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Screening Peptide/Protein Libraries Fused to the λ Repressor DNA-Binding Domain in *E. coli* Cells

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1. Introduction

The use of λ repressor fusions to study protein-protein interactions in *E. coli* was first described by Hu and others ***(1)***. Since then, the repressor system has been employed by several laboratories to screen genomic ***(2–5)*** and cDNA libraries ***(6)*** for homotypic or heterotypic interactions. λ repressor consists of distinct and separable domains: the N-terminal domain which has DNA binding activity and the C-terminal domain which mediates dimerization. The repressor fusion system is based on reconstituting the activity of the repressor by replacing the C-terminal domain with a heterologous oligomerization domain. The interaction is detected when the C-terminal domain forms a dimer (or higher order oligomer) with itself (homotypic interaction) or with a different domain from other fusion (heterotypic interaction) (*see* **Fig. 1**).

Repressor fusions are usually expressed from multicopy plasmids; for a detailed discussion of repressor fusion plasmids available from our laboratory *see* **ref. *7***. Similar plasmids have been constructed by other groups ***(5,8–10)*** with a variety of modifications. In all cases, unique restriction sites are available for cloning a desired insert in-frame with the N-terminal domain of repressor. **Table 1** lists the features of several of the repressor plasmid vectors in the literature.

The identification and characterization of homotypic or heterotypic interactions is done by fusing a target DNA (fragments from a specific gene of interest, or a genomic, cDNA, randomized, or rationally designed library) to the λ repressor DNA binding domain. Repressor fusion libraries are made by using appropriate vectors with standard cloning methods. Library construction is not discussed further in this chapter (*see* **Note 1**). Here, we focus on the evaluation

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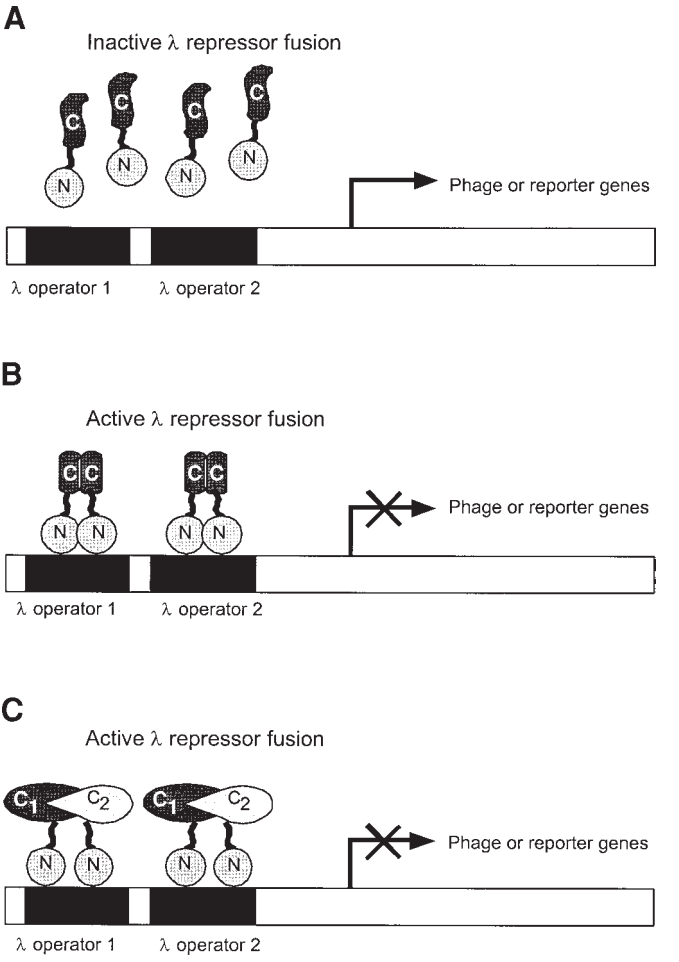

Fig. 1. The rationale of λ repressor fusions. Repressor fusions are used to detect protein-protein interactions in vivo. Protein or peptide targets are fused to the λ repressor DNA binding domain; these fusions can be evaluated for repressor activity using direct selection with λ phage, or a variety of reporter genes suitable for library screening. **(A)** Inactive repressor fusions are unable to bind its target DNA sequences (λ operators in promoters regulating phage or reporter genes). The expression of phage or reporter genes remains unaffected. In this case the fused peptide/protein is monomeric in vivo. **(B)** Active repressor fusions can be reconstituted when a dimeric peptide/protein is placed at the C terminus. The fusions are able to bind λ operators in the promoter and the reporter or phage genes are repressed. In this example the fusion is dimeric but a higher order oligomer can also reconstitute the activity of the repressor. **(C)** Heterodimers can also reconstitute the activity of the λ repressor. In this example, a target peptide (C_1) is encoded in a first plasmid and a peptide library is introduced in the cell by transformation. One of the library encoded peptides (C_2) is able to form a heterodimer with the target peptide reconstituting the activity of λ repressor.

of the resultant repressor fusions for repressor activity using either immunity to phage infection (*see* **Subheading 3.1.**) or a variety of reporters under λ repressor control (*see* **Table 2** and **Subheadings 3.2–3.4.**). Further screening is useful to ensure that the repressor activity of the fusion protein is dependent on the insert, especially when evaluating clones isolated by selection. A simple a high-throughput screening strategy based on nonsense suppression is described in **Subheading 3.5.**

2. Materials

Different subsets of the materials listed below are needed for the different protocols

2.1. General Use Media, Antibiotics, and Materials

1. Luria-Bertani (LB) broth and agar: Premixed LB broth (DIFCO, cat. no. 244620) and agar (DIFCO, cat. no. 244620) are prepared according to the vendors instructions.
2. 2XYT broth per L: 16 g tryptone, 10 g yeast extract, 10 g NaCl. Dissolve in 1 L distilled H_2O. Autoclave.
3. Antibiotics: Ampicillin 200 mg/mL in H_2O (1000X stock, use at a final concentration of 200 μg/mL); kanamycin 20 mg/mL in H_2O (1000X stock, use at a final concentration of 20 μg/mL).
4. Sterile 96-well microplates (clinical "V bottom").
5. Microplate replicator 96 pin (Boekel Model 140500).
6. Multichannel pipetter (8 or 12-channel) to handle volumes from 5–200 μL.
7. Sterile toothpicks.

2.2. Strains

Strains used are listed in **Table 3**. Different strains are used for each of the screening approaches described below.

2.3. For Phage Immunity Selections and Screens

1. AG1688 and JH787 (*see* **Note 2**).
2. λKH54 and λKH54h80 phage stocks at 10^9–10^{10} plaque forming units (pfu)/mL (*see* **Note 3**).
3. Tryptone broth per L: 10 g Tryptone, 5 g NaCl. Dissolve in 1 L H_2O. Autoclave.
4. Tryptone agar: 13 g Bacto-Agar/L of tryptone broth before autoclaving.
5. Tris-Magnesium (TM) buffer: 10 m*M* Tris-HCl, pH 8.0, 10 m*M* $MgSO_4$. Autoclave.
6. Tryptone top agar: 0.7 g Bacto agar/100 mL of tryptone broth before autoclaving.
7. Chloroform.
8. 15-cm LB plates containing ampicillin and kanamycin (*see* **Note 4**).
9. 100-mm LB Amp Kan plates containing 25 m*M* sodium citrate, added from a sterile 1M stock solution.

Table 1
Repressor Fusion Vectors Used for Peptide/Protein Library Screening

Name (size)	Promoter	Cloning sites/Comments	Ref.
pJH370	lacUV5	*Sal*I *Nde*I *Sac*I GCG GAG AGA TGG GTG TCG ACA CAT ATG AAA CAG CTG GAA GAC AAA GTT GAA GAG CTC TCT CTC TCT ACC CAC AGC TGT GTA TAC TTT GTC GAC CTT CTG TTT CAA CTT CTC GAG a e r w v s t H M K Q L E D K V E E L *Xho*I *Spe*I CTG TCT AAA AAC TAC CAC CTC GAG AAC GAA GTT GCG CGC CTG AAA AAA CTA GTT GGT GAC AGA TTT TTG ATG GTG GAG CTC TTG CTT CAA CGC GCG GAC TTT TTT GAT CAA CCA L S K N Y H L E N E V A R L K K L V G *Bam*H1 GAA CGT TGA GGA TCC CTT GCA ACT CCT AGG E R Opa	*(1)*

Original CI-GCN4 fusion construct. Also contains the ind1 *Hin*dIII site at position 117 of the linker between the N and C terminal domains. In principle, this could also be used to generate fusions with a shorter linker.

pJH391 lacUV5 *Sal*I *Sac*I *(17)*

7 kb

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GCG GAG AGA TGG GTG TCG AC GGATCGATCCC GTCCG TTT                GAG CTC
CGC CTC TCT ACC CAC AGC TG                                     CTC GAG
A   E   R   W   V   S                                          E   L
cI              130                    ... lacZ...             GCN4

                          XhoI                                      SpeI
CTG TCT AAA AAC TAC CAC CTC GAG AAC GAA GTT GCG CGC CTG AAA AAA CTA GTT GGT
GAC AGA TTT TTG ATG GTG GAG CTC TTG CTT CAA CGC GCG GAC TTT TTT GAT CAA CCA
L   S   K   N   Y   H   L   E   N   E   V   A   R   L   K   K   L   V   G

            BamH1
GAA CGT TGA GGATCC GGCTG CTAAC AAAGC CCGAA AGGAA GCTGA GTTGG CTGCT GCCAC
CTT GCA ACT CCTAGG CCGTC GATTG TTTCG GGCTT TCCTT CGACT CAACC GACGA CGGTG
E   R   Opa        T7 terminator
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pJH370 + a stuffer fragment that allows easier purification of backbone DNA cut with *Sal*I and *Bam*HI from singly cut vector DNA.

pJH391s lacUV5 *Bam*HI *(5)*

7 kB

Contains an S10 epitope tag to allow the identification of fusion proteins.

(continued)

Table 1 *(continued)*

Name (size)	Promoter	Cloning sites/Comments	Ref.
pLM99 3.4 kB	7107	*Sal*I *Sma*I *Sph*I *Bst*BI *Bgl*II *Bam*HI G TCG ACC CGG GCA TGC TTC GAA GAT CTT AAT TAA TTAAGGATCC C AGC TGG GCC CGT ACG AAG CTT CTA GAA TTA ATT AATTCCTAGG S T R A C F E D L N Ocr *Sal*I, *Sma*I, *Sph*I, *Bst*BI, *Bgl*II, *Bam*HI pLM99 (GenBank Acc. No. AF308739) contains a triple mutation in the cI DNA binding domain that makes the repressor a better activator at the P_{RM} promoter ***(20)*** without a detectable effect in DNA binding, an amber mutation at position 103 of the cI DBD and a FLAG epitope tag in the linker to allow the identification of fusion proteins. Expression of the fusion proteins is from the weak constitutive promoter 7107 ***(19)***.	*(7)*
pLM100 3.4 kb	7107	*Sal*I *Sma*I *Sph*I *Bst*BI *Bgl*II *Bam*HI G TCG ACG CCC GGG CAT GCT TCG AAG ATC TTA ATT AAT TAA GGATCC C AGC TGC GGG CCC GTA CGA AGC TTC TAG AAT TAA TTA ATT CCTAGG S T P G H A S K I L I N Ocr *Sal*I, *Sma*I, *Sph*I, *Bst*BI, *Bgl*II, *Bam*HI pLM100 (GenBank Acc. No. AF308740) is identical to pLM99 except for a frameshift at position 7 of the linker.	*(7)*

pLM101 3.4 kB	7107	*Sal*I *Sma*I *Sph*I *Bst*BI *Bgl*II *Bam*HI G TCG ACC CCG GGC ATG CTT CGA AGA TCT TAA TTAATTAAGGATCC C AGC TGG GGC CCG TAC GAA GCT TCT AGA ATT AATTAATTCCTAGG S T P G M L R R S Ocr *Sal*I, *Sma*I, *Sph*I, *Bst*BI, *Bgl*II, *Bam*HI pLM101 (GenBank Acc. No. AF308741) is identical to pLM99 except for a frameshift at position 7 of the linker.	***(7)***
pME10 2.8 kB	lacUV5	Multiple cloning site from pSP72 (Promega, Madison, WI) Contains the cI DBD amino acids 1-92.	***(10)***
pAC117	434 repressor	Contains the cI DBD amino acids 1-101.	***(9)***

Table 2
Reporters Available for Library Screening Using Repressor Fusions

Name	Reporter	Principle	Ref.
λ200	$O_R^+P_R$-*lacZ*	An active repressor fusion binds to the PR promoter, down-regulating the lacZ gene.	***(23)***
λ202	$O_R2^-P_R$-*lacZ*	An active repressor fusion binds to a single operator in the PR promoter, down-regulating the lacZ gene.	***(1)***
λ112O_sP_s	$O_s1^+O_s2^+P_s$-*cat-lacZ*	An active repressor fusion binds to two synthetic operators in a promoter, down-regulating the lacZ gene. Reporter used testing cooperative DNA binding of for repressor fuions to operator sites.	***(24)***
λXZ970	$O_s1^-O_s2^+P_s$-*cat-lacZ*	An active repressor fusion binds to a single synthetic operator in a promoter, down-regualting the *lacZ* gene. Reporter used for testing cooperative DNA binding of repressor fusions.	***(18)***
λLS100	$O_{434}^-O_s2^+P_s$-*cat-lacZ*	Same as above.	***(25)***
λLM58	$O_L^+P_L$-*cat-lacZ*	An active repressor fusion binds to the O_L1 and O_L2 operator in the P_L promoter, down-regulating the *cat* and *lacZ* genes.	***(7)***
λLM25	P_L-*GFP*	An active repressor fusion binds to the $O_L1$and O_L2 operator in the P_L promoter, down-regulating the GFPmut2 gene.	L. Mariño-Ramírez, unpublished.
λ$O_LP_LP_L$-amb sup tRNA in Q537	-amber suppressor tRNA	An active repressor fusion down-regulates the *lacZ* amber gene indirectly by repressing the transcription of an amber suppressor tRNA.	***(8)***

Table 3
***E. coli* Strains Used for Peptide/Protein Library Selection and Screening**

Strain	Genotype	Uses	Ref.
AG1688	[F'128 *lacIq lacZ*::*Tn*5] *araD139*, Δ(*ara-leu*)7697, Δ(*lac*)X74, *galE15*, *galK16*, *rpsL*(Str^R), *hsdR2*, *mcrA*, *mcrB1*	Host for libraries made with repressor fusion vectors lacking an amber mutation. Allows M13-mediated transduction.	***(26)***
JH371	AG1688 [λ200]	Same as AG1688. Allows screening with the *PR-lacZ* reporter (*see* **Table 2**).	***(1)***
JH372	AG1688 [λ202]	Same as AG1688. Allows screening with the *PR-lacZ* reporter (*see* **Table 2**).	***(1)***
JH787	AG1688 [ϕ80 Su-3]	Host for libraries made with repressor fusion vectors containing an amber mutation.	***(7)***
Q537	F^- *mcrA*, *mcrB, r*$^-$*k m+k, i, lac amU281, arg*Eam, *gal, rif, nal, sup0*	Allows screening with the P_L-amber suppressor tRNA reporter.	***(4)***
LM58[a]	JH787 [λLM58] [ϕ80 Su-3]	Allows screening with the P_L-*cat-lacZ* reporter. Allows amber suppression.	***(7)***
LM59[a]	AG1688 [λLM58]	Allows screening with the P_L-*cat-lacZ* reporter.	***(7)***
LM25	JH787 [λLM-GFP]	Allows screening with the P_L-*GFP* reporter.	L. Mariñ-Ramírez, unpublished.

2.4. For Screening with lacZ*-Based Reporters*

Materials for β-galactosidase assay of choice ***(11)***.

2.5. For Screening with Cat-Based Reporters

1. LM58 and/or LM59 (*see* **Note 5**).
2. Chloramphenicol 25 mg/mL in 100% ethanol (1000X stock, use at a final concentration of 25 μg/mL).
3. 15-cm LB plates containing ampicillin.
4. 15-cm LB plates containing ampicillin and chloramphenicol.

2.6. For Screening with Green Fluorescent Protein (GFP) Reporters

1. Repressor fusion libraries in LM25 (*see* **Note 6**).
2. 9-cm LB plates containing ampicillin and kanamycin.
3. LB-ampicillin-kanamycin broth.
4. Disposable analytical filter unit (NALGENE Cat. No. 140–4045).
5. Multiple-fluorophore purple/yellow low intensity beads (Spherotech Cat. No. FL-2060-2) (Working solution is 5 μL beads in 5 mL H_2O supplemented with 0.02% Sodium azide).
6. Flow cytometer FACSCalibur (Becton Dickinson).

2.7. Transfer of Plasmids by M13-Mediated Transduction

1. M13 rv-1 1×10^{11} pfu/mL (*see* **Note 7**).
2. 2XYT broth supplemented with ampicillin, kanamycin and 25 m*M* sodium citrate (if using colonies from phage selections).

3. Methods

Preparation of vector DNA, construction of libraries in repressor fusion vectors and transformation of competent cells can be done by a variety of standard molecular biology methods. The protocols below assume that you are starting with a freshly transformed or amplified library containing the desired inserts.

3.1. Selection or Screening for Phage Immunity

Cells expressing repressor activity are immune to λ infection. This provides a simple selection for active repressor fusions. Cells containing plasmids of interest are spread onto plates pre-seeded with phage. Any cells that lack repressor activity will be killed, and only the survivors need to be studied further.

Selection for active repressor fusions is done in the presence of two λ phage derivatives with different receptor specificities. λKH54 uses the LamB porin as the receptor for infection, whereas λKH54h80 is a ϕ80 hybrid phage that uses the TonB protein as the receptor. We estimate that double mutations resulting in simultaneous loss of both receptors occur at a frequency of around 10^{-9}, while the single mutations in each receptor occur at around 10^{-4}. Because the power of phage selection lies in its ability to process on the order of 10^7 clones/plate, the use of both phages is important to minimize the background of survivors due to host mutations.

Note that in freshly transformed cells, the intracellular concentration of repressor will be zero at the moment the plasmid is introduced, and the steady-state level of repressor will not be achieved for several generations after transformation. Thus, while plating a transformation directly on phage reduces the numbers of siblings recovered, there is a trade-off in a reduction in the recovery of active fusions.

1. Preseed plates by spreading approximately 10^8 phage each of λKH54 and λKH54h80. Allow the plates to dry briefly.
2. Plate cells from amplified or unamplified libraries onto plates containing λ phage. We have plated up to 10^7 cells from an amplified library on a single 150-mm plate. Allow plates to dry.
3. Incubate at 37°C overnight. Immune survivors should show up as single colonies the next day.
4. Pick colonies onto plates or into liquid cultures in microtiter plates containing sodium citrate (*see* **Note 8**).

3.2. Screening with lacZ Reporters

Repressor activity can also be evaluated using reporter constructs that place a screenable or selectable marker under the control of λ operators. Several reporters are available that use natural or artificial promoter-operators to drive *lacZ* expression under λ repressor control. However, these are generally based on strong promoters, and the repressed level of β-galactosidase is still high enough to give blue colonies on X-gal plates. Thus, it is necessary to screen transformants by enzyme assays. The protocol below is based on using the reporters λ200, λ202, λ112O_sP_s, λXZ970, or λLS100. The specialized uses of these reporters are described in **Table 2**.

1. Select transformants on LB Amp Kan plates.
2. Grow individual cultures of each transformant.
3. Assay for β-galactosidase activity using any of a variety of standard assays ***(11)***.

3.3. Screening with Chloramphenicol Acetyl Transferase (cat) Reporter

λLM58 carries a chloramphenicol reporter under the control of the P_L promoter, which can be down-regulated by an active repressor fusion (*see* **Table 2**). This allows simple screening on plates.

1. Select transformants on LB Amp Kan plates.
2. Replica plate or pick onto parallel LB Amp Kan plates in the presence and absence of 25 μg/mL chloramphenicol. Active fusions will be sensitive to chloramphenicol while inactive fusions will be resistant.

3.4. Green Fluorescent Protein (GFP) Reporter for the Screening of Active Repressor Fusions

λLM25 carries a GFPmut2 reporter is under the control of the P_L promoter, which can be repressed by an active repressor fusion (*see* **Table 1** and **Note 6**). The activity of a fluorescent reporter can be monitored by fluorescence-activated cell sorting (FACS); additionally FACS can be used to isolate a subpopulation of cells where the reporter has been repressed (*see* **Fig. 2**). For recent

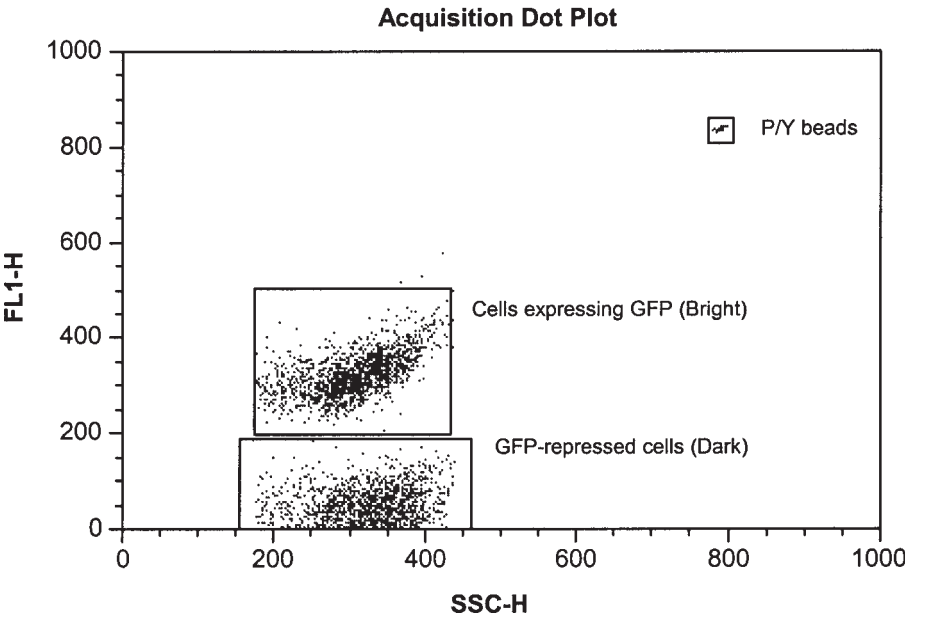

Fig. 2. Fluorescent-activated cell sorting of repressor fusion libraries. Repressor fusion librarires containing yeast genomic DNA were introduced into LM25 cells by electroporation and the libraries sorted as described in **Subheading 3.4.** The cells corresponding to the box labeled as GFP-repressed cells were collected, concentrated and plated as described in the text. A total of 81 cfuś were recovered and transduced into AG1688 (sup^0) and LM25 (supF). Forty three of these clones displayed an immune phenotype dependent on the insert; this fraction is similar to what is observed from this library when clones are isolated by phage selection.

reviews about the application of flow cytometry to various biological systems, *see* **ref.** ***12,13***. The expression level of the GFP reporter in the cell population is highly homogeneous, as detected by FACS. The homogeneous expression of the GFP reporter is due to the single copy lysogen carrying the reporter. This is important because multi-copy GFP reporters have great variations in the expression of reporters in a cell population.

1. Inoculate 3 mL LB-ampicillin-kanamycin broth with 1/100 vol of an amplified or unamplified library. Incubate at 37°C for 14 h.
2. Prepare 1 mL samples by diluting cells 10,000 fold with deionized water sterilized by filtration through a 0.2 μm filter.
3. Add purple/yellow low intensity beads (10 μL/mL of sample) as fluorescence control.
4. Sterilize the cell sorter by running 70% ethanol for 20 min followed by a wash with MilliQ water for 20 min. Perform cell sorting at a rate of less than 300 events/s

(collect light-scatter and green fluorescence data). Sort at least 50,000 events. Sort the fraction of cells with no detectable green fluorescence. Filtered MilliQ water was used as a sheath into which the cells were sorted.

5. Concentrate the sorted cells by filtration using a disposable analytical filter unit. Place the filter onto a 9-cm LB-ampicillin-kanamycin plate. Incubate at 37°C for 16 h (*see* **Note 9**).
6. Confirm immunity status of positive clones by transducing them into an appropriate background for evaluation by either phage or β-galactosidase assays.

3.5. Nonsense Suppression to Evaluate Insert-Dependence

It is important to check that the repressor activity expressed from a recombinant plasmid is actually due to the fusion of a self-assembly domain rather than some other plasmid mutation that increases expression of the N-terminal DNA-binding domain. Although this can be done by subcloning, conditional expression of the insert can be achieved by nonsense suppression when vectors pLM99-101 are used. These each contain an amber mutation at position 103 of the cI gene. Screening for repressor activity must be done in a host containing an amber suppressor, such as JH787 or LM58. These strains are paired with isogenic strains that are unable to suppress nonsense mutations, AG1688 and JH787, respectively.

1. Pick single colonies from one of the selections or screens above using sterile toothpicks and inoculate 150 µL of 2XYT-ampicillin-kanamycin broth + 25 m*M* sodium citrate (necessary if cells are from phage selection, *see* **Note 8**) in sterile 96-well microplates. Incubate at 37°C and grow for 16 h (*see* **Note 10**).
2. Mix 5 µL M13 rv-1 and 5 µL of each overnight culture. Incubate at 37°C for 10 min to allow phage to adsorb. Add 0.15 mL 2XYT+ 25 m*M* sodium citrate in sterile 96-well microplates broth. Grow for 6 h at 37°C.
3. Heat at 65°C for 20 min to kill *E. coli*. Spin the plates at 1000*g* for 15 min. Store the plate, which contains the M13 transducing phage stocks at 4°C.
4. Transfer the plasmid DNA containing the repressor fusions to an isogenic pair of strains, either AG1688 (Sup^0) and JH787 (SupF) or LM58 (SupF) and LM59(Sup^0) by M13 transduction. Mix 5 µL M13 transducing phage and 50 µL overnight culture from the SupF and Sup^0 strains. Incubate at 37°C for 30 min. Use the microplate replicator to transfer the transductions to LB-ampicillin plates. Incubate at 37°C overnight.
5. Screen the colonies for repressor activity by the appropriate method described above (phage immunity for AG1688 and JH787 or chloramphenicol sensitivity for LM58 and LM59).

4. Notes

1. Highly representative repressor fusion libraries are critical for a successful screening. In addition to methods described in popular cloning manuals ***(14,15)***,

construction of repressor fusion libraries have been described ***(3–5)***. Note that genomic libraries require higher coverage than is needed for genome sequencing because large numbers of fusion joints within every gene are needed for library saturation. Vectors pLM99-101 contain polylinkers that allow compatible ligation with a variety of blunt and sticky ends ***(16)***. For the generation of blunt ended fragments from the yeast genome, we have used DNA partially digested with *Cvi*TI (Megabase Research).

2. AG1688 ***(17)*** and JH787 (*see* Table 3) are both sensitive to λKH54 and λKH54h80. JH787, which contains an amber suppressor, should be used when the plasmid vector used for library construction contains an amber mutation, i.e., pLM99-101, between the cI DNA binding domain and the insert ***(7)*** to allow expression of the full-length fusions.
3. The KH54 deletion removes the cI gene, which is required for establishment and maintenance of lysogens. This is important because lysogens will pass as false positives in a library screen. The h80 substitution replaces λ genes with those of ϕ80. for this use, the relevant change replaces the receptor specificity of λ, which uses the LamB protein, with that of ϕ80, which uses the TonB protein. A mixture of phage is used to eliminate background due to spontaneous receptor mutants. Thus, for phage selection using this mixture of phage to be effective, the starting strain must contain wt alleles for both *lamB* and *tonB*.
4. Ampicillin selects for the plasmid vectors. Kanamycin selects for the F' episome in strains derived from AG1688. This F' carries the *lacI*^{q} allele needed to repress the expression of the fusion proteins expressed from the *lacUV5* promoter in pJH370 and pJH391. In addition, F functions are needed for M13-mediated transduction of the plasmids containing M13 origins (*see* **Subheading 3.5.**).
5. LM58 and LM59 are isogenic strains containing the chloramphenicol reporter carried by λLM58 (*see* **Table 2**). As with AG1688 and JH787, one strain (LM58) contains the SupF amber suppressor, while the other (LM59) is a nonsuppressor strain. The suppressor strain should be used for repressor fusion vectors that contain an amber mutation at position 103 in the cI DNA binding domain.
6. LM25 (JH787 [λLM-GFP]). λLM-GFP is λimm^{21} P_L-*GFP*. Constructed by recombination between λXZ1 ***(18)*** and Plasmid pLM10 (GenBank Acc. No. AF108217). This strain contains the GFPmut2 allele, which has been optimized for use with fluorescence-activated cell sorting (FACS) ***(19)***. GFPmut2 was cloned from pDS439 ***(20)*** under the control of the P_L promoter from phage λ. The P_L-*GFP* reporter is present in *E. coli* JH787 (*see* **Table 3**) as a single copy lysogen.
7. M-13 rv-1 ***(21)*** is used to transduce plasmids that contain an M13 ssDNA replication origin and M13 packaging signals ***(22)***. Phage stocks are prepared in the same manner as that used to prepare transducing stocks (*see* **Subheading 3.5.**) using a plasmid-free strain as the host. Mix 5 μL M13 rv-1 and 50 μL of a fresh overnight culture in a sterile test tube. Incubate at 37°C to preadsorb the phage. Add 5 mL 2XYT broth, incubate with aeration at 37°C for 6–8 h or overnight. Pellet cells by centrifugation. Save the supernatant. Pasteurize the phage stock by heating to 65°C for 20 min. Store at 4°C.

8. Sodium citrate chelates magnesium ions needed for phage infection. Citrate in the plates prevents reinfection by λ phage carried over from the selection plates.
9. Cells with reduced expression of GFP should contain active repressor fusions. The filter should have about 100 colonies. Adjust cell density to obtain isolated colonies if necessary.
10. Cultures in 96-well plates have a tendency to dry, to avoid this we incubate them for no longer than 16 h. Additionally, we incubate the culture plates on top of two plates that have been filled with distilled water and we keep a 500-mL beaker with distilled water in the incubator to increase humidity.

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