Clontech® Laboratories, Inc.

Matchmaker® Monoclonal Antibodies User Manual

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I. Introduction

Matchmaker Monoclonal Antibodies (mAbs) are purified from the serum-free media of mouse hybridoma cultures. These IgG mAbs can be used for probing Western blots (Burnette, 1981) prepared with protein isolated from transformed yeast cells. The immunoblotting procedure is based on standard protocols (Harlow & Lane, 1988) and modified for use with yeast proteins. Probing Western blots of yeast protein extracts with these mAbs yields a single, specific band (Figures 1 and 2 in Section III.D). Where possible, we generally recommend chemiluminescent methods because of greater sensitivity and because the blots can be washed and reprobed later. (Colorimetric substrates permanently stain the blot.) The Yeast Protocols Handbook (YPH; PT3024-1) provides supplemental protocols and information not covered in this User Manual.

GAL4 Monoclonal Antibodies

The GAL4 AD and GAL4 DNA-BD monoclonal antibodies bind specifically to the major activation domain (a.a. 768–881) or the DNA-binding domain (a.a. 1–147), respectively, of the yeast GAL4 protein (Printen & Sprague, 1994). Generally, neither GAL4 mAbs cross-react with other proteins extracted from *E. coli* or GAL4-deleted yeast strains.

GAL4 mAbs are useful in characterizing fusion proteins generated when using the high-expression cloning plasmids pGADT7 AD and pGADT7rec provided in Matchmaker Gold Yeast Two-Hybrid System (Cat. No. 630489) and Make Your Own "Mate & Plate™" Library System (Cat. No. 630490). The appropriate GAL4 mAb can be used to confirm that the fusion protein is being expressed in yeast, and that it has the expected molecular weight (Luban & Goff, 1995). In addition, Western blot analysis can be an important step in characterizing GAL4 AD fusion proteins identified as candidates for a positive interaction in a one- or two-hybrid library screening (Fields & Sternglanz, 1994) if the library was constructed in a high-expression plasmid such as pGADT7. Quantitation can be performed on a dot blot if an appropriate, purified control protein is available.

GAL4 AD mAb and DNA-BD mAb are able to detect 1–5 ng of purified GAL4 AD or DNA-BD peptide, respectively, on a dot blot under conditions similar to those specified in the Product Certificate of Analysis.

Note that expression from Matchmaker Gold Yeast Two-Hybrid System vectors pGBKT7 and pGADT7 AD can also be detected using our c-myc monoclonal antibody (Cat. No. 631206) and HA polyclonal antibody (Cat. No. 631207) respectively.

Table 1. List of Abbreviations

Abbreviation	Definition
AD	Activation domain
AD/library plasmid	Plasmid encoding a fusion of the GAL4 AD and a library insert
AD/library protein	A hybrid comprised of the GAL4 AD fused to a protein encoded by a library insert
AD vector	Plasmid encoding the GAL4 AD
DNA-BD	DNA-binding domain
DNA-BD/target plasmid [or bait plasmid]	Plasmid encoding the DNA-BD/target protein
DNA-BD/target protein [or bait protein]	A hybrid comprised of the GAL4 DNA-BD fused with the target protein
DNA-BD vector	Plasmid encoding the GAL4 DNA-BD
DO	Dropout (supplement or solution); a mixture of specific amino acids and nucleosides used to supplement SD base to make SD medium; DO supplements are missing one or more of the nutrients required by untransformed yeast to grow on SD medium. See the YPH, Appendix C, for recipe.
mAb	Monoclonal antibody
SD medium	Minimal synthetic dropout medium; is comprised of a nitrogen base, a carbon source (glucose unless stated otherwise), and a DO supplement. See the YPH, Appendix C, for recipe.

II. List of Components

All mAbs are provided in solution in storage buffer. Each aliquot is sufficient to probe at least two 50-cm^2 Western "mini"-blots (at least 10 blots total).

- 25 µg GAL4 AD Monoclonal Antibody (Cat. No. 630402); or
- 25 µg GAL4 DNA-BD Monoclonal Antibody (Cat. No. 630403)

III. Matchmaker mAbs Protocol

PLEASE READ THROUGH ENTIRE PROTOCOL BEFORE BEGINNING.

A. Preparation

1. Transformation of yeast host strain.
    - Please refer to your Matchmaker System User Manual or the YPH (Appendix F) for an appropriate yeast strain.
    - To obtain a detectable band on a Western blot, transform the host strain with a moderate-to-high expression plasmid (YPH, Table II).
    - We recommend a LiAc method for preparing yeast competent cells, such as our Yeastmaker™ Yeast Transformation System 2 (Cat. No. 630439).
2. Select transformant colonies as described in the transformation protocol, using the appropriate selection medium.
    - When selecting yeast cells transformed with the GAL4 DNA-BD vector (and derived plasmids), use SD/–Trp plates.
    - When selecting yeast cells transformed with the GAL4 AD vector (and derived plasmids), use SD/–Leu plates.

3. Preparation of log-phase yeast cultures
   - Prepare an overnight liquid culture in the appropriate SD selection medium; use these cultures to inoculate 50-ml expansion cultures in YPD.

NOTES:
- Please refer to the YPH (Chapter III.B and IV.B) for detailed instructions on preparing overnight yeast cultures and log-phase expansion cultures.
- Please refer to your Matchmaker System User Manual or the YPH, Appendix E, for guidelines on choosing an SD selection medium to use for the overnight culture.
- For the negative control overnight culture, inoculate 10 ml of SD/–Ura with an untransformed yeast colony.

4. We provide two alternative protocols in the YPH for the preparation of protein extracts from yeast cells (Chapters IV.C. and IV.D.). The results (i.e., protein yield and quality) for certain proteins may be better with one protocol than with the other; try both initially. The cell culture preparation method is the same for both protein extraction procedures.

B. Gel Electrophoresis and Blotting of Proteins

Reagents and Materials Required:

- Polyacrylamide/SDS mini-gel (Laemmli, 1970) (e.g., 12% resolving gel and 4–5% stacking gel.)
- Prestained protein molecular weight markers
- Suitable plastic containers for washing gels and blots
- Electroblotting transfer apparatus (e.g., BioRad Miniprotean II)
- Nitrocellulose (0.2-µm pore size, Schliecher & Schuell; or PVDF membranes) enough for ten 50-cm^2 blots.

NOTE: Proteins smaller than 20 kDa may pass through larger pore sizes.

- Transfer buffer (Prechill the buffer and add SDS just prior to use.)

	To prepare 1 liter:	
39 mM Glycine	2.9	g
48 mM Tris base	5.8	g
0.04% w/v SDS	0.4	g electrophoresis-grade SDS
20% v/v Methanol	200	ml 100% methanol
Deionized H_2O	___	To a final volume of 1.0 liter (Do not adjust pH)

1. Prepare a polyacrylamide/SDS mini-gel according to Laemmli (1970). A 1-mm 12% resolving gel with a 4–5% stacking gel works well for most proteins.
2. If protein samples have been frozen:
   - Quickly thaw samples by placing them in a 100°C water bath for 5 min.
   - Chill samples on ice.
   - Centrifuge samples in a microcentrifuge at 14,000 rpm for 2 min at 4°C to pellet debris. Protein extract is in the supernatant.
3. For each protein extract, load two wells on the gel, one with 20 µl and one with 40 µl of sample. Also, load at least one well with prestained protein molecular weight markers and one well with protein extract from nontransformed cells.

   Due to differences in expression levels for different fusion proteins, it is usually necessary to run a preliminary gel with a protein titration [corresponding to ~1–4 OD600 units of cells) to

determine optimal loading density. (Generally, 1 OD unit is sufficient.) However, even after the expression level for a particular fusion protein has been determined, it is safest to load two different amounts (e.g., 20 µl and 40 µl) the first time a new protein extract is run on a gel.

4. Electrophorese at 190–200 volts until the bromophenol blue dye front reaches the bottom of the gel (approx. 45 min).
5. Carefully remove gel from glass plates and place gel in a dish containing 100 ml of precooled (4°C) transfer buffer. Equilibrate gel in the buffer for approx. 45 min.
6. Set up the transfer apparatus in a cold room (4°C) according to the manufacturer's protocol.
7. Blot proteins onto the nitrocellulose paper according to the manufacturer's protocol. (For example, we use the BioRad Miniprotean II apparatus, placed in a cold room. We electrotransfer the proteins for 0.5 hr at 50V, followed by 1.5 hr at 100V, changing the ice pack two or three times during the transfer, as necessary.)
8. Wash the nitrocellulose blot 2 X (10–30 sec each) with deionized H_2O.
9. Confirm that the prestained molecular weight markers have transferred and mark their positions in pencil. (Blots may be stained in Ponceau S and rinsed with H_2O without interfering with the detection; (Sambrook & Russell, 2001.)
10. Proceed immediately to the next step: **do not allow the blot to dry out.**

C. Immunodetection

Reagents and Materials Required

- TBS buffer

	To prepare 1 liter of TBS buffer:	
20 mM Tris-HCl	20 ml	1 M stock solution (pH 7.6)
137 mM NaCl	137 ml	1 M stock solution
deionized H_2O	_____	To final volume of 1 liter

- TBST buffer (TBS buffer + 0.1% v/v Tween-20)
- Blocking buffer (Prepare fresh on the day of use.)
  TBST buffer +
  5% w/v Nonfat dry milk
  1% w/v Bovine serum albumin (BSA; Sigma, Factor V)
- Heat-sealable plastic bags and sealing apparatus
- Goat normal serum (Jackson ImmunoResearch, Cat. No. 005 000-001)
  Dilute 1:50 in Blocking buffer; use 2 ml diluted normal serum per 10 cm^2 of blot surface.

In addition, for detection using HRP-conjugated secondary enzyme you will need:

- HRP-conjugated Goat Anti-mouse IgG
  We recommend Jackson ImmunoResearch Laboratories' AP-conjugated whole IgG Goat Anti-mouse IgG (H+L) minus cross-reacted human, bovine, and horse serum proteins.
- x-ray film (e.g., Kodak BioMax MR)
- An appropriate HRP chemiluminescent detection system (e.g., Amersham ECL Western blot reagents Cat. No. RPN 2109)

For immunodetection using AP-conjugated secondary enzyme:

- AP-conjugated Goat Anti-mouse IgG
  We recommend Jackson ImmunoResearch Laboratories' HRP-conjugated whole IgG Goat Anti-mouse IgG (H+L) minus cross-reacted human, bovine, and horse serum proteins.
- An appropriate AP detection system (e.g., BCIP/NBT colormetric substrate as described in Sambrook and Russell, (2001) or a chemiluminescent detection system).

1. Prepare 100 ml of fresh Blocking buffer per 50 cm^2 of blot.
2. Soak the blot in 100 ml of Blocking buffer for 1 hr at room temperature (20–22°C), or overnight at 4°C, with constant gentle agitation. (This step is to block nonspecific antibody binding sites on the blot.)
3. Wash blot 3 times (5–10 min each time) using 100 ml of TBST for each wash.
4. Dilute the GAL4mAb (i.e., the primary antibody) in TBST. Allow 1 ml of diluted mAb solution per 10 cm^2 of blot surface, e.g., 5 ml for a 50-cm^2 blot using a heat-sealable bag for the probe incubation.
   - Use a final concentration of 0.5 µg/ml for the DNA-BD mAb.
   - Use a final concentration of 0.4 µg/ml for the AD mAb.
5. Place the blot in a heat-sealable plastic bag and add the diluted antibody solution. Seal bag, being careful to exclude bubbles.
6. Lay the bag on a flat platform shaker and place a lightweight book on top of it. Incubate at room temperature for 1 hr with gentle agitation.
7. Remove blot from bag and wash 3 times (5 min each) using 50–100 ml of TBST for each wash.
8. Dilute Goat Normal Serum 1:50 in Blocking buffer.
9. Place blot in a fresh heat-sealable plastic bag and add 2 ml of the diluted Goat Normal Serum per 10 cm^2 of blot to block nonspecific goat anti-rabbit antibody binding. Seal bag, being careful to exclude air bubbles.
10. Incubate at room temperature (20–22°C) for 30 min with gentle agitation on a platform shaker.
11. Remove blot from bag and wash 3 times (5 min each) using 50–100 ml fresh TBST for each wash.
12. Dilute the conjugated secondary antibody according to the manufacturer's recommendations. (For the HRP conjugate supplied by Jackson ImmunoResearch, we dilute 1:15,000 in TBST buffer; the AP-conjugate is diluted 1:5,000.) Prepare only the amount of diluted conjugate you need for the experiment. Allow 1 ml of the diluted HRP conjugate solution per 10 cm^2 of blot.
13. Place the blot in a fresh heat-sealable bag and add the diluted secondary antibody. Seal bag, being careful to exclude air bubbles.
14. Incubate at room temperature for 30 min with gentle agitation.
15. Remove blot from the bag and place it in a suitable container for washing. Wash blot four times (5 min each) using 50–100 ml TBST each wash.
16. Prepare the appropriate substrate and apply it to the blot according to the substrate manufacturer's directions.
17. (For chemiluminescent substrates) Expose the plastic-wrapped membrane to x-ray film. Typical exposure times are 1–30 min for xray film. Several films may be generated from the same membrane to obtain the optimal exposure time.
18. (For BCIP/NBT substrate) Color development will take approx. 1–6 hrs.

D. Analysis of Results

Compare the size of the fusion proteins with the molecular weight markers. The approximate sizes of the nonfused GAL4 DNA-BD, GAL4 AD, and selected control fusion proteins used in Matchmaker Gold Yeast Two-Hybrid System are shown in Table II. For comparison, typical results are shown in Figure 1.

Table 2. Approximate Sizes of GAL4 DNA-BD, and Control Fusion Proteins

Vector	Peptide or Fusion protein	Approx. size[a] (kDa)	Visualized with mAb
pGBKT7	GAL4 DNA-BD	22	GAL4 DNA-BD
pGBKT7-53	GAL4 DNA-BD/p53 hybrid	57	GAL4 DNA-BD
pGADT7 AD	GAL4 AD Vector	18b	GAL4 AD
pGADT7-T	GAL4 AD/SV40 T-antigen hybrid	89	GAL4 AD
pGBKT7-Lam	GAL4 DNA-BD/lamin hybrid	40	GAL4 DNA-BD

[a] The molecular weights shown were predicted from the corresponding sequence. Except as noted below, the predicted sizes are roughly equivalent to their relative sizes (Mr), as estimated on SDS-PAGE. The GAL4 DNA-BD and AD peptides encoded by other related vectors have slightly different molecular weights due to differences in the multiple cloning site.

[b] Mr is ~24 kDa.

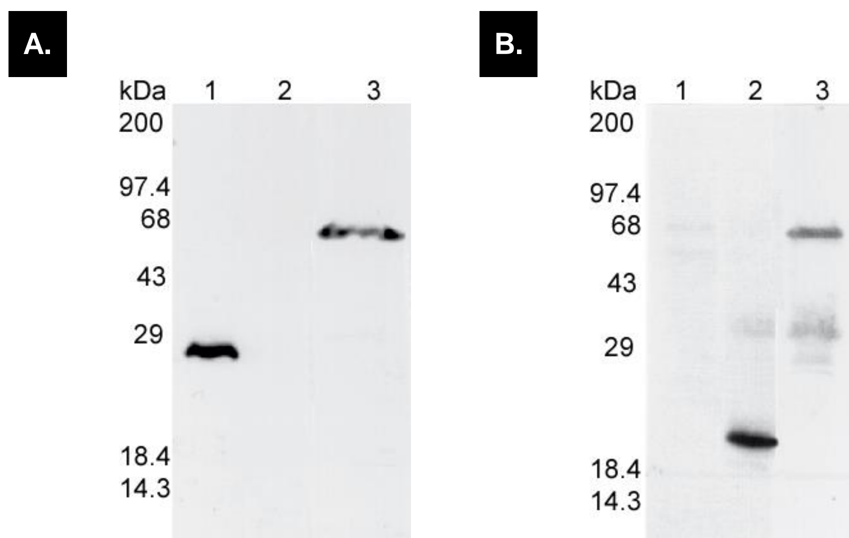

Figure 1. Western blots using the GAL4 DNA-BD and AD mAbs. Yeast strain CG-1945 was transformed (separately) with the indicated vector. Transformants were selected on the appropriate SD medium and soluble protein extracts were prepared using either the Urea/SDS method (Panel A) or the TCA method (Panel B). Protein samples equivalent to ~1–1.5 OD_{600} units of cells were electrophoresed on 12% polyacrylamide/SDS gels. Proteins were electroblotted from the gel to nitrocellulose filters (0.2 µm). The blots were probed with either the GAL4 DNA-BD mAb (0.5 µg/ml; Panel A) or the GAL4 AD mAb (0.4 µg/ml; Panel B), followed by HRP-conjugated polyclonal Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories; diluted 1:15,000 in TBST), as described in the protocol. Signals were detected using a chemiluminescent detection assay followed by an exposure to x-ray film. **Panel A.** Lane 1: pAS2 (a GAL4 DNA-BD vector). Lane 2: untransformed CG-1945 control. Lane 3: pVA3-1. **Panel B.** Lane 1: untransformed CG-1945 control. Lane 2: pACT2. Lane 3: pTD-1.

IV. Troubleshooting Guide

A. General Considerations

Optimal electrophoretic separation of proteins depends largely on the quality of the equipment and reagents used in the gel system, the manner in which the protein samples are prepared prior to electrophoresis, the amount of protein loaded on the gel, and the voltage conditions used during electrophoresis. These same considerations are important for the subsequent transfer of proteins to the nitrocellulose membrane where transfer buffer composition, temperature, duration of transfer, and the assembly of the blotting apparatus can all have profound effects on the quality of the resultant protein blot. Troubleshooting tips pertaining specifically to the isolation of proteins from yeast are in the YPH. The elecrophoretic and immunostaining conditions in this protocol were optimized for proteins prepared from yeast.

B. Few or No Immunostained Protein Bands On The Blot

1. Confirm the presence of protein bands in the gel (before transfer) by staining a parallel lane of the gel with Coomassie blue. (Note that once a gel has been stained with Coomassie blue, the protein bands will not transfer to a blot.)
2. Make sure that the nitrocellulose membrane is properly hydrated by prewetting the membrane in Transfer buffer. Omitting this step will prevent transfer of proteins from the gel to the membrane.
3. Make sure that the transfer "sandwich" is assembled correctly in the blotting apparatus. Check that the membrane is on the anode side of the gel during transfer (electrophoretic migration of the proteins occurs from the cathode to the anode).
4. Make sure there is good contact between the gel and the nitrocellulose during blotting. Eliminate all air bubbles between the membrane and the gel by rolling a Pasteur pipet across the top of the membrane covered with moistened filter paper.
5. Make sure that the gel is not being distorted during the transfer process. If necessary, increase equilibration time (Step III.B.5) and transfer buffer volume, to ensure thorough equilibration before the transfer. To avoid overheating the gel during transfer, change the ice packs more frequently.
6. Verify transfer of the proteins to the membrane by staining with Ponceau S (Sambrook & Russell, 2001; Harlow & Lane, 1988).
7. Use a microscope to ascertain that the yeast cells have been completely broken during the protein extraction procedure.

C. Bands Are Present, But Are Diffuse and/or Poorly Resolved

1. Make sure that the electrophoretic separation of proteins was done correctly. Check the anode and cathode buffer composition and pH. Check the composition of the separating and stacking gels. Check the setting used on the power supply.
2. The gel may be overloaded; load less protein per lane. Alternatively, use a thicker gel and increase equilibration and blot transfer times accordingly.
3. For SDS gels, make sure that the proteins are properly solubilized.
4. Check for air bubbles in the transfer sandwich.
5. The absence of smaller (<~20 kDa) proteins may indicate overtransfer through the nitrocellulose. Try using a lower voltage and/or a shorter transfer time. If you didn't already do so, use only 0.2-µm pore size nitrocellulose filters; in addition to the benefits of the smaller pore size, these filters have a larger protein-binding capacity.
6. The absence of larger proteins may indicate incomplete transfer to the nitrocellulose. Try using a higher voltage and/or a longer transfer time.

D. No Antiserum-specific Signal(s) On Blot

1. Check the dilution of the primary mAb. Nonspecific bands may indicate that the mAb is too concentrated; lack of the expected specific band(s) may indicate that the antiserum is too dilute.
2. Make sure that the appropriate secondary antibody was chosen and diluted properly.

E. Specific Signal Is Present, But Background Signals Are Excessive

1. The primary mAb and/or secondary antiserum may be too concentrated. To determine if the nonspecific signals are contributed by the secondary antibody, perform a control incubation in which the primary antibody is omitted from the procedure. (Add TBS in place of the primary antibody in Step IV.D.4.)
2. Decrease the film exposure time to bring antiserum-specific bands within the linear exposure range of the film.
3. Perform more thorough blot washings between incubations with the primary mAb and secondary antiserum and prior to addition of the substrate.
4. If there is a visible precipitate in the undiluted primary antibody, centrifuge this solution for 30 sec at maximum speed in a microcentrifuge. Carefully remove the supernatant, and use it for subsequent dilution in blocking buffer.

F. Several Bands Appear on the Blot Where a Single Protein Species is Expected

1. Make sure that the sample preparation protocol guards against protein degradation and/or proteolysis. Additional protease inhibitors and phosphatase inhibitors may be used as desired (see the YPH).
2. For reducing gels, make sure that the sample has been completely reduced with either dithiothreitol or 2-mercaptoethanol prior to loading the gel.

V. References

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