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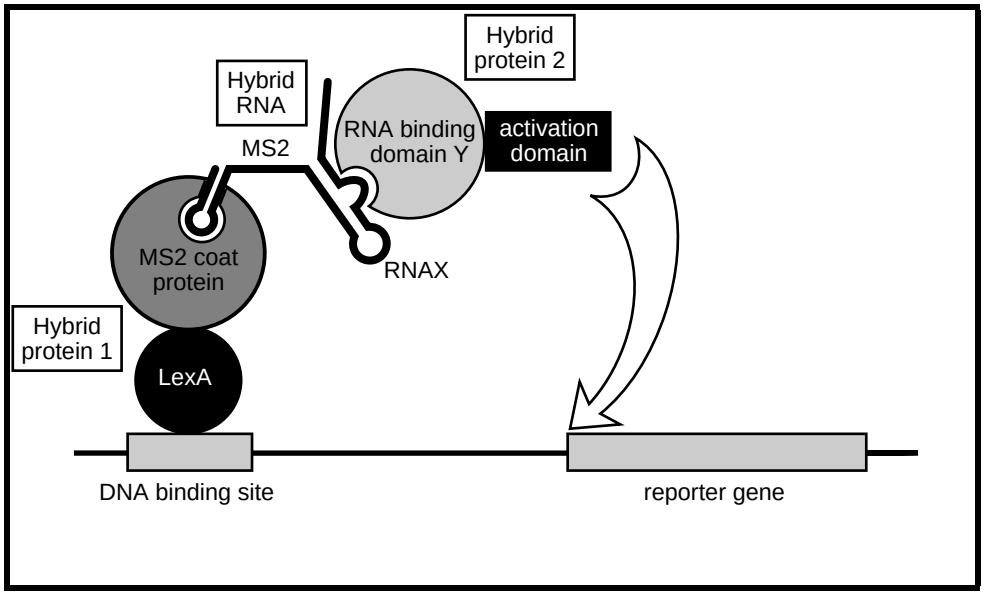
Dear Colleague,

Thanks for your interest in the Three-Hybrid System to detect RNA-protein interactions. The system was developed through a collaboration between Stan Fields' laboratory (University of Washington-Seattle) and my own. We are enclosing plasmids and strains that should enable you to get started using the system. Not all of the plasmids are yet designed optimally (i.e., few cloning sites, etc), but we thought it best to send this out to you now. *We ask you not to pass these reagents on to others, and that the reagents not be used for commercial purposes.*

If you have questions about the system, Dhruba Sengupta in Stan Fields' lab (206-616-4523; e-mail sengupta@genetics.washington.edu).

The arrangement of hybrid components is as drawn in the figure below. The LexA DNA binding domain is fused to the MS2 coat protein to form Hybrid Protein 1. Hybrid Protein 2 consists of the Gal4 activation domain linked to the RNA binding domain, Y, you wish to test. The Hybrid RNA consists of two MS2 RNA binding sites and the RNA sequence you wish to test, RNAX. Hybrid Protein 1 and the presence of MS2 sites in the Hybrid RNA are fixed, as is the Gal4 Activation Domain. RNAX and RNA Binding Domain Y vary.



We have now demonstrated that seven different known RNA-protein interactions can be detected using the method. The Kd's of these interactions range from approx 0.1 nM to 10 nM. We have not determined the relationship between Kd of the RNA-protein interaction and extent of transcriptional stimulation.

We and others have screened cDNA libraries to identify novel RNA binding proteins. In cDNA library screening, the large majority of false positives can be RNA-independent (probably due to protein-protein intereactions with the LexA-MS2 coat protein). We have developed a colony color

assay that helps to eliminate this class of false positives. The yeast strain, L40-coat is *ade2* mutant, so it normally turns pink after several days of growth. Since the RNA plasmids carry the wild type *ADE2* gene, yeast cells containing these plasmids remain white. This colony color difference enables us to distinguish RNA-dependent and RNA-independent positives. (see Section 4).

1. DESCRIPTION OF ENCLOSURES

We enclose the following. For each plasmid DNA, we are sending an aliquat of a maxiprep, and suggest you transform *E. coli* to get started. The yeast strain is sent as a slant.

- **Yeast strain YBZ1**. A derivative of *L40-coat* carrying a tandem, head-to-tail dimer of MS2 coat protein fused to a single LexA monomer. A nine-amino-acid linker (Gly-Ala-Pro-Gly-Ile-His-Pro-Gly-Met) was introduced to enable flexibility in orienting the two MS2 segments. In addition, each coat protein segment also carries a point mutation (N55K) that decreases the Kd of the RNA-protein interaction. The genotype of YBZ1 is *MATa, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2 :: (LexAop)-HIS3, ura3 :: (lexA-op)-lacZ, LexA-MS2 coat (N55K)*. Hook, B., D. Bernstein, et al. (2005). "RNA-protein interactions in the yeast three-hybrid system: affinity, sensitivity, and enhanced library screening." RNA **11**(2): 227-33.

- **pMS2-1 and pMS2-2**. These two plasmids can be used to facilitate making the Hybrid RNA construct (see below). **Both are TET^R vectors** (the AMP^R gene has been inactivated). DNA encoding the RNA sequence of interest can be cloned into these plasmids, then moved into pIIIEx426RPR (see below). pMS2-1 and pMS2-2 are based on BluescriptIIKS+ and do not contain any yeast genes or promoters. They simply allow you to place the RNA sequence of interest next to two MS2 coat protein recognition sites, then excise the relevant DNA fragment to be moved into pIIIEx426RPR. pMS2-1 and pMS2-2 differ only in the relative positions of the MS2 sites and your sequence of interest (see maps below).

- **pIII/MS2-1 and pIII/MS2-2**. These plasmids are yeast shuttle vectors, based on pIIIEx426RPR, for expressing an RNA containing your sequence of interest adjacent to two MS2 sites from an RNA polymerase III promoter (see below). They carry a *URA3* marker. An RNA sequence of interest can be cloned directly into the unique *SmaI* site of these plasmids, and then can be used in transformation.

- **pIIIA/MS2-1 and pIIIA/MS2-2**. These plasmids are similar to pIII/MS2-1 and pIII/MS2-2 but carry the *ADE2* gene in addition to *URA3* (see map). We now use these plasmids in cDNA library screening to help eliminate RNA-independent positives.

- **pACTII**. This plasmid (constructed by Steve Elledge's laboratory) can be used to create a hybrid protein in which a polypeptide of interest is linked to the *GAL4* Activation Domain. pACTII carries the *GAL4* Activation Domain followed by an HA epitope tag and a polylinker for cloning the gene of interst (see enclosed map for restriction sites). The HA epitope permits immunodetection of the hybrid protein. The hybrid protein is expressed from the *ADH* promoter. This multicopy plasmid carries a *LEU2* marker.

- **Positive controls: pIIIA/IRE-MS2 and pAD-IRP**. Plasmid pIIIA/IRE-MS2 expresses 5' IRE-MS2 3' Hybrid RNA from the yeast RNAseP promoter, and is on a multi-copy *URA3* plasmid. Plasmid pAD-IRP expresses the rabbit Iron Regulatory Protein fused to the Gal 4 Activation Domain. The plasmid is multi-copy and carries a *LEU2* marker. The parent vector for this plasmid is pACTII, constructed by Steve Elledge. pIIIA/IRE-MS2 and pAD-IRP, introduced into strain *L40-coat*, cause transcription activation of *HIS3* and *LacZ*. If you want to assay for *HIS3* transcriptional activation, include 3-aminotriazole to eliminate low levels of basal expression. For this combination of plasmids (pIIIA/IRE-MS2 and pAD-IRP), plate our transformants directly onto 5 mM 3-aminotriazole (see "Selecting for activation of *HIS3*" below).

All of the plasmids above are multi-copy.

The Three-Hybrid System is arranged so that Activation Domain libraries present in *LEU2*-containing vectors that already exist for Two-Hybrid Screens can be used directly in Three-Hybrid Screens.

The MS2 portion of the Hybrid RNAs contain two tandem MS2 sites. Both contain a mutation that increases the affinity of the interaction with MS2 coat protein. The sequence of the two sites is given in Bardwell and Wickens (1990) *Nucleic Acids Research* **18**, 6587-6594, and is contained in the "Hybrid RNA Sequence" page below.

Remember that pMS2-1 and pMS2-2 confer tetracycline, and not ampicillin, resistance.

2. PREPARING THE RNA PLASMID

Two routes of plasmid construction. We will term the DNA encoding your RNA sequence of interest, RNAX. RNAX can be cloned into the appropriate vector by either of two routes.

> Method 1. RNAX can be cloned into the unique *SmaI* site of pIIIA/MS2-1 or pIIIA/MS2-2. This will result in a 5' MS2-RNAX 3' (pIII/MS2-1) or 5' RNAX-MS2 3' (with pIII/MS2-2) Hybrid RNA, expressed from an RNA polymerase III promoter, in a high copy *URA3* vector.
>
> Method 2. RNAX can be cloned into the unique *SmaI* site of pMS2-1 or pMS2-2 (see the maps below). Fragments containing either 5' MS2-RNAX 3' or 5' RNAX-MS2 3' can then be moved into pIIIA/MS2-1.

Use of Method 1 saves one cloning step. However, it may be easier to determine the orientation and sequence of the Hybrid RNA in the pMS2 plasmids: the pMS2 plasmids have more restriction sites near the inserted RNA sequence, making it easier to determine the orientation of inserts (particularly with short inserts). In addition, inserts in the pMS2-1 and pMS2-2 plasmids can be sequenced using conventional T7 and T3 primers.

Deciding on order of sites. To decide which polarity (5' RNAX-MS2 3' *vs* 5' MS2-RNAX 3') is optimal, we recommend using an RNA folding program to see whether one arrangement is more likely to succeed than the other. In the IRE/IRP interaction, placing the MS2 sites after (3') of the IRE results in 2- to 3- fold more transcription than when the MS2 sites are first (5'). We do not know whether this is a general feature of Hybrid RNAs or a peculiarity of the IRE-containing RNAs. But in the absence of any other considerations, we would recommend making an RNAX-MS2 fusion.

The existence of possible RNA polymerase III terminators could determine which order of sites you prefer. A stretch of four or more T's in succession can function as an RNA polymerase III terminator. If the T's are at the 3' end of RNAX, it may be preferable to use the 5' MS2-RNAX 3' polarity. If the T's are in the middle of RNAX, then you may need to assess whether they present a problem. One way to do this is to clone RNAX between the IRE and MS2 sites and check whether the resulting RNA still works in the IRE/IRP assay. (There is a unique *SmaI* site in pIII/IRE-MS2 that can be used for this purpose.) If the IRE/IRP interaction still works, then the T's in RNAX probably will not be a problem; if it does not, then you may need to eliminate them.

3. SELECTING FOR ACTIVATION OF *HIS3*

For some experiments, you may want to select for *HIS3* expression. For this purpose, you may want to determine how much 3-aminotriazole to use by transforming your RNA plasmid together with an irrelevant Activation Domain plasmid, plating out on different concentrations of

3-aminotriazole. 5 mM 3-aminotriazole significantly retards growth due to basal levels of *HIS3* expression that occur in the absence of a second RNA-protein interaction; some RNAs tethered to the promoter (without any bound activation domain) may activate slightly, and so may require higher levels to eliminate the background. Excessively high concentrations will inhibit the growth of genuinely positive transformants.

4. cDNA LIBRARY SCREENING

Use either pIIIA/MS2-1 or pIIIA/MS2-2 as the hybrid RNA vector. This will enable you to distinguish RNA-dependent and RNA-independent positives. After transforming strain *L40-coat* with both the RNA plasmid and the cDNA library, plate on media selecting for the cDNA plasmid (*LEU2*) and for expression of the *HIS3* reporter, but not for the RNA plasmid (i.e. we use SD-leu-his plates). If activation of the reporter gene is independent of the RNA, a small percentage of the cells lose the RNA plasmid since it is not selected, and the colony becomes red or red-sectored (white colony with pink sectors). The desirable, RNA-dependent positives remain uniformly white. This is confirmed by subsequent selections against the RNA plasmid, using 5-FOA. Cells lacking the RNA plasmid should no longer activate the reporter. Such cells, "cured" of the RNA plasmid, can then be used as recipients for wild type and mutant RNAs in a subsequent transformation or mating.

5. USEFUL CITATIONS

1. The sequence of the MS2 coat protein binding sites used in our experiments is contained in Bardwell and Wickens (1990) *Nucleic Acids Research* **18**, 6587-6594.

2. David Engelke and colleagues constructed pIIIEx426RPR. That plasmid is described in Good and Engelke *Gene* **151**, 209-214 (1994).

3. The full sequence of pMS2-1, pMS2-2, PIIIA/MS2-1, and PIIIA/MS2-2 are available upon request. We can send the sequences by e-mail.

Our paper describing the Three-Hybrid System is attached.

Hybrid RNA Sequence

The following is the predicted RNA sequence of the RNA encoded by pIII/MS2-2.

GUUUUACGUUUGAGGCCUCGUGGCGCACAUGGUACGCUGUGGUGCUCGCG

RPR1 (RNase P RNA) leader

GCUGGGAACGAAACUCUGGGAGCUGCGAUUGGCAgaauuccggcuagaacuaggg

auccccgggcagcuugcaugccugcaggucgacucuagaaaacaugaggaucacccaugucugcaggu

SmaI MS2 sites

cgacucuagaaaacaugaggaucacccaugucugcaggucgacucuagaggaucgGAAUUCCCCC

AUAUCCAACUUCCAAUUUAAUCUUUCUUUUU

RPR1 (RNaseP RNA) terminator