Titration-free 454 sequencing using Y adapters

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We describe a protocol for construction and quantification of libraries for emulsion PCR (emPCR)-based sequencing platforms such as Roche 454 or Ion Torrent PGM. The protocol involves library construction using customized Y adapters, quantification using TaqMan-MGB (minor groove binder) probe–based quantitative PCR (qPCR) and calculation of an optimal template-to-bead ratio based on Poisson statistics, thereby avoiding the need for a laborious titration assay. Unlike other qPCR methods, the TaqMan-MGB probe specifically quantifies effective libraries in molar concentration and does not require specialized equipment. A single quality control step prior to emulsion PCR ensures that libraries contain no adapter dimers and have an optimal length distribution. The presented protocol takes ~7 h to prepare eight barcoded libraries from genomic DNA into libraries that are ready to use for full-scale emPCR. It will be useful, for example, to allow analyses of precious clinical samples and amplification-free metatranscriptomics.

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INTRODUCTION

Modern DNA sequencing technology has improved markedly in recent years. However, in many current technologies, sample library preparation before sequencing has surfaced as a key limiting factor. For instance, the current Roche 454 sequencing protocol for preparation of a shotgun library[1] requires 500 ng of DNA as starting material and includes a laborious titration assay. A faster library preparation protocol that can handle lower starting amounts would be desirable and particularly useful for, e.g., sequence analyses of precious clinical samples, cDNA sequencing of environmental samples for metatranscriptomics[2] without the need for amplification that may introduce biases[3], or microsatellite sequencing in population genetics[4]. The protocol presented here is based on our previous study that described a novel method for sequencing low-starting-amount materials[5].

Library preparation in most high-throughput sequencing technologies involves the ligation of universal adapter(s) to the ends of DNA sample fragments to enable PCR amplification[6–8]. Unlike linear adapters such as adapters A and B (used for 454 sequencing), a single Y adapter, proposed previously[9] and used for the Illumina sequencing, has several advantages. Given a 100% ligation efficiency, four double-stranded DNA (dsDNA) molecules would, on average, generate two properly appended dsDNA libraries using adapters A and B, whereas eight single-stranded DNA (ssDNA) libraries would be generated using a single Y adapter (**Fig. 1**). In addition, the Y adapters can only be ligated at the double-stranded stem end that enables a simultaneous incubation with all enzymes involved, thus eliminating the need for laborious and yield-reducing cleanup steps.

One of the main differences in current high-throughput DNA sequencing technology as compared with traditional Sanger sequencing is that sample template concentration is kept very low to avoid tedious microbial subcloning. Emulsion PCR–based sequencing uses many millions of water-in-oil droplets, each of which serves as a separated amplification compartment[6]. Sample library concentration is kept so low that the majority of the droplets libraries and an even smaller proportion contains mixed-molecule libraries in a stochastic manner that follows Poisson distribution, as shown by our sequencing data[5]. In addition, the enrichment step will select only those beads that have a library, but a too-low DNA-to-bead ratio will lead to insufficient amount of beads for sequencing, whereas a too-high ratio will lead to frequent occurrence of mixed library beads. Thus, one of the key factors for a successful experiment is to use an optimal amount of library for sequencing. It is important to keep in mind that the amount of library added is not linearly associated with the number of high-quality beads[5]. We recommend an input DNA-to-bead ratio of 0.08, which will result in 96% of the enriched beads having a single-copy template according to Poisson distribution, and will be sufficient for sequencing[5]. A higher proportion of 'nonpure' (mixed-copy) beads associated with higher DNA-to-bead ratio might, in addition, affect the consumption of nucleotide flows during sequencing and bioinformatics processes, such as image background and signal intensity normalizations. Apart from the predicted increase of mixed library beads, a higher ratio of input DNA to beads is also less tolerant of subtle pipetting errors[5].

Two quantitative PCR (qPCR) assays have previously been proposed to quantify libraries derived from trace amounts of starting material[10,11]. Besides requiring less library, as compared with UV spectrophotometry and fluorometry methods, qPCR assays also have the advantage of measuring the amount of effective library—as the total library typically contains a mixture of molecules that are amplifiable, amplifiable but inefficient, or nonamplifiable for various reasons[5]. The previous two methods are based on SYBR Green dye[10] qPCR and universal template TaqMan probe digital PCR[11], respectively. With the SYBR Green–based qPCR assay, there is no need to design and use the relatively expensive TaqMan probe. However, it measures the total mass of the library and requires transformation into copy numbers on the basis of amplicon size estimation by gel electrophoresis or Agilent Bioanalyzer. Furthermore, the precision (coefficient of variance (CV) of the estimates) of this

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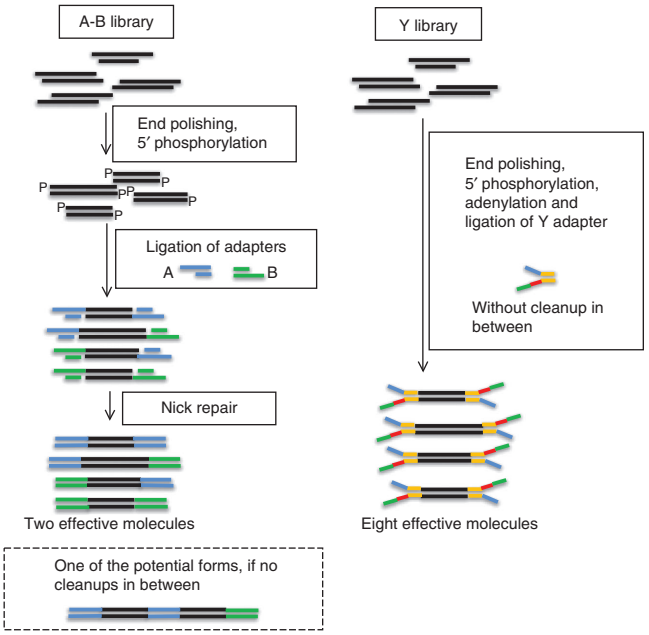

Figure 1 | A schematic illustration of the constructions of two types of libraries, A-B and Y. The A-B library construction method generates on average two effective double-stranded molecules (each appended with adapters A (blue) and B (green)) from four dsDNA molecules, given 100% ligation efficiency. The molecules appended with A-A and B-B are nonamplifiable because of the amplification-inhibiting hairpins formed between the complementary adapted sequences after denaturing and annealing steps. The Roche A-B library uses a biotinylated B adapter and two additional steps to generate two effective ssDNA molecules (see ref. 5). In contrast, the Y library construction method generates eight effective single-stranded molecules. The MGB-probe is in red and barcode in yellow.

the advantage of measuring the number of amplifiable molecules directly. The universal template TaqMan probe digital PCR is based on an 8-bp dual-labeled locked nucleic acid probe, complementary to the 5′-end tail of the customized amplification primer[11]. First, the digital PCR assay requires special equipment that is not widely accessible, such as Fluidigm's BioMark microfluidic device. Second, two rounds of quantifications are used: an initial crude quantification by qPCR to guide the dilution of the libraries for a more precise quantification by digital PCR, which renders a precision (CV 11.8%) higher than the initial qPCR alone (CV 21.2%)[11]. Our MGB probe-based assay[5] does not require special equipment and, therefore, is more accessible to ordinary laboratories. This MGB probe assay is at least as precise as digital PCR (CV 9.5% versus 11.8%). The MGB probe is a 20-bp-long probe complementary to the library molecule and is located next to the 3′ end of one of the amplification primers (**Fig. 2**). Having a probe targeting site between the amplification primers has the advantage that the amount of fluorescence signal is proportional to the number of library amplicons and not to the potential amplification primer dimers, which might be a potential source for less precise quantification with a probe-targeting part of an amplification primer. Further, a longer probe, as compared with a shorter one (8 bp), is more specific.

Limitations of the method should be acknowledged. The library quantification described here is based on a qPCR assay. Essentially, for all qPCR-based assays, amplification efficiency drops as the amplicon length increases. The best quantification method would be the one that best mimics the efficiency of emPCR. Either poorer or better efficiency than obtained by emPCR (depending on the emPCR system) could result in inaccurate estimation of the enrichment percentage after emPCR. This issue possibly pertains to all qPCR assays, including ours. It seems that our qPCR quantification method yields lower efficiency than the Roche Titanium emPCR method (data not shown) and results in ~50% more enriched beads than expected. However, as a typical bead recovery percentage is around 65–85%, the additional (~50%) beads more or less compensate for the bead loss during recovery and result in nearly the amount needed for loading onto the sequencing plate. Because qPCR amplification efficiency drops rapidly for long amplicons, and with the anticipated increase in read length in upgraded or

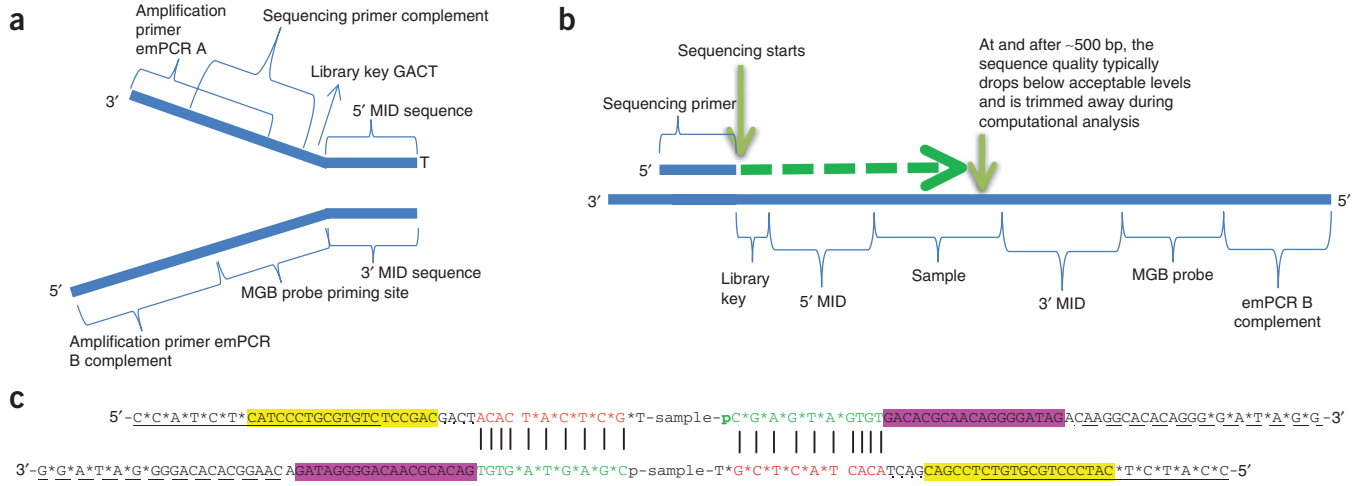

Figure 2 | Design of Y MID adapter. (**a**,**b**) Schematic illustrations of the Y MID adapter (**a**) and the sequencing process on a library molecule (**b**). (**c**) An example of two library molecules generated using one 'Y3' adapter. The emPCR primer A is underlined; emPCR primer B complement is shown by dashed underline. The sequencing primer is highlighted in yellow, the library key sequence is shown with dots underneath, the 5′ MID sequence is shown in

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new platforms in mind, we recommend empirically estimating the difference in enrichment percentage predicted by the qPCR method and one observed by titration assay once before applying the titration-free method routinely. Here we use a qPCR thermocycling program of ~1.5 h, which favors the complete extension of long amplicons. For applications of sequencing shorter amplicons (e.g., <150 bp), the thermocycling program can be shortened to <30 min, as in a typical Fast qPCR assay.

Experimental design

Multiplexing adapters (MID adapters), also referred to as barcodes, are essential as more and more projects require the pooling of samples. We present here a set of eight Y-barcoded adapters. The Y MID adapter has a stem of 10 bp, which serves as the barcode. It is important to have a short Y-adapter stem (just long enough to anneal and form dsDNA adapters at 25 °C for ligation to the sample DNA) to reduce the inhibitory effect during PCR amplification. The longer the stem, the stronger the inhibitory effect will be, assuming comparable G/C to A/T ratios. This inhibition is due to the potential formation of a hairpin from the complementary barcodes at both ends of an ssDNA molecule (**Fig. 2**). A 17-bp-long stem greatly inhibited PCR amplification (annealing temperature 60 °C) and showed no visible amplicons on agarose gel (data not shown). Each Y MID adapter has a unique stem and two universal branches and is created by annealing two oligonucleotides that share ten complementary nucleotides for the stem sequence (**Fig. 2**).

The barcodes were designed primarily using the barcodes selected from the 12 Roche 454 Rapid Library (RL) MID sequences. We designed two new barcode sequences, Ya1 and Ya2, containing the same number ($n = 17$) of nucleotide flows during sequencing as the RL MIDs. Selection and validation of the barcodes were performed in two steps. First, we used OligoAnalyzer (http://www.idtdna.com) to exclude ($n = 4$) by initial sequence analysis the RL MIDs that might form potential secondary structures (because of the introduction of the TaqMan-MGB probe complementary site) or that might have substantially different Gibbs free energy (ΔG) in the formation of the stem of Y adapters from the rest of the MID ΔGs. Second, experimental validation excluded two other RL MIDs barcodes (Y1 and Y12) that gave different proportions of sequencing yield than expected from the qPCR quantification. Because the qPCR quantification was applied to individual libraries, it cannot reflect possible interactions among the adapters when they are pooled in one reaction at a later stage. The remaining six RL MIDs barcodes and the two newly designed barcodes (Ya1 and Ya2) performed well in a panel.

Because sequencing starts from the 3′ end of a library molecule and sequencing errors accumulate as the polymerases extend toward its 5′ end, we designed the qPCR probe complementary site at the 5′ end of a library molecule so that it does not waste the sequencing capacity (**Fig. 2b**). Currently, the 454 Titanium sequencer generates read lengths of ~500 bp, whereas the library molecules are generally longer than 500 bp, indicating that most of the 5′ end of the library (3′ end of the read) is not sequenced at acceptable quality and, therefore, trimmed away. This can also be evidenced by the small difference in the total number of yielded bases between pre- and post-trimming of our customized adapter (711 versus 699 Mbp, see ANTICIPATED RESULTS).

For every experiment, it is useful to include a blank (no sample) library in parallel, starting from the first step until qPCR quantification and agarose gel electrophoresis. Because of background noise (most likely due to a trace amount of adapter dimers remaining in the library), the qPCR quantification may yield a value of hundreds to thousands of molecules in total for the blank library[12]. Agarose gel electrophoresis of the blank library qPCR amplicons would reveal a band corresponding to the size of an adapter dimer. This background can be ignored as it typically comprises <1% of a sample library. However, longer amplicons would indicate that contamination had been introduced in the upstream steps.

Size selection of DNA fragments is crucial. The Roche 454 Titanium platform is able to sequence, on average, 500 bp, and the selected DNA fragments should be longer than 500 bp to take advantage of the long read length. Conventional qPCR methods recommend using amplicons no longer than 150 bp to achieve good amplification efficiency. Using regular thermocycling conditions, the amplification efficiency drops for amplicons longer than 500 bp and drops profoundly for amplicons longer than 1,000 bp (data not shown). Fragments longer than 1,000 bp will consequently not be amplified well when mixed in one microdroplet with a 500-bp fragment, although their presence might not be a problem provided that the noise light signal from the 1,000-bp amplicons during sequencing is negligible. When a 1,000-bp fragment is amplified alone in a microdroplet, the number of amplicons on each bead will be much lower than normal (e.g., 100,000 amplicons of size 1,000 bp on one bead versus 1 million amplicons of size 500 bp on another bead), despite using the same thermocycling conditions. Conceivably, postsequencing light normalization is better for a library with a narrower size range, and we recommend removing DNA fragments longer than 900 bp for the current Roche 454 Titanium emPCR setting[13].

For an experiment in which a single sample is to be sequenced, the nonbarcoded Y adapter[5] can be used to save 10 bp for each read compared with the barcoded adapters. This Y adapter has a different key sequence (TCAG) than the barcoded ones (GACT). The Roche 454 pipeline, however, supports a simultaneous sequencing of libraries with different keys on one plate in physically separated regions; this can be done, for example, by using the nonbarcode Y adapter for one large-volume (LV) region (one sequencing plate consists of two LV regions) and eight different barcoded adapters for the other LV region.

MATERIALS

REAGENTS

▲ **CRITICAL** For all the reagents and buffers used, we have not noticed any adverse effect after storage in a freezer or refrigerator (per manufacturer's recommendations) for up to 1 year, except that any solutions containing

- DNA sample. Conventional methods (e.g., a variety of Qiagen DNA kits) from different types of sample can be used for DNA extraction.
- Oligonucleotides for Y adapters. We used 16 HPLC-purified oligonucleotides (Integrated DNA Technologies) to form eight Y adapters.

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TABLE 1 | Oligonucleotides used to form Y adapters.

Number	Sequence (5′–3′)
Y3	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACACT*A*C*T*C*G*T-3′
	5′-**p**C*G*A*G*T*AGTGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Y5	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACGAG*T*A*G*A*C*T-3′
	5′-**p**G*T*C*T*A*CTCGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Y8	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACGTA*C*T*G*T*G*T-3′
	5′-**p**C*A*C*A*G*TACGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Y9	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACGTA*G*A*T*C*G*T-3′
	5′-**p**C*G*A*T*C*TACGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Y10	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACTAC*G*T*C*T*C*T-3′
	5′-**p**G*A*G*A*C*GTAGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Y11	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTACTAT*A*C*G*A*G*T-3′
	5′-**p**C*T*C*G*T*ATAGTGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Ya1	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTCTACT*C*G*T*A*G*T-3′
	5′-**p**C*T*A*C*G*AGTAGGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G-3′
Ya2	5′-C*C*A*T*C*T*CATCCCTGCGTGTCTCCGACGACTGTACA*G*T*A*C*G*T-3′
	5′-**p**C*G*T*A*C*TGTACGACACGCAACAGGGGATAGACAAGGCACACAGGG*G*A*T*A*G*G

Complementary nucleotides that anneal to form the Y-adapter stem and barcode are shown in underlined text. Asterisks (*) indicate a phosphorothioate-modified bond, **p** indicates a phosphorylation.

- Oligos for qPCR. emPCR A 5′-CCATCTCATCCCTGCGTGTC-3′ (various vendors, salt purification); emPCR B 5′-CCTATCCCCTGTGTGCCTTG-3′ (Various vendors, salt purification); MGB probe 6FAM-CTATCCCCTGTTGCGTGTC-MGBNFQ (Applied Biosystems, HPLC purification)
- qPCR standards. The standards were prepared by cloning of an available library, PCR amplification of a single colony suspension, purification and dilutions, as described earlier[5]. Alternatively, a simple dilution method can be used[12] (see INTRODUCTION).
- UltraPure glycerol (Invitrogen, cat. no. 15514011, http://www.invitrogen.com)
- MinElute PCR purification kit (Qiagen, cat. no. 28004, http://www.qiagen.com)
- T4 DNA ligase (Enzymatics, cat. no. L603-LC-L, http://www.enzymatics.com)
- Klenow (3′→5′ exo-) (Enzymatics, cat. no. 01-LC-L, http://www.enzymatics.com)
- Taq DNA polymerase, recombinant (Invitrogen, cat. no. 10342-020, http://www.invitrogen.com)
- dNTP mix (Enzymatics, cat. no. N205L)
- AMPure XP beads (Agencourt, product no. A63880, http://www.beckmancoulter.com)
- TaqMan Fast Universal PCR Master Mix (Applied Biosystems, part no. 4352042, http://www.appliedbiosystems.com)
- End-repair mix (low concentration; Enzymatics, cat. no. Y914-LC-L, http://www.enzymatics.com)
- Tris-EDTA (TE) buffer (10×, BioUltra Molecular Biology Grade, pH 8.0; Sigma, cat. no. 93283, http://www.sigmaaldrich.com)
- Buffer PB (Qiagen, cat. no. 19066, http://www.qiagen.com)
- Water, Molecular Biology (Sigma, cat. no. W4502-1L, http://www.sigmaaldrich.com)
- Ethanol (BioUltra, for molecular biology; Sigma, cat. no. 51976, http://www.sigmaaldrich.com)
- GelPilot DNA loading dye (5×, Qiagen, cat. no. 239901, http://www.qiagen.com)
- GelRed nucleic acid gel stain (Biotium, cat. no. 41002, http://www.biotium.com/)

EQUIPMENT

- 7900HT Fast Real-Time PCR System or equivalent (Applied Biosystems, part no. 4329001, http://www.appliedbiosystems.com)
- Thermocycler
- Magnetic particle collector (MPC, DynaMag-2 magnet, cat. no. 123-21D, Invitrogen, http://products.invitrogen.com/ivgn/product/12321D)
- Nitrogen cylinder polyallomer tube (Beckman Coulter, part no. 357448, http://www.beckmancoulter.com) or other low-binding tube alternatives
- Nebulizers (Invitrogen, cat. no. K7025-05, http://www.invitrogen.com)
- PCR tubes
- Microcentrifuge tubes

REAGENT SETUP

Adapter annealing To a 200-µl PCR tube, add the following:

TE buffer (1×)	80 µl
Y adapter, top (100 µM)	10 µl
Y adapter, bottom (100 µM)	10 µl

Incubate at 95 °C for 1 min, 60 °C to 14 °C with −0.1 °C per second, 14 °C hold. Dilute the annealed adapters tenfold with 1× TE into working concentration (1 µM). This can be stored at −20 °C for at least 1 year.

Nebulizing buffer Nebulizing buffer is 10% (vol/vol) glycerol in 1× TE buffer. This buffer can be stored at 4 °C for at least 1 year.

PROCEDURE

DNA nebulization ● TIMING 1 h for eight samples

1| Add 590 µl of nebulizing buffer to a nebulizer. Add 10 µl of DNA sample. Connect the nebulizer to a nitrogen cylinder connected to a regulator and apply 30 psi for 1 min.

▲ **CRITICAL STEP** If larger sample volumes are used, the total volume should be adjusted to 600 µl.

2| Add 2.5 ml of PB buffer and mix by pipetting.

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4| Repeat Step 3 until all of the nebulized sample has been transferred to the spin column.

5| Add 700 µl of 70% (vol/vol) ethanol to the column, centrifuge at 10,000*g* for 1 min and discard the flow-through.

6| Elute the sample in 25 µl of 1× TE buffer according the manufacturer's instructions.
▲ **CRITICAL STEP** If you start with small amounts of DNA (less than 10 ng), low-binding tubes (e.g., polyallomer tubes), should be used throughout the library preparation, including for the storage of the library in the freezer.
▲ **CRITICAL STEP** The DNA nebulization steps (Steps 1–6) can be skipped if the starting sample is of low molecular weight (such as degraded archived formalin-fixed and paraffin-embedded tissue samples). The volume of AMPure XP beads used in the fragment size selection (Steps 7–11) should also be adjusted to avoid losing the sample fragments.

Fragment size selection ● TIMING 30 min for one sample, 5 more min for each additional sample
7| To the nebulized and purified DNA fragments, add an appropriate amount of AMPure XP beads (e.g., 11.5 µl of beads into 25 µl of sample to bind fragments longer than 900 bp; calibration is needed for each batch), and mix by pipetting.
▲ **CRITICAL STEP** Calibration of the AMPure XP beads should be done according to the manufacturer's instructions before library construction. The example given above was based on a calibration result showing that 11.5 µl of beads added to 25 µl of sample captured fragments longer than 900 bp, and that 14.5 µl of beads in 25 µl of sample captured fragments longer than 500 bp.

8| Transfer to a 1.5-ml tube and incubate at ambient temperature for 5 min.

9| Place the tube on the MPC. After the beads are pelleted (about 1 min), pipette the supernatant, which contains fragments shorter than 900 bp, into a new tube.

10| Add an appropriate amount of AMPure XP beads (e.g., 3.0 µl) to the tube, mix by pipetting and incubate at ambient temperature for 5 min.

11| Place the new tube on the MPC. After the beads are pelleted, pipette and discard the supernatant, which contains fragments shorter than 500 bp. The fragments that remain on the beads are in the size range of 500–900 bp.
▲ **CRITICAL STEP** Every time before you pipette the AMPure XP beads, you should vortex the bead tube thoroughly to obtain a homogeneous solution. The size selection is based on the amount of solution containing the AMPure XP beads, not the amount of beads *per se*, in relation to the sample volume. Fragments longer than 500 bp will remain on the beads in Step 11 when applying 58% (= 14.5/25) of beads, where 14.5 is the total volume of AMPure XP bead solution (11.5 µl from Step 7 plus 3.0 µl from Step 10) and 25 is the volume of sample from Step 6.

12| Add 500 µl of 70% (vol/vol) ethanol, incubate for 30 s and then pipette and discard the ethanol.

13| Repeat Step 12 once and remove any residual liquid drops at the bottom or on the walls of the tube.

14| Leave the tube open (on the MPC) to dry at ambient temperature for 2 min.

15| Remove the tube from the MPC and add 25 µl of 1× TE buffer (or 14 µl if starting with small amount of sample). Pipette to mix the bead pellet.

16| Place the tube back onto the MPC. After the beads are pelleted (about 1 min), collect the aqueous phase, which contains 500- to 900-bp-long fragments, into a new tube.

End-polishing, phosphorylation and dA extension ● TIMING 1 h

17| To a 200 µl PCR tube, add the following:

Size-selected DNA sample from Step 16 (add 1× TE up to 14 µl)	14.0 µl
dNTP mix (25 mM)	1.0 µl
SLOW ligation buffer, 10× (component of T4 DNA ligase kit)	2.5 µl
Buffer for Taq polymerase, 10× (Mg^{2+} free)	2.0 µl
End-repair mix (low concentration)	2.0 µl
Klenow exo-	0.5 µl
Taq polymerase	0.5 µl

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