GIBCO BRL®

Instruction Manual

GeneTrapper® cDNA Positive Selection System

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CAT. NO. 10356-020

LIFE TECHNOLOGIES™

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1.1 Important Information

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2 Overview

2.1 Hybridization Methods for cDNA Identification

The most popular screening method to identify cDNA clones is *in situ* hybridization. In this well-characterized technique (1), nucleic acid probes are hybridized to dense populations of bacteria or phage that have been grown on plates, transferred to membranes, and lysed to release the DNA. Because of the high density at which clones are plated, the isolation of pure clones requires secondary and tertiary screenings at decreasing clone densities. Generally, this process can only accommodate up to 10^6 clones and may take weeks or months to yield the desired clone.

2.2 Summary of the GeneTrapper® cDNA Positive Selection System

The GeneTrapper (patent pending) cDNA Positive Selection System facilitates the rapid (1 to 2 days) isolation of cDNA clones from DNA prepared from a cDNA library (representing 10^{12} DNA molecules). **Prior detection of the target cDNA in the library by PCR analysis will increase the likelihood of a successful result with the GeneTrapper System.** In this system (figure 1), an oligonucleotide, complementary to a segment of the target cDNA, is biotinylated at the 3´-end with biotin-14-dCTP using terminal deoxynucleotidyl transferase (TdT). Simultaneously, a complex population of ds phagemid DNA containing cDNA inserts (10^6 to 10^7 individual members) is converted to ssDNA using Gene II (phage F1 endonuclease) and (*E. coli*) Exonuclease III (Exo III). Hybrids between the biotinylated oligonucleotide and ssDNA are formed in solution and then captured on streptavidin-coated paramagnetic beads. A magnet is used to retrieve the paramagnetic beads from the solution, leaving nonhybridized ssDNA behind. Subsequently, the captured ssDNA target is released from the biotinylated oligonucleotide that remains attached to the paramagnetic beads.

After release, the desired cDNA clone is further enriched by using a nonbiotinylated target oligonucleotide to specifically prime conversion of the recovered ssDNA target to dsDNA. Following transformation and plating, typically, 20% to 100% of the colonies represent the cDNA clone of interest.

The GeneTrapper System provides several distinct advantages over PCR. Cloned, full-length cDNAs can be easily isolated by using the GeneTrapper System and one specific oligonucleotide of $\geqslant$16 nucleotides that is designed to anneal to the 5´-coding region. To obtain the same result from PCR would require sequence information at the 5´- and 3´-regions of the desired cDNA (two oligonucleotides) or a more difficult combined 3´ – 5´ procedure followed by a cloning procedure.

2.3 Oligonucleotide Design and Purification

For efficient cDNA capture and repair, certain principles of oligonucleotide design should be followed. Ideally, oligonucleotides should contain 25% A, 25% C, 25% G and 25% T, however the G+C content can vary from 50% to 60%. Oligonucleotides 20-24 nucleotides in length give optimal results (1). Higher percentages of G+C will increase the number of background colonies, and shorter oligonucleotide lengths will decrease the yield of desired cDNA clones.

Oligonucleotide sequence information determines the specificity of the clones obtained, since it dictates the specificity of the capture reaction. For the best results, oligonucleotides should be designed to the amino acid-encoding regions of

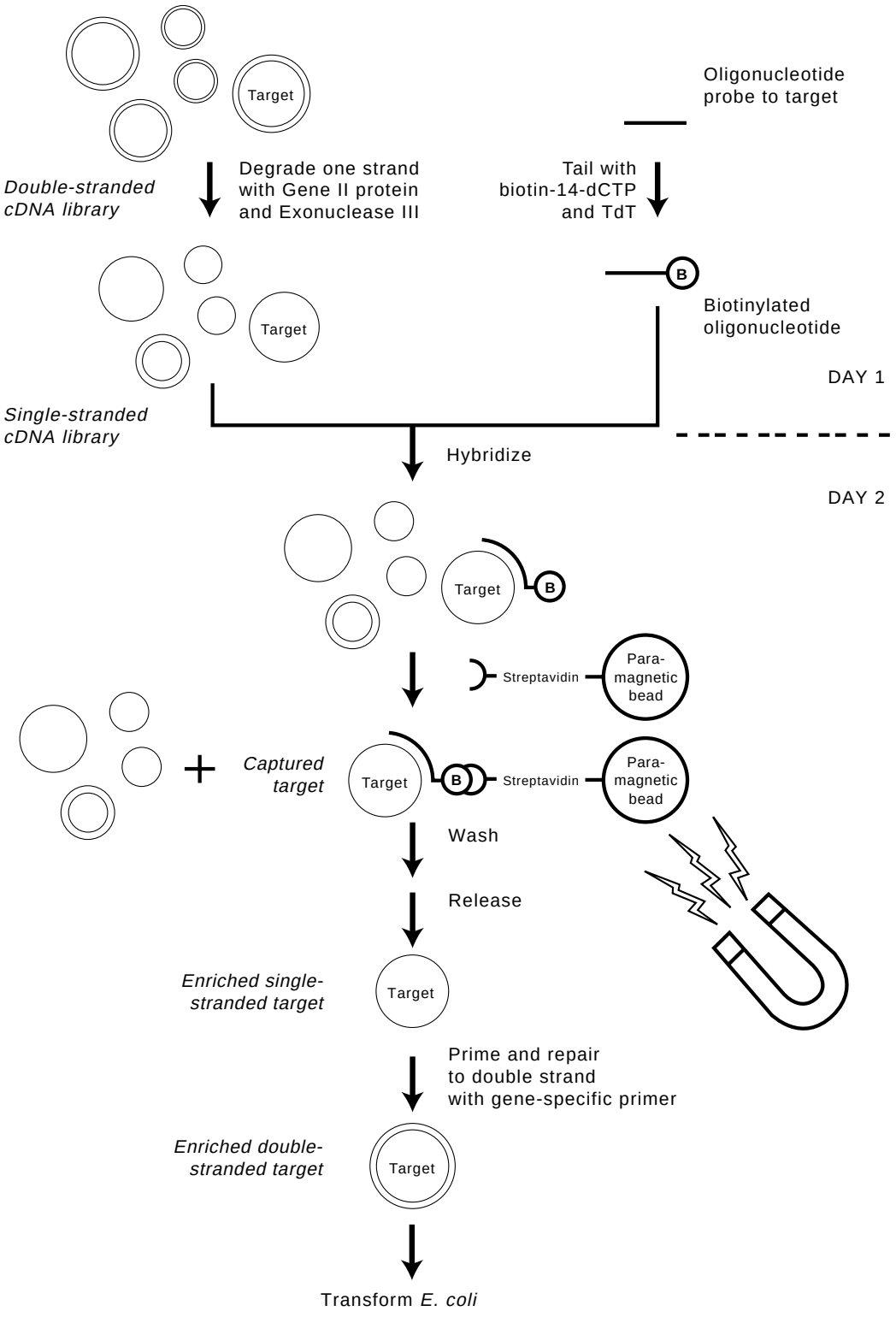

Figure 1. Overview of the GeneTrapper cDNA Positive Selection System procedure.

Overview

the cDNA. Avoid vector-like and repetitive sequences since large backgrounds will result. Use PCR primer-picking software to generate a list of candidate oligonucleotides, so that the likelihood of primer dimer and 3'-hairpin structures is minimized. Primer dimer formation will effectively reduce the concentration of oligonucleotide available for hybridization to target, and hairpins at 3'-ends will reduce or block biotinylation by TdT. Oligonucleotides suitable for use with the GENETRAPPER system can be selected from primer picking software candidates by additionally avoiding the following sequences: 1) the presence of more than one homopolymeric region of 3 bases (e.g. AAA, CCC, GGG, TTT), 2) the presence of any homopolymeric regions of 4 or more bases (e.g. AAAA, CCCC, GGGG, TTTT) and 3) the presence of more than one di-, tri- or tetranucleotide motif (e.g. CACACA..., GACGACGAC..., GATCGATC....). As with vector or repetitive sequences, the presence of the above sequences will greatly increase the background.

Care must be taken to select an oligonucleotide with the correct polarity. When using GIBCO BRL® SUPERSCRIPT™ cDNA Libraries, the oligonucleotide should be of the sense or transcribed strand orientation, since the single-stranded target DNA is the antisense or untranscribed strand orientation. The orientation of the plasmid vector f1 ori in a specific vector will determine the polarity of the ss phagemid DNA. If the target gene is cloned into multiple cloning sites (MCS) in the same orientation as the *lac*Z gene, sense strand (*i.e.*, the strand containing the ATG initiation codon for protein synthesis) sequence oligonucleotides will need to be used to capture ssDNA produced from pSPORT 1, pCMV•SPORT vectors (cDNA cloned into the *Not* I-*Sal* I region), pZL1 (λZIPLOX®), and Bluescript SK (+) cDNA libraries. Anti-sense (or non-ATG strand) oligonucleotides are necessary to capture ssDNA produced from pSPORT 2, Bluescript SK (-), and λZAP® II cDNA libraries. (For mass excision from λZAP cDNA libraries, see Section 5.1). These rules will hold for ssDNA produced by Gene II-Exo III digestion *in vitro*. If ssDNA is generated by *in vivo* phagemid production, oligonucleotides of the reverse polarity must be designed *(i.e.,* anti-sense oligonucleotides for pSPORT1, pCMV•SPORT vectors, etc.).

Oligonucleotides designed to the sequence information as close to the 5´-end as possible will tend to enrich for full-length cDNA clones. On the other hand, oligonucleotides containing sequence proximal to the 3´-end of the original mRNA will select partial, full length, and all other related cDNA clones (*i.e.,* spliced transcripts).

Many genes (*e.g.,* actin) are conserved among species and among members of gene families. Degenerate oligonucleotides designed to these conserved amino acid sequences can be used to isolate genes using the GENETRAPPER System. For example, degenerate oligonucleotides (*i.e.,* a mixture of D256 and D1024 sequences) fashioned from a portion of the CAT amino acid sequence enriched the control pSPORT 1-CAT DNA 1,000- to 5,000-fold (table 1).

To distinguish the clones of interest, screen the colonies by colony hybridization (see Section 3.15).

Table 1. Enrichment of the pSPORT 1-CAT cDNA from a 1:50,000 CAT:HeLa cDNA library with degenerate oligonucleotides.

Oligonucleotide[a]	Ap^r colonies/reaction	Cm^r colonies/reaction	% CAT clones
942	5.5×10^3	4.3×10^3	78.2
1051-D256	4.0×10^4	3.2×10^3	8
1051-D1024	5.2×10^4	1.2×10^3	2.3

[a] Oligonucleotide 942 was designed to the correct CAT peptide sequence and 1051-D256 (degenerate 256) and 1051-D1024 (degenerate 1024) are degenerate oligonucleotides designed to another CAT peptide sequence.

942: 5´–GACCGTTCAG CTGGATATTA CGGCC–3´
1051-D256: 5´–GTNTG(T/C)GA (T/C)GGNTT(T/C)CA (T/C)GTCGG–3´
1051-D1024 5´–GTNTG(T/C)GA (T/C)GGNTT(T/C)CA (T/C)GTNGG–3´

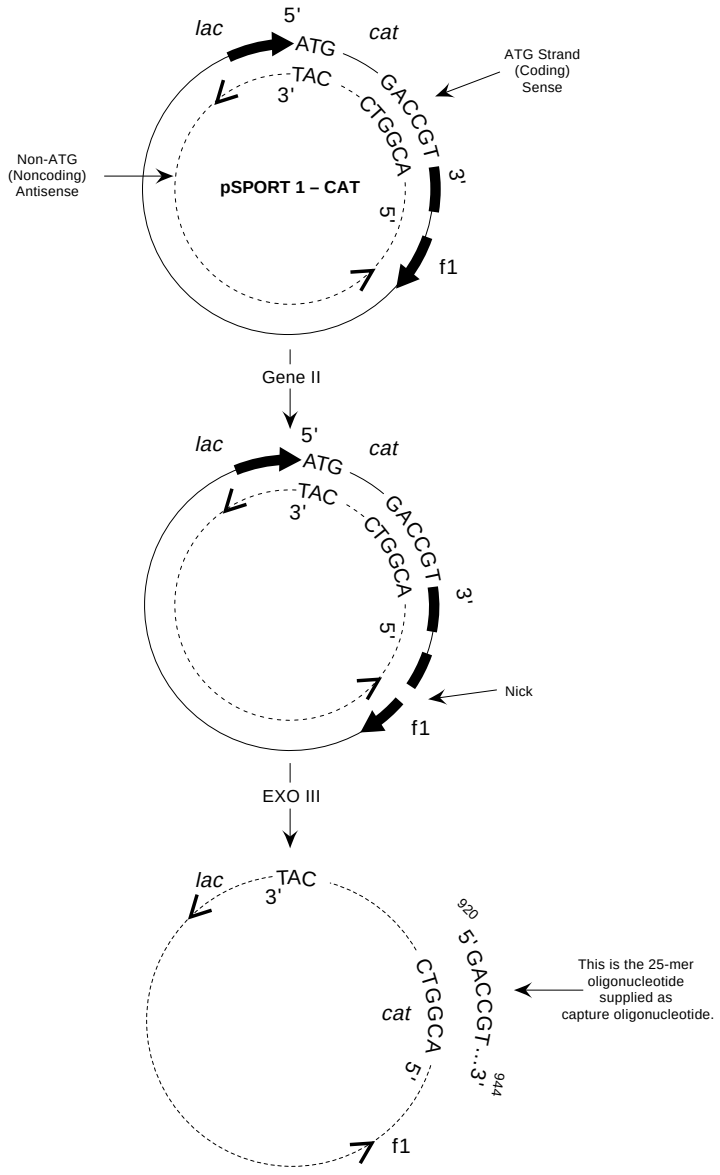

Figure 2. Overview of Gene II-Exo III digestion and CAT oligonucleotide binding.

2.4 Gene II-Exo III Digestion

The combined action of the Gene II-Exo III enzymes will convert ds phagemid DNA to ssDNA (figure 2). The replication initiator protein of bacteriophage f1 (Gene II) is a site-specific endonuclease that binds to the f1 ori in phagemid vectors (*e.g.,* pSPORT 1, pCMV•SPORT vectors, and pBluescript®) and nicks the viral strand of the supercoiled DNA (3, 4). After nicking by Gene II, Exo III (5) is used to digest the nicked strand from the 3´-end, leaving a ss covalently-closed DNA. Gene II-Exo III-prepared ssDNA is in the opposite polarity to ssDNA generated by *in vivo* phagemid production. This is an important consideration for determining the proper oligonucleotide sequence to be used in the GeneTrapper System (see Section 2.3).

Overview

Phagemid DNA must be sufficiently pure to be effectively digested by the Gene II and Exo III enzymes. The protocol in Section 3.3 must be followed exactly. Alternatively, the protocol in Section 5.2 can be used. Sufficient Gene II and Exo III are provided to pretest the purity of the dsDNA before starting the GeneTrapper System.

2.5 Repair of Captured cDNA Target

The oligonucleotide used to convert the captured ssDNA to dsDNA can be either the same sequence used for capture or another sequence in the gene of interest. This step enriches the selected population for target genes since dsDNA transforms bacterial cells with greater efficiency (20- to 100-fold) than any remaining ssDNA.

After capture and repair, abundant cDNA species represent a greater percentage of the positive clones than rare cDNA species (table 2). If the target cDNA clone is abundant (*e.g.,* actin), the repair step may be eliminated. If the percent representation is unknown, the repair step should be used to ensure adequate enrichment of the target cDNA. After transformation and plating, colonies are screened by PCR (see Section 3.12) or by colony hybridization (see Section 3.13) to confirm the identity of the clone of interest.

Table 2. Enrichment of different cDNAs from a human liver cDNA library after capture and repair.

Gene	Representation in the Original Library	Positive Clones (%)
DNAPϵ	~0.01%	70
eIF-4E	0.002%	56
RPA	0.002%	61

2.6 Transformation of Repaired DNA

Historically, electrocompetent *E. coli* have provided the highest number of colonies per transformation in the GeneTrapper procedure. Now with improved chemical transformation procedures, a comparable number of colonies can be obtained with UltraMAX™ DH5α-FT™ chemically competent *E. coli* (6). UltraMAX DH5α-FT competent cells have additional advantages in that the repaired DNA does not need to be ethanol-precipitated prior to transformation, nor is an electroporator required. The choice of transformation procedures is left to your preference.

2.7 Control CAT Oligonucleotide and Plasmid

These kit components can be used to test the oligonucleotide biotinylation reaction through cDNA capture and repair. The control sense strand CAT oligonucleotide anneals to the Gene II-Exo III-digested pSPORT 1-CAT DNA at map coordinates 920–944 (5'-GACCGTTCAG CTGGATATTA CGGCC-3', figure 2). The pSPORT 1-CAT DNA contains a 1.1-kb chloramphenicol acetyltransferase (CAT) gene. Typical results from cDNA capture and repair reactions using different CAT:HeLa ratios are shown in table 3. The percent positive CAT clone results reflect the results observed with real cDNA clones.

Table 3. Enrichment of the pSPORT 1-CAT cDNA from HeLa cDNA libraries after cDNA capture and repair.

Sample (CAT:HeLa)[a]	Ap^r colonies/reaction	Cm^r colonies/reaction	% CAT clones
1:5,000	1.84×10^4	1.75×10^4	95
1:50,000	5.5×10^3	4.3×10^3	78
1:500,000	2.0×10^3	600	30

[a] Numbers of molecules.

Methods

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3.1 Components

The components of the GeneTrapper cDNA Positive Selection System are listed below. Reagents are provided in sufficient quantities to perform 10 Gene II-Exo III digestions, 5 oligonucleotide biotinylations, 5 cDNA captures, and 10 repair reactions. Store the GeneTrapper System at –20°C.

Component	Amount
Terminal Deoxynucleotidyl Transferase (TdT)	25 µl
5X TdT Buffer	100 µl
Biotin-14-dCTP	30 µl
pSPORT 1-CAT DNA (1 ng/µl)	10 µl
CAT Oligonucleotide (0.5 µg/µl)	20 µl
Gene II	10 µl
10X Gene II Buffer	100 µl
Exonuclease III	20 µl
4X Hybridization Buffer	100 µl
Streptavidin Beads	270 µl
Wash Buffer	2.5 ml
3X Elution Buffer	50 µl
Glycogen (20 µg/µl)	20 µl
10 mM dNTP Mix	20 µl
Repair Enzyme	6 µl
10X Repair Buffer	100 µl
Manual	one

3.2 Additional Materials

The following items are required for use with the GeneTrapper System, but are *not* included in the system:

Equipment:

- water baths, heat blocks, or thermal cycler equilibrated at 30°C, 37°C, 55°C, 65°C, 70°C, 90°C, and 95°C
- bacterial incubator (capable of maintaining a temperature of 37°C and able to shake 500-ml to 2.8-L flasks at a setting of 275 rpm)
- side-baffled 1-L flask
- microcentrifuge capable of generating a relative centrifugal force of 14,000 × *g*
- Magna-Sep™ Magnetic Particle Separator or equivalent
- vertical gel electrophoresis apparatus

For electroporation:

- electroporation system and chambers (capable of generating a pulse length of 4 milliseconds and a field strength of 16,600 V/cm)
- electrotransformable cells, such as ElectroMAX® DH10B™ cells

For chemical transformation:

- high-efficiency chemically competent cells, such as UltraMax™ DH5α-FT™ cells

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Reagents:

- phenol:chloroform:isoamyl alcohol
- autoclaved, distilled water
- 1 M Tris-HCl (pH 7.5)
- absolute ethanol
- 70% ethanol
- 7.5 M ammonium acetate (store at 4°C)
- TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]
- formamide stop buffer (90% formamide, 0.5X TBE buffer, 0.1% bromophenol blue, 0.1% xylene cyanol)
- acrylamide
- bisacrylamide
- urea
- 10X TBE buffer (1 M Tris-base, 900 mM boric acid,10 mM EDTA)
- 10X TAE buffer [400 mM Tris-acetate (pH 8.3), 10 mM EDTA]
- ethidium bromide
- PD-10 column
- 1.1X PCR SUPERMIX [22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 μM dATP, 220 μM dCTP, 220 μM dGTP, 220 μM dTTP, recombinant *Taq* DNA Polymerase, stabilizers]
- silicone oil
- buffer I [15 mM Tris-HCl (pH 8.0), 10 mM EDTA]
- buffer I with RNase [15 mM Tris-HCl (pH 8.0), 10 mM EDTA, RNase A (100 μg/ml), RNase T1 (1,200 units/ml)]
- buffer II (0.2 M NaOH, 1% SDS)
- isopropanol (-20°C)
- Terrific Broth (See Section 5.5 for preparation)

Note: For best results, prepare Terrific Broth from individual components.

3.3 Preparation of dsDNA from a Plasmid cDNA Library

Note: Cesium chloride-purified dsDNA will yield the cleanest DNA (see Section 5.2). However, the procedure in Section 3.3 is less labor intensive and will work if followed exactly.

The production of pure dsDNA is essential for Gene II-Exo III digestion, so the instructions must be followed *exactly* as described.

1. Prepare buffer I with RNase. (RNase A and RNase T1 are required; see Section 3.2 for preparation.) Prepare buffer II at time of use.
2. Inoculate 100 ml of Terrific Broth containing 100 μg/ml ampicillin with 2.5×10^9 cells from an amplified library or 1×10^6 independent clones from a primary library in a 1-L side-baffled flask.
3. Grow the cells to saturation at 30°C.
4. Read the A_{590} of the culture. For accurate A_{590} determination, dilute cells 1:10 to 1:20 so that the observed value is between 0.2 and 0.8. Do not process more than 500 OD_{590} units.
5. At this scale, the procedure is performed in two 50-ml centrifuge tubes. Centrifuge at 4,800 × *g* for 15 min at 4°C. Pour off supernatant and proceed with the cell pellet.

Caution: The cells must be no more than 50 OD units/ml.

6. Resuspend the cell pellets in a total volume of 10 ml of buffer I with RNase. The cells must be less than 50 OD units/ml when resuspended.
7. Add 10 ml of buffer II to the resuspended cells. Invert to mix and incubate for 5 min at room temperature.
8. Add 10 ml of cold 7.5 M NH_4OAc to the cell mixture. Invert to mix and incubate on ice for 10 min. Centrifuge at 3,000 × *g* (4,000 rpm in an HS4 rotor) for 15 min at 4°C.
9. Pour the supernatant through cheesecloth or a milk filter into a fresh 50-ml centrifuge tube. Avoid the white flocculant material. Add an equal volume of cold isopropanol (-20°C), mix well, and centrifuge at 3,000 × *g* for 15 min at 4°C.

10. Discard the supernatant. Resuspend the pellet in 1 ml of buffer I with RNase and transfer it to a microcentrifuge tube. Clarify the solution by centrifugation at 4°C for 1 min at 14,000 × *g*. Transfer the supernatant to a fresh microcentrifuge tube.
11. Incubate the tube at 37°C for 30 min, then place it at 65°C for 5 min. Split the sample into two equal parts (~500 µl each) in 1.5-ml microcentrifuge tubes.
12. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each sample and vortex. Centrifuge at room temperature for 5 min at 14,000 × *g*. Transfer 450 µl of the upper aqueous phase to fresh microcentrifuge tubes. Avoid the interface.
13. Repeat the phenol:chloroform extraction 3 times. The interface should be clear. If not, repeat step 12 until clear.
14. Add an equal volume of isopropanol (-20°C) to each tube. Centrifuge at 4°C for 15 min at 14,000 × *g* and discard the supernatant.
15. Carefully add 500 µl of 70% ethanol to each tube. Centrifuge at 4°C for 5 min at 14,000 × *g*. Discard the supernatant. Dry the pellet at room temperature for 10 min.
16. Dissolve the two pellets in a total of 200 µl TE buffer. Make certain that the pellet is completely dissolved. **Note**: The ds plasmid DNA library concentration should be ~1 µg/µl.
17. Aliquot the dsDNA and store at –20°C after determining the concentration.

3.4 PAGE Purification of Oligonucleotides

Note: The CAT oligonucleotide that is provided with the system requires no additional purification. Even if your oligonucleotide was purified by HPLC, make sure it does not contain aborted synthesis products (*e.g.,* n-1, n-2, n-3).

Oligonucleotides contaminated by significant amounts of aborted synthesis products (*e.g.,* n-1, n-2, n-3) will yield a high percentage of background colonies if used for cDNA capture and repair. To avoid this problem, gel purify your oligonucleotide on a denaturing acrylamide preparative gel as described below. If your gene-specific oligonucleotide was previously purified by PAGE, proceed to step 8 of this section with 50 µg to 100 µg of your oligonucleotide.

CAUTION!

The purity of oligonucleotides used in GeneTrapper reactions dramatically affects the results. PAGE-purified oligonucleotides MUST be used. To ensure satisfactory results, purify oligomers as described below.

Commercially PAGE-purified custom oligonucleotides should be phenol/chloroform extracted and ethanol precipitated prior to tailing. After reconstituting the oligonucleotide in TE, follow the procedure below starting with step 13.

In addition, do NOT use machine-synthesized oligonucleotides that have had biotin added to them as part of the synthesis. These oligonucleotides cannot be gel-purified as described because biotinylated oligonucleotides will go to the interface when extracted with phenol/chloroform.

1. Assemble a gel mold using 1.5-mm spacers and comb (1.5-cm size wells).
2. Prepare a 12% (19:1 w/w) acrylamide:bisacrylamide, 8 M urea, 1X TBE buffer gel (see section 5.4 for gel preparation). For Models SA or S2, prepare 300 ml; for the V16, prepare 60 ml.
3. Dissolve 5 to 10 OD units (A_{260}) of oligonucleotide (33 µg = 1 OD) in 25 µl TE. Add an equal volume of formamide to the oligonucleotide.
4. Flush the gel wells and load the oligonucleotides. Leave a space between the oligonucleotides and load the formamide stop buffer with dyes into the first and the last lanes. Electrophorese the gel. For the Model S2 or SA, electrophorese at 40 watts or 600 to 700 V until the bromophenol blue has moved 15 to 20 cm. For the Model V16, electrophorese at 300 V.

Note: To avoid masking the oligonucleotide location, no dye is added to the oligonucleotide sample.

5. Transfer the gel to an intensifying screen. Examine the gel under short-wavelength UV. The oligonucleotide bands should be very easily visible as dark bands with the shorter sequences electrophoresing faster. Cut out the full length band with a razor blade.

6. Crush the gel slice and elute the oligonucleotide (1) in 1 ml of TE buffer overnight at 37°C with shaking.
7. Transfer the eluted solution to a fresh tube with a 1-ml pipette. Wash the gel slice with 200 µl of TE and combine with eluted solution. The transfer of some small gel slices will not affect the purification. Measure the total volume of the eluted oligonucleotide.
8. After washing a PD-10 column with 12 ml of autoclaved, distilled water, add the eluted oligonucleotide to the column and discard the flow-through.

Note: The void volume of the PD-10 column is 2.5 ml.

9. Wash the column with autoclaved, distilled water, using a volume equal to 2.5 ml minus the total volume of the eluted oligonucleotide which was loaded onto the column. Discard the wash flow-through.
10. Elute the oligonucleotide by adding 1 ml of autoclaved, distilled water to the column, and collect this fraction in a sterile tube. Add another 1 ml of autoclaved, distilled water and collect it in a second tube.
11. Dry the eluted oligonucleotide under vacuum.
12. Dissolve the eluted oligonucleotide in both tubes with a total volume of 100 µl of TE buffer.
13. Add 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1), vortex thoroughly, and centrifuge at room temperature for 5 min at 14,000 × *g* to separate the phases. Carefully remove 90 µl of the upper, aqueous layer and transfer it to a fresh 1.5-ml microcentrifuge tube.
14. Add 45 µl of 7.5 M NH_4OAc, followed by 350 µl of absolute ethanol (–20°C). Vortex the mixture thoroughly, store on dry ice for 10 min, and centrifuge at 4°C for 10 min at 14,000 × *g*.
15. Remove the supernatant carefully and overlay the pellet with 100 µl of 70% ethanol (–20°C). Centrifuge for 2 min at 14,000 × *g* and remove the supernatant.
16. Dry the oligonucleotide at room temperature for 10 min, or until residual ethanol has been evaporated, and dissolve in 60 µl of TE buffer. Determine the oligonucleotide concentration by OD_{260}. The oligonucleotide concentration should be greater than 0.5 µg/µl.

3.5 Biotinylation Reaction

A control CAT oligonucleotide is provided for verifying the biotinylation reaction. Simply perform a parallel biotinylation reaction with your oligonucleotide. **Caution**: Do not change or add more than the recommended amounts of Biotin-14-dCTP, as this will affect the biotinylation reaction (7, 8).

1. Add the following to a 1.5-ml microcentrifuge tube:

Note: Water should be added first, followed by the other components in the order shown**.**

Component	Amount
5X TdT Buffer	5 µl
oligonucleotide	3 µg
Biotin-14-dCTP	5 µl
autoclaved, distilled water	sufficient to bring the volume to 23 µl
TdT	2 µl

2. Vortex gently and centrifuge for 2 s at 14,000 × *g*.
3. Incubate for 1 h at 30°C.
4. Prepare a 15 × 17-cm 16% (19:1, w/w) acrylamide:bisacrylamide, 8 M urea, 1X TBE buffer gel using 0.7-mm spacers and comb (see Section 5.4 for gel preparation).

Note: No additional salt is needed. High concentrations of salt are present in the TdT buffer.

5. After 1 h, add 1 µl of Glycogen (20 µg/µl), 26 µl of 1 M Tris-HCl (pH 7.5), and 120 µl of ethanol to the oligonucleotide biotinylation reaction.
6. Vortex and store on dry ice for 10 min.
7. Centrifuge at 4°C for 30 min at 14,000 × *g*.
8. CAREFULLY and IMMEDIATELY remove the supernatant from the microcentrifuge tubes and layer 200 µl of 70% ethanol (–20°C) over the pellet. Centrifuge at 4°C for 2 min at 14,000 × *g*.

9. CAREFULLY remove the ethanol wash from the microcentrifuge tubes. Repeat the ethanol wash once and dry the pellets at room temperature for 10 min or until completely dry.
10. Dissolve the biotinylated oligonucleotide in 20 µl TE buffer.
11. To determine the labeling efficiency and the concentration of the labeled oligonucleotide, remove 4 µl for gel analysis and store the remainder at –20°C.

Note: After analysis proves sufficient labeling, the probe will be used in Section 3.9.

3.6 Analysis of the Oligonucleotide Biotinylation Reaction Products

1. Add 4 µl of formamide stop buffer to the 4 µl of biotinylated oligonucleotide. Vortex and centrifuge for 2 s at 14,000 × *g* to collect the contents of the tube.
2. Dilute the unbiotinylated oligonucleotide to 50 ng/µl, so that 4 µl contains 200 ng. Mix 4 µl of the dilution with 4 µl of formamide stop buffer.
3. Load samples with flat pipette tips onto the 16% gel prepared in Section 3.5, step 4.
4. Electrophorese for 1.5 h at 400 V or until the blue dye migrates to the middle of the gel.
5. Keeping the gel attached to one of the glass plates, stain the gel in ethidium bromide solution (0.5 µg/ml) for 15 min.
6. Transfer the gel to clear plastic wrap and photograph using transmitted ultraviolet light.

Note: If fluorescent background from ethidium staining obscures the oligonucleotide bands, destain for 10 min in water.

Compare the biotinylated products in the CAT Oligonucleotide control lane and the lane containing your oligonucleotide with the titrated, unbiotinylated CAT Oligonucleotide lanes. Greater than 80% of the oligonucleotides should be biotinylated (figure 3), and a range of biotin residues from 1 to 6 (3 to 4 should be dominant) should be added to the oligonucleotide. If the oligonucleotide does not biotinylate well, repeat the phenol:chloroform:isoamyl alcohol extraction of the nonbiotinylated oligonucleotide, biotinylation, and gel analysis. The biotinylated oligonucleotide concentration can be estimated by comparison to the lanes containing titrated CAT Oligonucleotide.

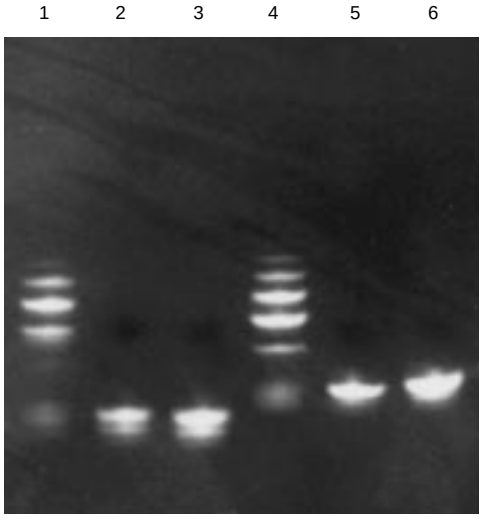

Figure 3. Gel analysis of an oligonucleotide biotinylation reaction. Biotinylated oligonucleotides were electrophoresed into a 16% acrylamide gel at 400 V for 1.5 h. Lane 1, 3 µl of labeled CAT Oligonucleotide; lane 2, 200 ng of unlabeled CAT Oligonucleotide; lane 3, 500 ng of unlabeled CAT Oligonucleotide; lane 4, 3 µl of labeled gene-specific oligonucleotide (GSO); lane 5, 200 ng of unlabeled GSO; and lane 6, 500 ng of unlabeled GSO.

3.7 Generation of ssDNA with Gene II and Exo III

Note: The quality of the dsDNA will determine the efficiency of the Gene II-Exo III digestion.

A control pSPORT 1-CAT DNA is provided for validating the system. It can be captured and enriched using the biotinylated CAT Oligonucleotide. If you are using the control, add 2 µl to 4 µl of the pSPORT 1-CAT DNA (1 ng/µl) to 50 µg of your dsDNA preparation. This amount should produce a CAT:library ratio of 1:12,000-1:25,000 and should not interfere with the capture of a specific gene from your library. To capture the pSPORT 1-CAT DNA, transfer 5 µg from this library to a tube separate from the target capture reaction.

Note: If more than one hybridization will be performed on one DNA preparation, this reaction can be scaled up 6- to 10-fold. Before scale-up, 5 µg of the dsDNA should be processed to verify that the Gene II - Exo III reaction works.

Prepare three water baths (30°C, 37°C, and 65°C) before starting this protocol.

1. For each hybridization, add the following to a 1.5-ml microcentrifuge tube at room temperature:

Component	Amount
10X Gene II Buffer	2 µl
ds phagemid cDNA (⩾1 µg/µl)	5 µg
autoclaved, distilled water	sufficient to bring the volume to 19 µl

Hint: Gene II is very thermal-sensitive to temperatures above –20°C. Therefore, for optimal results, add Gene II to the sample directly from the freezer.

2. To each reaction, add 1 µl of Gene II.
3. Vortex and centrifuge at room temperature for 2 s at 14,000 × *g* to collect the contents to the bottom of the tube.
4. Incubate in a 30°C water bath for 25 min.

Note: If your DNA is in a pBlueScript® vector, incubate at 30°C for 45 min.

5. Heat the mixture at 65°C for 5 min and immediately chill on ice for 1 min.
6. Transfer 1 µl of the mixture to a microcentrifuge tube containing 9 µl of TE buffer and 2 µl of gel loading dye. Save these samples on ice for the gel analysis.
7. To the remaining 19 µl in each tube, add 2 µl of Exo III. Vortex and centrifuge at room temperature for 2 s at 14,000 × *g*.

Note: If the reaction was scaled up, the Exo III volume must also be scaled up.

8. Incubate at 37°C for 60 min. During the incubation, prepare a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide in 1X TAE buffer for analysis of the Gene II and Exo III reactions.
9. Add an equal volume (20 µl) of phenol:chloroform:isoamyl alcohol (25:24:1), vortex thoroughly, and centrifuge at room temperature for 5 min at 14,000 × *g* to separate the phases.

Note: It is not necessary to ethanol precipitate to remove the residual phenol at this point.

10. Transfer 18 µl of the upper aqueous phase to a fresh 1.5-ml microcentrifuge tube. The ssDNA can be stored at 4°C for several days. STOP

Note: If the initial digestion was scaled up, transfer the aqueous phase to a fresh tube and aliquot 17 µl of the ssDNA to each microcentrifuge tube. Store on ice or at 4°C.

11. Transfer 1 µl to a microcentrifuge tube containing 9 µl of TE buffer and 2 µl of gel loading dye. Save on ice for gel analysis. **Do not proceed to Section 3.9 until gel analysis indicates that the dsDNA is converted to ssDNA by the Gene II-Exo III digestion.**

Note: The ssDNA is stable for several days at 4°C.

12. Load the samples for gel analysis onto a 0.8% agarose gel in 1X TAE or TBE buffer. Also electrophorese 250 ng of the original ds phagemid DNA and 1 µg of 1 Kb DNA Ladder. Electrophorese for 1.5 h at 80 V or 50 mA, or until the blue dye migrates 3-4 cm.

Note: Do not change the electrophoresis conditions. Rapid electrophoresis will affect gel analysis of digestion products.

3.8 Analysis of Gene II and Exo III Digestion Products

Note: Possible stopping points are indictaed by a STOP icon.

Compare the Gene II and Gene II-Exo III-treated samples to the undigested ds phagemid DNA (figure 4). More than 50% of the supercoiled DNA should be nicked by the Gene II protein and migrate as open-circle DNA (the slowly migrating band in lane 3). The nicked form of ds phagemid DNA generated by Gene II treatment should be completely converted to the ssDNA after the Exo III digestion (migrates faster than supercoiled).

If the dsDNA is converted to ssDNA, proceed to Section 3.9. If the dsDNA is not converted to ssDNA after Gene II-Exo III treatment, refer to Chapter 4.

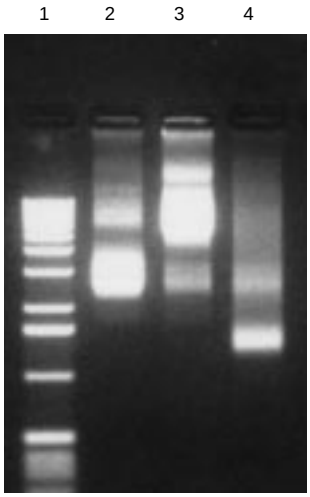

Figure 4. Gel Analysis of Gene II-Exo III-digested dsDNA. After digestion with Gene II or Gene II-Exonuclease III, dsDNA was electrophoresed into a 0.8% agarose-1X TAE gel for 1.5 h at 50 mA using a HORIZON® 11•14 apparatus. Lane 1, 1 Kb DNA Ladder; lane 2, 250 ng of undigested HeLa cDNA library; lane 3, 250 ng of Gene II-treated HeLa cDNA library; and lane 4, 250 ng of Gene II-Exonuclease III-treated HeLa cDNA library.

3.9 cDNA Capture Hybridization

The 4X Hybridization Buffer, Wash Buffer, and Streptavidin Beads should be stored at 4°C after thawing.

3.9.1 Oligonucleotide Hybridization

Note: If 3 µl of biotinylated oligonucleotide from the gel analysis is equal to 300 ng or 100 ng/µl, then dilute 1:5 to equal 20 ng/µl.

1. Dilute the biotinylated oligonucleotide to 20 ng/µl in TE buffer.
2. Incubate the 4X Hybridization Buffer for 2 min at 37°C, mix well, and add 6 µl to the remaining 17 µl of Gene II/Exo III-treated DNA. Mix by pipetting up and down.
3. Denature the DNA in a 95°C water bath for 1 min. Chill immediately on ice for 1 min.

Hint: To obtain the best results with degenerate oligonucleotides, use 2 to 5 ng per capture reaction.

4. Add 1 µl of diluted biotinylated oligonucleotide (20 ng) to the denatured DNA. Mix by pipetting up and down with a pipette setting of 10 µl. Incubate the tube at 37°C in a water bath or incubator for 1 h.

3.9.2 Streptavidin Bead Preparation

Note: If performing more than one hybridization, do not pool mixed beads into one tube.

1. Twenty min before the hybridization is complete, proceed with the treatment of the streptavidin paramagnetic beads. Gently mix the beads by pipetting until the beads at the bottom are completely resuspended.
2. Transfer 45 µl of the mixed beads to the bottom of a microcentrifuge tube for each reaction. Insert the tubes into the magnet and let sit for 2 min. Store the remaining paramagnetic beads at 4°C.

Caution: Do not let the beads dry out!

3. Retaining the tubes in the magnet, remove the supernatant by pipetting and immediately add 100 µl of TE buffer to the beads.
4. Remove the tube from the magnet and gently resuspend the beads by finger tapping or vortexing at the lowest setting. Re-insert the tube into the magnet.
5. After 2 min, remove the supernatant again. Resuspend the beads in 30 µl of TE buffer and place the tubes into a microcentrifuge tube rack.

3.9.3 cDNA Capture

Caution: Do not centrifuge the beads!

1. Remove the hybridization mixture from the 37°C water bath and centrifuge at room temperature for 2 s at 14,000 × *g*. Pipet the prepared paramagnetic beads into the mixture and gently mix by pipetting.

Note: At this step, a rotator (at a low setting), a Vortex Genie 2™ with microcentrifuge tube accessory starter set (Cat. No. 12-812A), at a setting of 2, or a rotating platform, at a setting of 205 rpm, can be used to keep the particles resuspended during the 30-min incubation.

2. Incubate the suspension for 30 min at room temperature. Gently mix the suspension frequently (every 2 to 3 min) by finger tapping or gently vortexing at the lowest setting for 10 s to resuspend the beads. Do not invert the tubes end-over-end or the beads may remain in the cap.
3. Insert the tubes into the magnet. After 2 min, remove and discard the supernatant.
4. Add 100 µl of Wash Buffer to the beads. Resuspend the beads by finger tapping or gently vortexing at the lowest setting (avoid splashing beads to sides of tube) and re-insert into magnet for 2 min. Remove and discard supernatant. Repeat this washing step one more time.
5. Add 100 µl of Wash Buffer to the beads. Resuspend the beads gently by pipetting up and down (do not vortex) and transfer the solution to a clean tube.
6. Insert the tubes into the magnet for 5 min. Remove and discard the supernatant from the paramagnetic beads. Immediately add 100 µl of Wash Buffer and finger tap or gently vortex. Insert the tubes into the magnet for 5 min.

Note: Keep the 1X elution buffer at room temperature.

7. During this 5 min, prepare only enough 1X elution buffer for the day. For each elution, mix 14 µl of TE buffer (pH 8.0) with 7 µl of the 3X Elution Buffer.
8. After the 5-min incubation, remove and discard the supernatant from the paramagnetic beads; add 20 µl of 1X elution buffer to the beads, and mix well by pipetting.
9. Incubate the beads for 5 min at room temperature. During the incubation, finger tap the beads for 10 s every minute. Insert the tube into the magnet and wait 5 min.

Caution: Do not transfer the beads!

10. Transfer and save the supernatant (contains the captured cDNA clone) to a fresh tube and resuspend the beads in 15 µl of TE buffer. Insert the tube into the magnet and wait 5 min.
11. Transfer the supernatant from the tube and combine it with the supernatant from step 10.
12. Insert the tube containing the combined supernatants into the magnet for 10 min to remove any remaining paramagnetic beads and transfer the supernatant to a fresh microcentrifuge tube. The transfer of some beads will not affect the efficiency of capture.
13. To the supernatant (~35 µl), add 1 µl of Glycogen, 18 µl of 7.5 M NH_4OAc, and 135 µl of ethanol (–20°C). Mix well and store on ice for 10 min or at 4°C overnight. STOP
14. Centrifuge at 4°C for 30 min at 14,000 × *g*.
15. Carefully remove the supernatant from the small pellets. Add 100 µl of 70% ethanol. Centrifuge at room temperature for 2 min at 14,000 × *g*. Remove the ethanol and dry the pellets at room temperature for 10 min or until dry.
16. Dissolve the pellets in 5 µl of TE buffer and store at 4°C. STOP

3.10 Repair of Captured cDNA

Note: If the captured cDNA is highly abundant, disregard Section 3.10; the DNA can be immediately electroporated into ELECTROMAX® Cells (see Section 3.11) or transformed into ULTRAMAX cells (see Section 3.12).

The repaired DNA can be transformed into either electrocompetent or ULTRAMAX DH5α-FT chemically competent cells with similar results. Follow steps 3.10.1, 3.10.2 and 3.11 for electroporation or steps 3.10.1 and 3.12 for chemical transformation.

3.10.1 Repair Reaction

1. Before starting the repair reaction, set up a thermal cycler for one cycle:

   90°C denature step for 1 min

   55°C annealing step for 30 s

   70°C extension step for 15 min

   Alternatively, three water baths or heat blocks can be set up at 55°C, 70°C and 90°C for the incubations.

Note: The oligonucleotide-primer can be the same oligonucleotide used in the cDNA capture hybridizations. Do not use the biotinylated oligonucleotide for this reaction!

2. Prepare a DNA primer/repair mix for each capture reaction by adding the following to the captured cDNA from step 16 of Section 3.9.3:

Component	Amount
captured DNA	5 µl
autoclaved, distilled water	11 µl
50 ng oligonucleotide (not biotinylated)	1 µl
10 mM dNTP Mix	0.5 µl
10X Repair Buffer	2 µl
Repair Enzyme	0.5 µl
final volume	20 µl

   Mix by pipetting and centrifuge at room temperature for 2 s at 14,000 × *g*.
3. Incubate the DNA primer/repair mix at 90°C for 1 min.
4. Transfer the mix to 55°C and incubate for 30 s.
5. Transfer the mix to 70°C and incubate for 15 min to allow primer extension.
6. Remove the tubes and centrifuge at room temperature for 2 s at 14,000 × *g*.
7. Store the repaired DNA at -20°C until needed. STOP
8. To electroporate the cells, proceed with section 3.10.2, then section 3.11.
9. To transform the DNA into chemically competent cells, proceed to Section 3.12.

3.10.2 Ethanol Precipitation of Repaired DNA

1. Precipitate the repaired DNA by adding 1 µl Glycogen, 11 µl of 7.5 M ammonium acetate, and 90 µl of –20°C ethanol to each tube. Vortex and place the tubes in ice for 10 min or at 4°C overnight. STOP
2. Centrifuge the tubes at 4°C for 30 min at 14,000 × *g*.
3. Carefully remove the ethanol from the small pellet and layer with 100 µl of 70% ethanol (–20°C). Centrifuge at 4°C for 2 min at 14,000 × *g*.
4. Remove all of the ethanol and dry the pellets at room temperature for 10 min or until dry.
5. Dissolve the pellets in 10 µl of TE buffer and store at 4°C. STOP

3.11 Electroporation

Note: Efficient transformation of ELECTROMAX DH10B cells requires a field strength of ~16.6 kV/cm and a pulse length of ~4 ms.

Note: The optimal electroporation voltage for the CELL-PORATOR System, in conjunction with the CELL-PORATOR Voltage Booster, is 2.5 kV using 23 µl of cells in a 0.15-gap chamber at settings of voltage: 400; capacitance 330 µF; impedance: low ohms; charge rate: fast; and resistance on voltage booster: 4,000 ohms.
The optimal electroporation voltage for ELECTROMAX cells in the BioRad Gene Pulser is 2.5 kV using 40 µl of cells in a 0.1-cm gap chamber at settings of 100 ohms and 25 µF.

3.11.1 Electroporation of Captured or Repaired DNA

1. Place 2 µl of each of the captured or repaired DNAs into autoclaved 1.5-ml microcentrifuge tubes on ice. Place 1 µl of the pUC control DNA, provided with the competent cells, into another tube.
2. Add the appropriate amount of electrotransformable cells, such as ELECTROMAX DH10B, (23 µl per transformation using the CELL-PORATOR Electroporation System or 40 µl per transformation using the BioRad Gene Pulser) to the tubes containing DNA. Mix by pipetting once and electroporate.
3. After electroporation, add 1 ml of S.O.C. medium to the electroporated cells, transfer them to a sterile 10-ml tube, and incubate for 1 h at 37°C with shaking.
4. For the captured or repaired cDNA samples, plate 100-µl and 200-µl aliquots onto LB plates containing 100 µg/ml ampicillin. Transfer the remaining cells to an autoclaved 1.5-ml microcentrifuge tube and centrifuge for 15 s, discard the supernatant, resuspend the cells in 200 µl of S.O.C. medium, and plate the entire electroporated sample onto one ampicillin plate.
5. For the pUC control DNA, dilute a portion 1:100, and plate 100 µl onto an LB plate containing 100 µg/ml ampicillin.
6. Incubate ampicillin plates overnight in a 37°C incubator.

Note: Mix the chloramphenicol thoroughly.

3.11.2 Electroporation of Captured or Repaired pSPORT 1-CAT DNA

Prepare the chloramphenicol plates (LB plates containing 7.5 μg/ml chloramphenicol and 100 μg/ml ampicillin) at least one day before the transformation is performed.

1. Use 2 µl of the captured or repaired pSPORT 1-CAT DNA reactions in an electroporation reaction, following the procedure in Section 3.11.1. Also electroporate 1 µl of the original CAT:dsDNA population (see Section 3.7) diluted to 10 ng/µl.
2. For the captured or repaired pSPORT 1-CAT DNA, plate 100 µl and 200 µl onto LB plates containing 7.5 µg/ml chloramphenicol and 100 µg/ml ampicillin and duplicate 100-µl aliquots onto LB plates containing ampicillin.
3. For the original CAT:dsDNA electroporation, dilute a portion to 1:1,000 and plate 10 µl and 100 µl onto ampicillin plates and 100 µl and 200 µl of undiluted sample onto chloramphenicol plates.
4. Incubate ampicillin plates and chloramphenicol plates overnight in a 37°C incubator.

3.12 Transformation with ULTRAMAX DH5α-FT Cells

3.12.1 Transformation of Captured or Repaired DNA

1. Remove competent cells from -70°C freezer; thaw on wet ice. Place the required number of 17 × 100 polypropylene tubes (Falcon 2059) on ice.
2. Immediately after thawing, gently mix cells, then aliquot 100 µl into chilled polypropylene tubes.
3. To determine transformation efficiency, add 5 µl (0.05 ng) control DNA to one tube containing 100 µl competent cells. Move the pipette through the cells while dispensing. Gently tap the tube to mix.
4. For each captured or repaired DNA reaction, mix 3 µl of the DNA into an individual tube of cells and store the remainder at -20°C. Gently tap the tubes to mix after addition of the DNA.
5. Incubate the cells on ice for 30 min.
6. Heat-shock cells for 45 s in a 42°C water bath; do not shake.
7. Place on ice for 2 min.
8. Add 0.9 ml of room temperature S.O.C medium.
9. Shake at 225 rpm (37°C) for 1 h.
10. Dilute the reaction containing the control plasmid DNA 1:400 with S.O.C. medium. Spread 100 µl of this dilution on LB or YT plates containing 100 μg/ml ampicillin.
11. For captured or repaired cDNA samples, plate 100 µl and 200 µl aliquots onto LB plates containing 100 µg/ml ampicillin. Transfer the remaining cells to an autoclaved 1.5-ml microcentrifuge tube and centrifuge for 15 s, discard the supernatant, resuspend the cells in 200 µl of S.O.C. medium, and plate the entire sample onto one ampicillin plate.
12. Incubate ampicillin plates overnight in a 37°C incubator.

3.12.2 Transformation of Captured or Repaired pSPORT 1-CAT DNA

Prepare the chloramphenicol plates (LB plates containing 7.5 μg/ml chloramphenicol and 100 μg/ml ampicillin) at least one day before the transformation is performed.

1. Transform 3 µl of each captured or repaired pSPORT 1-CAT DNA reactions, following the procedure in Section 3.12.1. Also transform 1 µl of the original CAT:dsDNA population (see Section 3.7) diluted to 10 ng/µl.
2. For the pSPORT 1-CAT DNA, plate 100 µl and 200 µl aliquots on LB plates containing 7.5 µg/ml chloramphenicol and 100 µg/ml ampicillin and duplicate 100-µl aliquots onto plates containing ampicillin.
3. For the CAT:dsDNA library transformation, dilute a portion to 1:1,000 and 1:10,000. Plate 100 µl of each dilution onto ampicillin plates and 100 µl and 200 µl of undiluted sample onto chloramphenicol plates.

4. Incubate ampicillin plates and chloramphenicol plates overnight in a 37°C incubator.

3.13 Calculation of Percent CAT-Positive Colonies

Count colonies on all of the plates from the pSPORT 1-CAT transformations. Calculate the percent positive for chloramphenicol resistance by dividing the total chloramphenicol-resistant colonies by the total ampicillin-resistant colonies in each transformation reaction. The percent of CAT-positive colonies should increase significantly after capture and repair when compared to the representation of CAT colonies in the original population (table 1).

3.14 Identification of Desired cDNA Clones by Colony PCR

The following conditions are optimized for PCR screening of cDNA clones obtained from GIBCO BRL SUPERSCRIPT cDNA Libraries, constructed in the pCMV•SPORT vector series. When screening clones from libraries constructed in other vectors, optimization of the $MgCl_2$ concentration, number of cycles, annealing time and temperature, extension time, primer sequence and enzyme conentration may be necessary.

1. Prepare a PCR master mix with the following components, 25 µl/clone:

Component	1X Reaction	10X Reaction
1.1X PCR SUPERMIX	22.5 µl	225 µl
primers, final concentration 200 nM/primer	variable	variable
autoclaved, distilled water to a final volume of	25 µl	250 µl

Note: Use aerosol-resistant pipette tips when going into stock PCR reagents so as to not contaminate the stocks.

2. Place 25 µl of master mix in the desired number of labeled tubes.
3. Using a micropipette tip or sterile toothpick, pick each colony into an individual tube containing master mix. Include one positive and one negative control. The recommended controls are 0.5 µl of library cDNA template (50 ng/µl) as a positive control and no template cDNA as a negative control.

Note: Label each colony or transfer the colonies to a fresh plate, so that the PCR results can be matched to the corresponding clone.

4. If using a thermal cycler withour a heated lid, add 1 drop of silicone oil to each reaction and centrifuge for 2 s at 14,000 × *g*.
5. Place the tubes in a prewarmed thermal cycler (94°C). Perform PCR using the following program:

   1 cycle: 94°C, 1 min

   30 cycles of:

   94°C, 30 s

   55°C, 30 s

   72°C, 1 min

Note: Inserts larger than 2 kb may require longer extension times.

6. Transfer 10 µl of each reaction to a new tube containing 2 µl 10X gel loading buffer.
7. Electrophorese the samples on a 2% (w/v) agarose gel prepared with 1X TAE buffer containing 0.5 µg/ml ethidium bromide.

3.15 Identification of Desired cDNA Clones by Colony Hybridization

1. Prepare colony filter lifts as described by Sambrook *et al.* (1).
2. Prehybridize the filters in a solution of 6X SET [0.9 M NaCl, 120 mM Tris-HCl (pH 7.8), 6 mM EDTA], 5X Denhardt's solution, 10 µg/ml tRNA, and 0.1% SDS for 1 h at 55°C.

Note: For degenerate oligonucleotide probes, hybridize at 37°C and wash at room temperature, 37°C, 42°C, and 47°C for 30 min. Proceed to step 5.

3. Hybridize the filters in the prehybridization solution containing a ^{32}P-end labeled oligonucleotide (3×10^5 to 1×10^6 counts/ml) for 16 h at 42°C.
4. After hybridization, sequentially wash the filters in a solution of 6X SSC [0.90 M NaCl, 90 mM sodium citrate (pH 7.0)], 0.1% SDS at room temperature, 37°C, 45°C, and 50°C for 30 min.

Note: Add fresh wash solution at each wash temperature used.

5. If excessive background counts remain, wash the filters in fresh wash solution at 55°C for an additional 30 min (52°C for degenerate oligonucleotide probes).

4

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
No (little) biotinylation of oligonucleotide	Oligonucleotide is not clean	Phenol:chloroform:isoamyl alcohol extract, ethanol precipitate, and relabel using protocols in Sections 3.4 and 3.5.
	3´ hairpin or dimer formation	Redesign oligonucleotide if there is complementarity at the 3´-end.
No (incomplete) digestion of dsDNA with Gene II and Exonuclease III	dsDNA is not pure enough	Incubate dsDNA with additional RNase and phenol extract using protocol in Section 3.3. Alternatively, more vigorous preparation of DNA using CsCl purification can be tried (see Section 5.2).
	OD_{590} of bacterial cells was greater than 500	Regrow, read OD_{590} and reisolate dsDNA using fresh buffers or use the protocol in Section 5.2.
	DNA:Gene II-Exo III ratio is suboptimal	Repeat with half as much DNA. **Caution**: Do not add more enzyme as excess glycerol will affect Exo III performance.
No (few) colonies obtained after captured plasmid-cDNA transformed	Transformation efficiency is low	Check the transformation efficiency of cells with control DNA. Switch to ELECTROMAX cells if using chemically competent cells.
	Incomplete repair reaction	Be certain of water bath settings and follow the protocol exactly.
	Oligonucleotide is <16 nucleotides in length	Increase oligonucleotide length to ⩾16 nucleotides.
High percent of background colonies after electroporation of the repaired DNA	Incomplete Gene II and Exonuclease III digestion	Reprocess dsDNA with RNase as above.
	Oligonucleotide is not pure	HPLC-purified oligonucleotides may not be sufficiently pure for use in the GENETRAPPER system. Gel-purify the oligonucleotide to remove aborted synthesis products (*e.g.*, n-1, n-2, n-3).
	Incomplete repair reaction	Check repair efficiency using the pSPORT 1-CAT control plasmid.
	Desired cDNA is rare	Use colony PCR to identify.
		Use a different oligonucleotide sequence (not capture oligo-nucleotide) for the repair reaction.
	Degenerate oligonucleotide was used for capture and repair	Decrease the sequence degeneracy. Decrease amount of oligonucleotide used in the capture reaction.
		Alternatively, a more vigorous preparation of ssDNA can be made. Use the protocol in Section 5.3.
	Desired cDNA sequence has the same polarity as the capture oligonucleotide	Recheck oligonucleotide polarity needed for the specific plasmid used and redesign.
	G+C content is >60%	Choose cDNA sequence that contains a more A +T rich region.
	Oligonucleotide contains extensive homopolymer tracts (*e.g.,* A_{16})	Choose cDNA sequence that contains a more alternating G, C, T, and A region.

Additional Protocols

5

5.1 Mass Excision of pBluescript Phagemid from a λZAP cDNA Library

For the best results with the GENETRAPPER System, the pBluescript phagemid must be excised and converted to double-stranded (ds) DNA, as described in the following procedure. If you use single-stranded (ss) DNA isolated directly after the phage packaging step (step 5.1.3), it will contain significant amounts of λ DNA, ds and ss helper phage DNA, and ds phage cDNA that will dramatically reduce the efficiency of the GENETRAPPER System.

The amplified library, *E. coli* strains, and helper phage necessary for this protocol are supplied with λZAP cDNA libraries. The XL1-Blue MRF′ cells are used for the production of helper and pBluescript phage cDNA. The XLOLR cells or the SOLR™ cells are functionally equivalent when used with λZAP cDNA libraries and can be used interchangeably to produce ds pBluescript cDNA.

5.1.1. Strain Maintenance

1. Using a sterile loop, streak each *E. coli* strain on an LB plate containing the appropriate antibiotic (15 μg/ml tetracycline for XL1-Blue MRF′ and XLOLR cells, 75 μg/ml kanamycin for SOLR cells). Incubate the plates at 37°C for 16 h.
2. Inoculate a single colony of XL1-Blue MRF′ and XLOLR into separate 50-ml conical tubes containing 15 ml of LB with 15 μg/ml tetracycline (75 μg/ml kanamycin for SOLR cells). Incubate the tubes at 37°C at 150 rpm for 7 to 8 h or until the cultures reach an OD_{590} ~0.6.
3. Transfer 10 ml of each culture to separate 50-ml conical tubes. Add 10 ml of freezing solution (60% SOB:40% glycerol v/v) to each culture tube. Mix well and dispense 1-ml aliquots into pre-marked cryotubes. Freeze the tubes in a dry-ice/ethanol bath. Store the *E. coli* stocks at -70°C.

5.1.2. Amplification of ExAssist™ Helper Phage

This protocol produces enough helper phage for an amplified library (10^9 cDNA clones). For mass excision from a primary library (1×10^6 clones), the helper phage, as supplied, is at the appropriate titer. Therefore, for a primary library, disregard this amplification step and proceed to step 5.1.3.

1. Thaw one tube of frozen XL1-Blue MRF′ cells (step 3 of section 5.1.1) and transfer 0.1 ml of cells into a 50-ml conical tube containing 15 ml 2X YT and 15 μg/ml tetracycline. Incubate at 37°C at 150 rpm for 8 h or until the culture reaches an OD_{590} ~0.5 (~3×10^8 cells/ml).
2. Add 10 ml of XL1-Blue MRF′ (a total of ~3×10^9 cells) to a 50-ml conical tube followed by 3×10^9 ExAssist helper phages.
3. Incubate at 37°C for 15 min without shaking.
4. Incubate at 37°C for 10 h with shaking at 150 rpm.
5. To remove the *E. coli* cells, heat the tube at 65°C for 15 min and then centrifuge at 4°C for 15 min at 4,000 × *g*. The supernatant containing the amplified ExAssist helper phage (~10^{12} pfu/ml) can be stored for up to a month at 4°C. Alternatively, dimethyl sulfoxide (DMSO) can be added to the supernatant to a final concentration of 7% DMSO and the amplified ExAssist helper phage can be stored at -70°C for 6 months.

Additional Protocols

5.1.3. Mass Excision of pBluescript Phagemid

This protocol can be used to mass excise ssDNA from an amplified or primary λZAP cDNA library. It is preferable to first amplify a primary library to obtain a stable, high-titer phage stock.

1. Inoculate 0.1 ml of XL1-Blue MRF´ stock cells (step 3 of 5.1.1) into a 250-ml autoclaved non-baffled flask containing 100 ml of 2X YT with 0.2% (w/v) maltose and 10 mM $MgSO_4$. Incubate the cells at 37°C at 200 rpm for 11 h or until the culture reaches an OD_{590} ~0.5-0.7 (~ 3×10^8 to 4×10^8 cells/ml).
2. Transfer 50 ml of cells (~1.5×10^{10} to 2×10^{10} cells) into two 50-ml conical tubes (25 ml/tube). Centrifuge the tubes at 2000 × *g* for 10 min at 4°C.
3. Using a 10-ml pipette, gently resuspend the two pellets in a total of 10 ml of 10 mM $MgSO_4$.
4. Transfer 7 ml (~1×10^{10} to 1.4×10^{10} XL1-Blue MRF´ cells) to a 50-ml conical tube. Incubate the tube for 10 to 15 min at 37°C without shaking to regrow the pili.

Note: For a primary λZAP library, use 10^6 pfu of the primary library, 10^7 XL1-Blue MRF´ cells and 10^8 pfu of ExAssist helper phage in a total volume of 7 ml of 10 mM $MgSO_4$.

5. Add 10^9 clones of the amplified λZAP cDNA library and 0.1 ml of amplified ExAssist helper phage (10^{11} pfu from step 5 of section 5.1.2). Incubate the excision mixture without shaking for 15 min at 37°C.
6. Transfer the excision mixture to a 125-ml non-baffled flask containing 28 ml of 2X YT (for a total volume of 35 ml) and continue incubating for 130 min at 37°C with shaking (200 rpm).
7. Heat the excision culture at 65°C for 15 min and transfer to a 50-ml conical tube.
8. Centrifuge the tube at 4°C for 15 min at 4,000 × *g*. Transfer the phagemid-cDNA library supernatant into a fresh 50-ml conical tube. The 1×10^9 clones of amplified library should yield a total of ~1×10^{12} excised phagemid (per 35 ml) and the 1×10^6 clones of primary library will yield a total of ~1×10^9 excised phagemid (per 35 ml). The excised phagemid can be stored at 4°C for one month.

5.1.4. Conversion to ds RF pBluescript cDNA

1. Thaw one tube of the XLOLR (or SOLR) cells and transfer 0.1 ml of cells into a 250-ml non-baffled flask containing 100 ml of 2X YT with 15 μg/ml of tetracycline (for SOLR cells use 75 μg/ml kanamycin). Incubate at 37°C at 200 rpm for 11 h or until the culture reaches an OD_{590} ~0.8.
2. For use with an amplified library, transfer the entire 100 ml culture into a 2-L flask containing 1 L of 2X YT with 15 μg/ml of tetracycline (or 75 μg/ml kanamycin) and incubate at 37°C at 250 rpm for 2.5 h or until the culture reaches an OD_{590} ~0.5 (a total cell population of ~3×10^{11}).

Note: For a primary library, transfer 80 ml of the culture into two 50-ml conical tubes and centrifuge at 2000 × *g* for 10 min at 4°C. Resuspend the pellets in a total volume of 37 ml of 10 mM $MgSO_4$ and proceed to step 5.

3. Transfer the 1-L culture into two autoclaved 500-ml centrifuge bottles and centrifuge at 2000 × *g* for 10 min at 4°C.
4. Resuspend (do not vortex) the pellets in a total volume of 100 ml of 10 mM $MgSO_4$.
5. Transfer the cell suspension into a 250-ml non-baffled flask and incubate without shaking for 10 to 15 min at 37°C to regrow the pili.
6. Add 3.5 ml of the phagemid-cDNA library supernatant (from step 8 of 5.1.3) (~10^{11} pfu of phagemid cDNA from the amplified library or ~10^8 pfu of phagemid cDNA from the primary library). Incubate without shaking for 15 min at 37°C.

Note: Clones are prone to deletions if the culture is grown at temperatures higher than 30°C.

7. Transfer the culture into a 2.8-L non-baffled flask containing 400 ml of 2X YT medium for the amplified library (or a 0.5-L non-baffled flask containing 160 ml of 2X YT medium for the primary library) and mix well.
8. Incubate the culture for 2 h at 30°C and 200 rpm. Add ampicillin to a final concentration of 100 μg/ml and continue incubating for 14 h at 30°C and 250 rpm.

Note: This method yields 150-250 μg of ds DNA. If the yield of ds DNA is significantly lower, the amplified ExAssist helper phage, the cDNA library, and the phage cDNA library should be titrated to determine the exact amounts to use in each section of the mass excision protocol.

9. Measure the OD_{590} of the culture. Use 500 OD_{590} units to make ds DNA using

the protocol described in Section 3.3.

5.2 Preparation of dsDNA

If the protocol in Chapter 3 does not result in DNA of sufficient purity, a CsCl purification method, as described by Sambrook et al. (1), may help improve the Gene II-Exo III reaction. Prepare a large preparation of ds plasmid DNA according to Sambrook (1) with the modifications listed below:

Note: Use 2.5 × 10^9 cfu from an amplified library.

1. Inoculate 200 ml of LB broth containing 100 µg/ml ampicillin with ⩾1 × 10^6 independent clones and incubate at 30°C overnight.
2. After lysis, but before purification or centrifugation in cesium chloride-ethidium bromide gradients, dissolve the plasmid DNA in 5 ml of TE buffer. If ELECTROMAX DH12S™ cells were used, incubate at 65°C for 10 min (to inactivate the *end*A protein).
3. After purification and dialysis, ethanol precipitate the plasmid DNA.
4. Resuspend the pellet in 200 µl of TE buffer, add 1 µl of a 20 mg/ml proteinase K solution, and incubate the reaction mixture at 42°C for 1 h.
5. Extract the plasmid DNA twice with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitate, and dissolve in 100 µl of TE buffer.

5.3 Preparation of ssDNA

Make a large scale ss plasmid DNA preparation from the plasmid-cDNA library.

1. Inoculate 200 ml LB broth containing 100 µg/ml ampicillin in a 1-L flask with ⩾1 × 10^6 primary transformants. Incubate the flask at 37°C with shaking (275 rpm) for 3 h.
2. Add 200 µl of M13KO7 helper phage (>1 × 10^{11} pfu/ml) to the culture, and continue to incubate the culture for 2 h.
3. Add 1.5 ml of 1% (w/v) kanamycin to the cells for a final concentration of 75 µg/ml. Incubate the infected cells for an additional 18 to 24 h at 37°C.
4. Centrifuge this culture at 16,000 × *g* for 15 min at 4°C.
5. Filter the supernatant through a 0.2-µm sterile filter into an autoclaved centrifuge bottle. Add 40 µl of DNase I (50 units/µl) and incubate at room temperature for 3 h. This step should remove any residual ds plasmid DNA contamination.
6. Transfer 100 ml of the supernatant to another centrifuge bottle. Add 25 ml of a solution containing 40% (w/v) PEG 4000, 2.5 M NaCl to each of the centrifuge bottles containing the supernatant.
7. Vortex the mixture, incubate on ice for 1 h, and centrifuge at 16,000 × *g* for 20 min at 4°C.
8. Carefully discard the supernatant. To fully drain off the remaining solution from the pellets, place the bottles on an angle, with the pellet side facing up, for 10 to 15 min. Remove the solution with a sterile Pasteur pipette.
9. Resuspend the pellets in a total volume of 2 ml of TE buffer. Add 10 µl of proteinase K solution (20 mg/ml), 20 µl of 10% SDS, and incubate this mixture at 45°C for 1 h.
10. Transfer the digested mixture to three microcentrifuge tubes, and extract four times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), precipitate with ethanol, and dissolve in 100 µl TE buffer.
11. Freeze the solubilized DNA at –20°C for 1 h and microcentrifuge at 14,000 × *g* for 15 min at 4°C.
12. Transfer the supernatant containing the ss plasmid DNA to a fresh tube, and discard the polysaccharide pellet. Store the ssDNA at 4°C.

13. Determine the DNA concentration (OD_{260}). On average, 100 to 200 µg of ssDNA can be obtained from 200 ml of cells.

5.4 Preparation of a 12% Polyacrylamide Gel

Note: Heating the gel mix at 65°C may be required to dissolve the urea. After heating, cool on wet ice for 3 min.

1. Prepare 300 ml of gel mix (12%) by adding 144 g of urea, 90 ml of 40% acrylamide [19:1, acrylamide:bis (stored at 4°C)], 30 ml of 10X TBE buffer, and water to a final volume of 300 ml.
2. After the urea is completely dissolved, add 2.0 ml of 10% ammonium persulfate and 200 µl of TEMED and mix well.
3. Pour into the glass sandwich. Insert the comb and clamp over the comb. The gel should be polymerized within 30 min.

5.5 Preparation of a 16% Polyacrylamide Gel

1. Prepare 30 ml of gel mix (16%) by adding 16.2 g of urea, 12 ml of 40% acrylamide [19:1, acrylamide:bis (stored at 4°C)], 3 ml of 10X TBE buffer, and 3 ml of water.
2. After the urea is completely dissolved, add 150 µl of 10% ammonium persulfate and 20 µl of TEMED and mix well.
3. Pour into the glass sandwich. Insert the comb and clamp over the comb. The gel should be polymerized within 30 min.

5.6 Media

2X YT

Component	Amount/liter
Bacto-Tryptone	16 g
Bacto-Yeast Extract	10 g
NaCl	5 g

Adjust to pH 7.5 with NaOH**.**

S.O.B.

Component	Amount/liter
Bacto-Tryptone	20 g
Bacto-Yeast Extract	5 g
NaCl	0.584 g
KCl	0.186 g

Adjust to pH 7 with NaOH.

Terrific Broth

Component	Amount
Bacto-Tryptone	12 g
Bacto-Yeast Extract	24 g
Glycerol	4 ml
Distilled water	900 ml

Combine materials and autoclave.

Component	Amount
KH_2PO_4 (monobasic)	2.3 g
K_2HPO_4 (dibasic)	12.5 g
Distilled water	100 ml

Combine materials and autoclave.

After stocks have cooled, combine the two stocks and mix well.

References

6

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7 Related Products

Product	Size	Cat. No
Combination Systems		
SuperScript Plasmid System and ElectroMAX DH10B Competent Cells	one set	19625-011
SuperScript Plasmid System and UltraMAX DH5α-FT Competent Cells	one set	18248-047
GeneTrapper cDNA Positive Selection System and ElectroMAX DH10B Competent Cells	one set	10356-038
GeneTrapper cDNA Positive Selection System and MagnaSep Magnetic Particle Separator	one set	10356-046
GeneTrapper cDNA Positive Selection System and ElectroMAX DH10B Competent Cells plus MagnaSep Magnetic Particle Separator	one set	10356-053
GeneTrapper cDNA Positive Selection System and UltraMAX DH5α-FT Competent Cells	one set	10356-145
GeneTrapper cDNA Positive Selection System and UltraMAX DH5α-FT Competent Cells plus MagnaSep Magnetic Particle Separator	one set	10356-137
SuperScript™ cDNA Libraries:		
Human Brain	2 × 0.5 ml	10418-010
Human Heart	2 × 0.5 ml	10419-018
Human Kidney	2 × 0.5 ml	10420-016
Human Leukocyte	2 × 0.5 ml	10421-014
Human Liver	2 × 0.5 ml	10422-012
Human Lung	2 × 0.5 ml	10424-018
Human Spleen	2 × 0.5 ml	10425-015
Human Testis	2 × 0.5 ml	10426-013
Human Fetal Brain	2 × 0.5 ml	10662-013
Rat Brain	2 × 0.5 ml	10653-012
Rat Liver	2 × 0.5 ml	10654-010
Mouse Brain	2 × 0.5 ml	10655-017
Mouse Liver	2 × 0.5 ml	10656-015
Mouse Kidney	2 × 0.5 ml	10657-013
Mouse Testis	2 × 0.5 ml	10658-011
Mouse 8.5-Day Embryo	2 × 0.5 ml	10664-019
Mouse 10.5-Day Embryo	2 × 0.5 ml	10665-016
Mouse 13.5-Day Embryo	2 × 0.5 ml	10666-014
Mouse 15.5-Day Embryo	2 × 0.5 ml	10667-012
Products for Purification		
Phenol	100 g	15509-011
	500 g	15509-037
Phenol:Chloroform:Isoamyl Alcohol, (25:24:1, v/v/v)	100 ml	15593-031
Products for Transformation		
UltraMAX DH5α-FT Competent Cells	5 × 0.1 ml	10643-013
MAX Efficiency® DH5α™ Competent Cells	1 ml	18258-012
MAX Efficiency DH10B Competent Cells	1 ml	18297-010
IPTG	1 g	15529-019
S.O.C. Medium	10 × 10 ml	15544-018
X-gal	100 mg	15520-034

Product	Size	Cat. No
Products for Electroporation		
ELECTROMAX DH10B Cells	5 × 0.1 ml	18290-015
ELECTROMAX DH12S Cells	5× 0.1 ml	18312-017
CELL-PORATOR *E. coli* Electroporation System	---	11613-015
CELL-PORATOR Electroporation System with 50 disposable microelectroporation chambers	---	11609-013
CELL-PORATOR Voltage Booster	---	11612-017
Disposable Microelectroporation Chambers	pkg. of 50	11608-031
Other Related Products		
SUPERSCRIPT II RNase H- Reverse Transcriptase	10,000 units	18064-014
Taq DNA Polymerase, Native	100 units	18038-018
	500 units	18038-042
	1,500 units	18038-067
Taq DNA Polymerase, Recombinant	100 units	10342-053
	500 units	10342-046
	1,500 units	10342-046
PCR SUPERMIX	100 reactions	10572-014
GENETRAPPER cDNA Positive Selection System	5 reactions	10356-020
SUPERSCRIPT Plasmid System for cDNA Synthesis and Cloning		
SUPERSCRIPT Choice System for cDNA Synthesis and Cloning		
Glycogen	100 µl	10814-010
1 Kb DNA Ladder	250 µg	15615-016
	1 mg	15615-024
1 Kb PLUS DNA Ladder™	250 µg	10787-018
	1 mg	10787-026
Acrylamide	100 g	15512-015
	500 g	15512-023
Agarose	100 g	15510-019
	500 g	15510-027
10 mg/ml Ethidium Bromide	10 ml	15585-011
10X TAE Buffer	1 L	15558-042
	4 L	15558-026
	10 L	15558-034
10X TBE Buffer	1 L	15581-044
	4 L	15581-036
	10 L	15581-028
1 M Tris-HCl (pH 7.5)	1 L	15567-027

Part No. 10356

Lot No. KDRP01-0998