

Dual-indexed library preparation of gDNA for Illumina sequencing

James S. Santangelo

Last updated: April 5, 2021

Table of contents

1	Version history	2
2	Setting up	3
2.1	Reagents	3
2.2	Lab equipment	3
2.3	Before starting	4
3	Protocol	4
3.1	DNA Shearing and Cleanup	4
3.1.1	Shearing	4
3.1.2	Post-shearing cleanup	5
3.2	End-repair and cleanup	5
3.2.1	End-repair	5
3.2.2	Post end-repair cleanup	6
3.3	A-tailing and cleanup	6
3.3.1	A-tailing	6
3.3.2	Post A-tailing cleanup	6
3.4	Adapter ligation and cleanup	6
3.4.1	Adapter ligation	6
3.4.2	Post-adapter ligation cleanup	7
3.5	Indexing PCR and cleanup	7
3.5.1	Indexing PCR	7
3.5.2	2nd indexing PCR and pooling (optional)	8
3.5.3	Post PCR cleanup	8
3.6	Pooling for sequencing	8
3.6.1	Creating equimolar dilutions	9
3.6.2	Creating equimolar pool	9
3.7	QC of final libraries	9
3.8	Testing the libraries	9

1 Version history

1.0.0.1 v1.2.1 (Latest)

- Minor typo fixes
- Fixed heading references

1.0.0.2 v1.2.0

- Added consumables to supplies and updated costs
- Updated DNA shearing conditions and added transfer step post-cleanup
- Updated Taq concentration for A-tailing
- Clarified creation of equimolar dilutions and final library pool
- Added QC steps for final library
- Minor clarifications and typo fixes

1.0.0.3 v1.1.1

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

1.0.0.4 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

1.0.0.5 v1.0.0 (July 10, 2019)

- Initial commit of protocol

2 Setting up

2.1 Reagents

Table 1: Reagents and consumables used during library prep, including supplier and catalog numbers. All costs are approximate.

Reagent	Supplier	Catalog #	Cost (CAD)	Cost/reaction
Bioruptor tubes	Diagenode	C30010020	527.33	0.53
PCR Tubes	VWR	93001-118	86.10	2.87
Pipette tips 200 uL	TR-222-C-L	Corning	41.54	0.83
Pipetter tips 10 uL	T-300-L	Corning	38.75	0.77
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	8.60

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 μ L 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 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 [Fisher2011] with "home-brewed" master mixes (Kobs1997; Meyer2010; Fisher2011) and SPRI beads (Rohland2012), and incorporates i5 and i7 Illumina indices into adapters through PCR (Glenn2019).

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 [Glenn2019]). These are diluted to 5 μ M.
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 [Glenn2019]). These are diluted to 5 μ M. 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 reactions (Rohland2012; reproduced in supplementary file: *Protocol for preparing an inexpensive substitute for AMPure* in [Glenn2019]). 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. Addition of 0.05% Tween-20 to this PEG/NaCl solution will greatly facilitate the bead washed
4. Prepared a fresh aliquot of 80% ethanol.

3 Protocol

3.1 DNA Shearing and Cleanup

3.1.1 Shearing

1. Normalize DNA to 10 ng/ μ L (quantified by Qubit) in 25 μ L of low-TE (10 mM Tris-HCl pH8 and 0.1 mM EDTA) in Bioruptor tubes. You can shear more than 25 μ L if you want multiple shots at the library prep per sample.
 - *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.
 - For our samples we used 3 cycles of 15 seconds ON, 30 seconds OFF on a Bioruptor Pico
 - *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. After shearing, move 25 μL of DNA to empty PCR tube and proceed to post-shearing cleanup.

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 $< \sim 250$ 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 using a pipette.
6. Perform another ethanol wash as in step 5.
7. Remove any residual ethanol using a 10 μL a pipette. 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 will begin to crack.
8. Elute DNA in 20 μL low-TE (or dH₂O). 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 ([@Fisher2011]).

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 [@Meyer2010]. All volumes in μL . Final concentrations calculated from total sample volume (i.e., master mix + sample) = 30 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/ μL	10	1.5	0.5
T4 POL	U/ μL	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 20 μL of each DNA sample. Mix thoroughly by pipetting or flicking.
3. Incubate reaction 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-8000/2.5M NaCl (i.e. SPRI bead solution without the beads) to samples. This is a 2.8X Bead:DNA ratio ([@Fisher2011]).
2. Perform bead cleanup as in 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 [@Kobs1997] and [this wiki from the Rausher lab at Duke](#) for details on A-tailing. All volumes in μL . Final concentrations calculated from total sample volume (i.e., master mix + sample = 30 μL)

Reagent	Units	Initial conc.	Vol.	Final conc.
Taq buffer	X	10	3	1
dATP	mM	10	0.6	0.2
Taq	U	5	0.75	0.125
MgCl ₂	mM	50	1.5	2.5
Water	NA	NA	4.15	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-8000/2.5M NaCl (i.e. SPRI bead solution without the beads) to samples. This is a 2.2X Bead:DNA ratio ([@Fisher2011]).
2. Perform bead cleanup as in 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 [Meyer2010]. All volumes in μL . Final concentrations calculated from total sample volume (i.e., master mix + adapters + sample = 35 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 [Before starting](#)) 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/2.5M 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 [3.1.2](#), eluting DNA in 20 μL of low-TE.
3. After elution, place beads on magnet and carefully transfer the supernatant to new tube/plate by slowly drawing up the supernatant with a pipette. 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 . Final concentrations calculated from total sample volume (i.e., master mix + primers + sample = 25 μL)

Reagent	Units	Initial conc.	Vol.	Final conc.
Phusion buffer	X	5	5	1
dNTP	mM	10	0.5	0.2
Phusion polymerase	U/ μL	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 [Glenn2019] for details on these primers.
 - 98 $^{\circ}\text{C}$ for 30 seconds
 - 8 to 14 cycles of:
 - 98 $^{\circ}\text{C}$ for 30 seconds
 - 65 $^{\circ}\text{C}$ for 30 seconds
 - 72 $^{\circ}\text{C}$ for 60 seconds
 - 72 $^{\circ}\text{C}$ 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 [Post PCR cleanup](#)) are too low, you can perform a second PCR reaction on the remaining pre-PCR library using the same conditions as above. These two reactions can then be pooled prior to cleaning, and then eluted in a lower volume to increase concentrations (see [Post PCR cleanup](#) below).

3.5.3 Post PCR cleanup

1. Add 1X the post-PCR DNA volume in SPRI bead solution to each sample. If you only performed a single PCR reaction, this should be approximately 25 μL . If you pooled two 25 μL PCR reactions, this should be approximately 50 μL of SPRI bead solution. This should remove primer dimers and other small fragments remaining from PCR.
2. Perform bead cleanup as in [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. This is done in two steps: (1) Creating equimolar dilutions for each sample, and (2) Creating an equimolar pool for all samples to be multiplexed on a single lane.

3.6.1 Creating equimolar dilutions

1. Create equimolar dilutions for each sample, diluting the concentration down to that of the least concentrated sample. For highly concentrated samples, serial dilution may be required. The script in step 1 will output the volume required for each library, in addition to the volume of low-TE required to create the equimolar pool. It will additionally indicate whether serial dilutions are necessary and any required volumes.
 - *Note:* [This script](#) can facilitate determining the library volumes required to create equimolar pools for sequencing.

3.6.2 Creating equimolar pool

1. Pipette the same volume from each sample (which are now normalized to the same concentration from the step above) into the appropriate tube. You should have 1 tube for each sequencing lane.
 - *Note:* To avoid pipetting error associated with using very small volumes, it's best to ensure that at least 2 μL is being taken from each sample when creating the equimolar pool.

3.7 QC of final libraries

Sequencing centers generally suggest that customers send any relevant library QC data along with their library shipments. We will perform these QC steps here.

1. Quantify the concentration of the final library pools to be sequenced using a Qubit. Quantify each lane separately.
2. Visualize the library on a gel
 - Use 50 bp Fermentas Gene Ruler
 - Use a 1.5% agarose gel with 3 μL of 1% Ethidium Bromide (EtBr) added to gel
 - Run gel for 60 minutes at 100 V with 3 μL EtBr added to buffer
3. (optional) Visualize fragment size distribution using an Agilent Bioanalyzer with a DNA 1000 High Sensitivity chip

3.8 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 occurred because we sheared DNA to a mean size of 300 bp, resulting adapter read-through of some smaller fragments during sequencing. We now recommend shearing to 500bp to minimize this. Nonetheless, few reads were lost and all remaining reads were high quality.

References

- Fisher, Sheila, Andrew Barry, Justin Abreu, Brian Minie, Jillian Nolan, Toni M Delorey, Geneva Young, et al. 2011. "A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries." *Genome Biology* 12: 1–15.
- Glenn, Travis C, Roger A Nilsen, Troy J Kieran, Jon G Sanders, Natalia J Bayona-Vásquez, John W Finger, Todd W Pierson, et al. 2019. "Adapterama I: Universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext)." *PeerJ* 2019 (10): 1–31. <https://doi.org/10.7717/peerj.7755>.
- Kobs, By Gary. 1997. "Cloning Blunt-End DNA Fragments Into the pGEM -T Vector Systems." *Promega Notes Magazine* 62 (62): 1–15.
- Meyer, Matthias, and Martin Kircher. 2010. "Illumina sequencing library preparation for highly multiplexed target capture and sequencing." *Cold Spring Harbor Protocol* 2010 (6): 1–10. <https://doi.org/10.1101/pdb.prot5448>.
- Rohland, Nadin, and David Reich. 2012. "Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture." *Genome Research* 22: 939–46. <https://doi.org/10.1101/gr.128124.111.22>.