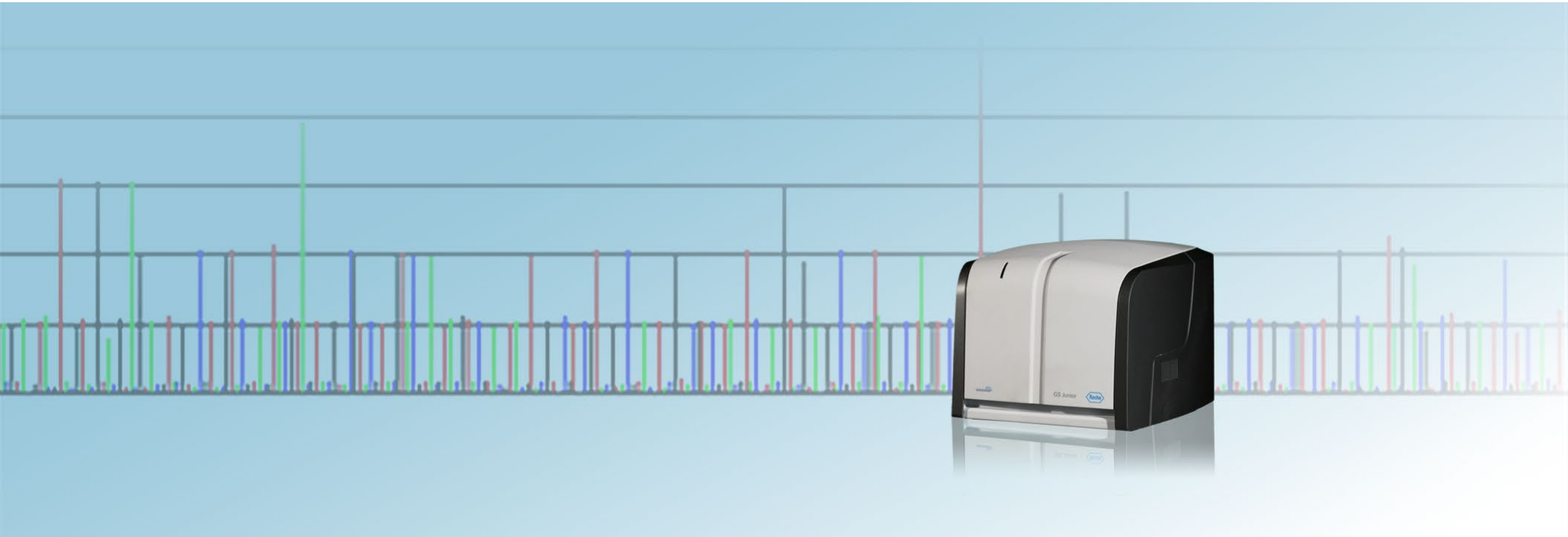
Paired End Library Preparation Method Manual, 20 kb and 8 kb Span

GS Junior Titanium Series

March 2012

	Instrument	Kit
✓	GS Junior	Junior
	GS FLX+	XL+
	GS FLX+	XLR70
	GS FLX	XLR70

For life science research only. Not for use in diagnostic procedures.



Paired End Library Preparation Method Manual, 20 kb and 8 kb Span

1 WorkFlow

The preparation of a GS Junior Titanium 20 kb or 8 kb span Paired End library comprises 14 major steps as described in Figure 1. Two procedures are described here. The 20 kb span Paired End protocol is shown in black, while the 8 kb span is in blue, when different.

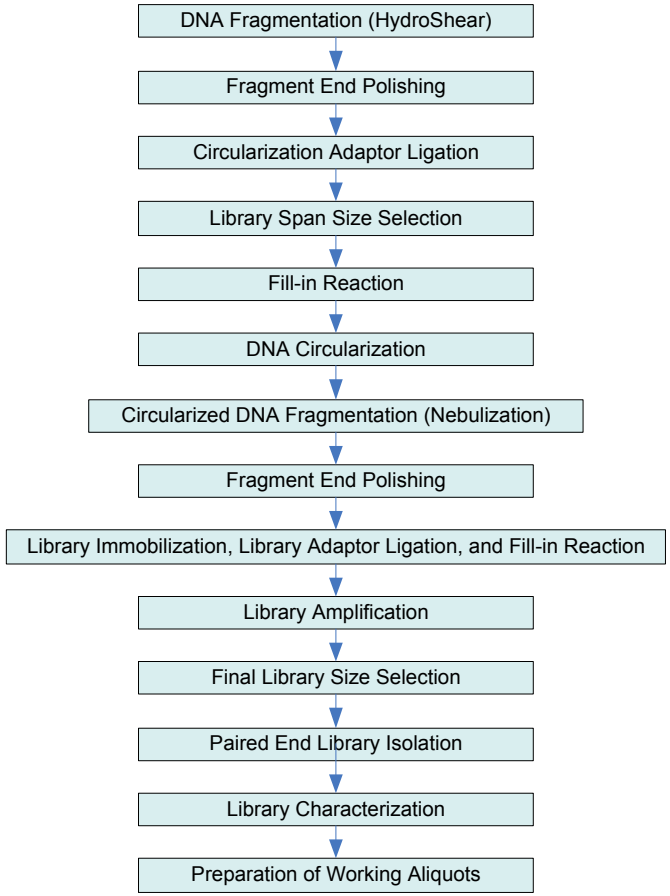


Figure 1: Workflow of the library preparation procedure for the GS Junior Titanium 20 kb and the 8 kb span Paired End libraries

2 Before you Begin

2.1 Sample DNA Preparation

1. Start with 30 µg (15 µg) of sample DNA (in Tris-HCl) in a microcentrifuge tube.
2. Add Tris-HCl to a final volume of 150 µl. Invert the tube 6 times. Do not vortex high molecular weight DNA and use wide orifice pipet tips when handling sample. DNA must be completely dissolved and free of particles before shearing.

2.2 Reagents and Kits

This protocol requires a GS FLX Titanium Library Paired End Adaptors Kit.

The GS Junior System Tables of Materials lists all other required items.

3 PROCEDURE

- In this manual, Tris-HCl refers to 10 mM Tris-HCl, pH 7.5-8.5 or Buffer EB, supplied in either QIAGEN kits, unless specified differently.
- Room temperature is +22 to +25ºC.

3.1 DNA Fragmentation (HydroShear)

1. Shear the DNA with the HydroShear apparatus, following the manufacturer's instructions. However, do not vortex nor heat the sample before shearing. Also, be aware that instructions in Section 6.4 of the HydroShear manual include steps that are contradictory to software prompts. At these steps, follow the manual's instructions and disregard the software prompt.
2. Test a small quantity of your sample, and run it on an agarose gel to determine the optimal shearing conditions.
3. We recommend using 20 cycles at a calibrated speed setting of 15 (16) with the large (standard) shearing assembly.

3.2 Fragment End Polishing

1. In a microcentrifuge tube, add the following reagents:

14	µl	Molecular Biology Grade Water
20	µl	10× PNK Buffer (free of precipitate. If any, warm buffer at 37°C and vortex.)
1	µl	Bovine Serum Albumin (20 mg/ml)
2	µl	ATP, lithium salt, pH 7 (100 mM)
8	µl	PCR Nucleotide Mix (10 mM each)
~140	µl	Sheared DNA

2. Mix by inverting the tube 6 times. Place the tube on ice and add the following enzymes:

5	µl	T4 DNA Polymerase (1 U/µl)
10	µl	Polynucleotide Kinase (PNK, 10 U/µl)
200	µl	Total volume

3. Mix by inverting the tube 6 times and incubate the polishing reaction at +**25°C** for **20 minutes**.
4. During the incubation, prepare a 0.5% Megabase agarose gel 1× TAE using a preparative comb as follows:
   - Use 150 ml of 0.5% agarose for a 13 × 16 cm gel bed, and add 15 µl of 10,000 × SYBR Safe for DNA visualization (do not use ethidium bromide). The sample well should be approx. 3 cm wide and framed by narrower wells on both sides, for molecular weight markers. See example of well size in Figure 3.
5. At the end of the incubation, purify the polished fragments IMMEDIATELY using the GenFind DNA Isolation Kit.
   a. Vortex the bottle of GenFind Binding Buffer to resuspend the GenFind beads.
   b. Add **300 µl** of GenFind Binding Buffer to the reaction tube.
   c. Place the tube on a tube rotator (LabQuake) to mix at **room temperature** for **5 minutes**.
   d. Place the tube on a Magnetic Particle Concentrator (MPC) until solution clears.
   e. Remove the supernatant and add **800 µl** of GenFind Wash Buffer 1. Invert the tube 5 times.
   f. Place the tube on a tube rotator at **room temperature** for **3 minutes**.

g. Repeat wash once as described in steps d. and f.
h. Place the tube on the MPC until the solution clears.
i. Remove the supernatant, and add **800 µl** of 70% ethanol **while** the tube is in the MPC.
j. Wait **1 minute** and then remove the supernatant.
k. Wash again with 70% ethanol as directed in steps h and i.
l. Carefully remove any residual ethanol and then remove tube from the MPC and air dry the pellet for no more than **2 minutes** at **+25°C**. In the event GenFind Beads appear wet after 2 minutes, continue with the protocol.
m. Add **80 µl** of 10mMTris-HCl pH 7.5 to 8.5, to the pellet and mix gently by inversion for **5 minutes**.
n. Place the tube in the MPC until the solution clears, then transfer the supernatant to a new microcentrifuge tube, using wide orifice pipette tip.

3.3 Circularization Adaptor Ligation

1. To the tube containing the purified, polished DNA, add the following reagents, in the order indicated:

~80	µl	Purified Polished DNA (already in the tube)
100	µl	Rapid Ligase Buffer, 2× Conc.
10	µl	Circularization Adaptors (20 µM)
190	µl	Total volume

2. Mix by gently inverting the tube.
3. Add **10 µl** of Rapid Ligase.
4. Mix by GENTLY inverting the tube and incubate the ligation reaction at +**25°C** for **15 minutes**.
5. At the end of ligation incubation, add*:

20 µl 10× BlueJuice gel loading buffer
2 µl 20% SDS

***Optional:** After adding the 10× BlueJuice gel loading buffer, but before adding the 20% SDS, the DNA sample can be stored at +4°C over night.

6. Heat at **65°C** for **10 minute**s and cool the tube on ice before loading on the gel.
7. Load the ligation reaction into a single 3 cm wide well on the 0.5% agarose.
8. Load a solution made of 2.5 µl of 1 kb DNA Extension Ladder with 11 µl of Tris HCl and 1.5 µl of 10× BlueJuice gel loading buffer in standard-width wells on either side of the sample.
9. Run the gel at **35 volts for 15-17 hours** (**85 volts for 4 hours**) at **+25°C**.
10. Do not expose the gel to any kinds of UV irradiation at any step during the procedure.

3.4 Library Span Size Selection

1. Examine the gel on a Transilluminator.

For the 8 kb procedure only: If a bright band below the 100 bp marker does not appear, do not proceed with the library. This indicates that Circularization Adaptors were not added in the previous section.

2. Cut the gel slice containing DNA fragments ranging from 17 kb to 25 kb (6.5 kb to 9.5 kb).

- Excising the brightest band on the gel, rather than the one of the desired size, will lead to a high yield library, of the cut size.
- Gel slices can be stored at +2 to +8°C for a month.

3. Extract DNA from the agarose gel slice by electroelution, using the Whatman Elutrap device according to the manufacturer's instructions, following **the option described below:**
    a. Place a BT2 membrane at point F to form the smallest trap for the device.
    b. Cut the gel into three pieces and stack the gel slices as close to the BT2 membrane as possible but never allow them to touch the membrane. If the gel touches the membrane, tilt the Elutrap to slide the gel off the membrane. Do not attempt to move the gel slice with tweezers as this might tear the membrane.
    c. Carefully load 600 µl of fresh 1× TAE into the trap. Place the device in the Elutrap electrophoresis chamber.
    d. Adjust the level of TAE buffer in the electrophoresis chamber so that the Elutrap device is submerged ¾ of the way.
    e. Fill the Elutrap internal chamber with fresh 1× TAE until the level is above the gel slices.
    f. Place the lid on the chamber and apply **150 volts** for **2 hours** to elute the DNA from the gel.
    g. Once elution is complete, reverse the current polarity and apply **150 volts** for **20 seconds** to release any material that may be attached to the BT1 membrane.
    h. Remove the eluted DNA from the trap using a wide orifice 250 µl pipette tip. On average, **600 to 800 µl** of solution are collected.

Optional: At this stage, the DNA sample can be stored at +2 to +8°C for one month.

4. Place the lid on the chamber and apply 150 volts for 2 hours to elute the DNA from the gel.
5. Once elution is complete, reverse the current polarity and apply 150 volts for 20 seconds to release any material that may be attached to the BT1 membrane.
6. Remove the eluted DNA from the trap using a wide orifice 250 µl pipette tip. On average, 600 to 800 µl of solution are collected.
7. **Optional:** At this stage, the DNA sample can be stored at +2 to +8°C for one month.
8. Concentrate the eluted DNA to **less than 40 µl**, using an Amicon Ultra-0.5 Centrifugal Filter Unit according to manufacturer's instruction.
9. Add **250 µl** of Tris-HCl to the column and concentrate the sample again to **less than 38 µl**.
10. Collect the concentrated eluted DNA, measure the exact volume with a wide orifice pipet tip, and adjust the volume to **38 µl** with Tris-HCl.

3.5 Fill-In Reaction

1. In a microcentrifuge tube, add the following reagents:

38	µl	Circularization-adapted DNA
5	µl	10× ThermoPol Buffer
4	µl	PCR Nucleotide Mix (10 mM each)
3	µl	*Bst* DNA Polymerase, Large Fragment (8 U/µl)
50	µl	Total volume

2. Mix by gently inverting the tube and incubate the fill-in reaction at +**50°C** for **15 minutes**.
3. Quantitate the DNA using the Quant-iT PicoGreen dsDNA Assay Kit, following the manufacturer's instructions.

- At this step, the **average DNA yield is greater than 400 ng. A minimum of 300 ng of DNA** is required to proceed with the preparation. If an excess of 300 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 300 ng required, it is preferable to start the procedure from the beginning.
- We strongly recommend use of a fluorescence based quantitation method, such as Quant-iT PicoGreen over UV absorbance quantitation.

Optional: At this stage, the DNA sample can be stored at +2 to +8°C over**night**

3.6 DNA Circularization

1. Prepare a stock of 1 M DTT by dissolving 1.54 g of 1,4-Dithiothreitol (DTT) in 10 ml of Molecular Biology Grade Water, followed by filtration.
2. Prepare a 300 ng aliquot of the filled-in DNA in a total volume of 80 µl (adjust volume with Molecular Biology Grade Water).
3. In a 0.2 ml tube, add the following reagents, in the order indicated:

10	µl	10× *Cre* Buffer
80	µl	Filled-in DNA (300 ng, from Step 2, above)
10	µl	*Cre* Recombinase (1 U/µl)
100	µl	Total volume

4. Mix by gently inverting the tube 6 times.
5. Run the following incubation program in a thermocycler:

37°C for 45 minutes
70°C for 10 minutes
4°C on hold

6. Prepare a fresh 100 mM DTT dilution from a 1 M DTT stock, as indicated below. Vortex and store on ice once done.

18	µl	Molecular Biology Grade Water
2	µl	DTT (1 M)
20	µl	Total volume

7. Add 1.1 µl DTT (100 mM).
8. Mix the reaction by inverting the tube 6 times, follow with a quick spin to collect the solution to the bottom of the tube.
9. Add the following reagents to the sample:

1.1	µl	ATP (100 mM)
5.0	µl	Plasmid-Safe ATP-Dependent DNase (10 U/µl)
3.0	µl	Exonuclease I (20 U/µl)

10. Mix gently, but completely, by inverting the tube.
11. Incubate the reaction at +37°C for 30 minutes.
12. Immediately following the incubation, add 8 µl of Carrier DNA to the sample and mix by gently inverting the tube 6 times. Do not substitute the supplied Carrier DNA with other material.

Optional: At this stage, the DNA sample can be stored at +2 to +8°C overnight.

3.7 Circularized DNA Fragmentation (Nebulization)

Hazardous Chemicals, *Compressed Nitrogen Gas*: This procedure involves the use of compressed nitrogen gas, an asphyxiating gas. When using compressed nitrogen gas, always follow standard safety procedures to minimize the potential hazards, as described in the Material Safety Data Sheet.

3.7.1 DNA Sample Preparation

1. Prepare a stock of Nebulization Buffer by mixing **5.31 ml** of Glycerol; **0.37 ml** of 1 M Tris-HCl, pH 7.5; **0.11 ml** of 0.5 M EDTA; and **4.21 ml** of Molecular Biology Grade Water.
2. Add **500 µl** of Nebulization Buffer to the bottom (cup) of a Nebulizer.
3. Transfer the sample to the Nebulization Buffer in the Nebulize. Swirl to mix.

3.7.2 Nebulizer Assembly

1. Press the pierced rubber stopper firmly into the Nebulizer top, and insert the Millipore filter unit into the stopper hole (Figure 2**A**).
2. Using sterile gloves, affix a Nebulizer Condensor tube around the Aspiration tube. To ensure proper function, make sure to push the Condensor tube all the way down around the base of the Aspiration tube, being careful not to rotate the Aspiration tube (Figure 2**A**).
3. Tightly screw the cup into the top of the Nebulizer, and connect one end of the Nebulizer tubing (included with the Nebulizer) to the Nebulizer's gas inlet (Figure 2**B**).
4. Transfer the Nebulizer to the externally vented nebulization hood.
5. Connect the loose end of the Nebulizer tubing to the nitrogen tank.

A B

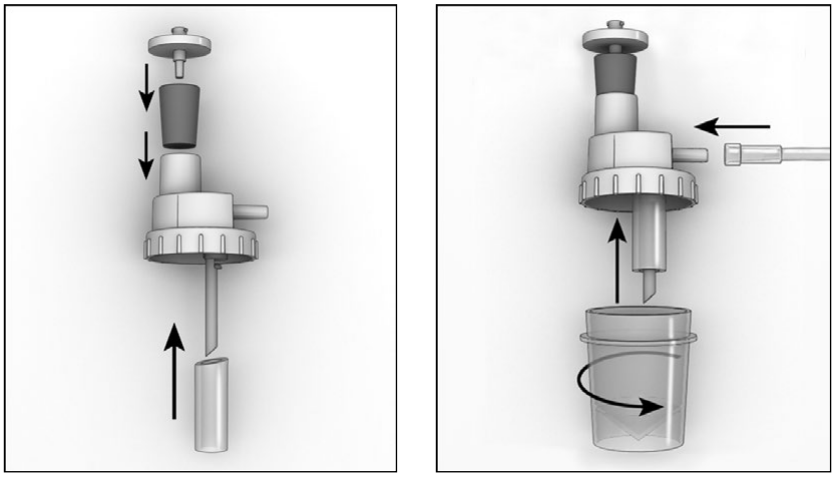


Figure 2: Assembling the nebulizer

3.7.3 DNA Nebulization and Collection/Purification of the Fragmented DNA

1. Direct **30 psi** (2.1 bar) of nitrogen through the Nebulizer for **2 minutes**, to nebulize the DNA.
2. After nebulization, turn off the nitrogen gas flow.
3. Disconnect the tubing from both the Nebulizer and the nitrogen tank.
4. Tap the Nebulizer on a table top to collect as much of the material as possible to the bottom of the cup.
5. Carefully unscrew the Nebulizer top and measure the volume of nebulized material. Total recovery should be greater than **300 µl.**
   - Do NOT collect any material that may have lodged outside the Nebulizer: this material may not have been completely fragmented and could cause problems later on.
6. Add **2.4 ml** of Qiagen's Buffer PBI directly into the Nebulizer cup and swirl to collect all material droplets and mix the sample.
7. Purify the nebulized DNA using **one** MinElute column.
   a. Load the column four times (approx. **750 µl** each). Spin for 15 seconds for each of the first three loading, and one minute for the last loading.
   b. Add **750 µl** of PE Buffer, centrifuge one minute, and discard the flowthrough.
   c. Elute with **25 µl** of Tris-HCl at room temperature.
8. Run **1 µl** of the purified nebulized material on an Agilent Bioanalyzer DNA 7500 LabChip to assess the quality of the DNA fragments.
9. The recovered nebulized material should have a peak around 500 bp (see Section 2 for a representative trace of the profile of the nebulized material).

3.8 Fragment End Polishing

1. Prepare a fresh 1 mg/ml dilution of Bovine Serum Albumin, from the 20 mg/ml stock. In a microcentrifuge tube, add the reagents below, vortex, and store on ice.

38	µl	Molecular Biology Grade Water
2	µl	Bovine Serum Albumin (20 mg/ml)
40	µl	Total volume

2. In a microcentrifuge tube, add the following reagents:

5.0	µl	Molecular Biology Grade Water
5.0	µl	10× PNK Buffer
5.0	µl	Bovine Serum Albumin (1 mg/ml)
0.5	µl	ATP, lithium salt, pH 7 (100 mM)
2.0	µl	PCR Nucleotide Mix (10 mM each)
23.0	µl	Nebulized DNA
5.0	µl	T4 DNA Polymerase (1 U/µl)
5.0	µl	Polynucleotide Kinase (PNK, 10 U/µl)
50.5	µl	Total volume

3. At this point, the DNA has been fragmented to ~500 bp fragments and it is no longer detrimental to vortex the sample or to use non wide-bore pipet tips.
4. Vortex and incubate the polishing reaction for **20 minutes** at +**25°C**.
5. IMMEDIATELY after, purify the polished products using a QIAquick column. Elute at **room temperature** with **50 µl** of Tris-HCl.

Optional: At this stage, the DNA sample can be stored at +2 to +8°C overnight.

3.9 Library Immobilization, Library Adaptor Ligation and Fill-In Reaction

3.9.1 Library Immobilization

1. Prepare a stock solution of 2× Library Binding Buffer by mixing **5.9 ml** of Molecular Biology Grade Water; **4.0 ml** of 5 M NaCl, and **0.1 ml** of 100× TE.
2. Transfer **25 µl** of Dynal M-270 Streptavidin beads to a new microcentrifuge tube.
3. Using the MPC, pellet the beads and remove the buffer.
4. Wash the beads **twice** with **50 µl** of 2× Library Binding Buffer, as follows:
   a. Add 50 µl of 2x Library Binding Buffer
   b. Take the microcentrifuge tube out of the MPC and vortex well to resuspend the beads
   c. Place the microcentrifuge tube in the MPC and wait for the beads to pellet
   d. Remove the supernatant
5. Resuspend the beads in **50 µl** of 2× Library Binding Buffer.
6. Add the **50 µl** of washed Dynal M-270 Streptavidin beads to the **50 µl** of polished, nebulized DNA.
7. Vortex well and place on a tube rotator at **room temperature** for **15 minutes**.
8. Using the MPC, wash the immobilized library **three times** with **500 µl** of TE Buffer. **Vortex well between each wash**.
9. Remove all remaining TE Buffer, and remove the tube from the MPC.

3.9.2 Library Adaptor Ligation

1. In a microcentrifuge tube, add the following reagents in the order indicated, and vortex:

15	µl	Molecular Biology Grade Water
25	µl	Rapid Ligase Buffer, 2× Conc.
5	µl	Library Adaptors (20 µM)
45	µl	Total volume

2. Add the **45 µl** of ligation mix to the tube containing the pellet of Paired End library beads. Vortex to resuspend the bead pellet.
3. Add **5 µl** of Rapid Ligase.
4. Vortex and place on a tube rotator at **room temperature** for **15 minutes**.
5. Using the MPC, wash the beads **three times** with **500 µl** of TE Buffer.
6. Quick spin and remove all remaining TE Buffer. Remove the tube from the MPC.

3.9.3 Fill-In Reaction

1. In a microcentrifuge tube, add the following reagents:

40	µl	Molecular grade water
5	µl	10× ThermoPol Buffer
2	µl	PCR Nucleotide Mix (10 mM each)
3	µl	*Bst* DNA Polymerase, Large Fragment (8 U/µl)
50	µl	Total volume

2. Add the **50 µl** from above to the tube of beads.
3. Vortex and incubate the fill-in reaction at +**50°C** for **15 minutes**.
4. Using the MPC, wash the beads **three times** with **500 µl** of TE Buffer. Quick spin and remove all remaining TE Buffer. Remove the tube from the MPC.
5. Resuspend beads in **20 µl** Tris-HCl.

3.10 Library Amplification

1. In a 200 µl thin-walled PCR tube, add the following reagents, in the order indicated:

22.5	µl	Molecular Biology Grade Water
5.0	µl	5× GC-RICH Reaction Buffer
6.0	µl	$MgCl_2$ (25 mM)
2.0	µl	PCR Nucleotide Mix (10 mM each)
2.0	µl	Amplification Primers (100 µM)
10.0	µl	"Adapted" Paired End library bead suspension
2.5	µl	GC-RICH Enzyme Mix (2 U/µl)
50.0	µl	Total volume

2. Vortex.
3. Run the following program in a thermocycler:

Hot Start Initiation	1x	3 minutes at	94°C
Amplification	20x	60 seconds at	94°C
		60 seconds at	60°C
		60 seconds at	72°C
Final Extension	1x	10 minutes at	72°C
On hold			4°C

This step uses half of the library beads prepared and usually produces ample amount of library DNA for sequencing. Leftover beads can be stored at -15 to –25°C for one month.

A DNA negative amplification control tube is recommended

3.11 Final Library Size Selection

Accuracy in pipetting during AMPure size selection is critical for appropriately sized libraries.

1. It is critical to the success of the procedure to use calibrated AMPure XP beads. See Section 3.4 for the calibration protocol.
2. Add **52 µl** of Tris-HCl to the **50 µl** of amplification reaction to bring the final volume a little over 100 µl.
3. Vortex and transfer **100 µl** of this amplification mixture to a new microcentrifuge tube.
4. Add *x* (µl) of AMPure XP beads to the tube, according to the calibrated Paired End cutoff value (PE cutoff value) of AMPure XP beads and to the equation:

$$x \text{ (in µl)} = (\text{PE cutoff value}) \text{ x } 100$$

5. Vortex.
6. Incubate without agitation at **room temperature** for **5 minutes**.
7. Using an MPC, pellet the beads against the wall of the tube.
8. Transfer the supernatant to a new microcentrifuge tube. Ensure that the supernatant volume equals to 100 µl + *x* µl (from Step 4). Adjust with Tris HCl if necessary.
9. Add **100 µl** of Tris-HCl to the transferred supernatant.
10. Add *y* µl of AMPure XP beads to the tube from Step 8 above:

$$y \text{ (in µl)} = x \text{ µl (from Step 4)} + 20 \text{ µl}$$

For example, if *x* equals 60 µl, add 60 + 20 = 80 µl (*y*) of AMPure XP beads.

11. Vortex.
12. Incubate without agitation at **+25ºC** for **5 minutes**.
13. Using an MPC, pellet the beads against the wall of the tube. Leave the tube in the MPC for all washes.
14. Remove the supernatant and wash the beads **twice** with **500 µl** of 70% ethanol, incubating for 30 seconds each time.
15. Quick spin and remove all the supernatant. Allow the AMPure XP beads to air dry for **2 minutes** at +**37ºC**.
    - The beads are dry when visible cracks start to form in the pellet. Do not over dry.
16. Remove the tube from the MPC, add **50 µl** of Tris-HCl and vortex to resuspend the beads.
    - This elutes the Paired End library from the AMPure XP beads.
17. Using the MPC, pellet the beads against the wall of the tube and transfer the **SUPERNATANT** to a new tube. You can discard the bead pellet.

3.12 Paired End Library Isolation (single-stranded)

Warning: Hazardous Chemicals - Sodium Hydroxide Solution (10 N)
Sodium hydroxide is a highly corrosive chemical that may cause burns if it contacts eyes or skin. Read the MSDS for handling precautions.

3.12.1 Immobilize the Library of Amplified Paired End Fragments

1. Transfer **50 µl** of Dynal M-270 Streptavidin beads to a new microcentrifuge tube.
2. Using a MPC, pellet the beads and remove the buffer.
3. Wash the beads **twice** with **100 µl** of 2× Library Binding Buffer, as follows:
    a. Add 100 µl of 2x Library Binding Buffer
    b. Take the microcentrifuge tube out of the MPC and vortex well to resuspend the beads
    c. Place the microcentrifuge tube in the MPC and wait for the beads to pellet
    d. Remove the supernatant
4. Resuspend the beads in **50 µl** of 2× Library Binding Buffer.
5. Add the **50 µl** of AMPure-selected PCR products to the washed Dynal M-270 Streptavidin beads.
6. Mix the solution well and place the microcentrifuge tube rotator at **room temperature** for **15 minutes**.
7. Using the MPC, wash the immobilized library **three times** with **500 µl** of TE.
8. Remove all remaining TE, and remove the tube from the MPC.

3.12.2 Isolate the ssDNA Paired End Library

1. Prepare a stock of Melt Solution by mixing **125 µl** of NaOH (10 N) in **9.875 ml** of Molecular Biology Grade Water.
2. In a microcentrifuge tube, prepare the neutralization solution by mixing **500 µl** of Qiagen PBI buffer and **10 µl** of 3 M sodium acetate, pH 5.2.
3. Add **50 µl** of Melt Solution to the washed library-carrying beads from above.
4. Vortex well and, using the MPC, pellet the beads away from the 50 µl supernatant.
    - At this point, the **supernatant** contains the single-stranded DNA Paired End library.
5. Carefully remove and transfer the **supernatant** to the freshly-prepared neutralization solution from Step 2, above.
6. Repeat Steps 3 through 5 (second **50 µl** Melt Solution wash of the beads), and pool the two Melt Solution washes together in the same tube of neutralization solution.
    - Buffer PBI contains a pH indicator. The solution should promptly return to its neutral / acidic yellow color after the addition of each Melt Solution wash and mixing. If it does not, add an extra 5 µl of the 3 M Sodium Acetate pH 5.2 solution to fully neutralize the melts.
7. Purify the neutralized Paired End library using a MinElute column:
    a. Do not use any additional PBI.
    b. Perform **two** PE washes to remove any salt.
    c. Elute with **20 µl** of TE at room temperature. Let the TE sit on the column for 1 minute prior to centrifugation.

3.13 Library Characterization

3.13.1 Library Quality Assessment and Physical Quantitation

1. Using the Agilent 2100 Bioanalyzer, prepare a RNA Pico 6000 LabChip according to the manufacturer's guidelines. Load **1 µl** of your library on to the chip.
   - See Section 3.3 for a representative trace and other expected characteristics of a Paired End library.
2. Assess the quality of the DNA library using the Bioanalyzer (Table 1):

Library Characteristic	Expected Result
Peak fragment length	between 500 nt and 600 nt*
Size cut-off	<10% below 300 nt
Adaptor dimer peak	None

Table 1: Quality assessment of Paired End libraries.

***Libraries with low (d 30%) or high (e 70%) GC content can show a peak fragment length increased or decreased by ~100 nt, respectively.**

3. Quantitate the mass of 1 µl of the GS Junior Titanium Paired End library using Quant-iT RiboGreen or assays with similar sensitivity.

3.13.2 Library Primary Dilution and Storage

1. From the RiboGreen quantitation results above, expressed in ng/µl, calculate the library concentration equivalence in molecules/µl, using the following equation:

$$\text{Molecules}/\mu\text{l} = \frac{(\text{Sample conc. in ng}/\mu\text{l}) \text{ x } (6.022 \text{ x } 10^{23})}{(328.3 \text{ x } 10^{9}) \text{x } (\text{avg. fragment length in nt})}$$

   …where 6.022 x 10^{23} is Avogadro's number (molecules/mole); 328.3 is the average molecular weight of nucleotides, in g/mole; and avg. fragment length is the result from the RNA 6000 LabChip profile from above. The GC content of the library will impact the estimated size determined by the RNA Pico 6000 LabChip. High GC libraries will appear smaller while low GC will appear larger than their actual size.
2. Dilute **2 µl** of the library to 1 x 10^{8} molecules/µl, in TE Buffer.
3. Store the concentrated library and, if not used immediately for the determination of the amount needed for the emPCR amplification procedure, the 1 x 10^{8} molecules/µl stock, at -15 to –25ºC.

3.14 Preparation of Working Aliquots

1. Prepare a library working dilution of 2 x 10^{6} molecules/ µl
2. Distribute this working stock into aliquots (*e.g.* 100 µl if you will be doing Large Volume emulsions; 25 µl if you will be doing Small Volume emulsions), and store them at -15 to -25ºC. (Re-freeze the leftover 1 × 10^{8} molecules/µl stock as well.)

4 APPENDIX

4.1 Example of an Agarose Gel Image from a Circularization Adapted DNA

Figure 3 shows typical circularization-adapted DNA samples (Section 3.3) run on a 0.5% agarose TAE gel at 35 volts for 16 hours (**A**, 20 kb) and 85 volts for 4 hours (**B**, 8 kb).

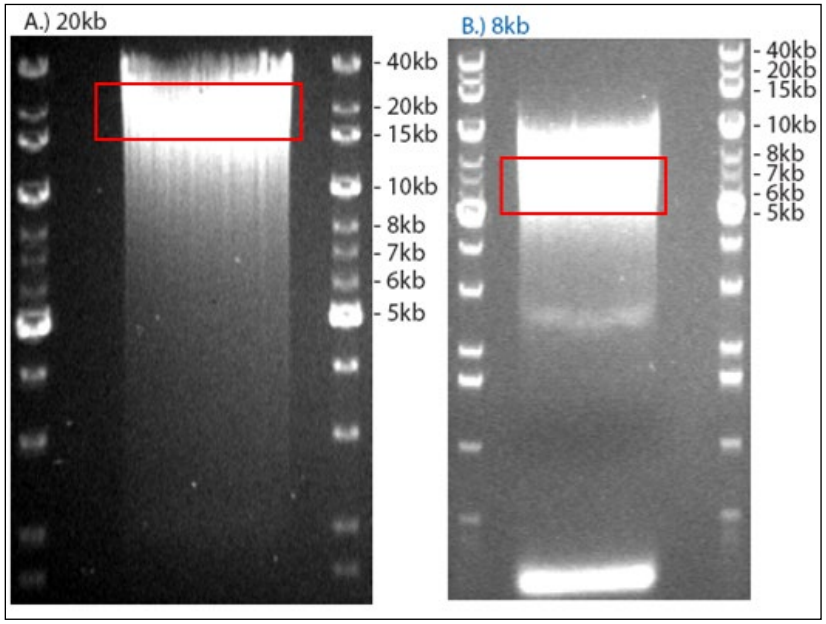


Figure 3: DNA samples that were fragmented following the 20 kb span Paired End library preparation procedure (A) or the 8 kb span procedure (B), circularization-adapted, and run on an 0.5% agarose gel containing 1× SYBR Safe. 2.5 µg of a 1 kb DNA extension ladder was used on both sides. Red rectangles indicate the optimal cutting area.

4.2 Example of an Agilent 2100 Trace of a Nebulized DNA Sample

Figure 4 shows a typical Agilent 2100 DNA 7500 LabChip profile for 1 µl of the material following nebulization of the circularized fragments and MinElute cleanup of a circularized Paired End library. The nebulized material should show as a single broad peak with a fragment size range of 300-700 bp (the Bioanalyzer tends to slightly overestimate the size of broad peaks). The peaks at 50 bp and 10 kb are internal markers.

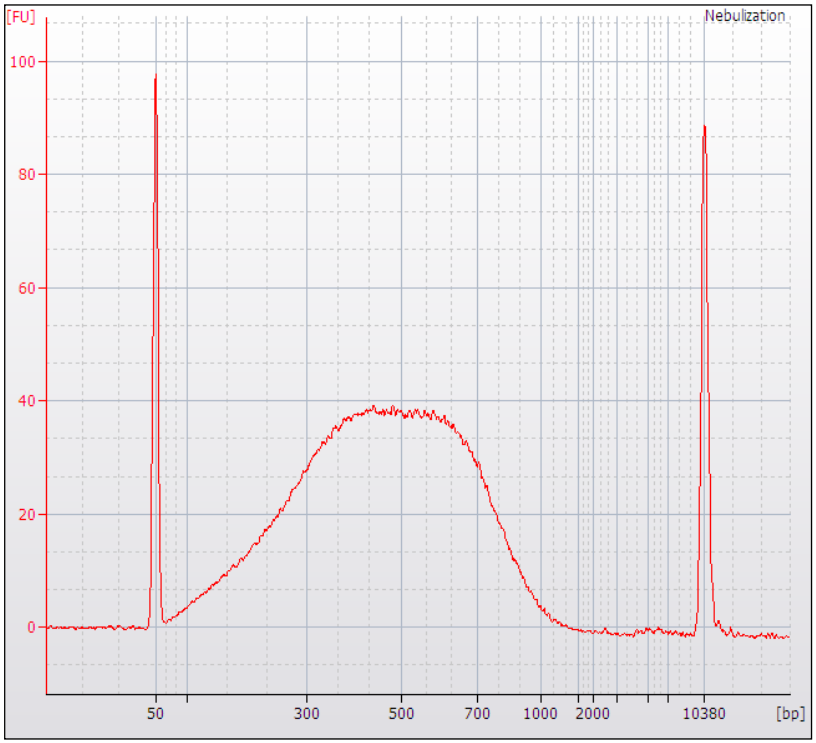


Figure 4: DNA 7500 LabChip profile of a nebulized and purified DNA sample for use in the preparation of a GS Junior Titanium Paired End library

4.3 Example of an Agilent 2100 Trace of a Final GS Junior Titanium Paired End Library

Figure 5 shows a typical Agilent 2100 RNA Pico 6000 LabChip profile for 1 µl of the final Paired End library material. The library should be seen as a narrow peak at ~500 bp (a small shoulder to the right of the peak is normal), and a concentration greater than 0.2 ng/µl (>4 ng total yield). The peak at 25 bp is an internal marker.

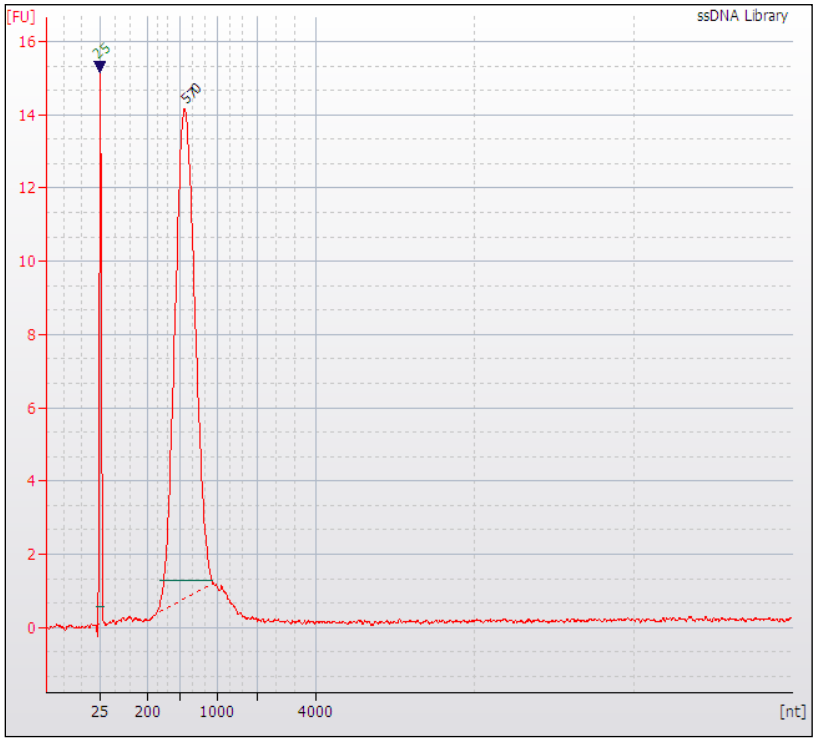


Figure 5: RNA Pico 6000 LabChip profile of a Paired End DNA library

4.4 AMPure XP Bead Calibration Procedure

In this library preparation protocol, AMPure XP beads are used to remove both undersize and oversized DNA fragments from the library.

Due to the high variability of AMPure XP beads lots in the size exclusion characteristics, each lot must be calibrated. A calibration assay procedure has been developed to determine the size exclusion characteristics of any given AMPure XP bead lot. This calibration will determine the ratio of AMPure XP beads to DNA sample (by volume) to use during library preparation, to create the optimal size library for sequencing in the Genome Sequencer System using the GS Junior Titanium chemistry.

In this calibration assay, a 100-1500 bp DNA ladder is incubated with AMPure XP beads in beads:DNA ratios ranging from 0.4:1 to 0.9:1 (vol:vol). Each bead:DNA ratio will provide different fragment size cut-off parameters. These cut-offs are assessed by running the DNA retained by the beads in each condition on an Agilent Bioanalyzer DNA 7500 LabChip, and recording the areas under the peaks (DNA concentration) in the 200-500 bp range. The optimal bead:DNA ratio to use for DNA libraries with the bead lot being tested is determined by comparing the results of this calibration assay with the empirically-derived optimal data provided below.

4.4.1 Procedure

1. Label eleven 1.7 ml tubes for the eleven bead:DNA ratios to be included in the assay, from 0.40 : 1 to 0.90 : 1, in increments of 0.05, per the table below.
2. In a new microcentrifuge tube, place **48 µl** of the 100-1500 bp DNA ladder, and dilute it with **1152 µl** of Molecular Biology Grade Water.
3. Aliquot **precisely 100 µl** of diluted DNA ladder to each labeled microcentrifuge tube.
4. Vortex the tube of AMPure XP beads vigorously and aliquot **900 µl** into a new microcentrifuge tube. You will use this aliquot for the rest of the procedure.

5. Using the same pipettor as used to measure the 100 µl aliquots of diluted DNA ladder, **add the appropriate amount of beads** to each sample. Make sure to:
    a. vortex the bead aliquot between each sample;
    b. change tips between each sample;
    c. pipette the beads slowly, making sure that you do not aspirate any air, and that there are no beads on the outside of the tip;
    d. dispense slowly such that ALL the beads are delivered into the sample.

Beads:DNA ratio (by volume)	Diluted DNA Ladder (µl)	AMPure XP Beads (µl)
0.40 : 1	100	40
0.45 : 1	100	45
0.50 : 1	100	50
0.55 : 1	100	55
0.60 : 1	100	60
0.65 : 1	100	65
0.70 : 1	100	70
0.75 : 1	100	75
0.80 : 1	100	80
0.85 : 1	100	85
0.90 : 1	100	90

Table 2: Volumes of Diluted DNA ladder and of AMPure XP beads for each Beads:DNA ratio of the assay

6. Vortex all the tubes, and incubate them at room temperature for **5 minutes**.
7. Using the MPC, pellet the beads against the wall of the tube. This may take several minutes due to the high viscosity of the suspension.
8. Remove the supernatant and wash the beads **twice** with **500 µl** of 70% ethanol, incubating for 30 seconds each time.
    - Larger DNA fragments will bind to the AMPure XP beads, with a decreasing size cut-off as the Bead:DNA ratio increases; the DNA species from the DNA ladder that are below the cut-off in each of the incubation conditions will thus be washed away in the next step.
9. Remove all the supernatant from each tube and allow the AMPure XP beads to air dry completely. To reduce drying time, place the tubes in a heating block at +37ºC. Visible cracks in the pellet are an indication the beads are dry.
10. Remove the tubes from the MPC, add 10 µl of Tris-HCl to each tube, and vortex to resuspend the beads.
11. Using the MPC, pellet the beads against the wall of the tube once more, and transfer the **supernatants** containing the **size-selected DNA ladder** to a set of new, appropriately labeled microcentrifuge tubes.
12. Separately, dilute **4 µl** of fresh DNA ladder with **6 µl** of Molecular Biology Grade Water
    - This aliquot of unprocessed, diluted DNA ladder will serve as a control.
13. Run 1 µl of each size-selected DNA ladder, including the control ladder, on a single Bioanalyzer DNA 7500 LabChip.

4.4.2 Analysis

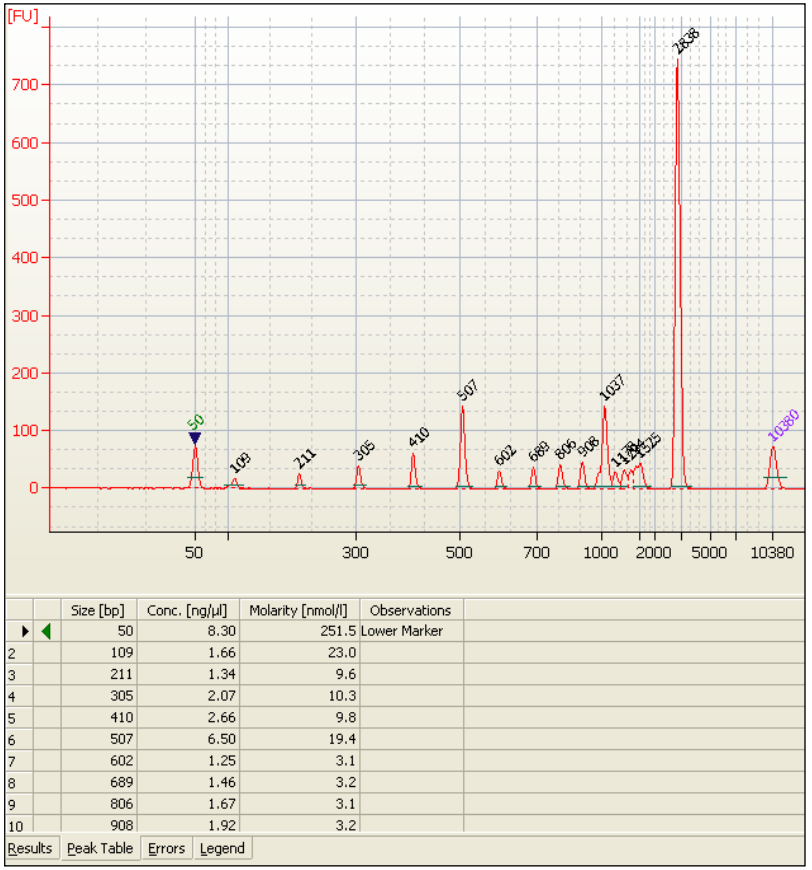
Results will show the gradual removal of small fragments from the DNA ladder samples, as the Beads:DNA ratio decreases (*i.e.* only large fragments bind to the beads at low bead ratio). To assess this, the DNA concentration of the 200 to 500 bp peaks is monitored in the 12 LabChip traces (including the non-selected control ladder). The peak at 900 bp should be fully retained in the whole range of bead:DNA ratios tested, and is used for normalization between the traces.

1. For each of the 12 traces, divide the DNA concentration (in ng/µl) of each of the following 4 peaks, by the DNA concentration of the 900 bp peak for that trace:
    a. 200 bp
    b. 300 bp
    c. 400 bp
    d. 500 bp
2. Compare the sets of 4 values for each of the 11 size-selected DNA ladders, with the values for columns 2 and 3 of Table 3.
    - **For the PE cutoff**, use the ratio of AMPure XP beads:DNA (by volume) that generated the set of peak ratio values most similar to the values given in column 2.
    - Make sure to also verify that the peak ratios in the control trace match the values given in column 3.

Peak ratio (DNA concentrations)	Optimal values for PE Cutoff	Values for the Control DNA Ladder
200/900	N/A	0.7
300/900	0.25	1.1
400/900	0.4	1.4
500/900	1.5	3.4

Table 3: Optimum ratios of the DNA concentration in the low molecular weight peaks to the 900 bp peak

Figure 6 below shows typical traces produced by the non-selected control DNA ladder, and AMPure XP bead size-selected DNA libraries.



	Size [bp]	Conc. [ng/µl]	Molarity [nmol/l]	Observations
	50	8.30	251.5	Lower Marker
2	109	1.66	23.0	
3	211	1.34	9.6	
4	305	2.07	10.3	
5	410	2.66	9.8	
6	507	6.50	19.4	
7	602	1.25	3.1	
8	689	1.46	3.2	
9	806	1.67	3.1	
10	908	1.92	3.2	

Results | Peak Table | Errors | Legend

Figure 6: Example DNA 7500 LabChip trace of a non-selected control ladder

- 10^9 total calculated molecules. This might increase library redundancy. If sufficient pre-circularized DNA remains, it is advised to repeat the protocol from that step.

Paired End Library Preparation Method Manual, 20 kb and 8 kb Span

Table of Contents

Published by
454 Life Sciences Corp.
A Roche Company
Branford, CT 06405
USA

© 2012 454 Life Sciences Corp.
All rights reserved.

For life science research only. Not for use in diagnostic procedures.
License disclaimer information is subject to change or amendment. For current license information on license disclaimers for a particular product, please refer to https://www.roche-applied-science.com/new/legal/index.jsp?id=legal_000000.

454, 454 LIFE SCIENCES, 454 SEQUENCING, GS FLX, GS FLX TITANIUM, GS JUNIOR, EMPCR, PICOTITERPLATE, PTP, NEWBLER, REM, GS GTYPE, GTYPE, AMPLITAQ, AMPLITAQ GOLD, FASTSTART, NIMBLEGEN, SEQCAP, MAGNA PURE, and CASY are trademarks of Roche.

SYBR is a registered trademark of Molecular Probes, Inc.
All other product names and trademarks are the property of their respective owners.

(6) 0312