

ViraPort cDNA Library Retroviral Supernatants

INSTRUCTION MANUAL

Revision A

BN #972000-12

For In Vitro Use Only
972000-12

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Agilent Technologies

Stratagene Products Division

11011 North Torrey Pines Road

La Jolla, CA 92037

Telephone (858) 373-6300

Order Toll Free (800) 424-5444

Technical Services (800) 894-1304

Internet techservices@agilent.com

World Wide Web www.stratagene.com

Europe

Location	Telephone	Fax	Technical Services
Austria	0800 292 499	0800 292 496	0800 292 498
Belgium	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 15775	0800 15740	0800 15720
France	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 919 288	0800 919 287	0800 919 289
Germany	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 182 8232	0800 182 8231	0800 182 8234
Netherlands	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 023 0446	+31 (0)20 312 5700	0800 023 0448
Switzerland	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 563 080	0800 563 082	0800 563 081
United Kingdom	00800 7000 7000	00800 7001 7001	00800 7400 7400
	0800 917 3282	0800 917 3283	0800 917 3281

All Other Countries

Please contact your local distributor. A complete list of distributors is available at www.stratagene.com.

ViraPort cDNA Library Retroviral Supernatants

CONTENTS

Materials Provided	1
Storage Conditions	1
Additional Materials Required	1
Notices to Purchaser	2
Safety Considerations	5
Undesired Production of Replication-Competent Retrovirus.....	6
Introduction	7
Overview of Replication-Defective Retroviral Gene Transfer Systems.....	8
Description of the pFB Retroviral Vector.....	10
Determination of Target Cell Transduction Efficiency Using the pFB-hrGFP Control Viral Supernatant	12
Determination of Target Cell Transduction Efficiency Using the pFB-Neo-LacZ Control Vector	13
Transduction of Target Cells with Control Viral Supernatant	14
Day 1: Preparing for Control Transduction.....	14
Day 2: Transducing the Target Cells.....	14
Day 4: Performing hrGFP Fluorescence Detection.....	15
Transduction of Target Cells with ViraPort cDNA Library Supernatants	16
Day 1: Preparing for Transduction of ViraPort retroviral cDNA library.....	17
Day 2: Transducing the Target Cells.....	17
Recovery of cDNA Clones from Positive Transductants	18
PCR Reaction.....	18
PCR Program.....	19
Troubleshooting	20
Preparation of Media and Reagents	21
References	21
Endnotes	21
MSDS Information	21

ViraPort cDNA Library Retroviral Supernatants

MATERIALS PROVIDED

Material provided	Quantity
Frozen tissue culture supernatant containing VSV-G pseudotyped retrovirus produced from an amplified pFB XR plasmid cDNA library ^a	$\geq 2 \times 10^6$ infectious virus particles
5' Retro primer (lyophilized) ^b	2.5 μ g
3' pFB primer (lyophilized) ^b	2.5 μ g
pFB-hrGFP control viral supernatant	$\geq 10^6$ infectious virus particles

^a ViraPort cDNA library retroviral supernatant was harvested from tissue culture producer cells, filtered with a 0.45 μ m filter and immediately frozen at -80°C . Store at -80°C immediately upon arrival.

^b Spin the tube briefly in a microcentrifuge to pellet the lyophilized primer in case it has become dislodged during shipping. For use in PCR, resuspend each primer in 25 μ l of TE buffer[§] to a final concentration of 100 ng/ μ l. For use in sequencing, dilute the 100 ng/ μ l stock 1:5 to a final concentration of 20 ng/ μ l.

STORAGE CONDITIONS

Retroviral Supernatant: -80°C Store immediately upon arrival!

pFB-hrGFP Control Viral Supernatant: -80°C Store immediately upon arrival!

Primers: -20°C

ADDITIONAL MATERIALS REQUIRED

NIH3T3 cells for validation of control viral supernatant

5-ml BD Falcon polystyrene round bottom tubes (BD Biosciences catalog #352054)

Growth medium for cell culture[§]

TE buffer[§]

DEAE-dextran solution[§]

TaqPlus Precision PCR System [Stratagene Catalog #600210]

6-well and 96-well tissue culture plates

100-mm tissue culture plates

[§] See *Preparation of Media and Reagents*

Revision A

© Agilent Technologies, Inc. 2009.

NOTICES TO PURCHASER

ViraPort Retroviral Products

Agilent hereby licenses Customer to use Agilent's ViraPort cDNA Library Retroviral Supernatants and/or pVPack Vectors, as the case may be, provided herewith (referred to hereinafter as the "Products") on the following terms and conditions. (For purposes of this Notice, "Customer" shall include any person or entity which ordered the Products or at any time uses the Products.) Customer's acceptance of delivery and/or use of the Products shall constitute Customer's binding agreement to the following terms and conditions. If Customer is unwilling to accept such terms and conditions, Agilent is willing to accept return of the Products prior to any use of the Products, for a full refund.

1. The Products shall be used solely for research purposes, solely on premises under the control of Customer, solely by employees or agents of Customer, and solely in compliance with all laws, regulations, rules and guidelines applicable to the Products and their use, testing, handling, or other disposition thereof, or otherwise applicable to Customer's activities hereunder.
2. Customer acknowledges and agrees that Customer is experienced and/or informed regarding the characteristics and use of MMLV-based retroviral products such as the Products, for what purposes the Products are to be used, and how the Products should be used.
3. THE PRODUCTS ARE EXPERIMENTAL IN NATURE AND ARE PROVIDED WITHOUT WARRANTIES OF ANY KIND, EXPRESS OR IMPLIED, INCLUDING, WITHOUT LIMITATION, WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Customer hereby waives, releases and renounces any and all warranties, guarantees, obligations, liabilities, rights and remedies, express or implied, arising by law or otherwise, with respect to the usefulness or freedom from defects of the products, including, but not limited to, (a) any implied warranty or merchantability or fitness for a particular purpose, (b) any implied warranty arising from course of performance, course of dealing or usage in the trade, and (c) any obligation, right, liability, claim or remedy for (1) loss of use, revenue or profit, or any other damages, (2) infringement of third party intangible property rights, and (3) incidental or consequential damages.
4. Customer shall bear all risks associated with the Products and their use, testing, handling or other disposition thereof, and all risks associated with Customer's activities under this Agreement. Customer hereby assumes all risks of damage or injury to Customer's facilities, employees or agents and to any third party arising from possession or use of the Products. Agilent shall have no liability to Customer, its employees or agents or to any third party, regardless of the form or theory of action (whether contract, tort or otherwise, including but not limited to, negligence and strict liability), for any direct, indirect, consequential, incidental or other damages arising out of or relating to the Products or this Agreement.
5. Customer may at any time properly dispose of the Products in a manner which ensures their prompt destruction and is consistent with all applicable laws, regulations, rules and guidelines.
6. No modification or waiver of any terms or conditions of this Notice shall be effective unless in a writing signed by Customer and an authorized representative of Agilent.

Vitality Fluorescent Protein Products Non-Commercial Research Use License For Nonprofit Entities

Agilent agrees to sell, and Licensee agrees to purchase, Agilent's Vitality fluorescent protein products provided herewith (referred to as the "Products") on the following terms and conditions. (For purposes of this License, "Licensee" shall include any person or entity which ordered the Products or at any time uses the Products.) LICENSEE'S ACCEPTANCE OF DELIVERY AND/OR USE OF THE PRODUCTS SHALL CONSTITUTE LICENSEE'S BINDING AGREEMENT TO THE FOLLOWING TERMS AND CONDITIONS. IF LICENSEE IS UNWILLING TO ACCEPT SUCH TERMS AND CONDITIONS, AGILENT IS WILLING TO ACCEPT RETURN OF THE PRODUCTS PRIOR TO ANY USE OF THE PRODUCTS, FOR A FULL REFUND.

1. The Products, containing DNA sequences encoding for fluorescent protein or variants thereof, are proprietary or exclusively licensed to Agilent and licensed hereunder for non-commercial research purposes only. Licensee may modify only the non-coding region outside of the nucleic acid encoding the fluorescent protein of the Products to facilitate non-commercial research. Licensee shall not have any rights to (i) modify the coding region of the nucleic acid encoding the fluorescent protein of the Products, (ii) offer the Products, or any component, derivative or modification thereof, for resale, or (iii) distribute, transfer, or otherwise provide access to, the Products, or any component, derivative or modification thereof, to any third party for any purpose or use.
2. Except as set forth above, no other rights, express or implied, are conveyed to Licensee. No rights are granted to Licensee to use the Products for (i) the provision of services to any for-profit third party (e.g., screening and profiling), (ii) diagnostic applications, (iii) methods employed in screens to evaluate compounds (e.g., high throughput screening ("HTS")), (iv) profiling chemicals for selectivity, bioavailability, drug metabolism or toxicity, (v) use *in vivo* in multicellular organisms (and methods therein) for gene therapy, (vi) quality control or quality assurance processes, including food and environmental testing, or (vii) use in manufacturing.
3. The Products shall be used solely on premises under the control of Licensee, and in compliance with all laws, regulations, rules and guidelines applicable to the Products and their use, testing, handling, or other disposition thereof, or otherwise applicable to Licensee's activities hereunder.
4. Title to the Products shall not transfer to Licensee.
5. Agilent warrants that, at the time of shipment, the Products will conform to the specifications which accompany the Products. This warranty limits Agilent's liability to replacement of the Products. AGILENT MAKES NO OTHER WARRANTIES, EXPRESS OR IMPLIED, WITH RESPECT TO THE PRODUCTS, INCLUDING ANY WARRANTIES OF MERCHANTABILITY OR FITNESS FOR ANY PARTICULAR PURPOSE OR THAT THE PRODUCTS DO NOT INFRINGE ANY PROPRIETARY RIGHTS OF ANY THIRD PARTY. Licensee hereby waives, releases and renounces any and all warranties, guarantees, obligations, liabilities, rights and remedies, express or implied, arising by law or otherwise, with respect to the usefulness or freedom from defects of the Products, including, but not limited to, (a) any implied warranty or merchantability or fitness for a particular purpose, (b) any implied warranty arising from course of performance, course of dealing or usage in the trade, and (c) any obligation, right, liability, claim or remedy for (1) loss of use, revenue or profit, or any other damages, (2) infringement of third party intellectual property rights, and (3) incidental or consequential damages.

6. Licensee agrees to bear all risks associated with the Products and their use, testing, handling or other disposition thereof, and all risks associated with Licensee's use of the Products purchased hereunder. Licensee hereby assumes all risks of damage or injury to Licensee's facilities, employees or agents and to any third party arising from possession or use of the Products. Agilent shall have no liability to Licensee, its employees or agents or to any third party, regardless of the form or theory of action (whether contract, tort or otherwise, including, but not limited to, negligence and strict liability), for any direct, indirect, consequential, incidental or other damages arising out of or relating to the Products or this License.

7. Licensee shall indemnify, defend and hold Agilent, its affiliates, distributors, suppliers, directors, officers, employees and agents, harmless from and against any and all claims, actions, demands, liabilities, damages and expenses (including attorneys' fees) relating to or arising out of any damage or injury, including, but not limited to, product liability and intellectual property infringement claims of any nature, alleged to have been caused by the Products or the use, testing, handling or other disposition thereof or Licensee's activities hereunder.

8. Licensee may at any time properly dispose of the Products in a manner which ensures their prompt destruction and is consistent with all applicable laws, regulations, rules and guidelines.

9. No modification or waiver of any terms or conditions of this License shall be effective unless in writing signed by Licensee and an authorized representative of Agilent. For information on acquiring a license to use the Products for commercial purposes, including commercial research purposes, please contact Stratagene Product Division Business Development, 11011 North Torrey Pines Road, La Jolla, California 92037, telephone number (858) 373-6300, facsimile number (858) 373-5300.

SAFETY CONSIDERATIONS

Note *The safety guidelines presented in this section are not intended to replace the BSL 2+ safety procedures already in place at your facility. The information set forth below is intended as an additional resource and to supplement existing protocols in your laboratory.*

The pFB vector harboring the cDNA expression library is a replication-defective MMLV-based vector. Virus produced using the plasmid libraries is pseudotyped with the VSV-G envelope protein, and is thus capable of infecting most dividing cell types, including human cells. Prior to use of the retroviral supernatant we strongly recommend that all users become thoroughly familiar with the safety considerations concerning the production and handling of retrovirus. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site <http://www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4s3.htm>. Although for most applications production and use of retroviral vectors fall within NIH Biosafety Level 2 criteria, cDNA expression libraries are expected to contain potentially oncogenic inserts at some frequency regardless of the state of the tissue from which the cDNA was derived, and should thus be handled with BSL 2+ precautions. The University of California, San Diego (UCSD) has an active retrovirus research group, the UCSD Vector Development Lab, with experience in safety practices for use with MMLV-derived vectors. The information they have can be found at <http://medicine.ucsd.edu/gt/MoMuLV.html>. For more information regarding BSL 2+ practices, it is strongly recommended that you also consult the UCSD Environmental Health and Safety Web site <http://www-ehs.ucsd.edu/ADENO.HTM>. Most potential hazards can be avoided by practicing good tissue culture technique. The use of gloves and disposable lab coats is strongly recommended when working with virus. Prior to thawing the supernatant, the area around the cap should be carefully inspected for any sign of leakage, and thoroughly wiped with 70% ethanol. Screw caps should be removed in the hood only, and any fluid around the outside lip of the tube or the inside surface of the cap should be carefully wiped with a tissue wetted with 70% ethanol, and the tissue should be disposed of in the hood (see below). When pipetting virus-containing supernatants and transferring plates or flasks to and from the laminar flow hood, the production of aerosols should be avoided. All pipets and plasticware should be disinfected for 15 minutes with 70% ethanol, 10% bleach, or other disinfectant recommended by the UCSD Vector Development Lab prior to removal from the laminar flow hood. It is recommended that prior to removal from the laminar flow hood, disposable plasticware should be UV irradiated for 20 minutes, enclosed and taped into a red biohazard bag inside the laminar flow hood, enclosed into a second bag outside the hood and autoclaved.

Undesired Production of Replication-Competent Retrovirus

The ViraPort retrovirus was produced by transiently transfecting tissue culture cells with the pFB XR plasmid cDNA library together with two additional vectors from which the Gag-Pol and VSV-G proteins are expressed (pVPack-GP and pVPack-VSV-G, respectively). The latter two packaging vectors were designed for minimal sequence overlap with each other and with the pFB vector used to make the libraries, in order to greatly reduce the probability of production of replication-competent retrovirus (RCR) by homologous recombination between the three vectors. Despite these precautions, there is nevertheless a low risk of RCR production. Although the 293-based cell line used to produce the retroviral supernatants has been tested for RCR production, the individual library supernatants have not been tested. In addition, the use of target cell lines harboring endogenous retrovirus capable of encapsidating vector proviral RNA (encoding the cDNA) can result in the undesirable spread of vector-derived virus. There are a number of published protocols describing assays for detection of RCR based on mobilization of a provirus containing a detectable marker (“marker rescue”) or by direct detection of reverse transcriptase activity in tissue culture supernatants. It is strongly recommended that prior to infection, all potential target cell lines should be first tested for the presence of endogenous retrovirus using one of the assays described.¹ Furthermore, stable cell lines transduced with ViraPort cDNAs that are to be extensively used beyond the short term expansion of clones for PCR-retrieval of the integrated cDNA should also be tested for RCR.

INTRODUCTION

Expression cloning enables the identification and isolation of a gene based on its function or phenotype in the absence of any prior knowledge of the sequence of the nucleic acid or protein. The use of retroviral vectors for expression cloning has several advantages over traditional methods. Recent advances in viral packaging systems ensure that virtually any mitotic cell type can be transduced with efficiencies approaching 100%. The copy number of individual cDNA expression cassettes can be easily controlled by varying the multiplicity of infection (MOI). Thus, populations of infected cells may be generated in which greater than 90% of the cells are transduced with 1–5 individual cDNAs per cell, greatly reducing the time and labor of isolating the gene of interest. A wide range of gene types have been cloned from complex cDNA libraries using functional assays that allow selection or screening for a specific trait (Table I).²

TABLE I

Examples of Genes Cloned by Expression Screening

Gene class	Selection/Screen
Cell surface proteins	FACS sorting
Extracellular receptors	FACS sorting
	Proliferation or survival
Growth factors	Factor-dependent growth
Oncogenes	Loss of contact-inhibition
Cell cycle proteins	Loss of contact-inhibition
	Phenotypic complementation
Signaling proteins	Loss of contact-inhibition
	Phenotypic complementation
Transcription factors	Reporter activation
Apoptosis inhibitors	Survival: resistance to apoptosis inducers
Metastasis-inducing genes	In vivo metastasis
	In vitro invasion
Differentiation-inducing genes	Phenotype: differentiation
Ion channels	Phenotype: ion-specific indicator or tracer

The high titer viral stock provided was produced from a cDNA plasmid library of high complexity inserted directionally into the replication-defective retroviral vector pFB. The target cell line of choice is transduced by simply thawing the viral supernatant, diluting in media for infection at the desired multiplicity, and adding the diluted virus directly to the cells. Two or more days following infection an appropriate selection or screen is applied to the cells, and positive transduced cells are clonally expanded. The cDNA insert is then recovered from isolated genomic DNA by PCR using the vector-specific primers provided, and subcloned for sequencing and further functional validation. If desired, the recovered cDNA may be used as probe to re-isolate clones directly from the original plasmid library from which the viral stock was derived. Plasmid libraries are available separately as *E. coli* glycerol stocks.

The ViraPort retrovirus was produced by transiently transfecting tissue culture cells with the pFB XR plasmid cDNA library together with two additional vectors from which the Gag-Pol and VSV-G proteins are expressed (pVPack-GP and pVPack-VSV-G, respectively). Because all of the *cis* and *trans* elements required to produce infectious virus are separated onto three plasmids, with minimal or no sequence overlap between the plasmids, there is a relatively low probability of production of replication-competent retrovirus (RCR) due to homologous recombination between the vectors (however, see *Safety Considerations*). This viral production system is considerably safer than the majority of stable producer cell lines or vector-based systems for which there is a large degree of homology between the packaging vector(s) and the retroviral expression vector.³

Overview of Replication-Defective Retroviral Gene Transfer Systems

Non-replicating retroviral vectors contain all of the *cis* elements required for transcription of mRNA molecules encoding a gene of interest, and packaging of these transcripts into infectious virus particles (Figure 1). The vectors are typically composed of an *E. coli* plasmid backbone containing a pair of 600 base pair viral long terminal repeats (LTRs) between which the gene of interest is inserted. The LTR is divided into 3 regions. The U3 region contains the retroviral promoter/enhancer. The U3 region is flanked in the 3' direction by the R region, which contains the viral poly-adenylation signal (pA), followed by the U5 region which, along with R, contains sequences that are critical for reverse transcription. Expression of the viral RNA is initiated within the U3 region of the 5' LTR and is terminated in the R region of the 3' LTR. Between the 5' LTR and the coding sequence for the gene of interest resides an extended version of the viral packaging signal ($\psi+$), which is required in *cis* for the viral RNA to be packaged into virion particles.

Recent advances in transfection technology have allowed the production of high titer viral supernatants following transient cotransfection of the viral vector together with expression vectors encoding the *gag*, *pol* and *env* genes (Figure 1),^{4,5} obviating the need for the production and maintenance of stable packaging cell lines.

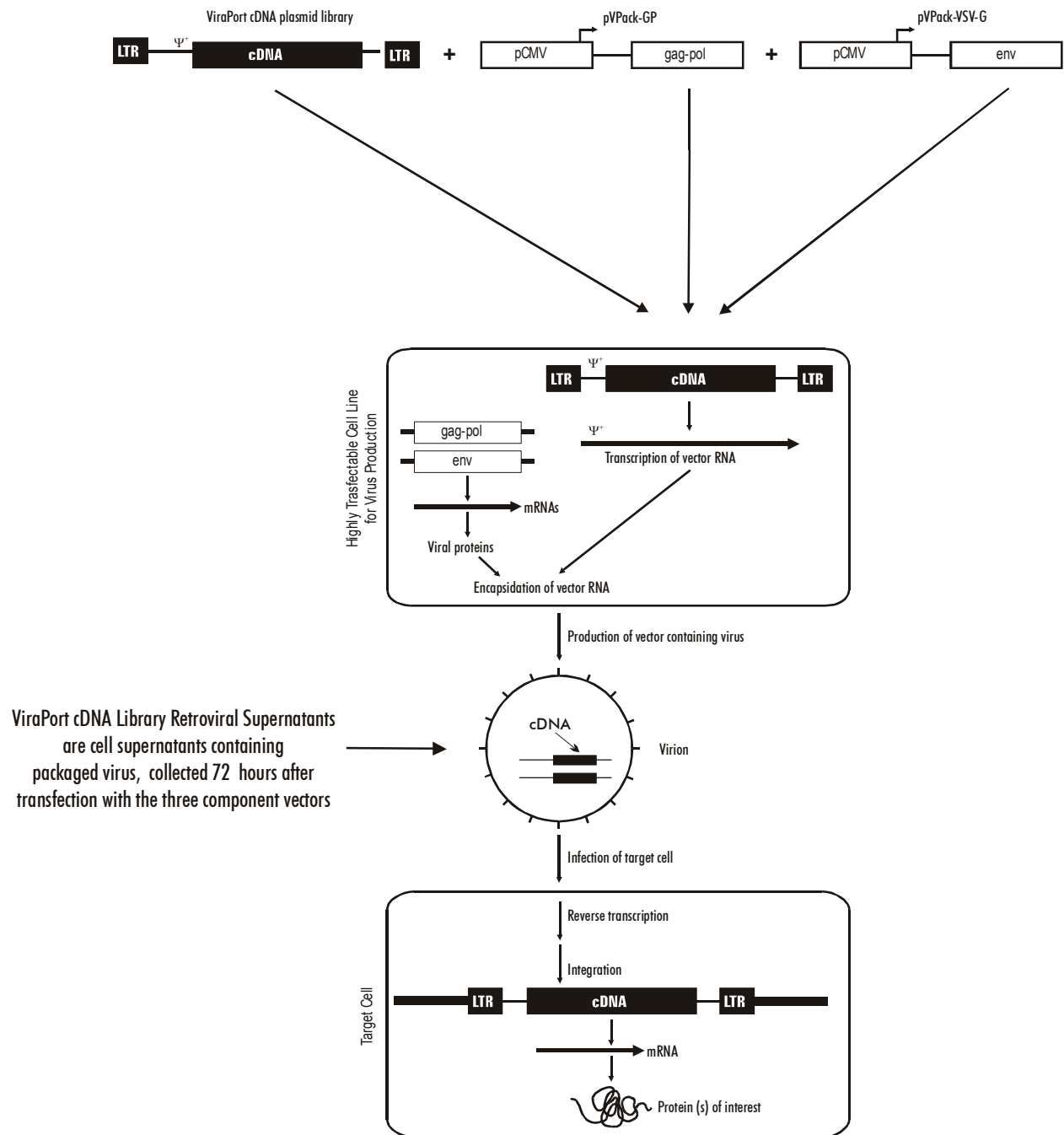


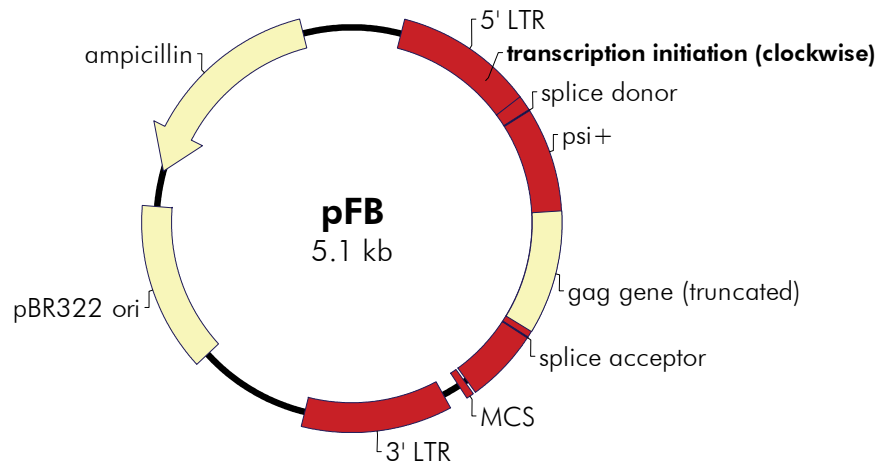
FIGURE 1 Production of a replication-defective retrovirus and subsequent infection of a target cell.

Description of the pFB Retroviral Vector

Note *The sequence for the pFB retroviral vector is available from at www.stratagene.com.*

The ViraPort retroviral supernatants were produced from plasmid cDNA libraries inserted into the vector pFB. The pFB vector contains an extended MMLV packaging signal ($\psi+$) that includes N-terminal sequences from the *gag* gene, and a multiple cloning site (MCS) that is located between the MMLV 5' and 3' long terminal repeat sequences (LTRs) (see Figure 2). The cDNA libraries are inserted directionally between the *EcoR* I and *Xho* I sites of the MCS. Proviral cDNA inserts are PCR-amplified from genomic DNA isolated from expanded positive transductants using the 5' Retro primer and 3' pFB primer provided with the kit.

In order to ensure insertion of large cDNAs, we chose the vector pFB as the backbone in the production of the cDNA library. The insert capacity of this MMLV-based replication-defective vector is 8.0 kb, because it does not contain extraneous sequence, e.g., antibiotic-resistance marker, between the LTRs. As a result, there is no direct method for measuring the infectious titer of the ViraPort retroviral supernatants. The given titer was determined by RT-PCR of RNA isolated directly from frozen retroviral supernatants, and comparison of the RT-PCR signal with that for a GFP-containing reference virus whose titer was determined by FACS analysis of transduced NIH3T3 cells after a single freeze-thaw cycle.



pFB Multiple Cloning Site Region (sequence shown 2057–2086)

Sal I EcoR I BamH I Xho I Not I
 | | | | |
 GTCGACGAATTCGGATCCTCGAGCGGCCGC

Feature	Position (bp)
5' -long terminal repeat (LTR)	209–760
transcription initiation (clockwise)	616
splice donor	818–822
ψ + extended viral packaging signal	760–2046
<i>gag</i> gene (truncated)	1236–1723
splice acceptor	1751–1753
5' retro primer binding site	2008–2028
multiple cloning site	2057–2086
3' pFB primer binding site	2121–2101
3' -long terminal repeat (LTR)	2163–2756
pBR322 origin of replication	3237–3904
ampicillin resistance (<i>bla</i>) ORF	4055–4912

FIGURE 2 Circular map, MCS, and features of the pFB retroviral vector.

Primers

Sequences for oligonucleotide primers that recognize regions flanking the multiple cloning site of the pFB vector are shown in the following table. These primers are suitable for both sequencing and PCR amplifying cDNA inserts in the pFB vector. Sequencing primers are generally used at a concentration of 20 ng/ μ l. PCR primers are generally used at a concentration of 100 ng/ μ l.

Primer	Coordinates	Sequence
5' Retro primer	2008–2028 bp	5'-GGCTGCCGACCCCGGGGGTGG-3'
3' pFB primer	2121–2101 bp	5'-CGAACCCAGAGTCCCGCTCA-3'

DETERMINATION OF TARGET CELL TRANSDUCTION EFFICIENCY USING THE pFB-hrGFP CONTROL VIRAL SUPERNATANT

The provided VSV-G pseudotyped pFB-hrGFP retroviral supernatant contains the coding sequence for the humanized recombinant green fluorescent protein and has been tested in NIH3T3, COS-7, CHO, 293, and HeLa cells, and consistently gives titers $\geq 10^7$ cfu/ml. Supercoiled pFB-hrGFP plasmid DNA is also available (Stratagene Catalog #240027).

The pFB-hrGFP control viral supernatant allows a qualitative assessment of the efficiency with which the target cell type is transduced by VSV-G pseudotyped retrovirus (see Figure 3 for pFB-hrGFP features). In addition, transduction of NIH3T3 cells with the pFB-hrGFP supernatant serves as an indicator of the integrity of the frozen ViraPort supernatants, e.g., in cases where the virus may have unknowingly undergone an additional freeze-thaw cycle prior to use, resulting in a significant decrease in titer. This latter application may be useful in that there is no direct method for determining the number of infectious particles in ViraPort supernatants that have undergone more than one freeze-thaw cycle. It is recommended that both the target cell intended to be used for the ViraPort screen and NIH3T3 cells are infected in parallel with pFB-hrGFP prior to the actual library screen. This allows both the quality of the ViraPort library supernatant (which should always be stored with the pFB-hrGFP supernatant) and the relative transduction efficiency of the target cell to be determined simultaneously. Direct comparisons between the cell lines based on hrGFP fluorescence should be made with caution however, as differences in fluorescence may be due to cell type-dependent differences in hrGFP expression rather than differences in transduction efficiencies.

Note *Prior to thawing the virus, users should be thoroughly familiar with the suggestions and Web sites described in Safety Considerations. All potential target cell lines should first be tested for the presence of endogenous retrovirus.*

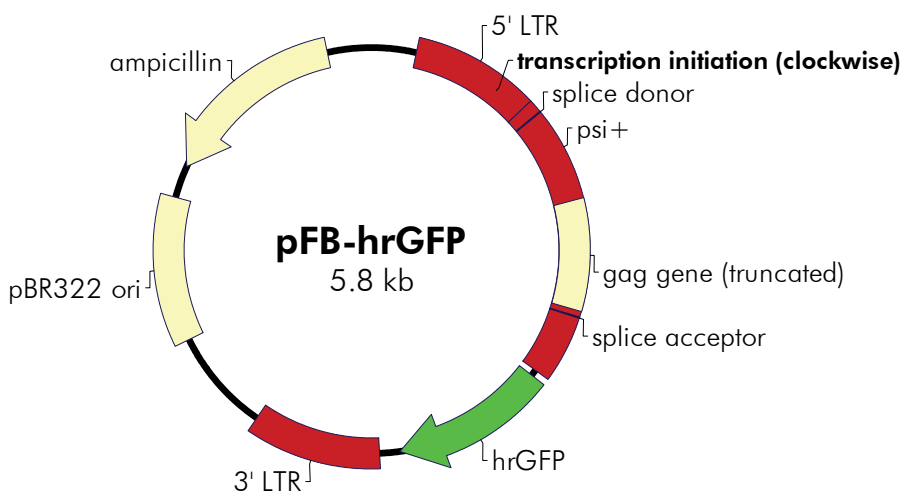


FIGURE 3 Circular map and features of the pFB-hrGFP control vector.

DETERMINATION OF TARGET CELL TRANSDUCTION EFFICIENCY USING THE PFB-NEO-LACZ CONTROL VECTOR

As an alternative to the pFB-hrGFP vector, the pFB-Neo-LacZ control vector can be used for determining the efficiency with which the chosen target cell is transduced; it also allows a quantitative assessment of viral promoter strength. Titer determination using the β -galactosidase gene may be carried out by fixing and staining cells with X-gal using the *In Situ* β -galactosidase Staining Kit (Stratagene Catalog #200384), and determining the number of blue cells as a percentage of the total number of visible cells in a field by light microscopy. Alternatively, β -galactosidase titers may be determined by Fluorescence Activated Cell Sorting (FACS)⁶ using the fluorescent substrate CMFDG (Molecular Probes, Eugene, OR). Titers may also be determined with this vector by G418-resistant colony formation from populations of cells infected with various dilutions of viral supernatant. For a quantitative determination of promoter strength in the target cell of choice, lysates from transduced cells may be assayed for β -galactosidase enzyme activity using the β -Galactosidase Assay Kit (Stratagene Catalog #200710). The pFB-Neo-LacZ gives titers on the order of 10^6 cfu/ml by visual inspection of *in situ* stained cells; this titer is generally 2-10-fold below that for the ViraPort library supernatants.

The pFB-Neo-LacZ vector is available as supercoiled plasmid DNA (Stratagene Catalog #240029) or as high-titer VSV-G pseudotyped supernatant (Stratagene Catalog #972001).

TRANSDUCTION OF TARGET CELLS WITH CONTROL VIRAL SUPERNATANT

This Control Transduction is performed to demonstrate that your particular cell line is able to be transduced, that the viral supernatants are able to be transduced, and to assess the quality of the viral supernatants.

Day 1: Preparing for Control Transduction

1. For both NIH3T3 cells and the target cells, seed 6 wells using 6-well tissue culture plates with 1×10^5 cells per well. This seeding density may vary with the target cell line; ~20% confluency at the time of infection is desirable.
2. Return the plates to the 37°C incubator overnight.

Day 2: Transducing the Target Cells

Prior to thawing the supernatant, the area around the cap should be carefully inspected for any sign of leakage, and thoroughly wiped with 70% ethanol. Media should be prepared and aliquoted into pre-labeled Falcon® 2054 polystyrene tubes prior to thawing the virus.

1. Quickly thaw the pFB-hrGFP (or pFB-Neo-LacZ) control supernatant by rapid agitation in a 37°C H₂O bath. Screw caps should be removed in the hood only, and any fluid around the outside lip of the tube or the inside surface of the cap should be carefully wiped with a tissue wetted with 70% ethanol, and the tissue should be disposed of in the hood. Thawed virus should be temporarily stored on ice if not used immediately.
2. Prepare a dilution series from 1:10 to 1:10⁴ in growth medium[§] (2.0 ml dilution per tube in 2054 tubes) supplemented with DEAE-dextran at a final concentration of 10 µg/ml (1:1000 dilution of the 10 mg/ml DEAE-dextran stock.[§]). Add 0.8–1.0 ml undiluted supernatant to an additional tube, and supplement with DEAE-dextran to 10 µg/ml.
3. Remove the plates containing the target cells (NIH3T3 cells and test target cells) from the incubator.
4. Remove and discard the medium from the wells. For tubes containing undiluted supernatant and for each dilution, add 1.0 ml per well to both the NIH3T3 and test target cell. Add 1.0 ml media (no virus) to the sixth well for an uninfected control. The remaining control supernatant should be aliquoted and refrozen at –80°C. It should be noted that the titer will drop, resulting in a loss of <50% of the remaining infectious particles with each subsequent freeze-thaw cycle.
5. Return the plates to the 37°C incubator and incubate for 3 hours.

[§] See *Preparation of Media and Reagents*.

6. After the 3 hour incubation, add an additional 1.0 ml growth medium to each well.
7. Return the plates to the 37°C incubator and allow 24–72 hours for analysis of expression of the respective reporter proteins.

Day 4: Performing hrGFP Fluorescence Detection

The hrGFP protein may be visualized by fluorescence microscopy or analyzed semi-quantitatively by flow cytometry, and compared to background fluorescence levels established by non-transduced target cells. In most cases, mammalian host cell lines transduced with the pFB-hrGFP control supernatant should show expression of hrGFP 24–72 hours after transduction. The hrGFP excitation/emission peaks are at 500 and 506 nm, respectively, and hrGFP can be visualized using standard FITC filters.

Specifications for hrGFP and EGFP Excitation and Emission Spectra

GFP Form ^a	Excitation/Emission Spectra Maxima (nm)
hrGFP	500/506
EGFP	488/509 ^b

^a Both forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

^b The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note *Filter sets compatible with the detection of hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see www.omegafilters.com):*

Exciter filter: XF1073

Emitter filter: XF3084

Beam splitter: XF2010

Microscope cube set with the exciter filter, emitter filter and beam splitter: XF100-2

TRANSDUCTION OF TARGET CELLS WITH VIRAPORT cDNA LIBRARY SUPERNATANTS

Prior to transduction of the library, some consideration should be given to the multiplicity of infection (MOI). At high MOIs for which 100% of the cells are transduced, the majority of infected cells will carry multiple integrants, of which only one is likely to be responsible for the selected phenotype. In this case each individual proviral cDNA will need to be PCR amplified, subcloned into a mammalian expression vector, and retested to determine which cDNA provided the desired phenotype. If the MOI is adjusted so that approximately 20% of the target cells are transduced, $\geq 90\%$ of the transduced cells will harbor a single integrant, with $\leq 10\%$ containing two integrants.⁷ While transduction at low MOI greatly simplifies the screening process, the number of target cells required for a single screen may be prohibitively high for very rare cDNAs. The MOI consideration is further complicated by the fact that once the library is thawed, freeze-thawing of the supernatant results in a loss of significantly less than 50% of the remaining infectious virus per freeze-thaw cycle. It is recommended that any viral supernatant that is not used immediately following the initial thaw be aliquoted and frozen at -80°C . In light of the reduction of viral titer with subsequent freeze-thaw cycles however, it is recommended that the user design the experimental strategy carefully for the initial screen. For genes for which there is little or no information regarding the abundance of the cDNA of interest, we recommend employing both strategies in a single screen. Positive transductants that are obtained with low MOI screens have a high probability of containing a single cDNA of interest after one round of screening. If no positive clones are obtained in a low MOI screen, positive transductants recovered from high MOI screens should harbor ≤ 10 cDNAs per cell, which can be subcloned and retested with relative ease. Note that the titer of the ViraPort supernatants is indirectly based on transduction of NIH3T3 cells with a reference virus (see *Description of the pFB Retroviral Vector*). Because the relative transduction efficiency between cell types may vary widely, it is generally prudent to carry out screens using as wide a range of supernatant dilutions as is practical.

Note *Prior to thawing the virus, users should be thoroughly familiar with the suggestions and Web sites described in Safety Considerations. All potential target cell lines should first be tested for the presence of endogenous retrovirus.*

Day 1: Preparing for Transduction of ViraPort retroviral cDNA library

1. Based on the desired MOI for the screen, seed the appropriate number of 100-mm plates with approximately 5×10^5 target cells per plate. This seeding density may vary with the target cell line; ~20% confluency at the time of infection is desirable. Note that for low MOI screens for which supernatants are diluted 1:100 or greater, only a fraction of the plated target cells will be transduced (e.g., if 10^6 cDNAs are to be screened at an MOI for which 20% of the cells are transduced, 10 plates should be seeded; see also *Description of the pFB Retroviral Vector* for notes on the titer of the ViraPort supernatant). It is recommended that one or two extra plates be included for a negative control “mock infection.”
2. Return the plates to the 37°C incubator overnight.

Day 2: Transducing the Target Cells

Prior to thawing the supernatant, the area around the cap should be carefully inspected for any sign of leakage, and thoroughly wiped with 70% ethanol. Media should be prepared and aliquoted into prelabeled 2054 tubes prior to thawing the virus.

1. Quickly thaw the ViraPort supernatant by rapid agitation in a 37°C H₂O bath. Screw caps should be removed in the hood only, and any fluid around the outside lip of the tube or the inside surface of the cap should be carefully wiped with a tissue wetted with 70% ethanol, and the tissue should be disposed of in the hood. Thawed virus should be temporarily stored on ice if not used immediately.
2. Based on the titer of the supernatant and the desired MOI, dilute the virus in the appropriate growth medium supplemented with DEAE-dextran at a final concentration of 10 µg/ml (1:1000 dilution of the 10 mg/ml DEAE-dextran stock. See *Preparation of Media and Reagents*). Prepare 3.0 ml diluted virus per 100-mm plate to be infected. A “mock cocktail” of growth medium plus DEAE-dextran may be prepared for a negative control. The remaining ViraPort supernatant should be aliquoted and refrozen at –80°C, however the titer is expected to drop significantly with subsequent freeze-thaw cycles.
3. Remove the plates containing the target cells from the incubator and discard the medium. For each plate, spread 3.0 ml diluted virus evenly over the cells. Return the plates to the 37°C incubator for 3 hours.
4. After the 3 hour incubation, add an additional 7.0 ml growth medium to each well, and return the plates to the 37°C incubator.

For most screens and selections, 48 hours is a sufficient amount of time to allow expression of the desired phenotype, and it is at this time that the selection pressure or screen is applied to the transduced cell population. The expression time may vary depending on the efficiency of gene expression or the nature of the phenotype, however. For certain genes it may be appropriate to apply the selection or screen at various time points following transduction (alternatively an appropriate control gene with an expected similar phenotype may be inserted into the pFB vector and tested prior to screening the library). In certain instances, the inclusion of 5 mM sodium butyrate and 1 μ M dexamethosone in the medium has been found to enhance expression from the MMLV promoter in transduced NIH3T3 cells.^{8,9}

RECOVERY OF cDNA CLONES FROM POSITIVE TRANSDUCTANTS

The method of isolation and clonal expansion of positive transductants depends on the nature of the selection or screen. If cells are to be selected directly on plates, colonies or foci should be expanded to provide enough cell mass for both propagation and freezing of the cell line and isolation of genomic DNA for PCR amplification of the proviral cDNA. For transductants selected by FACS, single cell dilutions may be seeded into 96-well plates and expanded in conditioned medium. However, if a sufficient number of positive cells are collected, the collection may be seeded in one or more 100-mm plates containing 10 ml of conditioned medium, and expanded as individual colonies.

For PCR recovery of the cDNA inserts, any standard protocol for isolation of genomic DNA from mammalian cells may be used. We have consistently had success with the PCR parameters described below.

PCR Reaction

Add the following components *in order* to a PCR tube:

- x μ l of sterile water to a final volume of 50 μ l
- 5.0 μ l 10 \times *TaqPlus Precision* buffer (see *TaqPlus Precision* PCR system)
- 0.5 μ l dNTP mix (25 mM each dNTP)
- x μ l purified genomic DNA (100–300 ng)
- 1.0 μ l 5' Retro primer (100 ng/ μ l)
- 1.0 μ l 3' pFB primer (100 ng/ μ l)
- 1.0 μ l *TaqPlus Precision* polymerase mixture (5 U/ μ l) (see *TaqPlus Precision* PCR system)

PCR Program

Cycles	Duration of cycle	Temperature
1	1 minute	95°C
40	1 minute	95°C
	1 minute	64°C
	5 minutes ^o	72°C
1	10 minutes	72°C

^o 1–2 minutes of extension time is recommended for each 1 kb of the target to be amplified. If the length of the cDNA is unknown, the extension time will need to be optimized.

TROUBLESHOOTING

Observation	Suggestion
<p>Low or no hrGFP fluorescence in cells infected with pFB-hrGFP supernatant</p>	<p>Low hrGFP fluorescence may be due to poor expression from the MMLV LTR in the target cell or poor transduction efficiency. In either case the same problem is likely to affect expression from the ViraPort proviral cDNAs. The relative efficiency of expression in the target cell line may be tested by direct transfection of pFB-hrGFP vector DNA alongside a CMV-based vector carrying the hrGFP gene. In the case that LTR expression is found to be significantly weaker than that for the CMV promoter, the use of 5 mM sodium butyrate and 1 μM dexamethosone may enhance expression in the target cell line.^{8,9} Poor hrGFP fluorescence due to poor transduction efficiency is more difficult to assess directly using the pFB-hrGFP virus. A qualitative comparison with transduced NIH3T3 cells may be made by isolation of genomic DNA from mass-infected target cells and infected NIH3T3 cells, and comparing PCR signal intensities using the vector-specific primers included with the kit.</p>
<p>No positive transductants recovered from screen or selection</p>	<p>The efficiency of recovery of positive transductants from the ViraPort library screen will depend on a) the efficiency of transduction and viral LTR-mediated gene expression in the chosen target cell line (see above); b) the choice of screen or selection for the desired phenotype; and c) the abundance of cDNAs with the desired phenotype in the chosen library. For an assessment of transduction and expression efficiency in the chosen target cell type, see above and the sections entitled <i>Determination of Target Cell Transduction Efficiency using the pFB-hrGFP Control Viral Supernatant</i> and <i>Determination of Target Cell Transduction Efficiency Using the pFB-Neo-LacZ Control Vector</i>. The optimal time for expression may vary depending on the efficiency of expression or the nature of the phenotype. For certain genes it may be appropriate to apply the selection or screen at various time points following transduction. Ideally, an appropriate control gene with an expected similar phenotype may be inserted into the pFB vector and tested prior to screening the library. The use of 5 mM sodium butyrate and 1 μM dexamethosone in the media may enhance expression from the MMLV promoter in the chosen target cell type.^{8,9}</p>
<p>No PCR product from genomic DNA isolated from clonally expanded positive transductants</p>	<p>The PCR conditions for amplification of proviral cDNA clones from genomic DNA work consistently well in our hands. Most DNA isolation kits and procedures should yield DNA preparations of sufficient purity for PCR amplification. The $A_{260/280}$ ratio of suitable quality DNA should be ~ 1.8. For cases in which no PCR product is recovered, PCR conditions should be optimized as recommended in the <i>TaqPlus Precision PCR</i> system manual. The user should also be aware that when amplifying cDNA clones from transductants harboring several inserts, there will likely be some bias in the PCR reaction toward smaller inserts, and thus large cDNAs may not be easily recoverable. If none of the recovered cDNAs exhibit the desired phenotype in a validation screen, it may be necessary to repeat the library screen using a lower MOI.</p>

PREPARATION OF MEDIA AND REAGENTS

Growth Medium DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum [FBS], 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine	DEAE-Dextran Stock Solution (10 mg/ml) 1 g DEAE-dextran [diethylaminoethyl-dextran, approx. mol. wt. 500,000], (10 mg/ml final concentration) Add 100 ml of high purity water, dissolve the DEAE-dextran, filter sterilize into a sterile container and keep sterile until required.
TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA	

REFERENCES

1. Cepko, C. (1996). Detection of Helper Virus in Retrovirus Stocks. In *Current Protocols in Molecular Biology*, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman et al. (Eds.). John Wiley and Sons, New York.
2. Kitamura, T. (1998) *Int J Hematol* 67(4):351-9.
3. Miller, A. D. (1997). Development and Applications of Retroviral Vectors. In *Retroviruses*, J. M. Coffin, S. H. Hughes and H. E. Varmus (Eds.), pp. 437-473. Cold Spring Harbor Laboratory Press, Plainview, NY.
4. Soneoka, Y., Cannon, P. M., Ramsdale, E. E., Griffiths, J. C., Romano, G. et al. (1995) *Nucleic Acids Res* 23(4):628-33.
5. Yang, S., Delgado, R., King, S. R., Woffendin, C., Barker, C. S. et al. (1999) *Hum Gene Ther* 10(1):123-32.
6. Fiering, S. N., Roederer, M., Nolan, G. P., Micklem, D. R., Parks, D. R. et al. (1991) *Cytometry* 12(4):291-301.
7. Onishi, M., Kinoshita, S., Morikawa, Y., Shibuya, A., Phillips, J. et al. (1996) *Exp Hematol* 24(2):324-9.
8. Pages, J. C., Loux, N., Farge, D., Briand, P. and Weber, A. (1995) *Gene Ther* 2(8):547-51.
9. Felts, K., Zaharee, K., Sundar, L., Limjoco, J., Waesche, A. et al. (2000) *Strategies* 13(1):15-18.

ENDNOTES

Falcon® is a registered trademark of Becton-Dickinson and Company.
GenBank® is a registered trademark of US Department of Health and Human Services.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at <http://www.stratagene.com/MSDS/>. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.