4. Nuclear Fast Red Staining Solution

A nuclear fast red counterstain is commonly used in studies using the blue β-galactosidase reaction product. To prepare the solution, five grams of aluminum sulfate and 0.1 gram of nuclear fast red powder (Sigma) are suspended in 100 ml of double distilled water. The solution is heated while stirring until the powder dissolves. The solution is cooled to room temperature and filtered through a 0.45 μm filter for use.

[29] Transduction of a Gene Expression Cassette Using Advanced Generation Lentiviral Vectors

By MICHELE DE PALMA and LUIGI NALDINI

Introduction

Lentiviral vectors (LVs) provide a powerful tool for gene transfer into both dividing and non-dividing cells (for a review see Vigna and Naldini [1]). They are able to stably transduce primary and terminally differentiated cells such as lymphocytes, hematopoietic stem cells, macrophages, neurons, and hepatocytes from different species, including rodents and primates. Moreover, LVs pseudotyped with the G protein envelope of the vesicular stomatitis virus (VSV-G) can be concentrated to high titers and allow efficient transduction of a wide range of tissues *in vivo*.[2–4]

Pseudotyped LVs are currently produced by transient transfection into 293T cells of a combination of plasmids encoding the required lentiviral packaging functions, the envelope of an unrelated virus that pseudotypes the particle, and the transfer vector (see [26] by Follenzi and Naldini). Different versions of these constructs and their combinations have been described and characterized, from HIV, SIV, and nonprimate lentiviruses. Here we refer to a late-generation vector system that we have contributed to develop from HIV-1 and which has been extensively characterized for performance and biosafety.[5–7] However, the approaches

[1] E. Vigna and L. Naldini, *J. Gene Med.* **2,** 308 (2000).
[2] L. Naldini, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono, *Science* **272,** 263 (1996).
[3] L. Naldini, U. Blomer, F. H. Gage, D. Trono, and I. M. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11382 (1996).
[4] T. Kafri, U. Blomer, D. A. Peterson, F. H. Gage, and I. M. Verma, *Nat. Genet.* **17,** 314 (1997).

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and methods described in this chapter can be applied to LVs produced by most types and sources of constructs, including by stable inducible packaging cell lines that are under advanced development in several laboratories.

Third-generation, replication-defective, pseudotyped HIV-1 derived vectors are produced by cotransfection of four types of plasmids into 293T cells[5]:

The core packaging construct, encoding the proteins and enzymes of the vector core, products of the HIV-1 *gag* and *pol* genes. Expression of this plasmid is conditional on expression of the Rev protein by a separate plasmid.

The Rev expression plasmid, containing the nonoverlapping HIV-1 *rev* cDNA.

The envelope construct, expressing the surface glycoprotein of an unrelated virus, most often VSV-G.

The transfer vector, containing the transgene expression cassette linked to the minimal HIV-1 sequences required for efficient encapsidation, reverse transcription, nuclear transport, and integration into the target cell chromatin.

At the time of transduction, only the transfer vector, which does not contain any viral genes, is integrated into the host cell genome.

This advanced vector design has alleviated most of the biosafety concerns associated with the use of vectors derived from HIV-1. In fact, the following crucial determinants of HIV-1 pathogenesis[8] have been removed from the constructs used to make vector:

The *env* gene, responsible for targeting T-helper lymphocytes.

All four accessory genes *vpr, nef, vif,* and *vpu,* whose crucial role in pathogenesis has been clearly demonstrated.

The *tat* gene responsible for the tremendous rate of expression of the HIV-1 genome in infected cells.

Consequently, the predicted biohazards of a replication-competent retrovirus originating during vector production by an unlikely series of illegitimate recombinations among the constructs would be substantially lower than, and different from,

[5] T. Dull, R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono, and L. Naldini, *J. Virol.* **72,** 8463 (1998).

[6] R. Zufferey, T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini, and D. Trono, *J. Virol.* **72,** 9873 (1998).

[7] A. Follenzi, L. E. Ailles, S. Bakovic, M. Geuna, and L. Naldini, *Nat. Genet.* **25,** 217 (2000).

[8] R. C. Desrosiers, *Nat. Med.* **5,** 723 (1999).

those associated with HIV-1, or even attenuated versions of primate lentiviruses currently evaluated for vaccine purposes.[9]

A further gain in vector biosafety and performance is obtained using a self-inactivating (SIN) transfer vector.[6,10,11] In the SIN-18 transfer vector construct,[6] for example, the region spanning the transcriptional enhancers and promoter of HIV-1, including the TATA box, was deleted from the 3′ LTR. Since the 3′ LTR is used as a template to generate both copies of the LTR in the integrated proviral form of the vector, the deletion results in transcriptional inactivation of both LTRs and prevents its mobilization and recombination in transduced cells.[12]

In this chapter, we describe the methods required to transduce an expression cassette for a transgene of interest into a selected target. The following experimental steps are discussed:

- Construction of the transfer vector carrying the desired expression cassette.
- Generation of the vector stock (these methods are discussed in detail in [26] by Follenzi and Naldini).
- Quality control of the vector stock.
- Transduction of the selected target cells.

For each step, the most relevant experimental parameters and limitations, the potential pitfalls, and the troubleshooting approaches are highlighted.

Construction of Transfer Vector Carrying Desired Expression Cassette

The genome of lentiviruses encodes a protein regulator of its own expression (Tat), which is essential for high-level transcription from the LTR. In its absence, as occurs in cells transduced by a LV, the LTR has a low basal transcriptional activity. Thus, most LVs incorporate an exogenous promoter to drive expression of the transgene. The simplest type of expression cassette is made by a promoter and the cDNA for the gene of interest. The promoter is most often located downstream to the HIV-derived intron of the vector (internal position). Promoters successfully used in LV are strong viral promoters such as the immediate early enhancer/promoter of the human cytomegalovirus (CMV)[3,4,13] and those of

[9] M. S. Wyand, K. Manson, D. C. Montefiori, J. D. Lifson, R. P. Johnson, and R. C. Desrosiers, *J. Virol.* **73,** 8356 (1999).

[10] H. Miyoshi, U. Blomer, M. Takahashi, F. H. Gage, and I. M. Verma, *J. Virol.* **72,** 8150 (1998).

[11] T. Iwakuma, Y. Cui, and L. J. Chang, *Virology* **261,** 120 (1999).

[12] A. A. Bukovsky, J. P. Song, and L. Naldini, *J. Virol.* **73,** 7087 (1999).

[13] H. Miyoshi, K. A. Smith, D. E. Mosier, I. M. Verma, and B. E. Torbett, *Science* **283,** 682 (1999).

endogenous housekeeping genes, such as phosphoglycerate kinase 1 (PGK)[7,14] and elongation factor 1α (EF1α),[15] which are expressed more ubiquitously but less powerfully. Promoters derived from oncoretroviral LTR have also been used.[16,17] If any of the above promoters is selected, the cDNA of the gene of interest is cloned by conventional DNA technology downstream to the promoter in the available unique cloning sites. The expression cassette must rely on the HIV-1 polyadenylation site in the vector 3′ LTR. Interestingly, the splice-suppressor activity of the Rev-RRE axis in producer cells may be exploited to deliver a cassette containing at least one intron into target cells.[17] This feature could represent a unique advantage of lentiviral over oncoretroviral vectors given the role of intervening sequences in controlling the efficiency of processing, export, and translation of RNA transcripts. Considering that the vector backbone of a SIN-18 vector is around 2 kb and that the HIV-1 genomic RNA is 9.18 kb, the size of the expression cassette should not be more than 7.5 kb. However, the actual size limits of LV remain to be investigated. If novel promoters, such as inducible and tissue-specific promoters, are to be used, the required regulatory sequences will be cloned downstream to the HIV-1 intron. Additional control elements, such as enhancer and matrix binding regions, may be introduced. Again, Rev activity in producer cells may enable the faithful delivery of complex DNA sequences containing cryptic splice sites that preclude transfer by oncoretroviral vectors.[18] Posttranscriptional regulatory elements enhancing the expression of the transgene may also be incorporated, such as an element from the 3′ end of the genome of the woodchuck hepatitis virus reported to enhance nuclear export and/or polyadenylation of the transcript and, consequently to increase its steady state in transduced cells.[19] Bicistronic expression cassettes containing an internal ribosome entry site (IRES) or two different promoters in tandem can also be constructed. If the expression cassette requires a polyadenylation site different from that of the vector, insertion in reverse orientation may be attempted. However, the accumulation of antisense transcripts in vector-producer cells may interfere with vector production.

An alternative design of the expression cassette is to introduce the promoter in place of the deleted transcriptional sequences of HIV-1 in the vector LTR. The heterologous sequences are merged with the residual viral sequences or hybrid versions containing larger portions of the HIV-1 promoter can be tested. The

[14] G. Guenechea, O. I. Gan, T. Inamitsu, C. Dorrell, D. S. Pereira, M. Kelly, L. Naldini, and J. E. Dick, *Mol. Ther.* **1,** 566 (2000).

[15] P. Salmon, V. Kindler, O. Ducrey, B. Chapuis, R. H. Zubler, and D. Trono, *Blood* **96,** 3392 (2000).

[16] S. K. Kung, D. S. An, and I. S. Chen, *J. Virol.* **74,** 3668 (2000).

[17] A. Ramezani, T. S. Hawley, and R. G. Hawley, *Mol. Ther.* **2,** 458 (2000).

[18] C. May, S. Rivella, J. Callegari, G. Heller, K. M. Gaensler, L. Luzzatto, and M. Sadelain, *Nature* **406,** 82 (2000).

[19] R. Zufferey, J. E. Donello, D. Trono, and T. J. Hope, *J. Virol.* **73,** 2886 (1999).

potential advantages of this type of design remain to be investigated. While the presence of intronic sequences within the primary vector transcript may enhance its processing, export, and translation, it is also possible that the suboptimal consensus of the HIV-1 splice acceptor sites and the presence of upstream RNA structures such as the TAR loop and the polyadenylation site in the 5′ R region may inhibit optimal expression. Moreover, the self-inactivating feature of the original vector is compromised.

The integrity of the expression cassette may be initially validated by transient transfection of the transfer vector construct (without the other plasmids) into 293T cells and scoring for expression of the transgene. In this setting, however, expression of the transgene is influenced by the strong constitutive promoter inserted upstream of the transfer vector. The activity of the internal promoter can be properly assessed only after transduction of the vector.

Generation of Vector Stock

These methods are discussed in detail in Chapter [26].

Quality Control of Vector Stock

Once a batch of vector is produced, it must be assayed for transducing activity, for the content of vector particles, for the absence of RCR, and for sterility. Knowledge of these parameters is required to properly set up and optimize transduction of the desired target *ex vivo* or *in vivo*. The screening of a lentiviral vector stock for the absence of RCR is a challenging task and is discussed elsewhere in this book (see [26]). Sterility of a vector stock is evaluated by testing aliquots in standard microbiological assays used for tissue culture.

Assaying Transducing Activity

LVs integrate into the host cell genome, thus allowing stable maintenance of the transgene in the progeny of transduced cells and providing a basis for long-term expression of the transgene. The transducing activity of the vector stock can be measured more easily when ubiquitous promoters and reporter transgenes that can be detected within individual cells are transferred. Using such vectors, reproducible assays can be set up to measure the concentration of infectious particles and the maximal transduction efficiency of the stock. The concentration of infectious particles is estimated by end-point titration of the vector stock on a standard well-infectable cell line (see protocol below). The assay is designed for high sensitivity to best approximate the number of infectious particles and to calculate their ratio to physical particles in the vector stock. It is well acknowledged that end-point titer per se has a poor predictive value on vector

performance in the transduction of primary targets. This parameter, however, can be combined with the measurement of physical particles to calculate the specific transducing activity, a useful indicator of vector performance (see below). End-point titration should always be accompanied by bulk vector assays that measure the maximal frequency of transduction obtained when high vector input is used (see protocol below). The gold standard of bulk vector assays is the measurement of the copy number of vector DNA integrated per genome of transduced cell (see protocol below). This assay provides conclusive evidence of transduction of the intact expression cassette and allows quantification independent of expression in the target cells. It permits proper comparisons to be made between the transducing activity of vectors differing for the promoter and the transgenes.

In most cases, the promoter of choice and/or the gene of interest do not allow proper evaluation of the vector stock in the titration assays mentioned above. In such case, it may be advisable to do one or more of the following:

Validate the production and purification methods using a reference vector construct and transducing a continuous cell line according to a standardized protocol.

Measure the particle content of the test vector stock (see below) and compare it to the reference vector.

When the gene product can be scored within the cells but the promoter of choice is expected to be selective, estimate transducing activity by titration in a permissive cell line in comparison with the reference vector stock.

In all other cases, verify bulk transduction activity by DNA analysis using a standard cell line as target.

Assaying Physical Components of Vector Particles

Several assays are available to measure the physical components of vector particles in a stock: the core viral proteins, the encapsidated vector RNA, and the reverse transcriptase (RT) activity. Because these assays are not dependent on transduction of cells, they provide no proof of activity of the stock. However, the measurement of the content of physical particles can be integrated with the information obtained by the transduction assays (end-point titer and DNA analysis) to calculate vector *infectivity* (or specific transducing activity), a reliable parameter to evaluate the quality of the vector stock.

The Gag capsid protein p24 is a major structural component of the HIV-1 core and, as a consequence, of all HIV-1 derived vectors. The total content of particles in the vector stock can be estimated by immunocapture of the mature core protein using highly sensitive and commercially available kits. Alternatively, the content of mature vector particles can be estimated by the RT activity of the suspension using an exogenous substrate. The advantages of the immunocapture assay are the

specificity, reproducibility, and yield of an absolute figure for the p24 concentration. It is important to verify the extent of association of the measured protein with particles by checking its sedimentation in the pellet after ultracentrifugation. A tentative estimate of the actual number of physical particles may then be made by calculating that an average of 2000 Gag molecules assemble the virion core. However, only electron microscopy or fluorescence microscopy after microfiltration and immunolabeling of the core proteins allow direct counting of individual particles.

Immunocapture assays for p24 also score, although less efficiently, the p24-containing Gag precursor polyprotein in immature particles. The presence of excess immature particles in a vector stock indicates a poorly developed production system. When new packaging systems are introduced, they should be validated for the yield of mostly mature vector particles by Western blot analysis of pelleted particles using antibodies against the viral core proteins.

The Gag p24 and RT assays described above do not distinguish between complete vector particles and noninfectious particles lacking some components such as the envelope or the vector RNA. The type of envelope protein and its level of incorporation in the particle not only control the target range, but also have a major influence on the stability and transduction efficiency of the vector. The content of encapsidated vector RNA in a vector stock is a good predictor of its transduction efficiency. Poor expression of unspliced vector RNA in producer cells and large size of the expression cassette may represent limiting factors for the efficiency of encapsidation of vector RNA. Methods to analyze RNA content can then be used to verify the effective packaging of vector RNA into particles by comparison with reference standard stocks or by copy number calculation. RNA can be extracted from pelleted vector particles using any RNA isolation kits, treated with DNase, and spotted onto nitrocellulose through a slot-blot apparatus. The nitrocellulose filter is then hybridized with a vector-specific riboprobe. For instance, serial dilutions of p24 equivalent of test and reference vectors are tested together with standards prepared using plasmid and carrier DNA, as shown for DNA analysis (see below). If radiolabeled probes are used, signal acquisition by storage phosphor screens and analysis of digital images allow comparing the standards with the loaded samples, and determining the RNA content per nanogram of p24. A content of RNA much lower than expected can be indicative of poor infectivity of particles. Real-time reverse PCR approaches can also be developed to score transfer vector-specific sequences. In all these methods, particular care must be exercised to eliminate residual plasmid DNA in the vector stock if transient transfection has been used for production.

Protocol for End-Point Titration of Vector Transducing Activity

End-point titration is performed by transducing a target cell line with serial dilutions of the vector preparation. In the simplest case, a reporter gene is cloned downstream of a constitutive promoter, as in the standard vector SIN-18.PGK.EGFP.

The PGK promoter drives expression of the enhanced green fluorescent protein (EGFP). The transduced cells can be analyzed by fluorescence-activated flow cytometry measuring the percentage of cells expressing EGFP in the total population and the mean fluorescence intensity (MFI) of positive cells. Since the PGK promoter is constitutively active in most cell types, every well-infectable cell line (for example HeLa or 293T cells) can be used for this analysis.

Several experimental parameters affect transduction when titering a vector preparation. The following protocol can be reasonably modified keeping in mind that what mainly influences transduction of cells in a dish is the concentration and not the total number of vector particles in the transduction medium.

1. Seed 1×10^5 cells/well in a six-well cell culture plate for as many tenfold serial dilutions of vector preparation. The cells are allowed to adhere and incubated at 37° for 24 h in appropriate medium. Before titration, the medium is replaced with 0.9 ml fresh medium containing 9 μg/ml polybrene.
2. Prepare serial tenfold dilutions of the viral stock, approximately ranging from 10^{-2} to 10^{-7} for concentrated vector and from undiluted to 10^{-5} for conditioned medium. Add 0.1 ml of each dilution to the cells. Incubate the cells at 37° for 12 h to allow transduction.
3. Change the medium and incubate the cells at 37° for an additional 72 h.
4. Wash cells with phosphate-buffered saline (PBS), than detach and fix, if required, in 1 ml of fixing solution [1% formaldehyde, 2% fetal bovine serum (FBS) in PBS].
5. Analyze cells by flow cytometry (fixed cells can be stored at 4° for a few days). Unfixed cells can be scored also for viability by exclusion of propidium iodide.

Mock-transduced cells are used as a standard control to gate the population of negative events. The titer is defined as number of transducing units per milliliter (TU/ml) of vector preparation, based on the assumption that a single vector copy integrated in the host genome will give a positive cell. Assuming that all the cells are equally susceptible to transduction, following the Poisson distribution for random independent events, a single transduction event, and not more, has occurred in most positive cells when the the percentage of positive cells in the total population is below 25%. It follows that the titer must be calculated from a sample corresponding to a vector dilution where positivity of cells ranges between 1% (to ensure an acceptable signal over the instrument noise) to 25%, in order not to underestimate the titer when multiple transduction events per cell have occurred. Proof of linearity must be obtained showing that different dilutions in the optimal testing range yield linear increase in transduction frequency. The equation to calculate titer is:

$$\text{Titer (TU/ml)} = (\text{number of cells at the time of vector addition}) \times (\%\text{EGFP-positive cells}/100) \times (\text{dilution factor})$$

When the transgene is different from EGFP, end-point titer can be performed staining cells expressing the transgene at steady state for flow cytometry using specific fluorochrome-conjugated antibodies or microscopy analysis. In the latter case, higher vector dilutions must be tested to allow scoring of positive cells. Vectors expressing reporter genes encoding for enzymatic activity such as LacZ can be titered by histochemical staining of transduced cell using chromogenic substrates precipitating inside the cells. Vectors expressing selectable markers can be titered testing transduced cells for long-term resistance to the selector drug. Resistant cells form isolated colonies at the highest dilutions of vector stock; for titration it is assumed that each drug-resistant colony results from a single transduction event.

End point titration is strongly affected by (1) the type of cell transduced; and (2) the promoter in the expression cassette.

1. Some cell lines are transduced less efficiently than others, thus leading to underestimation of the titer of the vector preparation. In contrast, other cell lines are remarkably susceptible to transduction. Such discrepancy implies that when transducing relatively refractory cells (such as some types of primary cells) using vectors titered on easily transduced cells, one must empirically employ a high number of TU to get measurable expression of the transgene (see below, the section on transduction of target cells).
2. The transcriptional activity of the promoter is strictly dependent on the cell type. This means that vectors containing weak, regulatable, or tissue-specific promoters cannot be titered according to the above-reported protocol. In this case, titration should be performed on cell lines in which transcription driven by that particular promoter is favored. If the vector is to be used for transcriptional targeting with tissue-specific expression, end-point titer must be specifically performed on the target cell line. To overcome such problems, vectors containing a second independent expression cassette can be designed, as in the hypothetical construct SIN-18.Promoter.Transgene.PGK.EGFP. However, this type of vector should be carefully tested, since promoter interference is likely to occur between the two expression cassettes.

Bulk Assay of Vector Transducing Activity: Maximal Frequency of Transduction

End-point titration should always be accompanied by bulk vector assays, i.e., by measuring the maximal frequency of transduction that is obtained when high vector input is used. Dose-response analysis shows a linear trend, in which the percentage of positive cells increases with the number of TU added, and then reaches a plateau, corresponding to the maximal frequency of transduction. In the region of the curve approaching the plateau, the MFI of positive cells should increase with increasing

vector doses, reflecting an increase in the average copy number of vector per transduced cell. Transduction of well-infectable cells with a vector stock of good infectivity should not plateau until it reaches a very high frequency of transduced cells, approaching 100%. If a vector stock of poor infectivity is used, the frequency of transduction may plateau at a lower percentage of cells and may not improve just by increasing the number of TU per cell, but only using a vector stock with higher infectivity. This limitation becomes particularly evident when target cells relatively resistant to transduction are used.

Bulk assays verify that excess noninfectious particles and other contaminants do not interfere with vector performance in experimental conditions more representative of the transduction of a gene of interest into most cells of a target population. Such interferences may be missed when highly diluted, low vector inputs are used and may explain the discrepancies mentioned above between a high end-point titer and a poor bulk transduction ability of a vector stock. If available, the cell types selected as target could be used directly to score bulk transduction activity. When using high input of vector, it is important to score cell viability (for instance by dye exclusion). Possible mechanisms of cytotoxicity at high vector dose are particle-mediated cell fusion (fusion-from-without), toxic levels of transgene expression, and excess integration events in the genome. If a fraction of bulk-transduced cells is lost, one could underestimate the transducing activity of a vector stock.

Bulk Assay of Vector Transducing Activity: Measurement of Copy Number of Vector Integrated per Genome

While end-point titer and maximal transduction frequency depend on both infectivity and transcriptional activity of the vector in the transduced cells, DNA analysis of transduced cells directly reveals the ability of the vector to integrate into the target cell genome, independent of transgene expression. DNA analysis is then crucial when vectors containing nonconstitutive promoters or transgenes that cannot be directly tested within the cells have to be validated. For instance, in some experimental conditions it is required to transduce the target cells with equal amounts of infectious particles of different vectors, as when testing transcription efficiency from a repertoire of expression cassettes in a given cell line. Real-Time PCR (TaqMan) and Southern blot analysis provide a method to normalize different vector stocks, independently of the type of construct, for the number of integration events per cell and thus can be used as basic titration assays more useful and reliable than end-point titer.

It has been shown that unintegrated lentiviral DNA can persist in transduced cells for few passages in culture and can serve as template for transgene expression during the first hours after transduction (our unpublished data and Ref. 20). Since

[20] D. L. Haas, S. S. Case, G. M. Crooks, and D. B. Kohn, *Mol. Ther.* **2,** 71 (2000).

unintegrated DNA is not responsible for long-term expression[2] and is lost with time, Southern blot analysis must be performed on DNA from cells that have been cultured for several passages. A stable cell line is transduced with two or more reasonably high doses of vector stock (for instance: 10 and 100 ng of p24 equivalent of vector per 10^5 HeLa cells). Transduction is performed as described for endpoint titer; 8 μg/ml of polybrene can be added to the medium to improve transduction efficiency. Cells are cultured for at least 5–7 passages and then lysed with TNE (10 m*M* Tris-HCl, pH 7.5; 100 m*M* NaCl; 1% SDS) in the presence of 200 μg/ml proteinase K overnight at 37°, followed by extraction and purification of genomic DNA with phenol-chloroform and ethanol; however, any standard or commercial kit-provided methods for genomic DNA extraction and purification can be adopted. For Southern analysis, genomic DNA is exhaustively digested with one or more restriction enzymes able to release a vector fragment spanning a sequence that, when probed, reveals its total content in transduced cells. Southern blot analysis can be performed using standard protocols. Ten to 20 μg of genomic DNA from each sample is separated on 1% agarose gel, transferred to a hybridization membrane, and probed for vector-specific sequences. A probe for an endogenous sequence is used to normalize DNA loading for each sample. Following hybridization, the membrane is washed with SSC/SDS solutions and exposed.

To calculate integrated proviral copy number one can use the DNA from a reference cell clone where a single or predetermined number of copies of vector is integrated in the genome. Alternatively, proviral copy number can be calculated reconstructing standard copy numbers with a vector plasmid DNA. To calculate the amount of plasmid DNA per microgram of genomic DNA to obtain a copy number equivalent of 1 per genome, determine the number of base pairs in the plasmid and perform the following calculation: μg of plasmid DNA equivalent to 1 copy per genome per μg of genomic DNA = base pairs in plasmid/base pairs in genome (6×10^9 bp/genome for euploid human cells and 12×10^9 bp/genome for euploid murine cells).

A standard curve is made by adding vector plasmid DNA equivalent to 10, 5, 3, 1, and 0.5 copies per genome to 10–20 μg of genomic DNA from untransduced cells, digesting the mixture as above, and comparing the standards with the loaded samples.

If radiolabeled probes are used, signal acquisition by storage phosphor screens and analysis of digital images allow quantification of vector DNA.

Calculation of Infectivity of Vector Stock

The infectivity of a vector preparation can be defined as the transducing activity per unit of physical particle, where the first parameter is expressed as transducing units (TU) per milliter, as obtained by end-point titration, or copy number of

integrated vector, as a result of real-time PCR or Southern blot analysis of bulk transduced cells.

Advanced versions of VSV-G pseudotyped LVs, carrying ubiquitous or strong viral promoters driving the expression of EGFP, can be routinely titered to $0.5–1.0 \times 10^8$ TU/ml (end-point titer on HeLa cells) in supernatants of transfected 293T cells. For such vectors, ultracentrifugation and concentration up to a thousandfold can be performed without significant loss of transducing activity, and titers of more than 10^{10} TU/ml can be obtained. Since 1 ng of p24 could theoretically contain 1.2×10^7 particles, if all the particles in the vector stock were infectious, a titer of 10^8 TU/ml would correspond to a p24 concentration of about 10 ng/ml. Indeed, a concentration in the range of 500–1000 ng p24/ml is more reasonably expected. This is due to two major reasons. The first reason is that end-point titer fails to estimate the real content of infectious particles in a vector preparation, because vector particles move by Brownian motion in the medium and only a fraction of them have the chance to get in contact with a cell in the monolayer and transduce it in the time window of the assay.[21] This operational limitation of the assay could be accounted by a correction factor calculated by mathematical means[22] and does not affect comparison of different batches of vectors. The second reason instead is crucially linked to the quality of the vector batch tested. In fact, the efficiency of packaging of infectious particles is lower than what is predicted by theoretical calculations, and a good fraction of the total p24 protein is not assembled into infectious virions. This is due to several factors. Some factors are intrinsic to the biological mechanism of viral assembly. Other factors are dependent on the limitations imposed by the vector design, which employs a fraction of the viral genome split among separate and independently regulated constructs. Optimization of the type and expression ratio of these constructs within vector producer cells is required to obtain a vector stock of acceptable infectivity. When a new transfer vector construct is introduced, further optimization of the production conditions may be needed.

All these factors add up in reducing the measurable infectivity of a vector stock, which can be calculated by the following equation:

$$\text{Infectivity} = (\text{TU/ml})/(\text{ng p24/ml}) = \text{TU/ng p24}$$

For instance, a titer of 1×10^8 TU/ml (end-point titer on HeLa cells) corresponding to a concentration of p24 of 500 ng/ml gives an infectivity of (1×10^8 TU/ml)/(500 ng p24/ml) = 2×10^5 TU/ng p24. Such a level of infectivity would

[21] S. P. Forestell, E. Bohnlein, and R. J. Rigg, *Gene Ther.* **2,** 723 (1995).

[22] S. Andreadis, T. Lavery, H. E. Davis, J. M. Le Doux, M. L. Yarmush, and J. R. Morgan, *J. Virol.* **74,** 3431 (2000).

be considered more than satisfactory, as values approaching 10^5 TU/ng p24 are acceptable for most applications. Infectivity can also be calculated referring to vector copy numbers. In this case, infectivity must be calculated multiplying the vector copy number/cell genome by the number of cell genomes present at the time of vector addition, per ng of p24.

$$\begin{aligned} \text{Infectivity} &= (\text{vector copy number/cell genome}) \\ &\quad \times (\text{number of cell genomes at the time of vector addition})/\text{ng p24} \\ &= \text{copy number/ng p24} \end{aligned}$$

The ratio between the infectivity estimated from end-point titration and the infectivity estimated by DNA analysis indicates the fraction of integrated vectors that allow expression of the transgene, or the minimal number of vector copies allowing detectable expression of the transgene within that cell type. Transgene silencing may occur because of random integration of the vector into heterochromatin and it may be induced by poorly understood genome surveillance mechanisms, particularly following long-term follow-up of transduced cells or selection and expansion of clones, as shown for MLV-derived vectors.[23] In such cases, the use of tissue-targeted promoters, minimal viral sequences (such as in SIN vectors), and chromatin insulators has been shown to improve the expression performance of retroviral vectors.[24]

Transduction of Target Cells

Once the vector stock has been proved to display an infectivity comprised in the indicated range and to obey the above-mentioned quality controls, it can be utilized to transduce the desired target cell *in vitro* or *in vivo*.

Cells can be transduced *in vitro* directly in their culture medium by adding the required amount of vector preparation. Polybrene can help transduction, but some primary targets do not tolerate the standard concentrations; other compounds, such as dextran sulfate and the fibronectin fragment CH-296 (Takara Shuzo, Osaka, Japan) may be tested. It is likely that the choice of viral envelope will affect the efficacy, if any, of these cofactors. Some quiescent cell types, such as lymphocytes, can be more efficiently transduced with addition of a growth or activation stimulus. Indeed, cells can be treated with cytokines or growth factors without any interference with the transduction performance.

Information obtained by titration of the vector stock by end-pointx analysis or by calculation of integrated copy number is exploited to design the transduction

[23] S. Halene and D. B. Kohn, *Hum. Gene Ther.* **11,** 1259 (2000).

[24] D. W. Emery, E. Yannaki, J. Tubb, and G. Stamatoyannopoulos, *Proc. Natl. Acad. Sci. U.S.A.* **97,** 9150 (2000).

protocol. A typical experiment is performed by testing increasing doses of vector calculated as TU/ml or as p24 equivalent of vector. To achieve high-frequency transduction of most target cell types, dose ranges from 10^6 to 10^8 TU/ml (end-point titer on HeLa) can be tested. These doses correspond to 10 to 1000 ng p24/ml of a vector with an infectivity of 10^5 TU/ng p24.

Critical Factors Affecting Transduction *in Vitro*

Vector Concentration

When the vector titer is calculated on a permissive cell line, refractory targets may need to be transduced employing a high number of TU per ml. One should take into account the fact that the expression *TU/cell,* which is equivalent to MOI (multiplicity of infection), is an arbitrary definition, because it does not consider the volume in which the transduction is performed, and therefore the particle concentration. Within this context, it is well acknowledged that vector concentration in the transduction medium is more important than the absolute number of particles available for each cell, since only a fraction of them come into contact with the target, especially when cells are cultured in monolayers in a large volume of medium. To increase the transduction efficiency, using a given amount of vector, cells must be transduced at a reasonably high density in the least volume, so that the possibility that vectors and cells encounter each other is enhanced. Other maneuvers that increase the chances of vector particles to come into contact with target cells, such as prolonged centrifugation of vector together with the cells (spinoculation), have been shown to increase transduction.[25] Haas *et al.*[20] have shown that the level of gene transfer into CD34+cells by VSV-G pseudotyped LVs is improved by increasing the concentration of vector particles, holding the MOI constant in the transduction medium, but not increasing the MOI when the vector concentration is held constant. This result supports the notion that the concept of MOI is misleading when defining the transduction conditions.

Transgene Expression and Effects of Pseudotransduction and Transient Expression from Unintegrated Vector

One crucial advantage of LVs is the independence of transduction from cell division. Thus, almost every cell in a population can be transduced by a single exposure to the vector. However, when saturation is observed below the required frequency of transduction, either a vector has a less than optimal infectivity, or some cells in a heterogenous population are refractory to transduction. These two

[25] U. O'Doherty, W. J. Swiggard, and M. H. Malim, *J. Virol.* **74,** 10074 (2000).

possibilities can be addressed performing more than a single round of transduction, which may result in a higher frequency of transduced cells.

Following transduction of a given target, the average expression level of the transgene is proportional to the vector input, provided that the transduction frequency has not reached the saturation threshold. This means that the average expression level depends on the copy number of integrated vectors. According to a random distribution of independent events, it is impossible to achieve the maximal transduction frequency with a predominance of single integration events. Therefore, a cell population transduced to the maximal frequency displays a relatively wide range of integration events per cell. As a consequence, the expression level of the transgene can be very variable and this may result in toxicity in a fraction of cells.

Evaluation of transgene expression should be obtained at steady-state level, a condition which is normally reached several days after transduction, based on the cell type, the promoter transcriptional activity, mRNA stability, and half-life of the transgene protein.

Pseudotransduction effects have been described. Pseudotransduction is due to direct transfer of the transgene protein by either its presence in vector supernatants or its incidental incorporation into the vector particles.[26] This phenomenon occurs with low efficiency and can be detected only at early stages, usually in the range of few hours, following transduction. Of major concern is the expression driven by unintegrated vectors. Nonintegrated circular vectors can persist in the cell nucleus and represent dead-end by-products of aborted vector transfer. These DNA molecules can express transgenes. The extent of expression from unintegrated vector can be directly demonstrated producing a vector stock by transfection of a packaging plasmid defective for the expression of the viral integrase.[2] Such a vector is unable to actively integrate into the host cell genome, except for the rare events due to non-integrase-mediated mechanisms. The absolute level of transgene expression from unintegrated vectors can be relatively high during the first days after transduction, but is shown to rapidly decrease with time when the cells are proliferating (transgene protein half-life plays a critical role in determining these kinetics).[20] In addition, integrated vs unintegrated vector DNA can be demonstrated by Southern analysis. To show unintegrated vector, an enzyme with a unique restriction site in the vector has to be used to digest the genome from transduced cells. Upon this treatment, while integrated DNA, because of random integration, appears in the form of a smear, unintegrated vector appears as a single band. DNA analysis can be therefore used to estimate the ratio between the two DNA forms and allow timing of clearance of the unintegrated vectors more reliably than by scoring expression.

[26] M. L. Liu, B. L. Winther, and M. A. Kay, *J. Virol.* **70,** 2497 (1996).

Concluding Remarks

Pseudotyped lentiviral vectors provide a powerful tool for several gene transfer applications. The above-discussed protocols serve to predict the potential performance of vector stocks, especially when they are intended for *in vivo* purposes, when high-titer and high-infectivity concentrated vector is required. Among the other parameters, the infectivity of a vector has proved to be a limiting-factor to efficient gene transfer in many applications, such as *ex vivo* transduction of lymphocytes and CD34 + hematopoietic progenitors and *in vivo* administration.[7] Late-generation of VSV-G pseudotyped LVs can be concentrated to titers of 10^{10} TU/ml, with an infectivity in the range of 10^5 TU/ng p24 (end-point titer on HeLa). When displaying such features and complying with the other quality controls discussed in this chapter, the vector preparations allow significant levels of gene transfer in challenging *in vivo* settings.

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[30] Construction, Purification, and Characterization of Adenovirus Vectors Expressing Apoptosis-Inducing Transgenes

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Introduction

Construction and large-scale production of recombinant adenovirus (rAd) vectors expressing proapoptotic transgenes, or any cytotoxic product in general, presents a special challenge to researchers. On the one hand, high levels of expression are often desired in the target cells, especially if the rAd vector is to be used as a therapeutic agent and is therefore required to kill the cells it infects as efficiently as possible. On the other hand, high levels of cytotoxic gene expression may be strongly deleterious to the packaging cell line in which the rAd vector is developed and propagated. In the most severe cases, total failure to obtain a viable vector after transfecting packaging cells with vector DNA results. However, transgene-related cytotoxic activity that does not kill the packaging cells outright can be even more problematic, since it is likely to place a strong selective pressure

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