invitrogen™
by life technologies™

pTracer™-CMV2

Catalog nos. V885-01, V885-20

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MAN0000666

User Manual

Table of Contents

Contents and Storage

Contents

This manual is supplied with the following products.

Catalog no.	Contents	Amount
V885-01	pTracer™-CMV2 Kit	20 µg (40 µl of vector at 0.5 µg/µL in TE buffer, pH 8.0)
	Zeocin™ antibiotic	1 g (100 mg/ml, 8 x 1.25 ml)
V885-20	pTracer™-CMV2 Vector	20 µg (40 µl of vector at 0.5 µg/µL in TE buffer, pH 8.0)

Shipping/Storage

Catalog no. V885-01 is shipped on dry ice. Upon receipt, store the plasmid and the Zeocin™ antibiotic at -20°C.

Catalog no. V885-20 is shipped at room temperature. Upon receipt, store the plasmid at -20°C.

Introduction

Product Description

pTracer™-CMV2 is a 6.2 kb mammalian expression vector derived from pcDNA3.1 and designed for visual detection of transformed *E. coli* cells and transfected mammalian cells. The vector contains the following elements:

Cycle 3-GFP, an improved GFP (Green Fluorescent Protein) gene (Crameri *et al.*, 1996), fused to the Zeocin™ resistance gene (see below) for convenient, noninvasive detection of transformed or transfected cells

- Human elongation factor 1α-subunit promoter (hEF-1α) for mammalian expression of the Cycle 3-GFP-Zeocin™ fusion (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
- A synthetic bacterial promoter, EM-7, for expression of Cycle 3-GFP in *E. coli*
- Human cytomegalovirus immediate-early (CMV) promoter for high-level expression of your gene in a wide range of mammalian cells
- Zeocin™ resistance for stable selection in mammalian cell lines
- Ampicillin and Zeocin™ resistance genes for selection of transformants in *E. coli*

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™.

Experimental Outline

Experimental Overview

The table below outlines the basic steps needed to clone and express your gene of interest in pTracer™-CMV2 and to visually detect transformed or transfected cells.

Step	Action	Page
1	Develop a cloning strategy and ligate your gene into pTracer™-CMV2. Use the diagram of the multiple cloning site on page 5.	5
2	Transform *E. coli* and select transformants on LB medium containing 50 to 100 µg/ml ampicillin or Low Salt LB containing 25 to 50 µg/ml 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.	6
5	Isolate pure plasmid DNA and transfect your cell line.	7
6	Allow the cells to recover for 24 to 96 hours.	8
7	Assay for fluorescence and estimate transfection efficiency.	8
8	Assay for optimal expression of your gene.	8
9	Optional: Select for stable cell lines using Zeocin™ and detect a homogeneous population of cells using fluorescence.	9

Zeocin™

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. Ordering information is provided below. Additional information is available from Technical Service (see page 12).

Item	Amount	Catalog no.
Zeocin™	1 g	R250-01
	5 g	R250-05

Applications of Zeocin™

Zeocin™ and the resistance gene (*Sh ble*) are used for selection in mammalian cells (Mulsant *et al.*, 1988); plants (Perez *et al.*, 1989); yeast (Baron *et al.*, 1992); and prokaryotes (Drocourt *et al.*, 1990). Suggested concentrations of Zeocin™ for selection in *E. coli* and mammalian tissue culture cells 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 requires that the concentration of NaCl be no more than 5 g/liter (< 90 mM).**

E. coli Strain

Many *E. coli* strains are suitable for the growth of this vector including. 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 from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10F´	5 x 80 µl	C665-55
One Shot® TOP10F´ (chemically competent cells)	20 x 50 µl	C3030-03

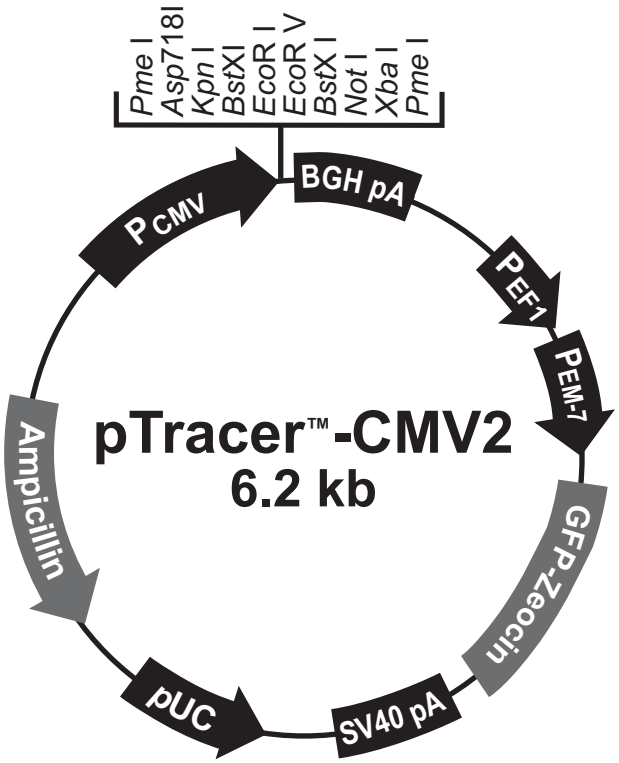
Important

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™-CMV2 vector.

pTracer™-CMV2 Vector

Map of pTracer™-CMV2

The figure below summarizes the features of the pTracer™-CMV2 vector. **The complete sequence for pTracer™-CMV2 is available for downloading from www.invitrogen.com** or from Technical Service (see page 12).



Comments for pTracer™-CMV2
6210 nucleotides

CMV promoter: bases 209-863
CMV forward priming site: bases 769-789
Multiple cloning site: bases 901-1010
BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1025-1223
EF-1a promoter: bases 1304-2487
EM-7 promoter: bases 2603-2658
GFP-Zeocin™ resistance gene: bases 2677-3753
    GFP ORF: bases 2677-3381
    Zeocin™ ORF (no Met): bases 3382-3753
SV40 polyadenylation sequence: bases 3883-4013
pUC origin: bases 4396-5069 (opposite strand)
Ampicillin resistance gene: bases 5214-6074 (opposite strand)

continued on next page

pTracer™-CMV2 Vector, continued

Features of pTracer™-CMV2

pTracer™-CMV2 contains the following elements. All features have been functionally tested. The multiple cloning site has been tested by restriction analysis.

Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Permits efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
CMV Forward priming site	Allows for sequencing through the insert
Multiple cloning site	Allows insertion of your gene for expression
BGH Reverse priming site	Allows for sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
Human EF-1α promoter	Allows high-level expression of the Cycle 3-GFP-Zeocin™ resistance gene fusion in mammalian cells (Goldman *et al.*, 1996; Mizushima and Nagata, 1990)
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 *E. coli* or transfected mammalian cells using fluorescence microscopy Selection of stable transfectants in mammalian cells
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection of vector in *E. coli*

Cloning into pTracer™-CMV2

Introduction

A diagram is provided below to help you ligate your gene of interest into pTracer™-CMV2. General considerations are listed below for additional information. Transform your ligation mixtures into *E. coli* using your method of choice and select transformants using ampicillin or Zeocin™.

Maintenance of pTracer™-CMV2

To propagate and maintain pTracer™-CMV2, transform a small amount of the supplied vector stock solution into a *rec*A, *end*A *E. coli* strain such as TOP10, TOP10F´, DH5α, JM109, or equivalent. Transformants are selected on LB plates containing 50 to 100 µg/ml ampicillin or Low Salt LB containing 25 to 50 µg/ml Zeocin™ (page 11). Be sure to prepare a glycerol stock for long-term storage.

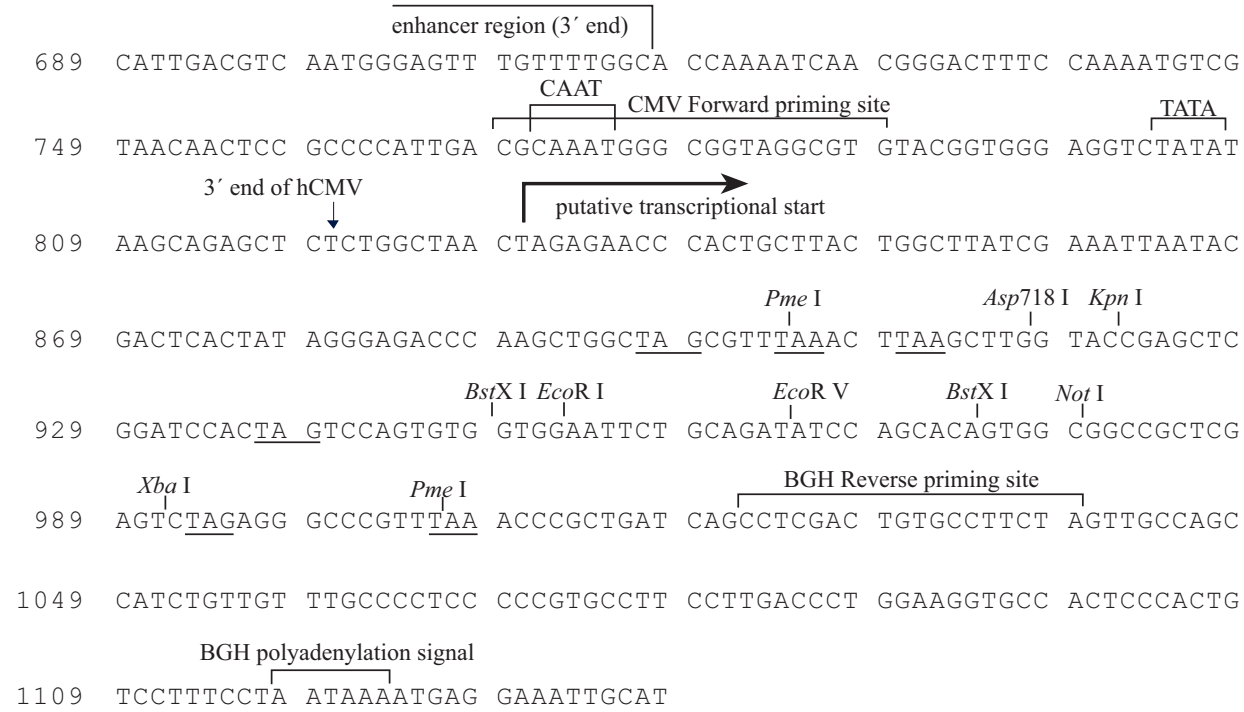
Important

pTracer™-CMV2 is a nonfusion vector. Your insert must contain a Kozak sequence (Kozak, 1987; Kozak, 1990) and a stop codon for proper expression of your gene.

The *Xba* I site contains an internal stop codon (TCTAGA).

Multiple Cloning Site of pTracer™-CMV2

Below is the multiple cloning site for pTracer™-CMV2. The 3´ end of the human cytomegalovirus (hCMV) promoter-enhancer is shown just upstream of the multiple cloning site. Restriction sites are labeled to indicate the cleavage site. Potential stop codons are shown underlined. The multiple cloning site has been confirmed by sequencing and functional testing.



Cloning into pTracer™-CMV2, continued

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain and select on LB plates containing 50 to 100 µg/ml ampicillin or Low Salt LB containing 25 to 50 µg/ml Zeocin™ (see page 11). Incubate overnight at 37°C and check for transformants.

Detection of 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. We recommend that you minimize exposure to UV light to prevent mutagenesis.

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).

We recommend that you sequence your construct with the CMV Forward and BGH Reverse primers to confirm that your gene is in the correct orientation for expression and contains an ATG and a stop codon. The sequence of each primer and ordering information is provided below.

Primer	Sequence	Catalog no.
CMV Forward	5´-CGCAAATGGGCGGTAGGCGTG-3´	N622-02
BGH Reverse	5´-TAGAAGGCACAGTCGAGG-3´	N575-02

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).

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™-CMV2. The next step is to isolate very clean DNA and transfect your cell line (see below). Before transfecting your cells, read **Detection of Fluorescence**, next page, 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™ (page 9). Selection with Zeocin™ is not necessary for transient expression. It is only necessary when selecting for stable transfectants.

Plasmid Preparation

Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipids, decreasing transfection efficiency. We recommend isolating DNA using the S.N.A.P.™ Miniprep Kit (Catalog no. K1900-01) for small-scale transfections. For isolation of larger amounts (10-200 µg), we recommend the S.N.A.P.™ MidiPrep Kit (Catalog no. K1910-01) or CsCl gradient centrifugation.

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* (Ausubel *et al.*, 1994).

There are a variety of methods available for mammalian cell transfection. Invitrogen offers the Calcium Phosphate Transfection Kit for mammalian transfection and Lipofectamine™ 2000 Reagent to optimize lipid-mediated transfection.

Catalog No.	Description
11668-027	Lipofectamine™ 2000 Reagent

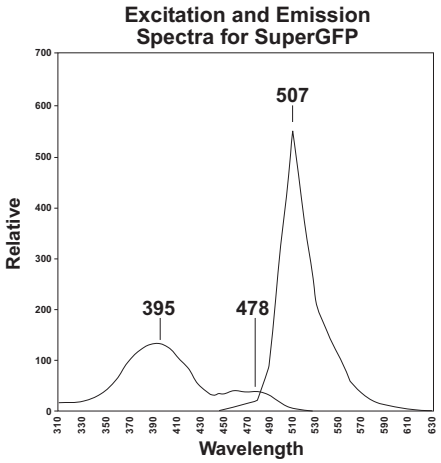
continued on next page

Mammalian Transfection, continued

Detection of 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.



Detection of Transfected Cells

After transfection, allow the cells to recover for 24 to 48 hours before assaying for fluorescence. 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 during viewing to alleviate this problem. If you plan to continue to culture your cells, remove the PBS and replace with fresh medium before returning the cells to the incubator.

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 to optimize expression of your gene of interest.

Transient Expression of Your Gene

We recommend that you perform a time course 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 to 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 transfer to microcentrifuge tubes. Be sure to include untransfected cells as a control for background activity.
- Store the cell pellet 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.
- 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 may wish to generate a stable cell line expressing your protein. To generate a stable cell line, first 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.

Determination of 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 x 10^5 cells per 60-100 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 choose to 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. Here are a few enzymes that may allow you to linearize your pTracer™-CMV2 construct:

Fsp I (5510); *Eam*1105 I (5288); *Pvu* I (5658); *Sca* I (5768); *Ssp* I (6092).

Selection of 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 appropriate concentration required for your cell line. Split the cells such that they 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.
5. Zeocin™-resistant 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.
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

Important

We have found that in cells transfected with pTracer™-CMV2, both fluorescence and expression of a heterologous gene are easily detected within 48 hours. Cell lines tested include COS, CHO, NIH3T3, and 293. Expression of the Cycle 3-GFP-Zeocin™ fusion and the heterologous gene may vary from cell line to cell line. Basic guidelines are provided below for troubleshooting any unexpected result.

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 transilluminator. 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. To check for background fluorescence, compare with a negative control.
3. A filter set was used that did not allow excitation at the best 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.

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 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 that the plasmid is not being maintained episomally. Plasmid DNA can be isolated from cells as described by Hirt, 1967 .
3. Be sure and isolate at least 50 independent foci as the location of integration may affect expression of both promoters.

Recipes

Low Salt LB Medium

If you wish to select bacterial transformants using Zeocin™, use the recipe below. For Zeocin™ to be active, the salt concentration of the medium must remain low (<90 mM) and the pH must be 7.5. 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.

Technical Service

Web Resources

Visit the Invitrogen website at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available at www.invitrogen.com/sds.

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Purchaser Notification

Introduction	Use of the pTracer™-CMV2 vector is covered under a number of different licenses including those detailed below.
Limited Use Label License No. 55: Cycle 3 GFP	The 'cycle 3' mutant GFP was produced by Maxygen, Inc. using DNA shuffling technology. Commercial licensing inquiries should be directed to: Affymax Research Institute, 4001 Miranda Avenue, Palo Alto, CA 94304, U.S.A.
Limited Use Label License No. 60: EF-1alpha Promoter	EF-1alpha promoter products are sold under license for research purposes only. The use of this product for any commercial purpose, including but not limited to, use in any study for the purpose of a filing of a new drug application, requires a license from: Mochida Pharmaceutical Co., Ltd., 7, Yotsuya 1-Chome, Shinjuku-Ku, Tokyo 160, Japan. Tel: 81-3-3225-5451; Fax: 81-3-3225-6091.
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Notes

Notes

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