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High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure

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1 Works for me dx.doi.org/10.17504/protocols.io.bscpnavn

ABSTRACT

Zika virus is a Flavivirus, transmitted via *Aedes* mosquitos, that causes a range of symptoms including Zika congenital syndrome. Zika has posed a challenging situation for health, public and economic sectors of affected countries. To quantitate Zika virus neutralizing antibody titers in serum samples, we developed a high throughput plate-based Zika virus reporter virus particle (RVP) assay that uses an infective, non-replicating particle encoding Zika virus surface proteins and capsid (CprME) and a reporter gene (*Renilla* luciferase). This is the first characterization of a Zika virus RVP assay in 384-well format using a Dengue replicon *Renilla* reporter construct. Serially diluted test sera were incubated with RVPs, followed by incubation with Vero cells. RVPs that have not been neutralized by antibodies in the test sera entered the cells and expressed *Renilla* luciferase. Quantitative measurements of neutralizing activity were determined using a plate-based assay and commercially available substrate. The principle of limiting the infection to a single round increases the precision of the assay measurements.

RVP EC_{50} titers correlated closely with titers determined using a plaque reduction neutralization test (PRNT) (R^2>95%). The plate-based Zika virus RVP assay also demonstrated high levels of precision, reproducibility and throughput. The assay employs identical reagents for human, rhesus macaque and mouse serum matrices. Spiking studies indicated that the assay performs equally well in different species, producing comparable titers irrespective of the serum species. The assay is conducted in 384-well plates and can be automated to simultaneously achieve high throughput and high reproducibility.

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Lab Protocol for Zika RVP Miconeutralization Assay Bohning et al 2021.docx

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Serially diluted test sera were incubated with RVPs, followed by incubation with Vero cells. RVPs that have not been neutralized by antibodies in the test sera entered the cells and expressed *Renilla* luciferase. Quantitative measurements of neutralizing activity were determined using a plate-based assay and commercially available substrate. The principle of limiting the infection to a single round increases the precision of the assay measurements.

RVP EC_{50} titers correlated closely with titers determined using a plaque reduction neutralization test (PRNT) (R^2>95%). The plate-based Zika virus RVP assay also demonstrated high levels of precision, reproducibility and throughput. The assay employs identical reagents for human, rhesus macaque and mouse serum matrices. Spiking studies indicated that the assay performs equally well in different species, producing comparable titers irrespective of the serum species. The assay is conducted in 384-well plates and can be automated to simultaneously achieve high throughput and high reproducibility.

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Title: High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure.

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INTRODUCTION

Neutralization of viral infectivity is one of the methods for determining the presence of functional antibodies in serum samples. Reporter virus particles (RVP) were purchased from Integral Molecular (Philadelphia, PA) Zika reporter virus particles (Z-RVPs) contain CprM/E from ZIKV strain SPH2015 packaged within the particle a Dengue-based replicon with the Renilla luciferase gene.

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The Z-RVPs express a Renilla luciferase reporter gene upon infection of permissive cells. The half maximal effective concentration EC50 titer of neutralizing antibodies is determined using a bioluminescent reaction which generates a glow-type luminescent signal by the interaction of the Renilla luciferase and coelenterazine substrate. The luminescent signal is measured using a luminescence enabled plate reader. Reduction in luminescent signal in the presence of serum indicates neutralization. This laboratory procedure (LP) reflects the experimental set up for an experiment with maximal six 384-well plates. The volumes listed throughout this LP can be changed based on the number of samples in the run.

Equipment, Materials and reagents

Table 1 Equipment

Equipment Description	Manufacturer/Vendor*
Sorvall Legend XTR Centrifuge	Thermo Scientific
Freezer ≤-65	Thermo Scientific
Freezer -20◦C + 10◦C	Thermo Scientific
Refrigerator 5◦C + 4◦C	Thermo Scientific
Water bath, 37◦C + 2◦C and 56+ 2◦C	Fisher Scientific
Biological safety cabinet, Class II, certified	Baker
Cell Culture Incubator 37+2◦C, 5+1% CO2	Thermo Scientific
Single channel pipettes, variable volumes, calibrated (e.g. P2, P20, P100, P200, P1000)	Thermo Scientific
8 or 12-channel pipettes, variable volumes, calibrated	Thermo Scientific
E1-Clip-tip electronic 8 channel pipettes, variable volumes, calibrated	Thermo Scientific
E1 - Clip-tip Equalizer 384 pipette 12 or 8 channel 30 µl volume, calibrated	Thermo Scientific
E1-Clip-tip Equalizer 384 pipette 12 or 8 channel 125µL volume, calibrated	Thermo Scientific
Pipet-Aid	Drummond
Countess II	Life Technologies
Enspire Reader	Perkin Elmer
Microcentrifuge	Eppendorf

**Equivalent manufacturers/vendors may be used.*

Table 2. Materials

Citation: (04/24/2021). High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure. https://dx.doi.org/10.17504/protocols.io.bscpnavn

Material Description	Manufacturer/Vendor*	Product Number*
Clip-tip 384 filter sterile tips (30 and 125µL volumes)	Thermo Scientific	94420103/94420153
Sterile 96-well polypropylene plates(untreated) U-bottom or V-bottom PCR plate	Greiner Bio-One/Thermo Scientific	650101/AB-1400
Adhesive foil PCR foil sealing sheets	Thermo Scientific	AB-0626
Clear plate seals	EXCEL Scientific	100SEALPLT
Sterile 15mL/50mL conical tubes	Falcon	352097/352070
125mL Sterile bottles	Nalgene	2019-0125
Tissue-culture treated 384-well plates white opaque bottom	Corning	3570
Sterile media reservoirs (25ml divided , 25mL or 50mL)	Vistalab Technologies	3054-1005/3054-1003/3054-1006
Sterile serological pipettes, assorted volumes (e.g. 5, 10, 25, and 50mL volumes)	Fisherbrand	13-678-11D/13-678-11E/13-678-11/13-678-11F
Tissue-Culture treated T175cm2 cell culture flask	Corning	10-126-61
Clip tip sterile filter pipet tips, assorted volumes (e.g. 20, 50, 200, 300, and 1000µL)	Thermo Scientific	94420213/94420253/94420313/94420513/94420713

*Equivalent manufacturers/vendors and/or product numbers may be used.

Table 3. Reagents

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Material Description	Manufacturer/Vendor*	Product Number*
1x Opti-MEM reduced serum media	Gibco	11058-021
1X DMEM	Corning	15-017-CV
0.05%Trypsin	Gibco	25300-054
1X DPBS	Gibco	14040141
L-Glutamine(200mM)	Gibco	25030-081
100x Penicillin (10,000 IU/mL)/ Streptomycin (10,000 µg/mL)	Gibco	15140-122
Fetal Bovine Serum, Heat Inactivated	Sigma Life Science	F4135-100ML
Negative control serum	Innovative Research	IGRS-SER
Positive control serum	Takeda	Anti-Dakar Rabbit serum
Wet Ice	N/A	N/A
Zika RVP (Dengue backbone)	Integral Molecular	variable
Renilla-Glo Luciferace Assay System	Promega	E2750

*Equivalent manufacturers/vendors and/or product numbers may be used.

Preparation of Media and Reagents

Assay Media

1. Prepare assay media (1X Opti-MEM): In a biosafety cabinet, aseptically add the following ingredients to the 500mL bottle of 1X Opti-MEM.
2. Remove 55 mL of 1X Opti-MEM.
3. Add 50 mLs of Heat Inactivated (HI) FBS.
4. Add 5 mLs of 100X Penicillin/Streptomycin.
5. Mix by inverting several times.
6. Label with ELN reference number, initials, preparation date, and expiration date (1 month from preparation date).
7. Mix by inverting several times, cover bottle in foil before storing at 4±5°C.

Vero BREL Growth Media

1. Prepare cell growth media for cells (1X DMEM): In a biosafety cabinet, aseptically add the following ingredients to the 1X DMEM.
2. Remove 55 mL of 1X DMEM
3. Add 50 mLs of Heat Inactivated FBS
4. Add 5 mLs of L-Glutamine
5. Label with initials, preparation date, and expiration date (1 month from preparation date).
6. Mix by inverting several times, cover bottle in foil before storing at 4±5°C.

Note: Serum serial dilutions and Transfer Dilution Series to daughter plate can be performed manually or by the Tecan Fluent Automated system. Additional information is available upon request.

Prepare Serum

1. Thaw samples on wet ice or in refrigerator and spin down using microcentrifuge (quick spin). Label 0.5-1.5 mL microcentrifuge tubes with appropriate sample IDs, and positive/negative controls.
2. Aliquot amount needed to heat inactivation (HI). Typically, 20 µL per sample and positive control, and 120 µL for negative control. (The control amounts and all the steps listed in this procedure are for a 6-plate assay; Sample number and assay volumes will vary per experiment).
3. HI aliquots in 56± 2°C water bath for 30±2 minutes. Serum samples prior to HI may go through 4 freeze-thaw cycles.
4. Spin down HI serum using microcentrifuge (quick spin).
5. Store HI serum on wet ice.

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6. Using excel document RVP Neut calc-Dilution Plate set up tab see Appendix 2.1 for reference. Obtain a 96-well dilution plate. Label dilution plates according to sample ID's and positive and negative controls.
7. For the positive control prepare a 1:10 dilution by adding 10 µL of HI serum and 90 µL of assay media in a 0.5-1.5 mL microcentrifuge tube. Vortex and spin down using microcentrifuge (quick spin).
8. Then prepare a 1:20 dilution in appropriate dilution plate well by adding 95 µL of assay media and 5 µL of the HI 1:10 diluted serum to column 1 of the 96-well dilution plate (final dilution of 1:200).
9. For samples 1-72 and negative control, prepare 1:8 dilution by adding 56 µL of assay media and 8 µL of HI serum in column 1 of the 96-well dilution plate.
10. Add 36 µL of assay media to columns 2-11 of the 96-well dilution plates. Refer to Figure 1 and Appendix 2.1 for dilution plate set up.
11. Perform 3-fold serial dilutions. Serial dilutions can be performed using a manual pipette or an electronic pipette:

- **Manual pipette dilutions**: Using a manual 8 channel pipette perform 3-fold serial dilutions. Pipet mix column 1, 5 times. Transfer 18 µL from column 1 to column 2, repeat across the plate to column 11. Pipette mix 5 times after each dilution. After dilutions are complete, discard last 18 µL from column 11.

- **Electronic pipette dilutions**: Using 8 channel electronic pipette perform 3-fold serial dilutions by the program below: Program E1 - Clip-tip Equalizer 96-well pipette 8 channel, 125 µl volume: Mix volume 18 µL; 0 µl volume overfill; Mix cycle 5 times; Speed 10; Fill 18.0 µL, speed 10; Dispense 18 µL; 1 time; speed 10. Change tips after each mixing cycle. After dilutions are complete, discard last 18 µL from column 11.

12. Cover 96-well dilution plate with adhesive seal. Spin dilution plates down at 300rpm for 1 minute.

Figure 1. Dilution Plate set-up

Transfer Dilution Series to Assay plate

1. Use the excel document RVP Neut Calc sheet -Assay Plate tab for the layout of the 384-well assay plate. See Figure 2 for 384-well assay plate layout and Appendix 2.2.
2. Obtain a white 384-well tissue culture plate.
3. To transfer the serially diluted serum in the 96-well dilution plate to the 384-well daughter plate:
4. Program E1 - Clip-tip Equalizer 384 pipette 12 channel, 30 µl volume:
   Aspirate of 22.5 µl volume; 2.5 µl volume overfill; aspirate speed 3
   Multi-dispense of 7.5 µl volume; dispense 3 times; speed 3.
   Purge speed 10.

5. In the 384-well daughter plate the positive control and samples 1-6, 13-18, 25-30, 37-42, 49-54 and 61-66, column 12 will contain the most concentrated dilution; column 2 will be the least concentrated dilution. This can be done by turning the 96-well dilution plate upside down (A1 corner is your bottom right) prior to adding the samples to the 384-well daughter plate. (Note sample numbers will vary according to number of samples, adjust as needed).
6. In the 384-well daughter plate the negative control and samples 7-12,19-24,31-36,43-48,55-60,67-72, column 13 will be the most concentrated dilution; column 23 will contain the least concentrated dilution. Position the 96-well dilution plate in the upright position (with the A1 corner in the upper left-hand corner) prior to adding the samples to the 384-well daughter plate. Refer to Figure 2 and Appendix 2.2 for example of the 384- well plate set up. (Note sample numbers will vary according to number of samples, adjust as needed).
7. Transfer serum dilution series from 96-well plate to 384-well daughter plate.
8. Aspirate the serum serial dilution from the 96-well plate. Dispense first 7.5 µL back into dilution plate. Multi-dispense 7.5 µL per sample into the 384-well daughter plate. Purge into waste and discard tips between samples. Repeat for all samples.
9. Spin 384-well daughter plates down at 300rpm for 2 minutes
10. Proceed to **RVP Addition and Dilution** or to **Assay Plate Seal & Storage.**

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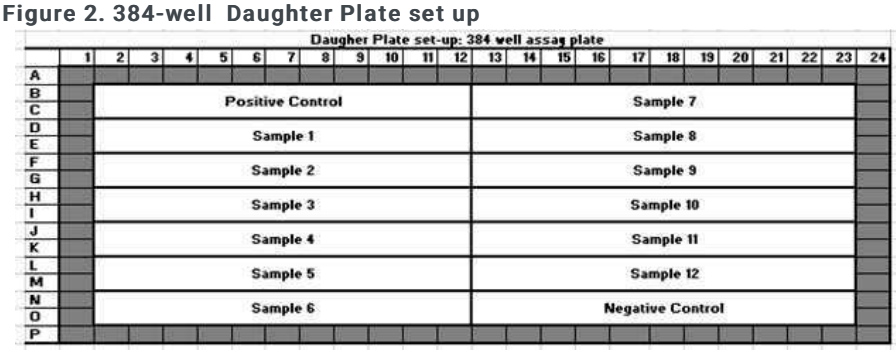
Figure 2. 384-well Daughter Plate set up



Assay Plate Seal & Storage

1. Seal the 384-well daughter plates with adhesive foil seal in biosafety cabinet.
2. Label plates with initials and date. Store assay plates at -20°C for no more than 4 days.
3. Proceed to RVP dilution and addition.

RVP Dilution and Addition

1. Use the excel document RVP Neut Calc- RVP tab to calculate RVP dilution (See Table 4 for reference and Appendix 1.1 for table formulas). Enter gray areas only. In OPTIMIZED RVP µL/WELL column add 2.5µL for RVP lot 229A. The RVP input amount should be assessed per RVP lot. In #WELLS/PLATES add the number of wells being utilized in one plate. In #PLATES enter 6 (total number plates ran per assay, will vary). In VOLUME DILUTED RVP/WELL (µL) enter 7.5. Volume Diluted RVP required (µL) will populate. Enter in the value from VOLUME DILUTED RVP REQUIRED (µL) to VOLUME PREP. VOLUME RVP (µL) and VOLUME ASSAY MEDIA (µL) will populate (Table 4).

Table 4. RVP Dilution Set up[1]

RVP#	Lot#	dilution	Optimized RVP ul/well	# wells/plates	# plates	Total # wells	Volume diluted RVP/well (ul)	Volume diluted RVP required (ul)	Volume Prep(uL)	Volume RVP(uL)	Volume Assay Media(uL)
SHP2015 Zika RVP	229A	12.0	2.5	322	6	1932	7.5	18113	18113	6038	12075

[1]See Appendix 1.1 to calculate formulas

1. If the 384-well daughter plates were stored in -20°C, equilibrate plates for 10-20 minutes at room temperature in the BSC.
2. Thaw 384-well daughter plates in a single layer. Do not stack plates.
3. Spin 384-well daughter plates down at 300rpm for 2 minutes.
4. In BSC, carefully remove foil from top of plate, then proceed to **RVP dilution.**

RVP Dilution

1. Collect required number of vials of RVP from VOLUME RVP (µL) from Table 4.
2. Quick thaw required number of RVP tubes in 37±2°C water bath for 2-3 minutes (5mL vials of RVP will take a bit longer to thaw). Immediately store on wet ice after thawing.
3. Pool all RVP single aliquot tubes into one size appropriate conical tube, mix gently by inversion 2-3 times.
4. Add ASSAY MEDIA VOLUME µL from Table 4 volume assay media in new sterile conical tube. Add RVP VOLUME µL from Table 1 volume pooled RVP to conical tube containing assay media.
5. Mix gently by inverting 3-4 times, store on wet ice. Use immediately.

RVP Addition

1. Transfer diluted RVP to sterile media reservoir, keep reservoir on wet ice.
2. Program E1-Clip-tip Equalizer 384 pipette 12 channel 30µL volume:
   Add 11 pipette tips to the pipettor.
   Aspirate of 22.5 µL volume; 2.5 µL volume overfill; aspirate speed 3;
   multi-dispense 7.5 µL dispense 3 times; speed 3;
   Purge speed 10.
1. Aspirate diluted RVP, multi-dispense the first 7.5 µL in reservoir, then multi-dispense 7.5 µL to the appropriate sample in the 384 well plate. Purge into waste and discard tips between samples. Continue with the rest of the plate adding the RVP one sample at a time
2. Spin down the 384-well daughter plate at 300rpm for 2 minutes.

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3. Immediately transfer plates to humidified 37+2◦C, 5% CO2. incubator Do not stack plates. Orient plates long edge parallel to door.
4. Incubate 384-well daughter plates for 60±2 minutes. Record start and stop time along with temperature in and out of the incubator.
5. Immediately proceed to cell culture harvest, cells will be added once the 60±2 incubation time is complete.

Assay Incubation

Cell Harvest

1. Place Vero BREL growth media and 0.05% Trypsin in 37±1°C water bath 10 minutes prior to splitting cells.
2. Obtain T-175 cell culture flasks from clean cell culture incubator.
3. Record cell passage number, harvest date and date last passaged in ELN
4. Wash flasks with 10 mL 1X DPBS twice.
5. Add 5 mL of 0.05% Trypsin to cell culture flask, place in (37+2◦C, 5% CO2 incubator) for 5 minutes. Check to be sure cells have detached from flask under microscope.
6. Add 5 mL of Vero BREL Growth media to tryspinized cells.
7. Transfer cells to 50 mL conical tube, centrifuge for 5 minutes, 130xG, at room temperature.
8. Aspirate supernatant from pelleted cells. Resuspend cells in 10 mL of assay media, per flask split.

Cell Count

1. Add 20 µL of cells to 20µL of trypan blue to cell countess. Record the lot number of Trypan blue.
2. Add 10 µL of each of mixture to both sides of a hemocytometer. Record the lot number of Cell countess chamber.
3. Count cell using Countess II
4. Record viability and cell count in RVP Neut Calc sheet -cells tab (Table 5 and Appendix 1.2 for formulas).

Cell Dilution

1. Use the excel document RVP Reagent Calc - Cells tab to calculate number of cells needed for the assay. Enter values in grayed areas only. Non-grayed areas will automatically populate. Refer to Table 5 for reference only.
2. Enter 4625 in cells well column.
3. Enter correct number of wells/plate used (example in this portion is 322 wells per 1-384 well plate).
4. Enter in number of plates used in # plates column.
5. Total wells, Volume required will automatically populate.
6. Round volume up if needed and enter value in Volume Prepared.
7. Cells required will automatically populate.
8. Average number of cells (mL) will automatically calculate once the two cell counts (cells/mL live) are recorded in the worksheet. The Assay media volume (mL) will also auto populate.
9. Keep concentrated cell stock on ice until 5 minutes are left of neutralization incubation.

Table 5. Cell Dilution Tab[1]

Vero Brell	N/A	N/A
Optimized Cells well	4625	N/A
cells / ml	308333	N/A
# wells/plate	322	N/A
# plates	6	N/A
Total wells	1932	N/A
Volume req.	38	mls
Volume Prepared	40	mls
Cells Req.	12333333	cells

Harvest Date	
Passage Number	
Date of Passage	
Flask Size	t175
Flask Media Volume	40

Set	Cells/mL total	Cells/ mL live	% live
count 1			
count 2			
Average (including additional dilution factor*)	NA	#DIV/0!	#DIV/0!

Amount of cells (mL)			Assay Media volume(mL)	
#DIV/0!	mL	in	#DIV/0!	mL

[1]See Appendix 1.2 for formulas to calculate

Cell Addition

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1. Add the amount of cells (mL) and assay media (mL) to the conical tube.
2. Resuspend cells by gently inverting 2-3 times.
3. Transfer diluted cells to sterile media reservoir in batches. Swirl conical tube before adding cells to the reservoir.
4. Program E1-Clip Tip Equalizer 384 pipette 12 channel:
   Aspirate of 105 µL volume; 10 µL volume overfill; speed 10
   Multi-dispense 15 µL volume; dispense 7 times; speed 3
   Purge speed 10.
1. Aspirate cell dilution, dispense first 15 µL in reservoir, multi-dispense to the next 6 wells in to the 384-well daughter plates. Purge in the waste. Discard between aspiration cycles.
2. Repeat step 5 until all wells containing samples have cells added.
3. Program E1-Clip Tip Equalizer 384 pipette 12 channel 125 µL volume:
   Aspirate of 120 µL volume; 12 µL volume overfill; speed 10;
   Multi-dispense 30 µL volume; dispense 4 times; speed 10;
   Purge speed 10.
1. Add 30 µL assay media to the empty wells. Add 30 µL of assay media to row A 1-24; P 1-24 and columns B1-BO and B24-O24 and column 1 B-O and column 24 B-O.
2. Spin plates down at plates 300rpm for 2 minutes.
3. Immediately transfer to humidified 37+2◦C, 5% CO2 incubator. Do not stack plates. Be sure that the plates are positioned in a consistent orientation, long side parallel to back of the incubator.
4. Incubate plates for 72+ 2hrs. Record incubation times.

Renilla-Glo Detection

Storage conditions for Renilla-Glo luciferase assay buffer: -20°C. 4 ◦C for up to 1 year; room temperature, 22◦C + 4◦C for up to 3 months. Prepare Renilla-Glo substrate-buffer immediately before use, reagent will lose 10% activity after 2 hours at room temperature.

1. Allow *Renilla*-Glo buffer to equilibrate to room temperature. Do not thaw above 25°C. *Renilla*-Glo buffer can be stored at room temperature for up 3 months once removed from -20°C. Record the date of thawing and date of expiration on the buffer bottle, ELN, and worksheet.
2. Record time and date when plates are taken out of incubator.
3. In biological safety cabinet, allow plates to equilibrate to room temperature for 20±5 minutes. No stacking of plates, spread them in one layer.
4. Use the excel document RVP Reagent Calc-*Renilla*-Glo Reagent tab to calculate *Renilla*-Glo buffer and substrate amounts. Enter in grayed areas only in Table 6. Enter in number of wells in #wells, and number of plates in assay in #plates. Buffer (mL), and Substate (mL) will auto populate (6 plates were used for example).
5. Quickly obtain *Renilla*-Glo substrate from -20°C freezer. In a 125 mL sterile bottle combine Buffer (mL) and Substrate (mL). Invert 3-4 times to mix.

Table 6. Renilla-Glo Buffer Preparation[1]

Renilla Glo Buffer	
#wells	322
#plates	6
Buffer (mL)	72.5
Substrate(mL)	0.725

[1]See Appendix 1.3 to calculate values

Renilla-Glo Reagent Addition

1. In a BSC, transfer *Renilla*-Glo substrate- buffer solution to sterile media reservoir.
2. Program E1-Clip-tip Equalizer 384 pipette 12 channel 125 µL volume:
   Aspirate 120 µL volume; 12 µL volume overfill; speed 5
   Multi-dispense 30 µL; dispense 4 times; speed 7
   Purge speed 10.
1. Aspirate *Renilla*-Glo Reagent, dispense first 30 µL back into reservoir, multi-dispense 30 µL to wells B2-O23 of the 384-well assay plates. Discard tips after each aspiration cycle.
2. Incubate plates in dark at room temperature for 15±2 minutes. Start timer once *Renilla*-Glo Reagent has been added to all plates. Record the order of addition *Renilla*-Glo into the plates in the ELN.
3. Read Plates on Enspire
4. If detecting multiple plates, load assay plates in cell stacker in order of *Renilla*-Glo reagent addition
5. Run program.

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6. Save raw data files.
7. Proceed to Zika RVP Microneutralization Assay for data regression and analysis.
8. Note: LP for analysis is available upon request.

Appendix

Appendix 1. Zika RVP Microneutralization Assay Calculation Formulas:

1.1 RVP Preparation:

- Dilution= (Total Volume)÷(Optimized RVP (uL/well))
- Optimized RVP amount per well
- #wells/plate=number of wells occupied with sample per assay plate
- # plates= number of plates utilized in experiment
- Total number of wells= (#wells/plate)×# plates
- Volume diluted RVP/well(µL)= 7.5 volume of RVP to add to each well
- Volume diluted RVP required required(µL) =(Total # wells×volume diluted RVP/(well(uL)))×1.25
- Volume Prepared (µL)= Diluted RVP required rounded to nearest whole number
- Volume RVP=(Volume Prepared÷Volume diluted RVP/well(uL))×Optimized RVP(uL/well)
- Volume Assay Media(µL)= Volume Prepared(uL)-Volume RVP (uL)

1.2 Cell Preparation

- Optimized cells/well=4625
- Cells/mL =((Optimized cells/well)/(Volume of cells added/well))*1000
- Total number of wells =Number of plates×Number of wells/plate
- Volume Required=(Total wells×15×1.3)/(1000)
- Volume Prepared =Round Volume Required up

1.3 Renilla-Glo Preparation

- #wells-numbers of wells per assay plate
- #plates- number of 384 plates used in the assay
- Buffer(mL)- =(#wells)×(#plates)x(0.03mL)×(1.25overage)
- Substrate(mL) =Buffer (mL)÷100

Appendix 2. RVP Neut Calc Excel Workbook

2.1 RVP Neut Calc - Dilution Plate set up Tab

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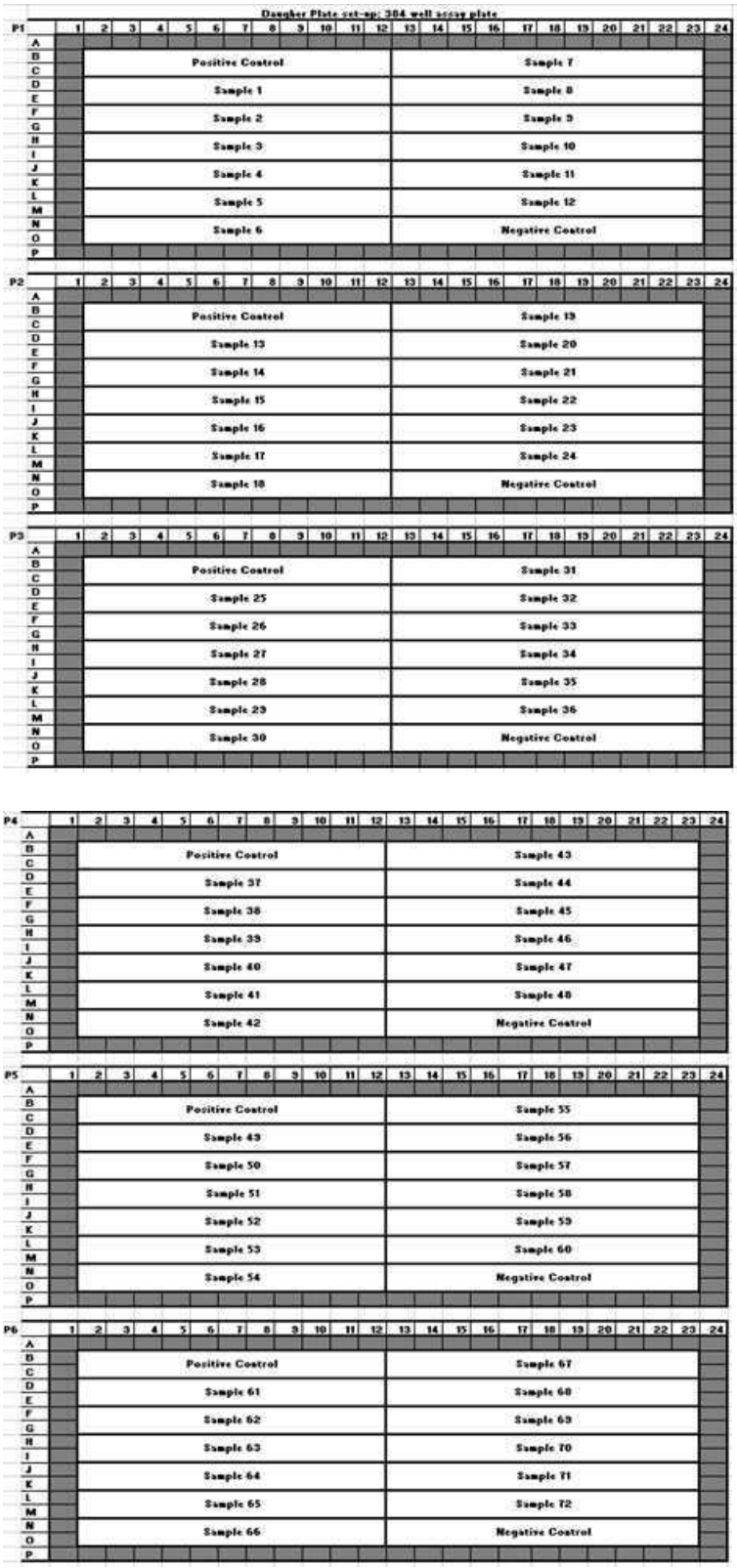
Serum sample pre-dilution worklist:				
Dilution Plate #	Sample ID	Barcode	Subject ID	Pre-dilution
Dilution Plate #1	Positive Control	N/A		
	1			
	2			
	3			
	4			
	5			
	6			
	7			
Dilution Plate #2	8			
	9			
	10			
	11			
	12			
	Negative Control	N/A		
	Positive Control	N/A		
	13			
Dilution Plate #3	14			
	15			
	16			
	17			
	18			
	19			
	20			
	21			
Dilution Plate #4	22			
	23			
	24			
	Negative Control	N/A		
	Positive Control	N/A		
	25			
	26			
	27			
Dilution Plate #5	28			
	29			
	30			
	31			
	32			
	33			
	34			
	35			
Dilution Plate #6	36			
	Negative Control	N/A		
	Positive Control	N/A		
	37			
	38			
	39			
	40			
	41			
Dilution Plate #7	42			
	43			
	44			
	45			
	46			
	47			
	48			
	Negative Control	N/A		
Dilution Plate #8	Positive Control	N/A		
	49			
	50			
	51			
	52			
	53			
	54			
	55			
Dilution Plate #9	56			
	57			
	58			
	59			
	60			
	Negative Control	N/A		
	Positive Control	N/A		
	61			
Dilution Plate #10	62			
	63			
	64			
	65			
	66			
	67			
	68			
	69			
Dilution Plate #11	70			
	71			
	72			
	Negative Control	N/A		
	N/A	N/A	N/A	N/A

2.2 RVP Neut Calc- Assay Plate Layout Tab

Citation: (04/24/2021). High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure. https://dx.doi.org/10.17504/protocols.io.bscpnavn



2.3 RVP Neut Calc- Neut Assay Conditions Tab

Cell Type	Neut Time (min)	Assay Time (hrs)
vero brel	60	72hrs

2.4 RVP Neut Calc- RVP Tab

Citation: (04/24/2021). High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure. https://dx.doi.org/10.17504/protocols.io.bscpnavn

Zika RVP

RVP#	Lot#	dilution	Optimized RVP ul/well	# wells/plates	# plates	Total # wells	Volume diluted RVP/well (ul)	Volume diluted RVP required (ul)	Volume Prep(uL)	Volume RVP(uL)	Volume Assay Media(uL)
SHP2015 Zika RVP	229A	12.0	2.5	322	6	1932	7.5	18113	18113	6038	12075

2.5 RVP Neut Calc v03- Cells Tab

Vero BrelI	N/A	N/A
Optimized Cells well	4625	N/A
cells / ml	308333	N/A
# wells/plate	322	N/A
# plates	6	N/A
Total wells	1932	N/A
Volume req.	38	mls
Volume Prepared	40	mls
Cells Req.	12333333	cells

Harvest Date	
Passage Number	
Date of Passage	
Flask Size	t175
Flask Media Volume	40

Set	Cells/mL total	Cells/ mL live	% live
count 1			
count 2			
Average (including additional dilution factor*)	NA	#DIV/0!	#DIV/0!

Amount of cells (mL)			Assay Media volume(mL)	
#DIV/0!	mL	in	#DIV/0!	mL

2.6 RVP Neut Calc- Renilla-Glo Reagent Tab

Renilla Glo Buffer	
#wells	322
#plates	6
Buffer (mL)	72.5
Substrate(mL)	0.725

Citation: (04/24/2021). High throughput Zika virus reporter virus particle microneutralization assay: Laboratory Procedure. https://dx.doi.org/10.17504/protocols.io.bscpnavn