xGen™ DNA Library Prep Kit MC
xGen DNA Library Prep Kit MC UNI

For research use only. Not for use in diagnostic procedures. | Version 2

REVISION HISTORY

Version	Release date	Description of changes
2	June 2022	Adjusted product size offerings
1	December 2021	Initial release

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OVERVIEW

The xGen™ DNA Library Prep Kits MC and MC UNI enables the construction of high complexity, next generation sequencing (NGS) libraries from double-stranded DNA (dsDNA) that has undergone DNA fragmentation by Covaris® shearing, or that does not require fragmentation (e.g., PCR amplicons). The workflow is designed for nanogram range inputs (1 ng to 1 µg) for direct sequencing or targeted sequencing using xGen Hybridization Capture Panels. With two enzymatic incubations and an optional PCR amplification, this two-hour workflow is ideal for high-throughput applications and is easily automated.

Protocol options

- The xGen DNA Library Prep Kit MC has an indexing by PCR workflow and includes xGen Stubby Adapters for compatibility with xGen Indexing Primers. PCR reagents are included in the kit; however, xGen Indexing Primers must be purchased separately.
- The xGen DNA Library Prep Kit MC UNI has an indexing by ligation workflow compatible with xGen Full-Length Adapters (not supplied in this kit, but available at the xGen Index Primers & Adapters page). This kit supports both a PCR-free workflow and includes P5/P7 amplification primers and polymerase for an optional library amplification step.

Alternatively, if an integrated enzymatic fragmentation step is preferred, the xGen DNA Library Kit EZ can be used.

To assess sample quality, quantification by qPCR using xGen Input DNA Quant Primers has been tested and shown to provide expected results. (Cat. No. 10009856; Contact applicationsupport@idtdna.com for more information about the Input DNA Quantification Assay).

xGen DNA Library Prep Kit MC and MC UNI workflows

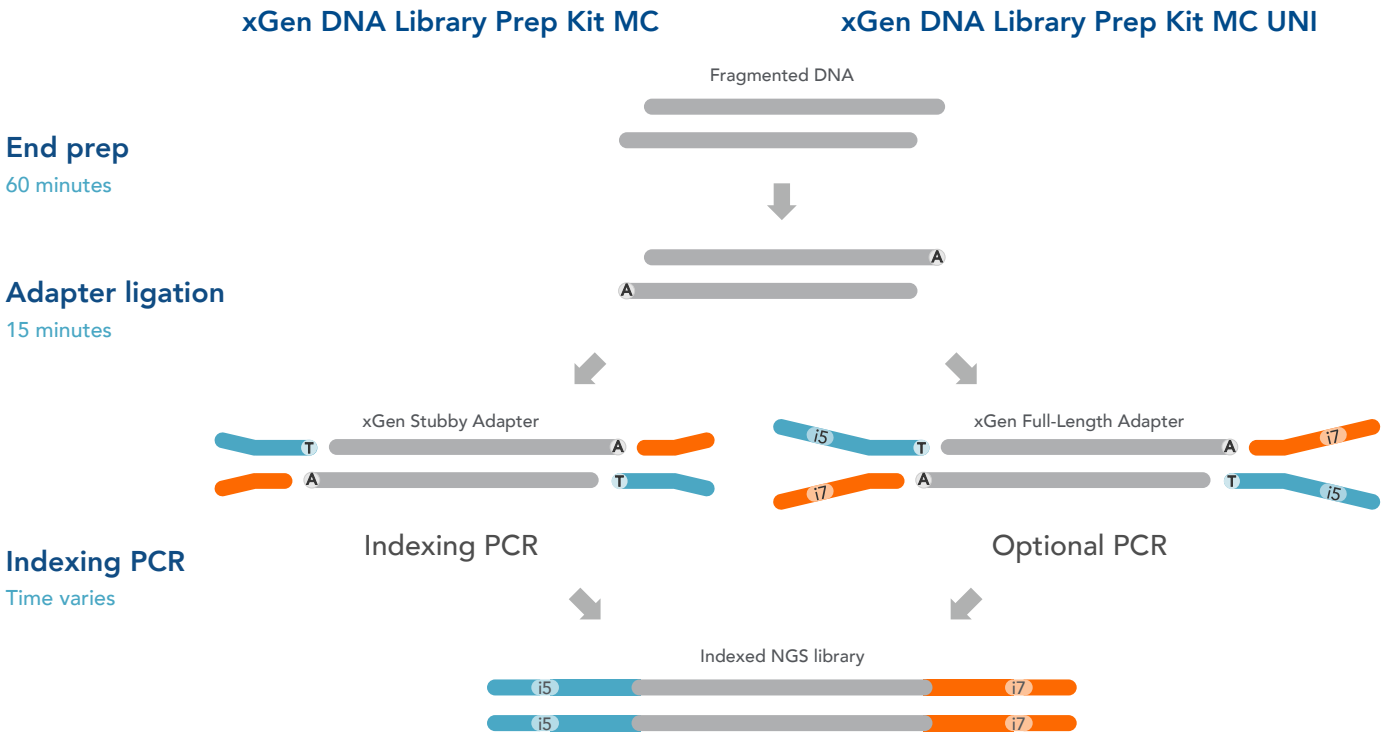

Figure 1. xGen DNA Library Prep Kit MC and MC UNI workflows. An NGS library is prepared from fragmented DNA in three main steps: 1) End prep consists of end repair, polishing, and dA-tailing of the fragmented DNA; 2) If using the xGen DNA Library Prep Kit MC (left), ligation of xGen Stubby Adapters is followed by 3) an indexing PCR step that generates the complete adapter sequence. If using the xGen DNA Library Prep Kit MC UNI (right), xGen Full-Length Adapters are ligated to the dA tails of the fragments. If necessary, an optional PCR amplification step can be added.

This protocol includes two enzymatic incubations, an optional PCR, and bead-based purification steps. This minimizes sample handling and reduces the overall library preparation time to two hours (with PCR). As depicted in Figure 1, the first incubation consists of end repair, polishing, and dA-tailing, performed in a single End Prep reaction. This is followed by ligation of either a stubby adapter (included in the xGen DNA Library Prep Kit MC, Figure 1, left) or a full-length adapter (purchased separately to be used with the xGen DNA Library Prep Kit MC UNI, Figure 1, right). The ligation step is followed by either an Indexing PCR step, if using the stubby adapter, or an optional, library amplification step if using full length adapters.

Note: Both kits are compatible with Normalase™ enzymatic treatment, an enzyme that creates equimolar libraries for NGS (see Appendix B: xGen Normalase instructions before starting your PCR setup).

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 stage	Component	16 rxn (µL)	96 rxn (µL)	Storage
End prep	• Buffer E1	83	496	–20°C
	• Enzyme E2	17	104	
	• Enzyme E3	17	104	
	• Enzyme E4	6	35	
Adapter ligation	• Buffer L1	317	1900	
	• Enzyme L2	105	632	
	• Reagent L3*	88	528	
PCR amplification	• PCR Master Mix	440	2640	
	• Reagent P2**	88	528	
	Low EDTA TE	6 mL	24 mL	Room temperature

*Reagent L3 is the xGen Stubby Adapter and is only supplied with the xGen DNA Library Prep Kit MC.
**Reagent P2 is the amplification primer pair P5/P7 and is only supplied with the xGen DNA Library Prep Kit MC UNI.

IDT—Customizable kit contents

Workflow component	Product name	Index number	Reaction size (rxn)	Catalog number
xGen core reagents	xGen DNA Library Prep Kit MC	N/A	16	10009861
	xGen DNA Library Prep Kit MC	N/A	96	10009819
	xGen DNA Library Prep Kit MC UNI	N/A	16	10009862
	xGen DNA Library Prep Kit MC UNI	N/A	96	10009820
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

* For index sequences, see the Index Sequence Master List. For adapter sequences, see Appendix C. 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 suppliers

Item	Supplier	Catalog #
0.2 mL PCR tubes or 96-well plates	Various suppliers	Varies
Aerosol resistant tips and pipette ranging from 1–1000 µL	Various suppliers	Varies
200–proof/absolute ethanol	Various suppliers	Varies
Nuclease-free water for preparation of 80% ethanol	Various suppliers	Varies
Fluorometric assays for library quantification	Various suppliers	Varies
qPCR library quantification kit	Roche/KAPA	Varies
High sensitivity Bioanalyzer® consumables, or equivalent	Agilent	Varies
SPRIselect® or Agencourt AMPure® XP beads, or equivalent	Beckman Coulter	Varies

Equipment

Item	Supplier	Catalog #
Covaris® ultrasonicator, or equivalent	Covaris	Varies
0.2 mL magnets for individual tubes and plates, or equivalent	Permagen	MSR812 MSP750
Fluorometer (Qubit™) or similar input DNA quantification instrument	Various suppliers	Varies
Bioanalyzer® or equivalent for library size determination	Agilent	Varies
Quantitative PCR instrument	Various suppliers	Varies
Microcentrifuge	Various suppliers	Varies
Programmable thermal cycler	Various suppliers	Varies
Vortex	Various suppliers	Varies

GUIDELINES

Storage and usage recommendations

- Upon receipt, store the reagents at –20°C except for Low EDTA TE, which is stored at room temperature.
- To maximize use of enzyme reagents when ready to use, 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 a shortage of enzyme reagents.
- After thawing reagents, briefly vortex (except the enzymes) to mix well. Spin all tubes in a microfuge to collect contents prior to opening.
- Assemble all reagent master mixes ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Always add reagents to the master mix in the specified order, as stated throughout the protocol.

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 the instructions below 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 tested with SPRIselect® beads (Beckman Coulter) and Agencourt AMPure® XP (Beckman Coulter). If other beads are used, solutions and conditions for DNA binding may differ.

Consider the information below to perform efficient size selection:

- Post-shearing, analyze the samples' size distribution by electrophoresis methods to determine the median fragment size of your dsDNA samples.
- The size selection process utilized in this protocol results in a Left Side Size Selection and is designed to produce an average fragment size of 350 bp for direct sequencing, or 200 bp for targeted sequencing.
- To customize size selection, refer to the recommendations found in the Beckman Coulter's SPRIselect User Guide or the application guide for your chosen size-selection beads.

Input DNA considerations

Input DNA quantification

- Determine dsDNA concentration using Qubit™, or a similar fluorometric method, as it measures the double-stranded, adaptable DNA content of your sample.
- A wide range of DNA concentrations (from 1 ng to 1 µg) have provided expected results with this kit.

DNA sonication

If working with samples that have already undergone (or do not require) fragmentation, omit this step. This protocol supports 200 bp and 350 bp sheared DNA in the Low EDTA TE buffer supplied with the kit. If the DNA volume post-shearing is less than 50 µL, add TE buffer to a final volume of 50 µL. Alternatively, samples can be diluted with the Low EDTA TE buffer. Other fragment sizes are compatible; contact applicationsupport@idtdna.com for further information.

Automation

- This protocol is readily automatable. A 10% overage volume of reagents is supplied to accommodate automation. Contact us at applicationsupport@idtdna.com if you require additional reagent overage volume or would like to learn about our custom packaging options.
- While IDT does not supply automated liquid handling instruments or consumables, our automation team collaborates with automation solution providers and customers to develop automated scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us at applicationsupport@idtdna.com for more information.

PROTOCOL

Perform end prep

1. Transfer fragmented dsDNA samples to a sterile 0.2 mL PCR tube and adjust the volume of the sample to a final volume of 50 µL using Low EDTA TE, if necessary.
2. Preset a thermal cycler according to the program listed below with the heated lid set to 70°C (a heated lid is required):

Temperature (°C)*	Time
4	Hold
20	30 min
65	30 min
4	Hold

*Thermal cycler lid at 70°C

3. Prepare the End Prep Master Mix in the order listed in the table below:

Reagents	Volume per sample (µL)
Low EDTA TE	3
• Buffer E1	4.7
• Enzyme E2	1
• Enzyme E3	1
• Enzyme E4	0.3
Total Master Mix	**10**
Fragmented DNA	50
Total volume	**60**

4. Thoroughly mix the Master Mix by moderate vortexing for 5 seconds.
5. Add 10 µL of premixed Master Mix to each sample and mix thoroughly by moderate vortexing for 5 seconds.
6. Spin down the sample tubes in a microfuge and place them in the chilled thermal cycler. Advance the program to the 20°C step.
7. Before completion of the thermal cycler program, prepare the Master Mix for the adapter ligation step. The samples should be kept at 4°C no more than one hour.

Important: Proceed to the adapter ligation step **within one hour** to avoid a loss in yield.

Perform adapter ligation

1. Preset a thermal cycler according to the program listed below with lid heating OFF.

Temperature (°C)*	Time
20	15 min

*Thermal cycler lid heating OFF

Important: When using DNA input <50 ng, Reagent L3 must be diluted in Low EDTA TE buffer to the appropriate concentrations, as outlined in the table below to achieve low levels of adapter dimer:

DNA input	Adapter
≥50 ng	No dilution
10 ng	10-fold (1:10)
1 ng	20-fold (1:20)

2. Use the appropriate table below based on the library prep kit and respective adapter type.
   - xGen DNA Library Prep Kit MC includes Reagent L3 that contains xGen Stubby Adapters
   - xGen DNA Library Prep Kit MC UNI is designed for ligation of xGen Full-Length Adapters

xGen DNA Library Prep Kit MC	
Reagents	Volume per sample (µL)
• Buffer L1	18
• Enzyme L2	6
• Reagent L3*	5
Total Master Mix	**29**
End-repair sample	60
Total volume	**89**

Note: Buffer L1 and Enzyme L2 can be prepared ahead of time and kept on ice. Add the Reagent L3 containing the xGen Stubby Adapter just before use.

xGen DNA Library Prep Kit MC UNI	
Reagents	Volume per sample (µL)
• Buffer L1	18
• Enzyme L2	6
Total Master Mix	**24**
End-repair sample	60
xGen Full-Length Adapter**	5 µL/sample
Total volume	**89**

** The xGen full-length adapters should be added individually to each sample to uniquely index each library. Use these adapters at a 15 µM stock concentration.

Note: Buffer L1 and Enzyme L2 can be prepared ahead of time and kept on ice.

When using the xGen DNA Library Prep Kit MC

1. Add 29 µL of the premixed Ligation Master Mix directly to the End Prep repair sample.
2. Thoroughly mix the Ligation reactions by moderate vortexing for 5 seconds. Spin down the sample tubes in a microfuge.
3. Place tubes in the thermal cycler. Advance the program to incubate samples at 20°C for 15 minutes with lid heating OFF.

When using the xGen DNA Library Prep Kit MC UNI

1. For xGen DNA Library Prep MC UNI users ONLY, add the xGen Full-Length Adapters individually to each sample.
2. Add 24 µL of the premixed Ligation Master Mix directly to the End Prep reaction mixture.
3. Thoroughly mix the Ligation reactions by moderate vortexing for 5 seconds. Spin down the sample tubes in a microfuge.
4. Place tubes in the thermal cycler. Advance the program to incubate samples at 20°C for 15 minutes with lid heating OFF.

Perform post-ligation cleanup

Important: Make sure the beads are at room temperature before you begin this procedure.

1. Purify the Adapter Ligation reaction using a SPRI bead suspension, magnetic rack, and freshly prepared 80% ethanol. Prepare enough 80% ethanol for ~1 mL per sample. Invert or briefly vortex beads to homogenize the suspension before use.
2. Add the specified SPRI bead volume to each sample. Mix by pipetting 10 times or until homogenous. Make sure no bead-sample suspension droplets remain on the sides of the tube.

Application	Average insert size (bp)	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
Direct Sequencing	350	89	72 (ratio: 0.8X)	21
Hybridization Capture	200	89	72 (ratio: 0.8X)	21

3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microfuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove and discard the supernatant, using a clean pipette tip, without disturbing the pellet.
6. Add 200 µL of freshly prepared, 80% ethanol solution to the pellet while it is still on the magnet. Be careful to not disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
7. Repeat step 6 once more for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microfuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Immediately add 21 µL of Low EDTA TE to resuspend the pellet without overdrying the beads; mix well by pipetting up and down until homogenous. If droplets of the resuspension are on the side of the tube, pulse-spin the tube in a microfuge to collect contents. After at least 2 minutes, place the tube on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).
10. Transfer 20 µL eluate to a new 0.2 mL PCR tube. Make sure that the eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on magnet, and transfer eluate again.

Safe Stop: Store the purified, post-ligation, cleaned libraries at –20°C if not proceeding directly to library amplification or PCR-free sequencing.

PCR-free libraries are now ready for quantification, which should be performed by qPCR to ensure accuracy. Note that PCR-free libraries cannot be accurately quantified or assessed for library size by electrophoretic methods.

Important: A second cleanup (using a 0.8X) may be beneficial when PCR-free libraries are prepared for direct sequencing on patterned flow cells with the potential for index hopping. If using unique, dual indexed adapters, this additional cleanup is not necessary.

Perform PCR amplification

The ligation step is followed by either an Indexing PCR step if using xGen Stubby Adapters (Reagent L3 supplied in xGen DNA Library Prep Kit MC), or an optional library amplification step if using xGen Full-Length Adapters (with xGen DNA Library Prep Kit MC UNI).

Note: Use the HiFi polymerase Master Mix supplied in the kits for both direct sequencing and pre-hybridization capture PCR workflows.

DNA Input	Minimum recommended cycles*	
	xGen DNA Library Prep MC	xGen DNA Library Prep MC UNI
1 µg	3†	0–3
100 ng	3†	0–3
10 ng	6–7	6–7
1 ng	9–10	9–10

* These recommendations are for direct sequencing (>4 nM). For downstream hybridization capture, more PCR cycles are needed to generate the recommended amount of >500 ng. See Appendix B for the recommended cycles to generate higher yields.

† When indexing by PCR, a minimum of 3 cycles is required to complete adapter sequences, irrespective of whether enough library is available following ligation.

1. Preset the following thermal cycler program, adjusting the number of cycles depending on the input amount and workflow (see table above).

Number of Cycles	Temperature (°C)*	Time
	98	2 min
Cycles based on previous table	98	20 sec
	60	30 sec
	72	30 sec
	72	1 min
	4	Hold

*Thermal cycler lid kept at 105°C

2. Depending on which adapter was used during the ligation step, follow Workflow A: Indexing PCR when xGen Stubby Adapters and xGen Dual Indexing Primer Pairs (xGen DNA Library Prep MC) were used; follow Workflow B: Library amplification when xGen Full-Length Adapters and Reagent P2 (P5/P7 amplification primers) were used.

Workflow A: Indexing PCR

Add indexing primers directly to the eluted library, add 25 µL PCR Master Mix to each sample (see table below). Mix by moderate vortexing for 5 seconds and spin down in a microfuge.

Note: If using xGen Normalase Indexing primer pairs, see Appendix B: xGen Normalase 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

Step A Master Mix	
Reagents	Volume per sample (µL)
• PCR Master Mix	25
Sample + Primer Mix	25
Total volume	**50**

Workflow B: Library amplification

For optimal amplification of fully indexed libraries, add Reagent P2 to the PCR Master Mix (see table below). Add 30 µL of the prepared PCR Master Mix to the eluted samples and mix by moderate vortexing for 5 seconds, then spin samples down in a microfuge.

xGen DNA Library Prep Kit MC UNI	
Reagents	Volume per sample (µL)
• PCR Master Mix	25
• Reagent P2	5
Total Master Mix	**30**
Eluted sample	20
Total volume	**50**

Place tubes in the thermal cycler and run the program from step 1.

Perform post-PCR cleanup

Important: Make sure the beads are at room temperature before you begin this procedure.

1. Purify the PCR amplification reaction using a SPRI bead suspension, magnetic rack, and freshly prepared 80% ethanol. Invert or briefly vortex beads to homogenize the suspension before use.
2. Add the specified SPRI bead volume to each sample. Mix by pipetting 10 times or until homogenous. Make sure that there are no bead-sample suspension droplets left on the sides of the tube.

Application	Average insert size (bp)	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
Direct sequencing	350	50	32.5 (ratio: 0.65X)	21
Hybridization capture	200	50	75 (ratio: 1.5X)	21

3. Incubate the samples for 5 minutes at room temperature.
4. Pulse-spin the samples in a microfuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
5. Remove and discard the supernatant without disturbing the pellet.
6. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Do not to disturb the pellet. Incubate for 30 seconds, then carefully remove the ethanol solution.
7. Repeat step 6 once more for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microfuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Without delay to avoid overdrying beads, add 21 µL of Low EDTA TE to resuspend the pellet, then mix well by pipetting up and down until homogenous. If droplets of the suspension are on the side of the tube, pulse-spin the tube in a microfuge to collect contents. After at least 2 minutes, place the tube on the magnetic rack and wait until the solution clears and a pellet is formed (~2 minutes).
10. Transfer 20 µL eluate volume to a new, 0.2 mL PCR tube. Make sure that the eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on magnet, and transfer eluate again.

    Important: If you are direct sequencing on patterned flow cells, perform a second cleanup to ensure optimal removal of the unincorporated primers, which can increase index hopping on patterned flow cells. Repeat steps 1–10 above using a 1.2X ratio. This second purification is not necessary when using either xGen Unique Dual Indexing primer pairs/adapters, or the xGen Normalase Module, or when libraries will go into an xGen Hybridization Capture.

Application	Average insert size	Sample volume (µL)	Bead volume (µL)	Elution volume (µL)
Direct sequencing	350 bp	20	24 (ratio: 1.2X)	20

Perform library quantification

The library is now ready for quantification, which can be performed using fluorometric methods (e.g., Qubit™) or qPCR. An electrophoretic chip (e.g., high sensitivity DNA Agilent Bioanalyzer kit) can be used to ensure desired library size and confirm quantification. Store libraries at –20°C.

APPENDIX A: RECOMMENDED PCR CYCLES

Use this table to guide the PCR cycles you will use when generating >500 ng of amplified library.

Input DNA into End Prep step	Number of cycles required to generate	
	100 ng library	1 µg library
1 µg	0–3*	2–3*
100 ng	2–3*	6–7
10 ng	7–9	11–13
1 ng	13–15	17–19

* When using the xGen DNA Library Prep Kit MC and indexing by PCR, a minimum of 3 cycles is required to complete adapter sequences, irrespective of whether enough library is available following ligation.

APPENDIX B: xGEN NORMALASE INSTRUCTIONS

Important: Carefully review this section and the **xGen Normalase Module** protocol before setting up your Normalase PCR. To achieve expected results, amplify each library using the xGen Normalase primer pairs with the appropriate number of cycles and thermocycling conditions as shown; this will provide a library yield of 12 nM or greater, in a 20 µL eluate.

For the xGen DNA Library Prep Kit MC

The xGen DNA Library Prep MC uses xGen Normalase CDI and UDI primer pairs.

1. To each sample, add 2 µL of each Normalase CDI primer* or 4 µL of Normalase UDI primer pairs† for a total volume of 24 µL (eluted DNA sample and primers). See Tables in step 2.

Indexing options	Reagents	Volume per sample (µL)
xGen Normalase CDI primer pairs	i5 primer i7 primer	2 2
xGen Normalase UDI primer pairs	Premixed primer pair	4

* Requires 1 µL Reagent R6 (refer to Tables in step 2)

† Requires 1 µL Reagent R7 (refer to Tables in step 2)

2. Assemble the PCR Master Mix on ice. Mix thoroughly and pulse-spin to collect contents. Add 26 µL of the Total Master Mix (PCR Master Mix + Reagent R7 OR PCR Master Mix + Reagent R6) to each sample tube, thoroughly mix, then pulse-spin to collect contents (50 µL total reaction volume).

xGen Normalase UDI primer pairs	
Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R7	1
Total Master Mix	**26**
Eluted sample and primers	24
Total volume	**50**

xGen Normalase CDI primer pairs	
Component	Volume per reaction (µL)
• PCR Master Mix	25
• Reagent R6	1
Total Master Mix	**26**
Eluted sample and primers	24
Total volume	**50**

3. Run the following thermal cycler program with the lid kept ON, adjusting the number of cycles depending on the input amount (see Table below).

Number of Cycles	Temperature (°C)*	Time
	98	2 min
Cycles based on next table	98	20 sec
	60	30 sec
	72	30 sec
	72	5 min
	4	Hold

*Thermal cycler lid kept ON

Use this table for the recommended minimum number of cycles for each input to provide ≥12 nM yields suitable for the xGen Normalase workflow:

DNA input (ng)	Minimum number of cycles for ≥12 nM
≥100	3
25	5
10	7
1	10

Note: The number of cycles required can vary not only based on the input amount, but also on the quality of the sample.

4. Proceed to Perform post-PCR cleanup.
5. Proceed to Normalase I, pooling, and Normalase II in the xGen Normalase Module protocol.

For the xGen DNA Library Prep Kit MC UNI

The xGen DNA Library Prep MC UNI uses Reagent R5 for Normalase PCR.

1. If you typically obtain the required minimum threshold (i.e., ≥12 nM following library amplification), use Normalase primers (Reagent R5 included in the xGen Normalase Module) and add one additional PCR cycle to your program.
2. If your library yields are ≥12 nM before amplification, a minimum of 3 cycles is still required to condition the libraries for downstream Normalase enzymology.
3. Assemble the PCR Master Mix using standard PCR reagents as shown in the table below, except substitute standard primers (Reagent P2) with 5 µL of Reagent R5. Thoroughly mix by moderate vortexing, pulse-spin to collect contents, then place in the thermal cycler.

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

4. Repeat steps 3–5 of the For the xGen DNA Library Prep Kit MC section.

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 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′

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

APPENDIX D: PRIMER SEQUENCES

As a reference, the primer sequences are listed below. These primers include full-length Illumina adapter and index sequences.

i7 primer: Replace 8 bases for CDI, and 8 or 10 bases for UDI **X's** with the **REVERSE COMPLEMENT** of the specified i7 index sequence in the Index Sequences Master List:

5´–CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX(XX)**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT–3´

i5 primer: Replace 8 bases for CDI and 8 or 10 bases for UDI **Y's** with the specified Forward Strand Workflow i5 index sequence in the Index Sequences Master List:

5´–AATGATACGGCGACCACCGAGATCTACAC**YYYYYYYYYY**ACACTCTTTCCCTACACGACGCTCTTCCGATCT–3´

To confirm the compatibility of your own primers with the xGen Amplicon workflow, contact applicationsupport@idtdna.com. To purchase custom Normalase Indexing Primers, contact your local sales representative or distributor.

APPENDIX E: TROUBLESHOOTING

Issue	Possible cause	Suggested remedy
Library migrates unexpectedly on Bioanalyzer	Over-amplification of library leads to the formation of heteroduplex structures that migrate abnormally. xGen UDI-UMI libraries without PCR will not migrate correctly due to the forked ends.	Perform the minimum number of PCR cycles necessary to avoid over-amplification. Quantify library by qPCR, as other quantification methods will not accurately detect heteroduplex library molecules.
Incomplete resuspension of beads after ethanol wash during purification steps	Overdrying of beads	Continue pipetting the liquid over the beads to break up clumps for complete resuspension. To avoid overdrying, 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 before pipetting.
Retention of liquid in pipette tip	Viscous reagents (e.g., Buffer L1) may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to make sure that all liquid is released from the pipette tip.
Unexpected increase in adapter dimers	Improper adapter dilution Improper bead purification Reagent L3 not added to the ligation master mix just before use.	Use the specified dilution for your input quantity. Use the specified bead volume particularly for the post-PCR purification. Add adapter to your ligation master mix just before use.

xGen DNA Library Prep Kit MC

xGen DNA Library Prep Kit MC UNI

Technical support: applicationsupport@idtdna.com