**UW Medicine - Pathology**

400-02-01-01

Amniotic Fluid Cultures

|  |
| --- |
| Adopted Date: 09/04/91  Review Date: 06/12/09  Revision Date: 08/01/12 |

PURPOSE

This procedure allows for prenatal cytogenetic diagnosis. Major indications for this test are advanced maternal age, elevated prenatal risk profile (PRP), abnormal ultrasound (including intrauterine growth retardation, IUGR), or listing of previous cytogenetic abnormality. Optimally, samples are obtained between 15-18 weeks gestational age. The goal for a final diagnosis is 7-12 days.

PROCEDURE

### Specimen Requirements

For Cytogenetics analysis, 15-30 ml of amniotic fluid obtained under sterile conditions in Corning tissue culture tubes.

For CGH Array, 15-20 ml of amniotic fluid or 2 T-25 flasks 100% confluent.

### Materials and Equipment

* + - 1. Sterile 100 x 15 mm square Petri dish
      2. T12.5 cm2 flasks (Falcon #35-3107, vented cap)
      3. Plastek coverslip kits (Martek #CSGK/F)
      4. Centrifuge
      5. 5% CO2 wet incubator at 37°C.
      6. Laminar flow hood [Biosafety Hood (BioGard)]
      7. Inverted microscope
      8. Thermotron
      9. 15-ml Conical centrifuge tubes with blue caps (Falcon #352095).
      10. Sterile pipettes
      11. MultiPrep Genie Robotic Harvester

### Reagents and Solutions

1. Growth medium:
   * Amniomax C-100 medium (Gibco Cat. #17001-082)
   * 90-m1 bottle supplemented by 15 m1 frozen portion (Gibco Cat. #17002-080), store refrigerated for no more than a week.
     1. Colcemid stock (10 µg/ml). (Gibco Cat. #15210-040)

For working solution: dilute in sterile H20 to 10%.

* + 1. In situ hypotonic solution: 80% of 0.8% sodium citrate + 20% 0.075 M KCl diluted in dH2O, kept at room temperature for 2 mo. (3.2g sodium citrate/400ml dH20 + 0.56g KCl/100ml dH2O)
    2. Carnoy's fixative (3 parts absolute methanol; 1 part glacial acetic acid), made fresh, kept at room temperature.
    3. Versene (Gibco Cat. #15040-066).
    4. Trypsin stock: Trypsin - EDTA 10X (Invitrogen #15400054) aliquotted and frozen in 10 ml aliquots. Working solution 10 ml stock + 90 ml sterile PBS (Gibco #14190-136). Keep 3 mo at 37°C.
    5. Mass culture hypotonic solution:

300 ml 0.4% KCl + 100 ml 0.4% Na citrate, keep at room temperature for 2 mo.

* + 1. BrdU working solution: 0.03 g BrdU (Sigma #B5002) in 10 ml HBSS (Gibco #14170-120). Aliquot in 1 ml lots and store in dark at -20°C for up to one year. Thaw just before use.
    2. Cytoseal 60 (Richard-Allan Scientific #8310-4)
    3. Sterile H2O (Baxter #2F7114)

### Procedure

### Day 0 Set-Up:

* + - 1. Make sure patient name and ID number match on all tubes and paperwork. If Cytogenetics was done previously on the family, office personnel will retrieve relevant files.
      2. Note any special studies ordered and forward specimen, if necessary.
      3. Is IFISH requested?
      4. Log information into log book, on culture sheet, into computer and on board.
    1. **Day 2**:
       1. Flood using 2-2.5ml of media
    2. **Day 4-6**:
       1. Feed (change the media) between 4 and 6 days using 2ml of media
    3. **Day 6-11**:
       1. Coverslip harvest done after 6-8 days or when ready.
       2. Flask harvest done after 9-11 days or when ready.

1. **Day 10-14**:
   1. Report of no growth when necessary

### Handling of Incoming Amniotic Fluid Specimens: Log In

1. Check the Cytogenetics Request form accompanying the specimen for accuracy and completeness. Check the specimen for sample collection container condition, color, quantity, proper labels, etc. If something is missing or specimen is not in good condition, (i.e., came in Monoject syringe, which is toxic, instead of BD syringe) notify supervisor immediately.
2. Look for indication. If special studies other than cytogenetics are requested make necessary arrangements for proper handling and distribution of material: biochemistry test for CHRMC or other facility, alpha fetoprotein test for Lab Medicine (see below) and CGH array, for example.
3. Log in: When a sample is received, it is logged in GCS and in the Amniotic Fluid logbook and an accession number is attributed: AF/last two digits of year/ consecutive 4-digit accession number, e.g., **AF04-0052**. All vessels are labeled with this number and the patient’s first initial and first three letters of last name (e.g., Jane Smith = **JSMI**) using a VWR alcohol-proof marking pen. Log the case in to GCS, write on the board and on worksheet. Look for indication. If previous cytogenetic studies were done on the patient or family, obtain previous reports or files. Record the quality of the sample (color, clarity, volume) in computer under specimen information.

**Specimens for IFISH:** See procedure for IFISH on uncultured amniocytes, SOP number 400-04-01-02.

**Specimens for AFP** are ***sometimes*** sent to us along with the fluid samples for Cytogenetics (packaged separately or together). The AFP specimens should be forwarded to Lab Medicine: Room NW-220 Community Services / Specimen Processing. For specimens from clinics outside of UWMC, save 0.5 ml of fluid, and refrigerate.

**Specimens for Biochemistry** for CHRMC or other facilities are sent parallel to ours most of the time. When that is not the case, cells are grown here along with our cultures and the material is shared between Cytogenetics and appropriate labs. If there is very little material, it should be used primarily for the most important test (to be discussed with physician).

**Specimens for array CGH** reference specimen requirements for array testing. If there is a question about priority, have office call physician or discuss with Cytogenetics Director.

### Culture of Cells

***Note***: No mouth pipetting. Adhere to sterile techniques. Use tissue culture hood [Biosafety Hood (BioGard)]. Use gloves. Use Multiple incubators to grow flasks and coverslips. Every effort should be made to avoid mislabeling of samples including ensuring that only one specimen is set up at a time (**ONE SAMPLE IN THE BIOSAFETY (BioGard) HOOD AT A TIME**).

***Note****:* Media used to set up cases has to be completed no less than 24 hr in advance. 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 case log book.

***Note:*** 5-6 coverslips should be set up. If there is a limited amount of specimen then 4 coverslips at minimum should be set up in addition to a backup flask.

1. **Day 0:**
   1. Prepare culture vessels: each case is set up on 4-6 coverslip kits and in one T-12.5 cm2 vented flask. Always use two different lots of media for each case (including different additives) and label bottles A and B.

***IMPORTANT:*** Before labeling coverslips and flask, check to make sure the patient name and accession number on the tubes matches the paper work and the information in log book.

* 1. Label Petri dishes (Martek kits) with accession number (AF/last two digits of year / dash / consecutive number e.g. **AF04-52**) and the patient’s first initial and first three letters of last name (e.g., Jane Smith = **JSMI**) using a VWR alcohol-resistant marking pen. Place dishes in larger, square Petri dish. These square dishes can then be put into 3-tier plexiglass towers.
  2. Complete culture sheet and label the request sheet(s), label tubes with case #. Log information in logbook including patient's name on the section where the patient's label will later be placed.
  3. Spin fluid in the specimen tubes (either 15 ml Corning tissue culture or 15ml polypropylene centrifuge tubes) for 8 min at 1000 rpm. ***(Note*: If AFP is requested, be sure to save 0.5 ml of unspun fluid, or supernatant after centrifuging, to separate tube.) If IFISH is ordered, save 5 ml of unspun fluid, and refrigerate, or harvest.** 
     1. Remove label from one tube and place in logbook next to corresponding case number. Complete entering information into logbook. Remove label from second tube and place on culture sheet. Remove label from third tube and place on back of culture sheet.

***IMPORTANT:*** Make sure that tubes from only one patient are in the setup hood at one time. Check that all patient information matches on all tubes and paperwork.

* + 1. After remaining fluid is spun, set up amniotic fluid as follows:

**For Two Specimen Tubes**

* + - 1. For Tube 1: Carefully remove almost all supernatant and re-suspend pellet with 1.0 ml type A media. Divide suspension equally between coverslips 1, 2 and 3. If it spills over the edge of the coverslip, immediately transfer to a new coverslip and add 0.3 ml media.
      2. For Tube 2: Carefully remove almost all supernatant and re-suspend pellet with 1.0 ml type B media. Divide suspension equally between coverslips 4, 5 and 6(optional). If it spills over the edge of the coverslip, immediately transfer to a new coverslip and add 0.3 ml media.
      3. Rinse specimen tubes with 3-5 ml of B media and place in labeled T-12.5 flask.

**For Three Specimen Tubes**

***Note:*** The tube with the smallest volume should be considered “tube 3”.

1. For Tube 1: Carefully remove almost all supernatant and re-suspend pellet with type A media to make a 1.5 ml total volume. Divide suspension between coverslips 1, 2 and 3, adding 0.5 ml to each coverslip. If it spills over the edge of the coverslip, immediately transfer to a new coverslip and add 0.3 ml media.
2. For Tube 2: Carefully remove almost all supernatant and re-suspend pellet in 1.5ml type B media to make a volume of 1.5 ml. Divide suspension between coverslips 4, 5 and 6 (optional) adding 0.5 ml to each coverslip.
3. For Tube 3: Remove supernatant and re-suspend pellet in 5ml of B media. Place cells in labeled T12 flask.
   1. ***Important:* After completing setup of each case, double check that the patient code and accession number on the coverslips and flasks matches the paperwork, tubes, and logbook.**
   2. Carefully place dishes and flask in appropriate incubators.
   3. Prepare folder and write case on the board and place folder in appropriate location in the office so billing and charges can to be entered.
      1. **Day 2:**

Flood coverslips with 2.5 ml media.

* + 1. **Days 4-5:**

Check for growth and feed with two different lots of media for each case. Note lack of growth on the culture sheet.

* + - 1. **Days 6-8** (or later if growth is inadequate, earlier if rush case):

1st harvest (see Harvest slides #1 below); always harvest only part of the case at a time. Feed the rest of the culture.

* + - 1. **Days 8-9:**

2nd harvest (see Harvest slides #1 below).

* + - 1. **Day 10:**
         * Feed Flasks
         * Report growth status on “growth status” forms for slow or no growth samples and give form to supervisor.

**Days 9-11:**

Harvest the flasks: (See Harvest flasks #2 below).

**Days 12-14:**

Report again no growth (if persistent) to the person signing out so the referring physician can be notified.

***Note****:* Should the case be kept in culture for some time after harvests (for special studies or for freezing cells), cultures should be fed every 3 days.

### Harvest Multi-Prep Genie Robotic Culture Harvester “Rainbow”

***Important:***

* + - 1. Do not harvest more than 2 coverslips on a case at the same time, do 2 harvests if necessary.
      2. When an array is performed on an AF case the AF flask should be harvested and pellet saved in case further testing is needed
      3. Routinely the flask is not harvested but should be kept for emergencies. (see “f.” below)

1. Multi-Prep Genie Robotic Culture Harvester

a. Switch on harvester by pressing the red rocker switch on the back

b. After the system has started up and found the “home” position, the main menu will be displayed. The main menu will show up when the vacuum pressure is ready. Now you can begin loading carousel plates.

c. Load Wash Stations with empty Petri dishes

d. Connect the hypotonic solution and fixative. Refer to the volume chart above Rainbow for the appropriate amounts of solutions based on number of coverslips ready for harvest

e. Check waste container, empty if necessary

f. On the main menu press 1 to un/load machine. Use arrow keys to rotate carousel. Load coverslips vertically on the carousels.

g. Once samples are loaded on machine close harvester doors and select #2- HARVEST on the console.

h. Enter desired protocol number followed by ENT button

i. Once the wash stations and sample dish locations have been detected, press ENT to continue. Be sure that the machine has detected the correct number of dishes.

j. if the harvester miscounts the samples, check to make sure all the dishes are level and repeat step h.

k. once complete, harvester will sound an alarm. Remove dishes and continue with the drying process.

***Note: In the event that Rainbow malfunctions, make a note of the current step by looking at the control console. After the current step is noted, turn machine off by using the power switch on the back of the machine. Remove dishes and continue with harvest. Write down the error on the error log sheet being sure to note the date and step of error.***

***Note:*** Use the Biosafety Hood (BioGard) for steps a and b and fume hood for steps c and d.

2. Manual coverslip harvest by in situ method as follows when clones are 0.5 - 1.5 mm diameter (6-8 days usually) showing mitotic activity:

* 1. Add 0.8 ml of 10% colcemid stock in H2O to a 1 ml aliquot of BrdU. Keep refrigerated until used up.
  2. Add 50 µl BrdU/colcemid solution to each coverslip late in day prior to harvest.
  3. The following morning remove the medium by aspiration. Add 2 ml of hypotonic solution 80% Na citrate / 20% KCl (see reagents, D.3) at 37°C for 19-25 min at room temperature. Note that hypo times can vary greatly depending on sample condition, temperature and humidity levels.
  4. Add slowly and drop-wise 0.5 ml of fixative (methanol:acetic acid, 3:1). Let sit 2 to 5 min at room temperature.
  5. Remove the fixative/hypotonic mixture by aspiration. Add 3 m1 of fixative. Let sit 15 min. Change fixative two more times for 10 min each. Remove fixative with suction. Dry coverslips in the Thermotron set at 31°C and 30% relative humidity. Remove coverslips from dishes and mount on labeled frosted glass slide using Cytoseal 60. Bake for 60 min in 95°C oven before banding.
  6. Emergency backup: The flask is kept in the event of inadequate number of clones for case analysis and sign out. If there is determined to be an inadequate number of clones for sign out, the flask is checked for harvest readiness.
     1. **Harvest flasks**
  7. Add 100ul of colcemid (stock 10 µg/ml) to the flask for 30 min at 37°C.
  8. Collect the media in a 15 ml conical centrifuge tube. Rinse flask 2 times with versene at 37°C (3 ml each) and collect the rinses in the same centrifuge tube. Add 1 ml of trypsin-EDTA at 37°C to the flask. When cells are detached (check in microscope and tap/smack bottom of flask on counter), transfer cells in trypsin to the same centrifuge tube. Make sure to leave a small amount of cells in the flask incase more cells needed. Spin tube at approximately 1000 rpm 8 min. Put 5 ml media back into flask and maintain until two weeks after case number is erased from board.
  9. Remove supernatant and flick tube to re-suspend the pellet. Add 8 ml of hypotonic (at 37°C) for 22 min. Add a few drops of fixative (methanol:acetic acid, 3:1) to the tube and mix. Spin 8 min at 1000 rpm.
  10. Remove the supernatant and flick the tube gently to re-suspend the pellet. Slowly add 6 ml of fixative and let it sit at room temperature for a minimum of 10 min.
  11. Centrifuge cells. Remove supernatant as close to the cell pellet as possible and add fresh fixative drop by drop until appropriate cell concentration is reached. Gently pipette to re-suspend and make slides.
  12. Bake slides for 60 min in 95°C oven before banding.

**Special considerations for aCGH**

***NOTE:* When an array is performed on an AF case the AF flask should be harvested and pellet saved in case further testing is needed**

***Note:* For high risk patients, we should receive 40ml of fluid. The Genetic Counselors will alert the lab when a case is considered high risk.**

***Array lab needs two fully confluent T25 flasks OR 3 70% confluent T25 flasks***

1. **Setup**
   1. Follow above set up procedure BUT harvest one coverslip as soon as possible to check for abnormalities.
   2. Set up two flasks:
      1. Use cells from one full tube for a flask—this will be for the CGH array if the karyotype is normal
      2. Second flask will be used as the back-up flask (same as protocol above)
   3. Expand flask cultures (see above) when ready. When two fully confluent flasks (or 3 70% flasks) are ready, trypsinize cells and spin to form a cell pellet. Wash once with sterile DPBS and spin again. Give spun, pelleted cells to the Cytogenetics array team (follow above procedure for trypsinizing flasks).

***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**.**

REFERENCE

1. Hoehn H, et al. Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. I. Clonal morphology and growth potential. *Pediat Res* 1974; 8:746‑754.
2. Hoehn H, et al. Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. II. Cytogenetic parameters as functions of clonal type and preparative techniques. *Clin. Genet.* 1975, 7:29-36.
3. Hoehn H, et al. Cultivated cells from diagnostic amniocentesis in second trimester pregnancies. III. The fetal urine as a potential source of clonable cells. *Humangenetik* 1975; 29:285‑290.
4. Schmid W. A technique for *in situ* karyotyping of primary amniotic fluid cell cultures. *Humangenetik* 1979; 30:325‑330.

Written By: Director Approval:

(Signature and Date) (Signature and Date)

­­­­­­­­­­­­­­­­­\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Cytogenetics Supervisor