Dual-indexed library preparation of gDNA for Illumina sequencing

James S. Santangelo

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1 Version history

1.0.0.1 v1.1.1 (Latest)

- Minor typo fixes
- Added details on performin 2nd PCR raction to increase concentrations
- Added details on pooling into equimolar pools for sequencing

1.0.0.2 v1.1.0

- Added colored links
- Minor typo and syntax changes
- Updated end-repair master mix
- Added post shearing bead cleanup
- Autogenerated references
- Added YAML header to generate table of contents, title, author, and last updated

1.0.0.3 v1.0.0 (July 10, 2019)

- Initial commit of protocol

2 Setting up

2.1 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**

2.2 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

2.3 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 proto-

col 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 Illumina indices into adapters through PCR (Glenn et al. (2019)).

This protocol assumes that you have:

1. Hydrated all of index-containing forward and reverse PCR primers (described in supplementary file: *Protocol for preparing 1.25 nmol iTru primer aliquots for use during library preparation* in Glenn et al. (2019)). These are diluted to 5 µM.
2. Annealed the adapter stubs (i.e., iTrusR2-stubRCp, iTrusR1-stub) to form the Y-yoke adapter (supplementary file:*Protocol for preparing double-stranded iTru adapters for use during library preparation* in Glenn et al. (2019)). 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 (2012); reproduced in supplementary file:*Protocol for preparing an inexpensive substitute for AMPure* in Glenn et al. (2019)). Be sure to also prepare a PEG solution lacking the actual SPRI beads (described in the same file, which will be used throughout the protocol.
4. Prepared a fresh aliquot of 80% ethanol. Addition of 0.05% Tween-20 to this PEG/NaCl solution will greatly facilitate the bead washed

3 Protocol

3.1 DNA Shearing and Cleanup

3.1.1 Shearing

1. Normalize DNA to 10 ng/µL (quantified by Qubit) in 25 µL of low-TE (10 mM Tris-HCl ph8 and 0.1 mM EDTA). You can shear more than 25 µL if you want multiple shots at the library prep per smaple.
    - *Note:* We've had success using lower concentrations if DNA quantity is an issue
2. Shear DNA by sonication to mean fragment size of ~500 bp.
    - *Note:* We sheared DNA using a Bioruptor Pico, but any acoustic shearing device would likely be fine (e.g., Covaris).
    - *Note:* It's a good idea to perform a few different rounds of shearing with different settings and running these on a gel and/or Bioanalyzer make sure you're getting the correct fragment size distribution.

3.1.2 Post-shearing cleanup

1. Add 20 µL of SPRI bead solution to each sample. This is a 0.8X Bead:DNA ratio, which is meant to remove fragment < ~250 base pairs.
2. Incubate at room temperature for 5 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 5 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 low-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)).

3.2 End-repair and cleanup

3.2.1 End-repair

1. Prepare the end-repair master mix below shown in table 2. Each reaction requires 10 µL. It's a good idea to prepare more than you'll need (e.g., enough for 9 samples if you're preparing 8 libraries)

   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	1
ATP	mM	10	3	1
dNTP	mM	10	0.3	0.1
T4 PNK	U/uL	10	1.5	0.5
T4 POL	U/uL	5	0.6	0.1
Water	NA	NA	1.6	NA
Total	NA	NA	**10**	NA

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).

3.2.2 Post end-repair cleanup

1. Add 84 µL of 20% PEG-8000/2.5 mM NaCl (i.e. SPRI bead solution without the beads) to samples. This is a 2.8X Bead:DNA ratio (Fisher et al. (2011)).
2. Perform bead cleanup as in sec. 3.1.2, eluting DNA in 20 µL of low-TE.

3.3 A-tailing and cleanup

3.3.1 A-tailing

1. Prepare the A-tailing master mix shown in table 3. Each sample 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.

3.3.2 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 sec. 3.1.2, eluting DNA in 20 µL of low-TE.

3.4 Adapter ligation and cleanup

3.4.1 Adapter ligation

1. Prepare the adapter ligation master mix shown in table 4. Each sample 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.
Ligase buffer	X	10	3.5	1
PEG-4000	mM	50	3.5	5
T4 Ligase	U	5	0.88	0.125
Water	NA	NA	2.12	NA
Total	NA	NA	**10**	NA

2. Add 5 µL of 5 µM adapter mix (see point 2 in sec. 2.3) 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

3.4.2 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 sec. 3.1.2, eluting DNA in 20 µL of low-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.

3.5 Indexing PCR and cleanup

3.5.1 Indexing PCR

1. Prepare the PCR master mix shown in table 5. Each reaction requires 12.5 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. (2019) 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

3.5.2 2nd indexing PCR and pooling (optional)

Because only 10 µL of the pre-PCR (i.e., ligated) library is used in the indexing PCR, each library prep allows for a total of two PCR reactions. If you find that final library concentrations (i.e., after cleaning, see sec. 3.5.3) are too low, you can perform a second PCR reaction on the remain pre-PCR library using the same conditions as above. These two reaction can then be pooled prior to cleaning, and then eluted in a lower volume to increase concentrations (see sec. 3.5.3 below).

3.5.3 Post PCR cleanup

1. Add 0.8X the post-PCR DNA volume in SPRI bead solution to each sampled. If you only performed a single PCR reaction, this should be approximately 20 µL. If you pooled two 25 µL PCR reactions, this should be approximately 40 µL of SPRI bead solution. This should remvove primer dimers and other small fragments remaining from PCR.
2. Perform bead cleanup as in sec. 3.1.2, eluting in 23 µL (i.e., 20 µL for sequencing and 3 µL for post-cleaning QC)
3. 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.

3.6 Pooling for sequencing

For multiplexed sequencing, dual-indexed libraries need to be pooled into equimolar ratio pools to ensure approximately equal sequencing coverage. [

1. This script can facilitate determining the library volumes required to create equimolar pools for sequencing.
2. Pipette the required volume of each cleaned, dual-indexed library into the appropriate tube. You should have 1 tube for each sequencing lane.

3.7 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 largely eliminated 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

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