3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Mouse Monoclonal Antibody Isotyping Reagents

Catalog Number **ISO2**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The Mouse Monoclonal Antibody Isotyping Reagents are intended for use in the qualitative isotype determination of mouse monoclonal antibodies. Antibodies in hybridoma cell culture supernatant, ascites fluid, or purified forms can be identified by Enzyme Linked Immunosorbent Assay (ELISA) or immunodiffusion (Ouchterlony) assays. The procedures are designed to make interpretation of results as straightforward as possible. Nevertheless, due to the nature of some samples being evaluated, careful attention must be paid to interpretation of test results.

These Mouse Monoclonal Antibody Isotyping Reagents may be used in Ouchterlony immunodiffusion or ELISA (capture, indirect, or antigen-mediated) procedures. The set of isotyping reagents may be used for the determination of mouse monoclonal antibody isotype as found in various forms including culture supernatant (neat or concentrated), ascites fluid, or purified form. To select the most suitable assay system, see Table 1.

Table 1.
Assay System Selection

	Assay Procedure			
Monoclonal Antibody	Ouchterlony Immuno-diffusion	Capture ELISA	Indirect ELISA	Antigen-Mediated ELISA
Culture Supernatant (neat)	–	+	–	+
Culture Supernatant (concentrated)	+	+	–	+
Ascites Fluid	+	–	+	+
Purified	+	+	+	+

It should be noted the antigen-mediated ELISA is the method of choice for isotyping monoclonal antibodies in ascites fluid by enabling the exclusive immobilization of the relevant antibody in the assay system.

Components

Sufficient reagents are provided to perform 40 tests by Ouchterlony immunodiffusion or 1000 tests by ELISA.

Goat Anti-Mouse IgG1 (Catalog Number M5532)	0.2 ml
Goat Anti-Mouse IgG2a (Catalog Number M5657)	0.2 ml
Goat Anti-Mouse IgG2b (Catalog Number M5782)	0.2 ml
Goat Anti-Mouse IgG3 (Catalog Number M5907)	0.2 ml
Goat Anti-Mouse IgM (Catalog Number M6157)	0.2 ml
Goat Anti-Mouse IgA (Catalog Number M6032)	0.2 ml

Reagents and Equipment Required, but Not Provided

For Ouchterlony immunodiffusion assays:

- Agarose slides or agarose on a support film
- Rinsing, staining, and destaining solutions

For ELISA:

- Multiwell plates (polystyrene, flat bottom)
- Buffers for coating, washing, and substrate
- Substrate and stopping solutions
- Enzyme labeled Goat Anti-Mouse IgG (Fab Specific), needed only for capture ELISA (Catalog Number A9917)
- Enzyme labeled Rabbit Anti-Goat IgG, needed for indirect or antigen-mediated ELISA (Catalog Number A5420)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Due to the sodium azide content a material safety data sheet (MSDS) for this product has been sent to the attention of the safety officer of your institution. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
The kit ships on wet ice and for continuous use, store at 2–8 °C.

For extended storage, the solutions may be frozen in working aliquots. Repeated freezing and thawing is not recommended. Storage in "frost-free" freezers is not recommended. If slight turbidity occurs upon prolonged storage, clarify the solution by centrifugation before use.

Procedures
Notes: Read instructions carefully.

Plan the procedure carefully to achieve the maximum use from this set of isotype specific reagents.

All pipettes must be accurate. Small deviations in the sampling of the reagents can cause great differences in the final readings and lead to inaccurate determinations.

The assay should be carried out at room temperature (20–25 °C). Other temperatures may cause erroneous results.

A. Ouchterlony Immunodiffusion Assay

Reagents and Equipment

- 0.1 M Tris-barbital buffer (5×):
  44.3 g/L Tris base (Catalog Number T1503)
  22.4 g/L Barbital (Catalog Number B0375)
  0.5 g/L Calcium lactate
  (Catalog Number L 2000)
  1.0 g/L Sodium azide (Catalog Number S2002)
  Dilute 1:5 before use.
- Agarose: Pure agarose powder
  (Catalog Number A6877)
- Polyethylene Glycol (PEG),
  Average molecular weight = 8,000
  (Catalog Number P2139)
- Staining Solution:
  2.5 g/L Crocein Scarlet 7B
  (Catalog Number C3643)
  150 mg/L Brilliant Blue R
  (Catalog Number B0149)
  5% (v/v) glacial Acetic acid
  (Catalog Number A6283)
  30 g/L Trichloroacetic Acid
  (Catalog Number T4885).
  Dissolve by warming to 60 °C for 30 minutes prior to addition of water. Filter through Whatman No. 1 filter paper.
- Rinsing solution: 3% NaCl
- Destaining solution: 0.3% Acetic Acid
- Microscope slides
- Microslide frames
- Support film, 0.2 mm thick, cut to fit the microslide frame (optional)
- Leveling table
- Gel punch for immunodiffusion (1 central well and 6 surrounding wells, 2 mm diameter holes 9 mm apart).
- Humidity chamber
- Vacuum pump
- Water bath (55–60 °C)

Ouchterlony Immunodiffusion Assay Procedure

1. Prepare a 2% agarose solution in Tris-barbital buffer. Boil and transfer to 55–60 °C water bath.
2. Prepare a 6% PEG solution in Tris-barbital buffer. Dissolve PEG by warming to 60 °C. Transfer to water bath. Avoid long incubation in the water bath. Do not boil.
3. Place the microslide frame (optional: place gel bond film over microslides) on the leveling table.
4. Mix equal volumes of agarose and PEG solutions. Keep in water bath at 55–60 °C.
5. Using a pipette, apply a small amount of the agarose-PEG solution between the slides and the frame, this will adhere the frame to the slide.
6. Pour 25–30 ml of the agarose-PEG solution on the slides. Allow to solidify and then store in a humidity chamber at 4 °C (up to 2 weeks).
7. Outline the immunodiffusion plan. The monoclonal antibody to be tested should be placed in the center well and the isotype specific reagents should be placed in the peripheral wells.
8. Punch holes in the agarose plate using the gel punch.
9. Using a vacuum pump, carefully aspirate agarose from holes.
10. Carefully pipette the reagents into appropriate holes according to the plan. Use 5 µl of each isotype specific reagent in the outer wells and 10 µl of the test specimen in the center well (concentration of the sample should be ~50 µg/ml, or a 1:100 dilution of ascites fluid). Avoid over-filling any reagent.
11. Place the agarose plate (or film) in a humidity chamber for at least 24 hours (an additional 24 hours incubation may produce clearer results). The precipitin lines will appear in a dark field light viewing box as dense, opaque white lines in the agarose layer.
12. For permanent results - Rinse the plates in 3% NaCl for 24 hours. This assures complete clearing of any unbound protein and renders the background of the plate a very light color after staining. Rinse twice in water for 1 hour each rinse. Cover the plates with filter paper to ensure even drying.
13. Dry plates in a drying oven at 45–50 °C or near a fan for 45–60 minutes.
14. Stain the plate for 30 minutes in staining solution.
15. Destain the plate with three successive washes in 0.3% acetic acid. Each wash requires 30 minutes. After destaining the precipitin lines should be stained blue-red against a light pink background. The plate may be dried for permanent record.

B. ELISA

Notes: The ELISA method is a highly sensitive technique. Avoid any mixing of reagents. Contamination caused by using the same pipette tips for two different reagents may lead to erroneous results. Different troughs or containers should be used for each reagent when using a multipipettor.

Polystyrene multiwell plates from various manufacturers may show differences in absorption properties and considerable lot-to-lot variations; therefore, it is recommended that an approved multiwell plate be used.

All ELISA results are qualitative and should be observed visually.

The ELISA procedures describe the use of enzyme labeled antibodies (see Reagents and Equipment Required, but Not Provided). Other equivalent antibodies may be used.

Reagents and Equipment

- Polystyrene multiwell plates or removable strip plates.

- Phosphate buffered saline (PBS) - 10 mM phosphate buffer with 150 mM NaCl, pH 7.4.

- Washing buffer: PBS containing 0.05% TWEEN® 20.

- Substrate for peroxidase:
  Buffer - 0.02 M sodium phosphate, pH 6.8
  Hydrogen Peroxide (H_2O_2)
  5-Aminosalicylic Acid (Catalog Numbers A3537 or A6178).
  Note: Other peroxidase substrates are also applicable.

- Stopping Solution: 3 N NaOH

- Peroxidase conjugated Rabbit Anti-Goat IgG (whole molecule) Affinity Isolated Antibody (Catalog Number A5420), needed for indirect or antigen-mediated ELISA.

- Peroxidase conjugated Goat Anti-Mouse IgG (Fab Specific) Affinity Isolated Antibody (Catalog Number A9917), needed only for capture ELISA.

Indirect ELISA Procedure

1. Dilute antibody to be tested in PBS; 1:5000 for ascites fluid and 1 μg/ml for a purified antibody (1.2 ml of diluted solution is needed for each sample to be tested).
2. Pipette 0.1 ml of the diluted antibody into each of 12 wells of a multiwell plate.
3. Incubate the plate (covered) for 1 hour at 37 °C.
4. Remove the coating solution. Wash 3 times with washing buffer.
5. Dilute an appropriate amount of the isotype specific reagents 1:1,000 in PBS (0.2 ml of diluted isotype specific reagents are required for each sample to be tested).
6. Add 0.1 ml of the each of the diluted isotype specific reagents to duplicate wells coated in step 2.
7. Incubate the plate at room temperature for 30 minutes.
8. Wash as in step 4.
9. Dilute the peroxidase labeled Rabbit anti-Goat IgG 1:5,000 in washing buffer (1.2 ml of diluted enzyme labeled antibody is needed for each sample being tested).
10. Add 0.1 ml of the enzyme labeled antibody to each well.
11. Incubate the plate at room temperature for 15 minutes.
12. Prepare the substrate as follows (1.2 ml of solution is needed for each sample being tested):
    a. Dissolve 5-Aminosalicylic acid at 1 mg/ml in substrate buffer.
    b. Prepare a fresh solution of 1% hydrogen peroxide in water.
    c. Add 0.1 ml of the 1% hydrogen peroxide solution for every 10 ml of the 5-Aminosalicylic Acid prepared.
13. At the end of the 15 minute incubation, wash the plate as in step 4.
14. Add 0.1 ml of the freshly prepared substrate to each well.
15. Incubate the plate at room temperature for 10–15 minutes. Development of a brown color in a well indicates a positive result.
16. Stop the reaction by adding 50 μl of 3 N NaOH to each well.
17. Inspect the plate visually.

Antigen-Mediated ELISA Procedure

1. Apply 0.1 ml of appropriately diluted antigen to coat each of the 12 wells of the multiwell plate. Incubate overnight at 4 °C or for 1 hour at 37 °C.
2. Remove coating solution. Wash 3 times with washing buffer.
3. Dilute monoclonal antibody to be tested to an appropriate dilution in PBS or apply the culture supernatant without dilution (1.2 ml is needed for each sample to be tested).
4. Pipette 0.1 ml of the sample to be tested to each of the coated wells.
5. Incubate the plate for 2 hours at room temperature.
6. Wash as in step 2.
7. Continue with steps 5–17 of the procedure for the Indirect ELISA.

Procedure for Capture ELISA

1. Dilute the isotype specific antibodies 1:1,000 in PBS (0.2 ml of each diluted antibody is needed for each sample to be tested).
2. Pipette 0.1 ml of each of the diluted antibodies to 2 wells of a multiwell plate.
3. Incubate the plate (covered) for 1 hour at 37 °C.
4. Remove the coating solution. Wash the plate 3 times with washing buffer.
5. Pipette 0.1 ml of the sample to be tested into each of the wells (use culture supernatant undiluted, dilute concentrated or purified samples diluted in PBS to 2–5 μg/ml).
6. Incubate the plate at room temperature for 1 hour.
7. Wash as in step 4.
8. Dilute the peroxidase labeled Goat Anti-Mouse IgG (Fab Specific) antibody 1:600 in washing buffer (1.2 ml of the diluted enzyme conjugated antibody is needed for each sample being tested).
9. Add 0.1 ml of the enzyme conjugated antibody to each well.
10. Incubate the plate at room temperature for 30 minutes.
11. Continue with steps 12–17 in the procedure for Indirect ELISA (please note in the Capture ELISA the enzyme conjugate is incubated on the plate for 30 minutes not 15, and incubation with the substrate is for 20–30 minutes).

Results

The described assays are designed to make interpretation of results straightforward; the antibody isotypes are visibly identified in both the Ouchterlony immunodiffusion and ELISA applications. Nevertheless, due to the nature of samples being evaluated, in many instances careful attention must be paid when the results are being interpreted.

For instance, considerable amounts of normal mouse immunoglobulins, especially of the abundant IgG1 isotype occur in ascites fluid along with the desired monoclonal antibody secreted by the hybridoma cells. The concentrations of specific monoclonal antibodies and other immunoglobulins being tested may vary with each hybridoma line when culture supernatant or ascites fluid are being used. Consequently, the appropriate assay protocol and interpretation of results must be considered. To select for the most appropriate assay procedure see Table 1.

References

1. Goding, J., Monclonal Antibodies, Principles and Methods, Academic Press, (London, UK: 1983).
2. Goding, J., J. Immunol. Meth., **39**, 285 (1980).
3. Gardner, I., Pathology, **17**, 64, (1985).
4. Coulter, A. et al., Med. Lab. Sci., **46**, 54 (1989).
5. Klein-Schneegans, A. et al., J. Immunol. Meth., **119**, 117 (1989).

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Troubleshooting Guide

Ouchterlony Double Diffusion		
Problem	**Possible Cause**	**Suggested Action**
No precipitation line observed.	Reagents omitted or not used in proper order.	Prepare and use checklist.
	Agarose slides misprepared.	Test with another antigen-antibody system.
	Concentration of antibody tested is too high or too low.	Redilute antibody.
	Hybridoma not secreting antibody.	Check antibody secretion.
Too many precipitation lines obtained.	More than one hybridoma clone in the sample.	Reclone the hybridoma.
	Ascites fluid contains other host derived antibodies.	Use higher dilution of ascites fluid to dilute out the contaminants, or switch to an antigen-mediated ELISA.
ELISA		
Problem	**Possible Cause**	**Suggested Action**
No color obtained in plate or signal is too weak.	Reagents omitted or not used in proper order.	Prepare and use checklist.
	Enzyme labeled second antibody other than recommended.	Use the recommended source for second antibody or determine working dilution for second antibody.
	Concentration of antibody tested too low.	Use higher concentration of sample.
	Hybridoma not secreting antibody.	Check antibody secretion.
	Inappropriate preservative such as sodium azide is present in the buffer.	Check buffer composition.
	Substrate solution prepared incorrectly.	Check substrate with labeled second antibody or other peroxidase reagent.
	Improper storage of kit.	Check protocol for storage.
Too many signals obtained.	More than one hybridoma in the sample.	Reclone the hybridoma.
	Ascites fluid contains host-derived antibodies.	Apply purified sample. Use higher dilution of ascites fluid to dilute out contaminants or switch an antigen-mediated ELISA.
	Very high concentration of antibody in sample may exhibit cross-reactivity with isotype specific or second antibodies.	Dilute the sample more than recommended in the protocol.
	Plate not properly washed.	Use proper washing technique.
	IgG1 specimen is singled out also by the IgG2a specific antibody giving positive results.	Disregard the IgG2a signal as this background appears occasionally. Consider only the IgG1 signal.
	IgG1 specific antibody labels the sample along with another isotype specific antibody (especially if the sample is ascites fluid).	Disregard the IgG1 signal as this indicates a host derived antibody.
	Enzyme labeled second antibody is other than recommended, resulting in non-specific binding.	Use the recommended source for for the antibody or determine the working dilution for the antibody used.