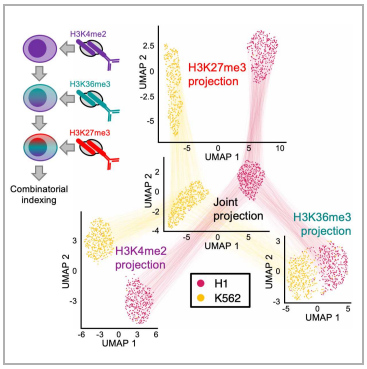


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**Protocol status:** Working
We use this protocol and it's working

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# Multiple Targets Identified via Tagmentation (MulTI-Tag) v1.0

Michael Meers
Lab[1]

[1]Department of Genetics, Washington University School of Medicine in St. Louis

Michael Meers Lab

## ABSTRACT

We introduce a public protocol for Multiple Targets Identified via Tagmentation (MulTI-Tag), a chromatin profiling approach that acertains the genomic enrichment of multiple chromatin protein targets in the same cellular context. Multi-Tag uses unique, antibody-specific barcodes incorporated into pA-Tn5-loaded adapters to sequentially tagment targets and read out protein identity via barcode sequencing. MulTI-Tag attains profiles of similar quality to CUT&Tag, and crucially it attains exemplary specificity of barcode-derived target identification. MulTI-Tag can be used to profile multiple chromatin targets in individual cells via plate-based combinatorial indexing approaches, and in principle can be used with microfluidic platforms.

## MATERIALS

- Chilling device (e.g.metal heat blocks on ice or cold packs in an ice cooler)
- Pipettors (e.g. Rainin Classic Pipette 1 mL, 200 µL, 20 µL, and 10 µL)
- Disposable tips (e.g. Rainin 1 mL, 200 µL, 20 µL)
- Disposable centrifuge tubes for reagents (15 mL or 50 mL)
- Standard 1.5 ml microfuge tubes
- 0.5 ml maximum recovery PCR tubes (e.g. Fisher cat. no. 14-222-294)
- Frozen native or lightly cross-linked whole cells or nuclei suspension (e.g. human K562 or H1 cells) prepared as described in the Appendix (Steps 52-64, cite: Kaya-Okur et al. (2020) Nature Protocols).
- Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, ca. no. BP531)
- Strong magnet stand (e.g. Miltenyi Macsimag separator, cat. no. 130-092-168)
- Vortex mixer (e.g. VWR Vortex Genie)
- Mini-centrifuge (e.g. VWR Model V)
- PCR thermocycler (e.g. BioRad/MJ PTC-200)
- Distilled, deionized or RNAse-free H2O (dH2O e.g., Promega, cat. no. P1197)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K+); Sigma-Aldrich, cat. no. H3375)
- 1 M Manganese Chloride (MnCl2; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride (CaCl2; Fisher, cat. no. BP510)

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- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na+); Sigma-Aldrich, cat. no. H3375)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S0266)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X-100)
- 0.5 M Ethylenediaminetetraacetic acid (EDTA; Research Organics, cat. no. 3002E)
- Antibody to an epitope of interest. Because in situ binding conditions are more like those for immunofluorescence (IF) than those for ChIP, we suggest choosing IF-tested antibodies if CUT&RUN/Tag-tested antibodies are not available
- CUTAC control antibody to RNA Polymerase II Phospho-Rpb1 CTD Serine-5 phosphate (PolIIS5P) or histone H3K4me2. We have obtained excellent results with these rabbit monoclonal antibodies: Cell Signalling Technology Phospho-Rpb1 CTD (Ser5), CST #13523 (D9N5I) and Epicypher H3K4me2 #13-0027.
- Secondary antibody, e.g. guinea pig α-rabbit antibody (Antibodies online cat. no. ABIN101961) or rabbit α-mouse antibody (Abcam cat. no. ab46540)
- Protein A/G–Tn5 (pAG-Tn5) fusion protein loaded with double-stranded adapters with 19mer Tn5 mosaic ends (Epicypher cat. no. 15-1117).
- 1 M Magnesium Chloride (MgCl2; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS) pH 8.5 (with NaOH)
- 1,6-hexanediol (Sigma-Aldrich cat. no. 240117-50G)
- N,N-dimethylformamide (Sigma-Aldrich cat. no. D-8654-250mL)
- NEBNext 2X PCR Master mix (New England Biolabs cat. no. ME541L)
- PCR primers: 10 µM stock solutions of i5 and i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. We do not recommend Nextera or NEBNext primers.
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- Thermolabile Proteinase K (New England Biolabs, cat. no. P8111S)
- SPRI paramagnetic beads (e.g. HighPrep PCR Cleanup Magbio Genomics cat. no. AC-60500)
- 10 mM Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)

## Reagent setup

1 **Bead binding buffer** Mix 200 µL 1M HEPES-KOH pH 7.9*, 100 µL 1M KCl, 10 µL 1M CaCl2 and 10 µL 1M MnCl2, and bring the final volume to 10 mL with dH2O. Store the buffer at 4 °C for up to several months. *HEPES-NaOH pH 7.5 is OK.

**Wash buffer** Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 µL 2 M spermidine and 250 µl 10% Triton-X100, bring the final volume to 50 mL with dH2O, and add 1 Roche Complete

Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 2 days.

**Wash 300 buffer** Add 750 µL 5M NaCl to 24.3 ml Triton-Wash buffer. Store at 4 °C for up to 2 days.

**TAPS wash buffer** Mix 1 mL dH2O, 10 µL 1 M TAPS pH 8.5, 0.4 µL 0.5 M EDTA (10 mM TAPS, 0.2 mM EDTA).

**TAPS-SDS buffer** (For 32 samples) Mix 20 µl 10% SDS and 2 µl 1 M TAPS pH 8.5 in 178 µl dH2O. Just before use add 20 µL Thermolabile Proteinase K (NEB cat. no. P8111S).

**Triton neutralization solution** Mix 30 µL 12.5% Triton X-100 with 370 µL ddH2O.

## Conjugate generation (4 hours)

2 Resuspend dehydrated, 5'-aminated (NH2) P5_i5 oligo in 1xPBS at a concentration of 200 µM.

3 Resuspend dehydrated Tn5MErev oligo (5'-[phos]CTGTCTCTTATACACATCT-3') in 1xPBS at a concentration of 200 µM.

4 Anneal P5_i5 oligo with an equal volume of Tn5MErev oligo to generate 100 µM P5_i5 adapter by incubating the mixture at 95˚C for 2 minutes on a thermal cycler, then ramping down the temperature my 0.5˚C every 30 seconds until it reaches 25˚C.

5 Use annealed P5_i5 adapter, along with 100 µg primary antibody purified in 1xPBS in the absence of BSA, glycerol, and sodium azide, to generate antibody-adapter conjugates at a molar ratio of 1:10 according to manufacturer's protocols (Abcam ab218260).

## Conjugate assembly (1 hour)

6 Resuspend free P5_i5 oligo in 1xTE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) at a concentration of 200 µM.

7 For bulk experiments: Resuspend free P7_i7 oligo in 1xTE at a concentration of 200 µM. For single cell experiments: Resuspend 96 uniquely barcoded P7_i7 oligos in 1xTE at a concentration of 200 µM.

8 Resuspend dehydrated Tn5MErev oligo (5'-[phos]CTGTCTCTTATACACATCT-3') in 1xTE at a concentration of 200 µM.

9 Anneal P5_i5 oligo with an equal volume of Tn5MErev oligo to generate free 100 µM P5_i5 adapter as described in step 4.

10 **For bulk experiments:** Anneal P7_i7 oligo with an equal volume of Tn5MErev oligo to generate free P7_i7 adapter as described in step 4. **For single cell experiments:** In each well of a 96 well plate, anneal one of 96 uniquely barcoded P7_i7 oligos with an equal volume of Tn5MErev oligo.

11 For each conjugate to be used, combine 0.5 µg antibody-i5 adapter conjugate with 1 µL 5 µM pA-Tn5 and 16 pmol free P5_i5 adapter in a minimal volume and incubate 30-50 minutes at RT.

12 **For bulk experiments:** Combine 1 µL pA-Tn5 with 32 pmol P7_i7 adapter in a minimal volume and incubate for 30-50 minutes at RT. **For single cell experiments:** In each well of a 96 well plate, combine 10 µL pA-Tn5 with 320 pmol of one of 96 uniquely barcoded P7_i7 adapters in a minimal volume and incubate for 30-50 minutes at RT. Seal the plate, store at 4˚C, and use for several future experiments.

13 Use assembled conjugates within 24 hours—when incubating overnight, conjugates should be assembled directly before incubation.

## MulTI-Tag (1+ days)

14 **For bulk experiments:** Wash 10 µL paramagnetic Concanavalin A beads (Bangs Laboratories) 2x with 1 mL Bead Binding Buffer, then resuspend in 10 µL Bead Binding Buffer. **For single cell experiments:** Wash 5 µL paramagnetic Wheat Germ Agglutinin (WGA)-MyOneC1 Dynabeads 2x with 1 mL Bead Binding Buffer, then resuspend in 5 µL Bead Binding Buffer. While this quantity of beads is sufficient for 50000 nuclei, combinatorial indexing experiments should prepare to use

an input of 2 million nuclei that will be distributed equally across a 96 well plate (~20833 nuclei/well) For example: you might prepare 8 samples of 250000 nuclei and 25 µL beads each, which will then be processed normally until step 26, at which point each sample will be aliquoted equally across 12 wells in the 96 well plate.

15 Add resuspended beads to 50000 nuclei while gently vortexing to immediately mix.

16 Incubate beads and nuclei with rotation for 10 minutes at RT.

17 Clear supernatant from nuclei-bead mix of magnet stand and resuspend in 50 µL Wash 300 buffer plus 2 mM EDTA and assembled conjugate of interest.

18 Incubate 1 hour at RT or overnight at 4˚C with nutation. To use more conjugates go to Step 13; upon addition of last conjugate go to Step 18.

19 Clear supernatant and wash 2x with 200 µL Wash 300 buffer.

20 Clear supernatant and add 50 µL Wash 300 buffer plus 10 mM MgCl2.

21 Incubate 1 hour at 37˚C.

22 Clear supernatant on magnet stand and wash 2x with 200 µL Wash 300 buffer plus 2 mM EDTA.

23 Repeat steps 11-17 for all conjugates to be added; after addition of the last conjugate, skip from Step 12 to Step 18.

24 Clear supernatant and add 100 µL Wash Buffer with 1:100 Secondary antibody.

25 Incubate 30 minutes-1 hour at RT with nutation.

26 Clear supernatant and Wash 1x with 200 µL Wash Buffer.

27 **For bulk experiments:** Clear supernatant and add 100 µL Wash 300 Buffer plus assembled P7_i7 adapter pA-Tn5. **For single cell experiments:** Clear supernatant and add Wash 300 buffer to samples such that the sum total volume of all samples is 26 µL * 96 (2.469 mL). Distribute 25 µL to each well in a Lo Bind 96 well plate, and add 2 µL uniquely barcoded P7_i7 adapter pA-Tn5 mix to each well using an 8-channel p20 micropipette.

28 Incubate 1 hour at RT with nutation.

29 **For bulk experiments:** Clear supernatant and wash 2x with 200 µL Wash 300 buffer. **For single cell experiments:** Clear supernatant and wash each well in 96 well plate 2x with 100 µL Wash 300 buffer.

30 Clear supernatant and add 50 µL Wash 300 buffer plus 10 mM MgCl2.

31 Incubate 1 hour at 37˚C.

32 Clear supernatant and add 100 µL TAPS wash buffer.

## Bulk MulTI-Tag DNA preparation (1.5 hours)

33 Clear supernatant and add 5 µL 10 mM TAPS-SDS buffer.

34 Incubate 1 hour at 58˚C.

35 Add 16 µL Triton neutralization solution and mix well by pipetting.

36 Add 2 µL each of forward and reverse barcoded PCR primers.

37 Add 25 µL NEBNext 2x PCR Master Mix and mix well with pipetting.

## Single cell MulTI-Tag DNA preparation (1.5+ hours)

38 Clear supernatant from columns 1-6 and, using wide-bore pipette tips and an 8-channel p200 micropipette, add 100 µL TAPS wash buffer to column 1.

39 Pipette up and down to dislodge all nuclei from the walls, then remove TAPS-nuclei mix and pipette into column 2.

40 Repeat step 39 for all columns 2 through 6 to ensure that all nuclei are resuspended and consolidated in column 6.

41 Repeat steps 38-40 to clear any remaining nuclei, resulting in 200 µL TAPS wash buffer per well in column 6.

42 Repeat steps 38-41 for columns 7-12.

43 Clear supernatant from column 6 and, using a wide-bore pipette tip, add 100 µL TAPS wash buffer to row A column 6.

44 Pipette up and down to dislodge all nuclei from the walls, then remove TAPS-nuclei mix and pipette into row B column 6.

45 Repeat step 44 for all rows B through H in column 6 to ensure that all nuclei are resuspended and consolidated in Row H column 6.

46 Repeat steps 43-45 to clear any remaining nuclei, resulting in 200 µL TAPS wash buffer per well in row H column 6.

47 Repeat steps 43-46 for column 12.

48 Transfer 200 µL nuclei mix from each of row H column 6 and row H column 12 into fresh, low-bind 1.7 mL tubes, then chill on 0˚C cold block on ice for 2 minutes.

49 To gently digest extracellular DNA to reduce nuclei/bead clumping, proceed to step 50. Otherwise, proceed to step 53.

50 Prepare RQ1 RNase-free DNase solution (Promega) by combining 10 µL 10x DNase buffer, 80 µL ddH2O, and 10 µL RQ1 DNase.

51 Clear supernatant and transfer empty tube back to 0˚C cold block. Squirt entire 100 µL of DNase solution onto beads, flick tube gently to mix, and incubate on cold block for 5-10 minutes.

52 Add 100 µL DNase stop solution and flick tube gently to mix.

53 Immediately transfer to magnet stand, clear supernatant and add 100 µL 10 mM TAPS.

54 Bring nuclei-bead solution up to 650 µL and add to 20-micron filter affixed to fresh Eppendorf tube; spin 5 minutes at 100xg and collect supernatant.

55 Prepare nuclei for nanowell/microfluidic platform of choice according to manufacturer's instructions.

## Amplification and cleanup (1.5 hours)

56 Amplify DNA using the following conditions:

1. 5 minutes at 58˚C
2. 5 minutes at 72˚C

3. 45 seconds at 98˚C
4. 15 seconds at 98˚C
5. 10 seconds at 60˚C
6. Repeat steps 4-5 13 times (14 total cycles)
7. 5 minutes at 72˚C
8. Hold at 8˚C

**57** Purify DNA once using 1.1x Ampure XP magnetic beads and resuspend in 25 µL 10 mM Tris-HCl pH 8.0 plus 1 mM EDTA.