.
3. Using sterile techniques, isolate tibia and femora from animals.
4. Clean the bones using 70% ethyl alcohol and sterile gauze.
5. Cut off joints and flush the bone marrow from each into a sterile, 15-ml Falcon centrifuge tube using Ham's F12 medium (3 ml).
6. Remove the debris using a sterile Pasteur pipette.
7. Centrifuge the cell suspension using table top centrifuge at 285 × *g* for 5 min.
8. Discard the supernatant.
9. Resuspend the pellet in 1 ml of fresh medium and aspirate a few times (to break up cell clumps).
10. Take 0.1 ml of cell suspension and make desirable dilutions.
11. Stain cells with crystal violet dye and count the cell number with a hemocytometer.
12. Suspend approximately one million cells in 5 ml of complete medium.

E. Set the cultures

1. Use sterile conditions during setting cultures.
2. Add 5 ml complete medium into sterile 30-ml Falcon flasks (25 $cm^2$).
3. Add required volume of cell suspension (one million cells).
4. Add 0.05 ml of BrdU solution (this is not needed for the chromosomal aberration study).
5. Cover the flasks with aluminum foil (not necessary for chromosomal aberration study).
6. Incubate in the incubator at 37° C.

F. Harvest the cultures

1. Perform all steps in subdued light until cells are fixed.
2. Add 0.05 ml colchicine to each flask 3 h before harvest (depending on the study, for SCE at 21 to 25 h and for chromosomal aberrations at 9 to 11 h of culturing).
3. After complete culture period, transfer the contents into 15-ml Falcon centrifuge tubes.
4. Rinse each flask with 3 ml Hank's balanced salt solution, aspirate, and then add the rinsate to the respective tubes.
5. Centrifuge the contents at 285 × *g* for 5 min.
6. Discard the supernatant.
7. Resuspend the pellet.
8. Slowly add 4 ml of hypotonic solution (0.075 *M* KCl) at 37° C while flicking bottom of the centrifuge tube.
9. Keep the tubes in the incubator (37° C) for 20 min.
10. Centrifuge the contents as before, remove the supernatant, and resuspend the pellet.
11. Slowly add (drop-by-drop) freshly prepared fixative and continue flicking the tube.
    a. Add in total 4 ml of fixative.
    b. Let the cell suspension stand for 10 min in the refrigerator.
12. Centrifuge and resuspend the cells in 4 ml of fresh fixative and allow to stand for 5 min in the refrigerator.
13. Repeat step 12.
14. Centrifuge and remove supernatant.
15. Add a few drops of fresh fixative and thoroughly mix the cells by flicking the tube.
16. Draw the cell suspension into a Pasteur pipette and drop the suspension (3 to 5 drops per slide, without overlapping) onto clean, cold (4°C), wet slides.
17. Tilt the slides and place them on a slide stand and allow to air dry overnight.
18. Label slides and store in dark.

F. Set the slide warmer
1. Make a rectangular pool (12 × 4″) on the slide warmer using silicone rubber adhesive.
2. Allow the adhesive to air dry for 2 to 3 d.
3. About 15 min before staining, fill the pool with Sorenson's buffer (60 to 70 ml).
4. Set the temperature control knob between 56 to 60° C and turn on both the slide warmer and the UV lamp.
5. Check correct temperature with thermometer on the slide warmer.

G. Stain the slides for sister chromatid differentiation
1. Add 5 ml Hoechst 33258 stock solution into a Couplin jar and dilute with 45 ml distilled water giving a concentration of 5.0 μg/ml of Hoechst 33258.
2. Add 2.5 ml of Giemsa stock solution into a Couplin jar and dilute with 47.5 ml Sorenson's buffer giving a concentration of 5% of Giemsa.
3. Stain slides for 15 min in Hoechst 33258 dye.
4. Rinse slides in distilled water.
5. Immerse slides in Sorenson's buffer (pH 6.8) on a slide warmer at 56 to 60° C and expose to black light (360 nm) for 15 min at a distance of 1 cm.
6. Rinse slides in distilled water.
7. Stain slides with freshly prepared 5% Giemsa for 10 to 15 min.
8. Rinse slides in distilled water; air dry.
9. Dip slides in xylene and mount cover slips over cells with Permount mounting medium.
10. Score SCEs in 30 to 50 chromosome spreads from each animal with oil-immersion, bright-field optics.

H. Stain the slides for chromosomal aberration analysis
1. Perform steps seven to nine of the sister chromatid differentiation procedure.
2. Score chromosomal aberrations in 100 chromosome spreads from each animal with oil-immersion, bright-field optics.

## IV. DISCUSSION

The assay described is unique in that the animals can be exposed to single or multiple doses of the test compound which can undergo metabolic activation in vivo. This activation is much more meaningful than that obtained with the addition of S9 fraction in the culture and is therefore more suitable for extrapolation of results to humans. The higher mitotic yields and their reproducibility in the mouse bone marrow cell culture depend on the use of Ham's F12 medium and mouse uterus extract. Flask cultures produced much higher mitotic yields than the tube cultures. This suggests that surface area is critical for growth and spread of cells (8). Because the cell cycle of the bone marrow is approximately 12 to 14 h, culture of cells for 24 to 28 h in the presence of BrdU helps differentiate sister chromatids for SCE analysis. When cells are exposed to BrdU and allowed to go through two rounds of DNA replication, each chromosome in the second metaphase ($M_2$) will consist of one partially BrdU substituted chromatid, and the other completely BrdU substituted chro-

matid. Such chromosomes, when stained with the fluorochrome dye, Hoechst 33258, followed by exposure to fluorescent light and Giemsa stain exhibit a darkly stained chromatid (unifilary BrdU-substituted) and a lightly stained chromatid (bifilary BrdU-substituted). SCEs appear as dark and light reciprocal switch points (Fig. 1 *A*, *B*). This system can also be used to study chromosomal aberrations with appropriate culture duration (Fig. 1 *C*) and to compare the in vivo and partial in vivo cytogenetic studies in mice. Further, this system can be used to analyze cytotoxicity of chemicals both in partial in vivo and in vitro studies using BrdU-cell cycle technique (9). The procedures used for harvesting of cultures, staining of slides, and analyses of

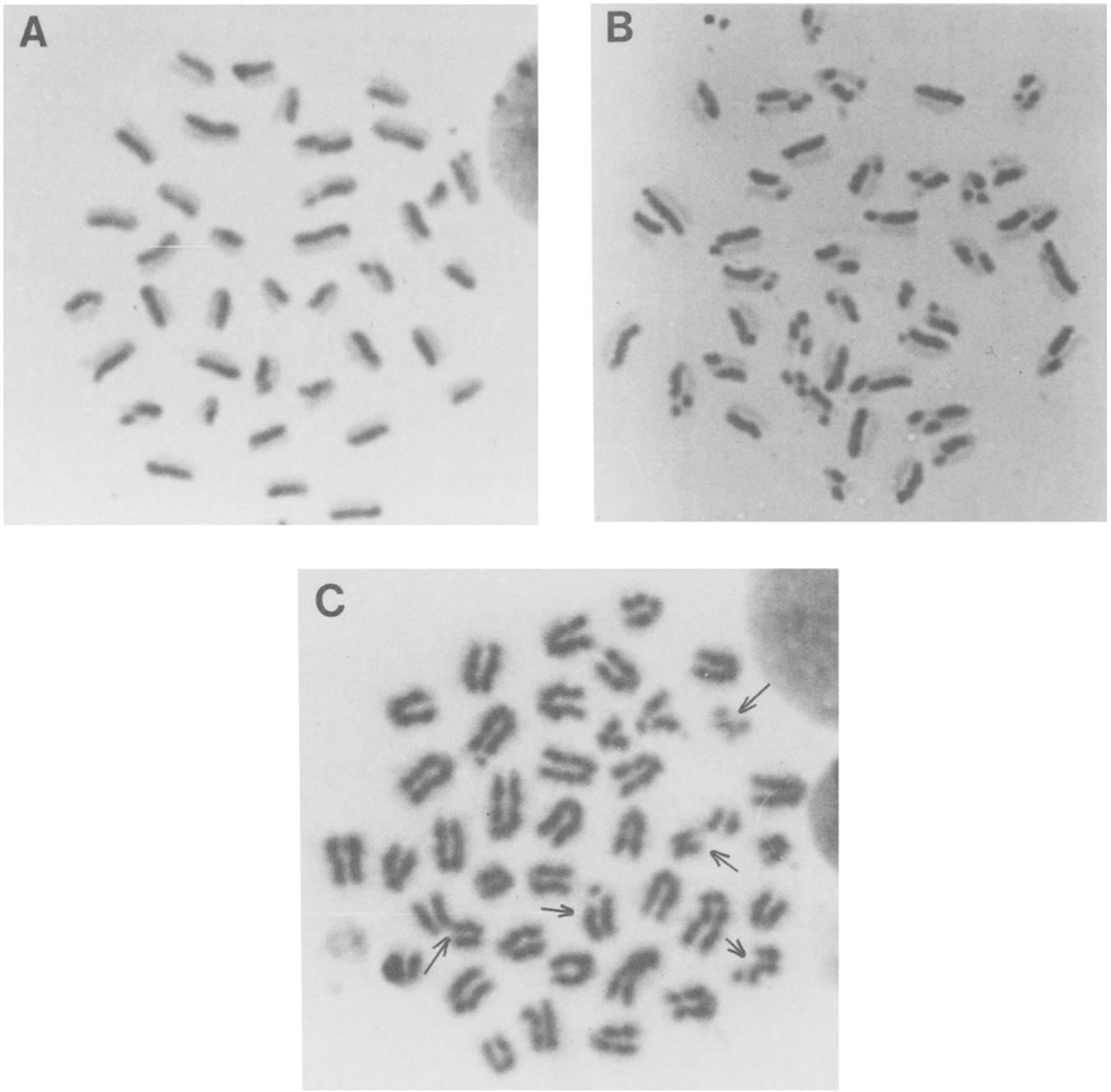


FIG. 1. *A*. Sister chromatid differentiation and spontaneous SCEs in bone marrow cell after BrdU incorporation in culture. *B*. SCEs induced in bone marrow cell after animal exposure to cyclophosphamide (20 mg/kg) and then cell culturing. *C*. Bone marrow cell with nondifferentiated chromatids for chromosomal aberration analysis. *Arrows* indicate chromatid break.

SCEs and chromosomal aberrations are according to earlier reports (3,10,11) with slight modification.

The reported procedure has been validated in our laboratory for both partial in vivo and in vitro conditions with different genotoxic agents (12).

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