I. Introduction

This protocol is for use with the SMARTer PrepX DNA Library Kit, 24 Samples (Cat. No. 640101), which accommodates rapid, walkaway automation of NGS library prep on the SMARTer™ Apollo™ Library Prep system. When run with the `PrepX ILMN 8` script, this kit can be used to process **three batches of up to eight samples per batch** into 220-, 320-, 520-, or 870-base-pair-read DNA libraries suitable for sequencing on Illumina® platforms. **Read this Protocol-At-A-Glance in its entirety before you begin with particular attention paid to the SMARTer Apollo System Best Practices.**

II. Workflow Overview

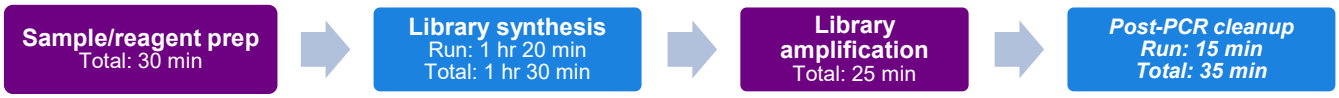


Figure 1. Library preparation workflow overview for one batch of up to eight samples on the SMARTer Apollo system with the SMARTer PrepX DNA Library Kit - A. Blue and purple boxes indicate steps performed on and off the SMARTer Apollo system, respectively. The italicized text indicates that the post-PCR cleanup requires a separate protocol. Run = the run time in minutes on the SMARTer Apollo system, if applicable. Total = total time in minutes spent, including thawing of reagents, reagent and equipment setup, heating and cooling of thermal blocks, incubation of reactions, and automated liquid-handling processes, if applicable.

III. List of Components

SMARTer PrepX DNA Library Kit, 24 Samples (Cat. No. 640101)	
Box 1. (Store at –20°C.)	
PrepX DNA Ligase Buffer, 24	350 µl
PrepX PCR Master Mix	700 µl
PrepX Complete Ligase Enzyme	3 x 15 µl
PrepX Complete (24 sample) Yellow Strips	24 x 4-tube yellow strips
Box 2. (Store at 4°C.)	
PrepX Molecular Grade Water	100 ml
PrepX Cleanup Beads	6.8 ml
PrepX 2.5M NaCl	10 ml

Additional Materials Required

Sequencing Indexes

- PrepX Complete ILMN Barcodes 1-24 (Cat. No. 640103)

SMARTer Apollo Consumables

The following consumables must be purchased separately from Takara Bio. and were used to validate protocols and scripts. **Do not make any substitutions.**

SMARTer Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
SMARTer Apollo Piercing Tips	640085	Box of 1,000 tips	8 tips
SMARTer Apollo Filter Tips	640084	Box of 960 tips	56 tips
SMARTer Apollo Reservoirs	640087	Box of 100 reservoirs	4 reservoirs
SMARTer Apollo Microtiter Plates	640083	Box of 25 plates	1 plate
SMARTer Apollo 1.1 mL MiniTubes	640088	Box of 960 minitubes	24 minitubes
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	5 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Box of 125 strips	5 strips

General lab equipment, reagents, and consumables

- Single-channel pipettes: 10 µl, 20 µl, 200 µl, and 1,000 µl
- Eight-channel pipettes (recommended): 20 µl and 200 µl
- Filter pipette tips: 2 µl, 20 µl, 200 µl, and 1,000 µl
- PCR thermal cycler
- Covaris instrument and related materials for DNA shearing
- DNA LoBind Tubes (Eppendorf, Cat. No. 0030108051)
- 100% ethanol (EtOH; molecular biology grade)

IV. SMARTer Apollo System Best Practices

- **Read this Protocol-At-A-Glance in its entirety before you begin.**
- **Before beginning library preparation, follow the Illumina index pooling guidelines to select the right barcode adapters for your set of samples.** (Barcode adapter sequences are in Appendix B.) To avoid index read failure due to registration failure, confirm that the combination of barcode adapter sequences you intend to use will maintain color balance for each base of the index read being sequenced. For more information, visit: https://www.illumina.com/informatics/sample-experiment-management/sequencing-experiment-setup.html
- Clean the work surfaces, including the retention plates, with 70% ethanol at least once a week.
- **Restart the instrument before every run. Also, between each subprotocol, perform a power cycle by turning the instrument off, waiting 1 min, and then turning it back on.**
- Discard any deformed plastics.
- Separate partial tube strips with scissors and remove resulting plastic overhangs.
- Spin down reagents before placing them on the deck to avoid air bubbles. **Bubbles at the bottoms of tubes must be removed to ensure accurate volume delivery.**
- Ensure plastics are properly seated on the deck surface **with caps/lids removed**. Be sure to push any tubes down completely and evenly prior to installing the metal retention plates.
- Empty the waste box before every run. **An accumulation of tips in the waste box may cause the run to fail.**

V. Protocols

A. Protocol: Sample and Reagent Prep

For each protocol, the corresponding step in the workflow diagram is indicated in with a green outline.

Materials Required

Reagents	Storage conditions	Source
Sample (DNA)	–20°C	User
Plate containing barcode adapters	–20°C	Takara Bio
PrepX DNA Ligase Buffer, 24	–20°C	Takara Bio
PrepX Complete Ligase Enzyme	–20°C	Takara Bio
PrepX Molecular Grade Water	4°C	Takara Bio
PrepX Cleanup Beads	4°C	Takara Bio
PrepX 2.5M NaCl	4°C	Takara Bio
70% ethanol (prepared fresh)	Room temperature	User

NOTE: PrepX Cleanup Beads need to come to room temperature before the container is opened. Therefore, **we strongly recommend preparing ~1-ml aliquots upon receipt** and then refrigerating the aliquots. Individual tubes can be removed for each experiment, allowing them to come to room temperature more quickly (≥30 minutes). This will also decrease the chance of bead contamination. Mix well to disperse the beads before adding them to your reactions. The beads are viscous, so pipette slowly.

SMARTer Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	5 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Bos of 125 strips	5 strips
SMARTer Apollo Microtiter Plates	640083	Box of 25 plates	5 plates

1. For each sample, prepare 1–100 ng of appropriately-sized DNA as follows:
   a) Quantify DNA using a Qubit Fluorometer or your preferred method.
   b) Assess the size using a fragment analyzer.
   c) (Optional) Further fragment the samples using a Covaris instrument according to the parameters in Appendix A and per the manufacturer's instructions.
   d) Quantify the DNA again, then prepare the samples at the desired input amount. We recommend using an input amount of 1–100 ng fragmented DNA in a volume of 15 µl (for a concentration of 0.067–6.67 ng/µl).
2. Prepare the barcode adapters as follows:
   a) Place the barcode adapter plate in a thermal cycler set to the following annealing conditions:

	Temperature	Time
Step 1	95°C	5 min
Step 2	70°C	15 min

NOTE: This protocol is designed for the generation of up to 8 libraries at a time, so no more than 8 barcodes are used during a given run. Store the plate with the remaining barcode adapters at –20°C until the next use. Do not repeat the annealing process.

   b) Move the plate from the thermal cycler to a benchtop and allow it to cool to room temperature.
   c) Add 6 µl of PrepX Molecular Grade Water to each well containing a barcode adapter (24 wells total, with a layout shown in Appendix B; **always change tips to avoid cross contamination**).
   d) Transfer one batch of barcode adapters into a fresh 8-tube strip. Cap and place on ice until used.

3. Prepare the barcode adapter master mix ("Adapter MM") for **each** of the desired barcode adapters (up to 8) as described in the table below. Add 15 µl of Adapter MM to each tube of a new SMARTer Apollo 0.2 ml PCR 8-tube strip. Mix thoroughly with a pipette. Cap and keep on ice.

Component	Volume/rxn
Barcode adapter (e.g., well A1)	2 µl
PrepX Molecular Grade Water	13 µl
Total volume	15 µl

4. Prepare the ligation master mix ("Ligation MM") in one PCR tube as described in the table below. Mix thoroughly with a pipette. Keep on ice until aliquoted.

Component	Volume/rxn	Volume/8 rxns + 1 rxn excess
PrepX DNA Ligase Buffer	12 µl	108 µl
PrepX Complete Ligase Enzyme	1 µl	9 µl
PrepX Molecular Grade Water	2 µl	18 µl
Total volume	15 µl	135 µl

5. On the benchtop, aliquot the reagents into the consumables as described in the table below:

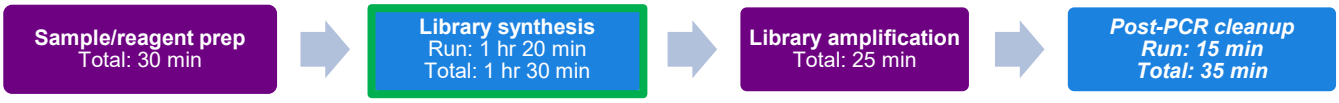
Component	Consumable	Volume/rxn
Sample	New SMARTer Apollo 8-tube strip	15 µl
Adapter MM	SMARTer Apollo 8-tube strip (from Step 4)	15 µl
Ligation MM	1 new SMARTer Apollo 8-tube strip	15 µl
PrepX Molecular Grade Water	1 SMARTer Apollo reservoir	15 ml
PrepX Cleanup Beads*	New SMARTer Apollo 8-tube strip	200 µl
PrepX 2.5M NaCl	SMARTer Apollo microtiter plate, Row 1 (Figure 2)	100 µl
70% ethanol	1 SMARTer Apollo reservoir	15 ml

*The PrepX Cleanup Beads should be warmed to room temperature and mixed well prior to use.

NOTES:

- The SMARTer Apollo system is specifically calibrated for the consumables indicated in the table above. Using alternative consumables may cause the run to fail.
- We recommend moving to the Library Synthesis protocol (Section V.B) immediately.

B. Protocol: Library Synthesis



Materials Required

Reagents	Current temperature	Source
Sample	On ice	User
Adapter MM	On ice	Section V.A.
Ligation MM	On ice	Section V.A.
PrepX Molecular Grade Water	Room temperature	Takara Bio
PrepX Cleanup Beads	Room temperature	Takara Bio
PrepX 2.5M NaCl	Room temperature	Takara Bio
70% ethanol	Room temperature	User
PrepX Complete (24 sample) Yellow Strips	On ice	Takara Bio

SMARTer Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
SMARTer Apollo Piercing Tips	640085	Box of 1,000 tips	8 tips
SMARTer Apollo Filter Tips	640084	Box of 960 tips	56 tips
SMARTer Apollo Reservoirs	640087	Box of 100 reservoirs	4 reservoirs
SMARTer Apollo Microtiter Plates	640083	Box of 25 plates	1 plate
SMARTer Apollo 1.1 mL MiniTubes	640088	Box of 960 minitubes	24 minitubes
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	5 strips
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Box of 125 strips	5 strips

1. Turn on the instrument or, if the instrument is already on, perform a power cycle by turning the instrument off, waiting 1 min, and then turning it back on.
2. Load SMARTer Apollo consumables onto the SMARTer Apollo work surface according to the layout in Figure 2. First, load the SMARTer Apollo consumables that do not initially hold reagents (table above). Just before the run, load the SMARTer Apollo consumables containing reagents, but not samples, onto the system. For information on the deck layout when processing fewer than eight samples, please see Appendix C.
3. To access the `PrepX ILMN 8` script, press **Library Prep** > **DNA > ILMN**. Then, under the PrepX ILM 8 heading, select the desired library insert size (**220 bp**, **320 bp**, **520 bp**, or **870 bp**). The **Cooling** indicator will appear.
4. When the **Cooling** indicator has disappeared, and the **Run** button has appeared, load the samples, reagents, and remaining SMARTer consumables onto the SMARTer Apollo deck following the layout shown in Figure 2 and the instructions shown on the touch screen.

NOTE: Ensure plastics are properly seated on the deck surface with caps carefully removed. Be sure to push any tubes down completely and evenly prior to installing the metal retention plates.

5. Install the metal retention plates on Blocks 3 and 4.
6. Empty the waste box and remove any used consumables from the system.

   NOTE: An accumulation of tips in the waste box may cause the run to fail.

7. Close the instrument door and press **Run.**

   NOTE: The run time is 80 minutes.

8. When the run is complete, remove the libraries from Block 3, Row 5 (Products), cap the tubes, and place them on ice. The final DNA library product volume should be ~15 µl per tube.
9. Turn off the instrument.

   SAFE STOPPING POINT: If you do not plan to proceed immediately to the Library Amplification protocol, the products in Block 3 can be capped and stored at –20°C for up to one week.

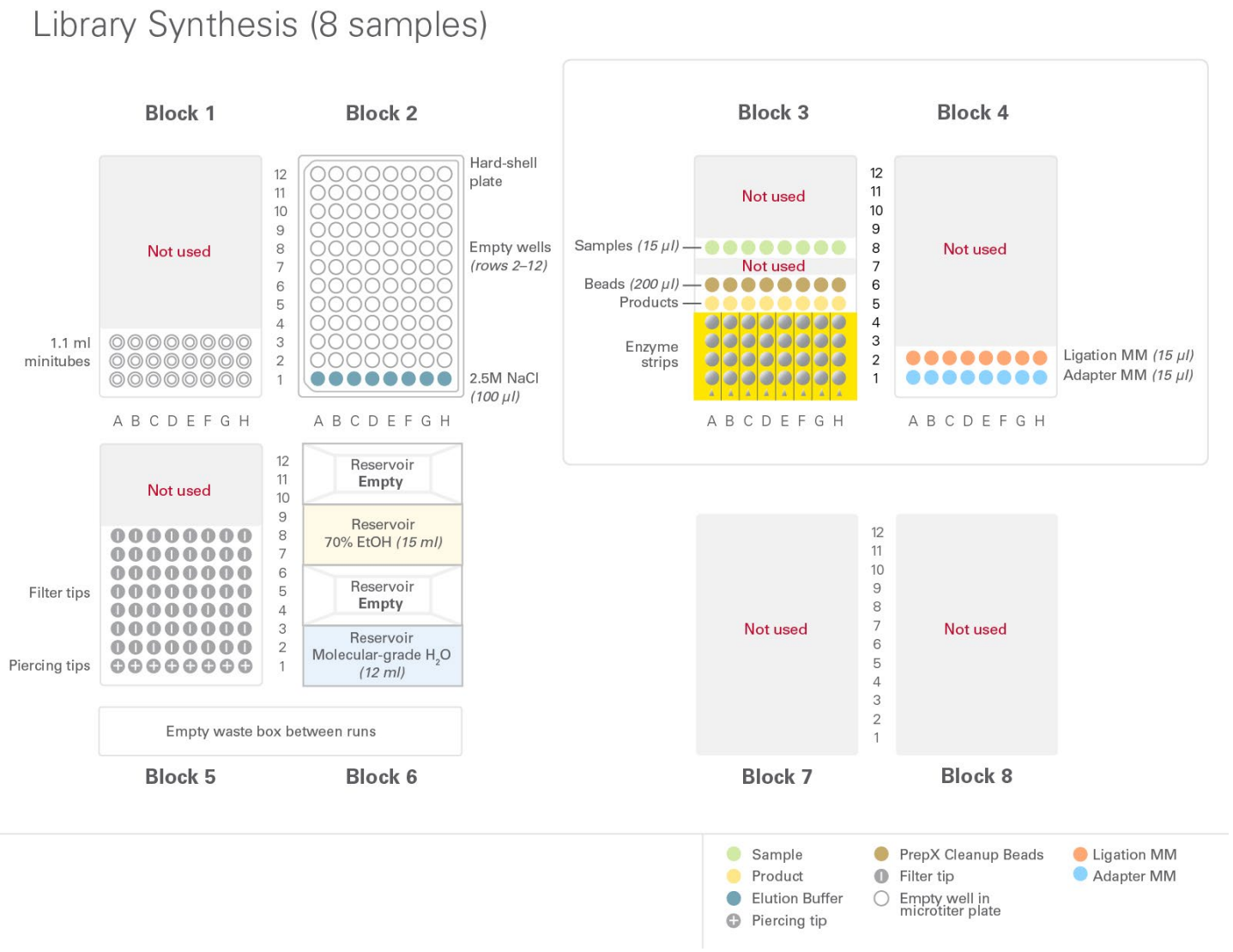
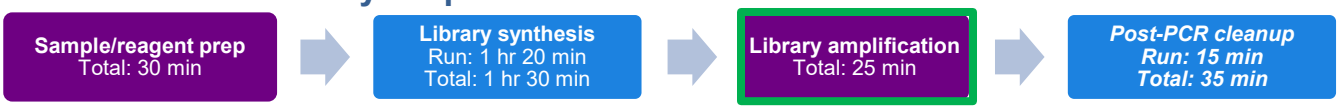


Figure 2. Deck layout for library synthesis from 8 samples. For this protocol, use script `PrepX ILMN 8`. Run time is 80 minutes.

C. Protocol: Library Amplification



Materials Required

Reagents	Current temperature	Source
Products (from Block 3, Row 5)	On ice	Section V.B.
PrepX PCR Master Mix	On ice	Takara Bio
PrepX PCR Primers	On ice	Takara Bio
PrepX Molecular Grade Water	On ice	Takara Bio

SMARTer Apollo consumables	Cat. No.	Quantity	Usage/8-rxn run
SMARTer Apollo 0.2 ml PCR 8-Tube Strips, Clear	640082	Box of 125 strips	1 strip
SMARTer Apollo Caps for 0.2 ml PCR 8-Tube Strips, Clear	640086	Box of 125 strips	1 strip

1. On ice, prepare the Amplification MM in a 1.5-ml tube, per the table below. Mix the Amplification Master Mix by vortexing gently, and then spin down. Aliquot 27 µl of this master mix into the wells indicated in Figure 2.

Component	Volume/rxn	Volume/8 rxns + 1 rxn excess
PrepX PCR Master Mix	25 µl	225 µl
PrepX PCR Primers	2 µl	18 µl
Total volume	27 µl	243 µl

NOTE: Do not change the volumes of the PrepX PCR Master Mix or the PrepX PCR primers.

2. Use the Amplification MM from Step 1 to prepare the PCR mixture as follows:

Component	Volume/rxn
Amplification Master Mix (from Step 1)	27 µl
Products (from Block 3, Row 5)	5 ng
PrepX Molecular Grade Water	Varies
Total volume	50 µl

3. Perform PCR using the following program:

98°C	30 sec
5–10 cycles:	
98°C	10 sec
60°C	30 sec
72°C	30 sec
72°C	300 sec
4°C	forever

NOTE: Depending on the sample type, the PCR conditions may need to be optimized.

4. Spin the tubes to collect the contents, and then bring each reaction volume up to 50 µl with PrepX Molecular Grade Water. Proceed to the **PrepX PCR Cleanup 8** protocol immediately **OR** PCR products may be stored at 4°C overnight.

Appendix A. Fragmentation Guidelines

Fragment the intact DNA according to the recommended settings below (for a Covaris S220 instrument). Depending on the instrument and sample type used, these settings may need to be optimized. Follow the manufacturer's instructions if using a different instrument.

Table I. Recommended Covaris S220 Instrument Settings for Desired Library Insert Sizes

Parameter	Recommended settings for library insert size		
	220 bp & 320 bp	520 bp	870 bp
Duty cycle	10%	5%	5%
Intensity	4	3	3
Cycles/burst	200	200	200
Time/cycle	9 sec	10 sec	10 sec
Number of cycles	8	5	4
Total process time	72 sec	50 sec	40 sec

Appendix B. SMARTer PrepX Complete ILMN Barcodes 1-24

SMARTer PrepX Complete ILMN Barcodes 1-24 (Cat. No. 640103) allows the preparation of up to 24 Illumina-ready DNA libraries using the SMARTer PrepX ILMN DNA Library Kit - A. Before beginning library preparation, follow the Illumina index pooling guidelines to select the right barcode adapters for your set of samples. To avoid index read failure due to registration failure, confirm that the combination of barcode adapter sequences you intend to use will maintain color balance for each base of the index read being sequenced. Barcode adapter sequences are listed in Table II. For more information, visit: https://www.illumina.com/informatics/sample-experiment-management/sequencing-experiment-setup.html

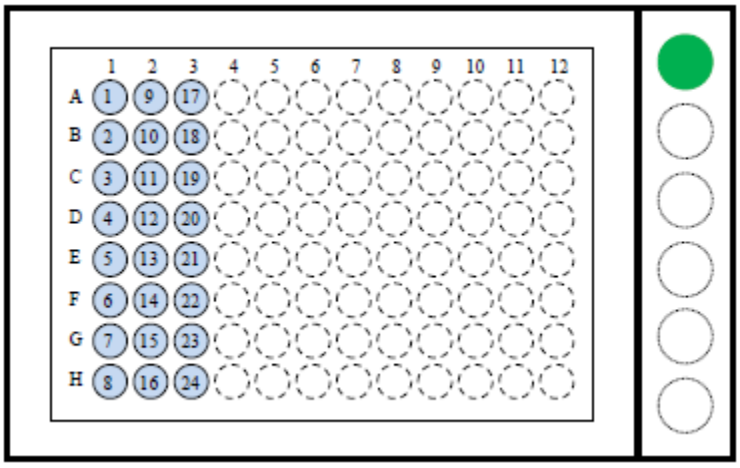


Figure 3. Contents of SMARTer PrepX Complete ILMN Barcodes 1-24. The green circle in the diagram represents the PrepX PCR Primers supplied in a tube with a green cap.

Table II. Barcode adapter sequences for the SMARTer PrepX Complete ILMN Barcodes 1-24. Barcode sequences are highlighted in red.

Index	Barcode adapter sequence (5'–3')
1	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CTCACG**ATCTCGTATGCCGTCTTCTGCTTG
2	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CAGCTT**ATCTCGTATGCCGTCTTCTGCTTG
3	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CGCTAC**ATCTCGTATGCCGTCTTCTGCTTG
4	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATTGTA**ATCTCGTATGCCGTCTTCTGCTTG
5	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TCCTAG**ATCTCGTATGCCGTCTTCTGCTTG
6	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGATCC**ATCTCGTATGCCGTCTTCTGCTTG
7	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TTGTCA**ATCTCGTATGCCGTCTTCTGCTTG
8	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGCTTT**ATCTCGTATGCCGTCTTCTGCTTG
9	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATCCGC**ATCTCGTATGCCGTCTTCTGCTTG
10	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CTGAAA**ATCTCGTATGCCGTCTTCTGCTTG
11	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CTGGCC**ATCTCGTATGCCGTCTTCTGCTTG
12	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TAATGT**ATCTCGTATGCCGTCTTCTGCTTG
13	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATTTCG**ATCTCGTATGCCGTCTTCTGCTTG
14	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TGTACG**ATCTCGTATGCCGTCTTCTGCTTG
15	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CAGTGT**ATCTCGTATGCCGTCTTCTGCTTG
16	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GACTCA**ATCTCGTATGCCGTCTTCTGCTTG
17	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATGACT**ATCTCGTATGCCGTCTTCTGCTTG
18	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GTAGGC**ATCTCGTATGCCGTCTTCTGCTTG
19	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGACCA**ATCTCGTATGCCGTCTTCTGCTTG
20	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TCAGCC**ATCTCGTATGCCGTCTTCTGCTTG
21	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ACGGTC**ATCTCGTATGCCGTCTTCTGCTTG
22	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TTCGAA**ATCTCGTATGCCGTCTTCTGCTTG
23	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGGTAC**ATCTCGTATGCCGTCTTCTGCTTG
24	GATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TATCAG**ATCTCGTATGCCGTCTTCTGCTTG

Appendix C. Deck Setup for Additional Reaction Sizes

Using the `PrepX ILMN 8` and `PrepX Cleanup 8` scripts, the SMARTer Apollo system can run up to eight samples per batch. If you are performing fewer than eight reactions, please use a specific deck layout based on the number of samples (Table VI).

Table III. Deck Layout Options for Various Sample Numbers

Setting up less than 8 samples	
# of samples	Columns to load
1	D or E*
2	D, E
3	C, D, E or D, E, F
4	C, D, E, F
5	B, C, D, E, F or C, D, E, F, G
6	B, C, D, E, F, G
7	A, B, C, D, E, F, G or B, C, D, E, F, G, H

*The user can choose between a deck setup centered around column D or E. The scripts are designed to accommodate either layout.

Appendix D. Library Validation

Figures 4 and 5 show example traces from pre-PCR and post-PCR library outputs using the 220-, 320-, 520-, or 870-bp size selection options.

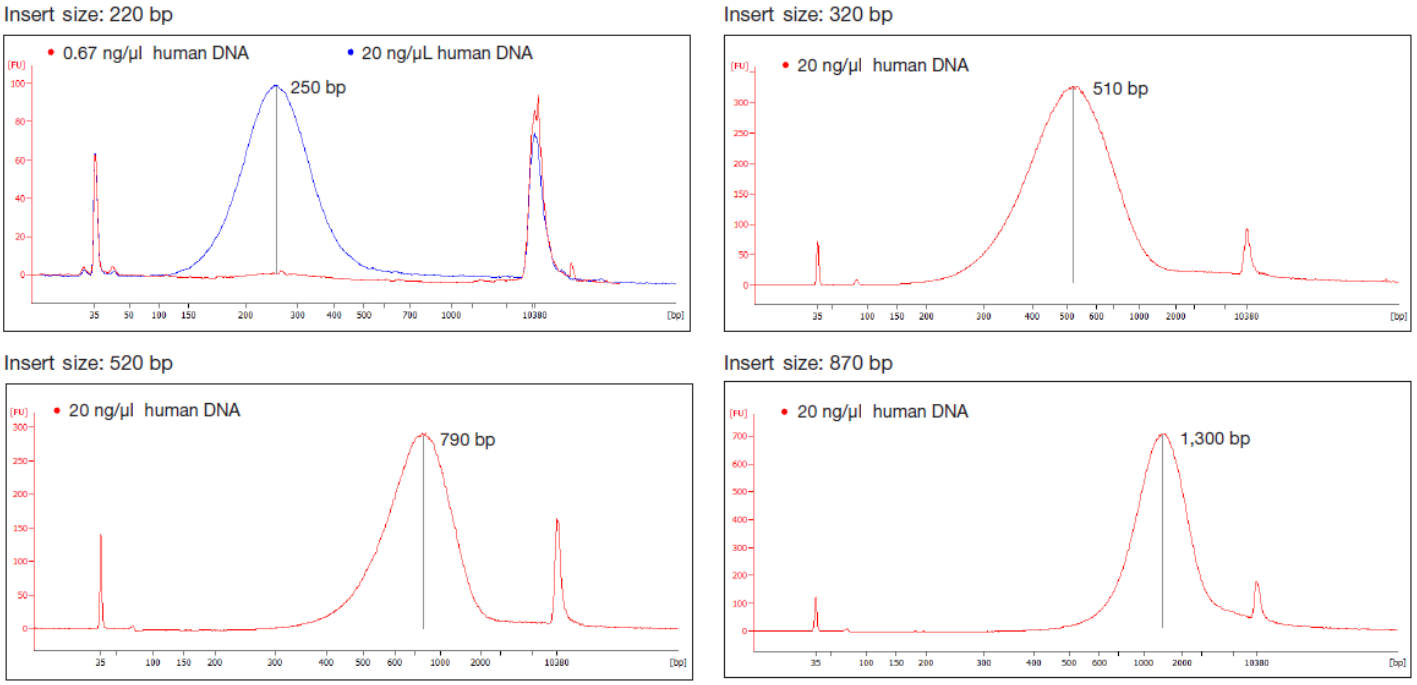


Figure 4. Bioanalyzer traces of pre-PCR library outputs. Covaris-fragmented human DNA was processed through the Protocol: Library Amplification (Section V.C) and validated using an Agilent 2100 Bioanalyzer and Agilent's High Sensitivity DNA Kit.

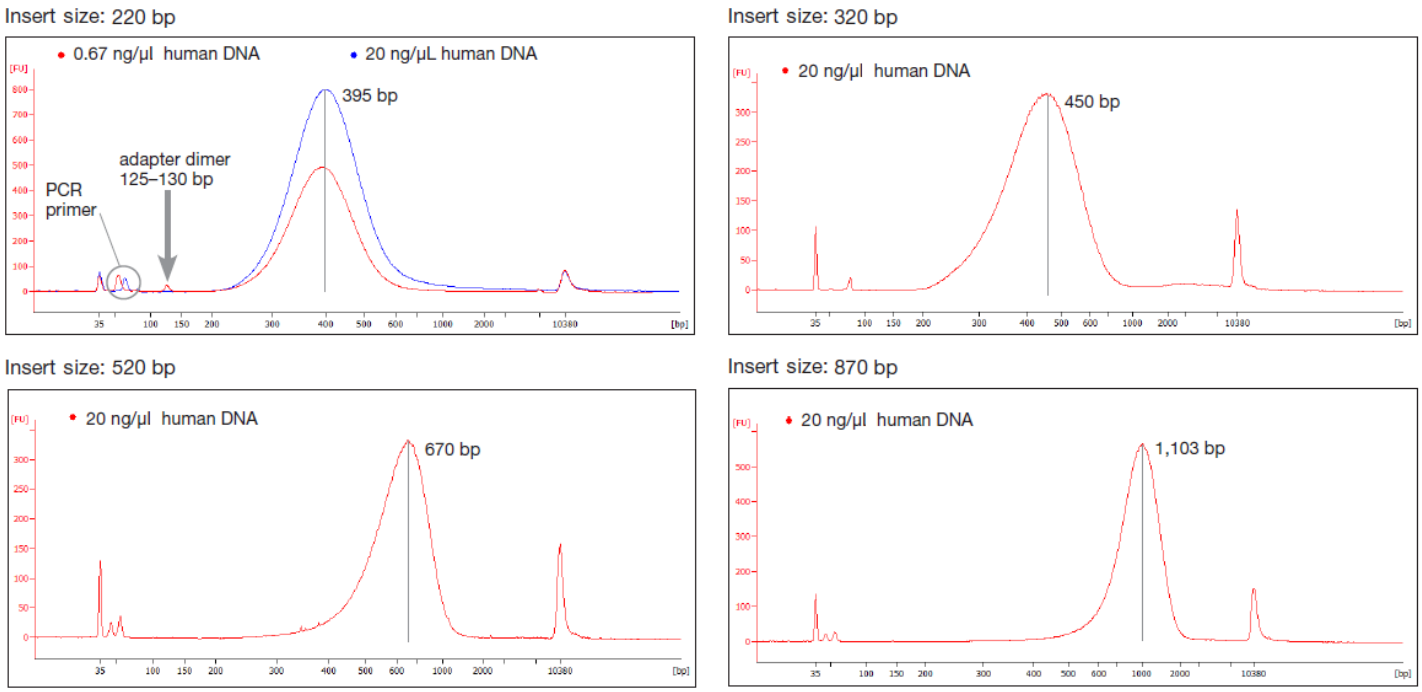


Figure 5. Bioanalyzer traces of post-PCR library outputs. The pre-PCR library outputs shown in Figure 5 were amplified in a commercial thermal cycler for a total of five cycles, then cleaned up using the **PrepX PCR Cleanup 8 - Protocol-At-A-Glance** protocol.

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