SIGMA-ALDRICH®

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Product Information

ANTI-FLAG® High Sensitivity, M2 coated 96-well plates

Catalog Number **P2983**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

Expression vectors that encode the DNA sequence for specific non-native proteins provide a convenient means for producing and purifying proteins of interest in a foreign organism. These expression vectors also allow specific peptide or protein sequences to be fused with the cloned protein. These protein fusion partners can help stabilize the expressed protein and/or increase its solubility. Fusion partners can also serve as affinity handles or tags, such as FLAG, *c-myc* or poly-Histidine that facilitate the capture, purification and detection of the recombinant protein.[1-5]

The ANTI-FLAG HS, M2 Coated Plate is a versatile platform designed for the capture and detection of recombinant FLAG fusion proteins isolated from FLAG protein expression systems. ANTI-FLAG M2, a mouse monoclonal IgG1 antibody, is covalently linked to the surface of a microtiter plate via the Fc portion of the antibody. This linkage provides a favorable orientation of the antibody on the surface of the plate affording higher binding capacity compared to that of a passively absorbed antibody surface.

Capture and detection of fusion proteins and their partners with affinity ligands immobilized on solid supports has become common practice. Supports being used today are agarose, microparticles, membranes and microtiter plates. ELISA, SDS-PAGE electrophoresis, Western Blot, MALDI mass spectroscopy, or affinity binding analysis may be used to assess the recombinant protein following transformation, growth, expression, and lysis of a host organism. In the event that many cell samples containing FLAG-linked fusion proteins are to be analyzed, the ANTI-FLAG HS, M2 Coated Plate offers greater speed and convenience over other methods. ANTI-FLAG HS, M2 Coated Plates are also powerful tools in High Throughput Screening (HTS) applications. The plates may be used for general screening of recombinant protein production, protein-protein interaction studies, protein-organic molecule interaction studies, signal transduction studies, and/or estimation of fusion protein content.

Component

The plate is supplied as a 96-well microtiter plate with clear sides and bottom.

Coating

ANTI-FLAG M2 mouse monoclonal antibody, IgG1, is coated at a reaction volume of 200 μl/well.

Blocking

The wells are pre-blocked for convenience at 275 to 300 μl/well with a complex solution containing bovine serum albumin.

Specificity

The plates are specific for the FLAG epitope (an octapeptide with the sequence N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C) regardless of its placement in the fusion protein: amino-terminal, Met-amino terminal, carboxy terminal or internal. Binding of the epitope is not Ca^{2+} dependent.

Sensitivity

Detection of 1 ng/well of a control fusion protein was observed in an ELISA format with p-Nitrophenyl Phosphate (pNPP) as a substrate.

Capacity

Capture of 100 to 300 ng/well of a FLAG fusion protein has been demonstrated.

Precautions and Disclaimer

- Regeneration and reuse of the plate is not recommended.
- This product is for laboratory use only, not for drug, household, or other use.

Storage/Stability

Store at 2-8 °C. If unopened, the ANTI-FLAG HS, M2 Coated Plate is stable for at least two years. If opened, the plate should be stored with desiccant at 2- 8 °C and used within two weeks.

Procedures

Binding and Elution of a FLAG Fusion Protein; Immunoprecipitation with SDS-Page Analysis:

Materials Required

- ANTI-FLAG HS, M2 Coated Plate, Product No. P 2983
- Cell lysate containing FLAG-fusion protein

1. Load up to 200 µl/well of cell lysate containing the FLAG fusion protein.
2. Cover with a 96-well plate cover or sealing tape.
3. Allow the cell lysate to incubate in the plate for a minimum of one hour at 37 °C or 2 hours at room temperature. If incubating overnight, place at 4 °C.
4. Remove the plate cover and wash the plate three times (300 µl per well) with an appropriate wash buffer (e.g., 100 mM Tris buffered saline, pH 8.2, containing 2 mM $MgCl_2$, 0.5 % Tween, for Alkaline Phosphatase conjugates).

   Note: Plate washing may be done with an automated plate washer or by hand with a multi-channel pipet. If washing by hand, remove any residual liquid by inverting the plate and gently patting it on lint-free paper towels.

5. Elute the protein by loading 30 µl of a 2X sample buffer containing 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue and 125 mM Tris HCl, pH 6.8 (Product Number S 3401).

   Note: Any one of several common elution buffers may be used to elute the FLAG fusion proteins from the surface of the plate. We use the buffer described above for preparation of FLAG fusion proteins for SDS-PAGE analysis, as described below.

6. Seal the plate with sealing tape.
7. Vortex for a minimum of 10 minutes, making certain that the buffer completely washes the sides of the wells. Caution: ensure that the buffer does not splash from the plate.
8. Load samples directly onto an SDS-PAGE gel and run per the manufacturer's recommendations.
9. Gels may be stained or Western Blot analysis may be performed using standard protocols.

Cell Lysate Screening

Materials Required

- ANTI-FLAG HS, M2 Coated Plate, Product No. P 2983
- Cell lysate containing FLAG-fusion protein
- Antibody against the fusion protein of interest and a labeled secondary antibody, if necessary
- Substrate for the detection of enzyme

Equipment Required

- Multi-channel (8- or 12-channel) pipet
- Spectrophotometer capable of reading 96-well microtiter plates at 405 nm

1. Prepare serial dilutions of the crude cell lysate containing the FLAG-fusion protein with a dilution buffer of choice (e.g., phosphate or Tris buffered saline containing 0.5 % Tween 20).

   Note: Initially, it is recommended that log dilutions of the cell lysate be done to determine the performance characteristics of the expression system.

2. Apply up to 200 µl per well of each diluted lysate, or as a negative control, apply up to 200 µl per well of non-expression cell lysate.
3. Cover with a 96-well plate cover or sealing tape.
4. Allow the cell lysate to incubate in the plate for a minimum of one hour at 37 °C or 2 hours at room temperature. If incubating overnight, place at 4 °C.

5. Remove the plate cover and wash the plate three times (300 µl per well) with an appropriate wash solution (e.g., phosphate or Tris buffered saline containing 0.5 % Tween 20).

   Note: Plate washing may be done with an automated plate washer or by hand with a multi-channel pipet. If washing by hand, remove any residual liquid by inverting the plate and gently patting it on lint-free paper towels.

6. Prepare a dilution of the primary antibody or antibody-enzyme conjugate using an appropriate diluent (e.g., 100 mM Tris buffered saline, pH 8.2, containing 1 % BSA, 2 mM $MgCl_2$, and 0.5 % Tween for Alkaline Phosphatase conjugates).
   Note: For detection using an antibody against the fusion protein, the primary and secondary antibody dilution must be determined empirically.

7. Apply a volume of the diluted antibody that is slightly larger than the original cell lysate volume (e.g. if 100 µl/ well of cell lysate was initially loaded, load 125 µl of the primary or secondary antibody per well). Cover the plate with a 96-well plate cover or sealing tape.

8. Allow the plate to incubate for a minimum of one hour at room temperature (18 °C to 26 °C).

   Note: Proceed to step 13 if an antibody-enzyme conjugate was used in steps 6-8. If an unlabeled primary antibody was used in steps 6-8, proceed to step 9

9. Remove the plate cover and wash the plate three times (300 µl per well) with an appropriate wash solution.

10. Prepare a dilution of the secondary antibody-enzyme conjugate using an appropriate diluent (e.g., 100 mM Tris buffered saline, pH 8.2, containing 1 % BSA, 2 mM $MgCl_2$, and 0.5 % Tween for Alkaline Phosphatase conjugates).
11. Apply a volume of the diluted secondary antibody that is equal to the volume of primary antibody used in Step 7. Cover the plate with a 96-well plate cover or sealing tape.

12. Allow the plate to incubate for a minimum of one hour at room temperature (18 °C to 26 °C).

13. Remove the plate cover and wash the plate three times (300 µl per well) with an appropriate wash solution (e.g., 100 mM Tris buffered saline, pH 8.2, containing 2 mM $MgCl_2$, 0.5 % Tween for Alkaline Phosphatase conjugates).

Detection of Bound Fusion Proteins

1. Prepare any necessary substrate and/or stop solutions per product instructions (e.g., Product No. N 7653).

2. Apply up to 200 µl of the appropriate substrate per well.

3. Using a 96-well plate reader, record signal per substrate manufacturer's recommendations (e.g., 405 nm for pNPP).

Troubleshooting Guides
Screening

Problem	Possible Cause	Solution
High Background	Crude cell lysate may contain enzymes capable of turning over substrate	Check the negative control, non-expressing cell lysate
	Antibody titer too high	Increase the dilution of primary and/or secondary antibodies
	Substrate incubation time too long or substrate is degraded	Decrease time exposed to substrate or utilize a fresh substrate
No signal	No fusion protein expressed	Re-evaluate the expression system and prepare a fresh culture for analysis
	Antibody/Antibody-enzyme conjugate inactive	Utilize a proven antibody/antibody-enzyme conjugate at appropriate titers
	Degraded substrate	Utilize a fresh stock of substrate
Low Signal	Low level of fusion protein	Re-evaluate the expression system and prepare a fresh culture for analysis
	Crude cell lysate too dilute	Reduce the lysate dilution factor
	Antibody titer too low	Reduce the dilution of primary and/or secondary antibodies
	Substrate incubation time insufficient	Increase exposure to substrate as long as background levels remain low
High Signal	Crude cell lysate too concentrated	Further dilute crude cell lysate
	Antibody titer too high	Increase the dilution of primary and/or secondary antibodies
	Substrate incubation time too long	Decrease exposure time to substrate

Immunoprecipitation

Problem	Possible Cause	Solution
Low Yield of Eluted Protein	Low Binding Efficiency	Increase the amount of lysate/protein in the binding step and/or extend the binding incubation time .
	Low elution efficiency	Allow longer incubation with the elution buffer. Check to see that the agitation of the elution buffer is sufficient to completely wash the well walls

REAGENT COMPATIBILITY TABLE

Reagent	Effect	Comments
Chaotropic agents (e.g. urea, guanidine HCl)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains these types of components since it will denature the M2 antibody and destroy its ability to bind the FLAG tagged proteins. Low concentrations of urea (1 M or less) can be used.
Reducing agents (e.g. DTT, DTE, 2-mercaptoethanol)	Reduces the disulfide bridges holding the M2 antibody chains together	**Do not** use any reagent that contains these types of components since it will reduce the disulfide linkages in the M2 antibody and destroy its ability to bind the FLAG tagged proteins.
Tween 20, 5% or less	Reduces non-specific protein binding	May be used up to recommended concentration of 5 % but do not exceed.
Triton X-100, 5% or less	Reduces non-specific protein binding	May be used up to recommended concentration of 5 % but do not exceed.
Igepal CA-630, 0.1% or less	Reduces non-specific protein binding	May be used up to recommended concentration of 0.1 % but do not exceed.
CHAPS, 0.1% or	Reduces non-specific	May be used up to recommended concentration of 0.1 % but do not

less	protein binding	exceed.
Digitonin, 0.2% or less	Reduces non-specific protein binding	May be used up to recommended concentration of 0.2 % but do not exceed.
Sodium chloride, 1.0 M or less	Reduces non-specific protein binding by reducing ionic interactions	May be used up to recommended concentration of 1.0 M but do not exceed.
Sodium dodecyl sulfate (SDS)	Denatures the immobilized M2 antibody	**Do not** use any reagent that contains this detergent in the loading and washing buffers since it will denature the M2 antibody and destroy its ability to bind the FLAG tagged proteins.
Deoxycholate	Interferes with M2 binding to FLAG proteins	**Do not** use any reagent that contains this detergent since it will inhibit the M2 antibody from binding to FLAG fusion proteins.

References

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3. Chiang, C.M. and Roeder, R.G., Pept. Res., **6**, 62 (1993).
4. Ausubel F.M., et.al. Current Protocols in Molecular Biology, pp. 10.15.1-10.16.29 (John Wiley and sons Inc., N.Y. 1998).
5. Harlow E. and Lane D. Antibodies, A Laboratory Manual, pp. 514-517, 541-542, 547-549 (Cold Spring Harbor Laboratory Press, NY, 1988).

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AH,RS,PHC 08/10-1

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