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Methods for Generating Shotgun and Mixed Shotgun/Paired End Libraries (with or without MID tags) for the 454 DNA Sequencer Titanium Picotiter Plates

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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 their analysis using these high-throughput approaches because the cost per instrument run is orders of magnitude more expensive than for typical Sanger dideoxynucleotide sequencing on fluorescent-based capillary systems. Although the methods provided by 454/Roche with the GS-20 and GS-FLX instruments using the Titanium picotiter plates are robust, in an effort to streamline and automate them, we now have incorporated the modifications that we introduced earlier in the FLX protocol into the most recent Roche Titanium picotiter plate protocol. As a result we have reduced the number of manual manipulations, automated portions of the library preparation procedure, and improved the overall yields for both shotgun and mixed shotgun/paired end libraries using the chemistry designed for use with the Titanium picotiter plates.

Key Words: Pyrosequencing, Shotgun, Paired-End, DNA Library

Introduction

Since their release in 2006 and 2007, the 454/Roche GS-20 and 454/Roche GS-FLX, respectively, have revolutionized the DNA sequencing field through their ability to relatively quickly generate large amounts of data 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 earlier modified these protocols and introduced a "combined shotgun and paired-end" protocol that requires fewer manipulations than the earlier methods and results in improved yields over the "paired-end only" protocol (Wiley, *et al.* 2009).

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 by monitoring the adenosine triphosphate (ATP) formed by reacting the released PPi with adenosine monophosphate (AMP) in an ATP sulfurylase catalyzed reaction using purified firefly luciferase (Nyrén, P and Lundin A.) as initially described (Nyrén P. 1987; Nyrén P, Pettersson B, Uhlén M. 1993). The pyrosequencing principles were presented in detail somewhat later (Ronaghi, M., M. Uhlén and P. Nyrén. 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) and subsequently released as commercial instruments initially by 454 Inc. and later by Roche Inc. Presently, these sequencing instruments require significant up-front manipulations that are described in detail in the initial publications (Margulies, *et al.* 2005), and briefly include shearing the DNA followed by enzymatic end-repair, the ligation of adapter sequences and then a second round of end-repair to yield a blunt ended DNA library that is quantitated. After 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 typically is assembled using the manufacturer supplied Newbler assembler.

"Shotgun" sequencing involves shearing DNA, typically using genomic or cloned DNA into smaller fragments that are sequenced individually (Roe 2004). Historically, in shotgun sequencing the target DNA first was sheared and after end-repair, was ligated into vectors that were transformed into competent cells to create a clone library from which individual cloned plasmids containing 2-4 Kbp inserts were isolated. Each end of the ligated DNA library inserts subsequently were 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 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) that required enrichment for DNA fragments that, when randomly sheared, contained a portion of a ligated "paired-end" tag and more recently the introduction of chemistry for even longer read lengths and a several fold increase in sample density on the newly released Roche Titanium picotiter plates. However, since the "paired-end" tag protocols require additional modifications to enrich in "paired-end"-containing DNAs and a large percentage of the fragments still contain fewer than 50 bases on at least one side of the linker, a large segment of the "paired-end" reads, even with the Titanium improvements were too short to be useful as "paired-ends" in the subsequent assembly and closure processes. Since this enrichment and the subsequent the steps for isolating a single stranded DNA-containing library were quite inefficient as they resulted a greatly reduced yield, as well as being labor intensive, these two steps were eliminated and other parameters were optimized, as described below, resulting in an optimized "combined shotgun and paired-end" sequencing protocol. In addition, the "shotgun" sequencing protocol as provided by the manufacturer also was similarly modified by eliminating the steps for isolating a single stranded DNA-containing library and other parameters were optimized, as also described below, that resulted in an optimized "shotgun" sequencing protocol. A schematic representation of both the "shotgun" and "combined shotgun and paired-end" modified Titanium sequencing protocols is shown in Figure 1.

Thus, our "combined shotgun and paired-end" sequencing protocol, an

amalgamation of the original "shotgun" and "paired-end" library protocols supplied by the manufacturer (Roche, Inc) and our optimized "shotgun" library preparation protocol described below, 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 454/Roche supplied protocols and that they represent years 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. More recently we have begun making our own library preparation reagents in an effort to reduce the cost of this process when sequencing 96 BACs on a single picotiter plate using 48 MID tagged BACs per half plate.

Basic Protocol 1 -Titanium

Shotgun Library Preparation

This protocol involves the preparation of a shotgun library for the 454/Roche GS-FLX. After shearing the DNA via nebulization (Roe, BA, Crabtree. JS and Khan, AS 1996) to obtain double stranded DNA fragments in the size range 500 to 800 base pairs, the ends are repaired, adapter sequences are ligated on each end, and again repaired to yield a blunt ended DNA library that then is quantified and diluted prior to amplification via emPCR. This protocol has been updated to cover the new Titanium chemistry as well as the now standard robotic manipulation of the enzymatic steps.

Materials

3-5 μg Purified DNA

GS DNA Library Preparation Kit (Roche, Inc.) components were replaced by the following reagents to reduce the cost of library making. This was especially worthwhile when preparing libraries for sequencing 96 MID tagged BACs on a single 2 quadrant picotiter plate on the GS FLX with Titanium chemistry.

10X TM buffer (500 mM Tris-HCl, pH 8.0, 150 mM $MgCl_2$ in sterile double distilled water i.e. Molecular Biology Grade water)

Nebulization buffer (200 ul 10x TM buffer, 0.5 ml sterile glycerol [ACS grade Glycerin Fisher Scientific #G33-500] brought to 2 ml with sterile ddH_2O)

10X polynucleotide kinase buffer included with the T4 polynucleotide kinase.

BSA (1 mg/ml) dilution from 10 mg/ml stock (included with T4 DNA polymerase)

25 mM ATP (included with the Plasmid-safe ATP-Dependent DNase)

10 mM each of the four dNTPs (Roche PCR Nucleotide Mix # 11581295001)

T4 polynucleotide kinase (30,000 U/ml) USB # 70031X

T4 DNA polymerase (3,000 U/ml) NEB # M0203L

Bst DNA polymerase, large fragment (8 U/ul) NEB # M0275L

(or T4 DNA polymerase (3 U/ul) NEB # M0203L and Klenow (5 u/ul) NEB # M0210L

Plasmid-safe ATP dependent DNase (10 U/ul) Epicentre # E3101K

Exonuclease I (10 U/ul) **70073Z 2500 UN**) Affymetrix-USB **70073Z 2500 UN**

Caliper Automated Microfluidics System, AMS-90, and SE30 DNA LabChip

Vacuum Drying System (National Appliance Co. Model 5831)

96 well 0.2 ml block Thermal Cycler (Applied Biosystems Model 9700)

Compressed Nitrogen - Research Grade

Dual-stage regulator

1/4" (6.35 mm) hose barb fitting with 1/4" (6.35 mm) NPT female compression fitting

Flexible tubing (1/4" (6.35 mm) ID), with stainless steel screw clamp

Microcentrifuge

Magnetic Particle Concentrator (Invitrogen #120-20D)

Isopropanol chilled to -80^{o}C

70% Ethanol

Wet Ice

Qiagen MinElute PCR Purification kit (Qiagen Inc # 28006)

Agencourt AMPure SPRI beads (Agencourt, Inc. # 000132)

Note: Unless specifically stated, all reagents, including the nebulizer and all listed buffers, enzymes and other reagents, such as nucleotide triphosphates, were obtained from the indicated suppliers rather than as components of the GS DNA Library Preparation Kit (Roche, Inc. # 04 852 265 001). The composition of the buffers was as described in the supplement to Margulies, *et al.* 2005.

DNA Nebulization

1. Prepare -20°C Isopropanol/Wet Ice bath.
2. Affix Nebulizer Condenser Tube around the Aspiration tube in the nebulizer.
3. Pipette 3-5 μg of dsDNA sample to bottom of 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 nebulizer in -20°C Isopropanol/Wet Ice bath.
8. Apply 30 psi of nitrogen for 2.5 minute at -20°C for genomic, BAC or Fosmid DNA
9. Measure amount of sample recovered. Ensure >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.

Concentration of DNA on a Qiagen MinElute centrifuge column

10. Add 2.5 ml of PBI Buffer to nebulizer containing the recovered sample
11. Obtain two Qiagen MinElute centrifuge columns. Add 750 μl of PBI Buffer/DNA mixture to each of the two columns. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute.
12. Discard flow-through.
13. Add 750 μl of remaining PBI Buffer/DNA sample to each of the two purification columns. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute.

14. Discard flow-through and add 750 μl of PE Buffer to each of the two purification columns. Microcentrifuge at full speed (>/=10,000 rpm) for 1 minute.
15. Discard flow-through. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute. Rotate column 180° and microcentrifuge an additional 30 seconds.
16. Place the Qiagen MinElute centrifuge columns into clean 1.5 ml microcentrifuge tubes. Add 12 μl of EB Buffer to center of each of the two columns. Let stand for 1 minute. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute to elute DNA.
17. Pool eluates from both columns for ~24 μl.

Sizing and Quality Assessment

18. Place the entire purified sample in Caliper AMS-90 microtiter plate. If needed, 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 manufacturers instructions.

Preparation of Enzyme Mixes

20. In a strip well, add the following reagents:

    11 μl double distilled, sterile, Molecular Biology Grade water

    5 μl 10X polynucleotide kinase buffer included with the purchased T4 polynucleotide kinase.

    5 μl BSA (1mg/ml) dilution from 10 mg/ml stock included with T4 DNA polymerase

    2 μl 25 mM ATP (Included with the Epicentre Plasmid-safe ATP-Dependent DNase)

    2 μl mixture containing 10 mM each of the 4 dNTPs Roche PCR Nucleotide Mix # 11581295001

    1 μl T4 Polynucleotide Kinase USB # 70031X

    1 μl T4 DNA polymerase NEB # M0203L

    27 μl total

21. In a microcentrifuge tube, add the following to generate the adaptor reaction mix:
    15 µl 2X Ligase Buffer (# 1 in Rapid DNA Ligation Kit Roche # 11635379001)
    5 µl Library Adaptors (These may be General adaptors or MID tagged adaptors)
    20 µl total
22. In a strip well, add the following to generate the ligation reaction mix
    10 ul 2x ligation Buffer (# 1 in Roche Rapid DNA Ligation Kit)
    5 ul Ligase (# 3 in Roche Rapid DNA Ligation Kit)
    15 ul Total
23. In a strip well, add the following to generate the fill-in reaction mix:
    40 µl Molecular Biology Grade water (ddH_20)
    5 µl 10x Fill-In Thermo-Pol BST polymerase buffer (or 1x TM buffer)
    2 µl dNTP mix (10 mM each)
    3 µl Fill-In BST polymerase large fragment (8 U/ul) (or 3 ul of T4 DNA polymerase (3 U/ul) and 2 ul Klenow (5 u/ul) although we use BST polymerase).
    50 ul Total
24. Place all strip wells in their respective places on the enzyme chilling station on the SciClone ALH
25. Place the DNA in a 96 well v-bottom plate into the input stack of the Twister II robot

The following steps cover the action of the SciClone ALH as it performs each reagent addition, enzyme reaction incubation and subsequent wash steps.

26. Add 27 ul polishing (fill-in) reaction to wells containing DNA, mix by shaking, and incubate the polishing reaction for 25 minutes at 25°C.
27. Purify on AMPure SPRI beads SPRI beads as follows:
    a. Add 25 ul of AMPure SPRI beads (use 50% the volume of SPIR beads).. Shake to mix.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
e. Dry for 5 min.
f. Remove tube from magnetic particle collector, add 17 µl EB Buffer, shake
g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate.

A and B Adapter Ligation and AMPure SPRI bead purification

28. Aspirate 15 ul ligation reaction mix and 15 ul adaptor reaction mix in the same tip and add to the DNA purified from the previous step. Incubate for 15 min. at 25˚C.
29. Purify on AMPure SPRI beads SPRI beads as follows:
    a. Add 25 ul of AMPure SPRI beads (use 50% the volume of SPIR beads).. Shake to mix.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
    e. Dry for 5 min.
    f. Remove tube from magnetic particle collector, add 25 µl EB Buffer, shake

       Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate.

Fill-In Reaction and AMPure SPRI bead purification

30. Add 50 ul fill-in reaction mix to wells containing DNA. Mix and incubate at 25˚C for 20 minutes.
31. Mix and incubate at 25˚C for 20 minutes.
32. Purify on AMPure SPRI beads SPRI beads as follows:
    a. Add 52 ul of AMPure SPRI beads. Shake to mix. What is the original volume of the solution here as this needs to be changed to reflect using 50% the volume of SPIR beads.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
e. Dry for 5 min.
f. Remove tube from magnetic particle collector, add 17 µl EB Buffer, shake
g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate. 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.

Library Quantitation and Dilution

33. Place the entire purified sample in Caliper AMS-90 or BioAnalyzer sample loading plate.
34. 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 or Agilent BioAnalyzer DNA 750 LabChip.according to manufacturers instructions.
35. Calculate molecules/µl from the ng/µl results from the Caliper AMS-90 or Agilent BioAnalyzer using the following formula:

$$\text{Molecules/µl} = \frac{(\text{Sample conc.;ng/µl}) \times (6.022 \times 10^{23}\ \text{molecules/mole})}{(656.6 \times 10^{9}\ \text{grams/mole}) \times (\text{avg. fragment length; nt})}$$

36. Dilute library stock 1:4 by adding ~39 µl of TE buffer to concentrated library prior to proceeding with the manufacturers recommended protocol for emPCR and subsequent sequencing on the GS-FLX. Note: The diluted library stock can be frozen at -20^{o}C and thawed at least 4 times over 1 year.

Basic Protocol 2

Shotgun/Paired End Library Preparation – Titanium

This protocol involves the preparation of a "shotgun/paired-end" library for the 454/Roche GS-FLX. This is based on an initial circularization of fragments sheared to

one of three different size ranges, 2 Kb to 4 Kb (~3 Kb) , 8 Kb to 10 Kb (~9 Kb) and ~20 Kb, in a GeneMachines® HydroShear® (Oefner, PJ, et al 1996) utilizing a common central linker to identify potential paired-end reads, followed by a second shearing in a nebulizer prior to adapter ligation, emPCR and sequencing.

Materials

3-5 µg Purified DNA in Tris Buffer or ddH_20 (Note the absence of EDTA)

GS FLX Titanium Paired End Adaptor Set (Roche Inc. #05 463 343 001)

The GS FLX Titanium General Library Preparation Kit (Roche # 05233747001 was replaced by reagents purchased from alternative suppliers to reduce the cost as described in Basic Protocol 1.

96 well 0.2 ml block Thermal Cycler (Applied Biosystems Model 9700)

Compressed Nitrogen - Research Grade

Caliper Automated Microfluidics System, AMS-90, and SE30 DNA LabChip

Vacuum Drying System (National Appliance Co. Model 5831)

Dual-stage regulator

1/4" (6.35 mm) hose barb fitting with 1/4" (6.35 mm) NPT female compression fitting

Flexible tubing (1/4" (6.35 mm) ID), with stainless steel screw clamp

GeneMachines® HydroShear® (Genomic Solutions # JHSH000000-1, equipped with either the HydroShear Standard (1Kb-9Kb) Shearing Assembly JHSH204004, the HydroShear Large (4Kp-40Kb) Shearing Assembly # JHSH204007 and Hydroshear 500 µl Syringe # JHSH010002)

Microcentrifuge

Magnetic Particle Concentrator (Invitrogen #120-20D)

Isopropanol chilled to -80^{o}C

70% Ethanol

Wet Ice

Qiagen MinElute PCR Purification kit (Qiagen Inc # 28006)

Agencourt AMPure SPRI beads (Agencourt, Inc. # 000132)

Plasmid-Safe ATP-Dependent DNase (Epicentre Biotechnologies #E3101K)

Cre Recombinase (NEB #M0298L)

Rapid Ligase Kit (Roche #11635379001)

Note: Unless specifically stated, all reagents, including the nebulizer and all listed buffers, enzymes and other reagents, such as nucleotide triphosphates, were obtained from the indicated suppliers rather than obtained as components of the GS DNA Library Preparation Kit (Roche, Inc. # 04 852 265 001). The composition of the buffers was as described in the supplement to Margulies, *et al.* 2005.

Detailed Protocol

DNA Fragmentation on HydroShear

1. Transfer the appropriate amount (5 ug for the ~3 Kb library, 15 ug for the ~9 Kb library, or 30 ug for the ~20 Kb library,) of sample DNA (in ddH_2O, 10 mM Tris-HCl, pH 7.5-8.5 (or Buffer EB supplied in either Qiagen kit) to a microcentrifuge tube.

   Note: For optimal performance, Roche recommends **not to use TE or a solution containing EDTA** for dissolving DNA and those that have been dissolved in a solution containing EDTA, such as TE, must be precipitated and resuspended in 10 mM Tris-HCl, pH 7.5-8.5, or Molecular Biology Grade Water (ddH_20). DNA samples dissolved in buffers with greater than 100 mM salt concentration should be precipitated and dissolved in 10 mM Tris-HCl, pH 7.5-8.5 without EDTA, or a similar low salt buffer or Molecular Biology Grade Water. Higher concentrations of salt will alter the shearing characteristics of the sample.
2. Add Tris Buffer (10 mM Tris-HCl, pH 7.5-8.5 without EDTA) to the appropriate final volume (200 ul for the ~3 Kb library or 150 ul for the ~9 Kb or ~20 Kb library). Vortex to mix and place the tube in an ice-water bath to cool to approximately 4 °C for 15 minutes to insure unbiased cleavage in the HydroShear
3. Shear the DNA in a HydroShear apparatus, following the manufacturer's instructions given in the User's 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 aperture shearing assembly, and produces an average fragment size of approximately 2.2 kb (an ~3 Kb library). For ~9 Kb libraries shear for 20 cycles at speed code 16 also with a standard aperture. For ~20 Kb libraries, shear for 20 cycles at speed code 15 with the large aperture shearing assembly. However, each Hydroshear 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.

Small Fragment Removal using AMPure SPRI beads (Skipped for titanium 8 and 20 kb libraries)

4. Measure the total DNA volume from the HydroShear, using a pipettor.
5. Add Tris buffer (10 mM Tris-HCl, pH 7.5-8.5 without EDTA) to a final volume of 200 µl.
6. Add 100 µl of AMPure SPRI beads (use 50% the volume of SPRI beads). Vortex to mix.
7. Incubate for 5 min at room temperature.
8. 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.
9. Remove supernatant and wash beads twice with 500 µl 70% Ethanol
10. Vacuum Dry completely for 10 min. Pellet is dry when visible cracks form.
11. Remove tube from magnetic particle collector, add 50 µl EB Buffer, vortex
12. Using the magnetic particle collector, pellet the beads and transfer supernatant containing 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.

Sizing and Quality Assessment

13. Assess the size and quality of the 2 Kb to 4 Kb fragments on either a BioAnalyzer DNA 750 LabChip or a Caliper AMS-90.
14. Verify that the size of fragments is in the ~3 Kb or ~9 Kb range by analysis on the Caliper AMS-90 using SE30 DNA LabChip or a Agilant BioAnalyzer according to

manufacturer's instructions. As an alternative for ~9 Kb fragments and for ~20 Kb fragments, determine their sizes by electrophoresis of an aliquot on a 0.8% agarose gel.

Fragment End Polishing and AMPure SPRI bead purification

15. In a microcentrifuge tube, add the following reagents, in the order indicated:
    - ddH_2O - 27 ul for ~3 Kb libraries or 16 ul for ~9 Kb and ~20 Kb libraries,
    - 10x polynucleotide kinase buffer - 10 ul for ~3 kb or 20 ul for ~9 Kb and ~20 kb libraries
    - BSA (10 mg/ml) - 1 ul for ~3 Kb libraries or 2 ul for ~9 Kb and ~20 Kb libraries,
    - ATP (25 mM) - 4 ul for ~3 kb or 8 ul for ~9 Kb and ~20 Kb libraries,
    - dNTPs (10 mM each) - 4 ul for ~3 kb or 8 ul for ~9 Kb and ~20 Kb libraries
    - Sheared DNA - 50 ul for ~3 kb or 140 ul for ~9 Kb and ~20 Kb libraries
    - T4 DNA polymerase (3,000 U/ml) - 2 ul for all libraries
    - T4 polynucleotide kinase (30,000 U/ml) - 2 ul for ~3 kb or 4 ul for ~9 Kb and ~20 Kb libraries
    - Final volume - 100 ul for ~3 kb libraries, or 200 ul for ~9 Kb and ~20 Kb libraries,
16. Mix by inversion and incubate the polishing reaction for 20 minutes at 25°C
17. Purify on AMPure SPRI beads as follows:
    a. Add the appropriate amount of AMPure SPRI beads (50 ul for ~3 kb, or 100 ul for ~9 Kb and ~20 Kb libraries (use 50% of the volume of SPRI beads). Vortex to mix.
    b. Incubate for 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 supernatant and wash beads twice with 500 µl 70% Ethanol.
    e. Vacuum Dry completely for 10 min. Pellet is dry when visible cracks form.
    f. Remove tube from magnetic particle collector, add the appropriate amount of EB Buffer (35 ul for ~3 Kb, or 80 ul for ~9 Kb and ~20 Kb libraries), vortex.

g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to a fresh 1.5 ml tube. If needed, the DNA can be stored on ice or refrigerated for several hours to overnight prior to hairpin adaptor ligation.

Circularization Adapter Ligation and AMPure SPRI bead purification.

18. In a microcentrifuge tube, add the following reagents, in the order indicated:
    2x Rapid Ligase Buffer - 50 ul for ~3 Kb or 100 ul for ~9 Kb and ~20 Kb libraries,
    Circularization Adaptors - 10 ul for all three libraries
    Sheared and Polished DNA - 35 ul for ~3 Kb or 80 ul for ~9 Kb and ~20 Kb libraries
    Total - 95 ul for ~3 Kb or 190 ul for ~9 Kb and ~20 K libraries
19. Mix by inversion.
20. Add the appropriate amount of Rapid Ligase. (5 ul for ~3 Kb or 10 ul for ~9 Kb and ~20 kb libraries)
21. Mix by inversion and incubate the ligation reaction at 25°C for 15 minutes.
22. Purify the products using AMPure SPRI size exclusion beads, as follows:
    a. Add the appropriate amount of AMPure SPRI beads (50 ul for ~3 Kb or 100 ul for ~9 Kb and ~20 kb libraries, use 50% the volume of SPIR beads). Vortex to mix.
    b. Incubate for 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 supernatant and wash beads twice with 500 µl 70% Ethanol
    e. Vacuum Dry completely for 10 min. Pellet is dry when visible cracks form.
    f. Remove tube from magnetic particle collector, add 40 ul of EB Buffer, vortex.
    g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to a fresh 1.5 ml tube. If needed, the DNA can be stored on ice or refrigerated for several hours to overnight.

Fill-in Reaction

In a microcentrifuge tube, add the following reagents:

40 µl Circularization-adapted DNA

5 µl 10x ThermoPol *Bst* DNA polymerase buffer

2 µl PCR Nucleotide Mix (10 mM each of the 4 dNTPs)

3 µl *Bst* DNA polymerase, large fragment (8 U/µl)

50 µl total volume

Mix by vortexing and incubate the fill-in reaction at **50°C** for **15 minutes or 25 deg C for 25 minutes**.

Alternatively:

a. In a strip well, add the following to generate the fill-in reaction mix:

40 µl Molecular Biology Grade water (sterile ddH20)

5 µl 10x Fill-In Thermo-Pol *Bst* DNA polymerase buffer (or 1x TM buffer)

2 µl dNTP mix (10 mM each)

3 µl Fill-In *Bst* polymerase, large fragment (8 U/ul) (or 3 ul of T4 DNA polymerase (3 U/ul) and 2 ul Klenow (5 u/ul, although we use the *Bst* polymerase)

50 ul Total

b. Place all strip wells in their respective places on the enzyme chilling station on the SciClone ALH

c. Place the DNA in a 96 well v-bottom plate into the input stack of the Twister II robot

The following steps cover the action of the SciClone ALH as it performs each reagent addition, enzyme reaction incubation and subsequent wash steps.

Add 27 ul polishing reaction to wells containing DNA, mix by shaking, incubate the polishing reaction for 25 minutes at 25°C, and then go on to step 23.

23. Purify the products using AMPure SPRI size exclusion beads, as follows:

h. Add the appropriate amount of AMPure SPRI beads (70 ul for ~3 Kb or 140 ul for

~9 Kb and ~20 Kb libraries). Vortex to mix. This can't be right. What is the initial volume here 100 or 50 ul??

i. Incubate for 5 min at room temperature.
j. 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.
k. Remove supernatant and wash beads twice with 500 µl 70% Ethanol
l. Vacuum Dry completely for 10 min. Pellet is dry when visible cracks form.
m. Remove tube from magnetic particle collector, add 52 ul of EB Buffer, vortex.

Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to a fresh 1.5 ml tube. If needed, the DNA can be stored on ice or refrigerated for several hours to overnight.

Quantitate the eluted DNA using a nano-drop method. At this step, the average DNA yield is greater than 200 ng. A minimum of 100 ng of DNA are required to proceed with the preparation. If an excess of 100 ng is obtained, the remainder DNA can be used in subsequent circularization events and should be stored at +4°C until needed. If the DNA yield does not reach the 100 ng required the resulting library will not meet the minimum specifications for Paired End libraries. If a minimum of 100 ng is not obtained, it is preferable to start the procedure from the beginning.

DNA Circularization

24. Prepare 100 mM DTT solution from 1 M DTT solution
25. Add the following to a 0.2 microcentrifuge tube in order:

    10 ul 10x Cre buffer
    80 ul Filled in DNA
    10 ul Cre recombinase (1 U/ul)
    100 ul total volume
26. Mix and incubate with the following program:

    37°C for 45 minutes, 70°C for 10 minutes, hold at 4°C
27. Add 1.1 ul 100 mM DTT, mix

28. Add the following to the reaction tube:

    4.4 ul ATP (25 mM)

    5 ul Plasmid-safe ATP dependent DNase (10 U/ul)

    6 ul Exonuclease I (10 U/ul)

29. Mix and incubate for 30 minutes at 37C

DNA Nebulization

24. Prepare -20^{o}C Isopropanol/Wet Ice bath
25. Affix Nebulizer Condenser Tube around the Aspiration tube in the nebulizer.
26. Pipette 50 µl of circularized dsDNA sample to bottom of nebulizer.
27. Add TE Buffer to a final volume of 100 μ l.
28. Add 500 μ l of Nebulization Buffer and mix by swirling.
29. Assemble the nebulizer ensuring condenser tube is included.
30. Submerge nebulizer in -20^{o}C Isopropanol/Wet Ice bath.
31. Apply 45 psi of nitrogen for 2.5 minute at -20^{o}C for 2 to 4 kb circularized DNA.
32. Measure amount of sample recovered. Ensure >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.

Concentration of DNA on a Qiagen MinElute centrifuge column

33. Purify on a Qiagen MinElute centrifuge column:
    a. Add 2.5 ml of PBI Buffer to nebulizer containing the recovered sample
    b. Obtain one Qiagen MinElute centrifuge columns. Add 750 μ l of PBI Buffer/DNA mixture to the columns. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute using a second Qiagen MinElute centrifuge column without sample as a blank counterbalance.
    c. Discard flow-through.
    d. Add the remaining PBI Buffer/DNA sample to the purification column in 750 ul aliquots. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute after each addition, discarding flow through.

e. Discard flow-through and add 750 μl of PE Buffer to the purification column. Microcentrifuge at full speed (>/=10,000 rpm) for 1 minute.
f. Discard flow-through. Microcentrifuge at full speed (>/= 10,000 rpm) for 1 minute. Rotate column 180° and Microcentrifuge an additional 30 seconds.
g. Place the Qiagen MinElute centrifuge column in clean 1.5 ml microcentrifuge tubes. Add 26 μl of EB Buffer to the column. Let stand for 1 minute. Microcentrifuge at full speed for 1 minute to elute DNA.

The following section concerns the ligation of A and B adaptors to the shotgun/paired-end library. It has been changed to reflect the now standard robotic manipulation as well as the new Titanium chemistry. All enzyme mix reagents are found in the Titanium shotgun library kit.

Enzyme mix Preparations

34. In a strip well, add the following reagents from the GS DNA Library preparation kit, in the order indicated to generate the polishing reaction mix:

    11 µl Molecular Biology Grade, sterile ddH2O
    5 µl 10x polynucleotide kinase buffer
    5 µl BSA (1 mg/ml diluted from 10 mg/ml stock)
    2 µl ATP (25 mM)
    2 µl dNTPs
    1 µl T4 DNA polymerase (3,000 U/ml)
    1 µl T4 polynucleotide kinase (30,000 U/ml)
    27 µl final volume

35. In a microcentrifuge tube, add the following to generate the adaptor reaction mix:

    15 µl 2X ligation buffer (supplied with the Rapid Ligation kit)
    5 µl Library Adaptors (These may be either the general adaptors or individual MID tagged adaptors)
    20 µl total

36. In a strip well , add the following to generate the ligation reaction mix:

    10 ul 2x ligation buffer
    5 ul Ligase
    15 ul total

37. In a strip well, add the following to generate the fill-in reaction mix:

    40 µl Molecular Biology Grade water (ddH_20)

    5 µl 10x Fill-In Thermo-Pol *Bst* DNA polymerase buffer

    2 µl dNTP mix

    3 µl Fill-In *Bst* DNA polymerase ,large fragment

    50 ul total volume

38. Place all strip wells in their respective places on the enzyme chilling station on the SciClone ALH
39. Place the DNA in a 96 well v-bottom plate into the input stack of the Twister II robot

The following steps cover the action of the SciClone ALH as it performs each enzyme reaction and subsequent wash

40. Add 27 ul polishing reaction to wells containing DNA, mix by shaking, and incubate the polishing reaction for 25 minutes at 25°C.
41. Purify on AMPure SPRI beads, as follows:
    a. Add 25 µl of AMPure SPRI beads (use 50% the volume of SPIR beads). Shake to mix.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
    e. Dry for 5 min.
    f. Remove tube from magnetic particle collector, add 17 µl EB Buffer, shake
    g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate.

A and B Adapter Ligation and AMPure SPRI bead purification

42. Aspirate 15 ul ligation reaction mix and 15 ul adaptor reaction mix in the same tip and add to the DNA purified from the previous step. Incubate for 15 min. at 25°C.

43. Purify on AMPure SPRI beads, as follows:
    a. Add 25 µl of AMPure SPRI beads (use 50% the volume of SPIR beads). Shake to mix.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
    e. Dry for 5 min.
    f. Remove tube from magnetic particle collector, add 25 µl EB Buffer, shake
    g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate.

Fill-In Reaction and AMPure SPRI bead purification

44. Add 50 ul fill-in reaction mix to wells containing DNA. Mix and incubate at 25°C for 20 minutes.
45. Purify on AMPure SPRI beads, as follows:
    a. Add 25 µl of AMPure SPRI(use 50% the volume of SPIR beads). Shake to mix.
    b. Incubate for 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 supernatant and wash beads twice with 200 µl 95% Ethanol
    e. Vacuum dry for 5 to 10 min. Pellet is dry when visible cracks form.
    f. Remove tube from magnetic particle collector, add 17 µl EB Buffer, shake
    g. Using the magnetic particle collector, pellet the beads and transfer supernatant containing purified DNA to the next column in the 96 well plate. If needed, the DNA can be stored on ice or refrigerated for several hours to overnight prior to library quantitation and dilution, skipping the ssDNA immobilization beads, lbrary immobilization, and subsequent amplification steps recommended by 454/Roche, to make and amplify ssDNA.

Library Quantitation and Dilution

46. Place all the purified sample in Caliper AMS-90 microtiter plate or the Agilent loading plate.
47. Verify that the size of fragments is in the 500 to 800 bp range by analysis on the Caliper AMS-90 using SE30 DNA LabChip or the Agilent BioAnalyzer or the Agilent BioAnalyzer according to manufacturers instructions.
48. Calculate molecules/µl from the ng/µl results from the Caliper AMS-90 or the Agilent BioAnalyzer using the molecules calculator spreadsheet.

$$\text{Molecules/µl} = \frac{\text{(Sample conc.;ng/µl) X } (6.022 \times 10^{23} \text{ molecules/mole})}{(656.6 \times 10^{9} \text{grams/mole}) \text{ X (avg. fragment length; nt)}}$$

49. Dilute library stock 1:4 by adding ~39 µl of TE buffer to the concentrated library prior to proceeding with the manufacturers recommended protocol for emPCR and subsequent sequencing on the GS-FLX Titanium plates. Note: The diluted library stock can be frozen at -20^{o}C and re-used for 1 year.

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, in an effort to streamline them for automation, we have incorporated several novel changes and deleted several extraneous steps. 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 that 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 that will be used for the subsequent emPCR. We initially added 70% of the original volume in SPRI beads, i.e add 70 ul of SPRI beads to 100 ul of DNA solution, but now have determined that adding 50% of the original volume in SPRI beads more efficiently removes DNA fragments shorter than ~300 bp. Although the MinElute centrifuge columns are extremely useful in numerous applications there is an approximately 20% loss of sample each time they are used according to the manufacturers instructions. In contrast, the AMPure SPRI beads have a recovery of between 90 and 95% depending on the number of small (less that 300 base pairs) DNA fragments when used according to the manufacturers 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, immediately after the nebulization steps we still include MinElute centrifuge columns 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 to enrich DNA molecules that contain a single A and B adapter ligated on each end of the fragment. This step again was deemed unnecessary, since enrichment after the emPCR will not select those DNA fragments

with identical adapters on each end. Fragments with A adapters ligated to both ends of the same fragment will not bind to the DNA binding beads, while those fragments with B adapters 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 by a factor of at least 2-fold.

When it was first introduced, the GS-FLX gave average read lengths at least 2 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 3 and one-half times that of the GS-20 (data not shown). To take advantage of the longer read lengths, we have replaced the Mme I digest that was recommended to generate paired ends with a second shearing step. This resulted in generating larger paired end fragments that when sequenced on the GS-FLX, gave longer reads that assisted the shotgun assembly process.

After implementing the above protocol alterations that streamlined the library-making process and increased the overall yield, the initial experiments showed that about 5% of the sequenced reads contained the paired-end linker sequence, which would be expected for an initial shearing length of about 4K bases and an average sequence length of 250 bases. Of these linker-containing sequences, almost 50% did not have at least 50 bases on one side of the linker. This results in one of the paired-end reads being too short to be useful in the subsequent assembly and closure processes. We therefore 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. As shown in Table 2, the ratio of paired end reads to shotgun reads is relative 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 and paired-end" protocol typically generates only 3-5% paired end reads for a typical GS-FLX sequencing run, when one considers the significant reduction in the labor-intensive library-making steps and an average of approaching 500,000 reads on a GS-FLX full picotiter plate, 15,000 to 25,000 usable paired ends is quite significant and useful in the assembly using the winter 2008 release of the 454/Roche Newbler assembler (version 1.1.03).

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 have baseline data using the recommended protocol before venturing out to try one with modifications. Although the protocol presented above has been thoroughly tested over the past year, it always is useful 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 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 12-13 ng even when starting with the recommended amount of input DNA. Although, recent advances in employing 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 initial DNA in the first shearing step. This increased amount results in improved yields. Also, as indicated in the manufacture-supplied literature, it should be noted that the maximum capacity of each Qiagen MinElute

centrifuge columns is 5 μg and two columns typically are used to concentrate the nebulized DNA.

Finally, it is important to note that the amount of AMPure SPRI beads used in each step is relative to the amount of DNA containing solution to which the AMPure SPRI bead suspension is being added. The optimum amount is 50% to 70% by volume of AMPure SPRI bead suspension relative to the DNA solution This percentage should be determined for each batch of SPRI beads using a 100bp ladder and analysis before and after SPRI purification of the Agilent or Caliper, but we use 50% throughout the protocols described here. Using too low a percentage of SPIR beads will cause an increase in the size exclusion limit as well as a decrease in the overall amount of DNA bound, and thus recovered due to the decreased concentration of PEG in the AMPure SPRI bead suspension while using to high a percentge will not efficiently remove the less than 300 bp fragments (Hawkins, *et al.* 1994).

Anticipated Results

The final product of these protocols is a library of double stranded DNA molecules with 454 adapters 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 and paired end library) the paired end tagged sequence that was inserted as a linker prior to circularization of the 2-4 Kb sheared DNA fragments. Figure 2 shows a typical genomic DNA sample that has been sheared using the HydroShear, sized and quantitated on a Caliper AMS-90.

After the generation of a mixed shotgun and paired end library it is common to have a significantly lower total amount of DNA. As illustrated in Figure 3, after generating a mixed “shotgun and paired-end” library, for the DNA sample shown in Figure 2, the overall yield of DNA was 12.6 ng. Table 1 shows the overall results for a sequencing run using the library shown in Figure 3. The amount of sequences lost to the “Too Short Primer” filter using the DNA library prepared using the above AMPure SPRI bead, double stranded, mixed “shotgun and paired-end” library making process was only $1/10^{th}$ of 1%, compared to order of magnitude higher values obtained when using the manufacturer recommended protocols.

Time Considerations

It is strongly recommended that both Basic Protocol 1, the "shotgun" library making protocol, and Basic Protocol 2, the mixed "shotgun and paired-end" library making protocol, are performed following the indicated recommended times.

In Basic Protocol 1, although the time required to complete the "shotgun" library making protocol only is approximately 3 hours, it is possible to pause the protocol at several places. For example, as noted in Step 9, 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. If needed, as indicated in Step 18, 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. Also as indicated in Step 28, the sample can be stored on ice or in the refrigerator for several hours to overnight prior to the library quantitation step. Once quantitated the library can be stored at -20 ^{o}C for as much as a year with multiple freezing and thawing if it necessary to perform additional emPCR amplifications.

In Basic Protocol 2, the time required to complete the mixed "shotgun and paired-end" library making takes approximately 2 days with an overnight incubation after Step 33 on the first day. 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 step, as indicated in steps 18g, 24g, 31g, 34g, 41g, 54g. 57g, and 60g.

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Figure Legends

Figure 1. Schematic representation of the "shotgun" and mixed "shotgun, paired-end" library making protocols. The left panel shows an outline of the steps needed to produce the "shotgun" library that subsequently is quantitated, amplified by emPCR and then sequenced on the 454/Roche GS-FLX sequencer, while the right panel shows the steps used in the "combined shotgun and paired-end" protocol. Note that after the circularized DNA was nebulized, the end polishing, adaptor ligation and fill-in was as shown in the "shotgun" protocol.

Figure 2. A typical genomic DNA sample sheared with the Hydroshear and analyzed on a Caliper AMS-90

Figure 3. A typical "combined shotgun and paired-end" library analyzed on a Caliper AMS-90.

Figure 1.

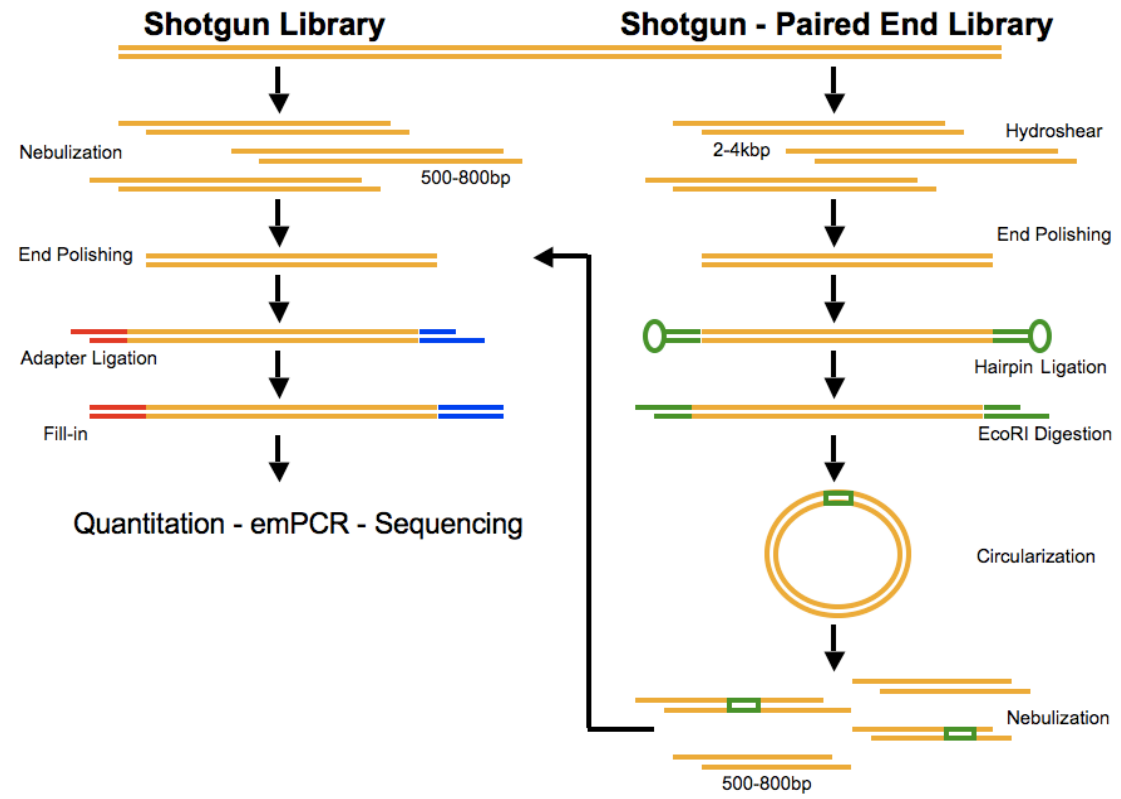
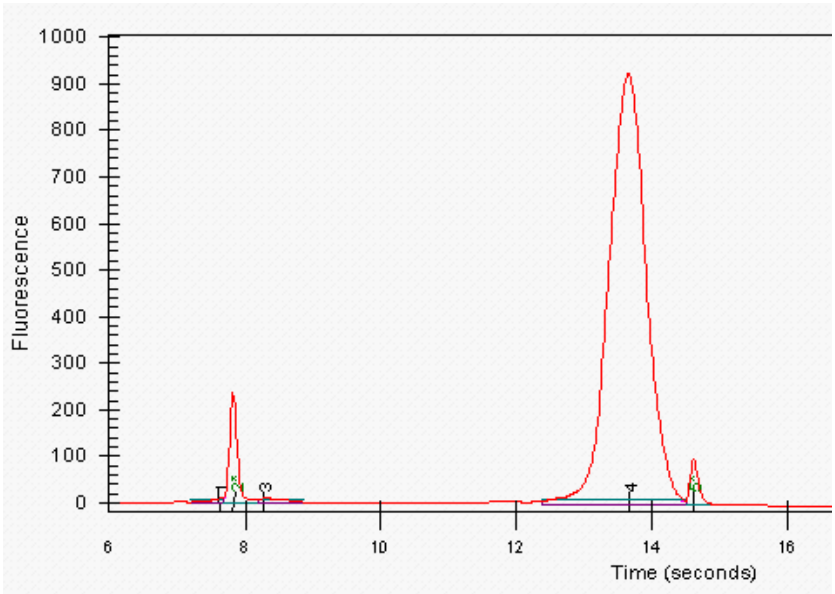
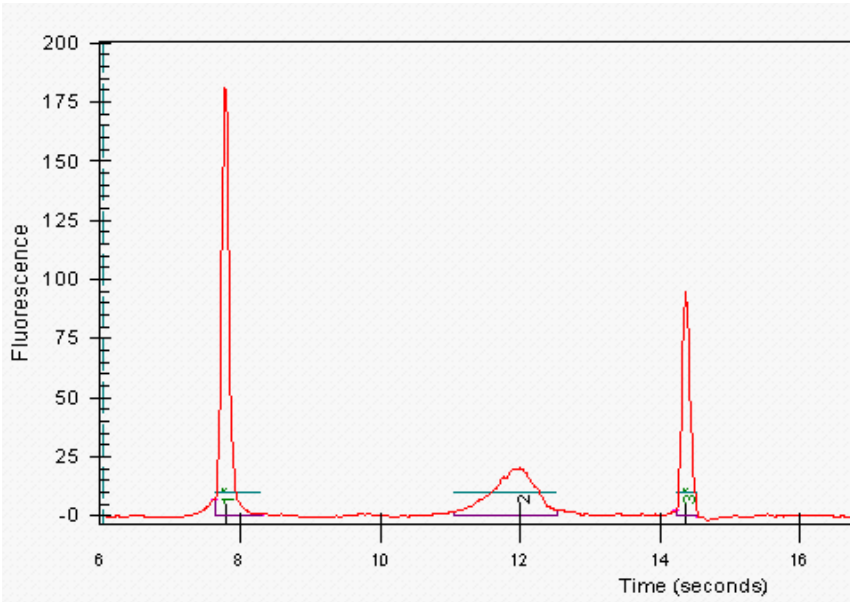

Shotgun Library
Shotgun - Paired End Library
Nebulization
500-800bp
End Polishing
Adapter Ligation
Fill-in
Quantitation - emPCR - Sequencing
Hydroshear
2-4kbp
End Polishing
Hairpin Ligation
EcoRI Digestion
Circularization
Nebulization
500-800bp

Figure 2.



Peak	Mig.Time(secs)	Size(BP)	Conc.(ng/ul)	Molarity(nmol/l)	Height	Area
1	7.63				11.6	2.7
2	7.82	15	2.50	252.5	238.0	32.1
3	8.27	77	0.23	4.5	11.0	5.2
4	13.67	2757	39.98	22.0	924.6	557.5
5	14.62	7000	2.50	0.5	94.6	12.5

Figure 3.



Peak	Mig.Time(secs)	Size(BP)	Conc.(ng/ul)	Molarity(nmol/l)	Height	Area
1	7.78	15	2.50	252.5	184.2	25.0
2	12.00	670	0.84	1.9	20.1	15.0
3	14.37	7000	2.50	0.5	96.2	12.8

Table 1. An overview of the sequencing results for the mixed shotgun and paired end library shown in Figures 1 and 2.

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

Table 2. Typical paired-end percentages from mixed shotgun and 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*	Expected % of Reads With Usable Paired Ends**
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***	17.47%	12.57%
3000	400***	11.73%	8.44%
4000	400***	8.83%	6.36%
6000	400***	5.91%	4.25%
8000	400***	4.44%	3.19%

Notes to Table 2:

* Because not all DNA-beads gave readable sequence and a portion of the tags on the paired-ends could not be discerned, the actual results were approximately 75% of the expected results and with typical sheared DNA in the 2-4 Kb range approximately 5% linker-contained recognizable tags instead of the expected 6.8%.

** Assuming 50 bases are required on each side of the paired-end linker are necessary to be recognized as a linker being present.

*** A 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.