

Creator™ SMART™ cDNA Libraries User Manual

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I. Introduction

Clontech's Creator SMART cDNA Libraries simplify the isolation of full-length cDNA clones and the subsequent transfer of genes of interest into a variety of expression vectors for functional analysis. Recovery of target genes is achieved through Cre-*loxP* recombination, which enables quick and precise gene transfer due to the unique properties of Cre recombinase. The Creator Libraries were generated for the National Cancer Institute from numerous cancer and normal tissues. For a complete listing of available libraries, please visit www.clontech.com.

Creator™ library clones can be used with multiple expression systems

With the Creator System (patent pending), you can simultaneously place your gene within multiple systems for functional analysis (Figure 1). Clontech offers Creator acceptor vectors for many of our most powerful systems (Table I). The Matchmaker Acceptor Vectors enable you to discover novel protein-protein interactions using the Matchmaker Two-Hybrid System 3. The Living Colors® Acceptor Vectors allow you to generate N-terminal fusions of your target gene to a fluorescent tag for protein localization studies. For dose-dependent inducible expression studies of your protein, transfer your gene into the acceptor vectors pLP-TRE2 or pLP-RevTRE, which are compatible with Tet and the retroviral RevTet Gene Expression Systems. The IRES Bicistronic Expression Acceptor Vectors have an IRES (internal ribosomal entry site) sequence and a constitutive CMV promoter to produce a bicistronic message for high expression of your protein in mammalian cells. Our PROtet Acceptor Vector yields high protein expression levels in bacterial cells for easy purification with TALON® resin. For more information on these and other Creator acceptor vectors, please refer to **vectors.clontech.com**.

Creator™ System overview

The Creator System allows the transfer of a target gene directly into multiple acceptor expression vectors without the need for subcloning. By using Cre recombinase, any gene cloned into a specialized cloning vector (pDNR-LIB for Creator Libraries) can be transferred into any acceptor vector for functional analysis (Figure 1). Cre recombinase mediates recombination between or within DNA sequences at specific locations called *loxP* sites (Sauer, 1994; Abremski et al., 1984). These sites consist of two 13-bp inverted repeats separated by an 8-bp spacer region (Figure 2). The 8-bp spacer region in the *loxP* site has a defined orientation that forces the target gene to be transferred in a fixed orientation and reading frame. Typically, 90–95% of the clones isolated by this method contain the correct insert, all of which are in the correct orientation.

The Creator donor vector pDNR-LIB contains two *loxP* sites that flank the 5' end of the library insert and the 5' end of an open reading frame for the

I. Introduction *continued*

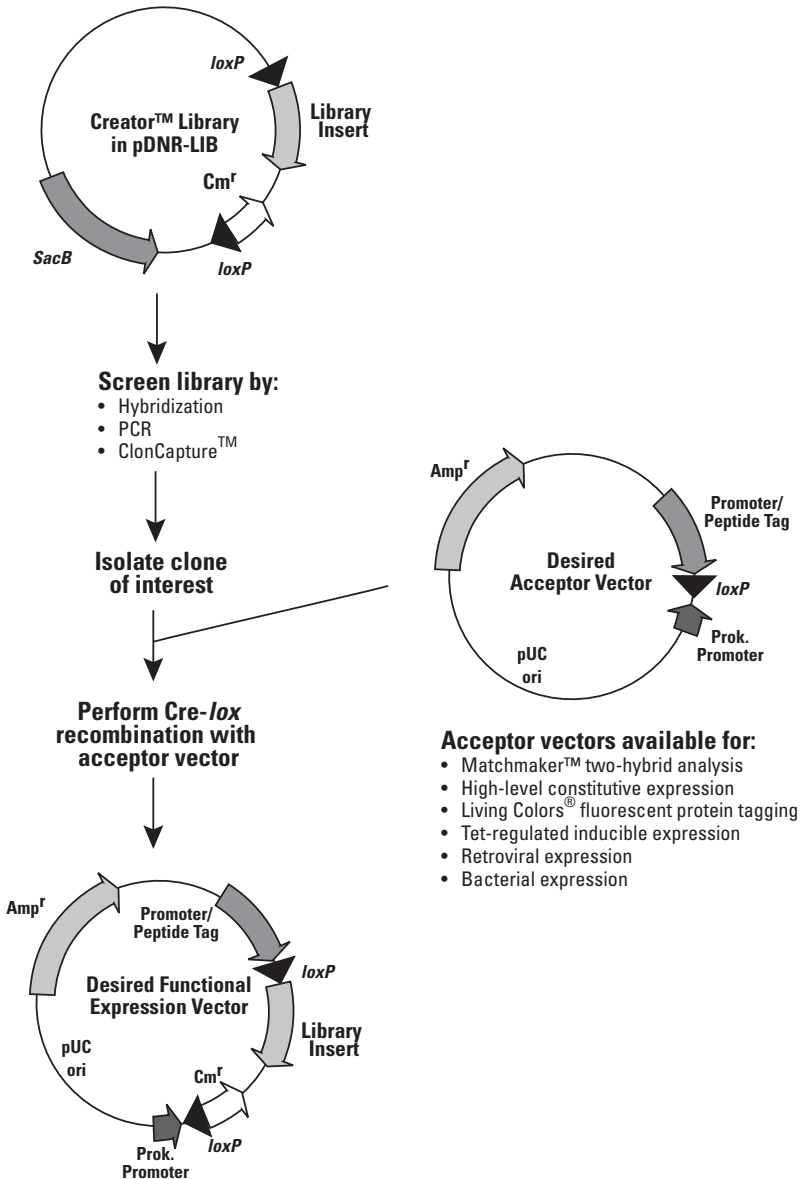


Figure 1. Creator™ library construction allows you to transfer your gene into multiple expression systems quickly and inexpensively.

I. Introduction *continued*

TABLE I. CREATOR™ ACCEPTOR VECTORS

Acceptor Vector	Promoter/Features	Functional Application
Matchmaker™ Vectors		
pLP-GADT7	ADH1/GAL4 activation domain	Express fusions to GAL4 AD to study protein interactions by two-hybrid screening.
pLP-GBKT7	ADH1/GAL4 DNA-binding domain	Express fusions to GAL4 DNA-binding domain to study protein interactions by two-hybrid screening.
pLP-CMV-Myc	CMV/C-terminal fusions to c-Myc	Express proteins identified by two-hybrid screening and purify and detect with Myc antibody.
Living Colors® Vectors		
pLP-EGFP-C1	CMV/C-terminal fusions to EGFP (green)	Express fusion to EGFP (enhanced green fluorescent protein) to study localization of the protein of interest in live cells; no dyes or cofactors required.
pLP-ECFP-C1	CMV/C-terminal fusions to ECFP (cyan)	Express fusion to ECFP (enhanced cyan fluorescent protein) to study localization of the protein of interest in live cells; no dyes or cofactors required.
pLP-EYFP-C1	CMV/C-terminal fusions to EYFP (yellow)	Express fusion to EYFP (enhanced yellow fluorescent protein) to study localization of the protein of interest in live cells; no dyes or cofactors required.
Bicistronic Vectors		
pLP-IRESneo	CMV/IRES, neo selection marker	Constitutive mammalian expression with single transcript for both gene of interest and neo selection marker.
pLP-IRES2-EGFP	CMV/IRES, EGFP selection marker	Constitutive mammalian expression with single transcript for both gene of interest and EGFP selection via cell sorting (flow cytometry).
Tetracycline-Regulated Expression Vectors		
pLP-TRE2	Inducible tet-responsive expression vector	High-level, regulated promoter for expression in mammalian cells.
pLP-RevTRE	Inducible tet-responsive promoter in retroviral expression vector with hyg selection	High-level, regulated retroviral expression in mammalian cells.
Retroviral Vector		
pLP-LNCX	CMV promoter in retroviral expression vector with neo selection	Constitutive retroviral expression.
Prokaryotic Expression Vector		
pLP-PROTet-6xHN	Inducible tet-responsive expression vector	High-level, regulated promoter for expression in bacteria.

I. Introduction *continued*

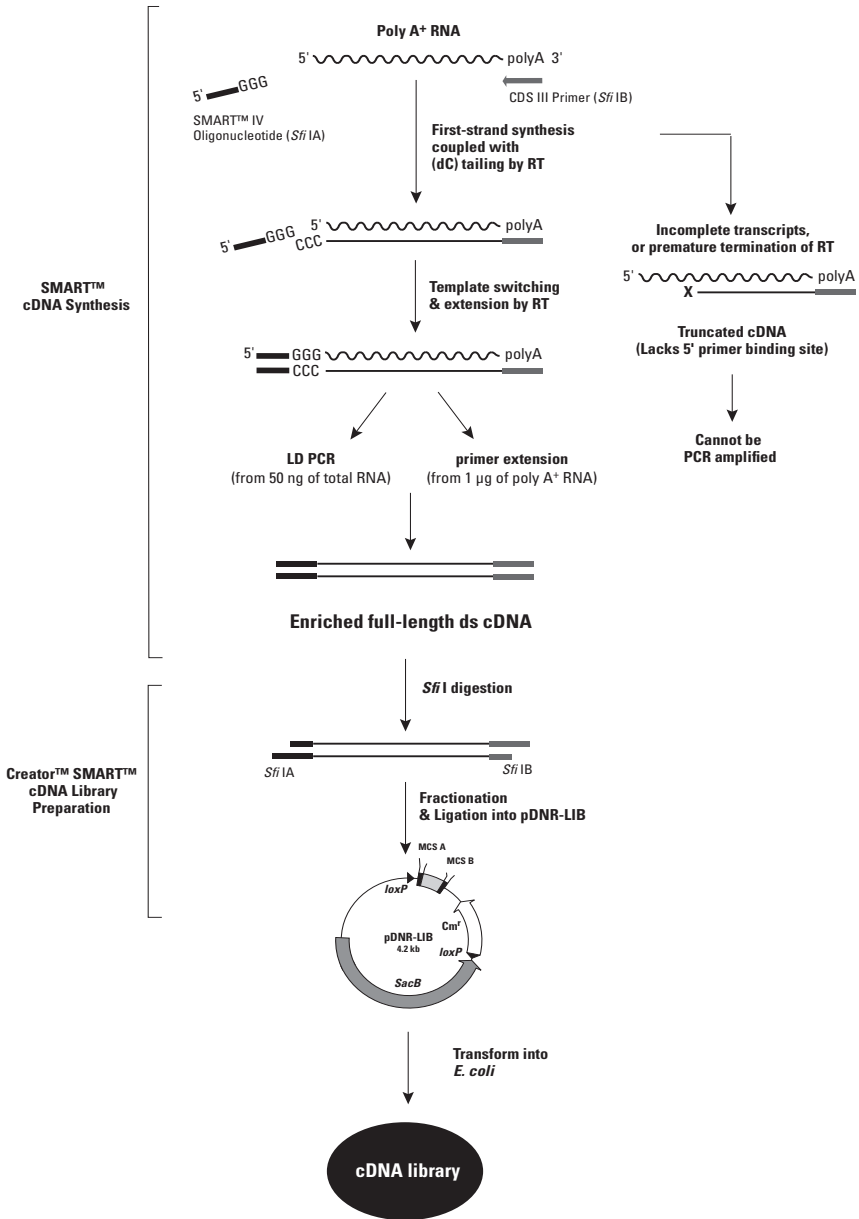


Figure 3. SMART™ cDNA synthesis and library construction.

I. Introduction *continued*

Creator™ SMART™ cDNA Library construction

Creator Libraries are constructed with cDNA synthesized using Clontech's SMART (Switch Mechanism At the 5' end of RNA Transcript) technology to ensure a high percentage of full-length clones. Traditional library construction methods typically yield shorter clones because cDNA synthesis occasionally terminates before the complete mRNA sequence is read by reverse transcriptase (RT). Premature termination is particularly common for long mRNAs, especially if the first-strand synthesis is primed with only oligo(dT) primers or if the mRNA contains abundant secondary structures which interfere with RT processivity. In addition, conventional cDNA cloning procedures use T4 DNA polymerase to generate blunt cDNA ends after second-strand synthesis. As a result, the 5' ends of genes in cDNA populations are under-represented and tend to be 5–30 nucleotides shorter than the original mRNAs.

The SMART protocol is designed to preferentially enrich for full-length cDNAs while eliminating T4 DNA polymerase and adaptor ligation (Figure 3). SMART libraries contain a higher percentage of full-length clones than libraries constructed by conventional methods (October 1998 Clontechniques) or other full-length cDNA synthesis protocols (Okayama & Berg, 1982; Kato et al., 1994). This procedure uses long distance PCR (LD PCR; Barnes, 1994) or primer extension and the unique SMART IV Oligonucleotide, which serves as a short, extended template at the 5' end of the mRNA, together with a modified oligo(dT) that primes the first-strand synthesis reaction to generate high yields of full length double-stranded (ds) cDNA.

When the RT reaches the 5' end of the RNA molecule, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA. The SMART IV Oligo, which has an oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide. The resulting full-length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo, which then serves as a universal priming site in the subsequent amplification by LD PCR or primer extension (Chenchik et al., 1998). Only those ss cDNAs having the universal priming site at the 5' end can serve as a template. Incomplete cDNAs and cDNA transcribed from polyA⁻ RNA will lack this priming site and will not be amplified. The cDNA is incubated with Sfi I and enriched for full-length clones by size fractionation on a low-melting-point agarose gel. The cDNA is then directionally cloned into the Sfi I A and B sites in pDNA-LIB to create a library (Figure 3). Recombinant plasmids are transfected into the DH10B strain of *E. coli*, which is specially prepared to accept plasmids with large inserts, and are ready to be screened for your target gene.

I. Introduction *continued*

Screening Creator™ Libraries

Creator libraries can be screened using any standard plasmid library screening protocol. This User Manual provides examples of three screening protocols: PCR screening, colony hybridization screening, and ClonCapture screening. Creator Libraries are supplied with M13 forward and reverse primers, which anneal to sites flanking pDNR-LIB inserts. These primers can be used in conjunction with gene-specific primers to screen the library for a target gene with PCR or for quickly screening insert sizes of positive clones. For optimal long-distance PCR amplification we recommend our Advantage® 2 Kit (Cat. Nos. 639207, 639206). ClonCapture offers the advantage of quickly isolating clones (including low- and medium abundance transcripts) based on a partial cDNA sequence by highly enriching the cDNA for the target gene. For additional information, go to www.clontech.com and select ClonCapture's product page.

Sequencing Clones

Depending on which downstream expression system you choose to study your gene, it may be critical that your clone contain an ATG start codon or be in frame with the *loxP* site. For example, genes transferred from the donor vector to the IRES or pLP-TRE2 and pLP-RevTRE acceptor vectors should include an initiating ATG codon for proper expression. Additionally, if you want to express your gene as a fusion with fluorescent proteins or epitopes tags, then the gene of interest must be in frame with *loxP*. Please refer to vectors.clontech.com for more information about the requirements of the various expression vectors. We recommend that you screen and sequence multiple clones to determine that you have the correct, full-length target gene inserted in frame with *loxP*. These characteristics will ensure compatibility with **all** acceptor vectors and expression systems. Libraries made with SMART cDNA can facilitate preliminary mapping of transcription start sites (Fromont-Racine, 1993) due to the high percentage of full-length clones.

Creator™ Kit Components

Creator SMART cDNA Libraries include 2 vials of the Creator library culture and the M13 forward and reverse primers. Cre recombinase is required for rapid transfer of identified clones into expression vectors and is sold separately (Cat. No. 631614). Please see the Related Products section for ordering information or visit our website at www.clontech.com.

II. List of Components

Store library culture at -80°C .

Store vector and primers at -20°C .

- 2 x 1 ml **Library culture** (in DH10B)
Supplied as 1.0-ml liquid bacterial cultures (DH10B in LB broth + 25% glycerol). Avoid multiple freeze/thaw cycles.
- 20 μM **M13 Forward Primer**
- 20 μM **M13 Reverse Primer**
- **Vector Information Packet** (PT3508-5)
- **Creator™ DNA Cloning Kit User Manual** (PT3460-1)

III. Additional Materials Required

The following materials are required but not supplied:

- **Cre Recombinase** (Cat. No. 631614)
- [optional] **Sfi I enzyme** (Sigma #R8256)
- **Chloramphenicol stock solution (Cm)**
30 mg/ml in 100% ethanol; 1000X. Store at -20°C .
- **Advantage® 2 PCR Kit** (Cat. Nos. 639207, 639206)
- **SOC Medium**
5 g/L Bacto-yeast extract
20 g/L Bacto-tryptone
0.54 g/L NaCl
Add 10 ml of a 250 mM solution of KCl, and adjust the pH to 7.0.
Autoclave. Prior to use, add 5 ml of 2 M MgCl_2 (autoclaved).
- **LB Broth**
10 g/L Bacto-tryptone
5 g/L Bacto-yeast extract
5 g/L NaCl
Adjust pH to 7.0 with 5 N NaOH. Autoclave.
- **LB/Cm agar plates**
Prepare LB as described above. Prior to autoclaving, add agar (15 g/L). After autoclaving, cool to 55°C and add chloramphenicol (30 $\mu\text{g}/\text{ml}$; final concentration). Pour into 10-cm plates and let harden. Invert plates and store at 4°C .

III. Additional Materials Required *continued*

- **LB/Cm/sucrose agar plates**

Prepare LB as described above. Prior to adjusting pH, add sucrose (7% w/v). Add agar (15 g/L) and autoclave for 20 minutes maximum (longer autoclaving times may cause sucrose to burn). After autoclaving, cool to 55°C and add chloramphenicol (30 µg/ml). Pour into 10-cm plates, and let harden. Then invert plates and store at 4°C.

- **LB + 25% glycerol**

750 ml	LB
250 ml	glycerol

Hybridization Screening

- **SSPE (20X) Buffer**

175.3 g	NaCl (3 M final)
27.6 g	NaH ₂ PO ₄ × H ₂ O (0.2 M final)
40 ml	0.5 M stock EDTA (0.02 M final)
900 ml	H ₂ O

Adjust to pH 7.4 with 10 N NaOH.
Add water to 1 L.

- **SSC (20X) Buffer**

175.3 g	NaCl (3 M final)
88.2 g	sodium citrate (0.3 M final)
900 ml	H ₂ O

Adjust to pH 7.0 with 1 M HCl.
Add water to 1 L.

- **ExpressHyb™** (Cat. Nos. 636831, 636832)
- **Whatman filter papers** (#1, #23MM and 3MM)
- **Nylon membranes** (such as Schleicher & Schuell's Nytran Plus)
- **0.5 M NaOH**
- **1 M Tris HCl, pH 7.5**
- **1 M Tris HCl, 1.5 M NaCl, pH 7.5**
- **Heat-sealable plastic bags**
- **NucleoBond® Plasmid Maxi Kit** (Cat. Nos. 635933, 635934, 635935)

IV. General Considerations

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

• Mass conversion of libraries

We strongly recommend against performing a mass conversion (transferring the entire library into a particular acceptor vector). Due to the nature of Cre-*loxP* recombination, the simultaneous transfer of many inserts is typically not efficient.

• Selective medium

Creator Libraries should be plated on LB/Cm plates for titering, amplification and hybridization screening procedures. After screening the Creator library, the insert can be easily transferred to various acceptor vectors using Cre Recombinase (Cat. No. 631614). Sucrose and chloramphenicol double selection is only required for initial selection of correct recombinants after transferring your target gene into an acceptor vector. Cells transformed with the parental donor vector or insert-lacking acceptor vectors are eliminated by sensitivity to sucrose and Cm. Only cells carrying the recombinant acceptor vector will survive on the selective medium. After selection, recombinant clones can be grown in the same medium used for propagation of the acceptor vector.

• Library titering

All libraries are provided at a minimum titer of 10^8 colony forming units (cfu)/ml; however most libraries have titers of 10^9 – 10^{11} cfu/ml. Titer is determined at the time of library construction and is indicated on the Product Analysis Certificate (PAC). The library should be retitered upon receipt to determine the most accurate titer before screening. A protocol for library titering is provided in Section V.A. Once the titer has been determined the library should be amplified for long-term storage. A protocol for library amplification is provided in Section V.C.

V. Library Titering and Amplification

Upon receipt, retiter the library according to the following protocol. The titer, or number of colony-forming units (cfu) per ml of culture, stated on the PAC was determined when the library was constructed. Titers are usually stable at -80°C for at least one year. If the library titer is at least ten-fold higher than the number of independent clones stated on the PAC, the library is representative, and you should proceed with screening. If the titer is $<10^8$ cfu/ml, please contact our Technical Service Department or your local distributor.

- Diluted libraries are always less stable than undiluted libraries.
- A 2–5-fold range in titer calculations is reasonable, especially if more than one person is doing the titering.
- Always use the recommended concentration of antibiotic in the medium to ensure plasmid stability.
- Use proper sterile technique when aliquoting and handling libraries.
- Design appropriate controls and include them during plasmid library growth to test for cross-contamination.

A. Library Titering Protocol

1. Prewarm three LB/Cm plates at 37°C (or 30°C) for 1–2 hr.
2. Thaw an aliquot of the library on ice.
3. Remove 1 μl of the library, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution A ($1:10^3$).
4. Remove 1 μl from Dilution A, and add it to 1 ml of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. This is Dilution B ($1:10^6$).
5. Add 1 μl from Dilution A to 50 μl of LB broth in a 1.5-ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto a prewarmed LB agar plate.
6. Remove 50- μl and 100- μl aliquots from Dilution B and spread onto separate LB/Cm plates.
7. Leave plates at room temperature for 15–20 min to allow the inoculum to soak into the agar.
8. Invert the plates and incubate at 37°C overnight.
9. Count the number of colonies to determine the titer (cfu/ml). Calculate the titer according to the following formulas:
 - colony # Dilution A $\times 10^3 \times 10^3 = \text{cfu/ml}$
 - (colony # Dilution B/plating volume) $\times 10^3 \times 10^3 \times 10^3 = \text{cfu/ml}$

V. Library Titer and Amplification *continued*

You must amplify premade Creator Libraries to obtain enough high quality plasmid for library screening using ClonCapture and for long-term storage of the library.

B. Determining the Number of Plates Required for Amplification

The exact number of LB/Cm agar plates required depends on the library size. Use the following calculation to determine the number of plates to use. Normally, use 3 times the size of the original library and plate at 20,000 cfu/150-mm plate.

Sample calculations:

$$(\# \text{ of independent clones} \times 3) = \# \text{ clones}$$

$$2 \times 10^6 \times 3 = 6 \times 10^6 \text{ clones}$$

$$\# \text{ of clones to screen/ colonies per plate} = \# \text{ of plates}$$

$$6 \times 10^6 / 20,000 = 300 \text{ plates}$$

- If the titer is 6×10^8 , determine how much of the library stock to spread on each plate.

$$\# \text{ clones/ library titer} = \mu\text{s of library to plate}$$

$$6 \times 10^6 / 6 \times 10^8 \text{ titer} = 10 \mu\text{l}$$

- Calculate the volume of media needed to plate 150 μl on each plate.

$$300 \text{ plates} \times 150 \mu\text{l} = 45 \text{ ml}$$

- Add 10 μl of the library to 45 ml of LB/Cm and spread 150 μl onto each of the 300 plates of LB/Cm.
- Allow the agar plates to dry at room temperature for 2–3 days, or at 30°C for 3 hr, prior to plating cells. Moisture droplets on the agar surface can lead to uneven spreading of cells.

C. Library Amplification Protocol

1. If you have not done so already, titer the plasmid library.
2. Plate the library directly on selective medium (LB/Cm plates) at a high enough density so that the resulting colonies will be nearly confluent (~20,000–30,000 cfu per 150-mm plate). Plate enough cfu to obtain at least 2–3X the number of independent clones in the library.

Notes:

- The number of independent clones is the number of independent colonies present in the library before amplification. The size of the library is stated on the PAC.
- To promote even growth of the colonies, continue spreading the inoculum over the agar surface until all visible liquid has been absorbed, and then allow plates to sit at room temperature for 15–20 min. If using glass beads to spread the colonies, shake the plate back and forth—not round and round.

V. Library Titer and Amplification *continued*

3. Invert the plates and incubate at 37°C for 18–20 hr.

Note: Growing the transformants on solid medium instead of in liquid culture minimizes uneven amplification of the individual clones.

4. Add 5 ml of LB + 25% glycerol to each plate and scrape the colonies into the liquid. Pool all the resuspended colonies in one flask and mix thoroughly.
 - Set aside five 1-ml aliquots of the library culture in case you wish to re-amplify the library at a later time. Store the aliquots at –80°C.
 - Divide the remainder of the library culture into 50-ml aliquots. Use one of the aliquots for ClonCapture as needed. Store the remainder of the library aliquots at –80°C. For use within one week, aliquots may be stored at 4°C.
5. If you are using ClonCapture to isolate genes of interest, use a 50-ml aliquot of the amplified library as described in Section VII.

VI. Library Screening

After the library has been titered and amplified, it must be screened to determine whether your gene of interest is present.

A. Screening a Library by PCR

The Creator Library should be screened using a gene-specific primer and one of the M13 primers to determine whether the target gene is present. M13 forward and reverse primers are provided with the Creator Libraries for this purpose. You can also use two gene specific primers to verify that your target gene is present. We recommend the Advantage 2 PCR Kit (Cat. Nos. 639207, 639206) for efficient long-distance DNA amplification.

1. Set up a 25 μ l PCR reaction.

Library culture	1 μ l
10X Advantage 2 PCR buffer	2.5 μ l
dNTP Mix (10 mM each)	0.5 μ l
Sense primer (20 μ M)	0.5 μ l
Anti-sense primer (20 μ M)	0.5 μ l
Water	19.5 μ l
50X Advantage 2 Polymerase Mix	0.5 μ l
Total volume	25 μl

2. Overlay each tube with 2 drops of mineral oil and amplify in a Perkin-Elmer Thermal Cycler 480 using the following program:

- 1 cycle:
 - 94°C 30 sec
- 20–25 cycles:
 - 94°C 30 sec
 - 68°C 2 min
- 1 cycle:
 - 68°C 5 min

3. Electrophorese 5 μ l of PCR product on a 1.2% TAE/agarose gel with DNA size markers. If no band appears, run five additional 2-step cycles.

VI. Library Screening *continued*

B. Hybridization Screening—General Considerations

Creator Libraries can be screened by hybridization with DNA probes (Section VI.C) or oligonucleotide probes (Section VI.D). In either case, we recommend that you prepare at least two replicate filters of each plate to be screened. Additionally, determine conditions that give the lowest background by performing a hybridization experiment with a blank filter or, preferably, a control filter containing nonrecombinant colonies.

The handbooks edited by Sambrook et al. (1989) and Ausubel et al. (1995) contain further information on hybridization using nucleic acid probes. Jacobs et al. (1988), Lathe (1985), Suggs et al. (1981), Wallace et al. (1981), and Wood et al. (1985) contain information on using oligonucleotide probes, and Woo (1979) has information about DNA probes.

Important notes:

- Use positively charge-modified, supported nylon filters (Schleicher & Schuell's Nytran Plus works well). Nitrocellulose filters are too brittle for hybridization screening.
- Your hybridization probe should contain at least 10^7 counts per minute (cpm). Its specific activity should be $>8 \times 10^8$ cpm/ μ g, and the final concentration of hybridization solution should be $1\text{--}2 \times 10^6$ cpm/ml.
- We recommend ExpressHyb™ Hybridization Solution (Cat. No. 636831) in place of standard prehybridization and hybridization solutions. ExpressHyb is a nonviscous, premade solution that gives you high sensitivity and low background. If you are using a rapid hybridization solution, follow the protocol accompanying it.

C. Replica Plating

1. Place 87-mm filters on 100-mm LB plates containing 30 μ g/ml Cm.
2. Determine the number of plates and based on titer, dilute bacteria into LB media to an appropriate volume for plating (see Section V.B).
3. Plate approximately $1\text{--}2 \times 10^4$ colonies on each plate, spreading the culture onto the filter. Alternatively, use 150-mm LB plates and appropriate filters. In this case, up to 50,000 colonies can be screened per plate required.
4. Incubate the plates for at least 8 hours at 37°C until the colonies reach 1.0 mm in diameter.
5. Remove the filter and place it colony side up onto a sterile Whatman #1MM filter paper on a flat glass surface.

VI. Library Screening *continued*

6. Prewet a new filter on an LB plate and place it over the library filter. Cover this with another Whatman #1 filter paper and press firmly with a glass plate (e.g., from a minigel apparatus).
7. With a small needle, mark the filter asymmetrically for future alignment.
8. Separate the master and replica filters and place them face up on LB-Cm plates.
9. Incubate both plates for 4–5 hours at 37°C. To make multiple replicates, repeat steps 4–9. Normally two copies of the library are screened in parallel with one probe.
10. Seal the master bacterial plate with parafilm and store at 4°C.

D. Hybridization Preparation

1. Place the filter replica from Section C colony side up, on the surface of Whatman #23 MM filter paper that has been prewetted with 0.5 M NaOH. Let sit for one minute.
2. Remove the filter and place it on another piece of Whatman filter paper prewetted with 1 M Tris-HCl (pH 7.5). Let sit for one minute.
3. Remove the filter and place it on a piece of Whatman filter paper prewetted with 1 M Tris-HCl, 1.5 M NaCl (pH 7.5). Let sit for one minute.
4. Place the filter into a solution of 1 M Tris-HCl, 1.5 M NaCl (pH 7.5). Remove the bacterial debris by gently rubbing with a gloved hand. Rinse the filter paper in the same solution and blot dry on paper towels.
5. Bake the filter for 1 hr at 80°C in a vacuum oven or UV crosslink the filter according to the manufacturer's instructions.

E. Hybridization Using Probes >200 bp

If your probe is less than 200 bp, skip to Section F. We recommend using ExpressHyb buffer for hybridization because it is not necessary to add sonicated salmon sperm DNA as a blocking agent.

1. Place ExpressHyb in a heat-sealable plastic bag. Use 10 ml for one filter or 15 ml for two. Use 2–3 ml extra for each additional filter you will be treating in the same plastic bag. However, do not put more than 15 filters in one bag.
2. Place filters in the plastic bag and seal it. Avoid trapping air bubbles. If you are placing multiple filters in a single bag, use enough solution to wet all filters. Do not allow filters to adhere to each other, and ensure that each filter is in direct contact with the solution.

VI. Library Screening *continued*

3. Incubate filters on a shaker in ExpressHyb at 42°C for 4 hr.
4. Denature the labeled DNA probe by heating at 100°C for 10 min. Immediately chill the probe on ice.
5. Cut one corner of the plastic bag and pour off the ExpressHyb. Make hybridization solution by mixing the same volume of fresh ExpressHyb with the probe.
6. Incubate the filters in hybridization solution at 42°C for 16–20 hr.
7. Carefully remove the filters from the plastic bag, and place the hybridization solution in your radioactive liquid waste.
8. Wash the filters at room temperature three times for 10 min each. Do not allow them to dry. Use a minimum of 15 ml of wash buffer for one filter, or 25 ml for two filters; use 5 ml extra for each additional filter.
9. Wash the filters in **1X SSC plus 0.05% SDS** at 65°C for 1 hr. Repeat once.
Note: If background is too high, you may wish to add a 1-hr wash in 0.2X SSC plus 0.1% SDS at 68°C. Monitor background with a Geiger counter; if you detect more than 1,000 cpm, wash further.
10. Blot-dry the filters on Whatman 3MM paper at room temperature.
Note: At this point, you may seal the filters individually in plastic bags to keep them moist. If they remain moist, you may wash them again to reduce background noise. Also, keep them moist if you plan to strip them for rehybridizing to a different probe.
11. Proceed to Section G for signal detection by autoradiography.

F. Hybridization Using Oligonucleotide Probes <200 bp

1. Place the required volume of ExpressHyb in a heat-sealable plastic bag. Use 10 ml for one filter, 15 ml for two, and 2–3 ml extra for each additional filter, with no more than 15 filters in one bag.
2. Place filters in the plastic bag and seal it. Avoid trapping air bubbles. If you are placing multiple filters in a single bag, use enough solution to wet all filters. Do not allow filters to adhere to each other, and ensure that each filter is in direct contact with the solution.
3. Determine the probe's estimated dissociation temperature (T_d). Incubate the filters at 20°C below this temperature for 4–6 hr on a platform shaker at ~60 rpm. For probes of length 14–27 nt, the T_d is:

$$T_d (\text{°C}) = 4(\text{G}+\text{C}) + 2(\text{A}+\text{T})$$

For longer probes:

$$T_d = 81.5^\circ\text{C} + 16.6 \text{ Log}_{10} [\text{Na}^+] + 0.41(\% \text{ G/C content}) - 500/n$$

n = the number of nucleotides

VI. Library Screening *continued*

You can decrease the T_d by approximately 13°C if you decrease the incubation solution's Na^+ concentration from 1 M (6X SSPE) to 0.2 M (1X SSPE).

- Denature the oligonucleotide probe: heat at 100°C for 2 min to release any secondary structure. Chill the probe quickly on ice.
- Cut one corner of the bag and pour off the solution. Make hybridization solution by mixing the same volume fresh ExpressHyb with the probe.
- Incubate the filters in the hybridization solution at the appropriate temperature for 16–20 hr.
- Carefully remove the filters from the plastic bag. Place the solution in your radioactive liquid waste container.
- Wash the filters under low-stringency conditions in a large volume of 2X SSC and 0.05% SDS for 1 hr. The wash buffer should be at room temperature or 30°C below the calculated T_d , whichever is higher.
Note: Use a minimum of 15 ml of wash buffer for one filter, 25 ml for two, and 5 ml extra for each additional filter.
- Replace the wash solution with fresh solution 20°C below the T_d . Wash filters under these higher stringency conditions for 15 min. If background is too high ($\geq 1,000$ cpm as measured with a Geiger counter), increase the high-stringency wash time to 30 min.
- Blot-dry the filters on Whatman 3MM paper at room temperature. See step E.10 for filter storage information.
- Proceed to Section G.

G. Signal Detection by Autoradiography

- Cut a piece of Whatman 3MM filter paper to the size of the x-ray film you will use. Tape duplicate filters on a single piece of 3MM filter backing. Place them alongside one another and in the same orientation.
- Mark the filter backing at three asymmetric locations with radioactive ink so that each filter can be aligned. Cover in plastic wrap.
- Expose filters to Kodak XAR film at –80°C for 16–24 hr. Use an intensifying screen to enhance the signal.
- After developing the film, align it with the filters to find positive colonies. If you cannot pick an isolated colony, remove an agar plug containing several colonies and place it in 1 ml of cold LB buffer. Briefly shake gently to allow bacteria to disperse into the media. Use of cold LB and cold storage is important to prevent further growth of the colonies. This will allow a better estimate of titer to be made.

VI. Library Screening *continued*

5. Replate to obtain 200–1,000 colonies on a 150-mm plate. Rescreen, pick an isolated colony and proceed.

VII. ClonCapture™ Screening

The Creator SMART cDNA libraries can be screened for your gene of interest with the ClonCapture DNA Selection Kit. ClonCapture uses RecA, a component of the homologous recombination pathway of *E. coli*, to isolate target clones from cDNA libraries without tedious screening. ClonCapture uses the unique pairing ability of RecA to form stable triple-stranded complexes between double-stranded library plasmids containing a target sequence and a 200–600 bp biotinylated probe based on homology. The captured enriched plasmids are then precipitated, resuspended, and transformed into bacteria. The resulting colonies are screened by colony hybridization and PCR to identify the desired clones. For a detailed protocol, consult the ClonCapture cDNA Selection Kit User Manual (PT3246-1), which can be obtained at www.clontech.com.

The ClonCapture procedure requires only 1 µg of a supercoiled plasmid cDNA library, which can be obtained by amplifying and purifying the Creator Library as detailed in Section V.C.

1. Prepare plasmid DNA from 50 ml of library culture. We recommend using a NucleoBond Plasmid Maxi Kit (Cat. Nos. 635933, 635934, 635935).
2. Check the concentration of plasmid DNA and run 0.25 µg–0.5 µg on an 0.8% TAE agarose gel. If your plasmid DNA contains ≥90% supercoiled DNA, there is no need for CsCl purification. If, however, the quality of the DNA is poor, you should purify through a CsCl gradient.

VIII. Analysis of Inserts

After positive clones have been identified, you should analyze the inserts to confirm you have isolated the correct clone, that you have the full-length cDNA, and that the insert is correctly oriented. For details on the requirements of the various expression vectors, please refer to **vectors.clontech.com**. Examples of insert analyses are given below, however any method may be used to determine their identity.

A. Analyzing Inserts by PCR

Once you have isolated positive clones, analyze the insert to ensure compatibility with all Creator acceptor vectors. The M13 primers provided with the libraries allow you to estimate the size of the insert. Gene-specific primers can be used to verify that you have isolated the correct clone.

- PCR amplification can yield sufficient material for characterizing inserts by restriction enzyme digestion.
 1. Pick well-isolated positive colonies from hybridization screening plates.
 2. Grow colonies in 100 μ l of LB/Cm medium at 37°C on a shaker until the medium becomes cloudy (usually 2–4 hr). Follow the PCR reaction parameters specified in Section VI.A.
 3. Estimate insert sizes by electrophoresing an 5- μ l sample of the PCR product on a 0.8% agarose gel with 1-kb ladder DNA size markers.

B. Analyzing Clones by Restriction Enzyme Digestion

Plasmid DNA can be isolated from culture using standard procedures (Sambrook et al., 1989).

1. Digest 5–10 μ l of purified recombinant plasmid with the appropriate restriction enzyme.
 - For Creator Libraries, we suggest using Sfi I. pDNR-LIB has two Sfi I sites that flank the insert, so the digestion should yield two DNA fragments: the insert and the pDNR-LIB backbone (see the Appendix). Incubate your DNA with 8 units Sfi I per 1 μ g DNA at 50°C for one hour to overnight.
2. Heat samples at 70°C for 10 min to inactivate the enzyme (only necessary if using an enzyme other than Sfi I as Sfi I is not heat inactivated).
3. Analyze the digest on a 0.8% agarose minigel.

VIII. Analysis of Inserts *continued*

4. If you see abundant undigested DNA, try the following steps:
 - a. Reduce the DNA concentration.
 - b. Increase the amount of restriction enzyme.
 - c. Digest the DNA overnight.
 - d. Combine the above digestions.
 - e. Further purify the plasmid DNA on a CHROMA SPIN™-400 Column.
 - f. Amplify the insert by PCR (see Section VI.A).

C. Sequencing the Insert

The target gene insert should be sequenced before proceeding with functional analysis to ensure that you have a full-length gene with the correct reading frame. Library clones may have stop codons in the 5' UTR (untranslated region) which will interfere with contiguous reading from the 5'-end of your gene. Isolated clones may need to have an ATG start codon (the acceptor vector information packet will indicate whether an ATG is needed for the system of interest and if the insert must be in frame with the *loxP* site).

IV. References

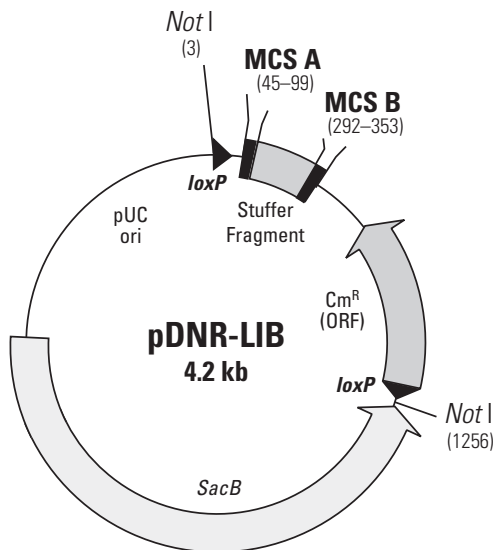
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IX. Related Products

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Product	Cat. No.
• Cre Recombinase	631614
• ExpressHyb™ Hybridization Solution	636831
	636832
	636833
• pDNR-LIB	631611
Mammalian Expression Vectors	
• pLP-IRESneo Acceptor Vector	631607
• pLP-CMV-Myc Acceptor Vector	631603
Matchmaker™ Vectors	
• pLP-GADT7 Acceptor Vector	630405
• pLP-GBKT7 Acceptor Vector	630406
Retroviral Expression Vectors	
• pLP-LNCX Acceptor Vector	631504
Bacterial Expression Vectors	
• pLP-PROTet-6xHN Acceptor Vector	631201
Inducible Mammalian Expression Vectors	
• pLP-TRE2 Acceptor Vector	631016
• pLP-RevTRE Acceptor Vector	631015
Additional Related Products	
• Advantage® 2 PCR Kit	639206
	639207
• Advantage® 2 Polymerase Mix	639201
	639202
• Human β -Actin Control Amplimer Set	639001
	639002
• Marathon-Ready™ cDNAs	many
• NucleoBond® Plasmid Maxi Kit	635933
	635934
	635935

Appendix: pDNR-LIB Vector Map



MCS A

45
loxP •
TTA TCA GTC GAC GGT ACC GGA **CAT ATG CCC GGG** AAT TCG GCC ATT ACG GCC **TGC AGG** ATC C **Stuffer fragment.**
SalI *NdeI* *SmaI* *EcoRI* *SfiI* *PstI* *BamHI*

MCS B 292

Stuffer fragment GG ATC CGG CCG CCT CGG CCC TTC GAG AAG CTT TCT AGA CCA TTC GTT TGG CGC GCG GGC CCA GTA GGT AAG TGA A **STOPs**
BamHI *SfiI* *XhoI* *Hind III* *XbaI* *Bsp120I* *ApaI*

Figure 4. pDNR-LIB Vector Map and MCS (unique sites are in bold). pDNR-LIB is used for the construction of cDNA libraries with inserts that can then be transferred into a Creator System acceptor expression vector. Cre recombinase (available separately) mediates the exchange of DNA sequences at specific locations known as *loxP* sites on both the donor and acceptor vectors. The pDNR-LIB vector contains two *loxP* sites which flank the 5' end of MCS A and the 5' end of the open reading frame encoding the chloramphenicol resistance gene (*Cm^r*). pDNR-LIB also contains the sucrase gene from *B. subtilis* (*SacB*) which provides selection against both incorrect recombinants and the parental donor vector after the recombination reaction with an acceptor vector.

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Notes

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