USER GUIDE

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SuperScript® Human Stem Cell cDNA Libraries

For isolating cDNAs, arraying cDNA, and gene expression

Catalog numbers A1030001, A1030101, A1030201, A1030301, A1030401, and A1030501

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General Information

Type of Libraries

This manual is supplied with the following SuperScript® cDNA Libraries in pCMV•SPORT6.1:

Product	Catalog no.
hEC Standard cDNA Library, N-tera2	A1030001
hEC Standard cDNA Library, 2102Ep	A1030101
hESC Standard cDNA Library, BG01v	A1030201
hESC Normalized cDNA Library, BG01	A1030301
hESC Normalized cDNA Library, BG02	A1030401
hESC Normalized cDNA Library, BG03	A1030501

Contents and Storage

Each SuperScript® Stem Cell cDNA Library in pCMV•SPORT6.1 is supplied in 80% SOB medium, 20% (v/v) glycerol.

Store the library at -80°C.

Library Specification

The general specifications for each type of Stem Cell cDNA Library in pCMV•SPORT6.1 are described below.

For more details, refer to the Certificate of Analysis included with the specific library.

Libraries	Features	Total Primary Clones	Av. Insert Size
Standard Amplified	• High representation • > 87% colonies contain insert	>1 x 10^9	>1.2 kb
Normalized, Full-length, Amplified	• High representation • 87% colonies contain insert • Percentage full-length is ≥ 60% • Fold reduction of the high abundance gene should ≥ 10	>1 x 10^9	>1.4 kb

Intended Use

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

Cell Line Information

Human embryonic stem cell lines (BG01, BG02, BG03 and BG01v) were cultured in hESC medium (DMEM-F12, 20% Knockout™ Serum Replacement, 1% Non-essential amino acid, 55 μM 2-Mercaptoethanol, 2 mM L-Glutamine, supplemented with 4 ng/ml basic FGF) conditioned by mitomycin C-inactivated mouse embryonic fibroblast cells (MEF) in a feeder-free fashion. Cells were passaged using collagenase at a ratio of 1:2-4 every 4-5 days.

Human embryonic carcinoma lines (NTera2 and 2102Ep) were cultured in DMEM with 10% FBS and passaged using trypsin at a ratio of 1:4-5 every 3-4 day. All embryonic stem cells are pluripotent.

Immunology markers and other information are summarized in the following table.

Cell Line	NTera2	2102Ep	BG01V	BG01	BG02	BG03
Source	Human malignant embryonic testicular carcinoma, subcloned	Primary human testicular teratocarcinoma, subcloned	Human blastocyst	Human blastocyst	Human blastocyst	Human blastocyst
Passage	9	55	67	65	35	18
Karyotype	hypotriploid	hyperdiploid	Abnormal 48XY 12+/17+	Normal 46XY	Normal 46XY	Normal 46XX
Gender	Male	Male	Male	Male	Male	Female
Pluripotent	Yes	Yes	Yes	Yes	Yes	Yes
Immunology Markers:						
SSEA-1	-	-	-	-	-	-
SSEA-3	N/A	N/A	+	+	+	+
SSEA-4	+	+	+	+	+	+
TRA 1-60	+	+	+	+	+	+
TRA 1-81	+	+	+	+	+	+
Oct-4	+	+	+	+	+	+
Alkaline Phosphatase	+	+	+	+	+	+

Accessory Products

Additional Products

The table below lists additional products that may be used with the Stem Cell cDNA Library.

Product	Quantity	Catalog no.
Terrific Broth	500 g	22711-022
T7 Promoter Primer	327 pmoles	N560-02
Sp6 Promoter Primer	342 pmoles	N550-02
2.5 mM dNTP Mix	1 ml	R725-01
Platinum® *Taq* DNA Polymerase	100 reactions	10966-018
Lipofectamine® 2000	1.5 ml	11668-019
pDONR™221	6 µg	12536-017
Phenol:Chloroform:Isoamyl Alcohol, (25:24:1, v/v/v)	100 ml	15593-031
Gateway® BP Clonase® II Enzyme Mix	20 reactions	11789-020
Gateway® LR Clonase® II Enzyme Mix	20 reactions	11791-020
One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells	20 reactions	C8540-03
One Shot® MAX Efficiency® DH10B™ T1 Phage Resistant Cells	20 reactions	12331-013
Not I	200 units	15441-025
*Eco*R I	5000 units	15202-013
REact® 3 Buffer	2 x 1 ml	16303-018
SP6 RNA Polymerase	500 units	18018-010
Library Transfer (BxP reactions) Service	--	11133-020
Library Transfer (LxR reactions) Service	--	11133-019

Introduction

Overview

Introduction

SuperScript® Human Stem Cell cDNA Libraries in pCMV•SPORT6.1 are suitable for isolating cDNAs, PCR of target sequences, cDNA sequencing, preparing cDNA arrays, and gene expression in stem cells.

SuperScript® Human Stem Cell cDNA Libraries are constructed using SuperScript® III Reverse Transcriptase to generate full-length and high-yield cDNA.

For the Normalized Full-Length Libraries, 5'CAP antibody was used to further enrich full-length cDNAs followed by subtraction based negative selection to normalize cDNA library, by which the high abundant genes are reduced and low abundant genes are increased.

Libraries may be screened using any cDNA positive selection system, PCR, or plate screening procedures, *in vitro* transcription analysis, or by functional analysis using the eukaryotic CMV promoter.

pCMV•SPORT 6.1

Important features of pCMV•SPORT6.1 are listed below.

- The CMV promoter for efficient **transient** expression of cloned cDNAs in eukaryotic cells
- An f1 origin for single-strand DNA production
- The SV40 poly A sequence for transcription termination and polyadenylation of mRNA
- Sp6 and T7 RNA polymerase promoter sites flanking the multiple cloning site for *in vitro* transcription of sense and anti-sense RNA
- The recombination sites, *att*B1 and *att*B2 for transfer of cDNA into Gateway®-compatible vectors
- Ampicillin resistance gene for selection of transformants in *E. coli*
- The pUC origin for high copy replication and maintenance of the plasmid in *E. coli*

For map of pCMV•SPORT6.1, see page 13.

Continued on next page

Overview, Continued

Note

The pCMV•SPORT6.1 vector does **NOT** contain a eukaryotic origin of replication or antibiotic resistance marker for stable expression in mammalian cells.

Preparation of SuperScript® Human Stem Cell cDNA Libraries

Each SuperScript® Human Stem Cell cDNA library is prepared as follows:

- mRNA is isolated using two steps. First, total RNA is isolated from tissues or cells using the TRIzol® Reagent. Second, polyA mRNA is isolated from total RNA using the FastTrack® MAG mRNA Isolation Kit (Life Technologies, K1580-02).
- First-strand cDNA is synthesized using SuperScript® III Reverse Transcriptase
- For full-length libraries, mRNA/cDNA hybrids were treated with RNAse I to digest single strand mRNAs followed by incubation with 5´ cap-antibody magnetic beads to enrich for full-length mRNA/cDNA hybrids
- Second-strand cDNA is synthesized using *E. coli* RNAse H, *E. coli* DNA polymerase I, and *E. coli* DNA ligase
- cDNA is blunt-ended using T4 DNA Polymerase and digested with *Not* I
- cDNA is size-selected using column chromatography or agarose gel electrophoresis
- Size-selected cDNA is directionally cloned into the *Not* I-*Eco*R V region of the vector (*Eco*R V site is destroyed during cloning)
- Ligation mixture is transformed into competent DH10B™ T1-Phage Resistant *E. coli* and the number of primary recombinants is determined
- To create normalized libraries, cDNA from the primary library was self-subtracted using excess biotinylated RNA from the same library

Genotype of DH10B™ T1

F′ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara*,*leu*)7697 *gal*U *gal*K *rps*L *nup*G *ton*A (confers phage T1 and T5 resistance)

Methods
Using the Stem Cell cDNA Library

Introduction

Each SuperScript® Human Stem Cell cDNA library may be screened using the PCR or plate screening procedures, by cDNA sequencing, cDNA arrays, *in vitro* transcription analysis using the Sp6 RNA polymerase, or by functional analysis using the eukaryotic CMV promoter.

General procedures for preparing DNA from the library, plate screening, and colony PCR are provided in this section. For detailed information on library screening refer to published references (Ausubel *et al.*, 1994; Sambrook *et al.*, 1989).

To verify library titer and insert size, see pages 15-16.

Plating the Library

Prepare LB plates containing 100 µg/ml ampicillin. To plate a library for screening or picking colonies:

1. Check the titer of the cDNA library on the Certificate of Analysis that is included with the library. If you wish to verify the library titer, see page 15.
2. Based on the library titer, dilute an aliquot of the library such that you can plate 250-500 clones on a 75 mm plate or 2500-5000 clones on a 150 mm plate. Use LB plates containing 100 µg/ml ampicillin.
3. Incubate the plates at 37°C overnight.
4. Use the isolated colonies for colony PCR screening (page 4) or use the method of choice for screening.

Plasmid Isolation

You may use the plasmid DNA isolation kit of choice (see **www.lifetechnologies.com/napq** for a selection of kits). We use the PureLink® HQ Mini Plasmid DNA Purification Kit (Cat. No. K2100-01).

If you are planning to use the plasmid DNA for *in vitro* transcription or other methods to synthesize RNA, you should extract and precipitate the DNA as described on page 18 to remove RNAses. Once you have obtained plasmid DNA you may use it in the application of choice.

For a basic plasmid isolation protocol, see the **Appendix**, page 17.

Continued on next page

Colony PCR Screening

Use this PCR procedure to screen for the presence of specific cDNA or identify desired cDNA clones.

You may use T7 and Sp6 primers (see below for sequences) from the vector or use gene-specific primers.

T7 promoter primer
5′-TAATACGACTCACTATAGGGAGA-3′
Sp6 promoter primer
5′-AGCTATTTAGGTGACACTATAG-3′

1. To 0.5 ml labeled microcentrifuge tube, add 10 µl TE.
2. Using a pipette tip, transfer individual colonies from the plated library (previous page) into separate tubes containing TE. Pipet up and down to mix.
3. Incubate the tubes in a prewarmed thermal cycler at 99°C for 5 minutes.
4. Incubate the tubes on ice for 2 minutes.
5. Centrifuge briefly and place the tubes on ice.
6. Prepare the following reaction mix or use the PCR SuperMix (Cat. No. 10572-014) and add 40 µl of reaction mix to each tube.

   1X PCR Buffer (contains no $MgCl_2$)

   0.2 mM dNTP mix

   0.5 µM primers

   2.4 mM $MgCl_2$

   2.5 units Platinum® *Taq* DNA polymerase
7. Bring the volume to 50 µl with sterile water.
8. Perform PCR using the following program:

Temperature	Time	Cycles
95°C	1 minute	1
94°C	15 seconds	40
60°C	1 minute	
72°C	1 minute	
72°C	5 minutes	1

9. Transfer 10 µl of each reaction to a new tube containing 2 µl 10X gel loading buffer.
10. Electrophorese the samples on a 1.5% agarose gel and analyze your results.

Continued on next page

Expression of Cloned cDNA

The CMV promoter in pCMV•SPORT6.1 vector enables **transient** expression of cloned cDNAs in mammalian cells. Library or individual clones may be screened using a functional assay of choice.

Note: The pCMV•SPORT6.1 vector does **NOT** contain any eukaryotic origin of replication or antibiotic resistance marker for stable expression in mammalian cells.

For expression of cDNA in bacterial, yeast, or insect expression system, you need to transfer the cDNA insert to an appropriate expression vector using the Gateway® Technology (next page). The complete library can be transferred into other different DEST vectors by BxP and LxR reactions. Conditions must be optimized depending on the vector of choice. Library transfer services are available (page iv).

Performing BP Reaction with cDNA Clone

Gateway® Technology

The, pCMV•SPORT6.1 contains *att*B1 and *att*B2 recombination sites flanking the cDNA cloning site. You can transfer the cDNA insert into other Gateway® compatible vectors for expression in different systems by performing a BP recombination reaction with a pDONR™ vector (page iv) to create an entry vector and the subsequent LR recombination reaction with a DEST vector of choice for expression in the desired system (page 10). For details on Gateway® technology, refer to the Gateway® Technology Manual with Clonase® II available on our Web site at **www.lifetechnologies.com** or contact Technical Support (see page 21).

BP Reaction to Entry Vector

To perform the BP recombination reaction, you will transfer the gene of interest into an *att*P-containing donor vector to create an *att*L-containing entry clone. Genes in the human stem cell cDNA clone are transferred to the donor vector backbone by mixing the DNAs with the Gateway® BP Clonase® II Enzyme Mix (Catalog no. 11789-020). The resulting recombination reaction is then transformed into *E. coli* and the entry clone is selected. Recombination between the *att*P (donor vector) and *att*B (cDNA expression clone) sites replaces the *ccd*B gene in the donor vector with the gene of interest and results in the formation of *att*L sites in the entry clone. Once you have obtained an entry vector containing the cDNA gene of interest you may then use the LR reaction to move the gene into the destination (expression) vector of choice. (page 10)

Important

The BP recombination reaction protocol included below is for performing a BP reaction with a specific cDNA clone. **Do not** use this BP recombination reaction to perform a BP reaction with the library. To perform a library transfer (BxP reactions), custom services are available (see page iv).

Continued on next page

Performing BP Reaction with cDNA Clone,
Continued

Note

For optimal efficiency, perform the BP recombination reaction using:

- **Linear** *att*B cDNA clone (see Gateway® Technology manual for linearizing the cDNA expression clone)
- **Supercoiled** *att*P-containing donor vector

Note: Supercoiled or relaxed *att*B substrates may be used, but will react less efficiently than linear *att*B substrates.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α ™, DH10B™, DH10B™T1, or equivalent for transformation. **Do not** use *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Materials Required

You will need the following materials:

- Linearized *att*B cDNA expression clone (40-100 fmole)
- pDONR™ vector (page iv; resuspend to 150 ng/µl with water)
- Gateway® BP Clonase® II Enzyme Mix (page iv, keep at -80°C until use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the Gateway® BP Clonase® II Enzyme Mix; thaw and keep on ice until use)
- pEXP7-tet positive control (50 ng/µl; supplied with the Gateway® BP Clonase® II Enzyme Mix)

Continued on next page

Performing BP Reaction with cDNA Clone,
Continued

Positive Control

pEXP7-tet is provided in the Gateway® BP Clonase® II Enzyme Mix as a positive control for the BP reaction. pEXP7-tet is an approximately 1.4 kb linear fragment and contains *att*B sites flanking the tetracycline resistance gene and its promoter (Tc^r). Using the pEXP7-tet fragment in a BP reaction with a donor vector results in entry clones that express the tetracycline resistance gene. Determine the efficiency of the BP recombination reaction by streaking entry clones onto LB plates containing 20 µg/ml tetracycline.

BP Reaction

BP Clonase® II Enzyme Mix is supplied as a 5X solution. If you wish to scale the reaction volume, make sure the BP Clonase® II Enzyme Mix is at a final concentration of 1X. For a positive control, use 100 ng (2 µl) of pEXP7-tet.

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

Linearized *att*B expression clone (40-100 fmol)	1-7 µl
Donor vector (150 ng/µl)	1 µl
TE buffer, pH 8.0	to 8 µl

2. Thaw on ice the BP Clonase® II Enzyme Mix for about 2 minutes. Vortex the BP Clonase® II Enzyme Mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 µl of BP Clonase® II Enzyme Mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return BP Clonase® II Enzyme Mix to -20°C or -80°C storage.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes. Proceed to transformation, next page.

Continued on next page

Performing BP Reaction with cDNA Clone,
Continued

Transformation

Note: Any competent cells (as long as they do not contain the F´ episome) with a transformation efficiency of >1.0 × 10^8 transformants/µg may be used.

1. Add 1 µl of each BP reaction into 50 µl of One Shot® Competent *E. coli*. Add 1 µl of pUC19 DNA (10 ng/ml) to a separate tube of cells as a positive control for transformation. Incubate on ice for 30 minutes.
2. Heat-shock cells by incubating at 42°C for 30 seconds.
3. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking.
4. Plate 20 µl and 100 µl of each transformation onto selective plates.

Expected Results

An efficient BP recombination reaction may produce hundreds of colonies (>1500 colonies if the entire BP reaction is used for transformation and is plated).

If you included the pEXP7-tet control in your BP recombination experiment, assess the efficiency of the BP reaction by streaking entry clones onto LB agar plates containing 20 µg/ml tetracycline after transformation into a suitable *E. coli* host. True entry clones should be tetracycline-resistant.

Expressing Your Recombinant Protein

Once you have obtained an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into an *att*R-containing destination vector of choice for expression in the desired system. Refer to the Gateway® Technology manual for performing the LR recombination reaction. Library transfer (LxR reaction) services are available (page iv).

Performing the LR Reaction

Introduction

After you have generated an entry clone, you will perform the LR recombination reaction to transfer the gene of interest into an *att*R-containing destination vector to create an *att*B-containing expression clone. For more information, please refer to the Gateway® Manual with Clonase® II.

E. coli Host

You may use any *rec*A, *end*A *E. coli* strain including OmniMAX™ 2-T1^R, TOP10, DH5α™, DH10B™or equivalent for transformation (see page iv for ordering information). **Do not** transform the LR reaction mixture into *E. coli* strains that contain the F′ episome (*e.g.* TOP10F′). These strains contain the *ccd*A gene and will prevent negative selection with the *ccd*B gene.

Positive Control

The pENTR™-gus plasmid is provided with the LR Clonase® II Enzyme Mix for use as a positive control for recombination and expression. Using the pENTR™-gus entry clone in an LR recombination reaction with a destination vector will allow you to generate an expression clone containing the gene encoding alpha-glucuronidase (*gus*) (Kertbundit *et al.*, 1991).

LR Clonase® II Enzyme Mix

LR Clonase® II Enzyme Mix is available separately from Life Technologies (Catalog no. 11791-020) to catalyze the LR recombination reaction. The LR Clonase® II Enzyme Mix combines the proprietary enzyme formulation and 5X LR Clonase Reaction Buffer previously supplied as separate components in LR Clonase® Enzyme Mix (Catalog no. 11791-019) into an optimized single tube format to allow easier set-up of the LR recombination reaction. Use the protocol provided on the next page to perform the LR recombination reaction using LR Clonase® II Enzyme Mix.

Continued on next page

Performing the LR Reaction, Continued

Materials Needed

You should have the following materials on hand before beginning:

- Purified plasmid DNA of your entry clone (50-150 ng/µl in TE, pH 8.0)
- Destination vector of choice (150 ng/µl in TE, pH 8.0)
- LR Clonase® II Enzyme Mix (keep at -20°C until immediately before use)
- TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA)
- 2 µg/µl Proteinase K solution (supplied with the LR Clonase® II Enzyme Mix; thaw and keep on ice until use)
- pENTR™-gus positive control (50 ng/µl; supplied with the LR Clonase® II Enzyme Mix)
- Appropriate competent *E. coli* host and growth media for expression
- S.O.C. Medium
- LB agar plates with the appropriate antibiotic to select for expression clones

Continued on next page

Performing the LR Reaction, Continued

LR Reaction

LR Clonase® II Enzyme Mix is supplied as a 5X solution. If you wish to scale the reaction volume, make sure the LR Clonase® II Enzyme Mix is at a final concentration of 1X. For a positive control, use 100 ng (2 µl) of pENTR™-gus.

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

Entry clone (50-150 ng)	1-7 µl
Destination vector (150 ng/µl)	1 µl
TE buffer, pH 8.0	to 8 µl

2. Thaw on ice the LR Clonase® II Enzyme Mix for about 2 minutes. Vortex the LR Clonase® II Enzyme Mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 µl of LR Clonase® II Enzyme Mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return LR Clonase® II Enzyme Mix to -20˚C or -80˚C storage.
5. Incubate reactions at 25˚C for 1 hour.
6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37 C for 10 minutes.

Transformation

Note: Any competent cells (as long as they do not contain the F´ episome) with a transformation efficiency of $>1.0 \times 10^8$ transformants/µg may be used.

1. Add 1 µl of each LR reaction into 50 µl of One Shot® Competent *E. coli*. Add 1 µl of pUC19 DNA (10 ng/ml) to a separate as a positive control for transformation. Incubate on ice for 30 minutes.
2. Heat-shock cells by incubating at 42°C for 30 seconds.
3. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking.
4. Plate 20 µl and 100 µl of each transformation onto selective plates.

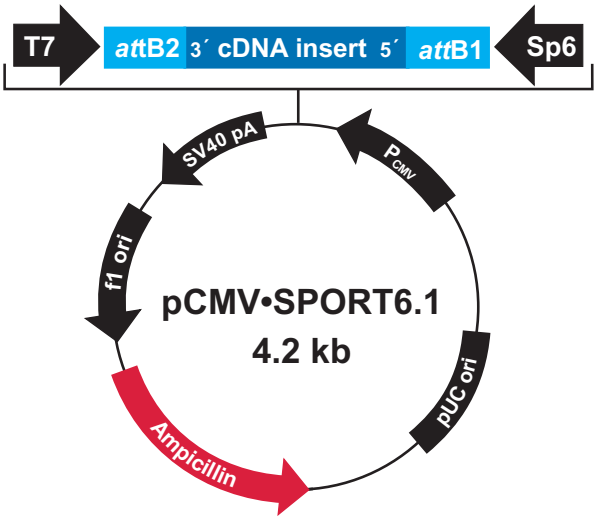
Expected Results

An efficient LR recombination reaction will produce >5000 colonies if the entire LR reaction is transformed and plated.

Appendix
pCMV•SPORT6.1

Map of pCMV•SPORT6.1

The figure below shows the map and features of pCMV•SPORT6.1 vector. **The complete sequence of pCMV•SPORT6.1 is available for downloading from our Web site (www.lifetechnologies.com) or by contacting Technical Support (page 21).**



Comments for pCMV•SPORT6.1 (no insert)
4177 nucleotides

SV40 early polyadenylation signal (c): bases 126-392
M13 Forward (-20) priming site: bases 664-679
T7 promoter/priming site: bases 691-710
*att*B2: bases 711-735
*att*B1: bases 807-831
Sp6 promoter/priming site (c): bases 833-850
M13 Reverse priming site (c): bases 861-877
CMV promoter (c): bases 920-1510
pUC origin: bases 1895-2562
Ampicillin (*bla*) resistance gene (c): bases 2729-3589
bla promoter (c): bases 3590-3641
f1 origin (c): bases 3721-4176

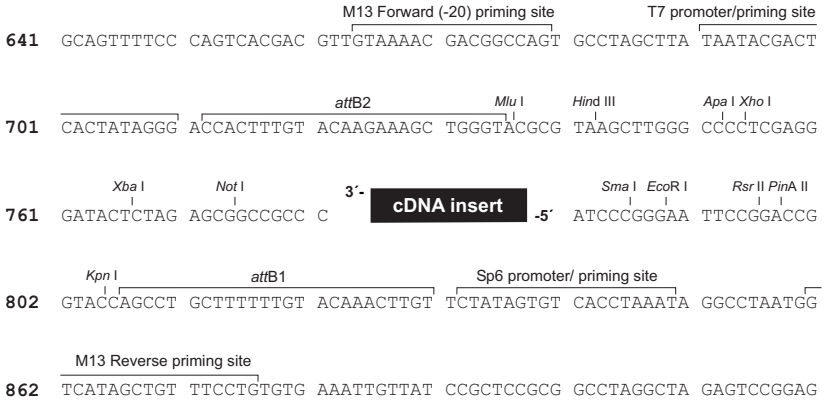
(c) = complementary strand

Continued on next page

pCMV•SPORT6.1, Continued

Cloning Site of pCMV•SPORT6.1

The cloning site for pCMV•SPORT6.1 is shown below. Restriction sites are labeled to indicate the cleavage site.



Additional Protocols

Checking the Library Titer

The library titer is included on the Certificate of Analysis shipped with the library.

If desired, you may check the library titer using the protocol described below.

1. Thaw an aliquot (one tube) of the Stem Cell cDNA Library.
2. To 100 µl of the library aliquot, add 900 µl SOC medium and mix well. This is the 10^{-1} dilution.
3. Prepare a series of dilutions with SOC medium as described below:

   For amplified library: Prepare 10^{-6}, 10^{-7}, and 10^{-8} dilutions of the library with SOC medium.
4. Plate 100 µl from each dilution on LB plates containing 100 µg/ml ampicillin.
5. Incubate the plates at 37°C overnight.
6. Count the number of colonies on each plate and calculate the titer as described below.

Calculating the Titer

The library titer is calculated using the formula:

Titer (cfu/ml) = No. of colonies x dilution factor x 10

For example: The number of colonies on a 10^{-3} plate is 100, then the library titer is 100 x 10^3 x10 = 1 x 10^6 cfu/ml.

Continued on next page

Additional Protocols, Continued

Verifying the Insert Size

You may verify the insert size by PCR or restriction enzyme analysis.

PCR

Perform colony PCR on 12-24 colonies as described on page 4 using T7 and Sp6 primers.

Restriction Enzyme Analysis

1. Plate the library to isolate individual colonies as described on page 3.
2. Prepare plasmid DNA from 12-24 colonies.
3. Prepare the following mix in each tube:

DNA sample	0.5-1 µg
10X REact® 3	2 µl
Not I	10-50 units
*Eco*R I	10-50 units
Sterile water	to 20 µl

4. Incubate at 37°C for 1-2 hours.
5. Analyze insert size on a 1% agarose gel using appropriate DNA markers.

Isolating Plasmid DNA

Materials Needed

- Terrific Broth (page 19 for a recipe)
- Stock ampicillin solution (~100 mg/ml)
- Buffer I with RNAse (page 19 for a recipe)
- Buffer II (page 20 for a recipe)
- 7.5 M ammonium acetate, chilled
- Isopropanol, chilled
- Phenol:Chloroform:Isoamyl alcohol (25:24:1)
- 70% ethanol

Preparing dsDNA from a Plasmid cDNA Library

1. Inoculate 100 ml Terrific Broth containing 100 µg/ml ampicillin with 2.5 x 10^6 cells from a primary library or 2.5 x 10^9 cells from an amplified library in a 500-ml flask.
2. Incubate the culture for 14-20 hours for an amplified library and 22-24 hours for a primary library at **30°C** with shaking at 275 rpm. **Clones are prone to deletions when the culture is grown at > 30°C.**
3. Read the A_{590} of the culture. For accurate A_{590} determination, dilute the cells 1:10-1:20, such that the observed value is between 0.2-0.8.
4. Process ~ 500 OD_{590} units in two 50 ml centrifuge tubes.
5. Centrifuge the tubes at 4800 x g for 15 minutes at 4°C. Discard the supernatant. If desired the cell pellet can be stored at -80°C until ready for use.
6. Resuspend the cell pellets in a total volume of 10 ml Buffer I with RNAse (cells must be < 50 OD units/ml).
7. Add 10 ml of Buffer II to the resuspended cells. Invert the tubes to mix the cells and incubate for 5 minutes at room temperature. Do not exceed 5 minutes.
8. Add 10 ml cold 7.5 M ammonium acetate. Invert tubes to mix and incubate for 10 minutes on ice.
9. Centrifuge the tubes at 3,000 x g for 15 minutes at 4°C. Pour the supernatant through cheesecloth or a clean, DNase-free, porous filter into a fresh 50-ml centrifuge tube. You should have ~25 ml supernatant in the tube. Avoid the white flocculent material. Turn to the next page for the remainder of the procedure.

Continued on next page

Isolating Plasmid DNA, Continued

Preparing dsDNA from a Plasmid cDNA Library, continued

10. Add an equal volume of cold isopropanol to the tube, mix well, and centrifuge the tubes at 3,000 x g for 15 minutes at 4°C. Discard the supernatant.
11. Resuspend the cell pellet in 1 ml of Buffer I with RNAse and transfer to a microcentrifuge tube.
12. Centrifuge the tubes at 14,000 x g for 1 minute at 4°C. Transfer the supernatant to a fresh microcentrifuge tube. Incubate the tube at 37°C for 10 minutes.
13. Incubate the tubes at 65 °C for 5 minutes. Split each sample into two equal parts (~ 500 µl each) in 1.5-ml microcentrifuge tubes and precipitate DNA (below).

Extracting and Precipitating DNA

1. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each sample from Step 13, above, and vortex the tubes for ~1 minute.
2. Centrifuge the tubes at 14,000 x g for 5 minutes at room temperature.
3. Avoiding the interface, transfer 450 µl of the upper (aqueous) phase to a fresh microcentrifuge tube.
4. Repeat the phenol:chloroform:isoamyl alcohol extraction at least twice. If an interface remains, repeat Steps 1-4 until the supernatant is clear.
5. Add an equal volume (~450 µl) of chilled isopropanol to each tube. Centrifuge the tubes at 14,000 x g for 15 minutes at 4°C. Discard the supernatant.
6. Add 500 µl 70 % ethanol to each tube. Centrifuge the tubes at 14,000 x g for 5 minutes at 4°C. Discard the supernatant.
7. Dry the pellet for 10 minutes at room temperature.
8. Completely dissolve the two pellets in 200 µl TE buffer. The plasmid DNA library concentration must be approximately 1 µg/µl.
9. Store the DNA at -20°C.

Continued on next page

Recipes

Terrific Broth

1. Dissolve the following reagents in 800 ml distilled water:

Tryptone	12 g
Yeast Extract	24 g
Glycerol	4 ml

2. Mix well and adjust the volume to 900 ml with distilled water.
3. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C.
4. Dissolve the following reagents in 80 ml of distilled water:

KH_2PO_4 (monobasic)	2.3 g
K_2HPO_4 (dibasic)	12.5 g

5. Mix well and adjust the volume to 100 ml with distilled water.
6. Autoclave on liquid cycle for 20 minutes. Allow solution to cool to ~55°C. Mix this solution with the solution prepared in Step 3.
7. After the media is cooled, add antibiotic to the desired concentration.
8. Store at 4°C.

Buffer I with RNAse

15 mM Tris-HCl, pH 8.0
10 mM EDTA
100 µg/ml RNAse A
1200 U/ml RNAse T1

1. To prepare 1 liter Buffer I, mix the following:

1 M Tris-HCl, pH 8.0	15 ml
0.5 M EDTA	20 ml
Sterile water	to 1000 ml

2. Mix well and store at room temperature.
3. Prepare 50 ml **fresh Buffer I with RNAse** as follows:

Buffer I (Step 2)	50 ml
RNAse T1 (1200 units/µl)	50 µl
RNAse A (10 mg/ml)	500 µl

4. Mix well and store on 4°C until use. Use Buffer I with RNAse for preparing DNA, page 17.

Continued on next page

Buffer II

0.2 M NaOH
1% SDS

1. To prepare 50 ml Buffer II, mix the following:

10% SDS	5 ml
10 N NaOH	1 ml
Sterile water	to 50 ml

2. Mix well and store at room temperature. Use Buffer II for preparing DNA, page 17.

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Gateway® Clone Distribution Policy

Introduction

The information supplied in this section is intended to provide clarity concerning Life Technologies' policy for the use and distribution of cloned nucleic acid fragments, including open reading frames, created using Life Technologies' commercially available Gateway® Technology.

Gateway® Entry Clones

Life Technologies understands that Gateway® entry clones, containing *att*L1 and *att*L2 sites, may be generated by academic and government researchers for the purpose of scientific research. Life Technologies agrees that such clones may be distributed for scientific research by non-profit organizations and by for-profit organizations without royalty payment to Life Technologies.

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