

Creator[™] Acceptor Vector Construction Kit User Manual

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I. Introduction & Protocol Overview

The Creator™ Gene Cloning & Expression System provides a revolutionary method for transferring a target gene directly into multiple expression vectors, without the need for time-consuming subcloning steps. The Creator System serves as a convenient gateway to analyzing your gene of interest using yeast two-hybrid studies, tetracycline-regulated expression, retroviral expression, fluorescent protein tagging, and many other applications (April 2000 Clontechiques). The **Creator™ Acceptor Vector Construction Kit** expands the possibilities of the Creator System by allowing you to make acceptor expression vectors tailored to your exact research needs.

The Creator System (patent pending) uses Cre-*loxP* site-specific recombination to catalyze the transfer of a target gene from a donor vector—a plasmid containing your gene of interest—to an acceptor vector, a plasmid containing regulatory elements of the desired host expression system (Figure 1). Creator

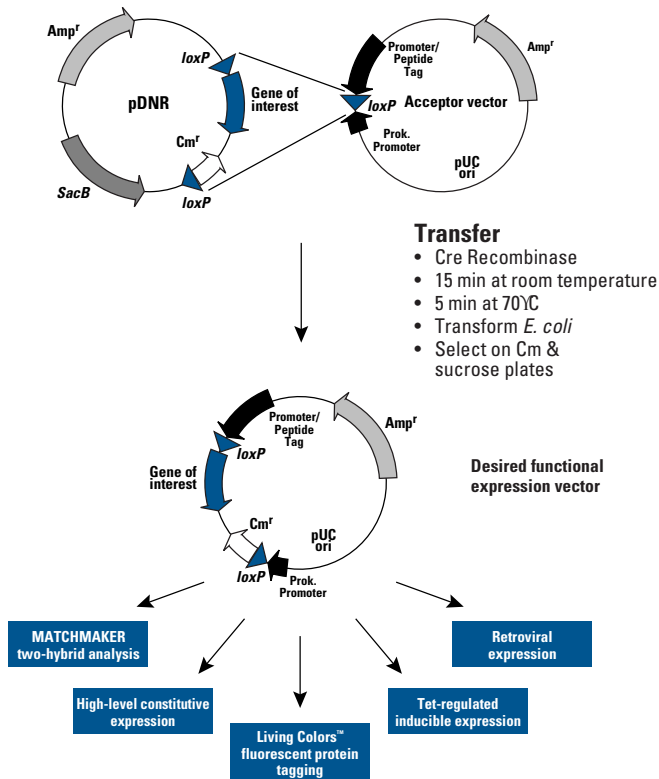


Figure 1. Flow chart of the Creator™ DNA Cloning reaction. Reagents for these procedures are included in the Creator™ DNA Cloning Kits.

I. Introduction & Protocol Overview *continued*

donor vectors (generalized by pDNR in Figure 1) contain two *loxP* sites, which flank the 5' end of the MCS and the 5' end of the open reading frame for the chloramphenicol resistance gene (Cm^r; Figure 1). Creator acceptor vectors contain a single *loxP* site, followed by a bacterial promoter. This promoter drives expression of the chloramphenicol marker after Cre-*lox*-mediated recombination, eliminating non-recombinant vectors. The gene of interest, once transferred, will become linked to the specific expression elements for which the Acceptor Vector was designed. The Creator Acceptor Vector Construction Kit provides all the necessary components to convert your conventional expression vectors into Creator-compatible Acceptor Vectors.

The key element of this kit is the 161 bp *loxP*-Promoter Cassette, which is carried on the 650-bp *loxP*-Promoter Template (Figure 2). To generate an Acceptor Vector, the cassette is first amplified through PCR, using primers bearing incorporated restriction sites (Figure 3). The PCR product is then cleaved with the appropriate restriction enzymes and the cassette ligated into your pre-cut target vector. The newly constructed plasmid is then transformed into bacteria for selection and amplification. Two control primers, one containing an *EcoR* I site and another containing a *Bam*H I site, are included with the kit. Alternatively, you may design your own primers, based on the control primers' sequences, to incorporate restriction sites specific for your target plasmid (see Section V). Furthermore, by using the sequence of the included control primers to design your primers, you eliminate the need for tedious optimization of PCR conditions.

Generate a Wide Range of Creator-Compatible Expression Vectors

The Creator Acceptor Vector Construction Kit can be used to generate a range of acceptor vectors specific for each researcher's needs. This kit allows researchers working with any expression system to take advantage of the speed and accuracy of the Creator cloning method, simply by linking the appropriate

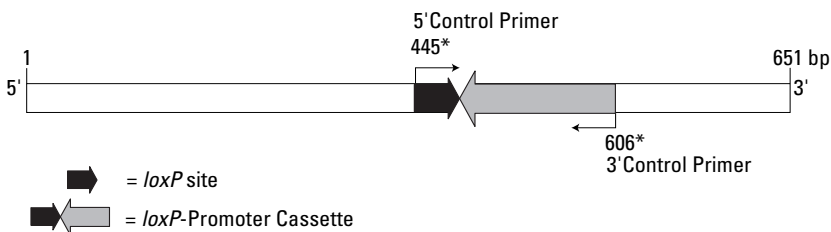


Figure 2. Diagram of the *loxP*-Promoter Template. The “outermost” annealing nucleotide position of the Control Primers (indicated by an asterisk [*]) in relation to the *loxP*-Promoter Template are indicated in the figure (not drawn to scale).

I. Introduction & Protocol Overview *continued*

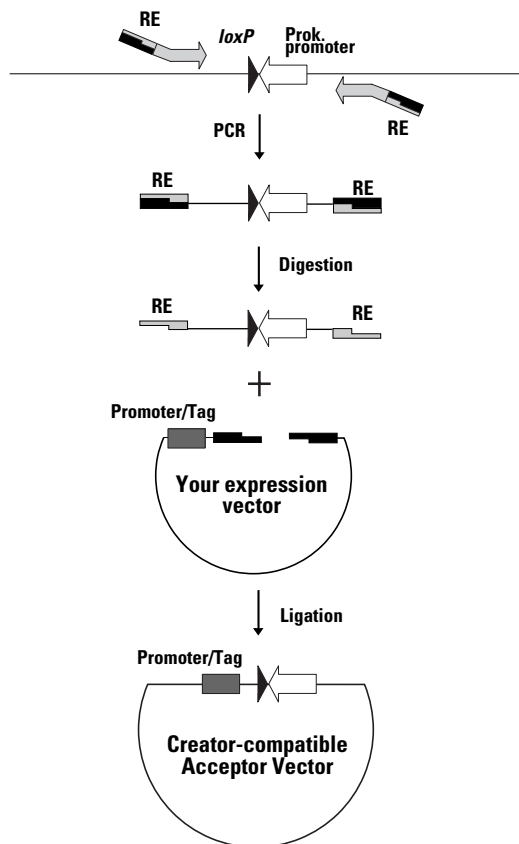


Figure 3. Flow chart of the Creator™ Acceptor Vector construction procedure. RE: Restriction enzyme site.

promoter to the *loxP*-Promoter Cassette. It is also possible to construct vectors with N-terminal tags such as glutathione S-transferase (GST) for bacterial expression of fusion proteins. In addition, the Creator Construction Kit can be used to make vectors that express an insert from a tissue specific promoter. The resulting expression vector can then be used to express a range of different reporter genes to track the promoter's unique expression pattern.

The acceptor expression vectors synthesized with the Creator Acceptor Vector Construction kit are compatible with all of our pDNR Donor Vectors. See Related Products (Section IX) for ordering information and a complete list of our Creator™ System products.

II. List of Components

Store NucleoSpin® Extraction Trial Kit at room temperature. Store all other components at –20°C.

- 10 µl **50X AdvanTaq DNA Polymerase**

Concentration in 50X mix	Component	Final rxn concentration
50 %	Glycerol	1.0 %
20 mM	Tris-HCl (pH 8.0)	0.4 mM
100 mM	KCl	2 mM
0.1 mM	EDTA (pH 8.0)	2.0 µM
0.25 %	Tween-20	0.005 %
0.25 %	Nonidet P-40	0.005 %
2 mM	Na-polyphosphate glass	40 µM

- 50 µl **10X AdvanTaq PCR Buffer**

Concentration in 10X mix	Component	Final rxn concentration
400 mM	Tricine-KOH (pH 8.0 at 25°C)	40 mM
160 mM	KCl	16 mM
45 mM	MgCl ₂	4.5 mM
37.5 µg/ml	BSA	3.75 µg/ml

- 10 µl ***loxP*-Promoter Template**

- 10 µl **50X dNTP Mix** (10 mM each nucleotide)

- 10 µl **5' Control Primer** (20 µM)

- 10 µl **3' Control Primer** (20 µM)

- 500 µl **PCR-Grade Water**

- 1 **NucleoSpin® Extraction Trial Kit**

- NucleoSpin Extraction Spin Columns

- 2-ml Collection Tubes

- Buffer NT2

- Buffer NT3 (Add 95% ethanol as indicated on the bottle label before use)

- Buffer NE

III. Additional Materials Required

The following materials are required but not supplied:

- [Optional] **Mineral oil** (We recommend Sigma Cat. No. M-3516.)
- **0.5-ml PCR reaction tubes** (We recommend GeneAmp 0.5-ml reaction tubes [Cat. No. N801-0737 or N801-0180].)
- **Thermal cycler**
- **Dedicated pipettors**
- **PCR pipette tips** suitable for the above pipettors and preferably equipped with hydrophobic filters.
- **TE buffer** (10 mM Tris [pH 7–7.5], 1 mM EDTA)
- **T4 DNA Ligase** (NEB Cat. No. M0202S)
- **DNA size markers** (100-bp and 1-kb DNA ladders)
- **DNA mass ladder** (low mass)
- **5X Stop/loading buffer** (Sambrook *et al.* [1989] provides several recipes.)
- **50X TAE electrophoresis buffer**

242.0 g	Tris base
57.1 ml	glacial acetic acid
37.2 g	Na ₂ EDTA•2H ₂ O

Add H₂O to 1 L.

- **Restriction enzymes and buffers** (*EcoR* I/*Bam* H I if using supplied primers; refer to Section V.)
- **Bovine serum albumin (BSA)**
- [Optional] **NucleoSpin® Extraction Kit** (Cat. No. 635961)
- **Electrocompetent or chemically competent cells**
(For ordering information, see Related Products.)
- **SOC Medium**

2 %	Tryptone
0.5 %	Yeast Extract
10 mM	NaCl
2.5 mM	KCl
10 mM	MgCl ₂ •6H ₂ O
20 mM	glucose

1. For 1 liter, dissolve 20 g of tryptone, 5 g of yeast extract, and 0.5 g of NaCl in 950 ml of deionized H₂O.
2. Prepare a 250 mM KCl solution by dissolving 1.86 g of KCl in deionized H₂O for a total volume of 100 ml. Add 10 ml of this stock KCl solution to the solution prepared in Step 1.

III. Additional Materials Required *continued*

3. Adjust pH to 7.0 with 5 M NaOH, then bring the volume to 980 ml with deionized H₂O.
 4. Prepare a 1 M solution of MgCl₂ by dissolving 20.33 g of MgCl₂•6H₂O in deionized H₂O for a total volume of 100 ml.
 5. Autoclave both solutions on liquid cycle at 15 lbs/sq. in. for 20 min.
 6. Meanwhile, make a 2 M solution of glucose by dissolving 36 g of glucose in deionized H₂O for a total volume of 100 ml. Filter-sterilize this solution.
 7. Let the autoclaved solutions cool to about 55°C, then add 10 ml of the filter-sterilized 2 M glucose solution and 10 ml of 1 M MgCl₂. Store at room temperature or 4°C.
- **LB broth**
 - 10 g/L Bacto-tryptone
 - 5 g/L Bacto-yeast extract
 - 5 g/L NaClAdjust pH to 7.0 with 5 N NaOH. Autoclave.

IV. General Considerations

- Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can produce nonspecific amplification; in some instances, contaminants can cause DNA bands even in the absence of added template DNA. We recommend that you use small aliquots of starting material to avoid contaminating your stocks. You should also use dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. In addition, we recommend setting up a negative control reaction that does not contain any template.
- Because of the small volumes used in PCR experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture and deliver the contents from the pipette tip into the mixture, then rinse the tip by pipetting up and down several times.
- For the highest transformation efficiency, we recommend using NucleoSpin® Plasmid Miniprep or Maxiprep Kits to obtain highly pure plasmid DNA (see Related Products for ordering information).

V. Primer Design

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions.

1. For the most reliable PCR results, we recommend that you design your primers based on the sequence of the Control Primers, changing only the non-annealing restriction site portion. By using the control primers as the basis for your amplification primers, you eliminate the need to optimize PCR conditions for your primers. The sequence of the Control Primers and *loxP*-Promoter Cassette are shown in Figure 4. The sequence of the entire *loxP*-Promoter Template is also available at **www.clontech.com**.
2. If you plan to construct an acceptor vector for expressing an introduced gene as a fusion protein, design your cloning strategy so that the upstream coding sequence in your expression vector will be in frame with the *loxP* site of the *loxP*-Promoter Cassette. The first codon of the *loxP* site is represented by the first three bases following the *EcoR* I site in the 5' Control Primer sequence (see Figure 4).

Note: The Creator Cloning System is currently not compatible with C-terminal tags/protein fusions.

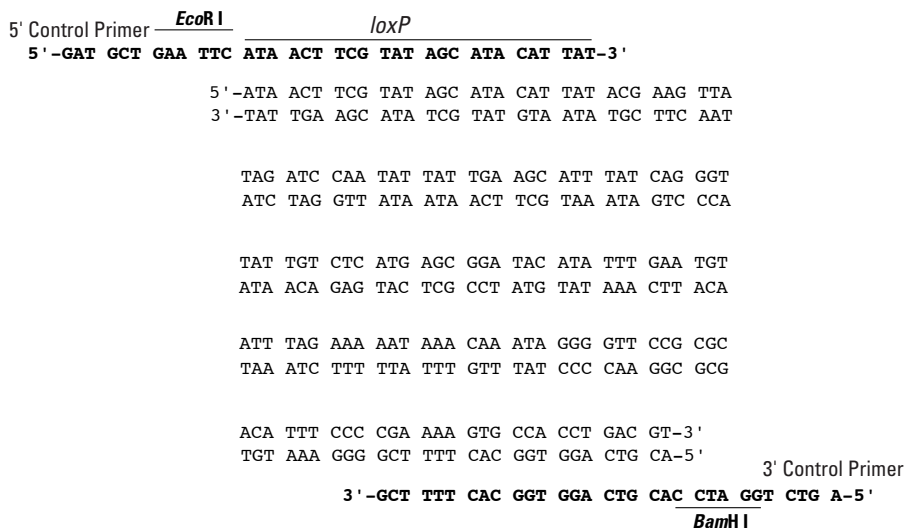


Figure 4. Sequence map of the *loxP*-Promoter Cassette. The map is shown in frame with the *loxP* site. The Control Primer sequences are shown in bold.

V. Primer Design *continued*

- To digest effectively, certain restriction enzymes require a greater number of bases surrounding the restriction site than are included in the design of the Control Primers. To ensure efficient cleavage when using these enzymes we recommend adding additional bases to the 5' ends of your primers.
- The *loxP*-Promoter Cassette lacks restriction sites for the following enzymes:

<i>Aat</i> II	<i>Blp</i> I	<i>Bss</i> S I	<i>Ehe</i> I	<i>Nci</i> I	<i>Scr</i> F I
<i>Acc</i> I	<i>Bpm</i> I	<i>Bst</i> 1107 I	<i>Fau</i> I	<i>Nco</i> I	<i>Sex</i> A I
<i>Afl</i> II	<i>Bsa</i> A I	<i>Bst</i> B I	<i>Fnu</i> 4H I	<i>Nde</i> I	<i>Sfa</i> N I
<i>Afl</i> III	<i>Bsa</i> B I	<i>Bst</i> E II	<i>Fok</i> I	<i>Ngo</i> M I	<i>Sfc</i> I
<i>Age</i> I	<i>Bsa</i> H I	<i>Bst</i> F5 I	<i>Fse</i> I	<i>Nhe</i> I	<i>Sfi</i> I
<i>Ahd</i> I	<i>Bsa</i> I	<i>Bst</i> N I	<i>Fsp</i> I	<i>Not</i> I	<i>Sgf</i> I
<i>Alu</i> I	<i>Bsa</i> J I	<i>Bst</i> X I	<i>Hae</i> II	<i>Nru</i> I	<i>Sgr</i> A I
<i>Alw</i> N I	<i>Bsa</i> W I	<i>Bsu</i> 36 I	<i>Hae</i> III	<i>Nsi</i> I	<i>Sma</i> I
<i>Apa</i> I	<i>Bsc</i> 4 I	<i>Cac</i> 8 I	<i>Hga</i> I	<i>Nsp</i> I	<i>Sna</i> B I
<i>Apa</i> L I	<i>Bse</i> R I	<i>Cla</i> I	<i>Hinc</i> II	<i>Pac</i> I	<i>Spe</i> I
<i>Apo</i> I	<i>Bsg</i> I	<i>Csp</i> 6 I	<i>Hind</i> III	<i>Pfl</i> M I	<i>Sph</i> I
<i>Asc</i> I	<i>Bsi</i> E I	<i>Cvi</i> J I	<i>Hinf</i> I	<i>Ple</i> I	<i>Srf</i> I
<i>Ase</i> I	<i>Bsi</i> HKA I	<i>Dde</i> I	<i>Hpa</i> I	<i>Pme</i> I	<i>Sse</i> 8387 I
<i>Asp</i> 718 I	<i>Bsi</i> W I	<i>Dra</i> I	<i>Hpa</i> II	<i>Pml</i> I	<i>Stu</i> I
<i>Ava</i> I	<i>Bsi</i> I	<i>Dra</i> III	<i>Hph</i> I	<i>Ppu</i> 10 I	<i>Sty</i> I
<i>Ava</i> II	<i>Bsm</i> B I	<i>Drd</i> I	<i>Kas</i> I	<i>Ppu</i> M I	<i>Swa</i> I
<i>Avr</i> II	<i>Bsm</i> F I	<i>Dsa</i> I	<i>Kpn</i> I	<i>Psh</i> A I	<i>Taq</i> I
<i>Bam</i> H I	<i>Bsm</i> I	<i>Eae</i> I	<i>Mae</i> III	<i>Psp</i> 1406 I	<i>Tfi</i> I
<i>Ban</i> I	<i>Bsp</i> 120 I	<i>Eag</i> I	<i>Mbo</i> II	<i>Pst</i> I	<i>Tse</i> I
<i>Ban</i> II	<i>Bsp</i> 1286 I	<i>Ear</i> I	<i>Mfe</i> I	<i>Pvu</i> I	<i>Tsp</i> 45 I
<i>Bbe</i> I	<i>Bsp</i> E I	<i>Ecl</i> 136 II	<i>Mlu</i> I	<i>Pvu</i> II	<i>Tsp</i> 509 I
<i>Bbs</i> I	<i>Bsp</i> LU11 I	<i>Eco</i> 47 III	<i>Mnl</i> I	<i>Rsa</i> I	<i>Tsp</i> R I
<i>Bbv</i> I	<i>Bsp</i> M I	<i>Eco</i> 57 I	<i>Msc</i> I	<i>Rsr</i> II	<i>Tth</i> 111 I
<i>Bcg</i> I	<i>Bsr</i> D I	<i>Eco</i> N I	<i>Mse</i> I	<i>Sac</i> I	<i>Xba</i> I
<i>Bcg</i> I	<i>Bsr</i> F I	<i>Eco</i> O109 I	<i>Msl</i> I	<i>Sac</i> II	<i>Xcm</i> I
<i>Bcl</i> I	<i>Bsr</i> G I		<i>Msp</i> A1 I	<i>Sal</i> I	<i>Xho</i> I
<i>Bfa</i> I	<i>Bsr</i> I	<i>Eco</i> R I	<i>Mwo</i> I	<i>Sap</i> I	<i>Xma</i> I
<i>Bgl</i> I	<i>Bss</i> H II	<i>Eco</i> R II	<i>Nae</i> I	<i>Sau</i> 96 I	<i>Xmn</i> I
<i>Bgl</i> II	<i>Bss</i> K I	<i>Eco</i> R V	<i>Nar</i> I	<i>Sca</i> I	

VI. Acceptor Vector Construction Procedure

A. Amplification of the *loxP*-Promoter Cassette

1. Place all components on ice and allow to thaw completely. Mix each component thoroughly before use.
2. Combine the following reagents in a 0.5-ml PCR tube, in the order shown.

Template Reaction	Negative Control	
19 μ l	21 μ l	PCR-Grade Water
2.5 μ l	2.5 μ l	10X AdvanTaq PCR Buffer
2 μ l	---	<i>loxP</i> -Promoter Template
0.25 μ l	0.25 μ l	5' Control Primer (20 μ M)
0.25 μ l	0.25 μ l	3' Control Primer (20 μ M)
0.5 μ l	0.5 μ l	50X dNTP Mix (10 mM ea of dATP, dCTP, dGTP, dTTP)
0.5 μ l	0.5 μ l	50X AdvanTaq DNA Polymerase
25 μl	25 μl	Total volume

3. Mix well and spin tube briefly to collect all the liquid in bottom of tube.
4. [If your thermal cycler does not have a "hot lid"]: Add 1–2 drops of mineral oil to prevent evaporation during cycling. A good "seal" of mineral oil should have a well-defined meniscus between the two phases. Cap the PCR tubes firmly.
5. Commence thermal cycling using the following parameters. These are general guidelines for use with "hot lid" thermal cyclers—the optimal parameters may vary with different thermal cyclers.
 - 95°C for 1 min
 - 16 cycles:

95°C	30 sec
60°C	2 min
 - 60°C for 15 min
6. Transfer a 5- μ l sample of your PCR reaction to a fresh tube and add 1 μ l of 5X stop/loading buffer. Analyze your sample(s) by electrophoresis on a 2.0% agarose/ethidium bromide gel, along with suitable DNA size markers.
7. **Expected results:** The reaction should produce a single major fragment of 161 bp (see Figure 5). No bands should be generated in the negative (i.e., no DNA template) control.

VI. Acceptor Vector Construction Procedure *continued*

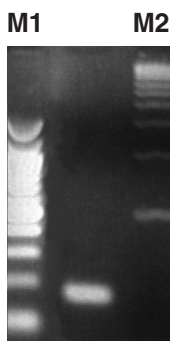


Figure 5. Amplification of the *loxP*-Promoter Cassette. A 5- μ l sample of the *loxP*-Promoter Cassette PCR reaction was analyzed on a 2% agarose/ethidium bromide gel. Lane M1: 100-bp DNA size marker. Lane M2: 1-kb DNA size marker.

B. Purification of PCR Product

To purify the PCR product from unincorporated nucleotides use the NucleoSpin Extraction columns and follow the procedure described below. Before use, be sure to add 95% ethanol directly to Buffer NT3 as specified on the bottle.

Note: It is not necessary to remove the mineral oil layer from the PCR sample. However, the oil layer can be removed by placing tubes at -20°C for about 30 min to freeze the aqueous layer. Remove the oil layer using a pipette.

1. Adjust the PCR reaction volume to 100 μ l with TE (pH 7–7.5).
2. Add 400 μ l of Buffer NT2 to the sample. Mix well.
3. Insert the NucleoSpin Extraction Spin Column into a 2-ml collection tube. Load the sample into the column. Centrifuge at maximum speed for 1 min at room temperature. Discard the flowthrough.
4. Reinsert the column in the 2-ml Collection Tube. Add 700 μ l of Buffer NT3 to the column. Centrifuge at maximum speed for 1 min. Discard the flowthrough.
5. Repeat Step 4.
6. Put the NucleoSpin column back in the 2-ml Collection Tube. Centrifuge at maximum speed for 1 min to remove residual Buffer NT3 from the membrane filter.

Note: The ethanol in Buffer NT3 inhibits enzymatic reactions and must be removed completely by performing this centrifugation step.

7. Place the NucleoSpin column in a clean 1.5-ml microcentrifuge tube. Let membrane dry for 1 min then add 50 μ l of Buffer NE. Let buffer soak membrane for 1 min, then centrifuge at maximum speed for 1 min.

VI. Acceptor Vector Construction Procedure *continued*

C. Quantification of PCR Product

Typical yield using the Control Primers provided is 50–100 ng/μl.

1. When the cycling is complete, analyze a 5-μl sample of the PCR product, alongside a low DNA mass ladder, on a 2.0% agarose/EtBr gel.
2. Photograph the gel and compare the band intensity of the PCR product to the band intensities of the mass standards to quantify PCR yield.

D. Digestion of PCR Product

1. Set up the following restriction enzyme digestion (substitute appropriate enzymes/buffers if you designed your own primers):

1–2	μg	purified PCR product (approximately 20 μl)
5	μl	10X restriction enzyme buffer (for <i>EcoR</i> I/ <i>BamH</i> I)
5	μl	10X BSA
1	μl	<i>EcoR</i> I restriction enzyme (10–20 units)
1	μl	<i>BamH</i> I restriction enzyme (10–20 units)

Adjust the final volume to 50 μl with sterile H₂O.

Note: The glycerol concentration in the mix will depend on the type and amount of enzyme(s) used for the digestion. To avoid “star” activity, reaction volumes should be increased proportionally (if necessary) to avoid glycerol concentrations > 5% (v/v). The amount of DNA should be maintained.

2. Mix well. Incubate at 37°C for 2 hr (adjust temperature and length of incubation if using restriction enzymes other than *EcoR* I and *BamH* I).

E. Purification of Digested *loxP*-Promoter Cassette

To purify the digested *loxP*-Promoter Cassette from the small cleaved cDNA fragments, use the NucleoSpin Extraction Kit and follow the procedure outlined below.

1. Adjust the digestion reaction volume to 100 μl with TE (pH 7–7.5).
2. Add 400 μl of Buffer NT2 to the digestion reaction. Mix well.
3. Insert the NucleoSpin Extraction Spin Column into a 2-ml collection tube. Load the sample into the column. Centrifuge at maximum speed for 1 min at room temperature. Discard the flowthrough.
4. Reinsert the column in the 2-ml Collection Tube. Add 700 μl of Buffer NT3 to the column. Centrifuge at maximum speed for 1 min. Discard the flowthrough.
5. Repeat Step 3.
6. Put the NucleoSpin column back in the 2-ml Collection Tube. Centrifuge at maximum speed for 1 min to remove residual Buffer NT3 from the membrane filter.

Note: The ethanol in Buffer NT3 inhibits enzymatic reactions and must be removed completely by performing this centrifugation step.

VI. Acceptor Vector Construction Procedure *continued*

7. Place the NucleoSpin column in a clean 1.5-ml microcentrifuge tube. Let membrane dry for 1 min then add 30 μ l of Buffer NE. Let buffer soak membrane for 1 min, then centrifuge at maximum speed for 1 min.
8. Quantify yield as described in Section VI.C.

F. Digestion and Purification of Expression Vector

Digest ~2.5 μ g of your expression vector with the appropriate enzymes. Purify your digested expression vector. [Optional] We recommend the following purification method:

1. Add 5X stop/loading buffer to the digest. Load the entire volume of the digestion onto a 12.5-cm x 20-cm agarose/ethidium bromide gel (use three 4.5-mm wide wells), along with suitable DNA size markers. Run the gel overnight at 22 volts, using a 30-cm electrophoresis apparatus.
 2. Excise and isolate the digested vector, using Clontech's NucleoSpin Extraction Kit (Cat No. 635961). Use a clean scalpel or razor blade to excise the DNA fragment from the agarose gel. Excise the fragment carefully to minimize the volume of agarose loaded onto the NucleoSpin membrane. Weigh the gel slice, and then transfer it to a clean microcentrifuge tube or 15-ml conical tube.
 3. For every 100 mg of agarose, add 300 μ l of Buffer NT1. Double the volume of NT1 for gels made with >2% agarose.
 4. Incubate the sample at 50°C for 10 min; briefly vortex the sample every 2–3 min during incubation.
 5. Insert the NucleoSpin Extraction Spin Column into a 2-ml collection tube. Load the sample into the column. Centrifuge at maximum speed for 1 min at room temperature. Discard the flowthrough.
 6. Reinsert the column into the 2-ml Collection Tube. Add 700 μ l of Buffer NT3 to the column. Centrifuge at maximum speed for 1 min. Discard the flowthrough.
 7. Repeat Step 6.
 8. Put the NucleoSpin column back in the 2-ml collection tube. Centrifuge at maximum speed for 1 min to remove residual Buffer NT3 from the membrane filter.
- Note:** The ethanol in Buffer NT3 inhibits enzymatic reactions and must be removed completely by performing this centrifugation step.
9. Place the NucleoSpin column in a clean 1.5-ml microcentrifuge tube. Let membrane dry for 1 min then add 50 μ l of Buffer NE. Soak membrane for 1 min, then centrifuge at maximum speed for 1 min.

Note: If your digested vector is 5–10 kb in length, we recommend warming Buffer NE to 70°C before use. After adding Buffer NE to the spin column, incubate the samples at room temperature for one minute to collect the purified fragment before centrifuging.

VI. Acceptor Vector Construction Procedure *continued*

- Determine yields on a 1.2% agarose gel as described in Section VI.C.

G. Ligation into the Expression Vector

- Set up the ligation, using 100 ng expression vector DNA and sufficient purified *loxP*-Promoter Cassette to give a 3:1, insert:vector molar ratio:

100 ng	Digested vector DNA
(10–15 ng, typically)*	Purified <i>loxP</i> -Promoter Cassette
0.5 µl	BSA
1.5 µl	10X Ligase buffer
0.5 µl	T4 DNA Ligase

Bring volume to 15 µl with sterile H₂O

* To calculate the correct amount of the *loxP*-Promoter Cassette for ligation to your expression vector, use the following formula:

$$\text{ng insert} = 300 \times \frac{161}{\text{Size of expression vector in base pairs}}$$

- Incubate for 3 hr at room temperature.
- [Optional] As a control, set up a parallel reaction, using only the digested vector. If colonies result following transformation, your vector is incompletely digested. Digest a fresh aliquot of your expression vector, using each restriction enzyme sequentially.

H. Transformation

Either electrocompetent or chemically competent cells may be used for transformation. The amount of the ligation reaction and competent cell suspension used per tube depends on the type of competent cells.

Notes:

- For either transformation method, be sure to include one tube with competent cells alone, as a control for (untransformed) host cell viability and antibiotic sensitivity, and one tube with a known quantity of uncut parental expression vector to determine transformation efficiency.
 - The transformation efficiency cells should be $>5 \times 10^7$ cfu/µg. If not, repeat with a fresh sample of cells.
- Transform 50 µl of electrocompetent cells with 1 µl of ligation mixture, or 100 µl of chemically competent cells with 10 µl of ligation mixture.
 - Add 950 µl of SOC medium (or LB broth) and grow cells at 37°C for 1 hr.
 - Plate 1/10 the volume of the cell culture on a 10-cm LB-agar containing the appropriate selection antibiotic for your vector.
 - Centrifuge the remaining cell culture at 6,000 rpm for 1 min. Resuspend pellet in 100 µl of SOC medium (or LB broth) and spread on a second selection plate. Incubate both selection plates overnight.

VI. Acceptor Vector Construction Procedure *continued*

5. The next day, pick 5 random colonies and isolate clones using a standard method of your choice (e.g., miniprep). Analyze clones by restriction digest with the same two restriction enzymes used for cloning to confirm your final construct. A fragment of 161 bp should be released when analyzed on a 2% agarose/ethidium bromide gel.
6. Sequence one of the clones to confirm that the correct sequence was amplified and cloned.

VII. Troubleshooting Guide

The simplicity of the Creator Acceptor Vector Construction Kit makes its use fairly straightforward. If you do not achieve typical results, this guide may help you to determine the source of the problem.

A. No PCR Product Observed

- | | |
|-----------------------------------|--|
| Too few cycles | Increase the number of cycles (3–5 additional cycles at a time). |
| PCR component missing or degraded | Use a checklist when assembling reactions. Perform a positive control reaction, using the Control Primers, to ensure that each component is functional. If the positive control does not work, repeat, replacing individual components to identify the faulty reagent. |

B. PCR Products are Smeared on Gel

- | | |
|-------------------|--|
| Too much template | Try a lower concentration of DNA template in the PCR reaction. |
| Contamination | Contamination most often results in extra bands or smearing. It is important to include a negative control (a control that replaces the DNA template with PCR-grade H ₂ O but still includes the primers) in every PCR experiment to determine if the PCR reagents, pipettes, or PCR reaction tubes are contaminated with previously amplified targets. We advise using commercially available aerosol-free pipette tips. |

C. No or Few Colonies

- | | |
|-------------------------------|--|
| Low transformation efficiency | You should obtain $>5 \times 10^7$ cfu/μg; otherwise use new, fresh competent cells. |
| Poor plasmid DNA quality | For best results, we recommend using NucleoSpin Plasmid Purification Products to obtain highly pure, miniprep plasmid DNA. For ordering information, see Related Products, Section IX. |

VIII. References

Clark, J. M. (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res.* **16**:9677–9686.

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Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual, Second Edition* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

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IX. Related Products

For the latest and most complete listing of all Clontech products, please visit www.clontech.com

• Creator™ pDNR-1 Cloning Kit	631615
• Creator™ SMART™ cDNA Libraries	many
• Cre Recombinase	631614
• pDNR-LIB	631611
• Creator™ Acceptor Vectors	many
• Creator™-Compatible RevTet-Off Retroviral Gene Expression System	631023
• Creator™-Compatible RevTet-On Retroviral Gene Expression System	631024
• Creator™-Compatible PROTet-6xHN Bacterial Gene Expression System	631204

Plasmid Purification Kits

• NucleoSpin® Plus Plasmid Miniprep Kit	635988
• NucleoSpin® Multi-8 Plasmid Kit	635973 635974
• NucleoSpin® Multi-8 Plus Plasmid Kit	635975 635976