ChAMP protocols

In situ methylation

Reagents:

- **GpC Buffer:** 50 mM Tris-HCl, 50 mM NaCl, pH 8.5 at 25 °C. 10× stock stable for years at RT.
- **GpC DTT:** Add DTT to 10 mM. Store at 4 °C; stable for 2 days.
- **PBST:** PBS + 0.1% Tween-20.

Recommended: Grow cells on Polylysine coated plates.
Use a T25 for direct (Nanopore) sequencing and a 96 well (10,000 cells, can be scaled down to single digit) for BSC-amplification (Illumina). Solution volumes are provided for T25. For 96 wells, 100 µl is used, unless otherwise noted.

1. Wash once with 2 ml of PBS (NOT PBST)

2. Fix with 1.5 ml of 0.1% FA in PBST for 5 min.
   Note: Can vary according to antibody and sample - check for optimal results via immunofluorescence. Do not exceed 1% FA for 5 min.

3. Immediately stop reaction with 1.5 ml 1 M Tris, 2.5 M glycine

4. Wash once with 2 ml of PBST

5. Permeabilize with 1.5 ml of PBS 0.5% triton x100 for 7 min
   Note: Permeabilization conditions can be adjusted depending on sample and antibody.

Good staining by immunofluorescence is essential for successful execution of the protocol.

6. Wash twice with 2 ml of PBST

7. Incubate at 65 °C for 10 min with PBST [50 µl for 96 well, 1 ml for T25]. Make sure the plate/flask is sealed.

Note: This improves DNA accessibility by denaturing DNA binding proteins. This will not completely remove the histones and will not affect most epitopes. Temperature can be lowered or this step can be omitted if it negatively affects immunofluorescence.

8. Block in BSA 5% (in PBST) and 0.1 M glycine for 30m, Room Temp (RT) [150 µl for 96 well, 1.5 ml for T25] with mild agitation. Optionally, incubate overnight 4 °C.

9. Incubate for 1 hour with primary antibody in PBST BSA 5% (1:200) at RT [150 µl for 96 well, 1.5 ml for T25] with mild agitation. Optionally, incubate overnight 4 °C.

Note: Primary antibody concentration can be adjusted, concentration similar to used in Immunofluorescence works well.

10. Wash 4 times with 2 ml of PBST over 45 min. (1 quick + 3 x 15 min washes).

11. One quick wash with 2 ml of PBS + 0.5% triton.

12. One quick wash with 2 ml of GpC buffer.

————————Everything in 4 °C from this point on————————

Note: high salt concentration and elevated temperature deactivate the MTase

13. Block with cooled GpC+DTT buffer with 5% BSA for 30m [150 µl for 96 well, 1.5 ml for T25] with mild agitation.

14. ChAMP mix:

Remove ChAMP aliquot from -80 °C and thaw on ice (20 ng/µl stock)
Make 1:50 dilution of ChAMP in GpC + DTT with 5% BSA

15. Aspire wells and add ChAMP premix [150 µl for 96 well, 1.5 ml for T25]

16. Incubate for 1 hour at 4 °C with mild agitation.

Note: Increase incubation time for thick samples.

17. Perform 6 washes over ~2h— all at 4 °C, with mild agitation:
    washes 1-2: 15 min with 1.5 ml of 1% NP-40 with GpC buffer + DTT + 5% BSA + salmon DNA (1 µg per well)
    washes 3-4: 15 min with 1.5 ml of 1% Tween 20 with GpC buffer + DTT + 5% BSA
    last 2 washes: 1.5 ml of GpC buffer + DTT (remaining hour)

    Note: Detergents needed to remove non-specific binding of ChAMP, reducing background significantly. Detergents tests as compatible with ChAMP include NP40, tween-20 and Triton X-100 and combination of all 3 at 1% each.

18. Incubate with GpC buffer + DTT with SAM 1:20 at 37 °C for 1 hour. [150 µl for 96 well, 1.5 ml for T25] (SAM stock: 32mM, final conc: 1.6mM)

19. Wash twice with 1.5 ml of PBST

20. Add PBST [50 µl for 96 well, 1.5 ml for T25]

21. Incubate at 65 °C overnight
    Note: For reverse crosslinking.

22. Add Proteinase K (20 mg/ml stock solution), incubate at 50 °C 2 h.
  [1 µl for 96 well / 5 µl for T25]

23. Deactivate Prot K by incubating at 95 °C for 15 min
    Note: omit this step for direct (Nanopore) sequencing, to reduce DNA shearing.

24. Check under a microscope cells are no longer present on surface of plate [if present, add more Proteinase K + 1% SDS and continue to digest at 50 °C]

25. Store DNA -20 °C for Illumina, and 4 °C for Nanopore sequencing.
    Note: For Illumina, SpeedVac if necessary. Read length may decrease with storage time for Nanopore.

Library Preparation for Nanopore Sequencing

1. Perform a phenol-Chloroform precipitation, followed by ampure XP bead cleanup, using a 1:1 beads to input ratio.

2. Use output for library prep (either tagmentation or ligation based).

Library Preparation for Illumina Deep Sequencing

1. BSC convert DNA (based on Zymo lightning, similar kits can be used)
Add 30 ng of DNA in 20 µl to 130 µl of lightning conversion reagent (Zymo).

Incubate in PCR

98 °C 8 min
54 °C 1.5 hours
4 °C <20 hours

Note: Amount of DNA can be adjusted, but should not exceed 100 ng.

Add 600 µl M-Binding Buffer to a Zymo-Spin™ IC Column in a collection tube. Add the bisulfite-converted sample to the column and invert several times to mix. Spin down (max speed for 30 seconds).

Discard the flow-through and add 100 µl M-Wash Buffer to the column. Spin down.

Add 200 µl L-Desulphonation Buffer to the column and let stand for 20 minutes. After the incubation, spin down.

Discard the flow-through. Add 200 µl M-Wash Buffer to the column and spin down. Repeat this wash step.

Add 10 µl Elution Buffer directly to the column matrix and let stand for 1 minute. Spin down.

2. Make a tube with 2 µl of cleaned Bisulfite-converted DNA, 2 µl of CutSmart buffer, 1 µl of P5_Rand primer, 1 µl of dNTP (10 mM) and 13 µl of DDW.

3. PCR 1:

1. 98 °C 2 min
2. 98 °C 30s
3. 8 °C 5 min

   Add 1 µl of Klenow -exo at each 8 °C step, wait for temperature to get to 8 °C before adding.
4. 16 °C 1 min

(Ramp rate: 0.1 °C/s)

5. 22 °C 1 min
   (Ramp rate: 0.1 °C/s)
6. 28 °C 1 min
   (Ramp rate: 0.1 °C/s)
7. 36 °C 1 min
   (Ramp rate: 0.1 °C/s)
8. 37 °C 5 min
9. Goto step 2 four times
   Note: 1 cycle of PCR is sufficient if greater than 100 cells

Hold at 4 °C

4. Clean DNA

Add 1 µl of Exonuclease I. Incubate at 37 °C for 15 minutes.
Note: Remove primers.
Deactivate Exonuclease I by Incubating at 85 °C for 10 minutes.
Mix the 1.8X PCR volume of AMPure XP beads, Pipette well
Wait 5 minutes
Use magnet to collect beads and remove supernatant
Wash beads twice with 80% EtOH
Air-dry for 5 minutes.
Elute in 10 µl of DDW

5. PCR 2: Make a tube with 9 µl of cleaned DNA, 2 µl of CutSmart buffer, 1 µl of P7_Rand primer, 1 µl of dNTP (10 mM) and 6 µl of DDW

Repeat PCR 1.
Note: Do a single cycle for greater than 100 cells, 5 cycles for less.

6. Clean DNA

Add 1 µl of Exonuclease I. Incubate at 37 °C for 15 minutes
Deactivate Exonuclease I by Incubating at 85 °C for 10 minutes.
Mix the 1.8X PCR volume of AMPure XP beads, Pipette well
Wait 5 minutes
Use magnet to collect beads and remove supernatant
Wash beads twice with 80% EtOH
Air-dry for 5 minutes.
Elute in 10 µl of DDW

7. PCR with P5_handle/P7_handle (10 cycles) using Bioline Red Readymix

Add to the 10 µl of eluted DNA

10 µl X2 MyTaq HS red mix or Zymo mix
1 µl P5_handle
1 µl P7_handle

PCR- Red mix
95 °C 3m
95 °C 30s
60 °C 30s
72 °C 20s
Goto (95 °C 30s) 9 times.
72 °C 5m

8. Clean DNA

Add 1 µl of Exonuclease I. Incubate at 37 °C for 15 minutes
Deactivate Exonuclease I by Incubating at 65 °C for 10 minutes. *keeps DNA double-stranded
Mix the 1.8X PCR volume of AMPure XP beads, Pipette well
Wait 5 minutes
Use magnet to collect beads and remove supernatant
Wash beads twice with 80% EtOH
Air-dry for 5 minutes.
Elute in 10 µl of GpC buffer
Measure using qubit (optional)

9. Re-methylation

Methylate DNA by adding 2 µl of x10 GpC buffer (with DTT), 1 µl SAM and the 3 µl ChAMP and 5 µl of GpC buffer, incubate at 37 °C for 1 hour. Incubate for 5 min at 65 °C.

10. Enrich for methylated DNA

Enrich for methylated DNA for example using Methylated-DNA IP Kit (D5101) by Zymo:

Add 25 µl of DNA Denaturing Buffer to a final volume of 50 µl.
Incubate at 98 °C for 5 minutes.
While the DNA is being denatured, mix in a 1.5 ml microcentrifuge tube:
250 µl MIP Buffer
15 µl of ZymoMag Protein A
2 µl Mouse Anti-5-Methylcytosine
Invert the tube 2-4 times to mix the antibody/Protein A mixture.
Add the denatured DNA immediately to the antibody/Protein A mixture after the incubation above is complete.

Incubate at 37 °C for 45 min on a shaking heat block.
Place tubes on a magnetic tube rack, allow time for the beads to cluster, then remove and discard the supernatant. 6.
Wash twice with 500 µl of MIP Buffer.
Wash with 500 µl of DNA Elution Buffer.
Wash with 250 µl of DNA Elution Buffer.
Add 15 µl of DNA Elution Buffer to each tube and resuspend the beads by gently flicking the tube or pipetting up and down. Transfer to a PCR tube and incubate at 80 °C for 5 minutes. Spin down and transfer the supernatant to new tube.
Measure using qubit, calculate enrichment ratio. (optional)

11. PCR with P5/P7 (half 10 cycles/ half 20 cycles).

Cycle number can be adjusted, depending on input DNA.
PCR-Red mix
95 °C 3m
    95 °C 30s
    60 °C 30s
    72 °C 20s
Goto (95 °C 30s) 9 or 19 times.
72 °C 5m

12. Run on a bioanalyzer/gel

PCR: Will notice a smear for the library- should be aiming for the library with the majority of the band between 300-500 bp.
Bioanalyzer: Will notice a more elongated curve that stretches out between 300-500 bp.
Note: PCR is usually sufficient to indicate a good library.

13. Clean up and quantify:

Mix the PCR reaction with 1.8X volume of AMPure XP beads, pipette well
Wait 5 minutes
Use magnet to collect beads and remove supernatant
Wash beads twice with 80% EtOH
Air-dry for 5 minutes.
Elute in 10 µl of DDW
Measure DNA concentration by nanodrop or qubit.

14. Proceed to deep sequencing.

Primers:

P5_rand	ACACTCTTTCCCTACACGACCCTCTTCCGATCTHHHHHHHHW
P7_rand	GGTGACTGGAGTTCAGACGTGTCCTCTTCCGATCTDDDDDDDW
P7_handle	GGTGACTGGAGTTCAGACGTG
P5_Handle	TTCCCTACACGACCCTCTTCCGATCT
P5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
P7_1_L	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
P7_2_L	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
P7_3_L	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
P7I1	CAAGCAGAAGACGGCATACGAGATATCACGGTGACTGGAGTTCAGACGTGTG
P7I2	CAAGCAGAAGACGGCATACGAGATCGATGTGTGACTGGAGTTCAGACGTGTG
P7I3	CAAGCAGAAGACGGCATACGAGATTTAGGCGTGACTGGAGTTCAGACGTGTG
P7I4	CAAGCAGAAGACGGCATACGAGATTGACCAGTGACTGGAGTTCAGACGTGTG
P7I5	CAAGCAGAAGACGGCATACGAGATACAGTGGTGACTGGAGTTCAGACGTGTG
P7I6	CAAGCAGAAGACGGCATACGAGATGCCAATGTGACTGGAGTTCAGACGTGTG
P7I7	CAAGCAGAAGACGGCATACGAGATCAGATCGTGACTGGAGTTCAGACGTGTG
P7I8	CAAGCAGAAGACGGCATACGAGATACTTGAGTGACTGGAGTTCAGACGTGTG
P7I9	CAAGCAGAAGACGGCATACGAGATGATCAGGTGACTGGAGTTCAGACGTGTG
P7I10	CAAGCAGAAGACGGCATACGAGATTAGCTTGTGACTGGAGTTCAGACGTGTG
P7I11	CAAGCAGAAGACGGCATACGAGATGGCTACGTGACTGGAGTTCAGACGTGTG
P7I12	CAAGCAGAAGACGGCATACGAGATCTTGTAGTGACTGGAGTTCAGACGTGTG

ChAMP Purification

Reagents:
GpC buffer: 50 mM NaCl; 50 mM Tris-HCl; 10 mM DTT; pH 8.5
Lysis buffer: GpC buffer + 0.5% Triton X-100 + 0.1mM EDTA + protease inhibitor [1 tablet of roche cOmplete tablets EASYpack / 50 ml]

1. ChAMP was expressed in T7 Express lysY E. coli (C3010, NEB).
    Note: It is essential to use bacteria that do restrict methylated DNA

2. Grow ChAMP bacteria overnight at 37 °C shaker in 50 ml of LB+ kanamycin (50 µg/ml).

3. Move 10 ml of overnight grown culture into 200 ml of LB+ 1 mM IPTG (no kanamycin) and grow for an additional 4h at 37 °C shaker.
    Note: ChAMP is readily detected without induction in both C3010 and 10G bacteria

4. Move cells into centrifuge bottles and cool on ice for 15 min.

————————————Everything at 4 °C or on ice from this point on————————————

5. Pellet cells in cooled centrifuge (4 °C) at 10,000 G for 15 min. Remove media.

6. Resuspend pellet in cold 10 ml of GpC buffer.

7. Pellet cells again in cooled centrifuge (4 °C) at 10,000 G for 15 min. Remove supernatant.

8. Resuspend pellet in 10 ml of cold lysis buffer and transfer to 50 ml tubes.

9. Incubated at 4 °C rotating for 1 hour.
    Note: Sonication may also be used here.

10. Centrifuge samples for 10 minutes at 5,500G in cool centrifuge (4 °C).

11. Prewash 0.5 ml of his-tag beads with GpC buffer, repeat once.

12. Take supernatant and incubate with his-tag beads for 1 hour at 4 °C rotating.
    Note: Samples of supernatant and pellet may be collected for additional Western blot QC.

13. Load a filter column with the bead solution.

14. Wash filter column with 5 ml of GpC buffer, followed by a wash with 5 ml of GpC buffer supplemented with 20 mM Imidazole. Elute the protein by adding in 2 ml 200 mM Imidazole GpC buffer, and elute the remaining protein with 2 ml of 1000 mM Imidazole GpC buffer. Collect the passthrough from each buffer in separate tubes.
    Note: Protein eluted between 200 and 1000 mM of Imidazole, with the earlier fractions containing more protein, and the later fractions being more pure.

15. Make single use aliquots at 4 °C (20 µl) and store right away in -80 °C. (Prechill tubes)

16. Quality control:
    A. Run a coomassie protein gel with the eluted fractions and a BSA standard. Successful purification will result in a strong and specific band of approximately 70kDa at the multi-nanogram/µl range.
    B. Run a DNA gel with the following reactions:
        1. 200 ng of a closed plasmid #positive control
        2. Closed plasmid incubated in a reaction buffer for 1 hour at 37 °C. Digested.
        3. Closed plasmid incubated in a reaction buffer, with SAM omitted. Digested #Negative control.

        As HaeIII is a 4-cutter, most plasmids will have multiple cut sites.

    A successful reaction will result in only coiled, supercoiled and linear forms of the plasmid in the positive control lane, predominantly the same forms in the test lanes (increase in linear form is acceptable) and complete digestion in the negative control lane.

Reaction buffer: GpC buffer supplemented with 10 mM DTT and 160 µM S-adenosylmethionine (SAM). Add cutsmart buffer and and perform function tests to assess enzyme concentration, purity and function.

Digestion reaction: Add 10X cutsmart buffer directly to the methylation reaction for a final concentration of 1X. Add 1µl of HaeIII. Incubate at 37 °C for 1 hour.

17. After the stock has passed quality control, it can be used without re-testing for at least 6 months, as long as it is kept at -80 °C.