T7•Tag® Monoclonal Antibody

About the Kits

T7•Tag Monoclonal Antibody	50 µg	69522-3
	250 µg	69522-4

Description

The T7•Tag Monoclonal Antibody is a mouse monoclonal antibody (IgG_{2b}, κ) directed against the 11 amino acid (MetAlaSerMetThrGlyGlyGlnGlnMetGly) gene *10* leader peptide expressed by many of the pET translation vectors as well as pSCREEN™, and pRSET vectors. Since the peptide is the natural amino terminal end of the T7 major capsid protein, the antibody also recognizes T7 bacteriophage (e.g. the T7Select® Vectors). The antibody is qualified for Western blotting, immunoprecipitation and immunohistochemistry (1–6).

The T7•Tag Monoclonal Antibody can detect as little as 1–10 ng on a Western blot and cross-reactivity with bacterial, insect or mammalian cells lysates is negligible. The 50 µg package provides enough antibody for 50 Western blots (10 × 10 cm) or 50 immunoprecipitation assays and a Positive Control Extract containing a 31.1 kDa T7•Tag fusion protein is provided for Western blots.

Components

- 50 or 250 µg T7•Tag Monoclonal Antibody
- 250 µl T7•Tag Positive Control Extract (containing 31.1 kDa T7•Tag protein)

Storage

Store all components at –20°C.

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Western Blotting

This protocol is for the transfer and detection of proteins on nitrocellulose membranes. Note that PVDF or other hydrophobic blotting membranes may require different blocking conditions (e.g. longer blocking incubations, higher concentration of blocking reagent).

T7•Tag Positive Control Extract

The T7•Tag Positive Control Extract is prepared from an E.coli λDE3 lysogen carrying a pSCREEN™-1b(+) recombinant that produces a 31,102 dalton protein containing the T7•Tag peptide. It is supplied at 2.5 ng protein/µl in 1X sample buffer (75 mM DTT, 62.5 mM Tris-HCl, 7.5% glycerol, 1.5 % SDS, 0.005% bromphenol blue, pH 6.8) and can be loaded directly on gels after heating. Loading 5 µl on a mini-gel should give a strong band at the indicated size using alkaline phosphatase/NBT/BCIP detection.

For blotting applications, use the T7•Tag Positive Control Extract to verify performance of the T7•Tag Monoclonal Antibody and of the detection system employed to produce the signal.

Preparation

1. For each standard 10 × 10 cm blot, prepare 20 ml of blocking solution, 3% BSA in 1X TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20).
2. For each standard 10 × 10 cm blot prepare 150 ml 1X TBST for primary and secondary antibody dilutions and washes.
3. Dilute the T7•Tag Monoclonal Antibody 1:10,000 in 1X TBST, e.g. add 1 ul antibody to 10 ml TBST.
4. Dilute the Goat Anti-Mouse IgG AP Conjugate (Cat. No. 69266-3) or HRP Conjugate (Cat. No. 71045-3) 1:5,000 in 1X TBST, e.g. add 4 µl of antibody into 20 ml TBST.

Protocol

1. The following steps should be performed at room temperature with gentle rocking or agitation during incubations. Use a clean tray and place the membrane protein-side up.
2. Run an SDS-PAGE gel of the T7•Tag fusion protein sample. If using the T7•Tag Positive Control Extract, heat the entire tube for 2 min at 37°C to solubilize the SDS. Transfer 5 µl of the extract to a tube and heat for 3 min at 70°C along with the target fusion protein samples. Spin to collect the sample at the bottom of the tube and load onto a gel. A strong sharp 31.1 kDa band will be detected in the Positive Control Extract sample. Load protein markers in an adjacent lane. Perfect Protein® (Cat. No. 69959-3) or Trail Mix™ (Cat. No. 70982-3) Western Markers are available from Novagen and require the S-protein Conjugate (AP; Cat. No. 69598-3, or HRP; Cat. No. 69047-3) for detection.
3. Transfer the proteins to nitrocellulose electrophoretically. Any standard device can be used according to the manufacturer's instructions. A standard transfer buffer is 25 mM Tris base, 192 mM glycine, pH 8.3, 20% methanol. If using the Perfect Protein or Trail Mix Western markers, the 150 and 225 kDa bands may transfer incompletely due to their large size. The 15 kDa band may not efficiently bind to the membrane (particularly 0.45 µ pore size nitrocellulose) due to its small size.
4. Remove the membrane from the blotting apparatus and incubate in 20 ml blocking solution at room temperature for 30 min.
5. Incubate the membrane for 30 min at room temperature with 10 ml T7•Tag antibody diluted 1:10,000.
6. Wash the membrane three times for 5 min using 20 ml 1X TBST with gentle shaking at room temperature.
7. Incubate the membrane for 30 min at room temperature with 20 ml Goat Anti-Mouse IgG AP or HRP Conjugate diluted 1:5,000.

Note: HRP conjugates cannot be used with blocking buffer containing sodium azide. Sodium azide inhibits HRP activity.

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8. Wash the membrane three times for 5 min using 20 ml 1X TBST with gentle shaking at room temperature.

Note: Washing steps should be performed in sufficient volume and repeated three times to assure complete removal of unbound conjugate. If background is evident, the blot can be washed several more times before adding additional substrate.

9. Develop the blot with chemiluminescent or colorimetric detection reagents (see below).

Chemiluminescent detection

CDP-Star® AP Substrate (Cat. No. 69086-3) or SuperSignal® HRP Substrate (Cat. No. 69059-3) are available from Novagen for sensitive chemiluminescent detection. Use 1.5 ml of the CDP-Star AP Substrate or 1 ml of the SuperSignal HRP Substrate per 10 × 10 cm blot. Prepare the SuperSignal Substrate working solution by briefly mixing equal parts of 2X Luminol/Enchancer and 2X Stable Peroxide Solution.

1. After the final washing step is complete, try to drain as much TBST from the membrane as possible. It is helpful to touch the corner of a dry paper towel to the edge of the membrane as it is held at an angle. Place the membrane protein-side up in a clean tray or on plastic wrap.
2. For a typical 10 × 10 cm blot, 1–1.5 ml of the chemiluminescent substrate working solution is sufficient. Prepare the substrate immediately before use. Wet the entire surface of the membrane with the appropriate substrate. Incubate the blot in the substrate at room temperature for 1 min.
3. Remove the membrane from the substrate. Drain any excess substrate from the membrane by touching the edge to a paper towel. Place the membrane in a Development Folder (Cat. No. 69137-3) or on a fresh sheet of plastic warp and fold the plastic over the membrane. Remove any bubbles between the plastic and the membrane. Gently remove any liquid from the exterior of the plastic.
   Optional: Place a gLOCATOR™ Luminescent Label (Cat. No. 69102-3) on a corner of the Development Folder. The gLOCATOR Luminescent Label has space to record blot-identifying data for future reference.
4. Place the wrapped membrane in a film cassette with autoradiographic film and expose for 1–10 min. An initial exposure time of 1 min is recommended. Longer exposures can be performed although the highest light output occurs in the first five minutes. Light output continues over several hours. Be careful not to move the film or membrane after initial placement or multiple images can result.

Colorimetric detection

AP Detection Reagent Kit (Cat. No. 69264-3) is available from Novagen for colorimetric detection and contains enough NBT, BCIP and 20X AP Buffer for 25 blots (10 × 10 cm).

1. Based on a 10 × 10 cm blot, prepare developing solution by combining 60 µl NBT (83 mg/ml nitro-blue tetrazolium in 70% (v/v) dimethylformamide) and 60 µl BCIP (42 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, toludinium slat in 100% deimethylformamide) to 15 ml of 1X AP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 1 mM $MgCl_2$).
2. Place the membrane protein side up in a clean tray and add the developing solution. Incubate the membrane at room temperature until purple color develops. Strong purple signal should appear within 2–10 minutes.
3. To stop the reaction, wash the blot thoroughly in deionized water and allow to air dry. Store dry blot at room temperature wrapped in plastic.

Dot Blot Protocol

1. Make serial dilutions of a prepared extract in 10mM Tris-HCl, 25 mM EDTA, pH 8.0 covering a range of 2 µg/ml – 200 µg/ml protein (make one additional series for the T7•Tag positive Control extract if desired).
2. Spot 1 µl of a T7•Tag fusion protein sample directly onto nitrocellulose. Allow to air dry for several minutes.

Note: The bromphenol blue dye in the sample buffer of the T7•Tag Positive Control Extract does not interfere with detection. It is washed away prior to the addition of the colorimetric or chemiluminescent reagents.

3. Proceed as described for steps 4–9 under the Western Blot Protocol.

Immunoprecipitation Protocol

This protocol is for the immunoprecipitation of T7•Tag fusion can be used for proteins synthesized *in vitro* using rabbit reticulocyte lysates or with cellular extracts. We recommend using Novagen's STP3® or Red Nova® Lysate for superior *in vitro* translation performance. For the preparation of extracts from mammalian or insect cells we recommend CytoBuster™ Protein Extraction Reagent (Cat. No. 71009-3)

This protocol uses *S. aureus* cells (e.g., Pansorbin®, Cat. No. 507858) as the precipitant for immune complexes. The T7•Tag antibody is mouse IgG_{2b}, which binds strongly to protein A. Other immobilized forms of protein A (Protein A Agarose, Protein G Plus/Protein A Agarose Suspension) or MagPrep® Anti-Mouse IgG Beads (Cat. No. 70996-3) can be substituted for fixed *S. aureus*.

Immunoprecipitation with Pansorbin

Perform all steps at 0–4°C

1. Shake the bottle of Pansorbin to resuspend. Remove 0.5 ml to a 1.5 ml tube. Spin at maximum speed in a microcentrifuge for 1 min. Remove the supernatant, resuspend pellet in 1 ml 1X wash buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, add 0.2 mM methionine if using radiolabeled protein). Spin again, resuspend in 0.5 ml 1X wash buffer and store on ice until use.
2. Add the desired amount of the completed translation reaction to each of two 1.5 ml tubes containing 0.5 ml 1X wash buffer on ice. One tube will be used for immunoprecipitation, while the other will serve as background (minus antibody). Mix gently.
3. Preabsorb the samples by adding 50 µl washed Pansorbin to each sample. Briefly vortex and then incubate on ice for 5 min. Spin at maximum speed in a microcentrifuge for 1 min. Remove supernatants to fresh tubes. This step is to remove materials that bind non-specifically to Pansorbin.
4. Add 1 µl T7•Tag Monoclonal Antibody to one of the tubes.
5. Mix and incubate the samples on ice for 60 min.
6. Add 50 µl washed Pansorbin to each tube. Vortex and incubate on ice for 60 min.
7. Spin at maximum speed in a microcentrifuge for 1 min. Remove supernatant, resuspend pellet in 0.75 ml 1X wash buffer (be sure to resuspend thoroughly), and spin again. Repeat wash a total of 3 times. After the last spin, remove as much supernatant as possible.
8. Add 50 µl 1X SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 2.5% 2-mercaptoethanol, 10% glycerol, 0.05% Bromphenol blue) to the pellet. Resuspend thoroughly, heat at 70°C for 3 min, and spin out the Pansorbin at maximum speed in a microcentrifuge for 5 min.
9. Load the supernatant on SDS gel and run it. For optimal results load the maximal amount of supernatant for the well size being used. Also include a lane of protein markers such as Trail Mix™ Western Makers which have pre-stained bands for convenience.

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Detection by autoradiography

1. After the gel is finished running, fix the proteins by soaking in 10% TCA or isopropanol:water:acetic acid 25:65:10 for 20 min.
2. Dry the gel and expose to X-ray film. Exposure times will vary based on the amount of radioactivity incorporated and the amount of sample used for immunoprecipitation. A strong signal should be observed under the following conditions:

   0.2-2 µg RNA template and 10-20 µCi of > 600 Ci/mmol ^{35}S-met in a 25 µl translation reaction

   10 µl of translation mix per immunoprecipitation reaction overnight (14–20 hr) exposure

   Sensitivity can be increased about 10-fold using fluorography. Soak the gel in a solution containing appropriate scintillants (e.g. Amplify (Amersham)) according to the manufacturer's instructions. Use intensifying screens during the exposure.

Detection by Western blot

1. Transfer the proteins to nitrocellulose electrophoretically. Any standard device can be used according to the manufacturer's instructions. A standard transfer buffer is 25 mM Tris base, 192 mM glycine, pH 8.3, 20% methanol. If using the Perfect Protein or Trail Mix Western markers, the 150 and 225 kDa bands may transfer incompletely due to their large size. The 15 kDa band may not efficiently bind to the membrane (particularly 0.45 µ pore size nitrocellulose) due to its small size.
2. Proceed with a Western blot protocol specific for the detection antibody.

References

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