

# Dissecting a Known RNA–Protein Interaction using a Yeast Three-Hybrid System

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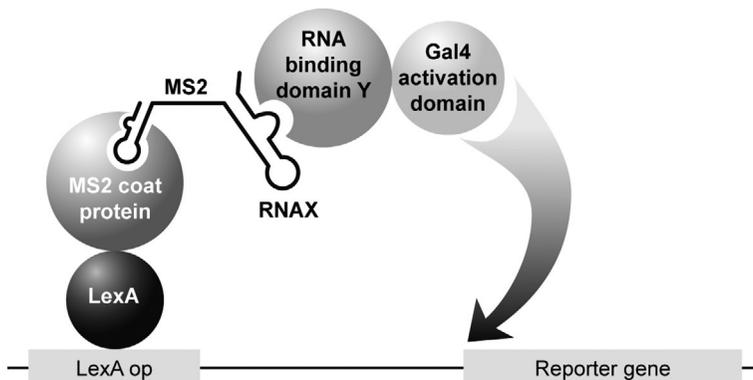
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## Abstract

The yeast three-hybrid system has been applied to known protein–RNA interactions for a variety of purposes. For instance, protein and RNA mutants with altered or relaxed binding specificities can be identified. Mutant RNAs can also be analyzed to better understand RNA-binding specificity of a specific protein. Furthermore, this system complements other biochemical techniques, for example, SELEX, co-immunoprecipitation and cross-linking experiments (see [UV crosslinking of interacting RNA and protein in cultured cells](#) and [PAR-CLIP \(Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation\): a step-by-step protocol to the transcriptome-wide identification of binding sites of RNA-binding proteins](#)).

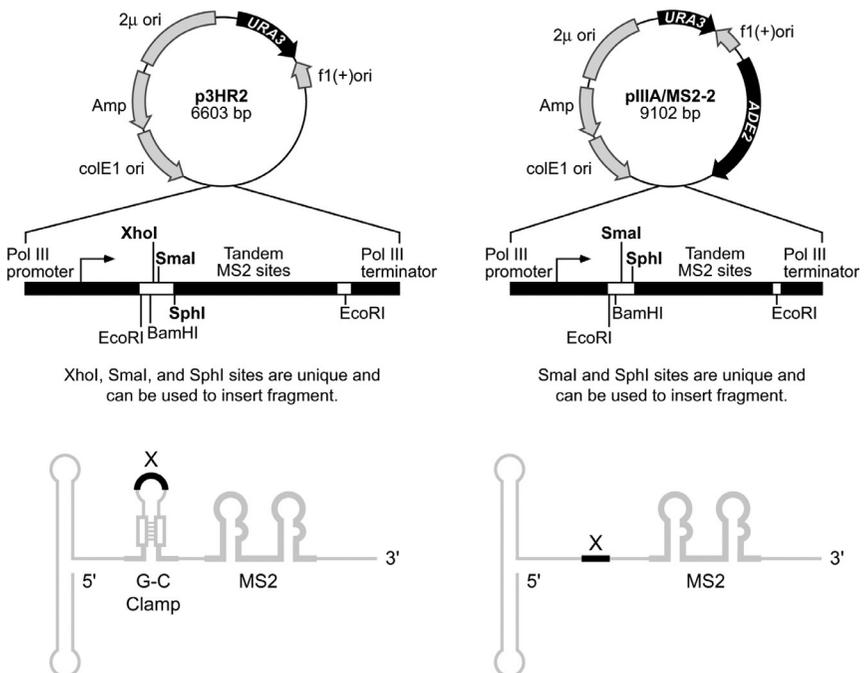
## 1. THEORY

The general strategy of the three-hybrid system is shown in [Fig. 10.1](#). DNA-binding sites are placed upstream of a reporter gene, which has been



**Figure 10.1** Three-hybrid system used to detect and analyze RNA–protein interactions. The diagram depicts the general strategy of the three-hybrid system. Specific protein and RNA components that are typically used are represented. For the sake of simplicity, the following points are not illustrated. In the YBZ-1 strain, both *lacZ* and *HIS3* reporter genes are present under the control of *lexA* operators (eight in the *lacZ* promoter and four in the *HIS3* promoter). The LexA protein binds as a dimer. The hybrid RNA contains two MS2 stem-loops and the MS2 coat protein binds one stem-loop as a dimer.

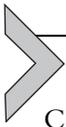
integrated into the yeast genome. The first hybrid protein consists of a DNA-binding domain linked to an RNA-binding domain. The RNA-binding domain interacts with its RNA-binding site in a bifunctional ('hybrid') RNA molecule. The other part of the RNA molecule interacts with a second hybrid protein 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. 10.1. 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. 10.2) consists of two MS2 coat protein-binding



**Figure 10.2** Plasmids for the expression of hybrid RNAs. p3HR2 displays the RNA sequence of interest in the loop of a G-C clamp (Stumpf et al., 2008), whereas pIIIA/MS2-2 does not (Zhang et al., 1997). The sequence of interest can be inserted into the unique restriction sites.

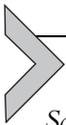
sites linked to the RNA sequence of interest, X. The second hybrid protein consists of the transcription activation domain of the yeast Gal4 transcription factor linked to an RNA-binding protein, Y.

Here, we consider a case in which RNA X and protein Y are known, and their interaction is assayed *in vivo*. This protocol is intended to analyze mutant RNAs and proteins. The output of the system – *HIS3* and *lacZ* activation – has been examined as a function of *in vitro* affinity using a specific RNA–protein interaction (Hook et al., 2005). When the protein FBF-1 was tested with various RNAs,  $K_d$  was linearly related to the log of  $\beta$ -galactosidase activity over the range measured, from 1 to 100 nM.



## 2. EQUIPMENT

Centrifuge  
Spectrophotometer  
Luminometer (e.g., Turner 20/20n Single Tube Luminometer, Promega)  
30 °C incubator-shaker  
Vortex mixer  
Micropipettors  
Micropipettor tips  
1-ml cuvettes  
Luminometer tubes  
Glass tubes (18 × 150 mm) with caps  
Petri dishes (100 mm)  
Nitrocellulose filter (Petri dish-sized)  
Whatman 3MM chromatography paper (Petri dish-sized)  
Parafilm  
Sterile toothpicks  
0.2- $\mu$ m filters  
1.5-ml microcentrifuge tubes



## 3. MATERIALS

*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., pIII/MS2-2 or p3HR2)  
Activation domain fusion plasmid (e.g., pACTII or pGADT7)

Synthetic dextrose (SD) dropout media  
 Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ )  
 Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )  
 Potassium chloride (KCl)  
 Magnesium sulfate ( $\text{MgSO}_4$ )  
 2-Mercaptoethanol  
 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal)  
 Dimethylformamide  
 3-Aminotriazole  
 Beta-Glo<sup>®</sup> reagent (Promega)  
 Liquid nitrogen

### 3.1. Solutions & buffers

#### Step 1A Z-buffer

Component	Final concentration	Stock	Amount
$\text{Na}_2\text{HPO}_4$	60 mM	1 M	60 ml
$\text{NaH}_2\text{PO}_4$	40 mM	1 M	40 ml
KCl	10 mM	1 M	10 ml
$\text{MgSO}_4$	1 mM	1 M	1 ml
2-Mercaptoethanol	50 mM	14.2 M	Add fresh

Add to 850 ml water. Adjust the pH to 7.0, if necessary. Add water to 1 l and filter sterilize.

#### X-Gal solution ( $20 \text{ mg ml}^{-1}$ )

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

#### Step 1C 3-Aminotriazole (1 M)

Dissolve 4.21 g 3-AT in 50 ml water. Pass through a  $0.2\text{-}\mu\text{m}$  filter to sterilize. Aliquot into tubes, wrap in foil, and store at  $-50^\circ\text{C}$ .



## 4. PROTOCOL

### 4.1. Duration

Preparation	About 5–9 days
Protocol	2–5 days

## 4.2. Preparation

The protein(s) of interest and desired RNA sequence(s) to be tested should be cloned into an activation domain fusion vector and hybrid RNA vector respectively (as a reference see Molecular Cloning). Here, we consider a case in which the activation domain vector carries a LEU2 marker and the RNA vector carries the URA3 marker.

Introduce both protein and RNA plasmids into YBZ-1 yeast using standard transformation protocols (Gietz and Woods, 2002, see also Chemical Transformation of Yeast). Transformants should be plated on SD–Leu–Ura medium (see *Saccharomyces cerevisiae* Growth Media).

## 4.3. Tip

*Yeast can be transformed with both plasmids simultaneously or the fusion protein plasmid can be transformed first, followed by the RNA plasmid. Usually, transforming the plasmids sequentially yields more transformants. However, the fusion protein plasmid is occasionally toxic to the yeast, requiring a cotransformation of the plasmids.*

## 4.4. Tip

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



## 5. STEP 1A ASSAYING INTERACTIONS: QUALITATIVE FILTER ASSAY FOR $\beta$ -GALACTOSIDASE ACTIVITY

### 5.1. Overview

The ‘strength’ of an RNA–protein interaction is gauged by assaying the activity of a reporter gene, in this case, *lacZ*.  $\beta$ -galactosidase activity can be assayed by measuring the conversion of a lactose analog to a chromogenic or luminescent product. This is a qualitative assay for  $\beta$ -galactosidase activity, carried out on yeast grown on a nitrocellulose filter.

### 5.2. Duration

2 days

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

**1A.2** Lyse cells by immersing the filter in liquid nitrogen for 5–20 s and thawing on the benchtop for 1 min.

- 1A.3** Repeat the freeze/thaw cycle twice.
- 1A.4** Place Whatman 3MM chromatography paper in a Petri dish and saturate it with 5 ml Z-buffer. Add 75  $\mu\text{l}$  of 20  $\text{mg ml}^{-1}$  X-Gal solution. Remove excess buffer in the Petri dish.
- 1A.5** Place the nitrocellulose filter containing the cells onto the saturated Whatman paper, with the cells side up. Seal the dish with Parafilm.
- 1A.6** Incubate the filter, with the colony side up, for 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.

### 5.3. Tip

*Pick multiple average-sized colonies for each inoculation. While this is not ideal microbiological practice, it has worked reproducibly.*

### 5.4. Caution

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

### 5.5. Tip

*X-Gal should be added to the Z-buffer fresh.*

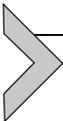
### 5.6. Tip

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

### 5.7. 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 and IRP interaction) to ensure that the assay works.*

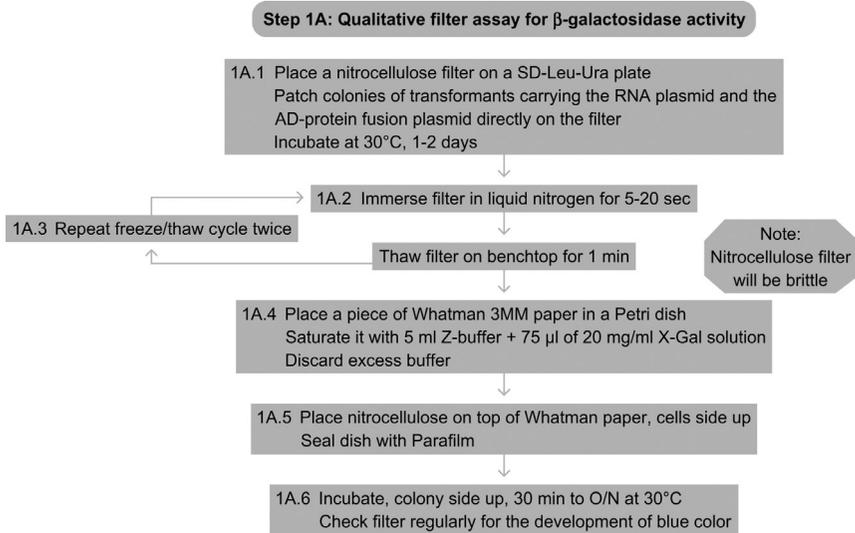
See [Fig. 10.3](#) for the flowchart of Step 1A.



## **6. STEP 1B ASSAYING INTERACTIONS: QUANTITATIVE SOLUTION ASSAY FOR $\beta$ -GALACTOSIDASE ACTIVITY**

### **6.1. Overview**

The ‘strength’ of an RNA–protein interaction is gauged by assaying the activity of a reporter gene, in this case, *lacZ*.  $\beta$ -galactosidase activity can be assayed by measuring the conversion of a lactose analog to a chromogenic or luminescent product. This is a quantitative liquid  $\beta$ -galactosidase assay



**Figure 10.3** Flowchart of Step 1A.

using the enzyme-coupled luminescent substrate, Beta-Glo<sup>®</sup>. This simple yet sensitive assay uses a luminometer to measure the output from the *lacZ* gene.

## 6.2. Duration

2 days

- 1B.1** Inoculate a 4 ml culture for each strain in selective media (SD–Leu–Ura) in triplicate. Grow the cultures to saturation on a rotary shaker (200 rpm) at 30 °C (1.5–2 days).
- 1B.2** Dilute the cultures 1:40 (100 µl in 4 ml fresh media) and let them grow for 2–2.5 h. Check 1 ml of the culture to ensure an OD<sub>660</sub> of 0.1–0.2.
- 1B.3** Thaw the Beta-Glo<sup>®</sup> reagent and let it warm to room temperature.
- 1B.4** Dispense 50 µl aliquots of Beta-Glo<sup>®</sup> reagent into enough microcentrifuge tubes for the assay. Add 50 µl of exponentially growing cells to the tubes. Vortex each tube for 10 s to ensure complete lysis.
- 1B.5** Incubate for 1 h at room temperature in the dark.
- 1B.6** Transfer 10 µl of the lysate to an appropriate luminometer tube.
- 1B.7** Integrate luminiscent signal for 1 s.
- 1B.8** Normalize signal to cell number (or 0.1 OD<sub>660</sub>) to yield an activity/cell (or activity/0.1 OD<sub>660</sub>) value.

### 6.3. Tip

*Pick multiple average-sized colonies for each inoculation. While this is not ideal microbiological practice, it has worked reproducibly.*

### 6.4. Tip

*Yeast cells tend to settle quickly. Vortex the cultures to ensure that they are mixed well before reading OD<sub>660</sub>.*

### 6.5. Caution

*The Beta-Glo<sup>®</sup> reagent is light sensitive. Thaw it in the dark.*

### 6.6. Tip

*Luminescence is stable from 30 min up to 4 h after adding the reagent.*

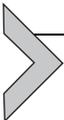
### 6.7. Tip

*Numerous variations on the liquid  $\beta$ -galactosidase assay exist. The protocol described here uses the enzyme-coupled luminescent substrate Beta-Glo<sup>®</sup> (Promega Corporation). This simple yet sensitive assay uses a luminometer to measure the output from the lacZ gene. The protocol described here uses a Turner 20/20n luminometer (Promega Corporation). Certain details, such as sample volumes, will vary depending on the instrument used.*

### 6.8. Tip

*This protocol can be modified to read a large number of assays by using a microplate luminometer.*

See [Fig. 10.4](#) for the flowchart of Step 1B.



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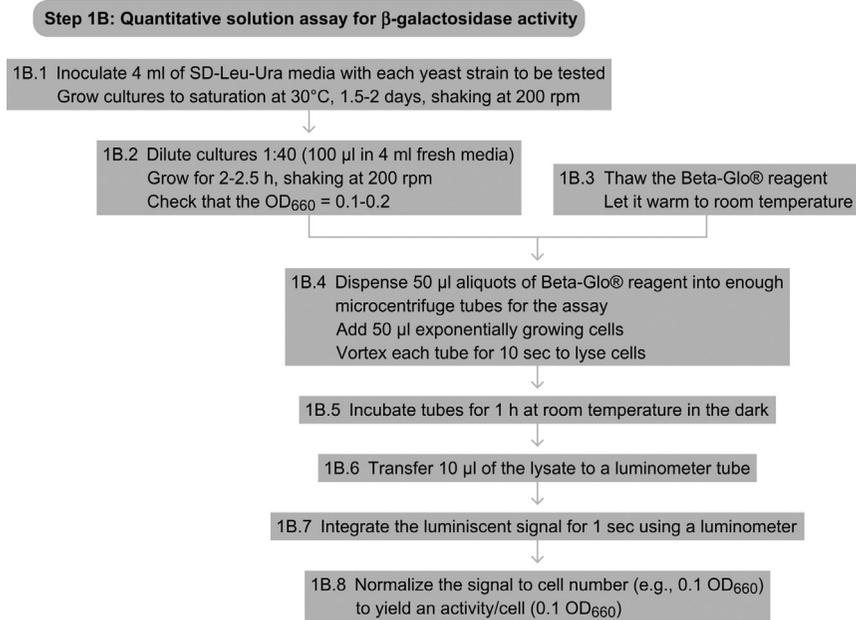
## **7. STEP 1C ASSAYING INTERACTIONS: 3-AMINOTRIAZOLE (3-AT) RESISTANCE ASSAY**

### **7.1. Overview**

The ‘strength’ of an RNA–protein interaction is gauged by assaying the activity of a reporter gene, in this case, *HIS3*. This step determines the level of resistance to 3-aminotriazole (3-AT), which monitors *HIS3* activity.

### **7.2. Duration**

3–5 days



**Figure 10.4** Flowchart of Step 1B.

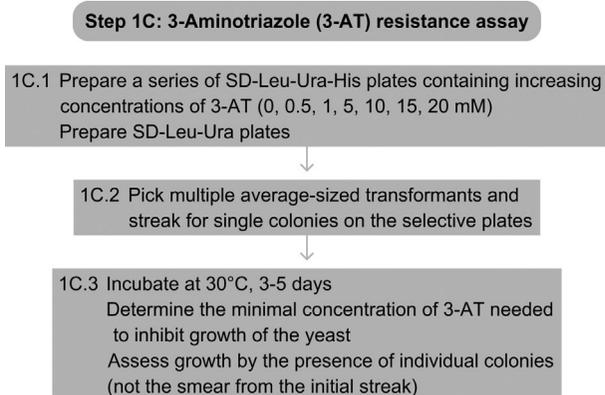
- 1C.1** Prepare a series of selective medium plates that lack histidine (SD–Leu–Ura–His) and contain increasing amounts of 3-AT (e.g., 0, 0.5, 1, 5, 10, 15, 20 mM). Also prepare selective medium plates containing histidine (SD–Leu–Ura) to serve as a control for the transformation.
- 1C.2** Pick multiple average-sized transformants and streak for single colonies on prepared plates.
- 1C.3** Incubate at 30 °C for 3–5 days. Determine the minimal concentration of 3-AT needed to inhibit growth of the yeast. Growth should be assessed by the presence of individual colonies and not the smear of yeast from the initial streak.

### 7.3. Tip

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

### 7.4. Tip

*While this is not ideal microbiological practice, it has worked reproducibly.*



**Figure 10.5** Flowchart of Step 1C.

## 7.5. Caution

*It is common to observe a few large colonies on high concentrations of 3-AT.*

See [Fig. 10.5](#) for the flowchart of Step 1C.

## REFERENCES

### Referenced Literature

- Gietz, R. D., & Woods, R. A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods in Enzymology*, 350, 87–96.
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- Bernstein, D. S., Buter, N., Stumpf, C., & Wickens, M. (2002). Analyzing mRNA–protein complexes using a yeast three-hybrid system. *Methods*, 26, 123–141.
- Stumpf, C. R., Opperman, L., & Wickens, M. (2008). Chapter 14. Analysis of RNA–protein interactions using a yeast three-hybrid system. *Methods in Enzymology*, 449, 295–315.

## Referenced Protocols in Methods Navigator

- UV crosslinking of interacting RNA and protein in cultured cells.
- PAR–CLIP (Photoactivatable Ribonucleoside–Enhanced Crosslinking and Immunoprecipitation): a step-by-step protocol to the transcriptome-wide identification of binding sites of RNA-binding proteins.
- Molecular Cloning.
- Chemical Transformation of Yeast.
- Saccharomyces cerevisiae* Growth Media.