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ProQuest™ Two-Hybrid System

USER GUIDE

A sensitive method for detecting protein-protein interactions

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The information in this guide is subject to change without notice.

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Product information

IMPORTANT! Before using this product, read and understand the information in the "Safety" appendix in this document.

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Contents and storage

Table 1 ProQuest™ Two-Hybrid System (Cat. No. PQ10001-01)

Component[1]	Storage
ProQuest™ Vectors	-80°C
ProQuest™ Control Vectors	-20°C
LR Clonase™ II	-20°C

[1] All components are shipped on dry ice.

Shipping and storage

The ProQuest™ Two-Hybrid System is shipped as described below. Upon receipt, store each item as detailed below.

Box	Component	Shipping	Storage
1	ProQuest™ Vectors	Dry ice	-80℃
2	ProQuest™ Control Vectors	Dry ice	-20℃
3	LR Clonase™ II	Dry ice	-20℃

ProQuest™ Two-Hybrid system reagents

The following reagents are included with the ProQuest™ Two-Hybrid System Reagents.

ProQuest™ Vectors Box (Store the reagents at -80℃)		
Reagent	**Composition**	**Amount**
pDEST™22	Liquid in TE Buffer, pH 8.0	6 µg
pDEST™32	Liquid in TE Buffer, pH 8.0	6 µg
MaV203 glycerol stock	YPAD + 20% glycerol	0.5 mL

ProQuest™ Control Vectors Box (Store the reagents at -20℃)		
Reagent	**Composition**	**Amount**
pEXP™-AD502	Liquid in TE Buffer, pH 8.0	1 µg
pEXP™32/Krev1	Liquid in TE Buffer, pH 8.0	10 µg
pEXP™22/RalGDS-wt	Liquid in TE Buffer, pH 8.0	10 µg
pEXP™22/RalGDS-m1	Liquid in TE Buffer, pH 8.0	10 µg
pEXP™22/RalGDS-m2	Liquid in TE Buffer, pH 8.0	10 µg

LR Clonase™ II Box (Store at -20℃ for up to 6 months. For long-term storage, store at -80℃.)		
Reagent	**Composition**	**Amount**
Gateway™ LR Clonase™ II Enzyme Mix	Proprietary	40 µL
Proteinase K Solution Solution	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% glycerol	40 µL
pENTR™-gus Positive Control	50 ng/µL in TE Buffer, pH 8.0	20 µL

Genotype MaV203

The genotype of MaV203 is as follows:

MaV203 (MATα, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10*::URA*3, *GAL*1::*lacZ*, *HIS*3$_{\text{UAS GAL1}}$*::HIS*3@*LYS*2, *can*1^{R}, *cyh*2^{R})

Accessory products

Introduction

The products listed in this section may be used with the ProQuest™ Two-Hybrid System. For more information, see (**thermofisher.com**).

MaV203 competent cells

We provide a glycerol stock of the MaV203 yeast strain. A protocol is provided to perform small-scale transformations using this stock. To limit your workload, purchase competent MaV203 cells, subclone scale. Alternatively, prepare competent cells using the *S. c*. EasyComp™ Kit, which can be frozen for later use. For large-scale applications, such as a forward two-hybrid library screen, we recommend obtaining MaV203 Competent Cells, Library Scale to get the highest transformation efficiency and to limit your workload.

Item	Amount	Catalog no.
MaV203 Competent Cells, Library Scale	2 x 0.55 ml	11281011
MaV203 Competent Cells, Subclone Scale	4 x 0.10 ml	11445012
S. c. EasyComp™ Kit	1 kit	K505001

Note: For your convenience, we have added a protocol in the (Appendix C, “Supplementary protocols”) to make your own large-scale competent cells using MaV203 cells provided with the kit.

Accessory products

Some of the reagents supplied in the ProQuest™ Two-Hybrid System and as well as other products suitable for use with the kit are available separately from **thermofisher.com**. Ordering information is provided below.

Item	Amount	Catalog no.
PureLink™ HQ Mini Plasmid DNA Purification Kit	100 preps	K210001
PureLink™ HiPure Plasmid Miniprep Kit	25 preps 100 preps	K210002 K210003
PureLink™ HiPure Plasmid Midiprep Kit	25 preps 50 preps	K210004 K210005
5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal),	100 mg 1 g	15520034 15520018
Denatured Sheared Salmon Sperm DNA	5 x 1 mL	15632011
One Shot™ TOP10 Electrocomp™ *E. coli*	10 reactions 20 reactions	C404050 C404052

(continued)

Item	Amount	Catalog no.
Platinum™ PCR SuperMix HiFi	100 reactions	12532016
Platinum™ PCR SuperMix	100 reactions	11306016
Platinum™ *Taq* DNA Polymerase	100 reactions	10966018
S.N.A.P.™ Gel Purification Kit	25 reactions	K199925
2.5 mM dNTP Mix	1 mL	R72501
Gateway™ LR Clonase™ II Enzyme Mix	20 reactions 100 reactions	11791020 11791100
Gateway™ BP Clonase™ II Enzyme Mix	20 reactions 100 reactions	11789020 11789100
Proteinase K Solution	100 mg 1g	25530015 25530031
pCR™8/GW/TOPO™ TA Cloning™ Kit	20 reactions	K250020
Gentamicin Reagent Solution (10 mg/mL), liquid	10 mL 10 x 10 mL	15710064 15710072
SOC Medium	10 x 10 mL	15544034
LB Agar, powder (Lennox L Agar)	500 g 2.5 kg	22700025 22700041
LB Broth Base, powder (Lennox L Broth Base)	500 g 2.5 kg	12780052 12780029

3-Aminotriazole

For selection of HIS^{+} transformants in *S. cerevisiae*, you will need to obtain 3-aminotriazole, which is available from Millipore Sigma, St. Louis, MO, (Catalog No. A8056).

Zymolyase™

For isolation of yeast DNA you need Zymolyase™ (1.5 U/µL), which is available from G-Biosciences, St.Louis, MO (Catalog no. 786-036).

Cycloheximide

For Plasmid shuffling (see “Plasmid shuffling” on page 94), you will need to obtain cycloheximide, which is available from Millipore Sigma, St. Louis, MO (Catalog No. C1988).

Yeast media

For yeast selective media, recipes are provided in Appendix B, “Recipes”.

2 General introduction

Overview

ProQuest™ Two-Hybrid System

The ProQuest™ Two-Hybrid System is a genetic method for detecting interactions between proteins *in vivo* in the yeast *Saccharomyces cerevisiae*. The ProQuest™ Two-Hybrid System draws on modifications by Chevray & Nathans and incorporates Gateway™ Technology.

Supported applications

The ProQuest™ Two-Hybrid System supports three types of applications:

- Verifying an interaction between two known proteins or protein domains for which there is a prior reason to expect an interaction (testing two-hybrid interactions); see Chapter 3, “Verifying interaction”.
- Screening a library for novel proteins that specifically interact with a known bait (forward two-hybrid library screen); see Chapter 4, “Forward Two-Hybrid library screen”.

Advantages of the ProQuest™ Two-Hybrid system

The ProQuest™ Two-Hybrid System is a system designed to enable detection of protein-protein interactions and has been modified to decrease false positives. The primary modifications include:

- Uses low-copy-number (*ARS*/*CEN*) vectors to control over-expression and increase reproducibility
- Contains three different reporter genes with independent promoters to rapidly weed out false positives
- Uses a reporter gene (*URA*3) that allows both positive and negative selection
- An extended panel of yeast control vectors to aid in setting up the experiments and evaluate results
- Incorporation of the Gateway™ Technology to allow rapid and easy generation of bait and prey constructs, and to facilitate down-stream applications

System components

The ProQuest™ Two-Hybrid System includes:

- Yeast expression vectors pDEST™22, pDEST™32, and pEXP™-AD502 for generation of GAL4 DNA Binding Domain (GAL4 DBD) and GAL4 Activation Domain (GAL4 AD) fusion proteins
- Reagents for production of the expression clones containing GAL4 DBD and GAL4 AD fusion proteins
- A glycerol stock of yeast strain MaV203, which is the two-hybrid yeast strain used
- Positive and negative controls for the two-hybrid assay

Gateway™ Technology

All yeast expression vectors in the ProQuest™ Two-Hybrid System are Gateway™-adapted to allow rapid and easy generation of bait and prey constructs, and to facilitate down-stream applications.

The Gateway™ Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems.

For a brief description of the Gateway™ Technology, see the **Appendix**, Appendix A, "Gateway™ recombination reactions"

Purpose of this manual

This manual provides the following information:

- An overview of the two-hybrid technology ("General description of the Two-Hybrid system" on page 15)
- Instructions to make your bait and prey plasmid ("Generating bait and prey plasmids" on page 31)
- Guidelines for testing the interaction between two proteins ("Recommended controls" on page 40)
- Guidelines for choosing the library you want to screen ("Choosing Two-Hybrid library" on page 49)
- Procedures to perform forward two-hybrid library screens ("Screening forward Two-Hybrid library" on page 55)

Important

The ProQuest™ Two-Hybrid System is designed to help you perform your two-hybrid analysis. The system has been designed to help you perform your experiment in the simplest, most direct fashion, but use of the system assumes that users are familiar with manipulating yeast and cloning.

General description of the Two-Hybrid system

Two hybrid proteins

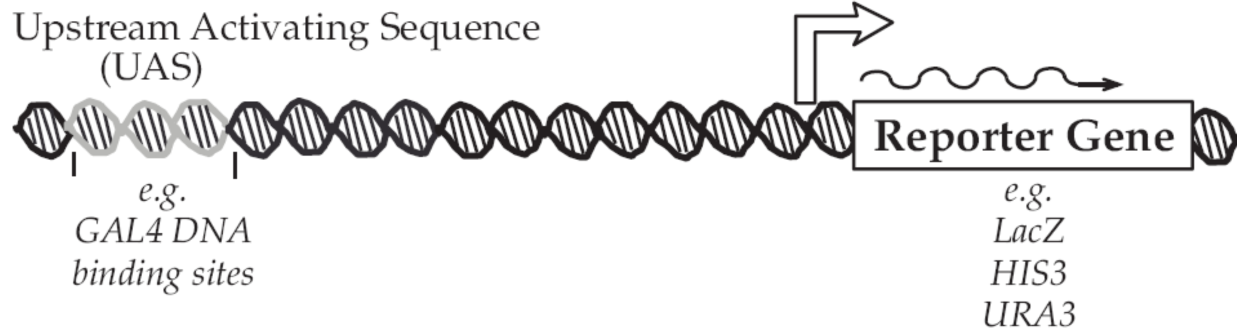
Two-hybrid or interaction trap systems exploit the fact that transcription factors are comprised of two domains, a DNA binding domain (DBD) and an activation domain (AD). Two separate hybrid proteins are constructed in two-hybrid screens. The first hybrid protein is the DBD/protein X fusion known as the "bait", while the second hybrid protein is the AD/protein Y fusion known as the "prey". These two hybrids are encoded on separate yeast expression plasmids, with independent selectable markers.

Figure 1 Bait

Figure 2 Prey

Reporters under control of UAS

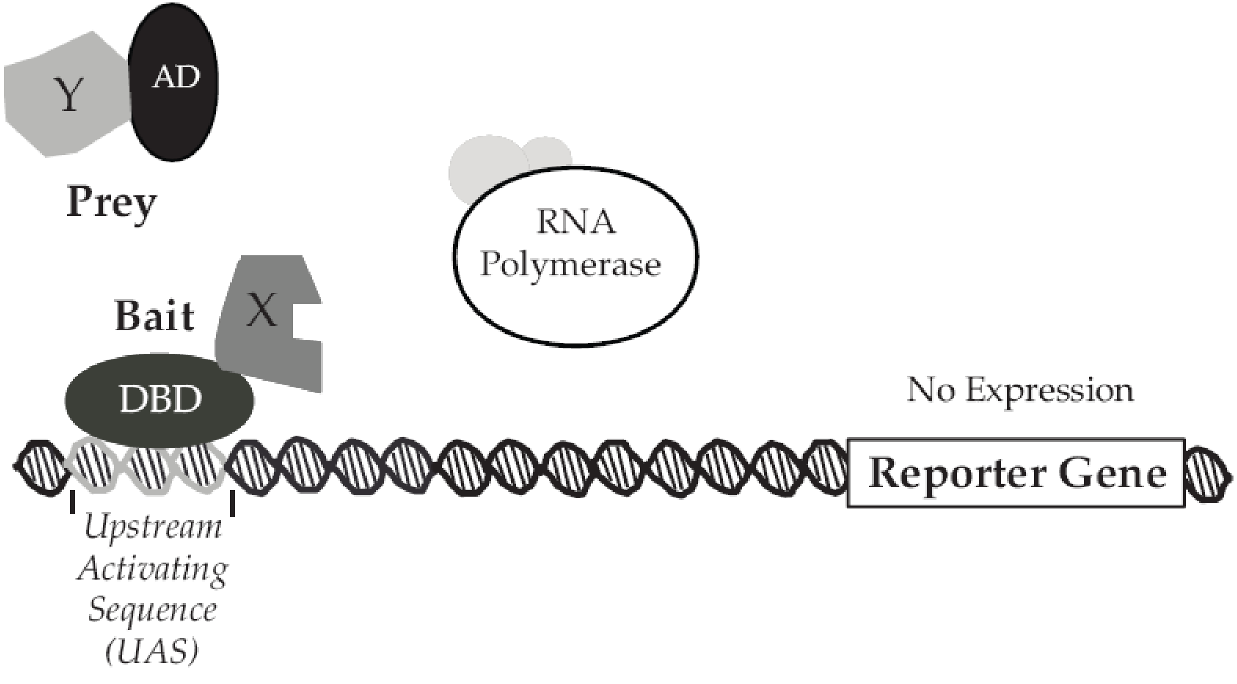
The yeast strain employed contains reporter genes, such as *lac*Z or auxotrophic markers such as *HIS*3 or *URA*3. The regulatory regions of these reporters have been engineered to contain the DNA binding sites (operator sequences) for the DBD/protein X fusion (bait). These operator sequences act as upstream activating sequences (UAS) in yeast.



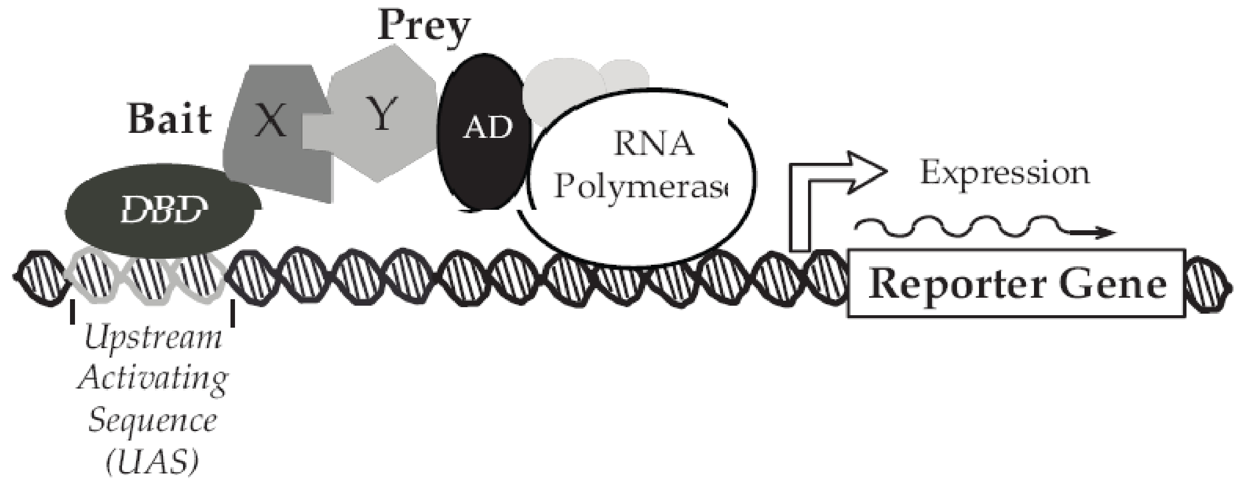
Note: Yeast two-hybrid strains have been specifically modified to contain these reporter genes. *wt* yeast strains cannot be used!

Interaction drives expression of reporters

The yeast strain used is transformed with the expression plasmids encoding the bait and prey. If protein X interacts with protein Y in the nucleus, this will bring the activation domain together with the DNA-binding domain to reconstitute transcriptional activation and result in expression of the reporter genes.



X and Y do not interact - no Reporter Gene Expression



X and Y interact - Reporter Gene Expressed!

Evaluating reporter gene expression

There are two main ways to check for positive interactions in yeast strains containing reporter genes:

- Positive interactions are detected by selecting on plates lacking the auxotrophic marker, such as Histidine or Uracil. Yeast cells containing plasmids that express interacting bait and prey proteins will grow and form colonies.
- Positive interactions are detected by assaying for enzyme activity, such as colorimetric assays for β-galactosidase activity. This is used to reduce false positives after selection for auxotrophs, or to measure interaction strength quantitatively.

Screening Two-Hybrid libraries

Two-hybrid libraries (i.e. prey libraries) consist of a collection of expression plasmids in which an Activation Domain is fused to individual cDNAs. Screening prey libraries will detect prey proteins that interact with the bait protein of interest. To perform the screen, transform the library and bait expressing plasmids into yeast. Cells containing a prey that interacts with the bait will form colonies on selective plates. Secondary screens, such as for β-galactosidase expression, will confirm the interaction.

False positives

Early two-hybrid systems suffered from false positives - candidate proteins identified as interacting but which do not truly interact or are biologically irrelevant. False positives can result from:

- Proteins containing regions with surfaces having low affinities for many different proteins, (e.g., large hydrophobic surfaces)
- Proteins that normally interact with a large number of proteins (e.g., heat shock proteins)
- Proteins containing regions functioning as activation domains
- Proteins affecting chromatin structure
- Proteins having low or nonspecific affinities for the promoter regions (or proteins bound there) that drive the expression of reporter genes

Limiting the false positives is essential in successful two-hybrid experiments.

Advanced™ Two-Hybrid systems

The ProQuest™ Two-Hybrid System has been extensively improved to limit false positives, as well as to allow performance of more advanced applications. For details about the ProQuest™ Two-Hybrid System and strategies to limit false positives, see "Reducing false positives" on page 21.

ProQuest™ Two-Hybrid system

Verifying Two-Hybrid interaction

Using the ProQuest™ Two-Hybrid System to verify an interaction between two known proteins, you will perform the following steps:

1. Construct bait plasmid
2. Construct prey plasmid
3. Transform yeast cells with bait and prey plasmid
4. Test reporter activity

Forward Two-Hybrid library screen

Using the ProQuest™ Two-Hybrid System for a forward two-hybrid library screen, you will perform the following steps:

1. Construct and test bait plasmid
2. Construct or obtain two-hybrid library
3. Transform yeast cells with bait plasmid and library
4. Select for reporter activity by growth on auxotrophic plates
5. Confirm interaction of positive prey plasmids

Note: The ProQuest™ Two-Hybrid System comes with a positive interaction control. However, a bait-specific positive interaction control may be an additional useful tool in testing your experiment. Construct a prey plasmid with a known interactor of the bait protein to use as a bait-specific positive control.

Cloning vectors

The ProQuest™ Two-Hybrid System includes these yeast expression vectors:

- pDEST™32 for generation of the bait plasmid
- pDEST™22 for construction of the prey plasmid, or for generation of a two-hybrid library by Gateway™ recombination
- pEXP™-AD502 for generation of a two-hybrid library by restriction cloning

Two-Hybrid control vectors

The ProQuest™ Two-Hybrid System includes four two-hybrid control plasmids based on the interaction of Krev1 (a.k.a. Rap1A; a member of the Ras family of GTP binding proteins) with RalGDS (the Ral guanine nucleotide dissociator stimulator protein. The RalGDS mutants RalGDS-m1 and RalGDS-m2 affect the interaction with Krev1 and were generated using the SureFrame™ Allele Library Construction Kit. The properties of these plasmids are summarized below.

Control plasmid	Backbone	Insert	Mutant	Role	Interaction with pEXP™32/Krev1
pEXP™32/ Krev1	pDEST™32	full-length rat Krev1	*wt*	Bait	not applicable
pEXP™22/ RalGDS-*wt*	pDEST™22	ras association domain of RalGDS, *wt*	*wt*	Prey	strong
pEXP™22/ RalGDS-m1	pDEST™22	ras association domain of RalGDS, m1	I77T[1]	Prey	weak
pEXP™22/ RalGDS-m2	pDEST™22	ras association domain of RalGDS, m2	L65P[1]	Prey	not detectable

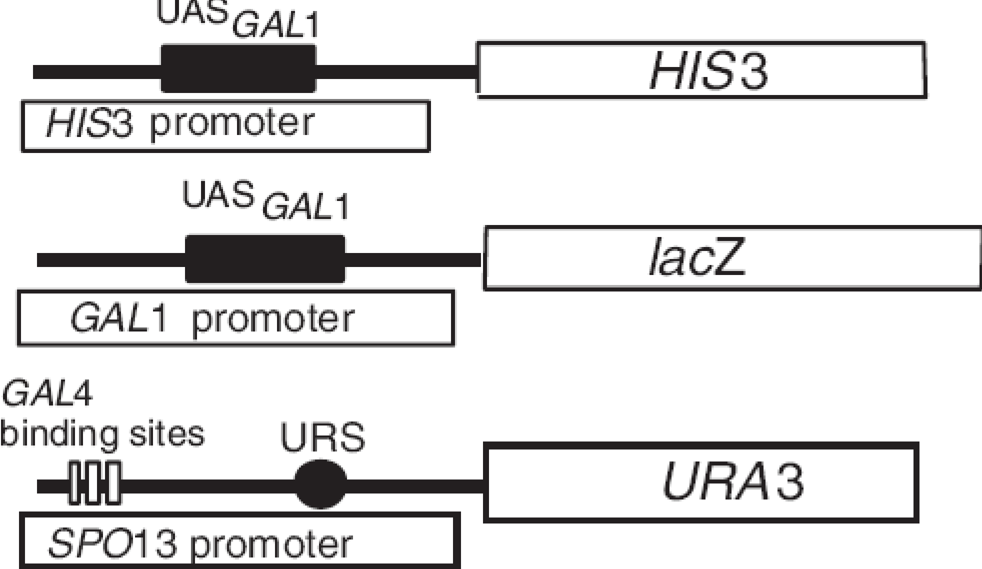
[1] Amino acid 65 and amino acid 77 in the insert correspond to amino acid 829 and amino acid 841 in the full-length RalGDS sequence, respectively.

The vectors pDEST™32 and pDEST™22 are suitable as negative two-hybrid controls.

- pDEST™32 as a negative control for bait plasmid
- pDEST™22 as a negative control for prey plasmid

MaV203 yeast strain

The ProQuest™ Two-Hybrid System uses the MaV203 yeast strain to serve as the host strain for the bait and prey plasmids. MaV203 contains single copies of each of three reporter genes (*HIS*3, *URA*3 and *lac*Z) that are stably integrated at different loci in the yeast genome. The promoter regions of *URA*3, *HIS*3, and *lac*Z are unrelated (except for the presence of GAL4 binding sites).



Reducing false positives

This system reduces false positives by:

- Including a third unrelated promoter that facilitates discrimination of artifactual reporter gene activation.
- Providing four phenotypes for assessing true interactors
- Using low-copy-number (*ARS/CEN*) vectors that reduce expression levels and toxicity

Three reporter genes

A major class of false positives is promoter-context dependent, e.g. the prey recognizes promoter sequences or other proteins bound to the promoter. In the ProQuest™ Two-Hybrid System, these false positives are reduced because three independent transcription events (from three distinct promoters) must occur at independent chromosomal loci.

Four phenotypes

Induction of the *HIS*3 and *URA*3 reporter genes by two-hybrid-dependent transcriptional activation allows cell growth on plates lacking histidine or uracil, respectively. Induction of the *lac*Z gene results in a blue color when assayed with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Two-hybrid-dependent induction of URA3 results in conversion of the compound 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Hence, cells containing interacting proteins grow when plated on medium lacking uracil, but growth is inhibited when plated on medium containing 5FOA.

	His⁺	β-Gal	Ura⁺	5FOA
Bait and Prey do not interact	–	White	–	+
Bait and Prey do interact	+	Blue	+	–

Low-Copy-Number vectors

In the ProQuest™ Two-Hybrid System, the low-copy-number (*ARS/CEN*) expression vectors express the bait and prey proteins at a relatively low level, which is beneficial for these reasons:

- Overexpression of the bait and prey hybrid proteins increases nonspecific interactions (false positives)
- Many proteins are toxic when overexpressed and interacting proteins may be missed at high expression levels (false negatives)
- *ARS/CEN*-based vectors provide more consistent plasmid copy numbers (versus the high variability of two micron-based vectors), leading to increased reproducibility of the reporter gene expression levels
- The consistent expression of fusion proteins at levels closer to physiological conditions is particularly valuable for detecting subtle differences, e.g. when characterizing mutations that disrupt known protein:protein interactions

Gateway™ compatibility

Incorporation of the Gateway™ Technology into the ProQuest™ System accelerates the cloning of genes into and out of the ProQuest™ Two-Hybrid vectors at several steps:

- Rapidly clone your gene of interest into the bait or prey plasmids using UltiMate™ ORF clones, previously established entry vectors, or a PCR amplification using Gateway™ primers
- Transfer libraries into prey plasmid with high efficiency and speed
- Move positive interactors into a variety of expression vectors for downstream protein expression and functional analysis

For more information on Gateway™ Recombination, see , Appendix A, “Gateway™ recombination reactions”.

Plasmid shuffling

The ProQuest™ Two-Hybrid System supports plasmid shuffling, which speeds up rescreening of positives after a library screen.

The yeast strain MaV203 is resistant to cycloheximide (cyh^r) due to the recessive *cyh2*^{r} allele. MaV203 cells containing bait plasmid, which contains the dominant *CYH2*^{S} gene, are sensitive to cycloheximide. Cells that have spontaneously lost the bait plasmid are selected using cycloheximide The resulting prey-only cells are made competent and re-transformed with the bait. Transformants are then selected and retested for the interaction of bait and prey.

Features of pDEST™32

pDEST™32 is the GAL4 DNA Binding Domain (GAL4 DBD) containing Gateway™ Destination Vector. This vector is used to clone your gene of interest in frame with the sequence encoding the GAL4 DBD (forming the bait).

This vector includes the following features:

- The constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene (*ADH*1) to drive expression of the GAL4 DBD bait fusion
- The sequence encoding the GAL4 DBD (amino acid 1-147) for fusion to your gene of interest
- Two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance gene (Cm^r) and a *ccd*B gene. Following the LR recombination reaction, the Cm^r and *ccd*B genes are replaced by the gene of interest and the *att*R sites are converted to *att*B sites. As a result, the gene of interest is now fused in frame with the DBD flanked by *att*B sites in the vector backbone.
- The *ARS*4/*CEN*6 sequence for replication and low-copy-number maintenance in yeast
- The *LEU*2 gene for selection in yeast on medium lacking leucine
- The dominant *CYH*2^S allele that confers sensitivity to cycloheximide in yeast (for plasmid shuffling)
- A pUC-based replication origin and gentamicin resistance gene (Gm^r) for replication and maintenance in *E. coli*

Features of pDEST™22

pDEST™22 is a GAL4 Activation Domain (GAL4 AD) containing Gateway™ Destination Vector. This vector is used to clone the second known gene of interest in frame with the sequence encoding the GAL4 AD (generating the prey).

This vector includes the following features:

- The constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene to drive expression of the GAL4 AD
- The sequence encoding the GAL4 Activation Domain (amino acid 768-881) fused to the nuclear localization signal from SV40 Large T antigen for fusion to your prey gene of interest
- Two recombination sites, *att*R1 and *att*R2, flanking a chloramphenicol resistance gene and a *ccd*B gene. Following the LR recombination reaction, the Cmr and *ccd*B genes are replaced by the gene of interest and the *att*R sites are converted to *att*B sites. As a result, the gene of interest is now fused in frame with AD flanked by *att*B sites in the Destination Vector backbone.
- The *ARS*4/*CEN*6 sequence for replication and maintenance at low-copy-number in yeast

- The *TRP*1 gene for selection in yeast on medium lacking tryptophan
- A pUC-based replication origin and ampicillin resistance gene for replication and maintenance in *E. coli*

Features of pEXP™-AD502

pEXP™-AD502 is an Activation Domain (AD) Gateway™ Expression Vector. This plasmid is used to construct a cDNA or genomic library for identifying proteins (preys) that interact with the fusion protein (bait).

Features of this vector include:

- The constitutive moderate-strength promoter and transcription terminator of the yeast Alcohol Dehydrogenase gene to drive expression of the GAL4 AD
- The sequence encoding the GAL4 Activation Domain (amino acid 768-881) fused to the nuclear localization signal from SV40 Large T antigen for fusion to your prey gene of interest
- Two recombination sites, *att*B1 and *att*B2, flanking a multiple cloning site, including *Sal* I and *Not* I sites for generation of cDNA libraries using the SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis and Plasmid Cloning.
- The *ARS*4/*CEN*6 sequence for replication and maintenance at low-copy-number in yeast
- The *TRP*1 gene for selection in yeast on medium lacking tryptophan
- A pUC-based replication origin and ampicillin resistance gene for replication and maintenance in *E. coli*

Note that pEXP™-AD502 is derived from pDEST™22.

Plasmid isolation

Yeast cells containing potentially interacting proteins harbor both bait and prey plasmids. It is desirable to isolate bait and prey plasmids separately in *E. coli* to confirm the interaction and further characterize the candidate clones. To facilitate the isolation in *E. coli*, the pDEST™32 vector encodes gentamicin resistance while the pDEST™22 and pEXP™-AD502 vector encodes ampicillin resistance. Plasmid DNA isolated from yeast cells containing bait and prey plasmids is introduced into *E. coli* by electroporation and transformants containing bait plasmids are selected with ampicillin.

Features of yeast strain MaV203

The yeast strain provided in the ProQuest™ System is MaV203 and contains the following features:

- A set of non-reverting auxotrophic mutations: *leu*2 and *trp*1 to allow selection for the bait and prey fusion vectors, and *his*3 for growth upon induction of the reporter gene *GAL*1*::HIS*3
- Deletions of the *GAL*4 and *GAL*80 genes encoding GAL4 and its repressor GAL80, respectively. In the absence of GAL80, galactose is not required for activation of GAL4-inducible promoters
- Three stably integrated single-copy GAL4-inducible reporter genes: *SPAL*10*::URA*3 integrated at *URA*3; $HIS3_{UAS\ GAL1}$*::HIS*3 integrated at *LYS*2; and *GAL1::lac*Z integrated at an unknown locus
- The recessive drug resistance marker *cyh*2^R for plasmid shuffling

Genotype MaV203

The genotype of MaV203 is as follows:

MaV203 (MATα, *leu*2-3,112, *trp*1-901, *his*3Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10*::URA*3, *GAL*1::*lac*Z, *HIS*$3_{UAS\ GAL1}$*::HIS*3@*LYS*2, *can*1^R, *cyh*2^R)

IMPORTANT! The yeast strain MaV203 is unique to the ProQuest™ Two-Hybrid System. Other strains used for two-hybrid analysis cannot be substituted.

Considerations in designing a Two-Hybrid screen

Introduction

Prior to beginning a two-hybrid screen, determine as much information regarding the protein of interest and those interactions that you expect to detect. Several issues that are of particular interest are listed below.

Transcriptional activator

The fusion of proteins containing domains capable of functioning as transcriptional activators to the GAL4 DBD will induce the reporter genes in the absence of interacting proteins and cannot be used in a typical two-hybrid screen. For example, roughly 0.1% of random *E. coli* sequences behave as transcriptional activation domains when fused to the GAL4 DBD. Other domain functions should also be considered; e.g., domains exhibiting repressor activity.

Possible solutions:

- A screen with segments of such proteins that lack these activities can conceivably be constructed and tested.
- Perform a swapped two-hybrid screen where the AD-fusion vector contains the test protein of interest and is used to screen a cDNA library constructed in the DBD-vector

Protein family

It is often useful to anticipate the number of interacting proteins one might expect to recover from a two-hybrid screen. Test proteins that are members of large protein families may interact with other members at varying degrees, generating a spectrum of reporter gene readout profiles. The prevalence of these proteins should be considered when determining the number of colonies required for a two-hybrid screen and the predicted strength of the reporter gene expression (e.g., strong interactors or weak interactors).

Expression pattern

The choice of which cDNA library to screen is critical and depends primarily upon the expression pattern of the protein used in the screen. To help in the selection of a cDNA library, verify by PCR the presence of a cDNA corresponding to the bait within the tissue of interest.

Interaction artifacts

The interaction of two fusion proteins in a two-hybrid screen is not necessarily an indication that these proteins interact *in vivo* under native conditions.

- Often only segments of the protein are analyzed, revealing (or masking) domains that might otherwise be unavailable.
- DBD or AD fusion proteins may bear little structural resemblance to the native protein.
- Many posttranslational modifications present in higher eukaryotic cells are absent (or incorrectly modified) in yeast, which may preclude or provide a basis for protein:protein interactions.
- Interactions can be mediated non-specifically (e.g., by large hydrophobic regions).
- Interactions can occur between proteins that are biologically irrelevant (e.g., the proteins exist in different cell types, compartments or at different times during development or cell cycle).

Confirmation of interaction

It is important to confirm the interactions between protein pairs detected in a two-hybrid screen by biochemical methods. Consider the following options:

- Ideally, the purified protein of interest and antibodies (preferably monoclonal) against the protein of interest are available for immunoprecipitation and Western blot experiments.
- In some cases, antibodies raised against the GAL4 DBD or AD regions, or other epitopes included in the fusion protein, can be used for immunoprecipitation and Western blot experiments.
- Finally, it is important to design in advance a functional test for the biological relevance of the protein:protein interactions.

Applications for ProQuest™ Two-Hybrid screen

Supported applications

The ProQuest™ Two-Hybrid System supports two types of applications:

- Verifying an interaction between two known proteins or protein domains for which there is a prior reason to expect an interaction
- Screening a library for novel proteins that specifically interact with a known bait (forward two-hybrid library screen)

Verifying interaction

If you want to verify an interaction between two known proteins (testing two-hybrid interactions); see Chapter 3, "Verifying interaction".

Forward Two-Hybrid library screen

If you want to screen a library for novel proteins that specifically interact with your bait (forward two-hybrid library screen); see Chapter 4, "Forward Two-Hybrid library screen".

Note: The ProQuest™ Two-Hybrid System comes with a positive interaction control. However, a bait-specific positive interaction control may be an additional useful tool in testing your experiment. Construct a prey plasmid with a known interactor of the bait protein to use as a bait-specific positive control We recommend that you start by verifying the interaction between your bait and this bait-specific positive interaction control, if available. This will allow you to familiarize with the system.

Verifying interaction

Introduction

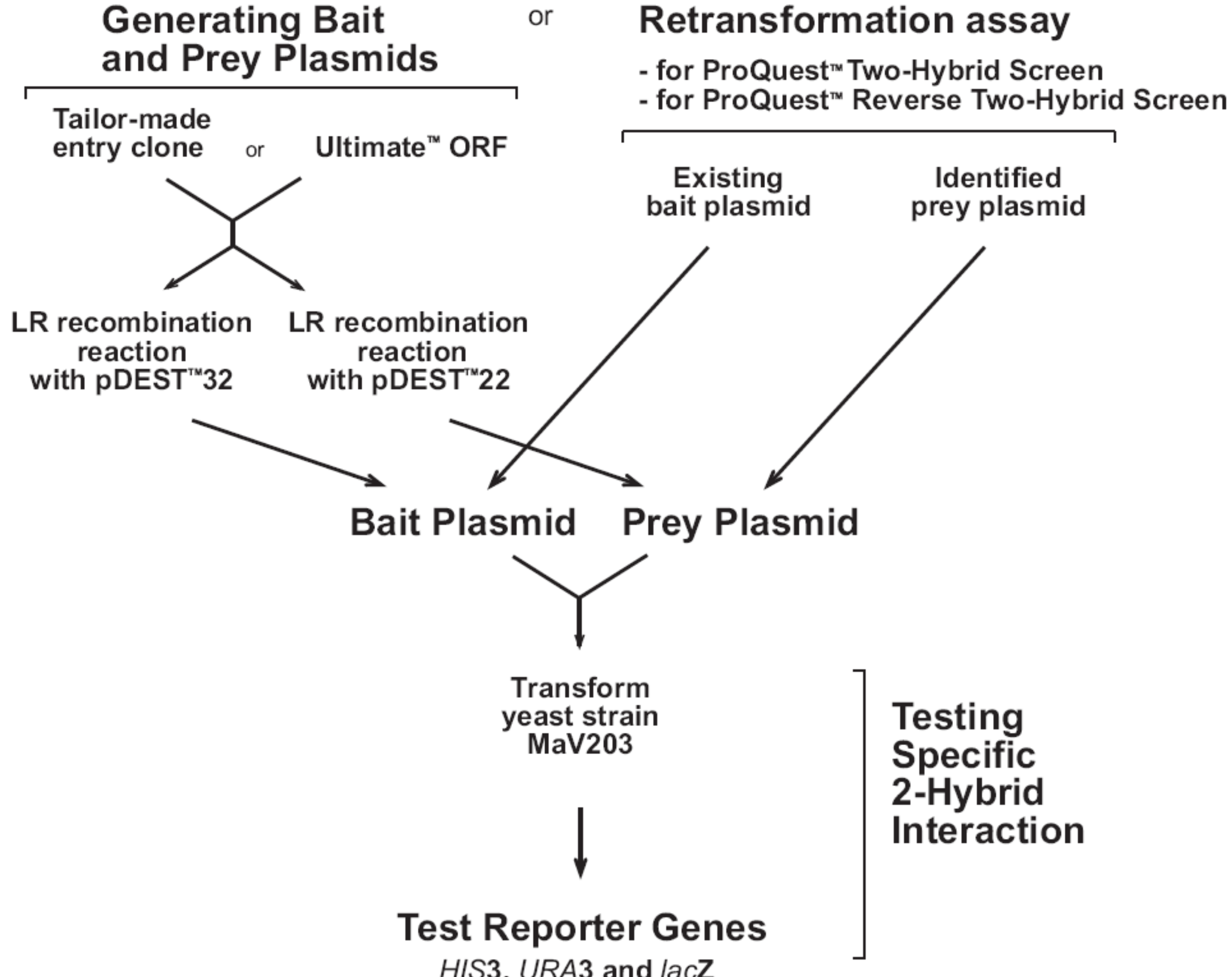
This chapter describes how to verify an interaction between two known proteins or protein domains for which there is a prior reason to expect an interaction.

The first part describes the construction of the required bait and prey plasmids ("Generating bait and prey plasmids" on page 31).

The second part describes how to test a specific two-hybrid interaction ("Recommended controls" on page 40).

Flowchart

The figure below illustrates the major steps necessary to verify an interaction using the ProQuest™ Two-Hybrid System.



Methods

Generating bait and prey plasmids

Overview

Introduction

This section describes how to generate specific bait and prey plasmids. The gene of interest for the **bait** plasmid is cloned into pDEST™32, resulting in pEXP™32 containing your gene of interest. The gene of interest for the **prey** plasmid is cloned into pDEST™22, resulting in pEXP™22 containing your gene of interest. pDEST™32 and pDEST™22 are Gateway™-adapted destination vectors.

Gateway™ Technology

The Gateway™ Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems.

For details about the Gateway™ Recombination Reactions, see Appendix A, “Gateway™ recombination reactions”.

Requirement of bait and prey

A specific bait plasmid is required for every application of this manual. A specific prey plasmid is required if you want to test a specific forward two-hybrid interaction. However, a specific prey plasmid of a known interactor of the bait is a valuable positive control when screening a forward two-hybrid library, and you should consider generating one.

Important

If you want to test a specific forward two-hybrid interaction between two proteins, we recommend constructing prey plasmids of each cDNA, and also bait plasmids of each cDNA. This way, you can perform the two-hybrid assay two ways: with protein A as bait and protein B as prey, and with protein B as bait and protein A as prey. This will generate more convincing data regarding the interaction you want to test.

Construction of plasmids

To construct your bait or prey plasmids, perform the following steps:

1. Identify or generate a suitable entry clone.
2. Perform an LR recombination reaction between entry clone and pDEST™32 or pDEST™22.
3. Transform competent cells.
4. Select the proper expression clone.

Entry clone

In order to generate the bait plasmid and the prey plasmid, you need an entry clone with your gene of interest flanked by *att*L1 and *att*L2 sites. Use an entry clone you generated for previous Gateway™ recombinations, or obtain an UltiMate™ ORF clone of your gene of interest (see **thermofisher.com** or contact Technical Service).

Generating a new entry clone

If you do not have an entry clone available, and an UltiMate™ ORF clone is not an option, you can generate your entry clone in a number of different ways:

- Perform a BP recombination reaction between an existing expression or cDNA clone with *att*B1 and *att*B2 sites and an appropriate donor vector (such as pDONR™221; see the Gateway™ Technology with Clonase™ II manual)
- Carry out a PCR on your gene of interest and use TOPO™ cloning into a suitable entry vector (such as pCR™8/GW/TOPO™ TA, Catalog no. K250020; see the pCR™8/GW/TOPO™TA Cloning™ Kit manual, or see the pENTR™ Directional TOPO™ Cloning Kits manual for directional cloning)
- Perform PCR with *att*B primers on your gene of interest and a BP recombination reaction with an appropriate donor vector (such as pDONR™221; see the Gateway™ Technology with Clonase™ II manual)
- Use restriction digestion to clone your gene of interest into an entry vector (see the Gateway™ pENTR™ Vectors manual)

The indicated manuals are available from **thermofisher.com** or by contacting Technical Service.

Reading frame and stop codons

- Make sure the reading frame of your entry clone is correct for insertion into pDEST™32 or pDEST™22. The insert is translated as a fusion with GAL4 DBD or GAL4 AD respectively. The frame should be as indicated below:

```
                    Thr Ser Leu Tyr Lys Lys Ala Gly
Entry   vector---N75-ACA AGT TTG TAC AAA AAA GCA GGC TNN NNN NNN NNN NNN
clone   vector---N75-TGT TCA AAC ATG TTT TTT CGT CCG ANN NNN NNN NNN NNN
                 |___________________________________||________________|
                                attL1                  GENE OF INTEREST
```

- We recommend including an in-frame stop codon at the end of the Open Reading Frame (ORF) of your gene of interest. This will prevent read-through into vector sequences, which may interfere with interaction.
- No in-frame stop codon should be present in between the *att*L1 sequence and the ORF of your gene of interest (i.e. immediately 5' of your gene of interest)

Note: UltiMate™ ORF clones meet all the conditions for LR recombination with pDEST™32 and pDEST™22.

Cloning site and recombination region of pDEST™32

Use the diagram below to help you clone your gene of interest into pDEST™32. Note the following features in the diagram below:

- The shaded region corresponds to those DNA sequences that will be transferred from the entry vector into pDEST™32 following recombination, forming the bait vector pEXP™32
- The reading frame for the GAL4 DBD is shown; the insert needs to be in frame with GAL4 DBD
- Sequences for suggested forward and reverse sequencing primers are shown

The complete sequence of pDEST™32 is available for downloading from (**thermofisher.com**) or by contacting Technical Service. For a map of pDEST™32, see Appendix D, "Maps and features of vectors".

```
1511   TTTCTGCACA ATATTTCAAG CTATACCAAG CATACAATCA ACTCCAAGCT TGAAGCAAGC CTCCTGAAAG

                       GAL4 DNA Binding Domain
1581   ATG AAG CTA CTG TCT TCT ATC GAA CAA GCA TGC GAT ATT TGC CGA CTT AAA AAG
       Met Lys Leu Leu Ser Ser Ile Glu Gln Ala Cys Asp Ile Cys Arg Leu Lys Lys
                              Suggested forward sequencing/PCR primer
1635   CTC AAG TGC TCC AAA GAA AAA CCG AAG TGC GCC AAG TGT CTG AAG AAC AAC TGG
       Leu Lys Cys Ser Lys Glu Lys Pro Lys Cys Ala Lys Cys Leu Lys Asn Asn Trp

1690   GAG TGT CGC TAC TCT CCC AAA ACC AAA AGG TCT CCG CTG ACT AGG GCA CAT CTG
       Glu Cys Arg Tyr Ser Pro Lys Thr Lys Arg Ser Pro Leu Thr Arg Ala His Leu

1743   ACA GAA GTG GAA TCA AGG CTA GAA AGA CTG GAA CAG CTA TTT CTA CTG ATT TTT
       Thr Glu Val Glu Ser Arg Leu Glu Arg Leu Glu Gln Leu Phe Leu Leu Ile Phe

1797   CCT CGA GAA GAC CTT GAC ATG ATT TTG AAA ATG GAT TCT TTA CAG GAT ATA AAA
       Pro Arg Glu Asp Leu Asp Met Ile Leu Lys Met Asp Ser Leu Gln Asp Ile Lys

1851   GCA TTG TTA ACA GGA TTA TTT GTA CAA GAT AAT GTG AAT AAA GAT GCC GTC ACA
       Ala Leu Leu Thr Gly Leu Phe Val Gln Asp Asn Val Asn Lys Asp Ala Val Thr

1905   GAT AGA TTG GCT TCA GTG GAG ACT GAT ATG CCT CTA ACA TTG AGA CAG CAT AGA
       Asp Arg Leu Ala Ser Val Glu Thr Asp Met Pro Leu Thr Leu Arg Gln His Arg

1959   ATA AGT GCG ACA TCA TCA TCG GAA GAG AGT AGT AAC AAA GGT CAA AGA CAG TTG
       Ile Ser Ala Thr Ser Ser Ser Glu Glu Ser Ser Asn Lys Gly Gln Arg Gln Leu

                                              2044
                                                       attB1
2013   ACT GTA TCG TCG AGG TCG AAT CAA ACA AGT TTG TAC AAA AAA GCA GGC T
       TGA CAT AGC AGC TCC AGC TTA GTT TGT TCA AAC ATG TTT TTT CGT CCG A cDNA INSERT
       Thr Val Ser Ser Arg Ser Asn Gln Thr Ser Leu Tyr Lys Lys Ala Gly

                 3867
         attB2
       ACCCAGCTTT CTTGTACAAA GTGGTTTGAT GGCCGCTAAG TAAGTAAGAC GTCGAGCTCT AAGTAAGTAA
       TGGGTCGAAA GAACATGTTT CACCAAACTA

                                                   Suggested reverse sequencing/PCR primer
3927   CGGCCGCCAC CGCGGTGGAG CTTTGGACTT CTTCGCCAGA GGTTTGGTCA AGTCTCCAAT CAAGGTTGTC

3997   GGCTTGTCTA
```

Cloning site and recombination region of pDEST™22

Use the diagram below to help you clone your gene of interest into pDEST™22. Note the following features in the diagram below:

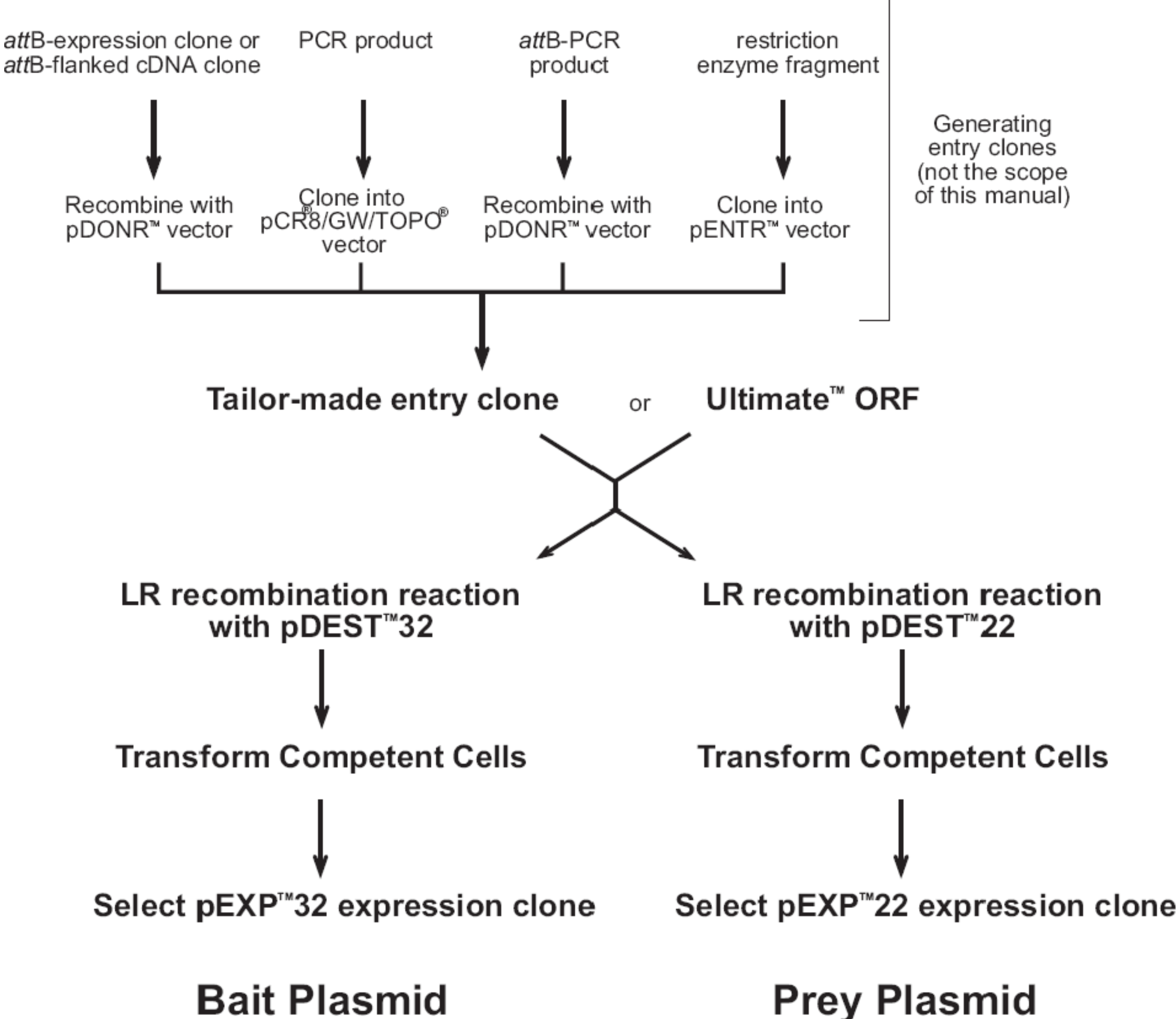
- The shaded region corresponds to those DNA sequences that will be transferred from the entry vector into pDEST™22 following recombination, forming the bait vector pEXP™22
- The reading frame for the GAL4 AD is shown; the insert needs to be in frame with GAL4 AD
- Sequences for suggested forward and reverse sequencing primers are shown

The complete sequence of pDEST™22 is available for downloading from (**thermofisher.com**) or by contacting Technical Service. For a map of pDEST™22, see "Features of vectors pDEST™32 and pDEST™22" on page 99.

```
1661 CCTTTCTTCC TTGTTTCTTT TTCTGCACAA TATTTCAAGC TATACCAAGC ATACAATCAA CTCCAAGCTT

              Nuclear Localization Signal
1731 ATG CCC AAG AAG AAG CGG AAG GTC TCG AGC GGC GCC AAT TTT AAT CAA AGT GGG AAT
     Met Pro Lys Lys Lys Arg Lys Val Ser Ser Gly Ala Asn Phe Asn Gln Ser Gly Asn
                                 GAL4 DNA Activation Domain
1788 ATT GCT GAT AGC TCA TTG TCC TTC ACT TTC ACT AAC AGT AGC AAC GGT CCG AAC CTC
     Ile Ala Asp Ser Ser Leu Ser Phe Thr Phe Thr Asn Ser Ser Asn Gly Pro Asn Leu

1845 ATA ACA ACT CAA ACA AAT TCT CAA GCG CTT TCA CAA CCA ATT GCC TCC TCT AAC GTT
     Ile Thr Thr Gln Thr Asn Ser Gln Ala Leu Ser Gln Pro Ile Ala Ser Ser Asn Val

1902 CAT GAT AAC TTC ATG AAT AAT GAA ATC ACG GCT AGT AAA ATT GAT GAT GGT AAT AAT
     His Asp Asn Phe Met Asn Asn Glu Ile Thr Ala Ser Lys Ile Asp Asp Gly Asn Asn
                                                  Suggested forward sequencing/PCR primer
1959 TCA AAA CCA CTG TCA CCT GGT TGG ACG GAC CAA ACT GCG TAT AAC GCG TTT GGA ATC
     Ser Lys Pro Leu Ser Pro Gly Trp Thr Asp Gln Thr Ala Tyr Asn Ala Phe Gly Ile

2016 ACT ACA GGG ATG TTT AAT ACC ACT ACA ATG GAT GAT GTA TAT AAC TAT CTA TTC GAT
     Thr Thr Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val Tyr Asn Tyr Leu Phe Asp

2073 GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG GGT GGG TCG AAT CAA ACA AGT
     Asp Glu Asp Thr Pro Pro Asn Pro Lys Lys Glu Gly Gly Ser Asn Gln Thr Ser
     2128     attB1                                    attB2   3951
2127 TTG TAC AAA AAA GCA GGC T                  ACCCAGCTTT CTTGTACAAA GTGGTTTGAT
     AAC ATG TTT TTT CGT CCG A    cDNA INSERT   TGGGTCGAAA GAACATGTTT CACCAAACTA
     Leu Tyr Lys Lys Ala Gly
3971 GGCCGCTAAG TAAGTAAGAC GTCGAGCTCT AAGTAAGTAA CGGCCGCCAC CGCGGTGGAG CTTTGGACTT

                            Suggested reverse sequencing/PCR primer
4041 CTTCGCCAGA GGTTTGGTCA AGTCTCCAAT CAAGGTTGTC GGCTTGTCTA CCTTGCCAGA AATTTACGAA
```

Experimental outline

The experimental outline for generating the bait and prey plasmids is shown below.



Create bait and prey plasmids using the LR recombination reaction

Introduction

This section explains how to create specific bait and prey plasmids using an existing entry clone and the destination vectors pDEST™32 and pDEST™22. To ensure that you obtain the best possible results, we suggest that you read this section and the next section entitled **Transforming Competent Cells with Bait and Prey Plasmids** before beginning.

Substrates for the LR recombination reaction

For most applications, we recommend performing the LR recombination reaction using a:

- **Supercoiled** *att*L1 and *att*L2-containing entry clone
- **Supercoiled** pDEST™32 or pDEST™22 (contains *att*R1 and *att*R2)

Note

Although the Gateway™ Technology manual has previously recommended using a linearized destination vector and entry clone for more efficient LR recombination, further testing at Thermo Fisher Scientific has found that linearization of destination vectors and entry clones is generally not required to obtain optimal results for any downstream application.

LR Clonase™ II Enzyme Mix

LR Clonase™ II enzyme mix is provided with the kit to catalyze the LR recombination reaction. The LR Clonase™ II enzyme mix combines the proprietary enzyme formulation and 5X LR Clonase™ Reaction Buffer previously supplied as separate components in LR Clonase™ enzyme mix (Catalog no. 11791019) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Use the protocol provided on the next page to perform the LR recombination reaction using LR Clonase™ II enzyme mix.

Note: You may perform the LR recombination reaction using LR Clonase™ enzyme mix, if desired. To use LR Clonase™ enzyme mix, follow the protocol provided with the product. Do not use the protocol for LR Clonase™ II enzyme mix provided on the next page.

Resuspending pDEST™32 and pDEST™22

The pDEST™32 and pDEST™22 vectors are supplied as 6 µg of plasmid DNA, liquid in TE Buffer, pH 8.0. To use the vector, resuspend in 40 µL of sterile water to obtain a 150 ng/µL stock.

Positive recombination control

The pENTR™-gus plasmid is provided with the LR Clonase™ II Enzyme Mix for use as a positive control for recombination and expression. Using the pENTR™-gus entry clone in an LR recombination reaction with a destination vector will allow you to generate an expression clone containing the gene encoding β-glucuronidase (*gus*).

Materials needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone for the bait and/or your entry clone for the prey (50-150 ng in TE, pH 8.0)
- pDEST™32 and/or pDEST™22 (both 150 ng/µL in TE, pH 8.0)
- LR Clonase™ II enzyme mix (keep at -20℃ until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)

- 2 µg/µl Proteinase K Solution solution (supplied with the LR Clonase™ II enzyme mix; thaw and keep on ice until use)
- Positive Recombination Control pENTR™-gus, if desired

Setting up the LR recombination reaction

1. Add the following components to 1.5 mL microcentrifuge tubes at room temperature and mix.

Note: To include a negative control, set up a second sample reaction and omit the LR Clonase™ II enzyme mix (see Step 4 on page 37).

Component	Forming Bait Plasmid			Forming Prey Plasmid		
	Sample	Negative Control	Positive Control	Sample	Negative Control	Positive Control
Entry clone for bait (50-150 ng/reaction)	1-7 µL	1-7 µL	--	--	--	--
Entry clone for prey (50-150 ng/reaction)	--	--	--	1-7 µL	1-7 µL	--
pDEST™32 (150 ng/µl)	1 µL	1 µL	1 µL	--	--	--
pDEST™22 (150 ng/µl)	--	--	--	1 µL	1 µL	1 µL
pENTR™-gus (50 ng/µl)	--	--	2 µL	--	--	2 µL
TE Buffer, pH 8.0	to 8 µL	to 10 µL	5 µL	to 8 µL	to 10 µL	5 µL

2. Remove the LR Clonase™ II enzyme mix from -20℃ and thaw on ice (~ 2 minutes).
3. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
4. Add 2 µL of LR Clonase™ II enzyme mix to the sample vial. Do not add LR Clonase™ II enzyme mix to the negative control vial. Mix well by vortexing briefly twice (2 seconds each time).
   Reminder: Return LR Clonase™ II enzyme mix to -20℃ immediately after use.
5. Incubate reactions at 25℃ for 1 hour.

Note: For most applications, 1 hour will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the recombination reaction can be extended up to 18 hours. For large plasmids (≥ 10 kb), longer incubation times (*i.e*. overnight incubation) will yield more colonies and are recommended.

6. Add 1 µl Proteinase K Solution to each reaction. Incubate 10 minutes at 37℃.
7. Proceed to transform a suitable *E. coli* host and select for expression clones.

Note: You may store the LR reaction at -20℃ for up to 1 week before transformation, if desired.

Transforming competent cells with bait and prey plasmids

Introduction

Competent *E. coli* cells are not provided with the ProQuest™ Two-Hybrid System. Below the prerequisites of appropriate host strains are indicated. You can order suitable competent cells from **thermofisher.com** or use your standard in-house competent cells if they are compatible.

E. coli host strain

You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1R, TOP10, DH5α™, DH10Bor equivalent for transformation. Other strains are suitable. Do not use *E. coli* strains that contain the F′ episome (*e.g*. TOP10F′) for transformation. These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

For your convenience, TOP10, DH5α™, and DH10B *E. coli* are available as chemically competent or electrocompetent cells from **thermofisher.com** (see table below).

Item	Quantity	Catalog No.
Library Efficiency™ DH5α™ *E. coli*	5 x 200 µL	18263012
One Shot™ TOP10 Chemically Competent *E. coli*	20 x 50 µL	C404003
One Shot™ MAX Efficiency™ DH10B T1 Phage Resistant Chemically Competent *E. coli*	20 x 50 µL	12331013
One Shot™ TOP10 Electrocomp™ *E. coli*	20 x 50 µL	C404052
ElectroMAX™ DH10B *E. coli*	5 x 100 µL	18290015

Procedure

Transform 1 µL of the LR reaction according to the protocol provided with your competent cells, and plate two concentrations of cells on 10 cm diameter LB agar plates with 10 µg/m Lgentamicin (for bait plasmids) or 100 µg/mL ampicillin (for prey plasmids). Let grow overnight at 37℃.

What you should see

If you use *E. coli* cells with a transformation efficiency of ≥ 1 x 10^8 cfu/µg, the LR reaction should give > 5000 colonies if the entire LR reaction is transformed and plated.

Analyzing transformants details

Analyzing transformants

To analyze positive clones, we recommend that you:

1. Pick 5-10 colonies and culture them overnight in LB or SOB medium containing 10 µg/mL gentamicin (for bait plasmids) or 100 µg/mL ampicillin (for prey plasmids).

2. Isolate plasmid DNA using your method of choice. To obtain pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit (Catalog no. K210002).

3. Perform restriction analysis to confirm the presence of the insert.

Note: *Bsr*G I cleaves within all *att* sites, and can be used to help characterize clones.

Sequencing

If you sequenced your entry clone, sequence analysis is not required. However, if you want to perform sequencing to confirm the reading frame of your expression clone, use primers that anneal 50-300 bp from the junction (either within the vector or the insert). Below are primers you can use to sequence bait and prey junctions.

Plasmid	Direction	Primer
Bait	forward	5'-AACCGAAGTGCGCCAAGTGTCTG-3'
Bait and Prey	reverse	5'-AGCCGACAACCTTGATTGGAGAC-3'
Prey	forward	5'-TATAACGCGTTTGGAATCACT-3'

If you want to design your own primer, you can download the sequence for pDEST™32 or pDEST™22 from our Web site, **thermofisher.com**. Make sure the 3' end of the primer is directed towards the junction you want to sequence.

Long-term storage

Once you have identified the correct expression clone, be sure to purify the colony and make a glycerol stock for long-term storage. We recommend that you store a stock of plasmid DNA at -20℃.

1. Streak the original colony out for a single colony on an LB plate containing 10 µg/mL gentamicin (for bait plasmids) or 100 µg/mL ampicillin (for prey plasmids).

2. Isolate a single colony and inoculate into 1-2 mL of LB containing 10 µg/ml gentamicin (for bait plasmids) or 100 µg/mL ampicillin (for prey plasmids).

3. Grow until the culture reaches stationary phase.

4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.

5. Store the glycerol stock at -80℃.

Testing specific Two-Hybrid interaction

Overview

Introduction

This section describes how to transform your bait and prey plasmid into MaV203 cells, and test activation of the three reporter genes. Use this chapter for three purposes:

- To test a specific interaction between two proteins
- Retransformation assay to confirm the interaction of your bait with a prey identified in the forward two-hybrid screen ("Screening forward Two-Hybrid library" on page 55)

Recommended controls

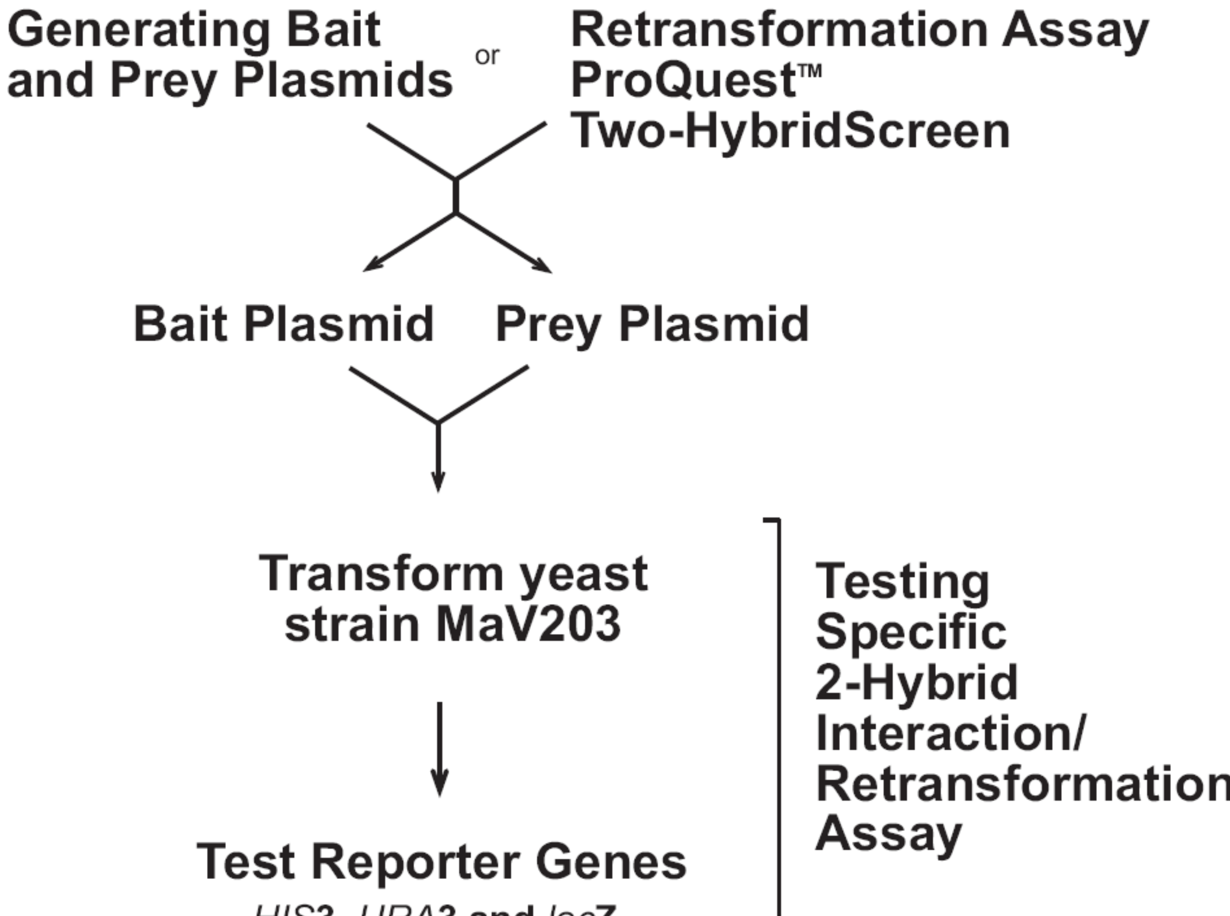
The following transformation, and interaction controls are recommended. Bait and prey plasmids are not provided with the system and need to be generated ("Generating bait and prey plasmids" on page 31); the other vectors are provided with the kit.

	LEU2 Plasmid	TRP1 Plasmid	Purpose
1	none	none	Negative transformation control
2	pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
3	pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
4	pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
5	pDEST™32	pDEST™22	Negative activation control
6	Bait plasmid	pDEST™22	Negative activation control; baseline
	If available:		
7	pDEST™32	Prey plasmid known to interact with bait	Negative activation control
8	Bait plasmid	Prey plasmid known to interact with bait	Bait-specific positive interaction control (if available)

Note: If you are testing multiple bait plasmids, perform a transformation with each bait plasmid for controls 6 and 8; label the controls 6a, 6b, 8a, 8b and so on.

Experimental outline

The experimental outline for Testing Specific Two-Hybrid Interaction is shown below.



Small scale yeast transformation

Competent yeast cells

The MaV203 yeast strain is provided with the kit to serve as the host for your bait and prey plasmids. Below we provide a small-scale protocol for transforming yeast cells. Prepare a new batch of competent cells for every transformation.

Alternatively, to limit your workload and increase the transformation efficiency, you may purchase the following products:

- MaV203 Competent Cells, Subclone Scale from **thermofisher.com** (Catalog no. 11445012). Use the transformation protocol provided with these cells.
- If you plan to do library scale transformations later on, you can purchase MaV203 Competent Cells, Library Scale from **thermofisher.com** (Catalog no. 11281011) and use one vial to perform multiple small-scale transformations. Per transformation, scale down to 25 µL competent cells, 1 µg DNA per plasmid, 180 µL PEG/LiAc, 10.8 µL DMSO. Otherwise, follow the transformation protocol provided with these cells.
- If you want to generate a large batch of competent cells that can be frozen, use the *S. c*. EasyComp™ Kit (Catalog no. K505001). Use the transformation protocol provided with this kit.

Note: There are other small-scale transformation methods that can be used.

Transformation guide

Perform two transformations per interaction pair you want to test:

- pDEST™32 and prey plasmid to test
- Bait plasmid and prey plasmid to test

Additionally, we recommend generating the controls indicated in the table in “Recommended controls” on page 40.

Select on SC-Leu-Trp plates. Store representative transformants in glycerol at -80℃ for future use.

Note: If you want to test a specific forward two-hybrid interaction between two proteins two ways (i.e. with protein A as bait and protein B as prey, and with protein B as bait and protein A as prey), you will perform four transformations per interaction.

Important

You need to generate fresh plates of controls 2-8 (see table in “Recommended controls” on page 40). If you have previously generated the controls and do not want to retransform them, streak a colony from the stored plates onto new SC-Leu-Trp plates and incubate for 48 hours at 30℃.

To initiate cultures from frozen yeast stocks, streak a small amount of frozen stock on a YPAD plate. Once growth is established, you may check the phenotype of each strain by streaking the strain on a minimal plate supplemented with the appropriate amino acids.

Keep glycerol stocks of all strains including transformed strains. If you use strains or transformants directly from plates be sure the plates are less than 4 days old.

Materials needed

Be sure to have the following reagents on hand before starting.

- YPAD
- 1X TE
- 1X LiAc (100mM Lithium Acetate/0.5X TE)
- Denatured sheared salmon sperm DNA (**thermofisher.com**, Catalog no. 15632011)
- Plasmid DNA to be transformed
- 1X LiAc/40% PEG-3350/1X TE
- DMSO. For best results, use fresh DMSO from an unopened bottle. DMSO that has been stored at -20℃ also works well.
- SC-Leu-Trp plates, for selection of yeast cells transformed with both the bait and prey plasmid, or LEU2 and TRP1 plasmid of the controls (see “Recommended controls” on page 40)
- Bait plasmid
- Prey plasmid known to interact with bait in yeast two-hybrid (if available)
- pDEST™32, pDEST™22, pEXP™32/Krev1, pEXP™22/RalGDS-wt, pEXP™22/RalGDS-m1, pEXP™22/RalGDS-m2 (supplied with the kit)

Preparing competent MaV203 cells

1. Inoculate 10 mL of YPAD with a colony of MaV203 and shake overnight at 30℃.
2. Determine the OD_{600} of your overnight culture. Dilute culture to an OD_{600} of 0.4 in 50 mL of YPAD and grow an additional 2-4 hours.
3. Pellet the cells at 2500 rpm and resuspend the pellet in 40 mL 1X TE.
4. Pellet the cells at 2500 rpm and resuspend pellet in 2 mL of 1X LiAc/0.5X TE.
5. Incubate the cells at room temperature for 10 minutes
6. Proceed immediately to transform the competent MaV203 cells

Transformation of competent MaV203 cells

The protocol below describes transformation of MaV203 yeast cells using your own prepared competent cells. To transform MaV203 cells using purchased MaV203 Competent Cells, or the *S. c.* EasyComp™ Kit, refer to the manual included with each product.

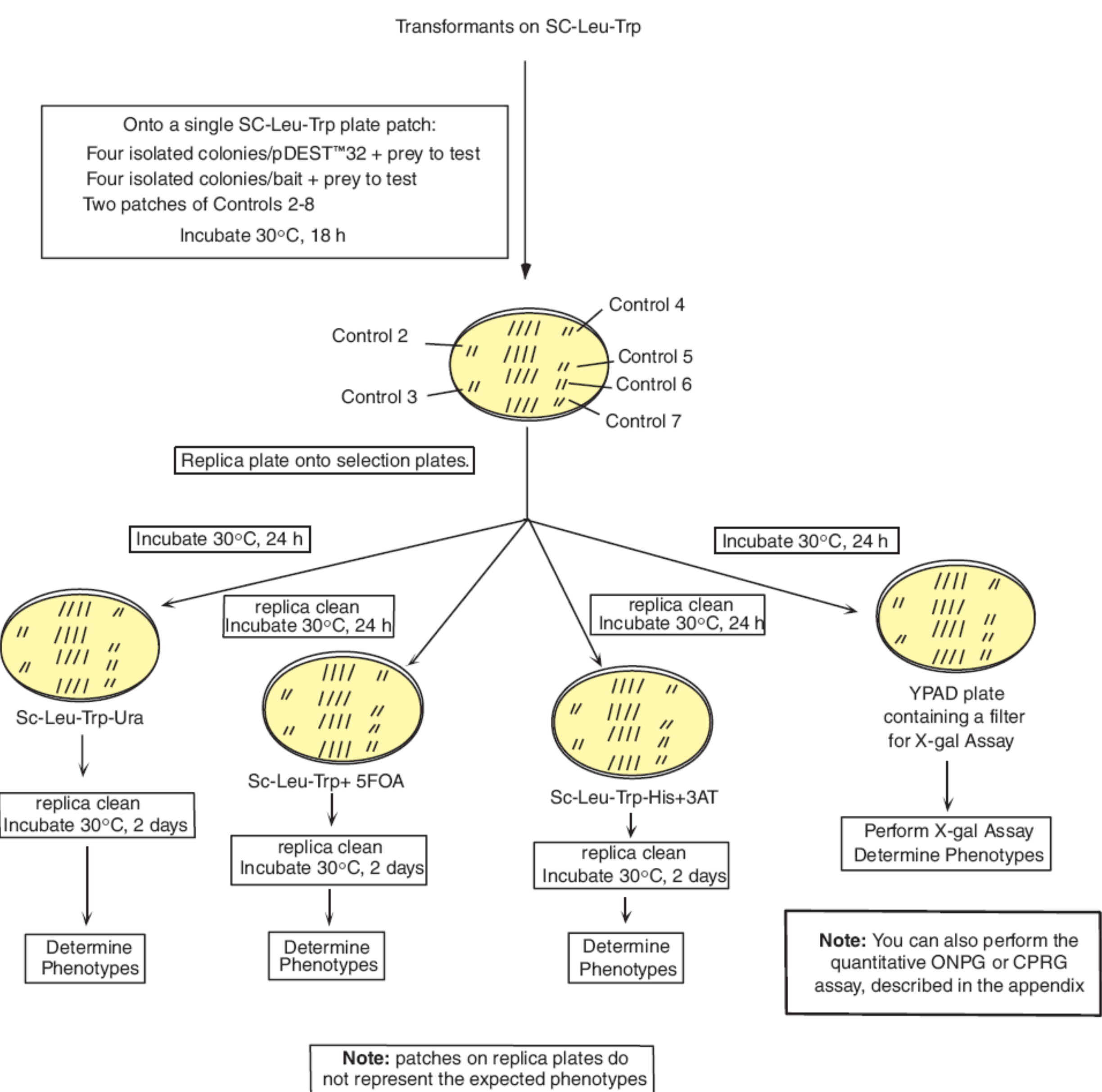
1. For each transformation, mix together 1 µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA with 100 µL of the yeast suspension from Step 5 on page 43, above.
2. Add 700 µL of 1X LiAc/40% PEG-3350/1X TE and mix well.
3. Incubate solution at 30℃ for 30 minutes.
4. Add 88 µL DMSO, mix well, and heat shock at 42℃ for 7 minutes.

5. Centrifuge in a microcentrifuge for 10 seconds and remove supernatant.
6. Resuspend the cell pellet in 1 mL 1X TE and re-pellet.
7. Resuspend the pellet in 50-100 µL TE and plate on a selective plate.

Characterization of transformants

Introduction

MaV203 cells that contain bait and prey proteins that strongly interact will induce all three reporter genes present in this system (*HIS*3, *URA*3, *lacZ*). Identify these colonies by a series of patching and replica plating steps onto the selection/screen plates, which are described in this section.



Note: For an explanation of controls 2-8, see "Recommended controls" on page 40

Required test plates

To test a specific interaction, use the plates described below for the assay you perform.

Test	*HIS*3 induction	*URA*3 induction	*URA*3 induction	β-Galactosidase induction
Assay	His auxotrophy	5FOA sensitivity	Uracil auxotrophy	X-gal assay
Plates used	SC-Leu-Trp-His+3AT	SC-Leu-Trp+5FOA	SC-Leu-Trp-Ura	YPAD
Concentrations	10 mM 3AT 25 mM 3AT 50 mM 3AT 100 mM 3AT	0.2% 5FOA	No Uracil	Not applicable

Note: For an explanation about the *HIS*3 inhibitor 3AT, refer to "Testing bait" on page 51

Materials needed

- Plates with transformants and controls (see table, "Recommended controls" on page 40)
- Fresh plates:
  - SC-Leu-Trp plates, to grow yeast containing the 2 plasmids to be tested
  - YPAD plates, for X-gal assays to test *lac*Z induction
  - SC-Leu-Trp-Ura plates to test *URA*3 induction
  - SC-Leu-Trp-His+3AT plates, to test *HIS*3 induction
  - SC-Leu-Trp+5FOA plates, to select yeast cells that do not induce *URA*3
- nitrocellulose or nylon membrane
- 30℃ incubator
- Autoclaved velvets for replica plating/cleaning
- X-gal (5-bromo-5-chloro-3-indolyl-β-D-galactoside)
- N,N-dimethyl formamide (DMF)
- Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0)
- 2-mercaptoethanol
- 125-mm Whatman™ 541 filter papers
- 15-cm petri dishes
- Forceps
- Liquid nitrogen

Generating master plates

1. Using an autoclaved toothpick or loop, patch onto a single SC-Leu-Trp plate the following:
   - Two isolated colonies of yeast controls 2-8 (see table, “Recommended controls” on page 40)
   - Four isolated colonies of transformants containing pDEST™32 and prey for an interaction you want to test (up to two clones can be analyzed per plate)
   - Four isolated colonies of transformants containing bait plasmid and prey for an interaction you want to test (up to two clones can be analyzed per plate)
2. If more than 2 preys need to be tested, use additional plates. On each plate include the yeast controls 2-8. Put transformants containing the same prey and pDEST™32 or bait plasmid on one plate.
3. Incubate plates for 18 hours at 30℃.

Note: If you want to test a specific forward two-hybrid interaction between two proteins two ways (i.e. with protein A as bait and protein B as prey, and with protein B as bait and protein A as prey), put both interaction pairs on the same plate.

Important

If you are unfamiliar with replica plating and replica cleaning, see Appendix C, “Supplementary protocols” before continuing this protocol. Replica cleaning is essential to reduce background.

Testing reporter genes

1. Replica plate onto the following plates, in the order listed. Be sure to make asymmetric marks on the plates and membrane to allow for realignment with the master plate. Replica clean where indicated.
   - YPAD containing a nitrocellulose or nylon membrane for an X-gal Assay
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp-His+3AT; replica clean
   - SC-Leu-Trp+5FOA; replica clean
2. Incubate all plates for ~24 hours at 30℃.
3. After 18 to 24 hours incubation of the YPAD plates containing a membrane, perform an X-gal Assay on the membrane as described below.
4. After incubation of the selection plates for 24 h, replica clean the following plates:
   - SC-Leu-Trp-Ura
   - SC-Leu-Trp+5FOA
   - SC-Leu-Trp-His+3AT

5. Incubate for 2 additional days at 30℃.

6. Compare the phenotypes of the transformants to yeast control 2-8 (see table, "Recommended controls" on page 40), and to the phenotype exhibited in the original screen. Weak phenotypic differences should be considered. A particular prey that activates target genes in the presence of DEST™32 is likely a false positive.

Factors influencing growth properties

The growth properties of yeast cells on the selection/screen plates can be influenced by several parameters in addition to the induction levels of the reporter genes:

- Cells approaching stationary phase exhibit different expression levels of the hybrid proteins from cells growing in exponential phase.
- As the number of cells transferred by replica plating increases, the phenotypic differences between positive and negative controls decrease. Replica cleaning dilutes and normalizes the number of cells plated.
- The amount of growth of yeast patches on a particular selection plate will vary dramatically between 2 and 6 days of growth. Correct incubation times are critical.

X-gal assay

1. For each membrane, dissolve 10 mg X-gal in 100 µL DMF. Combine 100 µL X-gal in DMF, 60 µL 2-mercaptoethanol and 10 mL Z buffer.

2. Stack two round 125-mm Whatman™ 541 filter papers in a 15-cm petri dish. Saturate with ~8 mL of the X-gal solution. Remove any air bubbles.

3. Using forceps, carefully remove the membrane from the surface of the YPAD plate. Completely immerse the membrane in liquid nitrogen for 20-30 seconds. Place the frozen membrane on top of the soaked Whatman™ filters colony side up. Remove any air bubbles. Tip the plates slightly and remove excess buffer.

4. Cover the plates and incubate at 37℃. Tip the plates at a slight angle so excess X-gal solution does not accumulate on the membrane. Monitor the appearance of blue color over a 24-h period. Score final results at 24 hours.

Strong interactors show blue color within 1 hour (e.g., yeast control 2; see table, "Recommended controls" on page 40). Weak interactors (e.g., yeast control 3) show blue color within 24 h, but can remain very faint blue to white.

Note

- When scoring the results, the membrane will be a mirror image of the master plate.
- Nitrocellulose membranes are fragile and can crack during freezing; therefore neutrally charged nylon membranes are recommended.
- Handle liquid nitrogen with care. Always wear thick gloves and goggles.
- ONPG will stain. Wear gloves during these procedures.

Quantitative β-Galactosidase assays

For quantitative measurements of β-galactosidase induction, perform the ONPG assay or the CPRG assay as described in the (“Quantitative β-Galactosidase assays in liquid cultures” on page 91).

Expected results testing specific Two-Hybrid interaction

Expected results

Yeast controls 2-4 were retransformed and replica plated on the indicated plates as described in “Generating master plates” on page 46. Growth was scored and an X-gal assay was performed after the recommended incubation times. The results are shown below.

Control 2 3 4
Interaction: Strong Weak Absent Assay:

SC-Leu-Trp

SC -Leu-Trp-His + 10 mM 3AT

SC -Leu-Trp-His + 25 mM 3AT

SC -Leu-Trp-His + 50 mM 3AT

SC -Leu-Trp-His + 100 mM 3AT

SC -Leu-Trp-Ura

SC -Leu-Trp + 0.2% 5FOA

X-gal Assay

Note: Control 2: pEXP™32/Krev1 + pEXP™22/RalGDS-wt
Control 3: pEXP™32/Krev1 + pEXP™22/RalGDS-m1
Control 4: pEXP™32/Krev1 + pEXP™22/RalGDS-m2

Forward Two-Hybrid library screen

Introduction

This chapter describes how to perform a forward two-hybrid library screen using the ProQuest™ Two-Hybrid System. This will enable you to identify new putative partners that interact with your bait (your gene of interest cloned into the GAL4 DBD vector).

In the first part you will determine the conditions to use for screening the library with your bait plasmids (“Testing bait” on page 51). This is absolutely **required** for all new bait plasmid constructs, since these conditions are impossible to predict.

The second part describes how to transform yeast strain MaV203 with bait and library plasmid to identify new putative interactors with your bait (“Screening forward Two-Hybrid library” on page 55).

Required reagents before starting

Before you start, you need to have:

- A bait plasmid, consisting of your gene of interest (or part thereof) cloned into pDEST™32 (in frame with the GAL4 DBD). If you do not have such a bait plasmid, generate one as explained in “Generating bait and prey plasmids” on page 31).
- A two-hybrid library suitable to use with ProQuest™ Two-Hybrid System. See below for available options.

Choosing Two-Hybrid library

There are a number of different sources for libraries available from Thermo Fisher Scientific:

- Use a ProQuest™ Three-Frame cDNA Library from Thermo Fisher Scientific. These are constructed to enrich for in-frame ORFs, and are especially suited for use in the ProQuest™ Two-Hybrid System.
- Other ProQuest™ Pre-made cDNA Libraries; see (**thermofisher.com**) or call Technical Service.
- Generate your own three frame two-hybrid library using a modified method for the **CloneMiner™ cDNA Library Construction Kit**. See Appendix C, “Supplementary protocols”.
- Generate your own two-hybrid library using the regular method for the CloneMiner™ cDNA Library Construction Kit.
- Generate your own two-hybrid library using restriction cloning of your cDNAs into the pEXP™-AD502 plasmid (included in the ProQuest™ Two-Hybrid System). See the SuperScript™ Plasmid System with Gateway™ Technology for cDNA Synthesis & Cloning manual for instructions, available for downloading from (**thermofisher.com**) or by contacting Technical Service. A map of the vector pEXP™-AD502 is provided in (Appendix D, “Maps and features of vectors”).

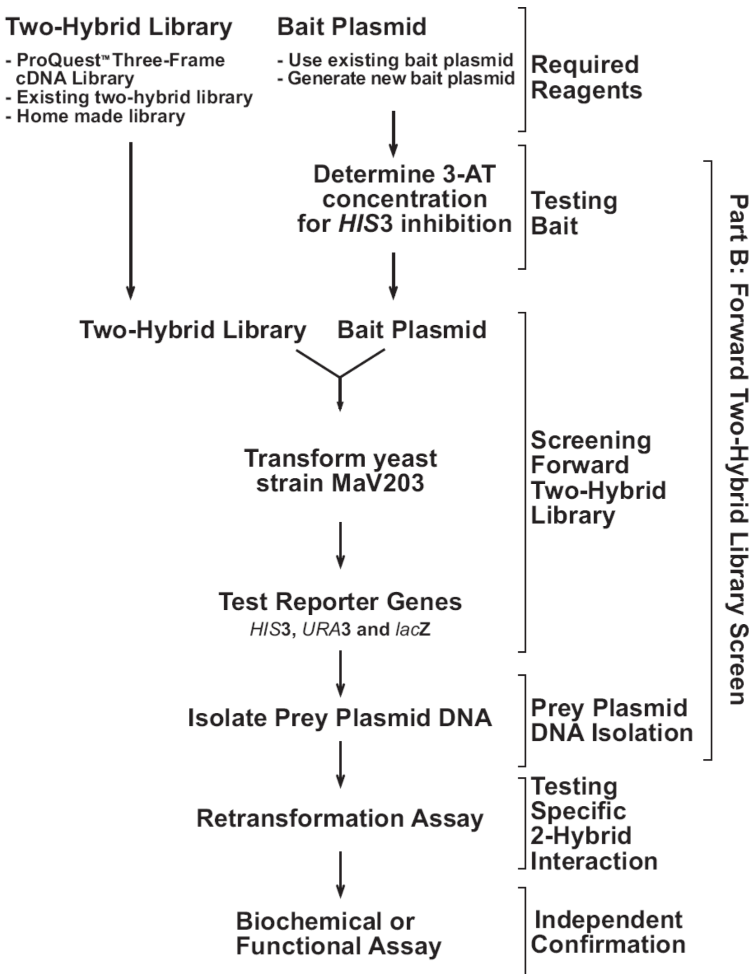
Note: Libraries from sources other than Thermo Fisher Scientific may be used. As a general rule, the library plasmid needs to contain a *TRP*1 selection marker and an activation domain upstream of the insert. We advise using a *ARS*4/*CEN*6 sequence for replication and maintenance at low-copy-number in yeast, and moderate strength promoter such as the yeast Alcohol Dehydrogenase gene (*ADH*1).

Bait-Specific positive interaction control

The ProQuest™ Two-Hybrid System comes with a positive interaction control. However, a bait-specific positive interaction control may be an additional useful tool in testing your experiment. You may construct a prey plasmid with a known interactor of the bait protein to use as a bait-specific positive control and test as explained in Chapter 3, "Verifying interaction" before starting the two-hybrid library screen. This will also get you familiar with the system.

Flowchart

The figure below illustrates the major steps necessary to perform a ProQuest™ Forward Two-Hybrid Library screen.



Methods

Testing bait

Overview

Introduction

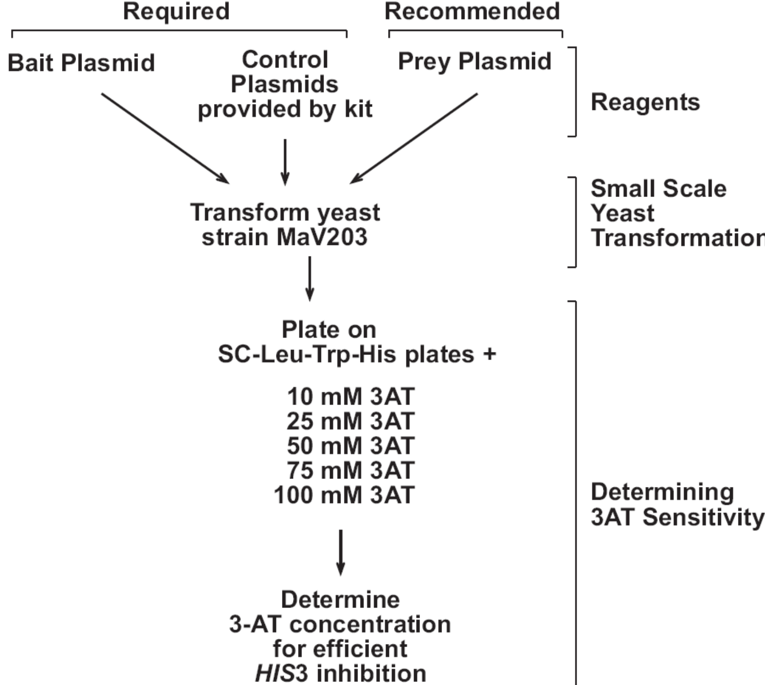
To maximize sensitivity of the *HIS*3 reporter gene, the MaV203 strain already expresses a basal level of *HIS*3. Additionally, bait proteins often contain a certain level of transcriptional activity. This is enough to initiate some transcription at the most sensitive reporter in the system, *HIS*3.

*HIS*3 encodes an enzyme involved in histidine biosynthesis, which can be specifically inhibited in a dose-dependent manner by 3-Amino-1,2,4-Triazole (3AT). By determining the threshold of resistance to 3AT and including that concentration of 3AT in plates lacking histidine, even slight increases in HIS3 reporter gene expression are detected. This enhances the likelihood of detecting weak protein:protein interactions.

This section describes how to transform your bait plasmid into MaV203 cells, test for self-activation, and determine the proper concentration of 3-amino-triazole (3AT) to suppress self-activation at the *HIS*3 gene.

Experimental outline

The experimental outline for Testing Bait is shown below.



Small scale yeast transformation

Transformation guide

Use this table as a guide for the transformations to perform. Select on SC-Leu-Trp plates, which selects for the presence of both the LEU2 and TRP1 plasmid. Store representative transformants in glycerol at -80℃ for future use.

LEU2 Plasmid		TRP1 Plasmid	Purpose
1	none	none	Negative transformation control
2	pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
3	pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
4	pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
5	pDEST™32	pDEST™22	Negative self-activation control
6	Bait plasmid	pDEST™22	Test of self-activation
	If available:		
7	pDEST™32	Known prey (if available)	Test of self-activation prey
8	Bait plasmid	Known prey (if available)	Positive interaction control

Note: The bait and prey plasmid have to be generated by the user as described before ("Generating bait and prey plasmids" on page 31); the other vectors are provided with the kit.

If you plan to screen a two-hybrid library using the **Preparing and Transforming Competent Cells (Library Scale)** protocol provided in this manual ("Preparing and transforming competent cells (Library Scale)" on page 87), we suggest you transfom MaV203 with the bait plasmid **without** prey plasmid or pDEST™22, and select on an SC-Leu plate. Store this plate at 4℃ and make a glycerol stock, as you use it in the Library Scale Yeast Transformation protocol to generate competent yeast cells containing the bait plasmid.

Competent yeast cells

The MaV203 yeast strain is provided with the kit to serve as the host for your bait and prey plasmids. Below we provide a small-scale protocol for transforming yeast cells. Prepare a new batch of competent cells for every transformation.

Alternatively, to limit your workload and increase the transformation efficiency, you may purchase the following products:

- MaV203 Competent Cells, Subclone Scale from **thermofisher.com** (Catalog no. 11445012). Use the transformation protocol provided with these cells.
- MaV203 Competent Cells, Library Scale from **thermofisher.com** (Catalog no. 11281011) and use one vial to perform multiple small-scale transformations (the other vial can be used in a large-scale library transformation). Per transformation, scale down to 25 µL competent cells, 1 µg DNA per plasmid, 180 µL PEG/LiAc, 10.8 µL DMSO. Otherwise, follow the transformation protocol provided with these cells.
- If you want to generate a large batch of competent cells that can be frozen, use the *S. c.* EasyComp™ Kit (Catalog no. K505001). Use the transformation protocol provided with this kit.

Note: There are other small-scale transformation methods that can be used..

Determining 3AT sensitivity

Introduction

In this section you will test the bait (GAL4 DBD fusion) for nonspecific activation. We assess the extent of self-activation on the reporter gene *HIS*3 by determining the concentration of *HIS*3 inhibitor 3AT necessary to repress growth. This concentration will later be used in library two-hybrid screening to suppress growth of yeast cells not containing interacting bait and prey.

Self-Activation

A suitable bait **should not:**

- Non-specifically transactivate the reporter constructs in the MaV203 strains.
- Interact with either the nuclear localization signal (NLS) or with the activation domain in pDEST™22.

Materials required

Be sure to have the following reagents and equipment on hand before proceeding. See Appendix B, “Recipes” for specific media recipes.

- 30℃ incubator
- Plates with strains generated in previous section (“Small scale yeast transformation” on page 42)
- 3-aminotriazole (Millipore Sigma, St. Louis, MO; Catalog No. 09540)
- SC-Leu-Trp plates containing 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM 3AT
- Autoclaved velvets for replica plating/cleaning

Important

If you are unfamiliar with replica plating and replica cleaning, see "Replica Plating/Replica cleaning" on page 89 before continuing this protocol. Replica cleaning is essential to reduce background.

Testing bait plasmid

1. After incubation, patch four different colonies containing bait plasmid and pDEST™22 (transformation 2, previous section, "Small scale yeast transformation" on page 42) on a single SC-Leu-Trp plate. On the same plate, patch two colonies each from interaction controls and activation controls (controls 2 to 8, see table in previous section, "Small scale yeast transformation" on page 42). Incubate for ~18 hours at 30℃.

2. Replica plate from this SC-Leu-Trp master plate onto SC-Leu-Trp-His plates containing 3AT at concentrations of 0 mM, 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM. Immediately replica clean the plates. Incubate for 24 hours at 30℃.

3. After incubation, replica clean again and incubate for 2 days (40-44 h) at 30℃. The lowest concentration of 3AT that inhibits the growth of the cells from transformation 2 (containing bait and pDEST™22) is the basal amount of 3AT added to all plates lacking histidine.

Analysis

If cells from transformation 2 (containing bait plasmid and pDEST™22) grow even in the presence of 100 mM 3AT, the bait plasmid likely encodes a protein that directly or indirectly self-activates the reporter genes in this system. Such plasmids are not suitable for use in the two-hybrid screen.

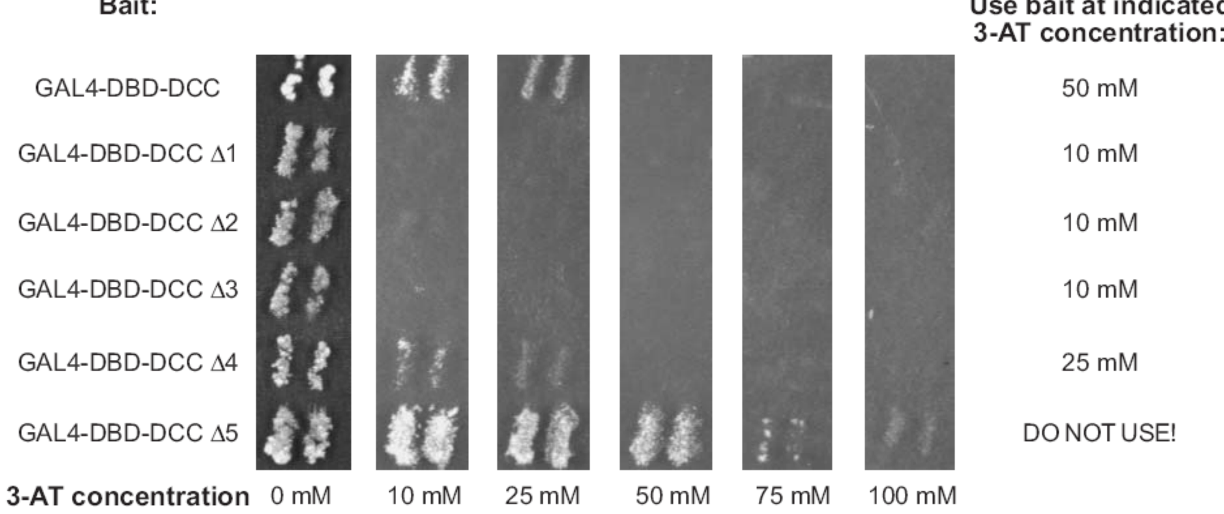
Alternatives for self-activating bait

If your bait is self-activating *HIS*3, try deletion derivatives of the original bait for a two-hybrid screen. Generate bait plasmids that lack parts of the coding sequence of your gene of interest, and test whether these self-activate.

Example of expected results

The coding sequence for the cytoplasmic domain of the human DCC gene (deleted in colorectal cancer) was fused to the GAL4 sequence encoding the DNA Binding Domain (GAL4 DBD-DCC) in pDEST™22. Derivatives were constructed that had increasing deletions at the 3' end (Δ 3, Δ 2, and Δ 1, respectively) or 5' end (Δ4 and Δ 5). Each of these constructs was introduced, along with a non-interacting prey plasmid, into MaV203 (selecting on SC-Leu-Trp plates). Self-activation was tested as described above on SC-Leu-Trp-His at 0 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM 3AT.

The baits were used in subsequent library screens at the indicated concentrations of 3AT except for GAL4 DBD-DCC Δ5. This bait was not used due to self-activation, i.e. growth at 100 mM 3AT.



Note: The DCC clones were the kind gift of Drs. G. Hu, M. Vidal and E. Fearon.

Screening forward Two-Hybrid library

Overview

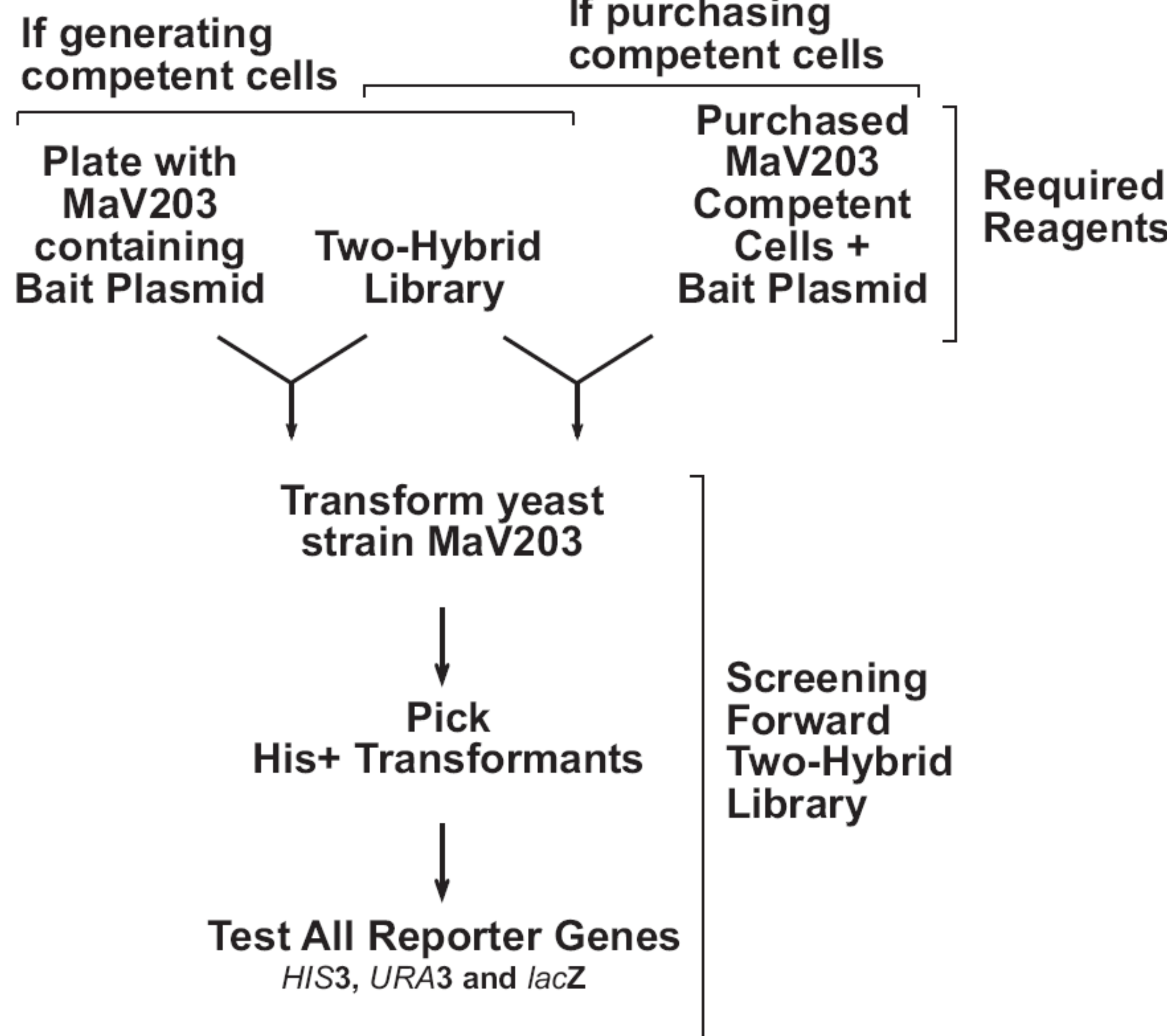
Introduction

This section describes how to transform your bait and library into MaV203 cells, and test activation of the three reporter genes. This will let you identify putative new interactors for your bait protein.

Note: If you do not have any experience performing two-hybrid screens, we suggest you first test a specific two-hybrid interaction as described in “Recommended controls” on page 40 using your bait and prey plasmids or the provided controls. This will allow you to get familiar with two-hybrid screens and the ProQuest™ Two-Hybrid System before performing the more technically challenging two-hybrid library screen.

Experimental outline

The experimental outline for Screening Forward Two-Hybrid Library is shown below.



Library scale yeast transformation

Introduction

This section describes how to perform a library transformation. We recommend that you screen > 10^6 yeast transformants for mammalian cDNA libraries. Therefore, use these protocols specifically written for library transformations, since other yeast transformation protocols do not yield enough transformants.

Competent yeast cells

To transform your bait and library plasmids into yeast, you need to obtain competent MaV203 cells. We recommend using MaV203 Competent Cells, Library Scale from **thermofisher.com** (Catalog no. 11281011). Use the transformation protocol provided in Appendix C, "Supplementary protocols". These cells have a very high transformation efficiency (> 2×10^5 transformants/µg library DNA) and are easy to work with.

If you do not want to purchase MaV203 Competent Cells, Library Scale from Invitrogen™ (Catalog no. 11281011), use the Preparing and Transforming Competent Cells (Library Scale) protocol for transforming yeast cells described in the Appendix, "Materials needed" on page 58. Prepare a new batch of competent cells for every transformation. This will take considerably more time and will not yield as high a transformation efficiency. To obtain a decent number of transformants, yeast cells already containing your bait have to be transformed with the library DNA, which will yield an efficiency of > 2×10^5 transformants/µg library DNA.

Important

If you perform the Library Scale Yeast Transformation protocol described in "Preparing and transforming competent cells (Library Scale)" on page 87, you need to have a SC-Leu plate with MaV203 transformed with bait plasmid without prey plasmid or pDEST™22. Transform MaV203 with bait plasmid as described in "Small scale yeast transformation" on page 42, and select on an SC-Leu plate.

3AT concentration

The optimum concentration of 3AT for your bait **must** be determined prior to doing a library transformation; see "Testing bait" on page 51.

Calculating transformation efficiency

The transformation efficiency is represented by the number of colonies per transformation reaction. Count colonies on the 10-cm SC-Leu-Trp plates, preferably on the plates having 20 to 300 colonies. Calculate the transformation efficiency by the following equation:

Number of colonies per transformation reaction =

Colonies on a plate x dilution factor × total volume / plated volume

For example, if 150 colonies are counted when 0.1 mL of a 1:100 dilution of the library screen (12 mL total volume) are plated, the calculation would be:

$$150 \times 100 \times \frac{12 \text{ ml total volume}}{0.1 \text{ ml plated}} = 1.8 \times 10^6 \text{ colonies / reaction}$$

A successful transformation for a library screen should have at least 1×10^6 transformants.

Characterization of His^+ transformants

Introduction

You should have identified a number of colonies that grow on plates without histidine. Some of these may be false positives (see "False positives" on page 17); others represent true interactors. MaV203 cells that contain bait and prey proteins that strongly interact will induce all three reporter genes present in this system (*HIS*3, *URA*3, *lac*Z). Identify these colonies by a series of patching and replica plating steps onto the selection/screen plates, which are described in this section.

Controls

Use yeast controls 2-8 generated in the previous chapter ("Small scale yeast transformation" on page 42). Below is indicated the purpose of these controls:

LEU2 Plasmid		TRP1 Plasmid	Purpose
2	pEXP™32/Krev1	pEXP™22/RalGDS-wt	Strong positive interaction control
3	pEXP™32/Krev1	pEXP™22/RalGDS-m1	Weak positive interaction control
4	pEXP™32/Krev1	pEXP™22/RalGDS-m2	Negative interaction control
5	pDEST™32	pDEST™22	Test of self-activation
6	Bait plasmid	pDEST™22	Negative interaction control
	If available:		
7	pDEST™32	Known prey (if available)	Negative interaction control
8	Bait plasmid	Known prey (if available)	Positive interaction control

Materials needed

- Plates with His^+ transfomants
- Yeast controls 2–8 generated in the previous chapter ("Small scale yeast transformation" on page 42)
- SC-Leu-Trp plates to grow yeast cells containing both bait and prey plasmid
- YPAD plates, to grow cells for assaying *lac*Z activity
- SC-Leu-Trp-His+3AT plates, to select for cells expressing *HIS*3. Use the 3AT concentration as determined in "Testing bait" on page 51
- SC-Leu-Trp-Ura plates, to select for cells expressing *URA*3
- SC-Leu-Trp+0.2% 5FOA plates, to select against cells expressing *URA*3
- nitrocellulose or nylon membrane
- 30℃ incubator
- Autoclaved velvets for replica plating/cleaning
- X-gal (5-bromo-5-chloro-3-indolyl-β-D-galactoside)
- N,N-dimethyl formamide (DMF)

- Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0)
- 2-mercaptoethanol
- 125-mm Whatman™ 541 filter papers and 15-cm petri dishes
- Forceps
- Liquid nitrogen

Generating master plates

1. Streak transformants that grow on SC-Leu-Trp-His+3AT on SC-Leu-Trp plates to isolate single purified colonies.
2. Prepare fresh colonies of the yeast controls 2–8 generated in the previous chapter ("Small scale yeast transformation" on page 42) by streaking a colony from the stored plates onto new SC-Leu-Trp plates.
3. Incubate plates for 48 hours at 30℃.
4. Using an autoclaved toothpick or loop, patch onto a single SC-Leu-Trp plate the following:
   - Two isolated colonies of yeast controls 2–8
   - Four isolated colonies of each potential positive clone (generally up to four clones can be analyzed per plate)
5. If more than 4 His+ clones were identified, use additional plates. On each plate include the 7 yeast controls.
6. Incubate plates for 18 hours at 30℃.

Note: Store the plates from Step 1 on page 59 containing the His^+ transformants at 4℃.

Testing reporter genes

Perform the assays described in "Testing reporter genes" on page 46, and "X-gal assay" on page 47, to test for *HIS*3, *URA*3 and *lac*Z expression.

Important

The use of large amounts of DNA used in the library transformation can result in multiple prey clones in a single transformant. Such transformants may show growth on 5FOA plates and care must be taken to identify and retest all candidate prey clones following isolation in *E. coli*. The use of 5 µg of each vector will reduce the number of transformants containing multiple copies of prey plasmids, but will also reduce transformation efficiency slightly.

Interpretation of phenotypes

Interpretation of the four reporter gene readouts is the most critical step in a two-hybrid screen. A summary of likely interpretations of observed phenotypes is provided below. A detailed description of the phenotypes is provided in "Interpretation of results" on page 66.

-His	*lacZ*	-Ura	0.2% 5FOA	Easily-Interpreted Phenotypes
no growth				False positive/background
growth	blue	no growth	no growth	Interactor, probably weak
growth	blue	growth	no growth	Interactor
growth	white	no growth	growth	Non-interactor

-His	*lacZ*	-Ura	0.2% 5FOA	One Inconsistent Phenotype
growth	blue	growth	growth	Probable interactor Possible mixed population*
growth	white	growth	no growth	Probable interactor (check *lacZ* expression using CPRG assay)

-His	*lacZ*	-Ura	0.2% 5FOA	Two Inconsistent Phenotypes
growth	blue	no growth	growth	Possible non-interactor Possible weak interactor (look carefully for any inhibition on 5FOA) Possible mixed population*
growth	white	growth	growth	Possible interactor (check *lacZ* expression using CPRG assay) Possible mixed population*; look carefully for inhibition on 5FOA
growth	white	no growth	no growth	Probable weak interactor (check *lacZ* expression using CPRG assay; confirm 5FOA phenotype)

- Intermediate levels of URA3 expression can result in both cell growth inhibition on 0.2% 5FOA and insufficient *URA*3 gene product to allow growth on plates lacking uracil. The $5FOA^S/Ura^-$ phenotype is often indicative of protein pairs that interact weakly.
- The strength of the three different reporter gene read-outs can vary dramatically between different pairs of interacting proteins.
- Colonies that are white in the X-gal assay may be tested in the more sensitive CPRG assay for β-galactosidase activity; see "Quantitative β-Galactosidase assays in liquid cultures" on page 91.

What to do next

Overview

Introduction

After you have isolated HIS+ transformants, confirm the interaction. Do this using two independent methods:

1. Repeat the two-hybrid assay with bait and prey plasmids to make sure that no mutations have been generated in the bait plasmid or yeast strain in the primary transformants. The phenotype of the primary transformants (*HIS*3, *URA*3 and *lacZ* expression) should be replicated in this new assay.

2. Perform a biochemical or functional assay to confirm the interaction of bait and prey in an independent assay.

Repeat Two-Hybrid assay

Several types of “false positives” can result in phenotypes resembling true interactions (e.g., a mutation in bait that converts it to a self activator). If the bait/prey interactions identified above are authentic, the reporter gene phenotype should be reproduced when prey is reintroduced into MaV203 with the original bait plasmid, but not when empty pDEST™32 is introduced. Two methods can be used with the ProQuest™ System:

- **Retransformation assay** (preferred method)
- **Plasmid shuffling** (faster method)

Retransformation assay

The Retransformation assay consists of the following steps:

1. Isolate the prey plasmid in *E. coli*, as described in “Prey plasmid DNA isolation” on page 63.

2. Perform restriction and sequence analysis.

3. Retransform the prey plasmid in MaV203 together with bait plasmids or controls, and test reporter gene expression (*HIS*3, *URA*3 and *lacZ*) as described in “Testing specific Two-Hybrid interaction” on page 40.

Note: Use 3AT concentration in SC-Leu-Trp plates as determined in “Testing bait” on page 51.

Required test plates

To validate potential interacting proteins identified in a two-hybrid screen, use the plates described below for the assay you perform.

Test	*HIS*3 induction	*URA*3 induction	*URA*3 induction	β-Galactosidase induction
Assay	His auxotrophy	5FOA sensitivity	Uracil auxotrophy	X-gal assay
Plates used	SC-Leu-Trp-His+3AT	SC-Leu-Trp+5FOA	SC-Leu-Trp-Ura	YPAD
Concentration used	3AT concentration as determined ("Testing bait" on page 51)	0.2% 5FOA	No Uracil	Not applicable

Plasmid shuffling

The plasmid shuffling method (see "Plasmid shuffling" on page 94) is fast and easy, but does not exclude false positives resulting from host cell mutations. Therefore the Retransformation Assay is the preferred method, but plasmid shuffling may be useful if you need to screen large numbers of HIS^+ transformants.

Sequence analysis

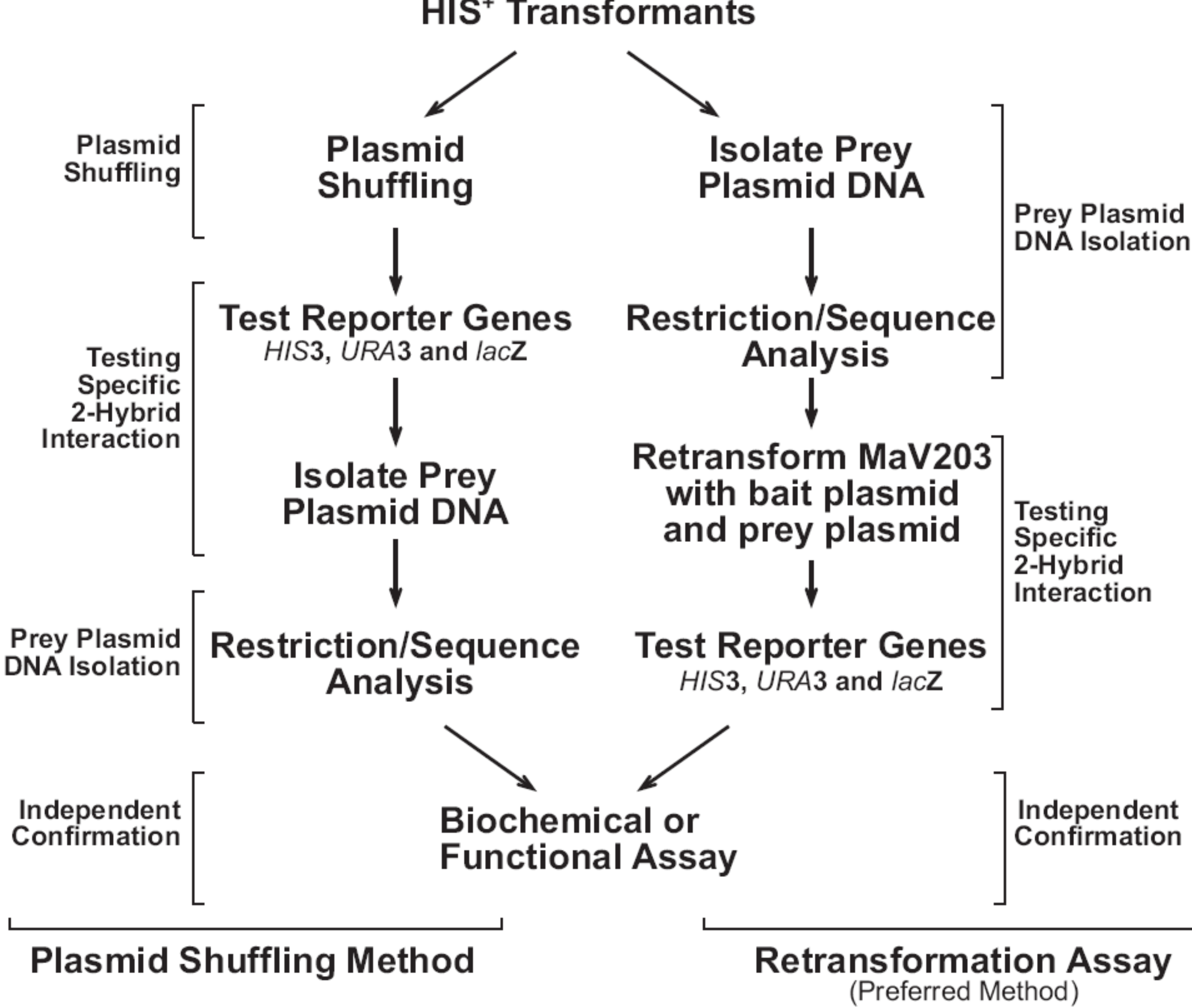
If you can repeat the interaction between bait and prey, sequence the insert. Make sure the insert is in frame with the GAL4 AD domain; if not the clone is likely a false positive.

Confirmation of interaction

If sequence information suggests the interaction may be valid or biologically relevant, perform a biochemical and/or functional assay to validate the interaction in an independent experiment. For suggestions, see "Confirmation of interaction" on page 62.

Experimental outline

The experimental outline for the steps to take after HIS+ transformants have been identified is shown below.



Prey plasmid DNA isolation

Introduction

Plasmid DNA isolated from yeast is typically not suitable for restriction analysis. Consequently, plasmids isolated from yeast are first introduced into *E. coli*, then miniprep DNA from the resulting transformants is characterized. Several quick yeast plasmid preparations have been described. The following method uses the PureLink™ HQ Mini Plasmid DNA Purification Kit , since it is a fast and reliable method for DNA isolation. Other purification kits are suitable.

Materials needed

- 30℃ shaker
- SC-Trp medium
- 1 x TE (10 mM Tris-HCl (pH 7.5), 1 mM EDTA)
- PureLink™ HQ Mini Plasmid DNA Purification Kit (Catalog no. K210001)
- Zymolyase™ (1.5 U/µl, Genotech, St.Louis, MO, Catalog no. 786-036)
- 2-mercaptoethanol
- Competent cells, such as One Shot™ TOP10 Chemically Competent *E. coli* cells (Catalog no. C404003)
- LB+100 µg/ml ampicillin medium and plates
- 37℃ incubator

Plasmid DNA extraction

IMPORTANT! This is a modified version of the protocol provided with the kit.

1. Suspend an isolated colony from a fresh plate in 3 ml SC-Trp and incubate at 30℃ with shaking for ~24 hours. OD_{660} should be in between 1.0–2.3.
2. Centrifuge the 3 mL culture at 1500 × *g* for 15 minutes at room temperature. Remove all residual liquid.
3. Resuspend the cells in 1 mL 1xTE.
4. Centrifuge the 3 mL culture at 1500 × *g* for 15 minutes at room temperature. Remove all residual liquid.
5. Resuspend the cells in 240 µL Resuspension Buffer containing RNase A (provided with the PureLink™ HQ Mini Plasmid DNA Purification Kit).
6. Add 10 µL Zymolyase™ (1.5 U/µL) and 5 µL 2-mercaptoethanol.
7. Incubate at 37℃ for 30 minutes.
8. Add 240 µL Lysis Buffer (provided with the PureLink™ HQ Mini Plasmid DNA Purification Kit); mix gently by inverting the tube 4–8 times.
9. Incubate for 3–5 minutes at room temperature (do not exceed 5 minutes).
10. Add 340 µL of Neutralization/Binding Buffer (provided with the PureLink™ HQ Mini Plasmid DNA Purification Kit), and immediately mix gently by inverting the tube 4–8 times.
11. Centrifuge for 10 minutes at maximum speed in a tabletop centrifuge to clarify the cell lysates.
12. Place a PureLink™ spin column inside a 2-mL collection tube. Pipette or decant the supernatant into the spin column.

13. Centrifuge the column at room temperature at 10,000–14,000 × *g* for 30-60 seconds, discard the flow through from the collection tube.

14. Add 650 µL of Wash Buffer prepared with ethanol to the column (provided with the PureLink™ HQ Mini Plasmid DNA Purification Kit).

15. Centrifuge the column at room temperature at 10,000–14,000 × g for 30-60 seconds. Discard the flow through from the collection tube.

16. Repeat the Wash steps 13 on page 65 and 14 on page 65.

17. Centrifuge the column at maximum speed for 2.5 minutes to remove the residual wash buffer.

18. Place the spin column in a clean 1.7-mL elution tube.

19. Add 70 µL of Elution Buffer (provided with the PureLink™ HQ Mini Plasmid DNA Purification Kit), or water to the center of the column.

20. Incubate the column at room temperature for 1 minute.

21. Centrifuge at maximum speed for 2 minutes.

Transforming *E. coli*

Use 5–10 µL of DNA to transform One Shot™ TOP10 Chemically Competent *E. coli* according to the procedure provided with the cells, or transform your in-house competent cells. Plate ~20% of the transformation mixture on LB agar plate + 100 µg/mL ampicillin. Grow overnight at 37℃.

Note: If you do not get enough colonies, transform 1 µL of DNA into electrocomp cells such as ElectroMAX™ DH10B cells (Catalog no. 18290015).

Analyzing transformants

To analyze positive clones, we recommend that you:

1. Pick 5–10 colonies and culture them overnight in LB or SOB medium containing 100 µg/mL ampicillin (for prey plasmids).

2. Perform restriction analysis to confirm the presence of the insert.

   Note: *Bsr*G I cleaves within all *att* sites, and can be used to help characterize clones.

3. Isolate plasmid DNA using your method of choice. To obtain pure plasmid DNA for automated or manual sequencing, we recommend using the PureLink™ HQ Mini Plasmid Purification Kit and the primers indicated below:

Plasmid	Direction	Primer
Prey	forward	5’-TATAACGCGTTTGGAATCACT-3’

Interpretation of results

Introduction

This section provides help in the interpretation of the results of a two-hybrid experiment.

Interpretation of growth is subjective

The interpretation of growth/slight growth/no growth on the various selection plates is quite subjective. Yeast controls 2–8 ("Recommended controls" on page 40) should show the expected growth profile on the selection plate being examined in order to make valid conclusions. If they do not, confirm that the plates were prepared correctly, that the cells of the controls are fresh and correctly ordered (2–8). If necessary, transform the controls again and use isolated fresh colonies on SC-Leu-Trp plates.

False positives

False positives have been defined as clones containing bait and prey that induce the reporter genes in a two-hybrid screen, but where bait and prey do not interact; or these interactions are biologically irrelevant. Several recent references have discussed false positives.

False positives in which bait or prey self-activate (e.g., mutations in bait that result in self-activation, or prey clones that activate transcription by binding to the promoters or proteins bound there) are usually eliminated by retesting prey with pDEST™32 (empty GAL4 DBD) and bait ("Recommended controls" on page 40). Swapping the DNA Binding Domain and Activation Domain between bait and prey provides strong evidence confirming the interaction. However, several examples have been described where swapping the DBD and AD impedes legitimate interactions. This is presumably due to steric issues, hence failure to retest in this experiment should not necessarily exclude the interaction.

On occasion, a false positive prey will not induce the reporter genes when tested with DBD alone, but will when an unrelated protein is fused to DBD. Therefore, it is useful to test candidate prey clones against an irrelevant bait, such as pEXP™32/Krev1 (if no interaction is expected with Krev1).

Certain proteins, such as those with low affinities for many different proteins (e.g., containing large hydrophobic domains) or those that may recognize structural features of proteins (e.g., heat shock proteins) may be identified as false positives in a two-hybrid screen. More complex structures, where bait and prey are bridged by a third protein or RNA, may also account for certain false positives.

It is critical to:

- Confirm that bait and prey interact by retesting the interaction and showing that it is specific to the test bait,
- Perform an independent assay such as co-immunoprecipitation, and
- Devise experiments to demonstrate that the interaction is biologically relevant.

Mixed populations

Mixed populations can result from numerous causes. Examples include:

- More than one prey plasmid in the cell resulting in one population that contains prey interacting with bait (no growth on 5FOA but growth on URA-plates), and another population that contains prey that does not interact with bait (giving rise to growth on 5FOA but not on URA-plates)
- Mutations or instability in bait, prey or *URA*3 reporter or promoter
- Carry over of cells from the -His+3AT plate that do not contain interacting bait and prey

If restriction analysis after **Prey Plasmid DNA Isolation** ("Prey plasmid DNA isolation" on page 63) indicates a mixed population, test all different isolated prey plasmids in the retransformation assay.

*HIS*3 and 3AT

In order to inhibit *HIS*3 at the threshold level, you should add 3AT to all SC-Leu-Trp-His plates. Yeast control 2–8 ("Recommended controls" on page 40) will show different growth patterns as the 3AT concentration is increased (see "Expected results testing specific Two-Hybrid interaction" on page 48). This must be considered when determining whether cells containing candidate interactors induce the HIS3 reporter gene. It is important to properly replica clean 3AT plates and to interpret the results at the indicated times. As the incubation time is increased beyond the indicated time, growth of cells not inducing HIS3 can become indistinguishable from those inducing the HIS3 reporter gene.

Master replica Plating/Cleaning

Difficulty in interpreting the reporter gene readouts is very often associated with replica plating and replica cleaning. These procedures take practice. The most important issues have been described in, "Replica Plating/Replica cleaning" on page 89. It is useful to practice with yeast controls 2–8 ("Recommended controls" on page 40) until the expected readouts are obtained consistently. Be sure plates and velvets are very dry. If controls 2–8 do not give the expected results, confirm that the plates were prepared correctly or transform the controls again and use isolated fresh colonies on SC-Leu-Trp plates. Always include controls 2–8 on each master plate (remember to keep the patches away from the edges of the plate) to confirm that the plates are correct, and the replica plating was successful. Alternatives to replica plating and replica cleaning are described in, Appendix C, "Supplementary protocols".

URA-Least sensitive selection

Growth on the SC-Leu-Trp-Ura plate is the least sensitive selection method and failure of candidate interactors to grow here should not exclude them as true interactors. This is because the *URA*3 promoter region contains the URS1 sequence that strongly represses transcription. Only strong protein:protein interactions induce this gene sufficiently to allow growth on SC-Ura plates. Furthermore, certain bait fusions, while showing strong induction of the *HIS*3 and *lac*Z reporter genes, may show weak induction of *URA*3.

Controls on 5FOA plates

Inhibition of growth on 5FOA is more sensitive than growth on SC-Leu-Trp-Ura plates. Compare the amount of growth of the candidate clone with yeast control 2–8 (see table in "Recommended controls" on page 40). Yeast control 3–7 should show good growth (little growth inhibition) on SC-Leu-Trp+0.2% 5FOA; and yeast control 2 and 8 should be completely inhibited. The replica cleaning step is critical for good results on 5FOA. If the controls do not show the expected results, confirm the amount of 5FOA added and the media composition.

X-gal assay

X-gal assays for examining induction of the *lac*Z reporter gene are quite sensitive if done correctly. Always use a fresh X-gal solution. The addition of excess X-gal/Z-buffer during incubation of the membrane will cause diffusion of the blue color into adjacent colonies. Use the minimal amount (7–8 mL) of buffer to saturate the paper filters. It is useful to tip the plate slightly during incubation to allow any excess buffer to accumulate below the filter papers. Yeast control 2 (see table in "Recommended controls" on page 40) gives a very strong blue reaction. Keep this patch at the bottom of the tipped plate to avoid diffusion into adjacent patches. Efficient cell lysis is critical. Cells are lysed by immersion in liquid nitrogen. Typically 20–30 seconds is sufficient. Be careful with the membrane after lysing the cells. The patches become viscous and touching them will cause smearing. Be certain that the patches are incubated facing up. Nitrocellulose filters often crack in liquid nitrogen. Yeast controls 2–8 must give the expected result to accurately interpret the results. Yeast control 3 should give a very faint blue when lysis is complete. For those candidate clones showing very weak or questionable *lac*Z activity, it is often useful to use the CPRG assay ("Quantitative β-Galactosidase assays in liquid cultures" on page 91) to confirm the phenotype.

Troubleshooting

Generating bait and prey plasmid

The table below lists some potential problems and possible solutions that may help you troubleshoot the Generating Bait and Prey Plasmid procedures.

Problem	Reason	Solution
Few or no colonies obtained from sample reaction **and** the transformation control gave colonies	Incorrect antibiotic used to select for transformants	Select for transformants on LB agar plates containing 10 µg/ml gentamicin (for bait plasmids) or 100 µg/ml ampicillin (for prey plasmids).
	Didn't use the suggested LR Clonase™ II enzyme mix or LR Clonase™ II enzyme mix wasinactive	• Make sure to store the LR Clonase™ II enzyme mix at -20℃ or -80℃. • Do not freeze/thaw the LR Clonase™ II enzyme mix more than 10 times. • Use the recommended amount of LR Clonase™ II enzyme mix • Test another aliquot of the LR Clonase™ II enzyme mix.
	Not enough transformation mixture plated	Increase the amount of *E. coli* plated.
Growth of yeast control 4-7 on SC-Leu-Trp-His+3AT	Plates not replica cleaned	Replica clean immediately after replica plating, and again after 24 hours incubation
	Inadequate replica cleaning	Review **Appendix**, "Replica Plating/Replica cleaning" on page 89. Immediately after replica cleaning, plate should contain no remaining visible cells.
	Too many cells transferred during replica plating	Review **Appendix**, "Replica Plating/Replica cleaning" on page 89. Transfer a minimal number of cells.

(continued)

Problem	Reason	Solution
Growth of yeast control 4-7 on SC-Leu-Trp-His+3AT	Incorrectly prepared 3AT plates	Review **Appendix**. Confirm that all stock solutions were fresh and prepared correctly. Confirm that the calculation for amount of 3AT addition was correct.
	Incorrect incubation times	Incubate plates no longer than 60 hours (40–44 hours is usually best). Colonies arising after 60 hours are not likely to be of interest.

Testing specific Interaction/ retransformation assay

The table below lists some potential problems and possible solutions that may help you troubleshoot testing a specific interaction and retransformation assay.

Problem	Reason	Solution
Failure to obtain transformants of MaV203 with DNA isolated from *E. coli*	Failure to add both bait and prey plasmids during transformation	Use bait and prey plasmids simultaneously in co-transformation procedures.
	Incorrect selection plates	Plate co-transformations on SC-Leu-Trp plates
Failure of candidate clones to reproduce the reporter gene activation observed in the original screen	Candidate clones were false positives	Candidate clones could have been mutants of bait that self-activate. See "Interpretation of results" on page 66 for additional information on false positives.
	Co-transformed pDEST™32 instead of bait plasmid	Retransform MaV203 with bait and prey plasmid.
	Multiple prey clones in the original 3AT^R transformants	Examine more ampicillin^R *E. coli* transformants for additional prey clones. Test each by reintroduction.

Testing bait

The table below lists some potential problems and possible solutions that may help you troubleshoot testing the bait experiments.

Problem	Reason	Solution
Failure of yeast controls 2, 3 or 8 to grow on SC-Leu-Trp-His+3AT	Incorrectly prepared 3AT plates	See above.
	Controls are too old or were mixed up	Return to the original DNA stocks provided, retransform on SC-Leu-Trp, and use fresh colonies.
	Uneven replica plating	When replica plating, maintain an even pressure across the entire surface of the master and selection plates. Uneven pressure can result in the failure of cells to transfer.
Failure of transformants to grow on any of the SC-Leu-Trp-His+3AT plates	Incorrectly prepared 3AT plates	See above.
	Strains being tested do not contain bait and prey	Confirm growth on SC-Leu-Trp plates.
	Uneven replica plating	See above.
Growth of MaV203 transformed with bait and pDEST™22 on all concentrations of 3AT	Bait self activates	Subclone segments of bait into pDEST™32 and retest.
	Incorrectly prepared 3AT plates	See above.
	Improper replica plating or replica cleaning	Review “Replica Plating/Replica cleaning” on page 89. Immediately after replica cleaning, plate should contain no remaining visible cells (although a faint haze may be present on 3AT transformation plates).
	Incorrect incubation times	See above.

Forward two hybrid screen

The table below lists some potential problems and possible solutions that may help you troubleshoot the forward two-hybrid screens.

Problem	Reason	Solution
No transformants on SC-Leu-Trp-His+3AT (candidate interactors)	Gene of interest not in frame with GAL4 DNA Binding Domain-encoding sequence	Sequence the DBD/test DNA junction.
	Poor quality cDNA library	Determine the percent of vectors containing inserts and their average size.
	Inadequate amount of cDNA library	Confirm concentration of library.
	Test DNA cloned into pDEST™32 lacks or masks a domain required for protein:protein interaction	Clone and test alternative segments of the test DNA (bait)
	cDNA library used does not contain proteins that interact with test protein X	Screen a cDNA library from an alternative tissue, developmental time point, or organism.
		Determine whether the bait protein is expressed in the library.
	Prey that interacts with bait may be toxic, unstable or require post-translational modification	Some posttranslational modifications cannot be accomplished in yeast.
		Make sure a cDNA library is constructed in pDEST™22 or pEXP™-AD502 and not in other high-copy-number AD-vectors.
	Bait may be toxic, unstable or require post-translational modification	Some posttranslational modifications cannot be accomplished in yeast.
		Subclone segments of bait into pDEST™32 and retest.
High background on SC-Leu-Trp-His+3AT	3AT concentration too low	Retest bait on various concentrations of 3AT
	Plates made incorrectly	Review Appendix B, “Recipes”

(continued)

Problem	Reason	Solution
High background on SC-Leu-Trp-His+3AT	Improper replica cleaning	Review **Appendix**, "Replica Plating/ Replica cleaning" on page 89. Immediately after replica cleaning, plate should contain no remaining visible cells (although a faint haze may be present on 3AT transformation plates).
	Improper incubation times	Do not incubate plates longer than 60 hours. Colonies arising after 60 hours are not likely to be of interest.

Isolation of prey plasmid

The table below lists some potential problems and possible solutions that may help you troubleshoot isolating the prey plasmid.

Problem	Reason	Solution
Failure to obtain enough *E. coli* transformants	*E. coli* not sufficiently competent	Use ElectroMAX™ DH10B cells for library.
	Too much DNA used	Use only 1 µL of DNA. Inhibitory compounds may reduce transformation efficiencies.
	Incorrect selection or concentration	Select for plasmid on LB+ampicillin (100 µg/mL).
	Alternative yeast DNA preparation procedure used	Use the method described in "Prey plasmid DNA isolation" on page 63. Other procedures designed for high-copy-number vectors may not work with the *ARS/CEN*-based vectors used here.
	DNA suspended in incorrect buffer	Electroporation is sensitive to ionic strength. Suspend DNA pellet in TE.

Gateway™ recombination reactions

Introduction

The Gateway™ Technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move your DNA sequence of interest into multiple vector systems.

Review the information in this section to briefly familiarize yourself with the Gateway™ recombination reactions. For details, refer to the Gateway™ Technology with Clonase™ II manual available from our web site at **thermofisher.com** or by contacting Technical Service .

Recombination reactions

Two recombination reactions constitute the basis of the Gateway™ Technology:

BP reaction

Facilitates recombination of an *att*B substrate (*att*B-PCR product or a linearized *att*B expression clone) with an *att*P substrate (donor vector) to create an *att*L-containing entry clone. This reaction is catalyzed by BP Clonase™ II enzyme mix.

LR reaction

Facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) to create an *att*B-containing expression clone. This reaction is catalyzed by LR Clonase™ II enzyme mix.

Characteristics of modified *att* sites

The wild-type λ *att* recombination sites have been modified in the Gateway™ System, thereby ensuring specificity of the recombination reactions to maintain orientation and reading frame. The modified *att* sites have the following characteristics:

Site	Length	Found in…
*att*B	25 bp	Expression vector Expression clone
*att*P	200 bp	Donor vector
*att*L	100 bp	Entry vector Entry clone
*att*R	125 bp	Destination vector

Specificity of modified *att* sites

The modified *att* sites have the following specificity.

- *att*B1 sites react only with *att*P1 sites
- *att*B2 sites react only with *att*P2 sites
- *att*L1 sites react only with *att*R1 sites
- *att*L2 sites react only with *att*R2 sites

Vectors in ProQuest™ system

Each of the vectors supplied in the ProQuest™ Two-Hybrid System is Gateway™-adapted, *i.e.* contains the appropriate *att* sites that allow site specific recombination to facilitate the transfer of heterologous DNA sequences between vectors.

Gateway™ vectors

There are four different types of Gateway™-adapted vectors available from Invitrogen™ to generate your desired entry and expression clones:

Gateway™ Vector	Vector Characteristics	Vectors in ProQuest™Two-Hybrid Systems
Donor Vector	Contains *att*P sites Used to clone *att*B-flanked PCR products and genes of interest to generate entry clones	pDONR™-Express
Entry vector	Contains *att*L sites Used to clone PCR products or restriction fragments that do not contain *att* sites to generate entry clones	--
Destination vector	Contains *att*R sites Recombines with the entry clone in an LR reaction to generate an expression clone Contains elements necessary to express the gene of interest in the appropriate system (*i.e. E. coli*, mammalian, yeast, insect)	pDEST™22 pDEST™32
Expression vector	Contains *att*B sites Used to clone PCR products or restriction fragments that do not contain *att* sites to generate expression clones Contains elements necessary to express the gene of interest in the appropriate system (*i.e. E. coli*, mammalian, yeast, insect)	pEXP™-AD502

Selection of Gateway™ vectors

To enable recombinational cloning and efficient selection of entry or expression clones, most Gateway™ vectors contain two *att* sites flanking a cassette containing:

- The *ccd*B gene (see below) for negative selection (present in donor, destination, and supercoiled entry vectors)
- Chloramphenicol resistance gene (Cm^R) for counterselection (present in donor and destination vectors)

After a BP or LR recombination reaction, this cassette is replaced by the gene of interest to generate the entry clone and expression clone, respectively.

*ccd*B gene

The presence of the *ccd*B gene allows negative selection of the donor and destination (and some entry) vectors in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard & Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g*. OmniMAX™ 2-T1R, DH5α™, TOP10). When recombination occurs (*i.e*. between a destination vector and an entry clone or between a donor vector and an *att*B-PCR product), the *ccd*B gene is replaced by the gene of interest. Cells that take up unreacted vectors carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Propagating Gateway™ vectors

Because of the lethal effects of the CcdB protein, all Gateway™ vectors containing the *ccd*B gene **must** be propagated in an *E. coli* strain that is resistant to CcdB effects. We recommend using the *ccd*B Survival T1^R *E. coli* strain which is resistant to CcdB effects.

One Shot™ *ccd*B Survival T1^R Chemically Competent *E. coli* are available from (Catalog no. C751003) for transformation.

Recipes

Recipe details

SC medium and plates

Synthetic Complete Medium:

Synthetic Complete medium consists of a nitrogen base, a carbon source, and a “dropout” solution containing essential amino acids, nucleic acids, trace elements and vitamins. For selection purposes, certain amino acids are omitted or “dropped out” (e.g., leucine, tryptophan, histidine) from the dropout solution. For liquid medium, the agar is omitted. Alternative recipes that use yeast nitrogen base with ammonium sulfate are available (Sherman).

1. Prepare the following solutions.
   40% glucose
   20 mM uracil
   100 mM histidine-HCl
   100 mM leucine
   40 mM tryptophan

2. Autoclave the 40% glucose and filter sterilize the amino acids. Store the amino acids in the dark or wrapped in foil.

3. Prepare an amino acid powder mix of purine and amino acids by mixing equal weights (for example 2–3 g for each compound) of the following: adenine sulfate, alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

4. The liquid medium and the agar are autoclaved in two separate 2-L flasks. For 2 L of medium, to one flask add:

Component	Amount
Yeast nitrogen base without amino acids	13.4 g
Amino acid powder mix	2.7 g

5. Add a clean stir bar, suspend in 1 L distilled water and adjust the pH to 5.9 with NaOH. Keep the stir bar in the flask to stir the medium after autoclaving.

6. To the second flask add 40 g of agar in 900 mL distilled water. The agar will be solubilized during autoclaving.

7. Autoclave both flasks for 20 minutes on the liquid setting.

8. After autoclaving, pour the contents of the flask containing agar into the flask containing medium. Cool in a 50℃ water bath for about 1 hour. Add 100 mL autoclaved 40% glucose.

9. Depending on the auxotrophies to be tested with the dropout medium, also add the appropriate amino acids (e.g., for SC-Leu, add all except Leucine):
   16 mL of 20 mM uracil
   16 mL of 100 mM histidine-HCl
   16 mL of 100 mM leucine
   16 mL of 40 mM tryptophan

LB (Luria-Bertani) medium and plates

Composition:

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

pH 7.0

1. For 1 liter, dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 950 ml deionized water.

2. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter.

3. Autoclave on liquid cycle for 20 minutes at 15 psi. Allow solution to cool to 55℃ and add antibiotic if needed.

4. Store at room temperature or at +4℃.
   For LB agar plates:

5. Prepare LB medium as above, but add 15 g/L agar before autoclaving.

6. Autoclave on liquid cycle for 20 minutes at 15 psi.

7. After autoclaving, cool to ~55℃, add antibiotic if needed, and pour into 10 cm plates.

8. Let harden, then invert and store at +4℃.

5FOA plates

Make 2xSC-Leu-Trp medium (SC-Leu-Trp medium with two times the final concentration of ingredients as described on the previous page) without agar. Cool to approximately 65℃. Add 5FOA as powder at either 0.05%, 0.1%, or 0.2%. Adjust pH to 4.5, filter sterilize. Combine with 4 % agar cooled to 65℃. Mix and pour plates.

3AT plates

Follow the previous recipe for SC-Leu-Trp-His medium. Cool to approximately 65℃. Add 3AT as powder. Stir a few minutes to dissolve, then pour plates without further adjusting the pH.

Cycloheximide plates

Follow the recipe in Appendix B, “Recipes” for YPAD or SC medium supplemented with the appropriate amino acids. Cool to approximately 65℃. Add cycloheximide (filter-sterilized stock at 10 mg/mL, stored at -20℃) to a final concentration of 10 µg/mL.

10xLiAc

1 M Lithium Acetate

Filter sterilize

10xTE

100 mM Tris-HCl

10 mM EDTA

pH 7.5

Autoclave

1X LiAc/0.5X TE

10 mL 10x LiAc

5 mL 10x TE

85 mL Sterile water

Filter sterilize

1X LiAc/1X TE

10 mL 10x LiAc

10 mL 10x TE

80 mL Sterile water

Filter sterilize

1X LiAc/40% PEG-3350/1X TE

10 mL 10x LiAc

5 mL 10x TE

40 g PEG-3350

Bring volume up to 100 mL with Sterile water

Filter sterilize

Z buffer

16.1 g $Na_2HPO_4 \cdot 7H_2O$ (or 8.52 g anhydrous)

5.5 g $NaH_2PO_4 \cdot H_2O$ (or 4.8 g anhydrous)

0.75 g KCl

0.246 g $MgSO_4 \cdot 7H_2O$ (or 0.12 g anhydrous)

Add sterile water up to 1000 mL

Adjust pH to 7.0

Filter sterilize

Supplementary protocols

Generating a cDNA library using Three-Frame

Three-Frame

In this section we provide instructions how to construct a three-frame library using the CloneMiner™ cDNA Library Construction Kit. For detailed instructions, refer to the manual for the CloneMiner™ cDNA Library Construction Kit, which is available for downloading from **thermofisher.com** or by contacting Technical Service.

Advantages of the Three-Frame libraries

A modified method is used in the construction of three-frame libraries. This provides the following advantages:

- Enriches for ORFs in all three reading frames
- Allows equal representation of all three frames
- Reduces 5'UTR regions that may contain stop codons
- Limits the 3' poly-Adenylation sequences

Three reading frame adapters

The Three-Frame cDNA libraries are constructed using three 5' adapters instead of the single adapter provided in the CloneMiner™ cDNA Library Construction Kit. These adapters differ by one or two nucleotides in length to permit expression of ORFs in all the 3 possible reading frames. This will enrich the cDNA library for in-frame ORFs.

The sequences of the 3 reading frame adapter oligos are as follows:

```
Reading Frame α (RFα):
5'-TCGTCGGGGACAACTTTGTACAAAAAAGTTGG-3'
3'- CCCCTGTTGAAACATGTTTTTTCAACCp-5'
Reading Frame β (RFβ):
5'-TCGTCGGGGACAACTTTGTACAAAAAAGTTGGA-3'
3'- CCCCTGTTGAAACATGTTTTTTCAACCTp-5'
Reading Frame γ (RFγ):
5'-TCGTCGGGGACAACTTTGTACAAAAAAGTTGGAA-3'
3'- CCCCTGTTGAAACATGTTTTTTCAACCTTp-5'
```

Note: Reading frame adapter RFα is the same as the adapter from the CloneMiner™ cDNA Library Construction Kit.

Reduced 5'UTRs

The CloneMiner™ cDNA Synthesis Kit contains a size fractionation step that generates cDNA free of adapters and other low molecular weight DNA. In the construction of the Three-Frame cDNA libraries, the largest cDNAs are also excluded, which serves to reduce 5' UTR regions that may contain stop codons. This is reflected in a smaller average insert size (1-1.5 kb) than the standard CloneMiner™ library construction method.

Reduced Poly-A sequences

The CloneMiner™ cDNA Synthesis Kit has been modified to reduce the length of 3' polyadenylation sequences. Two nucleotides (VN) have been added to the 3' end of the oligo d(T) primer to anchor the 1st strand cDNA synthesis to the start of the Poly-A tail. The sequence of oligo $d(T)_{25}VN$ primer is:

```
5'-Biotin-ACAACTTTGTACAAGAAAGTTGGGTGCGGCCGC (T)25VN -3'
```

Where V=C,G,A; N=C,G,A,T

Preparation of Three-Frame libraries

Three-Frame cDNA Libraries are prepared as follows:

1. mRNA is isolated using two steps. First, total RNA is isolated from tissues using the TRIzol™ Reagent. Second, mRNA is isolated from total RNA using the FastTrack™ MAG mRNA Isolation Kit (Catalog no. K158002).

2. cDNA is synthesized using a modified CloneMiner™ cDNA Library Construction System
   - First-strand cDNA is synthesized using Biotin-*att*B2-Oligo™(dT)-VN primer (see "Reduced Poly-A sequences" on page 83)
   - Second-strand cDNA is synthesized using *E. coli* RNase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase
   - Blunt-end cDNA is created using T4 DNA polymerase
   - cDNA is divided into three portions to be adapted with three different reading frame *att*B1 adapters (α, β and γ). Adapter β and γ contain one and two more base pair respectively at the C-terminal end of the adapter (see Appendix C, "Supplementary protocols").
   - Three frame cDNAs are separately size-selected using column chromatography (see Appendix C, "Supplementary protocols")
   - Size-selected cDNAs are separately cloned into the pDONR™222 vector through a Gateway™ BP recombination reaction
   - The BP recombination mix is transformed into ElectroMAX™ DH10B-$T1^R$ *E. coli* and the number of primary recombinants is determined

3. An equal amount of library DNA from recombinants generated in each reading frame is mixed and transferred into pDEST™22 vector by Gateway™ LR recombination

4. The LR recombination reaction is transformed into ElectroMAX™ DH10B-T1^R competent *E. coli* and number of primary recombinants is determined

5. The cDNA library is amplified once using a semi-solid procedure to minimize representational biases

Library scale yeast transformation (Purchased competent Cells)

Introduction

In this section, you will introduce your bait plasmid and a two-hybrid library into MaV203 using co-transformation (both vectors transformed simultaneously) of purchased MaV203 Competent Cells, Library Scale.

Library scale competent yeast cells

MaV203 Competent Yeast Cells, Library Scale have been developed for use with the ProQuest™ Two Hybrid System to facilitate library-scale transformations.

Each tube contains 550 µL competent yeast cells, enough for at least 2×10^6 colonies.

Transformants per screen

We recommend that you screen $>10^6$ yeast transformants for mammalian cDNA libraries. Therefore, one tube of library-scale competent cells is enough for:

- Two 250-µL aliquots for two independent library screens (over 1×10^6 transformants each)
- Two 250-µL aliquots for a single large library screen (over 2×10^6 transformants)

Note: 50 µL of competent cells is used for control purposes.

Materials needed

These materials are needed for a screen with two aliquots of 250-µL of competent cells:

- MaV203 Competent Cells, Library Scale plus provided transformation reagents (PEG/LiAc Solution; control plasmid pMAB37 DNA)
- Your bait plasmid and your two-hybrid library
- Forty 15-cm SC-Leu-Trp-His+3AT plates (the optimum concentration of 3AT for your bait must be determined prior to doing a library transformation; see "Recommended controls" on page 40)
- Eight 10-cm SC-Leu-Trp plates to determine the total number of transformants (six is enough for a single large library screen)
- Sterile 15 or 50 mL polypropylene tubes
- Sterile 1.5 mL microcentrifuge tubes
- 30℃ incubator
- 30℃ and 42℃ water bath

- DMSO. For best results, use fresh DMSO from an unopened bottle. DMSO that has been stored at -20℃ also works well.
- Autoclaved saline (0.9% NaCl)
- Autoclaved velvets for replica plating/cleaning

Important

- **Do not freeze thaw**. Competent yeast can only be thawed once without dramatic loss in competency.
- The optimum concentration of 3AT for your bait **must** be determined prior to doing a library transformation; see “Recommended controls” on page 40.

Transformation procedure

One tube of library-scale competent cells (550 µL) is enough for 2 library screens (250 µL each) and 1 positive and 1 negative control (25 µL each).

1. Thaw the PEG/LiAc Solution in a beaker containing room temperature water before the assay. Mix the solution well before dispensing.

2. Thaw competent cells by placing in a 30℃ water bath for 90 seconds. Do not allow the cells to remain at 30℃ longer than 90 seconds. Proceed immediately to Step 3 on page 85. Steps 3 on page 85, 4 on page 85, and 5 on page 85 can be done at room temperature.

3. Once the cells are completely thawed, invert the cells several times. Do not vortex the cells. Transfer cell volumes to tubes as indicated below:

	Screen 1	Screen 2	Positive Control	Negative Control
Tube	15 or 50 mL polypropylene	15 or 50 mL polypropylene	1.5 mL microcentrifuge	1.5 mL microcentrifuge
Cells	250 µL	250 µL	25 µL	25 µL

4. To each aliquot of cells, add the following plasmids and libraries.

Screen 1	Screen 2	Positive Control	Negative Control
10 µg bait plasmid	10 µg bait plasmid	4 µL pMAB37 DNA	no DNA
10 µg library DNA	10 µg library DNA	4 µL pMAB12 DNA	no DNA

Note: The total volume of DNA added to the library screen transformations must be ≤100 µL, (i.e. bait plasmid and library DNA concentration should be ≥0.2 µg/µL)

5. Add the indicated volume of PEG/LiAc Solution to each tube. Mix well until all of the components are homogeneous.

	Screen 1	Screen 2	Positive Control	Negative Control
PEG/LiAc	1.5 mL	1.5 mL	180 µL	180 µL
Mixing	Swirl polypropylene tubes		Invert microcentrifuge tubes	

6. Incubate for 30 minutes in a 30℃ water bath. Swirl the tubes occasionally (every 10 minutes) to resuspend the components.

7. Add the indicated amount of DMSO to each tube. Mix well.

	Screen 1	Screen 2	Positive Control	Negative Control
DMSO	88 µL	88 µL	10.8 µL	10.8 µL
Mixing	Swirl polypropylene tubes		Invert microcentrifuge tubes	

8. Heat shock the cells for 20 minutes in a 42℃ water bath. Swirl the tubes occasionally.

9. Centrifuge each tube as indicated. Carefully discard the supernatant.

	Screen 1	Screen 2	Positive Control	Negative Control
Centrifuge	Tabletop centrifuge		Microcentrifuge	
Time	5 minutes		5 seconds	
Speed	1800 rpm (640 × *g*)		1800 rpm (200-400 × *g*)	

10. Suspend each pellet in autoclaved saline (0.9% NaCl) by gently pipetting.

	Screen 1	Screen 2	Positive Control	Negative Control
Saline	8 mL	8 mL	1 mL	1 mL

Note: If you are performing a single large library screen with two 250-µL aliquots you can pool the two library screen transformations.

11. Remove 100 µL from each transformation and dilute 1:100 and 1:1000 in autoclaved saline. Plate 100 µL of each dilution on 10 cm SC-Leu-Trp plates.

12. For **each library screen**: plate 400 µL aliquots onto twenty 15 cm SC-Leu-Trp-His+3AT plates.

Note: Do not plate the controls on the 15-cm SC-Leu-Trp-His+3AT plates!

13. Incubate the plates for 3 days at 30℃.

14. Count the number on the 10-cm SC-Leu-Trp plates, preferably on the plates having 20 to 300 colonies. For calculating transformation efficiency, see below.

15. Replica clean each 15-cm SC-Leu-Trp-His+3AT plate containing the library screens.

Note: If you are unfamiliar with replica cleaning, see Appendix C, “Supplementary protocols”. Replica cleaning is essential to reduce background.

16. Incubate the plates for 2 to 3 more days at 30℃.

Preparing and transforming competent cells (Library Scale)

Introduction

This section describes how to perform a library transformation preparing your own competent cells. We recommend that you screen >10^6 yeast transformants for mammalian cDNA libraries. This protocol is specifically written for library transformations, since other yeast transformation protocols do not yield enough transformants.

Important

If you perform the **Preparing and Transforming Competent Cells (Library Scale)** protocol described in this section, you need to have a SC-Leu plate with MaV203 transformed with bait plasmid **without** prey plasmid or pDEST™22. Transform MaV203 with bait plasmid as described in "Small scale yeast transformation" on page 42, and select on an SC-Leu plate.

Materials needed

Be sure to have the following materials and reagents on hand before starting. Pay close attention to the number and type of plates required as well as the medium.

- Fresh SC-Leu plate of MaV203 transformed with bait plasmid (be sure the plate is less than 4 days old)
- Library DN A (30 µg)
- 50 µg carrier DNA (sheared salmon sperm or yeast tRNA)
- Thirty 15-cm SC-Leu-Trp-His+3AT selection plates to select for HIS+ transformants

Note: The optimum concentration of 3AT for your bait must be determined prior to doing a library transformation; see "Testing bait" on page 51.

- Two 10-cm SC-Leu-Trp plates to determine the number of transformants
- SC-Leu medium
- Sterile water
- 1X TE/1X LiAc
- 40% PEG-3350/1X LiAc/1X TE
- DMSO. For best results, use fresh DMSO from an unopened bottle. DMSO that has been stored at -20℃ also works well.
- Autoclaved saline (0.9% NaCl)
- Autoclaved velvets for replica plating/cleaning
- 50 ml conical centrifuge tubes
- 1.5 ml sterile microcentrifuge tubes
- 30℃ incubator and shaking incubator; 42℃ heat block

Preparing competent cells

1. Inoculate 20 mL of SC-Leu with MaV203 containing your bait plasmid. Grow overnight at 30°C.
2. In the morning, dilute culture into 300 mL SC-Leu to 2×10^6 cells/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains 2×10^7 cells/mL (OD_{600} = ~0.50).
3. Centrifuge 5 minutes at 1000–1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 mL sterile water and transfer to a 50 mL conical tube.
4. Centrifuge 5 minutes. at 1000-1500 × *g*. Decant supernatant and resuspend cells in 1.5 mL 1X TE/1X LiAc.
5. Proceed immediately to transform competent cells.

Transforming competent cells

1. Add 1 µg library DNA and 50 µg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5 mL microcentrifuge tubes. Add 50 µL of the resuspended yeast solution from Step 4 on page 88 to each tube.

   Note: The total volume of library and salmon sperm DNA added should be <20 µL and preferably <10 µL.

2. Add 300 µL of sterile 40% PEG-3350/1X LiAc/1X TE to each tube, and invert to mix thoroughly. Incubate 30 minutes at 30°C.
3. Add DMSO to 10% (~40 µL per tube) and invert to mix. Heat shock 10 minutes in 42°C heating block.
4. Remove 100 µL from one transformation tube and dilute 1:100 and 1:1000 in autoclaved saline. Plate 100 µL of each dilution on 10-cm SC-Leu-Trp plates.
5. Plate each transformation tube on a separate 15-cm selection plate.
6. Incubate the plates for 3 days at 30°C.
7. Count the number on the 10-cm SC-Leu-Trp plates, preferably on the plates having 20 to 300 colonies. For calculating transformation efficiency, see below.
8. Replica clean each 15-cm selection plate containing the library screens.

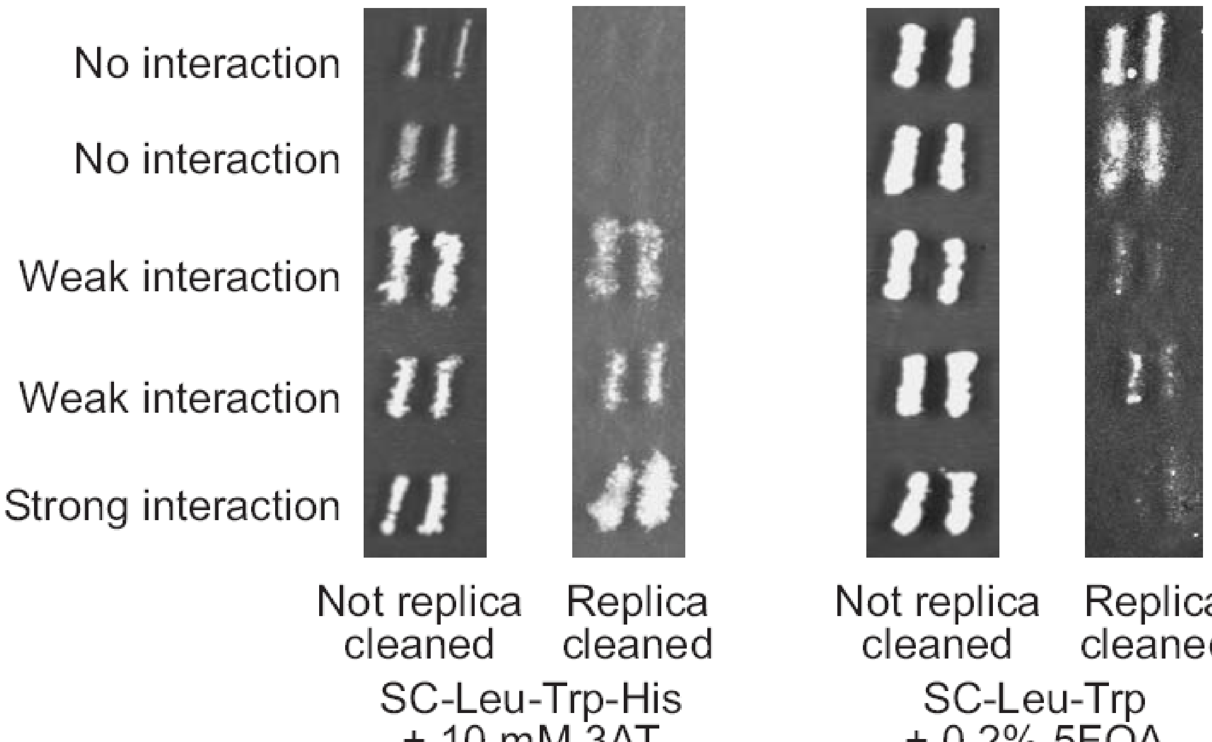
   Note: If you are unfamiliar with replica cleaning, see Appendix C, “Supplementary protocols”. Replica cleaning is essential to reduce background.

9. Incubate the plates for 2 to 3 more days at 30°C.

Replica Plating/Replica cleaning

Introduction

Replica plating is performed by gently pressing a master plate onto an autoclaved velvet to transfer the colonies or patches to a selection plate. Replica cleaning serves to remove excess cell material transferred to the selection plates. The figure below shows plates that have not been replica cleaned and plates that have been replica cleaned properly.



Essential tips

- It is crucial to transfer a minimal number of cells to the selection plates.
- The time of incubation of the master plate can affect the results. This is probably due to yeast cells approaching stationary phase, which can result in changing expression levels of the hybrid proteins.
- During replica plating, do not press the plate onto the replica velvet too hard. This smears the cells and increases surface moisture.
- Replica cleaning requires a fair amount of pressure in order to remove all excess cell material. After replica cleaning, the recipient plate should not contain visible cell material. At most, a shadow of cells should be observed when held to the light. If substantial cell material is present, repeat the replica clean procedure with a fresh velvet. It is often necessary to replica clean a plate 2–3 times to remove all of the cell material. A successfully replica cleaned plate will typically contain a faint imprint of the velvet on the surface of the agar.

- Too much moisture causes smearing of the patches. Be sure plates are dry. Allow the freshly poured agar plates to dry for 3 or 4 days before use.
- Prewarm plates at 30℃ to helps keep surface moisture to a minimum.
- Be sure the replica velvets are clean and very dry. Replica cleaning works poorly with moist velvets.
- If suitable, patch controls on every master plate, and verify the phenotypes on the selection plates to control the efficiency of the different steps.

Procedure for replica plating

After incubation of the master plate (18 h, 30℃), gently press the master plate with a light, consistent pressure onto an autoclaved velvet. Be sure to transfer only a slight haze of cells and avoid cell clumps on the velvet. This will make subsequent replica cleaning easier. The selection plate(s) of interest are then gently pressed onto this "inoculated" velvet to transfer the colonies or patches. A single "inoculated" velvet can be used to inoculate up to five selection plates. Place asymmetric marks on the master plate and selection plates to allow realignment.

Procedure for replica cleaning

Immediately following replica plating or following incubation, replica clean ("dilute") the cells on the selection plate by pressing a new autoclaved velvet onto the surface. A greater amount of pressure is required in this procedure than in replica plating.

Note: Where indicated, the plates should be replica cleaned again after an initial 24-h incubation.

Cleaning velvets

After replica plating or cleaning, remove the cell material from the velvets by light brushing with a bottle brush and immerse them in water. Autoclave them in the water with a 25-min cycle. Air dry the velvets by hanging them. Pack the velvets flat in aluminum foil, approximately 30/pack, and autoclave with a 20-min cycle and a 99-min drying cycle (if available). After several uses, the velvets can be machine washed using cold water. It is very important not to add soap. Machine dry using low heat. Pack the velvets as before and autoclave.

Alternatives to replica Plating/Cleaning

Replica plating/replica cleaning are the fastest, easiest and most reproducible methods for identifying candidate clones; however, alternatives can be used:

- Suspend candidate yeast colonies in sterile saline, and prepare 1:10 dilutions. Spot Samples from each of these dilutions (i.e., 10 µL) onto each of the selection plates (include SC-Leu-Trp), and following incubation, determine the amount of growth for each dilution. By comparing growth on SC-Leu-Trp to the same dilution on each selection plate, the extent to which the reporter gene is induced can be determined. With care, several candidate clones can be examined on each selection plate.
- A similar option is to streak candidate yeast colonies (using a sterile loop or toothpick) on each of the selection plates and, following incubation, determine the extent of growth.

Quantitative β-Galactosidase assays in liquid cultures

Introduction

Quantitative assays for β-galactosidase (β-gal) activity in liquid cultures can be performed using either o-nitrophenyl-β-D-galactopyranoside (ONPG) or Chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. As a substrate, CPRG is more sensitive and faster than ONPG and is therefore particularly useful for clones exhibiting weak or moderate expression levels of β-galactosidase.

Note

- It may be necessary to dilute or concentrate the cells to remain within the linear range of the assay.
- Both ONPG and CPRG will stain. Wear gloves during these procedures.

ONPG assay

For each strain, test 3 to 5 independent isolated colonies. Assay each sample extract in triplicate to reduce variability. The ONPG assay incubation requires 5 minutes to 24 hours. ONPG may not be appropriate for analysis of weak interactions.

1. Inoculate an isolated single colony into 2.5 ml SC-Leu-Trp using an entire yeast colony/tube. Incubate overnight with shaking (230–250 rpm) at 30℃.
2. Inoculate 5 ml YPAD medium with 1 mL culture giving a starting OD_{600} of ~0.5. Incubate at 30℃ with shaking (230–250 rpm) until the OD_{600} = 1.0-1.5.
3. Dissolve ONPG at 4 mg/mL in Z buffer with shaking for 1 to 2 hours.
4. Determine and record the final OD_{600}. Be sure the cells are well suspended (no clumps) by vortexing or by gently pipetting up and down. Accurate readings require $OD_{600} < 1.0$.
5. Place 1.5 mL culture in each of three 1.5-mL microcentrifuge tubes. Centrifuge at 14,000 × *g* for 30 seconds. Carefully remove and discard supernatant (avoid cell loss in all steps as this will affect the final activity calculations).
6. Resuspend each cell pellet in 1.5 mL Z buffer. Centrifuge at 14,000 × *g* for 30 seconds. Carefully remove and discard supernatant.
7. Resuspend each cell pellet in 300 µL Z buffer. Transfer 100 µl of the cell suspension to a fresh graduated microcentrifuge tube. Add autoclaved, acid-washed 0.5-mm glass beads to a final volume of 200 µl. Place the remaining 200 µl on ice for repeat assays if necessary.
8. Vortex 1-2 minutes.
9. Prepare a stock of 700 µL Z buffer + 1.9 µL 2-mercaptoethanol per sample to be assayed.
10. Set up a blank tube with 100 µL Z buffer.
11. Add 700 µl Z buffer + 2-mercaptoethanol to each extract and the blank.

12. Start timer. Immediately add 160 µL of ONPG in Z buffer to the reaction and blank tubes and place tubes in a 30℃ water bath.

13. Monitor color development. After a medium-yellow color develops, stop the reaction by addition of 400 µL 1 M Na_2CO_3 to each reaction and the blank. Record elapsed time. Reaction times vary from 5 minutes to 24 hours.

14. Centrifuge reaction tubes for 5 minutes at 14,000 × *g*.

Note: Cells can also be lysed by three cycles of immersion in liquid nitrogen until the cells are frozen (10 seconds), followed by a brief (90 seconds) incubation in a 37℃ water

15. Carefully transfer supernatants to clean cuvettes, avoiding all cellular debris.

16. Calibrate the spectrophotometer against the blank at OD_{420}.

17. Measure the OD_{420} of each sample. The linear range of this assay at OD_{420} is 0.02–1.0. For accuracy, the OD_{420} is best read between 0.3–0.7.

18. Calculate β-gal units, where 1 unit of β-gal is defined as the amount that hydrolyzes 1 mmol of ONPG to o-nitrophenol and D-galactose per minute:

    β-gal units = 1,000 x OD_{420}/(t x V x OD_{600}); where

    t = elapsed time (in minutes) of incubation

    V = volume of culture used in the assay (ml)

    OD_{420} = absorbance by o-nitrophenol (and light scattering by cell debris)

    OD_{600} = cell density at the start of the assay

CPRG assay

For each strain, test 3 to 5 independent isolated colonies. Assay each sample extract in triplicate to reduce variability. The CPRG assay typically requires 3 minutes to 24 hours. The CPRG assay is the preferred substrate for weak interactors.

1. Prepare Buffer 1, Buffer 2, and 6 mM $ZnCl_2$.
    - Buffer 1: For 100 mL, dissolve 2.38 g HEPES, 0.9 g NaCl, 0.065 g L-aspartate (hemi-Mg salt), 1.0 g BSA, and 50 µL Tween™ 20 in 75 mL distilled water and adjust the pH to 7.25–7.3. Adjust volume to 100 mL. Filter sterilize and store at 4℃ for up to 3 months.
    - Buffer 2: Dissolve 27.1 mg CPRG in 20 mL Buffer 1 (to give 2.23 mM CPRG). Filter sterilize and store in the dark at 4℃ for up to 3 months.

2. Inoculate an isolated single-colony into 2.5 mL SC-Leu-Trp using an entire yeast colony per tube. Grow overnight with shaking (230–250 rpm) at 30℃.

3. Inoculate 5 mL YPAD medium with 1.0 mL overnight culture giving a starting OD_{600} of ~0.5. Incubate at 30℃ with shaking (230-250 rpm) until the OD_{600}= 1.0-1.5.

4. Determine and record the final OD_{600}. Be sure the cells are well suspended (no clumps) by vortexing or by gently pipetting up and down. Accurate readings require OD_{600} <1.0.

5. Place 1.5 mL culture in each of three graduated 1.5-mL microcentrifuge tubes. Centrifuge at 14,000 × *g* for 30 seconds. Carefully remove and discard supernatant (avoid cell loss in all steps as this will effect the final activity calculations).
6. Suspend each cell pellet in 1.0 mL Buffer 1. Centrifuge at 14,000 g for 30 seconds. Carefully remove and discard supernatant.
7. Suspend each cell pellet in a final volume of 100 µL Buffer 1. Add autoclaved, acid-washed 0.5-mm glass beads to a final volume of 200 µL.
8. Vortex 1–2 min.
9. Prepare a buffer blank by combining 100 µL Buffer 1 and 900 µL Buffer 2.
10. Add 900 µL Buffer 2 to each sample. Vortex to mix thoroughly. Start timer/record time.
11. Monitor color development. After a rusty yellow to a red-brown color develops, stop the reaction by addition of 250 µL of 6 mM $ZnCl_2$ to the sample and the buffer blank. Record elapsed time. Reaction times can vary from seconds to 24 hours.
12. Centrifuge samples at 14,000 × *g* for 1 minutes to pellet cell debris.
13. Carefully transfer supernatants to clean cuvettes, avoiding all cellular debris.
14. Calibrate the spectrophotometer against the blank at OD_{574}.
15. Measure the OD_{574} of each sample. The linear range of this assay at OD_{574} is 0.25–1.8.
16. Calculate β-gal units, where 1 unit of β-gal is defined as the amount that hydrolyzes 1 mmol of CPRG to chloramphenicol red and D-galactose per minute:

    β-gal units = $1{,}000 \times OD_{574}/(t \times V \times OD_{600})$; where

    t = elapsed time (in minutes) of incubation

    V = volume of culture used in the assay (ml)

    OD_{574} = absorbance by chloramphenicol red (and light scattering by cell debris)

    OD_{600} = cell density at the start of the assay

Plasmid shuffling

Introduction

The pDEST™32 plasmid contains the CYH2^S gene. Cells harboring this plasmid do not grow when plated on medium containing cycloheximide. When MaV203cells transformed with bait and prey plasmid are plated on SC-Trp+cycloheximide, surviving cells will have spontaneously lost the single-copy bait plasmid and will contain only the prey plasmid; thus the cured cells will not grow when plated on SC-Leu. A transformation assay is then used to reintroduce bait into prey-containing cells. The reporter gene phenotype of the resulting transformants is determined.

Procedure for plasmid shuffling

1. From an SC-Leu-Trp master plate containing the patched cells with candidate interacting fusion proteins, streak colonies onto SC-Trp+cyh (10 µg/mL). Incubate 3-5 days at 30℃.
2. Suspend a good-sized isolated colony from the SC-Trp+cyh plate in ~50 µL autoclaved, distilled water and spread onto the center (~5 cm^2) of a 10-cm YPAD plate using an autoclaved loop or toothpick. Incubate overnight at 30℃. Repeat for each candidate clone.
3. For each clone, scrape the cells from the YPAD plate and completely suspend (by brief vortexing and pipetting up and down) in 5 mL autoclaved, distilled water. Add a sufficient amount of this cell suspension to 100 mL YPAD broth in a 500-ml flask to produce a final OD_{600} of 0.1. Reserve approximately 10 mL YPAD medium to use as a blank in the spectrophotometer.

   Note: Perform serial 1:10 dilutions in water of the 5-mL cell suspension then determine the OD_{600} of each dilution to allow an estimate of the amount of cell suspension required to produce the desired OD of 0.1. Accurate cell densities require that the measured OD < 1.0. Use plastic cuvettes.

4. Follow the sequential transformation procedure described in "Preparing and transforming competent cells (Library Scale)" on page 87. The method can be scaled down ten-fold.
5. For each transformation, combine 50 µL cells, 5 µl freshly boiled herring or salmon sperm carrier DNA and 100 ng of each plasmid DNA in an autoclaved 1.5-mL microcentrifuge tube. Mix gently by pipetting up and down. Add 300 µL PEG/LiAc solution and mix gently.
6. Incubate for 30 minutes in a 30℃ water bath.
7. Heat shock for 15 minutes in a 42℃ water bath.
8. Centrifuge in a microcentrifuge (6,000-8,000 × *g*) for 20–30 seconds at room temperature. Carefully remove the supernatant.
9. Suspend the cell pellet in 500 µL autoclaved, distilled water.
10. Plate 100 µL onto a SC-Leu-Trp selection plate. Incubate for 48–72 hours at 30℃.

11. Patch at least three transformants from each transformation onto an SC-Leu-Trp plate. Also patch yeast controls 2–8. Incubate for 24 hours at 30℃.

12. Replica plate this master plate onto SC-Leu-Trp-His+3AT, SC-Leu-Trp+0.2% 5FOA, SC-Leu-Trp-Ura, and YPAD+nylon or nitrocellulose membrane (for X-gal Assay) and treat as described in "Characterization of transformants" on page 44.

Maps and features of vectors

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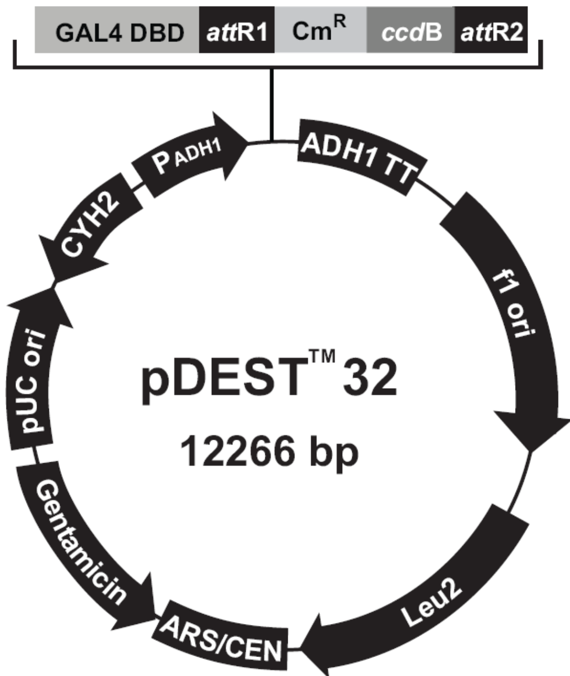
- pEXP™22-RalGDS-wt, m1, m2 103
- Map of pENTR™-gus 105

pDEST™32

Map of pDEST™32

The figure below shows the map of the pDEST™32 vector. The complete sequence of pDEST™32 is available for downloading from (**thermofisher.com**) or by contacting Technical Service.



Comments for pDEST™ 32 12266 nucleotides

ADH1 promoter: bases 103-1557

GAL4 DNA binding domain: bases 1581-2024

*att*R1 site: bases 2037-2161

Chloramphenicol resistance (Cm^R) gene: bases 2411-3070

*ccd*B gene: bases 3411-3716

*att*R2 site: bases 3757-3881

ADH1 transcription termination region: bases 4119-4276 f1 origin: bases 4603-5058

Leu2 gene: bases 5767-6861 ARS4/CEN6 origin: bases 7589-8107

Gentamicin resistance gene: bases 8452-8985 (c) pUC origin: bases 9833-10506

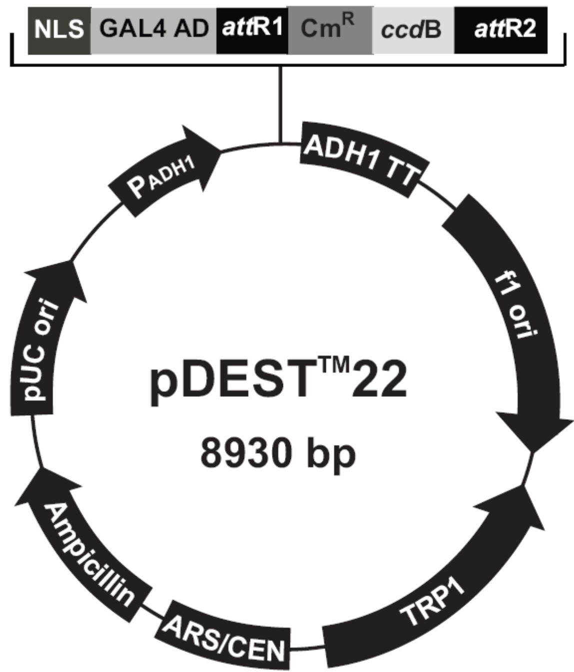
Cycloheximide sensitivity (CYH2): bases 11445-11894 (c)

(c) = complementary strand

pDEST™22

Map of pDEST™22

The figure below shows the map of the pDEST™22 vector. The complete sequence of pDEST™22 is available for downloading from (**thermofisher.com**) or by contacting Technical Service.



Comments for pDEST™22 8930 nucleotides

ADH1 promoter: bases 272-1726

Nuclear localization signal (NLS): bases 1734-1754

GAL4 DNA activation domain: bases 1761-2105

*att*R1 site: bases 2121-2145

Chloramphenicol resistance (Cm^R) gene: bases 2495-3154

*ccd*B gene: bases 3495-3800

*att*R2 site: bases 3841-3965

ADH1 transcription termination region: bases 4203-4360

f1 origin: bases 4687-5142

TRP1 gene: bases 5245-5919 (c)

ARS4/CEN6 origin: bases 6455-6972

Ampicillin (*bla*) resistance gene: bases 7104-7964

pUC origin: bases 8109-8782

(c) = complementary strand

Features of vectors pDEST™32 and pDEST™22

Features of vectors

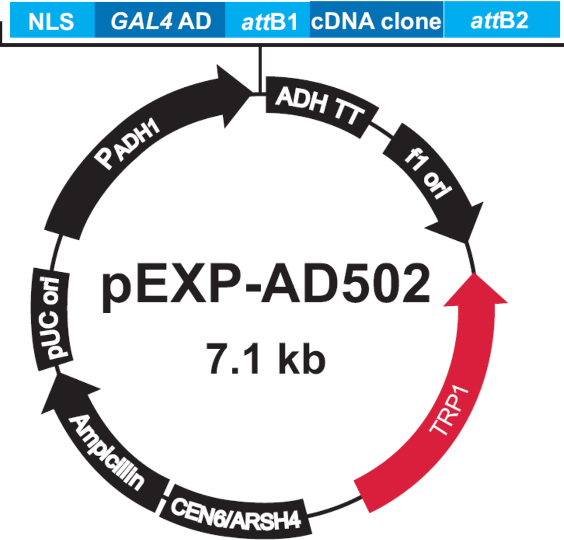
The table below shows the features of the pDEST™32 and pDEST™22 vectors.

Feature	Vector	Benefit
ADH1 promoter	pDEST™32, pDEST™22	Permits medium-level, constitutive expression of the gene of interest
GAL4 DBD coding sequence	pDEST™32	Allows bait protein to bind to GAL4 binding sites in promoters of reporter genes and activate reporters after prey recruitment
GAL4 AD coding sequence	pDEST™22	Allows prey protein to activate reporters after binding to bait protein
*att*R1 and *att*R2 sites	pDEST™32, pDEST™22	Bacteriophage λ-derived recombination sequences that allow recombinational cloning of a gene of interest in the expression construct with a Gateway™ destination vector.
Chloramphenicol resistance gene (Cm^R)	pDEST™32, pDEST™22	Allows counterselection of the plasmid.
*ccd*B gene	pDEST™32, pDEST™22	Permits negative selection of the plasmid.
ADH1 TT	pDEST™32, pDEST™22	Allows transcription termination and polyadenylation of mRNA.
f1 origin	pDEST™32, pDEST™22	Allows rescue of single-stranded DNA
LEU2 gene	pDEST™32	Permits selection for plasmid in transformed yeast cells
TRP1 gene	pDEST™22	Permits selection for plasmid in transformed yeast cells
ARS/CEN	pDEST™32, pDEST™22	Yeast centromere and replication origin: allows replication and maintenance of plasmid in yeast
Gentamicin resistance gene	pDEST™32	Permits selection for the plasmid in *E. coli*
Ampicillin resistance gene	pDEST™22	Permits selection for the plasmid in *E. coli*
pUC origin	pDEST™32, pDEST™22	Permits high-copy replication and maintenance in *E. coli*.
Cycloheximide Sensitivity Gene ($CYH2^S$)	pDEST™32	Lets you counter select against bait plasmid through plasmid shuffling

pEXP™-AD502

Map of pEXP™-AD502

The figure below shows the map of the pEXP™-AD502 vector. The complete sequence of pEXP™-AD502 is available for downloading from (**thermofisher.com**) or by contacting Technical Service.



Comments for pEXP™-AD502 (no nsert) 7146 nucleotides

ADH1 promoter: bases 64-1517

Initiation ATG: bases 1523-1525

SV40 nuclear localization signal (NIS): bases 1526-1548

GAL4 activation domain: bases 1556-1903

*att*B1: bases 1910-1934

*att*B2: bases 1979-2003

ADH1 transcription termination region: bases 2045-2511

f1 origin: bases 2703-3158

TRP1 gene (c): bases 3255-3929

TRP1 promoter (c): bases 3930-4031

CEN6/ARSH4 (c): bases 4461-4979

bla promoter: bases 5012-5116

Ampicillin (*bla*) resistance gene: bases 5111-5971

pUC origin: bases 6116-6789

(c) = complementary strand

MCS of pEXP™-AD502

The figure below shows the multiple cloning site of the pEXP™-AD502 vector. Use the diagram below to help you clone your gene of interest into pDEST™32. Note the following features in the diagram below:

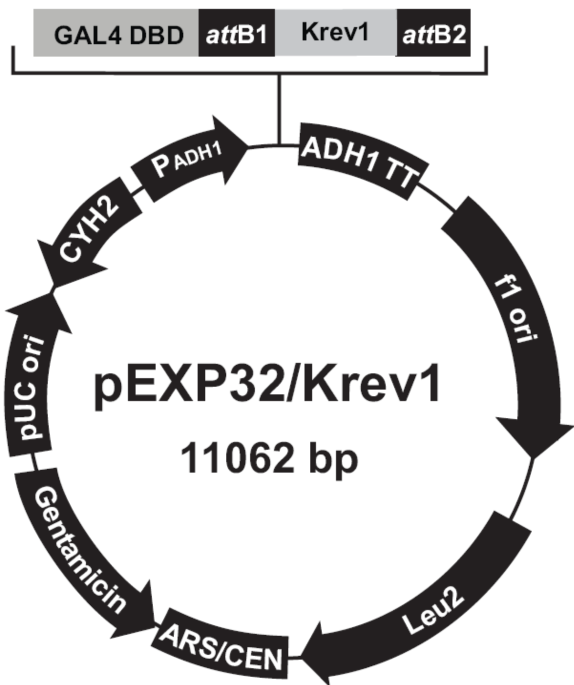
- The reading frame for the GAL4 AD is shown; the insert needs to be in frame with that
- Sequences for suggested forward and reverse sequencing primers are shown below

```
1451  TCCCTTTCTT CCTTGTTTCT TTTTCTGCAC AATATTTCAA GCTATACCAA GCATACAATC AACTCCAAGC

              SV40 nuclear localization signal (NLS)
1521  TT ATG CCC AAG AAG AAG CGG AAG GTC TCG AGC GGC GCC AAT TTT AAT CAA AGT GGG AAT
         Met Pro Lys Lys Lys Arg Lys Val Ser Ser Gly Ala Asn Phe Asn Gln Ser Gly Asn

                                 GAL4 DNA activation domain
1580  ATT GCT GAT AGC TCA TTG TCC TTC ACT TTC ACT AAC AGT AGC AAC GGT CCG AAC CTC
      Ile Ala Asp Ser Ser Leu Ser Phe Thr Phe Thr Asn Ser Ser Asn Gly Pro Asn Leu

1637  ATA ACA ACT CAA ACA AAT TCT CAA GCG CTT TCA CAA CCA ATT GCC TCC TCT AAC GTT
      Ile Thr Thr Gln Thr Asn Ser Gln Ala Leu Ser Gln Pro Ile Ala Ser Ser Asn Val

1694  CAT GAT AAC TTC ATG AAT AAT GAA ATC ACG GCT AGT AAA ATT GAT GAT GGT AAT AAT
      His Asp Asn Phe Met Asn Asn Glu Ile Thr ALa Ser Lys Ile Asp Asp Gly Asn Asn

                                                   Suggested forward sequencing/PCR primer
1751  TCA AAA CCA CTG TCA CCT GGT TGG ACG GAC CAA ACT GCG TAT AAC GCG TTT GGA ATC
      Ser Lys Pro Leu Ser Pro Gly Trp Thr Asp Gln Thr Ala Tyr Asn Ala Phe Gly Ile

1808  ACT ACA GGG ATG TTT AAT ACC ACT ACA ATG GAT GAT GTA TAT AAC TAT CTA TTC GAT
      Thr Thr Gly Met Phe Asn Thr Thr Thr Met Asp Asp Val Tyr Asn Tyr Leu Phe Asp

1865  GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG GGT GGG TCG ATC ACA AGT TTG TAC
      Asp Glu Asp Thr Pro Pro Asn Pro Lys Lys Glu Gly Gly Ser Ile Thr Ser Leu Tyr
                                                                 attB1
                          Sal I      SmaI  Eco RI   BglII   SpeI     Not I
1922  AAA AAA GCA GGC TTG TCG ACC CGG GAA TTC AGA TCT ACT AGT GCGGCCGC ACGCGTACCC
      Lys Lys Ala Gly Leu Ser Thr

                               Sst I                     Sst II
1983  AGCTTTCTTG TACAAAGTGG TGACGTCGAG CTCTAAGTAA GTAACGGCCG CCACCGCGGT GGAGCTTTGG
            attB2

                                                    Suggested reverse sequencing/PCR primer
2053  ACTTCTTCGC CAGAGGTTTG GTCAAGTCTC CAATCAAGGT TGTCGGCTTG TCTACCTTGC CAGAAATTTA

2123  CGAAAAGATG GAAAAGGG
```

pEXP™32-Krev1

Map of pEXP™32-Krev1

The figure below shows the map of the pEXP™32-Krev1 vector. The complete sequence of pEXP™32-Krev1 is available for downloading from (thermofisher.com) or by contacting Technical Service.



Comments for pEXP™32/Krev1 11062 nucleotides

ADH1 promoter: bases 103-1557

GAL4 DNA binding domain: bases 1581-2024

*att*B1 site: bases 2037-2061

Krev1 gene: bases 2082-2630

*att*B2 site: bases 2653-2677

ADH1 transcription termination region: bases 2915-3072

f1 origin: bases 3399-3854

Leu2 gene: bases 4563-5657

ARS4/CEN6 origin: bases 6385-6903

Gentamicin resistance gene: bases 7248-7781 (c)

pUC origin: bases 8629-9302

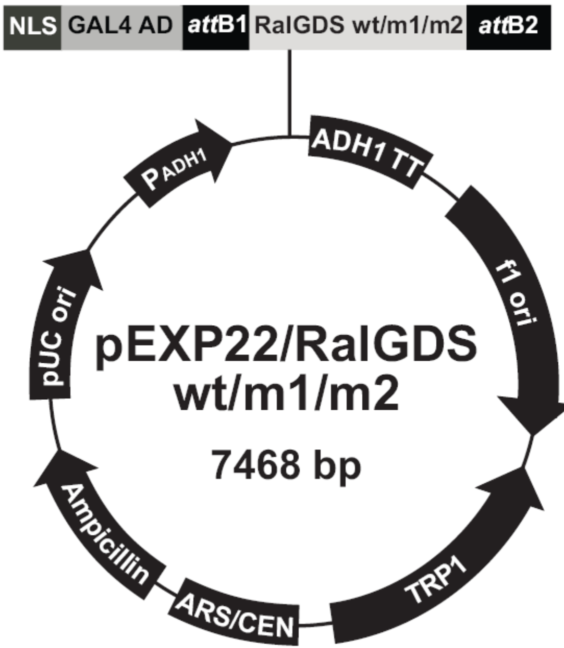
Cycloheximide sensitivity (CYH2): bases 10241-10690 (c)

(c) = complementary strand

pEXP™22-RalGDS-wt, m1, m2

Maps of pEXP™22-RalGDS-wt, m1, m2

The figure below shows the map of the pEXP™22-RalGDS-wt, pEXP™22-RalGDS m1, m2 vector. The complete sequence of pEXP™22/RalGDS-wt, pEXP™22/RalGDS-m1 and pEXP™22/RalGDS-m2 are available for downloading from (**thermofisher.com**) or by contacting Technical Service.



Comments for pEXP™22/RalGDSwt/m1/m2 7468 nucleotides

ADH1 promoter: bases 272-1726

Nuclear localization signal (NLS): bases 1734-1754

GAL4 DNA activation domain: bases 1761-2105

*att*B1 site: bases 2121-2145

RalGDS gene: bases 2166-2462

Note: For RalGDS gene, wt is the wildtype, m1 has I77T, and m2 has L65P

*att*B2 site: bases 2479-2503

ADH1 transcription termination region: bases 2741-2898

f1 origin: bases 3225-3680

TRP1 gene: bases 3783-4457 (c)

ARS4/CEN6 origin: bases 4993-5510

Ampicillin (*bla*) resistance gene: bases 5642-6502

pUC origin: bases 6647-7329

(c) = complementary strand

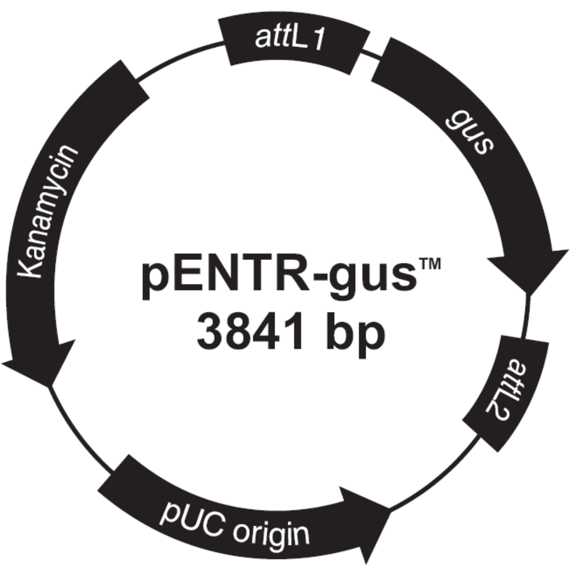
Map of pENTR™-gus

Description

pENTR™-gus is a 3841 bp entry clone containing the *Arabidopsis thaliana* gene for β-glucuronidase (*gus*). The *gus* gene was amplified using PCR primers containing *att*B recombination sites. The amplified PCR product was then used in a BP recombination reaction with pDONR201™ to generate the entry clone. For more information about the BP recombination reaction, refer to the Gateway™ Technology with Clonase™ II manual which is available for downloading from our Web site or by contacting Technical Services.

Map of control vector

The figure below summarizes the features of the pENTR™-gus vector. The complete sequence for pENTR™-gus is available from (**thermofisher.com**) or by contacting Technical Service.



Comments for pENTR™-gus™ 3841 nucleotides

*att*L1: bases 99-198 (complementary strand)

gus gene: bases 228-2039

*att*L2: bases 2041-2140

pUC origin: bases 2200-2873 (C)

Kanamycin resistance gene: bases 2990-3805 (C)

C = complementary strand

Supplemental information

Gateway™ Technology

Under the Gateway™ Technology Open Architecture Policy:

Academic and government researchers may create and freely distribute Gateway™ entry clones (containing attL1 and attL2 sites) and expression clones (containing attB1 and attB2 sites) for research use without licensing fees or royalties.

Any organization may now freely distribute Gateway™ entry clones created by Academic or Government researchers, for research use, without paying licensing fees or royalties, and may distribute Gateway expression clones created by Academic or Government researchers, for research use, for a nominal fee of up to US$10 per clone.

The rights to perform the Gateway™ recombinational cloning reaction, for research purposes, with these distributed clones is conveyed by the purchase of Invitrogen™ Gateway™ Clonase enzyme.

We will not assert a claim against the buyer of infringement based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer provided that no method claim in the corresponding patents were used in the manufacture of such product.

For more information, see **Gateway Open Architecture Policy**.

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! **Potential Biohazard.** Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! **BIOHAZARD.** Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

Documentation and support

Customer and technical support

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- Product documentation
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

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