STANDARD OPERATING PROCEDURE

Title: Evaluation using Indirect ELISA

SOP#: M-102

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1. PURPOSE

This procedure is to be used for the characterization of purified monoclonal antibody.

2. SCOPE

This document describes the procedure for the indirect Enzyme Linked ImmunoSorbant Assay (ELISA) that is used to assess the reactivity of the monoclonal antibodies for the recombinant antigens and peptides used as immunogens. It is possible to use this procedure for the qualification of the presence of endogenous protein in cell lysate but is not the primary intent. It is not to be used as the procedure for assessment of reactivity in unpurified monoclonal antibodies.

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. The interpretation of the results must be done by a person trained in the procedure and familiar with such interpretation.

4. EQUIPMENT

- Plate Shaker; Boekel Scientific Jitterbug 2 model 270400
- Plate Reader: Tecan Spark microplate reader (450nm wavelength)

5. MATERIALS

- Microtiter plates – medium binding; Costar Cat. #9017,
- Microtiter plates – high binding; Costar Cat. #9018
- Carbonate-Bicarbonate Coating Buffer (pre-mixed powder) – prepared according to manufacturer's directions; Thermo Scientific Cat. #28382

- Phosphate Buffered Saline (PBS), 10x solution (Fisher Scientific Cat. #BP399-1) diluted to 1x with deionized water to give 11.9 mM phosphate, 137mM NaCl, 2.7mM KCl, pH 7.4
- PBS – 0.05% Tween (PBST) prepared by addition of 500uL Tween 20; (Fisher Cat. #BP337-500) into 1 liter of PBS
- PBS – 0.05% Tween, 0.5% Milk (Blocking Buffer) prepared by addition of 5 grams of Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Cat. # 1706404XTU) into 1 liter of PBST
- 1-Step TMB Turbo Substrate, Pierce-Thermo catalog # 34022
- 0.18N Sulfuric acid; Thermo Scientific, cat.# N600.
- Polypropylene Tubes

6. REAGENTS

- Purified protein (Antigen) corresponding to the antibody.
- BSA-conjugated peptide corresponding to the antibody
- Peptide corresponding to the antibody
- Cell Lysate
- Purified monoclonal antibody corresponding to the Antigen, Peptide or Endogenous protein (such as cell lysate)
- 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

7. PROCEDURE

7.1. Antigen, Peptide or Cell Lysate Coating

7.1.1. Prepare a plate outline as in the diagram below. This format allows for up to 5 antibodies to be tested with one analyte and antibody dilution is tested in duplicate. Control wells are located in the last two columns, rows A-C.

	1	2	3	4	5	6	7	8	9	10	11	12	
	Ab 1		Ab 2		Ab 3		Ab 4		Ab 5				
	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	1:1K Cont	Blk	Blk	A
	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	1:10K Cont	Blk	Blk	B
	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	1:100K Cont	HRP NSB	HRP NSB	C
1:1K													D
1:10K													E
1:100K													F
1:1,000K													G
1:10,000K													H

7.1.2. Prepare a dilution of the antigen, BSA-peptide or cell lysate into the Carbonate Coating Buffer to give a final concentration of approximately 10µg/ml for antigen and peptide and 100ug/mL for cell lysate (need 100µl/number wells).

7.1.3. Mix thoroughly by vortex.

7.1.4. Add 100µl of diluted antigen, BSA-peptide, free peptide (10µg/ml stock) or cell lysate (100ug/mL) to each titration well (total 10 wells per antibody). These are indicated in the above diagram (Rows D-H).

7.1.5. Add 100µl of Carbonate Coating Buffer to each of the control wells as indicated in the above diagram (Rows A-C).

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7.1.6. Incubate the plate for 1 hour at 37°C with mild shaking. Alternatively, incubate 16-24 hours at 4 -8°C without shaking. For free-peptide, allow incubation to complete dryness on to the micro-titer plate (high-binding) by use of high heat forced air set to low.

7.1.7. For analyte samples apart of free peptide, remove the Carbonate Coating Solution by decanting and tapping plate gently on a paper towel.

7.1.8. Immediately, add 225µl of Blocking Buffer to all wells as a blocking agent to prevent non-specific binding.

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

7.1.10. Remove the Blocking Buffer by decanting and tapping plate gently on a paper towel.

7.1.11. Rinse and wash the wells 3 times with 250µl PBST. To do this, dispense PBST into wells and then gently decant/tap the plate on a paper towel. Repeat 3x.

7.2. Primary Antibody preparation and incubation

7.2.1. Prepare 1ml of intermediate stock solution (IS) to approximately 0.01 mg/ml in PBST. Do NOT use polystyrene tubes. Polypropylene tubes are preferred for all antibody dilution steps.

7.2.2. Prepare 5 dilution levels of each IS primary antibody in PBST as follows:

- 1:10 – 100µL of IS + 900µL of PBST
- 1:100 – 100µL of 1:10 + 900µL of PBST
- 1:1,000 – 100µL of 1:100 + 900µL of PBST
- 1:10,000 – 100µL of 1:1000 + 900µL of PBST
- 1:100,000 – 100µL of 1:10000 + 900µL of PBST

7.2.3. Add 100µl of each dilution as indicated in the diagram above (1:10, to rows A and D, 1:100 to Rows B and E, 1:1000 to Rows C and F, 1:10000 to Row G and 1:100000 to Row H).

7.2.4. Incubate for 30 minutes to 1 hour at 37°C with shaking.

7.2.5. Wash the plate: decant and rinse 3 times with PBST as described in 7.1.11.

7.3. Secondary antibody preparation and incubation

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

7.3.1.1. Add 5µl of the secondary antibody to 25ml of PBST. This will make sufficient secondary antibody for 2.5 microtiter plates. Note, the concentration of the secondary antibody may need to be adjusted depending on the individual antibody performance.

7.3.2. Add 100µl of secondary antibody to each well as indicated in the diagram (Rows A-H, columns 1-10). Additionally, include the 2 additional wells listed as “HRP NSB” in the plate diagram (Row C, columns 11 and 12).

7.3.3. Incubate the plate for 30 minutes to 1 hour @ 37°C with mild shaking.

7.3.4. Remove the secondary antibody solution by decanting and tapping plate gently on a paper towel.

7.3.5. Wash the plate: decant and rinse 3 times with PBST as described in 7.1.11.

7.4. Detection and Evaluation

7.4.1. Add 100µl of TMB substrate to each well of the plate including the wells in columns 11 and 12 (controls).

7.4.2. 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.

7.4.3. Add 100µl of 0.18N sulfuric acid to each well to stop the reaction. The color will change to yellow.

7.4.4. Read the absorbance in a microplate reader using a 450 nm wavelength.

7.4.5. Perform data reduction according to SOP D-100.

8. REFERENCED DOCUMENTS

8.1.1. Operation manual, Tecan Spark microplate reader

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

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

8.1.4. SOP D-100: Data reduction of Indirect ELISA results