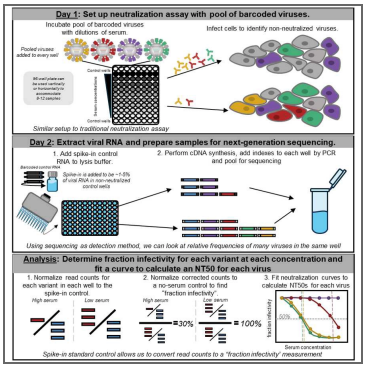

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Protocol status: Working
We use this protocol and it's working

Created: Jan 20, 2024

Sequencing-based Neutralization Assay for Influenza A Virus

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Rosario Araceli L Tarabi

ABSTRACT

Traditional neutralization assays for influenza virus test a single viral strain against a single serum sample in each measurement. Here we describe a sequencing-based approach for neutralization assays that can measure the titers of serum samples against many viruses at once using the same serum volume and workflow of a traditional neutralization measurement. This method relies on incorporating a nucleotide barcode into the hemagglutinin genomic segment of the influenza virus, pooling many barcoded viruses together, and then using Illumina sequencing to read out the neutralization of all of the viruses in the pool at once. Here we provide the step-by-step protocol for running these assays with serum samples.

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MATERIALS

(BEFORE DAY 1):

- Phosphate Buffered Saline **Thermo Fisher Scientific Catalog #28374** (1X)
- Receptor destroying enzyme (RDE) **Denka Seiken Co., Ltd**
- Barcoded influenza virus library containing HAs from strains of interest
- RNA spike-in control
- Serum samples

(DAY 1):

- D10 Media (recipe below)
- Influenza Growth Media (recipe below)
- Tissue-culture-treated 96-well plates
- Barcoded influenza virus library
- RDE-treated sera from before day 1
- Confluent 100 mm plate of MDCK-SIAT1 cells
- Trypsin-EDTA solution for dissociating cells from plate

D10 Media Recipe:

450 mL DMEM (High glucose)
50 mL Heat-inactivated Fetal Bovine Serum (FBS)
5 mL Penicillin Streptomycin Solution (10000 U/mL)
5 mL L-Glutamine (200 mM)

->sterile filter into a 500mL bottle

->store 4 °C

Influenza Growth Media Recipe:

20 mL BSA Fraction V Solution, 7.5%
5 mL Penicillin Streptomycin Solution (10000 U/mL)
500 µL Filtered Calcium Chloride (100 mg/mL)
50 µL Heat-Inactivated Fetal Bovine Serum (FBS)
475 mL Opti-MEM I

-> sterile filter into a 500mL bottle

-> store 4 °C

(DAY 2)

- iScriptTM cDNA synthesis kit **Bio-Rad Laboratories Catalog #170-8841**
-
iScript™ RT-qPCR Sample Preparation Reagent **Bio-Rad Laboratories Catalog #1708898**
- RNA spike-in control
- Gene specific cDNA primer (5' *CTCCCTACAATGTCGGATTTGTATTTAATAG*-3')
- Phosphate buffered Saline
-
KOD Hot Start DNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #71086-3**
- Forward Primer (5'-*GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCCCTACAATGTCGGATTTGTATTTAATAG*-3')
- Reverse Primer (5'-*AGTAGAAACAAGGGTGTTTTTCCTTATATTTCTGAAATCC*-3')
- Indexing Primer mixes (5 uM stocks of mixed Fwd and Rev Primers)
- 80% Ethanol (EtOH)
- Agencourt AmPure XP beads **Contributed by users Catalog #A63880**
- Elution buffer

SAFETY WARNINGS

Current and recent strains of human influenza virus are biosafety-level 2, but some historical human strains and animal strains are biosafety level 3 or higher. If you aren't sure of the biosafety level of the strains you are working with, check with your biosafety committee.

Several steps within the protocol are performed in a biosafety cabinet as they pose aerosol hazards. Prior to heat inactivation of the sera, all serum samples should be processed within a biosafety cabinet. In addition, as this protocol uses influenza A virus, setup for neutralization assays and preparation of cDNA reactions should be carried out in a biosafety cabinet. To reduce the hazard of working with aerosols, prepare a beaker of 1:10 dilution of bleach before work within the biosafety cabinet, immediately after pipetting any potential aerosol hazards, rinse pipette tips in the bleach solution by pipetting up and down. Seclude bleached tips to be disposed with other virus-contaminated materials as biohazardous waste.

Barcoded influenza HA library

1

Note

For this protocol, a pooled library of influenza virus strains which each contain a unique barcode sequence within the HA segment is needed. The reagents and protocols used to generate a barcoded influenza library are described in the associated manuscript. Prior to the start of this protocol, a barcoded influenza library must be obtained as well as a custom RNA spike-in control, which is used in the normalization of barcode count data in order to calculate neutralization titers from sequencing data.

(BEFORE DAY 1) RDE-treat & heat-inactivate sera

3h 40m

2

Note

It is important to treat serum with receptor destroying enzyme (RDE) prior to use in influenza neutralization assays. This process removes residual sialic acids from serum components that can otherwise inhibit HA-mediated infection. This protocol was modified slightly from Zost et al, 2017 (https://doi.org/10.1073/pnas.1712377114).

Keep in mind that the processes in these steps effectively dilute the final RDE-treated sera 1:4, and that dilution factor should be taken into account when calculating neutralization titers.

3 Thaw sera On ice

30m

4 Prepare RDE solution, by adding 20 mL 1X phosphate buffered saline (PBS) into one receptor destroying enzyme (RDE) bottle (VWR, Cat. No. 370013). Replace stopper and gently invert to mix. Sterile filter the solution through a 0.22 um filter prior to use. Aliquots of this RDE solution can be stored at -20 C for future use, if needed.

10m

5 Pipette RDE solution into labeled, externally threaded tubes, such that you can dilute the sera 1:4. For our sera samples, we added 300 µL RDE solution . Then, added 100 µL serum .

10m

6 To removes residual sialic acids in serum incubate serum and RDE at 37 °C 02:30:00 2h 30m

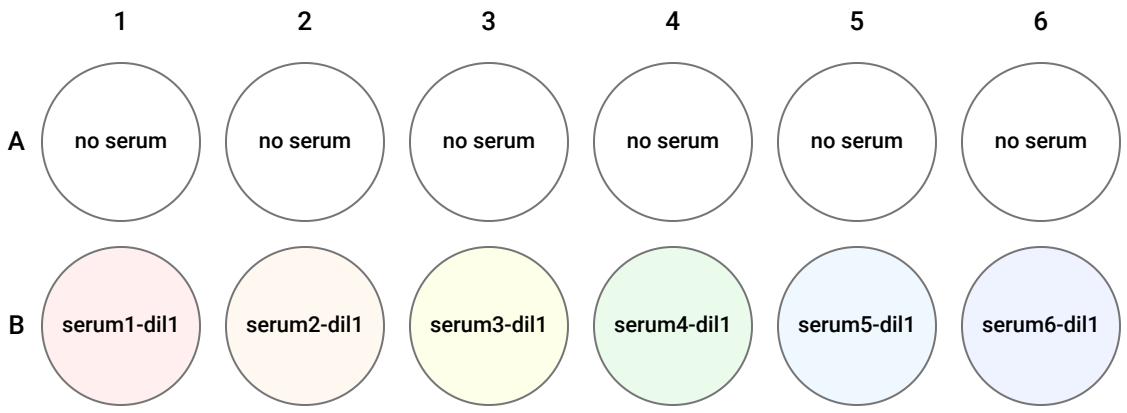
7 Then, to heat-inactivate the RDE and serum, incubate at 56 °C 00:30:00 30m

8 Move samples to ice, and then store heat-inactivated and RDE-treated serum samples at -80 °C until you are ready to begin the neutralization assays.

(DAY 1) Determine your plate setup

9 Different plate setups may be used depending on how many samples of sera you are testing, and how many dilutions you wish to run for each serum. For example, you could perform serial dilutions of serum down the plate vertically, running up to 12 serum samples with 7 different dilutions (plus a no-serum control) per serum sample. Or you could perform dilutions across the plate horizontally, running 8 serum samples with 11 different dilutions per serum-sample (plus a no-serum control). Additional plate set up designs are also feasible to meet the needs of your particular project.

Pictured below is an example plate setup for 12 serum samples with 7 concentrations of serum




1
2
3
4
5
6
C
serum1-dil2
serum2-dil2
serum3-dil2
serum4-dil2
serum5-dil2
serum6-dil2
D
serum1-dil3
serum2-dil3
serum3-dil3
serum4-dil3
serum5-dil3
serum6-dil3
E
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F
serum1-dil5
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G
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H
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serum4-dil7
serum5-dil7
serum6-dil7
7
8
9
10
11
12
A
no serum
no serum
no serum
no serum
no serum
no serum
B
serum7-dil1
serum8-dil1
serum9-dil1
serum10-dil1
serum11-dil1
serum12-dil1
C
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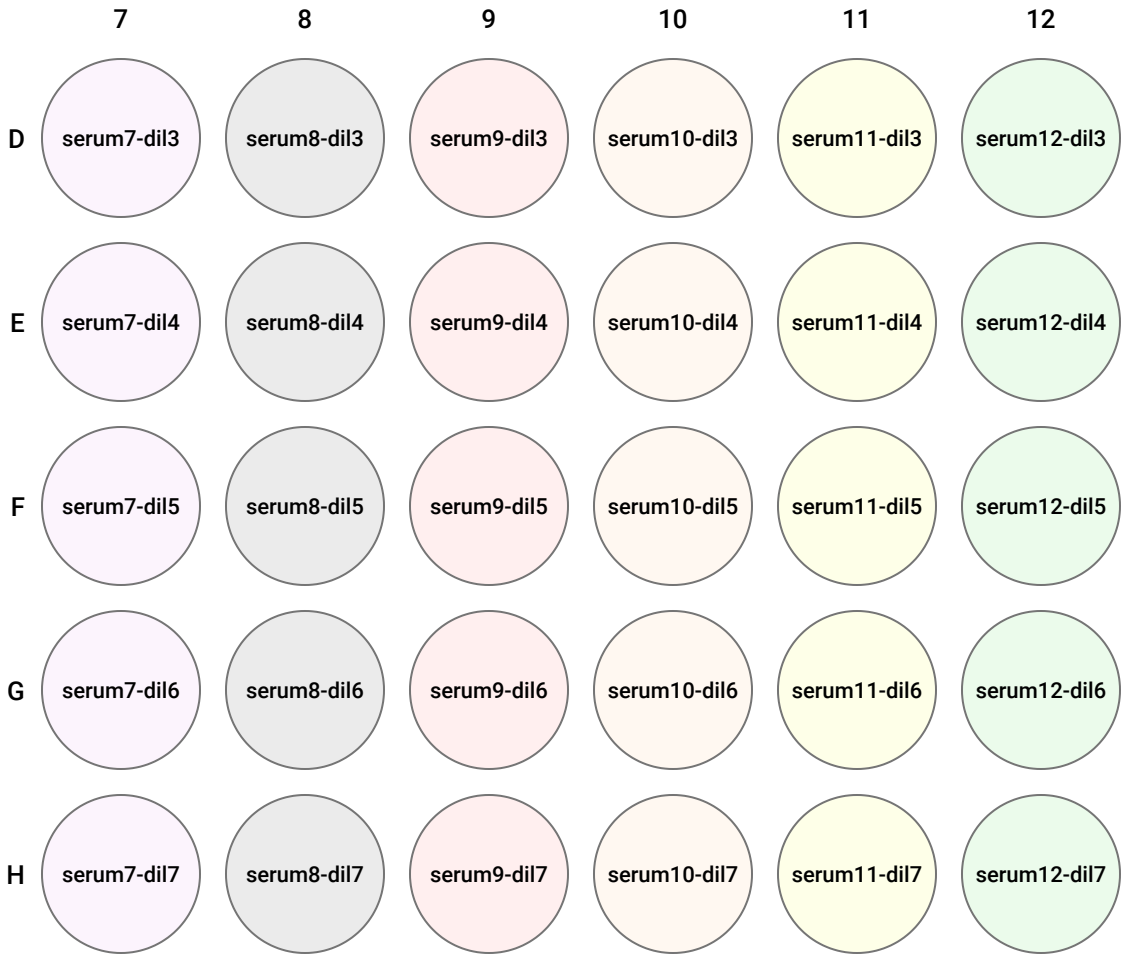
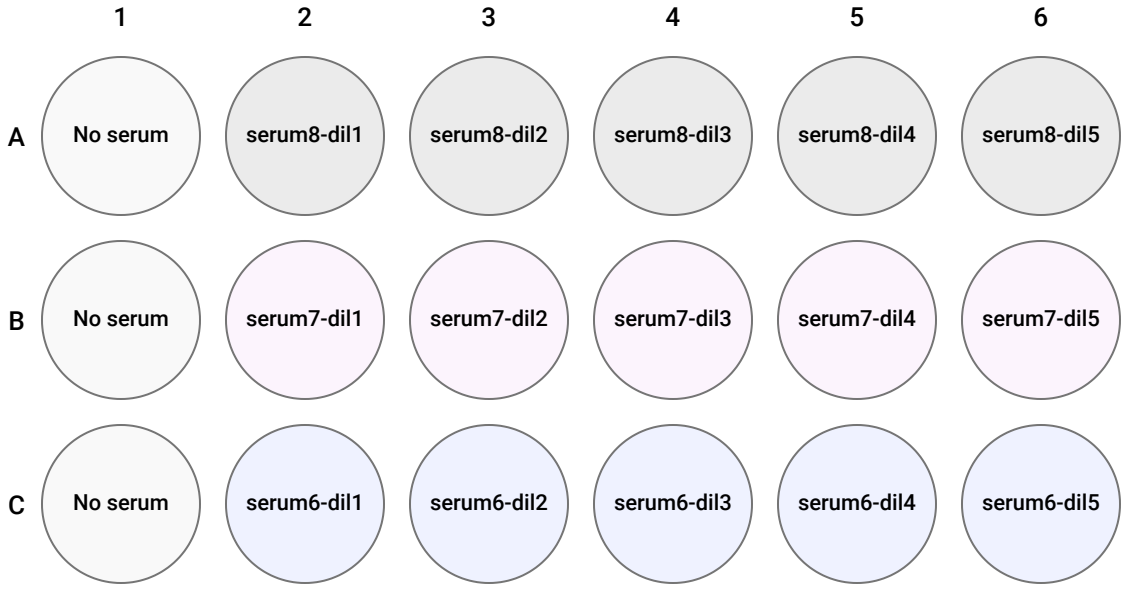
serum11-dil2
serum12-dil2



Pictured below is another example plate setup (8 serum samples with 11 concentrations of serum).




1
2
3
4
5
6
D
No serum
serum5-dil1
serum5-dil2
serum5-dil3
serum5-dil4
serum5-dil5
E
No serum
serum4-dil1
serum4-dil2
serum4-dil3
serum4-dil4
serum4-dil5
F
No serum
serum3-dil1
serum3-dil2
serum3-dil3
serum3-dil4
serum3-dil5
G
No serum
serum2-dil1
serum2-dil2
serum2-dil3
serum2-dil4
serum2-dil5
H
No serum
serum1-dil1
serum1-dil2
serum1-dil3
serum1-dil4
serum1-dil5
7
8
9
10
11
12
A
serum8-dil6
serum8-dil7
serum8-dil8
serum8-dil9
serum8-dil10
serum8-dil11
B
serum7-dil6
serum7-dil7
serum7-dil8
serum7-dil9
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serum7-dil11
C
serum6-dil6
serum6-dil7
serum6-dil8
serum6-dil9
serum6-dil10
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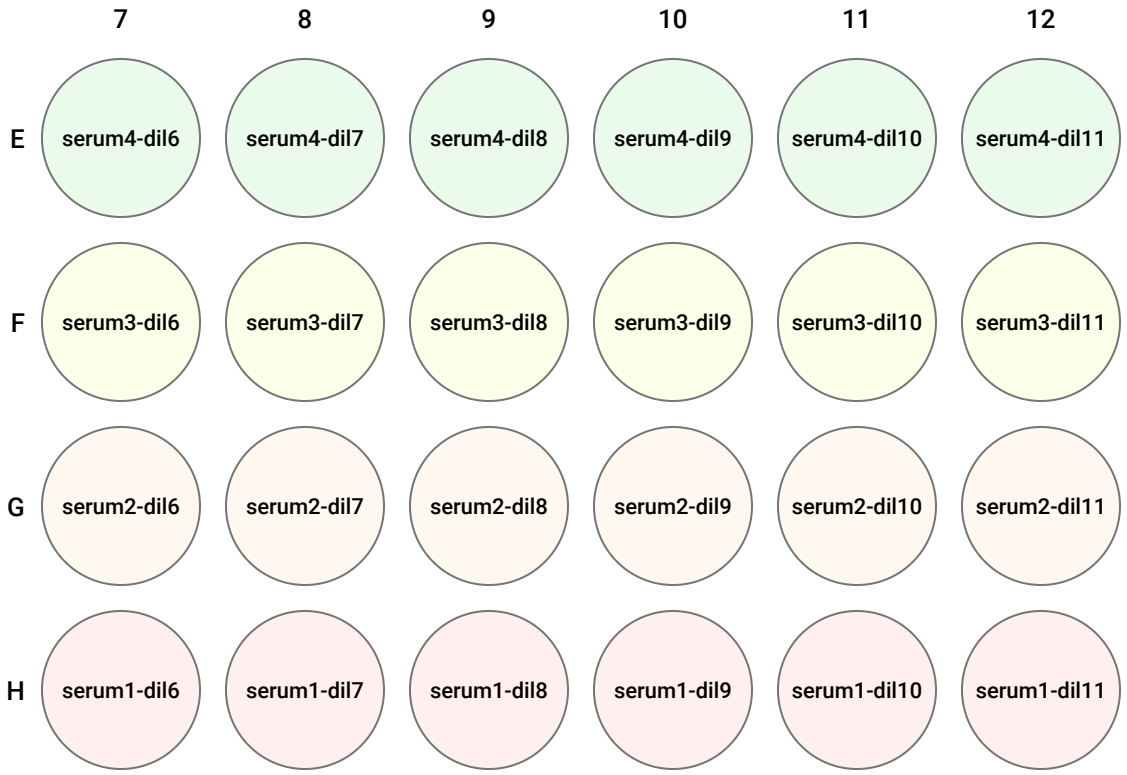
serum5-dil10
serum5-dil11



(DAY 1) Set up incubations of serum and virus

1h 20m

10

Safety information

The following steps should be carried out in a biosafety cabinet because they involve influenza virus. Current and recent strains of human influenza virus are biosafety level 2, but some historical human strains and animal strains are biosafety level 3 or higher. If you aren't sure of the biosafety level of the strains you are working with, check with your biosafety committee.

Prepare a beaker of 10% bleach immediately before performing any of the steps for rinsing pipette tips. Be sure to rinse tips here after interaction with virus samples, and seclude tips to be disposed with the rest of your lab's virus waste.

11 Thaw virus and RDE-treated sera Room temperature 00:30:00

30m

12 Pick an initial serum dilution factor for your assay: this will be the highest serum concentration tested, and other wells will contain serial dilutions of this concentration. You also need to choose what dilution factor to

use as you dilute the serum from one well to the next. For example, you might start with an initial dilution of 1:20 and perform 3-fold dilutions down the plate. Recall that the RDE treated sera has already been diluted 1:4 in the steps above.

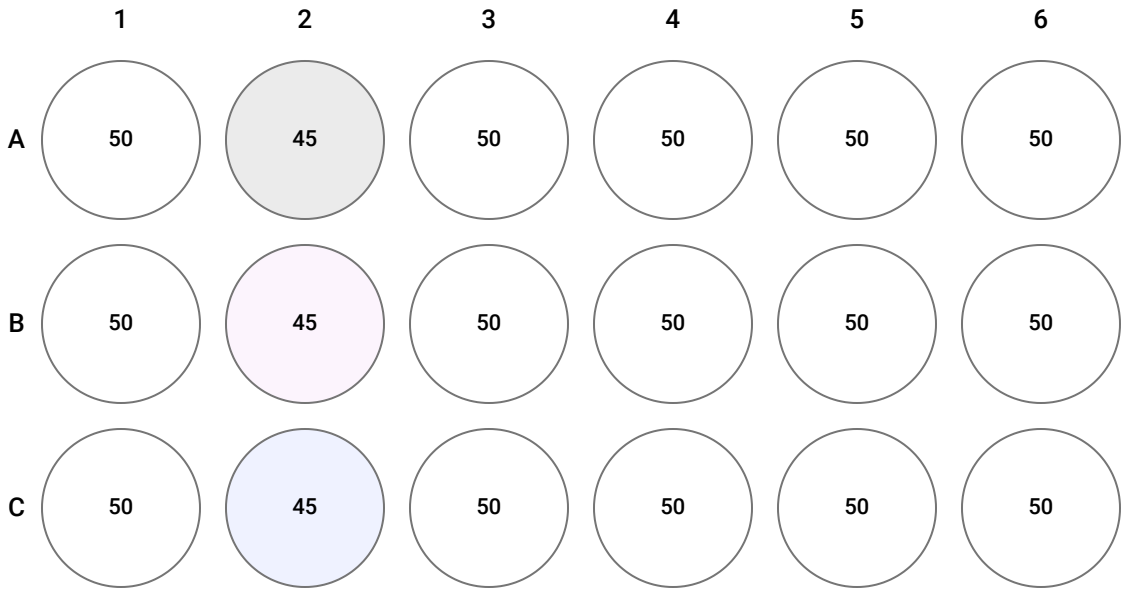
Note

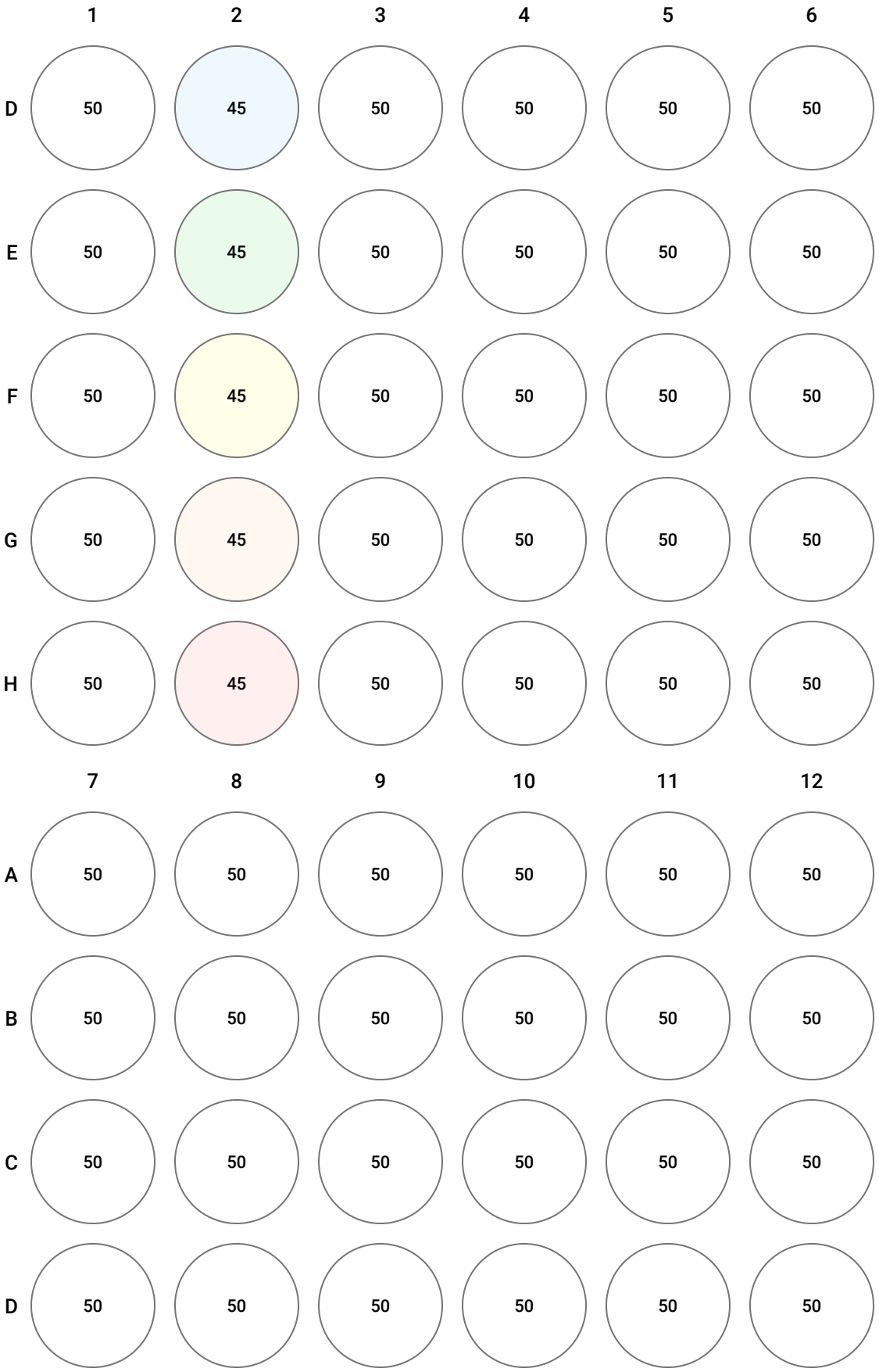
The best choice of the initial dilution and dilution-factor used will depend on the serum that is being tested with this method, as well as whether you want to always have the dilution range capture the neutralization titer 50% (NT50), or if just determining a bound on the NT50 is sufficient. If a larger range in NT50s is expected, and downstream analysis which requires that all NT50s measured are within range (such as assessing fold-change), a larger dilution factor might be preferred. If you are trying to measure small changes in neutralization titers within a given range, then a smaller dilution factor may be preferred, even though this may result in some NT50s being out of range of dilutions tested.

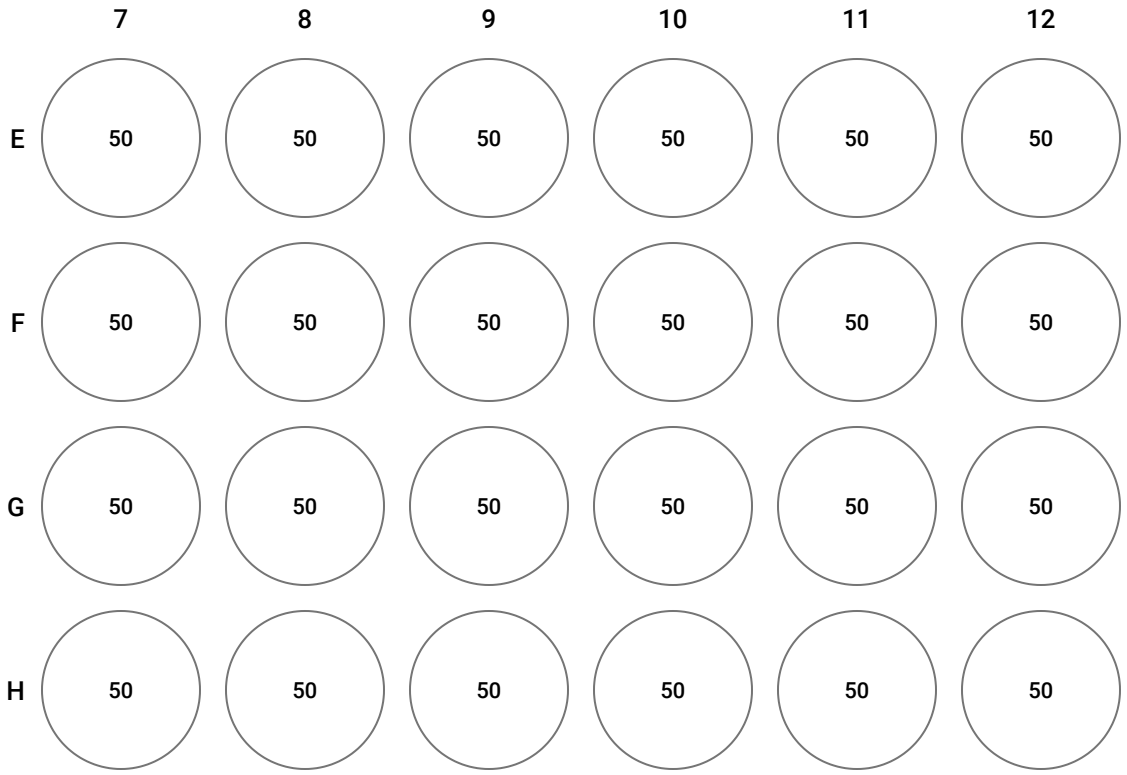
13 Add influenza growth media (Opti-MEM supplemented with 0.1% heat-inactivated FBS, 0.3% bovine serum albumin, 100 µg per mL of calcium chloride, 100 U per mL penicillin, and 100 µg per mL streptomycin) to your plate such that all rows contain either 50 uL or the volume needed for your initial dilution of sera. 5m

An example is shown below, using the setup described above where 8 serum samples are run horizontally across the plate. In this example, we use an initial dilution of serum of 1:20. As the serum has been diluted 1:4 during RDE treatment and will be diluted 1:2 with virus in a later step, you'd need to dilute the RDE-treated sera 2:5 for the initial dilution in the plate to obtain a final dilution of 1:20.

For this example, you would add 45 µL influenza growth media to the wells in your initial dilution column and 50 µL influenza growth media in every other well:




1 2 3 4 5 6
D 50 45 50 50 50 50
E 50 45 50 50 50 50
F 50 45 50 50 50 50
G 50 45 50 50 50 50
H 50 45 50 50 50 50
7 8 9 10 11 12
A 50 50 50 50 50 50
B 50 50 50 50 50 50
C 50 50 50 50 50 50
D 50 50 50 50 50 50



14 Add 30 µL RDE-treated serum to each well in column 2. This leaves you with a 1:10 sera dilution of the original serum (a 2:5 dilution of RDE-treated serum that was already diluted 1:4 during RDE treatment) in 75uL total volume in the wells in column 2. 5m

15 Perform serial 3-fold dilutions, pipetting 25 µL serum dilution from column 2 into column 3, and mixing by pipetting up and down several times. Repeat this step with column 3 into column 4 and so forth until you get to row 12, after which you will pipette the residual 25 uL from this column directly into bleach. After this step, you will have completed the serial 3-fold dilutions across the plate. You should still have no serum in column 1, and all 96 wells should now contain 50uL total volume. 5m

16 The appropriate amount of virus library needed for each plate is experimentally determined as the amount virus that can be added to the number of cells per well (50,000 MDCK-SIAT1 cells as described below) and 5m

still be in the linear range of viral transcriptional output changing with viral dilution. (See note for more detail). As 50 µL of virus will be added to each well, we first dilute the virus library that the appropriate amount of virus is in each 50 µL aliquot, and then add 50 µL virus dilution to each well (including the control, no serum row of wells).

Note

This method requires that the number of sequencing counts for each HA viral barcode normalized by the spike-in RNA counts be directly proportional to the number of virions encoding that barcode that infect cells. The amount of virus library used per well for this assay should be experimentally determined to meet this condition. The range at which this is true depends on the number of transcriptionally active viral particles per cell (MOI). At low to moderate MOI, increasing the number of virions infecting cells will result in more barcoded viral RNA produced, but at high MOIs the capacity of infected cells to produce viral RNA becomes saturated. To identify the amount of virus library where there is a linear relationship between the amount of input library and the resulting spike-in normalized HA counts, one should perform a dilution series, infecting wells with different amounts of virus library, adding a known concentration of RNA spike-in control and sequencing these samples to identify the highest amount of virus library that can be added and still result in a two-fold decrease in the counts of viral barcodes relative to spike-in control barcodes for every 2-fold dilution of virus library.

A description of how to prepare the RNA spike in control (a single stranded RNA molecule, which resembles barcoded HA vRNA, but with the sequence of GFP in place of the HA ectodomain) is provided in the associated manuscript.

17 Incubate plate of virus and serum 37 °C in CO2 incubator 01:00:00 1h

(DAY 1) Add cells to plate 30m

18 After the virus-serum mix has been incubated for 35 min of the 60 min incubation time, prepare cells to be added to the plate. First, aspirate media off of a confluent 100 mm plate of MDCK-SIAT1 cells. Wash the cells once with 2 mL PBS. Then, treat cells with 2 mL trypsin-EDTA, incubating at 37 °C in CO2 incubator until cells disassociate from the plate (this typically takes around 5-10 min, since MDCK-SIAT1 cells are highly adherent). Once the cells are mostly dissociated, pipetting gently can help dissociate the remainder. 12m

19

Note

We use MDCK-SIAT1 cells in this assay rather than MDCK-SIAT1-TMPRSS2 cells to limit secondary viral replication (TMPRSS2 cleaves HA in producing cells to activate it for infection). These cells adhere extremely well to tissue-culture plates, so make sure to allow for sufficient time to trypsin treat and wash such that these cells are ready after virus and serum have incubated for 1 hour.

20 Inactivate the trypsin by adding 4 mL D10 media , washing the cells off the plate and resuspending in this media. Transfer the resuspended cells to a conical tube. Centrifuge 300 x g, 00:03:00 , then aspirate the supernatant. Wash cells once with 5 mL influenza growth media , and then resuspend cells in 5 mL influenza growth media . Use a cell counter to determine cell concentration, and dilute cells to 1e6 cells/mL with more influenza growth media. You will need about 5.5 mL of cells at 1e6 cells/ml in influenza growth media for each 96-well plate that you wish to run. 8m

21 Once the plate with the serum and the virus library has been incubating for 1 hour total, add 50 µL 1e6 cells/mL in influenza growth media to each well. 10m

22 Return the plate containing cells, serum, and virus library to the 5% CO2 incubator. Incubate at 37 °C for 16:00:00 . This time allows non-neutralized viruses to infect cells and transcribe viral RNA. 16h

(DAY 2) cDNA synthesis 3h 7m

23

Note

In order to relate the counts of each barcoded vRNA in the sequencing data to the number of vRNA represented in the infected cells, we need to add a spike-in control at a known concentration to each well. We generate an RNA spike-in control using in vitro transcription. This construct is similar to the barcoded HA vRNA, but contains GFP in place of the HA ectodomain. A number of known barcodes are associated with the RNA spike-in control and these barcodes are used to normalize the counts for each barcoded HA in the eventual sequencing data. An detailed description of how to generate this construct is provided in the associated manuscript.

*We describe these next steps using the iScript Select Synthesis Kit and the iScript Sample Preparation Buffer from BioRad, which we have tested in this method for performing cDNA synthesis of viral RNA (vRNA) from infected cells without the need for RNA extraction. Other commercially available kits could be used for this method if preferred.

24 Approximately 15 hours into the incubation period of the cells and virus, start thawing reagents from the iScript Select Synthesis Kit (BioRad) needed to perform cDNA synthesis (5x reaction mix, nuclease-free water, GSP enhancer, gene-specific primer, iScript enzyme mix) and a 200 pM aliquot of the RNA spike-in control. Most of the reaction mix components can be thawed Room temperature but the the enzyme mix and the RNA spike-in control should be thawed On ice . 1h

25 When your RNA spike-in control is thawed, mix 55 µL of 200 pM RNA spike-in control into 5445 µL iScript sample preparation reagent , thereby diluting the spike-in control 1:100 for a final concentration of 2 pM. You will end up with 5.5 mL total volume of lysis buffer using these specific amounts, which is sufficient for one plate. Adjust the above amounts according to how many plates of neutralization assays you set up. 15m

26 When your cDNA synthesis reagents have thawed, prepare a cDNA synthesis mastermix. We use an 18 µL final reaction volume for each well, typically preparing sufficient mastermix for 100 reactions per plate to allow excess prior to distributing the the mastermix to PCR tubes. For one plate, your mastermix can be prepared like so: 15m

400 µL 5x reaction mix
900 µL nuclease-free water
200 µL GSP enhancer
200 µL gene-specific primer (2 uM stock)
100 µL iScript enzyme mix

Please adjust these amounts according to how many plates of neutralization assays you are running.

Aliquot 18 µL cDNA synthesis mastermix into 8-strip PCR tubes or a 96-well PCR plate.

Note

The gene-specific cDNA synthesis primer used is:
cDNA_Fwd: 5' *CTCCCTACAATGTCGGATTTGTATTTAATAG*-3'

This primer binds immediately upstream of the barcode in the vRNA.

27

Safety information

The following section should be carried out in a biosafety cabinet because the steps involve influenza virus. Current and recent strains of human influenza virus are biosafety-level 2, but some historical human strains and animal strains are biosafety level 3 or higher. If you aren't sure of the biosafety level of the strains you are working with, check with your biosafety committee.

Filter tips are used for these steps to prevent issues with aerosol hazards and reduce RNAse contamination.

Prepare a beaker of 10% bleach immediately before performing any of the steps for rinsing pipette tips. Be sure to rinse tips here after interaction with virus samples, and seclude tips to be disposed with the rest of your lab's virus waste.

28 Remove the supernatant from the plate using a multichannel pipette, taking care not to disturb the cells an 5m pipette this directly into bleach solution.

29 Wash the infected cells once with 150 µL PBS per well. This wash step removes any residual virus 10m from the supernatant and improves the cell lysis efficiency. Take care not to disturb the adhered cells when performing this wash step. When removing this liquid, pipette directly into bleach, and rinse tips with bleach solution. Examine the wells of the plate to ensure that residual liquid is completely removed from the wells of the plate prior to adding lysis buffer. This is essential as if residual liquid remains, this will dilute the spike-in control and result in noise in the measurements collected with this assay.

30 Add 50 µL lysis buffer (with the RNA spike-in control added) to each well. Wait 2-5 minutes for cells to lyse. Examine wells under the microscope to determine that cell lysis has occurred. This lysis buffer is gentle, expect that nuclei will remain adhered to the plate, though the cell borders will be less defined following lysis. 5m

31 After 2-5 minutes, transfer lysate to a new 96-well PCR plate so that the lysis of cells will not continue. 2m

32 Add 2 µL cell lysate to the strip tubes (or 96-well PCR plate) containing 18 µL iScript cDNA Select Synthesis mastermix 4m

33 Transfer the strip tubes (or 96-well PCR plate) containing cDNA synthesis reaction mixes to the thermocycler with the lid heated to 60 °C

Set the following protocol:

33.1 Incubate at 42 °C 01:00:00 1h

33.2 Inactivate enzymes at 85 °C 00:05:00 5m

33.3 Hold at 4 °C

*This is a potential stopping point, samples could be stored a -20 °C overnight, if needed before progressing to the next step of the assay.

(DAY 2) Round 1 PCR: adding overlap for indexing primers 35m

34 During cDNA synthesis, prepare Round 1 PCR mixes. 10m

Note

The following primers are used for Round 1 PCR:

Rnd1_Fwd: 5'-*GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCCCTACAATGTCGGATTTGTATTTAATAG*-3'
Rnd1_Rev: 5'-*AGTAGAAACAAGGGTGTTTTTCCTTATATTTCTGAAATCC*-3'

For one sample, the mix is as follows:

- 1.5 µL Forward Primer
- 1.5 µL Reverse Primer
- 25 µL KOD Hot Start Master Mix
- 17 µL H2O
- 5 µL template from cDNA synthesis

= 50 µL total volume

The following mix is enough to prepare for 96 samples:

- 150 µL Fwd Primer
- 150 µL Rev Primer
- 2500 µL KOD Hot Start Master Mix
- 1700 µL H2O

= 4500 µL mastermix total volume

Aliquot 45 µL mastermix per well in a 96-well PCR plate.

Add 5 µL template . Seal the 96-well PCR plate with a PCR plate seal that is appropriate for thermal cycling and cold storage, such as (**#MSF1001, BioRad**).

35 Set the PCR plate containing Round 1 PCR reactions in thermocycler with the lid heated to 100 °C

Set the following protocol:

35.1 Initial denaturation 2m

95 °C 00:02:00

35.2 Denaturation 20s

95 °C 00:00:20

35.3 Annealing 10s

56 °C 00:00:10

35.4 Extension 20s

70 °C 00:00:20

35.5 Repeat previous three PCR cycle steps for denaturation, annealing, and extension 19 more 15m 50s

35.6 Final extension 2m

70 °C 00:02:00

35.7 Hold 10 °C

*This is a potential stopping point, samples could be stored a -20 °C overnight, if needed before progressing to the next step of the assay.

(DAY 2) Round 2 PCR: adding indices 40m

36

Note

The steps below describe how to add 10 bp unique dual indices to the samples, which is the method we use in our lab for demultiplexing these samples after an Illumina NextSeq run. Alternate methods for indexing could be used.

For this step, we ordered dual indices based on the index sequences designed by Twist Biosciences (https://www.twistbioscience.com/resources/protocol/Unique%20Dual-Index-Sequences-reference-spreadsheets-and-sample-sheet-templates) inside custom indexing primers ordered through IDT which have the following format:

UDI_i7: 5'-*CAAGCAGAAGACGGCATACGAGATnnnnnnnnnnGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3*
UDI_i5: *5'-AATGATACGGCGACCACCGAGATCTACACnnnnnnnnnnACACTCTTTCCCTACACGACGCTCTTCCGATCT-*3'

Indexing primer stock mixes are prepared at 5 uM final concentration, with i7 and i5 indexes pooled at equal concentration (i.e. 2.5 uM UDI_i7 and 2.5 uM UDI_i5).

*Note, we observed an error in sequencing of barcodes that start with 'GG' in some wells where indexes also contained a 'GG' sequence. We advise that when designing the virus library for this method, that barcodes starting with 'GG' be avoided.

37 Prepare mix for Round 2 PCR. For one sample, the reaction mix is as follows: 20m

2.4 µL Indexing Primer Mix (5 uM stock, contains equal concentration mix of fwd and rev indexing primer pair)

20 µL KOD Hot Start Mastermix

16.6 µL H2O

1 µL Round 1 PCR product

= 40 µL total volume

We prepare a mastermix of KOD and H2O and apply this first to the plate, then add the primers. The following mix is enough to prepare for 96 samples:

2000 µL KOD

1660 µL H2O

= 3660 µL mastermix total volume

Transfer 36.6 µL mastermix into each well of a 96-well PCR plate.

Add 2.4 µL Indexing Primer Mix (each mix of primers should match the number assigned to your sample).

Add 1 µL Round 1 PCR result .

Seal the 96-well PCR plate with a PCR plate seal that is appropriate for thermal cycling and cold storage.

38 Set 96-well PCR plate containing the Round 2 reaction mixes in thermocycler with the lid heated to 100 °C
Set the following protocol:

38.1 Initial denaturation 2m
95 °C 00:02:00

38.2 Denaturation 20s
95 °C 00:00:20

38.3 Annealing 10s
66 °C 00:00:10

38.4 Extension 20s
70 °C 00:00:20

38.5 Repeat previous three PCR cycle steps for denaturation, annealing, and extension 19 more tim 16m

38.6 Final extension 2m

70 °C 00:02:00

38.7 Hold 10 °C

*This is a potential stopping point, samples could be stored a -20 °C overnight, if needed before progressing to the next step of the assay.

(DAY 2) Pooling & Preparing for Sequencing

1h 22m

39

Note

Lastly, we will pool the samples and run on an agarose gel to remove any residual indexing primers, and remove any Round1 PCR product from the Round2 sample. Following this step, a magnetic bead cleanup step is performed to insure that the sample is in an appropriate buffer for next-generation sequencing. The final band for the indexed sample should be 181 bps in length.

40 Prepare a 1% agarose gel. This can be done during Round 2 PCR.

41 Pool Round 2 PCR products at equal volume (enough so you have at least ~200uL total volume).

*We pool using a multichannel, first pooling all columns of the plate into a single 8-strip (5 uL from each sample), then pooling all 8 of the pools for each row of the plate. This will result in total volume of 480 uL per plate. It is not necessary to prepare this total volume for sequencing, only approximately ~100 uL needs to be gel extracted and bead purified for sequencing, but we have found it useful to pipette a larger volume (5 uL as opposed to 2 uL) per sample when pooling to ensure that all samples are included in the final pool.

42 Run 100 µL Round 2 PCR results pool on your gel from step 33 at 85 V 00:40:00 . We often run 30-40 µL of the sample per well on the agarose gel (with loading buffer), this results in 3-4 lanes of sample to be cut out and processed for sequencing.

40m

43

Note

Since you are submitting the results of this gel for sequencing, it is important to ensure it is exposed to as little contamination from prior runs and ultraviolet light as possible. With this in mind, we use a blue light and Sybr Safe Gel Stain (Invitrogen, S33102) for visualization of the gel. We also use a layer of plastic wrap between the gel and the light source that insures that the sample will not be contaminated by prior runs with the same indexing primers. If you are running multiple plates on the same day that were prepared with the same indexes, use separate gels for each plate.

Cut the band out of the gel, and extract the DNA using a Nucleospin Gel Extraction Kit (740609, Takara), or alternate gel extraction kit. Transfer the eluate to a strip tube for magnetic bead cleanup with AMPure XP SPRI Reagent (Beckman Coulter).

44 Perform a magnetic bead cleanup on your sample using AMPure XP SPRI Reagent to remove residual salts from the gel extraction:

44.1 Add 2X the volume of your sample of AMPure XP SPRI Reagent (i.e. if you eluted in 40 uL add 80 uL Ampure XP beads).

45 Incubate Room temperature 00:05:00 . 5m

45.1 Load strip of tubes onto magnetic block. Incubate 00:05:00 . 5m

45.2 Pull off liquid while being careful to avoid disrupting magnetic beads. Wash twice, using 150 µL 80% EtOH each time (again, taking care to not disrupt the magnetic beads). Extract all liquid from tube and wait for pellet to start drying. 5m

45.3 When the pellet is dry (but not so dry that it has started to crack, approximately 00:10:00, 15m
resuspend in 40 µL elution buffer . Remove strip of tubes from magnetic block and repeatedly pipette the liquid up and down to thoroughly mix the beads into the elution buffer. Incubate at Room temperature for 00:05:00 .

45.4 Place strip tubes on magnetic block, incubate 00:05:00 . 5m

45.5 Once the beads are adhered to the magnet, remove the residual liquid from the beads, careful n 1m
to disturb the beads, and transfer this liquid to new 1.5 uL tube.

46 Quantify total DNA concentration, for this we use a Qubit. We typically obtain 40 µL of sample at 3m
[M] 0.005 µg/µL

47 Label, appropriately dilute, and submit sample for sequencing as determined by the sequencing service yo 3m
are using. We use Illumina NextSeq for these runs (a P1 run for 1 plate and a P2 should be used for 2-4 plates). We submit the 181 bp amplicon for sequencing with a 50 bp read length. We aim to obtain an average coverage of between 500,000-1,000,000 reads/well. This is likely higher coverage than necessary, however, by over-sequencing we allow for variability in loading of the different wells, which is helpful given that we are not performing any sample normalization prior to pooling.

Data Analysis

48 Following demultiplexing of the sequencing run, sequencing data is analyzed using the modular analysis pipeline developed by the Bloom lab for processing high-throughput sequencing-based neutralization assays. This pipeline is available at https://github.com/jbloomlab/seqneut-pipeline

See associated manuscript for a detailed description of this analysis method.

Briefly, this pipeline takes in the FASTQ files from a sequencing run, calculates the counts for each barcoded viral variant and the RNA spike-in control in each well. The ratio of barcoded viral variant to spike-in control in each well containing serum is normalized to the ratio of barcoded viral variant to spike-in control in the no-serum control well to calculate a fraction infectivity. Then, neutralization titers are computed by fitting Hill-

curve style neutralization curves to the fraction infectivity values using the neutcurve package; see the documentation for the details of these curves. The titers represent the reciprocal serum dilutions at which half the viral infectivity is neutralized. For more details, please see the description at https://github.com/jbloomlab/seqneut-pipeline