Yeast Protocols Handbook

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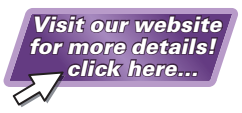

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I. Introduction

The Yeast Protocols Handbook provides background information and general yeast protocols that complement our system-specific User Manuals. The protocols in this Handbook have been optimized with our yeast-based Matchmaker™ Two-Hybrid and One-Hybrid Systems, and Matchmaker Libraries. The Yeast Protocols Handbook is especially useful for researchers who wish to use yeast as a vehicle for their molecular biology experiments, but have little or no prior experience working with yeast. For novice and experienced users alike, the Yeast Protocols Handbook will help you obtain the best possible results with your Matchmaker and other yeast-related products from Clontech.

This Handbook includes:

- detailed information on culturing and handling yeast
- information on the yeast promoters used in the Matchmaker Systems
- two protocols for preparing protein extracts from yeast
- quantitative and qualitative β-galactosidase assays (for use with *lacZ* yeast reporter strains)
- a simple, optimized protocol for isolating plasmids from yeast
- PCR amplification and yeast colony hybridization protocols for the rapid analysis of positive clones obtained in a library screening
- a small-scale, lithium acetate yeast transformation protocol
- additional protocols for working with certain yeast plasmids and host strains

The special application of yeast transformation for one- and two-hybrid library screening is covered in detail in each product-specific User Manual. The special application of yeast mating for library screening is covered in the Pretransformed Matchmaker Libraries User Manual.

About our yeast-based products

The Matchmaker GAL4 Two-Hybrid Systems (Cat No. K1604-1, K1605-1, 630303) and LexA Two-Hybrid System (Cat No. K1609-1) are complete kits for identifying and investigating protein-protein interactions *in vivo* using the yeast two-hybrid assay. The Matchmaker One-Hybrid System (Cat No. K1603-1) provides the basic tools for identifying novel proteins *in vivo* that bind to a target DNA sequence such as a *cis*-acting regulatory element. Matchmaker Two-Hybrid Systems are compatible with our pBridge Three-Hybrid Vector (Cat No. 630404) for the investigation of tertiary protein complexes. The Matchmaker Libraries are constructed in vectors that express inserts as fusions to a transcriptional activation domain, and are thus a convenient resource for researchers wishing to screen a library using the one- or two-hybrid assays. Pretransformed Matchmaker Libraries provide an even greater level of convenience for those wishing to perform a two-hybrid library screening without using large- or library-scale yeast transformations.

Clontech offers an extensive line of kits and reagents that support and complement the Matchmaker Systems and Libraries. The Yeastmaker™ Yeast Transformation Kit (Cat No. 630439) includes all the necessary reagents and protocols for efficient transformation using the lithium acetate method. Also available from Clontech: a selection of GAL4 DNA-binding domain (DNA-BD) and activation domain (AD) hybrid cloning vectors; the pGilda Vector for use with LexA-based two-hybrid systems; monoclonal antibodies and sequencing primers; and yeast media, including Minimal SD Base and many different formulations of Dropout (DO) Supplement. Finally, the pHA-CMV and pMyc-CMV Vector Set (Cat No. 631604) can be used to confirm protein interactions in mammalian cells.

For ordering information on these products, please see Chapter XI of this Handbook or the Clontech Catalog.

II. Introduction to Yeast Promoters

Yeast promoters and other *cis*-acting regulatory elements play a crucial role in yeast-based expression systems and transcriptional assays such as the Matchmaker One- and Two-Hybrid Systems. Differences in the promoter region of reporter gene constructs can significantly affect their ability to respond to the DNA-binding domain of specific transcriptional activators; promoter constructs also affect the level of background (or leakiness) of gene expression and the level of induced expression. Furthermore, differences in cloning vector promoters determine the level of protein expression and, in some cases, confer the ability to be regulated by a nutrient (such as galactose in the case of the *GAL1* promoter).

This chapter provides a brief introduction to several commonly used yeast promoters and *cis*-regulatory elements. For further information on the regulation of gene expression in yeast, we recommend the *Guide to Yeast Genetics and Molecular Biology* by Guthrie & Fink (1991; No. V2010-1); *Molecular Biology and Genetic Engineering of Yeasts*, edited by Heslot & Gaillardin (1992); Stargell & Struhl (1996); and Pringle *et al*. (1997; No. V2365-1).

UAS and TATA regions are basic building blocks of yeast promoters

The initiation of gene transcription in yeast, as in other organisms, is achieved by several molecular mechanisms working in concert. All yeast structural genes (i.e., those transcribed by RNA polymerase II) are preceded by a region containing a loosely conserved sequence (TATA box) that determines the transcription start site and is also a primary determinant of the basal transcription level. Many genes are also associated with *cis*-acting elements—DNA sequences to which transcription factors and other *trans*-acting regulatory proteins bind and affect transcription levels. The term "promoter" usually refers to both the TATA box and the associated *cis*-regulatory elements. This usage is especially common when speaking of yeast gene regulation because the *cis* regulatory elements are relatively closely associated with the TATA box (Yoccum, 1987). This is in contrast to multicellular eukaryotes, where *cis*-regulatory elements (such as enhancers) can be found very far upstream *or* downstream from the promoters they regulate. In this text, "minimal promoter" will refer specifically to the TATA region, exclusive of other *cis*-acting elements.

The minimal promoter (or TATA box) in yeast is typically approximately 25 bp upstream of the transcription start site. Yeast TATA boxes are functionally similar to prokaryotic Pribnow boxes, but are not as tightly conserved. Furthermore, some yeast transcription units are preceded by more than one TATA box. The yeast *HIS3* gene, for example, is preceded by two different TATA boxes: TR, which is regulated, and TC, which is constitutive (Mahadevan & Struhl, 1990). Yeast TATA boxes can be moved to a new location, adjacent to other *cis*-regulatory elements, and still retain their transcriptional function.

One type of *cis*-acting transcription element in yeast is upstream activating sequences (UAS), which are recognized by specific transcriptional activators and enhance transcription from adjacent downstream TATA regions. The enhancing function of yeast UASs is generally independent of orientation; however, it is sensitive to distance effects if moved more than a few hundred base pairs from the TATA region. There may be multiple copies of a UAS upstream of a yeast coding region. In addition, UASs can be eliminated or switched to change the regulation of target genes.

UAS and TATA regions can be switched to create novel promoters

The "mix and match" nature of yeast TATA boxes and UASs has been used to great advantage in yeast two-hybrid systems to create novel promoters for the reporter genes. (For general references on yeast two-hybrid systems, see Chapter X.) In most cases, the *lacZ, HIS3, ADE2 and LEU2* reporter genes are under control of artificial promoter constructs comprised of a TATA and UAS (or operator) sequence derived from another gene (Table I). In some cases, the TATA sequence and the UAS are derived from different genes; indeed, the LexA operator is a *cis*-acting regulatory element derived from *E. coli*.

For GAL4-based systems, either a native *GAL* UAS or a synthetic $UAS_{G\ 17\text{-}mer}$ consensus sequence

II. Introduction to Yeast Promoters *continued*

TABLE I. YEAST PROMOTER CONSTRUCTS USED TO REGULATE REPORTER GENE EXPRESSION IN MATCHMAKER PLASMIDS AND HOST STRAINS

Plasmid or host strain[a]	Reporter gene	Origin of UAS	UAS regulated by	Origin of TATA sequence	Expression level[b] Induced (uninduced)
CG-1945	*lacZ*	$UAS_{G\ 17\text{-}mer\ (x3)}$[c]	GAL4	*CYC1*	low
	HIS3	*GAL1*	GAL4	*GAL1*	high(slightlyleaky)
HF7c	*lacZ*	$UAS_{G\ 17\text{-}mer\ (x3)}$[c]	GAL4	*CYC1*	low
	HIS3	*GAL1*	GAL4	*GAL1*	high (tight)
Y190	*lacZ*	*GAL1*	GAL4	*GAL1*	high
	HIS3	*GAL1*	GAL4	*HIS3* (TC+TR)	high (leaky)
Y187	*lacZ*[d]	*GAL1*	GAL4	*GAL1*	high
SFY526	*lacZ*	*GAL1*	GAL4	*GAL1*	high
PJ69-2A	*HIS3*	*GAL1*	GAL4	*GAL1*	high (tight)
	ADE2	*GAL2*	GAL4	*GAL2*	high (tight)
AH109	*HIS3*	*GAL1*	GAL4	*GAL1*	high (tight)
	ADE2	*GAL2*	GAL4	*GAL2*	high (tight)
	lacZ	*MEL1*	GAL4	*MEL1*	low
EGY48	*LEU2*	LexA $op_{(x6)}$	LexA	*LEU2*	high
p8op-lacZ	*lacZ*	LexA $op_{(x8)}$	LexA	*GAL1*[e]	high
pHISi	*HIS3*	(none)[f]	(n.a.)	*HIS3* (TC+TR)	n.a.[f] (leaky)
pHISi-1	*HIS3*	(none)[f]	(n.a.)	*HIS3* (TC+TR)	n.a.[f] (leaky)
pLacZi	*lacZ*	(none)[f]	(n.a.)	*CYC1*	n.a.[f] (tight)

[a] See Appendices E & F for references.

[b] When induced by a positive two-hybrid interaction; "leaky" and "tight" refer to expression levels in the absence of induction.

[c] Conserved 17-bp palindromic sequence to which the GAL4 protein binds (Guthrie & Fink, 1991).

[d] Y187 probably contains two copies of the *lacZ* gene, judging by the strength of the signal in this strain and in the strains from which it was derived (Durfee *et al.*, 1993; Harper *et al.*, 1993).

[e] This is the minimal TATA region of the *GAL1* promoter; it does not include the *GAL1* UAS and therefore is not responsive to regulation by GAL4 protein.

[f] The Matchmaker One-Hybrid System vectors do not contain a UAS because they are used to experimentally test target elements inserted upstream of the minimal promoter for their ability to bind specific transcriptional activators. In the absence of inserted target elements, reporter gene expression is not induced; however, expression levels may be leaky, depending on the nature of the minimal promoter used in that vector.

***GAL1* UAS**	GAL1-bs1	TAGAAGCCGCCGAGCGG
	GAL1-bs2	GACAGCCCTCCGAAGGA
	GAL1-bs3	GACTCTCCTCCGTGCGT
	GAL1-bs4	CGCACTGCTCCGAACAA
***GAL2* UAS**	GAL2-bs1	CGGAAAGCTTCCTTCCG
	GAL2-bs2	CGGCGGTCTTTCGTCCG
	GAL2-bs3	CGGAGATATCTGCGCCG
	GAL2-bs4	CGGGGCGGATCACTCCG
	GAL2-bs5	CGGATCACTCCGAACCG
***MEL1* UAS**		CGGCCATATGTCTTCCG
UAS $_{G17\text{-}mer}$		CGGAAGACTCTCCTCCG

Figure 1. Sequence of the GAL4 DNA-BD recognition sites in the *GAL1, GAL2,* and *MEL1* UASs and the $UAS_{G\ 17\text{-}mer}$ consensus sequence (Giniger & Ptashne, 1988).

II. Introduction to Yeast Promoters *continued*

(Heslot & Gaillardin, 1992) provides the binding site for the GAL4 DNA-BD. For LexA-based systems, multiple copies of the LexA operator provide the binding site for the LexA protein. If you are putting together your own one- or two-hybrid system, you must make sure that the reporter gene's promoter will be recognized by the DNA-BD moiety encoded in your DNA-BD fusion vector.

Reporter genes under the control of GAL4-responsive elements

In yeast, the genes required for galactose metabolism are controlled by two regulatory proteins, GAL4 and GAL80, as well as by the carbon source in the medium (Guthrie & Fink, 1991; Heslot & Gaillardin, 1992). When galactose is present, the GAL4 protein binds to GAL4-responsive elements within the UAS upstream of several genes involved in galactose metabolism and activates transcription. In the absence of galactose, GAL80 binds to GAL4 and blocks transcriptional activation. Furthermore, in the presence of glucose, transcription of the galactose genes is immediately repressed (Johnston *et al.*, 1994).

The UASs of the 20 known galactose-responsive genes all contain one or more conserved palindromic sequences to which the GAL4 protein binds (Guthrie & Fink, 1991; Giniger *et al.* 1985; reviewed in Heslot & Gaillardin, 1992). The 17-mer consensus sequence, referred to here as $UAS_{G\ 17\text{-}mer}$, functions in an additive fashion, i.e., multiple sites lead to higher transcription levels than a single site (Giniger & Ptashne, 1988). The protein binding sites of the *GAL1, GAL2, MEL1* UASs, and the $UAS_{G\ 17\text{-}mer}$ consensus sequence, are shown in Figure 1.

The tight regulation of the *GAL* UASs by GAL4 makes it a valuable tool for manipulating expression of reporter genes in two-hybrid systems that are dependent on the GAL4 DNA-BD. However, in such systems, the yeast host strains must carry deletions of the *gal4* and *gal80* genes to avoid interference by endogenous GAL4 and GAL80 proteins; thus, no significant glucose repression is observed in these strains and no induction is observed unless a two-hybrid interaction is occurring. Therefore, nutritional regulation of *GAL* UASs is not a feature of GAL4-based two-hybrid systems. However, the host strain used in the LexA system does support galactose induction, as it is wild type for GAL4 and GAL80 functions.

In the GAL4-based MatchmakerTwo-Hybrid Systems, either an intact *GAL1, GAL2* or *MEL1* UAS or an artifically constructed UAS consisting of three copies of the 17-mer consensus binding sequence, is used to confer regulated expression on the reporter genes (Table I). The *HIS3* reporter of AH109, PJ69-2A, HF7c, and CG-1945, and the *lacZ* reporter ofY190,Y187, and SFY526 are all tightly regulated by the intact *GAL1* promoter (including the *GAL1* UAS and *GAL1* minimal promoter). In HF7c and CG1945, *lacZ* expression is under control of $UAS_{G\ 17\text{-}mer\ (x3)}$ and the extremely weak minimal promoter of the yeast cytochrome C1 (*CYC1*) gene. *lacZ* under the control of the intact *GAL1* promoter can be expressed at ~10X the level obtained with the $UAS_{G\ 17\text{-}mer\ (x3)}$/*CYC1* minimal promoter construct under similar induction conditons (Clontech Laboratories; unpublished data). Therefore, some weak or transient two-hybrid interactions may not be detectable in HF7c or CG1945 unless you use a highly sensitive β-galactosidase assay (such a liquid culture assay using a chemiluminescent substrate; Chapter VI.F). The *ADE2* reporter of PJ69-2A and AH109 is tightly regulated by the intact *GAL2* promoter, whose induction properties are similar to those of the *GAL1* promoter. In AH109, *lacZ* is under the control of the *MEL1* UAS and minimal promoter.The *MEL1* promoter is stonger than the $UAS_{G\ 17\text{-}mer\ (x3)}$/*CYC1* minimal promoter, but weaker than the *GAL1* promoter (Aho *et al.*, 1997).

Reporter genes under the control of a minimal *HIS3* promoter

The native yeast *HIS3* promoter contains a UAS site recognized by the transcriptional activator GCN4, and twoTATA boxes. GCN4 regulates one of theTATA boxes (TR), while the otherTATA box (TC) drives low-level constitutive expression of *HIS3* (Iyer & Struhl, 1995). TC is not regulated by the native GCN4-binding UAS, the *GAL1* UAS, or artificial UAS_G constructs (Mahadevan & Struhl, 1990; Hope & Struhl, 1986).

The *HIS3* reporter gene in yeast strainY190 is unusual among the GAL4 two-hybrid reporter gene

II. Introduction to Yeast Promoters *continued*

constructs in that it is under the control of the *GAL1* UAS and a minimal promoter containing both *HIS3* TATA boxes (Flick & Johnston, 1990). The result is high-level expression (due to the *GAL1* UAS) when induced by a positive two-hybrid interaction; this construct also exhibits a significant level of constitutive leaky expression (due to the *HIS3*TC). In contrast, in HF7c, CG-1945, PJ69-2A, and AH109 the entire *HIS3* promoter (including both TATA boxes) was replaced by the entire *GAL1* promoter, leading to tight regulation of the *HIS3* reporter in those strains (Feilotter *et al.*, 1994).

The *HIS3* reporter plasmids pHISi and pHISi-1 used in the Matchmaker One Hybrid System also have both of the *HIS3* TATA boxes present in the minimal promoter. By inserting a *cis*-acting element in the MCS, the regulated TATA box (TR) can be affected, but there is still a significant amount of constitutive, leaky expression due to the *HIS3*TC. The leaky *HIS3* expression of these one-hybrid plasmids is first used to help construct *HIS3* reporter strains, and later is controlled by including 3-aminotriazole in the medium to suppress background growth.

Reporter genes under the control of LexA operators

In LexA-based two-hybrid systems, the DNA-BD is provided by the entire prokaryotic LexA protein, which normally functions as a repressor of *SOS* genes in *E. coli* when it binds to LexA operators, which are an integral part of the promoter (Ebina *et al.*, 1983). When used in the yeast two-hybrid system, the LexA protein does not act as a repressor because the LexA operators are integrated *upstream* of the minimal promoter and coding region of the reporter genes. *LEU2* reporter expression in yeast strain EGY48 is under the control of six copies of the LexA operator (op) sequence and the minimal *LEU2* promoter. In the *lacZ* reporter plasmids, *lacZ* expression is under control of 1–8 copies of the LexA op (Estojak *et al.*, 1995) and the minimal *GAL1* promoter. Because all of the *GAL1* UAS sequences have been removed from the *lacZ* reporter plasmids (West *et al.*, 1984), this promoter is not regulated by glucose or galactose.

Promoters used to drive fusion protein expression in two-hybrid cloning vectors

The *ADH1* promoter (or a truncated version of it) is the promoter used to drive expression of the fusion proteins in most of the Matchmaker cloning vectors. The 1500-bp full-length *ADH1* promoter (Ammerer, 1983; Vainio, GenBank accession number: Z25479) leads to high-level expression of sequences under its control in pGADT7, pGAD GH, pLexA, and pAS2-1 during logarithmic growth of the yeast host cells. Transcription is repressed in late log phase by the ethanol that accumulates in the medium as a by-product of yeast metabolism.

Several Matchmaker cloning vectors contain a truncated 410-bp *ADH1* promoter (Table II). At one point, it was believed that only this portion was necessary for high-level expression (Beier & Young, 1982). In most vector constructs, however, this truncated promoter leads to low or very low levels of fusion protein expression (Ruohonen *et al.*, 1991; Ruohonen *et al.*, 1995; Tornow & Santangelo, 1990). This observation has been confirmed at Clontech by quantitative Western blots (unpublished data). The high-level expression reported by Beier & Young (1982) was apparently due to a segment of DNA derived from pBR322, which was later found to coincidentally enhance transcriptional activity in yeast (Tornow & Santangelo, 1990). In the Matchmaker vector pACT2, strong constitutive fusion protein expression is driven by the 410-bp truncated *ADH1* promoter adjacent to this enhancing pBR322 segment.

The DNA-BD cloning vector pGBKT7 used in Matchmaker Two-Hybrid System 3 contains a 700-bp fragment of the *ADH1* promoter. This trucated promoter leads to high-level expression, but no ethanol repression (Ruohonen *et al.*, 1991; Ruohonen *et al.*, 1995).

The AD cloning vector pB42AD and the alternative DNA-BD vector pGilda used in the Matchmaker LexA Two-Hybrid System utilize the full-length *GAL1* promoter to drive fusion protein expression. Because the LexA system host strain is wild-type for GAL4 and GAL80, fusion protein expression is regulated by glucose and galactose.

II. Introduction to Yeast Promoters *continued*

TABLE II. YEAST PROMOTER CONSTRUCTS IN THE MATCHMAKER CLONING VECTORS

Vectors[a]	Promoter	Regulation/ Relative Protein Expression Level	Signal Strength on Western blot [b]
p LexA, pGAD GH, pAS2-1, pAS2, pGADT7	*ADH1* (full-length)	Ethanol-repressed/High	+++
pACT2, pACT	*ADH1* (410 bp+)[c]	Constitutive/medium	++
pGAD GL	*ADH1* (410 bp)	Constitutive/low	+/– (weak)
pGAD424, pGAD10 pGBT9		Constitutive/ very low	(not detectable)
pGBKT7	*ADH1* (700 bp)	Consitutive/high	+++
pB42AD, pGilda	*GAL1* (full-length)	Repressed by glucose; induced (high-level) by galactose	(not detectable)[d] +++[d]
p8op-lacZ	*GAL1* (minimal)	Not regulated by glucose or galactose	(no data)

[a] See Appendix E for vector references.

[b] Unpublished data obtained at Clontech Laboratories using the appropriate GAL4 domain-specific mAb (Cat No. 630402 or Cat No. 630403). Soluble protein extracts were prepared from CG-1945 transformed with the indicated plasmid. Samples equivalent to ~1 OD_{600} unit of cells were electrophoresed and then blotted to nitrocellulose filters. The blots were probed with either GAL4 DNA-BD mAb (0.5 µg/ml) or GAL4 AD mAb (0.4 µg/ml) using 1 ml of diluted mAb per 10 cm^2 of blot, followed by HRP-conjugated polyclonal Goat Anti-Mouse IgG (Jackson Immunological Research; diluted 1:15,000 in TBST). Signals were detected using a chemiluminescent detection assay and a 2.5-min exposure of x-ray film. Signal intensities were compared to that of known amounts of purified GAL4 DNA-BD (a.a. 1–147) or GAL4 AD (a.a. 768–881).

[c] The truncated *ADH1* promoter in pACT2 is adjacent to a section of pBR322 which acts as a transcriptional enhancer in yeast.

[d] Data obtained using EGY48[p8op-lacZ] transformed with pGilda and grown in the presence of glucose or galactose, respectively (April 1997 *Clontechniques*); no data available for pB42AD.

III. Culturing and Handling Yeast

For additional information on yeast, we recommend Guthrie and Fink (1991) *Guide to Yeast Genetics and Molecular Biology* (Cat No. V2010-1).

A. Yeast Strain Maintenance, Recovery from Frozen Stocks, and Routine Culturing

1. Long-term storage

- Yeast strains can be stored indefinitely in YPD medium with 25% glycerol at –70°C. For storage >1 year, the temperature must be maintained below –55°C.
- Transformed yeast strains are best stored in the appropriate SD dropout medium to keep selective pressure on the plasmid. (See Appendix C.A for recipes and Appendix E for plasmid information.)

To prepare new glycerol stock cultures of yeast:

a. Use a sterile inoculation loop to scrape an isolated colony from the agar plate.

b. Resuspend the cells in 200–500 µl of YPD medium (or the appropriate SD medium) in a 1.5-ml microcentrifuge tube. Vortex tube vigorously to thoroughly disperse the cells. Add sterile 50% glycerol to a final concentration of 25%.

c. Tightly close the cap. Shake the vial before freezing at –70°C.

2. To recover frozen strains and prepare working stock plates:

a. Streak a small portion of the frozen glycerol stock onto a YPD (or appropriate SD) agar plate.

b. Incubate the plate at 30°C until yeast colonies reach ~2 mm in diameter (this takes 3–5 days). Use these colonies as your working stock.

c. Seal plates with Parafilm and store at 4°C for up to two months. Streak a fresh working stock plate from the frozen stock at 1–2-month intervals.

d. If you cannot recover the strain, the cells may have settled ; in this case, thaw the culture on ice, vortex vigorously, and restreak. The glycerol stock tube may be refrozen a few times without damaging the cells.

3. To prepare liquid overnight cultures:

a. Use only fresh (<2-months old) colonies from the working stock plate. Use one large (2–3-mm diameter) colony per 5 ml of medium. If colonies are small, or if you are inoculating a larger volume, use several colonies. **Important:** Vigorously vortex the medium for ~1 min to thoroughly disperse the cells.

Notes:

- Liquid cultures will grow slower than expected if clumps are present in the inoculum; cells in the interior of the clumps do not have access to the nutrients in the medium.
- If you are inoculating a volume greater than 1 ml, it is easier to disperse the clumps if the colonies are first placed in 1 ml of medium in a microcentrifuge tube, vortexed, and then transferred to the desired volume.
- When growing overnight cultures of yeast transformants, use the appropriate SD minimal medium to keep selective pressure on extrachromosomal plasmid(s).
- The growth in YPD of yeast strains carrying the *ade2-101* mutation will be enhanced by adding adenine hemisulfate (0.003% final concentration) to the medium (Appendix C.A). All of the host strains (except EGY48) used in the Matchmaker Systems carry this auxotrophic mutation.
- The growth of transformed PJ69-2A cells in SD/–Trp may also be enhanced by adding excess adenine to the medium (Appendix C.A).

b. Incubate at 30°C for 16–18 hr with shaking at 230–270 rpm. With most strains, this will yield a stationary phase culture (OD_{600} > 1.5).

Note: Different yeast strains grow at different rates. Growth rates may also be affected by the presence of fusion proteins in certain transformants. In addition, the doubling time of most strains growing in SD minimal medium is twice as long as in YPD.

c. If you need a mid-log phase culture, transfer enough of the overnight culture into fresh medium to produce an OD_{600} = 0.2–0.3. Incubate at 30°C for 3–5 hr with shaking (230–250 rpm). This will, with most strains, produce a culture with an OD_{600} ~0.4–0.6.

Note: Generally, YPD or YPDA may be used in this incubation. Because of the shorter incubation time, plasmid loss will not be significant. However, do not use YPD if you want to induce protein expression from the yeast *GAL1* promoter of a LexA system plasmid, e.g., pB42AD or pGilda; YPD contains glucose, which represses transcription from the *GAL1* promoter.

III. Culturing and Handling Yeast *continued*

B. Growth Selection for Transformation Markers and Reporter Gene Expression

Most yeast cloning vectors and control plasmids (including those provided in our Matchmaker Systems) carry at least one nutritional marker to allow for selection of yeast transformants plated on SD minimal medium lacking that specific nutrient. Furthermore, if you are cotransforming yeast with two or more different plasmids bearing different nutritional markers, the plasmids can be independently selected. Thus, the SD selection medium you choose for plating transformants depends generally on the purpose of the selection. Specific factors to consider in choosing the appropriate SD selection medium are:

- the plasmid(s) used and whether you are selecting for one or more plasmids
- whether you are selecting for colonies in which two hybrid proteins are interacting
- whether—and to what extent—the host strain is leaky for reporter gene expression
- whether you want to induce protein expression from the regulated *GAL1* promoter
- whether you intend to perform *in-vivo,* agar-plate β-galactosidase assays (for *lacZ* reporter expression in the LexA Two-Hybrid System).

Please refer to your system-specific User Manual for further information on choosing the appropriate SD selection media for particular plasmids, host strains, and applications.

IV. Preparation of Yeast Protein Extracts

A. General Information

We provide two alternative protocols for the preparation of protein extracts from yeast. The results (i.e., protein yield and quality) will vary depending on the protein and may be more successful with one protocol than with the other. Because it is difficult to predict which procedure will give better results, we provide two protocols for comparison. The cell culture preparation method (Section B) is the same for both protein extraction procedures.

Both extraction procedures address the two most challenging aspects of isolating proteins from yeast: 1) disrupting yeast cell walls; and 2) inhibiting the many endogenous yeast proteases. Yeast cell walls are tough and must be disrupted by a combination of physical and chemical means; methods that utilize glycolytic enzymes are not recommended for this application because they are often contaminated with proteases. Endogenous proteases must be counteracted with a cocktail of strong protease inhibitors (recipe in Appendix D.A). If you know your protein of interest is susceptible to a protease not inhibited by the recommended cocktail, add the appropriate inhibitor before using the mixture. You may also wish to add other inhibitors such as sodium fluoride to prevent dephosphorylation, if that is appropriate for your protein.

B. Preparation of Yeast Cultures for Protein Extraction

Reagents and Materials Required:

- YPD and appropriate SD liquid medium (Recipes in Appendix C.A)
- 20- and 50-ml culture tubes
- Ice-cold H_2O
- Dry ice or liquid nitrogen

1. For each transformed yeast strain you wish to assay in a Western blot, prepare a 5-ml overnight culture in SD selection medium as described in Section III.A, except use a single isolated colony (1–2 mm in diameter, no older than 4 days). Use the SD medium appropriate for your system and plasmids (Appendix E). Also prepare a 10-ml culture of an untransformed yeast colony in YPD or (if possible) appropriate SD medium as a negative control.
2. Vortex the overnight cultures for 0.5–1 min to disperse cell clumps. For each clone to be assayed (and the negative control), separately inoculate 50-ml aliquots of YPD medium with the entire overnight culture.
3. Incubate at 30°C with shaking (220–250 rpm) until the OD_{600} reaches 0.4–0.6. (Depending on the fusion protein, this will take 4–8 hr.) Multiply the OD_{600} (of a 1-ml sample) by the culture volume (i.e., 55 ml) to obtain the total number of OD_{600} units; this number will be used in Sections C & D. (For example, 0.6 x 55 ml = 33 total OD_{600} units.)

   Note: During late log phase the *ADH1* promoter shuts down and the level of endogenous yeast proteases increases.
4. Quickly chill the culture by pouring it into a prechilled 100-ml centrifuge tube halfway filled with ice.
5. Immediately place tube in a prechilled rotor and centrifuge at 1000 x g for 5 min at 4°C.
6. Pour off supernatant and resuspend the cell pellet in 50 ml of ice-cold H_2O. (Any unmelted ice pours off with the supernatant.)
7. Recover the pellet by centrifugation at 1,000 x g for 5 min at 4°C.
8. Immediately freeze the cell pellet by placing the tube on dry ice or in liquid nitrogen. Store cells at –70°C until you are ready to proceed with the experiment.

IV. Preparation of Yeast Protein Extracts *continued*

C. Preparation of Protein Extracts: Urea/SDS Method

(Figure 2; Printen & Sprague, 1994)

Reagents and Materials Required:

- 1.5-ml screw-cap microcentrifuge tubes
- Glass beads (425–600 µm; Sigma Cat No. G-8772)
- Protease inhibitor solution (Appendix D.A)
- PMSF stock solution (Appendix D.A)
- Cracking buffer stock solution (Appendix D.A)
- Cracking buffer, complete (Appendix D.A)

Note: Unless otherwise stated, keep protein samples on ice.

1. Prepare complete cracking buffer (Appendix D.A) and prewarm it to 60°C. Because the PMSF degrades quickly, prepare only the amount of cracking buffer you will need immediately. Use 100 µl of cracking buffer per 7.5 OD_{600} units of cells. (For example, for 33 total OD_{600} units of cells, use 0.44 ml of cracking buffer.)
2. Quickly thaw cell pellets by separately resuspending each one in the prewarmed cracking buffer.
   - If cell pellets are not immediately thawed by the prewarmed cracking buffer, place the tubes briefly at 60°C to hasten melting. To avoid risk of proteolysis, do not leave them longer than 2 min at 60°C.
   - Because the initial excess PMSF in the cracking buffer degrades quickly, add an additional aliquot of the 100X PMSF stock solution to the samples after 15 min and approximately every 7 min thereafter until Step 9, when they are placed on dry ice or are safely stored at –70°C or colder. (Use 1 µl of 100X PMSF per 100 µl of cracking buffer.)
3. Transfer each cell suspension to a 1.5-ml screw-cap microcentrifuge tube containing 80 µl of glass beads per 7.5 OD_{600} units of cells.

   Note: The volume of the glass beads can be measured using a graduated 1.5-ml microcentrifuge tube.
4. Heat samples at 70°C for 10 min.

   Note: This initial incubation at 70°C frees membrane-associated proteins. Thus, if you skip this step, membrane-associated proteins will be removed from the sample at Step 6 (high-speed centrifugation).
5. Vortex vigorously for 1 min.
6. Pellet debris and unbroken cells in a microcentrifuge at 14,000 rpm for 5 min, preferably at 4°C, otherwise at room temperature (20–22°C).
7. Transfer the supernatants to fresh 1.5-ml screw-cap tubes and place on ice (first supernatants).
8. Treat the pellets as follows:
   a. Place tubes in a 100°C (boiling) water bath for 3–5 min.
   b. Vortex vigorously for 1 min.
   c. Pellet debris and unbroken cells in a microcentrifuge at 14,000 rpm for 5 min, preferably at 4°C, otherwise at room temperature.
   d. Combine each supernatant (second supernatant) with the corresponding first supernatant (from Step 7).

   Note: If no supernatant is obtained, add more cracking buffer (50–100 µl) and repeat Steps 8.b & c.
9. Boil the samples briefly. Immediately load them on a gel. Alternatively, samples may be stored on dry ice or in a –70°C freezer until you are ready to run them on a gel.

IV. Preparation of Yeast Protein Extracts *continued*

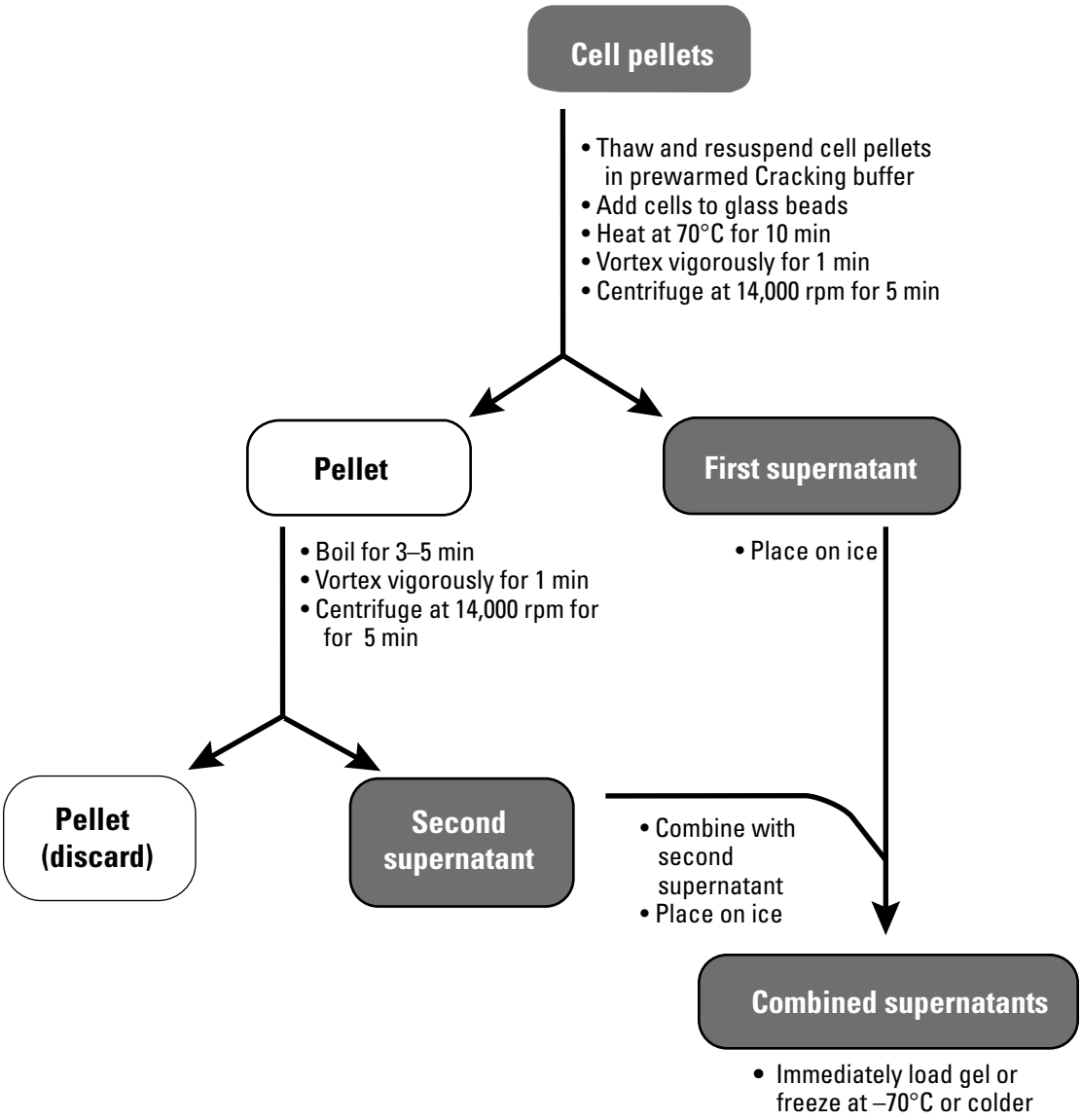

Figure 2. Urea/SDS protein extraction method.

IV. Preparation of Yeast Protein Extracts *continued*

D. Preparation of Protein Extracts: TCA Method

(Figure 3; Horecka, J., personal communication)

Reagents and Materials Required:

- 1.5-ml screw-cap microcentrifuge tubes
- Glass beads (425–600 µm; Sigma Cat No. G-8772)
- Protease inhibitor solution (Appendix D.A)
- PMSF Stock solution (Appendix D.A; *Add as necessary throughout the protocol.)*
- [Recommended] Bead Beater (BioSpec, Bartlesville, OK)

  Note: If you do not have access to a Bead Beater, a high-speed vortexer can be used instead. However, vortexing is not as effective as bead-beating at disrupting the cells.
- TCA buffer (Appendix D.A)
- Ice-cold 20% w/v TCA in H_2O (see Sambrook *et al.* [1989] for tips on preparing TCA solutions)
- TCA-Laemmli loading buffer (Appendix D.A)

Note: Unless otherwise stated, keep protein samples on ice.

1. Thaw cell pellets on ice (10–20 min).
2. Resuspend each cell pellet in 100 µl of ice-cold TCA buffer per 7.5 OD_{600} units of cells. (For example, for 33 total OD_{600} units of cells, use 0.44 ml of TCA buffer.) Place tubes on ice.
3. Transfer each cell suspension to a 1.5-ml screw-cap microcentrifuge tube containing glass beads and ice-cold 20% TCA. Use 100 µl of glass beads and 100 µl of ice-cold 20% TCA per 7.5 OD_{600} units of cells.

   Note: The volume of the glass beads can be measured using a graduated 1.5-ml microcentrifuge tube.
4. To disrupt cells, place tubes in a Bead-Beater and set speed at highest setting. Bead-beat the cells for 2 X 30 sec, placing tubes on ice for 30 sec in between the two bead-beatings. Place tubes on ice.

   Note: If you do not have access to a Bead-Beater, you can vortex the tubes vigorously at 4°C for 10 min; alternatively, you can vortex at room temperature for shorter periods (of 1 min each) at least 4 times, placing tubes on ice for 30 sec in between each vortexing. Place tubes on ice.
5. Transfer the supernatant above the settled glass beads to fresh 1.5-ml screw-cap tubes and place tubes on ice. This is the first cell extract.

   Note: The glass beads settle quickly, so there is no need to centrifuge tubes at this point.
6. Wash the glass beads as follows:
   a. Add 500 µl of an ice-cold, 1:1 mixture of 20% TCA and TCA buffer.
   b. Place tubes in Beat Beater and beat for another 30 sec at the highest setting. (Alternatively, vortex for 5 min at 4°C, or vortex 2 X 1 min at room temperature, placing the tube on ice for 30 sec in between the two vortexings.)
   c. Transfer the liquid above the glass beads (second cell extract) to the corresponding first cell extract from Step 5.
7. Allow any carryover glass beads to settle in the combined cell extracts ~1 min, then transfer the liquid above the glass beads to a fresh, prechilled 1.5-ml screw-cap tube.
8. Pellet the proteins in a microcentrifuge at 14,000 rpm for 10 min at 4°C.
9. Carefully remove supernatant and discard.
10. Quickly spin tubes to bring down remaining liquid. Remove and discard liquid using a pipette tip.
11. Resuspend each pellet in TCA-Laemmli loading buffer. Use 10 µl of loading buffer per OD_{600} unit of cells.

    Note: If too much acid remains in the sample, the bromophenol blue in the buffer will turn yellow. Generally, this will not affect the results of the electrophoresis.
12. Place tubes in a 100°C (boiling) water bath for 10 min.
13. Centrifuge samples at 14,000 rpm for 10 min at room temperature (20–22°C).
14. Transfer supernatant to fresh 1.5-ml screw-cap tube.
15. Load the samples immediately on a gel. Alternatively, samples may be stored on dry ice or in a –70°C freezer until you are ready to run them on a gel.

IV. Preparation of Yeast Protein Extracts *continued*

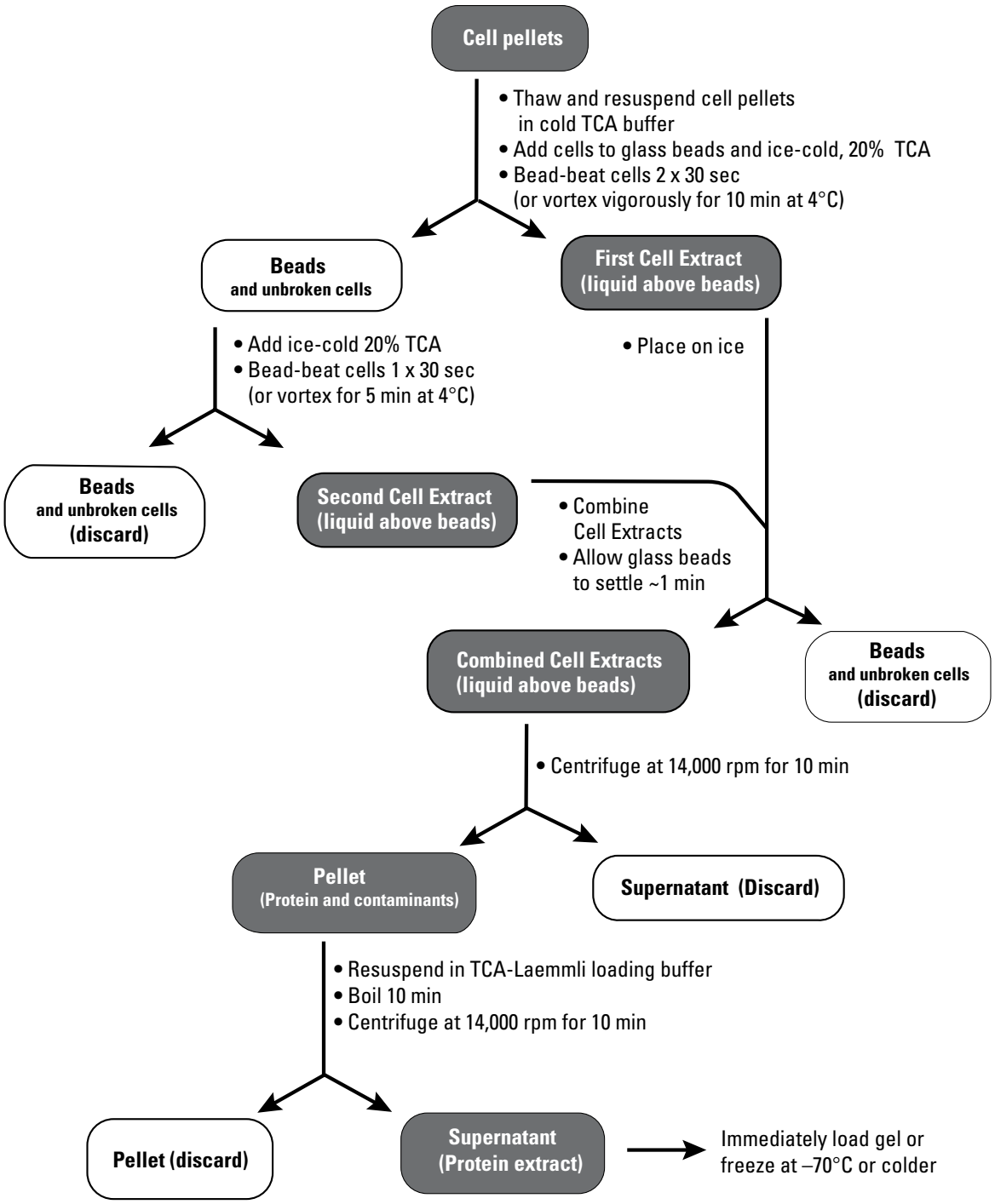

Figure 3. TCA protein extraction method.

IV. Preparation of Yeast Protein Extracts *continued*

E. Troubleshooting

Optimal electrophoretic separation of proteins depends largely on the quality of the equipment and reagents used in the gel system, the manner in which the protein samples are prepared prior to electrophoresis, the amount of protein loaded on the gel, and the voltage conditions used during electrophoresis. These same considerations are important for the subsequent transfer of proteins to the nitrocellulose membrane where transfer buffer composition, temperature, duration of transfer, and the assembly of the blotting apparatus can all have profound effects on the quality of the resultant protein blot. The following troubleshooting tips pertain to the isolation of protein from yeast. Information on running polyacrylamide protein gels and performing Western blots is available in published laboratory manuals (e.g., Sambrook *et al.*, 1989, or Ausubel *et al.*, 1987–96).

1. Few or no immunostained protein bands on the blot
   - The transfer of protein bands to the blot may be confirmed by staining the blot with Ponceau S.
   - The presence of protein bands in the gel (before transfer) may be confirmed by staining a parallel lane of the gel with Coomassie blue. (Note that once a gel has been stained with Coomassie blue, the protein bands will not transfer to a blot.)
   - The extent of cell wall disruption can be determined by examining a sample of treated cells under the microscope. Incomplete cell lysis will lower the protein yield.
2. Several bands appear on the blot where a single protein species is expected
   - Protein degradation and/or proteolysis may have occurred during sample preparation. Additional protease inhibitors may be used as desired. Also, make sure that in Steps C.8.a and D.12 (boiling the protein extracts), the samples are placed into a water bath that is **already boiling**. If samples are placed in the water before it has reached boiling temperature, a major yeast protease (Proteinase B) will be activated. (Proteinase B is a serine protease of the subtilisin family.)
   - Dephosphorylation of a normally phosphoryated fusion protein may have occurred during sample preparation. Sodium fluoride (NaF) may be added to the protease inhibitor stock solution to help prevent dephosphorylation (Sadowski *et al.*, 1991).
3. If you are running a reducing gel, make sure that the protein sample has been completely reduced with either dithiothreitol or 2-mercaptoethanol prior to loading the gel.

V. Yeast Transformation Procedures

A. General Information

LiAc-mediated yeast transformation

There are several methods commonly used to introduce DNA into yeast, including the spheroplast method, electroporation, and the lithium acetate (LiAc)-mediated method (reviewed in Guthrie & Fink, 1991). At Clontech, we have found the LiAc method (Ito *et al.*, 1983), as modified by Schiestl & Gietz (1989), Hill *et al.* (1991), and Gietz *et al.* (1992), to be simple and highly reproducible. This chapter provides detailed protocols for using the LiAc procedure in a standard plasmid transformation and in a modified transformation to integrate linear DNA into the yeast genome.

In the LiAc transformation method, yeast competent cells are prepared and suspended in a LiAc solution with the plasmid DNA to be transformed, along with excess carrier DNA. Polyethylene glycol (PEG) with the appropriate amount of LiAc is then added and the mixture of DNA and yeast is incubated at 30°C. After the incubations, DMSO is added and the cells are heat shocked, which allows the DNA to enter the cells. The cells are then plated on the appropriate medium to select for transformants containing the introduced plasmid(s). Because, in yeast, this selection is usually nutritional, an appropriate synthetic dropout (SD) medium is used.

Simultaneous vs. sequential transformations

The LiAc method for preparing yeast competent cells typically results in transformation efficiencies of 10^5 per µg of DNA when using a single type of plasmid. When the yeast is simultaneously cotransformed with two plasmids having different selection markers, the efficiency is usually an order of magnitude lower due to the lower probability that a particular yeast cell will take up both plasmids. (Yeast, unlike bacteria, can support the propagation of more than one plasmid having the same replication origin, i.e., there is no plasmid incompatibility issue in yeast.) Thus, in a cotransformation experiment, the efficiency of transforming each type of plasmid should remain at ~10^5 per µg of DNA, as determined by the number of colonies growing on SD medium that selects for only one of the plasmids. The *co*transformation efficiency is determined by the number of colonies growing on SD medium that selects for *both* plasmids and should be ~10^4 cfu/µg DNA.

Simultaneous cotransformation is generally preferred because it is simpler than sequential transformation—and because of the risk that expression of proteins encoded by the first plasmid may be toxic to the cells. If the expressed protein is toxic, clones arising from spontaneous deletions in the first plasmid will have a growth advantage and will accumulate at the expense of clones containing intact plasmids. However, if there is no selective disadvantage to cells expressing the first cloned protein, sequential transformation may be preferred because it uses significantly less plasmid DNA than simultaneous cotransformation. In some cases, such as when one of the two plasmids is the same for several different cotransformations, sequential transformations may be more convenient.

Scaling up or down

The small-scale yeast transformation procedure described here can be used for up to 15 parallel transformations, and uses 0.1 µg of each type of plasmid. Depending on the application, the basic yeast transformation method can be scaled up without a decrease in transformation efficiency. If you plan to perform a two-hybrid library screening, you will need a large or library-scale transformation procedure, which will require significantly more plasmid DNA. Please refer to your Matchmaker system-specific User Manual for further information on library screening strategies and specific protocols.

Integration vs. nonintegration of yeast plasmids

For most yeast transformations performed while using the Matchmaker Systems, it is not necessary or desirable to have the plasmid integrate into the yeast genome. (In fact, yeast plasmids do not efficiently integrate if they carry a yeast origin of replication and are used uncut.) However, there are two exceptions to this general rule, as explained in the respective system-specific User Manuals: (a) In the Matchmaker One-Hybrid System, the researcher must

V. Yeast Transformation Procedures *continued*

construct their own custom reporter plasmid and then integrate it into the yeast host strain before performing the one-hybrid assay. (b) In the Matchmaker LexA Two-Hybrid System, the p8op-lacZ reporter plasmid can be used either as an autonomously replicating plasmid or as an integrated plasmid, depending on the desired level of reporter gene expression. The primary reason for integrating a plasmid in some Matchmaker applications is to generate a stable yeast reporter strain in which only one copy of the reporter gene is present per cell, and thereby control the level of background expression. If you have an application that requires integration of a plasmid into the yeast genome, please see Section V.D.

Transformation controls

When setting up any type of transformation experiment, be sure to include proper controls for transformation efficiencies. In the case of simultaneous cotransformation, it is important to determine the transformation efficiencies of both plasmids *together*, as well as of *each type* of plasmid independently. That way, if the cotransformation efficiency is low, you may be able to determine whether one of the plasmid types was responsible (see Troubleshooting Guide, Section F). Therefore, be sure to plate an aliquot of the transformation mixture on the appropriate SD media that will select for only one type of plasmid. Example calculations are shown in Section V.E. When screening a library or performing a one- or two-hybrid assay, you will need additional controls, as explained your system-specific User Manual.

B. Reagents and Materials Required

Note: The Yeastmaker Yeast Transformation System (Cat No. 630439) contains all the solutions (except media, H_2O, and DMSO) required for yeast transformation. Yeastmaker reagents have been optimized for use in the Matchmaker One- and Two-Hybrid Systems.

- YPD or the appropriate SD liquid medium
- Sterile 1X TE/1X LiAc (Prepare immediately prior to use from 10X stocks; stock recipes in Appendix D.B)
- Sterile 1.5-ml microcentrifuge tubes for the transformation
- Appropriate SD agar plates (100-mm diameter)

  Notes:

  - Prepare the selection media and pour the required number of agar plates in advance. (See your system-specific User Manual or Appendix E for media recommendations.) Be sure to plan for enough plates for the control transformations and platings.
  - Allow SD agar plates to dry (unsleeved) at room temperature for 2–3 days or at 30°C for 3 hr prior to plating any transformation mixtures. Excess moisture on the agar surface can lead to inaccurate results due to uneven spreading of cells or localized variations in additive concentrations.
- Appropriate plasmid DNA in solution (check amounts required)
- Appropriate yeast reporter strain for making competent cells (check volume of competent cells required; Steps 1–11 of Section V.E will give you 1.5 ml, enough for 14–15 small-scale transformations)
- Herring testes carrier DNA (Appendix D.B)
- Sterile PEG/LiAc solution (Prepare only the volume needed, immediately prior to use, from 10X stocks; Appendix D.B)
- 100% DMSO (Dimethyl sulfoxide; Sigma Cat No. D-8779)
- Sterile 1X TE buffer (Prepare from 10X TE buffer; Appendix D.B)
- Sterile glass rod, bent Pasteur pipette, or 5-mm glass beads for spreading cells on plates.

V. Yeast Transformation Procedures *continued*

C. Tips for a Successful Transformation

- Fresh (one- to three-week-old) colonies will give best results for liquid culture inoculation. A single colony may be used for the inoculum if it is 2–3 mm in diameter. Scrape the entire colony into the medium. If colonies on the stock plate are smaller than 2 mm, scrape several colonies into the medium. See Chapter III.A for further information on starting liquid cultures from colonies and from a liquid culture inoculum.
- Vigorously vortex liquid cultures to disperse the clumps before using them in the next step.
- The health and growth phase of the cells at the time they are harvested for making competent cells is critical for the success of the transformation. The expansion culture (Step E.6) should be in log-phase growth (i.e., OD_{600} between 0.4 and 0.6) at the time the cells are harvested. If they are not, see the Troubleshooting guide (Section V.F).
- When collecting cells by centrifugation, a swinging bucket rotor results in better recovery of the cell pellet.
- **For the highest transformation efficiency (as is necessary for library screening), use competent cells within 1 hr of their preparation.** If necessary, competent cells can be stored (after Step E.11) at room temperature for several hours with a minor reduction in competency.
- To obtain an even growth of colonies on the plates, continue to spread the transformation mixtures over the agar surface until all liquid has been absorbed. Alternatively, use 5-mm sterile glass beads (5–7 beads per 100-mm plate) to promote even spreading of the cells.

D. Integrating Plasmids into the Yeast Genome

Important: Please read Section V.A for guidelines on when it is appropriate to use this procedure.

To promote integration of yeast plasmids, follow the small-scale LiAc transformation procedure (Section V.E below) with the following exceptions:

- Before transformation, linearize 1–4 µg of the reporter vector by digesting it with an appropriate restriction enzyme in a total volume of 40 µl at 37°C for 2 hr. Electrophorese a 2-µl sample of the digest on a 1% agarose gel to confirm that the plasmid has been efficiently linearized.

  Notes:
  - If the vector contains a yeast origin of replication (i.e., 2 µ ori), it will be necessary to remove it before you attempt to integrate the vector.
  - The vector should be linearized within the gene encoding the transformation (i.e., nutritional selection) marker. However, if the digestion site is within a region that is deleted in the host strain, the plasmid will not be able to integrate. Please refer to your product-specific User Manual for recommended linearization sites.
- At Step 12, add 1–4 µg of the linearized reporter plasmid + 100 µg of carrier DNA; for each reporter plasmid, also set up a control transformation with undigested plasmid (+ 100 µg carrier DNA).
- At Step 20, resuspend cells in 150 µl of TE buffer.
- Plate the entire transformation mixture on one plate of the appropriate SD medium to select for colonies with an integrated reporter gene.

E. Small-scale LiAc Yeast Transformation Procedure

1. Inoculate 1 ml of YPD or SD with several colonies, 2–3 mm in diameter.

   Note: For host strains previously transformed with another autonomously replicating plasmid, use the appropriate SD selection medium to maintain the plasmid (Appendix E).
2. Vortex vigorously for 5 min to disperse any clumps.
3. Transfer this into a flask containing 50 ml of YPD or the appropriate SD medium.
4. Incubate at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD_{600}>1.5).
5. Transfer 30 ml of overnight culture to a flask containing 300 ml of YPD. Check the OD_{600} of the diluted culture and, if necessary, add more of the overnight culture to bring the OD_{600} up to 0.2–0.3.

V. Yeast Transformation Procedures *continued*

6. Incubate at 30°C for 3 hr with shaking (230 rpm). At this point, the OD_{600} should be 0.4–0.6.
   Note: If the OD_{600} is <0.4, something is wrong with the culture (see Troubleshooting Section F.6).
7. Place cells in 50-ml tubes and centrifuge at 1,000 x g for 5 min at room temperature (20–21°C).
8. Discard the supernatants and thoroughly resuspend the cell pellets in sterile TE or distilled H_2O. Pool the cells into one tube (final volume 25–50 ml).
9. Centrifuge at 1,000 x g for 5 min at room temperature.
10. Decant the supernatant.
11. Resuspend the cell pellet in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc.
12. Add 0.1 µg of plasmid DNA and 0.1 mg of herring testes carrier DNA to a fresh 1.5-ml tube and mix.
    Notes:
    - For simultaneous cotransformation (using two different plasmids), use 0.1 µg of each plasmid (an approximately equal molar ratio), in addition to the 0.1 mg of carrier DNA.
    - For transformations to integrate a reporter vector, use at least 1 µg of linearized plasmid DNA in addition to the carrier DNA.
13. Add 0.1 ml of yeast competent cells to each tube and mix well by vortexing.
14. Add 0.6 ml of sterile PEG/LiAc solution to each tube and vortex at high speed for 10 sec to mix.
15. Incubate at 30°C for 30 min with shaking at 200 rpm.
16. Add 70 µl of DMSO. Mix well by gentle inversion. **Do not vortex**.
17. Heat shock for 15 min in a 42°C water bath.
18. Chill cells on ice for 1–2 min.
19. Centrifuge cells for 5 sec at 14,000 rpm at room temperature. Remove the supernatant.
20. Resuspend cells in 0.5 ml of sterile 1X TE buffer.
21. Plate 100 µl on each SD agar plate that will select for the desired transformants. To ensure that you will obtain a plate with well-separated colonies, also spread 100 µl of a 1:1000, 1:100, and 1:10 dilution on 100-mm SD agar plates. These will also provide controls for (co)transformation efficiency.
    Note: If you are performing a cotransformation, plate controls to check transformation efficiency and markers of each plasmid. On separate 100-mm plates, spread 1 µl (diluted in 100 µl H_2O) on medium that will select for a single type of plasmid.
22. Incubate plates, up-side-down, at 30°C until colonies appear (generally, 2–4 days).
23. To calculate the cotransformation efficiency, count the colonies (cfu) growing on the dilution plate from Step 22 above that has 30–300 cfu.

$$\frac{\text{cfu} \times \text{total suspension vol. (µl)}}{\text{Vol. plated (µl)} \times \text{dilution factor} \times \text{amt. DNA used (µg)}^*} = \text{cfu/µg DNA}$$

    * In a cotransformation, this is the amount of one of the plasmid types, not the sum of them. If you have used unequal amounts of two plasmids, use the amount of the lesser of the two.

    Sample calculation:
    - 100 colonies grew on the 1:100 dilution plate (dilution factor = 0.01)
    - plating volume: 100 µl
    - resuspension volume = 0.5 ml
    - amount of limiting plasmid = 0.1 µg

$$\frac{100 \text{ cfu} \times 0.5 \text{ ml} \times 10^3 \text{ µl/ml}}{100 \text{ µl} \times 0.01 \times 0.1 \text{ µg}} = 5 \times 10^5 \text{ cfu/µg DNA}$$

24. Pick the largest colonies and restreak them on the same selection medium for master plates. Seal plates with Parafilm and store at 4°C for 3–4 weeks.

V. Yeast Transformation Procedures *continued*

F. Troubleshooting Yeast Transformation

The overall transformation efficiency should be at least 10^4 cfu/µg for transformation with a single type of plasmid, and 10^3 cfu/µg for simultaneous cotransformation with two types of plasmids. If your cotransformation efficiency is lower than expected, calculate the transformation efficiency of the single plasmids from the number of transformants growing on the appropriate control plates. If the two types of plasmids separately gave transformation efficiencies >10^5 cfu/µg, switch to sequential transformation.

If the transformation efficiency for one or both of the separate plasmids is <10^5 cfu/µg, several causes are possible.

1. Suboptimal plasmid preparation

- Repeat the transformation using more (up to 0.5 µg) of the plasmid DNA that had the low transformation efficiency.
- Check the purity of the DNA and, if necessary, repurify it by ethanol precipitation before using it again.

2. Suboptimal carrier DNA

- If you are not already doing so, use Yeastmaker Carrier DNA, which is available separately (Cat No. 630440) or as part of the Yeastmaker Yeast Transformation System (Cat No. 630439), and has been optimized for high transformation efficiencies in this system.
- If transformation efficiencies are declining in successive experiments, the carrier DNA may be renaturing. Reboil the carrier DNA for 20 min, and then chill it quickly in an ice-water bath.

3. Suboptimal yeast competent cells

- Make sure that the expansion culture (Step E.6) was in log-phase growth at the time the cells were harvested for making competent cells. If the overnight culture (Step E.4) or expansion culture (Step E.6) grew slower than expected (or not at all), start over at Step E.1 by preparing a fresh overnight culture. Failure to thoroughly disperse the colony used for the inoculum will result in slow growth; see Section III.A.3. If you still have problems obtaining a healthy liquid culture, streak a fresh working stock plate (from the frozen glycerol stock) and inoculate with a fresh colony.
- Check the liquid medium to make sure it was made correctly. If you suspect that the medium or carbon source stock solutions have been over-autoclaved, remake fresh solutions and either filter sterilize them or adjust the autoclave settings appropriately before autoclaving.
- The addition of adenine hemisulfate to YPD (in Steps E.3 and E.5) will enhance the growth of yeast strains that contain the *ade2-101* mutation. All of our Matchmaker host strains (except EGY48) carry this mutation.
- Check the concentration of the resuspended competent cells (after Step E.11) using a hemocytometer. If the cell concentration is <1 x 10^9/ml, spin the cells down again (at 1,000 x g for 5 min) and resuspend them in a smaller volume of 1X TE/LiAc buffer.
- Occasionally, there is a contaminant in the water that can affect transformation efficiency and/or cell growth. Prepare all reagents using sterile, deionized, distilled water such as Milli-Q™-filtered. Confirm that your water purification system is functioning properly.

VI. α- and β-Galactosidase Assays

A. General Information

Considerations

- To reduce variability in liquid assays, assay five separate transformant colonies, and perform each assay in triplicate.
- It is important that the colonies to be assayed for α- and β-galactosidase activity are growing on the appropriate SD minimal medium. SD (dropout) medium is used to keep selective pressure on the hybrid plasmids and, in the case of the Matchmaker LexA Two-Hybrid System, the *lacZ* reporter plasmid up to the time the cells are lysed for the assay. The type of SD medium needed depends on the plasmids and host strains used. Furthermore, when working with a *lacZ* reporter under the control of the inducible *GAL1* promoter (such as in the LexA System), the SD medium must contain galactose (not glucose) as the carbon source. See the system-specific User Manual for media recommendations.

β-Galactosidase Assays

- X-gal must be used as the β-galactosidase substrate for solid-support assays because of its high degree of sensitivity. (X-gal is ~10^6-fold more sensitive than ONPG.) Although more sensitive than X-gal, Galacton-Star™ is not recommended for agar plate and filter assays because it gives troublesome background.
- The filter and liquid β-galactosidase assays described here use at least one freeze/thaw cycle in liquid nitrogen to lyse the yeast cell walls. Freeze-thaw cycles are a rapid and effective cell lysis method which permits accurate quantification of β-galactosidase activity (Schneider *et al.*, 1996).
- The **colony-lift filter assay** (Breeden & Nasmyth, 1985) used to measure β-galactosidase activity is primarily used to screen large numbers of cotransformants that survive the *HIS3* growth selection in a GAL4 two-hybrid or one-hybrid library screeening. It can also be used to assay for an interaction between two known proteins in a GAL4 two-hybrid system.
- The ***in vivo*, agar plate assay** is primarily used to screen large numbers of cotransformants for the expression of the *lacZ* reporter gene in a LexA two-hybrid library screening when the reporter gene is maintained on an autonomously replicating plasmid.The *in vivo* assay works for LexA transformants because of the *lacZ* reporter plasmid's high copy number and because of the preamplification step that normally precedes the β-galactosidase assay in this system. (Please refer to the Matchmaker LexA Two-Hybrid User Manual for more information on library screening.) Because of its relatively low sensitivity, the *in vivo*, agar plate assay is not suitable for screening transformants in a GAL4-based two-hybrid assay, or in a LexA-based two-hybrid assay when the reporter gene has been integrated into the host genome.
- Liquid cultures are assayed for β-galactosidase to verify and quantify two-hybrid interactions. Because of their quantitative nature, liquid assays can be used to compare the **relative** strength of the protein-protein interactions observed in selected transformants. However, there is no direct correlation between β-galactosidase activity and the K_d of an interaction (Estojak *et al.*, 1995). Furthermore, quantitative data cannot be compared between different host strains having different *lacZ* reporter constructs. In fact, due to promoter strength differences, it may be possible to quantitate the relative strength of interactions in some yeast strains (e.g., Y190, Y187), but not in others (e.g., CG-1945 or HF7c). (See Chapter II for a discussion of the promoters.)
- The liquid assays described here use one of three substrates: ONPG, CPRG, or a chemiluminescent substrate (Galacton-Star). The three substrates differ in their relative cost, sensitivity, and reproducibility. See Table III.

α-Galactosidase Assays

- The **α-Gal Quantitative Assay** is a sensitive colorimetric method for the detection and quantitation of yeast α-galactosidase activity resulting from expression of the *MEL1* reporter gene in our GAL4-based Matchmaker two-hybrid systems.

  MEL1 is a member of the *GAL* gene family, which, as a group, facilitates the uptake and utilization of galactose by the cell. Upon binding of GAL4 to the MEL1 upstream activating sequence (*MEL1* UAS), the *MEL1* gene product, α-galactosidase, is actively

VI. α- and β-Galactosidase Assays *continued*

expressed and secreted to the periplasmic space and culture medium where it catalyzes the hydrolysis of melibiose to galactose and glucose. The α-Gal Quantitative Assay allows you to specifically identify and measure this catalytic activity using *p*-nitrophenyl-α-D-galactoside (PNP-α-Gal), a colorless compound that yields a yellow product (*p*-nitrophenol) upon hydrolysis.

The α-Gal Quantitative Assay can be used to measure the extracellular α-galactosidase activity produced during the culture of any yeast strain that carries the *MEL1* gene. *MEL1* is endogenous to many but not all yeast strains. (Liljeström, 1985; Post-Beittenmiller *et al.*, 1984). Table IX provides a list of the GAL4-based Matchmaker yeast strains known to carry the *MEL1* gene. This list includes strain AH109 used in Clontech's Matchmaker Two-Hybrid System 3 (Cat No. 630303) and included with Clontech's GAL4-based cDNA libraries.

MEL1 has been shown to be a sensitive *in vivo* reporter for GAL4-based two-hybrid assays (Aho *et al.,* 1997). The quantitative nature of the α-Gal Assay makes it possible to compare the degree of *MEL1* expression in different Matchmaker two-hybrid host cell populations containing different pairs of interacting proteins, or to measure differences in the relative strength of binding between mutant forms of interacting proteins.

Principle of the α-Gal Quantitative Assay

In the α-Gal Quantitative Assay, the catalytic activity of α-galactosidase is monitored colorimetrically by measuring the rate of hydrolysis of the chromogenic substrate, *p*-nitrophenyl-α-D-galactoside (PNP-α-Gal). One of the products of this reaction, *p*-nitrophenol, displays a strong absorption band at 410 nm.

$$\text{PNP-}\alpha\text{-Gal} + H_2O \xrightarrow{\alpha\text{-galactosidase}} \underset{(\lambda_{max} = 410\ nm)}{\textit{p}\text{-nitrophenol}} + \text{D-galactose}$$

Because yeast naturally secrete α-galactosidase into the surrounding medium, it is more convenient to assay than β-galactosidase, an intracellular enzyme encoded by the *lacZ* reporter gene. The α-galactosidase assay is carried out by simply combining a small aliquot of cell-free culture media with a fixed volume of Assay Buffer; cell lysis is not necessary. After a prescribed incubation time (usually 60 min), the absorbance at 410 nm (OD_{410}), which is proportional to moles *p*-nitrophenol liberated, is recorded and used to calculate the concentration of α-galactosidase in milliunits/(ml x cell).

- To monitor *MEL1* expression directly on nutritional selection plates, use **X-α-Gal** (Cat No. 630407).
  X-α-Gal can be included in the medium before pouring plates or spread on top of the medium before plating liquid cultures. As α-galactosidase accumulates in the medium, it hydrolyzes
  X-α-Gal causing yeast colonies to turn blue. Instructions for preparing X-α-Gal indicator plates are given in the X-α-Gal Protocol-at-a-Glance (PT3353-2) supplied with each purchase of the substrate. Directions for use can also be downloaded from our website at **www.clontech.com**.

VI. α- and β-Galactosidase Assays *continued*

TABLE III. COMPARISON OF β-GALACTOSIDASE ASSAYS

Type of assay	Substrate	Protocol Section	Applications/Comments
In vivo, agar plate	X-gal in medium	VI.B	• Less sensitive than colony-lift assays; recommended only when the cells to be assayed contain many copies of the *lacZ* reporter gene (such as on a high-copy-number plasmid) • Convenient for large-scale experiments; screen many plates and colonies at the same time • Potential drawbacks: – Qualitative results only – Expensive if assaying many plates – Need to check for blue color development at several time intervals between 24 and 96 hr. – Background can be troublesome
Colony-lift, filter	X-gal on filter	VI.C	• Relatively sensitive; recommended when the cells to be assayed contain one or only a few copies of the *lacZ* reporter gene • Convenient for large-scale experiments; screen many plates and colonies at the same time • Relatively inexpensive to screen many plates • Get results quickly (in most cases, within a few hours) • Potential drawbacks: – Qualitative results only – More manipulations required than for *in vivo* assay
Liquid culture	ONPG	VI.D	• For assaying a small number of selected transformants • Less expensive than CPRG or Galacton-Star™ • Potential drawbacks: – May not be sensitive enough to quantify weak or transient two-hybrid interactions
Liquid culture	CPRG	VI.E	• For assaying a small number of selected transformants • 10-times more sensitive than ONPG • Potential drawbacks: – Less reproducible than ONPG for strong positive colonies because of CPRG's fast reaction rate
Liquid culture	Chemiluminescent (Galacton-Star™)	VI.F	• For assaying a small number of selected transformants • The most sensitive β-gal substrate • Potential drawbacks: – Relatively expensive – Requires luminometer or scintillation counter – Can give high background

Summary: Relative sensitivity of the five types of β-galactosidase assays:

[Least sensitive] → [Most sensitive]

X-gal (in agar plates)	ONPG (liquid assay)	CPRG (liquid assay)	X-gal (filter assay)	Galacton Star (liquid assay)

VI. α- and β-Galactosidase Assays *continued*

B. *In vivo* Plate Assay Using X-gal in the Medium (For LexA Systems only)

Reagents and Materials Required:

- Appropriate SD agar plates containing X-gal (80 mg/L) and 1X BU salts (Appendix C.A).

  Notes:
  - BU salts are included in the medium to maintain the optimum pH for β-galactosidase and to provide the phosphate needed for the assay.
  - **The X-gal should be incorporated into the medium before the plates are poured.** If the X-gal is spread over the surface of the agar plates, it can result in uneven distribution and thus localized variations in X-gal concentration. Also, the extra liquid on the plate surface (from spreading the X-gal) may lead to uneven spreading of the cell suspension and will delay absorption of the liquid.
  - X-gal is heat-labile and will be destroyed if added to hot (i.e.>55°C) medium.
  - Prepare the required number of plates in advance. Allow plates to dry (unsleeved) at room temperature for 2–3 days or at 30°C for 3 hr prior to spreading or streaking the cells. Excess moisture on the agar surface can lead to uneven spreading of cells.

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1. Streak, replica plate, or spread the transformants to be assayed on selection medium containing X-gal and BU salts.
   - When performing a two-hybrid library screening where very few of the cotransformants are expected to be positive for *lacZ* expression (or where it is difficult to predict the number of interactors), plate the cells at a high density. We recommend plating at two different densities to cover a range; e.g., 0.5 x 10^6 cfu on some (150-mm plates) and 2 x 10^6 on others.
   - When performing a two-hybrid assay where most or all of the individual colonies may be LacZ$^+$, spread 200–400 cfu per 100-mm plate.
2. Incubate plates at 30°C for 4–6 days.
3. Check plates every 12 hr (up to 96 hr) for development of blue color.

   Notes:
   - If you are performing a two-hybrid library screening using the Matchmaker LexA System, please see the User Manual for further information on identifying and storing LacZ$^+$ colonies.
   - Colonies grown on X-gal-containing medium will be somewhat smaller than those grown without X-gal.

C. Colony-lift Filter Assay

Reagents and Materials Required:

- Whatman No. 5 or VWR Grade 410 paper filters, sterile

  Notes:
  - 75-mm filters (e.g., VWR Cat No. 28321-055) can be used with 100-mm plates; 125-mm filters (e.g., VWR Cat No. 28321-113) can be used with 150-mm plates
  - Alternatively, 85- and 135-mm filters can be specially ordered from Whatman.
  - Nitrocellulose filters also can be used, but they are prone to crack when frozen.
- Forceps for handling the filters
- Z buffer (Appendix D)
- Z buffer/X-gal solution (Appendix D)
- X-gal stock solution (Appendix D)
- Liquid nitrogen

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1. For best results use fresh colonies (i.e., grown at 30°C for 2–4 days), 1–3 mm in diameter.

   Notes:
   - If only a few colonies are to be assayed, streak them (or spread them in small patches) directly onto master SD selection agar plates. Incubate the plates at 30°C for an additional 1–2 days, and then proceed with the β-galactosidase assay below.
   - Use the SD selection medium appropriate for your system and plasmids. When testing LexA transformants, be sure to use gal/raff induction medium.
2. Prepare Z buffer/X-gal solution as described in Appendix D.
3. For each plate of transformants to be assayed, presoak a sterile Whatman No. 5 or VWR grade 410 filter by placing it in 2.5–5 ml of Z buffer/X-gal solution in a clean 100- or 150-mm plate.

VI. α- and β-Galactosidase Assays *continued*

4. Using forceps, place a clean, dry filter over the surface of the plate of colonies to be assayed. Gently rub the filter with the side of the forceps to help colonies cling to the filter.
5. Poke holes through the filter into the agar in three or more asymmetric locations to orient the filter to the agar.
6. When the filter has been evenly wetted, carefully lift it off the agar plate with forceps and transfer it (colonies facing up) to a pool of liquid nitrogen. Using the forceps, completely submerge the filters for 10 sec.
   Note: Liquid nitrogen should be handled with care; always wear thick gloves and goggles.
7. After the filter has frozen completely (~10 sec), remove it from the liquid nitrogen and allow it to thaw at room temperature. (This freeze/thaw treatment is to permeabilizes the cells.)
8. Carefully place the filter, colony side up, on the presoaked filter (from Step C.3). Avoid trapping air bubbles under or between the filters.
9. Incubate the filters at 30°C (or room temperature) and check periodically for the appearance of blue colonies.
   Notes:
   - The time it takes colonies producing β-galactosidase to turn blue varies, typically from 30 min to 8 hr in a library screening. **Prolonged incubation (>8 hr) may give false positives.**
   - Yeast transformed with the β-galactosidase positive control plasmid will turn blue within 20–30 min. Most yeast reporter strains cotransformed with the positive controls for a two-hybrid interaction give a positive blue signal within 60 min. CG-1945 cotransformed with the control plasmids may take an additional 30 min to develop. If the controls do not behave as expected, check the reagents and repeat the assay.
10. Identify the β-galactosidase-producing colonies by aligning the filter to the agar plate using the orienting marks. Pick the corresponding positive colonies from the original plates to fresh medium. If the entire colony was lifted onto the filter, incubate the original plate for 1–2 days to regrow the colony.

D. Liquid Culture Assay Using ONPG as Substrate

Reagents and Materials Required:

- Appropriate liquid medium (Appendix C.A)
- 50-ml culture tubes
- Z buffer (Appendix D)
- Z buffer + β-mercaptoethanol (Appendix D)
- ONPG (Appendix D)
- 1 M Na_2CO_3
- Liquid nitrogen

1. Prepare 5-ml overnight cultures in liquid SD selection medium as described in Chapter III.A.3. Use the SD medium appropriate for your system and plasmids.
   Note: Be sure to use SD medium that will maintain selection on the plasmids used.
2. On the day of the experiment, dissolve ONPG at 4 mg/ml in Z buffer (Appendix D) with shaking for 1–2 hr.
3. Vortex the overnight culture tube for 0.5–1 min to disperse cell clumps. Immediately transfer 2 ml of the overnight culture to 8 ml of YPD (except for the LexA System).
   Note: For the LexA System, use the appropriate SD/Gal/Raff induction medium for the strains being assayed.
4. Incubate the fresh culture at 30°C for 3–5 hr with shaking (230–250 rpm) until the cells are in mid-log phase (OD_{600} of 1 ml = 0.5–0.8). Record the exact OD_{600} when you harvest the cells.
   Note: Before checking the OD, vortex the culture tube for 0.5–1 min to disperse cell clumps.
5. Place 1.5 ml of culture into each of three 1.5-ml microcentrifuge tubes. Centrifuge at 14,000 rpm (10,000 x g) for 30 sec.
6. Carefully remove supernatants. Add 1.5 ml of Z buffer to each tube and vortex until cells are resuspended.
7. Centrifuge cells again and remove supernatants. Resuspend each pellet in 300 µl of Z

VI. α- and β-Galactosidase Assays *continued*

buffer. (Thus, the concentration factor is 1.5 /0.3 = 5-fold).

Note: Differences in cell recoveries after this wash step can be corrected for by re-reading the OD_{600} of the resuspended cells.

8. Transfer 0.1 ml of the cell suspension to a fresh microcentrifuge tube.
9. Place tubes in liquid nitrogen until the cells are frozen (0.5–1 min).
10. Place frozen tubes in a 37°C water bath for 0.5–1 min to thaw.
11. Repeat the freeze/thaw cycle (Steps 9 & 10) two more times to ensure that the cells have broken open.
12. Set up a blank tube with 100 µl of Z buffer.
13. Add 0.7 ml of Z buffer + β-mercaptoethanol to the reaction and blank tubes. **Do not add Z buffer prior to freezing samples.**
14. Start timer. Immediately add 160 µl of ONPG in Z buffer to the reaction and blank tubes.
15. Place tubes in a 30°C incubator.
16. After the yellow color develops, add 0.4 ml of 1 M Na_2CO_3 to the reaction and blank tubes. Record elapsed time in minutes.

    Notes:

    - The time needed will vary (3–15 min for the single-plasmid, β-gal-positive control; ~30 min for a two-hybrid positive control; and up to 24 hr for weaker interactions).
    - The yellow color is not stable and will become more intense with time. You will need to run a new blank tube with every batch.
17. Centrifuge reaction tubes for 10 min at 14,000 rpm to pellet cell debris.
18. Carefully transfer supernatants to clean cuvettes.

    Note: The cellular debris, if transferred with the supernatant, will strongly interfere with the accuracy of this test.
19. Calibrate the spectrophotometer against the blank at A_{420} and measure the OD_{420} of the samples relative to the blank. The ODs should be between 0.02–1.0 to be within the linear range of the assay.
20. Calculate β-galactosidase units. 1 unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of ONPG to *o*-nitrophenol and D-galactose per min per cell (Miller, 1972; Miller, 1992):

$$\text{β-galactosidase units} = 1{,}000 \times OD_{420} / (t \times V \times OD_{600})$$

where: t = elapsed time (in min) of incubation

V = 0.1 ml x concentration factor*

OD_{600} = A_{600} of 1 ml of culture

* The concentration factor (from Step D.7) is 5. However, it may be necessary to try several dilutions of cells at this step (hence different concentration factors) to remain within the linear range of the assay.

E. Liquid Culture Assay Using CPRG as Substrate

Reagents and Materials Required:

- Appropriate liquid medium (Appendix C.A)
- 50-ml culture tubes
- Buffer 1 (Appendix D)
- Buffer 2 (Appendix D)
- CPRG (chlorophenol red-β-D-galactopyranoside; Roche Applied Science Cat. No. 10884308001)
- 3 mM $ZnCl_2$ (Filter sterilized to preserve for ~3 months)
- Liquid nitrogen

1. Prepare 5-ml overnight cultures in liquid SD medium as described in Chapter III.A.3. Use the SD selection medium appropriate for your system and plasmids.

   Note: Be sure to use SD medium that will maintain selection on the plasmids used.
2. Vortex the overnight culture tube for 0.5–1 min to disperse cell clumps. Immediately

VI. α- and β-Galactosidase Assays *continued*

transfer 2 ml of the overnight culture to 8 ml of YPD (except for LexA System).

Note: For the LexA System, use the appropriate SD/Gal/Raff induction medium for the strains being assayed.

3. Incubate fresh culture at 30°C for 3–5 hr with shaking (230–250 rpm) until the cells are in mid-log phase (OD_{600} of 1 ml = 0.5–0.8). Record the exact OD_{600} when you harvest the cells.

   Note: Before checking the OD, vortex the culture tube for 0.5–1 min to disperse cell clumps.
4. Place 1.5 ml of culture into each of three 1.5-ml microcentrifuge tubes. Centrifuge at 14,000 rpm (16,000 x g) for 30 sec to pellet the cells.
5. Carefully remove the supernatant, add 1.0 ml of Buffer 1, and vortex until cells are thoroughly resuspended.
6. Centrifuge at 14,000 rpm (16,000 x g) for 30 sec to pellet the cells.
7. Carefully remove the supernatant and resuspend the cells in 300 µl of Buffer 1. (The concentration factor is 1.5 /0.3 = 5-fold.)

   Note: Differences in cell recoveries after this wash step can be corrected for by re-reading the OD_{600} of the resuspended cells.
8. Transfer 0.1 ml of the cell suspension to a fresh microcentrifuge tube.
9. Place tubes in liquid nitrogen until the cells are frozen (0.5–1 min).
10. Place frozen tubes in a 37°C water bath for 0.5–1 min to thaw.
11. Repeat the freeze/thaw cycle (Steps 9 and 10) two times to ensure that all cells are broken open.
12. Add 0.7 ml of Buffer 2 to each sample and mix by vortexing. **Thorough mixing is critical to the assay.**
13. Record the time when Buffer 2 was added. This is the starting time.
14. Add 1 ml of Buffer 2 to a separate tube (this will be the buffer blank).
15. When the color of the samples is yellow/grey to red, add 0.5 ml of 3.0 mM $ZnCl_2$ to each sample and the buffer blank to stop color development. Record the stop time. (For very strong β-galactosidase-positive colonies, color development occurs within seconds; weak-to-moderate reactions take several hours to develop).
16. Centrifuge samples at 14,000 rpm for 1 min to pellet cell debris.
17. Transfer samples to fresh tubes.
18. Zero the spectrophotometer using the buffer blank and measure the OD_{578} of the samples. (An OD_{578} between 0.25 and 1.8 is within the linear range of the assay.)
19. Calculate β-galactosidase units. 1 unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of CPRG to chlorophenol red and D-galactose per min per cell (Miller, 1972; Miller, 1992):

$$\text{β-galactosidase units} = 1000 \times OD_{578} / (t \times V \times OD_{600})$$

where:

t = elapsed time (in min) of incubation

V = 0.1 x concentration factor*

OD_{600} = A_{600} of 1 ml of culture

* The concentration factor (from Step E.7) is 5. However, it may be necessary to try several dilutions of cells at this step (hence different concentration factors) to remain within the linear range of the assay.

F. Liquid Culture Assay Using a Chemiluminescent Substrate

Reagents and Materials Required:

- Appropriate liquid medium (Appendix C.A)
- 50-ml culture tubes
- Z buffer (Appendix D)
- Galacton-Star reaction mixture (Provided with the Luminescent β-galactosidase Detection Kit II)
- Liquid nitrogen
- Luminometer [or scintillation counter with single-photon-counting program]
- Optional: 96-well, opaque white, flat-bottom microtiter plates [Xenopore Cat No. WBP005]

VI. α- and β-Galactosidase Assays *continued*

- Optional: Purified β-galactosidase (for a standard curve)

Note: For best results, we recommend using the Luminescent β-galactosidase Detection Kit II (Cat No. 631712), which includes a reaction buffer containing the Galacton-Star substrate and the Sapphire II™ accelerator, positive control bacterial β-galactosidase, and a complete User Manual.

Chemiluminescent detection of β-galactosidase

It is important to stay within the linear range of the assay. High-intensity light signals can saturate the photomultiplier tube in luminometers, resulting in false low readings. In addition, low intensity signals that are near background levels may be outside the linear range of the assay. If in doubt, determine the linear range of the assay and, if necessary, adjust the amount of lysate used to bring the signal within the linear range. See Campbell *et al*. (1995) for a chemiluminescent β-galactosidase assay used in a yeast two-hybrid experiment.

1. Prepare 5-ml overnight cultures in liquid SD medium as described in Chapter III.A.3. Use the SD medium appropriate for your system and plasmids.

   Note: For qualitative data, a whole colony, resuspended in Z buffer, may be used for the assay directly. See instructions following this section.
2. On the day of the experiment, prepare the Galacton-Star reaction mixture. Keep buffer on ice until you are ready to use it.
3. Vortex the overnight culture tube for 0.5–1 min to disperse cell clumps. Immediately transfer at least 2 ml of the overnight culture to no more than 8 ml of YPD (except for the LexA System).

   Note: For the LexA System, use the appropriate SD/Gal/Raff induction medium for the strains being assayed.
4. Incubate the fresh culture at 30°C for 3–5 hr with shaking (230–250 rpm) until the cells are in mid-log phase (OD_{600} of 1 ml = 0.4–0.6).
5. Vigorously vortex the culture tube for 0.5–1 min to disperse cell clumps. Record the exact OD_{600} when you harvest the cells.
6. Place 1.5 ml of culture into each of three 1.5-ml microcentrifuge tubes. Centrifuge at 14,000 rpm (10,000 x g) for 30 sec.
7. Carefully remove supernatants. Add 1.5 ml of Z buffer to each tube and thoroughly resuspend the pellet.
8. Centrifuge at 14,000 rpm (10,000 x g) for 30 sec.
9. Remove the supernatants. Resuspend each pellet in 300 µl of Z buffer. (Thus, the concentration factor is 1.5 /0.3 = 5-fold.)
10. Read the OD_{600} of the resuspended cells. The OD_{600} should be ~2.5. If the cell density is lower, repeat Steps 5–9 , except resuspend the cells in <300 µl of Z buffer.
11. Vortex each cell suspension and transfer 100 µl to a fresh tube.

    Note: The remaining cell suspension can be stored at –70°C to –80°C.
12. Place tubes in liquid nitrogen for 0.5–1 min to freeze the cells.
13. Place frozen tubes in a 37°C water bath for 0.5–1 min to thaw.
14. Repeat freeze/thaw cycle (Steps 12 & 13) once to ensure that cells have been cracked open.
15. Warm to room temperature enough reaction buffer for the entire experiment.
16. Set up a blank tube with 25 µl of Z buffer.
17. [Optional] If you wish to obtain absolute as well as relative data, set up a series of β-galactosidase standard tubes containing 0.0005, 0.001, 0.003, 0.010, and 0.020 unit of β-galactosidase in 25 µl of Z-buffer.
18. Place 20–30 µl of each cell lysate in a separate sample tube (or into wells of an opaque 96-well, flat-bottom microtiter plate suitable for plate luminometers). If you are using a sample tube, the tube should hold at least 0.5 ml.

    Note: The amount of yeast extract required may vary depending upon the level of β-gal expression and the detection device used. Use 10–30 µl of extract for positive controls and 20–30 µl for experimental samples with potentially low levels of enzyme activity. It is important to vary the amount of extract to keep the signal within the linear range of the assay.
19. Add 200 µl of Galacton-Star reaction mixture to each sample tube or well and mix

VI. α- and β-Galactosidase Assays *continued*

gently.

20. Incubate at room temperature (20°–25°C) for 60 min.

    Note: Light signals produced during this incubation are stable for >1 hr; therefore, detection can be performed 1–2 hr after the incubation.

21. Centrifuge tubes at 14,000 rpm (16,000 x g) for 1 min at 4°C. (If you are using microtiter plates, centrifuge plates at 1,000 x g for 5 min in a specially adapted rotor.) Proceed directly to the appropriate detection steps for your assay: Step 22, 23, 24, or 27.
22. Detection using a tube luminometer
    a. Turn on the tube luminometer. Set the integration time for 5 sec.
    b. Calibrate the luminometer according to the manufacturer's instructions.
    c. If the sample is not already in a tube suitable for luminometer readings, transfer the entire solution from (Step 21) to an appropriate tube. Do not disturb the pellet.
    d. Place one sample at a time in the luminometer compartment and record the light emission (RLU) as a 5-sec integral. Use your blank sample as a reference when interpreting the data.
23. Detection using a plate luminometer

    After Step 21, simply record light signals as 5-sec integrals.
24. Detection using a scintillation counter
    a. Transfer the entire solution from Step 21 to a 0.5-ml microcentrifuge tube.

       Note: Plan to use scintillation counter adaptors that keep the tubes upright.
    b. Place the tube in the washer of the scintillation counter adaptor and place the adaptor in the machine's counting rack. Set the integration time for at least 15 sec.

       Note: Integration times <15 sec may not produce accurate results.
    c. To detect chemiluminescent signals, **use a single-photon-count program**. Consult your scintillation counter's manufacturer for further information about this software.
25. For detection methods described in Steps 22–24: Calculate the β-galactosidase activity in terms of RLU/OD_{600} unit of cell culture. (Note that Miller unit calculations are not possible using these methods.)
26. [Optional] If you have set up β-galactosidase standards, prepare a standard curve of RLU vs. the amount of β-galactosidase. Estimate the quantity of β-galactosidase in the unknown samples using the standard curve. Determine the amount of enzyme per OD_{600} unit of cell culture. The final OD_{600} units of cells assayed per sample is calculated as follows:

    OD_{600} (from Step 5) x vol (from Step 18) x conc. factor (from Step 9)
27. Detection by exposure of x-ray film

    Light emission can also be recorded by exposure of x-ray film to reaction samples in opaque 96-well flat-bottom microtiter plates. The relative intensity of the resulting spots on the film can be estimated by comparison to positive and negative controls. Note that x-ray film is several orders of magnitude less sensitive than a luminometer or scintillation counter.

    Overlay the microtiter plate with x-ray film, cover the film with plastic wrap, and place a heavy object such as a book on top to hold the film in place. Expose the film at room temperature for 5–30 min.

    Note: To compare samples accurately, they must be within the linear response capability of the x-ray film. We therefore recommend that you obtain several different exposures.

Qualitative Liquid Assay Using Galacton-Star as the Substrate

This alternative cell preparation method directly detects β-galactosidase activity in resuspended yeast colonies. It is recommended for detecting extremely weak *lacZ* transcriptional signals that cannot be detected by X-gal filter assays. For a +/– result, it is more labor-intensive than a filter assay. However, because of its greater sensitivity, it is less likely to give a false-negative result.

1. Grow colonies on the appropriate SD selection medium.
2. Transfer an entire large (2–3 mm), fresh (2–4-day-old) colony to a 0.5-ml tube containing 50 µl of Z buffer. If colonies are small, use several. At the same time, prepare a master

VI. α- and β-Galactosidase Assays *continued*

or reference plate of the colonies to be assayed.

3. Completely resuspend the colony in the Z buffer by repeatedly pipetting up and down.
4. Place tubes in liquid nitrogen for 0.5–1 min to freeze the cells.
5. Continue with Step 13 of the main procedure (VI.F) above.
6. Compare results with those of the negative control.

G. α-Gal Quantitative Assay

Reagents and Materials Required:

- Appropriate liquid synthetic dropout (SD) culture medium (Appendix C.A)
- 50-ml culture tubes
- PNP-α-Gal Solution (100 mM)
- 10X Stop Solution (Appendix D.F)
- 1X NaOAc Buffer (Appendix D.F)
- Assay Buffer (Appendix D.F)
- 1.5-ml cuvettes or 96-well, flat-bottom microtiter plates for OD_{410} measurements

Preparation of Samples

1. Inoculate 2–5 ml of liquid synthetic dropout (SD) medium, containing the appropriate dropout supplements, with a yeast colony expressing the pair of proteins being analyzed. It is advisable to set up triplicate cultures for each type of yeast colony being analyzed.

   Fresh (one- to three-week-old) colonies will give best results for liquid culture inoculation. A single colony may be used for the inoculation if it is 2–3 mm in diameter. Scrape the entire colony into the medium. If the colonies on the master plate are smaller than 2 mm, transfer several colonies into the medium.

   Examples:
   - For AH109 and Y190 cotransformants expressing interacting pairs of GAL4 BD and AD fusion proteins, inoculate into SD/–His/–Leu/–Trp.
   - For AH109 and Y190 cotransformants expressing non-interacting pairs of fusion protein constructs, inoculate into SD/–Leu/–Trp.
   - For Y187 tranformants expressing interacting or non-interacting proteins, inoculate into SD/–Leu/–Trp.
   - For non-transformed AH109, Y187, and Y190 host strains, inoculate into SD/–Ura.
2. Incubate at 30°C overnight (~16–18 hr) with shaking (250 rpm).
3. Vortex the cell culture tube for 0.5–1 min to disperse cell clumps, then transfer 1 ml of the suspension to a clean cuvette and record the OD_{600}. For accuracy, the OD_{600} should lie between 0.5–1.0. Dilute the cell suspension if necessary; remember to account for the dilution factor when making your final calculations (Step 11, below).
4. Place 1.0 ml of culture into a 1.5-ml microcentrifuge tube. Centrifuge at 14,000 rpm (10,000 x g) for 2 min or until the cells are completely pelleted.

   Note: It is important to ensure that cells and cellular debris are completely pelleted to minimize intereference from light scattering in the colorimetric assay below.
5. Carefully transfer the supernatant to a clean tube and store at room temperature for use in Step 6, below.

   Note: To minimize the loss of enzyme activity, we suggest proceeding with the colorimetric assay immediately once the cell-free superantant has been isolated.

Colorimetric Assay

Below we provide protocols for 1-ml and 200-µl assays. If you have access to a spectrophotometer equipped to read microtiter plates, you may find it more convenient to use the 200-µl assay protocol, which is intended for use with 96-well, flat-bottom microtiter plates.

Note: The experimental conditions and volumes of reagents used throughout the α-Galactosidase Quanitative Assay have been carefully tested and optimized for use in the 1-ml and 200-µl assay formats described below. Please follow as directed. Though we do not recommend changing the actual volumes of reagents used in the colorimetric assay, if the signal from an experimental sample exceeds the linear range of the assay, you can dilute the media supernatant before transferring an aliquot to the reaction tube or well. Remember to correct for individual sample volumes and dilutions when tabulating final results at Step 11.

5. Prepare a sufficient amount of Assay Buffer for all samples including controls, and allow to equilibrate to room temperature.

VI. α- and β-Galactosidase Assays *continued*

- For each 1-ml assay, you will need 24 µl Assay Buffer.
- For each 200-µl assay (96-well microtiter plate format), you will need 48 µl Assay Buffer.

Note: We recommend assaying each sample in triplicate. Be sure to include positive and negative controls in your assay. Also include a reagent blank in each assay to calibrate the spectrophotometer prior to reading the OD of your samples. A reagent blank is composed of sterile, unused culture media, Assay Buffer, and Stop Solution combined according to Steps 6–9.

	Assay Scale	
	200-µl	1-ml
6. Transfer cell culture medium supernatant (from Step 4) into a 1.5-ml microcentrifuge tube or into a well of a clear microtiter plate[a].	16 µl	8 µl
7. Add Assay Buffer to each sample.	48 µl	24 µl
8. Incubate at 30°C for 60 min. Be sure to cover microtiter plates with a lid or parafilm to prevent evaporation.		
9. Terminate the reaction by adding Stop Solution. **Note:** Use 1X Stop Solution for 1-ml assays and 10X Stop Solution for 200-µl assays.	136 µl of **10X**	960 µl of **1X**
10. Record the optical density of each sample at 410 nm (OD_{410})[b].	96-well plate	1.5-ml cuvette

[a] Add a corresponding volume of sterile, unused culture media to the reagent blank.

[b] Zero the spectrophotometer using the reagent blank and measure the OD_{410} of the experimental and control samples relative to the blank.

11. Calculate α-galactosidase units. One unit of α-galactosidase is defined as the amount of enzyme that hydrolyzes 1 µmole *p*-nitrophenyl-α-D-galactoside to *p*-nitrophenol and D-galactose in 1 min at 30°C in acetate buffer, pH 4.5 (Lazo *et al.*, 1978).

$$\text{α-galactosidase [milliunits/(ml x cell)]} = OD_{410} \times V_f \times 1{,}000/[(\varepsilon \times b) \times t \times V_i \times OD_{600}]$$

t = elapsed time (in min) of incubation

V_f = final volume of assay (200 µl or 992 µl)

V_i = volume of culture medium supernatant added (16 µl or 8 µl)

OD_{600} = optical density of overnight culture[a]

$\varepsilon \times b$ = *p*-nitrophenol molar absorbtivity[b] at 410 nm x the light path (cm)
= 10.5 (ml/µmol) for 200-µl format[b,c]
= 16.9 (ml/µmol) for 1-ml format where b = 1 cm[b,d]

[a] The optical density at 600 nm, recorded in Step 2, is used to normalize the OD_{410} of different media samples to the number of cells in each culture.

[b] The molar absorbtivity, though independent of concentration and light path, varies with the chemical properties (e.g., pH) of the solution. Because different strengths and quantities of Stop Solution are used to terminate the 200-and 1-ml reactions, the final pHs and, therefore, the molar absorbtivities are different for the two formats.

[c] Determined at Clontech using a SPECTRAmax® Microplate Spectrophotometer and Corning Costar UV-transparent, flat-bottom plates (Corning Cat No. 3635); Some microplate readers have pathlength correction capabilities to normalize absorbance values to those obtained when the light pathlength is 1-cm (e.g., using 1.5-ml cuvettes). With pathlength correction on, the molar absorbtivity, ε, of *p*-nitrophenol at 410 nm in the 200-µl format was determined to be 20.3 ml/µmol.

The well-diameter and, therefore, the light path (b) in other brands of 96-well plates may differ from that of the Corning plates used for these determinations. To determine ε x b in other plates, construct a plot of A_{410} versus concentration of *p*-nitrophenol (PNP) as follows. Using a 10 µmol/ml standard solution of *p*-nitrophenol (Sigma Cat No. 104-1), make 1:2 serial dilutions in water down to 0.02 µmol/ml PNP. Use these serial dilutions in place of medium supernatant in Step 6, above. Then follow Steps 7, 9, and 10 as directed; omit Step 8. At Step 7, use Assay Buffer that has been prepared by combining 2 volumes of 1X NaOAc with 1 volume of H_2O, **not** PNP-α-Gal. Finally, plot A_{410} versus concentration of PNP. According to the Beer-Lambert Law, the proportionality constant, ε x b, is equal to the slope of the straight line defined by these data.

[d] Determined at Clontech using 1.5-ml cuvettes

VII. Working With Yeast Plasmids

A. General Information

Isolating plasmid DNA from yeast is not trivial, primarily because of the tough cell wall. Furthermore, the relatively large size (>6 kb) and low copy number (~50/cell) of some yeast plasmids results in very low DNA yields, regardless of the plasmid isolation method used. In addition, plasmid DNA isolated from yeast is often contaminated by genomic DNA because yeast contain ~3X as much genomic DNA as *E. coli*, and the isolation method breaks the yeast chromosomes and releases them from cellular material.

There are several yeast plasmid isolation procedures currently in use. The various protocols differ primarily in the method used to break the cell walls. Here we provide the protocol that we optimized for our Yeastmaker Yeast Plasmid Isolation Kit (Cat No. 630441). This procedure, which was modified from the method of Ling *et al.* (1995), uses extensive digestion with lyticase to weaken the cell walls and SDS to burst the resulting spheroplasts. The DNA preps can be cleaned up using either CHROMA SPIN™ Columns or phenol:chloroform extraction followed by ethanol precipitation. If CHROMA SPIN™ Columns are used, this method takes <2 hr from cell pellets to purified plasmid, and is simple enough to be easily adapted for processing many samples simultaneously.

This purification method yields DNA of sufficient purity for use as a PCR template (Chapter VIII) or for transforming *E. coli* (Chapter VII.C). However, if you need a large quantity of plasmid, or very pure plasmid DNA, such as for sequencing or restriction enzyme digestion, you will have to transform *E. coli* and prepare plasmid using standard methods (Sambrook *et al.*, 1989).

Plasmid rescue via complementation of *E. coli* mutations

Plasmid isolation from yeast cotransformants is complicated by the presence of two (or more) types of plasmids in a single yeast colony. Nutritional selection of *E. coli* transformants bearing the yeast plasmid of interest can be an efficient way to "rescue" one type of plasmid from a mixture of plasmids bearing different nutritional transformation markers. For more information on plasmid rescue via transformation of *E. coli*, see Section VII.C.

B. Plasmid Isolation from Yeast

Reagents and Materials Required

The Yeastmaker Yeast Plasmid Isolation Kit (Cat No. 630441) provides the SDS and lyticase solutions, CHROMA SPIN-1000 DEPC-H_2O Columns, and 2-ml centrifuge tubes for use with the columns.

- Appropriate SD liquid or agar medium to keep selection on the plasmids (Appendix C.A; Appendix E).
- Sterile, 1.5-ml microcentrifuge tubes (or a 96-tube microtiter array, multichannel pipettors, and centrifuge adaptor for multiwell plates).
- 20% SDS
- Lyticase Solution (5 units/µl in TE buffer; store at 4°C for up to 2 months or at –20°C for up to 6 months. If colloidal material precipitates, mix the solution by inversion before using.)
- Recommended: CHROMA SPIN-1000 DEPC-H_2O Columns (Cat No. 636093) and 2-ml centrifuge tubes for use with the columns
- If you do not use CHROMA SPIN Columns, you will need materials to perform phenol:chloroform extraction and ethanol precipitation:
  - Phenol:chloroform:isoamyl alcohol (25:24:1; See Sambrook *et al.*, 1989, for information on preparing neutralized phenol solutions)
  - 10 M ammonium acetate
  - 95–100% ethanol

VII. Working With Yeast Plasmids *continued*

1. Prepare yeast cultures for lysis (Step a, b, or c below).
   a. From a solid patch of growth:
      i. Spread a thin film of yeast cells (~2-cm^2 patch) onto the appropriate SD agar medium.
      ii. Incubate plate at 30°C for 3–4 days. (The patch should show abundant yeast growth.)
      iii. Scrape up a portion of the patch (~10 mm^2) and resuspend the cells in 50 µl of sterile H_2O or TE in a 1.5-ml microcentrifuge tube.
   b. From a liquid culture:
      i. Inoculate a large (2–4-mm), fresh (2–4-day-old) yeast colony into 0.5 ml of the appropriate SD liquid medium. Vortex tube vigorously to completely break up the colony and resuspend the cells.
      ii. Incubate at 30°C overnight with shaking at 230–250 rpm.
      iii. Spin down the cells by centrifuging at 14,000 rpm for 5 min.
      iv. Carefully pour off the supernatant and resuspend pellets in the residual liquid (total volume ~50 µl).
   c. For semi-automated handling of a large number of samples:
      i. Place a large (2–4-mm), fresh (2–4-day-old) yeast colony into 0.5 ml of the appropriate SD liquid medium in separate wells of a 96-tube microtiter array. Vortex each tube vigorously to resuspend the cells. (Alternatively, use 0.5 ml of an overnight SD liquid culture instead of a yeast colony.)
      ii. Using a centrifuge adapted for multiwell plates, centrifuge the entire array at 1,000 x g for 5 min to pellet the cells.
      iii. Carefully pour (or draw) off supernatants and resuspend pellets in the residual medium (~50 µl) by vortexing or pipetting up and down.
2. Add 10 µl of lyticase solution to each tube. Thoroughly resuspend the cells by vortexing or repeatedly pipetting up and down.
3. Incubate tubes at 37°C for 30–60 min with shaking at 200-250 rpm.

   [Optional] Check a drop of the cell suspension under a phase contrast microscope (400X) for the progress of cell lysis by adding a drop of 20% SDS to the side of the coverslip. As they come into contact with the SDS, most cells should lose their refractile appearance and appear as "ghost-like" spheroplasts. If there are still many intact cells present, incubate the samples for another 30 min.
4. Add 10 µl of 20% SDS to each tube and vortex vigorously for 1 min to mix.
5. Put the samples through one freeze/thaw cycle (at –20°C) and vortex again to ensure complete lysis of the cells.
6. If necessary, samples can be stored frozen at –20°C. If samples have been frozen, vortex them again before using them.
7. Pour the entire contents of the tube from Step 5 above onto a prespun CHROMA SPIN-1000 Column and purify the plasmid DNA according to the CHROMA SPIN User Manual. Purified plasmid DNA will elute from the column.

   If you do not use CHROMA SPIN Columns, clean up the prep as follows:
   a. Bring the volume of the sample up to 200 µl in TE buffer (pH 7.0).
   b. Add 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
   c. Vortex at highest speed for 5 min.
   d. Centrifuge at 14,000 rpm for 10 min.
   e. Transfer the aqueous (upper) phase to a fresh tube.
   f. Add 8 µl of 10 M ammonium acetate and 500 µl of 95–100% Ethanol.
   h. Place at –70°C or in a dry-ice/ethanol bath for 1 hr.
   i. Centrifuge at 14,000 rpm for 10 min.
   j. Discard supernatant and dry the pellet.
   k. Resuspend pellet in 20 µl of H_2O.

VII. Working With Yeast Plasmids *continued*

Note: The amount of plasmid DNA recovered is small relative to the contaminating genomic DNA; therefore, it cannot be measured by A_{260} or seen on an agarose gel.

C. Transforming *E. coli* with Yeast Plasmids

We recommend using electroporation (Section C.1) when transforming *E. coli* with plasmids isolated from yeast because of the relatively high transformation efficiency that can be obtained. This is important because of the yeast genomic DNA that is present in yeast-isolated plasmids; the presence of genomic DNA reduces the transformation efficiency of the plasmids. However, if you choose to use chemically competent cells (Section C.2), it is essential that the cells be able to yield a transformation efficiency of at least 10^7 cfu/µg (of pUC19 DNA).

Nutritional selection of *E. coli* transformants

In the Matchmaker two-hybrid systems, cloning vectors carrying *HIS3, LEU2 ,* or *TRP1* markers can be selectively rescued by complementation of the *E. coli hisB, leuB, or trpC* mutations, respectively. (The yeast *HIS3, LEU2 ,* and *TRP1* genes are expressed well enough in *E. coli* to allow this complementation.) Furthermore, due to incompatibility of the *E. coli* plasmid replication origins used on the different vectors, only one plasmid construct will propagate in a given *E. coli* transformant plated on selection medium. Thus, there is no need to screen every *E. coli* transformant for the presence of the other (unwanted) plasmids.

If you plan to perform a nutritional selection for plasmid rescue, we recommend using *E. coli* srain KC8, which carries the *hisB, leuB,* and *trpC* mutations (K. Struhl, personal communication). KC8 Chemically Competent (Cat No. 630434) and Electrocompetent (Cat No. 630435) Cells are available from Clontech. HB101, which carries the *leuB* mutation (Bolivar & Backman, 1979), may be used to select for yeast plasmids bearing the *LEU2* marker only.

For nutritional selection of KC8 and HB101 transformants on M9 minimal medium, add a 1X mixture of amino acids (i.e., dropout [DO] supplement) lacking the specific nutrient that will allow selection of the desired plasmid (Appendix E). (The same DO supplements used for yeast SD medium can be used to supplement M9 minimal medium; see Appendix C for recipes).

- Because of its auxotrophic mutations, KC8 requires His, Leu, Trp, and thiamine for growth on minimal medium, unless one of these nutrients is specifically omitted for the selection.
- HB101 requires leucine, proline, and thiamine for growth on minimal medium, unless one of these nutrients is specifically omitted for the selection; note that HB101 is streptomycin resistant.
- Although optional, we recommend including ampicillin (50 µg/ml) in the medium to reduce background growth.

Any of the common *E. coli* host strains (e.g., DH5α; JM109) may be used if you prefer to select transformants by resistance to ampicillin rather than using a nutrional selection. However, because both the DNA-BD and AD plasmids will be represented in the *E. coli* transformant population (and not necessarily in equal proportions), many transformant colonies will need to be screened for the presence of the desired plasmid(s). The plasmids can be distinguished by restriction enzyme digestion or PCR amplification using AD vector-specific insert-screening primers.

Reagents and materials required

- *E. coli* competent cells (chemically competent or electrocompetent)
  Notes:
  - For methods to prepare electrocompetent *E. coli* cells, see Kaiser & Auer (1993), Dower *et al.* (1988), Chuang *et al.* (1995), and Sambrook *et al.* (1989). Alternatively, purchase premade chemically competent or electrocompetent *E. coli* cells from Clontech.
  - If you use the direct electroporation method of Marcil & Higgins (1992), the *E. coli* competent cells must be transformed at an efficiency of 10^9 cfu/µg (of pUC19 DNA) to work satisfactorily with yeast plasmids.
- For transformation of electrocompetent cells, you need an electroporator and a cuvette with a 0.1-cm gap.

VII. Working With Yeast Plasmids *continued*

- Yeast plasmid DNA (from Section B above)
- Sterile, 14-ml polypropylene conical tubes (e.g., Falcon™ Cat No. 2059)
- Hanahan's SOC medium or LB broth (Sambrook *et al*., 1989)
- LB/amp (50 µg/ml) agar plates for antibiotic selection or appropriately supplemented M9/amp plates for nutritional selection (Appendix C.B)
- Materials for isolating plasmid DNA from *E. coli.*

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1. Procedure for transforming electrocompetent *E. coli* KC8

a. Prepare or thaw electrocompetent *E. coli* cells.
b. Add 1–2 µl of yeast plasmid solution to 40 µl of electrocompetent cells on ice.
c. Transfer samples to a prechilled cuvette having a 0.1-cm gap. Perform the electroporation according to the manufacturer's instructions.
d. Add 1 ml of LB or (preferably) SOC medium with no antibiotic to the cuvette. Transfer the cell suspension to a 14-ml conical Falcon tube.
e. Incubate at 37°C for 1 hr with vigorous shaking (250 rpm).
e. Pellet cells by centrifuging at 2,500 rpm for 5 min in a tabletop centrifuge.
f. Discard supernatant and resuspend pellet in residual liquid.
g. Plate cells on supplemented M9/amp agar medium.
h. Incubate plates at 37°C for 24 hr (LB/amp selection only), or for 36–48 hr (for nutritional selection on M9 medium). If you do not recover any colonies, see the Troubleshooting tips below.
i. See Section C.3 for tips on plasmid isolation.

2. Procedure for transforming chemically competent *E. coli* KC8

Transformation efficiency is significantly affected by temperature. Therefore, prechill the 14-ml Falcon tubes and pipette tips to 4°C before using them.

a. Prepare the chemically competent cells or thaw them on ice.
b. Add 10 µl of yeast plasmid solution to a prechilled Falcon tube.
c. Add 100 µl of competent cells to the tube and mix well by gently tapping the tube.
d. Incubate on ice for 30 min.
e. Heat shock by transferring the tube to a 42°C water bath and incubating for 45–50 sec.
f. Chill on ice for 2 min.
g. Add 1 ml of LB broth or (preferably) SOC medium with no antibiotic.
h. Incubate at 37°C for 1 hr with vigorous shaking (250 rpm).
i. Pellet cells by centrifuging at 2,500 rpm for 5 min in a table-top centrifuge.
j. Discard supernatant and resuspend pellet in residual liquid.
k. Plate cells on appropriate medium (LB/amp or supplemented M9).
l. Incubate plates at 37°C for 24 hr (LB/amp selection only), or for 36–48 hr (for nutritional selection on M9 medium). Typically, 10–100 colonies will be seen on the plate for a successful transformation using plasmid isolated from yeast. If you do not recover any colonies, see the Troubleshooting tips below.
m. If you performed a parallel transformation using the control pUC19 DNA, calculate the transformation efficiency. (The competent cells should have been transformed with an efficiency of $\geq 1 \times 10^7$ cfu/µg. See Section V.E.23 for a sample calculation.)
n. See Section C.3 for tips on plasmid isolation.

3. Tips on Isolating plasmid DNA from the *E. coli* transformants

a. Use a standard plasmid mini-prep procedure to isolate plasmid DNA from the *E. coli* transformants (Sambrook *et al.*, 1989).

VII. Working With Yeast Plasmids *continued*

Notes:

- If you are using an *endA*$^+$ bacterial strain such as KC8 or HB101 as the host strain, extra care must be taken when preparing plasmid DNA because of the presence of endonuclease A. (See Sambrook *et al*. [1989]1:1.22–1.23.)
- Boiling lysis is not recommended for isolation of plasmids from *endA*$^+$ bacteria.
- If you are using a commercial plasmid preparation kit, follow the manufacturer's directions for host strains that are *endA*$^+$.
- If you plan to use the plasmid for sequencing or other applications requiring highly purified DNA, the plasmid should be extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol before use. Alternatively, CHROMA SPIN™+TE-400 Columns (Cat No. K1323-1) may be used to purify the plasmid.

b. To verify that you have obtained the correct plasmid, amplify the insert by PCR, digest it with *Alu* I or *Hae* III, and run a small sample on an agarose/EtBr gel. Compare the restriction digestion pattern with that of the original clone isolated from yeast.

4. Troubleshooting tips

a. If you do not obtain any transformants, you may need to improve the transformation effiency of the cells.

- If you performed a nutritional selection on M9 minimal medium, repeat the transformation, but plate the cells on LB/amp instead. (The recovery of new transformants is generally better on on LB/amp than on M9 medium.)Then replica plate the Amp^r transformants to the appropriate M9 minimal medium for selection of the desired plasmid and to verify that the undesired plasmid(s) have been lost. Note that it takes somewhat longer to see colonies on M9 medium than on LB.
- If you are not already doing so, use electrotransformation rather than chemical transformation; higher transformation efficiencies are usually obtained with electroporation.
- Use competent cells that are known to be transformed with a very high efficiency. (Both chemically competent and electrocompetent cells are available from Clontech.)

b. If you try the measures recommended in Section 4.a above and still do not recover any *E. coli* transformants the problem may be the plasmid preparation or the plasmid itself.

- The yeast plasmid preparation may have no plasmid DNA in it. Check the medium you used for the overnight cultures. It is important to use a medium that maintains selection on the desired plasmid. The working stock plate used as your inoculum source should also keep selection on the plasmid. When you repeat the plasmid isolation procedure, be sure to include the freeze/thaw cycle at Step VII.B.5 to ensure complete cell lysis.
- Check the concentration of total DNA in your plasmid prep using absorbance at 260 nm or by running a small sample (10 µl) on a gel. Although plasmid DNA makes up only a small fraction of the total DNA, you can at least confirm that you have DNA in your prep. The larger chromosomal DNA fragments should be visible on a 1% agarose/EtBr gel. (The limit of detection with EtBr staining is ~4 ng [Sambrook *et al.*, 1989, Appendix E.5].)
- Even if you have a substantial amount of DNA in your prep, there is a remote possibility that the plasmid of interest has integrated into the yeast chromosome and therefore cannot replicate autonomously when introduced into *E. coli*. If the plasmid's insert can be amplified by PCR (Chapter VIII), it may be possible to recover the insert by subcloning from the PCR product.
- The plasmid may encode a protein that is toxic to *E. coli.* Again, it may be possible to recover the insert by subcloning the PCR-amplified fragment.

VIII. Analysis of Yeast Plasmid Inserts by PCR

A. General Information

Sometimes a two-hybrid library screening results in many, even hundreds, of positive candidate clones. However, a few abundant insert sequences may account for the majority. Sorting colonies into groups will eliminate duplicates bearing the same plasmid insert and will save time in the subsequent analysis. The cDNA inserts from all plasmids encoding candidate interacting proteins can be amplified by PCR and sorted into groups based on restriction digestion patterns. After colonies have been sorted, a representative clone from each group can be transferred to a new master plate for further analysis.

To ensure efficient amplification of all inserts, regardless of size, we strongly recommend the use of long-distance (LD) PCR (Barnes, 1994; Cheng *et al.*, 1994) with the Advantage® 2 Polymerase Mix (Cat No. 639201). The Advantage® 2 PCR Kit (Cat No. 639206) provides a Advantage® 2 Polymerase Mix (which includes TaqStart™ Antibody), a 10X Advantage® 2 PCR Buffer, 50X dNTP, a positive control template, a mix of positive control primers, and a complete User Manual.

Clontech offers PCR primers designed to amplify inserts cloned into Matchmaker Two-Hybrid System vectors. The insert-screening amplimers hybridize to sequences flanking the multiple cloning site (MCS) of the respective vectors. If you purchase Matchmaker LD-Insert Screening Amplimers, we recommend that you use the LD-PCR protocol that accompanies that product. However, LD-Insert Screening Amplimers can also be used in conventional PCR using a single DNA polymerase to amplify inserts up to 3 kb (e.g., Ausubel *et al.*, [1995] Chapters 15.1 & 15.3).

- Matchmaker AD LD-Insert Screening Amplimers (Cat No. 630433) are for amplifying inserts in the GAL4 AD cloning vectors pGAD10, pGAD424, pGAD GL, pGAD GH, pGADT7, pACT, and pACT2.
- Matchmaker pB42AD LD-Insert Screening Amplimers (Cat No. 9108-1) are for amplifying inserts in the LexA system AD cloning vector pB42AD.
- Matchmaker DNA-BD Vector Insert Screening Amplimers (Cat No. 5417-1) are for conventional PCR amplification of inserts in the GAL4 DNA-BD cloning vectors pGBT9, pGBKT7, pAS2, and pAS2-1.
- Matchmaker LexA DNA-BD Insert Screening Amplimers (Cat No. 9109-1) are for conventional PCR amplification of inserts in pLexA and pGilda.

B. Tips For Successful PCR of Yeast Plasmid Templates

1. Optimization of thermal cycling parameters

   The optimal cycling parameters will vary with different templates, primers, experimental protocols, tubes, and thermal cyclers. Refer to the LD-Insert Screening Amplimers User Manual, Ausubel *et al.* (1995), or Roux (1995) for suggestions on optimizing PCR conditions. In some cases, "touchdown" PCR may be needed. We have found that touchdown PCR significantly improves the specificity of many PCR reactions in a wide variety of applications (Don *et al.*, 1991; Roux, 1995). Briefly, touchdown PCR involves using an annealing/extension temperature that is several degrees (typically 3–10°C) *higher* than the T_m of the primers during the initial PCR cycles (typically 5–10). The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles. The change can be performed either in a single step or in increments over several cycles; for example, use 72°C for the first five cycles, 70°C for the next 5 cycles, and 68°C for the remaining cycles.

2. Primer design

   Primer design is the single largest variable in PCR applications and the single most critical factor in determining the success or failure of PCR reactions. For best results, we recommend that you use LD-Insert-Screening Amplimers from Clontech. However, if you design your own primers, be sure to use sequences flanking the MCS. *Always check and recheck your primer design before constructing or ordering primers.*

   Length and G/C content: In general, primers should have a T_m of at least 70°C to achieve

VIII. Analysis of Yeast Plasmid Inserts by PCR *continued*

optimal results in a two-step cycling program with a 68°C annealing/extension step. Therefore, whenever possible, primers should be *at least* 22 nucleotides (nt) long (25–30-mers are preferred) and should have a GC content of 45–60%.

3. Thermostable polymerase

   The Advantage® 2 Polymerase Mixes are designed for LD PCR—i.e., they contain both primary and proofreading polymerases to permit amplification of virtually any insert, regardless of size. If you do not use an Advantage Polymerase Mix, you will need to prepare your own polymerase mix from commercially available, LD PCR-licensed DNA polymerases, such as *Taq* or AmpliTaq®. We also strongly recommend that you include TaqStart Antibody in the polymerase mix, for automatic hot start (see Section B.4 below). (TaqStart Antibody is premixed in the Advantage 2 Polymerase Mix.)

4. Use of antibody-mediated or conventional hot start

   To minimize nonspecific amplification, we strongly recommend that you perform hot start PCR. There are several methods available for hot start PCR, including those using wax beads (Chou *et al.*, 1992) or a manual hot start (D'Aquila *et al.*, 1991). TaqStart Antibody (Cat No. 639250, 639251) provides an automatic hot start when used with *Taq* or KlenTaq DNA Polymerase (Kellogg *et al.*, 1994). Antibody-mediated hot start with TaqStart Antibody is more convenient than manual hot start or wax-bead-mediated hot start, and has been proven to be at least as effective as the conventional methods.

5. Template quality

   a. Because of the exponential nature of PCR amplification, many conventional PCR applications such as screening cDNA inserts work well with templates of average or even low quality, including plasmid DNA isolated from yeast. Use 1-2 µl of yeast plasmid DNA preparation (from Section VII.B) per PCR.

   b. Be sure to use a *single, well-isolated* yeast colony when inoculating liquid cultures for preparation of plasmid from yeast (Chapter VII.B).

   c. If the yeast transformant contains more than one plasmid insert sequence, you may see multiple PCR bands. Restreak the yeast transformant on the appropriate SD medium that maintains selection on the desired plasmid(s) but not on their interactions (Appendix E). The extra generations of growth will allow segregation (i.e., loss) of some of the plasmids. After reconfirming the presence of positive plasmids using a β-gal colony-lift assay, repeat the plasmid isolation and PCR analysis on well-isolated colonies. In some cases, it may be necessary to transform *E. coli* with the yeast plasmid prep, and isolate plasmid from *E. coli* transformants to ensure a homogeneous plasmid preparation (Chapter VII.C).

6. Tips for characterizing PCR products

   a. Electrophorese 10 µl samples of the PCR product on an EtBr/0.8% agarose gel to confirm that the PCR worked and to determine if the plasmid prep contains multiple (nonhomogeneous) plasmids.

   b. Digest another 10-µl sample of each amplified insert with a frequent-cutter restriction enzyme, such as *Alu* I or *Hae* III, in a 20-µl volume reaction. Run these samples on an EtBr/1.8% agarose gel in parallel with DNA size markers for comparison.

7. Good PCR practices

   a. Prepare reactions with dedicated pipettors in a dedicated work space

      Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can produce nonspecific amplification; in some instances, contaminants can cause DNA bands even in the absence of added template DNA. We recommend that you set up your PCR reactions in a dedicated lab area or noncirculating containment hood and use dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. *Perform post-PCR analysis in a separate area with a separate set of pipettors.*

VIII. Analysis of Yeast Plasmid Inserts by PCR *continued*

b. Pipetting

Because of the small volumes used in PCR experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture, deliver the solution, and rinse the pipette tip by pipetting up and down several times.

c. Use a Master Mix

To reduce tube-to-tube variation, use a master mix whenever you set up multiple PCR reactions. If you wish, include the primers in the master mix also. If you are setting up several sets of parallel samples, assemble multiple master mixes (e.g., each with a different set of primers). *The master mix should be thoroughly mixed before use (i.e., vortexed without bubbling).*

d. Always include positive and negative controls (i.e., H_2O instead of DNA template). Positive controls are provided with all of Clontech's Insert Screening Amplimer Sets.

IX. Additional Useful Protocols

A. Yeast Colony Hybridization

Yeast colony hybridization is an efficient way to screen a large collection of library transformants for the presence of an abundant cDNA insert. Duplicate colonies bearing the same library plasmid can then be eliminated from further analysis. We have had success with this modification of the classic protocol of Grunstein and Hogness (1975; Kaiser, *et al.*, 1994; Ausubel *et al.*, 1994). In this procedure, colonies are directly lifted onto a nylon membrane. β-glucuronidase is used to break cell walls.

Reagents and Materials Required

- Appropriate SD agar plates that will keep selection on the plasmid(s) of interest (Appendix E or the system-specific User Manual)
- Labeled cDNA probe complementary to previously isolated cDNAs

  Note: Oligonucleotides, random-primed cDNAs, or PCR-generated fragments can be used as probes. Oligonucleotide probes may be advantageous if the cDNA is a member of a protein family to avoid inadvertently excluding related genes that are not identical to those initially obtained.
- 1 M sorbitol/20mM EDTA/50 mM DTT (prepare fresh)
- 1 M sorbitol/20 mM EDTA
- 0.5 M NaOH
- 0.5 M Tris-HCl (pH 7.5)/6X SSC (Ausubel *et al.*, 1994)
- 2X SSC (Ausubel *et al.*, 1994)
- 100,000 units/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma Cat No. G-7017)
- 82-mm circular nylon membrane, sterile
- Whatman 3 MM paper
- 80°C vacuum oven *or* UV cross-linker
- Additional reagents and equipment for bacterial filter hybridization (Ausubel *et al.*, 1994)

1. If you have not done so already, collect the colonies to be screened onto a master plate in a grid pattern to facilitate future identification of the colonies. Include a positive and negative control on each plate. Since this will be your master plate, it is important to use the appropriate SD agar medium to maintain selection on all plasmids (including any reporter plasmid). Incubate plate at 30°C for 2–4 days until colonies appear.
2. Prepare sorbitol/EDTA/DTT solution.
3. For each plate of colonies to be screened, presoak a Whatman 3 MM paper in the sorbitol/EDTA/DTT solution.
4. Using forceps, place a sterile, prelabeled, dry nylon membrane over the surface of the plate of colonies to be assayed. Gently rub the membrane with the side of the forceps to help colonies cling to the membrane.
5. Poke holes through the membrane into the agar in three or more assymetric locations to orient the membrane to the master plate.
6. When the membrane has been evenly wetted, carefully lift it off the agar plate with forceps and allow it to air dry briefly (~5 min). Place membrane, colony-side-up, on a presoaked sheet of Whatman 3 MM paper (from Step 3 above) and incubate for ~30 min.
7. Optional: Place membranes at –70°C for 5 min, then thaw at room temperature for one or more cycles to facilitate the disruption of the cell walls.
8. Dilute the β-glucuronidase 1:500 in sorbitol/EDTA. Use 2 µl (of the 100,000 units/ml β–glucuronidase stock) per ml of sorbitol/EDTA to give a final concentration of 200 units/ml). Allow 3–5 ml of diluted β-glucuronidase per filter to be screened.
9. For each membrane to be screened, cut another piece of Whatman 3 MM paper to fit inside a 100-mm petri dish. Place the paper disc in the dish containing the diluted β-glucuronidase to saturate the paper. Remove excess liquid.
10. Carefully layer the nylon membrane, colony side up, on top of the β-glucuronidase-soaked filter. Avoid trapping air bubbles in between the two layers. Cover the dish. Incubate the membrane on the filter for up to 6 hr at 37°C until >80% of the cells lack a cell wall.

IX. Additional Useful Protocols *continued*

Note: The extent of cell wall removal can be determined by removing a small quantity of cells from the filter to a drop of sorbitol/EDTA on a microscope slide, and observing directly with a phase-contrast microscope at ≥60X magnification. Cells lacking a cell wall are nonrefractile.

11. Place membrane on Whatman 3 MM paper saturated with 0.5 M NaOH for 8–10 min.
12. Place membrane on Whatman 3 MM paper saturated with 0.5 M Tris-HCl (pH 7.5)/6X SSC for 5 min. Repeat step 12 with a second sheet of presoaked Whatman 3 MM paper.
13. Place membrane on Whatman 3 MM paper saturated with 2X SSC for 5 min. Then place membrane on dry Whatman paper to air dry for 10 min.
14. Bake membrane at 80°C for 90 min in a vacuum oven or UV cross-link.
15. Proceed as for bacterial filter hybridization (Ausubel *et al.*, 1994).

B. Generating Yeast Plasmid Segregants

For some applications, it is useful to generate a segregant strain that has only a single type of plasmid from yeast cotransformants containing more than one kind of plasmid. There are several ways this can be accomplished. The most reliable but also most time-consuming way is to isolate the mixed plasmid DNA from yeast, use it to transform *E. coli*, isolate the desired plasmid from *E. coli* transformants, and transform the desired yeast host strain with the isolated plasmid DNA. Alternatively, the yeast cotransformant strain can be grown for several generations on SD medium that maintains selection on the desired plasmid only, as described in Section B.1 below. The search for yeast segregants can be significantly accelerated if you are working with a cycloheximide-resistant yeast host strain and the unwanted plasmid confers sensitivity to cycloheximide, as described in Section B.2 below. Cycloheximide counterselection is an option with the Matchmaker Two-Hybrid System 2 (Cat No. K1604-1), but cannot be used with the host strains provided with Pretransformed Matchmaker Libraries or the original Matchmaker System (Cat No. K1605-1).

1. Segregation by natural loss of an unselected plasmid
   a. Culture individual cotransformant colonies (separately) in 3 ml of the appropriate SD liquid selection medium for 1–2 days at 30°C with shaking (230–250 rpm). The medium must maintain selection on the plasmid of interest, but not on the plasmid you wish to lose. Under these conditions, the plasmids that are not selected for are lost at a rate of 10–20% per generation. Refer to Appendix E for information on yeast plasmid transformation/selection markers.
   b. Spread a diluted sample of this liquid culture on agar plates that will select for the desired plasmid.

IX. Additional Useful Protocols *continued*

c. Incubate the plate at 30°C for 2–3 days or until colonies appear.

d. Using sterile toothpicks or pipette tips, transfer 20–30 individual colonies (in an orderly grid fashion) to appropriate SD selection plates to verify that they have lost the unwanted plasmid and retained the plasmid of interest.

Note: Store the yeast segregants on the appropriate SD selection plates wrapped in Parafilm at 4°C for up to two weeks.

2. Cycloheximide counterselection of yeast segregants

Some yeast host strains, such as CG-1945 and Y190, carry the *cyh^{r}2* mutant allele and are cycloheximide resistant (Cyh^R; C. Giroux, personal communication, for CG-1945, and Harper *et al.*, 1993, for Y190). The wild-type *CYH^{s}2* gene is dominant to the *cyh^{r}2* mutant allele. Thus, when transformed with a plasmid such as pAS2-1 that contains the wild-type *CYH^{s}2* gene, the host strain will become sensitive to cycloheximide; this holds true for a Cyh^R host strain cotransformed with a *CYH^{s}2*-bearing plasmid and another plasmid that does not carry the *CYH^{s}2*. gene. Therefore, one can effectively select for yeast cells that have spontaneously lost the *CYH^{s}2*-bearing plasmid while retaining the other plasmid, simply by plating the cotransformants on the appropiate SD medium containing cycloheximide.

Note: The *CYH2* gene encodes the L29 protein of the yeast ribosome. Cycloheximide, a drug which blocks polypeptide elongation during translation, prevents the growth of cells that contain the wild-type *CYH2* gene. Cycloheximide resistance results from a single amino acid change in the CYH2 protein. Cells containing both the sensitive (wild-type) and the resistant (mutant) *CYH2* alleles fail to grow on medium containing cycloheximide. Therefore, the loss of a *CYH2*-containing plasmid can be selected for directly if the host carries the resistant allele chromosomally (Guthrie & Fink [1991], pp 306–307).

a. From each of the restreaked (Cyh^S) cotransformants of interest, pick a colony, 1–3 mm in diameter, and resuspend it in 200 µl of sterile H_2O. Vortex thoroughly to disperse the cells.

Note: Do not patch or streak cells from the colony over to the cycloheximide-containing medium. Cells transferred in this way are at too high a density for the cycloheximide selection to work.

b. Spread 100 µl of the cell suspension onto an SD/–Leu/+cycloheximide plate. Also spread 100 µl of a 1:100 dilution.

Note: The concentration of cycloheximide to use in the medium depends on the host strain. For example, use 1.0 µg/ml for CG-1945; 10.0 µg/ml for Y190.

c. Incubate the plate at 30°C until individual Cyh^R colonies appear. (This usually takes 3–5 days.)

d. Transfer the Cyh^R colonies to appropriate SD selection plates to verify that they have lost the *CYH^{s}2*-bearing plasmid and retained the plasmid of interest. Refer to Appendix E for information on yeast plasmid transformation/selection markers.

Note: These yeast clones are referred to as **Cyh^R segregants**. Store them on the appropriate SD selection plates wrapped in Parafilm at 4°C for up to two weeks.

C. Yeast Mating

Yeast mating is a convenient method of introducing two different plasmids into the same host cells, and, in some applications, can be used as a convenient alternative to yeast cotransformations (Bendixen *et al.*, 1994; Harper *et al.*, 1993; Finley & Brent, 1994). See Guthrie & Fink (1991) or Pringle *et al.* (1997) for information on the biology of yeast mating. The following small-scale protocol works well for creating diploids by yeast mating. If you wish to screen a Pretransformed Matchmaker Libary using yeast mating, please refer to the User Manual provided with those libraries for an optimized, library-scale mating protocol.

1. Preparation for yeast mating

a. If you have not done so already, generate an appropriate yeast strain containing the plasmid of interest.

b. Transform the chosen mating partner separately with the plasmids you wish to test in combination with the plasmid of interest. Be sure to include transformations with the appropriate negative and positive control plasmids, if applicable.

IX. Additional Useful Protocols *continued*

c. Select for transformants on the appropriate SD dropout medium.

d. For each plasmid of interest to be tested, set up pairwise yeast matings with transformants containing control plasmids. Use either the standard procedure (Section C.2) or the procedure adapted for microtiter (96-well) plates (Section C.3).

2. Yeast mating procedure (standard)

   a. Pick one colony of each type to use in the mating. Use only large (2–3-mm), fresh (<2-months old) colonies from the working stock plates.

   b. Place both colonies in one 1.5-ml microcentrifuge tube containing 0.5 ml of YPD medium. Vortex tubes to completely resuspend the cells.

   c. Incubate at 30°C overnight (20–24 hr) with shaking at 200 rpm.

   d. Spread 100-µl aliquots of the mating culture on the appropriate SD minimal media. Use double dropout to select for both plasmids and triple dropout to select for diploids in which a positive two-hybrid interaction is occurring. Proceed to step 4 below.

3. Yeast mating procedure (microtiter plate version)

   If you have many plasmids of interest to mate to several control strains, it may be more efficient to set up the matings in separate wells of a sterile, flat-bottom microtiter plate. In between steps, keep plate covered with a sterile lid.

   a. Aliquot 160 µl of YPD medium to each well.

   b. For each plasmid of interest to be tested, place a single transformant colony in a 1.5-ml microcentrifuge tube containing 1 ml of YPD. Vigorously vortex the tube to disperse the cells.

   c. For each type of control plasmid to be used, place several transformant colonies in 3 ml of YPD in a sterile, 10-ml conical tube. Vigorously vortex the tube to disperse the cells.

   d. Aliquot 20 µl of the cell suspension from Step 3.b into each well of a vertical column. Use a separate column for each plasmid of interest to be tested.

   e. Aliquot 20 µl of the cell suspension from Step 3.c into each well of a horizontal row. Use a separate row for each type of control plasmid.

   f. Place plate on a rotating platform shaker and incubate at 30°C for 6–18 hr at 200 rpm.

      Note: Do not rotate at a higher speed or the medium will spill out of the wells.

   g. Spread 100 µl of each mating culture on 100-mm plates containing the appropriate SD minimal medium and proceed to next step.

4. Incubate plates at 30°C for 3–5 days to allow diploid cells to form visible colonies.

5. Score for growth on the SD agar plates.

6. Confirm nutritional and reporter phenotypes of diploids

   To detect (or reconfirm) protein-protein interactions, assay the fresh diploid colonies from the SD selection plates (Step 4) above for β-gal activity using the colony-lift filter assay (Section VI.C). Discard any β-gal-positive colonies that contain the candidate library plasmid alone.

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XI. Matchmaker and Related Products

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• Yeastmaker™ Yeast Transformation System	630439
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• Yeastmaker™ Yeast Plasmid Isolation Kit	630441
• YPD Medium	630409
• YPD Agar Medium	630410
• Minimal SD Base (contains glucose)	630411
• Minimal SD Agar Base (contains glucose)	630412
• Minimal SD Base/Gal/Raf (contains galactose & raffinose)	630420
• Minimal SD Agar Base/Gal/Raf (contains galactose & raffinose)	630421
• Dropout (DO) Supplements for use with any SD Base	many
GAL4-based one- and two-hybrid systems and related products	
• Mammalian Matchmaker Two-Hybrid Assay Kit	630301
• Matchmaker One-Hybrid System	630302
• Matchmaker Two-Hybrid System 3	630303
• Matchmaker Co-IP Kit	630449
• pCMV-Myc & pCMV-HA Vector Set	631604
• Matchmaker cDNA & Genomic Libraries	many
• Matchmaker Pretransformed Libraries	many
• Matchmaker AD LD-Insert Screening Amplimer Set	630433
• GAL4 AD Monoclonal Antibody	630402
• GAL4 DNA-BD Monoclonal Antibody	5399-1
• pGBKT7 DNA-BD Vector	630403
• pGADT7 AD Vector	630442
• pLP-GBKT7 DNA-BD Creator™ Acceptor Vector	630406
• pLP-GADT7 AD Creator™ Acceptor Vector	630405
• pBridge™ Three-Hybrid Vector	630404
General cloning reagents	
• QUICK-Clone™ cDNA	many
• Genomic DNA	many
• *E. coli* KC8 Electrocompetent Cells	630435
• *E. coli* KC8 Chemically Competent Cells	630434
Miscellaneous related reagents	
• X-α-Gal	630407
• Luminescent β-gal Detection Kit II	631712
• Advantage® 2 PCR Kit	639206 639207
• Advantage® 2 Polymerase Mix	639201 639202
• CHROMA SPIN™ TE-400 Columns	636076

APPENDIX A. Glossary of Technical Terms

Note: *Many of these terms have other meanings in different contexts. For brevity, we have included only definitions relevant to this Yeast Protocols Handbook.*

allele: One of two or more forms that can exist at a given genetic locus (e.g., *his3-200* is a mutant allele and *HIS3* is a wild-type allele at the *his3* locus). In standard yeast nomenclature, mutant alleles are written in lower case italics, while wild-type alleles are written in upper case italics.

auxotroph: A strain of yeast or other microorganisms that will proliferate only when the medium is supplemented with some specific nutrient not normally required by the organism. For example, Trp^- yeast strains are auxotrophic for tryptophan (Trp); they require Trp in the medium.

***cis*-acting element (or *cis*-acting locus):** A DNA sequence that affects the transcriptional activity of genes located on the same DNA molecule, often via binding of regulatory proteins or factors.

confluent: When yeast or bacterial colonies growing on an agar plate are so numerous that the edges of the colonies touch each other.

clone: (a) A group of genetically identical cells or individuals derived by asexual division from a common ancestor. (b) A heterologous cDNA fragment inserted into a vector; also refers to copies of that original cDNA.

colony: A visible clone of cells growing on solid medium.

diploid: In yeast, a cell having two complete chromosome sets as a result of mating of haploid **a** and α strains. A cell can also be diploid for one particular gene or several genes, due to the presence of plasmids, or as a result of gene duplication.

dropout (DO) supplement: A mixture of several amino acids and nucleosides that must be added to minimal synthetic medium to support the growth of yeast strains that have defined nutritional requirements; typically, one or more specific nutrients is left (or "dropped") out of the DO supplement so that the resulting synthetic dropout (SD) medium will only support the growth of yeast that are able to synthesize that nutrient.

gene: (a) The fundamental physical unit of heredity, recognized through its variant alleles; (b) a DNA sequence that regulates and encodes a functional product, e.g., a polypeptide chain or an RNA molecule.

genetic complementation: The production of a wild-type phenotype when (a) two different mutations are combined in a diploid cell; or (b) when a wild-type allele on a plasmid is introduced into a cell bearing a defective chromosomal allele via yeast mating or transformation.

genome: The entire complement of genetic material in a cell excluding autonomously replicating plasmids and mitochondrial DNA.

genotype: Generally, a list of mutant alleles and exogenous genetic elements. Wild-type alleles are sometimes listed as well for clarity in a specific experimental context.

haploid: A cell having one chromosome set. A diploid cell or organism can also be haploid for a given gene due to chromosomal deletions.

hybridization probe: A defined nucleic acid segment which can be labeled and used to identify specific DNA clones bearing the complementary sequence via hybridization.

leaky mutant: A mutant that represents a partial rather than a complete inactivation of the wild-type function; leaky phenotypes can result from a mutation in the coding region or in the promoter region. In yeast one- and two-hybrid systems, some of the host strains are leaky for expression of certain auxotrophic markers (for example, *HIS3* expression in Y190).

mating types: A genetically haploid state of unicellular organisms that can reproduce sexually by cellular and nuclear fusion to produce a diploid organism. In *S. cerevisiae*, there are two mating types, **a** and α, which differ only physiologically and not in physical form.

mutant: An organism or cell carrying a mutation.

APPENDIX A. Glossary of Technical Terms *continued*

mutant allele: An allele differing from the allele found in the standard or wild type.

mutation: (a) The process that produces a gene or a chromosome differing from the wild type. (b) The DNA or amino acid change resulting from such a process.

operator: In bacteria, a DNA region that acts a binding site for a specific repressor protein and thereby exerts control over transcription of the adjacent structural gene or operon.

operon: In bacteria, a set of adjacent structural genes that are transcribed into a single mRNA molecule, plus the adjacent regulatory genes that affect transcription of the structural genes.

PCR: Polymerase chain reaction; a process by which a defined segment of DNA is exponentially replicated *in vitro* by the action of a thermostable DNA polymerase during repeated cycles of heating and cooling.

phenotype: The observable properties of an organism determined by the organism's genetic constitution (genotype) and the effects of the environment.

plasmid: A genetic element in bacteria or yeast that can replicate autonomously in the host cell. Some plasmids can also be inserted into the host's genome in defined natural or experimental situations, e.g., via transformation of linearized plasmid DNA.

promoter: A DNA sequence to which RNA polymerase complex binds and initiates transcription of an adjacent structural gene or gene cluster. In yeast, the promoter is typically comprised of at least one TATA box and other closely associated *cis*-regulatory elements (e.g., UASs).

prototroph: A strain of yeast or other microorganisms that will proliferate even if a particular nutrient is not supplied in the medium. For example, Trp^- yeast strains are prototrophic for Trp; they can synthesize their own Trp from other biomolecules and do not require it in the medium. A prototrophic transformation marker or reporter gene can be used to complement the corresponding auxotrophic allele in another strain.

segregation: Genetically, the production from a single cell of two daughter cells having distinct genotypes and phenotypes due to the separation of two alleles of a gene. In yeast, this can occur during sporulation or in transformant clones as a result of loss of a plasmid.

***trans*-acting element:** A gene that controls transcriptional activity of another gene through a diffusable gene product (protein) such as a repressor or activator.

transformation: The process of introducing foreign DNA into a cell.

transformation markers: Genetic alleles whose phenotypes identify the presence of a plasmid introduced into a cell; typically, such markers are genes that complement a nutritional requirement or confer resistance to an antibiotic.

UAS: Upstream Activating Sequence; yeast DNA sequences that control the initiation of transcription of adjacent structural genes via binding of specific regulatory proteins. An example is the binding of the yeast GAL4 transcriptional activator (or DNA-BD) to the UAS_G of the *GAL1* promoter.

wild type: The genotype or phenotype of an organism as it is found in nature or in a standard laboratory strain.

APPENDIX B. Yeast Genetic Markers Used in the Matchmaker Systems

TABLE IV. SELECTED YEAST GENES AND THEIR ASSOCIATED PHENOTYPES

	Allele		
Wild type	**Mutant**	**Phenotype of mutant**	
TRP1	*trp1-901*	Trp^-	Requires tryptophan (Trp) in the medium to grow, i.e., is a Trp auxotroph
LEU2	*leu2-3, 112*	Leu^-	Requires leucine (Leu) to grow, i.e., is a Leu auxotroph
HIS3	*his3-200*	His^-	Requires histidine (His) to grow, i.e., is a His auxotroph
URA3	*ura3-52*	Ura^-	Requires uracil (Ura) to grow, i.e., is a Ura auxotroph
LYS2	*lys2-801*	Lys^-	Requires lysine (Lys) to grow, i.e., is a Lys auxotroph
ADE2	*ade2-101*	Ade^-	Requires adenine (Ade) to grow; i.e., is an Ade auxotroph; in addition, confers a pink or red colony color to colonies growing on media low in adenine. The red pigment is apparently an oxidized, polymerized derivative of 5-aminoimidazole ribotide which accumulates in vacuoles (Smirnov *et al.,* 1967; Weisman *et al.,* 1987).
GAL4	*gal4-542* (or *gal4*Δ)	Gal^-	Deficient in regulation of galactose-metabolizing genes (Flick & Johnston, 1990; Johnston *et al.,* 1994)
GAL80	*gal80-538*	Gal^-	Deficient in regulation of galactose-metabolizing genes (GAL genes are constitutively expressed)
CYH^s2	cyh^r2	Cyh^r	Resistant to cycloheximide

TABLE V. MATCHMAKER REPORTER GENES AND THEIR PHENOTYPES

Reporter Gene	Gene Description	Positive Phenotype[a]	Negative Phenotype[a]
lacZ	Encodes β-galactosidase	$LacZ^+$ • Blue colony • β-gal activity above background	$LacZ^-$ • White colony • Undetectable or background level of β-gal activity
HIS3	Confers His prototrophy	His^+ • Grows on SD/–His	His^- • Does not grow on SD/–His[b]
LEU2	Confers Leu prototrophy	Leu^+ • Grows on SD/–Leu	Leu^- • Does not grow on SD/–Leu
ADE2	Confers Ade prototrophy	Ade^+ • Grows on SD/–Ade	Ade^- • Does not grow on SD/–Ade • Pink or red colony color when grown on medium (such as YPD) low in Ade

[a] Relative levels of background expression and reporter gene induction are dependent on the promoter constructs controlling them. See Chapter II for information on the promoters.

[b] 5–60 mM 3-AT may be required to suppress leaky *HIS3* expression in **certain** host strains and transformants and to obtain an accurate His^- phenotype.

APPENDIX C. Media Recipes

A. YEAST MEDIA

- **YPD medium**

  YPD Medium (Cat No. 630409) and YPD Agar Medium (Cat No. 630410) are available in powder form from Clontech. Our YPD Medium is a blend of peptone, yeast extract, and dextrose in optimal proportions for growth of most strains of *Saccharomyces cerevisiae*. See Chapter XI for ordering information. If you purchase Clontech's YPD media, prepare the medium according to the instructions provided. Alternatively, prepare your own YPD mixture as follows:

20	g/L	Difco peptone
10	g/L	Yeast extract
20	g/L	Agar (for plates only)

  - [Optional] For adenine-supplemented YPD (YPDA), add 15 ml of a 0.2% adenine hemisulfate solution per liter of medium (final concentration is 0.003%, in addition to the trace amount of Ade that is naturally present in YPD). Adenine hemisulfate tolerates autoclaving.

  Add H_2O to 950 ml. Adjust the pH to 6.5 if necessary, then autoclave. Allow medium to cool to ~ 55°C and then add dextrose (glucose) to 2% (50 ml of a sterile 40% stock solution). Adjust the final volume to 1 L if necessary.

  Note: If you add the sugar solution before autoclaving, autoclave at 121°C for 15 min; autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the medium. Note that YPD from Clontech already contains glucose.

  - [Optional] For kanamycin-containing medium, prepare YPD or YPDA as above. After autoclaved medium has cooled to 55°C, add 0.2–0.3 ml of 50 mg/ml kanamycin (final concentration 10–15 mg/L).

- **SD medium**

  Minimal SD Base and Minimal SD Agar Base, either with dextrose (glucose), or galactose + raffinose, are available from Clontech in powder form. (See Chapter XI for ordering information.) If you purchase Clontech's Minimal SD Base, prepare your SD/Dropout (DO) medium according to the instructions provided. For example, to prepare SD/–Leu/–Trp agar, you will need to combine SD Minimal Agar Base (Cat No. 630412) with –Leu/–Trp DO Supplement (Cat No. 630412).

  Alternatively, you can purchase yeast nitrogen base from another supplier (e.g., Difco Cat No. 0919-15-3) and prepare SD/DO medium as follows:

6.7	g	Yeast nitrogen base without amino acids
20	g	Agar (for plates only)
850	ml	H_2O
100	ml	of the appropriate sterile 10X Dropout Solution

  - Adjust the pH to 5.8 if necessary, and autoclave. Allow medium to **cool to ~ 55°C** before adding 3-AT, cycloheximide, additional adenine, or X-gal (see below).
  - Add the appropriate sterile carbon source, usually dextrose (glucose) to 2%, unless specified otherwise for your application. Adjust the final volume to 1 L if necessary.

    Notes:

    - If you add the sugar solution before autoclaving, autoclave at 121°C for 15 min; autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the medium. Note that SD Minimal Base from Clontech already contains a carbon source.
    - If you purchase galactose separately, it must be highly purified and contain <0.01% glucose.

  - [Optional] For 3-AT-containing medium, add the appropriate amount of 1 M 3-AT stock solution and swirl to mix well. The concentration of 3-AT used in the medium depends on the yeast strain and, to some extent, on the presence of transforming plasmid(s). See your system-specific User Manual for further information.

    Notes:

    - 3-AT is heat-labile and will be destroyed if added to medium hotter than 55°C.
    - 3-AT, a competitive inhibitor of the yeast *HIS3* protein (His3p), is used to inhibit low levels of His3p expressed in a leaky manner in some reporter strains (Fields, 1993; Durfee *et al.*, 1993).

APPENDIX C. Media Recipes *continued*

- [Optional] For cycloheximide-containing medium, add the appropriate amount of 1 mg/ml cycloheximide stock solution and swirl to mix well. The concentration of cycloheximide used in the medium depends on the yeast strain. See your system-specific User Manual for further information.

  Notes:
  - Cycloheximide is heat-labile and will be destroyed if added to medium hotter than 55°C.
  - Cycloheximide-containing medium is used for selection of yeast strains, such as Y190 and CG-1945, carrying the *cyh^{r}2* allele.
- [Optional] If you wish to add excess adenine to SD medium, add 15 ml of 0.2% adenine hemisulfate solution per liter of medium.
- Pour plates and allow medium to harden at room temperature. Store plates inverted, in a plastic sleeve at 4°C.

- **SD/Gal/Raf/X-gal plates**

  Prepare SD medium as described above except use 725 ml of H_2O and do not adjust the pH. Autoclave, and **cool to ~ 55°C**. Then add:

	Final concentration	To prepare 1 L of medium
Galactose	2%	50 ml of 40% stock
Raffinose	1%	25 ml of 40% stock
10X BU salts	1X	100 ml of 10X stock
X-Gal	80 mg/L	4 ml of 20 mg/ml

  Pour plates and allow medium to harden at room temperature. Store plates inverted, in a plastic sleeve, in the dark, at 4°C for up to two months. Adjust final volume to 1L if necessary.

  Notes:
  - Galactose must be highly purified and contain <0.01% glucose.
  - If the medium is too hot (i.e., >55°C) when the salt solution is added, the salts will precipitate. Also, X-Gal is heat labile and will be destroyed if added to hot medium.
  - **BU salts must be included in the medium** to adjust the pH to ~7, which is closer to the optimal pH for β-galactosidase activity, and to provide the phosphate necessary for the β-gal assay to work.
  - As the plates age, salt crystals will form in the medium. These do not affect the performance of the medium or the results of the β-galactosidase assay.
  - If you are assaying for expression of a *lacZ* reporter gene in a system that requires expression of a protein from an intact yeast *GAL1* promoter (such as in the Matchmaker LexA Two-Hybrid System), you must use 2% galactose + 1% raffinose as the carbon sources instead of glucose. If you are not using Clontech's SD/Gal/Raf Minimal Base, be sure to obtain high-quality galactose that is not contaminated by glucose.

Stock solutions for use with SD Media

- **1 M 3-AT (3-amino-1,2,4-triazole**; Sigma Cat No. A-8056); prepare in deionized H_2O and filter sterilize. Store at 4°C. Store plates containing 3-AT sleeved at 4°C for up to 2 months.
- **10X BU Salts**

  Dissolve the following components in 1 L (total) of H_2O:

  70 g $Na_2HPO_4 \bullet 7H_2O$

  30 g NaH_2PO_4

  Adjust to pH 7, then autoclave and store at room temperature.

- **Carbon sources**, filter sterilized or autoclaved:

  Note: Autoclave at 121°C for 15 min; autoclaving at a higher temperature, for a longer period of time, or repeatedly may cause the sugar solution to darken and will decrease the performance of the medium.

  • **40% Dextrose** (glucose) Store at 4°C.

  • **40% Galactose** (for LexA Two-Hybrid System; D(+) Galactose, e.g., Sigma Cat No. G-0750) Store at 4°C.

  • **40% Raffinose** (for LexA Two-Hybrid System) Store at 4°C.

- **1 mg/ml (1000X) CHX (Cycloheximide**; Sigma Cat No. C-7698); prepare in deionized H_2O, filter sterilize, and aliquot. Store at 4°C for up to 2 months. Store plates containing CHX sleeved at 4°C for up to 1 month.
- **50 mg/ml kan (kanamycin)**; prepare in deionized H_2O, filter sterilize, and aliquot. Store at –20°C for up to 1 month. Store plates containing kan sleeved at 4°C for up to 1 month.

APPENDIX C. Media Recipes *continued*

- **X-gal** (20 mg/ml in DMF) Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide. Store in the dark at –20°C.

- **10X Dropout (DO) Solution**

  A combination of a Minimal SD Base and a DO Supplement will produce a synthetic, defined minimal medium lacking one or more specific nutrients. The specific nutrients omitted depends on the selection medium desired. To prepare SD/–Leu/–Trp agar, for example, combine –Leu/–Trp DO Supplement (Cat No. 630417) with Minimal SD Agar Base (Cat No. 630412). (With this naming convention [e.g., SD/–Leu/–Trp], if a nutrient is not indicated as missing, it is assumed to be present in the medium.) Many of the commonly used DO Supplements can be purchased from Clontech. Instructions for preparing the corresponding SD/DO medium are printed on the products' labels.

  Alternatively, make your own dropout supplement by combining the nutrients listed below at the concentrations indicated to prepare a **10X Dropout Solution**. A 10X Dropout Solution contains all but one or more of these nutrients. Note that serine, aspartic acid, and glutamic acid are not included in this list because they make the medium too acidic and because yeast can synthesize these amino acids endogenously. However, if you wish to select for yeast strain AH109 using medium that lacks methionine (i.e., on SD/–Met), you should add aspartic acid to the 10X DO Solution—a 10X solution of aspartic acid is 1000 mg/L.

  10X dropout supplements may be autoclaved and stored at 4°C for up to 1 year.

Nutrient	10X Concentration	Sigma Cat. No.
L-Adenine hemisulfate salt	200 mg/L	A-9126
L-Arginine HCl	200 mg/L	A-5131
L-Histidine HCl monohydrate	200 mg/L	H-8125
L-Isoleucine	300 mg/L	I-2752
L-Leucine	1000 mg/L	L-8000
L-Lysine HCl	300 mg/L	L-5626
L-Methionine	200 mg/L	M-9625
L-Phenylalanine	500 mg/L	P-2126
L-Threonine	2000 mg/L	T-8625
L-Tryptophan	200 mg/L	T-0254
L-Tyrosine	300 mg/L	T-3754
L-Uracil	200 mg/L	U-0750
L-Valine	1500 mg/L	V-0500

  Example: To make one liter of 10X –Leu/–Trp DO Solution, combine the following:

  - 200 mg adenine hemisulfate
  - 200 mg arginine HCl
  - 200 mg histidine HCl monohydrate
  - 300 mg isoleucine
  - 300 mg lysine HCl
  - 200 mg methionine
  - 500 mg phenylalanine
  - 2000 mg threonine
  - 300 mg tyrosine
  - 200 mg uracil
  - 1500 mg valine

  1 L Dissolve components in 1 L deionized H_2O. Autoclave.

APPENDIX C. Media Recipes *continued*

B. *E. coli* MEDIA

- **Hanahan's SOC Medium**

	Final concentration	To Prepare One Liter
Bactotryptone	2%	20 g
Yeast extract	0.5%	5 g
NaCl	10 mM	10 ml of 1 M NaCl
KCl	2.5 mM	2.5 ml of 1 M KCl
$MgCl_2$*	10 mM	10 ml of 1 M $MgCl_2$• 6 H_2O
$MgSO_4$*	10 mM	10 ml of 1 M $MgSO_4$ • 7H_2O
Glucose*	20 mM	20 ml of 1 M glucose
Deionized H_2O	to 1 L	

* Before adding $MgCl_2$, $MgSO_4$, and glucose stock solutions, separately filter sterilize them using a 0.2-µm filter.

Add the bactotryptone, yeast extract, and NaCl to 900 ml of deionized H_2O; stir or shake until solutes have dissolved. Add the KCl. Adjust the pH to 7 with 5 N NaOH (~0.2 ml). Adjust the volume to 960 ml with deionized H_2O and autoclave. Just before use, add filter-sterilized $MgCl_2$, $MgSO_4$, and glucose.

- **LB broth**

Bacto-tryptone	10 g/L
Bacto-yeast extract	5 g/L
NaCl	5 g/L

Adjust pH to 7.0 with 5 N NaOH. Autoclave. Store broth at 22°C.

- **LB/amp agar plates**

Prepare LB broth (Sambrook *et al.*, 1989) as above. Add agar (18 g/L), autoclave, and cool to 50°C. Add ampicillin to 50 µg/ml. Pour plates and store at 4°C.

- **M9 minimal medium** for nutritional selection of *E. coli* transformants complemented by the wild-type yeast gene. For optimal recovery of KC8 and HB101 transformants, add a 1X mixture of amino acids (i.e., dropout [DO] supplement) lacking the specific nutrient that will allow selection of the desired plasmid. (The same DO supplements used for yeast SD medium can be used to supplement M9 minimal medium; see Appendix C.A for dropout recipe or purchase premixed DO Supplements from Clontech.) In addition, KC8 requires thiamine, and HB101 requires thiamine and proline, for growth on minimal medium.

  Prepare 900 ml of M9 medium as directed in Sambrook *et al.* (1989). To prepare agar plates, add agar (20 g/L) prior to autoclaving. After autoclaving, allow medium to cool to 55°C. Then add the following:
  - 1 ml of 50 mg/ml ampicillin stock
  - 1 ml of 1.0 M thiamine-HCl stock
  - 100 ml of an appropriate sterile 10X DO stock solution

  In addition, for HB101 cells only:
  - 4 ml of a 10 mg/ml stock of proline
- **Stock solutions for use with M9 or LB media**

  Ampicillin (50 mg/ml in H_2O). Store at 4°C no longer than 1 month.

  Thiamine-HCl (1 M, filter-sterilized)

  Proline (10 mg/ml, filter sterilized)

  10X DO stock solution (Appendix C.A)

APPENDIX D. Solution Formulations

A. For Preparation of Protein Extracts

- **Protease Inhibitor Solution** (concentrated)

  Always prepare solution fresh just before using. Place on ice to prechill.

		To prepare 688 µl:	Type of protease(s) inhibited:
Pepstatin A (Sigma Cat No. P4265)	0.1 mg/ml	66 µl of a 1 mg/ml stock solution* in DMSO	Carboxyl proteases
Leupeptin (Sigma Cat No. L2884)	0.03 mM	2 µl of a 10.5 mM stock solution*	Some thiol and serine proteases
Benzamidine (Sigma Cat No. B6506)	145 mM	500 µl of a 200 mM stock solution*	Trypsin, plasmin, and thrombin
Aprotinin (Sigma Cat No. A6279)	0.37 mg/ml	120 µl of a 2.1 mg/ml stock solution*	Some serine proteases

* Store the individual stock solutions as directed on the labels and follow label precautions.

- **PMSF (phenylmethyl-sulfonyl fluoride) stock solution** [100X*]

  Dissolve 0.1742 g PMSF (Sigma Cat No. P7626) in 10 ml isopropanol. Wrap tube in foil and store at room temperature. PMSF primarily inhibits serine proteases.

  * Although this is a 100X stock solution, the final concentration of PMSF is greater than 1X in some mixtures, i.e., PMSF is used in excess.

  Caution: PMSF is hazardous. Wear gloves. Handle with care and read label precautions.

- **Glass Beads** (425–600 µm; Sigma Cat No. G-8772)

For Urea/SDS Protein Extraction Method:

- **Cracking buffer stock solution**

			To prepare 100 ml:
Urea	8	M	48 g
SDS	5%	w/v	5 g
Tris-HCl [pH6.8]	40	mM	4 ml of a 1 M stock solution
EDTA*	0.1	mM	20 µl of a 0.5 M stock solution
Bromophenol blue	0.4	mg/ml	40 mg
Deionized H_2O			To a final volume of 100 ml

* EDTA primarily inhibits metalloproteases.

- **Cracking buffer** (complete): The following recipe is sufficient for one protein extract. Scale-up recipe as required.

  Prepare only the volume you need just before use.

  Because PMSF has a short half-life (~7 min) in aqueous solutions, you may need to add additional aliquots of PMSF during the course of the procedure. The initial excess PMSF in the Cracking buffer quickly degrades.

	To prepare 1.13 ml of complete Cracking buffer:
Cracking buffer stock solution	1 ml (recipe above)
β-mercaptoethanol	10 µl
Protease inhibitor solution	70 µl, prechilled (recipe above)
PMSF	50 µl of 100X stock solution

APPENDIX D. Solution Formulations *continued*

For TCA Protein Extraction Method:

- **20% w/v TCA in H_2O** (Store at 4°C; See Sambrook *et al*. [1989] for tips on preparing TCA solutions.)

- **TCA Buffer**

  Place on ice to prechill before use.
  Add the protease inhibitor solution and pMSF immediately prior to use.

			To prepare 10 ml of TCA buffer:
Tris-HCl (pH 8)	20	mM	200 µl of a 1 M stock solution
Ammonium acetate	50	mM	66.6 µl of a 7.5 M stock solution
EDTA	2	mM	40 µl of a 0.5 M stock solution
Deionized H_2O	9.7	ml	
Protease inhibitor solution	50	µl/ml	500 µl, prechilled (recipe above)
PMSF			100 µl of 100X stock solution

- **SDS/glycerol stock solution**

			To prepare 12 ml:
SDS	7.3%	w/v	3.5 ml of a 25% stock solution
Glycerol	29.1%	v/v	3.5 ml of 100%
Tris-base	83.3	mM	1.0 ml of a 1 M stock solution, not pH-adjusted
Bromophenol blue			Spatula tip-full
Deionized H_2O			To a final volume of 12 ml

- **Tris/EDTA solution**

			To prepare 10 ml:
Tris-base	200	mM	2.0 ml of 1 M stock solution, not pH-adjusted
EDTA	20	mM	0.4 ml of a 0.5 M stock solution
Deionized H_2O			7.6 ml

- **TCA-Laemmli loading buffer**

  Prepare fresh just prior to use.

	To prepare 1 ml:	
SDS/glycerol stock solution	480 µl	(Stock solution may need to be warmed to 60°C to reliquefy)
Tris/EDTA solution	400 µl	(Recipe above)
β-mercaptoethanol	50 µl	
PMSF	20 µl	PMSF stock solution (100X)
Protease inhibitor solution	20 µl	Prechilled (recipe above)
Deionized H_2O	30 µl	

APPENDIX D. Solution Formulations *continued*

B. For Transformation of Yeast

- **Herring testes carrier DNA (10 mg/ml)**

  Sonicated, herring testes carrier DNA in solution can be purchased separately (Cat No. 630440; see Chapter XI for ordering information), or can be prepared using a standard method (Sambrook *et al.*, 1989). Just prior to use, denature the carrier DNA by placing it in a boiling water bath for 20 min and immediately cooling it on ice. **Use only high-quality carrier DNA; nicked calf thymus DNA is not recommended.**

- **PEG/LiAc solution** (polyethylene glycol/lithium acetate)

  Prepare fresh just prior to use.

Final Conc.	To prepare 10 ml of solution	
PEG 4000	40%	8 ml of 50% PEG
TE buffer	1X	1 ml of 10X TE
LiAc	1X	1 ml of 10X LiAc

- **Stock solutions**

  50% PEG 3350 (Polyethylene glycol, avg. mol. wt. = 3,350; Sigma Cat No. P-3640) prepare with sterile deionized H_2O; if necessary, warm solution to 50°C to help the PEG go into solution.

  100% DMSO (Dimethyl sulfoxide; Sigma Cat No. D-8779)

  10X TE buffer: 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5. Autoclave.

  10X LiAc: 1 M lithium acetate (Sigma Cat No. L-6883) Adjust to pH 7.5 with dilute acetic acid and autoclave.

C. For β-galactosidase Filter Assays

- **Z buffer**

$Na_2HPO_4 \cdot 7H_2O$	16.1	g/L
$NaH_2PO_4 \cdot H_2O$	5.50	g/L
KCl	0.75	g/L
$MgSO_4 \cdot 7H_2O$	0.246	g/L

  Adjust to pH 7.0 and autoclave. Can be stored at room temperature for up to 1 year.

- **X-gal stock solution**

  Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL; Cat No. 8060-1) in N,N-dimethylformamide (DMF) at a concentration of 20 mg/ml. Store in the dark at –20°C.

- **Z buffer/X-gal solution**

100	ml	Z buffer
0.27	ml	β-mercaptoethanol (β-ME; Sigma Cat No. M-6250)
1.67	ml	X-gal stock solution

D. For Liquid β-galactosidase Assays with ONPG as Substrate

- **Z buffer** (see preceding section for recipe)
- **Z buffer with β-mercaptoethanol**
  To 100 ml of Z buffer, add 0.27 ml of β-mercaptoethanol.
- **ONPG (o-nitrophenyl β-D-galactopyranoside;** Sigma Cat No. N-1127**)**
  4 mg/ml in Z buffer. Adjust to pH 7.0 and mix well.
  Notes:
  - ONPG requires 1–2 hr to dissolve.
  - Prepare solution fresh before each use.

APPENDIX D. Solution Formulations *continued*

E. For Liquid β-galactosidase Assays with CPRG as Substrate

- **Buffer 1**

	To prepare 100 ml of solution
HEPES	2.38 g
NaCl	0.9 g
L-Aspartate [hemi-Mg salt; Sigma Cat No. A-9506]	0.065 g
BSA	1.0 g
Tween 20	50.0 µl

Dissolve the above components in 75 ml of deionized H_2O. Adjust pH to 7.25–7.30, then bring volume to 100 ml. Filter sterilize. Store at 4°C for up to 3 months.

- **Buffer 2** (20 ml)

Dissolve 27.1 mg of CPRG in 20 ml of Buffer 1 (final concentration of CPRG is 2.23 mM). Filter sterilize. Store at 4°C in the dark for up to 3 months.

F. For α-Gal Quantitative Assays

- **PNP-α-Gal Solution**

100 mM (*p*-nitrophenyl α-D-Galactopyranoside; Sigma Cat No. N0877) in deionized H_2O

For 10 ml, dissolve 301.3 mg of PNP-α-Gal in 10 ml of deionized H_2O. Filter sterilize.

Notes:
- Prepare solution fresh before each use.
- Keep the *p*-nitrophenyl α-D-Galactopyranoside solid anhydrous. Store in a dessicator at –20°C.

- **10X Stop Solution**

1 M Na_2CO_3 in deionized H_2O (Sigma Cat No. S7795)

- **1X NaOAc**

0.5 M sodium acetate, pH 4.5 (Sigma Cat No. S7545)

- **Assay Buffer**

Prepare Assay Buffer fresh, before each use, by combining 2 volumes 1X NaOAc Buffer with 1 volume PNP-α-Gal Solution [2:1 (v/v) ratio]. Mix well.

TABLE VI. MATCHMAKER TWO-HYBRID SYSTEM CLONING VECTORS

Vector	System[a]	Description[b]	Selection on SD Medium	Size (kb)	Diagnostic R.E. Sites (kb)	GenBank Accession No.	References (Plasmid name in reference)
pACT	MM Libraries	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r, HA epitope tag	–Leu	7.65	*Eco*R I (3.0, 3.05, 1.6)	not available	Durfee *et al.*, 1993; Elledge, pers. comm.
pACT2	GAL4 2H-2 & MM Libraries	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r, HA epitope tag	–Leu	8.1	*Hin*d III (7.3, 0.8)	U29899	Li *et al.*, 1994; Elledge, pers. comm.
pAS2-1[c]	GAL4 2H-2	*GAL4*$_{(1–147)}$ DNA-BD, *TRP1*, amp^r, *CYH^{s}2*	–Trp	8.4	*Hin*d III (4.6, 2.2, 0.9, 0.7)	U30497	Harper *et al.*, 1993
pB42AD	LexA 2H	acidic activator B42, *TRP1*, amp^r, HA epitope tag	–Trp	6.45	*Hin*d III (3.4, 2.1, 0.6, 0.35)	U89961	Gyuris *et al.*, 1993 (pJG4-5)
pGAD10	MM Libraries	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r	–Leu	6.6	*Hin*d III (5.9, 0.7)[d]	U13188	Bartel *et al.*, 1993a
pGAD424	GAL4 2H	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r	–Leu	6.6	*Hin*d III (5.9, 0.7)[d]	U07647	Bartel *et al.*, 1993a
pGAD GH	MM Libraries	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r	–Leu	7.9	*Hin*d III (7.1, 0.5, 0.3)	in submission	van Aelst *et al.*, 1993
pGAD GL	MM Libraries	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r	–Leu	6.9	*Hin*d III (6.1, 0.5, 0.3)	not available	van Aelst *et al.*, 1993
pGADT7	GAL4 2H-3	*GAL4*$_{(768–881)}$ AD, *LEU2*, amp^r, HA epitope tag	–Leu	8.0	*Hin*d III (7.1, 0.8)	in submission	Clontech
pGBKT7	GAL4 2H-3	*GAL4*$_{(1–147)}$ DNA-BD, *TRP1*, kan^r, c-Myc epitope tag	–Trp	7.3	*Hin*d III (4.9, 1.5, 0.9)	in submission	Clontech Louret *et al.*, 1997
pGBT9	GAL4 2H	*GAL4*$_{(1–147)}$ DNA-BD, *TRP1*, amp^r	–Trp	5.5	*Hin*d III (4.6, 0.9)	U07646	Bartel *et al.*, 1993a
pGilda	LexA 2H	LexA$_{(1–202)}$, DNA-BD, *HIS3*, amp^r	–His	6.6	*Hin*d III (0.2, 6.3)	in submission	Golemis *et al.*, 1996 Gimeno *et al.*, 1996
pLexA	LexA 2H	*LexA*$_{(1–202)}$, DNA-BD, *HIS3*, amp^r	–His	10.2	*Hin*d III (5.2, 4.8, 0.2)	U89960	Gyuris *et al.*, 1993 (pEG202)
pBridge	GAL4 2H, GAL4 2H-2, -3	*GAL4*$_{(1–147)}$ DNA-BD, *TRP1*, amp^r	–Trp	6.5	*Hin*d III (5.6, 0.9)	in submission	Tirode *et al.*, 1997

[a] Key to system abbreviations: GAL4 2H-2 = Matchmaker Two-Hybrid System 2 (Cat No. K1604-1); LexA 2H = Matchmaker LexA Two-Hybrid System (Cat No. K1609-1); GAL4 2H = Matchmaker Two-Hybrid System (Cat No. K1605-1); GAL4 2H-3 = Matchmaker Two Hybrid System 3 (Cat No. 630303). MM 1H = Matchmaker One-Hybrid System (Cat No. K1603-1). Some plasmids are also available separately.

[b] Additional vector information, restriction maps, and multiple cloning site (MCS) sequences are provided in the Matchmaker GAL4 Two-Hybrid System Vectors Handbook, the LexA Two-Hybrid System User Manual, and Vector Information Packets (provided with regular and Pretransformed Matchmaker Libraries).

[c] pAS2-1 is a derivative of the plasmid described in this reference; the plasmid was modified at Clontech. The *Eco*R I site is unique in pAS2-1.

[d] pGAD424 is linearized by digestion with *Sal* I; pGAD10 does not contain a *Sal* I site.

TABLE VII. MATCHMAKER TWO-HYBRID SYSTEM REPORTER AND CONTROL PLASMIDS

Vector[a]	System	Description	Selection on SD Medium	Size (kb)	Diagnostic R.E. Sites (kb)	References (Plasmid name in reference)
p8op-lacZ	LexA 2H	*lacZ* under control of $lexA_{op(x8)}$, *URA3*, amp^r	–Ura	~10.3	*Hin*d III (6.3, 2.1, 1.9)	Estojak *et al.*, 1995 (pSH18-34)
pB42AD-T	LexA 2H	SV40 large T-antigen$_{(87–708)}$ in pB42AD, *TRP1*, amp^r	–Trp	8.5	*Hin*d III (3.4, 2.1, 1.0, 0.9, 0.6, 0.5)	Li & Fields, 1993; Chien *et al.*, 1991
pCL1	GAL4 2H & 2H-2	wild-type full-length *GAL4* gene in aYCp50 derivative, *LEU2*, amp^r	–Leu	~15.3	*Hin*d III (~11.2, 2.8, 1.8)	Fields & Song, 1989
pGADT7-T	GAL4 2H-3	SV40 large T-antigen $_{(84–708)}$ in pGADT7, *LEU2*, amp^r	–Leu	10.0	*Xho* I/*Eco*R I (8.0, 2.0)	Clontech
pGBKT7-53	GAL4 2H-3	murine p53$_{(72-390)}$ in pGBKT7, *TRP1*, kan^r	–Trp	8.3	*Bam*HI/*Eco*R I (7.3, 1.0)	Clontech
pGBKT7-Lam	GAL4 2H-3	Human lamin C$_{(66-230)}$ in pGBKT7, *TRP1*, kan^r	–Trp	7.9	*Bam*HI/*Eco*R I (7.3, 0.57)	Clontech
pLAM5′	GAL4 2H	Human lamin C$_{(66–230)}$ in pGBT9, *TRP1*, amp^r	–Trp	6.0	*Hin*d III (4.7, 0.8, 0.6)	Bartel *et al.*, 1993a
pLAM5′-1	GAL4 2H-2	Human lamin C$_{(66–230)}$ in pAS2-1 *TRP1*, amp^r	–Trp	~9.0	*Hin*d III (4.6, 2.2, 0.9, 0.85, 0.4)	Bartel *et al.*, 1993a
pLexA-53	LexA 2H	murine p53$_{(72–390)}$ in pLexA *HIS3*, amp^r	–His	11.1	*Hin*d III (5.7, 5.2, 0.2)	Iwabuchi *et al.*, 1993
pLexA-Lam	LexA 2H	Human lamin C$_{(66–230)}$ in pLexA, *HIS3*, amp^r	–His	10.6	*Hin*d III (5.2, 4.3, 0.9, 0.2)	Bartel *et al.*, 1993a
pLexA-Pos	LexA 2H	*LexA/GAL4* fusion gene, *HIS3*, amp^r	–His	~13.5	*Hin*d III (6.0, 4.5, 3.0)	Golemis *et al.*, 1994 (pSH17-4)
pTD1	GAL4 2H	SV40 large T-antigen $_{(84–708)}$ in pGAD3F, *LEU2*, amp^r	–Leu	~15.0	*Hin*d III (12, 1.3, 1.2, 0.5)	Li & Fields, 1993; Chien *et al.*, 1991
pTD1-1	GAL4 2H-2	SV40 large T-antigen $_{(84–708)}$ in pACT2, *LEU2*, amp^r	–Leu	~10.0	*Hin*d III (7.3, 1.2, 1.0, 0.5)	Li & Fields, 1993;
pVA3	GAL4 2H	murine p53 $_{(72–390)}$ in pGBT9 *TRP1*, amp^r	–Trp	6.4	*Hin*d III (4.6, 1.8)	Iwabuchi *et al.*, 1993
pVA3-1	GAL4 2H-2	murine p53 $_{(72–390)}$ in pAS2-1 *TRP1*, amp^r	–Trp	9.4	*Hin*d III (4.6, 2.2, 1.7, 0.9)	Iwabuchi *et al.*, 1993 Chien *et al.*, 1991

[a] pB42AD-T, pLAM5′, pLAM5′-1, pLexA-53, pLexA-Lam, pTD1-1, and pVA3-1 are derivatives of the plasmids described in the indicated references; plasmids were modified at Clontech.

TABLE VIII. MATCHMAKER ONE-HYBRID SYSTEM CLONING, REPORTER & CONTROL PLASMIDS

Vector[a]	Description[b]	Selection on SD Medium	Size (kb)	Diagnostic R.E. Sites (kb)	Reference
p53HIS	*HIS3* under control of p53 binding sites in pHISi, *HIS3, URA3*, amp^r	**–Ura, –His**	6.7	*Eco*R I/*Xho* I[c] (5.7, 1.0)	Luo *et al.,* 1996
p53BLUE	*lacZ* under control of p53 binding sites in pLacZi, *URA3*, amp^r	**–Ura**	6.4	*Eco*R I/*Xho* I[c] (6.3, 0.1)	Luo *et al.,* 1996
pGAD53m	murine $p53_{(72–300)}$ fused to $GAL4_{(768–881)}$ AD, *LEU2*, amp^r	**–Leu**	7.6	*Eco*R I[d] (7.6)	Luo *et al.,* 1996
pHISi	*HIS3* under control of cloned target element, *URA3,* amp^r	**–Ura, –His**[e]	6.8	*Eco*R I/*Xho* I (5.7, 1.0)[f]	Alexandre *et al.,* 1993
pHISi-1	*HIS3* under control of cloned target element, amp^r	**–His**[e]	5.4	*Eco*R I/*Xho* I (4.4, 1.0)	Alexandre *et al.,* 1993
pLacZi	*lacZ* under control of cloned target element, *URA3,* amp^r	**–Ura**	6.9	*Eco*R I/*Xho* I (6.4, 0.04)[f]	Luo *et al.,* 1996

[a] In the one-hybrid system, a putative recognition sequence (the target DNA sequence) must be cloned into the MCS of one of the reporter plasmids. The construct is then used to generate the necessary yeast reporter strain for detecting specific DNA-protein interactions.

[b] Additional vector information, restriction maps, and multiple cloning site (MCS) sequences are provided in the Matchmaker One-Hybrid System User Manual.

[c] p53HIS and p53Blue are not cut by *Sma* I, which makes it possible to distinguish them from pHISi and pLacZi, each of which have a single *Sma* I site.

[d] pGAD53m is not cut by *Xho* I.

[e] Leaky *HIS3* expression in these plasmids permits its use as a selectable marker on SD/–His (without 3-AT).

[f] In addition, pHISi and pLacZi have a single *Sma* I site, which makes it possible to distinguish them from p53HIS and p53Blue, which are not cut by *Sma* I.

TABLE IX. YEAST REPORTER STRAINS IN THE MATCHMAKER ONE-AND TWO-HYBRID SYSTEMS

Strain	System	Genotype[a]	Reporter(s)[b]	Transformation Markers[c]	References
SFY526	GAL4 2H	*MATa, ura3-52, his3-200, ade2-101, lys2*-801, *trp 1-901, leu2-3, 112, can^r, gal4-542, gal80-538, URA3 : : GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	*lacZ*	*trp1, leu2*	Harper *et al.*, 1993
HF7c	GAL4 2H	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3 : : GAL4 $_{17\text{-mers(x3)}}$-CYC1$_{TATA}$-lacZ*	*HIS3, lacZ*	*trp1, leu2, cyh^{r}2*	Feilotter *et al.*, 1994; C. Giroux, personal communication
Y187	GAL4 2H-2, GAL4 2H-3, & PT Libraries[d]	*MATα, ura3-52, his3-200, ade 2-101, trp 1-901, leu 2-3, 112, gal4Δ, met$^-$, gal80Δ, URA3 : : GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ, MEL1*	*lacZ, MEL1*	*trp1, leu2*	Harper *et al.*, 1993
CG-1945[e]	GAL4 2H-2,	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3 : : GAL4 $_{17\text{-mers(x3)}}$-CYC1$_{TATA}$-lacZ*	*HIS3, lacZ*	*trp1, leu2, cyh^{r}2*	Feilotter *et al.*, 1994; C. Giroux, personal communication
Y190	GAL4 2H-2	*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh^{r}2, LYS2 : : GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, MEL1 URA3 : : GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ*	*HIS3, lacZ, MEL1*	*trp1, leu2, cyh^{r}2*	Harper *et al.*, 1993; Flick & Johnston, 1990
EGY48	LexA 2H	*MATα, ura3, his3, trp1, LexA$_{op\ (x6)}$-LEU2*	*LEU2*	*his3, trp1, ura3*	Estojak *et al.*, 1995
YM4271	LexA 2H & MM 1H	*MATa, ura3-52, his3-200, lys2-801, ade2-101, ade5, trp1-901, leu2-3, 112, tyr1-501,gal4Δ, gal80Δ, ade5 : : hisG*		*his3, trp1*	Liu *et al.*, 1993
PJ69-2A[f]	PT Libraries	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, MEL1*	*HIS3, ADE2, MEL1*	*trp1, ura3, leu2*	James *et al.*, 1996
AH109[f]	GAL4 2H-3	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, MEL1 GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	*HIS3, ADE2, lacZ, MEL1*	*trp1, leu2*	James *et al.*, 1996; Holtz, Unpublished

[a] The *trp1, his3, gal4, and gal80* mutations are all deletions; *leu2–3*, 112 is a double mutation. The *LYS2* gene is nonfunctional in the HF7c and CG-1945. See Chapter II for more information on the promoters of the reporter genes.

[b] See Table V for more information on reporter genes and their phenotypes.

[c] Genes that are used as selection markers in this system.

[d] PT Libraries = Pretransformed Matchmaker Libraries.

[e] CG-1945 is a derivative of HF7c (Feilotter *et al.*, 1994).

[f] The *ade2–101* gene of the precursor strain was replaced (by recombination) with the *GAL2-ADE2* reporter construct.

Notes

Notes