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Dear Colleagues,

Enclosed is the 2-hybrid system kit our lab has developed. We hope that you find it useful for your needs. As a favour, please use local sources for general yeast and bacteriological questions. Because all of these strains and reagents are unpublished, we ask that you do not distribute these reagents to others without permission and use them only for the purpose that was described in your letter. Good luck.

Sincerely,

Stephen J. Elledge

Strains

Y190 = MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112
+ URA3::GAL--> lacZ, LYS2::GAL(UAS)-->HIS3 cyh^r

Y187 = MATα gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 met⁻ URA3::GAL-->lacZ.

pAS1-CYH as DNA

pACT2 as DNA

pSE1111 (SNF4 fused to the activation domain of GAL4 in pACT) in bacteria.

pSE1112 (SNF1 fused to the DNA-binding domain of GAL4 in pAS1) in bacteria.

Both *SNF* genes are inserted as BamH1 fragments. These can serve as a positive control for X-gal staining and 3-AT resistance. Parental plasmids can be recovered by cutting with BamH1 and religating.

Y187/pAS1-CDK2

Y187/pAS1-SNF1

Y187/pAS1-p53 (From S. Fields)

Y187/pAS1-lamin (From S. Fields)

These are all for false positive elimination through the mating test. The more specificity fusions you test your positives with, the better you can trust the significance of your positives. I encourage you to gather your colleagues bait constructs as your false positive detectors.

Dear Colleague,

OD₆₀₀ 0.4 : 10⁸/ml
ON 10⁹/ml

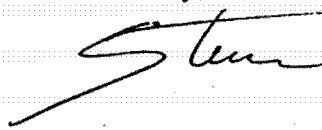
Enclosed you will find a cDNA library made in the lambda vector λ ACT (activation domain) and several other strains depending upon your request. The titer should be 1×10^{10} PFU/ml or greater, with 1×10^8 total recombinants, amplified only once from the packaging. Upon receipt, these phage should be titered, amplified to give a higher titer stock if necessary, and aliquotes can be frozen (9% DMSO, -70°C) for future use including DNA preparation. Libraries should be titered and amplified as phage on LE392 or equivalent bacterial host (NOT BNN132).

The cDNA was sized selected to be $>600\text{bp}$. The bacterial strain is BNN132 = JM107/ λ KC, a kan^r lambda lysogen containing the cre gene. Infection of this strain with the library will produce Amp^r colonies that have quantitatively excised the plasmid via cre-mediated lox recombination. I typically infect the library into log phase JM107/ λ KC cells and plate about 10^8 infected cells (10^8 phage in 3×10^8 cells) per large LB Amp plate. I usually absorb the phage for 30' at 30°C and then add LB and allow the cells plus phage to grow for an hour before plating. This allows the cells to express the bla gene and probably to undergo recombination. I usually plate about 10 large plates worth of library which is about 100 times the original number of recombinants. I have found no difference between plating at 30°C or 37°C . The λ KC phage has a wild type repressor. DNA can be prepared directly from the lysogens scraped from these plates. Occasionally I have observed tiny plaques on the lawn of lysogens. They are virulent mutants of λ ACT and I ignore them. I usually take these cells and resuspend them in terrific broth and grow them up a little further to get more DNA (6 liters gives 2 mg of plasmid DNA). Remember, these are pBR322 based plasmids so the copy number is not high. There is no real reason not to do the whole thing in liquid. I think that growth on plates presents less of a competitive situation for the clones, but it may not really be needed in actuality. For further information I suggest you read PNAS 88:1731-1734 on the λ YES-R system.

If λ ACT is included it will be as either plasmid DNA or as an extrachromosomal 42kb plasmid lysogen. The lysogen is ts due to the cI857 repressor so grow it at 28°C or lower. I generally purify it like a standard plasmid by growing several liters and double-banding it on CsCl. I find CsCl DNA to be the best for making libraries. Occasionally with other of my lambda vectors I have observed deletion plasmids taking over the strain. Just to be safe you might want to streak out a couple of different colonies from which to prepare DNA. I refer you to a paper on λ YES style vectors (PNAS 88:1731) of which λ ACT is one and the Genes and Development paper 7:555-569. If supplied as DNA, transform into E. coli and plate for plaques at 30°C . By picking a plaque into LB Amp(50) at 28°C , you can recover a lysogen and prep by CsCl.

Although this goes without saying, these libraries and strains should not be distributed to other researchers without my prior consent and should be used for only the experiments you described in your letter. Good luck.

Sincerely,



Stephen J. Elledge

Protein Interaction Cloning Outline

Transformation of library into Y190 (pAS-CYH-Target)



Recovery and plating on SC-his, leu, trp + 3-AT



X-GAL filter lift assay for β -galactosidase activity



Blue colonies selected, streaked for single colonies and retested for β -galactosidase activity w/X-GAL



Colonies struck on cycloheximide media to select for loss of pAS-CYH (test for loss of TRP1)



Trp- Leu+ colonies mated to pAS-X/Y187 strains and selected by growth on SC-leu, trp. Diploids screened for β -galactosidase activity w/X-GAL



Colonies failing to activate non-specific fusions in diploids are chosen for further analysis. Plasmid DNAs are recovered in *E. coli* and retested.



Secondary Assays

Protein Interaction Cloning Using the Two Hybrid System

Library scale transformation

Transform pACT-cDNA library DNA into recombinant Y190 that is already transformed with a pAS1-CYH2 DNA expressing the bait protein.

1. CsCl purified pACT-library is necessary.
2. Making competent Y190/pAS1 recombinant yeast.
 - a. Inoculate Y190/pAS1 recombinant yeast into 100 ml selective media. Incubate overnight at 30°C on shaker.
 - b. After overnight growth, OD₆₀₀ will be 1-2.
 - c. Dilute cells to 0.1-0.2 OD₆₀₀ or about 1/10 in 500 ml YEPD.
 - d. Incubate at 30°C on shaker to 0.7± 0.1 OD₆₀₀.
 - e. Spin 7 min, 5000 rpm (Sorvall).
 - f. Remove medium. Wash cells and transfer cells to a 50 ml centrifuge tube with distilled water, 22°C.
 - g. Spin 7 min, 3000 rpm.
 - h. Remove water and wash cells with 40 ml of LiSORB (100 mM lithium acetate, 10 mM Tris 8.0, 1 mM EDTA, 1.0 M sorbitol). Incubate 30 min at 30°C.
 - i. Prepare DNA
 1. Heat 400 µl 20 mg/ml herring testes DNA 95°C for 5-10 min.
 2. Add 1.6 ml LiSORB and vortex to mix. It must be homogeneous.
 3. Cool to 22°C.
 4. Mix 100 µg of pACT-cDNA library DNA with 900 µl of carrier DNA/LiSORB.
 - j. Prepare 40% PEG/LiTE
 1. Make at least 25 ml of 40% PEG in LiTE. That is 10 g of PEG3400 in a total volume of 25 ml. Dissolve completely before 0.2 µm filtration.
 - k. Spin down yeast 7 min at 3000 rpm.
 - l. Resuspend in 1.4 ml LiSORB.
 - m. Add pACT-cDNA library/carrier DNA/LiSORB mixture to cells and mix. Incubate 30 min at 30°C.
 - n. Add 9 volumes of 40% PEG in LiTE (should be about 20 ml) and mix. Incubate 30 min at 30°C.
 - o Heat shock 42°C for 15 min.
 - p Spin 10 min at 3000 rpm
 - q. Pipet off PEG.
 - r. Recover in 100 cc WHL dropout media for >1 hr, 30°C, in shaker

- s. Spin 7 min at 5000 rpm. Pipet off media except for 10 ml.
- t. Resuspend. Take out an aliquot. Dilute 1/1000. Spread 25, 50, 100 μ l per 100 mm plate of WL drop out agar plates. The number of colonies can be used to measure transformation efficiency. You should get at the order of magnitude of 10 million.
- u. Spread on selective WHL plus 3-amino-triazole agar plates with a glass loop (0.5 ml/plate).
- v. Incubate at 30°C. In 3 days, you should get colonies growing.

Protein Interaction Cloning Using the Two Hybrid System

Library transformation using Y190

Y190 = MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112
+ URA3::GAL--> lacZ, LYS2::GAL-->HIS3 cyh^r

After subcloning the gene of interest into pAS1-CYH or a similar GAL4 DNA-binding domain fusion vector, transform this construct into Y190 selecting for TRP1 in the case of pAS1. It should be noted that expression of the fusion protein should be verified by western blotting with anti HA antibodies available from BABCO if specific antisera are not available for your protein. Occasionally, a bona fide fusion is made and cannot be detected with HA (levels too low, but sufficient for screening).

The resulting strain should be checked for its growth properties on SC-His plates containing differing concentrations of 3-AT (3-aminotriazole, SIGMA, A8056) and on its ability to activate the lacZ reporter. These tests should be carried out relative to strains carrying pSE1112 alone. We have found that 3-AT concentrations of 25 mM to 50 mM are sufficient to select against pAS1 subclones that fail to activate transcription on their own. If your construct activates transcription alone, it cannot be used in this assay. If it fails to activate transcription, like most fusions, you may proceed to the library transformation step. Also check out the RNA transformation protocol we have included. It works a little more reproducibly in our hands.

1. Use a colony or overnight to inoculate 200 mls SC-Trp and grow overnight at 30°C. The use of a saturated overnight from the night before ensures that the 200 ml culture will be fully grown the next day.
2. Take OD A600 of above culture and inoculate 500 mls YEPD such that in ~2 generations (3-4 hrs) the A600=0.5 to 0.8. (Synthetic complete - Trp media is used to select for pAS1 but YEPD gives best transformation efficiencies)
3. Harvest cells at 5K for 10 minutes in a Sorvall centrifuge
4. Wash once with distilled water ~100 mls and resuspend in 50 mls LiSORB and incubate at 30°C for 15-30'.
5. Spin down as above and resuspend in ~625 µl LiSORB. Hold on ice.
6. Prepare carrier DNA mix.
 - Boil 200 µl 20 mg/ml sheared salmon sperm DNA for 7-10'
 - add 800 µl LISORB (room temp, RT)
 - mix by pipeting mixture up and down
 - cool to RT (ice can be used, but care must be taken to ensure that the Temp. does not go lower than RT or the mixture will gel)
 - add 40µg library DNA
7. Add ~100µl of above DNA mix to 100 µl cells from step 6.
8. Incubate at 30°C for 30 minutes (optional)
9. To 100 µl cells + DNA add 900 µl 40% PEG3350 in 100 mM LiAc/TE and incubate at 30°C for 30'. Plate 5 ul of cells to test transformation efficiency on SC-Trp, Leu.
10. Recovery: Pool cells and add to ~100ml SC--His,Trp, Leu liquid media, shake at 30°C for 1-3 hours; harvest cells and resuspend in ~6 mls of SC--His,Trp,Leu liquid media and plate ~300 µl per

150mm plate (SC--His,Trp, Leu+ 25 mm AT) or 50 mM 3-AT. (Plating directly from the PEG also works but is more messy.)

11. Colonies that grow after 3 to 5 days are then tested for β -galactosidase activity using the X-Gal colony filter assay described in the accompanying protocol. Blue colonies are taken for further study, they can often be taken directly from the filters in addition to the original plate.

Efficiency = $\sim 5 \times 10^4$ to 10^5 colonies/ μ g cDNA library.

For determination of efficiency plate 5 μ l before and after recovery on SC-Trp, Leu

LiSORB=100 mM LiOAC, 10 mM Tris pH 8, 1 mM EDTA, 1 M Sorbitol.

LiAcTE=100 mM LiOAC, 10 mM Tris pH 8, 1 mM EDTA (0.51g LiOAC, 0.5ml 1M Tris, 0.1ml 0.5M EDTA \rightarrow 50ul)

We have found that total yeast RNA works more reproducibly as a carrier but it is more work to prepare the RNA than DNA. That transformation protocol is included as well. We usually place the transformation mix in SC-His, Trp, Leu after the heat shock step and allow it to recover for 3 hours. This allows the transformants to be established and HIS3 transcription to be activated. The cells at this stage can be pelleted and resuspended in a smaller volume and plated directly or made 10% DMSO, and frozen at -70°C . Cells in PEG seem more fragile and often die when pelleted so the recovery step is useful. Cells lose less than half their viability when frozen and can be stored indefinitely. This is useful because they can be thawed and plated at an optimal density for screening/selection at your leisure. This protocol can be scaled up for 1 liter of cells or more.

The HIS3/3-AT selection sometimes works as a good selection and sometimes looks like more of an enrichment. We often see many micro colonies on the original selection plates. Occasionally they are 1% of the total Leu⁺Trp⁺ colonies. In most cases true positives continue to grow into large colonies while the micro-colonies seem to stop growing. The secondary screen for blues eliminates these micro colonies. The majority of His⁺ blue colonies are the large colonies that grow out. An enrichment of 100-fold is very useful because it allows you to screen 100 times as many colonies on a single plate so a whole library can be screened in only 20 large plates. We have also developed a GAL \rightarrow URA3 selection system which requires higher levels of 2 hybrid activated transcription than the His selection. That strain is available upon request.

Included in the kit is pSE1111 (SNF4 fused to the activation domain in pSE1107) and pSE1112 (SNF1 fused to the DNA-binding domain of GAL4 in pAS1). These are both inserted as BamHI fragments. These can serve as a positive control for X-gal staining and 3-AT resistance.

It should be noted that pAS1 alone can activate lacZ weakly. Therefore it is not a good negative control. pSE1112 is a better negative control. The weak activation of pAS1 appears to go away when genes are cloned into it. We do not understand why it is weakly activating alone, but we think it is likely due to sequences beyond the polylinker and which are of no consequence once cDNAs are cloned into it.

We have recently made improvements in the system. We have constructed a Y153 derivative, Y190, that is resistant to cycloheximide (2.5 μ g/ml) due to a mutation in the CYH2 gene. Y190 is now being supplied with the kit. This is a recessive drug resistance. When a plasmid carrying the wild type CYH2 gene is in the strain, cells become sensitive to cycloheximide. We have constructed a pAS1-CYH plasmid that contains the CYH2 gene. After a positive clone has been selected in the system, loss of the pAS1-CYH plasmid can be achieved by streaking on SC-Leu 2.5 μ g/ml cycloheximide media. It is probably a good idea to streak the colonies out on SC-Leu before streaking on cycloheximide media to allow plasmid loss and dilution of the CYH2 gene product. However, it does work streaking directly from SC-TrpLeu. The colonies that grow should be Trp⁻, but they should be checked for loss of the TRP marker, just to be safe and avoid CYH2 gene conversion events. This plasmid loss allows one to check

for plasmid dependency of lacZ activation as well as generating a strain that contains only the library plasmid, facilitating plasmid recovery into bacteria.

False positives do occur that appear to be dependent upon both plasmids. This is a reoccurring problem of the 2-hybrid system. You should give some thought to secondary criteria for distinguishing a true positive. False positives will light up many non-related pAS1 fusions, that is one of the definitions of a false positive. To eliminate false positives, we usually generate a strain that has lost the pAS plasmid but retains the library plasmid. This is done using the CYH trick or growing in YEPD, plating on SC-Leu and replica plating to SC-Trp to look for loss of pAS1. This strain (Leu⁺Trp⁻) is mated to a strain, Y187 = MAT α gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-->lacZ, that is of the opposite mating type but contains different unrelated fusions in pAS1 (Leu⁻Trp⁺) such as SNF1 (pSE1112), lamin, p53 (Lamin and p53 are provided by Stan Fields). Once your Y190 strain of interest has lost the pAS1-fusion plasmid (by plasmid loss or cycloheximide selection) it is Trp⁻Leu⁺ and can be mated to Y187 containing pAS1-X. Diploids can be selected by growth on SC-TrpLeu and then immediately tested for β -galactosidase activity in the filter screen assay. Colonies that activate lacZ expression significantly above background levels (pSE1112) probably contain Leu plasmids encoding false positives that non-specifically activate your fusion and should be disregarded.

We have used several different tests to detect *in vitro* binding of positives. One is to make a PCR primer to the library plasmid that has a T7 promoter placed in an appropriate position to place the insert of the library plasmid under T7 control. The PCR-derived fragments can then be directly added to a coupled transcription-translation system (TnT from Promega) and radiolabeled protein made. We usually add 6 μ l of a robust 30 cycle PCR reaction to 25 μ l of the translation mix (TnT). 5 μ l of this reaction mixture run on a SDS gel gives a readily detectible signal on an overnight exposure. This tells you the size of the fused protein and can be used to detect interaction *in vitro* with a GST-bait fusion.

The sequences of the PCR primers we have used successfully are:

1)

TAA TAC GAC TCA CTA TAG GGA GAC CAC ATG GAT GAT GTA TAT AAC TAT ~~CTA~~ TTC
T7 Promoter Met Gal4 Activation Domain

445 ng
ACT-1

2)

CTA CCA GAA TTC GGC ATG CCG GTA GAG GTG TGG TCA
In the ADH Terminator

ACT-2

A second test is to IP your protein out of yeast extracts and then use antibodies to the activation domain of GAL4 to detect binding of the fusion protein. Our GAL4 activation domain antibodies are currently being tested for this purpose. Unfortunately, they do not IP presently.

A third method is to switch the bait and prey in their respective plasmids, i.e. take the library insert out of pACT and insert it into pAS1, and place the original pAS1 insert into pACT. The majority of false positives will not interact in this test. It should be noted that some true positives may not activate for structural reasons, so only a positive result can be trusted. We have recently placed the pAS1 polylinker into pACT to facilitate this transfer, creating pACT2.

For general yeast protocols we recommend the Methods in Enzymology Vol. 194 "Guide to Yeast Genetics and Molecular Biology" by Guthrie and Fink and the Red book.

As with all protocols, this is probably not optimal and I encourage you to add variations. Let me know if any new variation makes for a simpler or more efficient protocol. For example, several people have said that direct plating of the transformation mix in peg onto selective plates works well for them. It is probably worth a try.

Our lab, Wade Harper's lab, and Stan Fields' lab have all successfully isolated clones from our λ ACT libraries using the HIS3 selection of Y153 and Y190 and have each contributed to these protocols. Good luck.

Protein Interaction Cloning Using the Two Hybrid System

Small scale transformation

Transformation using strain Y190

Y190 Genotype: MAT α gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112
+URA3::GAL--> lacZ, LYS::GAL-->HIS3 cyh^r

Preliminary steps

Clone the gene open reading frame into pAS1-CYH2 by standard *E. coli* based cloning methods. (Note that these methods apply to pAS1-CYH2 in regards to selective media and host strains. Check genotypes and genetic markers!)

Note: this method is for small scale transformation. For library screening, a high efficiency large scale method must be used.

Transform recombinant pAS1-CYH2 DNA into Y190.

1. Mini-preparations of DNA are used. (CsCl purified works better but is not really necessary).

2. Making competent Y190

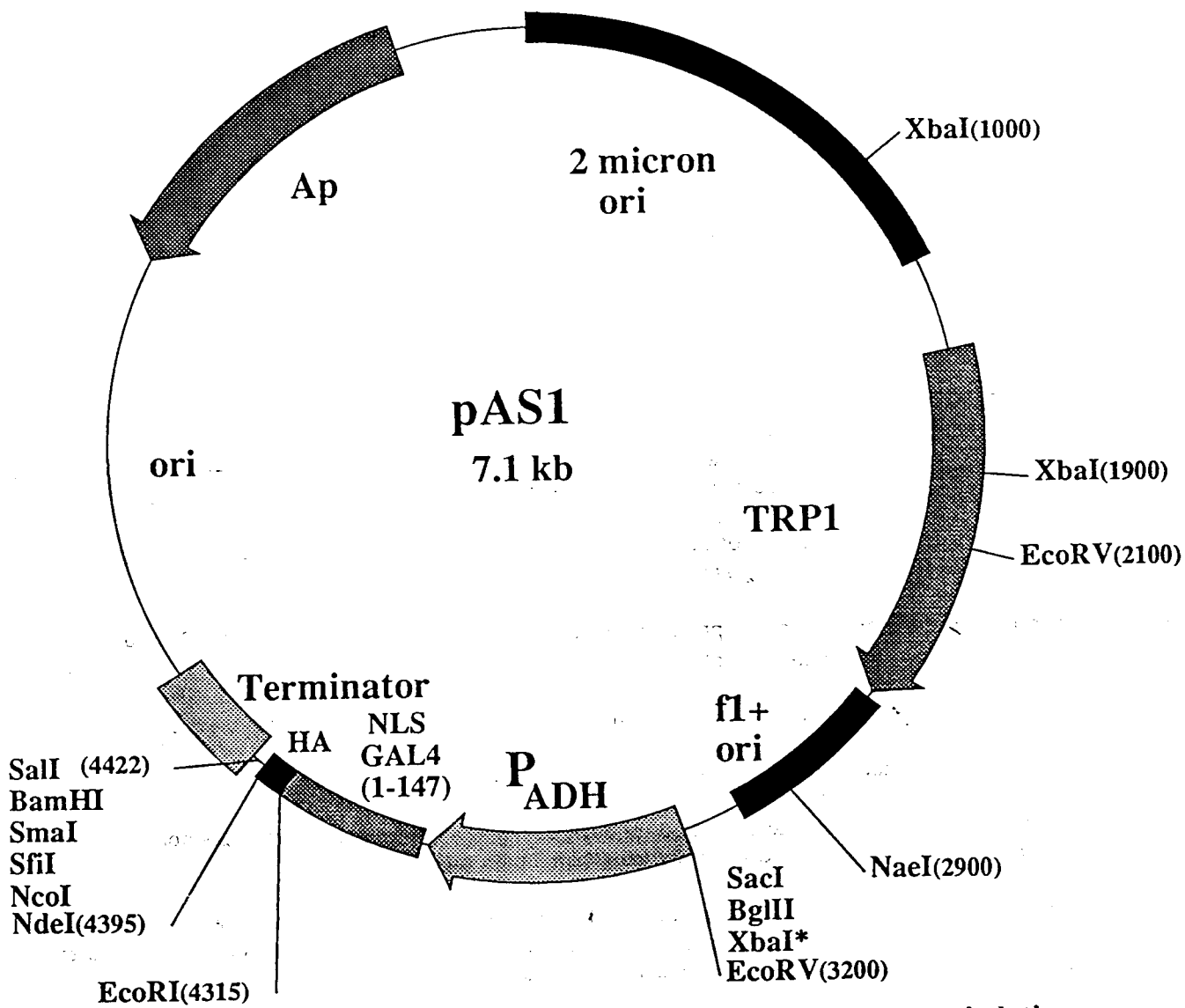
- a. Inoculate Y190 into YEPD media. Incubate overnight at 30°C on shaker.
- b. After overnight growth, OD₆₀₀ will be 1-2.
- c. Dilute cells to 0.1-0.2 OD₆₀₀ or about 1/10 in YEPD.
- d. Incubate at 30°C on shaker to 0.7± 0.1 OD₆₀₀.
- e. Spin 5 min, 2000 rpm (IEC centrifuge).
- f. Wash cells with distilled water, 22°C.
- g. Spin 5 min, 2000 rpm.
- h. Resuspend in 1/100 of original culture volume with LiTE (100 mM lithium acetate, 10 mM Tris 8.0, 1 mM EDTA).
- i. Frozen competent: add DMSO to final 10% (vol/vol) concentration. Measure volume because the yeast take up room. Fast freeze on dry ice/ethanol bath and store at -80°C. (Frozen competent are less efficient at transformation but still good enough).

3. Transforming Y190

- a. 50 μ l of yeast at 22°C (As little as 10 μ l can be used)
- b. Prepare DNA
 1. Carrier: 200 μ l of 20 mg/ml herring testing DNA. Heat >90°C for 5-10 min. Immediately add 800 μ l of LiSORB and vortex. The solution should be homogeneous. Cool to room temperature.
 2. Add 5 μ g plasmid DNA to 50 μ l of carrier.

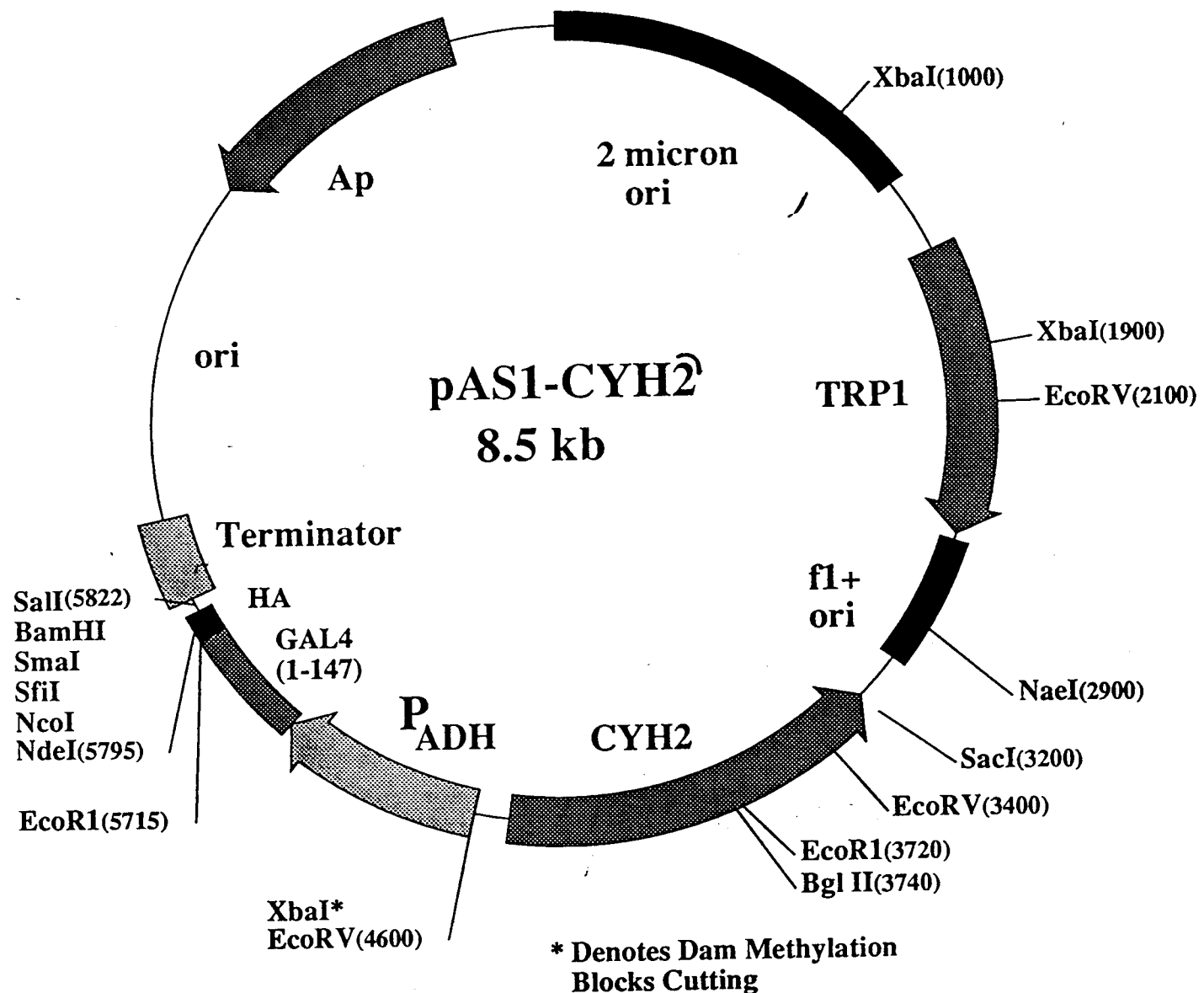
3. Add yeast to plasmid/carrier and mix. Incubate 30 min at 30°C.
- c. Add 450 μ l PEG3400 (40% wt/vol in LiTE or 8 g of PEG3400 mixed completely with about 13 ml LiTE and 0.22 μ m filter sterilized) and mix gently by pipetting.
 - d. Incubate 30°C for 30 min.
 - e. Heat shock 42°C for 10 min.
 - f. Pulse spin for 5 to 10 sec in Eppendorf microfuge
 - g. Pipet off PEG.
 - h. Recover in 1 cc DOB SC-Trp (or -Trp-Leu) for 1 hr, 30°C, in shaker (optional)
 - i. Pellet cells in microcentrifuge < 5 sec. Pipet off 0.8 ml.
 - j. Resuspend and spread on selective agar plates with a glass loop (0.1 ml/plate).
 - k. Incubate at 30°C

Note: for double transformants (i.e. a yeast with a pAS1-CYH2 already in it and now you want to introduce a pACT DNA), follow the same procedures to make competent yeast. Selective media will be double drop-out or triple drop-out with 3-amino-triazole when you plate out yeast at the very end (step 3j).



* denotes Dam methylation interference of cutting

CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA C
 NdeI SfiI NcoI SmaI BamHI SalI



Sfi I

CAT ATG GCC ATG GAG GCC CCG GGG ATC CGT CGA C

NdeI NcoI SmaI BamHI Sal I

* ↑ ↑ ↑ *

A XX · XXX ATG · xxx CCX · xxx

T XX · XXX TAC · xxx G GX · xxx

pAS1 HA epitope and polylinker reading frame

R1 M↓A Y P Y D ↓V P D Y A^{NheI}
 ↑GAA TTC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC TTG GGT GGT
 primer
SfiI
CAT ATG GCC ATG GAG GCC CCG GGG ATCC
 NdeI NcoI SmaI BamHI

pSE1107 Activation domain polylinker and reading frame

Bgl II BamH Bgl II
 GAG ATC TGG AAT TCG GAT CCT CGA GAG ATC TAT
 EcoRI XhoI

Sequence around the cloning site of pACT.

Mlu I Met
 GCGTATAACGCGTTTGGGAATCACTACAGGGATGTTTAATACCACTACAATGGATGATGT 57
 ↓ y N Y L F D D E D T P P N P K K
 ATAT AAC TAT CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA 108
 ↑
Bgl II BamH Bgl II
 GAG ATC TGG AAT TCG GAT CCT CGA GAG ATC TAT ACT-3
 EcoRI XhoI

GAATCGTAGATACTGAAAAACCCCGCAAGTTCACTTCAACTGTGCATCGTGCACCATCTC
 AATTTCTTTCATTTATACATCGTTTTGCCT