Preparing NGS libraries using the xGen™ DNA Library Prep Kit EZ and the Element Elevate™ Library Prep Workflow for sequencing on the Element AVITI™ System

The method presented here is provided by IDT. This method uses the xGen DNA Library Prep Kit EZ with the Element Elevate Library Prep Workflow which includes the Element Index and Adapter Kit and the Elevate Library Circularization Kit. This demonstrated protocol can be used as a starting point for creating next generation sequencing (NGS) libraries intended for downstream AVITI sequencing. This method can be used in similar experiments but may not be fully optimized for your application. IDT does not guarantee these methods, and application scientists at IDT can only provide general guidance with limited troubleshooting support. For more information and support for Element Biosciences products and sequencing on the AVITI System, see Element website.

Overview

The xGen DNA Library Prep Kit EZ from IDT is a fast and flexible solution designed to produce NGS libraries from a broad range of double-stranded DNA inputs. This demonstrated protocol describes the workflow for whole genome sequencing (WGS) using the xGen DNA Library Prep Kit EZ with the Element Elevate indexes and adapters. For sequencing, the Element AVITI System requires the generation of circularized libraries using AVITI-specific primer sequences. This protocol includes the Element Elevate Index and Adapter Kit workflow followed by the Elevate Library Circularization Kit workflow to convert the xGen DNA library fragments into circular templates for sequencing.

Note: Hybridization capture is not supported by this demonstrated protocol. If hybridization capture is required following library preparation, either follow the Adept demonstrated protocol or Contact Us to design custom xGen Blocking Oligos and a custom xGen Library Amplification Primer Mix.

There are four major activities outlined in this protocol:

- **Enzymatic preparation.** Performs fragmentation, end-repair, and dA-tailing of dsDNA.
- **Adapter ligation.** Performs ligation of Elevate adapters to the enzymatically prepped samples.
- **PCR amplification.** Amplify and index libraries using Element Index pairs.
- **Elevate circularization.** Prepare for AVITI sequencing with annealing, circularization, and digestion steps.

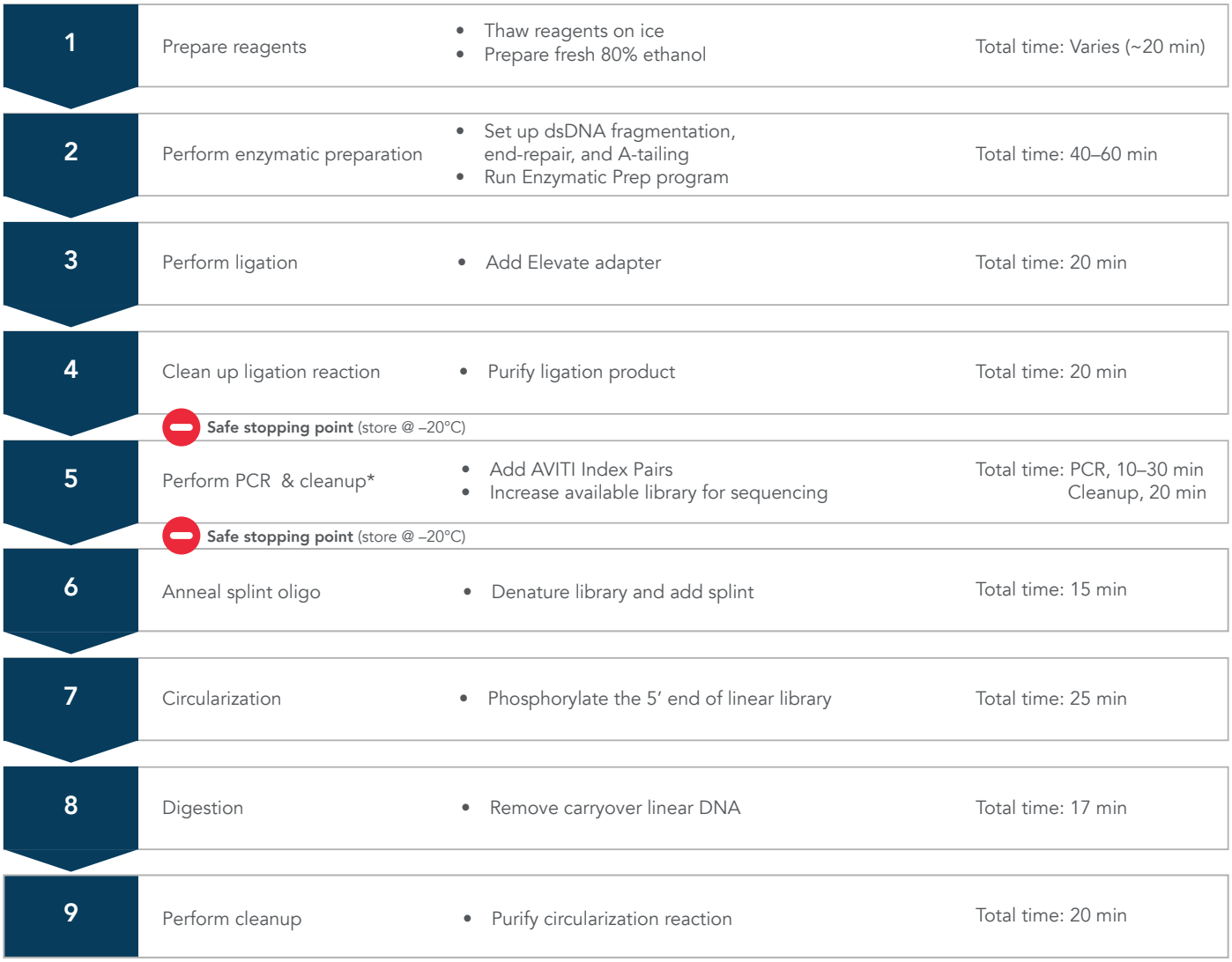

Figure 1. Workflow of the demonstrated protocol.

Consumables and equipment

Consumables—IDT

Item	Catalog #
xGen DNA Library Prep Kit EZ	10009821

Consumables—Other suppliers

Item	Supplier	Catalog #
Element Elevate Index and Adapter Kit	Element Biosciences	830-00005
Element Elevate Circularization Kit	Element Biosciences	830-00001
Absolute ethanol, 200 proof	Varies	Varies
Magnetic purification beads (choose one):		
SPRIselect™ purification beads, or equivalent	Beckman Coulter	B23317/B23318/B23319
AMPure® XP-PCR purification beads, or equivalent	Beckman Coulter	A63880 or A63881
Digital electrophoresis chips and associated reagents (choose one):		
Experion™ DNA 1K Analysis Kit, or equivalent	Bio-Rad™	700-7107
High Sensitivity DNA Kit	Agilent	5067-4626
High Sensitivity D1000 ScreenTape®, or equivalent	Agilent	5067-5584
Fluorometric DNA quantification assay kit		
Qubit™ dsDNA HS Assay Kit, or equivalent	Thermo Fisher Scientific	Q32851 or Q32854
Qubit dsDNA BR Assay Kit, or equivalent	Thermo Fisher Scientific	Q32850 or Q32853
PCR tubes, 0.2 mL	Varies	Varies
96-well, low-bind PCR plates		
Low-bind DNA tubes, 1.5 mL		
Aerosol-resistant tips and pipettes ranging from 2–1000 µL		

Equipment

Item	Supplier	Catalog #
Digital electrophoresis		
Experion™ Electrophoresis Station, or equivalent	Bio-Rad	700-7010
2100 Electrophoresis Bioanalyzer™, or equivalent	Agilent	G2939BA
2200 TapeStation™ System/4200 TapeStation System, or equivalent	Agilent	G2965AA or G2991AA
Qubit 4 Fluorometer, or equivalent	Thermo Fisher Scientific	Q33226
Magnet options (choose one):		
Magnetic Separator Plate	Permagen	MSP750
Magnetic PCR Strip Magnetic Separator Rack	Permagen	MSR812
Magnet for 1.5mL tubes or deep-well plate	Varies	Varies
Microcentrifuge		
Vortex		
Thermal Cycler		

Reagent handling

Important: Always store kit reagents at –20°C, except for the Low EDTA TE which can be stored at room temperature.

Note: The enzymes provided are temperature sensitive. Appropriate care should be taken during storage and handling. To maximize use of enzyme reagents, remove enzyme tubes from –20°C storage and place on ice for at least 10 minutes prior to pipetting. Attempting to pipette enzymes at –20°C may result in reagent loss.

Except for Buffer W1 and enzymes, briefly vortex the reagents after thawing them on ice. Spin all tubes in a microcentrifuge to collect contents before opening.

Thaw Buffer W1 (for Ligation Master Mix) at room temperature. Buffer W1 is viscous and requires special handling during pipetting. When ready for use, pipette slowly to draw the accurate quantity.

To create master mixes, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Add reagents to the master mix in the specified order as stated throughout the protocol. Once prepared, master mixes should be stored on ice until used.

DNA input considerations

The xGen DNA Library Prep Kit EZ works with a broad range of DNA inputs, ranging from 100 pg–1 µg. This kit can use high quality gDNA, amplicons, and formalin-fixed paraffin-embedded (FFPE) DNA. To quantify the concentration of low-quality human DNA samples, qPCR can be performed using the xGen Input DNA Quantification Primers (Cat. No. 10009856).

Important: For specific input quantities recommended in this protocol, refer to the total DNA quantified after fragmentation.

Fragmentation parameters

When utilizing a new lot of the fragmentation enzyme, you may experience variation in the required fragmentation times. Refer to your certificate of analysis (CoA) for specific fragmentation time recommendations for the lot number that you received. Fragmentation times provided in this protocol are for high quality samples. You may need to determine shorter fragmentation time for samples of compromised quality (e.g., FFPE).

EDTA in elution buffers

The enzymatic preparation reaction is sensitive to high concentrations of EDTA. A high concentration of EDTA, such as 1 mM in standard 1X TE buffer, will slow the reaction, resulting in larger insert sizes. Alternatively, no EDTA (i.e., if eluted in Tris buffer only) will result in faster fragmentation and smaller insert sizes. Our standard enzymatic prep conditions are determined using 0.1 mM EDTA TE [as provided in this kit (Low EDTA TE)].

If DNA is eluted in standard TE with 1 mM EDTA, perform a buffer exchange using a column or bead-based purification protocol. 3x SPRIselect (Beckman Coulter) is recommended for minimum loss of sample gDNA, although you may need to optimize your buffer exchange method depending on sample source. Alternatively, you can adjust the amount of Reagent K2 used in the Enzymatic Prep step to no more than 3X to achieve the desired fragment length (up to 4.5 µL of Reagent K2 per reaction).

If DNA is resuspended in 10 mM Tris (e.g., Buffer EB from Qiagen, 10 mM Tris-HCl, pH 8.5) or water without EDTA, Reagent K2 is not needed during Enzymatic Prep.

Note: For more information on automation, size-selection, and avoiding contamination see the full xGen DNA Library Prep Kit EZ protocol.

Protocol

Enzymatic prep

Important: Keep the Enzymatic Prep Master Mix and the DNA samples on ice until they are loaded in the thermal cycler to safeguard against fragmentation. Enzymes are active at room temperature and may fragment DNA to undesired sizes.

1. Transfer the DNA sample to a sterile 0.2 mL PCR tube. Adjust sample volume to a total of 19.5 µL using Low EDTA TE, then place the tube on ice.

Components	Volume per sample (µL)
Low EDTA TE	(19.5 – x)
DNA	x
Total volume	**19.5**

2. Set up the thermal cycler with the Enzymatic Prep program, as described below, with the lid set to 70°C (heated lid required).

Step	Temperature * (°C)	Time
Hold	4	∞
Fragmentation	32	Variable (see note)
Inactivation	65	30 minutes
Hold	4	Less than 1 hour

* Lid temperature needs to be set to 70°C.

Important: Fragmented samples can be kept at 4°C for no longer than 1 hour.

Note: See your Certificate of Analysis (CoA) for fragmentation time recommendations for the individual lot number that you received. Reaction times may be optimized for individual samples. Specifically, for sample inputs <25 ng, longer fragmentation times may be required.

3. Begin the Enzymatic Prep program by chilling the thermal cycler to 4°C.
4. Prepare the Enzymatic Prep Master Mix by adding the components in the order shown:

Enzymatic Prep Master Mix	
Components	Volume per sample (µL)
• Buffer K1	3.0
• Reagent K2	1.5
• Enzyme K3	6.0
Total volume	**10.5**

5. Vortex the Enzymatic Prep Master Mix for 5 seconds, then briefly centrifuge. Keep mix on ice until ready to use.

   Important: Ensure that the Enzymatic Prep Master Mix is mixed thoroughly before and after the addition of DNA samples to prevent incomplete fragmentation.

6. Add 10.5 µL of the premixed Enzymatic Prep Master Mix to each tube containing DNA samples and enough Low EDTA TE to reach a final volume of 30 µL.
7. Thoroughly vortex the sample tubes for 5 seconds.
8. Briefly centrifuge the sample tubes, then immediately place in the chilled thermal cycler and advance the Enzymatic Prep program to the 32°C fragmentation step.
9. While the Enzymatic Prep program runs, prepare the Ligation Master Mix.

Adapter ligation

1. Before starting adapter ligation, preset a thermal cycler according to the program listed below with lid heating **OFF.**

Ligation program		
Step	Temperature* (°C)	Time
Ligation	20	20 minutes
Hold	4	Hold

* Lid temperature should be OFF.

2. For DNA input <25 ng, dilute adapters (Element Adapter Mix) as shown:

   Important: Adapter dilution is necessary to achieve low levels of adapter dimer. For certain applications, adapter dilution may be adjusted to achieve best results.

DNA input	Adapter*
≥25 ng	No dilution
10 ng	10-fold (1:10)
1 ng	20-fold (1:20)
100 pg	30-fold (1:30)

3. Prepare the Ligation Master Mix. Add components in the order shown.

Enzymatic Prep Master Mix	
Components	Volume per sample (µL)
• Buffer W1	12
• Enzyme W3	4
• Element Adapter Mix*	5
Low EDTA TE	9
Total Master Mix	**30**
Fragmented sample	30
Total volume	**60**

* If preparing the Ligation Master Mix ahead of time, add the adapter to the Master Mix just prior to use.

Important: Slowly pipette the viscous Buffer W1 to avoid bubbles and to ensure accuracy.

4. When the Enzymatic Prep program is complete, add 30 µL pre-mixed Ligation Master Mix to the tubes containing fragmented DNA.
5. Thoroughly mix samples by moderate vortexing for 5 seconds and briefly centrifuge.
6. Place samples in the pre-programmed thermal cycler and run the Ligation program from step 1 of this section.
7. After the Ligation program is complete, proceed immediately to Post-ligation cleanup.

Post-ligation cleanup

Important: Make sure magnetic purification beads are equilibrated to room temperature before starting this section.

1. Prepare fresh 80% ethanol solution.
2. Vortex the beads until the solution is homogenous.
3. Add 48 µL of beads to each sample at room temperature (ratio of bead to sample is 0.8).
4. Thoroughly mix samples by moderate vortexing for 5 seconds, then briefly centrifuge.
5. Incubate the samples for 5 minutes at room temperature.
6. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).
7. Remove and discard the supernatant without disturbing the pellet (less than 5 µL may be left behind).
8. Add 180 µL of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. Be careful to not disturb the pellet.
9. Incubate for 30 seconds at room temperature, then carefully remove the ethanol solution using a pipette.
10. Repeat steps 8 and 9 for a second ethanol wash.
11. Quick spin the samples in a microcentrifuge, then place them back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube with a clean pipette tip.
12. Remove the samples from the magnetic rack.
13. Add 20 µL Low EDTA TE to the sample tubes.
14. Incubate the samples at room temperature for 2 minutes.
15. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
16. Carefully transfer the clear solution into a clean tube, being careful to avoid any bead carryover.

Safe Stop: DNA libraries can be stored overnight at –20°C.

PCR amplification

1. Set up the thermal cycler with the PCR program as shown below, with a heated lid set to 105°C. Adjust the number of cycles based on input amount and workflow. Note that a >17 nM yield is needed.

PCR amplification program			
Step	Cycles	Temperature (°C)	Time
Initial denaturation	1	98	45 seconds
Denaturation	Varies (see table Cycling recommendation for PCR-amplified workflows)	98	15 seconds
Annealing		60	30 seconds
Extension		72	30 seconds
Final extension	1	72	1 minute
Hold	1	4	∞

* Lid should be heated to 105°C.

Cycling recommendations*	
DNA input	Minimum recommended cycles for >17 nM
1 µg	3**
100 ng	4–5
10 ng	7–8
1 ng	11–12
100 pg	13–14

* Optimization may be needed to determine correct cycling conditions for different sample types.

**When indexing by PCR, a minimum of 3 cycles is required to attach adapter sequences, irrespective of whether a sufficient library amount is available following ligation.

2. Add the following components to the eluted library sample:

Components	Volume per reaction (µL)
• PCR Master Mix	25
Element Index Pairs	5
Sample	20
Total volume	50

3. Gently vortex for 5 seconds and briefly centrifuge.
4. Place samples into pre-programmed thermal cycler and run the PCR Amplification program.
5. When the PCR program is complete, vortex the room temperature beads until the solution is homogenous.
6. Proceed to Post-PCR cleanup.

For the master list of index sequences, see Run Manifest Workflow Guide (MA-0001) on Element website.

Post-PCR cleanup

1. Add the specified bead volume to each sample as shown:

Average insert size (bp)	Sample volume (µL)	Bead volume (µL)
350	50	32.5 (ratio: 0.65)
200	50	90 (ratio: 1.8)

2. Vortex sample tubes, then briefly centrifuge.
3. Incubate the samples for 5 minutes at room temperature.
4. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).
5. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet (less than 5 µL may be left behind).
6. Add 180 µL of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. Be careful to not disturb the pellet.
7. Incubate for 30 seconds at room temperature, then carefully remove the ethanol solution.
8. Repeat steps 4 and 5 for a second ethanol wash.
9. Quick spin the samples in a microcentrifuge, then place them back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube with a clean pipette tip.
10. Add 21 µL of Low EDTA TE to the sample tubes and mix well until homogenous.
11. Incubate sample tubes at room temperature for 2 minutes.
12. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).
13. Carefully transfer 20 µL clear solution (eluted DNA) into a clean tube, being careful to avoid any bead carryover.

    Safe Stop: Libraries can be stored overnight at –20°C.

14. The library is now ready for quantification, which can be performed using fluorometric methods (e.g., Qubit Fluorometer).

Elevate library circularization protocol for AVITI sequencing

For the remainder of the protocol, reagents from the Element Elevate Library Circularization Kit are required. The circularization protocol allows library inputs of 0.5 pmol in 30 µL (equivalent to 16.67 nM in 30 µL). Input amounts as low as 0.2 pmol can be used if needed; however, this may result in material loss. After quantification of linear libraries, normalize libraries to ≥16.67 nM and pool based on the sequencing throughput requirements needed for each sample. Up to 96 uniquely indexed libraries can be pooled for a single circularization reaction (do not pool libraries with same index pair). If the final pool contains >30 µL volume, a speed vac or bead-based method can be used to concentrate library to 30 µL. If the final pool contains <30 µL, add Elution Buffer to bring to 30 µL volume.

Note: For more information or troubleshooting, visit the Element website.

Anneal splint oligo

1. Ensure all reagents are thawed completely and remain on ice during the remainder of the protocol.
2. Briefly vortex Elevate Annealing Mix reagent and briefly centrifuge.
3. Set up the thermal cycler with the Elevate Annealing program, as described below, with the lid set to 105°C.

Step	Temperature * (°C)	Time
1	95	5 minutes
2	37	5 minutes
Hold	37	Hold

* Lid temperature needs to be set to 105°C.

4. Prepare the Annealing Mix by adding the components in the order shown to each well (containing library) of a 96-well plate:

Annealing Mix	
Components	Volume per sample (µL)
• Pooled linear library (at 16.67 nM)	30
• Elevate Annealing Mix	14
Total volume	44

5. Set a pipette to 33 µL and mix each reaction by pipetting 10x.
6. Seal the plate and briefly centrifuge.
7. Place samples into pre-programmed thermal cycler and run the Elevate Annealing program.
8. Once completed, remove the plate from the thermal cycler, centrifuge briefly and immediately proceed to the next step.

Circularize library

1. Gently flick Ligation Enzyme 1 to mix, then briefly centrifuge and store on ice.
2. Vortex Ligation Buffer to mix and briefly centrifuge.
3. Set up the thermal cycler with the Elevate Circularization program, as described below, with the lid set to 75°C.

Step	Temperature * (°C)	Time
1	37	10 minutes
2	65	10 minutes
Hold	4	Hold

* Lid temperature needs to be set to 75°C.

4. Prepare the Circularization Master Mix by adding the components in the order shown.

Circularization Master Mix	
Components	Volume per sample (µL)
• Ligation Buffer	5
• Ligation Enzyme 1	1
Total volume	6

5. Set pipette to 75% of Circularization Master Mix volume and mix by pipetting 10x while stored on ice.
6. Add 6 µL of Circularization Master Mix to each reaction, set pipette to 38 µL and mix each reaction 10x by pipetting.
7. Seal the plate and briefly centrifuge.
8. Place samples into pre-programmed thermal cycler and run the Elevate Circularization Program.
9. Once completed, remove the plate from the thermal cycler, centrifuge briefly, and immediately proceed to the next step.

Digest Linear DNA

1. Gently flick Digestion Enzyme 1 and Digestion Enzyme 2 to mix, briefly centrifuge, and store on ice.
2. Set up the thermal cycler with the Elevate Digestion program, as described below, with the lid set to 105°C.

Step	Temperature * (°C)	Time
1	37	10 minutes
2	80	2 minutes
Hold	4	Hold

* Lid temperature needs to be set to 105°C.

3. Prepare the Digestion Master Mix by adding the components in the order shown.

Digestion Master Mix	
Components	Volume per sample (µL)
• Digestion Enzyme 1	1
• Digestion Enzyme 2	1
Total volume	2

4. Set pipette to 75% of the Digestion Master Mix volume and mix by pipetting 10x while stored on ice.

5. Add 2 µL of Digestion Master Mix to each reaction, set pipet to 39 µL and mix each reaction by pipetting 10x.
6. Seal the plate and briefly centrifuge.
7. Place samples into pre-programmed thermal cycler and run the Elevate Digest Program.
8. Once completed, remove the plate from the thermal cycler, centrifuge briefly, and immediately proceed to the next step.

Cleanup

1. Prepare fresh 80% ethanol solution.
2. Transfer each reaction (52 µL) to a new deep-well plate or larger tube.
3. Vortex the room-temperatures beads until the solution is homogenous.
4. Add 104 µL of beads to each sample at room temperature (ratio of bead to sample is 2:1).
5. Mix samples vigorously (recommend 2 minutes of vortexing at 1500–1800 rpm for plates or pipetting 120 µL vigorously 10x times for tubes).
6. Incubate the samples for 5 minutes at room temperature.
7. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~3–5 minutes).
8. Remove and discard the supernatant using a clean pipette tip, without disturbing the pellet.
9. Add 200 µL of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. Be careful to not disturb the pellet.
10. Incubate for 30 seconds at room temperature, then carefully remove the ethanol solution.
11. Repeat steps 9 and 10 for a second ethanol wash.
12. Using a 10 µL or 20 µL pipette, remove residual ethanol.
13. Air-dry the beads unsealed or uncapped for 3–5 minutes until the pellet loses shines. Do not over dry beads.
14. Once dry, remove the samples from the magnet.
15. Add 32 µL of Elution Buffer to the sample tubes and mix well until homogenous (recommend vortexing plates at 1500–1800 rpm for 2 minutes, or for tubes set a pipette to 24 µL and mix by pipetting >10x until fully resuspended).
16. Incubate sample tubes at room temperature for 2 minutes.
17. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).
18. Carefully transfer 30 µL clear solution (eluted circularized library) into a clean tube, being careful to avoid any bead carryover.

    Safe Stop: Libraries can be stored at –20°C for ≤15 days.

19. The circularized library is now ready for quantification, which must be performed using qPCR with the **Element** provided standards and primers.

Example of data output

Example libraries were prepared using 100 ng of NA12878 DNA (Coriell Institute) using the xGen DNA Library Prep Kit EZ and the Element Elevate Library Prep Workflow. The sample was enzymatically fragmented to ~350 bp, and an additional ~135 bp of sequence was added to each library fragment via the ligation and indexing steps for an average expected fragment size of ~485 bp (post-ligation and indexing, prior to Adept circularization). A TapeStation High-Sensitivity DNA trace (Agilent) of an example library shows a final linear library fragment size peak at ~495 bp (Figure 2). Sequencing quality metrics for 24 libraries sequenced using the Element AVITI System after completing the combined xGen DNA Library Prep Kit EZ and Elevate Library Prep Workflow (2 x 150 paired-end reads) are shown in Table 1.

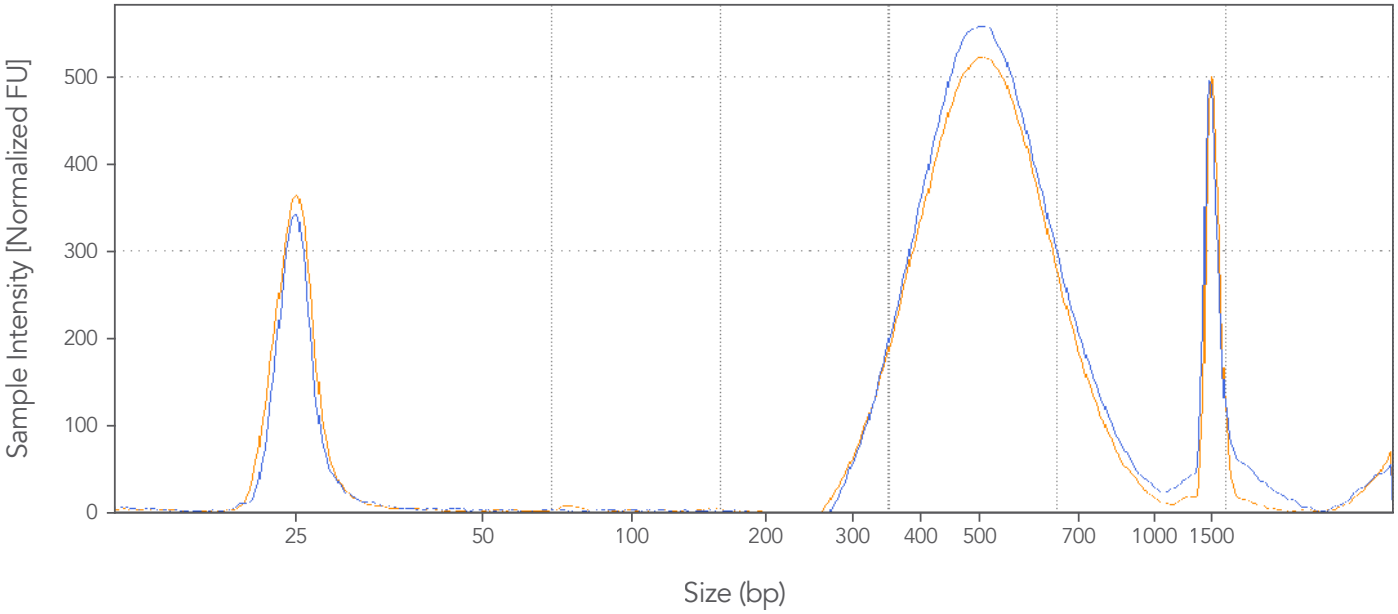

Figure 2. Representative Tapestation traces for 350 bp DNA fragments subjected to library preparation. The resulting library showed a peak fragment size of ~495bp; this is the expected size after adding the adapters and index pairs (~135 bp). The library generated using the xGen DNA Library Prep Kit EZ with the Elevate Index and Adapter Kit (blue) results in a similar profile to the library generated using the complete xGen DNA Library Prep Kit EZ with xGen UDI index primers (orange).

Table 1. Sequencing metrics obtained on the Element AVITI System.

Polonies	Q30 (%)	Index Assignment (%)	Total Yield (Gb)
631,472,939	97.4	96.4	182.698

Table 1. shows the sequencing metrics obtained on the Element AVITI System. The sequencing run included 24 libraries generated using the xGen DNA Library Prep Kit EZ with Element Elevate adapters and indexing primers.

xGen DNA Library Prep Kit EZ and Elevate Workflow for AVITI Sequencing

For more information, go to www.idtdna.com/ContactUs