USER GUIDE

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BsdCassette™ Vector

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

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Contents

Kit Contents and Storage

Shipping and Storage	The BsdCassette™ vector is shipped at room temperature. Store at –20°C.
Kit Contents	This manual is supplied with the BsdCassette™ vector (pCMV/Bsd). The vector (20 µg) is supplied at 0.5 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 in a total volume of 40 µL.
Blasticidin	Blasticidin is available separately for purchase. See page 11 for ordering information.
Product Use	**For research use only. Not intended for any human or animal therapeutic or diagnostic use.**

Introduction

Product Overview

Description of the System

The BsdCassette™ vector (pCMV/Bsd) is a small plasmid (3.6 kb) that contains a blasticidin expression cassette (BsdCassette™) flanked by 5´ and 3´ polylinkers. The BsdCassette™ contains two promoters and the resistance factor to the antibiotic blasticidin. Expression of the resistance factor is driven by a bacterial promoter (EM7) for expression in *E. coli*. In addition, the vector contains the mammalian promoter CMV upstream of the EM7 promoter for expression in mammalian cells.

Applications

The BsdCassette™ vector can be used for the following applications:

- The BsdCassette™ can be excised and transferred to the vector of choice to confer resistance to blasticidin.
- The vector can be used as a backbone to develop your own vector of choice.

Methods

Cloning into Polylinker

5´ Polylinker

If you wish to use the BsdCassette™ vector as a backbone for developing your own vector, use the diagram below to clone additional elements into the 5´ polylinker. Restriction sites are labeled to indicate the cleavage site. Refer to the map of the BsdCassette™ vector to identify available restriction sites (see the Appendix, pages 10 for a map of the BsdCassette™ vector). The nucleotide sequence for the BsdCassette™ vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 12).

```
                                  Apa I  Sfi I Nae I  Bgl II                Not I       EcoR V
1871 TGCTCACATG CTGGGCCCAG CCGGCCAGAT CTGAGCTCGC GGCCGCGATA
        Nhe I  Xho I              5´ end of CMV promoter
1921 TCGCTAGCTC GAGGTCCGTT ACATAACTTA CGGTAAATGG CCCGCCTGGC
```

3´ Polylinker

If you wish to clone additional elements into the 3´ polylinker, use the diagram below. Restriction sites are labeled to indicate the cleavage site. Refer to the map for the BsdCassette™ vector to identify available restriction sites (see the Appendix, pages 10 for a map of the BsdCassette™ vector). The nucleotide sequence for the BsdCassette™ vector is available for downloading from www.lifetechnologies.com or by contacting Technical Support (see page 12).

3´ Poly linker of pCMV/Bsd:

```
                SV40 early polyadenylation sequence
3159    GTGGTTTGTC CAAACTCATC AATGTATCTT ATCATGTCT
        EcoR I  Xma I Sma I BamH I Xba I   Sal I Acc I      Pst I    Sph I Hind III
3198      GAATTCC CGGGGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG

3245    GCACTGGCCG TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC

3295    CCAACTTAAT CGCCTTGCAG CACATCCCCC TTTCGCCAGC TGGCGTAATA

3345    GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG CAGCCTGAAT
             Kas I  Nar I
3395    GGCGAATGGC GCCTGATGCG GTATTTTCTC CTTACGCATC TGTGCGGTAT...
```

Selection in *E. coli*

Introduction

The following section contains guidelines for maintenance, propagation, and selection of the BsdCassette™ vector in *E. coli*.

E. coli Strain

Many *E. coli* strains are suitable for the propagation of the BsdCassette™ vector including TOP10 and TOP10F´. We recommend that you propagate the BsdCassette™ vector in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A).

For your convenience, TOP10 *E. coli* are available as chemically competent or electrocompetent cells for purchase (see page 11 for ordering).

E. coli Transformation

You may use any method of choice for transformation. Chemical transformation is the most convenient for many researchers. Electroporation is the most efficient and the method of choice for large plasmids.

Maintenance of BsdCassette™ Vector

The BsdCassette™ vector contains the ampicillin and blasticidin resistance genes to allow selection of the plasmids using ampicillin or blasticidin (see pages 9–10 for more information about the BsdCassette™ vector). For more information about blasticidin, refer to pages 7–8.

To propagate and maintain the BsdCassette™ vector, we recommend using the following procedure:

1. Use the supplied 0.5 µg/µL stock solution in TE, pH 8.0 to transform a *rec*A, *end*A *E. coli* strain like TOP10, TOP10F´, or equivalent.
2. Select transformants on LB agar plates containing 50–100 µg/mL ampicillin or 100 µg/mL blasticidin in Low Salt LB (see the next page for a recipe).
3. Prepare a glycerol stock of the plasmid for long-term storage (see the next page for a protocol).

For fast and easy microwaveable preparation of Low Salt LB agar containing ampicillin, imMedia™ Amp Agar is available for purchase (see page 11 for ordering).

Selection in *E. coli*

To facilitate selection of blasticidin resistant *E. coli*, the salt concentration of the medium must remain low (< 90 mM) and the pH must be 7.0. Prepare LB broth and plates using the recipe on the next page.

Failure to lower the salt content of your LB medium will result in non-selection due to inhibition of the drug unless a higher concentration of blasticidin is used.

Continued on next page

Selection in *E. coli*, Continued

Low Salt LB Medium

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 7.0 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 blasticidin to 100 µg/mL final concentration.
4. Store plates at 4°C in the dark. Plates containing blasticidin are stable for up to 2 weeks.

Analysis of Transformants

Plasmid DNA should be isolated from blasticidin-resistant transformants and analyzed to confirm the resulting construct.

- Select 10–20 transformants and isolate plasmid DNA.
- Analyze the DNA by restriction mapping or sequencing.
- After you have identified positive transformants, be sure to prepare a glycerol stock of each plasmid for long-term storage (see below for a protocol).

Preparing a Glycerol Stock

After you have identified transformants containing your plasmid of interest, be sure to purify the colony and make a glycerol stock for long-term storage. It is also a good idea to keep a DNA stock of your plasmid at –20°C.

1. Streak the original colony out on an LB plate containing 50 µg/mL ampicillin or 100 µg/mL blasticidin in Low Salt LB. Incubate the plate at 37°C overnight.
2. Isolate a single colony and inoculate into 1–2 mL of LB containing 50 µg/mL ampicillin or 100 µg/mL blasticidin in Low Salt LB.
3. Grow the culture to mid-log phase (OD_{600} = 0.5–0.7).
4. Mix 0.85 mL of culture with 0.15 mL of sterile glycerol and transfer to a cryovial.
5. Store at –80°C.

Selection in Mammalian Cells

Introduction

Before transfecting cells with the vector containing the BsdCassette™, it is important to test the sensitivity of your mammalian host cell line to blasticidin as natural resistance varies among cell lines. We recommend performing a 'kill curve' to determine the minimal amount of blasticidin that will prevent growth of your untransfected host cell line. **Refer to Determination of Blasticidin Sensitivity** below.

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 lipid complexing, decreasing transfection efficiency. We recommend isolating DNA using the PureLink® HiPure Miniprep Kit or the PureLink® HiPure Midiprep Kit (see page 11 for ordering information) or CsCl gradient centrifugation.

Determination of Blasticidin Sensitivity

To obtain a stable integrant, you need to determine the minimum concentration of blasticidin required to kill your untransfected host cell line. Typically, concentrations between 2–10 µg/mL blasticidin are sufficient to kill the untransfected host cell line. Test a range of concentrations (see below) to ensure that you determine the minimum concentration of blasticidin necessary to prevent growth of your untransfected cell line. Refer to pages 7–8 for instructions on how to prepare and store blasticidin. Use the guidelines below to perform a kill curve.

1. Plate or split a confluent plate so the cells will be approximately 25% confluent in a 60 mm or 100 dish. Prepare a set of 6 plates. **Note**: Other culture vessels may be used. Remember to adjust the number of cells as necessary.
2. The next day, substitute culture medium with medium containing varying concentrations of blasticidin (e.g. 0, 1, 3, 5, 7.5, and 10 µg/mL blasticidin).
3. Replenish the selective media every 3–4 days, and observe the percentage of surviving cells.
4. Count the number of viable cells at regular intervals to determine the appropriate concentration of blasticidin that prevents growth within 1-2 weeks after addition of the antibiotic.

Methods of Transfection

For established cell lines (e.g. HeLa, COS-1), consult original references or the supplier of your cell line for the optimal method of transfection. We recommend 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).

Methods for transfection include calcium phosphate (Chen and Okayama, 1987; Wigler *et al*., 1977), lipid-mediated (Felgner *et al*., 1989; Felgner and Ringold, 1989) and electroporation (Chu et al., 1987; Shigekawa and Dower, 1988). For high efficiency transfection in a broad range of mammalian cells, use Lipofectamine® 2000 Reagent available for purchase (page 11 for ordering). For more information on Lipofectamine® 2000 and other transfection reagents, visit www.lifetechnologies.com or contact Technical Support (page 12).

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Linearizing Vector for Stable Integration

To obtain stable transfectants, we recommend that you linearize your BsdCassette™-based vector before transfection. While linearizing your vector may not improve the efficiency of transfection, it increases the chances that the vector does not integrate in a way that disrupts the gene of interest or other elements required for expression in mammalian cells. If you decide to linearize your vector, use restriction enzymes that digest the DNA at sites within the non-eukaryotic DNA sequences of your vector.

Selection of Stable Integrants

Once you have determined the appropriate concentration of blasticidin to use for selection, you can generate a stable cell line with your BsdCassette™ construct. The transfection method and conditions will vary from cell line to cell line. The guidelines listed below are for transfection in a 100 mm plate. Other culture vessels may be used.

1. Transfect 10^6 cells with 20 µg of plasmid using the desired protocol. Remember to include a plate of untransfected cells as a negative control.
2. 24 hours after transfection, wash the cells once with 1X PBS and add fresh medium to the cells.
3. 48 hours after transfection, split the cells into fresh medium containing blasticidin at the pre-determined concentration required for your cell line. Split the cells such that they are no more than 25% confluent. If the cells are too dense, the antibiotic will not kill the untransfected cells. Antibiotics work best on actively dividing cells.
4. Feed the cells with selective medium every 3–4 days until foci can be identified.
5. Pick and expand at least 20 foci to test for expression of the protein of interest.

Troubleshooting

No Stable Transfectants Selected

Try the ideas below if you are unsuccessful in isolating stable transfectants.

1. If you excised the CMV BsdCassette™, and subcloned it into another vector, try transfecting your cell line with the original parent vectors (pCMV/Bsd) as a positive control.
2. If the parent vector fails to confer blasticidin resistance to your cell line, it may be that the promoter is not active in that cell line. You may try subcloning the blasticidin resistance gene behind another eukaryotic promoter that you know works in your cell line and testing for blasticidin resistance.
3. Check transfection efficiencies. Perform a transient transfection with a reporter construct to verify the transfection efficiency of your cell line.
4. Linearize construct before transfection. Integration may be occurring at the *bsd* gene.

Appendix

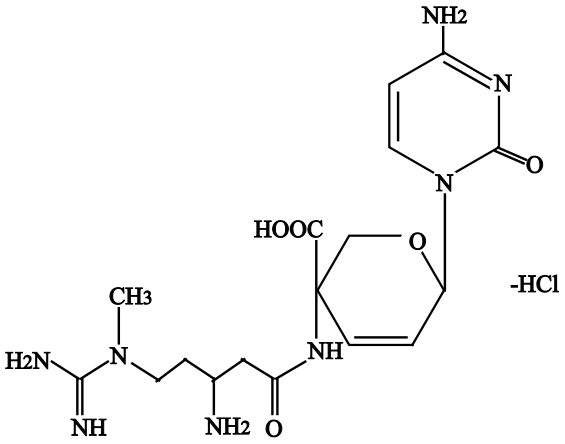
Blasticidin

Blasticidin

Blasticidin S HCl is a nucleoside antibiotic isolated from *Streptomyces griseochromogenes* which inhibits protein synthesis in both prokaryotic and eukaryotic cells (Takeuchi *et al*., 1958; Yamaguchi *et al*., 1965). Resistance is conferred by expression of either one of two blasticidin S deaminase genes: *bsd* from *Aspergillus terreus* (Kimura et al., 1994) or *bsr* from Bacillus cereus (Izumi *et al*., 1991). These deaminases convert blasticidin S to a non-toxic deaminohydroxy derivative (Izumi *et al*., 1991).

Molecular Weight, Formula, and Structure

The formula for blasticidin S is $C_{17}H_{26}N_8O_5$-HCl, and the molecular weight is 458.9. The diagram below shows the structure of blasticidin.



Handling Blasticidin

Always wear gloves, mask, goggles, and protective clothing (e.g. a laboratory coat) when handling blasticidin. Weigh out blasticidin and prepare solutions in a hood.

Continued on next page

Blasticidin, Continued

Preparing and Storing Stock Solutions

Blasticidin is available for purchase in 50 mg aliquots (see page 11 for ordering). Blasticidin is soluble in water. Sterile water is generally used to prepare stock solutions of 5–10 mg/mL.

- Dissolve blasticidin in sterile water and filter-sterilize the solution.
- Aliquot in small volumes suitable for one time use (see next to last point below) and freeze at –20°C for long-term storage or store at 4°C for short-term storage.
- Aqueous stock solutions are stable for 1–2 weeks at 4°C and 6–8 weeks at -20°C.
- pH of the aqueous solution should not exceed 7.0 to prevent inactivation of blasticidin.
- Do not subject stock solutions to freeze/thaw cycles (**do not store in a frost-free freezer**).
- Upon thawing, use what you need and discard the unused portion.
- Medium containing blasticidin may be stored at 4°C for up to 2 weeks.

Suggested Concentrations of Blasticidin

Blasticidin and the *bsd* gene are used for selection in mammalian cells, yeast, and prokaryotes (Takeuchi *et al*., 1958; Yamaguchi et al., 1965). Suggested concentrations of blasticidin to use for selection in *E. coli* and mammalian cells are listed below.

Organism	Blasticidin Concentration
E. coli	100 µg/mL in Low Salt LB* (see page 3)
Mammalian cells	2–10 µg/mL (depending on the cell line)

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

Features of BsdCassette™ Vector

Features

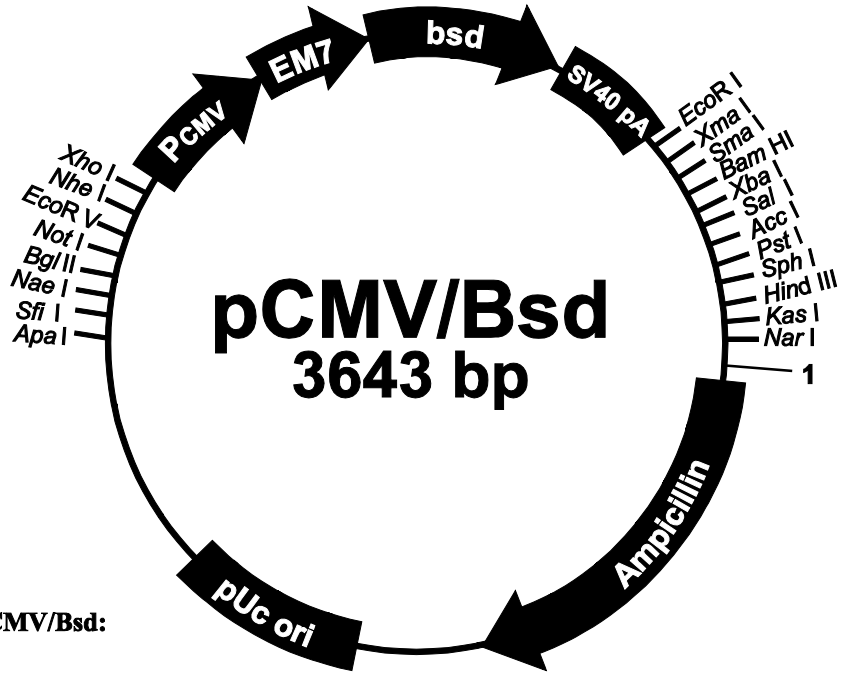
The table below describes the features of the BsdCassette™ vector. All features have been functionally tested and the vector completely sequenced.

Features	Benefit
5´ polylinker	Allows excision of the BsdCassette™ or subcloning of a heterologous promoter into pEM7/Bsd.
CMV promoter	Allows expression of the blasticidin resistance gene in mammalian cells. This is the human CMV immediate-early promoter (Towne strain) without intron A (Stenberg *et al.*, 1984; Thomsen *et al.*, 1984).
EM7 promoter	Synthetic promoter based on the bacteriophage T7 promoter for expression of the blasticidin resistance gene in *E. coli.*
Blasticidin (*bsd*) resistance gene	Allows selection by blasticidin in *E. coli*, yeast, plants, and mammalian hosts (Kimura *et al.*, 1994; Kimura and Yamaguchi, 1996).
SV40 early polyadenylation sequence	Allows efficient transcription termination and polyadenylation of mRNA in mammalian cells.
3´ polylinker	Allows excision of the BsdCassette™ or subcloning of additional elements.
bla promoter	Allows expression of the ampicillin (*bla*) resistance gene.
Ampicillin (*bla*) resistance gene	Allows selection of transformants in *E. coli.*
pUC origin	Allows high-copy number replication and maintenance in *E. coli.*

Map of pCMV/Bsd Vector

Map of pCMV/Bsd

pCMV/Bsd is a 3643 bp plasmid that expresses the blasticidin resistance gene using the bacterial EM7 promoter or the human CMV immediate-early promoter. The figure below summarizes the features of the pCMV/Bsd vector including the polylinkers which can be used to excise the BsdCassette™ or clone in additional elements. The vector sequence of pCMV/Bsd is available for downloading from www.lifetechnologies.com/support or by contacting Technical Support (page12).



Comments for pCMV/Bsd:
3643 nucleotides

bla promoter: bases 102-200
Ampicillin (*bla*) resistance gene: bases 201-1061
pUC origin: bases 1206-1879
5′ polylinker: bases 1883-1933
CMV promoter: bases 1939-2557
EM7 promoter: bases 2561-2627
Blasticidin (*bsd*) resistance gene: bases 2628-3026
SV40 early polyadenylation sequence: bases 3068-3198
3′ polylinker: bases 3198-3407

Accessory Products

Additional Products

The following additional products may be used with the BsdCassette™ vectors. For more information, visit www.lifetechnologies.com/support or contact Technical Support (see page 12).

Item	Quantity	Cat. no.
One Shot® TOP10 Chemically Competent *E. coli*	20 reactions	C4040-03
Electrocomp™ TOP10 (electrocompetent *E. coli*)	20 reactions	C664-55
imMedia™ Amp Agar	20 each	Q601-20
Lipofectamine® 2000 Reagent	1.5 mL	11668-019
PureLink® HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink® HiPure Plasmid Midiprep Kit	25 preps	K2100-04
Blasticidin	50 mg, powder	R210-01

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

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