

Preparation of DNA samples for analysis by *Mu*-Illumina

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Buffers

Low TE: 10 mM Tris pH7.5, 0.1 mM EDTA

10X T4 DNA ligase buffer (Promega; provided with the enzyme)

PB and **PE** buffers supplied with Qiaquick columns (Qiagen)

10X NEB 2 Restriction Enzyme Buffer (NEB)

60 mM Tris-HCl, 150 mM NaCl: to prepare 1 ml, combine 60 μ l 1M Tris pH 7.5, 75 μ l 2M NaCl, 865 μ l H₂O

Gel loading dye: 30% glycerol and a minimal amount of Bromophenol Blue and xylene cyanol to avoid masking DNA fluorescence

1% ethidium bromide (Fisher Scientific)

50X TAE : 242 g Tris Base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8, ddH₂O to bring volume to 1 liter)

20X SSC

PBS

2 X B&W Buffer: 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2M NaCl, 0.1% Tween20

To prepare 10 ml: 100 μ l 1 M Tris-HCl pH 7.5

20 μ l 0.5 M EDTA pH 7.5

6.66 ml 3 M NaCl

10 μ l Tween 20

3197 μ l ddH₂O

Enzymes and Reagents

T4 DNA ligase (3 U/ μ l) (Promega)

T4 DNA polymerase (3 U/ μ l) (NEB)

DNA polymerase I Klenow fragment **3'→5' Exo+** (NEB M0210)

Klenow fragment **3'→5' Exo(-)** (NEB M0212)

T4 Polynucleotide kinase (10 U/ μ l) (NEB)

Phusion High Fidelity Taq DNA Pol (NEB) and the GC Buffer it is supplied with.

100 mM rATP

dNTP mix (10 mM each)

1 mM dATP

“1 kb plus” DNA ladder (Invitrogen)

I-Block (Applied Biosystems) - a “protein-based blocking reagent”.

Dynabeads® M-270 Streptavidin (Invitrogen 653-05)

NuSieve GTG Agarose

QIAquick PCR purification kit (Qiagen 28104)

QIAquick Gel Extraction Kit (Qiagen 28704)

Quant-iT dsDNA HS Assay kit (Invitrogen) (0.2 –100 ng)

Specialized equipment

Bioruptor sonicator (Diagenode)

DynaMag magnet (Invitrogen)

Qubit Fluorometer (Invitrogen)

Oligodeoxynucleotides

Adapter oligo-ACGT : /5'Phos/cgtAGATCGGAAGAGCTCGTATGCCGTCTTCTGCTTG

Adapter oligo-ACGT: ACACTCTTTCCCTACACGACGCTCTTCCGATCTacgT

(lower case nts represent the barcode)

Barcode options: ACGT, CGTT, GTAT, TACT, AGCT, CTGT, GATT, TCAT, GGGT, CCCT, CAAT, ATTT

P5 oligo:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT

P7 oligo: CAAGCAGAAGACGGCATAACGAGCTCTTCCGATCT

Biotinylated Mu TIR oligo (5 μ M stock): /5' dual biotin /CGC CAW SGC CTC CAT TTC GTC

GAA TCCCBT BCB CTC TTC KTC YAT AAT GRC AAT TAT CTC

5' Dual biotin label, from IDT: Mod-code 52-Bio.

Standard Mixed Base Instructions

To use a Standard Mixed Base, simply type in the IUB symbol (from the table below) which represents the desired mix.

R	A,G
Y	C,T
M	A,C
K	G,T
S	C,G
W	A,T
H	A,C,T
B	C,G,T
V	A,C,G
D	A,G,T
N	A,C,G,T

Method

DNA can be extracted from plant tissue with any standard method. It should then be treated with ribonuclease A, extracted once with phenol-chloroform-isoamyl alcohol, and once with chloroform. The DNA is then ethanol precipitated, rinsed with 70% ethanol, and resuspended in Low TE to a concentration of 200-400 ng/ μ l.

1. DNA Shearing

Aim to shear DNA to an average size between 300 and 600 bp.

- Place 10 μ g genomic DNA in 100 μ l low TE in a 0.5 ml microfuge tube.
- Place tube in a Bioruptor sonicator. Set to 30 sec “on” and 30 sec “off”, and sonicate for 8 min.

The sample volume and the type of tube influence shearing efficiency, so these settings may need to be adjusted. Therefore, we recommend checking the DNA by running 1/20th of the sample on an agarose gel when initially establishing conditions. Once suitable conditions are established, they are reproducible and the DNA does need not to be checked each time.

2. Processing the termini of the DNA fragments.

These steps are based on the protocol suggested by Illumina to generate termini that are suitable for ligation to the adapters.

End-polishing and 5' phosphorylation:

- Set-up the following reaction in a thin-walled, 200 μ l tube suitable for PCR.

Sheared DNA (5 μ g)	50 μ l
Low TE	24 μ l
10X T4 DNA ligase buffer	10 μ l
100 mM rATP	1 μ l
dNTP mix (10 mM each)	4 μ l
T4 DNA polymerase (3 U/ μ l)	5 μ l
DNA pol I Klenow fragment, 3' \rightarrow 5' Exo+(5 U/ μ l)	1 μ l
T4 Polynucleotide kinase (10 U/ μ l)	<u>5 μl</u>
	100 μ l

Flick tube gently to mix, and quick spin to collect droplets.

- Incubate for 30 min at 20°C. (It can be convenient to do this in a PCR machine, but do NOT use the heated lid at this temperature.)
- Purify the DNA on a Qiaquick PCR purification kit according to manufacturer's instructions, except that the following buffer volumes should be used: 500 μ l PB and 700 μ l PE for the wash.
- Elute from the column with 35 μ l EB buffer supplied with the kit.

Addition of deoxyA to 3' termini:

- Assemble the following reaction in a thin-walled, 200 μ l tube suitable for PCR:

End-repaired DNA recovered from above:	32-35 μ l
10X NEB 2 restriction enzyme buffer	5 μ l
1 mM dATP	10 μ l
Klenow DNA pol <u>exo(-)</u> (5U/ μ l)	<u>3 μl</u>
	50 μ l

Flick to mix, quick spin to collect droplets.

Incubate 30 min, 37 °C. (If you use a PCR machine, use the heated lid to avoid condensation)

- Purify the DNA with the Qiaquick PCR purification kit, using 250 μ l PB, and washing with 700 μ l PE.
- Elute the DNA from the column with 42 μ l EB buffer supplied with the kit.

3. Anneal Adapters

Assemble the following reaction in a 0.5 ml microfuge tube:

Adapter oligo-1 (200 μ M)	1 μ l
Adapter oligo-2 (200 μ M)	1 μ l
60 mM Tris-HCl pH 7.5, 150 mM NaCl	1 μ l
(Final: 20 mM Tris-HCl pH 7.5, 50 mM NaCl and 400 pmoles of adapters)	

Incubate in a ~94 °C water bath in a beaker (~200 ml water) for 2 min.

Place the beaker on a benchtop and allow the bath to cool to room temp.

Quick spin to pellet condensed drops.

4. Ligate Adapters to the Prepared DNA Fragments

- Add the following to the tube containing the 3 μ l (400 pmoles) annealed adapters:

Processed DNA fragments	40 μ l
10X T4 DNA ligase buffer	5 μ l
100 mM rATP	1 μ l
T4 DNA ligase (3U/ μ l)	2 μ l (Add last)

Flick gently to mix, and quick spin to pellet liquid.

- Incubate 15 min at room temp (or in a 25°C bath if your room is unusually hot or cold)
- Purify on a Qiaquick PCR purification column, using 250 μ l PB and a 700 μ l PE wash.
- Elute from the column with 50 μ l EB

5. Gel purification to enrich DNA fragments of the correct size and to remove unligated adapters

- Pour an agarose gel: 2% NuSieve GTG Agarose in 1XTAE buffer containing ethidium bromide (1 μ l of a 1% ethidium bromide into 100 ml agarose solution).

**** It is important to use this type of agarose****

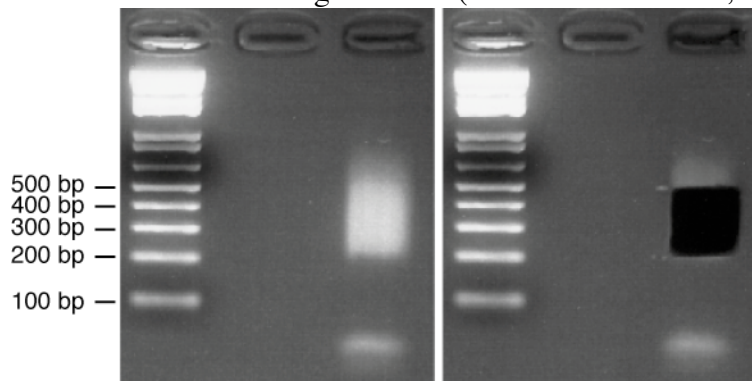
- Reduce the volume of the DNA sample from 50 μ l to 20 μ l by incubating in a Speed-Vac while spinning under vacuum, with **no heat**. This usually takes ~15 minutes. *Be careful not to start the vacuum until the sample is spinning, and to release the vacuum while the sample is still spinning!*

- Add 5 μ l Loading Dye containing minimal dye and load the gel. Run the “1 kb plus” ladder to provide size markers. ** To avoid cross-contamination, leave at least one empty lane between each DNA sample and next to the ladder. **

- Run gel at 70 volts for ~45 minutes (not too long, to minimize the size of the gel slice needed for elution).

- Excise a gel slice containing fragments between 220-500 bp, using a fresh scalpel blade. ** Do not take fragments smaller than 200 bp to avoid contamination by ligated adapters**

It should look something like this: (left- before excision; right- after excision)



- Extract the DNA from the gel slice using a QIAquick Gel Extraction Kit according to the manufacturer’s instructions. (e.g. for a 300 mg gel slice, use 900 μ l QG, place at 55 °C 10 min. Then add 300 μ l isopropanol, apply to column, follow QIAquick manual for washes (do ALL suggested washes), elute with 50 μ l.) (note: 800 μ l is the maximum to apply to the column so you may have to apply half of sample to the column, spin and then load the other half and spin).

- Take 1 μ l of the elution, and dilute 1:10 in low TE. Set aside for the PCR test to evaluate enrichment of *Mu*-sequences (see below: This is the “1st Biotin selection Load”). Use the remainder of the sample for the next step.

6. 1st Biotin-Mu Oligonucleotide Hybridization

Annealing the Mu-TIR oligo to the DNA fragments:

Biotin-MuTIR oligo (5 μ M stock)	1 μ l
Eluted DNA	<u>49 μl</u>
	50 μ l

*** To reduce the labor involved in subsequent sample processing, multiple samples can be pooled at this step. For example, if you have 5 differentially barcoded samples, combine the five DNAs (245 μ l) and 5 μ l Biotin-MuTIR oligo)****

- Heat to 95 °C (hot water in a beaker), 5 min
- Transfer to a 55 °C heat block
- Immediately add 50 μ l 2 X B&W (preheated to 55 °C)
- Incubate at 55 °C for 3 h with occasional mixing and flicking down droplets. (Don't let the sample cool during these mixing steps). Cover the tubes with a glass plate to reduce condensation during the incubation.

7. Preparing Streptavidin-coupled Dynabeads:

While the hybridization is incubating, prepare the magnetic streptavidin-coupled beads (Dynabeads) that will be used to capture the biotinylated *Mu* oligo. *Use a DynaMag magnet to collect the beads at all steps.*

- Prepare one ml of 2% I-Block Solution:

2% I Block	20 mg I-Block powder
0.5% SDS	50 μ l 10% SDS
1 X PBS	100 μ l 10X PBS
H ₂ O	850 μ l

Add 20 mg I-Block powder to 850 μ l water. Then add 100 μ l 10X PBS (RECIPE). Dissolve the I-Block by heating to ~65°C and vortexing vigorously. This can be difficult! Finally, add 50 μ l 10%SDS and mix gently to avoid foaming.

- “Block” sufficient Dynabeads M-270 Streptavidin for all samples. (140 μ l bead slurry/sample is sufficient for both biotin selections)
 - Wash beads 3X with 1 X B&W: ~1 ml/wash for a volume of beads sufficient for up to 10 samples; add the buffer, vortex, collect the beads with the magnet, pipet off the liquid, repeat.
 - Block beads with the 2% I Block Solution:
 - Use 1 ml I-Block solution for a volume of beads sufficient for up to 10 samples.
 - Incubate for 1 h at room temp with constant rotation.
 - Wash beads: Divide the bead slurry into two portions according to the following ratio: 100/140 of the sample will be used to capture the biotin-oligo DNA complexes, and 40/140 of the sample will be used for subsequent capture of free MuTIR oligo that elutes from the beads, and which otherwise can prime PCR and interfere with subsequent steps. These need to be washed in different buffers:

The “large” portion (100 μ l/140 μ l/sample should be washed 3X with 1 X B&W.
The “small” portion (40 μ l/140 μ l) should be washed 3X with low TE.

8. Binding the Biotin-MuTIR/DNA hybrids to Streptavidin-Dynabeads

- Add the Biotin-Mu /genomic DNA hybridization reaction to 1.5 ml microfuge tube containing 50 μ l (250 μ l if pooling 5 samples) of pelleted streptavidin beads (previously I-blocked and rinsed with 1 X B&W from above)
- Incubate 15 min. at room temp with constant agitation
During this incubation, preheat Wash solutions to 55 °C.
- Wash beads four times for 5 min. at 55 °C with 100 μ l Wash : 1XSSC, 0.1% SDS.
Pipet up and down after adding each wash to resuspend the beads. (500 μ l washes if pooling 5 samples)

Wash	
	<u>10 ml</u>
20XSSC	0.5 ml
20% SDS	50 μ l

- Resuspend beads in ~100 μ l 1 X SSC (to dilute out the SDS) and transfer to a new tube
Use the magnet with the fresh tube to separate the beads from the wash. Pipet off the liquid.
- Elute genomic DNA from the biotin Mu-oligo by adding 35 μ l low TE (For pooled samples still use 35 μ l for elution and continue the protocol as you would for an individual sample) and incubating 95 °C for 3 min. (In theory, this doesn't disrupt the biotin/streptavidin interaction, so most of the Mu oligo should stay bound to the beads.).
- Use the magnet to collect the beads. Transfer supernatant to a fresh tube and snap cool on ice for 2 min to avoid reannealing of any eluted Mu oligo to Mu TIRs.
- To remove any remaining Biotin-Mu oligo (which we found could prime PCR if not removed), combine supernatant with 20 μ l of pelleted streptavidin beads (the portion that was previously I-blocked and rinsed with **low TE**). Pipet up and down to mix, collect the beads with the magnet, and immediately pipet off the liquid (which contains your precious DNA sample) and transfer it to a new tube.

9. PCR amplification to bulk up DNA prior to second round of hybrid-selection

PCR cycles are minimized (12 cycles) to reduce bias against sequences that are difficult to PCR.

Template DNA (eluted from streptavidin beads)	30 μ l
5X Phusion High Fidelity DNA Pol (NEB) GC Buffer	10 μ l
2.5 mM dNTPs	4 μ l
P5 primer (25 μ M)	2 μ l
P7 primer (25 μ M)	2 μ l
DMSO	1.5 μ l
Phusion taq (NEB)	0.5 μ l

PCR cycle:
1. 98 C 30 sec
2. 98 C 10 sec
3. 65 C 30 sec
4. 72 C 30 sec
5. Go to step 2 11 times
6. 72 C 5 min
7. 4 C forever

Cleaning the PCR product

- Add 10 μ l 3M NaAc pH 5.2 to 250 μ l PB buffer supplied with the QIAquick PCR purification kit. (This is important to maintain the correct pH - as described in QIAquick protocol)

Continue with the QIAquick protocol, using 700 μ l of PE wash.

- Elute with 50 μ l EB

Take 1 μ l of the elution, and add to 9 μ l low TE. Set aside for the PCR test to evaluate enrichment of *Mu*-sequences (see below: This is the Post-Biotin 1/PCR 1 sample).

- Use the remaining 49 μ l of the sample for the next step.

10. 2nd Biotin Mu Hybridization

This is exactly the same as the first biotin Mu oligo/DNA hybridization (see above), except that **the final elution uses 70 μ l low TE.**

11. PCR amplification (15 cycles)

Template DNA eluted from streptavidin bead selection	30 μ l (1/2 of eluate)
5X Phusion GC Buffer	10 μ l
2.5 mM dNTP	4 μ l
P5 primer (25 μ M)	2 μ l
P7 primer (25 μ M)	2 μ l
DMSO	1.5 μ l
Phusion taq	0.5 μ l

PCR cycle:	1. 98 C 30 sec
	2. 98 C 10 sec
	3. 65 C 30 sec
	4. 72 C 30 sec
	5. Go to step 2 14 times
	6. 72 C 5 min
	7. 4 C forever

Use the QIAquick PCR purification kit as above, using 10 μ l 3M NaAc pH 5.2 to maintain optimal pH, and 250 μ l PB, 700 μ l PE wash)

- Elute with 50 μ l EB

- Remove 1 μ l of the eluate and add to 9 μ l low TE. Save for future Mu-enrichment test (this is Post-Biotin 2/PCR 2).

12. Gel Purification 2

- Reduce volume from 50 μ l to 20 μ l before loading on gel (speed-vac 15 min/no heat as before)
Add Loading dye (with minimal amount of dye)

Gel purify exactly as above (step 5), except that the final elution is in 30 μ l.

In brief, load samples on 2% NuSieve GTG Agarose (important to use this type), in 1XTAE, leaving at least one empty lane between ladder and sample. Run 70 volts for approximately 45 minutes (not very far to keep gel slice small).

- Cut out gel slice containing DNA fragments between 220-500 bp
- Gel purify using a QIAquick Gel Extraction Kit as before:
In brief- for a 300 mg gel slice, add 900 μ l QG, place at 55 °C 10 min, add 300 μ l isopropanol, apply to column, follow QIAquick manual for washes, **elute with 30 μ l**.

This eluate is your sample for Illumina sequencing**

If this gel shows that the second round of PCR is weak, then you can go back and re-PCR using the other 1/2 of the eluate for more cycles (eg: 20 cycles.)

13. Qubit Fluorometer (Invitrogen) – for determining the DNA concentration

****THIS STEP IS CRITICAL FOR OBTAINING HIGH YIELDS OF HIGH QUALITY SEQUENCE. Inaccurate quantification will result in clusters that are not spaced optimally in the flow cell. A nanodrop spectrophotometer is not sufficiently accurate. Do NOT use it!****

- Use Quant-iT dsDNA HS Assay kit (Invitrogen) (0.2 – 100 ng)
Use the tubes that are provided with the fluorometer.
Prepare sufficient volume of Quant-iT Assay Buffer for all samples plus two extra for the standards

Example for 8 samples:	10 X stock:	1990 μ l Quant-iT Buffer
		10 μ l Quant-iT Reagent
		2000 μ l

Mix:

198 μ l Quant-iT Buffer & Reagent + 2 μ l DNA sample for Illumina sequencing

190 μ l Quant-iT Buffer & Reagent + 10 μ l Standard 1

190 μ l Quant-iT Buffer & Reagent + 10 μ l Standard 2

- Let sit 2 min before measuring the concentration

Measure concentration of DNA using the Qubit Fluorometer

14. Diluting the DNA for Illumina sequencing.

For Illumina Sequencing, the DNA fragments should be at 10 nM and 10 μ l of sample are applied to each Illumina channel.

For a sample containing DNA fragments averaging 300 bp in size, your sample should be at 19.8 ng DNA/10 μ l, based on the following calculation:

$$\begin{aligned}10 \text{ nM} &= 0.01 \times 10^{-12} \text{ moles}/\mu\text{l} = (X \text{ gms}/\mu\text{l})(1 \text{ mole bp}/660 \text{ gm})(1/300 \text{ bp}) \\X &= 1.98 \times 10^{-9} \text{ gms}/\mu\text{l} \\X &= 1.98 \text{ ng}/\mu\text{l} \times 10 \mu\text{l} = 19.8 \text{ ng}/10 \mu\text{l}\end{aligned}$$

Dilute your final gel eluate in low TE to achieve this concentration (2 ng/ μ l).

If you are multiplexing, add an equal volume of each barcoded sample (at 2 ng/ μ l) to the final 10 μ l sample submitted for sequencing. If using 10 barcodes (our routine method), use 1 μ l of each.

PCR to test for enrichment of Mu-containing sequences

Samples:

- | | |
|---|-----------|
| 1. 1 st Biotin "Load" (1:10 dil) | 1 μ l |
| 2. Post-Biotin 1/PCR 1 (1:10 dil) | 1 μ l |
| 3. Post-Biotin 2/PCR 2 (1:10 dil) | 1 μ l |

Mu1 specific oligos: 192 bp product (internal to Mu1)

Oligos (0.5 μ g/ μ l):

RC Mu For 5'-CTT GTC TCC GTC GCC GCG CTC T-3'	0.5 μ l
RC Mu Rev 5'-CCC CGG CGT GGA GGC CCG AGA A-3'	0.5 μ l
5X Phusion GC Buffer	5 μ l
2.5 mM dNTP	2 μ l
DMSO	0.75 μ l
Phusion taq pol	0.5 μ l
H ₂ O	<u>15 μl</u>
	25 μ l

- PCR cycle:
1. 98 C 30 sec
 2. 98 C 10 sec
 3. 60 C 15 sec
 4. 72 C 15 sec
 5. Go to step 2 19 times
 6. 72 C 5 min
 7. 4 C forever

Example of results:

1st Biotin "load" (samples 1-6)	Post-Biotin1/PCR1 (samples 1-6)	Post-Biotin2/PCR2 (samples 1-6)
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