A simple protocol to detect interacting proteins by GST pull down assay coupled with MALDI or LC-MS/MS analysis.

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Method Article

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Abstract

Glutathione-S-transferase \(GST) tagged proteins have variety of applications including purification of fusion proteins. The GST pull-down assay is an _in vitro_ method used to determine a physical interaction between a GST tagged probe protein to an unknown or a known target protein. The procedure involves incubation of the GST fusion protein immobilized on glutathione-agarose beads with the total cell lysate. Complexes eluted from the beads are resolved by SDS-PAGE and analyzed by silver/comassie staining. The protein bands are subjected to in gel trypsin digestion followed by MALDI \(Matrix-assisted laser desorption/ionization) or LC-MS/MS \(Liquid chromatography- mass spectrometry) analysis of resulting peptides to identify the interacting proteins.

Introduction

Detection of functional complex formed by direct physical interaction between protein molecules forms the basis of understanding various signaling pathways as well as cross talk between different pathways. GST [1]pull-down assay is an important technique useful for both confirming the protein-protein interaction predicted by other research techniques \(e.g., co-immunoprecipitation, yeast two hybrid etc.) as well as for screening of novel /unknown protein interactions[2, 3]. Pull-down assays are a kind of affinity purification that not only enhance the efficiency of protein purification but also can be used simultaneously to perform a pull-down, or co-purification, of potential binding partners. In GST pull-down assay, a GST tagged bait protein is immobilized on glutathione-agarose beads followed by incubation with a protein source that contains putative "prey" proteins, such as a cell lysate. The source of prey protein at this step depends on whether the researcher is confirming a previously suspected protein-protein interaction or identifying an unknown interaction. The protein complex protein eluted from the beads are resolved by SDS-PAGE and stained with Comassie brilliant blue R250 or G250 or with Silver. The protein bands excised from the gel is subjected to in-gel tryptic digestion protocol [4, 5]. The digested peptides mixture are analyzed byMALDI-MS or LC-MS/MS \(termed “GeLCMS”). We describe herein a detailed protocol for GST pull down and in gel trypsin digestion suitable for identification of interacting proteins by MALDI-MS or LC-MS/MS.

Reagents

GST pull down assay • 50% slurry of Glutathione Sepharose 4B • Protein extraction buffer: 1mM DTT, 1mM PMSF, 1mM EDTA \(pH 8.0) in 1XPBS pH 7.4 • 1XPBS pH 7.4 • IPTG \(isopropyl-β-D-thiogalactoside) • LB broth • Lysozyme \(100mg/ml stock in water .Storage at -20ºc) • DNase \(10/ml stock solution in 50 mM HEPES, 250mM KCl, 1M DTT and 50% glycerol) • Triton X 100 \(0. 2%) • Glutathione elution buffer: 20mM reduced glutathione in 50mM Tris-HCl \(pH 8.0) **In-gel protein digestion for mass spectrometry** • Distaining and wash solution: 50mM ammonium bicarbonate in 50% Acetonitrile • Digestion Buffer: 50mM ammonium bicarbonate in water • Reducing Buffer: 10mM Dithiothreitol \(DTT) in 50mM ammonium bicarbonate \(freshly prepared) • Alkylating Buffer : 55mM iodoacetamide in 50mM ammonium bicarbonate \(freshly prepared) • Trypsin solution: To perform 10

reactions, 5 µl of trypsin \(0.2 µg/ µl) is taken and to it, 95 µl of digestion buffer is added. Mix it properly and then use. • Formic acid • Trifluoroacetic acid

Equipment

• 4 °C microcentrifuge. • Rotatory shaker incubator • Rocker shaker • Laminar flow hood • Micro centrifuge and Falcon tubes

Procedure

GST pull down assay _Preparation of bacterial cell lysate with expressed GST/ GST fusion protein_ • Inoculate 5ml of LB broth with ampicillin \(100 µg ml−1) in a culture vial with a single colony of BL21 strain of E.coli \(transformed with empty pGEX4T-2 or protein of interest cloned in pGEX4T-2) and incubate overnight at 37° C with shaking at 200 rpm. • Inoculate1% of overnight grown culture into 100 ml of LB ampicillin \(100 µg ml−1) in a conical flask \(capacity of 500 ml) and incubate at 37° C with shaking until O. D 600 of 0.6 is achieved. • Induce the culture with 0.3mM conc. of IPTG for 4 hrs. Harvest the cells by centrifugation at 5500 rpm at 4° C for 15 min. • Wash the cells twice with 1 X PBS. Resuspend the pellet in protein extraction buffer. Add lysozyme to the concentration of 1mg/ml and incubate on ice for 30 min. with occasional mixing in between. • Add Triton X 100 \(0. 2%) and DNase \(5µg/ml) to the above mix followed by vigorous shaking. Incubate for 1 hr at 4° C with gentle shaking to solubilize the fusion protein. • Spin at 10,000 rpm for 10 minutes. Clarify the above supernatant by filtration through 0.4µ filters. Note: Cell lysate from BL21 expressing GST \(Empty pGEX4T-2) is used as control to compare with the cell lysate expressing GST fusion protein. Both samples are processed simultaneously _Immobilization of bait protein_ \(GST/GST fusion protein) • Prepare 50% slurry of Glutathione Sepharose 4B from 75% slurry. Gently shake the vial of Glutathione Sepharose 4B resin to suspend the slurry. Transfer 1.33ml of slurry into a 15ml Falcon tube for generating 1ml bed volume. • Spin at 2100 rpm for 5min. Decant the supernatant carefully and add 10 bed volume of ice cold 1X PBS and mix well by inverting. Spin at 2100 rpm for 5 min. followed by decanting of supernatant. • Repeat the above step 2 more times. • Finally, add 1.0 ml of 1X PBS to the above slurry to prepare 50% slurry. • Incubate the clarified crude cell lysate expressing GST or GST fusion protein \(described above) with 50% slurry for 2-4 hours at 4° C with gentle shaking • Spin at 500 x g for 5 min. Wash the column three times with 10 bed volume of ice cold 1X PBS. _Incubation of cell lysate \(consisting of interacting prey protein) with immobilized bait protein_ • Prepare the cell lysate according to the source organism. For bacteria, the method described above can be followed. However, yeast cells can be disrupted by glass beads and plant cells can be crushed in liquid nitrogen using mortar and pestle. • Add the clarified cell lysate to the Glutathione Sepharose 4B column with immobilized GST/ GST fusion protein and incubate for 2-4 hours at 4ºC. • Spin at 500 x g for 5 min. Wash the column three times with 10 bed volume of ice cold 1X PBS. • Elute the interacting protein complex by incubating with 1 bed volume of glutathione elution buffer for 15 min. at RT and spin at 500 x g. • Repeat this step twice. Collect each elute separately. **Separation of interacting protein complex on SDS- PAGE gel** • The elutes thus obtained both from column containing

both immobilized GST and GST fusion proteins are loaded adjacent to each other on a 10% SDS PAGE gel with appropriate molecular weight marker. • Stain the gel with either silver stain or Comassie. • Excise the protein bands from the gel for further processing. **In-gel protein digestion for mass spectrometry** _Excising and destaining of gel pieces_ • Wash the gel with sufficient water and place it on a light box to excise the desired bands with a clean scalpel. • Cut the bands in to small pieces \(1x1 or 2x2mm) and transfer into a microcentrifuge tube containing water. Tube can be stored at 4ºC until further processing. • Add 200µl of destaining solution to the gel pieces and keep at 37ºC for 30 min. with slow shaking. • Discard distaining solution and repeat the step. • Add 200 µl 100% ACN to the gel pieces and incubate for 10 min with occasional shaking. The gel pieces should now appear white \(translucent). • Remove ACN from the tube. _Reduction and alkylation of gel pieces_ • Add 100-200 µl of reducing buffer and incubate at 60 ºC for 1 hr. • Remove the reducing buffer and allow to cool at room temperature. • Add 200 µl of alkylating buffer for 30 min. in dark at room temperature. • Remove and discard alkylating buffer • Wash the sample by adding 200 µl of destaining solution and incubate at 37ºC for 15 minutes. Then discard destaining solution. • Repeat the above step once more. _Shrink gel pieces_ • Shrink gel pieces by adding 50-100 µl of acetonitrile. Incubate sample for 15 minutes at room temperature. The gel pieces should now become white \(translucent). • Carefully remove acetonitrile and allow gel pieces to air dry for 5-10min. Trypsinization of proteins and recovery of fragments • Add 10 µl of the trypsin solution to the tube containing the shrunken gel pieces. Incubate in ice for 10 min and allow gel pieces to swell and absorb the trypsin solution. If 10 µl is not sufficient to cover and fully swell gel pieces, use additional 10ul. • Add 25 µl of digestion buffer to the tube. Incubate sample at 37ºC for four hrs or at 30 ºC overnight with shaking. _Extraction of peptides_ • Remove digestion mixture and place in a clean tube • To further extract peptides, add 10 µl of 0.5% trifluoroacetic acid or 1% formic acid solution to gel pieces and incubate for 5 minutes. Remove extraction solution and add to digestion mixture. This step also serves to inactivate trypsin. • Centrifuge to remove any gel particles to prevent clogging or damage of columns. Transfer to a fresh eppendorf and store at 4ºC.

Timing

GST pull down • Bacterial pre-culture:16 hrs • Time to attain O. D 600 of 0.6: 2-3 hrs • IPTG Induction: 4hrs • Preparation of cell lysate with GST/ fusion protein: 2 hrs. • Glutathione Sepharose 4B column column preparation: 20-25 min. • Immobilization of bait protein \(GST/GST fusion protein): 2-4 hrs • Incubation of cell lysate with immobilized bait protein: 2-4 hrs **In gel protein digestion** • Excising of protein bands : 3 -5 min per band • In-gel reduction and alkylation of proteins: 60 min • Destain gel pieces: 30 min • Saturating gel pieces with trypsin: 120 min • Digestion: 30 min to overnight • Extraction of peptide digestion products: 15 min

Troubleshooting

see the table 1

Anticipated Results

In SDS-PAGE gel, we expect to observe single band of purified GST \(≈ 28 kDa) in the elute obtained from immobilized GST column. In the elute obtained from immobilized GST fusion protein column, besides a band of fusion protein , we expect to get other band/ bands of different molecular weights which are expected to be the probable interacting partners of protein of interest. These bands should be present exclusively in the elute obtained from immobilized fusion protein sample and should be absent in GST control samples.

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