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# **Troubleshooting methods for the generation of novel pseudotyped viruses**

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## **Keywords**

Pseudotyped virus, pseudotypes, pseudoparticles, envelope glycoproteins

## **Future Perspectives**

The use of pseudotyped viruses has historically been limited to a small number of readily tractable viruses. However, the flexibility of pseudotyped viruses makes them attractive as safe surrogates for enveloped viruses requiring high bio-containment, for use in serological screening, assessing vaccine efficacy, targeted gene transduction, virus entry inhibition and receptor usage studies. Through the development of more standardized protocols, a broader range of reporter genes and backbone viruses, virus pseudotyping will become a central and powerful technique in the study and treatment of enveloped viruses.

## **Executive Summary**

A pseudotyped virus (PV) is a virus particle with an envelope protein originating from a different virus. The ability to dictate which envelope proteins are expressed on the surface has made pseudotyping an important tool for basic virological studies such as determining the cellular targets of the envelope protein as well as identification of potential antiviral compounds and measuring specific antibody responses. In this review, we describe the common methodologies employed to generate pseudotyped viruses (PVs) with a focus on approaches to improve the efficacy of PV generation.

## **What are pseudotyped viruses?**

In 1911, Peyton Rous published the first report of a non-cellular, filterable agent that could produce cancer - a sarcoma of chickens [1]. This agent was later named the Rous sarcoma virus (RSV) and was used to generate the first virus pseudotypes [2].

Rubin and colleagues [3] discovered that infectious RSV particles were not released from sarcoma cells during infection with RSV alone. However, if RSV-transformed sarcoma cells were co-infected with a helper avian leukosis virus, Rous-associated virus (RAV), then infectious RSV and RAV progeny were released (as RSV and RAV are antigenically-unrelated avian C-type retroviruses they could be distinguished). Importantly, it was also shown that the RSV particles produced possessed the RSV genome but the RAV outer 'coat', as determined by serology and cell tropism [2, 3]. They called these hybrid particles 'pseudotypes'.

A serendipitous property of retroviruses such as RSV is their natural ability to incorporate other cellular proteins, including the envelope proteins of other viruses [4-6]. The discovery that human immunodeficiency virus 1 (referred to as HIV in this review) effectively incorporates the envelope proteins from human T-lymphotrophic virus 1 (HTLV-1) [4] provided evidence that this virus is a tractable platform for making

recombinant PVs. Murine leukemia virus (MLV, also referred to as MuLV or Moloney murine leukemia virus, MoMLV) was independently discovered to assemble infectious particles with HTLV-1 envelope proteins [7].

Nomenclature has developed alongside molecular knowledge. The protein nucleocapsid encasing the ribonucleic acid (RNA) genome is now known as the 'core' and the outer membrane of these virus particles, derived from the host cell membrane during viral egress by 'budding', as an 'envelope'. Thus proteins embedded in the membrane become incorporated in the viral envelope and define particle antigenicity and cell tropism. Here, the term 'pseudotyped virus' (PV) is defined as an enveloped virus particle comprising a virus capsid/matrix/core surrounded by a cell-derived membrane bearing the foreign virus envelope protein(s) (VEP). The genetic material packaged by a PV does not encode a VEP. Therefore, a PV is capable only of entering and transducing a target cell, initiating nucleic acid replication without producing infectious particles. Depending upon end-user application, PVs have been variously referred to as pseudotypes, pseudo-viruses, pseudo-particles, virus pseudotypes, lenti- or retrovirus vectors, trans-complemented viruses, gene transfer vectors, reporter virus particles and virus-like particles. It is important to note that many of these terms have also been used to describe particles or techniques that do not fit the above definition. The term 'virus envelope protein' is used rather than 'virus glycoproteins' to include non-glycosylated viral surface proteins such as the mature form of the flavivirus M protein and exclude glycosylated non-structural proteins. Furthermore, the distinction between VEP and 'virus surface protein' distinguishes PVs from techniques involving non-enveloped viruses (e.g. adeno-associated virus vectors).

A functional PV can be generated by transfection of a 'producer' cell with three plasmids (Figure 1). The PV 'backbone' is a virus that provides both the core and the recombinant genome packaged by PVs. This combination is essential because genome packaging is mediated by specific interactions between sequences in the genome and the

core/matrix/capsid. The most commonly used PV backbones are HIV or MLV retroviruses for which the core expression plasmid encodes the *gag* and *pol* genes with no packaging signal. The recombinant genome is typically a reporter gene such as luciferase flanked by retroviral long terminal repeats (LTRs) and a packaging signal ( $\psi$ ). The third plasmid expresses the open reading frame of the VEP of the virus of interest with appropriate signal peptide.

## **Applications of pseudotyped viruses (PVs)**

The flexibility of PVs means that they are suitable for a wide range of applications. A key feature of PVs is that they cannot replicate apart from the reporter gene maintained as the genome; they are therefore widely used at ACDP/BSL 1 or 2 (regardless of the origin of the VEP) and have been particularly valuable in the study of highly pathogenic viruses.

### *PVs for research and therapeutic agent screening*

PVs offer the ability to rapidly generate key data in the characterization of virus–host interactions. The use of PVs has been of crucial significance in the rapid pace of research into the Middle Eastern Respiratory syndrome (MERS) outbreak [8-11]. During the recent Ebola outbreak, PVs were successfully used in high-throughput screening studies that helped in the identification of potential antivirals and filovirus entry inhibitors [12-14] as well as in the study of the viral life cycle and virus receptor interaction [15].

PVs have been used extensively to investigate the entry cascade of hepatitis C virus (HCV) for which, until recently, a robust *in vitro* cell-culture system did not exist for primary virus isolates. The two glycoproteins of HCV, E1 and E2, have been successfully expressed and incorporated into PVs, initially as modified glycoproteins into VSV backbones [16], then later as unmodified constructs using VSV [17], MLV [6] and HIV [18] backbones. The entry pathway of genetically diverse HCV strains was dissected

using these experimental systems, revealing a requirement for an array of receptors [19-23]. Both E1 and E2 are required for assembly of infectious PVs with E2 acting as a chaperone for E1 incorporation, interacting with both E1 and the retroviral core [24]. Using PVs to investigate the effects of specific point mutations on HCV entry also revealed key amino acids involved in receptor interactions [25-27]. However, comparison with authentic HCV viruses revealed that PVs are less tolerant to alteration *in vitro*.

### *PVs for measuring antibodies*

PVs have been used as surrogates of wild-type viruses in sensitive, high throughput neutralization assays (PVNAs), also referred to as pseudotyped particle- (pp-) or pseudotyped virus-based microneutralization assays (pv-MN). For most PVNAs, serial dilutions of sera are incubated with a pre-determined amount of a PV (quantified by the measurement of reporter gene expression) for one hour at 37°C. A fixed amount of virus-susceptible target cells is then added and reporter gene expression measured after an appropriate incubation period (e.g. 48 hours) [28]. The titer of antibody is typically expressed as the highest dilution of the sample that reduces reporter gene expression by either 50% (IC<sub>50</sub>) or 90% (IC<sub>90</sub>) compared to controls [29]. The PVNA is typically sample-sparing. The option to incorporate a number of different reporter genes means that PVs can be adapted to a multiplex serum screening format [30, 31].

Comparative serology studies for highly pathogenic avian influenza (HPAI) of the H5 subtype have shown that results from more traditional serological assays including hemagglutination inhibition, micro-neutralization and single radial hemolysis correlate well with those obtained by PVNA [32]. It is thought that a lower density of hemagglutinin (HA) on the surface of influenza PVs compared to wild-type virus means that a sub-set of antibodies are better able to access cross-reactive epitopes on the stalk of the VEP. The PVNA can therefore be used to measure antibodies generated by prototype 'universal vaccines' composed of HA stem fragments. These antibodies cannot

be measured by the classical hemagglutination inhibition test as this relies on measuring blocking of antibodies against the receptor-binding site on the globular head of the HA, which is missing [33, 34].

PVs have also been widely used to investigate neutralization of HCV entry. The PVNA was first established to test the neutralizing potency of sera [35-37] and monoclonal antibodies [38-42] against diverse strains of HCV. This revealed the existence of broadly-neutralizing antibodies generated during natural infection [43, 44] and following immunization with vaccines [45, 46]. Studies using PVs demonstrated that serum contains factors other than antibodies that have the potential to neutralize HCV entry [47-49], while the apolipoprotein components of serum can enhance HCV infectivity and protect against antibody-mediated neutralization [50, 51]. Direct comparison of HCV PVs and wild-type HCV viruses revealed that PVs are more resistant to antibody-mediated neutralisation [41], suggesting that PVNA might over-estimate the amounts of antibody required for clinical administration.

Retroviral PVs bearing the HIV-1 glycoproteins have also been used for high throughput PVNA and assessment of vaccine-induced immunity. Comparison of an array of different assays in multiple laboratories found that PVs were generally more sensitive to neutralization than replicating viruses [52, 53], confirming previous findings [54]. As such, PVs might not accurately determine the neutralization potency of inhibitors against a single virus strain, but provide a powerful and rapid method for comparing the neutralization sensitivity of many different strains.

### *PVs as vaccines*

The production of neutralizing antibodies is a hallmark of successful vaccination resulting in protection against virus infections (reviewed in [55]). As VEPs are the key targets of neutralizing antibodies, PVs capable of expressing different proteins might be a very useful platform for vaccine design. Indeed, immunization with VSV-based PVs has been

shown to protect mice against challenge with influenza [56]. However, disadvantages include the potential risk of genomic integration (see below) and lack of sustained antigen production, and greater efforts have been made to develop virus-like particle (VLP)-based vaccines (reviewed in [57]).

### *PVs for gene transfer*

The comparative ease with which VEPs can be swapped has been exploited to produce retrovirus delivery vectors for gene transfer where the integration of packaged transgene into the target cell genome enables longer-term gene expression compared with the episomal vectors, typified by many DNA viruses. The first gene therapy vectors were based on retroviruses with their *gag*, *pol* and *env* regions removed and replaced with a therapeutic gene and sometimes a selective marker. During vector production, the *gag*, *pol* and *env* protein products were supplied in *trans* by suitable stable packaging cell lines. Although the first gene therapy trial employed a retrovirus vector with its cognate envelope protein [58], soon after, heterologous retrovirus envelopes were used, primarily to enhance target cell transduction and thus therapeutic gene expression [59]. One issue with the original C-type retrovirus vectors (e.g. MLV) was that they were only able to transduce dividing cells. To address this issue, researchers turned to the lentiviruses (e.g. HIV), which do not require replicating cells for efficient transduction.

Another issue has been that of insertional mutagenesis as a result of genomic integration of the transfer gene causing activation of oncogenes [60]. Though a rare occurrence, it did manifest itself by producing leukemia in five patients several years after gene therapy for severe combined immunodeficiency [61]. It is believed that the viral control elements situated in the retrovirus LTRs activate host genes. Consequently, self-inactivating vectors have been designed which eliminate these elements [62].

In order to alter the natural cell tropism of transfer vectors, heterologous envelope glycoproteins have been employed with varying degrees of success [63]. VSV-G was used for pseudotyping retrovirus vectors [64] to broaden the range of target cells,



tissues and species, and has been widely adopted since. Conversely, pseudotyping provides the means to retarget viral vectors for transduction of particular targets, such as lung, central nervous system, kidney, liver and hematopoietic cells [63].

## **Considerations for developing novel pseudotyped viruses**

Many different viruses have been pseudotyped. However there is no single standardized experimental approach. There are many factors that need to be considered when generating a novel virus pseudotype, taking into account numerous aspects of the virus biology, the intended application and how tractable the virus is to forming PVs. These are expanded upon in the following sections and summarized in Figure 2.

The "plug and play" pseudotype construction model is exemplified by influenza PVs assembled using retroviral cores. With a toolkit of 6 plasmids and a streamlined optimization grid for transfection, most strains of influenza are currently amenable to pseudotyping onto retroviral cores [65-67]. Essentially these assembly processes fall broadly into three protocols with the one chosen being dependent on the HA to be pseudotyped and also the downstream application. The simplest system is employed for generating PVs from highly pathogenic avian influenza viruses of the H5 and H7 subtypes which possess multi-basic HA cleavage sites. These are ideal when only HA-mediated cell entry is required as there will be no neuraminidase (NA) on the released PVs [68, 69]. Inclusion of a plasmid encoding NA generates particles that more closely mimic their cognate wild-type virus, which is useful for surveillance and vaccine immunogenicity studies (whole virus vaccines). In order to effectively pseudotype HAs that possess a monobasic HA cleavage site (e.g. from seasonal human influenza viruses), a protease must be supplied (as described below).

### *Viral backbones for pseudotyping*

As previously mentioned, HIV or MLV retroviruses are the most commonly used backbones for PV production. The HIV backbone for PVs has undergone several iterations

to improve safety including the deletion of accessory proteins, promoter sequences and the provision of the HIV *rev* gene on a separate fourth plasmid (reviewed in [70]). Systems are also available whereby the reporter gene is encoded on the *gag-pol* plasmid reducing it to a two plasmid transfection system. When establishing a novel virus PV system it may be relevant to consider which generation of HIV backbone to use as it has been reported that *nef* co-expression enhanced PV assembly of VEPs from retroviruses, but not non-retroviruses, on the HIV backbone [71]. Two and three plasmid systems, comparable to the lentivirus system, exist for MLV. MLV is non-pathogenic in humans and has fewer accessory proteins compared to HIV. It was the preferred PV backbone prior to development of the second and third generation HIV systems but the two retrovirus backbones are now comparable in terms of safety and ease of use.

Not all VEPs are incorporated efficiently into HIV or MLV backbones [72]; the choice of backbone for pseudotyping a novel virus may be informed by reports of successful systems published for related viruses. The vesicular stomatitis virus (VSV) system, which was originally applied to the study of Ebola virus (EBOV) envelope proteins [73], provides an alternative. VSV is a single-stranded negative sense RNA virus encoding 5 genes that are transcribed on separate mRNAs, enabling deletion or substitution of any given gene for a heterologous gene. PVs can be generated by combining a recombinant VSV genome, in which the VSV-G gene has been deleted (rVSV- $\Delta$ G\*) and is replaced with a reporter gene, with an expression plasmid encoding the desired VEP(s).

Establishment of the rVSV- $\Delta$ G\* system is complex (Figure 3). In order to initiate replication of the rVSV- $\Delta$ G\* genome, cells must be transfected with the pVSV- $\Delta$ G\* genome plasmid and plasmids expressing the VSV nucleoprotein (N), phosphoprotein (P) and polymerase protein (L). Furthermore, initial transcription of the VSV- $\Delta$ G\* genome from the plasmid is controlled by the T7 RNA polymerase promoter (T7 pol). T7 pol is commonly provided by one of two methods: infection of the producer cells with a recombinant virus expressing T7 pol (modified vaccinia virus Ankara (MVA)-T7 or

fowlpox virus (FPV)-T7) or by using a clone of the BHK-21 cell line stably expressing T7 pol from a plasmid [74]. T7 helper viruses are preferred because they achieve higher levels of T7 pol activity compared to stable cell lines. The addition of a VSV-G expression plasmid results in the production of particles encapsidating the VSV- $\Delta$ G\* genome and coated with VSV-G, or VSV-pseudotyped rVSV. Infection of target cells (lacking the T7 pol and the VSV accessory protein expression plasmids) with these PVs leads to efficient replication of the VSV- $\Delta$ G\* genome and production of non-infectious  $\Delta$ Env-rVSV. Therefore transfection of cells with a heterologous VEP expression plasmid and subsequent infection with VSV-pseudotyped rVSV particles leads to the production of heterologous VEP-pseudotyped rVSV. However, due to the high efficiency of VSV-G pseudotyping, it is important to assess PV neutralization with antibodies against the heterologous virus envelope and VSV-G to confirm the composition of the PVs. A comprehensive method for the production of VSV-based PVs is given in [75].

### *Provision of proteases*

Many viruses make use of host cell proteases for the production or release of mature virus particles. Furthermore, protease usage can be a critical determinant of viral tropism [76]. For seasonal human influenza viruses, the requirement for protease-mediated cleavage of the hemagglutinin in order for it to become fusion competent is a well-documented trait [65, 77-79]. However, protease activation is also a well-studied component in the *Paramyxoviridae* (a family of negative sense single-strand RNA viruses), where the F protein precursor must be cleaved in order to facilitate maturation of the fusion protein [80], and in the *Coronaviridae* (a family of positive sense, single-strand RNA viruses) where proteases can be involved in both cleavage of the spike protein and facilitating release from the host cell [81-83]. Therefore, it may be necessary to supplement PV generation protocols with specific proteases. In many studies it has become common practice to co-transfect a protease-encoding plasmid alongside the other plasmids required for PV production [65, 77, 79, 84]. For influenza,

the most commonly used proteases when supplied as a plasmid are HAT and TMPRSS2 [77].

It is also possible to induce protease-mediated activation through the addition of purified protease to the culture medium or purified PV preparation. The addition of exogenous tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin has been demonstrated to facilitate activation of SARS-CoV [85, 86] MERS-CoV [10, 87] and human coronavirus 229E [88]. Exogenous TPCK trypsin is also used for influenza, but an additional step is required in order to deactivate the protease, using commercially available protease inhibitors, prior to inoculation of the PVs onto target cells [65, 77].

### *Signal peptides and transmembrane domains*

Appropriate signal peptides (SPs) and transmembrane domains (TMDs) are a key component of successful PV production as they contribute to sub-cellular targeting and membrane retention of the VEP. It is necessary to target VEPs to the endoplasmic reticulum to facilitate trafficking to sites of assembly. The exact site of retrovirus budding is yet to be completely defined. The cytoplasmic tails of VEPs have also been identified as important factors [89-91]. Gibbon ape leukemia virus (GaLV) required the cytoplasmic domains of MLV glycoproteins to form PVs on an HIV-1 backbone [92].

### *VEP alteration*

The diverse structure of VEPs, a characteristic used to divide them into three major classes (I-III), can be the cause of their inefficient incorporation into PVs and consequently poor viral titers. Issues such as localization to the Golgi complex may be overcome by splicing together different sections of heterologous VEPs, to generate chimeric proteins. The structure of a VEP can be crudely broken down into the ecto-, transmembrane and cytoplasmic domains. Ectodomains play the major role in cell

binding and antigenicity so any alterations could affect serological and tropism properties of the VEP so this domain should usually be maintained. However, several studies have shown that switching of the cytoplasmic domains is one mechanism by which PV titers can be improved [16, 93, 94]. Carpentier *et al.* conducted a detailed series of experiments to determine if titers of lentiviral PV bearing the rabies virus envelope protein (RABV-G) could be improved by engineering chimeric VEPs [94]. By swapping in the corresponding domains from VSV-G they were able to generate a series of chimeric VEPs and determine efficiency of VEP incorporation into PVs and infectivity. The only chimera that led to an increase in PV titer comprised the RABV-G ecto- and transmembrane domains with the VSV-G cytoplasmic domain. This work has been expanded by Bentley *et al.* who showed that RABV-G which previously gave no or very low/unusable PV titers could successfully be pseudotyped by swapping the cytoplasmic domain for that of VSV-G [95]. However, it is important to note that there is some variation within the literature about the exact sequence for the VSV cytoplasmic domain [16, 96, 97].

The reason chimeric VEPs result in better incorporation of VEP and higher PV titer is unclear. It could be due to a stronger interaction with the backbone matrix/capsid protein if the cytoplasmic domain being introduced is shorter as this may reduce steric hindrance. Alternatively, the chimeric VEPs may be trafficked more efficiently to the sites from which the backbone virus naturally buds.

Other modifications to VEPs can boost titers. Measles virus has two envelope proteins, F and H, and it has been shown that if the full-length wild-type VEPs are used then only very small quantities of infectious PV are produced [98]. However, if both VEPs are truncated, H $\Delta$ 24 and F $\Delta$ 30, levels of incorporation into PV and the PV titer are both markedly higher.

## **Optimizing PV titers**

The VSV glycoprotein (VSV-G) produces high titer PVs and is therefore commonly used as a positive control in developing novel PVs. Hepatitis B virus represents the other extreme where existing systems result in very low infectious PV titers [99].

### *Quantification of PVs obtained*

PV titer is most commonly determined by measuring reporter gene expression in susceptible target cell lines. However, if little or no expression is seen, it may be necessary to conduct further tests. For retrovirus-based PVs this appears to underestimate the number of cells that have undergone genome integration, due to variability of marker expression; PCR has been employed to determine this more precisely [100, 101]. Titration of viral genomic RNA from producer line supernatants conversely overestimates the number of infectious particles due to the presence of defective interfering particles. Titration of HIV-based PV preparations can also be performed by Gag (p24) protein ELISA [102] with infectivity defined as number of infectious units per unit of p24. Lastly, titration of several retroviral vectors based on reverse transcriptase activity via qPCR has recently been reported [103].

Virus particle analysis has historically been conducted via electron microscopy. However, new technologies (e.g. nanoparticle tracking analysis) have enabled rapid quantification of virus particles and determination of particle size [104]. The continued development of such systems may provide vital information on the efficacy of PV production, both in terms of quantity and quality.

If low titers of PV production are demonstrated, there are several measures that can be taken to boost these to more usable levels as described below. On the other hand, if no detectable titers are observed, it may be necessary to change the approach (for example using a different backbone or generating a chimeric VEP).

### *Concentration of PVs*

Ultracentrifugation combined with a sucrose cushion or gradient can be used to concentrate PVs [18, 105]. Concentration of PVs can also be performed using polyethylene glycol (PEG)-precipitation. PEG precipitation has advantages over ultracentrifugation, which requires expensive equipment, and low speed centrifugation, which can be time consuming. Optimization of incubation time and molecular weight and concentration of PEG used will all be required, however numerous commercial kits are available with specific protocols. An optimized method for MLV uses 8.5% (w/v) PEG 6000 for 90 minutes at 4°C followed by collection of the precipitate by centrifugation at 7,000 x *g* for 10 minutes [106, 107].

### *Influence of plasmid and codon optimization*

Codon optimization is used to increase the expression of a protein in organisms by increasing translational efficiency. Most amino acids are encoded by more than one codon with each codon recognized by a specific tRNA. Organisms have developed individual preferences for particular codons for a given amino acid (known as 'codon usage bias'). As the efficient generation of PVs requires good levels of VEP expression in the producer cells, codon optimization relevant for the given producer cell line may improve viral titers [108-111].

The plasmids used to encode the VEP(s) can also greatly influence expression levels and therefore PV titer [112, 113]. In order to maximize the level of VEP expression, plasmids with strong promoter elements are used such as pcDNA, pCAGGS or phCMV. Expression in these plasmids is driven by either the human cytomegalovirus major immediate early or beta-actin promoter, two of the strongest promoters that have been identified. As pcDNA- and phCMV-based plasmids are commercially available they negate downstream intellectual property conflicts.

The methods of producing PVs on both the VSV and retroviral backbones involve transfection of producer cells with multiple plasmids. Establishing successful transfection of plasmids in appropriate ratios appears to be important for successful generation of infectious PVs. Experiments with HIV-based PVs demonstrated that differences in infectivity occur with different amounts of the VEP plasmid [114]. This also occurs for MLV-based PVs, with the amount of VEP plasmid being finely tuned to the species of virus being pseudotyped [115]. Interestingly, maximal expression of VEP did not always correlate with maximal infectivity, suggesting that optimization of the ratio of expression of viral capsid genes to VEP might be important to generating infectious particles.

### *Use of PV packaging cell lines*

Cell lines stably expressing one or more components of the PV system have been developed to standardize and simplify PV production and reduce transfection costs.

Transfecting cells with a plasmid encoding a lentivirus or retrovirus backbone with functional LTRs but with a deletion for the packaging signal ( $\psi$ ) [116] results in 'PV packaging cells' that continually express high levels of the capsid and enzymes required for retrovirus-based PV production. Subsequent transfection of these cells with plasmids encoding a VEP gene and a reporter gene, with  $\psi$  signal and LTRs, results in the production of functional PVs. Specific packaging cell lines have also been generated by maintaining episomal replication of a plasmid encoding the backbone *gag-pol* genes using selection markers [117]. Packaging cell lines have been produced for a range of PV backbones, including HIV, MLV and FeLV, and are available commercially. More recently, a lentivirus packaging cell line was developed specifically to meet clinical approval standards for the generation of PVs for gene therapy by inserting the HIV *gag-pol* genes into the genome of 293FT cells using Cre recombinase mediated cassette exchange (RMCE) [118].



It is well known that VSV-pseudotyped retrovirus PVs result in high levels of reporter gene expression in producer cells due to transduction of the reporter gene at multiple sites in the genome [119]. Therefore, transduction of producer cells with VSV-pseudotyped retrovirus PVs packaging the gene encoding the VEP(s) for a novel virus will generate a cell line with potentially high levels of VEP expression. This may be appropriate for novel PV candidate viruses with poor envelope protein expression levels.

### *Improved interaction of PVs with target cells*

During development of entry models for HCV, large differences in PV transduction of target cells were observed [19, 114, 120], in particular PVs representing HCV genotype 3 were associated with lower infectivity ([19]; unpublished results). As a result, some methodologies have used polybrene, or 'spinoculation' to increase observed infectivity [114, 121].

As a result of their amino acid composition, VEPs are negatively charged. This can limit the initial and sometimes non-specific interaction between the VEP and molecules on the target cell surface [122]. Addition of polybrene (hexadimethrine bromide), a cationic polymer, to the cell culture media (typically at a final concentration of 8µg/ml) is thought to reduce the charge repulsion between the negatively charged VEPs and receptors such as sialic acid or increasing virus aggregation [123]. Spinoculation involves centrifuging VPs onto the target cell surface. Both methods have the effect of increasing observed signal, but increase the complexity of the downstream applications.

## **Conclusions**

The generation of PVs has facilitated progress in research involving highly pathogenic viruses and has been beneficial to research involving viruses for which *in vitro* culture methods have not been available. Despite the many success stories, pseudotyping of members of some virus families (e.g. the *Flaviviridae*) has yet to become a routine

procedure. Even within virus families for which PVs have been successfully generated, not all subtypes of strains can be predictably pseudotyped by the same methods. In summary, there are numerous factors to be taken into consideration when generating PVs. As the field develops, the range of choice of viral backbones and reporter genes is expanding and progress is being made towards the standardization of protocols for consistency, which is essential for some of the main applications of PVs. Novel viruses may pose technical barriers to pseudotyping, but the resultant PVs will provide a powerful tool to dissect aspects of virus binding and entry as well as in the development of antivirals and vaccines.

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\*\* The detailed experiments provided within this manuscript provide crucial information regarding which domain(s) of Rhabdovirus VEPs are important for efficient incorporation into PVs. It highlights the role the cytoplasmic C domain alone plays in generating high titer PVs.

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## **Figure legends**

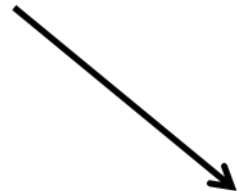
Figure 1. Schematic of the basic three-plasmid approach to generation of a pseudotyped virus with a retroviral core.

Figure 2. Summary diagram of factors to consider when generating a pseudotyped virus expressing a novel viral envelope protein.

Figure 3. Schematic of generation of a pseudotyped virus with a vesicular stomatitis virus (VSV) core.

**Plasmid 2: Reporter**

Packaging-Competent Reporter gene e.g. GFP or Luciferase



**Plasmid 1: Capsid/core/matrix**

Structural proteins plus genome-associated enzymes



**Plasmid 3: Virus envelope protein (VEP)**

VEP presented of the cell-derived membrane

