**UW Medicine - Pathology**

400-02-01-03

Peripheral Blood Cultures (PHA-Stimulated)

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| Adopted Date: 08/91  Review Date: 06/09  Revision Date: 08/12 |

PURPOSE

To detect constitutional chromosome abnormalities present in peripheral blood. Major indications include: multiple congenital abnormalities (MCA), developmental delay, mental retardation, specific chromosome syndromes, delayed puberty, multiple miscarriages (couple), history of chromosome abnormality, and abnormalities of sexual development.

PROCEDURE

### Specimen Requirements

* + - 1. **Adults and Children** (one month and older): 2-10 ml of venous blood into a syringe or vacutainer containing sodium heparin.
      2. **Newborn** (to about one month old): 1-2 ml of peripheral blood or cord blood in a syringe or vacutainer containing sodium heparin.

### Materials and Equipment

* + - 1. Dry 5% CO2 incubator
      2. Laminar flow hood [Biosafety Hood (BioGard)]
      3. Fume hood
      4. Centrifuge
      5. 15 ml centrifuge tubes (Falcon # 352095).
      6. Sterile pipettes.
      7. Corning T25 Flasks (430639, with vent cap)
      8. Hanabii PII Harvester

### Reagents and Solutions

* + - 1. Lymphocyte Growth Media for Peripheral Blood Cultures (complete RPMI 1640 with 20% Fetal Bovine Serum) 
         1. RPMI 1640 (Sigma R8758 with L-Glutamine) 100 ml
         2. Fetal Bovine Serum (Hyclone ALG14153) 20 ml
         3. Phytohemagglutinin (Murex 30852701) 3 ml
         4. Pen/Strep solution (Sigma P0781) 0.3 ml

***Note:*** One small aliquot of each bottle must be incubated at 37°C for at least 24 hr before using the media to make sure the media is not contaminated. Whenever possible, overlap new media lots between different cultures in a case to verify that a new lot can support growth. Record media lot in media preparation book. Media shelf life is about one month when stored at 4°C.

* + - 1. Colcemid 10 µg/ml (Gibco # 15210-040)
      2. PHA (Murex 30852701) HA-15 (once in solution good for 1 month)

Dilute Stock PHA with 5ml dH2O

* + - 1. 0.075 M KCl hypotonic solution (stored in incubator at 37°C, good for 2 weeks).
      2. 0.4% KCl hypotonic solution for thymidine culture (2g KCl in 500ml H20) good for 1 month at 4°C
      3. 3:1 methanol:glacial acetic acid ‑ made fresh and kept at room temperature.
      4. Actinomycin D (AMD) diluted to 100 µg/ml (Sigma − 5mg-A1410)
         1. Reconstitute with 1 ml H2O (to get approximately 5000 µg/ml).
         2. Remove 1.0 ml and dilute with 49 ml dH2O) (to get 100 µg/ml).
         3. Aliquot 5 ml each into 15 ml tubes and label.
         4. Store in freezer in AMD container. Good for 6 mo.

***Note:*** Actinomycin D is very sensitive to ultraviolet and fluorescent lights. It should be mixed, distributed and stored in total or semi-dark conditions. AMD is a carcinogen. Wear gloves.

* + - 1. Hanks’ Balanced Salt Solution (HBSS) (Gibco 14170-120)
      2. Sodium Heparin, 5000 USP units/ml (0.05 ml per 10 ml culture)

### Procedure: Culture / Harvest

* + - 1. **Log in:** Log in the blood logbook and GCS: Use PB/last two digits of year/consecutive number, 4-digit accession number, e.g., **PB10-0052**. Write case number and diagnosis on the white board. Blood can sit at room temperature for 2-3 days before being set up if immediate setup is not possible. Note indication to be sure of proper set up. If an indication for a specimen is not among the usual indications for this type of specimen or if an indication is ambiguous or unclear, the technologist must call or have front office staff call the sending physician to clarify before setup.

If CGH array is also ordered, create an MC number and follow procedure for CGH microarray set up and forward sample to array lab for DNA extraction.

* + - 1. **Culture Set up**

***Note:* ONE SAMPLE IN THE BIOSAFETY HOOD (B**i**oGard) AT A TIME** to avoid labeling errors.

**No mouth pipetting. Adhere to sterile techniques**. Use tissue culture hood [Biosafety Hood (BioGard)]. Wear gloves and lab coats. Use multiple incubators when possible. All glassware in contact with blood must be autoclaved after use

Labels generated from GCS must be double checked with labels on the specimen containers.

If the blood specimen is received in EDTA (purple top), centrifuge the whole blood. Discard the supernatant. Add 5-10 ml of media and spin again. Make the final volume same as initial specimen.

* + - * 1. Routine blood setup includes three 10ml cultures: A-72hr, B-72hr with Thymidine addition and C-72hr Thymidine. See table below for STAT set up or other non-routine specimens.
        2. Place label on all 3 culture vessels, T25 flasks or if limited specimen use a T12 flask.
        3. Pipet 9ml of RPMI into T25 flasks (10ml cultures) or 4.5ml in T12 flasks (5ml cultures)
        4. Add 1 ml heparinized whole blood per 10 ml culture or 0.5ml blood to 5ml cultures.
        5. Place in 37°C/5% CO2 incubator for 72 hrs (for non-routine, see table below)-use two incubators, culture A in one and cultures B and C in another
      1. **Routine Harvests:** Harvest routine samples after 72hrs of incubation. Use HANBI p-II robotic harvester (**400-07-01-06)** for A cultures whenever possible. B and C cultures require manual harvesting.
         1. Add 100 ul of colcemid stock (Gibco 10 µg/ml; final conc 0.1 µg/ml) to 10ml cultures, 50 ul to 5ml cultures and mix well. Transfer from flasks to 15ml tubes
         2. Return tubes to incubator for 30 min
         3. Place tubes into the harvester (see **Harvesting with Hanabi p-II: procedure 400-07-01-06) If performing a Manual Harvest for Routine continue to next step (d) For Thymidine cultures, see “E” below**
         4. Add pre-warmed (37°C) hypotonic solution (0.075 M KCl) to a final volume 12.0 ml. Cap tube, invert to mix thoroughly, and place in 37°C incubator for 12 min.
         5. Add 15 drops of fresh fixative 3:1 methanol:acetic acid to the hypotonic, invert the tube to mix thoroughly, and spin at 1200 rpm for 8 min. (Remove the supernatant hypotonic solution with a Pasteur pipette).
         6. Re-suspend cells thoroughly by vortex or agitation, then add 8 ml of fresh fixative (3:1 methanol:acetic acid) and mix well. Cap and let stand for a minimum of 30 min.
         7. Wash the cells three times, each time adding less fixative:

first wash - 10 ml

second wash – 8 ml

third wash – 5 ml

All fixative treatments are done at room temperature. Fixed cells are stored in freezer (for years) or refrigerator for a maximum of a week.

***Note:*** If newborn PB specimen received on Thursday, set up 48hr routine (A) culture & 48hr thymidine (B) & 72 hr C THY, keep in refrigerator overnight and transfer flask to incubator on Friday morning. If adult PB specimen received on Thursday, set up on Friday or setup Thursday and put in refrigerator overnight and transfer to incubator on Friday morning.

4. **STAT Harvesting**: After 24 hours, harvest STAT A cultures. After 48 hr harvest STAT B back-up cultures.

a. add colcemid for 60 min for STAT A culture

NOTE: only set up a 24 hour culture if absolutely necessary or requested by physician. Check with Director or Supervisor if unsure.

**Peripheral Blood Set-Up Summary Sheet**

|  |  |  |  |  |  |  |  |  |  |
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| **STAT - Set up A (24 hr), B (48 hr), C+thy (72 hr)/Prometaphase** | | | | | | | | (15 ml tubes) | |
| Amount of specimen | | **2.5 ml or more ↓** | | | **1.5 ml ↓ blood** | | | **1.0 ml ↓ Blood** | |
| **A—24 hr**  **1hr colcemid** | | A−4.5 ml RPMI 1640 + 0.5 ml blood | | | A−4.5 ml RPMI 1640 + 0.5 ml blood | | | A−4.5 ml RPMI 1640 + 0.5 ml blood | |
| B—48 hr | | B−9 ml RPMI 1640 + 1 ml blood | | | B−4.5 ml RPMI 1640 + 0.5 ml blood | | | B−No 48 hr culture | |
| C—72 hr (Thy) | | C−9 ml RPMI 1640 + 1 ml blood  Or 5ml culture + 0.5ml blood | | | C−4.5 ml RPMI 1640 + 0.5 ml blood | | | C−4.5 ml RPMI 1640 + 0.5 ml blood | |
| **Routine Baby (16 months or younger)/Prometaphase** | | | | | | | | **Important: 1) Clotted cases check with IM/BG** | |
| Amount of specimen | | **2.5 ml or more ↓** | | | | **1.5 ml ↓** | | **2) STAT cases check with IM/BG** | |
| A—48 hr | | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | 4.5 ml RPMI 1640 media + .5 ml blood | | \*If not enough specimen received, set up A and B only | |
| B—48 hr (Thy) | | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | NONE | | Add 0.3 ml of 1% thymidine after 24 hr | |
| C—72 hr (Thy) | | 9 ml RPMI 1640 (C) media + 1 ml blood | | | | T25 flask | | Add 0.4 ml of 1% thymidine after 48 hr | |
| **Routine (Patient >16 months)/Prometaphase** | | | | | | | |  | |
| A—72 hr | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | | T25 flask | |  | |
| B—72 hr (Thy) | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | | T25 flask | | Add 0.3 ml of 1% thymidine after 48 hr | |
| C—72 hr (Thy) | 9 ml RPMI 1640 (C) media + 1 ml blood | | | | | T25 flask | | Add 0.4 ml of 1% thymidine after 48 hr | |
| **FISH only or Family Follow-up to Amnio and Research cases** | | | | | | | | | |
| A—72 hr | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | | T25 flask | |  | |
| B—72 hr (Thy) | 9 ml RPMI 1640 (A+B) media + 1 ml blood | | | | | T25 flask | | Add 0.3 ml of 1% thymidine after 48 hr | |
| **Fanconi's Anemia Breakage Study (Congenital Aplastic Anemia)** | | | | | | | | | Add: | |
| A—72 hr | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube | | Nothing | |
| B—72 hr | | | | 9 ml RPMI + 1 ml blood | | | T25 flask | | 0.3 ml of 1% thymidine **after 48 hr** | |
| E—72 hr | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube (foil wrapped) | | 12.5 l-0.1 g/ml DEB **after 24 hr** | |
| F—96 hr (4 day) | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube (foil wrapped) | | 12.5 l-20 ng/ml mitomycin C\*\* | |
| G—96 hr (4 day) | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube (foil wrapped) | | 25 l-40 ng/ml mitomycin C\*\* | |
| **Control (same age and sex)** | | | | | | | | | Note: \*\*Add when set up specimen. | |
| E—72 hr | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube (foil wrapped) | | 12.5 l-0.1g/ml DEB after 24 hr | |
| G—96 hr (4 day) | | | | 4.5 ml RPMI + 0.5 ml blood | | | 15 ml tube (foil wrapped) | | 25 l-40 ng/ml mitomycin C | |
| **X-Inactivation/BrdU Study** (set up A, C and D. No B culture for X-inactivation only) | | | | | | | | | | |
| 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** | |
| **Array ― PB specimen (Routine + FISH)** | | | | | | | | | | |
| A1―72hrs | | | 4.5ml RPMI + 0.5ml blood | | | | | | T25 Flask | |
| A2―72hrs | | | 4.5ml RPMI + 0.5ml blood | | | | | | T25 Flask | |

### High Resolution Chromosomes Using Excess Thymidine (Prometaphase)

Chromosome elongation techniques produce a higher proportion of cells in prometaphase. The use of this procedure has led to the identification of a number of chromosome abnormalities characterized by small deletions, known as microdeletion syndromes, duplication, inversion, and translocation. This protocol synchronizes dividing cells by blocking the cells in S phase using excess thymidine. This blocks the process of cell division by interrupting a biosynthetic pathway. After 17 hrs, the cells are released from the block and harvested at different elapsed times to catch cells at the prometaphase state.

* + - 1. **Solutions**
         1. Lymphocyte Growth Media (Sigma RPMI 1640, same as for routine Peripheral Blood Cultures above)
         2. Thymidine solution, 1%

Thymidine 1g

Deionized H2O 100ml

Aliquot 10 ml of solution into centrifuge tubes. Store at –20°C for 1 year.

* + - * 1. Hank’s Balanced Salts Solution (HBSS), 1X
        2. Colcemid, 10 µg/ml
      1. **Thymidine Culture Initiation**

**On cultures B and C (see above for initial set up process)**

* + - * 1. **After 48 hr** of incubation, add 0.3 ml of 1% thymidine solution to the **B** culture and 0.4ml to the **C** culture. Addition as late in the day as possible is optimum, time being 3:00 pm. Document the time of thymidine addition on the Harvest Log, including date and technologist.
        2. Return the culture to the incubator for **an additional 17-24 hr**.
      1. **Thymidine Culture Harvesting** 
         1. Transfer the **B and C** culture contents to a 15 ml centrifuge tube, along with the culture label, and centrifuge at 1000 rpm for 8-10 min.
         2. Discard the supernatant and suspend the cell pellet in 10 ml of HBSS. Centrifuge at 1000 rpm for 8 min.

***Note:*** This rinsing step will release the block caused by the excess thymidine.

* + - * 1. Repeat the rinsing of the cell pellet with HBSS one time.
        2. After the last centrifugation, discard the supernatant and suspend the cell pellet in 9 ml of pre-warmed Complete RPMI 1640 Lymphocyte Growth Medium. Incubate an additional 3 hr 30 min to allow the cell cycle to continue.
        3. After 3hrs and 30mins add 0.1 ml AMD (100µg/ml) for 30 min at 37°C.
        4. Add 0.2 ml Colcemid for an additional 10 min at 37°C. Document the time of Colcemid addition on the Harvest Log.
        5. Centrifuge and re-suspend pellet by adding 10-12ml 0.04% KCl (hypotonic solution). Mix pellet well in hypotonic solution and return culture tubes to 37°C incubator for 30 min.
        6. Add 1.0ml of fixative (prefix)
        7. Centrifuge for 10 minutes and aspirate supernatant
        8. After hypotonic treatment and fixation steps of the Routine Harvesting Procedure for Lymphocyte Cultures.

***Note****:* The hypotonic treatment may be slightly increased to facilitate spreading of chromosomes and to increase the number of usable metaphases.

***\*Note:*** The Hanabi P-II robotic harvester can substitute for these manual harvest methods if there are no clots in the culture. Hanabi P-II is not used for Thymidine harvests. See Hanabi P-II Operation instructions.

### Slide Preparation

* + - 1. Clean slides in 95% EtOH.
      2. Set the temperature at 31-37°C and at 30% humidity for slide making in Thermotron. Settings will vary based on daily conditions, adjust as necessary to achieve optimal metaphase quality
      3. Scan culture tube barcode and print slide labels (refer to labeling procedure)
      4. Drop 2 drops cell suspension onto clean and wet slide at a slight angle. Wipe back and sides of slide with a paper towel
      5. Examine slides on low power (10 X) phase contrast and choose the slide giving best metaphase spreads, make additional slides as needed from that culture.
      6. Bake slides at 90oC for 1 hr before staining. Bake FISH slides for 20 minutes at 90oC.
      7. Band slides for G-banding. Use 45 seconds in Trypsin and 50 Seconds in Wrights stain. (note: trypsin and stain time vary)

***Notes:***

1. Water clearing method. For cultures which are (due to insufficient hypotonic treatment) so gelatinous after repeated washes in fixative that chromosome spreading is seriously hampered. This procedure gives a clean, white pellet and has no deleterious effect on chromosome morphology or banding.
   1. Suspend thoroughly fixed (2 hr or more) cells in 6 ml of fresh fixative.
   2. Add 2 ml distilled H2O and mix very thoroughly on vortex.
      1. Immediately spin down the cells and quickly resuspend cleaned pellet in 6 ml of fresh fixative.
      2. Wash once more with fixative and prepare slides.

***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