



# CloneMiner™ cDNA Library Construction Kit

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

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**User Manual**



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## Acknowledgements

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## Kit Contents and Storage

### Shipping/Storage

The CloneMiner™ 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	BP Clonase™ Enzyme Mix: <b>-80°C</b> All other components: <b>-20°C</b>
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	<b>-80°C</b>
cDNA Size Fractionation Columns	<b>+4°C</b>

### Number of Reactions

The CloneMiner™ 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 xi for ordering information.

### Components for cDNA Library Construction

The components for cDNA library construction are listed below. Store the BP Clonase™ enzyme mix at  $-80^{\circ}\text{C}$ . Store all other components at  $-20^{\circ}\text{C}$ .

Item	Composition	Amount
2.3 kb RNA control	0.5 $\mu\text{g}/\mu\text{l}$ in: 10 mM HEPES 2 mM EDTA, pH 7.2	15 $\mu\text{l}$
DEPC-treated Water	Sterile, DEPC-treated water	1 ml
Biotin- <i>att</i> B2-Oligo(dT) Primer	30 pmol/ $\mu\text{l}$ in DEPC-treated water	8 $\mu\text{l}$
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 $\mu\text{l}$
5X First Strand Buffer	250 mM Tris-HCl, pH 8.3 375 mM KCl 15 mM $\text{MgCl}_2$	1 ml
0.1 M Dithiothreitol (DTT)	in DEPC-treated water	250 $\mu\text{l}$

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## Kit Contents and Storage, continued

### Components for cDNA Library Construction, continued

Item	Composition	Amount
SuperScript™ II Reverse Transcriptase	200 U/μl in: 20 mM Tris-HCl, pH 7.5 1 mM EDTA 100 mM NaCl 0.01% NP-40 (v/v) 1 mM DTT 50% Glycerol (v/v)	50 μl
5X Second Strand Buffer	100 mM Tris-HCl, pH 6.9 450 mM KCl 23 mM MgCl <sub>2</sub> 0.75 mM β-NAD 50 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	500 μl
<i>E. coli</i> 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
<i>E. coli</i> 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
<i>E. coli</i> RNase H	2 U/μl in: 20 mM Tris-HCl, pH 7.5 100 mM KCl 10 mM MgCl <sub>2</sub> 0.1 mM EDTA 0.1 mM DTT 50 μg/ml BSA 50% Glycerol (v/v)	20 μl

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## Kit Contents and Storage, continued

### Components for cDNA Library Construction, continued

Item	Composition	Amount
T4 DNA Polymerase	5 U/ $\mu$ l in: 100 mM Potassium Phosphate, pH 6.5 10 mM $\beta$ -mercaptoethanol 50% Glycerol (v/v)	15 $\mu$ l
<i>attB1</i> Adapter	1 $\mu$ g/ $\mu$ l in: 10 mM Tris-HCl, pH 7.5 1 mM EDTA 0.1 M NaCl	70 $\mu$ l
5X Adapter Buffer	330 mM Tris-HCl, pH 7.6 50 mM MgCl <sub>2</sub> 5 mM ATP	70 $\mu$ l
T4 DNA Ligase	1 U/ $\mu$ l in: 100 mM Potassium Phosphate, pH 6.5 10 mM $\beta$ -mercaptoethanol 50% Glycerol (v/v)	50 $\mu$ l
pDONR™222 Vector	150 ng/ $\mu$ l vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	40 $\mu$ l
BP Clonase™ Enzyme Mix	Proprietary	80 $\mu$ l
5X BP Clonase™ Reaction Buffer	Proprietary	200 $\mu$ l
Proteinase K	2 $\mu$ g/ $\mu$ l in: 10 mM Tris-HCl, pH 7.5 20 mM CaCl <sub>2</sub> 50% Glycerol (v/v)	40 $\mu$ l
pEXP7-tet Control DNA	50 ng/ $\mu$ l in TE Buffer, pH 8.0	2 x 20 $\mu$ l
30% PEG/Mg solution	30% PEG 8000/30 mM MgCl <sub>2</sub>	2 x 1 ml

#### Biotin-*attB2*-Oligo(dT) Primer Sequence

The Biotin-*attB2*-Oligo(dT) Primer is biotinylated to block blunt-end ligation of the *attB1* Adapter to the 3' end of the cDNA during the adapter ligation step. The primer sequence is provided below with the *attB2* sequence in bold.

5'-Biotin-GGCGGCCGC**ACAAC**TTTGTACAAGAAAGTTGGGT(T)<sub>19</sub>-3'

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## Kit Contents and Storage, continued

### ***attB1* Adapter Sequences**

The double-stranded adapter is made by denaturation and slow annealing of the two oligonucleotides in annealing buffer. The *attB1* Adapter is supplied at 1 µg/µl. The sequence is provided below with the *attB1* sequence in bold.

5' - TCGTCGGGG**ACAAC**TTTGTACAAAAAAGTTGG - 3'  
 3' - CCCCTGTTGAAACATGTTTTTTCAACCp - 5'

### **DH10B™ T1 Phage Resistant Cells**

Four boxes of ElectroMAX™ DH10B™ T1 Phage Resistant Cells are provided with the kit. Transformation efficiency is >1 x 10<sup>10</sup> cfu/µg DNA. Each box includes the following items. Store at -80°C.

Item	Composition	Amount
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	--	5 x 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 +4°C)	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl <sub>2</sub> 10 mM MgSO <sub>4</sub> 20 mM Glucose	2 x 6 ml

### **Genotype of DH10B™ T1 Phage Resistant Cells**

F<sup>-</sup> *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74* *recA1* *endA1* *ara*Δ139 Δ(*ara, leu*)7697 *galU* *galK* λ<sup>-</sup> *rpsL* *nupG* *tonA*

### **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 +4°C.

## Accessory Products

### Introduction

The products listed in this section may be used with the CloneMiner™ cDNA Library Construction Kit. For more information, refer to our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or contact Technical Service (page 77).

### Additional Products

Many of the reagents supplied with the CloneMiner™ cDNA Library Construction Kit as well as other products suitable for use with the kit are available separately from Invitrogen. Ordering information is provided below.

Item	Quantity	Catalog no.
SuperScript™ II Reverse Transcriptase	2000 units	18064-022
	10,000 units	18064-014
	4 x 10,000 units	18064-071
BP Clonase™ Enzyme Mix	20 reactions	11789-013
	100 reactions	11789-021
LR Clonase™ Enzyme Mix	20 reactions	11791-019
	100 reactions	11791-043
ElectroMAX™ DH10B™ T1 Phage Resistant Cells	5 x 100 µl	12033-015
cDNA Size Fractionation Columns	3 columns	18092-015
<i>E. coli</i> DNA Ligase	100 units	18052-019
<i>E. coli</i> DNA Polymerase I	250 units	18010-017
T4 DNA Polymerase	50 units	18005-017
T4 DNA Ligase	100 units	15224-017
DEPC-treated Water	4 x 1.25 ml	10813-012
FastTrack® 2.0 mRNA Isolation Kit	6 reactions	K1593-02
Micro-FastTrack® 2.0 mRNA Isolation Kit	20 reactions	K1520-02
S.N.A.P.™ MiniPrep Kit	100 reactions	K1900-01
S.N.A.P.™ MidiPrep Kit	20 reactions	K1910-01
Kanamycin Sulfate	100 ml (10 mg/ml)	15160-054
RNase Away™ Reagent	250 ml	10328-011
5X Second Strand Buffer	0.5 ml	10812-014

### Gateway® Destination Vectors

A large selection of Gateway® destination vectors is available from Invitrogen 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 our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or contact Technical Service (page 77).



# Introduction

## Overview

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### Introduction

The CloneMiner™ cDNA Library Construction Kit is designed to construct high-quality cDNA libraries without the use of traditional restriction enzyme cloning methods. This novel technology combines the performance of SuperScript™ II Reverse Transcriptase with the Gateway® Technology.

Single-stranded mRNA is converted into double stranded cDNA containing *attB* sequences on each end. Through site-specific recombination, *attB*-flanked cDNA is cloned directly into an *attP*-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 3.

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### Features of the CloneMiner™ cDNA Library Construction Kit

Features of the CloneMiner™ cDNA Library Construction Kit include:

- SuperScript™ II reverse transcriptase for efficient conversion of mRNA into cDNA
  - Biotin-*attB2*-Oligo(dT) Primer for poly(A) mRNA binding and incorporation of the *attB2* sequence to the 3' end of cDNA
  - *attB1* Adapter for ligation of the *attB1* sequence to the 5' end of double-stranded cDNA
  - *attP*-containing vector (pDONR™222) for recombination with *attB*-flanked cDNA to produce an entry library through the Gateway® BP recombination reaction (see pages 73-74 for a map and list of features)
- 

### Advantages of the CloneMiner™ cDNA Library Construction Kit

Using CloneMiner™ cDNA Library Construction Kit offers the following advantages:

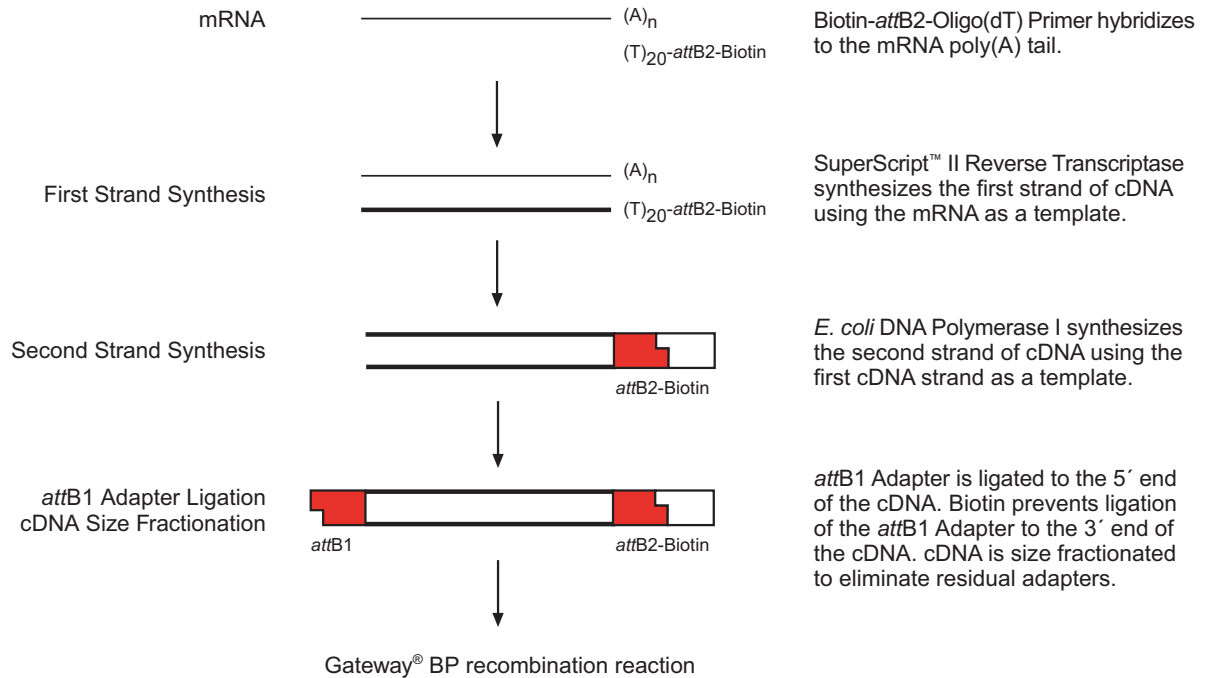
- Produces high yields of quality, double-stranded cDNA
  - Eliminates use of restriction enzyme digestion and ligation allowing cloning of undigested cDNA
  - Highly efficient recombinational cloning of cDNA into a donor vector results in a higher number of primary clones compared to standard cDNA library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

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# Overview, continued

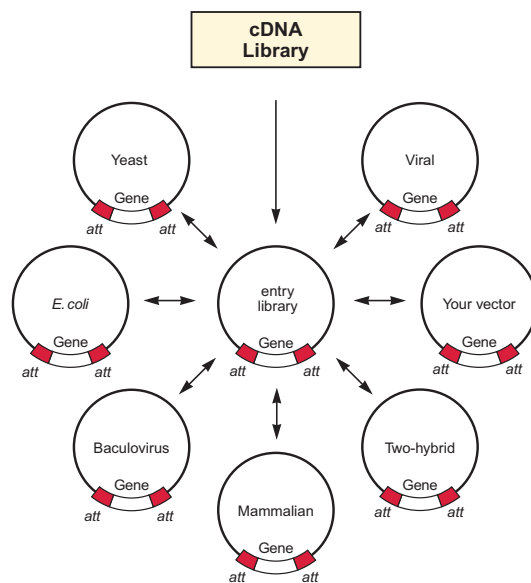
## Experimental Summary

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



## The Gateway® Technology

Gateway® is a universal cloning technology based on the site-specific recombination properties of bacteriophage lambda (Landy, 1989). The 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 the Gateway® Technology, see the next page.



# The Gateway<sup>®</sup> Technology

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## The Basis of Gateway<sup>®</sup>

The Gateway<sup>®</sup> 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). In the Gateway<sup>®</sup> Technology, 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 the Gateway<sup>®</sup> Technology. For detailed information, refer to the Gateway<sup>®</sup> Technology manual. This manual is available from our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or by contacting Technical Service (page 77).

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## 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.* Clonase<sup>™</sup> enzyme mix)

These components are discussed below.

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## 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.* Clonase<sup>™</sup> enzyme mix). The hallmarks of lambda recombination are listed below.

- 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, *attL* sites are comprised of sequences from *attB* and *attP* 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).

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# The Gateway<sup>®</sup> Technology, continued

## att Sites

Lambda recombination occurs between site-specific attachment (*att*) sites: *attB* on the *E. coli* chromosome and *attP* on the lambda chromosome. The *att* sites serve as the binding site for recombination proteins and have been well characterized (Weisberg and Landy, 1983). Upon lambda integration, recombination occurs between *attB* and *attP* sites to give rise to *attL* and *attR* 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<sup>™</sup> cDNA Library Construction Kit, the wild-type *attB* sites encoded by the *attB1* Adapter and Biotin-*attB2*-Oligo(dT) Primer and the wild-type *attP1* and *attP2* sites encoded by pDONR<sup>™</sup>222 have been modified to improve the efficiency and specificity of the Gateway<sup>®</sup> BP recombination reaction.

## ccdB Gene

The presence of the *ccdB* gene in pDONR<sup>™</sup>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 and Couturier, 1992), thereby inhibiting growth of most *E. coli* strains (e.g. DH5 $\alpha$ <sup>™</sup>, TOP10). When recombination occurs between pDONR<sup>™</sup>222 and the *attB*-flanked cDNA, the *ccdB* gene is replaced by the cDNA insert. Cells that take up nonrecombined pDONR<sup>™</sup>222 carrying the *ccdB* gene or by-product molecules retaining the *ccdB* gene will fail to grow. This allows high-efficiency recovery of the desired clones.

## Gateway<sup>®</sup> Recombination Reactions

Two recombination reactions constitute the basis of the Gateway<sup>®</sup> Technology. By using the CloneMiner<sup>™</sup> 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 *attB*-flanked cDNA with an *attP*-containing vector (pDONR<sup>™</sup>222) to create an *attL*-containing entry library (see diagram below). This reaction is catalyzed by BP Clonase<sup>™</sup> enzyme mix.



- **LR Reaction:** Facilitates recombination of an *attL* entry clone or entry library with an *attR* substrate (destination vector) to create an *attB*-containing expression clone or expression library (see diagram below). This reaction is catalyzed by LR Clonase<sup>™</sup> enzyme mix.





# Choosing a Library Construction Method

## Introduction

There are several ways to construct your cDNA library using the CloneMiner™ cDNA Library Construction Kit. You will need to decide between:

- Radiolabeling or not radiolabeling your cDNA
- Size fractionating your cDNA by column chromatography or by gel electrophoresis

We recommend radiolabeling your cDNA and size fractionating your cDNA by column chromatography. This section provides information to help you choose the library construction method that best suits your needs.

## Radiolabeling vs. Non-Radiolabeling

The table below outlines the advantages and disadvantages of the radiolabeling and non-radiolabeling methods. Use this information to choose one method to construct your cDNA library.

	Radiolabeling Method	Non-Radiolabeling Method
<b>Analyzing First Strand Synthesis</b>	Direct measure of cDNA yield and overall quality of the first strand	No knowledge of cDNA yield or quality until the library is constructed
<b>Determining cDNA Yields for Cloning</b>	Reliable quantitative method using scintillation counter	Qualitative, subjective method using agarose plate spotting assay
<b>Sensitivity of cDNA Detection</b>	Very sensitive to a wide range of cDNA amounts using scintillation counter	Sensitive in detecting 1–10 ng of cDNA per spot (see <b>Performing the Plate Spotting Assay</b> , page 54). Limited resolution for cDNA yields greater than 10 ng per spot (see <b>Performing the Plate Spotting Assay</b> , page 54).
<b>Experimental Time</b>	Time consuming filter washes, counting samples, performing calculations	DNA standards and plates for the plate spotting assay can be prepared in advance for several experiments, limited calculations
<b>Preparation</b>	Requires extensive preparation of reagents, equipment, and work area	Requires minimal preparation of DNA standards and agarose plates for the plate spotting assay
<b>Lab Environment</b>	Need to work in designated areas, dispose of radioactive waste, monitor work area, follow radioactive safety regulations	Regular lab environment with no radioactive hazards or radioactive safety regulations



### Note

Be sure to read the section entitled **Advance Preparation**, page 12, to prepare any necessary reagents required for your method of choice. If you will be using the radiolabeling method, also read the section entitled **Working with Radioactive Materials**, page 7. If you will be using the non-radiolabeling method, we recommend that you read the section entitled **Performing the Plate Spotting Assay**, page 54, before beginning.

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## Choosing a Library Construction Method, continued

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### Choosing a Size Fractionation Method

Size fractionation generates cDNA that is free of adapters and other low molecular weight DNA. Although we recommend size fractionating your cDNA by column chromatography, you may also size fractionate your cDNA by gel electrophoresis. Either method can be used with radiolabeled or non-radiolabeled cDNA. Refer to the guidelines outlined below and choose the method that best suits your needs.

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### Column Chromatography

Column chromatography is commonly used to size fractionate cDNA. Use the column chromatography method to generate a cDNA library with an average cDNA insert size of approximately 1.5 kb (if you start with high-quality mRNA). Columns are provided with the kit. Protocols to size fractionate radiolabeled or non-radiolabeled cDNA by column chromatography are provided in this manual.

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### Gel Electrophoresis

Use 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.

Protocols to size fractionate radiolabeled or non-radiolabeled cDNA by gel electrophoresis are provided in the CloneMiner™ cDNA Construction Kit Web Appendix. Because you will need to have additional reagents on hand, we recommend reading the Web Appendix before beginning. This manual is available from our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or by contacting Technical Service (page 77).

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### Important

The CloneMiner™ 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  $\lambda$ -*att* recombination system: see Ohara and Temple, 2001 and Ohara *et. al.*, 2002
  - mRNA handling techniques: see Chomczynski and Sacchi, 1987
-

# Working with Radioactive Material

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## Introduction

Read the following section if you will be constructing your cDNA library using a radiolabeled isotope. This section provides general guidelines and safety tips for working with radioactive material. For more information and specific requirements, contact the safety department of your institution.

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Use extreme caution when working with radioactive material. Follow all federal and state regulations regarding radiation safety. For general guidelines when working with radioactive material, see below.

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## General Guidelines

Follow these general guidelines when working with radioactive material.

- Do not work with radioactive materials until you have been properly trained.
  - Wear protective clothing, gloves, and eyewear and use a radiation monitor.
  - Use appropriate shielding when performing experiments.
  - Work in areas with equipment and instruments that are designated for radioactive use.
  - Plan ahead to ensure that all the necessary equipment and reagents are available and to minimize exposure to radioactive materials.
  - Monitor work area continuously for radiation contamination.
  - Dispose of radioactive waste properly.
  - After you have completed your experiments, monitor all work areas, equipment, and yourself for radiation contamination.
  - Follow all the radiation safety rules and guidelines mandated by your institution.
- 



## Important

Any material in contact with a radioactive isotope must be disposed of properly. This will include any reagents that are discarded during the cDNA library synthesis procedure (*e.g.* phenol/chloroform extraction, ethanol precipitation, cDNA size fractionation). Contact your safety department for regulations regarding radioactive waste disposal.

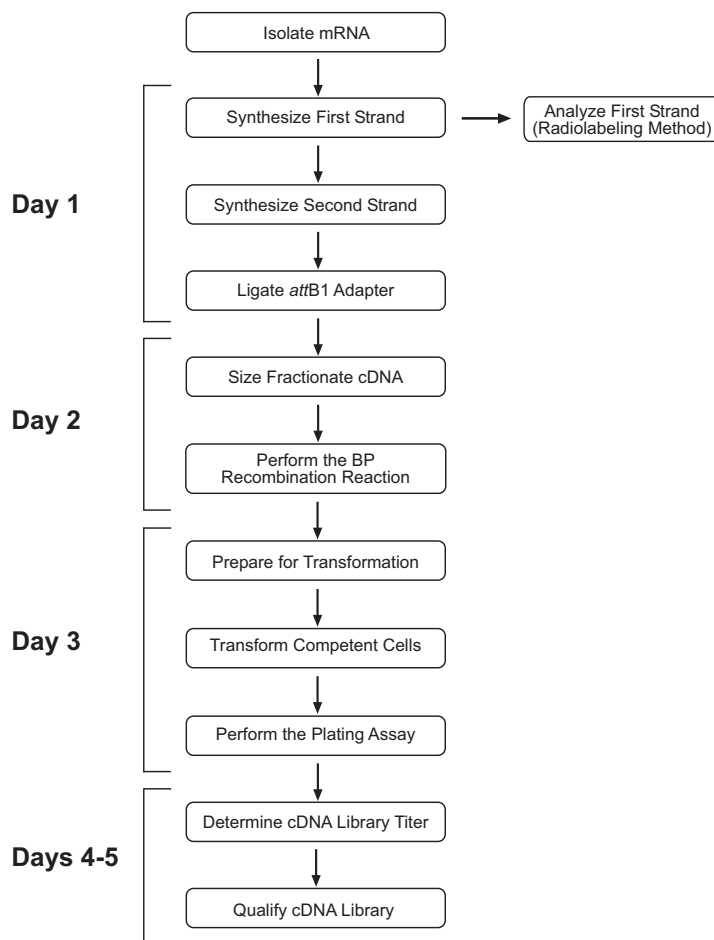
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# Experimental Timeline

## Introduction

The CloneMiner™ cDNA Library Construction Kit is designed to produce an entry library from your starting mRNA within three days. It will take an additional two 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 will not be following this timeline, be sure to plan ahead for convenient stopping points (see below for more information).

## Recommended Timeline



If you are performing the radiolabeling method, we recommend that you follow the timeline outlined above. Radiochemical effects induced by  $^{32}\text{P}$  decay in the cDNA can reduce transformation efficiencies over time.

## 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 the **uncentrifuged** ethanol precipitate at  $-20^{\circ}\text{C}$  to maximize cDNA stability.

# Experimental Overview

## Introduction

The experimental steps necessary to synthesize *attB*-flanked cDNA and to generate an entry library are outlined below. Once you have isolated your mRNA, 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	Radiolabeling Method Page	Non-Radiolabeling Method Page
1	1	Synthesize the first strand of cDNA from your isolated mRNA using the Biotin- <i>attB2</i> -Oligo(dT) Primer and SuperScript™ II RT.	14	14
	2	Synthesize the second strand of cDNA using the first strand cDNA as a template.	18	18
	3	Analyze the first strand reaction for cDNA yield and percent incorporation of [ $\alpha$ - <sup>32</sup> P]dCTP.	20	--
	4	Ligate the <i>attB1</i> adapter to the 5' end of your cDNA.	23	23
2	1	Size fractionate the cDNA by column chromatography to remove excess primers, adapters, and small cDNA.	26	47
	2	Perform the BP recombination reaction between the <i>attB</i> -flanked cDNA and pDONR™222.	31	52
3	1	Transform the BP reactions into ElectroMAX™ DH10B™ T1 Phage Resistant cells. Add freezing media to transformed cells to get final cDNA library.	35	35
	2	Perform the plating assay to determine the cDNA library titer.	40	40
4-5	1	Calculate the cDNA library titer using the results from the plating assay.	42	42
	2	Inoculate 24 positive transformants from the plating assay. Determine average insert size and percent recombinants by restriction analysis.	43	43
	3	Sequence entry clones to verify presence of cDNA insert, if desired.	45	45

# Methods

## Before Using the Kit

### Isolating mRNA

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#### 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.

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

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#### 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, a non-toxic solution available from Invitrogen (see page xi 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).

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#### mRNA Isolation

mRNA can be isolated from tissue, cells, or total RNA using the method of choice. We recommend isolating mRNA using the Micro-FastTrack™ 2.0 or FastTrack® 2.0 mRNA Isolation Kits available from Invitrogen (see page xi for ordering information).

Generally, 1 to 5 µg of mRNA will be sufficient to construct a cDNA library containing 10<sup>6</sup> to 10<sup>7</sup> primary clones in *E. coli*. 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.

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#### Important

It is very important to use the highest quality mRNA possible to ensure success. Check the integrity and purity of your mRNA before starting (see next page).

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## Isolating mRNA, continued

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### Checking the Total RNA Quality

To check total RNA integrity, analyze 1  $\mu\text{g}$  of your RNA by agarose/ethidium bromide 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
- 

### Checking the mRNA Quality

mRNA will appear as a smear from 0.5 to 12 kb. rRNA bands may still be faintly visible. If you do not detect a smear or if the smear is running significantly smaller than 12 kb, you will need to repeat the RNA isolation. Be sure to follow the recommendations listed on the previous page to prevent RNase contamination.

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# Advance Preparation

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## Introduction

Some of the reagents and materials required to use the CloneMiner™ cDNA Library Construction Kit are not supplied with the kit and may not be common lab stock. Refer to the lists below to help you prepare or acquire these materials in advance.

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## Note

Refer to the section entitled **Before Starting** at the beginning of each procedure for a complete list of required reagents.

---

## Materials Required for the Radiolabeling Method

You should have the following materials on hand before performing the radiolabeling method:

- [ $\alpha$ -<sup>32</sup>P]dCTP, 10  $\mu$ Ci/ $\mu$ l (Amersham Biosciences, Catalog no. PB.10205)
  - Glass fiber filters GF/C, 21 mm circles (Whatman, Catalog no. 1822 021)
  - Solvent-resistant marker (Fisher Scientific, Catalog no. 14-905-30)
  - 10% trichloroacetic acid + 1% sodium pyrophosphate (see page 63 for a recipe)
  - 5% trichloroacetic acid (see page 63 for a recipe)
- 

## Materials Required for the Non-Radiolabeling Method

You should have the following on hand before performing the non-radiolabeling method.

- SYBR® Gold Nucleic Acid Gel Stain, recommended (Molecular Probes, Catalog no. S-11494). Other stains are suitable. See page 54 for more information.
- 

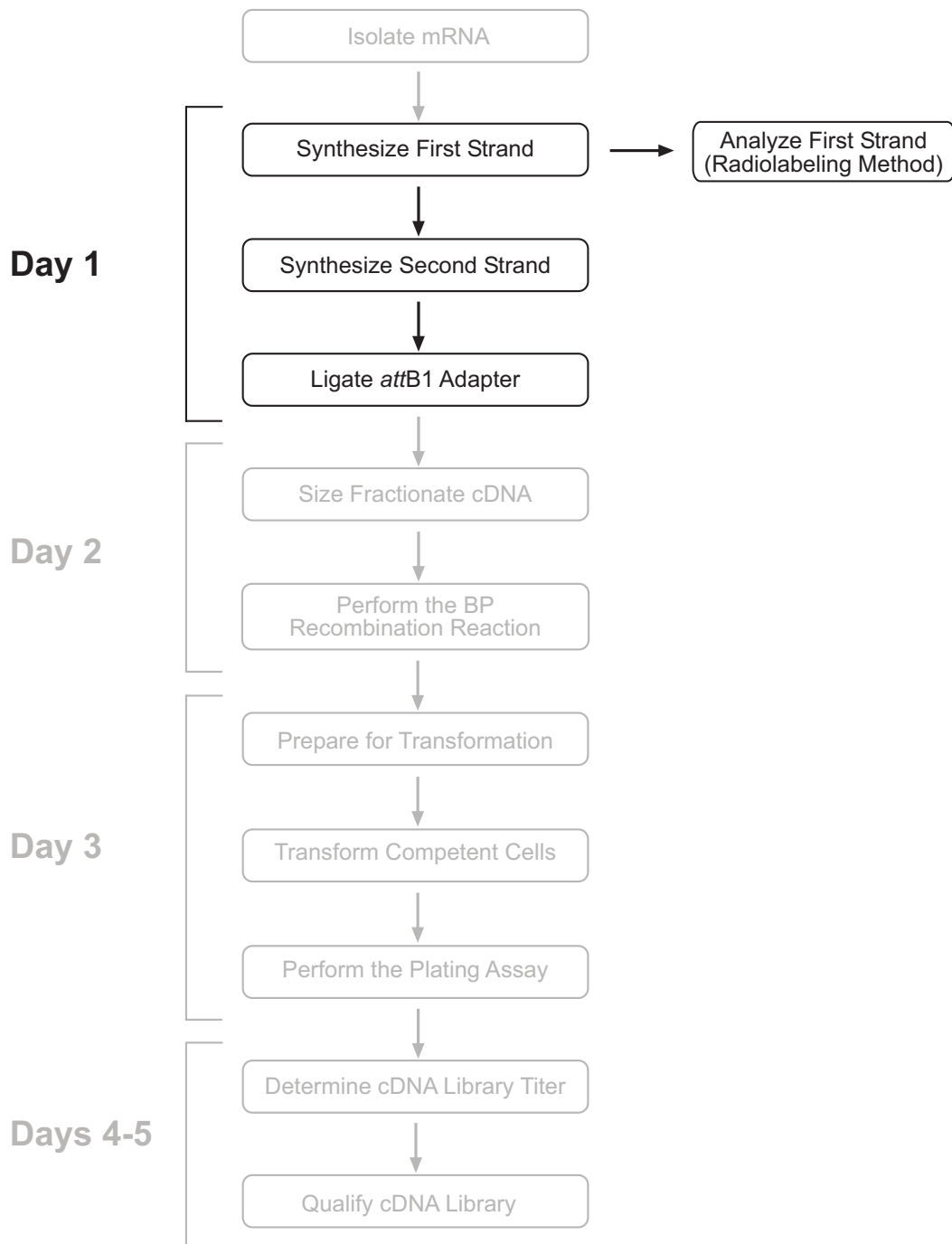
## Number of Reactions

This 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 will be performing control reactions (2.3 kb RNA control, pEXP7-tet control, BP negative control, and pUC 19 transformation control) each time you construct a cDNA library.

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## Day 1: Synthesizing cDNA with Flanking *attB* Sites



# Synthesizing the First Strand

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## 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™ II 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 **Synthesizing the Second Strand** (pages 18-19) and **Ligating the attB1 Adapter** (pages 23-24) 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 10. The amount of mRNA needed to prepare a library depends on the efficiency of each step. Generally, 1 to 5 µg of mRNA will be sufficient to construct a cDNA library containing 10<sup>6</sup> to 10<sup>7</sup> primary clones in *E. coli*.

---

## 2.3 kb RNA Control

We recommend that you include the 2.3 kb RNA control in your experiments to help you evaluate your results. The 2.3 kb RNA control is an *in vitro* transcript containing the tetracycline resistance gene and its promoter (Tc<sup>r</sup>).

---

## 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
  - Tubes should remain in the thermocycler or water bath when adding SuperScript™ II RT to minimize temperature fluctuations (see **Hot Start Reverse Transcription**, below)
- 

## Hot Start Reverse Transcription

Components of the first strand reaction are pre-incubated at 45°C before the addition of SuperScript™ II RT. Incubation at this temperature inhibits nonspecific binding of primer to template and reduces internal cDNA synthesis and extension by SuperScript™ II RT. For this reason, it is important to keep all reactions as close to 45°C as possible when adding SuperScript™ II RT.

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## Synthesizing the First Strand, continued

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

---

### Preparing [ $\alpha$ - $^{32}$ P]dCTP

If you will be labeling your first strand with [ $\alpha$ - $^{32}$ P]dCTP (10  $\mu$ Ci/ $\mu$ l), dilute an aliquot with DEPC-treated water to a final concentration of 1  $\mu$ Ci/ $\mu$ l. Use once and properly discard any unused portion as radioactive waste.

---

### Using the Non-Radiolabeling Method

If you prefer to construct a non-radiolabeled cDNA library, perform the following protocols substituting DEPC-treated water for [ $\alpha$ - $^{32}$ P]dCTP. For more information on the advantages and disadvantages of constructing a non-radiolabeled library, see page 5.

---

### Before Starting

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

#### Supplied with kit:

- 2.3 kb RNA control (0.5  $\mu$ g/ $\mu$ l) (optional)
- DEPC-treated water
- Biotin-*att*B2-Oligo(dT) Primer (30 pmol/ $\mu$ l)
- 10 mM (each) dNTPs
- 5X First Strand Buffer
- 0.1 M DTT
- SuperScript™ II RT (200 U/ $\mu$ l)

#### Supplied by user:

- High-quality mRNA (up to 5  $\mu$ g)
  - Thermocycler (recommended) or water bath, heated to 65°C
  - Ice bucket
  - [ $\alpha$ - $^{32}$ P]dCTP, diluted to 1  $\mu$ Ci/ $\mu$ l (radiolabeling method only)
  - Thermocycler (recommended) or water bath, heated to 45°C
  - 20 mM EDTA, pH 8.0 (radiolabeling method only)
- 

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## Synthesizing the First Strand, continued

### Diluting Your Starting mRNA

In a PCR tube or 1.5 ml tube, dilute your starting mRNA with DEPC-treated water according to the table below. **The total volume for your mRNA + DEPC-treated water will vary depending on the amount of starting mRNA.**

If you will be using the 2.3 kb RNA control supplied with the kit, add 5  $\mu$ l of DEPC-treated water to 4  $\mu$ l of the control mRNA for a total volume of 9  $\mu$ l and a final mRNA amount of 2  $\mu$ g.

Reagent	$\mu$ g of starting mRNA					Control
	$\leq 1$	2	3	4	5	
mRNA + DEPC-treated water	10 $\mu$ l	9 $\mu$ l	8 $\mu$ l	7 $\mu$ l	6 $\mu$ l	9 $\mu$ l (4 $\mu$ l of mRNA + 5 $\mu$ l of water)

### Priming Reaction

- To your diluted mRNA (mRNA + DEPC-treated water), add the Biotin-*att*B2-Oligo(dT) Primer and 10 mM dNTPs according to the following table.

Reagent	$\mu$ g of starting mRNA					Control
	$\leq 1$	2	3	4	5	
mRNA + DEPC-treated water	10 $\mu$ l	9 $\mu$ l	8 $\mu$ l	7 $\mu$ l	6 $\mu$ l	9 $\mu$ l
Biotin- <i>att</i> B2-Oligo(dT) Primer (30 pmol/ $\mu$ l)	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
10 mM (each) dNTPs	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l
<b>Total Volume</b>	<b>12 <math>\mu</math>l</b>	<b>11 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>9 <math>\mu</math>l</b>	<b>8 <math>\mu</math>l</b>	<b>11 <math>\mu</math>l</b>

- Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample.
- Incubate the mixture at 65°C for 5 minutes and cool to 45°C for 2 minutes. During these incubation steps, perform step 1 of the **First Strand Reaction**, below.

### First Strand Reaction

- Add the following reagents to a fresh tube.

**Note:** If you will be using the non-radiolabeling method, substitute DEPC-treated water for [ $\alpha$ -<sup>32</sup>P]dCTP.

5X First Strand Buffer	4 $\mu$ l
0.1 M DTT	2 $\mu$ l
[ $\alpha$ - <sup>32</sup> P]dCTP (1 $\mu$ Ci/ $\mu$ l)	1 $\mu$ l

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

*continued on next page*

## Synthesizing the First Strand, continued

### First Strand Reaction, continued

- After the priming reaction has cooled to 45°C for 2 minutes (step 3, previous page), add the mixture from step 1 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:

	µg of starting mRNA					Control
	≤1	2	3	4	5	
Total Volume	19 µl	18 µl	17 µl	16 µl	15 µl	18 µl

- Incubate the tube at 45°C for 2 minutes.
- With the tube remaining in the thermocycler or water bath, carefully add SuperScript™ II RT according to the following table. Note that this step may be difficult.

	µg of starting mRNA					Control
	≤1	2	3	4	5	
SuperScript™ II RT (200 U/µl)	1 µl	2 µl	3 µl	4 µl	5 µl	2 µl

The total volume should now be 20 µl regardless of the amount of starting mRNA.

- With the tube remaining in the thermocycler or water bath, mix the contents gently by pipetting. **Be careful to not introduce bubbles.** Incubate at 45°C for 60 minutes.
- If you are constructing a radiolabeled cDNA library, proceed to **First Strand Reaction Sample**, below. If you are constructing a non-radiolabeled cDNA library, proceed to **Synthesizing the Second Strand**, page 18.

### First Strand Reaction Sample

Follow the steps below to generate a sample for first strand analysis. We recommend analyzing the sample during an incubation step in the second strand reaction.

- After the first strand reaction has incubated at 45°C for 60 minutes (step 6, above), mix the contents gently by tapping and centrifuge for 2 seconds to collect the sample.
- Add 1 µl of the first strand reaction to a separate tube containing 24 µl of 20 mM EDTA, pH 8.0. Mix gently by pipetting and place on ice until you are ready to analyze the first strand reaction (see **Analyzing the First Strand Reaction**, page 20).
- Take the remaining 19 µl first strand reaction and proceed immediately to **Synthesizing the Second Strand**, next page.

# Synthesizing 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.

---

## Before Starting

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<sub>4</sub>OAc (ammonium acetate)
  - 100% ethanol
  - Dry ice or a -80°C freezer
  - 70% ethanol
- 

## Second Strand Reaction

Perform all steps quickly to prevent the temperature from rising above 16°C. If you radiolabeled your cDNA, we recommend that you perform the first strand analysis during the two hour incubation in step 2 of this protocol.

1. Place the first strand reaction tube containing 19 μl of cDNA (radiolabeling method) or 20 μl of cDNA (non-radiolabeling method) on ice. Keep the tube on ice while adding the following reagents.

DEPC-treated water	92 μl
5X Second Strand Buffer	30 μl
10 mM (each) dNTPs	3 μl
<i>E. coli</i> DNA Ligase (10 U/μl)	1 μl
<i>E. coli</i> DNA Polymerase I (10 U/μl)	4 μl
<i>E. coli</i> RNase H (2 U/μl)	1 μl
<b>Total volume</b>	<b>150 μl</b> (radiolabeling method) <b>151 μl</b> (non-radiolabeling method)

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## Synthesizing the Second Strand, continued

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### Second Strand Reaction, continued

2. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample. Incubate at 16°C for 2 hours. During this 2 hour incubation step, perform the first strand analysis if you are using the radiolabeling method (see **Analyzing the First Strand Reaction**, page 20).
  3. Add 2 µl of T4 DNA Polymerase to create blunt-ended cDNA. Mix the contents gently by pipetting and centrifuge for 2 seconds to collect the sample. Incubate at 16°C for 5 minutes.
  4. Add 10 µl of 0.5 M EDTA, pH 8.0 to stop the reaction. Proceed to **Phenol/Chloroform Extraction**, below.
- 

### Phenol/Chloroform Extraction

1. Add 160 µl of phenol:chloroform:isoamyl alcohol (25:24:1) and shake by hand thoroughly for approximately 30 seconds.
  2. Centrifuge at room temperature for 5 minutes at 14,000 rpm. Carefully remove the upper aqueous phase to a fresh 1.5 ml tube.
  3. Proceed to **Ethanol Precipitation**, below.
- 

### Ethanol Precipitation

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

Glycogen (20 µg/µl)	1 µl
7.5 M NH <sub>4</sub> OAc	80 µl
100% ethanol	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 14,000 rpm.
  3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µl of 70% ethanol.

**Note:** If you are performing the radiolabeling method, use a Geiger counter to monitor the supernatant for the presence of radioactivity. The majority of the radioactivity should be in the pellet and not in the supernatant.
  4. Centrifuge the sample at +4°C for 2 minutes at 14,000 rpm. 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 18 µl of DEPC-treated water by pipetting up and down 30–40 times. Centrifuge for 2 seconds to collect the sample. Transfer the sample to a fresh tube and place on ice.

**Note:** : If you are performing the radiolabeling method, use a Geiger counter to make sure you have resuspended and transferred all of the cDNA pellet. The majority of the radioactivity should be associated with the sample and not with the old tube.
  7. Proceed to **Ligating the attB1 Adapter**, page 23.
-

# Analyzing the First Strand Reaction

---

## Introduction

This section contains guidelines to help you determine the overall yield of your first strand cDNA and the percent incorporation of [ $\alpha$ - $^{32}$ P]dCTP. We recommend performing the following protocol and calculations during the second strand reaction incubation (step 2, page 19). This procedure can only be performed with radiolabeled cDNA libraries.

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied by user:

- Glass fiber filters GF/C, 21 mm circles (Whatman, Catalog no. 1822 021)
  - Solvent-resistant marker (Fisher Scientific, Catalog no. 14-905-30)
  - Heat lamp (optional)
  - Scintillation vials
  - Scintillation fluid
  - Beaker or plastic container
  - 10% TCA (trichloroacetic acid) + 1% sodium pyrophosphate (NaPPi), on ice (see page 63 for a recipe)
  - 5% TCA (trichloroacetic acid), on ice (see page 63 for a recipe)
  - 100% ethanol
  - Lab shaker
  - Scintillation counter
- 

## Preparing Filters

You will need two glass fiber filters for each first strand reaction sample.

1. Using a solvent-resistant marker, label filters to distinguish which one will be washed. For example, label the filters for the first sample as "1" and "1 W" where "W" stands for "washed."
  2. Mix the contents of the first strand reaction sample from step 2, page 17, by tapping the tube and centrifuge for 2 seconds to collect the sample.
  3. Spot 10  $\mu$ l aliquots onto each of the two glass fiber filters (*i.e.* on 1 and 1 W). Repeat for all samples if you are constructing more than one library.
  4. Dry filters under a heat lamp for 3 minutes or at room temperature for 10–15 minutes.
  5. Place the non-washed filter (*i.e.* labeled "1") directly into a labeled scintillation vial and add the appropriate volume of scintillation fluid. Mix well. Repeat for all non-washed filters.
- 

*continued on next page*



# Analyzing the First Strand Reaction, continued

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## Washing Filters

Use the following protocol to wash filters labeled with a "W." Use a beaker or plastic container that is large enough to hold 200 ml of reagent with adequate shaking. Multiple filters can be washed together in one container.

1. Place the container on top of a shaker and add the first reagent in the table below. Submerge filters using forceps and shake for the time indicated. Continue the wash steps according to the table below. Properly discard the washing solution each time before performing the next wash.

Wash	Reagent	Amount	Time
1	10% TCA + 1% NaPPi	200 ml	10 min
2	5% TCA	200 ml	10 min
3	5% TCA	200 ml	5 min
4	100% ethanol	200 ml	2 min

2. Dry washed filters under a heat lamp for 3 minutes or at room temperature for 10–15 minutes.
3. Place washed filters into individual, labeled scintillation vials and add the appropriate volume of scintillation fluid. Mix well.
4. Count both the washed and unwashed filters using a standard  $^{32}\text{P}$  scintillation program.
5. Proceed to **Overview of Calculations**, below.

---

## Overview of Calculations

The overall yield of the first strand reaction is calculated from the amount of acid-precipitable radioactivity. In order to perform this calculation, you must first determine the specific activity of the radioisotope in the reaction. You will be performing a series of calculations to determine:

- Specific activity (SA) of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$
- Yield of first strand cDNA
- Percent incorporation of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$

---

## Calculating the Specific Activity

The specific activity is defined as the counts per minute (cpm) of an aliquot of the reaction divided by the quantity (pmol) of the same nucleotide in the aliquot. The specific activity for  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  (used at  $1\ \mu\text{Ci}/\mu\text{l}$ ) is calculated using the equation below. Refer to page 65 for a sample calculation.

$$\begin{aligned}\text{SA (cpm/pmol dCTP)} &= \frac{(\text{cpm unwashed filter}/10\ \mu\text{l})}{(200\text{pmol dCTP}/10\ \mu\text{l})} \\ &= \frac{\text{cpm unwashed filter}}{200\text{pmol dCTP}}\end{aligned}$$

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*continued on next page*

## Analyzing the First Strand Reaction, continued

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### Calculating the First Strand cDNA Yield

Use the specific activity and the acid-precipitable radioactivity of the washed filter to calculate the cDNA yield using the equation below. Refer to page 65 for a sample calculation.

$$\begin{aligned}\text{cDNA Yield } (\mu\text{g}) &= \frac{(\text{cpm of washed filter}) \times (25\mu\text{l}/10 \mu\text{l}) \times (20\mu\text{l}/1 \mu\text{l}) \times (4 \text{ pmol dNTP}/\text{pmol dCTP})}{\text{SA} (\text{cpm}/\text{pmol dCTP}) \times (3030 \text{ pmol dNTP}/\mu\text{g cDNA})} \\ &= \frac{(\text{cpm of washed filter}) \times 50 \times (4 \text{ pmol dNTP}/\text{pmol dCTP})}{\text{SA} (\text{cpm}/\text{pmol dCTP}) \times (3030 \text{ pmol dNTP}/\mu\text{g cDNA})} \\ &= \frac{(\text{cpm of washed filter}) \times (200)}{\text{SA} \times (3030)}\end{aligned}$$

In the above equation, the numerator takes into account that 1/20 of the first strand reaction was removed for analysis. The numerator also takes into account that 10  $\mu\text{l}$  of the 25  $\mu\text{l}$  analysis sample was spotted on the washed filter.

---

### Calculating the Percent Incorporation of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$

Use the cDNA yield to calculate the percent incorporation of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  using the equation below. Refer to page 66 for a sample calculation.

$$\text{Percent Incorporation} = \frac{\text{cDNA yield } (\mu\text{g})}{\text{starting mRNA amount } (\mu\text{g})} \times 100$$

### What You Should See

The percent incorporation gives an estimate of the cDNA quality and reflects the quality of the starting mRNA. A first strand reaction demonstrating 20–50% incorporation of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$  will give a library with larger clones on average than a library with 10–20% incorporation.

If the first strand reaction shows an incorporation of 10% or less, your library will yield clones that are well below average in size and that are not highly representative of your starting mRNA. For these reasons, we recommend that you **do not** continue with your cDNA library construction if your first strand reaction shows less than 10% incorporation of  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ . Start again with higher quality mRNA.

If you would like to improve your percent incorporation of labeled dCTP, see the **Troubleshooting Guide**, page 61.

---

# Ligating the *attB1* Adapter

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## Introduction

Follow the guidelines in this section to ligate the *attB1* Adapter to the 5' end of your double-stranded cDNA.

---

## Before Starting

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

### Supplied with kit:

- 5X Adapter Buffer
- *attB1* 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
- 

## Protocol

1. Keep the tube containing 18 µl of your double-stranded, blunt-ended cDNA from step 6, page 19 on ice and add the following reagents:

5X Adapter Buffer	10 µl
<i>attB1</i> Adapter (1 µg/µl)	10 µl
0.1 M DTT	7 µl
<u>T4 DNA Ligase (1 U/µl)</u>	<u>5 µl</u>
<b>Total volume</b>	<b>50 µl</b>

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

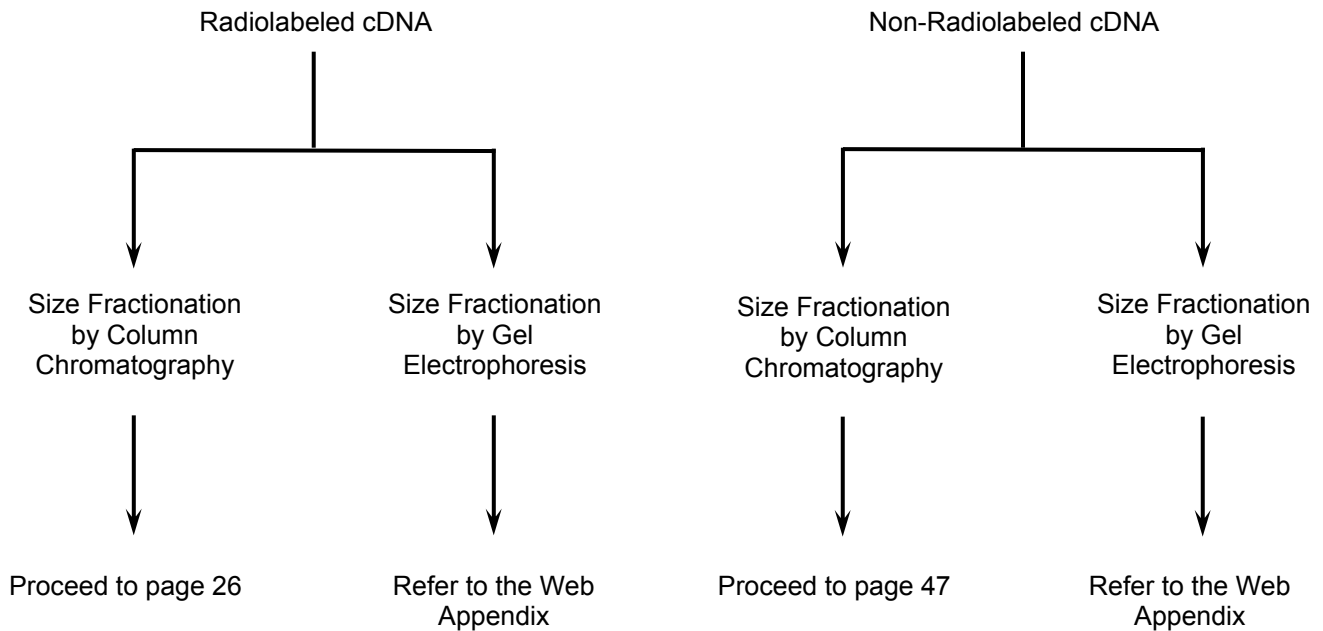
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## Ligating the *attB1* Adapter, continued

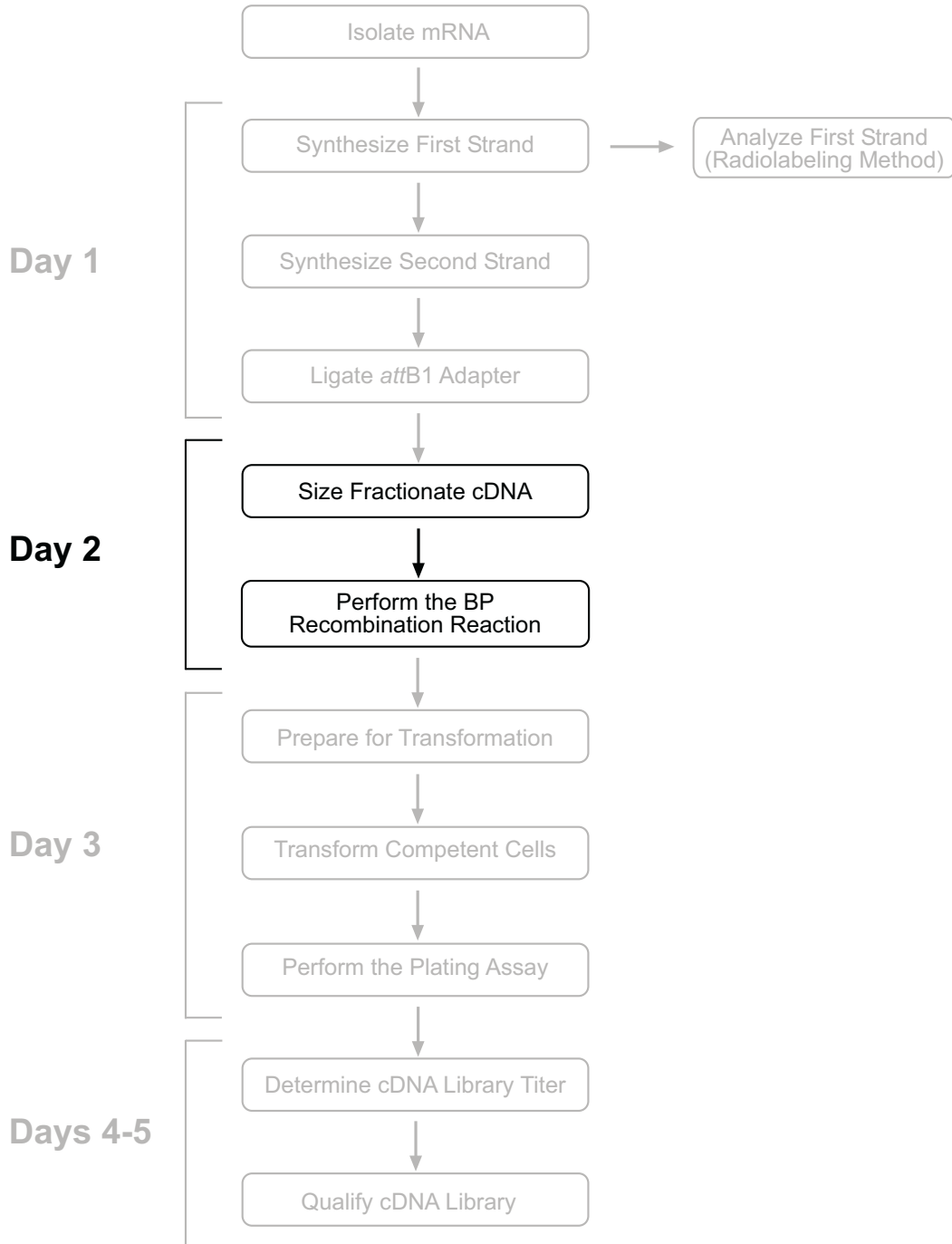
---

### The Next Step

After you have ligated the *attB1* Adapter to the 5' end of your double-stranded cDNA, you will need to size fractionate the cDNA. The protocol you will be performing depends on if your cDNA is radiolabeled and which fractionation protocol you will be performing. For more information on choosing a size fractionation protocol, see page 6. A flow chart is provided below to direct you to the next section.



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



# Size Fractionating Radiolabeled cDNA by Column Chromatography

---

## Introduction

Column chromatography optimizes size fractionation of the cDNA and makes the cloning of larger inserts more probable. Follow instructions closely using the columns supplied with the kit to produce the highest quality library possible.

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Use extreme caution when working with radioactive material. Follow all federal and state regulations regarding radiation safety. For general guidelines when working with radioactive material, see page 7.

---

## 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 and/or small 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.

---



## Important

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

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied with kit:

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

### Supplied by user:

- Ice bucket
  - Thermocycler (recommended) or water bath, heated to 70°C
  - TEN buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 25 mM NaCl)
  - Scintillation vials
  - Scintillation counter
  - 100% ethanol
  - 7.5 M NH<sub>4</sub>OAc (ammonium acetate)
  - Dry ice or -80°C freezer
  - 70% ethanol
  - TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- 

## Stopping the Ligation Reaction

1. Incubate the tube from step 2, page 23 at 70°C for 10 minutes to inactivate the ligase.
  2. Place the tube on ice.
- 

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# Size Fractionating Radiolabeled cDNA by Column Chromatography, continued

---

## Setting 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
- 

## Washing 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 will 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, pipette 0.8 ml of TEN buffer into the column and let it drain completely. Refer to the important note below for column specifications.
  3. Repeat the wash step three more times for a total of four washes and 3.2 ml of TEN buffer. Let the column drain until dry. Proceed to **Collecting Fractions**, below.
- 



## Important

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

---

## Collecting Fractions

When collecting fractions, we recommend wearing gloves that have been rinsed with ethanol to reduce static.

1. Label 20 sterile 1.5 ml tubes from 1 to 20. Place them in a rack 1 to 2 cm from the bottom of the column with tube 1 under the outlet of the column.
  2. Add 100  $\mu$ l of TEN buffer to the 50  $\mu$ l heat-inactivated cDNA adapter ligation reaction from step 1, previous page. 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. Move tube 2 under the column outlet and add 100  $\mu$ l of TEN buffer to the column. Collect the effluent into tube 2. Let the column drain completely.  
**Note:** It is important to make sure all of the effluent has drained from the column before adding each new 100  $\mu$ l aliquot of TEN buffer.
  5. Beginning with the next 100  $\mu$ l aliquot of TEN buffer, collect single-drop fractions into individual tubes starting with tube 3. Continue to add 100  $\mu$ l aliquots of TEN buffer until all 18 tubes (tubes 3–20) contain a single drop.
- 

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## Size Fractionating Radiolabeled cDNA by Column Chromatography, continued

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### Filling Out the Worksheet: Columns A, B, and C

A worksheet is provided to help you with your data recording (see page 75). Refer to page 67 for a sample worksheet to help you with your calculations.

1. Using a pipet, measure the volume in each tube. Use a fresh tip for each fraction to avoid cross-contamination. Record this value in column A of the worksheet.
  2. Calculate the cumulative elution volume with the addition of each fraction and record this value in column B.
  3. Identify the first fraction that exceeds a total volume of 600  $\mu\text{l}$  in column B. Do not use this fraction or any subsequent fractions for your cDNA library.  
**Important:** These fractions (corresponding to fractions 14 through 20 in the sample worksheet, page 67) contain increasing amounts of the *attB1* Adapter which will interfere with cloning reactions and will contaminate the library. We recommend discarding these tubes to avoid accidentally using them in the remainder of the protocol.
  4. Place each remaining capped tube directly into a scintillation vial. Do not add scintillation fluid. Obtain Cerenkov counts for each tube and record this value in column C.
- 

### Filling Out the Worksheet: Columns D and E

Cerenkov counts will appear above background after approximately 300  $\mu\text{l}$  of total volume (corresponding to fraction 5 in the sample worksheet, page 67).

1. For each fraction in which the Cerenkov counts exceed background, calculate the cDNA yield. Refer to **Calculating the Double Strand cDNA Yield**, below. Record this value in column D.
  2. Divide each cDNA amount in column D by the fraction volume in column A to determine the cDNA concentration for that fraction. Record this value in column E.
- 

### Calculating the Double Strand cDNA Yield

Cerenkov counts are approximately 50% of the radioactivity that would be measured in scintillant. Use the specific activity (SA) determined from the first strand reaction sample and the equation below to calculate the yield of double-stranded cDNA. Refer to page 68 for a sample calculation.

$$\begin{aligned}\text{Amount of ds cDNA (ng)} &= \frac{(\text{Cerenkov cpm}) \times 2 \times (4 \text{ pmol dNTP/pmol dCTP}) \times (1,000 \text{ ng/} \mu\text{g ds cDNA})}{\text{SA (cpm/pmol dCTP)} \times (1,515 \text{ pmol dNTP/} \mu\text{g ds cDNA})} \\ &= \frac{(\text{Cerenkov cpm}) \times 8}{\text{SA} \times (1.515)}\end{aligned}$$

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*continued on next page*



# Size Fractionating Radiolabeled cDNA by Column Chromatography, continued

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## Required cDNA Yield

You will need a final cDNA yield of at least 30 ng to perform the BP recombination reaction. Because you will lose approximately half of your sample during the ethanol precipitation procedure, we recommend that you pool a minimum of 60 ng of cDNA from your fractions. See below for guidelines on selecting and pooling cDNA fractions.

---

## Selecting and Pooling cDNA Fractions

The first fraction with detectable cDNA above background level contains the purest and largest cDNAs in the population. Because this fraction often does not contain enough cDNA for cloning, you may need to pool several fractions to reach a minimum of 60 ng of cDNA.

1. Using the worksheet, determine the cDNA yield in the first fraction containing detectable cDNA above background level.
2. If the cDNA yield in this fraction is less than 60 ng, add cDNA from subsequent fractions until 60 ng of cDNA is reached.

**Note:** The first 60 ng of cDNA from a column will make a library with a larger average insert size compared to a library made from the first 100 ng of cDNA. Use the values in column E to calculate the smallest volume needed from the next fraction to obtain the desired amount of cDNA for cloning.

---

## Ethanol Precipitation

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

Glycogen (20 µg/µl)	1 µl
7.5 M NH <sub>4</sub> OAc	0.5 volume ( <i>i.e.</i> 0.5 x volume of cDNA)
100% ethanol	2.5 volumes [ <i>i.e.</i> 2.5 x (volume of cDNA + NH <sub>4</sub> OAc)]

**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 14,000 rpm.
  3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150 µl of 70% ethanol.

**Note:** Use a Geiger counter to monitor the supernatant for the presence of radioactivity. The majority of the radioactivity should be in the pellet and not in the supernatant.
  4. Centrifuge the sample at +4°C for 2 minutes at 14,000 rpm. 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:** Use a Geiger counter to make sure you have resuspended and transferred all of the cDNA pellet. The majority of the radioactivity should be found in the fresh tube and not in the old tube.
- 

*continued on next page*

## Size Fractionating Radiolabeled cDNA by Column Chromatography, continued

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### Calculating the cDNA Yield

1. Place the capped tube containing the resuspended cDNA from step 6, previous page, directly into a scintillation vial. Do not add scintillation fluid. Obtain Cerenkov counts.
2. Determine the cDNA yield using the equation below. Refer to **Calculating the Double Strand cDNA Yield**, page 28 for the full equation.

$$\text{Amount of ds cDNA (ng)} = \frac{(\text{Cerenkov cpm}) \times 8}{\text{SA} \times (1.515)}$$

---

### What You Should See

You should have a final cDNA yield of approximately 30–40 ng to perform the BP recombination reaction. Using approximately 30–40 ng of cDNA in the BP reaction should produce a library containing 5–10 million clones.

If your cDNA yield is less than 30 ng, you may pool additional fractions and ethanol precipitate the cDNA. Resuspend any additional cDNA pellets using the cDNA sample from step 6, previous page.

Once you have the desired amount of cDNA, proceed to **Performing the BP Recombination Reaction with Radiolabeled cDNA**, next page.

---

# Performing the BP Recombination Reaction with Radiolabeled cDNA

---

## Introduction

General guidelines are provided below to perform a BP recombination reaction between your *attB*-flanked cDNA and pDONR™222 to generate a Gateway® entry library. We recommend that you include a positive control and a negative control (no *attB* substrate) in your experiment to help you evaluate your results. For a map and a description of the features of pDONR™222, see pages 73-74.

---

## Propagating pDONR™222

If you wish to propagate and maintain pDONR™222, we recommend using 10 ng of the vector to transform One Shot® *ccdB* Survival™ 2 T1<sup>R</sup> Chemically Competent Cells (Catalog no. A10460) from Invitrogen. The *ccdB* Survival™ 2 T1<sup>R</sup> *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccdB* 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<sup>r</sup>) flanked by *attB* sites. Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene.

---

## Recommended cDNA:pDONR™222 Ratio

For optimal results, we recommend using 30–40 ng of cDNA and 250 ng of pDONR™222 in a 10 µl BP recombination reaction. If the amount of cDNA you will be using is out of this range, make the following changes to the protocol on the next page:

- Adjust the amount of pDONR™222 such that there is an approximately 1:7 mass ratio of cDNA to pDONR™222
  - If you will be using less than 250 ng of pDONR™222, dilute an aliquot of the vector in order to have a large enough volume to accurately pipette
  - Adjust the amount of TE buffer, pH 8.0 to reach a final volume of 7 µl
  - If you will be using more than 4 µl of cDNA, increase the BP reaction to a final volume of 20 µl (see page 33)
- 

*continued on next page*

# Performing the BP Recombination Reaction with Radiolabeled cDNA, continued

## Before Starting

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

### Supplied with kit:

- pDONR™222 (150 ng/μl)
- pEXP7-tet control DNA (50 ng/μl)
- 5X BP Clonase™ Reaction Buffer
- BP Clonase™ enzyme mix (keep at –80°C until immediately before use)

### Supplied by user:

- attB-flanked cDNA (30–40 ng)
- TE buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)
- 25°C incubator

## BP Recombination Reaction

The following protocol uses 30–40 ng of cDNA and 250 ng of pDONR™222 in a 10 μl BP reaction. Use 30 ng of your 2.3 kb RNA control cDNA for the BP reaction. If the attB-flanked cDNA sample is greater than 4 μl, see the next page for necessary modifications.

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

Component	cDNA Sample	2.3 kb RNA Control	BP Negative Control	BP Positive Control
attB-flanked cDNA (30–40 ng)	X μl	X μl	--	--
pDONR™222 (150 ng/μl)	1.67 μl	1.67 μl	1.67 μl	1.67 μl
pEXP7-tet positive control (50 ng/μl)	--	--	--	0.5 μl
5X BP Clonase™ Reaction Buffer	2 μl	2 μl	2 μl	2 μl
TE buffer, pH 8.0	to 7 μl	to 7 μl	to 7 μl	to 7 μl

2. Remove the BP Clonase™ enzyme mix from –80°C and thaw on ice (~2 minutes).
3. Vortex the BP Clonase™ enzyme mix briefly twice (2 seconds each time).
4. Add 3 μl of BP Clonase™ 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 BP Clonase™ enzyme mix to –80°C immediately after use.

5. Incubate reactions at 25°C for 16–20 hours. Proceed to **Day 3: Transforming Competent Cells**, page 34.

*continued on next page*

## Performing the BP Recombination Reaction with Radiolabeled cDNA, continued

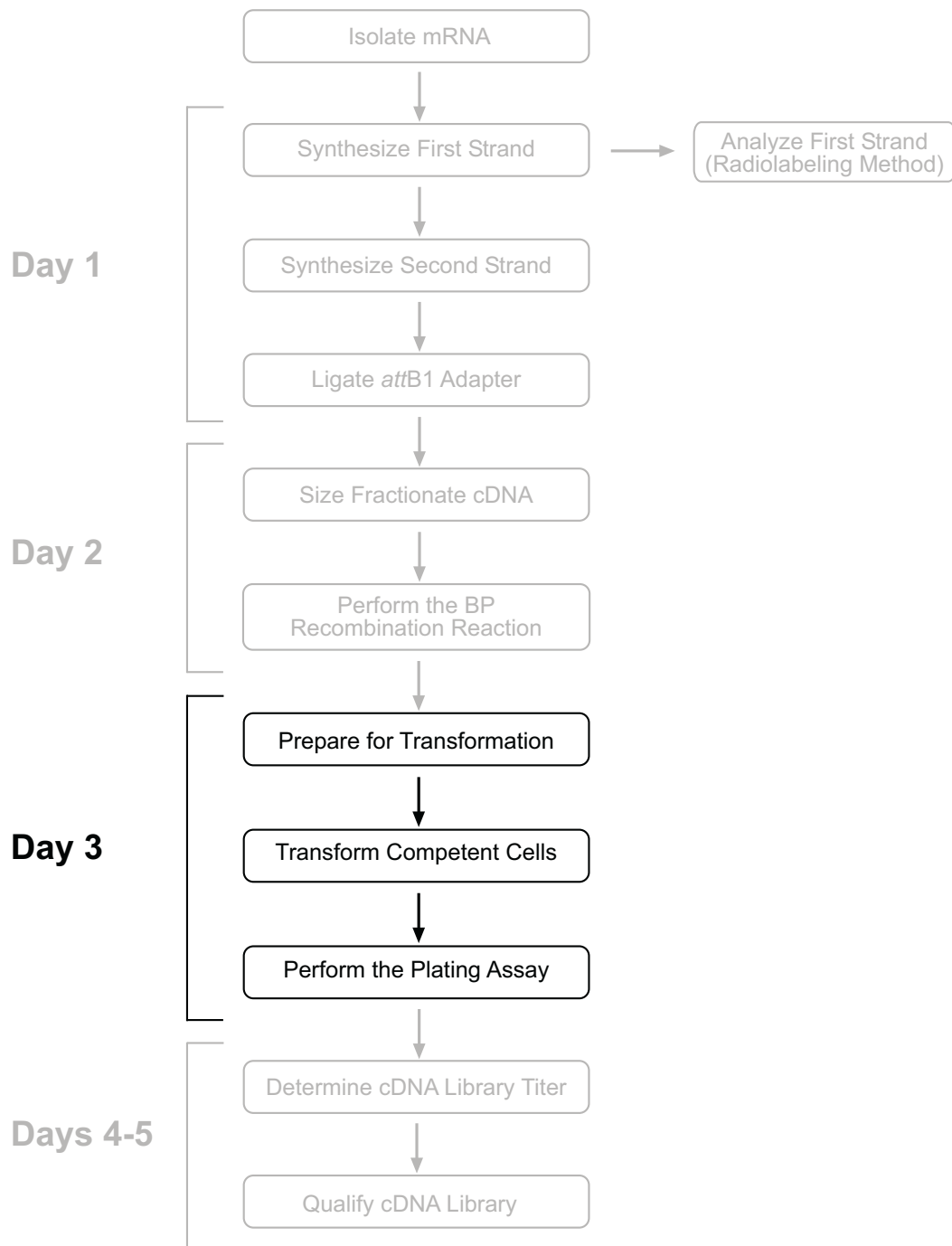
---

### Performing a 20 $\mu$ l BP Reaction

If you will be using more than 4  $\mu$ l of cDNA, you may increase the total BP reaction volume to 20  $\mu$ l. You will need to make the following changes to the protocol on the previous page:

- Add the appropriate amount of pDONR<sup>TM</sup>222 according to the recommended ratio (see **Recommended cDNA:pDONR<sup>TM</sup>222 Ratio**, page 31)
  - Add an additional 2  $\mu$ l of 5X BP Clonase<sup>TM</sup> Reaction Buffer (4  $\mu$ l total)
  - Add the appropriate amount of TE buffer to reach a final volume of 14  $\mu$ l
  - Add 6  $\mu$ l of BP Clonase<sup>TM</sup> enzyme mix
-

## Day 3: Transforming Competent Cells



# Preparing for Transformation

---

## Introduction

Once you have performed 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 ( $>1 \times 10^{10}$  cfu/ $\mu$ g DNA) making them ideal for generating cDNA libraries. Follow the guidelines below 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.

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied with kit:

- Proteinase K (2  $\mu$ g/ $\mu$ l)
- Glycogen (20  $\mu$ g/ $\mu$ l)
- pUC19 positive control (10 pg/ $\mu$ l)

### Supplied by user:

- BP recombination reactions (from step 5, page 32)
  - Water bath, heated to 37°C
  - Thermocycler or water bath, heated to 75°C
  - Sterile water
  - 7.5 M NH<sub>4</sub>OAc (ammonium acetate)
  - 100% ethanol
  - Dry ice or a -80°C freezer
  - 70% ethanol
  - 15 ml snap-cap tubes (*e.g.* Falcon™ tubes)
  - Ice bucket
- 

## Stopping the BP Recombination Reaction

1. To each BP reaction from step 5, page 32, add 2  $\mu$ l of proteinase K to inactivate the BP Clonase™ enzyme mix.
  2. Incubate the reactions at 37°C for 15 minutes then at 75°C for 10 minutes.
- 

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## Preparing for Transformation, continued

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### Ethanol Precipitation

1. To each tube, add reagents in the following order. Use sterile water. Do not use the DEPC-treated water provided with the kit.

Sterile water	90 $\mu$ l
Glycogen (20 $\mu$ g/ $\mu$ l)	1 $\mu$ l
7.5 M $\text{NH}_4\text{OAc}$	50 $\mu$ l
100% ethanol	375 $\mu$ l

If you performed a 20  $\mu$ l BP reaction, add 80  $\mu$ l of sterile water to each tube and add all other reagents as listed above.

**Note:** You may stop at this point and store the tube at  $-20^\circ\text{C}$  overnight if necessary.

2. Place the tube in dry ice or at  $-80^\circ\text{C}$  for 10 minutes. Centrifuge the sample at  $+4^\circ\text{C}$  for 25 minutes at 14,000 rpm.
3. Carefully remove the supernatant while trying not to disturb the cDNA pellet. Add 150  $\mu$ l of 70% ethanol.

Centrifuge the sample at  $+4^\circ\text{C}$  for 2 minutes at 14,000 rpm. Carefully remove the supernatant. Repeat the 70% ethanol wash. Remove as much of the remaining ethanol as possible.

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

Resuspend the cDNA pellet in 9  $\mu$ l of TE buffer by pipetting up and down 30–40 times.

---

### Preparing the Controls

You will be dividing your cDNA sample into six aliquots and transforming each aliquot into ElectroMAX<sup>™</sup> DH10B<sup>™</sup> competent cells. To reduce the amount of work, we recommend that you transform only two aliquots of the 2.3 kb mRNA, BP negative, and BP positive controls and one aliquot of the pUC19 control. Consider the following before preparing the controls:

- If arcing occurs during electroporation, the sample should be immediately discarded. 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.
- 

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## Preparing for Transformation, continued

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### Aliquoting 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 (2.3 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 table below. Place tubes on ice.

	<b>cDNA Library</b>	<b>2.3 kb RNA Control</b>	<b>BP Negative Control</b>	<b>BP Positive Control</b>	<b>pUC 19 Control</b>
<b>Number of 1.5 ml Tubes</b>	6	2	2	2	1
<b>Aliquot in Each Tube</b>	1.5 µl	1.5 µl	1.5 µl	1.5 µl	1.0 µl

5. Proceed to **Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells**, next page.
-

# Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells

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## 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. Once you have thawed 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.

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied with kit:

- ElectroMAX™ DH10B™ T1 Phage Resistant Cells (thaw on ice before use)
- S.O.C. medium (Invitrogen, Catalog no. 15544-034)

### 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 page 63 for a recipe)
- 

## Electroporator Settings

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

Voltage	2.0 kV
Resistance	200 Ω
Capacity	25 µF

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  $1 \times 10^{10}$  cfu/µg of pUC19 control DNA.

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*continued on next page*

# Transforming ElectroMAX™ DH10B™ T1 Phage Resistant Cells, continued

## Electroporation

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 recommendation below).

1. To one tube containing a DNA aliquot, add 50 µl of thawed ElectroMAX™ DH10B™ competent cells. **Mix gently by pipetting up and down two times. Be careful to not introduce bubbles into your sample.**
2. Transfer the entire contents of the tube from step 1, above, 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**, previous page). 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 2.3 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 one hour incubation at 37°C, pool all cells representing one 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 **Performing 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 two. Electroporate extra samples of competent cells. Make sure that you have enough ElectroMAX™ DH10B™ Cells to perform this troubleshooting step (see page xi for ordering information).

# Performing the Plating Assay

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied by user:

- cDNA library and control aliquots
  - S.O.C. medium (Invitrogen, Catalog no. 15544-034)
  - LB plates containing 50 µg/ml kanamycin (six for each cDNA library and BP reaction controls, warm at 37°C for 30 minutes)
  - LB plates containing 100 µg/ml ampicillin (two for pUC19 control, warm at 37°C for 30 minutes)
- 

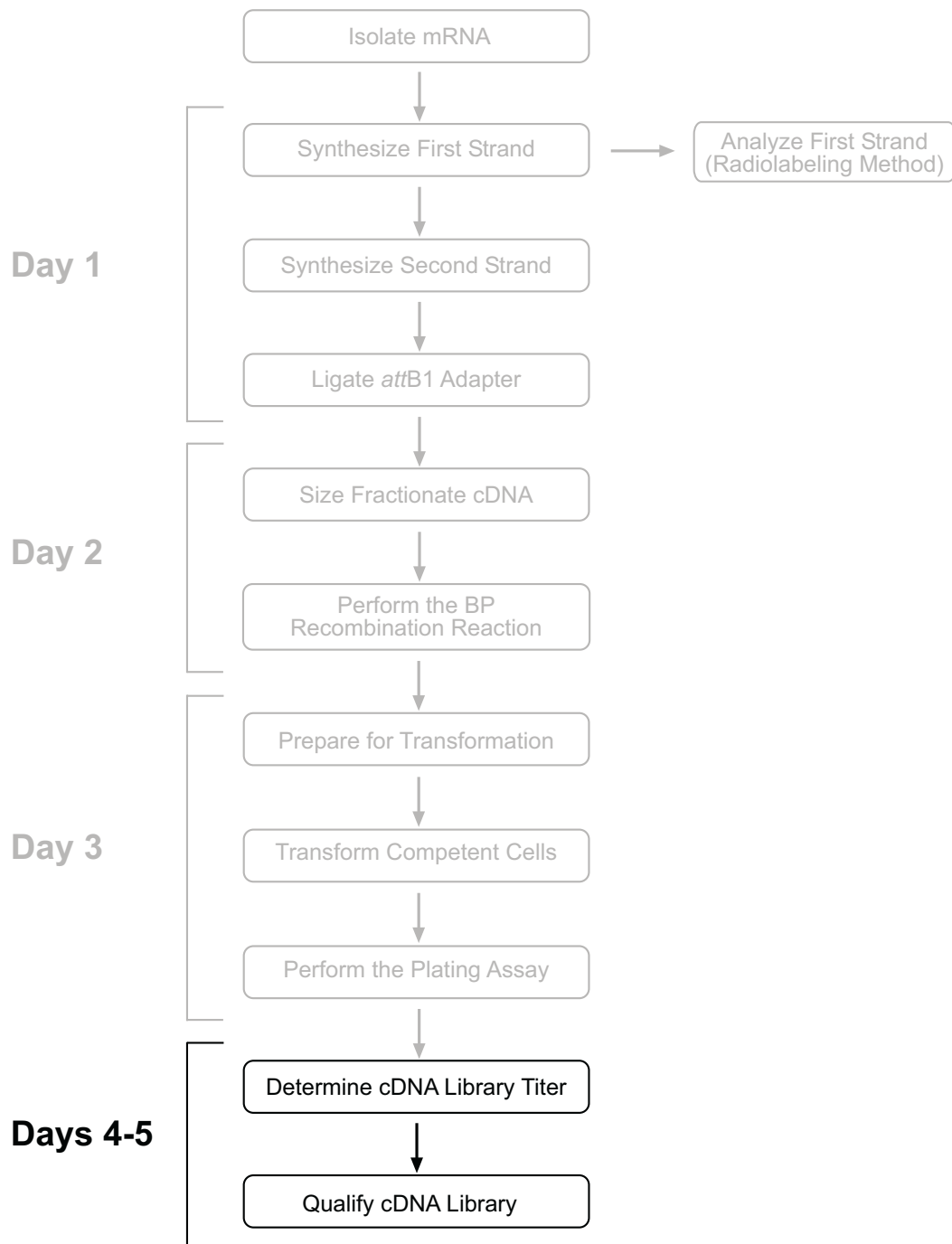
## Plating Assay

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 six prewarmed LB plates containing 50 µg/ml kanamycin for each cDNA library, 2.3 kb RNA control, BP negative control, and BP positive control. You will need two 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.
5. Proceed to **Days 4-5: Analyzing the cDNA Library**, next page.

	<b>cDNA Library</b>	<b>2.3 kb RNA Control</b>	<b>BP Negative Control</b>	<b>BP Positive Control</b>	<b>pUC 19 Control</b>
Dilutions	10 <sup>-2</sup> 10 <sup>-3</sup> 10 <sup>-4</sup>	10 <sup>-2</sup> 10 <sup>-3</sup> 10 <sup>-4</sup>	undiluted 10 <sup>-1</sup> 10 <sup>-2</sup>	10 <sup>-2</sup> 10 <sup>-3</sup> 10 <sup>-4</sup>	10 <sup>-2</sup> -- --
Amount to Plate of Each Dilution	2 x 100 µl	2 x 100 µl	2 x 100 µl	2 x 100 µl	2 x 100 µl
Total Number of LB + Kan Plates	6	6	6	6	--
Total Number of LB + Amp Plates	--	--	--	--	2

---

## Days 4-5: Analyzing the cDNA Library



# Determining the cDNA Library Titer

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## Introduction

Guidelines are provided below to determine the titer of your cDNA library. Refer to page 69 for a sample titer calculation.

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## Calculations

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

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

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.

$$\text{Total CFU (cfu)} = \text{average titer (cfu/ml)} \times \text{total volume of cDNA library (ml)}$$

**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.

---

## Expected Total CFUs

In general, a well represented library should contain  $5 \times 10^6$  to  $1 \times 10^7$  primary clones. If the number of primary clones is considerably lower for your cDNA library, see **Troubleshooting**, page 61.

---

## What You Should See

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

Control	Expected Titer	Expected Volume	Expected Total CFUs
2.3 kb RNA control	$\geq 1 \times 10^6$ cfu/ml	12 ml	$\geq 1 \times 10^7$ cfu
BP positive control	$\geq 1 \times 10^6$ cfu/ml	12 ml	$\geq 1 \times 10^7$ cfu
BP negative control	$\leq 0.3\%$ of BP positive control	12 ml	$\leq 0.3\%$ of BP positive control
pUC19 control	$\geq 1 \times 10^{10}$ cfu/ $\mu$ g DNA	--	--

---

# Qualifying 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.

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## General Molecular Biology Techniques

For help with restriction enzyme analysis, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 1989) or *Current Protocols in Molecular Biology* (Ausubel et al., 1994).

---

## Before Starting

You should have the following materials on hand before beginning:

### Supplied by user:

- Restriction enzyme *BsrG* I and appropriate buffer (New England Biolabs, Catalog no. R0575S)
  - 1 Kb Plus DNA Ladder, recommended (Invitrogen, Catalog no. 12302-011). Other DNA ladders are suitable.
  - Electrophoresis apparatus and reagents
- 

## Analyzing Transformants by *BsrG* I Digestion

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

- *attL* sites of your entry clone to give you the size of your insert (see page 46 for a diagram of the recombination region)
  - *attP* sites and *ccdB* gene in pDONR™222 to distinguish non-recombined pDONR™222 (see page 73 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 S.N.A.P.™ MiniPrep Kit (Catalog no. K1900-01) or the Concert™ 96 Plasmid Purification System (Catalog no. 12263-018) if you will be analyzing multiple libraries at a time.
  3. Digest 300–500 ng of plasmid DNA with *BsrG* I following the manufacturer's instructions. Also digest 250 ng of supercoiled pDONR™222 with *BsrG* I as a control.
  4. Electrophorese samples using a 1% agarose gel. Include a DNA ladder to help estimate the size of your inserts.
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## Qualifying the cDNA Library, continued

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### Expected Digestion Patterns

Use the following guidelines to determine the size of the cDNA inserts. Refer to page 70 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
- 

### Determining 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 70 for sample results.
  3. Add together 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

You should see an average insert size of  $\geq 1.5$  kb and at least 95% recombinants for your cDNA library.

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 10. To troubleshoot any of the cDNA synthesis steps, see **Troubleshooting**, page 61.

---

### The Next Step

If you wish to sequence entry clones, proceed to **Sequencing Entry Clones**, next page.

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 **Performing the LR Library Transfer Reaction**, page 57.

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# Sequencing Entry Clones

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## Introduction

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

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## 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 <i>attL1</i> )	M13 Forward (-20): 5'-GTAAAACGACGGCCAG-3'
Reverse primer (proximal to <i>attL2</i> )	M13 Reverse: 5'-CAGGAAACAGCTATGAC-3'

The M13 Forward (-20) and M13 Reverse Primers (Catalog nos. N520-02 and N530-02, respectively) are available separately from Invitrogen. For other primers, Invitrogen offers a custom primer synthesis service. For more information, visit our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or contact Technical Service (page 77).

**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)<sub>23</sub>N-3' where N is A, C, or G.

---

## General Guidelines

The AT rich *attL* 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
- 

## Sequencing 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<sup>TM</sup>222 x *attB*-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 *attB*-flanked cDNA into the pDONR<sup>TM</sup>222 vector by recombination. Non-shaded regions are derived from the pDONR<sup>TM</sup>222 vector.
- Bases 441 and 2686 of the pDONR<sup>TM</sup>222 sequence are marked.

M13 Forward (-20) priming site

321 GACGTTGTAA AACGACGGCC AGTCTTAAGC TCGGGCCCCA AATAATGATT TTATTTTGAC  
AGCCCCGGGT TTATTACTAA AATAAAACTG

381 TGATAGTGAC CTGTTCTGTTG CAACAAATTG ATGAGCAATG CTTTTTTTATA ATG CCA ACT  
ACTATCACTG GACAAGCAAC GTTGTTTAAC TACTCGTTAC GAAAAAATAT TAC GGT TCA

---

*attL1*

441 **BsrG I** 2686 **BsrG I**

440 TTG TAC AAA AAA GTT GGN --- cDNA --- NAC CCA ACT TTC TTG TAC AAA  
AAC ATG TTT TTT CGT CCN --- NTG GGT TGA AAG AAC ATG TTT

---

2696 GTT GGC ATT ATAAGAAAGC ATTGCTTATC AATTTGTTGC AACGAACAGG TCACTATCAG  
CAA CCG TAA TATTCTTTTCG TAACGAATAG TTAAACAACG TTGCTTGTCC AGTGATAGTC

---

*attL2*

2755 TCAAAATAAA ATCATTATTT GCCATCCAGC TGATATCCCC TATAGTGAGT CGTATTACAT  
AGTTTTATTT TAGTAATAAA CGGTAGGTCG

---

M13 Reverse priming site

2815 GGTCATAGCT GTTTCCTGGC AGCTCTGGCC CGTGTCTCAA AATCTCTGAT GTTACATTGC

## Appendix

# Size Fractionating Non-Radiolabeled cDNA by Column Chromatography

### Introduction

Column chromatography optimizes size fractionation of the cDNA and makes the cloning of larger inserts more probable. Follow instructions closely using the columns supplied with the kit to produce the highest quality library possible.



Because your cDNA is not labeled with [ $\alpha$ - $^{32}$ P]dCTP, you will need to estimate your cDNA yields using a plate spotting assay. You will be performing this assay throughout the size fractionation procedure. We recommend that you read the section entitled **Performing the Plate Spotting Assay**, page 54, before size fractionating your cDNA.

### How the Columns Work

Each column provided with the kit contains 1 ml of Sephacryl<sup>®</sup> S-500 HR resin. This porous resin traps residual adapters and/or small 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.



### Important

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

### Before Starting

You should have the following materials on hand before beginning:

#### Supplied with kit:

- cDNA Size Fractionation Columns
- Glycogen (20  $\mu$ g/ $\mu$ l)

#### Supplied by user:

- TEN buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 25 mM NaCl)
- 100% ethanol
- 7.5 M NH<sub>4</sub>OAc (ammonium acetate)
- Dry ice or -80°C freezer
- 70% ethanol
- TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA)

### Stopping the Ligation Reaction

1. Incubate the tube from step 2, page 23 at 70°C for 10 minutes to inactivate the ligase.
2. Place the tube on ice.

*continued on next page*

# Size Fractionating Non-Radiolabeled cDNA by Column Chromatography, continued

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## Setting 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
- 

## Washing 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 will 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, pipette 0.8 ml of TEN buffer into the column and let it drain completely. Refer to the important note below for column specifications.
  3. Repeat the wash step three more times for a total of four washes and 3.2 ml of TEN buffer. Let the column drain until dry. Proceed to **Collecting Fractions**, below.
- 



## Important

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

---

## Collecting Fractions

When collecting fractions, we recommend wearing gloves that have been rinsed with ethanol to reduce static.

1. Label 20 sterile 1.5 ml tubes from 1 to 20. Place them in a rack 1 to 2 cm from the bottom of the column with tube 1 under the outlet of the column.
  2. Add 100  $\mu$ l of TEN buffer to the 50  $\mu$ l heat-inactivated cDNA adapter ligation reaction from step 1, previous page. 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. Move tube 2 under the column outlet and add 100  $\mu$ l of TEN buffer to the column. Collect the effluent into tube 2. Let the column drain completely.  
**Note:** It is important to make sure all of the effluent has drained from the column before adding each new 100  $\mu$ l aliquot of TEN buffer.
  5. Beginning with the next 100  $\mu$ l aliquot of TEN buffer, collect single-drop fractions into individual tubes starting with tube 3. Continue to add 100  $\mu$ l aliquots of TEN buffer until all 18 tubes (tubes 3–20) contain a single drop.
- 

*continued on next page*

# Size Fractionating Non-Radiolabeled cDNA by Column Chromatography, continued

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## Filling Out the Worksheet: Columns A and B

A worksheet is provided to help you with your data recording (see page 76). Refer to page 71 for a sample worksheet to help you with your calculations.

1. Using a pipet, measure the volume in each tube. Use a fresh tip for each fraction to avoid cross-contamination. Record this value in column A of the worksheet.
2. Calculate the cumulative elution volume with the addition of each fraction and record this value in column B.
3. Identify the first fraction that exceeds a total volume of 600  $\mu\text{l}$  in column B. Do not use this fraction or any subsequent fractions for your cDNA library.

**Important:** These fractions (corresponding to fractions 14 through 20 in the sample worksheet, page 71) contain increasing amounts of the *attB1* Adapter which will interfere with cloning reactions and will contaminate the library. We recommend discarding these tubes to avoid accidentally using them in the remainder of the protocol.

---

## Filling Out the Worksheet: Columns C and D

You will be estimating the concentration and yield of your cDNA fractions using the plate spotting assay. Refer to **Performing the Plate Spotting Assay**, page 54 for detailed guidelines on preparing the plates and staining the DNA.

1. Using the **DNA Spotting Assay** protocol on page 56, spot 1  $\mu\text{l}$  of each fraction onto a prewarmed plate.
  2. Record the estimated cDNA concentration of each fraction in column C.
  3. Multiply the cDNA concentration in column C by the fraction volume in column A to determine the amount of cDNA for that fraction. Record this value in column D.
- 

## Required cDNA Yield

You will need a final cDNA yield of 75 ng to perform the BP recombination reaction. Because you will lose approximately half of your sample during the ethanol precipitation procedure, we recommend that you pool a minimum of 150 ng of cDNA from your fractions. See the next page for guidelines on selecting and pooling cDNA fractions.

---



### Note

If you have previously performed the BP recombination reaction using radiolabeled cDNA, note that the amount of non-radiolabeled cDNA required is greater (see page 29). This larger amount is due to the difference in scale between quantifying DNA by radioactivity using a scintillation counter and quantifying DNA by the plate spotting assay using the DNA standard. Thus, 30 ng of cDNA as measured by counts is roughly equivalent to 50–100 ng of cDNA as measured by comparison to the DNA standard.

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# Size Fractionating Non-Radiolabeled cDNA by Column Chromatography, continued

---

## Selecting cDNA Fractions

The first fractions containing detectable cDNA by the plate spotting assay contain the purest and largest pieces of cDNA in the population. You will want to use cDNA from these fractions for the BP recombination reaction.

We recommend that you also include the fraction preceding the first fraction with detectable cDNA. This fraction may contain large pieces of cDNA in quantities that are not visible using the plate spotting assay.

---

## Pooling cDNA Fractions

You will need to pool fractions together to obtain approximately 150 ng of cDNA. Start with the fraction preceding the first fraction containing detectable cDNA. Add cDNA from subsequent fractions until the desired amount of cDNA is reached.

**Note:** The first 150 ng of cDNA from a column will make a library with a larger average insert size compared to a library made from the first 300 ng of cDNA. Use the values in column C to calculate the smallest volume needed from the next fraction to obtain the desired amount of cDNA for cloning.

---

## Ethanol Precipitation

1. To the tube of pooled cDNA, add reagents in the following order:  
Glycogen (20 µg/µl)            1 µl  
7.5 M NH<sub>4</sub>OAc            0.5 volume (*i.e.* 0.5 x volume of cDNA)  
100% ethanol            2.5 volumes [*i.e.* 2.5 x (volume of cDNA +NH<sub>4</sub>OAc)]  
**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 14,000 rpm.
  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 14,000 rpm. 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 4.5 µl of TE buffer by pipetting up and down 30–40 times. Transfer the sample to a fresh tube.
- 

## Preparing Aliquots for the Plate Spotting Assay

1. Remove 0.5 µl of your cDNA sample from step 6, above, and add to 4.5 µl of TE buffer to make a 1:10 dilution.
  2. Remove 2.5 µl of the 1:10 dilution and add to 2.5 µl of TE buffer to make a 1:20 dilution. Proceed to **Estimating the cDNA Yield**, next page.
- 

*continued on next page*

## Size Fractionating Non-Radiolabeled cDNA by Column Chromatography, continued

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### Estimating the cDNA Yield

You will be estimating the concentration and yield of your cDNA sample using the plate spotting assay. Refer to **Performing the Plate Spotting Assay**, page 54 for detailed guidelines on preparing the plates and staining the DNA.

1. Using the **DNA Spotting Assay** protocol on page 56, spot 1  $\mu\text{l}$  of your 1:10 dilution and 1  $\mu\text{l}$  of your 1:20 dilution onto a prewarmed plate.
  2. Estimate the cDNA concentration of the diluted sample. Multiply this concentration by the dilution factor to get the cDNA concentration of your size fractionated cDNA.
  3. Determine the final cDNA yield by multiplying the cDNA concentration by the total volume in the tube.
  4. You may need to prepare additional dilutions of your samples for the plate spotting assay if your spots appear saturated (see Important Note on page 54).
- 

### What You Should See

You should have a final cDNA yield of approximately 75–100 ng to perform the BP recombination reaction. Using approximately 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 pool additional fractions and ethanol precipitate the cDNA. Resuspend any additional cDNA pellets using the cDNA sample from step 6, previous page.

Once you have the desired amount of cDNA, proceed to **Performing the BP Recombination Reaction with Non-Radiolabeled cDNA**, next page.

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# Performing the BP Recombination Reaction with Non-Radiolabeled cDNA

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## Introduction

General guidelines are provided below to perform a BP recombination reaction between your *attB*-flanked cDNA and pDONR™222 to generate a Gateway® entry library. We recommend that you include a positive control and a negative control (no *attB* substrate) in your experiment to help you evaluate your results. For a map and a description of the features of pDONR™222, see pages 73-74.

---

## Propagating pDONR™222

If you wish to propagate and maintain pDONR™222, we recommend using 10 ng of the vector to transform One Shot® *ccdB* Survival™ 2 T1<sup>R</sup> Chemically Competent Cells (Catalog no. A10460) from Invitrogen. The *ccdB* Survival™ 2 T1<sup>R</sup> *E. coli* strain is resistant to CcdB effects and can support the propagation of plasmids containing the *ccdB* 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<sup>r</sup>) flanked by *attB* sites. Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene.

---

## Before Starting

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

### Supplied with kit:

- pDONR™222 (150 ng/µl)
- pEXP7-tet positive control (50 ng/µl)
- 5X BP Clonase™ Reaction Buffer
- BP Clonase™ enzyme mix (keep at –80°C until immediately before use)

### Supplied by user:

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

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## Performing the BP Recombination Reaction with Non-Radiolabeled cDNA, continued

### BP Recombination Reaction

The following protocol uses 75–100 ng of cDNA and 250 ng of pDONR™222 in a 10 µl BP reaction. If the *attB*-flanked cDNA sample is greater than 4 µl, see below for necessary modifications.

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

Component	cDNA Sample	2.3 kb RNA Control	BP Negative Control	BP Positive Control
<i>attB</i> -flanked cDNA (75–100 ng)	X µl	X µl	--	--
pDONR™222 (150 ng/µl)	1.67 µl	1.67 µl	1.67 µl	1.67 µl
pEXP7-tet positive control (50 ng/µl)	--	--	--	0.5 µl
5X BP Clonase™ Reaction Buffer	2 µl	2 µl	2 µl	2 µl
TE buffer, pH 8.0	to 7 µl	to 7 µl	to 7 µl	to 7 µl

2. Remove the BP Clonase™ enzyme mix from –80°C and thaw on ice (~2 minutes).
3. Vortex the BP Clonase™ enzyme mix briefly twice (2 seconds each time).
4. Add 3 µl of BP Clonase™ 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 BP Clonase™ enzyme mix to –80°C immediately after use.
5. Incubate reactions at 25°C for 16–20 hours. Proceed to **Day 3: Transforming Competent Cells**, page 34.

### Performing a 20 µl BP Reaction

If you will be using 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 an additional 2 µl of 5X BP Clonase™ Reaction Buffer (4 µl total)
- Add the appropriate amount of TE buffer to reach a final volume of 14 µl
- Add 6 µl of BP Clonase™ enzyme mix

# Performing the Plate Spotting Assay

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## Introduction

If you are constructing a non-radioactive cDNA library, you will be estimating your cDNA yields using a plate spotting assay. Samples will be spotted on agarose and compared under UV light to spots containing known quantities of DNA. Guidelines are provided below to prepare the plates and to perform the assay.

---



## Important

The plate spotting assay is an assay to qualitatively determine the concentration and yield of your cDNA samples. Comparison of samples to the DNA standard is subjective and may vary from person to person. In addition, the plate spotting assay is limited in its range of cDNA detection. While you can detect as little as 1 ng of cDNA using SYBR<sup>®</sup> Gold Nucleic Acid Gel Stain (see **Choosing a Nucleic Acid Stain**, below), the assay cannot resolve an unlimited amount of cDNA. Generally, spots containing more than 50 ng of cDNA will appear equally stained under UV light.

---

## Choosing a Nucleic Acid Stain

DNA may be detected using ethidium bromide or SYBR<sup>®</sup> Gold Nucleic Acid Gel Stain available from Molecular Probes (Catalog no. S11494). We recommend using SYBR<sup>®</sup> Gold because it is 10-fold more sensitive than ethidium bromide for detecting DNA in electrophoretic gels.

Ethidium bromide staining requires preparing plates containing agarose plus ethidium bromide. SYBR<sup>®</sup> Gold staining requires preparing agarose-only plates followed by staining the plate using a SYBR<sup>®</sup> Gold solution. Guidelines are provided in this section for both stains.

---

## Using the pEXP7-tet Positive Control

Supercoiled pEXP7-tet DNA is included with the kit as a positive control for the BP recombination reaction. pEXP7-tet can also be used as a DNA standard for the plate spotting assay. The concentration of your cDNA samples can be estimated by comparison under UV light to known quantities of pEXP7-tet DNA.

---

## Number of Plates Needed

You will need two plates per library. One plate will contain each of your fractions and another plate will contain cDNA samples that were pooled and ethanol precipitated.

---

## Before Starting

You should have the following materials on hand before beginning.

### Supplied with kit:

- pEXP7-tet control DNA (50 ng/μl)

### Supplied by user:

- Polystyrene petri dishes, 100 × 15 mm
  - Ethidium bromide (optional, 10 mg/ml)
  - SYBR<sup>®</sup> Gold Nucleic Acid Gel Stain (recommended; Molecular Probes Catalog no. S11494)
  - 1% agarose in TAE buffer
- 

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# Performing the Plate Spotting Assay, continued

## Preparing Plates

1. Prepare a 100 ml solution of 1% agarose in 1X TAE buffer. Heat until agarose dissolves and let cool for a few minutes.  
If you will be staining your cDNA with ethidium bromide, proceed to step 2. If you will not be using ethidium bromide, skip to step 3.
2. Add 10  $\mu\text{l}$  of ethidium bromide (10 mg/ml) to the agarose solution for a final concentration of 1  $\mu\text{g}/\text{ml}$ . Swirl the solution to mix.
3. Pour the agarose solution into a petri dish just until the bottom is covered. This will be approximately 15 ml for a 100 x 15 mm plate.
4. Allow agarose to solidify at room temperature (keep plates in the dark if you are using ethidium bromide). Plates can be stored at +4°C for up to one month. Warm plates to room temperature before use.

## Preparing pEXP7-tet Control DNA

Serially dilute pEXP7-tet control DNA in TE buffer to final concentrations of:

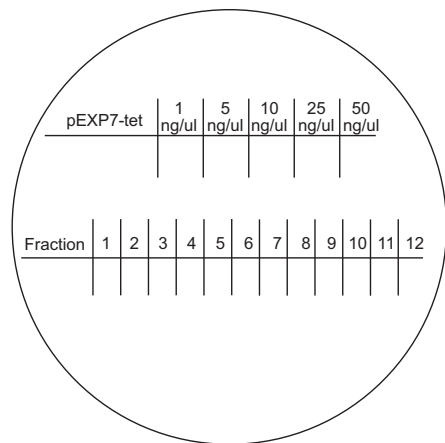
- 25 ng/ $\mu\text{l}$
- 10 ng/ $\mu\text{l}$
- 5 ng/ $\mu\text{l}$
- 1 ng/ $\mu\text{l}$

DNA standards can be stored at -20°C for up to 1 month.

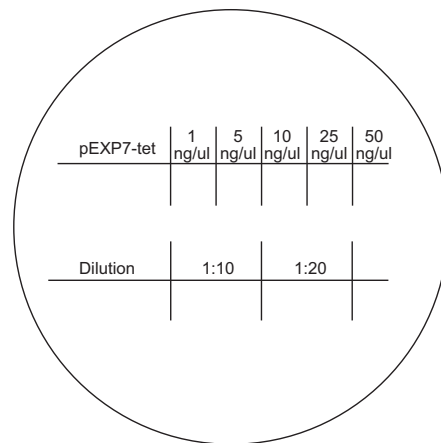
## Labeling Plates

Using a marker, label plates on the bottom side of the petri dish and indicate where the DNA standards and samples will be spotted (see below).

### Sample Plates for cDNA Size Fractionation by Column Chromatography



Column Chromatography Fractions



Dilutions of Pooled Fractions

*continued on next page*

# Performing the Plate Spotting Assay, continued

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## Guidelines

Consider the following points before performing the DNA plate spotting assay:

- Warm plates to room temperature before using
  - Do not reuse plates
  - Spot DNA standards and cDNA samples within 10 minutes of each other
- 

## DNA Spotting Assay

1. Onto a prewarmed plate, spot 1  $\mu$ l of each pEXP7-tet control DNA dilution. Avoid touching the agarose with the pipette tip. When the 1  $\mu$ l aliquot is released, capillary action will pull the small volume from the pipette tip onto the plate surface. Avoid formation of bubbles.
  2. Once the DNA standards are spotted, spot 1  $\mu$ l of each cDNA sample in a similar fashion.
  3. Allow spots to dry at room temperature for 5–15 minutes.
  4. If you are staining your samples with SYBR<sup>®</sup> Gold, proceed to **Staining Plates with SYBR<sup>®</sup> Gold**, below. If you are staining your samples with ethidium bromide, proceed to the next step.
  5. Remove the lid and visualize the plate under UV light and photograph. Note that the labels and samples will be in the reverse order.
  6. Using the known concentration of the DNA standards, estimate the amount of cDNA in each sample. Refer to page 72 for a sample plate.
- 

## Staining Plates with SYBR<sup>®</sup> Gold

1. Add 5  $\mu$ l of SYBR<sup>®</sup> Gold to 50 ml of TAE buffer to make a 1x stain. This solution can be stored in the dark per manufacturer's instructions.
  2. Remove the plate lid and pour the SYBR<sup>®</sup> Gold solution over the agarose until the entire plate is covered (approximately 15 ml). Place the plate in a box and wrap in foil to protect the solution from light.
  3. Shake the plate on a lab shaker for 20 minutes.
  4. Discard the stain in the appropriate waste. Air dry the plate.
  5. Remove the lid and visualize the plate under UV light and photograph. Note that the labels and samples will be in the reverse order.
  6. Using the known concentration of the DNA standards, estimate the amount of cDNA in each sample. Refer to page 72 for a sample plate.
-

# Performing the LR Library Transfer Reaction

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## Introduction

Once you have qualified your cDNA library and analyzed 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.

---

## Before Starting

You should have the following materials on hand before beginning.

### Supplied with kit:

- 30% PEG/Mg solution

### Supplied by user:

- S.N.A.P.™ MidiPrep Kit, recommended (Invitrogen, Catalog no. K1910-01)
  - 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)
  - LR Clonase™ enzyme mix (Invitrogen Catalog no. 11791-019)
  - 5X LR Clonase™ Reaction Buffer (supplied with LR Clonase™ enzyme mix)
  - Ice bucket
  - Proteinase K (2 µg/µl) (supplied with LR Clonase™ enzyme mix)
  - Sterile water
  - Glycogen (20 µg/µl)
  - 7.5 M NH<sub>4</sub>OAc
  - 100% ethanol
  - Dry ice or a -80°C freezer
  - 70% ethanol
  - ElectroMAX™ DH10B™ T1 Phage Resistant Cells or equivalent
- 

## Preparing Double-Stranded DNA

You may prepare plasmid DNA from your cDNA library using your method of choice. We recommend using the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01). Consider the following points when preparing your DNA:

- Inoculate  $5 \times 10^6$ – $1 \times 10^7$  cfu of your cDNA library into 50 ml of LB containing 50 µg/ml kanamycin
  - Grow the culture to an OD<sub>600</sub> of 1.0 (approximately 6 hours)
  - Use TE buffer, pH 8.0 to elute your DNA
- 

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## Performing the LR Library Transfer Reaction, continued

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**PEG Precipitation** After you have prepared 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  $\mu$ l of TE buffer. If you started with less than  $5 \times 10^6$  clones, resuspend the pellet in less TE buffer.
  4. Determine the DNA yield (see **Determining DNA Yield**, below).
  5. Dilute the DNA to 25 ng/ $\mu$ l. 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 60s).
- 

### Determining the DNA Yield

1. Dilute 5–10  $\mu$ l 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/ $\mu$ l.
- 

### 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/ $\mu$ l)	2 $\mu$ l	--	--
Positive control plasmid (25 ng/ $\mu$ l)	--	--	2 $\mu$ l
Destination vector (150 ng/ $\mu$ l)	3 $\mu$ l	3 $\mu$ l	3 $\mu$ l
5X LR Clonase™ Reaction Buffer	4 $\mu$ l	4 $\mu$ l	4 $\mu$ l
TE Buffer, pH 8.0	5 $\mu$ l	7 $\mu$ l	5 $\mu$ l
<b>Total volume</b>	<b>14 <math>\mu</math>l</b>	<b>14 <math>\mu</math>l</b>	<b>14 <math>\mu</math>l</b>

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*continued on next page*

## Performing the LR Library Transfer Reaction, continued

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### LR Library Transfer Reaction, continued

2. Remove the LR Clonase™ enzyme mix from –80°C and thaw on ice (~2 minutes).
  3. Vortex the LR Clonase™ enzyme mix briefly twice (2 seconds each time).
  4. Add 6 µl of LR Clonase™ enzyme mix to each sample. Mix well by vortexing briefly twice (2 seconds each time).  
**Reminder:** Return LR Clonase™ enzyme mix to –80°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**, below.
- 

### 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 <sub>4</sub> OAc	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 tube in dry ice or at –80°C for 10 minutes. Centrifuge the sample at +4°C for 25 minutes at 14,000 rpm.
  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 14,000 rpm. 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.
- 

### Transforming Competent *E. coli*

You may use any *recA*, *endA* *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 *ccdA* gene and will prevent negative selection with the *ccdB* 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 **Transforming Competent Cells**, page 34.

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*continued on next page*

## Performing the LR Library Transfer Reaction, continued

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### Analyzing the Expression Library

Follow the guidelines outlined in the section entitled **Analyzing 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 *BsrG* I which cuts within both *attB* sites of the expression library as well as within the *attR* sites and *ccdB* gene for non-recombined destination vectors
  - Digest and electrophorese your destination vector with no insert to determine the background *BsrG* I digestion pattern for your particular destination vector
- 

### What You Should See

When starting with  $\geq 5 \times 10^6$  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.

---



# 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.

Problem	Cause	Solution
Low cDNA yield or low incorporation of [ $\alpha$ - $^{32}$ P]dCTP after first strand synthesis (radiolabeling method only)	Insufficient starting mRNA	Quantitate the mRNA by measuring the $A_{260}$ , if possible. We recommend using 1–5 $\mu$ g of starting mRNA.
	Poorly prepared mRNA or degraded mRNA	Follow the recommendations for mRNA isolation and working with mRNA (see page 10).
	Old [ $\alpha$ - $^{32}$ P]dCTP or [ $\alpha$ - $^{32}$ P]dCTP not added	Do not use [ $\alpha$ - $^{32}$ P]dCTP that is more than 2 weeks old. Use fresh [ $\alpha$ - $^{32}$ P]dCTP. See page 15 for guidelines on preparing [ $\alpha$ - $^{32}$ P]dCTP.
	Essential reagent accidentally not added or not working	Perform the 2.3 kb RNA control reaction to verify that the correct reagents have been added and are working properly.
	Inaccurate incubation temperatures or temperature fluctuations	Perform the first strand reaction at 45°C. Keep reactions at 45°C when adding SuperScript™ II RT.
	SuperScript™ II RT stored incorrectly	Store SuperScript™ II RT at –20°C in a frost-free freezer.
Low cDNA yield after size fractionation by column chromatography	Faulty columns	Check each column to verify that it is working properly. See page 27 for column specifications.
	Samples run too quickly over columns	Let columns drain completely before adding additional buffer.
Low cDNA library titer with pUC19 transformation control working properly	cDNA of poor quality	Make sure the first strand reaction shows >15% percent incorporation of [ $\alpha$ - $^{32}$ P]dCTP (radiolabeling method only).
	Insufficient ligation of <i>attB1</i> Adapter	Perform the 2.3 kb RNA control reactions to verify the ligation step worked properly.
	Incorrect ratio of cDNA to pDONR™222	Refer to page 31 for the recommended ratio of cDNA to pDONR™222 for the BP reaction.

*continued on next page*

## Troubleshooting, continued

Problem	Cause	Solution
Low cDNA library titer with pUC19 transformation control working properly, continued	Insufficient amount of cDNA used in the BP recombination reaction	Use the minimum amount of cDNA required for the BP recombination reaction. Refer to page 29 for the radiolabeling method and page 49 for the non-radiolabeling method.
	BP Clonase™ enzyme mix is inactive or suggested amount was not used	<ul style="list-style-type: none"> <li>• Perform the pEXP7-tet positive control reactions to verify that BP Clonase™ enzyme mix is active</li> <li>• Test another aliquot of the BP Clonase™ enzyme mix</li> <li>• Make sure that you store the BP Clonase™ enzyme mix at –80°</li> <li>• Do not freeze/thaw the BP Clonase™ enzyme mix more than 10 times</li> <li>• Use the recommended amount of BP Clonase™ enzyme mix (see page 32)</li> </ul>
	Recombination reactions were not treated with proteinase K	Treat reactions with proteinase K before transformation.
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	Once you have thawed a tube of competent cells, discard any unused cells.
	Transformation performed incorrectly	Closely follow the electroporation protocol for ElectroMAX™ DH10B™ competent cells on page 39. If you are using another <i>E. coli</i> strain, follow the manufacturer's instructions.
	Electroporator parameters not optimized	Follow recommended settings for BioRad Gene Pulser® II and BTX® ECM® 630 on page 38 or optimize your own electroporator to achieve a transformation efficiency of $>1 \times 10^{10}$ cfu/μg DNA.
	Loss of transformation efficiency due to arcing	See recommendations on page 39 to reduce chances of arcing.

*continued on next page*

## Troubleshooting, continued

Problem	Cause	Solution
Low average insert size	cDNA of poor quality	Make sure the first strand reaction shows >15% percent incorporation of [ $\alpha$ - <sup>32</sup> P]dCTP (radiolabeling method only).
	Column fractions pooled beyond 600 $\mu$ l	Do not include these fractions as they will contaminate your cDNA library with <i>attB1</i> Adapter.
	Too much cDNA pooled from fractions	Make sure to pull only the minimum required amount of cDNA from the earliest column fractions. For recommendations on selecting and pooling cDNA fractions, see page 29 (radiolabeling method ) or page 50 (non-radiolabeling method).
Low percentage of recombinants	cDNA of poor quality	Make sure the first strand reaction shows >15% percent incorporation of [ $\alpha$ - <sup>32</sup> P]dCTP (radiolabeling method only).
	Insufficient amount of cDNA used in the BP recombination reaction	Use the minimum amount of cDNA required for the BP recombination reaction. Refer to page 29 for the radiolabeling method and page 49 for the non-radiolabeling method.
Unable to distinguish spots for the plate spotting assay (non-radiolabeling method only)	cDNA concentration in sample is too high	You will not be able to distinguish between spots containing more than 50 ng of cDNA. Spot 0.5 $\mu$ l of the sample or dilute an aliquot of the sample before spotting.
	Samples are not properly spotted	Make sure to not touch or pierce agarose with the pipette tip. Allow spots to dry at room temperature before staining with SYBR <sup>®</sup> Gold.
	Plates stored incorrectly or plates too old	Store agarose plates with ethidium bromide in the dark at +4°C. Store plates for no longer than one month. For best results, use fresh plates.
	Stain stored incorrectly or stain too old	Store stain according to manufacturer's instructions. When staining plates with SYBR <sup>®</sup> Gold, make sure to protect plate from light.

# Recipes

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## **10% Trichloroacetic Acid + 1% Sodium Pyrophosphate**

100% trichloroacetic acid (TCA) (see below)  
Sodium pyrophosphate decahydrate

1. Dissolve 10 g of sodium pyrophosphate in 750 ml of deionized water.
  2. Add 100 ml of 100% trichloroacetic acid (TCA).
  3. Bring final volume to 1 L with deionized water.
  4. Store at +4°C for up to 6 months.
- 

## **100% Trichloroacetic Acid**

This recipe is designed to hydrate one standard 500 g bottle of TCA crystals. If you wish to hydrate a different size bottle of TCA, adjust the volume sizes accordingly. **Use caution when handling TCA. TCA causes severe burns and is harmful if swallowed or inhaled.**

1. Add 227 ml of deionized water to a 500 g bottle of TCA.
  2. Cap the bottle tightly and invert the bottle slowly several times to dissolve the TCA thoroughly.
  3. Add a stir bar to the bottle and stir the solution until homogeneous. No further volume adjustment is required.
  4. Store at room temperature for up to one year.
- 

## **5% Trichloroacetic Acid**

1. Add 50 ml of 100% trichloroacetic acid to 950 ml of deionized water.
  2. Store at room temperature for up to 3 months.
- 

## **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 solution is homogeneous.
  2. Autoclave for 30 minutes on liquid cycle.
  3. Store at room temperature for up to 1 month.
-

# Sample cDNA Library

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## Introduction

In this section, we provide a sample experiment to illustrate the cDNA library construction process. This experiment starts with isolated mRNA and continues through construction and qualification of a radiolabeled cDNA library. All steps were performed according to the protocols in this manual.

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## Starting mRNA

3  $\mu\text{g}$  of high-quality HeLa cell mRNA

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## First Strand Analysis

A sample of the first strand reaction was removed and analyzed to determine specific activity, cDNA yield, and percent incorporation of [ $\alpha$ - $^{32}\text{P}$ ]dCTP. The unwashed and washed filters gave the following counts:

	Counts per Minute (cpm)
Unwashed Filter	45998
Washed Filter	2601

### Specific Activity

The specific activity was determined using the counts for the unwashed filter and the equation below:

$$\begin{aligned}\text{SA (cpm/pmol dCTP)} &= \frac{(\text{cpm unwashed filter}/10 \mu\text{l})}{(200\text{pmol dCTP}/10 \mu\text{l})} \\ &= \frac{(45998\text{cpm}/10 \mu\text{l})}{(200\text{pmol dCTP}/10 \mu\text{l})} \\ &= 230 \text{ cpm/pmol dCTP}\end{aligned}$$

### First Strand cDNA Yield

The first strand cDNA yield was determined using the counts for the washed filter, the calculated specific activity, and the equation below:

$$\begin{aligned}\text{cDNA Yield } (\mu\text{g}) &= \frac{(\text{cpm of washed filter}) \times (25\mu\text{l}/10 \mu\text{l}) \times (20\mu\text{l}/1 \mu\text{l}) \times (4 \text{ pmol dNTP}/\text{pmol dCTP})}{\text{SA (cpm/pmol dCTP)} \times (3030\text{pmol dNTP}/ \mu\text{g cDNA})} \\ &= \frac{(\text{cpm of washed filter}) \times 50 \times (4 \text{ pmol dNTP}/\text{pmol dCTP})}{\text{SA (cpm/pmol dCTP)} \times (3030\text{pmol dNTP}/ \mu\text{g cDNA})} \\ &= \frac{(\text{cpm of washed filter}) \times (200)}{\text{SA} \times (3030)} \\ &= \frac{2601 \times 200}{230 \times 3030} \\ &= 0.746 \mu\text{g cDNA}\end{aligned}$$

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*continued on next page*

## Sample cDNA Library, continued

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### First Strand Analysis, continued

#### Percent Incorporation

The percent incorporation of [ $\alpha$ - $^{32}$ P]dCTP was determined using the calculated first strand cDNA yield and the equation below:

$$\begin{aligned}\text{Percent Incorporation} &= \frac{\text{cDNA yield } (\mu\text{g})}{\text{starting mRNA amount } (\mu\text{g})} \times 100 \\ &= \frac{0.746 \mu\text{g cDNA}}{3 \mu\text{g starting mRNA}} \times 100 \\ &= 25\%\end{aligned}$$

The results of the first strand analysis are summarized below:

Specific Activity	230 cpm/pmol dCTP
cDNA Yield	0.746 $\mu$ g
Percent Incorporation	25%

---

### Size Fractionation by Column Chromatography

After *attB1* adapter ligation, the cDNA was size fractionated using column chromatography. The results are listed in the sample worksheet on the next page. Tube 5 was the first tube to give Cerenkov counts above background. Using the data for tube 5, we demonstrate below how the worksheet was filled out.

#### Tube 5 Example

The volume in tube 5 was measured to be 36  $\mu$ l (column A). Adding this volume to the previous cumulative volume (*i.e.* 306  $\mu$ l) gave a total volume of 342  $\mu$ l (column B). The Cerenkov count was 213 cpm (column C).

The double strand cDNA yield was determined using the count value from column C, the specific activity already calculated in the first strand analysis, and the equation below:

$$\begin{aligned}\text{Amount of ds cDNA (ng)} &= \frac{(\text{Cerenkov cpm}) \times 2 \times (4 \text{ pmol dNTP/pmol dCTP}) \times (1,000 \text{ ng/ } \mu\text{g ds cDNA})}{\text{SA (cpm/pmol dCTP)} \times (1515 \text{ pmol dNTP/ } \mu\text{g ds cDNA})} \\ &= \frac{(\text{Cerenkov cpm}) \times 8}{\text{SA} \times (1.515)} \\ &= \frac{(213) \times 8}{230 \times (1.515)} \\ &= 4.9 \text{ ng cDNA (column D)}\end{aligned}$$

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*continued on next page*

## Sample cDNA Library, continued

### Size Fractionation by Column Chromatography, continued

The concentration of cDNA was determined using the calculated cDNA yield and the value in column A.

$$\text{Concentration of cDNA (ng/}\mu\text{l)} = \frac{\text{amount of cDNA (ng)}}{\text{fraction volume (}\mu\text{l)}}$$

$$= \frac{\text{column D}}{\text{column A}}$$

$$= \frac{4.9 \text{ ng}}{36 \mu\text{l}}$$

$$= 0.136 \text{ ng/}\mu\text{l (column E)}$$

### Sample cDNA Library Worksheet

Tube	A Fraction Volume ( $\mu\text{l}$ )	B Total Volume ( $\mu\text{l}$ )	C Cerenkov Counts (cpm)	D Amount of cDNA (ng)	E Concentration of cDNA (ng/ $\mu\text{l}$ )
1	151	151	22	--	--
2	85	236	14	--	--
3	34	270	25	--	--
4	36	306	15	--	--
5	36	342	213	4.9	0.136
6	34	376	1136	26.1	0.77
7	35	411	2628	60.3	1.72
8	36	447	4114	94.5	2.625
9	36	483	4427	101.6	2.82
10	33	516	3614	83.0	2.52
11	36	552	2947	67.7	1.88
12	36	588	2139	49.1	1.36
13	36	624	1761	40.4	1.12
14	36				
15	36				
16	35				
17	36				
18	36				
19	36				
20	36				

*continued on next page*

## Sample cDNA Library, continued

---

### Selecting and Pooling Fractions

Fractions 5, 6, and part of fraction 7 were pooled together for a total of 61.1 ng of cDNA (see table below).

Fraction	Pooled Volume (μl)	Concentration of cDNA (ng/μl)	Amount of cDNA (ng)
5	36	0.136	4.9
6	34	0.77	26.1
7	17.5	1.72	30.1
<b>Total Pooled cDNA (ng)</b>			<b>61.1</b>

---

### Calculating the cDNA Yield

After ethanol precipitation, the pooled cDNA gave a Cerenkov count of 1538 cpm. cDNA yield was determined using the count value, the specific activity already calculated in the first strand analysis, and the equation below:

$$\begin{aligned}\text{Amount of ds cDNA (ng)} &= \frac{(\text{Cerenkov cpm}) \times 2 \times (4 \text{ pmol dNTP/pmol dCTP}) \times (1,000 \text{ ng/ } \mu\text{g ds cDNA})}{\text{SA (cpm/pmol dCTP)} \times (1515 \text{ pmol dNTP/ } \mu\text{g ds cDNA})} \\ &= \frac{(\text{Cerenkov cpm}) \times 8}{\text{SA} \times (1.515)} \\ &= \frac{(1538) \times 8}{230 \times (1.515)} \\ &= 35.3 \text{ ng cDNA}\end{aligned}$$

---

### BP Recombination Reaction

The entire 4 μl cDNA sample containing a total of 35.3 ng of cDNA was used in the BP recombination reaction. All other components were added according to the specifications on page 32. BP reactions were divided and transformed into six aliquots of ElectroMAX™ DH10B™ T1 Phage Resistant Cells.

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*continued on next page*



## Sample cDNA Library, continued

### Determining the cDNA Library Titer

The results of the plating assay are listed below.

Dilution	Amount Plated (μl)	Colonies Per Plate
10 <sup>-2</sup>	100 μl	654
10 <sup>-3</sup>	100 μl	54
10 <sup>-4</sup>	100 μl	7

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

$$\begin{aligned} \text{cfu/ml} &= \frac{\text{colonies on plate} \times \text{dilution factor}}{\text{volume plated (ml)}} \\ &= \frac{654 \text{ colonies} \times 100}{0.10 \text{ ml}} \\ &= 6.54 \times 10^5 \text{ cfu/ml} \end{aligned}$$

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.

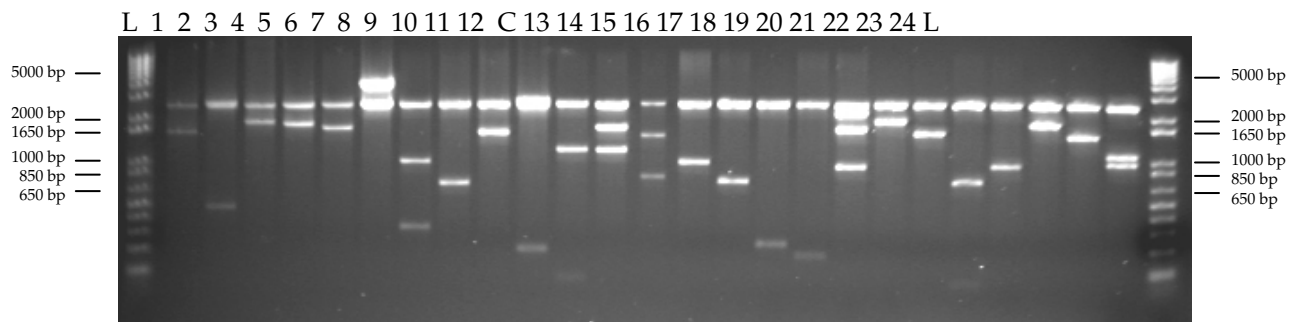
Dilution	Amount Plated (μl)	Colonies Per Plate	Titer (cfu/ml)	Average Titer (cfu/ml)	Total Volume (ml)	Total CFUs (cfu)
10 <sup>-2</sup>	100 μl	654	6.54 × 10 <sup>5</sup>	6.31 × 10 <sup>5</sup>	12	7.6 × 10 <sup>6</sup>
10 <sup>-3</sup>	100 μl	54	5.4 × 10 <sup>5</sup>			
10 <sup>-4</sup>	100 μl	7	7 × 10 <sup>5</sup>			

*continued on next page*

## Sample cDNA Library, continued

### Qualifying the cDNA Library

Plasmid DNA was isolated from 24 colonies using the S.N.A.P.<sup>™</sup> MiniPrep Kit from Invitrogen. 300–500 ng of plasmid DNA and 250 ng of supercoiled pDONR<sup>™</sup>222 were digested with *Bsr*G I and run on a 1% agarose gel stained with ethidium bromide. Results are shown below. Note that pDONR<sup>™</sup>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 from Invitrogen  
C = Digested pDONR<sup>™</sup>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

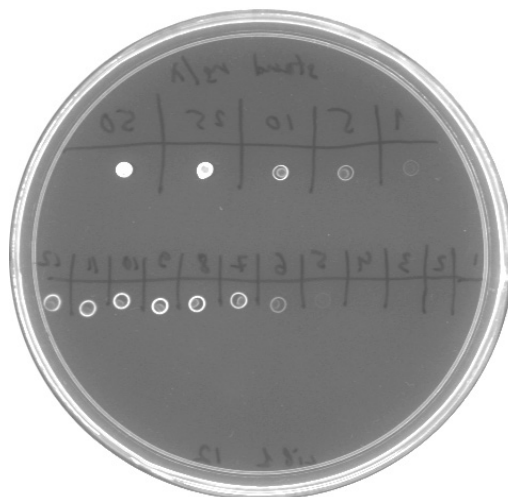
Number of Clones Analyzed	24
Number of Clones Containing Inserts	24
Percent Recombinants	100%
Average Insert Size (kb)	1.8
Insert Size Range (kb)	0.2–4.75

\*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.

# Sample Size Fractionation with Non-Radiolabeled cDNA

## Size Fractionation by Column Chromatography

A sample plate and worksheet is provided below to demonstrate how to estimate the yield of your non-radiolabeled cDNA. Samples were size fractionated by column chromatography and cDNA yields were estimated using the plate spotting assay. Refer to **Labeling Plates**, page 55 to see how plates were labeled. Note that samples are in the reverse order.



Serial dilutions of pEXP7-tet control DNA and column fractions 1–13 were spotted and stained with SYBR<sup>®</sup> Gold as described on page 54.

Tube	A Fraction Volume (μl)	B Total Volume (μl)	C Concentration of cDNA (ng/μl)	D Amount of cDNA (ng)
1	151	151	--	--
2	85	236	--	--
3	34	270	--	--
4	36	306	--	--
5	36	342	0.5	18
6	34	376	4	136
7	35	411	8	280
8	36	447	10	360
9	36	483	--	--
10	33	516	--	--
11	36	552	--	--
12	36	588	--	--
13	36	624	--	--
14	36			
15	36			
16	35			
17	36			
18	36			
19	36			
20	36			

*continued on next page*

## Sample Size Fractionation with Non-Radiolabeled cDNA, continued

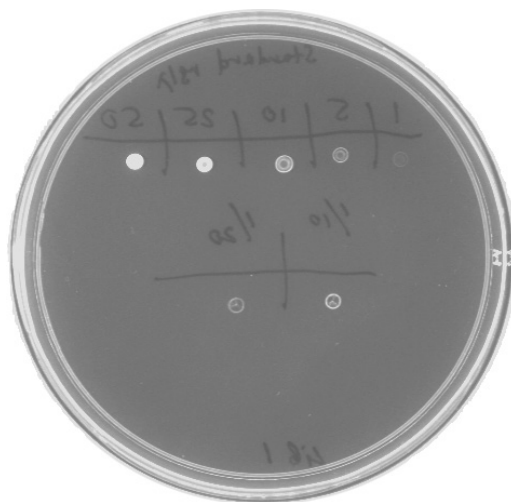
### Selecting and Pooling Fractions

Fractions 5, 6, and part of fraction 7 were pooled together for a total of 294 ng of cDNA (see table below).

Fraction	Pooled Volume ( $\mu\text{l}$ )	Concentration of cDNA ( $\text{ng}/\mu\text{l}$ )	Amount of cDNA (ng)
5	36	0.5	18
6	34	4	136
7	17.5	8	140
<b>Total Pooled cDNA (ng)</b>			<b>294</b>

### Estimating the cDNA Yield

After ethanol precipitating the pooled cDNA, cDNA yield was estimated using the plate spotting assay. Refer to **Labeling Plates**, page 55 to see how plates were labeled. Note that samples are in the reverse order.



Serial dilutions of pEXP7-tet control DNA and two dilutions of ethanol-precipitated cDNA were spotted and stained with SYBR<sup>®</sup> Gold as described on page 54.

	<b>1:10 Dilution</b>	<b>1:20 Dilution</b>
cDNA Concentration of Diluted Sample ( $\text{ng}/\mu\text{l}$ )	5	2.5
Final cDNA concentration ( $\text{ng}/\mu\text{l}$ )	50	50
Volume of cDNA ( $\mu\text{l}$ )	4	4
<b>Total cDNA Yield (ng)</b>	<b>200</b>	<b>200</b>

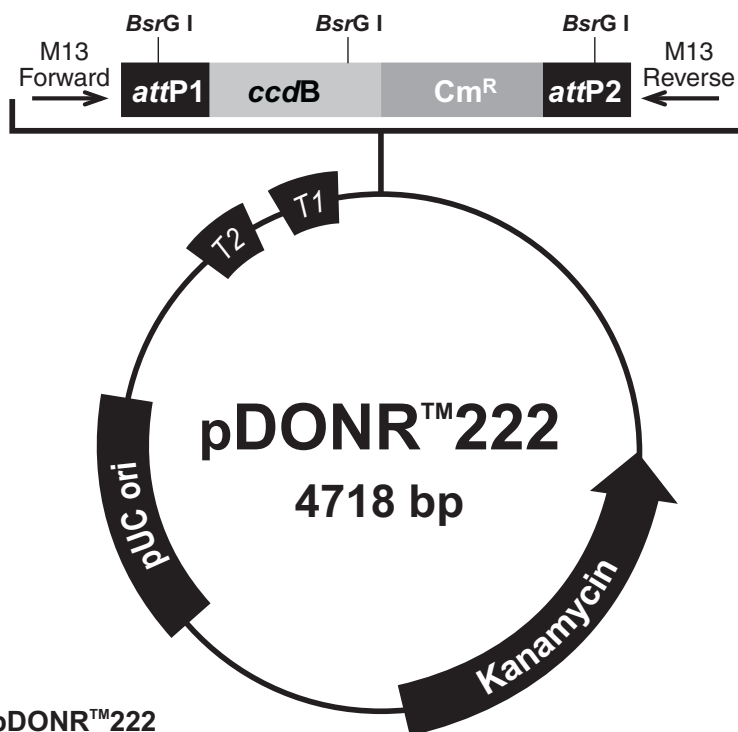
### BP Recombination Reaction

3  $\mu\text{l}$  of the cDNA sample containing a total of 150 ng of cDNA was used in the BP recombination reaction.

# 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 our Web site ([www.invitrogen.com](http://www.invitrogen.com)) or by contacting Technical Service (page 77).



### Comments for pDONR™222 4718 nucleotides

*rrnB* 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  
*attP1*: bases 360-591  
*BsrG I* restriction sites: bases 442, 1232, 2689  
*ccdB* gene: bases 987-1292 (c)  
Chloramphenicol resistance gene: bases 1612-2295 (c)  
*attP2*: 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
<i>rrnB</i> T1 and T2 transcription terminators	Protects the cloned gene from expression by vector-encoded promoters, thereby reducing possible toxicity (Orosz <i>et al.</i> , 1991).
M13 forward (-20) priming site	Allows sequencing in the sense orientation.
<i>attP1</i> and <i>attP2</i> sites	Bacteriophage $\lambda$ -derived DNA recombination sequences that permit recombinational cloning of <i>attB</i> -containing cDNA (Landy, 1989).
<i>BsrG</i> I restriction sites	Allows detection and size determination of cDNA inserts by restriction enzyme analysis.
<i>ccdB</i> 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 <i>E. coli</i> .
pUC origin	Allows high-copy replication and maintenance of the plasmid in <i>E. coli</i> .

# Experimental Worksheet for the Radiolabeling Method

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## Introduction

A worksheet is provided to help you with your record keeping and calculations. Before you record any data, we suggest you make several copies of this worksheet for use with additional cDNA synthesis reactions.

Tube	A Fraction Volume ( $\mu$ l)	B Total Volume ( $\mu$ l)	C Cerenkov Counts (cpm)	D Amount of cDNA (ng)	E Concentration of cDNA (ng/ $\mu$ l)
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

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# Experimental Worksheet for the Non-Radiolabeling Method

---

## Introduction

A worksheet is provided to help you with your record keeping and calculations. Before you record any data, we suggest you make several copies of this worksheet for use with additional cDNA synthesis reactions.

Tube	A Fraction Volume ( $\mu$ l)	B Total Volume ( $\mu$ l)	C Concentration of cDNA (ng/ $\mu$ l)	D Amount of cDNA (ng)
1				
2				
3				
4				
5				
6				
7				
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# Technical Service

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## World Wide Web



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## Technical Service, continued

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