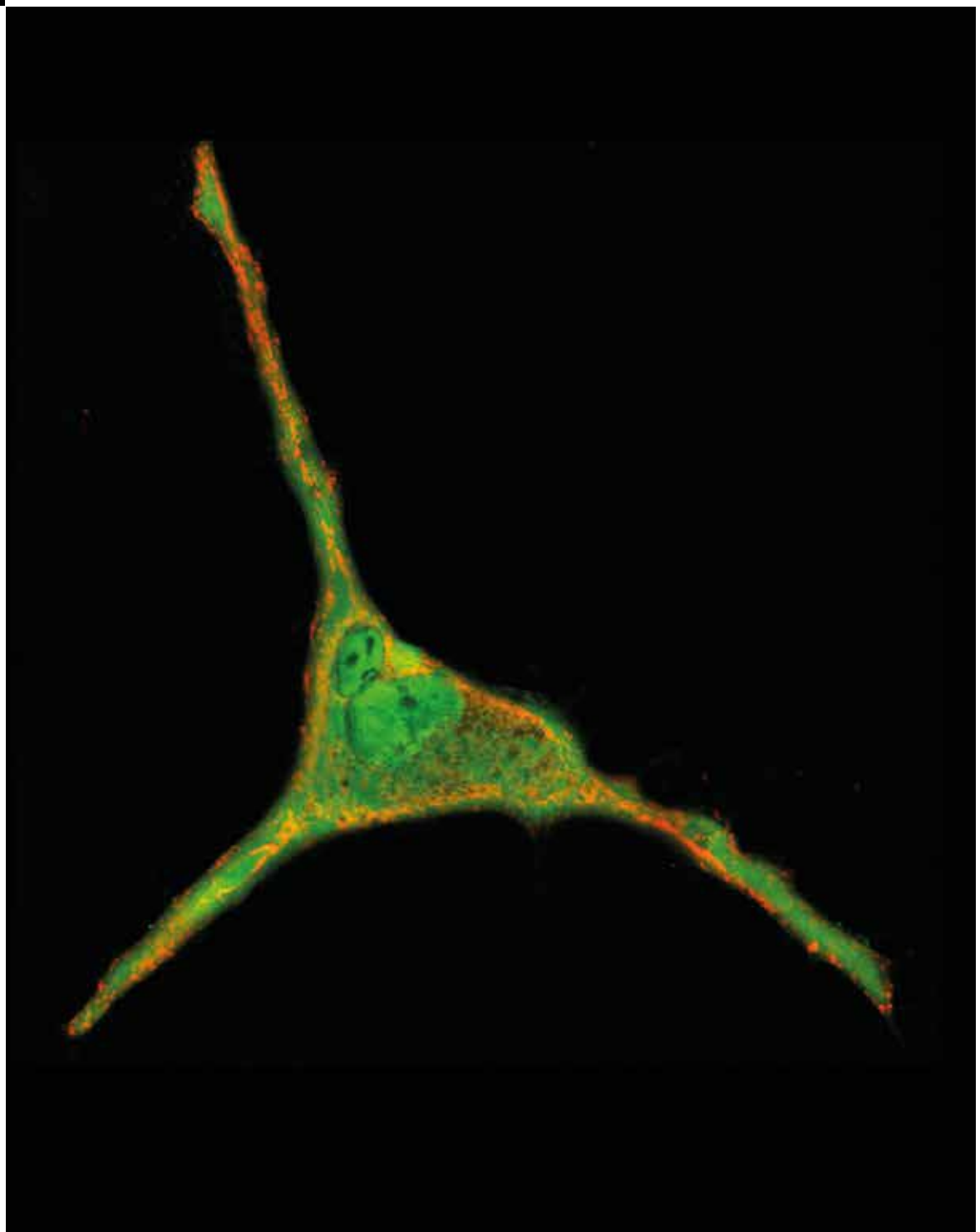
The COGEM formula revisited

Experimental validation of the reduction ratio formula for free lentiviral particles



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ONDERZOEKSRAPPORT

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Experimental validation of the reduction ratio formula for free lentiviral particles

COGEM report 2020-01

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Cover:

Lentiviruses are frequently used as gene transfer vehicles. Depicted is a human mesenchymal stromal cell in which a lentiviral vector was used to transfer and express a green fluorescent protein gene to allow tracing the cells. The cell has been stained with acridine orange to visualize the lysosomes.

Courtesy of LUMC Department of Cell and Chemical Biology, Leiden, The Netherlands

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Preface

Lentiviral vectors (LVVs) have proven to be very powerful genetic tools in biomedical research. Most LVVs are derived from the *human immunodeficiency virus* (HIV) type 1 and are designed to deliver and integrate a gene of interest into the genome of cells. Gene therapy using LVV-treated ("transduced") cells has rapidly found its way into clinical trials as it allows scientists to treat genetic disorders and provide cells with specific functions. In recent years, the design of LVVs has been considerably refined to enhance their safety profile, for example by separating the genes necessary for producing the LVVs onto additional plasmids and by including self-inactivating viral promoter sequences that significantly reduce gene expression after integration. Using these systems, LVVs can be produced that are free of contaminating replication-competent lentiviruses. Both for research and clinical applications of LVV-treated cells, preventing exposure to free infectious LVV particles that may remain from the vector inoculum, is an important element in the safety assessment.

The presence of free LVV particles in preparations of transduced cells depends on a number of factors, including their initial amount, their decrease (reduction ratio) during the half-life and their decrease due to washing and inactivating steps of the cells after transduction. As a guideline for researchers, COGEM drew up a formula to help estimating the number of free LVV particles in transduced cell preparations (COGEM-advies-CGM/090331-03, 2009). However, some elements in this formula, such as the reduction of free LVV particles by washing of the cells, are based on theoretical assumptions. The half-life of LVV particles pseudotyped with the Vesicular Stomatitis Virus G (VSV-G) protein for cell entry has been reported, but presently LVVs are frequently pseudotyped with other viral envelope proteins and the half-life of such vectors has not yet been reported. In addition, the effectiveness of inactivating steps may vary for the different LVV particles. The formula does also not take into account any decrease resulting from the cell transduction. Because experimental data are missing for a correct assessment, the current formula is a theoretical estimate of the reduction ratio of free LVV particles in transduced cell preparations. To improve the risk assessment of research and clinical applications of LVV-treated cells, COGEM commissioned the present research project to experimentally substantiate the factors that determine the number of free LVV particles in preparations of transduced cells. The researchers extensively studied the effects of experimental conditions and manipulations on the level of free vector particles. Their study included LVV particles pseudotyped with several regularly used envelope proteins for cell entry, which revealed differences in their half-life as well as in the effectiveness of inactivation steps. On these aspects the researchers report many new and important findings. The supervisory committee considers that the results of this research project indicate that many specific factors affect the

reduction ratio of free lentiviral particles. The formula therefore needs adaptation to fit the different LVVs and specific production conditions. Therewith there will be no “one formula fits all”. The current research provides a guideline for the field on how the COGEM formula, together with or without specific measurement methods, can be used in a more differentiated, accurate and prudent way.

Gene therapy using LVVs is promising for the treatment of a number of important disorders. A good safety profile of LVVs and LVV-treated cell products is therefore essential. We recommend that the results of this study be taken into account and that research into measurement and inactivation methods of free LVV particles be continued. The supervisory committee compliments the researchers on their many important contributions and trusts that the results will find their way into this scientific field and in particular will influence the safety assessments of researchers and COGEM.

Tjeerd Kimman - Chair of the Guidance Committee

The Guidance Committee of this project consisted of:

Dr. T.G. Kimman (Wageningen Bioveterinary Research, member of COGEM)
Dr. A.T. Das (Amsterdam University Medical Centers, member of COGEM)
Dr. N.P. van Til (University Medical Center Utrecht)
Dr. B.M te Riet (GMO office, National Institute for Public Health and the Environment)
A.T.A. Box, BSc (COGEM secretariat)

2. Management summary (in Dutch)

Lentivirale vectoren zijn bijzonder populair voor stabiele genetische modificatie van zoogdiercellen. Met de huidige productiesystemen kunnen virusconcentraten worden gemaakt die bestaan uit replicatie-defectieve lentivirale vectordeeltjes zonder dat deze preparaten replicatie-competente lentivirussen bevatten. De stabiele integratie van de lentivirale vector genomen in het gastheer DNA gebeurt op een goed-voorspelbare manier, hoewel de chromosomale locatie van de integratie niet gestuurd kan worden. De in het chromosoom-geïntegreerde vectoren kunnen na integratie niet worden gemobiliseerd, mits de zogenaamde zelf-inactiverende (SIN) vectoren worden gebruikt.

Lentivirale vectoren afgeleid van het humane immunodeficiëntie virus (HIV) worden het meest gebruikt. Veelal wordt het envelop-eiwit van HIV vervangen door een heteroloog envelopeiwit om de vectoren de capaciteit te geven cellen te infecteren die niet de HIV-receptor CD4 tot expressie brengen.

Voor het gebruik van lentivirale vectoren is een GGO vergunning dan wel een kennisgeving vereist. Voor het verkrijgen van een dergelijke vergunning of het doen van een kennisgeving is een risicobeoordeling nodig. Hierin moet aandacht geschonken worden aan de mogelijke aanwezigheid van replicatie-competente lentivirussen die zouden kunnen ontstaan bij de productie. Daarnaast moet worden ingegaan op de risico's van de cellulaire producten die worden gemaakt. Tenslotte moet worden ingegaan op de risico's die geassocieerd zijn met de aanwezigheid van vrije en infectieuze replicatie-defectieve lentivirale vectordeeltjes die overgebleven kunnen zijn uit het gebruikte vector-inoculum. Met de huidige vectorproductiesystemen, waarbij de benodigde helpergenen zijn gescheiden op 3 of meer plasmiden, kan het risico op de vorming van replicatiecompetente lentivirussen worden verwaarloosd. Echter, het risico op insertionele mutagenese kan niet worden uitgesloten, hoewel dit door het gebruik van de SIN vectoren sterk is gereduceerd. Daarnaast kan de overexpressie van het geïntroduceerde transgen negatieve consequenties hebben. Het is daarom van belang om ook de aard van het transgen te evalueren wanneer er gekeken wordt naar de effecten van vrije vector deeltjes. Vectoren met genen die coderen voor toxische of transformerende producten behoeven speciaal de aandacht.

Om onderzoekers behulpzaam te zijn die vrije vectordeeltjes niet afdoende konden kwantificeren, heeft de COGEM ca. 10 jaar geleden een formule ontwikkeld om de Reductie Ratio te bepalen. Deze ratio schat het verlies aan infectieuze titer van replicatie-defectieve vectordeeltjes op basis van een aantal inactiverende activiteiten. Deze omvatten spontane inactivering van de infectieuze deeltjes, degradatie van vectordeeltjes o.i.v. trypsine of complement en het verlies van vectoren door het

wassen of passeren van de gekweekte celculturen. De gecombineerde effecten van deze inactiverende stappen werden gerelateerd aan de hoeveelheid infectieuze vectordeeltjes in het inoculum. De voor de formule benodigde parameters werden grotendeels ontleend aan literatuurdata.

Recent is deze reductie-ratio benadering ook Europees onderschreven in een document 'Good Practice on the assessment of GMO-related aspects in the context of clinical trials with human cells genetically modified by means of retro/lentiviral vectors', dat in oktober 2019 ook door Nederland is onderschreven (https://ec.europa.eu/health/sites/health/files/files/advtherapies/docs/gmcells_gp_en.pdf).

In de literatuur werden destijds alleen data gevonden voor lentivirale vectoren die het VSV-G eiwit als envelopeiwit droegen. In de huidige studie werden de nieuwe data gegenereerd voor lentivirale vectoren met een aantal andere heterologe envelopeiwitten. Tevens werden proeven gedaan om de bestaande data met VSV-G envelopeiwit-dragende vectoren te confirmeren. Ook werden gebruikte aannamen kritisch geëvalueerd ten einde de bruikbaarheid uit te breiden en de onderbouwing van de formule verbeteren.

Dit leidde de aanvragers tot de volgende conclusies en constateringen.

- De VSV-G envelopeiwit-dragende vectoren zijn stabieler dan tot nu toe werd aangenomen. De halfwaardetijd van deze deeltjes werd bepaald op 34,7 uur, i.p.v. de 10,4 uur die eerder als norm werd gehanteerd.
- De efficiëntie van een was-stap is ca. 95% reductie massa voor een Ø10 cm petrischaal. Dit is identiek aan de gehanteerde norm.
- De efficiëntie van een was-stap op een Ø35 mm petrischaal is ca 98% reductie in massa, en dus hoger dan de 95 % die eerder werd aangenomen.
- Trypsine inactiveert de VSV-G envelopeiwit dragende deeltjes minder efficiënt dan eerder was aangenomen.
- Er wordt beargumenteerd dat verdisconteren van vector-inactivering door humaan complement onvoldoende is gestandaardiseerd voor opname in de formule zonder door de aanvragers aan te leveren validatiedata.
- De experimenteel verkregen reductiefactoren voor de verschillende parameters in de formule hangen af van de experimentele opzet en de condities waaronder een experiment is uitgevoerd. De hier gepresenteerde waarden kunnen dan ook alleen worden aangevoerd door aanvragers als hun experimentele condities overeenkomen met de hier gebruikte methoden.

- Tenslotte wordt aannemelijk gemaakt dat de titers zoals die worden bepaald met standaard titerbepalingen leiden tot een onderschatting van het werkelijke aantal aanwezige infectieuze lentivirale vectordeeltjes. Teneinde hiervoor te corrigeren verdient het aanbeveling de berekende hoeveelheid infectieuze vectordeeltjes in het inoculum (C_i) te vermenigvuldigen met 10.

Deze nieuwe parameters kunnen leiden tot toename van de berekende hoeveelheid infectieuze vrije vectordeeltjes afkomstig uit het inoculum. Voor toepassingen onder ingeperkt gebruik kan dat tot gevolg hebben dat getransduceerde cellen bijvoorbeeld vaker moeten worden gewassen of langer moeten worden gekweekt, indien een omlaagschaling naar een lager inperkingsniveau gewenst is. Voor toepassingen voor introductie in het milieu kan dat betekenen dat er eerder dan voorheen een milieurisicoanalyse wordt gevraagd met betrekking tot de aanwezigheid van dergelijke deeltjes. Hoewel elke aanvraag met grote zorgvuldigheid op de aanwezigheid van vrije vectordeeltjes en de daarbij komende mogelijke risico's moet worden geëvalueerd, lijkt deze stap overkomelijk en zal het in de meeste gevallen tot praktische toepassing van de vectoren kunnen komen. Immers, de kans op een significante blootstelling aan vrije vectordeeltjes wordt bij de meeste toepassingen ingeschat als erg klein en tevens is het effect dat kan worden verwacht van een blootstelling aan een beperkt aantal infectieuze replicatie-defectieve vector deeltjes van een SIN lentivirale vector zonder schadelijk transgen, zeer gering.

3. Summary

Lentiviral vectors are particularly popular for stable genetic modification of mammalian cells. With current production systems, virus concentrates can be made that consist of replication-defective lentiviral vector particles without containing any replication-competent lentiviruses. The stable integration of the lentiviral vector into the DNA of the host happens in a well-predictable manner, although the chromosomal location of the integration cannot be controlled. Once integrated into the chromosome the vectors cannot be mobilized, provided that the so-called self-inactivating (SIN) vectors are used.

Lentiviral vectors derived from the human immunodeficiency virus (HIV) are the most commonly used. Often the envelope protein of HIV is replaced by a heterologous envelope protein to give the vectors the ability to infect cells that do not express the HIV-receptor CD4.

A GMO permit is required for the use of lentiviral vectors (a notification is sufficient for certain applications under contained use in The Netherlands). An (environmental) risk analysis is required to obtain such a permit. In this analysis, attention must be paid to the possible presence of replication-competent lentiviruses that could arise during production. In addition, the (environmental) risks of the cellular products that are made must be addressed. Finally, the (environmental) risks associated with the presence of free and infectious replication-defective lentiviral vector particles that may be left over from the vector inoculum that is used for transduction of cells must be addressed. By using the current vector production systems, where the required helper functions are separated on 3 or more plasmids, the risk of the formation of replication-competent lentiviruses can be neglected. However, the risk of insertional mutagenesis cannot be excluded, although this is greatly reduced by the use of the SIN vectors. In addition, the overexpression of the introduced transgene can have negative consequences. It is therefore important to evaluate the nature of the transgene when looking at the effects of free vector particles. Vectors with genes encoding toxic or transforming products require special attention.

To assist researchers who could not adequately quantify these vector particles, COGEM developed a formula to determine the Reduction Ratio about 10 years ago. This ratio estimates the loss of infectious titer of replication-defective vector particles based on a number of inactivating activities. These include spontaneous inactivation of the infectious particles, degradation of vector particles under the influence of trypsin or complement and the loss of vectors by washing or passaging the transduced cell cultures. The combined effects of these inactivating steps were related to the

amount of infectious vector particles in the inoculum. The parameters required for the formula were largely derived from literature data.

Recently, this reduction-ratio approach was also endorsed in a European document in 'Good Practice on the assessment of GMO-related aspects in the context of clinical trials with human cells genetically modified by means of retro/lentiviral vectors', which was also endorsed in The Netherlands in October 2019 (https://ec.europa.eu/health/sites/health/files/files/advtherapies/docs/gmcells_gp_en.pdf).

At the time the formula describing the Reduction Ratio was conceptualized, literature data were only found for lentiviral vectors which carried the VSV-G protein as an envelope protein. In the current study, new data were generated for lentiviral vectors with a number of other heterologous envelope proteins. Additionally, experiments to confirm the existing data with VSV-G envelope protein-carrying vectors were performed. Moreover, assumptions that were used in the current formula were also critically evaluated in order to expand the usability and improve the foundation of the formula.

On the basis of these results the applicants could draw the following conclusion and observations.

- The VSV-G envelope protein-carrying vectors are more stable than previously thought. The half-life of these particles was determined to be 34.7 hours, instead of the 10.4 hours previously used as a standard.
- The efficiency of a washing step is approximately 95% reduction in mass for a Ø10 cm petri dish. This is identical to the standard currently used.
- The efficiency of a washing step on a Ø35 mm petri dish is around 98% reduction in mass, and therefore higher than the 95% previously assumed.
- Trypsin inactivates the VSV-G envelope protein-carrying particles less efficiently than previously assumed.
- It is being argued that vector inactivation by human complement is insufficiently standardized for inclusion in the formula without supplying of validation data by the applicants.
- The experimentally obtained reduction factors for the different parameters in the formula depend on the experimental design and the conditions under which an experiment was conducted. The values presented here can therefore only be employed by applicants if their experimental conditions correspond to the methods used here.

- Finally, it is made plausible that the titers as determined with standard titration methods lead to an underestimation of the actual number of infectious lentiviral vector particles that are present in a preparation. In order to correct for this, it is recommended to multiply the calculated amount of infectious vector particles in the inoculum (Ci) by 10.

These new parameters can lead to an increase in the calculated amount of infectious free vector particles from the inoculum. For applications under contained use, this may result in more extensive washing or prolonged culturing of transduced cells if a down-scaling to a lower containment level is desired. For deliberate release applications, this may imply that an environmental risk analysis is requested more often than before with regard to the presence of these particles. Although every application must be evaluated with great care for the presence of free vector particles and the possible risks involved, this step appears surmountable and will, in most cases, lead to the practical application of the vectors. After all, the chance of significant exposure to free vector particles is estimated to be very low in most applications and also the effect that can be expected from exposure to infectious replication-defective vector particles from a SIN lentiviral vector without harmful transgene, is very small.

4. Introduction

Retroviral and lentiviral vectors have become the method of choice for many applications that require stable genetic modification of mammalian cells in cell culture (Milone & O'Doherty, 2018). Lentiviral vectors have been predominantly derived from Human immunodeficiency virus 1 (HIV1). The popularity of lentiviral vectors stems from their propensity to integrate their genetic payload into the chromosomal DNA of their host cell. They do so in cycling cells as well as in mitotically quiescent cells.

There is a plethora of systems for the production of lentiviral vectors. With the more recent 2nd and 3rd generation production systems batches of replication-defective lentiviral vectors can be produced that are free of contaminating replication-competent lentiviruses (COGEM-advies-CGM/090331-03, 2009). To the best of our knowledge, there are no reports to date that describe the generation of replication-competent lentivirus during the production of replication-defective lentiviral vector stocks with the 2nd and 3rd generation systems.

While the HIV virus itself depends on the expression of the CD4 molecule on the surface of the target cell for productive infection, the lentiviral vectors are usually produced with an alternative envelope protein in their lipid membrane. The envelope of the lentiviral particle is 'pseudotyped' with the alternative envelope glycoprotein (Cronin, Zhang, & Reiser, 2005). This allows the transduction of cells lacking CD4 when the pseudotyped envelope protein engages its cognate receptor on the target cell.

A range of different envelope proteins have been employed for production of lentiviral vectors. These include the envelope glycoprotein of vesicular stomatitis virus (the VSV-G protein), the envelope protein of non-human retroviruses (e.g. the ecotropic retrovirus Murine leukemia virus (MuLV), the Gibbon ape leukemia virus (GALV), the feline endogenous RD114 retrovirus, Moloney murine leukemia virus 4070A, Moloney murine leukemia virus strain 10A1, the Rabies virus glycoprotein and the Measles virus hemagglutinin and fusion glycoproteins (Joglekar & Sandoval, 2017; Stitz et al., 2000; Tomas et al., 2019)). All but one of these receptors are capable of binding receptors on human cells. The exception is the ecotropic MuLV envelope glycoprotein which recognizes a receptor on murine cells only.

The VSV-G protein has been the most popular for pseudotyping the envelope proteins of lentiviral vectors. VSV-G binds LDL-Receptor family members, allowing these viruses to infect a wide range of cell types of many distinct host species, including rodent and human cells (Finkelshtein, Werman,

Novick, Barak, & Rubinstein, 2013). The others have been used predominantly for transduction of human blood and bone-marrow derived cells, as in these cells VSV-G pseudotyped vectors are less effective (Amirache et al., 2014). The ecotropic MuLV-envelope pseudotyped vectors have been used mainly for experiments in which lentiviral-vector transduction is to be limited to murine cells (Watson, Kobinger, Passini, Wilson, & Wolfe, 2002).

Lentiviral vectors are widely used in biomedical research under Contained-Use conditions. A key safety factor is the resistance of the split vector 2nd and 3rd generation production systems to the formation of replication-competent lentiviruses during production. These production systems allows the production of lentiviral vector stocks that are free of contaminating replication-competent lentiviruses (Dull et al., 1998; Schambach, Zychlinski, Ehrnstroem, & Baum, 2013; Zufferey et al., 1998). This has resulted in the permission to use HIV-derived lentiviral vectors and cells modified with such vectors under biosafety level 2 conditions (COGEM-advies-CGM/090331-03, 2009).

The so-called self-inactivating or SIN lentiviral vectors are integrated in the cells in a structure in which the major part of the U3 region in the long terminal repeat (LTR) is lost. As a result, the LTR cannot serve as a promotor and there is virtually no transcription initiating in the SIN LTR. As a consequence, the expression of the transgene is dependent on heterologous promoter inserted between LTRs. Transcription initiating at the internal promotor does not generate mRNAs that harbor the lentiviral packaging signal. Consequently, in absence of transcripts that harbor the packaging signal the integrated lentiviral vector cannot be mobilized upon superinfection by replication-competent lentiviruses (Zufferey et al., 1998).

It goes without saying that the production and handling of lentiviral vector particles, and the handling of cells modified with such vectors, can be associated with (environmental) risks and therefore the use of these vectors is subject to GMO guidelines and regulations. In contained-use applications the handling of lentiviral vector stocks produced with 2nd or 3rd generation vector lots requires a BSL-2 containment facility. This is based, amongst others, on the resistance of the production systems to generation of replication-competent vector stocks. The absence of detectable mobilization of SIN vectors by replication-competent lentiviruses have led to a practice in which the culture and maintenance of lentiviral vector-transduced cells is permitted under BSL-1 level once the risks of the viral-vector particles added to the cells has been reduced to a negligible level. There are few risk factors associated with handling lentivirus vector particles. With the vector production systems used to date, in which the helper functions are separated on 3 or more different plasmids, the risk of forming replication-competent lentiviruses during production has been eliminated. However, even in absence of replication there is a risk of insertional mutagenesis (Cavazzana-Calvo et

al., 2010). However, the use of SIN vectors reduces this risk factor considerably. In contrast to gamma-retrovirus derived vectors the lentivirus vectors integrate in the cellular DNA with a preference for the transcribed regions of genes. This strongly reduces the risk of oncogene activation in comparison to gamma-retrovirus vectors. In a retrospective study of recipients of SIN lentivirus vector modified stem cells, Fisher and collaborators demonstrated the absence of selective clonal expansion of particular gene modified clones in all of 33 recipients (Fischer, Hacein-Bey Abina, Touzot, & Cavazzana, 2015).

However, this does not eliminate the potential negative consequences of overexpression of the transgene. Therefore it remains essential to take the transgene into account when evaluating the potential effects of the free lentivirus vector particles. Vectors with genes encoding toxic or transforming products deserve special attention.

To obtain permission for working under BSL-1 level a stepwise risk assessment (ERA) is required. A key step in the ERA is assessment is the risk that may be associated with infectious replication-defective viral particles that remain from the initial vector inoculum used for generating the genetically modified cell product.

Reduction ratio = $(20^{W} \times 200^{I} \times 2^{2,4T})/C_i$

***The COGEM formula**. This formula calculates the reduction ratio, i.e. the fold decrease in amount of remaining infectious viral vectors divided by the amount of viral vectors in the inoculum. In the formula the parameter 'W' represents the number of times the cell culture was washed, 'I' signifies the number of inactivating washes with trypsin or human serum, 'T' is the culture time after the start of transduction in days. The factor 2.4 is based on the published half-life of VSV-G envelope pseudotyped infectious lentiviral vector particles at 37°C. 'C_i' is the measured amount of infectious viral particles in the inoculum.*

The reduction ratio represents the fold-decrease in the concentration of free infectious vector particles related to the amount of infectious viral vectors added to the culture. A ratio of 1 indicates that, based on the procedure used, the amount of free infectious virus particles present is reduced to 1/1 = 1. A reduction ratio of 100 indicates that 100 times more viral particles are expected to be inactivated than were initially present in the inoculum, meaning that 1/100 = 0.01 infectious particles are left on average in the culture.

Several years ago the COGEM proposed a formula that could be used for estimating the residual amount of free infectious viral particles in the cell product to be evaluated (COGEM-advies-CGM/090331-03, 2009). This formula should assist a risk analysis for standard infections in absence of actual experimental data. The COGEM formula calculates the residual amount of free infectious viral particles as function of the time, number of vector inactivating steps, and the number of washing steps.

While initially generated for use in risk assessments for contained use applications, more recently the formula has also been employed for estimating the number of residual viral vector particles in cell preparations for deliberate release applications. The required reduction ratio was initially set to 100, but more recently in some applications the use of a reduction ratio of 1 has been employed.

A basic assumption is that the starting amount of free infectious viral particles used to inoculate the cells is known. This assumes that the titer is assessed in a cell system that is maximally sensitive to the viral vector particles, and that most vector particles are detected in the biological assay.

In this study we performed experiments to expand the range of conditions with which the formula can be applied. In the studies, we included VSV-G envelope pseudotyped lentiviral vectors, as a comparator and to verify the literature data on which the current parameter values are based.

Most of the experiments performed for this study were biological assays in human 293T and mouse B77 cells transduced by envelope-pseudotyped lentiviral vectors equipped with a green fluorescent protein (GFP) expressing transgene. By flow cytometry analyses, the percentage of cells displaying GFP signal was assayed compared to non-transduced control cells. The flow cytometry analyses are linear, and will detect virtually all the transduction events, unless the virus lands in a transcriptionally silent area, or if the vector contains mutations that inactivate the reporter genes. These factors are probably of minute influence and are therefore neglected. Another limitation is that at higher transduction efficiencies (i.e. >60%) the GFP signal may slightly underestimate the true titers as at higher transduction frequencies an increasing fraction of the transduced cells will contain more than one vector copy.[1] The distribution of the frequencies will follow the Poisson distribution. Therefore

[1] The >60% value stems from the Poisson distribution: for a 60% transduction efficiency an MOI of 0.9 virus particles/cell is required. After one half-life, the MOI would be 0.45, yielding 36% positive cells. After two half-lives at the MOI would be 0.23, giving rise to 20% transduced cells. These values can be used to calculate an apparent half-life. The calculation demonstrates that we would *over*-estimate the true half-life by a maximally of approximately 10%. Thus if the infectivity decay is measured at minimally three timepoints spanning at least two half-lives and starting at a transduction frequency of <60% the measured half-live is overestimated by maximally 10%. This seems suited for the purpose.

we tried to keep our transduction frequencies below 40%. In the cases where higher percentages were found we opted not to account for doubly infected cells, as this would only marginally affect the measured half-lives of the viruses. Our data are therefore slightly over-estimating the vector's half-life.

In short we performed the following analyses.

- We assessed the half-life of lentiviral vector particles pseudotyped with other envelope proteins for which no half-life data are known in literature
- We sought to confirm published half-life data for the VSV-G envelope glycoprotein;
- We determined the efficiency of trypsin-mediated vector inactivation for the envelope-pseudotyped vectors;
- We measured the amount of residual medium for two dish-sizes, as a guide to estimate the efficiency of washing the cell cultures;
- We did not study the impact of complement-mediated vector inactivation, as literature data demonstrate that it is not feasible to provide broadly applicable efficiency guidelines.

In addition,

- We determined the effect of variations in the infection procedure on the apparent infectious viral particle titers;
- We measured how polybrene, often added to the culture medium for enhancing the efficiency of vector transduction, affects the vector half-life.

The outcomes of our study provide new parameters that can be used to estimate the amount of residual infectious vector particles for envelope-pseudotyped lentiviral vectors. This should aid the risk analyses that are performed in absence of experimental data that quantify the amount of residual infectious viral particles in specified experimental conditions.

5. Materials and methods

Acquiring the plasmids encoding the virus envelope proteins

Plasmids containing the gene for VSV-G glycoprotein (#8454), pCMV-VSV-G, rabies virus (strain SAD B19) glycoprotein (#15785) pHCMV-RabiesG, ecotropic Moloney murine leukemia virus envelope gene (#15802) pHCMV-EcoEnv, Moloney murine leukemia virus strain 10A1 envelope gene (#15805) pHCMV-10A1 and amphotropic 4070A gene (#15799) pHCMV-AmphoEnv were purchased from Addgene (www.addgene.org), online plasmid numbers are indicated above. Addgene is a non-profit plasmid repository and plasmids are provided by researchers. Plasmid pCMV-VSV-G was a kind gift from Bob Weinberg. All other Addgene derived plasmids were a kind gift from Miguel Sena-Esteves.

Plasmids encoding for the gibbon ape leukemia virus envelope protein (phCMV-GALV TR) and the feline endogenous virus RD114 envelope glycoprotein (phCMV-RD114 TR) were a kind gift from professor F. Staal, Leiden University Medical Center, Leiden, The Netherlands. Plasmids encoding the H and F glycoproteins of Measles virus stain Edmonston (pCG-HD24 and pCG-Fdel30) were a kind gift from professor F.-L. Cosset, Claude Bernard University, Lyon, France.

The helper plasmids encoding HIV-1 gag/pol (pMDLg-RRE) and, HIV-1 rev (pRSV-REV) were purchased from Addgene and were a kind gift of Didier Trono (respectively Addgene #12251 and #12253). Plasmid pRRL-cPPT-CMV-GFP-PRE-SIN (here named pLV-CMV-GFP) was a kind gift of Jurgen Seppen.

Production of lentiviral vectors

Third generation self-inactivating lentiviral transfer vectors containing a green fluorescent protein (GFP) transgene, the plasmids encoding the different envelope proteins and the two other helper plasmids (encoding HIV-1 gag/pol, HIV-1 rev) were co-transfected overnight into 60-70% confluent 293T cells (human embryonic kidney cells containing the SV-40 T-antigen) using the polyethyleneimine (PEI) method. Briefly, plasmids were mixed in the following concentration: 7.5 µg pCMV-[envelope gene], 11.4 µg pMDLg-RRE (gag/pol), 5.4 µg pRSV-REV and 13.7 µg pLV-CMV-GFP followed by the addition of 150 mM Opti-MEM® I (Gibco; Thermofisher Scientific, catalog # 31985070) to a total volume of 1 ml. In a second tube 114 µl PEI (1 mg/ml) and 150 mM Opti-MEM® I were mixed (total volume 1 ml). For obtaining lentiviral vectors with the envelop proteins of Measles virus, 6.23 µg of both plasmids pCMV-HD24 and pCMV-Fdel30 are added to the mixture, other plasmids quantities are kept as above. The content of the two tubes was gently mixed together, incubated at RT for 10 minutes and added to the culture medium (Dulbecco's Modified Eagle Medium (Gibco; Thermofisher Scientific) supplemented with 8 % FBS and penicillin-streptomycin) of

a T175 flask containing 293T cells. After overnight incubation (37°C / 5% CO_2) culture medium was replaced and supernatant was harvested after 48 and 72 hours post-transfection, centrifuged for 5 minutes at 845 x g at room temperature and subsequently passed through 0.45 µm pore-sized filters (Pall, Supor PES, Acrodisc, Supor polyethersulfone membrane, pore size 0.45 µm, diameter of filter unit 25 mm, art no 4614), aliquoted and stored at −80°C. The physical titers of the vector preparations were quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation, NY) and can be found in Appendix A.

Concentrated lentiviral vector stocks were obtained by ultracentrifugation of the filtered vector-containing culture medium. 31 ml of culture medium was added to a 35 ml polypropylene tube (Beckmann Coulter, 25 x 89mm, art. No 326823) onto a 4 ml 20% sucrose solution, placed into a Beckmann Sw32 Ti rotor and centrifuged at 50,000 x g for 2 hours at 4°C. Subsequently, the supernatant was removed and the pellet was resuspended in 0.6 ml $T_{50}N_{130}E_1$ buffer (50mM Tris-Cl, 130mM NaCl and 1mM EDTA; pH 7.8) by gently shaking overnight at 4 °C, aliquoted and stored at −80°C.

Residual culture medium volume on culture dishes

Ø35 mm or Ø10 cm (Greiner CELLSTAR®, Sigma Aldrich, catalog # resp. P6987 and 7612) culture dishes were weighed on an analytical balance before adding resp. 3 and 10 ml culture medium supplemented with 8% FBS and 8 µg/ml polybrene. Culture medium was aspirated by a resp. 5 and 10 ml serological pipette (Greiner CELLSTAR®, Sigma Aldrich, catalog # resp. 7615 and 7740) and dishes were weighed again. Next, the volume of culture medium was added again to the dishes and removed by an aspiration needle coupled to a vacuum system and dishes were weighed again. The density (specific weight) of the applied culture medium was determined by weighing 1 ml of culture medium in a microcentrifuge tube on an analytical balance. From the residual weight of the culture dishes and the density of the culture medium, the residual volume in the culture dishes was calculated.

Lentiviral vector transduction of 293T and B77 cells

GFP-transgene containing lentiviral vectors pseudotyped with the ecotropic Moloney murine leukemia virus envelope protein were assayed on B77 cells (non-producer B77 avian sarcoma virus transformed BALB/3T3 mouse embryonic cells). All other envelope-pseudotyped lentiviral vectors were assayed on 293T cells in 24-well format. By flow cytometry analyses, the percentage of cells displaying GFP signal was assayed compared to non-transduced control cells. In these cell-based assays (the assays for determining the half-life, trypsin sensitivity and the degree of cellular uptake of the different envelope-pseudotyped vectors) the quantity of vector particles that were being added

were based on volume. In these assays we aimed at a maximum of 30-40% GFP positive cells to ensure that the majority of cells is transduced by a single lentiviral vector particle. The p24 titer of the vector stocks was determined for comparison purposes only as it was impossible to correlate the physical particle titer to the biological activity of a vector stock with this method.

Half-life assay

The indicated volume of unconcentrated or concentrated envelope-pseudotyped lentiviral vectors was added to 250 µl culture medium into a 24-well culture plate and incubated at 37°C / 5% CO_2 for the indicated time. Subsequently, 10 µl culture medium containing 208 µg/ml polybrene was added (to obtain a final concentration of 8 µg/ml polybrene), the vector-containing medium was transferred to 60-70% confluent 293T or B77 cells and centrifuged for 90 minutes at 845 x g at 33°C. Next, 250 µl fresh culture medium was added and cells were placed at 37°C / 5% CO_2. Two days after transduction cells were harvested for flow cytometry analysis. From the graphical representation of the percentage of GFP-positive cells obtained by flow cytometry, the half-life was determined following the formula for exponential decay $N(t) = N_0 * e^{-\lambda t}$ in which $t_{½} = \ln 2 / \lambda$.

Residual lentiviral vectors in culture medium / Cellular uptake assay

The indicated volume of unconcentrated or concentrated envelope-pseudotyped lentiviral vector was added to 250 µl culture medium supplemented with 8 µg/ml polybrene into a 24-well culture plate with or without 60-70% confluent 293T or B77 cells. Subsequently, cells were centrifuged as above ("spin infection") or only incubated at 37°C / 5% CO_2. Vector-containing medium in culture plates without cells were placed at 37°C / 5% CO_2 without centrifugation. After 90 minutes 250 µl fresh culture medium was added to all wells and plates were incubated at 37°C / 5% CO_2. The next day, the culture medium was replaced and vector-containing medium was transferred to fresh cells again followed by a spin infection as described above. Two (secondary infection) or three (primary infection) days after transduction cells were harvested for flow cytometry analysis.

Trypsin inactivation assay

Small volumes of concentrated envelope-pseudotyped lentiviral vectors (5 – 15 µl) were incubated with 60 µl 0.05% trypsin-EDTA (from 0.5% Trypsin-EDTA, Gibco, ThermoFisher Scientific, catalog # 15400054) in PBS or PBS only in microcentrifuge tubes. After incubation for 5 minutes at 37°C trypsin was inactivated by the addition of 600 µl culture medium with 8 µg/ml polybrene and divided over 2 wells containing 60-70% confluent 293T or B77 cells (24-well format, 300 µl/well). Culture plates were centrifuged for 90 minutes at 845 x g at 33°C, 300 µl fresh culture medium was added and cells were incubated at 37°C / 5% CO2 for two days before flow cytometry analysis.

Flow cytometry analysis of lentiviral vector transduced cells

Cells were harvested in PBS and subsequently fixed in 4% paraformaldehyde for flow-cytometry analyses, resuspended in flow cytometry buffer (0.5% BSA and 2mM EDTA in PBS), and assayed on a BD LSRII flow cytometer. Per sample at least 10,000 living cells/events were measured and data were analyzed using FlowJo™ software version 10.

Statistical analyses

General standard deviations of duplo of triplo values were calculated using the *stdev* function in Microsoft Excel. Standard deviations of the reduction factors, consisting of a ratio between two values with their own standard deviations, were calculated using the formula:

$$\delta R = |R| \times \sqrt{((\delta X/X)^2 + (\delta Y/Y)^2)}$$

δR: standard deviation of the average reduction factor

|R|: average absolute value of the reduction factor

δX: standard deviation of the numerator of the reduction factor ratio

X: average value of the numerator of the reduction factor ratio

δY: standard deviation of the denominator of the reduction factor ratio

Y: average value of the denominator of the reduction factor ratio

Example cellular uptake of VSV-G envelope-pseudotyped lentiviral vectors:

Average % of GFP+ cells in 2nd infection with no cells in 1st infection: 39.7, ±1.2

Average % of GFP+ cells in 2nd infection with spin infection at 1st : 4.9, ±1.1

|R|: 39.7/4.9 = 8.1

δX: 1.2

X: 39.7

δY: 1.1

Y: 4.9

$\delta R = |8.1| \times \sqrt{((1.2/39.7)^2 + (1.1/4.9)^2)} = 1.8$

Standard deviations of the average reduction factors or percentage of residual vector activity of different experiments were calculated with the following formula:

$$\delta R = \sqrt{(((n_a-1) \times \delta X^2) + ((n_b-1) \times \delta Y^2))/((n_a-1)+(n_b-1))}$$

δR: standard deviation of the average reduction factor/% of residual vector activity

n_a: number of samples for value for first experiment with average value X

δX: standard deviation of average value X

n_b: number of samples for value for second experiment with average value Y

δY: standard deviation of average value Y

Example residual percentage of vector activity after trypsin treatment of VSV envelope-pseudotyped lentiviral vectors:

Average % of residual vector activity in first experiment: 107.8%, ±12.3%, 2 sample average

Average % of residual vector activity in second experiment: 99.2%, ±17.2%, 2 sample average

n_a: 2

δX: 12.3

n_b: 2

δY: 17.2

$$\delta R = \sqrt{(((2-1) \times 12.3^2) + ((2-1) \times 17.2^2)) / ((2-1)+(2-1)))} = 15.0\%$$

6. Results

In this results section we present the experimentally obtained data on the required parameters for determining the reduction ratio of free particles for envelope-pseudotyped lentiviral vectors. A total of eight lentiviral vectors pseudotyped with different envelope proteins were assessed (table R1). Each lentiviral vector is equipped with a green fluorescent protein (GFP) transgene to monitor the biological active ('infectious') status of the vector particle in the cells. The raw data of the experiments described in the result section can be found in Appendix A to F.

Full name of envelope protein	Abbreviation
Vesicular stomatitis virus G protein	**VSV-G**
Measles virus hemagglutinin and fusion glycoproteins	**Measles**
Gibbon ape leukemia virus envelope protein	**GALV**
Rabies virus glycoprotein	**Rabies**
Feline endogenous virus RD114 envelope glycoprotein	**RD114**
Moloney murine leukemia virus 4070A envelope protein (amphotropic)	**4070A**
Moloney murine leukemia virus strain 10A1 envelope protein (amphotropic)	**10A1**
Moloney murine leukemia virus envelope glycoprotein (ecotropic)	**MuLV**

Table R1 Eight envelope-pseudotyped lentiviral vectors were studied in this project

5.1 Residual culture medium volume on culture dishes

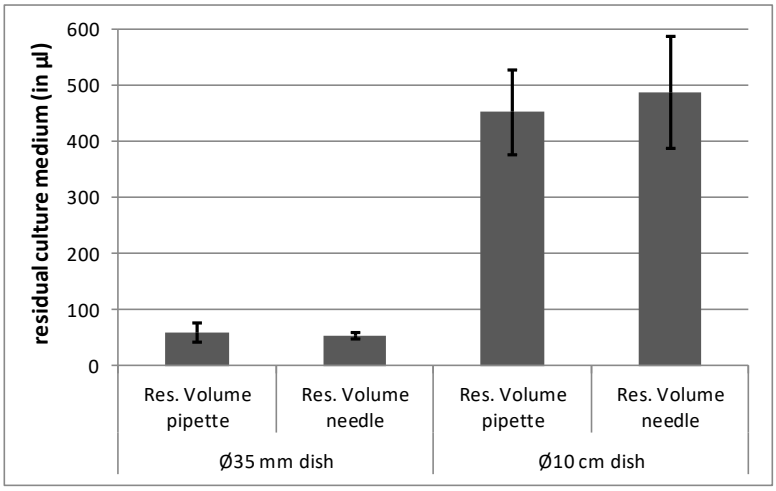
In the current formula for the reduction ratio of free lentiviral vector particles a factor 20 is used to describe the reduction of free lentiviral particles through the replacement of the culture medium as it was assumed that 5% of the volume remained on the culture dish upon aspiration of the culture medium.

We measured the residual culture medium volume on culture plates or dishes with a diameter of 35 mm or 10 cm as most of the lentiviral vector transduction in the laboratory are performed on cell culture systems with these dimensions. Importantly, we performed the experiments in the absence of vector particles in the culture medium and without cells in the culture dishes. Although this may influence the amount of residual culture medium on the dish, the volume of the cells themselves cannot be measured and may differ between cell types and cell culture conditions. In addition, the level of adhesion to cells and culture dishes may vary between the differently envelope-pseudotyped vector particles

In this study we added 3 ml and 10 ml of culture medium to resp. Ø35 mm and Ø10 cm culture dishes (n=10) and we calculated the absolute residual volume on the dish after aspiring the medium with a pipette or an aspiration needle (coupled to a vacuum system) (graph R1) and the residual volume as percentage of the input volume (graph R2). The raw data of the experiment are presented in Appendix B.

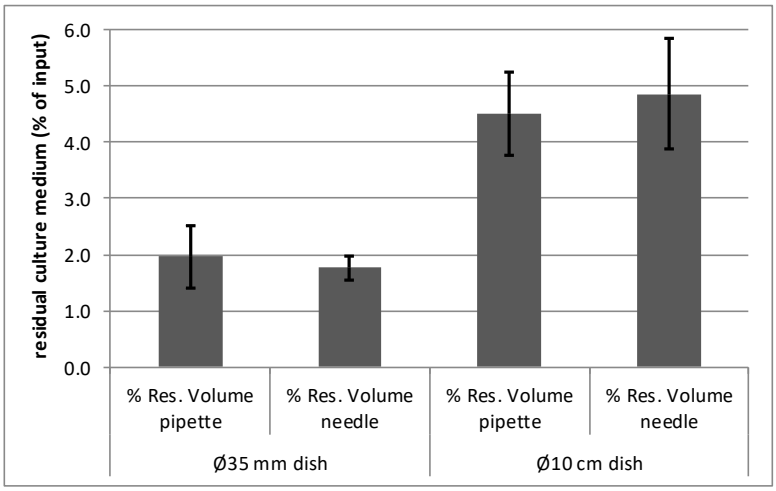
The residual volume on Ø35 mm dishes was on average 59 µl (±17) and 53 µl (±6) when the culture medium was aspirated with resp. a pipette or an aspiration needle and 451 µl (±75) and 486 µl (±99) on Ø10 cm dishes. Considering the standard deviation, this indicated that the residual volume was equal with both aspiration methods.

On Ø35 mm dishes on average respectively 2.0% (±0.6) and 1.8% (±0.2) of the input volume was left on the dish after aspiration of the medium with a pipette or an aspiration needle, resulting in a 50 times reduction of free lentiviral particles upon replacement of the culture medium. The residual volume on Ø10 cm dishes was on average respectively 4.5% (±0.8) and 4.9% (±1.0) of the input volume after aspiration with pipette or needle, indicating that the factor 20 used in the currently applied formula is correct for Ø10 cm dishes. These results also pointed out that the reduction factor to be used in the formula depends on the dimensions of the culture system. Therefore, we would like to suggest to consider the implementation of dimension-specific reduction factors in the formula, in accordance with the cell culture systems used in the experiment.



Graph R1

Residual volume of culture medium (µl) on Ø35 mm and 10 cm dishes after aspiring the medium with a pipette or aspiration needle ± standard deviation (n = 10 dishes).



Graph R2

Residual volume of culture medium as percentage of the input volume (resp. 3 ml and 10 ml) on Ø35 mm and 10 cm dishes after aspiring the medium with a pipette or aspiration needle ± standard deviation (n = 10 dishes).

5.2 Trypsin inactivation of envelope-pseudotyped lentiviral vectors

The enzyme trypsin is routinely used in the laboratory to dissociate attached cells from their culture dishes to be able to apply a treatment to the cells. Trypsin is an endopeptidase cleaving C-terminal of lysine and arginine residues in polypeptides. The enzyme is capable of cleaving the envelope proteins on lentiviral vector particles, thereby inactivating free floating particles in solution and dissociating bound, extracellular particles from cells, leaving them unable to transduce the target cells and ready to be washed away.

The current formula for the reduction ratio of free lentiviral vector particles describes a factor 200 to define the reduction of infectious vector particles caused by trypsin or human serum in combination with a washing step. This is based on the finding that 90% of wildtype HIV-1 virus is inactivated upon incubation with 0.01% trypsin for 10 minutes leading to a reduction factor of 10 for trypsin treatment only (resulting in factor 200 if multiplied with the effect of a washing step (factor 20)) (COGEM-advies-CGM/090331-03, 2009; Tang & Levy, 1991).

In the current study we investigated the effect of trypsin on the eight envelope-pseudotyped lentiviral vector particles. We incubated a small volume (5-15 µl) of purified serum-free particles with 60 µl 0.05% trypsin in PBS (or PBS only) for 5 minutes at 37 °C, conditions that are used in the daily cell culture routine. Trypsin was subsequently inactivated and the vector particles were added to 293T or B77 (for MuLV) cells to determine the percentage of particles that is able to transduce the cells, leading to a measurable GFP expression. Raw data of the analyses can be found in Appendix C.

We represented the average *residual activity* of the vector particles by dividing the percentage of GFP-positive cells in the trypsin-containing condition by the GFP-positive cells in the PBS only condition (which was set at 100% activity) (table R2). We also converted these data to a reduction factor as practiced in the formula (GFP+ cells in PBS only / GFP+ cells in PBS+trypsin) (table R3).

Residual envelope-pseudotyped lentiviral vector activity after trypsin treatment						
average residual lentiviral vector activity after trypsin treatment in % (relative to PBS control)						
VSV-G	Measles	GALV	Rabies	RD114	4070A	MULV
104%	23%	1%	61%	1%	26%	10%
±15%	±3%	±1%	±11%	±0.2%	±15%	±1%

Table R2 Average percentage of residual envelope-pseudotyped lentiviral vector activity after trypsin treatment, ± standard deviation is indicated in grey, n=2 independent experiments with technical duplicates, for Measles, RD114 and MuLV, n=3 with technical duplicates. (The 10A1-pseudotyped vector lost all GFP activity upon purification and was not included in these analyses.)

The average reduction factor by trypsin of the different vectors ranged from 1 (100% residual activity/no inactivation) up to 110 (1% residual activity) and therefore appears to depend for a large part on the vector's envelope protein.
The reduction factor found for three of the vectors in a pilot experiment deviated substantially from the values obtained in later experiments (Appendix C). We anticipate that the vector batch (Batch 2, see Appendix A) used for the pilot experiment was contaminated with fetal bovine serum, partly protecting the vectors from inactivation by trypsin. Therefore, we would like to stress that the experimentally determined reduction factors can be significantly lower in the presence of (trace amounts) of serum.

The reduction factor by trypsin inactivation appears to depend on the envelope protein of the vector and differs for most envelope-pseudotyped vectors considerably from the reduction factor of 10 that is applied in the current formula. We would like to suggest to consider the implementation of the vector-specific reduction factors in the formula.

Reduction factor by trypsin inactivation of envelope-pseudotyped lentiviral vectors						
VSV-G	Measles	GALV	Rabies	RD114	4070A	MULV
1	4	79	2	110	4	10

Table R3 Reduction factors of the envelope-pseudotyped lentiviral vectors based on inactivation by trypsin treatment

5.3 Half-life of pseudotyped lentiviral vectors

The impact of the number of culture days T in the current formula for the reduction ratio of free lentiviral vector particles is based on the notion that the vector particles lose their infectivity based on a stochastic process with a constant half-life. In the current formula the half-life of VSV-G pseudotyped particles is considered to be around 10 hours (0.42 days), leading to a reduction factor of 2.4. This number is based on a publication on the kinetic analyses of the stability of VSV-G pseudotyped lentiviral vector particles (Higashikawa & Chang, 2001).

In the current study we determined the half-life of the eight envelope-pseudotyped lentiviral vector particles under conditions that are representative for the daily routine in cell transduction experiments. Polybrene is routinely used to enhance the transduction efficiency of the lentiviral vector particles. In a pilot experiment (Appendix C) we investigated the effect of polybrene on the half-life of VSV-G pseudotyped particles by incubating the vector particles in culture medium (with FBS, without cells) with or without polybrene for several periods of time after which the vector-containing medium was transferred to cells. From the experiment it became clear that polybrene negatively affects the half-life of VSV-G pseudotyped vector particles, but this difference is not apparent if the vector particles are directly incubated with cells (without prior incubation of vector particles in culture medium in the absence of cells). This t=0 condition is the regular method to start a transduction experiment. Additionally, polybrene is also absent during the subsequent culturing of transduced cells while free infectious lentiviral vector particles could still be present. Moreover, determination of the half-life in absence of polybrene, which reduces the half-life of the vectors, represents the desired situation from a safety perspective. For these reasons we decided to study the half-life of the vector particles *without* polybrene.

We incubated lentiviral vector particles in culture medium containing 8% FBS at 37 °C (without cells) in a time series. At the end of the incubation time the vector containing medium was added to 293T of B77 cells to determine the percentage of particles that is able to transduce the cells, leading to a measurable GFP expression. From the graphical representation of these data the half-life ($t_{½}$) was determined following the formula for exponential decay $N(t) = N_0 * e^{-\lambda t}$ in which $t_{½} = \ln 2 / \lambda$ (table R4). The reduction factors derived from the half-life of the different vectors are presented in table R5.

The half-life of the differently pseudotyped lentiviral vector particles ranged from around 8 to around 40 hours and appears to depend, at least in part, on the envelope protein as the differences between the specific vectors were considerable. In our experiments the half-life of VSV-G pseudotyped lentiviral vector particles is around 35 hours and this value differs considerably from the half-life of

10.4 hours that is used in the current formula for the reduction factor of free lentiviral vector particles. Differences in assay method and assay conditions between the published and our data probably underlie the observed discrepancy in the half-life of VSV-G pseudotyped particles. This substantial influence of experimental set-up on the outcome of the half-life should be considered consciously upon implementing our obtained data in practice.

For the pseudotyped vector particles other than VSV-G we were not able to find any literature data for comparing purposes.

Considering the vector-specific half-life values, we would like to suggest to consider the implementation of the experimentally determined vector specific reduction factors in the formula.

Half life of envelope-pseudotyped lentiviral vectors (in hours)				
				average
VSV-G	34.7	34.7		**34.7** ±0.0
Meas	14.1	14.1		**14.1** ±0.0
GALV	7.6	9		**8.3** ±1.0
Rabies	16.1	14.7		**15.4** ±1.0
RD114	17.8	33	12.8	**21.2** ±10.5
4070A	38.5	34.7		**36.6** ±2.7
10A1		13.6	13.9	**13.8** ±0.2
MuLV	24.8	15.8	15.1	**18.6** ±5.4

Table R4 Half-life of vectors with different envelope proteins (independent experiments with technical triplicates), ± standard deviation is indicated in grey behind the average value.

Reduction factor half-life	
VSV-G	0.7
Measles	1.7
GALV	2.9
Rabies	1.6
RD114	1.1
4070A	0.7
10A1	1.7
MuLV	1.3

Table R5 Reduction factors suggested to be applied in the formula based on the half-life of the envelope-pseudotyped lentiviral vectors

5.4 Cellular uptake of envelope-pseudotyped lentiviral vectors

The current applied formula for the reduction ratio of free lentiviral vector particles does not include a parameter for the potential reduction of free vector particles by uptake of the particles in cells. Here, we first investigated the influence of the experimental set-up on the vector uptake in the cells and subsequently assessed whether the vector concentration in the culture medium actually decreased upon incubation with 293T or B77 cells. Typical transduction experiments using lentiviral vectors can be performed as stationary infection, where the cells with the vector containing cell culture medium is incubated relatively *motionless* at 37 °C/5% CO_2, or as spin infection where the culture plate is centrifuged to spin the particles down in the direction of the cells. We performed a stationary and a spin infection with three of the envelope-pseudotyped lentiviral vectors under equal circumstances and compared both conditions by calculating the ratio between the percentage of GFP-positive cells in the spin infection by the percentage in the stationary infection (table R6).

Increased cell transducion by lentiviral vectors upon spin infection						
	Spin infection		Stationairy infection		*Ratio spin/stationary infection*	
	% of GFP-positive cells					
VSV-G	51.5	±2.8	20	±0.8	**2.6**	±0.2
Rabies	25.4	±0.6	17.7	±1.5	**1.4**	±0.1
4070A	58.8	±1.8	10.1	±0.3	**5.8**	±0.2

Table R6 Increase in the percentage of GFP-positive cells in spin infection versus stationary infection with three envelope-pseudotyped lentiviral vectors. n=1 with technical triplicates and sextuplicates for VSV-G, ± standard deviation values are shown in grey.

From the data it became apparent that a spin infection with these three envelope-pseudotyped lentiviral vectors led to an increased percentage of GFP-positive cells compared to a stationary infection. The extend of the increase differed per pseudotyped lentiviral vector. This could be caused by an inherent difference between the envelop proteins or/and differences in assay conditions or vector batches.

Importantly, these results also showed that the apparent/measured amount of vector particles in the **initial inoculum** of a transduction experiment was strongly dependent on the experimental procedure that was used to determine the titer of the vector batch. Stationary infection procedures as well as (to a lesser extent) spin infection procedures underestimated the amount of vector particles in the inoculum (see also Table R7).

Another relevant parameter for the formula for the reduction ratio of free lentiviral vector particles is the potential decrease in free vector concentration in the cell culture medium. To address this, we transferred the culture medium of the infection described above to fresh cells and performed a second spin infection on each sample to maximize the amount of vector particles entering the cells. Next, we assayed the percentage of GFP-positive cells and calculated the ratio of GFP-positive cells between the vector containing culture medium in the absence of cells from the first 'infection' (to correct for the half-life of the pseudotyped lentiviral particle) and the vector containing culture medium from the first stationary infection. Also, we calculated the ratio of GFP-positive cells between the vector containing culture medium in the absence of cells from the first 'infection' and the vector containing culture medium from the first spin infection (table R7).

2nd infection - Reduction of vector concentration in stationairy infection						
medium from:	**1st infection - No cells**		**1st infection - Stationairy**		*Ratio no cells/stationary infection*	
	% of GFP-positive cells					
VSV-G	17.8	±0.6	22.6	±0.6	**0.8**	±0.0
Rabies	21.1	±1.2	21.6	±1.8	**1.0**	±0.1
4070A	41.3	±5.5	31.9	±3.6	**1.3**	±0.2

2nd infection - Reduction of vector concentration in spin infection						
medium from:	**1st infection - No cells**		**1st infection - Spin**		*Ratio no cells/spin infection*	
	% of GFP-positive cells					
VSV-G	17.8	±0.6	6	±0.6	**3.0**	±0.3
Rabies	21.1	±1.2	19.2	±2.0	**1.1**	±0.1
4070A	41.3	±5.5	28.3	±3.1	**1.5**	±0.3

Table R7 Reduction ratios in free vector concentrations after spin and stationary infections. n=1 with technical triplicates, ± standard deviation values are shown in grey.

From the second infection it became clear that the concentration of vector particles in the medium in a stationary infection did not significantly decrease as the ratio of GFP-positive cells between medium that is incubated with and without cells is around 1 for all assessed envelope-pseudotyped lentiviral vectors. For spin infections it appeared that there was, for some envelope-pseudotyped vectors, a decrease in vector concentration in the medium after a first infection round on cells. For VSV-G pseudotyped lentiviral vectors this ratio was most pronounced.

Next, we repeated the assay with seven of the envelope-pseudotyped lentiviral vectors for only the spin infection condition (table R8). These data demonstrated a decrease in free vector concentration

in the medium upon a spin infection for all assayed envelope-pseudotyped lentiviral vectors except for GALV. We did not investigate the cause of this deviant result, but it might be explained by the nature of vector-cell interactions of the GALV envelope protein. Although all other envelope-pseudotyped vectors did show a reduced concentration in the culture medium after a spin infection, the differences between the different vectors were considerable and also the reduction factors between two experiments with the same envelope-pseudotyped vectors varied substantially (e.g. VSV-G pseudotyped vectors showed a reduction ratio of respectively 3.0 and 8.1 in subsequent experiments). This showed that the inherent technical variations in the experimental procedure hampered the formulation of a generalized manner for estimating the reduction of vector concentration in the culture medium due to transduction of target cells. Therefore, we have the opinion that this factor could not be included in the COGEM formula for the reduction ratio of free lentiviral vector particles, unless data are provided that are obtained with the specific vector (batch), the precise infection procedure and the same batch of cells that would be used in the studies for which permissions are sought

Reduction of vector particles in medium			
2nd infection no cells/spin			
	reduction percentage		**reduction factor**
VSV-G	88%	±3%	**8.1**
Measles	78%	±11%	**4.5**
GALV	-	-	**0.3**
RD114	58%	±8%	**2.4**
4070A	47%	±7%	**1.9**
10A1	87%	±3%	**7.8**
MULV	29%	±3%	**1.4**

Table R8 Reduction in free vector particles after spin infections

7. Discussion

In this report we determined a number of parameters that could be used for estimating the number of residual envelope-pseudotyped infectious lentiviral vector particles.

A vector-independent parameter is the efficiency with which vector particles are washed away with the culture medium. Upon removal of tissue culture medium with a plastic pipette or a platinum aspiration needle, a limited amount of medium remains and adheres to the culture dish. We assessed the total amount of liquid remaining in the dish by determining the dry weight of the dish, and after addition of culture medium and removal of the culture medium, the wet weight. Assuming random distribution of the viral vector particles in the liquid, this should give an estimate of the fraction of the vector that is retained in the dish after removal of the medium. We found that in two frequently used dish sizes (i.e. 35 mm and 10 cm diameter dishes) the remaining fraction was respectively 2% and 5%. The values were identical for the plastic pipette and the aspiration needle. This implies removal of the medium reduces the amount of inoculum lentiviral vector particles by 20-fold on a Ø10 cm dish, and 50-fold on a Ø35mm dish. Every subsequent washing step can be expected to have a similar reduction in remaining lentiviral vector particle amounts. The 20-fold value is in agreement with the standard parameter proffered for the COGEM formula (COGEM-advies-CGM/090331-03, 2009).

Suggestion: It is feasible to differentiate in the efficiency of vector removal for the different dish sizes. For dish sizes other than 35 mm and 10 cm dishes applicants should provide data.

Trypsin treatment is known to inactivate VSV-G envelope pseudotyped lentiviral vectors (COGEM-advies-CGM/090331-03, 2009; Tang & Levy, 1991). We determined the inactivation efficiency of seven envelope-pseudotyped lentiviral vectors. In the experiments we used an amount of trypsin typically used to detached our cells form the culture dish (i.e. 5 min at 37°C). The efficiency differed substantially between the different envelope-pseudotyped lentiviral vectors and ranged from 1 to 100 % residual vector activity after trypsin treatment. The residual activity value for VSV-G-envelope pseudotyped particles (100 % in 5 minutes) showed that trypsin inactivation for these vectors is less efficient than initially assumed from the trypsin inactivation data derived from wild-type HIV-1.

Suggestion: Use the measured inactivation frequency in the formula. This would could be calculated as $(Wash * Tryp)^{I}$ *. In this formula Wash represents the fold-reduction of vector amount as result of washing, Tryp represents the fold reduction (reduction factor) by trypsin treatment as per table R3, and I the number of trypsin treatments.*

EXAMPLE

As an example, twice splitting 10 cm dishes of cells initially exposed to a measles-virus H/F-pseudotyped lentiviral vector in which all cells are retained would result in a (20 x 20 x 4)^2 that is a 2.6 x 10^6-fold reduction of the amount of infectious lentiviral vector particles. The medium from the cells is removed (20-fold reduction), the cells are washed with EDTA-buffer (20-fold reduction), trypsin is added and incubated for 5 minutes (4-fold reduction). The combined efficiency of the single procedure is 1600 fold. If the cells are passed twice following the procedure, the combined effect of the trypsinization is 1600^2 is a 2.6 x 10^6-fold reduction in virus titer.

It has been described that lentiviral vector particles cannot only be inactivated by trypsin, but also by exposure to human complement. (DePolo et al., 2000) described that human serum very efficiently inactivates VSV-G pseudotyped lentiviral vectors but that, in contrast, lentiviral vectors pseudotyped with the amphotropic 4070A envelope largely resist inactivation by human sera. This demonstrates that the efficiency of inactivation is dependent on the envelope protein. The same authors reported varying inactivation efficiencies with different serum batches. Also these authors mention varying degrees of VSV-G-pseudotyped vector inactivation by a heat-stable factor. This led these authors to suggest that VSV-G-specific antibodies may be underlying the donor to donor variation in complement-mediated vector inactivation efficiency.

The situation may even be more complex. The complement sensitivity has been reported to depend on the cell line used to produce the vector particles. (Takeuchi et al., 1996) demonstrated that Gal(alpha 1-3)Gal terminal carbohydrates are expressed by most mammals that these are absent in humans as humans lack a functional (alpha 1-3) galactosyltransferase gene. Anti-Gal(alpha 1-3)Gal antibodies present in human serum can inactivate retroviruses produced from animal cells that express (alpha 1-3) galactosyltransferase. Several reports demonstrated that VSV itself had reduced stability in human sera when were grown on cells that are (alpha 1-3) galactosyltransferase positive (Takeuchi et al., 1996; Thiry, Cogniaux-Le Clerc, Content, & Tack, 1978). The sensitivity of vector particles produced in (alpha 1-3) galactosyltransferase positive cells to human serum may be dependent on natural IgM antibodies recognizing Gal(alpha 1-3)Gal in the human sera (Beebe & Cooper, 1981).

The inherent variations in the capacity to inactivate VSV-G pseudotyped lentiviral vectors of human sera thwarts the definition of a robust rule-of-the-thumb estimate of the effectiveness of complement-mediated inactivation of lentiviral vectors. Also fact that the (alpha 1-3) galactosyltransferase status of the producer cells dictates the complement sensitivity is a complicating factor. Therefore it will be very

difficult to reliably predict the impact of complement treatment on residual vector-particle concentrations as intended by the COGEM formula.

Suggestion: Given a considerable donor-to-donor variability in the efficiency of complement-mediated vector inactivation, the efficiency of complement inactivation vectors particle can only be taken into account when experimental data are provided to validate the efficiency of vector inactivation with the specific serum batch used for the inactivation and vector particles produced with a production system identical to the one for which permission is sought.

Lentiviral vector particles have a finite half-life at 37°C. Although it is unknown what step in the lentiviral vector transduction pathway is inhibited when the particles lose their capacity to transduce cells, it is tempting to speculate that this is governed primarily by the envelope protein. This is based on the observation that particles that have their envelope pseudotyped with different heterologous envelope proteins vary in their half-lives. The half-lives of the various vectors are summarized in table R4. Remarkable is the relative stability of the VSV-G envelope-pseudotyped particles. The value of 34.7 hours is considerably higher than the value of 10.4 hours reported by (Higashikawa & Chang, 2001). While we can only speculate on the reason for the discrepancy, it may be caused by differences in the buffer conditions used. While we determined the half-life in standard bicarbonate-buffered tissue culture medium (i.e. DMEM supplemented with 8% FCS) Higashikawa and Chang studied the stability in 100mM TRIS.Cl buffer at pH=7.0. Moreover, the latter authors in the same report also report a half-life value of 321.2 minutes (5.35 hours) using apparently very similar conditions.

We also noted that inclusion of polybrene, a polycation often included in the lentiviral vector transduction medium to enhance the infection efficiency, affected the half-life of VSV-G envelope-pseudotyped lentiviral vector particles. In the absence of polybrene in the culture medium a half-life value of 49.5 hours was found, while in the presence of 8 µg/ml polybrene a value of 21.0 hours was found. Note that the values differ from the values we reported above. This is due to a small difference in the methodology used (i.e. storing the samples at -20°C for variable times which was done in the pilot experiments). The mechanism underlying for the effect of polybrene on vector half-life is unknown, but it is tempting to speculate that the particles may have a higher tendency to aggregate as a consequence of the electrostatic neutralization of the viral membranes. This would reduce the apparent infectious lentiviral vector particle titer.

Usually the polybrene is present for maximally one day following the addition of the viral vector preparation, after which the medium is refreshed with medium without polybrene. We decided to

use medium without polybrene for determining the stability of the viral vectors. On the one hand because the effect of polybrene on the half-life of VSV-G-pseudotyped vectors is not apparent if the vector particles are directly incubated with cells (without prior incubation of vector particles in culture medium in the absence of cells). This t=0 condition is the regular method to start a transduction experiment. Additionally, polybrene is also absent during the subsequent culturing of transduced cells while free infectious lentiviral vector particles could still be present. Moreover, determination of the half-life in absence of polybrene, which reduces the half-life of the vectors, represents the desired situation from a safety perspective, it could be seen as a 'worst-case' measurement.

For the viral vectors that were envelop pseudotyped with other envelope proteins the stability was measured only in the absence of polybrene. For all of the envelopes the half-life varied between 8.3 and 36.6 hours.

Suggestion: Given the variation observed in the half-lives of the infectious lentiviral vector particles the half-life for the VSV-G envelope pseudotyped lentiviral vector particles should be updated, and the half-lives for the other envelopes should be adopted to be used in the formula.

Currently, the physical titer of a lentiviral vector batch is routinely determined with a p24 ELISA assay, measuring vector-associated and free p24 protein in a stock, which is converted to ng of vector per volume. The use of this method automatically implies an overestimation of the amount of physical particles in a stock. Moreover, batch to batch variations in the amount of free p24 protein in the solution complicates the reliability of the obtained physical titer. Furthermore, with the current assay methods it is extremely complex to convert the physical vector titer into an infectious vector amount. In the research field a general rule of thumb for VSV-G-pseudotyped lentiviral vectors is widely in use: the addition of 1 ng of p24 to 2,500 cells is considered as an multiplicity of infection (MOI) of 1 (example: transduction of 100,000 cells with MOI 10 requires 400 ng of vector). This conversion factor was based on the values provided by Zufferey et al., who provided a range (1 ng p24 is the equivalent of 1000-5000 infectious units) (Zufferey et al., 1998). We confirmed this and found a value of 1 ng p24 is the equivalent of 2500 infectious units by diluting a CMV-GFP virus-vector stock with a known amount of p24 on 293T cells. The conversion factor works reasonably well in experiments on most cell lines.

Besides the uncertainties in the current assays to titer a vector batch, also experimental variations in titration procedures may influence the resulting titer. Sometimes the infection efficiency of lentiviral vectors is enhanced by centrifugation (e.g. the spin infection procedure). For three vectors we

determined the effect of a spin infection on the apparent titer of the vector stock. In 4070A-envelope pseudotyped vectors the gain of infectious titer upon spin-infection was 5.8 fold. In VSV-G pseudotyped lentiviral vectors the ratio between the spin and non-spin infection was found to be 2.6 fold. For Rabies envelope-pseudotyped particles this difference was less pronounced with a ratio of 1.4. While the reasons for these differences are unknown, the data imply the amount of input vector could be underestimated considerably if based on a stationary vector titration experiment. The effect is caused to the physical size of vector particles which prevent Brownian motion of the particles, preventing diffusion of the vectors. The only manner in which the vector particles can move in the dish is by fluid convection, which is the movement of the fluid itself (Nyberg-Hoffman, Shabram, Li, Giroux, & Aguilar-Cordova, 1997). The consequence of this is that the apparent titer of a vector suspension is strongly affected by the volume in which the experiment is performed. Doubling the volume but not the amount of virus can reduce the number of cells infected and thereby causing an underestimation of the apparent titer of up to two-fold. The consequence is the apparent titer of the lentiviral vector stocks may be a considerable underestimation of the actual amount of infectious lentiviral particles. This is of importance as the titer is used in COGEM's formula to calculate the reduction ratio. Given the 2.6 and 5.8-fold increase in apparent infectious viral particle titer, it seems reasonable to correct for this effect in the COGEM formula.

Suggestion: It seems appropriate to correct for the underestimation of the actual infectious lentiviral particle titer in the COGEM formula. This could be done by multiplying the measured amount of initial vector particles in the culture medium (C_i) by ten. By this multiplication, the uncertainty in determining the exact amount of infectious lentiviral vector particles in a stock solution can be largely intercepted.

Another factor that has been proffered as being neglected in the COGEM formula is the amount of vector that is taken up by the cells, and should (could) be subtracted from the amount of free-vector particles. We determined the residual amount of vector particles after infection using the spin infection procedure. Here we noted for most envelope-pseudotyped vectors a depletion as result of cellular uptake of infectious lentiviral particles after a spin-infection procedure (ranging from 0% (GALV) to 88% depletion), but the inter-experimental differences were substantial.

Suggestion: Given the inherent technical variations in the procedure and the transduction efficiency of cells it seems unfeasible and unwarranted to provide a robust and generalizable parameter for estimating the reduction of viral vector titer as result of vector loss due to transduction of the target cells. Hence this factor cannot be included in the COGEM formula, unless the experimental data are

provided which should be obtained with lentiviral vectors, cell systems, and infection procedure identical to those to be used in the studies for which permission is sought.

In this report we provide new data for the stability of envelope pseudotyped lentiviral vectors. These data can be used to estimate the number of residual infectious lentiviral vector particles after infection of cells in culture. The approach for calculation of the maximal amount of free vector particles via the COGEM formula is broadly applicable. However the COGEM report makes a notable exception for macrophages and dendritic cells. These cells can take up lentiviral vectors and release them several days later in an infectious form (COGEM-advies-CGM/090331-03, 2009). Washing and trypsinization of such cell cultures is therefore ineffective for removing internalized infectious vector particles. Hence the trypsinization and the washing steps cannot be taken into account. This mechanism was described for the HIV virus itself, and to the best of our knowledge not (yet) for envelope-pseudotyped lentiviral vectors (Wilflingseder, Banki, Dierich, & Stoiber, 2005; Wu, Chen, & Phillips, 2003).

Reversely, we are not aware of data disputing this internalization phenomenon for lentiviral vectors, and therefore, this exception should be kept in place.

The amount of free infectious vector particles remaining from the inoculum is of importance as these particles, together with replication competent lentiviral vectors potentially generated during production with early-generation production systems, and the resulting vector-modified cells constitute the three component that should be considered in the risk analysis that must precede any use of lentiviral vectors.
While the presence of replication competent lentiviruses in the vector stocks can be excluded with the third-generation production systems available to date, and the vector-modified cells have readily predictable phenotypes, the amount of the residual infectious replication-defective viral vector particles that remain from the inoculum needs to be quantified. However, investigators often have difficulties in quantifying this number in the cell product. To assist these researchers, COGEM offered a formula to estimate the Reduction Ratio and the maximal number of residual infectious lentiviral particles in the cell product (COGEM-advies-CGM/090331-03, 2009). If the Reduction Ratio exceeds the desired value, than the presence of infectious lentiviral vector particles remaining from the inoculum can be excluded.

If the desired Reduction Ratio is not achieved, then a formal risk analysis dedicated to risks associated with the presence to such particles is required, or alternatively, precautions should be

taken assuming infectious lentiviral particles are present (e.g. perform the work at BSL-2, wearing gloves). While this seems rather strict, it is difficult to conceive realistic scenario's in which the inadvertent exposure would lead to notable risks. This is even more so if strictly the environmental risks are considered, i.e. the risks beyond experimenters handling lentiviral-vector modified cell products in a contained use setting or beyond the patients receiving such cells as medicinal products in a deliberate release setting. The exposure to the free infectious-lentiviral vector particles can be anticipated to be very low in contained use applications, provided the material is handled in a careful manner. In a deliberate release setting there could be free infectious-lentiviral vector particles in the cell product that is administered to a patient. The likelihood that these free particles are shed and passed to third persons seems minute (Cesani et al., 2015). Also, the hazards associated with infectious vector particles being passed to third persons appear minimal, as it can be assumed that it would concern very small quantities. Additionally, the applicants are not aware of any literature data demonstrating a direct harmful effect of infection of human cells with a SIN lentiviral vector lacking a harmful insert. However, it should be noted that the availability of literature data on this topic is limited. This lack of data about possible scenarios demands caution and careful evaluation of individual applications using lentiviral vectors in a deliberate release setting. Taken together, the use of lentiviral vectors can be considered safe, even if products are handled that contain replication-defective infectious lentiviral vector particles. That being said, applicants should, at all times, aim for a minimal amount of free infectious lentiviral vectors particles in their products. As lentiviral vectors increasingly become the tool of choice for gene transfer, this offers excellent opportunities for collecting more data on using these new therapeutic entities in daily practice.

8. Conclusions

The main conclusion that can be drawn from the results that were obtained experimentally for this research project is that, generally, the reduction factor is strongly dependent on the experimental conditions that were used. Small variations in assay or storage circumstances, for example in the concentration of fetal bovine serum in the vector stock, can have a considerable impact on the resulting reduction factor. Hence, the revisited COGEM formula with the updated reduction factors can only be practiced under conditions similar to the assay conditions that were described here. In all other cases, and if researchers wish to include a reduction factor for the cellular uptake of the vectors, we suggest to the COGEM to encourage researchers to provide data that were obtained with the specific vector (batch), the precise infection procedure and the same batch of cells that would be used in the studies for which permissions are sought.

Taken together, we suggest to the COGEM to include the experimentally determined reduction factors (for the residual culture medium volume after a wash step, inactivation of the vectors by trypsin treatment and the half-life of the vectors) in the formula for the reduction ratio of free infectious lentiviral particles provided that experimental conditions are similar to the assays described here (also see footnotes in figure Cc.1). In addition, to correct for the underestimation of the actual infectious lentiviral particles in a vector stock, we suggest to multiply the measured amount of initial vector particles in the culture medium (C_i) by ten. By this multiplication, the inevitable uncertainty in determining the exact amount of infectious lentiviral vector particles in a stock solution can be corrected.

Figure Cc.1 shows the revisited COGEM formula for the reduction ratio of free lentiviral vector particles including the different reduction factors to be entered in the formula for the specific dish size or envelop-pseudotyped lentiviral vectors.

It seems prudent that the COGEM considers the new information in this report. The committee needs to judge whether the formula should to be updated according to the suggestions in the report. Here it seems necessary to consider updating the formula on the national as well as on the European level. In addition, it seems essential to consider a neighboring question, that is whether it is essential to exclude free replication defective lentivirus vector particles in all applications, or that a limited presence of such particles could be permitted in specific-contained use or deliberate release applications. If the desired Reduction Ratio is not achieved, then a formal environmental risk analysis dedicated to risks associated with the presence to such particles is required. Although caution is demanded, literature data on the hazards of these particles are limited and it is difficult to conceive realistic scenario's in which the inadvertent exposure of third parties would lead to notable risks.

Reduction ratio[1] = $(Wash^{W} \times (Wash \times Tryp)^{I} \times 2^{(HL \times T)})/(10 \times C_i)$

Wash:	reduction factor by wash step
W:	amount of wash steps
Tryp:	reduction factor by trypsin treatment
I:	amount of trypsin treatments following a wash step
HL:	reduction factor by half-life of the vector
T:	culture time in days
C_i:	measured amount of infectious vector particles in inoculum

Reduction factors					
Wash		***Tryp***[2]		***HL***	
Ø 35 mm dish	50	VSV-G	1	VSV-G	0.7
Ø 10 cm dish	20	Measles	4	Measles	1.7
		GALV	79	GALV	2.9
		Rabies	2	Rabies	1.6
		RD114	110	RD114	1.1
		4070A	4	4070A	0.7
		10A1	1	10A1	1.7
		MuLV	10	MuLV	1.3

1: The given formula for the reduction ratio of free, infectious lentiviral vector particles is only applicable to cultures of **adherent** cells.

2: Directly applicable if dissociation of cells is performed after removal of culture medium, thorough washing of cells with EDTA and in 0,05% trypsin, for at least 5 minutes at 37°C.

Table Cc.1 The revisited COGEM formula for the reduction ratio of free infectious lentiviral vector particles

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Appendix A - p24 ELISA results of envelope-pseudotyped lentiviral vectors

The concentration of p24 in unconcentrated (table A.1) and concentrated lentivirus (table A.2) vector samples was determined by ELISA.

Unconcentrated lentiviral vectors – Batch 1		
Full name of envelope protein	**Abbreviation**	**[p24] (ng/ml)**
vesicular stomatitis virus G protein	VSV-G	**367**
measles virus hemagglutinin and fusion glycoproteins	Measles	**575**
gibbon ape leukemia virus envelope protein	GALV	**241**
rabies virus glycoprotein	Rabies	**161**
feline endogenous virus envelope glycoprotein	RD114	**149**
Moloney murine leukemia virus 4070A envelope protein (amphotropic)	4070A	**417**
Moloney murine leukemia virus strain 10A1 envelope protein (amphotropic)	10A1	**560**
Moloney murine leukemia virus envelope glycoprotein (ecotropic)	MuLV	**221**

Concentrated lentiviral vectors – Batch 2		
Full name of envelope protein	**Abbreviation**	**[p24] (ng/ml)**
measles virus hemagglutinin and fusion glycoproteins	Measles	**4648**
gibbon ape leukemia virus envelope protein	GALV	**8731**
feline endogenous virus envelope glycoprotein	RD114	**10184**

Concentrated lentiviral vectors – Batch 3				
Envelope protein	**Unconcentrated [p24] (ng/ml)**	**Concentrated [p24] (ng/ml)**	**Recovery ratio**	**Concentration factor**
VSV-G	322	**9762**	0.74	30.3
Measles	910	**39011**	0.84	42.9
GALV	598	**16405**	0.56	27.4
Rabies	299	**9161**	0.62	30.6
RD114	603	**17601**	0.60	29.2
4070A	635	**22091**	0.77	34.8
10A1	547	**17781**	0.68	32.5
MuLV	604	**21955**	0.76	36.4
No envelope	842	**27176**	0.66	32.3

Appendix B - Residual culture medium volume in culture dishes

The density of the culture medium (table B.1) is determined first, to convert the weight of the residual culture medium in Ø35 mm and Ø10 cm dishes into a volumetric measure (table B.2 and B.3).

Weight of 1 ml culture medium (in grams)			
tube #	empty tubes	tubes with medium	weight culture medium
1	0.9469	1.9454	0.9985
2	0.9545	1.9508	0.9963
3	0.9380	1.9320	0.9940
4	0.9501	1.9445	0.9944
5	0.9535	1.9485	0.9950
average	0.9486	1.94424	0.9956
standard deviation	0.0066	0.0073	0.0018

density of culture medium		
µl of medium	weight	density (g/µl)
1000	0.99564	0.00099564

Weight of 1 ml Milli-Q water (in grams, for comparison purposes only)			
tube #	empty tubes	tubes with Milli-Q	weight Milli-Q
1	0.9335	1.9391	1.0056
2	0.9419	1.9490	1.0071
3	0.9396	1.9429	1.0033
4	0.9331	1.9401	1.0070
5	0.9452	1.9497	1.0045
average	0.93866	1.94416	1.0055
standard deviation	0.0053	0.0049	0.0016

density of Milli-Q		
µl of water	weight	density (g/µl)
1000	1.0055	0.0010055

Table B.1 Density of culture medium

Residual culture medium in Ø35 mm culture dishes after aspiration				
dish #	**Empty (g)**	**Pipette (g)**	**Res. Weight - Pipette (g)**	**Res. Volume - Pipette (in µl)**
1	3.292	3.365	0.072	73
2	3.282	3.359	0.076	77
3	3.292	3.342	0.051	51
4	3.282	3.324	0.042	42
5	3.325	3.363	0.038	38
6	3.290	3.327	0.037	37
7	3.324	3.394	0.069	70
8	3.325	3.403	0.078	78
9	3.309	3.381	0.072	73
10	3.302	3.354	0.052	53
average	3.30236	3.3611	0.059	**59**
standard deviation	0.017	0.026	0.016	17

dish #	**Needle (g)**	**Res. Weight - Needle (g)**	**Res. Volume - Needle (in µl)**
1	3.337	0.045	45
2	3.339	0.057	57
3	3.353	0.062	62
4	3.338	0.056	56
5	3.375	0.050	51
6	3.350	0.060	60
7	3.372	0.047	48
8	3.369	0.043	44
9	3.362	0.053	54
10	3.355	0.054	54
average	3.35511	0.05275	**53**
standard deviation	0.014	0.006	6

Empty	Weight empty dish (in gram)
Pipett	Weight dish after medium aspiration by pipette (in gram)
Needle	Weight dish after medium aspiration by needle (in gram)
Res. Weight - Pipette	Weight residual medium in dish after aspiration by pipette (in gram)
Res. Weight - Needle	Weight residual medium in dish after aspiration by needle (in gram)
Res. Volume - Pipette	Volume residual medium in dish after aspiration by pipette (in µl)
Res. Volume - Needle	Volume residual medium in dish after aspiration by needle (in µl)

Residual culture medium in Ø10 cm culture dishes after aspiration				
dish #	**Empty (g)**	**Pipette (g)**	**Res. Weight - Pipette (g)**	**Res. Volume - Pipette (in µl)**
1	17.776	18.070	0.294	295
2	17.705	18.082	0.377	379
3	17.746	18.263	0.517	519
4	17.732	18.226	0.494	496
5	17.746	18.243	0.497	499
6	17.769	18.252	0.483	485
7	17.723	18.210	0.487	489
8	17.736	18.134	0.398	400
9	17.713	18.239	0.526	528
10	17.794	18.213	0.419	421
average	17.744	18.1932	0.4492	**451**
standard deviation	0.028	0.071	0.075	75

dish #	**Needle (g)**	**Res. Weight - Needle (g)**	**Res. Volume - Needle (in µl)**
1	18.130	0.354	356
2	18.176	0.471	473
3	18.203	0.457	459
4	18.221	0.489	491
5	18.412	0.666	669
6	18.120	0.351	353
7	18.286	0.563	565
8	18.233	0.497	499
9	18.288	0.575	578
10	18.211	0.417	419
average	18.228	0.484	**486**
standard deviation	0.085	0.099	99

Empty	Weight empty dish (in gram)
Pipett	Weight dish after medium aspiration by pipette (in gram)
Needle	Weight dish after medium aspiration by needle (in gram)
Res. Weight - Pipette	Weight residual medium in dish after aspiration by pipette (in gram)
Res. Weight - Needle	Weight residual medium in dish after aspiration by needle (in gram)
Res. Volume - Pipette	Volume residual medium in dish after aspiration by pipette (in µl)
Res. Volume - Needle	Volume residual medium in dish after aspiration by needle (in µl)

Residual percentage of culture medium in Ø3 cm culture dishes after aspiration		
dish #	**Res. Volume - Pipette**	**Res. Volume (%)**
1	73	2.4%
2	77	2.6%
3	51	1.7%
4	42	1.4%
5	38	1.3%
6	37	1.2%
7	70	2.3%
8	78	2.6%
9	73	2.4%
10	53	1.8%
average	59	**2.0%**
standard deviation	17	0.6%
dish #	**Res. Volume - Needle**	**Res. Volume (%)**
1	45	1.5%
2	57	1.9%
3	62	2.1%
4	56	1.9%
5	51	1.7%
6	60	2.0%
7	48	1.6%
8	44	1.5%
9	54	1.8%
10	54	1.8%
average	53	**1.8%**
standard deviation	6	0.2%
Res. Volume - Pipette	Volume residual medium in dish after aspiration by pipette (in µl)	
Res. Volume - Needle	Volume residual medium in dish after aspiration by needle (in µl)	
Res. Volume (%) - Pipette	Residual percentage of culture medium in dish after aspiration by pipette (initial volume = 100%)	
Res. Volume (%) - Needle	Residual percentage of culture medium in dish after aspiration by needle (initial volume = 100%)	

Residual percentage of culture medium in Ø10 cm culture dishes after aspiration		
dish #	**Res. Volume - Pipette**	**Res. Volume (%)**
1	295	3.0%
2	379	3.8%
3	519	5.2%
4	496	5.0%
5	499	5.0%
6	485	4.9%
7	489	4.9%
8	400	4.0%
9	528	5.3%
10	421	4.2%
average	451	**4.5%**
standard deviation	75	0.8%
dish #	**Res. Volume - Needle**	**Res. Volume (%)**
1	356	3.6%
2	473	4.7%
3	459	4.6%
4	491	4.9%
5	669	6.7%
6	353	3.5%
7	565	5.7%
8	499	5.0%
9	578	5.8%
10	419	4.2%
average	486	**4.9%**
standard deviation	99	1.0%

Res. Volume - Pipette	Volume residual medium in dish after aspiration by pipette (in µl)
Res. Volume - Needle	Volume residual medium in dish after aspiration by needle (in µl)
Res. Volume (%) - Pipette	Residual percentage of culture medium in dish after aspiration by pipette (initial volume = 100%)
Res. Volume (%) - Needle	Residual percentage of culture medium in dish after aspiration by needle (initial volume = 100%)

Appendix C – Trypsin inactivation of envelope-pseudotyped lentiviral vectors.

Residual lentiviral vector activity after trypsin treatment - Pilot experiment						
	% GFP- positive cells					
	Measles - 15 µl conc. undil.		**RD114 - 15 µl conc. undil.**		**GALV - 10 µl conc. undil.**	
	+ trypsin	PBS only	+ trypsin	PBS only	+ trypsin	PBS only
	6.9	11.8	3.6	9.9	19.0	61.5
	6.5	12.3	2.5	10.7	19.1	63.5
	6.1	11.2	3.4	8.7		58.9
average	**6.5**	**11.8**	**3.2**	**9.8**	**19.1**	**61.3**
standard deviation	0.4	0.6	0.6	1.0	0.1	2.3
residual % of lentiviral vector activity after trypsin treatment (relative to PBS control)						
	55%		32%		31%	
fold decrease in lentiviral vector activity after trypsin treatment (PBS only/+trypsin)						
	1.8		**3.1**		**3.2**	

Residual lentiviral vector activity after trypsin treatment - Experiment 1						
	% GFP-positive cells					
	Measles - 10 µl undil.		**RD114 - 15 µl undil.**		**MULV - 15 µl undil.**	
	+	-	+	-	+	-
	4.3	21.2	0.3	16.8	5.3	38.8
	4.8	26.0	0.3	15.4	5.4	37.5
average	**4.6**	**23.6**	**0.3**	**16.1**	**5.3**	**38.2**
standard deviation	0.4	3.4	0.0	1.0	0.0	0.9
residual lentiviral vector activity after trypsin treatment in % (relative to PBS control)						
	19.4%		**1.8%**		**14.0%**	
standard deviation	3.2%		0.2%		0.4%	
	4.7	20.0	0.2	13.7	1.9	19.3
	5.8	20.8	0.2	13.7	1.7	17.2
average	**5.3**	**20.4**	**0.2**	**13.7**	**1.8**	**18.3**
standard deviation	0.7	0.6	0.0	0.0	0.1	1.5
residual lentiviral vector activity after trypsin treatment in % (relative to PBS control)						
	25.7%		**1.5%**		**10.0%**	
standard deviation	3.7%		0.3%		1.1%	
Average residual lentiviral vector activity in % (relative to PBS control)						
	22.6%		**1.7%**		**12.0%**	
standard deviation	3.4%		0.3%		0.8%	

[a] Experiment A and B are biological duplo's; different lentiviral vector aliquot of the same vector batch were used. As both experiments were performed simultaneously, they were considered as one experiment.

Residual lentiviral vector activity after trypsin treatment - Experiment 2												
% GFP-positive cells												
VSV-G - 15 µl 500x dil.		**Measles - 10 µl undil.**		**GALV - 10 µl 10x dil.**		**Rabies - 5 µl 10x dil.**		**RD114 - 15 µl undil.**		**4070A - 10 µl 10x dil.**		**MULV - 15 µl undil.**
+	-	+	-	+	-	+	-	+	-	+	-	+	-
23.3	24.0	5.1	19.9	0.5	44.2	17.2	29.0	0.07	16.3	3.2	19.7	2.9	25.8
24.9	20.7	6.3	20.8	1.5	53.3	20.8	23.4	0.05	13.2	7.7	14.2	2.7	24.9
24.1	**22.4**	**5.7**	**20.4**	**1.0**	**48.8**	**19.0**	**26.2**	**0.06**	**14.8**	**5.4**	**17.0**	**2.8**	**25.4**
1.1	2.3	0.8	0.6	0.7	6.4	2.5	4.0	0.01	2.2	3.2	3.9	0.1	0.6
residual lentiviral vector activity after trypsin treatment in % (relative to PBS control)													
107.8%		**28.0%**		**2.1%**		**72.5%**		**0.4%**		**32.1%**		**10.8%**	
12.3%		4.3%		1.4%		14.6%		0.1%		20.1%		0.6%	

Residual lentiviral vector activity after trypsin treatment - Experiment 3												
% GFP-positive cells												
VSV-G - 15 µl 500x dil.		**Measles - 10 µl undil.**		**GALV - 10 µl 10x dil.**		**Rabies - 5 µl 10x dil.**		**RD114 - 15 µl undil.**		**4070A - 10 µl 10x dil.**		**MULV - 15 µl undil.**
+	-	+	-	+	-	+	-	+	-	+	-	+	-
17.5	20.8	4.3	27.0	0.2	34.6	12.4	27.7	0.11	14.3	3.0	19.3	0.8	10.8
22.0	19.0	5.3	27.2	0.1	40.8	13.8	25.9	0.06	13.0	3.9	15.2	0.9	10.2
19.8	**19.9**	**4.8**	**27.1**	**0.2**	**37.7**	**13.1**	**26.8**	**0.09**	**13.7**	**3.4**	**17.3**	**0.8**	**10.5**
3.2	1.3	0.7	0.1	0.1	4.4	1.0	1.3	0.04	0.9	0.7	2.9	0.1	0.4
residual lentiviral vector activity after trypsin treatment in % (relative to PBS control)													
99.2%		**17.7%**		**0.4%**		**48.9%**		**0.6%**		**19.8%**		**8.0%**	
17.2%		2.4%		0.2%		4.4%		0.3%		5.0%		0.6%	

Appendix D – Half-life of VSV-G pseudotyped lentiviral vectors in presence/absence of polybrene

Half-life VSV-G pseudo-typed lentiviruses % GFP-positive cells				
non-spin infection - **with polybrene** - 8 µl unconcentrated VSV-G				
incubation time (h)	0	-	18	48
absolute incub. time (h)	0	15.4	18.8	42.5
	54.4	28.9	24.6	12.8
	53.5	32.0	24.6	12.9
	53.9	30.7	25.5	13.9
average	53.9	30.5	24.9	13.2
standard deviation	0.5	1.6	0.5	0.6
trend line: $y = a*e^{-bx}$	$y = 52.496e^{-0.014x}$			
half-life = LN(2)/b =	**21.0 hours**			

Half-life VSV-G pseudo-typed lentiviruses % GFP-positive cells				
non-spin infection - **no polybrene** - 8 µl unconcentrated VSV-G				
incubation time (h)	0	-	18	48
absolute incub. time (h)	0	15.42	18.78	42.53
	48.4	43.6	37.4	25.8
	52.9	42.0	38.6	31.0
	53.7	47.7	41.0	28.9
average	51.7	44.4	39.0	28.6
standard deviation	2.9	2.9	1.8	2.6
trend line: $y = a*e^{-bx}$	$y = 50.878e^{-0.033x}$			
half-life = LN(2)/b =	**49.5 hours**			

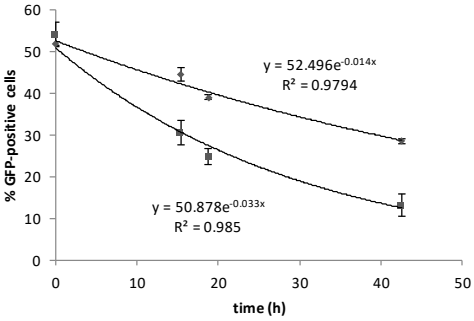
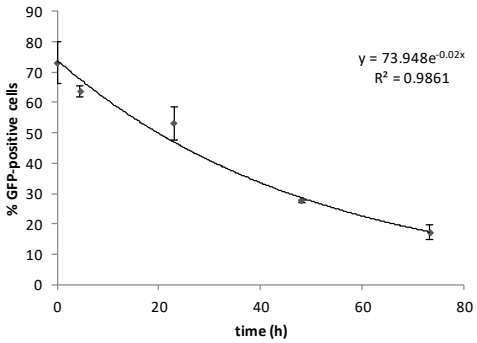


Table C.1 Half-life of VSV-G pseudotyped lentiviral vectors incubated in the presence of absence of 8 µg/ml polybrene

Appendix E – Half-life of different envelope-pseudotyped lentiviral vectors

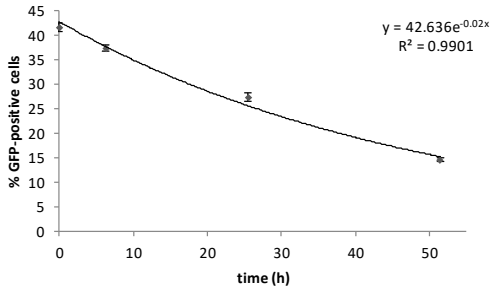
VSV-G pseudotyped lentiviral vectors – Experiment 1

Half-life VSV-G pseudo-typed lentiviruses					
% GFP-positive cells					
spin infection - no polybrene - 2,5 µl unconcentrated VSV-G					
incubation time (h)	0	5	24	48	72
absolute incub. time (h)	0	4.50	22.92	48.00	73.25
	77.4	62.1	53.9	27.8	14.6
	76.6	62.8	47.3	27.2	19.2
	65.1	65.7	58.3	N/A	17.8
average	73.0	63.5	53.2	27.5	17.2
standard deviation	6.9	1.9	5.5	0.4	2.4
trend line: $y = a*e^{-bx}$	$y = 73.948e^{-0.02x}$				
half-life = LN(2)/b =	**34.7 hours**				



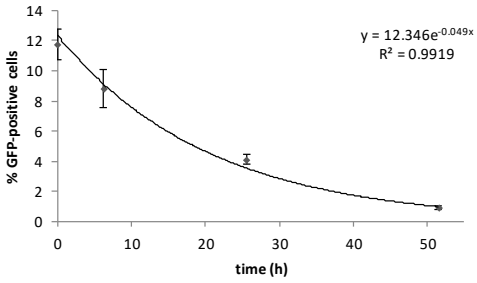
VSV-G pseudotyped lentiviral vectors - Experiment 2

Half-life VSV-G pseudo-typed lentiviruses				
% GFP-positive cells				
non-spin infection - no polybrene - 5 µl unconcentrated VSV-G				
incubation time (h)	0	6	24	48
absolute incub. time (h)	0	6.25	25.50	51.50
	41.2	36.5	26.6	14.6
	42.6	37.4	28.3	15
	40.9	37.7	27.1	14.3
average	41.6	37.2	27.3	14.6
standard deviation	0.9	0.6	0.9	0.4
trend line: $y = a*e^{-bx}$	$y = 42.636e^{-0.02x}$			
half-life = LN(2)/b =	**34.7 hours**			



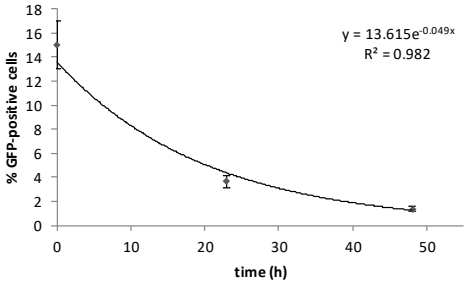
Measles pseudotyped lentiviral vectors – Experiment 1

Half-life Measles pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 15 µl concentrated Measles				
incubation time (h)	0	6	24	48
absolute incub. time (h)	0	6.25	25.5	51.5
	12.3	7.65	3.84	0.82
	12.4	8.69	4.48	0.93
	10.6	10.1	4.11	1.05
average	11.8	8.8	4.1	0.9
standard deviation	1.0	1.2	0.3	0.1
trend line: $y = a*e^{-bx}$	$y = 12.346e^{-0.049x}$			
half-life = LN(2)/b =	**14.1 hours**			



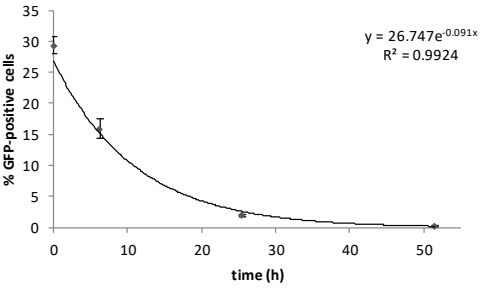
Measles pseudotyped lentiviral vectors – Experiment 2

Half-life Measles pseudo-typed lentiviruses			
% GFP-positive cells			
spin infection - no polybrene - 15 µl concentrated Measles			
incubation time (h)	0	24	48
absolute incub. time (h)	0	22.92	48
	17.3	3.98	1.59
	13.6	3.86	1.22
	14.1	3.1	1.36
average	15.0	3.6	1.4
standard deviation	2.0	0.5	0.2
trend line: $y = a*e^{-bx}$	$y = 13.615e^{-0.049x}$		
half-life = LN(2)/b =	**14.1**	**hours**	



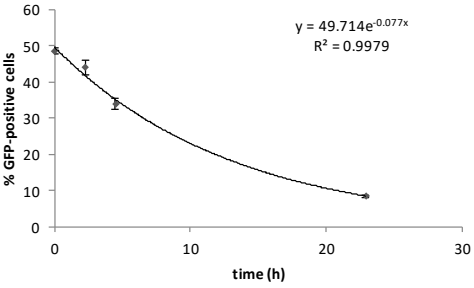
GALV pseudotyped lentiviral vectors – Experiment 1

Half-life GALV pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 5 µl concentrated GALV				
incubation time (h)	0	6	24	48
absolute incub. time (h)	0	6.25	25.5	51.5
	27.7	16.4	1.83	0.36
	30.3	17.2	2.16	0.26
	30.1	14.1	1.97	0.21
average	29.4	15.9	2.0	0.3
standard deviation	1.4	1.6	0.2	0.1
trend line: $y = a*e^{-bx}$	$y = 26.747e^{-0.091x}$			
half-life = LN(2)/b =	**7.6**	**hours**		



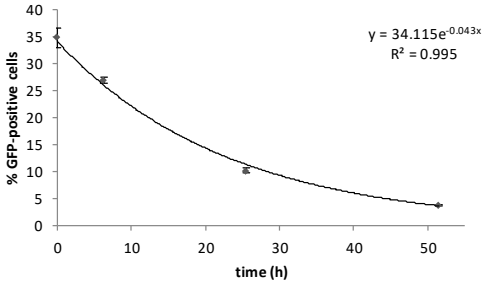
GALV pseudotyped lentiviral vectors – Experiment 2

Half-life GALV pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 5 µl concentrated GALV				
incubation time (h)	0	2	5	24
absolute incub. time (h)	0	2.25	4.5	22.92
	48.0	41.9	35.8	8.3
	49.6	46.1	33.8	8.5
	48.4	44.2	32.7	9.0
average	48.7	44.1	34.1	8.6
standard deviation	0.8	2.1	1.6	0.3
trend line: $y = a*e^{-bx}$	$y = 49.714e^{-0.077x}$			
half-life = LN(2)/b =	**9.0**	**hours**		



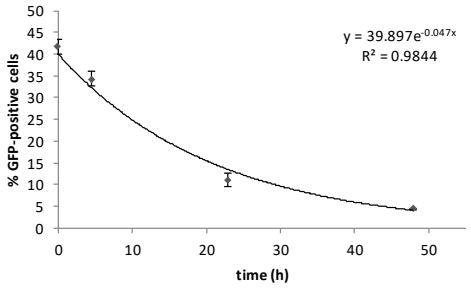
Rabies pseudotyped lentiviral vectors – Experiment 1

Half-life Rabies pseudo-typed lentiviruses				
% GFP-positive cells				
non-spin infection - no polybrene - 50 µl concentrated Rabies				
incubation time (h)	0	6	24	48
absolute incub. time (h)	0	6.25	25.5	51.5
	32.8	26.6	10.0	3.9
	35.8	27.7	9.9	3.9
	35.8	26.8	10.7	3.8
average	34.8	27.0	10.2	3.9
standard deviation	1.7	0.6	0.4	0.0
trend line: $y = a*e^{-bx}$	$y = 34.115e^{-0.043x}$			
half-life = LN(2)/b =	**16.1**	**hours**		



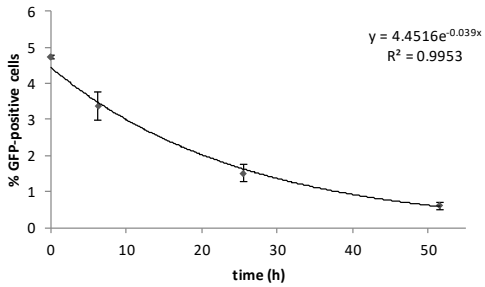
Rabies pseudotyped lentiviral vectors – Experiment 2

Half-life Rabies pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 15 µl concentrated Rabies				
incubation time (h)	0	5	24	48
absolute incub. time (h)	0	4.50	22.92	48
	40.1	34.4	9.3	4.4
	43.5	35.9	12.0	4.6
	41.7	32.5	12.0	4.5
average	41.8	34.3	11.1	4.5
standard deviation	1.7	1.7	1.5	0.1
trend line: $y = a*e^{-bx}$	$y = 39.897e^{-0.047x}$			
half-life = LN(2)/b =	**14.7**	**hours**		



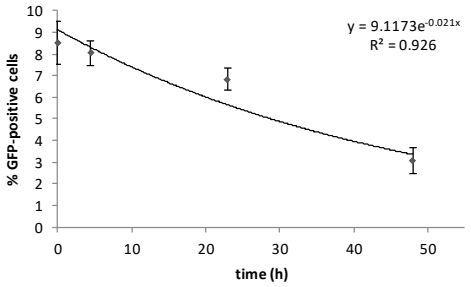
RD114 pseudotyped lentiviral vectors – Experiment 1

Half-life RD114 pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 15 µl concentrated RD114 batch 1				
incubation time (h)	0	5	24	48
absolute incub. time (h)	0	6.25	25.5	51.5
	4.8	3.7	1.4	0.7
	4.7	3.5	1.3	0.5
	4.7	2.9	1.8	0.6
average	4.7	3.4	1.5	0.6
standard deviation	0.1	0.4	0.2	0.1
trend line: $y = a*e^{-bx}$	$y = 4.4516e^{-0.039x}$			
half-life = LN(2)/b =	**17.8**	**hours**		



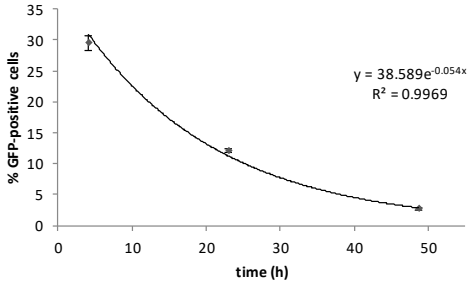
RD114 pseudotyped lentiviral vectors – Experiment 2

Half-life RD114 pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 15 µl concentrated RD114 batch 1				
incubation time (h)	0	5	24	48
absolute incub. time (h)	0	4.5	22.92	48
	7.6	7.6	6.9	3.1
	9.6	8.7	7.3	3.7
	8.4	7.8	6.3	2.5
average	8.5	8.0	6.8	3.1
standard deviation	1.0	0.5	0.5	0.6
trend line: $y = a*e^{-bx}$	$y = 9.1173e^{-0.021x}$			
half-life = LN(2)/b =	**33.0**	**hours**		



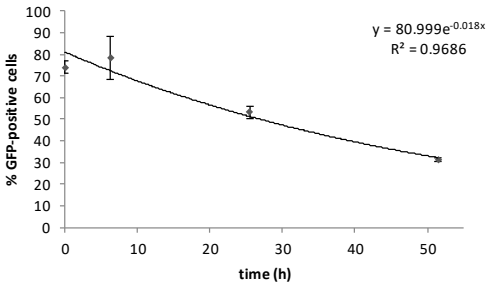
RD114 pseudotyped lentiviral vectors – Experiment 3

Half-life RD114 pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 15 µl concentrated RD114 batch 2				
incubation time (h)	0	5	24	48
absolute incub. time (h)	-	4.17	23	48.75
	N/A	29.6	12.5	2.5
	N/A	30.7	12.0	2.9
	N/A	28.3	12.0	2.9
average	N/A	29.5	12.2	2.8
standard deviation	N/A	1.2	0.3	0.2
trend line: $y = a*e^{-bx}$	$y = 38.589e^{-0.054x}$			
half-life = LN(2)/b =	**12.8**	**hours**		



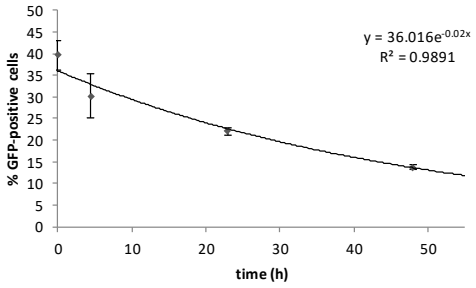
4070A pseudotyped lentiviral vectors – Experiment 1

Half-life 4070A pseudo-typed lentiviruses					
% GFP-positive cells					
spin infection - no polybrene - 80 µl unconcentrated 4070A					
incubation time (h)	0	5	24	48	
absolute incub. time (h)	0	6.25	25.5	51.5	
	72.6	87.9	50	31.6	
	72.4	79.7	56	31.7	
	77.1	68.1	53.4	30.5	
average	74.0	78.6	53.1	31.3	
standard deviation	2.7	9.9	3.0	0.7	
trend line: $y = a*e^{-bx}$	$y = 80.999e^{-0.018x}$				
half-life = LN(2)/b =	**38.5**	**hours**			



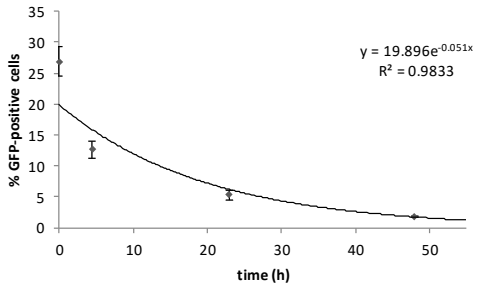
4070A pseudotyped lentiviral vectors – Experiment 2

Half-life 4070A pseudo-typed lentiviruses					
% GFP-positive cells					
spin infection - no polybrene - 25 µl unconcentrated 4070A					
incubation time (h)	0	5	24	48	72
absolute incub. time (h)	0	4.5	22.92	48	73.25
	41.0	24.6	21.6	13.6	8.7
	42.2	31.2	23.0	14.5	8.1
	35.6	34.7	21.6	13.2	7.9
average	39.6	30.2	22.1	13.8	8.2
standard deviation	3.5	5.1	0.8	0.7	0.4
trend line: $y = a*e^{-bx}$	$y = 36.016e^{-0.02x}$				
half-life = LN(2)/b =	**34.7**	**hours**			



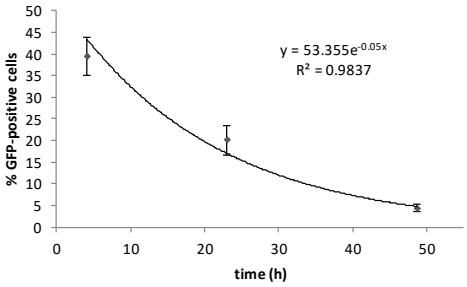
10A1 pseudotyped lentiviral vectors – Experiment 1

Half-life 10A1 pseudo-typed lentiviruses					
% GFP-positive cells					
spin infection - no polybrene - 50 µl unconcentrated 10A1					
incubation time (h)	0	5	24	48	72
absolute incub. time (h)	0	4.5	22.92	48	73.25
	29.4	11.1	6.2	1.8	0.5
	26.4	13.9	5.2	1.9	0.4
	24.7	13.0	4.7	1.8	0.6
average	26.8	12.7	5.4	1.8	0.5
standard deviation	2.4	1.4	0.8	0.1	0.1
trend line: $y = a*e^{-bx}$	$y = 19.896e^{-0.051x}$				
half-life = LN(2)/b =	**13.6**	**hours**			



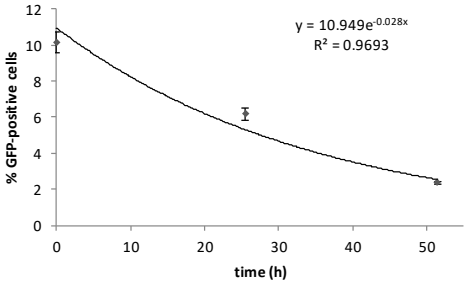
10A1 pseudotyped lentiviral vectors – Experiment 2

Half-life 10A1 pseudo-typed lentiviruses				
% GFP-positive cells				
spin infection - no polybrene - 50 µl unconcentrated 10A1				
incubation time (h)	0	5	24	48
absolute incub. time (h)	-	4.17	23	48.75
	N/A	34.5	16.2	3.8
	N/A	41.1	22.3	5.4
	N/A	42.7	21.7	4.0
average	N/A	39.4	20.1	4.4
standard deviation	N/A	4.3	3.4	0.8
trend line: $y = a*e^{-bx}$	$y = 53.355e^{-0.05x}$			
half-life = LN(2)/b =	**13.9**	**hours**		



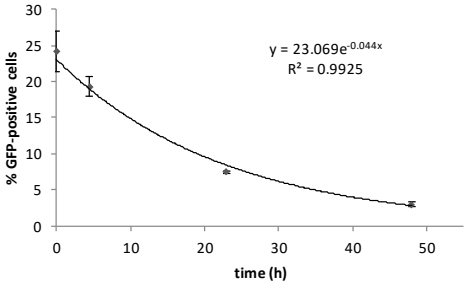
MuLV pseudotyped lentiviral vectors – Experiment 1

Half-life MuLV pseudo-typed lentiviruses % GFP-positive cells				
non-spin infection - no polybrene - 50 µl unconcentrated MULV				
incubation time (h)	0	5	24	48
absolute incub. time (h)	0	6.25	25.5	51.5
	9.5	N/A	6.0	2.4
	10.5	N/A	6.0	2.4
	10.5	N/A	6.5	2.3
average	10.2	N/A	6.2	2.4
standard deviation	0.6	N/A	0.3	0.1
trend line: $y = a*e^{-bx}$	$y = 10.949e^{-0.028x}$			
half-life = LN(2)/b =	**24.8**	**hours**		



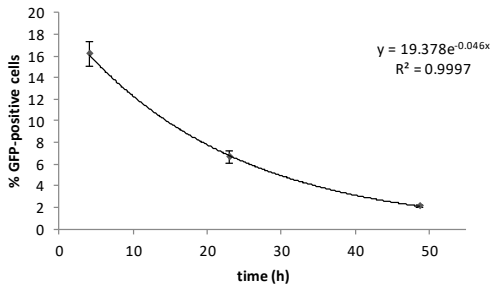
MuLV pseudotyped lentiviral vectors – Experiment 2

Half-life MuLV pseudo-typed lentiviruses % GFP-positive cells				
spin infection - no polybrene - 15 µl unconcentrated MULV				
incubation time (h)	0	5	24	48
absolute incub. time (h)	0	4.5	22.92	48
	27.0	17.7	7.3	3.0
	24.2	20.1	7.7	2.6
	21.4	20.0	7.4	3.3
average	24.2	19.3	7.5	3.0
standard deviation	2.8	1.4	0.2	0.3
trend line: $y = a*e^{-bx}$	$y = 23.069e^{-0.044x}$			
half-life = LN(2)/b =	**15.8**	**hours**		



MuLV pseudotyped lentiviral vectors – Experiment 3

Half-life MuLV pseudo-typed lentiviruses % GFP-positive cells				
spin infection - no polybrene - 15 µl unconcentrated MULV				
incubation time (h)	0	5	24	48
absolute incub. time (h)	-	4.17	23	48.75
	N/A	14.9	6.0	2.0
	N/A	16.6	6.9	2.0
	N/A	17.1	7.0	2.3
average	N/A	16.2	6.7	2.1
standard deviation	N/A	1.2	0.5	0.1
trend line: $y = a*e^{-bx}$	$y = 19.378e^{-0.046x}$			
half-life = LN(2)/b =	**15.1**	**hours**		



Appendix F – Cell uptake/Residual vector particles in medium

Raw data of table R6 and R7.

VSV-G					
	1st infection		2nd infection - all spin		
	non-spin	spin	1st non-spin	1st spin	1st no cells
	21.1	54.1	21.9	5.5	18.3
	19.1	54.8	22.9	5.7	17.9
	20.0	48.4	23.0	6.1	17.2
	19.4	53.0		6.5	
	19.4	50.1		5.5	
	20.7	48.5		6.9	
average	**20.0**	**51.5**	**22.6**	**6.0**	**17.8**
standard deviation	0.8	2.8	0.6	0.6	0.6
Increase in infectivity spin vs non-spin				**2.6** ±0.2	
Reduction factor vector in medium >					
2nd infection no cells/non spin				**0.8** ±0.0	
2nd infection no cells/spin				**2.9** ±0.3	

Rabies					
	1st infection		2nd infection - all spin		
	non-spin	spin	1st non-spin	1st spin	1st no cells
	17.9	25.1	20.3	17.3	19.7
	19.1	26.1	23.7	21.3	21.5
	16.2	25.0	20.8	18.9	22.0
average	**17.7**	**25.4**	**21.6**	**19.2**	**21.1**
standard deviation	1.5	0.6	1.8	2.0	1.2
Increase in infectivity spin vs non-spin				**1.4** ± 0.1	
Reduction factor vector in medium >					
2nd infection no cells/non spin				**1.0** ± 0.1	
2nd infection no cells/spin				**1.1** ± 0.1	

4070A					
	1st infection		2nd infection - all spin		
	non-spin	spin	1st non-spin	1st spin	1st no cells
	10.2	57.8	27.8	28.0	35.4
	10.3	57.8	33.9	31.5	42.2
	9.8	60.9	34.1	25.3	46.2
average	**10.1**	**58.8**	**31.9**	**28.3**	**41.3**
standard deviation	0.3	1.8	3.6	3.1	5.5
Increase in infectivity spin vs non-spin				**5.8** ±0.2	
Reduction factor vector in medium >					
2nd infection no cells/non spin				**1.3** ±0.2	
2nd infection no cells/spin				**1.5** ±0.3	

Raw data of Table R8.

VSV-G			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	71.8	6.1	39.8
	69.5	4.6	40.8
		4.0	38.4
average	**70.7**	**4.9**	**39.7**
standard deviation	1.6	1.1	1.2
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**87.6%**	±2.8%
Reduction factor		**8.1**	±1.8

RD114			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	4.6	1.2	2.7
	7.1	0.8	2.5
	8.0	1.2	2.6
average	**6.6**	**1.1**	**2.6**
standard deviation	1.8	0.2	0.1
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**58.2%**	±8.1%
Reduction factor		**2.4**	±0.5

GALV			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	10.9	8.3	3.4
	13.6	13.8	5.6
	16.2	18.8	4.1
average	**13.6**	**13.6**	**4.4**
standard deviation	2.7	5.3	1.1
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		-	-
Reduction factor		**0.3**	±0.1

Measles			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	25.3	0.5	1.8
	12.3	0.6	2.2
	12.5	0.2	1.8
average	**16.7**	**0.4**	**1.9**
standard deviation	7.4	0.2	0.2
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**77.8%**	±11.0%
Reduction factor		**4.5**	±2.3

4070A			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	54.9	32.5	71.6
	74.5	34.1	63.7
	82.1	34.0	56.0
average	**70.5**	**33.5**	**63.8**
standard deviation	14.0	0.9	7.8
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**47.4%**	±6.6%
Reduction factor		**1.9**	±0.2

MULV			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	50.1	4.8	6.6
	50.1	4.7	6.9
	47.8	5.0	6.8
average	**49.3**	**4.8**	**6.7**
standard deviation	1.3	0.1	0.2
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**28.5%**	±2.6
Reduction factor		**1.4**	±0.1

10A1			
	1st infection	2nd infection - all spin	
	spin	1st spin	1st no cells
	19.2	3.7	23.8
	32.5	2.8	26.2
	37.3	2.8	22.1
average	**29.7**	**3.1**	**24.0**
standard deviation	9.4	0.6	2.1
Reduction of vector particles in medium (2nd inf no cells/spin)			
Reduction percentage		**87.2%**	±2.6%
Reduction factor		**7.8**	±1.6

Appendix G – Research call COGEM

Oproep indienen projectofferte

Indienen van projectoffertes is mogelijk tot: 26 oktober 2018

Offertes richten aan: **F. van der Wilk, secretaris COGEM; info@cogem.net,**
(of Postbus 578, 3720 AN Bilthoven)

De Commissie Genetische Modificatie (COGEM) laat ter ondersteuning van haar werkzaamheden onderzoek door derden verrichten. Voor een van deze projecten wordt een uitvoerder gezocht. Geïnteresseerden worden opgeroepen projectoffertes in te dienen.
Inschrijving op opengestelde projecten is niet aan voorwaarden gebonden en staat open voor elke geïnteresseerde. Oproepen tot inschrijving worden onder meer op de COGEM website en in de e-mail nieuwsbrief gepubliceerd. De commissie streeft ernaar om tenminste drie offertes per project te ontvangen.
De offerte moet tenminste een duidelijke beschrijving bevatten van de voorgestelde werkzaamheden en een inzichtelijke begroting. Dit betekent dat er een duidelijke koppeling moet zijn tussen de begrote kosten en de voorgestelde werkzaamheden onder vermelding van het aantal ingeschatte uren en een specificatie van de uurtarieven.
Het project zal begeleid worden door een commissie van deskundigen. Deze begeleidingscommissie zal in aanwezigheid van de uitvoerders minimaal drie keer bijeenkomen. De uitvoerders zullen de resultaten van hun onderzoek presenteren in een vergadering van een van de subcommissies van de COGEM.

Het Dagelijks Bestuur van de COGEM neemt het besluit over toewijzing van projecten. De voorstellen worden beoordeeld op de volgende criteria:

- mate van aansluiting bij de onderzoeksvraag;
- competentie van het onderzoeksteam voor de uitvoering van het voorgestelde onderzoek;
- helderheid en (wetenschappelijke) kwaliteit van het voorgestelde onderzoek;
- uitvoerbaarheid van) het werkprogramma;
- prijs en kosteneffectiviteit.

De volgende projecten staan open voor inschrijving:

1) Titel: Experimentele onderbouwing van COGEM formule reductie vrije lentivirale vectordeeltjes
Binnen het biomedisch onderzoek wordt veel gebruik gemaakt van virale vectoren die zijn afgeleid van lentivirussen. Eén van de overwegingen in de risicoanalyse is of na transductie van cellen of weefsel nog zogenaamde vrije lentivirale vectordeeltjes aanwezig zijn. Blootstelling van derden, zoals laboratoriummedewerkers, aan vrije vectorvirusdeeltjes moet voorkomen worden, omdat ze bij besmetting mogelijk kunnen leiden tot schadelijke effecten. De aanwezigheid van vrije vectordeeltjes en hun uiteindelijke concentratie is afhankelijk van een aantal factoren, waaronder de halfwaardetijd van de virusvector en het aantal wasstappen van de cellen na infectie.
In 2009 heeft de COGEM een formule opgesteld om de afname (reductieratio) van het aantal vrije lentivirale vectordeeltjes in een preparaat te kunnen berekenen (COGEM advies CGM/090331-03).

Sommige elementen in deze formule berusten echter op theoretische aannames zoals de omvang van de reductie van vrije virusdeeltjes door wasstappen. De halfwaardetijd van lentivirale vectordeeltjes gepseudotypeerd met het VSV-G envelopeiwit is bekend, maar tegenwoordig worden ook vectoren gebruikt die gepseudotypeerd zijn met andere (virale) eiwitten en van deze vectordeeltjes zijn geen halfwaardetijden gepubliceerd. Daarnaast wordt in de formule geen rekening gehouden met het verlies van infectieuze vectordeeltjes bij de beoogde infectie van cellen. De formule is daarmee een theoretische berekening van het 'worst case scenario' en de werkelijke reductieratio ligt waarschijnlijk hoger.
Ter verdere verbetering van de milieurisicobeoordeling wil de COGEM daarom laten onderzoeken wat de halfwaardetijden zijn van een aantal van de meest toegepaste gepseudotypeerde lentivirale vectordeeltjes (pseudotypering met G-eiwit rabiësvirus, GALV env, mazelenvirus H- en F-eiwit, RD114 env, MuLV env, of amphotrope envelopeiwitten als 10A1, en 4070A), wat de daadwerkelijke reductie is van wasstappen en met welke reductie die gepaard gaat met de infectie van de cellen, rekening gehouden moet worden in de formule.
Gezien de toepassing van lentivirale vectoren om CAR-T cellen te modificeren en deze cellen vervolgens te infuseren in de mens wil de COGEM ook laten onderzoeken of net als bij VSV-G, andere pseudotyperingen ook leiden tot complement-gemedieerde inactivatie van de vectordeeltjes en of deze vectordeeltjes al dan niet trypsine-resistent zijn.
Doel van het project: Onderbouwing van elementen van de milieurisicobeoordeling met experimentele gegevens
Resultaat: gegevens over halfwaardetijden van gepseudotypeerde lentivirale vectordeeltjes, reductieratio's van wasstappen en celinfectie, en inactivatie.
Type onderzoek: laboratoriumonderzoek

Appendix H – Authorized project proposal

Experimentele bepaling van biologische eigenschappen van gepseudotypeerde lentivirus vectoren

Opdachtgever: COGEM

Leiden, 9 nov. 2018

Opgesteld door:	**LUMC,**	Telefoon:	Mailadres:
	Rob Hoeben &	**071 526 9241**	r**.c.hoeben@lumc.nl**
	Iris Dautzenberg		

Inhoudsopgave Projectvoorstel

1. Achtergrond

De COGEM en de overheid gebruiken een formule om de afname van de het aantal infectieuze virus deeltjes te schatten in een viruspreparaat dat gebruikt wordt om zoogdiercellen te transduceren. Wanneer de afname van de infectieuze virusconcentratie de input hoeveelheid virus overtreft met een factor 1 of 100 (afhankelijk van de gewenste handelingen) mogen de handelingen worden uitgevoerd op een lager inperkingsniveau.

In de gebruikte formule worden een aantal biologische eigenschappen van het virus meegewogen alsmede een aantal handelingen waarbij de hoeveelheid vrij-virus wordt beperkt, meegewogen. Deze gewogen factoren zijn:

1. de halfwaardetijd van de infectieuze vectordeeltjes bij 37°C,
2. de inactivering van het virus door humaan complement,
3. de inactivering van het virus door trypsine behandeling, en
4. het aantal wasstappen na blootstelling van cellen aan de virusvectoren.

De eerst drie eigenschappen worden in belangrijke mate bepaald door het eiwit dat op de mantel van het virus als envelop wordt gebruikt. In de literatuur zijn deze waarden wel te vinden voor lentivirale vectoren die gepseudotypeerd zijn met het Vesicular Stomatitis Virus G (VSV-G) eiwit. Voor andere als pseudotypering gebruikte eiwitten zijn deze waarden niet in de wetenschappelijke literatuur te vinden. Dat beperkt het toepassen van de 'COGEM formule' voor het schatten van de afname van de infectieuze titer van vrije vectordeeltjes met een andere dan de VSVg envelop.

De huidige formule van de reductieratio is:

$$(20^{W} \times 200^{I} \times 2^{2,4T})/Ci$$

Hierin is W het aantal wasstappen, I het aantal inactiverende wasstappen met trypsine of niet-geïnactiveerd humaan serum en T staat voor de kweektijd in dagen na transductie. De halfwaardetijd van een lentivirus bij een temperatuur van 37°C vormt de basis voor de factor 2,4 in de formule. De Ci is het gemeten oorspronkelijke aantal infectieuze virusdeeltjes in het inoculum.

2. Programma van eisen

Dit project is erop gericht de benodigde parameters te bepalen voor lentivirus vectoren gepseudotypeerd met een aantal regelmatig gebruikte envelopeiwitten.

De te onderzoeken envelop eiwitten zijn:

- vesicular stomatitis virus G protein (VSV-G) (te gebruiken als referentie)
- rabies virus glycoprotein (RV-G)
- gibbon ape leukemia virus envelope protein (GALV)
- measles virus (MV) Edmonston (Ed), hemagglutinin (H), and fusion (F) glycoproteins (gps) (H/F-LVs)
- feline endogenous virus RD114 envelope glycoprotein
- murine leukemia virus envelope glycoprotein (MuLV)
- murine leukemia virus 10A1 protein
- murine leukemis virus 4070A protein

3. Globale opzet van de werkzaamheden

a. De eerste activiteit binnen dit project zal gericht zijn op het **verkrijgen van de envelop genen** via commerciële of academische wegen.

b. Parallel hieraan worden **gestandaardiseerde analyse methoden** vastgesteld op basis waarvan de daadwerkelijke experimenten worden uitgevoerd

c. **Produceren, zuiveren en karakteriseren van vectorbatches**
Bovenstaande gepseudotypeerde lentivirussen zullen worden geproduceerd via standaard methoden op 293T cellen en worden gezuiverd met behulp van ultracentrifugatie op sucrose gradiënten volgens standaard protocollen. De titratie van de zuivere virussen zal gericht zijn op zowel de fysieke partikel concentratie alsmede de infectieuze virus concentratie in het preparaat.

Hierna zullen de volgende experimenten onafhankelijk van elkaar in **drievoud** worden uitgevoerd:

i. **Halfwaardetijd in kweekmedium bepalen van GFP-gen bevattend lentivirus met bovenstaande envelop eiwitten.**

Het virus zal in kweekmedium geïncubeerd worden op celkweektemperatuur (37 °C) voor t = 0, 1, 2, 4, 8, 12, 18 en 24 uur. Hierna zal de helft van het virus bevattende kweekmedium worden gebruikt in een p24 ELISA om de fysieke hoeveelheid van een

van de virus componenten (p24) te bepalen. De andere helft zal op de doelcellen worden gebracht voor een overnacht incubatiestap (18-24 uur), waarna het kweekmedium zal worden ververst. Achtenveertig uur na transductie zal het aantal GFP-positieve cellen worden geanalyseerd met behulp van flowcytometerie als een maat voor de infectieuze virus concentratie.

ii. **Reductieratio van het GFP-gen bevattend lentivirus bepalen bij iedere wasstap na een lentivirale infectie van cellen.**

In de huidige COGEM-formule die de reductieratio van vrije lentivirus deeltjes beschrijft, wordt aangenomen dat na iedere wasstap 5% van het oude kweekmedium en dus 5% van de virushoeveelheid zal achterblijven op de cellen. In dit project zal deze aanname op twee manieren verder worden onderzocht.

a. Een bekende infectieuze virus hoeveelheid zal volgens het gangbare protocol worden geïncubeerd op de doelcellen voor een periode van 18-24 uur. Na de incubatieperiode zal het kweekmedium worden vervangen en dit nieuwe kweekmedium zal direct worden getitreerd op zowel de concentratie infectieuze virus partikels (flowcytometerie) als fysieke partikels (p24 ELISA).
b. Eveneens zal de hoeveelheid achter gebleven medium in een kweekschaal met cellen worden bepaald met behulp van een kleurstof.
   Het reguliere celkweekmedium bevat de kleurstof fenolrood die bij neutrale, gelijkblijvende pH rood is. In het voorgestelde experiment zal de kleur van het kweekmedium kwantitatief worden gemeten in een spectrofotometer. Hierna zullen de cellen herhaaldelijk (5 maal) worden gewassen met PBS en zal de afname van het kleursignaal worden bepaald.

iii. **Reductieratio van het GFP-gen bevattende lentivirus door opname in cellen.**

Een bekende infectieuze virus hoeveelheid zal volgens het gangbare protocol worden geïncubeerd met of zonder de doelcellen op 37 °C. Op t = 0, 1, 2, 4, 8, 16 en 24 uur zal het kweekmedium worden getitreerd op zowel de concentratie infectieuze virus partikels (flowcytometerie) als fysieke partikels (p24 ELISA). Het verschil in virus titer tussen de samples geïncubeerd met en zonder cellen staat model voor de virusopname door de doelcellen.

iv. **Inactivering van het lentivirus door incubatie met humaan complement.** Onder standaard celkweekcondities worden het kweekmedium gecomplementeerd met 5-10% serum. Een bekende infectieuze virus hoeveelheid zal worden geïncubeerd met 10 % humaan serum (niet hitte geïnactiveerd, dus actief complement bevattend) voor t = 0, 2, 8, 12, 18 en 24 uur. Hierna zal zowel de infectieuze als de fysieke partikel titer van het lentivirus worden bepaald.

v. **Inactivering van het lentivirus door incubatie met trypsine.**

Onder standaard celkweekcondities worden vele celtypen van de kweekplaat gedissocieerd met behulp van een 0.05% trypsine oplossing. Een bekende infectieuze virus hoeveelheid zal worden geïncubeerd met 0.05 % trypsine 37 °C voor t = 0, 5, 10, 30 en 60 minuten. Hierna zal zowel de infectieuze als de fysieke partikel titer van het lentivirus worden bepaald.

4. Haalbaarheid en risico's

De te gebruiken methoden zijn alle operationeel in het lab. Een onzekere factor is de beschikbaarheid van de verschillende envelopeiwitten. De volgende genen coderend voor envelopeiwitten zijn al in ons lab beschikbaar, de overige zullen moeten worden verkregen van collega's.

- vesicular stomatitis virus G protein (VSV-G) (te gebruiken als referentie)
- gibbon ape leukemia virus envelope protein (GALV)
- measles virus (MV) Edmonston (Ed), hemagglutinin (H), and fusion (F) glycoproteins (gps) (H/F-LVs)
- murine leukemia virus envelope glycoprotein (MuLV)
- murine leukemis virus 4070A protein

Hoewel dat in vrijwel alle gevallen door collega's beschikbaar wordt gesteld, is dat tijdsduur vaak enigszins onvoorspelbaar. Zodra wij vernemen dat het project ons gegund wordt zullen wij de genen coderend voor de ontbrekende envelop aanvragen.

5. Verslaglegging

Tijdens het uitvoeren zijn twee tot drie bijeenkomsten met een door de COGEM te benoemen begeleidingscommissie voorzien teneinde de verwachtingen, proefopzet, resultaten en rapportage nader te bespreken. De experimentele methoden en de resultaten van de experimenten zullen schriftelijk worden gepresteerd in een rapport aan de COGEM. Tevens zullen de data desgewenst gepresenteerd worden in een mondelinge presentie voor de COGEM. Hierna zullen de verkregen data eveneens worden verwerkt in een wetenschappelijk Engelstalig manuscript en ter publicatie worden aangeboden in een internationaal tijdschrift met open-acces toegang.

Leiden, 9 november 2018

Namens de uitvoerders,

Prof. Dr. R.C. Hoeben
(projectleider)

Mw. Guillaine de Blecourt
(Manager Bedrijfsvoering Divisie 4)

Bijlage A: Project planning: Gannt Chart

Tijdschema van de geplande werkzaamheden. De uitvoerder verwacht in de periode van 9 maanden 2/3 van de tijd te gebruiken aan dit project. Hierop is ook de begroting gebaseerd.

Werkzaamheden	Tijd in maanden								
Vergaren van de plasmiden coderend voor de gewenste envelop eiwitten									
Opzetten van gestandaardiseerde analyse methoden									
Produceren, zuiveren en titreren van de lentivirussen gepseudotypeerd met het gewenste envelop eiwit									
Uitvoeren van de experimenten									
Verslaglegging									

Bijlage B: CVs van de uitvoerders

Name:	**Rob C. Hoeben**
Date of Birth	May 16, 1959
Place of Birth	Beverwijk, The Netherlands
Current Address	Gerbrandylaan 43, 2314 EX Leiden, The Netherlands
1978-1986	Studied Biology at the University of Utrecht. Graduation January 1986
1986-1991	PhD research at the Dept. of Medical Biochemistry of the Leiden University with Prof. dr A.J. van der Eb, Prof. dr E. Briët. Thesis: *Towards gene therapy in haemophilia A: Vectors for the expression of blood-clotting factor VIII in vivo, graduation November 6th, 1991.*
1991-1996	Post-doctoral position at the Department of Medical Biochemistry, Leiden University, working on 'The development of gene-transfer vectors for gene therapy of liver cancer and hepatic disorders'
1996-2000	Appointed as staff member and head of the Virus and Stem Cell Biology Lab. at the Dept. of Molecular Cell Biology, at the Leiden Univ. Med. Center
March 2000- present	Professor at the Leiden University and at the Department of Cell and Chemical Biology of the Leiden University Medical Center.

Professor Hoeben has a long-term interest in viruses and stem cells and their use for gene and stem-cell therapy. He made several influential contributions. He documented the unexpected hepatotoxicity of the HSVtk/GCV enzyme/prodrug combination in rat models for liver cancer and the involvement of mitochondria herein. Moreover, the '911' helper cell line was developed for research-grade production of adenoviral vectors and, in collaboration with scientists at Crucell (Leiden), his group established the PER.C6 cell line as a basis for a new adenovirus-production platform. This system has become a world standard for the production of clinical-grade biologicals. Current research is focused on the development of targeted adenovirus and reovirus vectors for treatment of cancer. His group operates the LUMC Viral Vector Facility, which produces research grade Adenovirus and Lentivirus vectors for groups in the Leiden area. Finally, Prof Hoeben, with Prof. Huizinga (Rheumatology) and Prof. R.G. Nelissen (Orthopaedic Surgery), has been conducting in the LUMC a clinical gene therapy study 'Gene Therapy in Aseptic Prosthetic Replacement Loosening: A Phase I study'. Prof. Hoeben is inventor of eleven patents granted by the USPTO and one by the EPO, covering adenoviruses and associated technology. He (co-)authored approximately 160 publications. Prof. Hoeben is member of The Netherlands Commission on Genetic Modification (COGEM).

Name:	**Iris J.C. Dautzenberg**
Date of Birth	May 13, 1983
Place of Birth	Geleen, The Netherlands
Current Address	Doctor van Noortstraat 74, 2266 HA Leidschendam, The Netherlands
2001 - 2008	Bachelor and Master study Medical Biotechnology at Wageningen University. Graduation: November 2008.
2008 - 2013	PhD research at the Dept. of Molecular Cell Biology at the Leiden University Medical Center (LUMC). Promotor: Prof. dr. R.C. Hoeben. PhD Thesis: *Forward and Reverse Genetics Strategies for Improving Oncolytic Reoviruses*. Graduation: June 14th, 2018
2015 – 2017	Employed by different companies including the GMP-certified analytical services provider Eurofins|MicroSafe laboratories in Leiden as project manager.
2017-June 2018	Scientific employee at the department of Cell and Chemical Biology, LUMC.
June 2018 – present	Post-doctoral researcher at the department of Cell and Chemical Biology, Leiden University Medical Center working on the clinical development of new oncolytic adenoviruses and reoviruses

During the Master study Medical Biotechnology dr. ir. Dautzenberg performed several internship in the field of virology and cancer therapy (Laboratory of Virology, Wageningen University; Norris Cancer Center, University of Southern California, LA, USA) and this triggered her passion for the oncolytic virotherapy; defeating cancer with viruses. Here final internship thesis under supervision of prof. R. Hoeben on oncolytic reoviruses (dept. of Molecular Cell Biology, Leiden University Medical Center), was granted the Van der Want thesis award for the best Master thesis 2008/2009.
She continued the research on oncolytic reoviruses and adenoviruses as a PhD student at the department of Molecular Cell Biology of the LUMC. Subsequently, she worked for different companies including the GMP-certified analytical services provider Eurofins|MicroSafe laboratories in Leiden as project manager, coordinating virology/biotechnology-related projects for (bio)medical companies.
In 2017 she returned to the LUMC resuming the research on oncolytic viruses, leading to defence of her PhD dissertation titled '*Forward and Reverse Genetics Strategies for Improving Oncolytic Reoviruses'* in 2018. Currently, her post-doctoral research is focused on developing innovative oncolytic adenoviruses and reoviruses for fast and feasible clinical application. Within the scope of this project she received the 1st ever ZonMW-Young Investigator Award 2018 from the Dutch Society for Gene and Cell Therapy (NVGCT) for a proposal on new delivery mechanisms for oncolytic viruses.