Frag-MaP experimental protocol

Seth D. Veenbaas 10/16/2023

Experiment Summary

Detect the RNA binding site of fully-functionalized fragments in B. subtilis total RNA.

Protocol Overview:

- Photocrosslink and extract RNA
- Partial Fragmentation of total RNA
- Click Azide-PEG3-Biotin
- Streptavidin bead enrichment (optional)
- Ethanol precipitation
- MaP RT
- Second strand synthesis
- NEB Ultra II adapter ligation kit
- Qubit
- Bioanalyzer
- Pool and dilute library
- Miseq

Photocrosslink and extract RNA (3 days)

Prepare ligand crosslinked total RNA for JuMP RT, and sequencing.

NOTE: All experiments should start fresh by streaking B. sub on plates.

- B. sub does not grow well from glycerol stocks or passaged cell pellets.
- B. sub does not store well at 4 °C because it enters a spore form.

Day 1: Plate cells

Materials:

- B. subtilis str. 168 glycerol stock
- LB Agar plate

Protocol:

1. Streak a plate with B. subtilis str. 168 to form single colonies.
2. Incubate plate at 30 °C overnight.

Day 2: Overnight culture

Materials:

- LB media (5 mL)
- Culture tube

Protocol:

1. Inoculate 5 mL LB media with a single colony from plate.
2. Grow cells overnight at 30 °C, 225 rpm.

Day 3: Probe cells and extract RNA (~10 hours)

Materials:

- LB media (50 mL)
- PBS buffer (100 mL)
- 50 mM ligand stock in DMSO (20 uL per sample)
- Sterile 6-well plastic plate (1 well per sample)
- lysozyme (60 mg)
- 1M Tris pH 7 (180 uL)
- 0.5 M EDTA (120 uL)
- Chloroform
- 100% ethanol
- Qiogen RNAeasy Plus Universial Mini kit

Protocol:

3. Inoculate 60 mL LB media with 3 mL cultured cells and grow cells to 0.50 OD600.

   NOTE: Expect cell growth to take ~2.5 hours.

Time point	#1 OD600	#2 OD600
	-	-

4. Buffer excahnge cells.
   - Gently pellet cells at 3200g for 10 min at 4 °C.
   - Carefully remove supernatant.
   - Wash cell pellet with 50 mL PBS buffer, repeat centrifugation, and carefully remove supernatant.
   - Resuspend cells in 50 mL PBS buffer.
5. Probe cells with functionalized ligands.
   - Combine 4,980 uL of cultured media with 20 uL of photo-reactive ligand (50 mM stock in DMSO) and pipette mix. Avoid prolonged light exposure.

     NOTE: final [ligand] is 200 uM. 250X dilution.

   - Incubate ligand-treated cells for 30 minutes at 30 °C and 225 rpm.

     NOTE: Keep solution in dark.

6. Cross-link functionalized ligands to RNA.
    - Transfer 5 mL cell solution to a sterile 6-well plastic plate.
    - Place uncover plate and place on ice
    - Irradiate with 365 nm light source and cross-link with 3 J/cm2.

      NOTE: This is nine minutes (or three max energy hits) 5-6 cm from the light source in a UVP crosslinker.

7. Create cell pellet.
    - Centrifuge to pellet the cells. 8,000xg for 10 min. at 4 °C.
    - Discard supernatant.

8. Prepare fresh lysis buffer.
    - (30 mM Tris pH 7, 10 mM EDTA, 10 mg/mL lysozyme):

Reagent	Amount
lysozyme	50 mg
1M Tris pH 7	150 uL
0.5M EDTA	100 uL
RNase inhibitor murine	50 uL
NF H2O	4700 uL
TOTAL	**5,000 uL**

9. Lysis cell pellet with lysozyme buffer.
    - Add 250 uL of buffer lysis to each cell pellet.
    - Incubate for 30 min. at RT.

10. Extract and purify total RNA from cells.
    - Use 1000 uL of Tri-reagent
    - Mix aggressively to aid in lysis
    - Lyse for 15 minutes
    - Add 250 uL of chloroform
    - Vortex and incubate at RT for 3-5 minutes
    - Centrifuge at 15000xg for 15 minutes at 4 C
    - **NOTE: Keep sample on ice until aqueous layer is recovered**
    - Add 100% ethanol to each recovered aqueous layer in equal parts (1:1)

- Follow Direct-zol RNA Miniprep Plus Kit instructions.
- Elute from column with 50 uL of NFW

NOTE: Yields of ~50-60 ug total RNA have been achieved per 5 mL culture probed.

11. Check RNA purity.
    - Measure absorbance ratio (A260/280 and A260/230) on nanodrop.

Culture	Sample ID	Yield (µg)	[RNA] (ng/µL)	A260/280	A260/230

NOTES:

- Samples diluted to 500 ng/µL and flash frozen.

Storage:

- RNA can be stored overnight at 4 °C for ~ 1 week.
- RNA can be flash-frozen in aliquots and stored at -80 °C for ~1 year.

Next Steps:

- Partial RNA Fragmentation
- MaP-RT

Sample Details:

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

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

Partial Fragmentation of total RNA (~0.5 hour)

Fragment RNA to around 150 nt average length.

Materials:

- Total RNA (10 µg)
- NEBNext® Magnesium RNA Fragmentation Module

Protocol:

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

Component	Volume
Purified total RNA (~10 µg)	18 µL
RNA Fragmentation Buffer (10X)	2 µL
Total	**20 µL**

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

More Information:

NEBNext® Magnesium RNA Fragmentation Module Protocol

Click Azide-PEG3-Biotin (~1 hour)

This prtocol is intented to add a vector to enable biotin-streptavin enrichment for crosslinked RNA.

Materials:

Multiple reagents amounts by the number of intended samples

Reagents	[Reagent]	[Final]	Amount
Total RNA-alkyne – 10X	~10 µg	~0.1 µM	18 µL
N3-Cy5 – 25X	50 µM	2 µM	2 µL
HEPES Buffer pH 7.5 – 25X	250 mM	10 mM	2 µL
DMSO	-	-	25 µL
Ascorbic Acid – 20X	10 mM	0.5 mM	2.5 µL
Cu(II): TBTA – 41.7X	10 mM	240 µM	1.2 µL
TOTAL	-	-	**50 µL**

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

Reagents	Amount
TOTAL	**5.0 mL**

2. Dilute RNA:

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

Reagents	[Reagent]	Final [RNA-Alkyne]	Amount
RNA-alkyne – 10X	75 ng/µL	~ 0.01 µM	17.3 µL
Nuclease free H2O	-	-	- µL
Subtotal	-	-	**17.3 µL**

3. Heat denature RNA:

    - Incubate RNA at 95 °C for 2 min.
    - Snap cool on ice for 2 min.

4. Add HEPES buffer and N3-PEG3-Biotin to tubes:

    - Add HEPES and N3-PEG3-Biotin.

Reagents	[Reagent]	[Final]	Amount
HEPES Buffer pH 7.5 – 25X	250 mM	10 mM	2 µL
N3-PEG3-Biotin – 25X	50 µM	2 µM	2 µL
Subtotal	-	-	**21.3 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
DMSO	-	-	25 µL
Subtotal	-	-	**46.3 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

Reagents	[Reagent]	[Final]	Amount
Total	-	-	**50.0 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).

9. Remove DMSO.
    - Dilute to ~500 µL with NF water (final [DMSO] is 5%).
    - Concentrate with Amicon Ultra 0.5mL Centrifugal filter spin column (10-50K NMWL cut off). Spin for 10 min at 14,000 g and 4 °C.

OPTIONAL:

- Wash column with ~500 µL of cold TE buffer
- Spin for 10 min at 14,000 g and 4 °C.

Results - Nanodrop:

Sample ID	[RNA] (ng/µL)	A260/280	A260/230
1D			
1M			
1Z			
1Q			
1B			
1H			
1G			
1J			
2D			
2M			
2Z			

Sample ID	[RNA] (ng/µL)	A260/280	A260/230
2Q			
2B			
2H			
2G			
2J			

Streptavidin bead enrichment (~1 hour)

Enrich crosslinked RNA via Biotin-Streptavidin pull-down.

Before starting:

- click Azide-3PEG-biotin to Alkyne-RNA and purify (RNAeasy Minelute)

Materials:

- 0.5M Tris pH 7.5
- 2M NaCl
- 10% Tween-20
- Streptavidin Magnetic Beads
- Formamide
- 0.5M EDTA

Protocol:

1. Prepare Bind Buffer (2X):

Component	[reagant]	[2X]	[Final]	Amount (µL)
Tris pH 7.5	1 M	100 mM	50mM	1,000
KCl	2 M	200 mM	100 mM	2,000
TWEEN-20	10%	0.02%	0.01%	20
NFW	-	-	-	6,980
TOTAL	**-**	**-**	**-**	**10,000**

2. Prepare Wash Buffer:

Component	[reagant]	[Final]	Amount (µL)
Bind Buffer	2X	1X	7,000
NFW	-	-	7,000
TOTAL	**-**	**-**	**14,000**

3. Prepare Elution Buffer:

Component	Concentration	[Final]	Amount (µL)
Formamide	99%	95%	960
EDTA (pH 8.2)	0.5M	10 mM	20
NFW	-	-	20
TOTAL	**-**	**-**	**1000**

4. Wash NEB Streptavidin Magnatic Beads.

    - Vortex beads until comletely suspended.
    - Add 50 µL of beads to a PCR strip.
    - Incubaute at RT for 5 minutes.
    - Apply magnet to beads and remove supernatant.
    - Resuspend beads in 100 µL of Wash Buffer (1X).
    - Apply magnet to beads and remove supernatant.
    - Repeat wash with Wash Buffer (1X).

5. Bind RNA to beads:

    - Add RNA-biotin to a strip of PCR tubes.
    - Add Bind Buffer (2X).
    - Add NEB Streptavidin Magnatic Beads.

Component	Concentration	[Final]	Amount (µL)
RNA-Biotin	-	5 µg	25
Bind Buffer	2X	1X	25
Streptavidin Beads	-	-	50
TOTAL	**-**	**-**	**100**

6. Incubate RNA-Biotin and Streptavidin Beads for at least 30 minutes at RT.

    - < 10 minutes for short oligios (~ 30 nucleotides).
    - ~ 15 minutes for fragmented total RNA.
    - ~ 30 minutes for full length total RNA.

7. Pull-down:

    - Immobilize beads with magnetic rack.
    - Remove and discard supernatant.

8. Wash beads 4 times:

    - Resuspend beads in 150 µL of Wash Buffer (1X).
    - Immobilize beads with magnetic rack.
    - Remove and discard supernatant.

9. Elute RNA:
    - Add 50 µL of Elution Buffer.
    - Heat tubes at 95 °C for 4 minutes.
    - Quickly transfer tubes to magnetic rack and immobilize beads.
    - Transfer RNA-biotin solution into a new tube.

More information:

- *A chemical probe based on the PreQ1 metabolite enables transcriptome-wide mapping of binding sites*

Next steps:

- Ethanol precipitation

Purify RNA - Ethanol Precipitation (~2 hours)

Purified RNA may need to be concentrated by precipitation for downstream applications. Precipitation of RNA with ethanol (or isopropanol) is the standard method to recover RNA from aqueous solutions.

Materials:

- RNA (> 10 ng/µL)*
- 100% Ethanol (or isopropanol)
- 70% Ethanol
- A salt from TABLE 1.

TABLE 1. Salt solutions for precipitating RNA

Salt	Stock (M)	[Final] (M)	Comments
Sodium Acetate	3 (pH 5.2)	0.3	Recommended.
Ammonium acetate	5	0.5	Use with isopropanol to remove free nucleotides.
Lithium Chloride	8	0.8	Avoid for downstream IVT or RT.

NOTE: Avoid Potassium salts if RNA will be exposed to dodecyl sulfate (SDS).

Protocol:

1. Combined components:
    - Add RNA to appropriately sized tube.
    - Add one of the salt solutions shown in Table 1.
    - Add 2.5–3.0 volumes of ice-cold ethanol (or 1 volume of isopropanol).
    - Mix the solution well.

Component	Concentration	Equivalents
RNA	>10 ng/µL*	1

Component	Concentration	Equivalents
Salt	10X	0.1
Ethanol	100%	2.9
Glycogen (*optional)	100X	-
TOTAL	**-**	**4**

*If [RNA] is < 10 ng/µL, add glycogen (final concentration of 50–150 µg/mL) to facilitate low concentration precipitation.

2. Precipitate RNA:
    - Store the ethanolic solution for 1 h to overnight at −20 °C.
    - RNA precipitation is faster and more complete at higher RNA concentrations.
3. Recover the RNA:
    - Centrifugation RNA solution at 12,000g–14,000g for 10 min at 4 °C to form pellet.
    - RNA pellet is is often invisible.
    - Decant the supernatant without disturbing the RNA pellet.
    - Carefully remove remaining traces of the supernatant with a disposable pipette tip.
4. Wash the RNA pellet (x2):
    - Add 0.5 mL of ice-cold 70% ethanol to pellet.
    - Centrifuge at maximum speed for 10min at 4 °C.
    - Repeat ethanol wash.
5. Dry RNA pellet:
    - Leave tube open at room temperature until the last traces of fluid have evaporated.
    - Do not dry the RNA pellet completely; otherwise, it can be difficult to dissolve the RNA.
6. Dissolve the RNA pellet:
    - Dissolve RNA in the desired volume of an RNase-free buffer (usually TE; pH between 6 and 7).
    - Rinse the walls of the tube well with the buffer.

Storage:

- RNA can be stored for up to 1 yr at −80 °C.

More information:

- Precipitation of RNA with Ethanol

Mutational profiling RT (~3 hours)

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 (~15 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.
9. Purify cDNA with G-50 column

More Information:

- First-Stand cDNA Synthesis Using SuperScript II RT

Second Strand Synthesis (~2 hours)

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. Into a tube combine:

Reagent	Amount
sscDNA	20 µL
nucleotide-free water	48 µL
10X 2nd Strand Reaction Buffer	8 µL
2nd Strand Enzyme Mix	4 µL
TOTAL	**80 µL**

2. Mix by pipetting.

3. Incubate at 16°C for 60 min.

Next Steps:

- Purify DNA
- HS DNA Qubit assay
- Nextera XT

DNA Library Prep (Adapter Ligation) - NEBNEXT Ultra II (~2 hours)

Materials:

- dsDNA (500 pg - 1 µg)
- NEBNext Ultra II End Prep Enzyme Mix
- NEBNext Ultra II End Prep Reaction Buffer
- NEBNext Ultra II Ligation Master Mix
- NEBNext Ligation Enhancer
- 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	50 µL
End Prep Reaction Buffer	7 µL
End Prep Enzyme Mix	3 µL
TOTAL	**60 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	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	60 µl
NEBNext Adaptor (diluted)	2.5 µl
NEBNext Ultra II Ligation Master Mix	30 µl
NEBNext Ligation Enhancer	1 µl
TOTAL	**93.5 µL**

- Mix thoroughly and spin down.
- Incubate at 20 °C for 15 minutes with lid heater off.
- Add 3 µl of (red) USER enzyme.
- 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

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
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	10–13

- 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
	Annealing/Extension	65 °C	75 seconds	3-15
Final Extension		65 °C	5 minutes	1
HOLD		4 °C	Hold	1

5. Cleanup of PCR Reaction - SPRI beads

Bead Ratio: 1.2x

1. Beads added to reaction mixture (1.2X)
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.

Notes:

- 12 cycles of PCR used
- Lengths are insert + adaptor (60 nt) + primers (60 nt)

Next Steps:

- Qubit
- Bioanalyzer

Qubit (~0.5 hours)

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

Material

- Qubit 1X dsDNA HS kit
  - Qubit tubes
  - 1X working solution
  - Standards 1 & 2

Protocol

1. Create standard 1 & 2:
   - 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

Bioanalyzer (~1 hour)

Determine the average length of nextera library.

Materials

- High sensitivity DNA reagants
- High sensitivity DNA chip
- Chip priming station

Protocol

- Follow Agilent protocol

Results

Sample ID	Avg. bp length	% of total

Pool and dilute library (~1 hour)

Dilute sample to the same molarity and pool for sequencing.

Material

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

Target library concentration for illumina v3 chemistry

2.00 nM [library] -> 10 pM [final]

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

3. Quantify the concentration of the pooled sample library using Qubit

4. Dilute the pooled library to the final concentration (ng/µL) calculated by illumina-mix-calculator.xlsx

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

5. Verify concentration via Qubit.

Sample ID	ng/µL	nM

Next Steps

MiSeq

MiSeq (~2 hours)

Normalized DNA sequencing libraries are pooled, denatured, diluted, loaded into a MiSeq reagent kit, and ran on the MiSeq

1. Post-run wash (if not already completed)
    - After the run, discard waste, reagent cartridge and PR2 bottle
        - Formamide must be removed from the reagent cartridge (Well #8)
    - Fill an empty PR2 bottle with 500 mL 0.5% Tween 20:
        - 25 mL 10% Tween 20
        - 475 MilliQ water
    - From PR2 bottle, put 5 mL of 0.5% Tween 20 into each well of the wash tray
    - Load wash tray, wash bottle, and waste bottle
    - Start post-run wash

2. Reboot computer and clear disk space
    - Make drive has at least 100 GB free
    - Shut-down windows through the start menu
    - Once complete, shut off power to the device for 30 seconds
    - Turn on power switch, device will reboot

3. Thaw reagent cartridge
    - Open MiSeq Reagent Kit Box 1 of 2 (stored at -20)
    - Thaw HT1 buffer on ice (or RT if impatient, then move to ice)

- Copy the serial number from the reagent cartridge (MS#######-#00V#)
- Thaw reagent cartridge in a room temperature water bath (~1 hr)
- Once completely thawed, store at 4C until loading

4. Pool, denature, and dilute libraries

- Prepare a fresh dilution of 0.2 N NaOH by mixing
  - 200 uL 1.0 N NaOH
  - 800 uL nuclease free water
- Dilute samples to 2.0 nM and combine in the desired ratio
- In a 1.7 mL tube, combine
  - 5 uL 2.0 nM library
  - 5 uL 0.2 N NaOH
- Briefly vortex and quick spin down (280 x g for 1 min)
- Incubate at room temp for at least 5 min
- Add 990 uL chilled HT1
- Sample library is at 10 pM in HT1 with 1mM NaOH

5. Clean flow cell

- Remove flow cell from MiSeq Reagent Kit Box 2 of 2 (4 C), handle by the edges
- Wash flow cell with water
- Carefully clean glass surface with lens paper
- Repeat until surface is free of smudges
- Wrap in lens paper until needed

6. Load cartridge and start run (load within 1 hour of denaturing samples)

- Flip cartridge 10x to ensure proper mixing
- Using a 1000 uL tip, poke open the hole for the sample
- Load 600 uL sample (avoid air bubbles), gently tap cartridge so libraries pool at bottom
- Follow the on-screen directions to insert the flow cell, reagent cartridge, and PR2 bottle

7. Post-run wash (perform ASAP, within 24 hours of run completion)

- After the run, discard waste, reagent cartridge and PR2 bottle
  - Formamide must be removed from the reagent cartridge (Well #8)
- Fill an empty PR2 bottle with 500 mL 0.5% Tween 20:
  - 25 mL 10% Tween 20
  - 475 MilliQ water
- From PR2 bottle, put 5 mL of 0.5% Tween 20 into each well of the wash tray
- Load wash tray, wash bottle, and waste bottle
- Start post-run wash

Sample details:

- Final library concentration should be ~10 pM
- Cluster density should be ~1000-1200

Kit details:

Box	Ref	Lot	Exp
1			
2			