pMyr XR Vector Kit

Instruction Manual

Catalog #217430

Revision B

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217430-12

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pMyr XR Vector Kit

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pMyr XR Vector Kit

MATERIALS PROVIDED

Material provided	Quantity
pMyr XR vector, digested with *Eco*R I–*Xho* I and treated with calf intestinal alkaline phosphatase (CIAP)	5 μg (0.1 μg/μl)
XR LacZ test insert (600 bp) digested with *Eco*R I and *Xho* I	30 ng (10 ng/μl)
cdc25H host strain	0.5 ml

STORAGE CONDITIONS

Vectors: –20°C
Yeast Strains: –80°C

HOST STRAINS AND GENOTYPES

Host strain	Genotype
cdc25H Yeast Strain	Matα *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^{+}$

ADDITIONAL MATERIALS REQUIRED

Chloramphenicol
Tryptone
Yeast extract
Bacto® peptone
Bacto® agar
Adenine sulfate
Tris-HCl
EDTA

NOTICE TO PURCHASER

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

INTRODUCTION

Background

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

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

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

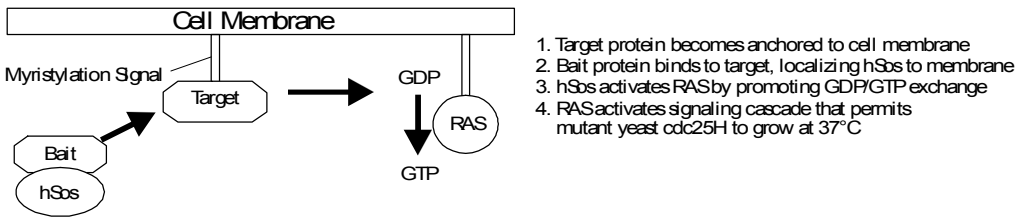


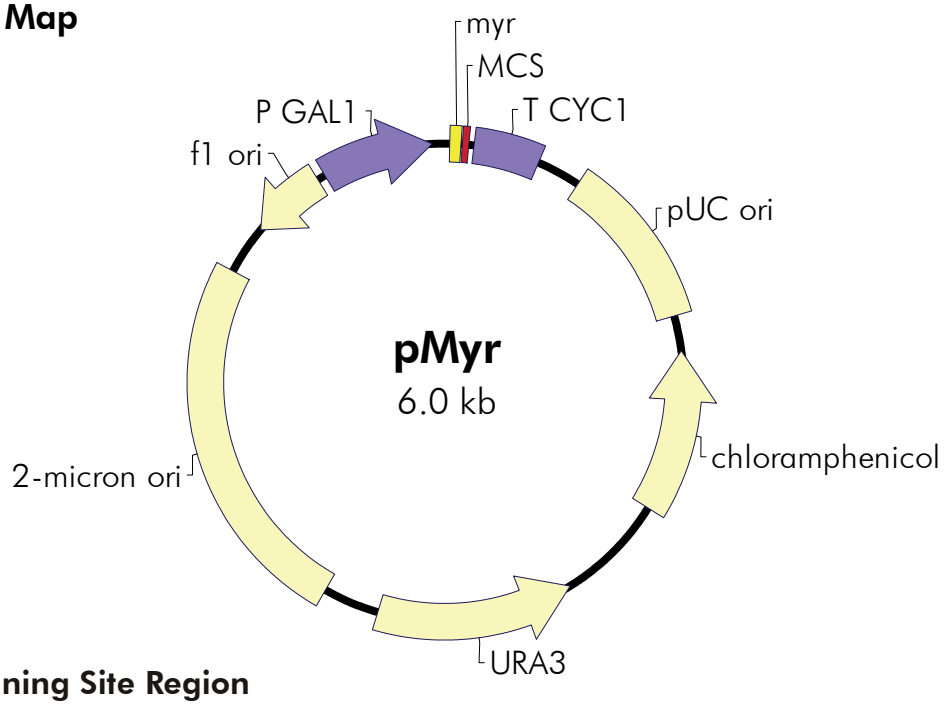
FIGURE 1 Schematic diagram of the Ras-signaling pathway utilized in the CytoTrap two-hybrid system.

VECTOR DESCRIPTION

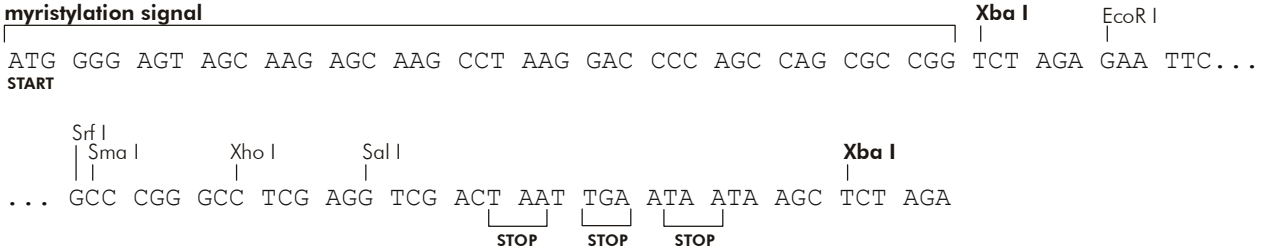
The pMyr XR vector contains DNA encoding the myristylation membrane localization signal (Myr) and is designed for constructing cDNA libraries that contain DNA inserts encoding target proteins. The *GAL1* promoter, driving expression of the Myr-target fusion is induced by adding galactose to the growth medium. Target proteins will be directed to and anchored in the yeast membrane (see Figure 1).

The pMyr XR vector contains the pUC and 2μ origins for replication in *E. coli* and yeast, respectively. The pMyr vector also carries the yeast biosynthetic gene *URA3*, for selection of yeast transformants based on nutritional requirements, and carries the chloramphenicol-resistance gene for selection in *E. coli*.

pMyr Vector Map



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



Note: *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	5524–5974

FIGURE 3 The pMyr vector. The complete vector sequence is available at *www.genomics.agilent.com*. The vector supplied in this kit has been digested with *Eco*R I and *Xho* I restriction enzymes, and does not contain the sequence between *Eco*R I and *Xho* I.

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)	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 Strain Genotype

Host strain	Genotype
cdc25H Yeast Strain (α)	*MATα 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*).

TARGET LIBRARY CONSTRUCTION

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

cDNA Libraries

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

VECTOR CONSTRUCTION

Insert Preparation, Ligation, and Transformation

The pMyr XR vector supplied is predigested with *Eco*R I and *Xho* I restriction enzymes and treated with calf intestinal alkaline phosphatase (CIAP). DNA encoding the target protein is prepared for insertion into the pMyr vector either by restriction digestion or PCR amplification. DNA encoding the target protein must be inserted so that the target protein is expressed in the same reading frame as the Myr protein.

Cloning Considerations

- The gene of interest should have ends compatible with the *Eco*R I/*Xho* I ends of the pMyr XR vector.
- Resuspend the prepared insert DNA in a volume of TE buffer[§] that will allow the concentration of the insert DNA to be the same as the concentration of the vector DNA (~0.1 µg/µl).

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

Ligating cDNA into the pMyr XR Vector

Note *For ligation, the ideal ratio of insert-to-vector DNA is variable; however, a reasonable starting point is 2:1 (insert-to-vector molar ratio), measured in available picomole ends. The ratio is calculated as follows:*

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

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 LacZ XR 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)
   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. Incubate the reaction tubes overnight at 12°C.

4. Perform a standard transformation of the ligation reactions into *E. coli* competent cells.

Preparing Agar Plates for Color Screening

To prepare LB agar plates for blue–white color screening, add 80 µg/ml X-gal, 20 mM IPTG, and the appropriate antibiotic to the LB agar.[§] Alternatively, 100 µl of 10 mM IPTG and 100 µl of 2% X-gal can be spread on the LB agar plates 30 minutes prior to plating the transformations. Prepare the IPTG in sterile dH_2O. Prepare the X-gal in dimethylformamide (DMF).

Determining the Number of Transformants

Plating

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

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

2. Plate 1 µl and 10 µl of each 1 ml sample transformation onto LB-chloramphenicol agar plates.

3. 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.*

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*).

YPAD Agar (30–40 Plates/Liter)

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

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–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)

LB Agar IPTG/X–gal Plates

80 µg/ml X-gal in dimethylformamide (DMF)
20 mM IPTG in sterile distilled water
add the IPTG and X–gal solutions to LB–chloramphenicol agar plates
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

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)
Note rATP is added separately in the ligation reaction

TE Buffer

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

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

Bacto® is a registered trademark of Difco Laboratories.
Parafilm® is a registered trademark of American Can Company.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.