Methyl-MAPS Protocol v3.0b

This is an updated version of the protocol found in:


For more information or questions about this protocol please contact:

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Note: This protocol is adapted from the 2x60 Mate-Pair Kit from LifeTech: “Fragment Library Preparation: 5500 Series SOLiD™ Systems User Guide”. Reagent product numbers can be found there.
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Time Line and Protocol Notes

McrBC & RE Digests 15 hours
End Repair and Ligate MP Adaptors 2 hours
Circularize DNA and Nick Translate 3 hours
Digest DNA with T7 and S1 2 hours
A-Tail and Ligate Y-Adaptor 2 hours
Trial Amplification 2 hours
Final Library Amplification 2 hours
Size-Select and Purify Final Library 3 hours
Total Time 32 hours

Notes:

• You can easily parallelize phases of this protocol, especially the digestions. The circularization and nick translation steps are highly time sensitive. I would not recommend handling more than 2 libraries at a time for this step. It can be tricky to handle more than 4 libraries at a time for subsequent bead steps for A-tailing and ligation steps.
• Be sure to use low retention tips and Eppendorf LoBind tubes throughout protocol
• SPRI Bead Tips
  o For SPRI beads prior to nick-translation, I either flick mix the tubes well or very gently vortex (only after all digestions).
  o Always use fresh 70% ethanol to wash SPRI beads.
  o To pipet accurately, I pipet the same volume of water twice and discard. Each time note how high water comes up pipet tip. Be sure beads come up to the same level and there are no air bubbles in the tip. Do not use a P-1000 to pipet beads.
  o Gently vortex stock bead tube between each pipetting.
• Do not stop at steps, unless protocol says it is okay to.
• You will likely run out of plasmid-safe buffer, enzyme and 100mM ATP and will need to supplement AB kit with additional. Plasmid-Safe is available from Epicentre.
• DO NOT use a nanodrop for estimating your initial DNA concentration. Use a Qubit, or some other DNA specific assay. Run starting gDNA on an agarose gel to ensure there is not degradation. DNA should run as a relatively tight high molecular weight band with little to no smearing.
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Methylation Sensitive- and Dependent-Digestion
In parallel, digest DNA with methylation-sensitive (RE = Acil, BstUI, HhaI, HpaII, and HpyCH4IV) and methylation-specific (McrBC) restriction enzymes to generate the methylated and unmethylated fraction libraries, respectively. Rounds of McrBC and RE can be done in parallel.

Note: Size selection is performed with final bead purification. You must titrate your SPRI beads with ladders to determine the correct amount to use with every lot number. This cutoff will vary with different bead lot numbers. You must be precise with the amount of SPRI beads added! We aim to set the size cutoff between 500 and 700 bp. Ideally you want a partial loss of 500-600 bp, no loss of 700+ bp and complete loss of 400 bp and less.

McrBC Digestion
Digest ≤5ug of DNA per tube. More than this can result in incomplete digestion. Use multiple tubes if >5ug of DNA is needed.

McrBC Round 1
1. Set up reactions in 1.5ml LoBind tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Y</td>
<td></td>
</tr>
<tr>
<td>NEB Buffer 2</td>
<td>5</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>0.5</td>
</tr>
<tr>
<td>GTP (100X)</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>43.5-X-Y</td>
</tr>
<tr>
<td>McrBC X</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X ul = ug DNA

2. Digest at 37°C for 4-6 hours.

AMPure DNA Purification (45 min, use FRESHLY MADE 70% EtOH)
1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Transfer 80ul of SPRI beads to reaction.
4. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
5. Place tube on magnet for 3-5 min, until solution is clear
6. While tube is on magnet, carefully remove clear supernatant and discard.
7. While tube is on magnet, add 500ul of 70% EtOH
8. Let beads settle for 1min.
9. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
10. Remove EtOH
11. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
12. Add 30µl of nuclease free H2O to beads
13. Vortex well and incubate at room temperature for 3 min
14. Place tube on magnet and wait 2-3 min until solution is clear
15. Remove supernatant to fresh tube. Be careful not to remove beads.

**STOPPING POINT: Store at 4°C**

**McrBC Round 2**
1. Set up reactions in 1.5ml LoBind tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>NEB Buffer 2 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>1</td>
</tr>
<tr>
<td>GTP (100X)</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>13.5-X</td>
</tr>
<tr>
<td>McrBC</td>
<td>X</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X µl = ug DNA

2. Digest at 37°C for 4-6 hours.

**AMPure DNA Purification** (45 min, use FRESHLY MADE 70% EtOH)
1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Transfer 80µl of SPRI beads to reaction.
4. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
5. Place tube on magnet for 3-5 min, until solution is clear
6. While tube is on magnet, carefully remove clear supernatant and discard.
7. While tube is on magnet, add 500µl of 70% EtOH
8. Let beads settle for 1min.
9. Remove EtOH and wash 1 more time with 500µl of 70% EtOH
10. Remove EtOH
11. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
12. Add 30µl of nuclease free H2O to beads
13. Vortex well and incubate at room temperature for 3 min
14. Place tube on magnet and wait 2-3 min until solution is clear
15. Remove supernatant to fresh tube. Be careful not to remove beads.

**STOPPING POINT: Store at 4°C**
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McrBC Round 3

1. Set up reactions in 1.5ml LoBind tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>NEB Buffer 2 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>0.5</td>
</tr>
<tr>
<td>GTP (100X)</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>14-X</td>
</tr>
<tr>
<td>McrBC</td>
<td>X</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50</td>
</tr>
</tbody>
</table>

X µl = ug DNA

2. Digest at 37°C for 2-3 hours.
3. Combine like McrBC libraries if needed.

Size Selection

1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Bring reaction volume to 100 ul if needed.
4. Transfer 55ul of SPRI beads to reaction (Titrate this with a ladder!).
5. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
6. Place tube on magnet for 3-5 min, until solution is clear
7. While tube is on magnet, carefully remove clear supernatant and discard.
8. While tube is on magnet, add 500ul of 70% EtOH
9. Let beads settle for 1 min.
10. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
11. Remove EtOH
12. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
13. Add 50ul of nuclease free H2O to beads
14. Vortex well and incubate at room temperature for 3 min
15. Place tube on magnet and wait 2-3 min until solution is clear
16. Remove supernatant to fresh tube. Be careful not to remove beads.

**STOPPING POINT: Store at 4° C**
**RE Digestion**
Digest ≤5ug of DNA per tube. More than this can result in incomplete digestion. Use multiple tubes if >5ug of DNA is needed.

**RE Round 1**
1. Set up reactions in 1.5ml LoBind tubes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Y</td>
</tr>
<tr>
<td>NEB Buffer 1 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>Water</td>
<td>45-2X-Y</td>
</tr>
<tr>
<td>HpaII</td>
<td>X</td>
</tr>
<tr>
<td>HpyCH4IV</td>
<td>X</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X ul = ug DNA

2. Digest at 37°C for 4-6 hours.

**AMPure DNA Purification** (45 min, use FRESHLY MADE 70% EtOH)
1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Transfer 80ul of SPRI beads to reaction.
4. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
5. Place tube on magnet for 3-5 min, until solution is clear
6. While tube is on magnet, carefully remove clear supernatant and discard.
7. While tube is on magnet, add 500ul of 70% EtOH
8. Let beads settle for 1min.
9. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
10. Remove EtOH
11. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
12. Add 30ul of nuclease free H2O to beads
13. Vortex well and incubate at room temperature for 3 min
14. Place tube on magnet and wait 2-3 min until solution is clear
15. Remove supernatant to fresh tube. Be careful not to remove beads.

**STOPPING POINT: Store at 4°C**
**RE Round 2**

1. Set up reactions in 1.5ml LoBind tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>NEB Buffer 3 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>BSA (100x)</td>
<td>0.5</td>
</tr>
<tr>
<td>Water</td>
<td>14.5-1.5X</td>
</tr>
<tr>
<td>AciI</td>
<td>X</td>
</tr>
<tr>
<td>HhaI</td>
<td>0.5X</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X ul = ug DNA

2. Digest at 37°C for 4-6 hours.

**AMPure DNA Purification** (45 min, use FRESHLY MADE 70% EtOH)

1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Transfer 80ul of SPRI beads to reaction.
4. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
5. Place tube on magnet for 3-5 min, until solution is clear
6. While tube is on magnet, carefully remove clear supernatant and discard.
7. While tube is on magnet, add 500ul of 70% EtOH
8. Let beads settle for 1 min.
9. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
10. Remove EtOH
11. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
12. Add 30ul of nuclease free H2O to beads
13. Vortex well and incubate at room temperature for 3 min
14. Place tube on magnet and wait 2-3 min until solution is clear
15. Remove supernatant to fresh 0.5 mL tube. Be careful not to remove beads.

**STOPPING POINT: Store at 4°C**
RE Round 3
1. Make digestion mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>NEB Buffer 2 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>Water</td>
<td>15-X</td>
</tr>
<tr>
<td>BstUI</td>
<td>X</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X ul = ug DNA

2. Digest at 60°C for 2-3 hours in the PCR machine.

AMPure DNA Purification (45 min, use FRESHLY MADE 70% EtOH)
1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Transfer 80ul of SPRI beads to reaction.
4. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
5. Place tube on magnet for 3-5 min, until solution is clear
6. While tube is on magnet, carefully remove clear supernatant and discard.
7. While tube is on magnet, add 500ul of 70% EtOH
8. Let beads settle for 1 min.
9. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
10. Remove EtOH
11. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
12. Add 30ul of nuclease free H2O to beads
13. Vortex well and incubate at room temperature for 3 min
14. Place tube on magnet and wait 2-3 min until solution is clear
15. Remove supernatant to fresh tube. Be careful not to remove beads.

STOPPING POINT: Store at 4° C
RE Round 4

1. Set up reactions in 1.5ml LoBind tubes.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>30</td>
</tr>
<tr>
<td>NEB Buffer 1 (10x)</td>
<td>5</td>
</tr>
<tr>
<td>Water</td>
<td>15-2X</td>
</tr>
<tr>
<td>HpaII</td>
<td>X</td>
</tr>
<tr>
<td>HpyCH4IV</td>
<td>X</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

X µl = ug DNA

2. Digest at 37°C for 2-4 hours.

SIZE SELECTION

AMPure DNA Purification  (45 min, use FRESHLY MADE 70% EtOH)

1. Let SPRI beads come to room temperature.
2. Vortex beads well to ensure a homogeneous solution.
3. Bring reaction volume to 100 ul if needed.
4. Transfer 55µl of SPRI beads to reaction (Titrate this if needed!).
5. Very gently vortex briefly to mix and put on rotator for 5 min (increase to 10 min if using small amounts of DNA)
6. Place tube on magnet for 3-5 min, until solution is clear
7. While tube is on magnet, carefully remove clear supernatant and discard.
8. While tube is on magnet, add 500ul of 70% EtOH
9. Let beads settle for 1min.
10. Remove EtOH and wash 1 more time with 500ul of 70% EtOH
11. Remove EtOH
12. Pulse spin and remove any remaining EtOH and dry 3 min at room temperature (do not over dry!)
13. Add 50ul of nuclease free H2O to beads
14. Vortex well and incubate at room temperature for 3 min
15. Place tube on magnet and wait 2-3 min until solution is clear
16. Remove supernatant to fresh tube. Be careful not to remove beads.

STOPPING POINT: Store at 4° C
**NOTE:** For the rest of the protocol use the 2x60 Mate-Pair Kit from LifeTech. Instructions are modified from “Fragment Library Preparation: 5500 Series SOLiD™ Systems User Guide”.

### End-repair the DNA

#### Quantitate Digested, Size Selected DNA

Quantitate the purified DNA using 1 μL of sample with the Qubit dsDNA HS Assay.

#### End-repair

For fast and efficient blunt-ended ligation, End Polishing E2 enzyme is used to convert DNA with damaged or incompatible 5′-protruding and/or 3′-protruding ends to 5′-phosphorylated, blunt-ended DNA. End Polishing E1 enzyme and ATP are also included for phosphorylation of the 5′-ends of the blunt-ended DNA for subsequent ligation.

1. For <5μg of starting material, combine and mix the components below in a LoBind tube. If >5 μg of starting material for mate-paired libraries with 1–3 kb inserts, scale up or set up parallel reactions:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size-selected DNA</td>
<td>50.0</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>17.0</td>
</tr>
<tr>
<td>5× Reaction Buffer</td>
<td>20</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>4.0</td>
</tr>
<tr>
<td>End Polishing E1</td>
<td>4.0</td>
</tr>
<tr>
<td>End Polishing E2</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

2. Incubate the mixture at room temperature (20–25°C) for 30 minutes.

3. Heat inactivate the enzymes at 75°C for 20 minutes.

4. Put the DNA on ice.
**Ligate MP Adaptors to the DNA**

This ligation step adds the MP Adaptors to the sheared, end-repaired DNA. The MP Adaptors are missing a 5’ phosphate at the non-joining end; as a result, there is a nick on each strand when the DNA is circularized. The MP Adaptors are included in double-stranded form in the SOLiD™ Mate-Paired Library Standard Adaptors module.

**Calculate the amount of adaptor to use**

Calculate the amount of adaptor needed, \( Y \), for the reaction based on the amount of DNA before the end-repair step:

\[
\{ \mu\text{g-to-pmol conversion factor } \} = 10^6 \text{ pg/ug} \times 1 \text{ pmol/660 pg} \times 1/\{\text{avg insert size}\}
\]

**NOTE:** Adjust avg insert size based on size selection. For defaults above, avg insert size = 700bp. I.e. conversion factor = 2.16.

\[
Y \mu\text{L adaptor} = \{\mu\text{g DNA}\} \times \{\mu\text{g-to-pmol conversion factor }\} \times 50 \times 1 \mu\text{L adaptor}/25 \text{ pmol}
\]

Note: If \( Y \leq 1 \mu\text{L} \), use 1 \( \mu\text{L} \) in the reaction.

Example: If the average insert size is 1500 bp and you have 1ug DNA then the conversion factor is 1 pmol/ug and \( Y = 2 \mu\text{L} \).

**Ligate the MP Adaptors to the DNA**

1. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End Repaired DNA</td>
<td>100</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>24 – 2 ( \times Y )</td>
</tr>
<tr>
<td>5× Reaction Buffer</td>
<td>10</td>
</tr>
<tr>
<td>ATP, 100 mM</td>
<td>1</td>
</tr>
<tr>
<td>MPR Adaptor (ds), 25 uM</td>
<td>( Y \dagger )</td>
</tr>
<tr>
<td>MPL Adaptor (ds), 25 uM</td>
<td>( Y \dagger )</td>
</tr>
<tr>
<td>T4 Ligase, 5U/ul</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>150</strong></td>
</tr>
</tbody>
</table>

\( \dagger \) If \( Y < 1 \mu\text{L} \), use 1 \( \mu\text{L} \).

2. Incubate the reaction mixture at room temperature (20–25°C) for 30 minutes.
Purify the DNA using Agencourt AMPure® XP Reagent

1. Resuspend the Agencourt AMPure® XP Reagent beads.

2. Bind the DNA to the Agencourt AMPure® XP Reagent:
   a. Prepare the bead suspension in the sample reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Reaction</td>
<td>150</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>150 †</td>
</tr>
<tr>
<td>Agencourt AMPure® XP Reagent</td>
<td>240 ‡</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>540</strong></td>
</tr>
</tbody>
</table>

   † Equal to 1 volume of sample reaction.
   ‡ Equal to 1.6 volumes of sample reaction.

   b. Vortex the beads for 15 seconds, then pulse-spin.
   c. Incubate the mixture at room temperature (20–25°C) for 5 minutes.
   d. Place the tube in a magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash the DNA 2 times. For each wash, keep the tube in the magnetic rack:
   a. Add 600 μL of 70% ethanol to the tube, without disturbing the beads.
   b. Keep the tube in a magnet for at least 1 minute, then remove and discard the supernatant without disturbing the beads.

4. Remove the tube from the magnet, pulse-spin the tube, return the tube to the magnetic rack, then remove and discard the supernatant with a 20-μL pipettor.

5. Open the tube, then dry the beads at room temperature (20–25°C) for 3 minutes.

6. Elute the DNA:
   a. Remove the tube from the magnet, then add 50 μL Elution Buffer. (E1)
   b. Vortex the beads for 15 seconds, pulse-spin, then incubate the beads at room temperature (20–25°C) for 3 minutes.
   c. Place the tube in magnet for at least 1 minute until the solution clears.
   d. Transfer the supernatant to a new 1.5-mL LoBind Tube.
**Quantitate Digested, Size Selected DNA**
Quantitate the purified DNA using 1 μL of sample with the Qubit dsDNA HS Assay.

**Assess DNA recovery**
If the recovery compared to starting unsheared genomic DNA is...
>5% then continue...
<5% Minimize loss in the following purification steps and evaluate the stringency of the first size selection. Life Technologies recommends >+/-10% of the target insert size.
< 50 ng – too little material to continue, restart and use more DNA.

**STOPPING POINT: Store at 4°C**

**Circularize the DNA by intra-molecular hybridization**
The mate-paired adaptor contains a blocking oligonucleotide to protect the 3’ overhangs of the MP Adaptors from self-annealing. At circularization, heat denaturation removes the blocking oligonucleotide. The DNA circularizes through intramolecular hybridization at low concentrations.

1. Fill all of the holes to be used in a heat block with water, then pre-heat the block to 70°C. Note: This can take up to an hour or more to equilibrate!

2. Calculate the total volume of the circularization reaction (T, μL), so that for a known concentration of DNA [DNA] (ng/μL) and known volume of DNA (V), the final concentration of DNA in the reaction is 0.5 ng/μL:  \( T = [\text{DNA}] \times V / 0.5 \)

**Example**
If \([\text{DNA}] = 5 \text{ ng/μL}\) and \(V = 50 \mu\text{L}\), then \(T = 500 \mu\text{L}\).

**Note**: Use multiple tubes if necessary and recombine at purification stage. Magnet can only bind ~ 1ml of solution total at a time. The LifeTech Protocol recommends not going above \(T=1000\), but when I tried this my libraries did not work. \(T > 1000\) yields successful libraries though.

3. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>V</td>
</tr>
<tr>
<td>10x Plasmid-Safe Buffer</td>
<td>(T/10)</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>(T-(T/10)-V)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>(T)</td>
</tr>
</tbody>
</table>
4. Incubate the reaction in the heat block at 70°C for 5 minutes, then place the reaction on ice for 5 minutes.

**IMPORTANT!** The incubation time is critical. Keep the time as close to 5 minutes as possible, then proceed to the next immediately to “Isolate the circularized DNA”.

**Isolate the circularized DNA**

*Treat the DNA with Plasmid-Safe™ DNase*

Plasmid-Safe™ DNase is used to eliminate uncircularized DNA. After Plasmid-Safe™ DNase-treatment, the DNA is purified using the Agencourt AMPure® XP Reagent.

1. Combine the following, where T=the total volume of the circularization reaction (μL):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circularized DNA</td>
<td>T</td>
</tr>
<tr>
<td>ATP (100 mM)</td>
<td>T/100</td>
</tr>
<tr>
<td>Plasmid-Safe DNase (10U/ul)</td>
<td>T/100</td>
</tr>
</tbody>
</table>

**Example**

If T = 800 μL, then:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circularized DNA</td>
<td>800</td>
</tr>
<tr>
<td>ATP (100 mM)</td>
<td>8</td>
</tr>
<tr>
<td>Plasmid-Safe DNase (10U/ul)</td>
<td>8</td>
</tr>
</tbody>
</table>

2. Incubate the reaction mixture at 37°C for 40 minutes.

**Purify the DNA using Agencourt AMPure® XP Reagent**

1. Resuspend the Agencourt AMPure® XP Reagent beads.

2. Bind the DNA to the Agencourt AMPure® XP Reagent:
   a. Prepare the bead suspension in the sample reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Reaction</td>
<td>T</td>
</tr>
<tr>
<td>Bead Dilution Buffer</td>
<td>0.7 x T</td>
</tr>
<tr>
<td>Agencourt AMPure®XP Reagent</td>
<td>0.3 x T</td>
</tr>
</tbody>
</table>
b. Vortex the beads for 15 seconds, then pulse-spin.
c. Incubate the mixture at room temperature (20–25°C) for 5 minutes.
d. Place the tube in a magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash the DNA 2 times. For each wash, keep the tube in the magnetic rack:
a. Add 600 μL of 70% ethanol to the tube, without disturbing the beads.
b. Keep the tube in a magnet for at least 1 minute, then remove and discard the supernatant without disturbing the beads.

4. Remove the tube from the magnet, pulse-spin the tube, return the tube to the magnetic rack, then remove and discard the supernatant with a 20-μL pipettor.

5. Open the tube, then dry the beads at room temperature (20–25°C) for 3 minutes.

6. Mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease Free Water</td>
<td>84</td>
</tr>
<tr>
<td>Nick Translation Buffer</td>
<td>10</td>
</tr>
</tbody>
</table>

7. Elute the DNA:
a. Remove the tube from the magnet, then add the 94 μL pre-mixed solution of Nick Translation Buffer to the tube of DNA.
b. *Gently* vortex the beads for 15 seconds, pulse-spin, then incubate the beads at room temperature (20–25°C) for 3 minutes.
c. Place the tube in magnet for at least 1 minute until the solution clears.
d. Transfer the supernatant to a new 0.2-mL PCR tube.

**IMPORTANT! Proceed to the next step immediately.**

**Quantitate the circularized DNA (OPTIONAL)**
Quantitate the purified DNA using 1 μL of sample with the Qubit dsDNA HS Assay. Set up tubes ahead of time, and measure amounts later.

**IMPORTANT! Proceed to the next step immediately.**
Nick-translate the circularized DNA

Nick translation using E. coli DNA polymerase I translates the nick into the genomic DNA region. The size of the mate-paired tags to be produced can be controlled by adjusting the reaction temperature and time. For convenience, for different mate-tag sizes, change the reaction time but keep the temperature constant. See Appendix for more information about changing the time for different size libraries.

IMPORTANT! Incubate the nick translation reaction at 5°C on a thermal cycler using the “No heated lid” feature. DNA polymerase I is very sensitive to slight changes in temperature. If your thermocycler does not have a “No heated lid” feature, leave the lid off. Before adding enzyme to the reaction mix for nick translation, chill the enzyme and the reaction mix separately in a thermocycler at 5°C for several minutes.

1. Combine in the 0.2-mL PCR tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-treated, purified DNA</td>
<td>93-94</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>5</td>
</tr>
</tbody>
</table>

2. Vortex the mix, then pulse-spin.

3. Incubate the mix without DNA polymerase I at 5°C in a thermocycler for 2–3 minutes. Use the “no heated lid” feature or leave the lid off.

4. In another 0.2-mL PCR tube, add 3 μL of DNA polymerase I, then pulse spin.

5. Incubate the DNA polymerase I at 5°C in a thermocycler for >1 minute. Use the “no heated lid” feature or leave the lid off.

6. Prepare 400μL of Binding Buffer (B2-S) with isopropanol (55%) in a 1.5-mL LoBind Tube.

7. Set the timer to 6 minutes. Note: The time for nick-translation depends on laboratory practice and thermocycler conditions. See Appendix for changing the time for different size libraries.

8. Transfer all of the reaction mix to the tube containing the DNA polymerase I incubating at 5°C, then pipet the total reaction mix up and down 5 times to mix. Use the “no heated lid” feature or leave the lid off.

9. Start the timer.

10. At the end of the incubation, immediately transfer the nick translation reaction to the
1.5-mL LoBind Tube, containing Binding Buffer (B2-S). Binding Buffer (B2-S) denatures the enzyme and stops the reaction.

**Purify the DNA with the SOLiD™ Library Micro Column Purification Kit**

1. Pre-spin an empty PureLink® Microcolumn in collection tube at 10,000×g for 1 minute. Verify the column membranes are intact and are not lifted or folded after the spin.

2. Load the DNA onto the PureLink® Micro columns:
   a. Mix the nick-translated DNA well in Binding Buffer (B2-S) with isopropanol (55%).
   b. Apply all of the mix to the PureLink® Micro column(s) in collection tube(s).
   c. Spin the column(s) at 10,000×g for 1 minute at room temperature, then discard the flow-through. dsDNA is bound to the column.

3. Wash the column(s):
   a. Return the PureLink® Micro column(s) to the same collection tube(s).
   b. Add 650 μL of Wash Buffer (W1) with ethanol to wash the column(s).
   c. Spin the column(s) at 10,000×g for 1 minute at room temperature, then discard the flow-through.
   d. Spin the column(s) at 14,000 × g at room temperature to remove residual wash buffer.

4. Elute the DNA:
   a. Transfer the column(s) to clean 1.5-mL LoBind tube(s).
   b. Add 25 μL of Elution Buffer (E1) to the center of the column(s) to elute the DNA, then let the column(s) stand for 1 minute.
   c. Spin the column(s) at 14,000×g for 1 minute at room temperature.
   d. Add the eluate from the last spin back to the column(s), then let the column(s) stand for 1 minute.
   e. Spin the column(s) at 14,000 × g for 1 minute at room temperature.

5. If necessary, pool the eluted DNA into one 1.5-mL LoBind Tube.

**STOPPING POINT: Store at 4°C**
Digest the DNA with T7 Exonuclease and S1 Nuclease

T7 exonuclease recognizes the nicks within the circularized DNA. With its 5’ - 3’ exonuclease activity, T7 exonuclease digests the unligated strand away from the tags creating a gap in the sequence. This gap creates an exposed single-stranded region that is more easily recognized by S1 Nuclease, so the mate-paired tags can be cleaved from the circularized template. S1 nuclease leaves 5’ phosphorylated products.

Digest the DNA with T7 exonuclease

1. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>25</td>
</tr>
<tr>
<td>10x Buffer 4</td>
<td>5.0</td>
</tr>
<tr>
<td>T7 exonuclease</td>
<td>2.0</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>18.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50</strong></td>
</tr>
</tbody>
</table>

2. Incubate the reaction mixture at 37°C for 15 minutes.
3. Heat inactivate the T7 exonuclease at 70°C for 20 minutes.
4. Chill the reaction on ice for 5 minutes.

Digest the DNA with S1 nuclease

1. Freshly dilute 1 μL of S1 Nuclease to 50U/μL with S1 Nuc (nuclease) Dilution Buffer. Note: Concentration is variable between kits!

2. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>50</td>
</tr>
<tr>
<td>3M NaCl</td>
<td>1.7</td>
</tr>
<tr>
<td>S1 Nuclease (Diluted)</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>53.7</strong></td>
</tr>
</tbody>
</table>

3. Incubate the reaction mixture at 37°C for 45 minutes.

**IMPORTANT! Proceed to the next step immediately.**
Purify the DNA using Agencourt AMPure® XP Reagent

1. Resuspend the Agencourt AMPure® XP Reagent beads.
2. Bind the DNA to the Agencourt AMPure® XP Reagent:
   a. Prepare the bead suspension in the sample reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Reaction</td>
<td>53</td>
</tr>
<tr>
<td>Agencourt AMPure® XP Reagent</td>
<td>95†</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>148</strong></td>
</tr>
</tbody>
</table>

† Equal to 1.8 volumes of sample reaction.

b. Vortex the beads for 15 seconds, then pulse-spin.
   c. Incubate the mixture at room temperature (20–25°C) for 5 minutes.
   d. Place the tube in a DynaMagTM-2 magnetic rack for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash the DNA 2 times. For each wash, keep the tube in the magnetic rack:
   a. Add 600 μL of 70% ethanol to the tube, without disturbing the beads.
   b. Keep the tube in a magnet for at least 1 minute, then remove and discard the supernatant without disturbing the beads.

4. Remove the tube from the magnet, pulse-spin the tube, return the tube to the magnetic rack, then remove and discard the supernatant with a 20-μL pipettor.

5. Open the tube, then dry the beads at room temperature (20–25°C) for 3 minutes.
6. Elute the DNA:
   a. Remove the tube from the magnet, then add 50 μL Elution Buffer. (E1)
   b. Vortex the beads for 15 seconds, pulse-spin, then incubate the beads at room temperature (20–25°C) for 3 minutes.
   c. Place the tube in magnet for at least 1 minute until the solution clears.
   d. Transfer the supernatant to a new 1.5-mL LoBind Tube.

**STOPPING POINT: Store at 4°C**
Add A-Tail to the digested DNA
Adding a dA tail to the S1-nuclease-treated DNA by A-Tailing Enzyme II increases the efficiency of ligation to P1-T and P2-T Adaptors.

1. Combine to prepare the dA-tailing mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7/S1 Digested DNA</td>
<td>50</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>36</td>
</tr>
<tr>
<td>Nick Translation Buffer</td>
<td>10</td>
</tr>
<tr>
<td>dA + dNTP Mix</td>
<td>1.0</td>
</tr>
<tr>
<td>A-Tailing Enzyme II</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

2. Incubate the reaction mix at 37°C for 30 minutes.

3. Add 5.0μL of 0.5M EDTA to the dA-tailing mix to stop the reaction.

4. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stopped dA-tailing mix</td>
<td>105</td>
</tr>
<tr>
<td>Bead Binding Buffer</td>
<td>200</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>95</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>400</strong></td>
</tr>
</tbody>
</table>
Bind the library molecules to streptavidin beads

Prewash the beads
Dynabeads® MyOneTM Streptavidin C1 specifically bind to the biotin-labeled MP Adaptor in the library molecules to purify the library from side products.

1. Prepare 1×BSA solution (amounts are per library):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100x BSA</td>
<td>5</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>495</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>500</strong></td>
</tr>
</tbody>
</table>

2. Vortex the tube of Dynabeads® MyOne™ Streptavidin C1, then transfer 50μL of the beads into a 1.5-mL LoBind Tube.

3. Add 500μL of Bead Wash Buffer to the 50μL of solution of beads, vortex the beads for 15 seconds, then pulse-spin.

4. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

5. Add 500μL of 1×BSA and vortex for 15 seconds, then pulse-spin the tube.

6. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

7. Add 500μL of Bead Binding Buffer. Vortex the beads for 15 seconds, then pulse-spin.

**IMPORTANT! Proceed to next step only after the A-tailing of the DNA is stopped.**

8. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

Bind the library DNA molecules to the beads
1. Add the entire 400μL of solution of library DNA in Bead Binding Buffer to the pre-washed beads, then vortex for 15 seconds.

2. Rotate the solution at room temperature (20–25°C) for 30 minutes, then pulse-spin.
Wash the bead-DNA complex

1. Prepare 1× Reaction Buffer. For one sample:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Reaction Buffer</td>
<td>120</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>480</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>600</strong></td>
</tr>
</tbody>
</table>

2. Place the tube with the bead-DNA complex in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash the beads 3 times. For each wash:
   a. Resuspend the beads in 500 µL of Bead Wash Buffer. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

4. Wash and resuspend the beads:
   a. Resuspend the beads in 500 µL of 1× Reaction Buffer. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.
   c. Resuspend the beads in 86 µL of 1× Reaction Buffer.

Ligate Y-Adaptor to the DNA

The ligated library molecules are bound to streptavidin beads, washed, and purified from ligation by-products.

1. Ligate the Y-Adaptor to the bead-bound DNA:
   a. Combine:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-bead complex</td>
<td>86</td>
</tr>
<tr>
<td>Y-Adaptor (15 uM)</td>
<td>2.7</td>
</tr>
<tr>
<td>T4 DNA Ligase, 5 U/µl</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

   b. Rotate the reaction mixture at room temperature (20–25°C) for 30 minutes.
   c. Place the tube in the magnet for >1 minute until the solution clears, then remove and discard the supernatant.
2. Wash the beads 3 times. For each wash:
   a. Resuspend the beads in 500 μL of Bead Wash Buffer. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash and resuspend the beads:
   a. Resuspend the beads in 500 μL of Buffer E1. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.
   c. Resuspend the beads in 30 μL of Buffer E1.

**STOPPING POINT: Store bead-DNA complex at 4°C from here on out.**

**Trial-amplify the library**

The library is trial-amplified using Illumina Index Primers with the Platinum® PCR Amplification Mix. Trial amplification determines the number of PCR cycles to be used for final library amplification without overamplification. Choose the number of PCR cycles from the trial PCR so that the amplified library is just visible on 2% E-Gel® EX Gel. Platinum® PCR Amplification Mix contains a proofreading enzyme for high-fidelity amplification.

1. Prepare a PCR master mix for amplification reactions:

<table>
<thead>
<tr>
<th>Component†</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Amplification Mix</td>
<td>47</td>
</tr>
<tr>
<td>PCR 1.0 Primer (25 uM)</td>
<td>1.9</td>
</tr>
<tr>
<td>Index Primer (25 uM)</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>50.8</strong></td>
</tr>
</tbody>
</table>

† I have had better success not adding PCR 2.0 Primer per the usual Illumina instructions.

2. Vortex the PCR master mix. For the negative control, transfer 23 μL of the PCR master mix to a PCR tube. Label the tube “PCR #0”.

3. Add remaining 25 μL of PCR master mix to PCR tube labelled “PCR #1”. Add 2 μL DNA-bead complex solution. Mix by pipetting up and down.
4. Run on Thermocycler:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>Denature</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Cycling†</td>
<td>Denature</td>
<td>94°C</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>64°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>Extend</td>
<td>70°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Holding</td>
<td>Extend</td>
<td>70°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Holding</td>
<td>-</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

† Run for 18 cycles if using 1-2 ug starting DNA. You may want to try 16 if using more DNA. If you need to amplify 20 cycles to get a band for trial PCR this is fine. Much more than that and I would be concerned about library complexity and would remake the library.

5. Check products on 2% Agarose TBE Gel with a 50 bp or 25 bp ladder as a standard. Library size is size on gel minus ~155 bp for adaptors.

Example Trial PCR Result:
Lane 1: 25bp ladder
Lane 2: Successful Library; Top band (smeared) is library. Bottom bands are adaptor dimers.
Lane 3: Questionable Library
Lane 4: 100 bp ladder
**Amplify the library**

The library is amplified using Library PCR Primers 1 and 2 with the Platinum® PCR Amplification Mix, which includes a proofreading enzyme for high-fidelity amplification. Reduce the number of cycles as much as possible and use the entire nick-translated DNA-complex for amplification to get maximum representation of the library and to avoid PCR-related biases due to differential amplification of library molecules. Platinum® PCR Amplification Mix contains a proofreading enzyme for high-fidelity amplification.

**Perform PCR on the library**

1. Prepare a master mix for amplification reactions:

<table>
<thead>
<tr>
<th>Component†</th>
<th>Volume (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Amplification Mix</td>
<td>329</td>
</tr>
<tr>
<td>PCR 1.0 Primer (25 uM)</td>
<td>13.3</td>
</tr>
<tr>
<td>Index PCR Primer (25 uM)</td>
<td>13.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>355.6</strong></td>
</tr>
</tbody>
</table>

† I have had better success not adding PCR 2.0 Primer per the usual Illumina instructions. Also, you may be able to half or third the amount of master mix you use for this step. You will have more beads per reaction in this case. Beads can interfere with PCR conditions, so it is probably not a good idea to decrease the number of PCR cycles. I would not do this if you are starting the protocol with only 1-2 ug DNA per library, but might be worth a try if you are starting with more input DNA.

2. Place the tube of DNA-bead complex in a magnet for ≥1 minute until the solution clears.

3. With a 20-μL pipettor, carefully remove and dispose of the supernatant until ~5μL of Elution Buffer (E1) remains above the beads. Do not draw beads into the pipettor tip.

4. Add the master mix from step 1 to the beads.

5. Vortex the beads for 15 seconds, then aliquot the suspension to new PCR tube (~28 ul per tube).
6. Run on Thermalcycler:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holding</td>
<td>Denature</td>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Cycling†</td>
<td>Denature</td>
<td>94°C</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td>Anneal</td>
<td>64°C</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>Extend</td>
<td>70°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Holding</td>
<td>Extend</td>
<td>70°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Holding</td>
<td>-</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

† Base cycles on trial PCR.

**Recover DNA-bead complex**

1. Combine all reactions. Pellet beads for > 1 minute on a magnet. Remove supernatant to new tube and save for purification.

2. Wash the beads 3 times. For each wash:
   a. Resuspend the beads in 500 μL of Bead Wash Buffer. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.

3. Wash and resuspend the beads:
   a. Resuspend the beads in 500 μL of Buffer E1. Vortex the beads for 15 seconds, then pulse-spin.
   b. Place the tube in the magnet for at least 1 minute until the solution clears, then remove and discard the supernatant.
   c. Resuspend the beads in 30μL of Buffer E1.

4. Store beads at 4°C. PCR can be re-run if necessary.

**Purify the DNA with the SOLiD™ Library Micro Column Purification Kit**

1. Pre-spin empty PureLink® Microcolumn in collection tubes at 10,000×g for 1 minute before use.

2. Add 4 volumes of Binding Buffer (B2-L) with isopropanol (40%) to 1 volume of sample (supernatant from previous section). Mix well.

3. Load the DNA onto the PureLink® Microcolumn:
   a. Apply all of the PCR sample with beads to the PureLink® Micro column in collection tube.
b. Spin the column at 10,000×g for 1 minute at room temperature, then discard the flow-through. dsDNA is bound to the column.
c. Ensure that the entire PCR sample has been loaded onto the column(s).

4. Wash the column(s):
   a. Return the PureLink® Micro column to the same collection tube.
   b. Add 650 µL of Wash Buffer (W1) with ethanol to wash the column.
   c. Spin the column at 10,000×g for 1 minute at room temperature, then discard the flow-through.
   d. Spin the column at 14,000 × g to remove residual wash buffer.

5. Elute the DNA:
   a. Transfer the column to a clean 1.5-mL LoBind Tube.
   b. Add 25 µL of Elution Buffer (E1) to the center of the column to elute the DNA, then let the column stand for 1 minute.
   c. Spin the column at 14,000×g for 1 minute at room temperature.
   d. Add the eluate from the last spin back to the column, then let the column stand for 1 minute.
   e. Spin the column(s) at 14,000 × g for 1 minute at room temperature.

Quantitate Digested, Size Selected DNA
Quantitate the purified DNA using 1 µL of sample with the Qubit dsDNA BR Assay.

STOPPING POINT: Store purified PCR product at 4°C.

Purification of Library Molecules
We use a 1% agarose TBE gel but you can use a precast agarose Flashgels or recovery E-Gels for this step. Use a Ficoll based loading dye so that gel bands run cleanly. Leave at least one blank well between the marker and the sample and between each sample to avoid contamination when cutting the DNA from the gel.

Agarose Gel Separation
1. Prepare a 1.0% low-melt agarose gel with 1X TBE buffer with ethidium bromide.
2. Preload wells that will have ladder or sample with 1ul DNA loading dye. Let gel sit 5 minutes to ensure no wells are leaking.
3. Load no more than 10 ug DNA per well. Load the minimum number of wells to increase recovery.
4. Load 100bp and 25 bp DNA ladders.
5. Run the gel at 5V/cm. You may need to run the gel for quite some time to get good separation.

6. Lay the gel on plastic wrap on the gel box to prevent DNA contamination. Photograph the gel with a ruler lying on top.

7. Using a ruler, make a straight cut across the gel to cut out the library band (library band size will depend on the nick translation time used).

8. Put each segment into a LoBind tube. Weigh each gel slice by using an empty tube to tear the balance.

QIagen Gel Purification

Note: You may need to use a minElute column instead of a Qiaquick column and elute on a smaller volume if you need a more concentrated sample for sequencing.

1. Add 3 volumes Buffer QG to 1 volume of gel.

2. Dissolve the gel at 37°C, mixing frequently until gel slice completely dissolved. Quick spin to clear liquid from lid.

3. Add 1 gel volume of isopropyl alcohol. Mix by inverting tube several times. Quick spin to clear liquid from lid.

4. Apply up to 750ul of the sample to the column.

5. Let the columns stand for 2 minutes at room temperature.

6. Centrifuge the columns at 10,000xg (13,000 rpm) for 1 minute. Discard the flow-through after spinning and place the QIAquick columns back into the same collection tubes.

7. Add additional DNA/QG buffer/isopropanol mix if there is some remaining. Each column can filter 400mg of gel (or 2000ul mix). Repeat until all mix filtered. If >2ml, use multiple columns.

8. Add 500ul QG Buffer to each column to remove traces of gel. Spin 1 minute at 10,000xg (13,000 rpm) and discard flow through.

9. Add 750ul wash Buffer PE to each column to wash the column.

10. Centrifuge the columns at 10,000xg (13,000 rpm) for 1 minute. Discard the flow through.

11. Repeat: Centrifuge the columns at 10,000xg (13,000 rpm) for 2 minutes to remove residual wash buffer.

12. Air-dry the columns for 2 minutes to evaporate any residual alcohol.

13. Transfer the columns to clean 1.5mL LoBind tube.
14. Add 30ul Buffer EB (10mM Tris pH 8.0) to each column to elute the DNA. Let the columns stand for 2 minutes prior to centrifugation.

15. Centrifuge the columns at 10,000xg (13,000 rpm) for 1 minute.

16. If gel slices had to be split into multiple columns, pool the identical fractions of flow-through DNA into one tube.

**DNA Quantification and QC**

1. Quantitate the final library product as needed for sequencer (We Qubit using the BR assay).

2. Using an Agilent Bioanalyzer, run 1ul of the library (or a dilution of the library if stock over 50ng/ul) to determine DNA concentration and purity of library. You should have one clean peak in the trace.

3. If needed, you can boost the final amount of DNA, can put the final library product through 2 PCR cycles (in several tubes). Only do this if you are sure the complexity of the library is fine, but you don’t have enough material.
Appendix

Nick Translation Times
To really tune this you will need to do some experiments on your own as the exact times can vary according to lab practice and the actual thermalcycler used. The standard deviation in fragment sizes also increases as the times get longer. For Methyl-MAPS we really want to sequence all the way to the end of the fragments, thus it is important to tune the upper bound (not just the mean) of the library size to be less than the fragment size selected for sequencing.

The following info is from ABI R&D:

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<th>Mean Tag Length (bp)</th>
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