

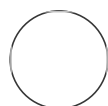


APR 07, 2023

Mouseome Cloning and PhIP-seq protocol

 Forked from [Falciparome_PhIP-seq_protocol](#)Elze Rackaityte¹, Joe DeRisi¹¹University of California, San Francisco

DeRisi Lab



Elze Rackaityte

University of California, San Francisco

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DOI:

dx.doi.org/10.17504/protocols.io.kxygx9144g8j/v1

Document Citation: Elze Rackaityte, Joe DeRisi 2023. Mouseome Cloning and PhIP-seq protocol. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.kxygx9144g8j/v1>

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Created: Feb 14, 2023**Last Modified:** Apr 07, 2023**DOCUMENT integer ID:**
77008

ABSTRACT

Protocol used for cloning mouseome into T7 genome for PhIP-seq

Protocol used for Mousome PhIP-seq in the *Rackaityte et. al study*

Part 1 - Phage Library Preparation

(Adapted from Aug 2018 protocol by Jay Rajan, Derisi Lab)

Complex oligonucleotide library packaging in bacteriophage T7

This protocol describes starting from the oligo pool of the library to packaging it in T7 phage.

Step 1: Oligonucleotide pool amplification (Day 1)

Reagents

2x Agilent oligonucleotide pools; resuspended at 0.2 nM

Phusion polymerase

5X Phusion reaction buffer

Nuclease free H₂O

10mM dNTP

Primers

10 uM Forward cloning primer (10bp of flanking sequence+EcoR I site +T+ 5' linker); aim for T_m of 65 C

10 uM Reverse cloning primer (10bp of flanking sequence+Hind III site+ reverse complement of 3'linker)

A	B
5' linker :	gtggtcaata cggaattctg ctgcagttgt aggaggtgt
3' linker:	ATCCTGAGCTAAGCTTGAGGCCATGGCATATG CTTA

Primer sequences for amplifying mouse T7 library

1.Set up PCR reactions in either 50ul volume. Given expected nucleic acid loss after bead purification, in general would shoot for at least 4 x 50ul reactions per oligo pool.

2.PCR reaction conditions (50 ul reaction; scale quantities appropriately for volume)

5X Phusion buffer-10ul

10uM Fwd Cloning Primer-1ul

10uM Rev Cloning Primer-1ul

10mM dNTP-1ul

Phusion-1ul

0.2nM Oligonucleotide pool-0.5ul

H₂O-35.5ul

PCR program

98 x 30s

For 20 cycles:

98 x 5s

70 x 20s

72 x 15s

72 x 2 minutes

10 x infinity

3. While the PCR is running, warm Ampure XP beads to room temperature

4. Clean up the PCR with 1 volume room temperature Ampure XP beads using the standard protocol. After the second 70% ethanol wash, do a quick spin of the beads in a microcentrifuge (30s, at least 8000g) to 'pellet' any residual alcohol. Place back on magnet and remove the small amount of remaining alcohol.

5. Allow beads to dry for 5 minutes, remove from magnet

6. Resuspend beads in 40ul nuclease-free H₂O, incubate at room temperature for 2 minutes

7. Place back on magnet for 1 minute, remove supernatant

5. Quantify DNA by Qubit

6. Run 5 ul of the sample on a 1-2% agarose gel to confirm that only a single band of the expected size is present before going on to the restriction digest.

Step 2: Restriction digestion and insert purification (Day 1)

1. Set up a 50ul restriction digest using the template DNA prepared in Step 1:

Template X ul (1 ug)

NEB 10X Cut-Smart buffer-5ul

NEB EcoR I – HF - 1ul

NEB Hind III – HF - 1ul

H₂O 50ul – X ul

2. Incubate in the thermal cycler, without lid heating, at 37 C for 1 hour

3. Heat inactivate at 65 C for 10 minutes, then place on ice or store at 4 C

4. Purify the restriction digest using 1 volume of Ampure XP beads, as described above in step 1.

5. Use the Bioanalyzer to confirm the expected 20 bp shift between cut and uncut insert.

6. Set up a 5ml overnight culture of E coli strain BLT-5403, can pick a colony from a plate streaked from the glycerol stock, or inoculate directly from glycerol stock (scrape pipet tip, and pipet up and down into 5ml of LB-Carb in an overnight culture tube).

IMPORTANT: AT NO POINT SHOULD THE AMPLIFIED TEMPLATE DNA BE EXPOSED TO UV (E.G. FROM THE GELDOC). THIS DRAMATICALLY REDUCES CLONING EFFICIENCY AND CAN INTRODUCE BIAS INTO THE LIBRARY.

Step 3: Cloning into T7 backbone (Day 2)

1. Set up the ligation reaction using NEB QuickLigase or T4 DNA ligase. For a single Agilent oligonucleotide pool (238,068 oligos), did 4 packaging reactions. The more packaged phage one has to start with, the better the library will be in terms of representation. Aiming for $\geq 10^8$ (~500X overage) phage for a single pool ensures excellent library representation. Setting up the ligations as individual reactions (4 x 5ul reactions vs 1 x 20ul reaction) works well for

the cloning.

T4 ligase buffer (10x):0.5 ul
T7 vector arms (.02 pmol):1 ul
Insert (0.06 pmol):x ul
T4 ligase:1 ul
H₂O:2.5 ul – x ul

Total - 5ul

2. Incubate at 16 C overnight (~12H) in the thermal cycler without lid heating and heat inactivate at 65 C x 10 minutes.
3. Place the ligation reaction on ice immediately or store at 4 C until use

Step 4: Packaging (Day 2)

Four packaging reactions as below were performed in 30ul volumes each.

1. Pipet 5 ul of ligation into a clean Eppendorf tube. Add 25 ul of packaging extract to the 5 ul, stirring the mixture with the pipet tip 10-20 times. Do not pipet up and down. Repeat the same procedure for the number of packaging reactions that are being done.
2. Incubate at room temperature for 2 hours (on bench top)
3. Quench the packaging reaction with 270 ul of cold LB-Carb medium and place on ice.
4. Titer the packaging reaction with a plaque assay
5. Calculate the number of E. coli cells needed to inoculate phage at an MOI of 0.001. For example, if the titer of the packaging reaction is 1×10^8 pfu/ml, would need 1×10^{11} E coli. At an OD₆₀₀ of 0.5, there are 2×10^8 E coli/ml. So 500 ml of OD₆₀₀ E coli equals 1×10^{11} cells.
6. Use the remainder of the overnight culture from Step 2 to inoculate the volume of E coli required for library amplification and grow to an OD₆₀₀ of 0.5 in the 37 C shaking incubator.

Step 5: Liquid lysate amplification (Day 2)

1. Inoculate the OD₆₀₀ 0.5 E coli cultures with the combined packaging reactions.
2. Incubate the inoculated cultures in the 37 C shaking incubator and monitor for lysis. Typically, lysis occurs 1-1.5H post-inoculation, though it can sometimes take longer. Do not let the lysis go any longer than necessary.
3. Add 0.1 volumes of 5M NaCl to stabilize the phage lysate (e.g. 50ml of 5M NaCl to 500 ml of lysate)
4. Clarify the stabilized lysate by centrifuging in 500ml polycarbonate Oakridge tubes at 15,000g x 30 minutes at 4 C. Filter the clarified lysate into a glass wide-mouth bottle (make sure the o-ring is intact) using 0.2 uM bottle-top filter units.
5. Add 5X PEG-NaCl solution to the filtered, clarified lysate to a final concentration of 1X (e.g. add 140 ml of 5X PEG-NaCl to 550 ml of stabilized lysate and 10ml of LB to bring to a total volume of 700ml). Store the bottle at 4C in the cold room overnight, with or without agitation.

6. The PEG solution will be turbid the next day. Divide the PEG-containing solution across 500 ml Oakridge polycarbonate tubes and spin at 15,000g x 1H to ensure that all of the PEG precipitate pellets on the side of the centrifuge tube.
7. Carefully decant the supernatant, taking care to not disturb the pellet. Resuspend the pellet in a volume of SM buffer equivalent to the original culture volume (e.g. 500ml of SM if the original culture volume was 500ml) and place in a fresh wide-mouth glass bottle (again, with o-ring intact). Reducing the volume (e.g. by half) will result in a more concentrated prep.
8. Add an autoclaved magnetic stir-bar and stir overnight at 4 C at the minimum speed required to circulate the entire volume.
9. The resuspended PEG precipitate will be slightly turbid the next day, but there should be no large particles. Filter the solution into a wide-mouth glass bottle using bottle-top 0.2 μ m filters. As before, 1 bottle-top filter is good for about 200ml of solution.
10. Store the filtrate in glass bottle at 4C. Freeze down stocks of the library by adding 0.1 volumes of autoclaved 80% glycerol. Lysate stocks should be stored at -80C.

Step 6 : Sequence Alignment (Day 3-4)

Pick 10-20 plaques from phage titer plates into 50 μ L of PCR-grade water.

Bake phage plaque to prep for PCR at **70°C for 15 mins**

Send for Sanger sequencing using primers outside of the insert site to confirm that the insert is in-frame and correctly oriented

A	B	C
SF2	ACGGTGGTCTTCGCCAG	Forward universal T7 primer for amplifying cloned insert for Sanger Sequencing
SR2	CATATAGTTCCTCCTTTCAG CA	Reverse universal T7 primer for amplifying cloned insert for Sanger Sequencing

Primers for Sanger sequencing of phage

Step 7: Phage Library sequencing (Day 3-4)

Paired-end 150 bp can be done to cover the entire peptide sequence in the read. Dephasing primers are used to sequence the insert off the linker.

Mouse forward dephasing (mix equimolar):

A	B
Step1_0N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCGCTGCAGTTGTAGGAGGTGTC

A	B
Step1_1N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNTTCGCTGCAGTTGTAGGAGGTGT C
Step1_2N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNTTCGCTGCAGTTGTAGGAGGTG TC
Step1_3N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTTCGCTGCAGTTGTAGGAGGT GTC

Mouse reverse dephasing (mix equimolar):

A	B
Step1_0N_primer_mous e_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTTGAGGCCATGGCATATGCTTA
Step1_1N_primer_mous e_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGCTTGAGGCCATGGCATATGCTTA
Step1_2N_primer_mous e_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGCTTGAGGCCATGGCATATGCTT A
Step1_3N_primer_mous e_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGCTTGAGGCCATGGCATATGCT TA

Cycling conditions

In a 50 ul reaction

5X Phusion buffer 10 ul

P7 Forward 10 uM 1 ul

P5 Reverse 10 uM 1 ul

10 mM dNTP 1 ul

Template (50ng)x ul

H₂O 36 – x ul

Phusion 1 ul

98 C x 30s

98 C x 5s, 70 C x 20s, 72 C x 15s x - 10 cycles

72 C x 2"

10 C x infinite hold

1. Bake phage lysate diluted 1:4 in PCR-grade water to prep for PCR at **70°C for 15 mins** in thermocycler
2. Perform PCR
3. Size check the PCR product and perform next generation sequencing. Nova-seq was performed on the mouseome library and sequencing to at least 500x coverage.

Step 8: Sequence Alignment (Day 5)

At discretion of end user, since analytical pipeline/data analysis tools used may vary.

Solutions

SM Buffer (from Phage on Tap)

Makes 1L

5.8 g NaCl

2.0g MgSO₄·7H₂O

50ml 1M Tris-HCl, pH 7.4

H₂O to 1L

5X PEG-8000/NaCl

200g PEG-8000

150g NaCl

H₂O to 1L

Part 2 - Immunoprecipitation protocol

(Adapted from 2016 protocol by Caleigh Mandel-Brehm, DeRisi lab)

This protocol was done in plate format for high-throughput processing of samples

PROTOCOL:

Day 0 - Blocking:

- 1ml blocking buffer (3% BSA fraction V in 1X TBS-Tween) added to each well in a 2ml deep-well 96 well plate.
- Cover with foil seal and set for rotating overnight in the overhead in the cold room.

Day 1 - Set up IP round 1:

- Pipette out the blocking buffer.
- Add 500uL of stock Phage-Library (10¹⁰ pfu/ml) to each well. Allow 10' to mix thoroughly overhead at 4 degrees.
- Add appropriate amount of human sample or commercial antibody. If mouse plasma, stock the sample in 1:1 storage buffer (0.04% NaN₃, 40% Glycerol, 40mM HEPES to preserve antibody integrity). Then, make required dilution of that stock in 1x PBS and use 1ul in the IP. 1ul of 1:1 dilution of plasma was used in the study. For GFAP samples, 1ug of GFAP was added.
- Set for rotating overnight in the overhead in the cold room.

Day 2 - Round 1 IP

1. Grow up E.Coli (BLT 5403) and make sure you have a non-contaminated stock before moving forward with IP'ing with A/G beads.
2. Wash protein A/G beads (Dynabeads, Thermo Fisher Cat No 10002D and 10004D, respectively) with Tris/NP-40, 3 x 3' minutes at 4 degrees overhead. Use 25ul of A Slurry and 25 uL of G slurry per IP - mix and wash them in TNP-40. After three washes, resuspend the beads in a final volume of 25ul Tris/NP-40.
3. Add 25 ul of the washed A/G bead mixture into each well.
4. Immunoprecipitate with beads for 1 hour, overhead at 4 degrees. **Do not prolong this step.**
5. Using a magnetic rack, wash the IP 5x for 10' minutes each, overhead at 4 degrees with 500ul cold RIPA buffer to each well
6. During one of the washes, remove the *E. coli* (OD between 0.4-0.7). Aliquot 1mL into each well in a new 96-well deep well plate.
7. After the 5th RIPA wash, resuspend beads in 125 ul LB/CARB
8. Add the total volume of resuspended beads to the deep well plate with 1 mL of E. Coli in LB/CARB OD600= 0.5. Incubate 37C with orbital shaking, sealed with a gas permeable seal. Solution should clarify within an hour and half to two hours.
9. After the ecoli has clarified- remove from 37, place on bench. Add 5.0 M NaCl for a final molarity of 0.5 NaCl
10. Spin 3000xg 1h 4C and take 500ul lysate to repeat another round of IP.
11. Save 100ul of lysate for sequencing.
 - Set up Round 2 IP with 500ul lysate from Round 1 and repeat the next day.
 - Set up Round 3 IP with 500ul lysate from Round 2 and repeat the next day.
 - From Round 2 and Round 3 lysate, prepare phage DNA and submit for sequencing as described in Part 3.

Reagents:

Tris NP-40

10mM Tris HCl [pH 7.4]

140mM NaCl

0.1% NP-40

Filter sterilized

Blocking buffer

3% BSA fraction V in 1x TBS-Tween

RIPA buffer

10mM Tris-HCl (pH 7.4)

1% Triton-X
0.1% SDS
140mM NaCl

Part 3 - NGS Library preparation from IPs

(Adapted from protocol by Sara Vazquez and Sabrina Mann, DeRisi lab)

Library prep will be a 2-step PCR in which in PCR1, Tru Seq adapters will be appended and in the second PCR, barcodes with Illumina P5 and P7 adapters will be added. You can go from PCR 1 directly into PCR2 without any form of purification.

PCR 1 – 13 cycles

Mouse forward dephasing (mix equimolar):

A	B
Step1_0N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTCGCTGCAGTTGTAGGAGGTGTC
Step1_1N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNTTCGCTGCAGTTGTAGGAGGTGT
Step1_2N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNTTCGCTGCAGTTGTAGGAGGTG
Step1_3N_primer_mouse_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNTTCGCTGCAGTTGTAGGAGGTGTC

Mouse reverse dephasing (mix equimolar):

A	B
Step1_0N_primer_mouse_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCTTGAGGCCATGGCATATGCTTA
Step1_1N_primer_mouse_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGCTTGAGGCCATGGCATATGCTTA
Step1_2N_primer_mouse_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGCTTGAGGCCATGGCATATGCTTA
Step1_3N_primer_mouse_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGCTTGAGGCCATGGCATATGCTTA

PCR 2 – 5 cycles

Template – 1ul of PCR1

Primers:

Primes to the Truseq regions of the PCR 1 amplicon and appends barcodes along with Illumina P5/P7 adapters.

Fwd - Illumina P5 + custom barcode + TruseqRead1

AATGATACGGCGACCAACGAGATCTACACbarcodeACACTCTTTCCCTACACGACGCTCTTCCGATCT

Rev - Illumina P7 + custom barcode + TruseqRead2

CAAGCAGAAGACGGCATACGAGATbarcodeGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Reaction

Template + water - ul

5x HF buffer - 10ul

10uM primer mix - 1ul (or 5uM - 2ul)

Phusion - 0.5ul

10mM dNTP - 1ul

50 ul

PCR program

98 x 30s

13cycles for PCR 1 and 5 cycles for PCR 2

98 x 5s

68x 20s

72 x 20s

72 x 2 minutes

10 x infinity

Primers for DNA Amplification:

- Following PCR2, pool equal volumes of all samples together and size check the PCR product.
- Bead purify the pool and submit for NGS.

