CHAPTER ELEVEN

Identifying Proteins that Bind a Known RNA Sequence Using the Yeast Three-Hybrid System

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Abstract
The yeast three-hybrid system can be used to identify a protein partner of a known RNA sequence by screening a cDNA library fused to a transcription activation domain, with a hybrid RNA as ‘bait.’ Most commonly, such screens are performed to identify proteins that interact with a given RNA in vivo.

1. THEORY
The general strategy of the three-hybrid system is shown in Fig. 11.1. DNA-binding sites are placed upstream of a reporter gene, which has been integrated into the yeast genome. The first hybrid protein consists of a DNA-binding domain linked to an RNA-binding domain. The RNA-binding domain interacts with its RNA-binding site in a bifunctional
A "hybrid" RNA molecule. The other part of the RNA molecule interacts with a second hybrid protein consisting of another RNA-binding domain linked to a transcription activation domain. When this tripartite complex forms at the promoter, the reporter gene is turned on. Reporter expression can be detected by phenotype or simple biochemical assays. The specific molecules used most commonly for three-hybrid analysis are depicted in Fig. 11.1.

The DNA-binding site consists of a 17-nucleotide recognition site for the *Escherichia coli* LexA protein and is present in multiple copies upstream of both the *HIS3* and the *lacZ* genes. The first hybrid protein consists of LexA fused to bacteriophage MS2 coat protein, a small polypeptide that binds to a short stem-loop sequence present in the bacteriophage RNA genome. The hybrid RNA (depicted in more detail in Fig. 11.2) consists of two MS2 stem-loops and the MS2 coat protein binds one stem-loop as a dimer. The second hybrid protein consists of the transcription activation domain of the yeast Gal4 transcription factor linked to an RNA-binding protein, Y.

By introducing a cDNA library, cognate protein partners of a known RNA sequence can be identified. Secondary screens of the initial ‘positives’ are needed to winnow down candidates for further analysis and to eliminate false positives. Here, we consider a case in which the activation domain library carries a *LEU2* marker and the hybrid RNA vector (pIIIA) carries the *ADE2* and *URA3* markers. When the RNA interacts with the protein

![Figure 11.1](image-url)
produced from a cDNA, the HIS3 reporter gene is expressed and the yeast grow on media lacking histidine and/or containing 3-aminotriazole (a competitive inhibitor of the HIS3 gene product). Additional screens are performed using the lacZ reporter and colorimetric assays.

2. Equipment

- Centrifuge
- 30 °C incubator-shaker
- Petri dishes
- Micropipettors
- Micropipettor tips
- Nitrocellulose filter (Petri dish-sized)
- Whatman 3MM chromatography paper (Petri dish-sized)
- Sterile toothpicks
- Parafilm
3. MATERIALS

cDNA–activation domain fusion library
_Saccharomyces cerevisiae_ strain YBZ-1: _MATa, ura3-52, leu2-3, -112, his3-200, trp1-1, ade2, LYS2::(LexAop)-HIS3, URA3::(lexAop)-lacZ, and LexA-MS2 MS2 coat (N55K)
Hybrid RNA plasmid (e.g., pIIIA/MS2-2)
Synthetic dextrose (SD) dropout media (liquid and plates)
3–Aminotriazole (3–AT)
5–Fluoroorotic acid (5–FOA)
Sodium phosphate dibasic (Na2HPO4)
Sodium phosphate monobasic (NaH2PO4)
Potassium chloride (KCl)
Magnesium sulfate (MgSO4)
2-Mercaptoethanol
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)
Dimethylformamide
Zymolyase (G-Biosciences)
Promega Wizard Plus SV Miniprep kit
Liquid nitrogen

3.1. Solutions & buffers

**Step 2** 3–Aminotriazole (1 M)

Dissolve 4.21-g 3–AT in 50-ml water. Pass through a 0.2-μm filter to sterilize. Aliquot into tubes, wrap in foil, and store at −50 °C.

**Step 5** Z-buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
<th>Stock</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2HPO4</td>
<td>60 mM</td>
<td>1 M</td>
<td>60 ml</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>40 mM</td>
<td>1 M</td>
<td>40 ml</td>
</tr>
<tr>
<td>KCl</td>
<td>10 mM</td>
<td>1 M</td>
<td>10 ml</td>
</tr>
<tr>
<td>MgSO4</td>
<td>1 mM</td>
<td>1 M</td>
<td>1 ml</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>50 mM</td>
<td>14.2 M</td>
<td>Add fresh</td>
</tr>
</tbody>
</table>

Add to 850-ml water. Adjust pH to 7.0, if necessary. Add water to 1 l and filter-sterilize.
X-Gal solution (20 mg ml\(^{-1}\))

Dissolve 0.4-g X-Gal in 20-ml dimethylformamide (in a fume hood). Aliquot into tubes, wrap in foil, and store at \(-20^\circ\)C.

4. PROTOCOL

4.1. Duration

<table>
<thead>
<tr>
<th>Preparation</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol</td>
<td>1–2 months</td>
</tr>
</tbody>
</table>

4.2. Preparation

Obtain appropriate activation domain library either from individual laboratories or commercial sources.

Transform the hybrid RNA plasmid into YBZ-1 yeast using the LiAc method (Gietz and Woods, 2002, see Chemical Transformation of Yeast).

Prepare sufficient plates listed in subsequent steps for the selection (see Saccharomyces cerevisiae Growth Media).

See Fig. 11.3 for the flowchart of the complete protocol.

5. STEP 1 PILOT TRANSFORMATION TO DETERMINE EXPECTED TRANSFORMATION EFFICIENCY

5.1. Overview

Perform a pilot transformation to determine the expected transformation efficiency. This allows one to calculate how much to scale up the transformation in order to obtain the desired number of transformants.

5.2. Duration

3–4 days

1.1 Transform cells from a single YBZ-1 yeast colony carrying the RNA plasmid with the activation domain library using the LiAc method (Gietz and Woods, 2002, see Chemical Transformation of Yeast).
Select transformants by plating on SD–Leu–Ura medium. Incubate the plates at 30 °C for 3–4 days.

1.2 Count the number of colonies from a single transformation (~10^8 cells) to determine transformation efficiency (number of transformants/µg DNA/10^8 cells).

1.3 Calculate the number of transformants desired to obtain sufficient coverage of the library. For example, to be 95% confident that a library with 10^6 clones is entirely screened, one must search through 3 × 10^6 transformants as determined by the following equation:

\[ N = \ln(1 - p) / \ln (1 - (1/u)) , \]

where \( N \), transformants; \( p \), probability (% confidence); \( u \), library size.
1.4 Based on the transformation efficiency from Step 1.2, determine the scale of transformation required to obtain the desired number of transformants in Step 1.3.

5.3. Tip
Steps 1 and 2 (pilot transformation and determination of 3-AT concentration to use) can be carried out simultaneously.

5.4. Tip
Yeast can be transformed with both plasmids simultaneously or sequentially. Usually, transformation of the plasmids sequentially yields more transformants. However, transformation of a single plasmid is occasionally toxic to the yeast, requiring a cotransformation of both plasmids.

5.5. Tip
For starters, transform with 0.1–1.0-μg library plasmid DNA. The amount of DNA has to be optimized to obtain a high transformation efficiency.

5.6. Tip
For a detailed yeast transformation protocol, refer to the Gietz Lab webpage (http://home.cc.umanitoba.ca/~gietz/index.html).

See Fig. 11.4 for the flowchart of Step 1.

**Step 1: Pilot transformation to determine expected transformation efficiency**

1.1 Transform cells from a single YBZ-1 yeast colony carrying the hybrid RNA plasmid with the cDNA-AD library using the LiAc method
Plate cells on SD-Leu-Ura plates
Incubate at 30°C, 3-4 days

1.2 Count the number of colonies from a single transformation (~1x10^8 cells)
Determine transformation efficiency: # transformants/μg DNA/10^8 cells

1.3 Calculate the number of transformants needed to give the desired coverage of the library (e.g., to be 95% confident of completely screening a library of 10^6 clones, you must search through 3 x 10^6 transformants)

1.4 Based on the transformation efficiency, determine the scale of the transformation needed to obtain the desired number of transformants

Figure 11.4 Flowchart of Step 1.
6. **STEP 2 DETERMINE 3-AT CONCENTRATION TO BE USED IN SELECTION**

6.1. Overview

3-AT is a competitive inhibitor of the **HIS3** gene product and is typically added to select for strong interactions. In this step, one can determine how much 3-AT to include in the selection, such that a balance between suppressing background activation and permitting growth of ‘real’ positives can be achieved.

6.2. Duration

4–6 days

2.1 Prepare a series of selective medium plates that lack histidine (SD–Leu–Ura–His) and contain increasing amounts of 3-AT (e.g., 0, 0.5, 1, 2, 3, and 5 mM). Also prepare selective medium plates containing histidine (SD–Leu–Ura). The SD–Leu–Ura–His plates are used to assess the level of background activation by the RNA in the absence of a protein. The SD–Leu–Ura plates serve as a control for the transformation.

2.2 Transform cells from a single yeast colony carrying the RNA plasmid with an ‘empty’ activation domain plasmid. Plate transformants on the prepared plates from Step 2.1. Incubate at 30 °C for 3–5 days.

2.3 Determine the minimal concentration of 3-AT that resulted in no growth of yeast transformants. That would be a suitable starting concentration for the selection experiment.

6.3. Tip

3-AT is light-sensitive. Keep plates in the dark when possible.

6.4. Tip

If colonies are observed even at high concentrations of 3-AT, the RNA likely autoactivates the reporter even in the absence of an interacting protein and is not suitable for the selection experiment.

6.5. Tip

The addition of 3-AT to the medium decreases the number of false positives by demanding a more stringent interaction between the RNA and protein. However,
3-AT concentrations that are too high can result in the loss of colonies containing legitimate RNA–protein interactions. See Fig. 11.5 for the flowchart of Step 2.

7. STEP 3 INTRODUCE THE cDNA LIBRARY

7.1. Overview

The cDNA library is introduced into YBZ-1 yeast which has been transformed with the hybrid RNA plasmid. Transformants are selected on SD–Leu–His and containing a predetermined amount of 3-AT from Step 2.

7.2. Duration

7–10 days

3.1 Transform cells from a single yeast colony carrying the RNA plasmid with the activation domain library using the LiAc method (Gietz and Woods, 2002, see Chemical Transformation of Yeast). Based on the expected transformation efficiency from Step 1 and the number of transformants desired, the scale of transformation should be adjusted accordingly.

3.2 Calculate the total number of transformants. Save 50 µl of transformed cells from Step 3.1 and make serial dilutions in sterile water (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). Plate 100 µl per dilution on SD–Leu–Ura medium and incubate at 30 °C for 3–4 days. Once colonies are visible, count the number of colonies and calculate the total number of transformants. The confidence level at which the entire library is screened can be calculated using the formula in Step 1.3.
3.3 Plate the remaining transformants on SD–Leu–His medium which contains a predetermined amount of 3-AT from Step 2. Incubate at 30 °C for 7 days (or more, if necessary) until colonies start to form.

7.3. Tip

Maintenance of the RNA plasmid (i.e., selection on URA3) is not demanded. This permits cells that can activate HIS3 independent of the RNA to lose the RNA plasmid, permitting the colony color screen below.

See Fig. 11.6 for the flowchart of Step 3.

8. STEP 4 ELIMINATE RNA-INDEPENDENT FALSE POSITIVES BY COLONY COLOR

8.1. Overview

Two classes of positives are obtained from the initial transformation: RNA-dependent and RNA-independent. To facilitate elimination of the RNA-independent false positives, we exploit the ADE2 gene on the pIIIA hybrid RNA plasmid. The host strain is an ade2 mutant. When the level of adenine in the medium is low, cells attempt to synthesize adenine de novo and accumulate a red purine metabolite due to lack of the ADE2-encoded enzyme. This accumulation renders the cells pink or red in color. In contrast, cells carrying the wild-type ADE2 gene are white.

8.2. Duration

1–2 days

4.1 RNA-independent positives do not require the RNA plasmid to activate the HIS3 gene and so can lose the plasmid. These false positives will yield pink colonies or white colonies with pink sectors. These colonies should be discarded.
4.2 For RNA-dependent positives, selection for HIS3 indirectly selects for maintenance of the RNA plasmid, which carries the ADE2 gene; thus, these transformants are white. Pick the white colonies (typically a few large and many small colonies) and patch onto SD–Leu–Ura plates to select for the cDNA and RNA plasmids. 

4.3 White colonies that are able to grow are selected for further analysis in Step 5.

8.3. Tip

*Incubation of the initial transformation plates at 30 °C usually allows enough time for the color to develop. If color development is not strong after a week, incubation of the plates at 4 °C overnight can help.*

8.4. Tip

*Most of the small white colonies will turn out to be RNA-independent false positives and fail to grow.*

See Fig. 11.7 for the flowchart of Step 4.

9. STEP 5 ASSAY β-GALACTOSIDASE ACTIVITY

9.1. Overview

As a secondary selection, the level of β-galactosidase activity is determined by performing a qualitative β-galactosidase activity.
9.2. Duration

1–2 days

5.1 Place a nitrocellulose filter on a plate containing selective medium (SD–Leu–Ura) and patch transformants directly on the filter. Incubate cells at 30 °C for 1–2 days.

5.2 Lyse cells by immersing the filter in liquid nitrogen for 5–20 s and thawing on the benchtop for 1 min.

5.3 Repeat freeze/thaw cycle twice.

5.4 Place a piece of Whatman 3-MM chromatography paper in a Petri dish and saturate it with 5-ml Z-buffer. Add 75 μl of 20 mg ml⁻¹ X-Gal solution. Discard excess buffer in Petri dish.

5.5 Overlay the nitrocellulose filter containing cells onto the saturated Whatman paper, cells side up. Seal dish with parafilm.

5.6 Incubate, colony side up, 30 min to overnight at 30 °C. Examine the filters regularly. A strong interaction (such as that between IRE and IRP) should turn blue within 30 °min.

9.3. Tip

Typically, 50 positives can be patched onto a 100-mm diameter Petri dish.

9.4. Caution

After freezing and thawing, the nitrocellulose filter is brittle and may crack easily.

9.5. Tip

X-Gal should be added fresh. Keep stock solution in the dark as it is light-sensitive.

9.6. Tip

Ensure good contact between the nitrocellulose filter and the Whatman paper.

9.7. Tip

With protracted incubation, weak interactions eventually yield a blue color. For this reason, it is important to examine the filters periodically to determine how long it takes for the color to develop. It is also helpful to include a positive control (e.g., IRE–IRP interaction) to ensure that the assay works.

See Fig. 11.8 for the flowchart of Step 5.
**10. STEP 6 CURE THE RNA PLASMID AND TEST POSITIVES FOR RNA DEPENDENCE**

### 10.1. Overview

Most, but not all, of the RNA-independent false positives are eliminated by the colony color assay in Step 2. To ensure that the positives are RNA-dependent, the RNA plasmid is removed by counterselection against \textit{URA3}. Cells will be plated on media containing 5-fluoroorotic acid (5-FOA). 5-FOA is converted by the \textit{URA3} gene product to 5-fluorouracil, which is toxic. Cells lacking \textit{URA3} can grow in the presence of 5-FOA if uracil is provided, while cells containing \textit{URA3} cannot. Expression of \textit{lacZ} is then monitored. Candidates that fail to activate the reporter genes are further analyzed.

### 10.2. Duration

4–5 days

1. **6.1** Replica-plate the positives from Step 4 to SD–Leu plates. Incubate the plates at 30 °C for 1 day, allowing the cells to lose the RNA plasmid.

2. **6.2** Replica-plate the cells from Step 6.1 onto SD–Leu + 0.1% 5-FOA plates. Incubate the plates at 30 °C for 2–3 days.
6.3 Cells that grow can be streaked on SD–Ura plates to confirm the loss of the RNA plasmid. A single pass through 5-FOA counterselection is usually sufficient.
6.4 Assay β-galactosidase activity as in Step 5.
6.5 Select the candidates that fail to activate lacZ for further analysis. Discard false positives which activate lacZ (blue color) in the absence of the RNA plasmid.

See Fig. 11.9 for the flowchart of Step 6.

11. STEP 7 ISOLATE PLASMIDS FOR AUTOACTIVATION TEST AND SEQUENCING

11.1. Overview

Once candidates are identified, the cDNA plasmid must be recovered from yeast and introduced into E. coli (see Transformation of Chemically Competent E. coli or Transformation of E. coli via electroporation). The plasmids can then be amplified in E. coli for use in future applications. cDNA plasmids must be transformed back into a yeast strain containing an ‘empty’ RNA plasmid to test for RNA-independent activation (see Chemical Transformation of Yeast). Expression of either the HIS3 or lacZ reporter gene is monitored.
11.2. Duration

3–5 days

7.1 Inoculate each positive clone (from patched positives in Step 6.2) in 5-ml SD–Leu medium and grow to saturation (24–48 h) at 30 °C in a rotary shaker (200 rpm).

7.2 Pellet 2-ml cells at 5000 × g. Resuspend the pellet in 250 μl of Cell Resuspension Buffer.

7.3 Spheroplast yeast with 5-μl Zymolyase for 2 h at 37 °C.

7.4 Add 250-μl lysis buffer and invert to mix.

7.5 Incubate for 5 min at room temperature, followed by 5 min at 65 °C. Cool to room temperature.

7.6 Add 10 μl of alkaline protease solution and incubate for 10 min at room temperature.

7.7 Add 350 μl of lysis buffer and invert to mix.

7.8 Centrifuge at >16 000 × g for 10 min.

7.9 Add lysate to the binding column. Centrifuge at >16 000 × g for 1 min.

7.10 Wash the column with 700-μl wash buffer. Centrifuge at 16 000 × g for 1 min. Discard the flow-through.

7.11 Wash the column with 500-μl wash buffer. Centrifuge at >16 000 × g for 1 min. Discard the flow-through.

7.12 Centrifuge at >16 000 × g for 2 min to remove the residual buffer.

7.13 Elute the plasmid DNA in 100-μl water.

7.14 Transform the plasmid DNA into E. coli using conventional methods. Plate transformants on medium containing the appropriate antibiotic to select for the cDNA plasmid.

7.15 Perform colony PCR to identify clones carrying the cDNA plasmid (see Colony PCR). Isolate plasmids from E. coli using conventional methods.

7.16 Transform each cDNA plasmid back into a yeast strain containing an ‘empty’ RNA plasmid.

7.17 Assay transformants for the expression of the lacZ reporter, as described in Step 5.

7.18 Discard false positives that activate lacZ in the absence of your specific RNA sequence. Identify the remaining cDNAs by sequencing the plasmids isolated from E. coli.

11.3. Tip

This step is a modified version of the Promega Wizard Plus SV Miniprep kit.
11.4. Tip
Since either RNA or cDNA plasmids can be present in E. coli, colonies can be screened for the plasmid of interest by colony PCR. If different antibiotic resistance markers are present on each plasmid, most colonies should contain the cDNA plasmid.

11.5. Tip
Alternatively, HIS3 expression can be monitored as described in Dissecting a known RNA-protein interaction using a yeast three-hybrid system.

11.6. Tip
Compare the cDNA sequences to common sequence databases to identify protein partners (e.g., BLAST).
See Fig. 11.10 for the flowchart of Step 7.

12. STEP 8 DETERMINE BINDING SPECIFICITY USING MUTANT AND CONTROL RNAs

12.1. Overview
Clones that require the RNA plasmid to activate the reporter genes can then be analyzed to determine whether they interact sequence specifically with the RNA.

12.2. Duration
3–4 days

8.1 Introduce small, directed (point) mutations in the hybrid RNA (e.g., mutating a nucleotide of known importance in the RNA) that disrupt the interaction between the RNA and the protein (see Site-Directed Mutagenesis).

8.2 Transform the candidate cDNA plasmids into a yeast strain containing either the wild-type or mutant hybrid RNA plasmids.

8.3 Assay transformants for the expression of the lacZ reporter qualitatively, as described in Step 5, to determine the sequence specificity of the positive clones.

12.3. Tip
In this case, it may be helpful to know the identity of the candidates from the screen and use this information to guide mutagenesis studies.
Step 7: Isolate plasmids for autoactivation test and sequencing

7.1 Inoculate positive clones (from patched positives in step 6.2) in 5 ml SD-Leu medium Grow at 30°C, with shaking (200 rpm) for 24-48 h

7.2 Pellet 2 ml of cells at 5000 x g Resuspend pellet in 250 μl Cell Resuspension Buffer

7.3 Add 5 μl zymolase Incubate at 37°C, 2 h to make spheroplasts

7.4 Add 250 μl Lysis Buffer Invert to mix

7.5 Incubate 5 min at room temperature Incubate 5 min at 65°C Cool to room temperature

7.6 Add 10 μl of alkaline protease solution Incubate 10 min at room temperature

7.7 Add 350 μl Lysis Buffer Invert to mix

7.8 Centrifuge at >16,000 x g, 10 min

7.9 Add lysate to binding column Centrifuge at >16,000 x g, 1 min

7.10 Wash column with 700 μl Wash Buffer Centrifuge at >16,000 x g, 1 min Discard flow through

7.11 Wash column with 500 μl Wash Buffer Centrifuge at >16,000 x g, 1 min Discard flow through

7.12 Centrifuge at >16,000 x g, 2 min

7.13 Elute plasmid DNA in 100 μl water

7.14 Transform plasmid DNA into competent E.coli Plate cells on agar plates containing the proper antibiotic to select for the cDNA-AD plasmid

7.15 Identify clones carrying the cDNA-AD plasmid by colony PCR isolate plasmid DNA using a miniprep kit

7.16 Transform each cDNA-AD plasmid back into a yeast strain containing an "empty" hybrid RNA plasmid

7.17 Assay transformants for the expression of lacZ (Step5)

7.18 Discard false positives that activate lacZ in the absence of the specific RNA sequence Sequence the remaining positive clones (plasmid DNA isolated from E. coli) to identify the cDNA

Figure 11.10 Flowchart of Step 7.
12.4. Tip
If no subtle mutations are available, rudimentary analyses such as using deletions or antisense RNA can be helpful.

12.5. Tip
If a quantitative comparison between protein and various RNAs is desired, a quantitative β-galactosidase assay is provided in Dissecting a known RNA-protein interaction using a yeast three-hybrid system. See Fig. 11.11 for the flowchart of Step 8.

13. STEP 9 FUNCTIONAL TESTS OR ADDITIONAL SCREENS

13.1. Overview
Almost invariably, additional steps will be needed to identify those positives that are biologically meaningful. Each screen is unique. The interactions being analyzed and the organisms being studied will determine what additional steps need to be taken to determine the biological relevance of each interaction. It is not surprising, given that the assay is performed outside of most biological contexts, that some specific, high-affinity interactions may not be relevant to the biology of the system being studied.

REFERENCES

Referenced Literature


**SOURCE REFERENCES**


**Referenced Protocols in Methods Navigator**

Chemical Transformation of Yeast.
*Saccharomyces cerevisiae* Growth Media.
Transformation of Chemically Competent *E. coli*.
Transformation of *E. coli* via electroporation.
Colony PCR.
Dissecting a known RNA-protein interaction using a yeast three-hybrid system.
Site-Directed Mutagenesis.