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1 An optimised method for the production of MERS-CoV spike expressing viral pseudotypes

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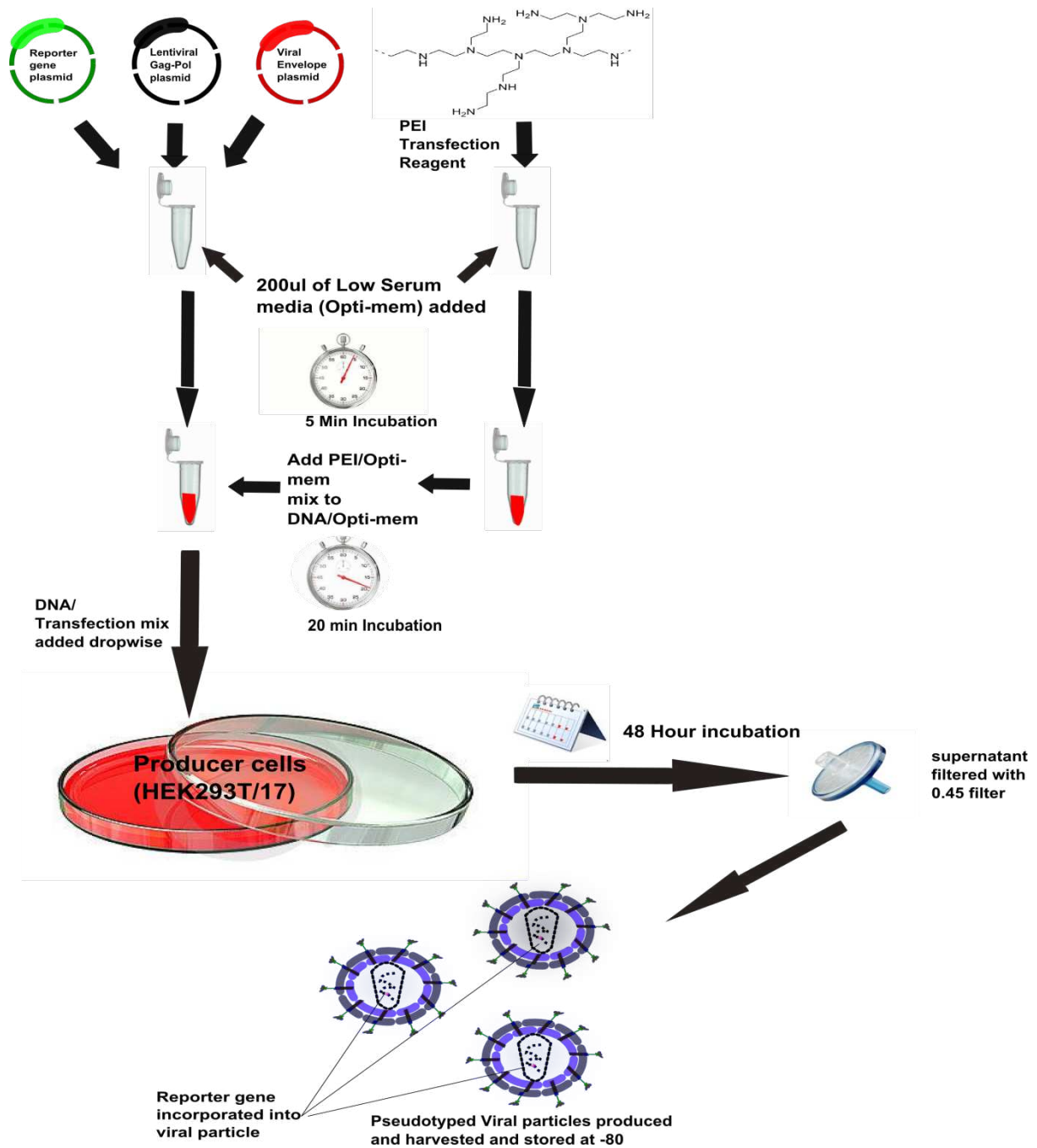
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6 Method Name: Optimised MERS pseudotype production

7

8 Visual Abstract



11 **Abstract**

12 The production and use of pseudotyped viral particles is widely established for many viruses, and
13 applications in the fields of serology and vaccine development are manifold. Viral pseudotypes have
14 proven to be powerful tools to study the effects of viral evolution on serological outcomes, viral
15 tropism and immunogenicity studies [1–7]. Pseudotyped viruses are chimeric constructs in which the
16 outer (surface) glycoprotein(s) of one virus is combined with the replication-defective viral “core” of
17 another virus [8, 9]. Pseudotypes allow for accurate, sequence-directed, sensitive antibody
18 neutralization assays and antiviral screening to be conducted within a low biosecurity facility and offer
19 a safe and efficient alternative to wildtype virus use [8].

20 The protocol outlined here represents a rapid and reliable method for the generation of high-titre
21 pseudotype viral particles with the MERS-CoV spike protein on a lentiviral core, and is adapted from
22 previously published protocols [1, 7, 10]. This protocol is optimised for transfection in a 100mm petri
23 dish with 7ml of supernatant harvested, however it can be readily scaled to different production
24 volumes.

25
26 This protocol has a number of advantages including.

- 27 1. Use of readily available reagents
- 28 2. Consistent, high virus titres
- 29 3. Rapid generation of novel glycoproteins for research into strain variation

30 31 **Method Details**

32 33 Materials and equipment

- 34
- 35 • HEK 293T/17 cells (ATCC® CRL-11268™)
- 36 • Dulbecco’s modified Eagle medium with Glutamax (Cat. No. 31966-021) Supplemented with
37 10% Foetal bovine serum and 1% penicillin/Streptomycin (P/S)
- 38 • Trypsin-EDTA (0.05%), phenol red (Cat. No. 25300-054)
- 39 • Gibco Reduced Serum media Opti-MEM® (Cat. No. 31985-047)
- 40 • Optional: TC20™ Automated Cell Counter (Cat. No. 145-0102EDU)
- 41 • Branched Polyethyleneimine solution at concentration of 1mg/ml (Cat. No. 408727)
- 42 • Sterile syringes (10ml)
- 43 • Millex-HA 0.45 µm filters (Cat. No. SLHAM33SS)
- 44 • Rabbit Polyclonal Antibody to Novel coronavirus (HCoV-EMC/2012) Spike protein
45 (SinoBiological Cat. No. 40069-RP02)
- 46 • Nunc® UpCell™ Surface cell culture dish (Manufacturer No. 174902)
- 47 • Microcentrifuge tube Safe-Lock write-on graduated with lid latch 1.5mL

48
49 Note: All steps should be carried out in a class II biosafety cabinet to avoid contamination.

50 51 Plasmids

52
53 Glycoprotein expression plasmid: pCAGGS-MERS-CoV Spike (Note: The MERS-CoV Spike protein
54 should be codon optimised)

55 Lentiviral vector plasmid expressing firefly luciferase: pCSFLW [11]

56 Second-generation lentiviral packaging construct plasmid: p8.91 (expressing HIV-gag) [12]

57

58 **Transfection Steps**

59

60 **Timeline: Transfection -24 hours.**

61

62 1. 293T/17 cells should be subcultured into 100mm petri dishes at a ratio that will yield 70-90%
63 confluence at the time of transfection. In our hands this protocol yields similar results
64 regardless of petri dish size when supernatant yield is equivalent.

65

66 **Timeline: Day of Transfection.**

67

68 2. DMEM/10% FBS/1% P/S and Opti-MEM® should be pre-warmed to 37°C using a water bath or
69 similar.

70 3. Prepare and label two sterile 1.5ml microcentrifuge tubes (tube 1 and tube 2) per transfection.

71 4. Add the following plasmids (0.9:1:1.5 envelope:core:vector ratio) for transfection to tube 1:

72 a. pCAGGS-MERS-CoV spike: 0.9 µg

73 b. p8.91-lentiviral vector: 1.0 µg

74 c. pCSFLW: 1.5 µg

75 5. Add 200µl Opti-MEM® to the plasmid DNA mix (tube 1).

76 6. Add 200µl Opti-MEM® and 35 µl of 1mg/ml PEI to tube 2.

77 7. Incubation Step. Mix both tubes by gently flicking and incubate for 5 min at room temperature
78 (RT).

79 8. After incubation, pipette the Opti-MEM®/PEI solution from tube 2 into the Opti-MEM®/DNA
80 solution in tube 1.

81 9. Incubation Step. Gently flicking the tube to mix every 3-4 min, incubate the tube at RT for 20
82 min.

83 10. While transfection mix is incubating, the culture media on the 293T/17 cells should be removed
84 and 7ml of fresh DMEM/10% FBS/1% P/S added. It is important at this point to add media
85 slowly to one side of the dish to avoid detaching adherent cells.

86 11. After 20 min incubation, pipette the DNA/Opti-MEM®/PEI solution onto the 293T/17 cells by
87 adding dropwise over the complete area of the plate. Swirl the plates gently to ensure even
88 dispersal.

89 12. Incubation Step. Incubate the plate at 37°C, 5% CO₂ overnight (o/n). In our hands incubation
90 times of between 12-16 hours result in equivalent final pseudotype production titres.

91

92 **Timeline: 12-16 hours post transfection**

93

94 13. Post o/n incubation the media on the cells should be changed and 7ml fresh DMEM/10%
95 FBS/1% P/S added. Add media slowly to one side of the plate to avoid cell detachment.

96 14. Incubate the plates 37°C 5% CO₂ o/n for 32-36 hours.

97

98 **Timeline: 44-52 hours post transfection**

99

100 15. Supernatant containing the viral pseudotype particles are harvested using a 10ml sterile
101 syringe and then filtered into falcon tubes via a syringe driven Millex HA-0.45µm filter.

102 16. Store all filtered supernatant at -80°C. It is recommended that supernatant is stored as aliquots
103 to avoid multiple freeze thaw cycles. **Note:** Supernatant may be stored at 4°C for up to one
104 week with no detectable loss of titre.

105

106 17. **Optional Step:** Additional media may be added to cells to allow a second harvest 18-24 hours
107 later by adding further DMEM/10% FBS/1% P/S. In this case extreme care must taken in initial
108 pseudotype collection (step 15) to avoid damage to cell monolayer. We have observed that
109 cells in poor health after first harvest yield significantly less viral particles upon second harvest.

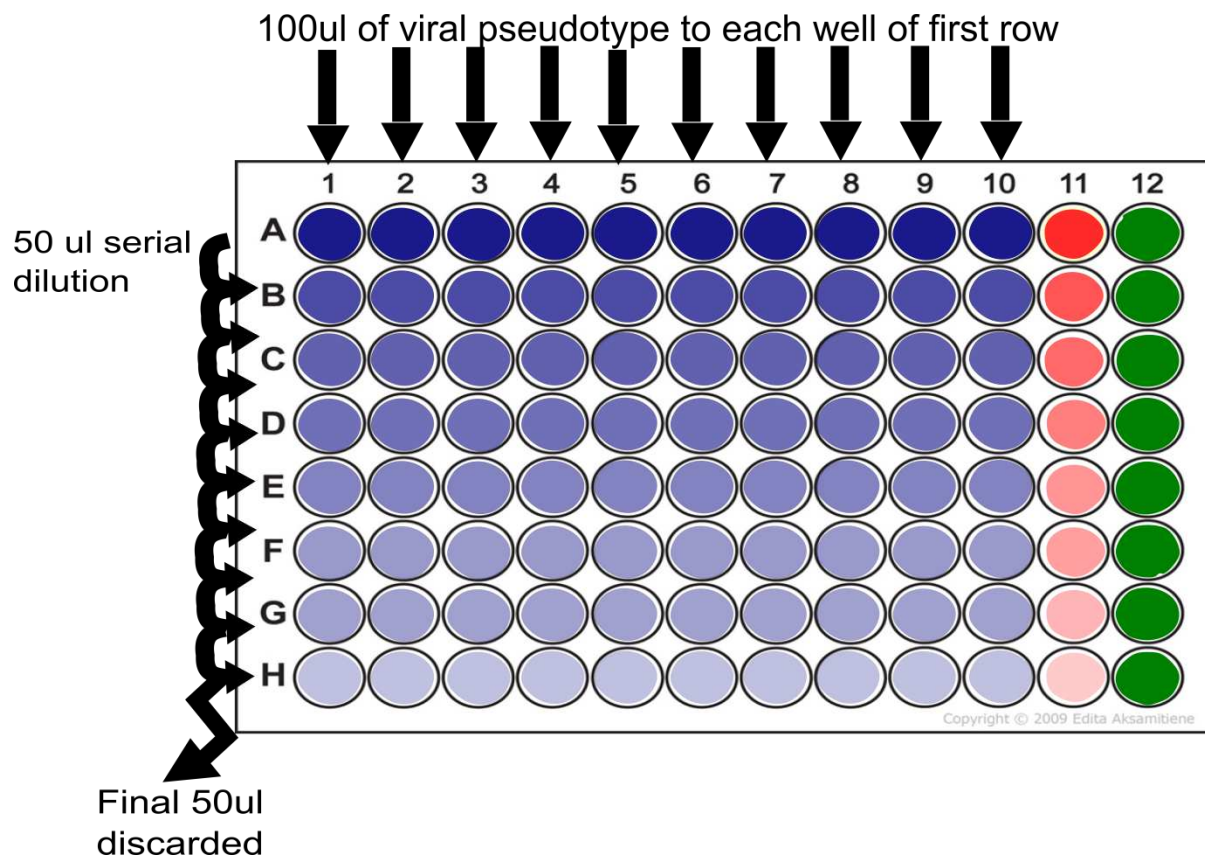
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111 **Note:** A control pseudotype virus may be created by following the steps outlined above but leaving
112 out the pCAGGS-MERS-CoV spike construct. This produces particles that do not express the viral
113 surface glycoprotein and therefore should be unable to transduce target cells (Δ -env control).

114 Titration Steps (Figure 1)

115 **Note:** Titration consists of transduction of reporter (in this case firefly luciferase) into target
116 cells mediated by the viral glycoprotein expressed on the viral pseudotype. Controls for
117 titrations are provided via the inclusion of “cell only” and “ Δ -env” columns.

- 118 1. In a 96 well white plate add 50 μ l of DMEM/10% FBS/1% P/S to the entire column of “cell
119 only” control (see Figure 1 column 12).
- 120 2. Add 50 μ l of DMEM/10% FBS/1% P/S from row B to H that are to contain pseudotyped virus
121 or Δ -env control.
- 122 3. Add 100 μ l of MERS pseudotype virus supernatant to each well of row A (excluding control
123 columns) and add 100ul of Δ -env to column 11 (See Figure 1).
- 124 4. Remove 50 μ l from row 1 virus-containing wells and perform 1:2 serial dilutions down all wells
125 below.



126

127 Figure 1: Serial dilution step showing addition of 100ul of pseudotype virus supernatant to each well
128 of row A and dilution of 50ul taken from this well to row B. This process is continued to end of plate
129 (row H) at which point the final 50ul is discarded. Δ-env control is indicated in red (column 11) and cell
130 only control is indicated in green (column 12).

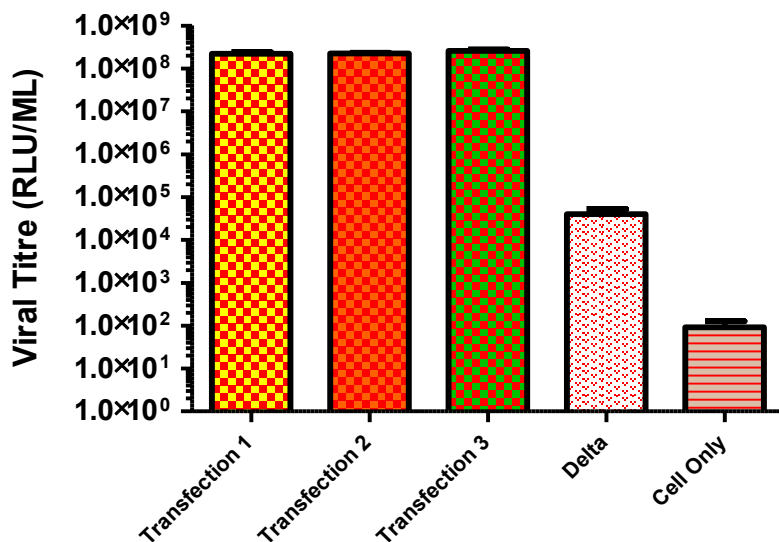
- 131 5. With each dilution step use pipette to mix 8 times up and down.
- 132 6. After completing serial dilution the final 50 μl from the last well of each column should be
133 discarded. **Note:** at this point each well should contain 50μl of mixed DMEM and viral
134 supernatant.
- 135 7. Prepare a plate of susceptible target cells (Huh-7) (preferentially subcultured 1:4 48 hours
136 before):
- 137 a. Remove media from plate
 - 138 b. Wash the plate with 2 ml of trypsin and discard trypsin
 - 139 c. Add additional 2 ml of trypsin to the plate to detach cells
 - 140 d. After cells have detached add DMEM/10% FBS/1% P/S to the plate to quench trypsin
141 activity
 - 142 e. Count cells using TC20™ Automated Cell Counter or haemocytometer and add 1×10^4
143 cells in a total volume of 50 μl to each well
- 144 8. Centrifuge plate for 1 minute at 500rpm if there are droplets on the sides of the wells
- 145 9. Incubate the plate for 48 hours at 37°C 5% CO₂
- 146 10. Read plate using Bright Glo™ Luciferase assay system or equivalent

147

148

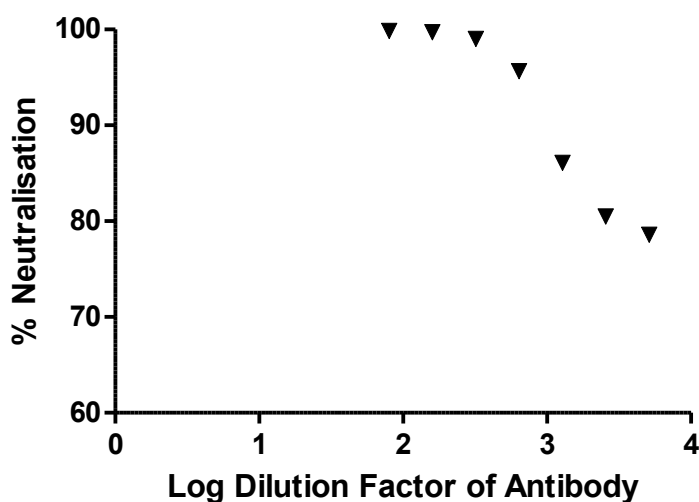
149 Method validation and transfection results

150 Figure 2 displays data recorded from multiple transfections indicating consistency of results. Results
151 are measured in Relative Luminescence Units (RLUs) as measured using a GloMax® 96 Microplate
152 Luminometer and the Bright Glo™ Luciferase assay system. The pseudotype particles generated in
153 the absence of viral envelope (Delta) show increased luciferase activity compared to cell only in part
154 due to transformation method used to discern RLU per ML. The presence of some carry-over
155 luciferase within viral particles is also likely to generate an increase in RLU values recorded.



156 Figure 2: Pseudotype production titres from three replicates of optimised transfection protocol using codon optimised MERS-
157 CoV Spike.
158

159 Figure 3 shows percentage neutralization of the MERS-CoV pseudotype with commercially produced
160 anti-MERS spike antibody. Figure clearly indicates that as the dilution factor increases, so the
161 percentage neutralization decreases, 100% neutralization indicates that RLU values at this
162 concentration are equivalent to a delta envelope control.



163
164 Figure 3: Anti-MERS-Spike Antibody (Rabbit Polyclonal Antibody to Novel coronavirus (HCoV-EMC/2012) Spike protein)
165 neutralizes MERS viral pseudotype entry into Huh7 cells.

166
167
168

	Transfection 1	Transfection 2	Transfection 3	Cell Only
Mean/ml Titre	2.2E+08	2.3E+08	2.6E+08	91

169 Table 1: Mean RLU calculated per ml of viral supernatant for three pseudotype production runs. The protocol outlined here
170 provides a rapid and consistent method for the generation of high-titre viral pseudotype particles expressing the MERS-CoV
171 spike protein suitable for further downstream applications. Efficient knock-down of pseudotype virus entry using a polyclonal
172 antibody directed against the spike glycoprotein (Figure 3) demonstrates potential utility for vaccine immunogenicity and
173 Mab/antiviral screening. The use of readily available reagents should facilitate increased reproducibility.

174 Keywords

175 MERS coronavirus; lentiviral pseudotype; virus neutralization

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