BacterioMatch II Two-Hybrid System Vector Kit

Instruction Manual

Catalog #240065

Revision C

Research Use Only. Not for Use in Diagnostic Procedures.

240065-12

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BacterioMatch II Two-Hybrid System Vector Kit

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CRITICAL SUCCESS FACTORS

Before beginning experiments using the BacterioMatch II two-hybrid system vector kit, 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 C

BacterioMatch II Two-Hybrid System Vector Kit

MATERIALS PROVIDED

Materials provided	Amount
BacterioMatch II two-hybrid system plasmids[a]	
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[a]	
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
XL1-Blue MRF′ Kan, glycerol stock[b] (host strain for propagating pBT and pTRG recombinants)	0.5 ml

[a] Store at –20°C.

[b] Store at –80°C. Genotype: *Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F′ proAB lacI^{q}ZΔM15* Tn*5 (Kan^{r})]*.

STORAGE CONDITIONS

All Plasmids: –20°C
XL1-Blue MRF′ Kan glycerol stock: –80°C

ADDITIONAL MATERIALS REQUIRED

BacterioMatch II Validation Reporter Competent Cells (Catalog #200192)
14-ml BD Falcon® polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
Components for minimal, his-dropout bacteriological media,§ including:
- M9 salts§ (Qbiogene, Cat. # 3037-032)
- 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)

NOTICE TO PURCHASER

U.S. Patent No. 5,925,523, covering the BacterioMatch two-hybrid system, is licensed exclusively by Agilent Technologies, Inc. Research use of the BacterioMatch two-hybrid system by commercial entities requires a license from Agilent Technologies, Inc. For license information, please contact: Director of Business Development at (858) 535-5400.

§ See *Preparation of Media and Reagents*.

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.

The BacterioMatch II Two-Hybrid System Vector Kit (Catalog #240065) is designed for the characterization of protein-protein interactions between a pair of proteins cloned in the pBT (bait) and the pTRG (target) vectors. 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.

For two-hybrid screening using the pBT-bait plasmid to identify protein-protein interaction partners from a pTRG cDNA plasmid library, we offer the BacterioMatch II Two-Hybrid System Library Construction Kit (Agilent Catalog #200412) and the BacterioMatch II Two-Hybrid System Library Construction Kit with Electrocompetent Cells (Agilent Catalog #200414). A variety of BacterioMatch II premade-library kits are also available; for a complete list, visit www.genomics.agilent.com.

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.

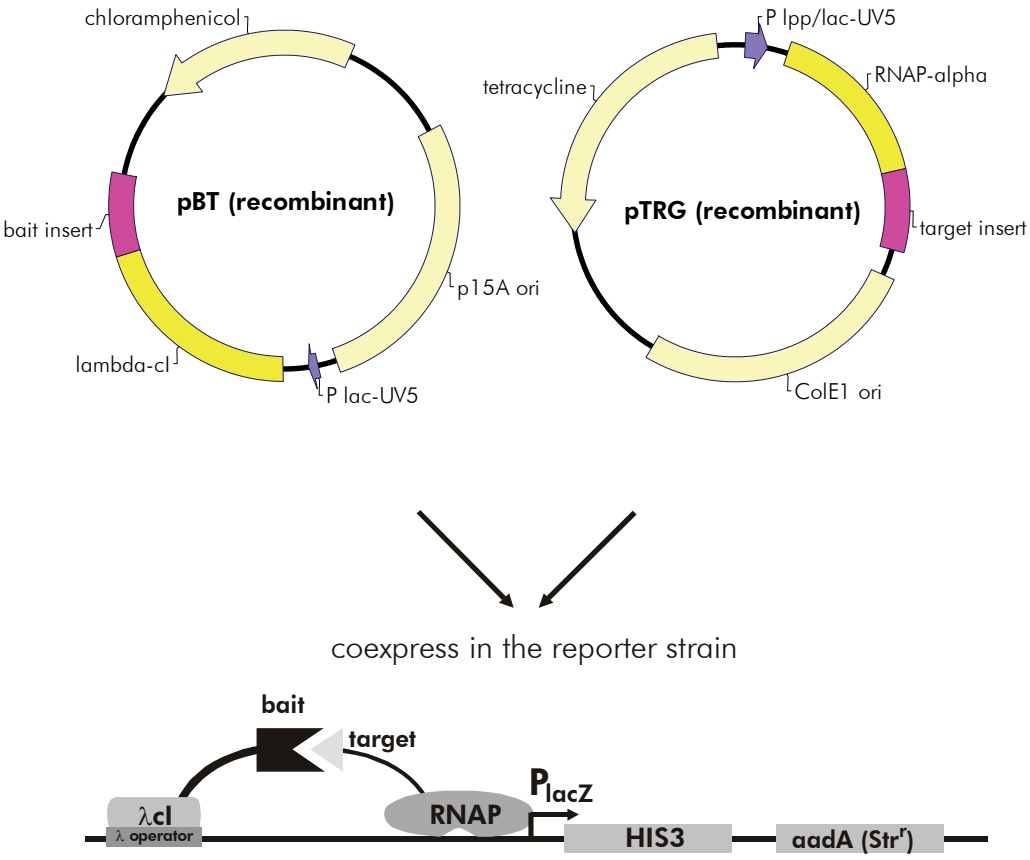


Figure 1 Schematic of the BacterioMatch II two-hybrid system dual reporter construct

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, 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 (chemically-competent)	Library screening (cotransformation of pBT-bait and the pTRG library)	≥7.5 × 10^{7} cfu/μg pUC18	6 × 500 μl aliquots
200192	BacterioMatch II Validation Reporter Competent Cells (chemically-competent)	Interaction assays, expression testing, validation of screen isolates	≥1 × 10^{7} cfu/μg pUC18	5 × 200 μl aliquots
200195	BacterioMatch II Electrocompetent Reporter Cells (electroporation-competent)	Library screening (cotransformation of pBT-bait and the pTRG library) with increased efficiency compared to chemically-competent reporter cells	≥1 × 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. From a 5 ml LB-chloramphenicol culture (34 μg/ml chloramphenicol), yields of approximately 200 ng are obtained.

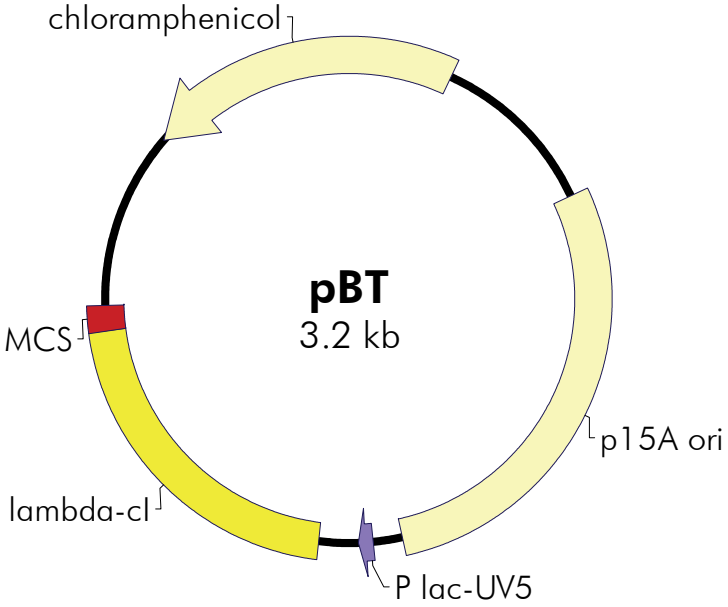
The 4.4 kb target plasmid, pTRG (Figure 3), carries the low-copy ColE1 replication origin and confers tetracycline resistance. The plasmid directs transcription of the amino-terminal domain of RNA polymerase α subunit through a multiple cloning site at the 3′ end of the α subunit gene and is under the 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. The arrangement of *Eco*R I and *Xho* I restriction sites in the pTRG MCS makes the plasmid compatible with inserts produced using the Agilent cDNA Synthesis Kit (Catalog #200401). From a 5 ml LB-tetracycline culture (12.5 μg/ml tetracycline), yields of approximately 1 μg are obtained.

Both bait and target plasmids contain the *Not* I restriction site in the MCS which encodes a short alanine linker to facilitate the orientation and folding of the fused bait and target proteins.

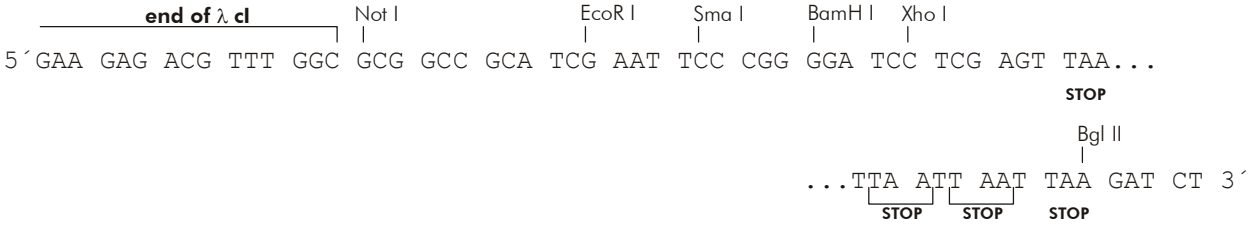
Note *The pTRG vector (with or without insert present), as well as recombinant pBT plasmids, can be toxic to the bacterial host. To minimize host toxicity, propagate all forms of the pBT and pTRG plasmids using a host strain containing the* $lacI^q$ *gene (such as the XL1-Blue MRF′ strain provided). 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)

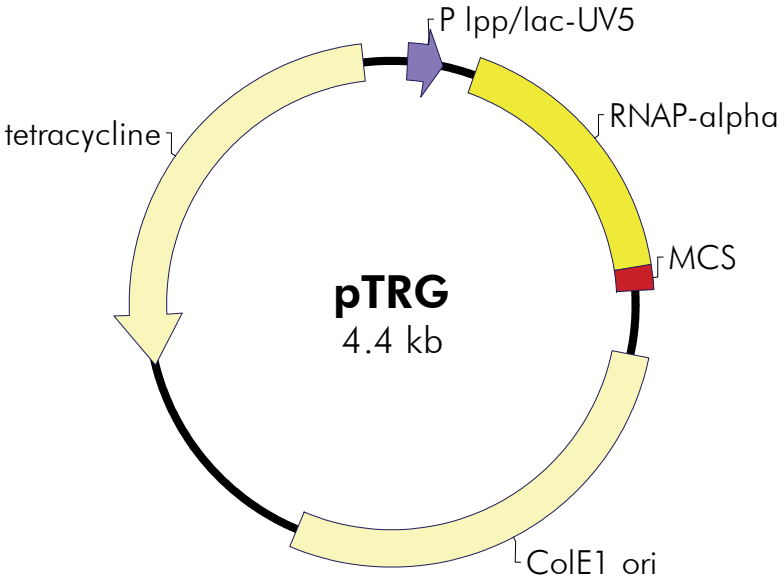


Feature	Position
chloramphenicol resistance ORF	2770–219
p15A origin of replication	581–1493
lac-UV5 promoter	1556–1586
λ cI ORF	1631–2341
pBT forward primer [5' TCCGTTGTGGGGAAAGTTATC 3']	2291–2311
multiple cloning site	2342–2394
pBT reverse primer [5' GGGTAGCCAGCAGCATCC 3']	2419–2436

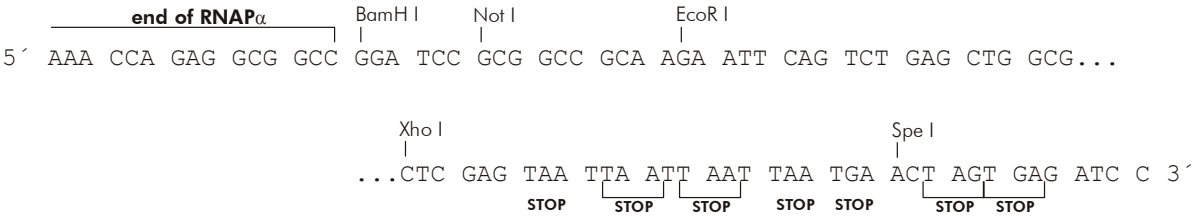
Note *The nucleotide sequence and list of restriction sites for the pBT bait plasmid can be found at* www.genomics.agilent.com.

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.genomics.agilent.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

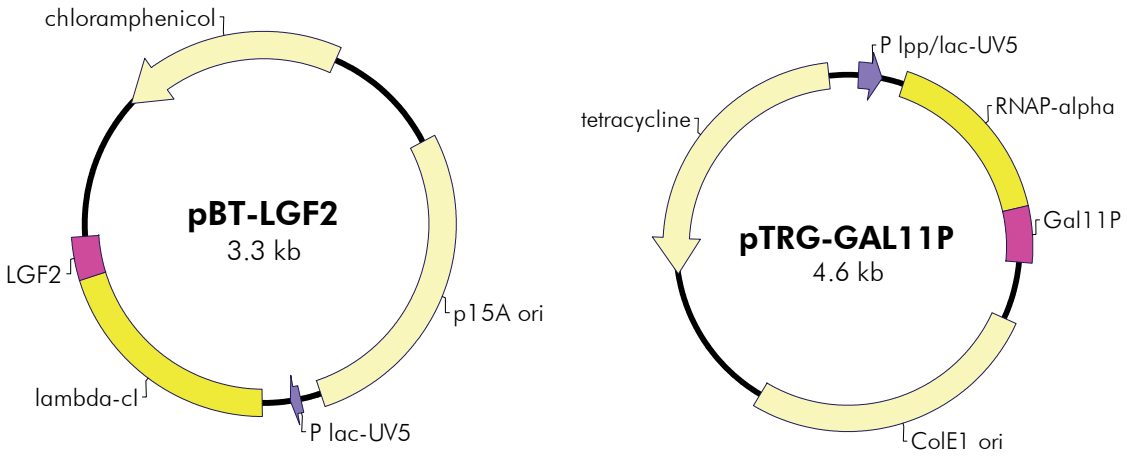


FIGURE 4 Circular maps of the interaction control plasmids

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.

RECOMBINANT PBT AND PTRG PLASMID CONSTRUCTION AND VALIDATION

Cloning into the pBT and pTRG Plasmids

Bait and Target Insert Preparation

DNA is prepared for insertion into the pBT or pTRG plasmids 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. DNA encoding the target protein must be inserted in frame with the RNAPα 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). In the MCS of the pTRG plasmid, the *Bam*H I, *Not* I, *Eco*R I, *Xho* I, and *Spe* I sites are unique (see Figure 3).

If the insert DNA is phosphorylated, we suggest dephosphorylating and agarose-gel isolating the digested pBT and pTRG 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.

Constructing cDNA Libraries in the pTRG Plasmid

For two-hybrid screening of a pTRG plasmid cDNA library, we offer BacterioMatch II two-hybrid system library construction kits that include either chemically-competent reporter cells (Agilent Catalog #200412) or electrocompetent reporter cells (Agilent Catalog #200414). In addition to a complete set of BacterioMatch II vectors, control plasmids and reporter cells, these kits provide the reagents required to prepare a unidirectional pTRG cDNA plasmid library. The kit features our cDNA Synthesis Kit (also available separately, Agilent Catalog #200401), with optimized reagents for converting mRNA to cDNA inserts that are suitable for unidirectional insertion into the pTRG plasmid.

A variety of BacterioMatch II premade libraries, prepared from mRNA originating from a number of different organisms and cell types, are also available. For a list of premade pTRG plasmid cDNA libraries, visit www.genomics.agilent.com.

For complete library construction and library screening protocols, see the appropriate BacterioMatch II library construction kit manual, available online at http:// www.genomics.agilent.com.

Ligation of DNA Inserts into the pBT and pTRG Plasmids

1. Digest 2 µg of both the pBT plasmid and the pTRG plasmid separately in 50-µl reaction volumes.

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

2. Extract each 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 for each DNA sample.

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

5. To each of the tubes, 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 pellets twice with 70% (v/v) ethanol and then dry the pellets.

7. Resuspend the pellet in each tube in the appropriate 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 plasmids have 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:

$$X \text{ ng of insert} = \frac{(\text{number of base pairs of insert}) \times (100 \text{ ng of vector})}{Y \text{ bp of vector (either pBT or pTRG)}}$$

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. **Set up this ligation panel for each pBT and pTRG construct.**

Suggested Ligation Reactions

Ligation Reaction Components	Experimental	Control		
	1	2[a]	3[b]	4[c]
Prepared 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 Ligation Reactions

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

1. Use the ligation reactions 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 potentially toxic bait and target proteins.** This limits the selection against individual bait or target proteins. Also, the strain must be chloramphenicol- and tetracycline sensitive to allow selection for pBT and pTRG transformants, respectively.

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

3. Plate the recombinant pTRG transformants on LB-tetracycline agar[§] plates.

4. 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 and the pTRG Plasmids

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 appropriate primers shown in the table below). Identify recombinants containing the DNA insert in frame with the λcI protein (in the case of recombinant pBT) or with the RNAPα protein (in the case of recombinant pTRG).

Primers for use with pBT and pTRG

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'
pTRG forward primer	5'- TGG CTG AAC AAC TGG AAG CT- 3'
pTRG reverse primer	5'- ATT CGT CGC CCG CCA TAA - 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 both recombinant pBT and pTRG is expected to be low due to the low copy number of both of these plasmids.

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 and pTRG in separate transformation reactions and (2) plasmid DNA purified from sequence-verified pBT and pTRG recombinants in separate transformation reactions. Follow the transformation protocol supplied with the reporter competent cells, with the plating modifications given below. General guidelines for expression analysis follow.

Note *When preparing pBT or pTRG 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 specific protocol modifications for low-copy number plasmids suggested by the purification kit manufacturer). The pBT plasmid is present at about 5–10 copies per cell and the pTRG plasmid is present at about 20–30 copies per cell.*

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

- 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.

- Grow individual pTRG-containing colonies in 2 ml of M9+ His-dropout broth containing 12.5 µg/ml tetracycline at 37°C overnight. Use these cultures to inoculate fresh 2-ml cultures of M9+ His-dropout broth containing 12.5 µg/ml tetracycline 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 bait or target protein. Recombinant bait protein expression may also be verified by Western blot analysis using a lambda cI antibody.

  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 or target 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.*

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

Preparation of Recombinant pBT and pTRG 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 protein of interest, purify the recombinant pBT and pTRG plasmids in sufficient quantity for the subsequent experiments. Grow cultures of XL1-Blue MRF´ Kan cells harboring the verified plasmids, 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 and the pTRG plasmid are maintained at about 5–10 and 20–30 copies per cell, respectively. When preparing pBT or pTRG 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).*

TESTING SELF-ACTIVATION BY RECOMBINANT PBT OR PTRG

Prior to performing two-hybrid interaction assays using the recombinant bait and target plasmids (pBT and pTRG, each containing their respective genes of interest) it is prudent to determine whether a specific recombinant is suitable for detecting protein-protein interactions in the BacterioMatch II two-hybrid system. Cotransformation of the BacterioMatch II reporter strain using recombinant pBT plasmid (containing the bait gene of interest) and the empty pTRG vector should not produce a significant number of colonies on Selective Screening Medium (5 mM 3-AT). Likewise, cotransformation of the reporter strain using recombinant pTRG plasmid (containing the target gene of interest) and the empty pBT vector should not produce a significant number of colonies on this medium. If significant growth is observed in either of the cotransformation experiments, the recombinant 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 (Agilent Catalog #200192) to complete this set of control experiments. Follow the* Cotransformation Protocol *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 both for assays with individual pTRG target plasmids and for library screens 0.1–1% of total cotransformants verifies suitability of bait for assays with individual pTRG target plasmids; troubleshooting required prior to use in library screens >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) + recombinant pTRG (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 target plasmids)
	Selective Screening Medium (5 mM 3-AT)	Tests whether a given target protein is able, on its own, to activate the reporter cassette	≤1% of total cotransformants verifies suitability of target for assays with individual pBT bait plasmids >1% of total cotransformants indicates self-activation or media problem. Check 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

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 ≥1 × 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.

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

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*, 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 Recombinant pBT or pTRG Self-Activation Tests

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 and target vectors for BacterioMatch II two-hybrid assay experiments and library screens.

    If either the bait or target 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 or target protein.

PERFORMING PROTEIN-PROTEIN INTERACTION ASSAYS

To assay for a two-hybrid interaction between a specific bait and target plasmid pair, cotransform the BacterioMatch II validation reporter competent cells with the plasmid pairs listed in Table III. For these experiments, complete steps 1–14 of the protocol in *Cotransformation Protocol*, and then proceed to the *Protein-Protein Interaction Assay Plating and Analysis* section, below.

Notes *The protein-protein interaction assays must be performed in the BacterioMatch II two-hybrid reporter strain, containing the reporter gene cassette. Do not substitute another strain.*

For two-hybrid assays, bacterial cell growth is performed on minimal medium using a 37°C incubation temperature. Any follow-up steps that require growth on rich medium should employ a 30°C incubation temperature.

TABLE III

Cotransformations for Detecting Protein-Protein Interactions

Plasmids	Purpose	Expected Results
pBT-LGF2 + pTRG-Gal11^{P} (50 ng each)	Positive control demonstrating the behavior of a robust interaction pair	Significant growth (10^4–10^5 cfu) should be observed on Selective Screening Medium (5 mM 3-AT)
recombinant pBT + pTRG (empty vector) (50 ng each)	Negative control indicative of background growth due to activation by the recombinant bait alone or to reporter strain background growth	Few or no colonies should be observed on Selective Screening Medium (5 mM 3-AT)
pBT (empty vector) + recombinant pTRG (50 ng each)	Negative control indicative of background growth due to activation by the recombinant target alone or to reporter strain background growth	Few or no colonies should be observed on Selective Screening Medium (5 mM 3-AT)
recombinant pTRG + recombinant pBT (50 ng each)	Tests for interaction between the bait and target proteins	Growth on Selective Screening Medium (5 mM 3-AT) indicates a putative positive interaction pair

Protein-Protein Interaction Assay Plating and Analysis

1. After completing steps 1–14 of the *Cotransformation Protocol*, plate 100-µl aliquots of each of the cotransformation mixtures on both Nonselective Screening Medium (no 3-AT) and on Selective Screening Medium (5 mM 3-AT).

   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.*

2. 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.

3. Score the plates. Interactions between a pair of hybrid proteins, including the Gal4 + Gal11^{P} hybrid protein pair, are indicated by growth of colonies on Selective Screening Medium (5 mM 3-AT). The strength of the interaction typically correlates with the ratio of colonies obtained on the selective plates compared to on the nonselective plates.

   Notes *Reporter strain cotransformants of various sizes may appear on the plates.*

   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****.*

   Certain bait-target interactions may require cofactors, such as metal ions, that are not present in the minimal media at sufficient levels to allow detection of the interaction. It may be possible to supplement the media with the relevant cofactor(s) in order to increase transcriptional activation to detectable levels.

   For a given plasmid pair, if colonies are observed on Nonselective Screening Medium (no 3-AT) but not on Selective Screening Medium (5 mM 3-AT), cotransformation was achieved but the protein pair does not demonstrate an interaction that is detectable by this two-hybrid assay.

If colonies are not observed on Nonselective Screening Medium (no 3-AT) or on Selective Screening Medium (5 mM 3-AT), then cotransformation was not successful. Perform troubleshooting to determine whether both plasmids are of high quality and that the correct quantity was added. Perform transformation of the reporter strain with the pUC18 control plasmid to verify the transformation efficiency of the reporter strain, using the transformation protocol provided with the reporter strain competent cells. See *Troubleshooting* for additional suggestions.

Preserving Putative Positive Colonies

After identifying putative positive colonies on the Selective Screening Medium (5 mM 3-AT) plates, patch several colonies for each interaction pair onto LB-Tetracycline/Chloramphenicol agar plates.[§] These patched plates serve as the primary source of plasmid DNA for the putative positive colonies in subsequent analyses.

Verification of Positives using the Streptomycin Resistance Reporter

Activation of the second reporter gene, *aadA*, encoding streptomycin resistance, may be used as a verification of the interaction between the bait and target proteins. To assay for transcriptional activation of the *aadA* reporter, patch cells from a putative positive colony from a Selective Screening Medium (5mM 3-AT) plate onto a Dual Selective Screening Medium (5 mM 3-AT + Strep) plate.[§] 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 (e.g. 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 assay. 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.

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

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.
The yield of target plasmid is low	The copy number of the target plasmid is low in the host strain. Increase the volume of culture or apply a standard chloramphenicol 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 construction of bait or target plasmids, transformation results in few colonies or in small, slow-growing colonies	Use the XL1-Blue MRF´ Kan strain provided with the kit for bait and target vector 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 or target 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 and/or target 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 or target plasmid in rich medium prior to Western blot analysis. Growth in rich medium may increase the abundance of the 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 preparing and transforming 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.
No colonies on Selective Screening Medium (5 mM 3-AT) plates following cotransformation with a known interacting protein pair	Protein is not expressed; ensure the gene of interest is ligated in frame with the fusion protein; ensure that the DNA used in the cotransformation is of high quality.
	The bait or target fusion protein may be toxic to the reporter strain host cells. Examine the growth properties of the cotransformants on Nonselective Screening Medium (no 3-AT). If growth is weak on the nonselective medium, try reducing the amount of IPTG inducer added to the medium in order to reduce the fusion gene expression levels. Test whether fusion proteins containing truncated versions of the proteins of interest are less toxic to the reporter strain host.
	The bait or target fusion protein may be unstable or improperly folded. Construct fusion proteins containing different domains of the proteins of interest.
	The interaction may not be robust enough to be detected on 5mM 3-AT. Reduce the amount of 3-AT and re-test the plasmid pair.
	The interaction may require certain protein modifications that are not made in *E. coli* cells.
	The interaction may require a cofactor, such as a metal ion, that is not present in the media in sufficient quantity. Test supplementation of the screening media recipes with any suspected small-molecule cofactors.
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.

M9 salts are commercially available from Qbiogene (Catalog #3037-032) or may be prepared using standard bacteriological media recipes. The components in 1 liter water should be: Na_2HPO_4: 67.8 g, KH_2PO_4: 30 g, NaCl: 5 g, NH_4Cl: 10 g. Qbiogene M9 salts prepared as directed will yield a 5× solution, and should be doubled for use with this kit.

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.

$M9^+$ 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

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 (BD/Clontech, Cat. #630415)

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.*

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 (Sigma, Cat. #A-8056) 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 Media and Other Reagents

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)

Rich Media and Other Reagents (Continued)

10× Ligase Buffer	TE 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*	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.***	

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ENDNOTES

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MSDS INFORMATION

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