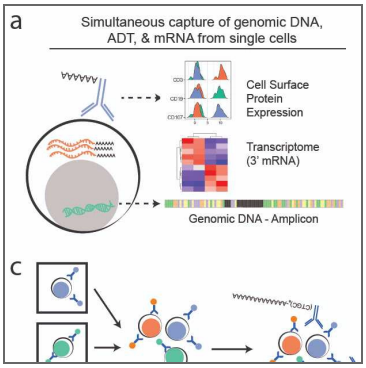

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We use this protocol and it's working

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MINECRAFTseq V1

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ABSTRACT

Genetic studies have identified thousands of individual disease-associated non-coding alleles, but identification of the causal alleles and their functions remain critical bottlenecks. Even though CRISPR-Cas editing has enabled targeted modification of DNA, inefficient editing leads to heterogeneous outcomes across individual cells, limiting the ability to detect functional consequences of disease alleles. To overcome these challenges, we present a multi-omic single cell sequencing approach that directly identifies genomic DNA edits, assays the transcriptome, and measures cell surface protein expression. We apply this approach to investigate the effects of gene disruption, deletions in regulatory regions, and non-coding single nucleotide polymorphisms.

ATTACHMENTS

ExtendedFigure2.jpg

IMAGE ATTRIBUTION

In progress

PROTOCOL integer ID: 95823

Keywords: Single cell multiomics, CRISPR editing

Lysis plate generation

1h

1 In a PCR clean hood, prepare a lysis mix containing the following reagents:

	A	B	C	D
	Reagent	Reaction Concentration	Volume (ul)	384-well plate volume (ul)
	Nuclease-free Water		0.158	60.672
	TritonX-100 (%)	0.2	0.02	7.68
	DTT(mM)	1.2	0.012	4.608
	dNTP (mM)	6	0.24	92.16
	Betaine (M)	1	0.2	76.8
	dCTP (mM)	9	0.09	34.56
	RRI	1.2	0.03	11.52
	OligoDT (uM)	2.5	0.25	96
	Total volume (ul)		1	384

Note

Each OligoDT has a unique barcode per well. This should be added separately on a per-well basis using a multi-channel or a liquid handling platform.

Seal the plates with an aluminum PCR seal and spin down for 1 minute at 1000g, 4°C.

Proceed immediately to the next step or store the plate on ice to avoid denaturing of Recombinant RNase Inhibitor.

- For long-term storage, store the plates in -20 °C .

Single cell sorting

3h

2 Sort single cells into wells containing 1 µL of the lysis buffer.

- Cells can be stained with fluorophore-conjugated and oligoconjugated antibodies. Indexing flow cytometry can be used to collect this information to hash multiple conditions.

- This can be achieved in any number of ways. We routinely use the BIGFOOT from Thermofisher for rapid and accurate sorting into 384 well plates.
- We have discovered that staining with PI or 7-AAD seems to interfere with downstream applications. We would not recommend doing this.

Immediately after sorting, seal the plate with an aluminum PCR seal and spin down for 1 minute at 1000g, 4 °C .

Place the plate containing single cells on dry ice if processing multiple plates until ready to transfer them all into a -80 °C freezer.

RNA denaturation

3 Thaw the plates containing single cells at room temperature for 1 minute.

Make sure the aluminum PCR seal is still intact. Re-seal if necessary.

Incubate the plate in a thermocycler with a heated lid for 3 minutes at 72°C, followed by a 4 °C hold step.

RT-PCR

4

Note

- The RT-PCR master mix should be prepared in a clean hood.
- It is recommended to use fresh stocks of Template Switch Oligos (TSO).
- Keeping stocks and master mixes on ice is crucial to prevent degradation.

Prepare the following RT-PCR master mix:

- These volumes account for a 15% pipette error.

	A	B	C	D
	Reagent	Reaction Concentration	Volume (ul)	384-well plate volume (ul)
	Nuclease-free Water		1.47	647.61
	TSO (uM)	1.8	0.05	19.87
	dNTP (mM)	0.3	0.15	66.24
	5X KAPA High Fidelity Buffer (X)	1	1.00	441.6
	Betaine (M)	0.8	0.80	353.28

	A	B	C	D
	DTT (mM)	4.8	0.24	105.98
	MgCl2 (mM)	9.7	0.05	21.42
	MaximaH Minus RT Enzyme (U)	2	0.05	22.08
	RRI (U)	0.8	0.10	44.16
	KAPA HOTSTART HIFI Enzyme (U)	2	0.10	44.16
	Total volume (ul)		4.00	1766.4

Add 4 µL of the master mix into each well of the 384-well plate. Seal with an aluminum PCR seal and proceed to RT incubation. This can be done with any number of liquid handling/dispensing systems. In our hands, both the Bravo and MANTIS systems have been applied to distribute master mixes.

In a thermocycler with a heated lid, start the RT step:

	A	B	C	D
	RT step	Step	Temperature (°C)	Time
		1	50	60 min
		2	85	5 min
		3	4	Hold

After the RT step, remove the plate from the thermocycler and place it on ice.

Add primers for amplification of genomic DNA and ADT:

	A	B	C	D
	Reagent	Reaction Concentration (uM)	Volume (ul)	384-well plate volume (ul)
	ADT (ul)	0.05	0.05	22.08
	Genomic Specific (ul)	0.2	0.05	22.08
	Total volume (ul)		0.10	4.00

Note

We have discovered that the following primers cannot be present in the RT step. In our hands, this step is performed with an IDOT liquid dispensing machine or MANTIS.

Seal the plate with an aluminum PCR seal and spin down for 1 minute at 1000g, 4 °C.

In a thermocycler with a heated lid, start the following PCR program:

	A	B	C	D
	Step	Temperature (°C)	Time	
	1	98	5 min	
	2	98	20 sec	Cycle for 16-22 cycles
	3	65	20 sec	
	4	72	6 min	
	5	72	5 min	
	6	4	Hold	

Note

Cycling time depends on the input populations. Cell lines are cycled from 16-20 cycles and primary cells from 20-22 cycles.

Store plates at -20 °C until continuing with nested genomic DNA, pooling, and cleanup.

Nested genomic DNA PCR

5 In a sterile hood, prepare a PCR master mix using the following reagents:

- These volumes account for a 15% pipette error.

	A	B	C	D
	Reagent	Final Concentration	Volume (ul)	384-well plate volume (ul)
	Water		1.95000	865.605
	dNTP (mM)	0.3	0.15000	66.585
	5X KAPA High Fidelity Buffer (X)	1	1.00000	443.9
	KAPA HOTSTART HIFI Enzyme (U)	2	0.10000	44.39
	Genomic Capture/p7 (Blocked) specific primer	0.4	0.40000	177.56
	Total volume (ul)		3.60000	1598.04

Add 3.6 µL of the master mix to each well in a clean 384-well plate.

Seal the plate with an aluminum PCR seal and spin down for 1 minute at 1000g, 4 °C.

Add 0.4 µL of barcoded capture primers to each well.

Note

Capture primers are used to identify each well, similar to the barcoded oligoDT. These primers bind a common sequence on the inner genomic DNA primers and can be used in any experiment. See the diagram and paper for a visual description.
- We have done this step using the Bravo Liquid Handling Platform.

Thaw the pre-amplified RT-PCR plate from -20 °C. Making sure that the aluminum seal is still intact, spin down for 1 minute at 1000g, 4 °C.

Aliquot 1 µL of the pre-amplified RT-PCR plate into the 384-well plate containing the master mix to perform the nested PCR step.

Note

- It is crucial to keep careful track of the plate orientation, otherwise, the analysis will be difficult. For example, make sure to transfer the contents of the RT-PCR plate well # A1 to the plate containing the master mix at well # A1.
- We have done this step using the Bravo Liquid Handling Platform.

Store the pre-amplified RT-PCR plate at -20 °C as it will be used in **step 7 (cDNA Library Preparation)**.

Seal the plate containing the master mix with an aluminum PCR seal and proceed to the PCR step:

	A	B	C	D
	Step	Temperature (°C)	Time	
	1	98	5 min	
	2	98	20 sec	20 Cycles
	3	65	20 sec	
	4	72	30 sec	
	5	72	5 min	
	6	10	Hold	

After the PCR step, proceed to pool the samples and SPRI clean of genomic DNA or store at -20 °C.

Genomic DNA Library Preparation

6

Note

The following contains steps for genomic DNA pooling, SPRI clean-up, ExoI and ExoVII nuclease treatment, re-SPRI, P5-P7 amplification reaction, and the final SPRI purification in preparation for sequencing.

DNA Pooling:

- If the plate was stored at -20°C, thaw for 1 minute.
- After making sure that the aluminum seal is still intact, spin down for 1 minute at 1000g, 4°C.
- Pool 2ul of the product from each well into a clean 1.5ml tube. This can be performed on a Bravo liquid handling platform.

SPRI clean-up:

From the pool, take 315ul for SPRI clean-up and store the rest at -20°C. SPRI clean at 1.2X.

- To 315ul of product, add 378ul of room temperature SPRI.
- Incubate at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 40ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 5 minutes.
- While keeping the tube on the magnet, transfer 37ul of the supernatant to a new tube. This is the gDNA amplicon.

Measure DNA concentration on a QuBit. Proceed to ExoI and ExoVII treatments and P5-P7 amplification reaction.

- There is often no point in checking distribution at this point because the product might not be detectable.

ExoVII treatment:

- This step is done to remove the excess capture primers and OligoDT.
- Dilute the gDNA amplicons to 2ng/ul in Nuclease-free water.
- Use 10ul of the 2ng/ul gDNA amplicon to perform ExoI nuclease reactions in PCR plates.
- Prepare a master mix containing the following reagents: **SOMEONE DOUBLECHECK THESE CALCULATIONS AND REAGENTS**

	A	B
	Reagent	Volume per reaction (ul)

	A	B
	ExoVII 5X buffer	4
	ExoVII	1
	Water	5
	Total volume (ul)	10

- Seal the plate containing the master mix with an aluminum PCR seal and follow the following steps using a thermocycler:

	A	B	C
	Step	Temperature (°C)	Time
	1	37	30
	2	95	10
	3	4	Hold

re-SPRI:

This step is done to remove the ExoVII nuclease. Take 20ul for SPRI clean-up and store the rest at -20°C. SPRI clean at 1.2X.

- To 20ul of product, add 24ul of room temperature SPRI.
- Incubate at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 11ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 5 minutes.
- While keeping the tube on the magnet, transfer 10ul of the supernatant to a new plate for immediate P5-P7 amplification reaction.

P5-P7 Amplification:

Prepare the following master mix for the P5-P7 amplification reaction:

- To 10ul of gDNA amplicon, add 10ul of the master mix **without the P5 and P7 primers.**
- Add 2.5ul of the P5 primer and 2.5ul of the unique P7 primer and mix.

	A	B	C
	Reagent	Final Concentration	Volume (ul)
	gDNA amplicon	2ng/ul	10
	DNA P5 Primer	2uM	2.5
	Unique HPLC DNA P7 Primer	2uM	2.5

	A	B	C
	5X Q5 Buffer	5X (NEB reagent)	5
	dNTP	10uM	1
	Q5 HotStart Enzyme		0.5
	Nuclease-free water	NA	3.5
	Total volume (ul)		25

Note

- Keep track of the libraries and barcodes in an Excel sheet.
- Keep careful track of the barcodes used for the DNA P7 primers.

Seal the plate with an aluminum PCR seal and proceed to the amplification reaction in a thermocycler:

	A	B	C	D
	Step	Temperature (°C)	Time	
	1	98	3 min	
	2	98	15 sec	16 cycles
	3	65	20 sec	
	4	72	45 sec	
	5	72	10 min	
	6	4	Hold	

Pool the products from the amplification reaction and proceed to the last DNA SPRI clean-up step. **HOW MUCH TO POOL**

Final SPRI clean-up:
SPRI clean at 1.2X.
Example:

- To 25ul of product, add 30ul of room temperature SPRI.
- Incubate at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 10ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 2 minutes.
- Save the eluent - this is the **DNA P5/P7**.

Spec on a Qubit. Proceed to check on a Tape Station. **(EXAMPLE PHOTO HERE)**

Proceed to sequencing.

Note

Sequencing guidelines:
- The DNA products are designed to be sequenced on a MiSEQ using 300 cycles.
- Read 2 will often contain the information needed to identify the CRISPR-induced mutation
- Make sure to request a high phiX spikein given that all reads will be similar. Read 1 will contain the well barcode.

cDNA Library Preparation

7

Note

The following contains steps for cDNA pooling, SPRI clean-up, ExoI nuclease treatment, re-SPRI, Nextera tagmentation, P5-P7 amplification reaction, and the final SPRI purification in preparation for sequencing.

cDNA Pooling:
- Thaw the pre-amplified RT-PCR plate from -20°C for 1 minute at room temperature.
- Making sure that the aluminum seal is still intact, spin down for 1 minute at 1000g, 4°C.
- Pool 2ul of the product from each well into a clean 1.5ml tube. This can be performed on a Bravo liquid handling platform.
- Label the tube as **RT-PCR.**

SPRI clean at 0.6X:
- To 315ul of product, add 190ul of room temperature SPRI.
- Incube at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, save 300ul of the supernatant into a new tube (which will be used in **step 8 ADT Library Preparation**).
- Discard the rest of the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made Ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.

- Take the tube off the magnet and resuspend in 21ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 5 minutes.
- While keeping the tube on the magnet, transfer 20ul of the supernatant to a new 96-well plate. This is the full-length cDNA (which can be labeled as **cDNA**).

Spec on a QuBit. Check the distribution of cDNA libraries on DS5000 tape, one plate per primer. Banding is ok. **(WE SHOULD INCLUDE AN EXAMPLE PHOTO HERE)**

ExoI treatment:

- To 18ul of the cDNA sample, add 2ul of ExoI and 5ul of Q5 (5X buffer).
- Mix and incubate in a thermocycler:

	A	B	C
	Step	Temperature (°C)	Time
	1	37	5 min
	2	85	5 min
	3	4	Hold

Note

Since ExoI is thermolabile, there is no need to re-SPRI. However, we have found that the first SPRI is not as efficient in size exclusion (ideally, <500bp). So we recommend doing a second SPRI after the ExoI treatment.

re-SPRI:

Transfer to a 1.5ml tube and proceed to re-SPRI at 0.6X:

- To 25ul of product, add 15ul of room temperature SPRI.
- Incube at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, save 300ul of the supernatant into a new tube. Discard the rest.
- While keeping the tube on the magnet, wash twice with 150ul of 80% freshly made Ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 21ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 5 minutes.
- While keeping the tube on the magnet, transfer 20ul of the supernatant to a new tube. This is the full-length cDNA (which can be labeled as **cDNA**).

Proceed to tagmentation and P5-P7 amplification reaction.

Nextera Tagmentation:

Note

- Keep track of the libraries and barcodes in an Excel sheet.
- Keep careful track of the barcodes used for the ADT P7 primers.

- Dilute the full-length cDNA to 2ng/ul in Nuclease-free water.
- In a 96-well PCR plate, add 5ul of Illumina Tagment DNA TDE1 buffer.
- Add 2.5ul of the 2ng/ul cDNA.
- Pipette to mix.
- Add 2.5ul of Illumina Amplicon Tagment Mix.
- Pipette to mix. Seal with aluminum PCR seal. Spin down for 1 minute at 1000g, 4°C.
- Run the following program in a thermocycler:

	A	B	C
	Step	Temperature (°C)	Time
	1	55	5 min
	2	10	Hold

- Immediately when the reaction stabilizes at 10°C, add 2.5ul of Illumina Neutralize Tagment buffer.
- Pipette to mix. Seal with aluminum PCR seal. Spin down for 1 minute at 1000g, 4°C.
- Incubate for 7 minutes at room temperature to deactivate the Tn5.
- Meanwhile, prepare the following master mix:

	A	B	C
	Reagent	Final Concentration	Volume (ul)
	RNA P5 Primer	2uM	2.5
	Nextera PCR mix		7.5
	Total volume (ul)		10

P5-P7 Amplification:

- When the incubation is done, add 2.5ul of unique RNA P7 primer.
- Then, add 10ul of the master mix.
- Seal with aluminum PCR seal and proceed to the amplification step in a thermocycler:

	A	B	C	D
	Step	Temperature (°C)	Time	
	1	72	3 min	
	2	95	30 sec	16 cycles
	3	95	10 sec	
	4	55	30 sec	

	A	B	C	D
	5	72	5 min	
	6	10	Hold	

Pool the products from the amplification reaction and proceed to the last cDNA SPRI clean-up step. **HOW MUCH TO POOL**

Final SPRI clean-up:
SPRI clean at 0.8X.
Example:

- To 25ul of product, add 20ul of room temperature SPRI.
- Incubate at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 10ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 2 minutes.
- Save the eluent - this is the **RNA P5/P7**.

Spec on a Qubit. Proceed to check on a Tape Station. **(EXAMPLE PHOTO HERE)**

Proceed to sequencing.

Note

Sequencing guidelines:

- The RNA products are designed to be sequenced on a NEXT or NOVAseq.
- Read 1 must be 26 cycles.
- Read 2 should be as long as possible.

ADT Library Preparation

8

Note

The following contains steps for ADT SPRI clean-up, ExoI and ExoVII nuclease treatment, re-SPRI, P5-P7 amplification reaction, and the final SPRI purification in preparation for sequencing.

SPRI clean at 1.4X:

- To 300ul of product, add 420ul of room temperature SPRI.
- Incube at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made Ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 40ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 5 minutes.
- While keeping the tube on the magnet, transfer 37ul of the supernatant to a new tube. This is the ADT (which can be labeled as **ADT**).

Spec on a QuBit. Proceed to ExoI and ExoVII treatments and P5-P7 amplification reaction.

- There is often no point in checking distribution at this point because the product might not be detectable.

ExoI treatment: DOUBLECHECK REAGENTS (IS IT JUST EXOI AND WATER + SAMPLE)

- This step is done to remove the excess capture primers and OligoDT.
- Dilute the ADT sample to 2ng/ul in Nuclease-free water.
- Use 10ul of the 2ng/ul ADT sample to perform Exo nuclease reactions in PCR plates.
- Prepare a master mix containing the following reagents: **SOMEONE DOUBLECHECK THESE CALCULATIONS**

	A	B
	Reagent	Volume per reaction (ul)
	ExoI	4
	Water	6
	Total volume (ul)	10

Seal the plate containing the master mix with an aluminum PCR seal and follow the following program using a thermocycler:

	A	B	C
	Step	Temperature (°C)	Time
	1	37	30
	2	95	10
	3	4	Hold

Proceed to P5-P7 amplification. There is no need to SPRI after the ExoI reaction as the ExoI nuclease is thermolabile.

P5-P7 Amplification:

Prepare the following master mix for the P5-P7 amplification reaction:

- To 10ul of the sample containing ADT, add 10ul of the master mix **without the P5 and P7 primers.**
- Add 2.5ul of the P5 primer and 2.5ul of the unique P7 primer and mix.

	A	B	C
	Reagent	Final Concentration	Volume (ul)
	ADT sample	2ng/ul	10
	ADT P5 Primer	2uM	2.5
	Unique HPLC ADT P7 Primer	2uM	2.5
	5X Q5 Buffer	5X (NEB reagent)	5
	dNTP	10uM	1
	Q5 HotStart Enzyme		0.5
	Nuclease-free water	NA	3.5
	Total volume (ul)		25

Note

- Keep track of the libraries and barcodes in an Excel sheet.
- Keep careful track of the barcodes used for the ADT P7 primers.

Seal the plate with an aluminum PCR seal and proceed to the amplification reaction in a thermocycler:

	A	B	C	D
	A	B	C	D
	Step	Temperature (°C)	Time	
	1	98	3 min	
	2	98	15 sec	
	3	65	20 sec	
	4	72	45 sec	
	5	72	10 min	
	6	4	Hold	

Pool the products from the amplification reaction and proceed to the last ADT SPRI clean-up step. **HOW MUCH TO POOL**

Final SPRI clean-up:

SPRI clean at 1.6X.
Example:

- To 25ul of product, add 40ul of room temperature SPRI.
- Incubate at room temperature for 10 minutes.
- Place the tube on a magnet for 5 minutes.
- While keeping the tube on the magnet, discard the supernatant.
- While keeping the tube on the magnet, wash twice with 80% freshly made ethanol. Wait for 30 seconds. Remove ethanol carefully without touching the product.
- While keeping the tube on the magnet, dry the tube for 10-15 minutes.
- Take the tube off the magnet and resuspend in 10ul of Nuclease-free water. Incubate for 2-5 minutes.
- Place the tube on the magnet for 2 minutes.
- Save the eluent - this is the **ADT P5/P7**.

Spec on a Qubit. Proceed to check on a Tape Station. **(EXAMPLE PHOTO HERE)**

Proceed to sequencing.

Note

Sequencing guidelines:

- The ADT products are designed to be sequenced with the RNA libraries at ~ 1-5% of the final library.
- Read 1 must be at least 26 cycles and read 2 should at least be 15 cycles.

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