

Chapter 8

Pseudotyped Viruses for Influenza



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Abstract We have developed an influenza hemagglutinin (HA) pseudotype (PV) library encompassing all influenza A (IAV) subtypes from HA1-HA18, influenza B (IBV) subtypes (both lineages), representative influenza C (ICV), and influenza D (IDV) viruses. These influenza HA (or hemagglutinin-esterase fusion (HEF) for ICV and IDV) pseudotypes have been used in a pseudotype microneutralization assay (pMN), an optimized luciferase reporter assay, that is highly sensitive and specific for detecting neutralizing antibodies against influenza viruses. This has been an invaluable tool in detecting the humoral immune response against specific hemagglutinin or hemagglutinin-esterase fusion proteins for IAV to IDV in serum samples and for screening antibodies for their neutralizing abilities. Additionally, we have also produced influenza neuraminidase (NA) pseudotypes for IAV N1-N9 subtypes and IBV lineages. We have utilized these NA-PV as surrogate antigens in in vitro assays to assess vaccine immunogenicity. These NA PV have been employed as the source of neuraminidase enzyme activity in a pseudotype enzyme-linked lectin assay (pELLA) that is able to measure neuraminidase inhibition (NI) titers of reference antisera, monoclonal antibodies, and postvaccination sera. Here we show the production of influenza HA, HEF, and NA PV and their employment as substitutes for wild-type viruses in influenza serological and neutralization assays. We also introduce AutoPlate, an easily accessible web app that can analyze data from pMN and pELLA quickly and efficiently, plotting inhibition curves and calculating half-maximal concentration (IC_{50}) neutralizing antibody titers. These serological techniques coupled with user-friendly analysis tools are faster, safer, inexpensive alternatives to classical influenza assays while also offering the reliability and reproducibility to advance influenza research and make it more accessible to laboratories around the world.

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Abbreviations

HA	Hemagglutinin
HEF	Hemagglutinin-esterase fusion
HPAI	Highly pathogenic avian influenza
HRP	Horseradish peroxidase
IAV	Influenza A virus
IBV	Influenza B virus
IC ₅₀	Half-maximal inhibitory concentration
ICV	Influenza C virus
ID ₅₀	Half-maximal inhibitory dilution
IDV	Influenza D virus
IIV	Inactivated influenza vaccine
LAIV	Live attenuated influenza vaccine
LPA	Lectin peanut agglutinin
LPAI	Low pathogenic avian influenza
LV	Lentiviral vectors
NA	Neuraminidase
NI	Neuraminidase inhibition
OD	Optical density
pELLA	Pseudotype enzyme-linked lectin assay
pMN	Pseudotype microneutralization assay
PV	Pseudotype virus
RIV	Recombinant influenza vaccine
SA	Sialic acid

8.1 Introduction

Influenza viruses belong to the *Orthomyxoviridae* family and have segmented, negative sense, single-stranded, enveloped RNA as genetic material [1, 2]. Within this family, there are three influenza types that circulate in humans, influenza A, B, and C [3, 4]. Influenza D, on the other hand, mainly affects cattle and swine with the latter being a preferred reservoir [5]. Influenza A virus (IAV) is classified into subtypes, while influenza B virus (IBV) is divided into lineages based on host range and different combinations of glycoproteins on their surface, most notably HA and NA [6]. IAV and IBV are ever-present threats, causing seasonal epidemics worldwide. Influenza seasons in both the northern and southern hemispheres cause morbidity and mortality to afflicted populations as well as considerable burden to economies [7–9].

The IAV and IBV lipid membrane surfaces are dominated by influenza's two major membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [6, 10, 11] (Fig. 8.1). The surface glycoprotein HA is responsible for viral

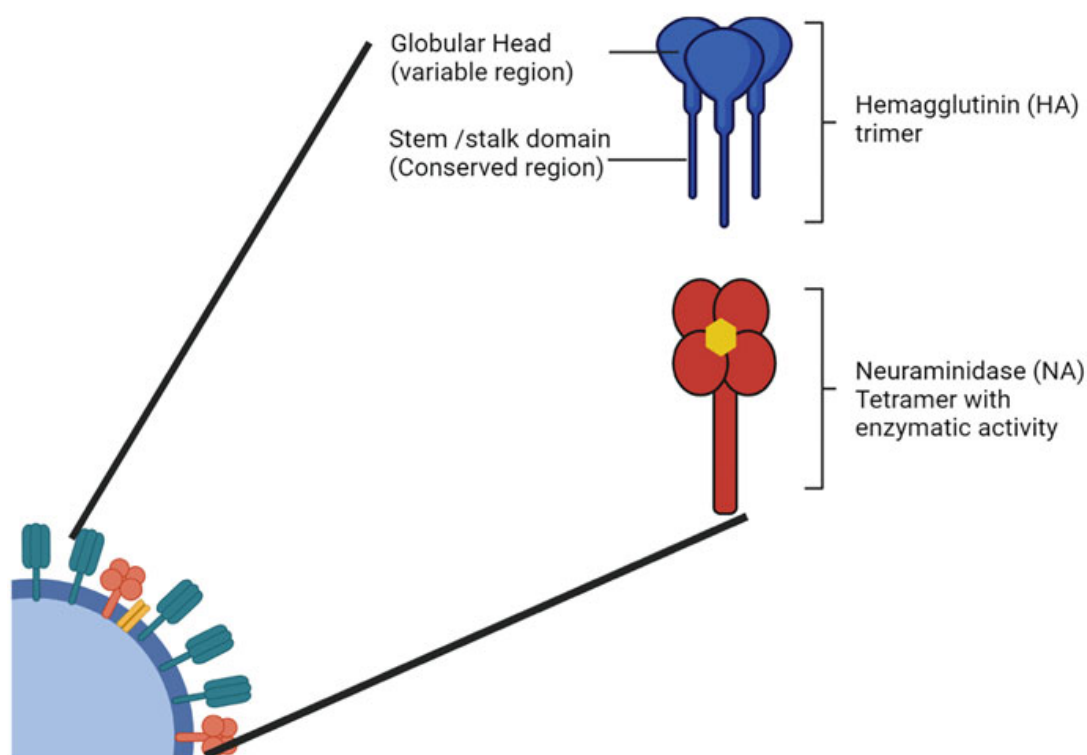


Fig. 8.1 The hemagglutinin (HA) and neuraminidase (NA) glycoproteins present on the surface of influenza A (IAV) and B (IBV) viruses. These proteins are responsible for viral entry and egress, respectively, and are the focus of studies to ascertain mechanisms of neutralization and inhibition and assess antiviral capacity of anti-influenza antibodies and drugs. (Image created using Biorender)

cell attachment and entry into host cells via sialic acid receptors [6, 10], while NA is known for its enzymatic activity that enables the release of virus progeny during the later stages of infection [11–13]. Alternatively, influenza C virus (ICV) and influenza D virus (IDV) have a single surface glycoprotein called hemagglutinin-esterase fusion (HEF) replacing HA and NA found in IAV and IBV [14]. ICV and IDV also express the enzyme esterase that has a similar enzymatic function to neuraminidase.

Influenza A viruses (IAV) are known to cause the most severe and common infections in its hosts compared to IBV and ICV as it is able to infect a wide host range from humans to other mammals, in addition to its known reservoir, the avian species [15, 16]. Influenza A viruses can be classified into combinations of 18 HA and 11 NA subtypes. These subtypes are clustered into two phylogenetic groups: Group 1 comprising H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18, and Group 2 comprising H3, H4, H7, H10, H14, and H15 [17]. IAV zoonoses from wild birds to domestic poultry have been identified as the leading cause of its spread into mammals, specifically swine and humans [18]. These avian zoonotic viruses are not readily transmitted from their natural avian reservoir to humans, but nonetheless this has still occurred in the past, with a virus undergoing antigenic drift or, worse, antigenic shift with possible acquisition of new genes from influenza viruses from other species leading to the emergence of a novel virus that can start a pandemic [18–20]. While there are many genetically distinct subtypes from recombination of HA

and NA in IAV, only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes have caused human pandemics that have been recorded in history [18, 20, 21].

In recent years, the spread of highly pathogenic avian influenza (HPAI) viruses such as H5 and H7 in poultry and wild bird populations has led to devastating effects with flocks being culled and, in the case of human spillovers, death [22–25]. HPAI viruses that are known to have the requisite polybasic insertional mutation at the HA cleavage site making them highly pathogenic in poultry are currently restricted to the H5 and H7 subtypes [26, 27]. Although these HPAI have caused sporadic deaths in humans, none of these strains was easily transmitted from person to person [26, 28–30], and their acquisition of genes from other circulating influenza viruses does not seem apparent [21]. It is known that these viruses require further adaptation in order to adapt to humans and cause widespread transmission [21, 25]. Infection by low pathogenic avian influenza (LPAI) viruses such as H9 or H10 has also been reported, but their effects are not as pronounced as H5 or H7 HPAI [31, 32].

Influenza B virus (IBV), on the other hand, is responsible for around 25% of all seasonal influenza infections and has additionally been shown to infect seals and pigs [33–35]. IBV co-circulates with IAV in humans as two distinct lineages, defined as the B/Yamagata/16/1988-like viruses and B/Victoria/2/1987-like viruses [36]. The two lineages are defined by genetic and antigenic differences of their major surface glycoprotein hemagglutinin (HA). Probably due to low reporting during the COVID pandemic and a host of other factors, only B/Victoria-like lineage infections have been diagnosed in the last 3 years [37].

Vaccination remains the cornerstone of influenza prevention and treatment. Commonly, human flu vaccines are designed to protect against four types of influenza virus that officially designated laboratories around the world have predicted to be in circulation during the upcoming season [33]. There are different types of flu vaccines available, and there are recommendations for use for each one of them. Available influenza vaccines include quadrivalent inactivated influenza vaccine [IIV4], recombinant influenza vaccine [RIV4], or live attenuated influenza vaccine (LAIV4) [33, 38, 39]. No preference is expressed for any influenza vaccine over another, but there are recommendations about who would be better suited for each type of vaccine accounting for factors such as age, location, and prior exposure.

Seasonal influenza vaccines must protect against and restrict virus transmission of H1N1, H3N2, and influenza B viral strains from both Victoria and Yamagata lineages currently circulating in humans globally. These candidate vaccine viruses are identified using painstaking, laborious, and very time-critical methods the season prior. In parallel, discovery of new vaccine antigens that could pave the way for longer protection that would last more than a single season and can cover a broad range of IAV and IBV viruses is now the holy grail of influenza vaccine research [40, 41].

The hemagglutination inhibition assay (HI) and the single radial hemolysis assay (SRH) have been the gold standards for assaying seroprotective titers and amounts of specific antigens (mostly hemagglutinin), respectively, in vaccines. However, these

classical methods have many limitations [42, 43]. As such, innovative methods that can improve on these traditional assays and provide advantages such as ease of production and access, safety, reproducibility, and specificity are in the works. We have developed and fine-tuned an influenza pseudotyping platform technology that utilizes lentiviral vectors (LV) to act as surrogate viruses that can be used to screen vaccination sera, small molecule drugs, and monoclonal and polyclonal antibodies, to name a few, that can target the influenza hemagglutinin head and stem domains and neuraminidase [44–46]. These pseudotype viruses (PV) undergo abortive replication and do not give rise to replication-competent progeny [47–49]. While it is logistically possible to deal with low pathogenic strains of influenza, studies on novel strains and those that are not part of the seasonal influenza repertoire require higher containment facilities as well as highly trained and qualified personnel, hampering discovery and innovation.

Together with the HI assay, virus neutralization assays are standard in influenza research and development laboratories and are employed to detect immune responses against influenza elicited by vaccination or natural exposure in human and animal models. Using influenza PV in place of wild-type viruses, we have developed and optimized a pseudotype neutralization assay (pMN) that can quantify viral neutralization by serum neutralizing antibodies [44, 45, 50]. Unlike the HI which only detects anti-HA head antibodies that bind around the receptor binding site, the pMN can also detect HA stalk directed antibodies, making it more sensitive and versatile [51–56]. The pMN can be applied to many enveloped RNA viruses as it measures how much of the virus is able to transduce cells in the presence of an inhibitor which can be serum, antibodies, small molecules, drugs, or antivirals. In recent years, the pMN has also become crucial to characterizing functional immune responses during the ongoing SARS-CoV-2 pandemic [57, 58], indicating its remarkable applicability to new and emerging viruses, including those on the WHO Blueprint list.

In addition to HA neutralization, assays that measure neuraminidase inhibition are also important in future vaccine approaches. Neuraminidase has been a successful target for antiviral drugs and has been shown to control influenza infection *in vivo* [59–61]. Together with HA, NA is considered a prime candidate to be part of a “universal” or “cross-subtype” influenza vaccine [40, 41, 62]. Currently licensed inactivated influenza vaccines contain NA, but the quality and stability of NA in these preparations have not been fully investigated [63]. Anti-NA antibodies are not considered as classically neutralizing as they do not block infection but instead reduce viral spread [64]. A MUNANA substrate-based assay [65] has been utilized in the past to indirectly measure neuraminidase enzymatic activity; however, this assay employs hazardous chemicals making it unsuitable for day to day testing in research laboratories. Safer and more straightforward alternatives such as the enzyme-linked lectin assay (ELLA) [66–68] and fluorescence-linked MUNANA based assays (e.g., NA-Star/NA Fluor™) have since been developed to quantify NA enzymatic activity as an indirect measure of antibody mediated inhibition of viral egress from infected cells.

Neuraminidase PV have already been successfully used in place of reassortant virus or Triton X-treated wild-type virus in the ELLA assay for N1 and N2 subtypes [67]. Therefore, we have produced an NA-PV library encompassing IAV N1-N11 and IBV from the Victoria-like (B/Vic) and Yamagata-like (B/Yam) lineages for use in a pseudotype ELLA (pELLA) assay. Like their HA PV counterparts, these NA-PV can be effectively employed in pELLA to assess NA inhibition of reference anti-NA antisera, monoclonal antibodies (mAb), and anti-NA antibodies generated through vaccination and/or natural immunity without the requirement for containment above BSL 2.

After the pMN and pELLA assays have been run, the next challenge is to analyze the data and convert them into meaningful results showing neutralization potency and viral inhibition of immune responses. These are usually reported as half-maximal concentration (IC_{50}) in the case of antibodies or half-maximal dilution (ID_{50}) for antisera. This is obtained by measuring neutralization along a dilution series of antibody, serum, or drug in log scale, giving rise to a dose-response curve following a classic S shape which is well represented by the 4-parameter log-logistic regression curve [69]. IC_{50}/ID_{50} is then defined as the concentration/dilution which gives 50% neutralization or inhibition of the curve.

For the pMN, the main output is raw luminescence (or fluorescence) data that can be exported as CSV or Excel files for ease. Values are shown as relative luminescence units (RLU) for each well [45, 50, 51]. For pELLA, it is optical density (OD) collected at 450 nm. These raw outputs have to be copy-pasted from their source files to a chosen statistics or software package where reformatting is required and errors can accumulate. There are proprietary and open-source tools available for these analyses, but most of them require a bioinformatics or coding background to use, while some of the popular software come with expensive licenses.

As an alternative approach to data analysis that we have evaluated and tested with both the pMN and pELLA, we present AutoPlate [70]. AutoPlate employs a user-friendly interface which involves adding experimental metadata to the system with a few clicks bypassing the need for opening individual files and copying blocks of raw data, automatically reformatting the data into the standard pMN and pELLA plate layouts, and performing statistical analysis. AutoPlate produces publication-ready figures and also allows users to export data for further analysis into R or GraphPad prism. AutoPlate is open source and can be accessed through an online Shiny app or installed as an R package that is available at <https://github.com/PhilPalmer/AutoPlate>.

In this book chapter, we highlight the production and use of lentiviral vectors pseudotyped with influenza hemagglutinins in microneutralization assays (pMN) and influenza neuraminidase in enzyme lectin-linked assays (pELLA) as a safe and sensitive alternative to study specific antibody responses elicited by natural influenza infection or vaccination against certain antigens (Fig. 8.2). These versatile tools allow evaluation of broad and cross-subtype neutralizing antibody responses that may inform further preclinical studies involving antigen discovery, vaccination dosing regimens in animal models, and predictive antigen and vaccine designs for evolving viruses and future pandemics.

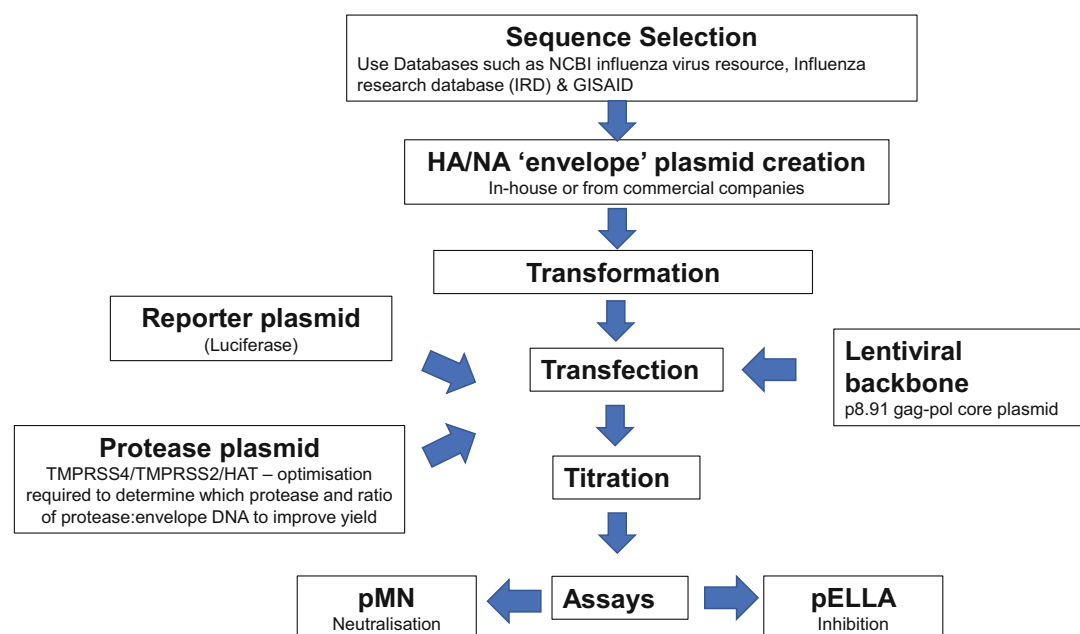


Fig. 8.2 Overall workflow for influenza pseudotype virus (PV) production and downstream assays. PV can be employed to assess anti-influenza neutralization (pMN) and inhibition (pELLA)

8.2 Production of Pseudotyped Viruses and Developing Assays Based on Pseudotyped Viruses

8.2.1 Materials

HEK293T/17 cells (ATCC: CRL-11268^a).

MDCKII cells (ECACC: 00062107): required as target cells for H17/18 PV.

ST cells (ATCC: CRL-1746TM): required as target cells for ICV/IDV HEF PV.

Dulbecco's Modified Essential Medium (DMEM) (PANBiotech P04-04510).

Heat-inactivated fetal bovine serum (PANBiotech P30-8500).

Penicillin-streptomycin (PenStrep) (Sigma Aldrich, Dorset, UK P4333).

Opti-MEMTM (Thermo Fisher Scientific 31985062).

FuGENE® HD Transfection Reagent (ProMega E2312).

Exogenous Neuraminidase (Sigma N2876).

Nunc F96 MicroWell white opaque polystyrene plates (Thermo Fisher Scientific 136101).

VACUSIP benchtop aspiration system (Integra 159010).

Bright-GloTM luciferase assay system (Promega PR-E2650).

Phosphate buffered saline (Sigma D8662).

GloMax® Navigator (ProMega GM2000).

Nunc MaxisorpTM flat-bottom 96-well plates (Thermo Fisher 44-2404-21).

Fetuin (Sigma F3385).

KPL coating buffer (Sera Care 50-84-00).

TWEEN® 20 (Sigma Aldrich P1379).

Bovine serum albumin (Sigma Aldrich A3294).

BRAND® 96-well microplate, U-bottom (Merck BR701330-100EA).

Lectin from *Arachis hypogaea* (peanut) peroxidase conjugate (Sigma L7759).

1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher 34029).

8.2.2 *Protocols for Production of Pseudotyped Viruses and Developing Assays*

8.2.2.1 **Production of Influenza Hemagglutinin (HA) and Neuraminidase (NA) Pseudotypes (PV)**

Day 1

1. Seed 4×10^5 HEK 293 T/17 cells per well of a 6-well plate in 2 mL complete DMEM (10% heat-inactivated FBS, 1% PenStrep).
2. Incubate cells at 37 °C, 5% CO₂ overnight.

Day 2

3. Replace media with fresh 2 mL complete DMEM per well. Incubate plates at 37 °C, 5% CO₂.
4. Prepare transfection mixture as per Table 8.1. Briefly, the following plasmids are needed depending on the desired PV: HA encoding plasmid, NA encoding plasmid, protease-encoding plasmid, luciferase reporter plasmid pCSFLW [71], and p8.91 gag-pol (Gag-Pol expression plasmid [72]). For ICV/IDV, a HEF encoding plasmid is required.

Note 1: For transfection of low pathogenicity avian influenza (LPAI) and other subtypes with a monobasic cleavage site, an additional plasmid expressing

Table 8.1 Amounts of influenza HA and/or HA-NA transfection components needed per well of a 6-well plate

Solutions/plasmids	Amount	Type of PV
OptiMEM	100 µL	HA, HA-NA
p8.91	250 ng	HA, HA-NA
pCSFLW	375 ng	HA, HA-NA
HA (pEVAC)	10 ng (50 ng for HA18)	HA
HA (pI.18/phCMV1)	50–500 ng	HA
NA (pEVAC)	10 ng	HA-NA
NA (pI.18/phCMV1)	50–500 ng	HA-NA
Protease-encoding plasmid	2.5–500 ng	LPAI HA
FuGENE® HD	3 µL per µg total plasmid DNA	HA, HA-NA

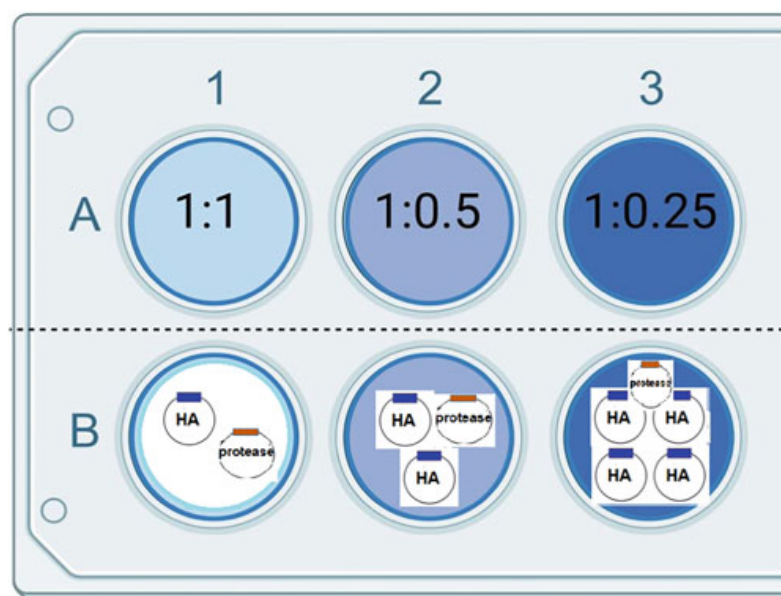


Fig. 8.3 PV yield optimization by changing the ratio of protease plasmid to HA plasmid DNA. PV titers can be increased by transfecting in a “checkerboard” approach with different proteases (e.g., HAT, TMPRSS4, and TMPRSS2). Protease plasmid is added at a ratio of 1:1, 1:0.5, and 1:0.25 to HA plasmid DNA for rapid optimization in a 6-well plate format. (Image created in BioRender)

either type II transmembrane protease serine 2 (TMPRSS2) [73], type IV transmembrane protease serine 4 (TMPRSS4) [74], or human airway trypsin-like protease (HAT) [73] is also needed. Optimization of amount of protease to be added may be required [44] (Fig. 8.3).

Note 2: For NA-PV, we found that co-transfection with 10 ng H11 encoding A/red shoveler/Chile/C14653/2016 (H11) in our plasmid of choice (pEVAC) with 10 ng NA encoding plasmid in pEVAC produces the best results when used in pELLA [46].

5. Combine components from Table 8.1 in OptiMEM as indicated and add FuGENE® HD dropwise followed by incubation for 15 min.
6. Add the plasmid DNA-OptiMEM mixture to the cells with constant swirling.

Note 3: HA and NA are presented (Table 8.1) in different plasmid vectors (i.e., pEVAC, pI.18, pHCMV1) to demonstrate that the choice of plasmid will impact the amount of DNA required for transfection to obtain optimal PV production titers.

Day 3

7. At least 8 h post-transfection, add 1 unit of exogenous bacterial neuraminidase per well for HA PV only (with the exception of the H18 subtype which requires an N11 plasmid). There is no need to add this to HA-NA-PV transfection mixtures.

Day 4

8. Collect supernatants 48 h post-transfection by passing through a 0.45 μm cellulose acetate filter. Store PV viruses at $-80\text{ }^{\circ}\text{C}$.

8.2.2.2 Titration of Influenza Hemagglutinin (HA) PV

1. Perform titration experiments in Nunc F96 MicroWell white opaque polystyrene plates.
2. Add 100 μL of the HA PV to Row A of a 96-well plate. This should ideally be done in duplicate; e.g., Virus 1 will be in wells A1 and A2, Virus 2 in wells A3 and A4, etc. Leave wells A11 and A12 blank (Fig. 8.4).
3. Add 50 μL complete DMEM to all wells in the plate (Row B-H) including Columns 11 and 12.
4. Serially dilute HA PV twofold across the plate by transferring 50 μL of the HA PV from Row A to Row B, until Row H. Discard the last 50 μL .
5. Add 100 μL 1×10^4 HEK 293 T cells to each well (for H17/H18, use MDCKII, and for ICV/IDV, use ST cells).
6. Incubate plates at $37\text{ }^{\circ}\text{C}$, 5% CO_2 for 48 h.
7. After 48 h, discard liquid on the plate using VACUSIP.
8. Thaw Bright-Glo[®] luciferase assay substrate and dilute 1:1 with PBS.
9. Add diluted 25 μL Bright-Glo[®] to each well. Incubate plates for 5 min at room temperature.
10. Read plates on the GloMax[®] Navigator (Promega) using the Promega GloMax[®] Luminescence Quick-Read protocol.
11. Determine viral pseudotype titer in relative luminescence units/mL (RLU/mL).

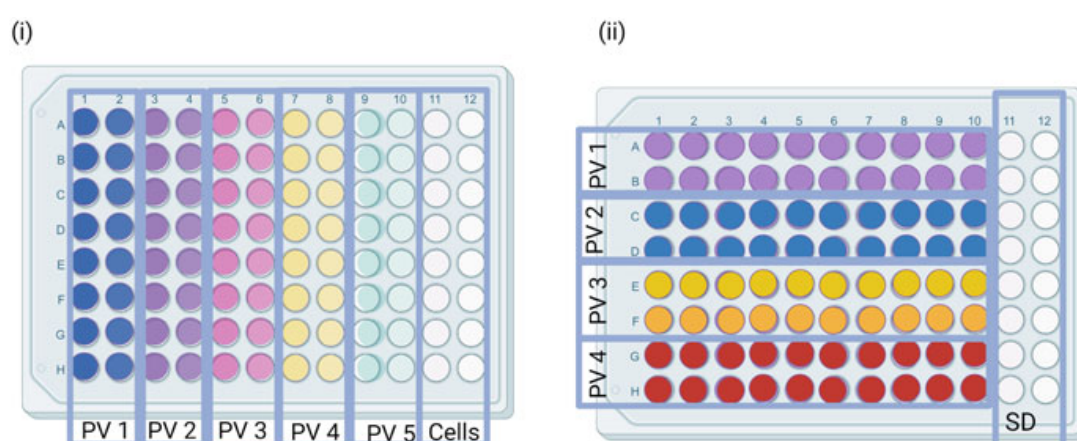


Fig. 8.4 Plate layout for titration of HA and NA-PV. (i) HA PV titration. It is recommended to titrate a maximum of 5 HA PV per plate with inclusion of cell-only controls. (ii) NA-PV titration. It is recommended to titrate a maximum of 4 NA-PV per plate with “blank” sample diluent (SD) only controls

Note 4: Luminescence output from the GloMax® Navigator is in RLU; therefore, dilution factors must be applied to raw data to calculate for RLU/mL. For example, for 50 μ L of pseudotype virus added, conversion to RLU/mL requires multiplication of the raw values with 20; the next values from the twofold dilution require multiplication by 40, the next by 80, and so on.

8.2.2.3 Titration of H11_Neuraminidase (H11_NA) PV via Enzyme Linked Lectin Assay (pELLA)

1. Coat clear Nunc Maxisorp™ flat-bottom 96-well plates with 100 μ L per well of 25 μ g/mL fetuin in 1X KPL coating buffer (diluted from 10X stock). Incubate overnight at 4 °C.
2. The next day, wash plates three times with wash buffer (WB) (0.5% (v/v) Tween-20 in PBS).
3. Add 240 μ L of H11_NA(X) (X indicating any N1-N9) PV to well A1 of a U-bottom 96-well mixing plate. Subsequent virus should be added to well B1, then to C1, and so forth.
4. Add 120 μ L sample diluent (SD) (1% (w/v) bovine serum albumin (BSA), 0.5% (v/v) Tween-20 in PBS) to all other wells of the mixing plate.
5. Serially dilute H11_NA(X) PV twofold by transferring 120 μ L of PV from well A1 to well A2, then from well A2 to well A3, and so forth, across the plate until Column 10. Do this for all other rows containing PV (Fig. 8.4).
6. Discard the last wash from the fetuin plate. Transfer 50 μ L of the PV dilutions from the mixing plate to two rows of fetuin plate. Each row of PV from the mixing plate will account for duplicate rows in the fetuin plate.
7. Leave Columns 11 and 12 blank, as these should only contain SD (no PV control).
8. Add 50 μ L SD to all wells. Total volume of liquid in each well should be 100 μ L.
9. Incubate plates overnight at 37 °C, 5% CO₂.
10. The next day, wash plates six times with WB.
11. Add 100 μ L of conjugate (1 μ g/mL lectin from *Arachis hypogaea* (peanut) peroxidase conjugate in conjugate diluent (1% (w/v) BSA in PBS)) to all wells.
12. Incubate plates at room temperature for 2 h with shaking (225 rpm).
13. Wash plates three times with WB.
14. Add 100 μ L 1-Step™ Ultra TMB Substrate and incubate in the dark with shaking (225 rpm) for 10 min.
15. Stop reaction by addition of 100 μ L 0.1 M H₂SO₄ per well.
16. Read optical density at 450 nm (OD₄₅₀) using a microplate reader.
17. Normalize readings to 100% and 0% OD₄₅₀, and the dilution that results in 90% OD₄₅₀ is selected as the PV dilution input for inhibition assays.

8.2.2.4 Pseudotype Microneutralization (pMN) Assay Using HA PV (Fig. 8.5)

1. Dilute samples to starting concentrations. Monoclonal antibody concentrations used are usually in the range of 0.5 ng/mL–1000 ng/mL, and serum and antiserum samples are recommended to be initially diluted 1:20 or 1:50 in 50 μ L complete DMEM.
2. Similar to HA PV titration, 100 μ L of the samples are serially diluted twofold across a Nunc F96 MicroWell white opaque polystyrene plate starting from well A2.
3. Leave Column 1 for controls, with A1 to E1 for HA PV-only controls and F1 to H1 for cell-only controls.
4. Add 50 μ L of PV at a titer of 1.0×10^6 RLU/well as determined via titration to all wells except for F1-H1.

Note 5: Adding the PV to the mAb or serum dilutions will double the final dilution of mAb or sera. For example, if you started with a 1:20 dilution, the final dilution that would be used for computations would be 1:40, to take into account addition of the PV.

5. Incubate plate for 1 h at 37 °C, 5% CO₂.
6. Afterward, add 50 μ L of 1.5×10^4 HEK293T/17 cells to each well (for H17/H18, use MDCKII and for ICV/IDV, use ST cells).
7. Incubate plate for 48 h at 37 °C and 5% CO₂.
8. After 48 h, discard media and add 25 μ L Bright-Glo® luciferase assay substrate to each well.

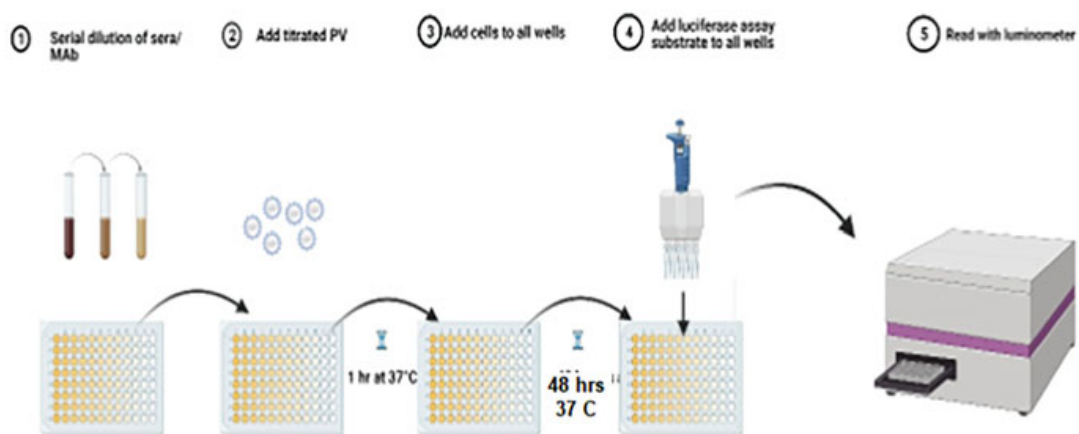


Fig. 8.5 Graphical representation of pMN assay. Sera or mAbs are serially diluted and added to 96-well plate. Titrated PV is diluted and added to all wells, except cell-only controls, and incubated for 1 h before addition of target cells. Plates are incubated for 48 h before addition of luciferase substrate. The final readout of luminescence production is obtained and the IC₅₀ determined. (Image created in BioRender)

9. Read plate using the GloMax® Navigator (ProMega) using the Promega GloMax® Luminescence Quick-Read protocol.
10. Calculate half-maximal inhibitory dilution or concentration (IC_{50}). A detailed analysis is described in Ferrara, 2018 [50].

8.2.2.5 Inhibition of H11_NA(X) PV by Antisera and Monoclonal Antibodies via Enzyme-Linked Lectin Assay (pELLA) (Fig. 8.6)

1. Dilute samples to starting concentrations. Monoclonal antibody concentrations used are usually in the range of 0.5 ng/mL–32 µg/mL, and serum and antiserum samples are recommended to be initially diluted 1:10 or 1:20 in SD.
2. Similar to H11_NA(X) PV titration, 240 µL of the samples are serially diluted twofold across a U-bottom 96-well mixing plate with the highest concentrations in Column 1 and the most diluted in Column 10. Leave Columns 11 and 12. Column 11 will be the PV-only control (0% inhibition), and Column 12 will be the SD only control (100% inhibition).

Note 6: Steps are similar to H11_NA(X) PV titration, but this time instead of PV being titrated, it is the sample (usually mAbs or antisera) that is diluted in the mixing plate and transferred to the fetuin plate.

3. Repeat steps 4–9 as described in Sect. 8.2.2.3.
4. Add 50 µL of the H11_NA(X) PV that resulted in 90% OD_{450} as determined in Sect. 8.2.2.3 to all wells of the fetuin-coated plate except for Column 12 which contains SD only (100% inhibition).
5. Add 50 µL and 100 µL of SD to Columns 11 and 12, respectively.
6. Repeat steps 9–17 as described in Sect. 8.2.2.3.
7. Calculate the IC_{50} as the inverse dilution of serum or antibody concentration that resulted in 50% inhibition of NA activity.

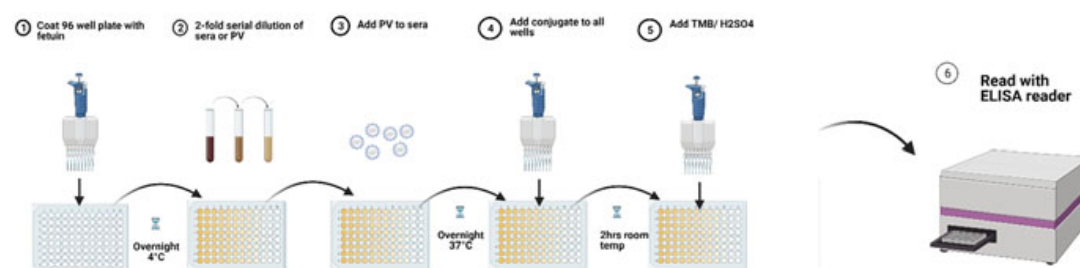


Fig. 8.6 Graphical representation of pELLA assay. Plates are coated with fetuin overnight, and then the sera/titrated PV mixture is added to wells; detection of NA enzymatic activity assessed by breakdown of fetuin to galactose, detected by binding to HRP conjugated peanut lectin agglutinate. Final readout of color change following addition of TMB reported as OD_{450} and the IC_{50} determined. (Image created in BioRender)

8.2.2.6 AutoPlate Analysis

To facilitate high-throughput analysis of pMN and pELLA data, the AutoPlate web app was developed (Fig. 8.7). This enables researchers to produce publication-ready figures quickly. AutoPlate is a user-friendly application software which employs R based technology.

1. Access AutoPlate web app by following the link <https://shinyapps.io/AutoPlate/>.
2. Follow instructions on the quick start guide that can be accessed here: <https://philpalmer.github.io/AutoPlate/index.html#web-application-quick-start>.
3. Detailed step by step protocol can be accessed in Palmer et al., 2022 [70].

8.3 Commentary

8.3.1 Background

The entire process from PV production to neutralization and inhibition assays has been streamlined into a high-throughput system [44–46] which decreases time required from commencing experiments to obtaining data that produce results comparable to wild-type virus assays. Our system can be used at BSL-2, requiring setup in an MSC class II cabinet to retain sterility for the duration of the assay. This negates the need for BSL-3 facilities and trained staff while maximizing flexibility. This platform makes it possible to produce new/emerging viral strains, including those which are classified as BSL-3, i.e., possible pandemic and/or actual pandemic strains, relatively quickly and for IAV, IBV, ICV, and IDV.

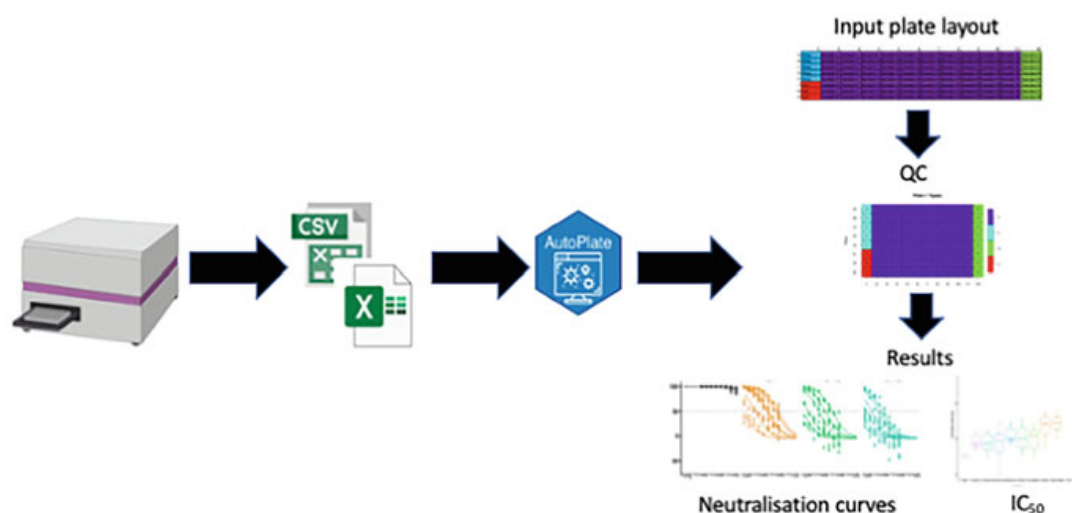


Fig. 8.7 Graphical representation of AutoPlate software workflow. Data is exported from the luminometer as csv files which are annotated and computationally processed within AutoPlate. The output of the software is IC_{50} titers and publication-ready inhibition curves

Transfection is designed to be carried out in a 6-well plate format which allows for optimization that can then be scaled up to tissue culture flasks. The pMN assay described herein can evaluate the neutralizing activity of both polyclonal (sera) and monoclonal anti-influenza antibodies. The system uses readily available cell lines (HEK293T for HA1–16 and IBV, MDCKII for HA17 and HA18 and ST cells for ICV and IDV) which do not require any modifications. Readout is luminescence-based, and the reporter gene is already incorporated into the lentiviral pseudotype produced. This can also be done using FACS analysis, by encoding a fluorescence-based reporter such as GFP. As the whole HA protein is presented in native conformation, antibodies can bind to both the HA head and the stalk as happens in vivo, an approach which is being assessed for novel vaccine and drug design [17, 75, 76]. The pELLA assay described in this chapter evaluates the ability of anti-influenza polyclonal and monoclonal antibodies as well as NA inhibitory drugs to restrict the enzymatic activity of influenza NA and therefore viral egress [46]. The PV used for the pELLA assay display both NA and HA on the surface, and therefore, the effect of steric hindrance of NA via non-neutralizing antibodies which bind to HA stalk exhibits more sensitivity than other methods [77]. As with our pMN assay, results are comparable to traditional assays, e.g., MUNANA substrate-based [46], but without the use of hazardous chemicals or expensive kits, and reassortant virus or Triton X-treated wild-type virus ELLA assays [67]. Additionally, pELLA requires an ELISA plate reader to obtain results, an instrument commonly found in standard laboratories. Both the pMN and pELLA assays utilize a 96-well plate format which can be used to carry out an initial screen of up to 40 samples in duplicate or a serial dilution of five samples per plate with cell-only and PV-only controls and reference antiserum (if required).

8.3.2 Critical Parameters and Troubleshooting

There are several factors which need to be considered to obtain optimal results for pseudotype production and both the pMN and pELLA assays. Firstly, the cells used for transfection and pMN should be kept at a consistent density and passaged three times a week. It is advisable to check the confluence and overall health of the cells before carrying out transfection or pMN and ensure that all reagents are within date and media recipes are followed exactly. Cells should be in a monolayer and at about 70% confluence during transfection. Generally, with experience, operators will be able to determine a maximum number of passages (this would be around passage 30) that will produce consistent results. Accurate counting of cells is critical, and where an automated cell counter is available, the viability of cells can be monitored. Additionally, FBS batch variation should be considered, and where possible, it is best practice to test batch variability on PV titers and purchase a large amount of a favorable lot from manufacturers.

With regard to transfection specifically, the plasmid utilized for the surface glycoprotein (s) “envelope,” i.e., HA/NA plasmid, can influence yield and will affect

the amount of plasmid required for PV production. If different plasmids are used for different influenza subtypes, this should be considered. The selection of protease (to cleave HA) is important as this will affect PV yield. We have previously described the proteases which are optimal for each subtype [44]. It should also be noted that some PV will not require a protease plasmid (i.e., those with HPAI HA). The 6-well plate checkerboard approach enables operators to test combinations of the ratio of envelope (HA and/or NA) plasmid to protease plasmid. Titration of PV for neutralization assays is critical, and operators should aim for a minimum titer of 5×10^7 RLU/mL (with the GloMax) and shouldn't be concerned if this requires several attempts of varying the amount of envelope plasmid and amount and type of protease plasmid. When assessing NA enzymatic activity of PV for inhibition assays, this should be calculated as 90% OD₄₅₀, but we found that the dilution that gives a minimum OD₄₅₀ of 2.5 should be employed for the assay to work consistently. All PV should be stored at -80°C , and where possible, aliquots should not be freeze-thawed repeatedly especially the NA-PV prepared for the pELLA assay as enzymatic activity decreases with each freeze-thaw cycle.

Specifically, regarding the pELLA assay, it is critical that all reagents are stored correctly and all buffer components are measured accurately, adhering to the recipes stated herein and not kept for longer than a week. Plates need to be coated with fetuin at least 18 h before use although plates can be kept for up to 1 month at 4°C . However, it is advisable to check that plates are covered sufficiently with a plate cover or tightly fitted lid and that the fetuin has not evaporated prior to use as this can affect results. This will be evident from a consistently low OD obtained from a column or row on the plate. It is also important, as with ELISA assays, that the plate is not allowed to dry out completely at any step as this will also result in lower OD. It is also essential to carry out all washing steps as stipulated in our protocol as this will limit the effects of nonspecific binding. The conjugate diluent should be prepared fresh on the day of the experiment. If using a plate washer, it is advised to make sure this is cleaned as per manufacturer's guidelines at the end of each day and any waste and remaining buffer are removed.

For both the pMN and pELLA, conditions for the assay such as cell density and health, media used, quality control of reagents, incubation times, and strict adherence to protocol, to name a few, should be consistent to allow comparison between timepoints and to assure assay to assay reproducibility. It is also advised that longitudinal samples should be assessed with PV from the same production batches where possible.

Additionally, when performing both assays, the controls detailed in this chapter should be included on every plate. This serves several functions: firstly, when analyzing data, the results will normalize the conditions on each plate, allowing for comparison between plates processed on the same day or different days. Secondly, these controls can highlight any operative or technical issues, especially if a standard antiserum is included, as the IC₅₀ can be tracked over long periods of time. Additionally, for both assays, we recommend that a negative control serum is included in each study to assess if a background is observed as this can be corrected

in final cutoff calculations. This may be important if using serum from nonhuman species; we have tested and used successfully sera from goat, mice, bat, and ferrets in these assays [44–46, 78]. Where possible, the same luminometer/plate reader should be used for related samples. Instruments should be serviced regularly to ensure they are working optimally. If all these considerations are adhered to and assays are performed systematically, highly robust results are obtained with this versatile high-throughput system.

8.3.3 *Understanding Results*

The final readout of the PV production titer presented herein for HA pseudotypes is relative luminescence units/ml. The pseudotype viruses have been encoded with firefly luciferase in their lentiviral vector genome, and after entry into target cells, the measured expression of luciferase is proportional to the number of cells transduced. For pMN, the levels of luminescence detected in test wells are compared to the signal from untransduced “cell-only” control wells (representing 100% neutralization) and “PV-only” control wells (representing 0% neutralization), where neutralizing antibodies/sera are not present. A lack of or reduction in luminescence is indicative of neutralizing antibody activity. This indirect measurement permits calculation of neutralizing antibody titers which are reported as IC₅₀ dilution. This IC₅₀ can then be compared between groups at single or multiple time points following vaccination, treatment, or infection.

The final readout of the pELLA presented herein is relative optical density at 450 nm (OD₄₅₀). This assay measures the enzymatic activity of NA by assessing the ability of NA to cleave the terminal sialic acid (SA) residue from the fetuin substrate that is coated on test plates. This gives rise to the production of galactose which in turn is recognized specifically by lectin peanut agglutinin (LPA). The LPA used here is conjugated to horseradish peroxidase (HRP) which in the presence of TMB produces a detectable color change which is measured as OD₄₅₀. The effect of antibodies or drugs which inhibit this enzymatic reaction can be indirectly measured following incubation with the NA-PV. The OD₄₅₀ detected in test wells is compared to the signal from sample diluent-only control wells (representing 100% inhibition) and “PV-only” control wells (representing 0% inhibition), where neutralizing antibodies/sera are not present. A lack of change or reduction in OD₄₅₀ is indicative of the presence of inhibiting antibodies or substances. This indirect measurement permits calculation of inhibition titers which are reported as IC₅₀ dilution. As with the pMN assay, this IC₅₀ can then be compared between groups at single or multiple time points following vaccination, treatment, or infection. We suggest that all samples are assessed in duplicate with control wells present on each plate in triplicate.

8.3.4 Time Considerations

The lead time for receipt of HA and NA plasmids varies depending on the manufacturer employed, but usually this is approximately 2 weeks. The transfection process requires the cells to be seeded overnight, and PV can be harvested in 48 h or extended to 72 h. The pMN assay is read 48 h after initial setup, and the pELLA assay takes 24 h to complete as it only requires overnight incubation. It is recommended that a plate washer is used for the pELLA assay, especially when more than five plates are assessed at one time to maximize efficiency.

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