pBluescript II XR cDNA Library Construction Kit

INSTRUCTION MANUAL

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Revision A.01

For In Vitro Use Only
200455-12

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pBluescript II XR cDNA Library Construction Kit

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pBluescript II XR cDNA Library Construction Kit

Caution ***DO NOT*** *substitute the reagents in this kit with reagents from another kit. Component substitution may result in lower-efficiency library construction.*

MATERIALS PROVIDED

Materials provided[a]	Quantity	Storage
pBluescript II SK (+) XR vector (20 ng/ µl) [*Eco*R I–*Xho* I digested, treated with calf intestinal alkaline phosphatase (CIAP)][b]	55 µl	–20°C
KAN SR fragment (test insert) (10 ng/ µl, 1.3 kb)	3 µl	–20°C
XL10-Gold ultracompetent cells[c,d] (yellow tubes)	10 × 100 µl	–80°C
pUC18 DNA control plasmid (0.1 ng/µl in TE buffer)	10 µl	–20°C
XL10-Gold β-Mercaptoethanol mix (β-ME)	50 µl	–80°C
First-strand reagents		
AccuScript reverse transcriptase (AccuScript RT)	15 µl	–20°C
RNase Block Ribonuclease Inhibitor (40 U/µl)	200 U	–20°C
First-strand methyl nucleotide mixture (10 mM dATP, dGTP, and dTTP plus 5 mM 5-methyl dCTP)	15 µl	–20°C
First-strand buffer (10×)	75 µl	–20°C
Linker–primer (1.4 µg/µl)	10 µl	–20°C
Test poly(A)$^+$ RNA (0.2 µg/µl)	5 µg	–20°C
Diethylpyrocarbonate (DEPC)-treated water	500 µl	–20°C
Second-strand reagents		
Second-strand buffer (10×)	150 µl	–20°C
Second-strand dNTP mixture (10 mM dATP, dGTP, and dTTP plus 26 mM dCTP)	30 µl	–20°C
Escherichia coli RNase H (1.5 U/µl)	15 U	–20°C
Escherichia coli DNA polymerase I (9.0 U/µl)	500 U	–20°C
Sodium acetate (3 M)	250 µl	–20°C
Blunting reagents		
Blunting dNTP mixture (2.5 mM dATP, dGTP, dTTP, and dCTP)	115 µl	–20°C
Cloned *Pfu* DNA polymerase (2.5 U/µl)	25 U	–20°C
Ligation reagents		
*Eco*R I adapters (0.4 µg/µl)	18 µg	–20°C
Ligase buffer[e] (10×)	250 µl	–20°C
rATP[e] (10 mM)	100 µl	–20°C
T4 DNA ligase[e] (4 U/µl)	140 U	–20°C

(Table continues on next page)

[a] Enough reagents are included to generate five vector-ligated constructs.

[b] After thawing, aliquot and store at –20°C. Do not pass through more than two freeze–thaw cycles. For short-term storage, store at 4°C for 1 month.

[c] Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Tet^R Hte [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* (Tet^R) Amy Cam^R].

[d] On arrival, store the ultracompetent cells immediately at –80°C. Do not place the cells in liquid nitrogen.

[e] These reagents are used more than once in the reaction.

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(table continued from the previous page)

Materials provided	Quantity	Storage
Phosphorylation reagents		
T4 polynucleotide kinase (5 U/µl)	50 U	–20°C
Ligase buffer[e] (10×)	250 µl	–20°C
rATP[e] (10 mM)	100 µl	–20°C
Xho I digestion reagents		
Xho I (40 U/µl)	600 U	–20°C
Xho I buffer supplement	250 µl	–20°C
Column reagents (shipped separately at 4°C)		
Sepharose CL-2B gel filtration medium	10 ml	4°C
Column-loading dye	17.5 µl	4°C
STE buffer (10×)	10 ml	4°C
Connector tubing (1-inch i.d., 3/16-inch o.d., and 1/32-inch wall)	1 × 4 cm	Rm Temp

[e] These reagents are used more than once in the reaction.

STORAGE CONDITIONS

XL10-Gold Ultracompetent Cells: –80°C
XL10-Gold β-Mercaptoethanol Mix: –80°C
pUC18 DNA Control Plasmid: –20°C
pBluescript II XR Vector: –20°C
KAN SR Fragment (Test Insert): –20°C
Other Reagents: –20°C
Sepharose® CL-2B Gel Filtration Medium: 4°C
Column-Loading Dye: 4°C
STE buffer: 4°C

ADDITIONAL MATERIALS REQUIRED

Certain reagents recommended in this instruction manual are potentially dangerous and present the following hazards: chemical (DEPC, phenol, chloroform, methylmercury hydroxide, and sodium hydroxide), radioactive (^{32}P-labeled radioisotope), or physical (high-voltage electrophoresis systems). The researcher is advised to take proper precautions and care with these hazards and to follow the safety recommendations from each respective manufacturer.

Reagents and Solutions

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

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

Ethanol (EtOH) [70%, 80%, and 100% (v/v)]
Sterile distilled water (dH_2O)
[α-^{32}P]Deoxynucleoside triphosphate ([α-^{32}P]dNTP) (800 Ci/mmol) ([α-^{32}P]dATP, [α-^{32}P]dGTP, or [α-^{32}P]dTTP may be used; do not use [α-^{32}P]dCTP)
SeaPrep agarose [FMC, Rockland, Maine (Catalog #50302)]

Equipment

Ribonuclease (RNase)-free microcentrifuge tubes
Disposable plastic 10-ml syringes, sterile (e.g., B-D® 10-cc syringe with a Luer Lok® tip)
Disposable 18 gauge, 1½-inch needles, sterile (e.g., B-D® *PrecisionGlide*® needle)
Disposable plastic 1-ml pipets, negatively graduated and sterile [e.g., Falcon® 7520 1-ml serological pipet (1 ml)]
Pasteur pipet
Portable radiation monitor (Geiger counter)
Water baths (8°, 12°, 16°, 30°, 37°, 42°, 65°, 70°, and 72°C)
Microcentrifuge
Micropipet and micropipet tips
Vacuum evaporator
Incubator (30° and 37°C)
Falcon® 2059 polypropylene tubes

INTRODUCTION

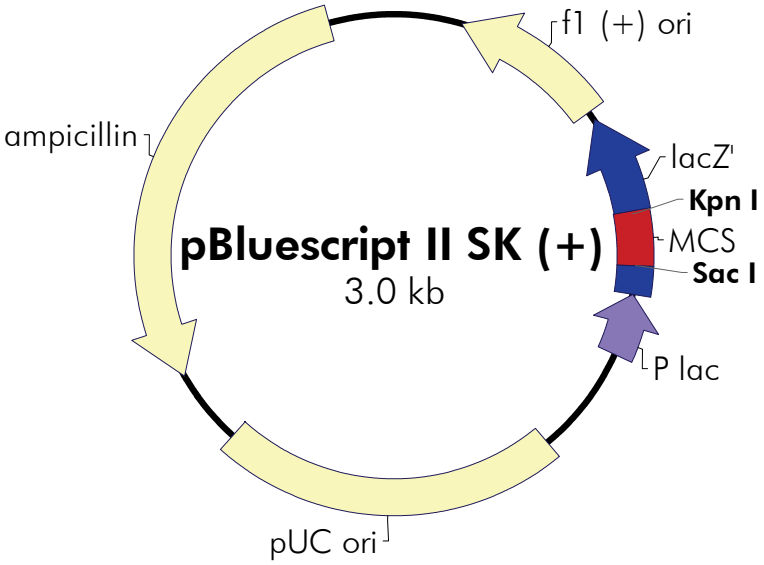
The construction of cDNA libraries is fundamental in discovering new genes and assigning gene function. cDNA libraries constructed directly into plasmid vectors are convenient for plasmid-based functional screening, expressed sequence tagged (EST) sequencing, normalization, and subtraction techniques. The pBluescript II library construction kit is designed for generating directional cDNA libraries in the pBluescript II SK (+) vector. Libraries constructed in the pBluescript II SK (+) vector can be screened with a DNA probe or an antibody probe in *E. coli*. When screening libraries with a functional assay, directional cloning increases the probability of finding clones two-fold when compared to non-directional libraries. With this system, cDNA is synthesized and fractionated by a drip-column method to increase clonal yield and cDNA insert length. cDNA inserts are ligated into the pBluescript II SK (+) vector to generate a plasmid library. The cDNA plasmid library is then transformed into XL10-Gold ultracompetent cells. Libraries generated with XL10-Gold cells increase the probability of obtaining full-length clones.

The pBluescript II SK (+) vector is a high-copy-number pUC-based plasmid with ampicillin resistance and the convenience of blue–white color selection. The vector supplied in this kit is predigested with *Eco*R I and *Xho* I restriction enzymes. The multiple cloning site (MCS) contains unique restriction enzyme recognition sites organized with alternating 5´ and 3´ overhangs to allow serial exonuclease III/mung bean nuclease deletions.[1] T3 and T7 RNA polymerase promoters flank the polylinker for in vitro RNA synthesis. The choice of promoter used to initiate transcription determines which strand of the DNA insert will be transcribed. *Bss*H II sites flank the T3 and T7 promoters. This rare six-base restriction enzyme allows the insert plus the RNA promoters to be excised and used for gene mapping.

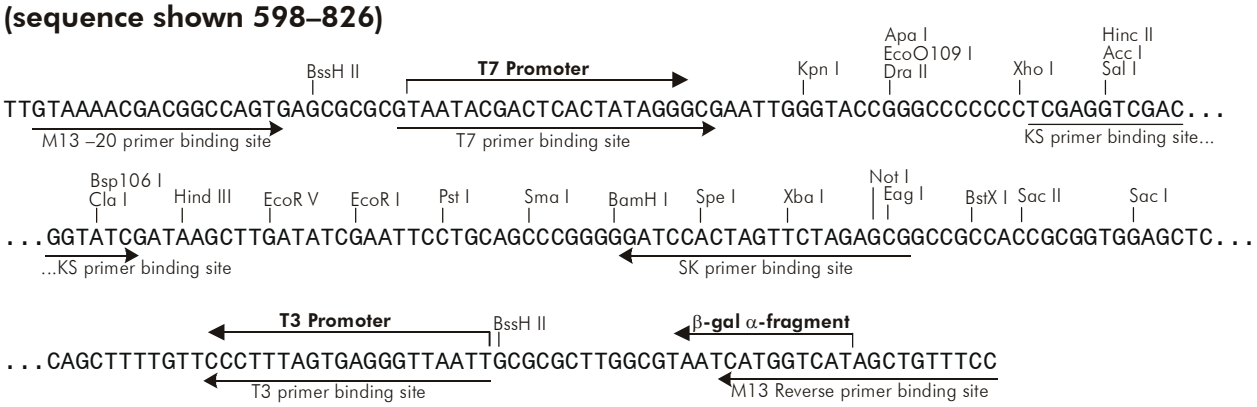
Expression in the pBluescript II SK (+) vector is driven by the *lac* promoter, which is repressed in the presence of the LacI protein and is inducible by isopropyl-β-D-thio-galactopyranoside (IPTG). In bacteria expressing the *lac*ZΔM15 mutation and *lacI*, such as XL10-Gold cells, colonies containing vector without insert will be blue in the presence of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) and IPTG. Ampicillin-resistant colonies containing vector with insert will be white and can express the inserted gene as a fusion protein. The MCS and T7 and T3 RNA polymerase promoter sequences are present in the N-terminal portion of a *lac*Z gene fragment. There are 36 amino acids from the MET sequence to the *Eco*R I site. There are a total of 131 amino acids, but this is interrupted by the large MCS.

The pBluescript II XR vector can be rescued as single-stranded (ss) DNA. pBluescript II phagemids contain a 454-bp filamentous f1 phage intergenic region (M13 related), which includes the 307-bp origin of replication. The (+) orientation of the f1 intergenic region allows the rescue of lacZ' sense ssDNA by a helper phage. This ssDNA can be used for dideoxynucleotide sequencing (Sanger method) or site-directed mutagenesis. Additional sequence and restriction site information for the pBluescript II SK (+) vector is available from the Stratagene website (*http://www.stratagene.com*).

pBluescript II SK (+) Vector Map



pBluescript II SK (+) Multiple Cloning Site Region (sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication	135–441
β-galactosidase α-fragment coding sequence (lacZ′)	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

FIGURE 1 The pBluescript II SK (+) phagemid vector. The complete sequence and list of restriction sites are available at *www.stratagene.com*. The vector sequence is also available from the GenBank® database (accession #X52328). The vector supplied in this kit is predigested with *Eco*R I and *Xho* I restriction enzymes.

cDNA Insert Preparation and Ligation

Background for Preparation of cDNA Libraries

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 a self-replicating plasmid vector. Once the information is available in the form of a cDNA library, individual processed segments of the original genetic information can be isolated and examined with relative ease.

The pBluescript II XR cDNA library construction kit uses a hybrid oligo(dT) linker–primer that contains an *Xho* I restriction site. Messenger RNA is primed in the first-strand synthesis with the linker–primer and 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 reverse transcriptase delivers the highest reverse-transcription accuracy while promoting full length cDNA synthesis. AccuScript reverse transcriptase 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 infect an McrA$^-$ McrB$^-$ strain (e.g., the XL10-Gold cells supplied with the system) when using the pBluescript cDNA library construction kit. After passing the library through XL10-Gold cells, the DNA is no longer hemimethylated and can be grown on McrA$^+$ McrB$^+$ strains (e.g., XL1-Blue strain).

cDNA Synthesis

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

This oligonucleotide was designed with a "GAGA" sequence to protect the *Xho* I restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the pBluescript vector in a sense orientation (*Eco*R I–*Xho* I). The poly(dT) region binds to the 3´ poly(A) region of the mRNA template, and AccuScript RT 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 dCTP becoming incorporated in the second strand. This ensures that the restriction sites in the linker–primer will be susceptible to restriction enzyme digestion. The uneven termini of the double-stranded cDNA are chewed back or filled in with cloned *Pfu* DNA polymerase, and *Eco*R I adapters are ligated to the blunt ends of the cDNA fragments. The adapters have the sequence shown below.

5´-OH-AATTCGGCACGAGG-3´
3´-GCCGTGCTCC-**P-5´**

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

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

The ligated DNA is then transformed into XL10-Gold cells. **Since most *E. coli* strains digest DNA containing 5´-methyl dCTP, it is important to transform into this McrA⁻ McrB⁻ strain to obtain the highest yield.**

Note *An outline of the pBluescript II XR cDNA library construction kit is provided (see Figure 2). If you plan to be away from the project for 1 or 2 days, it is best to schedule the synthesis such that the cDNA remains in the ligation reaction. Even though the majority of ligation is complete in the time recommended by the procedure, the provided ligase is extremely active and will continue to find and ligate available ends. Although most investigators wish to produce their cDNA libraries as rapidly as possible, it is important to remember that extended ligations and overnight precipitations can increase the yield.*

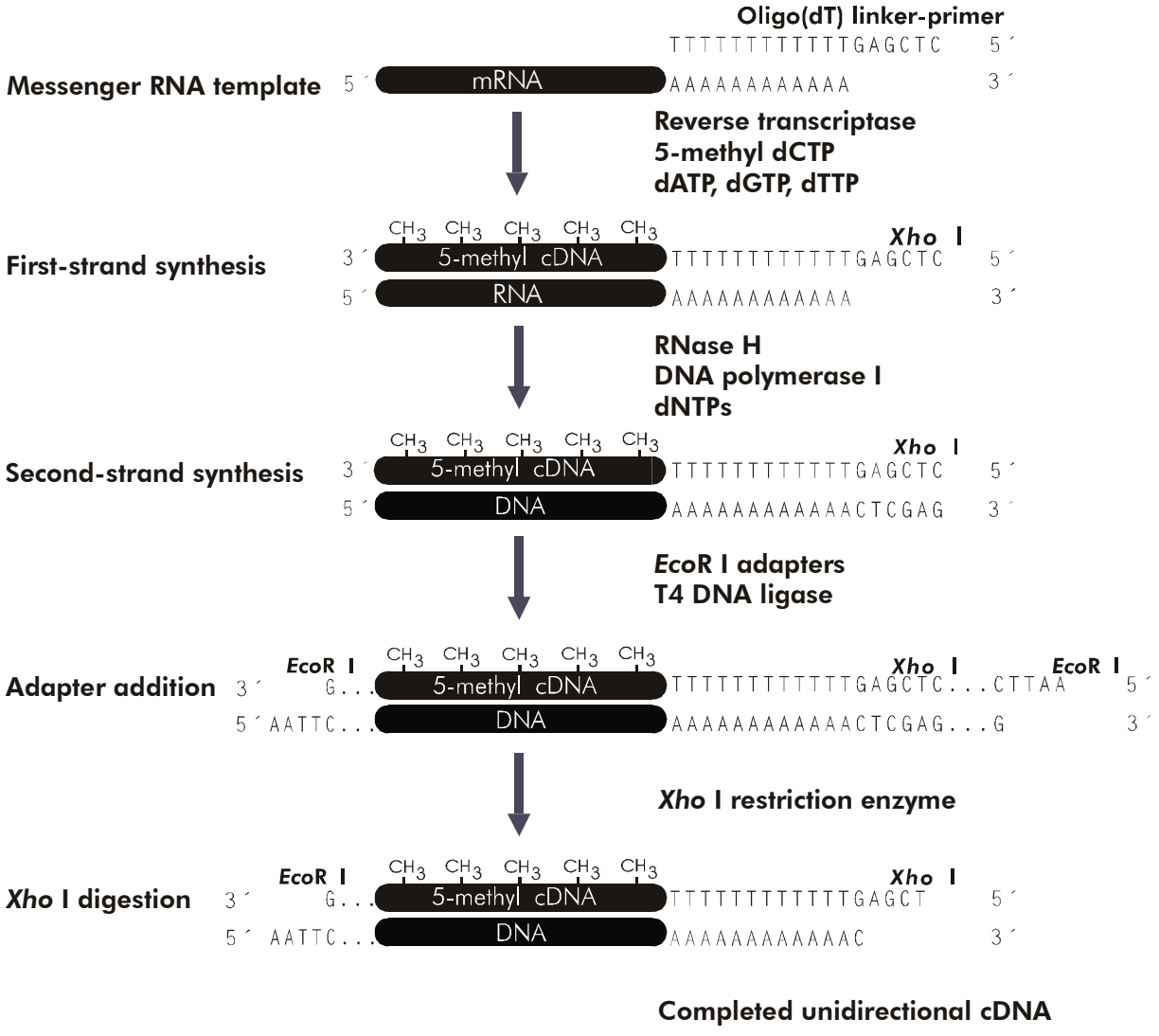


FIGURE 2 cDNA synthesis flow chart.

Generation of cDNA Inserts

Notes ***DO NOT*** *substitute the reagents in this kit with reagents from another kit. Component substitution may result in lower-efficiency library construction.*

The following protocol uses radiolabeled nucleotides in control first- and second-strand synthesis reactions to assess the quality and the size of the cDNA synthesis products. An alternative protocol, using SYBR Green II staining instead of ^{32}P *labeling, is available in a Technical Note on the Stratagene website:* http://www.stratagene.com/lit_items/cDNA_Synthesis_Kit-Using_SYBR®.pdf.

The following protocol is optimized for 5 µg of poly(A)$^+$ RNA.

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 II: RNA Purification and Quantitation*). The Stratagene RNA Isolation Kit (Stratagene Catalog #200345) uses the guanidinium thiocyanate–phenol–chloroform extraction method,[2] which quickly produces large amounts of undegraded RNA. To isolate mRNA, we offer the Absolutely mRNA Purification Kit (Stratagene Catalog #400806).

- If poor first-strand synthesis suggests problems associated with secondary structure (e.g., hairpinning), treatment with methylmercury hydroxide (CH_3HgOH) is recommended to relax secondary structure (see *Appendix III: 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 pBluescript II XR 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. Preheat two water baths to 42° and 72°C, respectively.

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

   Note *AccuScript RT 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-^{32}P]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-^{32}P]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 *Appendix III: Alkaline Agarose Gels*). Both first- and second-strand reactions should be run on the same gel after the second-strand reaction has been blunted and resuspended in the *Eco*R I adapters (see step 17 in *Blunting the cDNA Termini*).

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 dH_2O (DEPC-treated water is not required)
   2 µl of [α-^{32}P]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 mix 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. Gently mix 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 Stratagene RNA Isolation Kit because this phenol is 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 by gentle pipetting in 9 µl of *Eco*R 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, fewer 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 IV: Alkaline Agarose Gels*).

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

Ligating the *Eco*R I Adapters

1. Add the following components to the tube containing the blunted cDNA and the *Eco*R 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 *Eco*R 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*).

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 *Equipment* section in *Additional Materials Required*).

Assembling the Drip Column

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 3 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 the connecting 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 connecting tubing (~1/8-inch i.d.) snugly connects most disposable 1-ml pipets and the ends of all B-D® 10-cc syringes with the Luer Lok® tips.*

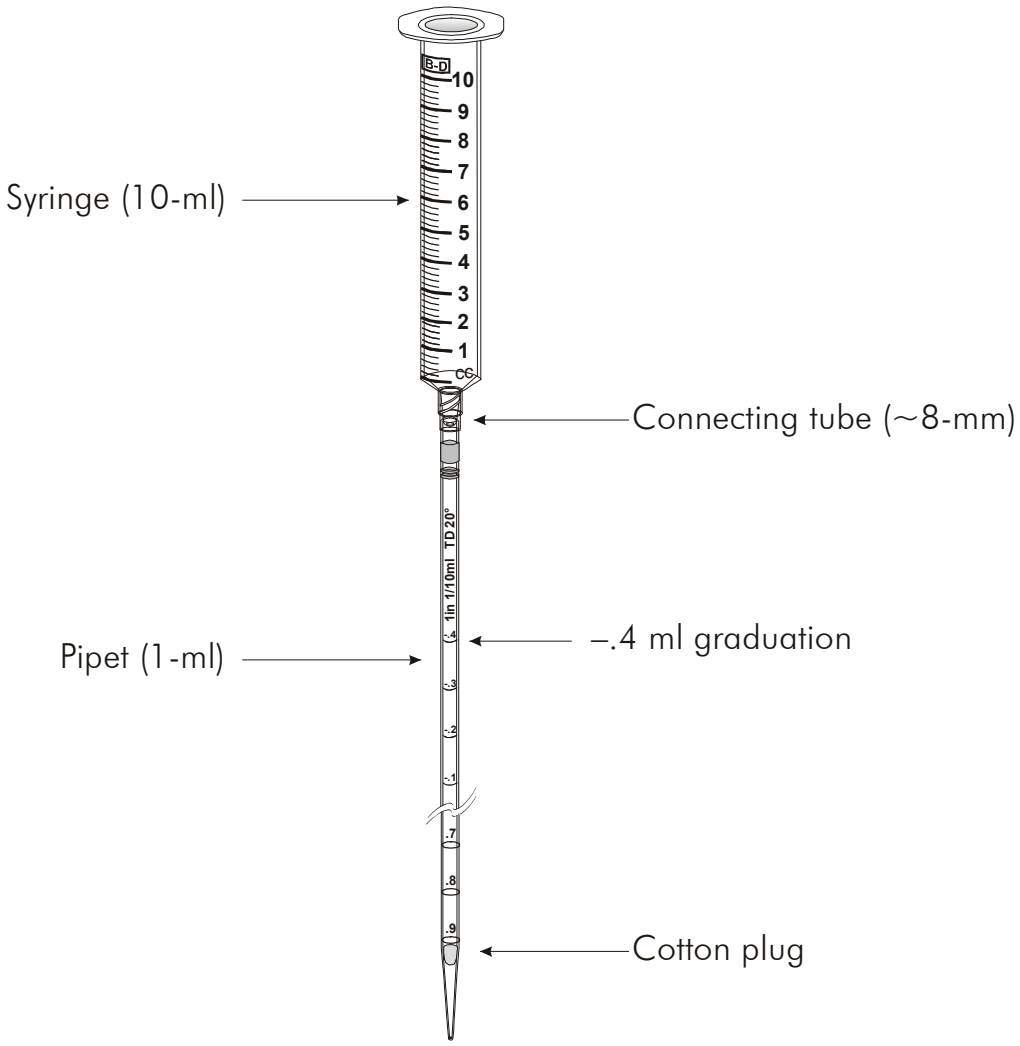


FIGURE 3 Assembly of the drip columns.

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.

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.

    Note *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-free 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 should be electrophoresed on a 5% nondenaturing acrylamide gel and exposed to x-ray film overnight** 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 80% (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 5.5 µl of sterile water. Mix by pipetting up and down.

Quantitate the cDNA before proceeding (see *Appendix IV: Ethidium Bromide Plate Assay—Quantitation of DNA*). Best results are usually obtained by ligating 10 ng of cDNA/20 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.

Ligating cDNA into the Plasmid Vector

We recommend performing a pilot ligation and transformation for each sample to establish the ligation efficiency of the test insert and cDNA with the pBluescript vector. The ligation and transformation reactions may then be scaled up and optimized to achieve a primary library consisting of a target number of transformants.

1. Set up a control ligation to ligate the test insert into the pBluescript vector as follows:

   1.0 µl of the pBluescript vector (20 ng)
   1.0 µl of KAN SR test insert (10 ng)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   1.5 µl of water

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

2. To prepare the sample ligation, add the following components:

   X µl of resuspended cDNA (~10 ng)
   0.5 µl of 10× ligase buffer
   0.5 µl of 10 mM rATP (pH 7.5)
   1.0 µl of the pBluescript vector (20 ng/µl)
   X µl of water for a final volume of 4.5 µl

   Then add

   0.5 µl of T4 DNA ligase (4 U/µl)

   Note *In all ligations, the final glycerol content should be less than 5% (v/v).* ***Do not exceed 5% (v/v) glycerol!***

3. Incubate the reaction tubes overnight at 12°C or for up to 2 days at 4°C.

4. After ligation is complete, transform XL10-Gold ultracompetent cells with the ligation reactions. XL10-Gold is a McrA$^-$ and McrB$^-$ strain and does not restrict methylated DNA. **Use of any other cell line may result in dramatically reduced transformation efficiency**.

Transformation Guidelines

Storage Conditions

Ultracompetent 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. Ultracompetent cells should be placed at –80°C directly from the dry ice shipping container.

Aliquoting Cells

When aliquoting, keep ultracompetent cells on ice at all times. It is essential that the Falcon® 2059 polypropylene 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 at least 100 µl of ultracompetent cells/transformation. Using a smaller volume will result in lower efficiencies.

Use of Falcon® 2059 Polypropylene Tubes

It is important that Falcon® 2059 polypropylene tubes are used for the transformation protocol, since other tubes may be degraded by the β-mercaptoethanol used in step 3 of the *Transformation Protocol*. In addition, the incubation period during the heat-pulse step is critical and has been optimized specifically for the thickness and shape of the Falcon 2059 polypropylene tubes.

Use of β-Mercaptoethanol

β-Mercaptoethanol (β-ME) has been shown to increase transformation efficiency. The XL10-Gold β-Mercaptoethanol mix provided in this kit is diluted and ready to use. For optimum efficiency, use 4 µl of the β-ME mix. (Using an alternative source of β-ME may reduce transformation efficiency.)

Length and Temperature of the Heat Pulse

There is a defined window of highest efficiency resulting from the heat pulse during transformation. Optimal efficiencies are observed when cells are heat pulsed for 30 seconds. Heat pulsing for at least 30 seconds is recommended to allow for slight variations in the length of incubation. Efficiencies decrease when incubating for <30 seconds or for >40 seconds. Do not exceed 42°C.

Transformation Protocol

1. Thaw the XL10-Gold ultracompetent cells on ice.

2. Gently mix the cells by hand. Aliquot 100 µl of the cells into a prechilled 15-ml Falcon 2059 polypropylene tube.

3. Add 4 µl of the XL10-Gold β-ME mix provided with the kit to the 100 µl of bacteria. (Using an alternative source of β-ME may reduce transformation efficiency.)

4. Swirl the contents of the tube gently. Incubate the cells on ice for 10 minutes, swirling gently every 2 minutes.

5. Add the entire ligation reaction (from step 3 of *Ligating cDNA into the Plasmid Vector*) to the cells and swirl gently.

   Note *As a transformation control, add 1 µl of pUC18 plasmid (diluted 1:10 in high quality water) to another 100-µl aliquot of the cells and swirl gently.*

6. Incubate the tubes on ice for 30 minutes.

7. Preheat NZY$^+$ broth[§] in a 42°C water bath for use in step 10.

8. Heat pulse the tubes in a 42°C water bath for 30 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 preheated (42°C) NZY$^+$ broth to each tube and incubate the tubes at 37°C for 1 hour with shaking at 225–250 rpm.

Notes *For plating quantities of pilot ligations see* Determining the Number of Transformants.

The cells may be concentrated by centrifuging at 1000 rpm for 10 minutes if desired. Resuspend the pellet in 200 µl of NZY$^+$ broth and plate.

When transforming the control pUC18 DNA, plate 5 µl of the transformation mixture in a 200-µl pool of NZY$^+$ broth on LB–ampicillin agar plates.[§]

250 colonies may be expected from each 5-µl control transformation to yield ≥5×10^{9}cfu/µg.

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

Determining the Number of Transformants

Plating

1. Plate 1 µl and 10 µl of each 1-ml pilot transformation onto LB-ampicillin agar plates.
2. Plate 1 µl and 10 µl of the kanamycin-resistant test insert transformation on LB-kanamycin agar plates.

Greater than 20 colonies should be observed from the 1-µl plating of the test insert transformation.

Count the number of ampicillin-resistant colonies on the 1-µl plate (from step 1 above) and multiply that number by 1000.

Example *200 colonies/µl × 1000 µl = 2.0 × 10^5 total cfu.*

Count the number of ampicillin-resistant colonies on the 10-µl plate (from step 1 above) and multiply that number by 100.

Example *2000 colonies/10 µl × 1000 µl = 2.0 × 10^5 total cfu.*

Scaling Up the Ligations and Transformations

1. Perform individual ligations according to the ligation protocol to reach the target primary library size desired. The number of ligations necessary may vary with each insert and should be based upon efficiencies realized with each pilot reaction.
2. Transform 100 µl of XL10-Gold ultracompetent cells individually with each ligation reaction (see *Transformation Protocol*).
3. Pool the individual transformation reactions after the one hour incubation (step 10 of *Transformation Protocol*).

   Note *This pool is the primary library. Store the primary library at 4°C and amplify as soon as possible (*see Amplifying the pBluescript cDNA Library*).*
4. Plate 1 µl and 10 µl of the pooled transformations onto selection plates to determine the total number of primary transformants.

Verifying the Insert Percentage and Size

Individual colonies can be examined to determine the percentage of vectors with inserts and the average insert size by either PCR directly from the colony with T3 and T7 primers or by restriction analysis of individually prepared plasmid DNA.

The XL10-Gold strain allows blue-white selection for the pBluescript II vector due to *lac*ZΔM15 complementation of the F´ episome. The color selection may be seen when plating on LB plates containing 100 µg/ml of ampicillin, 80 µg/ml of fresh X-gal, and 20 mM IPTG. Alternatively, plates for color selection can be made by spreading 100 µl of 40 mM IPTG and 100 µl of 2% X-gal on LB-ampicillin plates 30 minutes prior to plating transformants. X-gal should be prepared in dimethyl formamide and IPTG prepared in sterile, distilled H_2O (store stock solutions at –20°C until use.) Colonies containing vectors without inserts will be blue after incubation for 12–18 hours at 37°C. Colonies with vectors containing inserts will remain white. Further enhancement of the blue color may be obtained by placing plates at 4°C for 2 hours following overnight growth at 37°C.

Amplifying the pBluescript cDNA Library

The primary library may now be plated and screened; however, amplification of the library is desirable to produce a large and stable quantity of the library. Do not perform more than one round of amplification, as slower-growing clones may be significantly underrepresented.

We recommend amplifying plasmid libraries in 500-ml bottles of 2× LB agarose[§] using the semi-solid amplification method.[3,4] The libraries are amplified in suspension which allows for three-dimensional, uniform colony growth. This reduces the potential for under-representation of particular clones due to the overgrowth of some colonies during the expansion process that accompanies direct plating methods.

Note *Each 500-ml bottle of 2× LB agarose can accommodate ~5 × 10^5 primary cfu. To amplify a library of 1 × 10^6 primary cfu, two bottles are necessary.*

1. On a heated stir plate, using a large stir bar, combine 1.35 g of SeaPrep agarose with 450 ml of 2× LB[§] in each 500-ml autoclavable bottle. Stir until the agarose is in solution.
2. Autoclave the bottles and stir bars for 30 minutes.
3. Allow the bottles to cool to 37°C in a water bath (~1 hour).
4. Transfer the bottles to a stir plate and add 100 µg/ml of ampicillin.
5. Add up to 5 × 10^5 cfu/bottle of primary library and stir for several minutes.

   Note *The bottles must be handled very carefully at this stage. Avoid swirling or bumping the bottles as this may cause the microcolonies to fall out of suspension. The bottles must be incubated without disturbance or representation of the amplified library may be compromised.*

6. Tighten the bottle caps and incubate the bottles for 1 hour in an ice water bath (0°C). The water level in the ice bath should be even with the media level in the bottle.
7. Carefully remove the bottles from the ice bath and gently dry the bottles. Loosen the bottle caps and incubate for 40–45 hours at 30°C. (Incubation at 30°C reduces under-representation of slower growing clones.)
8. Pour the contents of the bottles into sterile 250-ml centrifuge bottles and spin at 10,000 × *g* for 20 minutes at **room temperature**. (Equilibrate the rotor to room temperature several hours prior to centrifugation. Using rotors stored at 4°C will cause the agar to solidify.)

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

9. Remove the semi-solid agarose supernatant and resuspend the pellets in 25 ml of 2× LB–glycerol (12.5%)[§] per 250-ml centrifuge bottle. Remove 100 µl for estimation of library titer and further characterization. Pipet the remainder into 1-ml aliquots. Store at –80°C.

10. Perform 6 serial dilutions with 100 µl of the amplified library diluted into 900 µl of LB medium. Plate 10 µl of the 10^{-5} and 10^{-6} dilutions onto selection plates. Amplification of a primary library containing 1×10^6 total transformants should result in a stable library of at least 1×10^9 total transformants.

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

APPENDIX I: RNA PURIFICATION AND QUANTIFICATION

RNA Purification

The Stratagene RNA Isolation Kit, using the guanidinium thiocyanate–phenol–chloroform extraction method,[2] is strongly recommended for total RNA isolation. This method is rapid, yet it produces large amounts of high-quality, undegraded RNA.

Although AccuScript RT 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.[5] 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 a poly(A)$^+$ RNA sample is added to 498 ml of water (e.g., $OD_{260} = 0.1$). Therefore,

$$0.1\,\text{OD unit} \times \left(\frac{500}{2}\ \text{dilution factor}\right) \times 40\,\mu\text{g of RNA/ml} = 1000\,\mu\text{g of RNA/ml or } 1\,\mu\text{g of RNA/}\mu\text{l}$$

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 poly(A)$^+$ RNA is below 1.5 µg/synthesis reaction, the RT may synthesize unclonable hairpin structures. If the amount of poly(A)$^+$ RNA is above 7 µg, the percentage of cDNAs that are full length may decrease. The pBluescript cDNA library construction kit is optimized for 5 µg of poly(A)$^+$ RNA, 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*).[6] 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 CH_3HgOH.
5. Incubate at room temperature for 1 minute.
6. Add 4 µl of 700 mM β-mercaptoethanol (see *Preparation of Media and Reagents*).
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 α-^{32}P dNTP to the amount of dNTP in the first- or second-strand reaction. Normally the second-strand band will be 1/10 to 1/20 the intensity of the first-strand band.*

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

- The absence of any buffering capacity in the "buffer" reduces the speed at which the sample can be run.
- The thickness of the typical undried agarose gel causes the radioactive emissions to be scattered to a degree that 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 that 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 condition causes the blue dye to fade.*

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

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 dH_2O 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.

TROUBLESHOOTING

Observations	Suggestions
Poor first-strand synthesis	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	Ensure that AccuScript RT activity is not inhibited. 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.
	Ensure that quantity of mRNA is sufficient. 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.
	Ensure that [α-^{32}P]dNTP is not degraded or contaminated. If [α-^{32}P]dNTP is heated, is left at room temperature too long, or is contaminated, it may not incorporate into cDNA, giving a false indication that synthesis is not occurring.
Poor second-strand synthesis	Ensure that the gel results are interpreted correctly. Control RNA will show distinctly different intensity between the first and second strand. This is due to the relative amounts of α-^{32}P to the amount of NTP in the first- or second-strand reaction. Normally, the second strand band will have only 1/10 to 1/20 the intensity of the first-strand band.
	Always mix and spin the enzymes in a microcentrifuge immediately before use. Vortex the buffers vigorously until no precipitate is visible.
	See the previous suggestions for *Poor first-strand synthesis*.
No first-strand synthesis, but good second-strand synthesis	Ensure that there is no DNA contamination within the RNA preparation.
Hairpinning	Ensure that the incubation temperatures are not 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.
	Ensure that quantity of mRNA is sufficient. 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.
	Some sources of RNA may have secondary structure (e.g., tumors, some plants, etc.). The RNA may have to be treated with methylmercury hydroxide to relax the secondary structure (see *Appendix II: Treating with Methylmercury Hydroxide*).
	Ensure that the quantity of DNA polymerase is not too high. 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.
Low counts in the drip column fractions	The number of counts per second per fraction may vary from 0 to 250 cps and yield primary libraries of $>1 \times 10^6$ pfu. Most of the counts remaining in the drip column are from unincorporated [α-^{32}P]dNTP. Verify the quantity of cDNA on the EtBr plate.
Poor ligation	Ensure that the glycerol concentration is not excessive. Do not use excess ligase. Also, do not submerge the pipet tip completely in the enzyme solution as additional enzyme will adhere to the outside of the pipet tip.

PREPARATION OF MEDIA AND REAGENTS

10× Alkaline Buffer (per 50 ml)

3 ml of 5.0 M NaOH
2 ml of 0.5 M EDTA
45 ml of water

10× STE Buffer

1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

LB Agar (per Liter)

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

LB Broth (per Liter)

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

2× LB Agarose

450 ml of 2× LB
1.35 g of SeaPrep agarose

Mix on a heated stir plate using a large stir bar until the agarose is in solution

2× LB–Glycerol [12.5% (v/v)]

175 ml of 2× LB broth
25 ml of glycerol [100% (v/v)]

Filter sterilize and store for up to 2 months at room temperature

2× LB Broth (per Liter)

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

LB–Ampicillin Agar (per Liter)

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

LB–Ampicillin Broth (per Liter)

1 liter of LB broth, autoclaved
Cool to 55°C
Add 10 ml of 10-mg/ml filter-sterilized ampicillin

LB–Kanamycin Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

10× MOPS Buffer

200 mM 3-[*N*-morpholino]propane-sulfonic acid (MOPS)
50 mM sodium acetate
10 mM EDTA
Adjust to a final pH of 6.5–7.0 with NaOH
Do not autoclave

Alkaline Agarose 2× Loading Buffer

200 µl of glycerol
750 µl of water
46 µl of saturated BPB[‖]
5 µl of 5 M NaOH

10× Ligase Buffer

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

Note *rATP is added separately in the ligation reaction*

NZY⁺ Broth (per Liter)

10 g of NZ amine (casein hydrolysate)
5 g of yeast extract
5 g of NaCl
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.5 using NaOH
Autoclave
Add the following filer-sterilized supplements prior to use:

12.5 ml of 1 M $MgCl_2$
12.5 ml of 1 M $MgSO_4$
20 ml of 20% (w/v) glucose (or 10 ml of 2 M glucose)

Column-Loading Dye

50% (v/v) glycerol
10% (v/v) 10× STE buffer
40% (w/v) saturated BPB[‖]

TE Buffer

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

Formaldehyde Gel Loading Buffer

720 µl of formamide
160 µl of 10× MOPS buffer
260 µl of 37% formaldehyde
100 µl of sterile water
100 µl of EtBr (10 mg/ml)
80 µl of sterile glycerol
80 µl of saturated BPB[‖] in sterile water

Note *The loading buffer is not stable and should be made fresh on the day of use*

Nondenaturing Acrylamide Gel (5%)

Mix the following in a vacuum flask

5 ml of 10× TBE buffer
8.33 ml of a 29:1 acrylamide–bis-acrylamide solution
36.67 ml of sterile deionized H_2O

De-gas this mixture under vacuum for several minutes
Add the following reagents

25 µl of TEMED
250 µl of 10% ammonium persulfate

[‖] 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.

REFERENCES

1. Short, J. M. and Sorge, J. A. (1992) *Methods Enzymol* 216:495–508.
2. Chomczynski, P. and Sacchi, N. (1987) *Anal Biochem* 162(1):156–9.
3. Kreiegler, M. (1990). In *Gene Transfer and Expression: A Laboratory Manual*, pp. 131–132. Stockton Press, New York.
4. Hanahan, D., Jessee, J. and Bloom, F. R. (1991) *Methods Enzymol* 204:63–113.
5. Krug, M. S. and Berger, S. L. (1987) *Methods Enzymol* 152:316–25.
6. Payvar, F. and Schimke, R. T. (1979) *J Biol Chem* 254(16):7636–42.

ENDNOTES

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.