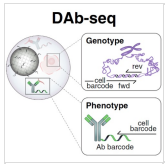

Version 1

Oct 27, 2020

DAb-seq: Single-Cell DNA and Antibody Sequencing V.1

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1 *Works for me* This protocol is published without a DOI.

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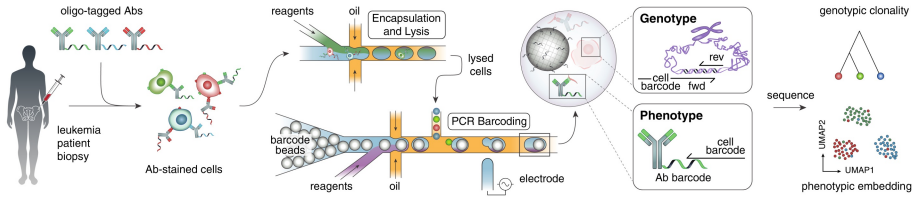
ABSTRACT

Studies of acute myeloid leukemia rely on DNA sequencing and immunophenotyping by flow cytometry as primary tools for disease characterization. However, leukemia tumor heterogeneity complicates integration of DNA variants and immunophenotypes from separate measurements. Here we introduce DAb-seq, a novel technology for simultaneous capture of DNA genotype and cell surface phenotype from single cells at high throughput, enabling direct profiling of proteogenomic states in tens of thousands of cells. To demonstrate the approach, we analyze the disease of three patients with leukemia over multiple treatment timepoints and disease recurrences. We observe complex genotype-phenotype dynamics that illustrate the subtlety of the disease process and the degree of incongruity between blast cell genotype and phenotype in different clinical scenarios. Our results highlight the importance of combined single-cell DNA and protein measurements to fully characterize the heterogeneity of leukemia.



Graphical overview of the DAb-seq protocol.

ATTACHMENTS

Supplementary Table 1_DNA panel primer sequences.xlsx

Supplementary Table 6_Library preparation primer sequences.xlsx

Supplementary Table 2_Antibody supplier list and tag sequences.xlsx

Tapestri Single-Cell DNA AML User Guide PN_3354A1.pdf

PROTOCOL CITATION

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single-cell, DNA sequencing, acute myeloid leukemia, droplet microfluidics, multiomics

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GUIDELINES

This protocol is compatible with Mission Bio V1 chemistry only.

MATERIALS TEXT

Antibody conjugation reagents and supplies

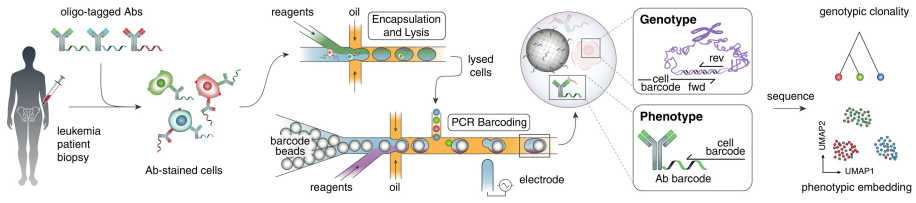
- D-PBS
- Monoclonal antibodies (various suppliers)
- DBCO-PEG5-NHS Ester linker (Click Chemistry Tools, cat. no. A102P)
- Amicon 50 kDa filter (Millipore Sigma, cat. no. UFC505024)
- Agilent Bioanalyzer Protein 230 Kit

DAb-seq workflow reagents and supplies

- PBS-F (D-PBS + 5% FBS)
- 5 mL DNA LoBind tubes (Eppendorf, cat. no. 0030108310)
- Human TruStain FcX (BioLegend, cat. no. 422301)
- Dextran sulfate (Research Products International, cat. no. D20020)
- Salmon sperm DNA (Invitrogen, cat. no. 15632011)
- Mission Bio Tapestri instrument and AML kit
- Ampure XP beads (Beckman Coulter, cat. no. A63881)
- Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher, cat. no. 65001)
- Agilent Bioanalyzer High Sensitivity DNA Kit
- Sequencing kit (Illumina)

ABSTRACT

Studies of acute myeloid leukemia rely on DNA sequencing and immunophenotyping by flow cytometry as primary tools for disease characterization. However, leukemia tumor heterogeneity complicates integration of DNA variants and immunophenotypes from separate measurements. Here we introduce DAb-seq, a novel technology for simultaneous capture of DNA genotype and cell surface phenotype from single cells at high throughput, enabling direct profiling of proteogenomic states in tens of thousands of cells. To demonstrate the approach, we analyze the disease of three patients with leukemia over multiple treatment timepoints and disease recurrences. We observe complex genotype-phenotype dynamics that illustrate the subtlety of the disease process and the degree of incongruity between blast cell genotype and phenotype in different clinical scenarios. Our results highlight the importance of combined single-cell DNA and protein measurements to fully characterize the heterogeneity of leukemia.



Graphical overview of the DAb-seq protocol.

Conjugation of antibodies to oligonucleotide barcodes 1d

1 Resuspend each monoclonal antibody to **100 µg** in **100 µl** PBS.

Antibody must be ordered in protein-free, azide-free buffer, preferably plain PBS. Resuspended from lyophilized stock with trace amounts of trehalose (e.g. from R&D Systems) is also acceptable. For a list of antibodies used in the DAb-seq publication, see Supplemental Table 2 attachment.

2 10m

Prepare [M]**25 Milimolar (mM)** DBCO/PEG/NHS linker in DMSO. After adding DMSO, vortex at **Room temperature** for **00:10:00** to allow linker to dissolve completely.

For long-term storage, aliquot DBCO linker in **5 µl** volumes and store at **-20 °C** .

3 Dilute DBCO linker to [M]**2.5 Milimolar (mM)** in water.

4 To each 100 uL antibody solution, add **1.07 µl** of 2.5 mM DBCO linker. Pipet up and down to mix.

5 Incubate linker/antibody solution for **02:00:00** at **Room temperature** . 2h

6 Add **400 µl** PBS to linker/antibody solution and transfer to 50 kDA Amicon filter.

7 Centrifuge **14000 x g, 00:10:00** . Discard flow-through.

8 Add **180 µl** PBS to filter and invert filter in new collection tube. Centrifuge **1500 x g, 00:05:00** to elute.

9 Resuspend lyophilized oligonucleotide to [M]**200 Micromolar (µM)** in PBS.

Oligos must have 5' azide group (IDT code: /5AzideN/) and be HPLC-purified. For full oligo sequence, including barcodes, see Supplemental Table 2 attachment.

10 Add **6.67 µl** oligo to the approximately **200 µl** of filtered antibody solution.

11 Incubate at **4 °C** **Overnight** . 2h

16-20 h is an acceptable incubation time.

12 Add **300 µl** PBS to antibody-oligo conjugate and transfer to 50 kDa Amicon filter.

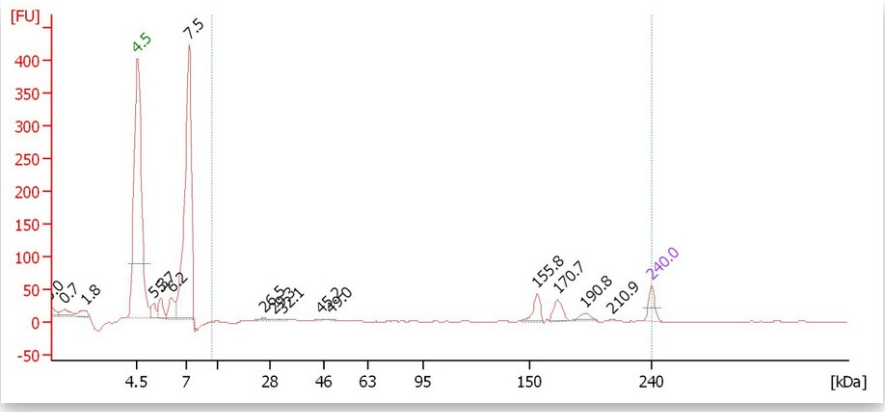
13 Centrifuge **14000 x g, 00:10:00** . Discard flow-through.

14 Repeat Step 13 two additional times, adding **500 µl** PBS for each wash. This is a total of three washes.

15 Add **30 µl** PBS to filter and invert filter in new collection tube. Centrifuge **1500 x g, 00:05:00** to elute.

16 Collect ~50 uL eluant and store in Protein LoBind tube at **4 °C** .

17 Verify conjugation using a Bioanalyzer Protein 230 kit or equivalent gel electrophoretic assay. A representative Bioanalyzer Protein 230 trace is shown below. A peak representing the unconjugated antibody is visible at ~155 kDa. Larger peaks represent antibodies conjugated to one, two, or more oligos.



Representative Bioanalyzer Protein 230 trace for oligo-antibody conjugate. Non-denaturing conditions were used in this run.

Staining cells 1d

18 Collect cells from culture or thaw from frozen according to cell-specific protocols. Resuspend cells in 5-10 mL PBS-F (D-PBS + 5% FBS) and count. It is highly recommended to measure cell viability with trypan blue or other exclusion assay.

Cells should be >90% viable before staining to ensure minimal cell loss during the washing steps.

19 Spin down 2 million cells in a 15 mL DNA LoBind tube for **400 x g, 00:04:00** . Aspirate the supernatant and resuspend the cell pellet in **180 µl** PBS-F.

It is helpful to grate the tube gently against the TC hood to loosen the cell pellet prior to the addition of buffer.

20 Add **10 µl** BioLegend Human TruStain FcX blocking solution, **4 µl** of a **[M]1 % (m/v)** dextran sulfate solution, and **4 µl** of **[M]10 mg/ml** salmon sperm DNA. Pipet gently to mix.

21 Incubate **00:10:00** **On ice** . 10m

22 Add **0.5 µg** of each antibody-oligo conjugate and incubate **00:30:00** **On ice** . 30m

23 Perform five washes to remove excess unbound antibody. For each wash, add **5 mL** PBS-F to the tube and centrifuge **400 x g, 00:04:00** .

24 After final wash, resuspend cells in Mission Bio Cell Buffer at a final concentration of 3M cells/mL.

Cell encapsulation and barcoding on the Tapestri instrument 1d

25 Follow cell encapsulation and barcoding procedure as described in the attached Mission Bio document: "Tapestri Single-Cell DNA AML User Guide".

Follow sections "Encapsulate Cells" (page 18) through "UV Treatment and Targeted PCR Amplification" (page 31). The DAb-seq protocol for these sections are unchanged from the conventional DNA-only workflow.

Cleanup barcoded DNA and antibody products

26 ⚠

Perform Steps 6.1 through 6.7 in the attached Mission Bio protocol. **DO NOT** discard the Ampure XP supernatant in Step 6.8, as this contains the short antibody tags. Instead, for each tube, transfer the supernatant to a new 1.5 mL DNA LoBind tube.

27 Finish DNA library cleanup as described in the Mission Bio protocol. Elute in 30 uL water and use a Qubit hsDNA assay to measure the concentration of barcoded DNA product in each tube. Transfer the DNA product to PCR tubes and store at **-20 °C** prior to library PCR.

Typical concentrations are between 0.5 and 2.0 ng/µl.

28 For antibody tag cleanup, add biotinylated capture oligonucleotide to the tube supernatants from Step 26 to a final concentration of **[M]0.6 Micromolar (µM)** .

The capture probe sequence is /5Biosg/GGCTTGTTGTGATTCGACGA/3C6/, using IDT codes.

29 Heat the supernatant-probe solution to **95 °C** for **00:05:00** to denature the PCR product, then snap cool on ice for probe hybridization. Allow tubes to cool for **00:05:00** on ice. 10m

30 For each sample tube, wash **10 µl** of magnetic streptavidin beads two times in **1 mL** D-PBS, allowing beads to bind to the magnet between washes. Resuspend beads in **10 µl** D-PBS and add to each tube. Pipet to mix.

31 Incubate tubes **00:15:00** at **Room temperature** with rotation to allow streptavidin-biotin binding. 15m

32 Place the tubes on a magnet and allow beads to separate.

33 Wash beads two times in **1 mL** D-PBS and resuspend in 30 µL water. Transfer the bead solutions to PCR tubes and store at **-20 °C** prior to library PCR.

Library preparation PCR

34 Prepare **50 µl** library PCR reactions for each tube, and for each of the DNA and antibody tag libraries:

DNA panel libraries:

- **25 µl** Mission Bio Barcoding Mix
- **5 µl** P5 primer (**4 Micromolar (µM)**)
- **5 µl** P7 primer (**4 Micromolar (µM)**) - *DNA panel specific*
- **4 ng** of barcoded DNA product in **15 µl** water

Antibody tag libraries:

- **25 µl** Mission Bio Barcoding Mix
- **5 µl** P5 primer (**4 Micromolar (µM)**)
- **5 µl** P7 primer (**4 Micromolar (µM)**) - *Antibody tag specific*
- **15 µl** bead-bound antibody tag product

Ensure all combinations of DNA panel and antibody tag libraries have unique P5 and P7 barcodes. The DNA panel and antibody tag libraries have different P7 primers, which are listed in the attachment "Supplementary Table 6: Library preparation primer sequences".

35 Amplify the DNA panel and antibody tag libraries according to the following protocol, using **10 cycles** for the DNA panel and **20 cycles** for the antibody tags.

Step	Temperature	Time	Cycle
1	95 °C	3 min	
2	98 °C	20 sec	**10** (DNA) or **20** (Ab tags)
3	62 °C	20 sec	
4	72 °C	45 sec	
5	72 °C	2 min	
6	4 °C	HOLD	

Library preparation PCR protocol.

Library cleanup and quantification

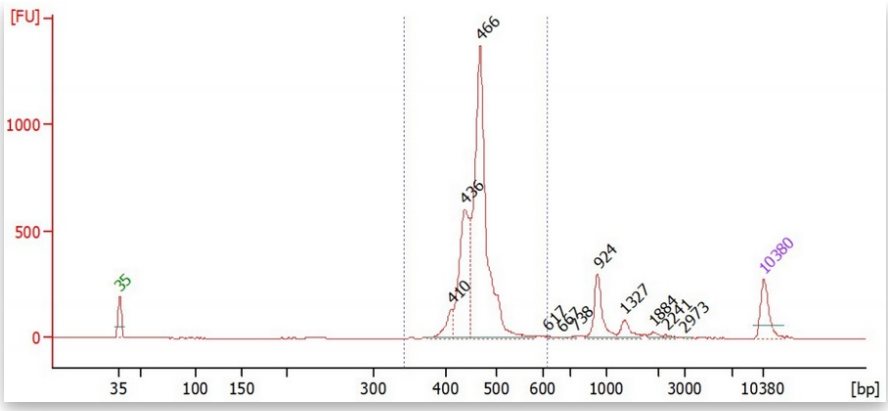
36 Perform library cleanup for both the DNA panel and antibody tag libraries as described in the attached Mission Bio document (Steps 7.10 to 7.25): "Tapestri Single-Cell DNA AML User Guide". In Step 7.13, use **35 µl** Ampure XP beads instead of **31.5 µl** .

37 Quantify the concentration of DNA panel and antibody tag libraries using the Qubit hsDNA assay.

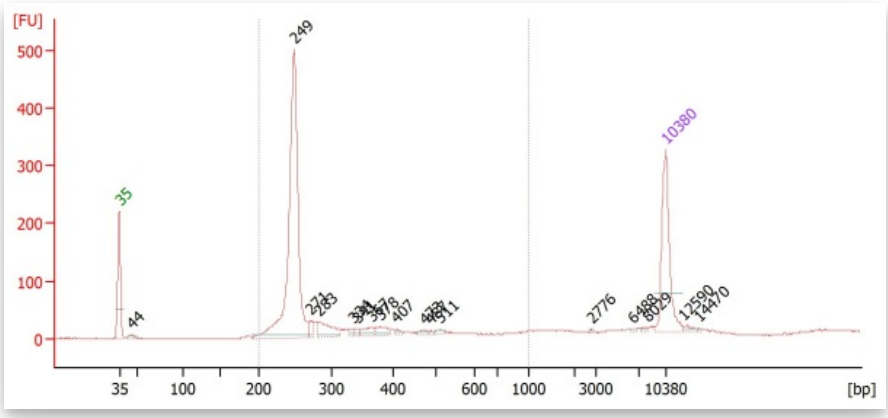
Typical concentrations are between 5 and 20 ng/uL.

38 Run **1 ng** of each library on a Bioanalyzer High-Sensitivity DNA chip or comparable TapeStation assay.

All libraries should be free of primer-dimers (<80 bp). The antibody tag library fragment should appear as a sharp peak around 250 bp. For the DNA panel libraries, the fragments should be distributed between 400 and 500 bp.



Representative DNA panel library, run on a Bioanalyzer High-Sensitivity DNA chip.



Representative antibody tag library, run on a Bioanalyzer High-Sensitivity DNA chip.

Next-generation sequencing

39 Pool the libraries for sequencing, the protocol for which varies depending on Illumina sequencing platform. It is most cost-effective to sequence the DNA panel and antibody libraries using separate kits, as the antibody tags require fewer cycles (~80 cycles for antibody tags vs. 300 for DNA panel). A custom Read 1 primer is required in both libraries (see note below).

The amount of reads to allot for each library depends on the number of cells sequenced and number of targets in the DNA and antibody panels. As a rule of thumb, allotting 100X coverage for each DNA and antibody target per cell should yield sufficient depth for genotyping and antibody tag counting.

Mission Bio V1 chemistry requires a custom Read 1 primer with the following sequence: GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAG. It should be HPLC purified.

Data processing

40 The raw FASTQ files are analyzed by the DAb-seq pipeline available at: https://github.com/AbateLab/DAb-seq. See the README for detailed instructions on setting up and running the pipeline.