Chapter Three: Pull-down Assays

CHAPTER 3

PULL-DOWN ASSAYS

Contents **Page**

Pull-down assays overview

Pull-down assays probe interactions between a protein of interest that is expressed as a fusion protein (e.g., bait) and the potential interacting partners (prey).

In a pull-down assay one protein partner is expressed as a fusion protein (e.g., bait protein) in *E. coli* and then immobilized using an affinity ligand specific for the fusion tag. The immobilized bait protein can then be incubated with the prey protein. The source of the prey protein depends on whether the experiment is designed to confirm an interaction or to identify new interactions. After a series of wash steps the entire complex can be eluted from the affinity support using SDS-PAGE loading buffer or by competitive analyte elution, then evaluated by SDS-PAGE.

Successful interactions can be detected by Western blotting with specific antibodies to both the prey and bait proteins, or measurement of radioactivity from a [^{35}S] prey protein.

REFERENCES

Bacterial expression of both bait and prey proteins

1. Simader, H. *et al.* (2006) *Nuc. Acids. Res.* **34**, 3968–79.
2. Phillips, P. *et al.* (2006) *J. Biol. Chem.* **281**, 4903–10.
3. Reinert, L. (2006) *Cancer. Res.* **66**, 8994–9001.
4. Onken, B. *et al.* (2006) *Proc. Natl. Acad. Sci.* **103**, 9045–50.
5. Li, H. *et al.* (2006) *J. Biol. Chem.* **281**, 14748–55.

Bacterial expression of bait (GST fusions) and mammalian cell expression of prey

1. Osler, M. *et al.* (2005) *J. Cell Sci.* **118**, 4667–78.
2. Ingham, R. *et al.* (2005) *Mol. Cell. Biol.* **25**, 7092–106.
3. Hirst, J. *et al.* (2005) *Mol. Biol. of the Cell* **16**, 2554–65.
4. Wißmüller, S. *et al.* (2006) *Nuc. Acids Res.* **34**, 1735–44.
5. Chan, W. *et al.* (2005) *J. Biol. Chem.* **25**, 23741–47.

Bacterial expression of bait (polyhistidine fusions), mammalian cell expression of prey

1. Vermeulen, M. *et al.* (2006) *Mol. Cell. Biol.* **26**, 5226–36.
2. Shao, H. *et al.* (2006) *J. Immunol.* **176**, 2933–41.
3. Hublitz, P. *et al.* (2005) *Genes and Development* **19**, 2912–24.
4. Desterro, J. *et al.* (2005) *Mol. Biol. Cell* **16**, 5115–26.

Pull-down assay formats

GST pull-downs

Bacterial expression of both bait and prey proteins

The most commonly used method to generate a bait protein is expression as a fusion protein contain a GST (glutathione-S-transferase) tag in *E. coli.* This is followed by immobilization on particles that contain reduced glutathione which binds to the GST-tag of the fusion protein. The primary advantage of a GST tag is that it can increase the solubility of insoluble or semi-soluble proteins expressed in *E. coli.*

An alternative fusion tag such as 6X polyhistidine can also be used to express the prey or bait protein in *E. coli.* Using this format, large amounts of bait and prey protein partners can be expressed. Since both proteins are expressed in a prokaryotic environment, interactions that require folding or modification of one or both partners may not occur.

Bacterial expression of bait protein. Mammalian expression of prey protein (Figure 3)

In this format the bait protein is expressed as a GST fusion protein in *E. coli* followed by immobilization on particles containing reduced glutathione.

Expression of endogenous or recombinant prey proteins in eukaryotic cells increases the probability that they will be properly modified or folded. These modifications can be critical for successful protein:protein interactions to occur.

Mammalian cell extracts containing the expressed prey protein are prepared and allowed to interact with the immobilized GST fusion bait protein.

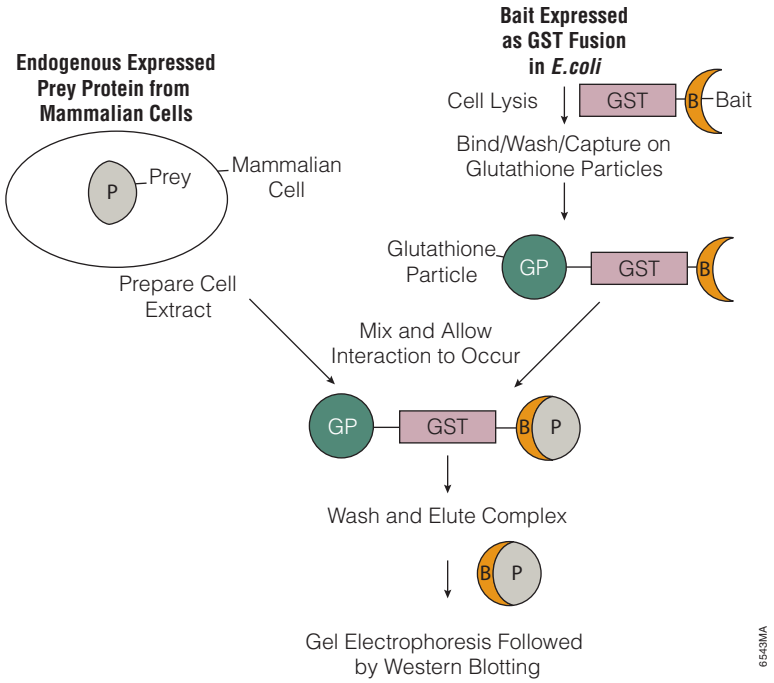

Figure 3. Schematic of pull-down assay using bacterial expression of bait protein and mammalian expression of prey protein. The sequence of the bait protein is cloned into a vector containing a GST tag and the appropriate elements for growth and expression in *E. coli.* Following expression the GST bait fusion protein is purified using glutathione particles, which bind to the GST tag. A cellular extract is prepared from mammalian cells containing the prey protein. An aliquot of the cell extract is then allowed to interact with the bound GST bait fusion protein for several hours. After washing away non-specifically bound proteins the complex is eluted by adding reduced glutathione and analyzed by Western blotting.

Bacterial expression of bait protein. Mammalian cell-free expression of prey protein (Figure 4)

Utilizing this format requires that the bait protein be expressed as a (GST) fusion protein in *E. coli* and immobilized on a support resin. Mammalian, cell-free expression systems are used to generate a non-fusion prey protein. Expressed prey proteins are then allowed to interact with the immobilized GST fusion bait proteins. When using cell-free expression systems prey proteins can be expressed in 1-2 hours and used without purification. This convenience enables the rapid characterization of several different prey protein domains created by site-directed mutagenesis. The effects of specific mutations on the interaction can then be evaluated.

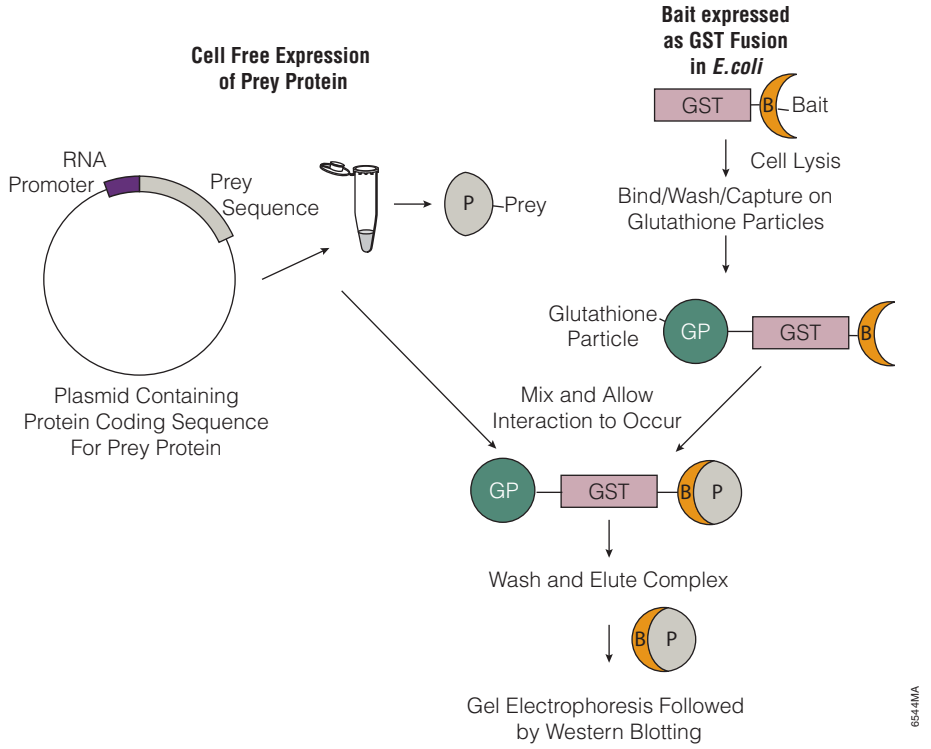

Figure 4. Schematic of pull-down assay using bacterial expression of bait protein and mammalian cell-free systems for the expression of prey protein. The sequence of the bait protein is cloned into a vector that contains the GST tag and the appropriate elements for growth and protein expression in *E. coli*. Following expression the GST bait fusion protein is immobilized using glutathione particles which bind to the GST tag. The sequence of the prey protein is cloned into a vector containing the appropriate elements for expression using a mammalian-based cell-free expression system. An aliquot of the expressed prey protein is then allowed to interact with the bound GST bait fusion for several hours. After washing away non-specifically bound proteins the prey is eluted and analyzed typically by Western blotting. Using an alternative procedure the prey may be expressed as [^{35}S]-labeled protein and detected directly in the gel.

REFERENCES (CONTINUED)

Bacterial expression of bait protein (GST fusions) mammalian cell-free expression systems as the source of prey protein

1. Liang, S. and Lutz, C. (2006) *RNA* **12**, 111–21.
2. Gu, X. *et al.* (2006) *J. Bio. Chem.* **281**, 17882–89.
3. Cohen, R. *et al.* (2006) *J. Clin. Endocrinol. Metab.* **91**, 239–47.
4. Hoberg, J. *et al.* (2006) *Mol. Cell. Biol.* **26**, 457–71.

HaloTag pull-downs using mammalian cell-free expression systems or mammalian cell extracts

1. Urh, M. *et al.* (2005) *Promega Notes* **92**, 21–4.
2. Los, G.V. *et al.* (2005) *Cell Notes* **11**, 2–6.

Pull-down assay formats

HaloTag-based pull-downs

Mammalian cell expression of both bait and prey proteins (Figure 5)

The properties of the HaloTag fusion protein provide some important advantages that increase the chance of successfully isolating interacting partners. First the HaloTag protein provides covalent attachment to a resin containing a HaloTag ligand (e.g., HaloLink Resin). This covalent linkage enables extensive washing to remove nonspecifically bound proteins, which can be problematic for most pull-down experiments.

Using this format, cells are transfected with HaloTag bait fusion protein and allowed to form complexes with endogenously expressed prey proteins. Cells are lysed and the complexes captured by adding the HaloLink Resin. After a series of washing steps the prey are detected by gel analysis followed by Western blotting.

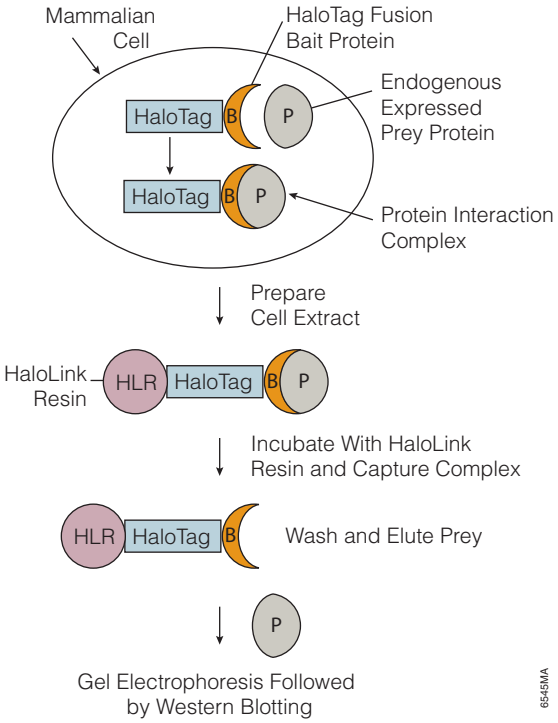

Figure 5. Schematic of HaloTag pull-down assay using mammalian cells to express both the prey and the bait proteins. The protein coding sequence of the bait protein is cloned into a HaloTag vector containing the necessary elements for growth and protein expression in mammalian cells. This recombinant vector is then transfected into the appropriate mammalian cell line. The HaloTag bait fusion interacts and forms a complex with the prey protein. Whole cell extracts are then prepared. The complex is then immobilized using the HaloLink Resin which forms a covalent bond with the HaloTag fusion tag. After extensive washing of the immobilized complex to remove non-specific proteins, the prey is eluted and typically analyzed by Western blotting or mass spectrometry.

Mammalian cell-free expression of both bait and prey proteins (Figure 6)

Mammalian cell-free expression systems can also be used to express both bait and prey proteins. The use of GST or polyhistidine fusion proteins can be problematic in cell-free expression systems because of high background. These technical issues can be resolved by expressing the bait protein as a HaloTag fusion protein.

Using this approach, the prey protein is expressed as a [^{35}S] or fluorescent-labeled protein containing no fusion tag. The bait protein is expressed as HaloTag fusion. The protein complex is allowed to form and then captured (using HaloLink Resin) and washed. The prey is then eluted from the complex and analyzed by gel electrophoresis.

When to use pull-down assays

Since pull-down assays can utilize prey proteins expressed in several different ways, such as endogenously in mammalian cells or in cell-free expression systems this technique can be used as either a discovery or a characterization tool.

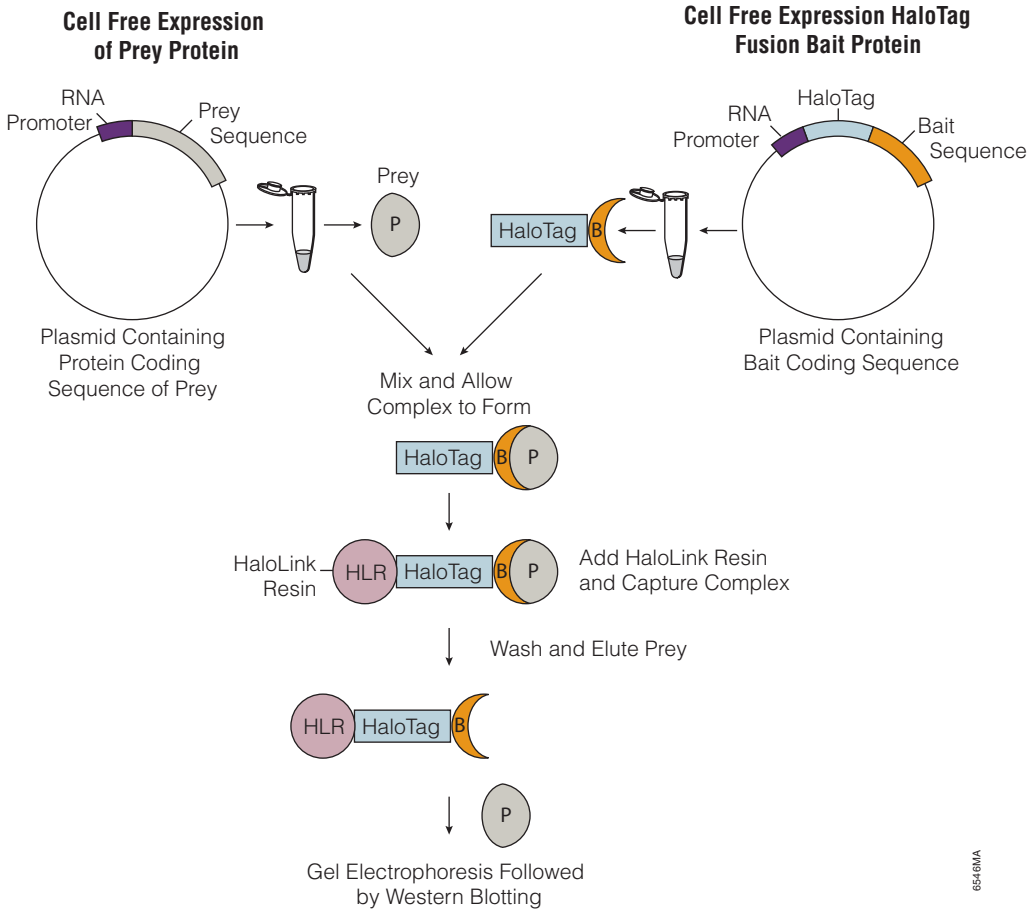

Figure 6. Schematic of pull-down assay using cell-free expression systems to express both bait and prey proteins. The protein-coding sequences of the bait and prey proteins are cloned into individual vectors containing the necessary elements for expression using mammalian-based cell-free systems. Proteins are expressed and aliquots from each reaction are mixed and the complex is allowed to form. The complex is then immobilized using the HaloLink Resin which forms a covalent bond with the HaloTag fusion tag. After extensive washing of the immobilized complex to remove non-specific proteins, the prey is eluted and typically analyzed by Western blotting. Using an alternative procedure the prey may be expressed as [^{35}S]-labeled protein and detected directly in the gel.

Reagent requirements for GST-based pull-downs

- Glutathione particles
- 2X SDS gel loading buffer
- SDS polyacrylamide gel
- Appropriate GST vector containing coding sequences for bait protein
- Source of prey protein (e.g., cell-free expression system, mammalian cells)
- Western blotting/antibodies (if using non-labeled prey)
- [^{35}S] methionine (if using labeled prey proteins from a cell-free expression system)

Reagent requirements for HaloTag pull-down assays in mammalian cells

- Transfection reagent
- Mammalian cell line expressing prey protein
- Appropriate HaloTag vector containing coding sequences for bait protein
- HaloLink Resin
- Antibodies to prey and bait proteins
- Western blotting reagents

Reagent requirements for HaloTag pull-down assays using cell-free expression systems

- 2X SDS gel loading buffer
- SDS polyacrylamide gel
- Cell-free expression system
- Appropriate HaloTag vector containing protein-coding sequences for the bait protein
- Appropriate vector containing protein-coding sequences for the prey protein
- HaloLink Resin
- Western blotting (if using non-labeled prey)
- [^{35}S] methionine (if using labeled prey)