Grow'n'Glow ACE1 Two-Hybrid System 'Complete Kit'

Order #
ACE01

Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Contents

The Grow'n'Glow ACE1 two-hybrid system was
developed by Dr. Thomas Munder at the Hans-Knöll-Institut, Jena, Germany.

General knowledge: Users of the kit should be familiar with basic molecular biology and microbiological techniques!

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1. Introduction

1.1. General

The yeast two-hybrid system[1] or interaction trap[2] has rapidly become a widely used technique in molecular biology. It is now the method of choice to identify protein-protein interactions from either cDNA libraries or known gene sequences[3]. The method relies on the transactivation of reporter genes in *Saccharomyces cerevisia*e to identify positive interactions. In common two hybrid systems, growth selection via activation of either a HIS3 or LEU2 reporter gene is used in conjunction with a second reporter gene, such as LacZ, which expresses β-galactosidase. The use of two reporter genes enables discrimination of false positives which activates only the auxotrophic marker. Yeast colonies that survive the growth selection scheme are chosen, re-streaked and tested for expression of the LacZ gene by means of a filter assay or growth on minimal medium plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). This latter step can often be time-consuming depending on the number of primary transformants obtained.

In order to simplify and accelerate the screening process of the two-hybrid systems and improve selection of positive clones, a yeast strain was constructed containing both the gene encoding the green fluorescent protein (GFP) from the bioluminescent cnidarian *Aequorea victoria*[4] and the metallothionein encoding CUP1 gene[5].

1.2. Background

MoBiTec's ACE1-based Grow'n'Glow yeast two-hybrid system is a version of a system originally developed by Fields and Song[1]. The yeast two-hybrid system has proven to be a powerful tool for identifying proteins from an expression library which can interact with one's protein of interest. The technology is based on the fact that many eukaryotic transcriptional activators, such as GAL4, consist of two physically separable modular domains, one DNA-binding domain and one transcriptional activation domain. In order to activate transcription, they have to be physically tethered to one another, and neither of them can activate transcription on its own. The same basic idea is followed in ACE1-based yeast two-hybrid systems where the DNA-binding and transcriptional activation domains of the yeast transcription factor ACE1 are used[6,7]. Binding of this transcription factor to DNA is dependent on the presence of copper ions that induce a conformational change in the N-terminus of ACE1, allowing the protein to interact with DNA[5].

The conventional reporter gene generally used with yeast two-hybrid systems is β-galactosidase.

The new Grow'n'Glow ACE1 System was developed at the Hans Knöll Institute in Jena as a more versatile and more accurate version of the yeast two-hybrid system, since it is using the reporter gene GFP. The practical advantages of this fluorescent protein over β-galactosidase are substantial. In a typical β-Gal screen, individual surviving yeast colonies are picked and then tested for β-galactosidase activity either by a filter assay or growth on minimal medium plates containing X-Gal. Both procedures are often labor-intensive, especially when hundreds of yeast colonies are obtained. In contrast, when GFP is used as the reporter, the selection plates containing the yeast colonies are simply placed under a UV lamp (without the lid!) and positives become immediately identifiable

by their green fluorescence. In summary, the GFP gene, stably integrated in the yeast chromosome, is a valuable addition to the family of reporter genes for two-hybrid systems and makes a secondary screening of yeast colonies faster and more cost-effective than conventional LacZ assays. Detection of protein-protein interactions via the green fluorescent protein provides global screening of colonies without bias and can be adapted to most yeast-based two-hybrid strategies. In addition, the copper resistance-mediating CUP1 reporter gene, also stably integrated in the yeast chromosome, allows a more stringent and even modulatory selection of positive clones on media containing copper ions.

Discrimination between strong or weak protein interactions at an early step can be achieved by different copper concentrations in the medium. Since only those cells expressing well-interacting proteins can endure the toxicity of copper ions, the stringency of the CUP1-mediated growth selection can be modulated by different concentrations of $CuSO_4$. Strong interactors should be distinguished readily from weak interactors by comparing CUP1 expression: the growth of yeast cells expressing low-affinity interacting proteins will be much more depressed on copper-containing medium than that of cells expressing proteins with a high binding affinity to one another. Furthermore, weak interactors determine weak GFP expression: the GFP signal of those cells is greatly reduced compared to cells expressing strongly interacting hybrid proteins.

Furthermore, this novel two-hybrid system is also suited for the development of high-throughput screening assays to screen for compounds affecting the interaction between therapeutic relevant proteins. Since the GFP reporter expression is initiated solely by the addition of copper ions (binding of the ACE1BD to DNA is dependent on the presence of copper ions!), incubation of cells with an inhibitory drug followed by the addition of copper should give clearly reduced GFP activities. In non-inducible systems, the reporter gene (e.g. β-galactosidase) is expressed during the whole fermentation process of the yeast cells before the addition of the inhibitory compound. Thus, the already existing level of the reporter protein makes it difficult to measure any inhibitory effect.

1.3. GFP

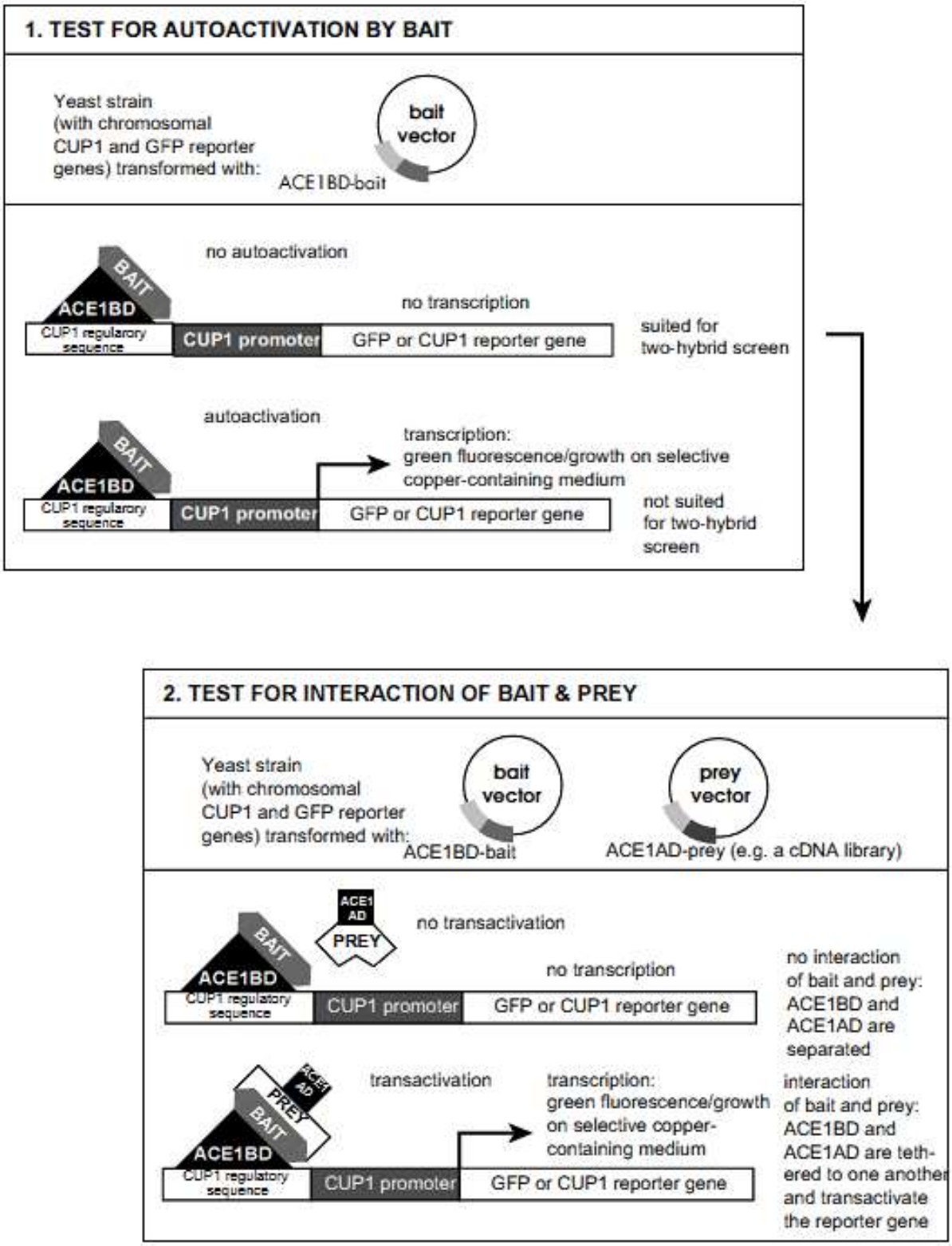
The GFP of the jellyfish *A. victoria* is activated *in vivo* by an energy transfer via the Ca^{2+}-stimulation of the photoprotein aequorin[8]. The blue light generated by aequorin excites GFP and results in the emission of green light. GFP itself consists of 238 amino acids (Mr = 27 kDa) and is synthesized as an apoprotein in which post-translational formation of the chromophore occurs in an O_2-dependent manner independent of any other gene products[9,4]. It maximally absorbs light at 395 nm and has an emission peak of 509 nm. The nonsubstrate requirement for GFP activity makes this protein an attractive reporter for gene expression studies and this utility was initially demonstrated in both prokaryotes (*Escherichia coli*) and eukaryotes (*Caenorhabditis elegans*)[9]. It has subsequently been used to monitor gene expression in many organisms including mouse[10],*Drosophila*[11], zebrafish embryos[12], *Arabidopsis*[13] and yeast[14]. In addition to the non-invasiveness of GFP detection (long-wave UV light) the protein is very stable, non-toxic and resistant to photobleaching.

These properties make GFP a viable alternative to traditional reporter genes such as β-galactosidase (LacZ), β-glucuronidase (GUS), chloramphenicol acetyltransferase (CAT) or firefly luciferase which require substrate for their detection. Several modifications of the wild-type GFP cDNA have been engineered with optimized codon usage, improved fluorescence activity and red-shifted variants with altered excitation maxima intended for fluorescence microscopy[4]. The *A. victoria* GFP variant GFPuv is optimized for maximal fluorescence by UV-light excitation making it fluoresce 18 times brighter than wildtype GFP while retaining identical excitation and emission wavelength maxima.

The GFP reporter allows a very sensitive direct detection in living cells without the time-consuming necessity of preparing cell extracts. Clones expressing interacting proteins are easily detected by placing the plate with the colonies under a UV hand lamp in the dark room. Since the detection of the GFP signal does not require cell lysis it allows the monitoring of reporter gene activity as a function of time, especially if a fluorescence spectrophotometer is used for measurement of whole cell fluorescence.

Legend to flow chart on page 7: Identification of molecular interactions with the Grow'n'Glow ACE1 Two-Hybrid System. To test whether the bait protein activates the reporter genes on its own ("autoactivation"), only the bait vector with the target gene istransformed into yeast. (Alternatively, you can co-transform yeast strain ITH5 with the bait vector carrying your target gene and the "empty" prey vector.) Clones growing on medium containing $CuSO_4$ and/or fluorescing green under UV-light are not suitable for a two-hybrid screen. Clones which do not grow on $CuSO_4$-containing medium and which do not fluoresce green, can be further tested for interaction of a bait and a prey protein in a two-hybrid screen. For this purpose, the bait vector and the prey vector (e.g. a cDNA library) are transformed into yeast. If the expressed bait and prey proteins are interacting, the DNA binding and transactivation domains of ACE1 are tethered to one another and transactivate the reporter genes CUP1 and GFP (both integrated in the yeast chromosome).

2. Schematic Overview of the Grow'n'Glow ACE1 System



3. Advantages of the Grow'n'Glow ACE1 System

- protein-protein interaction is detected directly by visualization under UV light
- allows to select for different protein interaction strengths
- greatly reduces amount of time & effort needed to screen a cDNA library
- allows immediate inspection of yeast colonies for gene activation
- non-invasive, chemical-free and cost-free assay
- stable GFP expression; GFP may be used as sole reporter gene
- no requirement for external substrates
- higher threshold for detection of protein-protein interactions
- fewer false positives by stringent selection for copper resistance
- growth selection can be modulated by different $CuSO_4$ concentrations
- suited for the development of high-throughput screening assays

4. Kit Components: "Complete Kit"

The Grow'n'Glow ACE1 Two Hybrid System is offered as a "Complete Kit" for scientists starting to establish the two-hybrid technology.

Grow'n'Glow Two-Hybrid System "Complete Kit":

Vectors*		Primers*
pTY137	5 µg	5'-BAITprimer 500 pmole
pTM114	5 µg	5'-PREYprimer 500 pmole
pTY143	5 µg	3'-PREYprimer 500 pmole
pTM125	5 µg	
pTY139	5 µg	

Host Strain
Yeast strain ITH5 1 ml

Available on request (not included in the kit): pre-transformed strains
- **ITH11** (ITH5 transformed with pTY137 and pTM114)
- **ITH16** (ITH5 transformed with pTY143 and pTM114)
- **ITH17** (ITH5 transformed with pTY137 and pTM125)
- **ITH12** (ITH5 transformed with pTY143 and pTM125)
- **ITH15** (ITH5 transformed with pTY139)

* Before use, reconstitute plasmid DNA in TE buffer (pH 8).

Order information see chapter 10.

NOTE: You may not require all of the components listed below. Read the manual carefully to determine which components will best suit your needs!

4.1. Yeast Strains

The provided yeast strain has the following genotype:
ITH5 (*MATa ura3-52 his3-171 trp1-289 ace1::TRP1 leu2-3,-112 LEU2::pTY99 CUP1^r*)

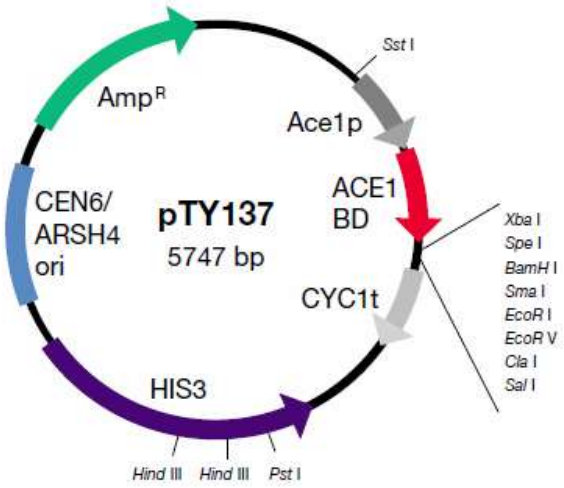
The strain contains a CUP1 gene cassette (multiple copies of the CUP1 gene) as well as the GFP reporter gene (under control of the Cup1 promoter) stably integrated on its chromosome. The CUP1 gene cassette allows growth selection via activation by ACE1BD/ACE1AD as well as a test for autoactivation of a reporter gene by the bait protein.

See chapter 6 for growth and maintenance of yeast.

4.2. DNA Vectors

4.2.1. Bait Plasmid pTY137

The bait vector pTY137 is used to generate fusions of the ACE1 binding domain with a bait (target) protein. Fusion protein expression is controlled by the constitutive Ace1 promoter (Ace1p).
For selection in yeast, the vector contains the HIS3 (histidine) marker and the ars/cen origin of replication. For propagation in *E. coli* an ampicillin resistance (Amp^R) is present. CYC1t, terminator sequences of the cytochrome c1 oxidase gene.



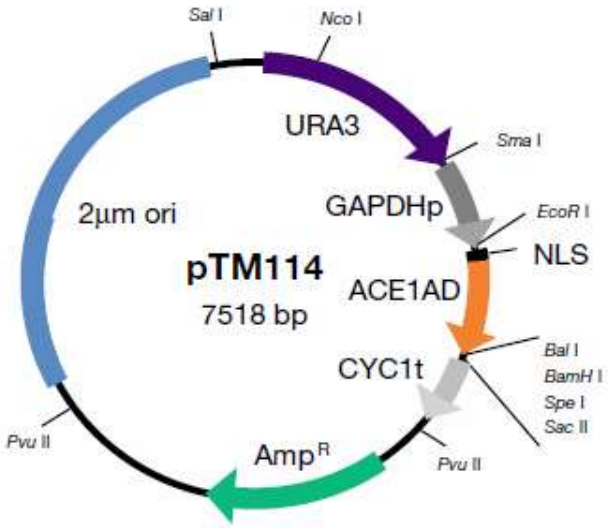
pTY137 polylinker:

AGG **TCT AGA ACT AGT GGA TCC CCC GGG** CTG CAG **GAA TTC GAT ATC** AAG CTT **ATC GAT** ACC **GTC GAC**
XbaI SpeI BamHI SmaI EcoRI EcoRV ClaI SalI

Polylinker of the vector indicating the open reading frame. The shown restriction endonucleases only cut within the polylinker (single cutters).

4.2.2. Prey Plasmid pTM114

The prey vector pTM114 is used to express cDNAs or other coding sequences inserted into the multiple cloning site as translational fusions to a cassette consisting of the SV40 nuclear localization sequence (NLS) and the ACE1 activation domain. Fusion protein expression is controlled by the strong constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. For selection in yeast, the vector contains the URA3 selectable marker and the 2 µm origin of replication; for propagation in *E. coli* an ampicillin (Amp^R) resistance.



pTM114 polylinker:

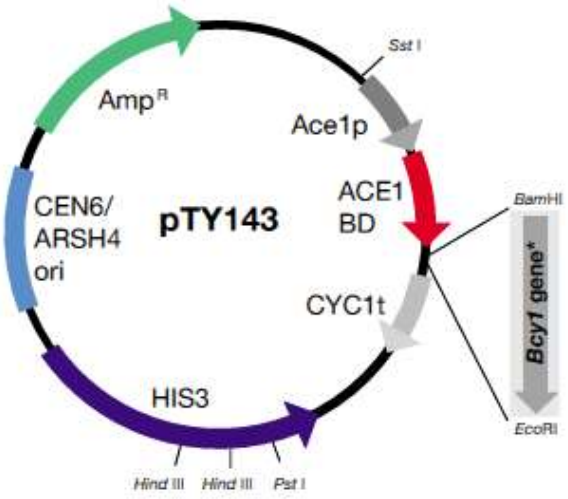
TGG CCA GCG **GAT CCA CTA GT**T CTA GAG CGG CCG CCA **CCG CGG**
BalI BamHI SpeI SacII

Polylinker of the vector indicating the open reading frame. The shown restriction endonucleases only cut within the polylinker (single cutters).

4.2.3. Control Plasmids

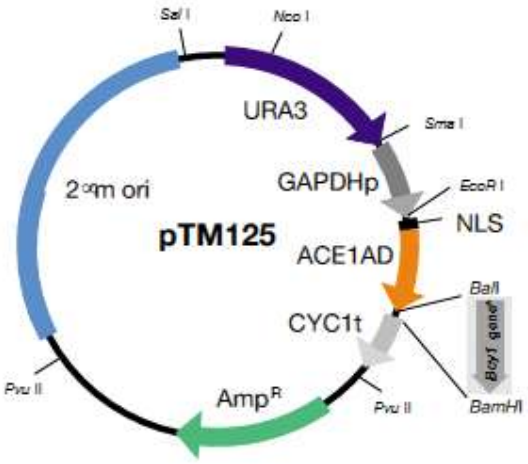
Bait control:

The bait control plasmid pTY143 is a derivative of pTY137 and carries the coding sequences for the N-terminal dimerization part of Bcy1 gene (encoding the regulatory subunit of the yeast cAMP-dependent protein kinase) fused to the ACE1BD. pTM143 serves as positive control in combination with the prey control vector pTM125. Since both control plasmids carry the coding sequences for the interacting N-terminal dimerization part of Bcy1.



Prey control:

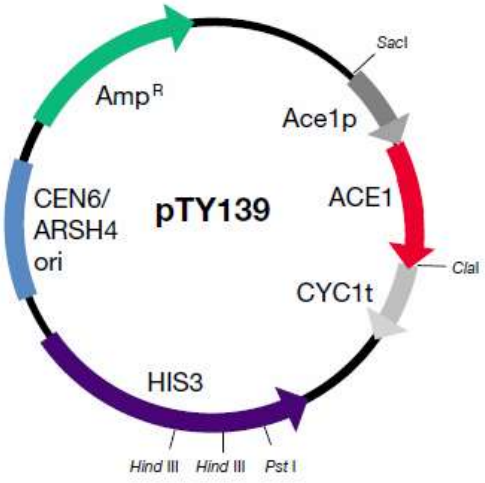
The prey control plasmid pTM125, a derivative of pTM114 also carries the coding sequences for the N-terminal dimerization part of the Bcy1gene (encoding the regulatory subunit of the yeast cAMP-dependent protein kinase) which is here fused to the ACE1AD.



* N-terminal part of the Bcy1 gene

Positive control:

The control plasmid pTY139 can activate transcription of the GFP reporter gene itself and enables its host to grow on copper-containing medium. It contains the entire Ace1 coding sequence (DNA binding and activation domain) under control of the Ace1 promoter and a HIS3 marker.



4.3. Primer

5'-BAITprimer:

5'-ATC AAA GGG AGG GTC ATG-3'

(5' forward primer for sequencing the junction between ACE1BD and the bait gene of pTY137).

5'-PREYprimer:

5'-ACT ACG AAC ACA GCA C-3'

(5' forward primer used to determine the reading frame and identity of positive clones in pTM114; can be used with 3' PREYprimer to amplify clone by PCR*).

3'-PREYprimer:

5'-GAC CTA GAC TTC AGG T-3'

(3' primer used to determine the identity of positive clones in pTM114; can also be used with 5' PREYprimer to amplify a clone by PCR*).

* Polymerase chain reaction: PCR is a patented process owned by Hoffmann-La Roche AG.

5. Materials Required, but not Supplied

Note: The specific materials listed below are the ones we have tested in the Grow'n'Glow System. For order information see chapter 10. Similar items from other sources may be interchangeable.

1. *Grow'n'Glow Yeast Growth Media* (see chapter 5.1.1.)
2. *Grow'n'Glow Bacterial Growth Media* (see chapter 5.1.2.)
3. Ampicillin, Roche Molecular Biochemicals order # 835269
4. For yeast transformations:

   Grow'n'Glow High Efficiency Yeast Transformation Kit (MoBiTec order # 2200-1) or, alternatively, *Grow'n'Glow Fast and Easy Yeast Transformation Kit* (MoBiTec order # 2100-1)

5. *Grow'n'Glow Yeast Plasmid Isolation Kit* (order # 2069-2)

5.1. Recipes for Media

For optimal results, we highly recommend to use the *Grow'n'Glow Yeast and Bacterial Growth Media* offered by MoBiTec (see chapter 5.1.1. and 5.1.2.) which are optimized for two hybrid systems. Rich medium YPD and the standard Wickerham yeast nitrogen base with carbon source optimized for *S. cerevisiae* can be added to water and autoclaved without the need to make concentrated solutions of vitamins, trace elements, salts or carbon sources. The powder dropout base formulation which is called DOB (Drop Out Base) medium or DOBA (Drop Out Base with Agar) is a complete standard Wickerham yeast nitrogen base with a carbon source. A complete supplemented synthetic defined medium is easily made by mixing two powders, DOB (or DOBA) and CSM (Complete Supplement Mixture). The formulation of CSM is a dropout supplement for virtually all strains of *S. cerevisiae* containing different combinations of amino acids, adenine and uracil. Cells grow vigorously in DOB supplemented with CSM.

These media are very easy to handle and are delivered as powder. Some of our media are available in small bags with the appropriate amount of powder for 0.5 liter medium (10 bags per package are sufficient for 10 x 0.5 liter medium). Just add water and autoclave - ready!

For order information see chapter 10.

5.1.1. Grow'n'Glow Yeast Growth Media

Notes:
DOB = Drop Out Base
DOBA = Drop Out Base with Agar
"-URA" signifies: medium lacks uracil
"-HIS" signifies: medium lacks histidine

a) YPD and YPD agar (rich medium; 20 g peptone, 10 g yeast extract, 20 g glucose, pH 6.5, 17 g agar per liter):

YPD broth:
Pour the entire content of a YPD broth bag (MoBiTec order # 4001-1) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to at least 37 °C. Ready for use.

YPD agar:
Pour the entire content of a YPD agar bag (MoBiTec order # 4001-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to 55 °C. Pour into plates. Ready for use.

b) YNB -URA and/or -HIS (selective medium) with glucose:

DOB (glucose):
26.7 g/l (1.7 g YNB, 5 g ammonium sulfate, 20 g glucose)
DOBA: DOB (glucose) with 17 g agar per liter

CSM (Complete Supplement Mixture):
The formulation of CSM is a dropout supplement for virtually all strains of *S. cerevisiae* containing different combinations of amino acids, adenine and uracil. Cells grow vigorously in DOB or DOBA supplemented with CSM.

DOB -URA (glucose):
Pour the entire content of a DOB glucose bag and 0.385 g CSM -URA (MoBiTec order # 4511-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to at least 37 °C. Ready for use.

DOB -HIS (glucose):
Pour the entire content of a DOB glucose bag and 0.385 g CSM -HIS (MoBiTec order # 4510-3) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to at least 37 °C. Ready for use.

DOB -URA -HIS (glucose):
Pour the entire content of a DOB glucose bag and 0.375 g CSM-URA -HIS (MoBiTec order # 4520-3) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to at least 37 °C. Ready for use.

DOBA -HIS (glucose):
Pour 21.85 g DOBA glu and 0.385 g CSM-HIS (MoBiTec order # 4510-3) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to 55 °C. Pour into plates. Ready for use.

DOBA -URA (glucose):
Pour 21.85 g DOBA glu and 0.385 g CSM-URA (MoBiTec order # 4511-2) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to 55 °C. Pour into plates. Ready for use.

DOBA -URA -HIS (glucose):
Pour 21.85 g DOBA glu and 0.375 g CSM-URA-HIS (MoBiTec order # 4520-3) into a 0.5 l flask, add 500 ml H_2O. Autoclave. Cool to 55 °C. Pour into plates. Ready for use.

5.1.2. Grow'n'Glow Bacterial Growth Media

a) LB Medium:
Pour the entire content of an LB medium bag (MoBiTec order # 3002-1) into a 0.5 l flask add 500 ml H_2O. Autoclave. Cool to at least 37 °C. Ready for use.

b) LB Agar Medium:
Pour the entire content of an LB agar medium bag (MoBiTec order # 3002-2) into a 0.5 l flask add 500 ml H_2O. Autoclave. Cool to 55 °C. Pour into plates. Ready for use.

c) LB Amp Medium (ampicillin selection):
Cool the LB medium (a, above) to 37 °C and add 4 ml of 25 mg/ml ampicillin (in distilled water, filter-sterilized) per liter of medium. Mix.

d) LB Amp Agar Medium (ampicillin selection):
Cool the LB agar medium above to 55 °C and add 4 ml of 25 mg/ml ampicillin (in distilled water, filter-sterilized) per liter of medium. Mix. Pour into plates.

Recipes for preparing *E. coli* growth media are described in Sambrook *et al.* (1989)[15] and Ausubel *et al.* (1997)16, recipes for the preparation of yeast growth media in Ausubel *et al.* (1997) and Guthrie and Fink (1991)[17].

6. Growth and Maintenance of Yeast

The yeast strain (*Saccharomyces cerevisiae*; see chapter 4.1.) in our "Complete Kit" are provided in YPD medium with 20% glycerol and can be maintained indefinitely at -80 °C.

Streak the yeast strains (do not thaw the strains ever) on YPD plates. Start cultures from single colonies and grow in YPD medium at 30 °C for 2 - 3 days.

General remarks: Yeast can be grown on plates or in liquid culture, like *E. coli.* **However, antibiotics, which work on *E. coli*, do not work on yeast, making good sterile technique mandatory when working with yeast.** The optimum growth temperature for yeast is 28 - 32 °C. The growth rate is relatively rapid, with a doubling time of 90 - 120 minutes. Budding yeast is very amenable to genetic and molecular biological methods due to its ability to be transformed by foreign DNA and its highly efficient system of homologous recombination.

Note that binding of the ACE1BD to DNA is dependent on the presence of copper ions. Thus, for selection of clones expressing interacting proteins media has to be supplemented with $CuSO_4$ (≥ 10 µM).

7. Grow'n'Glow Two-Hybrid System Protocol

7.1. Constructing the Hybrid Gene ACE1BD-Bait

Using standard recombinant DNA techniques, subclone your bait protein gene in the correct orientation into the polylinker of pTY137 (see chapter 4.2.1.). Design the subcloning of the bait gene such that it fuses in-frame with ACE1BD.

We strongly recommend verifying the sequence of the ACE1BD-bait junction with the sequencing primer (5'-BAITprimer) provided in the "Complete Kit" to make sure that a ACE1BD-bait fusion protein is going to be expressed correctly.

Note: We highly recommend testing your bait fusion protein in the assays below before performing a full-scale screen of e.g. a cDNA library constructed in the prey vector.

7.2. Autoactivation of CUP1

A two-hybrid system library screen does not have to be performed, if the bait protein activates the reporter genes on its own ("autoactivation"). Since CUP1 is the reporter used in the initial screen, it is important not to have a high background of colonies arising due to autoactivation of the CUP1 gene. Also, for some baits, the CUP1 reporter in ITH5 may be more sensitive than the GFPuv reporter. Therefore, the ability of the bait to autoactivate the CUP1 reporter should be tested before performing a large screen. To test for autoactivation by your bait fusion protein, transform yeast strain ITH5 with the bait vector containing your bait gene in the correct reading frame.

7.2.1. Yeast Transformation Protocol

We recommend the *Grow'n'Glow High Efficiency Yeast Transformation Kit* (order # 2200-1). However, for the above described testing of autoactivation other transformation procedures can be used as well (see Appendix I).

a) Use the protocol supplied with the *Grow'n'Glow High Efficiency Yeast Transformation Kit* using 200 ng of plasmid DNA for transformation or the procedure described in Appendix I (up to step j).
b) Spread 50 - 100 µl of each transformation onto separate DOBA (glu) –HIS plates.
c) Incubate at 30 °C for 2 - 3 days.
d) Streak 4 colonies from each plate onto another DOBA (glu) -HIS plate.
e) Incubate at 30 °C 1 - 2 days.
f) Perform a CUP1 autoactivation assay as follows (see 7.2.2.):

7.2.2. Testing Procedure for CUP1 Autoactivation

a) Transfer a colony of ITH5 containing the bait plasmid into 0.5 ml of sterile distilled water.
b) Vortex.
c) Dilute 100 µl into 1 ml of sterile distilled water.
d) Vortex: this is "Dilution 1".

e) Do three more serial 1:10 dilutions ("Dilutions 2-4") such that:

if "Dilution 1" is considered "undiluted",
"Dilution 2" = 1:10 diluted,
"Dilution 3" = 1:100 diluted, and
"Dilution 4" = 1:1000 diluted.

f) Plate 100 µl of each of "Dilutions 1-4" onto DOBA (glu) -HIS plates and onto DOBA (glu) -HIS plates supplemented with 30 µM $CuSO_4$.
g) Incubate at 30 °C for 1 - 3 days. You should see colonies on the -HIS plates, but not on the -HIS plates containing $CuSO_4$.

Note: If you do obtain many colonies on the -HIS plates containing $CuSO_4$, then your bait is autoactivating and you should perform the assays again using $CuSO_4$ concentrations higher than 30 µM (30 - 100 µM). If you do not obtain the expected results with a more stringent selection for copper resistance, you can try to subclone parts of your bait gene in order to delete those sections responsible for the autoactivation activity. If you are failing again to obtain the expected results with this control assay, you should not attempt a two-hybrid screening with the bait anymore.

Test	Plasmids	Growth
Autoactivation Test	Bait plasmid pTY137 with insert	?
Positive control	Control plasmid pTY139	+
Negative control	Bait plasmid without insert	-

Table: Expected results of CUP1 autoactivation by the bait protein cloned into pTY137. Yeast is grown on DOBA (glu) -HIS plates supplemented with 30 µM $CuSO_4$.

7.3. Autoactivation of GFP

A two-hybrid system library screen does not have to be performed, if the bait protein activates the reporter genes on its own. To test for autoactivation by your bait fusion protein, transform yeast strain ITH5 with the following combinations of vectors:

Plasmids	Plates	Expected Results
pTY137 (with insert) **(Test for GFP autoactivation)**	DOBA (glu) -HIS	No Fluorescence
pTY137 (without insert) **(Negative control)**	DOBA (glu) -HIS	No Fluorescence
pTY137 (with insert) + pTM114 (without insert) **(Test for GFP autoactivation)**	DOBA (glu) -URA -HIS	No Fluorescence
pTY139 **(Positive control)**	DOBA (glu) -HIS	Fluorescence
pTY143 + pTM125 **(Positive control)**	DOBA (glu) -URA -HIS	Fluorescence

Table: Expected results of testing for GFP autoactivation by the bait protein cloned into pTY137.

Expected results:

The colonies containing pTY139 or pTY143 + pTM125 should emit green light, the colonies containing bait plasmid pTY137 (without insert) or pTY137 + pTM114 (without insert) should not fluoresce green, and the colonies containing bait plasmid pTY137 (with insert) may or may not emit green light. If yeast colonies with your bait protein do not fluoresce green, then the bait does not autoactivate reporter gene expression and can be used for screening. If the clones containing your bait protein (or the bait protein and the "empty" prey vector pTM114) do emit green light in the above assay, then you must subclone only parts of the gene encoding your protein into bait plasmid pTY137 and test for a part that does not autoactivate.

Once you are convinced that your bait fusion can bind to CUP1 regulatory sequences within the CUP1 promoter without autoactivating either of the two reporter genes, then you are ready to perform a large-scale library screen.

7.4. Large-Scale Library Screen Protocol

This step is very critical. Use standard recombinant DNA techniques to construct your cDNA library in the prey vector. Clone the cDNA fragments in the correct orientation into the polylinker of pTM114 (see chapter 4.2.3.). Design the subcloning of the cDNA fragments such that they fuse in-frame with ACE1AD to make sure that ACE1AD-prey fusion proteins are going to be expressed correctly. The number of recombinants transformed with the prey clones should be as high as possible. We therefore recommend the use of the *Grow'n'Glow High Efficiency Yeast Transformation Kit* (MoBiTec order # 2200-1) for obtaining the best results, since it contains an optimized library transformation procedure.

Otherwise, you can try an up-scaling of the procedure (as described in Appendix II) with the following information:

To screen up to 10^6 independent clones you should use 100 µg library plasmid DNA for transformation of one liter yeast culture. When screening a prey library to find new proteins that interact with the bait protein, the bait and prey plasmids (see chapter 4.2.) can either be transformed into the yeast strain simultaneously or sequentially. We recommend the following strategy:

a) First introduce the bait plasmid and plate onto selective DOBA (glu) –HIS plates.
b) One yeast colony from the DOBA (glu) -HIS plates is re-streaked onto a DOBA-HIS plate. One colony is then selected and propagated for transformation with the prey library (or one prey plasmid with your insert of interest).
c) Dilute 100 µl transformation suspension (see Appendix II j) in 900 µl sterile distilled water and spread the diluted suspension onto 10 DOBA (glu)-HIS -URA plates (150 mm) supplemented with 30 µM $CuSO_4$. Spread serial dilutions of the transformation suspension onto a DOBA (glu) -HIS -URA plate without $CuSO_4$ to determine the transformation efficiencies (see Appendix III, k-l).

Note: The transformation of the yeast strain with pTY137 is performed as small-scale transformation and the library transformation as a large-scale transformation.

7.5. Green Fluorescent Protein (GFP) Assay Protocol

The copper-containing DOBA (glu) -HIS -URA plates grown with the putative positive colonies are exposed to standard UV-light in a darkroom. The colonies showing green fluorescence are putative (most probably) positive. These colonies are isolated by streaking them onto new DOBA (glu) -HIS -URA plates supplemented with $CuSO_4$ (master plate) and by incubating them for 24 - 72 hours at 30 °C until colonies appear, which are then checked again for GFPuv expression under a UV-lamp. The colonies that appear positive at this point are further characterized (see 7.6.).

Note: Check colonies on plates (without lid!) for fluorescence using a UV hand lamp in a darkroom.

Caution

When using ultraviolet (UV) radiation, it is important to protect yourself with a full-length UV-blocking face shield and other appropriate skin-covering garments. In order to avoid damage to the DNA, it is best to minimize the exposure time of the yeast to the UV light. The shortest duration possible is the best. Exposing any plate of yeast to UV for more than 2 minutes total does not provide reliable results. Maintain sterile technique whenever possible when working with yeast.

Disclaimer: MoBiTec is not responsible or liable for any harm or damage to any person or to any DNA as a result of exposure to UV radiation.

7.6. Further Characterization of Putative Positives

Putative positive colonies are picked from the master plate and are inoculated in 3 ml DOB (glu) -HIS -URA medium supplemented with $CuSO_4$ and are grown overnight. Freeze 1 ml of every culture in 20% glycerol at -80 °C. If the number of potential positives is small (<50), then all should be recovered and further characterized. If >50 potential positives are obtained, then you should characterize the first 50 that arise and freeze the rest in 1 ml aliquots at -80 °C in 20% glycerol.

7.6.1. Plasmid Isolation from Yeast

To isolate DNA from the potential positives we recommend the *Grow'n'Glow Yeast Plasmid Isolation Kit* to obtain best results (MoBiTec order # 2069-1). Alternatively, you can use the procedure in Appendix III.

7.6.2. Transformation of *E. coli* with Plasmids Isolated from Yeast

Use a standard transformation procedure ($CaCl_2$ or electroporation procedures; Sambrook *et al*., 1989[15]; Ausubel *et al*., 1997[16]) or the protocol listed in Appendix IV:

Use 5 µl plasmid DNA (for *Grow'n'Glow Yeast Plasmid Isolation Kit* users) to transform *E. coli*, spread the transformation solution onto LB-Amp agar plates and incubate at 37 °C overnight. Colonies arising at this stage contain either the bait- or the prey plasmid.

7.6.3. Prey Plasmid Identification by PCR

Follow the protocol a) to f):

a) Inoculate 6 colonies from each plate in 2 ml LB-Amp medium. Grow overnight at 37 °C.
b) 5 µl culture are transferred to a PCR tube and centrifuged for 5 sec at maximal speed. Discard supernatant. Add 30 µl PCR-mix and mix.

PCR-mix:

20 pmole	3'-PREYprimer
20 pmole	5'-PREYprimer
3 µl	10 X dNTP (2.5 mM dNTP)
3 µl	10 X Taq DNA polymerase buffer
	Taq DNA polymerase (use appropriate amount as suggested by supplier)
H_2O up to 30 µl	

c) Run the reaction in a thermal-cycler as follows:

1 cycle	2 min, 95 °C		
20 cycles	30 sec, 95 °C	30 sec, 60 °C	120 sec, 72 °C
1 cycle	5 min, 72 °C		

d) Load the PCR reactions on an agarose gel. Clones with an amplified fragment contain a prey plasmid with an insert of interest*.
e) Go back to the cultures (see point 7.6.3.a). Isolate plasmids from the cultures using the *Grow'n'Glow Yeast Plasmid Isolation Kit* (MoBiTec order #2069-1), which can also be used for bacterial plasmid purification. Alternatively, use another appropriate method for plasmid purification.
f) Sequence the prey plasmid insert with the 5'-PREYprimer. Use the DNA sequence to search against a database of choice.

* Performing a PCR with the 5'-PREYprimer and 3'-PREYprimer using the prey plasmid (carrying no insert) as template results in a 237 bp DNA fragment.

7.7. Other Verification Methods of Protein-Protein Interaction

The protein-protein interaction should always be confirmed by an independent method. One such method is purifying your bait and prey proteins, e.g., by means of a suitable protein expression and purification system and co-immunoprecipitation analysis using specific antibodies against your bait and prey proteins.

Moreover, you can also verify your results by switching cloning vectors (i.e. moving the library insert from the AD to the DNA-BD-vector and vice versa, and then repeat the two-hybrid assay) or using a different two-hybrid system (e.g. MoBiTec's LexA-based Grow'n'Glow GFP Two-Hybrid System, # GNGK01).

Another possibility is generating mutations of your prey (by deletions, substitutions or frame-shift mutations of the gene) and assaying the relative strength of the interactions using the GFP reporter assay and/or growth selection on media containing different concentrations of copper ions.

8. Literature

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9. Appendix

Appendix I: Small Scale Yeast Transformation Procedure

a) Grow a 5 ml culture of yeast in YPD medium at 30 °C with shaking (for 24 hours). Inoculate by picking a colony from a streaked ITH5 plate.
b) Measure the OD_{600} of a 1:10 dilution of the overnight culture. Calculate the OD_{600} of the 5 ml culture and use that to inoculate a 60 ml YPD culture to an OD_{600} = 0.1. Grow at 30 °C with vigorous shaking.
c) Once the OD_{600} = 0.5 - 0.7 (approximately 4 - 6 hours after inoculation), pellet the cells by spinning the culture at 1500 x g for 5 minutes. Resuspend in 20 ml of sterile distilled water, spin again at 1500 x g, and resuspend the pellet in 5 ml of 1 x TE/LiOAc.
d) Spin again at 1500 x g, and resuspend the pellet in 300 µl of 1 x TE/LiOAc.
e) Boil the carrier DNA 3 times 5 minutes and quickly chill on ice. This is essential for obtaining a maximum efficiency of transformation.
f) Add 250 ng of each plasmid DNA (e.g. pTY137 + insert and pTM114 + insert) to 25 µg of denatured carrier DNA (10 µl) and mix.
g) Add 50 µl resuspended cells from step d) to the DNA (10 µl) from step f).
h) Add 340 µl of 1 x TE/LiOAc/PEG, mix by inversion, and put the tubes at 30 °C (with or without shaking) for 30 minutes.
i) Incubate at 42 °C (without shaking) for 15 minutes.
j) Add 600 µl sterile distilled water, spin at 1500 x g in a microcentrifuge for 5 minutes, pour off the supernatant, and resuspend each pellet in 100 µl of sterile distilled water.
k) Dilute 10 µl of each transformation suspension in 990 µl sterile distilled water and spread 100 µl of this dilution onto separate appropriate selective DOBA plates (e.g. -HIS or -URA). Incubate at 30 °C for 2 - 3 days. Calculate the number of transformants obtained by counting the number of colonies on the plate. 100 colonies on the plate corresponds to an efficiency of 2 x 10^4/µg plasmid DNA (when transforming with e.g. 250 µg of bait and 250 µg of prey plasmid).
l) Streak 4 colonies from each plate onto another DOBA (glu) -HIS -URA plate. Incubate at 30 °C 2 - 3 days. Perform a GFP assay with a UV-lamp in a darkroom.

Appendix II: Large-Scale Yeast Transformation Procedure

a) Grow a 30 ml culture of yeast in YPD medium at 30 °C with shaking (for 24 hours). Inoculate by picking a colony from a streaked ITH5 plate.
b) Measure the OD_{600} of a 1:10 dilution of the overnight culture. Calculate the OD_{600} of the 30 ml culture and use that to inoculate 2 x 500 ml YPD culture to an OD_{600} = 0.1. Grow at 30 °C with vigorous shaking.
c) Once the OD_{600} = 0.5 - 0.7 (approximately 4 - 6 hours after inoculation), pellet the cells by spinning the culture at 1500 x g for 5 minutes. Resuspend in 2 x 50 ml of sterile distilled water, transfer to two 50 ml sterile conical tubes, spin again, and resuspend the two combined pellets in 20 ml of 1 x TE/LiOAc.
d) Spin again at 1500 x g for 5 minutes, and resuspend the pellet in 5 ml of 1 x TE/LiOAc.
e) Boil the carrier DNA 3 times for 5 minutes and quickly chill on ice. This is essential for obtaining a maximum efficiency of transformation.
f) Add 500 ng of each plasmid DNA to 25 µg of denatured carrier DNA (10 µl) and mix. (For large-scale transformation of yeast cells containing already bait plasmid DNA [pTY137 + insert] with library plasmid DNA use 1 µg of pTM114 based plasmid library DNA and 50 µg of carrier DNA. Do not use more than 1 µg of library DNA per tube

since multiple plasmids can enter the same yeast cell and give confusing results in later analyses).

g) Add 50 µl resuspended cells from step d) to the DNA (10 µl) from step f).
h) Add 340 µl of 1 x TE/LiOAc/PEG, mix by inversion, and put the tubes at 30 °C (with gentle or no agitation) for 30 minutes.
i) Incubate at 42 °C (without shaking) for 15 minutes.
j) Add 600 µl water to each tube, spin at 1500 x g in a microcentrifuge for 5 minutes, pour off the supernatant, and resuspend each pellet in 100 µl of sterile distilled water.
k) Dilute 10 µl of each transformation suspension in 990 µl sterile distilled water and spread 100 µl of this dilution onto separate appropriate selective DOBA plates (e.g. -HIS -URA). Incubate at 30 °C for 2 - 3 days until colonies appear.
l) Calculate the number of transformants obtained by counting the number of colonies on the plate. 200 colonies on the plate corresponds to an efficiency of 2 x 10^4/µg library DNA. Thus, 100 µg library DNA will yield 100 x 2 x 10^4 = 2 x 10^6 transformants). A saturating screen of e.g. a mammalian library requires at least 2 x 10^6 transformants.
m) Streak 4 colonies from each plate onto another DOBA (Glu) -HIS -URA plate. Incubate at 30 °C 2 - 3 days. Perform a GFP assay with a UV-lamp (see 7.5).

Note: For yeast transformation procedures, especially electrotransformation, (electroporation) see Ausubel *et al*. (1997).

Materials:

10 X TE:	100 mM Tris-HCl (pH 7.5) 10 mM EDTA
10 X LiOAc:	1 M lithium acetate
Polyethylene Glycol:	50 % (w/v) Polyethylene Glycol-3350 (in H_20)
1 X TE/LiOAc:	Right before use, mix 1 part 10 X TE 1 part 10 X LiOAc 8 parts sterile distilled water
1 X TE/LiOAc/PEG:	Right before use, mix 1 part 10 X TE 1 part 10 X LiOAc 8 parts 50 % PEG-3350
Carrier DNA:	Sonicated salmon or herring sperm DNA (Sigma) 20 mg/ml (size < 10 kb)

Appendix III: Isolation of Plasmids from Yeast

a) Grow a yeast colony in 2 ml DOBA (glu) -HIS -URA overnight or until culture shows abundant yeast growth.
b) Spin down 1.5 ml at 14,000 g for 1 minute.
c) Discard supernatant.
d) Resuspend in 200 µl lysis-buffer.
e) Add 100 µl of phenol and 100 µl of chloroform/isoamylalcohol (see below).
f) Add 0.3 g (~ 200 µl) glass beads (see below).
g) Vortex at maximal speed for at least 5 minutes.
h) Centrifuge at 14,000 g for 10 minutes.
i) Transfer supernatant carefully to fresh tube.
j) Add 8 µl 10 M ammonium acetate. Mix.
k) Add 500 µl 96% ethanol.
l) Place at -20 °C for at least 30 minutes or 10 minutes at -70 °C.
m) Centrifuge at 14,000 g for 10 minutes.
n) Discard supernatant and dry pellet.
o) Resuspend in 20 µl sterile TE buffer.

Materials:

Lysis-buffer: 2% Triton X-100
1% SDS
100 mM NaCl
10 mM Tris-HCl, pH 8.0
1 mM EDTA

TE-buffer: 10 mM Tris-HCl, pH 7.5
1 mM EDTA

Phenol: Tris-saturated (pH 8.0)

Chloroform/Isoamylalcohol: 24:1 (v/v)
Glass-beads: acid washed 400 - 600 nm glass-beads; Sigma

Appendix IV: Transformation of competent *E. coli* Cells

Competent cells:

a) Inoculate one colony of the *E. coli* strain in 2.5 ml SOB-medium (see below) and incubate overnight at 37 °C.

Note: Use a freshly streaked plate with your *E. coli* strain of choice.

b) Incubate 250 ml SOB with 2.5 ml overnight culture. Incubate at 37 °C and 200 rpm.
c) Once an OD_{550} of about 0.4 to 0.5 is reached (after about 2 to 2.5 hours), place cells on ice for 15 minutes. It is very important to keep the temperature at 0 °C unless otherwise stated.
d) Pellet cells by centrifugation. Spin 10 minutes at 6,000 rpm (3,000 g) at 4 °C.
e) Discard the supernatant.
f) Resuspend in 80 µl ice-cold RF I-buffer (see below).
g) Place on ice for 20 minutes.
h) Spin 6,000 rpm for 10 minutes at 4 °C.
i) Discard the supernatant. Resuspend the pellet in 20 ml ice-cold RF-II buffer (see below).
j) Aliquot the cells in 200 µl aliquots (Eppendorf tubes) on ice. Quick-freeze immediately in liquid nitrogen and place at -80 °C until use.

Materials:

SOB:
2% (w/v) Bacto Tryptone
0.5% (w/v) yeast extract
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$

RF I-buffer:
100 mM RbCl (rubidiumchloride)
30 mM KAc, pH 7.5
10 mM $CaCl_2$
15% (w/v) glycerol (87%)
Adjust to pH 5.8 with 0.2 M acetic acid.
Add 50 mM $MnCl_2$.

RF II-buffer:
10 mM MOPS
10 mM RbCl
75 mM $CaCl_2$
15% (w/v) glycerol (87%)
Adjust to pH 6.8 with 1 M NaOH.

Transformation Procedure:

a) Thaw the frozen, competent cells at 4 °C and store on ice.
b) Add plasmid DNA.
c) Incubate on ice for 30 - 60 minutes.
d) Incubate in a water bath preheated to 42 °C for exactly 2 minutes.
f) Add 1 ml LB medium.

g) Incubate at 37 °C for one hour.
h) Plate the transformation solution onto selective media (LB medium supplemented with 100 µg/ml ampicillin).
i) Incubate overnight (or 8 - 16 hours) at 37 °C.

Note: For *E. coli* transformation procedures, especially electrotransformation, (electroporation) see Ausubel *et al*. (1997)16 or Sambrook *et al*. (1989)[15]. Protocols for the isolation of plasmid DNA from *E. coli* can also be found in both manuals.

10. Order Information, Shipping and Storage

Order#	Product	Quantity
ACE01	Grow'n'Glow ACE1 Two Hybrid System 'Complete Kit':	
	pTY137, lyophilized DNA	5 µg
	pTM114, lyophilized DNA	5 µg
	pTY143, lyophilized DNA	5 µg
	pTM125, lyophilized DNA	5 µg
	pTY139, lyophilized DNA	5 µg
	5'-BAITprimer	500 pmole
	5'-PREYprimer	500 pmole
	3'-PREYprimer	500 pmole
	Yeast strain ITH5, glycerol stock	1 ml
shipped on dry ice; vectors & primers store at 4 °C; yeast strains store at -20 °C		

Related Products

Order#	Product	Quantity
GNGK01	Grow 'n' Glow Two-Hybrid System 'Complete Kit'	Kit
GNGK02	Grow 'n' Glow Two-Hybrid System 'Basic Kit'	Kit
GNGK03	Grow 'n' Glow One-Hybrid System	Kit
2100-1	Grow 'n' Glow Yeast Fast & Easy Transformation Kit	200 transf.
2200-1	Grow 'n' Glow High Efficiency Yeast Transformation Kit	250 transf.
2069-2	Grow 'n' Glow Yeast Plasmid Isolation Kit	100 preps
STAQ02	Supertaq	250 U

Grow'n'Glow Yeast and Bacterial Growth Media:

Order #	Description	Amount
Bags for 0.5 liter medium each:		
4001-1	YPD broth bags (with 25 g for 0.5 liter medium each)	10 bags
4001-6	YPD broth bags	10 x 10 bags
4001-2	YPD agar bags (with 33.5 g for 0.5 liter medium each)	10 bags
4001-7	YPD agar bags	10 x 10 bags
3002-1	LB medium bags (with 12.5 g for 0.5 liter medium each)	10 bags
3002-6	LB medium bags	10 x 10 bags
3002-2	LB agar bags (with 20 g for 0.5 liter medium each)	10 bags
3002-7	LB agar bags	10 x 10 bags
Powder:		
4510-3	CSM -HIS supplement	10 g
4511-2	CSM -URA supplement	10 g
4520-3	CSM -HIS -URA supplement	10 g

* DOB = Drop Out Base **DOBA = Drop Out Base with Agar

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