Protocol of construction of Blunt-End Illumina Libraries

Single index blunt-end Illumina library construction for ancient and historical samples, following Meyer and Kircher 2010 (Meyer oligos and NEBNext E6070L kit). Protocol provided by Nicolas Dussex (contact: [nicolas.dussex@gmail.com](mailto:nicolas.dussex@gmail.com) )

Key Considerations:

1. Use low-binding Eppendorf tubes
2. Avoid keeping finished libraries out of the fridge for too long, and avoid freeze thaw cycles
3. Indexed primers are diluted to 100 µM (stock), then make 1:10 dilutions to working stock (10µM/µL), this working solution cannot be kept
4. Don’t vortex solutions after adding enzymes, rather mix by flicking the tubes

**Buffer Preparation (This is done once, and then again when you have run out)**

1. Make Oligo Hybridisation Buffer (for 200 rxns)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Concentration** | **Volume** |
| NaCl | 500 mM | 19.76 mL when stock is 0.5 M |
| Tris-Cl pH 8 (Trizma from Sigma) | 10 mM | 0.2 mL when stock is 1 M |
| EDTA pH 8 | 1 mM | 0.04 mL when stock is 0.5 M |

1. Make Adapter Hybridisation Mix (100 µL for 200 rxns) as follows
   1. Make P5 hybridisation mix (200 µM)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Final concentration** | **Volume (μL)** |
| IS1\_adapter\_P5.F (500 μM) | 200 μM | 40 |
| IS3\_adapter\_P5+P7.R (500 μM) | 200 μM | 40 |
| Oligo Hybridization Buffer (10X) | 1X | 10 |
| ddH2O |  | 10 |

* 1. Make P7 hybridisation mix (200 µM)

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Final concentration** | **Volume (μL)** |
| IS2\_adapter\_P7.F (500 μM) | 200 μM | 40 |
| IS3\_adapter\_P5+P7.R (500 μM) | 200 μM | 40 |
| Oligo Hybridization Buffer (10X) | 1X | 10 |
| ddH20 |  | 10 |

1. Mix and incubate the reactions for **10 sec at 95ºC, followed by a ramp from95ºC to 12ºC at a rate of 0.1ºC/sec (eg. -1º/10sec)**

**This is saved as Adapter1**

1. Combine both reactions to get a ready-to-use mix, with 100 µM of each adapter. This stock can be saved in the freezer

**Step 1:** **DNA Quality Check and Sonication**

1. Run 2 µl DNA on 0.8% w/v agarose gel, ensure you run the 1kb+ DNA ladder as well as the size standard
2. Based on the size of the DNA fragments, determine whether or not sonication is necessary
   1. For very high MW DNA I normally sonicate for 30 - 45 minutes
   2. For mid range 15 mins
   3. Something in between 10 mins
3. Collect Ice, turn on vacuum centrifuge cold trap, place ice in sonicator to pre-cool and while downstairs get the necessary sonication tubes from the draw by the door
4. In the sonication tubes, place your DNA and make up to 100 µl final volume with milliQ
5. After approx 1 hour of precooling, take the first sonication batch downstairs (12 samples can be run at a time)
6. Add precooled milliQ (from the fridge) to the sonicator until the water fill line, ensure that there isn’t too much ice left
7. Add tubes to the rotor, place in machine. Ensure sonicator is set to 30/90 (on/off), and M intensity.
8. Turn of for desired time, ensure you place the warning signs on the door, and if staying you must wear ear protection.
9. Once time is up, remove samples from sonicator. If doing additional rounds, place more ice in the container and wait to cool some more before continuing.
10. Transfer the sample to clean low-bind eppendorf tube, and vacuum centrifuge until dry
11. Resuspend sample in 50 µl milliQ, leave in fridge overnight or RT for a few hours to ensure resuspension.

**Step 2: Blunt-End Repair**

1. Remove reagents from freezer, ensure ATP is covered in foil, and all enzyme are in the cold block.
2. Prepare master mix

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Final Concentration** | **Volume/Sample (µl)** | **X13** |
| Tango Buffer (10X) | 1X | 4 | 52 |
| dNTPs (25 mM) | 100 µM | 0.16 | 2.08 |
| ATP (100 mM) | 1 mM | 0.4 | 5.2 |
| T4 PNK (10 U/µL) | 0.5 U/µL | 2 | 26 |
| T4 DNA Polymerase | 0.1 U/µL | 0.8 | 10.4 |
| MilliQ |  | 12.64 | 164.32 |

1. Add 20 µl master mix to clean labelled tubes (small PCR tubes)
2. Add 20 µl Sonicated DNA
3. Briefly spin tubes
4. Incubate **15 min at 25ºC, followed by 5 min at 12ºC** (Saved as Nouser)
5. While running, take MinElute Columns from fridge AND turn on incubator to 37ºC
6. Place on ice or immediately proceed to the next step (total rxn volume: 40 µL)

**Step 3: MinElute Reaction Clean-Up**

1. Add 40µl reaction from above step to new low-bind eppendorf tubes
2. Apply **200 µL** PB buffer, mix by pipetting
3. Add to column
4. Spin 13,000 rpm for 1 min
5. Discard waste and change to new collection tube
6. Add **700 µL** of PE Buffer to column
7. Spin 13,000 rpm for 1 min
8. Discard waste and change to new collection tube
9. Add **700 µL** of PE Buffer to column
10. Spin 13,000 rpm for 1 min
11. Discard waste and change to new collection tube
12. Spin 13,000 rpm for 1 min to dry column
13. Change to new labelled low-bind eppendorf tube
14. Leave column with open lid for 5 min to dry out column
15. Add **22 µL** of EB buffer to column
16. Incubate **5 min at 37ºC**
17. Elute DNA by spinning down for 1 min at 13,000 rpm

**Step 4: Adapter Ligation**

1. Remove reagents from freezer. Check the buffer (T4 ligase buffer) has no precipitate after thawing, if present warm to 37ºC and vortex until precipitate is dissolved
2. Oligo hybridisation mix needs to me diluted 1:10 before adding to master mix. Note: This dilution needs to be made fresh each time. (1.5 ul oligo, 13.5 ul MQ X13 samples)
3. Prepare master mix:

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Final Concentration** | **Volume/reaction µL** | **X13** |
| T4 DNA Ligase Buffer (10X) | 1X | 4 | 52 |
| PEG-4000 (50%) | 5% | 4 | 52 |
| Adapter mix dilution\* (Oligo hybridisatio) | 10 pmol | 1 | 13 |
| T4 DNA Ligase (5U/µL) | 0.125 U/µL | 1 | 13 |
| MilliQ |  | 10 | 130 |

\*1µL of 1:10 dilution in TE or H2O from the stock

1. Add 20 µl master mix to new tubes (small tubes for thermocycler)
2. Add 20µl of cleaned DNA from step 3
3. Incubate for **30 min at 22ºC (Saved as Adapter2)**
4. While this is running, remove MinElute Columns from fridge, so they are room temperature for next step AND Turn on Incubator to 37ºC

**Step 5: MinElute Reaction Clean-Up**

1. Add 40µl reaction from above step to new low-bind eppendorf tubes
2. Apply **200 µL** PB buffer, mix by pipetting
3. Add to column
4. Spin 13,000 rpm for 1 min
5. Discard waste and change to new collection tube
6. Add **700 µL** of PE Buffer to column
7. Spin 13,000 rpm for 1 min
8. Discard waste and change to new collection tube
9. Add **700 µL** of PE Buffer to column
10. Spin 13,000 rpm for 1 min
11. Discard waste and change to new collection tube
12. Spin 13,000 rpm for 1 min to dry column
13. Change to new labelled low-bind eppendorf tube
14. Leave column with open lid for 5 min to dry out column
15. Add **22 µL** of EB buffer to column
16. Incubate **5 min at 37ºC**
17. Elute DNA by spinning down for 1 min at 13,000 rpm

**Step 6: Adapter Fill-In**

1. Remove reagents from freezer, ensure the thermopol reaction buffer has not precipitate before starting, if present warm to 37ºC and vortex
2. Prepare master mix

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Final Concentration** | **Volume/reaction (µL)** | **X13** |
| Thermopol Reaction (Isothermal amp buffer) buffer (10X) | 1X | 4 | 52 |
| dNTPs (25 mM) | 250 µM | 0.4 | 5.2 |
| Bst Polymerase, LF (8U/µL) | 0.3 U/µL | 1.5 | 19.5 |
| MilliQ | --- | 14.1 | 183.3 |

1. Add 20 µl master mix to labelled tubes (small PCR tubes)
2. Add 20µl DNA from Step 5, briefly spin
3. Incubate **20 min 37ºC**, followed by **80ºC for 20 min** (heat kill) (Saved as Fill-In)

**Safe Stopping point – Libraries can be stored in the freezer overnight or until ready to do the next step. I normally transfer to clean labelled low-bind eppendorfs as they store better in the boxes.**

**Step 7: Indexing PCR Test**

1. Remove Index combinations from the freezer. Prepare 1:10 dilutions. These can not be kept overnight. (1ul primer, 9 ul MQ)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | ID-i7 | Index-i7 | ID-i5 | Index-i5 |
| 1 | i7-1 | TCGCAGG | i5-89 | TAGTTCC |
| 2 | i7-2 | CTCTGCA | i5-90 | TGGCAAT |
| 3 | i7-3 | CCTAGGT | i5-91 | CGTATAT |
| 4 | i7-6 | ATGGAGA | i5-94 | GTACTAT |
| 5 | i7-8 | GCTCGAA | i5-96 | CGCAGCC |
| 6 | i7-12 | TTGAAGT | i5-100 | GTTATAC |
| 7 | i7-15 | CGACCTG | i5-92 | GCTAATC |
| 8 | i7-18 | TGCGTCC | i5-106 | TTGGTCA |
| 9 | i7-19 | GAATCTC | i5-107 | GTTGCAT |
| 10 | i7-21 | ACGCAAC | i5-130 | GACCGAT |
| 11 | i7-26 | GATGCCA | i5-114 | AGCAATC |
| 12 | i7-28 | AGATAGG | i5-116 | CAACTCT |
| 13 | i7-32 | AATAGTA | i5-120 | GTTACCG |
| 14 | i7-36 | TGGAATA | i5-137 | ACGCGGA |
| 15 | i7-43 | AAGGTCT | i5-143 | CCTCGCC |
| 16 | i7-52 | CGACGGT | i5-142 | AATATAG |
| 17 | i7-58 | ACGTATG | i5-135 | CGGACGT |
| 18 | i7-66 | AGAGCGC | i5-149 | CTTGGAA |
| 19 | i7-70 | GACGATT | i5-144 | TTAATAG |
| 20 | i7-93 | GACTTCT | i5-148 | CCAAGTC |
| 21 | i7-101 | CCTTAAT | i5-132 | GTAAGCC |
| 22 | i7-103 | TACTCGC | i5-140 | CGGTAAG |
| 23 | i7-105 | TAAGTAA | i5-145 | CCGAAGC |
| 24 | i7-128 | GATCGTC | i5-146 | TCGTTAT |
| 25 | i7-168 | CTCCAGT | i5-133 | GCCATGC |
| 26 | i7-172 | CTCAGAT | i5-131 | ACTTGCG |
| 27 | i7-196 | CCGTCCG | i5-124 | TCTCCTA |
| 28 | i7-199 | ACCTTCC | i5-127 | CGCTATT |
| 29 | i7-200 | TTCCGAG | i5-129 | ACGGCAG |
| 30 | i7-13 | ACTATCA | i5-134 | ATAACGT |
| 31 | i7-7 | CTCGATG | i5-95 | CGAGATC |
| 32 | i7-9 | ACCAACT | i5-97 | GAGAGGC |
| 33 | i7-10 | CCGGTAC | i5-98 | GCTTCAG |
| 34 | i7-11 | AACTCCG | i5-99 | ATATCCA |
| 35 | i7-14 | TTGGATC | i5-102 | CGCCAAC |
| 36 | i7-16 | TAATGCG | i5-104 | AGCGCCA |
| 37 | i7-20 | CATGCTC | i5-108 | ATCCTCT |
| 38 | i7-22 | GCATTGG | i5-110 | ATATGAT |
| 39 | i7-24 | CAATATG | i5-112 | AAGAACG |
| 40 | i7-25 | TGACGTC | i5-113 | CCGTTGA |
| 41 | i7-27 | CAATTAC | i5-115 | GCTCCGT |
| 42 | i7-29 | CCGATTG | i5-117 | AGACTCC |
| 43 | i7-30 | ATGCCGC | i5-118 | CTATCTT |
| 44 | i7-31 | CAGTACT | i5-119 | AAGCAGT |
| 45 | i7-193 | GAGGTTG | i5-121 | CCTAACG |
| 46 | i7-194 | TATGAGT | i5-122 | ATCATAA |
| 47 | i7-195 | CTTCGTT | i5-123 | TGATAAC |
| 48 | i7-197 | AACGTTA | i5-125 | CAGAGCA |
| 49 | i7-198 | GCATATT | i5-126 | CGGCTGG |
| 50 | i7-5 | GCAAGAT | i5-173 | AAGACGA |
| 51 | i7-17 | AGGTACC | i5-186 | TATCAAC |
| 52 | i7-23 | GATCTCG | i5-158 | TGAGAGA |
| 53 | i7-201 | AACCGCA | i5-150 | TGAAGCT |
| 54 | i7-202 | AGTCCTC | i5-183 | CCATGAG |
| 55 | i7-203 | ACGACTT | i5-136 | GCGAGTA |
| 56 | i7-204 | ATCCATA | i5-138 | GTCTAAT |
| 57 | i7-205 | CGGTCTC | i5-175 | AACTGAC |
| 58 | i7-206 | CTCATCG | i5-174 | ACCGCTC |
| 59 | i7-207 | TCGCGTT | i5-139 | GAAGCGT |
| 60 | i7-208 | CTTGACC | i5-188 | GACGTAC |

1. Prepare PCR master mix (Always do 1x negative reaction for the first library test)

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Final Concentration** | **Volume/reaction (µL)** | **X13** |
| AccuPrime reaction mix (10X) | 1X | 2.5 | 32.5 |
| AccuPrime Pfx | --- | 0.5 | 6.5 |
| milliQ |  | 16.75 | 227.5 |

1. Add 20.5 µl Mix master
2. Add **0.75 µl** of Index Primer 1
3. Add **0.75 µl** of Index Primer 2
4. Add **3 µl** DNA
5. Spin tubes down
6. Use following thermal cycler program - Saved as Index

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature (ºC)** | **Time** |  |
| Initial Denaturation | 95 | 2 min |  |
| Denaturation | 95 | 15 sec |  |
| Annealing | 60 | 30 sec | 15 cycles \* |
| Elongation | 68 | 60 sec |  |
| Hold | 4 | ∞ |  |

1. Make 1.2% w/v agarose gel, Run 5 µl of PCR product on gel at 100V for 2 hours, ensure you also run 1kb+ ladder for sizing. It's important you can determine the size of the library and you can distinguish between primer and library bands as the size is key to pooling the samples.
2. Based on the brightness of the library determine how many cycles for the next round. If too bright, reduce to 12, too weak increase to 18, if ok leave at 15

**Step 8: Indexing PCR**

1. If new index dilutions are needed, prepare first
2. Here we are preparing 5x reactions per sample
3. Prepare PCR master mix

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent** | **Final Concentration** | **Volume/reaction (µL)** | **X61** |
| AccuPrime reaction mix (10X) | 1X | 2.5 | 152.5 |
| AccuPrime Pfx | --- | 0.5 | 30.5 |
| milliQ |  | 16.75 | 1067.5 |

1. Add 20.5 µl Mix master
2. Add **0.75 µl** of Index Primer 1
3. Add **0.75 µl** of Index Primer 2
4. Add **3 µl** DNA
5. Spin tubes down
6. Use following thermal cycler program - Saved as Index. \*Run the number of cycles you determined were optimum in the test

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature (ºC)** | **Time** |  |
| Initial Denaturation | 95 | 2 min |  |
| Denaturation | 95 | 15 sec |  |
| Annealing | 60 | 30 sec | 15 cycles \* |
| Elongation | 68 | 60 sec |  |
| Hold | 4 | ∞ |  |

**Step 9: Size Selection and Purification**

Notes: (before start protocol)

Aliquot 20 µl of UV\_treated TE buffer to low-binding Eppendorf tubes

Add 1 µl of 1% Tween 20 for every 20 µl of TE buffer (final tween conc in tubes 0.05%)

Protect from sunlight by wrapping the tubes in aluminium foil (impt for the tween not the eluted libraries)

IMPORTANT: Always use clean fresh 80% ethanol from clean and pure 99% ethanol and thoroughly vortex before use

1. Remove beads from fridge at least 30 mins before starting
2. Prepare TE-tween mix by taking 800µl UV-treated TE buffer, and adding 40 µl 1% Tween. Cover eppendorf with foil
3. Prepare *fresh* 80% Ethanol (45 mL 95% made up to 50 with milliq)
4. Pull together all 6 PCR reactions of each library in a clean low bind tube
5. Allocate 100 µl of PCR product to another tube low bind tube
6. Add 50 µl Speedbeads to PCR product (0.5X volume), ensure the beads are well mixed before adding (this is critical)
7. Vortex, pulse spin and incubate at room temperature for 5 minutes
8. Place tubes on magnetic rack for 1 minute (or until all beads have migrated to wall and supernatant is clear)
9. Carefully remove supernatant to new low binding tube without disturbing the pellet
10. The pellet contains long fragments that you don’t need (as precaution add 20 µl TE buffer to pellet and store in the fridge)
11. Add 270µl speedbeads to supernatant (1.8X volume, again ensure bead mixture is mixed before adding, crucial step)
12. Vortex, pulse spin, and incubate at room temperature for 10 mins
13. Place tube on rack and incubate for 3 minutes or until beads have migrated to wall and supernatant is clear
14. While on Rack, Discard supernatant, keeping the pellet (beads are attached to the fragments you want)
15. While on Rack, add 500 µl ETOH (80%?), leave for 1 min, then remove by pipetting
16. Add 500 µl ETOH, leave 1 min and remove by pipetting
17. Add 500 µl ETOH, leave 1 min and remove by pipetting
18. Remove from rack, and very quick pulse spin to collect ethanol at bottom of tube
19. Place back on magnetic rack, when beads have migrated to wall, open carefully to stop ethanol moving, and remove any excess with small pipette
20. Leaving the tubes open on the rack, air dry to remove all ethanol. This step is critical and any carryover ethanol will interfere with downstream sequencing etc. The pellet should look like mud when dry, if it is shiny wait until it is no longer. It is better to overdry your samples rather than underdry
21. Remove tube from rack and add 36 µl TE-tween buffer, mix by pipetting.
22. vortex for 20 sec
23. Pulse spin and incubate at room temperature for 5 minutes
24. Place on magnetic rack for 5 minutes or until the supernatant is clear.
25. Remove 3 µl supernatant and transfer to set of tubes labelled 1 - 60 for picogreen analysis (must be low-bind tubes)
26. Remove the remainder of the library to clean low-bind tubes, being very careful not to disturb the beads. If you do, pulse spin sample and place back on rack until supernatant is clear again. It is crucial you don’t carry over any of the beads, as this tube now contains your final library. Store in the freezer until pooling.