Sindbis
Expression System

Catalog no. K750-01

Sindbis
Expression System

Version E
180402
25-0121

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Shipping and Storage

The Sindbis Expression System is shipped on dry ice. Please store the BHK cells in liquid nitrogen. The αMEM medium should be stored at +4°C. All other components (see below) are stored at -20°C.

Vector, Primer, and DNA Template

The components listed below should be stored at -20°C. A forward sequencing primer is included to confirm the presence and orientation of your cloned fragment.

DNA	Amount
pSinRep5 plasmid	20 µg, 1 µg/µl in TE, pH 7.5
DH-BB template	5 µg, 0.5 µg/µl in DEPC-treated TE, pH 7.5, linearized
DH(26S) template	5 µg, 0.5 µg/µl in DEPC-treated TE, pH 7.5, linearized
Sindbis Forward Sequencing Primer	0.1 µg/µl in water, 20 µl, 330 pmoles total 5´-AGCATAGTACATTTCATCTG-3´

The DH-BB and DH(26S) helper DNA templates are also available separately (Catalog nos. D170-05 and D175-05, respectively).

InvitroScript™Cap SP6 *in vitro* Transcription Kit

The InvitroScript™Cap SP6 *in vitro* Transcription Kit is specially designed for the routine synthesis of large amounts of capped mRNA transcripts from the SP6 promoter. The ribonucleotide mixture has been optimized to produce maximum yields of capped RNA transcripts. The kit contains enough reagents for twenty-five 20 µl reactions. Store this kit at -20°C. **Do not store in a frost-free freezer.** (The warming and cooling cycles of frost-free freezers are detrimental to enzymes.)

Reagent	Composition	Volume Supplied
10X Enzyme Mix	Placental RNase inhibitor SP6 RNA polymerase (proprietary)	55 µl
10X Transcription Buffer	(proprietary)	60 µl
2X Ribonucleotide Mix	10 mM each of ATP, CTP, UTP 2 mM GTP and 8 mM Cap Analog	260 µl
20 mM GTP	--	2 x 30 µl
Gel Loading Buffer	80% formamide 0.1% xylene cyanol 0.1% bromophenol blue 2 mM EDTA	1 ml
RNase-free, deionized water	--	1 ml
SinRep/*lac*Z template	5 µg, 0.5 µg/µl in DEPC-treated TE, pH 7.5, linearized	10 µl

The InvitroScript™Cap Kit is also available separately (Catalog no. K755-01).

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Baby Hamster Kidney Cells

One vial of 3 x 10^6 baby hamster kidney (BHK) cells are supplied in 1 ml of αMEM, 10% fetal bovine serum, 10% DMSO. Store in liquid nitrogen upon receipt. BHK cells are also available separately (Catalog no. R700-01).

αMEM Medium with L-glutamine

500 ml αMEM medium with 2mM L-glutamine is supplied with the Sindbis Expression System. It is recommended for the growth of BHK cells. Store αMEM medium with 2mM L-glutamine at +4°C. Additional αMEM with 2mM L-glutamine is available from Invitrogen in 500 ml bottles (Catalog no. Q400-01).

pSinHis

pSinHis (Catalog no. V970-20) is available for use with the Sindbis Expression System. This vector contains an N-terminal Xpress™ tag that allows simple purification and rapid detection of fusion proteins. The vector is provided in three reading frames to facilitate cloning.

Materials Supplied by the User

The following materials are required for use with this kit. Other materials may be necessary depending on the particular experiment. See the experiment of interest for the materials required.

- CO_2 incubator, 37°C, 5% CO_2
- αMEM medium
- Fetal bovine serum (FBS)
- Tissue culture grade 200 mM L-glutamine
- Phosphate-buffered saline (PBS) with divalent cations (see **Recipes**, page 34)
- Phosphate-buffered saline, divalent cation free, RNase-free (see **Recipes**, page 34)
- Tissue culture flasks and plates (75 cm^2 flasks, 175 cm^2 flasks, 35 mm plates)
- Electroporator or liposomes for transfection
- Electroporation cuvettes, sterile (0.4 cm)
- microcentrifuge tubes, RNase-free

Technical Service

For Technical Service, please call, write, fax or E-mail:

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Technical Service

If you need help with your Sindbis Expression Kit, please call our Technical Services Department at 1-800-955-6288 (U. S.) or +31 (0) 594 515 175 (Europe).

Thank You

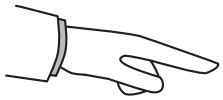
Invitrogen thanks you for your purchase of the Sindbis Expression Kit. We hope this furthers your research goals and allows you to obtain your results. Please call us if we may be of service.

Safety of Sindbis Virus

The low level of pathogenicity of Sindbis virus in humans has allowed it to be classified as a Biosafety Level-2 (BL-2) agent by the NIH Recombinant DNA Advisory Committee. Before synthesizing any constructs, experiments should be cleared through your institutional biosafety committee. All personnel working with the Sindbis Expression System should be properly trained to work with BL-2 level organisms. BL-2 precautions include the use of laminar flow hoods, laboratory coats, gloves, and decontamination of infectious wastes. Sindbis virus can be inactivated by organic solvents, bleach, or autoclaving. In addition, the components of the Sindbis Expression System have been designed to guard against any potential health threats (see **Risk Assessment**, below).

Risk Assessment

Experiments involving transfection of the recombinant RNA (replicon RNA) pose no health risk since replication occurs only within the cells receiving the RNA with no concomitant release of recombinant particles. Co-transfection of the replicon RNA with the helper RNAs, DH-BB or DH(26S), results in the release of particles which can infect new cells. Particles produced by these transfections have little or no plaque forming unit (pfu) capability associated with them. Although the replicon RNA is the predominant RNA found in these particles, the helper RNAs themselves are packaged at low levels (Bredenbeek *et al*., 1993). Therefore, the replicon particles that are thought to be virus free have the potential for being infectious and appropriate caution should be used (see **Safety of Sindbis Virus**, above). Recombination between Sindbis RNA molecules has been reported (Ausubel, *et al*., 1994); however, recombination when using DH-BB or DH(26S) in transfected BHK cells occurs very rarely and only when the transfection efficiency is poor. The production of recombinant virus is probably suppressed under conditions in which most cells are transfected with the recombinant RNA (Bredenbeek *et al*., 1993).



NOTE

A handbook of safety guidelines, *Biosafety in Microbiological and Biomedical Laboratories* (stock number 017-040-00523-7) is available through the U.S. Government Printing Office at (202) 512-2356, or write to: Superintendent of Documents, U.S. GPO, Washington, D. C. 20402.

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Introduction

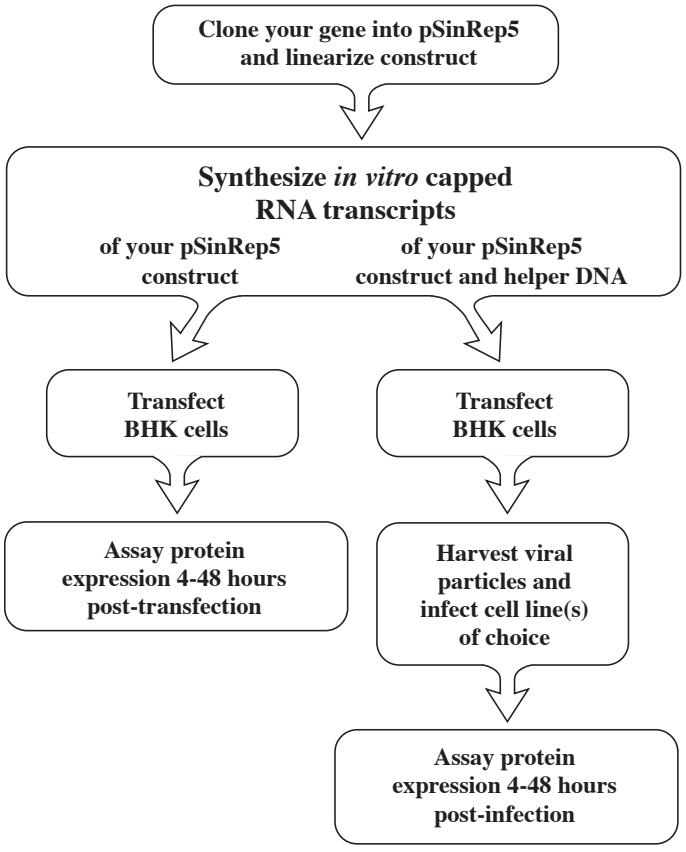
Overview

Introduction

The Sindbis Expression System is a transient expression system that can produce protein in many eukaryotic cell lines (Schlesinger, 1993; Xiong, *et al*., 1989). In this expression system, the Sindbis virus life cycle is exploited to produce recombinant protein. Briefly, your gene of interest is ligated into a specially designed plasmid vector (pSinRep5), under control of the Sindbis subgenomic promoter. This DNA construct is subsequently used to make genome-length RNA transcripts (recombinant RNA) *in vitro*. Once these capped and polyadenylated RNA transcripts are introduced into the cell cytoplasm by transfection, the RNA acts as a messenger RNA and is translated, producing the replication enzymes. These replication enzymes synthesize both genomic and subgenomic RNA molecules in the cytoplasm. Normally, the subgenomic RNA would encode the structural proteins required for RNA packaging and virus assembly; however, these have been removed and replaced with the gene of interest. The subgenomic RNA becomes the most abundant message in the transfected cells and promptly recruits most of the host's translational machinery for its own use, resulting in high levels of the desired protein (Liljeström and Garoff, 1991; Strauss and Strauss, 1994). For more details on the Sindbis virus life cycle, please see page 31.

Experimental Outline

The flow chart below describes the steps you need to perform for expression of the gene of interest using the Sindbis Expression System.



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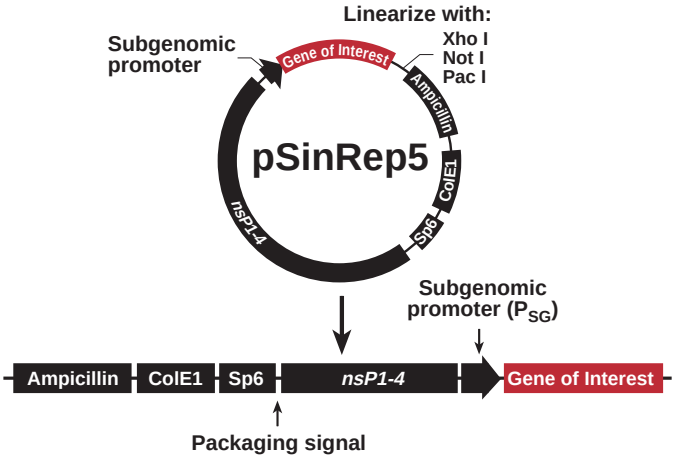
Transfection of Cells with Sindbis RNA

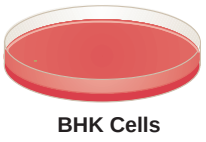
Transfection is used to introduce the recombinant RNA into the cytoplasm of the cell and is useful to quickly study expression of the desired gene product. Please note that no virus particles are produced because there are no structural proteins synthesized to package the RNA. The diagram below illustrates how transfected, recombinant RNAs produce recombinant protein.

1. **Clone gene into pSinRep5 and linearize resulting construct.**

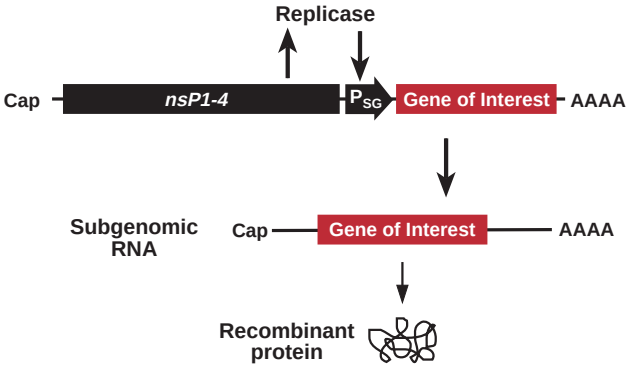


2. **Synthesize capped *in vitro* transcripts of recombinant RNA.**

3. **Transfect recombinant RNA into BHK cells.**



4. **The subgenomic RNA transcript is replicated by the products of the *nsP1-4* genes (replicase). Translation of subgenomic RNA results in expression of your protein.**



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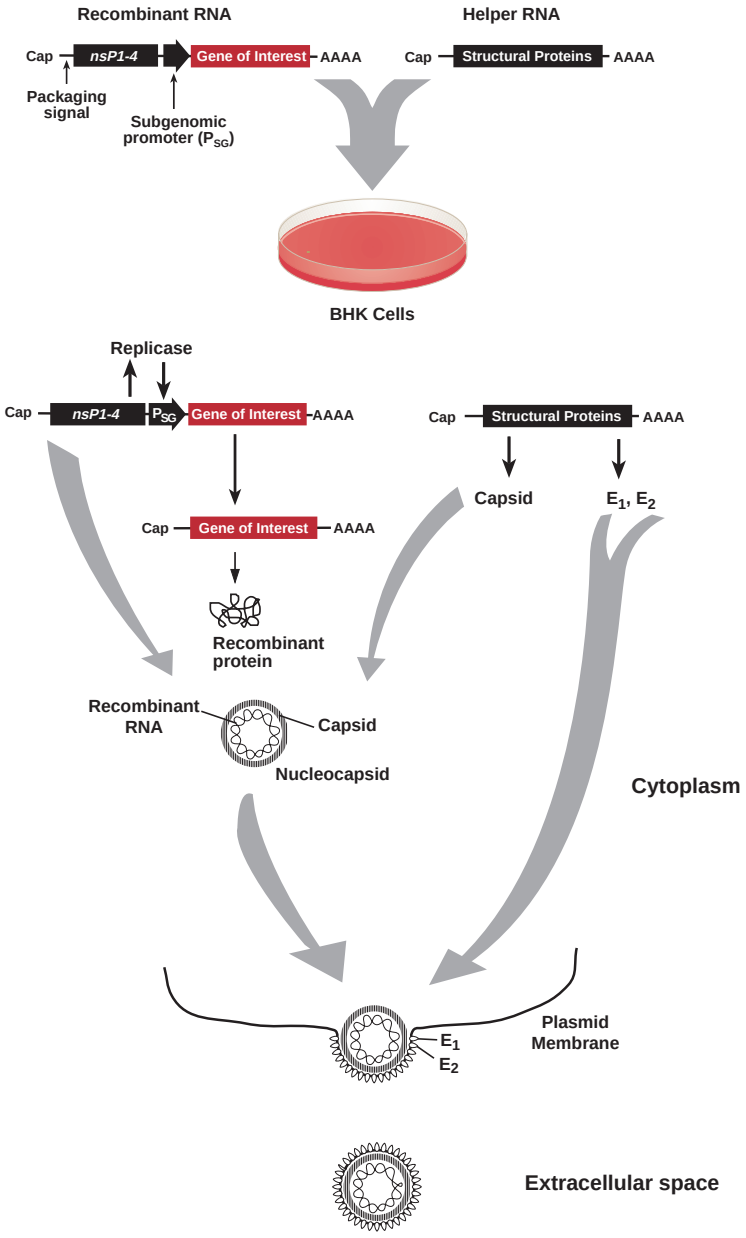
Production of Sindbis Pseudovirions

Infection (transduction) uses virus-like particles (pseudovirions) to deliver the recombinant RNA into the cytoplasm. Infection using Sindbis virus is a simple and very efficient method to deliver the recombinant RNA molecules to a variety of cell types that may be difficult to transfect using standard techniques.

Production of pseudovirions is accomplished by transfecting cells with the recombinant RNA and a helper RNA that provides the Sindbis structural proteins *in trans*. Particles released by the transfected cells contain only the recombinant RNA and are ready to infect new cells for expression studies. These virions will undergo only one round of infection as they do not contain the helper RNA which encodes the structural proteins. The diagram below illustrates how recombinant viral particles are produced.

1. **Synthesize capped *in vitro* transcripts of recombinant RNA and helper RNA.**
2. **Transfect recombinant RNA and helper RNA into BHK cells.**
3. **RNA transcripts are replicated by products of the *nsP1-4* genes. Recombinant protein and structural proteins are expressed (capsid, E_1, and E_2).**
4. **Recombinant RNA is packaged by the capsid protein to make the nucleocapsid.**
5. **Viral glycoproteins E_1 and E_2 are processed and transported to the plasma membrane.**
6. **Nucleocapsid associates with the E_1 and E_2 glycoproteins embedded in the plasma membrane. Viral particles bud into the medium.**



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Protein Expression Using Sindbis Virus

The table below provides references for heterologous protein expression using Sindbis virus expression technology:

Protein	Reference
β-galactosidase	Bredenbeek, *et al*., 1993
Chloramphenicol acetyl transferase	Hahn, *et al*., 1992; Olson, *et al*., 1992; Xiong, *et al*., 1989
Hemagglutinin A and cytotoxic T lymphocyte epitopes	Hahn, *et al*., 1992
Hepadnavirus proteins	Huang and Summers, 1991
Luciferase	Johanning, *et al*., 1995
Tissue Plasminogen Activator	Huang, *et al*., 1993

Description

pSinRep5 is a 9951 bp vector used to generate recombinant RNA molecules for transfection or infection of eukaryotic cell lines. It contains the Sindbis virus nonstructural protein genes 1-4 (*nsP1-4*) for replicating RNA transcripts *in vivo*, the promoter for subgenomic transcription, and a multiple cloning site. The multiple cloning site (*Xba* I, *Mlu* I, *Pml* I, *Sph* I, *Stu* I, and *Apa* I) allows insertion of your gene of interest behind the subgenomic promoter for expression in BHK cells or the cell line of choice. Three unique restriction sites (*Xho* I, *Not* I, and *Pac* I) located 3´ to the multiple cloning site allow linearization of the plasmid prior to *in vitro* transcription. The recombinant RNA transcripts are synthesized using the SP6 promoter and transfected into BHK cells.

Features of pSinRep5

The important elements of pSinRep5 are described in the following table. All features have been functionally tested.

Features	Benefits
SP6 Promoter	Allows *in vitro* transcription of the Sindbis *nsP1-4* genes and sequences containing the subgenomic promoter and the gene of interest
Non-structural genes (*nsP1-4*)	Code for the replicase proteins for *in vivo* replication of recombinant and subgenomic RNAs
Subgenomic Promoter	Allows transcription of heterologous genes
Multiple Cloning Site	Permits insertion of the gene of interest into pSinRep5 behind the subgenomic promoter
Sindbis forward sequencing priming site	Permits sequencing of your insert using the Sindbis Forward Sequencing Primer
PolyA Sequence (37 adenylate nucleotides)	Provides a polyA tail to stabilize the recombinant RNA transcript
3´ Restriction Sites	Three unique restriction sites allow linearization of the template for the production of discrete, monomeric *in vitro* transcripts
Ampicillin resistance gene	Confers resistance to ampicillin for selection in *E. coli*
ColE1 origin	For high copy number replication and growth in *E. coli*

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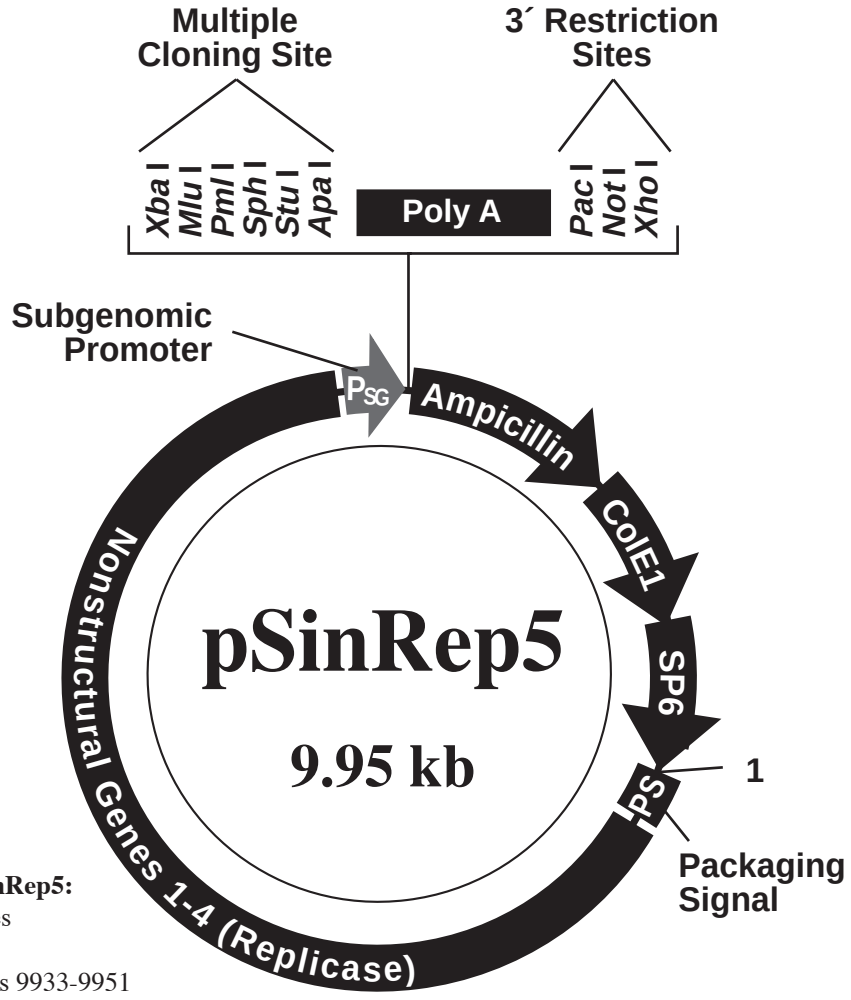
pSinRep5, continued

Map of pSinRep5

The figure below summarizes the features of the pSinRep5 vector. The complete nucleotide sequence is provided after page 44. Details of the multiple cloning site are shown on page 14.



Comments for pSinRep5:
9951 nucleotides

SP6 promoter: bases 9933-9951
Nonstructural genes: bases 60-7598
Subgenomic promoter (P_{SG}): bases 7580-7603
Forward Sindbis sequencing priming site: bases 7604-7623
Transcriptional start: base 7598
Multiple cloning site: bases 7647-7689
PolyA tail: bases 7997-8033
3 ´ Restriction sites: bases 8047-8067
Ampicillin resistance gene: bases 8227-9085
ColE1 origin: bases 9232-9861

Description

DH-BB [**D**efective **H**elper, deleted between **B**spM II and **B**amH I (see Bredenbeek, *et al*., 1993)] is a 6729 bp DNA template that contains the genes for the four structural proteins required for packaging of the Sindbis viral genome (capsid, p62, 6K, and E1, see page 31).

Cotransfection with DH-BB

When RNA from DH-BB is co-transfected with the recombinant RNA from pSinRep5 (or pSinHis), expression of the structural proteins *in trans* from the DH-BB RNA transcript allows packaging of the recombinant RNA into virions (pseudovirions). These virions may be isolated and stored for future experiments. Please note that DH-BB does not contain a packaging signal, so it will not form a defective interfering particle or be packaged with the recombinant RNA. This ensures that all pseudovirions produced will only contain recombinant RNA and can undergo only one round of infection (Bredenbeek, *et al*., 1993).

Preparation of DH-BB

The template has been linearized at the *Xho* I site and is supplied ready for *in vitro* transcription.

Additional DH-BB helper DNA template is available from Invitrogen (Catalog no. D170-05).

Features of DH-BB

The important elements of DH-BB are described below. All elements have been functionally tested.

Features	Benefits
Subgenomic Promoter	Allows expression of the structural genes
Structural Genes	Encode the four structural proteins required for packaging of the recombinant RNA
PolyA Sequence (37 adenylate nucleotides)	Provides a polyA tail to stabilize the recombinant RNA transcript when it is transfected into cells
Linearized Template	Allows convenient production of *in vitro* RNA transcripts

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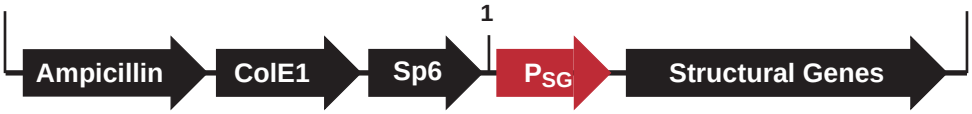
DH-BB Template, continued

Map of DH-BB

The figure below summarizes the features of the DH-BB template.



Comments for DH-BB:

6729 nucleotides

Ampicillin resistance gene: bases 5005-5863
ColE1 origin: bases 6010-6639
SP6 promoter: bases 6711-6729
Subgenomic promoter (P_{SG}): bases 671-694
Structural genes: bases 738-4472
Capsid: bases 738-1529
p62 (E2 & E3): bases 1530-2990
6K: bases 2991-3155
E1: bases 3456-4472
Poly A sequence: bases 4795-4831

Description

DH(26S) is a 8189 bp DNA template that contains the genes for the four structural proteins (capsid, p62, 6K, and E1, see page 31) required for packaging the Sindbis viral genome. The E2 and E1 glycoprotein sequences of DH(26S) are derived from a neurovirulent Sindbis virus cDNA (Lustig *et al*., 1988). This helper DNA may be more efficient than DH-BB at infecting some cell lines. It may be useful to create pseudovirions with both helper templates to determine which will work best in a particular cell line. Please see page 23 for more information on producing recombinant pseudovirions.

Co-transfection with DH(26S)

When RNA from DH(26S) is co-transfected with the recombinant RNA from pSinRep5 (or pSinHis), expression of the structural proteins *in trans* from the DH(26S) RNA transcript allows the recombinant RNA to be packaged into virions (pseudovirions). These virions may be isolated and stored for future experiments. Please note that DH(26S) RNA is packaged inefficiently compared to recombinant RNA. Co-transfection of recombinant RNA and DH(26S) produces high titers of particles which yield few or no plaque forming units (pfu), indicating that most of the virions produced contain only the recombinant RNA and therefore can undergo only one round of infection (Bredenbeek, *et al*., 1993).

Preparation of DH(26S)

The template has been linearized at the *Xho* I site and is supplied ready for *in vitro* transcription.

Additional DH(26S) helper DNA template is available from Invitrogen (Catalog no. D175-05).

Features of DH(26S)

The important elements of DH(26S) are described below. All elements have been functionally tested.

Features	Benefits
Subgenomic Promoter	Allows expression of the structural genes
Structural Genes	Encode the four structural proteins required for packaging of the recombinant RNA
PolyA Sequence (37 adenylate nucleotides)	Provides a polyA tail to stabilize the recombinant RNA transcript when it is transfected into cells
Linearized Template	Allows convenient production of *in vitro* RNA transcripts

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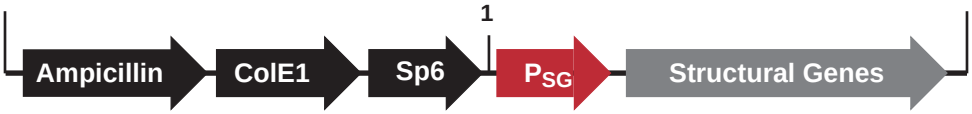
DH(26S) Template, continued

Map of DH(26S)

The figure below summarizes the features of the DH(26S) template.



Comments for DH(26S):

8189 nucleotides

Ampicillin resistance gene: bases 6465-7323
ColE1 origin: bases 7470-8099
SP6 promoter: bases 8171-8189
Subgenomic promoter (P_{SG}): bases 2073-2096
Structural genes: bases 2140-5874
Capsid: bases 2140-2931
p62 (E2 & E3): bases 2932-3123
6K: bases 4393-4557
E1: bases 4558-5874
Poly A sequence: bases 6197-6233

Description

SinRep/*lac*Z is a 13104 bp linearized DNA template containing the *lac*Z gene. An *Xba* I-*Hin*d III fragment containing the *lac*Z gene was cloned into the *Xba* I-*Pml* I sites of pSinRep5. The construct is supplied linearized at the *Xho* I site. Cells may be transfected with a SinRep/*lac*Z transcript and assayed for expression of β-galactosidase (see page 38). SinRep/*lac*Z may be co-transfected with either DH-BB or DH(26S) to determine which template yields optimal infection and the highest expression level of β-galactosidase in your cell line (see page 23).

Map of SinRep/*lac*Z

The figure below summarizes the features of the SinRep/*lac*Z template.

Comments for SinRep/LacZ:

13104 nucleotides

Ampicillin resistance gene: bases 11380-12238
ColE1 origin: bases 12385-13014
SP6 promoter: bases 13086-13104
Nonstructural genes: bases 60-7598
Subgenomic promoter (P_{SG}): bases 7580-7603
*lac*Z ORF: bases 7655-10664

Methods

Culturing BHK Cells

Introduction

Use the procedures below to initiate and maintain a culture of BHK cells. These cells are provided to get you started with the Sindbis Expression System. Other cell lines may be infected with Sindbis pseudovirions.

Description of BHK Cells

The BHK cell line was derived from baby hamster kidney. The cells have a tendency to clump in complete medium. In general, this is not a problem except when preparing the cells for electroporation (see page 19). In this case, care must be taken to avoid clumps. PBS is required to keep the cells from clumping during electroporation. Additional BHK cells are available from Invitrogen (Catalog no. R700-01).

General Cell Handling

- **All solutions and equipment that come in contact with the cells must be sterile.**
- Always use proper sterile technique in a laminar flow hood.
- All incubations are performed in a humidified, 37°C, 5% CO_2 incubator.
- The medium for BHK cells is αMEM.
- Complete medium for BHK cells is αMEM + 2 mM L-glutamine + **5% fetal bovine serum** (see page 34). FBS does not need to be heat inactivated for use with BHK cells.

  Note: 10% FBS makes the cells grow too fast resulting in complete lysis and cell death when infected with Sindbis virus.
- Use cells that are 80-90% confluent for all experiments.
- Before starting experiments, be sure to have cells established and also have some frozen stocks on hand.
- For general maintenance of cells, pass BHK cells when they are 80-90% confluent (1-2 days) and split at a 1:5 dilution. For example, transfer 2 ml of a 10 ml cell suspension to a new 175 cm^2 flask.
- Cells may be passaged 60-70 times before re-starting a culture from frozen stocks.

Before Starting

Be sure to have the following solutions and supplies available:

- 15 ml sterile, conical tubes
- 5, 10, and 25 ml sterile pipettes
- Cryovials
- Hemacytometer
- αMEM medium
- Tissue culture grade 200 mM L-glutamine
- Complete αMEM medium (αMEM + 2 mM L-glutamine + 5% FBS)
- αMEM medium + 2 mM L-glutamine + 10% FBS + 10% DMSO (freezing medium)
- Table-top centrifuge (+4°C)
- 175 cm^2 flasks and 35 mm plates (other flasks and plates may be used)
- PBS with cations
- Trypsin/versene (EDTA) solution (BioWhittaker) or other trypsin solution

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Initiating Cell Culture from Frozen Stock

The following protocol is designed to help you initiate a cell culture from a frozen stock. Note that the vial of BHK cells supplied with the kit contains 3 x 10^6 cells.

1. Remove the vial of cells from the liquid nitrogen and thaw quickly at 37°C.
2. Just before the cells are completely thawed, decontaminate the outside of the vial with 70% ethanol, and transfer the cells to a 15 ml sterile, conical tube.
3. Add 9 ml of prewarmed (37°C) complete αMEM medium dropwise to cells.
4. Centrifuge in a table-top centrifuge at 250 x g for 5 minutes at +4°C. Decant the medium. (This removes the DMSO from the cells.)
5. Resuspend the cells in 10 ml of complete αMEM, transfer to a 75 cm^2 flask, and incubate at 37°C. Incubation for 1-2 days should yield an 80-90% confluent monolayer.

Passaging the BHK Cells

1. When cells are ~80-90% confluent, remove all medium from the flask.
2. Wash cells once with 10 ml PBS with cations (see **Recipes**, page 34) to remove medium. Serum contains inhibitors of trypsin.
3. Add 5 ml of trypsin/versene (EDTA) solution to the monolayer and incubate 1-2 minutes at room temperature until cells detach. Check the cells under a microscope and confirm that most of the cells have detached. If cells are still attached, incubate a little longer until most of the cells have detached.
4. Once the cells have detached, briefly pipet the solution up and down to break up clumps of cells.
5. Add 5 ml of complete αMEM to stop trypsinization.
6. Transfer 2 ml of the cell suspension in Step 5 to a 175 cm^2 flask. Add complete αMEM medium to 30 ml total volume for each new 175 cm^2 flask and incubate in a humidified, 37°C, 5% CO_2 incubator.

Repeat Steps 1-6 as necessary to expand cells.

Freezing the BHK Cells

Before starting, label cryovials and place on ice. Prepare freezing medium: αMEM containing 2 mM L-glutamine, 10% FBS, and 10% DMSO.

1. When cells are ~80% confluent in a 175 cm^2 flask, remove the medium and wash the cells one time with 10 ml PBS with cations.
2. Add 5 ml of trypsin/versene (EDTA) solution and incubate 1-2 minutes until cells detach.
3. Once cells have detached, briefly pipet solution up and down to break up clumps of cells.
4. Add 5 ml of complete αMEM to stop trypsinization. Count a sample of cells in a hemacytometer.
5. Pellet all of the cells at 250 x g for 5 minutes in a table top centrifuge at +4°C.
6. Resuspend the cells at a density of 3 x 10^6 cells/ml in freezing medium (see above).
7. Aliquot 1 ml of the cell suspension per vial. Place vials at -20°C for 2-3 hours.
8. Transfer vials to a -70 or -80°C freezer and hold overnight.
9. Transfer vials to liquid nitrogen for long term storage.

Cloning into pSinRep5

General Molecular Biology Techniques

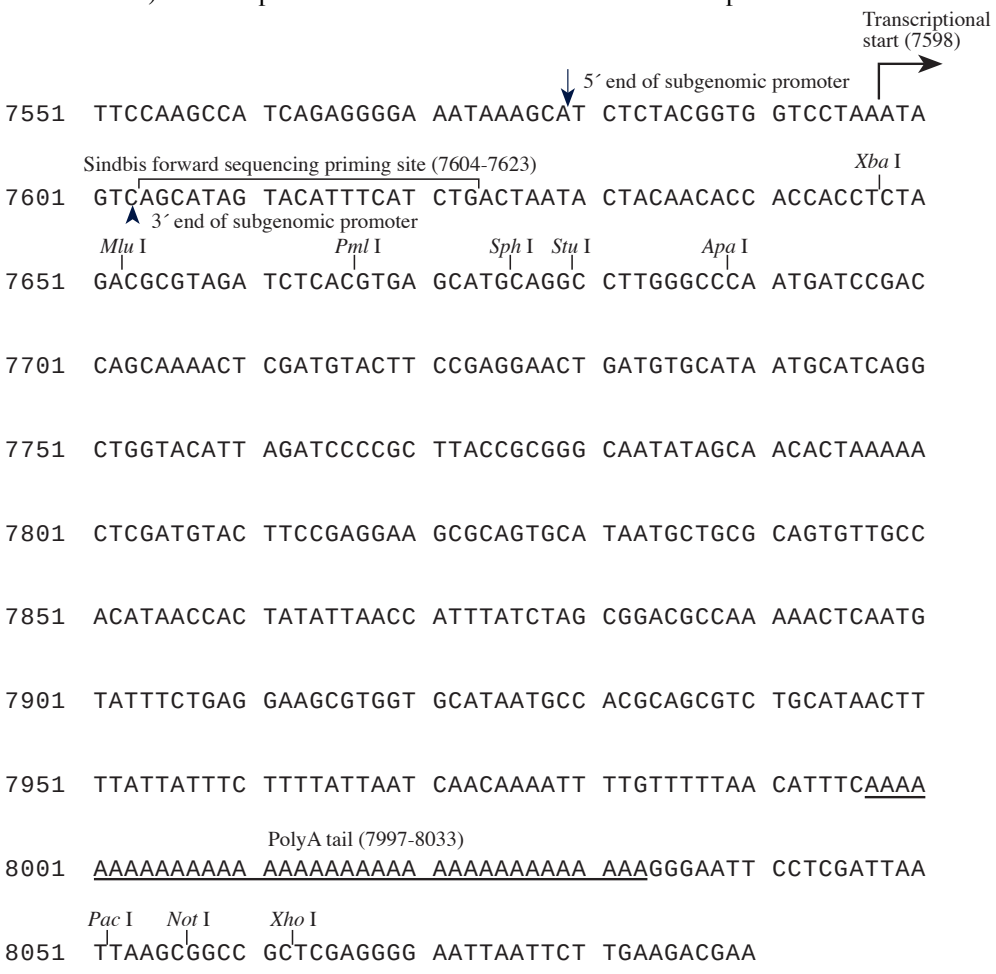
For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, DNA sequencing, and DNA biochemistry, please see *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al*., 1989) or *Current Protocols in Molecular* Biology (Ausubel, *et al*., 1994).

Maintenance of pSinRep5

In order to propagate and maintain pSinRep5, we recommend that you transform the vector into a *rec*A, *end*A *E. coli* strain like TOP10 (Catalog no. C664-55), DH5α, or equivalent. Select on LB plates containing 50-100 μg/ml ampicillin.

Cloning into pSinRep5

The subgenomic promoter region and multiple cloning site of pSinRep5 is included below to help you ligate your gene into pSinRep5. The entire subgenomic promoter is present in pSinRep5 (Raju and Huang, 1991). It is recommended to include a Kozak consensus sequence in your insert for proper initiation of translation of your gene (Kozak, 1990) and a stop codon for termination of the recombinant protein.



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E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. TOP10, DH5α) and select on LB plates containing 50-100 µg/ml ampicillin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the Sindbis Forward Sequencing Primer to confirm that your gene is in the correct orientation for expression and has a Kozak translation initiation sequence and a stop codon.

Important

The plasmid DNA must be free of RNA and RNase before performing *in vitro* transcription. RNA will inhibit transcription and may lower transfection efficiencies. RNase will degrade your transcripts.

Plasmid Preparation

Once you have your recombinant construct, you will need to prepare plasmid for *in vitro* transcription. You will need at least 1 µg of DNA for each *in vitro* transcription reaction. We recommend purifying your DNA using CsCl gradient centrifugation or using the plasmid miniprep procedure on page 40. This method uses RNase to eliminate RNA and a Proteinase K digest to remove the RNase. It is very important to eliminate RNase to avoid degradation of your transcripts. After isolation of plasmid DNA, be sure to determine the concentration of your sample using UV absorbance, fluorescence, or the DNA DipStick™ Kit (Catalog no. K5632-01).

In vitro Transcription of DNA Templates

Purpose

The purpose of these procedures is to generate recombinant RNA (and helper RNA) for transfection of BHK cells. You will use purified, linearized pSinRep5 (or pSinHis) containing your gene as the DNA template to produce recombinant RNA with the InvitroScript™Cap SP6 *in vitro* Transcription Kit. The recombinant RNA produced will be capped and have a polyA tail, so when it is transfected into the cells, it will be treated as messenger RNA.

InvitroScript™Cap SP6 *in vitro* Transcription

The InvitroScript™Cap SP6 *in vitro* Transcription Kit produces large amounts of polyadenylated RNA transcripts from the SP6 promoter using linearized DNA templates. The kit contains a GTP cap analog to produce capped RNA transcripts. The reaction conditions have been optimized to yield high levels of capped RNA in the presence of high nucleotide concentrations. Additional InvitroScript™Cap Kits are available from Invitrogen (Catalog no. K755-01).

Helper RNA

If you wish to package your recombinant RNA to produce pseudovirions, you will need to supply the structural proteins *in trans*. These proteins are encoded on the DH-BB and DH(26S) templates. These templates are supplied linearized and can be used directly in the *in vitro* transcription reaction with no further preparation. The helper RNA from these templates and your recombinant RNA may be co-transfected into BHK cells to produce pseudovirions (see page 23). These pseudovirions may be used to infect BHK or other cell lines.

Control RNA

Linearized SinRep/*lac*Z template is included in the InvitroScript™Cap SP6 *in vitro* Transcription Kit as a transcription control and a positive control for expression. This template contains the *lac*Z gene which expresses β-galactosidase. The template can be used directly in the *in vitro* transcription reaction with no further preparation.

Experimental Outline

The table below outlines the steps needed to prepare recombinant RNA.

Step	Action
1	Digest 2-20 µg of pSinRep5 containing your gene of interest with the appropriate 3´ restriction enzyme to linearize the plasmid.
2	Phenol extract and ethanol precipitate the DNA template. Resuspend DNA at 0.5 µg/µl in RNase free TE.
3	Set up the *in vitro* transcription reaction with recombinant pSinRep5, helper RNA, or SinRep/*lac*Z (control template). Incubate the reaction for 2 hours at 37°C.
4	Analyze the resulting RNA on a 1% agarose gel.

General Molecular Biology Techniques

For help with restriction enzyme digests, please see *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al*., 1989) or *Current Protocols in Molecular Biology* (Ausubel, *et al*., 1994).

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Important

Because you will be generating RNA, it is very important that any solution that comes in contact with the RNA be made with diethylpyrocarbonate-treated (DEPC) water and autoclaved (Sambrook, *et al*., 1989). All plasticware should be sterile. Be sure to wear gloves .

Before Starting

You will need to have available the following solutions and supplies:

- Restriction enzymes and buffers
- Phenol/chloroform (1:1)
- 0.5 M EDTA
- RNase-free 5 M ammonium acetate
- 100% ethanol
- RNase-free water or TE buffer
- 37°C incubator

In vitro Transcription

Linearization of the DNA template prior to *in vitro* transcription is necessary to create a homogeneous population of RNA molecules. There are three enzymes that you can use to linearize the DNA: *Xho* I, *Not* I, and *Pac* I. Choose the enzyme that does not cut in your gene to linearize the DNA template. Each *in vitro* transcription reaction requires 1 µg of linearized template. **Note**: The DH-BB, DH(26S) and SinRep/*lac*Z templates are provided linearized. These templates may be added directly to the transcription reaction at Step 5.

1. Linearize 2-20 µg of your pSinRep5 construct by restriction digestion using *Xho* I, *Not* I, or *Pac* I.
2. Terminate the restriction digest with 1/20 volume of 0.5 M EDTA.
3. Phenol extract the restriction digest and ethanol precipitate the DNA with 1/10 volume of 5 M ammonium acetate and 2 volumes of 100% ethanol.
4. Resuspend the DNA to a concentration of 0.5 µg/µl in RNase-free TE or water. You may wish to check an aliquot of your linearized template on an agarose gel to analyze quantity and quality.
5. Set up the *in vitro* transcription reaction at room temperature in a microcentrifuge tube by mixing the following reagents. If you wish to quantitate the transcription reaction, refer to page 37.

RNase-free water	2 µl
Linearized DNA template at 0.5 µg/µl (1 µg)	2 µl
2X Ribonucleotide Mix	10 µl
20 mM GTP*	2 µl
10X Transcription Buffer†	2 µl
10X SP6 Enzyme Mix	2 µl
Final Volume	20 µl

*Inclusion of extra GTP is needed to ensure full-length transcript (see next page)
†Do not set up the reaction on ice as the 10X Transcription Buffer will precipitate.

6. Mix the reaction gently and incubate for 2 hours at 37°C. A typical reaction should yield 10-20 µg of RNA from 1 µg of linearized template. Termination of the reaction is not required for transfection. If you wish to store the RNA, see Step 8, next page.

continued on next page

In vitro Transcription, continued

7. Check the quality of the RNA by mixing 1 µl of the transcription reaction with 3 µl of the gel loading dye, heating for 3-5 minutes at 80°C-90°C, and running on a 1% agarose gel.

   Note: RNA run on the agarose gel can be visualized by staining with ethidium bromide. Since this gel is nondenaturing, this procedure will only reflect the quality and quantity of the RNA. A nondenaturing gel cannot be used to accurately determine the size of the RNA. You may wish to compare the **quality** of the RNA band with DNA standards such as digested λDNA. The RNA band should be discreet and relatively thick in comparison to the DNA bands. Please note that you may see dimers of single-stranded RNA.

8. Proceed to **Transfection**, next page. Alternatively, you may aliquot the RNA in 10 µl samples and freeze at -80°C for storage.

Important

Since the Sindbis RNA transcript is greater than 10 kb, you must add 1-2 µl of 20 mM GTP in the transcription reaction as the concentration of GTP will become limiting during the reaction. Adding GTP will decrease the fraction of transcripts containing the cap (since it decreases the ratio of the cap analog to GTP), but will increase the yield of full-length product.

Purpose

The purpose of this section is to supply guidelines for transfection of RNA into BHK cells and other eukaryotic cells. Electroporation is strongly recommended as conditions may be optimized to obtain nearly 100% transfection frequency. We use electroporation exclusively at Invitrogen to transfect cells with recombinant Sindbis RNA.

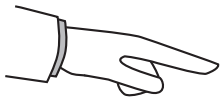
Transfection Using Liposomes

It is possible to transfect cells using liposome-mediated transfection. Please refer to *Current Protocols in Molecular Biology*, pages 16.20.5-16.20.6 for a protocol or Johanning, *et al.*, 1995. You will need 9-20 μg of liposomes per each 35 mm plate of cells to transform. Please note that the transfection efficiency may be much lower than with electroporation.

Before Starting

You will need the following solutions and equipment:

- 175 cm^2 flasks
- αMEM medium + 2 mM L-glutamine (no FBS)
- complete αMEM medium (see **Recipes**, page 34)
- PBS **with cations** (see **Recipes**, page 34)
- RNase-free PBS **without cations** (see **Recipes**, page 34)
- Trypsin/versene (EDTA) solution
- Tabletop centrifuge
- Hemacytometer
- Electroporation device (be sure to use a device that can transform eukaryotic cells and not just bacteria. You will need settings to give a field strength of 2125 V/cm with a capacitance of 50 μF)
- 0.4 cm, sterile electroporation cuvettes



NOTE

If you wish to transfect cells with your recombinant RNA and the helper RNA to produce pseudovirions, please see page 23 before continuing.

Preparation of Cells

We generally use 175 cm^2 flasks to grow cells for electroporation. One to two flasks provide enough BHK cells for two electroporations. It is very important to keep the BHK cells from clumping as this will prevent RNA from entering cells and lowering your transfection efficiency.

1. Grow BHK cells in a 175 cm^2 tissue culture flask containing 30 ml complete αMEM and grow until the monolayer is approximately 90% confluent and composed of approximately 10^7 cells. This should take one to three days.
2. Aspirate medium and wash the cells once with room temperature PBS **with cations**.
3. Add 5 ml of the trypsin/versene (EDTA) solution and incubate 1-2 minutes until cells detach. Briefly pipet the solution to obtain a single-cell suspension. Monitor under a microscope until a single-cell suspension has been obtained.
4. Add 5 ml complete αMEM medium to stop trypsinization. Transfer cells to a sterile 15 ml conical tube.

continued on next page

Preparation of Cells, continued

5. Centrifuge the cells at 400 x g for 5 minutes at room temperature or +4°C and aspirate supernatant. Resuspend cells in 10 ml αMEM containing 2 mM L-glutamine (**no serum**).

   If you find that there are still some clumps, keep the tube upright and let the clumps settle to the bottom of the tube (1-2 minutes). Carefully remove the cell suspension from the clumps of cells.
6. Centrifuge the cells at 400 x g for 5 minutes at room temperature or +4°C and aspirate supernatant. Resuspend cells in 10 ml RNase-free PBS **without cations**. Determine the number of cells with a counting chamber.
7. Centrifuge cells at 400 x g for 5 minutes at room temperature or +4°C and aspirate supernatant. Resuspend cells in RNase-free PBS **without cations** at a concentration of 10^7 cells/ml. Proceed immediately to **Electroporation of Cells**, below. BHK cells prepared for electroporation cannot be stored; they must be used immediately.

Important

Cells must be resuspended in PBS that is

- RNase-free to prevent RNA degradation
- Without cations to prevent arcing during electroporation

Guidelines for Electroporation

Actual settings are provided below for Invitrogen's Electroporator II. If you are using another electroporation device, please consult the manufacturer's instructions. If you are using another cell line, you will have to optimize electroporation conditions to achieve high transfection efficiencies. Please see **Electroporation Parameters to Optimize**, next page.

Controls

We recommend transforming BHK cells with SinRep/*lac*Z control RNA as a positive control for expression. Remember to also include a "no RNA" control.

Electroporation of Cells

1. Place 0.5 ml (~10^7 cells/ml) of cell suspension from Step 7, above, into a 0.4 cm electroporation cuvette.
2. Add 5-10 μl of the transcription reaction (5-10 μg of RNA, from Step 8, page 18) to the cell suspension, place cap on cuvette and mix thoroughly by inverting.
3. Set up your electroporation device to give a field strength of 2125 V/cm (voltage divided by cuvette width) with 50 μF capacitance. Settings are provided below for Invitrogen's Electroporator II. See Liljeström, *et al*., 1991 for settings for Bio-Rad Gene Pulser.

Power Supply Settings	Electroporator II Settings
Voltage: 850V	Capacitance: 50 μF/1800V
Current: 50 mA	Resistance: ∞ Ω
Power: 50W	

4. Place the cuvette in the electroporator and pulse the cell suspension twice. Wait until the unit recharges itself before pulsing again. Place cells on ice for 5 minutes to allow cells to recover.

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Electroporation of cells, continued

5. Transfer electroporated cells (0.5 ml) to 9.5 ml of complete αMEM medium. Rinse the cuvette with the cell suspension to collect all the cells.
6. Plate the cells (2 ml on a 35 mm plate, 5 ml on a 60 mm plate, or 10 ml on a 100 mm plate). Expression can be observed as early as 4 hours and as late as 72 hours post-transfection. Proceed to **Analysis of Protein Expression** page 24.

Assay for β-galactosidase

If you transfected the positive control RNA derived from SinRep/*lac*Z, you may stain your transfected cells *in situ* for β-galactosidase activity (page 38). Cell-free lysates may also be prepared and assayed for β-galactosidase activity using orthonitrophenyl-β-D-galactoside (ONPG), page 39.

Expression in Cells Other Than BHK Cells

The Sindbis Expression System can be used to express recombinant protein in a variety of cell lines. BHK cells, however, have been shown to yield the maximum levels of protein. To study the effects of your gene in another cell line, we recommend that you:

- Optimize transfection to maximize the number of cells expressing your recombinant RNA
- Perform a time course of expression as host cell functions affect the Sindbis life cycle
- Determine which helper DNA template will yield the most efficient expression
- Use viral particles to infect cell line

Electroporation Parameters to Optimize

All mammalian cell electroporations are generally performed using 0.4 cm cuvettes. The voltage and capacitance must be optimized for each cell line used. The resistance is determined by the electroporation buffer and the volume in which the cells are suspended. Note that the conditions to electroporate RNA are much different than those for DNA. Other parameters to optimize are:

Electroporation buffer

- Use "High salt" (sterile PBS without cations) when electroporating cells with RNA

Voltage

- For high salt buffer, use 200-1200 V. We recommend varying the voltage first when optimizing conditions for transfection of RNA

Capacitance

- For high salt buffer, start with 50 μF and increase to lengthen pulse

Volume

- Start with 500 μl and decrease to 250 μl to increase resistance or increase to 800 μl to reduce resistance.

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Electroporator II and Cuvettes

Invitrogen's Electroporator II is a simple electroporation device that utilizes common electrophoresis power supplies for the efficient electroporation of both prokaryotic and eukaryotic cells. This makes it a versatile, affordable choice for electroporating any kind of cell. We also carry electroporation cuvettes to electroporate bacteria, yeast, or mammalian cells. Please call us at 1-800-955-6288 (U. S.) or +31 (0) 594 515 175 (Europe) for more information.

Product	Size	Catalog no.
Electroporator II	with 10 cuvettes (0.1, 0.2, or 0.4 cm)	S1670-01, -02, or -04
Cuvettes (50)	0.1 cm	P410-50
	0.2 cm	P450-50
	0.4 cm	P460-50

Purpose

You may wish to produce pseudovirions containing your recombinant RNA. Pseudovirions may be used to infect a variety of different cell lines to optimize expression in the cell line of choice or to infect large scale cultures for production and purification of your protein. To package your recombinant RNA, you will first need to co-transfect your recombinant RNA with the helper RNA made from either the DH-BB or DH(26S) template. If you do not have RNA from any of these three plasmids, you will need to synthesize RNA using the InvitroScript™ Cap SP6 *in vitro* Transcription Kit (see page 16).

Once you have obtained pseudovirions, you will need to determine the optimal amount of virus needed to infect cells. This may vary between cell lines and needs to be optimized.

Experimental Outline

The table below outlines the steps needed to produce pseudovirions.

Steps	Description
1	Prepare RNA transcripts from pSinRep5 containing your gene (or SinRep/*lac*Z) and the DH-BB or DH(26S) template (structural genes) using the InvitroScript™ Cap SP6 *in vitro* Transcription Kit
2	Transfect a 1:1 v/v mixture of the two RNAs into BHK cells
3	24-36 hours posttransfection, harvest the medium containing the pseudovirions
4	Serially dilute the viral solution and infect cells to empirically determine the optimal dilution needed for expression of your protein
5	Virus may be frozen in 0.5 ml aliquots for future use

Positive Control

You may wish to produce pseudovirions containing the SinRep/*lac*Z recombinant RNA as a positive control.

Helper DNA Templates

Both DH-BB and DH(26S) helper DNA templates are included in the Sindbis Expression System. Each DNA template encodes different E2 and E1 glycoproteins which affect the ability of the virus to infect a given cell line. Some cell types may be infected better with pseudovirions produced from one helper DNA over the other. We recommend producing pseudovirions of the SinRep/*lacZ* control with both the DH-BB and DH(26S) templates to determine which helper DNA yields optimal infection and the highest expression of β-galactosidase in your cell line. In general, DH(26S) is more efficient at infection and, therefore, yields higher expression levels than DH-BB.

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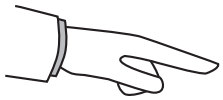
Production of Recombinant Sindbis Pseudovirions, continued

Before Starting

Be sure to have on hand the following reagents:

- RNA transcript of pSinRep5 plasmid containing desired insert (see page 17, steps 1-8)
- SinRep/*lac*Z RNA (see page 17, steps 1-8)
- DH-BB or DH(26S) Helper RNA (see page 17, steps 1-8)
- BHK cells, 80-90% confluent, ready for electroporation (see page 19, steps 1-7)
- Complete αMEM (containing 2 mM L-glutamine and 5% FBS)
- αMEM (or PBS) + 2 mM L-glutamine + 1% FBS
- PBS **with cations**
- 35 mm tissue culture plates
- If you wish to use liposomes, please see page 19



NOTE

All solutions and equipment coming into contact with cells must be sterile. Always use proper sterile technique. All incubations are performed in a humidified, 37°C, 5% CO_2 incubator unless otherwise noted.

Obtaining Pseudovirions

You should have capped RNA transcripts of your construct and helper DNA, and BHK cells ready for transfection before starting this procedure.

1. Mix the RNAs in a 1:1 v/v ratio. Typically, equal amounts of the *in vitro* transcription reactions (usually 5-10 µl of each) result in the highest yield of pseudovirions.
2. Place 0.5 ml (~10^7 cells/ml) of the cell suspension into a 0.4 cm electroporation cuvette.
3. Add 10-20 µl of the RNA mixture (10-20 µg of RNA) to the cell suspension, place cap on cuvette and mix thoroughly by inverting.
4. Set up your electroporation device to give a field strength of 2125 V/cm (voltage divided by cuvette width) and a capacitance of 50 µF. See page 20 for more information.
5. Place the cuvette in the electroporator and pulse the cell suspension twice. Wait until the unit recharges itself before pulsing again.
6. Transfer electroporated cells (0.5 ml) to 9.5 ml of complete αMEM medium. Rinse the cuvette with the cell suspension to collect all the cells.
7. Plate the cells (2 ml on a 35 mm plate, 5 ml on a 60 mm plate, or 10 ml on a 100 mm plate). Plate out **all** of the transfected cells. This will ensure the maximum amount of pseudovirion containing medium.
8. 24-36 hours posttransfection, remove the medium from the cells. Remove any loose cells by centrifuging the medium for 10 minutes at 2000 x g in a table top centrifuge at +4°C. Save the supernatant as this contains the pseudovirions.
9. Take 0.5 ml aliquots of the supernatant and freeze in an ethanol/dry ice bath. Store at -80°C. Freezing the virus in aliquots is recommended since repeated freeze-thawing reduces virus infectivity. Proceed to **Infecting Cells with Sindbis Pseudovirions**, next page.

continued on next page

Determining Actual Virus Titer

Since the recombinant virus can initiate only one round of infection, the titer of the packaged stock cannot be determined by a conventional plaque assay. However, the amount of recombinant virus needed for high level expression can be determined empirically by performing an expression experiment using dilutions of virus (see next page); therefore, it is not necessary to determine the actual titer of the virus stock.

If you wish to determine the actual titer of your virus stock, you must use indirect immunofluorescence. You will need a specific antibody to your protein. Different dilutions of the virus stock are used to infect cells and heterologous protein expression is detected by the antibody. The percentage of cells that score positive for fluorescence multiplied by the dilution factor gives the virus titer. For a complete protocol consult (Ausubel, *et al*., 1994, page 16.20.9).

Infecting Cells with Sindbis Pseudovirions

This procedure allows you to determine the optimal amount of virus needed to infect BHK or any other cell line. You will need to make serial dilutions of the virus to empirically determine the optimal amount of virus for protein expression. This protocol may also be used with other cells using medium appropriate for the cell line in question.

1. Grow BHK cells in complete αMEM to approximately 70-80% confluency in a 35 mm tissue culture plate.

   Plating 2 x 10^5 cells in a 35 mm tissue culture dish will give the required cell density the next morning. Cells may also be plated the same day the transfection is to be performed. 3 x 10^5 cells per 35 mm dish will be 80% confluent after six hours.

2. Prepare dilutions of your viral stock to empirically determine the best dilution of virus for expression.

Dilution	Viral Stock	αMEM (or PBS) + 2mM L-glutamine + 1% FBS
Undiluted	N/A	N/A
1:10	100 µl	900 µl
1:100	10 µl	990 µl

3. Aspirate medium from the cells and wash with PBS.

4. Use the table below to determine the amount of virus needed to infect cells in 35 mm, 60 mm, and 100 mm plates. Mix viral stock or dilution with αMEM (or PBS) + 2mM L-glutamine + 1% FBS and add to cells.

Plate Size	Viral Dilution	αMEM (or PBS) + 2mM L-glutamine + 1% FBS	Total Volume (µl)
35 mm	100 µl	200 µl	300
60 mm	150 µl	300 µl	450
100 mm	250 µl	500 µl	750

5. Incubate for one hour at room temperature or 37°C (whichever is more convenient). Gently rock the plates every 15 minutes to ensure the monolayer remains completely covered with the virus solution. You may wish to place the plates on a very slow rocker (1-2 up and down motions per minute). Do not let cells dry out.

continued on next page

Infecting Cells with Sindbis Pseudovirions, continued

6. After the 1 hour incubation, add αMEM + 2mM L-glutamine + 1% FBS to plates as indicated below and incubate at 37°C for 24 hours in a humidified, 5% CO_2 incubator.

Plate Size	αMEM + 2mM L-glutamine + 1% FBS
35 mm	2 ml
60 mm	4 ml
100 mm	10 ml

7. After 24 hours, assay the cells for expression of your protein or β-galactosidase. To analyze expression, proceed to **Analysis of Protein Expression**, page 28.

Important

Adding αMEM + 2mM L-glutamine + 1% FBS (instead of 5%) allows the cells to express protein over a longer period of time without cytopathic effects.

NOTE

The incubation in Step 5, previous page, can also be performed at a lower temperature (+4°C). Low temperature prevents endocytosis, allowing maximal binding of Sindbis pseudovirions to the plasma membrane. Shifting the temperature to 37°C after the one hour incubation allows synchronous infection by Sindbis and promotes the shut down of host cell protein synthesis (Frolov and Schlesinger, 1994).

Concentration of Pseudovirions

If you find that expression is low even with undiluted virus, you may have to concentrate the pseudovirions prior to infection. You may use medium containing pseudovirions from transfected cells or thaw frozen viral stock. The particles are sedimented by ultracentrifugation onto a sucrose cushion. This requires an ultracentrifuge and a SW-41, or equivalent, rotor. To make the sucrose solutions:

- 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA
- 55% sucrose (w/v) in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA
- 20% sucrose (w/v) in 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA

1. If starting from a transfection, remove medium from the transfected BHK cells (page 24, Step 8). Centrifuge medium for 15 minutes at 2000 x g in a tabletop centrifuge at +4°C. This will clear the medium of any remaining cells and cellular debris. You should have at least 8 ml of medium containing pseudovirions.
2. If starting with frozen pseudovirion stock, thaw enough aliquots to yield 8 ml.
3. If the volume of pseudovirions is less than 8 ml, use 50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA to bring the volume to 8 ml.
4. Place 1 ml of the 55% sucrose solution in a SW-41 centrifuge tube.
5. Carefully layer 3 ml of the 20% sucrose solution onto the 55% sucrose cushion.
6. Layer 8 ml of your sample on top of the 20% sucrose solution. Fill tube about 2 mm from top.

continued on next page

Concentration of Pseudovirions, continued

7. Centrifuge 90 minutes at 30,000 rpm (~160,000 x g) in a Beckman SW-41 rotor to sediment the viral particles onto the 55% sucrose cushion.
8. Carefully aspirate the top fraction containing the medium and remove 2 ml of the 20% sucrose solution.
9. Collect the virus band from the 20%/55% interface in a total volume of 1 ml. Divide band into 50 to 100 µl aliquots and freeze on dry ice or in liquid nitrogen. Store at -80°C. Determine the amount of virus needed to infect as described on page 25.

If you find that you have to concentrate pseudovirions routinely to maximize infection, you may wish to re-examine your transfection conditions. The more efficient your transfection, the more pseudovirions you will harvest. You may try changing to another method of transfection or optimizing your present method.

Infecting Other Cell Lines With Recombinant Sindbis Virions

We recommend using BHK cells to produce pseudovirions as these cells yield the maximum amount of pseudovirions. This viral stock may be used to infect the cell line of choice. We recommend that you:

- Perform a time course of expression to determine the point of maximum expression in your cell line (4-72 hours after infection).
- Optimize the amount of virus needed to obtain maximum expression. You may find that different amounts of virus may affect expression.

Analysis of Protein Expression

Introduction

For most proteins expressed in the Sindbis Expression System, expression levels are in the range of microgram of recombinant protein per mg total cell protein. For CAT expression in C6/36 mosquito cells and BHK cells, levels of CAT reached 3% of the total cellular protein (Olson, *et al*., 1992; Xiong, *et al*., 1989). In some cases, it is possible to visualize your protein using SDS-PAGE and Coomassie blue staining. Comparison of the transfected cell lysate with an untransfected lysate facilitates identification of your protein.

Alternatively, western blotting (if you have antibody) or metabolic labeling using a radioisotope such as [^{35}S]-methionine can be used to visualize your protein (Frolov and Schlesinger, 1994). Since the Sindbis replication cycle inhibits host protein synthesis, the expressed protein should be the prominent band on the resulting autoradiogram. Purification protocols such as immunoprecipitations should not be necessary to identify the expressed protein.

Before Starting

You will need the following solutions and equipment:

- 35 mm plate with transfected BHK cells, 80-90% confluent
- PBS **with cations**
- 1.5 ml microcentrifuge tubes
- Microcentrifuge
- 250 mM Tris-Cl, pH 8.0
- Nonidet P-40 detergent (10% stock)
- 250 mM Tris-Cl, pH 8.0, 1% NP-40, 150 mM NaCl
- Dry ice/ethanol bath
- 37°C temperature block or water bath
- SDS-PAGE sample buffer, solutions, and apparatus (prepare an SDS-polyacrylamide gel that will resolve your protein)

Preparation of Cell Lysates

There are a number of ways to prepare cell lysates for analysis on SDS-PAGE. The following two procedures (with and without detergent) are included for your convenience. One 35 mm plate (~5 x 10^5 cells) is sufficient for each sample to be tested. It is important to include a plate of untransfected (uninfected) cells as a negative control and a plate of cells transfected (infected) with recombinant RNA made from SinRep/*lac*Z as a positive control.

1. Wash cells two times with half the initial culture volume of PBS.
2. Scrape cells using a rubber policeman into 1 ml PBS **with cations.** Transfer to a 1.5 ml microcentrifuge tube.

continued on next page

Preparation of Cell Lysates, continued

3. Centrifuge 1-2 minutes at maximum speed in a microcentrifuge. Decant the supernatant. At this point you may proceed to the **left** column for cell lysis **without** detergent or to the **right** column for cell lysis **with** detergent.

Step	Without Detergent	With Detergent
4.	Resuspend cell pellet in 30-50 µl 250 mM Tris-Cl, pH 8.0	Resuspend cell pellet in 30-50 µl 250 mM Tris-Cl, pH 8.0, 1% NP-40, 150 mM NaCl.
5.	Freeze cell suspension in a dry ice/ethanol bath and then thaw at 37°C. Repeat two more times.	Incubate at 37°C for 10 minutes, vortex, and then place on ice.

6. Pellet the cell debris by centrifuging 1-2 minutes at maximum speed in a microcentrifuge. Transfer supernatant to a new tube.
7. Add your SDS-PAGE sample buffer of choice and boil 5 minutes. Load 20-25% of your sample onto an SDS-PAGE and process according to your own procedure.
8. If solution is too viscous to load samples, shear DNA by passing through a 22 gauge needle 5-10 times.

SDS-PAGE Analysis

Compare the lane containing the sample from the untransfected (uninfected) cells with the lane containing the sample from cells expressing the recombinant protein. For expression in BHK cells, you may be able to see a discrete, overexpressed band at the correct molecular weight for your protein. However, in other cell lines, it may be necessary to use a western blot or metabolic labeling (see below) to visualize the recombinant protein.

Metabolic Labeling

This procedure is designed to quickly determine whether your protein is expressed. You may assay cells starting 4 hours postinfection or posttransfection; however, you may wish to wait until 24 hours after infection or transfection to ensure expression of your construct. You will need the following reagents:

- Methionine-free MEM (not αMEM), available from ICN or LTI
- αMEM containing 2 mM L-glutamine **only (no FBS)**
- [^{35}S]-methionine, 10 µCi/µl
- PBS
- SDS-PAGE loading buffer, solutions, and apparatus
- Film for autoradiography

To label cells in a 35 mm plate, use the following protocol.

1. Wash the cells two times with half the initial culture volume of PBS.
2. Incubate the monolayer at 37°C in 400 µl methionine-free medium for 20-30 minutes.
3. Add 5-10 µCi [^{35}S]-methionine and incubate the cells for 20-30 minutes at 37°C.
4. Add 1 ml of αMEM containing 2 mM L-glutamine (no FBS) to dilute isotope. Incubate for 10 minutes.
5. After the 10 minute incubation, wash the monolayer three times with PBS and collect the monolayer in 1 ml PBS using a rubber policeman to scrape cells.
6. Pellet the cells by centrifugation and dissolve the cell pellet in 25-50 µl SDS-PAGE loading buffer.
7. Analyze one-fifth of the sample (5-10 µl) on an SDS-PAGE and autoradiograph.

continued on next page

Troubleshooting

Use the following table to troubleshoot protein expression in BHK cells. Be sure to run a sample from cells transfected with RNA from SinRep/*lac*Z. The cells should produce a discrete, overexpressed band at ~117 kDa for β-galactosidase when analyzed on a Coomassie-stained SDS-PAGE gel.

If...	And....	Then
you do not see an overexpressed band for your protein on Coomassie blue-stained SDS-PAGE gel....	the control protein, β-galactosidase, is expressed,....	expression of your protein may be low. Perform a time course to optimize expression.
	you have antibody to your protein....	perform a western blot to determine if your protein is expressed.
	you do **not** have antibody to your protein,...	use a functional assay or the metabolic labeling protocol described on page 29 to test for expression of your protein.
	expression of your protein is still not detectable by western blot or metabolic labeling or is very low,....	you may wish to sequence your construct if you have not already done so to confirm that your gene has a Kozak initiation ATG and a stop codon.
	the control protein is **not** expressed,....	the RNA preparation was of poor quality or transfection (or infection) was not efficient.
you are trying to produce pseudovirions	the expression of your protein and the control protein is low,....	try infecting with a different helper virus.

Appendix

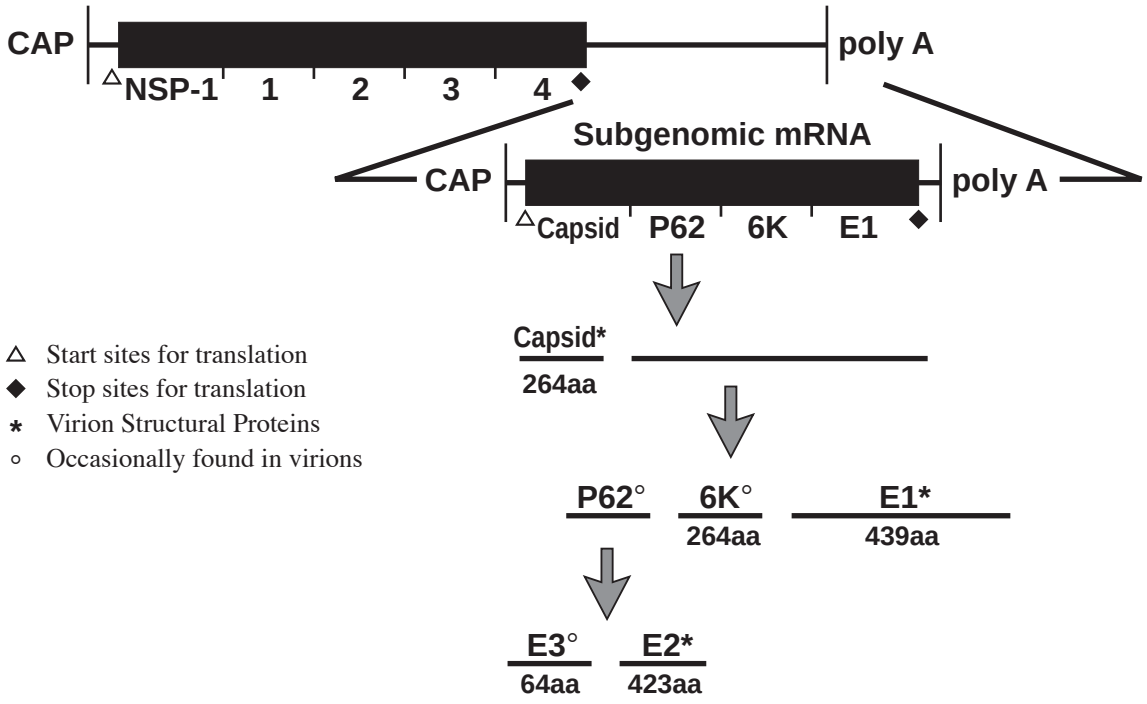
Sindbis Virus Life Cycle

Alphaviruses

The Sindbis virus is a member of the alphavirus family. These viruses are small-enveloped viruses with single-stranded RNA genomes. The RNA genome has a positive polarity and replicates in virtually all eukaryotic cells. A quick review of the Sindbis life cycle is provided below for your convenience (Schlesinger and Schlesinger, 1990). For more information about Sindbis replication, please refer to (Peters and Dalrymple, 1990; Schlesinger, 1993; Strauss and Strauss, 1994).

Sindbis Genome and Expression of Viral Proteins

The complete sequence of the Sindbis genome has been determined and is approximately 11,700 nucleotides (Rice and Strauss, 1981; Strauss, *et al*., 1984). During replication two mRNA species are produced, the full-length genomic RNA and the smaller subgenomic RNA. The 5´ ends of both transcripts are capped with 7-methylguanosine and the 3´ ends are polyadenylated. The genomic RNA transcript codes for the replication, or non-structural, proteins (*nsP1-4*) while the subgenomic transcript codes for the structural proteins required for assembly and budding of the virus (capsid, p62, 6K, and E1).



Infection

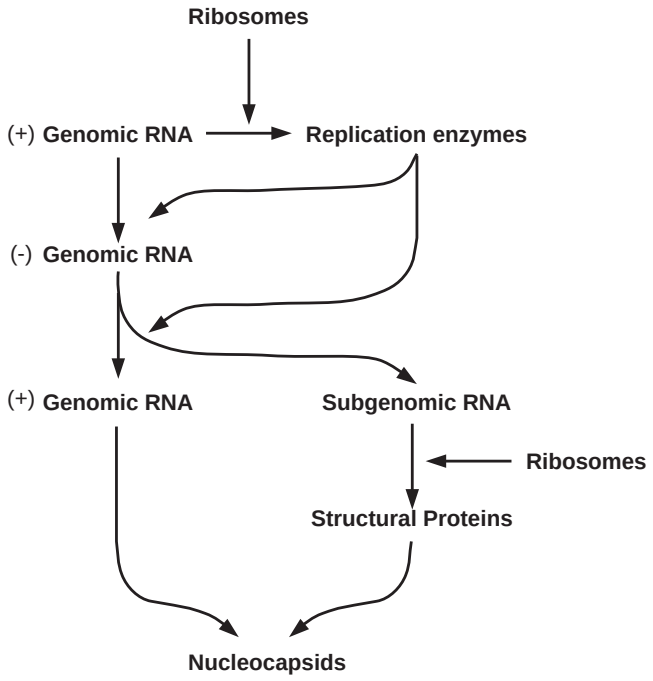
Virus particles bind to the cell membrane through glycoprotein spikes (E1 and E2) in the viral membrane. Once bound to the cell surface, Sindbis virions are thought to be endocytosed into the cell cytoplasm by coated vesicles. These vesicles become uncoated and acidify, promoting fusion of the viral membrane with the vesicle membrane and releasing the nucleocapsid into the cytoplasm. Endocytosis may not be the only mode of entry. Sindbis virus may also fuse directly with the plasma membrane to release the nucleocapsid into the cytoplasm. (Fan and Sefton, 1978).

continued on next page

Sindbis Virus Life Cycle, continued

Translation and Replication

The nucleocapsid is released from the viral RNA genome by an as yet undefined mechanism, and the RNA genome functions as a mRNA in the cytoplasm of the cell. Ribosomes bind to the genomic RNA (the plus-strand) and produce the replication enzymes necessary for the production of minus-strand RNA, additional genomic, plus-strand RNA, and subgenomic, plus-strand RNA. The minus strand is used as a template to make more genomic-length RNA (plus-strand). Once enough genomic-length RNA has been synthesized, the minus-strand is used to generate the subgenomic plus-strand RNA.



Temporal Regulation

Production of plus- and minus-strand genomic RNA occurs during the first 3-3.5 hours after infection. Minus-strand synthesis then stops while plus-strand genomic and subgenomic RNA synthesis continues. Most of the genomic, plus-strand RNA molecules become packaged into nucleocapsids, so there is an excess of subgenomic RNA molecules over genomic molecules (10:1). The subgenomic RNA molecules recruit nearly all the host's ribosomes to produce the viral structural proteins (Strauss and Strauss, 1994).

Production of Structural Proteins

The structural polypeptides result from several proteolytic cleavages that occur as the nascent polyprotein is translated. The capsid protein is the first protein released, and it rapidly interacts with genomic, plus-strand RNA to form the nucleocapsid. The next protein, p62 is translated and translocated into the endoplasmic reticulum (ER). The polyprotein is again cleaved, releasing p62 into the endoplasmic reticulum. Further translation creates another signal for membrane insertion to direct the E1 protein into the endoplasmic reticulum. E1 and p62 quickly form a heterodimer in the ER and are processed further (see next page).

continued on next page

Maturation and Transport

As p62 and E1 proteins move through the ER and the Golgi apparatus, the following modifications occur:

- Glycosylation of p62 and E1 in the ER
- Acylation of both p62 and E1 in the ER
- Proteolysis of p62 to yield E2 and E3 in the Golgi

It is not known what role these modifications play in the viral life cycle. They do not seem to function as signals for sorting or plasma membrane localization. These post-translational modifications may have some role in promoting fusion of the virus with host membranes and promoting interactions with the host lipids or the viral nucleocapsids. E1 and E2 become the glycoprotein spikes in mature virus while the E3 protein is rarely found in Sindbis virions.

Assembly and Budding

Assembly of the virion occurs at the plasma membrane (Scheefers, *et al*., 1980; Simons and Garoff, 1980; Smith and Brown, 1977). The heterodimer of E1 and E2 inserts into the plasma membrane. From electron micrographs, almost all of the E1/E2 glycoprotein is immobilized in patches of budding virus. It is thought that the E2 cytoplasmic tail provides the binding site for the nucleocapsid. This interaction between E2 and the nucleocapsid is thought to initiate the actual budding and release of the virion.

Recipes

Complete αMEM Medium

αMEM medium with 2mM L-glutamine is available from Invitrogen in 500 ml bottles (Catalog no. Q400-01). To make **complete αMEM medium**, add FBS to a final concentration of 5%.

Phosphate Buffered Saline With Cations

For washing cells only. The solution does not need to be RNase-free.

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
0.9 mM $CaCl_2$-$2H_2O$
0.5 mM $MgCl_2$-$6H_2O$

1. Dissolve:
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   0.13 g $CaCl_2$-$2H_2O$
   0.10 g $MgCl_2$-$6H_2O$
   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter and autoclave for 20 minutes on liquid cycle.
4. Store at +4°C or room temperature.

Phosphate Buffered Saline Without Cations, RNase-free

For electroporation of cells:

137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

1. Dissolve:
   8 g NaCl
   0.2 g KCl
   1.44 g Na_2HPO_4
   0.24 g KH_2PO_4
   in 800 ml deionized water.
2. Adjust pH to 7.4 with concentrated HCl.
3. Bring the volume to 1 liter.
4. Add 1 ml diethylpyrocarbonate (DEPC) and stir overnight to make the solution RNase-free.
5. Autoclave for 20 minutes on liquid cycle to sterilize and inactivate the DEPC.
6. Store at +4°C or room temperature.

continued on next page

Formaldehyde/ Glutaraldehyde Solution

2% formaldehyde
0.2% glutaraldehyde
in PBS without cations, pH 7.4

1. Make fresh and prepare only what you need. Use 1 ml of reagent per 35 mm plate, 2 ml per 60 mm plate, and 5 ml per 100 mm plate.
2. For 10 ml of reagent, mix

   0.54 ml 37% formaldehyde
   40 µl 50% glutaraldehyde
   9.42 ml PBS without cations, pH 7.4
3. Use immediately.

X-Gal Reagent for Staining Cells

1 mg/ml X-Gal
4 mM potassium ferricyanide ($K_3Fe(CN)_6$)
4 mM potassium ferrocyanide ($K_4Fe(CN)_6$-$3H_2O$)
2 mM magnesium chloride, hexahydrate
in PBS (without cations), pH 7.4

Prepare just enough solution to stain the number of plates you desire. You will use 1 ml of reagent to stain a 35 mm plate, 2 ml for a 60 mm plate, and 5 ml for a 100 mm plate. Multiply by the number of plates you are staining to determine the total volume of reagent needed.

1. Prepare 10 ml each of the following stock solutions. Solutions are stable indefinitely if stored as indicated.
   - X-gal: 20 mg/ml in dimethylformamide (DMF). Dissolve 200 mg in 10 ml DMF and store at -20°C.
   - Potassium Ferricyanide and Potassium Ferrocyanide: 0.4 M each in deionized water. Dissolve 1.32 g of potassium ferricyanide and 1.69 g of potassium ferrocyanide in 10 ml deionized water. Store at -20°C.
   - Magnesium Chloride: 200 mM in deionized water. Dissolve 0.4 g in 10 ml deionized water and store at room temperature or -20°C.
2. For 10 ml of reagent, mix together:

   0.5 ml of 20 mg/ml X-Gal stock solution
   0.1 ml of the potassium ferricyanide/ferrocyanide stock solution
   0.1 ml of the magnesium chloride stock solution
   9.3 ml of PBS without cations
3. Solution is ready for use.

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continued on next page

Recipes, continued

Z Buffer

60 mM Na_2HPO_4-$7H_2O$
40 mM NaH_2PO_4-H_2O
10 mM KCl
1 mM $MgSO_4$-$7H_2O$
50 mM β-mercaptoethanol
pH 7.0

1. Dissolve: 16.1 g Na_2HPO_4-$7H_2O$
   5.5 g NaH_2PO_4-H_2O
   0.75 g KCl
   0.246 g $MgSO_4$-$7H_2O$
   2.7 ml β-mercaptoethanol
   in 950 ml deionized water.
2. Adjust pH to 7.0 with either NaOH or HCl and bring the volume up to 1 liter with water.
3. Do not autoclave! Store at +4°C.

ONPG Solution

4 mg/ml in 100 mM phosphate buffer, pH 7.0

1. Dissolve 1.61 g Na_2HPO_4-$7H_2O$
   0.55 g NaH_2PO_4-H_2O
   in 90 ml deionized water.
2. Adjust pH to 7.0 with either NaOH or HCl and add 400 mg of ONPG. Stir to dissolve and bring the volume up to 100 ml with water.
3. Store at -20°C away from light.

---

1 M Sodium Carbonate

Dissolve 12.4 g sodium carbonate in 100 ml of deionized water. Store at room temperature.

Purpose

The purpose of the following procedure is to determine the quantity of RNA produced using the InvitroScript™ CAP SP6 *in vitro* Transcription Kit (page 16). Please note that this method will tend to over estimate the amount of RNA in the sample because it is difficult to remove all of the free radioactive-labeled nucleotides.

Before Starting

You will need to prepare or have on hand the following reagents and supplies:

- [α-^{32}P] UTP, 10 μCi/μl
- G-50 Sephadex spin columns (Pharmacia)
- 37°C heat block or water bath
- Whatman 3MM paper
- Scintillation cocktail

Important

The concentration of GTP limits the amount of RNA that can be synthesized; therefore, it is important to use a tracer other than GTP.

Procedure

1. After setting up the *in vitro* transcription reaction (page 17, Step 5), withdraw 5 μl of the reaction and add 0.5 μl [α-^{32}P] UTP.
2. Incubate for 2 hours at 37°C.
3. Add RNase-free, deionized water to 100 μl to stop the reaction.
4. Remove 50 μl of the sample and centrifuge through a G-50 spin column according to manufacturer's instructions. This will remove unincorporated nucleotides. Flow-through will contain mRNA (incorporated [α-^{32}P] UTP). Reserve the remaining sample (50 μl) to determine the total (incorporated + unincorporated) amount of radioactivity in the transcription reaction.
5. Take 5 μl aliquots from the two RNA samples in Step 4, spot on individually labeled Whatman 3MM paper disks, and allow to dry.
6. Add the papers to scintillation cocktail and count in a scintillation counter. The ratio of the incorporated to total radioactivity from Step 4 represents the fraction of radiolabeled nucleotide incorporated into RNA.

Calculation of Yield

Incorporation of 1% of the ^{32}P counts corresponds to 1.3 μg of RNA synthesized using the InvitroScript™ CAP SP6 *in vitro* Transcription Kit. Use the counts determined in Steps 1-5, above, to calculate the yield of mRNA.

Calculation of yield:

$$\frac{\text{Incorporated [}\alpha\text{-}^{32}\text{P] UTP}}{\text{Total [}\alpha\text{-}^{32}\text{P] UTP}} \times 100 \times 1.3\ \mu\text{g RNA} = \mu\text{g RNA per 20 } \mu\text{l reaction}$$

SinRep/*lac*Z (Incorporated) 150079 cpm
SinRep/*lac*Z (Total) 890989 cpm

(150079/890989) (100) (1.3 μg) = 21.9 μg/20 μl reaction

Note that this gives the yield in a 20 μl reaction; therefore if the reaction volume is doubled so is the calculated yield. The theoretical yield of a 20 μl reaction is 40 μg.

Staining Cells for β-galactosidase Expression

Introduction

The SinRep/*lac*Z positive control template can be used to check expression levels and transfection efficiencies. A quick way to do this is to stain the transfected cells directly in the tissue culture dish. Comparison of the number of stained cells on a transfected or infected plate to an untransfected plate can be used to determine the efficiency of transfection. Alternatively, a transfected or infected cell lysate can be assayed for β-galactosidase activity.

Before Starting

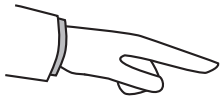
You will need the following reagents and equipment:

- 35 mm plates with transfected or infected cells, 7-16 hours posttransfection
- PBS with cations
- 2% formaldehyde, 0.2% glutaraldehyde in PBS, pH 7.3 (See **Recipes**, page 34)
- X-gal Reagent (see **Recipes**, page 34)

Staining Cells for β-galactosidase Activity

At any point 4 hours posttransfection, you may stain the cells for β-galactosidase activity. Please note that this procedure will kill the cells. Also, to detect β-galactosidase activity at early time points, it may be necessary to incubate the cells in the X-gal reagent for longer periods to detect color development (Steps 6 and 7). Optimal expression may not occur until later time points.

1. Aspirate the tissue culture medium from the dish containing the cells to be stained.
2. Rinse twice with PBS using half the volume of the initial culture medium.
3. Fix the cells in 2% formaldehyde, 0.2% glutaraldehyde in PBS, pH 7.3 for 10 minutes at room temperature.
4. Rinse twice with half the volume of the initial culture medium of PBS.
5. Stain with the X-gal reagent using half the volume of the initial culture medium at 37°C and incubate for 15 minutes.
6. After incubating for 15 minutes, check the cells under the microscope for the development of blue color in the cells.
7. If cells are not blue, return the cells to 37°C and keep checking until you see a blue color develop. Color development should be visible within 30-60 minutes after addition of the X-gal reagent.



NOTE

For your convenience, the β-Gal Staining Kit (Catalog no. K1465-01) is available from Invitrogen. The kit contains all the reagents described above to stain whole cells for β-galactosidase expression.

Introduction

In the β-galactosidase assay, the lactose analog orthonitrophenyl-β-D-galactoside (ONPG), is added to the cell lysate (Miller, 1972, p. 403). ONPG is cleaved by β-galactosidase to produce the ONP anion which produces a yellow color under basic conditions. The assay is monitored by observing the change in absorbance at 420 nm using a UV-VIS spectrophotometer. A protein assay is also performed for each sample in order to normalize the number of β-galactosidase units per mg total protein (Bollag and Edelstein, 1991).

Before Starting

The solutions for the assay are found in the **Recipe** section, page 34. You will need to make the following solutions:

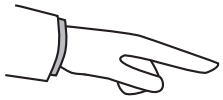
- 4 mg/ml ONPG
- "Z" Buffer
- 1 M Sodium carbonate

Assay for β-galactosidase Protein

1. To test each lysate (see **Analysis of Protein Expression**, page 28), dilute 1:100 with Z buffer. Take 1-10 µl and transfer to a fresh microcentrifuge tube.
2. Add distilled, deionized water to a final volume of 30 µl.
3. Add 66 µl of 4 mg/ml ONPG and 204 µl "Z" buffer. Mix by gently flicking the tube and centrifuging briefly.
4. Incubate the tube at 37°C for 30 minutes. You should see a faint yellow color develop if β-galactosidase is present.
5. To stop reaction, add 500 µl of 1 M sodium carbonate.
6. Read the absorbance at 420 nm against a blank without lysate. Be sure to assay a sample of the untransfected cell lysate as a control.
7. Be sure to assay at least three different volumes of lysate. Changes in absorbance should be linear with respect to the amount of lysate assayed. If it is not, you will not get an accurate determination of expression. The most common error is using too much lysate which will cause you to overestimate the expression levels. Decrease the amount of lysate until absorbance is linear with the amount of lysate.
8. Once you have determined you are assaying the lysate accurately, determine the protein concentration of the lysate, and calculate the activity of the lysate using the following formula:

   β-galactosidase Units = $(OD_{420} \times 380)/ t / mg$ protein assayed

   where t = the time of incubation in minutes at 37°C, 380 is a conversion factor to convert absorbance to µmoles of ONPG, and mg protein is the amount of protein assayed. Be sure to subtract the background activity of the untransfected cell lysate.



NOTE

For your convenience, the β-Gal Assay Kit (Catalog no. K1455-01) is available from Invitrogen. The kit contains all the reagents described above to assay cell lysates for β-galactosidase expression.

Miniprep Plasmid Preparation

Introduction

Generally, the cleaner the template DNA, the greater the yield from the *in vitro* transcription reaction. Template DNA purified on a CsCl gradient gives the highest yields. However, the miniprep procedure below yields DNA template of sufficient quality for *in vitro* transcription. **The procedure uses a Proteinase K digestion step to ensure that all ribonuclease is degraded prior to the *in vitro* transcription reaction.**

Before Starting

You will need the following reagents and equipment:

- ~1.5 ml of bacterial culture (*E. coli* transformed with pSinRep5 containing your gene)
- Sterile 50 mM glucose, 10 mM EDTA, pH 8, 25 mM Tris-HCl, pH 8.0
- 0.2 N NaOH, 1% SDS (make fresh)
- 3 M potassium/5 M acetate (for 100 ml, mix 60 ml 5 M potassium acetate and 11.5 ml glacial acetic acid with 28.5 ml deionized water)
- 100% ethanol
- 10 mg/ml RNase
- TE with 20 µg/ml RNase (Add 1 µl of 10 mg/ml RNase to 500 µl TE)
- *Xho* I, *Not* I, or *Pac* I restriction enzyme and appropriate buffer
- 20 mg/ml Proteinase K
- Phenol/chloroform
- 5 M ammonium acetate (RNase-free)
- RNase-free water

Isolation of Nucleic Acid and RNase Digestion

The procedure below starts with the basic alkaline miniprep procedure to isolate nucleic acid.

1. Centrifuge 1.5 ml of the bacterial culture at room temperature for 30 seconds. Remove all the medium.
2. Add 110 µl of 50 mM glucose, 10 mM EDTA, pH 8, 25 mM Tris-HCl, pH 8.0 to the pellet and vortex vigorously to fully resuspend the bacterial pellet.
3. Add 220 µl of 0.2 N NaOH, 1% SDS, invert the tube 10 times to mix, and incubate on ice for 1 minute (or longer).
4. Add 165 µl 3 M potassium/5 M acetate, vortex for 10 seconds, and incubate for 5 minutes on ice.
5. Centrifuge at +4°C for 5 minutes at maximum speed in a microcentrifuge.
6. Transfer the supernatant to a tube containing 1 ml ethanol, invert several times to mix, and incubate on ice for 5 minutes.
7. Centrifuge at +4°C for 5 minutes at maximum speed. Decant supernatant, centrifuge briefly, and aspirate off residual supernatant.
8. Resuspend nucleic acid pellet in 50 µl TE buffer containing 20 µg/ml RNase. Vortex vigorously and incubate for 5 minutes at 37-42°C. Make sure pellet is solubilized.

continued on next page

Restriction-Proteinase K Digestion

After removing RNA, the plasmid is ready to be linearized prior to *in vitro* transcription. Choose a restriction enzyme that will linearize the plasmid without cutting your gene. After restriction digestion, Proteinase K is added to remove all enzymes. The digest is then extracted with phenol/chloroform and the DNA ethanol precipitated. The volume of the restriction digest should be at least 2-3 times the volume of the miniprep used. For example, it your miniprep volume is 10 µl, perform the restriction digest in a 20-30 µl volume.

1. Digest miniprep DNA (all or part) with *Xho* I, *Not* I, or *Pac* I. Digest at 37°C for at least 1 hour, using 0.3-0.8 units of restriction enzyme per microliter of miniprep DNA.
2. Add a 1/10 volume of Proteinase K (20 mg/ml), mix by pipetting, and incubate at 50°C for at least 30 minutes.
3. Extract the digest once with phenol/chloroform and remove the top, aqueous phase to a new tube.
4. Add 1/10 volume of 5 M ammonium acetate (RNase-free) and 2 volumes of ethanol. Incubate at -20°C for at least 15 minutes.
5. Centrifuge the solution at maximum speed in a microcentrifuge for 15 minutes at +4°C. Decant supernatant, centrifuge briefly, and remove all the ethanol.
6. Resuspend DNA pellet in 10-20 µl RNase-free water per 50 µl of miniprep nucleic acid per 1.5 ml bacterial culture.
7. Determine concentration of the DNA. You will need 0.5-1.0 µg of template DNA per a 20 µl *in vitro* transcription reaction. This will be about 1-3 µl of the 10-20 µl preparation in Step 6.

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Citations

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