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DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER

Purpose

- This procedure describes the steps involved in acquiring and analyzing DNA content using Navios Flow Cytometer.

Workplace 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 LGM 8000, 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 LGM 8005, specifically, but not limited to, exposure to body fluids.
- For proper handwashing, see LGM 8010, specifically, but not limited to, proper handwashing.
- For proper infection control, see LGM 8004, specifically, but not limited to, proper use of gloves.
- For proper handling of regular and infectious waste, see LGM 8006, specifically, but not limited to proper disposal of regular and biohazardous waste.
- For proper cleaning of work area, see LGM 8007 - 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 LGM 8012.

Policy

- Navios Flow cytometer shall be used for flow cytometry data acquisition and analysis of paraffin embedded tissue, fresh tissue, and other body fluid cell suspension.
- Multicycle AV for Windows Version 328 will be used as software for DNA cell cycle analysis.
- SCPMG Flow cytometry Laboratory currently performs DNA content analysis, hematopoietic neoplasm, solid tumors: tissues derived from products of conception. Hydatidiform mole is extremely useful in distinguishing partial hydatidiform mole from complete hydatidiform mole. When combined with clinical history and careful gross and microscopic examination, DNA ploidy analysis is useful and strong indicator of diagnosis.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

Specimen Requirement

- See SOP# LBTM 240.2 for processing and staining de-paraffinized cell suspension for DNA ploidy.
- See SOP# LBTM 240.3 for processing and staining body fluids cell suspension for DNA ploidy.

Reagent, Materials and Equipment

Reagents:

- DNA Prep™ Beckman Coulter, PN 6607055 100 tests.
- CEN (Chicken Erythrocyte Nuclei) CEN Singlet Cytometry Control BioSure® Catalog 1013
- Deionized Water
- Bleach
- IsoFlow Sheath Fluid, Beckman Coulter, PN 8547008
- FlowClean cleaning agent, PN A64669

Materials:

- Polyproplylene tubes 12X75mm.

Equipment:

- Navios Flow Cytometer

Quality Control

- To prepare instrument for DNA acquisition and analysis perform Daily Start-Up Procedure for Navios flow cytometer: SOP # LBTM 200.40
- Refer to SOP LBTM #200.42 daily verification optical alignment and the integrity of fluidics, standardization of light and fluorescence intensity.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

Procedure

How to standardize the Navios cytometer with Chicken Erythrocyte Nuclei (CEN) and normal peripheral blood lymphocytes and acquisition of DNA samples.

Step	Action
1	At the **Resource Explorer**, click on **Protocol** icon, from the BT Lab acquisition folder, drag **DNA.pnl** into the **Acquisition Manager** Window
2	Enter Carousel number in the **Carousel #** column.
3	Enter Sample number in the **Sample ID** column.
4	To start, press **F9.**
5	Click on **Cytometer** from the main menu, click on **Cytometer control,** and click on **Setup Mode.**
6	Click on the **Detector tab** from the cytometer control, and click on **Volts** (FL3)
7	On the **FL3 INT Lin** window, position the mouse near the 0 origin of the plot to show the big sigma sign. Right click to show histogram statistics.
8	Position the histogram cursor **B** on the diploid peak do the CEN
9	At the **Cytometer control** window, click on **FL3 Volts.** Adjust voltage using the scroll bar, until the CEN X-mean is 85.0 ± 1.
10	Click **Acq.Setup** tab on the **Cytometer Control,** click **Setup Mode** to acquire the CEN events.
11	Record adjusted CEN or CRBC mean on the DNA Quality Control, Internal Standard/Linearity Check, Form F#247.
12	Acquire 2nd tube on the carousel containing a mixture of CEN and peripheral blood lymphocytes.
13	Position the G1 peak of the CEN using histogram cursor labeled B. Also, position the Diploid peak of the normal lymphocytes, using histogram cursor labeled C.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

Step	Action
14	After the acquisition of the 2nd tube containing the mixture of Normal blood lymphocytes and CEN, record the following parameters on Form # F247: a) Record X-mean of NPB Lymphs on the Mean peak channel column and X-mean of CRBC or CEN on the Mean peak channel column. b) Calculate the ratio of Normal CL/CRBC, and record on Form # F247. c) Record the HPCV of Go/G1 of normal peripheral blood lymphocyte (CL) on the Form #F247.
15	When all parameters are adjusted, continue the acquisition process with DNA samples.

How to analyze paraffin embedded DNA samples (Diploid) using Multicycle for Windows.

Step	Action
1	At the desktop, double click **Multicycle for Windows (AV DNA Analysis Software Version 328)** icon.
2	Click **File** and Click **Open**. **Note**: The folder that contains the DNA histograms is in the **LMD** folder. See below is tree to describe the file destination hierarchy. **My Computer/Local Disk C/BeckmanCoulter/Users/BT Lab/LMD**
3	Once LMD folder is reached, double click the folder and select the file to be analyzed., choose, FCS and click Open. At Parameter Selection choose: FL3 Peak LIN- FL3 PEAK LIN, Click, Ok (twice)
4	Click **Analyze** at the main menu, and click **Autofit** or press **F7.**
5	To refit histogram, Click **Analyze**, and click **Refit Histogram.** a) To adjust the position G1 mean, click the **G1 mean** on the **Analyze One Cell Cycle** window, position the mouse cursor to the center of the G1 peak and right click mouse button. b) To adjust the G2 mean, click the **G2 mean** and position the mouse cursor to the center of the G2 and left click mouse button.
6	Click **OK** on the Analyze On Cell Cycle window.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

Step	Action
7	To calculate percentage of background aggregate and debris (BAD) click Confidence Model—Aggregate. **Note:** % Background aggregate and debris should less than 20%, if it is greater than >20%, it should be referred to the pathologist for possible re-cut and re-staining of sample due preponderance of events in the histogram between the lowest G1 and the highest G2 that are debris and aggregates, as modeled by the analysis program.
8	To print histogram: a) Go to **Options** Menu, choose Display #2. b) Choose **Annotation:** Enter 270 for Angle, Type Diploid, ok c) To Edit Text right click, Annotated text, make changes, and choose, **Print** and choose Print Current Window.

How to analyze paraffin embedded DNA samples (Aneuploid) using Multicycle for Windows.

Step	Action
1	Double click **Multicycle for Windows** icon, click **File** from the main menu, click, **Open** and choose file to be analyzed.
2	At WinCycle main menu, click **Analyze** and click **AutoFit.**
3	To adjust G1 Peak, Click Analyze, click ReFit Histogram.
4	At the **Analyze Two cell Cycles window,** click **G1 Mean**.
5	Position the mouse cursor at the center of the G1 Peak and right click the mouse.
6	The value of the **G2 Mean** should approximately twice as much as the G1 Mean, if not adjust the position of the G2 Mean.
7	To adjust the position of the G1 aneuploid peak, click on **A1 Mean**, and position the mouse cursor at the center of the peak and right click the mouse.
8	**A2 Mean** should be located about twice the value of the **A1 Mean.** Adjust if necessary.
9	Click the confidence model: **Aggregate** to show results of Background Aggregate and Debris. BAD should be less the 20 minutes. **Note:** % Background aggregate and debris must less than 20%, if it is greater than >20%, it must be referred to the pathologist for possible re-cut and re-staining of sample due preponderance of events in the histogram between the lowest G1 and the highest G2 that are debris and aggregates, as modeled by the analysis program.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

How to analyze fresh tissue DNA samples (Diploid) using Multicycle for Windows.

Step	Action
1	At the desktop, double click **Multicycle for Windows** icon.
2	Click **File** and Click **Open**. **Note**: The folder that contains the DNA histograms is in the **HST** folder. See below is tree to describe the file destination hierarchy. **My Computer/Local Disk C/BeckmanCoulter/Users/BT Lab/LMD**
3	Once HST folder is reached, double click the folder and select the file to be analyzed.
4	Click **Analyze** at the main menu, and click **Autofit** or press **F7.**
5	To refit histogram, Click **Analyze**, and click **Refit Histogram.** c) To adjust the position G1 mean, click the **G1 mean** on the **Analyze One Cell Cycle** window, position the mouse cursor to the center of the G1 peak and right click mouse button. d) To adjust the G2 mean, click the **G2 mean** and position the mouse cursor to the center of the G2 and left click mouse button.
6	Click on **Background Fit**, and de-select **Sliced Nuclei.** Click, **OK.**
7	Click **OK** on the Analyze On Cell Cycle window.

How to analyze fresh tissue DNA samples (Aneuploid) using Multicycle for Windows.

Step	Action
1	Double click **Multicycle for Windows** icon, click **File** from the main menu, click, **Open** and choose file to be analyzed.
2	At WinCycle main menu, click **Analyze** and click **AutoFit.**
3	To adjust G1 Peak, Click Analyze, click ReFit Histogram.
4	At the **Analyze Two cell Cycles window,** click **G1 Mean**.
5	Position the mouse cursor at the center of the G1 Peak and right click the mouse.
6	The value of the **G2 Mean** should approximately twice as much as the G1 Mean, if not adjust the position of the G2 Mean.
7	To adjust the position of the G1 aneuploid peak, click on **A1 Mean**, and position the mouse cursor at the center of the peak and right click the mouse.
8	**A2 Mean** should be located about twice the value of the **A1 Mean.** Adjust if necessary.
9	Click on **Background Fit**, and de-select **Sliced Nuclei.** Click, **OK.**

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

10	Click the confidence model: Aggregate to show results of Background Aggregate and Debris. BAD should be less the 20%. **Note:** % Background aggregate and debris should less than 20%, if it is greater than >20%, it should be referred to the pathologist for possible re-cut and re-staining of sample due preponderance of events in the histogram between the lowest G1 and the highest G2 that are debris and aggregates, as modeled by the analysis program.

How annotate DNA histogram files:

Step	Action
1	At the main menu, click on **Options** and select **Add New Annotations.**
2	To label the diploid peak: a) Angle: enter 270 degrees. b) Font: Select **Arial font**, size **12**, and select **Bold.** c) Click **OK.**
3	If applicable label aneuploid peak. a) Angle: enter 270 degrees b) Font: Select **Arial** font, size **14**, and select **Bold.**
4	To label Name, Medical Record number, and DNA case number. a) Angle: 0 degrees b) Font: Select **Arial** font, size **14**, and select **Bold.**

Interpretation

- Auto fit option performs automated cell cycle analysis:
  - Automatic peak detection and analysis of 1, 2 3 cell cycles.
    Each peak is displayed with designation on the histogram graph:

Diploid	First Aneuploid Population	2nd Aneuploid population
G1, G2	A1, A2	B1, B2

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

Note: The computer estimates of each of the six peaks is indicated by colored vertical line over the histogram, and by a number to the right of the analysis option items.[G1mean:], [G1mean:], [A1mean;], [A2mean:], [B1mean;], [B2mean:]. The position of the peaks should be examined to ensure that each is near to the desired peak position. An exact estimate is not required. Each of the 6 peak positions can be revised the mouse cursor.

(If one place the G1 peak over the A1 or B1 or vice-versa, the peak assignment will be switched)

- Background debris fitting
- Least squares fitting of cell cycle model.
- Confidence analysis of cell cycle data.

Note: Since most data contains some aggregates, Model #4 + (Aggregates) is selected after Autofit option is executed.

- Sliced Nucleus Debris Modeling is recommended method for fitting debris. Nuclei can be cut in to pieces by sectioning paraffin blocks or by mincing tissue. Sectioning and mincing produce a characteristic flatter of continuum of noise to the left side of the peaks. The sliced nucleus is added to the histogram as debris.
- Sliced Nuclei option is used in analysis of material from paraffin block. If an aneuploid peak with a D.I is ≤ 1.5 cut nuclei of this population will overlap the S phase of the diploid population yielding a falsely elevated S of diploid. If D.I is ≤ 1.5, report Average S- Phase, and if DI is > 1.5 examine histogram, and if there is no significant overlap, report regular S-phase.
- Zero order of S phase (rectangular) is used for histograms that show preponderance of background debris. The zero order S phase option allows the tilt of the data in the S phase region to be assigned in the debris area not to the S phase itself.

- 2^{nd} order S phase (curved) is suitable for experimental analysis of cultured cells and may result in an overestimation of S phase.
- First order S phase (trapezoidal) is for histograms of highest quality where there is no ambiguity between overlap of G1 and S or S and G2 phase.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

- **DNA Histogram Interpretation Guidelines:**

  - DNA ploidy status of tumors is defined as the amount of DNA relative to normal. The DNA index (DI) is a value given to express the amount of DNA content relative to normal and is calculated by the following equation:

  - DI = mean or modal channel no of DNA aneuploid Go/G1 peak ÷ mean or modal channel of DNA diploid Go/G1 peak.

  - Definition of terms:

Term	D.I. Value	Range
Diploid (normal DNA content)	1.00	0.9-1.10
Hypodiploid	<1.00	<0.90
Hyperdiploid	>1.00	>1.10
Tetraploid	=2.00	1.90-2.10
Near Tetrapoid	1.85-1.90	Or 2.10 – 2.15
Aneuploid	DI does not equal to 1.00	
Multiploid	More than 2 Go/G1 peak populations.	

  - DNA diploid is reportable if:
    - Only one cycling population is seen.
    - The CV is less than 5% for fresh.
    - The CV is less than 7% for paraffin tissue.
    - Review of the fresh tissue cytospin reveals at least 15% tumor cells.
    - Visually have low debris.
    - Fluorescent scope evaluation is satisfactory.

  - DNA aneuploidy is reportable if:
    - If two resolvable peaks are identified.
    - The CV is more than 5% for fresh.
    - The CV is more than 7% for paraffin tissue.
    - At least a 10% tumor peak is found with corresponding G2M.

  - DNA tetraploid is reportable if

    - 15% to 20% of the total events collected are in the 4N peak position with visible G2M.
    - Fluorescent microscope review of stained cells shows no aggregated cells.

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- Tetraploid with 25% in 4N position without a visible G2M , no aggregated cells, and the cytospin is adequate.
- **Note: liver and endocrine tissues can be normally tetraploid.**

- Questionable/borderline ploidy is reportable if:
  - Small peak falling short of 10% tumor cells, problem of artifact or low tumor concentration.
  - Left of right shoulder in the diploid peaks (asymmetrical peaks).
  - Diploid peak position cannot be identified.
  - High B.D. or B.A.D.% greater than 35%.

- Uninterpretable ploidy analysis:
  - All debris.
  - Cell yield insufficient, less than 10,000 cells collected (paraffin embedded tissue)
  - Poor resolution, CV greater than 5% for fresh or frozen tissue greater than 7% for paraffin embedded tissue.

- Cycle statistics is reportable if:
  - Sufficient population (more than 10,000 cells collected) to allow poly model (peak substraction model) to produce accurate statistics.
  - Only if CV is less than or equal to 5% for fresh or frozen or 7% for paraffin embedded tissue.
  - If B.D. or B.A.D is less than or equal to 20% (S-Phase is reportable).

- Hydatidiform mole is extremely useful in distinguishing partial hydatidiform mole from complete hydatidiform mole. When combined with clinical history and careful gross and microscopic examination, DNA ploidy analysis is useful and strong indicator of diagnosis.
- Partial moles are usually triploid while complete moles are diploid or tetraploid. However, most products of conception are diploid by flow cytometry, diploid histogram does not suggest complete hydatidiform mole unless supported clinically and microscopically

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Reporting and Documentation

- The results of the DNA ploidy analysis are given to the clinicians by the assigned pathologist.
- DNA requisitions, DNA histograms and DNA worksheets are forwarded back to the BT Laboratory for filing.

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

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Controlled Document

- Form # F045 Requisition Form: DNA Ploidy Analysis for Flow Cytometry
- Form # F247: DNA Quality Control Internal Standard/Linearity Check.
- Worksheet for DNA Diagnostic Worksheet (Printed from BT Lab Program).
- LGM 8000 – Safety Practices.
- LGM 8004 - Infection Control.
- LGM 8005 – Universal Body Substance Precautions.
- LGM 8006 – Handling of Regular and Infectious Waste.
- LGM 8007 – Cleaning Work Areas.
- LGM 8010 – Handwashing Policy.
- LGM 8012 – Storage and Disposal of Chemical Hazardous Waste.

Uncontrolled Document

- Banez El, et. al. (1992) False aneuploidy in benign tumor with a high lymphocyte content. Human Pathology 23: 1244-1251.
- Rabinovitch, Peter S. (2002) Multicycle AV for Windows. *Multicycle DNA Content and Cell Cycle Analysis Software.* Phoenix Flow Systems. San Diego, CA.
- Multicycle for Windows. (2004) Help Section of the Software: Version 3, University of Washington.
- Wersto R.P. Liblit R. L., and Koss L.G. (1989). Flow cytometric DNA analysis of human solid tumors. A review of the interpretation of DNA histograms. Human Pathology 22:1085-1098.
- Lage, JM and Popek EJ. 1993. The role of DNA flow cytometry in evaluation of partial and complete hydatidiform moles and hydropic abortions. Semi Annual Diagnostic Pathology 1993 Aug;10(3):263-74.
- Clinical Medicine: Pathology, 2008, 1:61-67.
- Rafael Nunez: DNA Measurement and Cell Cycle Analysis by Flow Cytometry: Current Issues Molecular Biology, (2001), 3(3):61-70. Caiseter Academic Press, 2001

Self Assessment

- The an ideal results of Background Aggregate and Debris should be:
  a) > 20%
  b) < 20%
  c) does not matter

DNA CONTENT ACQUISITION AND ANALYSIS USING NAVIOS FLOW CYTOMETER, continued

- **TRUE or FALSE:** Sliced Nuclei option is used in analysis of material from paraffin block. If an aneuploid peak with a D.I is ≤ 1.5 cut nuclei of this population will overlap the S phase of the diploid population yielding a falsely elevated S of diploid.

- **TRUE or FALSE:** Zero order of S phase (rectangular) is used for histograms that show preponderance of background debris. The zero order S phase option allows the tilt of the data in the S phase region to be assigned in the debris area not to the S phase itself.

- **TRUE or False:** Cycle statistics is reportable if only if CV is less than or equal to 5% for fresh or frozen or 7% for paraffin embedded tissue.
- **TRUE or False**: Uninterpretable ploidy analysis may due to overwhelming presence of debris, insufficient cell yield (<10,000 cells collected) or poor resolution, CV >5% for fresh or frozen tissue or greater than 7% for paraffin embedded tissue.

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