**UW Medicine - Pathology**

400-02-01-04

Neoplasia Cultures

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| Adopted Date: 09/04/91Review Date: 06/11/06Revision Date: 1/25/2013 |

PURPOSE

This procedure is designed to provide a Cytogenetics diagnosis from a bone marrow, blood or other sample from a patient with a hematological malignancy. Neoplasia cultures can aid in identifying or verifying a specific type of leukemia, lymphoma, or other neoplastic disorder. This information may be useful in deciding the appropriate treatment for the patient.

PROCEDURE

The general indications for hematological malignancy include the following:

1. APL (AML-M3) is treated as a STAT (see notes)
2. R/O chronic myelogenous leukemia (CML).
3. R/O Philadelphia chromosome (Ph1)
4. Preleukemia or any other leukemia (ALL, AML, CLL, etc.)
5. T-Cell leukemia and lymphoma
6. Plasma cell disorders, Multiple Myeloma, Waldenstoms
7. Lymphoma: Hodgkins, NHL, Burkitt's, DLBCL, Follicular.
8. Any aplastic, dysplastic, myeloproliferative condition, etc.
9. Any neutropenia, cytopenia, thrombocytopenia, ITP, Polycythemia Vera
10. Elevated blasts in blood.
11. Elevated WBC or unexplained anemia.

***Notes:***

* AML-M3 (Acute Promyelocytic Leukemia) is treated as a STAT and the 24-hr harvest analyzed for the t(15;17). A direct t(15;17) FISH probe is done whenever indicated by physician.
* T-cell neoplasia cases are studied with PHA (D culture). In case of doubt, consult physician.
* **CLL** and **Plasma cell** disorder cases have an additional culture set up and stimulated with DSP-30/IL-2 (culture H).
* If the specimen is a follow-up to determine if an abnormality is constitutional vs acquired, it should be set up as a peripheral blood family follow-up (PB) sample and not as a neoplasia (NE) sample.

### Specimen Requirements

* + 1. **Bone Marrow:** When possible at least 1.0 ml of bone marrow in a sodium heparin vacutainer is requested, depending on cell count lesser amounts could be sufficient. Samples received in RPMI or transport media are also acceptable. Clotted specimens, or samples received in other anticoagulants, require additional processing, see section for Compromised Specimens.
		2. **Blood:** The optimal amount is 5-10 ml in a sodium heparin vacutainer tube. **See step 5 below.**
		3. **Bone core**: The specimen should immediately be placed in UWMC Cytogenetics transport or other sterile transport or culture media.
		4. **Other tissues:** These methods will work on biopsy specimens including pleural effusions, bone core, lymph node and spleen biopsies, as long as a free cell suspension can be made from the tissue. Lymphoblastoid cell lines may be harvested as described here, but the optimal hypotonic time may vary from 5-25 min. Use 5-10 x 106 cells in log phase growth per harvest.
		5. When to accept blood instead of bone marrow for leukemia:
			1. CML (Ph1)
				- elevated counts, even small, does not need 5% blasts
				- not treated (e.g., by hydroxyurea)
				- pathology shows early cells

Acute Leukemia

elevated counts, needs 5% blasts

if cannot get marrow (for specific reason) always get blood if any blasts at al (even if less than 5-10%)

CLL

* use for stimulated cultures
	1. The specimen should be maintained at room temperature and sent as soon as possible to the lab in either the aspirating syringe or sterile vacutainer tube.
	2. Results may be compromised by a variety of factors, including a long transit time, clotted specimen, exposure to cold or hot temperatures, cell count, a poor tap, and/or sample collected and transported in tubes with incorrect anticoagulant (i.e., EDTA-purple top or other). If a sample does arrive in an EDTA tube, it is spun down immediately, transferred to a 15-ml tube and washed 2X in RPMI, see section for Compromised Specimens.

### B. Materials and Equipment

1. T25 Corning flasks (Corning Cat. #25100-25) T12.5 flask (Falcon Cat# 353018).
2. 50-ml (Falcon Cat. # 2070) and 15-ml centrifuge tubes (Sarstedt 62.554.002pp).
3. For bone core biopsy, spleen or lymph nodes and other tissue: Petri dish, scalpels and disposable pipettes.
4. Laminar flow hood [Biosafety Hood (BioGard)]
5. Inverted microscope
6. Hemocytometer (Fuch's Rosenthal Ultraplane 1/16 sq mm 0.2mm deep)
7. Fume hood

### Reagents and Solutions

1. Media
	1. MARROW MAX (Gibco Cat# 12260-014) (Thaw and use as is; this is complete media.) warm media to room temperature or 37°C.
	2. Prior to use, new bottles of media must be sterility checked. Do so by taking a 0.5-1ml aliquot in a 15ml tube, do not fully tighten cap to allow for proper air flow, and place in the incubator at 37°C for at least 24 hr. Check for discoloration and cloudiness. Do not use media if discoloration or cloudiness is present.
	3. New lots of media must be QC’d prior to use.
2. Phytohemagglutinin (PHA) used for T-cell cases (Murex Cat. #HA-15) good for 1 month after reconstitution stored at 4°C.
3. Hypotonic: 0.075 M KCl at 37°C. good for 1 month stored at 37°C.
4. 10-5 M methotrexate (Sigma Cat. #A6770.) (MTX) Working Solution Good for 2 wk at 4°C.
	1. Prepare 25 mg/ml.
	2. Solution 1. Use 1.0 ml of 25 mg/ml solution and dilute with 9 ml distilled H2O. Store frozen 1 year.
	3. Solution 2. Dilute 1.0 ml of Solution 1 with 9 ml of distilled H2O.
	4. 10-5 M MTX Working Solution: Dilute 0.1 ml Solution 2 with 4.9 ml distilled H2O to get Working Solution of 1.1 x 10-5 M.
5. 10-3 M thymidine (Sigma Cat. #T9250) (dT) Working Solution (Store frozen: good for 6 mo.)
	1. M thymidine stock solution. Dissolve 242 mg of thymidine in 10 ml of H2O. Aliquot into 1 ml portions. Store frozen for 1 yr.
	2. 10-3 M thymidine working solution. Dilute 0.1 ml of 0.1 M thymidine stock solution with 9.9 ml of H2O. This solution is 10-3 M thymidine. Aliquot in 2.5 ml portions. Store frozen for 6 mo.
6. Fixative: 3:1 methanol:acetic acid (made fresh and kept at room temperature)
7. Colcemid (Karyomax, Gibco Cat. #15210-040).
8. H culture cocktail. Interleukin 2 (IL-2) 10,000U in 1 ml (Fisher/ Roche 11011456001); DSP-30 oligonucleotide from IDT (5'- T\*C\*G\* T\*C\*G\* C\*T\*G\* T\*C\*T\* C\*C\*G\* C\*T\*T\* C\*T\*T\* C\*T\*T\* G\*C\*C -3') 200μM. Make 150μl aliquots from 50μl DSP-30 and 100 μl IL-2 and freeze. Use 150 μl per 5ml culture.  [CLL cocktail worksheet](file:///%5C%5Cpathology%5Cfiles%5Cgroups%5CCytogenetics%5CShared%20Files%5CLab%20Files%5CCLL-mitogenCocktail-IL2%2BDSP30.xls)

### Procedure

***Note****:* See specifics for Bone Core Biopsy, or Lymph Node/Spleen tissue biopsy, below. For clotted specimens or samples received in EDTA or other anticoagulant, see section for Compromised Specimens. See attached Neoplasia Set-Up Summary Sheet. 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 HOOD (BioGard) AT A TIME**).

1. **Log in**: Upon receiving, attribute an NE/last 2 digits of year/ consecutive number, 4-digit accession number, e.g., **NE04-0052**, log into NE book, and on the board. Look for indication and make sure the request form is complete. All bone marrow, blood, lymph node and spleen specimens for diagnosis of neoplastic or pre-neoplastic diseases are logged in NE log book and to GCS.
2. **Culturing**

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

* 1. Count the number of cells per ml in sample using LeukoChek
		1. Puncture the cap diaphragm with the protective shield on the pipet assembly.
		2. Remove the shield and fill the tube with whole blood or bone marrow. Be sure the capillary tube fills completely (20ul) when full, the capillary action will stop. Gently wipe away excess blood or bone marrow from capillary.
		3. Squeeze reservoir slightly to expel air, maintain pressure on the reservoir while inserting the pipet full of blood or bone marrow into the reservoir. Simultaneously cover the top opening of the capillary tube holder with finger
		4. Release pressure from the reservoir and then from the capillary tube opening, this will cause the fluid to be drawn into the diluent
		5. Gently squeeze the reservoir to rinse capillary tube being careful not to spill any blood or bone marrow and mix carefully with finger over top of opening
		6. Remove pipet assembly, invert and place capillary on the top of the cap, making the capillary device a dropper
		7. Discard 3 or 4 drops and expel solution into a hemocytometer for counting leukocytes and platelets
		8. Count leukocytes in 5 squares on the hemocytometer. Recommended to count 4 corner squares and one in the middle

N=\_\_\_ (cell count) x100/103 =\_\_\_\_x106 cells/ml

10/Cell count= amount of specimen added to a culture 10 ml culture. Use half of that volume for 5 ml cultures

 ***Note:*** Do not use the WBC number if given, always count.

***Note****:* Optimum cell density is 1 x 106 cells per ml culture medium.

* 1. Label vessels with patient labels printed from GCS. Set up two 10 ml cultures (A and B) in T25 Corning flasks. Set up a 5 ml culture (C) in T12.5

If insufficient cells are available to set up all cultures, prioritize as follows:

* For Routine setup- top priority culture A, second priority B, third priority C.
* For Multiple Myeloma, Waldenstom's or plasma cell disorders-top priority H, second A, third B.
* For CLL-top priority H, second A, third B, fourth C.
* For T-cell-top priority D, second A, third B, fourth C.

If the cell count in bone marrow is below 10 x 106 ml, don't use more than 1.0 ml marrow per culture or when the count is particularly low, take the plasma and buffy coat and set up the standard 10 x 106 cells in culture: to obtain the plasma and buffy coat, let the specimen stand 2-3 hr or centrifuge 10-20 min at 1000 rpm. Remove entire plasma and buffy coat to another sterile tube (a few red cells won't hurt), count cells again and set up cultures with this fraction. Several ml of plasma and buffy coat may be used per culture. For T-cell leukemia samples, set up additional 5 ml culture (D) and add 0.1 ml PHA. For CLL, set-up additional 5 ml culture (H) with 150μl DSP-30/IL-2 cocktail.

These methods are designed to give cultures with a standard and adequate number of WBCs, while minimizing the number of RBCs, which tend to interfere with the hypotonic treatment and create a mess in the fixative. The number of RBCs in 0.5 ml of blood or marrow will not interfere with harvest quality, but if more than 1 ml specimen per culture is used, the RBCs should be reduced by taking the plasma and buffy coat.

* 1. Incubate at 37°C in 5% CO2, for the period of time described below:

Harvest a one-day unstimulated culture (16-25 hr, flask A) and a two day unstimulated culture (flask C). Harvest an MTX-synchronized harvest (flask B) on day 1 or 2.

***Note***: Cultures for myeloma, Waldenstrom’s and other plasma cell diseases, incubate and harvest: A—24 hr; B—48 hr; H—72 hr.

* 1. Specimens that need attached cell culture for long-term culturing (L culture). Set up one 5 ml culture with TR media. This culture goes into TR incubator and will be monitored and harvested by TR personnel. Specimens that need "L" culture are:
1. Lymph nodes with a diagnosis of lymphadenopathy.
2. Any tissue sample with a diagnosis of Ewings sarcoma.
3. Any tissue mass (not lymph nodes), even with diagnosis of leukemia or lymphoma.
4. Any sample with question of carcinoma as well as routine neoplasia diagnosis like lymphoma or myeloma.
	1. **Methotrexate synchronization:** Add 0.1 ml of 10-5 M methotrexate to flask B late on the day of initiation or late on day 1 of culture (0-8 hr or 24 hr in culture). Record time on daily worksheet.
	2. **Harvesting**
		1. Flasks A and C: Routine Neoplasia Harvest (Manual)\*
			1. Add 0.1 ml of colcemid stock (1.0 µg/ml; Gibco) to flask A and 0.05 ml to flask C for 2-day harvest (final concentration 0.07 µg/ml). Record time on daily worksheet.
			2. Mix thoroughly and incubate at 37°C for 30 min.
			3. Transfer flask content to a 50-ml/15-ml conical plastic centrifuge tube.
			4. Spin at 1000 rpm in clinical centrifuge for 10 min.
			5. Remove supernatant media with a Pasteur pipette. Resuspend cells in remaining media; add 10-12ml of pre-warmed (37°C) hypotonic solution 0.075 M KC1.
			6. Invert tube to mix thoroughly and place in 37°C incubator for 12 min. Invert tubes once during the hypotonic treatment to keep the cells well suspended.
			7. Add 20 drops of fresh fixative (3:1 methanol:acetic acid) per 25 ml of hypotonic, or 10 drops per 12 ml of hypotonic, invert the tube to mix thoroughly and spin at 1000 rpm for 8 min.
			8. Remove the supernatant hypotonic solution with a Pasteur pipette.
			9. Gently resuspend cells by flicking. Then add 1 ml fixative (3:1 methanol:acetic acid), and mix thoroughly.
			10. Bring volume to 8 ml with fixative and mix well. Cap and let stand at room temperature for 30-60 min. Transfer from 50 ml centrifuge tube to 15 ml.
			11. Wash the cells three times, each time adding less fixative:
				* First wash ‑ 6 ml
				* Second wash ‑ 4 ml
				* Third wash ‑ 2 ml

***Note:*** All fixative steps are done at room temperature. Fixed cells are stored in freezer (for years) or in refrigerator for a week maximum.

1. Flask B: Methotrexate Synchronized Harvest (Manual)\*
	1. About 17 hr after adding the MTX block, add 0.1 ml 10-3 M thymidine to flask B (final concentration of dT 10-5M). Do not wash MTX out of culture. Record time on daily worksheet.
	2. Return culture to incubator for 4 hr 20 min.
	3. Add 0.1 ml colcemid stock, incubate for 10 min and proceed with hypotonic and fixative steps as in Routine Harvest above. Record time on daily worksheet.
2. For stat APL or other Direct Harvests:
	1. Do a cell count on the specimen as usual for Neoplasia set-up.
	2. Add the amount you would normally inoculate to a 15ml tube with 12ml of pre-warmed 0.075M KCl hypotonic and incubate at 37ºC for 12 minutes.
	3. Add approximately 1ml of fix, dropwise and mix gently and centrifuge.
	4. Slowly add 5-10ml of fix and centrifuge.
	5. Label slides with printed slide labels
	6. Re-suspend cell pellet in small amount of fix and drop two slides.
	7. Bake slides 20 minutes at 95ºC.
	8. Place slides in 2xSSC at 73ºC for 1min.
	9. Prepare probe for use: #194 PML/RARA-dual fusion. Mix 1µl probe, 2µl of H2O and 7µl hybridzation buffer.
	10. Dehydrate slides with 70%,80%,95% ethanol series.
	11. Dry and apply probe to one slide, coverslip, seal with rubber cement.
	12. Hybridize on Hybrite using program: 75 °C for 2minutes, 37 °C overnight.
	13. The next morning, wash FISH slides and analyze right away.
	14. Set up at least one backup culture
3. For "D" cultures or PHA-stimulated marrow or blood, harvest on day 3 (~72 hours) using routine neoplasia harvesting methods

***\*Note:*** The Hanabi P-II robotic harvester can substitute for these manual harvest methods if there are no clots in the culture. See Hanabi P-II Operation instructions in the NW125G drawer.

* 1. **Neoplastic analysis**
1. B cell malignancies
	1. Unstimulated culture:

**A:**  24 hr harvest

**B:** 24 hr harvest with methotrexate

**C:** 48 hr harvest

* Analyze a total of 20 cells utilizing at least 5 cells from each of the harvests (A, B, and C). If abnormal cells are found, all 20 cells can be from the best culture.
* If typical abnormality relevant to the disease (e.g., Ph1 in CML) is found in the first 5 cells, analyze partially 15 more cells for this anomaly unless the indication is tumor progression. In that case, analyze 20 cells completely.
* If the indication is relapse of disease, look for previous specific abnormality in 10 cells completely analyzed, 10 cells partially analyzed and 30 cells scanned in the microscope.
	1. For CLL: IL-2/DSP-30 stimulated culture-H: 72 hr harvest. Analyze 20 cells first, in addition to the 20 cells from the routine cultures as above.
		1. T cell malignancies
1. As above. Analyze 20 cells from PHA-stimulated culture.

***Note***: Preliminary diagnosis: 5 cells analyzed (including 3 drawings or photo analysis). If the 5 cells are normal, extend to 10 cells.

* 1. Neoplasia cases requiring additional scoring. Score 30 additional cells in neoplasia cases when the following is found:
1. one cell with a structural abnormality
2. one cell with a trisomy
3. one cell with monosomy 5 or 7 in MDS and AML
4. one cell with monosomy 13 in multiple myeloma
5. two cells with monosomy 5, 7, 12, 13, 17, 18, 20, X, Y for all indications, except multiple myeloma
6. two cells with monosomy for the same chromosome (any chromosome) in multiple myeloma
7. do not disregard cells with hypodiploidy or hyperdiploidy, especially in multiple myeloma

***Note***: If possible, score the additional cells from the same culture in which the anomaly was detected. If there is insufficient material from this culture, use another one if possible.

### Bone Core Biopsy, Tissue Mass, Carcinomas w/ Hematological Diagnosis

### Aseptically transfer bone marrow core from transport tube to polystyrene Petri dish. Pipette transport media back into transport tube. Add 0.5 ml fresh media to sample, then mince with scalpels to release cells. Centrifuge transport media at 1000 rpm for 8 min.

### Divide minced biopsy between two 15ml Falcon tubes for 5 ml cultures A and B. A cell count is not usually possible. Rinse Petri dish with 2 ml fresh medium and divide between cultures. Discard supernatant from transport tube and add mixed pellet evenly to A and B tubes. Agitate vigorously to release cells. Add media to bring each culture to 5 ml. For Ewing's Sarcoma, Carcinoma diagnosis, save some cells to set up for an L culture. The L culture is given to Tumor techs to grow, harvest and make slides.

### Harvest flasks at 24 and 48 hr, as usual for two-culture specimen. Use routine harvest procedures, modified slightly. Use 8 ml hypotonic instead of 12 ml, and add 6 ml fixative instead of 8 ml before letting specimen stand for 30-60 min.

### Lymph Node/Spleen Biopsy

Same as for Bone Core Biopsy (see above), except count cells if after mincing specimen, the media appears cloudy and full of cells. If this happens, set up at standard concentration of 1 x 106 cells per ml culture. Do not use collagenase.

***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**.**

### Compromised Specimens

* + 1. **Specimens received in incorrect anticoagulant**

Anticoagulants other than sodium or lithium heparin are toxic to cells grown for cytogenetic analysis. Therefore, blood and bone marrow samples drawn in tubes containing EDTA (purple top) or other anticoagulants need to be washed prior to set up in order to remove as much of the toxic agents as possible.

1. Materials
	1. Disposable volumetric pipettes
	2. Falcon 15-ml centrifuge tube
	3. Hanks’ or RPMI media (without additives)
2. Procedure
	1. Gather all equipment and media
	2. Aseptically transfer the specimen to 15-ml centrifuge tube
	3. Add media to a total volume of 10 ml and mix well.
	4. Centrifuge at 1000 rpm for 8-10 min.
	5. Aseptically remove and discard supernatant with volumetric pipette.
	6. Flick button in tube to loosen pellet.
	7. Re-suspend pellet in media twice the volume of original sample. Mix well.
	8. Centrifuge again 8-10 min.
	9. Aseptically remove and discard supernatant.
	10. Flick pellet and re-suspend in RPMI to original volume, mixing well.
	11. Count cells and continue processing as routine sample.
	12. Make note that sample was washed under *Specimen Comments* in computer.

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APPENDIXES

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| **Neoplasia Set-up Summary Sheet**  |
| **Count****Calculate****Set up** | Use the Leukocheck kitCount # of cells in 5 large hemocytometer squares (this # is N) N x 100 x 1,000 = M (cells/ml of original specimen)10/M x 106 = volume (ml) of specimen needed for a 10ml culture. See table below |
|  |  |
| **Stat - APL, AML-M3, t(15;17)** |  |
| DH-Direct Harvest | Prewarmed hypotonic (0.075M KCl) | 5 ml, 15 ml tube |
| A—24 hr culture | Marrow Max (Lot 1) | 10 ml, T25 flask |
| B—24 hr culture | Marrow Max (Lot 2) | 10 ml, T25 flask |
| C—48 hr culture | Marrow Max (Lot 2) | 5 ml, T12.5 flask |
|  |  |
| **Routine** |  |
| A—24 hr culture | Marrow Max (Lot 1) | 10 ml, T25 flask |
| B—24 hr culture | Marrow Max (Lot 2) | 10 ml, T25 flask |
| C—48 hr culture | Marrow Max (Lot 2) | 5 ml, T12.5 flask |
|  |
| **T-cell** |  |
| A—24 hr culture | Marrow Max (Lot 1) | 10 ml, T25 flask |
| B—24 hr culture | Marrow Max (Lot 2) | 10 ml, T25 flask |
| C—48 hr culture | Marrow Max (Lot 2) | 5 ml, T12.5 flask |
| D—72 hr culture | Marrow Max (Lot 1) add 0.1ml PHA | 5 ml, T12.5 flask |
|  |
| **CLL, SLL, PLL** |  |
| A—24 hr culture | Marrow Max (Lot 1) | 10 ml, T25 flask |
| B—24 hr culture | Marrow Max (Lot 2) | 10 ml, T25 flask |
| C—48 hr culture | Marrow Max (Lot 2) | 5 ml, T12.5 flask |
| H – 72 hr culture | Marrow Max (Lot 1) add 150ul IL2-DSP30 | 5 ml, T12.5 flask |
|  |
| **Myeloma, Waldenstrom’s, Plasma Cell, Mast Cell Disorder, MGUS, Hairy Cell Leukemia, Paraproteinemia, bone lesion, kappa light chain**  |  |
| A—24 hr culture | Marrow Max (Lot 1) | 10 ml, T25 flask |
| B—48 hr culture | Marrow Max (Lot 2) | 10 ml, T25 flask |
| H—72 hr culture | Marrow Max (Lot 2) add 150 ml IL2-DSP-30 | 5 ml, T12.5 flask |
| **NOTE**: Set up long term culture "L" for Carcinomas, Tissue mass, Adenopathy, Ewing's sarcoma. Use TR media **[page: 8]**.**NOTE**: On Saturdays, the B culture must be a 48 hr culture, as there is no MTX harvest on Sunday**NOTE**: For clotted specimens or samples in EDTA, see section for Compromised Specimens **[page: 8]**.**FOR ALL "B" CULTURES**Add 0.1 MTX in the late afternoon. Add 0.1 ml dT to release the MTX 17 hr later.For example: If MTX is added at 3 pm, add dT at 8 am to release. |

Written By: Director Approval:

(Signature and Date) (Signature and Date)

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 Cytogenetics Supervisor