

1. Pull-down

I. Process workflow

- a. Preparation of mixtures
- b. Interaction, washing, elution
- c. SDS-PAGE
- d. Staining and destaining

II. Motivation

One of the basic and simple questions regarding the functioning of proteins in the cell is which proteins like each other? The protein molecular machines are composed of more or less flexible amounts of each component. The presence of specific gadget may greatly influence the working conditions of protein machine. To uncover the rules of its proper assembling scientists use pull-down assays where single components are tested for their ability to bind each other. In the following experiment we will test if protein X is able to bind protein Y and test different conditions which could influence the binding.

III. Theoretical background

Elucidating gene function involves determining the function of each gene's encoded protein product. In the cell, proteins participate in extensive networks of protein:protein interactions. These interactions take the form of dynamic "protein machines", which assemble and disassemble in concert with an ever-changing influx of intra, inter and extracellular cues. A preliminary step in understanding protein structure and function is to determine which proteins interact with each other, thereby identifying the relevant biological pathways. The pull-down technique has become an invaluable tool for the life scientist interested in studying cellular pathways via protein:protein interactions.

Pull-down assays overview

Pull-down assays probe interactions between a protein of interest (e.g., bait) and the potential interacting partners (prey). One protein partner is expressed usually as a fusion protein (e.g., bait protein) and then immobilized using an affinity ligand specific for the fusion tag. It 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 and then evaluated by SDS-PAGE. Successful interactions can be detected by Western blotting with specific antibodies to both the prey and bait proteins, or by direct staining of the proteins

in the gel, when purified proteins are used for pull-down assay. Alternatively, the protein band isolation from a polyacrylamide gel and subsequent tryptic digestion of the isolated protein can be analysed by mass spectrometric identification of digested peptides. This technique can be used as either a discovery or a characterization tool and could be considered as a form of affinity purification.

The Pull-Down Assay as a Confirmatory Tool

Confirmation of previously suspected interactions typically utilizes a prey protein source that has been expressed in an artificial protein expression system. This allows the researcher to work with a larger quantity of the protein than is typically available under endogenous expression conditions and eliminates confusing results, which could arise from interaction of the bait with other interacting proteins present in the endogenous system that are not under study. Protein expression system lysates (i.e., *E. coli* or baculovirus-infected insect cells), *in vitro* transcription/translation reactions, and previously purified proteins are appropriate prey protein sources for confirmatory studies.

The Pull-Down Assay as a Discovery Tool

Discovery of unknown interactions contrasts with confirmatory studies because the research interest lies in discovering new proteins in the endogenous environment that interact with a given bait protein. The endogenous environment can entail a plethora of possible protein sources but is generally characterized as a complex protein mixture considered to be the native environment of the bait protein. Any cellular lysate in which the bait is normally expressed, or complex biological fluid (i.e. blood, intestinal secretions, etc.) where the bait would be functional, are appropriate prey protein sources for discovery studies.

Critical Components of Pull-Down Assays

Bait proteins for pull-down assays can be generated either by linking an affinity tag to proteins purified by traditional purification methods or by expressing recombinant fusion-tagged proteins. The purified protein can be tagged with a protein-reactive tag (e.g., Sulfo-NHS-LC-Biotin) commonly used for such labeling applications. If a cloned gene is available, molecular biology methods can be employed to subclone the gene to an appropriate vector with a fusion tag (e.g., 6xHis or glutathione S-transferase, GST). Recombinant clones can be overexpressed and easily purified, resulting in an abundance of bait protein for use in pull-down assays.

Interactions can be stable or transient and this characteristic determines the conditions for optimizing binding between bait and prey proteins. Stable interactions make up most cellular structural features but can also occur in enzymatic complexes that form identifiable structures and does not disassemble over time. Since these interactions often contribute to cellular structure, the dissociation constant between proteins is usually low, correlating to a strong interaction. Strong, stable protein complexes can be washed extensively with high ionic strength buffers to eliminate any false positive results due to nonspecific interactions. If the complex interaction has a higher dissociation

constant and is a weaker interaction, the interaction strength and thus protein complex recovery can be improved by optimizing the assay conditions related to pH, salt species and salt concentration. Problems of nonspecific interactions can be minimized with careful design of appropriate control experiments.

Transient interactions are usually associated with transport or enzymatic mechanisms and are defined by their temporal interaction with other proteins. These interactions are more difficult to identify using physical methods like pull-down assays because the complex may dissociate during the assay. They often require cofactors and energy via nucleotide triphosphates hydrolysis. Incorporating cofactors and nonhydrolyzable NTP analogs during assay optimization can serve to 'trap' interacting proteins in different stages of a functional complex that is dependent on the cofactor or NTP.

The working solutions for washing and binding are physiologic in pH and ionic strength, providing a starting point from which specific buffer conditions for each unique interacting pair can be optimized.

Elution of the Bait-Prey Complex

Identification of bait-prey interactions requires that the complex is removed from the affinity support and analyzed by standard protein detection methods. The entire complex can be eluted from the affinity support by utilizing SDS-PAGE loading buffer or a competitive analyte specific for the tag on the bait protein. SDS-PAGE loading buffer is a harsh treatment that will denature all protein in the sample, and it restricts analysis to SDS-PAGE. This method may also strip excess protein off the affinity support that is nonspecifically bound to the matrix, and this material will interfere with analysis. Competitive analyte elution is much more specific for the bait-prey interaction because it does not strip proteins that are nonspecifically bound to the affinity support. This method is non-denaturing; thus, it can elute a biologically-functional protein complex, which could be useful for subsequent studies.

An alternative elution protocol allows selective elution of prey proteins while the bait remains immobilized. This is accomplished using a step-wise gradient of increasing salt concentration or a step-wise gradient of decreasing pH. A gradient elution is not necessary once the critical salt concentration or pH has been optimized for efficient elution. These elution methods are also non-denaturing and can be informative in determining relative interaction strength.

Importance of Control Experiments for the Pull-Down Assay

In all pull-down assays, carefully designed control experiments are absolutely necessary for generating biologically significant results. A negative control consisting of a non-treated affinity support (minus bait protein sample, plus prey protein sample) helps to identify and eliminate false positives caused by nonspecific binding of proteins to the affinity support. The immobilized bait control (plus bait protein sample, minus prey protein sample) helps identify and eliminate false positives caused by nonspecific binding of proteins to the tag of the bait protein. The immobilized bait control also serves as a positive control to verify

that the affinity support is functional for capturing the tagged bait protein.

IV. Design of the experiment

a. Preparation of mixtures

Solutions and reagents:

- **2xT buffer:** 50 mM Tris-HCl, 20 % glycerol, 1 mM EDTA
- **3 M KCl**
- **1000 x NP-40:** 10 % nonidet P-40
- **1000 x DTT:** 1 M dithiothreitol
- **Sterile distilled H₂O:**
- **Protein X**
- **Protein Y**

Protocol:

- All the next steps have to be performed on ice. Place three 1,5 ml microcentrifuge tubes on ice and mark the tubes. Pipette the individual components of the reaction according the desired concentrations. The final concentration in the control tube has to be 1x T buffer, 1x DTT, 1x NP-40, 0,1 M KCl, 0,5 µg of protein X. The next tube has the same composition with the addition of protein Y. And the last one has 1M KCl. Pipette the water as first and proteins as last components. The total volume of reaction is 25 µl. Transfer 5 µl from the reactions to fresh 1,5 ml tube containing 5 µl of 6x SDS-PAGE loading buffer (input fraction).

b. Interaction, washing, elution

Solutions and reagents:

- **Gluthation sepharose beads**
- **6x SDS-PAGE loading buffer:**

Protocol:

- Pipette 75 µl of gluthation sepharose beads to 1,5 ml microcentrifuge tube. Add 1 ml of washing buffer carrying the concentrations 1x T buffer, 1x DTT, 1x NP-40, 0,1M KCl. Mix gently and spin 1 minute 2000 rpm in the microcentrifuge. Discard supernatant using blue ip avoiding to disturb the beads pellet. Keep the beads wet by keeping some liquid above. Repeat 2 times more, except that before the last centrifugation distribute the mixture to three tubes (333 µL aliquots). Add the reactions mixed in previous protocol to the beads. Keep the mixtures on ice for 20-30 minutes with periodic occasional mixing. Centrifuge the samples and carefully transfer 5 µl of the supernatant to fresh 1,5 ml tubes containing 5 µl of SDS-PAGE loading buffer (flow fraction). Washing of the beads

fraction is done by three times adding 1 ml of washing buffer. At the end use very thin tip to remove all the liquid. Add 20 µl of SDS-PAGE loading buffer (beads fraction).

c. SDS-PAGE

Solutions and reagents:

- **1 x SDS-PAGE buffer:** 25 mM Tris, 192 mM glycine, 0.1% SDS, pH8.3
- **12 % SDS polyacrylamide gel:**

Resolving gel-composition for 8 gels - total volume:	36 ml
30 % acrylamide solution (29:1 acrylamide:bisacrylamide)	14,4 ml
Resolving buffer (1,5 MTris-HCl, pH 8,8)	4,5 ml
20 % SDS	0,180 ml
15 % APS	0,213 ml
Distilled H ₂ O	16,7 ml
TEMED	0,036 ml

Stacking gel (3,75%)-composition for 8 gels - total volume:	12 ml
30 % acrylamide solution (29:1 acrylamide:bisacrylamide)	1,5 ml
Resolving buffer (1,5 MTris-HCl, pH 8,8)	3 ml
20 % SDS	0,060 ml
15 % APS	0,071 ml
Distilled H ₂ O	7,34 ml
TEMED	0,036 ml

Protocol:

- Prepare the 10% SDS-PAGE gel according the above described recipe.
- Boil all the tubes having SDS-PAGE loading buffer for 4 minutes and load on the gel. Run the electrophoresis for 40 minutes at constant 210V in 1x SDS-PAGE running buffer.

d. Staining and destaining

Solutions and reagents:

- **Coomassie blue solution:** 0,2 % Coomassie brilliant blue R-250, 25 % methanol, 12,5 % acetic acid
- **Destain solution:** 25 % methanol, 12,5 %acetic acid

Protocol

- Disassemble the glasses and carefully transfer the gel to the tray with coomassie blue solution. Let the gel on the low speed rotary shaker for 20 minutes. Transfer the gel to fresh tray and wash quickly with distilled water. Add destain solution to submerge the gel and place again on the shaker. Monitor the appearance of protein bands.

V. References

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VI. Question

How did the higher concentration of KCl in the solution influence the binding of the two studied proteins? Explain why.