

Preparation of Human Leukocyte Cultures for Chromosome Analysis

1. Preparation of culture medium
 - a. Use medium 199 or RPMI 1640 + 20% FCS, 50 ug/ml Gentamicin
 - b. Add 0.4 λ undiluted PHA-P/ml medium or 2 λ per ml medium of PHA-P diluted 1:5 in medium 199.
2. Preparation of culture
 - a. Draw 15 ml blood and defibrinate with glass beads in sterile container.
 - b. Dilute defibrinated blood 1:2 with saline or BSS.
 - c. Slowly layer 20 ml diluted blood over 10 ml of ficoll isopaque gradient (24 parts 9% ficoll: 10 parts 33.9% Isopaque in dist. H₂O)
 - d. Centrifuge at 1200 rpm 35 min. room temp.
 - e. Draw off white cell layer with bent tip pasteur pipet.
 - f. Dilute aspirant 3X volume with BSS.
 - g. Centrifuge 1200 rpm for 15 minutes.
 - h. Resuspend in medium, count, dilute to 106 lymphs/ml.
3. Incubation of cultures

Incubate @ 37°C for 3 days. culture cells + PHA at .25 x 10⁶ cells per micro-culture plate well.
4. Harvesting of cultures
 - a. For 3 day cultures. 4 hours prior to sacrificing add Colcemid (Gibco stock solution at 10 ug-/cc) to give a final concentration of 0.6 ug/cc. Shake plate to mix and reincubate for 4 hours.

For 4 day cultures with overnight exposure of Colcemid. At end of third day, add Colcemid to give a final concentration of 0.2 ug/cc. Shake to mix Colcemid, reincubate for 16-20 hours.
 - b. After Colcemid treatment, aspirate cultures into 2 cc conical (siliconized) centrifuge tubes. Wash wells 2X with .2 cc PBS.
 - c. Centrifuge (speed 7 for 3 minutes) Discard supernatant.
 - d. Add 1/2 cc of 37°C warmed 1.0% NaCitrate or a solution of .45% KCl + .45% NaCitrate, or a solution of 1 part medium to-3 parts dist. H₂O.

Add hypotonic slowly with shaking, then pipet vigorously.
Add an additional 1/2 cc hypotonic and incubate @ 37°C for 10 minutes.
 - e. Add .4 cc of freshly prepared fixative and resuspend the cells. Fixative is 3 parts absolute Methanol; 1 part Glacial Acetic Acid.
 - f. Centrifuge at speed 7 for 3 minutes. Discard supernatant.
 - g. Add 2 ml fixative, let cells soak in pellet for 5 minutes.
 - h. Resuspend with pipet.
 - i. Centrifuge at speed 7 for 3 minutes. Discard supernatant.
 - j. Add fresh fixative and resuspend, let stand 5 min at room temp.
 - k. Repeat steps i and j again.
 - l. Resuspend in .2 ml of fixative.
5. Preparation of slides
 - a. From a height of approx. 1-2 ft., drop a small drop of cell suspension on a clean slide and air dry.
6. Staining of slides
 - a. Giemsa stain for 10 min., rinse in dist. H₂O, air dry.
 - b. 2% aceto-orcein stain (2 drops per slide), place coverslip on slide, invert slide & press between filter papers to squeeze out stain & air bubbles. Seal coverslip with nail polish.