Lyophilisation of influenza, rabies and Marburg lentiviral pseudotype viruses for the development and distribution of a neutralisation-assay based diagnostic kit

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Abstract

Pseudotype viruses (PVs) are chimeric, replication-deficient virions that mimic wild-type virus entry mechanisms and can be safely employed in neutralisation assays, bypassing the need for high biosafety requirements and performing comparably to established serological assays. However, PV supernatant necessitates -80°C long-term storage and cold-chain maintenance during transport, which limits the scope of dissemination and application throughout resource-limited laboratories. We therefore investigated the effects of lyophilisation on influenza, rabies and Marburg PV stability, with a view to developing a pseudotype virus neutralisation assay (PVNA) based kit suitable for affordable global distribution. Infectivity of each PV was calculated after lyophilisation and immediate reconstitution, as well as subsequent to incubation of freeze-dried pellets at varying temperatures, humidities and timepoints. Integrity of glycoprotein structure following treatment was also assessed by employing lyophilised PVs in downstream PVNAs. In the presence of 0.5M sucrose-PBS cryoprotectant, each freeze-dried pseudotype was stably stored for 4 weeks at up to 37°C and could be neutralised to the same potency as unlyophilised PVs when employed in PVNAs. These results confirm the viability of a freeze-dried PVNA-based kit, which could significantly facilitate low-cost serology for a wide portfolio of emerging infectious viruses.

Keywords

Retroviral pseudotype viruses, lyophilisation, neutralising antibodies, serological assays

1 Introduction

The impact of emerging and re-emerging viral diseases on global health is becoming increasingly apparent year on year. Influenza (family *Orthomyxoviridae*, genus *Influenzavirus A*, species *Influenza A virus*) remains one of the viruses most likely to cause high morbidity and mortality in human populations, after significant outbreaks of H5N1 and H7N9 subtypes beginning in 1997 and 2013 respectively, and the low pathogenic but highly transmissible 2009 H1N1 pandemic virus (Yuen et al., 1998; WHO, 2010; Gao et al., 2013). This threat persists with the first human cases of H6N1 and H10N8, and the recent discovery of diverse H17N10 and H18N11 subtypes in bat reservoirs (Tong et al., 2013; Wei et al., 2013; To et al., 2014). Similarly, rabies (family *Rhabdoviridae*, genus *Lyssavirus*, species *Rabies virus*) is a globally ubiquitous virus, present on all continents other than Antarctica, and responsible for over 60,000 deaths per year, primarily of children in resource-limited areas of Asia and Africa (WHO, 2013). Once symptoms occur, rabies has a close to 100% case fatality rate, the highest of any viral infection. Indeed, only a handful of people have survived following development of clinical symptoms and most of those had neurological sequelae (Jackson, 2013). Sporadic outbreaks of Marburg virus (family *Filoviridae*, genus *Marburgvirus*, species *Marburg marburgvirus*) in the Democratic Republic of the Congo in 1999-2000, and then in Angola in 2004-2005 (respective mortality rates of 83% and 90%), as well as small Ugandan outbreaks more recently, serve to remind us that spillover events into human populations from unexpected viral sources can create serious public health concerns (Brauburger et al., 2012). Therefore, options for monitoring the spread and curtailing the outbreak severity of pathogenic viruses are vitally important.

Serological assays that can detect and quantify antibody responses raised against antigenic surface glycoproteins enable the evaluation of potential vaccines and antiviral treatments, as well as sero-surveillance to monitor the epidemiological movements of a virus, thus contributing to international public health initiatives. Serology compliments direct virus isolation or reverse

transcription polymerase chain reaction (RT-PCR) diagnosis, by enabling the identification of an acute viral infection after the temporary viremic stage has passed (Papenburg et al., 2011).

However, conventional serological assays possess drawbacks which detrimentally affect their efficiency. Importantly, most require the use of infectious wild-type virus, necessitating expensive, specialized biosafety level 3 or 4 (BSL-3 or -4) laboratories which are not readily available, especially in resource-limited areas. Hemagglutination inhibition (HI) assays, used routinely for influenza, suffer from variability caused by different erythrocytes and inhibitory factors, as well as low sensitivity. ELISA-based assays do not require the use of wild-type virus, but are also hindered by low sensitivity and cross-reactivity between samples. Furthermore, both HI and ELISA cannot differentiate between virus neutralising and non-neutralising antibody responses (Mather et al., 2013). Virus neutralisation assays, such as plaque reduction neutralisation test (PRNT) and fluorescent antibody virus neutralisation (FAVN) assay, can measure virus neutralising antibody (VNAb) responses with high sensitivity and specificity levels but also require high biosafety for assay preparation, and in some cases are time-consuming and suffer from low-throughput (Cliquet et al., 1998; Mather et al., 2013).

A potential solution to these issues is the utilisation of retroviral pseudotype viruses (PVs). PVs are composed of the structural and enzymatic core of one virus combined with heterologous envelope glycoproteins (Temperton and Wright, 2009). Manipulations to the genomic RNA of the lentiviral core create a replication-defective PV that encapsulates a quantifiable reporter gene. Transduction of a permissible target cell line is dependent upon the ability of the envelope glycoprotein to engage its cellular receptor in a process that mimics wild-type virus entry mechanisms. If this is successful, the reporter gene can be integrated into the host cell genome and subsequently expressed. Resultant levels of reporter protein in transduced cells can be measured, giving a readout equivalent to viral titre. Pseudotype virus neutralisation assays (PVNAs) attain

93 comparable, if not higher, sensitivity and specificity results than many traditional serological assays
94 (Desvaux et al., 2012).

95 In order to maximise the utility of the pseudotype assay system, multiplexing of PVNAs has
96 been demonstrated which permits simultaneous quantification of VNAb responses against several
97 PVs (each harbouring a different reporter gene i.e. renilla and firefly luciferase, or GFP and RFP) in
98 the same assay, sparing valuable reagents such as serum samples (Wright et al., 2010). The flexibility
99 of reporter genes that can be incorporated into PVs further customises the assay. Luciferase and GFP
100 reporters enable highly quantitative readouts but require expensive reagents and/or equipment.
101 However, infection by PVs that encapsulate *lacZ* (expressing β-galactosidase) or secreted alkaline
102 phosphatase (SEAP) reporter genes can be quantified by adding colorimetric substrates such as
103 ONPG, CPRG or p-nitrophenyl phosphate and measuring color change with an ELISA plate reader or
104 by eye (Wright et al., 2009; Kaku et al., 2012).

105 Multiplexing, as well as selecting 'low-cost' reporter genes, considerably reduces the cost-
106 per-assay burden of the pseudotype platform. However, the high expenses associated in optimal
107 transportation and storage can be an inhibitory obstacle in the international distribution of PVNAs.
108 Despite pseudotype studies being conducted on field serum from resource-poor tropical countries,
109 and reports of viruses that circulate in tropical regions being successfully pseudotyped (Wright et al.,
110 2009; Kishishita et al., 2013), there appear to have been no published studies involving the carrying
111 out of pseudotype neutralisation assays in tropical countries, especially in rudimentary laboratories
112 without air-conditioning or access to reliable freezer units.

113 The aim of this study was to ascertain the viability of lyophilising pseudotype viruses with a
114 view to developing a PVNA-based kit. Pseudotype stability was monitored after subjection to
115 environmental conditions likely experienced in the production, transit and usage of such a kit,
116 especially to tropical countries. PV titres were also assessed subsequent to lyophilisation and

117 immediate reconstitution, as well as incubating freeze-dried pellets at a variety of temperatures and
118 humidities before reconstitution.

119 2 Materials and Methods

120 *2.1 Viruses and cells*: The virus isolates pseudotyped in this study were influenza
121 A/H5N1/Vietnam/1194/2004 strain (Genbank accession number ABP51976), rabies virus (RABV)
122 strain Evelyn Rokitniki Abseleth (ERA; UniProtKB/Swiss-Prot code ABN11294) and the Lake Victoria
123 strain of Marburg virus (MARV; Genbank accession number DQ447649). Previously, the influenza HA
124 gene and RABV G gene of these isolates were both sub-cloned into the pI.18 expression vector (Cox
125 et al., 2002). The Marburg GP gene within the pCAGGS expression vector was a kind gift from
126 Graham Simmons (Blood Systems Research Institute, San Francisco, CA, USA).

127 Human embryonic kidney 293T clone 17 (HEK293T/17; ATCC CRL-11268) (Pear et al., 1993)
128 cells were used for all transfections and as a target cell line for titration and neutralisation assays
129 involving H5 pseudotype virus. Baby hamster kidney 21 cells (BHK-21; ATCC CRL-10) (Stoker and
130 MacPherson, 1964) were used as a target cell line for RABV and MARV pseudotype virus assays. Both
131 cell lines were cultured at 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (Life
132 Technologies, UK) supplemented with 15% foetal bovine serum (FBS) and 1% penicillin/streptomycin
133 (Sigma Aldrich, UK).

134 *2.2 Serum samples:* For use in H5 PVNAs, a sample from a panel of ten sera extracted from
135 chickens vaccinated with an inactivated, monovalent, adjuvanted H5N2 vaccine
136 (A/chicken/Mexico/232/94/CPA strain) was selected. Previous studies have confirmed its
137 seropositivity by HI (a titre of 1:1024 with a homologous H5N2 test antigen) and PVNA, against an H5
138 A/Vietnam/1194/2004 luciferase PV (Terregino et al., 2010; Molesti et al., 2013). To neutralise RABV
139 pseudotypes, serum was used from a human subject vaccinated on days 0, 7 and 21 with the
140 inactivated Rabipur vaccine (Novartis Vaccines, Germany).

2.3 Production of pseudotype viruses: The generation of all lentiviral pseudotype viruses was performed as detailed previously (Temperton et al., 2007; Wright et al., 2008). 24 hours prior to transfection, approximately $4x10^6$ HEK293T/17 cells were seeded into sterile $10cm^3$ tissue culture plates (Nunc™ Thermo Scientific, UK). The HIV *gag-pol* plasmid, pCMV-Δ8.91 (Zufferey et al., 1997) and the firefly luciferase reporter construct pCSFLW ((Capecchi et al., 2008) based on pHR'SIN-cPPT-SGW outlined in (Demaison et al., 2002)) were transfected simultaneously with either the influenza HA, rabies G or Marburg GP expression vectors at a ratio of 1:1.5:1 (core:reporter:envelope) using the Fugene6 lipid-based reagent (Promega, UK). At 24 hours post-transfection, the cells were incubated with fresh media. For H5 transfections, exogenous recombinant neuraminidase from *Clostridium perfringens* (Sigma Aldrich, UK) was also added at this stage. Pseudotype supernatants were harvested at 48 hours after transfection and passed through a 0.45µm pore filter (Millex®, Millipore, Billerica, MA, USA), before being prepared for lyophilisation. Remaining supernatant was aliquoted and stored at -80°C.

2.4 Lyophilisation of pseudotype viruses: Individual samples of pseudotype virus were mixed with a sucrose-PBS cryoprotectant solution at a 1:1 v/v ratio to a 1M-0.1M range of molarities. Importantly, all lyophilisation was carried out in low surface-tension polypropylene microcentrifuge tubes (Caesa Lab, Canada), to prevent binding of the virus glycoproteins to the inside surface of the tubes, and subsequent loss of pseudotype titre, during freeze-drying. Once prepared, virus samples were pre-frozen at -80°C. Immediately prior to lyophilisation, a second, pierced lid, made of standard polypropylene, was applied to each sample tube to allow for moisture release. All lyophilisation was carried out overnight in a FreeZone 2.5 litre freeze-drying chamber (Labconco, Kansas City, MO, USA) at a temperature of -50°C and a pressure of <0.133mBar. If the lyophilised pellets were stored for a sustained length of time after freeze-drying, the standard polypropylene pierced lid was removed from the sample tube, and the original low surface-tension polypropylene lid was replaced. Likewise, in the instances where the pellets were stored at a constant humidity as well as temperature, the sample tubes were kept in a sealed, humidified incubator unit, controlled

by a humidistat. DMEM + GlutaMAX (with the same supplementation as for the cell culture) were attempted for all reconstitution of lyophilised pellets, except for Figure 6 where distilled, nuclease-free H_2O was also used.

2.5 Pseudotype titration and neutralisation assays: Titration and neutralisation assays were performed in 96-well plates and based upon previously described protocols (Temperton et al., 2007; Wright et al., 2009; Scott et al., 2012), but adapted for the use of reconstituted, lyophilised pseudotype. For titration assays, 1:2 serial dilutions of reconstituted pseudotype were incubated with $1x10^4$ HEK293T/17 or BHK-21 cells for 48 hours before measuring relative luminescence units per ml (RLU/ml). For the neutralisation assay, serum samples were serially diluted (ranging from 1:40 to 1:81920) and incubated with $1x10^6$ RLU of reconstituted pseudotype (as calculated from the titration assay) for 1hr at 37°C to permit antibody attachment to surface virus glycoproteins. $1x10^4$ HEK293T/17 or BHK-21 cells were then added to each well and incubated for 48 hours, prior to taking a chemiluminescent readout. In all instances, Bright-Glo luciferase assay reagent (Promega, UK) and a Glomax 96 luminometer (Promega, UK) were used to quantify luciferase reporter expression.

2.6 Statistical analysis: Pseudotype transduction titres were calculated by converting RLU readout values at a range of assay dilutions into RLU/ml, before determining the arithmetic mean and standard deviation. PVNA raw data was normalised as % neutralisation between mean values for a virus only control (equivalent to 0% neutralisation or 100% infection) and a cell only control (equivalent to 100% neutralisation or 0% infection), then IC_{50} and IC_{90} values were calculated using non-linear regression analysis (log [inhibitor] vs normalised response – variable slope). All data manipulation was performed on GraphPad Prism 5 (GraphPad software, San Diego, CA, USA).

3 Results

190 High titre lentiviral pseudotype particles were generated bearing the envelope glycoproteins
191 from influenza A/H5N1/Vietnam/1194/2004, rabies ERA and Marburg Lake Victoria strains.
192 Transduction efficiency of the pseudotypes into HEK293T/17 cells (for influenza H5) and BHK-21 cells
193 (for RABV and MARV) was evaluated, and luciferase expression was observed at $2.04x10^{10}$, $8.21x10^{9}$
194 and $7.46x10^{9}$ RLU/ml, respectively (Figure 1). All titration assays included two negative controls: Δ
195 envelope glycoprotein (ΔEG), which is a PV bearing no viral envelope glycoprotein, and a non-
196 transduced cell only control.

197 Next, pseudotype supernatants were mixed with a stepwise dilution series of sucrose-PBS
198 solutions (1M, 0.5M, 0.25M, 0.1M) which acts as a cryoprotectant during lyophilisation. Supernatant
199 was also lyophilised in pure PBS solution containing no sucrose, which is referred to as 0M sucrose-
200 PBS. After overnight freeze-drying, lyophilised pellets were immediately reconstituted and
201 transduction efficiency measured in a titration assay. Less than $1\log_{10}$ of decrease in viral titre,
202 measured in RLU/ml, was observed with H5, RABV and MARV pseudotypes at all cryoprotectant
203 concentrations, when compared to their non-lyophilised counterparts (Figure 2a-2c). Levels of titre
204 retention are therefore sufficient for these lyophilised PVs to be taken forward into PVNA assays. As
205 PV titre was retained following reconstitution of recently lyophilised pellets, regardless of sucrose-
206 PBS concentration, freeze-drying for subsequent experiments was carried out at 1M, 0.5M and 0M
207 cryoprotectant molarities.

208 To ascertain the stability of PV aliquots stored in the freeze-dried state, individual lyophilised
209 pellets in 1M, 0.5M and 0M cryoprotectant were incubated for varying durations at the following
210 temperatures: -80°C, -20°C, +4°C, +20°C, +37°C/70% relative humidity (RH) and +37°C/95% RH. After
211 1, 2 and 4 weeks, freeze-dried pellets of PV were reconstituted and titrated as previously described
212 (subsections 2.4 and 2.5) to calculate viral titre in RLU/ml. Generally, PV titre retention was high for
213 all lyophilised H5 (Figure 3a-3c), RABV (Figure 4a-4c) and MARV (Figure 5a-5c) samples that were
214 stored at the lowest temperatures, but as the storage temperature increased, PV samples freeze-

dried in the absence of cryoprotectant degraded significantly, with transduction efficiency decreasing to that of ΔEG. Interestingly, relative humidity (RH) seems to play a role in viability of lyophilised PV pellets, with 1M- and 0.5M-cryoprotected samples stored for 4 weeks generally retaining functional virus titre up to 37^{o}C and 70% RH, but heavily degrading in a 95% humidified atmosphere at the same temperature.

It is possible that reconstituting in supplemented DMEM results in an accumulation of soluble culture medium components in the pseudotype sample which may affect downstream employment in serological assays. To address this issue, we reconstituted H5, RABV and MARV pseudotypes (immediately after lyophilisation, in the presence of 0.5M sucrose-PBS) with distilled, nuclease-free H_2O and DMEM (with supplementation described in subsection 2.1), before comparing their transduction ability into corresponding target cell lines with a titration assay (Figure 6). Levels of pseudotype titre retention were very similar with either reconstitution solution, indicating that possible culture medium nutrient accumulation when using DMEM to reconstitute lyophilised pseudotypes does not have an adverse effect on pseudotype infectivity. However, water could viably be used as an alternative solution for resuspension of freeze-dried pseudotypes, but it is uncertain whether this would detrimentally affect the health of the target cell lines in titration and neutralisation assays, due to insufficient volumes of fresh DMEM.

The ability for lyophilised PVs to transduce target cells indicates that the influenza A, RABV and MARV envelope glycoproteins do not structurally deteriorate during the freeze-drying process, especially in the receptor-binding domains. However, in order to assess the structural integrity in the antigenic epitopes of the glycoproteins, neutralisation assays were also carried out using serum samples confirmed as antibody-positive against H5 and RABV strains. VNAb IC_{50} and IC_{90} titres (the reciprocal of the highest serum dilution still able to confer 50% and 90% virus neutralisation) were compared between lyophilised and immediately reconstituted H5 and RABV pseudotypes, and their

239 'fresh', unlyophilised counterparts, with no discernible reduction in the capability of antibody-
240 mediated neutralisation observed (Table 1).

241 **4 Discussion**

242 It has been well-documented that retroviral pseudotypes are valid, reliable alternatives to
243 wild-type virus for serological applications (Temperton and Wright, 2009). Advantageous qualities of
244 the PVNA platform include the ability to conduct the assay in BSL-1 laboratories, as well as the
245 availability to perform multiplex assays and incorporate 'low-cost' reporter genes into
246 pseudoparticles, which all reduce the cost implications and increase the potential ubiquity of
247 pseudotyping (Wright et al., 2009, 2010; Kaku et al., 2012). However, the current necessity to store
248 aliquots of PV supernatant at -80°C and to maintain the cold-chain during PV transportation present
249 serious monetary obstacles for laboratories to acquire such reagents, especially if on a limited
250 budget.

251 Here, we have demonstrated the viability of lyophilisation as an alternative, cost-effective
252 state for the storage and distribution of pseudotype viruses. In the presence of cryoprotectant, H5
253 influenza, rabies and Marburg PV supernatant retain very high levels of infectivity following freeze-
254 drying and reconstitution. Subsequent freeze-dried pseudotype pellets can generally withstand
255 incubation for 4 weeks at a range of temperatures up to 37°C, and incubation in a 'tropical climate'
256 (37°C and 95% relative humidity) for 2 weeks, whilst maintaining a viral titre sufficient for
257 employment in downstream neutralisation assays. This confirms the stability and glycoprotein
258 integrity of lyophilised PVs throughout environmental conditions likely to be experienced within the
259 production, dissemination and storage of a PVNA-based kit. Furthermore, both H5 influenza and
260 rabies reconstituted pseudotypes were neutralised by VNAb-positive serum samples to the same
261 potency as their 'fresh', unlyophilised counterparts, indicating that antigenic epitopes on each virus
262 glycoprotein do not structurally deteriorate during lyophilisation, thus ratifying the suitability of
263 freeze-dried PVs from a serological viewpoint.

With regard to the wider implications for PVNA-based serology kits, the findings reported in this study are also encouraging. The survival and usability of somatic cells after freeze-drying and reconstitution has already been established, with positive implications for many areas of biomedicine (Loi et al., 2008). Indeed, the opportunity to produce samples of pseudotype virus and candidate cell line as stable, dried pellets would considerably facilitate global distribution of a multi-component PVNA kit, at a fraction of the current expenditure for overseas shipping and storage on dry ice. Additionally, the high cost of purchasing frozen cell line ampoules from certified repositories can be avoided by incorporating lyophilised cells into such a kit. Overall, the utilisation of PVNA-based kits would significantly ameliorate logistic dilemmas surrounding vaccine evaluation and serological surveillance, especially for laboratories situated in resource-poor countries where many emerging viral infections are prevalent.

Virus lyophilisation as a stable means of storage is certainly not a novel phenomenon, with the process being acknowledged for decades (Tyrrell and Ridgwell, 1965). Studies involving wild-type virus freeze-drying generally concur with this one in several aspects by, for instance, demonstrating the ability to store lyophilised foot and mouth disease virus at 4°C for 1 year (Fellowes, 1965) and freeze-dried poliovirus preparations at 37°C for 5 days (Berge et al., 1971). Infectivity tests were also undertaken on pseudorabies virus lyophilised in a number of suspension media, with glutamate formulations mixed with sucrose or dextran proving the most cryoprotective (Scott and Woodside, 1976). Furthermore, the viability of freeze-dried viral vector formulations has been investigated for gene therapy applications. Retroviral vectors have recovered with more than 90% infectivity post-lyophilisation in the presence of sucrose cryoprotectant (Shin et al., 2010), with adenoviral vectors only showing negligible drops in titre following freeze-drying and storage at ambient temperatures (Croyle et al., 2001). Likewise, lyophilsed influenza virosomes retained both structure and function after 12 weeks' storage at 4°C (Wilschut et al., 2007). In comparison, pseudotyped retroviral vectors rapidly decreased in titre following three to five freeze-thaw cycles (Higashikawa and Chang, 2001),

289 which further reinforces how preferable lyophilisation is for employment in VNAb-based serological
290 kits.

291 Relative humidity (RH) plays a pivotal role in storage stability of dried pseudotype pellets,
292 with high RH levels proving detrimental to PV recovery and transduction potential. Certainly for wild-
293 type influenza virus, it has been confirmed that both virus transmission and infectivity are
294 significantly decreased in highly humid atmospheres, which could be an attributive factor for its
295 seasonal fluctuation (Lowen et al., 2007; Noti et al., 2013). It has previously been postulated that
296 viruses with a high lipid content are more sensitive to high RH (Assar and Block, 2001).

297 Investigation of further parameters would be necessary before a robust, reliable PVNA-
298 based kit could be trialled and clinically utilised. Firstly, existing data would need to be extrapolated
299 by testing freeze-dried pellet storage stability over longer durations i.e. six months, one year and
300 three years, as well as comparing other candidate suspending media to sucrose-PBS to ensure
301 maximum efficiency of cryoprotection. Employing freeze-dried pseudotypes in PVNAs against larger
302 panels of sera, before drawing comparisons against not only unlyophilised pseudotypes, but also
303 established serological assays using live virus, would be vital to assess accordance in VNAb titres
304 between assays. To increase PVNA kit flexibility and customisation, it would also be important to
305 assess the sensitivity of other commonly used pseudotype virus cores to lyophilisation and
306 subsequent stability studies. Another consideration is to accurately simulate conditions during an
307 international transit journey, thus ascertaining the ability for lyophilised PVs to cope with harsh
308 temperature and atmospheric fluctuations between, for example, an aeroplane cargo deck and
309 tropical climate conditions.

310 **5 Conclusion**

311 In this study, it is shown H5 influenza, rabies and Marburg pseudotype viruses can be stably
312 stored in a lyophilised state for 4 weeks at temperatures up to 37°C, in the presence of at least 0.5M

sucrose-PBS as a cryoprotectant, and retain much of their infectivity once reconstituted and employed in virus neutralisation assays. This confirms the viability of producing a freeze-dried PVNA-based kit, which would considerably facilitate the execution of vaccine evaluation and sero-surveillance studies, especially in countries without access to BSL-3/-4 containment laboratories or constant cold-chain storage facilities, and ultimately permit the development of improved serological control measures for many emerging viral infections.

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Figure Legends

Figure 1: Infectivity of pseudotyped lentiviral vectors displaying influenza H5 A/Vietnam/1194/2004 HA, RABV ERA G and MARV Lake Victoria GP glycoproteins. Pseudotype transduction titers are expressed as mean ±SD of relative luminescent units per ml (RLU/ml). Δ envelope glycoprotein, HEK293T/17 cell only and BHK-21 cell only negative controls are also shown.

Figure 2: Transduction retention of pseudotype viruses with (A) influenza H5 A/Vietnam/1194/2004 HA, (B) RABV ERA G and (C) MARV Lake Victoria GP envelope glycoproteins following lyophilisation at a gradient of sucrose-PBS cryoprotectant molarities. Relative PV transduction titers are shown as mean ±SD of relative luminescent units per ml (RLU/ml).

Figure 3: Effect of lyophilisation and pellet incubation on infectivity of H5 pseudotyped lentiviral vectors. Freeze-dried PVs displaying H5 A/Vietnam/1194/2004 HA were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are displayed as mean ±SD of relative luminescent units per ml (RLU/ml).

Figure 4: Effect of lyophilisation and pellet incubation on infectivity of RABV pseudotyped lentiviral vectors. Freeze-dried PVs displaying RABV ERA G glycoproteins were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are expressed as mean ±SD of relative luminescent units per ml (RLU/ml).

Figure 5: Effect of lyophilisation and pellet incubation on infectivity of MARV pseudotyped lentiviral vectors. Freeze-dried PVs displaying MARV Lake Victoria GP glycoproteins were stored in either 1M, 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype transduction titres are shown as mean ±SD of relative luminescent units per ml (RLU/ml).

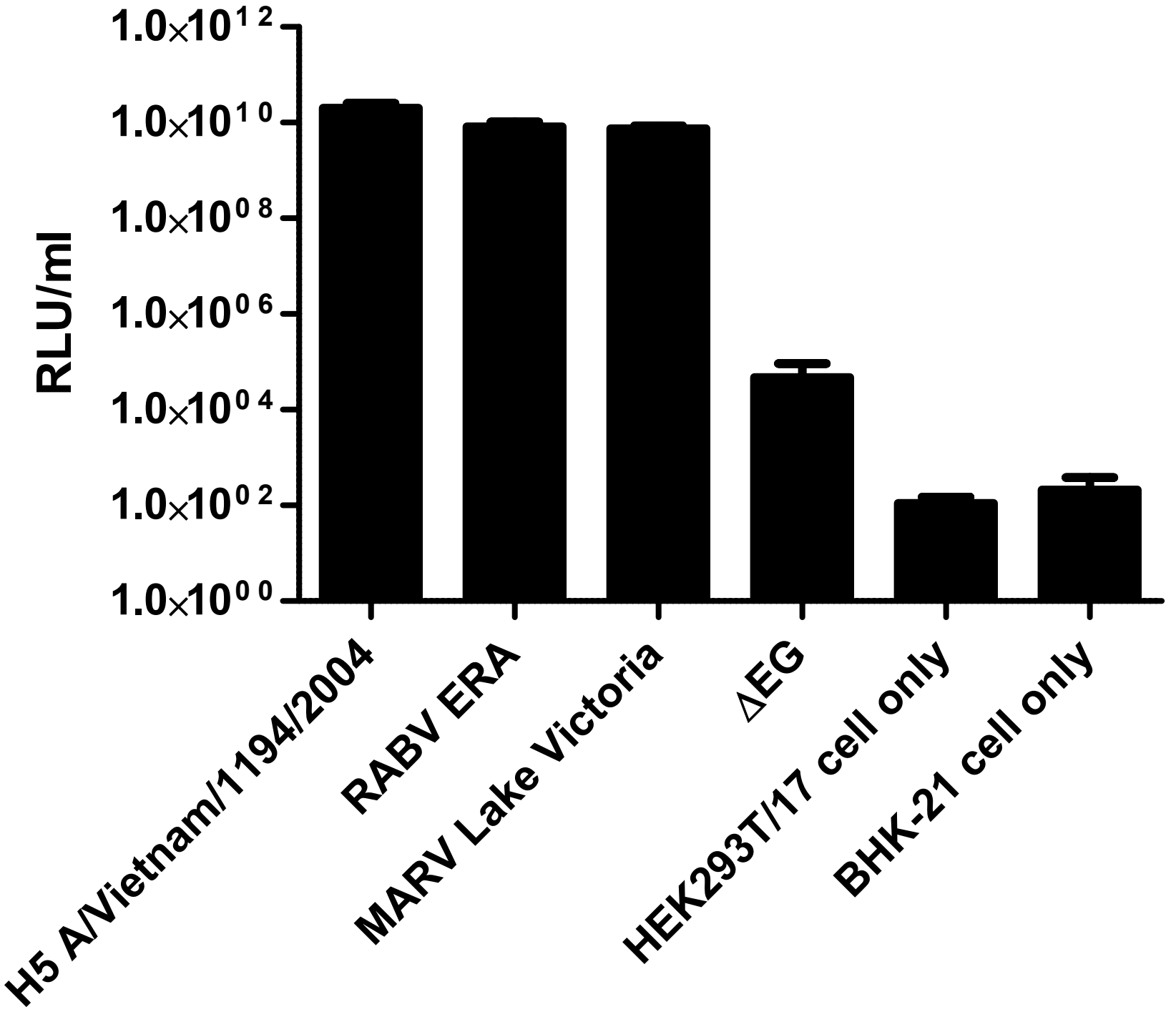
Figure 6. Influence of reconstitution solution on H5, RABV and MARV pseudotyped lentiviral vectors. Freeze-dried PVs with influenza H5 A/Vietnam/1194/2004 HA, RABV ERA G or MARV Lake Victoria GP envelope glycoproteins were reconstituted in either distilled, nuclease-free H_2O or supplemented DMEM culture medium before utilisation in a titration assay. Pseudotype transduction titres are shown as mean ±SD of relative luminescent units per ml (RLU/ml). Unlyophilised pseudotype positive controls, and Δ envelope glycoprotein, HEK293T/17 cell only and BHK-21 cell only negative controls are also shown.

Tables

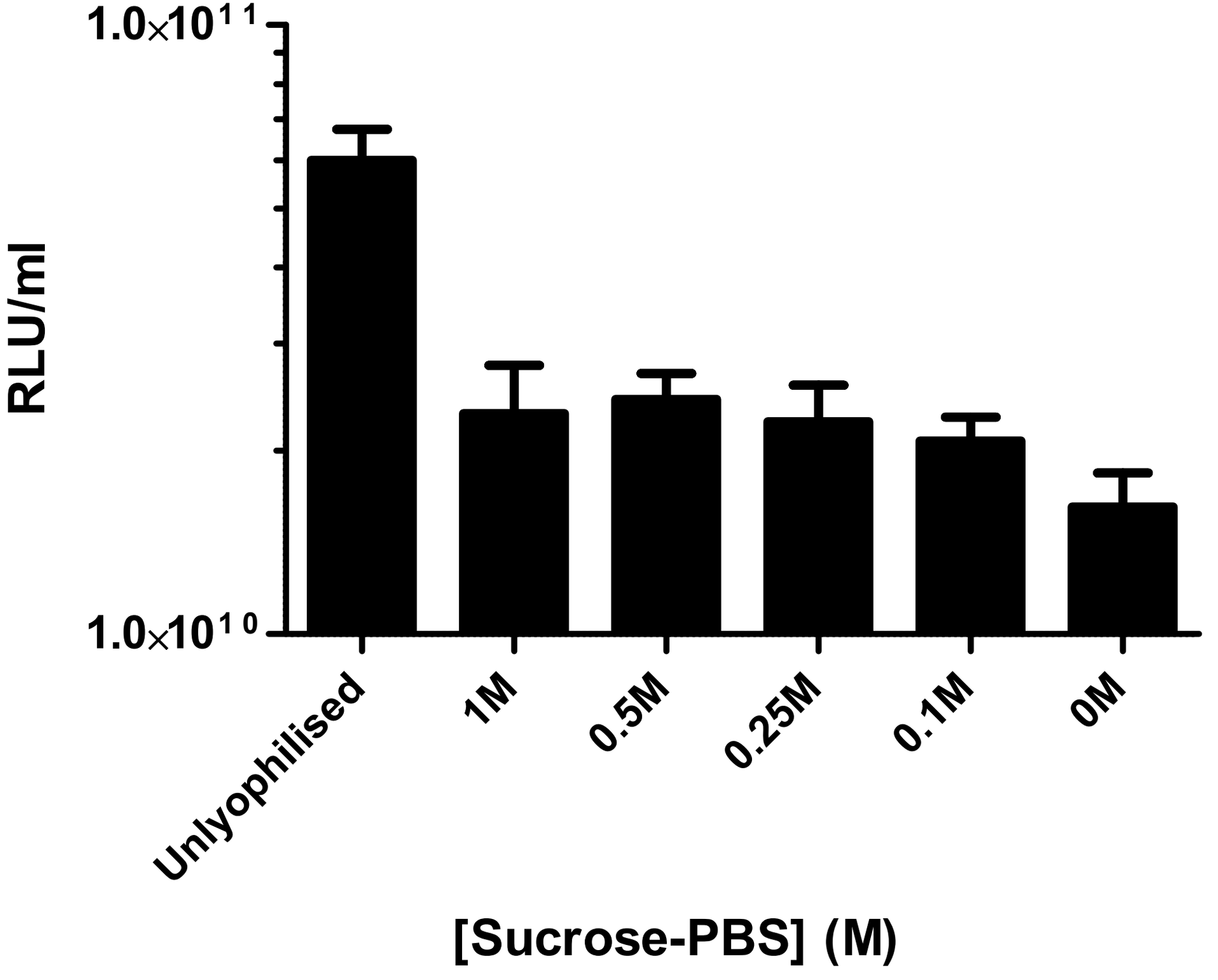
Pseudotype virus (PV)	IC_{50}	IC_{90}
H5 A/Vietnam/1194/2004 - lyophilised	2560-5120 (4679)	1280-2560 (1390)
H5 A/Vietnam/1194/2004 - unlyophilised	2560-5120 (3919)	1280-2560 (1345)
RABV ERA – lyophilised	40960-81920 (44072)	5120-10240 (6381)
RABV ERA – unlyophilised	20480-40960 (27324)	5120-10240 (6222)

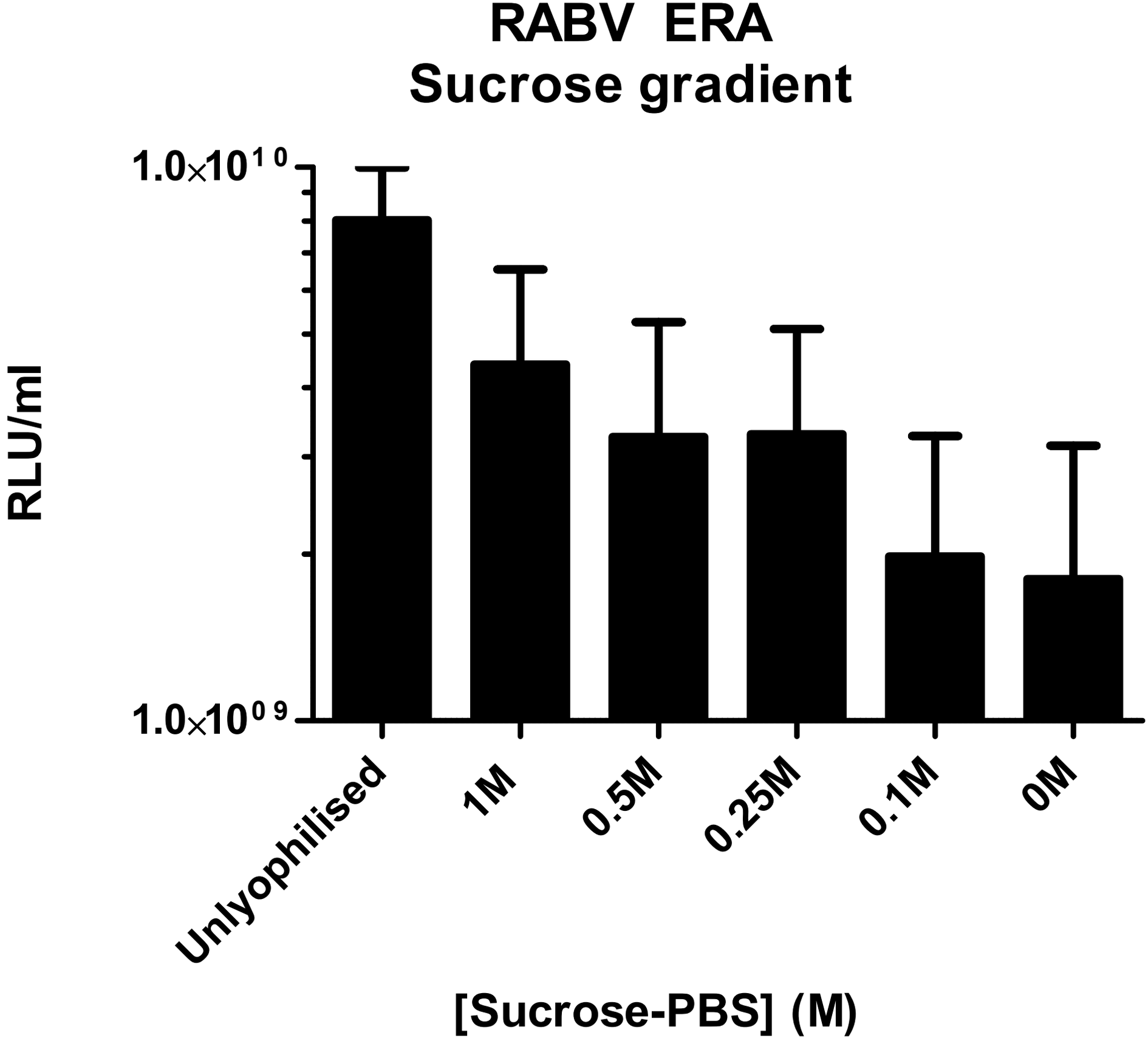
Table 1: Comparison of neutralising antibody titres against untreated and lyophilised pseudotyped lentiviruses. Half maximal inhibitory concentration (IC_{50}) and 90% inhibitory concentration (IC_{90}) values of confirmed antibody-positive antisera against H5 influenza and RABV pseudotypes before and after lyophilisation. VNAb titres were calculated using GraphPad Prism 5 software and are displayed as serum assay dilutions. Exact IC_{50} and IC_{90} values are also shown in parentheses.

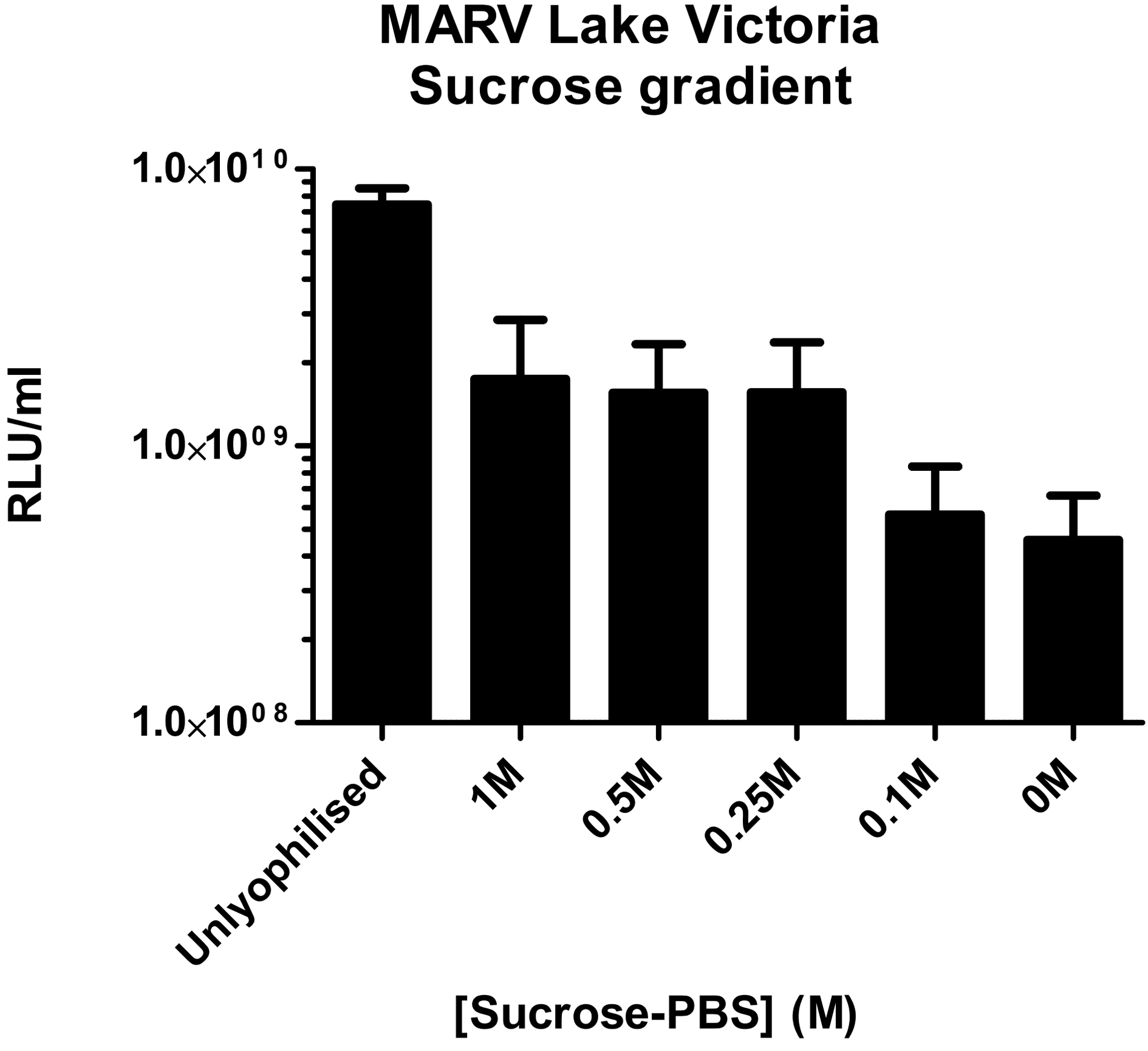
H5 A/Vietnam/1194/2004, RABV ERA and MARV Lake Victoria PV titres

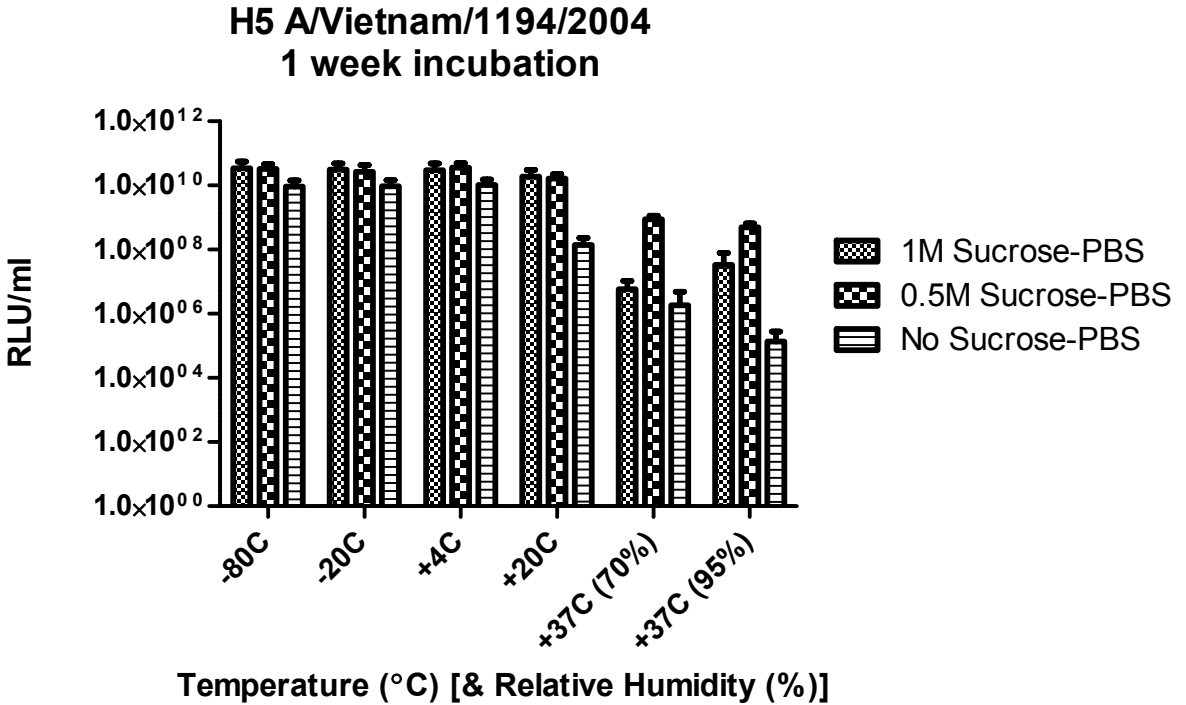


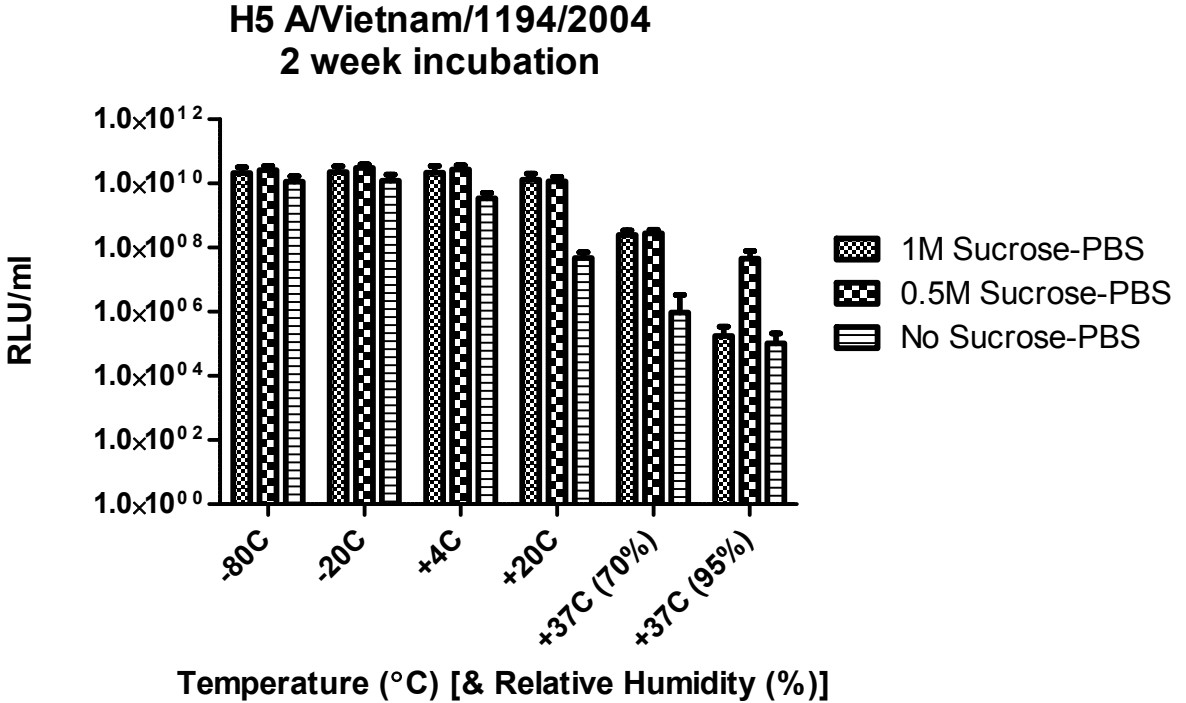
H5 A/Vietnam/1194/2004
Sucrose gradient

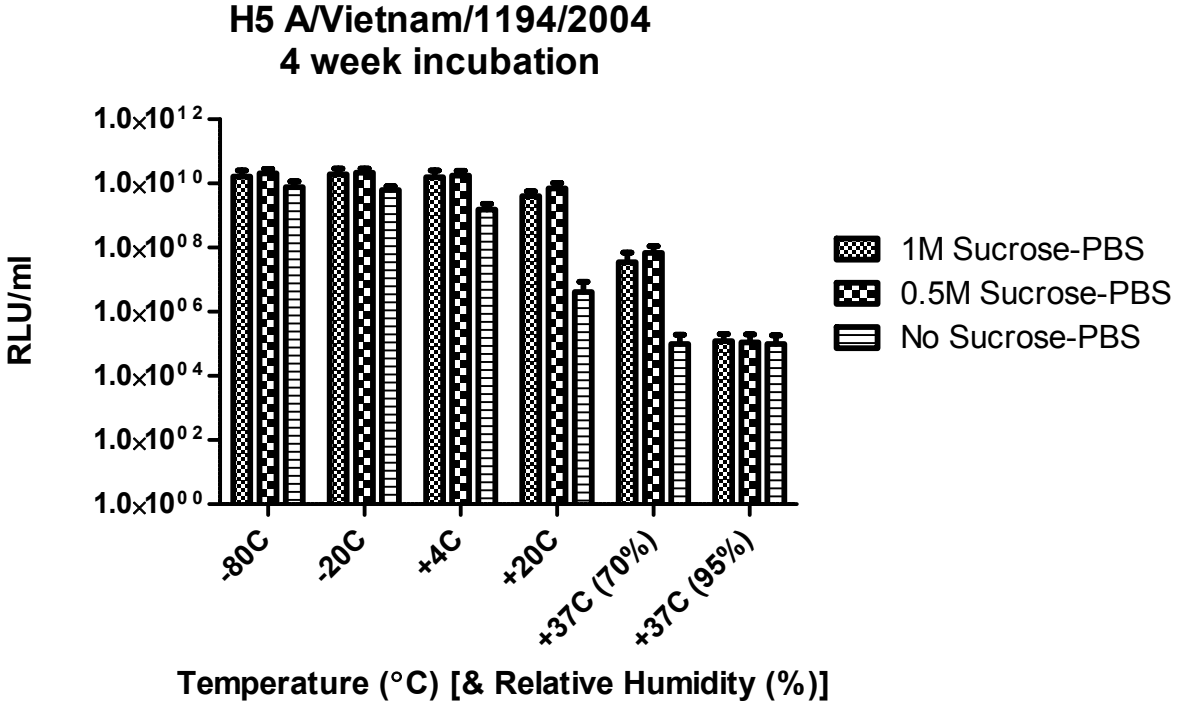


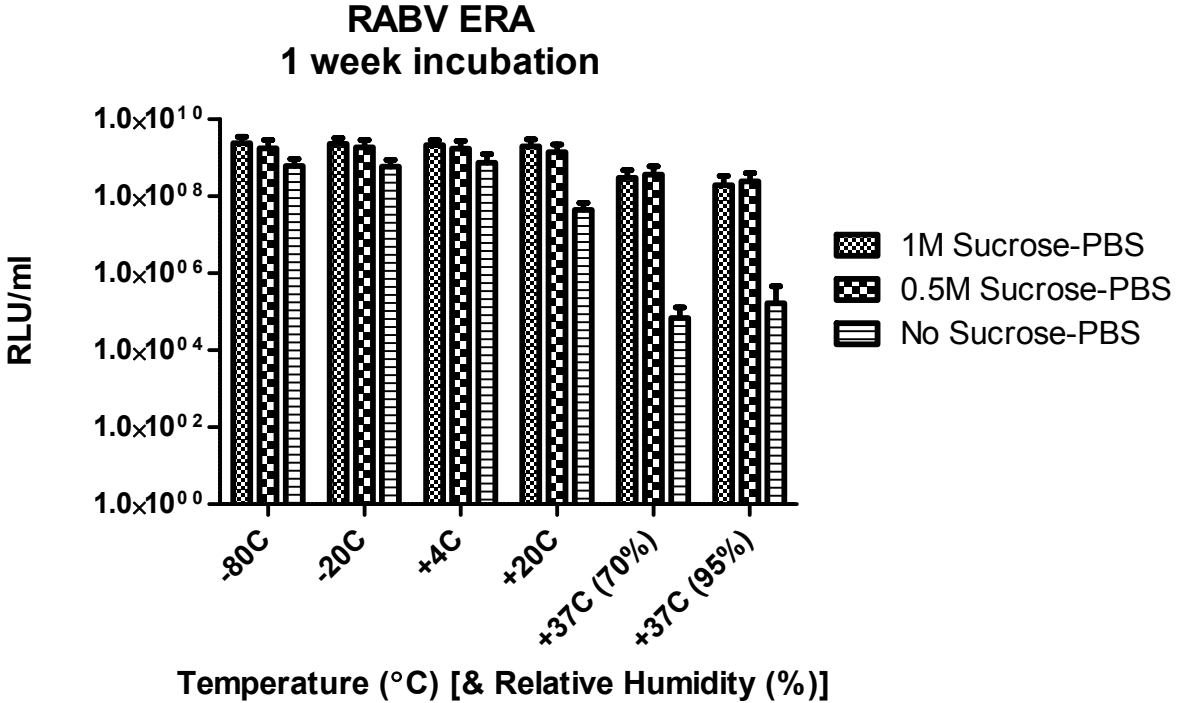

RABV ERA
Sucrose gradient
1.0×10 10
1.0×10 09
RLU/ml
Unlyophilised
1M
0.5M
0.25M
0.1M
0M
[Sucrose-PBS] (M)

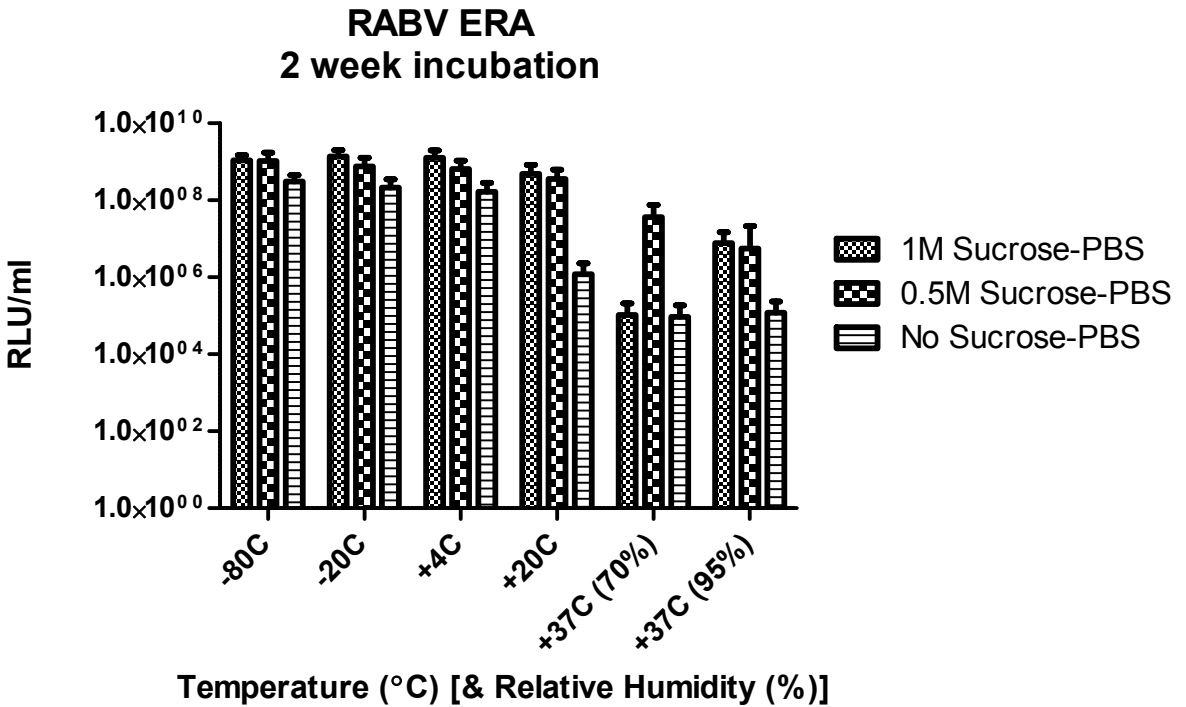

MARV Lake Victoria
Sucrose gradient
RLU/ml
1.0×10 10
1.0×10 09
1.0×10 08
Unlyophilised
1M
0.5M
0.25M
0.1M
0M
[Sucrose-PBS] (M)

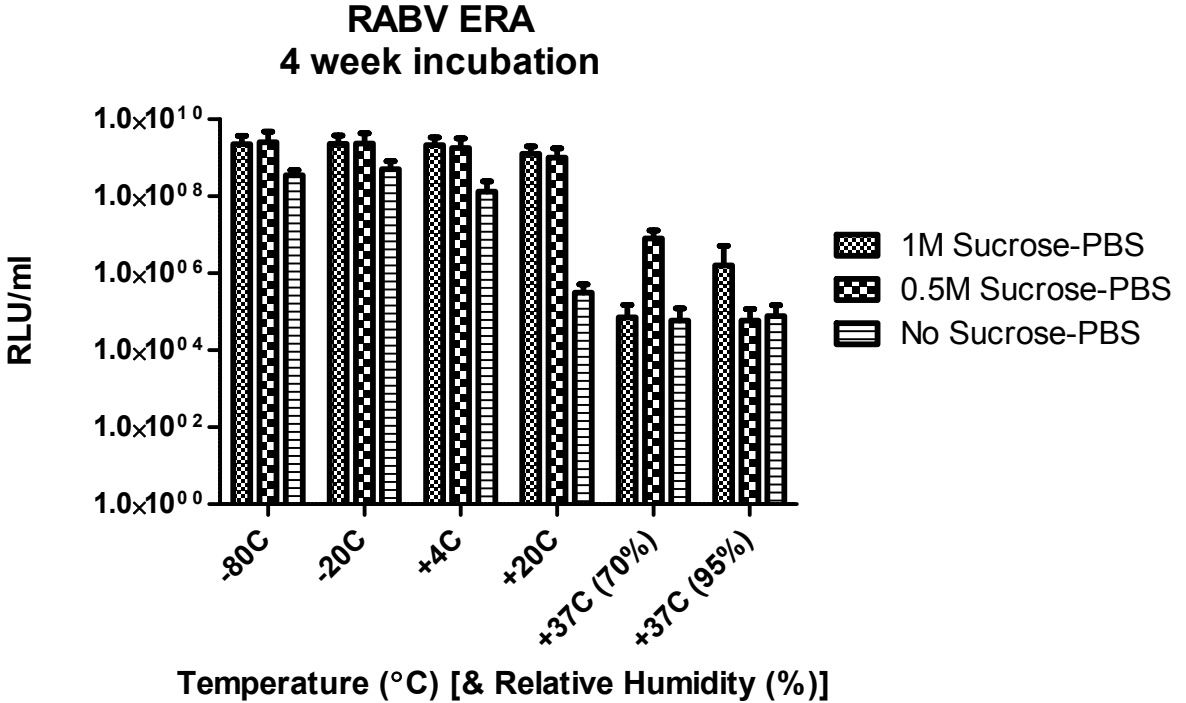

H5 A/Vietnam/1194/2004
1 week incubation
RLU/ml
1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS

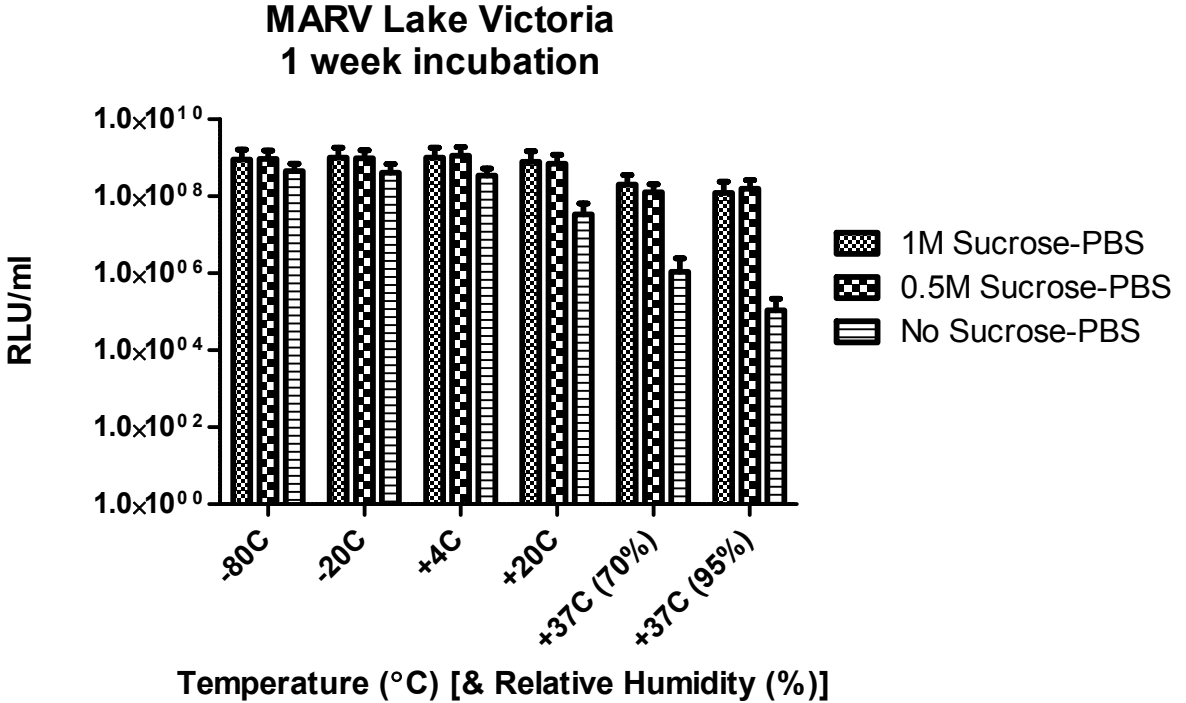

H5 A/Vietnam/1194/2004
2 week incubation
RLU/ml
1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS

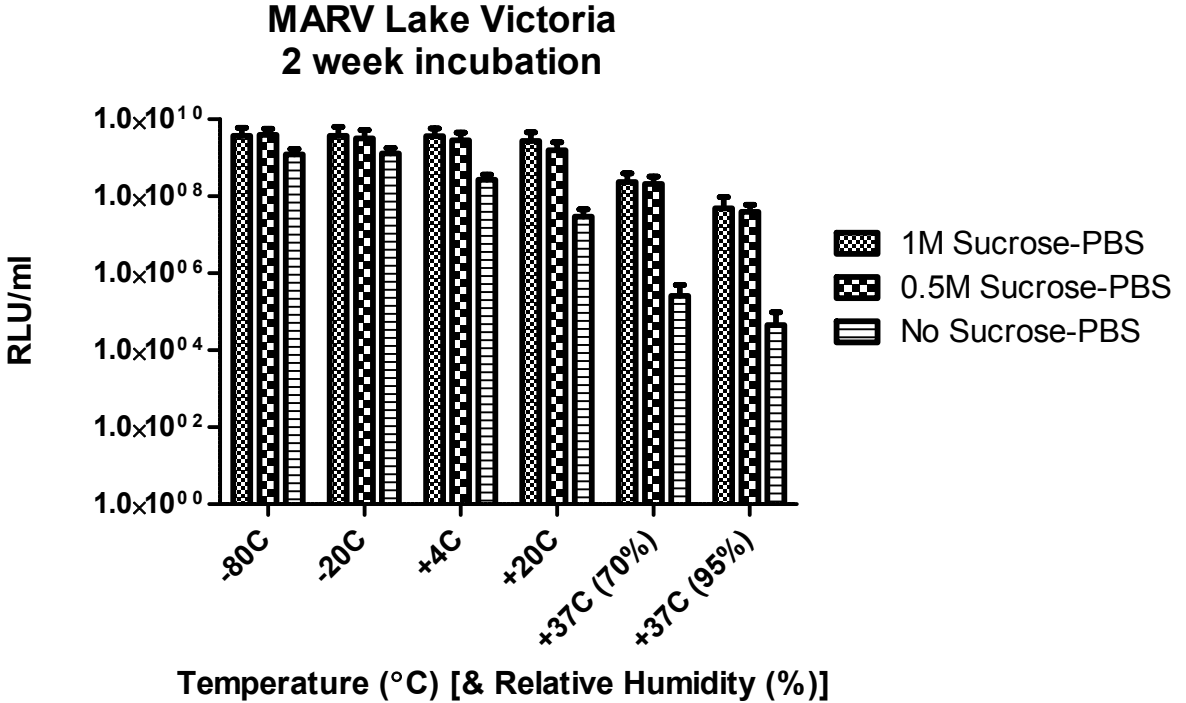

H5 A/Vietnam/1194/2004
4 week incubation
RLU/ml
1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS

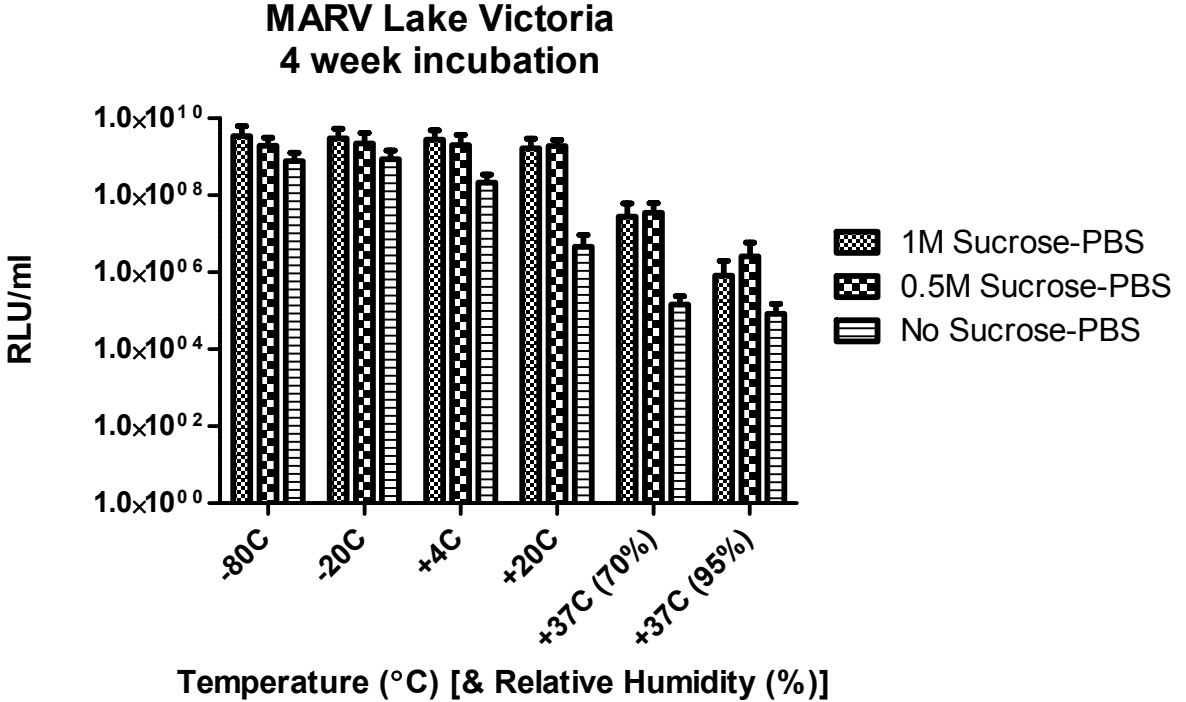

RABV ERA
1 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS

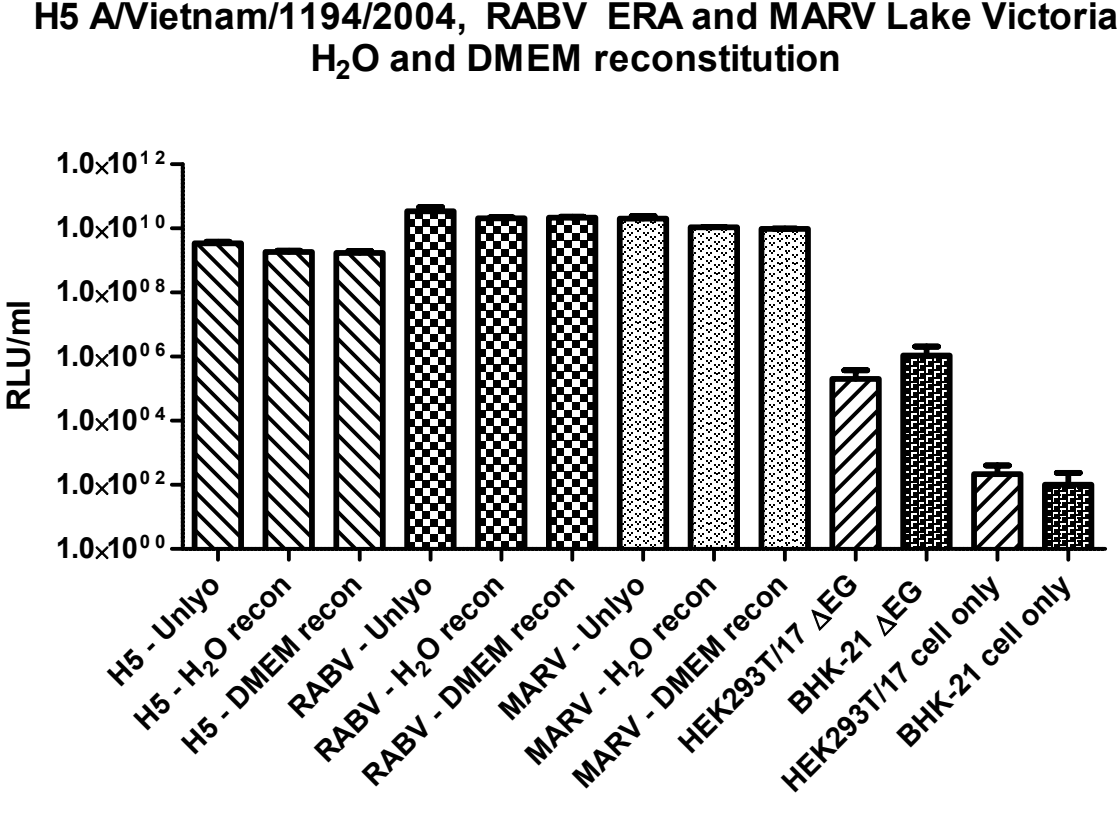

RABV ERA
2 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS


RABV ERA
4 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS


MARV Lake Victoria
1 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS


MARV Lake Victoria
2 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS


MARV Lake Victoria
4 week incubation
RLU/ml
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
1.0x10^02
1.0x10^00
-80C
-20C
+4C
+20C
+37C (70%)
+37C (95%)
Temperature (°C) [& Relative Humidity (%)]
1M Sucrose-PBS
0.5M Sucrose-PBS
No Sucrose-PBS


H5 A/Vietnam/1194/2004, RABV ERA and MARV Lake Victoria
H2O and DMEM reconstitution
RLU/ml
1.0×10^12
1.0×10^10
1.0×10^08
1.0×10^06
1.0×10^04
1.0×10^02
1.0×10^00
H5 - Unlyo
H5 - H2O recon
H5 - DMEM recon
RABV - Unlyo
RABV - H2O recon
RABV - DMEM recon
MARV - Unlyo
MARV - H2O recon
MARV - DMEM recon
HEK293T/17 ΔEG
BHK-21 ΔEG
HEK293T/17 cell only
BHK-21 cell only