FLAG® Western Detection Kit

INSTRUCTION MANUAL

Catalog #200470
Revision A

For In Vitro Use Only
200470-12

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FLAG® Western Detection Kit

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FLAG® Western Detection Kit

MATERIALS PROVIDED

Materials provided[a]	Quantity
Anti-FLAG M2 antibody[b–d]	200 µg
Goat anti-mouse antibody, AP conjugated, immunoaffinity purified, polyclonal[c]	100 µl
Sterile, filtered fraction V bovine serum albumin (BSA) [30% (w/v) in saline]	15 ml
Nitroblue tetrazolium (NBT)[b] [75 mg/ml in 70% *N,N*-dimethylformamide (DMF)]	2 ml
5-Bromo-4-chloro-3-indolyl phosphate (BCIP)[b] (50 mg/ml in 100% *N,N*-DMF)	1.5 ml

[a] Sufficient materials are provided for ten 100-mm nitrocellulose membranes.

[b] Also available separately [Anti-FLAG M2 antibody, Stratagene Catalog #200471 (200 µg), #200472 (1 mg); BCIP (5-bromo-4-chloro-3-indolyl phosphate, Stratagene Catalog #300045 (1.5 ml); NBT (nitroblue tetrazolium), Stratagene Catalog #300130 (2 ml)].

[c] Please see the *Certificate of Analysis* for the concentration.

[d] Immediately on receipt, store the anti-FLAG M2 antibody at –20°C and the goat anti-mouse antibody at 4°C. Avoid multiple freeze–thaw cycles. Aliquot and fast freeze the primary and secondary antibodies for long-term storage.

STORAGE CONDITIONS

BSA: –20°C
Anti-FLAG® M2 Antibody: –20°C

Note *For best results, store the anti-FLAG M2 antibody at –20°C.*

Goat Anti-Mouse Antibody: 4°C
NBT: 4°C
BCIP: 4°C

Revision A

ADDITIONAL MATERIALS REQUIRED

Western Blotting and Antibody Probing of Electroblots

Laemmli sample buffer (1×)§
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) system
Nitrocellulose membranes (available from Stratagene, Catalog #420106, #420107, and #420108)
Transfer buffer (1×)§
Blocking solution§ or bovine lacto transfer technique optimizer (BLOTTO)§
Tris-buffered saline (TBS)§

Color Development Reaction

Notes *Do not prepare the color development solution containing the NBT and BCIP until just prior to use in the color development reaction [see* Preparation of the Color Development Solution Containing Nitroblue Tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP) *subsection of* Color Development Reaction*].*

Color development solution§
Tris-buffered saline (TBS)
Stop solution§
Whatman® 3MM paper

Dot Blot Assay

Strip of nitrocellulose membrane (1 × 6 cm)
Blocking solution or BLOTTO
Tris-buffered saline (TBS)
Color development solution
Whatman® 3MM paper
Stop solution

§ See *Preparation of Reagents*.

NOTICES TO PURCHASER

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INTRODUCTION

Epitope tagging technology is a powerful method for the analysis of protein structure and function both in vivo and in vitro, and the FLAG® peptide (DYKDDDDK)[1] is one of the most widely used epitope tags in the field of molecular biology, cell biology, and genetics for protein labeling.[1,2,3,4] The Stratagene FLAG® western detection kit includes an anti-FLAG® M2 antibody that can detect recombinant fusion proteins with the FLAG epitope located at either the N- or C-terminal, as well as recombinant fusion proteins with the FLAG epitope located internally. The anti-FLAG M2 antibody can detect as little as 5 ng of FLAG epitope-tagged recombinant fusion protein. The anti-FLAG M2 antibody is highly specific, with low cross-reactivity with prokaryotic and eukaryotic proteins.[3,4]

The FLAG western detection kit utilizes the anti-FLAG M2 antibody as the primary antibody and an alkaline phosphatase (AP)-conjugated goat anti-mouse antibody as the secondary antibody. The signal is detected by a color development solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The FLAG western detection kit can be used to monitor expression of FLAG-tagged proteins in prokaryotic and eukaryotic cells, to follow FLAG epitope-tagged protein during protein purification steps, and to identify proteins following immunoprecipitation. In addition, the FLAG western detection kit provides a rapid and sensitive way to detect proteins expressed in the ESP yeast protein expression and purification system (Stratagene Catalog #251600).

WESTERN BLOTTING[5,6] AND ANTIBODY PROBING OF ELECTROBLOTS

Note *Unless otherwise indicated, conduct all procedures at room temperature on a shaker.*

1. Boil the antigenic protein samples in 1× Laemmli sample buffer for 2 minutes and load the samples and a molecular-weight marker into separate lanes of an appropriate percentage SDS–polyacrylamide gel for analysis. Resolve the gel by SDS–PAGE.

2. Equilibrate a nitrocellulose membrane in 1× transfer buffer by soaking the membrane for 20 minutes.

   Note *Never touch the nitrocellulose membranes with bare hands; always use gloves or forceps. Also, do not allow the nitrocellulose membranes to dry during any step.*

3. Electroblot the antigenic protein samples onto the nitrocellulose membrane either at 30 V and ~200 mA or at the voltage and approximate current specified by the manufacturer of the transfer apparatus for 1 hour in 1× transfer buffer.

4. Following electrophoretic transfer of the proteins onto the nitrocellulose membrane, block the membrane by incubating the nitrocellulose membrane in blocking solution, using at least 25 ml of blocking solution for an 8- × 6-cm membrane, with gentle rocking at room temperature for a minimum of 1 hour.

   Notes *Blocking may also be performed overnight at 4°C.*

   BLOTTO may be substituted for the blocking solution throughout the western blotting, antibody probing, and the optional dot blot assay procedures when blocking the membrane. ***Do not use BLOTTO to dilute the anti-FLAG M2 antibody or the goat anti-mouse antibody.***

5. Thoroughly wash the nitrocellulose membrane twice with a generous amount of TBS for a minimum of 2 minutes/wash.

   Notes *If nitrocellulose membranes are combined during the wash steps, less of the wash solution can be used. Separate the nitrocellulose membranes with forceps at each change to ensure adequate exposure of each membrane.*

6. For an 8- × 6-cm membrane, add 20 µg of the anti-FLAG M2 antibody to at least 10 ml of TBS and incubate the membrane in this antibody dilution at room temperature for a minimum of 1 hour.

7. Thoroughly wash the nitrocellulose membrane twice with a generous amount of TBS for a minimum of 2 minutes/wash.

8. Dilute the goat anti-mouse antibody 1:5000 in blocking solution.

9. Gently rock the nitrocellulose membrane in the goat anti-mouse antibody dilution, using at least 10 ml for an 8- × 6-cm nitrocellulose membrane, at room temperature for a minimum of 1 hour.

10. Thoroughly wash the nitrocellulose membrane three times with a generous amount of TBS for a minimum of 5 minutes/wash and then proceed to the *Color Development Reaction.*

COLOR DEVELOPMENT REACTION

Preparation of the Color Development Solution Containing Nitroblue Tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP)

Prepare the color development solution containing NBT and BCIP just prior to use as outlined in the following steps.

1. Dilute 40 µl of NBT (75 mg/ml) in 10 ml of color development solution to a final concentration of 0.3 mg/ml for a standard 8- × 6-cm nitrocellulose membrane.

2. Add 30 µl of BCIP (50 mg/ml) dropwise to a final concentration of 0.15 mg/ml.

   Note *If a precipitate forms, warm the color development solution containing the NBT and BCIP to 37°C and pass this solution through a 0.2-µm filter.*

3. Protect the color development solution containing the NBT and BCIP from light and leave the solution at room temperature until ready for use in step 2 of the following section.

Color Development on the Nitrocellulose Membrane

Note *Allow the color development reaction to proceed in the dark.*

1. Remove the nitrocellulose membrane from the final TBS wash (see step 11 of *Western Blotting and Antibody Probing of Electroblots*) and blot off any excess moisture with Whatman® 3MM paper.

2. Immerse the nitrocellulose membrane in the color development solution containing NBT and BCIP. Allow the color development reaction to proceed in the dark until the positive reactions are clearly visible (usually within minutes).

   Notes *Extended times for color development can result in high backgrounds. If extended times (1–5 hours) are required for the color development reaction, store the nitrocellulose membranes in the dark and check the membranes periodically to minimize the background.*

   If a visible precipitate forms during the color development reaction that is not associated with specific dots or bands, replace the color development solution containing NBT and BCIP with a freshly prepared solution and allow the color development reaction to continue.

3. Remove the color development solution containing NBT and BCIP from the nitrocellulose membrane and rinse the membrane with TBS.

4. Terminate the color development reaction by immersing the nitrocellulose membrane in stop solution.

5. Following completion of the color development reaction, air dry the nitrocellulose membrane and store the membrane protected from light.

If desired, the optional *Dot Blot Assay* may be performed to quickly and simply determine if the recombinant fusion protein has been produced.

DOT BLOT ASSAY (OPTIONAL)

The dot blot assay assesses the ability of the anti-FLAG M2 antibody dilution and the goat anti-mouse antibody dilution to detect different levels of recombinant fusion protein. The dot blot assay also verifies the reaction of the NBT and BCIP with the AP-conjugated goat anti-mouse antibody.

1. Cut out a 1- × 6-cm strip of nitrocellulose membrane. Using a soft pencil, mark the strip into six equal squares measuring ~1 cm^2.

2. Prepare the nitrocellulose membrane with serial protein dilutions, anti-FLAG M2 antibody, and goat anti-mouse antibody as follows:

   a. Spot 1 µl of each of the following serial protein dilutions onto separate squares of the strip: 100 ng/µl, 10 ng/µl, 5 ng/µl, and 1 ng/µl.

   b. Spot 1 µl of the anti-FLAG M2 antibody diluted in TBS to a concentration of 10 ng/µl onto a separate square of the strip.

   c. Spot 1 µl of the goat anti-mouse antibody diluted 1:5000 in blocking solution onto a separate square of the strip.

3. Air dry the nitrocellulose membrane for 5 minutes.

4. Immerse the strip in blocking solution for 1 hour at room temperature to block nonspecific protein-binding sites.

5. Thoroughly wash the nitrocellulose membrane two to three times for 2 minutes each in a generous amount of TBS.

6. Prepare 10 ml of the anti-FLAG M2 antibody diluted in TBS to a concentration of 2 µg/ml and incubate the strip in the anti-FLAG M2 antibody dilution at room temperature for 1 hour.

7. Thoroughly wash the nitrocellulose membrane two to three times for 2 minutes each in a generous amount of TBS.

8. Prepare 10 ml of the goat anti-mouse antibody diluted 1:5000 in blocking solution and gently rock the strip in the goat anti-mouse antibody dilution at room temperature for a minimum of 1 hour.

9. Thoroughly wash the nitrocellulose membrane three times with a generous amount of TBS for a minimum of 5 minutes/wash.

10. Prepare the color development solution containing NBT and BCIP as outlined in the following steps:

    a. Dilute 40 µl of NBT (75 mg/ml) in 10 ml of color development solution to a final concentration of 0.3 mg/ml.

    b. Add 30 µl of BCIP (50 mg/ml) dropwise to a final concentration of 0.15 mg/ml.

       Note *If a precipitate forms, warm the color development solution containing the NBT and BCIP to 37°C and pass this solution through a 0.2-µm filter.*

    c. Protect the color development solution containing the NBT and BCIP from light and leave the solution at room temperature until ready for use in step 12 of this section.

11. Remove the strip from the final TBS wash and blot off any excess moisture with Whatman 3MM paper.

12. Immerse the nitrocellulose membrane in color development solution containing NBT and BCIP. Allow the color development reaction to proceed in the dark until the positive reactions are clearly visible. Check the membrane every minute for a positive reaction.

13. Rinse the residual precipitated dye from the strip with a generous amount of TBS.

14. Terminate the color development reaction by immersing the nitrocellulose membrane in stop solution.

15. Air dry the nitrocellulose membrane and store the membrane protected from light.

TROUBLESHOOTING

Western Blotting, Antibody Probing of Electroblots, and Color Development Reaction

Observations	Suggestions
The signal is not visible on the nitrocellulose membrane	Ensure that the goat anti-mouse antibody or the color development solution is fresh and/or properly prepared
	To test the activity of the working dilution of the goat anti-mouse antibody, add 1 ml of the color development solution containing NBT and BCIP with 1 ml of the goat anti-mouse antibody diluted in TBS as indicated on the *Certificate of Analysis*; if no color precipitates within 30 minutes, repeat this test with a fresh dilution of the goat anti-mouse antibody If color does appear, proceed to the *Dot Blot Assay* to check the protein, the anti-FLAG M2 antibody, and the goat anti-mouse antibody
	Verify the FLAG sequence in the protein
Excessive background on the nitrocellulose membrane is present	If the nitrocellulose membrane shows excessive background, check that the blocking solution or BLOTTO is properly prepared and ensure that the nitrocellulose membrane is thoroughly washed
	Check to ensure that that the anti-FLAG M2 antibody is properly diluted in TBS
	Check to ensure that that the goat anti-mouse antibody is properly diluted in the blocking solution

Dot Blot Assay (Optional)

Note *The observations in this section are addressed by performing the optional* Dot Blot Assay.

Observations	Suggestions
The protein is not visible on the dot blot strip, while the anti-FLAG M2 antibody probe is visible	Because the protein is folded, the FLAG epitope tag, which is located in the interior of the protein molecule, is not making contact with the anti-FLAG M2 antibody probe; perform western blotting noting that the denaturing characteristics of the gel should cause the protein to unfold, making the FLAG epitope tag available for detection by the anti-FLAG M2 antibody
	Verify the FLAG sequence in the protein
The anti-FLAG M2 antibody probe is not visible on the dot blot strip, while the goat anti-mouse antibody is visible	Use a freshly prepared dilution of the anti-FLAG M2 antibody probe or replace the probe completely with a new anti-FLAG M2 antibody probe
The goat anti-mouse antibody is not visible on the dot blot strip	To test the activity of the working dilution of the goat anti-mouse antibody, add 1 ml of the color development solution containing NBT and BCIP with 1 ml of the goat anti-mouse antibody diluted in TBS as indicated on the *Certificate of Analysis*; if no color precipitates within 30 minutes, repeat this test with a fresh dilution of the goat anti-mouse antibody

PREPARATION OF REAGENTS

Stock Solutions

Use sterile concentrated stock solutions prepared with deionized, double-distilled water (ddH_2O) or the equivalent. The stock solutions necessary for all reagent preparations in the FLAG western detection kit are as follows:

Stock solutions	Sterilization method
1 M Tris-HCl (pH 7.5)	Autoclave
1 M Tris-HCl (pH 9.5)	Autoclave
1 M Tris-HCl (pH 2.9)	Autoclave
5 M NaCl	Autoclave
1 M $CaCl_2$	Autoclave
1 M $MgCl_2$	Filter sterilize

Standard Reagents

Note *Use freshly prepared reagents. The suggested working volume is 10 ml for each 8- × 6-cm nitrocellulose membrane.*

Blocking Solution
1% (w/v) BSA in TBS

TBS
50 mM Tris-HCl (pH 7.5)
150 mM NaCl

BLOTTO
5 g of nonfat dry milk
1 ml of 1% (w/v) thimerosal–H_2O (Sigma Catalog #T 5125)
Adjust the volume to 100 ml with TBS
Store at 4°C for 7–10 days

Color Development Solution
100 mM Tris-HCl (pH 9.5)
100 mM NaCl
5 mM $MgCl_2$

Laemmli Sample Buffer (2×)
250 mM Tris-HCl (pH 6.8)
8% (w/v) SDS
40% (v/v) glycerol
0.01% (w/v) bromphenol blue dye
200 mM β-mercaptoethanol

Stop Solution
20 mM Tris-HCl (pH 2.9)
1 mM $CaCl_2$

Transfer Buffer (1×)
12 mM Tris-HCl (pH 8.3)
96 mM glycine
20% (v/v) methanol

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1. Hopp, T., Prickett, K., Price, V., Libby, R., March, C. *et al.* (1988) *BioTechnology* 6:1204-1210.
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3. Su, X., Prestwood, A. K. and McGraw, R. A. (1992) *Biotechniques* 13(5):756-62.
4. Smit, M. J., Timmerman, H., Alewijnse, A. E., Punin, M., van den Nieuwenhof, I. *et al.* (1995) *Biochem Biophys Res Commun* 214(3):1138-45.
5. Harlow, E. and Lane, D. (Eds.). (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.
6. Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc Natl Acad Sci U S A* 76(9):4350-4.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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FLAG® Western Detection Kit

Catalog #200470

QUICK-REFERENCE PROTOCOL

Western Blotting and Antibody Probing of Electroblots

- Boil the protein samples in 1× Laemmli sample buffer for 2 minutes, load the samples and a molecular-weight marker into separate lanes of a SDS–polyacrylamide gel, and resolve by SDS–PAGE
- Equilibrate a nitrocellulose membrane in 1× transfer buffer for 20 minutes
- Electroblot the protein samples onto the nitrocellulose membrane either at 30 V and ~200 mA or at the voltage and approximate current specified by the manufacturer of the apparatus for 1 hour in 1× transfer buffer
- Block the nitrocellulose membrane in blocking solution with gentle rocking at room temperature for a minimum of 1 hour
- Wash the membrane twice with TBS for a minimum of 2 minutes/wash
- Add 20 µg of the anti-FLAG M2 antibody to at least 10 ml of TBS and incubate the membrane in this antibody dilution at room temperature for a minimum of 1 hour
- Wash the membrane twice with TBS for a minimum of 2 minutes/wash
- Dilute the goat anti-mouse antibody 1:5000 in blocking solution
- Gently rock the nitrocellulose membrane in the goat anti-mouse antibody dilution at room temperature for a minimum of 1 hour
- Wash the membrane three times with TBS for a minimum of 5 minutes/wash

Color Development Reaction

- Prepare the color development solution containing NBT and BCIP
- Blot off any excess moisture and immerse the nitrocellulose membrane in color development solution containing NBT and BCIP until the positive reactions are clearly visible
- Rinse the membrane with TBS
- Immerse the nitrocellulose membrane in stop solution
- Air dry the membrane and store protected from light