CytoTrap XR Library Construction Kit

Instruction Manual

Catalog #200444

Revision B

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200444-12

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CytoTrap XR Library Construction Kit

Contents

CytoTrap XR Library Construction Kit

MATERIALS PROVIDED

Materials provided	Quantity
Vectors[a]	
pSos vector, supercoiled	20 µg (1 µg/µl in TE buffer)
pMyr XR vector, digested with *Eco*R I–*Xho* I and treated with calf intestinal alkaline phosphatase (CIAP)	5 µg (0.1 µg/µl in TE buffer)
pMyr vector, supercoiled[b]	20 µg (1 µg/µl in TE buffer)
Test insert[c]	
XR LacZ test insert (600 bp) digested with *Xho* I and *Eco*R I	30 ng (10 ng/µl in TE buffer)
Control plasmids[c]	
pSos MAFB control plasmid (positive control)	20 µg (1 µg/µl in TE buffer)
pMyr MAFB control plasmid (positive control)	20 µg (1 µg/µl in TE buffer)
pSos Col I control plasmid (negative control)	20 µg (1 µg/µl in TE buffer)
pMyr Lamin C control plasmid (negative control)	20 µg (1 µg/µl in TE buffer)
pMyr SB (positive control)	20 µg (1 µg/µl in TE buffer)
Host strains[d]	
cdc25H host strains (α and *a*)	0.5 ml each
XL10-Gold Kan ultracompetent cells[e]	10 × 100 µl
pUC18 DNA control plasmid (0.1 ng/µl in TE buffer)	10 µl
XL10-Gold β-Mercaptoethanol mix (β-ME)	50 µl
cDNA synthesis kit	1 kit

[a] On arrival, store the pMyr XR vector at –20°C. After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month.

[b] Store the pMyr and pSos vectors at –20°C.

[c] Store at –20°C.

[d] Store at –80°C.

[e] On arrival, store the competent cells immediately at –80°C. Do not place the cells in liquid nitrogen.

STORAGE CONDITIONS

XL10-Gold Kan Ultracompetent Cells: –80°C
XL10-Gold β-Mercaptoethanol Mix: –80°C
pUC18 DNA Control Plasmid: –20°C
Vectors: –20°C
Yeast Strains: –80°C
Column Reagents: 4°C

Revision B

cDNA Synthesis Kit

Reagents provided[a]	Quantity	Storage temperature
First-strand reagents		
AccuScript reverse transcriptase (AccuScript RT)	15 µl	–20°C
RNase Block Ribonuclease Inhibitor (40 U/µl)	200 U	–20°C
First-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP)	15 µl	–20°C
First-strand buffer (10×)	75 µl	–20°C
Linker–primer (1.4 µg/µl)	10 µl	–20°C
Test poly(A)$^+$ RNA (0.2 µg/µl)	5 µg	–20°C
Diethylpyrocarbonate (DEPC)-treated water	500 µl	–20°C
Second-strand reagents		
Second-strand buffer (10×)	150 µl	–20°C
Second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP)	30 µl	–20°C
Escherichia coli RNase H (1.5 U/µl)	15 U	–20°C
Escherichia coli DNA polymerase I (9.0 U/µl)	500 U	–20°C
Sodium acetate (3 M)	250 µl	–20°C
Blunting reagents		
Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)	115 µl	–20°C
Cloned *Pfu* DNA polymerase (2.5 U/µl)	25 U	–20°C
Ligation reagents		
*Eco*R I adapters (0.4 µg/µl)	18 µg	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
T4 DNA ligase[b] (4 U/µl)	140 U	–20°C
Phosphorylation reagents		
T4 polynucleotide kinase (5 U/µl)	50 U	–20°C
Ligase buffer[b,c] (10×)	250 µl	–20°C
rATP[b] (10 mM)	100 µl	–20°C
Xho I digestion reagents		
Xho I (40 U/µl)	600 U	–20°C
Xho I buffer supplement	250 µl	–20°C
Column reagents		
Connecting tubing d (1/8-inch i.d., 3/16-inch o.d., and 1/32-inch wall)	1 × 4 cm	Room temperature or 4°C
Sepharose CL-2B gel filtration medium[d]	10 ml	4°C
Column-loading dye[c,d]	17.5 µl	4°C
STE buffer[c] (10×)	10 ml	–20°C

[a] Enough reagents are included to generate five vector-ligated constructs.

[b] These reagents are used more than once in the reaction.

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

[d] The Sepharose CL-2B gel filtration medium and the column-loading dye are shipped separately at 4°C.

ADDITIONAL MATERIALS REQUIRED

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (DEPC, phenol, chloroform, methylmercury hydroxide, and sodium hydroxide), radioactive (^{32}P-labeled radioisotope), 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 Agilent 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)
[α-^{32}P]Deoxynucleoside triphosphate ([α-^{32}P]dNTP) (800 Ci/mmol) ([α-^{32}P]dATP, [α-^{32}P]dGTP, or [α-^{32}P]dTTP may be used; do not use [α-^{32}P]dCTP)
Acid-washed glass beads (425–600 µm)
VWRbrand™ 3-mm glass beads (VWR Scientific, Westchester, Pennsylvania, Catalog #26396-630)
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)
Yeast extract
Bacto® peptone
Bacto® agar
Yeast nitrogen base
Ammonium sulfate
SeaPrep® ultralow gelling temperature agarose [FMC, Rockland, Maine (Catalog #50302)]
IPTG
X-gal
Sos1 antibody [BD Biosciences (Catalog #610095 or #610096)]
XL1-Blue electroporation-competent cells
pMyr –specific primers
Cell lysis buffer§
Dextrose
Galactose
Raffinose
LiSORB§
NZY$^+$ broth§
PEG/Lithium acetate solution§
SU buffer§
Salmon sperm DNA§
YPAD broth§
Yeast lysis solution§
10× Dropout solution§

§ See *Preparation of Media and Reagents*.

Sequencing Primers and Sequences

Sequencing primers[a]	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′

[a] 5′ primer is at the 5′ end of the MCS and 3′ primer is at the 3′ end of the MCS.

Equipment

Nondenaturing acrylamide gel, 5% (see *Preparation of Media and Reagents*).
Replica plating mold (150 mm) and velvet squares
Ribonuclease (RNase)-free microcentrifuge tubes
Disposable plastic 10-ml syringes, sterile (e.g., 10-cc BD® syringe with Luer Lok® tip or equivalent)
Disposable 18-gauge, 1½-inch needles, sterile (e.g., BD *PrecisionGlide*® needle or equivalent)
Disposable plastic 1-ml pipets, negatively graduated and sterile [e.g., 1-ml BD Falcon® disposable polystyrene serological pipet (BD Biosciences Catalog #357250) or equivalent]
Pasteur pipet
Portable radiation monitor (Geiger counter)
Water baths (4°, 8°, 12°, 16°, 30°, 37°, 42°, 65°, 70°, and 72°C)
Microcentrifuge
Micropipet and micropipet tips
Vacuum evaporator
Incubator (37°C)
14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
96-well plates
Whatman® No. 1 qualitative filter paper, Grade 1 {Fisher Scientific, Pittsburgh, Pennsylvania [Catalog #09-805C (7 cm diameter) and #09-805F (12.5 cm diameter)]}
VWRbrand™ qualitative filter papers, Grade No. 413 [VWR Scientific, Westchester, Pennsylvania, Catalog #28310-026 (7.5 cm diameter)] and #28310-106 (12.5 cm diameter)]

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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:

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INTRODUCTION

The CytoTrap XR library construction 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 researchers to study protein interactions that cannot be assayed by using 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.

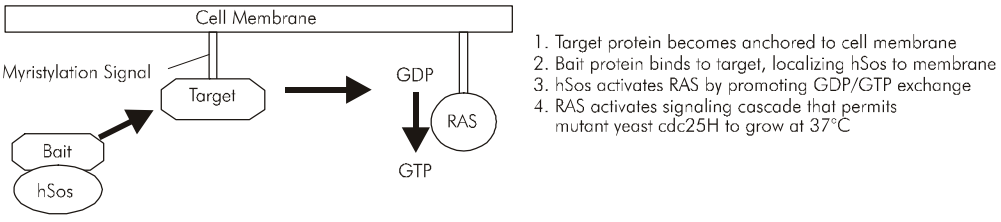


FIGURE 1 Schematic diagram of the Ras-signaling pathway utilized in the CytoTrap two-hybrid system.

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 pMyr XR vector has been digested with *Eco*R I and *Xho* I and dephosphorylated with CIAP. 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).

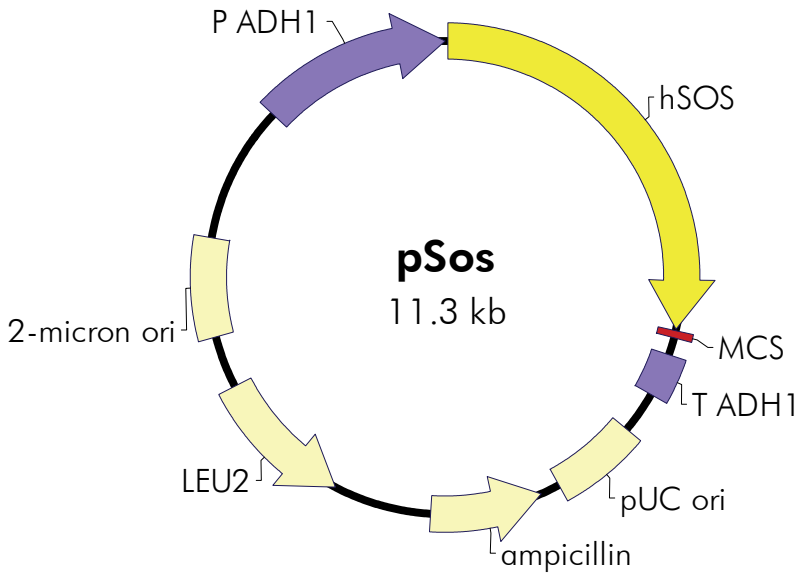
Both pSos and pMyr vectors contain the pUC and 2µ origins for replication in *E. coli* and yeast, respectively. The pSos and pMyr vectors also carry yeast biosynthetic genes *LEU2* and *URA3*, respectively, for selection of yeast transformants based on nutritional requirements. The pSos vector contains 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

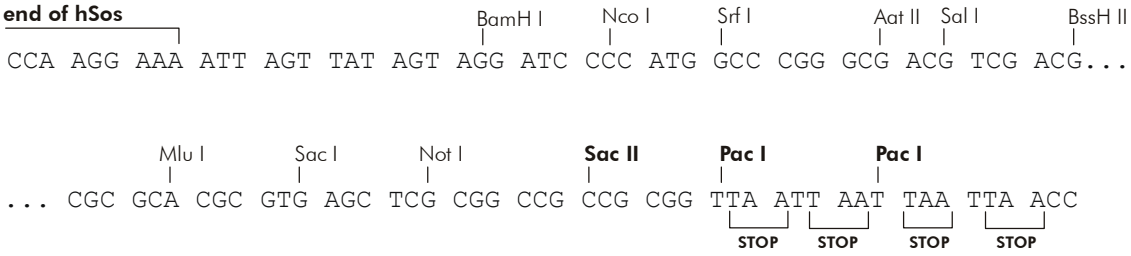
pSos	pMyr
Bam HI	*Eco*R I
Nco I	*Srf* I/*Sma* I
Srf I	*Xho* I
Aat II	*Sal* I
Sal I	
*Bss*H II	
Mlu I	
Sac I	
Not I*	

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

pSos Vector



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

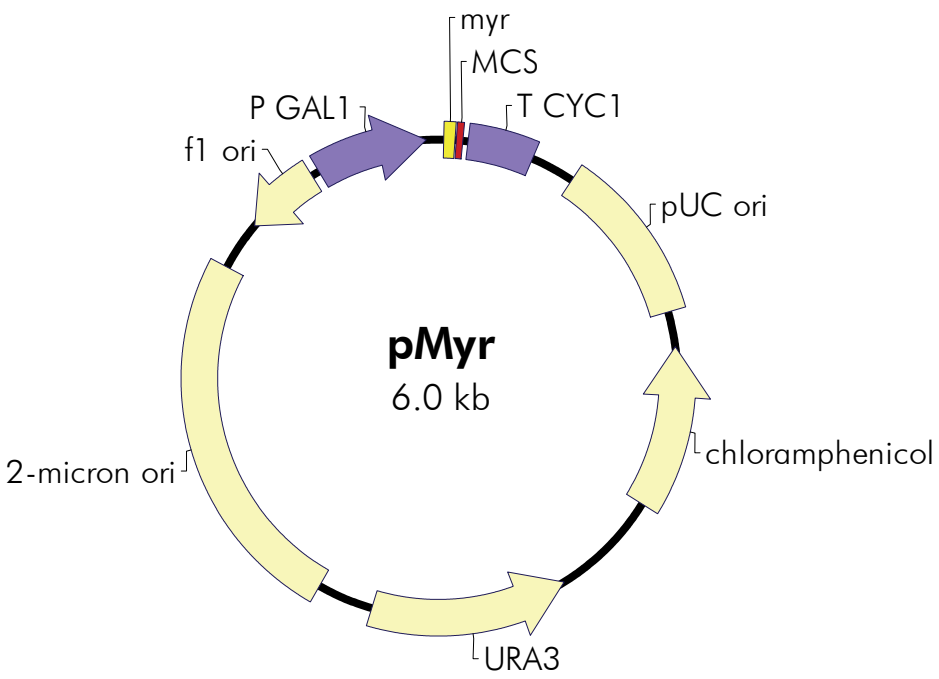


Notes: *The **Sac II** and **Pac I** sites in the pSos MCS (shown in bold) are not unique. A stop codon is present in all three reading frames.*

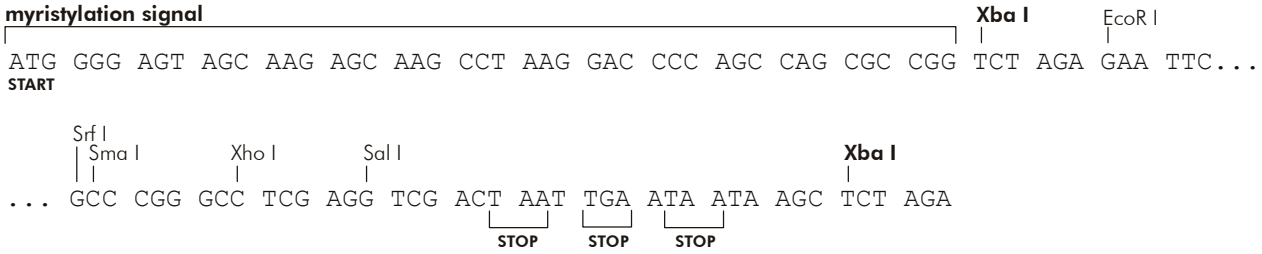
Feature	Nucleotide Position
hSos ORF	24–3209
multiple cloning site	3223–3276
yeast *ADH1* terminator	3413–3741
pUC origin of replication	4064–4731
ampicillin resistance (*bla*) ORF	4882–5739
yeast *LEU2* selection marker ORF	6498–7589
2μ yeast origin of replication	7993–8747
yeast *ADH1* promoter	9806–11259

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)	*MAT***a** *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

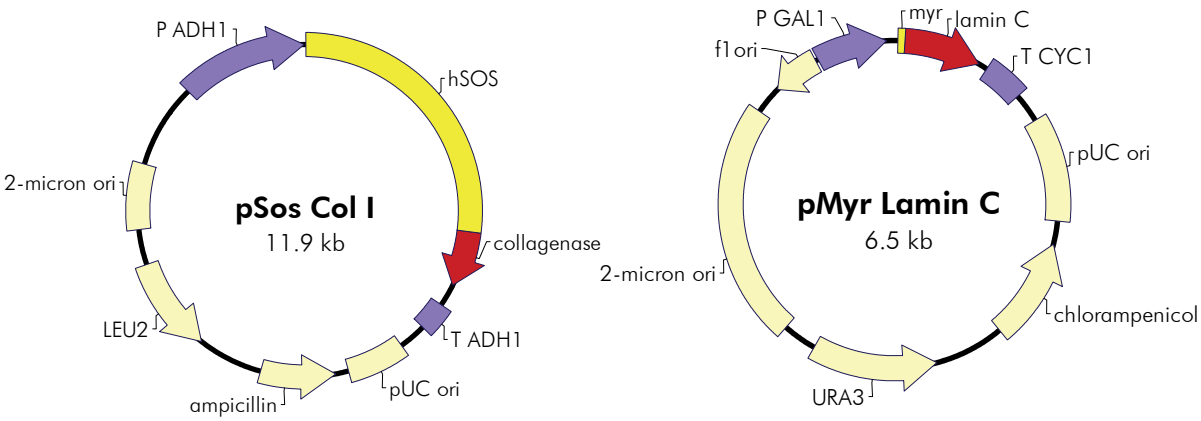


FIGURE 4 Circular maps of the pSos Col I and pMyr Lamin C control plasmids.

Positive Controls

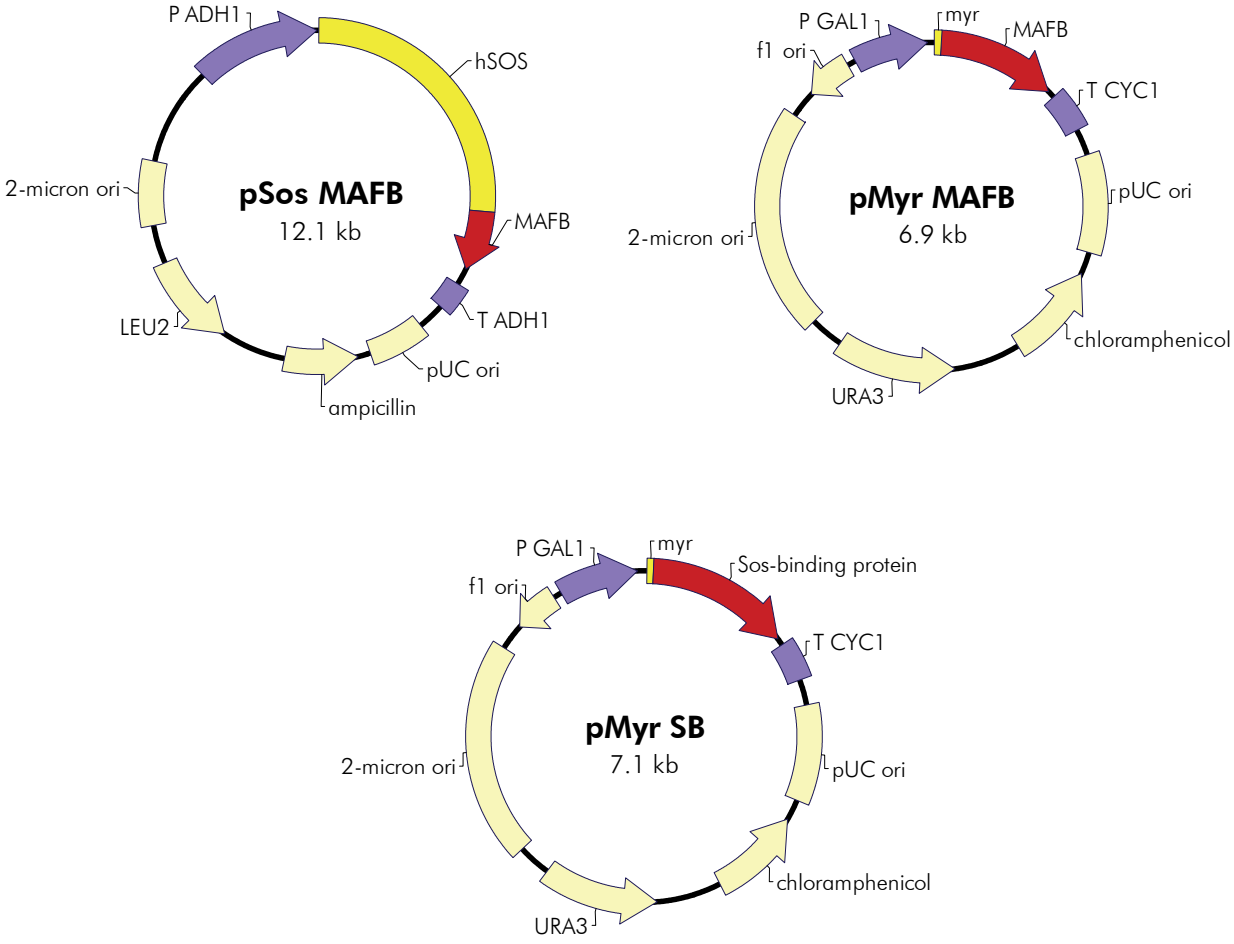


FIGURE 5 Circular maps of the pSos MAFB, pMyr MAFB, and pMyr SB positive control plasmids.

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	+	+	–	+

pMYR TARGET VECTOR CONSTRUCTION

Background

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. Figure 6 gives an overview of the cDNA synthesis procedure.

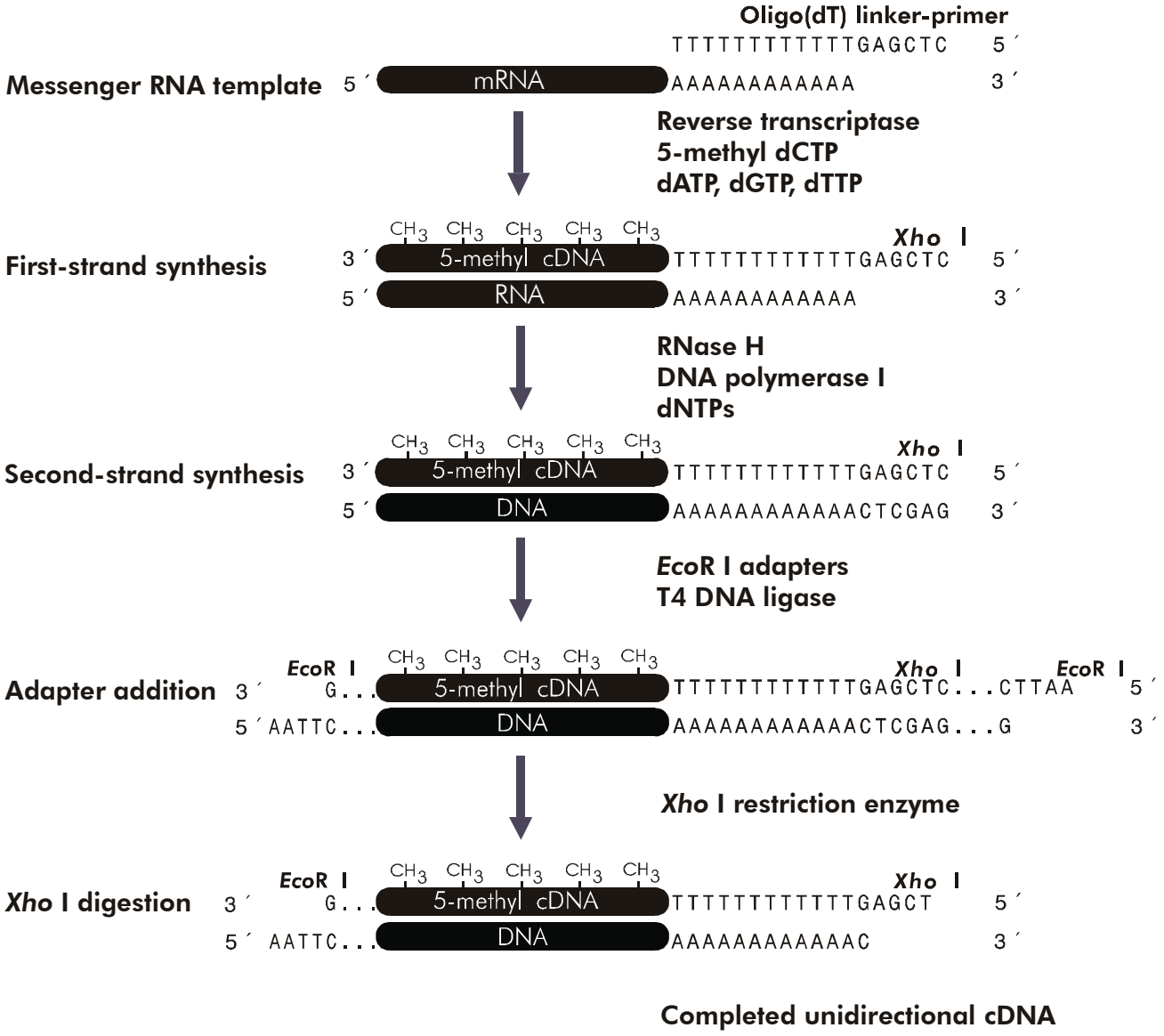


FIGURE 6 cDNA synthesis flow chart.

cDNA Libraries

Complementary DNA inserts to be ligated into the pMyr vector are prepared from mRNA. The cDNA Synthesis Kit provides the reagents required to convert mRNA to cDNA inserts prior to unidirectional insertion into the pMyr XR vector. The protocols for preparing cDNA inserts are found in *cDNA Insert Preparation and Ligation*.

cDNA Insert Preparation and Ligation

Background for Preparation of cDNA Libraries

Complementary DNA libraries represent the information encoded in the mRNA of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then is inserted into the pMyr XR vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease. Therefore, cDNA library construction provides a method by which the transcription and processing of mRNA can be examined and interpreted.

The cDNA Synthesis Kit uses a hybrid oligo(dT) linker–primer that contains an *Xho* I restriction site. First-strand synthesis is primed with the linker–primer and is reverse-transcribed using AccuScript reverse transcriptase and 5-methyl dCTP.

AccuScript reverse transcriptase (AccuScript RT) is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3'-5' exonuclease. AccuScript reverse transcriptase delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript reverse transcriptase delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy. These advantages make AccuScript RT the enzyme of choice for applications involving the preparation of accurate, full-length, cDNA transcripts, including first-strand cDNA synthesis and library construction.

The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, which protects the cDNA from digestion with certain restriction endonucleases such as *Xho* I. Therefore, on *Xho* I digestion of the cDNA, only the unmethylated site within the linker–primer is cleaved.

Hemimethylated DNA introduced into an McrA$^+$ McrB$^+$ strain would be subject to digestion by the *mcrA* and *mcrB* restriction systems. Therefore, it is necessary to initially transform an McrA$^-$ McrB$^-$ strain (e.g., the XL10-Gold Kan strain supplied with the CytoTrap system) when using the cDNA Synthesis Kit. After passing the library through XL10-Gold Kan cells, the DNA is no longer hemimethylated and can be grown on McrA$^+$ McrB$^+$ strains (e.g., XL1-Blue strain).

cDNA Synthesis

The yield, length, and accuracy of cDNA transcripts is enhanced with the use of AccuScript RT, an engineered version of the Moloney murine leukemia virus reverse transcriptase combined with a proofreading 3'-5' exonuclease. First-strand cDNA synthesis begins when AccuScript RT, in the presence of nucleotides and buffer, finds a template and a primer. The template is mRNA and the primer is a 50-base oligonucleotide with the following sequence:

5´-GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTT-3´
"GAGA" Sequence *Xho* I Poly(dT)

This oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the pMyr XR vector in a sense orientation (*Eco*R I–*Xho* I) with respect to the P_{GAL1}. The poly(dT) region binds to the 3´ poly(A) region of the mRNA template, and AccuScript RT begins to synthesize the first-strand cDNA.

The nucleotide mixture for the first strand contains normal dATP, dGTP, and dTTP plus the analog 5-methyl dCTP. The complete first strand will have a methyl group on each cytosine base, which will protect the cDNA from restriction enzymes used in subsequent cloning steps.

During second-strand synthesis, RNase H nicks the RNA bound to the first-strand cDNA to produce a multitude of fragments, which serve as primers for DNA polymerase I. DNA polymerase I "nick-translates" these RNA fragments into second-strand cDNA. The second-strand nucleotide mixture has been supplemented with dCTP to reduce the probability of 5-methyl-dCTP becoming incorporated in the second strand. This ensures that the restriction sites in the linker–primer will be susceptible to restriction enzyme digestion. The uneven termini of the double-stranded cDNA are nibbled back or filled in with cloned *Pfu* DNA polymerase, and *Eco*R I adapters are ligated to the blunt ends. The adapters have the sequence shown below.

5´-OH-AATTCGGCACGAGG-3´
3´-GCCGTGCTCCp-5´

These adapters are composed of 10- and 14-mer oligonucleotides, which are complementary to each other with an *Eco*R I cohesive end. The 10-mer oligonucleotide is phosphorylated, which allows it to ligate to other blunt termini available in the form of cDNA and other adapters. The 14-mer oligonucleotide is kept dephosphorylated to prevent it from ligating to other cohesive ends. After adapter ligation is complete and the ligase has been heat inactivated, the 14-mer oligonucleotide is phosphorylated to enable its ligation into the dephosphorylated vector arms.

The *Xho* I digestion releases the *Eco*R I adapter and residual linker–primer from the 3´ end of the cDNA. These two fragments are separated on a drip column containing Sepharose® CL-2B gel filtration medium. The size-fractionated cDNA is then precipitated and ligated to the pMyr XR vector.

Generation of cDNA Inserts

Note *The following protocol has been optimized for 5 μg of poly(A)$^+$ RNA per reaction.*

Protocol Guidelines

- The quality and quantity of the mRNA used is of fundamental importance to the construction of a large, representative cDNA library (see *Appendix II: RNA Purification and Quantitation*). The Agilent RNA Isolation Kit (Catalog #200345) uses the guanidinium thiocyanate–phenol–chloroform extraction method,[6] which quickly produces large amounts of undegraded RNA. To isolate mRNA, we offer the Absolutely mRNA Purification Kit (Catalog #400806).

- Secondary structure of mRNA may cause the synthesis of truncated cDNAs. To relax secondary structure, treatment with methylmercury hydroxide (CH_3HgOH) is recommended (see *Appendix III: Methylmercury Hydroxide Treatment*).

- It is imperative to protect the RNA from any contaminating RNases until the first-strand cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

- When removing aliquots of any of the enzymes used in the cDNA synthesis protocol, flick the bottom of the tube to thoroughly mix the enzyme solution. Do not vortex the enzyme stock tubes.

Synthesizing First-Strand cDNA

1. Prepare water baths at 16°, 42°, and 72°C.

2. Thaw the radioactive [α-^{32}P]dNTP (do not use [^{32}P]dCTP) and all nonenzymatic first-strand components. Keep the radioactive dNTP on ice for use in step 6 and in the second-strand synthesis. Briefly vortex and spin down the contents of the nonenzymatic tubes. Place the tubes on ice.

   Note *AccuScript reverse transcriptase is temperature sensitive and should remain at –20°C until the last moment.*

3. The final volume of the first-strand synthesis reaction is 50 µl. The volume of added reagents and enzymes is 14 µl, thus the mRNA template and DEPC-treated water should be added in a combined volume of 36 µl. For the control reaction, prepare the following annealing reaction with 25 µl (5 µg) of test RNA and 11 µl of DEPC-treated water.

4. In an RNase-free microcentrifuge tube, add the following reagents in order:

   5 µl of 10× first-strand buffer
   3 µl of first-strand methyl nucleotide mixture
   2 µl of linker–primer (1.4 µg/µl)
   X µl of DEPC-treated water
   1 µl of RNase Block Ribonuclease Inhibitor (40 U/µl)

5. Mix the reaction and then add *X* µl of poly(A)$^+$ RNA (5 µg). Mix gently.

6. Allow the primer to anneal to the template for 10 minutes at room temperature. During the incubation, aliquot 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol) into a separate tube for the control.

7. Add 3 µl of AccuScript RT to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction should now be 50 µl.

8. Mix the sample gently and spin down the contents in a microcentrifuge.

9. **Transfer 5 µl of the first-strand synthesis reaction to the separate tube containing the 0.5 µl of the [α-^{32}P]dNTP (800 Ci/mmol). This radioactive sample is the first-strand synthesis control reaction.**

10. Incubate the first-strand synthesis reactions, including the control reaction, at 42°C for 1 hour.

11. Prepare a 16°C water bath for second-strand synthesis. If a water bath with a cooling unit is not available, use a large Styrofoam® container with a lid. Fill the container three-quarters full with water and adjust the temperature to 16°C with ice. Cover the container with a lid.

12. After 1 hour, remove the first-strand synthesis reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reaction on ice. Store the radioactive first-strand synthesis control reaction at –20°C until ready to analyze by alkaline agarose gel electrophoresis (see *Appendix IV: Alkaline Agarose Gels).* The radioactive first-strand reaction will be gel-analyzed alongside a radioactive sample of the second-strand reaction after the second strand reaction has been blunted and resuspended in the *Eco*R I adapters (see step 17 in *Blunting the cDNA Termini*).

Synthesizing Second-Strand cDNA

1. Thaw all nonenzymatic second-strand components. Briefly vortex and spin in a micro-centrifuge before placing the tubes on ice.

   Note *It is important that all reagents be < 16°C when the DNA polymerase I is added.*

2. Add the following components in order to the 45-µl nonradioactive, first-strand synthesis reaction on ice:

   20 µl of 10× second-strand buffer
   6 µl of second-strand dNTP mixture
   114 µl of sterile dH_2O (DEPC-treated water is not required)
   2 µl of [α-^{32}P]dNTP (800 Ci/mmol)

3. Add the following enzymes to the second-strand synthesis reaction:

   2 µl of RNase H (1.5 U/µl)
   11 µl of DNA polymerase I (9.0 U/µl)

4. Gently vortex the contents of the tube, spin the reaction in a microcentrifuge, and incubate for 2.5 hours in a 16°C water bath. Check the water bath occasionally to ensure that the temperature does not rise above 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector.

5. After second-strand synthesis for 2.5 hours at 16°C, immediately place the tube *on ice.*

Blunting the cDNA Termini

1. Add the following to the second-strand synthesis reaction:

   23 µl of blunting dNTP mix
   2 µl of cloned *Pfu* DNA polymerase

2. Quickly vortex the reaction and spin down in a microcentrifuge. Incubate the reaction at 72°C for 30 minutes. **Do not exceed 30 minutes!!**

3. Thaw the 3 M sodium acetate.

   Note *Since radioactivity can leak out between the lid and body of some micro-centrifuge tubes during the vortexing and precipitation steps, wrap a small piece of Parafilm laboratory film around the rim of the microcentrifuge tube to prevent leakage.*

4. Remove the reaction and add 200 µl of phenol–chloroform [1:1 (v/v)] and vortex.

   Note ***Do not use the low-pH phenol from the Agilent RNA Isolation Kit because this phenol is too acidic and may denature the DNA.*** *The phenol must be equilibrated to pH 7–8.*

5. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube. Be careful to avoid removing any interface that may be present.

6. Add an equal volume of chloroform and vortex.

7. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube.

8. Precipitate the cDNA by adding the following to the saved aqueous layer:

   20 µl of 3 M sodium acetate
   400 µl of 100% (v/v) ethanol

   Vortex the reaction.

9. Incubate the precipitation reaction overnight at –20°C.

10. In order to orient the direction of precipitate accumulation, place a mark on the microcentrifuge tube or point the tube hinge away from the center of the microcentrifuge as an indicator of where the pellet will form.

11. Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C.

12. Avoid disturbing the pellet and carefully remove and discard the radioactive supernatant in a radioactive waste container.

    Note *The conditions of synthesis and precipitation produce a large white pellet. The pellet accumulates near the bottom of the microcentrifuge tube and may taper up along the marked side of the tube.*

13. Gently wash the pellet by adding 500 µl of 70% (v/v) ethanol to the side of the tube away from the precipitate. **Do not mix or vortex!**

14. Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature with the orientation marked as in step 10.

15. Aspirate the ethanol wash and dry the pellet by vacuum centrifugation.

16. Resuspend the pellet in 9 µl of *Eco*R I adapters and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend. To ensure that the cDNA is completely in solution, transfer the cDNA to a fresh microcentrifuge tube. Monitor the now empty tube with a handheld Geiger counter. If the cDNA is in solution, few counts should remain in the empty tube.

17. **Transfer 1 µl of this second-strand synthesis reaction to a separate tube. This radioactive sample is the second-strand synthesis control reaction.** Analyze the samples of the first- and second-strand synthesis reactions on an alkaline agarose gel at this point. It is important to determine the size range of the cDNA and the presence of any secondary structure (see *Appendix IV: Alkaline Agarose Gels*).

    Note *The second-strand synthesis reaction can be stored overnight at –20°C.*

Ligating the *Eco*R I Adapters

1. Add the following components to the tube containing the blunted cDNA and the *Eco*R I adapters:

    1 µl of 10× ligase buffer
    1 µl of 10 mM rATP
    1 µl of T4 DNA ligase (4 U/µl)

2. Spin down the volume in a microcentrifuge and incubate overnight at 8°C. Alternatively, the ligations can be incubated at 4°C for 2 days.

3. In the morning, heat inactivate the ligase by placing the tubes in a 70°C water bath for 30 minutes.

Phosphorylating the *Eco*R I Ends

1. After the ligase is heat inactivated, spin the reaction in a microcentrifuge for 2 seconds. Cool the reaction at room temperature for 5 minutes.

2. Phosphorylate the adapter ends by adding the following components:

    1 µl of 10× ligase buffer
    2 µl of 10 mM rATP
    5 µl of sterile water
    2 µl of T4 polynucleotide kinase (5.0 U/µl)

3. Incubate the reaction for 30 minutes at 37°C.

4. Heat inactivate the kinase for 30 minutes at 70°C.

5. Spin down the condensation in a microcentrifuge for 2 seconds and allow the reaction to equilibrate to room temperature for 5 minutes.

Digesting with *Xho* I

1. Add the following components to the reaction:

   28 µl of *Xho* I buffer supplement
   3 µl of *Xho* I (40 U/µl)

2. Incubate the reaction for 1.5 hours at 37°C.

3. Add 5 µl of 10× STE buffer and 125 µl of 100% (v/v) ethanol to the microcentrifuge tube.

4. Precipitate the reaction overnight at –20°C.

5. Following precipitation, spin the reaction in a microcentrifuge at maximum speed for 60 minutes at 4°C.

6. Discard the supernatant, dry the pellet completely, and resuspend the pellet in 14 µl of 1× STE buffer.

7. Add 3.5 µl of the column-loading dye to each sample.

The sample is now ready to be run through a drip column containing Sepharose CL-2B gel filtration medium (see *Size Fractionating*).

Size Fractionating

Before attempting the experimental protocols outlined within this section, please read this section in its entirety in order to become familiar with the procedures. Review of the *Troubleshooting* section may also prove helpful. The drip columns should be prepared and the cDNA should be eluted in 1 day. Because a full day is required to complete these procedures, gathering all necessary materials in advance is recommended (see the *Equipment* section in *Additional Materials Required*).

1. Perform the following preparatory steps while assembling the drip columns:

   a. Remove the Sepharose CL-2B gel filtration medium and the 10× STE buffer from refrigeration and equilibrate the two components to room temperature.

   b. Prepare 50 ml of 1× STE buffer by diluting 10× STE buffer 1:10 in sterile water.

2. Assemble the drip columns as outlined in the following steps (see Figure 7 for a diagram of the final setup):

   Note *Wear gloves while assembling the drip columns.*

   a. Remove the plastic wrapper from the top of a sterile 1-ml pipet.

b. Using a sterile needle or a pair of fine-tipped forceps, **carefully** tease the cotton plug out of each pipet, leaving a piece of the cotton plug measuring ~3–4 mm inside. Cut off the external portion of the cotton plug.

c. Push the remaining 3- to 4-mm piece of the cotton plug into the top of each pipet with the tip of the needle or forceps.

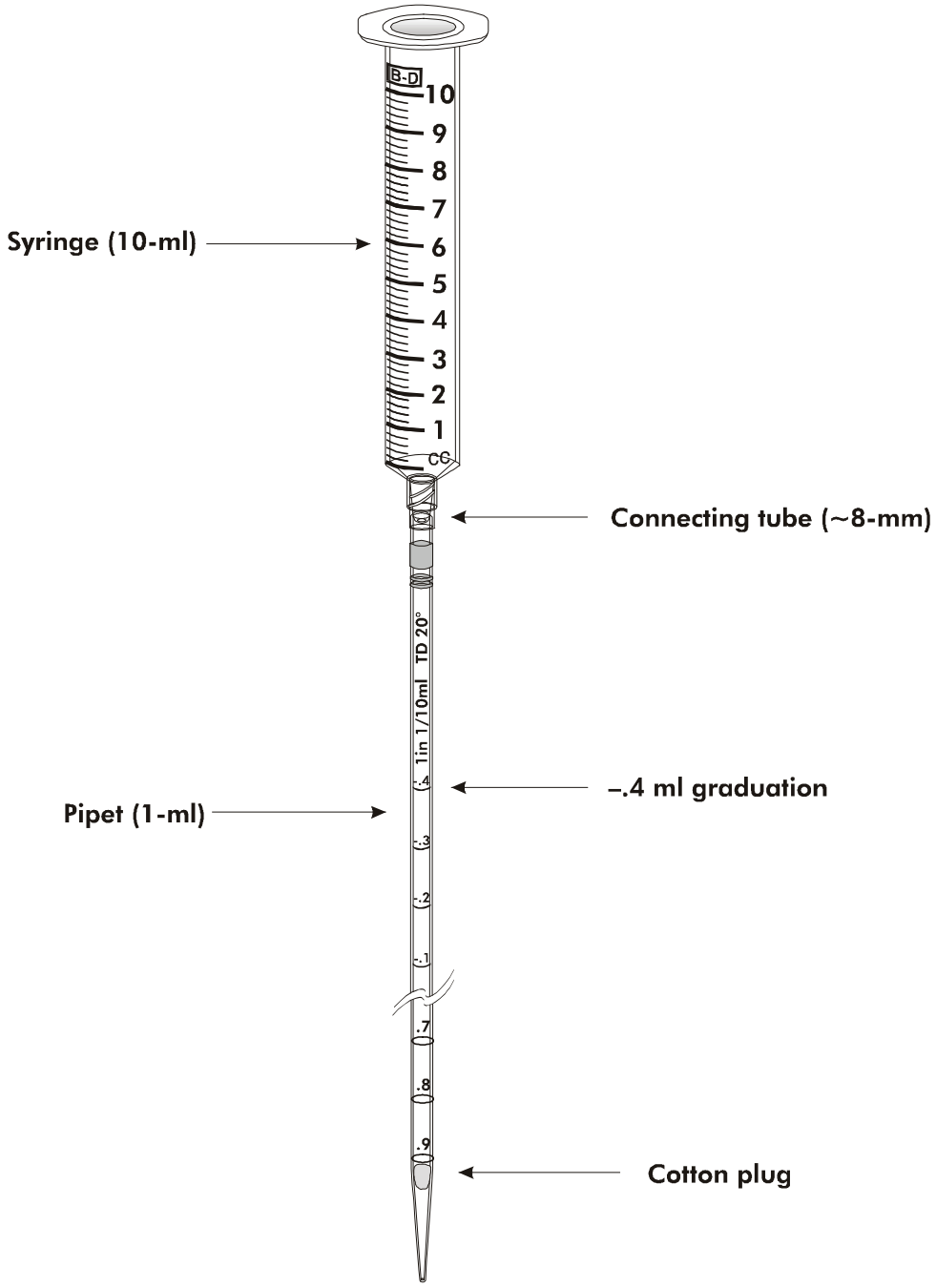


FIGURE 7 Assembly of the drip columns.

d. Cut a small piece of plastic tubing measuring ~8 mm. Use this small tube to connect the 1-ml pipet to the 10-ml syringe. First attach one end of the connecting tube to the pipet and then connect the other end to the syringe. There should be no gap between the pipet and the syringe when joined by the connecting tube.

Note *The inside diameter of the plastic tubing (~1/8-inch i.d.) snugly connects most disposable 1-ml pipets and the ends of all BD® 10-cc syringes with the Luer Lok® tips.*

e. Rapidly and forcefully push the plunger into the syringe to thrust the cotton plug down into the tip of the pipet.

Note *It may take several attempts to drive the cotton all the way down into the tip of the pipet. However, pushing the cotton plug as far down into the pipet tip as possible is important in order to achieve optimal separation of the cDNA fractions.*

f. Remove the plunger from the syringe. Because the syringe functions as a buffer reservoir for the drip column, leave the syringe firmly attached to the pipet throughout the remainder of the size fractionation procedure.

3. Locate a support for the assembled drip column. Butterfly clamps or a three-fingered clamp on a ring stand can be used.

Loading the Drip Column

1. Load the drip column with a uniform suspension of Sepharose CL-2B gel filtration medium as outlined in the following steps:

   a. Immediately prior to loading the drip column, gently mix the Sepharose CL-2B gel filtration medium by inversion until the resin is uniformly suspended.

   b. Place the column in the ring stand. Fill a glass Pasteur pipet with ~2 ml of 1× STE buffer. Insert the pipet as far into the drip column as possible and fill the column with the buffer.

Notes *If the 1× STE buffer flows too quickly through the column, stem the flow by affixing a yellow pipet tip to the end of the column. Make sure to remove the pipet tip prior to loading the column with the Sepharose CL-2B gel filtration medium.*

If bubbles or pockets of air become trapped in the STE buffer while filling the column, remove the trapped air prior to packing the column with the resin. To remove the bubbles or air, re-insert the Pasteur pipet into the top of the column and gently pipet the STE buffer in and out of the pipet until the trapped air escapes through the top of the column.

c. Immediately add a uniform suspension of Sepharose CL-2B gel filtration medium to the column with a Pasteur pipet by inserting the pipet as far into the column as possible. As the resin settles, continue adding the Sepharose CL-2B gel filtration medium. Stop adding the resin when the surface of the packed bed is ¼ inch below the "lip of the pipet." The lip of the pipet is defined as the point where the pipet and the syringe are joined.

Notes *If air bubbles form as the resin packs, use a Pasteur pipet as described in step 1b to remove the blockage. Failure to remove bubbles can impede the flow of the column and result in a loss of the cDNA.*

If the preparation of Sepharose CL-2B gel filtration medium settles and becomes too viscous to transfer from the stock tube to the column, add a small volume (~1–5 ml) of 1× STE buffer to resuspend the resin.

2. Wash the drip column by filling the buffer reservoir (i.e., the syringe) with a minimum of 10 ml of 1× STE buffer. As the column washes, the buffer should flow through the drip column at a steady rate; however, it may take at least 2 hours to complete the entire wash step. After washing, do not allow the drip column to dry out, because the resin could be damaged and cause sample loss. If this occurs, pour another column.

Note *If a free flow of buffer is not observed, then bubbles or pockets of air have become trapped in the drip column. In this case, the column must be repacked. If cDNA is loaded onto a column on which a free flow of buffer is not observed, the sample could become irretrievably lost.*

3. When ~50 µl of the STE buffer remains above the surface of the resin, immediately load the cDNA sample using a pipettor. Gently release the sample onto the surface of the column bed, but avoid disturbing the resin as this may affect cDNA separation.

4. Once the sample enters the Sepharose CL-2B gel filtration medium, fill the connecting tube with buffer using a pipettor.

Note *Do not disturb the bed while filling the connecting tube with buffer.*

Gently add 3 ml of 1× STE buffer to the buffer reservoir by trickling the buffer down the inside wall of the syringe. Do not squirt the buffer into the reservoir because this will disturb the resin, resulting in loss of the sample.

5. As the cDNA sample elutes through the column, the dye will gradually diffuse as it migrates through the resin. Because the dye is used to gauge when the sample elutes from the column, monitor the progress of the dye, or the cDNA sample could be irretrievably lost.

Collecting the Sample Fractions

The drip column containing the Sepharose CL-2B gel filtration medium separates molecules on the basis of size. Large cDNA molecules elute first followed by smaller cDNA and finally unincorporated nucleotides. Using a handheld monitor, two peaks of radioactivity can generally be detected during the course of elution. The first peak to elute from the column represents the cDNA. Due to the conditions of label incorporation during second-strand synthesis, the cDNA is not extremely radioactive; therefore, the counts per second may be barely above background levels. In contrast, the second peak to elute from the column is highly radioactive as this is the unincorporated radioactive nucleotides. Although this material elutes from the column in parallel with the dye, unincorporated nucleotides are usually not collected because the cDNA has already eluted from the column.

For standard cDNA size fractionation (>400 bp), collect ~12 fractions using the procedure described in this section. The progression of the leading edge of the dye through the column will be used as a guideline to monitor collection; however, the drops collected from the column should be monitored for radioactivity using a handheld Geiger counter. Until the fractions have been assessed for the presence of cDNA on a 5% nondenaturing acrylamide gel (see *Preparation of Media and Reagents*), do not discard any fractions based on the quantity of radioactivity detected.

1. Using a fresh microcentrifuge tube to collect each fraction, begin collecting **three drops** per fraction when the leading edge of the dye reaches the **–0.4-ml** gradation on the pipet.

2. Continue to collect fractions until the trailing edge of the dye reaches the **0.3-ml** gradation. A minimum of 12 fractions, each containing ~100 µl (i.e., three drops), should be collected. Alternatively, fractions can be collected until the radioactive unincorporated nucleotides begin to elute. In either case, monitor the fractions for the presence of radioactivity to determine whether the cDNA has eluted successfully. If no counts are detected, continue collecting the fractions until the peak of unincorporated nucleotides is recovered.

3. Before processing the fractions and recovering the size-fractionated cDNA, **remove 8 µl of each collected fraction and save for later analysis. These aliquots will be electrophoresed on a 5% nondenaturing acrylamide gel** to assess the effectiveness of the size fractionation and to determine which fractions will be used for ligation.

Processing the cDNA Fractions

In this section of the size fractionation procedure, the fractions collected from the drip column are extracted with phenol–chloroform and are precipitated with ethanol to recover the size-selected cDNA. The purpose of the organic extractions is to remove contaminating proteins; of particular concern is kinase, which can be carried over from previous steps in the synthesis. Because kinase often retains activity following heat treatment, it is necessary to follow the extraction procedures.

1. Begin extracting the remainder of the collected fractions by adding an equal volume of phenol–chloroform [1:1 (v/v)].

2. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

3. Add an equal volume of chloroform.

4. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

5. To each extracted sample, add a volume of 100% (v/v) ethanol that is equal to twice the individual sample volume.

   Note *The 1× STE buffer contains sufficient NaCl for precipitation.*

6. Precipitate overnight at –20°C.

7. Spin the sample in the microcentrifuge at maximum speed for 60 minutes at 4°C. Transfer the supernatant to another tube. To ensure that the cDNA has been recovered, use a handheld Geiger counter to check the level of radioactivity present in the pellet. If the majority of the radiation is detected in the supernatant, repeat the centrifugation step; otherwise, discard the supernatant.

8. Carefully wash the pellet with 200 µl of 70% (v/v) ethanol, ensuring that the pellet remains undisturbed. *Do not mix or vortex!* Spin the sample in a microcentrifuge at maximum speed for 2 minutes at room temperature. Remove the ethanol and verify that the pellet has been recovered by visual inspection or with the handheld Geiger counter. Vacuum evaporate the pellet for ~5 minutes or until dry. Do not dry the pellet beyond the point of initial dryness or the cDNA may be difficult to solubilize.

9. Using a handheld Geiger counter verify that the cDNA has been recovered and record the number of counts per second (cps) that is detected for each fraction.

10. If <30 cps is detected, resuspend each cDNA pellet in 3.5 µl of sterile water. If the value is >30 cps, resuspend the cDNA in 5 µl of sterile water. Mix by pipetting up and down.

Quantitate the cDNA before proceeding (see *Appendix V: Ethidium Bromide Plate Assay—Quantitation of DNA*). Best results are usually obtained by ligating 60 ng of cDNA/0.1 µg of vector. Place the remaining cDNA at –20°C for short term storage only. The cDNA is most stable after ligation into the vector and may be damaged during long-term storage.

Ligating cDNA into the pMyr XR Vector

1. Set up a control ligation to ligate the test insert into the pMyr XR vector as follows:

   0.3 µl of the pMyr XR vector (0.1 µg/µl)
   1.0 µl of XR LacZ test insert (10 ng/µl)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   2.2 µl of distilled water

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

2. To prepare the sample ligation, add the following components:

   X µl of resuspended cDNA [Use ~2:1 molar ratio of insert to vector (~60 ng)]
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   1.0 µl of the pMyr XR vector (0.1 µg/µl)
   X µl of distilled water for a final volume of 4.5 µl

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

3. Flick the tube gently to mix, then spin down briefly.

4. Incubate the reaction tubes overnight at 12°C.

XL10-GOLD KAN TRANSFORMATION GUIDELINES

Storage Conditions

Ultracompetent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Ultracompetent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the 14-ml BD Falcon polypropylene round-bottom tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use at least 100 µl of ultracompetent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon polypropylene tubes

It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by the XL10-Gold β-ME used in step 3 of *Transforming XL10-Gold Cells with the pMyr cDNA Library*. In addition, the incubation period during the heat-pulse step is critical and has been calculated for the thickness and shape of the 14-ml BD Falcon polypropylene tube.

Use of β-Mercaptoethanol

β-Mercaptoethanol (β-ME) has been shown to increase transformation efficiency. The XL10-Gold β-mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 4 µl of the β-ME mix. (Using an alternative source of β-ME may reduce transformation efficiency.) Do not use XL10-Gold β-ME mix for the preparation of yeast competent cells.

Length of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat pulsed for 30 seconds. Heat pulsing for at least 30 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease when incubating for <30 seconds or for >40 seconds. Do not exceed 42°C.

Preparing the Agar Plates for Color Screening

To prepare the plates for blue–white color screening, add X-gal and IPTG to molten LB agar according to the recipe in *Preparation of Media and Reagents*. Alternatively, 100 µl of 10 mM IPTG (stock prepared in sterile distilled water) and 100 µl of 2% X-gal (stock prepared in dimethylformamide) can be spread on the LB agar plates[§] 30 minutes prior to plating the transformations.

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

TRANSFORMING XL10-GOLD CELLS WITH THE PMYR CDNA LIBRARY

Transformation Protocol

1. Thaw the XL10-Gold Kan ultracompetent cells on ice.

2. Gently mix the cells by flicking the tube. Aliquot 100 µl of the cells into a prechilled 14-ml BD Falcon polypropylene tube.

3. Add 4 µl of the XL10-Gold β-ME mix provided with the kit to the 100 µl of bacteria. (Using an alternative source of β-ME may reduce transformation efficiency.)

4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.

5. Add 1 µl of the ligation reaction (from step 3 of *Ligating cDNA into the Plasmid Vector*) to the cells and swirl gently. (For the **control ligation** add 1 µl to 100 µl cells.)

   Note *As a transformation control, add 1 µl of pUC18 plasmid (diluted 1:10 in high quality water) to another 100-µl aliquot of the cells and swirl gently.*

6. Incubate the tubes on ice for 30 minutes.

7. Preheat NZY$^+$ broth (see *Preparation of Media and Reagents*) in a 42°C water bath for use in step 10.

8. Heat pulse the tubes in a 42°C water bath for 30 seconds. The duration and temperature of the heat pulse is *critical* for obtaining the highest efficiencies. Do not exceed 42°C.

9. Incubate the tubes on ice for 2 minutes.

10. Add 0.9 ml of preheated (42°C) NZY$^+$ broth to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

Notes *For quantities of pilot ligations to plate, see* Determining the Number of Transformants.

The cells may be concentrated by centrifuging at 200 × g for 3–5 minutes if desired. Resuspended the pellet in 200 µl of NZY$^+$ broth and plate.

When transforming the control pUC18 DNA, plate 5 µl of the transformation mixture in a 200-µl pool of NZY$^+$ broth on LB-ampicillin agar[§] plates. 250 colonies may be expected from each 5-µl of the pUC18 control transformation to yield ≥5×10^{9}cfu/µg.

Determining the Number of Transformants

Plating

1. Plate 1 µl and 10 µl of each 1 ml pilot transformation onto LB-chloramphenicol[§] agar plates.

2. Plate 1 µl and 10 µl of the XR LacZ test insert transformation on LB-chloramphenicol agar plates.

3. Incubate the plates overnight at 37°C.

   Note *Greater than 30 colonies should be observed from the 1-µl plating of the test insert transformation.*

4. Pick 50 colonies, transfer them to an LB-chloramphenicol plate with X-gal and IPTG, and incubate the cells at 37°C. Blue colonies contain the test insert.

   Count the number of chloramphenicol-resistant colonies on the 1-µl plate (from step 1 above) and multiply that number by 1000.

   Example *200 colonies/1 µl × 1000 µl = 2.0×10^5 total cfu.*

   Count the number of chloramphenicol-resistant colonies on the 10-µl plate (from step 1 above) and multiply that number by 100.

   Example *2000 colonies/10 µl × 100 µl = 2.0×10^5 total cfu.*

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

Scaling Up the Ligations and Transformations

Perform individual ligations according to the ligation protocol to reach the desired primary target library size. The number of ligations necessary may vary with each insert and should be based upon efficiencies realized with each pilot reaction. The ligation reaction may be scaled up to 25 µl using the following *Protocol for Scaling Up Ligation Reactions*.

Protocol for Scaling Up Ligation Reactions

Ligation

1. To scale up the ligation reaction, add the following components:

   X µl of resuspended cDNA (Use ~2:1 molar ratio of insert to vector)
   2.5 µl of 10× ligase buffer
   2.5 µl of 10 mM rATP (pH 7.5)
   5.0 µl of the pMyr XR vector (0.1 µg/µl)
   X µl of distilled water for a final volume of 22.5 µl

2. Then add 2.5 µl of T4 DNA ligase (4 U/µl).

3. Flick the tube gently to mix, then spin down briefly.

4. Incubate the reaction tubes overnight at 12°C.

Transformation

1. Transform 100 µl of XL10-Gold Kan ultracompetent cells individually with 1–3 µl of the ligation reaction (see *Transformation Protocol*). The highest number of transformants per nanogram of cDNA is obtained when using 1 µl of ligation reaction per 100 µl of competent cells.

2. Pool the individual transformation reactions after the one hour incubation (step 10 of *Transformation Protocol*).

   Note *This pool is the primary library. Store the primary library at 4°C if amplifying within 1–2 days* (*see* Amplifying the pMyr cDNA Library*).*

3. Plate 1 µl and 10 µl of the pooled transformations onto selection plates to determine the total number of primary transformants.

4. After the number of cfu/µl is determined, plate the rest of the pooled transformants on 15-cm LB chloramphenicol plates. Each plate should contain approximately 20,000–30,000 colonies.

5. Incubate overnight at 37°C

6. Add 6 ml LB medium to each plate and gently scrape the bacteria with a spreader to form a dense suspension.

7. Wash each plate with additional 2 ml LB medium to recover residual bacteria.

8. Pool bacteria in a single sterile flask with a cap.

9. Take half of the pooled bacteria and perform an alkaline lysis/CsCl gradient purification of the plasmid DNA. This DNA is suitable for transformation of yeast.

10. To the remainder of the pooled bacteria, add 0.2 volumes of 80% glycerol and mix thoroughly by inverting the flask.

11. Dispense 1-ml aliquots into sterile 1.5-ml microcentrifuge tubes and freeze at –80°C. These aliquots can be used to inoculate liquid culture for preparation of additional DNA. They can be also used for further amplification by plating on LB-chloramphenicol plates (repeat steps 4 through 8) or by semi-solid agar method.

Verifying the Insert Percentage and Size

Individual colonies can be examined to determine the percentage of vectors with inserts and the average insert size by either PCR directly from the colony with Myr-specific primers or by restriction analysis of individually prepared plasmid DNA. The LacZ test insert is 600 bp in length.

Suggested Sequencing Primers

Sequencing primers[a]	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′

[a] 5′ primer is at the 5′ end of the MCS and 3′ primer is at the 3′ end of the MCS.

Amplifying the pMyr cDNA Library

Amplification of the library is desirable to produce a large and stable quantity of the library. Do not perform more than one round of amplification, as slower-growing clones may be significantly underrepresented.

We recommend amplifying plasmid libraries in 500-ml bottles of 2× LB agarose (see *Preparation of Media and Reagents*) using the semi-solid amplification method.[7,8] The libraries are amplified in suspension, which allows for three-dimensional, uniform colony growth. This reduces the potential for under-representation of particular clones due to the overgrowth of some colonies during the expansion process that accompanies direct plating methods.

Notes *Each 500-ml bottle of 2× LB agarose can accommodate ~5 × 10^5 primary cfu. To amplify a library of 1 × 10^6 primary cfu, two bottles are necessary.*

We have observed that the use of SeaPrep agarose is critical to ensure optimal amplification.

1. On a heated stir plate, using a large stirbar, combine 1.35g of SeaPrep agarose with 450 ml of 2× LB broth (see *Preparation of Media and Reagents*) in each 500-ml autoclavable bottle. Heat and stir until the agarose is in solution.

2. Autoclave the bottles and stir bars for 30 minutes.

3. Allow the bottles to cool to 37°C in a water bath (~1 hour).

4. Transfer the bottles to a stir plate and add 50 µg/ml of chloramphenicol.

5. Add up to 5×10^5 cfu/bottle of primary library and stir for several minutes.

   Note *The bottles must be handled very carefully at this stage. Avoid swirling or bumping the bottles as this may cause the microcolonies to fall out of suspension. The bottles must be incubated without disturbance or representation of the amplified library may be compromised.*

6. Tighten the bottle caps and incubate the bottles for 1 hour in an ice water bath (0°C). The water level in the ice bath should be even with the media level in the bottle.

7. Carefully remove the bottles from the ice bath and gently dry the bottles. Loosen the bottle caps and incubate for 40–45 hours at 30°C. (Incubation at 30°C reduces under-representation of slower growing clones.)

8. Pour the contents of the bottles into sterile 250-ml centrifuge bottles and spin at $10{,}000 \times g$ for 20 minutes at **room temperature**. (Equilibrate the rotor to room temperature several hours prior to centrifugation. Using rotors stored at 4°C will cause the agar to solidify.)

9. Remove the semi-solid agarose supernatant and resuspend the pellets in 25 ml of 2× LB-glycerol (12.5%) (see *Preparation of Media and Reagents*) per 250-ml centrifuge bottle. Remove 100 µl for estimation of library titer and further characterization. Pipet the remainder into 1-ml aliquots. Store at –80°C.

10. Perform 6 serial dilutions with 100 µl of the amplified library diluted into 900 µl of LB medium. Plate 10 µl of the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions onto selection plates. This amplification should result in approximately a 1000-fold increase in stable transformants.

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**.

Dephosphorylate the digested pSos vector with CIAP[9] prior to ligation with the insert DNA. 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 ammonium acetate 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*) such that the concentration of the vector DNA is the same as the concentration of the insert DNA (~0.1 µg/µl).

8. Calculate the amount of insert required for the ligation reaction (see equation below). The ideal molar ratio of insert-to-vector DNA 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:

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

9. Ligate the prepared vector and insert DNA fragments according to the protocol in the table below, which includes three control ligations. Incubate the ligation reaction mixtures overnight at 12°C.

Suggested Ligation Reactions

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*.

10. 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, verify 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.

YEAST TRANSFORMATION

Cotransformation of Bait and Target Plasmids

We recommend introducing the bait and target plasmids 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[10] 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

In order to gain familiarity with the protocol and expected results, users that are 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. After the protocols are familiar, we recommend including 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 8 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, perform the protocol 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.*

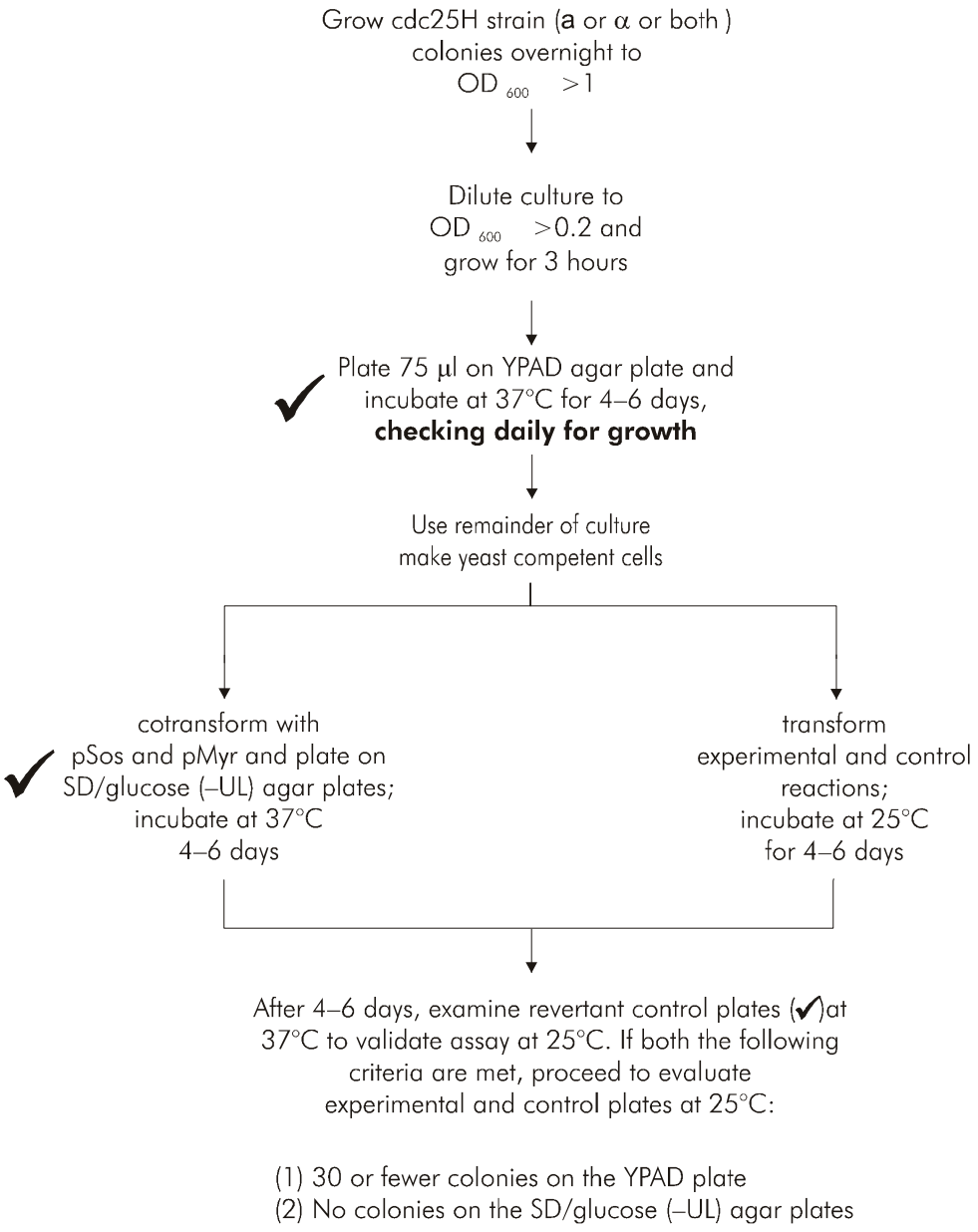


FIGURE 8 Strategy for preparation and phenotypic verification of yeast competent cells

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 *We recommend generating 4–5 independent preparations of yeast competent cells derived from independent colonies, due to the ability of the cdc25H strain to produce revertants of the temperature-sensitive phenotype during growth.*

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.

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 1×10^6 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.

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

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/Lithium Acetate 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 sterile 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 0.5×10^3–1×10^4 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 *(1^{st} column) and transformants in mating type* α *(2^{nd} 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). 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 35 µl 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 9. Before each library screening, prepare fresh yeast competent cells as described in *Preparation of Yeast cdc25H Competent Cells.* . It is necessary to test 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.

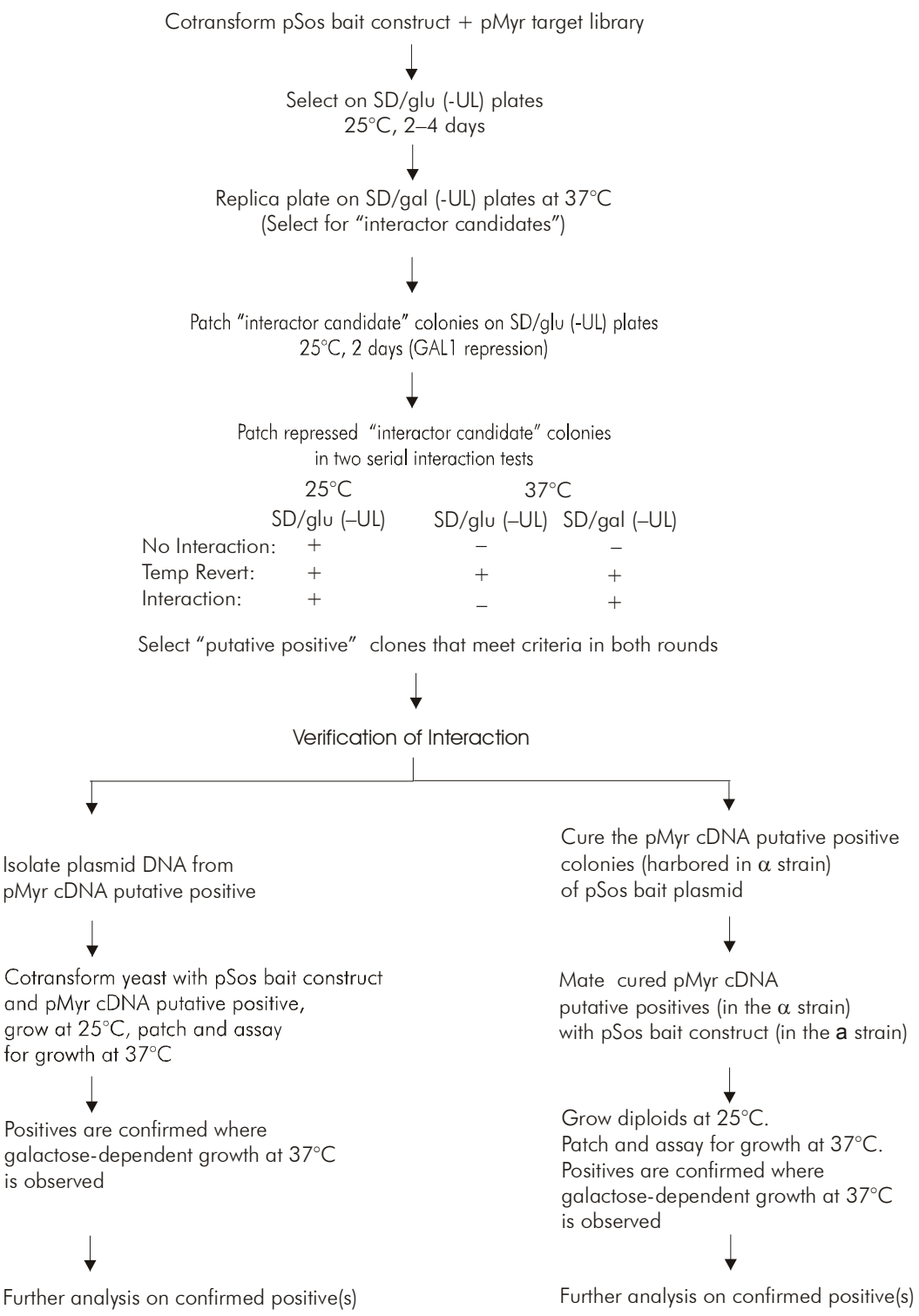


FIGURE 9 Library screening procedure for the CytoTrap two-hybrid system.

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 1×10^4–2×10^4 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 sodium acetate (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.[11,12]

APPENDIX I: GENERAL COMPARISON OF *Escherichia coli* VERSUS YEAST HOST STRAINS

Quality/feature	Host strain	
	Escherichia coli	**Yeast**
Doubling time	20 minutes	>1 hour
Complex media (nonselective)	LB and NZY	YPAD
Chemically defined media (selective)	M9	SD
pH	7 (neutral)	5.8 (acidic)
Growth temperature	37°C	25°C
Antibiotic sensitivity	Sensitive to most antibiotics	Resistant to most antibiotics including ampicillin
Selection method for presence of plasmid	Add antibiotic to media	Remove amino acid from media
Colonial morphology	Small, flat colonies	Large, rounded colonies
Cell diameter	1 μm	3–5 μm
Odor	Musty, pungent	Bread dough

APPENDIX II: RNA PURIFICATION AND QUANTIFICATION

RNA Purification

The Agilent RNA Isolation Kit, using the guanidinium thiocyanate–phenol–chloroform extraction method,[6] is strongly recommended for total RNA isolation. This method is rapid, yet produces large amounts of high-quality, undegraded RNA.

Although AccuScript reverse transcriptase is not inhibited by ribosomal RNA (rRNA) and transfer RNA (tRNA) contamination, it is advisable to select the poly(A)$^+$ fraction. The amounts of rRNA and tRNA vastly outnumber amounts of mRNA and will decrease the efficiency of the cDNA synthesis. Poly(A)$^+$ RNA is selected on oligo(dT) cellulose columns.[13] Some protocols call for the addition of SDS in the purification steps. Sodium dodecyl sulfate is a powerful enzyme inhibitor and helps prevent degradation of the RNA by RNases, but its presence can also inhibit the enzymes required for cDNA synthesis. If the mRNA intended for use with this kit is suspended in an SDS solution, the RNA must be phenol extracted and ethanol precipitated.

Ribonucleases A and T1 are widely used in almost all molecular biology labs and are nearly indestructible. Ribonucleases are produced by microbes and have also been found in the oils of the skin. Make an effort to use tubes and micropipet tips which have been handled only with gloves. Use freshly autoclaved and baked tips and tubes. Usually these precautions are sufficient, but to be absolutely certain that microcentrifuge tubes and other components intended for use with RNA are not contaminated, the components can be treated with DEPC. Diethylpyrocarbonate is extremely toxic and should be handled with care. Submerge the microcentrifuge tubes in a 0.1% (v/v) DEPC-treated water solution. Leave the beaker of submerged tubes in a fume hood overnight and then dispose of the DEPC-treated water. Autoclave the microcentrifuge tubes for at least 30 minutes. Even though the tubes may still have a sweet DEPC odor, the DEPC is completely inactivated by this procedure. Place the tubes in a drying oven overnight. Equipment which cannot be treated by DEPC can be rinsed in a freshly mixed 3% (v/v) hydrogen peroxide solution, followed by a methanol rinse. Remember, once the RNA is converted to first-strand cDNA, RNases are no longer a concern. Caution should still be exercised in maintaining a sterile, DNase-free environment.

RNA Quantification

RNA can be quantified by measuring the optical density of a dilute RNA solution. The conversion factor for RNA at the wavelength of 260 nm is 40 µg/ml/OD unit as shown in the example below.

Two microliters of an unquantified poly(A)$^+$ sample is added to 498 ml of water ($OD_{260} = 0.1$).

$$0.1 \text{ OD unit} \times \left(\frac{500}{2} \text{ dilution factor}\right) \times 40 \text{ µg / ml} = 1000 \text{ µg / ml or } 1 \text{ µg / µl}$$

If a sample has significant rRNA contamination, the actual amount of mRNA available for cDNA conversion will be overestimated by this procedure.

If the amount of mRNA is below 1.5 µg/synthesis reaction, the RT may synthesize unclonable hairpin structures. If the amount of mRNA is above 7 µg the percentage of cDNAs which are full length may decrease. The cDNA Synthesis Kit provided with the CytoTrap system has been optimized for 5 µg of mRNA, but successful libraries have been generated using the minimums and maximums described here.

Secondary structure may be a problem with certain RNAs, particularly plant and tumor mRNAs. These samples can be treated with methylmercury hydroxide (see *Appendix III: Methylmercury Hydroxide Treatment*). Treatment with methylmercury hydroxide requires heating the RNA to 65°C. If the RNA contains even a minute amount of RNase, the RNase activity will increase by several orders of magnitude with the increased temperature and significantly degrade the RNA. Treatment with methylmercury hydroxide is therefore recommended only if the RNA is free of RNases.

APPENDIX III: METHYLMERCURY HYDROXIDE TREATMENT

Warning *Methylmercury hydroxide is an extremely toxic chemical. Wear gloves and use with caution in a fume hood.*

1. Resuspend the mRNA in 20 µl of DEPC-treated water.
2. Incubate at 65°C for 5 minutes.
3. Cool to room temperature.
4. Add 2 µl of 100 mM CH_3HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 µl of 700 mM β-mercaptoethanol.
7. Incubate at room temperature for 5 minutes.

APPENDIX IV: ALKALINE AGAROSE GELS

Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix.

Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first- and second-strand cDNA.

Note *The test cDNA sample will run as a tight band at 1.8 kb and will show distinctly different intensity between the first and second strands. This is due to the relative ratio of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally the second strand will be only 1/10 to 1/20 the intensity of the first-strand band.*

Alkaline agarose gels differ from conventional gels in the following ways:

1. The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.

2. The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree which makes a clear autoradiograph difficult to interpret.

The following alternative methods help avoid these complications.

The Slide Technique

The easiest and least expensive method is to use a 5 × 7.5-cm glass slide, position a minigel comb over it with high tension clips, and add 10 ml of molten alkaline agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel which can be exposed without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. To improve the resolution, pat the gel dry with several changes of Whatman 3MM paper after electrophoresis is complete.

To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.

The Vertical Alkaline Agarose Technique

Vertical alkaline agarose gels can be produced using a vertical gel apparatus with 1.5-mm spacers. Since the alkaline agarose gels do not have sufficient friction to remain bound to ordinary glass, a frosted glass plate or gel bond must be used with the vertical apparatus. The combs normally used for acrylamide can be used with this apparatus, if the outside teeth are wrapped in tape to prevent the comb from sinking more than 1.2 cm into the agarose.

The 55°C agarose will solidify almost immediately on contact with the cold glass plates, so it is essential to load the mold rapidly with a 60-ml syringe. The comb should already be in the mold, and if it is necessary to reposition the comb, do it immediately after the gel is poured. In order to reduce the possibility of destroying the wells when pulling out the comb, place the solidified gel in a –20°C freezer for 5 minutes immediately prior to removing the comb. When pulling out the comb, it is essential to avoid a vacuum between the teeth and the well. Vacuum can be detected when the well distorts from its normal square shape. When a vacuum occurs, push the comb to separate the glass plates and break the vacuum. After the samples have been run and the glass plates are ready to be opened, slide the unfrosted glass plate off of the alkaline agarose gel instead of prying the plate away from the gel. Pat the gel dry several times using several pieces of Whatman 3MM paper.

Note *To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.*

Conventional Submerged Gels

These gels will require drying either by blotting or through the use of a gel dryer.

Caution ***Even when multiple layers of absorbent paper are placed under the gel, free nucleotides can easily contaminate the drying apparatus. These gels should be poured as thin as possible and should be dried without heat, if time permits, and should never be dried above 40°C.***

Protocol

The following formula makes 80 ml of 1% (w/v) alkaline agarose for electrophoresis of cDNAs in the 1–3 kb size range.

Melt 0.8 g of agarose in 72 ml of water. Allow the agarose to cool to 55°C. During this time, assemble the gel apparatus. Add 8 ml of 10× alkaline buffer[§] to the cooled agarose, swirl to mix, and pour the agarose immediately. If buffer is added before the correct temperature is reached, the agarose may not solidify.

Load the sample in an equal volume of alkaline agarose 2× loading buffer[§]. Run the gel with 1× alkaline buffer at 100 mA and monitor the system for heat. If the apparatus becomes warmer than 37°C, the amperage should be reduced. The migration of the BPB in alkaline agarose is similar to the migration in regular agarose and should be run to at least one-half or three-quarters distance of the gel.

Note *The alkali condition causes the blue dye to fade.*

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

APPENDIX V: ETHIDIUM BROMIDE PLATE ASSAY—QUANTITATION OF DNA

An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

Preparation of Ethidium Bromide Plates

Note *Prepare the EtBr plates under a fume hood.*

Prepare 100 ml of a 0.8% (w/v) agarose and Tris-acetate media. Cool the molten agarose to 50°C and then add 10 µl of EtBr stock solution (10 mg/ml). The EtBr stock solution is prepared in dH_2O and is stored in the dark at 4°C. Swirl to mix the EtBr stock solution and pour the solution into 100-mm petri dishes using ~10 ml/plate. Allow the plates to harden and incubate the plates at 37°C to dry, if necessary. These plates may be stored in the dark at 4°C for up to 1 month.

Preparation of Standards

Using a DNA sample of known concentration, make seven serial dilutions in 100 mM EDTA to cover the range from 200 to 10 ng/µl. These standards may be stored at –20°C for 3 months.

Plate Assay for Determination of DNA Concentration

Using a marker, label the petri dish to indicate where the sample and the standards (200, 150, 100, 75, 50, 25, and 10 ng/µl) will be spotted.

Thaw the standards and carefully spot 0.5 µl of each standard onto the surface of a prepared EtBr plate. Be careful not to dig into the surface of the plate. Let capillary action pull the small volume from the pipet tip to the plate surface and do not allow a bubble to form. Change pipet tips between each standard.

After spotting all of the standards, immediately spot 0.5 µl of the cDNA sample onto the plate adjacent to the line of standards. Allow all spots to absorb into the plate for 10–15 minutes at room temperature. Remove the lid and photograph the plate using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates.

Standards and unknowns must be spotted within 10 minutes of each other.

TROUBLESHOOTING

cDNA Synthesis

Observation	Suggestion
Poor first-strand synthesis	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	Minute amounts of SDS or lithium in the RNA will inhibit the first-strand synthesis reaction. Do not use these in the RNA preparations. Multiple phenol–chloroform extractions will sometimes remove the inhibitors.
	Ensure that sufficient mRNA is present. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	Ensure that the [α-^{32}P]dNTP is not contaminated or degraded, causing poor label incorporation into cDNA, and falsely indicating poor cDNA synthesis. Protect the [α-^{32}P]dNTP from heat and leave it at room temperature for the minimum time required.
Poor second-strand synthesis	Interpret the gel results correctly. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally, the second strand will have only 1/10 to 1/20 the intensity of the first-strand band.
	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously.
	No first-strand synthesis. See the previous suggestions for *Poor first-strand synthesis*.
No first-strand synthesis, but good second-strand synthesis	Ensure that there is no DNA contamination in the RNA preparation.
Hairpinning	Do not allow incubation temperatures above 16°C . Add second-strand synthesis reaction components to the first-strand reaction mix on ice and then transfer the reaction mixture directly to 16°C for incubation. After incubation, place the samples on ice immediately.
	Ensure that sufficient mRNA is present. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation.
	Some sources of RNA may have secondary structure (e.g., tumors, some plants, etc.). The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure (see *Appendix III: Methyl-mercury Hydroxide Treatment*).
	Ensure the correct amount of DNA polymerase is being used. Use a calibrated pipet to measure the enzyme. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.
Low counts in the drip column fractions	The number of counts per second per fraction may vary from 0 to 250 cps and yield primary libraries of $>1 \times 10^6$ pfu. Most of the counts remaining in the drip column are from unincorporated [α-^{32}P]dNTP. Verify the quantity of cDNA on the EtBr plate.
Poor ligation	Do not use excess ligase as this introduces an inhibitory amount of glycerol into the reaction. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.

Two-Hybrid Vector System Screening

Observation	Suggestion
More than 30 colonies appear on non-transformed plates when incubated at 37°C > 4 days	The culture contains a high number of temperature-sensitive revertants or a yeast strain which 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 *Preparation of Yeast Host Strain* 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*.
Cotransformants of pSos MAFB and pMyr MAFB 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.
Cotransformants of pSos Col I and pMyr Lamin C grow at 37°C	Verify that the correct control plasmid pair is used.
Transformants show no galactose-dependent growth at 37°C	Verify the nucleotide sequence of the Sos and insert DNA to ensure the bait protein is expressed.
	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 expression of the bait protein is known.
	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).

Plasmid Isolation from Yeast

Observation	Suggestion
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

10× Alkaline Buffer (per 50 ml)

3 ml of 5.0 M NaOH
2 ml of 0.5 M EDTA
45 ml of deionized H_2O

2× LB Broth (per Liter)

20 g of NaCl
20 g of tryptone
10 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

Alkaline Agarose 2× Loading Buffer

200 µl of glycerol
750 µl of water
46 µl of saturated BPB*
5 µl of 5 M NaOH

* To make saturated BPB, add a small amount of bromophenol blue crystals to water and vortex. Centrifuge the sample briefly and look for the presence of an orange pellet. If a pellet is seen, the solution is saturated. If not, add more crystals and repeat the procedure.

2× LB Agarose

450 ml of 2× LB
1.35 g of SeaPrep agarose
Mix on a heated stir plate using a large stir bar until the agarose is in solution

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 Agar IPTG/X–gal Plates

To LB-chloramphenicol agar, autoclaved and cooled to 55°C add:
X-gal (stock made in dimethylformamide (DMF)) to a final concentration of **80 µg/ml** and
IPTG (stock made in sterile distilled water) to a final concentration of **20 mM**

or

Spread 100 µl of 10 mM IPTG and 100 µl of 2% X-gal on LB–chloramphenicol agar plates 30 minutes prior to plating the transformations

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)

NaOH/β-ME Buffer

1.85 M NaOH
7.5% β-Mercaptoethanol

TE Buffer

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

Column-Loading Dye

50% (v/v) glycerol
10% (v/v) 10× STE buffer
40% (w/v) saturated BPB*

* for saturated BPB, see footnote in recipe for *Alkaline Agarose 2× Loading Buffer*, above

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)

1.4 M β-ME (yeast competent cells)

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

LiSORB (per Liter)

100 mM lithium acetate
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

Nondenaturing Acrylamide Gel (5%)

Mix the following in a vacuum flask

5 ml of 10× TBE buffer
8.33 ml of a 29:1 acrylamide–bis-acrylamide solution
36.67 ml of sterile deionized H_2O

De-gas this mixture under vacuum for several minutes
Add the following reagents
25 µl of TEMED
250 µl of 10% ammonium persulfate

NZY+ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:
12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

10× STE Buffer

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

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.

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

PEG/Lithium Acetate Solution

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

2× LB Glycerol (12.5%) (per 100 ml)

2 g NaCl
2 g tryptone
1 g yeast extract
Add deionized H_2O to a final volume of 50 ml
Adjust to pH 7.0 with 5 N NaOH
Autoclave
Add 50 ml autoclaved glycerol (25% v/v)

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

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
Dry the plates at room temperature for 2–3 days
Store the plates in a sealed bag

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.

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ENDNOTES

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