SIGMA®
CHEMICAL COMPANY

SIGMA IMMUNOTYPE™
MOUSE MONOCLONAL ANTIBODY ISOTYPING KIT

Stock No. ISO-1
Directions For Use

PROCEDURE OVERVIEW

Isotyping Strip	Biotinylated Antibody	ExtrAvidin® Peroxidase	Substrate		
			Buffer	Chromagen	H2O2
Vial 1	Vial 2	Vial 3	Vial 4a	Vial 4b	Vial 4c
Place strip in assay tube	Fill separate mixing tube with 2 ml PBS-BSA	Fill separate mixing tube with 2 ml PBS-BSA	Fill separate mixing tube with 2 ml PBS-BSA		
↓	↓	↓		↓	
Add 2-3 ml mouse hybridoma supernatant	Add 1 drop of vial 2	Add 1 drop of vial 3	Add two drops from vial 4a, mix; add two drops from vial 4b, mix; add one drop from vial 4c, mix.		
↓	↓	↓		↓	
Incubate 30 minutes	Pour contents into assay tube	Pour contents into assay tube	Place strip in clean assay tube, pour 2-3 ml substrate into assay tube		
↓	↓	↓		↓	
Wash once with PBS-T-BSA for 5 minutes	Incubate 5 minutes	Incubate 5 minutes	Incubate until first sign of isotype. Remove from tube and allow to air dry		
↓	↓	↓		↓	
	Wash once with PBS-T-BSA for 5 minutes	Wash once with PBS-T-BSA and once with PBS, 5 minutes per wash	Remove strip from tube and immerse in 0.1 M NaOH for 1-2 minutes, then rinse with distilled water.		

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INTRODUCTION

Sigma Immunochemicals' ImmunoType™ Kit is intended for the rapid, sensitive and specific determination of mouse monoclonal antibody isotypes in culture supernatants. The kit contains a novel antibody-capturing strip and the reagents required for isotype determination, supplied in easy-to-use dropper vials. Only a small volume (2ml) of a hybridoma culture supernatant is required.

The procedure is designed to make interpretation of results as straightforward as possible. Assay time required is less than 1 hour. The antibody isotype is identified by the self-descriptive strip which may be stored for permanent record keeping.

No Pipetting Required!
No Reagent Preparation Required!

PRINCIPLE OF APPLICATION

The ImmunoType Kit consists of nitrocellulose membrane strips bound to an inert support, on which the entire assay is carried out. Required reagents are supplied in easy to use dropper vials. The precoated strip specifically captures the relevant mouse immunoglobulin. After incubation, a highly sensitive biotin-avidin-enzyme detection system is used to identify the mouse immunoglobulin isotype and is revealed by self-description. A positive control is also incorporated into the strip serving as an internal quality control. Undesired non-specific absorptions are prevented by pre-treatment of the strip and the use of appropriate buffers. System sensitivity is 1 µg/ml of mouse monoclonal antibody in culture supernatant.
The kit may be used for the determination of mouse monoclonal antibody isotype in small volumes of culture supernatants already in the initial stages of hybridoma isolation. It is also applicable for the determination of isotypes in diluted ascites fluid. However, this application is not recommended due to the presence of host-derived antibodies which may give multiple isotype signals.

MATERIALS SUPPLIED

Vial	Description	Quantity
1	Isotyping Strips	10 strips
2	Biotinylated Purified Antibody to Mouse Immunoglobulins	1 ml
3	ExtrAvidin®-Peroxidase	1 ml
4a	Substrate Buffer (2.5M Acetate buffer, pH 5.0)	1 ml
4b	Substrate Chromogen (3-amino-9-ethyl-carbazole in N,N-dimethyl formamide; AEC-DMF)	1 ml
4c	Substrate 2% Hydrogen Peroxide (H_2O_2)	1 ml

MATERIALS NOT SUPPLIED

1. PBS-T-BSA: Phosphate Buffered Saline (PBS) containing 0.05% Tween 20 (Sigma Product No. P-1379) and 1% BSA (Bovine Serum Albumin, Sigma Product No. A-7906).
2. 12x75mm tubes with caps (5 required/test)
3. Orbital shaker platform (optional)
4. Forceps

STORAGE CONDITIONS: Store at 0-5°C. **Do Not Freeze**

The kit reagents have been tested using the procedure described in this guide. The quantity of reagents supplied is sufficient for 10 assays.

Please refer to Material Safety Data Sheet (MSDS) for this kit with regards to cautions and handling of kit components.

PRECAUTIONS

1. Read instructions carefully before beginning the assay.
2. Plan assay carefully to achieve the maximum use from this kit.
3. This kit is highly sensitive. Utmost precaution should be taken to avoid any mixing of reagents and tubes. Any contamination caused by using the same tube for the preparation of different reagents may lead to erroneous results.
4. Additions from the easy-to-use dropper vials must be accurate. Small deviations in the sampling of reagents can cause great differences in the final readings and lead to inaccurate determinations.
5. The assay should be carried out at room temperature (20-25°C). Other temperatures may cause erroneous results, since reactions are affected by temperature.
6. Avoid touching the strip, use forceps.
7. Improper handling of kit reagents (e.g. excessive shaking of hydrogen peroxide) may accelerate material decomposition.
8. Hydrogen peroxide is corrosive and may cause burns. Avoid contact with eyes, skin and clothing.
9. This kit is intended for RESEARCH USE ONLY, not for use in diagnostic procedures.

ASSAY PROCEDURES

A. Main Steps of Assay
   1. Addition of sample being tested.
   2. Immunodetection by biotinylated antibody.
   3. Linkage of ExtrAvidin®-Peroxidase to biotinylated antibody.
   4. Color development.

B. Notes
   1. The assay should be carried out at room temperature (20-25°C). Other temperatures may cause erroneous results.
   2. Prepare all the reagents in separate tubes.
   3. For best performance, the use of an orbital shaking device during the incubation and washing steps is recommended. However, manual agitation or even gentle shaking of the test tube will usually give satisfactory results if an orbital shaker is not available.
   4. To prepare PBS-T-BSA buffer use the following procedure:
      a. Dissolve one PBS tablet (Sigma Product No. P-4417) in 200ml distilled or deionized water.
      b. Add 100µl Tween 20 (Sigma Product No. P-1379).
      c. Add 2gm bovine serum albumin (BSA, Sigma Product No. A-7906).
      d. Mix thoroughly.

C. Method

1. Use forceps to remove one strip from storage tube. Place strip into a 12x75mm test tube (use one strip per tube for each hybridoma supernatant).
2. Add 2-3 ml of hybridoma supernatant to the test tube (2ml is sufficient to overlay the strip if a shaker is being used).
3. Incubate for 30 minutes (during each incubation the strip should be immersed in the culture supernatant).
4. Decant or aspirate the supernatant and wash the strip (leave strip in the assay tube) once with 3-4ml of PBS-T-BSA for 5 minutes. Discard any remaining wash buffer from the assay tube.
5. Prepare the biotinylated second antibody by adding 1 drop from vial No. 2 into a separate tube containing 2ml of PBS-T-BSA (this is equivalent to a 1:50 dilution).
6. Pour the diluted biotinylated second antibody into the assay tube. Incubate for 5 minutes.
7. Decant or aspirate the antibody and wash the strip (leave the strip in the assay tube) once in 3-4ml of PBS-T-BSA for 5 minutes. Discard any remaining wash buffer from the assay tube.
8. Prepare the ExtrAvidin®-Peroxidase by adding 1 drop from vial No. 3 into a separate tube containing 2ml of PBS-T-BSA (this is equivalent to a 1:50 dilution).
9. Pour the diluted ExtrAvidin®-Peroxidase into the assay tube. Incubate for 5 minutes.
10. Decant or aspirate the ExtrAvidin®-Peroxidase and wash the strip (leave the strip in the assay tube) once with 3-4ml of PBS-T-BSA and finally in PBS, 5 minutes each wash. Discard the remaining wash buffer from the assay tube.
11. Prepare the substrate solution:
    In a separate test tube add in order:
    - 4ml water
    - 2 drops from vial 4a (substrate buffer), mix
    - 1 drop from vial 4b (substrate chromogen), mix
    - 1 drop from vial 4c (2% H_2O_2), mix
12. Pour the substrate solution into the assay tube. Incubate until a clear, red, insoluble signal (+) is obtained for the positive control. The isotype signal may appear in as little as 60 seconds. If the signal fails to appear within this time allow the reaction to continue until a signal does appear, up to a maximum of 10 minutes (see troubleshooting). A slight reddish background on the strip is considered normal.
13. As soon as the isotype signal appears immediately remove the strip from the tube.
14. Dry the strip between sheets of filter paper.
15. For a permanent record store the labeled strip in a plastic sleeve. Protect from light.

INTERPRETATION OF RESULTS

The assay described above is designed to make interpretation of results straightforward; the antibody isotype is visibly identified on the isotyping strip by self-description. The positive control incorporated into the strip serves as an internal quality assurance device for the reagents being used. Due to the nature of specimens being tested, careful consideration is required when results are evaluated. For instance, the amount of antibody in the test sample may vary with hybridoma type when culture supernatants are being used. Consequently, the appropriate assay protocol and interpretation has to be considered. In the case of faulty or unexplainable results, refer to the following troubleshooting guide.

REFERENCES

1. Goding, J.W., "Monoclonal Antibodies, Principles and Practice", Academic Press, London (1983).
2. McDougal, J.S., et al., J. Immunol. Meth., **63**, 281-290 (1983).
3. Beyer, C.F., J. Immunol. Meth., **67**, 79-87 (1984).

TROUBLESHOOTING

Problem 1: No color obtained on strip or signal is too weak.

Possible Cause	Suggested Action
a. Reagents omitted or not used in proper order.	a. Prepare and use checklist.
b. Inappropriate preservative such as sodium azide is present in the buffers.	b. Check buffer composition.
c. Substrate solution incorrectly prepared.	c. Check substrate with labeled second antibody or other peroxidase reagent.
d. Improper storage of kit reagents.	d. Store at 0-5°C.
e. Insufficient agitations and/or incubation.	e. Apply appropriate agitation and/or increase incubation periods.

Problem 2: Too many signals obtained.

Possible Cause	Suggested Action
a. More than one hybridoma clone in specimen.	a. Reclone hybridoma.
b. Strip is not properly rinsed.	b. Use proper washing.
c. IgG1 specimen is also singled out as IgG2a antibody therefore positive signals are obtained for IgG1 and IgG2a.	c. Disregard the IgG2a signal as this background appears occasionally. Consider the IgG1 signal only.
d. Ascites fluid containing host-derived antibodies.	d. Use a purified specimen, higher dilutions of ascites fluid to dilute out the contaminants or use culture supernatants.

Problem 3: Clear signal is obtained for only the positive control.

Possible Cause	Suggested Action
a. Concentration of antibody tested is too low.	a. Use higher concentration of specimen.
b. Hybridoma is not secreting antibody.	b. Check antibody secretion.