



DeRisi Lab Phage Immunoprecipitation Sequencing (PhIP-Seq)

Hannah Kortbawi¹, Caleigh Mandell-Brehm², Brian O'Donovan³, Saravazquez¹, Madhuraraghavan¹, Elze Rackaityte¹, Grace Wang¹, Aditi Saxena¹, David Yu¹, Joseph Derisi¹

¹University of California San Francisco; ²Yale School of Medicine; ³Delve Bio

DeRisi Lab

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Hannah Kortbawi
University of California San Francisco

ABSTRACT

This protocol describes the process of Phage Immunoprecipitation Sequencing (PhIP-Seq) as it is performed in the DeRisi lab. It is the basis of many published papers from our group and is used for the discovery of protein targets of antibodies. The technique uses T7 bacteriophage that display a library of tiled peptide fragments that cover a human or microbial proteome. Human sera or monoclonal antibody controls are incubated with a phage display library and antibodies are immunoprecipitated using protein A/G beads. The purified phage library is then amplified in *E. coli* and re-incubated with sera, typically using three rounds of enrichment. Enriched phage DNA is then sequenced to identify antibody reactivity. In this way, a proteomic assay (immunoprecipitation) is converted into a genomic assay, leveraging the significant advantages of next-generation sequencing counting statistics.

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MATERIALS

Equipment

Pipet-Aid

Multi-channel pipettes that can fit 20uL (0.5-10uL LTS) , 200uL (20-200uL LTS or Universal) and 1000uL (100-1200uL LTS or Universal) filter tips

- 20 uL tips: At least 8 boxes per plate (1 box per round of IP for adding serum to peptidome/lysate, 1 box for diluting phage lysate, 2 boxes for transferring lysate into PCR I and PCR I into PCR II, 1 box for PCR II barcoding primers, and 3 rows of 12 for adding PCR I MM into plate, PCR II MM into plate, and pooling barcoded PCR II products)
- 200 uL tips: At least 4 boxes per plate per round of IP (1 for adding AG beads to peptidome+serum, 1 for transferring beads in LB to *E. coli*, 1 for transferring lysate into 96-well PCR plate for library prep, and 3 rows of 12 for adding LB to beads, adding NaCl to lysate, and adding water to plate to dilute phage lysate for library prep)
- 1000 uL tips: At least 7 boxes per plate per round of IP (6 for RIPA washes, 1 for transferring lysate to new blocked plate, and 1 row of 12 for aliquoting BSA, 1 row for adding peptidome, 5 rows for adding RIPA for washes, 1 row for aliquoting *E. coli*)

Pipets (Universal or LTS is fine)

Rocker in 4°C walk-in fridge

Magnetic stir plate

Bunsen burner w/ tubing

37°C shaker(s) that can accommodate 125mL and 1L flasks and 2mL deep well plates

Magnetic rack(s) that can fit 15mL, 50mL conicals and 1.5mL tubes

Spectrophotometer that can measure OD₆₀₀ of E.coli w/ cuvettes

96-Well Microtiter Plate Magnetic Separation Rack (NEB Cat# S1511S)

Qubit

Bioanalyzer

Materials

Graduated cylinders

1L Erlenmeyer flasks

125mL Erlenmeyer flasks

Serological pipets

Parafilm

Magnetic stir bars

Cuvettes

MicroAmp Adhesive Film Applicator (Thermo Scientific Cat# 4333183)

Foil seals (Bio-Rad Cat# MSF1001)

Rubber 96-well Imperma Mats (Genesee Cat#22-552)

0.22um PVDF filter 1L Stericups (Millipore Cat#S2GPU11RE)

Round 96 well, 2mL plates (Genesse Science Cat# 22-485)
Square 96 well, 2mL pyramid bottom plates (VWR Cat# 75870-796)
Air-O-Seal gas permeable seals (Thomas Scientific Cat#1149R80)
Hard-Shell 96-Well Polypropylene PCR Plates, Low Profile, Thin Wall, Skirted (Bio-Rad Cat# HSP9601)
20uL, 200uL and 1000uL LTS filter tips
Invitrogen Qubit Assay Tubes (Invitrogen Cat# Q32856)

Reagents

MilliQ H₂O
Pierce 20X TBS Tween 20 Buffer 500mL (Thermo Scientific Cat# 28360)
Bovine Serum Albumin Fraction V (Sigma-Aldrich Cat# 10735094001)
NaCl (Fisher Cat# S271)
AccuGENE 1 M Tris HCl Buffer (Lonza Cat# 51237)
TERGITOL solution Type NP-40 (Sigma-Aldrich Cat# NP40S-100ML)
SDS Solution, Molecular Biology Grade (10% w/v) (Promega Cat# V6553)
Triton X-100 (Sigma-Aldrich Cat# X100-100ML)
Glycerol for molecular biology, >99% (Sigma-Aldrich Cat# G5516-100mL)
1M HEPES (Thermo Scientific Cat# 15630080)
NaN₃ (Sigma-Aldrich Cat#S2002-100G)
10X PBS -Ca and -Mg (Thermo Scientific Cat# AM9624)
Carbenicillin 100mg/mL
Corning 1 L Millers LB Broth Corning Cat# 46-050-CM
BLT 5403 *E. coli* glycerol stock (EMD Millipore Cat# 69142-0.2ML; stored in -80; upon first use, make multiple personal glycerol stocks from first culture you make - don't keep taking from the lab stock)
Titered phage library (**Appropriate titer for human peptidome library is at least 1 x 10¹⁰ pfu/mL.** Titer decreases by ~1 log every 3 months that a library is stored at 4°C. A stored library should be re-titered before use if stored for more than a few months.)
Storage buffer (SB)
Glial Fibrillary Acidic Protein, Polyclonal antibody (Agilent Cat# Z033429-2)
DynaBeads Protein A for Immunoprecipitation (Thermo Scientific Cat# 10008D)
DynaBeads Protein G for Immunoprecipitation (Thermo Scientific Cat# 10009D)
Nuclease-Free Water (Thermo Scientific Cat# AM9937)
Phusion High-Fidelity DNA Polymerase (NEB Cat# M0530L)
Deoxynucleotide (dNTP) Solution Mix (NEB Cat# N0447L)
Flag and Strep pooled primers
TruSeq Dual Index Sequencing Primers in 96-well plates
AMPure XP beads (Beckman Coulter Cat# A63881)
200 Proof Ethanol (Koptec Cat#64-17-5)

Qubit 1X dsDNA HS Assay Kit (Thermo Scientific Cat# Q33230)
Agilent High Sensitivity DNA Kit (Agilent Cat# 5067-4626)

BEFORE START INSTRUCTIONS

Design and set up plates

1. Determine how many plates you will be doing based on number of samples, relevant healthy controls, GFAP antibody control (1:10 in SB), A/G bead-only control wells, and canary (no-bead, no-sample) control wells. Because individuals possess unique auto-reactive antibodies, we recommend using numerous (≥ 50) healthy control samples to account for non-disease-related enrichment - see [Vazquez et al. 2022](#).
2. Design plate layout: for multiple-plate and multiple-batch runs, have a few duplicate healthy controls across plates and batches to help control for batch effect. Every plate should have A/G bead-only wells (no serum), and every batch of plates should have multiple canary wells. For patient cohorts with multiple disease statuses, disease status/outcome should be randomized within plates. For an example of a quality control validation plate layout, see [attached spreadsheet](#).
3. Create working plates: aliquot serum into 96-well PCR plates (Bio-Rad Cat# HSP9601) and dilute 1:1 in [storage buffer](#) (SB). Serum should be arrayed in the plate layout planned for PhIP-Seq assay (put diluted GFAP in appropriate well, and leave A/G bead and canary wells empty). 5 uL of serum in 5 uL SB is a good amount in case assay needs to be repeated.
4. Store plates flat at 4°C until needed.

Make sure you have enough supplies and reagents

(See Materials tab)

Before Starting

1 Make buffers

- 1.1 Make 3% BSA blocking solution in 1X TBS-Tween
 - 1 mL of BSA is needed per well for blocking plates
 - Use [spreadsheet](#) for volume and recipe calculations

1.2 Make TNP40

- Very little TNP40 is needed per IP run - making 500 mL of TNP40 will last several runs of phage
- Use [spreadsheet](#) for volume and recipe calculations

1.3 Make RIPA buffer

- 2.5 mL of RIPA is used per well round of IP (5 washes x 500 uL RIPA per wash)
- Use [spreadsheet](#) for volume and recipe calculations
- Store RIPA at 4°C until ready to use

1.4 Make 5M NaCl

- 78 uL of 5M NaCl is used per well per round of IP
- Use [spreadsheet](#) for volume and recipe calculations

1.5 Prepare work space.

Spray work surface with 1:10 dilution of bleach and thoroughly wipe down before starting

2 Block plates.

Plates are blocked with BSA to reduce non-specific binding of antibodies to phage.

Block enough plates for 3x the number of plates of samples you are running.

BSA should be made fresh for every PhIP-Seq experiment.

2.1 Decant 3% BSA solution into a sterile reagent reservoir

Add 1 mL BSA per well into a round-bottom 2mL 96-well deep well plate [see materials] using a multichannel

*Tips do not need to be changed between rows or plates at this step

2.2 Seal plates thoroughly with foil seals and incubate on rocker at 4°C overnight or for 4-6 hours at room temp.

Day 1

3 Re-spray work surface with 10% bleach and thoroughly wipe down before starting For all following steps, work next to a lit Bunsen burner at all times

4 Prepare overnight *E. coli*(BLT5403) stock

*This should be prepared from frozen for each day

- 4.1 Add 20-25 mL LB-carb into an autoclaved 125 mL Erlenmeyer flask

*Flame all flask and bottle openings upon opening and closing containers

- 4.2 Using a sterile loop to scrape some *E. coli* from a **frozen** BLT5403 *E. coli* stock

*It is critical to start all overnight cultures from the glycerol stock

- 4.3 Incubate in 37°C shaking incubator overnight.

It can be useful to start two overnight cultures in case one fails

5 Incubate peptidome with serum

- 5.1 Change gloves and re-clean your work surface with bleach.

- 5.2 Discard BSA from one of the blocked plates by upside-down flick over the sink.

Bring the plate back to the bench and blot the top dry using a clean paper towel. Do not wipe, only blot.

- 5.3 Pour appropriate volume of peptidome into a sterile reagent reservoir (50mL per plate).

Use multichannel P1000 to add 500 uL peptidome to each well of the plate using filter tips.

5.4 Change gloves

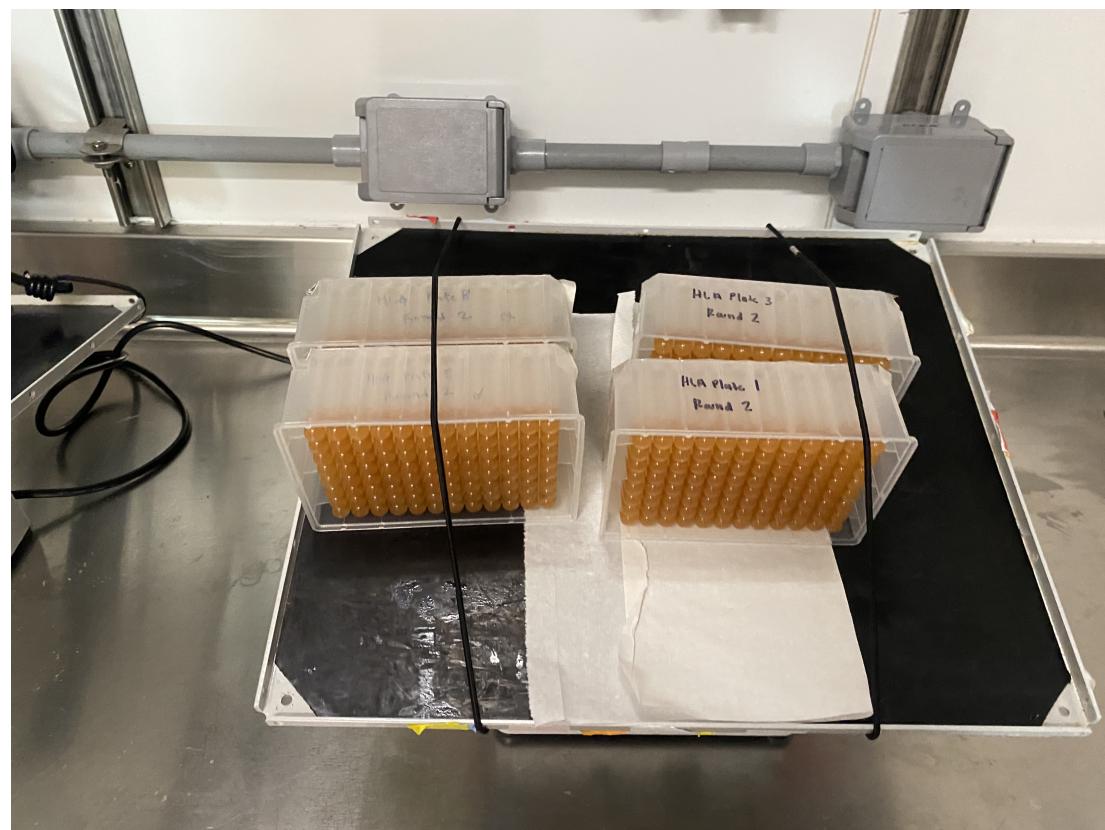
5.5 Add 1 uL of 1:1 serum in storage buffer (SB) to each well containing peptidome following the pre-defined plate map.

- Use LTS tips and dispense serum directly into phage solution. Do not pipette up and down to mix.
- To avoid contamination, when removing tips, bring pipette straight up from wells, move to the right so that the tips do not hover over any other rows, and eject tips into a bench height trash can.
- **DO NOT** eject tips into benchtop waste container. This can aerosolize droplets from the tips or otherwise create splash that can contaminate other wells of the plate. Ejecting tips below the benchtop minimizes exposure.

5.6 Seal plates very well with **rubber seals**. A mechanical plate sealer can be used to improve ease. Visually inspect each plate to ensure complete sealing.

Rubber seals can be autoclaved on wet cycle after use for re-use.

5.7 Incubate plates on their side on the rocker at 4°C overnight. Use tape or other means to keep plates securely positioned on their sides.



Plates are secured on their sides on a rocker for all mixing steps.

Days 2-4: IP and Washes (rounds 1-3)

6 Prepare A/G beads

6.1 Bleach work station and work over flame at all times.

The flame creates local updraft so no particles fall into wells. If a Bunsen burner is not available, use an alcohol burner.

6.2 Resuspend protein A and protein G beads by gentle swirling and end-over-end mixing. Avoid introducing bubbles.

***NEVER** vortex because this can damage the beads.

- 6.3 Add A and G in a 1:1 mix to a 15 mL or 50 mL conical tube.
12.5 μ L of each bead type is needed per well. Make extra to account for pipetting error: 1 plate = 1.3 mL each bead
- 6.4 Place tube of A/G bead mixture on block magnet on ice. Any high magnetic field magnet will work - you can tape the tube to the magnet if you do not have a stand.
- 6.5 Using a new 2 mL serological pipette attached to vacuum manifold tubing, aspirate supernatant. Re-sheath the pipette in the plastic casing between washes to maintain sterility.
*A new tip should be used each day, but changing between washes isn't necessary.
- 6.6 *Do not allow beads to dry out!
Immediately add cold TNP40 to beads at a volume enough to cover bead pellet and resuspend gently using serological pipette. Avoid adding bubbles.
- 6.7 Repeat the previous two steps twice more for a total of 3 washes.
- 6.8 Resuspend beads in cold TNP40 (volume proportional to IPs being done)
A good rule of thumb is 25 μ L per well. Add extra TNP40 proportional to the amount of extra beads pipetted in 6.3: if 1.3 mL of each bead was used, resuspend washed beads in 2.6 mL TNP40.

7 Add A/G Beads to peptidome+serum

- 7.1 Spin down plates at 1000 RCF for 1 minute at 4°C to pull down liquid from top of seals.
Visually inspect liquid levels in the plate from the side - if there are different heights in different wells, the plate was not correctly sealed and you should not proceed with the assay.

- 7.2** Add 25 uL washed A/G beads to each well containing sample+library using a P200 multichannel pipette. Airdrop beads over the wells.

Remember to leave A/G beads out of canary wells

Mix beads in reservoir by gentle side-to-side rocking between each plate to avoid settling.

- 7.3** Seal plates very well with rubber seals.

- 7.4** Mix on rocker at 4°C for 1 hour.

*DO NOT EXCEED 1 hour of incubation - 50 minutes to 1 hour of bead binding is okay. Start washing first group of plates (if doing more than 2) at 50 minutes. Longer incubation may increase nonspecific binding.

8 While the beads and samples are incubating, prepare new *E. coli* culture.

8.1 Change gloves and bleach station

- 8.2** Aliquot enough LB-Carb for 550 uL per well into a flask and add 1 mL *E. coli* from overnight culture.

*Make sure to use a flask that can hold 5x the volume of culture used

*Flame all flask and bottle openings upon opening and closing

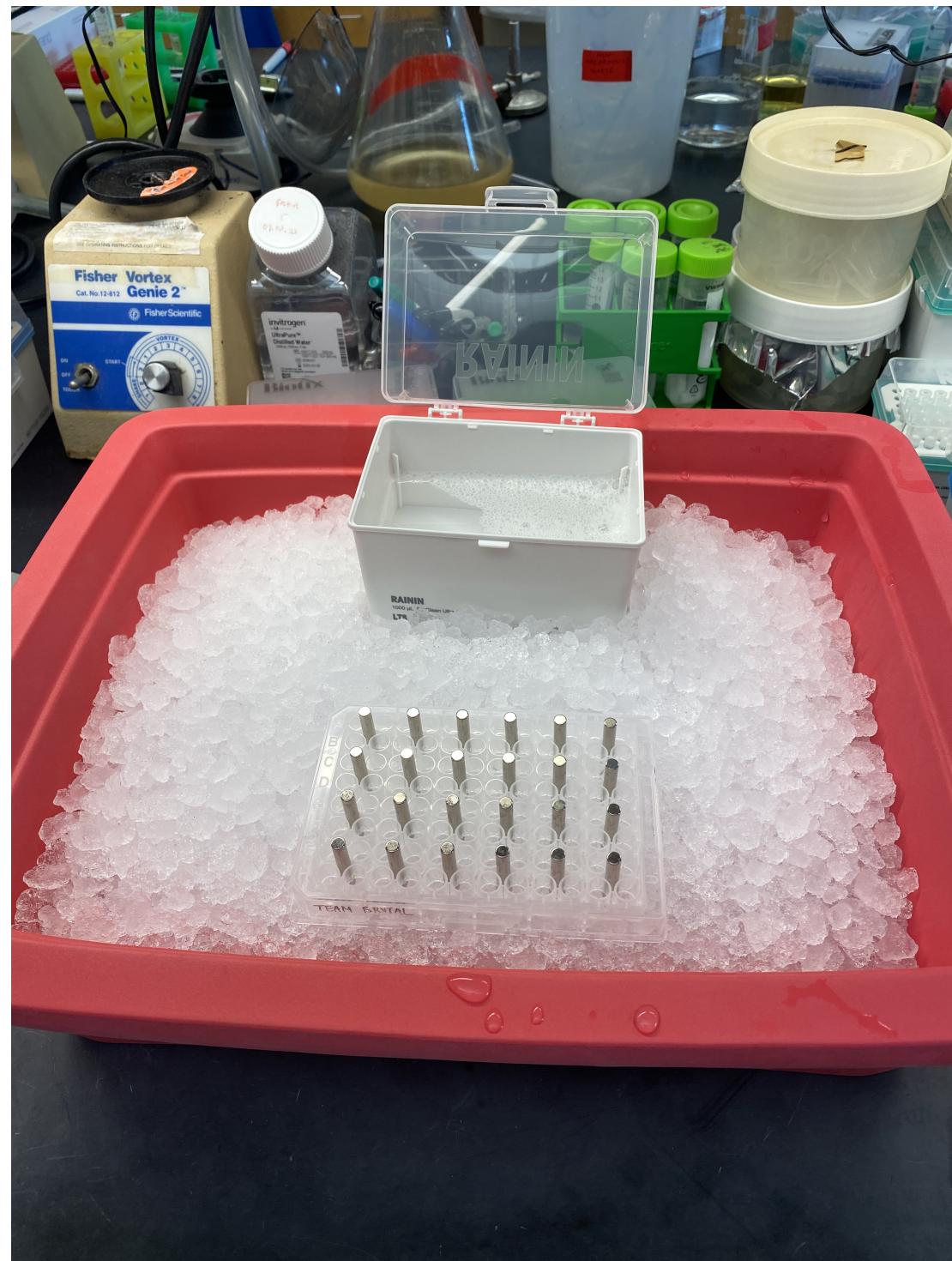
- 8.3** Incubate *E. coli* culture in 37 degree incubator with rotational shaking.

9 While the beads and samples are incubating, prepare wash station

9.1 Change gloves and bleach station**9.2 Unwrap P1000 tip boxes - 7 boxes per plate****9.3 Set up wash station:**

- Place a 96-well plate magnet and an empty, clean P1000 tip box (tip holder insert removed) on ice in a rectangular ice bucket.
- Place RIPA buffer on ice in a nearby ice bucket

Decant RIPA into empty P1000 tip box (used as reagent reservoir) and close the lid - *do this right before bead incubation is over*



RIPA is stored in a sterile empty tip box within close distance of the plate magnet. The RIPA reservoir should be closed while previous wash is being removed from the plate.

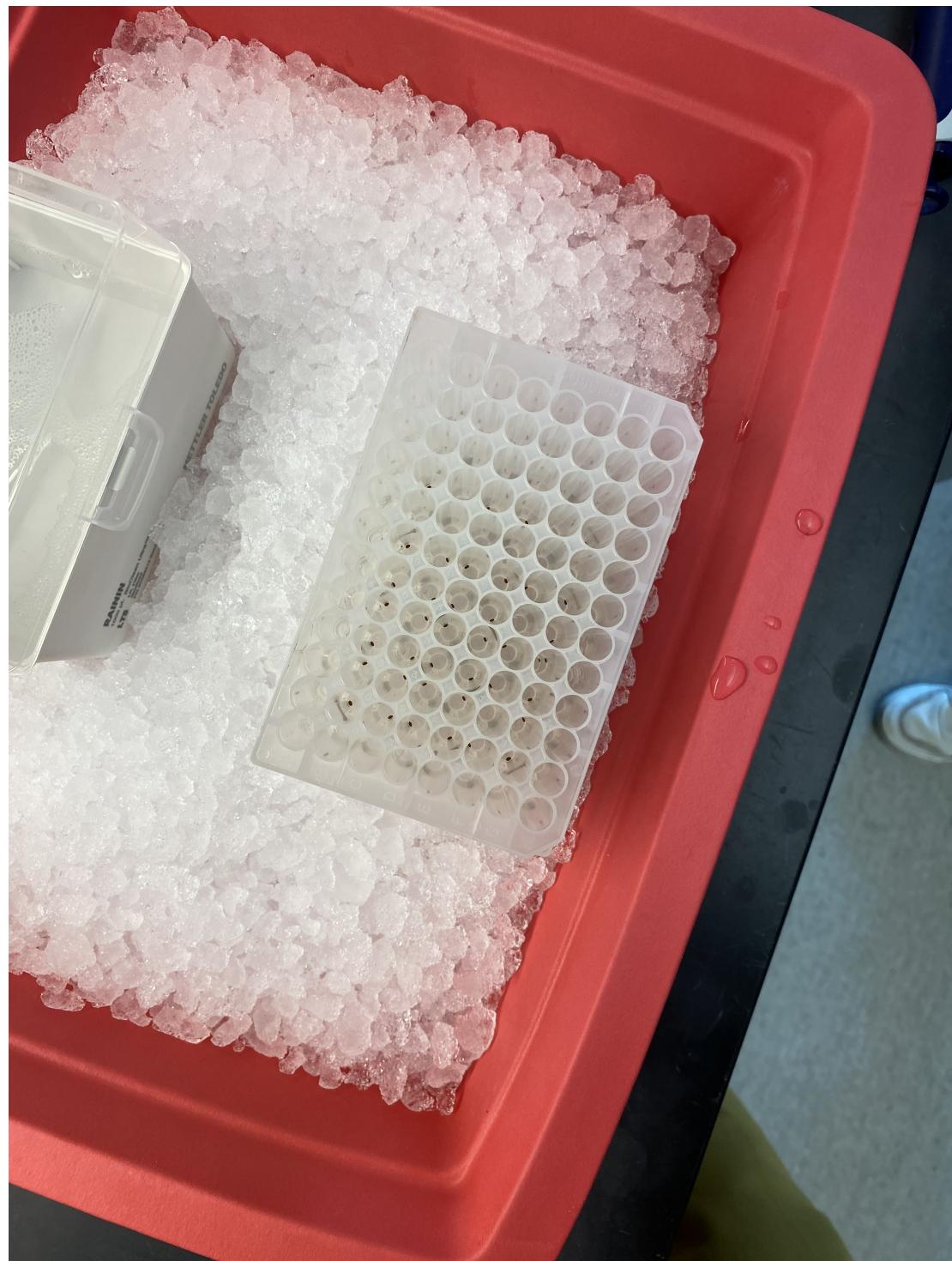
- 9.4** Tape a large plastic bucket to the inside of a large, waist-height garbage can next to your bench. Fill it with ~1 cm of 10% bleach.

10 Wash phage-bound beads

10.1 Spin plate at 800-1000 RCF for 1 minute to pull down liquid from the top of the seal in each well

10.2 Place plate on top of magnet on ice and allow the beads to pellet. Remove rubber seal very carefully, slowly peeling the seal back across the face of the plate, **not upwards**. This is to avoid creating a momentary vacuum in the wells, which could cause liquid to aerosolize. No liquid should be visible on the inside of the seal.

*If any liquid appears to fling up from the seal, IMMEDIATELY stop removing the seal, re-seal, and spin down again. The run may be contaminated at this point - consider re-starting.



When plate is correctly placed on magnet, beads will rapidly pellet on the sides of the wells, as shown here. Check that the beads have pelleted before removing liquid from the wells.

10.3

Using a multichannel set to 600 μL , remove all supernatant from each well.

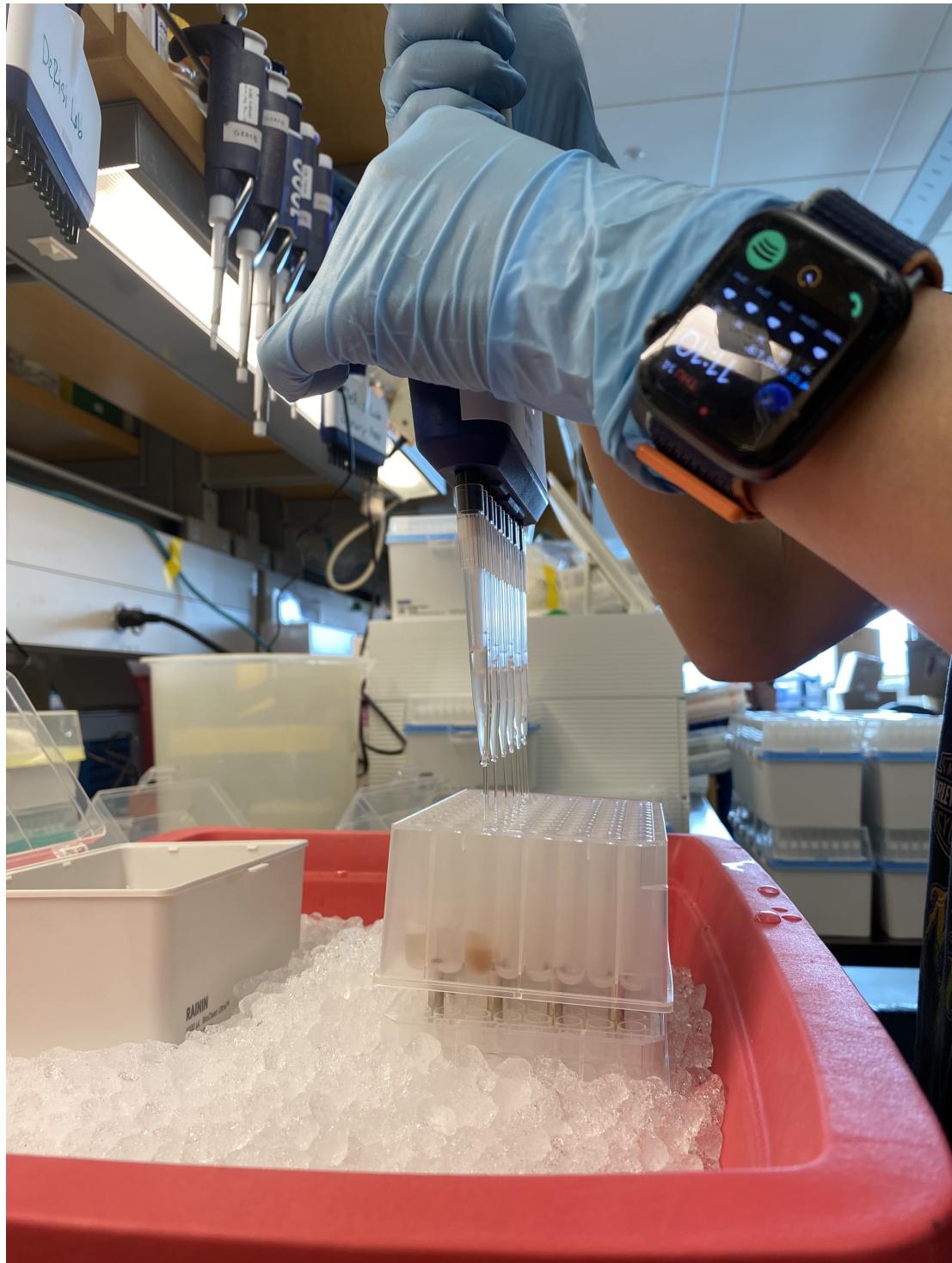
- For subsequent washes, set pipette to 500 μL .
- Pipette from opposite side of well from the bead pellet to avoid touching beads

Discard supernatant into a waste bucket containing bleach in bench height garbage can. Eject tips into garbage can. DO NOT carry tips over other rows of the plate, as supernatant may drip into other wells.



Discard supernatant into a reservoir with bleach below the height of the bench and then eject tips into a trash can below the height of the bench.

- 10.4** Open the lid of the RIPA reservoir and pick up 500 uL using a fresh set of tips. Carefully airdrop into each row, using the same tips for the entire plate (as long as tips do not touch any wells).



When pipetting RIPA, dispense buffer in a continuous stream to avoid splashing. Airdrop at least 1cm above the plate.

- 10.5 Carefully blot the top of the plate with a clean paper towel to remove any drips.
- 10.6 Seal plate tightly with rubber seal and mark plate to keep track of wash number.
- 10.7 Gently invert plate to resuspend beads, and then place plate on rocker for 5-10 minutes (10 min maximum) at 4°C.
- 10.8 Repeat the RIPA wash for the next plate as the previous one is rocking.
- 10.9 *Repeat for a total of 5 washes per plate*
 - Each addition of RIPA counts as a wash
 - Aim for no more than 2 hours for all 5 washes so the sample is not in RIPA for too long
 - When doing multiple plates, batch them in twos for easy centrifuging (e.g., six plates would be done in three pairs of two at a time)
- 11 Around the 4th incubation, check the OD₆₀₀ of the *E. coli* culture - 0.3-0.6 is good.
- 11.1 *Change gloves and bleach bench or aliquot on a different bench entirely for this step!
When *E. coli* has grown to correct OD, decant it into a sterile 50 mL reagent reservoir or a clean, empty P200 tip box.
- 12 **Amplify enriched phage**

- 12.1** After the 5th wash, remove all supernatant (set pipette to >500 uL), taking care to not touch the pellet, then remove plate from the magnet

- 12.2** Pour LB-carb into a sterile reagent reservoir (enough for 150 uL per well)
Immediately add 150 uL LB-carb to each well by airdrop (no need to change tips between rows as long as tips do not touch plate at all). Do not allow beads to dry out.

- 12.3** Add 550 uL *E. coli* to each well of a square-well 2 mL deep-well plate.
There is no need to change tips between wells at this step.

- 12.4** Use a multichannel P200 to mix each row of LB and beads, and then transfer to the plate with *E. coli*.
 - Be sure to get all beads transferred to the new plate - it is okay if the mixture gets frothy at this point, as the immunoprecipitated phage does not have to be bound to the beads and can just be in LB
 - Try to add the LB-carb/beads from each row to the *E. coli* around the same time so lysis occurs uniformly across the plate.
 - Start an off-plate canary, as well: aliquot 1 mL of *E. coli* culture into a glass test tube.

- 12.5** Seal with a gas permeable seal and incubate in a 37°C shaker for 1.5-2 hours.
Place off-plate canary tube into shaker at the same time, too.

- 12.6** Stop incubation when lysis occurs:
 - Try to catch the clarification as soon as possible
 - Place plate on 96-well magnet on the bench top (with flame on!) before checking for lysis to pellet beads to allow for easier observation of clarification vs. turbidity.
 - Compare lysis to on-plate canary well - the on-plate canary should remain turbid, indicating no lysis, while wells with patient samples should be cleared. This tells you that there has not been splashing between wells.
 - Compare lysis to off-plate canary - the off-plate canary should also remain turbid, which tells you that the original *E. coli* stock is not contaminated by phage.

- 12.7 Add 78 uL of 5M NaCl to each well of the clarified culture (to a final molarity of 0.5M).
- 12.8 Spin plate at 4°C for 1 hour at 3220 RCF (or max speed) for 1 hour to pellet *E. coli* cell debris.
- 12.9 While plate is spinning, empty out BSA from second round of plates as described previously.
- 12.10 After spinning is complete, use a multichannel P200 to take out 50-100 uL lysate from the **top** of the wells, avoiding debris at the bottom and transfer this lysate to a 96-well PCR plate to be stored at 4°C. This is your Round 1 Lysate.
*We often only transfer and keep 50 uL of round 2 and round 3 lysate for sequencing. Keeping 50 uL of round 1 lysate is optional.
- 12.11 Transfer 500 uL of lysate per well to new blocked deep-well plate. Again, slowly and carefully aspirate liquid from the **top** of the wells, avoiding any *E. coli* debris and beads.
If debris/beads are picked up, the plate can be spun again for 15 min to re-pellet and lysate transfer can be attempted again.
- 12.12 Add 1 uL of 1:1 serum:SB from working plate (should be exact same plate configuration as added in round 1) to the lysate in the Round 2 deep-well plate.
- 12.13 Store remaining lysate and bacterial pellet in the square well plate, sealed with a foil seal, at 4°C until library prep, sequencing, and QC have been completed.
- 12.14 Incubate serum+phage on 4°C rocker overnight.

12.15 Change gloves and bleach station.

12.16 Grow a new overnight stock of *E. coli* (from frozen) for the next day, as described in step 4.

12.17 Repeat steps 6-12 for two more rounds of IP for a total of **3 rounds**

Day 5: Library Prep

13 Prepare lysate

13.1 Bleach station and work over flame until PCR II is complete

13.2 Dilute phage lysate 1:4 in sterile, nuclease-free water (10 uL in 30 uL) in PCR plates.

13.3 Heat lysate at 70°C for 15 minutes.

While lysate is heating, prepare the PCR I master mix (see step 14).

Proceed immediately to PCR I after heating lysate. Do not let lysate sit for more than a few hours - DNA will degrade and PCR will fail.

14 PCR I

- 14.1** Use the [spreadsheet](#) to scale the PCR mix for the number of samples being prepped. Make at least 5-10% more master mix than the number of samples you have.
- PCR reaction volumes can be 12.5, 25, or 50 uL. All work and 12.5 uL is plenty for library prep.

- 14.2** Prepare the PCR I master mix.
- Change gloves after pipetting phage lysate.
 - Add water, 5X Phusion buffer, dNTPs, and Strep and Flag primers first.
 - Add polymerase from -20°C when you are ready to aliquot the master mix.
 - Make 10uM STREP and FLAG [primers](#) from 100uM stock fresh for each run.

- 14.3** Aliquot master mix into new PCR plate

- 14.4** Add volume of heated phage lysate proportional to volume of PCR reaction (see spreadsheet) to PCR I master mix in PCR plate, seal with foil seal, vortex, and thermal cycle with the following conditions:

	A	B	C
Step		Temp (C)	Time
Initial denature	98	30s	
13 Cycles	98	5s	
	70	20s	
	72	15s	
Final extension	72	2min	
Hold	10	Infinite	

While PCR I is running, prepare the PCR II mix (see step 15)

15 PCR II

- 15.1** Use the [spreadsheet](#) to scale the PCR mix for the number of samples being prepped. Make at least 5-10% more master mix than the number of samples you have.
- PCR reaction volumes can be 12.5, 25, or 50 μ L. All work and 12.5 μ L is plenty for library prep.

- 15.2** Prepare the PCR II master mix.
- Add water, 5X Phusion buffer, and dNTPs first.
 - Add polymerase from -20°C when you are ready to aliquot the master mix.
- 15.3** Aliquot master mix into new PCR plate.

- 15.4** Aliquot TruSeq 12bp dual index primers into appropriate wells using the volume proportional to the volume of the PCR reaction (see spreadsheet).
- *Carefully record which barcode IDs go into each plate. Do not re-use barcode plates across multiple plates destined for the same sequencing run.
 - *To avoid cross-contamination, seal barcode plates after use and do not reuse.
- 15.5** Add volume of PCR I proportional to the volume of the PCR reaction (see spreadsheet) to PCR II master mix in PCR plate, seal with foil seal, vortex, and thermal cycle with the following conditions:

	A	B	C
Step		Temp (C)	Time
Initial denature	98		30s
5 cycles	98		5s
	70		20s
	72		15s
Final extension	72		2min
Hold	10		

16 Pool samples

16.1 Mix PCR II samples using a P20 multichannel and transfer 10-12.5 uL of PCR II (using the same tips) to a 50 mL reagent reservoir.

16.2 Mix well in reagent reservoir by gentle side-to-side agitation and then transfer pooled samples to clean DNA LoBind Eppendorf tubes. (One plate will fit into a 5 mL tube with enough room for bead cleaning volumes, but not into a 1.5 mL tube.)

17 Bead clean

17.1 Allow beads to sit at room temperature for 30 minutes prior to use.

17.2 Calculate the total volume of your pooled samples (e.g., 100 uL).

Use AMPure XP beads at a 0.9x ration of beads to total volume of sample. E.g., if the sample is 100 uL, the appropriate bead volume is 90 uL.

17.3 Prepare fresh 80% EtOH (do not use stored diluted EtOH).

17.4 Gently resuspend beads by swirling and end-over-end mixing, but avoid generating bubbles. Add 0.9x of room temperature beads to DNA and mix well. Do not vortex.

17.5 Incubate sample at RT for 5 minutes.

- 17.6 Place samples on magnetic rack and incubate for another 5 minutes (beads should be completely pelleted).
- 17.7 Carefully remove supernatant without disturbing the bead pellet.
- 17.8 While keeping the sample on the magnetic rack, add enough 80% EtOH to submerge the pellet of each sample.
- 17.9 Incubate for 30 seconds, then remove supernatant.
- 17.10 Repeat EtOH wash for a total of 2 washes.
- 17.11 Place tube on magnetic rack and then sharply rap rack on the bench top to release remaining drop of EtOH from the pellet and then remove supernatant again.
- 17.12 Air-dry beads on magnetic rack until no EtOH is left (~ 5 min)

17.13 Remove tube from the magnetic rack. Elute DNA from beads in desired volume of 0.1x TE buffer, 10mM Tris-HCl, or nuclease-free water plus 3 uL of dead volume.
*A good rule of thumb is 50 uL of elution buffer per plate (plus 3 uL)

17.14 Vortex to mix beads and elution buffer.

17.15 Incubate for 2 minutes at room temperature off the magnetic rack.

17.16 Place on magnetic rack and pellet ~5 minutes, until solution is clear.

17.17 Remove desired volume of eluent and transfer to a clean nuclease-free DNA LoBind tube.
*Label the lid of this tube very clearly

18 Qubit library

18.1 Dilute samples 1:10

18.2 Prepare Qubit working solution (dilute HS Reagent 1:200 in HS buffer, scaled to the number of samples + 2 extra reactions for standards)

18.3 Aliquot 190 uL Qubit working solution into 2 tubes (one for each standard) and 199 uL working solution into tubes for each cleaned library.

18.4 Add 10 uL standard to appropriate tubes and 1 uL 1:10 diluted library to appropriate tubes.

18.5 Vortex samples thoroughly for 5 seconds each.

*Qubit will not correctly estimate concentration if samples are not vortexed.

19 Bioanalyzer

19.1 Notes

- Allow all reagents and samples to equilibrate to room temperature 30 minutes before use.
- Protect dye and dye mixtures from light. Remove light covers only when pipetting.
- Always insert the pipette tip to the bottom of the well when dispensing the liquid.
- Use loaded chips within 5 minutes after preparation

19.2 Allow HS DNA dye concentrate (blue) and HS DNA gel matrix (red) to equilibrate to RT for 30 min.

19.3 Add 15 uL HS DNA dye concentrate (blue) to HS DNA gel matrix (red). Vortex and spin down.

19.4 Transfer to a spin filter and centrifuge at 2240 g ± 20% for 15 min. Protect solution from light (wrap in foil). Store at 4°C. Use prepared gel-dye mix within 6 weeks of preparation.

- 19.5 Allow the gel-dye mix to equilibrate to room temperature for 30 min before use.
- 19.6 Put a new High Sensitivity DNA chip on the chip priming station.
- 19.7 Pipette 9 μL of gel-dye mix in the well marked Dark G.
- 19.8 Make sure that the plunger is positioned at 1 mL and then close the chip priming station.
- 19.9 Press plunger until it is held by the clip.
- 19.10 Wait for exactly 60 s then release clip.
- 19.11 Wait for 5 s, then slowly pull back the plunger to the 1 mL position.
- 19.12 Open the chip priming station and pipette 9 μL of gel-dye mix in the wells marked Light G.

- 19.13** Pipette 5 μ L of marker (green) in all sample and ladder wells. Do not leave any wells empty.
- 19.14** Pipette 1 μ L of High Sensitivity DNA ladder (yellow) in the well marked Ladder.
- 19.15** In each of the 11 sample wells pipette 1 μ L of sample (used wells) or 1 μ L of marker (unused wells).
- 19.16** Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
- 19.17** Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.
- 19.18** Results from Bioanalyzer will indicate the percentage between 300-700 basepairs and average basepair. Human peptidome library prep should show a peak around 349 bp.
- 20** Sequence using a sequencer with a maximum reads per run that will allow for 2 million reads per sample.