Chapter 9

Optimization of small-scale production of Zika virus envelope glycoprotein by transient expression in HEK293 cells for ELISA

Young Chan Kim[1,2] (young.kim@some.ox.ac.uk)

Maud Dumoux[3] (maud.dumoux@diamond.ac.uk)

Raymond J Owens[2,4] (ray@strubi.ox.ac.uk)

Arturo Reyes-Sandoval[1]* (arturo.reyes@ndm.ox.ac.uk).

[1] The Jenner Institute, Nuffield Department of Medicine, University of Oxford, The Henry Wellcome Building for Molecular Physiology, Roosevelt Drive, Oxford, UK

[2] Division of Structural Biology, University of Oxford, Wellcome Centre for Human Genetics, Roosevelt Drive, Oxford, UK

[3] Diamond Light Source Ltd., Diamond House, Harwell Campus, Didcot, UK

[4] Research Complex at Harwell, Rutherford Appleton Laboratory, Harwell Campus, Didcot UK

* Corresponding author: Arturo Reyes-Sandoval. Email: arturo.reyes@ndm.ox.ac.uk. The Jenner Institute, University of Oxford, Old Road Campus Research Building. Roosevelt Drive, Oxford, OX3 7DQ, UK. Phone: +44 (0) 1865 287811.

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Abstract

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus which has recently caused global epidemics with its association with congenital Zika syndrome such as severe microcephaly. The recombinant ZIKV envelope (Env) glycoprotein is useful for immunological applications such as serodiagnosis of ZIKV infection and for monitoring immune responses in preclinical and clinical ZIKV vaccine developments. In this chapter, we describe the optimization of production of Zika virus envelope glycoprotein in Human Embryonic Kidney (HEK 293T) cells by small-scale expression followed by large-scale protein production. Small-scale expression of HEK 293T cells allows screening of a large number of vectors simultaneously to select the vectors with best secretory profiles for scale-up in Expi293 mammalian system to maximise the protein yield followed by purification for research and clinical applications.

Keywords: Zika virus, envelope protein, HEK 293, CD4 fusion tag, protein production, protein purification.

1. Introduction

Optimum expression of proteins with high purity levels can be useful reagents for a wide array of applications such as immunological assays, protein-based vaccines or therapeutics and structural studies. Protein expression in mammalian cells is preferred to *E. coli* cells for correct post-translational modifications. Various commercially available Zika virus (ZIKV) envelope (Env) glycoproteins are expressed in insect cells, reaching a purity of approximately 85% but yet they are not easily affordable in developing countries endemic with ZIKV.

In this chapter, protocols for the production, purification and characterisation of secreted ZIKV Env glycoproteins by transient transfection of HEK 293 cells and optimisation for its use in ELISA for ZIKV serodiagnosis are described. The methods are exemplified by reference to the work by Kim et al [1]. ZIKV Env is the primary target for neutralizing antibodies during ZIKV infection and involved in the receptor binding and viral entry into host cells [2, 3]. In order to produce recombinant ZIKV Env glycoproteins for ZIKV serodiagnosis and monitoring of humoral responses in both mice and humans vaccinated with Env-based vaccines, the secretory profile of various constructs (prM-Env, Env only, and truncations of Env) with or without a rat CD4 fusion tag at the C-terminus were tested in small-scale expression screening to select the construct with the best secretion profile. The best secretion vector was chosen to be expressed at larger scale using the Expi293 mammalian transient expression system and purified by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography (SEC) to obtain highly purified fractions containing ZIKV envelope proteins (Env-CD4 and Env) as seen in the Figure 1. These ZIKV Env-CD4 recombinant proteins were characterised by western blotting (WB) using a pan flavivirus antibody, the sera from ChAdOx1 ZIKV prME_ΔTM vaccinated mice [4] and a ZIKV Env monoclonal antibody (Figure 2). ZIKV Env recombinant protein was then used as coating reagent for ELISA assay using sera from ZIKV-infected patients to detect patient-derived ZIKV antibodies and optimised for sero-diagnosis of ZIKV infection (Figure 3).

2. Materials

2.1 Small-Scale Cell Transfection

1. HEK 293T cells.

2. Cell culture medium: Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Foetal Calf Serum (FCS), non-essential amino acids (1:100) and L- Glutamine (1:100).

3. Trypsin- Ethylene dinitrilotetra-acetic acid (Trypsin- EDTA).

4. Phosphate buffered saline (PBS): 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4.

5. T175 tissue culture flask.

6. Plasmid DNA (*see* **Note 1**) with an A_{260} /A_{280} ratio of greater than 1.8 (*see* **Note 2**).

7. Transfection reagent.

8.V-well micro-titre plate.

9. 24-well tissue culture plate.

10. Tissue culture incubator at 37°C and 5% CO_2.

2.2 Large-Scale Cell Transfection

1. Expi293™ cells.

2. Expi293™ Expression Medium.

3. OPTI-MEM medium.

4. Expifectamine™ transfection reagent. (*see* **Note 3**).

5. Enhancer 1 and 2.

2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting (WB)

1. 2× SDS-PAGE sample loading buffer: 100 mM Tris-HCl pH 6.7, 40 mg/ml sodium dodecyl sulphate (SDS), 2 mg/ml bromophenol blue, 20% v/v glycerol, in water.

2. Heating block

3. SDS-PAGE MOPS running buffer.

4. SDS-PAGE gel.

5. Nitrocellulose or PVDF membrane.

6. Protein stain.

7. Phosphate buffered saline with Tween (PBST): 0.01 M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, 0.05% v/v Tween 20, pH 7.4.

8. 5% milk in PBST

9. Primary antibody solution: 1:2000 Anti-His_6 mouse monoclonal antibody or 1:500 dilutions of a mouse anti-flavivirus group antigen monoclonal antibody (Millipore) or 1:500 dilutions of mice sera following a vaccination with ChAdOx1 ZIKV prME_ΔTM [4] or 1:1000 anti-Zika Env monoclonal antibody (mouse mAb to Zika Env protein, Aalto BioReagents).

10. Secondary antibody solution: 1:5,000 Anti Mouse-Goat IgG Peroxidase conjugate.

11. Western blotting detection system.

2.4 Large Scale Glycoprotein Purification

1. 5 ml HisTrap FF column.

2. HiLoad 16/600 Superdex 200 column.

3. ÄKTA purification system such as ÄKTAxpress™

4. Nickel Wash buffer: 50mM Tris-HCl, 500mM NaCl, 30mM imidazole, pH 7.5 (*see* **Note 4**).

5. Nickel Elution Buffer: 50mM Tris-HCl, 500mM NaCl, 500mM imidazole, pH 7.5.

6. Size Exclusion Buffer: 20mM Tris-HCl, 200mM NaCl, pH 7.5.

7. 96 deep well plate

2.5 Enzyme-linked immunosorbent assay (ELISA)

1. 96-well Elisa plates.

2. Phosphate buffered saline (PBS): 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4.

3. Phosphate buffered saline with Tween (PBST): 0.01 M phosphate buffer, 0.0027M potassium chloride, 0.137M sodium chloride, 0.05% v/v Tween 20, pH 7.4.

4. Protein-Free Blocking Buffers.

5. Anti-Human IgG-alkaline phosphatase-conjugated antibody.

6. Buffer solution: Prepare pNPP substrate at 1mg/mL by dissolving each pNPP tablet in 20ml Diethanolamine Substrate Buffer. Leave to dissolve at RT in the dark for one hour.

7. Elisa plate reader.

8. Naïve control sera from healthy volunteers.

9. Zika patient samples.

3. Methods

Small-scale expression allows for rapid assessment of expression level from a large number of plasmids in parallel. This parallel expression screening in HEK 293T was used in the work of Kim et al [1] to screen constructs encoding different lengths of ZIKV prM-Env and Env. A protocol for the comparative small-scale expression screening of plasmids using HEK 293T cells is given in Section 3.1 followed by methods for sample preparation and Western blot analysis of secreted product in Section 3.2. The expression vectors showing the best secretion profile can then be scaled-up for production and purification in Section 3.3 and 3.4.

3.1 Transfection of Cells in 24 well plates for small-scale expression screening

1. Seed 1 ml of HEK 293T cells into a 24-well tissue culture plate at a density ~ 1.5 $\times 10^5$ cells / ml to give ~ 70% confluency after 24 h incubation at 37ºC.

2. After 24 h incubation, mix an appropriate amount of transfection reagent (depending on manufacturer's instructions) in DMEM supplemented with 1mM glutamine and 1 × non-essential amino acids in a V-well micro-titre plate.

3. Add ~ 1μg of plasmid DNA and mix thoroughly by mixing up and down for ~10 times and incubate for 30 min at room temperature.

4. Carefully aspirate the medium from the cell layer in the 24-well plate and discard.

5. Add sufficient cell culture medium to the DNA / transfection reagent cocktail to achieve a final volume of 1 ml. Add to the plated cells.

6. Incubate the cells for 72 h at 37°C in a 5% CO_2/95% air incubator.

3.2 Harvest and Western Blot Analysis of Secreted Proteins

1. To analyse the expression of secreted proteins, harvest the culture supernatant from the cells in the 24 well plate by centrifuging at 17,000 × g for 10 min. Mix an equal volume of supernatant and 2× SDS-PAGE sample loading buffer and denature the protein by heating to 95°C for 3 minutes.

2. Run the samples on an SDS-PAGE gel and transfer to a nitro-cellulose or PVDF membrane using standard Western blotting procedures (*see* **Note 5**).

3. Block the membrane for at least 30 min with 5% milk in PBST at room temperature.

4. Remove the blocking solution and add 1:2000 Anti-His_6 mouse monoclonal antibody as the primary antibody and incubate for 1h at room temperature on a shaker (*see* **Note 6**).

5. Wash the membrane three times in PBST for 5 min each time.

6. Remove the wash buffer and incubate the membrane for 1 h in the secondary antibody solution at room temperature on a shaker.

7. Wash the membrane three times in PBST for 5 min followed by two washes for 5 min each in PBS.

8. To visualise the protein, remove excess buffer from the membrane and cover with Western blotting detection system reagent for 5 min to develop the blot.

9. Signal is detected using a chemiluminescent Western blot imaging system.

3.3 Large-Scale Transient Expression

1. Seed Expi 293 cells in a roller bottle with 300 ml culture volumes at a density of ≥ 1.0-1.5 × 10^6 cells/ml at ~ 95% viability for 24 h.

2. Determine cell count and viability and prepare the transfection as below. Transfect if cell count ≥ 1.5-2.0 × 10^6 cells/ml at ~ 95% viability.

3. Prepare transfection cocktail as follows: Solution A - Add 800 µl of Expifectamine transfection reagent in a Falcon tube and dilute with 15 ml OPTI-MEM medium. Incubate for 5 min at room temperature. Solution B - Add 300 µg of DNA in a tube and dilute with 15 ml OPTI-MEM medium.

4. Mix solution A and B and incubate for 10 min at room temperature (*see* **Note 7**).

5. From the 300 ml suspension culture to be transfected, remove 30 ml of cells before adding the transfection cocktail to make up the final volume of 300 ml culture.

6. Incubate transfected cells for 16 - 18 hours at 37ºC.

7. After 16-18 h, add 1.5 ml of Enhancer 1 and 15 ml Enhancer 2 to the culture and return to the incubator and incubate for 2 days (*see* **Note 3**).

8. Determine cell count and viability. Harvest media by spinning in 500 ml centrifuge bottle for 45 min at 6000 x g. Filter sterilise using a 500 ml 0.45 µm bottle top filter and proceed to purification.

3.4 Automated purification protocol using the ÄKTAxpress™ for large-scale glycoprotein purification

1. Using an AKTAxpress™ unit, equilibrate the HiLoad16/600 Superdex 200 column with 2 column volumes of Size Exclusion Buffer.

2. After equilibration of the size exclusion column, insert the inlet tubes A1 and A2 into Nickel Wash Buffer and wash the pumps manually to fill the lines with buffer.

3. Insert A3 into Nickel Elution Buffer and insert the outlet tube F3 into a duran glass flask that is at least 1.5 times the sample volume and place a 96 deep well plate in the fraction collector.

5. Insert a pre-charged 5 ml HisTrap FF column into column position 1.

6. Carefully remove the line A2 from the Nickel Wash Buffer and insert it into the flask containing the sample.

7. Using a Method Run in the System Control section of the UnicornTM software, run the glycoprotein purification programme described in Nettleship *et al.* [5] (*see* **Note 8**).

3.5 SDS-PAGE Analysis of Large-Scale Purified Proteins

1. Analyse the A280 trace of the size exclusion profile and make a note of the fractions that contain protein, as represented by a peak in the A280 trace.

2. Take 10 µl from each fraction-of-interest and mix with 10 µl of 2× SDS-PAGE sample loading buffer. Denature the protein by heating the sample to 95ºC for 3 min.

3. Run the samples on an SDS-PAGE gel until the dye reaches the bottom. Wash the gel three times in water for 1 min each time and add 20 ml of protein stain and incubate at room temperature with rocking for at least 15 min.

4. To de-stain the gel, remove the stain and incubate the gel in water at room temperate with rocking for 15 min or more until a clear background is obtained.

5. Combine fractions containing the protein of interest by SDS-PAGE.

3.6 Western Blot Analysis of Large-Scale Purified Proteins

1. To characterise the purified proteins, similar western blot is carried out according to the protocol in Section 3.2 using a pan flavivirus antibody, the sera from ChAdOx1 ZIKV prME_ΔTM vaccinated mice and a ZIKV Env monoclonal antibody as the primary antibody.

3.7 Serodiagnosis of ZIKV by ELISA

Purified ZIKV Env is used as a coating reagent for immunoassay (ELISA) using sera from ZIKV-infected patients from an endemic area to detect patient-derived ZIKV antibodies and compared to the commercially available ZIKV Env (Figure 3).

1. Coat Elisa plates with 5 μg/ml antigen diluted in PBS; add 50μL per well (*see* **Note 9**).
2. Leave plates at room temperature on bench overnight.
3. Wash plates 6 times with PBST by adding PBST to completely fill plates to the top and discarding it.
4. Block plates with 300 μl per well Blocking Buffer for 2h at room temperature.
5. Dilute sera sample starting at 1:300 in PBST.
6. Discard blocking solution.
7. Add 50 μl of PBST to wells in rows B-H.
8. Add 75 μl of sera to wells in row A and serially dilute 3-fold down plate in PBST by transferring 25 μl into row below, mixing 20 times and transferring again. Throw away the 25 μl taken out of row H. Final volume should be 50 μl per well.
9. Leave 1h at RT.

10. Wash plates 6 times with PBST by adding PBST to completely fill plates to the top and discarding it.

11. Add 50 µl per well the detecting Ab– Anti-Human IgG-alkaline phosphatase-conjugated antibody diluted 1/5000 in PBST (5 mL per plate + 1 µL Ab). Leave 1h at RT.

12. Wash plates 6 times with PBST by adding PBST to completely fill plates to the top and discarding it.

13. Add pNPP substrate, 100 µl per well. (*see* **Note 10**).

14. Cover with foil and leave for 15 minutes to develop at room temperature. (*see* **Note 11**).

15. Read OD at 405nm on Elisa plate reader.

16. Serum antibody endpoint titers were defined by absorbance value three standard deviations greater than the average OD405 of control sera pool.

Notes

1. The selected prM-Env gene was cloned into pOPINTTGneo or pOPINTTGneo-3C-CD4 expression vectors which produce soluble ZIKV Env proteins with a C-terminal His tag or a C-terminal rat CD4 fusion and His tag.

2. DNA for transfection needs to have an A_{260}/A_{280} ratio of > 1.8. This can be obtained using standard commercial kits such as the PureLink HiPure Expi Plasmid Megaprep kit.

3. As an alternative to using the ExpiFectamin™ kit, 300 ml culture volume of cells are transfected by adding 30 ml of OPTI-MEM containing 300 µg of DNA and 1.6 ml of PEI Max 40kDa (1 mg/mL). The following day, 5 ml of 350 mM Valrpoic acid, 2 ml 1M sodium propionate and 5.5 ml of 45% w/v glucose are added to enhance expression.

4. The buffers are vacuum filtered using 0.45 µm filters to remove unwanted dust and bubbles.

5. Trans-Blot Turbo Mini Nitrocellulose Transfer Packs were used to transfer Env proteins from gels using the Trans-Blot Turbo Transfer System in Kim et al [1]. Alternatively, standard western blotting method using PVDF membranes can be used as described in Mahmood et al [6].

6. The amount of antibody depends on the size of the container the membrane is in, for example, 1:2000 dilution of Anti-His_6 mouse monoclonal antibody in 30 ml 5% milk PBST can be made by adding 15 µl of antibody in 30 ml 5% PBST.

7. Incubation longer than 10 min can result in loss of transfection efficiency.

8. The full method for the glycoprotein purification is written out in Nettleship et al [5] and can be copied into the Method Editor section of the Unicorn™ software.

9. Concentration of coating ZIKV Env may vary depending on antigen used.

10. Add substrate at least 1 minute apart to each plate so that all plates can be developed for the same length of time and OD values are read sequentially on the ELISA reader.

11. Development time is antigen/user dependent

References

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Figures

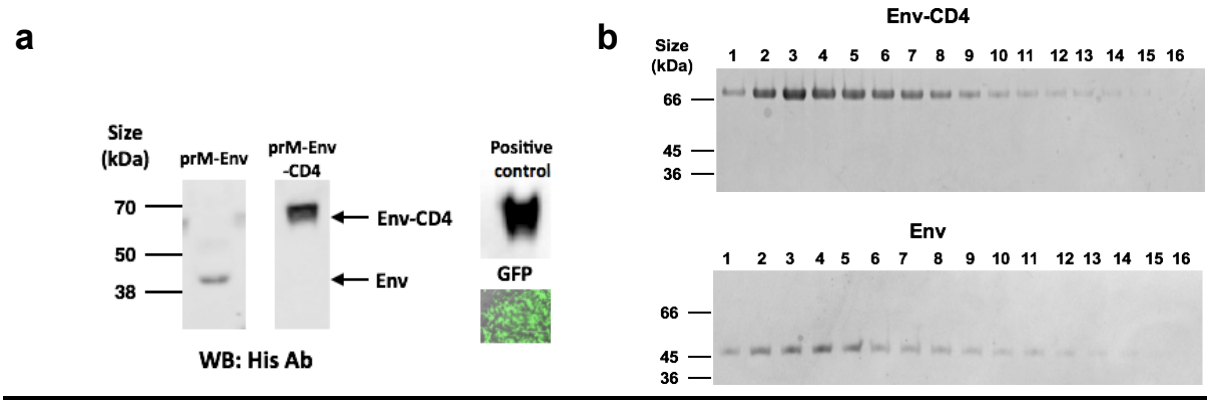
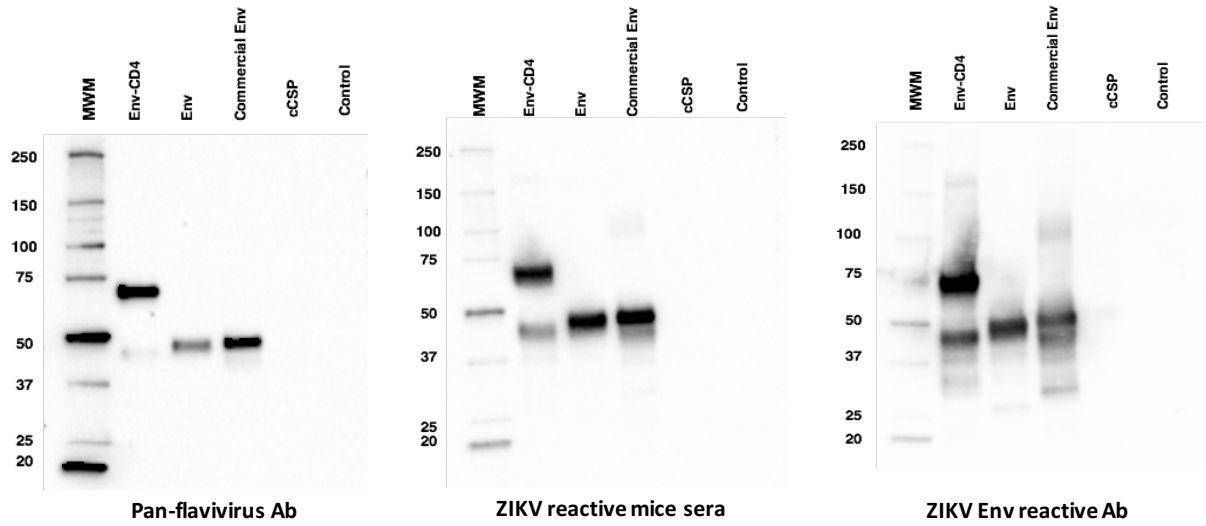


Figure 1 Production and purification of Zika Envelope proteins

a). An example of western-blot of Env-CD4 and Env secreted from HEK293T in small-scale expression screening detected with anti-His antibodies. The ectodomain of cell surface receptor sFcεRIα was the positive control and GFP was the negative control. b). Coomassie Brilliant Blue staining (CBB) of the fractions of purified Env-CD4 and Env proteins are shown (reproduced from [1] with permission from Nature Springer).



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312 **Figure 2 Characterisation of ZIKV Env proteins**

313 Western blot of the purified ZIKV Env-CD4 and Env proteins using a pan-flavivirus antibody,
314 sera from ChAdOx1 ZIKV prME_ΔTM immunized mice and Zika Env monoclonal antibody.
315 For Env-CD4, the top band corresponds to Env-CD4 protein and the bottom band to Env
316 protein which is formed following cleavage from Env-CD4. An unrelated malaria protein
317 (cCSP) was used as a negative control for the ZIKV-Env specific antibodies. Negative control
318 is cell-free media. (reproduced from [1] with permission from Nature Springer)

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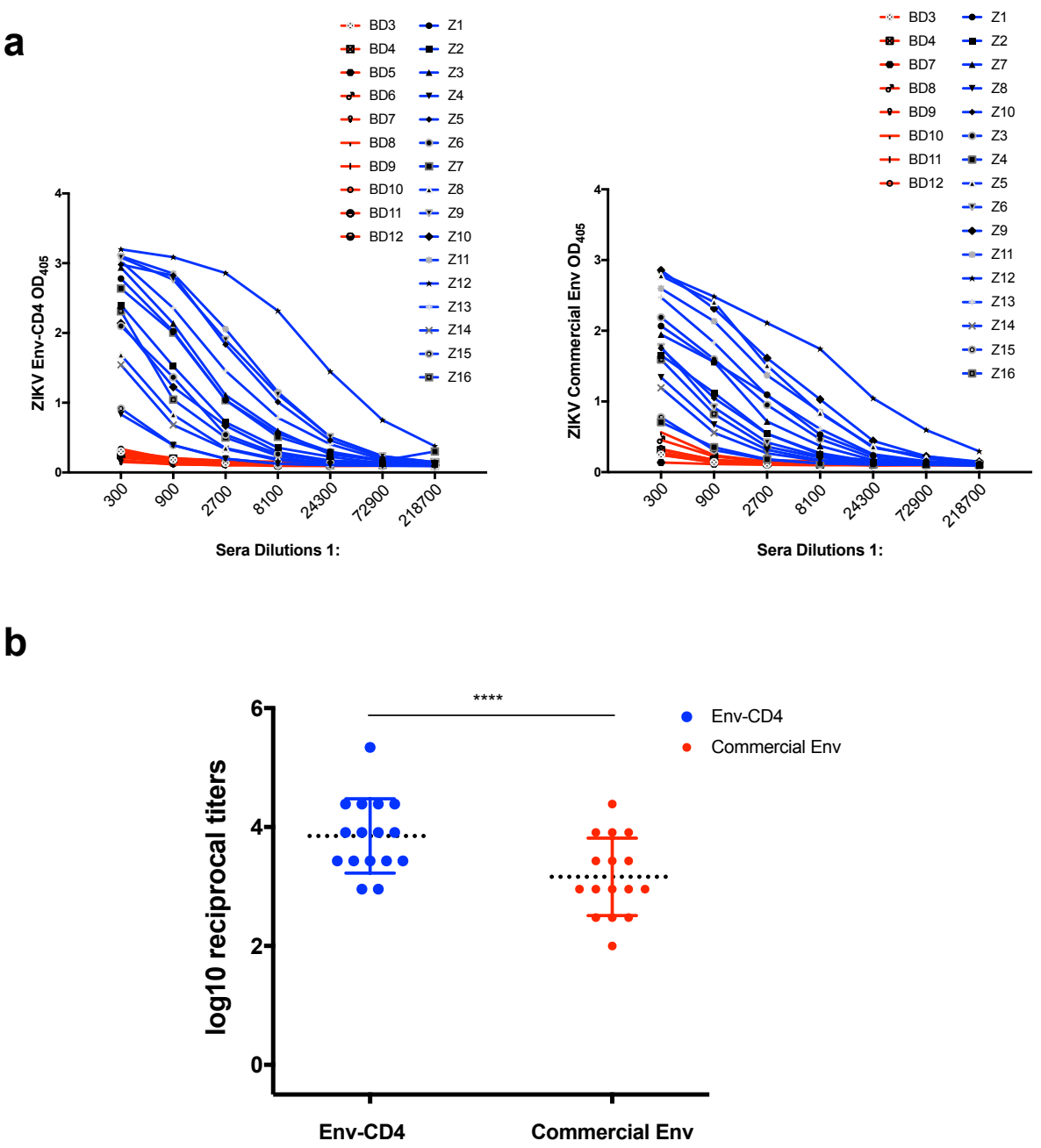

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322 **Figure 3 ELISA assays to assess the reactivity of human sera from patients in a ZIKV**
323 **endemic region of Mexico**. Sera from Zika patients from Mexico (blue) and sera obtained
324 from healthy donors (red). a). The graph of ZIKV Env-CD4 and commercial Env OD405
325 against sera dilutions. b). The graph shows the endpoint reciprocal titers for ZIKV patients
326 using Env-CD4 and commercial Env proteins. P value (<0.0001) was determined by pairwise
327 t-tests. (reproduced from [1] with permission from Nature Springer)