bio-protocol

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VirScan: High-throughput Profiling of Antiviral Antibody Epitopes

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Abstract

Profiling the specificities of antibodies can reveal a wealth of information about humoral immune responses and the antigens they target. Here, we present a protocol for VirScan, an application of the phage immunoprecipitation sequencing (PhIP-Seq) method for profiling the specificities of human antiviral antibodies. Accompanying this protocol is a video of the experimental procedure. VirScan and, more generally, PhIP-Seq are techniques that enable high-throughput antibody profiling by combining high-throughput DNA oligo synthesis and bacteriophage display with next-generation sequencing. In the VirScan method, human sera samples are screened against a library of peptides spanning the entire human viral proteome. Bound phage are immunoprecipitated and sequenced, identifying the viral peptides recognized by the antibodies. VirScan Is a powerful tool for uncovering individual viral exposure histories, mapping the epitope landscape of viruses of interest, and studying fundamental mechanisms of viral immunity.

Keywords: VirScan, PhIP-Seq, Bacteriophage display, Synthetic biology, High-throughput screening, Serology, Antibody, Epitope, Virus, Immunology

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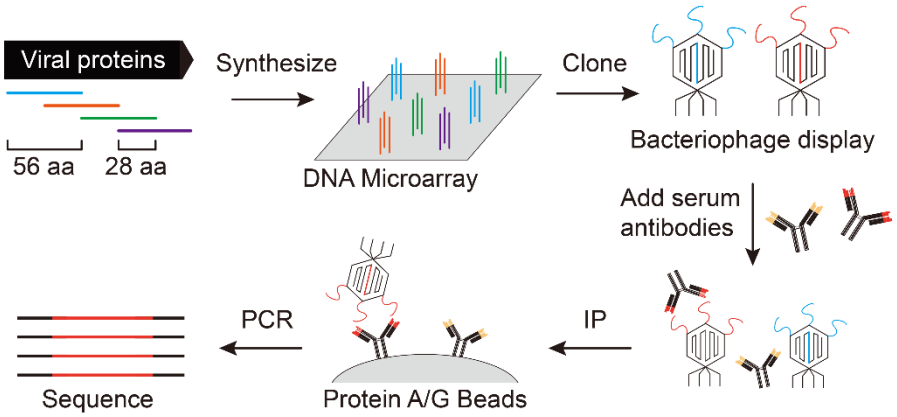
Graphical abstract:



Background

VirScan (Xu *et al.*, 2015) is based on a general technology called phage immunoprecipitation sequencing (PhIP-Seq) (Larman *et al.*, 2011; Mohan *et al.*, 2018; Mandel-Brehm *et al.*, 2019, Garrett *et al.*, 2020). In PhIP-Seq, a proteome-scale library of peptides is designed, and DNA oligos encoding these peptides are synthesized and cloned into a T7 bacteriophage display system. Each phage encodes the sequence of one peptide in its genome and displays the same peptide on its surface, thus linking genotype with phenotype (Smith and Petrenko, 1997; Kosuri *et al.*, 2010). For each PhIP-Seq reaction, the phage display library is mixed with a sample containing human antibodies, and the antibodies bind to their cognate epitopes on the phage surface. Then the phage-antibody complexes are immunoprecipitated and unbound phage are washed away. PCR amplification and high-throughput sequencing of the insert DNA from bound phage reveal the peptides targeted by antibodies in the sample. Whereas the original PhIP-Seq assays were performed using a phage display library of peptides derived from the human proteome to detect autoantibodies, VirScan employs a library of peptides derived from the human virome to identify the specificities of antibodies targeting viral antigens.

A comparison of the advantages and disadvantages of PhIP-Seq relative to peptide or protein microarrays for high-throughput epitope profiling is provided in Mohan *et al.* (2018). In brief, PhIP-Seq enables higher throughput, less expensive, and more highly programmable assays relative to peptide and whole protein microarrays. A disadvantage of PhIP-Seq compared with protein microarrays is that the experimental procedure takes longer to perform since its readout involves next-generation sequencing. As with all peptide-based epitope profiling methods, PhIP-Seq generally does not enable detection of discontinuous epitopes or epitopes that involve post-translational modifications.

A comprehensive article describing the PhIP-Seq protocol has been published (Mohan *et al.*, 2018) and is a valuable resource to those interested in using VirScan technology. The present article serves to supplement that resource by presenting videos of the experimental protocol (Videos 1–5) and including information relevant for VirScan-specific data analysis (Supplementary materials). This protocol does not contain information related to the design and generation of the VirScan phage display library, as these methods have been covered in depth by Mohan *et al.* (2018) and Xu *et al.* (2015). This article assumes that the researcher has access to the VirScan library and focuses on the downstream experimental procedures, namely, phage-antibody complex formation, immunoprecipitation, and preparation of DNA libraries for next-generation sequencing, which are also depicted in the videos (Videos 1–5). Further, this protocol covers VirScan-specific data analysis steps, including hits by virus calculation, virus score calculation, and determination of virus seropositivity.

The VirScan protocol may be modified with supplemental libraries, alternative immunoprecipitation reagents, and input samples other than human serum to address a broader set of scientific questions. Alanine scanning and saturation mutagenesis libraries may be designed to enable high-resolution mapping of antibody epitopes, as performed in Shrock *et al.* (2020) and Chen *et al.* (2021). The standard immunoprecipitation reagents, Protein A and

Protein G, may be replaced with isotype-specific secondary antibodies to profile antibody isotypes other than IgG, such as IgA or IgE, as performed in Shrock *et al.* (2020) and Chen *et al.* (2021). The protocol may be used with serum samples from several mammalian species other than humans, including mice and non-human primates, since the Protein A and Protein G bind to mouse and non-human primate IgG as well as human IgG (Borriello *et al.*, 2022). Finally, antibody-containing samples other than serum, including saliva, breast milk, and supernatant from cultured B cells, may be used as input samples for VirScan.

VirScan has been used successfully in many applications, including to estimate the number of viral species to which individuals have been exposed (Xu *et al.*, 2015); to show that infection by measles virus diminishes the preexisting antibody repertoire, leaving individuals vulnerable to reinfection to pathogens (Mina *et al.*, 2019); to study the maternally derived antibody repertoire in human infants (Pou *et al.*, 2019); to investigate the effects of CART therapy directed against CD19 on the antiviral antibody repertoire (Hill *et al.*, 2019); to uncover a putative viral etiology of the rare neurological condition Acute Flaccid Myelitis (Schubert *et al.*, 2019), to map SARS-CoV-2 linear epitopes with high resolution and determine humoral immune correlates of COVID-19 severity (Shrock *et al.*, 2020; Zamecnik *et al.*, 2020), and to provide evidence that Epstein-Barr virus infection increases risk for subsequent development of multiple sclerosis (Bjornevik *et al.*, 2022).

Materials and Reagents

1. Pipette Tips SR LTS 20 µL F 960A/5 (Rainin, catalog number: 17005860), storage temperature: room temperature
2. Pipette Tips SR LTS 200 µL F 960A/5 (Rainin, catalog number: 17005859), storage temperature: room temperature
3. Pipette Tips SR LTS 1,200 µL F 768A/4 (Rainin, catalog number: 17007084), storage temperature: room temperature
4. Disposable Serological Pipets, Polystyrene, Sterile, Plugged, 5 mL (*e.g.*, VWR, catalog number: 89130-896), storage temperature: room temperature
5. Disposable Serological Pipets, Polystyrene, Sterile, Plugged, 10 mL (*e.g.*, VWR, catalog number: 89130-898), storage temperature: room temperature
6. Disposable Serological Pipets, Polystyrene, Sterile, Plugged, 25 mL (*e.g.*, VWR, catalog number: 89130-900), storage temperature: room temperature
7. Disposable Serological Pipets, Polystyrene, Sterile, Plugged, 50 mL (*e.g.*, VWR, catalog number: 89130-902), storage temperature: room temperature
8. Reagent Reservoirs, Sterile (*e.g.*, Corning, Costar, catalog number: 4870), storage temperature: room temperature
9. Sterile Filter Storage Bottles/Receivers (*e.g.*, Thermo Fisher, Nalgene, catalog number: 455-0500), storage temperature: room temperature
10. Deep Well Plate, 96-well, PP, 1.1 mL, Standard, U-Bottom (Cole-Parmer, BrandTech, catalog number: EW-07904-04), storage temperature: room temperature
11. Kimtech Science Kimwipes Delicate Task Wipes (Kimberly-Clark, catalog number: 34155), storage temperature: room temperature
12. Sealing paddle (USA Scientific, catalog number: 2928-7355), storage temperature: room temperature
13. MicroAmp Optical Adhesive Film (Thermo Fisher, Applied Biosystems, catalog number: 4311971), storage temperature: room temperature
14. Colored Labeling Tape, Rainbow Pack (Fisher Scientific, Fisherbrand, catalog number: 15-901-10R), storage temperature: room temperature
15. PCR Plate, 96-well (*e.g.*, VWR, catalog number: 82006-704), storage temperature: room temperature
16. Bravo Lab Disposable Pipette Tips (Agilent, catalog number: 19477-022), storage temperature: room temperature
    Note: These are necessary if performing magnetic bead washes using the Agilent Bravo.
17. Nunc 96-Well Polypropylene DeepWell Storage Plates, sterile (Thermo Fisher, Thermo Scientific, catalog number: 260251), storage temperature: room temperature

18. Nalgene Disposable Polypropylene Robotic Reservoirs, sterile (Thermo Fisher, Thermo Scientific, catalog number: 1200-1301), storage temperature: room temperature
    Note: These are necessary if performing magnetic bead washes using the Agilent Bravo.
19. Corning 96-well Clear V-Bottom 2 mL Polypropylene Deep Well Plate, sterile (Corning, catalog number: 3960), storage temperature: room temperature
    Note: These are necessary if performing magnetic bead washes using the Agilent Bravo.
20. MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (Thermo Fisher, Applied Biosystems, catalog number: 4346906), storage temperature: room temperature
    Note: These are necessary if performing qPCR using the Applied Biosystems Fast 7500 system.
21. Qubit Assay Tubes (Thermo Fisher, Invitrogen, catalog number: Q32856), storage temperature: room temperature
22. (Optional) IgG (Total) Human ELISA Kit (*e.g.*, Thermo Fisher, Invitrogen, catalog number: BMS2091), storage temperature: 4°C
23. Tris Buffered Saline with Tween 20 (TBST-10X) (Cell Signaling, catalog number: 9997), storage temperature: room temperature
24. Bovine Serum Albumin (BSA) (VWR, catalog number: 0332-500G), storage temperature: 4°C
25. PBS, pH 7.4 (*e.g.*, Thermo Fisher, catalog number: 10010023), storage temperature: room temperature
26. VirScan T7 phage display library (Available upon request, storage temperature: -80°C)
    Note: Based on T7Select Packaging Kit (Millipore-Sigma, catalog number: 70014) storage temperature: -80°C.
27. UltraPure 1M Tris-HCI, pH 8.0 (Thermo Fisher, Invitrogen, catalog number: 15568025), storage temperature: 4°C
28. NaCl (5 M), RNase-free (Thermo Fisher, Invitrogen, catalog number: AM9759), storage temperature: room temperature
29. Magnesium sulfate solution (Millipore Sigma, catalog number: M3409-100ML), storage temperature: room temperature
30. Chloramphenicol (Millipore Sigma, catalog number: C0378-100G), storage temperature: room temperature for powder or -20°C for reconstituted solution
31. Kanamycin B sulfate salt (Millipore Sigma, catalog number: B5264-250MG), storage temperature: -20°C for powder and for reconstituted solution
32. NP-40 Surfact-Amps Detergent Solution (Thermo Fisher, catalog number: 85124), storage temperature: room temperature
33. Dynabeads Protein A for Immunoprecipitation (Thermo Fisher, Invitrogen, catalog number: 10008D), storage temperature: 4°C
34. Dynabeads Protein G for Immunoprecipitation (Thermo Fisher, Invitrogen, catalog number: 10009D), storage temperature: 4°C
35. UltraPure 1 M Tris-HCI Buffer, pH 7.5 (Thermo Fisher, Invitrogen, catalog number: 15567027), storage temperature: 4°C
36. UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher, Invitrogen, catalog number: 10977023), storage temperature: room temperature
37. Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, catalog number: M0493L) storage temperature: -20°C
38. dNTP Set (100 mM) (Thermo Fisher, Invitrogen, catalog number: 10297018), storage temperature: -20°C
39. TaqMan Gene Expression Master Mix (Thermo Fisher, Applied Biosystems, catalog number: 4369016), storage temperature: 4°C
40. UltraPure Agarose (Thermo Fisher, Invitrogen, catalog number: 16500100), storage temperature: room temperature
41. UltraPure DNA Typing Grade 50× TAE Buffer (Thermo Fisher, Invitrogen, catalog number: 24710030), storage temperature: room temperature
42. QIAquick Gel Extraction Kit (250) (QIAGEN, catalog number: 28706), storage temperature: room temperature
43. QIAquick PCR Purification Kit (250) (QIAGEN, catalog number: 28106), storage temperature: room temperature
44. Qubit dsDNA HS Assay Kit (Thermo Fisher, Invitrogen, catalog number: Q32851), storage temperature: mixed,

room temperature and 4°C

45. HPLC-purified primers (IDT, storage temperature: -20°C)

Primer name	Primer sequence (5' – 3')
IS7	ACACTCTTTCCCTACACGACTCCAGTCAGGTGTGATGCTC
IS8	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCCGAGCTTATCGTCGTCATCC
IS4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACTCCAGT
Index primer	CAAGCAGAAGACGGCATACGAGATNNNNNNNGTGACTGGAGTTCAGACGTGT
5'NEST-qPCR	TCGGGGATCCAGGAATTC
3'NEST-qPCR	CGTCGTCATCCTTGTAATCG
NEST_probe	/56-FAM/TAATCGCGG/ZEN/CCGCAAGCTTGTC/3IABkFQ/
T7-Illumina-READ1-A	TGCTCGGGGATCCAGGAATTCCGCTGCGT

Note: Orthogonal 7 nt barcodes for the Index primer are available upon request.

46. Phage extraction buffer (Recipe listed below), storage temperature: 4°C
47. PhIP-Seq Wash Buffer (Recipe listed below), storage temperature: 4°C

Equipment

1. Pipet-Lite Multi Pipette L12-20XLS+ (Rainin, catalog number: 17013808)
2. Pipet-Lite Multi Pipette L12-200XLS+ (Rainin, catalog number: 17013810)
3. E4 Pipette Multi E12-1200XLS+ (Rainin, catalog number: 17014499)
4. Portable Pipet-Aid XP Pipette Controller (Drummond, catalog number: 4-000-101)
5. Rotator (*e.g.*, Barnstead/Thermolyne, model: 415110)
6. Benchtop Centrifuge with swinging-bucket rotor assembly and microplate carrier (*e.g.*, Beckman Coulter, model: Allegra X-15R or Avanti J-15R, swinging-bucket rotor assembly: SX4750A or JS-4.750 , microplate carrier: SX4750)
7. Bravo NGS Automated Liquid Handling Platform (Agilent, catalog number: G5573AA)
8. 96-Well Microtiter Plate Magnetic Separation Rack (NEB, catalog number: S1511S)
   Note: This is necessary if performing magnetic bead washes manually.
9. Thermal cycler (*e.g.*, Bio-Rad, model: C1000 Touch with 96-well Fast Reaction Module, catalog number: 1851196)
10. 96-well Aluminum Block For 0.2 mL Tubes (Universal Medical, catalog number: 81001)
11. 7500 Fast Dx Real-Time PCR Instrument, with laptop computer (Thermo Fisher, Applied Biosystems, catalog number: 4406984)
12. Qubit 4 Fluorometer (Thermo Fisher, Invitrogen, catalog number: Q33238)

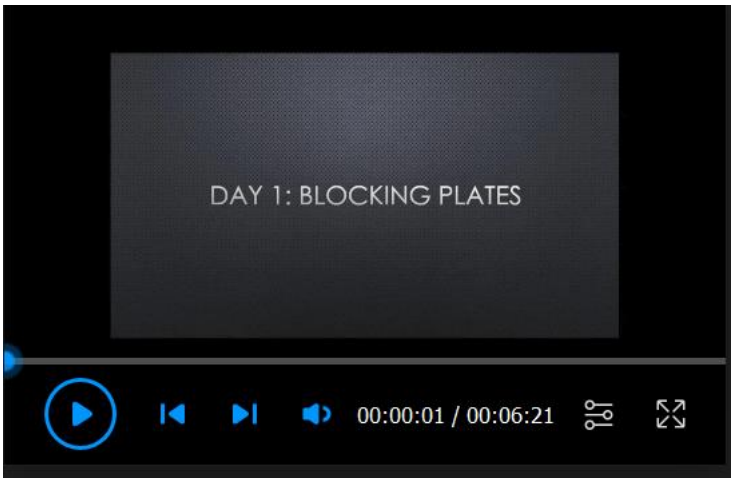
Software

1. bowtie (Langmead *et al.*, 2009)
2. samtools (Li *et al.*, 2009)
3. python (Python Software Foundation. Python Language Reference, version 2.7. Available at http://www.python.org)
4. gcc (GNU Compiler Collection, version 6.2.0. Documentation at https://gcc.gnu.org/onlinedocs/gcc.pdf)
5. R (R Core Team, 2017)

Procedure

Video 1. Introduction

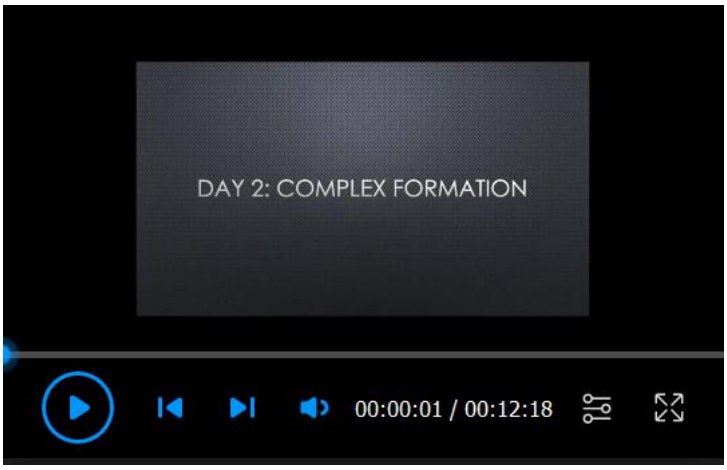
A. Block plates (Video 2)



Video 2. Blocking plates

1. Prepare 100 mL of TBST 3% BSA for every 96-well deep well plate (Cole-Parmer) to be blocked. Transfer to reagent reservoir.
2. Add 1 mL of TBST 3% BSA to each well of 96-well deep well plate.
3. Blot top of plate with kimwipes to remove excess liquid.
4. Seal plate well with MicroAmp optical adhesive film.
   Note: Plate may be sealed by pressing outward from the center of plate to eliminate large air bubbles, then by using a sealing paddle to eliminate all remaining air pockets.
5. Invert plate several times to ensure the liquid is moving throughout the plate.
6. Tape 96-well deep well plate to the plate rotator at 4°C and rotate end-over-end overnight.

B. Phage-antibody complex formation (Video 3)



Video 3. Phage-antibody complex formation

1. Thaw VirScan T7 phage library on ice. Characteristics of the library are shown in Table 1.
2. Prepare 110 mL of diluted T7 phage library for each 96-well deep well plate. See Table 2 for information on how to prepare the diluted phage library.
3. Mix very well.

Table 1. VirScan T7 phage library characteristics

Complexity (version Vir3)	115,753 members
Desired final concentration	2×10^5 pfu/mL per member of the library, or approximately 2×10^{10} pfu/mL

Table 2. Preparing diluted phage library

Component	Stock concentration	Final concentration	110 rxns
VirScan phage library	9.9×10^{10} pfu/mL (this may vary by batch)	2×10^{10} pfu/mL	22.2 mL
chloramphenicol	50 mg/mL (1,000×)	50 µg/mL (1×)	110 µL
kanamycin	50 mg/mL (1,000×)	50 µg/mL (1×)	110 µL
Phage extraction buffer			To 110 mL

4. Make aliquots of serum diluted to 0.2 µg/µL in PBS in 96-well PCR plates.
   Notes:
   a. *Concentration of IgG in human serum is generally 5–10 µg/µL. Dilute 2 µL of serum in 98 µL of PBS (1:50 dilution) to reach a concentration of approximately 0.2 µg/µL human IgG. Mix well.*
   b. *If needed, the concentration of IgG in a sample can be measured by IgG ELISA.*
   c. *Serum samples are typically run in duplicate.*
   d. *Eight no-serum controls are typically included for each run.*
5. Pour out blocking solution from 96-well deep well plates into sink. Flick plates several times to remove all blocking solution.
6. Blot the surface of the plate with a kimwipe to remove liquid.
7. Add 1 mL of diluted phage library to each well.
8. Blot the surface of the plate with a kimwipe to remove excess liquid.
9. Add sera containing 2 µg of IgG to each well, or 10 µL of the 0.2 µg/µL plate previously prepared.
10. Blot the surface of the plate with a kimwipe to remove excess liquid.
11. Seal plates extremely well with a new MicroAmp optical adhesive film, using a paddle. Make sure no air bubbles remain between wells.
12. Invert plate several times to ensure that liquid is moving throughout the plate. Secure plates on rotator at 4°C and rotate with end-over-end mixing for 20 h or overnight.

13. Seal plate with diluted serum samples and store at -80°C.

C. Immunoprecipitation (Video 4)

Video 4. Immunoprecipitation

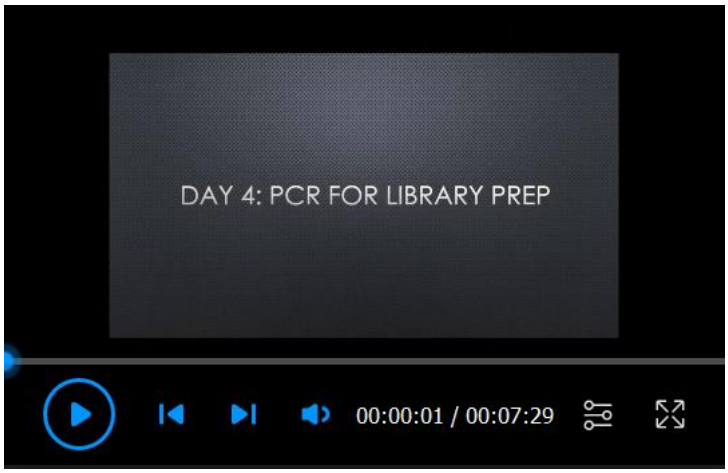
1. Centrifuge 96-well deep well plate at 500 × *g* for 3 min to collect liquid away from seal.
2. Tightly hold down plate while removing seal. Avoid splashing and cross-contamination between wells.
3. Resuspend Protein A and Protein G Dynabeads by shaking bottles until there are no remaining beads settled at the bottom.
4. For each 96-well deep well plate, add 2 mL of Protein A and 2 mL of Protein G Dynabeads to a reagent reservoir and mix with a serological pipette.
5. Add 40 µL of Protein A/G to each well of the 96-well deep well plate.
6. Blot the surface of the plate with a kimwipe to remove excess liquid.
7. Seal plate with a new MicroAmp optical adhesive seals and tape plate to a rotator. Rotate for 4 h at room temperature or overnight at 4°C.
8. Take one plate off at a time for washes. Centrifuge the 96-well deep well plate at 500 × *g* for 3 min to collect liquid away from the seal.
9. Perform three washes using a liquid handling robot, as shown in Video 4, washing with 170 µL of PhIP-Seq Wash Buffer each time. At one point during the washes, transfer beads to a new 96-well deep well plate (Thermo Fisher) to avoid phage that may have bound non-specifically to the wells of the original plate. The Bravo protocol file is available in the Supplementary materials.
    a. Alternatively, perform washes manually.
        i. Place the plate on a magnetic separation rack.
        ii. Let plate sit for 2 min to allow beads to collect. You should be able to see the solution become clear.
        iii. Aspirate the liquid from each well. Switch tips after each well to avoid cross-contamination.
        iv. When aspirating, make sure to hold the plate flush against the magnetic rods to avoid aspirating the beads.
        Note: Adjust the direction depending on where the magnetic rod sits relative to the wells.
        v. After aspirating the liquid from each row of wells, add 400 µL of PhIP-Seq Wash Buffer to the empty wells to prevent the beads from drying out.
        vi. Remove the plate from the magnetic separation rack and use a multichannel pipettor to resuspend the beads in all the wells by pipetting up and down 10 times.
        vii. Repeat steps i-iv for a total of three washes. During the first wash, transfer the beads to the new 96-well deep well plate.
        viii. Cover the plate with a new MicroAmp optical adhesive seal and centrifuge the beads at 500 × *g*

for 1 min. Aspirate any remaining liquid.

10. Resuspend the beads in each well in 40 µL of sterile water and transfer to PCR plate. Seal plate.
11. Spin PCR plate with resuspended beads in centrifuge for ~10 s, until centrifuge reaches 50 × *g*, to collect beads off the sides of the wells.
12. Heat plate to 95°C for 10 min to lyse T7 phage.
13. Store plate at -80°C for up to a week or proceed directly to library preparation for next-generation sequencing.

D. Library preparation for next-generation sequencing (Video 5)

Note: When setting up PCRs, keep PCR plate on aluminum block on ice at all times. Keep all reagents on ice at all times.



Video 5. Library preparation for next-generation sequencing

1. Thaw frozen beads on ice, then centrifuge at 1,000 × *g* for 2 min.
2. Make PCR1 master mix, mix well, and transfer to a reservoir on ice.

Component	Stock concentration	Final concentration	1 rxn (µL)	110 rxns (µL)
Sterile water			2.68	294.8
Reaction Buffer	5×	1×	6.0	660
dNTPs	10 mM	0.3 mM	0.90	99
Primer IS7	100 µM	0.2 µM	0.06	6.6
Primer IS8	100 µM	0.2 µM	0.06	6.6
Q5	2 U/µL	0.02 U/µL	0.30	33
Template	2×	1×	20	
Total			30 µL	

3. Aliquot 10 µL of PCR1 master mix to each well of a new 96-well PCR plate. Keep plate on aluminum block on ice at all times.
4. Resuspend beads by pipetting and add 20 µL of beads to corresponding wells. Mix well by pipetting.

Note: If sequencing input library, mix 5 µL of input library and 15 µL of sterile water and use this as the template for the PCR1 instead of 20 µL of resuspended beads.

5. Spin PCR1 plate in centrifuge for ~10 s, until centrifuge reaches 50 × *g*, then immediately remove plate and return to aluminum block on ice.
6. Run PCR1.

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	30 s
28 Cycles total	98°C	5 s
	66°C	10 s
	72°C	30 s
Final Extension	72°C	2 min
Hold	4–10°C	

7. Make PCR2 master mix, mix well, and transfer to reagent reservoir.
   Notes:
   a. *Sample multiplexing is achieved using barcoded PCR2 RV primers (Index primers).*
   b. *Index primers are diluted to 2.5 µM and kept in a 96-well plate.*

Component	**Stock concentration**	**Final concentration**	**1 rxn (µL)**	**110 rxns (µL)**
Sterile water			4.55	500.5
Reaction Buffer	5×	1×	2.0	220
dNTPs	10 mM	0.3 mM	0.3	33
Primer IS4	100 µM	0.5 µM	0.05	5.5
Index primer	2.5 µM	0.5 µM	2.0	
Q5	2 U/µL	0.02 U/µL	0.1	11
Template	2×	1×	1.0	
Total			10 µL	

8. Distribute 7 µLof PCR2 master mix to each well of a new 96-well PCR plate.
9. Add 2 µL of appropriate index primers (diluted to 2.5 µM) to corresponding wells.
10. Add 1 µL of appropriate PCR1 product to corresponding wells as template.
11. Mix PCR reactions by running the paddle rapidly across the bottom of PCR plate a few times, thus agitating the wells. Spin PCR plate in centrifuge for ~10 s until centrifuge reaches 50 × *g*, then immediately return plate to aluminum block on ice.
12. Run PCR2.

STEP	TEMP	TIME
Initial Denaturation	98°C	30 s
Eight cycles total	98°C	5 s
	68°C	10 s
	72°C	30 s
Final Extension	72°C	2 min
Hold	4–10°C	

Note: Steps 13–19 are quality control steps to verify that there is an amplicon in all appropriate wells.

13. Dilute PCR2 product 1:40,000 in sterile water.
    a. Serially dilute 2 µL of PCR2 product in 398 µL of sterile water (1:200 dilution) twice.
14. Make qPCR master mix, mix well, and transfer to reservoir.

Component	Stock concentration	Final concentration	1 rxn (µL)	110 rxns (µL)
Sterile water			8.75	962.5
Universal Mix	2×	1×	10	1100
3' NEST qPCR primer	100 µM	0.5 µM	0.1	11
5' NEST qPCR primer	100 µM	0.5 µM	0.1	11
NEST qPCR probe	100 µM	0.25 µM	0.05	5.5
PCR2 template, diluted 1:40,000			1.0	
Total			20 µL	

15. Distribute 19 µL of qPCR master mix to each well of a 96-well qPCR plate.
16. Add 1 µL of appropriate PCR2 product, diluted 1:40,000, to corresponding wells as template.
17. Mix qPCR reactions by running paddle rapidly across bottom of PCR plate a few times, thus agitating the wells. Spin in centrifuge for ~10 s, until centrifuge reaches 50 × *g*.
18. Run qPCR.

STEP	TEMP	TIME
1 Cycle	50°C	2 min
	95°C	10 min
40 Cycles	95°C	15 s
	60°C	2 min

19. If a well fails to amplify by qPCR, run out the corresponding PCR1 and PCR2 products to diagnose the problem. If necessary, redo PCR1 and/or PCR2.
20. Pool 2 µL of each sample of PCR2 in a reservoir, mix, and transfer to 1.5 mL microfuge tube.
    Notes:
    a. *Pool samples from individual plates separately.*

b. *If sequencing input library, add 10× the volume of any given sample into the final pool, i.e., if pooling 2 µL of each sample, add 20 µL of the input library to the pool.*

21. Run 40 µL of pooled PCR2 products from each plate on a 2% agarose TAE gel.
22. Gel extract correct size band using QIAgen Gel Extraction Kit.
    Notes:
    a. *The expected amplicon size for the T7 VirScan library is 376 bp.*
    b. *There may be a faint band directly below the correct-size band. Do not extract this faint lower band, as it contains products with truncated or missing Primer IS4 or Index Primer sequences.*
23. PCR purify gel-extracted samples using QIAquick PCR Purification kit.
    Note: PCR purification is performed after gel extraction to ensure greater purity of the sample prior to next-generation sequencing.
24. Quantitate DNA using dsDNA HS Qubit assay, then pool equal amounts (ng) of each plate.

E. Next-generation sequencing

1. Submit the pooled library for sequencing. The following sequencing parameters are required:
   Note: We generally sequence pooled libraries of 192 samples at a core facility with an Illumina NextSeq 500 instrument and the NextSeq 500/550 High Output Kit v2.5 (75 Cycles), which yields ~400M reads. We order single-read, single-index sequencing, detailed below.
   a. Read 1: 75 cycles
      Note: Only 50 cycles are required, but we typically order 75 cycles and truncate the reads during the Data Analysis steps.
   b. Index I7: 7 cycles
   c. Sequencing depth: 1M reads/sample.
   d. Custom sequencing primer for Read 1: T7-Illumina-READ1-A

Data analysis

Notes:

a. *In the instructions below, lines of code are bolded. These instructions are for use on a computing cluster using SLURM.*
b. *Example VirScan data for two serum samples and their technical replicates are provided (Supplementary materials). Data files include a sample legend, fastq files, BAM files, alignment report files, indexed BAM files, counts files, count.combined files (counts summed across four lanes of a Nextseq 500 flow cell), a counts table (count.combined data presented in a table format; summed counts for no-serum controls are provided in the column "input"), a Z-score table (again, summed counts for no-serum controls are present in the column 'input'), a hits_combined table, and virus scores files.*

A. Align sequencing reads to a reference file

1. Use the reference fasta file for the VirScan library ("vir3.fasta") (Supplementary materials) and generate index files with the .ebwt extension. Run the following commands:
   module load gcc/6.2.0

   module load bowtie/1.2.2

   bowtie-build vir3.fasta vir3
2. Align sequencing reads to the reference file. See "script.align.sh" and edit as needed (Supplementary materials). The output file is a file that ends in ".bam"
   Notes:

a. *Sequencing reads are typically distributed as fastq files. These fastq files are stored in a subdirectory called "raw.data".*
b. *In "script.align.sh", "bowtie -3 25" trims 25 nucleotides off the 3' end of each sequencing read. This is done if sequencing reads are 75 nucleotides in length. The reference file only includes the first 50 nucleotides of each member of the library, so the sequencing reads must be trimmed down to 50 nucleotides to align correctly to the reference.*
c. *In "script.align.sh", replace "path_to_vir3_reference_fasta_and_index_files" with the appropriate path.*

./ script.align.sh

3. Check the alignment report file that ends in ".out"
   Note: Typically, >85% of the reads align to the reference file.
4. Index files with the following commands. The output file is a file that ends in ".bai"
   module load gcc/6.2.0

   module load samtools/1.3.1

   for i in raw.data/*.bam; do samtools index $i; done
5. Count indexes with the following commands. The output is a file that ends in ".count.csv"
   module load gcc/6.2.0

   module load samtools/1.3.1

   for i in raw.data/*.bam; do samtools idxstats $i | cut -f 1,3 | sed -e '/^*\t/d' -e '1 i id\tSAMPLE_ID' | tr "\\t" "," >${i%.bam}.count.csv; done
6. Gzip the counts files with the following command.
   for i in raw.data/*.csv; do gzip $i; done
7. Create a directory called "log_directory" with the following command.
   mkdir log_directory
8. I f the same sample is run on two or more lanes of a flow cell and separate files are provided for each flow cell, combine the counts files from the different lanes using the following commands. These commands require the python script "combine_two_lanes.py" to be copied to the folder where you are running the commands (Supplementary materials).
   Note: In the code below, the samples were run on four lanes of an Illumina Nextseq 500 flow cell. The suffix of each count file is "L001_R1_001.count.csv.gz" if the count file was from the first lane of the flow cell, "L002_R1_001.count.csv.gz" if the count was from the second lane of the flow cell, etc.
   module load gcc/6.2.0

   module load python

   for i in raw.data/*L001_R1_001.count.csv.gz; do python combine_two_lanes.py $i ${i%1_R1_001.count.csv.gz}2_R1_001.count.csv.gz_ ${i%1_R1_001.count.csv.gz}1_2_R1_001.count.csv; done

   for i in raw.data/*L003_R1_001.count.csv.gz; do python combine_two_lanes.py $i ${i%3_R1_001.count.csv.gz}4_R1_001.count.csv.gz_ ${i%3_R1_001.count.csv.gz}3_4_R1_001.count.csv; done

   for i in raw.data/*L001_2_R1_001.count.csv; do python combine_two_lanes.py $i ${i%1_2_R1_001.count.csv}3_4_R1_001.count.csv_ ${i%1_2_R1_001.count.csv}1_2_3_4_R1_001.count.combined.csv; done
9. Gzip the count.combined files with the following command.

for i in raw.data/*1_2_3_4_R1.count.combined.csv; do gzip $i; done

B. Calculate Z-scores

Note: To perform the Z-score analysis, count.combined files are merged into a table, and columns corresponding with no-serum controls are summed in a column called "input".

1. Edit the R script "Zscore_analysis.R" to include the path to the count.combined table file and the desired path to the output file, then run the script (Supplementary materials). The packages "mmR_0.1.0" and "virScanR_0.1.0.9000" are required (Supplementary materials).
   Note: The file "Zscores_vir3" contains the results after this step (Supplementary materials).
2. A Z-score of at least 3.5 in both technical replicates of a sample is required to call a peptide a "hit".
   Note: The file "hits_combined_vir3_3.5_cutoff" contains the results after this step (Supplementary materials).

C. Calculate virus scores

1. Create a directory called "hits". In this directory should be .csv files for each sample with "True" or "False" values for each peptide ID, depending on whether the peptide scored as a hit (Z-score > 3.5) in both technical replicates of a sample or not. These files may be created by splitting each column of the "hits_combined_vir3_3.5_cutoff" file into a separate files (Supplementary materials).
2. Generate virus scores files using the following code:
   Note: The "VIR3_clean" file provides the annotations for the oligos" (Supplementary materials). There are 115,753 oligos in the Vir3 library. Some protein fragments are identical in different viruses, and in these case there are multiple rows in the "VIR3_clean" file that correspond to a single oligo. To identify the viral source of a given peptide, look for the row(s) in the VIR3_clean file with the "id" value of the given peptide.

   for i in hits/*.csv.gz; do python calc_scores_nofilter.py $i VIR3_clean.csv.gz Species 7 >virus_scores_$i; done

D. Determining virus seropositivity

1. A sample is determined to be seropositive for a virus if the virus_score > VirScan_viral_threshold and if at least one public epitope from that virus scores as a hit. The file "VirScan_viral_thresholds" contains the thresholds for each virus (Supplementary materials).
   Note: Public epitope annotations are available upon request.

Recipes

1. Phage extraction buffer

20 mM Tris-HCl, pH 8.0
100 mM NaCl
6 mM $MgSO_4$
Store at 4°C

2. PhIP-Seq Wash Buffer

50 mM Tris-HCl, pH 7.5
150 mM NaCl
0.1% NP-40
Store at 4°C

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Original research papers from which this protocol was derived: Larman *et al.* (2011), Xu *et al.* (2015), and Mina *et al.* (2019).
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Competing interests

S.J.E. is a founder of TSCAN Therapeutics, MAZE Therapeutics, Mirimus, and ImmuneID. S.J.E. serves on the scientific advisory board of Homology Medicines, TSCAN Therapeutics, MAZE Therapeutics, XChem, and is an advisor for MPM, none of which impact this work. E.L.S. was a consultant for ImmuneID. S.J.E. is an inventor on a patent application filed by the Brigham and Women's Hospital (US20160320406A) that covers the use of the VirScan library to identify pathogen antibodies in blood.

Ethics

Human specimens were collected in accordance with the local protocol governing human research after obtaining informed written consent from the donors. Secondary use of all human samples for the purposes of this work was exempted by the Brigham and Women's Hospital Institutional Review Board (protocol number 2013P001337).

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