Protocol

Bimolecular Affinity Purification (BAP): Tandem Affinity Purification Using Two Protein Baits

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INTRODUCTION

The tandem affinity purification (TAP) procedure was pioneered in yeast for the purpose of purifying and characterizing protein complexes. While affinity purification is relatively easy to perform, nonspecific protein interactions can plague the identification of true interacting partners of the given bait utilized in the purification. To alleviate this problem, two sequential affinity purification steps are employed in the TAP procedure. Since its inception in yeast, TAP has gone through many adaptations and has been employed multiple times in diverse organisms, including mammalian systems. In all these approaches, two out of many possible affinity moieties are employed and are usually expressed as a fusion polypeptide in the amino or carboxyl-terminal region of the protein bait. In this protocol, we describe a variation on the TAP procedure in which the affinity moieties are placed on two different proteins of a molecular complex to isolate or detect components present in the complex. This variation, which we refer to as bimolecular affinity purification (BAP), is suited for the identification of specific molecular complexes marked by the presence of two known components.

RELATED INFORMATION

This procedure is a variation of the conventional TAP procedure (Fig. 1). The protocol will focus on two affinity moieties: glutathione s-transferase (GST) and a biotinylation peptide from transcarboxylase. The protocol was used to isolate a protein complex containing both GCN5 (general control of nutrition 5) and COMMD1 (copper metabolism MURR1 domain containing 1) and confirms that this complex also interacts with the NF-κB (nuclear factor kappa B) subunit RelA (Mao 2009).

A method similar to the calcium phosphate precipitation in Step 3 can be found in **Calcium-Phosphate-Mediated Transfection of Eukaryotic Cells with Plasmid DNAs** (Sambrook and Russell 2006).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <**!**>, and recipes for reagents marked with <**R**>.

Reagents

Bait DNA sequences

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Cite as: Cold Spring Harb Protoc; 2009; doi:10.1101/pdb.prot5318

<R>BAP wash buffer
Biotin (4 mM) (cell-culture tested)
$CaCl_2$ (2 M) (cell-culture tested)
Cell culture medium

For HEK293 cells, we utilize Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

Cells, HEK293
Expression vectors:
- GST fusions: pEBG or pEBB-C-GST (or an equivalent expression system)
- Biotinylation target peptide: pEBB-C-TB (or an equivalent expression system)

<R>Glutathione elution buffer for BAP
Glutathione resin (immobilized onto agarose or Sepharose beads)
GST antibody
<R>HBSS for BAP (2X)
L-Glutathione, reduced (>98% purity)
<R>Phosphate-buffered saline (PBS) (1X, pH 7.4)

Prepare PBS without added calcium or magnesium salts.

Reagents for Western blotting (see Step 13)
<R>Triton lysis buffer (complete)
Streptavidin, horseradish peroxidase (HRP)-conjugated
Strepavidin resin (immobilized onto agarose or Sepharose beads)

Equipment

Cell scraper, 3-cm blade
Centrifuge chromatography columns, 22-mL total volume (Pierce 89898)
Centrifuges:
- Benchtop centrifuge with cooling system for 1.5-mL microcentrifuge tubes
- Tabletop centrifuge with a fixed-angle rotor that can reach 15,000*g* (e.g., Eppendorf 5810R centrifuge and F34-6-38 rotor).

Conical tubes, 50-mL, able to withstand 15,000*g* (Corning 430828)
Equipment for Western blotting (see Step 13)
Incubators (CO_2) for cell culture
Inverted microscope
Laminar flow hood
Microcentrifuge tubes, 1.5-mL
Pipette tips

Use tips with a wide opening when pipetting resuspended beads. Alternatively, cut conventional plastic tips with a razor blade (see Step 7.ii).

Pipettes (serological) and pipette aid
Tissue culture plates, 15-cm
Tube rotator

METHOD

Construct Cloning

1. Use standard molecular cloning techniques to insert the coding sequences of the desired baits in frame with the affinity tags.

   One of the baits is fused to GST in either the amino- or carboxyl-terminal position (using the expression vectors pEBG or pEBB-C-GST, or an equivalent expression system). The second bait is expressed fused to the Tobacco Etch Virus (TEV) protease site preceding the biotinylation target peptide (TB tag) of Propionibacterium freudenreichii *transcarboxylase (using the pEBB-TB vector or an equivalent expression plasmid). The biotinylation target peptide must be positioned in the carboxyl-terminus of the fusion protein for optimal results.*

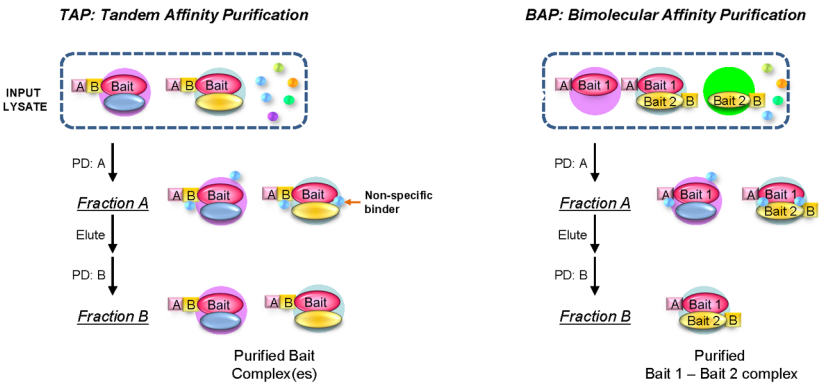

FIGURE 1. Schematic representation of the TAP and BAP procedures. Both procedures are based on two sequential affinity purification steps, but in TAP, both affinity moieties are fused to one bait (indicated as *A* and *B* in the diagram). In the BAP procedure, these affinity moieties are fused to two different baits. (For color figure, see doi: 10.1101/pdb.prot5318 online at www.cshprotocols.org.)

Protein Expression and Harvest

2. Seed 7 × 10^6 HEK 293 cells per 15-cm plate (containing 20 mL of cell culture medium) ~18 h prior to transfection.

   The method of expression of the bait proteins can be of critical importance to the success of the procedure. Variables to keep in mind are the cell type to be used and the level of expression that will be achieved. Generally, transient overexpression through plasmid transfection provides a rapid and scalable approach to this procedure but carries the risk of purifying nonspecific factors. For liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis, 10 plates are usually required, but smaller scale experiments may be performed in other circumstances (e.g., Western blot analysis). Stable expression, particularly when coupled with stable RNA interference of the endogenous protein, may overcome the concerns of acute transfection but requires larger-scale purifications.

3. Transfect cells using the conventional calcium phosphate method as follows. Perform all steps in a laminar flow hood. (For an alternative transfection method, see **Calcium-Phosphate-Mediated Transfection of Eukaryotic Cells with Plasmid DNAs** [Sambrook and Russell 2006]).

   Most HEK 293 cells are highly transfectable by calcium phosphate. The amounts of plasmid depend on the expression efficiency of the each individual bait but <10 µg is preferred.

   i. Aliquot plasmid DNA into a tube (e.g., if transfecting 10 plates using 20 µg of DNA per plate, add 200 µg of plasmid DNA to a 50-mL conical tube).

   ii. Add enough transfection reagents to make a total of ~1-2 mL of DNA:calcium phosphate mixture per plate (no more than 10% of the culture medium on the plate). For each 1 mL final transfection solution, use 61 µL of 2M $CaCl_2$, DNA, and enough ultrapure water to reach a total volume of 500 µL.

   The transfection mixture for all plates is best made as a single large batch.

   iii. To the DNA:$CaCl_2$:water mixture, add an equal volume of 2X HBSS using a pipette aid and a serological pipette (for 1 mL, this would amount to 500 µL of 2X HBSS). Continue to press on the pipette aid for 30 sec to deliver air bubbles to the reaction mixture. Let the solution stand for ~5 min.

   This will ensure a fine and homogenous DNA:calcium phosphate precipitate for optimal transfection efficiency.

   iv. Gently mix the transfection solution (swirling the solution in the tube by hand) and apply to the plates using a serological pipette. Apply the appropriate amount of solution per plate as evenly spaced drops and then gently tilt the plates to mix the medium and the transfection reagent.

   Confirm the presence of calcium phosphate crystals through an inverted microscope.

4. Replace medium ~8 h post-transfection with fresh warm medium supplemented with biotin (4 µM).

Low levels of biotinylation will occur even when the medium is not supplemented. However, optimal biotinylation requires additional biotin supplementation of the medium for expressed fusion proteins.

5. Two days post-transfection, collect the transfected cells (these steps are done at the bench):
   i. Aspirate the medium from each plate and add 1.5 mL of cold 1X PBS per plate.
   ii. Scrape the cells with a soft cell scraper and transfer to a 50-mL conical tube.
   iii. Rinse plate with an additional 1.5 mL of PBS and transfer to the 50-mL tube.
   iv. Combine all 10 plates into one 50-mL conical tube.
   v. Centrifuge at ~300*g* for 5 min (4°C).
6. Prepare a cleared lysate:
   i. Aspirate PBS and resuspend the cell pellet in complete Triton lysis buffer, using a total of 10 mL (or 1 mL per plate).
   ii. Incubate on ice for 20 min.
   iii. Centrifuge at 15,000*g* for 10 min at 4°C using a fixed-angle rotor.
   iv. Transfer the supernatant and repeat a second time, ensuring that the lysate is free of insoluble debris.
   v. Save an aliquot (~100 µL or 1% of the supernatant).

   Proceed immediately to the protein purification. Do not store the lysates in the freezer, because certain protein complexes will not be preserved if the samples are not processed immediately.

Protein Purification

7. Pre-equilibrate glutathione (GSH) resin column:
   i. Add 1 mL of 50% glutathione agarose beads to the chromatography column.
   ii. Wash the column with 5 mL of complete Triton lysis buffer (10X volume of beads).

   Cut the tip of the plastic pipette with a razor blade, or use special pipette tips that have a wide opening when resuspended beads are to be pipetted.
8. Bind first bait to the GSH column:
   i. Cap the bottom of the column, load the cell lysate, and then cap the top of the column.
   ii. Rotate the column for 1-3 h at 4°C.
   iii. Let the lysate flow through by gravity (or through centrifugation at 500*g* for 5 min).
   iv. Save an aliquot of the flow-through (100 µL or 1%) to ensure that the binding to the GSH column resulted in efficient depletion of the first bait from the lysate.
9. Wash the column four times with 20 mL of complete Triton lysis buffer, rotating for 5 min at 4°C between each wash. During the last wash, save an aliquot of the beads by removing 200 µL (1%) of the resuspended beads prior to draining the wash buffer.

   Given the large volume used in these washes, centrifugation is more efficient than gravity flow to drain the column.
10. Elute the column:
    i. Cap the bottom of the column, add 2 mL of glutathione elution buffer to the drained beads in the column, and then cap the top of the column.
    ii. Rotate the column for 20 min at 4°C.
    iii. Drain the column, saving the eluate, and repeat the elution step three additional times.
    iv. Pool all four eluates into a single batch. Save a sample of the GSH eluate (~80 µL or 1% of the eluate). After elution is complete, resuspend the beads in 5 mL of BAP wash buffer and save a sample of the beads post-elution (50 µL or 1% of the resuspended beads).

11. Pre-equilibrate the streptavidin (SA) agarose column:

    i. Load the column with ~25 µL of bead volume for every 100 mg of total initial input lysate utilized at the beginning of the affinity purification.

    ii. Apply 5 mL of BAP wash buffer and allow to drain by gravity flow.

12. Bind the second bait to the SA column:

    i. Apply GSH eluate to the SA column and rotate for 1-3 h at 4°C.

    ii. Drain the column by gravity flow.

    iii. Wash the SA agarose with 10 mL of BAP wash buffer five times.

    iv. After the last wash, resuspend the beads in 1 mL of BAP wash buffer and transfer the beads to a fresh 1.5-mL microcentrifuge tube.

    v. Set aside 10 µL (or 1%) of the bead suspension.

    vi. Precipitate the beads by centrifugation at 300*g* for 5 min at 4°C.

    vii. Add enough BAP wash buffer to keep the beads as a 50% slurry. Store the final purification product at 4°C.

    The stability of the sample at 4°C depends on the protein complex and the desired application in which this complex will be utilized.

    This is a stopping point in the procedure.

Quality Control

A quality-control Western blot should be performed to ensure that proper precipitation and elution of each one of the baits has occurred.

13. Load equal amounts of the following paired samples (13.i-13.iii) and the final SA precipitate (13.iv):

    i. Initial input and GSH column flow-through.

    This will provide a sense of the ability of the first column to deplete the first bait from the lysate.

    ii. GSH beads before and after elution.

    This will demonstrate binding of the GST-tagged bait and proper elution from the column at the end of the glutathione elution step.

    iii. GSH eluate and SA agarose flow-through.

    This should demonstrate the ability of the second column to deplete the TB-tagged bait from the GSH eluate.

    iv. Final SA precipitate.

    This sample ensures that the second bait was precipitated by the streptavidin beads as expected, and also confirms that coprecipitation of the first bait has occurred. Also, the proportion of the first bait that is bound to the second bait can be estimated, taking first into account the efficiency of the last precipitation (as noted in Step 13.iii), and then comparing the amount of the first bait offered to the final column (GSH eluate) and the amount that is ultimately recovered (final SA precipitate).

14. Perform immunoblotting for each bait.

    In addition to antigen-specific antibodies, an antibody to GST and streptavidin-HRP can be similarly used for detection. Finally, immunoblotting for expected interacting partners of the protein complex is an additional quality control step that can be employed.

    See Troubleshooting.

Final Analysis

15. Analyze the samples to identify novel interacting partners present in the protein complex. Examples are as follows:

    - Trypsin digestion on beads will release peptides for protein identification using LC/MS-MS. We would recommend avoiding all detergents in the second affinity purification steps as described here if this approach is chosen.

- Digestion of the sample by TEV protease will release the complex into solution, which can then be precipitated with acetone and trichloroacetic acid for further elimination of detergent contaminants prior to trypsin digestion and LC/MS-MS. The duration and temperature required for optimal TEV cleavage may vary for each individual fusion protein and should be optimized ahead of time. A good starting point is a 2-h digestion at 14°C, but longer digestion or room temperature may be required.
- The entire sample can be loaded onto an SDS-PAGE gel for band excision and LC/MS-MS identification. The beads should be precipitated and the supernatant thoroughly and carefully aspirated. The beads are then resuspended in 3X gel-loading buffer (~30-50 µL of buffer per 25 µL of bead volume).

TROUBLESHOOTING

Problem: Bait 1 did not elute from the GSH column.
[Step 14]
Solution: The elution of bait 1 depends on the presence in the elution buffer of glutathione and a mild detergent (0.1% Triton X-100 in this case). Consider the following:

1. A single elution step has variable efficiency, and sequential elution steps as described here are advisable for most baits, although this will dilute the final eluate.
2. Make sure that the glutathione has been stored properly (at 4°C, with desiccation).

Problem: Bait 2 was not precipitated in the final purification.
[Step 14]
Solution: There are at least two possible explanations:

1. Bait 2 did not coprecipitate with bait 1 in the initial GSH precipitation. This should be evident when looking at the sample from the GSH column prior to elution as well as in the GSH eluate. The BAP procedure presumes that the ability of both baits to interact is well established. However, the affinity tags and their positioning may impair the interaction between two proteins. If the tags are thought to be affecting the interaction between the baits, there are two potential steps that can be taken. Either the GST tag can be moved to the opposite end of bait 1, or other elutable affinity tags such as tandem epitope tags can be utilized instead of GST (Table 1).
2. Precipitation of bait 2 may be impaired if the fusion protein is not properly biotinylated in vivo. Although bait 2 may be expressed well, in some cases biotinylation of the transcarboxylase

Table 1. Various affinity moieties available for protein purification and their characteristics

Affinity moiety	Amino acid length (molecular mass)	Affinity (K_D)	Precipitation reagent	Elution method
His_6	6 (0.8 kDa)	1 mM	Nickel	Imidazole
FLAG peptide	8 (1 kDa)	0.1-100 nM[a]	Antibody	FLAG peptide
HA peptide	9 (1.1 kDa)	0.1-100 nM[a]	Antibody	HA peptide
Myc peptide	10 (1.2 kDa)	0.1-100 nM[a]	Antibody	Myc peptide
Biotin "mimicking" peptides (SBP, Strep-tag, etc.)	8-35 (1-4 kDa)	2-40 nM	Streptavidin	Biotin
Calmodulin binding peptide of MLCK2	26 (3 kDa)	2 nM	Calmodulin	Calcium chelation
Biotinylation target peptide	75-90 (10 kDa)	1 ftM	Streptavidin	Protease cleavage
Tandem IgG binding domains of Protein A or Protein G	~125 (14 kDa)	10 nM	Human IgG	Protease cleavage
Glutathione S-transferase (GST)	224 (26 kDa)	80 nM	Glutathione	Glutathione

[a]Reported range for the K_D for antibody-antigen interactions. The actual K_D for antibodies against these peptides has not been published.

peptide is deficient in certain fusion proteins, particularly when placed in the amino terminus. Thus, we recommend using this peptide always as a carboxyl-terminal fusion. To confirm proper biotinylation of bait 2, compare immunoblots performed with a protein-specific antibody and with streptavidin-HRP. (If the protein is expressed but poorly biotinylated, the signal from streptavidin-HRP will be dramatically lower.)

DISCUSSION

Advances in affinity-based protein purification techniques have resulted in relatively simple purification protocols that are generally accessible to molecular biology laboratories. A commonly utilized format is the TAP procedure, in which protein purification and mass spectrometry analysis are coupled to identify novel protein complexes (Rigaut 1999; Puig 2001; Burckstummer 2006). However, the purification of a given protein bait by this procedure does not mean that a single homogenous molecular complex has been isolated, and such consideration may be critical in certain circumstances. The protocol presented here is intended for the purification of protein complexes marked by the presence of two components and therefore can be tailored to represent a homogenous population (Fig. 1). In addition, the BAP procedure is simple and accessible to laboratories that are unable to do more complicated chromatographic separation techniques, but for whom the homogeneity of the sample is important. BAP requires the concurrent expression of two distinct baits and therefore its efficiency may be dramatically affected by differences in relative expression levels of the baits. Similarly, the tags fused to the proteins might alter the complex composition, enzyme activity, or cell homeostasis.

Therefore, it is of major importance that proper functionality of the baits is confirmed in the actual expression system prior to the purification. In this regard, while the protocol presented here is based on the GST and TB tags, a number of other affinity moieties could potentially be used with the appropriate modifications in the binding and elution conditions required (Table 1). Given the very high affinity of biotin and streptavidin, the TB tag is an excellent choice for the second purification step in this protocol. This procedure allows for simple confirmation of ternary complexes between the two baits and a potential third interacting partner, as was the case in our study of the GCN5-COMMD1 complex and its interaction with the NF-κB subunit RelA (Mao 2009). However, depending on the relative stoichiometry of the interaction between the two baits in cells and the scale of the purification performed, significant amounts of the purified complex can be isolated that will be suitable for MS-based identification of novel interaction partners.

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