

PROTOCOL

Chrometra AB ExM and AB ExM Post

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Product Information

Description	Polymerizable Fluorescent Antibody Reagent
Use	Reagent for staining of antibodies in microscopy.
Contents	Small (S): 1 antibody-labelling reaction Large (L): 3 antibody-labelling reactions

Introduction

Chrometra Antibody ExM for Antibody staining in polymeric gels

The Chrometra AB ExM Direct kit is used to introduce labels onto antibodies and proteins in a single step. By including a polymerizable moiety into the structure, the fluorescent conjugate becomes permanently bound to the surrounding matrix upon polymerization.

Kit Contents

- 1 or 3 vials of AB ExM reagent
- 1 or 3 vials of Dye reagent (for AB ExM Post)
- 1 or 3 purification columns
- Reaction buffer (Borate Buffer, pH 8.5)

Additional Materials Required

- Phosphate Buffered Saline (PBS), 1x, pH 7 to 7.5
- DMSO

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Storage and Safety

Upon receipt store product at -20°C/ -4°F.

Your conjugate can be stored at 4°C/ 39.2°F for limited time. For longer storage the conjugate can be stored at -20°C/ -4°F, and a cryoprotectant (e.g. 50% glycerol) can be added. The conjugate should always be stored in the dark.

Preparing the reagents

Prior to use, dissolve the reagent by adding minimal DMSO (10 µl) to a vial, followed by brief mixing.

Please note that the antibody should be in a suitable buffered solution prior to reaction. Suitable buffers are borate buffer and PBS, and should not contain primary amines. Limited amounts of standard antibody buffer components are tolerated, such as glycerine (<50%), EDTA and Sodium Azide (<0,1%). Tris containing buffers are tolerated but not recommended. Nucleophilic components should be avoided, and this includes primary amines (amino acids (e.g. glycine, ethanolamine) and thiols (mercaptoethanol, DTT). Should your antibody solution contain any of these components, an exchange to a suitable buffer through size exclusion or gel filtration is highly recommended prior to reaction.

As for all conjugation reactions, low reagent concentration hinders effective reaction, with kit components optimized for an antibody concentration of 1 to 2 mg/ml. When more concentrated, the solution can be diluted with reaction buffer or any other buffer meeting the requirements described above.

Labelling of the antibody

1. Prepare the reaction tube by adding up to 100 µg of antibody in a suitable buffer to the reaction tube.
2. Add the AB ExM reagent solution to the antibody.
3. Mix thoroughly by vortexing.
4. Incubate for 1h at 37°C/ 98.6°F or at room temperature overnight.
5. Purify the activated antibody through spin-column (See procedure below).

Purification through spin-column

1. Remove the column's bottom closure. Loosen cap (but do not remove).
2. Place column into a collection tube. Centrifuge at 1500 g for 1 minute.
3. Discard flow through and replace column in collection tube.
4. Add 300 µl of reaction buffer on top of the resin, taking care to not disturb the resin.
5. Centrifuge at 1500 g for 1 minute.

6. Discard flow through and replace column in collection tube.
7. Repeat step two more times. The last time, centrifuge for 2 minutes instead of 1 minute.
8. Blot the bottom of the column and transfer the column to a new collection tube.
9. Apply sample on top of the resin, allowing the solution to flow into the desalting resin bed. Add a stacker volume of reaction buffer (15 µl) on top of the column, to maximize protein recover. after the sample has completely entered the desalting resin bed.
10. Centrifuge for 2 minutes at 1500 g. The flow through contains the purified sample.

Example procedure for immunostaining

1. Apply blocking buffer ((1xPBS, 10% FBS, 1% Tween-20) for 15 minutes.
2. Apply the primary antibodies at the concentration suggested by the manufacturer or determined by optimization in blocking/permeabilization solution for 1h at RT.
3. Wash 3x for 5 minutes with blocking/permeabilization buffer.
4. Apply the secondary antibodies at the concentration suggested by the manufacturer or determined by optimization in blocking/permeabilization solution for 1h at RT.
5. Wash 3x for 5 minutes with blocking/permeabilization buffer.
6. Wash 2x with PBS to remove BSA and triton X-100 from the sample.

Example gelation and digestion procedure (4x Expansion)

1. Incubate samples in 0.1 mg/ml general linker (e.g.; Acrylink NHS (0.35 mM), Acrylink TFP (0.3 mM), AcX (0.35 mM)) in PBS for 90 min at RT.
2. Wash the cells with PBS buffer (3 times, for 5 minutes).
3. Prepare gelation mix (monomer + initiators): 2 M NaCl, 8.625% (w/w) sodium acrylate, 2.5% (w/w) acrylamide, 0.15% (w/w) N,N'-methylenebisacrylamide, 0.01% 4-hydroxy-TEMPO.
4. Immediately before use, add 0.15% (w/w) TEMED and 0.15% (w/w) APS to the required aliquot. Mix gently.
5. Quickly rinse each sample with 50 µL of the freshly initiated monomer solution to pre-infuse.
6. Assemble the sample in a gelation chamber.
7. Cover the sample in gelation solution.
8. Polymerize in a humidified incubator at 37 °C/ 98.6°F for 90 min (avoid evaporation).
9. Digest by incubating the gels with Proteinase K, 8 U/mL in digestion buffer (50 mM Tris, pH 8.0; 1 mM EDTA; 0.5% Triton X-100; 0.8 M guanidine HCl) at 37 °C for 3 h.

10. Optional: nuclear counterstain: Stain with DAPI, 1 µg/mL in PBS for 1 min; Wash the cells with PBS buffer (3 times).
11. Optional: Post Gelation staining procedure (see below).
12. Transfer gels to deionized H₂O for 10 min.
13. Replace with fresh H₂O and repeat 4x (or until dimension change plateaus) to reach full 4x expansion.

Post Gelation staining procedure (For use with AB ExM Post)

1. After completing immunostaining, gelation and digestion are to be performed according to standard ExM procedures (see above for example), resulting in gels with incorporated reactive groups.
2. After digestion, rinse the gel once with 1× PBS.
3. Add the matched staining reagent (1 unit, see section "Dye Concentration" below). Mix gently by pipetting.
4. Incubate the sample for 1 hour at 37°C/ 98.6°F, or overnight at room temperature. Shield from light.
5. Wash the gel with PBS buffer (3 times).
6. The gel is now ready for imaging or swelling (steps 12-13 of "Example gelation and digestion procedure above).

Tips and troubleshooting

- To avoid photobleaching of dilute dyes, minimize the exposure of fluorescently labelled specimens to light.
- If Borate Buffer precipitates during storage, solubilize it by warming at 37-50°C/ 98.6-122°F and vigorously vortexing the vial.
- To reduce the amount of antibody needed per immunostaining, coverslip with cultured cells can be flipped facing downwards onto a 80 µL droplet of blocking/permeabilization solution containing antibody, on a piece of Parafilm inside humidified chamber (only when cells were cultured on a coverslip).
- Alternative buffers for Antibody labeling are PBS and 0.2M carbonate-bicarbonate buffer, pH 9.4.
- Inefficient incorporation of the dye in the polymeric matrix can contribute to low signal after sample clearing and swelling. When low signal intensity is observed, grafting cross-linking benefits from gentle polymerization (through the addition of 4-hydroxy TEMPO) and prolonged reaction times.

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