REAGENT VALIDATION FILE

Cell lines

The vendor provides key quality control information (referenced in manuscript) and we determined these reagents to be mycoplasma free (see attached figure).

Viruses

- Zika virus (ZIKV) strain H/PF/2013 was originally obtained from the US Centers for Disease Control (CDC) and used in all experiments involving ZIKV.
- All four dengue virus (DENV) serotypes (DENV1 strain: WestPac74, GenBank: MW945952.1; DENV2 strain: S-16803, GenBank: GU289914.1; DENV3 strain: CH53489, GenBank: DQ863638.1; DENV4 strain: TVP-360, GenBank: KU513442.1) were originally obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, Maryland, USA).
- Low passage, stocks cultured in mycoplasma-free cells are used in all experiments. RT-PCR testing is detailed in attached slides.

Monoclonal antibodies

- A9E and G9E: These two monoclonals (mAbs) were produced by LakePharma and used as in a previously published study.[1] Prior to use in assays for this article, we compared serologic reactivity of new lots of these mAbs to previous stocks. We confirmed the previously reported specificity and potency of Zika virus neutralization, confirming binding to Zika but a complete lack of binding or neutralization to dengue virus (**Figure 1**).
- 4G2: This is a cross-reactive mouse anti-flavivirus antibody commonly used in flavivirus research. Large batches are column purified, protein concentration quantitated, and reactivity validated in focus assays and ELISA.

Purified proteins

- Recombinant E domain III of Zika virus was obtained from Dr. Aravinda de Silva and Dr. Premkumar Lakshmanane, via the protein production core at University of North Carolina at Chapel Hill, which provides validation reports for reagents produced.

➔ MH Collins worked on the team that first reported the utility of the recombinant protein as a diagnostic antigen:

Premkumar, L., Collins, M., Graham, S., Liou, G.-J. A., Lopez, C. A., Jadi, R., Balmaseda, A., Brackbill, J. A., Dietze, R., Camacho, E., De Silva, A. D., Giuberti, C., dos Reis, H. L., Singh, T., Heimsath, H., Weiskopf, D., Sette, A., Osorio, J. E., Permar, S. R., *et al.* Development of envelope protein antigens to serologically differentiate Zika from dengue virus infection. *J. Clin. Microbiol.* JCM.01504-17 (2017) doi:10.1128/JCM.01504-17.

➔ The method of Zika E domain III has been updated to improve assay robustness and the Collins Lab has independently and successfully implemented this version of the assay in more recent work:

Adams, C., Jadi, R., Segovia-Chumbez, B., Daag, J., Ylade, M., Medina, F. A., Sharp, T. M., Munoz-Jordan, J. L., Yoon, I. K., Deen, J., Lopez, A. L., de Silva, A. M. & Premkumar, L. Novel Assay to Measure Seroprevalence of Zika Virus in the Philippines. *Emerg. Infect. Dis.* **27**, 3073–3081 (2021).

Zepeda, O., Espinoza, D. O., Martinez, E., Cross, K. A., Becker-Dreps, S., de Silva, A. M., Bowman, N. M., Premkumar, L., Stringer, E. M., Bucardo, F. & Collins, M. H. Antibody Immunity to Zika Virus among Young Children in a Flavivirus-Endemic Area in Nicaragua. *Viruses* **15**, (2023).

Other reagents

Other reagents such as secondary antibodies, alkaline phosphatase conjugation kits, and substrate for ELISA assays are obtained from vendors who provide quality control information. Vendors and catalogue numbers are referenced in the manuscript per journal guidance.

Validation of Key Reagents

Natural infection by Zika virus but not DNA vaccination consistently elicits antibodies that compete with two potently neutralising monoclonal antibodies targeting distinct epitopes

Cell line validation

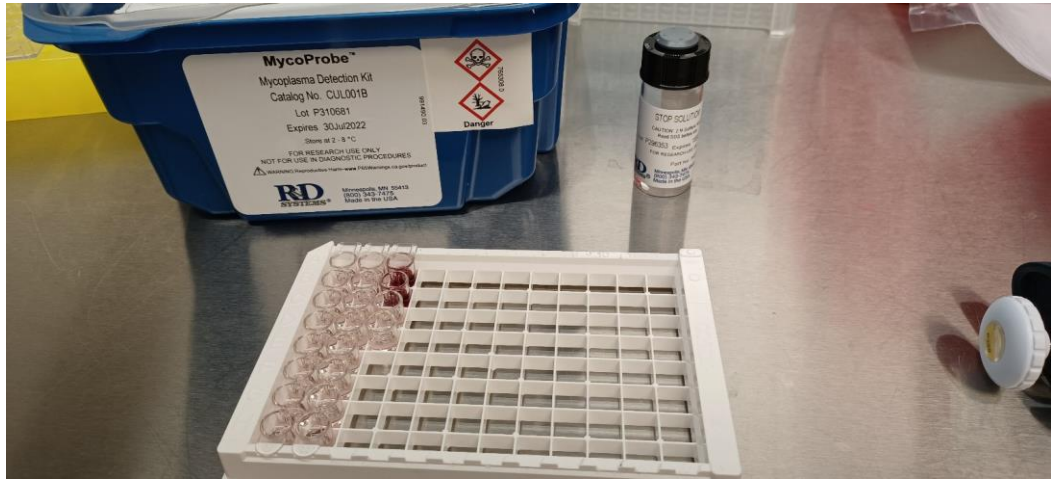
Validation information for Vero-81 cells and C6/36 cells provided by the vendor (ATCC) is referenced in the Methods section of the manuscript.

Further quality control information is available on vendor's website: https://www.atcc.org/products/ccl-81?matchtype=b&network=g&device=c&adposition=&keyword=%2Bvero%20%2Bcell%20%2Bline%20%2Batcc&gad=1&gclid=CjwKCAjw-b-kBhB-EiwA4fvKrAi_IIjDsbtGAhXHPbo-LTG2m4U-op32maE_l4voJCN9rpBoiVKhHBoCvFcQAvD_BwE

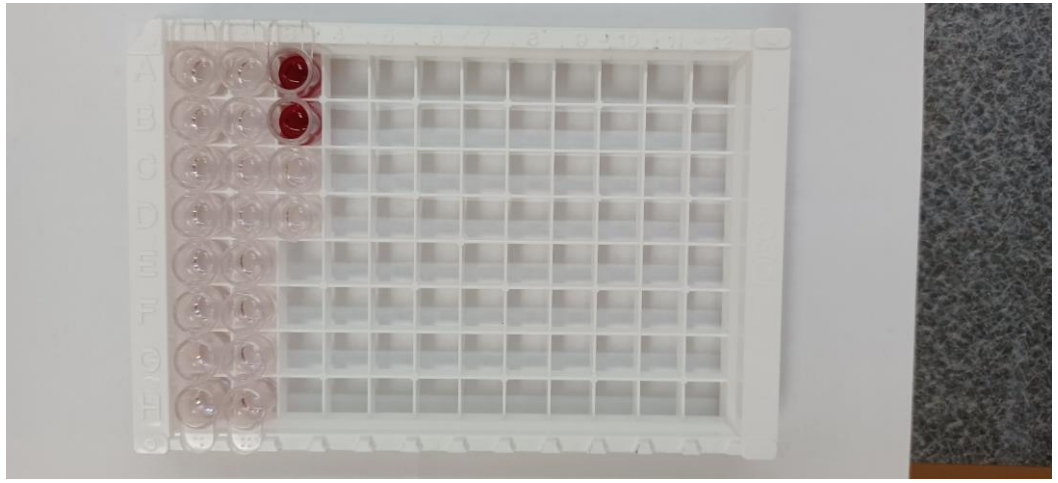
Mycoplasma testing performed in house during the time experiments for this work were being performed (2020-2022).

Cell line validation experiment – 10MAR2022

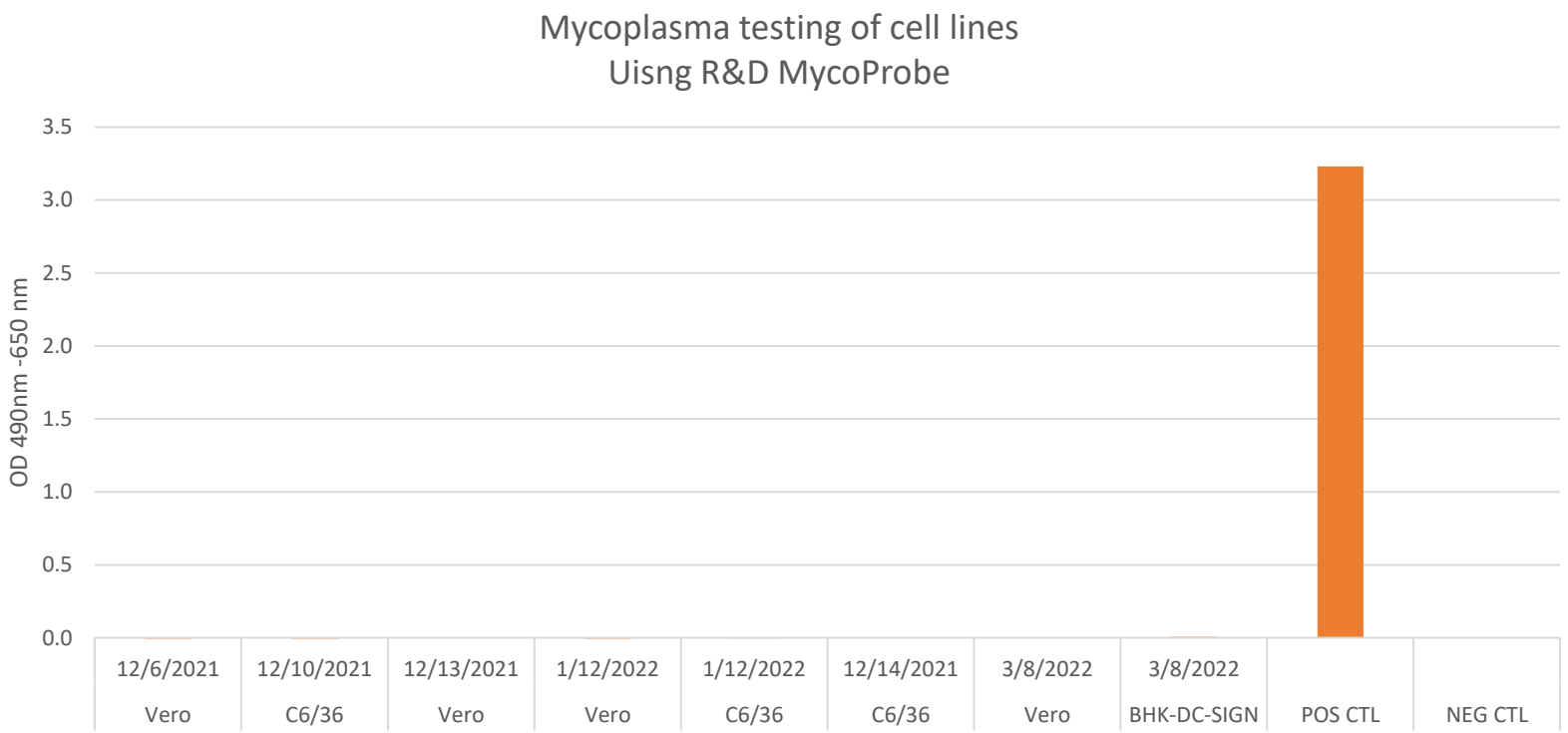
Mycoplasma testing using R&D MycoProbe kit



MycoProbe kit plate appearance and example set up



Vero and C6/36 cells in Collins Lab are mycoplasma free



Cell type	Date	OD 490nm	OD 650 nm		OD 490-650		OD - Neg Ctl	
Vero	12/6/2021	0.072	0.039		0.033		-0.006	
C6/36	12/10/2021	0.0715	0.039		0.033		-0.007	
Vero	12/13/2021	0.078	0.04		0.038		-0.001	
Vero	1/12/2022	0.072	0.0395		0.033		-0.007	
C6/36	1/12/2022	0.0755	0.041		0.035		-0.005	
C6/36	12/14/2021	0.079	0.042		0.037		-0.002	
Vero	3/8/2022	0.0795	0.0415		0.038		-0.001	
BHK-DC-SIGN	3/8/2022	0.087	0.041		0.046		0.007	
POS CTL		3.434	0.1635		3.271		3.232	
NEG CTL		0.0805	0.0415		0.039		0.000	

Validation of viruses (in-house)

	Análisis Denv Multiplex	Verde	Amarillo	Orange	Red	
		DENV-1	2	DENV-3	DENV-4	
	Selected	5.33	10	10	5	
	Crop 1st 5	Sí	Sí	Sí	Sí	Resultado Final
	Slope Correct	Sí	Si	"No	No	
	Threshold	0.03	0.05	0.025	0.025	
	Agua	N	N	N	N	Neg
Controles	DENV-1 4.0 ssDNA	29.43	N	N	N	DENV-1
	DENV-2 4.0 ssDNA	N	32.44	N	N	DENV-2
	DENV-3 4.0 ssSNA	N	N	30.19	N	DENV-3
	DENV-4 4.0 ssDNA	N	N	N	31.58	DENV-4
4	4 1:100 in PBS 10.19.2018	21.87	N	N	N	DENV-1
7	7 1:100 in PBS 03.08.2019	22.68	N	N	N	DENV-1
2.23.2110 DE	Daniel's Culture	21.69	N	N	N	DENV-1
3.8.2111 DE	Daniel's Culture	20.85	N	N	N	DENV-1

Our colleague Dr. Jesse Waggoner kindly performs molecular testing to verify the viral species of our viral culture and exclude cross-contamination by other viruses commonly cultured in the lab. The image to the left shows validation of representative cultures from each of the four dengue serotypes. Dr. Waggoner is an expert in molecular assays detecting RNA viruses, and his lab uses state-of-the art multiplex testing, based on assays originally described in the two publications below:

1. Waggoner, J. J., Abeynayake, J., Sahoo, M. K., Gresh, L., Tellez, Y., Gonzalez, K., Ballesteros, G., Pierro, A. M., Gaibani, P., Guo, F. P., Sambri, V., Balmaseda, A., Karunaratne, K., Harris, E. & Pinsky, B. A. Single-reaction, multiplex, real-time rt-PCR for the detection, quantitation, and serotyping of dengue viruses. *PLoS Negl. Trop. Dis.* **7**, (2013).

2. Waggoner, J. J., Gresh, L., Mohamed-Hadley, A., Ballesteros, G., Davila, M. J. V., Tellez, Y., Sahoo, M. K., Balmaseda, A., Harris, E. & Pinsky, B. A. Single-Reaction Multiplex Reverse Transcription PCR for Detection of Zika, Chikungunya, and Dengue Viruses. *Emerg. Infect. Dis.* **22**, 1295–1297 (2016).

Additionally, virus identity and monoclonal antibody specificity are mutually validated in binding ELISA. We also observe a consistent growth kinetic and plaque (focus) morphology overtime, which is unique to each virus.

Validation of monoclonal antibodies (commercial)

LakePharma The Biologics Company

COA
SR-21834

Process Summary and Specifications

- Protein A Affinity Chromatography
- The purified material was filtered through 0.2 µm filter and aliquoted in a biosafety cabinet
- Sterility testing was not performed. Sterility is not guaranteed
- Storage at -80°C is highly recommended for storage over 14 days

Protein Name	E3-A-10C (A9E)
Lot Number	TP40715F
Extinction Coefficient at A280 ((mg/mL)-1 cm-1)	1.588
Protein Concentration (mg/mL)	5.13
Endotoxin	<1 EU/mg
Physical State	Liquid
Formulation Buffer	100 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 6.0

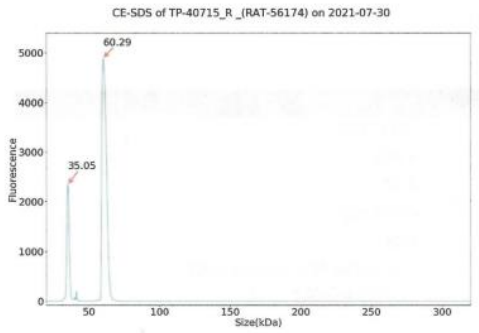
Generated by:
Signed by Henry Chen on 2021-08-03 at 03:12:15 PM PDT
Approved by Quality Control:

LakePharma The Biologics Company

CE-SDS
SR-21834

Lot #: TP40715F

CE-SDS Electropherogram Reducing:



LakePharma The Biologics Company

COA
SR-21834

Process Summary and Specifications

- Protein A Affinity Chromatography
- The purified material was filtered through 0.2 µm filter and aliquoted in a biosafety cabinet
- Sterility testing was not performed. Sterility is not guaranteed
- Storage at -80°C is highly recommended for storage over 14 days

Protein Name	D1-G-10F (G9E)
Lot Number	TP40714F
Extinction Coefficient at A280 ((mg/mL)-1 cm-1)	1.666
Protein Concentration (mg/mL)	5.01
Endotoxin	<1 EU/mg
Physical State	Liquid
Formulation Buffer	100 mM HEPES, 100 mM NaCl, 50 mM NaOAc, pH 6.0

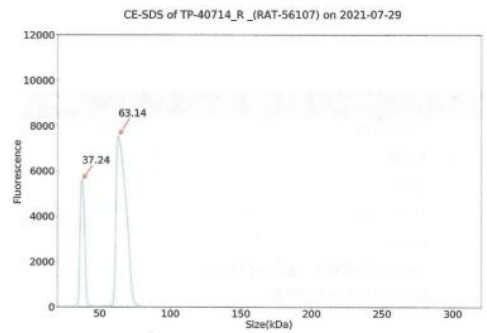
Generated by:
Signed by Alexis Tang on 2021-07-30 at 07:59:20 AM PDT
Approved by Quality Control:

LakePharma The Biologics Company

CE-SDS
SR-21834

Lot #: TP40714F

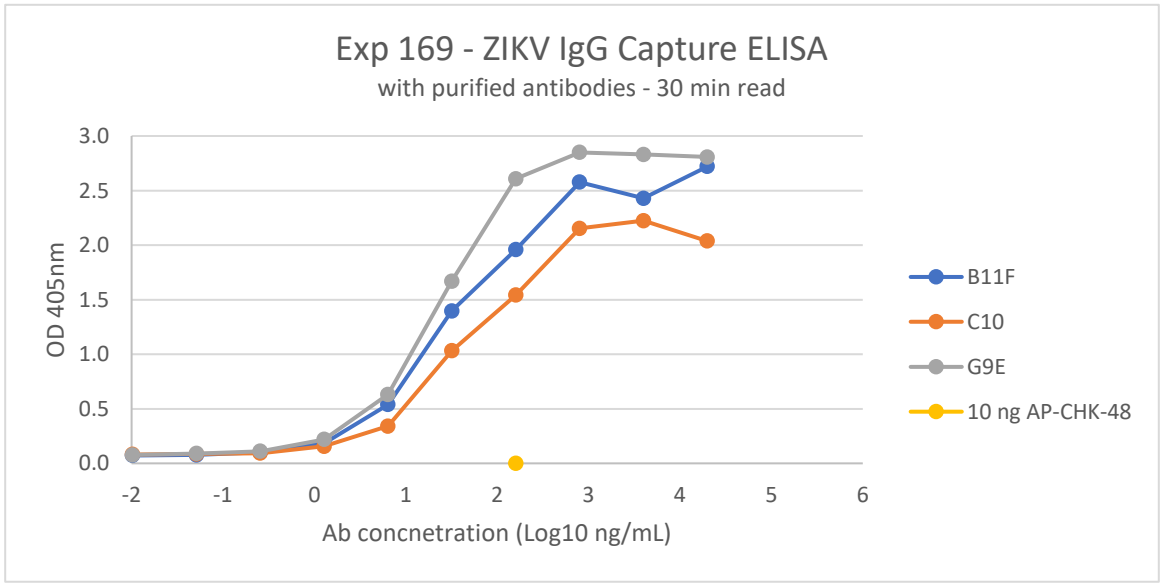
CE-SDS Electropherogram Reducing:

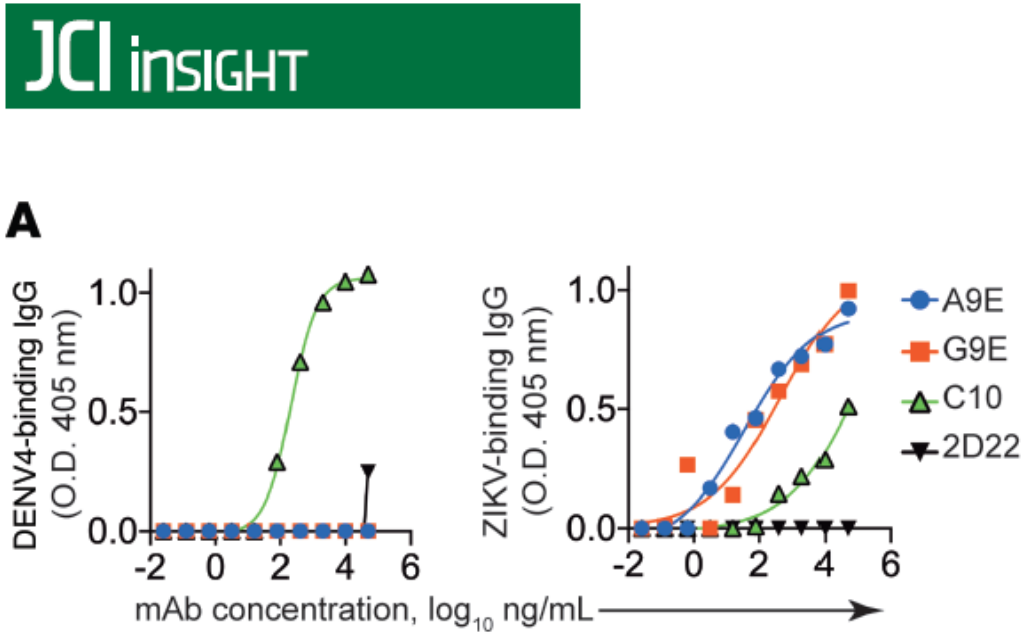


Note: We have obtained multiple stocks of A9E and G9E from Lake Pharma since their discovery in 2016, with reliable performance. This slide includes quality control information provided by the vendor. Our in-house reactivity validation follows.

Validation of G9E reactivity to ZIKV

Two batches of each mAb exhibit consistent IC50 for neutralizing Ab ~1-20 ng/uL, similar to what we previously published. On the Left, G9E is tested with other mAb known to react (or not) with ZIKV over a dilution series. B11F is ZIKV-specific; C10 is cross-reactive to DENV and ZIKV; CHK-48 reacts with the alphavirus chikungunya but not flaviviruses (included as negative control for the assay). The same reactivity pattern is observed for A9E (not shown).



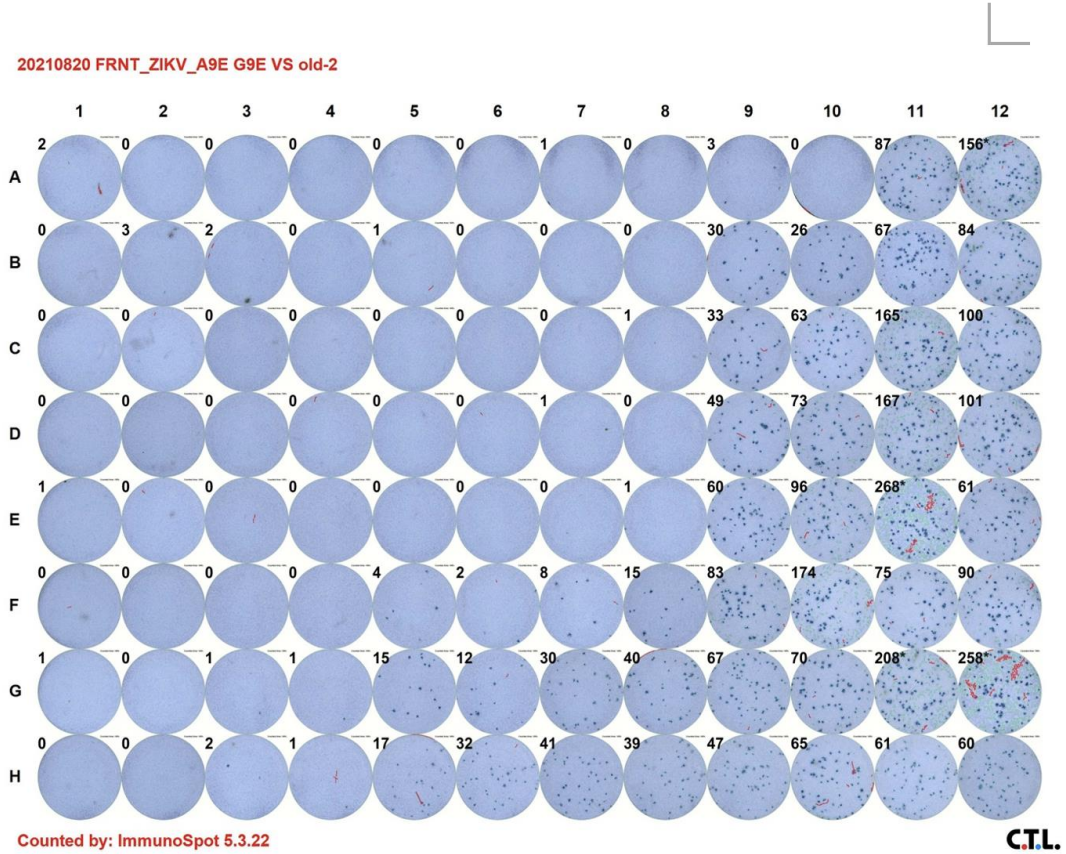


Figures from: JCI Insight. 2019;4(8):e124588. https://doi.org/10.1172/jci.insight.124588.

Validation of A9E and G9E neutralization of ZIKV

Two batches of each mAb exhibit consistent IC50 for neutralizing Ab ~1-20 ng/uL, similar to what we previously published.

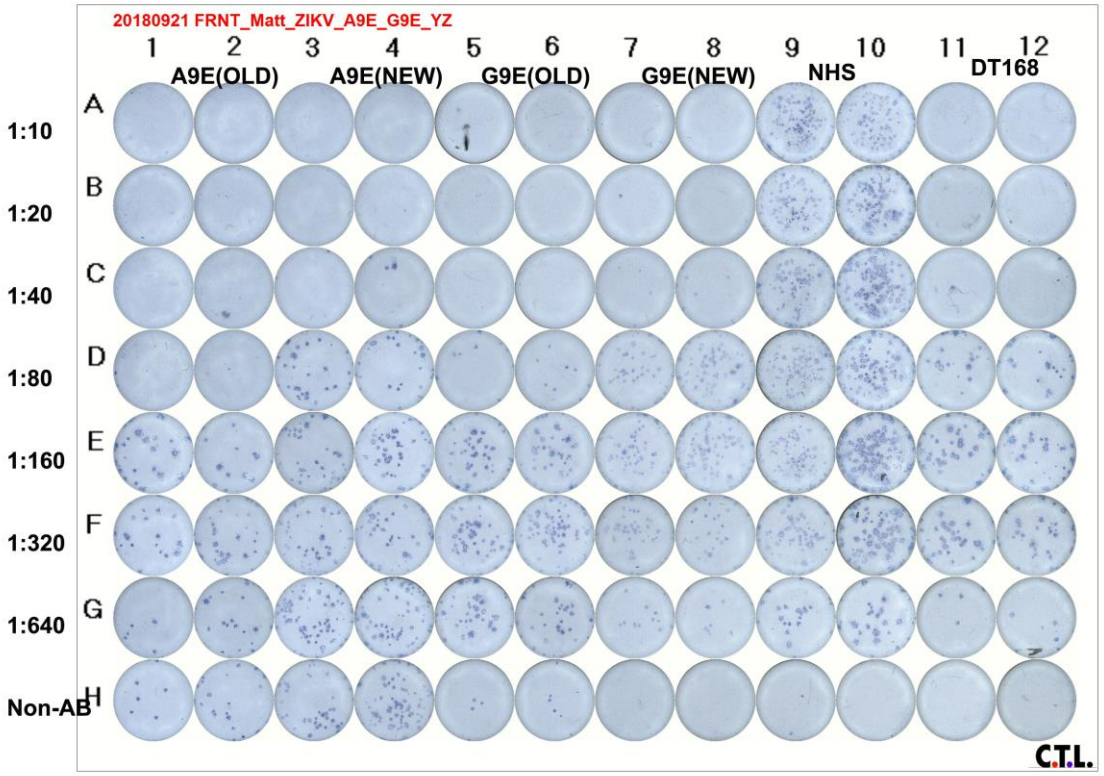
0	5mg/ml	ug/ml	ng/ml
1	1:100	50	50,000.00
2	1:400	12.5	12,500.00
3	1:1600	3.125	3,125.00
4	1:6400	0.78125	781.25
5	1:25600	0.1953125	195.31
6	1:102400	0.04882813	48.83
7	1:409600	0.01220703	12.21
8	1:1638400	0.00305176	3.05



A9E 9/12/18	A9E 7/30/21	G9E 9/12/18	G9E 7/30/21	AVE 032	TWS 024

Validation of A9E and G9E specificity

Two batches of these mAb neutralize ZIKV



Two batches of these mAb DO NOT neutralize DENV4