BacterioMatch II Two-Hybrid System XR Plasmid cDNA Library

INSTRUCTION MANUAL

BN #982000

Revision A

For In Vitro Use Only

982000-12

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BacterioMatch II Two-Hybrid System XR Plasmid cDNA Library

CONTENTS

CRITICAL SUCCESS FACTORS

Before beginning experiments using the BacterioMatch II two-hybrid system, please read the following general guidelines. Following these recommendations is critical to the success of your experiments.

1. It is important that the strain used to propagate pBT and pTRG plasmids contains the *lacI*^{q} gene. The XL1-Blue MRF´ Kan strain supplied in this kit as a glycerol stock is an appropriate host for propagation of pBT and pTRG. Do not substitute another strain when growing these plasmids.

2. pBT and pTRG are both low-copy-number plasmids; pBT is present at 5–10 copies per cell and pTRG is present at about 20–30 copies per cell. Depending on the yield of plasmid desired, it may be necessary to purify plasmid DNA from a larger volume of culture. It is also suggested that, during purification of plasmid DNA, protocol modifications recommended by the purification kit manufacturer for low-copy-number plasmids are employed.

3. The BacterioMatch II system uses a new *HIS*3-*aadA* reporter cassette. Detection of protein-protein interactions is based on transcriptional activation of the *HIS3* reporter gene, which allows growth in the presence of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3 enzyme. Prepare the media exactly as described in *Preparation of Media and Reagents*. In particular, ensure that the Selective Screening Medium plates contain the correct amount of 3-amino-1,2,4-triazole (3-AT). Small deviations from the media recipes can result in significant variation in experimental results.

Revision A

BacterioMatch II Two-Hybrid System XR Plasmid cDNA Library

MATERIALS PROVIDED

Materials provided	Quantity
Amplified premade library constructed in the pTRG plasmid and harbored in XL1-Blue MRF´ Kan cells[a]	1 ml
BacterioMatch II two-hybrid system plasmids[b]	
pTRG target plasmid, supercoiled, 10 µg [1 µg/µl in TE buffer (Catalog #240066)]	10 µl
pBT bait plasmid, supercoiled 10 µg [1 µg/µl in TE buffer (Catalog #240067)]	10 µl
BacterioMatch II two-hybrid system control plasmids[b]	
pTRG-Gal11^{P} control plasmid, supercoiled, 150 ng [50 ng/µl in TE buffer]	3 µl
pBT-LGF2 control plasmid, supercoiled, 150 ng [50 ng/µl in TE buffer]	3 µl
BacterioMatch II Screening Reporter Competent Cells (Catalog #200190)[c]	6 × 500 µl
β-mercaptoethanol (β-ME)	75 µl
pUC18 plasmid DNA (0.1 ng/µl in TE buffer)	10 µl
BacterioMatch II Validation Reporter Competent Cells (Catalog #200192)[c]	5 × 200 µl
β-mercaptoethanol (β-ME)	25 µl
pUC18 plasmid DNA (0.1 ng/µl in TE buffer)	10 µl
Host Strain for propagating pBT and pTRG recombinants, XL1-Blue MRF´ Kan; glycerol stock[c]	0.5 ml

[a] BacterioMatch II libraries have been amplified one time and frozen in LB-glycerol [15% (v/v)]. Store at –80°C immediately upon arrival. Consult the Certificate of Analysis for library titer (cfu/ml).

[b] Store the plasmids at –20°C.

[c] Store at –80°C.

STORAGE CONDITIONS

Premade Library, BacterioMatch II Screening/Validation Reporter Competent Cells, XL1-Blue MRF´ Kan glycerol stock : –80°C

BacterioMatch II Two-Hybrid System Plasmids: –20°C

ADDITIONAL MATERIALS REQUIRED

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
components for minimal, his-dropout bacteriological media,[§] including 10× M9 salts[§], adenine HCl (Sigma, Cat. #A-8751), –His Dropout Supplement (BD/Clontech, Cat. #630415)
chloramphenicol
tetracycline
streptomycin
isopropyl-1-thio-β-D-galactopyranoside (IPTG),
3-amino-1,2,4-triazole (3-AT, Sigma, Catalog #A-8056)
dimethyl sulfoxide (DMSO)
water baths (12°C and 42°C)
incubators (30°C and 37°C)

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

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HOST STRAINS AND GENOTYPES

XL1-Blue MRF′ Kan (strain harboring the pTRG cDNA library) and XL1-Blue MRF′ Kan glycerol stock	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*5* (Kan^{r})]
BacterioMatch II Two-Hybrid System Reporter Strain Competent Cells	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 hisB supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *lacI*^{q} *HIS3 aadA* Kan^{r}]

Note *The BacterioMatch II Two-Hybrid System Reporter Strain contains the* supE44 *mutation, an amber suppressor mutation that allows some readthrough of TAG stop codons with insertion of glutamine.*

INTRODUCTION

Our exclusive BacterioMatch II two-hybrid system is an efficient method for detecting protein-protein interactions *in vivo*. The BacterioMatch II two-hybrid system is based on a methodology developed by Dove, Joung, and Hochschild of Harvard Medical School and further refined by Joung and Pabo of the Massachusetts Institute of Technology.[1, 2, 3] This *E. coli*-based system offers advantages over our original BacterioMatch two-hybrid system. Featuring a new *HIS*3-*aadA* reporter cassette, the BacterioMatch II system offers the ability to screen libraries for harder-to-find binding partners with reduced background. Detection of protein-protein interactions is based on transcriptional activation of the *HIS3* reporter gene, which allows growth in the presence of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor of His3 enzyme. Positives are verified by using the *aadA* gene, which confers streptomycin resistance, as a secondary reporter.

In addition to two-hybrid screening, the bait and target plasmids may also be used for the characterization of protein-protein interactions between a known protein pair. Once an interaction pair has been identified, the system may be used to map the interaction domain of either the bait or target protein after site-directed mutagenesis. To facilitate these experiments, we offer the BacterioMatch II Two-Hybrid System Vector Kit (Catalog #240065) designed for the characterization of protein-protein interactions between a pair of proteins cloned in the pBT (bait) and the pTRG (target) vectors.

Advantages of the Bacterial Two-Hybrid System

While the yeast two-hybrid system has been widely and successfully exploited, a method that utilizes *E. coli* is valuable for many reasons: *E. coli* grows much faster than yeast, it is transformed with higher efficiency so larger numbers of interactions can be more rapidly and easily screened, and isolating plasmid DNA from *E. coli* is easier than DNA isolation from yeast. Furthermore, using *E. coli* for two-hybrid screening reduces the chance that the host harbors a eukaryotic homologue of one of the interacting protein partners. Some eukaryotic regulatory proteins, such as cell cycle checkpoint proteins and signal transduction pathway proteins, may be toxic in yeast because they interfere with the function of yeast homologues; presumably they would not be as harmful in *E. coli*. For the same reason, use of a bacterial system could also reduce the number of false positives observed. Although some heterologous proteins could be toxic to an *E. coli* host, and bacteria lack the ability to perform some posttranslational modifications, an *E. coli* two-hybrid system makes for an important experimental alternative.

OVERVIEW OF THE INTERACTION

The BacterioMatch II two-hybrid system detects protein-protein interactions based on transcriptional activation. A protein of interest (the bait) is fused to the full-length bacteriophage λ repressor protein (λcI, 237 amino acids), containing the amino-terminal DNA-binding domain and the carboxyl-terminal dimerization domain. The corresponding target protein is fused to the N-terminal domain of the α-subunit of RNA polymerase (248 amino acids). The bait is tethered to the λ operator sequence upstream of the reporter promoter through the DNA-binding domain of λcI. When the bait and target interact, they recruit and stabilize the binding of RNA polymerase at the promoter and activate the transcription of the *HIS3* reporter gene. A second reporter gene, *aadA*, encoding a protein that confers streptomycin resistance, provides an additional mechanism to validate the bait and target interaction (Figure 1).

Due to the tendency of both the λ repressor protein and the N-terminal domain of the α-subunit of RNA polymerase to dimerize, this system might not be optimal for the analysis of proteins that self-associate unless their interaction with other protein partners depends on the oligomerization.

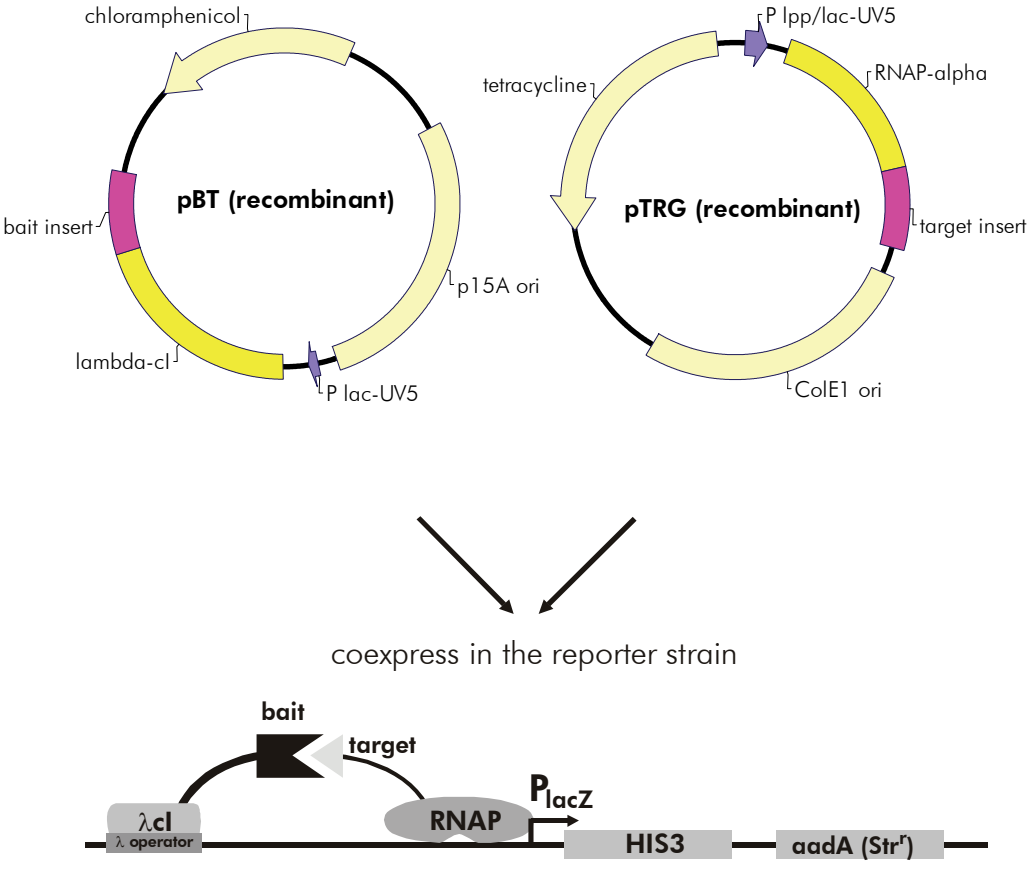


Figure 1 Schematic of the BacterioMatch II two-hybrid system dual reporter construct

Positive Selection Using *HIS3* Transcriptional Activation

Transcriptional activation of the *HIS3* gene is used as the initial test for interaction of the bait and target hybrid proteins. *HIS3* encodes a component of the histidine biosynthetic pathway that complements a *hisB* mutation in the reporter strain. The *HIS3* gene product is produced from the reporter gene cassette at low levels in the absence of transcriptional activation, allowing the reporter strain to grow on minimal medium lacking histidine.

The compound 3-amino-1,2,4-triazole (3-AT) acts as a competitive inhibitor of the *HIS3* gene product. In the presence of 5 mM 3-AT, the reporter strain is unable to grow on media lacking histidine.

When the reporter strain is cotransformed with hybrid bait and target proteins that interact, the RNA polymerase is recruited to the promoter, activating the transcription of *HIS3*. Growth of the reporter strain on media lacking histidine and containing 5 mM 3-AT occurs when transcriptional activation increases expression of the *HIS3* gene product to levels that are sufficient to overcome the competitive inhibition by 3-AT. This allows for positive selection for plasmids encoding interacting proteins on media containing 5 mM 3-AT.

The reporter gene cassette (Figure 1) is present in the BacterioMatch II reporter strain on an F´ episome. The cassette contains the *HIS3* gene, conferring the ability to grow on media containing 3-AT, and the *aadA* gene, conferring resistance to streptomycin. The activatable promoter in the reporter gene cassette is a modified *lac* promoter that contains a single λ operator (O_R2) centered at position –62, replacing the CRP-binding site originally associated with the *lac* promoter. This modified *lac* promoter is not inducible by IPTG. The Shine-Dalgarno translational signal is also included upstream of the reporter genes.

Reporter Strain

The BacterioMatch II two-hybrid system uses a reporter strain derived from our XL1-Blue MR strain, providing a high transformation efficiency in a restriction minus host. The reporter strain harbors *lac*I^q on the F´ episome to repress synthesis of the bait and target in the absence of IPTG, but a basal level of transcription of these proteins is always occurring.

Note *The BacterioMatch II reporter strain contains the supE44 mutation, an amber suppressor mutation that allows some readthrough of TAG stop codons with insertion of glutamine.*

Three varieties of the BacterioMatch II reporter strain competent cells are available in formats that are optimized for specific applications.

Catalog #	Description	Applications	Efficiency	Format
200190	BacterioMatch II Screening Reporter Competent Cells (provided; chemically-competent)	Library screening (cotransformation of pBT-bait and the pTRG library)	$\geq 7.5 \times 10^7$ cfu/µg pUC18	6 × 500 µl aliquots
200192	BacterioMatch II Validation Reporter Competent Cells (provided; chemically-competent)	Interaction assays, expression testing, validation of screen isolates	$\geq 1 \times 10^7$ cfu/µg pUC18	5 × 200 µl aliquots
200195	BacterioMatch II Electrocompetent Reporter Cells (available separately; electroporation-competent)	Library screening (cotransformation of pBT-bait and the pTRG library) with increased efficiency compared to chemically-competent reporter cells	$\geq 1 \times 10^9$ cfu/µg pUC18	5 × 100 µl aliquots

PLASMIDS

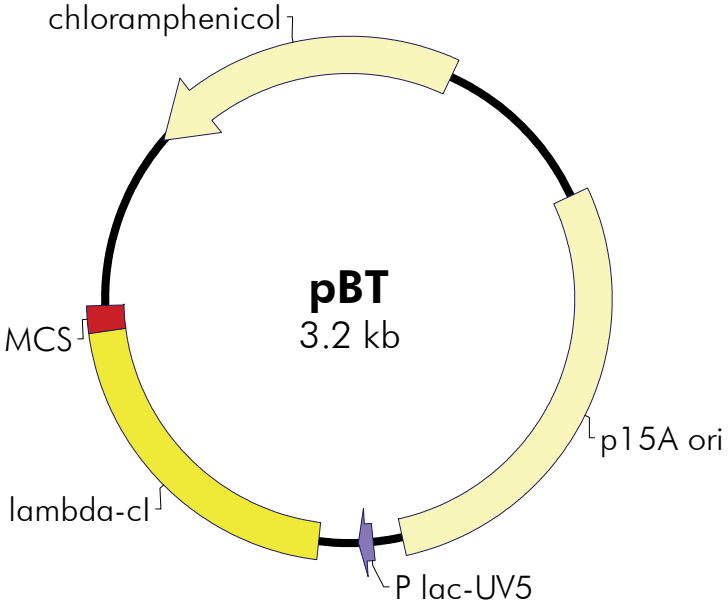
The pBT bait plasmid and the pTRG target plasmid are designed to allow detection of protein-protein interactions when used to cotransform a host strain containing the appropriate reporter gene cassette. The 3.2 kb pBT bait plasmid (Figure 2) carries a low-copy p15A replication origin and confers chloramphenicol resistance. The plasmid encodes the full-length bacterial phage λ cI protein under the control of the IPTG-inducible *lac-UV5* promoter. The multiple cloning site* contains several restriction sites to facilitate fusion gene construction. The *Not* I restriction site in the MCS encodes a short alanine linker to facilitate the orientation and folding of the fused bait protein. From a 5 ml LB-chloramphenicol culture (34 μg/ml chloramphenicol), yields of approximately 200 ng DNA are obtained.

The 4.4 kb target plasmid, pTRG (Figure 3), carries the low-copy ColE1 replication origin and confers tetracycline resistance. The plasmid directs transcription of the amino-terminal domain of RNA polymerase α subunit through a multiple cloning site at the 3´ end of the α subunit gene and is under the control of the IPTG-inducible, tandem promoter *lpp/lac-UV5*. The expression level from the *lpp/lac-UV5* promoter operator is higher than the levels of *lac-UV5*-driven expression. cDNA library inserts are ligated unidirectionally into the pTRG plasmid between the *Eco*R I and *Xho* I sites. This cloning scheme leaves the *Not* I site intact; the *Not* I site encodes a short alanine linker to facilitate the orientation and folding of the fused cDNA library inserts. For the empty pTRG vector, yields of approximately 1 μg DNA are obtained from a 5 ml LB-tetracycline culture (12.5 μg/ml tetracycline).

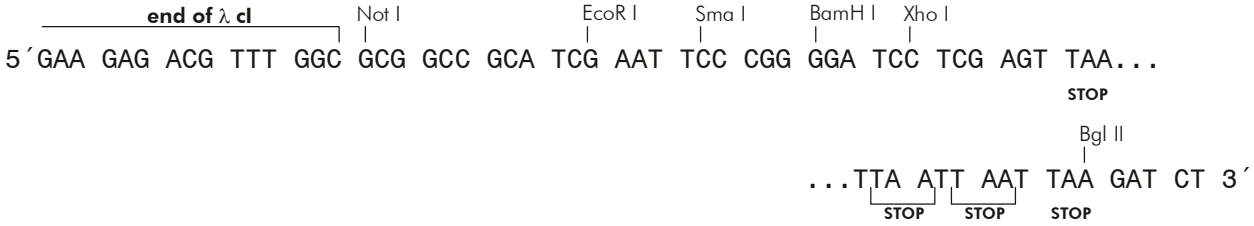
Note *The pTRG vector (with or without insert present), as well as recombinant pBT plasmids, can be toxic to the bacterial host. To minimize host toxicity, propagate all forms of the pBT and pTRG plasmids using a host strain containing the* lacI^{q} *gene (such as the XL1-Blue MRF´ strain provided). When growing cells harboring either plasmid on rich medium, incubate the cells at 30°C, in order to further reduce any potential toxicity. For growth on minimal medium, incubate the cells at 37°C.*

* Sites in the MCS of the pBT and pTRG are not directly compatible with each other. If swapping the insert between the two plasmids is desired, indirect cloning methods will have to be employed.

pBT Vector Map



pBT Multiple Cloning Site Region
(sequence shown 2327–2394)

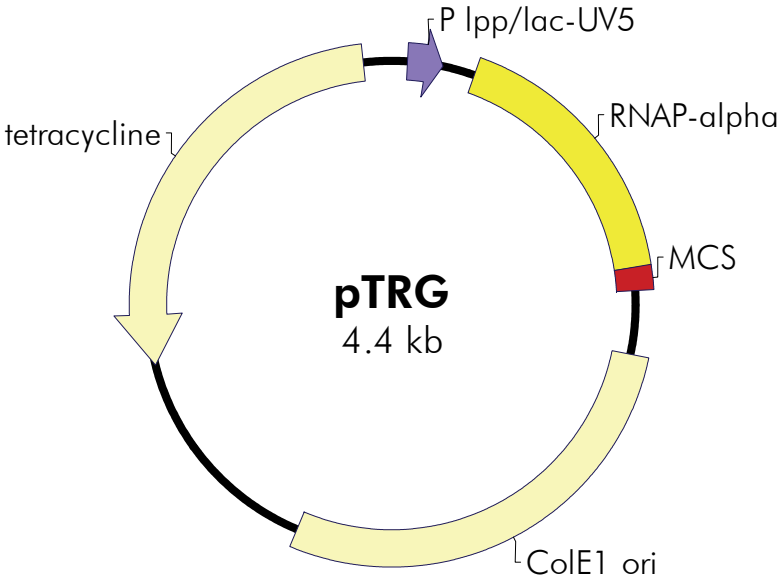


Feature	Position
chloramphenicol resistance ORF	2770–219
p15A origin of replication	581–1493
lac-UV5 promoter	1556–1586
λ cI ORF	1631–2341
pBT forward primer [5' TCCGTTGTGGGGAAAGTTATC 3']	2291–2311
multiple cloning site	2342–2394
pBT reverse primer [5' GGGTAGCCAGCAGCATCC 3']	2419–2436

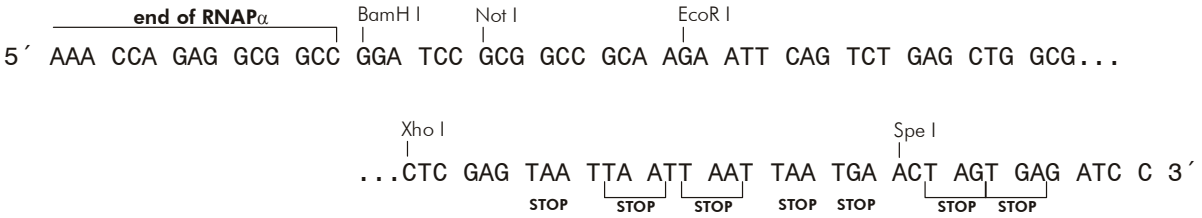
Note *The nucleotide sequence and list of restriction sites for the pBT bait plasmid can be found at* www.stratagene.com.

FIGURE 2 The pBT bait plasmid

pTRG Vector Map



pTRG Multiple Cloning Site Region
(sequence shown 978–1065)



Feature	Position
lpp promoter	47–76
lac-UV5 promoter	119–148
RNAPα ORF	243-992
pTRG forward primer [5' TGGCTGAACAACTGGAAGCT 3']	913–932
multiple cloning site	993–1058
pTRG reverse primer [5' ATTCGTCGCCCGCCATAA 3']	1102–1119
ColE1 origin of replication	1243–2475
tetracycline resistance ORF	3120–4310

Note *The nucleotide sequence and list of restriction sites for the pTRG target plasmid can be found at* www.stratagene.com.

FIGURE 3 The pTRG target plasmid

Control Plasmids

Description

The BacterioMatch II two-hybrid system includes the pTRG-Gal11^{P} and the pBT-LGF2 positive control plasmids (Table I and Figure 4).

TABLE I
Description Of Control Plasmids

Control plasmid	Description
pBT-LGF2	interaction control plasmid encoding the dimerization domain (40 amino acids) of the Gal4 transcriptional activator protein
pTRG-Gal11^{P}	interaction control plasmid encoding a domain (90 amino acids) of the mutant form of the Gal11 protein

Control Plasmid Maps

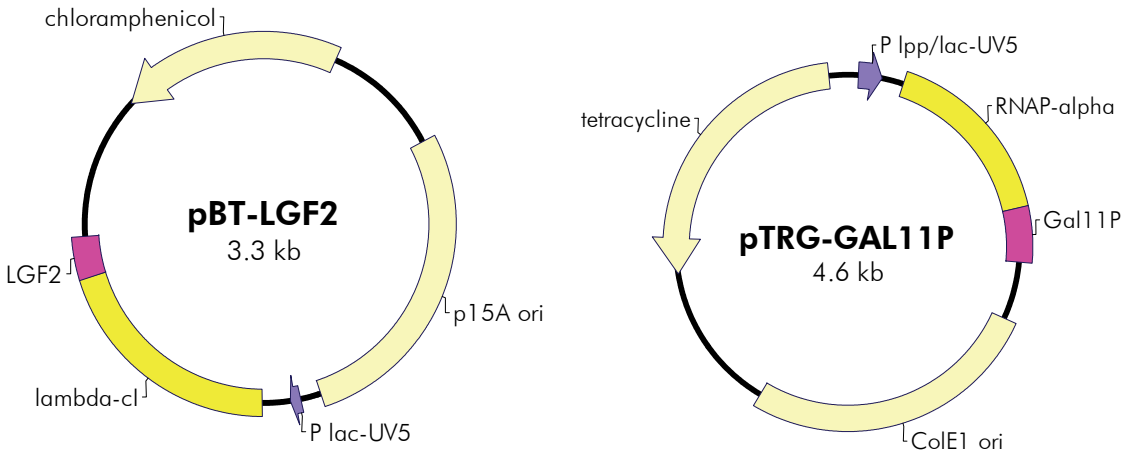


FIGURE 4 Circular maps of the interaction control plasmids

Applications

The dimerization domain of the yeast transcriptional activator Gal4 and a domain derived from a mutant form of Gal11 protein, called Gal11^{P}, have been shown to interact in *E. coli* cells.[1] These polypeptides are expressed by the pBT-LGF2 and the pTRG-Gal11^{P} control plasmids respectively. When these two plasmids are used to cotransform the BacterioMatch II two-hybrid system reporter strain competent cells, a positive interaction is indicated by the growth of colonies on M9^{+} His-dropout medium containing 5 mM 3-AT.

OVERVIEW OF THE TARGET cDNA LIBRARY CONSTRUCTION METHOD

cDNA Synthesis using StrataScript Reverse Transcriptase

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

First-strand synthesis is primed with a linker–primer and is reverse transcribed using StrataScript reverse transcriptase (StrataScript-RT) and 5-methyl dCTP. StrataScript-RT is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) without any detectable RNase H activity. Cloned StrataScript RT is purified from recombinant *E. coli* containing a genetically engineered mutant MMLV-RT gene. A point mutation in the highly conserved residue of the RNase H region results in the loss of undesired RNase H degradative activity without affecting the desired reverse transcriptase function. The result is a nuclease-free mutant of MMLV-RT that can produce larger yields of full-length cDNA transcripts than wild-type MMLV-RT, which possesses substantial RNase H^- activity. These advantages make StrataScript RT the enzyme of choice for applications involving the preparation of full-length, complete cDNA transcripts, including first-strand cDNA synthesis and library construction.

Preparation of cDNA Inserts for Ligation into the pTRG Plasmid

An overview of the cDNA synthesis scheme that was used for preparation of the cDNA library is presented in Figure 5.

First-strand cDNA synthesis begins when StrataScript RT, in the presence of nucleotides and buffer, finds a template and a primer. The template is mRNA and the primer is a 50-base oligonucleotide with the following sequence:

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

This oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the pTRG XR plasmid in a sense orientation (*Eco*R I–*Xho* I) with respect to the *lpp/lac-UV5* promoter driving target gene expression. The poly(dT) region binds to the 3´ poly(A) region of the mRNA template, and StrataScript reverse transcriptase begins to synthesize the first-strand cDNA.

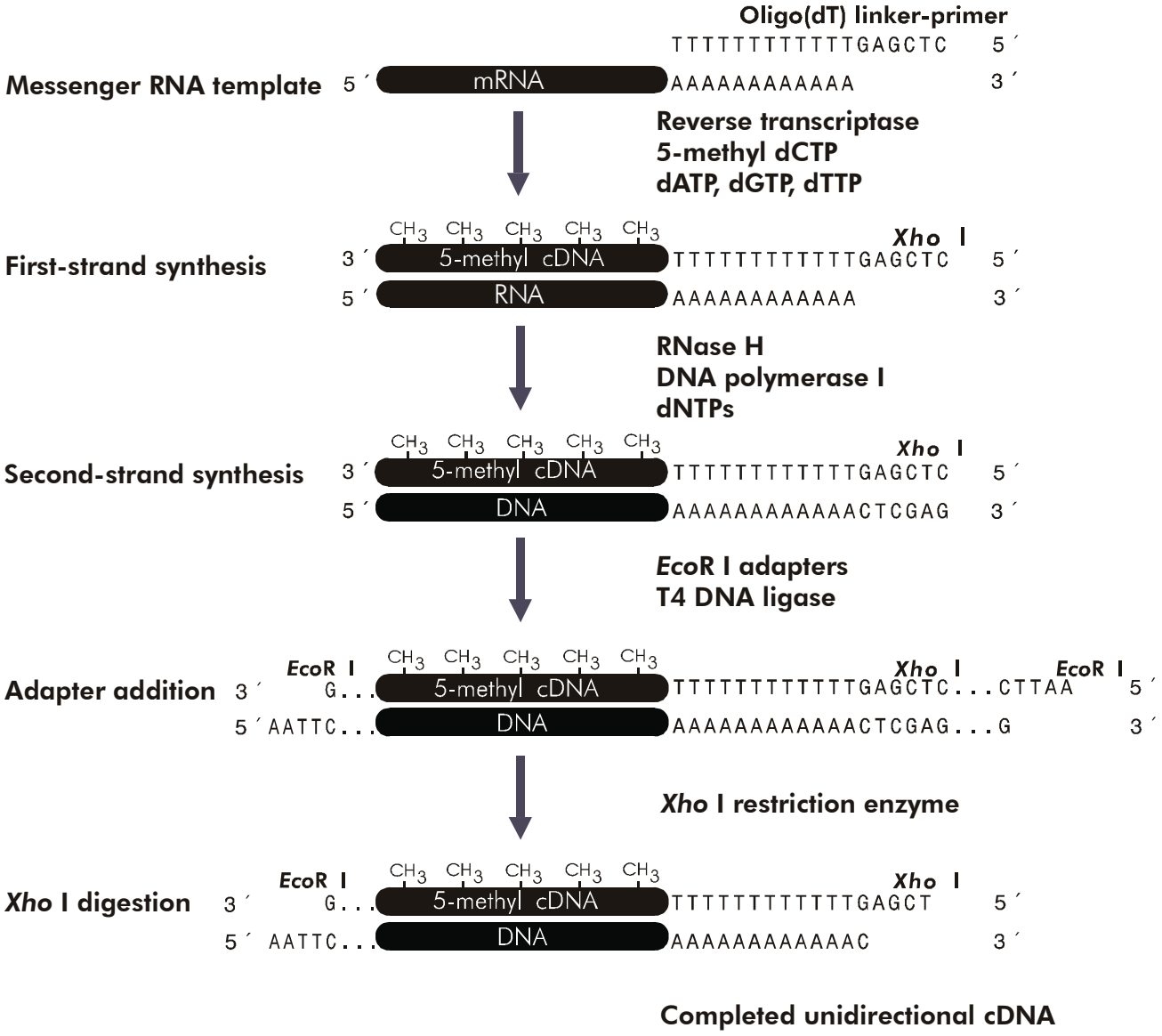


FIGURE 5 cDNA synthesis flow chart

The nucleotide mixture for the first strand contains normal dATP, dGTP, and dTTP plus the analog 5-methyl dCTP. The complete first strand will have a methyl group on each cytosine base, which will protect the cDNA from restriction enzymes used in subsequent cloning steps. The incorporation of 5-methyl dCTP versus normal dCTP has been tested with Klenow polymerase, *E. coli* DNA polymerase I, *E. coli* DNA polymerase III, avian myeloblastosis virus reverse transcriptase (AMVRT), MMLV-RT, and StrataScript reverse transcriptase on various templates and has proved to be an equally acceptable substrate for the enzymes listed.

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

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

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

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

The XL1-Blue MRF´ Kan strain is transformed with the pTRG cDNA library and is supplied in the form of a glycerol stock.

BAIT PLASMID CONSTRUCTION AND VALIDATION

Bait Insert Preparation

DNA is prepared for insertion into the pBT plasmid either by restriction digestion or PCR amplification. DNA encoding the bait protein must be inserted so that the bait protein is expressed in the same reading frame as the λcI protein. In the MCS of the pBT plasmid, the *Not* I, *Eco*R I, *Sma* I, *Bam*H I, *Xho* I, and *Bgl* II, sites are unique (see Figure 2).

If the insert DNA is phosphorylated, we suggest dephosphorylating and agarose-gel isolating the digested pBT plasmid DNA prior to setting up the ligation reaction. If more than one restriction enzyme is used, the background can be reduced further by selective precipitation using ammonium acetate, eliminating the small fragment from the MCS that results from digestion.

Ligating DNA Inserts into the pBT Plasmid

1. Digest 2 µg of the pBT plasmid in a 50-µl reaction volume.

   Optional *If appropriate, treat the digested plasmid DNA with alkaline phosphatase (following the manufacturer's instructions) and isolate using agarose gel electrophoresis.*

2. Extract the digestion reaction with an equal volume of phenol–chloroform until a clear interface is obtained.

3. Repeat the extraction with an equal volume of chloroform.

4. Add an equal volume of 4 M ammonium acetate to the aqueous phase.

5. Add 2 volumes of 100% (v/v) ethanol at room temperature. Immediately spin in a microcentrifuge at room temperature to precipitate the plasmid DNA.

6. Wash the pellet twice with 70% (v/v) ethanol and then dry the pellet.

7. Resuspend the pellet in a volume of TE buffer (see *Preparation of Media and Reagents*) such that the concentration of the plasmid DNA is the same as the concentration of the insert DNA (~0.1 µg/µl).

8. Verify that the plasmid has been digested completely using agarose gel electrophoresis.

9. Calculate the amount of insert required (see equation below). The ideal molar ratio of insert-to-vector DNA is variable; however, 2:1 or 3:1 ratios are recommended. The amount of insert DNA required for a 1:1 ratio is calculated as follows:

$$X \text{ ng of insert} = \frac{(\text{number of base pairs of insert}) \times (100 \text{ ng of vector})}{3210 \text{ bp (pBT plasmid)}}$$

10. Ligate the prepared vector and insert DNA fragments according to the protocol in the table below. Incubate the ligation reaction mixtures overnight at 12°C.

Suggested Ligation Reactions

Ligation Reaction Components	Experimental	Control		
	1	2[a]	3[b]	4[c]
Prepared pBT plasmid DNA (0.1 µg/µl)	1.0 µl	1.0 µl	1.0 µl	0 µl
Prepared insert (0.1 µg/µl)	*Y* µl to make a 2:1 or a 3:1 ratio	0 µl	0 µl	1.0 µl
10 mM rATP (pH 7.0)	1.0 µl	1.0 µl	1.0 µl	1.0 µl
10× ligase buffer[§]	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.5 µl	0 µl	0.5 µl
Double-distilled water (to 10 µl)	*X* µl	*X* µl	*X* µl	*X* µl

[a] Control 2 is used to test the effectiveness of the digestion and alkaline phosphatase treatment (if performed) of the plasmid.

[b] Control 3 is used to test whether the plasmid was digested completely and whether residual uncut plasmid remains.

[c] Control 4 is used to test whether the insert alone is contaminated with the plasmid DNA.

Transformation of the pBT Ligation Reaction

Note *In this section, bacteria are grown on* ***rich medium****, using a* ***30°C incubation temperature****.*

1. Use an aliquot from the ligation reaction to transform the XL1-Blue MRF´ Kan strain (provided with the kit as a glycerol stock). Protocols for preparing and transforming *E. coli* competent cells can be found in reference 4. **It is important to use a host strain (such as XL1-Blue MRF´ Kan) that contains the *lacI*^{q} gene in order to reduce the expression of a potentially toxic bait protein.** Also, the strain must be chloramphenicol-sensitive to allow selection for pBT transformants.

2. Plate the recombinant pBT transformants on LB-chloramphenicol agar[§] plates.

3. Incubate the plates at 30°C for approximately 24 hours. Colonies will be tiny and may require additional growth at 30°C. Incubation at 30°C is necessary to minimize potential toxicity.

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

Verifying the Insert in the pBT Plasmid

To verify insert cloning, prepare miniprep DNA from isolated colonies. Use this DNA to perform restriction analysis, DNA sequence analysis, or PCR analysis (using the primers shown in the table below). Identify recombinants containing the DNA insert in frame with the λcI protein.

Primers for use with pBT

Primer	Sequence
pBT forward primer	5'- TCC GTT GTG GGG AAA GTT ATC - 3'
pBT reverse primer	5'- GGG TAG CCA GCA GCA TCC - 3'

Verifying Expression

Note *In this section, bacterial cell growth is performed either on rich medium using a 30°C incubation temperature or in minimal medium using a 37°C incubation temperature.*

It is often possible to detect insert expression using Western blot analysis, although protein expression from the recombinant pBT plasmid is expected to be low due to the plasmid's low copy number.

Note ***Perform expression analysis in the BacterioMatch II two-hybrid reporter strain,*** *as this is the strain that will ultimately be used to detect the interaction of target and bait. Do not substitute another strain for protein expression experiments.*

For expression analysis, transform BacterioMatch II validation reporter competent cells (Catalog #200192) side-by-side with (1) non-recombinant pBT vector and (2) the sequence-verified recombinant pBT plasmid. Follow the transformation protocol supplied with the reporter competent cells, using the plating modifications given below. General guidelines for expression analysis follow.

Note *When preparing pBT plasmid DNA for transformation into the reporter strain, use protocol modifications for low-copy-number plasmids (i.e. grow a larger culture volume and implement any protocol modifications for low-copy number plasmids suggested by the DNA purification kit manufacturer).*

- Plate the reporter strain pBT transformants on LB-chloramphenicol agar[§] plates. Incubate the plates at 30°C for approximately 24 hours.

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

- Grow individual pBT-containing colonies in 2 ml of M9+ His-dropout broth[§] containing 25 µg/ml chloramphenicol at 37°C overnight. Use these cultures to inoculate fresh 2-ml cultures of M9+ His-dropout broth containing 25 µg/ml chloramphenicol and 10 µM IPTG, to induce protein expression. (Optimizing the IPTG concentration in the 10–100 µM range may be beneficial for some fusion proteins.) Grow the cells at 37°C until the OD_{600} is 0.5–0.6.
- Mix 20 µl of each induced culture with 20 µl of 2× SDS gel sample buffer.[§] Boil the samples for 5 minutes, and resolve using SDS-PAGE. Expression of the recombinant proteins may be verified by Western blot analysis with an antibody that immunoreacts with either the λcI protein or the protein of interest encoded by the insert.

Notes *When comparing protein expression in different strains (different reporter strain transformants) by Western analysis, it is important to grow all of the bacterial cultures to a similar density before preparing the cell lysates.*

Failure to detect expression of the bait fusion protein with a given antibody need not preclude use of the fusion protein-encoding vector. Some proteins may be expressed at levels that are too low for detection by Western blotting with a particular antibody but be expressed at levels sufficient for transcriptional activation of the reporter genes. Verify that the bait or target protein is in frame with the fusion protein before proceeding. See Troubleshooting *for additional suggestions.*

Preparation of Recombinant pBT DNA

Note *In this section, bacteria should be grown in* ***rich medium****, using a* ***30°C incubation temperature****.*

Following sequence validation and, optionally, confirmation of the expression of the fusion protein, purify the recombinant pBT plasmid in sufficient quantity for the subsequent experiments. Grow a culture of XL1-Blue MRF´ Kan cells harboring the verified plasmid, and then use standard alkaline lysis protocols[4] or commercially available plasmid DNA purification columns to prepare the DNA. Both methods usually produce plasmid DNA of sufficient quality for subsequent experiments.

Note *The pBT plasmid is maintained at about 5–10 copies per cell. When preparing plasmid DNA, use protocol modifications for low-copy-number plasmids (i.e. grow a larger culture volume and implement any protocol modifications for low-copy number plasmids suggested by the purification kit manufacturer).*

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

TESTING SELF-ACTIVATION BY RECOMBINANT pBT PRIOR TO SCREENING

Prior to performing a two-hybrid screen using the recombinant bait plasmid (pBT containing the bait gene of interest) it is necessary to determine whether the bait fusion protein is capable of activation of the reporter cassette in the absence of an interaction partner. To test this, the BacterioMatch II reporter strain is cotransformed with the recombinant pBT plasmid and the empty pTRG vector. This cotransformation pair should not produce a significant number of colonies on Selective Screening Medium (5 mM 3-AT). If significant growth on 5 mM 3-AT is observed in this cotransformation experiment, the specific bait is not suitable for use in the BacterioMatch II two-hybrid system without selective alteration of certain residues or regions.

Notes *Use BacterioMatch II validation reporter competent cells (Catalog #200192) to complete this set of control experiments. Follow the* Cotransformation Protocol (Validation Reporter Competent Cells) *on the following pages. Do not substitute another host strain for this control experiment.*

In this section, bacteria are grown on ***minimal medium*** *using a* ***37°C incubation temperature****.*

TABLE II

Cotransformations Carried Out in this Experiment

Plasmids	Plates	Purpose	Expected Results and Guidelines for Interpretation
recombinant pBT + pTRG (empty vector) (50 ng each)	Nonselective Screening Medium (no 3-AT)[§]	Measures number of cotransformants	10^2–10^3cfu per 200 µl of 1:100 dilution plated (efficiency varies for different bait plasmids)
	Selective Screening Medium (5 mM 3-AT)[§]	Tests whether the bait protein is able, on its own, to activate the reporter cassette	≤0.1% of total cotransformants verifies absence of bait self-activation and suitability of bait for library screens 0.1–1% of total cotransformants indicates that troubleshooting is required prior to use in library screens; bait is suitable for assays with individual pTRG target plasmids >1% of total cotransformants indicates bait self-activation or media problem; check pBT +pTRG-Gal11^P negative control cotransformation results to assess media quality
pBT (empty vector) + pTRG-Gal11^P (50 ng each)	Nonselective Screening Medium (no 3-AT)	Measures number of cotransformants	~10^3 cfu per 200 µl of 1:100 dilution plated
	Selective Screening Medium (5 mM 3-AT)	Negative control (non-interacting pair) used to verify media quality	≤0.1% of total cotransformants obtained is expected result; verifies that 3-AT selection is working properly >0.1% of total cotransformants indicates failure of 3-AT selection. Troubleshoot media preparation and repeat experiment

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

Cotransformation Protocol (Validation Reporter Competent Cells)

Note *It is important that the agar plates used in this transformation do not have excessive surface moisture, which can lead to an increase in the appearance of false positives. To achieve proper moisture levels, place the plates in a 37°C incubator with lids ajar for 30 minutes to 1 hour, until the moisture on the lid and agar surface has just evaporated. Avoid overdrying the plates, which results in delayed colony formation.*

If desired, test the transformation efficiency of the reporter strain competent cells using the pUC18 plasmid DNA provided with the competent cells in parallel with the cotransformations performed in this experiment. Follow the transformation protocol provided with the reporter strain competent cells for the pUC18 efficiency verification. Expect an efficiency of $\geq 1 \times 10^7$ *cfu/μg pUC18 DNA.*

1. Pre-warm SOC medium to 42°C.

2. Thaw the BacterioMatch II validation reporter competent cells on ice. Gently mix the cells by tapping the tube. Once thawed, aliquot 100 µl of cells into the appropriate number of prechilled 14-ml BD Falcon tubes. Prepare one aliquot for each pBT/pTRG test pair. **It is critical to use 14-ml BD Falcon polypropylene round-bottom tubes for this procedure.**

3. Add 1.7 µl of the β-ME provided with the reporter strain competent cells to each 100-µl aliquot of cells. Swirl the tube contents gently. **Do not substitute with another preparation of β-ME.**

4. Incubate the tubes on ice for 10 minutes, swirling every 2 minutes.

5. To each tube, add 50 ng each of the appropriate pBT bait vector plus pTRG target vector. Swirl the tubes gently.

6. Incubate the tubes on ice for 30 minutes, swirling gently after the first 15 minutes of incubation.

7. Swirl each tube gently, and then heat-pulse the tubes in a 42°C water bath for 35 seconds. **The duration and temperature of the heat pulse is critical for obtaining the highest efficiencies.**

8. Incubate the tubes on ice for 2 minutes.

9. Add 0.9 ml of pre-heated SOC medium to each tube.

10. Incubate the tubes at 37°C with shaking at 225 rpm for 90 minutes.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

11. Spin down the cells in a tabletop centrifuge at 2000 × *g* for 10 minutes. This step may be completed either at room temperature or at 4°C.

12. Aspirate the supernatant, taking care to avoid disturbing the pellet. To remove the rich medium, wash the cells once by resuspending the cells in 1 ml of room temperature M9+ His-dropout broth.

13. Collect the cells as described in step 11, gently aspirate the supernatant, and then resuspend the cells in a fresh 1 ml of M9+ His-dropout broth.

14. Incubate cells at 37°C with shaking at 225 rpm for 2 hours. This allows the cells to adapt to growth in minimal medium prior to plating.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

15. Plate each of the cotransformation mixtures on the appropriate media. For the cotransformation experiments listed in Table II, proceed to step 16. For other experiments that employ the *Cotransformation Protocol (Validation Reporter Competent Cells)*, proceed to the relevant section of the manual for plating instructions.

    Note *It is important to plate the recommended volume and to spread the mixture evenly on the plates as this promotes even colony distribution. Using sterile glass beads to spread the cells on the plates may facilitate even distribution.*

Plating and Analysis of the pBT-Bait Self-Activation Test

16. For each of the cotransformations listed in Table II, plate 200 µl of cells from the adapted outgrowth culture on Selective Screening Medium (5 mM 3-AT) plates.

17. For plating on Nonselective Screening Medium (no 3-AT), remove 100 µl of cells from the adapted outgrowth culture. Use this aliquot to prepare a 1:100 dilution of the culture in M9+ His-dropout broth. Plate 20-µl and 200-µl aliquots of the diluted cell suspension on Nonselective Screening Medium (no 3-AT) plates.

18. Incubate the plates at 37°C for 24 hours. If colonies are not apparent, transfer the plates to room temperature and continue to incubate the plates in a dark location (to preserve the tetracycline) for an additional 16 hours. This secondary incubation may allow the growth of cells containing toxic proteins or weak interactors.

19. Count the cfu obtained on the Nonselective Screening Medium (no 3-AT) plate that contains the appropriate number of colonies for counting (optimally 10–1000 cfu). This value is used to determine the number of cotransformants obtained.

    Significant numbers of colonies should be observed, indicating that the reporter strain competent cells were successfully cotransformed with the plasmid pair. The number of colonies obtained is variable for different plasmid pairs.

20. Count the cfu obtained on the Selective Screening Medium (5 mM 3-AT) plate.

    Note *It is normal for reporter strain transformants of various sizes to appear on both Nonselective- and Selective Screening Medium plates.*

21. Calculate the percentage of cotransformants that are able to grow on Selective Screening Medium (5 mM 3-AT) for each plasmid pair. When performing this calculation, take into account the 100-fold dilution, as well as the different amounts of the dilution plated. It is generally useful to standardize the calculations to the cfu expected from 200 µl of undiluted cell suspension, since this single amount was used for plating on selective medium. See below for two sample calculations from two theoretical experiments.

Calculation 1: Number of cotransformants in 200 µl of culture:

cfu obtained on Nonselective Screening Medium (no 3-AT) plate	**Dilution factor**	**Plating volume factor (relative to 200 µl)**	**Adjusted cotransformants from 200 µl culture**
50 cfu (20 µl plated)	× 100	× 10	50,000 cfu
100 cfu (200 µl plated)	× 100	× 1	10,000 cfu

Calculation 2: Percent of cotransformants able to grow on 5 mM 3-AT:

Adjusted cotransformants (200 µl culture)	**cfu obtained on Selective Screening Medium (5 mM 3-AT) (200 µl culture)**	**Percent of cotransformants able to grow on 5 mM 3-AT**
50,000 cfu	20 cfu	0.04%
10,000 cfu	5 cfu	0.05%

22. Compare the results of these calculations to the guidelines presented in Table II, to assess the suitability of the bait plasmid for BacterioMatch II two-hybrid library screens.

    If the bait protein is found to be unsuitable for BacterioMatch II two-hybrid system experiments, it may be possible to identify the regions/residues of the protein responsible for the non-specific interaction. Alteration of these residues may be used to create a suitable hybrid bait protein.

PREPARATION OF DNA FROM THE pTRG PLASMID cDNA LIBRARY

Description of *E. coli* Host Strain and Genotype

The cDNA library is harbored in the XL1-Blue MRF´ Kan strain. This strain is a restriction minus (McrA$^-$, McrCB$^-$, McrF$^-$, Mrr$^-$, HsdR) derivative of XL1-Blue supercompetent cells.[5, 6] XL1-Blue MRF´ Kan cells are deficient in all known restriction systems [Δ*(mcrA)183,* Δ*(mcrCB-hsdSMR-mrr)173*], are endonuclease deficient (*endA1*), and are recombination deficient (*recA*). The *mcrA, mcrCB*, and *mrr* mutations prevent cleavage of cloned DNA that carries cytosine and/or adenine methylation, which is often present in eukaryotic DNA and cDNA.[7, 8, 9] The McrA and McrCB systems recognize and restrict methylated cytosine DNA sequences. The Mrr system recognizes and restricts methylated adenine DNA sequences. It has been shown that the Mrr system also restricts methylated cytosine DNA sequences with a specificity differing from that of McrA and McrCB. This activity has been named McrF. This McrF activity against methylated cytosines has been shown to be equal to or greater than the restriction activity of the McrA and McrCB systems.[6] All of these systems (McrA, McrCB, McrF, Mrr, and HsdR) have been removed from XL1-Blue MRF´ Kan. The *hsdR* mutation prevents the cleavage of cloned DNA by the *Eco*K (*hsdR*) endonuclease system, and the *recA* mutation helps ensure insert stability. The *endA1* mutation greatly improves the quality of plasmid miniprep DNA.

Library Amplification and DNA Purification

To screen for bait and target protein-protein interactions, it is necessary to cotransform the BacterioMatch II two-hybrid system reporter strain competent cells with recombinant bait and cDNA library target plasmid DNA. Protocols in previous sections describe the construction, verification, and purification of bait plasmid DNA. The protocol below describes library amplification and plasmid DNA purification from the pTRG cDNA library using LB-tetracycline plates. If preferred, it is also possible to amplify the library using the semi-solid agar method.[10, 11]

Notes *For this procedure, the BacterioMatch II two-hybrid system XR cDNA library glycerol stock must be thawed completely.* ***Before thawing the glycerol stock,*** *consult the Certificate of Analysis for titer (cfu/ml) of the library and plan your plating scheme. Re-freezing the glycerol stock results in a significant loss of titer.*

In this section, bacteria are grown on ***rich medium*** *using a* ***30°C incubation temperature****.*

The basic protocol below involves plating the library on 150-mm round plates. Instructions are also provided for using 25-cm × 25-cm square plates, which are larger and allow the growth of a greater number of colonies on each plate.

Plating the Library for Amplification

Planning the Plating Scheme

Plating density: The library should be plated a density of ~20,000–30,000 colonies per 150-mm LB-tetracycline agar plate, or ~100,000 colonies per 25-cm × 25-cm square LB-tetracycline agar plate.

Amount of library glycerol stock per plate: Consult the Certificate of Analysis included with your BacterioMatch II two-hybrid system XR cDNA library for the library titer. Use this titer to calculate the volume of the glycerol stock to spread on each plate to achieve the appropriate number of colonies per plate.

Volume of SOC per plate: After calculating the volume of the glycerol stock per plate, calculate the amount of SOC medium required to bring the total spreading volume to 250 µl for 150 mm plates or to 1 ml for 25-cm × 25-cm square plates.

Number of plates: Determine the number of plates required to achieve the number of individual colonies you plan to harvest. For example if you are using 150-mm plates and plan to harvest 1×10^6 individual colonies, you will need to plate 20,000–30,000 colonies on each of 35–50 plates.

Summary of Plating Recommendations

Plate Type	Colony Number per Plate	Total Volume Spread per Plate (Glycerol Stock + SOC Diluent)
150-mm round	20,000–30,000	250 µl
25-cm × 25-cm square	100,000	1 ml

Performing the Plating

1. Completely thaw the 0.5-ml glycerol stock on ice. Invert the thawed stock several times to ensure the cells are completely resuspended. Plate according to step 2, below, and immediately re-freeze the remaining library stock in aliquots (see step 4).

2. On each LB-tetracycline agar plate, place a pool of SOC medium of the calculated volume and then pipette the appropriate volume of the cDNA library glycerol stock into the SOC pool. Spread the mixture evenly over the surface of the agar using a sterile spreader.

   If using 150-mm plates, on each plate spread ~2–3 × 10^4 cfu, using a total spreading volume (volume of glycerol stock + volume of SOC) of 250 µl.

   If using 25-cm × 25-cm square plates, on each plate spread ~10^5 cfu, using a use a total spreading volume (volume of glycerol stock + volume of SOC) of 1 ml.

3. Incubate the plates at 30°C for approximately 24 hours.

   Note *Increasing the incubation time (up to 30 hours) may increase the recovery of plasmids containing longer cDNA inserts.*

4. Aliquot the remainder of the library glycerol stock in several sterile, RNase/DNase free microcentrifuge tubes and re-freeze at –80°C.

   Note *Significant loss in titer will result following re-freezing. Prior to plating more of the library from this re-frozen glycerol stock, thaw one of the aliquots and plate serial dilutions from the thawed aliquot. Calculate the titer for the remaining aliquots from these serial dilution plates. Use this titer information to plan any later rounds of library amplification.*

Harvesting the Amplified Library

1. To prepare to harvest the library, place on ice the sterile container to be used to collect the amplified, pooled library. Pipet a small amount of liquid medium into the bottom of the container to prevent the dense bacterial paste from adhering to the container.

2. Using a wide, sterile scraper (for example a cell scraper, cell lifter, or rubber policeman), gently scrape all of the bacteria to one edge of the first 150-mm plate. Lift or scrape the dense paste from the edge into the chilled, sterile container.**

3. Repeat step 2 for all additional plates, ensuring that bacteria from all plates are pooled into the same sterile container.

4. Add enough SOC medium to the collected bacterial paste to allow pipetting. Keep the volume of added medium as small as possible.

5. Pipet the harvested bacteria up and down repeatedly but gently until the cells are completely resuspended. The cell paste will be quite dense; this step requires some time. Record the volume of resuspended cells. This is the amplified library.

6. Plate 2 µl and 5 µl of the resuspended amplified library onto LB-tetracycline plates and incubate the plates at 30°C for 24 hours to determine the size of the amplified library (cfu/ml).

** Some researchers prefer to add a small volume of liquid medium (e.g. SOC medium) to the plate before scraping or prefer to "wash" the plate with liquid medium after scraping. If this is desired, keep the volume of liquid medium added to a minimum, using just enough to allow pipetting during the resuspension step (~0.5 ml per 15-cm round plate and ~2 ml to a 25-cm × 25-cm plate). If scraping 25-cm × 25-cm plates, it is possible to use a very wide steel spatula or putty knife, which can be sterilized by ethanol flaming. Scraping square plates with a steel spatula, like scraping round plates with other tools, can be performed with or without added liquid medium.

7. Use half of the pooled bacteria to purify plasmid DNA using a commercially available maxiprep DNA column or alkaline lysis/CsCl gradient purification method. This DNA is suitable for use in transformation of the BacterioMatch II reporter strain competent cells.

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

9. Dispense 1-ml aliquots into sterile 1.5-ml microcentrifuge tubes and freeze at –80°C. These aliquots can be used to inoculate liquid culture for preparation of additional DNA. Do not perform additional rounds of amplification, as slower-growing clones may be significantly underrepresented.

LIBRARY SCREENING

Screening the Library: Overview

Figure 6 and Tables III and IV illustrate the steps to be performed in this section and describe the media that will be used during library screening.

Note *The protocols presented in this section are appropriate for library screening using the chemically-competent BacterioMatch II screening reporter strain cells (catalog #200190) provided with this kit. Screening may also be performed using BacterioMatch II electrocompetent reporter cells (catalog #200195), which offer higher cotransformation efficiencies. For screening protocols appropriate for the electrocompetent reporter cells, see* Appendix: Library Screening Using Electrocompetent Reporter Cells.

The screening experiment consists of plating recombinant pBT + pTRG cDNA library cotransformants on selective medium in sufficient numbers to cover the cDNA library complexity. (The number of cotransformation reactions required to cover the library is determined by performing a pilot cotransformation.) Colonies obtained on the initial screening plates [Selective Screening Medium (5 mM 3-AT)] are enriched on a second selective plate. Putative positive colonies are then validated using streptomycin resistance as a secondary reporter.

Perform a negative control cotransformation with pBT-bait (containing the bait gene insert) + pTRG (empty vector) alongside the library cotransformation. This negative control transformation should be carried through the screening protocol in parallel with the library screen.

As a positive control, cotransform the reporter strain with pBT-LGF2 + pTRG-Gal11^{P}. The positive control is performed in conjunction with the pilot cotransformation using a smaller cotransformation volume.

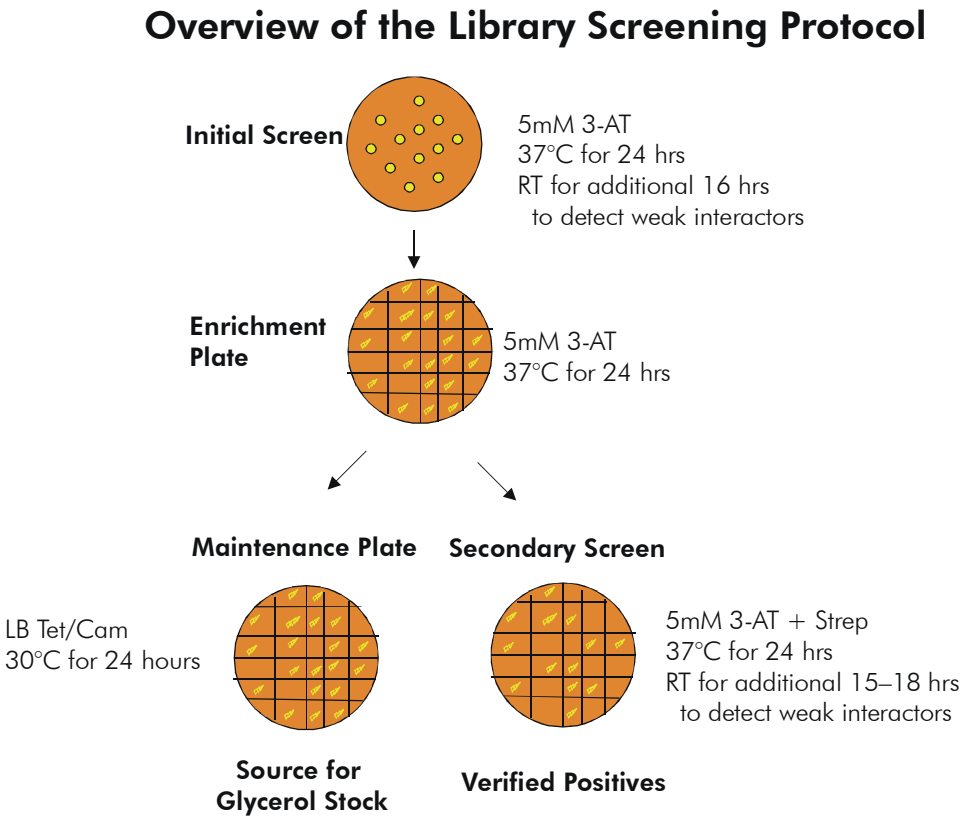


FIGURE 6 Overview of the library screening process

TABLE III

Overview of Plasmid Pairs for Library Screen Experiments

Plasmids	Purpose	Scale of Cotransformation Reaction	Growth expected on 5 mM 3-AT?
pBT-LGF2 + pTRG-Gal11^{P} (50 ng each)	Positive control. Coexpression in the reporter strain of Gal4 (encoded by LGF2) and Gal11^{P} demonstrates the appearance of a robust interaction	1 × 100 µl	yes
recombinant pBT + pTRG cDNA library (50 ng each)	Pilot library cotransformation to test the cotransformation efficiency for the specific recombinant pBT (containing the bait gene of interest) and the pTRG cDNA plasmid library	1 × 100 µl	N/A (not plated)
recombinant pBT + pTRG cDNA library (200 ng each per transformation reaction)	Library screen to test for interaction between the bait protein and target proteins in the cDNA library; colonies on Selective Screening Medium (5 mM 3-AT) indicate putative interaction	N × 500 µl, where N= number of reactions determined by pilot cotransformation	yes, if interactors are present in the library
recombinant pBT + pTRG empty vector (200 ng each)	Negative control. Growth observed on Selective Screening Medium (5 mM 3-AT) indicates the background expected from the bait protein in the absence of an interaction partner; insignificant growth is expected	1 × 500 µl	no (few or no colonies expected)

TABLE IV

Media used in BacterioMatch II Two-Hybrid Library Screening and Validation Experiments

Medium	Purpose
Selective Screening Medium (5 mM 3-AT) plates	Identification and enrichment of putative interactors based on *HIS3* transcriptional activation
Nonselective Screening Medium (no 3-AT) plates	Verification of cotransformation and calculation of cotransformation efficiency
Dual Selective Screening Medium (5 mM 3-AT + Strep) plates[§]	Verification of interaction based on transcriptional activation of the aadA gene, conferring streptomycin resistance
LB-Chloramphenicol/Tetracycline plates [§]	Maintenance of putative positives
LB-Tetracycline plates	Isolation of positive pTRG plasmids for retransformation
LB-Chloramphenicol plates	Verification of chloramphenicol sensitivity (indicating loss of pBT) prior to isolation of pTRG plasmids for retransformation
SOC liquid medium	Outgrowth following cotransformation
$M9^{+}$ His-dropout broth	Adaptation to minimal medium following outgrowth and prior to plating

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

Pilot Library Cotransformation and Positive Control Cotransformation

Note *In this section, bacterial cell growth is performed on minimal medium using a 37°C incubation temperature.*

Use one 500-µl aliquot of BacterioMatch II screening reporter competent cells (Stratagene Catalog #200190) to perform both the pilot library cotransformation and the positive control cotransformation. Follow the *BacterioMatch II Screening Reporter Competent Cells Cotransformation Protocol (100-µl Reactions)*, below, for this set of experiments.

For the pilot library cotransformation, transform the BacterioMatch II reporter strain competent cells with 50 ng of the recombinant pBT bait vector plus 50 ng of the pTRG target cDNA library DNA. Use the results of this pilot cotransformation to calculate the number of cotransformation reactions required to achieve library coverage.

For the positive control cotransformation, transform the BacterioMatch II reporter strain competent cells with 50 ng of pBT-LGF2 bait vector plus 50 ng of pTRG-Gal11^{P} target vector.

BacterioMatch II Screening Reporter Competent Cells Cotransformation Protocol (100-µl Reactions)

Note *It is important that the agar plates used in this transformation do not have excessive surface moisture, which can lead to an increase in the appearance of false positives. To achieve proper moisture levels, place the plates in a 37°C incubator with lids ajar for 30 minutes to 1 hour, until the moisture on the lid and agar surface has just evaporated. Avoid overdrying the plates, which results in delayed colony formation.*

If desired, test the transformation efficiency of the reporter strain competent cells using the pUC18 plasmid DNA provided with the competent cells in parallel with the cotransformations performed in this experiment. Follow the transformation protocol provided with the competent cells for the pUC18 efficiency verification. Expect an efficiency of ≥7.5 × 10^{7} cfu/µg pUC18 DNA.

1. Pre-warm SOC medium to 42°C.

2. Thaw one tube (500-µl) of BacterioMatch II screening reporter strain competent cells on ice. Gently mix the cells by tapping the tube. Once thawed, aliquot 100 µl of cells into the appropriate number of prechilled 14-ml BD Falcon tubes. **It is critical to use 14-ml BD Falcon polypropylene round-bottom tubes for this procedure.**

   Prepare one aliquot for each pBT/pTRG test pair, including the pilot library cotransformation and the positive control pBT-LGF2 and pTRG-Gal11^{P} plasmid pair.

3. Add 1.7 µl of the β-ME provided with the reporter strain competent cells to each 100-µl aliquot of cells. Swirl the contents of tube gently. Do not use a different β-ME preparation.

4. Incubate the tubes on ice for 10 minutes, swirling every 2 minutes.

5. To the cotransformation reaction tubes, add 50 ng each of the appropriate pBT bait vector plus pTRG target vector. Swirl the tubes gently.

6. Incubate the tubes on ice for 30 minutes, swirling gently after the first 15 minutes of incubation.

7. Swirl each tube gently, and then heat-pulse the tubes in a 42°C water bath for 35 seconds. **The precise temperature and duration of the heat pulse are critical determinants of cotransformation efficiency.** These two variables should be controlled as precisely as possible.

8. Incubate the tubes on ice for 2 minutes.

9. Add 0.9 ml of pre-heated SOC medium to each tube.

10. Incubate the tubes at 37 °C with shaking at 225 rpm for 90 minutes.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

11. Spin down the cells in a tabletop centrifuge at 2000 × *g* for 10 minutes. This step may be completed either at room temperature or at 4 °C.

12. Aspirate the supernatant, taking care to avoid disturbing the pellet. To remove the rich medium, wash the cells once by resuspending the cells in 1 ml of room temperature $M9^+$ His-dropout broth.

13. Collect the cells as described in step 11, gently aspirate the supernatant, and then resuspend the cells in a fresh 1 ml of $M9^+$ His-dropout broth.

14. Incubate cells at 37°C with shaking at 225 rpm for 2 hours. This allows the cells to adapt to growth in minimal medium prior to plating.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

15. Plate each of the cotransformation mixtures on the appropriate media, as indicated in the following sections.

    Note *It is important to plate the recommended volume and to spread the mixture evenly on the plates as this promotes even colony distribution. Using sterile glass beads to spread the cells on the plates may facilitate even distribution.*

Plating and Evaluating the Positive Control Cotransformation

After completing step 14 of the *BacterioMatch II Screening Reporter Competent Cells Cotransformation Protocol (100 µl)*, perform the following plating and analysis steps.

1. Plate 100-µl aliquots of the pBT-LGF2 + pTRG-Gal11^{P} cotransformation cell suspension on both Nonselective Screening Medium (no 3-AT) and on Selective Screening Medium (5 mM 3-AT).

2. Incubate the plates at 37°C for 18–24 hours.

3. Score the plates for growth. Interactions between a pair of hybrid proteins, including the Gal4 + Gal11^{P} hybrid protein pair, are indicated by growth on Selective Screening Medium (5 mM 3-AT). For the positive control transformation, a similar number of colonies are expected on the selective and nonselective plates. (Expect 10^4–10^5 cfu on each plate.)

4. Store the plates at 4°C for reference during the library screening experiments and to retain a stock of positive control cotransformants.

Note *The interaction of the Gal4-Gal11^{P} hybrid pair is robust and is readily detectable in the BacterioMatch II two-hybrid system.* ***It is important to note that cells harboring weaker interactors are expected to grow more slowly, requiring longer incubation times for colony development****.*

Plating and Evaluating the Pilot Cotransformation

After completing step 14 of the *BacterioMatch II Screening Reporter Competent Cells Cotransformation Protocol (100 µl)*, perform the following plating and analysis steps.

1. Remove 100 µl of cells from the adapted outgrowth culture from step 14 of the transformation protocol and use this aliquot to perform two 10-fold serial dilutions in M9^{+} His-dropout broth.

2. From the 100-fold dilution, plate 100 µl on each of three Nonselective Screening Medium (no 3-AT) plates (100 mm).

3. Incubate the plates at 37°C, inverted, for 24 hours. (Colonies may first become visible at 16–18 hours, but the plates should be incubated for the full 24 hours before colonies are counted.)

4. Count the colonies on each of the Nonselective Screening Medium (no 3-AT) plates. Each plate contains 1/1000th of the total cell suspension volume from the 100-µl pilot reaction.

5. Calculate the total cotransformants expected per 500-µl transformation reaction by multiplying the cfu obtained on a single plate by 5000. Average the results obtained for the three plates to derive the expected number of cotransformants for each 500-µl library cotransformation.

6. Determine the number of 500-µl cotransformation reactions required to achieve library coverage. For example, if 2.5×10^5 cotransformants are expected from each 500-µl of competent cells and the library contains 10^6 clones, four transformation reactions are required to cover the library once. In this example, the initial screen plating requires approximately 20, 150-mm Selective Screening Medium (5 mM 3-AT) plates.

Performing the BacterioMatch II Two-Hybrid Interaction Library Screen

Note *For library screens, most bacterial cell growth is performed on* ***minimal medium*** *using a* ***37°C incubation temperature****. The exceptional steps that require growth on rich medium employ a 30°C incubation temperature.*

Library Screening Transformation Protocol for the BacterioMatch II Screening Reporter Competent Cells (500 µl Reactions)

After evaluating the results of the pilot library cotransformation, perform a large-scale cotransformation of the reporter strain with recombinant pBT plus the pTRG cDNA library DNA, using the calculated number of 500-µl cotransformation reactions. Like the pilot cotransformation, this screen cotransformation should be completed using BacterioMatch II screening reporter competent cells (Catalog #200190). Use the protocol below for the large-scale screen cotransformation. (Do not use the protocol for the 100-µl pilot experiment.)

1. Pre-warm SOC medium to 42°C.

2. Thaw the appropriate number of aliquots of the BacterioMatch II screening reporter competent cells on ice. Gently mix the cells by tapping the tube. Once thawed, transfer 500 µl of cells into the appropriate number of prechilled 14-ml BD Falcon tubes**. It is critical to use 14-ml BD Falcon polypropylene round-bottom tubes for this procedure.**

3. Add 8.5 µl of the β-ME mix provided with the kit to each tube. Swirl the contents of tubes gently. **Do not use a different β-ME preparation.**

4. Incubate the tubes on ice for 10 minutes, swirling every 2 minutes.

5. To each tube, add 200 ng of the pTRG cDNA library and 200 ng of recombinant pBT. Swirl the tubes gently to mix.

6. Incubate the tubes on ice for 30 minutes. Swirl the tubes gently after the first 15 minutes of incubation.

7. Swirl the tubes gently and then heat-pulse the reactions at 42°C for 55 seconds. **The precise temperature and duration of the heat pulse are critical determinants of cotransformation efficiency.** These two variables should be controlled as precisely as possible.

8. Incubate the tubes on ice for 2 minutes.

9. Add 2.5 ml of pre-warmed SOC medium (at 42°C) to the tubes. Transfer each of the cell suspensions to separate 50-ml conical tubes for outgrowth.

10. Incubate the conical tubes at 37°C with shaking at 225 rpm for 90 minutes.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

11. Spin down the cells in a tabletop centrifuge at 2000 × *g* for 10 minutes. This step may be completed either at room temperature or at 4°C.

12. For each tube, aspirate the supernatant gently, taking care to avoid disturbing the pellet. In order to remove residual rich medium, wash the cells twice. For the first wash, resuspend the cells in 3 ml of room-temperature M9$^+$ His-dropout broth and then collect the cells as described in step 11.

13. For the second wash, aspirate the supernatant gently, and then resuspend the cells in a fresh 3 ml of M9+ His-dropout broth. Re-collect the cells (as described in step 11) and remove the wash medium by gentle aspiration.

14. For each tube, resuspend the cells in 1.5 ml of M9$^+$ His-dropout broth.

15. Incubate cells at 37°C with shaking at 225 rpm for 2 hours. This allows the cells to adapt to growth in minimal medium prior to plating.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

16. During the adaptation period (see step 15), evaluate and correct the surface moisture of the agar plates. It is important that the plate surface is not too wet to absorb the cell mixture. To achieve proper moisture levels, place the plates in a 37°C incubator with lids ajar for 30 minutes to 1 hour, until the moisture on the lid and agar surface has just evaporated. Avoid overdrying the plates, which results in delayed colony formation.

17. Pool the adapted outgrowth cultures from step 15 into one tube. Swirl the tube well to mix.

Plating the Library Screen Cotransformants

18. Remove an aliquot of the transformation mixture pool to determine the cotransformation efficiency. Remove 100 µl from the pool and prepare a 100-fold dilution by performing two 10-fold serial dilutions in $M9^+$ His-dropout broth. From the 100-fold dilution, plate 100 µl on each of three 100-mm Nonselective Screening Medium (no 3-AT) plates. Determine the cotransformation efficiency, as well as the total number of colonies screened, from these plates.

19. Plate the remainder of the pool, in ~300-µl aliquots, onto 150-mm Selective Screening Medium (5 mM 3-AT) plates.

    Note *To allow single colony formation, the total number of cotransformants per 150-mm plate should not exceed 2 x 10^5.*

20. Incubate the plates from steps 18 and 19 at 37 °C, inverted, for 24 hrs.

Enrichment of Putative Interactors

21. At the 24 hour time point, pick all colonies from the screening plates and patch each colony onto a 100-mm Selective Screening Medium (5 mM 3-AT) plate to enrich for the cotransformants (enrichment plate #1). Mark the positions of the picked colonies on the 150-mm screening plates, and then incubate the plates at room temperature, in a dark location for an additional 15–16 hours. This secondary incubation may allow the growth of cells harboring plasmids that encode toxic proteins or weak interactors. After the room temperature incubation, pick the late-arising colonies from the screening plates and patch onto a second Selective Screening Medium (5 mM 3-AT) plate (enrichment plate #2).

22. After each of the enrichment plates are patched, incubate each plate at 37°C for 16–18 hours, until the patched colonies appear. Continue with the processing and analysis of both enrichment plates #1 and #2 at the appropriate times.

Verification of Positives using the Streptomycin Resistance Reporter (Secondary Screen)

1. Pick cells from the enrichment plate patches and re-patch the cells onto both a Dual Selective Screening Medium (5 mM 3-AT + Streptomycin) plate and an LB-Tetracycline/Chloramphenicol plate for secondary screening and colony preservation, respectively. As indicators of growth expected on the Dual Selective Screening Medium, include patches of positive control cotransformants (pBT-LGF2 + pTRG-Gal11^{P}, taken from a Selective Screening Medium plate) and negative control cotransformants (recombinant pBT + pTRG empty vector, taken from a Nonselective Screening Medium plate).

Notes *Patching should be performed by lightly brushing cells from the toothpick onto the surface of the agar plate. Avoid piercing the surface of the agar with the toothpick during patching.*

Use fresh colonies from the Selective Screening Medium (5mM 3-AT) plates for patching on Dual Selective Screening Medium (5 mM 3-AT + Strep) plates. Colonies should be maintained on the selective medium for less than three days prior to patching on the dual selective medium.

Do not use cells maintained on rich medium for the aadA reporter gene activation secondary screen. If it is necessary to use cells kept on rich medium, adapt the cells first by growth on Nonselective Screening Medium and then by growth on Selective Screening Medium prior to patching on the Dual Selective Screening Medium plates. Switching cells from rich medium to minimal medium results in a growth delay, and the cells must be adapted to growth on minimal medium prior to testing the activation of the aadA reporter gene.

2. Incubate the secondary screen and the maintenance plates at 37°C for 24 hours. Record the growth pattern at the 24 hour time point.

3. Transfer both the secondary screen (5 mM 3-AT + Streptomycin) and the maintenance plates to room temperature and incubate the plates for additional 15–18 hrs in a dark location. Record the growth pattern. Store the maintenance plate at 4°C for up to 10 days.

   Note *As soon as interactor candidates have been validated to an acceptable confidence level, generate a glycerol stock for each cotransformant.*

4. Identify the colonies that grow on Dual Selective Screening Medium (5 mM 3-AT + Streptomycin). Using cells from a glycerol stock or the maintenance plate, perform further analysis to confirm the interaction and identify the pTRG plasmid that confers reporter gene activation.

Validation of Detected Protein-Protein Interactions: Retransformation of Isolated Target Plasmid Plus Bait Plasmid

It is necessary to verify the interaction between the bait fusion protein and the putative positive target proteins (isolated from the pTRG cDNA library screening described above). Demonstration of plasmid linkage, by retransformation of the reporter strain with a purified recombinant pTRG plasmid plus the recombinant pBT plasmid, is a required early validation step and is described here.

Purification of Plasmid DNA from the 3-AT Resistant Colonies Isolated During Library Screening

Note *The pTRG plasmid is present in the host at about 20–30 copies per cell. When preparing plasmid DNA, use suggested protocol modifications for low-copy-number plasmids. In order to obtain sufficient DNA for downstream analysis, including sequencing, a larger volume of overnight culture may be required for each putative positive isolate.*

1. Using cells from a glycerol stock or from colonies on a fresh LB-Tetracycline/Chloramphenicol maintenance plate, inoculate 2-ml cultures of LB broth supplemented with tetracycline (12.5 µg/ml).

   Note *No chloramphenicol should be included in the cultures to allow the loss of the bait plasmid during cell growth.*

2. Incubate the cultures at 30°C overnight with shaking at 225 rpm.

   Note *Ensure that the shaker speed does not exceed 250 rpm.*

3. Pellet the bacteria from up to 5 ml of culture by centrifugation at 3000 × *g* for 10 minutes at 4°C.

4. Prepare the plasmid DNA from the bacterial pellet using the method of choice. Standard alkaline lysis protocols[4] or commercially available plasmid DNA purification columns usually produce sufficient quality plasmid DNA. We offer the StrataPrep Plasmid Miniprep Kit (Catalog #400761), which is appropriate for this purpose. Protocol modifications to enhance recovery of low-copy-number plasmids should be employed.

Isolation of Putative Positive pTRG Clones from pBT Bait Plasmid

Since the DNA isolated from the putative positive colony contains both recombinant pBT and recombinant pTRG, it is necessary to separate the recombinant pTRG from the recombinant pBT. To separate the two plasmids, use the plasmid DNA isolated in step 4 above to transform XL1-Blue MRF´ Kan cells, provided in this kit as a glycerol stock. **It is important that the strain contains the *lacI*^{q} gene, but does not contain a tetracycline resistance gene that would interfere with detection of the true transformants.** Protocols for preparing and transforming *E. coli* competent cells can be found in reference 4.

1. Use 1 µl of purified plasmid DNA from each positive interactor clone to transform the XL1-Blue MRF´ Kan strain.

2. For each clone, after outgrowth of the transformation, plate 10 µl of the recovered cells onto 100-mm LB-tetracycline agar plates. (First, add 90 µl of SOC broth onto the center of each plate. Pipet 10 µl of cell suspension into the pool of SOC broth, and then spread the mixture across the plate.)

3. Incubate the plates at 30°C, inverted, for 17–30 hours.

4. Remove the plates from the incubator and patch colonies from each plate onto each of two plates: one plate containing LB-chloramphenicol and the other containing LB-tetracycline.

5. Identify colonies that are tet^r cam^s, indicating that the cells harbor only recombinant pTRG plasmid DNA. (Typically 80% of the patched colonies display this phenotype.) Culture cells containing each isolated putative positive target plasmid (tet^r cam^s) overnight at 30°C in LB broth containing 12.5 µg/ml tetracycline.

6. Use standard alkaline lysis protocols[4] or commercially available plasmid DNA purification columns to purify the plasmid DNA. Protocol modifications to enhance recovery of low-copy-number plasmids should be employed.

Retransformation of the Reporter Strain with the Isolated Target Plasmids

Cotransform the reporter strain using each purified target plasmid paired with the recombinant pBT plasmid. To verify dependence on the bait protein, also cotransform each purified target plasmid paired with the empty pBT vector. Together, these experiments serve to confirm that an observed interaction may be attributed to a specific isolated pTRG plasmid and to show that the individual target protein is not able to activate the reporter genes in the absence of the bait protein.

1. Perform the cotransformations using BacterioMatch II Validation Reporter Competent Cells (Catalog #200192). Follow the *Cotransformation Protocol (Validation Reporter Competent Cells)* in the manual section *Testing Self-Activation by Recombinant pBT Prior to Screening*. Complete steps 1–14 using two separate 100-µl aliquots of cells for each purified target plasmid, including the following two cotransformations and plating the cotransformation reactions on each of the indicated media plates:

Plasmids	Plates	Purpose
recombinant pBT + purified pTRG (containing a single cDNA) (50 ng each)	Selective Screening Medium (5 mM 3-AT)	Verification that the observed interaction is attributable to the isolated pTRG cDNA plasmid
	Nonselective Screening Medium (no 3-AT)	Verification of successful cotransformation and maintenance of cells cotransformed with the pTRG clone plus recombinant pBT
pBT (empty vector) + purified pTRG (containing a single cDNA) (50 ng each)	Selective Screening Medium (5 mM 3-AT)	Verification that the observed interaction is dependent on the bait protein
	Nonselective Screening Medium (no 3-AT)	Verification of successful cotransformation to allow interpretation of the Selective Screening Medium (5 mM 3-AT) results

2. Typically, this stage of analysis involves several individual putative target interactors, and analysis is most conveniently performed using a spotting assay. Prepare the appropriate number of both Nonselective Screening Medium (no 3-AT) and Selective Screening Medium (5 mM 3-AT) plates with grids suitable for spotting a 4-µl aliquot of each of the cotransformation reactions. For the spotting assay, after completing step 14 (adaptation of the cells at 37°C for 2 hours), spot a 4-µl aliquot of the cells onto both Nonselective Screening Medium (no 3-AT) and Selective Screening Medium (5 mM 3-AT). Store the remainder of the cell suspension at 4°C for potential re-plating after analysis of the spotting assay (See *Analysis of Retransformation Results*.)

   If only a few putative target interactors are being analyzed at this stage, plating a larger volume of each cotransformation reaction on separate plates may be preferable. In this case, spread 100 µl of the cotransformation reactions on both Nonselective Screening Medium (no 3-AT) and Selective Screening Medium (5 mM 3-AT) plates.

3. Incubate the plates at 37°C overnight.

Analysis of Retransformation Results

Those clones that reproducibly grow on Selective Screening Medium (5 mM 3-AT) when cotransformed with the bait protein and that fail to grow on Selective Screening Medium (5 mM 3-AT) when cotransformed with the empty pBT vector are verified positives and should be analyzed further.

If a clone fails to grow on Selective Screening Medium (5 mM 3-AT) when cotransformed with the bait protein, the following possibilities should be considered. False negative results may be generated in the retransformation spotting assay by a low cotransformation efficiency, by a weak interaction, or by a combination of these factors. To address these possibilities, we recommend plating 100 µl of the cotransformation reactions (stored at 4°C) on both Nonselective Screening Medium (no 3-AT) and Selective Screening Medium (5 mM 3-AT) plates. This ensures that an adequate number of cells are plated to accurately analyze the success or failure of cotransformation and to allow the detection of a weak interaction. If, upon examination of the Nonselective Screening Medium (no 3-AT) plates, the cotransformation efficiency appears to be low, the cotransformation should be repeated for this plasmid pair.

If cotransformation with the plasmid pair is demonstrated on the nonselective plates (no 3-AT), but no growth is observed on the selective plates (5 mM 3-AT), the positive activation phenotype observed in the original screen may be pTRG plasmid-independent. These clones may be dismissed from further analysis because they are likely to be false positives.

Further Analysis of Verified Positives

To identify the protein encoded by the target DNA, the nucleotide sequence of the target DNA can be determined and compared to nucleotide sequence databases to identify related proteins. In addition, the target DNA can be used as a hybridization probe to screen libraries for full-length target DNA clones and for clones with high homology to the target DNA.

TROUBLESHOOTING

Observation	Suggestion
The yield of bait plasmid is low	The copy number of the bait plasmid is low in the host strain. Increase the volume of culture or apply a standard spectinomycin treatment to the culture (see reference 4). Ensure that the host strain contains the *lacI*^{q} gene and that growth is performed at 30°C.
During bait plasmid construction, transformation results in few colonies or in small, slow-growing colonies	Use the XL1-Blue MRF´ Kan strain provided with the kit for bait plasmid propagation to ensure the presence of the *lacI*^{q} gene. Ensure that growth is performed at 30°C during plasmid production. Both of these measures decrease any potential toxicity of individual bait proteins to the host strain.
	Ensure that an appropriate protocol was used to transform the XL1-Blue MRF´ Kan strain. The several transformation protocols provided in this manual differ and were optimized for a specific host strain and a specific cell volume.
	In some cases, diluting the ligation reaction 1:5 prior to the transformation results in higher transformation efficiencies.
Efficiency of bait and target plasmid cotransformation into the reporter strain is low	Evaluate whether the low efficiency may be attributed to one of the two plasmids. When repeating the cotransformation, plate aliquots of the cotransformation reaction on both LB-chloramphenicol (to determine the efficiency for the bait plasmid) and LB-tetracycline (to determine the efficiency for the target plasmid). If one of the plasmids demonstrates a low efficiency, reassess the concentration and purity of the corresponding DNA preparation. In addition, plate the cotransformation reaction on LB-Tetracycline/Chloramphenicol agar plates to assess the potential of the reporter strain to be cotransformed with the specific bait and target plasmid pair.
	Check the concentration and purity of each plasmid preparation by spectrophotometry and agarose gel electrophoresis. If either plasmid is damaged or impure, perform additional rounds of plasmid purification and concentration or prepare new plasmid DNA stocks.
	Ensure that the reporter strain competent cells are stored and handled properly. Competent cells must be stored at the bottom of a –80°C freezer and should be handled as described in the cotransformation protocol.
The bait protein is not detected in Western blot analysis	Verify that the insert DNA is in the same reading frame as the fusion protein.
	If the antibody does not have a sufficiently high affinity for the protein, the protein may be expressed but may not be detectable.
	Try growing the reporter strain transformed with the bait plasmid in rich medium prior to Western blot analysis. Growth in rich medium may increase the abundance of the bait protein to detectable levels.
	Optimize the induction conditions including IPTG concentration (10–100 μM) and induction time.
	Increase the amount of cell extract used in the SDS-PAGE/Western blot procedure.
The positive control plasmids do not give the expected results in the reporter strain	Verify that the correct control plasmid pairs are used.
	Ensure that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation procedure. The protocol is optimized using these tubes.
	Use sterile technique when handling the BacterioMatch II reporter strain competent cells to avoid contamination of the culture with different *E. coli* strains.

Unexpectedly large number of colonies on Selective Screening Medium (5 mM 3-AT) plates containing negative control	3-AT concentration on the plate is too low. Ensure that the correct amount of 3-AT was added to the plates (see *Preparation of Media and Reagents*).
	Ensure that the plates were prepared exactly according to the recipe in *Preparation of Media and Reagents*. In particular, ensure that the medium includes all of the selective agents (chloramphenicol, tetracycline, and 3-AT), that the medium is allowed to cool to 50°C prior to 3-AT addition, and that the plates were stored properly (at 4°C, covered in foil, for up to one month).
	Ensure that the plates are incubated for approximately 24 hours at 37°C.
Colonies on Selective Screening Medium (5 mM 3-AT) plates grow slowly	Weak interaction pairs generate slow-growing colonies.
	Ensure that the plates are not too dry.
Colonies obtained from Selective Screening Medium (5 mM 3-AT) plates do not grow on Dual Selective Screening Medium (5 mM 3-AT + Strep) plates	The colony may be a false positive. Resistance to 5 mM 3-AT may not be dependent on two-hybrid transcriptional activation of the *HIS3* gene.
	The interaction may not be robust enough to be detected on 12.5 µg/ml streptomycin. Reduce the amount of streptomycin to 10 µg/ml and re-test the plasmid pair.

PREPARATION OF MEDIA AND REAGENTS

Minimal Media for BacterioMatch II Two-Hybrid Assays

Notes *3-AT is heat-labile and will be inactivated if added to medium at a temperature >55°C.*

All media components should be added in the order listed.

10× M9 salts are commercially available from Qbiogene (Catalog #3037-032) or may be prepared using standard bacteriological media recipes.

M9 Media Additives (sufficient for 500 ml of M9+ medium)

Prepare Solution I and Solution II separately, by mixing the components listed **in order**. Add Solution II to Solution I and then mix well. This mixture may then be added to the different variants of $M9^+$ media.

Solution I:

10 ml of 20% glucose (filter-sterilized)
5 ml of 20 mM adenine HCl (filter-sterilized)
50 ml of 10× His dropout amino acid supplement

Note: *Sterilize the 10× His dropout supplement by autoclaving at 121°C for **15 minutes** prior to addition to Solution I. Do not exceed 15 minutes.*

Solution II:

0.5 ml of 1 M $MgSO_4$
0.5 ml of 1 M Thiamine HCl
0.5 ml of 10 mM $ZnSO_4$
0.5 ml of 100 mM $CaCl_2$
0.5 ml of 50 mM IPTG

Note: *Filter-sterilize the thiamine-HCl and the IPTG prior to use. Sterilize the remaining components of Solution II using the method of choice.*

$M9^+$ His-dropout Broth (500 ml)

In a 500-ml flask, combine and mix well:
380 ml of sterile, deionized H_2O
50 ml of 10× M9 salts
To this mixture, add one preparation of M9 Media Additives (67.5 ml, see separate recipe)
Store at 4°C for up to one month
Bring to room temperature prior to use

Nonselective Screening Medium (500 ml)

In a 500-ml flask, combine:
380 ml deionized H_2O
7.5g Bacto agar
Autoclave at 121°C for 20 minutes
Cool the agar to 70 °C, and then add 50 ml of 10× M9 salts
Combine one preparation of M9 Media Additives (67.5 ml, see separate recipe) with the following agents:
0.5 ml of 25 mg/ml chloramphenicol
0.5 ml of 12.5 mg/ml tetracycline
When the agar mixture has cooled to 50°C, **immediately** add the M9 Media Additives/agent mixture to the agar
Pour into petri dishes (~25 ml/100-mm plate)
After the agar solidifies, wrap the plates in aluminum foil
Store plates at 4°C for up to 1 month

Note: *It is important to add the M9 Media Additives/agent mixture to the agar at 50°C. Cooling the agar to lower temperatures prior to addition may interfere with the even distribution of the selective agents in the agar mixture.*

Minimal Media for BacterioMatch II Two-Hybrid Assays (Continued)

3-AT Stock Solution, 1 M (10 ml)

Dissolve 840.8 mg of 3-AT in 10 ml of DMSO
Aliquot into 4 × 2.5 ml tubes
Store at –20°C for up to one month

Selective Screening Medium (5 mM 3-AT) (500 ml)

In a 500-ml flask, combine:
7.5g Bacto agar
380 ml H_2O
Autoclave at 121°C for 20 minutes
Cool the agar to 70°C, and then add 50 ml of 10× M9 salts
Combine one preparation of M9 Media Additives (67.5 ml, see separate recipe) with the following agents:
0.5 ml of 25 mg/ml chloramphenicol
0.5 ml of 12.5 mg/ml tetracycline
2.5 ml of 1 M 3-AT (dissolved in DMSO)
When the agar mixture has cooled to 50°C, **immediately** add the M9 Media Additives/agent mixture to the agar
Pour into petri dishes (~25 ml/100-mm plate; ~60 ml for 150-mm plate)
After the agar solidifies, wrap the plates in aluminum foil
Store plates at 4°C for up to 1 month

Notes: *Use freshly- prepared plates for library screening experiments.*

It is important to add the M9 Media Additives/agent mixture to the agar at 50°C. Cooling the agar to lower temperatures prior to addition may interfere with the even distribution of the selective agents in the agar mixture.

Dual Selective Screening Medium (5 mM 3-AT + Strep)(500 ml)

In a 500-ml flask, combine:
7.5g Bacto agar
380 ml H_2O
Autoclave at 121°C for 20 minutes
Cool the agar to 70 °C, and then add 50 ml of 10× M9 salts
Combine one preparation of M9 Media Additives (67.5 ml, see separate recipe) with the following agents:
0.5 ml of 25 mg/ml chloramphenicol
0.5 ml of 12.5 mg/ml tetracycline
2.5 ml of 1 M 3-AT (dissolved in DMSO)
0.5 ml of 12.5 mg/ml streptomycin
When the agar mixture has cooled to 50°C, **immediately** add the M9 Media Additives/agent mixture to the agar
Pour into petri dishes (~25 ml/100-mm plate)
After the agar solidifies, wrap the plates in aluminum foil
Store plates at 4°C for up to 1 month

Note: *It is important to add the M9 Media Additives/agent mixture to the agar at 50°C. Cooling the agar to lower temperatures prior to addition may interfere with the even distribution of the selective agents in the agar mixture.*

Rich (Complete) Media

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm plate; 60 ml for 150-mm plate)

LB–Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 45°C
Add 1.25 ml of 10-mg/ml tetracycline (prepared in 50% EtOH)
Pour into petri dishes (~25 ml/100-mm plate; 60 ml for 150-mm plate)
Store plates in a **dark, cool place or cover plates with foil** if left out at room temperature for extended time periods as tetracycline is light-sensitive

LB–Chloramphenicol Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 45°C
Add 2.5 ml of 10-mg/ml chloramphenicol (prepared in 100% ethanol)
Pour into petri dishes (~25 ml/100-mm plate; 60 ml for 150-mm plate)

LB–Chloramphenicol/Tetracycline Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 45°C, and then add:
0.5 ml of 25 mg/ml chloramphenicol
0.5 ml of 12.5 mg/ml tetracycline
Pour into petri dishes (~25 ml/100-mm plate; 60 ml for 150-mm plate)
Store plates in a **dark, cool place or cover with foil** if left out at room temperature as tetracycline is light-sensitive

SOB Medium (per Liter)

20.0 g of tryptone
5.0 g of yeast extract
0.5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Autoclave
Add 10 ml of filter-sterilized 1 M $MgCl_2$ and 10 ml of filter-sterilized 1 M $MgSO_4$ prior to use

SOC Medium (per 100 ml)

Note *This medium should be prepared immediately before use.*

2 ml of filter-sterilized 20% (w/v) glucose or 1 ml of filter-sterilized 2 M glucose
SOB medium (autoclaved) to a final volume of 100 ml

LB Broth (per Liter)

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

LB–Ampicillin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 10 ml of 10-mg/ml-filter-sterilized ampicillin
Pour into petri dishes (~25 ml/100-mm plate)

Bait Plasmid Construction Reagents

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

TE Buffer

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

2× SDS gel sample buffer

100 mM Tris-HCl (pH 6.5)
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

Note *Add dithiothreitol to a final concentration in the 2× buffer of 200 mM prior to use.* ***This sample buffer is useful for denaturing, discontinuous acrylamide gel systems only.***

APPENDIX: LIBRARY SCREENING USING ELECTROCOMPETENT REPORTER CELLS

Screening the Library: Overview

Figure 7 and Tables V and VI outline the steps to be performed in this section and describe the media that will be used during library screening.

The screening experiment consists of plating recombinant pBT + pTRG cDNA library cotransformants on selective medium in sufficient numbers to cover the cDNA library complexity. (The number of cotransformation reactions required to cover the library is determined by performing a pilot cotransformation.) Colonies obtained on the initial screening plates [Selective Screening Medium (5 mM 3-AT)] are enriched on a second selective plate. Putative positive colonies are then validated using streptomycin resistance as a secondary reporter.

Perform a negative control cotransformation with pBT-bait (containing the bait gene insert) + pTRG (empty vector) alongside the library cotransformation. This negative control transformation should be carried through the screening protocol in parallel with the library screen.

As a positive control, cotransform the reporter strain with pBT-LGF2 + pTRG-Gal11^{P}. The positive control is performed in conjunction with the pilot cotransformation using a smaller cotransformation volume.

TABLE V

Overview of Library Screen Experiments (Electrocompetent Reporter Cells)

Plasmids	Purpose	Scale of Cotransformation Reaction	Growth expected on 5 mM 3-AT?
pBT-LGF2 + pTRG-Gal11^{P} (50 ng each)	Positive control. Gal4 (encoded by LGF2) and Gal11^{P} demonstrate the appearance of a robust interaction	1 × 40 µl electrocompetent cells	yes
recombinant pBT + pTRG cDNA library (50 ng each)	Pilot library cotransformation to test the cotransformation efficiency for the specific recombinant pBT (containing the bait gene of interest) and the pTRG cDNA plasmid library	1 × 40 µl electrocompetent cells	N/A (not plated)
recombinant pBT + pTRG cDNA library (50 ng each per transformation reaction)	Library screen to test for interaction between the bait protein and target proteins in the cDNA library; colonies on Selective Screening Medium (5 mM 3-AT) indicate putative interaction	N × 40 µl, where N= number of electroporation reactions determined by pilot cotransformation	yes, if interactors are present in the library
recombinant pBT + pTRG empty vector (50 ng each)	Negative control. Growth observed on Selective Screening Medium (5 mM 3-AT) indicates the background expected from the bait protein in the absence of an interaction partner; insignificant growth is expected	1 × 40 µl	no (few or no colonies expected)

TABLE VI

Media used in BacterioMatch II Two-Hybrid Library Screening and Validation Experiments

Medium	Purpose
Selective Screening Medium (5 mM 3-AT) plates	Identification and enrichment of putative interactors based on *HIS3* transcriptional activation
Nonselective Screening Medium (no 3-AT) plates	Verification of cotransformation and calculation of cotransformation efficiency
Dual Selective Screening Medium (5 mM 3-AT + Strep) plates	Verification of interaction based on transcriptional activation of the *aadA* gene, conferring streptomycin resistance
LB-Chloramphenicol/Tetracycline plates	Maintenance of putative positives
LB-Tetracycline plates	Isolation of positive pTRG plasmids for retransformation
LB-Chloramphenicol plates	Verification of chloramphenicol sensitivity (indicating loss of pBT) prior to isolation of pTRG plasmids for retransformation
SOC liquid medium	Outgrowth following cotransformation
$M9^{+}$ His-dropout broth	Adaptation to minimal medium following outgrowth and prior to plating

Pilot Library Cotransformation and Positive Control Cotransformation

Note *In this section, bacterial cell growth is performed on* ***minimal medium*** *using a* ***37°C incubation temperature****.*

Use one 100-µl aliquot of BacterioMatch II electrocompetent reporter cells to perform both the pilot library cotransformation and the positive control cotransformation. Follow the *BacterioMatch II Electrocompetent Reporter Cells Cotransformation Protocol*, below, for this set of experiments.

Because each cDNA library has a unique composition in terms of plasmid size and transformation efficiency, it is necessary to perform a pilot library cotransformation to determine the number of cotransformants expected from a single electroporation reaction. First, results from the pilot cotransformation are used to determine the number of cotransformation reactions required to achieve library coverage. Second, the results provide an estimate of the expected concentration of cfu/ml at the conclusion of the procedure, which is required to determine the plating scheme for the screen. For the pilot reaction, transform the BacterioMatch II reporter strain competent cells with 50 ng of the recombinant pBT bait vector plus 50 ng of the pTRG target cDNA library.

For the positive control cotransformation, transform the BacterioMatch II reporter strain competent cells with 50 ng of pBT-LGF2 bait vector plus 50 ng of pTRG-$Gal11^{P}$ target vector.

BacterioMatch II Electrocompetent Reporter Cells Cotransformation Protocol

Note *It is important that the agar plates used in this transformation do not have excessive surface moisture, which can lead to an increase in the appearance of false positives. To achieve proper moisture levels, place the plates in a 37°C incubator with lids ajar for 30 minutes to 1 hour, until the moisture on the lid and agar surface has just evaporated. Avoid overdrying the plates, which results in delayed colony formation.*

1. Pre-warm SOC medium to 37°C. For each cotransformation reaction, pre-chill one sterile electroporation cuvette (0.1-cm gap) and one sterile 1.5-ml microcentrifuge tube thoroughly on ice.

2. Set the electroporator to a voltage setting of 1.5 kV (15 kV/cm field strength). Set the resistance to 400 Ω and the capacitance to 25 μF.

3. Add 50 ng each of the appropriate pBT bait vector plus pTRG target vector to a prechilled 1.5-ml microcentrifuge tube. Keep the tube on ice.

4. Thaw the BacterioMatch II electrocompetent reporter cells on ice. Gently mix the cells by tapping the tube. Once thawed, aliquot 40 μl of cells into each microcentrifuge tube, mixing the cells and the DNA by a few rounds of gentle repeated pipetting. Keep the tubes on ice throughout the procedure.

5. Transfer the cell-DNA mixture to a chilled electroporation cuvette, tapping the cuvette until the mixture settles evenly to the bottom.

6. Slide the cuvette into the electroporation chamber until the cuvette sits flush against the electrical contacts.

7. Pulse the sample once, then quickly remove the cuvette. Immediately add 960 μl of SOC medium (held at 37°C) to resuspend the cells.

8. Transfer the cells to a sterile 14-ml BD Falcon polypropylene round-bottom tube. Incubate the tube at 37°C for 90 minutes with shaking at 225–250 rpm.

   Note *Ensure that the shaker speed does not exceed 250 rpm.*

9. Spin down the cells in a tabletop centrifuge at 2000 × *g* for 10 minutes. This step may be completed either at room temperature or at 4°C.

10. Aspirate the supernatant, taking care to avoid disturbing the pellet. To remove the residual rich medium, wash the cells twice. For each wash, resuspend the cells in 1 ml of room temperature $M9^{+}$ His-dropout broth, collect the cells as described in step 9, and then gently aspirate the supernatant.

11. After aspirating the supernatant from the second wash step, resuspend the cells in a fresh 1.0 ml of M9+ His-dropout broth.

12. Incubate cells at 37°C with shaking at 225 rpm for 2 hours. This allows the cells to adapt to growth in minimal medium prior to plating.

    Note *Ensure that the shaker speed does not exceed 250 rpm.*

13. Plate each of the cotransformation mixtures on the appropriate media, as indicated in the following sections.

    Note *It is important to plate the recommended volume and to spread the mixture evenly on the plates as this promotes even colony distribution. Using sterile glass beads to spread the cells on the plates may facilitate even distribution.*

Plating and Evaluating the Positive Control Cotransformation

After completing step 12 of the *BacterioMatch II Electrocompetent Reporter Cells Cotransformation Protocol* perform the following plating and analysis steps.

1. Remove 100 µl of cells from the pBT-LGF2 + pTRG-Gal11^{P} cotransformation adapted outgrowth culture from step 12 of the transformation protocol. Prepare a 1/1000 dilution of this sample by performing three, 10-fold serial dilutions in M9^{+} His-dropout broth.

2. From the 1000-fold dilution, plate 100 µl on both Nonselective Screening Medium (no 3-AT) and on Selective Screening Medium (5 mM 3-AT).

3. Incubate the plates at 37°C for 18–24 hours.

4. Score the plates for growth. Interactions between a pair of hybrid proteins, including the Gal4 + Gal11^{P} hybrid protein pair, are indicated by growth on Selective Screening Medium (5 mM 3-AT). For the positive control transformation, a similar number of colonies are expected on the selective and nonselective plates. (Expect 10^{3}–10^{4} cfu on each plate.)

5. Store the plates at 4°C for reference during the library screening experiments and to retain a stock of positive control cotransformants.

    Note *The interaction of the Gal4-Gal11^{P} hybrid pair is robust and is readily detectable in the BacterioMatch II two-hybrid system.* ***It is important to note that cells harboring weaker interactors are expected to grow more slowly, requiring longer incubation times for colony development****.*

Plating and Evaluating the Pilot Cotransformation

After completing step 12 of the *BacterioMatch II Electrocompetent Reporter Cells Cotransformation Protocol*, perform the following plating and analysis steps to determine the number of cotransformants obtained in the pilot.

1. Remove 100 µl of cells from the adapted outgrowth culture from step 12 of the transformation protocol. Prepare a 1/1000 and a 1/10,000 dilution of this sample by performing the required number of 10-fold serial dilutions in $M9^+$ His-dropout broth.
2. From each of the 1000-fold and 10,000-fold dilutions, plate 100 µl on each of three Nonselective Screening Medium (no 3-AT) plates (100-mm).
3. Incubate the plates at 37°C, inverted, for 24 hours. (Colonies may first become visible at 16–18 hours, but the plates should be incubated for the full 24 hours before colonies are counted.)
4. Select the dilution that produced the better number of cfu for counting (preferably >50 and <1000 cfu), and count the colonies on each of the three plates.
5. Calculate the total cotransformants expected per electroporation reaction. For the 1/1000 dilution, multiply the cfu obtained on a single plate by 10^4. For the 1/10,000 dilution, multiply the cfu obtained on a single plate by 10^5. Average the results obtained for the three plates to derive the expected number of cotransformants for each 40-µl library cotransformation.
6. Determine the number of cotransformation reactions required to achieve library coverage. For example, if 5×10^5 cotransformants were obtained from the pilot cotransformation and the library contains 10^6 clones, two transformation reactions are required to cover the library once.
7. Determine the appropriate plating scheme for the large-scale cotransformation that is performed in the next section of the manual.

   We suggest plating up to 2×10^5 cotransformants per 150-mm screening plate, in a volume of approximately 200 µl. To achieve this, a cell density of $\leq 1 \times 10^6$ cotransformants per ml is required at the conclusion of the cotransformation protocol.

   If the pilot cotransformation produced $>10^6$ cotransformants, plan to dilute the suspension to an expected final concentration of 1×10^6 cfu per ml, and then to spread 200 µl of the diluted suspension on each 150-mm plate.

   If the pilot cotransformation produced fewer than 10^6 cotransformants, then plating 200 µl of the final cell suspension per 150-mm plate, without any dilution, is appropriate.

Performing the BacterioMatch II Two-Hybrid Interaction Library Screen

Note *For library screens, bacterial cell growth is performed on **minimal medium** using a **37°C incubation temperature**.*

Library Screening Transformation Protocol for the BacterioMatch II Reporter Electrocompetent Cells

After evaluating the results of the pilot library cotransformation, perform a large-scale cotransformation of the electrocompetent reporter strain with recombinant pBT plus the pTRG cDNA library DNA, using the number of 40-µl electroporation reactions required for library coverage. After initiating step 12 of the transformation protocol, proceed to the steps below.

1. During the adaptation period (see step 12), evaluate and correct the surface moisture of the agar plates. It is important that the plate surface is not too wet to absorb the cell mixture. To achieve proper moisture levels, place the plates in a 37°C incubator with lids ajar for 30 minutes to 1 hour, until the moisture on the lid and agar surface has just evaporated. Avoid overdrying the plates, which results in delayed colony formation.

2. If more than one electroporation was performed to achieve library coverage, pool the adapted outgrowth cultures from step 12 into one tube, swirling the tube well to mix the cultures.

3. If the expected density of cotransformants in the outgrowth cultures is greater than 1×10^6/ml (based on results from the pilot cotransformation), then dilute the suspension, using $M9^+$ His-dropout broth, to an expected final density of 1×10^6/ml.

Plating the Library Screen Cotransformants

4. To determine the cotransformation efficiency and determine the number of cotransformants screened, remove 100 µl from the pool and prepare a 1000-fold dilution by performing three 10-fold serial dilutions in $M9^+$ His-dropout broth. From the 1000-fold dilution, plate 100 µl on each of three 100-mm Nonselective Screening Medium (no 3-AT) plates.

5. Plate the remainder of the pool, in ~200-µl aliquots, onto 150-mm Selective Screening Medium (5 mM 3-AT) plates.

   Note *To allow single colony formation, the total number of cotransformants per 150-mm plate should not exceed 2×10^5.*

6. Incubate the plates from steps 4 and 5 at 37 °C, inverted, for 24 hrs. Proceed to *Enrichment of Putative Interactors* (step 21) in *Performing the BacterioMatch II Two-Hybrid Interaction Library Screen* in the main section of the manual. (A secondary incubation of the screening plates at room temperature should be performed, as detailed in step 21.)

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ENDNOTES

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