Dual-indexed library preparation of gDNA for Illumina sequencing

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Setting up

Reagents

Table 1: Reagents used during library prep, including supplier and catalog numbers. All costs are approximate and don't include lab consumables (e.g., tubes, tips, plates, etc.).

Reagent	Supplier	Catalog #	Cost (CAD)	Cost/reaction
dNTP	Fisher	Fermentas R1121	265	0.04
ATP	Fisher	Fermentas R0441	52.25	0.07
Tango buffer	Fisher	Fermentas BY5	14.3	0.01
T4 Polynucleotide Kinase	Fisher	EK0032	249	0.17
T4 DNA polymerase	Fisher	EP0062	249	0.33
Nuclease free water	Fisher	NA	33.2	0.02
dATP	Fisher	LS10216018	61.41	0.04
Taq	Fisher	LS18038067	276.22	0.55
iTrusR2-stubRCp	IDT	NA	65.4	0.01
iTrusR1-stub	IDT	NA	35.4	0.003
T4 DNA ligase	Fisher	Fermentas EL0011	279.67	0.28
Sera-Mag SpeedBeads	Fisher	09-981-123	686	0.23
PEG-8000	VWR	97061-098	64.83	0.02
EDTA	VWR	97062-656	61.14	0.02
Tris Buffer	VWR	97062-974	70.82	0.02
Tween 20	VWR	97061-384	64.23	0.02
NaCl	VWR	CA12001-384	56.34	0.02
50 bp DNA ladder	Fisher	FERSM0371	63.75	0.08
ITru5_Group1_8-Indices	IDT	NA	198.64	0.59
ITru7_Group1_12-Indices	IDT	NA	255.56	0.82
Phusion HiFi polymerase	NEB	M0530S	116.19	0.29
Total	NA	NA	NA	**3.63**

Lab equipment

1. PCR thermocycler with heated lid
2. Gel rigs
3. 96-well plate magnet (e.g., Dynamag-96 well #12331D)

4. Racks for 0.2 mL PCR tubes or plates
5. PCR plates or tubes
6. 10 and 100 µL multichannel or singlechannel pipettes

Before starting

This protocol is used for preparing dual-indexed, Illumina-compatible gDNA libraries for whole genome sequencing. It doesn't rely on any commercial kits; rather, the protocol uses home-made master mixes for all reactions to minimize costs. References will be provided throughout to credit the original publications.

Briefly, this protocol uses an "on-bead" library preparation (Fisher *et al.* 2011) with "home-brewed" master mixes (Kobs 1997; Meyer and Kircher 2010; Fisher *et al.* 2011) and SPRI beads (Rohland and Reich 2012), and incorporates i5 and i7 Ilummina indices into adapters through PCR (Glenn *et al.* 2016).

This protocol assumes that you have:

1. Hydrated all of index-containing forward and reverse PCR primers (described in supplementary file S6 in Glenn *et al.* 2016). These are diluted to 5 µM.
2. Annealed the adapter stubs (i.e., iTrusR2-stubRCp, iTrusR1-stub) to form the Y-yoke adapter (Supplementary S7 in Glenn *et al.* 2016). These are diluted to 5 µM. This is referred to as the *adaptor mix* throughout the protocol_
3. Prepared the SPRI bead solution, which replaces Ampure XP beads for cleaning enzymatic reations (Rohland and Reich 2010; reproduced in supplementary file S8 in Glenn *et al.* 2016). Be sure to also prepare a PEG solution lacking the actual SPRI beads (file S8 in Glenn *et al.* 2016), which will be used throughout the protocol.
4. Prepared a fresh aliquot of 80% ethanol.

Protocol

Step 1: DNA shearing

1. Normalize DNA to 40 ng/µL (quantified on Nanodrop).
    - *Note:* 20 ng/µL would probably be fine.
2. Shear DNA by sonication to mean fragment size of ~500 bp.
    - *Note:* We initially sonicated to 300 bp usng a Bioruptor Pico following the manufacturer's protocol. However, this resulted in adaptor contamination in some of the reads, which had to be trimmed. We have not yet figured out the protocol to shear to 500 bp on a Bioruptor Pico since Diagenode doesn't provide this.

Step 2: End-repair

1. Prepare the end-repair master mix below shown in table 2. Prepare 10 µL × 1.2 × # samples of master mix. Each reaction requires 10 µL.

   Table 2: End-repair master mix from Meyer and Kircher (2010). All volumes in µL

Reagent	Units	Initial conc.	Vol.	Final conc.
Tango buffer	X	10	3.5	1
ATP	mM	10	3.5	1
dNTP	mM	10	0.35	0.1
T4 PNK	U/uL	10	1.75	0.5
T4 POL	U/uL	5	0.7	0.1
Water	NA	NA	0.2	NA
Total	NA	NA	NA	**10**

2. Pipette 10 µL of master mix in to 25 µL of each DNA sample. Mix throughly by pipetting or flicking.
3. Incubate reation in thermocycler using the following conditions (from Fisher *et al.* 2011):
   - 15 mins at 12°C followed by 15 mins at 25°C (30 mins total).

Step 3: Post end-repair cleanup

1. Add 100 µL of SPRI bead solution to each sample. This is a 2.8X Bead:DNA ratio, which keeps dsDNA fragments but removes leftovers from end-repair master mix. Mix throughly by pipetting or flicking.
2. Incubate at room temperature for 5 to 15 minutes.
3. Place samples on magnetic rack and allow the beads to bind to the side of the tube. The supernatant should be clear.
4. Remove and discard the supernatant (the DNA is bound to the beads so won't be lost).
5. Add 80 µL of freshly prepared 80% ethanol. Let sit on magnetic rack for 30 to 60 seconds and discard ethanol.
6. Perform another ethanol wash as in step 5.
7. Remove any residual ethanol with a pipette tip. Let samples sit uncovered at room temperature for 2 to 10 minutes or until all ethanol has evaporated.
   - **Important:** Do not let beads dry out too much as this will reduce yield. Beads should be matte brown (not glossy). Dry beads with begin to crack.
8. Elute DNA in 20 µL TE (or dH20). Pipette mix to homogenize beads and TE. The DNA will now be in solution.

Note: The beads from the above reaction will be kept through all subsequent enzymatic

reactions and reused for all SPRI bead cleanups (except PCR). The beads do not interfere with the reactions (Fisher *et al.* 2011).

Step 4: A-tailing

1. Prepare the A-tailing master mix shown in table 3. Prepare 10 µL × 1.2 × # samples of master mix. Each reaction requires 10 µL.

   Table 3: See Kobs (1997) and this wiki from the Rausher lab at Duke for details on A-tailing. All volumes in µL

Reagent	Units	Initial conc.	Vol.	Final conc.
Taq buffer	X	10	3	1
dATP	mM	10	0.6	0.2
Taq	U	5	3	0.5
MgCl2	mM	50	1.5	2.5
Water	NA	NA	1.9	NA
Total	NA	NA	**10**	NA

2. Add 10 µL of master mix to 20 µL each DNA sample.
3. Incubate in thermocycler for 30 minutes at 70°C.

Step 5: Post A-tailing cleanup

1. Add 66 µL of 20% PEG-8000/2.5 mM NaCl (i.e. SPRI bead solution without the beads) to samples. This is a 2.2X Bead:DNA ratio (Fisher *et al.* 2011).
2. Perform bead cleanup as in step 3, eluting DNA in 20 µL of TE.

Adapter ligation

1. Prepare the adapter ligation master mix shown in table 4. Prepare 10 µL × 1.2 × # samples of master mix. Each reaction requires 10 µL.

   Table 4: Adapter ligation master mix from Meyer and Kircher (2010). All volumes in µL

Reagent	Units	Initial conc.	Vol.	Final conc.
Taq buffer	X	10	3	1
dATP	mM	10	0.6	0.2
Taq	U	5	3	0.5
MgCl2	mM	50	1.5	2.5
Water	NA	NA	1.9	NA

Reagent	Units	Initial conc.	Vol.	Final conc.
Total	NA	NA	**10**	NA

2. Add 5 µL of 5 µM adapter mix (see Before starting above) to each DNA sample.
3. Add 10 µL of master mix to each sample. Pipette mix thoroughly. The total volume should now be 35 µL.
4. Incubate in thermocycler for 30 minutes at 25°C

Post adapter ligation cleanup

1. Add 31.5 µL of 20% PEG/2.5 mM NaCl (i.e. speedbeads without the beads) to samples. This is a 0.9X bead:DNA ratio. This will get rid of smaller DNA fragments (e.g., < ~250 bp) like adapter dimers that may have formed during the ligation reaction.
2. Perform bead cleanup as in step 3, eluting DNA in 20 µL of TE.
3. After elution, place beads on magnet and transfer supernatant to new tube/plate. The DNA is in the supernatant. This will be used for indexing PCR.

Indexing PCR

1. Prepare the PCR master mix shown in table 5. Prepare 12.5 µL × 1.2 × # samples (plus controls)of master mix. Each reaction requires 10 µL.

   Table 5: PCR master mix. All volumes in µL

Reagent	Units	Initial conc.	Vol.	Final conc.
Phusion buffer	X	5	5	1
dNTP	mM	10	0.5	0.2
Phusion polymerase	U/uL	2	0.25	0.02
Water	NA	NA	6.75	NA
Total	NA	NA	**12.5**	NA

2. Add 12.5 µL of master mix to empty PCR tubes/plates corresponding to the number of samples to be amplified and indexed.
3. Add 10 µL of DNA sample to each well containing PCR master mix.
4. Add 1.25 µL of forward primer and 1.25 µL of reverse primer to each sample.
   - *Note:* Each sample should receive a unique combination of forward and reverse primers since these will incorporate the indices into the libraries. 8 forward + 12 reverse primers can uniquely tag 96 individuals.

5. Perform PCR using the following conditions. See Glenn *et al.* (2016) for details on these primers.
    - 98 °C for 30 seconds
    - 8 to 14 cycles of:
        - 98 °C for 30 seconds
        - 65 °C for 30 seconds
        - 72 °C for 60 seconds
    - 72 °C for 1 minute

Post-PCR cleanup

1. Add 25 µL of 20% PEG/2.5 mM NaCl (i.e. speedbeads without the beads) to samples. This is a 1X bead:DNA ratio which should primer dimers and other small fragments remaining from PCR.
2. QC library by visualizing on a gel using the 50 bp Fermentas ladder or by passing through an Agilent Bioanalyzer with a DNA 1000 high sensitivity chip.

Note: Samples can now be quantified via Nanodrop or (preferably) Qubit/Picogreen. If pooling for sequencing or capture, normalize DNA concentrations to 20 ng/µL and create pools.

Testing the libraries

We tested the protocol above by shotgun sequencing two white clover (*Trifolium repens*) genomes to 35X coverage on a lane of HiSeq 4000. FastQC reports for both sequencing libraries are available HERE. While the reports show some warnings, these can be larger explained by the adapter trimming performed using bbduk. The adapter contamination occured because we sheared DNA to a mean size of 300 bp, resulting adapter read-though of some smaller fragments during sequencing. We now recommend shaering to 500bp to minimize this. Nonetheless, few reads were lost and all remaining reads were high quality.

References

1. Kobs, G. 1997. Cloning Blunt-End DNA Fragments Into the pGEM ® -T Vector Systems. *Promega Notes Magazine* 16: 15-20. LINK
2. Fisher, S. *et al.* 2011. A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biology* 12: 1-15. LINK
3. Glenn, Travis C. *et al.* 2016. Adapterama I: Universal stubs and primers for thousands of dual-indexed Illumina libraries (iTru & iNext) 3. *bioRxiv*. LINK
4. Meyer, L., and Kircher, M. 2010. Illumina Sequencing Library Preparation for Highly Multiplexed Target Capture and Sequencing. *Cold Spring Harbor Protocols* 2010(6). LINK

5. Rohland, N., and Reich, D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Research* 22: 939-946. LINK