xGen™ Methyl-Seq DNA Library Prep Kit

REVISION HISTORY

Version	Release date	Description of changes
4	June 2023	Updated enzyme Y2 handling conditions and specified master mixes in more detail
3	June 2022	Adjusted product size offerings
2	March 2022	Updated names for adapters only
1	December 2021	Initial release

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OVERVIEW

The xGen Methyl-Seq DNA Library Prep Kit enables the preparation of high-complexity, next generation sequencing (NGS) libraries from bisulfite-converted DNA for Illumina® platforms.

The xGen Methyl-Seq DNA Library Prep Kit has been tested for whole genome bisulfite sequencing (WGBS) using genomic DNA, cfDNA, and formalin-fixed, paraffin-embedded (FFPE) samples. Libraries can be prepared from as little as 100 pg of high-quality starting input DNA. The kit has been tested down to 1 ng of cell-free DNA (cfDNA).

Targeted methylation sequencing using xGen Custom Hybridization Capture Panels has demonstrated compatibility with bisulfite-converted genomic DNA and cfDNA.

The xGen Adaptase™ technology used in this xGen Methyl-Seq Kit is compatible with single-stranded DNA (ssDNA), making it an ideal choice for NGS library prep from denatured DNA fragmented by bisulfite conversion. By using single-stranded, bisulfite-converted DNA molecules as input, the xGen Methyl-Seq Kit overcomes the significant loss of library complexity associated with conventional library preparations. Users of the xGen Methyl-Seq DNA Library Kit can use DNA inputs that were formerly prohibitively low, while maintaining high library complexity.

Supported applications

- Whole Genome Bisulfite Sequencing (WGBS)
- Targeted bisulfite sequencing with xGen Custom Hybridization Capture Panels*
- Bisulfite-converted DNA enriched by ChIP or other methods
- Reduced Representation Bisulfite Sequencing (RRBS)
- Sequencing of ancient DNA samples, when retention of fragments containing uracil nucleotides is desired

* Contact Us to consult with the IDT design team for methyl-specific hybridization panel designs

Workflow

The xGen Methyl-Seq DNA Library Prep Kit workflow, shown in Figure 1, utilizes four steps to produce a final, indexed library from bisulfite converted ssDNA input fragments.

The steps are as follows:

1. An Adaptase reaction simultaneously performs tailing and ligation of R2 Stubby Adapter to 3′ ends in a highly efficient, template-independent manner.
2. An Extension reaction generates a complementary uracil-free library molecule.
3. The Ligation reaction adds R1 Stubby Adapter to the uracil-free strand.
4. An Indexing PCR incorporates sample indexes and sequences needed for Illumina sequencing. Indexing primers are sold separately. The entire workflow takes about 1.5 hours before Indexing PCR.

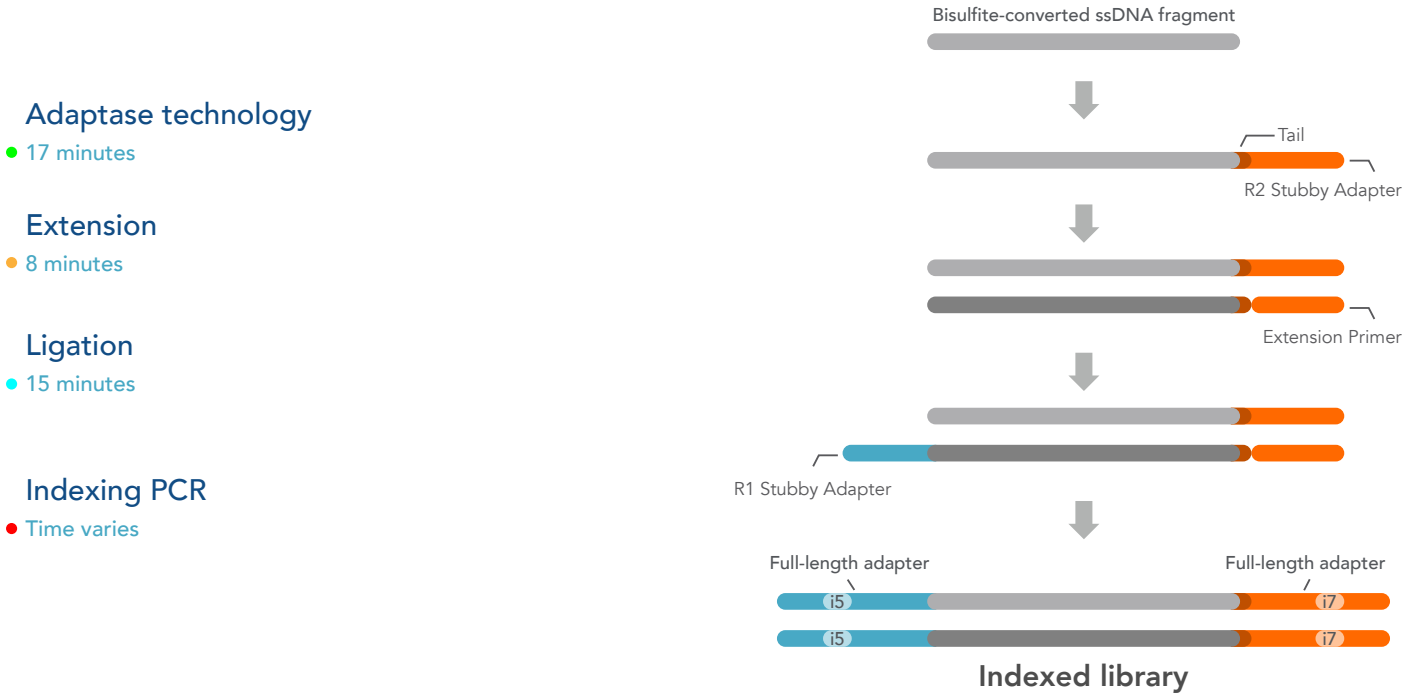

Figure 1. xGen Methyl-Seq DNA Library Prep Kit workflow. This kit utilizes four steps for library prep as described in the text.

CONSUMABLES AND EQUIPMENT

These kits contain sufficient reagents for the preparation of 16 or 96 libraries (10% excess volume provided).

Consumables from IDT—Kit contents

Workflow step	Component	16 rxn (µL)	96 rxn (µL)	Storage
Adaptase	• Buffer G1	71	423	–20°C
	• Reagent G2	71	423	
	• Reagent G3	44	264	
	• Enzyme G4	18	106	
	• Enzyme G5	18	106	
	• Enzyme G6	18	106	
Extension	• Reagent Y1	35	212	
	• Enzyme Y2	739	4436	
Ligation	• Buffer B1	53	317	
	• Reagent B2	176	1056	
	• Enzyme B3	35	212	
Indexing PCR	• Buffer R1	176	1056	
	• Reagent R2	71	423	
	• Enzyme R3	18	106	
Other reagents	Low EDTA TE	6000	24000	Room temperature

IDT—Customizable kit contents

Component	Product name	Index #	Reaction size (rxn)	Catalog #
xGen Core Reagents	xGen Methyl-Seq DNA Library Prep Kit	N/A	16	10009860
	xGen Methyl-Seq DNA Library Prep Kit	N/A	96	10009824
xGen Normalase Module (optional)	xGen Normalase Module	N/A	96	10009793
xGen CDI Primers	xGen CDI Primers	D501-D508/ D701-D712	96	10009815
xGen Normalase CDI Primers*	xGen Normalase CDI Primers	D501N-D508N/ D701N-D712N	96	10009794
xGen Normalase UDI Primer Plates*	xGen Normalase UDI Primer Plate 1	SU001-SU096	96	10009796
	xGen Normalase UDI Primer Plate 2	SU097-SU192	96	10009797
	xGen Normalase UDI Primer Plate 3	SU193-SU288	96	10009798
	xGen Normalase UDI Primer Plate 4	SU289-SU384	96	10009799
	xGen Normalase UDI Primer Set 1	SU001-SU384	4x96	10009795
	xGen Normalase UDI Primer Set 2	SU385-SU768	4x96	10009800
	xGen Normalase UDI Primer Set 3	SU769-SU1152	4x96	10009811
	xGen Normalase UDI Primer Set 4	SU1153-SU1536	4x96	10009812

* Normalase primers are compatible with both Normalase and non-Normalase workflows.

Consumables—Other suppliers

Item	Supplier	Catalog #
SPRISelect® or AMPure® XP beads or equivalent	Beckman Coulter	B23317/B23318/ B23319/A63880/ A63881/A63882
Digital electrophoresis chips and associated reagents (choose one):		
Experion® DNA 1K Analysis Kit	Bio-Rad	700-7107
High Sensitivity DNA Kit	Agilent	5067-4626
High Sensitivity D1000 ScreenTape® or equivalent	Agilent	5067-5584
KAPA Biosystems® Library Quantification Kit–Illumina/Universal, or equivalent	Roche	KK4824
KAPA HiFi ReadyMix (required if performing targeted hyb capture), or equivalent	Roche	KK2602
High-fidelity, uracil-tolerant polymerase (use only for Indexing PCR of >100 ng input)*, such as KAPA HiFi HotStart® Uracil+ReadyMix (Roche), or equivalent	KAPA Biosystems	KK2801/KK2802
EZ DNA Methylation-Gold Kit® or equivalent	Zymo Research	D5006
Unmethylated Lambda DNA	Promega	D1521
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851 or Q32584
PhiX or other high-complexity library for loading purposes	Various suppliers	Varies
96-well PCR Plates	Various suppliers	Varies
DNA LoBind Tubes, 1.5 mL	Various suppliers	Varies
PCR tubes, 0.2 mL	Various suppliers	Varies
Serological pipettes (5 mL–25 mL)	Various suppliers	Varies
50 mL conical tubes	Various suppliers	Varies
Aerosol-resistant, low-retention pipettes and tips, 2–1000 µL	Various suppliers	Varies
Absolute ethanol (200 proof)	Various suppliers	Varies
Nuclease-free water	Various suppliers	Varies

* Use of this polymerase is not compatible with Normalase primers or the Normalase workflow and is only required when using DNA inputs of >100 ng.

Equipment

Item	Supplier	Catalog #
Covaris® ultrasonicator	Covaris	varies
0.2 mL magnets for individual tubes and plates	Permagen	MSR812 (tubes) or MSP750 (plates)
Absorbance-based input quantification instrument (e.g., NanoDrop™ or equivalent)	Various suppliers	Varies
Digital electrophoresis instrument equivalent for library size determination	Various suppliers	Varies
Quantitative PCR instrument	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Programmable thermal cycler	Various suppliers	Varies
Vortex	Various suppliers	Varies

GUIDELINES

Reagent handling

- Store the xGen Methyl-Seq DNA Library Prep Kit reagents at –20°C, with the exception of the xGen Low EDTA TE Buffer, which is 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 mL of 80% ethanol 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 optimized 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.

- The size selections utilized in this protocol perform a Left Side Size Selection to remove small fragments and unused adapter. For customizing size selection, use Beckman Coulter's SPRISelect User Guide for desired conditions not included in this protocol.

DNA input considerations

For direct sequencing applications, the xGen Methyl-Seq Kit has been tested for an input range of 100 pg to 100 ng DNA into the fragmentation step and up to 1 ng cfDNA into the bisulfite conversion step. For xGen Hybridization Capture using **xGen Custom Hybridization Capture Panels**, a DNA input range of 1–100 ng is recommended.

Input quantities recommended in this protocol refer to the total DNA quantified prior to fragmentation and/or bisulfite conversion. Input DNA concentration may be assessed by fluorometric reading (Qubit). Accurate quantification of DNA is important for determining the number of PCR cycles required at the final step of the workflow. If sample material is limited and input quantification cannot be performed, apply the number of cycles recommended for the lowest input quantity.

Consider genome complexity and sample quality when choosing input DNA quantity. Although libraries may be successfully prepared from ultra-low inputs, reduced representation of genome complexity may be observed.

If using >100 ng of input material, substitute our Indexing PCR high-fidelity polymerase with a high-fidelity, uracil-tolerant polymerase for indexing PCR, such as KAPA HiFi HotStart Uracil+ ReadyMix. However, use of a uracil-tolerant polymerase during Indexing PCR of inputs >100 ng is not compatible with Normalase indexing primers or the Normalase workflow. Please reach out via **Contact Us** for more information.

Unmethylated lambda gDNA must be spiked into the sample before fragmentation to assess bisulfite conversion efficiency. For samples that do not require fragmentation (such as cfDNA), lambda gDNA may be separately fragmented to a size similar to fragments in the sample. We recommend a spike-in level of 0.1–0.5% (w/w), as specified in the **Standards and Guidelines for Whole Genome Shotgun Bisulfite Sequencing**.

For RRBS samples, we recommend using at least 100 ng of input gDNA, as a significant amount of sample loss is expected during the restriction enzyme digestion and size selection steps.

DNA fragmentation

When working with high molecular weight DNA, the DNA must be fragmented prior to bisulfite conversion. Fragmentation may be performed via sonication to produce 350 bp DNA fragments. This kit can be used with Covaris sheared DNA. After shearing, samples will be further fragmented to an average of 170–200 bp during bisulfite conversion; however, no further fragmentation is observed during bisulfite conversion when working with cfDNA samples.

Notes:

For RRBS applications, the MspI restriction enzyme will be used to digest the gDNA, followed by DNA purification and isolation of small fragments (100–220 bp). Therefore, fragmentation by Covaris or an equivalent DNA shearing kit is not necessary.

Other enzymatic fragmentation methods are not recommended for methylation sequencing or for use with this kit.

Omitting DNA shearing prior to bisulfite conversion alone produces a broader size distribution of fragments, resulting in larger library molecules that cluster poorly on Illumina flow cells. This often leads to low cluster density and loss of library complexity. Therefore, we do not recommend omitting the fragmentation step.

When implementing this workflow, we recommend analysis of the sheared DNA samples using electrophoretic methods, prior to both bisulfite conversion and library preparation.

Optional concentration step

The specified input volume for the EZ DNA Methylation-Gold kit is 20 µL. If needed, concentrate samples with Zymo Research DNA Clean & Concentrator or other method and elute in appropriate volume of xGen Low EDTA TE Buffer.

Bisulfite conversion

The xGen Methyl-Seq Kit can be used with EZ DNA Methylation-Gold Kit, following the manufacturer's instructions. The QIAGEN EpiTect® Bisulfite kit, or other conversion kits that contain nucleic acid carriers, are not recommended. Input quantities and PCR cycling recommendations are based on 75% recovery from the EZ DNA Methylation-Gold Kit. If using another bisulfite conversion kit, become familiar with percent DNA recovery to ensure a sufficient quantity of bisulfite-converted DNA for library construction.

This library prep requires an input DNA volume of 15 µL. Be sure to note the volume for final elution of bisulfite-converted DNA in xGen Low EDTA TE Buffer to prevent sample over-dilution.

When implementing this workflow, we recommend quantifying the amount of DNA recovered using an absorbance-based method to measure ssDNA, e.g., NanoDrop™ (Thermo Fisher Scientific). Be sure to test input quantities that will be within the detectable range of your spectrophotometer.

Automation

This protocol is readily automatable. A 10% overage volume of reagents is supplied to accommodate automation. Contact Us if you require additional reagent overage volume or would like to learn about our custom packaging options.

Tip: While IDT does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop and qualify automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact Us to discuss automating your xGen Methyl-Seq DNA Library Kit with your automated liquid handling system.

PROTOCOL

Perform Adaptase™ step

1. Add 15 µL of each fragmented sample into a low nucleic acid binding PCR plate or 0.2 mL PCR tube.

   Note: If sample volume <15 µL, use Low EDTA TE to bring the volume up to 15 µL.

2. For each sample, make the following Adaptase Master Mix.

Component	Volume per sample (µL)
Low EDTA TE	11.5
• Buffer G1	4.0
• Reagent G2	4.0
• Reagent G3	2.5
• Enzyme G4	1.0
• Enzyme G5	1.0
• Enzyme G6	1.0
Total volume	25.0

3. Pulse-vortex the Adaptase Master Mix for 10 seconds, then briefly centrifuge. Keep on ice.
4. Place the samples in the pre-heated thermal cycler and run the Denaturation program.

Denaturation Program		
Step	Temperature (°C)*	Time
1	95°	2 min

* Set the lid temperature to 105°C.

5. After the Denaturation program ends, immediately place samples on ice for 2 minutes. Proceed directly to the Adaptase Master Mix addition step.
6. Add 25 µL of Adaptase Master Mix to each sample and mix by pipetting or gentle vortexing. Briefly centrifuge.
7. Place the samples in the pre-heated thermal cycler and run the Adaptase program.

Adaptase Program		
Step	Temperature (°C)*	Time
1	37	15 min
2	95	2 min
3	4	Hold

* Thermal cycler lid set to 105°C.

8. While the Adaptase program runs, prepare the Extension Master Mix.

Component	Volume per sample (µL)
• Reagent Y1	2
• Enzyme Y2*	42
Total volume	44

*Gently rock Enzyme Y2 at room temperature for 5 minutes, or until any solutes appear to be in solution. Place back on ice for remainder of use.

9. Pulse-vortex the Extension Master Mix for 10 seconds, then briefly centrifuge. Keep the Master Mix on ice.
10. After the Adaptase program reaches 4°C, proceed immediately to the Perform extension.

Perform extension

1. Add 44 µL of Extension Master Mix to each sample. Mix by pipetting or gentle vortexing. Briefly centrifuge.
2. Place the samples in the thermal cycler and run the Extension program.

Extension Program		
Step	Temperature (°C)*	Time
1	98	1 min
2	62	2 min
3	65	5 min
4	4	Hold

* Set the lid temperature to 105°C.

Perform post-extension cleanup

Input	Cleanups	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
cfDNA or ≥10 ng gDNA	Single cleanup	84	101 (ratio: 1.2X)	15
<10 ng gDNA	1st cleanup	84	101 (ratio: 1.2X)	50
	2nd cleanup	50	60 (ratio: 1.2X)	15

1. Add the specified bead volume of SPRISelect beads to each well. Mix by vortexing. Briefly centrifuge.
2. Incubate the samples at room temperature for 5 minutes, off the magnet.
3. Place the samples on a magnetic rack until a pellet forms and the solution clears (approximately 2 minutes).

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, the yield and quality of the sequencing library may suffer.
4. Remove and discard the cleared supernatant. Be careful not to remove any beads.
5. Keeping the samples on the magnet, add 200 µL of 80% ethanol, then incubate for 30 seconds. Remove and discard the supernatant.
6. Repeat the previous step for a second wash with 80% ethanol.
7. Briefly centrifuge samples and place back on the magnet.
8. Use a P20 pipette tip to remove any residual ethanol.
9. Remove samples from magnet, then add the specified elution volume of Low EDTA TE buffer. Gently vortex to resuspend the pellet.
10. Allow the samples to incubate at room temperature for 5 minutes to elute DNA off the beads.
11. Place the samples on a magnet and wait for the liquid to clear completely for 1–2 minutes.

    Note: Depending on the strength of your magnet, you may need to wait longer.
12. Carefully transfer the specified elution volume of eluted DNA into a new well or tube. If necessary, perform a second cleanup.
13. Proceed to Ligation step or pause here.

    Safe Stop: Samples can be briefly stored at 4°C until ready to proceed or at –20°C if overnight storage is necessary.

Perform ligation

1. For each sample, make the following Ligation Master Mix.

Component	Volume per sample (µL)
• Buffer B1	3
• Reagent B2	10
• Enzyme B3	2
Total volume	15

2. Pulse vortex the Ligation Master Mix for 10 seconds, then briefly centrifuge. Keep on ice.
3. Add 15 µL of Ligation Master Mix to each sample. Mix by pipetting or gentle vortexing. Briefly centrifuge.
4. Place the samples in the thermal cycler and run the Ligation program.

Ligation Program		
Step	Temperature °C*	Time
1	25	15 min
2	4	Hold

* Set the lid temperature to OFF.

Post-ligation cleanup

Input	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
cfDNA	30	36 (ratio: 1.2X)	20
gDNA	30	30 (ratio: 1.0X)	20

1. Add the specified Bead Volume of SPRISelect beads to each well. Mix by vortexing. Briefly centrifuge.
2. Incubate the samples at room temperature for 5 minutes off the magnet.
3. Place the samples on a magnetic rack until a pellet forms and the solution clears (approximately 2 minutes).

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, the yield and quality of the sequencing library may suffer.

4. Remove and discard the cleared supernatant. Be careful not to remove any beads.
5. While keeping the samples on the magnet, add 200 µL of 80% ethanol, then incubate for 30 seconds. Remove and discard the supernatant.
6. Repeat the previous step for a second wash with 80% ethanol.
7. Briefly centrifuge samples and place them back on the magnet.
8. Use a P20 pipette tip to remove any residual ethanol.
9. Remove samples from the magnet, then add the specified elution volume of Low EDTA TE buffer. Gently vortex to resuspend the pellet.
10. Allow the samples to incubate at room temperature for 5 minutes to elute DNA off the beads.
11. Place the samples on a magnet and wait for the liquid to clear completely for 1–2 minutes.

    Note: Depending on the strength of your magnet, you may need to wait longer.

12. Carefully transfer 20 µL of eluted DNA into a new well or tube. Proceed to Indexing PCR or pause here.

    Safe Stop: Samples can be briefly stored at 4°C until ready to proceed or at –20°C if overnight storage is necessary.

Perform indexing PCR

1. Add indexing primers directly to each sample. If using Normalase indexing primers, volumes will differ. See Appendix B and the Normalase Kit protocol for specific instructions.

Indexing options	Reagents	Volume per sample (µL)
xGen UDI Primer Pairs	Pre-mixed primer pair	5.0
xGen CDI Primer Pairs	i5 primer	2.5
	i7 primer	2.5

Note: The indexing primers are provided separately, as part of the Indexing Kit. See the Index Master List for index sequences. If using alternative indexing primers, Contact Us to ensure that they are compatible with this protocol.

2. For each sample, make the following Indexing PCR Master Mix. Enzyme R3 should be added to the PCR Master Mix just before use.

Component	Volume per sample (µL)
Low EDTA TE	10
• Buffer R1	10
• Reagent R2	4
• Enzyme R3	1
Total volume	25

3. Pulse-vortex the PCR Master Mix for 10 seconds, then briefly centrifuge.
4. Add 25 µL of Indexing PCR Master Mix to each sample. Mix by pipetting or gentle vortexing. Briefly centrifuge.
5. Place the samples in the thermal cycler and run the Indexing PCR program.

Indexing PCR Program			
Step	Temperature (°C)*	Time	Cycles
1	98	30 sec	
2	98	10 sec	Based on sample input (see table below)
	60	30 sec	
	68	60 sec	
3	4	Hold	

* Set the lid temperature to 105°C.

Sample input size	# of cycles
100 ng	4–6
10 ng	7–9
1 ng	11–13
100 pg	14–16

The number of cycles needed to produce a library concentration high enough for direct sequencing will depend on DNA input quantity and quality. Approximate guidelines are indicated above, but the exact number of cycles required must be determined for the sample.

Important: If preparing libraries for use in downstream xGen Hybridization Capture, additional PCR cycling is required. See Appendix A for further considerations.

6. After the program completes, proceed to Post-Indexing PCR cleanup.

Perform post-indexing PCR cleanup

Input	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
cfDNA	50	40.0 (ratio: 0.8X)	20
gDNA	50	42.5 (ratio: 0.85X)	20

1. Add the specified Bead Volume of SPRISelect beads to each well. Mix by vortexing. Briefly centrifuge.
2. Incubate the samples at room temperature for 5 minutes off the magnet.
3. Place the samples on a magnetic rack until a pellet is formed and the solution clears (approximately 2 minutes).

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, the yield and quality of the sequencing library may suffer.
4. Remove and discard the cleared supernatant. Be careful not to remove any beads.
5. While keeping the plate on the magnet, add 200 µL of 80% ethanol, then incubate for 30 seconds. Remove and discard the supernatant.
6. Repeat the previous step for a second wash with 80% ethanol.
7. Briefly centrifuge samples and place them back on the magnet.
8. Use a P20 pipette tip to remove any residual ethanol.
9. Remove samples from the magnet, then add the specified elution volume of Low EDTA TE buffer. Gently vortex to re-suspend the pellet.
10. Allow the samples to incubate at room temperature for 5 minutes to elute DNA off the beads.
11. Place the samples on a magnet and wait for the liquid to clear completely for 1–2 minutes.

    Note: Depending on the strength of your magnet, you may need to wait longer.
12. Carefully transfer entire eluate of the sample into a new well or tube.
13. Optionally, if direct sequencing on patterned flow cells, perform the following second cleanup to ensure optimal removal of the unincorporated primers, which can increase index hopping. This second purification is not necessary if using xGen Unique Dual Indexing primer pairs, the xGen Normalase Module, or downstream xGen Hybridization Capture.

Input	Number of cleanups	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
cfDNA	1st cleanup	50	40.0 (ratio: 0.8X)	50
	2nd cleanup	50	40.0 (ratio: 0.8X)	20
gDNA	1st cleanup	50	42.5 (ratio: 0.85X)	50
	2nd cleanup	50	42.5 (ratio: 0.85X)	20

Library quantification

Accurate library quantification is essential to properly load the sequencing instrument. Libraries can be quantified using electrophoretic, fluorometric, or qPCR-based methods. Electrophoretic-based methods also allow examination of library molecule size distribution.

Considerations for sequencing

Bisulfite-converted DNA libraries are relatively low in base composition complexity due to C depletion. Therefore, it is important to assess the need for PhiX or other balanced, high-complexity library spike-ins. Please see Illumina's current recommendations based on your chosen sequencing instrument.

Data analysis and informatics

The xGen Adaptase technology, used in the xGen Methyl-Seq DNA Library Kit, adds a low-complexity dinucleotide tail with an average length of 8 bases to the 3' end of each fragment during the addition of the first NGS adapter molecule. If these tails are not trimmed bioinformatically from the sequencing data, it is normal and expected to observe them at the beginning of Read 2 (R2). When read length is close to fragment size, the tail may also be observed toward the end of Read 1 (R1) data.

To ensure optimal mapping efficiency, bioinformatic trimming of the low-complexity Adaptase tail from these libraries may be required, depending on the aligner used. For specific tail trimming recommendations, Contact Us.

Many informatics pipelines already include trimming of up to 10 bases from the beginning of both R1 and R2. Trimming eliminates any synthetic cytosine methylation introduced due to filling in overhangs during end-repair steps of conventional dsDNA library preparation, or due to low-quality bases caused by bisulfite treatment.

APPENDIX

Appendix A: Protocol adjustments for targeted hybridization capture

Hybridization capture using custom xGen Hyb Capture panels enables sequencing of specific regions of the methylome for deeper sequencing of regions of interest and lower sequencing costs. The only modifications required for compatibility with this protocol are to use KAPA HiFi HotStart® ReadyMix (Roche) for Indexing PCR and to use additional PCR cycles to achieve library yields ≥500 ng.

Following library quantification, a capture probe set designed to target your regions of interest is hybridized to complementary library fragments (**Contact us** to consult with IDT NGS Design Team for methyl-specific hybridization panel designs). See the **Cost-effective targeted methyl-seq using an xGen Custom Hyb Capture Panel and the xGen Methyl-Seq DNA Library Prep Kit Application Note** for information regarding design considerations due to bisulfite conversion of DNA template. The captured library is then sequenced.

For hybridization capture using the xGen Hyb Capture workflow, 500 ng of each library is required. To generate >500 ng of each library, follow the provided protocol adjustments below:

Indexing PCR: In place of the provided PCR Master Mix, use KAPA HiFi HotStart ReadyMix and follow manufacturer thermal cycling conditions. If input >100 ng, use a uracil-tolerant mix, such as KAPA HiFi HotStart Uracil+ReadyMix.

Note: Use of uracil-tolerant polymerase during Indexing PCR is not compatible with Normalase indexing primers or Normalase workflow.

PCR cycling: To achieve the ≥500 ng library yield necessary for hybridization capture, follow the recommended PCR cycles below. This step may require sample-specific optimization; increase the number of PCR cycles to increase yields as needed.

Sample type	DNA input (ng)	PCR cycles
gDNA	1	18
gDNA	10	14
gDNA	50	13
gDNA	100	10
cfDNA	1	21
cfDNA	5	18
cfDNA	10–25	16–17
FFPE	25	15

Appendix B: Normalase™ instructions

Review this section and the **xGen Normalase Module Protocol** before setting up a Normalase PCR. 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 ≥12 nM in a 20 µL eluate.

Normalase indexing primers complete the adapter sequences, amplify, and condition libraries for downstream Normalase steps. Assemble your reaction using standard PCR reagents as shown below, but substitute standard indexing primers with i5 and i7 Normalase Indexing Primers.

Note: Use of uracil-tolerant polymerase during Indexing PCR is not compatible with Normalase indexing primers or Normalase workflow.

Add 2 µL of each Normalase combinatorial dual index primer, or 4 µL of a Normalase unique index primer pair, to each sample for a total volume of 24 µL.

Indexing options	Reagents	Volume per sample (µL)
Normalase UDI	Pre-mixed primer pair	4
Normalase CDI	i5 primer i7 primer	2 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 total reaction volume). Place samples in the thermocycler.

Normalase CDI

Component	Volume per reaction (µL)
PCR Master Mix	25
Reagent R6	1
Total Master Mix	26
Eluted sample + primers	24
Total volume	**50**

Normalase UDI

Component	Volume per reaction (µL)
PCR Master Mix	25
Reagent R7	1
Total Master Mix	26
Eluted sample + primers	24
Total volume	**50**

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

PCR Program			
Step	Temperature (°C)	Time	
1	98	30 sec	
2	98	10 sec	Perform X cycles*
	60	30 sec	
	68	60 sec	
3	68	5 min	
4	4	Hold	

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

DNA input	Recommended PCR cycles
100 ng	8
10 ng	11
1 ng	15
100 pg	18

The number of cycles required may vary based on the input amount, as detailed above. Recommendations above are for high-quality input DNA.

Proceed to Perform post-indexing PCR cleanup.

Proceed to Normalase I, Pooling, and Normalase II in the Normalase Kit Protocol.

Appendix C: Indexed adapter sequences

The full-length adapter sequences are 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′

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

Appendix D: Troubleshooting guide

Problem	Possible cause	Suggested remedy
Incomplete resuspension of beads after ethanol wash during the cleanup steps	Overdrying of beads	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
Shortage of enzyme reagents	Pipetting enzymes at –20°C	Allow enzyme reagents to equilibrate on ice for 10 minutes prior to pipetting.
Low library yields	Inaccurate input quantification	Quantify your gDNA with Qubit prior to bisulfite conversion. For cfDNA and FFPE, refer to section "DNA Input Considerations" here for detailed information.
	Low recovery from bisulfite conversion	Quantify DNA before and after bisulfite conversion. Add more DNA into conversion or increase number of PCR cycles.
	Low quality sample	Use the Alu primers (sold separately) to determine integrity of initial human DNA sample and adjust input quantity accordingly. Increase the number of PCR cycles.
	Overdrying of beads	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
	Use of the included polymerase for pre-hybridization PCR amplification or for DNA inputs >100 ng	For libraries going into hybridization capture, in place of the provided PCR Master Mix, use KAPA HiFi HotStart Master Mix and follow manufacturer cycling conditions. If input >100 ng, use uracil-tolerant mix. For more information, Contact Us.
Low mapping rates or low bisulfite conversion rates	Failing to bioinformatically trim Adaptase tails	Refer to the Tail trimming for better data technical note.

xGen™ Methyl-Seq DNA Library Prep Kit

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