Frag-MaP with Enrichment (azide beads)

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Goal:

Enrich rRNA photo-crosslinked to fully-functionalized fragments in B. subtilis total RNA.

Protocol Overview:

- Fragment total RNA
- Click chemistry
- RNA-alkyne enrichment
- MaP-RT
- Second strand synthesis
- NEB Ultra II library prep
- Pool and dilute

Sample Details:

RNA provided from Frag-MaP Cell Probing 4 experiment (200 µM).

Sample ID	Replicate	Ligand	[RNA] (ng/µL)	i7 index	i5 index

Partial Fragmentation of total RNA

Fragment RNA to around 200 nt average length.

Materials:

- Total RNA (13.5 µg)
- NEBNext® Magnesium RNA Fragmentation Module

Protocol:

1. Mix the following components in a sterile PCR tube:

Component	Volume

Component	Volume
Purified total RNA (~13.5 µg)	27 µL
RNA Fragmentation Buffer (10X)	3 µL
Total	**30 µL**

2. Incubate in a preheated thermal cycler for 2 minutes at 94°C.
3. Transfer tube to ice immediately.
4. Add 3 µl 10X RNA Fragmentation Stop Solution.
5. Cleanup with Monarch RNA cleanup kit.

More Information:

NEBNext® Magnesium RNA Fragmentation Module Protocol

Monarch RNA Cleanup kit

Protocol:

- Follow manufacture protocol.
- Elute in 12.5 µL

Notes:

- The protocol for seperation of Large and Small RNA into Fractions did not produce better results than the standard protocol.

Click chemistry with alkyne-RNA & azide-oligo primer using 50% DMSO denaturant

This protocol is intented to add steric bulk to increase MaP-RT mutation efficency at fully functionalized fragment crosslink sites.

Materials:

Multiple reagents amounts by the number of intended samples

Reagents	[Reagent]	[Final]	Amount
Total RNA-alkyne – 4X	~13.5 µg	~0.4 µM	12 µL
N3-PEG-magbead – 5X	50 µmol/mL	0.5 µM	10 µL
DMSO	-	-	25 µL
Ascorbic Acid – 20X	10 mM	500 µM	2.5 µL
Cu(II): TBTA – 41.7X	10 mM	240 µM	1.2 µL

Reagents	[Reagent]	[Final]	Amount
TOTAL	-	-	**50.7 µL**

- N3-PEG-magbead 50 µmol/mL were purchased from Bangs Laboratories, Inc.

Protocol:

1. Prepare fresh 10 mM Ascorbic acid stock 20X:
    - Combine reagents.
    - Sparge solution with N2 and use immediately.

Reagents	Amount
Ascorbic acid	9.0 mg
NF Water	5.0 mL
TOTAL	**5.0 mL**

2. Dilute RNA:
    - Add RNA-alkyne and water to a strip of PCR tubes.

Reagents	[Reagent]	Final [RNA]	Amount
RNA-alkyne – 10X	1000 ng/µL	~ 0.4 µM	12 µL
Nuclease free H2O	-	-	- µL
Subtotal	-	-	**12 µL**

3. Heat denature RNA:
    - Incubate RNA at 95 °C for 2 min.
    - Snap cool on ice for 2 min.
4. Add N3-PEG-Magbead to tubes:

Reagents	[Reagent]	[Final]	Amount
N3-PEG-Magbead – 5X	50 µmol/mL	0.5 µM	10 µL
Subtotal	-	-	**22 µL**

NOTE: Batch samples can be kept on ice after HEPES addition.

5. Denature RNA by adding DMSO:
    - Add DMSO to each sample.
    - Briefly vortex.

Reagents	[Reagent]	[Final]	Amount

Reagents	[Reagent]	[Final]	Amount
DMSO	-	-	25 µL
Subtotal	-	-	**47 µL**

6. Start click reaction:
    - Add ascorbic acid and Cu(II)/TBTA mix to samples.

Reagents	[Reagent]	[Final]	Amount
Ascorbic Acid – 20X	10 mM	0.5 mM	2.5 µL
Cu(II): TBTA – 41.7X	10 mM	240 uM	1.2 µL
Total	-	-	**50.7 µL**

    NOTE: Initiate reaction within 5 minutes of adding DMSO (RNA stays denatured for ~30 minutes).

7. Incubate reaction.
    - Cover reaction.
    - Incubate at 40 °C for ~20 minutes.

    NOTE: No further conversion after 1 h and more RNA degradation occurs.

8. Quench reaction.
    - Add 1.0 µL of 0.5 M EDTA to each sample (50 µL).

RNA-Alkyne enrichment

Enrich crosslinked RNA via azide-magbead pull-down.

Before starting:

- click Azide-PEG-magbeads to Alkyne-RNA.

Materials:

- 0.5M Tris pH 7.5
- 2M NaCl
- 10% Tween-20
- 0.5M EDTA

Protocol:

1. Prepare Binding/Wash Buffer:

Component	[reagant]	[Final]	Amount (µL)

Component	[reagant]	[Final]	Amount (µL)
NaCl	2 M	1 M	12,500
Tris pH 7.5	1 M	10mM	250
EDTA	0.5 M	1 mM	50
TWEEN-20	10%	0.01%	25
NFW	-	-	12,175
TOTAL	**-**	**-**	**25,000**

2. Pull-down beads:
    - Immobilize beads with magnetic rack.
    - Remove and discard supernatant.
3. Wash beads 3 times:
    - Resuspend beads in 200 µL of Wash Buffer (1X).
    - Immobilize beads with magnetic rack.
    - Remove and discard supernatant.
    - Repeat (x3)
4. Resuspend beads:
    - Resuspend beads in 7.5 µL of NFW

Mutational profiling RT (DMS-optimized)

This is protocol is based on Smola et.al., 2015. It has been optimized for the synthesis of long cDNA from DMS modified RNA.

Before starting:

Modify RNA Purify RNA

Materials:

- 0.5 M Tris pH 8.0
- 0.75 M KCl
- 0.1 M DTT
- RNA (1 ng to 5 µg of total RNA)
- 2 µM RT primer (50 to 250 ng random primer)
- 10 mM dNTP mix
- 5 M betaine
- 40 mM MnCl2
- SuperScript II

Protocol:

1. Create fresh 10X NTP minus:

Reagent	Amount
1 M Tris ph 8.0	20 uL
2 M KCl	15 uL
1 M DTT	4 uL
NF Water	1 uL
TOTAL	**40 uL**

2. For each RNA sample combine:

Reagent	Amount
RNA (~10 ng)	7.5 uL
nonamer (~100 ng)	0.5 uL
10 mM dNTPs	2 uL
TOTAL	**10 µL**

3. Denature and anneal ribosome by incubating at 90°C for 5 min, then cool on ice.

4. Create the following master mix:

   (multiply by number of samples+1)

Reagent	Amount
10X NTP minus	2 uL
5 M Betaine	4 uL
40 mM MnCl2	3 uL
TOTAL	**9 uL**

5. Add 9 µL of master mix to each sample.

6. Incubate at 25°C for 2 min.

7. Add 1 µL of SuperScript II.

8. Set Thermocycler to this program:

   - Incubate at 25°C for 10 min.
   - Incubate at 42°C for 90 min.
   - 10 cycles of 50°C for 2 min and 42°C for 2 min.
   - Deactivate at 70°C for 10 min.

- Hold at 4°C.

Notes

- Thermocycler had an error. Held at 25 °C for 3 hours before starting protocol.

More Information:

- First-Stand cDNA Synthesis Using SuperScript II RT

Second Strand Synthesis

From first strand synthesis product, a mix of enzymes polymerizes a second cDNA from the first cDNA of a DNA-RNA hybrid, then digests away the RNA.

Before starting:

- first strand synthesis
- cDNA purification

Materials:

- NEBNext second strand synthesis enzyme mix
- NEBNext second strand synthesis 10x buffer
- Fresh 80% EtOH

Protocol:

Always keep 2nd Strand Enzyme cold (below 16°C)

1. Pull-down cDNA product on beads:
    - Immobilize beads with magnetic rack.
    - Remove and discard supernatant.
2. Prepare reaction:
    - Resuspend cDNA on-ice
    - Pipette mix

Reagent	Amount
sscDNA	(on-bead)
nucleotide-free water	32 µL
10X 2nd Strand Reaction Buffer	4 µL
2nd Strand Enzyme Mix	2 µL
TOTAL	**40 µL**

3. Incubate at 16°C for 60 min.

Next Steps:

- Purify DNA
- HS DNA Qubit assay
- NEBNext Ultra II library prep

cDNA purification - AMpure beads

Material:

- AMpure beads (equalibriate to RT)
- Fresh 80% ethanol
- Magnetic holder

Bead Ratio:

1.8X

Protocol:

1. Beads added to reaction mixture (1.8X)
2. Incubate at room temp. for 5 min.
3. Place in magnetic holder for 5 min.
4. Remove most of the susernatant
5. Wash beads with 80% ethanol (30 seconds) twice.
6. Add 20 µL of water, resuspend pellet, and incubate for 5 minutes.
7. Remove and keep supernatant.

More Information:

- NGS Beads for DNA Size Selection

Qubit

Quantify the dscDNA product from second strand synthesis via Qubit HS DNA assay.

Material

- Qubit tubes
- Working solution
- Standards 1 & 2

Protocol

1. Create standards:
    - Add 190 µL of working solution into Qubit tube
    - Add 10 µL of standard
    - Equalibrate to RT
2. Create samples:
    - Add 198 µL of working solution into Qubit tube

- Add 2 µL of cDNA
- Equalibrate to RT

3. Incubate for 2 minutes before reading concentrations

Results:

Sample ID	[dscDNA] ng/µL

DNA Library Prep (Adapter Ligation) - NEBNEXT Ultra II

Materials:

- dsDNA (500 pg - 1 µg)
- (green) NEBNext Ultra II End Prep Enzyme Mix
- (green) NEBNext Ultra II End Prep Reaction Buffer
- (red) NEBNext Ultra II Ligation Master Mix
- (red) NEBNext Ligation Enhancer
- (blue) NEBNext Ultra II Q5 Master Mix
- NEBNext Oligo Kit
- 80% Ethanol

Protocol:

1. Prepare Ends.

    - Add the following components into a PCR tube:

Components	Volume
dsDNA	25 µL
End Prep Reaction Buffer	3.5 µL
End Prep Enzyme Mix	1.5 µL
TOTAL	**30 µL**

    - Mix thoroughly and spin down.
    - Inbucate in thermal cycler with lid set to > 75 °C:

Step	Time (min.)	Temperature (°C)
Prep ends	30	20
Denature	30	65
End	HOLD	4

2. Adapter Ligation. (Adds ~60 nt)

- Dilute adaptors based on input DNA quantity (from NEBNext Oligo Kit):

Input DNA	Adpator Dilution	Working [Adapter]
101-1000 ng	No Dilution	15 µM
5 - 100 ng	1:10	1.5 µM
0.5 - 5 ng	**1:25 (recommended)**	**0.6 µM**

- Add the following components directly into the End Prep Reaction Mixture:
  - **NOTE: Do NOT premix Adapter with Master Mix or Ligation Enhancer.**

COMPONENT	VOLUME
End Prep Reaction Mixture	30 µl
(red) NEBNext Adaptor (diluted)	1.25 µl
(red) NEBNext Ultra II Ligation Master Mix	15 µl
(red) NEBNext Ligation Enhancer	0.5 µl
TOTAL	**47.75 µL**

- Mix thoroughly and spin down.
- Incubate at 20 °C for 15 minutes with lid heater off.
- Add USER enzyme.

COMPONENT	VOLUME
Ligation reaction mixture	47.75 µL
(red) USER enzyme	1.50 µl
TOTAL	**49.25 µL**

- Mix throughly.
- Incubate at 37 °C for 15 minutes with the lid heater set to > 47 °C

3. Cleanup of Adaptor-ligated DNA - SPRI beads

Bead Ratio: 1.4x (or lower)

1. Beads added to reaction mixture (1.4X)
2. Incubate at room temp. for 5 min.
3. Place in magnetic holder for 5 min.
4. Remove most of the supernatant
5. Wash beads with 80% ethanol (30 seconds) twice.
6. Add 17 µL of water, resuspend pellet, and incubate for 5 minutes.
7. Remove and keep supernatant.

4. PCR Amplification (Adds ~60 nt)

- Add the following components to a PCR strip:

COMPONENT	VOLUME
Adaptor Ligated DNA Fragments	15 µl
(blue) NEBNext Ultra II Q5 Master Mix	25 µl
Index Primer/i7 Primer	5 µl
Universal PCR Primer/i5 Primer	5 µl
TOTAL	**50 µl**

- Mix thoroughly and spin down.
- Choose number of PCR cycles using the table below:

INPUT DNA	# OF CYCLES (YIELD ~100 ng)
100 ng	3
50 ng	3–4
10 ng	6–7
5 ng	7–8
1 ng	9–10
0.5 ng	10–11+

- Perform PCR amplification using the following PCR cycling conditions:

STEP	PCR Cycle Step	TEMP	TIME	CYCLES
Initial Denaturation		98 °C	30 seconds	1
PCR cycles:				
	Denaturation	98 °C	10 seconds	3-15 **(15 recommended)**
	Annealing/Extension	65 °C	75 seconds	3-15 **(15 recommended)**
Final Extension		65 °C	5 minutes	1
HOLD		4 °C	Hold	1

5. Cleanup of PCR Reaction - SPRI beads

Bead Ratio: 0.95x

1. Beads added to reaction mixture (0.95X)
2. Incubate at room temp. for 5 min.
3. Place in magnetic holder for 5 min.
4. Remove most of the supernatant
5. Wash beads with 80% ethanol (30 seconds) twice.
6. Add 20 µL of water, resuspend pellet, and incubate for 5 minutes.
7. Remove and keep supernatant.

6. Repeat step 5

Bead Ratio: 0.95x

Notes:

- Length of DNA = Insert (~150) + adaptor (60 nt) + primers (60 nt) = 270
- Frag-MaP usually required 15 PCR cycles

Next Steps:

- Qubit
- Bioanalyzer

Qubit

Quantify the dscDNA product from second strand synthesis via Qubit HS DNA assay.

Material

- Qubit tubes
- Working solution
- Standards 1 & 2

Protocol

1. Create standards:
    - Add 190 µL of working solution into Qubit tube
    - Add 10 µL of standard
    - Equalibrate to RT
2. Create samples:
    - Add 196 µL of working solution into Qubit tube
    - Add 4 µL of cDNA
    - Equalibrate to RT
3. Incubate for 2 minutes before reading concentrations

Results

Sample ID	[dscDNA] ng/µL

Sample ID	[dscDNA] ng/µL

Agilent Tape Station

Determine the average length of sequencing library.

Materials

- High sensitivity D1000 DNA sample buffer
- High sensitivity D1000 DNA ladder
- High sensitivity D1000 DNA screentape
- Tape station tubes and caps

Specification

Analytical specifications	High Sensitivity D1000 ScreenTape assay
Sizing range	35–1000bp
Sizing accuracy	±10 %
Quantitative range	10–1000 pg/µL

Protocol

1. Allow D1000 Reagents to equilibrate to room temperature.

2. Prepare ladder (position: A1)

COMPONENT	VOLUME
D1000 Sample buffer	2 µl
Ladder	2 µl
TOTAL	**4 µl**

3. Prepare samples (position: A2-B8)

    - Dilute samples to < 1 ng/µL in NFW

COMPONENT	VOLUME
D1000 Sample buffer	2 µl

COMPONENT	VOLUME
Sample	2 µl
TOTAL	**4 µl**

4. Apply caps to tube strips and/or foil seals to 96-well sample plates.
5. Vortex sample and spin down.
6. Load Screentape and tubes/plate into the tape station instrument.

Results

Sample ID	Avg. bp length	% of total

Pool and dilute library

Dilute sample to the same molarity and pool for sequencing.

Material

- Illumina-mix-calculator.xlsx
- Sample_Sheet.xlsx

Target library concentration

300 pM

Protocol

1. Input data into illumina-mix-calculator.xlsx.
    - Avg. bp length (Bioanalyzer)
    - ng/µL (Qubit)
    - % Non-dimer (bioanalyzer)
    - Target library concentration (nM)

    NOTE: Recomended minimum volume: 3 µL

2. Pool sample library using the volumes calculated by illumina-mix-calculator.xlsx.

Sample ID	[non-dimer] (nM)	Amount to pool (µL)

3. Dilute the sequencing library to 2 nM using qubit measurements.
    - Best practice: 10 µL sample + 190 µL qubit working solution.
    - Store sequencing libraries at 2 nM.

Pooled library	ng/µL	pM
Storage concentration		

4. Dilute 2 nM sequencing library to target sequencing concentration using qubit measurements.
    - Best practice: 10 µL sample + 190 µL qubit working solution.
    - Prepare dilution fresh before each sequencing run.

Pooled library	ng/µL	pM
Target concentration		

Results:

Libary Name	ng/µL	pM

Next Steps

NextSeq

NextSeq

Materials:

- low-bind/low-retention microcentrifuge tubes
- NextSeq1000/2000 kit (P1 or P2):

Component	Storage location
Reagent cartiage	Freezer E
Flow cell	Deli fridge
RSB with TWEEN 20	Deli fridge

Workflow

1. Thaw Kit
2. Create v2 sample sheet
3. Pool and dilute library
4. Load cartridge
5. Sequence
6. Dispose of cartridge

Thaw kit

- Select a kit appropriate for the length of your library:

Read length	Kit type	Reads passing filter	Run time
100	P1	100 M	10 hr.
	P2	400 M	13 hr.
200	P2	400 M	21 hr.
300	P1	100 M	19 hr.
	P2	400 M	29 hr.
600	P1	100 M	34 hr.
	P2	400 M	44 hr.

- Thaw guidelines for 100, 200, or 300-cycle kits:

Method	Minimum Time to Complete Thaw	Do Not Exceed	Storage of Thawed Cartridge
25°C Controlled Water Bath	6 hours	8 hours	72 hours at 2-8°C
Refrigerator	6 hours at Room Temp + 12 hours at 2-8°C	72 hours at 2-8°C	60 hours at 2-8°C
Room Temperature	9 hours	16 hours	72 hours at 2-8°C

More information:

- Illumina kit specifications
- Illumina Thaw guidelines (also includes info for 600-cycle kits)

Create v2 sample sheet

The Nextseq can get sample information from a basespace run or a manual v2 sample sheet.

NOTE: Both indexes (i7 and i5) should be written in the forward direction, reference Nextseq index reference tables

Make a sample sheet in Basespace (recommended):

1. Log into Illumina Basespace and navigate to:

   BaseSpace > Runs > New Run > Run Planning

2. Input Run settings. (Recommended: select Local Secondary Analysis.)

3. Input Configuration. (Recommended: select Not Specified for Library Prep Kit & Index Adapter Kit)

4. Input read lengths and sample information.

   - Maximum number of cycles per kit:

Kit	**Max total cycles**	**Example max for dual indexes: Read 1 ; Index 1 ; Index 2 ; Read 2**
100	136	Y60;I8;I8;Y60
200	236	Y110;I8;I8;Y110
300	336	Y160;I8;I8;Y160
600	636	Y310;I8;I8;Y310

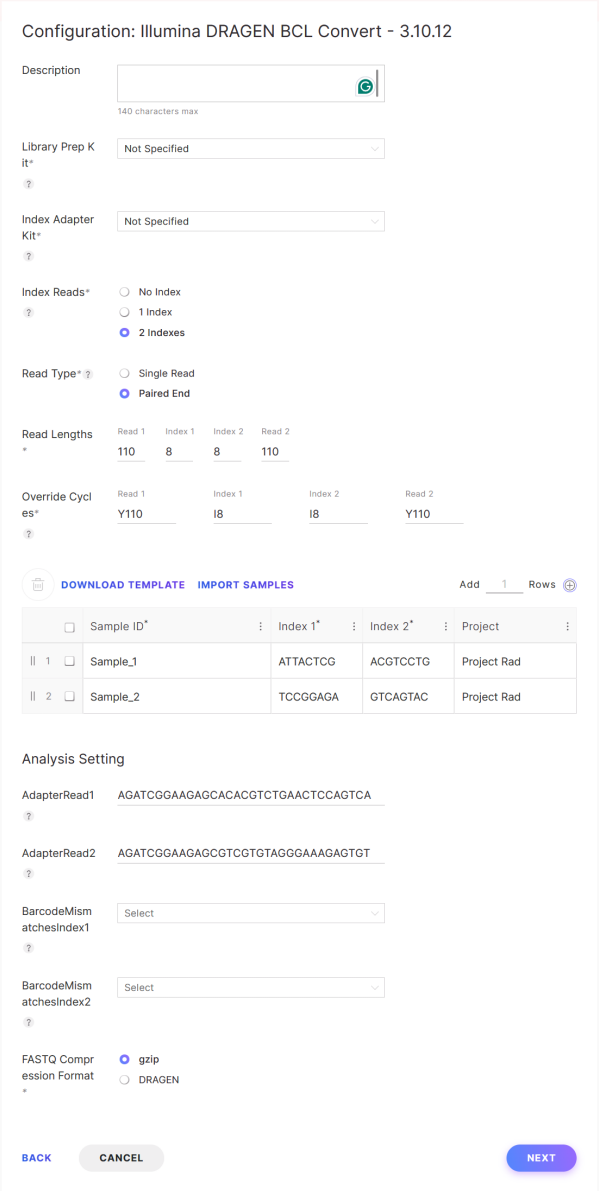
   - Index sequences:

     - Use the dropdown lists in the lab v2 Sample Sheet Template
     - Use Nextseq index reference tables
     - Indexes should be written in the forward direction (bases as they appear in the adaptor in the 5′-3′ direction)

   - Adaptor reads:

AdpterRead1	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA
AdapterRead2	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

5. Example configuration for a P2 200 cycle kit:



Manual v2 sample sheet:

- Input required information in highlighted columns of the lab v2 Sample Sheet Template
- Save the sample sheet as a .csv (comma delimited) file
- Transfer the .csv file to the Nextseq via flash drive or through the lab server

More information:

- Sample sheets for the NextSeq 1000/2000

Pool and dilute library (Onboard Denature and Dilute)

1. Measure sample concentration using Qubit HS dsDNA
2. Measure sample average length using the Bioanalyzer HS DNA kit or Tapestation
3. Use the mix calculation spreadsheet to normalize and pool libraries
    - Input required information in highlighted columns
4. Dilute pooled library:

- Use RSB TWEEN 20 to the dilute pooled library to 2 nM in a low-bind tube.
- Measure concentration using Qubit HS dsDNA (recommend sample volume ≥ 4 µL)

5. Dilute to loading concentration:

Library	Loading concentration	2 nM Library Volume	2 nM PhiX Volume	RSB with TWEEN-20 Volume
Standard starting concentration	650 pM	9.5 µL	0.5 µL	20.8 µL
Frag-Jump & Frag-MaP	250-300 pM	9.5 µL	0.5 µL	62.7 µL

- Recommended: 1-5 % PhiX (improved run metrics and troubleshooting)

More information:

- Illumina: library dilution protocol

Load cartridge

1. Remove the thawed cartridge from the bag.
2. Invert the cartridge 10 times (along long axis) to mix reagents.

3. Insert the flow cell into the front of the cartridge. (listen for an audible click)
    - The grey tab covering the flowcell must be removed before sequencing
4. Pierce the Library reservoir with a P1000 tip and push the foil to the edges to enlarge the hole.
5. Add the diluted library to the **bottom** of the reservoir. (Avoid touching the foil)
    - Add 20 µL of the diluted library (for onboard denature and dilution)

More information:

- Load consumables into the cartridge

Sequence

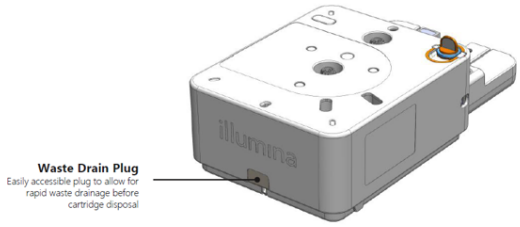
1. Start run with Illumina control software.
2. Input sample details:
    - Log into basespace and select planned run
    - Use manual v2 sample sheet
3. Load cartridge (orient flowcell toward instrument)
4. Click to proceed with run (twice)
5. Run statistics are available after cycle 25

More information:

- Initiating a Sequencing Run

Dispose of Cartridge

1. Eject Cartridge from Nextseq
    - Should be completed within 48 hours of run completion to prevent leaking
2. Remove the drain plug on the rear of the cartridge



3. Drain unwanted material into the aqueous unwanted materials carboy
    - Cartridge contains formamide
4. Return the drain plug
5. Recycle cartridge (optional):
    - Use the cartridge recycling tool to disassemble the cartridge
    - Recycle white plastics
    - Trash black plastics

Library composition

Library	Concentration	%

Reagent cartridge

Kit	Ref	Lot	SN

Kit	Ref	Lot	SN
P2 200			

Flow cell

Kit	Ref	Lot	SN
P2 200			

Run setup

Loading concentration	PhiX %	Denature	Read 1	Read 2	Index 1	index 2
300 pM	5	On-board	110	110	8	8

Run metric

% Loading concentration	Average %Q30	% PF	Total reads PF

Files

Mix calculator

Samplesheet