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# Protein-protein interaction assays: eliminating false positive interactions

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Many methods commonly used to identify and characterize interactions between two or more proteins are variations of the immobilized protein-protein interaction assay (for example, glutathione S-transferase (GST) pulldown and coimmunoprecipitation). A potential, and often overlooked, problem with these assays is the possibility that an observed interaction is mediated not by direct contact between proteins, but instead by nucleic acid contaminating the protein preparations. As a negatively charged polymer, nucleic acid (often cellular RNA) can adhere to basic surfaces on proteins, and thereby mediate interactions between an immobilized bait protein and a target protein. The contaminating nucleic acid may cause a false positive result in protein-protein interaction assays or may contribute to general background. Alternatively, in relatively rare cases, the presence of nonspecific nucleic acid can inhibit protein-protein interaction<sup>1</sup>. In general, contaminating nucleic acid can be especially problematic with proteins under study that naturally bind RNA or DNA (for example, transcription factors), although nucleic acid can mediate apparent protein-protein interactions in other systems as well. A simple and convenient method for decreasing false positives and background owing to contaminating nucleic acid is to treat the protein preparations with micrococcal nuclease<sup>1-3</sup>. Micrococcal nuclease (also known as S7 nuclease) cleaves single- and double-stranded DNA and RNA with no sequence specificity<sup>4</sup>. This protocol describes a GST pulldown assay that incorporates micrococcal nuclease treatment of both the immobilized GST-bait protein and the target protein preparations. The strategy can be adapted easily to proteins with other tags and immobilized using other methods. The target protein can be derived from many different sources—for example, a cell extract containing a native protein of interest; a recombinant protein expressed in *Escherichia coli*, insect cells or mammalian cells; a protein produced in an *in vitro* translation system; or a highly purified protein. The steps required to treat protein preparations with micrococcal nuclease are straightforward and can be incorporated into any immobilized protein-protein interaction assay.

## MATERIALS

### REAGENTS

*E. coli* extracts containing expressed GST and GST-fusion proteins

Glutathione Sepharose 4B (Amersham)

Micrococcal nuclease (EMD Biosciences—Calbiochem)

Target protein (in crude extracts or purified)

TGEM(0.1): (0.1 refers to the NaCl concentration) 20 mM Tris-HCl (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.1% NP-40, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M NaCl (add the DTT and PMSF on the day of the experiment)

TGEM(1.0): (1.0 refers to the NaCl concentration) 20 mM Tris-HCl (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 1 M NaCl (add the DTT and PMSF on the day of the experiment)

TGMC(0.1): (0.1 refers to the NaCl concentration) 20 mM Tris-HCl (pH 7.9), 20% glycerol, 5 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 0.1 M NaCl (add the DTT and PMSF on the day of the experiment)

### EQUIPMENT

Aspirator or vacuum line

Nutator mixer (Clay Adams)

SDS-PAGE mini apparatus and power supply

Syringe (1 ml), equipped with 25-gauge needle (Becton Dickinson). *To minimize the risk of needlestick injury, blunt the end of the 25-gauge needle by cutting off the tip with wire cutters (just below the upper edge of the slanted opening of the needle so that aspiration is not obstructed).*

## PROTOCOL

### Preparation of the resin

#### PROCEDURE

1| To carry out the interaction assays, the bait proteins must be bound to beads. Transfer a portion of glutathione Sepharose 4B resin to a microcentrifuge tube. Use an amount of resin that is at least twice the minimum amount needed for all reactions to be performed (extra prewashed resin can be stored at 4 °C for future use). We typically use 10 µl of resin per reaction as it is difficult to work with smaller volumes of beads.

*Glutathione Sepharose 4B resin is supplied in 20% ethanol. Before use, the resin must be washed and prepared as a slurry consisting of one part beads and three parts TGEM(0.1) buffer as described in Steps 2–4.*

2| Collect the beads by centrifugation at 800g at 15–25 °C for 2 min (faster and longer spins can fragment the beads). Remove the supernatant by aspirating carefully with a 25-gauge needle attached to a 1-ml syringe and an aspirator or vacuum line.

*Alternatively, instead of aspiration, supernatants can be removed from the bead pellets with pipet tips. This is problematic, however, because beads will stick to the outside of the plastic tip causing a progressive decrease in the volume of beads during multiple washes.*

#### ▲CRITICAL STEP

3| Wash the beads four times with ten bead volumes of TGEM(0.1). To perform each wash, add TGEM(0.1), mix gently, centrifuge at 800g for 2 min and aspirate the supernatants.

#### ▲CRITICAL STEP

4| After the last wash add three bead volumes of TGEM(0.1) and store on ice.

*After the last wash, the packed bead volume is most easily estimated by visually comparing the bead pellet to a series of microcentrifuge tubes containing known volumes of water.*

5| Thaw extracts containing bait proteins and dilute with an appropriate portion of ice-cold TGEM(0.1), such that 40 µl of bait protein solution can be used per binding reaction.

*Two protein extracts are required at minimum: one containing the GST-bait protein and a second containing control GST protein. To minimize background, use as little immobilized bait protein as possible. We typically use 500 ng of immobilized bait protein per reaction.*

#### ▲CRITICAL STEP

6| Centrifuge the diluted protein extracts at 16,000g at 4 °C for 20 min. Transfer supernatants to fresh microcentrifuge tubes on ice.

#### ▲CRITICAL STEP

7| Distribute 40 µl of resin slurry (10 µl of packed beads) from Step 4 into two 0.5-ml microcentrifuge tubes on ice.

*Alternatively, if multiple target proteins will be tested, a larger volume of each immobilized bait protein resin can be prepared and distributed into microcentrifuge tubes after Step 13.*

#### ➔TROUBLESHOOTING

8| Add 40 µl of diluted extracts containing GST-bait to one tube of resin and GST to another.

9| Close the lids tightly and mix gently so that the beads are dispersed in solution, not settled at the bottom of the tubes.

10| Place the tubes in a Nutator and mix at 4 °C for 2 h.

11| Centrifuge the tubes at 800g at 4 °C for 2 min. Remove the supernatants by aspiration.

12| Wash the beads two times with 100 µl (ten bead volumes) of ice-cold TGEM(1.0) and two times with 100 µl (ten bead volumes) of ice-cold TGEM(0.1). To perform each wash, add buffer, centrifuge at 800g at 4 °C for 2 min and aspirate the supernatants.

#### ➔TROUBLESHOOTING

### Preparation of bait protein extracts

### Immobilization of bait proteins

13| After the last wash add 20  $\mu$ l (two bead volumes) of ice-cold TGMC(0.1) to each tube.

14| Thaw the extract containing the target protein and dilute with an appropriate portion of ice-cold TGMC(0.1) or dialyze into TGMC(0.1) so that 30  $\mu$ l can be used per binding reaction. *Regardless of source, the target protein must be suspended in a buffer similar to TGMC(0.1) for micrococcal nuclease treatment.*

15| Centrifuge the extract at 16,000g at 4 °C for 20 min. Transfer supernatants to fresh tubes.  
**▲CRITICAL STEP**

16| Add 1 U of micrococcal nuclease to each tube of 30  $\mu$ l of immobilized bait protein (Step 13) and to each tube of 30  $\mu$ l target protein extract (Step 15). If the immobilized bait protein and target protein extracts are in larger volumes, add micrococcal nuclease to a final concentration of 0.033 U/ $\mu$ l.

17| Mix the tubes gently and incubate at 30 °C for 10 min. During the incubation, gently mix the samples containing the beads every few minutes. When the incubation is complete, place the tubes on ice.  
*The micrococcal nuclease does not need to be inactivated.*

**➔TROUBLESHOOTING**

18| Set up binding reactions by adding 30  $\mu$ l of micrococcal nuclease-treated target protein to micrococcal nuclease-treated immobilized bait protein slurries (also 30  $\mu$ l) on ice. Set up a control binding reaction in parallel using GST only as bait protein.  
*Place the remainder of target protein samples on ice. These will be used later as input samples for SDS-PAGE.*

19| Close lids tightly and gently mix so that beads are dispersed in solution.

20| Place the tubes on a Nutator and mix at 4 °C for 2 h.  
**➔TROUBLESHOOTING**

21| Centrifuge the samples at 800g at 4 °C for 2 min. Remove the supernatants by aspiration. *Alternatively, the unbound target protein can be analyzed. Instead of aspirating, use a pipet tip to carefully transfer the majority of each supernatant to a fresh microcentrifuge tube on ice. Be careful not to touch the bead pellet with the pipet tip.*

22| Wash the pellets four times, each time with 100  $\mu$ l (ten bead volumes) of ice-cold TGEM(0.1).  
**■ PAUSE POINT** If SDS-PAGE is to be performed at a later time, the tubes containing resin can be stored at -80 °C. In this case, place the input target protein and unbound target protein samples at -80 °C also.  
**▲CRITICAL STEP**

23| Prepare the samples for SDS-PAGE. Add 10  $\mu$ l of SDS sample buffer to each bead sample. As a control, mix an appropriate amount of input target protein with SDS sample buffer.  
*The amount of input target protein sample to use depends on the detection limit of the assay, but 10% of the amount added to each binding reaction (3  $\mu$ l) is usually sufficient. The unbound target protein (from Step 21) can be prepared similarly to the input sample.*

24| Heat the samples at 95 °C for 2 min and then collect the beads by centrifugation.

25| Load 10  $\mu$ l of the supernatant from each bead sample onto the SDS gel as follows:

**Preparation of the target protein**

**Treatment with micrococcal nuclease**

**Incubation of the target protein with immobilized bait protein**

**Analysis of bound protein by SDS-PAGE**





Lane	Sample
1	Molecular weight markers
2	GST-bait–target interaction
3	GST–target interaction
4	10% unbound target protein from GST-bait reaction
5	10% unbound target protein from GST reaction
6	10% input target protein

**26** | Analyze the proteins resolved by gel electrophoresis with Coomassie or silver staining. Staining allows the relative amounts of immobilized bait proteins and in some cases bound target proteins to be analyzed. In many cases, it will not be possible to visualize the bound target protein by staining. If Western blots will be performed, the samples can be split between two SDS gels: one that is used for staining and a second that is used for the Western blot.

**▲CRITICAL STEP**

**TROUBLESHOOTING TABLE**

PROBLEM	SOLUTION
Step 7 <b>It is difficult to accurately and reproducibly pipet resin.</b>	When pipetting resin from a stock tube to other tubes, keep a uniform slurry by frequently (but gently) flicking the bottom of the tube. Do not vortex the resin. In addition, because agarose-based beads will stick to the plastic pipet tip during transfer it is best to ‘precoat’ a pipet tip with resin before using the tip to transfer resin to multiple tubes. This is done by sucking up a desired volume of resin slurry from the stock tube into a pipet tip and ejecting the solution back into the same tube.
Step 12 <b>We observe high background binding of target protein to control beads.</b>	For some target proteins, background binding to beads can be decreased by increasing the NaCl or NP-40 concentration in the binding reaction or wash buffer.
Step 17 <b>Immobilized bait protein and target protein are degraded during micrococcal nuclease treatment.</b>	It is possible that proteases contaminating the protein preparations will degrade the bait and target proteins because of the higher temperature used for micrococcal nuclease treatment. If this occurs, additional protease inhibitors can be added to the TGMC(0.1) buffer.
Step 20 <b>An anticipated interaction is not observed.</b>	The amount of bait protein immobilized on the beads and the length of the incubation time can be increased to facilitate the detection of weak interactions.

**CRITICAL STEPS**

**Step 2** It is important that the resin does not dry out during aspiration. The end of the 25-gauge needle should be brought almost to the top of the bead pellet, but should not touch the beads. A small amount of buffer should be left on the beads after aspiration and the lids of the tubes should not be left open for long without adding additional buffer.

**Step 3** Slurries of agarose-based beads should not be vortexed because the force can fragment the beads. Instead, mix resin slurries by flicking the tubes such that beads are dispersed in solution. The beads can also be dispersed by forcefully pipetting wash buffer into the beads: place the pipet tip about halfway into the microcentrifuge tube (well above the beads) and dispense the buffer onto the bead pellet quickly.

**Step 5** It is important that the amount (number of moles) of bait protein is the same in all reactions. This can be established by performing a pilot experiment in which extracts containing bait proteins are incubated with beads, bound protein is visualized by SDS-PAGE and Coomassie staining, and the amount of protein on the beads is estimated by comparison to known amounts of marker proteins. The extracts are then diluted such that ~500 ng of each protein will be immobilized on 10 µl of beads.

**Steps 6** It is important to remove any insoluble material resulting from freeze-thaw and dilution. In the absence of these steps, the insoluble material can precipitate with the beads and lead to substantial background in the interaction assay.

**Step 15** See step 6.

**Step 22** Washes are critical for decreasing background target protein on the beads. Additional or more stringent washes can be performed if necessary (also see Troubleshooting for Step 12).

**Step 26** If Western blot analysis must be used to visualize the target protein, portions of the reactions should also be analyzed by Coomassie staining to ensure that the amount of immobilized bait protein is similar in all reactions when the assay is complete. Staining allows detection of unequal immobilization of bait protein or loss of bait protein (for example, owing to loss of beads or proteolysis). With cruder samples it might also be necessary to perform control reactions in which immobilized bait proteins are incubated in the absence of target protein. These beads can be analyzed along with other samples to ensure that putative target protein bands are not artifactual contaminants.

### COMMENTS

Treatment with ethidium bromide also has been found to reduce false positive results mediated by contaminating DNA in protein-protein interaction assays<sup>5</sup>. Ethidium bromide intercalates between stacked bases in the double helix and thus decreases the affinity with which DNA-binding proteins bind DNA. Ethidium bromide has the advantage that treatment with it does not require incubation at higher temperatures. Whereas the presence of ethidium bromide disrupts protein interactions mediated by DNA, micrococcal nuclease treatment has the advantage that it can eliminate false positives and high background that results from contamination by any nucleic acid, single- and double-stranded RNA and DNA. Treatment with micrococcal nuclease can be applied easily to interaction assays using other methods of immobilization. For example, resin pellets from immunoprecipitation can be treated with micrococcal nuclease to release molecules coprecipitated via contaminating nucleic acid. Magnetic beads can also be used instead of agarose beads.

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### SOURCE

This protocol was provided directly by the authors listed on the title page. For further details on the composition of media, gel solutions, standard buffers and standard procedures see Sambrook, J. & Russell, D.W., eds. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001; <http://www.cshlpress.com/link/molclon3.htm>).

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