NOTICES

Limitations of use

For research use only. Not for use in diagnostic procedures. Unless otherwise agreed to in writing, IDT does not intend these products to be used in clinical applications and does not warrant their fitness or suitability for any clinical diagnostic use. Purchaser is solely responsible for all decisions regarding the use of these products and any associated regulatory or legal obligations.

Safety data sheets pertaining to this product are available upon request.

TECHNICAL SUPPORT

Visit https://www.idtdna.com/pages/support for a helpful answers to frequently asked questions or contact us directly at applicationsupport@idtdna.com.

REVISION HISTORY

Version	Release Date	Description of Changes
1	December 2024	*Initial Release*

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OVERVIEW

The xGen™ 2S™ DNA Library Prep Kits prepare high complexity next generation sequencing libraries from damaged and low input double stranded (dsDNA) samples that have undergone DNA fragmentation. The workflow is designed to support a broad range of input quantities (10 pg to 1 µg) for whole genome and metagenome sequencing, direct sequencing of enriched samples such as ChIP and Hi-C, or targeted sequencing by hybridization capture. Unique end repair capabilities of 5' and 3' termini improve ligation efficiency of damaged samples while sequential ligation overcomes the requirement for adapter titration, thereby maintaining efficient ligation of low nanogram and picogram input quantities.

The xGen™ 2S™ PCR-free and xGen™ 2S™ Plus kits contain the same library prep reagents and volumes for end repair and ligation steps, and the xGen™ 2S™ Plus Kit additionally includes a high fidelity, proofreading polymerase and reagents for library amplification.

SUPPORTED APPLICATIONS AND SAMPLE TYPES

The xGen™ 2S™ DNA Library Prep Kit is suitable for the following sample types:

- Damaged DNA samples* such as DNA extracted from chemically fixed / cross-linked cells
- Enriched DNA from low cell number ChIP and Hi-C processing
- Low input environmental / metagenomic DNA samples
- Limiting DNA samples for oncology or other research studies
- Direct sequencing of DNA libraries or targeted sequencing using hybridization capture following library preparation**
- PCR-free sequencing

* For heavily nicked or denatured samples, use the xGen ssDNA and Low Input DNA Library Kit for best results
** For hybridization capture, perform library amplification with a polymerase that can support up to 1 µg library yield

xGen 2S DNA LIBRARY WORKFLOW

- The xGen™ 2S™ DNA Library Prep Kits have up to five enzymatic steps. These include:
- Repair I: 5' and 3' ends are dephosphorylated to prevent chimera formation and improve adapter ligation to 3' ends.
- Repair II: additional 3' end repair and polishing of 3' and 5' overhangs.
- Ligation I: the full-length i7 or truncated Read 2 adapter is ligated to the 3' ends of the dsDNA substrate.
- Ligation II: 5' end repair and ligation of the full-length i5 or truncated Read 1 adapter to 5' ends of the dsDNA substrate.
- PCR: library yield is increased using a high fidelity, proofreading polymerase, also adds indexing if using truncated adapters.

Bead-based purifications are used to remove unused adapters and change buffer composition between steps. This protocol is 'with bead', where the beads added to the first purification step are retained and re-used throughout the workflow with the addition of PEG-NaCl. This enables a single tube workflow with better sample recovery since tube transfers are not required and minimizes the bead volume and plastic consumable usage per sample as well.

Note: Normalase primers can be used during PCR for compatibility with Normalase library normalization (see the PCR step and Appendix A)

WORKFLOW OPTIONS

- **Indexing by Ligation Workflow**
  The xGen™ 2S™ DNA Library Prep Kits have an indexing by ligation workflow (Figure 1 Left) compatible with xGen™ 2S™ Full-Length Adapters for Illumina® sequencing (supplied separately, see Consumables from IDT-Reagents section). This workflow supports PCR-free sequencing from a minimum DNA Input of 100 ng, or if using lower inputs down to the picogram range, PCR amplification can be used to increase library yield using the 2S Plus kit or if performing hybridization capture, the 2S™ PCR-free kit with a polymerase of choice for library amplification. The Indexing by ligation workflow is also used for incorporating molecular identifier (MID) indexed adapters for subsequent error correction during data analysis. The MID adapters enable up to 96-plex multiplexed sequencing. With PCR, this workflow is compatible with the Normalase and Pre-Hyb Normalase Modules using the included R5 primers.

- **Indexing by PCR Workflow**
  The xGen™ 2S™ DNA Library Prep Kits also have an indexing by PCR workflow (Figure 1 Right). PCR amplification can be performed when using the 2S™ Plus Kit or the 2S™ PCR-free kit with a polymerase of choice when performing hybridization capture. The kits are compatible with xGen™ 2S™ Truncated adapters attached during the ligation steps. This is followed by library amplification using xGen Indexing Primers to complete the adapter sequences and add sample-specific indexes (truncated adapters and indexing primers supplied separately, see Consumable from IDT-Reagents section). This workflow enables up to 1536-plex multiplexed sequencing. This workflow is compatible with the Normalase and Pre-Hyb Normalase Modules using Normalase Indexing primers, supplied separately from the normalization modules.

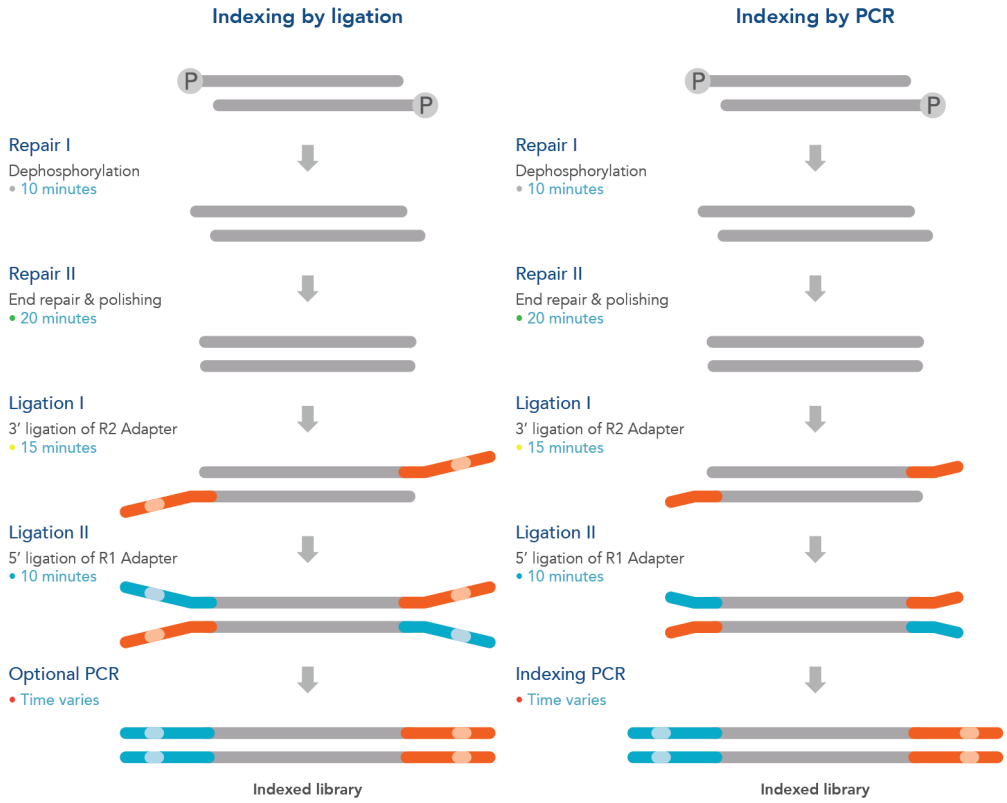

Figure 1. xGen™ 2S™ DNA Library Prep Kit Workflow. Up to five steps are performed and explained in detail in this protocol.

CONSUMABLES AND EQUIPMENT

These kits contain sufficient reagents for the preparation of 24 or 96 libraries (10% excess volume provided).

IMPORTANT: If using indexing primers, it is necessary to order both xGen™ 2S™ Truncated Adapters for the ligation steps as well as indexing primers for library amplification. Indexed adapters are also supplied separately, see below:

Consumables from IDT—Kit Contents

Workflow Stage	Component	24 rxn (µL)	96 rxn (µL)	Storage
Repair I	Buffer W1	158	634	–20°C
	Enzyme W2	28	106	–20°C
Repair II	Buffer G1	132	528	–20°C
	Reagent G2	344	1374	–20°C
	Enzyme G3	28	106	–20°C
	Enzyme G4	28	106	–20°C
Ligation I	Buffer Y1	80	316	–20°C
	Reagent Y2 or Reagent Y2 TruY2*	-	-	–20°C
	Enzyme Y3	53	212	–20°C
Ligation II	Buffer B1	132	528	–20°C
	Reagent B2 – MID or Reagent B2 TruB2*	-	-	–20°C
	Reagent B3	238	950	–20°C
	Enzyme B4	28	106	–20°C
	Enzyme B5	53	212	–20°C
	Enzyme B6	28	106	–20°C
Library Amplification	Reagent R1 or Indexing Primers	-	-	–20°C
	Reagent R2	106	424	–20°C
	Buffer R3	264	1056	–20°C
	Enzyme R4	28	106	–20°C
Additional Reagents	Low EDTA TE	20 mL	20 mL	Room Temp
	PEG NaCl Solution	20 mL	20 mL	Room Temp

* Provided separately with an indexing kit.

Consumables from IDT—Reagents

Workflow Component	Product Name	Index Number	Reaction Size (rxn)	Catalog Number
xGen™ Core Reagents	xGen™ 2S™ PCR-Free DNA Library Prep Kit	N/A	24 96	10009874 10009875
	xGen™ 2S™ Plus DNA Library Prep Kit	N/A	24 96	10009877 10009878
xGen™ Normalase Modules* (optional)	xGen™ Normalase Module xGen™ Pry-Hyb Normalase Module	N/A	96	10009793 10017913
xGen™ 2S Truncated Adapters	xGen™ 2S™ Truncated Adapters	N/A	96	10009908
xGen™ 2S™ MID Adapter Sets S1-S4*	xGen™ 2S™ MID Adapter S1 xGen™ 2S™ MID Adapter S2 xGen™ 2S™ MID Adapter S3 xGen™ 2S™ MID Adapter S4	S701-724 S725-748 S749-772 S773-796	96	10009903 10009904 10009905 10009906
xGen™ 2S™ MID Adapter Sets A, B, and A+B	xGen™ 2S™ MID Adapter Set A xGen™ 2S™ MID Adapter Set B xGen™ 2S™ MID Adapter Set A+B	See Indexing Guide	48 48 96	10009900 10009901 10009902
xGen™ Normalase UDI Primer Plates	xGen™ Normalase UDI Primer Plate 1 xGen™ Normalase UDI Primer Plate 2 xGen™ Normalase UDI Primer Plate 3 xGen™ Normalase UDI Primer Plate 4 xGen™ Normalase UDI Primer Set 1 xGen™ Normalase UDI Primer Set 2 xGen™ Normalase UDI Primer Set 3 xGen™ Normalase UDI Primer Set 4	SU001-SU096 SU097-SU192 SU193-SU288 SU289-SU384 SU001-SU384 SU385-SU768 SU769-SU1152 SU1153-SU1536	96 96 96 96 384 384 384 384	10009796 10009797 10009798 10009799 10009795 10009800 10009811 10009812

* For index sequences, see the Index Sequence Master List. For custom indexing options, contact us at applicationsupport@idtdna.com.

Note: Normalase primers are compatible with both Normalase and non-Normalase workflows.

Consumables from Other Vendors

Item	Supplier	Catalog Number
SPRIselect® or AMPure® XP beads	Beckman Coulter	B23317/B23318/B23319 or A63880/A63881/A63882
Kapa HiFi HotStart Polymerase (suggested for library amplification when using hybridization capture)	Roche	KK2601, KK2602
Aerosol-resistant pipette tips ranging from 1 to 1000 µL	Various Suppliers	Varies
0.2 mL PCR tubes or 96-well plates	Various Suppliers	Varies
1.5 mL microcentrifuge tubes	Various Suppliers	Varies
200 proof (absolute) ethanol (molecular biology grade)	Various Suppliers	Varies
Nuclease-free water (molecular biology grade)	Various Suppliers	Varies
Reagents for qPCR-, electrophoretic-, or fluorometric-based library quantification assay for Illumina® libraries	Various Suppliers	Varies

Equipment

Item	Supplier	Catalog Number
Permagen® Magnetic Separator, or similar	Permagen®	MSR812, MSP750
Instrument for qPCR-, electrophoretic-, or fluorometric-based library quantification assay for Illumina® libraries	Various Suppliers	Varies
Microcentrifuge	Various Suppliers	Varies
Vortex	Various Suppliers	Varies
Programmable thermal cycler	Various Suppliers	Varies
Pipettes ranging from 1 to 1000 µL capacity	Various Suppliers	Varies

GUIDELINES

Reagent Handling

- Store the xGen™ 2S™ DNA Library Prep Kit reagents at –20°C, except for the xGen™ Low EDTA TE Buffer and PEG NaCl, which are stored at room temperature.

Note: The enzymes provided in this kit are temperature sensitive and appropriate care should be taken during storage and handling.

- For all non-enzyme reagents, thaw on ice, then briefly vortex to mix well. Remove enzyme tubes from –20°C storage and place on ice for 10 minutes before use. Attempting to pipette enzymes at –20°C may result in reagent shortage. Spin all tubes in a microcentrifuge to collect contents before opening.
- To create master mixes, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Add reagents in the order listed ON ICE, then pulse vortex to mix and briefly centrifuge.
- Prepare a fresh 80% ethanol solution using 200 proof/absolute ethanol and nuclease-free water. Approximately 2.0 mL of 80% solution will be used per sample.

Avoid Cross-Contamination

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.

Follow these instructions 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 tips between each sample.

Size Selection During Cleanup Steps

This protocol has been performed with SPRIselect beads (Beckman Coulter) but can also be used with AMPure XP beads (Beckman Coulter) or equivalent. However, if other beads are used, solutions and conditions for DNA binding may differ. Consider the information below for performing efficient size selection.

- Post-shearing, confirm the size distribution of each sample by electrophoretic methods to determine the median fragment size of your dsDNA samples.
- The size selection steps in this protocol perform a Left Side Size Selection and are designed to remove unused oligonucleotides and small DNA fragments to select the specified Insert size.
- To customize size selection, please use Beckman Coulter's SPRIselect User Guide for desired conditions not included in this protocol.

DNA Input Considerations

- For direct sequencing applications, the xGen™ 2S™ DNA Library Kits have been tested for a wide range of inputs from 10 pg to 1 µg. For best results when amplifying libraries, a max input of 250 ng is recommended.
- DNA concentration may be assessed using Qubit or other fluorometric method, as it measures the double-stranded, adaptable DNA content of your sample.
- For low-quality human DNA samples, we suggest quantification with our ALU repeat-based qPCR assay using the xGen Input DNA Quantification Primers to accurately assess the usable amount of amplifiable human DNA in the samples and their integrity.

DNA Fragmentation

DNA should be an appropriate size before library construction. When working with high molecular weight DNA, the DNA must be fragmented prior to library preparation. This step is not needed for samples that have already undergone chromatin shearing or are otherwise fragmented, such as cell-free DNA. This workflow was developed with Covaris shearing and supports 200, 350, and 450 bp sheared DNA in low EDTA TE (10 mM Tris, 0.1 mM EDTA). Other shear sizes and methods are compatible, please contact us at applicationsupport@idtdna.com.

PROTOCOL

This is a “with bead” protocol. SPRI beads that are added to clean up the Repair I reaction will be retained and re-used throughout subsequent enzymatic reactions and cleanups using PEG NaCl supplied with the kit. Instead of adding new beads, PEG NaCl is added to the recycled beads to cause the negatively charged DNA to bind with the carboxyl groups on the bead surface. As immobilization is dependent on the concentration of PEG and salt in the reaction, the volumetric ratio of beads to DNA is critical. After each cleanup step, the beads are resuspended in the next reaction mix. This enables a single tube workflow that improves sample recovery since tube transfers are not required and reduces the bead volume and plastic consumables used per sample as well.

Important: Prepare the reaction mixes in advance to ensure that each mix is added without delay to prevent over-drying of the beads following each cleanup step. Assemble all reaction mixes ON ICE.

Perform Repair I

1. Add 40 µL of each fragmented sample to a sterile 0.2 mL PCR tube. Adjust sample volume to 40 µL using Low EDTA TE if necessary.

2. For each sample, make the following Repair I Master Mix.

Repair I Master Mix	
Component	**Volume per sample (µL)**
Low EDTA TE	13
• Buffer W1	6
• Enzyme W2	1
Total Volume	**20**

3. Pulse-vortex the Master Mix for 10 seconds, then briefly centrifuge. Keep the prepared mix on ice until ready to use.

4. Add 20 µL of Repair I Master Mix to each sample. Mix by gently pipetting and briefly centrifuge.

5. Run the following thermal cycler program based on sample type:

Repair I Program				
Sample Type	**Step**	**Temperature (°C)**	**Time**	**Lid Temperature**
cfDNA	1	37	5 min	105°C
	2	65	2 min	
	3	37	5 min	
All Other Inputs	1	37	10 min	Off

6. While the Repair I program runs, prepare the Repair II Master Mix.

Repair II Master Mix	
Component	**Volume per sample (µL)**
Low EDTA TE	30
• Buffer G1	5
• Reagent G2	13
• Enzyme G3	1
• Enzyme G4	1
Total Volume	**50**

7. Pulse-vortex the Master Mix for 10 seconds, then briefly centrifuge. Keep the prepared mix on ice until ready to use.

8. When the Repair I program is completed, proceed immediately to Perform Repair I cleanup.

Perform Repair I Cleanup

Note: Before starting cleanup, make sure you have prepared the Repair II Master Mix for timely bead re-suspension.

1. Clean up the Repair I reaction using magnetic rack, SPRI bead suspension, and enough freshly prepared ethanol for approximately 2 mL per sample.

2. Add the specified SPRI bead volume to each sample based on the table below.

Application	dsDNA Input	Insert Size	Sample Volume (µL)	Bead Volume (µL)
Direct Sequencing	cfDNA	All Sizes	60	84 (ratio: 1.4X)
	Less than 10 ng gDNA	All Sizes	60	84 (ratio: 1.4X)
	10 ng – 250 ng* gDNA	200 bp	60	60 (ratio: 1.0X)
		350 bp	60	54 (ratio: 0.9)
		450 bp	60	42 (ratio: 0.7)
Hybridization Capture	All Inputs	All Sizes	60	108 (ratio: 1.8)

* For PCR-Free libraries up to 1 µg DNA can be used.

3. Vortex sample tubes, 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 is formed (~2 minutes).

6. Remove and discard the supernatant 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. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
8. Repeat step 6 once more for a second wash with the 80% ethanol solution.
9. 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.
10. Remove the samples from the magnetic rack and proceed immediately to Perform Repair II

Perform Repair II

1. Add 50 µL of the pre-mixed Repair II Master Mix to the beads for each sample.
2. Pipette mix a minimum of 10 times until homogenous, then briefly centrifuge.
3. Run the following thermal cycler program:

Repair II Program			
Step	**Temperature* (°C)**	**Time**	**Lid Temperature**
1	20	20 min	OFF

4. While the Repair II program runs, prepare the Ligation I Master Mix. Refer to the table below when using either an indexed i7 adapter (left table, Reagent Y2) for indexing by ligation or a truncated adapter (right table, Reagent TruY2) for indexing by PCR.

Indexing by Ligation Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	20
• Buffer Y1	3
• Enzyme Y3	2
Total Master Mix	**25**
Sample	Beads
• Reagent Y2*	5
Total Volume	**30**

Indexing by PCR Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	20
• Buffer Y1	3
• Enzyme Y3	2
• Reagent Y2 TruY2**	5
Total Master Mix	**30**
Sample	Beads
Total Volume	**30**

* Indexed i7 adapter should be added to each sample to uniquely index each library.
** Truncated adapter (Reagent TruY2) should be added directly to the Master Mix just prior to use.

5. Proceed immediately to Perform Repair II cleanup when the thermocycler program is completed.

Perform Repair II Cleanup

Note: Before starting cleanup, make sure you have prepared the Ligation I Master Mix for timely bead re-suspension.

1. Cleanup the Repair II reaction using PEG NaCl solution.

2. Add the specified PEG NaCl volume to each sample based on the table below:

Application	dsDNA Input	Insert Size	Sample Volume (µL)	PEG NaCl Volume (µL)
Direct Sequencing	cfDNA	All Sizes	50	60 (ratio: 1.2X)
	Less than 10 ng gDNA	All Sizes	50	60 (ratio: 1.2X)
	10 ng – 250 ng gDNA	200 bp	50	42.5 (ratio: 0.85X)
		350 bp	50	37.5 (ratio: 0.75X)
		450 bp	50	27.5 (ratio: 0.55X)
Hybridization Capture	All Inputs	All Sizes	50	82.5 (ratio: 1.65X)

3. Vortex sample tubes, 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 is formed (~2 minutes).
6. Remove and discard the supernatant 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. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
8. Repeat step 6 once more for a second wash with the 80% ethanol solution.
9. 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.
10. Remove the samples from the magnetic rack and proceed immediately to Perform Ligation I.

Perform Ligation I

1. Add 25 µL (indexing by ligation) or 30 µL (indexing by PCR) of pre-mixed Ligation I Master Mix to the beads for each sample. Add 5 µL of the Indexed i7 adapter (Reagent Y2) to each sample if indexing by ligation.
2. Pipette mix a minimum of 10 times until homogenous, then briefly centrifuge.
3. Run the following thermal cycler program:

Ligation I Program			
Step	Temperature* (°C)	Time	Lid Temperature
1	25	15 min	OFF

4. While the Ligation I program runs, prepare the Ligation II Master Mix. Refer to the table below when using either a MID adapter (left table) for indexing by ligation or truncated adapter (right table, TruB2) for indexing by PCR. Keep the prepared mix on ice until ready to use.

Indexing by Ligation Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	30
• Buffer B1	5
• Reagent B2 – MID	2
• Reagent B3	9
• Enzyme B4	1
• Enzyme B5	2
• Enzyme B6	1
Total Master Mix	**50**
Sample	Beads
Total Volume	**50**

Indexing by PCR Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	30
• Buffer B1	5
• Reagent B2 TruB2	2
• Reagent B3	9
• Enzyme B4	1
• Enzyme B5	2
• Enzyme B6	1
Total Master Mix	**50**
Sample	Beads
Total Volume	**50**

5. After the ligation program is complete, proceed immediately to Ligation I cleanup.

Perform Ligation I Cleanup

Note: Before starting cleanup, make sure you have prepared the Ligation II Master Mix for timely bead re-suspension.

1. Clean up the Ligation I reaction using PEG NaCl solution.

2. Add the specified PEG NaCl volume to each sample based on the table below:

Application	dsDNA Input	Insert Size	Sample Volume (µL)	PEG NaCl Volume (µL)
Direct Sequencing	cfDNA	All Sizes	30	31.5 (ratio: 1.05X)
	Less than 10 ng gDNA	All Sizes	30	25.5 (ratio: 0.85X)
	10 ng – 250 ng gDNA	All Sizes	30	36 (ratio: 1.2X)
Hybridization Capture	All Inputs	All Sizes	30	49.5 (ratio: 1.65X)

3. Vortex sample tubes, 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 is formed (~2 minutes).

6. Remove and discard the supernatant 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. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.

8. Repeat step 6 once more for a second wash with the 80% ethanol solution.

9. 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.

10. Remove the samples from the magnetic rack and proceed immediately to Perform Ligation II.

Perform Ligation II

1. Add 50 µL of pre-mixed Ligation II Master Mix to the beads for each sample.
2. Pipette mix a minimum of 10 times until homogenous, then briefly centrifuge.
3. Run the following thermal cycler program:

Ligation II Program			
Step	**Temperature* (°C)**	**Time**	**Lid Temperature**
1	40	10 min	OFF

4. Proceed immediately to Perform ligation II cleanup.

Perform Ligation II Cleanup

1. Cleanup the Ligation I reaction using PEG NaCl solution.
2. Add the specified PEG NaCl volume to each sample based on the table below:

Application	dsDNA Input	Insert Size	Sample Volume (µL)	PEG NaCl Volume (µL)
Direct Sequencing	cfDNA	All Sizes	50	52.5 (ratio: 1.05X)
	Less than 10 ng gDNA	All Sizes	50	42.5 (ratio: 0.85X)
	10 ng – 250 ng gDNA	All Sizes	50	60 (ratio: 1.2X)
Hybridization Capture	All Inputs	All Sizes	50	82.5 (ratio: 1.65X)

3. Vortex sample tubes, 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 is formed (~2 minutes).
6. Remove and discard the supernatant 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. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
8. Repeat step 6 once more for a second wash with the 80% ethanol solution.
9. 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.
10. Add 21 µL of low EDTA TE buffer to sample tubes and mix well until homogeneous.
11. Incubate sample tubes at room temperature for 2 minutes.
12. Place the samples back on the magnetic rack until the solution clears and a pellet is formed (~2 minutes).

13. Carefully transfer 20 µL of eluted DNA into a clean tube. If magnetic beads are present, place on magnet for 2 minutes and transfer eluate again.

Safe Stop: Store freshly prepared libraries at 4°C (or long term at –20°C).

Perform PCR Amplification

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

Library amplification is optional if using at least 100 ng input and full length, indexed adapters. Indexing PCR is required if using truncated adapters to complete the adapter sequence and index each library.

Important: The number of cycles required to produce a library for sequencing will depend on input and quality. In the case of low-quality samples, the number of cycles required may vary based on the quality of the sample and amount of usable DNA present. Approximate guidelines for high-quality DNA are indicated below. The exact number of cycles required must be determined by the user.

Note: If performing hybridization capture, amplify libraries with a polymerase that can produce up to 1 µg library yield (see Consumables from other vendors section). Please refer to vendor specific thermocycling instructions to achieve the desired library yield for pre-hybridization capture pooling. If using Normalase Indexing primers for Pre-Hyb Normalase to achieve equimolar pooling, add 1–2 additional PCR cycles to achieve the necessary yield compared to standard Indexing Primers.

1. Set up the thermocycler 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 sample type.

PCR Amplification Program			
Step	**Cycles**	**Temperature* (°C)**	**Time**
Activate Enzyme	1	98	30 sec
Denaturation	Varies based on sample input (see table below)	98	10 sec
Annealing		60	30 sec
Extension		68	60 sec
Hold	1	4	Hold

Sample Input	Recommended Minimum PCR Cycles for Direct Sequencing
100 ng	3
10 ng	6
1 ng	9
100 pg	12
10 pg	15
10 ng cfDNA	0–2
1 ng cfDNA	5–6

* Set the lid temperature to 105°C

2. Prepare the PCR Master Mix by following the directions in 2a when indexing by ligation and using Reagent R1 or step 2b when indexing by PCR and using truncated TruY2 and TruB2 adapters and Indexing Primers.

a. For optional library amplification of fully indexed libraries, add 30 µL of the PCR Master Mix listed in the table below to the eluted library. Mix by moderate vortexing for 5 seconds and briefly centrifuge.

Indexing by Ligation PCR Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	10
• Reagent R1	5
• Reagent R2	4
• Buffer R3	10
• Enzyme R4	1
Total Master Mix	**30**
Sample	20
Total Volume	**50**

b. For Indexing PCR, add 25 µL of PCR Master Mix listed in the table below to the eluted library. Add a unique combination of indexing primers using the table below as a guideline. Mix by moderate vortexing for 5 seconds and briefly centrifuge.

Indexing Option	Reagents	Volume Per Reaction (µL)
xGen Unique Dual Indexing (UDI)	Pre-mixed primer pair	5
xGen Normalase Unique Dual Indexing (N-UDI)	Pre-mixed primer pair	4
	Reagent R7	1

Indexing by PCR Master Mix	
Component	**Volume Per Sample (µL)**
Low EDTA TE	10
• Reagent R2	4
• Buffer R3	10
• Enzyme R4	1
Total Master Mix	**25**
Sample	20
Indexing Primers	5
Total Volume	**50**

3. Place samples into pre-programmed thermal cycler and run the PCR Amplification Program.

4. When PCR program Is complete, proceed to Perform post-PCR cleanup.

Perform Post-PCR Cleanup

1. Clean up the PCR reaction using SPRI bead suspension.

2. Add the specified SPRI bead volume to each sample based on the table below.

Application	dsDNA Input	Insert Size	Sample Volume (µL)	Bead Volume (µL)
Direct Sequencing	cfDNA	All Sizes	50	42.5 (ratio: 0.85X)
	Less than 10 ng gDNA	All Sizes	50	42.5 (ratio: 0.85X)
	10 ng – 250 ng gDNA	All Sizes	50	70 (ratio: 1.4X)
Hybridization Capture	All Inputs	All Sizes	50	90 (ratio: 1.8X)

3. Vortex sample tubes, then briefly centrifuge.

4. Incubate samples for 5 minutes at room temperature.
5. Place the samples on a magnetic rack until the solution clears and a pellet Is formed (~2 minutes).
6. Remove and discard the supernatant 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. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
8. Repeat step 6 once more for a second ethanol wash with the 80% ethanol solution.
9. Quick spin samples in a microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube.
10. Add 21 µL of Low EDTA TE buffer for direct sequencing or 21 µL of nuclease-free water for hybridization capture to prevent EDTA from interfering with downstream applications and mix well until homogeneous.
11. Incubate sample tubes at room temperature for 2 minutes.
12. Place the samples back on the magnetic rack until the solution clears and a pellet Is formed (~2 minutes).
13. Carefully transfer 20 µL of eluted DNA to a clean tube. If magnetic beads are present, place on magnet for 2 minutes and transfer eluate again.

Second Post-PCR Purification (Optional)

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

1. At the end of the first clean up, resuspend beads in 50 µL of Low EDTA Buffer and incubate for 1–2 minutes. DO NOT transfer elute to a new tube but proceed directly to a second cleanup by adding PEG NaCl to the bead elute based on the table below.

Application	dsDNA Input	Insert Size	Sample Volume (µL)	PEG NaCl Volume (µL)
Direct Sequencing	cfDNA	All Sizes	50	52.5 (ratio: 1.05X)
	Less than 10 ng gDNA	All Sizes	50	42.5 (ratio: 0.85X)
	10 ng – 250 ng gDNA	All Sizes	50	60 (ratio: 1.2X)
Hybridization Capture	All Inputs	All Sizes	50	90 (ratio: 1.8X)

2. Vortex sample tubes, then briefly centrifuge.
3. Incubate samples for 5 minutes at room temperature.
4. Place the samples on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove and discard supernatant without disturbing the pellet (less than 5 µL will 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 not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.

7. Repeat step 6 once more for a second ethanol wash with the 80% ethanol solution.
8. Quick spin samples in a microcentrifuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube.
9. Add 21 µL of Low EDTA TE buffer for direct sequencing or 21 µL of nuclease-free water for hybridization capture to prevent EDTA from interfering with downstream applications and mix well until homogeneous.
10. Incubate sample tubes at room temperature for 2 minutes.
11. Place the samples back on the magnetic rack until the solution clears and a pellet Is formed (~2 minutes).
12. Carefully transfer 20 µL of eluted DNA to a clean tube. If magnetic beads are present, place on magnet for 2 minutes and transfer eluate again.

APPENDIX A: NORMALASE PCR INSTRUCTIONS

Please review this section and the Normalase Kit protocol before setting up your Normalase PCR for you xGen™ 2S™ libraries. To achieve expected results, amplify each library using Normalase primers with the appropriate number of cycles and thermocycling conditions shown below to obtain a library yield of 12 nM or greater in a 20 µL eluate.

Normalase PCR Setup:

For Indexing by Ligation, use Normalase Primers (Reagent R5)

1. If you typically obtain the required minimum threshold (≥12 nM following library amplification), simply utilize Normalase primers (Reagent R5 included in the Normalase Kit) and add one additional PCR cycle to your program.

2. If prior to amplification of your library yields are ≥12 nM, a minimum of 3 cycles is still required to condition the libraries for downstream Normalase enzymology.

3. Assemble your PCR Master Mix using standard PCR reagents as shown in the table below, except substitute standard primers (Reagent R1 included with indexed adapters) with 5 µL of Reagent R5. Thoroughly mix by moderate vertexing, pulse spin to collect contents and place in the thermocycler.

PCR RXN	Master Mix
Sample: 20 µL Master Mix: 30 µL **Final Volume: 50 µL**	Low EDTA TE: 10 µL
	• Reagent R2: 4 µL
	• Buffer R3: 10 µL
	• Enzyme R4: 1 µL
	• Reagent R5: 5 µL
	Final Volume: 30 µL

4. Run the following thermocycler program, adjusting the number of cycles depending on the input amount and sample quality. Lid heating set to 105°C.

Temperature	Time
98°C	30 Sec
98°C	10 Sec
60°C	30 Sec
68°C	60 Sec
Perform X Cycles*	
68°C	5 min
4°C	Hold

* The recommended minimum number of cycles for each input to provide ≥12 nM yields suitable for the Normalase workflow is as follows:

DNA Input (ng)	Minimum number of cycles for ≥12 nM
≥100	4
10	8
1	11
0.1	14
0.01	17

5. Proceed to Post-PCR Cleanup step in this protocol, then proceed to Normalase I, Pooling, and Normalase II in the Normalase Kit Protocol.

For Indexing by PCR, use Normalase Indexing Primers

Normalase indexing primers complete the adapter sequences, amplify and condition libraries for downstream Normalase steps. Assemble using standard PCR reagents as shown below, except substitute standard indexing primers with Normalase Indexing Primers and Reagent R7 (supplied together).

1. To each sample library (20 µL eluate), add 4 µL of Normalase Unique Dual Indexing Primers for a total volume of 24 µL.

Indexing Option	Reagents	Volume per sample
Normalase UDI	Pre-mixed i5 and i7 primer pair	4.0 µL

2. Assemble the PCR master mix on ice. Mix thoroughly and pulse spin to collect contents. Add 26 µL of the mix to each sample tube, mix thoroughly and pulse spin to collect contents (50 µL final PCR volume) and place In the thermocycler.

PCR RXN	UDI Master Mix per sample
Sample + Primers: 24 µL Master Mix: 26 µL **Final PCR Volume: 50 µL**	Low EDTA TE: 10 µL • Reagent R2: 4 µL • Buffer R3: 10 µL • Enzyme R4: 1 µL • Reagnet R7: 1 µL **Master Mix: 26 µL**

3. Use the same thermocycling instructions as above for Indexing by Ligation.

APPENDIX B: INDEXED ADAPTER SEQUENCES

The full-length adapter sequences are below, where the X and Y text indicates the location of the index sequences where X = 6 base for Set A and B, 8 base for Set S1-S4 and 10 base for UDIs, and where Y= 9 base N sequence for the MID or a 10 base Index for the UDIs. The sequences below represent the adapter sequences following completion of the indexing PCR step or indexing by ligation.

Index 1 (i7) adapters: 10 base Index shown but can be 6, 8 or 10 bases depending on the chosen set:

5’ – GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXXXXATCTCGTATGCCGTCTTCTGCTTG – 3’

Index 2 (i5) adapters: 10 base Index shown but can be 9 or 10 bases depending on the chosen set:

5’ – AATGATACGGCGACCACCGAGATCTACACYYYYYYYYYYACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3’

Refer to the Index Sequences Master List for index sequences for preparing your Illumina sequencing sample sheet on the instrument of your choice.

APPENDIX C: TROUBLESHOOTING GUIDE

Problem	Possible Cause	Suggested Remedy
Library migrates unexpectedly on Bioanalyzer.	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally. PCR-Free libraries migrate abnormally due to partially single stranded adapters.	Quantify library by qPCR, as other quantification methods will not accurately detect heteroduplex library molecules. Perform the minimum number of PCR cycles necessary to avoid over-amplification.
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. Make Sure to prepare master mixes in advance so bead resuspension can be performed without delay, following 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., enzymes) 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 bead purification. Lower than expected input DNA quantity.	Use the specified bead volume particularly for the post-PCR purification. Carefully quantify input to ensure appropriate cleanup steps are performed.