pSFV1 Eukaryotic Expression Vector

Cat. No.: 18448-019 **Size: 10 µg**

Conc.: 500 µg/ml Store at -20°C.

Description:

The pSFV1 Eukaryotic Expression Vector is a novel DNA expression system based on the Semliki Forest Virus (SFV) replicon which has a broad host range and a high level of expression efficiency (1). In contrast to other protein expression systems, the SFV expression system has several advantages, including direct cloning into the vector and proper glycosylation of the recombinant protein. The SFV expression system is easy to use. The DNA of interest is cloned into the pSFV1 plasmid vector that serves as a template for *in vitro* synthesis of recombinant RNA, and the RNA is then transfected into tissue culture cells with DMRIE-C Reagent, Lipofectin® Reagent or by electroporation. The recombinant RNA in the cell drives its own replication and capping, resulting in production of large amounts of heterologous protein while inhibiting host protein synthesis. High transfection efficiencies and expression of heterologous proteins have been achieved by transfection of pSFV1 *in vitro* transcribed RNA into CHO-K1 and BHK-21 cells (2). The plasmid pSFV3-*lacZ* contains the *E. coli* β-galactosidase gene, *lacZ*, and is included as a control plasmid to optimize transfection conditions.

Notes:

1. Caution: The gene of interest cloned into pSFV1 should not contain an *Spe* I restriction site since *Spe* I is used to linearize the recombinant plasmid prior to the *in vitro* RNA transcription reaction. If the gene of interest does contain a *Spe* I site, *Sap* I may be used as an alternate linearization site.
2. The gene that is cloned into pSFV1 must contain a signal for initiation of translation (Kozak sequence [3] and AUG). Alternatively, pSFV3 (1) can be used as a cloning vector: pSFV3-*lacZ* is pSFV3 with the *E. coli lacZ* gene cloned into the pSFV3 *Bam*H I cloning site.
3. The pSFV1 vector contains 72% of the wild type SFV genome (1).
4. All procedures employing the pSFV1 Eukaryotic Expression Vector must comply with NIH recombinant DNA and biosafety guidelines.

Components:

	Part No.	Amount
Plasmid pSFV1	Y01571	20 µl
Plasmid pSFV3-lacZ	Y01572	20 µl

Storage Buffer:

10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA

Quality Control:

Purity, DNA structure and selected restriction endonuclease sites are verified by agarose gel electrophoresis.

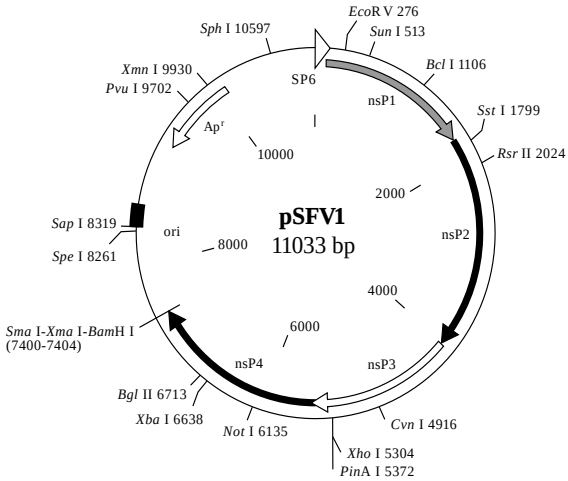
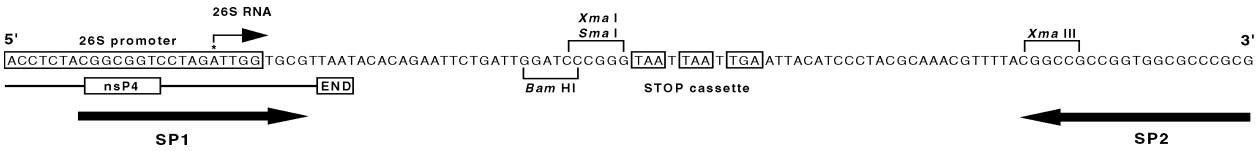


FIGURE 1:. Map and restriction sites for pSFV1 Eukaryotic Expression Vector. The Multiple Cloning Site (MCS) contains a *Bam*H I - *Sma* I - *Xma* I polylinker cassette. Restriction endonucleases that cleave pSFV1 once are shown on the outer circle. The nucleotide position refers to the 5′ base of the recognition sequence.



The sequence has not been confirmed by sequence analysis. It was assembled from the known sequence of fragments used to construct the vector. Vector sequences, restriction information, and maps can be found in the Vector Data area of our web site, www.invitrogen.com

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FIGURE 2: Polylinker region of pSFV1 (1). The position of the promoter for the subgenomic 26S RNA is boxed and the first nucleotide transcribed is indicated by an asterisk. The *Bam*H I - *Sma* I - *Xma* I polylinker cassette is situated 31 bases downstream of the transcription initiation site. The three translation stop codons in all three reading frames following the polylinker are boxed. Sequencing primers (SP) to check both ends of an insert can be designed to hybridize either to the promoter region (SP1), or to the region following the stop codon cassette (SP2).

NOTE: The *Xma* III site is not unique.

Enzymes which do not cut pSFV1:

Apa I	*Avr* II	*Bsm* I	*Bss*H II	*Bst*B I	*Cla* I
Esp I	*Nde* I	*Nru* I	*Nsi* I	*Sfi* I	

Cloning Procedure**:**

The pSFV1 Eukaryotic Expression Vector contains three unique cloning sites (*Bam*H I, *Sma* I, and *Xma* I). DNA fragments cloned into these sites must encode their own Kozak sequence and AUG translation initiation site. If the cloned sequence does not contain the required sequences for initiation of translation, pSFV3 may be used as a cloning vector (pSFV3 may be generated from pSFV3*lac*Z after deleting the *lac*Z fragment with *Bam*H I). The distance between the 5' end of the inserted sequence and the initiation codon is not critical (sequences up to 1 kb from the initiation site can be expressed); however, it is suggested to keep this distance as small as possible (<100 bp). The *Spe* I linearization site must be maintained in its position. *Sap* I may be used as an alternate linearization site. DH5α™ are competent cells and are the recommended *E. coli* host. Plasmid preparations may be grown under optimal conditions using Terrific Broth (4) containing 0.4% glycerol and 100 µg/ml ampicillin. Plasmid instability is infrequently encountered. Any instability problems may be minimized by growing *E. coli* clones at 30°C or by using the *E. coli* strain Stbl2™.

Preparation of mRNA *in vitro*: ***All manipulations and reactions should be done using RNase-free reagents****

NOTE: It is recommended to transcribe RNA from the pSFV3-*lac*Z control plasmid first in order to optimize mRNA synthesis and transfection conditions.

1. Linearize 2 to 10 µg of recombinant SFV plasmid DNA with *Spe* I (Cat.No. 15443-013) at 37°C (or optimally at 50°C) for 60 min in a total volume of 20 µl. (Alternatively, *Sap* I may be used). The insert may not contain a recognition site for *Spe* I or *Sap* I.
2. Add 80 µl H_2O and phenol-extract the DNA.
3. Add 10 µl 3 M sodium acetate (pH 4.8-5.4) to the aqueous phase and ethanol precipitate the DNA. Wash and dry DNA pellet. Resuspend DNA pellet in TE [10 mM Tris-HCl, 0.1 mM EDTA (pH 7.5)] to ~0.6 µg/µl.
4. Add the following in a 1.5-ml tube:

20 µl NTP mix*	
10 µl 5X SP6 Buffer (provided with SP6)	
5 µl 10 mM $m^7G(5')ppp(5')G$ RNA Capping Analog	(Cat.No. 15619-018)
5 µl 10 mM DTT (provided with SP6) (mix after adding)	
5 µl Human Placental Ribonuclease Inhibitor (50 units) (mix after adding)	(Cat.No. 15518-012)
2.5 µl linearized pSFV1 recombinant DNA (from step 3) (~1.5 µg)	
2.5 µl SP6 RNA polymerase (~38 units)	(Cat.No. 18018-010)
50 µl Total volume	

* NTP Mix (2.5 mM ATP, 2.5 mM CTP, 2.5 mM UTP, 1.25 mM GTP) is prepared by combining the following in a 1.5-ml tube:

100 µl of 10 mM ATP	(Cat.No. 18330-019)
100 µl of 10 mM CTP	(Cat.No. 18331-017)
100 µl of 10 mM UTP	(Cat.No. 18333-013)
50 µl of 10 mM GTP	(Cat.No. 18332-015)
50 µl of Autoclaved distilled H_2O	
400 µl Total volume (store at -20°C)	

5. Incubate at 37°C for 1-2 h.

6. Agarose gel analysis of *in vitro* transcribed RNA: Add a 2-µl aliquot of RNA to 5 µl of H_2O and add 3 µl of loading buffer. Run 5 µl of the RNA dilution on a 0.5% agarose TBE gel (containing 0.01% ethidium bromide). Use the 1 Kb DNA ladder (Cat.No. 15615-016), or λ DNA/*Hin*d III Fragments (Cat.No. 15612-013) as a size marker and quantitation standard. Run the RNA 2-3 cm into the gel for quantitation and to check quality of the RNA.
   NOTE: RNA transcribed from pSFV3-*lac*Z should migrate alongside a 2.7-kb DNA band.
   pSFV1 Recombinant RNA should be >90% of a single species and should migrate as a distinct band. The amount of RNA recovered should be ~12 to 25 µg per transcription reaction. If RNA yields are low (<12 µg), transfection efficiency will not be optimal (<90%).
   **** RNA is now ready to be used. Prior to cationic lipid-mediated transfection, however, the RNA may need to be further purified by isopropanol precipitation for optimal results.***
7. Isopropanol precipitation of *in vitro* transcribed RNA:
   a. Add 52 µl H_2O to the remaining 48 µl of the transcription reaction (from step 4)
   b. Add 10 µl 3 M sodium acetate (pH 4.8-5.4)
   c. Add 72 µl isopropanol (-20°C) and let stand for 10 min at room temperature
   d. Centrifuge 10 min at 4°C
   e. Wash RNA pellet with 200 µl 70% ethanol. Centrifuge, carefully remove supernatant, dry RNA pellet, and resuspend with 50 µl of TE [10 mM Tris-HCl, 0.1mM EDTA (pH 7.5)].
8. Store RNA at -70°C.

Transfection of Cells with SFV3-*lac*Z RNA Using Cationic Lipid Reagent:

High-efficiency transfection (>90% β-gal positive cells) of SFV3-*lac*Z RNA has been accomplished with the following cell types: BHK-21 (C-13; ATCC, CCL 10), CH0-K1 (ATCC, CCL 61), COS-7 (ATCC, CRL 1651), and HOS (ATCC, CRL 1543) (1,2). Other cell types may also be transfected at high efficiency by optimizing conditions. We recommend optimizing transfection conditions using RNA transcribed from pSFV3-*lac*Z by following the optimization protocol below. For the cell lines listed above, in 35-mm plates or wells, 10 µl of Lipofectin® Reagent and 5-10 µl (~2.5 to 5 µg) of recombinant RNA has given optimal results. In more recent studies, RNA transfections performed with DMRIE-C reagent have resulted in higher transfection efficiencies in BHK-21, COS-7, and CHO-K1 cells (6). Larger tissue culture plates can be used by scaling up all volumes and amounts in proportion to the surface area.

It is recommended that transfection conditions be optimized for each cell type using RNA transcribed from pSFV3-*lac*Z.

1. Plate an appropriate number of cells ($1.5\text{-}3 \times 10^5$) in 35-mm wells of six-well plates in 2 ml of complete growth medium. Cells should be ~80% confluent the following day at the time of transfection.
2. Wash cells in each well with 2 ml of Opti-MEM® I Reduced Serum Medium (room temp.).
3. Preparation of RNA-Cationic Lipid Complexes:

a. Add 1.0 ml Opti-MEM® I (room temp.) to each of six 12 × 75-mm polystyrene tubes.
b. Add DMRIE-C Reagent (0, 3, 6, 9, 12, 15 µl) to each of the tubes containing Opti-MEM® I, and vortex briefly (Mix lipid reagent well before use; see note). (Lipofectin® Reagent can also be used but may not give optimal results).
c. Add 2.5 - 5.0 µg RNA (5-10 µl) to each tube in step 3b, and vortex briefly.
   NOTE: DMRIE-C Reagent is a lipid suspension that may settle with time. Mix thoroughly by inverting the tube 5-10 times before removing a sample to ensure that a homogenous sample is taken.

4. Immediately add RNA-Lipid complexes (from step 3c) to washed cells.
5. Incubate 4 h at 37°C, then replace transfection medium with complete growth medium.
   NOTE: The time of exposure of cells to RNA-Lipid complexes, as well as the amount of RNA added to cells should be adjusted for each cell type.
6. To determine optimal transfection conditions with SFV3-*lac*Z RNA, allow cells to express RNA for 16 h and analyze cells for β-galactosidase activity using X-gal as a substrate (5); see protocol below for *in situ* β-gal staining.
7. Analyze protein levels at 12, 24, 36, 48, and 72 h post-transfection to determine optimal time to recover protein of interest.

Electroporation of SFV3-*lac*Z RNA:

The following electroporation protocol was optimized for BHK-21 cells (C13; ATCC, CCL 10). It is recommended that electroporation conditions be optimized in the user's laboratory for each cell type using RNA transcribed from pSFV3-*lac*Z.

1. BHK-21 cells are cultured in GMEM containing glutamine (2 mM), tryptose phosphate broth (10%), 10 mM HEPES (pH 7.3), 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% fetal bovine serum at 37°C with 5% CO_2, in 160 cm^2 tissue-culture flasks. Harvest cells at confluency. Cells should double every 10-12 h so that a 160-cm^2 flask will yield $2\text{-}4 \times 10^7$ cells at confluency.
2. Remove medium and wash with 5 ml Versene (0.53 mM EDTA in PBS) at room temperature. Wash flask by tilting, then remove wash solution.
3. Add 5 ml 0.25% trypsin, 1 mM EDTA (room temp.) and leave on cells 2-3 min. Detach cells by firmly tapping side of flask. There will be some cell clumps but these will disrupt easily.
4. Add 8 ml complete GMEM growth medium to cells. Triturate cells by pipetting up and down 3 times to get a single-cell suspension.
5. Transfer cell suspension to a 50-ml conical tube and centrifuge at 400 × *g* for 3 min (4°C).Remove medium, add 10 ml GMEM containing 2 mM glutamine (without FBS, tryptose phosphate broth, HEPES, and pen/strept) and pipette cells up and down 3 times. Centrifuge as above.Repeat step 6.
6. Resuspend cells to give ~1.25×10^7 cells/ml in GMEM containing 2 mM glutamine. Count cells and ensure that they are a single-cell suspension. If necessary, disrupt any remaining cell clumps as in step 4.
7. Add 20 µl (5-10 µg) of pSFV1 recombinant RNA to a 1.5-ml microcentrifuge tube.

8. Prior to electroporation, add 0.8 ml of cell suspension (~1×10^7 cells) to the 1.5-ml tube containing RNA.
   NOTE: Electroporate immediately after combining the cells with the RNA. Do not allow the cells and RNA to remain at room temperature longer than necessary (<1 min).
9. Immediately transfer cell suspension and RNA to electroporation chamber.
   Electroporate at 225 V (688 V/cm), 800 μF, Low Ω
   Pulse twice and allow cells to stand five min after electroporation.
10. Transfer cells to 23 ml of GMEM complete medium. Rinse electroporation chamber completely with an additional 1.0 ml of medium and combine with the 23-ml cell suspension (24 ml final volume).
11. To analyze β-gal expression after electroporation, plate cells in 35-mm wells at 1-2 × 10^5 cells/well (~500 μl of cell suspension from step 12) and allow cells to express SFV3-*lac*Z RNA for 16 h. Analyze cells for β-galactosidase activity using X-gal as a substrate (5); see protocol below for *in situ* β-gal staining.
12. To evaluate expression of protein of interest, plate the entire cell suspension from step 12 in a 75-cm^2 tissue culture flask. Analyze cells for protein of interest at 12, 24, 36, 48, and 72 h to determine the optimal time to recover protein.
    NOTE: The Bio Rad Gene Pulser® apparatus may also be used after establishing optimal electroporation conditions. BHK-21 cells (C-13, ATCC CCL 10) have been electroporated at 850 V (2125 V/cm), 25 μF, pulsed twice, no pulse controller. Cells were recovered as above but washed once and resuspended in PBS without calcium and magnesium.

In Situ β-Galactosidase Staining with X-Gal:

To assay β-galactosidase expression, cells in each well are rinsed with PBS and fixed for five min in 2% formaldehyde, 0.2% glutaraldehyde in PBS (or for 5 min with -20°C methanol), rinsed twice with PBS, and stained 2 h to overnight with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS (5). Evaluate transfection efficiency by determining the percent of positively stained cells.

Optimization of Protein Expression:

High levels of protein expression are achievable using the pSFV1 Eukaryotic Expression Vector when conditions are optimized to give high transfection efficiency. The optimization protocols given above are provided to establish optimal transfection conditions. For maximum protein expression, transfection efficiency should be greater than 90% (β-gal positive cells for SFV3-*lac*Z RNA). With lower efficiencies, the untransfected cells will rapidly divide and eventually outgrow the transfected population. This will result in lower levels of specific protein production and a lower percentage of the total protein represented by the specific protein of interest. CHO-K1 and BHK-21 cells have resulted in the highest level of specific protein expression following transfection with SFV3-*lac*Z RNA. The expression of recombinant protein in transfected cells may be enhanced by adding 50-200 mM KCl to the medium at 0-4 h post-transfection (7). The KCl concentration and the time of KCl addition should be optimized for each cell type and protein of interest.

The following recommendations are made to achieve maximal protein expression:

1. Begin to monitor cell viability 24 h after transfection. Add or replace fresh medium as necessary. As a consequence of transfection, some cells will detach from the culture plate; however, these cells may contain high levels of the protein of interest. Collect cells in suspension as well as the attached cells to recover the maximum amount of protein.
2. To monitor protein synthesis, pulse-labeling experiments should begin at 9 h post-transfection to determine synthesis rate.
3. To recover protein, lyse cells directly using a 1% NP-40 or 0.1% Triton® X-100 detergent solution in buffer.
4. Add 50-200 mM KCl to the medium at 0-4 h post-transfection to enhance SFV RNA-derived protein expression.
5. SFV Recombinant RNA may also be packaged into Recombinant SFV particles which are then used to infect cells (1,8). In cells that do not transfect with RNA at high efficiency, infection with recombinant SFV may give better results.

References:

1. Liljeström, P. and Garoff, H. (1991) *Bio/Technology 9*, 1356.
2. Ciccarone, V., Bergland, P., Liljeström, P., and Jessee, J. (1993) *Focus® 15.4*, 103.
3. Kozak, M. (1989) *J. Cell Biol. 108*, 229.
4. Tartof, K.D., and Hobbs, C.A. (1987) *Focus® 9.2*, 12.
5. Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986) *EMBO J. 5*, 3133.
6. Ciccarone, V., Anderson, D., Lan, J., Schifferli, K., and Jessee, J. (1995) *Focus® 17*, 84.
7. Garry, R.F. (1994) *J. Gen. Virol. 75*, 411.
8. Ciccarone, V., Anderson, D., and Jessee, J. (1994) *Focus® 16*, 94.