USER GUIDE

invitrogen™
by life technologies™

pTracer™-SV40

Catalog numbers V870-01, V871-20

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

life technologies™

Contents

Kit Contents and Storage

Kit Contents

This manual is supplied with the following products.

Catalog no.	Contents	Amount
V870-01	pTracer™-SV40	0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL
	Zeocin™ antibiotic	1 g (100 mg/mL, 8 × 1.25 mL)
V871-20	pTracer™-SV40 only	0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL

Shipping/Storage

Catalog no. V870-01 is shipped on dry ice. Upon receipt, store the plasmid and the Zeocin™ antibiotic at –20°C.

Catalog no. V871-20 is shipped at room temperature. Upon receipt, store the product at –20°C.

Product Use

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

Introduction

Product Overview

Description of the System

pTracer™-SV40 is a 4.2 kb mammalian expression vector derived from pZeoSV2 and designed for visual detection of transformed *E. coli* cells and transfected mammalian cells. It yields high-level stable and transient expression of the gene of interest in mammalian cells. The vector contains the following features:

- Cycle 3-GFP, an improved GFP (Green Fluorescent Protein) gene (Crameri *et al.*, 1996), fused to the Zeocin™ resistance gene for selection in mammalian cells
- Human cytomegalovirus immediate-early (CMV) promoter drives expression of the Cycle 3-GFP-Zeocin™ resistance gene fusion in mammalian cells
- SV40 early promoter drives high-level expression of the gene of interest in a wide range of mammalian cells
- Selection in both mammalian cells and *E. coli* using Zeocin™
- Episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS7)

Description of Cycle 3-GFP

The Cycle 3-GFP gene used in this vector is described in Crameri *et al.*, 1996. In this paper, the codon usage was optimized for expression in mammalian cells and three cycles of DNA shuffling were used to generate a mutant form of GFP that has the following characteristics:

- Excitation and emission maxima that are the same as wild-type GFP (395 nm and 478 nm for primary and secondary excitation, respectively, and 507 nm for emission)
- High solubility in *E. coli* for visual detection of transformed cells
- >40-fold increase in fluorescent yield over wild-type GFP

The Cycle 3-GFP gene is fused to the Zeocin™ resistance marker to correlate GFP fluorescence with resistance to the antibiotic Zeocin™.

Continued on next page

Product Overview, Continued

Experimental Overview

The table below outlines the basic steps needed to clone and express your gene of interest in pTracer™-SV40 and to visually detect transformed or transfected cells.

Step	Action	Page
1	Develop a cloning strategy and ligate your gene into pTracer™-SV40. Use the diagram of the multiple cloning site on page 5.	5
2	Transform *E. coli* and select transformants on Low Salt LB containing 25 to 50 µg/ Zeocin™.	6
3	Visually detect transformed cells using a transilluminator or hand-held UV lamp.	6
4	Analyze transformants for the presence and orientation of the insert.	7
5	Isolate pure plasmid DNA and linearize for mammalian transfection.	8
6	Transfect your cell line and allow the cells to recover for 24 to 48 hours.	8
7	Assay for fluorescence and estimate transfection efficiency.	9
8	Assay for optimal expression of your gene.	9
9	Optional: Select for stable cell lines using Zeocin™ and detect a homogeneous population of cells using fluorescence.	10

Zeocin™

Zeocin™ is a member of the bleomycin/phleomycin family of antibiotics isolated from *Streptomyces* (Berdy, 1980). It shows strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cell lines. It is particularly well-suited for selection of mammalian stable cell lines. See page 14for ordering. Additional information is available from Technical Support (page 15).

Applications of Zeocin™

Zeocin™ and the resistance gene (*Sh ble*) are used for selection in mammalian cells (Mulsant *et al.*, 1989); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in mammalian tissue culture cells and *E. coli* are listed below:

Organism	Zeocin™ Concentration and Selective Medium
E. coli	25–50 µg/mL in **low salt** LB medium*
Mammalian cells	50–1000 µg/mL (depends on cell line)

***Efficient selection in *E. coli* requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).**

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E. coli Strain

Many *E. coli* strains are suitable for the growth of this vector including TOP10F´, DH5αF´, and JM109. We recommend that you propagate vectors containing inserts in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells for purchase (see page 14 for ordering).

Important

DO NOT USE any *E. coli* strain that contains the complete Tn*5* transposable element (i.e. DH5αF´IQ™, SURE, SURE2). This transposon encodes the *ble* (bleomycin) resistance gene which will confer resistance to Zeocin™, preventing selection of colonies containing the pTracer™-SV40 vector.

Methods

Cloning into pTracer™-SV40

Introduction

A diagram is provided on page 5 to help you ligate your gene of interest into pTracer™-SV40. General considerations are listed below for additional information. When you are ready to transform *E. coli*, refer to page 6 for special information on selecting transformants using Zeocin™.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Maintaining pTracer™-SV40

To propagate and maintain pTracer™-SV40, use the supplied stock solution in TE pH 8.0 to transform a *rec*A, *end*A *E. coli* strain such as TOP10, TOP10F´, DH5α™, JM109, or equivalent. Transformants are selected on Low Salt LB containing 25–50 µg/mL Zeocin™ (see page 6). Be sure to prepare a glycerol stock for long-term storage.

Important

pTracer™-SV40 is a nonfusion vector allowing constitutive mammalian expression of the gene of interest. Your insert should contain an initiation codon, a Kozak sequence (see **References**), and a stop codon for proper expression of your protein in mammalian cells.

Note

1. Using excess *Eco*R I to digest the plasmid will cause this enzyme to cut at sites other than its particular recognition site ("star activity"). Be sure to use as few units as possible to complete the digestion.
2. pTracer™-SV40 does not encode the ampicillin resistance gene; therefore, selection for recombinants can be done only with Zeocin™ (see page 6).

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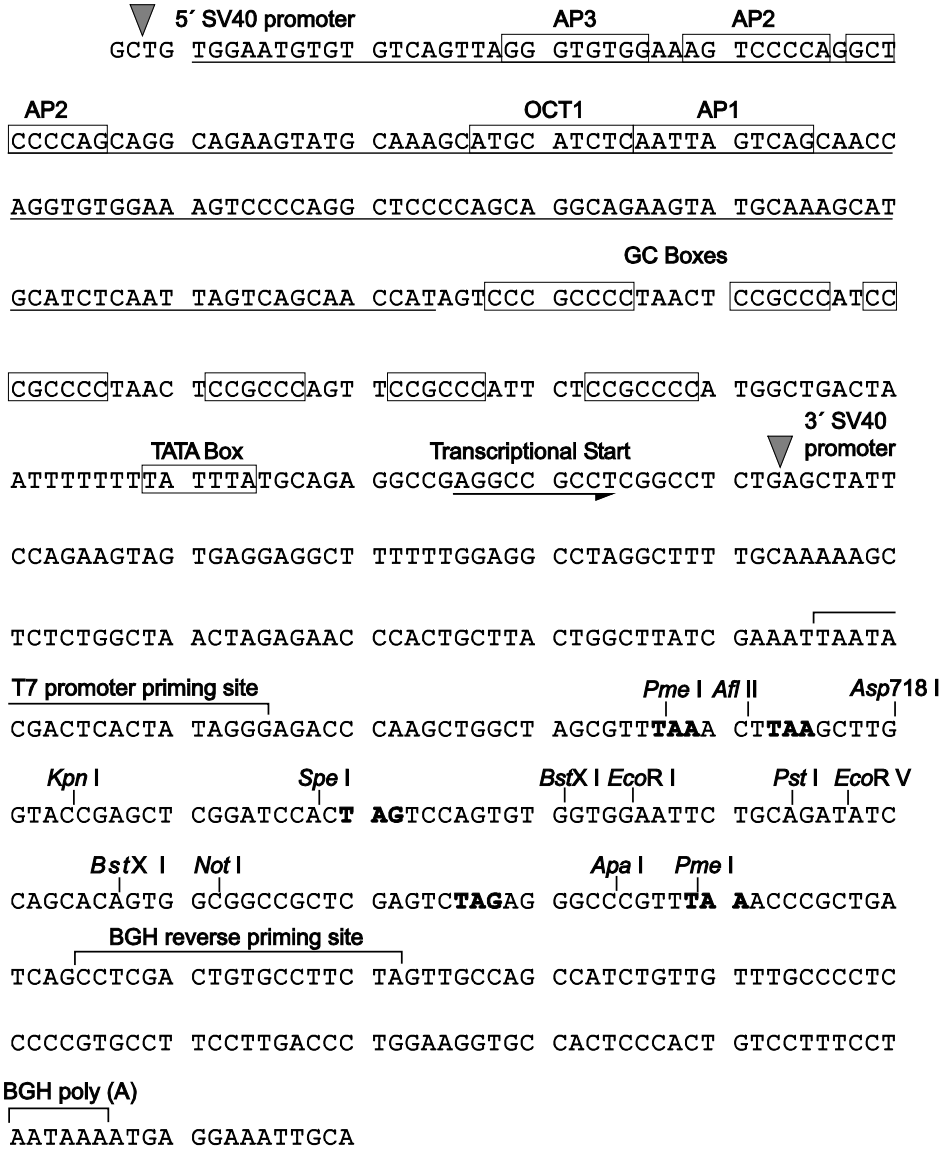
Cloning into pTracer™-SV40, Continued

Cloning into pTracer™-SV40

The graphic below shows the SV40 early enhancer/promoter and the multiple cloning site of pTracer™-SV40. The underlined regions at the 5´ end of the fragment containing the SV40 promoter are the two 72 bp enhancer repeats. Boxed sequences represent the binding sites of general transcription factors. AP1 indicates the Jun-Fos binding site, while GC boxes are where SP1 binds. The binding sites for AP2, AP3, and OCT1 are also shown. Restriction sites are labeled to indicate the cleavage site. Potential stop codons are shown in bold. The multiple cloning site has been confirmed by sequencing and functional testing.



Transformation into *E. coli*

Introduction

At this point, you have ligation mixtures that are ready to be transformed into *E. coli*. The transformation mix is plated on **Low Salt** LB medium (see below) containing 25 to 50 µg/mL Zeocin™. Transformants are isolated and analyzed for the presence and orientation of insert. After obtaining the desired recombinant plasmid, proceed to transfection of your mammalian cell line.

IMPORTANT! Low Salt LB Medium with Zeocin™

For maximal activity of Zeocin™, the salt concentration of LB medium must remain low (<90 mM) and the pH must be 7.5. Prepare LB broth and plates using the following recipe. Note the lower salt content of this medium. **Failure to lower the salt content of your LB medium will result in non-selection due to inactivation of the drug.**

Low Salt LB Medium:

10 g Tryptone; **5 g NaCl;** 5 g Yeast Extract

1. Combine the dry reagents above and add deionized, distilled water to 950 mL. Adjust pH to 7.5 with 1 N NaOH. Bring the volume up to 1 liter. For plates, add 15 g/L agar before autoclaving.
2. Autoclave on liquid cycle at 15 psi and 121°C for 20 minutes.
3. Allow the medium to cool to at least 55°C before adding the Zeocin™ to 25 µg/mL final concentration.
4. Store plates at 4°C in the dark. Plates containing Zeocin™ are stable for 1–2 weeks.

Transformation

Transform pTracer™-SV40 containing your insert into TOP10F´ or similar *E. coli* strain using your preferred method. Remember the following important points:

- Add either Low Salt LB or LB medium to the cells after heat shock or electroporation to allow them to recover.
- Plate on **Low Salt LB medium** with 25 µg/mL Zeocin™ and incubate overnight at 37°C.

Detecting Transformed *E. coli*

After overnight incubation, transformed *E. coli* can be detected by placing the plates on a transilluminator or with a hand-held UV lamp set on the long UV wavelength setting. Transformed colonies should be easily detected by bright green fluorescence. Select 10–20 clones and analyze for the presence and orientation of your insert.

Note: Fluorophore formation in *E. coli* grown at 37°C under aerobic conditions has a $T_{1/2}$ of about 95 minutes. It has been reported that *E. coli* containing Cycle 3-GFP grow 2- to 3- fold faster than *E. coli* containing wild-type GFP. This is presumably because of the reduced toxicity of the soluble Cycle 3-GFP (Crameri *et al.*, 1996).

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Transformation into *E. coli*, Continued

Sequencing is recommended to confirm the presence of a Kozak sequence and a stop codon. The T7 Promoter and BGH reverse primer sequences can be used to sequence across the multiple cloning site. Refer to the diagram on page 5 for the sequence and location of the primer binding sites.

For your convenience, we offer the T7 Promoter Primer (see page 14 for ordering) as well as a custom primer synthesis service. For more information, refer to www.lifetechnologies.com or contact Technical Support (see page 15).

Mammalian Transfection and Transient Expression

Introduction

At this point, you should have a positive clone with your fragment inserted in the correct orientation for expression in pTracer™-SV40. The next step is to isolate very clean DNA and transfect your cell line (see below). Before transfecting your cells, read **Detecting Fluorescence**, page 9, for information about using fluorescence to detect transfected cells. After mammalian transfection, the cells may be harvested and assayed for transient expression, or split and selected for stable transformants using the appropriate concentration of Zeocin™ (see page 10). Selection with Zeocin™ is not necessary for transient expression. It is only necessary when selecting for stable transfectants.

Preparing the Plasmid for Transfection

Plasmid DNA must be of high quality and free of contaminants. Contaminated DNA can be toxic to many cell lines. We recommend the PureLink® HiPure Miniprep Kit to isolate up to 30 µg of very pure plasmid DNA (see page 14 for ordering). For larger amounts, we recommend the PureLink® HiPure MidiPrep Kit or CsCl gradient centrifugation (see page 14).

Methods of Transfection

For established cell lines (e.g. HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. It is recommended that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology* (**Reference** section).

There are a variety of methods available for mammalian cell transfection. We offer Lipofectamine® and a large variety of reagents for mammalian transfection (see page 14 for ordering). For more details on the transfection products, visit www.lifetechnologies.com or contact Technical Support (see page 15).

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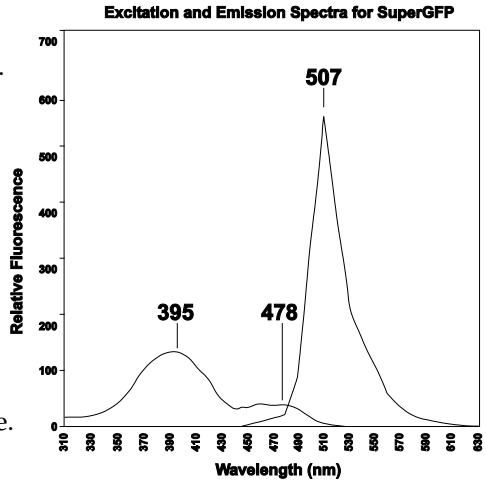
Mammalian Transfection, Continued

Detecting Fluorescence

To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of Cycle 3-GFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at these wavelengths yields a fluorescent emission peak with a maximum at 507 nm (see below).

Use of the best filter set will ensure that the optimal regions of the Cycle 3-GFP spectra are excited and passed (emitted). For example, the FITC filter set that we use excites Cycle 3-GFP with light from 460 to 490 nm, which covers the secondary excitation peak. The filter set passes light from 515 to 550, allowing detection of most of the GFP fluorescence. Standard FITC filters easily suit most purposes; however, it is important to keep in mind that fluorescence will be affected by the sample assayed and the filter you choose.



For general information about GFP fluorescence and detection, refer to Current Protocols in Molecular Biology.

Detecting Transfected Cells

After transfection, allow the cells to recover for 24 to 48 hours before assaying for fluorescence. **Note**: Most media fluoresce because of the presence of riboflavin (Zylka and Schnapp, 1996) and may interfere with detection of Cycle 3-GFP fluorescence. Medium can be removed and replaced with PBS to alleviate this problem.

Estimate the total number of cells before assaying for fluorescence. Then check your plate for fluorescent cells. You can use fluorescence to estimate transfection efficiency and normalize any subsequent assay for your gene of interest.

Cells can be incubated further in order to optimize expression of your gene of interest (see below).

Transient Expression

It is recommended that a time course be performed to determine the optimal time to assay for transient expression of the gene of interest as expression of the marker does not necessarily correlate with expression of your gene. **Optimal times may vary from 24–96 hours from the time of transfection depending on cell line.**

- Harvest 10^6–10^7 cells (one 100 mm plate at 80% confluence), pellet, and transfe to microcentrifuge tubes. Be sure to include untransfected cells as a control for background activity.
- The pellet of cells may be stored at –80°C if the assay cannot be performed immediately.
- To lyse the cell pellet, freeze-thaw 3 times in 250 mM Tris-HCl, pH 7.5 (or similar).
- The crude lysate is centrifuged, and the supernatant transferred to clean tubes to assay for expression of the gene of interest. The lysate may be stored at –80°C.

Isolation of Stable Transfectants

Introduction

Once your gene is expressed in your cell line, you can generate a stable cell line expressing your protein. To do this, you need to determine the minimum concentration of Zeocin™ needed to prevent growth of untransfected cells. This concentration of drug will be used to select for stable transfectants. In general, it takes 2 to 6 weeks to select foci with Zeocin™, depending on the cell line. You want to be able to isolate several foci to expand into stable cell lines. Be sure to use buffered medium as Zeocin™ is sensitive to changes in pH.

Determining Zeocin™ Sensitivity

To determine the minimal concentration of Zeocin™ required to prevent growth of the parental cell line use the protocol below:

1. Plate or split a confluent plate so there are approximately 2.5×10^5 cells per 60 mm or 100 mm dish. Prepare 7 plates and add varying concentrations of Zeocin™ (0, 50, 100, 250, 500, 750, and 1000 µg/mL) to each plate.
2. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
3. Count the number of viable cells at regular intervals to determine the appropriate concentration of Zeocin™ that prevents growth.

Linearizing Vector for Stable Integration

To obtain stable transfectants, you may linearize your vector before transfection. While linearizing your vector may not improve your chances of obtaining stable transfectants, it ensures that the vector does not integrate in a way that disrupts the gene of interest. Following is list of enzymes that may allow you to linearize your pTracer™-SV40 construct: *Alw*N I (1229), *Bgl* II (2296), *Bsp*H I (1533), *Sna*B I (2587), and *Ssp* I (2255).

Selecting Stable Integrants

Once you have determined the appropriate Zeocin™ concentration to use (see above), you can generate a stable cell line with your construct. You can use fluorescence to monitor development of foci and ensure a homogeneous population of cells.

1. Transfect 10^6 cells with 20 µg of vector using the desired protocol and plate onto 100 mm culture plates. Remember to include a plate of untransfected cells as a negative control.
2. 24 hours after transfection, wash the cells one time with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing Zeocin™ at the pre-determined concentration required for your cell line. Split the cells into four to eight 100 mm plates such that the cells are no more than 25% confluent.
4. Feed the cells with selective medium every 3–4 days until foci can be identified. Use fluorescence to monitor developing foci for expression.
5. When transfected cell foci are formed, the colonies may be picked using either cloning rings (if the colonies are isolated and large enough) or a pipette tip (if the colonies are small) and transferred to either 96- or 48-well plates. Grow cells to near confluence before expanding to larger wells or plates.
6. Test clones for expression of your protein. Positive clones can be expanded further into large microtiter wells and then into flasks or plates as desired and re-tested to confirm expression.

Troubleshooting Guidelines

Low or No Fluorescence

1. Check your original *E. coli* transformant by growing a 2–5 mL culture to saturation and assay for fluorescence by using a hand-held UV lamp or holding it over the transillluminator. If you detect fluorescence, your construct is fine, and you need to consider the other possibilities below.
2. High background fluorescence because of riboflavin in the culture medium. Replace medium with 1X PBS to eliminate background fluorescence.
3. A filter set was used that did not allow excitation at the optimal wavelength or permit detection of the emitted fluorescence. Check the filter set you are using.
4. Transfection efficiency is too low to allow detection of transfected cells. Optimize your transfection conditions or try another method.
5. Expression of Cycle 3-GFP may be low depending on the cell line used. See table below for time of expression and detection of Cycle 3-GFP and heterologous gene expression.

Cell Line	Detectable Fluorescence in..	Detection of Heterologous Expression in....
293	>24 hours	>48 hours
NIH3T3	>24 hours	>48 hours
CHO	>12 hours	>48 hours
COS	>12 hours	>48 hours
HeLa	>24 hours	>48 hours

No Transient Expression

Make sure there is an initiation codon in a proper Kozak consensus sequence (see **References**) for eukaryotic expression. Be sure there is also a stop codon.

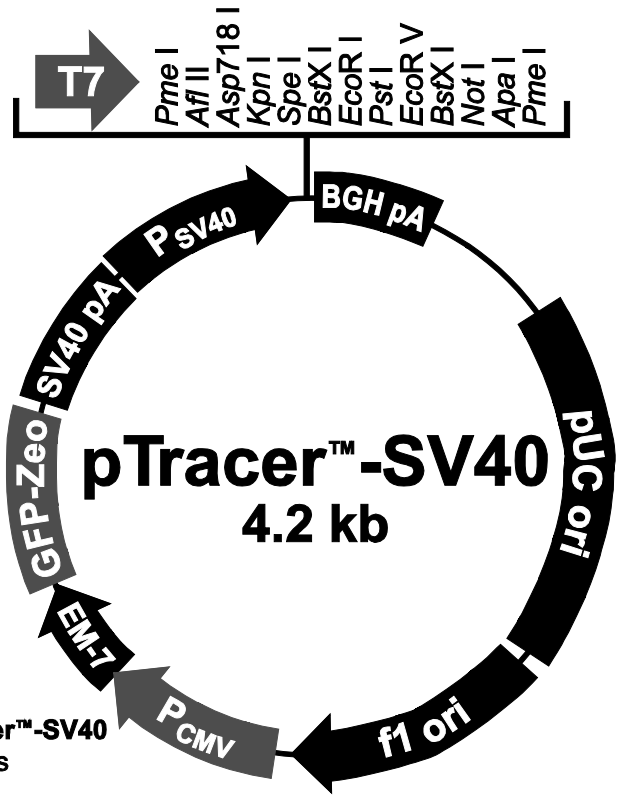
No Stable Expression

1. Confirm integration of your construct by either isolating genomic DNA and performing a Southern blot or PCR to see if your gene is present.
2. Confirm transcription by isolating mRNA and performing a Northern or RT-PCR to test for the expression of your gene. **Note**: Be sure plasmid has been eliminated from your cell line.
3. Be sure and isolate at least 50 independent foci as the location of integration may affect expression of both promoters.

pTracer™-SV40

pTracer™-SV40 Map

The figure below summarizes the features of the pTracer™-SV40 vector. The nucleotide sequence for pTracer™-SV40 can be downloaded from www.lifetechnologies.com or obtained by calling Technical Support (page 15).



Comments for pTracer™-SV40
4217 nucleotides

SV40 promoter: bases 10-356
T7 promoter: bases 401-420
Multiple Cloning Site: bases 439-548
BGH reverse priming site: bases 560-577
BGH polyadenylation signal: bases 560-773
pUC origin: bases 812-1537
f1 origin: bases 1780-2290
CMV promoter: bases 2304-2930
EM-7 promoter: bases 2931-2997
GFP-Zeocin™ ORF: bases 2998-4074
GFP ORF bases: 2998-3702
Zeocin™ resistance gene ORF bases: 3703-4074
SV40 polyadenylation signal: bases 4082-4217

Features of pTracer™-SV40

The important elements of pTracer™-SV40 are described in the following table. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

Features	Function
Simian Virus (SV40) early enhancer/promoter	Provides high-level expression of the gene of interest
T7 promoter priming site	Allows sequencing of insert
Multiple cloning site (MCS)	Permits insertion of gene of interest for expression
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
pUC origin	Replication, maintenance, and high copy number in *E. coli*
f1 origin	Rescue of single-strand DNA (anti-sense) for mutagenesis and sequencing
Human cytomegalovirus (CMV) immediate-early promoter/enhancer*	Permits efficient, high-level expression of the Cycle 3-GFP-Zeocin™ resistance gene fusion (Andersson *et al*., 1989; Boshart *et al*., 1985; Nelson *et al*., 1987)
EM-7 promoter	Permits efficient expression of the Cycle 3-GFP-Zeocin™ resistance gene fusion in *E. coli*
Cycle 3-GFP-Zeocin™ resistance gene fusion	Visual detection of transformed or transfected cells using fluorescent microscopy Selection of transformants in *E. coli* and stable mammalian cell lines
SV40 polyadenylation signal	mRNA stability and transcription termination
SV40 origin	Allows replication in cells expressing large T antigen

Appendix

Accessory Products

Detecting Fusion Protein

Additional products may be used with the pTracer™-SV40. Ordering information is provided below.

Item	Quantity	Catalog no.
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
Ultracomp™ TOP10F´ (chemically competent cells)	5 × 300 µL	C665-03
One Shot® TOP10F´ (chemically competent cells)	21 × 50 µL	C3030-03
T7 Promoter Primer	2 µg	N560-02
PureLink® HiPure Miniprep Kit	100 preps	K2100-03
PureLink® HiPure Midiprep Kit	25 preps	K2100-04
Zeocin™	1 g	R250-01
	5 g	R250-05
Lipofectamine® 2000	1.5 mL	11668-019

Technical Support

Obtaining support

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

At the website, you can:

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

Safety Data Sheets (SDS)

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

Certificate of Analysis

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

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Limited Use Label License: (Cycle 3) Mutant GFP

The (cycle 3) mutant GFP gene was produced by Maxygen, Inc. using the DNA shuffling technology. Crameri, A., Whitehorn,E.A., and Stemmer, W.P.C. (1996) Improved Green Fluorescent Protein by Molecular Evolution Using DNA Shuffling. *Nature Biotechnology*, 14: 315-319.

Limited Use Label License: GFP

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Notes

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