

Retroviral Pseudotypes – From Scientific Tools to Clinical Utility

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Advanced article

Article Contents

- Introduction
- General Method
- Analysis of Receptor Binding and Virus Entry and Exit
- Antiviral Screening
- Pseudotype Neutralisation Assays and Immunogenicity Testing
- Pseudotypes as Immunogens and for Gene Therapy
- Notes
- Acknowledgements

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Retroviral pseudotypes are important research and diagnostic tools for basic and clinical virology studies, facilitating the detailed investigation of individual genes, cellular receptors, antibody responses, serosurveillance and antiviral therapies. Importantly, pseudotypes enable the study of highly pathogenic viruses, without the need for high containment. Their use as gene therapy vectors is widely documented, but other uses, once less well known, are becoming more prominent. The substitution of envelope proteins expressed on the virion surface enables pseudotypes to be employed as surrogates for wildtype viruses in antibody neutralisation or antiviral screening assays and for the study of cell-virus receptor interactions. In addition, they are increasingly being utilised as vaccine immunogens, expressing the antigen either on the particle surface or as a transfer gene for cellular expression. These studies demonstrate the potential for using pseudotypes for both scientific and clinical applications.

Introduction

The standard definition of a pseudotype is a hybrid virus particle consisting of a protein nucleocapsid ('core') encasing a ribonucleic acid (RNA) genome, with the core itself being encapsulated in a lipid 'envelope' membrane derived from the host cell.

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Importantly, this envelope gained when cores exit from the cell by 'budding' is studded with proteins derived from *other* viruses. Many of these heterologous envelope proteins are antigenic targets for the host immune system. In pseudotypes, one or more of these envelope proteins may derive from study viruses. Many pseudotypes also carry foreign genes, called 'transfer' genes, engineered into their genome. When in the presence of susceptible cells, the envelope proteins bind to cell receptors permitting cellular entry, eventually resulting in transfer gene expression. Retroviruses have been extensively exploited as cores for pseudotyping. The name retrovirus derives from the Latin for 'behind' or 'backward' (*retro-*) and this highlights their key characteristic, the ability to reverse transcribe their dimeric single-stranded RNA genome into a double-stranded deoxyribonucleic acid (dsDNA) copy, which is subsequently integrated into the cell genome via the use of viral and cellular enzymes. For retroviral pseudotypes, this usually leads to expression of the transfer/reporter gene, the latter being readily quantifiable. Thus, reporter gene expression directly correlates with efficiency of viral envelope/receptor interaction, and conversely whether individual antibody responses or antiviral agents could interfere with the entry and replication process of the native virus. **See also: [Retroviral Replication; Vesicular Stomatitis Virus](#)**

Current pseudotyping protocols are based on several decades of vector development, often for use in gene therapy. The first retroviral vectors were based on native gammaretroviruses, such as Moloney mouse leukaemia virus (MLV). To produce such retrovirus vectors, a stable cell line containing the retroviral genome minus the packaging signal needed for genome encapsidation was first generated. These cells were then transfected with a plasmid construct containing the packaging signal and all *cis*-acting sequences necessary for virus particle assembly, but in which the genes required for genome replication and nucleocapsid formation had been removed (often replaced by a gene of interest – the transfer gene). Thus, all genes needed for retrovirus particle assembly were provided by the cell line in *trans*. However, recombination between newly produced retroviral vector and integrated helper virus sequences occasionally led to production of replication-competent viruses using these systems (Sakuma *et al.*, 2012). In order to reduce the production of replication-competent

virus, novel packaging cell lines were developed where the helper virus elements were introduced and genomically integrated separately, requiring only a final transient transfection of the transfer gene construct to generate the replication-deficient retrovirus particles required (Pear *et al.*, 1993). Nevertheless, replication-competent virus remained detectable. This situation was notably improved by adopting a triple-plasmid system where the structural and replication/integration genes (*gag-pol* plus regulatory/accessory proteins), envelope protein gene (*env*) and marker/reporter genes were present on separate plasmids which were transiently transfected into producer cell lines (Soneoka *et al.*, 1995; Naldini *et al.*, 1996). Simultaneous expression of these genes permits assembly of new viral particles, but multiple recombination events would be necessary to produce replication-competent virus. The particles are able to ‘transduce’ susceptible cells, integrate the marker gene, but are unable to produce new virus progeny (hence viral transduction rather than infection).

A myriad of more refined systems have since been developed, often aimed at improving safety, particularly for the use of human immunodeficiency virus (HIV) vectors in gene therapy (Sakuma *et al.*, 2012). Adaptations have included the removal of viral accessory genes (HIV-1 *Vif*, *Vpr*, *Vpu* and *Nef*), important in natural infection, but not in vectors. Subsequently, to limit interference with host gene expression following vector integration, ‘self-inactivating’ systems were engineered. Next, to avoid the need for transient transfection in large-scale vector production, continuous retrovirus producer cell lines were established (Ikeda *et al.*, 2003). Finally, to further reduce recombinational events, a four-plasmid system to generate vectors was designed, where the HIV *rev* regulatory gene was placed on a separate plasmid. The *tat* regulatory gene was also deleted from the *gag-pol* construct, as the *tat*-dependent U3 promoter driving transgene expression is replaced with a strong constitutive promoter. The latter adaptation has been adopted in the three-plasmid system, which remains the most widely used to generate most retroviral pseudotypes to this day (Figure 1).

Retroviruses have proven to be particularly useful for pseudotyping, and owing to their ability to stably integrate ‘therapeutic’ transfer genes into a target cell, they have been extensively developed as gene therapy vectors. Furthermore, the exchange of surface envelope proteins has enabled altered tropism or ‘tropism switch’ in pseudotypes, permitting targeting of specific cell types. **See also: Gene Therapy: Technology; Retroviral Vectors in Gene Therapy; Retroviruses in Human Gene Therapy; Lentiviral Vectors in Gene Therapy**

Representatives of all seven genera of retroviruses have been employed in pseudotyping studies but to date only gammaretroviral or lentiviral pseudotypes are widely used. Lentiviruses are a genus of the *Retroviridae* family, which unlike gammaretroviruses, can infect non-proliferating cells, which makes them amenable for gene therapy applications involving highly differentiated or quiescent cells (e.g. in G₀ cell cycle phase) including muscle or neurons. The most common lentivirus vector used for pseudotyping is HIV-type 1 (HIV-1), although simian immunodeficiency virus (Schnell *et al.*, 2000) and equine infectious anaemia virus (Mitrophanous *et al.*, 1999), among others, have occasionally been employed. Lastly, *Rhabdoviridae*, such

as vesicular stomatitis virus, have also been extensively utilised as pseudotype cores, but is not the subject of this review.

Pseudotypes have frequently been employed as tools for basic virus research, as they possess a number of distinct advantages over using wildtype viruses:

1. Binding and entry of cells is entirely receptor-mediated; therefore, pseudotyped particles can be engineered to specifically target certain cell types, which have been extensively exploited in gene therapy studies. For example, pseudotyping with the vesicular stomatitis virus G protein serves to widen the host range (Naldini *et al.*, 1996), whereas pseudotyping with the hepatitis B envelope confers hepatotropism (Sung and Lai, 2002). As only foreign envelope proteins are expressed on the pseudotype envelope, a more specific study of virus–cell receptor interaction can be undertaken without the influence of other proteins from the wildtype virus. Research using pseudotypes has led to the elucidation of virus entry mechanisms and identification of virus receptors (see Table 1).
2. A dsDNA copy of a chosen transfer gene is integrated into the host chromosome resulting in relatively stable and long-term gene expression, useful for short-term investigations, screening assays and gene therapy.
3. Using the envelope proteins of highly pathogenic viruses in the pseudotype system removes the need for handling wildtype viruses making it a much safer alternative, and requiring a much lower level of biological containment.
4. Pseudotypes are relatively stable, having a half-life of several days at 37 °C and of several weeks at room temperature. This stability can be influenced by the combination of vector and envelope glycoprotein (Wright *et al.*, 2009; Mather *et al.*, 2014; Sawoo *et al.*, 2014).

General Method

Generation of retroviral pseudotypes is achieved through the introduction of cloned versions of foreign envelope protein gene(s), core retroviral genes and transfer gene (e.g. reporter or therapeutic gene) concurrently into producer cells, normally highly transfectable cell lines such as human embryo kidney (HEK) 293 clone 17 T cells (American Type Culture Collection #CRL-11268) (Pear *et al.*, 1993).

1. *The envelope plasmid.* Envelope gene(s) of the study virus are cloned into an appropriate expression plasmid. Genes are usually derived via polymerase chain reaction amplification of viral cDNA using specific primers or from custom gene synthesis. Some expression vectors are commercially available and utilise different, usually strong constitutive gene promoters [e.g. from the human cytomegalovirus (CMV) immediate early gene], which can influence the efficacy of pseudotype generation. In general, simple RNA viruses require the cloning of the envelope gene alone, whereas more complex viruses require additional accessory envelope sequences. To date, attempts to pseudotype DNA

Table 1 Selected examples of the generation and application of retroviral pseudotypes from basic scientific research to translational studies

Virus family	Virus species (envelope protein)	Cell tropism	Receptor identification and study	PV antibody neutralisation	Seroprevalence	Antiviral screening	Vaccine evaluation	Experimental vaccine
<i>Arenaviridae</i>	Lymphotropic choriomeningitis virus (Miletic <i>et al.</i> , 1999), Lassa virus (Radoshitzky <i>et al.</i> , 2007)	Miletic <i>et al.</i> (1999)	Reignier <i>et al.</i> (2006), Radoshitzky <i>et al.</i> (2007)	Miletic <i>et al.</i> (1999), Rodrigo <i>et al.</i> (2011)		Rodrigo <i>et al.</i> (2011), Madrid <i>et al.</i> (2013), Wang <i>et al.</i> (2014)		Zhang <i>et al.</i> (2014)
<i>Bunyaviridae</i>	Hantaan virus, La Crosse virus (Ma <i>et al.</i> , 1999)	Ma <i>et al.</i> (1999)		Ma <i>et al.</i> (1999)		Gehring <i>et al.</i> (2014)		Yu <i>et al.</i> (2013)
<i>Coronaviridae</i>	SARS virus (Simmons <i>et al.</i> , 2004; Wang <i>et al.</i> , 2004), MERS virus (Zhao <i>et al.</i> , 2013)	Simmons <i>et al.</i> (2004)	Wang <i>et al.</i> (2004), Hou <i>et al.</i> (2010)	Temperton <i>et al.</i> (2005), Zhao <i>et al.</i> (2013)	Perera <i>et al.</i> (2013)		(Zhao <i>et al.</i> , 2013)	
<i>Filoviridae</i>	Ebola virus (Wool-Lewis and Bates, 1998; Chan <i>et al.</i> , 2000), Marburg virus (Chan <i>et al.</i> , 2000)	Wool-Lewis and Bates (1998), Chan <i>et al.</i> (2000)	Chan <i>et al.</i> (2000)			Madrid <i>et al.</i> (2013), Gehring <i>et al.</i> (2014), Wang <i>et al.</i> (2014)		
<i>Flaviviridae</i>	Hepatitis C virus (Bartosz <i>et al.</i> , 2003b), tosch <i>et al.</i> , 2003b)	Bartosz <i>et al.</i> (2003b), Cormier <i>et al.</i> (2004)	Cormier <i>et al.</i> (2004)	Bartosz <i>et al.</i> (2003a), Cormier <i>et al.</i> (2004)	Bartosz <i>et al.</i> (2003a)	Yu <i>et al.</i> (2012)		
<i>Orthomyxoviridae</i>	Influenza A viruses (Dong <i>et al.</i> , 1992; Temperton <i>et al.</i> , 2007)	Zhang <i>et al.</i> (2010)	Shelton <i>et al.</i> (2013)	Corti <i>et al.</i> (2011), Yang <i>et al.</i> (2014)	Wallerström <i>et al.</i> (2014)	Su <i>et al.</i> (2008), Basu <i>et al.</i> (2014), Wang <i>et al.</i> (2014), Aljofan <i>et al.</i> (2009)	(Alberini <i>et al.</i> , 2009)	Szécsi <i>et al.</i> (2006), Tao <i>et al.</i> (2012)
<i>Paramyxoviridae</i>	Measles virus (Funke <i>et al.</i> , 2008), Nipah and Hendra viruses (Aljofan <i>et al.</i> , 2009)	Khetawat and Broder (2010)	Funke <i>et al.</i> (2008)					
<i>Retroviridae</i>	Human T lymphotropic virus (Landau <i>et al.</i> , 1991), MLV (Watson <i>et al.</i> , 2002) HIV (Kim <i>et al.</i> , 2001)	Watson <i>et al.</i> (2002)	Deng <i>et al.</i> (1997)	Kim <i>et al.</i> (2001), McCoy <i>et al.</i> (2012)	Simek <i>et al.</i> (2009)	Forsman <i>et al.</i> (2008)	(Pegu <i>et al.</i> , 2014)	Breckpot <i>et al.</i> (2010)
<i>Rhabdoviridae</i>	Vesicular stomatitis virus (Naldini <i>et al.</i> , 1996), rabies virus (Wright <i>et al.</i> , 2008), Mokola lyssaviruses (Desmaris <i>et al.</i> , 2001)	Desmaris <i>et al.</i> (2001), Mentis <i>et al.</i> (2006)		Wright <i>et al.</i> (2008)	Wright <i>et al.</i> (2010)	Both <i>et al.</i> (2013)	(Wright <i>et al.</i> , 2009)	

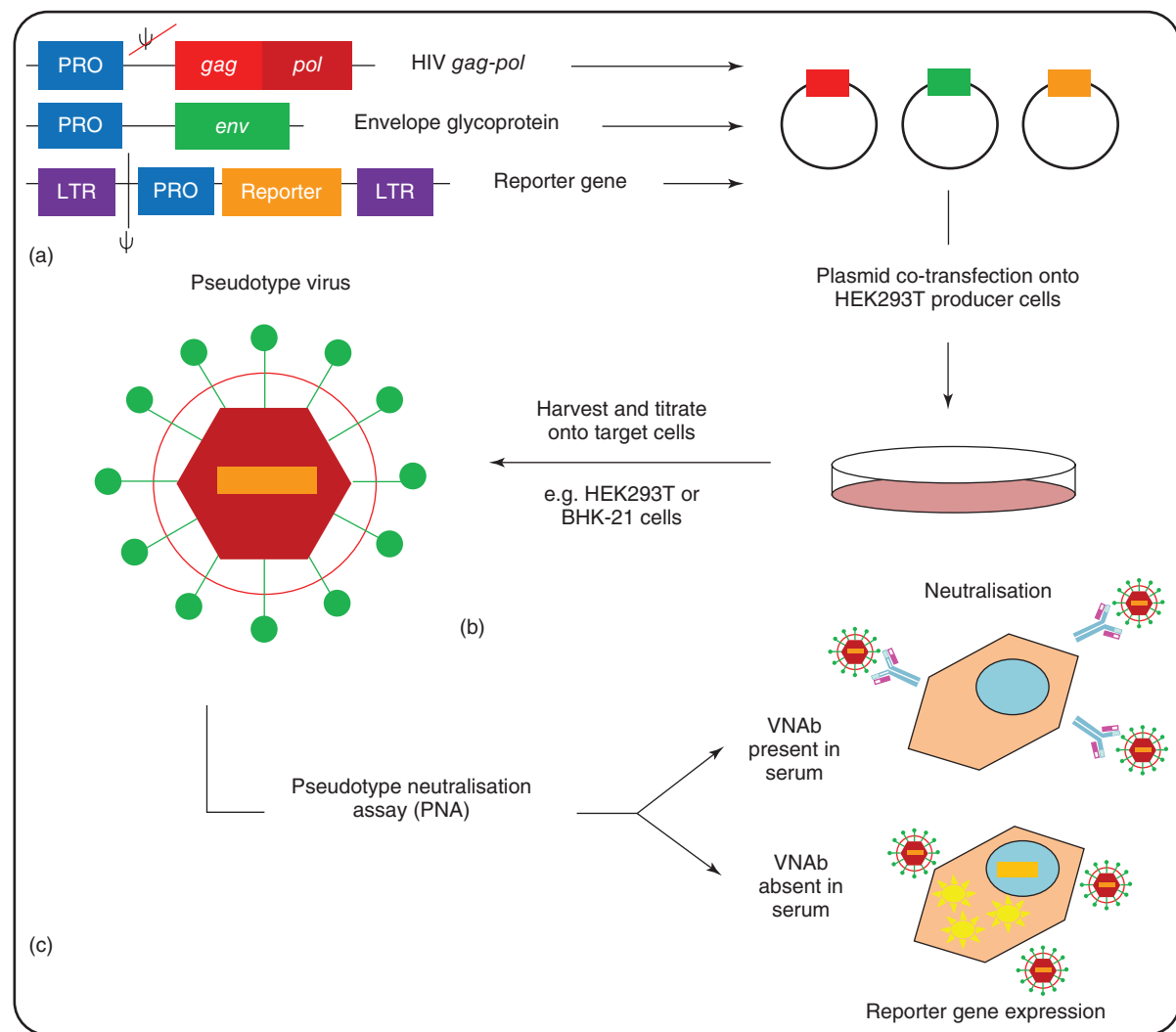


Figure 1 Schematic of a triple-plasmid co-transfection system for generation of retrovirus pseudotypes, and use to measure antibody neutralisation. (a) Plasmid DNA expression vectors carrying the HIV *gag-pol* 'core', envelope glycoprotein (*env*) and reporter genes (e.g. luciferase, GFP and β -galactosidase) are transfected into producer cells (e.g. HEK293T). PRO indicates a (usually constitutive) gene promoter. LTR represents the retroviral long terminal repeats involved in vector integration into host cell DNA. (b) Pseudotype virus (PV) supernatants are harvested 48–72 h post-transfection and relative PV titres are ascertained by measuring reporter expression in transduced target cells. (c) PVs can then be used in neutralisation assays (PNAs) to quantitate virus neutralising antibody (VNAb) responses in serum samples from individuals following natural infection or vaccination. The deletion and insertion of the packaging signal is indicated by the Ψ (psi) symbol. PRO, promoter; LTR, long terminal repeat.

- viruses onto retroviral cores have failed with the exception of hepatitis B virus (which is a pararetrovirus, because of its incomplete dsDNA genome and reverse transcriptase activity). These pseudotypes were shown to exhibit hepatotropism (Sung and Lai, 2002).
2. *The retroviral gag-pol plasmid.* The *gag* and *pol* genes encode polypeptides which are subsequently cleaved to release, respectively, structural proteins (including matrix, capsid and nucleocapsid) found within the core, and proteins involved in viral replication (protease, reverse transcriptase and integrase) responsible for processing the structural proteins, converting the ssRNA viral genome into dsDNA and ensuring integration (of the transfer gene) into the host cell

genome. In addition, in a lentiviral *gag-pol* construct, the *rev* gene is included. The Rev protein is involved in the export of viral mRNAs from nucleus to cytosol for translation.

3. *The transfer/reporter plasmid.* This is the gene that is stably integrated into the host cell DNA, from where the gene is expressed via various cis-acting transcriptional elements. The transfer plasmid contains a packaging signal upstream of the gene to ensure incorporation of viral RNA containing the gene into the viral core (see Ψ in **Figure 1**) during pseudotype generation.

It should be noted that particles can be produced by transfection of a *gag-pol* plasmid without the envelope glycoprotein and/or

transfer gene. Those lacking the glycoprotein (often termed delta or Δenv or 'bald') will bud but will be non-infectious and are thus routinely used as control viruses in transduction experiments.

In addition to increasing safety by reducing recombination events that can lead to replication-competent virus production, using three plasmids also offers increased flexibility. For example, as individual plasmids can be replaced, this permits easy swapping of retrovirus core, envelope glycoprotein or reporter.

Once the cellular machinery has transcribed and translated the transfected genes, an RNA dimer of the transfer gene (region between the long terminal repeats; LTR on **Figure 1**) is incorporated into the pseudotype via the packaging signal. As the transfer plasmid is the only one engineered to contain a packaging signal, no other nucleic acids are incorporated into the mature pseudotype particle. A domain at the N-terminus of Gag targets the nucleocapsid to the cell plasma membrane, into which the envelope protein(s) has been inserted. The pseudotype particles budding from the cell are encapsulated in the cell membrane, which forms the viral envelope.

Pseudotyped viruses are released into the producer cell culture medium. This supernatant can be titrated onto target cells to measure the concentration of functional particles. These attach to the cells via envelope protein–receptor interaction, followed by membrane fusion and internalisation. The pseudotype genome, bearing the transfer/reporter gene is integrated into the host cell DNA, from where it is expressed. The level of reporter gene expression correlates with the level of transduction by viable particles. As only the transfer gene is present in the pseudotype, no viral proteins are produced in target cells, so further pseudotype production and propagation does not occur. This is a key feature that renders safety in working with pseudotypes compared to working with the wildtype virus. Green fluorescent protein (GFP)-based pseudotypes are readily titrated using fluorescence microscopy or flow cytometry, luciferase pseudotypes by luminometry and beta-galactosidase (β -gal) pseudotypes by colour reaction (detailed below). The titres obtained are highly dependent on the cell type chosen for the assay as receptor density may differ between cell types. As an example, transduction of influenza haemagglutinin (HA)-based retroviral pseudotypes depends on sialic acid receptor type (α -2,3 sialic acid receptors are predominantly used by avian influenza strains and α -2,6 sialic acid receptors by mammalian strains) and density [different Madin–Darby canine kidney (MDCK) cell lines have different ratios of 2,3 and 2,6 receptors]. Also important is the density of HA on the surface of the pseudotype particle that may differ from strain to strain (Tao *et al.*, 2012). Other methods for standardisation of retroviral pseudotypes include qRT-PCR to determine the number of genome copies, ELISA measurement of retroviral core p24, Gag or envelope glycoprotein, RT activity assays to semiquantitate virus titre and Nanoparticle Tracking Analysis to ascertain viral particle numbers.

Once successfully generated, pseudotypes can be exploited as tools to study both basic virology and for translational studies in serology and the development and assessment of novel antiviral therapies. A number of such applications are detailed in the following sections (see also **Table 1**).

Analysis of Receptor Binding and Virus Entry and Exit

Retroviral and lentiviral vectors pseudotyped with foreign envelopes have many advantages over the wildtype virus when used as tools to study viral entry. First and foremost, the use of retroviral pseudotypes can have major safety advantages when studying cellular attachment and entry of high-containment viral pathogens such as Ebola, Severe Acute and Middle East Respiratory Syndrome-coronaviruses (SARS-CoV, MERS-CoV), lyssaviruses (e.g. rabies) and highly pathogenic (avian) influenza viruses (H5N1, H7N1 and H7N9) (see **Table 1**). Pseudotyped particles can also be safely and easily modified; nucleotide substitutions, deletions and insertions in envelope glycoprotein genes can be introduced (by site-directed mutagenesis) and changes subsequently evaluated. Functional studies of the envelope proteins in a foreign context allow the isolation of the viral entry process from the other steps in viral replication. It also enables the study of individual envelope proteins to be studied (e.g. influenza virus HA or rabies virus G) or in combination with others (influenza neuraminidase (NA) and its cognate HA).

Retroviral pseudotypes can also be used for the identification of virus–cell receptor targets and for the detailed study of receptor binding (see **Table 1**). The first step in the identification of a viral receptor using this technology involves the construction of a retroviral pseudotype as described earlier, where the transfer gene is a selectable marker (e.g. neomycin/G418). The second step is to identify two cell lines; a cell line that is refractory to entry by this pseudotyped virus and another cell line that is susceptible. A complementary deoxyribonucleic acid (cDNA) expression library is constructed from the susceptible cells and transfected into the refractory cells. Any resulting cells that display the receptor protein(s) correctly in the plasma membrane will now be permissive to transduction by the pseudotype and can be selected for via the marker gene. The cDNAs within these populations can subsequently be sequenced to determine which genes are present. The SARS-CoV receptor was identified as the angiotensin-converting enzyme 2 (ACE2) using a modification of this expression cloning technique (Wang *et al.*, 2004). **See also: Virus Host Cell Receptors; Severe Acute Respiratory Syndrome (SARS)**

Retroviral pseudotypes have also been used to study specific aspects of viral binding and entry (**Table 1**). For example, a SARS pseudotype was utilised to investigate tropism in a range of primate cells, and the pH-dependence of entry mediated via S glycoprotein–endosome fusion (Simmons *et al.*, 2004).

In addition to virus entry studies, retroviral pseudotypes have been widely used to study innate antiviral responses such as the post-entry restriction factors, TRIM5 α (tripartite motif 5 α) (Bae and Jung, 2014) and apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) (Bae and Jung, 2014) that have been shown to be potent antiviral proteins. In these cases, retroviral function is studied by taking advantage of a foreign envelope, VSV-G, which has a wider host range and cell tropism than those of parental retroviral envelopes. Recently, retroviral pseudotypes bearing envelope glycoproteins from lyssaviruses and influenza viruses have been used to study the activity of interferon-inducible transmembrane protein 3 (IFITM3), an

effector protein of the innate immune system, in many animal species including chickens (Smith *et al.*, 2013), bats and pigs (Benfield *et al.*, 2015).

In addition, by incorporating the *gag-pol* genes from HIV-infected patients into the pseudotype system, resistance to antiretroviral drugs such as protease and integrase inhibitors can be measured (Parry *et al.*, 2009). Retroviral pseudotypes have also been used to study Tetherin, a cellular protein that inhibits the release of HIV by anchoring the virus to the cell (Bae and Jung, 2014). **See also: Retroviral Replication; HIV Life Cycle and Inherited Co-Receptors**

Antiviral Screening

Retroviral and lentiviral pseudotypes are readily exploitable for the development of novel entry and exit assays which can be used for the screening of new antiviral compounds and for the study of antiviral drug resistance (see **Table 1**). Cell-based antiviral assays have been developed for influenza virus that enable the measurement of inhibition of pseudotype virus (PV) entry (for HA inhibitors) (Basu *et al.*, 2014) and PV exit (for NA inhibitors) (Su *et al.*, 2008). In the former study, pseudotypes were used in a high-throughput assay to screen 100 000 unique small molecules for agents that could inhibit the transduction of target cells by these particles, identifying two potent novel compounds. Subsequent characterisation revealed that they both bound to the HA stem, in a non-overlapping manner, to inhibit endosomal fusion. They also showed synergy with the currently licensed neuraminidase inhibitor (NAI), oseltamivir. Owing to the relative sequence conservation in the HA stem (compared to the head), the possibility exists that such compounds may inhibit many strains and subtypes of influenza, for example, oseltamivir-resistant H1N1 isolates.

For the influenza exit assay, the requirement of NA for the release of HA-pseudotyped particles from the surface of producer cells was exploited for the development of an assay to evaluate NAI activities by measuring the inhibition of PV production at different NAI concentrations. This assay was used to determine the IC₅₀ values of oseltamivir and zanamivir against N1 group NAs and oseltamivir-resistant mutants. IC₅₀ values obtained correlated strongly with those determined using the MUNANA ‘gold standard’ assay (Su *et al.*, 2008). Similar assays have been developed for HIV, rabies, Lassa and Marburg viruses (Saeed *et al.*, 2006; Wang *et al.*, 2014). New antiviral drugs are being developed that target the proteases that cleave the influenza HA (namely HAT and TMPRSS2), preventing its activation and subsequent virus infectivity. Influenza pseudotypes that necessitate co-transfection of such protease genes in order to effect transduction ability (those with a single arginine at the cleavage site) could play a significant role in the evaluation of such drugs (Ferrara *et al.*, 2012).

Given the unexpected occurrence of some virus outbreaks and the frequent lack of licensed vaccines or therapeutics, rapid screening of existing approved drug libraries is becoming more common place. Pseudotypes have been employed in small molecule screening in order to determine efficacy against the

new pathogen. For example, shortly before the recent unprecedented Ebola virus outbreak in West Africa, one group used lentiviral pseudotypes to screen >1000 FDA approved compounds for antiviral activity (Madrid *et al.*, 2013). Pseudotypes have additionally been used for high-throughput screening of (llama-derived) antibodies, to assess neutralising potency against influenza (Hultberg *et al.*, 2011) and HIV (McCoy *et al.*, 2012).

See also: Antiviral Drugs

Pseudotype Neutralisation Assays and Immunogenicity Testing

Virus-neutralising antibodies do not exclusively bind to proteins expressed on the surface of virus particles, but inhibit the process of virus multiplication. Studies on influenza have shown that neutralising antibodies can interfere with virus infectivity at a number of stages in the virus life cycle, both extracellular and intracellular (Brandenburg *et al.*, 2013). Unfortunately, many standard serological assays measure only antibody binding (HI and ELISA), rather than the inhibition of viral infectivity. As a result, neutralisation assays have been developed to allow for sensitive detection of functional antibody responses. For high-containment viruses (such as SARS-CoV, influenza H5N1, rabies and Ebola), however, these assays are not widely applicable owing to the requirement for high biosafety laboratory facilities and specially trained personnel. Using retroviral and lentiviral particles pseudotyped with the envelopes of such pathogens as ‘surrogate viruses’ for use in neutralisation assays is one way of circumventing this issue. Using a pseudotype strategy, only the envelope protein(s) of the virus is required, with no possibility of recombination or native virus escape. These pseudotypes undergo abortive replication and are unable to give rise to replication-competent progeny.

See also: Antibody Function

Safety considerations aside, pseudotypes are excellent serological reagents for virus neutralisation assays as the virions can contain a reporter gene and bear heterologous viral envelope proteins on the surface. The transfer of these reporter genes to target cells depends on the function of the viral envelope protein; therefore, the titre of neutralising antibodies against the envelope can be measured by a reduction in reporter gene transfer and expression. PV neutralisation assays have now been developed for a wide range of RNA viruses, from numerous virus families (**Table 1**). For HIV, Ross River virus, hepatitis C, SARS-CoV, influenza and rabies virus, these assays have been shown to be very sensitive and specific, and the neutralising antibody titres obtained correlate strongly with the respective wildtype virus assays (Kim *et al.*, 2001; Bartosch *et al.*, 2003a; Temperton *et al.*, 2005, 2007; Wang *et al.*, 2008; Wright *et al.*, 2009; Scott *et al.*, 2012; Molesti *et al.*, 2013, 2014; Yang *et al.*, 2014). These assays are also readily adaptable to a high-throughput format for use in large sero-surveillance studies and vaccine immunogenicity evaluation. For this purpose, an automated pseudotype production facility has been described, which has the capacity to produce 1000 mL of virus per week under Good Clinical Laboratory Practice conditions (Schultz *et al.*, 2012). **See also: Animal Viruses Pathogenic for Humans; Virus Discovery in the 21st Century**

Pseudotype-based influenza neutralisation assays have been shown to be highly efficient for the measurement of broadly-neutralising antibodies making them ideal serological tools for the study of cross-reactive responses against multiple subtypes with pandemic potential (Corti *et al.*, 2011). Antibodies directed specifically against the HA stalk have been shown to confer protection against multiple HA subtypes and form the basis of new strategies for universal vaccine development (Krammer *et al.*, 2015). The production of PVs with chimeric HAs made from mismatched globular heads and stalks allows the readout of antibody responses directed specifically against either HA1 or HA2 making them instrumental for immunogenicity testing of universal vaccines. These assays will also be crucial for the study of homo- and hetero-subtypic responses within an individual, or at the population level.

In order for these pseudotype assays to have wide applicability within laboratories with different resource levels, the availability of multiple reporter systems is highly desirable. The HIV GFP reporter plasmid pCSGW described for use in viral neutralisation assays (Temperton *et al.*, 2005, 2007; Wright *et al.*, 2008) has been modified to express alternative reporters. These include firefly luciferase (Wright *et al.*, 2008), renilla luciferase (Alberini *et al.*, 2009) and β -gal (Wright *et al.*, 2009). Of these, the luciferase reporter-based assays are the easiest to use in terms of user interaction and downstream data analysis. However, owing to the cost of the substrate reagents and necessity for specialised equipment (i.e. a luminometer), these assays may have reduced applicability for laboratories in resource-limited regions. GFP-based assays do not require any extra reagents but do necessitate specialised equipment (fluorescent microscope, 96-well plate reader/flow cytometry facility). The β -gal-based assays are most cost effective as β -galactoside reagents are readily available at low cost and specialised equipment is unnecessary (just a light microscope). The expression of β -gal can be quantified by counting infected cells following staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). However, there are two colorimetric substrates, ONPG (*o*-nitrophenyl- β -D-galactopyranoside) and CPRG (chlorophenol red- β -D-galactopyranoside) that allow the assays to be evaluated using a microplate reader. Recent reports describe multiplex assays for the study of neutralising antibodies that are directed against two viruses using pseudotypes carrying different luciferase reporters, firefly and renilla. Lyssavirus, influenza and coronavirus pseudotypes have been exploited for the development of such assays (Wright *et al.*, 2010; Molesti *et al.*, 2014; Zhou *et al.*, 2011). These permit the measurement of neutralising antibody responses against two antigenically distinct glycoproteins in the same serum/plasma sample thus increasing the amount and quality of serological data that can be acquired from valuable sera.

The pseudotypes constructed from pathogenic viruses of global health importance lend themselves to the establishment (pre-outbreak) of a pseudotype resource library consisting of virus envelope glycoprotein expression vectors and their cognate retroviral pseudotypes. In order to prioritise the strains to include in a library, the following could be utilised: data mining of WHO/OIE/ProMed news feeds, conference abstracts, social networks, peer-reviewed literature (human/animal

outbreaks/surveillance data/pre-clinical vaccine studies), bioinformatics/phylogenetic analysis and new antigenic cartography tools (Fouchier and Smith, 2010). This information can subsequently inform custom gene synthesis of surface glycoproteins from candidate viruses and subcloning into expression vectors. Once nucleic acid sequence and expression has been confirmed, retroviral vector systems can be used for pseudotyping as detailed above. A comprehensive retroviral pseudotype resource would lend itself to optimisation of pseudotype neutralisation assay protocols and standardisation of PV stocks depending on end-user requirements and laboratory facilities and to the training of specialist users. While there may be a degree of amino acid mismatch, a comprehensive pseudotype library would permit a surrogate cross-reactive strain to be employed as a preliminary measure until the outbreak strain had been sequenced and deposited in the NCBI sequence database to allow for synthetic gene synthesis. For the shipping and deployment of pseudotype neutralisation assays in resource-limited regions, their encapsulation into kit form is desirable. An experimental system containing lyophilised viruses has recently been described for influenza, Marburg and rabies (Mather *et al.*, 2014).

In summary, there are major advantages to using pseudotypes for the study of humoral immune responses to viral infection:

1. The assays are serum-sparing: typically 2–10 μ L of serum is required for each replicate, 5–25 fold less than for wildtype virus assays.
2. No virus culture is necessary as the serological antigen is entirely synthetic, derived by custom synthesis or PCR.
3. There is a wide choice of available reporters depending on end-user requirement.
4. Pseudotypes are easily amenable to high-throughput platforms.
5. Using pseudotypes as a surrogate for wildtype virus removes the need to handle dangerous pathogens in high biocontainment facilities.
6. As the pseudotyped glycoprotein is the only viral antigen routinely incorporated into the pseudotype particle, what is measured by this assay is antibody solely directed against this protein. This assay can thus be used as a tool for dissecting out (or delineating) responses observed using wildtype virus neutralisation assays in parallel.
7. Multiple studies have demonstrated high sensitivity, specificity and strong correlation of antibody titres when neutralisation assays using pseudotypes are compared with those employing wildtype viruses.

However, there are also potential disadvantages in using pseudotypes over wildtype viruses in neutralisation assays:

1. The density of envelope protein on the pseudotype surface is unknown and may not be analogous to that found on the respective wildtype virus. If the envelope protein density is significantly lower on the pseudotype, it may be more readily neutralised by antibodies. However, as there is a high correlation of neutralising antibody titres among the assays using wildtype virus or pseudotypes, this suggests that this may not be an issue when using polyclonal sera.

For influenza, however, this issue paradoxically presents a significant advantage as it has been demonstrated that pseudotypes are more sensitive than wildtype viruses at detecting broadly neutralising antibodies that bind to the stalk region of HA (Rappuoli and Dormitzer, 2012). Pseudotypes are thus playing a central role in the development of universal vaccines/antiviral drugs.

2. Some viruses bud internally so that their envelopes are retained on the endoplasmic reticulum (e.g. flaviviruses). For this reason, these viruses may produce low viral titres when their envelopes are used in the pseudotype system. This can be rectified in some cases by concentrating the virus via centrifugation, or via the use of “leaky” overexpression which effects expression of the glycoprotein at the plasma membrane (Simmons *et al.*, 2004; Perera *et al.*, 2013).
3. The ability of several innate host restriction factors, such as the TRIM and IFITM family of proteins, has been well documented to inhibit lentiviral or retroviral replication. This raises the possibility that cell lines routinely used for assays with wildtype virus will be refractory for infection using pseudotypes. The effect of this altered tropism on neutralisation assay titres is as yet unknown.

Pseudotypes as Immunogens and for Gene Therapy

Retroviral pseudotypes have recently been used as a source of antigen in vaccines. Retroviral vectors carrying the surface proteins of influenza (H5N1 and H7N1) and HIV-1 have been shown to function as immunogens resulting in the induction of humoral (influenza and HIV) and cellular responses (HIV) in animal models (Szécsi *et al.*, 2006; Breckpot *et al.*, 2010). For the influenza vaccines, retroviral virus-like particles were made which incorporated HA, NA and M2 from H5N1 and H7N1 subtypes. These particles induced rapid, subtype-specific neutralising antibody production in mice.

For the HIV-1 vaccine, a novel MLV/HIV-1 pseudotype vector was constructed and shown to induce HIV-1-specific humoral (HIV-1 Env-specific antibodies) and cellular immune responses [Rev and Env-specific cytotoxic T-lymphocyte (CTL)-response] in rhesus macaques (Szécsi *et al.*, 2006).

These novel vaccine immunogens can be prepared rapidly upon availability of viral RNA and could potentially be used as animal vaccines (influenza; pigs and domestic poultry), or for the generation of panels of neutralising antibodies targeted against newly emerging viral strains (influenza and HIV).

The use of retroviral pseudotypes for gene delivery has already had major impact on both experimental and clinical gene therapies. They can also be used to study the effect of introducing foreign proteins into a cell, as pseudotype transduction results in the long-term stable expression of the integrated gene. The foreign gene may be a reporter gene (GFP, luciferase or lacZ), as discussed earlier, a therapeutic gene (as is the case for gene therapy applications) or indeed any gene that is required to be stably expressed in a given cell line/type. To achieve efficient and sustained expression of foreign genes delivered by retroviral vectors,

there are several options for the design of the plasmid. The foreign gene may be directly expressed from the retroviral promoter in the construct's 5'-LTR or from a heterologous promoter downstream of the 5'-LTR, such as from the CMV immediate early or human β -actin genes. If expression of two genes is required, then a spliced message, an internal ribosome entry site (IRES), or even a dual promoter vector may be used. Lastly, cell-specific promoters can be used to regulate gene expression and alternative envelopes can be used which allow targeting of specific cells (Bischof and Cornetta, 2010). **See also: Lentiviral Vectors in Gene Therapy; Retroviral Vectors in Gene Therapy; Retroviruses in Human Gene Therapy**

Notes

‘Plasmid’ and ‘construct’ are used interchangeably throughout this article.

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Further Reading

Centers for Disease Control and Prevention (2015) *Diseases and Conditions*. <http://www.cdc.gov/DiseasesConditions/>