# CytoTrap Vector Kit

## INSTRUCTION MANUAL

Catalog #217438 (CytoTrap Vector Kit)
#217431 (pMyr vector)
#217433 (pSos vector)
Revision A

**For In Vitro Use Only**
217438-12

# LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

# ORDERING INFORMATION AND TECHNICAL SERVICES

## United States and Canada

**Agilent Technologies**
**Stratagene Products Division**
11011 North Torrey Pines Road
La Jolla, CA 92037
**Telephone** (858) 373-6300
**Order Toll Free** (800) 424-5444
**Technical Services** (800) 894-1304
**Internet** techservices@agilent.com
**World Wide Web** www.stratagene.com

## Europe

| Location | Telephone | Fax | Technical Services |
|---|---|---|---|
| **Austria** | 0800 292 499 | 0800 292 496 | 0800 292 498 |
| **Belgium** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 15775 | 0800 15740 | 0800 15720 |
| **France** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 919 288 | 0800 919 287 | 0800 919 289 |
| **Germany** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 182 8232 | 0800 182 8231 | 0800 182 8234 |
| **Netherlands** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 023 0446 | +31 (0)20 312 5700 | 0800 023 0448 |
| **Switzerland** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 563 080 | 0800 563 082 | 0800 563 081 |
| **United Kingdom** | 00800 7000 7000 | 00800 7001 7001 | 00800 7400 7400 |
| | 0800 917 3282 | 0800 917 3283 | 0800 917 3281 |

## All Other Countries

Please contact your local distributor. A complete list of distributors is available at www.stratagene.com.

# CytoTrap Vector Kit

# Contents

# CytoTrap Vector Kit

## MATERIALS PROVIDED

| Materials provided | Catalog #217438 | Catalog #217431 | Catalog #217433 |
|---|---|---|---|
| Vectors[a] | | | |
| pSos vector, supercoiled (Catalog #217433) (1 µg/µl in TE buffer) | 20 µg | — | 20 µg |
| pMyr vector, supercoiled (Catalog #217431) (1 µg/µl in TE buffer) | 20 µg | 20 µg | — |
| Control plasmids[a] | | | |
| pSos MAFB control plasmid (positive control) (1 µg/µl in TE buffer) | 20 µg | — | — |
| pMyr MAFB control plasmid (positive control) (1 µg/µl in TE buffer) | 20 µg | — | — |
| pSos Col I control plasmid (negative control) (1 µg/µl in TE buffer) | 20 µg | — | — |
| pMyr Lamin C control plasmid (negative control) (1 µg/µl in TE buffer) | 20 µg | — | — |
| pMyr SB control plasmid (positive control) (1 µg/µl in TE buffer) | 20 µg | — | — |
| Host strains[b] | | | |
| cdc25H host strains (α and a) | 0.5 ml each | 0.5 ml each | 0.5 ml each |

[a] Store at –20°C.

[b] Store at –80°C.

## STORAGE CONDITIONS

**Vectors:** –20°C
**Yeast Strains:** –80°C

### Host Strains and Genotypes

| Host strain | Genotype |
|---|---|
| cdc25H Yeast Strain (α) | *MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^+$ |
| cdc25H Yeast Strain (a) | *MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^+$ |

Revision A

# ADDITIONAL MATERIALS REQUIRED

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (phenol, chloroform, and sodium hydroxide) or physical (high-voltage electrophoresis systems). The researcher is advised to take proper precautions and care with these hazards and to follow the safety recommendations from each respective manufacturer.

## Reagents and Solutions

Phenol–chloroform [1:1 (v/v)] and chloroform

***Note*** *Do not use the low-pH phenol from the Stratagene RNA Isolation Kit because this phenol is too acidic and will denature the DNA.*

Ethanol (EtOH) [70% and 100% (v/v)]
Sterile distilled water ($dH_2O$)
Acid-washed glass beads (425–600 µm)
VWRbrand™ 4-mm glass beads [VWR Scientific, Westchester, Pennsylvania (Catalog #26396-633)]
β-mercaptoethanol (for preparation of yeast competent cells, dilute β-ME stock 1:10 (from 14.2 M to 1.4 M) with $dH_2O$ just prior to use)
Sos1 antibody [BD Biosciences (Catalog #610095 or 610096)]
Yeast extract
Bacto® peptone
Bacto® agar
Yeast nitrogen base
Ammonium sulfate

## Equipment

50-ml conical tubes
Replica-plating mold (150 mm) and velvet squares
Microcentrifuge tubes
Microcentrifuge
Micropipet and micropipet tips
96-well plates

# NOTICE TO PURCHASER

U.S. Patent No. 5,776,689 entitled "Protein Recruitment System" and covering this two-hybrid system is owned by The Regents of the University of California and Baylor College of Medicine. Use of the two-hybrid system by commercial entities will require a license from The Regents of the University California. For license information, please contact:

University of California, San Diego
Technology Transfer Office, Mailcode 0910 9500 Gilman Drive
La Jolla, California 92093-0910
Telephone: (619) 534-5815
Facsimile: (619) 534-7345

# INTRODUCTION

The Stratagene CytoTrap vector kit provides a novel method for detecting protein-protein interactions in vivo. The CytoTrap two-hybrid system is based upon generating fusion proteins whose interaction in the yeast cytoplasm activates the Ras-signaling pathway, inducing cell growth. These properties of the CytoTrap system enable the study of protein interactions that cannot be assayed by conventional two-hybrid or interaction trap systems. These include proteins that are transcriptional activators or repressors, proteins that require post-translational modification in the cytoplasm, and proteins that are toxic to yeast in conventional two-hybrid systems.

The CytoTrap system uses the yeast *S. cerevisiae* temperature-sensitive mutant strain cdc25H (see *Host Strains and Genotypes*), which contains a point mutation at amino acid (aa) residue 1328 of the *CDC25* gene.[1] *CDC25* is the yeast homologue of the human Sos (hSos) gene, encoding a guanyl nucleotide exchange factor that binds and activates Ras, beginning the Ras signal transduction pathway. The *cdc25* mutation present in the cdc25H strain prevents growth at 37°C, but allows normal growth at the permissive temperature (25°C). The CytoTrap system is based on the ability of the human Sos protein (hSos), to complement the *cdc25* defect and to activate the yeast Ras-signaling pathway. Expression of hSos and its subsequent localization to the plasma membrane allows the cdc25H yeast strain to grow at 37°C. The localization of hSos to the plasma membrane occurs through the interaction of two-hybrid proteins.

DNA encoding the protein of interest (bait protein) is cloned into the pSos vector MCS, generating a fusion protein of hSos and the bait protein. DNA encoding another protein of interest (target protein) or an expression library is cloned into the pMyr vector MCS and expressed as a fusion protein with a myristylation sequence that anchors the fusion protein to the plasma membrane. These fusion proteins are coexpressed in the cdc25H yeast strain, and the yeast cells are incubated at the restrictive temperature of 37°C. If the bait and target proteins physically interact, the hSos protein is recruited to the membrane, thereby activating the Ras-signaling pathway and allowing the cdc25H yeast strain to grow at 37°C.

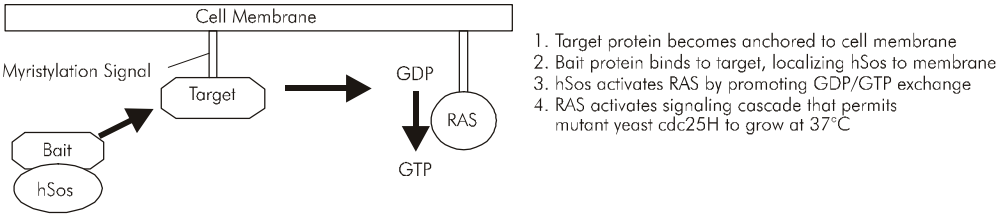



**FIGURE 1** Schematic diagram of the Ras-signaling pathway utilized in the CytoTrap two-hybrid system.

# VECTORS

The pSos and pMyr vectors are designed for constructing and expressing gene fusions with the hSos protein and a myristylation signal, respectively. The pSos vector contains DNA encoding amino acids (aa) 1 to 1067 of the hSos gene and unique 3′ cloning sites.[2] It is used for constructing a bait plasmid containing a DNA insert encoding a bait protein. The *ADH1* promoter driving expression of the hSos-bait fusion is constitutively active (see Figure 2).

The pMyr vector contains DNA encoding the myristylation membrane localization signal (Myr) and unique 3′ cloning sites and is used for constructing plasmids or cDNA libraries that contain DNA inserts encoding target proteins. The *GAL1* promoter, driving expression of the Myr-target fusion is induced by adding galactose to the growth medium. Target proteins will be directed to and anchored in the yeast membrane (see Figure 3).

Both pSos and pMyr vectors contain the pUC and 2μ origins for replication in *E. coli* and yeast, respectively. The pSos and pMyr vectors also carry yeast biosynthetic genes *LEU2* and *URA3*, respectively, for selection of yeast transformants based on nutritional requirements. The pSos vector contains the ampicillin-resistance gene and the pMyr vector contains the chloramphenicol-resistance gene to rapidly distinguish between the two vectors when recovering plasmids from *E. coli*. Table I contains a list of unique restriction sites for pSos and pMyr.

**TABLE I**
**Unique Restriction Sites in the MCS**

| pSos | pMyr |
|---|---|
| *Bam* HI | *Eco*R I |
| *Nco* I | *Srf* I/*Sma* I |
| *Srf* I | *Xho* I |
| *Aat* II | *Sal* I |
| *Sal* I | |
| *Bss*H II | |
| *Mlu* I | |
| *Sac* I | |
| *Not* I* | |

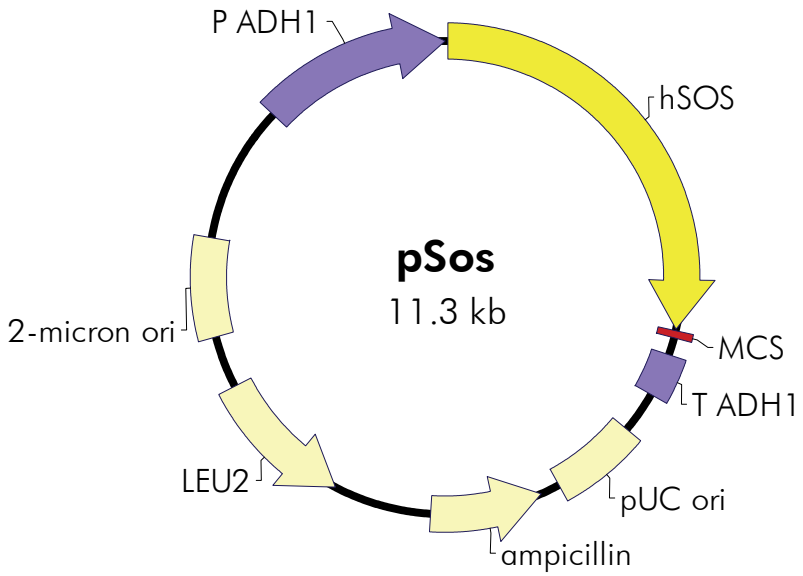
* To ensure complete digestion with *Not* I, use 10U enzyme per μg DNA and incubate the digestion reaction mixture overnight at 37°C.

**Suggested Sequencing Primers**

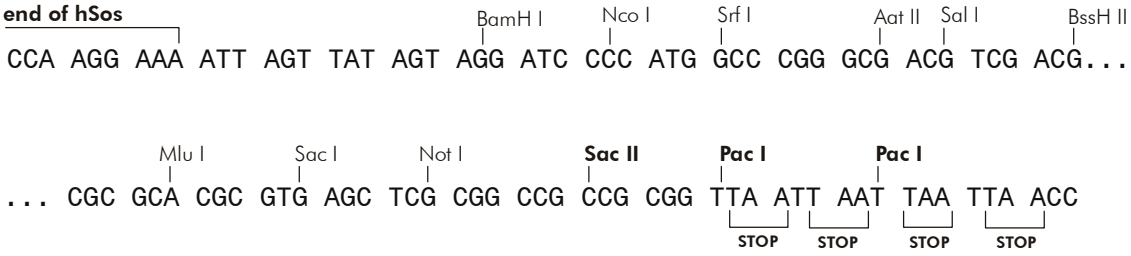
| Sequencing primers* | Sequence |
|---|---|
| Sos 5′ primer | 5′-CCAAGACCAGGTACCATG-3′ |
| Sos 3′ primer | 5′-GCCAGGGTTTTCCCAGT-3′ |
| Myr 5′ primer | 5′-ACTACTAGCAGCTGTAATAC-3′ |
| Myr 3′ primer | 5′-CGTGAATGTAAGCGTGACAT-3′ |

* 5′ primers are at the 5′ end of the MCS and 3′ primers are at the 3′end of the MCS.

## pSos Vector




## pSos Multiple Cloning Site Region
**(sequence shown 3201–3299)**

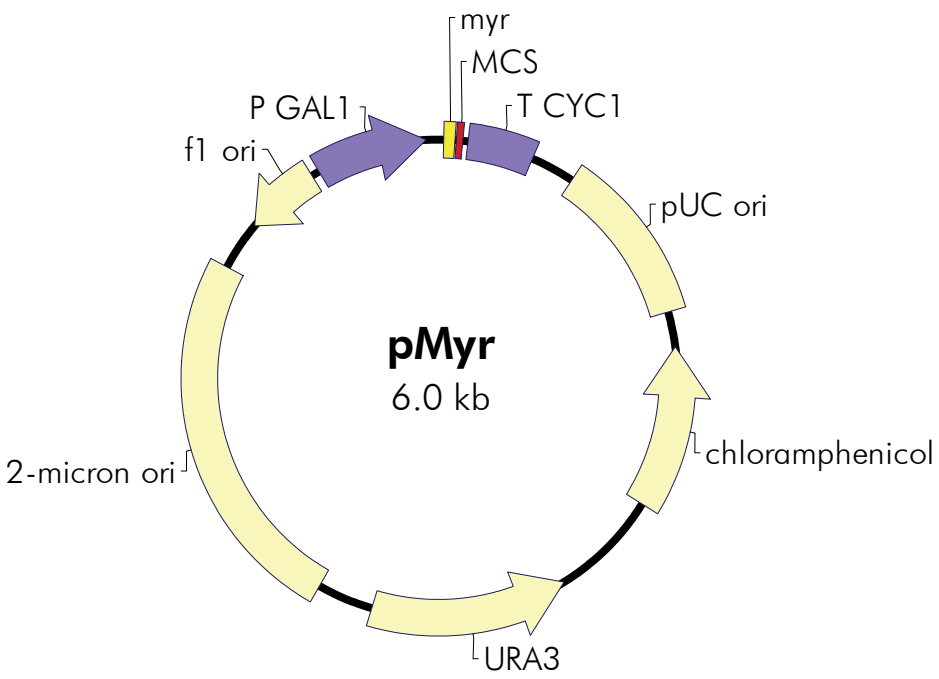



***Notes:*** *The **Sac II** and **Pac I** sites in the pSos MCS (shown in bold) are not unique. A stop codon is present in all three reading frames.*

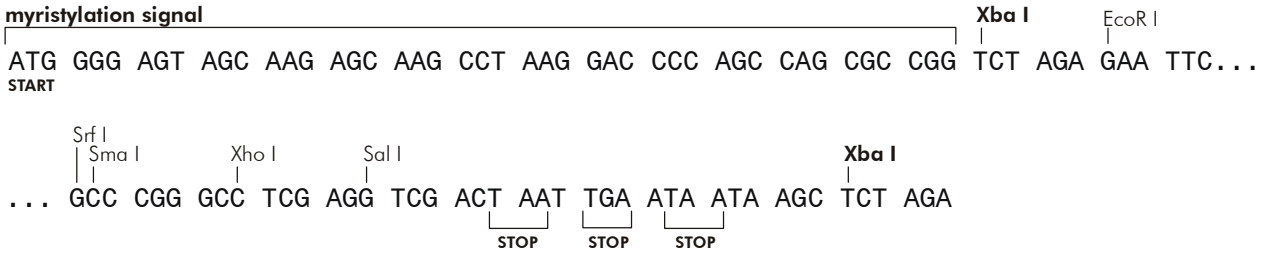
| Feature | Nucleotide Position |
|---|---|
| hSos ORF | 24–3209 |
| multiple cloning site | 3223–3276 |
| yeast *ADH1* terminator | 3413–3741 |
| pUC origin of replication | 4064–4731 |
| ampicillin resistance (*bla*) ORF | 4882–5739 |
| yeast *LEU2* selection marker ORF | 6498–7589 |
| 2μ yeast origin of replication | 7993–8747 |
| yeast *ADH1* promoter | 9806–11259 |

**FIGURE 2** The pSos vector

## pMyr Vector




## pMyr Multiple Cloning Site Region
**(sequence shown 22–120)**




***Notes:*** *The **Xba I** sites in the pMyr MCS (shown in bold) are not unique. A stop codon is present in all three reading frames.*

| Feature | Nucleotide Position |
|---|---|
| myristylation signal | 22–66 |
| multiple cloning site | 73–98 |
| yeast *CYC1* terminator | 121–387 |
| pUC origin of replication | 568–1235 |
| chloramphenicol resistance ORF | 1378–2034 |
| yeast *URA3* selection marker ORF | 2482–3282 |
| 2μ yeast origin of replication | 3512–4983 |
| f1 origin of ss-DNA replication | 5183–5489 |
| yeast *GAL1* promoter | 5527–5977 |

**FIGURE 3** The pMyr vector

# PREPARATION OF YEAST HOST STRAIN

**Note** *The temperature-sensitive phenotype of the cdc25H host strain reverts during yeast growth, and the reversion frequency is increased by growing the strain at temperatures above 25°C. It is critical to establish a frozen glycerol stock of cdc25H cells, then to minimize the number of generations between retrieval from the freezer stock and final two-hybrid interaction assays.*

The yeast host strain has been sent as a glycerol stock. Refer to the table below for the appropriate media for completing procedures in this section.

| Host strain | Agar plate for yeast streak | Medium for yeast glycerol stock |
|---|---|---|
| cdc25H (α or *a*) | YPAD Agar[a] | YPAD Broth[a] |

[a] See *Preparation of Media and Reagents*.

## Establishing an Agar Plate Yeast Streak

Place the provided yeast host strain glycerol stock vials at –80°C immediately. Prepare agar plate yeast streaks from the provided glycerol stocks to use as working stocks of the cdc25H strains.

**Notes** *The host yeast strains should be stored immediately at –80°C. Avoid repeated thawing of the yeast strains in order to maintain extended viability.*

*It is critical to grow the cdc25H yeast at room temperature (22–25°C). Higher temperatures induce mutational revertants.*

1. Obtain cells from the glycerol stock by scraping off splinters of solid ice with a sterile wire loop or sterile inoculating stick.

2. Streak the splinters onto a YPAD agar plate.

3. Incubate the plate at room temperature (22–25°C) until colonies appear (~4 days).

4. Seal the plate with Parafilm® laboratory film and store the plate at 4°C for up to 1 week.

5. Restreak the yeast culture from the –80°C glycerol stock onto a fresh plate every week.

## Preparation of a –80°C Yeast Glycerol Stock

1. In a sterile 50-ml conical tube, inoculate 10 ml of YPAD broth with one colony (grown for a minimal number of generations) from a YPAD plate. Grow the cells to late log phase ($OD_{600}$ = 0.8–1.0) at room temperature (22–25°C).

2. Add 4.5 ml of a sterile solution of 50% glycerol in liquid YPAD (prepared as 5 ml of glycerol + 5 ml of YPAD broth) to the yeast culture from step 1. Mix well.

3. Aliquot the glycerol-containing cell suspension into sterile centrifuge tubes (1 ml/ tube). This preparation may be stored at –80°C for more than 2 years.

4. Verify the temperature-sensitive growth phenotype of the new yeast stock to confirm that the strain has not reverted during growth. Streak a sample of the new glycerol stock on two YPAD agar plates. Incubate one plate at room temperature (22–25°C) and the second plate at 37°C. Observe both plates daily for 4 days; no growth should be observed on the plate incubated at 37°C.

## Host Strains and Genotypes

| Host strain | Genotype |
|---|---|
| cdc25H Yeast Strain (α) | *MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^+$ |
| cdc25H Yeast Strain (a) | *MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^+$ |

## Verification of Yeast Host Strain Marker Phenotype

The phenotype of the yeast host strain should be verified as outlined below prior to performing the CytoTrap system assays.

1. Prepare four sets of SD agar plates using the appropriate 10× dropout solutions (see *Synthetic Minimal Medium* in *Preparation of Media and Reagents*) to test the cdc25H yeast strain for the following nutritional requirements: tryptophan (Trp), leucine (Leu), histidine (His), and uracil (Ura). Streak yeast from the –80°C glycerol stock onto each of the four agar "dropout" plates (as outlined in steps 1 and 2 of *Establishing an Agar Plate Yeast Streak*) and incubate the plates at room temperature (22–25°C) for 4–6 days.

2. Simultaneously streak a sample of the same glycerol stock onto a YPAD agar plate, and incubate the plate at room temperature (22–25°C) for 4–6 days.

3. After the phenotype has been verified (growth on the YPAD plate and no growth on any of the four SD agar dropout plates), use colonies from the YPAD plate to inoculate medium for the preparation of competent yeast cells (see *Preparation of cdc25H Yeast Competent Cells*).

# CONTROL PLASMIDS

## Description

The CytoTrap system includes two negative control plasmids (Figure 4) and three positive control plasmids (Figure 5). The pSos Collagenase I (pSos Col I) control plasmid expresses the Sos protein and amino acids 148–357 of murine 72-kDa type IV collagenase.[3] The pMyr Lamin C control plasmid expresses the myristylation signal fused to human lamin C (aa 67–230).[4] pSos MAFB expresses the Sos protein and full-length MAFB as a hybrid protein.[5] The pMyr MAFB control plasmid expresses a hybrid protein that contains the myristylation signal fused to full-length MAFB. The pMyr SB control plasmid expresses the myristylation signal fused to a Sos-binding protein. Table II provides a summary of the features of each control plasmid.

**TABLE II**
**Description of Control Plasmids**

| Control plasmid | Insert description | Genotype |
|---|---|---|
| pSos Col I | Murine 72 kDa type IV collagenase (aa 148–357) | *LEU2*, Amp$^r$ |
| pMyr Lamin C | Human Lamin C (aa 67–230) | *URA3*, Cam$^r$ |
| pSos MAFB | Full length MAFB | *LEU2*, Amp$^r$ |
| pMyr MAFB | Full length MAFB | *URA3*, Cam$^r$ |
| pMyr SB | Sos-binding protein | *URA3*, Cam$^r$ |

## Negative Controls

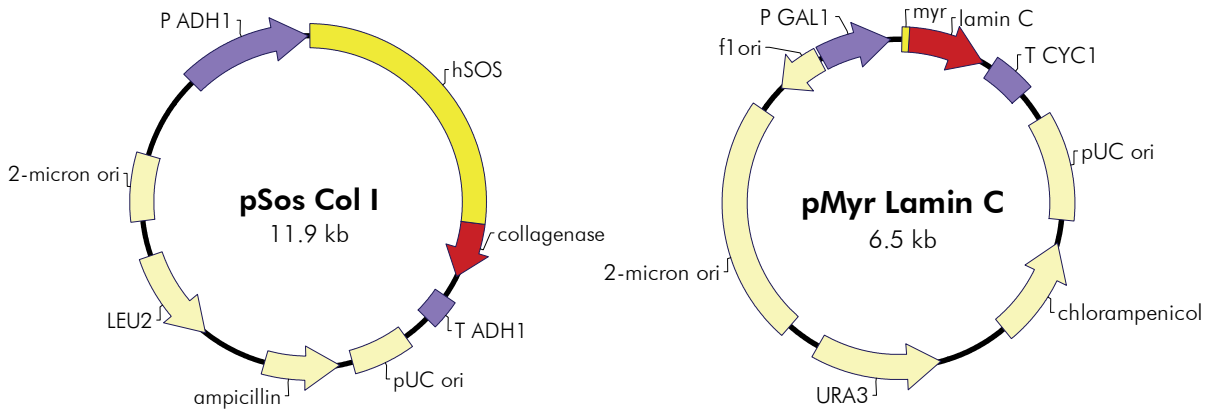



**FIGURE 4** Circular maps of the pSos Col I and pMyr Lamin C control plasmids.

## Positive Controls

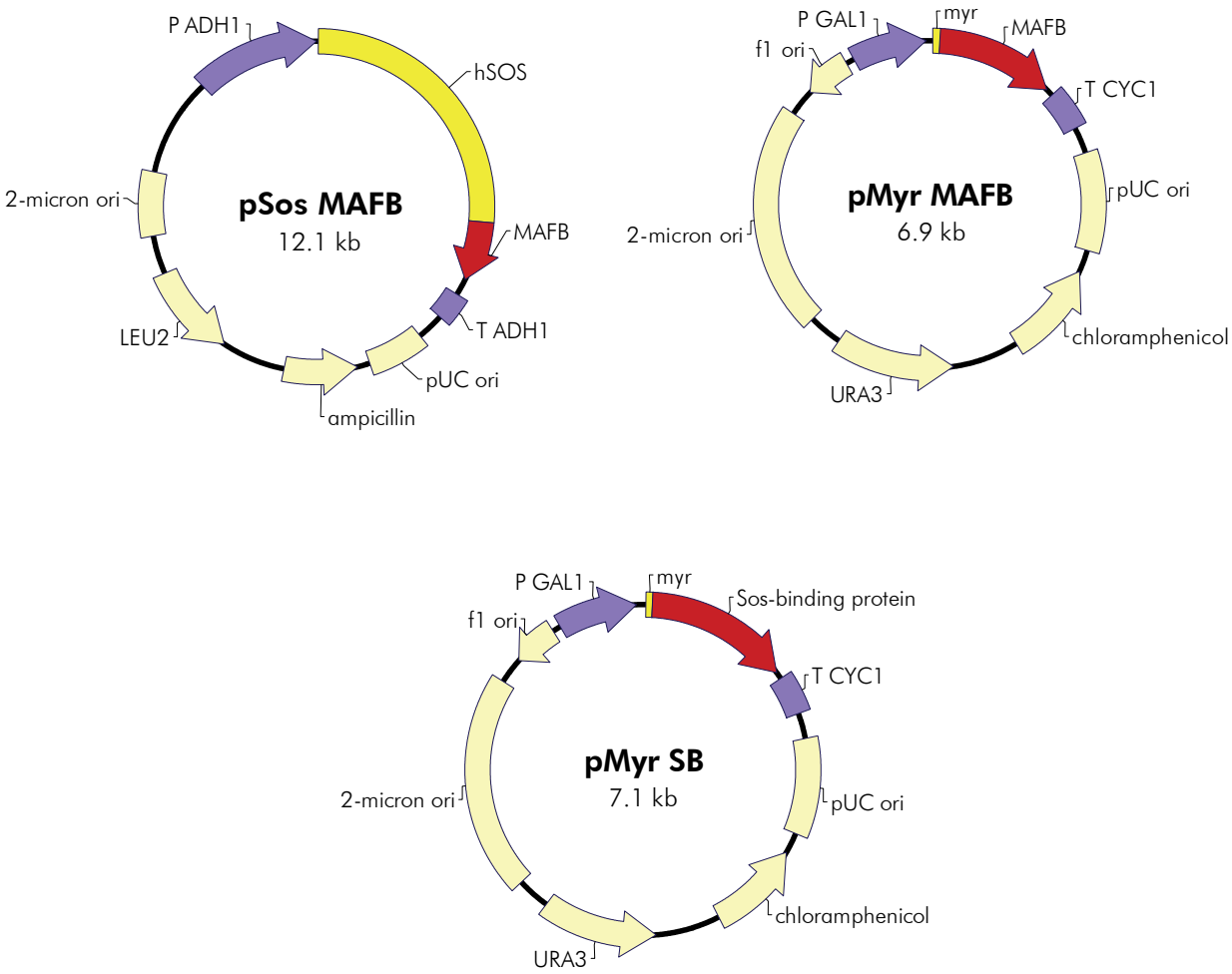



**FIGURE 5** Circular maps of the pSos MAFB, pMyr MAFB, and pMyr SB positive control plasmids.

## Applications

The CytoTrap control plasmids are used in pairwise combination as positive and negative controls for the rescue of the temperature-sensitive phenotype of cdc25H strain. The protein products expressed from pSos MAFB and pMyr MAFB interact with each other in vivo. Interaction of the hybrid proteins localizes hSos to the cell membrane, activating the Ras pathway, and permitting growth of cdc25H mutants at the restrictive temperature of 37°C. The pSos MAFB + pMyr Lamin C plasmid pair, and the pSos Col I + pMyr MAFB plasmid pair serve as negative controls, since the protein products produced in each of these two pairwise combinations do not interact in vivo (thus co-transformation does not enable growth of cdc25H mutants at 37°C). The pMyr SB plasmid expresses a Sos-binding protein fused to the myristylation signal. The Sos-binding protein interacts with the Sos protein. Thus cdc25H yeast cotransformed with pMyr SB and pSos grow at 37°C. Cotransformation of pMyr SB with pSos bait plasmid can be used to verify that the Sos bait fusion protein is properly localized in the cytoplasm.

## Expected Results

The expected results for transformation of control plasmids in pairwise combination into the cdc25H strain when plated on selective media and assayed for growth at 37°C are outlined in Table III. Expression of the pMyr fusion protein is induced by the presence of galactose and is repressed by the presence of glucose in the growth medium.

**TABLE III**
**Expected Results for Interaction of the Control Plasmids**

| Control plasmids | | SD (–UL)/25°C | | SD (–UL)/37°C | |
|---|---|---|---|---|---|
| **Sos fusion** | **Myr fusion** | **Glucose** | **Galactose** | **Glucose** | **Galactose** |
| MAFB | MAFB | + | + | – | + |
| Col I | MAFB | + | + | – | – |
| MAFB | Lamin C | + | + | – | – |
| MAFB | SB | + | + | – | + |

# BAIT PLASMID CONSTRUCTION

## Vector and Insert Preparation, Ligation and Transformation

DNA encoding the bait protein is prepared for insertion into the pSos vector either by restriction digestion or PCR amplification. DNA encoding the bait protein must be inserted so that the bait protein is expressed in the same reading frame as the hSos protein. In the MCS of the pSos vector, the *Hin*d III, *Bam*H I, *Nco* I, *Srf* I, *Aat* II, *Sal* I, *Bss*H II, *Mlu* I, *Sac* I, and *Not* I sites are unique; **however, the *Pac* I, *Sac* II, *Xba* I, and *Sma* I sites are not**.

To reduce the background, dephosphorylate digested pSos vector with CIAP prior to ligation with the insert DNA.[6] If more than one restriction enzyme is used, the background can be reduced further by selective precipitation using ammonium acetate, eliminating the small fragment that appears between the two restriction enzyme sites.

1. Digest 5 µg of the vector DNA in a final volume of 50 µl.

2. Extract with an equal volume of phenol–chloroform until a clear interface is obtained.

3. Repeat the extraction once with an equal volume of chloroform only.

4. Add an equal volume of 4 M $NH_4OAc$ to the aqueous phase.

5. Add 2.5 volumes of 100% (v/v) ethanol at room temperature. Immediately spin in a microcentrifuge at room temperature to precipitate the vector DNA.

6. Wash the pellet once with 70% (v/v) ethanol.

7. Resuspend the pellet in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the vector DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

8. Ligate the prepared vector and insert DNA fragments according to the protocol in the table below. Incubate the ligation reaction mixtures overnight at 12°C.

   ***Note*** *The ideal ratio of insert-to-vector DNA for ligation is variable; however, a reasonable starting point is 1:1 (insert-to-vector molar ratio), measured in available picomole ends. This is calculated as follows:*

$$\text{Picomole ends / microgram of DNA} = \frac{2 \times 10^6}{\text{number of base pairs} \times 660}$$

See below for a suggested ligation protocol, which includes three control ligations:

| Ligation Reaction Components | Experimental | | Control | | |
|---|---|---|---|---|---|
| | 1[a] | 2[a] | 3[b] | 4[c] | 5[d] |
| Prepared vector (0.1 µg/µl) | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl | 0 µl |
| Prepared insert (0.1 µg/µl) | *X* µl | *X* µl × 2 | 0 µl | 0 µl | 1.0 µl |
| 10 mM rATP (pH 7.0) | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl |
| 10× ligase buffer[§] | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl | 1.0 µl |
| T4 DNA ligase (4 U/µl) | 0.5 µl | 0.5 µl | 0.5 µl | 0 µl | 0.5 µl |
| Double-distilled water (to 10 µl) | *Y* µl | *Y* µl | *X* µl | *X* µl | *X* µl |

[a] Experimental samples 1 and 2 vary the insert-to-vector ratio.

[b] Control sample 3 tests the effectiveness of vector digestion and CIAP treatment.

[c] Control sample 4 tests for residual uncut vector in the vector preparation.

[d] Control sample 5 tests to ensure the insert alone is not contaminated with the vector DNA.

[§] See *Preparation of Media and Reagents*.

9. Transform each of the ligation reaction mixtures 1–5 (above) into *E. coli* competent cells.

## Verifying Bait Insert Cloning and Expression

Select isolated colonies for miniprep analysis to identify transformed colonies containing the pSos vector with the DNA insert. The nucleotide sequence of the cloning junctions and DNA insert should be determined to verify that the bait protein will be expressed in frame with the Sos domain and that the DNA insert does not contain mutations.

Expression of the bait protein may be verified by Western blot analysis using an antibody that immunoreacts either with the protein expressed from the DNA insert or with the hSos protein (see *Verifying Bait Protein Expression*). However, if the antibody used fails to detect expression of the bait protein, the bait protein may still be useful for detecting two-hybrid interactions. The ability of the antibody to detect the bait protein is dependent on several factors including the affinity of the antibody for the bait protein and the expression level of the bait protein. After analyzing expression of the bait protein by western blot analysis, the cytoplasmic localization of the bait protein can be verified by cotransformation of pMyr SB and the pSos bait plasmid followed by patching on galactose-containing medium and assaying for growth at 37°C.

## Verification of Bait Plasmid Suitability for CytoTrap Interaction Assays

Prior to initiating a CytoTrap two-hybrid assay or screen using a particular bait, we recommend verifying that the pSos-bait fusion does not interact with the myristylation signal in the absence of an interaction partner. The pSos bait plasmid (containing the gene of interest) must be cotransformed into the yeast host with either pMyr or pMyr Lamin C to establish that the

bait protein does not interact with the myristlyation signal provided by these negative control plasmids. Perform the co-transformation according to the protocols outlined in *Yeast Transformation*, below. After cotransformation, incubate the plates initially at room temperature (22–25°C) to allow colony formation. Patch colonies arising at room temperature onto fresh plates containing galactose, and assay for growth at 37°C. If the bait plasmid cotransformed with the pMyr empty vector or pMyr Lamin C can induce cdc25H yeast growth at 37°C, then the bait plasmid is unsuitable for detecting protein-protein interactions in the CytoTrap system. Induction of growth of the yeast host at 37°C by the bait plasmid may also occur if the bait protein contains sequences that target them to the membrane. This problem may be resolved by deleting portions of the bait protein, however deletions may also eliminate portions of the protein required for interaction with the target protein.

# TARGET PLASMID OR LIBRARY CONSTRUCTION

## Single Target Plasmid Construction

DNA encoding the target protein is prepared for insertion into the pMyr vector either by restriction digestion or PCR amplification. DNA encoding the target protein must be inserted into the pMyr vector so that the target protein is expressed in the same reading frame as the Myr coding sequences. In the MCS of the pMyr vector, the *Hin*d III, *Eco*R I, *Srf* I, *Sma* I, *Xho* I, and *Sal* I sites are unique; **however, the *Xba* I and *Spe* I sites are not**.

For cloning protocols, follow the recommendations given in *Vector and Insert Preparation, Ligation and Transformation* in the manual section *Bait Plasmid Construction.*

## Target Library Construction

The CytoTrap system is particularly useful for the identification of novel target proteins from a cDNA library that interact with a bait protein, and for the subsequent determination of protein domains or amino acids critical for the interaction. Specific mutations, insertions, or deletions that affect the encoded amino acid can be introduced into DNA encoding the target protein, and the mutant target proteins can be assayed for protein–protein interaction with the bait protein.

### cDNA Libraries

DNA inserts to be ligated into the pMyr vector may be prepared from either mRNA or genomic DNA. If the inserts are to be prepared from mRNA, the Stratagene cDNA Synthesis Kit is highly recommended. The cDNA Synthesis Kit provides the reagents required to convert mRNA to cDNA inserts prior to unidirectional insertion into the pMyr vector. cDNA inserts produced with the cDNA Synthesis Kit have *Eco*R I and *Xho* I sites at the 5´ and 3´ ends, respectively, suitable for unidirectional insertion into *Eco*R I plus *Xho* I-digested pMyr vector. The protocol for inserting prepared DNA may be found in *Insert Preparation, Ligation and Transformation*.

# YEAST TRANSFORMATION

## Cotransformation of Bait and Target Plasmids

The bait and target plasmids may be introducted into the cdc25H yeast strain by cotransformation. This strategy is recommended because it allows results to be generated 5 days faster than a sequential transformation strategy[7] and because it limits the number of generations of growth prior to the interaction assay, and thus reduces the incidence of cdc25H reversion leading to false positives. Cotransformation is especially useful when the bait plasmid is toxic to the yeast cells, thereby hindering the preparation of competent cells containing the bait plasmid. Toxicity of the bait protein can be determined by comparing growth curves of cdc25H yeast competent cells containing the bait plasmid and cdc25H yeast competent cells containing the pSos Col I plasmid grown in selective media.

Plasmids may also be transformed singly into yeast competent cells, followed by mating of the bait plasmid-containing and target plasmid-containing yeast strains. If you are performing single transformations, transform each plasmid into both mating types (*a* and α) of cdc25H to facilitate subsequent mating.

## Transformation of Control Plasmids

Users new to the yeast two-hybrid system should transform the control plasmids (described in Table IV) into the cdc25H strain prior to the initial transformation of the bait and target plasmids to gain familiarity with the protocol and expected results. After the protocols are familiar, the user should carry out at least one positive and one negative control transformation in parallel with each bait and target plasmid transformation, as indicators of yeast growth levels in the presence and absence of interacting proteins. For the control plasmid transformation, follow the procedures outlined *in Preparation of cdc25H Yeast Competent Cells* and *Transforming Yeast and Detecting Protein-Protein Interactions,* using the control plasmid combinations outlined in Table IV.

## Preparation of cdc25H Yeast Competent Cells

Figure 6 shows an overview of the stages for preparing yeast competent cells. This protocol outlines a strategy for verifying that the cell culture used to prepare competent cells does not contain revertants of the cdc25H temperature-sensitive phenotype. If revertants have arisen during the preparation of competent cells, the competent cell preparation is not suitable for CytoTrap two-hybrid experiments.

***Notes*** *A number of specialized media and reagents are required for the protocols in this and subsequent sections of the procedure. Consult the* Preparation of Media and Reagents *section for detailed recipes and instructions for media and reagent preparation.*

*This protocol yields 6.8 ml of yeast competent cells, which is enough for the initial transformations (see Table IV). For transforming a pMyr cDNA library, 10.5 ml of yeast competent cells is required. Instead of scaling up the protocol, we recommend carrying out the protocol given below in two parallel flasks/tubes, producing 2 × 6.8 ml of yeast competent cells.*

1. Prepare a fresh plate of cdc25H (*a* or α, see ***Note*** below) from the glycerol stock by scraping off splinters of solid ice with a sterile wire loop. Streak the splinters onto a YPAD agar plate. Incubate the plate at room temperature (22–25°C) until colonies appear (~4 days). Alternatively use a previously prepared plate (less than one week old).

   ***Note*** *Prepare competent cells from both mating types (*a *and α), if performing single plasmid transformations that will be mated.*

2. Pick 4–5 cdc25H yeast colonies (from a plate that is less than one week old) into separate 1.5-ml microcentrifuge tubes containing 1 ml YPAD. Vortex vigorously until cell clumps are completely dispersed.

   ***Note*** *Due to the ability of the cdc25H strain to produce revertants of the temperature-sensitive phenotype during growth, generating 4–5 independent preparations of yeast competent cells is recommended.*

3. Transfer the yeast cell suspensions into 250-ml flasks, each containing 50 ml YPAD. Incubate at room temperature (22–25°C) with shaking at 220–250 rpm for 14–16 hours.

4. Measure the $OD_{600}$ of the cultures. It must be > 1. If the $OD_{600}$ is < 1, continue incubating the culture and monitoring the $OD_{600}$. If after 19 hours the $OD_{600}$ is not greater than 1, it is necessary to start again with step 2 above, making sure that the yeast plate from which the colonies are picked is not more than one week old.

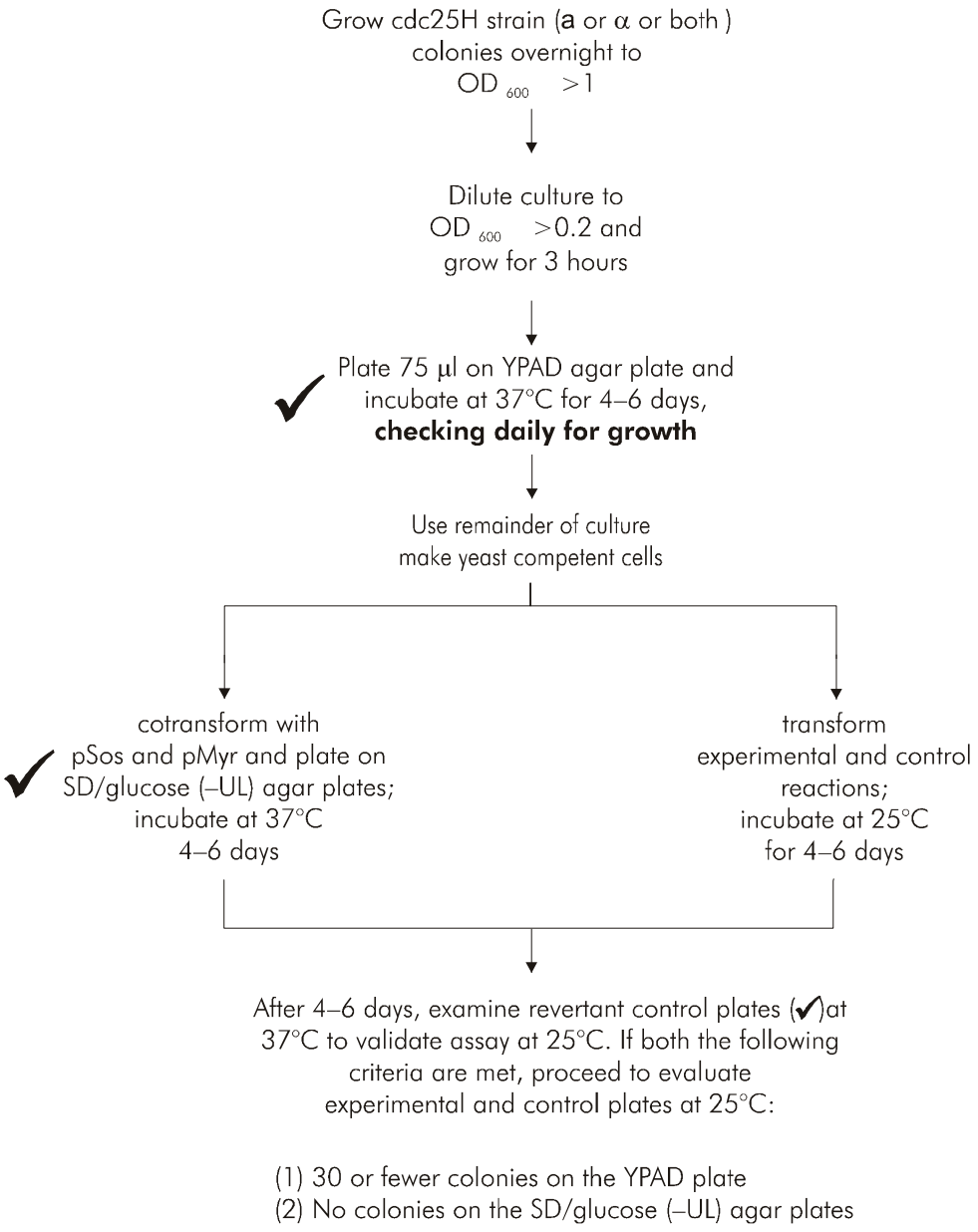



**FIGURE 6** Strategy for preparation and phenotypic verification of yeast competent cells

5. Prepare dilutions of the overnight cultures in 1- or 2-liter flasks for a total diluted culture volume of 300 ml. Use the appropriate amount of fresh YPAD medium and of the overnight cultures such that $OD_{600}$=0.2. Incubate the cultures at room temperature (22–25°C) with shaking at 220–250 rpm for 3 hours.

6. Measure the $OD_{600}$ of the cultures. It must be > 0.7. Plate 75 µl (approximately $1 \times 10^6$ cells) of each culture on a YPAD agar plate, seal the plates with Parafilm, and incubate the plates at 37°C. Observe the plates daily for 4–6 days, checking for temperature-sensitive revertants. If, up to the 6th day of incubation, more than 30 colonies appear on a plate, the yeast competent cell preparation corresponding to that plate is unreliable and all transformants arising from these competent cells are invalid (see *Evaluation of Control Plates to Determine Success of Yeast Competent Cell Production*).

7. Pellet the remaining volume of the yeast cultures by centrifugation at 1000 × g for 5 minutes at room temperature. Discard the media and resuspend the yeast cell pellets in 50 ml of $dH_2O$ by repeated pipetting with a 10-ml pipet. Spin the yeast cells at 1000 × *g* for 10 minutes at room temperature.

8. Discard the supernatant and resuspend the yeast cell pellet in 50 ml of LiSORB.[§] Incubate the cell suspension at room temperature for 30 minutes.

9. During this 30-minute incubation of the yeast cells, for each of the independent yeast cultures, place 400 µl of 20 mg/ml sheared salmon sperm DNA[§] in a boiling water bath and incubate for 10 minutes. After boiling, add 600 µl of LiSORB to each 400 µl salmon sperm DNA and mix by pipetting. Cool the salmon sperm DNA mixture to room temperature.

10. At the end of the 30-minute incubation, pellet the yeast cells by spinning at 1000 × *g* for 5 minutes at room temperature. Resuspend the yeast cell pellets in 300 µl of LiSORB.

11. Add 600 µl of salmon sperm DNA mixture from step 9 to the 300 µl of yeast cells from step 10. Mix thoroughly but gently by pipetting.

12. Add 5.4 ml of PEG/LiOAc solution[§] and 530 µl of DMSO to each cell preparation. Mix thoroughly but gently by pipetting. Aliquot 500 µl of competent cells into one microcentrifuge tube and make additional aliquots of 100 µl in separate microcentrifuge tubes. (For the transformations outlined in Table IV, 10 × 100 µl aliquots are required). Best results are obtained if the aliquots of yeast competent cells are used immediately. Do not freeze at –80°C for later use.

[§] See *Preparation of Media and Reagents*.

## Transforming Yeast and Detecting Protein-Protein Interactions

1. Prepare the yeast transformation mixtures outlined in Table IV below in microcentrifuge tubes. Add plasmid DNA in the combinations listed (or singly if the resulting transformants will be mated) to separate aliquots of freshly prepared cdc25H yeast competent cells (see *Preparation of cdc25H Yeast Competent Cells*).

   ***Note*** *Single plasmid transformations should be performed in both mating types to facilitate subsequent mating.*

**TABLE IV**
**Transformations for Detecting Protein-Protein Interactions**

| Number | Plasmid(s) | Amount of Plasmid | Volume of Yeast Competent Cells | Medium for Plating |
|---|---|---|---|---|
| 1[a] | pSos + pMyr | 2 µg each | 500 µl | see step 9 |
| 2 | pSos MAFB | 100 ng | 100 µl | SD/glucose (–L)* |
| 3 | pMyr SB | 100 ng | 100 µl | SD/glucose (–U)* |
| 4 | pMyr Lamin C | 100 ng | 100 µl | SD/glucose (–U)* |
| 5[b] | pSos MAFB + pMyr MAFB | 300 ng each | 100 µl | SD/glucose (–UL)* |
| 6[c] | pSos MAFB + pMyr Lamin C | 300 ng each | 100 µl | SD/glucose (–UL)* |
| 7[c] | pSos Col I + pMyr MAFB | 300 ng each | 100 µl | SD/glucose (–UL)* |
| 8[b] | pSos MAFB + pMyr SB | 300 ng each | 100 µl | SD/glucose (–UL)* |
| 9 | pSos Bait | 100 ng | 100 µl | SD/glucose (–L)* |
| 10[c] | pSos Bait + pMyr Lamin C | 300 ng each | 100 µl | SD/glucose (–UL)* |
| 11[d] | pSos Bait + pMyr SB | 300 ng each | 100 µl | SD/glucose (–UL)* |

[a] This cotransformation is used to determine the number of yeast revertants and the transformation efficiency.
[b] These cotransformations serve as positive controls.
[c] These cotransformations serve as negative controls.
[d] This cotransformation is a control that confirms the integrity of the pSos vector (the SB protein expressed from pMyr SB interacts with the Sos protein expressed from pSos Bait to rescue the growth at 37°C when plated on SD/galactose (–UL)).
* For complete plating instructions, see step 10.

2. Add 2 µl of 1.4 M β-mercaptoethanol to each tube. Mix the contents of each tube thoroughly but gently by inversion or tapping.

3. Incubate the transformation suspensions at room temperature for 30 minutes with occasional tapping.

4. Heat-shock the transformation suspensions at 42°C for 20 minutes.

5. Place the transformation suspensions on ice for 3 minutes.

6. Collect the cells by centrifugation for 30 seconds at 14,000 rpm at room temperature. Remove and discard the supernatant from each tube.

7. Resuspend cells in 0.5 ml of 1 M sorbitol.

8. Spread each transformation mixture on agar plates as indicated in Table IV and in steps 9 and 10 below. Spread cells by dropping approximately 10 non-acid washed glass beads on a plate, then add the transformation mixture, cover the plate, and shake or swirl until the mixture is spread on the surface of the plate. Once the plate is dry, pour off the glass beads.

   **Note** *It is very important to use glass beads for spreading to achieve even distribution of yeast colonies on the plates (see* Additional Materials Required*).*

9. For transformation 1, plate 10 µl and 100 µl of the cells on separate 150 mm SD/glucose (–UL) agar plates (see *Preparation of Media and Reagents*) and incubate these plates at room temperature (22–25°C). These platings are used to determine cotransformation efficiency. Plate the remainder of transformation reaction 1 on a 150-mm SD/glucose (–UL) agar plate. Incubate the inverted plate at 37°C, observing the plate daily for 4–6 days. This plate is used to check for temperature-sensitive revertants (refer to *Evaluation of Control Plates to Determine Success of Yeast Competent Cell Production*).

10. For transformations 2–11, plate the entire transformation reaction on a 100-mm SD/glucose plate (either SD/glucose (–U), SD/glucose (–L) or SD/glucose (–UL) according to Table IV). Incubate the inverted plates at room temperature (22–25°C) until colonies are visible (4–6 days).

## Evaluation of Control Plates to Determine Success of Yeast Competent Cell Production

1. Evaluate the quality of the cdc25H competent cell preparation by determining the frequency of temperature-sensitive revertants (see steps 2 and 3) and the transformation efficiency (see step 4) for the preparation.

2. Two control plates are used to test for temperature-sensitive revertants. The first control plate, from *Preparation of cdc25H Yeast Competent Cells*, step 6, contains a 75 µl aliquot of the yeast culture used to prepare competent cells. If, after incubation at 37°C for 4–6 days, this plate contains more than ~30 colonies, the culture contained a high number of temperature-sensitive revertants or a contaminant which is not cdc25H. The observation of more than 30 colonies on this plate invalidates any transformations performed using the corresponding competent cell preparation.

3. The second reversion control plate, from transformation #1 in Table IV, contains cells cotransformed with pSos and pMyr. After incubation of this plate 37°C for 4–6 days, no colonies should appear. Colonies present on this plate indicate that the cells used for the transformation contained temperature-sensitive revertants or were not cdc25H. This observation invalidates the transformations performed to detect protein-protein interactions with the corresponding competent cell preparation.

4. If the reversion control plates meet the criteria in steps 2 and 3 above, evaluate the transformation efficiency for the competent cell preparation. Count the number of colonies on plates from transformation #1 (Table IV) incubated at room temperature (22–25°C). For accurate counting, there should be at least 30 and no more than 300 cfu/plate.

5. Calculate the cotransformation efficiency using the following equation. The transformation efficiency should be at least $0.5 \times 10^3$–$1 \times 10^4$ cfu/μg.

$$\frac{\text{Number of cfu} \times \text{Total suspension volume (500 μl)}}{\text{Volume of transformation plated (μl)} \times \text{Amount of DNA used (2μg)}} = \text{cfu/μg DNA (Transformation efficiency)}$$

6. If the reversion control and transformation efficiency results are satisfactory, proceed to *Evaluation of Transformations Prepared to Detect Protein-Protein Interactions*. (If single plasmid transformations were performed, proceed to *Yeast Mating of Single Plasmid Transformants*.)

## Evaluation of Transformations Prepared to Detect Protein-Protein Interactions

***Note*** *This section describes the processing of transformation reactions prepared according to* Table IV*, in* Transforming Yeast and Detecting Protein-Protein Interactions.

1. Transformation 1 was used to calculate transformation efficiency and to test for reversion of the temperature-sensitivity of cdc25H. The plates derived from this transformation will not be used further.

2. Transformants from reactions 2, 3, 4, and 9, plated in step 10 of *Transforming Yeast and Detecting Protein-Protein Interactions,* contain only single plasmids, and the colonies from these plates will not be assayed for two-hybrid interactions at 37°C. Colonies from these plates can be used in mating experiments (see *Yeast Mating of Single Plasmid Transformants*). If storage of the transformants is desired, wrap the plates in Parafilm and store them at 4°C.

3. Examine plates containing transformations 5–8, 10, and 11 (plated in step 10 of *Transforming Yeast and Detecting Protein-Protein Interactions* and incubated at room temperature*),* and compare the growth at room temperature (22–25°C) to that expected (see Table V).

4. Select at least three colonies from each of these transformations (5–8, 10, and 11) for transfer to SD/glucose (–UL) and SD/galactose (–UL)[§] to test for protein-protein interactions that allow growth at 37°C.

5. For each colony to be picked, aliquot 25 µl of sterile $H_2O$ to wells of sterile 96-well plates. Transfer each colony to be screened to separate wells, resuspending the yeast colony in the sterile $H_2O$.

6. Spot 2.5 µl of the yeast/ $H_2O$ suspensions onto each of two SD/galactose (–UL) agar plates and two SD/glucose (–UL) agar plates.

7. Incubate one plate of each type at 37°C. Keep the second plate of each type at room temperature (22–25°C) for 5 days.

8. Score the growth at 37°C after at least 5 days' incubation time. The SD/galactose (–UL) agar plates that are incubated at 37°C may require up to 7–10 days' incubation time for yeast colonies to appear. The expected results are outlined in Table V.

**TABLE V**
**Expected Results for Yeast Transformations**

| Number | Plasmid (s) Transformed | SD Glucose [(–UL), (–U), or (–L) as appropriate] /25°C | SD (–UL)/37°C (after spotting)* | |
|---|---|---|---|---|
| | | | Glucose | Galactose |
| 1 | pSos + pMyr | + | N.A. | N.A. |
| 2 | pSos MAFB | + | N.A. | N.A. |
| 3 | pMyr SB | + | N.A. | N.A. |
| 4 | pMyr Lamin C | + | N.A. | N.A. |
| 5 | pSos MAFB + pMyr MAFB | + | – | + |
| 6 | pSos MAFB + pMyr Lamin C | + | – | – |
| 7 | pSos Col I + pMyr MAFB | + | – | – |
| 8 | pSos MAFB + pMyr SB | + | – | + |
| 9 | pSos Bait | + | N.A. | N.A. |
| 10 | pSos Bait + pMyr Lamin C | + | – | – |
| 11 | pSos Bait + pMyr SB | + | – | + |

* Only the cotransformations are spotted and grown at 37°C

[§] See *Preparation of Media and Reagents*.

## Mating of Single Plasmid Yeast Transformants

### Yeast Mating on Plate Procedure (Standard)

1. Aliquot 25 µl of autoclaved $H_2O$ to wells of sterile 96 well plates. Pick one colony of each transformant to be mated into a separate well. See Table VI below for the mating combinations and expected results.

   **Note** *Table VI illustrates one mating experiment between transformants in mating type* a *(1st column) and transformants in mating type* α *(2nd column). It is not necessary that the mating take place exactly as outlined as long as the two plasmids to be tested for interaction are harbored in cells of opposite mating type.*

**TABLE VI**
**Yeast Mating Combinations for Single Plasmid Transformants**

| Yeast Transformation | | SD (–UL)/25°C | | SD (–UL)/37°C (after patching) | |
|---|---|---|---|---|---|
| Mating Type (*a*) | Mating Type (α) | Glucose | Galactose | Glucose | Galactose |
| pSos MAFB[a] | pMyr SB | + | + | – | + |
| pSos MAFB[b] | pMyr Lamin C | + | + | – | – |
| pSos Bait[c] | pMyr SB | + | + | – | + |

[a] This mating experiment serves as a positive control.

[b] This mating experiment serves as a negative control.

[c] This mating experiment is a control that confirms the integrity of the pSos vector (the SB protein expressed from pMyr SB interacts with the Sos protein expressed from pSos Bait to rescue the growth at 37°C when plated on SD/galactose (–UL)).

2. Spot 2.5 µl of each of the two yeast/$H_20$ suspensions to be mated onto the same position on a YPAD plate. Incubate the plate at room temperature (22–25°C) for 24 hours.

3. Aliquot 25 µl of autoclaved $H_20$ to wells of sterile 96 well plates. Transfer mated cells from each mating patch of the YPAD plate into a separate well.

4. Spot 2.5 µl of each of the yeast/$H_20$ suspensions on two SD/glucose (–UL) and two SD/galactose (–UL) agar plates. Transfer one plate of each type to 37°C. Keep the second plate of each type at room temperature (22–25°C). Score the growth at 37°C after at least 5 days' incubation time. The SD/galactose (–UL) agar plates that are incubated at 37°C may require up to 7–10 days' incubation time for yeast colonies to appear.

### Yeast Mating in Solution Procedure (Microtiter Plate Version)

1. Aliquot 200 µl of YPAD broth to wells of sterile 96 well plates. Pick one colony of the pair of transformants to be mated into the same well. See Table VI above for the mating combinations and expected results.

2. Incubate the plate on a rotating platform shaker (at 220–250 rpm) at room temperature (22–25°C) for 24 hours.

3. Spot 20 µl of each mating culture on two SD/glucose (–UL) and two SD/galactose (–UL) agar plates. Transfer one plate of each type to 37°C. Keep the other plates at room temperature (22–25°C). Score the growth at 37°C after at least 5 days' incubation time. The SD/galactose (–UL) agar plates that are incubated at 37°C may require up to 7–10 days' incubation time for yeast colonies to appear.

## VERIFYING BAIT PROTEIN EXPRESSION

### Protein Purification

1. Place 5 ml of SD/glucose (–L) media in a 50-ml conical tube. Inoculate the medium with a single colony of cdc25H transformed with the pSos bait plasmid.

2. Incubate the yeast culture at room temperature (22–25°C) with vigorous shaking (~250 rpm) until the culture is saturated (2–3 days, until $OD_{600}$>1.0).

3. Pellet the yeast cells by spinning the culture at 1000 × *g* for 5 minutes at room temperature.

4. Prepare the protein sample using one of the two following protocols.

#### Protocol A

1. Resuspend the yeast cell pellet in 200 µl of Cell Lysis Buffer for Protein Isolation (see *Preparation of Media and Reagents*) containing freshly added protease inhibitors:

   1 mM PMSF
   10 µg/ml aprotinin
   1 µM pepstatin A
   100 µM leupeptin
   1 µg/ml chymostatin

2. Vortex the cells for 5 minutes at 4°C with an equal volume of acid-washed glass beads (0.5 mm in diameter, Stratagene Products Division). Monitor cell lysis by phase-contrast microscopy until >70% of the yeast cells appear transparent (i.e., ruptured and void of cellular contents).

3. Collect the lysate by centrifugation at 12,000 × *g* for 5 minutes at 4°C.

4. Transfer the supernatants to fresh 1.5-ml screw-cap tubes and place on ice.

5. Add 100 µl of Cell Lysis Buffer for Protein Isolation to the pellet/glass beads and vortex vigorously for 5 minutes at 4°C.

6. Collect the lysate by centrifugation at 12,000 × *g* for 5 minutes at 4°C.

7. Combine each supernatant with the corresponding first supernatant.

### Protocol B

1. Resuspend the yeast cell pellet with 1 ml of cold $dH_2O$.

2. Add 150 µl of freshly-made NaOH/β-ME buffer[§] to the cell suspension.

3. Vortex the cells for 30 seconds and incubate on ice for 15 minutes.

4. Vortex again then add 150 µl of 55% TCA (in water). Vortex and place the cells on ice for 10 minutes.

5. Collect the protein extracts by centrifugation at 12,000 × *g* for 10 minutes at 4°C. Remove the supernatant and centrifuge again to remove any residual supernatant.

6. Resuspend the pellet in 300 µl of SU buffer.[§] (Add 1–2 µl of Tris base if the solution turns yellow.) Vortex to resuspend the protein pellet. Heat at 65°C for 3 minutes prior to loading to SDS/PAGE.

## Western Analysis

Analyze by standard western blot techniques using the mouse monoclonal anti-Sos antibody (BD Biosciences) or an antibody that immunoreacts with the bait protein.

[§] See *Preparation of Media and Reagents.*

# LIBRARY SCREENING

An overview of the library screening process is shown in Figure 7. Before each library screening, prepare fresh yeast competent cells as described in *Preparation of Yeast cdc25H Competent Cells.* We recommend testing for temperature-sensitive revertants every time competent cells are prepared (see *Evaluation of Control Plates to Determine Success of Yeast Competent Cell Production*).

Initially, the pMyr cDNA library and pSos bait cotransformant colonies are selected at permissive temperature, then candidate interactors are identified by transferring the cotransformants to 37°C. "Putative positives" are identified among the candidates by two rounds of testing for galactose-dependent growth at 37°C. The putative positives are subjected to further analysis to verify the interaction. Verification strategies include either cotransformation of naive cdc25H yeast with purified plasmid DNA from the putative positive colony and the bait plasmid or curing the putative positive colonies of the bait plasmid, and mating the cured strains to a naïve cdc25H strain harboring the bait plasmid. If mating is to be performed, prepare cdc25H *a* strain competent cells and transform them with pSos Col I, pSos MAFB, and the pSos bait construct so that each of these single transformants is available to mate with the pMyr putative positive cDNA clones (harbored in the cdc25H α strain). If storage of the transformants is desired, wrap the plates in Parafilm and store at 4°C.

## Cotransformation and Identification of Putative Positive Interactors

1. Add 40 µg of pSos bait construct, 40 µg of pMyr cDNA plasmid library and 200 µl of 1.4 M β-mercaptoethanol to 10 ml of freshly prepared cdc25H (α) yeast competent cells in a 50-ml conical tube.

2. Mix the contents thoroughly but gently by inversion to ensure a homogenous mixture.

3. Transfer the contents into 20 separate microcentrifuge tubes.

4. As a negative control, in a separate microcentrifuge tube, add 2 µg of pSos plasmid, 2 µg of pMyr cDNA plasmid library, and 10 µl of 1.4 M β-mercaptoethanol to 500 µl of freshly prepared yeast competent cells.

5. Incubate the transformation mixtures at room temperature (22–25°C) for 30 minutes with occasional mixing.

6. Heat shock the transformation mixtures at 42°C for 20 minutes.

7. Place the transformation mixtures on ice for 3 minutes.

8. Collect the cells by centrifugation for 30 seconds at 14,000 rpm at room temperature (22–25°C). Remove the supernatant.

9. Resuspend cells in 0.5 ml of 1 M sorbitol.

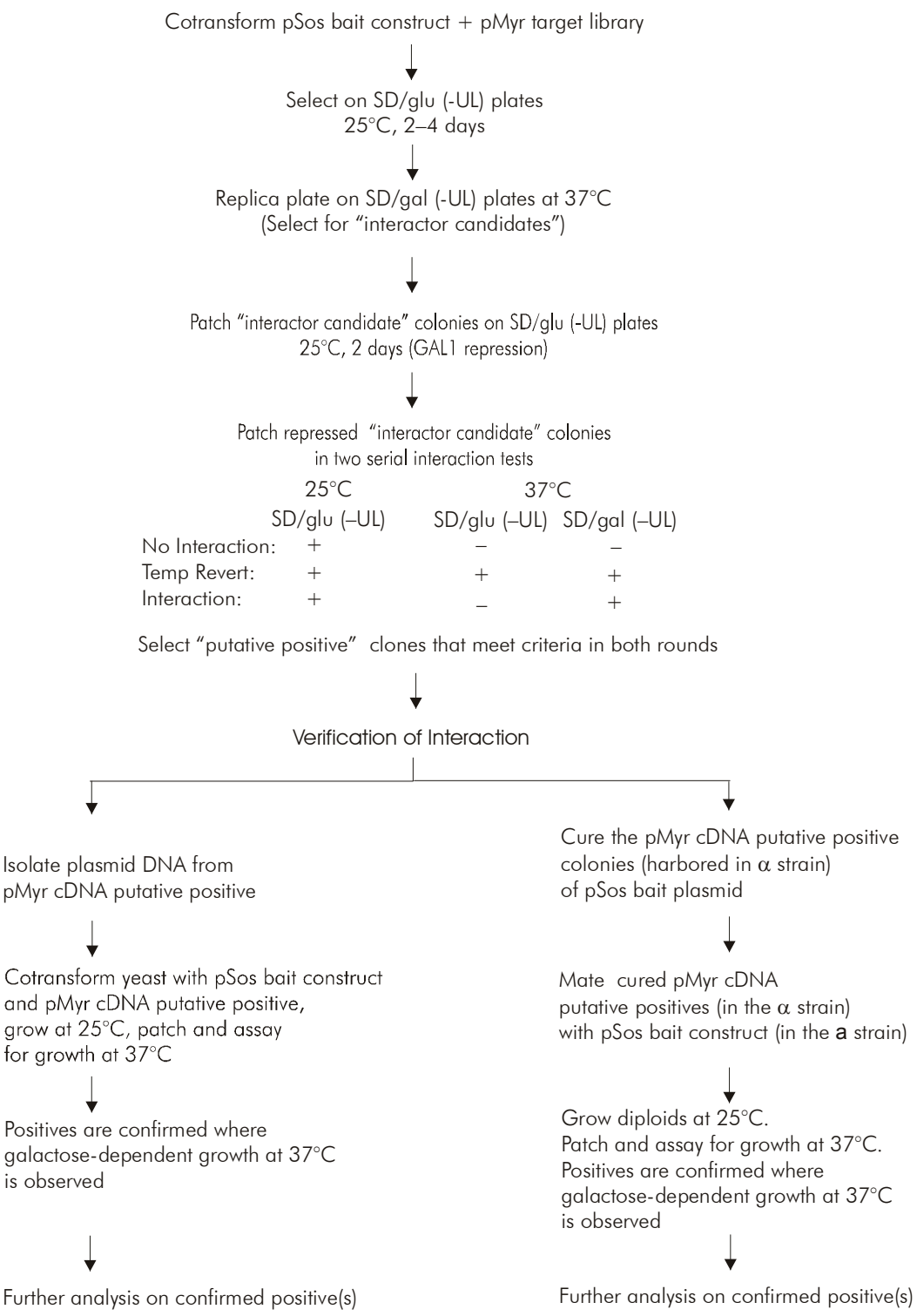



**FIGURE 7** Library screening procedure for the CytoTrap two-hybrid system.

10. Plate the entire transformation reaction in each tube on a 150-mm SD/glucose (–UL) agar plate. Drop approximately 10 non-acid washed glass beads on the plate, add the transformation reaction mixture, cover the plate, and shake or swirl the plate until the mixture is spread out. Once the plate is dry, pour off the glass beads.

    **Note** *It is very important to spread the cells using glass beads to achieve an even distribution of yeast colonies on the plates (see* Additional Materials Required*).*

11. Incubate the inverted plates at room temperature (22–25°C) for 48 hours. Increased incubation time will increase the sensitivity of detection but will also greatly increase background levels of temperature-sensitive revertants. The reversion frequency will be evaluated in step 15. Do not incubate the plates for more than 4 days.

    **Note** *It is important that the plates be kept at or below 25°C. Higher temperatures may induce mutational revertants.*

12. Replica plate the transformants onto SD/galactose (–UL) agar plates. Although no colonies are visible at 48 hours, the small colonies may be copied to the new plates by replica plating with the application of firm and even pressure. Incubate the plates at 37°C. Colonies should start to appear after 3 days.

13. Keep the SD/glucose plates at room temperature (22–25°C) to determine the transformation efficiency. Approximately $1 \times 10^4$–$2 \times 10^4$ colonies should form on each plate. To calculate the cotransformation efficiency, use the following equation.

$$\frac{\text{Number of cfu} \times \text{Total suspension volume (500 µl)}}{\text{Volume of transformation plated (µl)} \times \text{Amount of DNA used (2µg)}} = \text{cfu/µg DNA (Transformation efficiency)}$$

14. After 6 days, evaluate the pSos vector and pMyr cDNA control transformation replicas on SD/galactose (–UL). The number of colonies arising from the pSos vector and pMyr cDNA transformation growing at 37°C provides an estimate of the numbers of false positive clones from the cDNA library and of temperature-sensitive revertants.

15. Also after 6 days, pick colonies (interactor candidates) from the experimental library screen transformation replicates on galactose incubated at 37°C. In order to repress *GAL1* promoter-driven expression from the pMyr library prior to interaction tests, patch cells from the interactor candidate colonies onto an SD/glucose (–UL) agar plate (candidate patch plate), and incubate the plate at 22–25°C for 48 hours. Return the original galactose transformation replica plates to 37°C after picking colonies, since some additional colonies may appear much later (10 days).

16. After the 48 hour incubation (see step 15) patch cells from the SD/glucose (–UL) candidate patch plates onto two fresh SD/glucose (–UL) plates and one SD/galactose (–UL) plate. As a primary test to identify interactors among the candidates, incubate one SD/glucose (–UL) and the SD/galactose (–UL) plate at 37°C for approximately 48 hours. Keep the other SD/glucose (–UL) agar plate at room temperature (22–25°C) as a re-patching source plate.

17. After the 48 hour incubation, evaluate the primary interaction test plates (see step 16), identifying the patches growing at 37°C on SD/galactose (–UL) plates, but not on SD/glucose (–UL) plates. Perform a secondary interaction test by re-patching the interactor candidates from the re-patching source plate kept at 22–25°C (see step 16) onto another set of one SD/glucose (–UL) and one SD/galactose (–UL) agar plate, and incubate both plates at 37°C for 48 hours. The candidates producing patches that grow on SD/galactose (–UL) plates but not on SD/glucose (–UL) plates at 37°C in both the primary and secondary interaction tests should be considered "putative positive" clones and analyzed further (see *Verification of Interaction*).

# VERIFICATION OF INTERACTION

Verify the interaction between the pSos bait fusion protein and the putative positive interacting target proteins using one of the following two methods. Method A involves isolating pMyr library plasmids from putative positive clones and using them to retransform cdc25H cells with either the original bait or with irrelevant baits. Method B uses yeast mating as an alternative to yeast cotransformations.

## Method A: Yeast Cotransformation

### Isolation of pMyr cDNA Plasmid DNA from Yeast

Plasmid DNA can be isolated from yeast in sufficient quality and quantity to transform *E. coli* by the following procedure. This procedure yields a mixture of intact plasmid DNA and fragmented chromosomal DNA; therefore, the resultant plasmid DNA is not of sufficient purity for gel analysis.

1. Inoculate 5 ml of SD/glucose (–UL) media in a 50-ml conical tube with each of the putative positive clones.

2. Incubate the culture at room temperature (22–25°C) with vigorous shaking (~250 rpm) until the culture is saturated (2-3 days, $OD_{600}$>1.0).

3. Pellet the yeast culture at 1000 × *g* for 5 minutes at room temperature.

4. Resuspend the yeast pellet in 0.3 ml of Yeast Lysis Solution for DNA Isolation (see *Preparation of Media and Reagents*).

5. Transfer the suspension to a 1.5-ml microcentrifuge tube.

6. Add 50 µl of acid-washed glass beads (0.5 mm) and 300 µl of phenol/chloroform to the microcentrifuge tubes.

7. Vortex vigorously for one minute.

8. Spin the suspension at 14,000 × *g* for 5 minutes at room temperature.

9. Transfer the top aqueous phase containing the DNA to a new microcentrifuge tube.

10. Precipitate the DNA with 600 µl of 100% (v/v) ice-cold ethanol at –20°C overnight or at –80°C for 15 minutes.

11. Spin the suspension at 14,000 × *g* for 10 minutes at 4°C.

12. Decant the supernatant.

13. Wash the DNA pellet with 1 ml of 70% (v/v) ethanol and centrifuge at 14,000 × *g* for 5 minutes at room temperature.

14. Decant the supernatant and dry the DNA pellet under vacuum.

15. Resuspend the DNA pellet in 40 µl of $dH_2O$.

16. Precipitate the DNA with 4.8 µl of 3 M NaOAc (pH 5.2) and 100 µl of ethanol.

17. Repeat steps 11–14.

18. Resuspend the DNA pellet in 20 µl of $dH_2O$.

19. Transform high-efficiency electroporation-competent *E. coli* cells and select for pMyr cDNA plasmid by plating on LB-chloramphenicol agar plates.

20. Identify colonies that contain the pMyr cDNA plasmid by preparing miniprep DNA from isolated colonies from the LB-chloramphenicol agar plates and subjecting the DNA to restriction digest analysis.

## Verification of Specificity of Protein–Protein Interactions

To verify the specificity of the interaction between the bait and target proteins, transform yeast and plate on selective media as indicated in Table VII. Assay the transformants for the ability to grow at 37°C on SD/galactose agar plates.

1. Prepare and transform the yeast competent cells as described in *Preparation of Yeast cdc25H Competent Cells*. Cotransform the yeast competent cells with the plasmids outlined in Table VII, using 300 ng of **each** plasmid in each cotransformation reaction.

2. Plate each transformation reaction on separate 100-mm SD/glucose (–UL) plates. Incubate the inverted plates at room temperature (22–25°C) until colonies are visible (4–6 days).

3. Patch the transformants that grow on the SD/glucose (–UL) plates to two SD/glucose (–UL) and two SD/galactose (–UL) plates. Incubate one of each type of plates at room temperature (22–25°C) and at 37°C for > 4 days.

4. Determine the growth phenotype of the cotransformants and compare results to the expected results in Table VII.

**TABLE VII**

**Verification of the Specificity of the Interaction between the Bait and Target Proteins**

| Yeast transformation | SD Glucose (–UL)/25°C | SD (–UL)/37°C(after patching) | |
|---|---|---|---|
| | | Glucose | Galactose |
| pSos MAFB + pMyr MAFB[a] | + | – | + |
| pSos MAFB + pMyr Lamin C[b] | + | – | – |
| pSos Bait + pMyr cDNA putative positive[c] | + | –* | +* |

[a] This cotransformation serves as a positive control.

[b] This cotransformation serves as a negative control.

[c] Perform this transformation for each putative positive. There may be more than one.

* The combination of "– growth" on glucose and "+ growth" on galactose is confirmation of the putative positive.

## Method B: Yeast Mating

A segregant cdc25H (α) strain that contains only the pMyr cDNA plasmid is generated from the putative positive clones by a curing process. cdc25H strain (α) cells, containing the pMyr cDNA plasmid, are then mated with cdc25H strain (*a*) cells, transformed with the pSos bait plasmid or transformed with irrelevant baits. The diploids are then scored for galactose-dependent growth at 37°C.

### Generating Yeast Plasmid Segregants (Curing) for Mating

1. Culture individual cdc25H "putative positive" cotransformant colonies* (separately) in 3 ml of SD/glucose (–U) liquid medium for 2 days at room temperature (22–25°C) with shaking.

   * Obtained in step 16 of *Library Screening*.

2. Spread approximately 300–500 µl of the yeast culture on SD/glucose (–U) agar plates. Incubate the plates at room temperature (22–25°C) for 3–4 days or until colonies appear.

3. Using sterile pipette tips, transfer 30 individual colonies (in an orderly grid fashion) to SD/glucose (–U) and SD/glucose (–L) agar plates. Colonies that grow on SD/glucose (–U) agar plates but not on SD/glucose (–L) plates have lost the pSos plasmid and retained the pMyr cDNA plasmid. These colonies can be used for mating with a cdc25H strain (*a*) harboring any of the pSos plasmids (pSos Col I, pSos MAFB, and the pSos bait construct).

### Yeast Mating on Plate Procedure (Standard)

1. Aliquot 25 µl of sterile $H_2O$ to the wells of sterile 96 well plates. Pick colonies of each strain to be mated and transfer cells from individual colonies into separate wells. Strains to be mated include cdc25H (α) segregants containing only pMyr cDNA plasmids (see procedures above for the curing process) and cdc25H (***a***) transformants containing either pSos Col I, pSos MAFB, or the pSos bait plasmid. (The ***a*** strain transformants of the pSos plasmids were prepared previously and plated on SD/glucose (–L); see introductory section under the heading *Library Screening*.)

2. Spot 2.5 µl of the yeast/$H_2O$ suspension of both mating partners onto the same position on a YPAD plate. Incubate the plates at room temperature (22–25°C) for approximately 24 hours.

3. Aliquot 25 µl of autoclaved $H_20$ to wells of sterile 96 well plates. Transfer mated cells from each mating patch of the YPAD plate into a separate well.

4. Spot 2.5 µl of each of the yeast/$H_20$ suspensions on two SD/glucose (–UL) and two SD/galactose (–UL) agar plates. Transfer one plate of each type to 37°C. Keep the second plate of each type at room temperature (22–25°C). Score the growth at 37°C after at least 5 days' incubation time. The SD/galactose (–UL) agar plates that are incubated at 37°C may require up to 7–10 days' incubation time for yeast colonies to appear. The expected results are shown in Table VIII.

### Yeast Mating in Solution Procedure (Microtiter Plate Version)

1. Aliquot 200 µl of YPAD medium to wells of sterile 96 well plates. Pick a cdc25H (α) segregant containing only the pMyr cDNA plasmid (see procedures above for the curing process) and a colony of the appropriate cdc25H (***a***) transformant (containing either pSos Col I, pSos MAFB, or pSos bait plasmid) into the same well. Repeat this process for each of the combinations to be mated (see Table VIII).

2. Place the plate on a rotating platform shaker and incubate at room temperature (22–25°C) for 24 hours at 220–250 rpm.

3. Spot 20 µl of each mating culture on two SD/glucose (–UL) and two SD/galactose (–UL) agar plates. Transfer one plate of each type to 37°C. Keep the second plate of each type at room temperature (22–25°C). Score the growth at 37°C after at least 5 days' incubation time. The SD/galactose (–UL) agar plates that are incubated at 37°C may require up to 7–10 days' incubation time for yeast colonies to appear. The expected results are shown in Table VIII.

**TABLE VIII**
**Verification of the Specificity of the Interaction between the Bait and Target Proteins**

| Yeast transformation | SD (–UL)/25°C | | SD (–UL)/37°C (after patching) | |
|---|---|---|---|---|
| | Glucose | Galactose | Glucose | Galactose |
| pMyr cDNA (putative positive) + pSos Col I[a] | + | + | – | – |
| pMyr cDNA (putative positive) + pSos MAFB[a] | + | + | – | – |
| pMyr cDNA (putative positive) + pSos Bait | + | + | –* | +* |

[a] These matings serve as negative controls. Perform both of them for each putative positive tested.

* The combination of "– growth" on glucose and "+ growth" on galactose is confirmation of the putative positive.

If transformants do not give the expected results, see *Troubleshooting*.

To identify the protein encoded by the target DNA, the nucleotide sequence of the target DNA can be determined and compared to protein and nucleotide sequence databases to identify related or homologous proteins. Oligonucleotide primers can be used to determine the nucleotide sequence of the target DNA. In addition, the target DNA can be used as a hybridization probe to screen the plasmid library for full-length target DNA clones and for clones with high homology to the target DNA. Discussion regarding further verification of protein–protein interactions can be found in numerous publications.[8, 9]

# TROUBLESHOOTING

## Two-Hybrid Vector System Screening

| Observation | Suggestion |
|---|---|
| More than 30 colonies appear on non-transformed plates when incubated at 37°C for at least 4 days | The culture contains a high number of temperature-sensitive revertants or a yeast strain that is not cdc25H. Discard the culture |
| The bait protein is not detected in Western blot analysis | Ensure the insert DNA is in the same reading frame as the Sos sequence |
| | The insert DNA is expressed at levels too low to be detectable with the antibody used. A low level of bait protein may be adequate in the two-hybrid assay. If the nucleotide sequence encoding the bait protein is correct, continue with the two-hybrid screening |
| | If the antibody does not have a sufficiently high affinity for the bait protein, the bait protein may be expressed but may not be detectable. If the nucleotide sequence encoding the bait protein is correct, continue with the two-hybrid screening |
| Transformation with the bait plasmid alone results in colonies at 37°C | The Sos bait fusion protein alone can localize to the membrane and activate the Ras-signaling pathway. Subclone portions of the bait protein (see *Yeast Transformation and Assay for Growth Phenotype*) |
| The control plasmids do not give the expected results | Verify that correct control plasmid pairs are used |
| | Verify the phenotype of a yeast colony as described in *Yeast Host Strain Phenotype* and prepare new yeast competent cells using the same yeast colony |
| | Use sterile technique when preparing and transforming the yeast competent cells to avoid contamination of the culture with different yeast strain(s) or with *E. coli* |
| The transformants of the pSos MAFB and pMyr MAFB control plasmid pair do not grow at 37°C | Verify that media was made correctly to select for both control plasmids |
| | Verify that the correct control plasmid pair is used |
| The transformants of the pSos Col I and pMyr Lamin C control plasmid pair grow at 37°C | Verify that the correct control plasmid pair is used |
| Transformants show no GAL dependent growth at 37°C | Verify the nucleotide sequence of the Sos and insert DNA in pSos to verify the integrity of the bait protein coding sequences |
| | It is possible that the target proteins exist at a low frequency in the library; prepare and screen additional cotransformants; screen a different library |
| | It is possible that the target proteins do not exist in the library. Screen a library in which the bait protein is a known member |
| | Verify the pH of the SD agar plates using a pH indicator strip |
| | Vary the fusion point of the Sos and the bait protein to avoid problems caused by steric inhibition |
| | Verify that the competent cells containing the bait plasmid were used to transform the target plasmid(s) |
| Absence of $Amp^r$ or $Cam^r$ colonies when *E. coli* is transformed with DNA isolated from yeast | Transform *E. coli* with a greater volume of isolated DNA or reisolate plasmid DNA to insure sufficient yield of plasmid DNA from yeast plasmid isolation |
| | Continue incubation of the transformants to check for slow growth rate of the $Cam^r$ transformants |
| No discernible bands following restriction analysis of the recovered plasmid DNA | Transform *E. coli* with plasmid DNA isolated from yeast before restriction analysis to ensure that the plasmid DNA is not contaminated with yeast chromosomal DNA |

## PREPARATION OF MEDIA AND REAGENTS

### Synthetic Glucose Minimal Medium [SD/Glucose (–UL)] (per Liter)

1.7 g of yeast nitrogen base without amino acids
5 g of ammonium sulfate
20 g of dextrose
add 17 g of Bacto agar for SD dropout agar plates

Adjust the total volume to 900 ml with $dH_2O$
Autoclave for 15 minutes at 121°C, cool to 55°C. Add 100 ml of the appropriate filter-sterilized 10× dropout solution (see *10× Dropout Solution*).

### Synthetic Galactose Minimal Medium [SD/Galactose (–UL)] (per Liter)

1.7 g of yeast nitrogen base without amino acids
5 g of ammonium sulfate
20 g of galactose
10 g of raffinose
add 17 g of Bacto agar for SD dropout agar plates

Adjust the total volume to 900 ml with $dH_2O$
Autoclave for 15 minutes at 121°C, cool to 55°C. Add 100 ml of the appropriate filter-sterilized 10× dropout solution (see *10× Dropout Solution*).

### Cell Lysis Buffer for Protein Isolation

140 mM NaCl
2.7 mM KCl
10 mM $Na_2HPO_4$
1.8 mM $KH_2PO_4$
1 % Triton® X-100 containing freshly added protease inhibitors:
1 mM PMSF
10 µg/ml aprotinin
1 µM pepstatin A
100 µM leupeptin
1 µg/ml chymostatin

### LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized $H_2O$ to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate)

### LB–Ampicillin Agar (per Liter)

1 liter of LB agar, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

### LB–Chloramphenicol Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 3 ml of 10-mg/ml-filter-sterilized chloramphenicol
Pour into petri dishes (~25 ml/100-mm plate)

### 1.4 M β-ME (yeast competent cells)

Dilute stock -mercaptoethanol 1:10 with sterile $dH_2O$ just prior to use

### NaOH/β-ME Buffer

1.85 M NaOH
7.5% β-Mercaptoethanol

## Salmon Sperm DNA

Boil 400 µl of 20 mg/ml sheared salmon sperm DNA* for 10 minutes
Add 600 µl of LiSORB to the salmon sperm DNA and mix by pipetting
Cool the salmon sperm DNA mixture to room temperature (not below room temperature or the mixture will gel)

* Sonicate or randomly shear the salmon sperm DNA. For higher efficiency, phenol–chloroform extract and resuspend in TE buffer at a concentration of 20 mg/ml. Store the aliquots at –20°C. Before use, boil the salmon sperm DNA for 5 minutes.

## LiSORB (per Liter)

100 mM LiOAc
10 mM Tris-HCl (pH 8.0)
1 mM EDTA
1 M sorbitol
Add $dH_2O$ to a volume of 1 liter
Verify that the pH is 8.0
Autoclave
Store at room temperature

## PEG/LiOAc Solution

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)
100 mM LiOAc (pH 7.5)
40% (w/v) PEG 3350
Autoclave

## TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

## YPAD Agar (30–40 Plates/Liter)

1% yeast extract
2% Bacto peptone
2% dextrose
2% Bacto agar
40 mg adenine sulfate
Autoclave at 121°C for 20 minutes
Pour into petri dishes (~25 ml/100-mm plate)
Dry plates at room temperature for 2–3 days
Store plates in a sealed bag

## SU Buffer

5% (w/v) SDS
8 M Urea
125 mM Tris-HCl (pH 6.8)
0.1 mM EDTA
0.005% (w/v) bromophenol blue

Store at –20°C
Add 15 mg of DTT/ml of SU buffer prior to use

## YPAD Broth

1% yeast extract
2% Bacto peptone
2% dextrose
40 mg adenine sulfate
Add deionized $H_2O$ to a final volume of 1 liter
Autoclave at 121°C for 20 minutes

## Yeast Lysis Solution for DNA Isolation

2.5 M LiCl
50 mM Tris-HCl (pH 8.0)
4% Triton X-100
62.5 mM EDTA

## 10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

***Note*** *rATP is added separately in the ligation reaction*

## 10× Dropout Solution

To prepare the appropriate 10× dropout solution for the desired synthetic selection medium, simply omit the appropriate component as indicated in the footnote to Table IX that follows. All amino acids and nutrients can be autoclaved with the exception of threonine and aspartic acid, which must be filter sterilized. After sterilization, the 10× dropout solutions can be stored in 100-ml aliquots at 4°C for up to 1 year.

**TABLE IX**
**Formulation of 10× Dropout Solution**

| Components[a] | Weight (mg/liter) | Sigma Catalog # |
|---|---|---|
| L-Isoleucine | 300 | I 2752 |
| L-Valine | 1500 | V 0500 |
| L-Adenine hemisulfate salt | 200 | A 9126 |
| L-Arginine HCl | 500 | A 5131 |
| L-Histidine HCl monohydrate | 200 | H 8125 |
| L-Leucine | 1000 | L 8000 |
| L-Lysine HCl | 500 | L 5626 |
| L-Methionine | 200 | M 9625 |
| L-Phenylalanine | 500 | P 2126 |
| L-Threonine[b] | 2000 | T 8625 |
| L-Tryptophan | 500 | T 0254 |
| L-Tyrosine | 500 | T 3754 |
| L-Uracil | 200 | U 0750 |
| L -Glutamic acid | 1000 | G 1251 |
| L -Aspartic acid[b] | 1000 | A 9256 |
| L -Serine | 400 | S 4500 |

[a] The omission of Leu from the 10× dropout solution selects for the pSos plasmid or any other vector that expresses the *LEU2* gene. The omission of Ura from the 10× dropout solution selects for the pMyr plasmid or any other vector that expresses the *URA3* gene. The omission of both Leu and Ura from the 10× dropout solution selects for both plasmids.

[b] Add these amino acids only after autoclaving the 10× dropout solution.

## REFERENCES


1. Petitjean, A., Hilger, F. and Tatchell, K. (1990) *Genetics* 124(4): 797–806.
2. Chardin, P., Camonis, J. H., Gale, N. W., van Aelst, L., Schlessinger, J., *et al.* (1993) *Science* 260(5112): 1338–43.
3. Reponen, P., Sahlberg, C., Huhtala, P., Hurskainen, T., Thesleff, I., *et al.* (1992) *J Biol Chem* 267(11): 7856–62.
4. Bartel, P. L., Chien, C.-T., Sternglanz, R. and Fields, S. (1993) In *Cellular Interactions in Development: A Practical Approach*, pp. 153–179. Oxford Univ. Press, Oxford, England.
5. Kataoka, K., Fujiwara, K. T., Noda, M. and Nishizawa, M. (1994) *Mol Cell Biol* 14(11): 7581–91.
6. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., *et al.* (1987). *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
7. Callahan, M., Jerpseth, B., Mullinax, R. L. and Greener, A. (1995) *Strategies* 8(2): 45–46.
8. Germino, F. J. and Moskowitz, N. K. (1999) *Methods Enzymol* 303: 422–50.
9. Phizicky, E. M. and Fields, S. (1995) *Microbiol Rev* 59(1): 94–123.


## SUPPLEMENTAL REFERENCES


1. Allen, J. B., *et al*. (1995) *Trends Biochem. Sci.* 20: 511–516.
2. Aronheim, A., *et al*. (1994) *Cell* 78: 949–961.
3. Aronheim, A., *et al*. (1997) *Mol. Cell. Biol.* 17: 3094–3102.
4. Cohen, S., *et al*. (1981) *Nature (London)* 294: 182–184.
5. Colicelli J., *et al*. (1989) *Proc. Natl. Acad. Sci.* USA 86: 3599–3603.
6. Chien, C.-T., *et al*. (1991) *Proc. Natl. Acad. Sci.* USA 88: 9578–9582.
7. Estojak, J., *et al*. (1995) *Mol. Cell. Biol.* 15: 5820–5829.
8. Fields, S. and Song, O.-K. (1989) *Nature (London)* 340: 245–246.
9. Fields, S. and Sterngianz, R. (1994) *Trends Genetics* 10: 286–292.
10. Gimble, F. S. and Sauer, R. T. (1989) *J. Mol. Biol.* 206: 29–39.
11. Kataoka, K., *et al*. (1994) *Mol. Cell. Biol.* 14: 7581–7591.
12. Leuther, K. K. and Johnston, S. (1992) *Science* 256: 1333–1335.
13. McNabb, D. S., *et al*. (1995) *Genes Dev.* 9: 47–58.
14. Melcher, K. and Johnston, S. (1995) *Mol. Cell. Biol.* 15: 2839–2848.
15. Pabo, C. O., *et al*. (1979) *Proc. Natl. Acad. Sci.* USA 76: 1608–1612.
16. Ptashne, M., *et al*. (1976) *Science* 194: 156–161.
17. Quilliam, L. A., *et al*. (1995) *BioEssays* 17: 395–404.
18. Reece, K. S. and Phillips G. J. (1995) *Gene* 165: 141–142.
19. Robinson, L. J., *et al*. (1987) *Science* 235: 1218–1221.
20. Swaffield, J. C., *et al*. (1992) *Nature (London)* 374: 88–91.
21. Thevelein, J. M. (1991) *Mol. Microbiol.* 5: 1301–1307.
22. Harper, J. W., *et al.* (1993) *Cell* 75: 805–816.

## ENDNOTES

Bacto® is a registered trademark of Difco Laboratories.
Parafilm® is a registered trademark of American Can Company.
Triton® is a registered trademark of Rohm and Haas Co.
VWRbrand is a trademark of VWR Scientific.

## MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.