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

3

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21 **Abstract**

22 Pseudotype viruses (PVs) are chimeric, replication-deficient virions that mimic wild-type virus entry
23 mechanisms and can be safely employed in neutralisation assays, bypassing the need for high
24 biosafety requirements and performing comparably to established serological assays. However, PV
25 supernatant necessitates -80°C long-term storage and cold-chain maintenance during transport,
26 which limits the scope of dissemination and application throughout resource-limited laboratories.
27 We therefore investigated the effects of lyophilisation on influenza, rabies and Marburg PV stability,
28 with a view to developing a pseudotype virus neutralisation assay (PVNA) based kit suitable for
29 affordable global distribution. Infectivity of each PV was calculated after lyophilisation and
30 immediate reconstitution, as well as subsequent to incubation of freeze-dried pellets at varying
31 temperatures, humidities and timepoints. Integrity of glycoprotein structure following treatment
32 was also assessed by employing lyophilised PVs in downstream PVNAs. In the presence of 0.5M
33 sucrose-PBS cryoprotectant, each freeze-dried pseudotype was stably stored for 4 weeks at up to
34 37°C and could be neutralised to the same potency as unlyophilised PVs when employed in PVNAs.
35 These results confirm the viability of a freeze-dried PVNA-based kit, which could significantly
36 facilitate low-cost serology for a wide portfolio of emerging infectious viruses.

37 **Keywords**

38 Retroviral pseudotype viruses, lyophilisation, neutralising antibodies, serological assays

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44 **1 Introduction**

45 The impact of emerging and re-emerging viral diseases on global health is becoming
46 increasingly apparent year on year. Influenza (family *Orthomyxoviridae*, genus *Influenzavirus A*,
47 species *Influenza A virus*) remains one of the viruses most likely to cause high morbidity and
48 mortality in human populations, after significant outbreaks of H5N1 and H7N9 subtypes beginning in
49 1997 and 2013 respectively, and the low pathogenic but highly transmissible 2009 H1N1 pandemic
50 virus (Yuen et al., 1998; WHO, 2010; Gao et al., 2013). This threat persists with the first human cases
51 of H6N1 and H10N8, and the recent discovery of diverse H17N10 and H18N11 subtypes in bat
52 reservoirs (Tong et al., 2013; Wei et al., 2013; To et al., 2014). Similarly, rabies (family *Rhabdoviridae*,
53 genus *Lyssavirus*, species *Rabies virus*) is a globally ubiquitous virus, present on all continents other
54 than Antarctica, and responsible for over 60,000 deaths per year, primarily of children in resource-
55 limited areas of Asia and Africa (WHO, 2013). Once symptoms occur, rabies has a close to 100% case
56 fatality rate, the highest of any viral infection. Indeed, only a handful of people have survived
57 following development of clinical symptoms and most of those had neurological sequelae (Jackson,
58 2013). Sporadic outbreaks of Marburg virus (family *Filoviridae*, genus *Marburgvirus*, species *Marburg*
59 *marburgvirus*) in the Democratic Republic of the Congo in 1999-2000, and then in Angola in 2004-
60 2005 (respective mortality rates of 83% and 90%), as well as small Ugandan outbreaks more
61 recently, serve to remind us that spillover events into human populations from unexpected viral
62 sources can create serious public health concerns (Brauburger et al., 2012). Therefore, options for
63 monitoring the spread and curtailing the outbreak severity of pathogenic viruses are vitally
64 important.

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

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

71 However, conventional serological assays possess drawbacks which detrimentally affect
72 their efficiency. Importantly, most require the use of infectious wild-type virus, necessitating
73 expensive, specialized biosafety level 3 or 4 (BSL-3 or -4) laboratories which are not readily available,
74 especially in resource-limited areas. Hemagglutination inhibition (HI) assays, used routinely for
75 influenza, suffer from variability caused by different erythrocytes and inhibitory factors, as well as
76 low sensitivity. ELISA-based assays do not require the use of wild-type virus, but are also hindered by
77 low sensitivity and cross-reactivity between samples. Furthermore, both HI and ELISA cannot
78 differentiate between virus neutralising and non-neutralising antibody responses (Mather et al.,
79 2013). Virus neutralisation assays, such as plaque reduction neutralisation test (PRNT) and
80 fluorescent antibody virus neutralisation (FAVN) assay, can measure virus neutralising antibody
81 (VNA_b) responses with high sensitivity and specificity levels but also require high biosafety for assay
82 preparation, and in some cases are time-consuming and suffer from low-throughput (Cliquet et al.,
83 1998; Mather et al., 2013).

84 A potential solution to these issues is the utilisation of retroviral pseudotype viruses (PVs).
85 PVs are composed of the structural and enzymatic core of one virus combined with heterologous
86 envelope glycoproteins (Temperton and Wright, 2009). Manipulations to the genomic RNA of the
87 lentiviral core create a replication-defective PV that encapsulates a quantifiable reporter gene.
88 Transduction of a permissible target cell line is dependent upon the ability of the envelope
89 glycoprotein to engage its cellular receptor in a process that mimics wild-type virus entry
90 mechanisms. If this is successful, the reporter gene can be integrated into the host cell genome and
91 subsequently expressed. Resultant levels of reporter protein in transduced cells can be measured,
92 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 subsection 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% CO₂ 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).

141 2.3 *Production of pseudotype viruses*: The generation of all lentiviral pseudotype viruses was
142 performed as detailed previously (Temperton et al., 2007; Wright et al., 2008). 24 hours prior to
143 transfection, approximately 4×10^6 HEK293T/17 cells were seeded into sterile 10cm^3 tissue culture
144 plates (Nunc™ Thermo Scientific, UK). The HIV *gag-pol* plasmid, pCMV- $\Delta 8.91$ (Zufferey et al., 1997)
145 and the firefly luciferase reporter construct pCSFLW ((Capecchi et al., 2008) based on pHR'SIN-cPPT-
146 SGW outlined in (Demaison et al., 2002)) were transfected simultaneously with either the influenza
147 HA, rabies G or Marburg GP expression vectors at a ratio of 1:1.5:1 (core:reporter:envelope) using
148 the Fugene6 lipid-based reagent (Promega, UK). At 24 hours post-transfection, the cells were
149 incubated with fresh media. For H5 transfections, exogenous recombinant neuraminidase from
150 *Clostridium perfringens* (Sigma Aldrich, UK) was also added at this stage. Pseudotype supernatants
151 were harvested at 48 hours after transfection and passed through a $0.45 \mu\text{m}$ pore filter (Millex®,
152 Millipore, Billerica, MA, USA), before being prepared for lyophilisation. Remaining supernatant was
153 aliquoted and stored at -80°C .

154 2.4 *Lyophilisation of pseudotype viruses*: Individual samples of pseudotype virus were mixed
155 with a sucrose-PBS cryoprotectant solution at a 1:1 v/v ratio to a 1M-0.1M range of molarities.
156 Importantly, all lyophilisation was carried out in low surface-tension polypropylene microcentrifuge
157 tubes (Caesa Lab, Canada), to prevent binding of the virus glycoproteins to the inside surface of the
158 tubes, and subsequent loss of pseudotype titre, during freeze-drying. Once prepared, virus samples
159 were pre-frozen at -80°C . Immediately prior to lyophilisation, a second, pierced lid, made of
160 standard polypropylene, was applied to each sample tube to allow for moisture release. All
161 lyophilisation was carried out overnight in a FreeZone 2.5 litre freeze-drying chamber (Labconco,
162 Kansas City, MO, USA) at a temperature of -50°C and a pressure of $<0.133 \text{mBar}$. If the lyophilised
163 pellets were stored for a sustained length of time after freeze-drying, the standard polypropylene
164 pierced lid was removed from the sample tube, and the original low surface-tension polypropylene
165 lid was replaced. Likewise, in the instances where the pellets were stored at a constant humidity as
166 well as temperature, the sample tubes were kept in a sealed, humidified incubator unit, controlled

167 by a humidistat. DMEM + GlutaMAX (with the same supplementation as for the cell culture) were
168 attempted for all reconstitution of lyophilised pellets, except for Figure 6 where distilled, nuclease-
169 free H₂O was also used.

170 *2.5 Pseudotype titration and neutralisation assays:* Titration and neutralisation assays were
171 performed in 96-well plates and based upon previously described protocols (Temperton et al., 2007;
172 Wright et al., 2009; Scott et al., 2012), but adapted for the use of reconstituted, lyophilised
173 pseudotype. For titration assays, 1:2 serial dilutions of reconstituted pseudotype were incubated
174 with 1x10⁴ HEK293T/17 or BHK-21 cells for 48 hours before measuring relative luminescence units
175 per ml (RLU/ml). For the neutralisation assay, serum samples were serially diluted (ranging from 1:40
176 to 1:81920) and incubated with 1x10⁶ RLU of reconstituted pseudotype (as calculated from the
177 titration assay) for 1hr at 37°C to permit antibody attachment to surface virus glycoproteins. 1x10⁴
178 HEK293T/17 or BHK-21 cells were then added to each well and incubated for 48 hours, prior to
179 taking a chemiluminescent readout. In all instances, Bright-Glo luciferase assay reagent (Promega,
180 UK) and a Glomax 96 luminometer (Promega, UK) were used to quantify luciferase reporter
181 expression.

182 *2.6 Statistical analysis:* Pseudotype transduction titres were calculated by converting RLU
183 readout values at a range of assay dilutions into RLU/ml, before determining the arithmetic mean
184 and standard deviation. PVNA raw data was normalised as % neutralisation between mean values
185 for a virus only control (equivalent to 0% neutralisation or 100% infection) and a cell only control
186 (equivalent to 100% neutralisation or 0% infection), then IC₅₀ and IC₉₀ values were calculated using
187 non-linear regression analysis (log [inhibitor] vs normalised response – variable slope). All data
188 manipulation was performed on GraphPad Prism 5 (GraphPad software, San Diego, CA, USA).

189 **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 2.04×10^{10} , 8.21×10^9
194 and 7.46×10^9 RLU/ml, respectively (Figure 1). All titration assays included two negative controls: Δ
195 envelope glycoprotein (Δ EG), 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 , $+4^\circ\text{C}$, $+20^\circ\text{C}$, $+37^\circ\text{C}/70\%$ relative humidity (RH) and $+37^\circ\text{C}/95\%$ 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-

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

220 It is possible that reconstituting in supplemented DMEM results in an accumulation of
221 soluble culture medium components in the pseudotype sample which may affect downstream
222 employment in serological assays. To address this issue, we reconstituted H5, RABV and MARV
223 pseudotypes (immediately after lyophilisation, in the presence of 0.5M sucrose-PBS) with distilled,
224 nuclease-free H₂O and DMEM (with supplementation described in subsection 2.1), before comparing
225 their transduction ability into corresponding target cell lines with a titration assay (Figure 6). Levels
226 of pseudotype titre retention were very similar with either reconstitution solution, indicating that
227 possible culture medium nutrient accumulation when using DMEM to reconstitute lyophilised
228 pseudotypes does not have an adverse effect on pseudotype infectivity. However, water could viably
229 be used as an alternative solution for resuspension of freeze-dried pseudotypes, but it is uncertain
230 whether this would detrimentally affect the health of the target cell lines in titration and
231 neutralisation assays, due to insufficient volumes of fresh DMEM.

232 The ability for lyophilised PVs to transduce target cells indicates that the influenza A, RABV
233 and MARV envelope glycoproteins do not structurally deteriorate during the freeze-drying process,
234 especially in the receptor-binding domains. However, in order to assess the structural integrity in the
235 antigenic epitopes of the glycoproteins, neutralisation assays were also carried out using serum
236 samples confirmed as antibody-positive against H5 and RABV strains. VNAb IC₅₀ and IC₉₀ titres (the
237 reciprocal of the highest serum dilution still able to confer 50% and 90% virus neutralisation) were
238 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.

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

275 Virus lyophilisation as a stable means of storage is certainly not a novel phenomenon, with
276 the process being acknowledged for decades (Tyrrell and Ridgwell, 1965). Studies involving wild-type
277 virus freeze-drying generally concur with this one in several aspects by, for instance, demonstrating
278 the ability to store lyophilised foot and mouth disease virus at 4°C for 1 year (Fellowes, 1965) and
279 freeze-dried poliovirus preparations at 37°C for 5 days (Berge et al., 1971). Infectivity tests were also
280 undertaken on pseudorabies virus lyophilised in a number of suspension media, with glutamate
281 formulations mixed with sucrose or dextran proving the most cryoprotective (Scott and Woodside,
282 1976). Furthermore, the viability of freeze-dried viral vector formulations has been investigated for
283 gene therapy applications. Retroviral vectors have recovered with more than 90% infectivity post-
284 lyophilisation in the presence of sucrose cryoprotectant (Shin et al., 2010), with adenoviral vectors
285 only showing negligible drops in titre following freeze-drying and storage at ambient temperatures
286 (Croyle et al., 2001). Likewise, lyophilised influenza virosomes retained both structure and function
287 after 12 weeks' storage at 4°C (Wilschut et al., 2007). In comparison, pseudotyped retroviral vectors
288 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

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

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322 **References**

- 323 Assar, S.K., Block, S.S., 2001. Survival of Microorganisms in the Environment, in: Block, S.S. (Ed.),
324 Disinfection, Sterilization and Preservation. Lippincott, Williams and Wilkins, pp. 1221–1242.
- 325 Berge, T.O., Jewett, R.L., Blair, W.O., 1971. Preservation of enteroviruses by freeze-drying. *Appl.*
326 *Microbiol.* 22, 850–3.
- 327 Brauburger, K., Hume, A.J., Mühlberger, E., Olejnik, J., 2012. Forty-five years of Marburg virus
328 research. *Viruses* 4, 1878–927.
- 329 Capecchi, B., Fasolo, A., Alberini, I., Baudner, B., Crotta, S., Temperton, N.J., Montomoli, E., Del
330 Guidice, G., Rappuoli, R., 2008. Use of Pseudotyped Particles Expressing Influenza
331 A/Vietnam/1194/2004 Hemagglutinin in Neutralization Assays, in: Katz, J. (Ed.), *Options for the*
332 *Control of Influenza VI*. International Medical Press, London, pp. 303–305.
- 333 Cliquet, F., Aubert, M., Sagné, L., 1998. Development of a fluorescent antibody virus neutralisation
334 test (FAVN test) for the quantitation of rabies-neutralising antibody. *J. Immunol. Methods* 212,
335 79–87.
- 336 Cox, R.J., Mykkeltvedt, E., Robertson, J., Haaheim, L.R., 2002. Non-lethal viral challenge of influenza
337 haemagglutinin and nucleoprotein DNA vaccinated mice results in reduced viral replication.
338 *Scand. J. Immunol.* 55, 14–23.
- 339 Croyle, M.A., Cheng, X., Wilson, J.M., 2001. Development of formulations that enhance physical
340 stability of viral vectors for gene therapy. *Gene Ther.* 8, 1281–90.
- 341 Demaison, C., Parsley, K., Brouns, G., Scherr, M., Battmer, K., Kinnon, C., Grez, M., Thrasher, A.J.,
342 2002. High-level transduction and gene expression in hematopoietic repopulating cells using a

343 human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector
344 containing an internal spleen focus forming virus promoter. *Hum. Gene Ther.* 13, 803–13.

345 Desvaux, S., Garcia, J.M., Nguyen, T.D., Reid, S.A., Bui, N.A., Roger, F., Fenwick, S., Peiris, J.S.M., Ellis,
346 T., 2012. Evaluation of serological tests for H5N1 avian influenza on field samples from
347 domestic poultry populations in Vietnam: consequences for surveillance. *Vet. Microbiol.* 156,
348 277–84.

349 Fellowes, O.N., 1965. Freeze-Drying of Foot-and-Mouth Disease Virus and Storage Stability of the
350 Infectivity of Dried Virus At 4 C. *Appl. Microbiol.* 13, 496–9.

351 Gao, R., Cao, B., Hu, Y., Feng, Z., Wang, D., Hu, W., Chen, J., Jie, Z., Qiu, H., Xu, K., Xu, X., Lu, H., Zhu,
352 W., Gao, Z., Xiang, N., Shen, Y., He, Z., Gu, Y., Zhang, Z., Yang, Y., Zhao, X., Zhou, L., Li, X., Zou,
353 S., Zhang, Y., Li, X., Yang, L., Guo, J., Dong, J., Li, Q., Dong, L., Zhu, Y., Bai, T., Wang, S., Hao, P.,
354 Yang, W., Zhang, Y., Han, J., Yu, H., Li, D., Gao, G.F., Wu, G., Wang, Y., Yuan, Z., Shu, Y., 2013.
355 Human infection with a novel avian-origin influenza A (H7N9) virus. *N. Engl. J. Med.* 368, 1888–
356 97.

357 Higashikawa, F., Chang, L., 2001. Kinetic analyses of stability of simple and complex retroviral
358 vectors. *Virology* 280, 124–31.

359 Jackson, A.C., 2013. Current and future approaches to the therapy of human rabies. *Antiviral Res.* 99,
360 61–7.

361 Kaku, Y., Noguchi, A., Marsh, G.A., Barr, J.A., Okutani, A., Hotta, K., Bazartseren, B., Fukushi, S.,
362 Broder, C.C., Yamada, A., Inoue, S., Wang, L.-F., 2012. Second generation of pseudotype-based
363 serum neutralization assay for Nipah virus antibodies: sensitive and high-throughput analysis
364 utilizing secreted alkaline phosphatase. *J. Virol. Methods* 179, 226–32.

365 Kishishita, N., Takeda, N., Anuegoonpipat, A., Anantapreecha, S., 2013. Development of a
366 pseudotyped-lentiviral-vector-based neutralization assay for chikungunya virus infection. *J.*
367 *Cinical Microbiol.* 51, 1389–95.

368 Loi, P., Matsukawa, K., Ptak, G., Clinton, M., Fulka, J., Nathan, Y., Arav, A., 2008. Freeze-dried somatic
369 cells direct embryonic development after nuclear transfer. *PLoS One* 3, e2978.

370 Lowen, A.C., Mubareka, S., Steel, J., Palese, P., 2007. Influenza virus transmission is dependent on
371 relative humidity and temperature. *PLoS Pathog.* 3, 1470–6.

372 Mather, S., Scott, S., Temperton, N., Wright, E., King, B., Daly, J., 2013. Current progress with
373 serological assays for exotic emerging/re-emerging viruses. *Future Virol.* 8, 745–755.

374 Molesti, E., Milani, A., Terregino, C., Cattoli, G., Temperton, N.J., 2013. Comparative serological
375 assays for the study of h5 and h7 avian influenza viruses. *Influenza Res. Treat.* 2013, 286158.
376 doi:10.1155/2013/286158

377 Noti, J.D., Blachere, F.M., McMillen, C.M., Lindsley, W.G., Kashon, M.L., Slaughter, D.R., Beezhold,
378 D.H., 2013. High humidity leads to loss of infectious influenza virus from simulated coughs.
379 *PLoS One* 8, e57485.

- 380 Papenburg, J., Baz, M., Hamelin, M.-È., Rhéaume, C., Carbonneau, J., Ouakki, M., Rouleau, I., De
381 Serres, G., Boivin, G., 2011. Evaluation of serological diagnostic methods for the 2009 pandemic
382 influenza A (H1N1) virus. *Clin. Vaccine Immunol.* 18, 520–2.
- 383 Pear, W.S., Nolan, G.P., Scott, M.L., Baltimore, D., 1993. Production of high-titer helper-free
384 retroviruses by transient transfection. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8392–6.
- 385 Scott, E.M., Woodside, W., 1976. Stability of pseudorabies virus during freeze-drying and media .
386 Stability of Pseudorabies Virus During Freeze-Drying and Storage : Effect of Suspending Media.
- 387 Scott, S., Molesti, E., Temperton, N., Ferrara, F., Böttcher-Friebertshäuser, E., Daly, J., 2012. The use
388 of equine influenza pseudotypes for serological screening. *J. Mol. Genet. Med.* 6, 304–8.
- 389 Shin, S., Salvay, D., Shea, L., 2010. Lentivirus delivery by adsorption to tissue engineering scaffolds. *J.*
390 *Biomed. Mater.* 93, 1252–1259.
- 391 Stoker, M., MacPherson, I., 1964. Syrian hamster fibroblast cell line BHK21 and its derivatives.
392 *Nature* 203, 1355–7.
- 393 Temperton, N.J., Hoschler, K., Major, D., Nicolson, C., Manvell, R., Hien, V.M., Ha, D.Q., de Jong, M.,
394 Zambon, M., Takeuchi, Y., Weiss, R. a, 2007. A sensitive retroviral pseudotype assay for
395 influenza H5N1-neutralizing antibodies. *Influenza Other Respi. Viruses* 1, 105–12. d
- 396 Temperton, N.J., Wright, E., 2009. Retroviral Pseudotypes, in: *Encyclopedia of Life Sciences.* John
397 Wiley & Sons, Chichester.
- 398 Terregino, C., Toffan, A., Cilloni, F., Monne, I., Bertoli, E., Castellanos, L., Amarin, N., Mancin, M.,
399 Capua, I., 2010. Evaluation of the protection induced by avian influenza vaccines containing a
400 1994 Mexican H5N2 LPAI seed strain against a 2008 Egyptian H5N1 HPAI virus belonging to
401 clade 2.2.1 by means of serological and in vivo tests. *Avian Pathol.* 39, 215–22.
- 402 To, K.K.W., Tsang, A.K.L., Chan, J.F.W., Cheng, V.C.C., Chen, H., Yuen, K.-Y., 2014. Emergence in China
403 of human disease due to avian influenza A(H10N8) - Cause for concern? *J. Infect.* 68, 205–215.
- 404 Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J.,
405 Chen, L.-M., Johnson, A., Tao, Y., Dreyfus, C., Yu, W., McBride, R., Carney, P.J., Gilbert, A.T.,
406 Chang, J., Guo, Z., Davis, C.T., Paulson, J.C., Stevens, J., Rupprecht, C.E., Holmes, E.C., Wilson, I.
407 a, Donis, R.O., 2013. New world bats harbor diverse influenza A viruses. *PLoS Pathog.* 9,
408 e1003657. doi:10.1371/journal.ppat.1003657
- 409 Tyrrell, D.A., Ridgwell, B., 1965. Freeze-drying of certain viruses. *Nature* 206, 115–6.
- 410 Wei, S.-H., Yang, J.-R., Wu, H.-S., Chang, M.-C., Lin, J.-S., Lin, C.-Y., Liu, Y.-L., Lo, Y.-C., Yang, C.-H.,
411 Chuang, J.-H., Lin, M.-C., Chung, W.-C., Liao, C.-H., Lee, M.-S., Huang, W.-T., Chen, P.-J., Liu, M.-
412 T., Chang, F.-Y., 2013. Human infection with avian influenza A H6N1 virus: an epidemiological
413 analysis. *Lancet Respir. Med.* 1, 771–778.
- 414 WHO, 2010. Pandemic (H1N1) 2009 Update 112. Global Alert and Response. Geneva.
- 415 WHO, 2013. Rabies Fact Sheet No. 99. Geneva.

- 416 Wilschut, J., de Jonge, J., Huckriede, A., Amorij, J.-P., Hinrichs, W.L.J., Frijlink, H.W., 2007.
417 Preservation of influenza virosome structure and function during freeze-drying and storage. *J.*
418 *Liposome Res.* 17, 173–82.
- 419 Wright, E., Hayman, D.T.S., Vaughan, A., Temperton, N.J., Wood, J.L.N., Cunningham, A.A., Suu-Ire,
420 R., Weiss, R.A., Fooks, A.R., 2010. Virus neutralising activity of African fruit bat (*Eidolon helvum*)
421 sera against emerging lyssaviruses. *Virology* 408, 183–9.
- 422 Wright, E., McNabb, S., Goddard, T., Horton, D.L., Lembo, T., Nel, L.H., Weiss, R.A., Cleaveland, S.,
423 Fooks, A.R., 2009. A robust lentiviral pseudotype neutralisation assay for in-field
424 serosurveillance of rabies and lyssaviruses in Africa. *Vaccine* 27, 7178–86.
- 425 Wright, E., Temperton, N.J., Marston, D.A., McElhinney, L.M., Fooks, A.R., Weiss, R. a, 2008.
426 Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: a cross-
427 species comparison. *J. Gen. Virol.* 89, 2204–13.
- 428 Yuen, K.Y., Chan, P.K., Peiris, M., Tsang, D.N., Que, T.L., Shortridge, K.F., Cheung, P.T., To, W.K., Ho,
429 E.T., Sung, R., Cheng, A.F., 1998. Clinical features and rapid viral diagnosis of human disease
430 associated with avian influenza A H5N1 virus. *Lancet* 351, 467–71.
- 431 Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L., Trono, D., 1997. Multiply attenuated lentiviral vector
432 achieves efficient gene delivery in vivo. *Nat. Biotechnol.* 15, 871–5.

433 **Figure Legends**

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

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

442 Figure 3: Effect of lyophilisation and pellet incubation on infectivity of H5 pseudotyped lentiviral
443 vectors. Freeze-dried PVs displaying H5 A/Vietnam/1194/2004 HA were stored in either 1M, 0.5M or
444 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2
445 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype
446 transduction titres are displayed as mean \pm SD of relative luminescent units per ml (RLU/ml).

447 Figure 4: Effect of lyophilisation and pellet incubation on infectivity of RABV pseudotyped lentiviral
448 vectors. Freeze-dried PVs displaying RABV ERA G glycoproteins were stored in either 1M, 0.5M or
449 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week, (B) 2
450 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype
451 transduction titres are expressed as mean \pm SD of relative luminescent units per ml (RLU/ml).

452 Figure 5: Effect of lyophilisation and pellet incubation on infectivity of MARV pseudotyped lentiviral
453 vectors. Freeze-dried PVs displaying MARV Lake Victoria GP glycoproteins were stored in either 1M,
454 0.5M or 0M sucrose-PBS cryoprotectant at a variety of temperatures and humidities for (A) 1 week,
455 (B) 2 weeks and (C) 4 weeks before reconstitution and employment in a titration assay. Pseudotype
456 transduction titres are shown as mean \pm SD of relative luminescent units per ml (RLU/ml).

457 Figure 6. Influence of reconstitution solution on H5, RABV and MARV pseudotyped lentiviral vectors.
458 Freeze-dried PVs with influenza H5 A/Vietnam/1194/2004 HA, RABV ERA G or MARV Lake Victoria
459 GP envelope glycoproteins were reconstituted in either distilled, nuclease-free H₂O or supplemented
460 DMEM culture medium before utilisation in a titration assay. Pseudotype transduction titres are
461 shown as mean \pm SD of relative luminescent units per ml (RLU/ml). Unlyophilised pseudotype
462 positive controls, and Δ envelope glycoprotein, HEK293T/17 cell only and BHK-21 cell only negative
463 controls are also shown.

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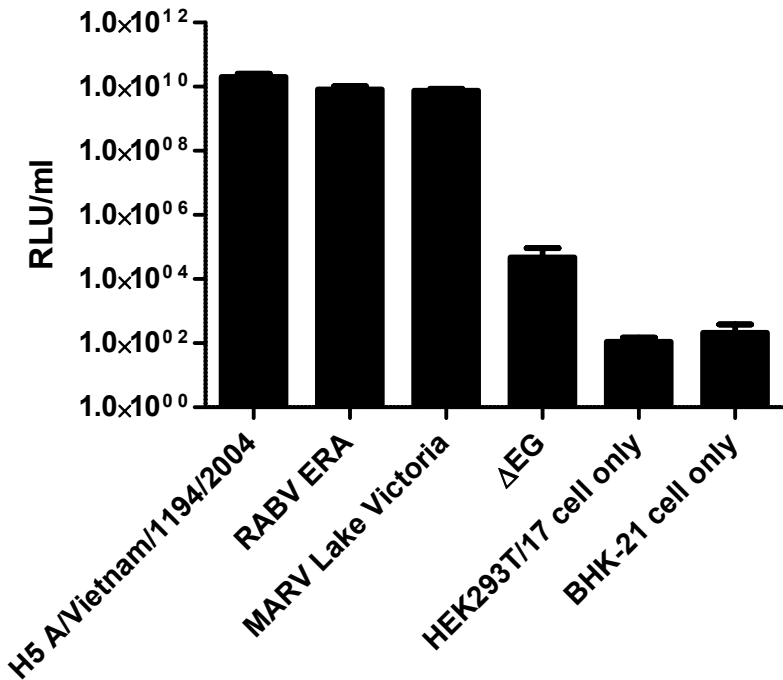
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470 **Tables**

	Pseudotype virus (PV)	IC₅₀	IC₉₀
471			
472			
473	H5		
474	A/Vietnam/1194/2004 - lyophilised	2560-5120 (4679)	1280-2560 (1390)
475	H5		
476	A/Vietnam/1194/2004 - unlyophilised	2560-5120 (3919)	1280-2560 (1345)
477	RABV ERA – lyophilised	40960-81920 (44072)	5120-10240 (6381)
478	RABV ERA – unlyophilised	20480-40960 (27324)	5120-10240 (6222)
479			

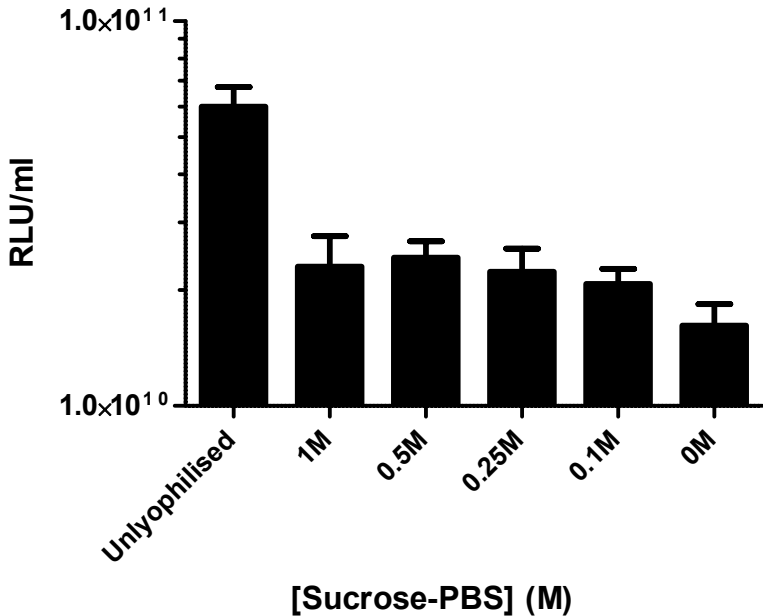
480 Table 1: Comparison of neutralising antibody titres against untreated and lyophilised pseudotyped
 481 lentiviruses. Half maximal inhibitory concentration (IC₅₀) and 90% inhibitory concentration (IC₉₀)
 482 values of confirmed antibody-positive antisera against H5 influenza and RABV pseudotypes before
 483 and after lyophilisation. VNAb titres were calculated using GraphPad Prism 5 software and are
 484 displayed as serum assay dilutions. Exact IC₅₀ and IC₉₀ values are also shown in parentheses.

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

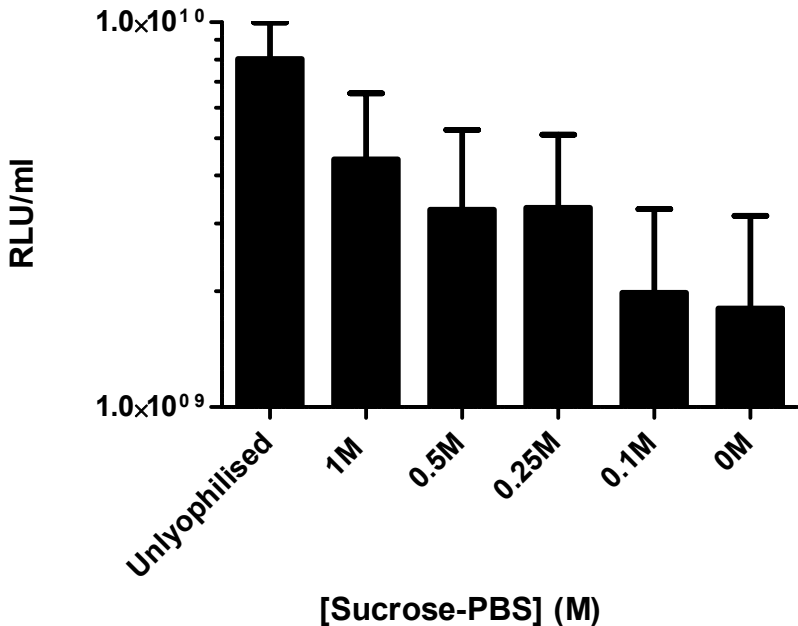


H5 A/Vietnam/1194/2004

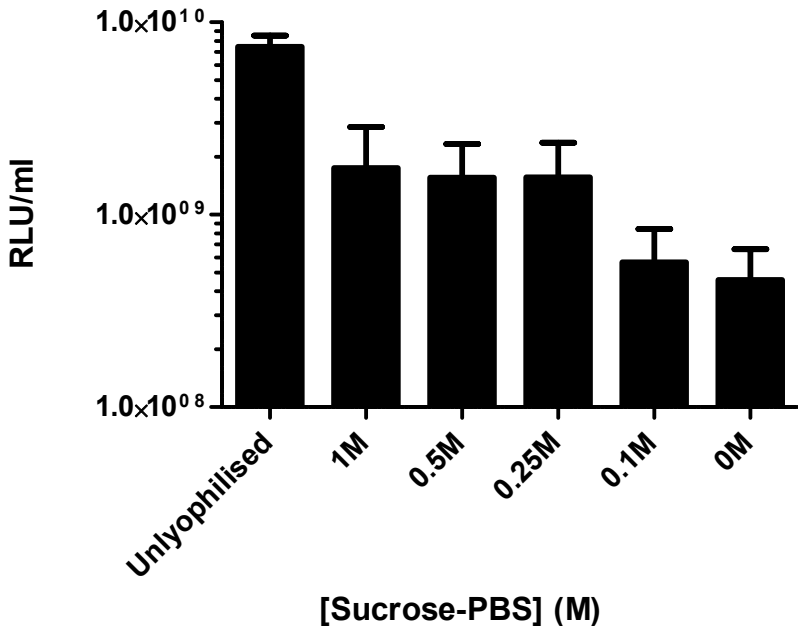
Sucrose gradient



RABV ERA Sucrose gradient

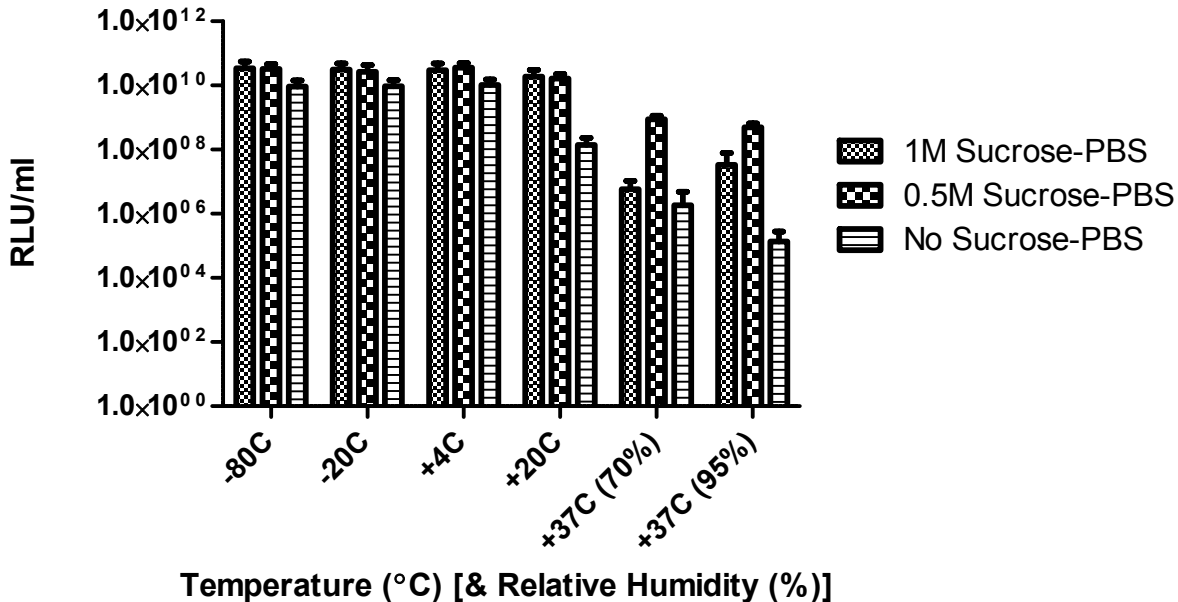


MARV Lake Victoria Sucrose gradient



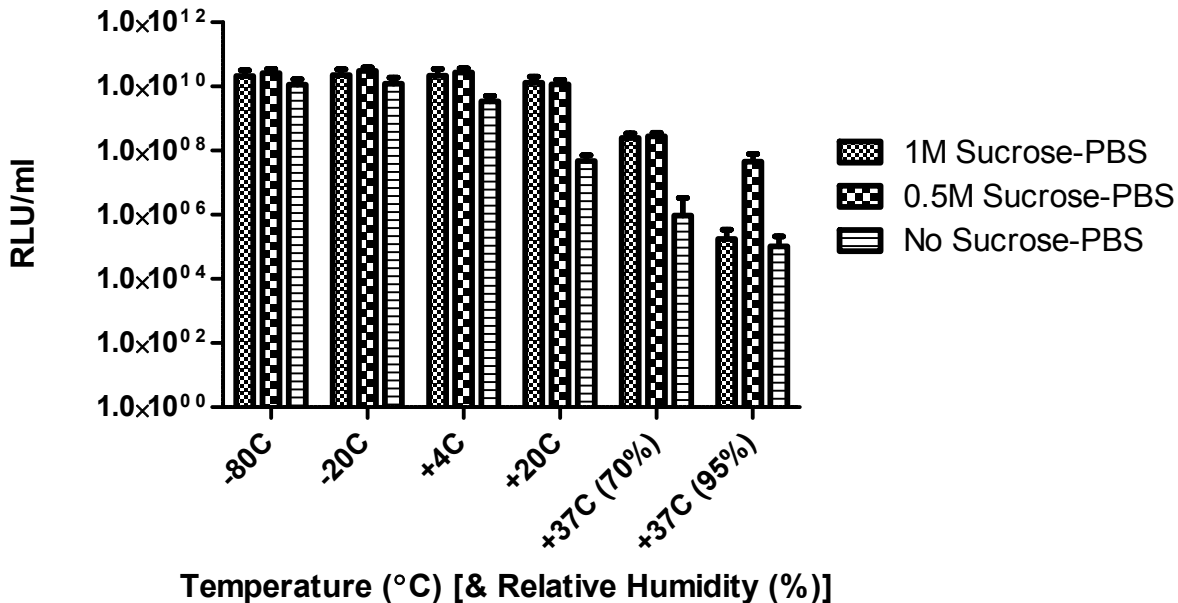
H5 A/Vietnam/1194/2004

1 week incubation



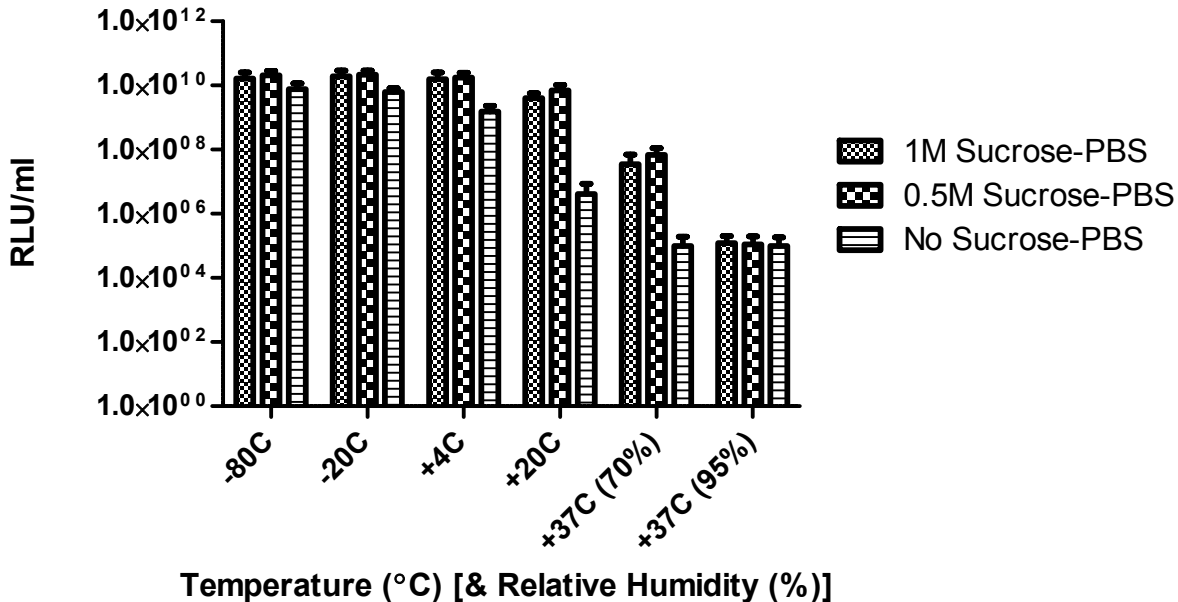
H5 A/Vietnam/1194/2004

2 week incubation



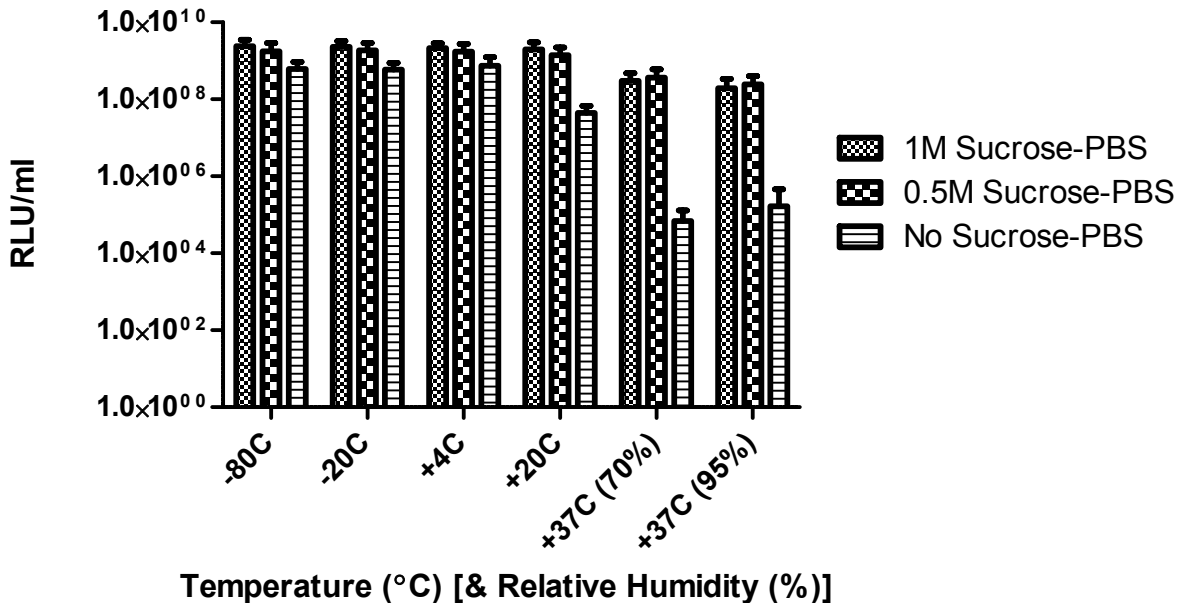
H5 A/Vietnam/1194/2004

4 week incubation



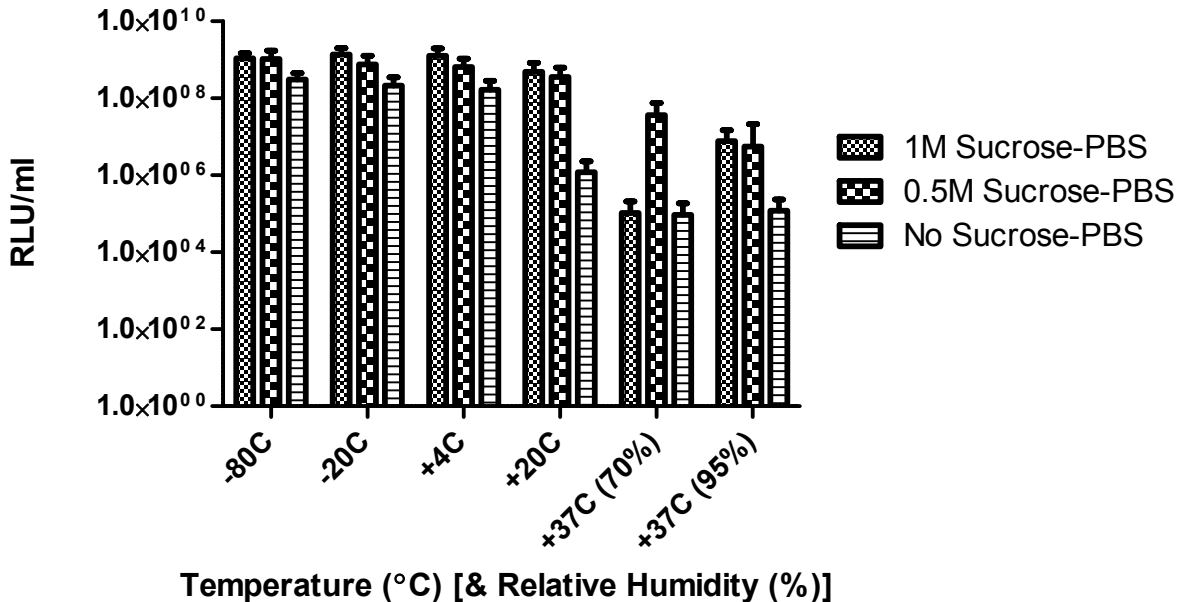
RABV ERA

1 week incubation



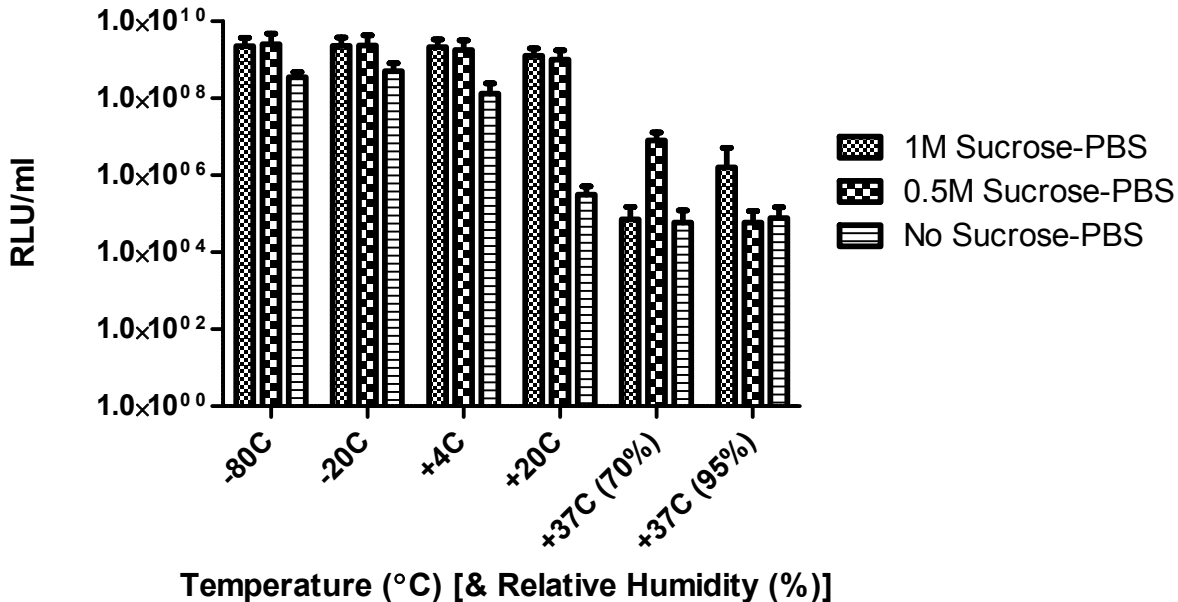
RABV ERA

2 week incubation



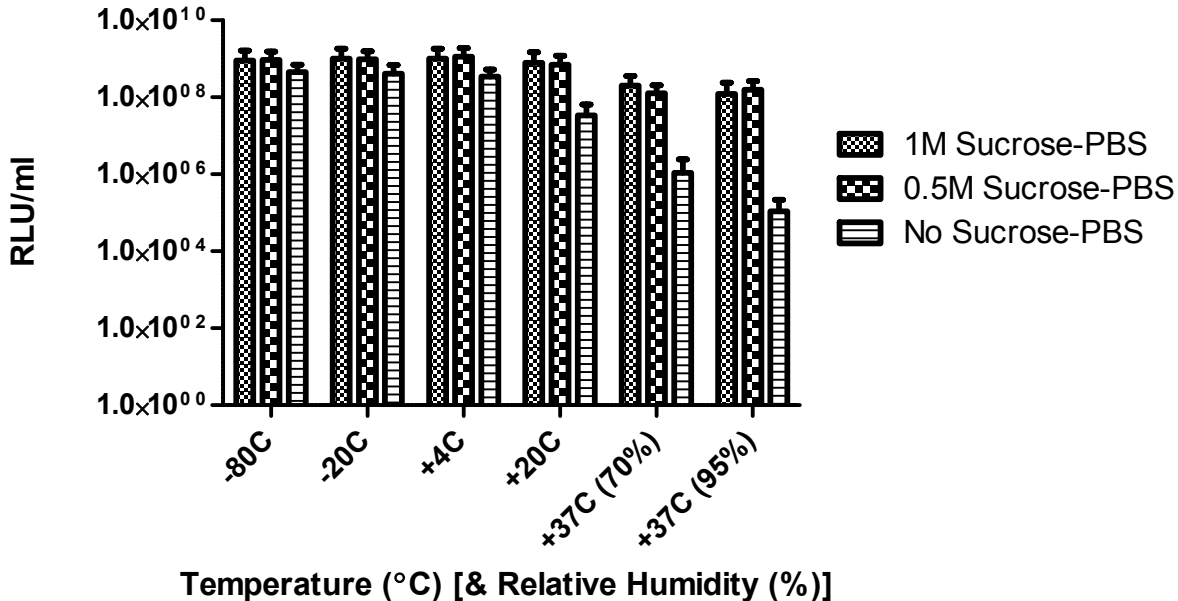
RABV ERA

4 week incubation



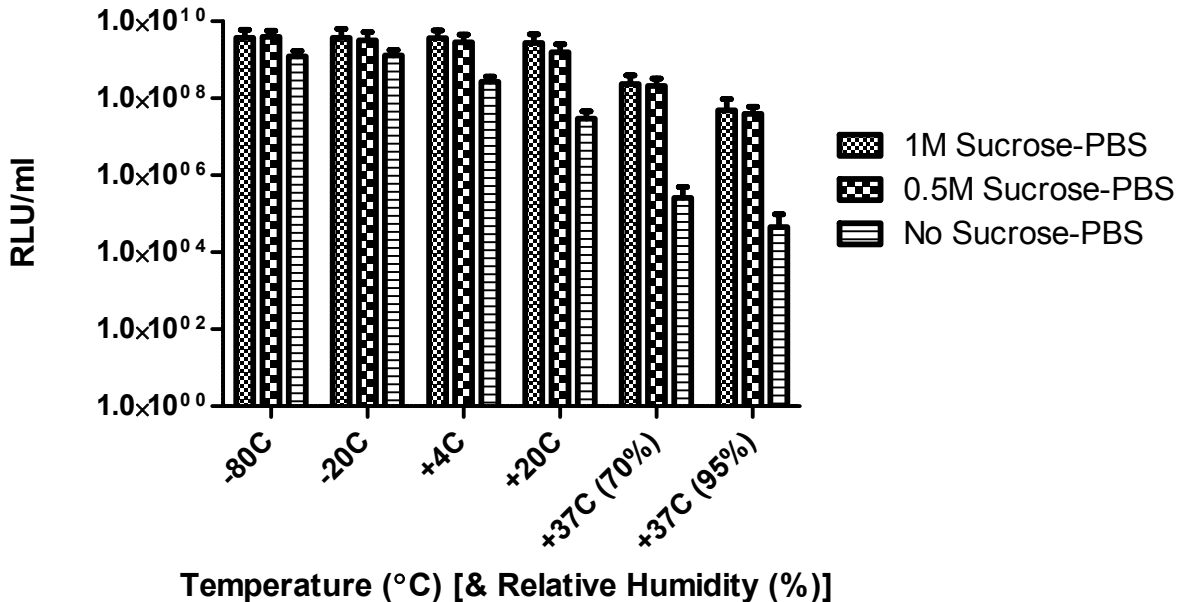
MARV Lake Victoria

1 week incubation

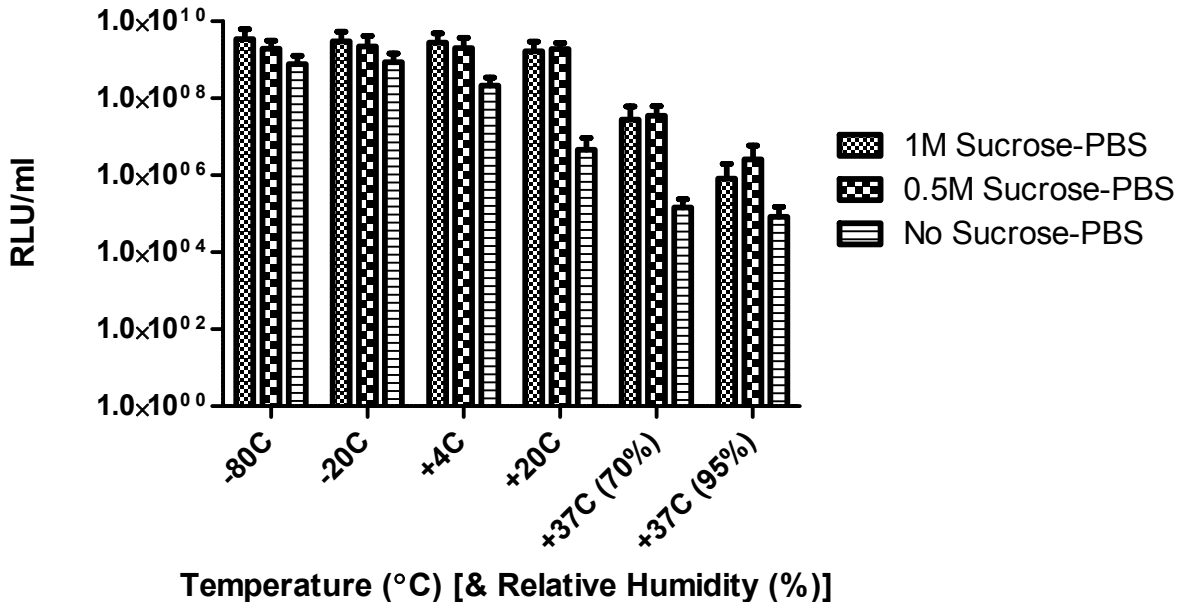


MARV Lake Victoria

2 week incubation



MARV Lake Victoria 4 week incubation



H5 A/Vietnam/1194/2004, RABV ERA and MARV Lake Victoria H₂O and DMEM reconstitution

