Mouse Typer®
Sub-Isotyping Kit

Instruction Manual

Protocol for Mouse Typer Sub-Isotyping Kit (Catalog Number 172-2051) and Mouse Typer Sub Isotyping Panel (Catalog Number 172-2055)

BIO-RAD

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Section 1
Introduction

1.1 Background

The growth and widespread use of mouse monoclonal antibody technology have created a need for a fast, accurate, and simple means of determining immunoglobulin class and sub-class. Several classes of mouse monoclonal antibody, all structurally different depending on their heavy chain composition, have been described.[1] Identification is essential since chemical and biological properties of the various classes are unique. They differ in their solubility and electrophoretic properties, in their susceptibility to cleavage enzymes, and in their reactivity with protein A.[2,3]

The Mouse Typer sub-isotyping kit is an extremely convenient kit for identifying mouse immunoglobulin class and sub-class in tissue culture supernatant and ascitic fluids. The ELISA based kit uses a panel of ultra pure reagents to determine mouse sub-isotypes: IgG_1, $IgG2_a$, $IgG2_b$, IgG_3, IgM, IgA, χ chain, and λ chain. It comes complete with all the essential reagents for 800 extremely sensitive tests (100 typings). The included protocol and troubleshooting guide guarantee efficient and accurate assays.

Determining a mouse monoclonal antibody's class with the Mouse Typer kit is fast and simple. Antigen is first adsorbed to a microplate, then treated with specific monoclonal antibody. Bound monoclonals are separately reacted with each of the Mouse Typer rabbit anti-mouse panel reagents. Immunoglobulin class and sub-class are then determined with Bio-Rad's goat anti-rabbit (H + L) horseradish peroxidase (HRP) conjugate and peroxidase substrate system. Color development occurs immediately, with positive wells having absorbances 3-6 times that of corresponding negatives. Using Bio-Rad's Model 3550 or Model 3550-UV Microplate Reader, results are easily quantitated in less than 60 seconds.

1.2 Materials Required

Kit Components:

Catalog Number	Product Description	Quantity/ Package
172-2055	**Mouse Typer Sub-Isotyping Panel**, includes ultra pure rabbit anti-mouse subclass specific anti-serum to mouse IgG_1, $IgG2_a$, $IgG2_b$, IgG_3, IgM, IgA, χ chain, and λ chain.	10 ml each
172-1019	**EIA Grade Affinity Purified Goat Anti-Rabbit IgG** (H + L), human adsorbed, horseradish peroxidase conjugate (GAR-HRP).	1 ml
172-1064	**Peroxidase Substrate System**, contains 2,2′-Azino-di (3-ethyl-benzthiazoline sulfonate [6]) and hydrogen peroxide.	200 ml

1.3 Storage and Stability

	Temperature	Shelf Life
Mouse Typer Sub-Isotyping Panel	4 °C	1 year
GAR-HRP Antibody Conjugate	4 °C	1 year
Peroxidase Substrate System	4 °C	>1 year

1.4 Reagents and Equipment Not Included

1. Specific mouse monoclonal antibody, culture or ascites fluid, and its cognate antigen.
2. Sodium chloride (NaCl), sodium phosphate dibasic heptahydrate (Na_2HPO_4-$7H_2O$), sodium phosphate monobasic (NaH_2PO_4-H_2O), ACS Reagent Grade.
3. Tween 20, EIA Grade (Bio-Rad catalog number 170-6531).
4. Bovine Serum Albumin BSA (Sigma catalog number A 9647).
5. Thimerosal, (Sigma catalog number T 5125).
6. Oxalic acid, dihydrate (J. T. Baker catalog number 0230-1).

7. 96-well polystyrene microplates (Bio-Rad catalog number 224-0096).
8. Pipet tips (Bio-Rad catalog number 223-9302, nonsterile).
9. Octapette® pipet, 100 ml (Bio-Rad catalog number 224-4800).
10. Reagent reservoirs (Bio-Rad catalog number 224-4872).
11. Automatic ELISA plate reader (Bio-Rad Model 3550 Microplate Reader, catalog number 170-6601 or Model 3550-UV Microplate Reader, catalog number 170-6638, or Model 550 Microplate Reader, catalog number 170-6750).

Section 2
Sub-Isotyping Assay

2.1 Solutions

The solution volumes that follow are recommended for an ELISA assay typing of 10 mouse monoclonal antibodies in one microtitration plate.

Phosphate buffered saline, PBS, 300 ml	(0.01 M phosphate buffer, pH 7.2) Add 0.105 g sodium monobasic phosphate, 0.600 g sodium dibasic phosphate, and 2.550 g sodium chloride to 250 ml distilled, deionized water. Adjust to pH 7.2 with HCl and bring to 300 ml with water.
Phosphate buffered saline wash solution, PBS-Tween, 200 ml	Divide the above solution into 100 and 200 ml fractions. Add 0.10 ml Tween 20 to the 200 ml fraction.
Antigen solution, 10 ml	Dissolve or dilute antigen preparation in 10 ml PBS to a final concentration of 1-10 µg/ml.

Blocking solution, 30 ml	1% BSA-PBS. Add 0.3 g BSA to 30 ml PBS. Adjust pH to 7.2.
Antibody conjugate solution, 10 ml	Dilute GAR-HRP conjugate 1:3,000 by adding 3.3 µl to 10 ml of PBS-Tween.
Peroxidase substrate solution, 10 ml	Mix 9 ml solution A with 1 ml solution B. **Prepare fresh prior to use and use immediately.**
Color stopping solution, 50 ml	(2% oxalic acid) Add 1 g oxalic acid dihydrate to 50 ml distilled, deionized water.

Note: If stock solutions of PBS, PBS-tween, and blocking solution are made, include the bacteriostat thimerosal at a concentration of 0.01%. Avoid the use of sodium azide, as it is an inhibitor of peroxidase activity.

2.2 General Recommendations

1. **Assay Incubation Temperature:** All incubation steps are performed at room temperature (23-25 °C), with the microplate covered to prevent evaporation. For convenience, any step may be carried out overnight at 4 °C. Incubation times can be decreased to 0.5 hr if done at 37 °C.
2. **Reagent Purity:** All reagents should be ACS or EIA Grade. Chemical impurities, as well as poor water quality, can cause enzyme inhibition and/or increased backgrounds.
3. **Antigen Adsorption:** Coat the immunoassay microtitration plates with 0.1 to 1.0 mg antigen per well. The optimal concentration should be determined empirically prior to subtyping. Antigen adsorption is a function of concentration, diluent, type of assay plate, and purity of sample.[4] Whole cell antigens also can be used for sub-isotyping.[4]

4. **Monoclonal Antibody Sample:** To insure proper typing of culture media samples, **do not** dilute when applying to microplates. When using ascites fluid, dilute at least 1:1,000 with PBS-tween before testing. Serial dilutions may be necessary to establish the working dilution of ascites fluid required for the best signal-to-noise ratio in the typing immunoassay.
5. **GAR-HRP Antibody Conjugate:** Bio-Rad's affinity purified antibodies should be used at recommended dilutions. These products give excellent signal-to-noise ratios while using less reagent. More antibody may be used, but this could result in higher backgrounds with minimal increase in detection sensitivity.
6. **Background:** Nonspecific background reactions are usually the result of low-purity second antibody and/or using excessive conjugate antibody concentrations. Always wash plates thoroughly, especially after the conjugate incubation. Tween 20 is essential in all wash steps after blocking. At a 0.05% concentration, it will not disrupt antigen-antibody interactions.

For further assistance in determining sub-isotyping of monoclonal antibodies, contact Bio-Rad Technical Services (in the USA 1-800-424-6723) or your local technical representative.

2.3 Procedure

Before starting the assay, read through the entire protocol.

1. Adsorb antigen (Ag) to the microplate by adding 100 µl Ag solution to all wells (0.1-1.0 µg Ag/well). Cover the plate and incubate at room temperature for at least 1 hour.

   Note: If the Ag coated plates are not used immediately, cover and store at 4 °C. Antigen solution should contain 0.01% thimerosal. Plates can be stored for up to 1 month.

2. Remove any unbound Ag by flooding the wells of the assay plates with PBS. Use a plastic wash bottle or an automatic plate washer. When using a wash bottle, fill each well, soak for 15 seconds, then vigorously shake off solution in a sink (flick-washing). Repeat 2 times.
3. To prevent nonspecific binding, fill all the wells with 300 µl blocking solution(1% BSA-PBS). Let stand at room temperature for 30 minutes, then flick-wash the plate 3x with PBS-tween.
4. Add hybridoma culture fluid (undiluted) or ascites (diluted) samples, 100 µl/well, using the suggested format outlined in Figure 1. Six wells of columns 2-11 are used (1 sample per column). Column 1 is reserved for substrate blank and column 12 for positive control (*i.e.*, mouse serum). Cover and incubate the plate for 1 hour at room temperature.
5. Empty the plates of hybridoma supernatant or diluted ascites fluid. Flick-wash the plate 3x with PBS-Tween.
6. Add appropriate rabbit anti-mouse panel reagents using the format in Figure 1. All of rows A-H are filled with respective panel reagent, 100 µl/well. Incubate the covered microplate for 1 hour at room temperature.

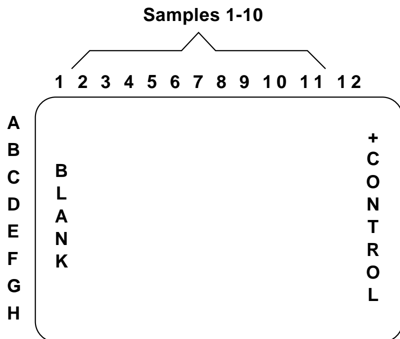

Fig. 1. Suggested format for sub-isotyping 10 samples on 1 microplate.

7. Empty the plates of panel reagents and flick-wash 5x with PBS-tween.
8. With the exception of column 1, fill the wells with diluted goat anti-rabbit horseradish peroxidase conjugate, 100 µl/well. Cover and incubate for 1 hour.
9. During the incubation with conjugate, prepare peroxidase substrate solution (see Section 2.1).
10. Wash off unreacted conjugate solution by flick-washing the plate 4x with PBS-tween. Wash an additional time with PBS. Invert and tap the plate over paper toweling to rid the wells of excess wash solution. Add 100 µl peroxidase substrate solution. Positive reactions will appear immediately. Identification of mouse sub-isotype can be assessed after a 10 to 30 minute room temperature incubation.
11. Stop color development by adding 100 µl/well 2% oxalic acid color stopping solution. Results can be documented using the Model 3550, 3550-UV, or Model 550 Microplate Reader at 415 nm.

Note: When assessing color development using Model 3550, Model 550, or the Model 3550-UV Microplate Reader, wash the bottom of the assay plate thoroughly with distilled water and wipe dry with non-lint toweling.

Section 3
Troubleshooting Guide

Problem	Probable Cause	Recommended Solution
A. High background.	1. Insufficient washing after conjugate antibody incubation.	1. Wash each well 6-7x and increase soak cycles to 30 seconds.
	2. Insufficient blocking after antigen adsorption.	2. Increase blocking step to 60 minutes.
	3. Tween 20 absent from washes.	3. Include Tween 20 in all washes and solutions after blocking.
	4. GAR-HRP conjugate concentration too high.	4. Use recommended dilutions. Generally, the less dilute, the higher the background.
	5. Color developed too long.	5. Decrease color development time by one-half.
	6. Substrate too old (high green color at working dilution).	6. Use fresh solution A and solution B
	7. Whole cell antigens have endogenous peroxidase activity.	7a. Use extracted antigens. 7b. Use other enzyme conjugated antibodies. 7c. Destroy endogenous activity by incubating adsorbed Ag with mixture of methanol/ H_2O_2 (99 ml methanol, 1 ml 30% H_2O_2), 100 µl/well for 1 hr.

Problem	Probable Cause	Recommended Solution
B. No reaction or weak color development.		
1. Horseradish peroxidase substrate solution inactive (Note 1).	a. Improper storage of reagents.	a. Store peroxidase substrate at 4 °C.
	b. Substrate solutions hydrolyzed due to age.	b. Use fresh peroxidase substrate solutions.
2. Goat anti-rabbit horseradish peroxidase conjugate is inactive or nonsaturating (Note 2).	a. Antibody improperly stored.	a. Store at 0-4 °C. Avoid bacterial contamination and repeated freeze thaw cycles.
	b. Nonsaturating concentrations of monoclonal or conjugate antibody used in incubations.	b. If possible , increase concentration of monoclonal, conjugate, or both. Use caution, the less the conjugate is diluted the greater the nonspecific color produced.
3. Monoclonal antibody solution is inactive or nonsaturating (note 3).	a. Monoclonal improperly stored.	a. Avoid bacterial contamination and heat inactivation.
	b. Antibody titer too low.	b. Increase amount of hybridoma culture media added to plates (*i.e.* 200 µl). May also be necessary to increase conjugate antibody concentration.
	c. Tween 20 deteriorates reactivity of antibodies.	c. Eliminate Tween 20 from all solutions and buffers except wash after blocking. Could result in increased backgrounds.

Problem	Probable Cause	Recommended Solution
C. **Monoclonal exhibits multiple sub-isotype (*i.e.* assay shows one clone to be both an IgG1 and IgM).**	1. Impure sample.	1. Use fresh source of culture supernatant. Purify media or ascites fluid.[5-7]
	2. Sample too concentrated	2. Dilute supernanants and ascites fluid, repeat test.
	3. Sample is polyclonal.	3. Re-clone hybridoma cells by limited dilution,[8] and repeat assay.

Notes to Troubleshooting Guide

1. Activity Test for Horseradish Peroxidase Substrate Solution.

   Combine 1.0 ml of substrate solution with 10 ml of antibody conjugate. If no color develops in 5 minutes, substrate solution is at fault.

2. Activity Test for Antibody Conjugate Solution.

   Combine 1.0 ml of horseradish peroxidase substrate solution (tested above) and 1.0 ml of diluted antibody conjugate solution. If no color develops in 5 minutes, the conjugate is suspect. Repeat procedure using fresh conjugate antibody dilution.

3. Activity Test for Monoclonal Antibody Solution.

   Use RID, Ouchterlony precipitation, or ELISA assay to determine reactivity. Repeat procedure with more concentrated monoclonal antibody solution.

Section 4
References

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