

Great EscAPe™ SEAP User Manual



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PT3057-1 (PR9Y3393)
Cat. Nos. 631704, 631736,
631737 & 631738
Published 09 November 2009

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I. Introduction

The **Great EscAPe SEAP Chemiluminescence and Fluorescence Detection Kits** are versatile tools for the systematic analysis of eukaryotic promoters and enhancers. The Great EscAPe system uses SEAP—a secreted form of human placental alkaline phosphatase (Berger *et al.*, 1988)—as a reporter molecule to monitor the activity of promoters and enhancers. Such sequences can be cloned into the pSEAP2-Basic Vector (also available separately; Cat. No. 631715). The chemiluminescent substrate CSPD (PubChem CID No. 424756) and the fluorescent substrate, 4-methylumbelliferyl phosphate (MUP), enable researchers to monitor expression of the SEAP reporter gene using simple, sensitive, nonradioactive assays of secreted phosphatase activity (see Figure 1). The chemiluminescent assay can detect as little as 10^{-13} g of SEAP protein, making it one of the most sensitive enzymatic reporters available. In side-by-side assays, the fluorescent assay is 10- to 100-fold less sensitive than the chemiluminescent assay, but comparable to assays for firefly luciferase and suitable for all but the most demanding systems. Both assays are linear over a 10^4 -fold range of enzyme concentrations, making them particularly well suited for comparative analyses.

The SEAP reporter gene encodes a truncated form of the placental enzyme that lacks the membrane anchoring domain, thereby allowing the protein to be efficiently secreted from transfected cells. Changes in levels of SEAP activity detected in the culture medium have been shown to be directly proportional to changes in intracellular concentrations of SEAP mRNA and protein (Berger *et al.*, 1988; Cullen & Malim, 1992). SEAP has the unusual properties of being extremely heat stable and resistant to the phosphatase inhibitor L-homoarginine (Cullen & Malim, 1992). Therefore, endogenous alkaline phosphatase activity can be eliminated by pretreatment of samples at 65°C and incubation with this inhibitor.

The secreted nature of SEAP provides several advantages for the use of this enzyme as a transcription reporter:

- Preparation of cell lysates is not required for analysis.
- The kinetics of gene expression can be studied simply, by repeatedly collecting culture medium from the same samples.
- Transfected cells are not disturbed by measurement of SEAP activity in the medium, so a single set of cultures can be used both for the SEAP assay and for further investigations such as RNA and protein analysis.
- Background from endogenous alkaline phosphatase is almost absent in the culture medium following pretreatment.
- Sample collection from the culture medium can be automated by growing cultures and performing the assays in 96-well plates.

I. Introduction *continued*

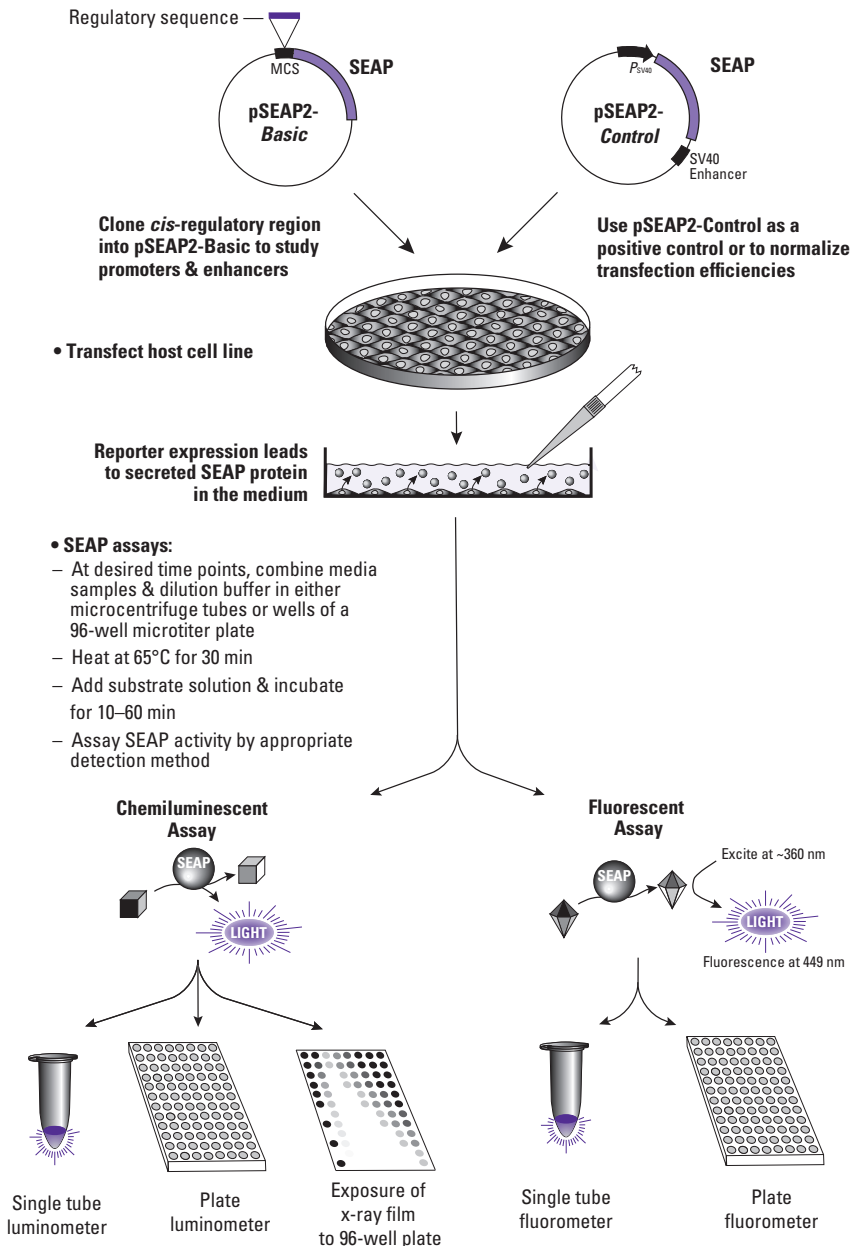


Figure 1. The Great EscAPe SEAP Assay procedure.

I. Introduction *continued*

Great EscAPe SEAP2 Reporter Vectors

The SEAP2 Reporter Vectors have been designed to provide maximal flexibility in studying regulatory sequences from the gene of interest. For vector maps and multiple cloning site (MCS) sequences, see Appendix A or visit our web site at www.clontech.com.

- **pSEAP2-Basic** (Cat. No. 631715) lacks eukaryotic promoter and enhancer sequences, and it contains an MCS that allows promoter DNA fragments to be inserted upstream of the SEAP gene. Enhancers can be cloned into either the MCS or unique downstream sites. A transcription blocker sequence upstream of the MCS reduces the potential read-through of upstream promoters which are part of the backbone.
- **pSEAP2-Control** (Cat No. 631717) is pSEAP2-Basic with the constitutive SV40 early promoter inserted upstream of the SEAP gene and the SV40 enhancer inserted downstream. pSEAP2-Control expresses SEAP in most cell types and provides an important positive control; in particular, it can be useful for normalizing transfection efficiencies.

The pSEAP2 Vectors incorporate a number of improvements over the original Great EscAPe vectors, such as including an enhanced Kozak consensus translation initiation site (Kozak, 1987); and removing the SV40 small-t intron, which would permit cryptic splicing and reduced expression in some genes and/or cell types (Huang & Gorman, 1990; Evans & Scarpulla, 1989). Additional improvements include switching from the early to late polyadenylation signal of SV40, which typically causes a fivefold increase in mRNA levels (Carswell & Alwine, 1989); and removing extraneous sequences from the 3' untranslated region of the SEAP mRNA. A synthetic transcription blocker is located upstream of the SEAP transcription unit and reduces background transcription (Eggermont & Proudfoot, 1993). The new vectors are also smaller and feature expanded multiple cloning sites.

II. Additional Materials Required

Great EscAPe SEAP assays can be carried out either in 0.5 ml microcentrifuge tubes or 96-well microtiter plates (for high-throughput applications; see Section V).

Chemiluminescence detection of SEAP activity can be performed using either a tube or plate luminometer, or via exposure of x-ray film to reactions carried out in 96-well microtiter plates. Fluorescence detection of SEAP activity can be performed with either a tube or plate fluorometer.

The following types of microtiter plates and accessories are recommended for high-throughput applications:

- **Falcon™ 96-well PCR Reaction Plates, low profile** (Cat. No. 352134)
These clear plates, which contain conical wells, are recommended for sample preparation.
- **Microlite 1 Luminescence Microtiter 96-well plates, Thermo Electron, Flat-Bottom** (VWR Scientific Products, Cat. No. 62403-124).
These opaque white plates, which contain flat-bottom wells, are recommended for chemiluminescent assays and detection.
- **AluminaSeal™ PreCut Adhesive Foil Sealing Film** (Diversified Biotech, Cat. No. ALUM-1000). This film is recommended for sealing both types of 96-well plates.
- Luminometer (for chemiluminescent assays) or fluorometer of choice.

III. Experimental Design and Sample Preparation

PLEASE READ THROUGH ENTIRE PROTOCOL BEFORE BEGINNING

A. Transfecting Mammalian Cells with SEAP Expression Vectors

1. Transfection techniques

The pSEAP2 Vectors may be transfected into mammalian cells by a variety of techniques. Clontech recommends using our high-efficiency transfection reagent, Xfect™ (Cat. Nos. 631317 & 631318). This reagent is compatible with both SEAP assays, but the procedure of choice will depend primarily on the type of cell being transfected. Different cell lines may vary by several orders of magnitude in their ability to take up and express exogenous DNA. Moreover, a method that works well for one type of cultured cell may be inferior for another. When working with a cell line for the first time, compare the efficiencies of several transfection protocols using the pSEAP2-Control Vector as described in Section III.B.

2. Transfection considerations

a. Perform transfections in triplicate

Each different construct should be transfected (and subsequently assayed) in triplicate to minimize variability among treatment groups. The primary sources of such variability are differences in transfection efficiencies.

b. Normalize for transfection efficiency

When monitoring the effect of promoter and enhancer sequences on gene expression, it is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection (Sambrook & Russell, 2001). This is easily done by cotransfecting a second plasmid that constitutively expresses an activity that can be clearly differentiated from SEAP. The level of the second enzymatic activity can then be used to normalize the levels of SEAP among different treatment groups. We recommend using the Ready-To-Glow™ Dual Secreted Reporter System (Cat. Nos. 631734 & 631735) for easy normalization. This system includes the same SEAP 2.0 vectors and assay reagents, plus vectors and assay reagents to measure *Metridia* secreted luciferase activity.

III. Experimental Design and Sample Preparation *continued*

Note on effects of SV40 large T antigen (COS cells): The specific level of expression for the pSEAP2 Vectors is likely to vary in different cell types. This is particularly true for cell lines containing the SV40 large T antigen, such as COS cells. The large T antigen promotes replication of the SV40 origin, sequences that are found in the promoter region of the pSEAP2-Control Vector. The combination of the large T antigen and SV40 origin leads to a higher copy number of these vectors in COS cells, which in turn may result in increased expression of the SEAP reporter gene relative to vectors lacking the SV40 origin.

B. Proper Use of Controls

1. Negative controls

A negative control is necessary to measure the background signal associated with the cell culture media. This can be determined by assaying 25 μ l of culture medium from cells transfected with the pSEAP2-Basic Vector, which contains the SEAP gene without a promoter or enhancer. The values obtained from such controls should be subtracted from experimental results.

2. Positive controls

a. Positive control for transfection and expression of exogenous DNA

A positive control is necessary to confirm transfection and expression of exogenous DNA and to verify the presence of active SEAP in the culture media. Expression and secretion of functional SEAP in transfected cells can be confirmed by assaying 25 μ l of culture medium from cells transfected with the pSEAP2-Control Vector, which contains the SEAP structural gene under transcriptional control of the SV40 promoter and enhancer. Cells transfected with this plasmid should yield high activity within 48–72 hours after transfection.

b. Positive control for detection method

The provided Positive Control Placental Alkaline Phosphatase can be used to confirm that the detection method is working. To do this, simply add 2 μ l of the Positive Control Alkaline Phosphatase to 23 μ l of culture medium from untransfected cells. This should yield a strong positive signal. A dilution series of the positive control enzyme can also be used to determine the linear range of the assay.

3. Normalizing transfection efficiencies

It is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection. See Section IIIA.2.b or Sambrook & Russell (2001) for more information.

III. Experimental Design and Sample Preparation *continued*

C. Staying Within the Linear Range of the Assay

It is important to stay within the linear response range of the assay. The linear range can be determined by assaying a dilution series of the Positive Control Placental Alkaline Phosphatase. High intensity signals can exceed the optimal range of detection instruments, resulting in incorrect, low readings. In addition, low intensity signals that are near background levels may fall outside the linear response range of the assay. Therefore, the target amount of SEAP in the assay should be adjusted to bring the signal within the linear range. For signals that are too intense, this can be achieved by diluting the cell culture media prior to assay. For low signals, the amount of SEAP may be increased by improving the transfection efficiency, starting with a greater number of cells, or increasing the volume of media assayed.

D. Sample Preparation for Chemiluminescent and Fluorescent SEAP Assays

For transient transfection assays, SEAP is generally detected in the cell culture medium 12–18 hr after transfection, with maximal levels detected 48–72 hours after transfection. This range is suggested only as a starting point, as optimal times for collecting samples will vary for different cell types, cell densities, and the nature of the particular experimental conditions.

The following procedure should be used to prepare samples of conditioned medium for use in both chemiluminescent and fluorescent assays. It is suitable for use with adherent cells and cells in suspension.

1. Collect 25 μ l of conditioned cell culture medium (in triplicate) and transfer to microcentrifuge tubes (or a fresh 96-well plate). Use 96-well plates with conical wells (see Section II) for sample preparation.

Note: For chemiluminescence assays, we recommend Microlite™ 1 Luminescence Microtiter 96-well plates (VWR Scientific Products, Cat. No. 62403-124).

2. Centrifuge at 12,000 \times g for 30 sec to pellet any cells present in the culture medium.

3. Transfer supernatant to a fresh microcentrifuge tube or 96-well plate.

Note: If necessary, the plate can be stored for future analysis. Cap tubes or cover plates with adhesive aluminum foil and store at -20°C until you are ready to perform the chemiluminescence or fluorescence assay (Parts IV and V, respectively).

IV. Chemiluminescent SEAP Assay

A. Chemiluminescent SEAP Assay Protocol

As noted in Section III, each construct should be transfected and subsequently assayed in triplicate. It may be necessary to dilute some samples in order to stay within the linear range of the assay. The linear range can be determined by assaying a dilution series of Positive Control Placental Alkaline Phosphatase (see Section III.C).

1. Prepare 1X Dilution Buffer by diluting the 5X Dilution Buffer 1:5 with ddH₂O.
2. Allow the SEAP Substrate Solution to equilibrate to room temperature (22–25°C).
3. Add 75 µl of 1X Dilution Buffer to each sample (from Step III.D.3) in the 96-well microtiter plate.
4. Seal the plate with adhesive aluminum foil or a regular 96-well lid and incubate the diluted samples for 30 min at 65°C using a heat block or water bath. Seal well and use extra care when incubating in a water bath.
5. Cool the samples on ice for 2–3 min, then equilibrate to room temperature.
6. Add 100 µl of SEAP Substrate Solution to each sample. Incubate for 10–60 min (30 min is recommended) at room temperature before reading.

IV. Chemiluminescent SEAP Assay *continued*

B. Chemiluminescence Detection Methods

1. Detection using a tube luminometer

If the assay is performed in a tube suitable for luminometer readings the sample may be placed directly in the instrument after Step IV.A.6 and measurements taken following a minimum 10 min incubation. If the assay is not performed in a suitable tube, transfer the entire solution from Step A.9 to the appropriate luminometer tube and place in the instrument. Record light signals as 5 to 15 sec integrals.

2. Detection using a plate luminometer

- a. If the assay was not performed in a luminometer-compatible microtiter plate, transfer the entire solution from each well in Step IV.A.6 to a suitable plate and place it in the instrument.
- b. Record light signals with the manufacturer's recommended luminometer settings.

3. Detection by exposing x-ray film

If a luminometer is not available, light emission may be recorded by exposure of x-ray film to opaque white 96-well flat-bottom microtiter plates containing chemiluminescent SEAP assay samples (see Section III). This detection procedure yields spots on the film, which can be quantitated by comparison to positive and negative control incubations.

- a. Perform the entire SEAP assay in opaque white 96-well flat-bottom microtiter plates.
- b. After at least a 10 min incubation at Step IV.A.6, place the microtiter plate over a piece of x-ray film, cover the plate and film with Saran wrap, and place a heavy object such as a book on top to hold the film in place.
- c. Expose the film for 5–30 min at room temperature.

Note: For comparisons between samples, it is critical to remain within the linear response range of the x-ray film. In order to avoid misleading results, it is recommended that several different film exposure times be utilized for each microtiter plate.

V. Fluorescent SEAP Assay

A. Fluorescent SEAP Assay Protocol

As noted in Section III, each construct should be transfected and subsequently assayed in triplicate. It may be necessary to dilute some samples in order to stay within the linear range of the assay. The linear range can be determined by assaying a dilution series of the Positive Control Placental Alkaline Phosphatase (see Section III.C).

Note: This format is for 96-well plates. If performing the assay in microcentrifuge tubes, the volumes can be increased.

1. Allow a enough Assay Buffer (see Step 7) for performing the entire experiment to equilibrate to room temperature.
2. Prepare the required amount of 1X Dilution Buffer (see Steps V.A.4, 8 & 9) by diluting a sufficient volume of the provided 5X stock solution 1:5 with ddH₂O just prior to use and allowing it to equilibrate to room temperature.
3. Thaw samples of cell culture medium, and place 25 µl of each sample into a separate well of a 96-well plate.
4. Add 25 µl of 1X Dilution Buffer to each 25 µl sample and mix gently using a vortex mixer with a plate adaptor.
5. Seal the sample wells of 96-well plates with adhesive aluminum foil (See Section II), and then incubate the diluted samples for 30 min in a 65°C incubator. (If you are performing the assay in microcentrifuge tubes, you can use a heating block or water bath.)
6. Cool samples to room temperature by placing on ice for 2–3 min, and then equilibrating to room temperature.
7. Add 97 µl of Assay Buffer to each sample well and incubate for 5 min at room temperature.
8. Prepare enough of a 1 mM MUP working dilution for your experiment (see Step V.A.9) by diluting the a sufficient volume of the provided 10 mM MUP Fluorescent Substrate 1:10 in 1X Dilution Buffer just prior to use.
9. Add 3 µl of the 1 mM MUP working dilution to each sample, and incubate for 60 min **in the dark** at room temperature.

V. Fluorescent SEAP Assay *continued*

B. Fluorescence Detection Methods

The excitation and emission peaks of MUP fluorescence are 360 nm and 449 nm, respectively.

1. Detection using a plate fluorometer

If the assays are performed in a 96-well flat-bottom microtiter plate suitable for plate fluorometers (i.e., black walls, transparent bottom), fluorescence can be measured directly using a PerSeptive Biosystems Cytofluor II Fluorescence Multiwell Plate Reader with the gain set at 58–68.

2. Detection using a tube fluorometer

If the assay is performed in a suitable tube for fluorometer readings, the sample may be placed directly in the instrument after Step V.A.9. Otherwise, transfer the entire solution from Step V.A.9 to an appropriate tube and place in the instrument.

VI. Troubleshooting Guide

A. Determining the Linear Range of the Assay

If in doubt about the linear range of the assay, prepare and assay a dilution series using the Positive Control Placental Alkaline Phosphatase.

B. Little or No Signal From Transfected Cells

1. Ensure that the assay conditions are correct and that the detection method is working by assaying the positive control enzyme.
2. Ensure that the transfection efficiency has been optimized by using pSEAP2-Control (or a suitable alternative) as an internal positive control for SEAP expression.
3. Increase the number and/or density (or concentration) of cells used in transfections.
4. If background signals from negative controls (i.e., cells transfected with pSEAP2-Basic) are low, increase the volume of media assayed from experimental cultures from 25 μ l to 50–75 μ l. (The 1X Dilution Buffer added at the next step should be adjusted accordingly.)
5. Increase the post-transfection interval prior to collecting media samples.
6. If you are concerned about potential inhibitory activity in your sample, collect an additional 25 μ l of conditioned media (Step III.D.1), add 2 μ l of Positive Control Placental Alkaline Phosphatase and continue with the Sample Preparation and SEAP Assay protocols (Parts III and either IV or V).
7. If assaying with x-ray film, try increasing the film exposure time.
8. For detection via a tube or plate luminometer, refer to the instrument instructions for methods to increase the sensitivity of light detection.

C. High Background Signals

1. Ensure that all intact cells and cellular debris are removed from the conditioned media by centrifugation in Step III.D.2. This step is particularly important for suspension cultures.
2. Ensure that the diluted media samples are heated for the full 30 min at 65°C as specified in Step IV.A.4 or V.A.5.
3. If the signal is too high, the volume of media assayed from experimental cultures can be reduced.
4. Alternatively, media supernatant can be diluted with fresh complete serum-containing or serum-free media.
Note: If diluting with fresh serum-containing media, please ensure heat inactivation of any endogenous alkaline phosphatase by incubation at 65°C for 30 min.
5. If possible, after transfection grow cells in media containing minimal serum. Serum levels >10% (v/v) may increase background.

VI. Troubleshooting Guide *continued*

D. Signal is too High, Exceeding the Linear Range of the Assay

1. This problem is easily corrected by either assaying a lower volume of conditioned medium, or by diluting the samples using fresh complete serum-containing or serum-free media.

Note: If diluting with fresh serum-containing media, please ensure heat inactivation of any endogenous alkaline phosphatase by incubation at 65°C for 30 min.

2. One can perform a time course after induction by collecting and assaying media supernatant at different time points to find a suitable time point at which the signal falls in the linear range.

E. Recommended Sequencing Primers

We recommend the following primers for sequencing inserts cloned into the pSEAP2 Vectors. (These primers are not available from Clontech.)

To sequence from the TB region into the MCS:

5'-CTAGCAAATAGGCTGTCCC-3'
(5057–5076 in pSEAP2-Control)

To sequence from the 5' region of the SEAP ORF into the MCS:

5'-CCTCGGCTGCCTCGCGTTCC-3'
(376–356 in pSEAP2-Control)

To sequence fragments inserted into restriction sites downstream of the SEAP ORF:

5'-GCCTTCGCCGCCTGCCTGGAG-3'
(1708–1728 in the 3' region of the SEAP gene)

VII. References

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VIII. Related Products

For a complete listing of all Clontech products,
please visit www.clontech.com.

Appendix A: Plasmid Maps & Multiple Cloning Sites

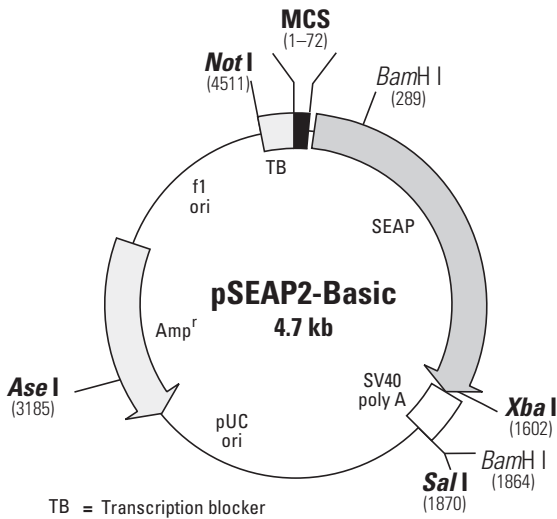


Figure 2. Map and multiple cloning site of pSEAP2-Basic. pSEAP2-Basic lacks eukaryotic promoter and enhancer sequences. The MCS allows promoter DNA fragments to be inserted upstream of the SEAP gene. Enhancers can be cloned into either the MCS or unique downstream sites. Unique restriction sites are in bold.

The Great EscAPe vectors contain the SV40 late polyadenylation signal inserted downstream of the SEAP coding sequences to ensure proper and efficient processing of the transcript in eukaryotic cells. A synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites, reduces background transcription (Eggermont & Proudfoot, 1993). The vector backbone contains an f1 origin for single-stranded DNA production and a pUC19 origin of replication and an ampicillin resistance gene for propagation in *E. coli*. The multiple cloning site region is identical in both pSEAP2-Basic & pSEAP2-ControlVectors except for a 209 bp promoter fragment that has been inserted between the Bgl II and Hind III sites in pSEAP2-Control.

The complete sequence information for the Great EscAPe SEAP2 Vectors can be downloaded from our web site at www.clontech.com.

Appendix A: Plasmid Maps & MCSs continued

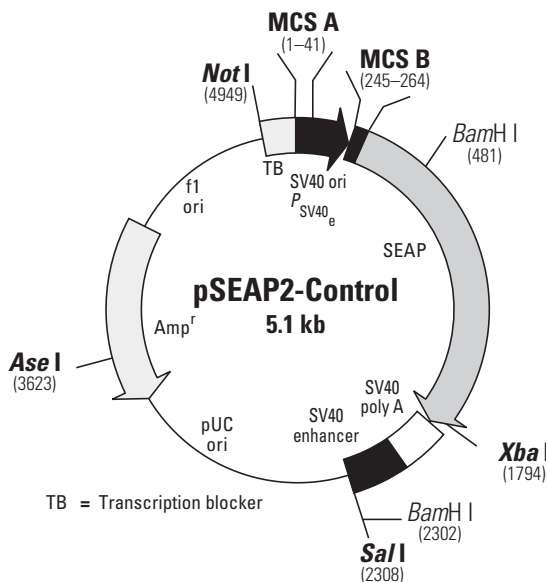


Figure 3. Map and multiple cloning site of pSEAP2-Control. pSEAP2-Control is pSEAP2-Basic with the SV40 early promoter inserted upstream of the SEAP gene and the SV40 enhancer inserted downstream. pSEAP2-Control expresses SEAP in most cell types and provides an important positive control in most experiments. Unique restriction sites are in bold.

See the description of pSEAP2-Basic (Figure 2) for information about elements common to both Great EscAPe vectors.

The complete sequence information for the Great EscAPe SEAP2 Vectors can be downloaded from our web site at www.clontech.com.

Notes

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