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4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER

Purpose & Principle

- This procedure describes the steps in performing cell surface marker study for 4-color Acute Leukemia immunophenotyping) for whole blood, bone marrow aspirate, effusion, solid tissue cell suspension, or other body fluids using panel of monoclonal antibodies conjugated with fluorochromes such Fluorescein Isothiocyanate (FITC), Phycoerythrin, (PE), Phycoerythrin-Texas Red® X (ECD), Phycoerythrin-Cy5 (PC5).
- Viable cell suspension is incubated with specific monoclonal antibodies, with their respective isotype control, and antigens on the surface of the cell will react with the presence of their respective antibodies. The red cells are lysed with ImmunoPrep® and the sample is acquired and analyzed using Navios cytometer.
- The Navios is an instrument for phenotyping surface markers of white blood cells. The scatter plots of cells are measured and analyzed as cells pass through one LASER beam in a single-fine using the method of hydrodynamic focusing.
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Work Place Safety:

All laboratory employees are expected to maintain a safe working environment and an injury-free workplace. Laboratory employees are responsible for their own safety, the safety of others and adhering to all departmental and medical center safety policies and procedures.

- For standard precautions and safety practices in the laboratory; see LAMC-PPP-0123 specifically, but not limited to, equipment safety, proper body mechanics, sharps exposure and proper use of personal protective equipment (PPE).
- For Universal Body Substance precautions, see LAMC-PPP-0128, specifically, but not limited to, exposure to body fluids.
- For proper handwashing, see LAMC-PPP-0132, specifically, but not limited to, proper handwashing.
- For proper infection control, see LAMC-PPP-LGM 8004, specifically, but not limited to, proper use of gloves.
- For proper handling of regular and infectious waste, see LAMC-PPP- 0127, specifically, but not limited to proper disposal of regular and biohazardous waste.
- For proper cleaning of work area, see LAMC-PPP-0130 - Cleaning Work Areas.
- For proper handling of chemicals and reagents, see the Chemical Hygiene Plan.
- For proper storage and disposal of chemical hazardous waste, see LAMC-PPP-0134.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

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Policy

- Navios Flow cytometer shall be used for flow cytometry data acquisition and analysis of bone marrow, peripheral blood and other body fluids.
- Flow Cytometry Lab uses components of ImmunoPrep reagent kit within the kit lot only.
- To ensure availability of monoclonal antibodies for testing minimum re-order level is 2 vials for most commonly used monoclonal Mab. Notify manager if reagent is below re-order level.

Specimen Requirement

- Use cell suspension or body fluid less than 48 hours old.
    - K_3EDTA blood – 5m. ACD and heparin is acceptable of if EDTA anti-coagulated specimen is not available.
    - Sodium heparinized bone marrow aspirate – 1-2ml. Wash once with Modified PBS before processing.
    - Effusion (pericardial, pleural, or peritoneal) treated with anticoagulant. 10 to 2 ml.
    - Cell suspension of washed or mashed solid tissue in cell media. 5 to 10ml.
    - Body fluid (such as CSF, synovial, etc.), untreated – 2.5 ml. If bloody, add anticoagulant.
- Obtain WBC count, if available.
- Maintain and transport specimen at room temperature (18-22 ^{0}C) in a leak-proof container. Avoid temperature <10 ^{0}C and >37 ^{0}C.
- Take note on the worksheet and report on the form if the following occur:
    - If the specimen is hot or cold but not obviously hemolyzed or frozen.
    - If the specimen is hemolyzed or frozen.
    - If clots are visible.
    - If the specimen is >48 hours old at the time it arrives at the laboratory.
- Do viability testing. Refer to SOP LAMC-PPP-0570.

Reagent, Materials, and Equipment

Reagents:

- Deionized Water
- Bleach
- IsoFlow Sheath Fluid, Beckman Coulter, PN 8547008

- Coulter Clenz cleaning agent, PN 8546930
- Phosphate buffered saline, pH =7.4. Sigma Catalog # P-3813

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Preparation:

Step	Action
1	Take 1 packet (foil pouch) and dissolve in 1 liter of deionized water. Yield: 0.01 M PBS pH= 7.4
2	Store in a 1 liter amber dispenser at room temperature.

- Modified wash media:

  Preparation: (Prepare weekly)

Step	Action
1	Take 500 ml of RPMI 1640-500 (Irvine Scientific Catalog # 9161 500ml)
2	Weigh 5grams of Albumin crystals from Bovine serum (Sigma, Catalog # A7906-50G)
3	Dissolve 5 grams of Albumin crystals in to 500ml of RPMI 1640.

- Albumin bovine 22% solution, 50 ml. Sigma A-7034 (Store at room temperature)
- ImmnoPrep® Reagent System, Beckman Coulter Catalog # 7546999
  - ImmunoPrep A (215ml) Contents: Formic Acid 1.2 ml/L & Stabilizer
  - ImmunoPrep B (95ml) Contents: Sodium Bicarbonate, 6.0g/L, NaCl, 14.5g/L, $NaSO_4$, & Stabilizer.
  - ImmunoPrep C (36ml) Contents: Paraformaldehyde = 10.0g/L, & Buffer

Note: Flow Cytometry Lab uses components of ImmunoPrep Reagent kit within the kit lot only.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

Monoclonal Antibody Reagents:

Reagent	Catalog #	Manufacturer	ul/test
CD33-FITC	340678	Becton Dickinson	6ul
CD13-PE	340686	Becton Dickinson	6ul
CD45-ECD	IM2710U	Beckman Coulter	6ul
CD34-PC5	IM2648	Beckman Coulter	6ul
HLA-DR-FITC	340688	Becton Dickinson	6ul
CD64-PE	IM3601	Beckman Coulter	6ul
CD14-PC5	IM2640	Beckman Coulter	6ul
TdT-FITC (Terminal deoxynucleotidyl trans ferase) *	F7139	Dako	6ul
MPO-PE (Myeloperoxidase)*	IM3455	Beckman Coulter	6ul
CD3-ECD*	IM2705U	Beckman Coulter	6ul
CD3-PC5*	IM3456	Beckman Coulter	6ul
CD19-ECD*	IM2708U	Beckman Coulter	6ul
CD45-Krome Orange	A96416	Beckman Coulter	5ul
CD10-FITC	IM2720	Beckman Coulter	6ul
CD20-PE	IM1835U	Beckman Coulter	6ul
CD19-PC5	IM2643	Beckman Coulter	6ul
CD2-FITC	IM0442	Beckman Coulter	6ul
CD5-PE	IM0469	Beckman Coulter	6ul
CD7-PC5	IM3613U	Beckman Coulter	6ul
CD38-PE	IM2371	Beckman Coulter	6ul
Neg.Ctrl-FITC/Neg.Ctrl-PE/CD45-ECD	IM3489U	Beckman Coulter	6ul
Neg.Ctrl-FITC/Neg.Ctrl-PE/Neg.Ctrl.-ECD*	IM3488U	Beckman Coulter	6ul
IgG1-PC5	PN IM2663	Beckman Coulter	6ul

* **Note:** B & T Flow cytometry laboratory performs intracellular immunophenotyping method on TdT, MPO, CD3, and CD19. Refer to LAMC-PPP-0569 intracellular immunophenotyping method.

Materials:

- Polyproplylene tubes 12X75mm., FisherScientific, Catalog # 14-959-5
- Disposable plastic centrifuge, 15ml., FisherScientific, Catalog # 35-2099
- Disposable Petri dishes, size 100 x 15mm., FisherBrand Catalog # 08-757-12
- Pipette Eppendorf
- Pipet tips (Finntip ® 250 Universal) Catalog # 9400260

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

Materials (continued)
- Transfer pipette, 1ml Graduated, FisherScientific, Catalog # 13-711-9A
- Test tube racks.

Equipment:

- Beckman Coulter Cytomics Navios flow cytometer
- Beckman/Coulter TQ Prep
- Beckman Refrigerated Centrifuge
- Vortex Mixer
- Labconco Type A2 Biosafety Cabinet
- Non-CO_2 Incubator at 37^0C

Quality Control

See associated Standard Operating Procedures:
- Daily Start-up Procedure for Navios Flow Cytometer, LAMC-PPP-0535.
- Quality Control Procedure for Navios Flow Cytometer, LAMC-PPP-0537
- Shutdown Procedure for Navios Flow Cytometer, LAMC-PPP-0536
- Navios Flow Cytometer, Preventive Maintenance, LAMC-PPP-0541

How to perform Auto Setup Schedule to set for instrument setting on lymphoma/leukemia panels.

Note: Perform the following steps after running Flow Check bead and AutoSetup procedure for 4-color T-cell enumeration.

Step	Action
1	From the Main menu, click on **Tools** and choose AutoSetup Scheduler.
2	From AutoSetup Scheduler window, choose **4c leuk-C**
3	Enter carousel number and click on **Schedule**.
4	The instrument will acquire AutoSetup tubes.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

How to obtain quality control results from AutoSet-up procedure for lymphoma/ leukemia panels.

Step	Action
1	From the main screen, click on **LJ icon**
2	To display LJ charts: Click on **Template,** and Select template, and choose leukemia lymphoma qc (the LJ of Verify tubes) click **Open.**
4	Instrument will display current LJ chart. Check for outliers.
5	Click **Data table** and inspect numerical values of outliers.
6	If outliers are present, close **Data table.** Point and click on the outlier on the **LJ** chart.
7	Type in action taken "to repeat" and click **Save Comment.**
8	Repeat AutoSetup procedure.
9	If all quality control data are within limits, go the the LJ chart, repeated quality control item and type in "repeated" and click **Save Comment.**
10	Exit the Report Generator window.

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Procedure:

- **Sample preparation for testing**: Dilution of ficolled blood and ficolled bone marrow specimen:

Note: For bone marrow specimen and peripheral blood: To ensure ideal flow rate, the WBC count should be from 5,000 to 10,000 cells/cu mm. WBC above 15,000 requires adjustment of the cell concentration to an appropriate level.

WBC COUNT	DILUTION (with PBS)
15,000 up to 30,000	1:2
30,000 up to 40,000	1:3
40,000 up to 60,000	1:5
60,000 up to 100,000	1:6
100,000 up to 200,000	1:10
Above 200,000	1:20

- **Sample preparation: Other body fluid or suspension:**

Step	Action
1	Transfer the sample to a labeled plastic centrifuge tube.
2	Wash sample, once, with wash media and centrifuge for 5 minutes at 1500RPM.
3	Resuspend the sample in 5ml. of wash media and incubate at 37^0C for 30 minutes.
4	Layer sample over 1ml of 22% albumin solution in a labeled 15ml centrifuge tube and centrifuge for 5 minutes at 1500 RPM.
5	Wash cell suspension again with modified wash media and resuspend in 2 ml of modified wash media.
6	Obtain cell count and adjust to 10 million cells/ml.

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Acute Leukemia Panel Antibody Combinations:

FITC	PE	ECD	PC5	Stain (volume)
CD33	CD13	CD45	CD34	6ul each
HLA-DR	CD64	CD45	CD14	6u each
CD2	CD5	CD45	CD7	6ul each

FITC	PE	ECD	PC5	Krome Orange
CD10	**CD22**	**CD19**	**CD20**	**CD45**

FITC	PE	ECD	PC5	Krome Orange
IgG1	**IgG1**	**IgG1**	**IgG1-PC5**	**CD45**
TdT	**MPO**	**CD19**	**CD3**	**CD45**

Acute Myelocytic Leukemia (AML)Panel:

FITC	PE	ECD	PC5	Stain (volume)
CD33	CD13	CD45	CD34	6ul each
HLA-DR	CD64	CD45	CD14	6u each

FITC	PE	ECD	PC5	Krome Orange
IgG1	**IgG1**	**IgG1**	**IgG1-PC5**	**CD45**
TdT	**MPO**	**CD19**	**CD3**	**CD45**

Acute Lymphocytic Leukemia (ALL) Panel:

FITC	PE	ECD	PC5	Stain (volume)
HLA-DR	CD64	CD45	CD14	6u each
CD10	CD20	CD45	CD19	6ul each
CD2	CD5	CD45	CD7	6ul each

FITC	PE	ECD	PC5	Krome Orange
IgG1	**IgG1**	**IgG1**	**IgG1-PC5**	**CD45**
TdT	**MPO**	**CD19**	**CD3**	**CD45**

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

T-Cell /NK-Cell Lymphoma Panel:

FITC	PE	ECD	PC5	Stain (volume)
CD8	CD4	CD3	CD7	6ul of CD8/CD4/CD3 + 6ul of CD7PC5.
CD25	CD5	CD19	CD3	6ul each
CD57	CD56	CD3	CD2	6ul each
CD2	CD5	CD45	CD7	6ul each

Note: B & T Flow cytometry laboratory performs intracellular immunophenotyping method on TdT, MPO, CD19, and CD3. Refer to LBTM 220.21 for 4-color intracellular immunophenotyping method.

Staining bone marrow, peripheral blood, and non-bloody body fluids with monoclonal antibodies:

Step	Action
1	Label 12 x 75 mm. plastic tube with BT case number and label the tube with the antibody.
2	Label 12 x 75 mm. plastic tube with BT case number and label it also with corresponding isotypic control of antibody on step 1.
3	Pipette (6ul) of monoclonal antibody on the tube labeled with antibody (tube from Step 1) (See tables of antibodies for volume used for staining)
4	Pipette (6ul) of isotypic control reagent on the tube labeled with isotypic control (tube from step 2)
5	Add 100ul (2 drops—using graduated transfer pipette) of sample (adjusted to optimal concentration) into each labeled tube.

Step	Action
6	Vortex, load stained tubes in a carousel, and incubate stained samples in the dark for 30 minutes at room temperature.
7	After incubation, load carousel containing stain tube in to the TQ-Prep Workstation.
8	Press start button of the TQ-Prep to process the samples.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

Acquisition and analysis of specimen using Navios flow cytometer.

Step	Action
1	At Resource explorer, click on the panel icon.
2	Click on **BT Lab** folder.
4	Scroll file list and look for the appropriate panel.
3	Drag desired panel into the Acquisition Manager. Check to make sure the proper combination of reagents is in the panel list, otherwise modified the list.
4	Enter carousel number into the **Carousel No.** column.
5	Enter specimen BT lab number in the **Sample ID** column.
6	Open MCL cover, and load the carousel to be acquired and analyzed. Close carousel cover.
7	For optimization of sample acquisition of isotype control, press **F9.** See next section for details on sample optimization.

Optimization of sample detector settings:

Step	Action
1	Optimization of isotype control: Click on **Cytometer** from the main menu, click on **Cytometer Control,** and click on **Setup Mode.**
4	Adjust gate A to encompass dim staining CD45 cells.
5	Inspect the positions of the quadrant markers and histogram markers from all windows of the protocol. Adjust if necessary.
6	After the three elements are adjusted: optimal placement of the gate of CD45 dim staining cells (Gate A), placement of quadrant markers, and histogram markers, click on **Acq. Setup** tab and deselect **Setup Mode.**
7	Press **F9** to acquire samples. **Note**: All protocols have stop criteria: at least 5,000 events in the gate or 300 seconds acquisition time. Acquire samples until stop criteria is met.
8	Continue to acquire subsequent samples, and during the acquisition, ensure gating consistency and optimal placement of quadrant markers and histogram markers. **Note:** For under-lysed samples re-stain and re-acquire samples.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

Retrieval and re-analysis of LMD files.

Step	Action
1	At the **Resource Explorer** window, click **LMD** icon, click on **BT Lab folder.**
2	Select desired file from the file list and drag the file into the empty space of the **Acquisition Manager.**
3	To adjust gate size: a) Click on the maximize window located on the upper right portion of the window.
3	b) Use the mouse and double click on the any portion of the gate. c) To increase the gate size, find and drag a little square outside the gate and drag it way from the gate until the desired size of the gate is reached. d) To decrease the gate size, drag the little square towards the center of the gate until desired gate size is reached. e) Click **Restore down** to return to original size of the window.
4	To adjust the quadrant marker(s): a) Click on the quadrant market window. b) At the intersection of the quadrant marker, adjust the markers to the desired quadrant marker position.
5	To adjust the histogram marker(s): a) Point the left most side of the histogram marker and drag the leftmost portion of the histogram marker to its desired position.
6	Click on the flow page window at the main menu.
7	To print the re-analyzed file, click the print icon from the main menu.

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

Interpretation

- Print-outs of immunophenotyping histograms shall be submitted to the assigned pathologist for interpretation of flow cytometry results together with accompanying sample slides.

Reporting and Documentation

- To detect clerical errors, analytical, and unusual laboratory results, a CLS aside from the one who acquired and analyzed the data from the cytometer, will review histogram and immunophenotyping results. The CLS who reviewed the results shall initial the worksheet containing the immunophenotyping report before release from the laboratory.
- Print-out of immunophenotyping histograms shall be submitted to the assigned pathologist together with accompanying stained slides.
- Results of the percentage positivity of monoclonal antibodies may be communicated through the telephone or faxed to the assigned pathologist upon his/her request.

Controlled Document

- LAMC-FORM-0070 – BT Marker Request Form
- LAMC-FORM-0090 -Lymphoma/Leukemia Daily Quality Control.
- LAMC-FORM-0105 Flow Set Navios 4C
- LAMC-PPP-0123 - Safety Practices.
- LAMC-PPP-0128 – Universal Body Substance Precautions (UBSP).
- LAMC-PPP-0132 – Handwashing Policy.
- LAMC-PPP-0127 – Infection Control.
- LAMC-PPP-0130 – Cleaning Work Areas.
- LAMC-PPP-0129 – Handling of Regular and Infectious Waste.

Uncontrolled Document

- Bertie, L.M., Charlton, B.J., Kirkley, B., Schiffgens, J., Wilson, J.I. & Woodcock, S.M., Clinical Laboratory Technical Procedure Manuals; Approved Guideline—Fourth Edition. Clinical Laboratory Standards Institute. Vol 22, Number 5. Retrieved November 17, 2005. http://flowsite.hitchcock.org/procman/

4-COLOR CELL SURFACE MARKER STUDY (ACUTE LEUKEMIA IMMUNOPHENOTYPING) BY FLOW CYTOMETRY USING NAVIOS FLOW CYTOMETER, continued

- Navios Flow Cytometer,(November 2009) Beckman Coulter Inc. Fullerton, CA
- Kussic, S.J., & Wood, B.L. (2003). Four-color flow cytometry identifies virtually all cytogenetically abnormal bone marrow samples in the work-up of non-CML myeloproliferative disorders. Americal Journal of Pathology. 120, 854-856.

Self Assessment

- The incubation time after addition of cells to the monoclonal antibodies:
  a) 30 minutes
  b) 20 minutes
  c) 5 minutes
- Files to be re-analyzed can be found from:
  a) BT Lab folder or BT Flow Cytometry folder from the list in the LMD icon.
  b) Common folder from list on the LMD icon.
  c) Common folder from list on the Histogram icon.

- For sample acquisition optimization, gate adjustment can be accomplished by:
  a) Double click any portion of the gate and use an outside square (vertex) to adjust the size of the gate.
  b) Double click any portion of the gate and use any vertex of the polygonal gate.
  c) Click on any portion of the gate and use any vertex of the polygonal gate.

- For sample optimization before acquisition, name three essential elements that need to be inspected and adjusted.
  a) Detector settings, gate on mononuclear cells, and position of quadrant markers.
  b) Detector settings, gate on mononuclear cells, and position of histogram markers.
  c) Gate on dim staining CD45 cells, position of quadrant markers, and position of histogram markers.

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