xGen™ DNA Library Prep EZ Kit
xGen DNA Library Prep EZ UNI Kit

REVISION HISTORY

Version	Release date	Description of changes
3	June 2024	Correction to consumables information for UDI primer plates and update to PCR cleanup information
2	July 2022	Adjusted product size offerings and added "Equipment" table
1	December 2021	Initial release

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INTRODUCTION

The xGen DNA Library Prep EZ Kits are designed to produce next-generation sequencing (NGS) libraries from a broad range of double-stranded DNA inputs (100 pg to 1 µg). This protocol describes the workflow for whole genome sequencing (WGS) and xGen Hybridization Capture for targeted sequencing. It also includes enzymatic fragmentation to streamline the workflow for high-throughput research applications and automation.

- The xGen DNA Library Prep EZ Kit has an indexing by PCR workflow and includes the xGen Stubby Y Adapter (xGen Indexing Primers supplied separately).
- The xGen DNA Library Prep EZ UNI Kit has an indexing by ligation workflow and is compatible with xGen full-length, indexed Y adapters (known as xGen UDI-UMI Adapters, supplied separately) and supports PCR-free sequencing with an optional PCR workflow.

Both kits produce libraries of equivalent complexity and quality; the only difference is the indexing workflow option. Also, both kits include the PCR amplification reagents.

The xGen DNA Library Prep EZ Kits support the following research applications:

- Whole genome sequencing (WGS)
- Hybridization capture of targeted genomic regions (e.g., exome)
- Metagenomic sequencing
- PCR-free sequencing
- Detection of germline inherited SNVs and indels
- Low-frequency somatic variation detection of SNVs and indels
- Copy number variation detection

OVERVIEW

The xGen DNA Library Prep EZ Kits streamline NGS sample preparation of dsDNA for sequencing on Illumina® platforms. The kits provide rapid DNA fragmentation and library construction to generate libraries for sequencing (Figure 1). Detailed instructions are provided for obtaining a mean aligned insert of 350bp or 200bp for direct or targeted sequencing. A protocol amendment is available for larger insert sizes (up to 550 bp) to guide you in using the xGen Deceleration Module.

Note: Both the xGen DNA Library Prep EZ and xGen DNA Library Prep Kit EZ UNI Kits are compatible with xGen Normalase™ workflow (see Appendix A: Perform the xGen Normalase Module for instructions).

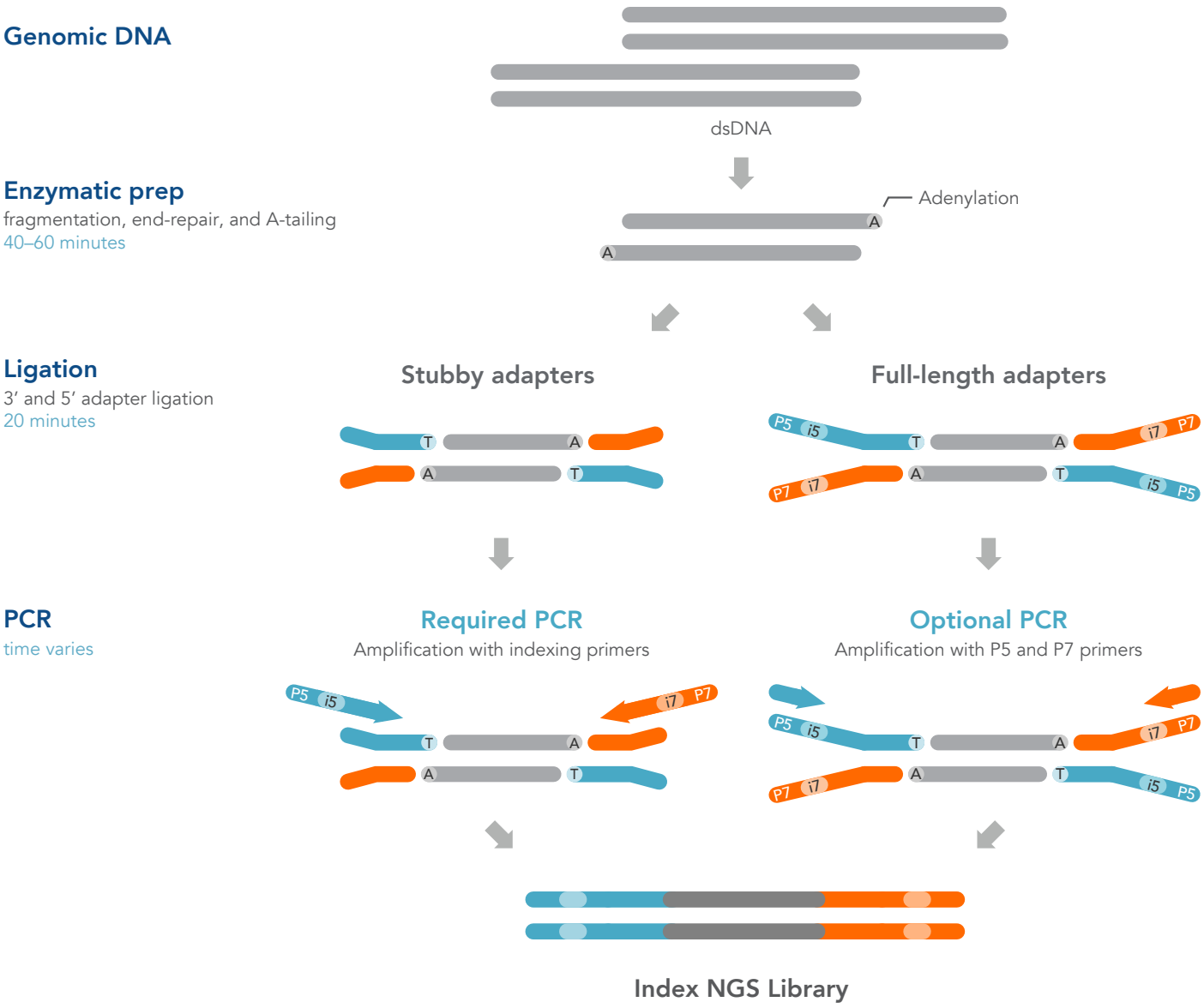

Figure 1.The xGen DNA Library Prep EZ and EZ UNI library preparation steps.

WORKFLOW

This workflow contains minimal enzymatic incubations and bead-based cleanups, thereby reducing the sample handling and overall library preparation time to under 2 hours before library amplification. There are three major activities outlined in this protocol:

- **Enzymatic preparation.** Performs fragmentation, end-repair, and dA-tailing of dsDNA in a single reaction. The final fragmentation profile is dependent on both incubation temperature and time.
- **Adapter ligation.** Performs ligation of either full-length indexed or stubby Y adapters. When using full-length indexed adapters, the final PCR step is optional and can be used to increase library yields. Ligation with the Stubby Y Adapter requires amplification with indexing primers to incorporate sample indexing sequences and to add the flow cell attachment sequences, P5 and P7, for Illumina® sequencing.
- **PCR amplification.** Amplify libraries based on the adapter and DNA input used.

1	Prepare reagents	• Thaw reagents on ice • Prepare fresh 80% ethanol	Total time: Varies (~20 min)
2	Perform enzymatic preparation	• Set up dsDNA fragmentation, end-repair, and A-tailing • Run Enzymatic Prep program	Total time: 40–60 min
3	Perform ligation	• Add full-length or stubby adapters	Total time: 20 min
4	Clean up ligation reaction	• Purify ligation product	Total time: 20 min
	Safe stopping point (store @ –20°C)		
5	Perform PCR & cleanup*	• Add index sequences (for stubby adapters only) • Increase available library for sequencing	Total time: PCR, 10–30 min Cleanup, 20 min
	Safe stopping point (store @ –20°C)		
6	Perform purification†	• For size selection (PCR-free)	Total time: 20 min

PCR-free
library construction workflow steps, 1–4 & 6

1. Prepare reagents
2. Perform enzymatic preparation
3. Perform ligation
4. Clean up ligation reaction
6. Perform purification†

PCR-amplified
library construction workflow steps, 1–5

1. Prepare reagents
2. Perform enzymatic preparation
3. Perform ligation
4. Clean up ligation reaction
5. Perform PCR & cleanup*

* Use of stubby adapter requires PCR to add index sequences to the library.
† Required for PCR-free.

CONSUMABLES AND EQUIPMENT

Kit contents—P/N 10009863, 10009821, 10009864, 10009822

Kits contain sufficient reagents for the preparation of 16, 96, or 384 libraries (10% excess volume provided).

	Components	16 rxn	96 rxn	Storage
End prep	• Buffer K1	53 µL	317 µL	–20°C
	• Reagent K2	80 µL	476 µL	
	• Enzyme K3	106 µL	634 µL	
Adapter ligation	○ Buffer W1	201 µL	1218 µL	
	○ Enzyme W3	67 µL	424 µL	
	○ Reagent W5*	87 µL	528 µL	
PCR amplification	• PCR Master Mix	436 µL	2640 µL	
	• Reagent R1**	87 µL	528 µL	
Additional reagents	Low EDTA TE	20 mL	20 mL	Room temperature

* Reagent W5 is the Stubby Y adapter and is only supplied with xGen DNA Library Prep EZ (Cat. Nos.; 10009863, 10009821).

**Reagent R1 is the amplification primers and is only supplied with xGen DNA Library Prep Kit EZ UNI (Cat Nos.; 10009864, 10009822).

Consumables—IDT

Workflow component	Product name	Index number	Reaction size	Catalog number
xGen core reagents	xGen DNA Library Prep EZ Kit	N/A	16 rxn	10009863
	xGen DNA Library Prep EZ Kit	N/A	96 rxn	10009821
	xGen DNA Library Prep EZ UNI Kit	N/A	16 rxn	10009864
	xGen DNA Library Prep EZ UNI Kit	N/A	96 rxn	10009822
xGen Normalase Module (optional)	xGen Normalase™ Module	N/A	96 rxn	10009793
xGen Deceleration Module (optional)	xGen Deceleration Module	N/A	96 rxn	10009823
xGen CDI Primers*	xGen CDI Primers	S701-S796/ D501-D508	96 rxn	10009815
xGen Normalase CDI primers*	xGen Normalase CDI Primers	D701-D712/ D501-D508	96 rxn	10009794
xGen Normalase UDI primer plates*	xGen Normalase UDI Primer Plate 1	SU001-SU096	96 rxn	10009796
	xGen Normalase UDI Primer Plate 2	SU097-SU192	96 rxn	10009797
	xGen Normalase UDI Primer Plate 3	SU193-SU288	96 rxn	10009798
	xGen Normalase UDI Primer Plate 4	SU289-SU384	96 rxn	10009799
	xGen Normalase UDI Primer Set 1	SU001-SU384	4 x 96 rxn	10009795
	xGen Normalase UDI Primer Set 2	SU385-SU768	4 x 96 rxn	10009800
	xGen Normalase UDI Primer Set 3	SU769-SU1152	4 x 96 rxn	10009811
	xGen Normalase UDI Primer Set 4	SU1153-SU1536	4 x 96 rxn	10009812

*For index sequences, see the Index Sequence Master List. For adapter sequences, see Appendix C. For custom indexing options, contact us.

Note: xGen Normalase CDI and UDI primers are compatible with Normalase and non-Normalase workflows.

Consumables—Other suppliers

Item	Supplier	Catalog number
Absolute ethanol (200 proof)	Various suppliers	Varies
Purification beads		
SPRIselect™ purification beads, or equivalent	Beckman Coulter	B23317/B23318/B2331
Agencourt® 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
KAPA Biosystems® Library Quantification Kit – Illumina® Universal, or equivalent	Roche	KK4824
Nuclease-Free Water	Various suppliers	Varies
PCR tubes, 0.2 mL		
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
qPCR system	Various Suppliers	Varies
Magnet options (choose one):		
Magnetic Separator Plate	Permagen	MSP750
Magnetic PCR Strip Magnetic Separator Rack	Permagen	MSR812
Microcentrifuge	Various Suppliers	Varies
Vortex		
Thermal Cycler		

Reagent handling

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

Note: The enzymes provided in this kit 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.

Avoid cross-contamination

Important: To reduce the risk of DNA and library contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed. We recommend taking these steps to avoid cross-contamination:

- Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
- Use barrier pipette tips to avoid exposure to potential contaminants.
- Always change pipette tips between each sample.

Size selection during cleanup steps

This protocol has been optimized with SPRISelect® beads (Beckman Coulter) but can also be used with Agencourt® AMPure® XP beads (Beckman Coulter) or equivalent bead-based nucleotide purification products. If other beads are used, solutions and conditions for DNA binding may differ. Consider these points before performing dsDNA size selection:

- Post-enzymatic fragmentation, analyze the size distribution by electrophoretic methods to determine the fragment size of your dsDNA samples.
- Left side size selection is recommended for this protocol.
- To customize size selection, use Beckman Coulter's SPRISelect User Guide for conditions not discussed here.

DNA input considerations

This kit works with a broad range of DNA inputs, ranging from 100 pg–1 µg, however, for PCR-free sequencing, use a minimum of 100 ng of DNA input with full-length indexed adapters. Our kit can use:

- **High Quality gDNA:** Quantify with Qubit or similar fluorometric method.
- **Formalin-fixed paraffin-embedded (FFPE) DNA:** Quantify using xGen Input DNA Quantification Primers (e.g., Human Alu primers). See the Input DNA Quantification Assay for more details.
- **Amplicons:** Quantify with Qubit or similar fluorometric method.

For high quality samples, dsDNA concentration can be determined using Qubit (Thermo Fisher Scientific), or a similar fluorometric method, to measure the size and concentration of your DNA samples.

To quantify the concentration of low-quality human DNA samples, qPCR can be performed using the xGen Input DNA Quantification Primers (Cat. No. 10009856). Our primers will help you accurately assess the usable amount of genomic DNA in the samples and its integrity. The xGen Input DNA Quantification Primers are for research use only (RUO).

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.

Tip: To find the CoA, enter the lot number of your kit (found on the bottom of the packaging) at idtdna.com/COA. Alternatively, read the CoA provided with your kit on the label inside the product box for lot-specific fragmentation parameters.

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).

Additionally, the xGen Deceleration Module can be used with this kit to achieve larger aligned insert sizes of 550 bp if needed.

EDTA in elution buffers

The enzymatic preparation reaction is sensitive to high concentrations of EDTA, which is usually introduced by elution buffers in the final steps of the DNA extraction or purification process. A high concentration of EDTA, such as 1 mM in standard TE buffer, will slow the reaction, resulting in larger insert sizes. Alternatively, no EDTA (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) and requires 1.5 µL of Reagent K2 for fragmentation.

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® from Beckman Coulter is recommended for minimum loss of sample gDNA). 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 or water (e.g., Buffer EB from Qiagen, 10 mM Tris-HCl, pH 8.5) without EDTA, Reagent K2 is not needed during Enzymatic Prep.

Automation

This protocol is amenable to automation. A 10% overage volume of reagents is supplied in the xGen DNA Library EZ kits to accommodate automation. IDT does not supply automated liquid handling instruments or consumables but collaborates with automation solution providers and customers to develop optimized scripts for use of our kits with liquid handling platforms. Contact your instrument vendor or contact us if you plan to use this kit with your automated liquid handling system.

Note: The IDT xGen Deceleration Module can be used to enable room temperature reaction setup and fragmentation times suitable for high-throughput use.

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 reaction (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 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 (e.g., Reagent W5 for xGen DNA Library Prep EZ, or full-length Indexed Y Adapters for xGen DNA Library Prep EZ UNI), 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)

* For xGen DNA Library Prep EZ UNI, non-diluted full-length indexed Y adapters are used at a 15 µM stock concentration.

3. Prepare the Ligation Master Mix by referring to the tables below when using either a Stubby Y adapter (left table, Reagent W5) or full-length indexed Y adapter (right table). Add components in the order shown.

xGen DNA Library Prep Kit EZ Master Mix	
Components	Volume per reaction (µL)
○ Buffer W1	12
○ Enzyme W3	4
○ Reagent W5 (stubby adapter)*	5
Low EDTA TE	9
Total Master Mix	**30**
Fragmented sample	30
Total volume	**60**

xGen DNA Library Prep EZ UNI Master Mix	
Components	Volume per reaction (µL)
○ Buffer W1	12
○ Enzyme W3	4
Low EDTA TE	9
Total Master Mix	**25**
Fragmented sample	30
Full-length Indexed Y Adapter**	5 (Unique Y Adapter added to each sample)
Total volume	**60**

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

**Full-length indexed adapters should be added to each sample to uniquely index each library.

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

4. When the Enzymatic Prep program is complete, add pre-mixed Ligation Master Mix to the tubes containing fragmented DNA:
   - 30 µL for xGen DNA Library Prep EZ
   - 25 µL for xGen DNA Library Prep EZ UNI
5. For xGen DNA Library Prep EZ UNI samples ONLY, add the full-length Indexed Y Adapters individually to each sample.
6. Thoroughly mix samples by moderate vortexing for 5 seconds and briefly centrifuge.
7. Place samples in the pre-programmed thermal cycler and run the Ligation program from step 1 of this section..
8. After the ligation program is complete, proceed immediately to Post-ligation cleanup.

Post-ligation cleanup

Important: Make sure magnetic 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, 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 Low EDTA TE to the sample tubes based on your application as shown:

Application	Elution volume (µL)
Direct sequencing	20
PCR-free direct sequencing (>100 ng input)	50
Hybridization capture	20

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.

If using Stubby Y Adapters (xGen DNA Library Prep EZ) for indexing PCR, or if performing an optional amplification step for libraries with full-length Indexed Y Adapters (xGen DNA Library Prep EZ UNI), proceed to PCR amplification.

Size selection for direct sequencing of PCR-free xGen DNA Library Prep Kit EZ UNI libraries (optional)

For PCR-free libraries created with the xGen DNA Library Prep Kit EZ UNI product, size selection can be done using the following purification.

1. Vortex beads until the solution is homogenous.
2. To the 50 µL eluate from the post-ligation cleanup, add 32.5 µL of beads (ratio of bead to sample is 0.65).
3. Vortex each sample to mix, then briefly centrifuge.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).
6. Remove and discard the supernatant using a clean pipette tip. Avoid disturbing the pellet (less than 5 µL may be left behind).
7. Add 180 µL of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. Be careful to not to disturb the pellet.
8. Incubate the sample tubes for 30 seconds, then carefully remove the ethanol solution.
9. Repeat steps 7 and 8 for a second ethanol wash.
10. Quick spin the samples in a microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube with a clean pipette tip.
11. Remove the samples from the magnetic rack.
12. Add 21 µL of Low EDTA TE to the sample tubes and mix well by pipetting until homogenous.
13. Incubate samples at room temperature for 2 minutes.
14. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
15. Carefully transfer 20 µL of supernatant containing eluted DNA into a clean tube, being carful to avoid any bead carryover.
16. Store freshly prepared libraries at –20°C.

Note: PCR-free libraries cannot accurately be quantified by fluorometric methods or assessed for library size by electrophoretic methods.

PCR amplification

Note: If you plan to use the xGen Normalase Module, see Appendix A: xGen Normalase Module PCR amplification instructions for specific instructions on the recommended number of PCR cycles and cycling conditions specific for Normalase PCR.

Use the IDT PCR Master Mix supplied in the xGen DNA Library Prep EZ Kits for both direct sequencing and pre-hybridization capture PCR workflows.

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.

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 for PCR-amplified workflows				
DNA input	Minimum recommended cycles for >4 nM* with 350 bp insert		Minimum recommended cycles for >500 ng* with 200 bp insert	
	xGen DNA Library Prep EZ	xGen DNA Library Prep EZ UNI	xGen DNA Library Prep EZ	xGen DNA Library Prep EZ UNI
1 µg	3**	0–3	3**	1–2
100 ng	3**	0–3	5	5
10 ng	6–7	6–7	9	9
1 ng	9–10	9–10	12	12
100 pg	11–12	11–12	15	15

* Additional PCR cycles are recommended to generate >12 nM for Normalase treatment (Refer to Appendix A).

**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. Prepare the PCR Master Mix by following the directions in **2a** when using the xGen DNA Library Prep Kit EZ (indexing primers) or step **2b** when using the xGen DNA Library Prep Kit EZ UNI (P5 and P7 primers).

   a. For Indexing PCR, add the indexing primers directly to the eluted library following these guidelines. Add 25 µL PCR Master Mix to each sample. Mix by moderate vortexing for 5 seconds and briefly centrifuge.

Indexing options	Components	Volume per reaction (µL)
xGen Unique Dual Indexing (UDI)	Pre-mixed primer pair	5
xGen Normalase Unique Dual Indexing (N-UDIs)	Pre-mixed primer pair	4
	Reagent R7	1
xGen Combinatorial Dual Indexing (CDI)	i5 primer	2.5
	i7 primer	2.5
xGen Normalase Combinatorial Dual Indexing (N-CDI)	Pre-mixed primer pair	4
	Reagent R6	1

xGen DNA Library Prep EZ Master Mix			
xGen UDIs or CDIs		xGen Normalase UDIs or CDIs	
Components	Volume per reaction (µL)	Components	Volume per reaction (µL)
• PCR Master Mix	25	• PCR Master Mix + Reagent R6 or R7	26
Sample + primer mix	25	Sample + primer mix	24
Total volume	50	Total volume	50

   b. For optional amplification of fully indexed libraries, prepare PCR Master Mix by following these guidelines. Add 30 µL of the prepared PCR Master Mix to the eluted samples, then mix by moderate vortexing for 5 seconds and briefly centrifuge.

xGen DNA Library Prep EZ UNI Master Mix	
Components	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R1	5
Total Master Mix volume	30
Eluted sample	20
Total volume	50

3. Place samples into preprogrammed thermal cycler and run the PCR Amplification Program.
4. When the PCR program is complete, vortex the room temperature SPRIselect beads until the solution is homogenous.
5. Proceed to Post-PCR cleanup.

PCR cleanup

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

Application	Average insert size (bp)	Sample volume (µL)	Bead volume (µL)
Direct sequencing	350	50	32.5 (ratio 0.65)*
Hybridization capture	200	50	90 (ratio: 1.8)

* Although 350 bp insert size is suggested for direct sequencing and 200 bp for hybridization capture, both insert sizes are compatible with either application, depending on the desired read length and sample processing.

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, 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 is 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 (i.e., Qubit™ Fluorometer) or qPCR.

    Note: If direct sequencing on patterned flow cells, proceed to Second post-PCR purification to remove excess primers, which can increase index hopping on patterned flow cells. This second purification is not necessary if you are using xGen Normalase, xGen Hybridization capture, or xGen Unique Dual Indexing Primers and Adapters.

Second post-PCR purification (optional)

Complete this section to remove excess primers from PCR-amplified libraries that are to be directly sequenced on a patterned flow cell.

1. Vortex beads until the solution is homogenous.
2. To the 20 µL sample from the Post-PCR Cleanup section, add 24 µL of beads (ratio of bead to sample is 1.2).
3. Vortex each sample to mix, then briefly centrifuge.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the samples on a magnetic rack until the solution clears and a pellet has formed (~2 minutes).

6. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet (less than 5 µL may be left behind).
7. 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.
8. Incubate the sample tubes for 30 seconds, then carefully remove the ethanol solution.
9. Repeat steps 7 and 8 for a second ethanol wash.
10. Quick spin the samples in a microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube with a clean pipette tip.
11. Remove the samples from the magnetic rack.
12. Add 21 µL of Low EDTA TE to the sample tubes and mix well by pipetting, until homogenous.
13. Incubate samples at room temperature for 2 minutes.
14. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
15. Carefully transfer 20 µL of supernatant containing eluted DNA into a clean tube, being careful to avoid any bead carryover.
16. Store freshly prepared libraries at –20°C.

Example of data output

Example libraries were prepared from NA12878 Coriell DNA (Coriell Institute) using the xGen DNA Library Prep Kit EZ. The sample was fragmented enzymatically to create 350 bp inserts. The prepared inserts were ligated to xGen Stubby Y adapters that add ~135 bp of sequence onto each ~350 bp library fragment. The final expected size of the library fragments is ~560 bp. In Figure 2, Agilent High-Sensitivity DNA traces show fragmented DNA (red line) prior to adapter ligation, and final library fragments (blue line).

Representative library traces: 350 bp fragmentation

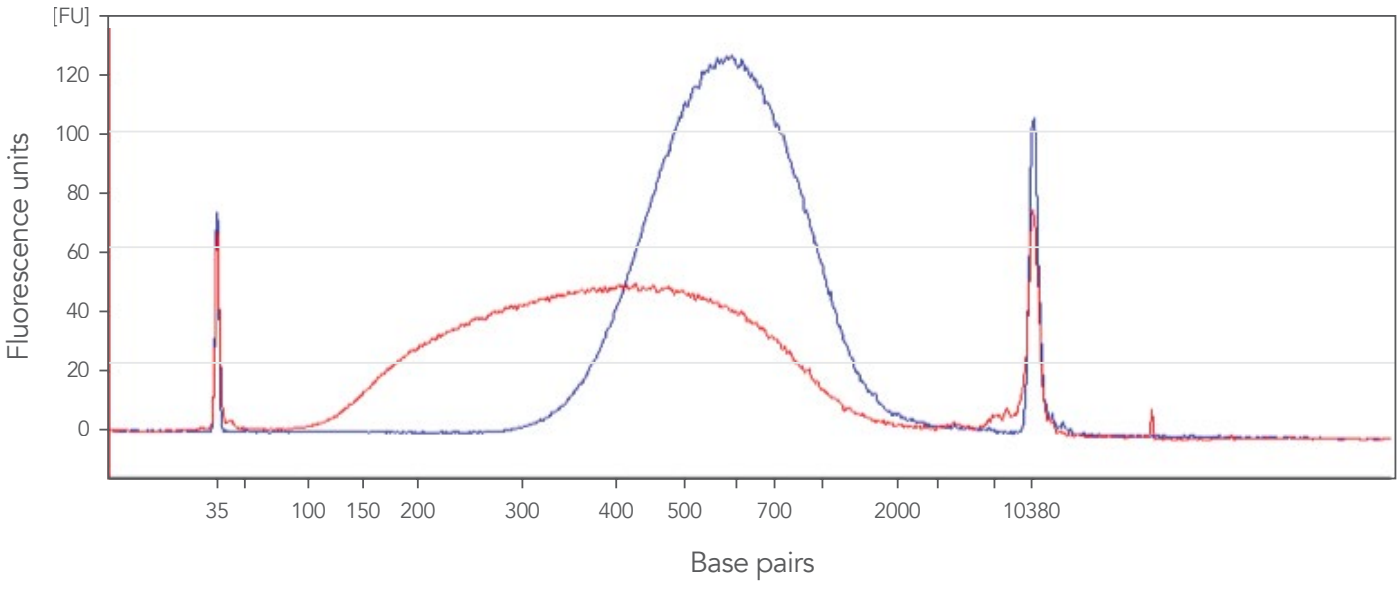

Figure 2. Representative Bioanalyzer traces for 350 bp DNA fragments (red) subjected to library preparation. The resulting library (blue) showed a peak height of ~560 bp; this is the expected library size after adding the adapters (~135 bp).

APPENDIX A: xGEN NORMALASE™ MODULE PCR AMPLIFICATION INSTRUCTIONS

Tip: Review this section and the **xGen Normalase Module protocol** before setting up your PCR. To achieve expected data output, amplify each library using Normalase primers with the appropriate number of cycles and thermocycling conditions as shown below, to obtain a library yield of 12 nM, or greater, in a 20 µL eluate.

Since the xGen Normalase Module is compatible with xGen DNA Library Prep EZ and xGen DNA Library Prep EZ UNI, there are slightly different conditions needed for the two options. Please ensure that you follow the correct section below.

xGen DNA Library Prep EZ Kit—with Normalase indexing primers

Normalase indexing primers complete the adapter sequences, amplify, and condition libraries for downstream Normalase steps. Assemble master mix using PCR reagents as shown.

1. To every 20 µL sample, add 2 µL each of Normalase Combinatorial Dual Index (CDI) primer or 4 µL of Normalase Unique Dual Index Primer Pair for a total volume of 24 µL.
2. Assemble the PCR Master Mix on ice. Thoroughly mix, then pulse spin tubes in a microcentrifuge to collect contents. Add 26 µL of the mix to each sample tube, then mix thoroughly and pulse spin to collect contents (50 µL total reaction volume) before placing in the thermal cycler.

xGen Normalase UDI PCR Master Mix	
Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R7	1
Total Master Mix volume	**26**
Eluted sample	20
xGen Normalase UDI primer pairs	4
Total volume	**50**

xGen Normalase CDI PCR Master Mix	
Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R6	1
Total Master Mix volume	**26**
Eluted sample	20
xGen Normalase CDI primers (i5 and i7) (D701–D712/ D501–D508)	2 (each)
Total volume	**50**

3. Run the following thermal cycler program, adjusting the number of cycles depending on the input amount and sample quality. Set thermal cycler lid temperature to 105°C.

Normalase PCR Amplification Program			
Step	Cycles	Temperature (°C)	Time
Initial denaturation	1	98	45 seconds
Denaturation	Varies (see table "Cycling recommendation for Normalase PCR workflows"	98	15 seconds
Annealing		60	30 seconds
Extension		72	30 seconds
Final extension	1	72	5 minute
Hold	1	4	∞

* Lid should be heated to 105°C

Cycling recommendations for Normalase Module use	
DNA input (ng)	Minimum number of cycles for ≥12 nM of 350 bp insert
≥100	3
25	5
10	7
1	10
0.1	13

4. Go to Post-PCR cleanup and follow the cleanup steps.
5. Proceed to Normalase I, Pooling, and Normalase II in the xGen Normalase Module protocol.

xGen DNA Library Prep EZ UNI Kit—with Reagent R5 (Universal Normalase Primers)

Notes:

- If you typically obtain the required minimum threshold (i.e., ≥12 nM following library amplification), use Normalase primers (Reagent R5) and add one additional PCR cycle to your program.
- If library yields are <12 nM prior to PCR, a minimum of 3 cycles is required to condition the libraries for downstream Normalase enzymology.

1. Assemble the PCR Master Mix using PCR reagents as shown in the table below, except substitute standard primers (Reagent R1) with 5 µL of Reagent R5. Thoroughly mix by moderate vortexing, then pulse spin to collect contents and place in the thermal cycler.

Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R5	5
Total Master Mix volume	30
Eluted sample	20
Total volume	50

2. Repeat steps 3–5 of the xGen Normalase Module PCR amplification instructions for the xGen DNA Library Prep EZ protocol above.

APPENDIX B: RECOMMENDED PCR CYCLES FOR HYBRIDIZATION CAPTURE

The recommendations below are based on using 200 bp of fragmented, Qubit-quantified, high-quality NA12878 Coriell DNA with the xGen DNA Library Prep EZ Kit. After Stubby Y adapter ligation, xGen Normalase PCR was performed with xGen Normalase Indexing primers.

Note: If you are using reduced quality DNA samples, additional PCR cycles may be necessary.

DNA input into xGen DNA Library Prep EZ and xGen DNA Library Prep EZ UNI (ng)	Minimum recommended PCR cycles to reach >500 ng yield with 200 bp insert
100	8
25	10
10	11
1	14

APPENDIX C: INDEXED ADAPTER SEQUENCES

The full-length adapter sequences are listed below. The underlined text indicates the location of the index sequences, which are 8 bp for CDI, and 8 or 10 bp for UDI. These sequences represent the adapter sequences following completion of the indexing PCR step.

Index 1 (i7) Adapters

5′ – GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX(XX)ATCTCGTATGCCGTCTTCTGCTTG – 3′

Index 2 (i5) Adapters

5′ – AATGATACGGCGACCACCGAGATCTACACYYYYYYYY(YY)ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3′

For the master list of sequences, see Index Sequences Master List found on the xGen Indexing page. The sequences will help in preparing your Illumina® sequencing sample sheet on the instrument of your choice.

APPENDIX D: TROUBLESHOOTING

Issue	Possible cause	Suggested solution
Library migrates unexpectedly on Bioanalyzer.	Over-amplification of library leads to the formation of heteroduplex structures that migrate slowly.	• Quantify library by qPCR, as other quantification methods will not accurately quantify heteroduplex library molecules. • Perform a lower number of PCR cycles than necessary to avoid over-amplification.
	Broad library size distribution for 350 bp fragmentation profile	• If using the Agilent High Sensitivity DNA system, traces for a library insert of 350 bp fragmentation run larger than expected at a ~560 bp node. This is due to the broad size distribution of the library; however, smaller inserts will preferentially cluster. When sequenced, a mean aligned insert size of 350 bp will be obtained.
	Migration behavior overestimates library size of PCR-free libraries due to partially single-stranded adapters.	• 200 bp insert, PCR-free libraries should migrate to a ~500 bp peak on a High Sensitivity Chip. • 350 bp insert, PCR-free libraries should migrate to a ~800 bp peak on a High Sensitivity Chip.
DNA is under fragmented (larger than expected molecular weight).	Input DNA was in a buffer with greater than 0.1 mM EDTA.	• Use a buffer exchange column or bead-based clean-up before fragmentation or use up to 3x volume of Reagent K2 in your fragmentation reaction.
	Improper mixing of reagents.	• Ensure fragmentation mixture is adequately mixed prior to and after adding to DNA samples.
DNA is over fragmented (smaller than expected molecular weight).	Reaction left at room temperature.	• Ensure the Enzymatic Prep master mix and the DNA sample are kept on ice until placed onto the pre-chilled thermal cycler.
	Sample integrity compromised.	• Fragmentation time must be optimized for DNA samples that are not high quality (e.g., FFPE). For more information, refer to the xGen Deceleration Module.
Incomplete resuspension of beads after ethanol wash during purification steps.	Over-drying of beads.	• Continue pipetting the liquid over the beads to break up clumps for complete resuspension. To avoid over-drying, resuspend beads immediately after the removal of residual ethanol.
Shortage of enzyme reagents.	Pipetting enzymes at –20°C	• Place enzyme reagents on ice for 10 minutes prior to pipetting.
Retention of liquid in pipette tip.	Viscous reagents (i.e., Buffer W1) may stick to pipette tip, especially for non-low retention tips.	• Pipette up and down several times to ensure all liquid is released from the pipette tip.
Unexpected increase in adapter dimers.	Improper adapter dilution.	• Use the specified dilution for your input DNA quantity.
	Improper bead purification.	• Use the specified bead volume, particularly during post-PCR purification.
	Reagent W5 not added to the ligation master mix just before use.	• Add Reagent W5 (stubby Y adapter) to the Ligation Master Mix just before use.

xGen™ DNA Library Prep EZ Kit

xGen DNA Library Prep EZ UNI Kit

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

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