

Methods for Generating Shotgun and Mixed Shotgun/Paired-End Libraries for the 454 DNA Sequencer

Graham Wiley,¹ Simone Macmil,¹ Chunmei Qu,¹ Ping Wang,¹ Yanbo Xing,¹ Doug White,¹ Jianfeng Li,¹ James D. White,¹ Alexander Domingo,² and Bruce A. Roe¹

¹University of Oklahoma, Norman, Oklahoma

²Roche Diagnostics, Indianapolis, Indiana

ABSTRACT

With the introduction of massively parallel, microminiature-based instrumentation for DNA sequencing, robust, reproducible, optimized methods are needed to prepare the target DNA for analysis using these high-throughput approaches because the cost per instrument run is orders of magnitude more than for typical Sanger dideoxynucleotide sequencing on fluorescence-based capillary systems. The methods provided by the manufacturer for genome sequencing using the 454/Roche GS-20 and GS-FLX instruments are robust. However, in an effort to streamline them for automation, we have incorporated several novel changes and deleted several extraneous steps. As a result of modifying these sample preparation protocols, the number of manual manipulations has also been minimized, and the overall yields have been improved for both shotgun and mixed shotgun/paired-end libraries. *Curr. Protoc. Hum. Genet.* 61:18.1.1-18.1.21. © 2009 by John Wiley & Sons, Inc.

Keywords: pyrosequencing • shotgun • paired-end • DNA Library

INTRODUCTION

Since their release in 2006 and 2007, the 454/Roche GS-20 and 454/Roche GS-FLX sequencers have revolutionized the DNA sequencing field through their ability to generate large amounts of data relatively quickly using massively parallel pyrosequencing after emulsion PCR (emPCR)-based DNA library amplification (Margulies et al., 2005). Robust protocols have been made available by 454 Life Sciences and Roche Diagnostics for shotgun (Margulies et al., 2005), paired-end only (Ng et al., 2006), and amplicon-based (Dahl et al., 2007) DNA sample preparation and sequencing. We now have modified these protocols and introduced a large-insert (2- to 4-kbp fragments), combined shotgun/paired-end protocol that requires fewer manipulations than the earlier methods and results in improved yields over the paired-end only protocol.

The DNA sequencing method implemented on the 454/Roche GS-20 and 454/Roche GS-FLX sequencers is based in part on the detection of inorganic pyrophosphate (PPi) that is released during the enzymatic incorporation of a deoxynucleotide triphosphate at the 3' end of a DNA primer. PPi release is monitored using purified firefly luciferase (an ATP sulfurylase catalyzed reaction; Nyrén and Lundin, 1985; Nyrén, 1987; Nyrén et al., 1993) to measure adenosine triphosphate (ATP) formed by the reaction of released PPi with adenosine monophosphate (AMP). The pyrosequencing principles were presented in detail somewhat later (Ronaghi et al., 1998), and more recently this DNA sequencing approach has been microminiaturized and massively parallelized, i.e., multiplexed to sequence hundreds of thousands of templates simultaneously (Margulies et al., 2005).

High-Throughput Sequencing

18.1.1

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The approach has been incorporated into commercial sequencing instruments, initially by 454 and later by Roche.

Presently, these instruments require significant up-front manipulations that are described in detail in the initial publications (Margulies et al., 2005). Briefly, they include shearing the DNA, followed by enzymatic end-repair, ligation of adaptor sequences, and then a second round of end-repair to yield a blunt-ended DNA library. After quantitation, dilution, and amplification via emulsion PCR (emPCR), the DNA is loaded onto a 454/Roche GS-FLX sequencer for massively parallel pyrosequencing, and the resulting sequence data is typically assembled using the manufacturer-supplied Newbler assembler.

Shotgun sequencing involves shearing DNA, typically using genomic or cloned DNA, into smaller fragments, which are sequenced individually (Roe, 2004). Historically, in shotgun sequencing, the target DNA first was sheared and, after end-repair, ligated into vectors and transformed into competent cells to create a clone library from which individual cloned plasmids containing 2- to 4-kbp inserts were isolated. Subsequently, each end of the ligated DNA library inserts was sequenced using the forward and reverse universal DNA sequencing primers to yield the paired ends for each of the sequenced, cloned inserts.

However, as initially described (Margulies et al., 2005), the individual sequence reads could not be paired, as the 100 to 250 base sequences from each end of the sheared ~500- to 1000-base fragments is not long enough to overlap. This limitation then led to the development of a paired-end tagging approach that was adapted to the 454/Roche massively parallel pyrosequencer (Ng et al., 2006). This approach required enrichment for DNA fragments that, when randomly sheared, contained a portion of a ligated paired-end tag. However, almost 50% of the paired-end tag-enriched DNAs did not have 50 bases or more on at least one side of the hairpin linker, and a large segment of the paired-end reads were too short to be useful in the subsequent assembly and closure processes.

Since this enrichment and the subsequent steps for isolating a single-stranded DNA-containing library were quite inefficient (they resulted a greatly reduced yield), as well as being labor intensive, these two steps were eliminated and other parameters were adjusted, as described in the optimized combined shotgun/ paired-end sequencing protocol (Basic Protocol 2). In addition, the shotgun sequencing protocol provided by the manufacturer was similarly modified by eliminating the steps for isolating a single-stranded DNA-containing library and optimizing other parameters (described in Basic Protocol 1), which resulted in an optimized shotgun sequencing protocol. A schematic representation of both the shotgun and the combined shotgun/paired-end sequencing protocols is shown in Figure 18.1.1.

Thus, our combined shotgun/paired-end library protocol (Basic Protocol 2)—an amalgamation of the original shotgun and paired-end library protocols supplied by the manufacturer (Roche) and our optimized shotgun library preparation (Basic Protocol 1)—yields both shotgun and paired-end reads from the same library. After emPCR (Nakano et al., 2003; Margulies et al., 2005) and sequencing, the fragments containing the central linker region can be identified to generate paired-end information for ordering and orienting the resulting assembled contigs.

It is important to note that these protocols deviate somewhat from the current protocols supplied by 454/Roche and that they represent over a year of optimization in the authors' laboratory. These protocols greatly streamline the overall DNA sample preparation process, as well as increase the final yield of library DNA. A more complete explanation of the changes is presented in the Commentary section following the protocols.

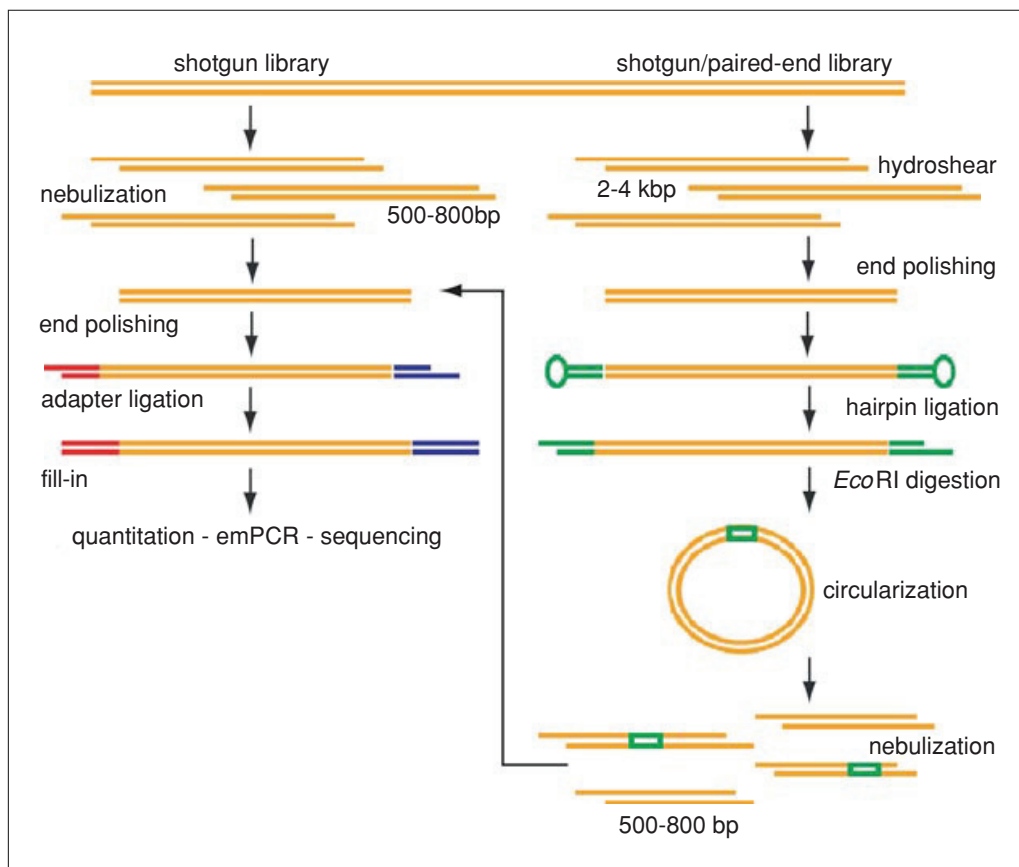


Figure 18.1.1 Schematic representation of the shotgun and mixed shotgun/paired-end library protocols. The left panel shows an outline of the steps needed to produce the shotgun library, which is subsequently quantitated, amplified by emPCR, and then sequenced on the 454/Roche GS-FLX sequencer. The right panel shows the steps used in the combined shotgun/paired-end protocol. Note that after the circularized DNA is nebulized, the end polishing, adaptor ligation, and fill-in is as shown in the shotgun protocol.

SHOTGUN LIBRARY PREPARATION

This protocol involves the preparation of a shotgun library for genome sequencing using the 454/Roche GS-FLX. After shearing the DNA via nebulization (Roe et al., 1996) to obtain double stranded DNA fragments in the size range 500 to 800 bp, the ends are repaired, adaptor sequences are ligated on each end, and the ends are repaired again to yield a blunt-ended DNA library, which then is quantified and diluted prior to amplification via emPCR.

Materials

- Isopropanol, chilled to -80°C
- GS DNA Library Preparation Kit (Roche), including:
 - Nebulizer, with condenser and aspiration tubes
 - TE buffer
 - Nebulization buffer
 - 10 $\times$ polishing buffer
 - BSA
 - ATP
 - dNTP mix
 - T4 polynucleotide kinase
 - T4 DNA polymerase
 - 2 $\times$ ligase buffer

BASIC PROTOCOL 1

High-Throughput Sequencing

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A and B adaptors

Ligase

Molecular biology grade water

10× Fill-In polymerase buffer

Fill-In polymerase

3 to 5 µg purified, double-stranded DNA: e.g., see Roe et al. (1996), Roe (2004),

APPENDIX 3B, APPENDIX 3C, or use a commercially available DNA isolation kit

Compressed nitrogen (research grade)

MinElute PCR Purification Kit (Qiagen), including:

PBI buffer

MinElute centrifuge columns

PE buffer

EB buffer

AMPure SPRI beads (Agencourt)

70% (v/v) ethanol

Dual-stage regulator on the nitrogen gas cylinder connected to the nebulizer using:

1/4-in. (6.35-mm) hose barb fitting with 1/4-in. (6.35-mm) NPT female

compression fitting

1/4-in. (6.35-mm) i.d. flexible tubing, with stainless steel screw clamp

1.5-ml microcentrifuge tubes

Microtiter plate for automated microfluidics system (Caliper)

Automated microfluidics system: AMS-90 and SE30 DNA LabChip (Caliper)

Magnetic particle concentrator (Invitrogen)

Vacuum drying system (e.g., model 5831; National Appliance)

NOTE: Most of the reagents in this protocol were obtained as components of the GS DNA Library Preparation Kit (Roche). The components of the kit are based on those described in the supplement to Margulies et al. (2005).

NOTE: All microcentrifugations are performed at room temperature.

Nebulize DNA

1. Prepare a -20°C isopropanol/wet ice bath, using -80°C isopropanol.

We mix -80°C isopropanol and ice, and the temperature reaches -20°C in ~ 1 min. If we start with room temperature isopropanol mixed with ice, it takes ~ 5 min of stirring to cool the mixture to -20°C .

2. Affix the nebulizer condenser tube around the aspiration tube in the nebulizer.
3. Pipet 3 to 5 µg double-stranded DNA sample to the bottom of the nebulizer.
4. Add TE buffer to a final volume of 100 µl.
5. Add 500 µl of nebulization buffer, and mix by swirling.
6. Assemble the nebulizer, ensuring condenser tube is included.
7. Submerge the nebulizer in a -20°C isopropanol/wet ice bath.
8. Apply 30 psi of nitrogen, using a dual-stage regulator, for 2.5 min at -20°C for genomic, BAC, or fosmid DNA.
9. Measure the volume of sample recovered. Ensure that >300 µl are recovered before proceeding.

Once the nebulization is complete, the sheared DNA can be stored in the nebulizer in an ice-water bath for several hours prior to concentration on the Qiagen MinElute centrifuge columns.

Concentrate DNA on centrifuge columns

10. Add 2.5 ml PBI buffer to the nebulizer containing the recovered sample.
11. Add 750 μ l PBI buffer/DNA mixture to each of two Qiagen MinElute centrifuge columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
12. Discard the flow-through.
13. Add 750 μ l of the remaining PBI buffer/DNA sample to each of the two purification columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
14. Discard the flow-through and add 750 μ l of PE buffer to each of the two purification columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
15. Discard the flow-through. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm). Rotate the column 180°, and microcentrifuge an additional 30 sec.
16. Place the columns into clean 1.5-ml microcentrifuge tubes. Add 12 μ l EB buffer to center of each of the two columns. Let stand 1 min. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm) to elute the DNA.
17. Pool the eluates from both columns (~ 24 μ l).

Assess DNA size and quality

18. Place the entire purified sample in a Caliper AMS-90 microtiter plate.
If necessary, the DNA-containing plate can be stored on ice or refrigerated for several hours to overnight prior to or after determining the fragment sizes in step 19.
19. Verify that the size of fragments is in the 500- to 800-bp range by analysis on the Caliper AMS-90 using a SE30 DNA LabChip, according to manufacturer's instructions.

End-polish DNA fragments and bead-purify

20. For a 50- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube:

- 24 μ l purified, nebulized DNA fragments (~ 2.5 μ g)
- 5 μ l of 10 $\times$ polishing buffer
- 5 μ l BSA
- 5 μ l ATP
- 2 μ l dNTP mix
- 5 μ l T4 polynucleotide kinase
- 5 μ l DNA polymerase.

Mix, and incubate 30 min at 25°C (room temperature).

21. Purify the DNA, using AMPure SPRI beads as follows:
 - a. Add 35 μ l AMPure SPRI beads to the DNA, and vortex to mix.
 - b. Incubate 5 min at room temperature.
 - c. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
 - d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
 - e. Vacuum dry completely (10 min).
The pellet is dry when visible cracks form.
 - f. Remove the tube from the magnetic particle collector, add 15 μ l EB buffer, and vortex.

- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

Ligate A and B adaptors and bead-purify DNA

22. For a 40- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube:

15 μ l nebulized, polished DNA
20 μ l of 2 $\times$ ligase buffer
1 μ l A and B adaptors
4 μ l ligase.

Mix, and incubate 15 min at 25°C (room temperature).

23. Purify the DNA, using AMPure SPRI beads as follows:

- a. Add 28 μ l AMPure SPRI beads, and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove the tube from the magnetic particle collector, add 25 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

NOTE: The steps for preparation of library immobilization beads and for library immobilization (recommended by 454/Roche) have been eliminated, as we no longer make single-stranded DNA (see the Commentary for the rationale for skipping this step), but go directly to the fill-in reaction step.

Carry out fill-in reaction and bead-purify DNA

24. For a 50- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube:

25 μ l purified DNA with ligated adaptors
15 μ l molecular biology grade water
5 μ l 10 $\times$ Fill-In polymerase buffer
2 μ l of 10 mM dNTP mix
3 μ l Fill-In polymerase

Mix and incubate 20 min at 25°C (room temperature).

25. Purify the DNA, using AMPure SPRI beads as follows:

- a. Add 35 μ l AMPure SPRI beads and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove the tube from the magnetic particle collector, add 15 µl EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads, transfer the supernatant containing purified the DNA to a fresh 1.5-ml tube.

At this point the sample can be stored on ice or in the refrigerator for several hours to overnight prior to the following library quantitation step.

Quantitate and dilute library

26. Place the entire purified sample in a Caliper AMS-90 microtiter plate.
27. Verify that the size of fragments is in the 500- to 800-bp range by analysis on the Caliper AMS-90, using a SE30 DNA LabChip according to manufacturer's instructions.
28. Calculate molecules/µl from the ng/µl results from the Caliper AMS-90 using the following formula.

$$\text{Molecules/}\mu\text{l} = \text{sample concentration (ng/}\mu\text{l)} \times (6.022 \times 10^{23} \text{ molecules/mole}) / (656.6 \times 10^9 \text{ grams/mole}) \times \text{average fragment length (nucleotides)}$$

29. Dilute the library stock 1:4 by adding ~39 µl TE buffer to the concentrated library, prior to proceeding with the manufacturer's recommended protocol for emPCR and subsequent sequencing on the GS-FLX.

NOTE: The diluted library stock can be stored up to 1 year at -20°C and thawed and refrozen at least four times over the year.

SHOTGUN/PAIRED-END LIBRARY PREPARATION

This protocol describes the preparation of a shotgun/paired-end library for the 454/Roche GS-FLX. This is based on an initial circularization of a fragment sheared to 2 to 4 kb in a GeneMachines HydroShear apparatus (Oefner et al., 1996), utilizing a common central linker to identify potential paired-end reads, followed by a second shearing prior to adaptor ligation, emPCR, and sequencing.

Materials

- 3-5 µg purified, double-stranded DNA in TE buffer: e.g., see Roe et al. (1996), Roe (2004), APPENDIX 3B, APPENDIX 3C, or use a commercially available DNA isolation kit
- TE buffer (APPENDIX 2D)
- AMPure SPRI beads (Agencourt)
- 70% (v/v) ethanol
- MinElute PCR purification kit (Qiagen), including:
 - EB buffer
 - MinElute centrifuge columns
 - PBI buffer
 - PE buffer
- 32 mM S-adenosylmethionine (SAM) stock (New England Biolabs)
- Molecular biology grade water
- 10× *Eco*RI methylase buffer and 40,000 U/ml *Eco*RI methylase (New England Biolabs)
- 10 mg/ml bovine serum albumin (BSA; New England Biolabs)
- 30,000 U/ml T4 polynucleotide kinase and 10× polynucleotide kinase buffer (USB)
- 10 mM (each) dNTP mix (see APPENDIX 2D)
- 3000 U/ml T4 DNA polymerase (New England Biolabs)

BASIC PROTOCOL 2

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2× Rapid Ligase buffer and Rapid Ligase (Roche)
 Hairpin linker (GS DNA Paired End Adaptor Kit, Roche)
 100 mM ATP (*APPENDIX 2D*)
 5000 U/ml lambda exonuclease (New England Biolabs)
 10,000 U/ml T7 exonuclease (New England Biolabs)
 5000 U/ml exonuclease I (New England Biolabs)
 20,000 U/ml *EcoRI*
 10× NEBuffer 4 (New England Biolabs)
 Isopropanol, chilled to -80°C
 GS DNA Library Preparation Kit (Roche), including:
 Nebulizer, with condenser and aspiration tubes
 TE buffer
 Nebulization buffer
 Polishing buffer
 BSA
 ATP
 dNTP mix
 T4 polynucleotide kinase
 T4 DNA polymerase
 2× ligase buffer
 A and B adaptors
 Ligase
 Molecular biology grade water
 10× Fill-In polymerase buffer
 Fill-In polymerase
 Compressed nitrogen (research grade)
 1.5-ml microcentrifuge tube
 GeneMachines HydroShear apparatus, equipped with the HydroShear large
 assembly and HydroShear syringe (Genomic Solutions)
 Magnetic particle concentrator (Invitrogen)
 Vacuum drying system (e.g., model 5831; National Appliance)
 Microtiter plate for the automated microfluidics system (Caliper)
 Automated microfluidics system: AMS-90 and SE30 DNA LabChip (Caliper)
 Thermal cycler *or* heating block
 12°C refrigerator
 Dual-stage regulator on the nitrogen gas cylinder connected to the nebulizer using:
 $\frac{1}{4}$ -in. (6.35-mm) hose barb fitting with $\frac{1}{4}$ -in. (6.35-mm) NPT female
 compression fitting
 $\frac{1}{4}$ -in. (6.35-mm) i.d. flexible tubing, with stainless steel screw clamp

NOTE: Most of the reagents used in this protocol were obtained as components of the GS DNA Library Preparation Kit (Roche). The components of the kit are based on those described in the supplement to Margulies et al. (2005).

NOTE: All microcentrifugations are performed at room temperature.

Fragment DNA

1. Transfer 5 μg of sample DNA (in TE buffer) to a 1.5-ml microcentrifuge tube.
2. Add TE buffer to a final volume of 200 μl . Vortex to mix, and place the tube in an ice-water bath to cool to $\sim 4^{\circ}\text{C}$ for 15 min to insure unbiased cleavage.

At higher temperatures, more AT base pairs are broken than GC base pairs (see Roe et al., 1996; Roe, 2004)

3. Shear the DNA in a HydroShear apparatus, following the manufacturer's instructions in the user manual.

In particular, be aware that instructions in section 6.4 of the HydroShear manual include steps that are contradictory to software prompts. The authors' validated procedure uses 20 cycles at a speed setting of 10 in the standard shearing assembly, and has produced an average fragment size of ~2.2 kb. However, each assembly should be tested individually for fragmentation size range, per the manufacturer's recommendation.

After shearing, the DNA can be stored in an ice-water bath or refrigerator for several hours to overnight.

Remove small DNA fragments

4. Estimate the total volume of the DNA solution from the HydroShear, using a 200- μ l micropipettor.
5. Add TE buffer to adjust the final volume to 200 μ l.
6. Add 100 μ l of AMPure SPRI beads, and vortex to mix.
7. Incubate 5 min at room temperature.
8. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
9. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
10. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

11. Remove tube from magnetic particle collector, add 50 μ l EB buffer, and vortex.
12. Using the magnetic particle collector, pellet the beads and transfer supernatant containing the purified DNA to a fresh 1.5-ml tube.

At this point the DNA can be stored in an ice-water bath or refrigerator for several hours to overnight prior to sizing and quality assessment.

Assess DNA size and quality

13. Place purified sample in Caliper AMS-90 microtiter plate.
14. Verify that the size of fragments is in the 2- to 4-kb range by analysis on the Caliper AMS-90, using SE30 DNA LabChip according to manufacturer's instructions.

Methylate *EcoRI* sites

15. Prepare a fresh 3.2 mM dilution of *S*-adenosylmethionine (SAM), from the 32 mM stock, using molecular biology grade water. For a 20- μ l reaction, combine 18 μ l of water with 2 μ l of 32 mM SAM in a 1.5-ml microcentrifuge tube. Vortex to mix, and store on ice.
16. For a 100- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube in the order indicated:

30 μ l molecular biology grade water
10 μ l 10 $\times$ *EcoRI* methylase buffer
5 μ l of 3.2 mM SAM
50 μ l sheared DNA
5 μ l of 40,000 U/ml *EcoRI* methylase.

Mix by vortexing, and incubate the methylation reaction 30 min at 37°C, using the thermal cycle or a heating block.

17. Purify the methylated DNA fragments, using AMPure SPRI beads as follows:

- a. Add 70 μ l AMPure SPRI beads. Vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove tube from magnetic particle collector, add 26 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to the fragment end polishing.

End-polish DNA fragments and bead-purify

18. Prepare a fresh 1 mg/ml dilution of BSA from the 10 mg/ml stock, using molecular biology grade water. For a 40- μ l reaction, combine 36 μ l of water with 4 μ l of 10 mg/ml BSA stock in a 1.5-ml microcentrifuge tube. Vortex to mix, and store on ice.
19. Prepare a fresh 10 mM dilution of ATP from the 100 mM stock, using molecular biology grade water. For a 20- μ l reaction, combine 18 μ l of water with 2 μ l of 100 mM ATP in a 1.5-ml microcentrifuge tube. Vortex to mix, and store on ice.
20. For a 50- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube in the order indicated:

5 μ l 10 $\times$ polynucleotide kinase buffer
5 μ l of 1 mg/ml BSA (from step 18)
5 μ l of 10 mM ATP (from step 19)
2 μ l of (10 mM each) dNTP mix
26 μ l sheared, methylated DNA
5 μ l of 3000 U/ml T4 DNA polymerase
2 μ l of 30,000 U/ml T4 polynucleotide kinase.

Mix by vortexing, and incubate 15 min at 12°C.

21. Immediately continue incubation at 25°C (room temperature) for an additional 15 min.
22. Purify the DNA, using AMPure SPRI beads as follows:
 - a. Add 35 μ l AMPure SPRI beads and vortex to mix.
 - b. Incubate 5 min at room temperature.
 - c. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for subsequent wash step.
 - d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
 - e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove the tube from the magnetic particle collector, add 15 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads, transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to hairpin linker ligation.

Ligate the hairpin linker, select with exonuclease, and bead-purify DNA

23. For a 95- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube in the order indicated:

50 μ l of 2 $\times$ rapid ligase buffer
 30 μ l hairpin linker
 15 μ l sheared, methylated, and polished DNA

Mix by vortexing.

24. Add 5 μ l rapid ligase, and mix by vortexing. Incubate the ligation reaction 15 min at 25°C (room temperature).
25. To remove any unreacted hairpin linker, add the following exonucleases to the ligation reaction:

2 μ l of 5000 U/ml lambda exonuclease
 2 μ l of 10,000 U/ml T7 exonuclease
 4 μ l of 5000 U/ml exonuclease I.

Mix by vortexing and incubate the exonuclease reaction 30 min at 37°C.

26. Further purify the products, using AMPure SPRI size exclusion beads as follows:

- a. Add 76 μ l of AMPure SPRI beads and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove tube from magnetic particle collector, add 50 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to digestion with EcoRI.

Digest DNA with EcoRI and bead-purify

27. For a 100- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube in the order indicated:

30 μ l molecular biology grade water
 10 μ l of 10 $\times$ NEBuffer 4
 ~50 μ l hairpin-adapted DNA fragments
 10 μ l of 20,000 U/ml EcoRI.

Mix by vortexing and incubate 16 hr (or overnight) at 37°C.

28. Purify the *Eco*RI digestion products, using AMPure SPRI size exclusion beads as follows:

- a. Add 70 μ l of AMPure SPRI beads, and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove the tube from the magnetic particle collector, add 50 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to DNA circularization.

Circularize DNA and bead-purify

29. For a 195- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube in the order indicated:

- 123 μ l molecular biology grade water
- 20 μ l of 10 $\times$ NEBuffer 4
- 2 μ l of 100 mM ATP stock solution
- 50 μ l *Eco*RI-digested DNA.

Mix by vortexing.

30. Add 5 μ l of rapid ligase. Mix by vortexing, and incubate 60 min at 25°C (room temperature).

31. Add the following exonucleases to the ligation reaction to remove any excess non-circularized DNA:

- 2 μ l of 5000 U/ml lambda exonuclease
- 2 μ l of 10,000 U/ml T7 exonuclease
- 4 μ l of 5000 U/ml exonuclease I.

Mix by vortexing, and incubate 30 min at 37°C.

32. Purify the selected ligation products, using AMPure SPRI size exclusion beads as follows:

- a. Add 146 μ l of AMPure SPRI beads, and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol.
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove the tube from the magnetic particle collector, add 50 μ l EB buffer, and vortex.
- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to shearing in the nebulizer.

Nebulize DNA

33. Prepare a -20°C isopropanol/wet ice bath, using -80°C isopropanol and ice.
34. Affix the nebulizer condenser tube around the aspiration tube in the nebulizer.
35. Pipet 50 μ l of the circularized double-stranded DNA sample to the bottom of the nebulizer.
36. Add TE buffer to a final volume of 100 μ l.
37. Add 500 μ l of nebulization buffer, and mix by swirling.
38. Assemble the nebulizer, ensuring that the condenser tube is included.
39. Submerge the nebulizer in a -20°C isopropanol/wet ice bath.
40. Apply 45 psi of nitrogen, using a dual-stage regulator, for 2.5 min at -20°C for 2- to 4-kb circularized DNA.
41. Measure the volume of sample recovered. Ensure that >300 μ l are recovered before proceeding.

Once the nebulization is complete, the sheared DNA can be stored in the nebulizer in an ice-water bath for several hours prior to concentration on the Qiagen MinElute centrifuge columns.

Concentrate DNA on centrifuge columns

42. Purify on a Qiagen MinElute centrifuge column:
 - a. Add 2.5 ml of PBI buffer to the nebulizer containing the recovered sample
 - b. Obtain two Qiagen MinElute centrifuge columns. Add 750 μ l of PBI Buffer/DNA mixture to each of the two columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
 - c. Discard the flow-through.
 - d. Add 750 μ l of the remaining PBI buffer/DNA sample to each of the two purification columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
 - e. Discard the flow-through, and add 750 μ l of PE buffer to each of the two purification columns. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm).
 - f. Discard flow-through. Microcentrifuge 1 min at full speed ($\geq 10,000$ rpm). Rotate the column 180° , and microcentrifuge for an additional 30 sec.
 - g. Place the Qiagen MinElute centrifuge columns into clean 1.5-ml microcentrifuge tubes. Add 13 μ l of EB buffer to the center of each of the two columns. Let stand for 1 min. Microcentrifuge 1 min at full speed to elute the DNA.
 - h. Pool eluates from both columns for ~ 26 μ l.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior fragment end polishing.

End-polish DNA fragments and bead-purify

43. For a 53- μ l reaction, combine the following reagents from the GS DNA Library Preparation Kit in a 1.5-ml microcentrifuge tube in the order indicated:

- 5 μ l of 10 $\times$ polynucleotide kinase buffer
- 5 μ l BSA solution
- 5 μ l ATP solution
- 2 μ l dNTP mix
- 26 μ l sheared, methylated DNA
- 5 μ l T4 DNA polymerase
- 5 μ l T4 polynucleotide kinase.

Mix by vortexing, and incubate the polishing reaction 30 min at 25°C (room temperature).

44. Purify the DNA, using AMPure SPRI beads as follows:

- a. Add 35 μ l of AMPure SPRI beads. Vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol
- e. Dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove tube from magnetic particle collector, add 15 μ l EB buffer, and vortex
- g. Using the magnetic particle collector, pellet the beads, and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior ligating the A and B adaptors.

Ligate A and B adaptors and bead-purify

45. For a 40- μ l reaction, combine the following in a 1.5-ml microcentrifuge tube:

- 15 μ l nebulized, polished DNA
- 20 μ l of 2 $\times$ ligase buffer
- 1 μ l A and B adaptors
- 4 μ l ligase.

46. Mix and incubate 15 min at 25°C (room temperature).

47. Purify on AMPure SPRI beads, as follows:

- a. Add 28 μ l of AMPure SPRI beads, and vortex to mix.
- b. Incubate 5 min at room temperature.
- c. Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- d. Remove the supernatant, and wash the beads twice with 500 μ l of 70% ethanol
- e. Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- f. Remove tube from magnetic particle collector, add 15 μ l EB buffer, and vortex.

- g. Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

NOTE: *The steps for preparation of library immobilization beads and for library immobilization (recommended by 454/Roche to make the single-stranded DNA) have been eliminated.*

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to the fill-in reaction.

Carry out fill-in reaction and bead-purify DNA

48. In a 1.5-ml microcentrifuge tube add the following:

25 µl purified DNA with ligated adaptors
15 µl molecular biology grade water
5 µl of 10× Fill-In polymerase buffer
2 µl of 10 mM (each) dNTP mix
3 µl Fill-In polymerase.

Mix, and incubate 30 min at 25°C (room temperature).

49. Purify the DNA, using AMPure SPRI beads, as follows:

- Add 35 µl of AMPure SPRI, and vortex to mix.
- Incubate for 5 min at room temperature.
- Using the magnetic particle collector, pellet the beads against the wall of the tube. Keep the tube of beads in the magnetic particle collector for the subsequent wash step.
- Remove the supernatant, and wash the beads twice with 500 µl of 70% ethanol.
- Vacuum dry completely (10 min).

The pellet is dry when visible cracks form.

- Remove the tube from the magnetic particle collector, add 15 µl EB buffer, and vortex.
- Using the magnetic particle collector, pellet the beads and transfer the supernatant containing the purified DNA to a fresh 1.5-ml tube.

If necessary, the DNA can be stored on ice or refrigerated for several hours to overnight prior to library quantitation and dilution.

Quantitate and dilute library

50. Place all the purified sample in a Caliper AMS-90 microtiter plate.
51. Verify that the size of fragments is in the 500- to 800-bp range by analysis on the Caliper AMS-90 using a SE30 DNA LabChip, according to manufacturer's instructions.
52. Calculate molecules/µl from the ng/µl results from the Caliper AMS-90 using the molecules calculator spreadsheet.

Molecules/µl = sample concentration (ng/µl) × (6.022 × 10²³ molecules/mole)/(656.6 × 10⁹ grams/mole) × average fragment length (nucleotides)

53. Dilute the library stock 1:4 by adding ~39 µl of TE buffer to the concentrated library, prior to proceeding with the manufacturer's recommended protocol for emPCR and subsequent sequencing on the GS-FLX.

NOTE: *The diluted library stock can be stored up to 1 year at -20°C and reused.*

COMMENTARY

Background Information

Sample preparation, prior to loading the 454/Roche GS-20 and 454/Roche GS-FLX, requires numerous labor-intensive steps (Margulies et al., 2005). As with any protocol, every step should be optimized to obtain the highest yield. Although the manufacturers' suggested sample preparation protocols are quite robust, we have incorporated several novel changes and deleted several extraneous steps in an effort to streamline them for automation. The result is that we have optimized the sample preparation protocol, minimized the manual manipulations, and thereby improved the overall yields.

One of the initial modifications we introduced was to replace the Qiagen MinElute centrifuge columns with an Agencourt AMPure SPRI bead-based purification. This change not only results in a 4- to 5-fold increase in yield, but also significantly reduces the concentration of small DNA fragments in the final DNA library. Although the MinElute centrifuge columns are extremely useful in numerous applications, there is an ~20% loss of sample each time they are used according to the manufacturer's instructions. In contrast, the AMPure SPRI beads have a recovery of between 90% and 95%, depending on the number of small (<300 bp) DNA fragments, when used according to the manufacturer's instructions. These small fragments amplify much more readily in the subsequent emPCR steps and increase the number of unwanted shorter sequence reads. Although the AMPure SPRI beads are effective, we still use MinElute centrifuge columns immediately after the nebulization steps to concentrate the DNA fragments, as they more efficiently concentrate the ~600 µl of nebulized DNA than would be feasible with the AMPure SPRI beads.

In addition to replacing several manufacturer recommended MinElute centrifuge-column steps with AMPure SPRI bead-based purification, we also removed the manipulations that were recommended for enriching DNA molecules that contain a single A and B adaptor ligated onto each end of the fragment. This step was deemed unnecessary because enrichment after the emPCR will not select those DNA fragments with identical adaptors on each end. Fragments with A adaptors ligated to both ends of the same fragment will not bind to the DNA binding beads, while those fragments with B adaptors ligated to both ends

of the same fragment will not be enriched after emPCR.

Another modification we introduced to further optimize this DNA library preparation method was to eliminate the steps that resulted in generating a single-stranded DNA library, as it, too, was unnecessary. By eliminating this step, the yields of the final shotgun DNA library preparation were increased at least 2-fold.

When it was first introduced, the GS-FLX sequencer gave average read lengths at least two and one-half times longer than the GS-20. The authors' laboratory has since developed a software modification to extend this read length even further to approximately three and one-half times that of the GS-20 (data not shown). To take advantage of the longer read lengths, we have replaced the *MmeI* digest that was recommended for generating paired ends with a second shearing step. This resulted in generation of larger paired-end fragments, which when sequenced on the GS-FLX gave longer reads that assisted the shotgun assembly process.

After implementing the protocol alterations described, the streamlined library-making process increased the overall yield, with the initial experiments showing ~5% of the sequenced reads having the paired-end linker sequence (which would be expected for an initial shearing length of ~4kb) and an average sequence length of 250 bases.

With the paired-end linker length of 44 bases, the expected percentage of reads that would contain the entire linker sequence is:

$$\% \text{ contains linker} = 100 \times (1 + \text{read length} - 44) / (\text{paired-end fragment length} + 44)$$

For example: $5.12\% = 100 \times (1 + 250 - 44) / (4000 + 44)$

Of these linker-containing sequences, almost 50% did not have at least 50 sequenced bases on each side of the linker. This results in one end of the paired-end reads being too short to be useful in the subsequent assembly and closure processes. Therefore, we investigated eliminating the paired-end library immobilization and PCR enrichment step after end-repair and ligation of their A and B shotgun adaptors. As a result, a library containing a mixture of both shotgun and paired-end DNA fragments was generated. Thus, instead of removing the fragments that lack the internal linker to generate a strictly paired-end library,

these fragments are retained in the library and used to generate shotgun style reads. The expected percentage of these mixed reads that are usable paired-end reads (i.e., containing the paired-end linker sequence plus at least 50 bases on each side of the linker) is:

$$\% \text{ paired ends} = 100 \times [1 + \text{read length} - 44 + (2 \times 50)] / (\text{paired-end fragment length} + 44)$$

For example: $2.65\% = 100 \times [1 + 250 - 44 + (2 \times 50)] / (4000 + 44)$

As shown in Table 18.1.1, the ratio of paired-end reads to shotgun reads is related to the length of the circularized DNA prior to shearing in the nebulizer and to the sequenced read lengths. Therefore, after random shearing, larger circularized DNA fragments will generate a greater number of smaller fragments without the internal linker sequence. Conversely, longer read lengths provide more sequences with the internal linker and therefore more usable paired ends. Although this combined shotgun/paired-end protocol typically generates only 3% to 5% paired-end reads for a typical GS-FLX sequencing run, the 15,000 to 25,000 usable paired ends generated in the assembly (using the winter 2008

release of the 454/Roche Newbler assembler, version 1.1.03) is quite significant and useful. This is particularly noteworthy when one considers the significant reduction in the labor-intensive library-making steps and an average approaching 500,000 reads on a full GS-FLX picotiter plate.

As stated previously, the modifications made to these protocols also make them much more amenable to automation. The authors' laboratory has successfully automated the shotgun library preparation, requiring only minimal human interaction. Using a Caliper SciClone advanced liquid handling system, as well as a Twister II robotic plate positioner, up to 16 libraries may be constructed in parallel with the input of nebulized, Qiagen MinElute centrifuge column-purified DNA. This automation also may be used for the post-nebulization manipulations of the shotgun/paired-end protocol (manuscripts in preparation).

Critical Parameters and Troubleshooting

As with any protocol that deviates from that provided by the manufacturer, one always should be cautious and generate baseline

Table 18.1.1 Typical Paired-End Percentages from Mixed Shotgun/Paired-End Libraries with Various Lengths of the First DNA Shearing and the Average Sequenced Read Length

First shearing average length	Average sequenced read length	Expected % of reads containing paired-end linker ^a	Expected % of reads with usable paired ends ^b
2000	250	10.13%	5.23%
3000	250	6.80%	3.52%
4000	250	5.12%	2.65%
6000	250	3.42%	1.77%
8000	250	2.57%	1.33%
2000	400 ^c	17.47%	12.57%
3000	400 ^c	11.73%	8.44%
4000	400 ^c	8.83%	6.36%
6000	400 ^c	5.91%	4.25%
8000	400 ^c	4.44%	3.19%

^aBecause not all DNA/beads gave readable sequences and a portion of the tags on the paired ends could not be discerned, the actual results were ~75% of the expected results and, with typical sheared DNA in the 2- to 4-kb range, ~5% linker-contained recognizable tags, instead of the expected 6.8%.

^bAssuming that 50 bases on each side of the paired-end linker are necessary to be recognized as a linker being present to allow the two ends to be incorporated into the assembly properly.

^cA read length of 400 bases is shown for estimating effects of longer read-lengths that might be provided by possible future GS-FLX upgrades, although no specific longer read-length protocol is presently available from 454/Roche in the GS-FLX Titanium upgrade. However, this has not been tested in the authors' lab.

data using the recommended protocol before venturing to try modifications. Although the protocol presented above has been thoroughly tested over the past year, it always is better to be a bit cautious when attempting modified protocols.

With this caveat in mind, the most critical parameter is the ability to quantitate the final DNA library that subsequently will be used in the emPCR steps, as recommended by 454/Roche. The initial amount of DNA sample for the first shearing step should be as close as possible to the recommended 3 to 5 μg . The overall final DNA library yield, with our improved, optimized protocol, is of the order of 12 to 13 ng, even when starting with the recommended amount of input DNA. Although, recent advances in quantitative PCR have made it possible to generate a shotgun library when

only small amounts of starting DNA are available (Meyer et al., 2008), it is advisable to use upwards of 10 μg of initial DNA in the first shearing step. This increased amount results in improved yields. Also, as indicated in the manufacturer-supplied literature, it should be noted that the maximum capacity of each Qia-gen MinElute centrifuge columns is 5 μg , and two columns typically are used to concentrate the nebulized DNA.

Finally, note that the amount of AMPure SPRI bead suspension used in each step is proportional to the amount of DNA-containing solution to which the AMPure SPRI bead suspension is being added. The optimum volume of the AMPure SPRI bead suspension is 70% of the volume of the DNA solution. Any less than this will cause an increase in the size exclusion limit, as well as a decrease in the

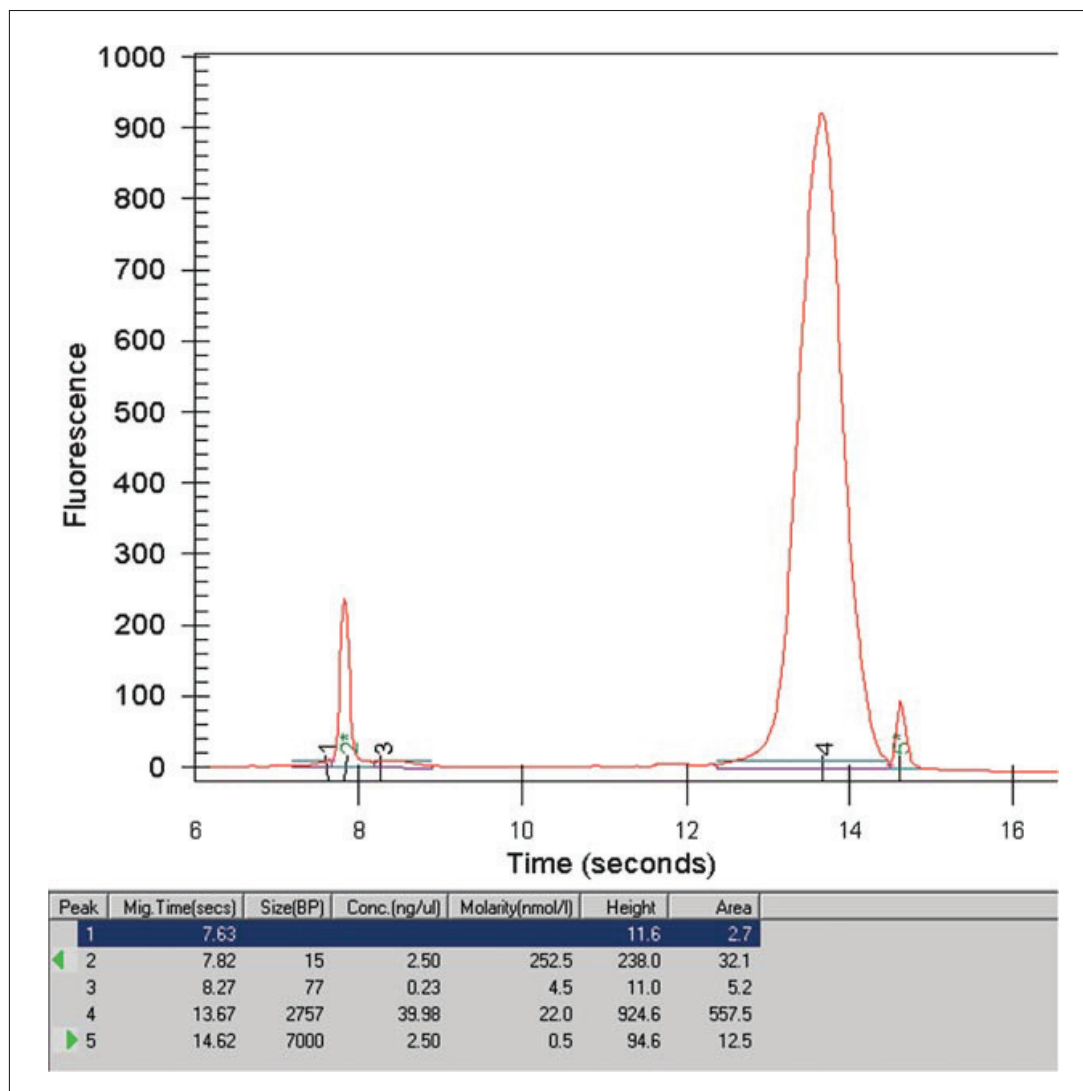


Figure 18.1.2 Results from a typical genomic DNA sample sheared with the Hydroshear and analyzed on a Caliper AMS-90.

overall amount of DNA bound, and thus recovered, due to the decreased concentration of PEG contributed by the AMPure SPRI bead suspension (Hawkins et al., 1994).

Anticipated Results

The final product of these protocols is a library of double-stranded DNA molecules with 454 adaptors attached to each end. These molecules are ready to be sequenced, as the DNA fragments in the library contain the 454 primer binding sites and (in the case of the mixed shotgun/paired-end library) the paired end-tagged sequence that was inserted as a linker prior to circularization of the 2- to 4-kb sheared DNA fragments. Figure 18.1.2 shows a typical genomic DNA sample that has been sheared using the HydroShear and then sized and quantitated on a Caliper AMS-90.

After the generation of a mixed shotgun/paired-end library, it is common to have a significantly lower total amount of DNA. After generating a mixed shotgun/paired-end library for the DNA sample shown in Figure 18.1.2, the overall yield of DNA was 12.6 ng. Table 18.1.2 shows the overall results for a sequencing run using the library shown in Figure 18.1.3. Using the DNA library prepared with the AMPure SPRI bead, double-stranded DNA, mixed shotgun/paired-end process, the amount of sequences lost to the Too Short Primer filter was only 1/10th of 1%, compared to order of magnitude higher values obtained when using the manufacturer recommended protocols.

Time Considerations

The following timing considerations are strongly recommended for performing Basic

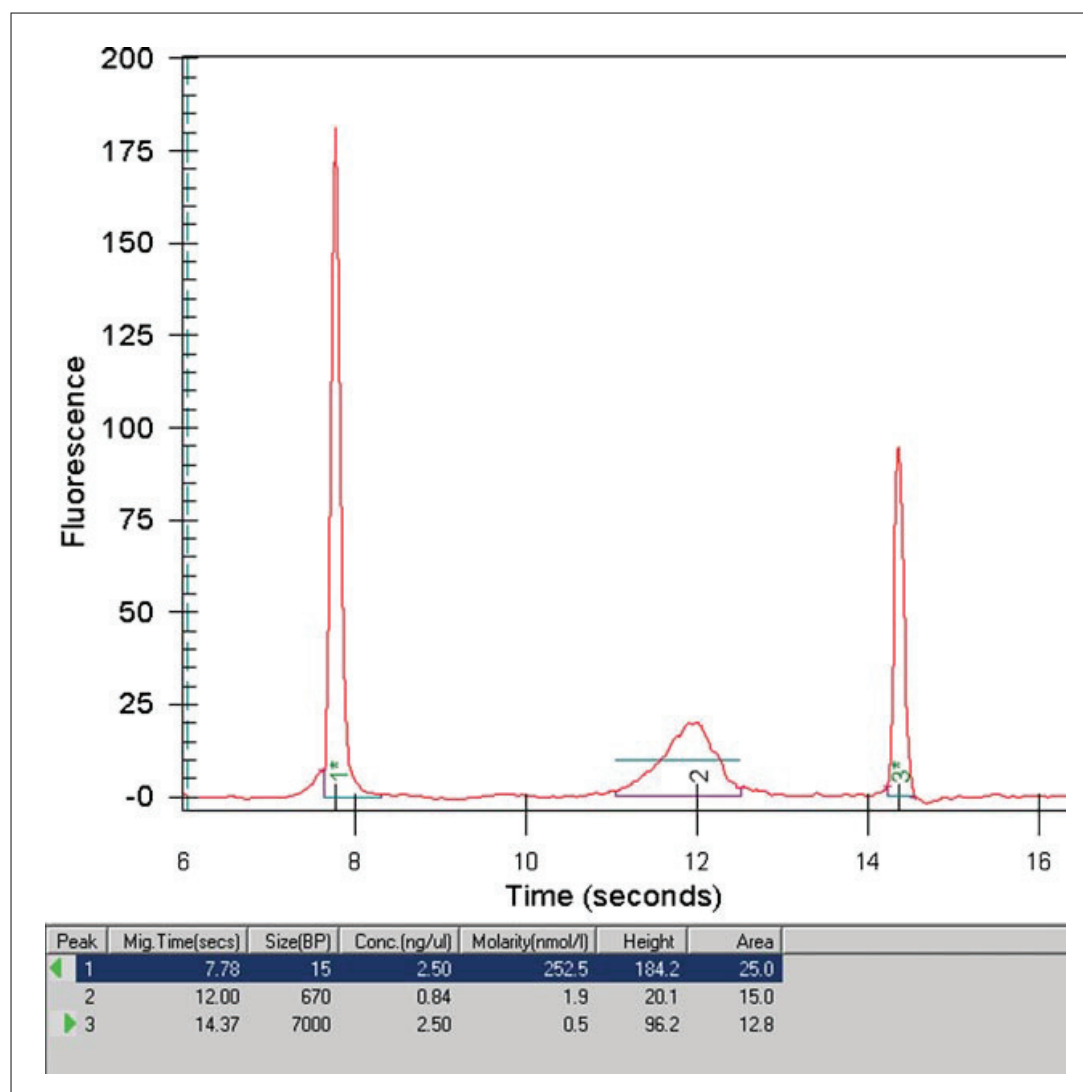


Figure 18.1.3 Result from a typical combined shotgun/paired-end library analyzed on a Caliper AMS-90.

Table 18.1.2 Overview of Sequencing Results for the Mixed Shotgun/Paired-End Libraries Shown in Figures 18.1.1 and 18.1.2

454/Roche parameter ^a	Value
Key Sequence	TCAG
Beads Loaded	100,000
Key Pass	42,616
Dot Failed	6,013
Mixed Failed	6,210
Trimmed Too Short Quality	7,198
Trimmed Too Short Primer	50
Total Passed Filtering	23,145
% Passed Filtering	54.3
Total Bases Sequenced	7,390,032
Average Read Length	319.2
Average Phred Quality	26.5

^aParameters obtained from output files from the sequencer provided by the sequencer-associated software. See the 454/Roche sequencer operating manual for definitions of the parameters.

Protocol 1 (the shotgun library protocol) and Basic Protocol 2 (the mixed shotgun/paired-end library protocol).

In Basic Protocol 1, although the time required to complete the shotgun library (after isolating and purifying the DNA) is only ~3 hr, it is possible to pause the protocol at several places. For example, once the nebulization is complete (step 9), the sheared DNA can be stored in the nebulizer in an ice-water bath for several hours prior to concentration on the Qiagen MinElute centrifuge columns. If necessary, the DNA-containing plate (step 18) can be stored on ice or refrigerated for several hours to overnight prior to or after determining the fragment sizes in step 19. The sample can also be stored on ice or in the refrigerator for several hours to overnight prior to the library quantitation step (step 28). Once quantitated, the library can be stored at -20°C for up to a year, with multiple freezing and thawing cycles, if it necessary.

In Basic Protocol 2, the time required to complete the mixed shotgun/paired-end library takes ~2 days with an overnight incubation after step 27. Although it is recommended that the time line given in the protocol be followed closely, it is possible to store the DNA in an ice-water bath or refrigerator for several hours to overnight as indicated after shearing the DNA in step 3 and after each AMPure SPRI bead purification (steps 17g, 22g, 26g, 28g, 32g, 44g, 47g, and 49g).

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