

STANDARD OPERATING PROCEDURE

Title: Well-base "Reverse Phase" Protein Array

SOP#: M-105

Version #: 2

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1. PURPOSE

The purpose of this document is to describe the procedure for performing a "reverse phase" protein array to determine specificity of antibodies in development as well as provide data on the expression pattern of the target protein in the NCI60 cell line panel.

2. SCOPE

This procedure may be used for testing antibodies, or screening expression of proteins with a qualified antibody.

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

- MESO QuickPlex SQ 120 reader; Meso-Scale Discovery (MSD)
- Multi-Array 96 HB, SECTOR Plate (MSD, Cat. #L15XB)
- Titer Plate Shaker (Barnstead Lab-Line, model 4625 or equivalent)
- Microplate Washer; BioTek™ ELx50™
- Rocking Platform (GenHunter Corporation or equivalent)
- Precision pipettes

5. MATERIALS

- PBST

-0.5 ml of Tween 20 (Bio-RAD, Cat. #170-6531)

-100 ml of 10x PBS (Quality Biological, Cat. #119-069-101)
-899.5 ml with Ultra Pure Water (K.D Medical, Cat. #RGF-3410)

- MSD-T Read Buffer (4x) w/Surfactant (Meso-Scale Discovery, Cat. #R92TC-2)
- Blotting Grade Blocker non-fat dry milk (Bio-Rad, Cat. #170-6404)
- BSA (MP Biomedicals, Cat. #160069)
- Pierce® RIPA Buffer (ThermoFisher Scientific, Cat. #89900)
- Pierce® BCA Protein Assay Kit (ThermoFisher Scientific, Cat. # 23225)

6. REAGENTS

- Purified monoclonal antibody to be tested
- Secondary Antibody: Goat anti-mouse Sulfo-tagged antibody (500 µg/ml, Meso-Scale Discovery, Cat. #R32AC-5).
- Anti-GAPDH Monoclonal Antibody, Clone 6C5 (EMD Millipore, Cat. #CB1001)

7. PROCEDURE

7.1. Protein Array Screening of 3 cell lines

- 7.1.1. Organize antibodies to be evaluated in Excel format by antigens.
- 7.1.2. Using the DTP Compare online database for NCI60 cell lines (http://dtp.nci.nih.gov/mtargets/mt_index.html), choose three cell lines that indicate high, medium, and low mRNA expression for the protein targeted by the antibody.
- 7.1.3. Pipet 5 µl of chosen cell line lysate at 100ng/µl to the wells of the Multi-Array plate.
 - 7.1.3.1. Cell lines were grown by standard protocol, scraped for harvesting, lysed in equal volumes of RIPA buffer with added protease inhibitor and stored at -80°C per manufacturer's instructions.
 - 7.1.3.2. Protein concentrations were determined by BCA Protein Assay per manufacturer's instructions.
- 7.1.4. After adding the cell line lysate to the 96 well plate, air dry the plate at room temperature for a minimum of 2 hours (up to overnight at room temperature).

- 7.1.5. Add 150 μ l per well of 5% nonfat milk in PBST solution to block the plate. Incubate the plate for 1 hour in the Titer plate shaker with gentle agitation (setting 2 on dial).
- 7.1.6. Wash the plate 5x with PBST using the microplate washer at 300 μ l per well.
- 7.1.7. Add 25 μ l per well of the target antibody (1:1000 to 1:10000 dilution), diluted in a 5% BSA in PBST solution. The typical concentration of target antibodies is approximately 1 mg/ml. (most commonly between 0.8 and 1.2 mg/ml)
- 7.1.8. Incubate the plate over night at 4°C under gentle agitation using the rocking platform at 2 shakes per minute.
- 7.1.9. Repeat the wash as outlined in step 7.1.6.
- 7.1.10. Add 25 μ l per well of secondary antibody (1:2000 dilution), diluted in a 5% nonfat dry milk in PBST solution.
- 7.1.11. Incubate the plate for 1 hour and 30 minutes at room temperature with agitation on the Titer plate shaker with gentle agitation (setting 2 on dial).
- 7.1.12. Repeat the wash as outlined in step 7.1.6.
- 7.1.13. Add 150 μ l of MSD-T Read Buffer (Dilute 1:4 in Ultra Pure Water) to each well and allow the plate to incubate for 2 minutes at room temperature.
- 7.1.14. Read the plate using the MSD MESO QuickPlex SQ 120 reader according to manufacturer's instructions.
- 7.1.15. Protein Array Re-screen (if necessary)
 - 7.1.15.1. Minimum signal should be two times background (background ~50 counts). Determine if results correlate with mRNA expression. A general trend approach is sufficient correlation. If the protein array data does not recapitulate a general low, moderate, strong trend seen in the RNA data then the protein array screening assay is repeated with three different cell lines of the NCI60 found by means of DTP Compare.
 - 7.1.15.2. If the data again fails to conform to the data viewed on DTP Compare after two attempts, discontinue the use of the antibody.

7.2. Protein Array Screening of NCI60

- 7.2.1. If the signal strength of the antibody conforms to the known gene expression profile of the cell line, and the maximum

signal is more than twice background, repeat the assay with the antibody against the entire NCI60 panel.

- 7.2.2. Pipet 5 μ l of the cell line lysate for each cell line of the NCI60 into 4 wells of the MSD plate and repeat protein array screening steps 7.1.4-7.1.6.
- 7.2.3. Once completed, add 25 μ l of target antibody (1:1000 to 1:10000 dilution) diluted in 5% BSA in PBST to the first 2 wells containing the specific cell line of the NCI60. In the other 2 wells add 25 μ l of GAPDH (1:10000) diluted in 5% BSA in PBST.
- 7.2.4. Repeat this process for the entire NCI60 panel, leaving a blank row to be used as a background for PBST.
- 7.2.5. Repeat protein array screening steps 7.1.8 – 7.1.14
- 7.2.6. Data Evaluation
 - 7.2.6.1. Protein array data can be expressed in the arbitrary units of the assay, or as a ratio of a secondary marker, such as GAPDH.
 - 7.2.6.1.1. The advantage of presenting it in relationship to GAPDH or other housekeeping gene is that this provides some comparison to what might be encountered with Western Blot analysis.
 - 7.2.6.2. The data presented on the reagent's web portal (<http://antibodies.cancer.gov/>) is normalized by a reference GAPDH expression level utilizing anti-GADPH and the same protocol.
 - 7.2.6.3. The results of this normalization are then normalized to a mean equal to 1.00 and standard deviation equal to 0.5.
 - 7.2.6.3.1. Levels below 0.5 should be interpreted as weak or negative expression.
 - 7.2.6.3.2. Levels above 1.5 should be interpreted as strong, positive expression.

8. REFERENCED DOCUMENTS

- 8.1. Chung, J-Y, Lee, S-J, Ylaya K, Braunschweig T, Traicoff JL, and Hewitt SM. (2008) A well-based reverse-phase protein array applicable to extracts from formalin fixed paraffin embedded tissue. *Proteomics, Clinical Applications* 2: 1539-47.
- 8.2. Instruction manual, ThermoFisher Scientific, RIPA lysis
- 8.3. Instruction manual, Pierce Biosciences, BCA Assay

- 8.4. Operation manual, BIO-TEK, plate washer
- 8.5. Operation manual, Barnstead Lab-Line, Titer Plate Shaker
- 8.6. Operation manual, Meso-Scale Discovery, MESO QuickPlex SQ 120 Reader
- 8.7. Operation manual, GenHunter Corporation. Rocking Platform