LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

Email
techservices@agilent.com

World Wide Web
www.genomics.agilent.com

Telephone

<table>
<thead>
<tr>
<th>Location</th>
<th>Telephone</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States and Canada</td>
<td>800 227 9770</td>
</tr>
<tr>
<td>Austria</td>
<td>01 25125 6800</td>
</tr>
<tr>
<td>Benelux</td>
<td>02 404 92 22</td>
</tr>
<tr>
<td>Denmark</td>
<td>45 70 13 00 30</td>
</tr>
<tr>
<td>Finland</td>
<td>010 802 220</td>
</tr>
<tr>
<td>France</td>
<td>0810 446 446</td>
</tr>
<tr>
<td>Germany</td>
<td>0800 603 1000</td>
</tr>
<tr>
<td>Italy</td>
<td>800 012575</td>
</tr>
<tr>
<td>Netherlands</td>
<td>020 547 2600</td>
</tr>
<tr>
<td>Spain</td>
<td>901 11 68 90</td>
</tr>
<tr>
<td>Sweden</td>
<td>08 506 4 8960</td>
</tr>
<tr>
<td>Switzerland</td>
<td>0848 8035 60</td>
</tr>
<tr>
<td>UK/Ireland</td>
<td>0845 712 5292</td>
</tr>
<tr>
<td>All Other Countries</td>
<td>Please visit <a href="http://www.genomics.agilent.com">www.genomics.agilent.com</a> and click Contact Us</td>
</tr>
</tbody>
</table>
# BacterioMatch II Two-Hybrid System Library Construction Kit

## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Critical Success Factors</td>
<td>1</td>
</tr>
<tr>
<td>Materials Provided: BacterioMatch II Two-Hybrid System</td>
<td>2</td>
</tr>
<tr>
<td>Storage Conditions</td>
<td>2</td>
</tr>
<tr>
<td>Materials Provided and Storage Conditions: cDNA Synthesis Kit</td>
<td>3</td>
</tr>
<tr>
<td>Additional Materials Required</td>
<td>4</td>
</tr>
<tr>
<td>BacterioMatch II Two-Hybrid System Assays and Screens</td>
<td>4</td>
</tr>
<tr>
<td>cDNA Library Construction</td>
<td>4</td>
</tr>
<tr>
<td>Notice to Purchaser</td>
<td>4</td>
</tr>
<tr>
<td>Host Strains and Genotypes</td>
<td>5</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Advantages of the Bacterial Two-Hybrid System</td>
<td>6</td>
</tr>
<tr>
<td>Overview of the Interaction</td>
<td>7</td>
</tr>
<tr>
<td>Positive Selection Using $HIS3$ Transcriptional Activation</td>
<td>8</td>
</tr>
<tr>
<td>Reporter Strain</td>
<td>9</td>
</tr>
<tr>
<td>Plasmids</td>
<td>10</td>
</tr>
<tr>
<td>pBT Vector Map</td>
<td>11</td>
</tr>
<tr>
<td>pTRG Vector Map</td>
<td>12</td>
</tr>
<tr>
<td>Control Plasmids</td>
<td>13</td>
</tr>
<tr>
<td>Bait Plasmid Construction and Validation</td>
<td>14</td>
</tr>
<tr>
<td>Bait Insert Preparation</td>
<td>14</td>
</tr>
<tr>
<td>Ligating DNA Inserts into the pBT Plasmid</td>
<td>14</td>
</tr>
<tr>
<td>Verifying Expression</td>
<td>16</td>
</tr>
<tr>
<td>Preparation of Recombinant pBT DNA</td>
<td>17</td>
</tr>
<tr>
<td>Testing Self-Activation by Recombinant pBT Prior to Screening</td>
<td>18</td>
</tr>
<tr>
<td>Cotransformation Protocol (Validation Reporter Competent Cells)</td>
<td>19</td>
</tr>
<tr>
<td>cDNA Library Construction in the pTRG Plasmid</td>
<td>22</td>
</tr>
<tr>
<td>Background</td>
<td>22</td>
</tr>
<tr>
<td>Protocol: Generating the cDNA Inserts</td>
<td>26</td>
</tr>
<tr>
<td>Protocol: Ligating the cDNA Library into the pTRG Plasmid</td>
<td>38</td>
</tr>
</tbody>
</table>
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 HIS3-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.
### BacterioMatch II Two-Hybrid System Library Construction Kit

**MATERIALS PROVIDED: BACTERIOMATCH II TWO-HYBRID SYSTEM**

<table>
<thead>
<tr>
<th>Materials Provided</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BacterioMatch II two-hybrid system plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pTRG XR target plasmid, digested with EcoRI and XhoI, 1.5 μg, (0.1 μg/μl in TE buffer)</td>
<td>15 μl</td>
</tr>
<tr>
<td>pTRG target plasmid, supercoiled, 10 μg (1 μg/μl in TE buffer [Catalog #240066])</td>
<td>10 μl</td>
</tr>
<tr>
<td>pBT bait plasmid, supercoiled 10 μg (1 μg/μl in TE buffer [Catalog #240067])</td>
<td>10 μl</td>
</tr>
<tr>
<td><strong>Test insert</strong></td>
<td></td>
</tr>
<tr>
<td>XR Amp test insert (~1 kb) digested with EcoRI and XhoI, 50 ng (10 ng/μl in TE buffer)</td>
<td>5 μl</td>
</tr>
<tr>
<td><strong>BacterioMatch II two-hybrid system control plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pTRG-Gal11 control plasmid, supercoiled, 150 ng [50 ng/μl in TE buffer]</td>
<td>3 μl</td>
</tr>
<tr>
<td>pBT-LGF2 control plasmid, supercoiled, 150 ng [50 ng/μl in TE buffer]</td>
<td>3 μl</td>
</tr>
<tr>
<td>Host strain for cDNA library construction, XL1-Blue MRF’ Kan Library Pack</td>
<td></td>
</tr>
<tr>
<td>Competent Cells</td>
<td>5 × 500 μl</td>
</tr>
<tr>
<td>pUC18 control plasmid (0.1 ng/μl in TE buffer)</td>
<td>10 μl</td>
</tr>
<tr>
<td>β-mercaptoethanol for XL1-Blue MRF’ Kan Library Pack (2-mercaptoethanol Library Pack)</td>
<td>50 μl</td>
</tr>
<tr>
<td><strong>BacterioMatch II Screening Reporter Competent Cells</strong></td>
<td></td>
</tr>
<tr>
<td>(Catalog #200190)</td>
<td>6 × 500 μl</td>
</tr>
<tr>
<td>β-mercaptoethanol (β-ME)</td>
<td>75 μl</td>
</tr>
<tr>
<td>pUC18 plasmid DNA (0.1 ng/μl in TE buffer)</td>
<td>10 μl</td>
</tr>
<tr>
<td><strong>BacterioMatch II Validation Reporter Competent Cells</strong></td>
<td></td>
</tr>
<tr>
<td>(Catalog #200192)</td>
<td>5 × 200 μl</td>
</tr>
<tr>
<td>β-mercaptoethanol (β-ME)</td>
<td>25 μl</td>
</tr>
<tr>
<td>pUC18 plasmid DNA (0.1 ng/μl in TE buffer)</td>
<td>10 μl</td>
</tr>
<tr>
<td>Host Strain for propagating pBT and pTRG recombinants, XL1-Blue MRF’ Kan; glycerol stock</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

a Store at –20°C.
b Store at –80°C.

**STORAGE CONDITIONS**

- All Plasmids: –20°C
- XL1-Blue MRF’ Kan Library Pack Competent Cells: –80°C
- BacterioMatch II Screening/Validation Reporter Competent Cells: –80°C
- XL1-Blue MRF’ Kan glycerol stock: –80°C

**Note**  See the following page for Materials Provided and Storage Conditions for the cDNA Synthesis Kit.
### MATERIALS PROVIDED AND STORAGE CONDITIONS: cDNA SYNTHESIS KIT

<table>
<thead>
<tr>
<th>Reagents Provided&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantity</th>
<th>Storage Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>First-strand reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AccuScript reverse transcriptase (AccuScript RT)</td>
<td>15 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>RNase Block Ribonuclease Inhibitor (40 U/μl)</td>
<td>200 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>First-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP)</td>
<td>15 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>First-strand buffer (10×)</td>
<td>75 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Linker–primer (1.4 μg/μl)</td>
<td>10 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Test poly(A)⁺ RNA (0.2 μg/μl)</td>
<td>5 μg</td>
<td>–20°C</td>
</tr>
<tr>
<td>Diethylpyrocarbonate (DEPC)-treated water</td>
<td>500 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Second-strand reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second-strand buffer (10×)</td>
<td>150 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP)</td>
<td>30 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td><em>Escherichia coli</em> RNase H (1.5 U/μl)</td>
<td>15 U</td>
<td>–20°C</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DNA polymerase I (9.0 U/μl)</td>
<td>500 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>Sodium acetate (3 M)</td>
<td>250 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Blunting reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)</td>
<td>115 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Cloned <em>Pfu</em> DNA polymerase (2.5 U/μl)</td>
<td>25 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>Ligation reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoR I adapters (0.4 μg/μl)</td>
<td>18 μg</td>
<td>–20°C</td>
</tr>
<tr>
<td>Ligase buffer&lt;sup&gt;b&lt;/sup&gt; (10×)</td>
<td>250 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>rATP&lt;sup&gt;b&lt;/sup&gt; (10 mM)</td>
<td>100 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>T4 DNA ligase&lt;sup&gt;b&lt;/sup&gt; (4 U/μl)</td>
<td>140 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>Phosphorylation reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4 polynucleotide kinase (5 U/μl)</td>
<td>50 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>Ligase buffer&lt;sup&gt;b&lt;/sup&gt; (10×)</td>
<td>250 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>rATP&lt;sup&gt;b&lt;/sup&gt; (10 mM)</td>
<td>100 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Xho I digestion reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xho I (40 U/μl)</td>
<td>600 U</td>
<td>–20°C</td>
</tr>
<tr>
<td>Xho I buffer supplement</td>
<td>250 μl</td>
<td>–20°C</td>
</tr>
<tr>
<td>Column reagents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Connecting tubing d (1/8-inch i.d., 3/16-inch o.d., and 1/32-inch wall)</td>
<td>1 × 4 cm</td>
<td>Room temperature or 4°C</td>
</tr>
<tr>
<td>Sepharose&lt;sup&gt;c&lt;/sup&gt; CL-2B gel filtration medium&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10 ml</td>
<td>4°C</td>
</tr>
<tr>
<td>Column-loading dye&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>17.5 μl</td>
<td>4°C</td>
</tr>
<tr>
<td>STE buffer&lt;sup&gt;c&lt;/sup&gt; (10×)</td>
<td>10 ml</td>
<td>4°C</td>
</tr>
</tbody>
</table>

<sup>a</sup> Enough reagents are included to generate five vector-ligated constructs.

<sup>b</sup> These reagents are used more than once in the reaction.

<sup>c</sup> See Preparation of Media and Reagents.

<sup>d</sup> The Sepharose CL-2B gel filtration medium and the column-loading dye are shipped separately at 4°C.
**ADDITIONAL MATERIALS REQUIRED**

**BacterioMatch II Two-Hybrid System Assays and Screens**

14-ml BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)

Components for minimal, his-dropout bacteriological media\(^\ast\), including:
- M9 salts\(^\ast\) (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 bath (12°C and 42°C)
Incubators (30°C and 37°C)

**cDNA Library Construction**

Phenol–chloroform [1:1 (v/v)] and chloroform

**Note**  Do not use the low-pH phenol from the Agilent RNA Isolation Kit because this phenol is too acidic and will denature the DNA.

\[^{-32}P\]Deoxynucleoside triphosphate ([\(^{-32}P\)dNTP] (800 Ci/mmol) ([\(^{-32}P\)dATP, [\(^{-32}P\)dGTP, or [\(^{-32}P\)dTTP may be used; do not use [\(^{-32}P\)dCTP)

Disposable plastic 10-ml syringes, sterile (e.g., 10-cc BD® syringe with Luer Lok\(^{\circledR}\) tip or equivalent)
Disposable 18-gauge, 1½-inch needles, sterile (e.g., BD PrecisionGlide\(^{\circledR}\) needle or equivalent)
Disposable plastic 1-ml pipets, negatively graduated and sterile [e.g., 1 ml BD Falcon\(^{\circledR}\) disposable polystyrene serological pipet (BD Biosciences Catalog #357250) or equivalent]
Pasteur pipets
Nondenaturing acrylamide gel, 5%\(^\ast\)
Portable radiation monitor (Geiger counter)
Water baths (4°C, 8°C, 12°C, 16°C, 37°C, 42°C, 70°C, and 72°C)
Whatman\(^{\circledR}\) No. 1 qualitative filter paper, Grade 1 [Fisher Scientific, Pittsburgh, Pennsylvania [Catalog #09-805C (7 cm diameter) and #09-805F (12.5 cm diameter)]

**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.

\(^\ast\) See *Preparation of Media and Reagents.*
**Host Strains and Genotypes**

| XL1-Blue MRF’ Kan Library Pack Competent Cells 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 lacIq ZΔM15 Tn5 (Kan' )] |
| 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' lacIq HIS3 aadA Kan'] |

*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 *HIS3-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 is particularly useful for the identification of novel proteins from a cDNA library that interact with a specific bait protein. The BacterioMatch II Two-Hybrid System Library Construction Kit (Catalog #200412) is designed for two-hybrid screening using the pBT-bait plasmid to identify protein-protein interaction partners from a pTRG cDNA plasmid library. We also offer the BacterioMatch II Two-Hybrid System Library Construction Kit with Electrocompetent Cells (Catalog #200414), allowing cotransformation of the pBT-Bait plasmid and the pTRG plasmid library into the reporter strain at higher efficiencies using electrocompetent reporter cells. The electrocompetent reporter cells are also available separately (Catalog #200195). A variety of BacterioMatch II premade-library kits are also available; for a complete list, visit www.genomics.agilent.com.

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 lacIq 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.

<table>
<thead>
<tr>
<th>Catalog #</th>
<th>Description</th>
<th>Applications</th>
<th>Efficiency</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>200190</td>
<td>BacterioMatch II Screening Reporter Competent Cells (provided; chemically-competent)</td>
<td>Library screening (cotransformation of pBT-bait and the pTRG library)</td>
<td>≥7.5 × 10⁷ cfu/µg pUC18</td>
<td>6 × 500 µl aliquots</td>
</tr>
<tr>
<td>200192</td>
<td>BacterioMatch II Validation Reporter Competent Cells (provided; chemically-competent)</td>
<td>Interaction assays, expression testing, validation of screen isolates</td>
<td>≥1 × 10⁷ cfu/µg pUC18</td>
<td>5 × 200 µl aliquots</td>
</tr>
<tr>
<td>200195</td>
<td>BacterioMatch II Electrocompetent Reporter Cells (available separately; electroporation-competent)</td>
<td>Library screening (cotransformation of pBT-bait and the pTRG library) with increased efficiency compared to chemically-competent reporter cells</td>
<td>≥1 × 10⁹ cfu/µg pUC18</td>
<td>5 × 100 µl aliquots</td>
</tr>
</tbody>
</table>
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 EcoRI and XhoI restriction sites in the pTRG MCS makes the plasmid compatible with inserts produced using the provided cDNA Synthesis Kit. 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 lacIq 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**

- **chloramphenicol**
- **p15A ori**
- **P lac-UV5**

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

5’ GAA GAG ACG TTT GGC GCG GCC GCA TCG AAT TCC CGG GGA TCC TCG AGT TAA...

...TTA ATT AAT TAA GAT CT 3’

<table>
<thead>
<tr>
<th>Feature</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloramphenicol resistance ORF</td>
<td>2770–219</td>
</tr>
<tr>
<td>p15A origin of replication</td>
<td>581–1493</td>
</tr>
<tr>
<td>lac-UV5 promoter</td>
<td>1556–1586</td>
</tr>
<tr>
<td>λ cl ORF</td>
<td>1631–2341</td>
</tr>
<tr>
<td>pBT forward primer [5’ TCCGTGTGGGAAAGTTATC 3’]</td>
<td>2291–2311</td>
</tr>
<tr>
<td>multiple cloning site</td>
<td>2342–2394</td>
</tr>
<tr>
<td>pBT reverse primer [5’ GGGTAGCCAGCAGCATCC 3’]</td>
<td>2419–2436</td>
</tr>
</tbody>
</table>

**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**

![Diagram of pTRG Vector Map](image)

**pTRG Multiple Cloning Site Region**
(Sequence shown 978–1065)

```
5' AAA CCA GAG GCG GCC GGA TCC GCG GCC GCA AGA ATT CAG TCT GAG CTG GCG...
```

Table: Feature and Position

<table>
<thead>
<tr>
<th>Feature</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>lpp promoter</td>
<td>47–76</td>
</tr>
<tr>
<td>lac-UV5 promoter</td>
<td>119–148</td>
</tr>
<tr>
<td>RNAPα ORF</td>
<td>243–992</td>
</tr>
<tr>
<td>pTRG forward primer [5’ TGGCTGAACAAGTGAAGCT 3’]</td>
<td>913–932</td>
</tr>
<tr>
<td>multiple cloning site</td>
<td>993–1058</td>
</tr>
<tr>
<td>pTRG reverse primer [5’ ATTCGTCGCCCAGGCAAT 3’]</td>
<td>1102–1119</td>
</tr>
<tr>
<td>ColE1 origin of replication</td>
<td>1243–2475</td>
</tr>
<tr>
<td>tetracycline resistance ORF</td>
<td>3120–4310</td>
</tr>
</tbody>
</table>

**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-Gal11P and the pBT-LGF2 positive control plasmids (Table I and Figure 4).

TABLE I
Description of Control Plasmids

<table>
<thead>
<tr>
<th>Control plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBT-LGF2</td>
<td>interaction control plasmid encoding the dimerization domain (40 amino acids) of the Gal4 transcriptional activator protein</td>
</tr>
<tr>
<td>pTRG-Gal11P</td>
<td>interaction control plasmid encoding a domain (90 amino acids) of the mutant form of the Gal11 protein</td>
</tr>
</tbody>
</table>

Control Plasmid Maps

Applications
The dimerization domain of the yeast transcriptional activator Gal4 and a domain derived from a mutant form of Gal11 protein, called Gal11P, have been shown to interact in *E. coli* cells. These polypeptides are expressed by the pBT-LGF2 and the pTRG-Gal11P 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.
**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, EcoRI, SmaI, BamHI, XhoI, and BglII 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**

<table>
<thead>
<tr>
<th>Ligation Reaction Components</th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepared pBT plasmid DNA (0.1 μg/μl)</td>
<td>1.0 μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Prepared insert (0.1 μg/μl)</td>
<td>Y μl to make a 2:1 or a 3:1 ratio</td>
<td>0 μl 0 μl 1.0 μl</td>
</tr>
<tr>
<td>10 mM rATP (pH 7.0)</td>
<td>1.0 μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>10× ligase buffer§</td>
<td>1.0 μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>T4 DNA ligase (4 U/μl)</td>
<td>0.5 μl</td>
<td>0.5 μl 0 μl 0.5 μl</td>
</tr>
<tr>
<td>Double-distilled water (to 10 μl)</td>
<td>X μl</td>
<td>X μl</td>
</tr>
</tbody>
</table>

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* 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 \( \lambda cI \) protein.

### Primers for use with pBT

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBT forward primer</td>
<td>5'- TCC GTT GTG GGG AAA GTT ATC - 3'</td>
</tr>
<tr>
<td>pBT reverse primer</td>
<td>5'- GGG TAG CCA GCA GCA TCC - 3'</td>
</tr>
</tbody>
</table>

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 using an antibody that immunoreacts either with the bait protein encoded by the insert or with the \( \lambda cI \) protein. Note, however, that 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. (See Preparation of Media and Reagents.) 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.

♦ 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. Do Western blot analysis with an antibody that immunoreacts with either the bait protein encoded by the insert or the λcI protein.

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 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>
<thead>
<tr>
<th>Table II</th>
<th>Cotransformations Carried Out in this Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td>Plates</td>
</tr>
<tr>
<td>recombinant pBT + pTRG (empty vector) (50 ng each)</td>
<td>Nonselective Screening Medium (no 3-AT)⁹</td>
</tr>
<tr>
<td></td>
<td>Selective Screening Medium (5 mM 3-AT)⁹</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>pBT (empty vector) + pTRG-Gal11⁷ (50 ng each)</td>
<td>Nonselective Screening Medium (no 3-AT)</td>
</tr>
<tr>
<td></td>
<td>Selective Screening Medium (5 mM 3-AT)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁹ 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:**

<table>
<thead>
<tr>
<th>cfu obtained on Nonselective Screening Medium (no 3-AT) plate</th>
<th>Dilution factor</th>
<th>Plating volume factor (relative to 200 μl)</th>
<th>Adjusted cotransformants from 200 μl culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 cfu (20 μl plated)</td>
<td>× 100</td>
<td>× 10</td>
<td>50,000 cfu</td>
</tr>
<tr>
<td>100 cfu (200 μl plated)</td>
<td>× 100</td>
<td>× 1</td>
<td>10,000 cfu</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Adjusted cotransformants (200 μl culture)</th>
<th>cfu obtained on Selective Screening Medium (5 mM 3-AT) (200 μl culture)</th>
<th>Percent of cotransformants able to grow on 5 mM 3-AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000 cfu</td>
<td>20 cfu</td>
<td>0.04%</td>
</tr>
<tr>
<td>10,000 cfu</td>
<td>5 cfu</td>
<td>0.05%</td>
</tr>
</tbody>
</table>

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.
cDNA Library Construction in the pTRG Plasmid

Background

The BacterioMatch II two-hybrid system is particularly useful for the identification of novel (target) proteins from a cDNA library, which interact with a bait protein, and for the subsequent determination of protein domains or amino acids critical for the interaction. Specific mutations, insertions, or deletions that affect the encoded amino acids can be introduced into DNA encoding the target protein, and the mutant target proteins can be assayed for the protein–protein interaction with the bait protein.

Overview of the cDNA Library Construction Method

DNA inserts to be ligated into the pTRG plasmid may be prepared from mRNA. The provided Agilent cDNA Synthesis Kit includes the reagents required to convert mRNA to cDNA inserts ready for unidirectional insertion into the pTRG XR (predigested) plasmid.

Figure 5 shows an overview of the cDNA synthesis procedure. Production of DNA inserts using the cDNA Synthesis Kit results in cDNA molecules with EcoRI and XhoI sites at the 5’ and 3’ ends, respectively. This allows for unidirectional insertion into the EcoRI and XhoI-digested pTRG XR plasmid provided with the kit.

The protocols for preparing cDNA inserts are found in Protocol: Generating the cDNA Inserts, and the protocols for ligation into the predigested vector are found in Protocol: Ligating the cDNA Library into the pTRG Plasmid.
**FIGURE 5** cDNA synthesis flow chart.
cDNA Insert Preparation Background

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 plasmid. 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. Therefore, cDNA library construction provides a method by which the transcription and processing of mRNA can be examined and interpreted.

The cDNA Synthesis Kit uses a hybrid oligo(dT) linker–primer that contains an Xho I restriction site. First-strand synthesis is primed with the linker–primer and the mRNA is reverse-transcribed using AccuScript reverse transcriptase and 5-methyl dCTP.

AccuScript reverse transcriptase (AccuScript RT) is a novel Moloney murine leukemia virus reverse transcriptase (MMLV-RT) derivative combined with a proofreading 3'-5' exonuclease. AccuScript RT delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript RT delivers greater than three-fold higher accuracy compared to leading reverse transcriptases, representing a significant advancement in cDNA synthesis accuracy. These advantages make AccuScript RT the enzyme of choice for applications involving the preparation of accurate, full-length, cDNA transcripts, including first-strand cDNA synthesis and library construction.

The use of 5-methyl dCTP during first-strand synthesis hemimethylates the cDNA, which protects the cDNA from digestion with certain restriction endonucleases such as Xho I. Therefore, on Xho I digestion of the cDNA, only the unmethylated site within the linker–primer is cleaved.

Hemimethylated DNA introduced into an McrA+ McrB+ strain would be subject to digestion by the mcrA and mcrB restriction systems. Therefore, it is necessary to initially transform an McrA− McrB− strain (e.g., the XL1-Blue MRF+ Kan Library Pack Competent Cells supplied with the BacterioMatch II two-hybrid system) when using the cDNA Synthesis Kit.
**CDNA Synthesis Background**

The yield, length, and accuracy of cDNA transcripts is enhanced with the use of AccuScript RT, an engineered version of the Moloney murine leukemia virus reverse transcriptase combined with a proofreading 3'-5' exonuclease. First-strand cDNA synthesis begins when AccuScript 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:

\[ 5' \text{GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAG} \ldots \text{GAGA} \text{Xho I Poly(dT)} \ 3' \]

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 (EcoR I–Xho I) with respect to the \( p_{\text{GAL1}} \). The poly(dT) region binds to the 3’ poly(A) region of the mRNA template, and AccuScript reverse transcriptase begins to synthesize the first-strand cDNA.

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.

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 dCpT 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 \( \text{Pfu} \) DNA polymerase, and EcoR I adapters are ligated to the blunt ends. The adapters have the sequence shown below.

\[ 5' \text{OH–AATTCCGGCACGAGG} \ldots \text{3'} \]

\[ 3' \text{GCCGTGCTCCp} \ldots \text{5'} \]

These adapters are composed of 10- and 14-mer oligonucleotides, which are complementary to each other with an EcoR 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 EcoR 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.
Protocol: Generating the cDNA Inserts

Note  The following protocol has been optimized for 5 µg of poly(A)\(^+\) RNA per reaction.

Protocol Guidelines

♦ The quality and quantity of the mRNA used is of fundamental importance to the construction of a large, representative cDNA library (see Appendix I: RNA Purification and Quantitation). Our RNA Isolation Kits use the guanidinium thiocyanate–phenol–chloroform extraction method,\(^5\) which quickly produces large amounts of undegraded RNA. To isolate mRNA, we offer the Absolutely mRNA Purification Kit.

♦ Secondary structure of mRNA may cause the synthesis of truncated cDNAs. To relax secondary structure, treatment with methylmercury hydroxide (CH\(_3\)HgOH) is recommended (see Appendix II: Methylmercury Hydroxide Treatment).

♦ It is imperative to protect the RNA from any contaminating RNases until the first-strand cDNA synthesis is complete. Wear fresh gloves, use newly autoclaved pipet tips, and avoid using pipet tips or microcentrifuge tubes that have been handled without gloves. Ribonuclease A cannot be destroyed by normal autoclaving alone. Baking or DEPC treatment is recommended.

♦ When removing aliquots of any of the enzymes used in the cDNA synthesis protocol, flick the bottom of the tube to thoroughly mix the enzyme solution. Do not vortex the enzyme stock tubes.
Synthesizing First-Strand cDNA

1. Prepare water baths at 16°, 42°, and 72°C.

2. Thaw the radioactive [α-32P]dNTP (do not use [32P]dCTP) and all nonenzymatic first-strand components. Keep the radioactive dNTP on ice for use in step 6 and in the second-strand synthesis. Briefly vortex and spin down the contents of the nonenzymatic tubes. Place the tubes on ice.

   **Note**  AccuScript reverse transcriptase is temperature sensitive and should remain at –20°C until the last moment.

3. The final volume of the first-strand synthesis reaction is 50 μl. The volume of added reagents and enzymes is 14 μl, thus the mRNA template and DEPC-treated water should be added in a combined volume of 36 μl. For the control reaction, prepare the following annealing reaction with 25 μl (5 μg) of test RNA and 11 μl of DEPC-treated water.

4. In an RNase-free microcentrifuge tube, add the following reagents in order:

   - 5 μl of 10× first-strand buffer
   - 3 μl of first-strand methyl nucleotide mixture
   - 2 μl of linker–primer (1.4 μg/μl)
   - X μl of DEPC-treated water
   - 1 μl of RNase Block Ribonuclease Inhibitor (40 U/μl)

5. Mix the reaction and then add X μl of poly(A)+ RNA (5 μg). Mix gently.

6. Allow the primer to anneal to the template for 10 minutes at room temperature. During the incubation, aliquot 0.5 μl of the [α-32P]dNTP (800 Ci/mmol) into a separate tube for the control.

7. Add 3 μl of AccuScript RT to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction should now be 50 μl.

8. Mix the sample gently and spin down the contents in a microcentrifuge.

9. **Transfer 5 μl of the first-strand synthesis reaction to the separate tube containing the 0.5 μl of the [α-32P]dNTP (800 Ci/mmol). This radioactive sample is the first-strand synthesis control reaction.**

10. Incubate the first-strand synthesis reactions, including the control reaction, at 42°C for 1 hour.
11. Prepare a 16°C water bath for second-strand synthesis. If a water bath with a cooling unit is not available, use a large Styrofoam® container with a lid. Fill the container three-quarters full with water and adjust the temperature to 16°C with ice. Cover the container with a lid.

12. After 1 hour, remove the first-strand synthesis reactions from the 42°C water bath. Place the nonradioactive first-strand synthesis reaction on ice. Store the radioactive first-strand synthesis control reaction at –20°C until ready to resolve by electrophoresis on an alkaline agarose gel (see step 17 in Blunting the cDNA Termi). The radioactive first-strand reaction will be gel-analyzed alongside a radioactive sample of the second-strand reaction after the second strand reaction is blunted and resuspended in the EcoRI adapters.

**Synthesizing Second-Strand cDNA**

1. Thaw all nonenzymatic second-strand components. Briefly vortex and spin in a micro-centrifuge before placing the tubes on ice.

   **Note** It is important that all reagents be < 16°C when the DNA polymerase I is added.

2. Add the following components in order to the 45-μl nonradioactive, first-strand synthesis reaction on ice:

   - 20 μl of 10× second-strand buffer
   - 6 μl of second-strand dNTP mixture
   - 114 μl of sterile dH₂O (DEPC-treated water is not required)
   - 2 μl of [α-³²P]dNTP (800 Ci/mmol)

3. Add the following enzymes to the second-strand synthesis reaction:

   - 2 μl of RNase H (1.5 U/μl)
   - 11 μl of DNA polymerase I (9.0 U/μl)

4. Gently vortex the contents of the tube, spin the reaction in a microcentrifuge, and incubate for 2.5 hours in a 16°C water bath. Check the water bath occasionally to ensure that the temperature does not rise above 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector.

5. After second-strand synthesis for 2.5 hours at 16°C, immediately place the tube on ice.
**Blunting the cDNA Termini**

1. Add the following to the second-strand synthesis reaction:
   
   - 23 μl of blunting dNTP mix
   - 2 μl of cloned *Pfu* DNA polymerase

2. Quickly vortex the reaction and spin down in a microcentrifuge. Incubate the reaction at 72°C for 30 minutes. **Do not exceed 30 minutes!!**

3. Thaw the 3 M sodium acetate.

   **Note** Since radioactivity can leak out between the lid and body of some micro-centrifuge tubes during the vortexing and precipitation steps, wrap a small piece of Parafilm® laboratory film around the rim of the microcentrifuge tube to prevent leakage.

4. Remove the reaction and add 200 μl of phenol–chloroform [1:1 (v/v)] and vortex.

   **Note** Do not use the low-pH phenol from the Agilent RNA Isolation Kit because this phenol is too acidic and may denature the DNA. The phenol must be equilibrated to pH 7–8.

5. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube. Be careful to avoid removing any interface that may be present.

6. Add an equal volume of chloroform and vortex.

7. Spin the reaction in a microcentrifuge at maximum speed for 2 minutes at room temperature and transfer the upper aqueous layer, containing the cDNA, to a new tube.

8. Precipitate the cDNA by adding the following to the saved aqueous layer:
   
   - 20 μl of 3 M sodium acetate
   - 400 μl of 100% (v/v) ethanol

   Vortex the reaction.

9. Precipitate overnight at –20°C.

10. In order to orient the direction of precipitate accumulation, place a mark on the microcentrifuge tube or point the tube hinge away from the center of the microcentrifuge as an indicator of where the pellet will form.
11. Spin in a microcentrifuge at maximum speed for 60 minutes at 4°C.

12. Avoid disturbing the pellet and carefully remove and discard the radioactive supernatant in a radioactive waste container.

**Note**  
*The conditions of synthesis and precipitation produce a large white pellet. The pellet accumulates near the bottom of the microcentrifuge tube and may taper up along the marked side of the tube.*

13. Gently wash the pellet by adding 500 μl of 70% (v/v) ethanol to the side of the tube away from the precipitate. **Do not mix or vortex!**

14. Spin in a microcentrifuge at maximum speed for 2 minutes at room temperature with the orientation marked as in step 10.

15. Aspirate the ethanol wash and dry the pellet by vacuum centrifugation.

16. Resuspend the pellet in 9 μl of EcoR I adapters and incubate at 4°C for at least 30 minutes to allow the cDNA to resuspend. To ensure that the cDNA is completely in solution, transfer the cDNA to a fresh microcentrifuge tube. Monitor the now empty tube with a handheld Geiger counter. If the cDNA is in solution, few counts should remain in the empty tube.

17. **Transfer 1 μl of this second-strand synthesis reaction to a separate tube. This radioactive sample is the second-strand synthesis control reaction.** We strongly recommend running the samples of the first- and second-strand synthesis reactions on an alkaline agarose gel at this point. It is important to determine the size range of the cDNA and the presence of any secondary structure (see Appendix III: Alkaline Agarose Gels).

**Note**  
*The second-strand synthesis reaction can be stored overnight at –20°C.*

### Ligating the EcoR I Adapters

1. Add the following components to the tube containing the blunted cDNA and the EcoR I adapters:

   1 μl of 10× ligase buffer  
   1 μl of 10 mM rATP  
   1 μl of T4 DNA ligase (4 U/μl)

2. Spin down the volume in a microcentrifuge and incubate overnight at 8°C. Alternatively, the ligations can be incubated at 4°C for 2 days.

3. In the morning, heat inactivate the ligase by placing the tubes in a 70°C water bath for 30 minutes.
**Phosphorylating the EcoR I Ends**

1. After the ligase is heat inactivated, spin the reaction in a microcentrifuge for 2 seconds. Cool the reaction at room temperature for 5 minutes.

2. Phosphorylate the adapter ends by adding the following components:
   - 1 μl of 10× ligase buffer
   - 2 μl of 10 mM rATP
   - 5 μl of sterile water
   - 2 μl of T4 polynucleotide kinase (5.0 U/μl)

3. Incubate the reaction for 30 minutes at 37°C.

4. Heat inactivate the kinase for 30 minutes at 70°C.

5. Spin down the condensation in a microcentrifuge for 2 seconds and allow the reaction to equilibrate to room temperature for 5 minutes.

**Digesting with Xho I**

1. Add the following components to the reaction:
   - 28 μl of Xho I buffer supplement
   - 3 μl of Xho I (40 U/μl)

2. Incubate the reaction for 1.5 hours at 37°C.

3. Add 5 μl of 10× STE buffer§ and 125 μl of 100% (v/v) ethanol to the microcentrifuge tube.

4. Precipitate the reaction overnight at –20°C.

5. Following precipitation, spin the reaction in a microcentrifuge at maximum speed for 60 minutes at 4°C.

6. Discard the supernatant, dry the pellet completely, and resuspend the pellet in 14 μl of 1× STE buffer.

7. Add 3.5 μl of the column-loading dye§ to each sample.

The sample is now ready to be run through a drip column containing Sepharose CL-2B gel filtration medium (see *Size Fractionating*).

§ See *Preparation of Media and Reagents.*
Size Fractionating

Before attempting the experimental protocols outlined within this section, please read this section in its entirety in order to become familiar with the procedures. Review of the Troubleshooting section may also prove helpful. The drip columns should be prepared and the cDNA should be eluted in 1 day. Because a full day is required to complete these procedures, gathering all necessary materials in advance is recommended (see the cDNA Library Construction section in Additional Materials Required).

1. Perform the following preparatory steps while assembling the drip columns:
   a. Remove the Sepharose CL-2B gel filtration medium and the 10× STE buffer from refrigeration and equilibrate the two components to room temperature.
   b. Prepare 50 ml of 1× STE buffer by diluting 10× STE buffer 1:10 in sterile water.

2. Assemble the drip columns as outlined in the following steps (see Figure 6 for a diagram of the final setup):
   
   **Note**  
   Wear gloves while assembling the drip columns.

   a. Remove the plastic wrapper from the top of a sterile 1-ml pipet.
   b. Using a sterile needle or a pair of fine-tipped forceps, carefully tease the cotton plug out of each pipet, leaving a piece of the cotton plug measuring ~3–4 mm inside. Cut off the external portion of the cotton plug.
   c. Push the remaining 3- to 4-mm piece of the cotton plug into the top of each pipet with the tip of the needle or forceps.
   d. Cut a small piece of plastic tubing measuring ~8 mm. Use this small tube to connect the 1-ml pipet to the 10-ml syringe. First attach one end of the connecting tube to the pipet and then connect the other end to the syringe. There should be no gap between the pipet and the syringe when joined by the connecting tube.
   
   **Note**  
   The inside diameter of the plastic tubing (~1/8-inch i.d.) snugly connects most disposable 1-ml pipets and the ends of all BD® 10-cc syringes with the Luer Lok® tips.
   
   e. Rapidly and forcefully push the plunger into the syringe to thrust the cotton plug down into the tip of the pipet.
   
   **Note**  
   It may take several attempts to drive the cotton all the way down into the tip of the pipet. However, pushing the cotton plug as far down into the pipet tip as possible is important in order to achieve optimal separation of the cDNA fractions.
f. Remove the plunger from the syringe. Because the syringe functions as a buffer reservoir for the drip column, leave the syringe firmly attached to the pipet throughout the remainder of the size fractionation procedure.

3. Locate a support for the assembled drip column. Butterfly clamps or a three-fingered clamp on a ring stand can be used.

**FIGURE 6** Assembly of the drip columns.
Loading the Drip Column

1. Load the drip column with a uniform suspension of Sepharose CL-2B gel filtration medium as outlined in the following steps:

   a. Immediately prior to loading the drip column, gently mix the Sepharose CL-2B gel filtration medium by inversion until the resin is uniformly suspended.

   b. Place the column in the ring stand. Fill a glass Pasteur pipet with ~2 ml of 1× STE buffer. Insert the pipet as far into the drip column as possible and fill the column with the buffer.

   Notes If the 1× STE buffer flows too quickly through the column, stem the flow by affixing a yellow pipet tip to the end of the column. Make sure to remove the pipet tip prior to loading the column with the Sepharose CL-2B gel filtration medium.

      If bubbles or pockets of air become trapped in the STE buffer while filling the column, remove the trapped air prior to packing the column with the resin. To remove the bubbles or air, re-insert the Pasteur pipet into the top of the column and gently pipet the STE buffer in and out of the pipet until the trapped air escapes through the top of the column.

   c. Immediately add a uniform suspension of Sepharose CL-2B gel filtration medium to the column with a Pasteur pipet by inserting the pipet as far into the column as possible. As the resin settles, continue adding the Sepharose CL-2B gel filtration medium. Stop adding the resin when the surface of the packed bed is ¼ inch below the “lip of the pipet.” The lip of the pipet is defined as the point where the pipet and the syringe are joined.

   Notes If air bubbles form as the resin packs, use a Pasteur pipet as described in step 1b to remove the blockage. Failure to remove bubbles can impede the flow of the column and result in a loss of the cDNA.

      If the preparation of Sepharose CL-2B gel filtration medium settles and becomes too viscous to transfer from the stock tube to the column, add a small volume (~1–5 ml) of 1× STE buffer to resuspend the resin.
2. Wash the drip column by filling the buffer reservoir (i.e., the syringe) with a minimum of 10 ml of 1× STE buffer. As the column washes, the buffer should flow through the drip column at a steady rate; however, it may take at least 2 hours to complete the entire wash step. After washing, do not allow the drip column to dry out, because the resin could be damaged and cause sample loss. If this occurs, pour another column.

**Note** If a free flow of buffer is not observed, then bubbles or pockets of air have become trapped in the drip column. In this case, the column must be repacked. If cDNA is loaded onto a column on which a free flow of buffer is not observed, the sample could become irretrievably lost.

3. When ~50 μl of the STE buffer remains above the surface of the resin, immediately load the cDNA sample using a pipettor. Gently release the sample onto the surface of the column bed, but avoid disturbing the resin as this may affect cDNA separation.

4. Once the sample enters the Sepharose CL-2B gel filtration medium, fill the connecting tube with buffer using a pipettor.

**Note** Do not disturb the bed while filling the connecting tube with buffer.

Gently add 3 ml of 1× STE buffer to the buffer reservoir by trickling the buffer down the inside wall of the syringe. Do not squirt the buffer into the reservoir because this will disturb the resin, resulting in loss of the sample.

5. As the cDNA sample elutes through the column, the dye will gradually diffuse as it migrates through the resin. Because the dye is used to gauge when the sample elutes from the column, monitor the progress of the dye, or the cDNA sample could be irretrievably lost.

**Collecting the Sample Fractions**
The drip column containing the Sepharose CL-2B gel filtration medium separates molecules on the basis of size. Large cDNA molecules elute first followed by smaller cDNA and finally unincorporated nucleotides. Using a handheld monitor, two peaks of radioactivity can generally be detected during the course of elution. The first peak to elute from the column represents the cDNA. Due to the conditions of label incorporation during second-strand synthesis, the cDNA is not extremely radioactive; therefore, the counts per second may be barely above background levels. In contrast, the second peak to elute from the column is highly radioactive as this is the unincorporated radioactive nucleotides. Although this material elutes from the column in parallel with the dye, unincorporated nucleotides are usually not collected because the cDNA has already eluted from the column.
For standard cDNA size fractionation (>400 bp), collect ~12 fractions using the procedure described in this section. The progression of the leading edge of the dye through the column will be used as a guideline to monitor collection; however, the drops collected from the column should be monitored for radioactivity using a handheld Geiger counter. Until the fractions have been assessed for the presence of cDNA on a 5% nondenaturing acrylamide gel (see Preparation of Media and Reagents), do not discard any fractions based on the quantity of radioactivity detected.

1. Using a fresh microcentrifuge tube to collect each fraction, begin collecting three drops per fraction when the leading edge of the dye reaches the –0.4-ml gradation on the pipet.

2. Continue to collect fractions until the trailing edge of the dye reaches the 0.3-ml gradation. A minimum of 12 fractions, each containing ~100 μl (i.e., three drops), should be collected. Alternatively, fractions can be collected until the radioactive unincorporated nucleotides begin to elute. In either case, monitor the fractions for the presence of radioactivity to determine whether the cDNA has eluted successfully. If no counts are detected, continue collecting the fractions until the peak of unincorporated nucleotides is recovered.

3. Before processing the fractions and recovering the size-fractionated cDNA, remove 8 μl of each collected fraction and save for later analysis. These aliquots will be electrophoresed on a 5% nondenaturing acrylamide gel to assess the effectiveness of the size fractionation and to determine which fractions will be used for ligation.

Processing the cDNA Fractions

In this section of the size fractionation procedure, the fractions collected from the drip column are extracted with phenol–chloroform and are precipitated with ethanol to recover the size-selected cDNA. The purpose of the organic extractions is to remove contaminating proteins; of particular concern is kinase, which can be carried over from previous steps in the synthesis. Because kinase often retains activity following heat treatment, it is necessary to follow the extraction procedures.

1. Begin extracting the remainder of the collected fractions by adding an equal volume of phenol–chloroform [1:1 (v/v)].

2. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.

3. Add an equal volume of chloroform.

4. Vortex and spin in a microcentrifuge at maximum speed for 2 minutes at room temperature. Transfer the upper aqueous layer to a fresh microcentrifuge tube.
5. To each extracted sample, add a volume of 100% (v/v) ethanol that is equal to twice the individual sample volume.

**Note**  *The 1× STE buffer contains sufficient NaCl for precipitation.*

6. Precipitate overnight at −20°C.

7. Spin the sample in the microcentrifuge at maximum speed for 60 minutes at 4°C. Transfer the supernatant to another tube. To ensure that the cDNA has been recovered, use a handheld Geiger counter to check the level of radioactivity present in the pellet. If the majority of the radiation is detected in the supernatant, repeat the centrifugation step; otherwise, discard the supernatant.

8. Carefully wash the pellet with 200 μl of 70% (v/v) ethanol, ensuring that the pellet remains undisturbed. *Do not mix or vortex!* Spin the sample in a microcentrifuge at maximum speed for 2 minutes at room temperature. Remove the ethanol and verify that the pellet has been recovered by visual inspection or with the handheld Geiger counter. Vacuum evaporate the pellet for ~5 minutes or until dry. Do not dry the pellet beyond the point of initial dryness or the cDNA may be difficult to solubilize.

9. Using a handheld Geiger counter verify that the cDNA has been recovered and record the number of counts per second (cps) that is detected for each fraction.

10. If <30 cps is detected, resuspend each cDNA pellet in 3.5 μl of sterile water. If the value is >30 cps, resuspend the cDNA in 5 μl of sterile water. Mix by pipetting up and down.

**We strongly recommend quantitating the cDNA before proceeding** (see Appendix IV: Ethidium Bromide Plate Assay—Quantitation of DNA). Best results are usually obtained by ligating 25–35 ng of cDNA/30 ng of vector. Place the remaining cDNA at −20°C for short term storage only. The cDNA is most stable after ligation into the vector and may be damaged during long-term storage.
Protocol: Ligating the cDNA Library into the pTRG Plasmid

Note During library construction, bacterial cell growth is performed on rich medium using a 30°C incubation temperature.

Pilot Ligation of the cDNA Library into the pTRG Plasmid

1. To prepare the sample ligation, add the following components to an RNase/DNase-free microcentrifuge tube:

   \[X\text{μl of resuspended cDNA [Use 3:1 molar ratio of insert to vector (~25–35 ng)]}\]
   
   0.6 μl of 10× ligase buffer
   
   0.6 μl of 10 mM rATP (pH 7.5)
   
   30 ng of pTRG XR plasmid (0.1 μg/μl)
   
   \[X\text{μl of distilled water for a final volume of 5.4 μl}\]

To prepare the positive control ligation, add the following components to an RNase/DNase-free microcentrifuge tube:

   1.5 μl of XR Amp test insert (15 ng)
   
   0.6 μl of 10× ligase buffer
   
   0.6 μl of 10 mM rATP (pH 7.5)
   
   30 ng of pTRG XR plasmid (0.1 μg/μl)
   
   \[X\text{μl of distilled water for a final volume of 5.4 μl}\]

To each ligation reaction tube add

   0.6 μl of T4 DNA ligase (4 U/μl)

2. Flick the tube gently to mix, then spin down briefly.

3. Incubate the reaction tubes overnight at 12°C.

4. Transform the XL1-Blue MRF’ Kan Library Pack Competent Cells, provided in this kit and available separately (Catalog #200138), according to the protocol below.
Transformation Guidelines for XL1-Blue MRF’ Kan Library Pack Competent Cells

Storage Conditions
Competent cells are sensitive to even small variations in temperature and must be stored at the bottom of a –80°C freezer. Transferring tubes from one freezer to another may result in a loss of efficiency. Competent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells
When aliquoting, keep competent cells on ice at all times. It is essential that the 14-ml BD Falcon tubes are placed on ice before the cells are thawed and that the cells are aliquoted directly into the prechilled tubes. It is also important to use the volume of competent cells specified in each protocol (100 μl or 500 μl per transformation). Using a smaller volume will result in lower efficiencies.

Use of 14-ml BD Falcon Polypropylene Round-Bottom Tubes
It is important that 14-ml BD Falcon polypropylene round-bottom tubes are used for the transformation protocol, since other tubes may be degraded by β-ME. In addition, the duration of the heat-pulse step is critical and has been optimized for the thickness and shape of these tubes.

Use of β-Mercaptoethanol
β-Mercaptoethanol (β-ME) has been shown to increase transformation efficiency. The β-ME Library Pack provided with the cells is diluted and ready to use. For optimum efficiency, use 2 μl of the β-ME Library Pack provided. Using an alternative source of β-ME may reduce transformation efficiency. Do not use the β-ME provided with the BacterioMatch II reporter strain competent cells.

Length of the Heat Pulse
There is a defined window of highest efficiency resulting from the heat pulse during transformation, depending on the volume of the competent cells. Optimal efficiencies are observed when a 100-μl aliquot of cells is heat pulsed for 33 seconds. For a 500-μl aliquot of cells, optimal efficiencies are observed following a 50-second heat pulse. Deviation from the optimal, even by 1 second, results in a significant reduction in efficiency. Do not exceed 42°C for either size aliquot of cells.
Transforming XL1-Blue MRF` Kan Library Pack Competent Cells with the pTRG cDNA Library

Transformations Carried Out in this Experiment

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Plates</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ligation reaction of pTRG plasmid and cDNA library</td>
<td>LB-tetracycline§</td>
<td>to create the pTRG cDNA library</td>
</tr>
<tr>
<td>ligation reaction of pTRG and Amp test insert</td>
<td>LB-ampicillin§</td>
<td>to determine efficiency of transformation using a ligated test insert</td>
</tr>
<tr>
<td>pUC18 (optional)</td>
<td>LB-ampicillin§</td>
<td>to calculate the transformation efficiency of the XL1-Blue MRF` Kan competent cells using supercoiled plasmid</td>
</tr>
</tbody>
</table>

Pilot Transformation Protocol for Library Pack Competent Cells

1. Pre-warm SOC medium§ to 42°C.

2. Thaw a 500-μl aliquot of XL1-Blue MRF` Kan Library Pack Competent Cells* on ice.

   **Note** The XL1-Blue MRF’ Kan strain contains the necessary lacIq gene. Do not substitute another strain for this transformation.

3. Immediately after thawing, gently mix the cells by flicking the tube. Aliquot 100 μl of cells into each of three prechilled 14-ml BD Falcon polypropylene round-bottom tubes. [Two aliquots are required in order to perform the pilot transformation in duplicate. The third aliquot is used for the control Amp test insert ligation.]

   **Optional** If the pUC18 transformation efficiency control is to be performed, aliquot 100μl of cells to a fourth tube.

4. Add 2 μl of the β-ME Library Pack provided with the kit to each 100 μl aliquot of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)

5. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes. Do not exceed 10 minutes.

6. Add 1.6 μl of the appropriate ligation reaction (from Pilot Ligation of the cDNA Library into the pTRG Plasmid) to each aliquot of cells and swirl gently.

   **Optional** For the transformation control, add 1 μl (100 pg) of pUC18 to the cells and swirl gently.

§ See Preparation of Media and Reagents.
* Additional competent cells are available separately (Catalog #200138).
7. Incubate the tubes on ice for 30 minutes.

8. Heat pulse the tubes in a 42°C water bath for 33 seconds. The duration and temperature of the heat pulse is critical for obtaining the highest efficiencies. Do not exceed 42°C.

9. Incubate the tubes on ice for 2 minutes.

10. Add 0.9 ml of prewarmed SOC medium to each tube, and allow the cells to recover by incubating the tubes at 30°C for 90 minutes with shaking at 225 rpm.

**Determining the Number of Transformants for the Pilot cDNA Library Ligation**

1. Place 100-μl pools of SOC medium on four separate 100-mm LB-tetracycline agar plates.

   a. Transfer 2 μl of recovered cell culture from the first pilot cDNA library transformation tube into one of the pools of SOC medium. Repeat with 5 μl of recovered cell culture from the first pilot cDNA library transformation. Ensure that the mixture is spread evenly on the plates.

   b. Transfer 2 μl of recovered cell culture from the second pilot cDNA library transformation tube into one of the pools of SOC medium. Repeat with 5 μl of recovered cell culture from the second pilot cDNA library transformation. Ensure that the mixture is spread evenly on the plates.

2. For the positive control ligation/transformation (Amp test insert), first place a 100-μl pool of SOC medium on each of two LB–ampicillin agar plates. Add 5 μl of the transformed cells to one pool of SOC medium and 10 μl to the other. Spread the mixtures evenly.

3. For the optional pUC18 transformation, first place a 100-μl pool of SOC medium on an LB–ampicillin agar plate. Add 2.5 μl of the transformed cells to the pool of SOC medium. Spread the mixture evenly.

4. Incubate all plates at 30°C for approximately 24 hours. Incubation at 30°C is necessary to minimize the generation of undesired mutants and to ensure good library representation.

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

5. For the optional pUC18 transformation, 250 colonies indicates a transformation efficiency for the XL1-Blue MRF’ Kan Library Pack Competent Cells of $1 \times 10^9$ cfu/μg pUC18 DNA.
6. Examine the plates containing the cDNA library transformations.

Count the number of tetracycline-resistant colonies on the 2-μl plate and on the 5-μl plate from the first pilot transformation (from step 1a above) and calculate total cfu for the transformation using the formula below:

\[
\frac{\text{[number of colonies]}}{\text{[volume of transformation plated (μl)]}} \times \frac{\text{[total volume of transformation (μl)]}}{\text{[volume of transformation plated (μl)]}} = \text{total cfu}
\]

Repeat the calculation after counting the numbers of colonies from the 2-μl and 5-μl plates from the second pilot transformation. The two values obtained for total cfu from the duplicate pilot experiments should be in the same range. Average them to obtain a value for average total cfu.

7. Examine the plates containing the positive control ligation/transformation (Amp test insert). Consult the pTRG XR Vector Certificate of Analysis for the lot-specific efficiency for transformation of pTRG XR vector plus Amp test insert ligation into the XL1-Blue MRF’ Kan Library Pack Competent Cells. (An efficiency of approximately 1 × 10^6 /μg pTRG XR is expected, resulting in ~300 cfu/10 μl transformation mixture.)

Verifying the Insert Percentage and Size

Inserts in individual colonies can be examined by either PCR directly from the colony with pTRG-specific primers or by restriction analysis of plasmid DNA prepared from individual colonies. If a sufficient number of individual colonies is tested in this way, the percentage of colonies containing inserts may be estimated and the insert size for the library may be sampled.

**Primers for use with pTRG**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTRG forward primer</td>
<td>5’- TGG CTG AAC AAC TGG AAG CT- 3’</td>
</tr>
<tr>
<td>pTRG reverse primer</td>
<td>5’- ATT CGT CGC CCG CCA TAA - 3’</td>
</tr>
</tbody>
</table>
Scaling Up the Ligations and Transformations for pTRG cDNA Library Construction

Perform the required number of ligation/transformation reactions to reach the desired target primary library size. (A representative library includes at least $1 \times 10^6$ cfu). Use the scaled-up Library Ligation and Library Transformation protocols below, which are equivalent to 5 pilot ligation/transformation reactions. If more than five reaction equivalents are required to achieve the desired library size, perform multiple rounds of the scaled-up protocols. The number of ligation/transformation reactions necessary may vary and should be based upon the efficiency realized with the pilot protocol.

**Example** If a single pilot transformation reaction is calculated to yield $10^5$ colonies and the target primary library size is $10^6$ clones, ten pilot ($100$-$\mu$l) reaction equivalents, or two of the scaled-up ($500$-$\mu$l) transformation reactions are required. Each of the two $500$-$\mu$l transformation reactions requires $8$ $\mu$l of the ligation reaction. Therefore, a single scaled-up ($30$-$\mu$l) ligation reaction is sufficient in this example. (If greater than three of the $500$-$\mu$l transformation reactions are required, perform multiple $30$-$\mu$l ligation reactions.)

**Library Ligation**

1. To scale up the ligation reaction to a $30$ $\mu$l volume, add the following components:
   - $X$ $\mu$l of resuspended cDNA [Use 3:1 molar ratio of insert to vector (~$145$–$155$ ng)]
   - $3.0$ $\mu$l of $10\times$ ligase buffer
   - $3.0$ $\mu$l of $10$ mM rATP (pH 7.5)
   - $1.5$ $\mu$l of pTRG XR plasmid ($150$ ng; $0.1$ $\mu$g/$\mu$l)
   - $X$ $\mu$l of distilled water for a final volume of $27.0$ $\mu$l

2. Then add $3.0$ $\mu$l of T4 DNA ligase (4 U/$\mu$l).

3. Flick the tube gently to mix, then spin down briefly.

4. Incubate the reaction tubes overnight at 12°C.

**Library Transformation**

1. Pre-warm SOC medium to 42°C.

2. Thaw the $500$-$\mu$l aliquot of XL1-Blue MRF´ Kan Library Pack Competent Cells* on ice.

* Additional competent cells are available separately (Catalog #200138).
3. Immediately after thawing, gently mix the cells by flicking the tube. Transfer 500 μl of cells into a prechilled 14-ml BD Falcon polypropylene round-bottom tube.

**Optional** To test the efficiency of the XL1-Blue MRF’ Kan Library Pack Competent Cells, prepare an additional 100-μl aliquot and perform the pUC18 transformation along with the library transformations. Follow the pUC18 transformation parameters in the pilot protocol above, Pilot Transformation Protocol for Library Pack Competent Cells, regarding heat pulse, SOC medium outgrowth, plating, and incubation.

4. Add 10 μl of the β-ME Library Pack provided with the kit to the 500 μl of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)

5. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes. Do not exceed 10 minutes.

6. Add 8 μl of the scaled-up ligation reaction to the cells and swirl gently.

7. Incubate the tubes on ice for 30 minutes.

8. Heat pulse the tubes in a 42°C water bath for 50 seconds. The duration and temperature of the heat pulse is critical for obtaining the highest efficiencies. Do not exceed 42°C.

9. Incubate the tubes on ice for 2 minutes.

10. Add 4.5 ml of pre-warmed SOC medium to each tube. Pool the transformation reactions by transferring all of the liquid from each 14-ml tube to a single, sterile 50-ml conical tube. Allow the cells to recover by incubating the conical tube at 30°C for 1.5 hours with shaking at 225 rpm.

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

This is the primary library.

11. Plate 2 μl and 5 μl of the pooled transformation reactions on 100-mm LB-tetracycline plates. (Pipet the small volumes into 100 μl pools of SOC medium prior to spreading the cells.) Use these plates to determine the total number of primary transformants, using the calculations described in Determining the Number of Transformants for the Pilot cDNA Library Ligation, above.
12. Plate the rest of the pooled transformants on 150-mm LB-tetracycline plates. Plate approximately 250 μl of culture on each plate and spread the liquid evenly over the surface of the agar. This plating volume ensures that the colonies are not overly crowded on the plate. If using 25-cm × 25-cm plates, 1 ml of culture per plate may be used.

13. Incubate the plates at 30°C for approximately 24 hours. Incubation at 30°C is necessary to minimize the generation of undesired mutants and to ensure good library representation.

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

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 it.

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 is 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 approximately 24 hours at 30°C to determine the size of the amplified library (cfu/ml) using the calculations described in Determining the Number of Transformants for the Pilot cDNA Library Ligation, above.

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.

** 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.
LIBRARY SCREENING

Screening the Library: Overview

Figure 7 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.

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-Gal11P. The positive control is performed in conjunction with the pilot cotransformation using a smaller cotransformation volume.

Overview of the Library Screening Protocol

![Overview of the Library Screening Protocol Diagram]

**FIGURE 7** Overview of the library screening process
### TABLE III
Overview of Plasmid Pairs for Library Screen Experiments

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Purpose</th>
<th>Scale of Cotransformation Reaction</th>
<th>Growth expected on 5 mM 3-AT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBT-LGF2 + pTRG-Gal11&lt;sup&gt;p&lt;/sup&gt; (50 ng each)</td>
<td>Positive control. Gal4 (encoded by LGF2) and Gal11&lt;sup&gt;p&lt;/sup&gt; demonstrate the appearance of a robust interaction</td>
<td>1 × 100 μl</td>
<td>yes</td>
</tr>
<tr>
<td>recombinant pBT + pTRG cDNA library (50 ng each)</td>
<td>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</td>
<td>1 × 100 μl</td>
<td>N/A (not plated)</td>
</tr>
<tr>
<td>recombinant pBT + pTRG cDNA library (200 ng each per transformation reaction)</td>
<td>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</td>
<td>N × 500 μl, where N= number of reactions determined by pilot cotransformation</td>
<td>yes, if interactors are present in the library</td>
</tr>
<tr>
<td>recombinant pBT + pTRG empty vector (200 ng each)</td>
<td>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</td>
<td>1 × 50 μl</td>
<td>no (few or no colonies expected)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Medium</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selective Screening Medium (5 mM 3-AT) plates</td>
<td>Identification and enrichment of putative interactors based on HIS3 transcriptional activation</td>
</tr>
<tr>
<td>Nonselective Screening Medium (no 3-AT) plates</td>
<td>Verification of cotransformation and calculation of cotransformation efficiency</td>
</tr>
<tr>
<td>Dual Selective Screening Medium (5 mM 3-AT + Strep) plates&lt;sup&gt;§&lt;/sup&gt;</td>
<td>Verification of interaction based on transcriptional activation of the aadA gene, conferring streptomycin resistance</td>
</tr>
<tr>
<td>LB-Chloramphenicol/Tetracycline plates&lt;sup&gt;§&lt;/sup&gt;</td>
<td>Maintenance of putative positives</td>
</tr>
<tr>
<td>LB-Tetracycline plates</td>
<td>Isolation of positive pTRG plasmids for retransformation</td>
</tr>
<tr>
<td>LB-Chloramphenicol plates</td>
<td>Verification of chloramphenicol sensitivity (indicating loss of pBT) prior to isolation of pTRG plasmids for retransformation</td>
</tr>
<tr>
<td>SOC liquid medium</td>
<td>Outgrowth following cotransformation</td>
</tr>
<tr>
<td>M9&lt;sup&gt;+&lt;/sup&gt; His-dropout broth</td>
<td>Adaptation to minimal medium following outgrowth and prior to plating</td>
</tr>
</tbody>
</table>

<sup>§</sup> 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 (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. 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-Gal11p target vector.

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

Notes  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 × 10⁷ 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-Gal11p 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-Gal11p 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 + Gal11p 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 10⁴–10⁵ 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-Gal11p 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 $2.5 \times 10^5$ 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. Recollect 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 $2 \times 10^5$.

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-Gal11p, 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 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 lacIq 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<sup>+</sup> cam<sup>+</sup>, 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<sup>+</sup> cam<sup>+</sup> ) overnight at 30°C in LB broth containing 12.5 μg/ml tetracycline.

6. Use standard alkaline lysis protocols<sup>4</sup> 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:

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

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

## cDNA Synthesis

<table>
<thead>
<tr>
<th>Observations</th>
<th>Suggestions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor first-strand synthesis</td>
<td>Ensure the reaction components are mixed and soluble. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible. Minute amounts of SDS or lithium in the RNA will inhibit the first-strand synthesis reaction. Do not use these in the RNA preparations. Multiple phenol–chloroform extractions will sometimes remove the inhibitors. The reaction may not contain sufficient mRNA. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation. The reaction may contain degraded $[\alpha-^{32}P]dNTP$. Protect the $[\alpha-^{32}P]dNTP$ from heat and leave at room temperature for the minimum time required. In addition, ensure that the $[\alpha-^{32}P]dNTP$ is not contaminated with unlabeled dNTPs, causing poor label incorporation into cDNA, and falsely indicating poor cDNA synthesis.</td>
</tr>
<tr>
<td>Poor second-strand synthesis</td>
<td>Confirm the interpretation of the gel results. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of $\alpha-^{32}P$ to the amount of NTP in the first- or second-strand reaction. Normally, the second strand will have only 1/10 to 1/20 the intensity of the first-strand band. Ensure the reaction components are mixed and soluble. Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible. Poor first strand synthesis will affect second-strand synthesis. See the previous suggestions for Poor first-strand synthesis.</td>
</tr>
<tr>
<td>No first-strand synthesis, but good second-strand synthesis</td>
<td>Ensure that there is no DNA contamination within the RNA preparation. Hairpinning This can result when incubation temperatures are higher than 16°C. Add second-strand synthesis reaction components to the first-strand reaction mix on ice and then transfer the reaction mixture directly to 16°C for incubation. After incubation, place the samples on ice immediately. The reaction may not contain sufficient mRNA. Optical density readings may be obscured by contaminating rRNA or DNA and may give a false indication of the amount of mRNA used in the synthesis. Repeat the mRNA preparation. The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure (see Appendix II: Methylmercury Hydroxide Treatment) to reduce the secondary structure present in some sources of RNA. Ensure that the appropriate amount of DNA polymerase is used. Use a calibrated pipet to measure the enzyme. Do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.</td>
</tr>
<tr>
<td>Low counts in the drip column fractions</td>
<td>Verify the quantity of cDNA on the EtBr plate. The number of counts per second per fraction may vary from 0 to 250 cps and yield primary libraries of $&gt;1 \times 10^6$ cfu. Most of the counts remaining in the drip column are from unincorporated $[\alpha-^{32}P]dNTP$.</td>
</tr>
<tr>
<td>Poor ligation</td>
<td>The use of excessive ligase results in an increase in the concentration of glyceral in the reaction and this can be inhibitory for ligation. Ensure that the correct volume of ligase is used and that the pipet tip does not become submerged in the enzyme solution during pipetting; this will result in additional enzyme adhering to the outside of the pipet tip.</td>
</tr>
<tr>
<td>Observation</td>
<td>Suggestion</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>The yield of bait plasmid is low</strong></td>
<td>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&lt;sup&gt;q&lt;/sup&gt; gene and that growth is performed at 30°C.</td>
</tr>
<tr>
<td><strong>During bait plasmid construction, transformation results in few colonies or in small, slow-growing colonies</strong></td>
<td>Use the XL1-Blue MRF&lt;sup&gt;+&lt;/sup&gt; Kan strain provided with the kit for bait plasmid propagation to ensure the presence of the lacI&lt;sup&gt;q&lt;/sup&gt; 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&lt;sup&gt;+&lt;/sup&gt; 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.</td>
</tr>
<tr>
<td><strong>Efficiency of bait and target plasmid cotransformation into the reporter strain is low</strong></td>
<td>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.</td>
</tr>
<tr>
<td><strong>The bait protein is not detected in Western blot analysis</strong></td>
<td>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.</td>
</tr>
<tr>
<td><strong>The positive control plasmids do not give the expected results in the reporter strain</strong></td>
<td>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.</td>
</tr>
<tr>
<td>Unexpectedly large number of colonies on Selective Screening Medium (5 mM 3-AT) plates containing negative control</td>
<td>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.</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Colonies on Selective Screening Medium (5 mM 3-AT) plates grow slowly</td>
<td>Weak interaction pairs generate slow-growing colonies. Ensure that the plates are not too dry.</td>
</tr>
<tr>
<td>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</td>
<td>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.</td>
</tr>
</tbody>
</table>
# 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: Na₂HPO₄: 67.8 g, KH₂PO₄: 30 g, NaCl: 5 g, NH₄Cl: 10 g. Qbiogene M9 salts prepared as directed will yield a 5× solution, and should be doubled for use with this kit.

<table>
<thead>
<tr>
<th>M9 Media Additives (sufficient for 500 ml of M9+ medium)</th>
<th>M9⁺ His-dropout Broth (500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>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.</td>
<td>In a 500-ml flask, combine and mix well: 380 ml of sterile, deionized H₂O 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</td>
</tr>
</tbody>
</table>

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 MgSO₄
0.5 ml of 1 M Thiamine HCl
0.5 ml of 10 mM ZnSO₄
0.5 ml of 100 mM CaCl₂
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 H₂O
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)

<table>
<thead>
<tr>
<th>3-AT Stock Solution, 1 M (10 ml)</th>
<th>Dual Selective Screening Medium (5 mM 3-AT + Strep) (500 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolve 840.8 mg of 3-AT (Sigma, Cat. #A-8056) in 10 ml of DMSO</td>
<td>In a 500-ml flask, combine:</td>
</tr>
<tr>
<td>Aliquot into 4 × 2.5 ml tubes</td>
<td>7.5g Bacto agar</td>
</tr>
<tr>
<td>Store at –20°C for up to one month</td>
<td>380 ml H₂O</td>
</tr>
<tr>
<td></td>
<td>Autoclave at 121°C for 20 minutes</td>
</tr>
<tr>
<td></td>
<td>Cool the agar to 70 °C, and then add 50 ml of 10x M9 salts</td>
</tr>
<tr>
<td></td>
<td>Combine one preparation of M9 Media Additives (67.5 ml, see separate recipe) with the following agents:</td>
</tr>
<tr>
<td></td>
<td>0.5 ml of 25 mg/ml chloramphenicol</td>
</tr>
<tr>
<td></td>
<td>0.5 ml of 12.5 mg/ml tetracycline</td>
</tr>
<tr>
<td></td>
<td>2.5 ml of 1 M 3-AT (dissolved in DMSO)</td>
</tr>
<tr>
<td></td>
<td>When the agar mixture has cooled to 50°C, immediately add the M9 Media Additives/agent mixture to the agar</td>
</tr>
<tr>
<td></td>
<td>Pour into petri dishes (~25 ml/100-mm plate; ~60 ml for 150-mm plate)</td>
</tr>
<tr>
<td></td>
<td>After the agar solidifies, wrap the plates in aluminum foil</td>
</tr>
<tr>
<td></td>
<td>Store plates at 4°C for up to 1 month</td>
</tr>
<tr>
<td>Notes: Use freshly-prepared plates for library screening experiments.</td>
<td><strong>Note:</strong> 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.</td>
</tr>
</tbody>
</table>

*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 H₂O 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 H₂O to a final volume of 1 liter
- Autoclave
- Add 10 ml of filter-sterilized 1 M MgCl₂ and 10 ml of filter-sterilized 1 M MgSO₄ 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 H₂O 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)
### cDNA Library and Bait Plasmid Construction Reagents

<table>
<thead>
<tr>
<th><strong>10× STE Buffer</strong></th>
<th><strong>TE Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M NaCl</td>
<td>10 mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>200 mM Tris-HCl (pH 7.5)</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>100 mM EDTA</td>
<td></td>
</tr>
</tbody>
</table>

**10× Ligase Buffer**
- 500 mM Tris-HCl (pH 7.5)
- 70 mM MgCl₂
- 10 mM dithiothreitol (DTT)

**Note**: *rATP is added separately in the ligation reaction*

<table>
<thead>
<tr>
<th><strong>Column-Loading Dye</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>50% (v/v) glycerol</td>
</tr>
<tr>
<td>10% (v/v) 10× STE buffer</td>
</tr>
<tr>
<td>40% (w/v) saturated BPB*</td>
</tr>
</tbody>
</table>

*To make saturated BPB, add a small amount of bromophenol blue crystals to water and vortex. Centrifuge the sample briefly and look for the presence of an orange pellet. If a pellet is seen, the solution is saturated. If not, add more crystals and repeat the procedure.*

<table>
<thead>
<tr>
<th><strong>2× SDS gel sample buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM Tris-HCl (pH 6.5)</td>
</tr>
<tr>
<td>4% SDS (electrophoresis grade)</td>
</tr>
<tr>
<td>0.2% bromophenol blue</td>
</tr>
<tr>
<td>20% glycerol</td>
</tr>
</tbody>
</table>

**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.*

<table>
<thead>
<tr>
<th><strong>Nondenaturing Acrylamide Gel (5%)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix the following in a vacuum flask</td>
</tr>
<tr>
<td>5 ml of 10× TBE buffer</td>
</tr>
<tr>
<td>8.33 ml of a 29:1 acrylamide–bis-acrylamide solution</td>
</tr>
<tr>
<td>36.67 ml of sterile deionized H₂O</td>
</tr>
<tr>
<td>De-gas this mixture under vacuum for several minutes</td>
</tr>
<tr>
<td>Add the following reagents</td>
</tr>
<tr>
<td>25 μl of TEMED</td>
</tr>
<tr>
<td>250 μl of 10% ammonium persulfate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Alkaline Agarose 2× Loading Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>200 μl of glycerol</td>
</tr>
<tr>
<td>750 μl of water</td>
</tr>
<tr>
<td>46 μl of saturated BPB[^1]</td>
</tr>
<tr>
<td>5 μl of 5 M NaOH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>10× Alkaline Buffer (per 50 ml)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ml of 5.0 M NaOH</td>
</tr>
<tr>
<td>2 ml of 0.5 M EDTA</td>
</tr>
<tr>
<td>45 ml of deionized H₂O</td>
</tr>
</tbody>
</table>
APPENDIX I: RNA PURIFICATION AND QUANTIFICATION

RNA Purification

We highly recommend using the RNA Isolation Kit or the guanidinium thiocyanate–phenol–chloroform extraction method to isolate total RNA. This method is rapid, yet it produces large amounts of high-quality, undegraded RNA.

Although AccuScript reverse transcriptase is not inhibited by ribosomal RNA (rRNA) and transfer RNA (tRNA) contamination, it is advisable to select the poly(A)+ fraction. The amounts of rRNA and tRNA vastly outnumber the mRNA and will decrease the efficiency of the cDNA synthesis. Poly(A)+ RNA is selected on oligo(dT) cellulose columns. Some protocols call for the addition of SDS in the purification steps. Sodium dodecyl sulfate is a powerful enzyme inhibitor and helps prevent degradation of the RNA by RNases, but its presence can also inhibit the enzymes required for cDNA synthesis. If the mRNA intended for use with this kit is suspended in an SDS solution, the RNA must be phenol extracted and ethanol precipitated.

Ribonucleases A and T1 are widely used in almost all molecular biology labs and are nearly indestructible. Ribonucleases are produced by microbes and have also been found in the oils of the skin. Make an effort to use tubes and micropipet tips which have been handled only with gloves. Use freshly autoclaved and baked tips and tubes. Usually these precautions are sufficient, but to be absolutely certain that microcentrifuge tubes and other components intended for use with RNA are not contaminated, the components can be treated with DEPC. Diethylpyrocarbonate is extremely toxic and should be handled with care. Submerge the microcentrifuge tubes in a 0.1% (v/v) DEPC-treated water solution. Leave the beaker of submerged tubes in a fume hood overnight and then dispose of the DEPC-treated water. Autoclave the microcentrifuge tubes for at least 30 minutes. Even though the tubes may still have a sweet DEPC odor, the DEPC is completely inactivated by this procedure. Place the tubes in a drying oven overnight. Equipment which cannot be treated by DEPC can be rinsed in a freshly mixed 3% (v/v) hydrogen peroxide solution, followed by a methanol rinse. Remember, once the RNA is converted to first-strand cDNA, RNases are no longer a concern. Caution should still be exercised in maintaining a sterile, DNase-free environment.

RNA Quantification

RNA can be quantified by measuring the optical density of a dilute RNA solution. The conversion factor for RNA at the wavelength of 260 nm is 40 μg/ml/OD unit as shown in the example below.

Two microliters of an unquantified poly(A)+ sample is added to 498 ml of water (OD\text{260} = 0.1).

\[
0.1 \text{ OD unit} \times \left(\frac{500}{2} \text{ dilution factor}\right) \times 40 \mu g / ml = 1000 \mu g / ml \text{ or } 1 \mu g / \mu l
\]
If a sample has significant rRNA contamination, the actual amount of mRNA available for cDNA conversion will be overestimated by this procedure.

If the amount of mRNA is below 1.5 μg/synthesis reaction, the RT may synthesize unclonable hairpin structures. If the amount of mRNA is above 7 μg the percentage of cDNAs which are full length may decrease. The cDNA Synthesis Kit provided with the BacterioMatch II two-hybrid system has been optimized for 5 μg of mRNA, but successful libraries have been generated using the minimums and maximums described here.

Secondary structure may be a problem with certain RNAs, particularly plant and tumor mRNAs. These samples can be treated with methylmercury hydroxide (see Appendix II: Methylmercury Hydroxide Treatment). Treatment with methylmercury hydroxide requires heating the RNA to 65°C. If the RNA contains even a minute amount of RNase, the RNase activity will increase by several orders of magnitude with the increased temperature and significantly degrade the RNA. Treatment with methylmercury hydroxide is therefore recommended only if the RNA is free of RNases.

**APPENDIX II: METHYLMERCURY HYDROXIDE TREATMENT**

**Warning** Methylmercury hydroxide is an extremely toxic chemical. Wear gloves and use with caution in a fume hood.

1. Resuspend the mRNA in 20 μl of DEPC-treated water.
2. Incubate at 65°C for 5 minutes.
3. Cool to room temperature.
4. Add 2 μl of 100 mM CH₃HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 μl of 700 mM β-mercaptoethanol.
7. Incubate at room temperature for 5 minutes.
APPENDIX III: ALKALINE AGAROSE GELS

Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix.

Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first- and second-strand cDNA.

Note  The test cDNA sample will run as a tight band at 1.8 kb and will show distinctly different intensity between the first and second strands. This is due to the relative ratio of $\alpha^{32P}$ to the amount of NTP in the first- or second-strand reaction. Normally the second strand will be only 1/10 to 1/20 the intensity of the first-strand band.

Alkaline agarose gels differ from conventional gels in the following ways:

1. The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.

2. The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree which makes a clear autoradiograph difficult to interpret.

The following alternative methods help avoid these complications.

The Slide Technique

The easiest and least expensive method is to use a 5 × 7.5-cm glass slide, position a minigel comb over it with high tension clips, and add 10 ml of molten alkaline agarose near the upper center of the slide. The surface tension of the solution will prevent overflow and produce a small, thin gel which can be exposed without further drying. Do not allow the teeth of the comb to overlap the edge of the plate or the surface tension may be broken. To improve the resolution, pat the gel dry with several changes of Whatman 3MM paper after electrophoresis is complete.

To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.
The Vertical Alkaline Agarose Technique

Vertical alkaline agarose gels can be produced using a vertical gel apparatus with 1.5-mm spacers. Since the alkaline agarose gels do not have sufficient friction to remain bound to ordinary glass, a frosted glass plate or gel bond must be used with the vertical apparatus. The combs normally used for acrylamide can be used with this apparatus, if the outside teeth are wrapped in tape to prevent the comb from sinking more than 1.2 cm into the agarose. The 55°C agarose will solidify almost immediately on contact with the cold glass plates, so it is essential to load the mold rapidly with a 60-ml syringe. The comb should already be in the mold, and if it is necessary to reposition the comb, do it immediately after the gel is poured. In order to reduce the possibility of destroying the wells when pulling out the comb, place the solidified gel in a –20°C freezer for 5 minutes immediately prior to removing the comb. When pulling out the comb, it is essential to avoid a vacuum between the teeth and the well. Vacuum can be detected when the well distorts from its normal square shape. When a vacuum occurs, push the comb to separate the glass plates and break the vacuum. After the samples have been run and the glass plates are ready to be opened, slide the unfrosted glass plate off of the alkaline agarose gel instead of prying the plate away from the gel. Pat the gel dry several times using several pieces of Whatman 3MM paper.

Note  To prevent radioactive contamination of film cassettes, seal the wet gels in airtight hybridization bags. Be careful not to trap any air in the hybridization bag which could lift the film away from the gel and cause blurring.
Conventional Submerged Gels

These gels will require drying either by blotting or through the use of a gel dryer.

Caution Even when multiple layers of absorbent paper are placed under the gel, free nucleotides can easily contaminate the drying apparatus. These gels should be poured as thin as possible and should be dried without heat, if time permits, and should never be dried above 40°C.

Protocol

The following formula makes 80 ml of 1% (w/v) alkaline agarose for cDNAs in the 1- to 3-kb size range.

Melt 0.8 g of agarose in 72 ml of water. Allow the agarose to cool to 55°C. During this time, assemble the gel apparatus. Add 8 ml of 10× alkaline buffer§ to the cooled agarose, swirl to mix, and pour the agarose immediately. If buffer is added before the correct temperature is reached, the agarose may not solidify.

Load the sample in an equal volume of alkaline agarose 2× loading buffer§. Run the gel with 1× alkaline buffer at 100 mA and monitor the system for heat. If the apparatus becomes warmer than 37°C, the amperage should be reduced. The migration of the BPB in alkaline agarose is similar to the migration in regular agarose and should be run to at least one-half or three-quarters distance of the gel.

Note The alkali conditions cause the blue dye to fade.

§ See Preparation of Media and Reagents.

72 BacterioMatch II Two-Hybrid System Library Construction Kit
APPENDIX IV: ETHIDIUM BROMIDE PLATE ASSAY—QUANTITATION OF DNA

An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

Preparation of Ethidium Bromide Plates

Note Prepare the EtBr plates under a fume hood.

Prepare 100 ml of a 0.8% (w/v) agarose and Tris-acetate media. Cool the molten agarose to 50°C and then add 10 μl of EtBr stock solution (10 mg/ml). The EtBr stock solution is prepared in dH₂O and is stored in the dark at 4°C. Swirl to mix the EtBr stock solution and pour the solution into 100-mm petri dishes using ~10 ml/plate. Allow the plates to harden and incubate the plates at 37°C to dry, if necessary. These plates may be stored in the dark at 4°C for up to 1 month.

Preparation of Standards

Using a DNA sample of known concentration, make seven serial dilutions in 100 mM EDTA to cover the range from 200 to 10 ng/μl. These standards may be stored at –20°C for 3 months.

Plate Assay for Determination of DNA Concentration

Using a marker, label the petri dish to indicate where the sample and the standards (200, 150, 100, 75, 50, 25, and 10 ng/μl) will be spotted.

Thaw the standards and carefully spot 0.5 μl of each standard onto the surface of a prepared EtBr plate. Be careful not to dig into the surface of the plate. Let capillary action pull the small volume from the pipet tip to the plate surface and do not allow a bubble to form. Change pipet tips between each standard.

After spotting all of the standards, immediately spot 0.5 μl of the cDNA sample onto the plate adjacent to the line of standards. Allow all spots to absorb into the plate for 10–15 minutes at room temperature. Remove the lid and photograph the plate using a UV lightbox. Compare the spotted sample of unknown concentration with the standards.

Do not reuse the plates.

Standards and unknowns must be spotted within 10 minutes of each other.
REFERENCES


ENDNOTES

BD®, Falcon®, Luer Lok®, and PrecisionGlide® are registered trademarks of Becton Dickinson and Company.
Parafilm® is a registered trademark of American Can Company.
Sepharose® is a registered trademark of Pharmacia Biotech AB.
Styrofoam® is a registered trademark of Dow Chemical Co.
Whatman® is a registered trademark of Whatman Ltd.

MSDS INFORMATION

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