

Yeast Two-Hybrid

This protocol is for transforming the DNA-BD/target plasmid and the AD/library plasmids into yeast sequentially.

A. Transforming the DNA-BD/target plasmid into yeast:

Preparation of Yeast Competent Cells:

1. Inoculate several colonies, 2-3 mm in diameter, into 0.5 ml YPD.
2. Vortex vigorously to disperse any clumps.
3. Transfer this into a flask or tube containing 3 ml YPD.
4. Incubate at 30 C for 16-18 hr with shaking at 250 rpm to stationary phase ($OD_{600} > 1.5$).
5. Transfer enough overnight culture to produce an $OD_{600} = 0.2-0.3$ into 15 ml YPD.
6. Incubate at 30 C for 3 hr with shaking at 230-270 rpm.
7. Centrifuge the cells at 1000g for 5 min at RT.
8. Discard the supernatant and vortex to resuspend the cell pellet in 3 ml H₂O.
9. Decant the supernatant.
10. Resuspend the cell pellet in freshly prepared, sterile 0.1 ml 1 TE/LiAc.

Transformation of Yeast Competent Cells:

1. Add 0.1 μ g DNA-BD vector construct and 0.1 mg herring testes carrier DNA to a tube and mix.
2. Add 0.1 ml yeast competent cells to the tube and mix.
3. Add 0.6 ml sterile PEG/LiAc solution and vortex to mix.
4. Incubate at 30C for 30 min with shaking (200rpm).
5. Add DMSO to 10% (70 μ l) and mix gently by inversion.
6. Heat shock in a 42 C water bath for 15 min.
7. Chill cells on ice (1-2 min).
8. Pellet cells by centrifugation for 5 sec at 14K rpm (swinging bucket rotor best).
9. Remove the supernant.
10. Resuspend cells in 0.5 ml 1TE buffer.

Plating Transformation Mixtures:

1. Plate 100 μ l of 1:1000, 1:100, 1:10 and 1:1 dilution on SD/-Trp plates (100 mm plates).
2. Incubate plates, colony side down, at 37C until colonies appear. Then seal the master plates with Parafilm and store at 4C for up to 3-4 weeks.

β-galactosidase Assays:

This step is to test whether the bait itself has leaky. Best result will be obtained using fresh colonies 1-3 mm in diameter.

1. Presoak one sterile Whatman #5 or VWR grade 410 filter for each plate of transformants to be assayed in Z buffer/X-gal solution as follows:
 - a) Add 1.75 ml of Z buffer/X-gal solution to a clean 100-mm plate. (Use 3.5 ml for 150- mm plate.)
 - b) Layer a 75-mm filter onto the liquid to soak it up.
2. Place a clean, dry filter over the surface of the agar plate containing transformants.
3. Freeze/thaw to permeabilize the cells as follows:
 - a) As soon as the filter has wetted from the agar, carefully lift it off the agar plate with forceps, and transfer it with colonies facing up into a pool of liquid nitrogen.
 - b) Using forceps, completely submerge the filters for 10 sec or until uniformly frozen.
 - c) Remove filter and thaw it at RT.
4. Carefully place the filter, colony side up, on the presoaked filter (from step 1 above). Do not trap air bubbles under or between filters.
5. Incubate the filters at 30C and check periodically for the appearance of blue colonies.

B. Transforming the DNA-AD/library plasmid into DNA-BD/ target plasmid-containing yeast:

Preparation of Yeast Competent Cells:

1. Inoculate several colonies, 2-3 mm in diameter, into 0.5 ml SD/-Trp growth media.
2. Vortex vigorously to disperse any clumps.
3. Transfer this into a flask or tube containing 50 ml SD/-Trp growth media.
4. Incubate at 30 C for 16-18 hr with shaking at 250 rpm to stationary phase ($OD_{600} > 1.5$).
5. Transfer enough overnight culture to produce an $OD_{600} = 0.2-0.3$ into 300 ml YPD.
6. Incubate at 30 C for 3 hr with shaking at 230-270 rpm.
7. Centrifuge the cells at 1000g for 5 min at RT.
8. Discard the supernatant and vortex to resuspend the cell pellet in 25-50 ml H₂O.
9. Decant the supernatant.
10. Resuspend the cell pellet in freshly prepared, sterile 1.5 ml 1 TE/LiAc.

Transformation of Yeast Competent Cells:

1. Add 10-50 μg DNA-AD vector construct and 2 mg herring testes carrier DNA to a tube and mix.
2. Add 1 ml yeast competent cells to the tube and mix.
3. Add 6 ml sterile PEG/LiAc solution and vortex to mix.

4. Incubate at 30C for 30 min with shaking (200rpm).
5. Add DMSO to 10% (700 μ l) and mix gently by inversion.
6. Heat shock in a 42 C water bath for 15 min. Swirl occasionally to mix.
7. Chill cells on ice (1-2 min).
8. Pellet cells by centrifugation for 5 min at 1000g (swinging bucket rotor best).
9. Remove the supernant.
10. Resuspend cells in 5 ml 1TE buffer.

Plating Transformation Mixtures:

1. Spread 100 μ l of 1:1000, 1:100, and 1:10 dilution on SD/-Trp/-Leu plates for transformation efficiency controls (100 mm plates).
2. Spread 1 μ l (diluted in 100 μ l of H₂O) on SD/-Trp and SD/-Leu plates to check transformation efficiency of each plasmid (100-mm plates).
3. Spread the remaining transformation suspension on SD/-Trp/-Leu/-His/+3-AT plates (200 μ l per 150-mm plate).

Notes:

- a) Use 5 mM 3-AT for CG-1945, and 25 mM 3-AT for Y190, unless your DNA-BD/target protein tittered otherwise.
 - b) If using CG-1945 and low-expression vectors such as pGBT9 and pGAD424, 3-AT should be eliminated from the selection plates.
4. Incubate plates, colony side down, at 30C until colonies appear. Then seal the master plates with Parafilm and store at 4C for up to 3-4 weeks.
 5. Calculate the transformation efficiency and estimate the number of clones screened.
 6. Choose positive AD/library clones for further analysis.

Notes:

- a) After 2-3 days, some His⁺ colonies will be visible on the library screening (SD/-Trp/-Leu/-His/+3-AT) plates, but plates should be incubated for 5-10 days to allow slower growing colonies (i.e., weak positives) to appear. Ignore the small, pale colonies that may appear after 2 days but never grow to >2 mm in diameter. True His⁺ colonies are robust and can grow to >2 mm in diameter.
 - b) Not all of the transformants surviving this selection will be true two-hybrid positives. The most common class of false positives can be eliminated by screening for expression of the second reportor gene.
7. Streak out His⁺ colonies on fresh SD/-Trp/-Leu master plates and grow for 2-4 days at 30C until colonies are at least 1 mm in diameter. At this point, you can perform a β -galactosidase filter assay on the fresh colonies using a sterile filter. After lifting the colonies for the β -galactosidase assay, place the master plates at 30C for 1-2 days to allow the colonies to regrow. Then seal the master plates with Parafilm and store at 4C for up to 3-4 weeks.

β -galactosidase Assays:

1. Presoak one sterile Whatman #5 or VWR grade 410 filter for each plate of transformants to be assayed in Z buffer/X-gal solution as follows:

- a) Add 1.75 ml of Z buffer/X-gal solution to a clean 100-mm plate. (Use 3.5 ml for 150- mm plate.)
 - b) Layer a 75-mm filter onto the liquid to soak it up.
2. Place a clean, dry filter over the surface of the agar plate containing transformants.
3. Poke holes through the filter into the agar in three or more asymmetric locations to orient the filter to the agar.
4. Freeze/thaw to permeabilize the cells as follows:
 - a) As soon as the filter has wetted from the agar, carefully lift it off the agar plate with forceps, and transfer it with colonies facing up into a pool of liquid nitrogen.
 - b) Using forceps, completely submerge the filters for 10 sec or until uniformly frozen.
 - c) Remove filter and thaw it at RT.
5. Carefully place the filter, colony side up, on the presoaked filter (from step 1 above). Do not trap air bubbles under or between filters.
6. Incubate the filters at 30C and check periodically for the appearance of blue colonies.

Notes:

- a) The time it takes colonies producing β -galactosidase to turn blue varies, especially with Y190. It will typically take from 30 min to 8 hr in a library screening. Prolonged incubation (>8 hr) tends to give false positives.
 - b) Yeast transformed with pCL1 (the wild-type GAL4 control) will turn blue within 20-30 min. Y187 or Y190 cotransformed with pVA3-1 and pTD1-1 should give a positive blue signal within 60 min; CG-1945 cotransformed with the same controls may take an additional 30 min to develop. If the controls do not behave as expected, check the reagents and repeat the assay.
7. Identify the β -galactosidase-producing colonies by aligning the filter to the agar plate using the orienting marks.
8. Pick the corresponding positive colonies from the original plates, and transfer them to fresh medium.

Separation of library plasmid from bait plasmid:

1. For each candidate clone, inoculate 5 ml of SD/-Trp liquid medium with a single yeast transformant colony.
2. Incubate at 30C for a few days with shaking at 250 rpm (change medium one or two times).
3. Spread 100 μ l of 1:1000 dilution on SD/-Trp plate.
4. Incubate plates, colony side down, at 30C until colonies appear.
5. Streak out each colony on fresh SD/-Leu and SD/-Trp/-Leu plates, Incubate plates, colony side down, at 30C.
6. Pick up the colony which can grow on SD/-Leu plate, but cannot grow on SD/-Trp/-Leu plate, transfer it to fresh SD/-Leu liquid medium.

Plasmid isolation from yeast cells:

1. Inoculate 5 ml of SD/-leu liquid medium with a single yeast transformant colony.
2. Incubate at 30C for at least 20 hr with shaking at 250 rpm (until the culture is saturated).
3. Pellet the cells by spinning at 1,000g for 5 min at RT.
4. Decant the supernatant. Vortex to resuspend the pellet in residual liquid, and transfer to a 1.5-ml microcentrifuge tube.
5. Add 0.2 ml of yeast lysis solution.
6. Add 0.2 ml of phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads. Vortex for 2 min.
7. Spin at 14,000 rpm for 5 min at RT.
8. Transfer the supernatant to a clean 1.5-ml tube.
9. Add 1/10 volume of 3M NaOAc, pH5.2, and 2.5 volumes of ethanol to precipitate the DNA.
10. Wash the pellet with 70% ethanol and dry under vacuum.
11. Resuspend the DNA pellet in 20 µl of TE buffer.

Materials required:

1. 1 M 3-amino-1,2,4-triazole (3-AT; Sigma #A-8056), dissolve in H₂O and filter-sterilize.
2. 40% dextrose, autoclaved or filter-sterilized.
3. 10mg/ml Carrier DNA, Sonicated, herring testes carrier DNA in solution can be purchased separately (#K1606-A). Just prior to use, denature the carrier DNA by placing it in a boiling water bath for 20 min and immediately cooling it on ice.
4. 1 PEG/LiAc solution: Prepare fresh just prior to use.

	Final Concentration	To prepare 10 ml of solution
PEG 4000	40%	8 ml of 50% PEG
TE buffer	1	1 ml of 10 TE
LiAc	1	1 ml of 10 LiAc

5. 1 TE/LiAc: prepare just before use from 10 solutions.
6. 50% PEG 4000 (Polyethylene glycol, avg. MW=3,350; Sigma #P-3640). Filter-sterilize or autoclave.
7. DMSO (Dimethyl sulfoxide; Signa #D-8779)
8. 10 TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, adjust pH to 7.5, and autoclave.
9. 10 LiAc: 1 M Lithium acetate (Sigma #L-6883), adjust pH to 7.5 with dilute acetic acid, and autoclave.
10. Z buffer

Na ₂ HPO ₄ .7H ₂ O	16.1g/L
NaH ₂ PO ₄ .H ₂ O	5.5g/L
KCl	0.75g/L
MgSO ₄ .7H ₂ O	0.246g/L

Adjust pH to 7.0 and autoclave. Prepare fresh as needed.

11. X-gal stock solution

Dissolve 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; #8060-1) in N,N-dimethylformamide(DMF) at 20 mg/ml. Store in the dark at -20°C .

12. Z buffer/X-gal solution Prepare fresh as needed.

100 ml Z buffer

0.27 ml β -mercaptoethanol (Sigma #M-6250)

1.67 ml X-gal stock solution