STANDARD OPERATING PROCEDURE

Title: Immunohistochemistry (IHC)/Tissue Microarray (TMA) of NCI60 Cell Line Array (CMA)

SOP#: M-106

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1. PURPOSE

The purpose of this document is to describe the procedure for immunohistochemistry (IHC), tissue microarray (TMA), and antibody titration. This immunohistochemistry protocol can be used with novel antibodies, but starting titers of antibody must be developed from other information, such as preliminary data from the Well-based "Reverse Phase" Protein Array screen of the NCI60 panel of protein lysates (SOP, M-105).

2. SCOPE

This procedure may be used for general immunohistochemical staining and antibody titration.

3. RESPONSIBILITIES

It is the responsibility of person(s) performing this procedure to be familiar with laboratory safety procedures. The interpretation of results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

- Leica Autostainer model XL
- Dako Autostainer model LV-1 or equivalent
  Note: The use of automated stainers is not required, however assay reproducibility and throughput is significantly enhanced by their use.
- Vegetable Steamer (Black & Decker or equivalent)
- Coplin Jars with lids

5. MATERIALS

- Hitology Reagents:
  - Xylenes; Mallinckrodt Chemicals, Cat. #8668-16
  - Alcohols; Sigma-Aldrich, Cat. #459844
- Antibody Dilutent; Dako, Cat #S0809
- IHC Wash Buffer; Dako, Cat #S3006
  - Dilute 1:10 with Deionized Water
- End Enzyme Block
  - 3% peroxidase blocking reagent; Dako, Cat. #S2001
- Protein Block
  - Protein block serum-free buffer; Dako, Cat. #X0909
- Antigen Retrieval Buffer
  - Target retrieval high pH; Dako, Cat. #S3307
  - Dilute 1:10 with Deionized Water
- Primary Reagent
  - Primary antibody dilute w/antibody diluent; Dako, Cat. #S0809
- Secondary Reagent (LSAB)
  - Biotinylated link universal; Dako, Cat. #K0690
- Tertiary Reagent (LSAB)
  - Streptavidin-HRP; Dako, Cat. #K0690
- Substrate (LSAB): Working Solution is 1 ml Substrate Buffer and 1 drop of chromagen
  - DAB+ Substrate buffer; Dako, Cat. #K3468
  - DAB+ Chromogen; Dako, Cat. #K3468
- Gill Hematoxylin
  - Lerner-2 hematoxylin; Lerner Laboratories, Cat. #1931413
- Bluing Reagent; Thermo Scientific Cat #ACC93666
- Coverslip media
  - Cytoseal XYL Mounting Medium; Richard Allan Scientific, Cat. #8312-4

6. REAGENTS

- Purified monoclonal antibodies to be tested
- NCI60 Cell Line Array (CMA), Tissue Microarray (TMA)

7. PROCEDURE

7.1.

7.1.1. If the maximum signal of the protein array screen is 5× background (~250 counts) as determined in SOP M-105, proceed to IHC of NCI60. If signal is less than 5× background, hold antibody evaluation.

7.1.2. To determine an estimated starting titer - calculate the average raw signal of the NCI60 panel from the protein array (SOP M-105).

7.1.2.1. For average signals of less than 500 arbitrary units, start diluting the antibody at 1:50.

7.1.2.2. For average signals greater than 500 arbitrary units, first determine an estimated antibody titer by multiplying by 7.5. Then choose 2 additional titers, one which is 5× less the original titer and another that is 5× greater. (Example - estimated starting titer 1:1000, additional titers to test : 1: 200 and 1:5000)

- Do not use a titer less than 1:50.

7.1.3. Dilute the target antibody in antibody diluent to represent the chosen titers defined in 7.1.2.

7.1.4. Obtain slides containing 5 μm sections of the entire NCI60 (NCI60 CMA).

7.1.5. Deparaffinize (remove paraffin) the slide using the autostainer XL (Leica).

Program of autostanier

Solution	Time (Min)
Xylene	2
Xylene	2
Xylene	2
100% ETOH	2
100% ETOH	2
95% ETOH	2
95% ETOH	2
70% ETOH	2
Water	2

7.1.6. Prepare 200 ml 1x Antigen Retrieval Buffer and then preheat the solution for 20 minutes in the vegetable steamer.

- Dilute 10x Antigen Retrieval Buffer 1:10
  - 180 ml deionized water
  - 20 ml Antigen Retreival Buffer

7.1.7. Place Slides in Coplin Jars with lid and Incubate the deparaffinized slides for 20 minutes in the vegetable steamer.

7.1.8. Cool down the slides at room temperature for 20 minutes.

7.1.9. Rinse the slides under running deionized water.

7.1.10. Place the slide in 200 ml IHC Wash Buffer solution for 10 minutes

- Dilute 10x Wash Buffer 1:10 using deionized water

7.1.11. Treat slides with LSAB labeling system (Dako) using the Dako autostainer

Dako Autostainer Program

Solution	Time(min)
-Rinse (Wash Buffer)	
-End Enzyme Block	10
-Rinse (Wash Buffer)	
-Protein Block:	10
-Primary Reagent	120
-Rinse (Wash Buffer)	
-Secondary Reagent	15
-Rinse (Wash Buffer)	
-Tertiary Reagent	15
-Rinse (Wash Buffer)	
-Substrate	20

7.1.12. Place slides on Autostainer (Leica) for hematoxylin counterstaining

Hematoxlin Counterstain

Solution	Time(Min)
Water	1
Hematoxylin	30 sec
Water	1
Bluing Reagent	30 sec
Water	1
70% ETOH	2
95% ETOH	2
100% ETOH	2
100% ETOH	2
Xylene	2
Xylene	2
Xylene	2

7.1.13. Place coverslip on the slides with cytoseal XYL Mounting medium using no more than three drops.

7.1.14. Interpretation is based on nuclear, cytoplasmic or membranous staining (or any combination there of) of the individual cells. Cell lines are scored 0, 1, 2, or 3 for no, weak, moderate and strong staining patterns respectively. Choice of correct titration of the antibody is based on maximum dynamic range of staining - negative to positive in different cell lines, combined with uniformity of staining within cell lines at the minimum dilution to obtain this staining pattern.

8. REFERENCED DOCUMENTS

8.1. Operation manual, Leica Autostainer model XL

8.2. Operation manual, Dako Autostainer model LV-1

8.3. Tissue Microarray (T-MTA-6A)

(www.cancer.gov/tarp)

8.4. NCI60 Cell Line Array (CMA)

Braunschweig T, Chung J-Y and Hewitt SM**.** (2005) Tissue Microarrays: Bridging the Gap between Research and the Clinic. *Exp Rev Prot* **2** (3) 325-36.

8.5. SOP M-105: Well-based “Reverse Phase” Protein Array