**UW Medicine - Pathology**

400-02-01-05

Skin Fibroblast (ST) Cultures

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| Adopted Date: 08/09/91Review Date: 06/12/09, 5/19/11Revision Date: 03/16/07, 9/30/13 |

PURPOSE

To provide cytogenetic diagnoses on skin biopsies, stillborns and products of conception. Skin biopsies can help determine the presence of mosaicism.

PROCEDURE

### Specimen Requirements

* + - 1. Skin biopsy: 2-4 mm2 (approximately 2 mm x 2 mm) sample of skin collected sterile and placed in sterile vials with 1-3 ml of transport medium (\*see below). Separate vials needed if more than one biopsy site taken, Specimen should **NOT BE PUT** in formaldehyde, alcohol or saline.
			2. Abortus material (spontaneous abortion, fetus, placenta, etc.): A small amount of tissue in sterile vial(s) with 1-3 ml transport medium (\*see below). When tissues are identifiable, the type of specimen preferred is from the fetus such as skin, or organ (liver, kidney, etc). **DO NOT PUT** specimen in formaldehyde or alcohol. Advise not to ship entire fetus and to refrigerate if not shipped immediately.
			3. Autopsy: Skin and organ is preferred, each tissue should be in separate, sterile vials with transport medium (\*see below), and labeled accordingly. **DO NOT PUT** specimen in formaldehyde, alcohol or saline. Refrigerate if not shipped immediately.

\***Transport Medium**: 100 ml alpha MEM, 10 ml fetal calf serum, 2.7 ml of 1.0 M HEPES buffer, 1ml of penicillin-streptomycin-glutamine, 0.2ml normocin. We can supply transport medium in individual vials (be sure caps are tight; store in refrigerator, shelf life is 3mo at 4°C; do not freeze). The following media are acceptable alternatives if shipping time will not exceed 24 hr: lactated Ringer's solution, viral transport medium, or fetal bovine serum. **DO NOT USE** normal saline, 5% dextrose, or tissue culture medium buffered with bicarbonate.

### Materials and Equipment

* + - 1. 60-mm Petri dishes
			2. Sterile fine forceps (watchmaker's forceps or dissecting needles may be used)
			3. Flask (T25 cm2)
			4. Clean glass microscope slides
			5. 22-µg Millipore filter
			6. Disposable scalpels (Bard Parker Cat. #371610)
			7. Sterile pipettes: 1-ml, 5-ml & 10-ml and sterile Pasteur pipettes.
			8. Sterile 15-cc centrifuge tubes with caps (Falcon 2095 or equivalent)
			9. Standard centrifuge
			10. Syringes, 10-ml and 1-ml
			11. Inverted microscope
			12. Laminar flow hood [Biosafety Hood (BioGard)]
			13. 5% CO2 wet and dry incubator at 37°C
			14. Balance
			15. pH meter
			16. 25-cm2 sterile flasks
			17. 100X 15-mm sterile square plastic Petri dishes
			18. 50-ml centrifuge tubes
			19. Waterproof marking pen
			20. Hemocytometer
			21. Nalgene Filter chamber (150ml 0.2um)

### C. Reagents and Solutions

1. Alpha MEM Earle’s salts nucleosides (Irvine Scientific cat. #9144)
2. Amniomax C-100 (Gibco)
3. Fetal bovine serum
4. Penicillin-streptomycin-glutamine (Gibco Cat. #10378-016)
5. Trypsin (p.25% (Gibco Cat. #610-5050AG)
6. Dulbecco's phosphate-buffered saline (D-PBS) (Gibco Cat. #310-4190AG)
7. Collagenase pretreatment solution (Worthington Type 2) aliquots
8. Colcemid (Gibco Cat. #120-5210AD)
9. Carnoy's fixative (3 parts absolute methanol: 1 part glacial acetic acid)
10. Versene (Gibco Cat. #15040‑066)
11. Trypsin stock: 20 ml trypsin ‑ EDTA 10X (Gibco Cat. #610-5405) rehydrated with 20 ml autoclaved distilled H2O working solution 10 ml stock + 90 ml PBS. Good for 6 months at room temperature
12. Normocin (Invivogen Cat# ant-nr-0)
13. Fungin (for tissue wash)
14. 1 M HEPES buffer (Gibco Cat# 15630-080)
15. Solid Tissue Medium

***Note***: Prepare two different lots at a time, labeled A and B to use in different vessels for each case.

* 1. 100 ml alpha MEM (with Earle's salts and nucleosides) (Irvine Scientific Cat. #9144)
	2. 20 ml fetal calf serum
	3. 100 ml Amniomax C-100 (Gibco Cat. #17001-017) + Amniomax supplement (Cat. #17002-105)
	4. 2 ml Pen/Strep + L-glutamine (Gibco BRL Cat# 10378-016) (stock = 10,000 U/ml pen; 10,000 µg/ml strep; 20.2 g/ml glut)
	5. 0.4 ml Normocin

***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 case log book. Media shelf life is about 2 weeks when stored at 4°C.

1. Tissue Wash
	1. 100 ml PBS (or Hank's)
	2. 0.2 ml pen/strep
	3. 0.2 ml Normocin
	4. 0.4 ml Fungin (10 mg/ml stock)
	5. Shelf life = 1 mo @ room temperature, 2 mo @4°C
2. Hypotonic Solution
	* 1. 1.2 g potassium chloride (KCl)
		2. 0.4 g Na3C6 sodium citrate dihydrate (Na3C6H507•2H20) in 400 ml double distilled H20
		3. Shelf life is 6 months at room temperature

18. Collagenase pretreatment:

 Reconstitute 1 vial (100mg) collagenase in 100 ml Alpha MEM bottle. Mix well.

 Filter using a Nalgene filter chamber (150ml, .2µm).

 Aliquot 4ml into 15ml blue top tubes. Label with a 6 month expiration and put in freezer door rack. (Lab tech usually has these prepared)

### Set-Up Procedure

Set up should be done under sterile conditions, in laminar flow hood [Biosafety Hood (BioGard)]. Wear gloves. No mouth pipetting. 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**).

1. **Log in**: Upon receipt of specimen assign an ST/last two digits of year/ consecutive number, 4-digit accession number, e.g., **ST04-0052**. The sample should be set up as soon as possible, refrigerate if leaving overnight. Log in the ST log book, on the board, and in the GCS computer program. Look for indication. If previous cytogenetic studies were done on the patient or family, obtain previous reports or files.

***Note****:* In case of several specimens from the same patient, assign an independent case number for each specimen if the study is for mosaicism. If different specimens from the same patient are sent as back-ups, assign the same number as that assigned to the main specimen, followed by A, B, C, D....

***Note****:* If the specimen received is a product of conception, inspect the contents in a Petri dish under the dissecting microscope to look for chorionic villi. Separate some of the villi and set them up using the chorionic villi protocol (second of this section). Fix the remaining tissues in the enclosed fixative vial provided and take them with the Anatomic Pathology Request form to the gross room (#NW211, telephone #598-4286), if other studies are requested. Otherwise, dispose of the remaining biohazardous and hazardous material following the guidelines for disposal.

**E. Culture Set-Up**

* 1. Label 2-4 coverslips with case (accession) number and the patient’s first initial and first three letters of last name (e.g., Mary Jones = **MJON**) and the letter of the coverslip A-D. If there are multiple tissue types, only set up 2 coverslips per tissue type.
	2. Wash tissue 2X in sterile PBS with antibiotics (tissue wash). Cut or scrape away subcutaneous fat in first wash. Remove tissue to 60 mm dish.
	3. Cut specimen into 2 x 2 mm pieces with 2 scalpels. Put .5 ml collagenase solid tissue (1X) into each dish. Mince tissue with scalpel blades. Transfer to tube with collagenase.
	4. Incubate specimens in collagenase in 5% CO2, 37°C incubator, sit for 1-3 hr, checking after 30-40 minutes and mixing well to help dissolve tissue. Centrifuge for 10 min at 1000 rpm. Decant supernatant. Re-suspend in 5 ml of solid tissue medium. Plate 0.5 ml to each coverslip. If there are two tubes (different tissue types) divide the specimens into equal number of coverslips, not exceeding 6 for each case. Incubate specimens in 5% CO2, 37°C incubator.
	5. Place 0.5-1ml of tissue in a T12 or T25 flask to use as back up
	6. Pipet 4-4.5ml of media into flask an place in incubator
	7. After setup, leftover tissue should be disposed of in biohazard waste. Fetuses and large amounts of POC (>50 ml) should be given to autopsy to process. Call Autopsy at 598-4205.

 **Secondary Culture**

* + - 1. From coverslips cells may be transferred either to T25 flasks or T75 cm2 flasks, (Falcon or Corning) depending on necessity (freezing or other studies).
			2. Trypsinization is accomplished by washing culture in versene, adding 1 ml of trypsin/EDTA incubating at 37°C, watching the cells get rounded (about 5 min) and start to detach (do not overtrypsinize!) then inactivating trypsin with medium (16% FCS).
			3. Take the trypsinzied cells and transfer into a flask or onto other coverslips as in culture set up.

**I-FISH**

* + - * 1. If FISH for aneuploidy is requested, a direct harvest may be performed on some of the remaining specimen from set up. A small cell pellet, about 0.50cc after collagenase exposure is adequate to process for I-FISH. Also, if there is adequate cell attachment onto coverslips, the floating cells from coverslips may be taken off and used for I-FISH.
				2. Place specimen in 15ml centrifuge tube.
				3. Add 5 ml of hypotonic (at 37°C) for 18 min. Add a few drops of fixative (methanol:acetic acid, 3:1) to the tube and mix. Spin 8 min at 1000 rpm.
		1. Remove the supernatant and flick the tube gently to re-suspend the pellet. Slowly add 5 ml of fixative and let it sit at room temperature for a minimum of 10 min.
		2. See procedure for 400-04-01-02 Interphase Fluorescence in Situ Hybridization (FISH) on Uncultured Amniocytes Procedure for processing. The sample is at Step D Hybridization.

**aCGH**

* + - * 1. If microarray is requested as reflex or first order, a portion of solid tissue is split with the array lab. 30-50mg of tissue is needed. If there is inadequate sample to split, two confluent T25 flasks or three 70% confluent T25 flasks are grown up for the array studies.

 **Culture maintenance**

**a. Day one** after set up, flood each coverslip with 2.5 ml of room temperature solid tissue media.

**b. Day two-three** check coverslip for cell attachment

**c. Day three-four** continue checking for cell attachment, wash and feed cells with fresh media

Change media in the flask at this time as well

**d**. The afternoon before each coverslip is ready for harvest, add 100 µl BrdU/colcemid to the flask and 50 µl to each coverslip. Use 100 µl BrdU/colcemid if harvesting a flask.

* + 1. Harvest

### F. 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 ST case the ST 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.**

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 correct 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:

**Manual Harvest**

* 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 19-21 min at room temperature.
	4. Add slowly and dropwise 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.

**G. Harvest flasks**

1. Add 100ul of colcemid (stock 10 µg/ml) to the flask for 30 min up to 3 hours at 37°C.
2. 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 in case 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.
3. 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.
4. 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.

***Note****:* No mouth pipetting. Adhere to sterile techniques. Use tissue culture hood [Biosafety Hood (BioGard)]. Use gloves. Use multiple incubators when possible. All glassware in contact with blood must be autoclaved after use. Bleach, fix or autoclave all labware in contact with specimen. 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.

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Written By: Director Approval:

(Signature and Date) (Signature and Date)

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 Cytogenetics Supervisor

**UW Medicine - Pathology**

 **Cytogenetics - UWMC**

**SIGNATURE PAGE FOR POLICIES AND PROCEDURES**

Procedure / Policy Title: Skin Fibroblast (ST) Cultures

Procedure / Policy Number: 400-02-01-05

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