



Apr 02, 2025

Version 1

MINECRAFTseq V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.dm6gpz8ojlzp/v1>

Yuriy Baglaenko¹

¹University of Cincinnati Department of Pediatrics



Majd Al Suqri

Brigham and Women's Hospital

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.dm6gpz8ojlzp/v1>

Protocol Citation: Yuriy Baglaenko 2025. MINECRAFTseq. protocols.io

<https://dx.doi.org/10.17504/protocols.io.dm6gpz8ojlzp/v1>

Manuscript citation:

Baglaenko, Yuriy, et al. "Defining the Function of Disease Variants with CRISPR Editing and Multimodal Single Cell Sequencing." *bioRxiv*, 2024, <https://doi.org/10.1101/2024.03.28.587175>.

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working.

Created: April 01, 2024

Last Modified: April 02, 2025

Protocol Integer ID: 97613

Keywords: MINECRAFTseq, functional variant, eQTL, base editing, multi-omic , multimodal functional genomic single cell assay, minecraftseq genetic study, genomic dna edit, functional consequences of disease allele, disease allele, heterogeneous outcomes across individual cell, specific effects of individual snp, gene disruption, causal variation in primary human cell, autoimmune variant, thousands of individual disease, cell surface protein expression, identification of the causal allele, crispr, modification of dna, individual snp, effects of gene disruption, causal allele, individual cell, understanding of complex human disease, complex human disease, primary human cell, targeted modification, cell, dna, sequencing approach, deletions in regulatory region, individual disease, genomic dna

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. We identify the specific effects of individual SNPs, including the state-specific effects of an IL2RA autoimmune variant in primary human T cells. Multimodal functional genomic single cell assays including DNA sequencing bridge a crucial gap in our understanding of complex human diseases by directly identifying causal variation in primary human cells.

Attachments



[steps.png](#)

270KB

Materials

REAGENTS and EQUIPMENTS

Some key plastic labware items and consumables

Tips, 30uL, sterile filter Bravo (Agilent, cat.no. AGI-30.F)

Tips, 70uL, sterile filter Bravo (Agilent, cat.no. AGI-70.F)

Eppendorf PCR plate 384-wells (Eppendorf, cat.no. 951020711)

Flow tubes with cell strainer snap cap (Corning, cat.no. 352235)

Qubit 1X dsDNA high sensitivity (HS) assay kit (Fisher Scientific, cat.no. Q33231)

Qubit assay tubes (ThermoFisher Scientific, cat.no. Q32856)

RNaseZap RNase Decontamination Wipes (ThermoFisher Scientific, cat.no. AM9786)

Agilent 4200 TapeStation loading tips (Agilent, cat.no. 5067-5599)

D1000 ScreenTape (Agilent, cat.no. 5067-5582) with D1000 Reagents (Agilent, cat.no. 5067-5583)

D5000 ScreenTape (Agilent, cat.no. 5067-5588) with D5000 Reagents (Agilent, cat.no. 5067-5589)

MINECRAFTseq

All primers, adapters, and oligonucleotides (IDT or Genewiz, various)

DNase/RNase-free water (ThermoFisher Scientific, cat.no. 10977015)

Triton X-100 solution (Sigma-Aldrich, cat.no. 9036-19-5)

dCTP solution 100mM (ThermoFisher Scientific, cat.no. R0151)

dNTP (ThermoFisher Scientific, cat.no. R0182)

KAPA HiFi HotStart plus dNTPs 100U (Roche, cat.no. 07958889001)

Betaine 5M solution (MilliporeSigma, cat.no. B0300-5VL)

USB dithiothreitol (DTT) 0.1M solution (ThermoFisher Scientific, cat.no. 707265ML)

MgCl₂ 1M (ThermoFisher Scientific, cat.no. AM9530G)

Maxima reverse transcriptase (200U/μL) (ThermoFisher Scientific, cat.no. EP0743)

Recombinant RNase inhibitor (RRI) (Takara, cat.no. 2313B)

CleanNGS 500mL (CleanNA, cat.no. CNGS-0500)

Thermolabile exonuclease I (NEB, cat.no. M0568L)

Exonuclease VII (NEB, cat.no. M0379L)

Q5 HotStart high-fidelity DNA polymerase (NEB, cat.no. M0493L)

Nextera XT DNA library prep kit (24 samples) (Illumina, cat.no. FC-131-1024)

Liquid handlers and dispensers

To accurately and quickly dispense small volumes into multiple 384-well plates, we utilized advanced liquid handlers and dispensers, such as the BRAVO, I.DOT, and MANTIS. These automated systems offer precision and consistency, which are critical for minimizing variability during library preparation that requires precise reagent handling. The BRAVO platform allowed for flexible liquid handling across different formats, while the I.DOT system enabled ultra-low volume dispensing with minimal waste, making it ideal for applications requiring very small volumes. The MANTIS dispenser provided high-throughput capability with the added benefits of rapid dispensing

times and reduced setup complexity. We followed the manufacturer's guidelines for configuring each system, ensuring that the workflows were fully integrated into our experimental setups.

Before start

The reaction components listed in the provided tables are specifically optimized for a 384-well plate format. We recommend adding an additional 12-15% when preparing the final master mix volumes. When preparing the reaction mixtures, it is highly recommended to work on ice to maintain reagent stability. We also used liquid handlers and dispensers (BRAVO, MANTIS, and I.DOT) to accurately measure out reagents.

Lysis plate generation

2h

- 1
 - A cell lysis reaction mixture, excluding the unique OligoDT barcodes, was prepared according to the setup in Table 1, in a clean and RNase-free environment.
 - Add 0.75uL of the lysis mixture to each well of a new 384-well plate.
 - When preparing multiple plates, first aliquot a larger volume of the lysis reaction mixture into one plate using a multichannel pipette.
 - OligoDT barcodes are stored in a plate format. After thawing on ice, briefly spin the plate for 1 minute at 1000×g at 4°C.
 - Add 0.25uL of OligoDT to each well of the plates already containing the lysis mixture. Each well will have a unique OligoDT barcode.
 - If preparing multiple plates, add the appropriate OligoDT volume to the plate containing the large volume of lysis reaction mixture. Then distribute the combined mixture evenly across the new additional plates using BRAVO system.
 - Ensure that each well in the lysis plates contains a total volume of 1uL.

	A	B	C	D
	Reagent	Start Conc	Final Conc	Vol per well (uL)
	Water	-	-	0.158
	TritonX-100 (%)	10	0.2	0.020
	DTT (mM)	100	1.2	0.012
	dNTP (mM)	25	6	0.240
	Betaine (M)	5	1	0.200
	dCTP (mM)	100	9	0.090
	RRI (U/uL)	40	1.2	0.030
	OligoDT (uM)	10	2.5	0.250

Table 1: Concentration (conc) and volume (vol) of each reagent used to setup the lysis plates.

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. We recommend preparing lysis plates a few hours before sorting or the day before and sorting immediately afterward. Prior to sorting, store lysis plates at -20°C for no longer than 24 hours.

- The plates were sealed and spin down for 1 minute at 1000xg at 4°C. Proceed immediately to the next step, sorting cells.

Single cell sorting

3h

- 2
 - Prior to sorting cells were hashed and/or stained with appropriate antibodies.
 - Cells were then sorted into lysis plates using the Bigfoot Spectral cell sorter with standard compensation.
 - Immediately after sorting, the plates were spun at 1000xg for 1 minute, flash-frozen on dry ice, and stored in -80°C until further library processing.



Note

When sorting, keep the cells and plates at 4°C. We recommend using a sorter that maintains this temperature. Lysis plates containing cells can be stored at -80°C for up to one month.

Reverse transcription (RT) and initial genomic DNA (gDNA) amplification PCR reaction

4h

- 3
 - A reaction master mix without the ADT additive or genomic DNA primers was setup using Table 2 below in a clean and RNase-free environment.
 - Lysis plates with cells are quickly incubated at 72°C for 3 minutes, followed by a 4°C hold step. Then plates were briefly spin at 1000xg at 4°C.



Note

It is recommended to use fresh stocks of TSO from -80°C, stored as aliquots. Keeping stocks and master mixes on ice is crucial to prevent degradation. Additionally, since mRNA degrades quickly, it is highly recommended to keep everything on ice and move as quickly as possible. For all PCR and incubation steps, we used a thermocycler with a heated lid.

	A	B	C	D
	Reagent	Start Conc	Final Conc	Vol per well (uL)
	Water	-	-	1.47
	TSO (uM)	200	1.8	0.05
	dNTP (mM)	10	0.3	0.15

A	B	C	D
5X KAPA High Fidelity Buffer (X) with Mg	5	1	1.00
Betaine (M)	5	0.8	0.80
DTT (mM)	100	4.8	0.24
MgCl ₂ (mM)	1000	9.7	0.05
MaximaH Minus RT Enzyme (U/uL)	200	2	0.05
RRI (U/uL)	40	0.8	0.10
KAPA HotStart HiFi Enzyme (U/uL)	1	0.02	0.10
Primers to add after RT reaction step			
ADT additive primers (uM)	5	0.05	0.05
Specific genomic DNA primers (uM)	20	0.2	0.05

Table 2: RT and initial genomic DNA amplification reaction components.

- Add 4uL of the master mix into each well of the 384-well lysis plates with cells. Here we used the BRAVO or MANTIS systems to distribute the master mix.
- Seal plate and using a thermocycler proceed to RT step following the program described in Table 2.1.

A	B	C	D	E
RT Step	Step	Temp	Time	
	1	50°C	1hour	
	2	85°C	5min	
	3	4°C	hold	
PCR Step				
	1	98°C	5min	
	2	98°C	20sec	21 cycles
	3	65°C	20sec	
	4	72°C	6min	
	5	72°C	5min	

	A	B	C	D	E
		6	4°C	hold	

Table 2.1: PCR cycler setup for Table 2 reaction components.

- After the RT step, remove the plate from the thermocycler and briefly spin down at 1000xg at 4°C.
- Add primers for amplification of genomic DNA and ADT using Table 2 above. Here we used I.DOT to add the primers. Seal the plate and spin down for 1 minute at 1000xg at 4°C.
- In a thermocycler start the PCR step program from Table 2.1. After the PCR step this gives you the RT-PCR plate.
- Store plates at -20°C until continuing with nested genomic DNA, pooling, and cleanup.

Note

Additional primers to perform the nested gDNA during the RT-PCR steps were previously tested and resulted in gDNA loss. We highly recommend taking an aliquot of the RT-PCR product to conduct the nested gDNA amplification as indicated in the step below.

Nested gDNA amplification PCR reaction

3h

- 4
 - Prepare a reaction master mix without the capture primers, following the setup in Table 3, in a clean environment.
 - Add 3.2uL of this mixture to each well of a new 384-well plate.
 - When preparing multiple plates, first aliquot a larger volume of the master mix into one plate using a multichannel pipette.
 - The unique capture primers are stored in plate format. After thawing on ice, briefly spin down the plate for 1 minute at 1000xg at 4°C.
 - Add 0.8uL of the capture primers to each well of the plates already containing the master mix. Each well will have a unique capture primer.



A	B	C	D
Reagent	Start Conc	Final Conc	Vol per well (uL)
Water	-	-	1.55
dNTP (mM)	10	0.3	0.15
5X KAPA High Fidelity Buffer (X) with Mg	5	1	1.00

	A	B	C	D
	KAPA HotStart HiFi Enzyme (U/uL)	1	0.02	0.10
	Genomic nested capture/P7 primers (uM)	5	0.4	0.40
	Unique capture barcode primer (Unblocked) (uM)	2.5	0.4	0.80

Table 3: Nested gDNA amplification reaction components.

- If preparing multiple plates, add the appropriate volume of capture primers to the plate containing the large volume of master mix, ensuring that each well has unique capture primers. Distribute the combined mixture evenly across the new additional plates using the BRAVO system.
- Ensure that each well in the plates contains a total volume of 4uL.

Note

Capture primers are used to identify each well, with cells uniquely labeled. These primers bind to a common sequence on the inner genomic DNA primers and are versatile for use in various experiments. Refer to the diagram and the accompanying paper for a visual description.

- Thaw the RT-PCR plate from -20 °C, from the previous step on ice and spin down for 1 minute at 1000xg at 4°C.
- Aliquot 1uL of the RT-PCR product into the 384-well plate containing the master mix with the capture primers. Here we used BRAVO to add the RT-PCR product.
- Seal the plate and proceed to the PCR step outlined in Table 3.1. After the PCR step this gives you the gDNA plate.

Note

It is essential to carefully track the plate orientation to avoid complications during analysis. For instance, ensure that the contents of well A1 from the RT-PCR plate are accurately transferred to well A1 of the plate containing the master mix.

	A	B	C	D
	Step	Temp	Time	
	1	98°C	5min	
	2	98°C	20sec	20 cycle s
	3	65°C	20sec	

	A	B	C	D
	4	72°C	30sec	
	5	72°C	5min	
	6	4°C	hold	

Table 3.1: PCR cycler setup for Table 3 reaction.

- Store the RT-PCR plate back at -20°C, as it will be used in the subsequent steps for cDNA and ADT library preparations.
- After the PCR step, proceed to genomic DNA library preparation or store the gDNA plate at -20°C.

gDNA library preparation

3h

5 Pooling and 1st SPRI clean-up at 1.2X



- If the gDNA plate was stored at -20°C, thaw it on ice and centrifuge for 1 minute at 1000xg at 4°C.
- Pool 2uL of product from each well (this step can be performed using a BRAVO system).
- Take 315uL from the pooled product and add 378uL of room temperature SPRI beads. Store the remaining pooled product at -20°C.
- Incubate at room temperature for 10 minutes, then place the tube on a magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for 5–10 minutes.
- Remove the beads from the magnet and resuspend in 40uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place the tube on the magnet for 5 minutes. While on the magnet, carefully transfer 37uL of the supernatant to a new tube. This contains the gDNA amplicon product.
- Measure gDNA concentration on a QuBit. Proceed to ExoI/VII treatment (to remove excess primers) and P5-P7 amplification.

Note

Allow the SPRI beads to equilibrate to room temperature for at least 30 minutes; the exact time may vary depending on the volume. When washing the SPRI beads bound to the product, ensure that fresh ethanol is prepared and avoid allowing the beads to dry out completely. There is often no point in checking distribution gDNA product at this point because the product might not be detectable.

Exonuclease treatment and 2nd SPRI clean-up at 1.2X



- Using Table 4, set up the Exo/VII reaction with 10uL of 2ng/uL gDNA amplicon.
- Ensure that the exonuclease reaction has total volume of 20uL.

A	B	C	D
Reagent	Start Conc	Final Conc	Vol (uL)
ExoVII buffer (X)	5	1	4
ExoVII (U/uL)	10	0.5	1
Thermolabile ExoI (U/uL)	20	4	4
Water	-	-	1

Table 4: gDNA exonuclease reaction setup.

- Incubate the gDNA exonuclease reaction according to the program outlined in Table 4.1.

A	B	C
Step	Temp	Time
1	37°C	30min
2	85°C	5min
3	4°C	hold

Table 4.1: Exonuclease incubation setup for Table 4 reaction.

- After incubation, take 20uL of the product for SPRI clean-up and store the remainder at -20°C.
- Add 24uL of room temperature SPRI beads to the product and let it sit at room temperature for 10 minutes.
- Place on magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for about 2 minutes.
- Remove the beads from the magnet and resuspend in 11uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place back on magnet and transfer 10uL of the supernatant containing the gDNA product for P5-P7 amplification in the next step.

P5-P7 amplification and final SPRI clean-up at 1.2X

- To 10uL of the gDNA amplicon product (post-exonuclease and 2nd SPRI clean-up), add 10uL of P5-P7 reaction mix, including 2.5uL each of the unique DNA P7 adapter and the common DNA P5 adapter, as outlined in Table 5.

	A	B	C	D
	Reagent	Start Conc	Final Conc	Vol (uL)
	Sample (post exo/2nd SPRI)	-	-	10
	DNA P7 Unique (uM)	2	0.2	2.5
	DNA P5 Common (uM)	2	0.2	2.5
	Q5 buffer (X)	5	1	5
	dNTP (mM)	10	0.4	1
	Q5 HotStart (U/uL)	2	0.04	0.5
	Water	-	-	3.5
	Total volume (uL)	-	-	25

Table 5: gDNA P5-P7 reaction setup.

- Ensure that the P5-P7 reaction has total volume of 25uL.

Note

Each gDNA plate will use a unique DNA P7 adapter along with a common DNA P5 adapter. Carefully track the libraries and barcodes used to ensure proper identification and organization when pooling plates for the final libraries.

- Proceed to the PCR step outlined in Table 5.1 for P5-P7 gDNA library amplification. This step produces the gDNA P5-P7 product.

	A	B	C	D
	Step	Temp	Time	
	1	98°C	3min	
	2	98°C	15sec	16 cycles
	3	65°C	20sec	

	A	B	C	D
	4	72°C	45sec	
	5	72°C	10min	
	6	4°C	hold	

Table 5.1: PCR cycler setup for Table 5 reaction.

- Pool 5uL of gDNA P5-P7 product per plate prior to SPRI clean-up.
- To 25uL of product (i.e. pool volume from 5 gDNA plates) add 30uL of room temperature SPRI beads and let it sit at room temperature for 10 minutes.
- Place on magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for about 2 minutes.
- Remove the beads from the magnet and resuspend in 12uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place back on magnet and transfer 12–10uL of the supernatant containing the final gDNA library.
- Measure the concentration using a Qubit and evaluate library quality using a TapeStation or Bioanalyzer. Refer to Diagram 1 for an example of gDNA final library quality.
- Proceed to sequencing gDNA library!

Note

The gDNA fragment sizes depend on the amplicon size of the targeted locus or region of interest. The concentration should be greater than 10ng/uL. If the concentration is lower, check the P5-P7 concentrations before SPRI clean-up, as there may not have been sufficient amplification. Properly label and store the libraries at -20°C until they are submitted for sequencing.

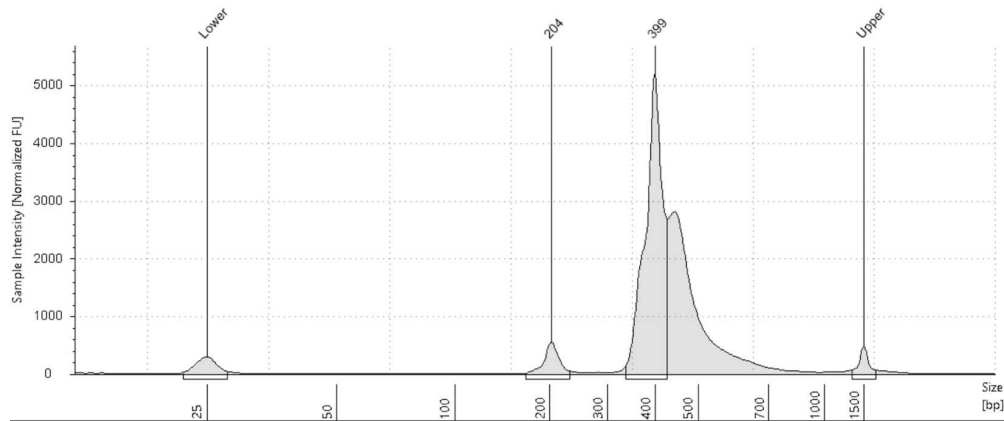


Diagram 1: Example of a final gDNA library. The library size depends on the amplicon size, and ideally, primer and adapter dimers should be minimal.

cDNA library preparation

2h

6 Pooling and 1st SPRI clean-up at 0.6X



- Thaw the RT-PCR plate on ice and centrifuge for 1 minute at 1000xg at 4°C.
- Pool 2uL of product from each well (this step can be performed using a BRAVO system).
- Take 315uL from the pooled product and add 190uL of room temperature SPRI beads. Store the remaining pooled product at -20°C.
- Incubate at room temperature for 10 minutes, then place the tube on a magnet for 5 minutes
- While on the magnet, **save 300uL of the supernatant** (will be used for ADT library preparation step). Discard the rest of the supernatant.
- Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for 5–10 minutes.
- Remove the beads from the magnet and resuspend in 21uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place the tube on the magnet for 5 minutes. While on the magnet, carefully transfer 18–20uL of the supernatant to a new tube. This contains the full-length cDNA product.
- Proceed to ExoI treatment (to remove excess primers).

Note

Allow the SPRI beads to equilibrate to room temperature for at least 30 minutes; the exact time may vary depending on the volume. SPRI clean-up on the cDNA to select for large fragments (>400bp). During the washing step, ensure fresh ethanol is prepared, and avoid letting the beads dry out completely.

**Exonuclease treatment and 2nd SPRI clean-up at 0.6X**

- Using Table 6, set up the Exol reaction with 18uL of cDNA product.
- Ensure that the exonuclease reaction has total volume of 25uL.

	A	B	C	D
	Reagent	Start Conc	Final Conc	Vol (uL)
	Q5 buffer (X)	5	1	5
	Thermolabile Exol (U/uL)	20	1.6	2

Table 6: cDNA exonuclease reaction setup.

- Incubate the cDNA exonuclease reaction according to the program outlined in Table 6.1.

	A	B	C
	Step	Temp	Time
	1	37°C	5min
	2	85°C	5min
	3	4°C	hold

Table 6.1: Exonuclease incubation setup for Table 6 reaction.

- After incubation, take 25uL of the product for SPRI clean-up.
- Add 15uL of room temperature SPRI beads to the product and let it sit at room temperature for 10 minutes.
- Place on magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for about 2 minutes.
- Remove the beads from the magnet and resuspend in 21uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place back on magnet and transfer 20uL of the supernatant containing the cDNA product.
- Measure cDNA concentration using a Qubit and evaluate plate quality using a TapeStation or Bioanalyzer. Refer to Diagram 2 for an example of cDNA plate quality.
- Proceed to tagmentation followed by P5-P7 amplification.

Note

Since ExoI is thermolabile, 2nd SPRI clean-up is not strictly necessary. However, the 1st SPRI clean-up is often less efficient at size exclusion. Therefore, we recommend performing a second SPRI clean-up after the ExoI treatment. Additionally, because mRNA is susceptible to degradation, prior to tagmentation checking the cDNA quality in the plate is helpful for troubleshooting potential issues.

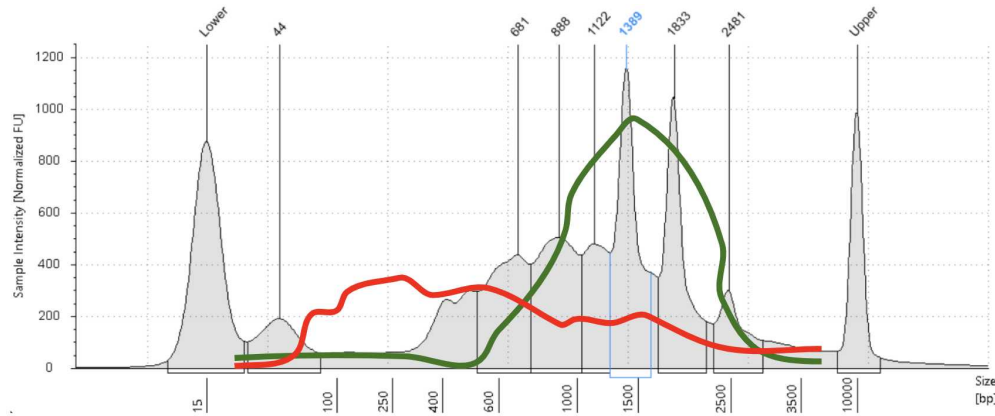


Diagram 2: Example of a cDNA plate quality after exonuclease treatment and 2nd SPRI clean-up. At this stage, there should be minimal primer dimers. An ideal plate should display full-length cDNA in the green region, while signals in the red region indicate degradation, suggesting suboptimal cDNA plate quality.

Tagmentation of full-length cDNA

- Dilute the full-length cDNA to 2ng/uL in nuclease-free water.
- Add 5uL of TD (Tagment DNA) buffer to 2.5uL of the diluted cDNA (2ng). Pipette to mix thoroughly.
- Add 2.5uL of ATM (Amplicon Tagment Mix), pipette to mix, and briefly spin down the mixture.
- Incubate the reaction at 55°C for 5 minutes, then hold at 10°C.
- Immediately upon stabilizing the reaction at 10°C, add 2.5uL of NT (Neutralize Tagment) buffer and pipette to mix thoroughly.
- Incubate at room temperature for 7 minutes, then immediately proceed to P5-P7 amplification.

Note

Too much ATM can lead to over-tagmentation, which can negatively affect the quality of your cDNA library.

P5-P7 amplification and final SPRI clean-up at 0.8X

- To the 12.5uL of cDNA (post-tagmentation), add 12.5uL of P5-P7 reaction mix, including 2.5uL each of the unique RNA P7 adapter and the common RNA P5 adapter, as outlined in Table 7.

A	B	C	D
Reagent	Start Conc	Final Conc	Vol (uL)
Sample (post tag)	-	-	12.5
RNA P7 Unique (uM)	2	0.2	2.5
RNA P5 Common (uM)	2	0.2	2.5
NPM (Nextera PCR Mix)	-	-	7.5
Total volume (uL)	-	-	25

Table 7: cDNA P5-P7 reaction setup.

- Ensure that the P5-P7 reaction has total volume of 25uL.

Note

Each cDNA plate will use a unique RNA P7 adapter along with a common RNA P5 adapter. Carefully track the libraries and barcodes used to ensure proper identification and organization when pooling plates for the final libraries.

- Proceed to the PCR step outlined in Table 7.1 for P5-P7 cDNA library amplification. This step produces the cDNA P5-P7 product.

A	B	C	D
Step	Temp	Time	
1 (gap repair)	72°C	3min	
2	95°C	30sec	
3	95°C	10sec	16 cycles
4	55°C	30sec	
5	72°C	45sec	
6	72°C	5min	

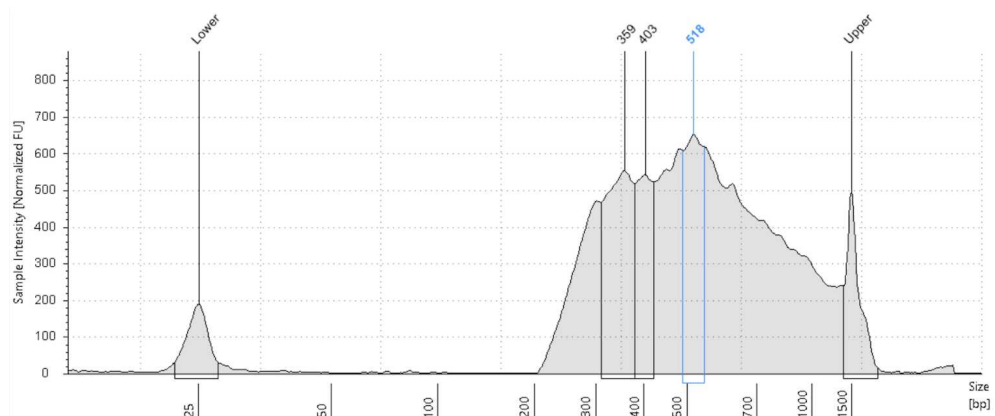
A	B	C	D
7	4°C	hold	

Table 7.1: PCR cycler setup for Table 7 reaction.

- Pool 5uL of cDNA P5-P7 product per plate prior to SPRI clean-up.
- To 25uL of product (i.e. pool volume from 5 cDNA plates) add 20uL of room temperature SPRI beads and let it sit at room temperature for 10 minutes.
- Place on magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for about 2 minutes.
- Remove the beads from the magnet and resuspend in 12uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place back on magnet and transfer 12–10uL of the supernatant containing the final cDNA library.
- Measure the concentration using a Qubit and evaluate library quality using a TapeStation or Bioanalyzer. Refer to Diagram 3 for an example of cDNA final library quality.
- Proceed to sequencing cDNA library!

Note

The cDNA library should have a uniform fragment size to ensure better sequencing coverage and reduce biases during data analysis. If the band size is too high, re-tagmentation may be necessary. The concentration should be greater than 10ng/uL. If the concentration is lower, verify the P5-P7 concentrations before SPRI clean-up, as insufficient amplification may have occurred. Properly label the libraries and store them at -20°C until they are submitted for sequencing.

**Diagram 3:** Example of a final cDNA library. An ideal cDNA library should have a size range of 300–500bp, with minimal primer and adapter dimers.

ADT library preparation

2h

7 Pooling and 1st SPRI clean-up at 1.4X



- To 300uL of product (supernatant from cDNA library preparation step), add 420uL of room temperature SPRI beads.
- Incubate at room temperature for 10 minutes, then place the tube on a magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for 5–10 minutes.
- Remove the beads from the magnet and resuspend in 40uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place the tube on the magnet for 5 minutes. While on the magnet, carefully transfer 37uL of the supernatant to a new tube. This contains the ADT product.
- Measure ADT concentration on a Qubit. Proceed to ExoI treatment (to remove excess primers) and P5-P7 amplification.

Note

Allow the SPRI beads to equilibrate to room temperature for at least 30 minutes; the exact time may vary depending on the volume. When washing the SPRI beads bound to the product, ensure that fresh ethanol is prepared and avoid allowing the beads to dry out completely.

Exonuclease treatment

- Using Table 8, set up the ExoI reaction with 18uL of 2ng/uL ADT product.
- Ensure that the exonuclease reaction has total volume of 25uL.

A	B	C	D
Reagent	Start Conc	Final Conc	Vol (uL)
Q5 buffer (X)	5	1	5
Thermolabile ExoI (U/uL)	20	1.6	2

Table 8: ADT exonuclease reaction setup.

- Incubate the ADT exonuclease reaction according to the program outlined in Table 8.1.



A	B	C
Step	Temp	Time
1	37°C	5min
2	85°C	5min
3	4°C	hold

Table 8.1: Exonuclease incubation setup for Table 8 reaction.

Note

There is no need to SPRI after the Exol reaction as the Exol nuclease is thermolabile.

P5-P7 amplification and final SPRI clean-up at 1.6X

- To the 5uL of ADT product (post-exonuclease), add 20uL of P5-P7 reaction mix, including 2.5uL each of the unique ADT P7 adapter and the common ADT P5 adapter, as outlined in Table 9.

A	B	C	D
Reagent	Start Conc	Final Conc	Vol (uL)
Sample (post exo)	-	-	5
ADT P7 Unique (uM)	2	0.2	2.5
ADT P5 Common (uM)	2	0.2	2.5
Q5 buffer (X)	5	0.8	4
dNTP (mM)	10	0.4	1
Q5 HotStart (U/uL)	2	0.04	0.5
Water	-	-	9.5
Total volume (uL)	-	-	25

Table 9: ADT P5-P7 reaction setup.

- Ensure that the P5-P7 reaction has total volume of 25uL.

Note

Each ADT plate will use a unique ADT P7 adapter along with a common ADT P5 adapter. Carefully track the libraries and barcodes used to ensure proper identification and organization when pooling plates for the final libraries.

- Proceed to the PCR step outlined in Table 9.1 for P5-P7 ADT library amplification. This step produces the ADT P5-P7 product.

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

Table 9.1: PCR cyclers setup for Table 9 reaction.

- Pool 5uL of ADT P5-P7 product per plate prior to SPRI clean-up.
- To 25uL of product (i.e. pool volume from 5 ADT plates) add 40uL of room temperature SPRI beads and let it sit at room temperature for 10 minutes.
- Place on magnet for 5 minutes.
- While on the magnet, discard the supernatant. Wash the beads twice with 80% ethanol, carefully removing the ethanol without disturbing the product. Allow the beads to air dry for about 2 minutes.
- Remove the beads from the magnet and resuspend in 12uL of nuclease-free water. Incubate at room temperature for 2–5 minutes.
- Place back on magnet and transfer 12–10uL of the supernatant containing the final ADT library.
- Measure the concentration using a Qubit and evaluate library quality using a TapeStation or Bioanalyzer. Refer to Diagram 4 for an example of ADT final library quality.
- Proceed to sequencing ADT library!

Note

The expected ADT library size is approximately 200bp. A smaller peak around 150bp may also be observed, likely corresponding to concatenated primers and/or primer dimers. While these are challenging to remove, they should not significantly interfere with the overall ADT library quality. The library concentration should be greater than 10ng/uL. If the concentration is lower, verify the P5-P7 primer concentrations before the SPRI clean-up step, as insufficient amplification may have occurred. Ensure that the libraries are properly labeled and stored at -20°C until submission for sequencing.

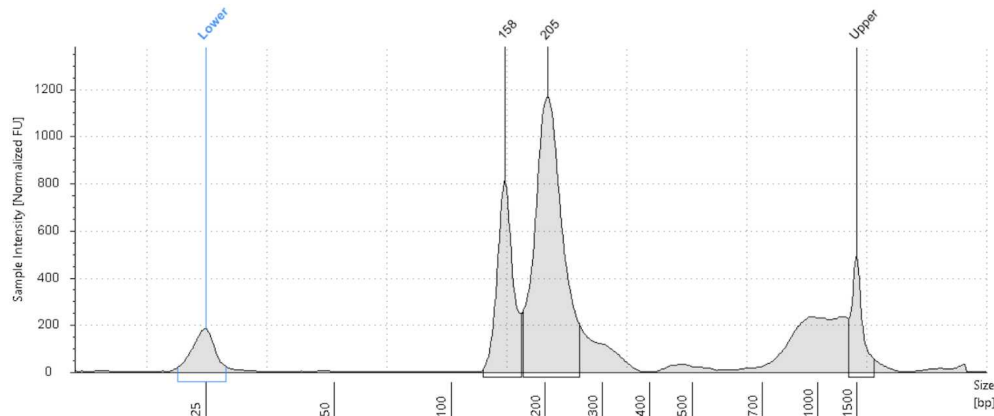


Diagram 4: Example of a final ADT library. The expected library size is approximately 200bp, with minimal primer and adapter dimers for optimal quality.

Sequencing

30m

8 gDNA

- The gDNA products are designed for sequencing on a MiSeq using a 300-cycle kit, with Read 1 set to 26 cycles and Read 2 set to 50 cycles.
- Use high PhiX spike-in of approximately 25%, as the reads are expected to be highly similar.

Note

Read 1 will contain the well barcode and Read 2 will contain the information needed to identify the CRISPR-induced mutation.

cDNA and ADT

- The cDNA and ADT products are designed to be sequenced together on a NextSeq or NovaSeq platform.
- Spike the ADT library into the cDNA library at 1–5% of the final library concentration.



- Ensure that Read 1 is at least 26 cycles and Read 2 as long as possible, a least above 15 cycles.

Congratulations you have completed MINECRAFTseq!