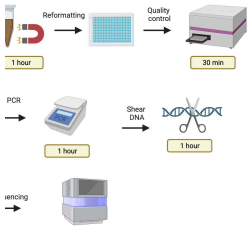
Nov 20, 2024

Minibulk v2 (modified Prime-seq)



Forked from prime-seq

DOI
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Janjic, A., Wange, L.E., Bagnoli, J.W. et al. Prime-seq, efficient and powerful bulk RNA sequencing. Genome Biol 23, 88 (2022). https://doi.org/10.1186/s13059-022-02660-8

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

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Abstract

Cost-efficient library generation by early barcoding has been central in propelling single-cell RNA sequencing. Here, we optimize and validate prime-seq, an early barcoding bulk RNA-seq method. We show that it performs equivalently to TruSeq, a standard bulk RNA-seq method, but is fourfold more cost-efficient due to almost 50-fold cheaper library costs. We conclude that prime-seq is currently one of the best options to set up an early barcoding bulk RNA-seq protocol from which many labs would profit.

WEHI advanced Genomics facility amendments from Prime-seq:
The plasticware has been changed from 96w plates to 384w and volumes have been reduced 1/4. The reverse transcription of Prime-Seq does not use RNase inhibitors, which I have included.
The primers used for full length cDNA amplification have been taken from 10x Genomics chemistry. This adds compatibility with ONT sequencing (FLT-Seq) developed by Jafar and compatibility with 10x Genomics indexing primers set TT.

Guidelines

- All reagents and plastic-ware can be found in the 'Materials' section.
- Use only RNase free supplies and clean all surfaces and tools with RNase Away prior to working
- Make sure all steps involving cell lysate and RNA before reverse transcription are carried out swiftly and on ice.
- All primer sequences are listed below:

	A	B	C	D	E	F
	Oligo	**Vendor**	**Purification**	**Working Conc.**	**Sequence**	**Notes**
	MB2_KF_DS RT primer	IDT	Standard Desalting	100 µM	ACACGACGCTCTTCCGATCTNNNNNNNNYRNNNYRNNN[10nt_BC]TTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	
	Biotin RAP-TSO	Sigma	RNase-Free HPLC	100 µM	/5Biosg/AAGCAGTGGTATCAACGCAGAGTACrGrGrG	Biotin reduces concatemers
	RAP_PCR-1_Fwd	IDT	Standard Desalting	10 µM	CTACACGACGCTCTTCCGAT*C*T	PCR1 primer
	10uM RAP-FL_Rev	IDT	Standard Desalting	10 µM	AAGCAGTGGTATCAACGCAG*A*G	PCR1 primer
	tenX_adaptTop	IDT	Standard Desalting	1.5 µM	5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCAC	Orded as pre-duplexed DNA
	tenX_adaptBot	IDT	Standard Desalting	1.5 µM	GCTCTTCCGATC*T	Orded as pre-duplexed DNA

Specific barcoded oligodT (MB2_Kx_DS_RT primer):

Base design is based off Karst et al., https://doi.org/10.1038/s41592-020-01041-y

- MB2 = protocol
- KF = Karst design forward UMI = NNNNNNNNYRNNNYRNNN
- KR = Karst design reverse UMI = NNNNNNNNRYNNNRYNNN
- Forward and reverse designs to improve complexity in Illumina sequencing
- DS = David Squire designed the well barcodes and thermodynamic properties
- RT = reverse transcription

mb_v2_384w_v1.xlsx

Materials

MATERIALS

- DNase I Reaction Buffer - 6.0 ml **New England Biolabs** **Catalog #**B0303S
- DNase I (RNase-free) - 1,000 units **New England Biolabs** **Catalog #**M0303S
- SPRIselect reagent kit **Beckman Coulter** **Catalog #**B23317
- Deoxynucleotide Solution Mix - 40 umol of each **New England Biolabs** **Catalog #**N0447L
- Exonuclease I (E.coli) - 3,000 units **New England Biolabs** **Catalog #**M0293S
- 5 M Sodium chloride (NaCl) **Sigma Aldrich** **Catalog #**S5150-1L
- Maxima H Minus Reverse Transcriptase (200 U/uL) **Thermo Fisher Scientific** **Catalog #**EP0752
- SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific** **Catalog #**AM2696
- NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads - 24 rxns **New England Biolabs** **Catalog #**E6177S
- EDTA **Sigma Aldrich** **Catalog #**E7889
- Ethanol absolute **Carl Roth** **Catalog #**9065.4
- KAPA HiFi 2x RM **Kapa Biosystems** **Catalog #**KR0370
- UltraPure DNase/RNase Free Distilled Water **Catalog #**10977-049
- Buffer EB **Qiagen** **Catalog #**19086
- Trizma hydrochloride solution **Sigma Aldrich** **Catalog #**T2694
- Aluminium seals for cold storage **Catalog #**391-1275
- PCR Seals **Thermo Scientific** **Catalog #**AB0558
- Dual Index Kit TT Set A **10x Genomics** **Catalog #**1000215

Protocol materials

Deoxynucleotide Solution Mix - 40 umol of each **New England Biolabs Catalog #**N0447L Materials

NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads - 24 rxns **New England Biolabs Catalog #**E6177S Materials

SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 Materials

EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E7889 Materials

Aluminium seals for cold storage **Catalog #**391-1275 Materials

Buffer EB **Qiagen Catalog #**19086 Materials

Trizma hydrochloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T2694 Materials

PCR Seals **Thermo Scientific Catalog #**AB0558 Materials

5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S5150-1L Materials

Exonuclease I (E.coli) - 3,000 units **New England Biolabs Catalog #**M0293S Materials

SUPERaseIN RNase Inhibitor **Thermo Fisher Scientific Catalog #**AM2696 Materials

Ethanol absolute **Carl Roth Catalog #**9065.4 Materials

UltraPure DNase/RNase Free Distilled Water **Catalog #**10977-049 Materials

Dual Index Kit TT Set A **10x Genomics Catalog #**1000215 Materials, Step 64

DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #**M0303S Materials

KAPA HiFi 2x RM **Kapa Biosystems Catalog #**KR0370 Materials

Maxima H Minus Reverse Transcriptase (200 U/uL) **Thermo Fisher Scientific Catalog #**EP0752 Materials

DNase I Reaction Buffer - 6.0 ml **New England Biolabs Catalog #**B0303S Materials

Safety warnings

Please follow all Manufacturer safety warnings and recommendations.
Keep a separation of pre and post PCR steps

Before start

Wipe bench surfaces with PCR clean wipes and keep working environment clean.

Preparation

12m

1 Clean all surfaces and pipettes with PCR clean wipes

5m

2 Turn on a lab oven to 50 °C and another to 65 °C

3 Thaw frozen buffers and primers on ice

10m

4 Prepare fresh 80% EtOH

2m

5 Prepare 900 µL diluted AMPure RNA XP beads: 1:4 in SPRI buffer per tube
225 µL RNA beads to 675 µL SPRI buffer

6 Input to minibulk v2 is extracted RNA or sorted cells.
It is essential, however, that the samples either have the same input or that they are normalized after the RNA is extracted, otherwise sequencing depth per sample will be impacted.

STEP CASE

Extracted RNA

76 steps

Follow this case if you are testing samples that have already been **RNA extracted,** were DNase treated, and have been normalized to the same input. The step here will guide you in preparing RNA-seq libraries and sequencing.

Example: 1 ng of total RNA from a cell line

Sample Preparation

7 Normalize the samples so they are all the same concentration.

Note

Minimum: 100 pg
Maximum: 4 ng

8 Transfer 1.2 µL of the **normalized RNA** to a new plate. Return the unnormalized RNA to the freezer.

9 You may store the sample plates at -80 °C at this point

Reverse Transcription

30m

10 Prepare **Reverse Transcription Mix**
Store on ice until use

	A	B	C	D
	Reagent	**Well uL**	**384w plate + 20%**	Conc
	UltraPure Water	0.5625	285.1	
	Maxima RT Buffer (5x)	0.5	253.4	1x
	100mM dNTPs	0.0125	6.3	500nM
	100uM Biotin RAP-TSO	0.025	12.7	1uM
	SuperaseIN	0.125	63.4	1U
	Maxima H Minus RT	0.075	38.0	1U
	Total (incl RNA)	2.5	1267.2	

11 Remove 384w plates from -80C freezer, thaw on ice and quick spin

12 Incubate 65 °C for 00:05:00
After this step increase the oven to 80 °C for later use

13 Add 1.3 µL **Reverse Transcription Mix** to each well with the FlexDrop

14 Incubate for 01:00:00 at 50 °C

1h

15 Incubate for 00:10:00 at 80 °C

10m

cDNA Pooling & Purification

40m

16 Pool all wells of each plate by inversion into a SBS reservoir using a centrifuge
400 x g, 4°C, 00:02:00
collect into a 5mL LoBind tube.

17 Measure the approximate volume in each cDNA pooled tube by measuring by pipette
Should be less than 870 µL

18 Perform 1x SPRI ratio cleanup with SPRI beads **diluted** 1:4 in SPRI buffer

19 Incubate for 00:05:00 at Room temperature to allow binding of the cDNA onto beads

20 Wash with 2 mL of **80% EtOH** while the tube is on the magnet. Discard the supernatant

> **Note**
>
> Volume of EtOH should be adjusted depending on the number of samples. More samples will require more EtOH to cover the beads completely.

21 Repeat wash step once more

22 Remove tubes from magnet and quick spin in centrifuge.
Use a large centrifuge for 5mL tubes if necessary

23 Air dry beads for 00:02:00

2m

> **Note**
>
> Daniel prefers 2 minutes to 5 minutes to reduce risk of overdrying. It is important to regularly check the beads and avoid over-drying.

24 Elute the beads in 45 µL of **UltraPure Water**

25 Incubate for 00:05:00 at RT off the magnet
Incubate for 00:02:00 on the magnet and transfer 43 µL to a new PCR tube or plate

Exonuclease I Treatment

26 Add 2 µL of ExoI Buffer (10x) and 1 µL of Exonuclease I. Incubate as follows:

	A	B	C
	Step	**Temp**	**Time**
	Digest	37	20
	Heat inactivation	80	10
	Storage	4	Infinite

27 Perform 0.8x SPRI bead size selection with 40 µL undiluted SPRI beads.

28 Incubate for 00:05:00 at Room temperature to allow binding of the cDNA onto beads

29 Place the tube on the magnet stand for 00:05:00 and discard supernatant

30 Wash with 180 µL of 80% EtOH while the tube is on the magnet, discard the supernatant

31 Repeat wash step once more

32 Air dry beads for 00:02:00

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly
check the beads and avoid over-drying.

33 Elute the beads in 22 µL of UltraPure Water

34 Incubate for 00:05:00 at RT off magnet
00:02:00 on magnet then transfer 20 µL to a new PCR tube or plate

Full length cDNA Amplification 30m

35 Prepare **Pre Amplification Mix**

	A	B
	Reagent	**1x**
	KAPA HiFi 2x RM	25 µL
	10uM RAP_PCR-1_Fwd	2 µL
	10uM RAP-FL_Rev	2 uL
	UltraPure Water	1 µL
	Total	**30 µL**

36 Add 30 µL **Pre Amplification Mix** to 20 µL cDNA sample

37 Incubate the Pre Amplification PCR as follows:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98 C	3 min	1 cycle
	Denaturation	98 C	15 sec	10 - 18 cycles*
	Annealing	65 C	30 sec	

	A	B	C	D
	Elongation	72 C	4 min	
	Final Elongation	72 C	10 min	1 cycle
	Storage	4 C	∞	

Note

Adjust the number of cycles based on input (sample number, cell number, or concentration).
As a general guide we recommend:
Daniel's experience is it is better to err on more PCR cycles for this full length PCR and less for the 2nd library PCR.

	A	B
	Total RNA Input	**Cycles**
	1ng	18
	10 ng	16
	50 ng	14
	100 ng	12
	>500 ng	10

cDNA Bead Purification

30m

38 Mix sample with 40 µL **SPRIselect** for a 0.8x SPRI to sample ratio

39 Incubate for 00:05:00 at 20 °C (Room Temp)

40 Place the tube on the magnet stand until clear (~3 min) and discard supernatant

41 Wash with 180 µL of **80% EtOH** while the tube is on the magnet, discard the supernatant

42 Repeat wash step once more

43 Air dry beads for 00:02:00 2m

> **Note**
>
> Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

44 Elute cDNA in 12 µL **UltraPure Water**

45 Incubate for 00:05:00 at RT and transfer 10 µL to a new PCR tube or plate

> **Note**
>
> **Stopping Point.** cDNA samples can be safely stored at -20 °C and protocol can be continued at a later date.

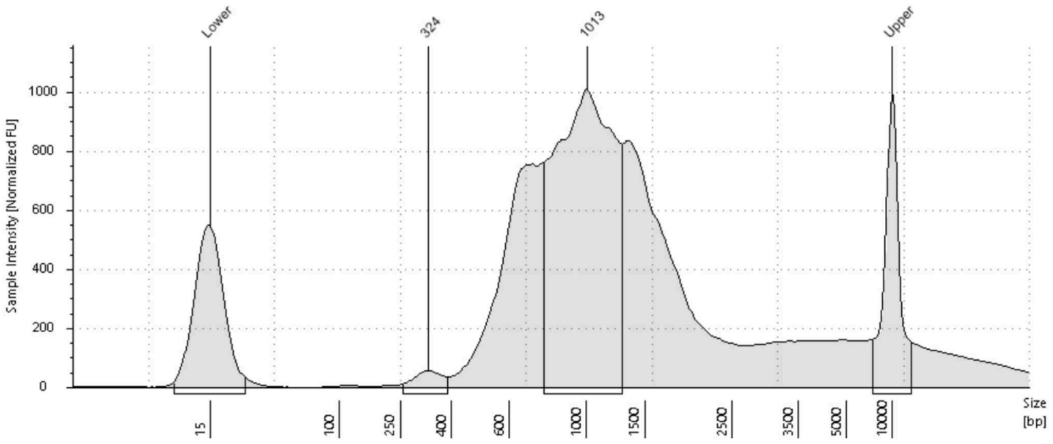
cDNA Quantification and Quality Check 20m

46 Quantify the cDNA using Qubit DNA HS following the manufacturer's protocol. Use 2 µl of clean cDNA for quantification.

47 Quality check the cDNA using the Tapestation D5000 tape.

> **Note**
>
> Passing the cDNA quality check does not guarantee that the data will be of high quality, however, if the cDNA fails the quality check it will usually not yield good libraries and will therefore generate lower quality data.

Expected result



Example tapestation D5000 HS

Library Preparation

15m

48 The input to library preparation is amplified full length cDNA from each pooled 384w plate.

Note

Before starting, read the library preparation section carefully as there are a few steps that are very time sensitive.

49 Prepare **Fragmentation Mix**

Take note of the total cDNA input in ng as it will be relevant in later PCR step

	A	B
	Reagent	**1x**
	Ultra II FS Reaction Buffer	1.4 µL
	Ultra II FS Enzyme Mix	0.4 µL
	Amplified cDNA	2.5 µL
	Water	1.7 µL
	Total	**6 µL**

Note

Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

Note

Vortex the Ultra II FS Enzyme Mix for 5-8 seconds prior to adding to master mix for optimal performance.

50 Vortex the **Fragmentation Mix** for 00:00:05 and incubate in thermocycler immediately

51 Incubate the Fragmentation reaction in a thermocycler as follows:

	A	B	C
	Step	**Temperature**	**Time**
	Pre-Cool	4 C	∞
	Fragmentation	37 C	5 min
	A Tailing and Phosphorylation	65 C	30 min
	Storage	4 C	∞

Note

Set heated lid to 75° C. Make sure the lid is at the correct temperature before you start the reaction.
After placing your samples, skip the 4C step to begin the 37C incubation once you have added your samples.

Adapter Ligation

45m

52 Prepare **Adapter Ligation Mix**

	A	B
	Reagent	**1x**
	NEBNext Ultra II Ligation Master Mix	6 µL
	NEBNext Ligation Enhancer	0.2 µL
	1.5uM IDT custom adapter (pre-annealed tenX_top and tenX_bottom)	0.5 µL
	Total	**6.7 µL**

53 Add 6.7 µL **Adapter Ligation Mix** to each fragmentation sample

54 Incubate for 00:15:00 at 20 °C in a thermocycler
Set heated lid to off

55 Add 7.7 µL Buffer EB to Samples

56 Mix samples with 16 µL **SPRI select beads** for a 0.8x SPRI ratio

> **Note**
>
> The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

57 Incubate for 00:05:00 at 20 °C (Room Temp)

58 Place the plate on the magnet stand until clear and discard supernatant.

59 Wash with 180 µL of **80% EtOH** while the plate is on the magnet. Discard the supernatant

60 Repeat wash step once more

61 Air dry beads for 00:02:00 2m

> **Note**
>
> Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

62 Elute samples in 22 µL water for 00:05:00 off magnet 7m

Incubate 00:02:00 on magent

Transfer 20 µL sample to a new tube

Library PCR 30m

63 Prepare **Library PCR Mix** by adding 25 µL Kappa HiFi polymerase to the 0.2mL tube containing purified adapter ligated sample.

64 Add 5 µL of Dual Index Kit TT Set A **10x Genomics Catalog #**1000215 to each sample.

Take care to change tips and avoid cross contamination

> **Note**
>
> Ensure a different index for each samples as this is the unique index that will be used for demultiplexing libraries.

65 Incubate the **Library PCR** reaction as follows:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98 C	30 sec	1 cycle
	Denaturation	98 C	20 sec	8 cycles *
	Annealing	54 C	30sec	
	Elongation	72 C	20 sec	

	A	B	C	D
	Final Elongation	72 C	2 min	1 cycle
	Storage	4 C	∞	

Note

Adjust the number of cycles based on cDNA input.
As a general guide we recommend:

	A	B
	cDNA Input	**Cycles**
	20 ng	8
	10 ng	9
	5 ng	10

Note

Daniel found the more PCR cycles used the more the library distribution skews to the left. Better to err on the fewer PCR cycles side.

Library Double Size Selection

1h

66 Mix Index PCR with 25 µL 0.5x ratio **SPRI select beads**

67 Incubate for 00:05:00 at 20 °C (Room Temp)

68 Place the plate on the magnet stand until clear and **transfer** 75 µL **supernatant to clean well**.

Note

Be careful **NOT** to discard! This is your library.

69 Mix supernatant with 15 µL total 0.8x ratio **SPRI select beads**

Note

The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

70 Incubate for 00:05:00 at 20 °C (Room Temp)

71 Place the plate on the magnet stand until clear and discard supernatant.

72 Wash with 180 µL of **80% EtOH** while the plate is on the magnet, discard the supernatant

73 Repeat wash step once more

74 Air dry beads for 00:02:00 2m

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

75 Elute in 22 µL **UltraPure Water**.

76 Incubate for 00:05:00 and then place on magnet until clear.

Transfer 20 µL eluted library to new tube.

Note

Stopping point. The libraries can be safely stored at -20 °C until they will be QCed and sequenced.

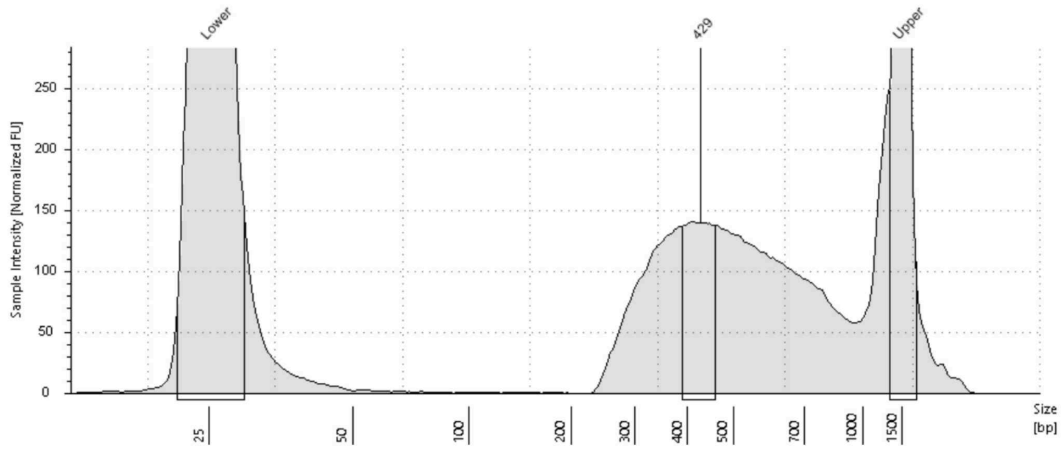
Library QC and quantification

30m

77 Quantify and quality control the library using the Tapestation D1000 kits.

Expected result

Libraries will typically exceed 1-5 ng/µl concentration



Sample on D1000 tape after 8 PCR cycles

Sequencing

78 Samples should be submitted according to your Sequencing Facility specifications.

At least **8 cycles** are required for the Index Read 1 (**i7**)

28 cycles for Read 1 (BC+UMI).
Read 2 (DNA) should be adjusted based on the quality of the genome being mapped to, but for human and mouse 50 cycles is sufficient.

Some potential sequencing options:

	A	B	C	D	E	F
	Sequencer	**Read 1**	**Read 2**	**Index Read (i7)**	**Index Read (i5)**	**Kit**
	NovaSeq	28	90	10	10	NovaSeq SP v1.5 100 cycle
	NextSeq 2000	28	52	10	10	NextSeq 2000 P3 50 cycles
	HiSeq	28	110	10	0	HiSeq 3000/4000 150 cycles

Bioinformatic preprocessing

6h

79 The fastqs may be preprocessed with your pipeline of choice. I prefer zUMIs where the parameters are:

```
file1:
    name: path_to_read1.fastq.gz
    base_definition:
      - BC(19-28)
      - UMI(1-18)
file2:
    name: path_to_read2.fastq.gz
    base_definition:
      - cDNA(1-90)
```

79.1 When I have multiple plates in a run I have the bclconvert script write the index reads to file and concatenate fastqs from all plates into a single file:

```
cat *I1_001.fastq.gz > combined_S1_I1_001.fastq.gz
cat *R1_001.fastq.gz > combined_S1_R1_001.fastq.gz
cat *R2_001.fastq.gz > combined_S1_R2_001.fastq.gz
```

79.2 I then use the index read as the first part of the well barcode:

```
sequence_files:
  file1:
    name: combined_S1_I1_001.fastq.gz
    base_definition:
      - BC(1-8)
  file2:
    name: combined_S1_R1_001.fastq.gz
    base_definition:
      - BC(19-28)
      - UMI(1-18)
  file3:
    name: combined_S1_R2_001.fastq.gz
    base_definition:
      - cDNA(1-90)
```

79.3 You will need to create a well barcode whitelist by concatenating the index reads used to all well barcodes:

```
# Read index read 1 barcodes
plateBC <- read.csv(here::here(
  "i7_only_Kit_TT_Set_A.csv"
))

# Read well barcodes
wellBC <- read.csv(here::here(
  "barcodeOrder_v1.csv"
))

# Define the vectors to be concatenated
vector1 <- plateBC$i7_8nt
vector2 <- wellBC$Well_BC

# Get all combinations
combinations <- expand.grid(vector1, vector2)

# Concatenate the combinations
combinations <- as.data.frame(paste(combinations$Var1,
combinations$Var2, sep=""))

# Write the result to a CSV file
write.csv(combinations, file = here::here("all_sample_BCs.csv"),
          row.names = FALSE, quote = F, col.names = FALSE)
```

all_sample_BCs.csv is the barcode_file in the zUMIs yaml