STANDARD OPERATING PROCEDURE

Title: Evaluation using Indirect ELISA

SOP#: M-102

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1. PURPOSE

This purpose of this procedure is to evaluate the binding of purified monoclonal antibody and corresponding antigen in an endpoint indirect Enzyme Linked Immunosorbent Assay (ELISA).

2. SCOPE

This document describes the procedure for an indirect ELISA that is used to assess the reactivity of a monoclonal antibody to its antigen. The antigen can be a purified recombinant protein and/or a synthetic peptide (only as BSA-conjugated). Evaluation of unpurified monoclonal antibody (e.g.: hybridomas supernatants) and antigen reactivity is out of the scope of this procedure.

3. RESPONSIBILITIES

It is the responsibility of the person(s) performing this test to be familiar with lab safety procedures and to have basic laboratory skills. Data analysis and interpretation of the results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT AND MATERIALS

- Plate Shaker; Boekel Scientific Jitterbug 2 model 270400
- Plate Reader: Tecan Spark microplate reader (450nm wavelength)
- Plate washer: BioTek- 50/TS- microplate washer
- Microtiter plates – medium binding; Costar Cat. #9017,
- Polypropylene Tubes 15 mL sterile, disposable with cap - Thomas (Cat. # 2602A19)
- Polypropylene Tubes 50 mL sterile, disposable with cap – Thomas (Cat. # 2602A26)

- Multichannel pipette -L- 300 (20-300) -12 channel- Rainin pipet lite-XLS
- Multichannel pipette -L-20 -12 channel- Rainin pipet lite-XLS
- Reagent Reservoirs-high impact polystyrene, 50ml, sterile, Thomas (Cat. #-8600A58)

5. REAGENTS

- Carbonate-Bicarbonate Coating Buffer (pre-mixed powder) – prepared according to manufacturer's directions; Thermo Scientific Cat. #28382
- 1-Step TMB Turbo Substrate, Pierce-Thermo Cat. # 34022
- 0.18N Sulfuric acid; Thermo Scientific, Cat.# N600.
- Antigen:
    - Purified recombinant protein corresponding to the antibody;
    - BSA-conjugated peptide corresponding to the antibody.
- Purified monoclonal antibody corresponding to the Antigen (recombinant protein or BSA-conjugated peptide)
- Goat anti-mouse - horseradish peroxidase (GAM-HRP) labeled antibody; Jackson ImmunoResearch Laboratories Cat. # 115-035-166
- Goat anti-rabbit - horseradish peroxidase (GAR-HRP) labeled antibody; Jackson ImmunoResearch Laboratories Cat. # 111-035-144
- Phosphate Buffered Saline, 10x solution (Fisher Scientific Cat. #BP399-1)
- Tween 20; (Fisher Cat. #BP337-500)
- Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Cat. # 1706404XTU)
- Deionized H_2O (In-house H_2O purification system, Lab C1002)

6. PROCEDURE

6.1. Preparation of buffers

6.1.1. **PBS 1X**: Phosphate Buffered Saline, 10x solution diluted to 1x with deionized water to give 11.9 mM phosphate, 137mM NaCl, 2.7mM KCl, pH 7.4. Prepare fresh on day of use.

6.1.2. **PBS-T**: PBS – 0.05% Tween 20, prepared by addition of 500uL Tween 20 into 1 liter of PBS. Prepare fresh on day of use.

6.1.3. **Blocking Buffer**: PBS – 0.05% Tween 20, 0.5% Milk prepared by addition of 5 grams of Blotting Grade Blocker Non-Fat Dry Milk into 1 liter of PBS-T. Prepare fresh on day of use.

6.1.4. **Carbonate Bicarbonate (Coating) Buffer** – Dissolve content of a pouch of dry-blend powder in 500 mL of deionized water, to obtain 0.2M carbonate-bicarbonate buffer at pH 9.4. The carbonate bicarbonate (coating) buffer can be kept at room temperature for up to 1 month.

6.2. Antigen (Purified protein or BSA-Peptide) Coating

One 96-well plate can accommodate the screening of up to three different primary antibodies, because each 4-columns block will be used for one primary antibody.

6.2.1. Prepare a plate outline as seen in the Figure 1 below:

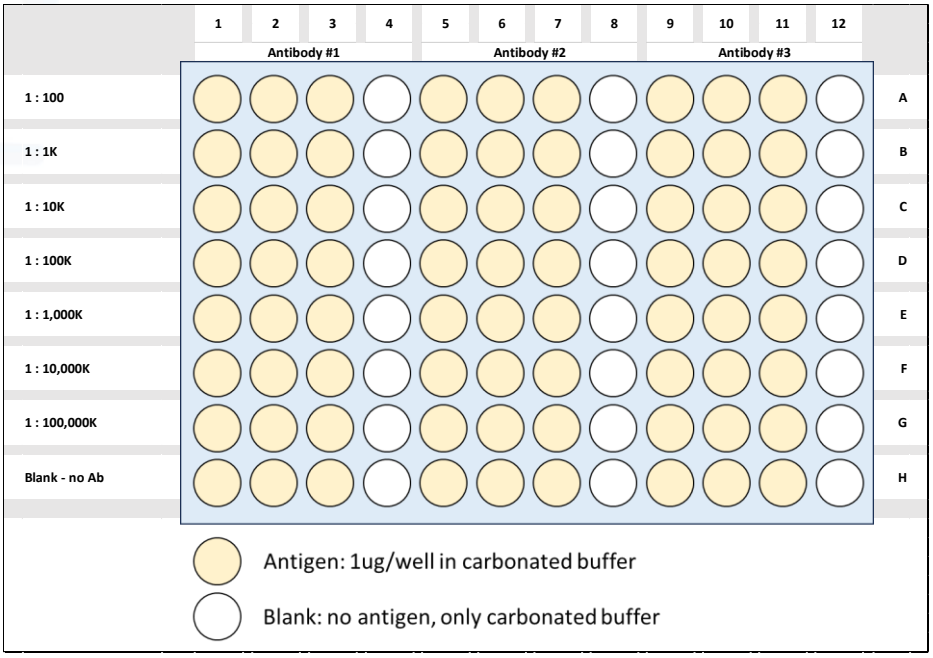

Figure 1.

6.2.2. Prepare a dilution of the antigen (protein or peptide) in Carbonate Bicarbonate Coating Buffer to give a final concentration of 10 µg/ml. For each primary antibody to be tested, prepare 3.0 mL of diluted antigen which is sufficient for 24 wells.

6.2.3. Mix thoroughly by vortexing.

6.2.4. Carefully transfer the diluted antigen (10 µg/ml) to a 50 mL plastic reservoir.

6.2.5. For primary Antibody #1, add 100 uL of diluted antigen to columns one, two, and three as outlined in Figure 1 (Section 6.2.1.) using a multi-channel pipette.

6.2.6. For primary Antibody #1. add 100 µl of Carbonate Coating Buffer to column 4, the no antigen control well, as indicated in Figure 1 (Section 6.2.1.) using a multichannel pipette.

6.2.7. Incubate the plate for one hour at 37°C with mild shaking at 350 RPM on the Jitterbug-2 plate shaker. Alternatively, incubate the plate for 16-24 hours at 4 – 8°C without shaking.

6.2.8. Wash the wells with 250 uL/well of PBS-T using the program named “ELISA protocol” on the BioTek microplate plate washer. Repeat 3x.

6.2.9. Immediately add 225 µl of Blocking Buffer using a multichannel pipette to all test and control wells to prevent non-specific binding.

6.2.10. Incubate for 30 minutes to 1 hour at 37°C with mild shaking at 350 RPM using a plate shaker.

6.2.11. Wash the wells with 250 µl PBS-T/well using the plate washer program named “ELISA protocol”. Repeat 3x.

6.3. Primary Antibody preparation and incubation

Prepare 0.5 mL of each test antibody/(ies) diluted to 0.1 mg/mL (0.1 mg/mL = 100,000,000 pg/mL) in 1X PBS-T in a polypropylene tube. Then perform a serial dilution according to the scheme below:

Antibody	Starting concentration (pg/mL)	Final concentration (pg/mL)	Fold Dilution
1 to 100	1.00E+08	1.00E+07	10
1 to 1000	1.00E+07	1.00E+06	10
1 to 10,000	1.00E+06	1.00E+05	10
1 to 100,000	1.00E+05	1.00E+04	10
1 to 1,000,000	1.00E+04	1.00E+03	10
1 to 10,000,000	1.00E+03	1.00E+02	10
1 to 100,000,000	1.00E+02	1.00E+01	10

6.3.1. Using a multichannel pipette, add 100µl of each Antibody dilution as indicated in Figure 1 (Section 6.2.1.) to

the appropriate wells. Use row H as Antibody control blank wells, and add only PBS-T buffer.

6.3.2. Incubate for 30 minutes to 1 hour at 37°C with shaking at 350 RPM.

6.3.3. Wash the plate using the plate washer with PBS-T as described in 6.2.8.

6.4. Secondary antibody preparation and incubation

6.4.1. Prepare a 1:5000 dilution of the GAM-HRP or GAR-HRP antibody (secondary antibody) corresponding to the primary antibody species.

6.4.1.1. Add 5µl of the secondary antibody to 25ml of PBS-T in 50 mL conical tube. This is sufficient secondary antibody solution for the full plate. Note, the concentration of the secondary antibody may need to be adjusted depending on the individual antibody performance.

6.4.2. Add 100µl of secondary antibody to each used well using a multichannel pipette.

6.4.3. Incubate the plate for 30 minutes to 1 hour @ 37°C with mild shaking at 350 RPM.

6.4.4. Wash the plate: decant and rinse 3 times with PBST as described in 6.2.8

6.5. Detection

6.5.1. Add 100µl of TMB substrate to each used well using a multichannel pipette.

6.5.2. Incubate at room temperature until sufficient blue color develops in the test wells. This time is usually 5 minutes, but an additional 2 minutes may be required for weak antibodies.

6.5.3. Add 100µl of 0.18N sulfuric acid to each well to stop the reaction using a multichannel pipette. The color will change to yellow.

6.5.4. Read the absorbance on the Tecan microplate reader using a 450 nm wavelength.

6.5.5. Export the excel result file to the appropriate project folder on the ACL Shared Drive.

6.6. Data analysis

6.6.1. Perform data analysis using a software program such as GraphPad Prism according to the following steps.

6.6.2. In GraphPad, create a new Project file by selecting an 'XY' data table with the options X – "Numbers" and Y – "Enter 3 replicate values in side-by-side sub columns".

6.6.2.1. In the Excel result file, subtract the value from the blank-treated well from each of the triplicate values from the wells treated with the matched Ab dilution. For example: [Well A1-Well A4], [Well A2 - Well A4], [Well A3 – Well A4], [Well B1-Well B4], etc.

6.6.2.2. Copy and paste the blank subtracted values into the corresponding Y1, Y2, and Y3 value cells in the new GraphPad file.

6.6.2.3. Add the seven final antibody concentrations (pg/mL) to the corresponding X value cells in the new GraphPad file: 1 x 10^7 to 10.

6.6.2.4. In GraphPad, select Analyze -> Data Processing ->Transform Concentrations X.

6.6.2.5. Select Transform Concentrations X and select 'OK'.

6.6.2.6. In the Parameters tab, check mark the Transform to Logarithms box and select the base of logarithms to log10 (common). Select 'OK'. The antibody concentration transformed values are 7 to 1.

6.6.2.7. Fit the data using the "Sigmoidal, 4PL X is log (concentration)" function to interpolate an IC50 (or EC50) value. Generate a graph that contains error bars for each triplicate value. The EC50 value is indicative of the binding strength of the antibody. The lower is the EC50 value, the stronger is the binding strength.

Note: the values from Row H are not included in the analysis. They are used as negative controls to verify the absence of non-specific binding due to secondary antibody interacting with the wells (for example H1-H3) coated with antigen or the blank well with no antigen (for example H4). If absorbance is measured in row H, it is not possible to evaluate the data properly and the experiment should be repeated.

7. REFERENCED DOCUMENTS

7.1.1. Operation manual, Tecan Spark microplate reader

7.1.2. Operation manual, Boekel Scientific Jitterbug 2 shaker/ incubator

7.1.3. Instructions for use, Pierce 1-Step TMB Turbo Substrate