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BIND&MODIFY: Long-range single-molecule mapping of chromatin modification in eukaryotes. V.2

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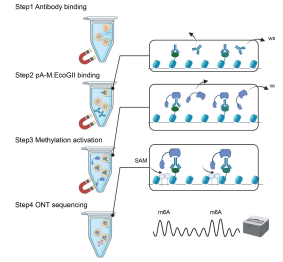
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We are still developing and optimizing this protocol

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Disclaimer

The authors declare no competing interests.

Abstract

Here we describe a powerful method, BIND&MODIFY, for probing histone modifications and transcription factors at single molecular level. This is the Protocol V2. Our approach used the recombinant fused protein A-M.EcoGII, which tethers the methyltransferase M.EcoGII to the protein binding sites and locally labels the neighboring DNA regions via artificial methylations. This method could reveal single-molecule heterogenous histone modification status and CpG methylation at the same time, and could enable quantify the correlation between the distal elements. Further applications based on this method's concept could be applied to probe multiple protein binding events on the same single molecular DNA. The method proposed herein may soon become an essential tool for third-generation sequencing in the future.

Materials

1.5ml DNA LoBind tubes (Eppendorf, 0030108051)

Digitonin (Abcam, ab141501)

Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma, 5056489001)

Bovine Serum Albumin (BSA), 20mg/ml, (NEB, B9000S)

Spermidine (Sigma, S0266)

Glycine (Sigma, 50046)

Pierce 16% Formaldehyde (w/v) (Thermo Scientific, 28908)

Polyvinylpyrrolidone, MW 40,000 (Sigma, PVP40)

Sodium metabisulfite (Sigma, 9000)

Polyethelene Glycol 8000(PEG) (Fisher Scientific, BP233-100)

RNase A, 10mg/ml (Thermo Scientific, EN0531)

Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma, P3803)

S-adenosylmethionine (SAM), 32mM (NEB, B9003S)

Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, BP531)

Sera-mag SpeedBeads carboxylate modified magnetic particles, 5% solids, 50 mg/mL, Hydrophobic, (VWR, 10204-666) (GE Healthcare Life Sciences, 44152105050350)

DNase/RNase-Free Distilled Water (Invitrogen 10977)

KCl, 2M, Rnase free (Invitrogen, AM9640G)

NaCl, 5M, Rnase free (Invitrogen, AM9759)

CaCl₂, 1M (Sigma, 21115)

MnCl₂, 1M (Sigma, M1787)

Tris, 1M, pH 8.0 (Thermoscientific, AM9856)

HEPES, 1M, pH 7.5 (Thermoscientific, J60712AK)

Potassium acetate, 3M, pH 5.5 (Invitrogen, AM9610)

Sodium acetate, 3M, pH5.2 (Invitrogen, R1181)

Triton X-100 (Sigma, X100)

EDTA, 0.5M, pH 8.0 (Invitrogen, 15575)

SDS, 10% (Invitrogen, 15553)

Tween-20 (Sigma, P9416)

Primary antibody, targeting histone modification and DNA binding protein. For example, α -H3K27me3 rabbit monoclonal antibody (CST, 9733), α -CTCF rabbit polyclonal antibody (Millipore, 07-729-25ul).

Secondary antibody, Guinea Pig anti-Rabbit IgG (Heavy & Light Chain) antibody (Antibodies-Online, ABIN101961).

pA-M.EcoGII Plasmid Amino Acid Sequence:



SLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDQAQ
APKADNKFNKEQQNAFYEILHLPNLTEEQRNGFIQSLKDDPSVSKEILAEAKKLNDQAQPKDDDKEFMLNTVKISSCELINAD
CLEFIRSLPENSVDLIVTDPPYFKVKPEGWDNQWKGDDDYLKWLDQCLAQFWRVLKPAGSLYLFCGHRLASDIEIMMRERF
SVLNHIIWAKPSGRWNGCNKESLRAYFPATERILFAEHYQGYPYRPKDAGYEAKGRALKQHVMAPLIAYFRDARAALGITAKQI
ADATGKKNMVPHWFSASQWQLPNESDYLKLQSLFARVAEEKHQERGELEKPHHQLVSTYSELNRKYMELLSEYKNLRRYFG
VTVQVPYTDVWTYKPVQYYPGKHPCEKPAEMLQQIISASS

pA-M.EcoGII Plasmid DNA Expression Sequence:

AGCTTAAAAGATGACCCAAGCCAAAGTGCTAACCTATTGTCAGAAGCTAAAAAGTTAAATGAATCTCAAGCACCGAAAG
CGGATAACAAATTCAACAAAGAACAACAAAATGCTTTCTATGAAATCTTACATTTACCTAACTTAAACGAAGAACAACGC
AATGGTTTCATCCAAAGCCTAAAAGATGACCCAAGCCAAAGCGCTAACCTTTTAGCAGAAGCTAAAAAGCTAAATGATG
CTCAAGCACCAAAAAGCTGACAACAAATTCAACAAAGAACAACAAAATGCTTTCTATGAAATTTTACATTTACCTAACTTA
ACTGAAGAACAACGTAACGGCTTCATCCAAAGCCTTAAAGACGATCCTTCAGTGAGCAAAGAAATTTTAGCAGAAGCTA
AAAAGCTAAACGATGCTCAAGCACCAAAAAGATGACGATAAAGAATTCATGCTTAATACTGTAAAAATATCCAGTTGTGAG
TTAATCAACGCCGACTGCCTGGAATTTATCCGGTCGTTACCCGAAAATTCTGTTGACCTGATAGTCACGGACCCGCCGT
ACTTTAAAGTGAAGCCCCGAGGGCTGGGATAACCAGTGGAAGGGCGACGATGATTACCTGAAGTGGCTGGACCAGTGTG
TGGCGCAGTTCTGGCGGGTGCTGAAACCTGCCGGAAGTCTTTACCTGTTCTGTGGCCATCGCCTGGCATCTGACATTG
AAATCATGATGCGTGAACGCTTCAGTGTGCTGAACCATATTATCTGGGCAAAGCCGTCCGGACGCTGGAACGGGTGCA
ACAAGGAAAGCCTGCGGGCGTATTTCCCCGCCACAGAGCGCATTCTGTTTCGCGGAACATTATCAGGGGCGGTATCGCC
CGAAAGATGCCGGGTATGAGGCGAAGGGCAGGGCACTGAAACAGCATGTGATGGCCCCGCTGATTGCTTACTTTTCGTG
ATGCGCGCGCTGCCCTGGGATAACGGCAAACAGATTGCAGATGCCACAGGAAAGAAAAACATGGTGCCGCACTGG
TTCAGTGCCAGTCAGTGGCAGCTACCGAACGAAAGCGATTATCTGAAATTACAGTCGCTGTTTGCCCGGGTGCCAGAA
GAGAAACATCAGCGCGGGGAACTGGAAAAGCCACACCACAGCTGGTCAGCACATACAGTGAGCTGAACCGGAAGTAT
ATGGAAGTGTGAGTGAATATAAAAATTTGCGGCGGTATTTTCGGTGTGACGGTGCAGGTGCCGTACACCGATGTGTGGA
CGTATAAACCGGTGCAGTACTATCCAGGGAAACATCCGTGCGAAAAACCGGCAGAAATGCTGCAGCAGATAATCAGCG
CAAGTAGTCGTCCTGGTGATCTGGTTGCGGATTTTTTCATGGGGTCGGGTTCAACGGTAAAAGCGGCGATGGCACTGG
GGCGTCGTGCGATTGGTGTGAGCTGGAGACCGGACGTTTTGAGCAGACAGTCAGGGAAGTTCAGGATTTAATCGTT

OVERVIEW

- 1 This protocol describes step-by-step guidelines for BIND&MODIFY method.

Protocol v2.

BIND&MODIFY method is based on the indirect labelling of DNA regions bound to the protein of interest (with antibody) using an engineered recombinant fusion protein, protein A-M.EcoGII (pA-M.EcoGII), whose methyltransferase activity can be locally controlled. Firstly, BIND&MODIFY method shows comparable distribution of histone modifications (H3K27me3, H3K4me3) and DNA binding protein (CTCF) with conventional ChIP-seq method. Secondly, BIND&MODIFY method resolves histone modification in complex genomic region, phases the epigenome, and uncovers epigenomic heterogeneity at single molecular level. Furthermore, BIND&MODIFY method reveals long-distance correlation between genome regulators. We believe BIND&MODIFY method to become one powerful tool for probing DNA binding protein and their regulatory mechanisms in the upcoming long-read sequencing technology arsenal.

With regard to previous published version,
BIND&MODIFY: Long-range single-molecule mapping of chromatin modification in eukaryotes

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To increase the methylation efficiency, the following changes were applied and used for targeting CTCF for experiments.

The major changes were:

- (1) introduce Protein A block step before antibody binding;
- (2) reduce primary antibody binding time from overnight to 2h at 4 degree;
- (3) add 0.5mM spermidine in the methylation buffer;
- (4) increase of methylation reaction time from 30min to 120min, and replenish 32mM SAM at 30min, 60min, 90min;
- (5) add 1ng/ul dsDNA 1ng/ul in the activation buffer(optional).

We refer this BIND&MODIFY protocol as Protocol v2

Step1 Antibody binding

Step2 pA-M.EcoGII binding

Step3 Methylation activation

Step4 ONT sequencing

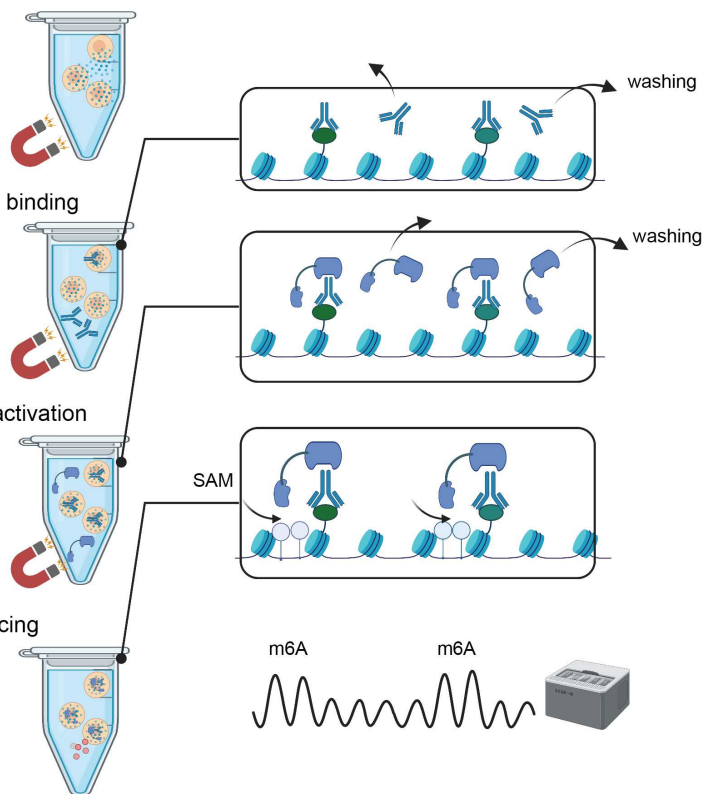


Figure1 Overview of BIND&MODIFY method

REAGENT SETUP

2h

- 2 Digitonin (5%): Dissolve 100mg digitonin in 2 ml DMSO. Aliquote at 50ul per PCR tube and freeze at -20. Avoid freeze-thaw cycles.
Caution: Digitonin is toxic and avoid any direct contact with skin or during breath. Use full PPE including a mask, lab coat and gloves while handling with digitonin. DMSO can penetrate through the skin.

Binding Buffer: Prepare fresh and store the buffer at 4°C for 6 months.

	A	B	C	D
		A	B	C
1		Reagent	Quantity	Final Concentration
2		1M HEPES pH 7.9	200ul	20mM

	A	B	C	D
	3	1M KCl	100ul	10mM
	4	1M CaCl ₂	10ul	1mM
	5	1M MnCl ₂	10ul	1mM
	6	H ₂ O	9750ul	
	7	Total	10ml	

Wash Buffer: Prepare fresh, and store the buffer at 4°C up to 1 week.

	A	B	C	D
		A	B	C
	1	Reagent	Quantity	Final Concentration
	2	1M HEPES pH 7.5	1.0ml	20mM
	3	5M NaCl	1.5ml	150mM
	4	2M Spermidine	12.5ul	0.5mM
	5	Roche Proteinase Inhibitor cocktail tablet	1 tablet	1X
	6	H ₂ O	47.5ml	
	7	Total	50ml	

Dig-wash Buffer: Prepare fresh, store the buffer at 4°C up to 2 days.

	A	B	C	D
		A	B	C
	1	Reagent	Quantity	Final Concentration
	2	2% Digitonin	400ul	0.02%
	3	20% BSA	200ul	0.1%



	A	B	C	D
	4	1X Wash Buffer	39.6ml	
	5	Total	40ml	

Tween-wash Buffer: Prepare fresh, store the buffer at 4°C up to 2 days.

	A	B	C	D
		A	B	C
	1	Reagent	Quantity	Final Concentration
	2	1M HEPES-KOH, pH 7.5	1000ul	20mM
	3	5M NaCl	1500ul	150mM
	4	2M Spermidine	12.5ul	0.5mM
	5	Roche Proteinase Inhibitor cocktail tablet	1 tablet	1X
	6	20% BSA	250ul	0.1%
	7	100% Tween-20	50ul	0.1%
	8	Total	50ml	

Methylation Buffer: Prepare fresh. Add SAM only before the methylation reaction.

	A	B	C	D
		A	B	C
	1	Reagent	Quantity	Final Concentration
	2	10X CutSmart Buffer	30ul	1x
	3	50X Roche Proteinase Inhibitor cocktail	6ul	1x
	4	5% Digitonin	3ul	0.05%



	A	B	C	D
	5	32mM SAM	7.5ul	800uM
	6	20% BSA	1.5ul	0.10%
	7	2M Spermidine	0.125ul	0.05mM
	8	10ng/ul dsDNA(optional)	1ul	1ng/ul
	9	H2O	250ul	
	10	Total	300ul	

Digestion Buffer: Prepare fresh.

	A	B	C	D
		A	B	C
	1	Reagent	Quantity	Final Concentration
	2	Polyvinylpyrrolidone 40	0.1g	1%
	3	Sodium metabisulfite	0.1g	1%
	4	5M NaCl	1.0ml	0.5M
	5	1M Tris-HCl, pH 8.0	1.0ml	0.5M
	6	0.5M EDTA	1.0ml	50mM
	7	20% SDS	625ul	1.25%
	8	H2O	6375ul	
	9	Total	10ml	

Mix and incubate at 65°C during at least 30 minutes. The solution need to be clear before use.



Serapure Beads Solution: Store the solution at 4°C for 1 month. 4ml serapure beads wash 4 times with water to remove sodium azide, then resuspend in 10ml serapure beads solution.

	A	B	C	D
		A	B	C
1		Reagent	Quantity	Final Concentration
2		50% PEG8000	3.6 ml	18%
3		5M NaCl	2 ml	1M
4		1M Tris-HCl, pH 8.0	0.1ml	10mM
5		0.5M EDTA	20ul	1mM
6		100% tween 20	5ul	0.05%
7		H2O	4275ul	
8		Total	10ml	

Bead Binding Buffer: Store the solution at 4°C for 1 week.

	A	B	C	D
		A	B	C
1		Reagent	Quantity	Final Concentration
2		50% PEG 8000	4ml	20%
3		5M NaCl	6ml	3.0M
4		Total	10ml	

Mix until the solution becomes clear. If PEG8000 is not dissolved, it can lead to a poor yield because PEG8000 makes gDNA to bind to the beads.

Bead Washing Buffer: Prepare fresh.

	A	B	C	D
		A	B	C



	A	B	C	D
1		Reagent	Quantity	Final Concentration
2		Absolute Ethanol	35ml	70%
3		H2O	15ml	
4		Total	50ml	

Cells Preparation

1h 30m

- 3 Harvest fresh cells at room temperature.

Note: Use Eppendorf DNA LowBind tube during the whole protocol to reduce cell/DNA loss.

- 4 Centrifuge cells at 300g for 5 min at 4°C.

- 5 Resuspend the cells in 1ml cold PBS, repeat step 5-step 6 twice.

- 6 Resuspend the cells in 900ul cold PBS. Count the cells. For lightly fixed cells, go to step 7.

Normally this protocol works for 5×10^5 cells per methylation reaction. Aliquot 4×10^6 cells per centrifuge tube for 8 tube methylation reaction.

- 7 Add freshly prepared 1% formaldehyde into the resuspended cells (100ul into 900ul cells), and put at room temperature for 10 min.

- 8 Stop the crosslinking by adding 1.25M glycine to twice molar ratio of formaldehyde (60ul into 1ml cell fixing reaction).



9 Centrifuge cells at 500g for 5 min at 4°C. Carefully remove all the liquids from the supernatant with 1000ul pipette tip followed by 100ul and 10ul pipette tip.

10 Resuspend the cells with **Wash Buffer** and count the fixed cells.

Normally this protocol works for 5×10^5 cells per methylation reaction. Aliquot 4×10^6 cells per centrifuge tube for 8 tube methylation reaction.

Bind cells or nuclei to Concanavalin A-coated beads

30m

11 Gently vortex and resuspend the ConA beads slurry, 10ul of the ConA beads would be enough for 5×10^5 cells. The following is for 4 samples.

12 Aliquot 90ul ConA beads slurry into 1ml **Binding Buffer** in a 1.5ml tube and mix by pipetting. Put the tube on a magnetic stand to clear (1-2min).

13 Remove the liquid completely on the magnetic stand. Add 1ml **Binding Buffer** and mix by pipetting. Quick spin the tube to remove the liquid from the cap.

14 Put the tube on a magnetic stand to clear, remove the liquid, and resuspend in 90ul **Binding Buffer** (10ul per sample) and place the activated beads slurry at room temperature until cells are prepared.

15 Carefully add in 90ul **Binding Buffer** containing ConA beads into the tube containing 4×10^6 cells prepared from step 6 or step 10. Place on end-over-end rotator for 10min.

16 Centrifuge cells at 500g for 5 min at 4°C. Carefully remove all the liquids from the supernatant with 1000ul pipette tip followed by 100ul and 10ul pipette tip.

17 Carefully add in 1ml **Dig-wash Buffer** to each of the tubes, place the tubes on ice for 5min. Check 10ul of the cells with Trypan Blue staining. If the cell permeabilization was successful, continue to the next steps.

Protein A Block

2h

18 Quick spin the tube to remove the liquid from the cap. Place the tube on a magnetic stand to clear, remove the liquid.

19 Resuspend the cells in 200ul ice cold **Tween-wash Buffer** with gentle vortexing.



- 20 Add 2.5ul 50ug/ul protein A. Place on end-over-end rotator at room temperature for 10min.

Primary Antibody Binding

2h

- 21 Quick spin the tube to remove the liquid from the cap. Place the tube on a magnetic stand to clear, remove the liquid.
- 22 Resuspend the cells in 400ul ice cold **Tween-wash Buffer** with gentle vortexing. Divide into eight 1.5ml Eppendorf LowBind tubes, and 50ul each tube. Scale up or down based on specific applications.
- 23 Add 0.5-1.0ul of specific primary antibody (H3K27me3 or CTCF) into each tube with gentle vortexing.

Note: We use 1:50-1:100 primary antibody dilution as recommended by CUT&TAG protocol.

- 24 Place the tube on end-over-end rotator at 4°C for 2h.
- 25 Quick spin the cells with primary antibody. Place the cells with primary antibody on a magnetic stand, carefully remove the solution.
- 26 Add 1ml **Tween-wash Buffer**. Invert the tube 10 times to resuspend the beads.
- 27 Repeat step 23-25 twice.

pA-M.EcoGII Binding

1h

- 28 Add 10ul pA-M.EcoGII recombinant enzyme into 190ul **Tween-wash Buffer**, mix gently by pipetting.
- 29 Quick spin the tube from step 28. Place the tube on a magnetic stand, carefully remove the solution.
- 30 Add the pA-M.EcoGII containing buffer to the cells with gentle vortexing.



- 31 Place the tube on end-over-end rotator at room temperature for 1h.
- 32 Quick spin the cells with pA-M.EcoGII. Place the cells with pA-M.EcoGII on a magnetic stand, carefully remove the solution.
- 33 Add 1ml **Tween-wash Buffer**. Invert the tube 10 times to resuspend the beads.
- 34 Repeat step 30-31 twice.

Methyltransferase Activation

2h

- 35 Quick spin the tube from step 34. Place the tube on a magnetic stand, carefully remove the solution.
- 36 Add 300ul **Methylation Buffer**. Invert the tube 10 times to resuspend the beads.
- 37 Incubate at 37°C thermomixer at 1000rpm for 120min. Supplement 7.5ul 32mM SAM at 30min, 60min, and 90min. Proceed to step 38 with DNA extraction by PCI, or proceed to step 46 with DNA extraction by Serapure Beads.

DNA extraction 1:PCI

2h

- 38 Take the tube from step 37. To stop the methylation reaction, add 10ul 0.5M EDTA, 1.5ul 20% SDS, and 5.0ul 20mg/ml Proteinase K to each tube.
- 39 Mix by vortexing at highest speed for 5s. Incubate at 55°C water bath overnight until the solution is clear.

Note: Increase incubation time if the solution is viscous or cloudy.
- 40 Add 300ul PCI and vortexing at highest speed for 30s. Invert the tube 10 times to mix thoroughly. Centrifuge at 16000g for 5min.



- 41 Transfer the upper liquid aqueous phase to a new 1.5ml centrifuge tube. Add 300ul chloroform. Invert the tube 10 times. Centrifuge at 16000g for 5min.
- 42 Transfer the upper liquid aqueous phase to a new 1.5ml centrifuge tube. Add 1/10 volume of 3M sodium acetate solution.
- 43 Add 2.5x volume of ice cold 100% ethanol in the solution to precipitate DNA. Incubate the tube at -20 overnight.
- 44 Centrifuge at 16000g for 10min. Discard the supernatant and rinse the pellet with 70% cold ethanol.
- 45 Air-dry the pellet. Dissolve in TE buffer.

DNA extraction 2: Serapure Beads

2h

- 46 Alternatively, the DNA extraction can be down with Serapure Beads method, this results in better recovery of genomic DNA in higher N50, which would benefit ONT sequencing.
- 47 Take the tube from step 37, place the tube on a magnetic stand, remove all the solution.
- 48 Add 600ul pre-warmed **Digestion Buffer**, plus 4.0ul 10mg/ml RNase A. Mix thoroughly immediately by pipetting up and down 10 times with a wide-bore tip.
- 49 Add 10.0ul 20mg/ml Proteinase K. Incubate the tube at 55°C water bath overnight.
- 50 Add 200µl (or 1/3 of the lysis buffer volume) of 5M potassium acetate and mix by inverting the tube 20 times in order to obtain a homogenous solution to fully precipitate the proteins and the polysaccharides that will complex with SDS. It is important to incubate at 4°C after the addition of potassium acetate.
- 51 Centrifuge at 5000g for 10 minutes at 4°C. Transfer the supernatant to a new 1.5 ml tube without disturbing the pellet.
- 52 Add one volume of **Bead Binding Buffer** and 1:18 (v:v) of Serapure beads previously prepared (vortex the beads solution for 20 seconds before use to ensure that the beads are completely resuspended).



- 53 Mix by inverting the tube 20 times. Incubate with a gentle mixer for 10 minutes at room temperature.
- 54 Quick spin the tube to remove the liquid from the cap. Place the tube in a magnetic stand until the solution becomes clear (2-3min). The actual time required to collect beads may vary according to samples.
- 55 Remove the supernatant without disturbing the beads pellet. Add 1 ml of **Bead Wash Buffer**, remove the tube from the magnetic rack and mix by inverting the tube 20 times.
- 56 Quick spin the tube to remove the liquid from the cap. Place the tube in a magnetic stand until the solution becomes clear (2-3min).
- 57 Repeat step 53-54.
- 58 Quick spin the tube to remove the liquid from the cap. Place the tube in a magnetic stand to remove the remaining **Bead Wash Buffer**.
- 59 Let the beads air-dry for 1 minute with the cap open. Do not let the beads dry more than 1 minute as this will significantly decrease elution efficiency.
- 60 Add 80 µl of TE buffer preheated to 50°C.
- 61 Resuspend the beads by flicking the tube. It is important that the beads are not aggregated.
- 62 Quick spin the tube. Place the tube in the magnetic rack. Let the solution to become clear. If DNA solution is highly concentrated, it can take a long time. In this case, it is recommended to let the tube in the magnetic rack overnight or to add more elution buffer.
- 63 Transfer 75 µl of the eluted gDNA solution in a new tube.

ONT library preparation and sequencing

3d

- 64 Follow Oxford Nanopore protocol of LSK109 for adaptor ligation.



- 65 Load 500ng of the ligated DNA to R9.4.1 flow cell. Use the Flow Cell Wash Kit to wash the flow cell.
- 66 Reload the flow cell every 24h.