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PREPARATION OF SPECIMEN FOR DNA CONTENT ANALYSIS ON PARAFFIN-EMBEDDED TISSUE

Purpose

The purpose of this procedure is to describe the steps of preparing sample for DNA ploidy analysis on paraffin embedded tissue.

In general tumors showing DNA aneuploidy have a worse prognosis than those tumors without aneuploidy. For evaluation of solid tumors, DNA content analysis is useful prognostic indicator.

DNA content analysis involves the isolation of nuclei from paraffin embedded tissue, and then analyzed by flow cytometry. Tissue sections (50 microns) are cut from the tissue blocks using microtome and then dewaxed using xylene or other histology clearing solvent. The sections are rehydrated by sequentially immersing the tissue sections in a series of ethanol/water solutions. Singe cell suspensions are prepared by digesting the tissue in an enzymatic buffer (trypsin). The cells are then treated with RNAse to destroy the double stranded RNA and the cell suspension is reacted with propidium iodide (intercalating nucleic acid stain). The laboratory uses flow cytometry to measure DNA content to determine the ploidy of the sample. MulticycleAV for Windows (DNA analysis software) is used to calculate percentage of cells in the S-phase and G2+M phase of the cell cycle.

Worksafety

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-0127, specifically, but not limited to, proper use of gloves.
- For proper handling of regular and infectious waste, see LAMC-PPP-0129, 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.

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Policy

- DNA laboratory request form may be obtained from SCPMG Flow cytometry Laboratory at 8-363-8542. Complete all requested information in the form.
- Turn-around time: 7 days upon receipt of the specimen. DNA ploidy is a batch tests, and lab does not accept rush cases.
- Kaiser SCPMG Flow Cytometry laboratory located at 4867 Sunset Blvd., First Floor, Room 1714 (Tie lie: 8-363-8542) performed DNA ploidy analysis, and service is available from Monday through Friday (8:30AM to 5:30PM), Request can be ordered by the pathologist and/or oncologist.
- For multiple reagent kit, use only the components within kit lot.

Specimen Requirement

- Paraffin block which contain tumor from the Histology Laboratory.
  Note: if more than one block of tumor, submit two tumor blocks.
  a) Site of tumor – 3 each of 50 micron section.
  b) Site of normal tissues -3 each of 50 micron section (to be used as a control-optional).

- Only tissue in paraffin block fixed with 10% formalin is suitable.

Send the following:
a) Competed DNA analysis request form.
b) H & E sections.
c) Paraffin blocks
d) Corresponding pathology report.

Reagent, Materials and Equipment

Materials:
- Plastic tubes (polystyrene) 12 x 17mm. with caps.
- Plastic conical centrifuge tubes, 15ml.
- Adjustable micropipet with tips.
- Aluminum foil.
- Microspoon.
- Microspatula.
- Microscope slides.
- Slide holder/rack.
- Slide stainer.
- Tissue forceps.

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Materials, continuation:

- Transfer pipets.
- Test tubes racks.
- Hemacytometer.
- Nylon tissue bags (Cat#66740013) –Shandon Inc.
- Nylon mesh: 37 micron Miami Aqua Culture

Reagents:

Modified Phosphate Buffered Solution: (PBS), pH 7.3.

- Stock solution
  - K2P04 = 188g.
  - NaH2P04 = 33g
  - NaCl = 180g
  - D.I. H20 to make 1 liter (store at room temperature)
- Working solution
  - Stock solution = 40ml.
  - D.I. H20 to make 1 liter. (store at room temperature).

- Trypsin Buffer
  - To 500ml D.I. H20 add:
    - 60.55 mg TRIS
    - 522.3 mg spermine tetrachloride
    - 822.3 mg. trisodium citrate
  - q.s. D.I. H20 to 1 liter adjust 7.6±0.2 using HCL solution, 1 M. add the following:
    - 1ml NON-IDET p-40 detergent.
    - 2.5g trypsin.
  - Mix thoroughly and refrigerate.
  - Stable for one month.
- Reagent alcohol or ethanol.
- Xylene or histology clearing solvent.
- Powder gelatin- J.T. Baker Inc. (Cat# 2124-.01)
- Chick erythrocyte nuclei control cell (CEN) – Biosure: Part #1013.

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- DNA-Prep Reagent Kit – Coulter (Cat#6604452)
    - DNA-Prep LPR (22ml).
        - 0.1% potassium cyanide.
        - 0.1% NaN3.
        - Nonionic detergents.
        - Saline.
        - Stabilizers.
- **Note: For multiple reagent kit, use only the components within kit lot.**
    - **Equipment:**

        - DNA-Prep Stain (3X70ml)
            - 0.5% propidium iodide
            - RNase [Type III-A bovine pancreas (4 IU/ml)
            - NaN3
            - Saline
            - Stabilizers
- **Note: For multiple reagent kit, use only the components within kit lot.**
    - **Equipment:**
        - Navios Flow cytometer.
            - Navios Flow Cytometer: AV50338
            - Navios Flow Cytometer: AV40313
        - HM 3350S Thermo Fisher microtome
        - Beckman refrigerated centrifuge—GPR
        - Incubator: oven, 60 degrees C
        - Water bath, 37 degrees C
        - Tissue flotation bath, 37 degrees C
        - Vacuum apparatus
        - Vortex mixer.
        - Biosafety Cabinet

Quality Control

- A suspension of chicken erythrocyte nuclei (CEN) will be run each time to calibrate the instrument mean channels.
- A "normal" lymphocyte control will be run with CEN to calculate ratio of the diploid peaks of chicken erythrocyte nuclei and normal lymphocyte control.

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Procedure:

Microdissection and sectioning of paraffin cut tissues:

Note: Paraffin cut tissues will be processed by microdissection. Microdissection is a process of dissecting specific tumor areas of the block to maximize the recovery of the tumor tissue. Both the 'normal' and 'pathologic' areas can be picked out from the same block. The hematopathologists will mark using marking pen the location of the tumor and normal tissue areas. All blocks must be reviewed by the hematopathologist. A representative cut (6 microns) stained with H&E must be submitted for hematopathologist review.

Step	Action
1	After the hematopathologist review of H&E slides, specimen (tissue block) and matched slides are sent to B&T Lab for cutting and processing. H&E slides have been marked and circled with instructions for microdissection.
2	Before cutting, turn on the tissue flotation bath so it will equilibrate to the desired temperature of 37 degrees C. Add a microspoonful of gelatin powder to make the tissue sections stick well to the microscope slides.
3	Place tissue blocks in an ice pan or spray it will tissue freezing aerosol before cutting.
4	Set microtome thickness to 50 microns. Turn upper left knob for adjustment.
5	Place tissue block on the microtome holder.
6	Cut at least 3 nice sections of the tissue block.
7	Fish out sections and place them in the tissue flotation bath.
8	Flatten sections and transfer them to labeled microscope slides. Blot dry, then air dry.
9	Place slides in slide holder and incubate in the incubator or oven at 60 degrees C for 10 minutes. Let it cool for five minutes and re-incubate for 10 minutes. This will melt out unwanted paraffin and facilitate better adhesion of tissue sections to the microscope slides. Note: For paraffin embedded needle biopsy tissue block. a) Scoop the entire tissue biopsy with a microspatula. b) Place the scooped tissue inside a labeled nylon biopsy bag (use one bag per specimen).

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Removal of paraffin and re-hydration of tissue:

Step	Action
1	Place microscope slides with cut sections of tissue block into the slide rack; or clamp nylon tissue bag with scooped tissue biopsy with tissue forceps.
2	Sequentially immerse the sample into the following solutions. **Note**: Transfer the sample from solution to solution very gently to avoid washing tissue off the slide.

Wash	Solution	Volume	Times
1	Americlear (clearing solvent)	200 ml Americlear	10 min.
2	Americlear (clearing solvent)	200 ml Americlear	10 min.
3	100% ETOH (reagent alcohol)	200 ml ETOH	10 min.
4	90% ETOH (reagent alcohol)	180 ml ETOH + 20ml DI water.	10min.
5	70% ETOH	140 ml ETOH + 60ml DI water	10min.
6	50% ETOH (reagent alcohol)	100ml ETOH + 100 ml DI water	10min.
7	100% DI water	200ml DI water	10 min
8	100% DI water	200ml DI water	10min.

Trypsin incubation of rehydrated tissue sample:

Step	Action
1	Label one 15 ml plastic conical centrifuge tube per sample.
2	Into each labeled tube aliquot 5ml of trypsin buffer (store in the refrigerator).
3	After the final wash (second D.I. water wash), gently scrape the tissue from each slide or each tissue bag into the properly labeled tube. **Note:** A microspatula may be used to push the tissue in to the conical tube. For microdissection – scrape only the specified circled area.

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Step	Action
4	Vortex each tube thoroughly, approximately 30 seconds per tube.
5	Cork each tube and place in to a 37 degrees water bath for incubation for 16-20 hours. Occasional vortexing is recommended.
6	Remove the tubes from the water bath and bring to room temperature. Vortex thoroughly.

Preparation of single cell suspension:

Step	Action
1	To stop the trypsin, add 10ml of Modified PBS to each tube (bring volume up to 15ml).
2	Centrifuge the tubes for 5 minutes at 1500 RPM. Remove supernatant, leaving approximately 1.0ml in the tube.
3	Repeat step 1 and 2.
4	Gently mesh the tissue (use a plastic transfer pipette head to agitate the tissue sample), then q.s. to 2.0ml with Modified PBS.
5	The suspensions are filtered to remove large aggregates of tissue and debris. a) Label three 12 x 75 plastic tubes per specimen. b) Filter suspension through a 210 micro nylon mesh into the first tube, then through a 37 nylon mesh into the second and third tubes. c) Check cell volume and q.s. to 2.0 ml with Modified PBS, if necessary.

How to perform cell count:

Step	Action
1	Use Propidium Iodide to stain the nuclei. (From DNA Prep Stain Kit)
2	Prepare a 1:2 dilution as follows: a) Mix cells thoroughly. b) Put 0.05 ml cell suspension into a labeled 12 X75 mm plastic tube. c) Add 0.05 ml Propidium and vortex. d) Let the solution sit for a few minutes before counting (at least 2 minutes.).
3	Use the hemacytometer to count the cells. To perform the cell count, count the four large outer squares.

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Step	Action
4	**To calculate the number of cells counted, use the following formula:** (#cell counted divided by #squares counted) (2 dilution) (10 depth) (1000)mm/mL^3. Example: (300/4) (2) (10) (1000) = 1.5 x 10^6 cells/ml. To obtain the final concentration of 1 x 10^6 cells/ml, the test sample should be suspended in 3.0 ml of Modified PBS. (Just obtain 1ml of aliquot of the cell suspension with 1 x 10^6 cells for staining. 3.0 x 10^6 cells available x 1ml/ 1 x 10^6 cells = 3ml of cells suspension available. **Note:** A final concentration for approximately 0.5- 1.0 x 10^6 / ml of staining buffer is needed for optimal results by flow cytometer. If this yield a final cell count of less than 0.5- 1.0 x 10^6 cells/ml, use only half the amount of lysing and staining reagents required.
5	**Staining of cell suspension.** a) Centrifuge the aliquot cell suspension at 1500 r.p.m. for 5 minutes at room temperature. Carefully decant all the supernatant. b) Add 100 ul of DNA-Prep LPR while vortexing and continue vortexing for 10 seconds. c) Using a disposable pipette, slowly add 1 ml of DNA –Prep Stain (Propidium Iodide) using vortex mixer. d) Incubate stained cells for 30 minutes at 4 degrees C.
4	**Preparation of normal lymphocyte control.** a) Obtain 100ul of "normal" peripheral blood control in a labeled tube. Specimen must be in EDTA and kept at room temperature. b) Add 100ul of DNA-Prep LPR while vortexing and continue vortexing for 10 seconds. c) Using a disposable pipette, add slowly, 1ml of DNA-Prep stain (Propidium Iodide and vortex as you add the reagent).

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Step	Action
5	e) Incubate cells stained with Propidium Iodide for 30 minutes at 4 degrees C.

Step	Action
5	**Preparation of chick erythrocyte nuclei (CEN) as an internal standard.** a) Put 2 drops of CEN in a labeled tube. CEN's are kept in a refrigerator. b) Add 100ul of DNA-Prep LPR while vortexing and continue vortexing for 10 seconds. c) Using a disposable pipette slowly add 1ml of DNA-Prep Stain (Propidium Iodide) and vortex solution as you add. d) Incubate stained cells with PI for about 30 minutes at 4 degrees C.
6	**Analysis of stained cells by flow cytometer.** a) Filter all cell suspensions through a 37 micron nylon mesh prior to acquisition of data using Navios cytometer. b) Refer to procedure # LAMC-PPP-0530.

Interpretation

- Not applicable for this procedure.

Reporting and Documentation

- Use LAMC-FORM-0066 for reporting cell count.
- Form LAMC-FORM-0068: Requisition form for DNA ploidy analysis.

Controlled Document

Document Number	Document Name
LAMC-PPP-0123 LGM 8000	Safety Practices
LAMC-PPP-0128 LGM 8005	Universal Body Substance Precautions (UBSP)
LAMC-PPP-0132 LGM 8010	Handwashing Policy
LAMC-PPP-0127	Infection Control
LAMC-PPP-0130	Cleaning Work Areas
LAMC-PPP-0530	DNA Content and Analysis Using Navios Cytometer.

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LAMC-PPP 0219	Handling of Regular and Infectious Waste
LAMC-PPP-0134	Storage and Disposal of Chemical Hazardous Waste
LAMC-FORM-0066	DNA Cell count Worksheet
LAMC-FORM-0068	Requisition Form: DNA Ploidy Analysis by Flow Cytometry

Uncontrolled Document

Coulter DNA-Prep Reagent Insert (2003). Cell lysing and permeabilizing, DNA staining and DNA reference Reagents. Coulter Immunology, Hialeah, FL 33010.

Barlogie b., et. al. 1983. Flow cytometry in clinical cancer research. Cancer Research 43:3982-3997.

Hedly, D. et. al. (1983). Method of analysis of cellular DNA content of paraffin embedded pathologic material using flow cytometry. The Journal of Histochem. And Cytochem. 31.31 (11) 1333-1335.

Hiddleman W., et. al. (1984). Convention on Nomenclature for DNA Cytometry. Cytometry S:445-446. 1984.

Krishan, A. (1975). Rapid flow cytofluorometic analysis of mammalian cell cycle by propidium iodide staining. J. Cell Biology. 66, 188-193.

Schutte, B. et.al. (1985). Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin embedded tissue. Cytometry 6, 26-30.

Vindelow, L. (1977). Flow Microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and staining of nuclei. Virchow Archi Cell Path. 24:227-242, 1977.

Vindelow, L. (1983). Detergent-Trypsin method for the preparation of nuclei for flow cytometric DNA analysis cytometry. 3,(5) 227-323.

Self Assessment

1. Trypsin incubation of rehydrated tissue is done by:

   a) Incubation of DNA sample at 37 degrees C for 16-20 hours.
   b) Incubation of DNA sample at room temperature for 16-20 hours.
   c) Incubation of DA sample at 4 degrees C for >20 hours.

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2. The ideal concentration of cells for DNA analysis is:
   a) Approximately 0.5-1.0 x 10^6 cells/ml.
   b) Approximately 10-20 x 10^6 cells/ml.
   c) Approximately 20-30 x 10^6 cells/ml.

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