USER GUIDE

invitrogen™
by life technologies™

CloneMiner™ II cDNA Library Construction Kit

High-quality cDNA libraries without the use of restriction enzyme cloning techniques

Catalog Number A11180

Revision Date 23 March 2012

Publication Part Number A11233

MAN0001697

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

life technologies™

Contents

Kit Contents and Storage

Shipping and Storage

The CloneMiner™ II cDNA Library Construction Kit is shipped on dry ice. Upon receipt, store the components as detailed below. All components are guaranteed for six months if stored properly.

Item	Storage
Components for cDNA Library Construction	**–30°C to –10°C**
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	**–85°C to –68°C**
cDNA Size Fractionation Columns	**2°C to 8°C**

Number of reactions

The CloneMiner™ II cDNA Library Construction Kit provides enough reagents to construct five cDNA libraries. While some reagents are supplied in excess, you may need additional reagents and materials if you wish to perform more than 5 reactions. You may also need additional electrocompetent *E. coli* cells if you perform control reactions each time you construct a cDNA library. See page 49 for ordering information.

Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Components for cDNA Library Construction

The components for cDNA library construction are listed below. Store all components at –30°C to –10°C.

Item	Composition	Amount
2.0 kb RNA control	0.5 µg/µL in: 10 mM HEPES 2 mM EDTA, pH 7.2	15 µL
DEPC-treated Water	Sterile, DEPC-treated water	1 mL
Biotin-*att*B2-Oligo(dT) Primer	30 pmol/µL in DEPC-treated water	8 µL
10 mM (each) dNTP	10 mM dATP 10 mM dGTP 10 mM dCTP 10 mM dTTP in 1 mM Tris-HCl, pH 7.5	20 µL
5X First Strand Buffer	250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $MgCl_2$	1 mL
0.1 M Dithiothreitol (DTT)	in DEPC-treated water	250 µL

Continued on next page

Components for cDNA Library Construction, Continued

Item	Composition	Amount
SuperScript® III Reverse Transcriptase	200 U/µL in: 20 mM Tris-HCl, pH 7.5 0.1 mM EDTA 100 mM NaCl 0.01% NP-40 (v/v) 1 mM DTT 50% Glycerol (v/v)	40 µL
5X Second Strand Buffer	100 mM Tris-HCl, pH 6.9 450 mM KCl 23 mM $MgCl_2$ 0.75 mM β-NAD 50 mM $(NH_4)_2SO_4$	500 µL
E. coli DNA Ligase	10 U/µL in: 10 mM Tris-HCl, pH 7.4 50 mM KCl 0.1 mM EDTA 1 mM DTT 0.2 mg/mL BSA 50% Glycerol (v/v) 0.1% Triton® X-100 (w/v)	10 µL
UltraPure™ Glycogen	20 µg/µL in RNase-free water	45 µL
E. coli DNA Polymerase I	10 U/µL in: 50 mM Potassium Phosphate, pH 7.0 100 mM KCl 1 mM DTT 50% Glycerol (v/v)	50 µL
E. coli RNase H	2 U/µL in: 20 mM Tris-HCl, pH 7.5 100 mM KCl 10 mM $MgCl_2$ 0.1 mM EDTA 0.1 mM DTT 50 µg/mL BSA 50% Glycerol (v/v)	20 µL

Continued on next page

Components for cDNA Library Construction, Continued

Item	Composition	Amount
T4 DNA Polymerase	5 U/µL in: 100 mM Potassium Phosphate, pH 6.5 10 mM β-mercaptoethanol 50% Glycerol (v/v)	15 µL
*att*B1 Adapter	1 µg/µL in: 10 mM Tris-HCl, pH 7.5 1 mM EDTA 0.1 M NaCl	70 µL
5X Adapter Buffer	330 mM Tris-HCl, pH 7.6 50 mM $MgCl_2$ 5 mM ATP	70 µL
T4 DNA Ligase	1 U/µL in: 100 mM Potassium Phosphate, pH 6.5 10 mM β-mercaptoethanol 50% Glycerol (v/v)	50 µL
pDONR™ 222 Vector	150 ng/µl vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	40 µL
Gateway® BP Clonase® II Enzyme Mix	Proprietary	40 µL
Proteinase K	2 µg/µL in: 10 mM Tris-HCl, pH 7.5 20 mM $CaCl_2$ 50% Glycerol (v/v)	40 µL
pEXP7-tet Control DNA	50 ng/µL in TE Buffer, pH 8.0	2 × 20 µL
30% PEG/Mg solution	30% PEG 8000/30 mM $MgCl_2$	2 × 1 mL

Biotin-*att*B2-Oligo(dT) Primer Sequence

The Biotin-*att*B2-Oligo(dT) Primer is biotinylated to block blunt-end ligation of the *att*B1 Adapter to the 3′ end of the cDNA during the adapter ligation step. The primer sequence is provided below, with the *att*B2 sequence in bold.

5′-Biotin-GGCGGCCGC**ACAACTTTGTACAAGAAAGTTGGGT**$(T)_{19}$-3′

Continued on next page

*att*B1 Adapter Sequences

The double-stranded adapter is made by denaturation and slow annealing of the two oligonucleotides in annealing buffer. The *att*B1 Adapter is supplied at 1 µg/µL. The sequence is provided below with the *att*B1 sequence in bold.

5′-TCGTCGGGG**ACAACTTTGTACAAAAAAGTTGG-**3′
3′-CCCC**TGTTGAAACATGTTTTTTCAACC**p-5′

DH10B™ T1 Phage Resistant Cells

One box of ElectroMAX™ DH10B™ T1 Phage Resistant Cells is provided with the kit. Transformation efficiency is >1 × 10^{10} cfu/µg DNA. Each box includes the following items. Store at −85°C to −68°C.

Item	Composition	Amount
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	—	5 × 100 µL
pUC19 Control DNA	10 pg/µL in: 5 mM Tris-HCl 0.5 mM EDTA, pH 8	50 µL
S.O.C. Medium (may be stored at room temperature or 2°C to 8°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose	2 × 6 mL

Genotype of DH10B™ T1 Phage Resistant Cells

F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G *ton*A

cDNA Size Fractionation Columns

Two boxes containing three disposable columns each are provided with the kit for a total of six columns. Each column contains 1 mL of Sephacryl® S-500 HR prepacked in 20% ethanol. Store columns at 2°C to 8°C.

Introduction

About the Kit

Kit usage

The CloneMiner™ II cDNA Library Construction Kit is designed to construct high-quality cDNA libraries without using traditional restriction enzyme cloning methods. This novel technology combines the performance of SuperScript® III Reverse Transcriptase with the Gateway® Technology. Libraries can be created with as little as 50 ng of mRNA as starting material. PCR amplification is not required, thus avoiding bias that may be introduced by amplification.

Single-stranded mRNA is converted into double stranded cDNA containing *att*B sequences on each end. Through site-specific recombination, *att*B-flanked cDNA is cloned directly into an *att*P-containing donor vector without the use of restriction digestion or ligation.

The resulting Gateway® entry cDNA library can be screened with a probe to identify a specific entry clone. This clone can be transferred into the Gateway® destination vector of choice for gene expression and functional analysis. Alternatively, the entire entry cDNA library can be shuttled into a Gateway® destination vector to generate an expression library. For more information on the Gateway® Technology, see page 10.

Features of the CloneMiner™ II cDNA Library Construction Kit

Features of the CloneMiner™ II cDNA Library Construction Kit include:

- SuperScript® III reverse transcriptase for efficient conversion of mRNA into cDNA
- Biotin-*att*B2-Oligo(dT) Primer for poly(A) mRNA binding and incorporation of the *att*B2 sequence to the 3′ end of cDNA
- *att*B1 Adapter for ligation of the *att*B1 sequence to the 5′ end of double-stranded cDNA
- *att*P-containing vector (pDONR™ 222) for recombination with *att*B-flanked cDNA to produce an entry library through the Gateway® BP recombination reaction (see pages 57–58 for a map and list of features)

Advantages of the CloneMiner™ II cDNA Library Construction Kit

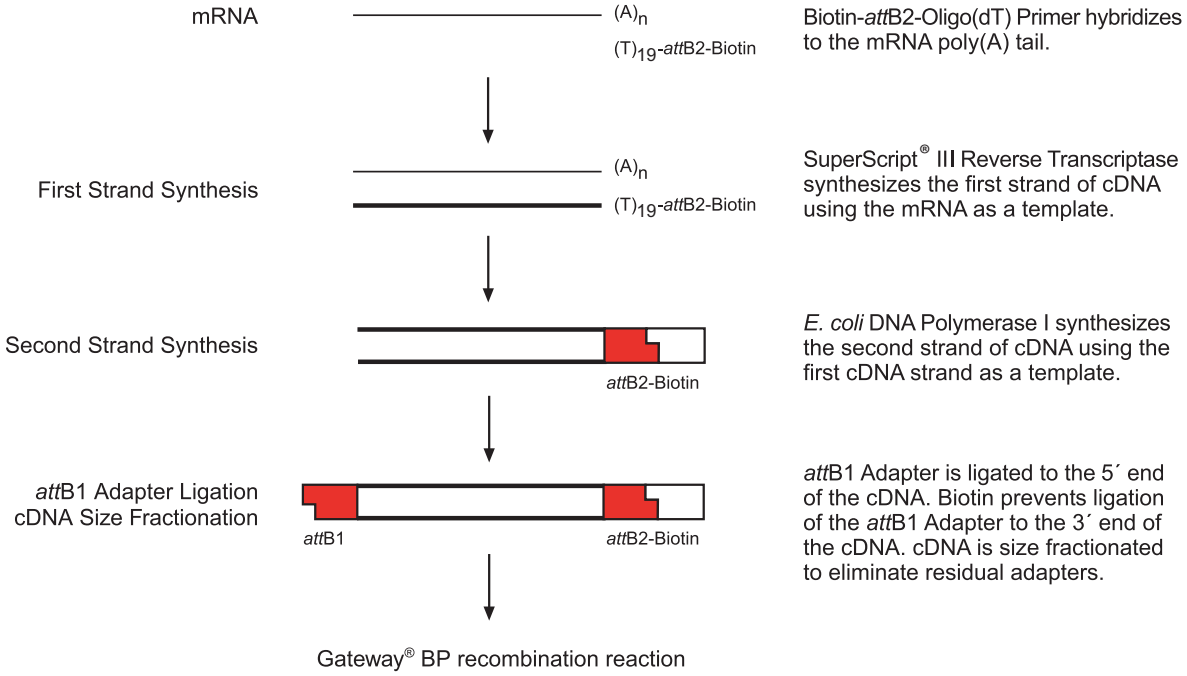
Using CloneMiner™ II cDNA Library Construction Kit offers these advantages:

- Produces high yields of quality, double-stranded cDNA
- Eliminates use of restriction enzyme digestion and ligation allowing cloning of undigested cDNA
- Allows the creation of nano-quantity cDNA libraries
- Highly efficient recombinational cloning of cDNA into a donor vector results in a higher number of primary clones compared to standard cDNA library construction methods (Ohara & Temple, 2001)
- Reduces number of chimeric clones and reduces size bias compared to standard cDNA library construction methods (Ohara & Temple, 2001)
- Enables highly efficient transfer of your cDNA library into multiple destination vectors for protein expression and functional analysis

Continued on next page

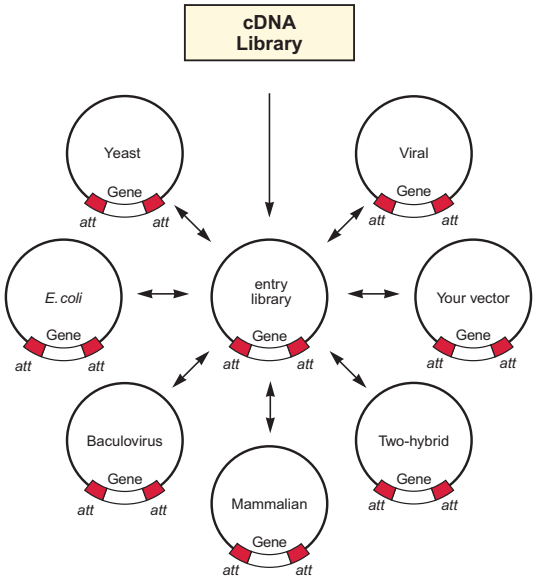
Experimental Summary

The following diagram summarizes the cDNA synthesis process of the CloneMiner™ II cDNA Library Construction Kit.



Gateway® Technology

Gateway® is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). Gateway® Technology provides a rapid and highly efficient way to move DNA sequences into multiple vector systems for functional analysis and protein expression. For more information on Gateway® Technology, see the next page.



Gateway® Technology

Description of Gateway® Technology

Gateway® Technology is based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E. coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). The components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985). This section provides a brief overview of Gateway® Technology. For detailed information, refer to the Gateway® Technology manual. This manual is available from **www.lifetechnologies.com/support** or by contacting Technical Support (page 60).

Recombination Components

Lambda-based recombination involves two major components:

- The DNA recombination sequences (*att* sites) **and**
- The proteins that mediate the recombination reaction (i.e. Gateway® Clonase® enzyme mix)

Characteristics of recombination reactions

Lambda integration into the *E. coli* chromosome occurs via intermolecular DNA recombination that is mediated by a mixture of lambda and *E. coli*-encoded recombination proteins (i.e. Gateway® Clonase® enzyme mix). The hallmarks of lambda recombination are:

- Recombination occurs between specific (*att*) sites on the interacting DNA molecules.
- Recombination is conservative (i.e. there is no net gain or loss of nucleotides) and does not require DNA synthesis. The DNA segments flanking the recombination sites are switched, such that after recombination, the *att* sites are hybrid sequences comprised of sequences donated by each parental vector. For example, *att*L sites are comprised of sequences from *att*B and *att*P sites.
- Strand exchange occurs within a core region that is common to all *att* sites (see next page).

For more detailed information about lambda recombination, see published references and reviews (Landy, 1989; Ptashne, 1992).

Continued on next page

Gateway® Technology, Continued

att Sites

Lambda recombination occurs between site-specific attachment (*att*) sites: *att*B on the *E. coli* chromosome and *att*P on the lambda chromosome. The *att* sites serve as the binding site for recombination proteins and have been well characterized (Weisberg & Landy, 1983). Upon lambda integration, recombination occurs between *att*B and *att*P sites to give rise to *att*L and *att*R sites. The actual crossover occurs between homologous 15 bp core regions on the two sites, but surrounding sequences are required as they contain the binding sites for the recombination proteins (Landy, 1989).

In the CloneMiner™ II cDNA Library Construction Kit, the wild-type *att*B sites encoded by the *att*B1 Adapter and Biotin-*att*B2-Oligo(dT) Primer and the wild-type *att*P1 and *att*P2 sites encoded by pDONR™ 222 are modified to improve the efficiency and specificity of the Gateway® BP recombination reaction.

*ccd*B Gene

The presence of the *ccd*B gene in pDONR™ 222 allows negative selection of the donor vector in *E. coli* following recombination and transformation. The CcdB protein interferes with *E. coli* DNA gyrase (Bernard & Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (*e.g.* DH5α™, TOP10). When recombination occurs between pDONR™ 222 and the *att*B-flanked cDNA, the *ccd*B gene is replaced by the cDNA insert. Cells that take up nonrecombined pDONR™ 222 carrying the *ccd*B gene or by-product molecules retaining the *ccd*B gene will fail to grow. This allows high-efficiency recovery of the desired clones.

Gateway® Recombination Reactions

Two recombination reactions constitute the basis of the Gateway® Technology. By using the CloneMiner™ II cDNA Library Construction Kit, you can take advantage of these two reactions to clone and shuttle your cDNA library into a destination vector of choice.

BP Reaction: Facilitates recombination of *att*B-flanked cDNA with an *att*P-containing vector (pDONR™ 222) to create an *att*L-containing entry library (see the following diagram). This reaction is catalyzed by Gateway® BP Clonase® II enzyme mix.

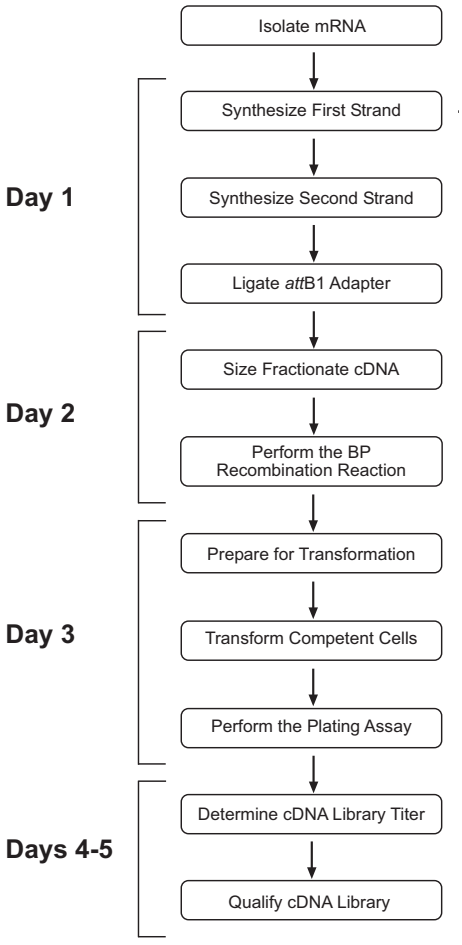
LR Reaction: Facilitates recombination of an *att*L entry clone or entry library with an *att*R substrate (destination vector) to create an *att*B-containing expression clone or expression library (see the following diagram). This reaction is catalyzed by Gateway® LR Clonase® or Gateway® LR Clonase II® enzyme mix.

Experimental Timeline

Introduction

The CloneMiner™ II cDNA Library Construction Kit is designed to produce an entry library from your starting mRNA within 3 days. It will take an additional 2 days to determine the titer and quality of the cDNA library. Note that this manual is organized according to the recommended timeline below. If you are not following this timeline, be sure to plan ahead for convenient stopping points (see below for more information).

Recommended timeline



Optional stopping points

If you cannot follow the recommended timeline, you may stop the procedure during any ethanol precipitation step. These steps occur during second strand synthesis and size fractionation, and are noted as optional stopping points. When stopping at these points, always store the cDNA as an **uncentrifuged** ethanol precipitate at –20°C to maximize cDNA stability.

Experimental Overview

Introduction

The experimental steps necessary to synthesize *att*B-flanked cDNA and to generate an entry library are outlined below. After isolating your mRNA (page 15), you will need a minimum of 3 days to construct a cDNA library. For more details on each step, refer to the indicated pages for your specific method.

Day	Step	Action	Page
1	1	Synthesize the first strand of cDNA from your isolated mRNA using the Biotin-*att*B2-Oligo(dT) Primer and SuperScript® III RT.	18
	2	Synthesize the second strand of cDNA using the first strand cDNA as a template.	22
	3	Ligate the *att*B1 adapter to the 5′ end of your cDNA.	25
2	1	Size fractionate the cDNA by column chromatography to remove excess primers, adapters, and small cDNA.	28
	2	Perform the BP recombination reaction between the *att*B-flanked cDNA and pDONR™ 222.	32
3	1	Transform the BP reactions into ElectroMAX™ DH10B™ T1 Phage Resistant cells by electroporation. Add freezing media to transformed cells to get final cDNA library.	35
	2	Perform the plating assay to determine the cDNA library titer.	40
4–5	1	Calculate the cDNA library titer using the results from the plating assay.	42
	2	Inoculate 24 positive transformants from the plating assay. Determine average insert size and percent recombinants by restriction analysis.	43
	3	Sequence entry clones to verify presence of cDNA insert, if desired.	45

Methods

Before Starting

Important

The CloneMiner™ II cDNA Library Construction Kit is designed to help you construct a cDNA library without the use of traditional restriction enzyme cloning methods. Use of this kit is geared towards those users who have some familiarity with cDNA library construction. We highly recommend that users possess a working knowledge of mRNA isolation and library construction techniques before using this kit. For more information about these topics, refer to the following published reviews:

- cDNA library construction using restriction enzyme cloning: see Gubler and Hoffman, 1983 and Okayama and Berg, 1982
- cDNA library construction using the λ-*att* recombination system: see Ohara and Temple, 2001 and Ohara *et. al.*, 2002
- mRNA handling techniques: see Chomczynski and Sacchi, 1987

Choosing a library construction method

There are several ways to construct your cDNA library using the CloneMiner™ II cDNA Library Construction Kit. Choices include:

- whether or not to radiolabel your cDNA
- the scale of the library (nano-quantity vs. standard)
- size fractionation method (column vs. gel)
- how to determine cDNA yields

We recommend that you follow the protocol as described in this manual, but descriptions are provided for optional protocols, which can be performed, depending upon your needs.

Radiolabeling vs. non-radiolabeling

Radiolabeling your cDNA is an optional protocol for the CloneMiner™ II cDNA Library Construction Kit. The radiolabeling protocol allows direct measurement of cDNA yield and overall quality of the first strand reaction. Review the CloneMiner™ cDNA Construction Kit Web Appendix at **www.lifetechnologies.com** for the advantages and disadvantages of each method.

Nano-quantity vs. standard libraries

The CloneMiner™ II cDNA Library Construction Kit allows you to create libraries with as little as 50 ng of starting mRNA. **Nano-quantity libraries** are prepared from <0.5 µg starting mRNA. Reaction volumes are halved for generation of nano-quantity libraries compared to standard libraries. **Standard libraries** are created with 0.5–5 µg of mRNA.

Number of reactions

This kit provides enough reagents to construct 5 cDNA libraries. While some reagents are supplied in excess, you may need additional reagents and materials if you wish to perform more than 5 reactions, or if you are starting with larger quantities of mRNA. You may also need additional electrocompetent *E. coli* cells if you will be performing control reactions (2.0 kb RNA control, pEXP7-tet control, BP negative control, and pUC19 transformation control) each time you construct a cDNA library.

Isolate mRNA

Introduction

You will need to isolate high-quality mRNA using a method of choice prior to using this kit. Follow the guidelines provided below to avoid RNase contamination.

Aerosol-resistant pipette tips are recommended for all procedures. See the following section for general recommendations for handling mRNA.

General Handling of mRNA

When working with mRNA:

- Use disposable, individually wrapped, sterile plasticware
- Use only sterile, RNase-free pipette tips and RNase-free microcentrifuge tubes
- Wear latex gloves while handling all reagents and mRNA samples to prevent RNase contamination from the surface of the skin
- Always use proper microbiological aseptic technique when working with mRNA
- You may use RNase *AWAY*® Reagent, or RnaseZap® (see page 49 for ordering information), to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see *Current Protocols in Molecular Biology* Ausubel *et al.*, 1994 or *Molecular Cloning: A Laboratory Manual* Sambrook *et al.*, 1989.

Isolate mRNA

You can isolate mRNA from tissue, cells, or total RNA using your method of choice. We recommend isolating mRNA using the FastTrack® MAG Micro mRNA Isolation Kit or the FastTrack® MAG Maxi mRNA Isolation Kit (see page 49 for ordering information).

Generally, 1–5 µg of mRNA is sufficient to construct a cDNA library containing 10^6–10^7 primary clones in *E. coli*. Smaller amounts of mRNA down to 50 ng can also be used to construct nano-quantity libraries containing ~10^5–10^6 primary clones.

Resuspend isolated mRNA in DEPC-treated water and check the quality of your preparation (see next page). Store your mRNA preparation at –80°C. We recommend aliquoting your mRNA into multiple tubes to reduce the number of freeze/thaw cycles.

Important

The CloneMiner™ II cDNA Library Construction Kit allows you to create cDNA libraries with small amounts of starting mRNA, but unless you are trying to create nano-quantity libraries (due to limited quantities of starting material), it is recommended to proceed with library creation only when you have enough material to perform the standard reaction, to assure adequate representation of primary clones.

To ensure success, use the highest quality mRNA possible. Check the quantity, integrity, and purity of your mRNA before starting (see page 16).

Continued on next page

Important

If you are creating a **standard library**, use 0.5–5 ug of starting mRNA. If you do not have enough mRNA, perform a new isolation with additional tissue.

For **nano-quantity libraries**, it is not always feasible to check the mRNA yield or quality. In such cases, determine the quality, and quantity of the total RNA. Estimate the amount of mRNA as being 1–5% of the total RNA. Use 50–500 ng of mRNA to generate a nano-quantity library.

Check the RNA yield

Use the following general protocol to calculate the yield of total or mRNA using A_{260} absorbance:

1. Aliquot 2 µL of the RNA into a clean UV cuvette and add 198 µL of TE Buffer for a 1:100 dilution.
2. Blank a UV/visible spectrophotometer using TE Buffer, and then measure the sample at 260 nm.
3. The A_{260} reading should fall within the standard specification for the spectrophotometer (typically 0.01–1.0 OD). If it falls outside this range, adjust the dilution and rescan. If the A_{260} reading is too low, use a lower dilution; if it's too high, use a higher dilution.
4. Calculate the yield of RNA using the formula below:

   RNA yield (µg/µL) = A_{260} × 0.04 µg/µL RNA × Dilution factor

The dilution factor is 100 for the dilution described in this procedure. For example, if you diluted 2 µL of mRNA at 1:100, and the A_{260} is 0.5, then 0.5 × 0.04 µg/µl RNA × 100 = 2 µg/µL.

Check the total RNA quality

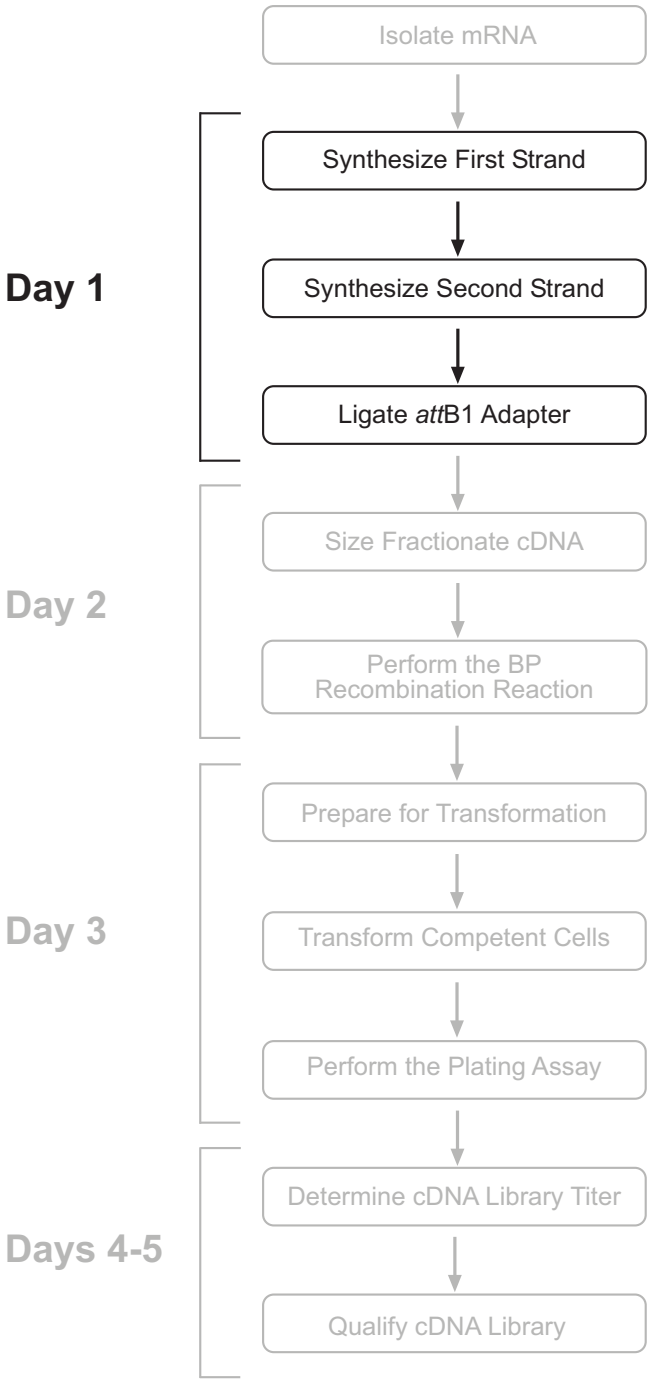
To check total RNA integrity, analyze 1 µg of your RNA by agarose gel electrophoresis. You should see the following on a denaturing agarose gel:

- 28S rRNA band (4.5 kb) and 18S rRNA band (1.9 kb) for mammalian species
- 28S band should be twice the intensity of the 18S band

Check the mRNA quality

To check quality, analyze 100 ng of your mRNA using a Bioanalyzer, or by performing agarose gel electrophoresis using a 1% E-Gel® EX Gel (see page 49 for ordering information). Your mRNA will appear as a smear, with the greatest intensity in the range of 1–3 kb. Some rRNA bands may still be faintly visible. If you do not detect a smear, or if the majority of the smear is significantly less than 1 kb, you will need to repeat the RNA isolation. Be sure to follow the recommendations listed on page 15 to prevent RNase contamination.

Day 1: Synthesize cDNA with Flanking *att*B Sites



Synthesize the First Strand

Introduction

This section provides detailed guidelines for synthesizing the first strand of cDNA from your isolated mRNA. The reaction conditions for first strand synthesis catalyzed by SuperScript® III RT have been optimized for yield and size of the cDNAs. To ensure that you obtain the best possible results, we suggest you read this section and the sections entitled **Synthesize the Second Strand** (pages 22–24) and **Ligate the *att*B1 Adapter** (pages 25–26) before beginning.

Important

cDNA synthesis is a multi-step procedure requiring many specially prepared reagents which are crucial to the success of the process. Quality reagents necessary for converting your mRNA sample into double-stranded cDNA are provided with this kit. To obtain the best results, **do not substitute any of your own reagents for the reagents supplied with the kit.**

Starting mRNA

To successfully construct a cDNA library, it is crucial to start with high-quality mRNA. For guidelines on isolating mRNA, see page 15. The amount of mRNA needed to prepare a library depends on the efficiency of each step. Using this kit, anywhere from 50 ng to 5 µg of mRNA is used to construct cDNA libraries containing 10^5–10^7 primary clones in *E. coli*.

2.0 kb RNA control

We recommend that you include the 2.0 kb RNA control in your experiments to help you evaluate your results.

Guidelines

Consider the following points before performing the priming and first strand reactions:

- We recommend using no more than 5 µg of starting mRNA for the first strand synthesis reaction
- Both the amount of DEPC-treated water used to dilute your mRNA and the total volume of your reactions will depend on the concentration of your starting mRNA
- We recommend using a thermocycler rather than a water bath both for ease and for accurate temperatures and incubation times
- Keep the tubes in the thermocycler or water bath when adding SuperScript® III RT to minimize temperature fluctuations

If you are constructing multiple libraries, we recommend making a cocktail of reagents to add to each tube rather than adding reagents individually. This reduces the time required for the step and also reduces the chance of error.

Continued on next page

Synthesize the First Strand, Continued

Required materials

Keep all reagents on ice until needed.

Supplied with kit:

- *Optional:* 2.0-kb RNA control (0.5 µg/µL)
- DEPC-treated water
- Biotin-*att*B2-Oligo(dT) Primer (30 pmol/µL)
- 10 mM (each) dNTPs
- 5X First Strand Buffer
- 0.1 M DTT
- SuperScript® III RT (200 U/µL)

Supplied by user:

- High-quality mRNA (up to 5 µg)
- Thermocycler (recommended) or water bath, heated to 70°C
- Ice bucket
- Thermocycler (recommended) or water bath, for stepwise incubations at 45°C, 50°C, and 55°C

Continued on next page

Dilute your starting mRNA

Dilute your starting mRNA with DEPC-treated water to the final volume indicated in the following table. Perform the dilution in an RNase-free 1.5-mL tube for **standard reactions** (0.5–5 µg starting mRNA), or an RNase-free 0.2-mL tube for **nano-quantity reactions** (<0.5 µg starting mRNA).

The total volume for your mRNA + DEPC-treated water varies with the amount of starting mRNA to account for the amount of SuperScript® III RT required for the first strand reaction.

Reagent	µg of starting mRNA					
	<0.5 (Nano)	**0.5–1**	**2***	**3**	**4**	**5**
mRNA + DEPC-treated water	5 µL	11 µL	10 µL	9 µL	8 µL	7 µL

*If you use the 2.0-kb RNA control supplied with the kit, add 6 µL of DEPC-treated water to 4 µL of the control mRNA (2 µg) for a total volume of 10 µL.

Priming reaction

1. Add the Biotin-*att*B2-Oligo(dT) Primer to your diluted mRNA (from **Dilute your starting mRNA**), according to the table below:

Reagent	Nano	Standard
Biotin-*att*B2-Oligo(dT) Primer (30 pmol/µL)	0.5 µL	1 µL

2. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample.
3. Incubate the mixture at 70°C for 7 minutes and allow it to gradually cool to 45°C over 15–30 minutes by ramping the temperature down if using a PCR machine, or turning the heat off if using a heat block. During these incubation steps, perform step 1 of the **First Strand Reaction**, next page.

Continued on next page

Synthesize the First Strand, Continued

First strand reaction

1. Add the following reagents to a fresh tube, according to the starting amount of mRNA used for the reaction:

Reagent	Nano	Standard
5X First Strand Buffer	2 µL	4 µL
0.1 M DTT	1 µL	2 µL
10 mM (each) dNTPs	0.5 µL	1 µL

2. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample, and place on ice until ready for use.

3. After the priming reaction (from step 3, page 20) has cooled to 45°C, add the mixture from step 1 of this procedure to the priming reaction tube. **Be careful to not introduce bubbles into your sample.** The total volume in the tube should now correspond to the following table:

Reagent	µg of starting mRNA					
	<0.5 (Nano)	**0.5–1**	**2**	**3**	**4**	**5**
Total Volume	9 µL	19 µL	18 µL	17 µL	16 µL	15 µL

4. Incubate the tube at 45°C for 2 minutes.

5. Keep the tube in the thermocycler or water bath, and carefully add SuperScript® III RT according to the following table.

Reagent	µg of starting mRNA					
	<0.5 (Nano)	**0.5–1**	**2**	**3**	**4**	**5**
SuperScript® III RT (200 U/µL)	1 µL	1 µL	2 µL	3 µL	4 µL	5 µL

   The total volume for all standard reactions should be 20 µL. For nano-quantity reactions, the total volume should be 10 µL.

6. With the tube still in the thermocycler or water bath, mix the contents gently by pipetting. **Be careful to not introduce bubbles**.

7. Incubate the reaction tube in stepwise increments as follows:

   45°C 20 minutes
   50°C 20 minutes
   55°C 20 minutes

Proceed to **Synthesize the Second Strand**, page 22.

Synthesize the Second Strand

Introduction

This section provides guidelines for synthesizing the second strand of cDNA. Perform all steps quickly to prevent the temperature from rising above 16°C.

Required materials

You should have the following materials on hand before beginning. Keep all reagents on ice until needed.

Supplied with kit:

- DEPC-treated water
- 5X Second Strand Buffer
- 10 mM (each) dNTPs
- *E. coli* DNA Ligase (10 U/µL)
- *E. coli* DNA Polymerase I (10 U/µL)
- *E. coli* RNase H (2 U/µL)
- T4 DNA Polymerase (5 U/µL)
- Glycogen (20 µg/µL)

Supplied by user:

- Ice bucket
- Thermocycler (recommended) or water bath at 16°C
- 0.5 M EDTA, pH 8.0
- Phenol:chloroform:isoamyl alcohol (25:24:1)
- 7.5 M NH_4OAc (ammonium acetate)
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol

Continued on next page

Synthesize the Second Strand, Continued

Second strand reaction

Perform all steps quickly to prevent the temperature from rising above 16°C.

1. Place the first strand reaction tube containing your cDNA on ice. Keep the tube on ice while adding the following reagents.

Reagent	Nano	Standard
DEPC-treated water	45.5 µL	91 µL
5X Second Strand Buffer	15 µL	30 µL
10 mM (each) dNTPs	1.5 µL	3 µL
E. coli DNA Ligase (10 U/µL)	0.5 µL	1 µL
E. coli DNA Polymerase I (10 U/µL)	2 µL	4 µL
E. coli RNase H (2 U/µL)	0.5 µL	1 µL

   The total volume should be 150 µL for standard reactions, and 75 µL for nanoscale reactions.

2. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample.
3. Incubate the tube at 16°C for 2 hours.
4. Add of T4 DNA Polymerase to create blunt-ended cDNA.

Reagent	Nano	Standard
T4 DNA Polymerase	1 µL	2 µL

   Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample.

5. Incubate at 16°C for 5 minutes.
6. Add 0.5 M EDTA, pH 8.0 to stop the reaction.

Reagent	Nano	Standard
0.5 M EDTA, pH 8.0	5 µL	10 µL

Proceed to **Phenol/Chloroform Extraction**, next page.

Continued on next page

Phenol/Chloroform extraction

1. Add phenol:chloroform:isoamyl alcohol (25:24:1) and shake by hand thoroughly for approximately 30 seconds.

Reagent	Nano	Standard
phenol:chloroform:isoamyl alcohol (25:24:1)	80 µL	160 µL

2. Centrifuge at room temperature for 5 minutes at 16,000 × g. Carefully remove the upper aqueous phase, and transfer the layer to a fresh 1.5-mL tube. Be sure not to carry over any phenol during pipetting.

   Proceed to **Ethanol Precipitation**, the following section.

Ethanol precipitation

1. To the aqueous phase, add reagents in the following order:

Reagent	Nano	Standard
Glycogen (20 µg/µL)	0.5 µL	1 µL
7.5 M NH_4OAc	40 µL	80 µL
100% ethanol	300 µL	600 µL

   Note: You may stop at this point and store the tube at –20°C overnight if necessary.

2. Place the tube in dry ice or at –80°C for 10 minutes. Centrifuge the sample at 4°C for 25 minutes at 16,000 × g.

3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.

4. Centrifuge the sample at 4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.

5. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.

6. Resuspend the pellet in DEPC-treated water by pipetting up and down 30–40 times. Use the following amounts of DEPC-treated water depending upon your starting amount of mRNA.

Reagent	Nano	Standard
DEPC-treated water	11 µL	22 µL

7. Centrifuge for 2 seconds to collect the sample. Transfer the sample to a fresh tube and place on ice.

Proceed to **Ligate the *att*B1 Adapter**, page 25.

Ligate the *att*B1 Adapter

Introduction

Follow the guidelines in this section to ligate the *att*B1 Adapter to the 5′ end of your double-stranded cDNA.

Required materials

Keep all reagents on ice until needed.

Supplied with kit:

- 5X Adapter Buffer
- *att*B1 Adapter (1 µg/µL)
- 0.1 M DTT
- T4 DNA Ligase (1 U/µL)

Supplied by user:

- Ice bucket
- Thermocycler (recommended) or water bath at 16°C

Ligation protocol

1. Keep the tube containing your double-stranded, blunt-ended cDNA from step 6, page 24 on ice and add the following reagents:

Reagent	Nano	Standard
5X Adapter Buffer	5 µL	10 µL
*att*B1 Adapter (1 µg/µL)	2 µL	4 µL
0.1 M DTT	4 µL	8 µL
T4 DNA Ligase (1 U/µL)	3 µL	6 µL

2. Mix the contents gently by pipetting. Incubate at 16°C for 16–24 hours.

Continued on next page

Ligate the *att*B1 Adapter, Continued

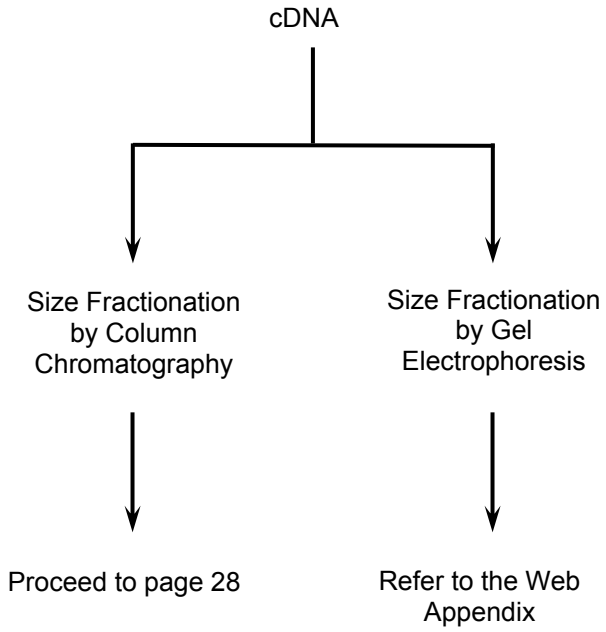
The next step

After ligating the *att*B1 Adapter to the 5′ end of your double-stranded cDNA, you will need to size fractionate the cDNA to generate cDNA that is free of adapters and other low molecular weight DNA.

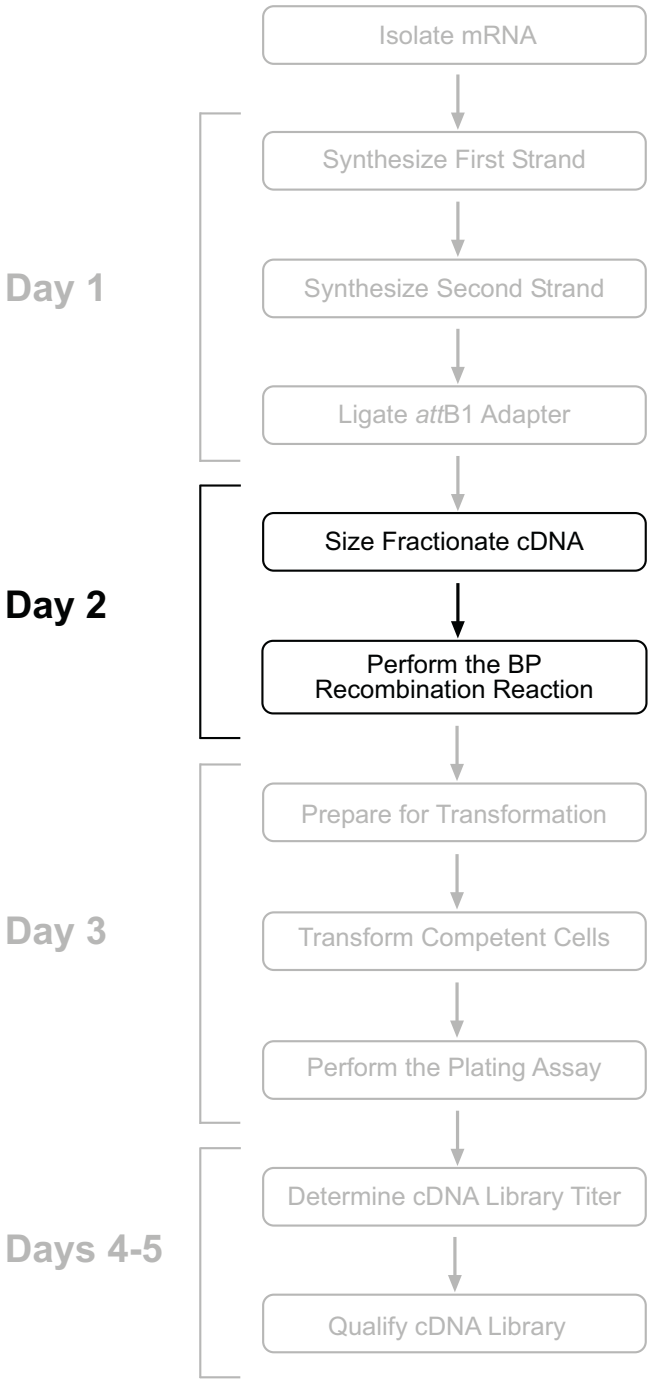
We recommend performing size fractionation by column chromatography (page 28), but depending upon your needs, you may also size fractionate your cDNA by gel electrophoresis.

The gel electrophoresis method to generate a cDNA library with a larger average insert size (>2.0 kb) or to select cDNA of a particular size (see the CloneMiner™ cDNA Construction Kit Web Appendix for details).

A flow chart is provided below to direct you to the next step.



Day 2: Size Fractionating cDNA by Column Chromatography and Performing the BP Recombination Reaction



Size Fractionate cDNA by Column Chromatography

Column chromatography

Column chromatography is commonly used to size fractionate cDNA. This method makes the cloning of larger inserts more probable, and generates a cDNA library with an average cDNA insert size of approximately 1.5 kb (when starting with high-quality mRNA).

Columns are provided with the kit. Follow instructions closely using the columns supplied with the kit to produce the highest quality library possible.

How the columns work

Each column provided with the kit contains 1 mL of Sephacryl® S-500 HR resin. This porous resin traps residual adapters, primers, and/or small truncated cDNAs (<500 bp) and prevents them from contaminating the library. Larger molecules bypass the resin and elute quickly while smaller molecules are retained within the resin and elute more slowly. Thus, earlier eluted fractions contain larger cDNA fragments than later fractions.

Required materials

Supplied with kit:

- cDNA Size Fractionation Columns
- Glycogen (20 µg/µL)

Supplied by user:

- cDNA sample
- TEN buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 25 mM NaCl)
- 100% ethanol
- 7.5 M NH_4OAc (ammonium acetate)
- Dry ice or –80°C freezer
- 70% ethanol
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)

Continued on next page

Size Fractionate cDNA by Column Chromatography, Continued

Important

If you are constructing more than 1 cDNA library, only add 1 cDNA adapter ligation reaction per column.

Stop the ligation reaction

1. Incubate the tube from step 2, page 25 at 70°C for 10 minutes to inactivate the ligase.
2. Place the tube on ice.

Set up the column

Keep the following points in mind when setting up a fractionation column:

- Anchor the column securely in a support stand
- Place a rack containing 1.5-mL tubes below the column
- The outlet of the column should be 1- to 2-cm above the 1.5-mL tubes
- You will need to be able to freely move the rack under the column

Wash the column

cDNA size fractionation columns are packed in 20% ethanol, which must be completely removed before adding your cDNA sample. Follow the steps below to remove the ethanol from the columns. The washing steps take approximately 1 hour.

1. With the column attached to a support stand, remove the **top cap first** followed by the bottom cap. Allow the ethanol to drain completely by gravity.
2. Once the column stops dripping, pipet 0.8 mL of TEN buffer into the column and let it drain completely. Refer to the following **Important** note for column specifications.
3. Repeat the wash step 3 more times for a total of 4 washes (3.2 mL) of TEN buffer. Let the column drain until dry. Proceed to **Collect fractions**, page 30.

Important

If the flow rate is noticeably slower than 30–40 seconds per drop, or the drop size from the column is not approximately 25–35 µL, do not use the column. The integrity and resolution of the cDNA may be compromised if the column does not meet these specifications.

Continued on next page

- Rinse gloves with ethanol before collecting fractions to reduce static
- Make sure all of the effluent has drained from the column before adding each new aliquot of TEN buffer

Collect fractions

1. Label 3 sterile 1.5-mL tubes from 1–3. Place them in a rack 1–2 cm from the bottom of the column with tube 1 under the outlet of the column.
2. Add 100 µL of TEN buffer to the 50 µL heat-inactivated cDNA adapter ligation reaction from step 1 from **Wash the column,** page 29. Mix gently by pipetting and centrifuge for 2 seconds to collect the sample.
3. Add the entire sample to the column and let it drain into the resin bed. Collect the effluent into Tube 1.
4. Add another 100 µL of TEN buffer to the column and let it drain into the resin bed. Collect the effluent in Tube 1.
5. Move tube 2 under the column outlet and add 240 µL of TEN buffer to the column. Collect the effluent into tube 2. Let the column drain completely.

   Note: To enrich for larger cDNA, use 160 µL of TEN buffer instead of 240 µL for elution into Tube 2.
6. Move Tube 3 under the column outlet and add 80 µL of TEN buffer to the column. Collect the effluent into Tube 3. Let the column drain completely.

   Note: Tubes 2 and 3 contain double strand cDNA molecules. The largest molecules are eluted from the column first, Tube 2 contains more large-size cDNA than Tube 3. Usually, cDNA eluted in Tube 2 are enough to generate a representative cDNA library that contains 10^6 primary clones, but occasionally, more cDNA may elute in the subsequent fraction. Keep Tube 3 for possible later use, depending upon the result of your library from Tube 2 and amount of starting mRNA.
7. *Optional*: Determine your cDNA quantity using a Bioanalyzer, NanoDrop, or plate spotting assay (see CloneMiner™ cDNA Construction Kit Web Appendix)

Take Tube 2 and proceed to **Ethanol Precipitation**, page 31.

Important

Save the sample in Tube 3. **Do not combine** Tube 3 with Tube 2 because Tube 3 may be potentially contaminated with adapters and primers which may result in background colonies upon plating (i.e., vectors with only *att*B1 and *att*B2, or polyA sequence inserts).

Generate an independent cDNA library from the contents of Tube 3 when:

- Creating a nano-quantity library
- The starting mRNA is much less than 1 µg
- The library does not contain the requisite number of primary clones
- More primary clones are needed
- Smaller cDNA inserts are needed (this protocol does not work for cloning of small RNA or miRNA)

Continued on next page

Size Fractionate cDNA by Column Chromatography, Continued

Ethanol Precipitation

1. To the tube of pooled cDNA, add reagents in the following order:

Reagent	Amount	
Glycogen (20 µg/µL)	1 µL	
7.5 M NH_4OAc	0.5 volume	(i.e. 0.5 × volume of cDNA)
100% ethanol	2.5 volumes	[i.e. 2.5 × (volume of cDNA +NH_4OAc)]

   Note: You may stop at this point and store the tube at –20°C overnight if necessary.

2. Place the tube in dry ice or at –80°C for at least 30 minutes. Centrifuge the sample at 4°C for 30 minutes at 16,000 × g.
3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.
4. Centrifuge the sample at 4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
5. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
6. Resuspend the cDNA pellet in 4 µL of TE buffer by pipetting up and down 30–40 times. Transfer the sample to a fresh tube.

   Note: If you perform analysis of your cDNA yield, you may wish to resuspend the cDNA pellet in a larger volume of TE buffer so that less cDNA is used up for analysis. Take the volumes of reagents required to perform the BP reaction into account when determining the volume for resuspending your cDNA.

Estimate the cDNA yield

You may determine the yield of the cDNA after column purification and ethanol precipitation. Note that 1 µL of your cDNA will be used in this procedure, reducing the amount of cDNA that you have to perform the BP recombination reaction.

For **nano-quantity libraries** the quantity of cDNA is too small to detect, so proceed directly to the BP recombination reaction.

Determine yield by analyzing 1 µL of your cDNA (from step 6 of **Ethanol Precipitation**) using a Bioanalyzer, NanoDrop, or by performing a plate spotting assay (see CloneMiner™ cDNA Construction Kit Web Appendix for detailed guidelines).

What you should see

A typical final cDNA yield is approximately 75–100 ng, starting from 1–2 µg of starting mRNA. Using 75–100 ng of cDNA in the BP reaction should produce a library containing 5–10 million clones.

If your cDNA yield is less than 75 ng, you may generate a second cDNA library using the contents from Tube 3 (see step 6, page 30).

Perform the BP Recombination Reaction

Introduction

General guidelines are provided below to perform a BP recombination reaction between your *att*B-flanked cDNA and pDONR™ 222 to generate a Gateway® entry library. We recommend that you include a positive control and a negative control (no *att*B substrate) in your experiment to help you evaluate results. For a map and a description of the features of pDONR™ 222, see pages 57–58.

Propagate pDONR™ 222

If you wish to propagate and maintain pDONR™ 222, we recommend using 10 ng of the vector to transform One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells (see page 49 for ordering information). The *ccd*B Survival™ 2 T1^R *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccd*B gene. To maintain the integrity of the vector, select for transformants in media containing 50 µg/mL kanamycin and 30 µg/mL chloramphenicol.

Note: DO NOT use general *E. coli* cloning strains including TOP10 or DH5α™ for propagation and maintenance as these strains are sensitive to CcdB effects. **DO NOT** use the ElectroMAX™ DH10B™ competent cells provided with this kit.

Positive Control

pEXP7-tet control DNA is included with this kit for use as a positive control for the BP reaction. pEXP7-tet contains an approximately 1.4 kb fragment consisting of the tetracycline resistance gene and its promoter (Tc^r) flanked by *att*B sites. Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene.

Required materials

Keep all reagents on ice until needed.

Supplied with kit:

- pDONR™ 222 (150 ng/µL)
- pEXP7-tet positive control (50 ng/µL)
- Gateway® BP Clonase® II enzyme mix (keep at –20°C until immediately before use)

Supplied by user:

- *att*B-flanked cDNA (75–100 ng)
- TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- 25°C incubator

Insert to vector ratio

The insert to vector ratio for performing the BP recombination reaction can range anywhere from 1:1–1: 5. If you determined your cDNA yield (page 31) and have >100 ng of cDNA, you can adjust the amount of insert to vector so that it corresponds to a ratio of 1:3–1:2.5 instead of using the amounts given in the protocol.

Continued on next page

BP recombination reaction

Perform the BP recombination reaction using the *att*B-flanked cDNA insert with a pDONR™ 222 vector in a total of 10 µL. If the *att*B-flanked cDNA sample is greater than 4 µL, see the following instructions for performing the reaction in a total of 20 µL.

1. Add the following components to a sterile 1.5-mL microcentrifuge tube at room temperature and mix.

Reagent	Nano*	Standard
*att*B-flanked cDNA	X µL	X µL
pDONR™ 222 (150 ng/µL)	1 µL	2 µL
TE buffer, pH 8.0	to 7 µL	to 7 µL

*Use these conditions when performing the BP recombination reaction using the contents of Tube 3 from cDNA fractionation.

For control reactions, use the following reaction volumes:

		BP Control	
Component	RNA Control	Negative Control	Positive Control
*att*B-flanked cDNA	X µL	—	—
pDONR™ 222 (150 ng/µL)	1.67 µL	1.67 µL	1.67 µL
pEXP7-tet positive control (50 ng/µL)	—	—	0.5 µL
TE buffer, pH 8.0	to 7 µL	to 7 µL	to 7 µL

2. Remove the Gateway® BP Clonase® II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Vortex the Gateway® BP Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 3 µL of Gateway® BP Clonase® II enzyme mix to each sample. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample. The total volume in each tube should now be 10 µL.

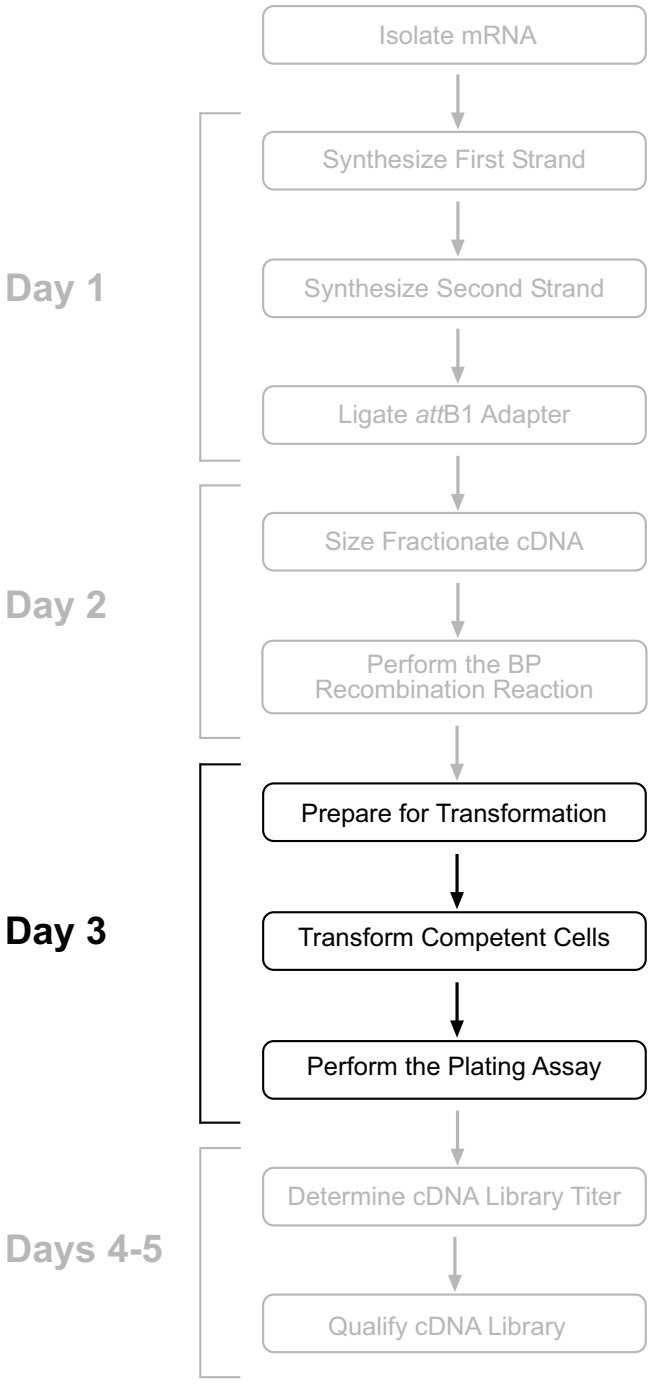
   Reminder: Return Gateway® BP Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate reactions at 25°C for 16–20 hours. Proceed to **Day 3: Transform Competent Cells**, page 35.

Perform a 20 µL BP reaction

If you have more than 4 µL of cDNA, you may increase the total BP reaction volume to 20 µL. You will need to make the following changes to the above protocol:

- Add TE buffer to reach a final volume of 14 µL (step 1)
- Add 6 µL of Gateway® BP Clonase® II enzyme mix (step 4)

Day 3: Transform Competent Cells



Prepare for Transformation

Introduction

After performing the BP recombination reaction, you will inactivate the reaction with proteinase K, ethanol precipitate the cDNA, and transform it into competent *E. coli*. The ElectroMAX™ DH10B™ T1 Phage Resistant Cells provided with the kit have a high transformation efficiency (>1 × 10^{10} cfu/µg DNA), making them ideal for generating cDNA libraries. Follow the following guidelines to prepare for the transformation procedure.

Transformation control

pUC19 plasmid is included to check the transformation efficiency of ElectroMAX™ DH10B™ T1 Phage Resistant Cells. Transform 10 pg of pUC19 using the protocol on page 39.

Required materials

Supplied with kit:

- Proteinase K (2 µg/µL)
- Glycogen (20 µg/µL)
- pUC19 positive control (10 pg/µL)

Supplied by user:

- BP recombination reactions (from step 5, page 33)
- Water bath, heated to 37°C
- Thermocycler or water bath, heated to 75°C
- Sterile water
- 7.5 M NH_4OAc (ammonium acetate)
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol
- 15-mL snap-cap tubes (e.g. Falcon® tubes)
- Ice bucket

Continued on next page

Prepare for Transformation, Continued

Stop the BP recombination reaction

1. Add 2µL of proteinase K to each BP reaction from step 5, page 33, to inactivate the Gateway® BP Clonase® II enzyme mix.
2. Incubate the reactions at 37°C for 15 minutes, then at 75°C for 10 minutes.

Ethanol precipitation

1. Add sterile water to the BP reaction mix to bring it to a final volume of 100 µL. **Do not** use the DEPC-treated water provided with the kit to increase the volume.
2. Add reagents in the following order to each tube:

Reagent	Amount
Glycogen (20 µg/µL)	1 µL
7.5 M NH_4OAc	50 µL
100% ethanol	375 µL

Note: You may stop at this point and store the tube at –20°C overnight if necessary.

3. Place the tube in dry ice or at –80°C for 10 minutes. Centrifuge the sample at 4°C for 25 minutes at 16,000 × g.
4. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µL of 70% ethanol.
5. Centrifuge the sample at 4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
6. Dry the cDNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
7. Resuspend the cDNA pellet in 9 µL of TE buffer by pipetting up and down 30–40 times.

Prepare the controls

You will be dividing your cDNA sample into 6 aliquots and transforming each aliquot into ElectroMAX™ DH10B™ competent cells. To reduce the amount of work, we recommend that you transform only 2 aliquots of the 2.0-kb mRNA, BP negative, and BP positive controls and 1 aliquot of the pUC19 control. Consider the following before preparing the controls:

- If arcing occurs during electroporation, discard the sample immediately. You will need to repeat the electroporation.
- You may prepare in advance additional aliquots, tubes, cuvettes, and reagents for any additional electroporations you may have to perform. See page 39 for recommendations for reducing arcing during electroporation.

Continued on next page

Aliquot samples

1. Label six 1.5-mL tubes for each cDNA library sample. For example, if you are constructing multiple libraries, label tubes for library A: A1, A2, A3, etc.
2. Label two 1.5-mL tubes for each of the cDNA library controls (2.0-kb mRNA, BP positive, and BP negative controls). For the pUC19 transformation control, label one 1.5-mL tube.
3. For each 1.5-mL tube from steps 1 and 2, label a duplicate 15-mL snap-cap tube (e.g. Falcon® tube).
4. Aliquot cDNA library samples and controls into the appropriate tubes according to the following table. Place the tubes on ice.

	cDNA Library	2.0 kb RNA Control	BP Negative Control	BP Positive Control	pUC 19 Control
Number of 1.5 mL Tubes	6	2	2	2	1
Aliquot in Each Tube	1.5 µL	1.5 µL	1.5 µL	1.5 µL	1.0 µL

5. Proceed to **Transform ElectroMAX™ DH10B™ T1 Phage Resistant Cells**, page 38.

Transform ElectroMAX™ DH10B™ T1 Phage Resistant Cells

Note

Each box of ElectroMAX™ DH10B™ T1 Phage Resistant Cells consists of 5 tubes containing 100 µL of competent cells each. Each tube contains enough competent cells to perform 2 transformations using 50 µL of cells per transformation. After thawing a tube of competent cells, discard any unused cells. **Do not** re-freeze cells as repeated freezing/thawing of cells may result in loss of transformation efficiency.

Required materials

Supplied with kit:

- ElectroMAX™ DH10B™ T1 Phage Resistant Cells (thaw on ice before use)
- S.O.C. medium (also available separately, see page 49 for ordering information)

Supplied by user:

- Ice bucket
- 0.1-cm cuvettes (on ice)
- Electroporator
- 37°C shaking incubator
- 15-mL snap-cap tubes (e.g. Falcon® tubes)
- Freezing media (60% S.O.C. medium:40% glycerol, see the following section for a recipe)

Freezing media

60% S.O.C. medium:40% glycerol

1. Combine 60 mL of S.O.C. medium and 40 mL of glycerol and stir until the solution is homogeneous.
2. Autoclave for 30 minutes on liquid cycle.
3. Store at room temperature for up to 1 month.

Electroporator settings

If you are using the BioRad Gene Pulser® II or BTX® ECM 630, we recommend the following settings:

Voltage	2.2 kV*
Resistance	200 Ω
Capacity	25 µF

*If the sample arcs at this voltage setting, recover the sample from the cap by tapping the cuvette, and redo the electroporation on the same cuvette at 2.0 kV.

If you are using another electroporator, you will need to optimize your settings using the pUC19 control DNA provided with the kit. The transformation efficiency of the ElectroMAX™ DH10B™ T1 Phage Resistant Cells should be at least 1×10^{10} cfu/µg of pUC19 control DNA.

Continued on next page

Transform ElectroMAX™ DH10B™ T1 Phage Resistant Cells, Continued

Electroporate

We recommend that you electroporate your controls first followed by your cDNA samples. This will allow you to troubleshoot any arcing problems before you electroporate your cDNA samples (see the following **Recommendation**).

1. To 1 tube containing a DNA aliquot, add 50 µL of thawed ElectroMAX™ DH10B™ competent cells. **Mix gently by pipetting up and down 2 times**. **Be careful to not introduce bubbles into your sample**.
2. Transfer the entire contents of the tube from step 1 of this procedure to a cold 0.1-cm cuvette. Distribute the contents evenly by gently tapping each side of the cuvette. **Be careful to not introduce bubbles into your sample**.
3. Electroporate the sample using your optimized setting (see **Electroporator Settings**, page 38). If your sample arcs, discard the sample immediately and repeat the electroporation with another aliquot. You will need to electroporate a minimum of 2 aliquots for the 2.0-kb RNA, BP negative, and BP positive controls and 1 aliquot for the pUC19 control.
4. Add 1 mL of S.O.C. medium to the cuvette containing electroporated cells. Using a pipette, transfer the entire solution to a labeled 15-mL snap-cap tube.
5. Repeat Steps 1–4 for all sample aliquots.
6. Shake electroporated cells for at least 1 hour at 37°C at 225–250 rpm to allow expression of the kanamycin resistance marker.
7. After the 1-hour incubation at 37°C, pool all cells representing 1 library into a 15-mL snap-cap tube.
8. Determine the volume for all cDNA libraries and controls and add an equal volume of sterile freezing media (60% S.O.C. medium: 40% glycerol).

   Note: Do not add freezing media to the pUC19 control. Mix by vortexing. Keep on ice. **This is the final cDNA library.**
9. Remove a 200 µL sample from each library and controls and place in 1.5-mL tubes for titer determination. Keep on ice.
10. **Store cDNA libraries at –80°C.** You may divide your library into multiple tubes to reduce the number of freeze/thaw cycles.
11. Proceed to **Perform the Plating Assay**, page 40.

If you experience arcing during transformation, try one of the following:

- Make sure the contents are distributed evenly in the cuvette and there are no bubbles.
- Reduce the voltage normally used to charge your electroporator by 10%.
- Make sure to ethanol precipitate the BP reaction prior to electroporation to reduce the salt concentration.
- Dilute the 1.5-µL aliquots with water and divide the sample in 2. Electroporate extra samples of competent cells. Make sure that you have enough ElectroMAX™ DH10B™ Cells to perform this troubleshooting step (see page 49 for ordering information).

Perform the Plating Assay

Required materials

Supplied by user:

- cDNA library and control aliquots
- S.O.C. medium (see page 49 for ordering information)
- LB plates containing 50 µg/mL kanamycin (6 for each cDNA library and BP reaction controls, warm at 37°C for 30 minutes)
- LB plates containing 100 µg/mL ampicillin (2 for pUC19 control, warm at 37°C for 30 minutes)

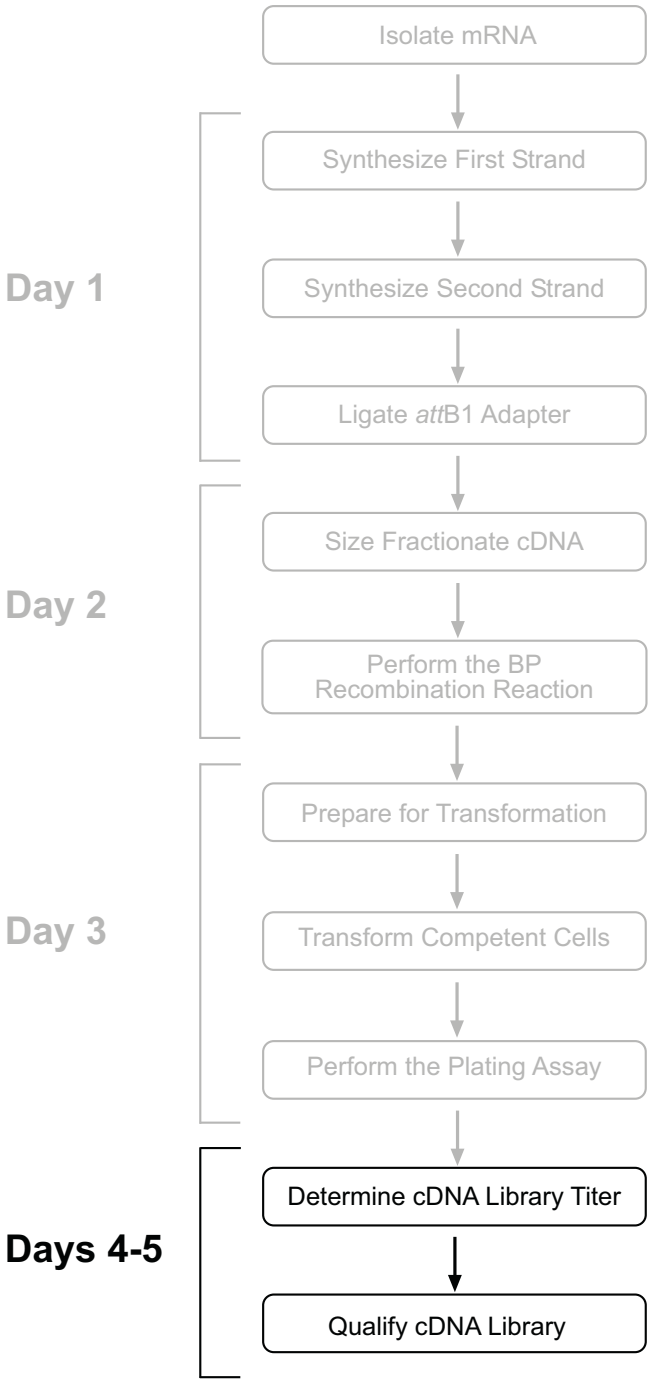
Plating Assay

1. Serially dilute your sample aliquots with S.O.C. medium according to the table below. For each 1:10 serial dilution, add 100 µL of the sample to 900 µL of S.O.C. medium.
2. You will be plating your serial dilutions in duplicate. You will need 6 prewarmed LB plates containing 50 µg/mL kanamycin for each cDNA library, 2.0-kb RNA control, BP negative control, and BP positive control. You will need 2 prewarmed LB plates containing 100 µg/mL ampicillin for the pUC19 transformation control.
3. Plate 100 µL of each dilution onto prewarmed LB plates containing the appropriate antibiotic.
4. Incubate plates overnight at 37°C.

Proceed to **Days 4–5: Analyze the cDNA Library**, page 41.

	cDNA Library	2.0-kb RNA Control	BP Negative Control	BP Positive Control	pUC 19 Control
Dilutions	10^{-2}	10^{-2}	undiluted	10^{-2}	10^{-2}
	10^{-3}	10^{-3}	10^{-1}	10^{-3}	—
	10^{-4}	10^{-4}	10^{-2}	10^{-4}	—
Amount to Plate of Each Dilution	2 × 100 µL	2 × 100 µL	2 × 100 µL	2 × 100 µL	2 × 100 µL
Total Number of LB + Kan Plates	6	6	6	6	—
Total Number of LB + Amp Plates	—	—	—	—	2

Days 4-5: Analyze the cDNA Library



Determine the cDNA Library Titer

Calculations

1. Using the results from the plating assay, page 40, and the equation below, calculate the titer for each plate.

$$\textbf{cfu/mL} = \frac{\textit{colonies on plate} \times \text{dilution factor}}{\text{volume plated (mL)}}$$

2. Use the titer for each plate to calculate the average titer for the entire cDNA library.

3. Use the average titer and the equation below to determine the total number of colony-forming units.

   Total CFU (cfu) = average titer (cfu/mL) × total volume of cDNA library (mL)

   Note: If you completed 6 electroporations for your cDNA library, the total volume will be 12 mL. For the controls, you will need to extrapolate the total number of colony-forming units using a total volume of 12 mL. Refer to page 55 for a sample titer calculation.

Expected Total CFUs

In general, a well represented library should contain 5×10^6 to 1×10^7 primary clones. If the number of primary clones is considerably lower for your cDNA library, see **Troubleshooting**, page 47.

What you should see

See the following table for expected titers and expected total colony-forming units for the control reactions.

Control	Expected Titer	Expected Volume	Expected Total CFUs
2.0 kb RNA control	≥ 1×10^6 cfu/mL	12 mL	≥ 1×10^7 cfu
BP positive control	≥ 1×10^6 cfu/mL	12 mL	≥ 1×10^7 cfu
BP negative control	≤ 0.3% of BP positive control	12 mL	≤ 0.3% of BP positive control
pUC19 control	≥1×10^{10} cfu/µg DNA	—	—

Qualify the cDNA Library

Introduction

It is important to qualify the cDNA library to determine the success of your cDNA library construction. Determining the average insert size and percentage of recombinants will give you an idea of the representation of your cDNA library.

Required materials

Supplied by user:

- Restriction enzyme *Bsr*G I and appropriate buffer (New England Biolabs, Cat. no. R0575S)
- 1 Kb Plus DNA Ladder, recommended (see page 49 for ordering information). Other DNA ladders are suitable.
- Electrophoresis apparatus and reagents

Analyze transformants by *Bsr*G I digestion

You will be digesting positive transformants with *Bsr*G I to determine average insert size and percentage of recombinants. *Bsr*G I sites generally occur at a low frequency making it an ideal restriction enzyme to use for insert size analysis. *Bsr*G I cuts within the following sites:

- *att*L sites of your entry clone to give you the size of your insert (see page 46 for a diagram of the recombination region)
- *att*P sites and *ccd*B gene in pDONR™ 222 to distinguish non-recombined pDONR™ 222 (see page 57 for a map)

Restriction digest

We recommend that you analyze a minimum of 24 positive clones to accurately determine average insert size and the percentage of recombinants.

1. Pick 24 colonies from the plating assay and culture overnight in 2 mL LB containing 50 µg/mL of kanamycin.
2. Isolate plasmid DNA using your method of choice. We recommend using the PureLink® Quick Plasmid MiniPrep Kit or the PureLink® 96 Plasmid Purification System (see page 49 for ordering information) if you will be analyzing multiple libraries at a time.
3. Digest 300–500 ng of plasmid DNA with *Bsr*G I following the manufacturer's instructions. Also digest 250 ng of supercoiled pDONR™ 222 with *Bsr*G I as a control.
4. Electrophorese samples using a 1% agarose gel. Include a DNA ladder to help estimate the size of your inserts.

Continued on next page

Qualify the cDNA Library, Continued

Expected digestion patterns

Use the following guidelines to determine the size of the cDNA inserts. Refer to page 56 for a sample electrophoresis.

- The pDONR™ 222 control will show a digestion pattern of 3 bands of the following lengths:

  2.5 kb
  1.4 kb
  790 bp

- Each cDNA entry clone should have a vector backbone band of 2.5 kb and additional insert bands
- Make sure to digest enough plasmid DNA to be able to visualize smaller insert bands (<300 bp)
- Make sure to run the gel long enough to distinguish bands representing insert sizes of approximately 2.5 kb from the 2.5 kb vector backbone band

Determine average insert size and % recombinants

1. Identify clones containing inserts using the guidelines outlined above.
2. For clones containing inserts, use the DNA ladder to estimate band sizes. If there are multiple bands for a single cDNA entry clone, add all band sizes to calculate the insert size. Do not include the 2.5 kb vector backbone band in your calculations. Refer to page 56 for sample results.
3. Add the insert sizes for all clones. Divide this number by the number of clones containing inserts to calculate the average insert size for your cDNA library.
4. Divide the number of clones containing inserts by the number of clones analyzed to determine the percent recombinants.

What you should see

For **standard cDNA libraries**, you should see an average insert size of ≥1.5 kb and at least 95% recombinants. For **nano-quantity libraries**, and libraries generated from the contents of the column fraction in Tube 3 (page 30), the size will be smaller.

If the average insert size or percent recombinants of your library clones is significantly lower, the cDNA going into the BP recombination reaction is either of poor quality or is insufficient in quantity. For guidelines on isolating quality mRNA, see page 15. To troubleshoot any of the cDNA synthesis steps, see **Troubleshooting**, page 47.

The next step

To sequence entry clones, proceed to **Sequence Entry Clones**, page 45.

You may screen your cDNA library to identify a specific entry clone and use this entry clone in an LR recombination reaction with a destination vector to generate an expression clone. Refer to the Gateway® Technology manual to perform an LR recombination reaction using a single entry clone.

Alternatively, you may transfer your cDNA library into a destination vector to generate an expression library for functional analysis. For detailed guidelines, refer to **Perform the LR Library Transfer Reaction**, page 51.

Sequence Entry Clones

Introduction

You may sequence entry clones generated by BP recombination using dye-labeled terminator chemistries including DYEnamic™ energy transfer or BigDye® reaction chemistries.

Sequencing primers

To sequence inserts in entry clones derived from BP recombination with pDONR™ 222, we recommend using the following sequencing primers. Refer to the following page for the location of the primer binding sites.

Forward primer (proximal to *att*L1)	M13 Forward (–20): 5′-GTAAAACGACGGCCAG-3′
Reverse primer (proximal to *att*L2)	M13 Reverse: 5′-CAGGAAACAGCTATGAC-3′

The M13 Forward (–20) and M13 Reverse Primers are available separately (see page 49 for ordering information). For more information about a custom primer synthesis service, visit www.lifetechnologies.com/support or contact Technical Support (page 60).

Note: If you experience difficulty using the M13 Reverse Primer to sequence entry clones, we recommend using an alternative reverse primer that hybridizes to the poly A tail of your cDNA insert. Design your reverse primer such that it is 5′-$(T)_{23}$N-3′ where N is A, C, or G.

General guidelines

The AT rich *att*L sites in the entry clones may decrease the efficiency of the sequencing reactions. To optimize your sequencing reactions, we recommend the following:

- Plasmid DNA sample should be of good quality and purity (OD_{260}/OD_{280} = 1.7–1.99)
- During plasmid preparation, elute plasmid using deionized water instead of TE buffer

Sequence using BigDye® chemistry

To sequence entry clones using the BigDye® chemistry, we recommend the following:

- Dilute plasmid DNA with deionized water to a final concentration of 100 ng/µL
- Use at least 700 ng of DNA
- Use 3.2 pmoles of primers
- Follow PCR conditions as specified in the BigDye® sequencing kit

Continued on next page

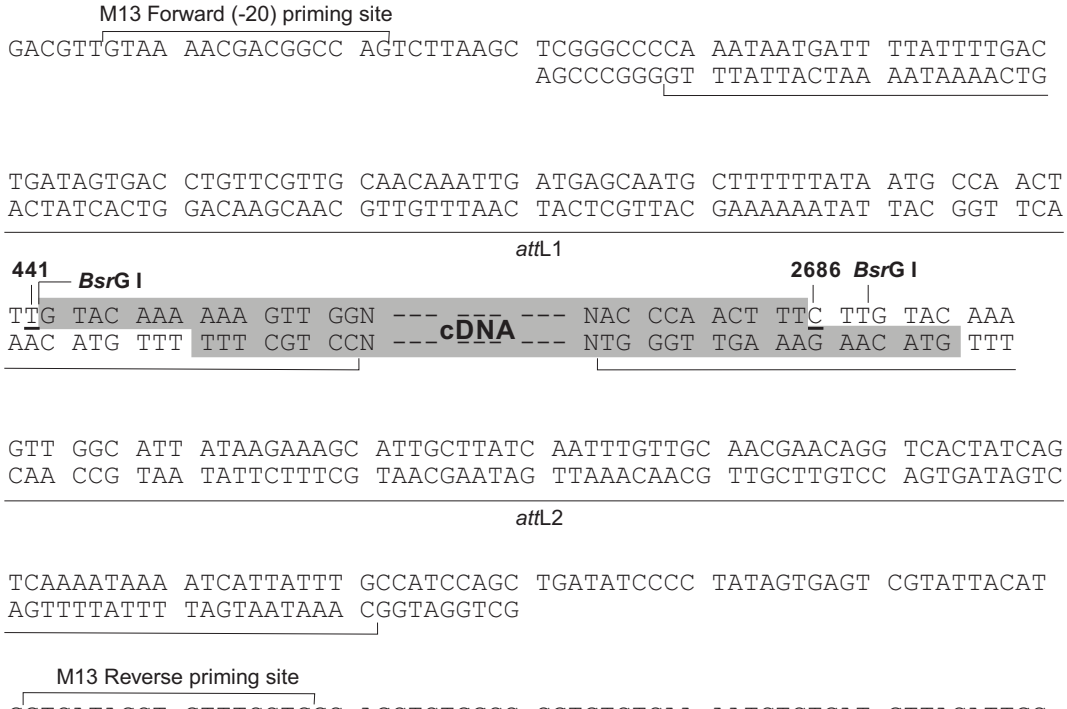
Sequencing Entry Clones, Continued

Recombination region

The recombination region of the entry library resulting from pDONR™ 222 × *att*B-flanked cDNA is shown below.

Features of the Recombination Region:

- Restriction sites are labeled to indicate the actual cleavage site.
- Shaded regions correspond to those DNA sequences transferred from the *att*B-flanked cDNA into the pDONR™ 222 vector by recombination. Non-shaded regions are derived from the pDONR™ 222 vector.
- Bases 441 and 2686 of the pDONR™ 222 sequence are marked.



Troubleshooting

Introduction

The following table lists some potential problems and possible solutions that may help you troubleshoot various steps during cDNA library construction. Note that the starting mRNA quality is a key factor that will affect the outcome of your results.

Observation	Cause	Solution
Low cDNA library titer (pUC19 transformation control working properly)	Insufficient starting mRNA	Quantitate the mRNA by measuring the A_{260}, if possible. We recommend using 1–5 µg of starting mRNA.
	Poorly prepared mRNA or degraded mRNA	Follow the recommendations for mRNA isolation and working with mRNA (see page 15).
	Essential reagent accidentally not added or not working	Perform the 2.0-kb RNA control reaction to verify that the correct reagents have been added and are working properly.
	Insufficient cDNA yield for BP recombination reaction	• Construct a library from the contents of the column fraction in Tube 3 (page 30). • Make sure enzymes used in reaction are kept at the appropriate temperatures. • Maximize the yield during precipitation and fractionation. • Check cDNA recovery after size fractionation by Bioanalyzer.
	Insufficient ligation of *att*B1 Adapter	Perform the 2.0-kb RNA control reactions to verify the ligation step worked properly.
	Poor Electroporation efficiency	• ElectroMAX™ DH10B™ should not be thawed for longer than 20 minutes on ice. • Verify electroporator settings. If using electroporators other than those mentioned on page 38, optimize the settings using a control plasmid to >1 × 10^{10} cfu/µg DNA.
	Gateway® BP Clonase® II enzyme mix inactive	• Perform positive control reaction to verify activity of enzyme mix. • Test a different aliquot of Gateway® BP Clonase® II enzyme mix. • Store Gateway® BP Clonase® II at −20°C in a frost-free freezer.

Continued on next page

Observation	Cause	Solution
Low cDNA library titer, Continued	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
Low average insert size	mRNA sample partially degraded	• Follow the recommendations for mRNA isolation and working with mRNA (see page 15). • Always resuspend RNA in DEPC-treated water.
Low percentage of recombinants	Insufficient amount of cDNA used in the BP recombination reaction	Use the minimum amount of cDNA required for the BP recombination reaction.
Low cDNA yield	Inaccurate incubation temperatures or temperature fluctuations	Perform the first strand reaction starting at 45°C. Keep reactions at 45°C when adding SuperScript® III RT.
	SuperScript® III RT inactive	• Store SuperScript® III RT at –20°C in a frost-free freezer. • Verify enzyme has not expired.
Low cDNA yield after size fractionation	Faulty columns	Check each column to verify that it is working properly. See page 29 for column specifications.
	Samples run too quickly over columns	Let columns drain completely before adding additional buffer.
Few or no colonies obtained from the pUC19 transformation control	ElectroMAX™ DH10B™ competent cells stored incorrectly	Store competent cells at –80°C.
	Loss of transformation efficiency due to repeated freeze/thawing	After a tube of competent cells has been thawed, discard any unused cells.
	Transformation performed incorrectly	Follow the electroporation protocol for ElectroMAX™ DH10B™ competent cells on page 39. If you are using another *E. coli* strain, follow the manufacturer's instructions.
	Electroporator parameters not optimized	Verify electroporator settings. If using electroporators other than those mentioned on page 38, optimize the settings using a control plasmid to $>1 \times 10^{10}$ cfu/µg DNA.
	Loss of transformation efficiency due to arcing	See recommendations on page 38–39 to reduce chances of arcing.

Appendix

Accessory Products

Additional products

Many of the reagents supplied with the CloneMiner™ II cDNA Library Construction Kit as well as other products suitable for use with the kit are available separately. Ordering information is provided in the following table. For more information, refer to www.lifetechnologies.com/support or contact Technical Support (page 60).

Item	Quantity	Catalog no.
SuperScript® III Reverse Transcriptase	2,000 units	18080-093
	10,000 units	18080-044
	4 × 10,000 units	18080-085
Gateway® BP Clonase® II Enzyme Mix	20 reactions	11789-020
	100 reactions	11789-100
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
	100 reactions	11791-043
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	5 × 100 µL	12033-015
cDNA Size Fractionation Columns	3 columns	18092-015
E. coli DNA Ligase	100 units	18052-019
E. coli DNA Polymerase I	250 units	18010-017
T4 DNA Polymerase	50 units	18005-017
T4 DNA Ligase	100 units	15224-017
Ribonuclease H	30 units	18021-014
UltraPure™ DEPC-treated Water	4 × 1.25 mL	10813-012
One Shot® *ccd*B Survival™ 2 T1^R Chemically Competent Cells	10 transformations	A10460
FastTrack® MAG Micro mRNA Isolation Kit	12 reactions	K1580-01
FastTrack® MAG Maxi mRNA Isolation Kit	6 reactions	K1580-02
PureLink® Quick Plasmid MiniPrep Kit	50 reactions	K2100-10
PureLink® HiPure Filter Plasmid MidiPrep Kit	25 reactions	K2100-14
PureLink® 96 Plasmid Purification System	4 × 96 preps	12263-018
Kanamycin Sulfate (10 mg/mL)	100 mL	15160-054
RNase *AWAY*® Reagent	250 mL	10328-011
RnaseZap®	250 mL	AM9780
E-Gel® EX Gel, 1%	10-Pak	G4010-01
E-Gel® iBase™ and E-Gel® Safe Imager™ Combo Kit	1 kit	G6465
5X Second Strand Buffer	0.5 mL	10812-014
SOC Medium	10 × 10 mL	15544-034
1 Kb Plus DNA Ladder	250 µg	10787-018
M13 Forward (–20) Primers	2 µg	N520-02
M13 Reverse Primers	2 µg	N530-02

Continued on next page

Gateway® destination vectors

A large selection of Gateway® destination vectors is available to facilitate expression of your cDNA library in virtually any protein expression system. For more information about the vectors available and their features, refer to **www.lifetechnologies.com/support** or contact Technical Support (page 60).

Perform the LR Library Transfer Reaction

Introduction

After qualifying your cDNA library and analyzing the entry clones, you can perform the LR recombination reaction to transfer your cDNA library into any Gateway® destination vector of choice. If you will be creating an expression library, you will need to follow the guidelines provided in this section for preparing DNA and for performing the LR recombination reaction.

Alternatively, you may screen your cDNA library to identify a specific entry clone and use this entry clone in an LR recombination reaction with a destination vector to generate an expression clone. Refer to the Gateway® Technology manual to perform a standard LR recombination reaction using a single entry clone.

Required materials

Supplied with kit:

- 30% PEG/Mg solution

Supplied by user:

- PureLink® MidiPrep Kit, recommended (see page 49 for ordering information)
- LB media containing 50 µg/mL kanamycin
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- Your cDNA library
- Destination vector of choice (150 ng/µL)
- Gateway® LR Clonase® II enzyme mix (see page 49 for ordering information)
- Ice bucket
- Proteinase K (2 µg/µL) (supplied with Gateway® LR Clonase® II enzyme mix)
- Sterile water
- Glycogen (20 µg/µL)
- 7.5 M NH_4OAc
- 100% ethanol
- Dry ice or a –80°C freezer
- 70% ethanol
- ElectroMAX™ DH10B™ T1 Phage Resistant Cells or equivalent

Prepare double-stranded DNA

You may prepare plasmid DNA from your cDNA library using your method of choice. We recommend using the PureLink® MidiPrep Kit (see page 49 for ordering information). Consider the following points when preparing your DNA:

- Inoculate 5×10^6–1×10^7 cfu of your cDNA library into 50 mL of LB containing 50 µg/mL kanamycin
- Grow the culture to an OD_{600} of 1.0 (approximately 6 hours)
- Use TE buffer, pH 8.0 to elute your DNA

Continued on next page

Perform the LR Library Transfer Reaction, Continued

PEG precipitation

After preparing plasmid DNA from your cDNA library, precipitate the DNA using the 30% PEG/Mg solution provided with the kit.

1. Precipitate the entire eluate with 0.4 volumes of the 30% PEG/Mg solution. Mix well by pipetting.
2. Centrifuge at room temperature for 15 minutes at 13,000 rpm. Carefully remove the supernatant.
3. Dry the pellet at room temperature for 10 minutes. Resuspend the pellet in 50 µL of TE buffer. If you started with less than 5×10^6 clones, resuspend the pellet in less TE buffer.
4. Determine the DNA yield (see **Determine the DNA Yield**).
5. Dilute the DNA to 25 ng/µL. You will need 50 ng of DNA for one LR recombination reaction. You should have enough DNA to perform several LR recombination reactions, if desired (see page 54).

Determine the DNA yield

1. Dilute 5–10 µL of the plasmid DNA sample and read the O.D. using a spectrophotometer at 260 nm.
2. Determine the concentration using the equation below:

   [DNA] = (A_{260}) (0.05 mg/mL) (dilution factor)
3. Determine the total yield by multiplying the concentration by the volume of DNA.
4. Dilute the DNA to 25 ng/µL.

LR Library transfer reaction

If you have a positive control plasmid for the LR recombination reaction, we recommend including it in your experiment to help you evaluate your results.

1. Add the following components to a sterile 1.5-mL microcentrifuge tube at room temperature and mix.

Component	Sample	Negative Control	Positive Control
cDNA entry library (25 ng/µL)	2 µL	—	—
Positive control plasmid (25 ng/µL)	—	—	2 µL
Destination vector (150 ng/µL)	3 µL	3 µL	3 µL
TE Buffer, pH 8.0	9 µL	11 µL	9 µL
Total volume	**14 µL**	**14 µL**	**14 µL**

Continued on next page

Perform the LR Library Transfer Reaction, Continued

LR Library transfer reaction, Continued

2. Remove the Gateway® LR Clonase® II enzyme mix from –20°C and thaw on ice (~2 minutes).
3. Vortex the Gateway® LR Clonase® II enzyme mix briefly twice (2 seconds each time).
4. Add 6 µL of Gateway® LR Clonase® II enzyme mix to each sample. Mix well by vortexing briefly twice (2 seconds each time).

   Reminder: Return Gateway® LR Clonase® II enzyme mix to –20°C immediately after use.
5. Incubate reactions at 25°C for 16–20 hours.
6. Add 2 µL of the proteinase K solution to each reaction. Incubate the reactions at 37°C for 15 minutes, then at 75°C for 10 minutes.
7. Proceed to **Ethanol Precipitation**, in the following section.

Ethanol precipitation

1. To the LR reaction, add reagents in the following order. Be sure to use sterile water and not DEPC-treated water.

Sterile water	80 µL
Glycogen (20 µg/µL)	1 µL
7.5 M NH_4OAc	50 µL
100% ethanol	375 µL

   Note: You may stop at this point and store the tube at –20°C overnight if necessary.
2. Place the tube in dry ice or at –80°C for 10 minutes. Centrifuge the sample at 4°C for 25 minutes at 16,000 × g.
3. Carefully remove the supernatant trying not to disturb the pellet. Add 150 µL of 70% ethanol.
4. Centrifuge the sample at 4°C for 2 minutes at 16,000 × g. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.
5. Dry the DNA pellet in a SpeedVac® for 2–3 minutes or at room temperature for 5–10 minutes.
6. Resuspend the DNA pellet in 9 µL of TE buffer by pipetting up and down 30–40 times.

Transform competent *E. coli*

You may use any *rec*A, *end*A *E. coli* strain including TOP10, DH5α™, DH10B™ or equivalent for transformation. **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (e.g. TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

We recommend using ElectroMAX™ DH10B™ T1 Phage Resistant Cells for maximum transformation efficiency. If you will be using ElectroMAX™ DH10B™ T1 Phage Resistant cells, follow the guidelines outlined in the section entitled **Transform Competent Cells**, page 32.

Continued on next page

Analyze the expression library

Follow the guidelines outlined in the section entitled **Analyze the cDNA library**, page 41, to determine the titer, average insert size, and percent recombinants of your expression library. We recommend that you:

- Analyze transformants by digesting with *Bsr*G I which cuts within both *att*B sites of the expression library as well as within the *att*R sites and *ccd*B gene for non-recombined destination vectors
- Digest and electrophorese your destination vector with no insert to determine the background *Bsr*G I digestion pattern for your particular destination vector

What you should see

When starting with ≥5×10^6 cfu from your cDNA entry library, you should obtain $5 \times 10^6 – 1 \times 10^7$ primary clones from one LR recombination reaction. If the number of primary clones is considerably lower for your expression library, you may perform additional LR recombination reactions using any remaining plasmid DNA from your entry library.

The average insert size and percentage of recombinants of your expression library should be maintained from your cDNA entry library.

Examples of Results

Introduction

In this section, some examples are provided to illustrate how to determine titer and qualify the cDNA library. All steps were performed according to the protocols in this manual.

Determine the cDNA library titer

The results of the plating assay are listed below.

Dilution	Amount Plated (µL)	Colonies Per Plate
10^{-2}	100 µL	654
10^{-3}	100 µL	54
10^{-4}	100 µL	7

The titer for each plate was determined using the results of the plating assay and the equation below. For the 10^2 dilution:

$$\text{cfu/mL} = \frac{\text{colonies on plate} \times \text{dilution factor}}{\text{volume plated (ml)}}$$

$$= \frac{654\,\text{colonies} \times 100}{0.10\,\text{ml}}$$

$$= 6.54 \times 10^5\ \text{cfu/mL}$$

The titer for each plate was used to calculate the average titer of the cDNA library.

The total colony-forming units was determined by multiplying the average titer by the total volume of the cDNA library. In this experiment, 6 electroporations were performed to result in a total volume of 12 mL.

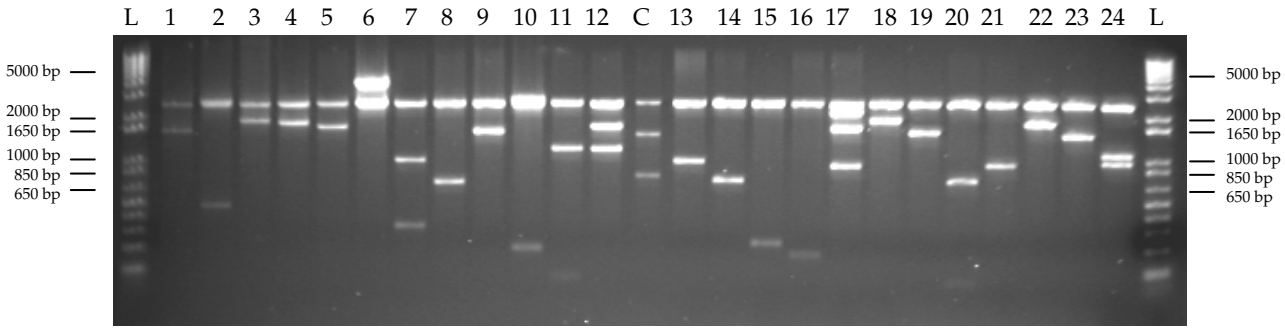
The calculated titers and total number of colony-forming units are shown below.

Dilution	Amount Plated (µL)	Colonies Per Plate	Titer (cfu/mL)	Average Titer (cfu/mL)	Total Volume (mL)	Total CFUs (cfu)
10^{-2}	100 µL	654	6.54×10^5			
10^{-3}	100 µL	54	5.4×10^5	6.31×10^5	12	7.6×10^6
10^{-4}	100 µL	7	$7x\ 10^5$			

Continued on next page

Qualify the cDNA library

Plasmid DNA was isolated from 24 colonies using the PureLink® Quick Plasmid MiniPrep Kit. 300–500 ng of plasmid DNA and 250 ng of supercoiled pDONR™ 222 were digested with *Bsr*G I and run on a 1% agarose gel stained with ethidium bromide. Results are shown in the following picture and table. Note that pDONR™ 222 (lane C) gives a digestion pattern of 2.5 kb, 1.4 kb, and 790 bp when digested with *Bsr*G I.



L = 1 Kb DNA Plus Ladder
C = Digested pDONR™ 222

Clone	Band Size (kb)	Insert Size (kb)	Clone	Band Size (kb)	Insert Size (kb)
1	1.5	1.5	13	0.9	0.9
2	0.5 + 2.5*	3.0	14	0.7	0.7
3	2.0	2.0	15	0.25	0.25
4	1.9	1.9	16	0.2	0.2
5	1.8	1.8	17	0.85 + 1.6 + 2.3	4.75
6	3.9	3.9	18	2.0	2.0
7	0.35 + 1.1	1.45	19	1.6	1.6
8	0.76	0.76	20	0.1 + 0.7	0.8
9	1.6	1.6	21	0.9	0.9
10	0.25 + 2.5	2.75	22	1.9	1.9
11	0.15 + 1.2	1.35	23	1.5	1.5
12	1.2 + 1.9	3.1	24	0.9 + 1.1	2.0

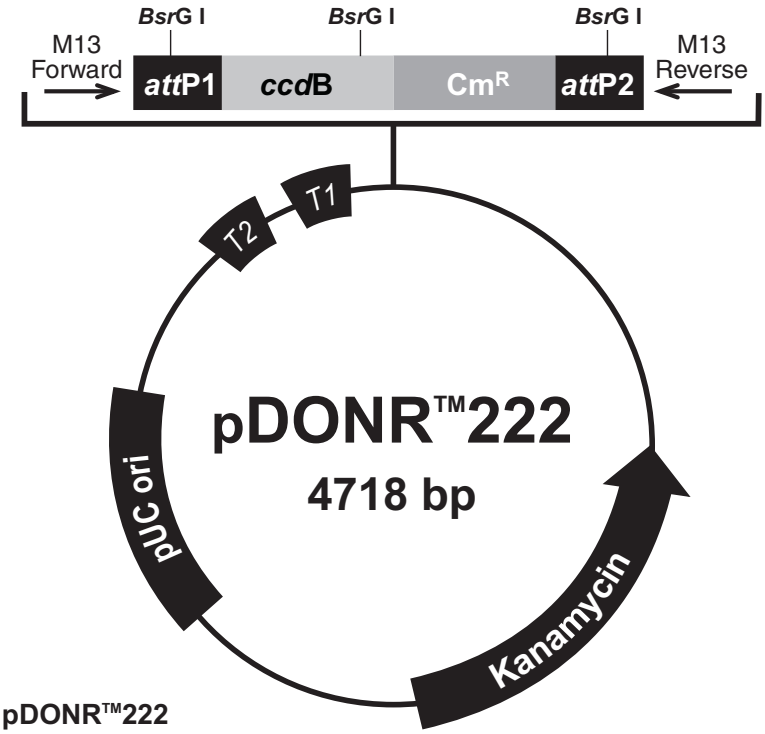
Number of Clones Analyzed	24
Number of Clones Containing Inserts	24
Percent Recombinants	100%
Average Insert Size (kb)	1.8
Insert Size Range (kb)	0.2–4.75

*Upon further electrophoresis, the 2.5-kb band was shown to be a double band consisting of the 2.5 kb vector backbone band and a 2.5-kb band resulting from *Bsr*G I digestion of the insert.

Map and Features of pDONR™ 222

pDONR™ 222 Map

The map below shows the elements of pDONR™ 222. The complete sequence of pDONR™ 222 is available from www.lifetechnologies.com/support or by contacting Technical Support (page 60). See page 59 for a map of the vector after the BP recombination reaction.



Comments for pDONR™222
4718 nucleotides

*rrn*B T2 transcription termination sequence: bases 58-85 (c)
rrnB T1 transcription termination sequence: bases 217-260 (c)
M13 Forward (-20) priming site: bases 327-342
*att*P1: bases 360-591
*Bsr*G I restriction sites: bases 442, 1232, 2689
*ccd*B gene: bases 987-1292 (c)
Chloramphenicol resistance gene: bases 1612-2295 (c)
*att*P2: bases 2543-2774 (c)
M13 Reverse priming site: bases 2816-2832
Kanamycin resistance gene: bases 2899-3714 (c)
pUC origin: bases 4045-4718

(c) = complementary strand

Continued on next page

Map and Features of pDONR™ 222, Continued

Features of the Vector

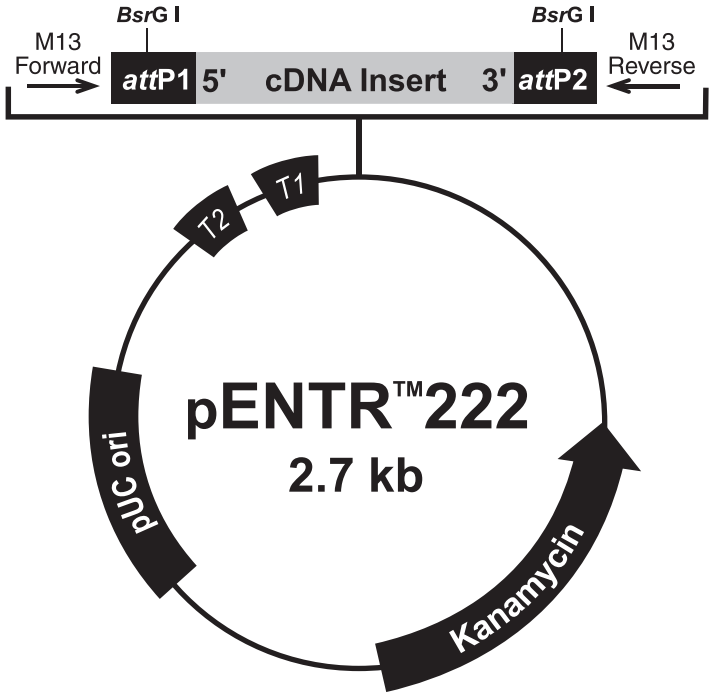
pDONR™ 222 (4718 bp) contains the following elements. All features have been functionally tested.

Feature	Benefit
*rrn*B T1 and T2 transcription terminators	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz *et al.*, 1991).
M13 forward (–20) priming site	Allows sequencing in the sense orientation.
*att*P1 and *att*P2 sites	Bacteriophage λ-derived DNA recombination sequences that permit recombinational cloning of *att*B-containing cDNA (Landy, 1989).
*Bsr*G I restriction sites	Allows you to detect and determine the size of cDNA inserts by restriction enzyme analysis.
*ccd*B gene	Allows negative selection of the plasmid.
Chloramphenicol resistance gene	Allows counterselection of the plasmid.
M13 reverse priming site	Allows sequencing in the anti-sense orientation.
Kanamycin resistance gene	Allows selection of the plasmid in *E. coli*.
pUC origin	Allows high-copy replication and maintenance of the plasmid in *E. coli*.

Map and Features of pENTR™ 222

pENTR™ 222 Map

The map below shows the elements of pENTR™ 222. See page 46 for information on the sequence flanking your cDNA insert.



Technical Support

Obtaining support

For the latest services and support information for all locations, go to www.lifetechnologies.com/support.

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (techsupport@lifetech.com)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

Safety Data Sheets (SDS)

Safety Data Sheets (SDSs) are available at www.lifetechnologies.com/support.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.lifetechnologies.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

Limited warranty

Life Technologies Corporation is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about a Life Technologies product or service, contact our Technical Support Representatives.

All Life Technologies products are warranted to perform according to specifications stated on the certificate of analysis. The Company will replace, free of charge, any product that does not meet those specifications. This warranty limits the Company's liability to only the price of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. The Company reserves the right to select the method(s) used to analyze a product unless the Company agrees to a specified method in writing prior to acceptance of the order.

Life Technologies makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore the Company makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, report it to our Technical Support Representatives.

Life Technologies Corporation shall have no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

Purchaser Notification

Limited Use Label License No. 54: *ccd*B-Fusion Vectors

ccdB selection technology is described in Bernard et al., "Positive Selection Vectors Using the F Plasmid ccdB Killer Gene" Gene 148 (1994) 71-74. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). For licensing information for use in other than research, please contact: Out Licensing, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, California 92008; Phone (760) 603-7200 or e-mail outlicensing@lifetech.com.

Limited Use Label License: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial services of any kind, including, without limitation, reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact **outlicensing@lifetech.com** or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York

Bernard, P., and Couturier, M. (1992) Cell Killing by the F Plasmid CcdB Protein Involves Poisoning of DNA-Topoisomerase II Complexes. *J. Mol. Biol. 226*, 735-745

Bushman, W., Thompson, J. F., Vargas, L., and Landy, A. (1985) Control of Directionality in Lambda Site Specific Recombination. *Science 230*, 906-911

Chomczynski, P., and Sacchi, N. (1987) Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Anal. Biochem. 162*, 156-159

Gubler, U., and Hoffman, B. J. (1983) A Simple and Very Efficient Method for Generating cDNA Libraries. *Gene 25*, 263-269

Landy, A. (1989) Dynamic, Structural, and Regulatory Aspects of Lambda Site-specific Recombination. *Ann. Rev. Biochem. 58*, 913-949

Ohara, O., Nagase, T., Mitsui, G., Kohga, H., Kikuno, R., Hiraoka, S., Takahashi, Y., Kitajima, S., Saga, Y., and Koseki, H. (2002) Characterization of Size-Fractionated cDNA Libraries Generated by the in vitro Recombination-Assisted Method. *DNA Res. 9*, 47-57

Ohara, O., and Temple, G. (2001) Directional cDNA Library Construction Assisted by the in vitro Recombination Reaction. *Nucleic Acids Res. 29*, e22

Okayama, H., and Berg, P. (1982) High-Efficiency Cloning of Full-Length cDNA. *Mol. Cell. Biol. 2*, 161-170

Orosz, A., Boros, I., and Venetianer, P. (1991) Analysis of the Complex Transcription Termination Region of the *Escherichia coli rrnB* Gene. *Eur. J. Biochem. 201*, 653-659

Ptashne, M. (1992) *A Genetic Switch: Phage (Lambda) and Higher Organisms*, Cell Press, Cambridge, MA

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory Press, Plainview, New York

Weisberg, R. A., and Landy, A. (1983) in *Lambda II* (Hendrix, R. W., Roberts, J. W., Stahl, F. W., and Weisberg, R. A., eds), pp. 211-250, Cold Spring Harbor Press, Cold Spring Harbor, NY