xGen™ Normalase™ Module

Table of contents

Overview 4

xGen Normalase Module workflow 5

- Workflow options 5

Normalase Module workflow 6

- Specifications 7

Consumables and equipment 8

- Consumables from IDT—Kit contents 8
  - xGen Normalase Module 8
- Consumables from IDT—Reagents 8
- Consumables from other suppliers 9
- Equipment 9

Important guidelines 10

- Meeting the 3X minimum threshold 10
- Normalase PCR recommendations 10
- Optional adjustments to final library pool 11
- Preparing reagent mixes and ethanol 11
- Automation 11

Protocol 12

- Perform Normalase PCR 12
  - When indexing by Adapter Ligation 12
  - When indexing by Normalase PCR with UDI primers 12
  - When indexing by Normalase PCR with CDI primers 13
  - Thermal cycler program 13
- Perform post-PCR cleanup 14
- (Optional) Quantification 14
- Perform the Normalase I step 15
- Perform equal volume library pooling 16

Perform the Normalase II step 17

Perform Normalase inactivation 17

Calibration of xGen Normalase output 18

Appendix A: Low-plex pooling recommendations 19

Normalase II reaction 19

Normalase inactivation 20

Appendix B: High sample volume pooling recommendations 21

Normalase II step 22

Normalase inactivation 22

Appendix C: Indexed adapter sequences 23

Appendix D: Troubleshooting 23

OVERVIEW

The xGen™ Normalase™ Module is an enzymatic normalization method for multiplexed sequencing of next-generation sequencing (NGS) libraries. This method streamlines library normalization by eliminating individual sample quantification and by performing equal volume pooling. The resulting pool has a sample read depth with a CV ≤10%, which is more uniform than library pools generated using qPCR quantification, fluorometric assay, and normalization beads.

The xGen Normalase technology is designed for sample types and library preparation workflows that produce consistent amplified library yields of ≥12 nM per library. The library must also include an amplification step using Normalase primers. Normalase chemistry can be adjusted to a lower minimum yield threshold of 6 nM for sample types or workflows that produce lower library yields.

See this table or simple calculator for library concentration in ng/µL.

Library yield (nM)	200 bp insert*† (ng/ µL)	350 bp insert*† (ng/ µL)
12	2.48	3.6
6	1.24	1.8

* 135 bp adapter used for calculating ng/µL; all quantities require 20 µL volume.

† 617.96 was used for average base pair MW.

xGEN NORMALASE MODULE WORKFLOW

This protocol has the following steps:

- **Normalase PCR** performs amplification to a 3X minimum yield of ≥12 nM (or ≥6 nM) using Normalase primers
- **The Normalase I step** enzymatically selects a 4 nM library fraction (or 2 nM if using ≥6 nM option).
- **Equal volume pooling** of libraries for multiplexed sequencing.
- **The Normalase II step** enzymatically generates an equal molar library pool

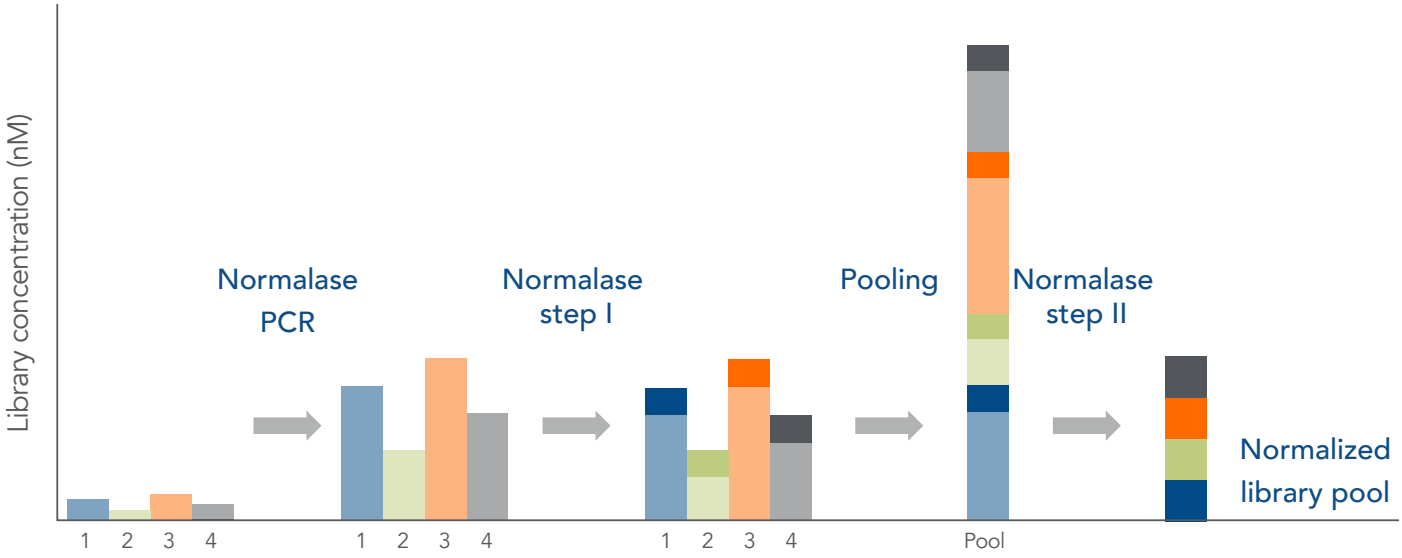

Figure 1. The xGen Normalase Module generates an equimolar library pool for multiplexing. Normalized libraries are produced in four main steps. First, Normalase PCR increases the initial library concentrations. Second, Normalase I treatment selects a 4 nM library fraction. Third, the samples are pooled in equal amounts for multiplexed sequencing. Finally, the Normalase II step generates the final pool of libraries. Four libraries following adapter ligation are shown on the first through third step, where Normalase PCR produces ≥12 nM library yields using Normalase primers, followed by the Normalase I step (where the 4 nM selected fraction is shaded darker at the top of the bars in this figure). Then, equal volume pooling and the Normalase II step produce an equal molar (normalized) library pool.

Workflow options

The xGen Normalase technology is compatible with library preparation workflows that add index sequences via adapter ligation with full-length, indexed adapters, and Normalase primers (Reagent R5, included in the Normalase Module) to amplify and condition libraries for downstream enzymology.

xGen Normalase technology is also compatible with library preparation workflows that add index sequences by PCR with stubby adapters and Normalase indexing primers (supplied separately from the Normalase Module) to index, amplify, and condition libraries for downstream enzymology. These include:

- xGen Normalase Combinatorial Dual Indexing Primers with Reagent R6 (up to 96-plex)
- xGen Normalase Unique Dual Indexing Primer PLATES with Reagent R7 (up to 1536-plex)

The following polymerases have been tested with Normalase PCR and have provided results as expected. Use of materials, other than those listed, may provide unexpected results.

- HiFi Polymerases supplied in xGen Library Prep Kits
- KAPA® HiFi HotStart ReadyMix (Roche)
- NEBNext® Ultra II Q5 Master Mix

Contact applicationsupport@idtdna.com for questions regarding compatibility with the xGen Normalase workflow.

NORMALASE MODULE WORKFLOW

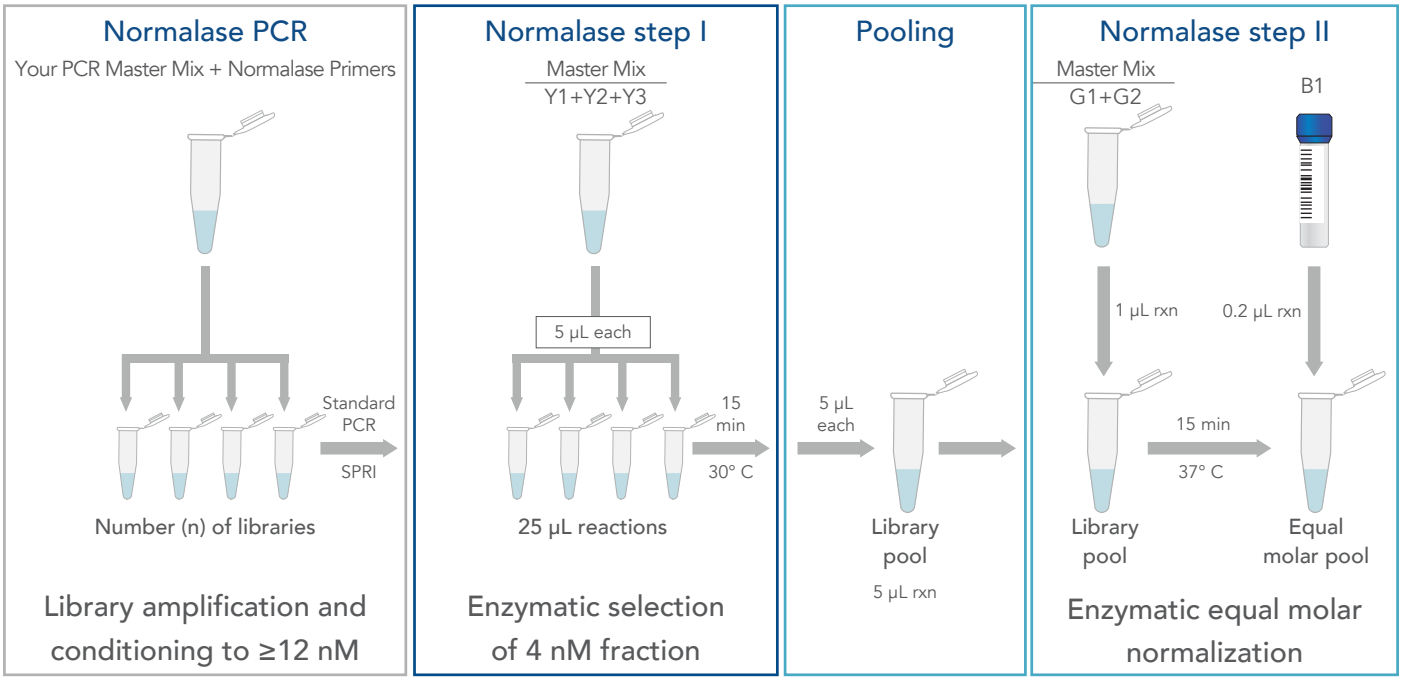

Figure 2: The Normalase Module workflow. The end-to-end workflow consists of four steps, including a PCR, two Normalase reactions, and sample pooling.

The Normalase Module workflow consists of four main steps:

1. **Normalase PCR:** This step replaces primers in conventional library amplification with Normalase primers, but is otherwise the same as a conventional library amplification. Even if library yields have met the 12 nM minimum before PCR amplification, performing a minimum of 3 cycles is required to condition each library for downstream Normalase enzymology. Similarly, a minimum of 3 cycles for PCR amplification is required if using the alternative 6 nM normalization workflow to condition the libraries for downstream enzymology.
2. **Normalase I reaction:** Following Normalase PCR and purification, Normalase I Master Mix is added to samples and incubated at 30°C for 15 minutes. During this step, the Normalase enzyme selects a 4 nM library fraction. If using the alternative 6 nM workflow, the Normalase I Master Mix is adjusted to enzymatically select a 2 nM library fraction; subsequent steps are performed according to the standard protocol.

   Tip: For best results, process all samples that will be pooled together for co-sequencing with the same Normalase I Master Mix.

   Very accurately pipette both Reagent Y2 and the Normalase I Master Mix during this step.
3. **Equal volume pooling:** During this step, 5 µL of each library to be co-sequenced is pooled into a single tube. Note your selected sequencing instrument loading concentration and volume requirements as well as the number of samples to be pooled for multiplexed sequencing. These factors may require a different pooling volume; see Appendix A and B for alternate pooling guides if needed.
4. **Normalase II reaction:** The library pool is incubated with Normalase II Master Mix, and then Reagent B1 for inactivation, to produce a final equal molar library pool of 4 nM (or 2 nM). The final pool is then quantified by qPCR to determine an accurate concentration before loading on the sequencer, but not by fluorometric or electrophoretic methods, due to the incompatibility of ssDNA library content.

Specifications

The xGen Normalase product specification is defined by the cluster density of a Normalase pool when loaded on a MiSeq™ (Illumina) v2 flow cell at a concentration of 12 pM. The expected result is to achieve a 1000–1200 K/mm2 cluster density and CV ≤ 10% within a pool (using xGen DNA EZ libraries with an average 200 bp insert size). Across Illumina® platforms, library types, insert sizes, and correct loading concentration are all necessary to achieve the number of reads required for your research application and supported by the chosen flow cell.

xGen Normalase technology is suitable for libraries prepared for direct sequencing (e.g., whole genome, whole transcriptome). xGen Normalase technology is not compatible with PCR-free sequencing.

Reliable results are obtained using libraries with uniform size distributions generated by Covaris® shearing or enzymatic fragmentation (i.e., 200–350 bp inserts) and bead-based size selection. Libraries with broad or variable size distribution (e.g., some transposase-based workflows) that demonstrate size-dependent clustering effects on Illumina® sequencers, which are independent of molarity, may have more variable results.

CONSUMABLES AND EQUIPMENT

For library preparation workflows that add indexes via adapter ligation with full-length, indexed adapters, an xGen Normalase Module is required for compatibility with the Normalase workflow. For those workflows, R5 primers are used in place of standard amplification primers.

For library preparation workflows that add indexes via PCR, an xGen Normalase Module and xGen Normalase indexing primers are required for compatibility with the Normalase workflow.

Note: Normalase indexing primers are supplied separately and used in place of standard indexing primers.

Consumables from IDT—Kit contents

xGen Normalase Module

Workflow steps	Component	Volume (µL) 96 rxns	Storage
Normalase PCR	• Reagent R5	528	–20°C
Normalase I step	• Buffer Y1	454	
	• Reagent Y2	21	
	• Enzyme Y3	52	
Normalase II step	• Buffer G1*	506	
	• Enzyme G2*	21	
	• Reagent B1*	105	
Additional reagent	Low EDTA TE	2 X 1200	Room temperature

* These reagents are provided at a 5-fold excess plus overage (when pooling 5 µL) so that library pooling and subsequent steps can be repeated for flexibility in re-pooling and re-sequencing.

Component	Volume (µL) 96 rxns	Storage
• Reagent R6	105	–20°C
i5 Primer	26	
i7 Primer	18	

**If using Normalase Unique Dual Indexing (UDI) Primer Plates, see the separate Index Plate Protocol for reagent volumes that are supplied, as well as handling instructions.

Consumables from IDT—Reagents

Item	Catalog number
xGen Library Prep Kit	Varies
xGen UDI-UMI indexed adapters (16 to 96-plex) if indexing by adapter ligation and using a library prep kit compatible with T-overhang adapters*	10006914 10005903
xGen Normalase Module	10009793, 100010159
If indexing by PCR, choose one:	10009794
xGen Normalase CDI Primers (96-plex)	10009795-10009800
xGen Normalase UDI Primer PLATES (96 to 1536-plex)	10009811-10009812

* xGen DNA Library Kit EZ UNI and xGen DNA Library Kit MC UNI, for example.

Consumables from other suppliers

Item	Supplier	Catalog Number
HiFi Polymerase, if not supplied with the library prep kit: Kapa™ HiFi™ HotStart Readymix NEBNext® Ultra™ II Q5® Master Mix	Roche, Kapa NEB, various	KK2602 M0544
qPCR-based library quantification kit for determining final library pool concentration (Kapa™)	Roche	KK4824
Qubit® or another fluorometric-based assay for determining individual library concentration prior to Normalase	Thermo Fisher Scientific	Q32851/Q32854
Magnetic beads for the cleanup step: SPRISelect™ beads (or other beads recommended by your xGen Library Prep Kit)	Beckman Coulter	B23317/B23318/B23319 A63880/A63881/A63882
0.2 mL PCR tubes	Various	Varies
1.5 mL microcentrifuge tubes (if high pooling volume is required)	Various	Varies
Aerosol-resistant, low retention pipette tips, 2 to 1000 µL	Various	Varies
200-proof/absolute ethanol (molecular biology-grade)	Various	Varies
Nuclease-free water (molecular biology-grade)	Various	Varies

Equipment

Item	Supplier	Catalog number
Magnetic rack for the cleanup step Permagen® 0.2 mL PCR strip or plate magnet, or equivalent magnet	Permagen	MSR812 MSP750
Microcentrifuge	Various	Varies
Vortex machine	Various	Varies
Programmable thermal cycler	Various	Varies
Fluorometer—Qubit® or other quantification	Various	Varies
Heat block that adjusts from 37–95 °C (if a high pooling volume is required)	Various	Varies
Real time thermal cycler (for qPCR library pool quantification)	Various	Varies

IMPORTANT GUIDELINES

Meeting the 3X minimum threshold

To ensure that all library yields have a concentration ≥12 nM in the 20 µL eluate following amplification with Normalase primers, accurate quantification of input material for library prep is important to determine the required number of PCR cycles to reach the minimum threshold. Similarly, ensure that each library has a concentration ≥6 nM in 20 µL eluate if using the alternative, lower threshold normalization workflow.

For xGen library preparation workflows compatible with Normalase treatment, refer to the xGen Library Prep Kit protocol for the minimum recommended number of Normalase PCR cycles to obtain a library concentration ≥12 nM for the supported input quantities.

Important: Achieving the minimum threshold for every library is required to achieve expected results. Libraries that do not meet this threshold will be under-represented during cluster generation and will impact the percent coefficient of variation (%CV) of the sequencing data.

Normalase PCR recommendations

When indexing by adapter ligation, use Reagent R5 during the Normalase PCR step:

- If you typically obtain the required minimum threshold, simply replace your conventional primers with Normalase primers (Reagent R5) and add one additional PCR cycle to your program. If, prior to amplification, your library yields are ≥12 nM (or 6 nM, if following the lower minimum threshold option), a minimum of 3 cycles is still required to condition the libraries for downstream Normalase enzymology. If using compatible xGen library prep kits, see the Normalase section in the library kit protocol Appendix section for the recommended minimum number of PCR cycles and other instructions.

When indexing by Normalase PCR, use Normalase Indexing Primers and Reagent R6 or R7 during the Normalase PCR step:

- Normalase indexing primers complete the adapter sequences, as well as amplify and condition libraries for downstream Normalase steps. If using compatible xGen library prep kits, see the Normalase section in the library kit protocol Appendix for the recommended minimum number of PCR cycles and other instructions. If using kits from other suppliers, be familiar with the number of cycles required to achieve the required minimum threshold following library amplification and add 1 or more additional cycles when using Normalase Indexing Primers instead of conventional indexing primers.

For index specific sequences, see the Index Sequences Master List. For adapter sequences, see Appendix C of this protocol.

Important: When using the xGen RNA Library Kit, see the xGen Normalase Module section in the RNA kit protocol for a required modification when using Normalase Indexing primers.

Optional adjustments to final library pool

If you have chosen the lower 6 nM concentration option, which is formulated to select a 2 nM fraction of each library, but require a higher pool concentration for your sequencing instrument, perform a 2.0X bead-based cleanup to concentrate the pool and then proceed to qPCR quantification and loading of the sequencing instrument. Since libraries are single stranded after the Normalase workflow, performing a 2.0X bead ratio retains single stranded molecules. This process can also be performed to further concentrate a 4 nM pool using the standard protocol.

Important: When performing a 2.0X SPRI to concentrate a library pool, the Normalase inactivation can be eliminated if the bead cleanup is performed directly after the Normalase II step.

Preparing reagent mixes and ethanol

To prepare master mixes, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss.

- To prepare reagent master mixes for Normalase PCR, Normalase I, and Normalase II steps, ensure the reagent vials and enzymes are thawed on ice. After thawing reagents, briefly vortex reagents (except the enzymes) to mix them, and spin tubes in a microcentrifuge to collect contents prior to opening.
- Prepare the master mixes at room temperature and always add reagents in the specified order. If preparing in advance, store master mixes on ice until use, then add to samples at room temperature. Master mixes can be prepared and used at room temperature if prepared just before use.
- Prepare a fresh, 80% ethanol solution using 200 proof/absolute ethanol and nuclease-free water. Approximately 0.5 mL of 80% ethanol solution will be used per sample.

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 volumes or for custom packaging options.

While Integrated DNA Technologies does not supply automated liquid handling instruments or consumables, our Automation Team collaborates with automation solution providers and customers to develop and qualify optimized scripts for use of our kits with liquid handling platforms routinely used in NGS library preparation. Contact us at applicationsupport@idtdna.com to discuss automating the xGen Normalase workflow with your automated liquid handling system.

PROTOCOL

Perform Normalase PCR

When indexing by Adapter Ligation

Assemble Master Mix using standard PCR reagents, as indicated in your xGen Library Kit protocol, except include 5 µL of Reagent R5 (Normalase primers) instead of the standard primers. Thoroughly mix by moderate vortexing and pulse-spin to collect contents. Add 30 µL Master Mix to each sample, mix by vortexing, pulse-spin, and place samples in the thermal cycler.

Note: The xGen Library Kits include two different polymerases: KAPA HiFi HotStart ReadyMix (Cat. No. KK2602) and NEBNext Ultra II Q5 Master Mix (Cat. No. M0544).

Component	PCR rxn volume (µL)
Sample	20
Polymerase Master Mix	25
• Reagent R5	5
Master Mix volume	**30**
Final volume	**50**

When indexing by Normalase PCR with UDI primers

Assemble the Master Mix using standard PCR reagents, as indicated in your xGen Library Kit protocol, but also add 1 µL Reagent R7. Thoroughly mix samples by moderate vortexing and pulse-spin to collect contents.

Add 4 µL each of a unique Normalase UDI Primer Pair (delivered premixed) to each sample. Add 26 µL Master Mix to each sample, mix by gentle vortexing, pulse-spin, and place samples in the thermal cycler.

Component	PCR rxn volume (µL)
Sample	20
UDI Primer Pair	4
Sample volume	**24**
Master Mix	25
• Reagent R7	1
Master Mix volume	**26**
Final volume	**50**

When indexing by Normalase PCR with CDI primers

1. Assemble the Master Mix by using standard PCR reagents, as indicated in your xGen Library Kit protocol, but also add 1 µL Reagent R6. Thoroughly mix by moderate vortexing, and pulse-spin to collect contents.
2. Add 2 µL each of i5 and i7 of a unique Normalase UDI primer to each sample. Then add 26 µL Master Mix to each sample, mix, pulse-spin, and place in the thermal cycler.

Component	PCR rxn volume (µL)
Sample	20
CDI i5 Primer	2
CDI i7 Primer	2
Sample volume	**24**
Master Mix	25
• Reagent R6	1
Master Mix volume	**26**
Final volume	**50**

Thermal cycler program

Refer to the Normalase Module section in the Appendix section of your xGen Library Kit protocol and follow the recommended PCR thermal cycler program listed for Normalase PCR.

If using an alternative polymerase, see the table below for recommended thermal cycler program settings.

Kapa HIFI HotStart ReadyMix	NEBNext Ultra II Q5 Master Mix*
Heated lid at 105°C	
98°C for 45 seconds PCR cycles:	98°C for 30 seconds PCR cycles:
98°C for 15 seconds	98°C for 10 seconds
60°C for 30 seconds	60°C for 30 seconds
72°C for 30 seconds	72°C for 60 seconds
Final extension of 72°C for 5 minutes	
Hold at 4°C"	

* If using Q5, add one additional cycle to your standard number of PCR cycles per sample.

Perform post-PCR cleanup

Important: Make sure the magnetic beads are at room temperature before starting.

1. Vortex beads to homogenize the suspension before use.

   Note: Use the same post-PCR bead ratio as recommended by your xGen Library Kit to achieve consistent results.

2. Add beads to each sample, according to the ratio recommended in the xGen Library Kit. Mix the sample and beads by moderate vortexing. Pulse-spin the mixture in a microcentrifuge.
3. Incubate samples for 5 minutes at room temperature.
4. Place samples 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 (less than 5 µL may be left behind).
6. Add 200 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 with a clean pipette tip.
7. Repeat the previous step for a second wash with the 80% ethanol solution.
8. Pulse-spin the samples in a microcentrifuge and place them back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube using a 200 µL pipette tip.
9. Remove samples from magnetic rack. Add 22 µL of Low EDTA TE buffer and resuspend the pellet. Mix well until homogenous by pipetting up and down.
10. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
11. Carefully transfer 20 µL of the sample to a new 0.2 mL PCR tube, without carry-over of any beads.

    Safe Stop: If needed, store libraries overnight at –20°C post-Normalase PCR cleanup.

(Optional) Quantification

If you wish to confirm that library yields have achieved the minimum threshold of >12 nM (or 6 nM), perform a library quantification (i.e., fluorometric, electrophoretic, or qPCR) before proceeding with the Normalase I step.

Quantification is recommended when implementing xGen Normalase technology to ensure that the sample type and PCR cycles used can reach the minimum yield threshold by the end of this protocol.

Perform the Normalase I step

If the 12 nM threshold has been met for all libraries after Normalase PCR, proceed with the standard instructions. If not, a Normalase I modification can be performed to enable a lower 6 nM threshold (see below).

1. Preset a thermal cycler program as listed below.

Normalase I Program
15 min at 30˚C with open lid or lid heating OFF

2. Prepare the Normalase I Master Mix as described in the table below. The mix can be prepared at room temperature and stored on ice until use, if prepared in advance. Ensure that it is thoroughly mixed by moderate vortexing, followed by a pulse-spin to collect contents.

   Important: If utilizing the lower threshold 6 nM yield concentration, prepare a 2-fold dilution of Reagent Y2 using an equal volume of low EDTA TE, then proceed with preparation of the Normalase I Master Mix as described in the table below:

Reagent	Volume per library	24 Libraries (µL)	96 Libraries (µL)
• Buffer Y1	4.3	103.2	412.8
• Reagent Y2*	0.2	4.8	19.2
• Enzyme Y3	0.5	12	48
Total volume	5.0	120	480

* For the 6 nM option, dilute Reagent Y2 2-fold with an equal volume xGen Low EDTA TE Buffer. The Normalase I Master Mix should be built for a minimum 10 reactions to ensure pipetting accuracy.

Important: The Normalase I Master Mix should be made for a minimum of 10 reactions to ensure pipetting accuracy. For best results, process all samples that will be pooled together for co-sequencing with the same Normalase I Master Mix. Samples prepared with different Normalase I Master Mixes may result in variation in final normalization volumes and lead to a higher %CV than expected.

3. Using a calibrated pipette, add 5 µL of Normalase I Master Mix into each 20 µL library eluate at room temperature. Thoroughly mix by moderate vortexing for 5 seconds.
4. Spin down the sample tube in a microcentrifuge, then place in the thermal cycler and run the program.

   Safe Stop: If needed, store libraries overnight at –20°C after performing the Normalase I step.

Perform equal volume library pooling

Sufficient Normalase II reagents are supplied so that this step can be repeated to enable various re-pooling combinations. Only 5 µL of post-Normalase I library (out of 25 µL volume) is used for pooling. Also note that the stability of normalized library pools is limited, with a maximum storage time of four weeks, since the resulting normalized pools contain single-stranded DNA. Therefore, if re-sequencing is required after four weeks, re-pool the Normalase I libraries and repeat the Normalase II step and Normalase inactivation.

Notes:

- If you are pooling fewer than 5 libraries, see Appendix A for low-plex pooling recommendations.
- If pooling 5 µL per sample does not generate a normalized pool of sufficient volume for instrument loading, see Appendix B for pooling recommendations.

Important: There is no minimum or maximum limit to the number of samples that can be placed into a single pool. However, see the following recommendations:

- Consider your desired number of reads for each sample and only pool those samples together that have the same required sequencing depth. For example, samples each requiring 10 million reads can be pooled together, whereas samples requiring 100 million reads should be combined in a separate pool. Thus, you can adjust your ratio of pools when loading the instrument to achieve the desired sequencing depth for each pool.
- Additionally, consider index compatibility as well as insert size. Combine libraries of comparable insert sizes that can be co-sequenced to avoid size-dependent clustering effects that are independent of molarity and can lead to higher variation in sample representation (%CV) in the output sequencing data. Also, do not pool libraries with index combinations that have not been assessed for co-sequencing, as demultiplexing errors and loss of data may result.

1. After completing the Normalase I thermal cycler program, generate a library pool (or pools) by placing 5 µL of each individual library into a 0.2 mL PCR tube. Up to 30 libraries can be pooled together (achieves up to a final volume of 186 µL). Alternatively, use a 1.5 mL microcentrifuge tube when pooling greater than 30 libraries, as the volume will exceed the PCR tube maximum volume.

   Note: To ensure even pooling, use a calibrated pipette to produce the best results.

2. Thoroughly mix using a vortex machine, spin the library pools in a microcentrifuge, and proceed to the Normalase II step.

Perform the Normalase II step

1. Preset a thermal cycler program, as listed below. Alternatively, if using a 1.5 mL microcentrifuge tube, set a heat block at 37°C.

Normalase II Program	Heat block (1.5 mL microcentrifuge tube)
15 min at 37˚C with open lid or lid heating OFF	15 min at 37˚C

2. Prepare Normalase II Master Mix. The Master Mix can be stored on ice until use, then added to pooled libraries at room temperature.

Reagents*	Volume per library (µL)	24 libraries (µL)	96 libraries (µL)
• Buffer G1	0.96	23.04	92.16
• Enzyme G2	0.04	0.96	3.84
Total volume	1	24	96

Note: Prepare Normalase II Master Mix for 24 samples, even if you are processing less than 24 samples, to avoid pipetting extremely low volumes. For best results, use a calibrated P2 pipette for adding Enzyme G2

3. Add 1 µL of Normalase II Master Mix to the tube for each library in the pool.
4. Mix well by vortexing for 5 seconds, then pulse-spin the library pools in a microcentrifuge.
5. Place the library pools in the thermal cycler or place the 1.5 mL microcentrifuge tubes into the 37˚C heat block. After 15 minutes, proceed to Perform Normalase inactivation.

Perform Normalase inactivation

1. Following the Normalase II reaction, preset a thermal cycler program as listed below. Alternatively, if using a 1.5 mL microcentrifuge tube, set a heat block at 95°C.

Normalase Inactivation Program	Heat block (1.5 mL microcentrifuge tube)
Hold at 95°C	
2 min at 95°C with lid kept at 105°C	2 min at 95°C
Hold at 4°C	

2. Add 0.2 µL of Reagent B1, multiplied by the total number of libraries within each prepared pool, to the pooled libraries. See examples listed in the table below:

Reagent	Volume per library (µL)	24-plex pool (µL)	96-plex pool (µL)
• Reagent B1	0.2	4.8	19.2

3. Place the library pools in the thermal cycler or place the 1.5 mL microcentrifuge tubes into the heat block at 95°C, to incubate the library pools.

Important: Do not incubate the samples longer than 2 minutes.

4. The final multiplexed library pools are now equivalent in molarity. Proceed to (Optional) Quantification of your Normalase pool and sequencing (see Calibration of xGen Normalase output). It is not necessary to perform an additional bead-based cleanup before sequencing.

Note: Final pools contain single stranded DNA and can be stored at –20°C for up to four weeks before sequencing.

Calibration of xGen Normalase output

The standard Normalase I enzymology is formulated to select a 4 nM fraction from each library. The Normalase product specification is defined by cluster density of the final Normalase pool when loaded onto a MiSeq v2 flow cell at 12 pM, assuming 4 nM, to achieve a 1000–1200 K/mm2 cluster density and CV ≤ 10% within a pool (using xGen EZ DNA libraries with an average insert size of 200 bp).

To obtain the expected sequencing results, you can perform a qPCR quantification on your final Normalase pool(s). Final library pools are ssDNA and cannot be quantified by dsDNA-based fluorometric methods or electrophoretic fragment analysis.

Calibration of Normalase output to your qPCR assay, sequencer loading procedure, and clustering output is required due to variation across different qPCR assays and laboratory practices. Across Illumina® platforms, library types, insert sizes, and correct loading concentration are all necessary to achieve the number of reads required for your research application and supported by the chosen flow cell.

Once you have calibrated your sequencer loading procedure to the Normalase output and have established that your samples meet the minimum threshold for the Normalase workflow, qPCR of the final pool is optional, but recommended. For example, a Normalase workflow error may have occurred that would lead to unexpected results (see Appendix D: Troubleshooting).

APPENDIX A: LOW-PLEX POOLING RECOMMENDATIONS

Use the following recommendations for pooling, the Normalase II step, and Normalase inactivation if pooling less than 5 libraries, or if using post-hybridization capture library pools. These pooling recommendations help avoid pipetting errors from dispensing extremely small volumes.

Number of libraries/pools	Volume per library/pool (µL)	Total volume (µL)	Normalase II Master Mix (µL)	Reagent B1 (µL)
1*	25	25	5	1
2	12.50			
3	8.30			
4	6.25			
5	5			

* Do not proceed with the above recommendations unless the sequencing will be performed within the next four weeks. Final libraries are single stranded and can be stored at –20°C for up to four weeks before sequencing.

1. Following the Normalase I incubation, generate a library pool (or pools) by carefully placing the specified amount of each individual library, as shown in the table above, into a 0.2 mL PCR tube. Use a calibrated pipette for the best results.
2. Thoroughly mix samples, spin the library pools in a microcentrifuge, then proceed to the Normalase II reaction.

Normalase II reaction

1. Preset a thermal cycler program for 15 minutes at 37°C with open lid or lid heating OFF.
2. Prepare Normalase II Master Mix (listed in the table below). The Master Mix can be stored on ice until use, and then added to pools at room temperature.

Reagents*	Volume per pool (µL)	5 pools (µL)
• Buffer G1	4.8	24
• Enzyme G2	0.2	1
Total volume	5	25

* Prepare Normalase II Master Mix for a minimum of 5 pools, even if you are processing less than 5 pools, in order to avoid pipetting extremely low volumes.

Normalase inactivation

1. Following the Normalase II reaction, preset a thermal cycler program as listed below.

Normalase inactivation program
Hold at 95°C
2 min at 95°C with lid kept at 105°C
Hold at 4°C

2. Add 1 µL of Reagent B1 to each pool.
3. Place the library pools, with Reagent B1, in the thermal cycler.
4. Your final multiplexed library pools are now at an equivalent molarity. Proceed to the (Optional) Quantification section of your Normalase pool and sequencing.

Notes:

- Final pools contain single-stranded DNA and can be stored at –20°C for up to four weeks before sequencing. For longer term storage, refer to the safe stop point following post-Normalase PCR purification, and perform the pooling and subsequent steps as indicated above before sequencing.
- For library pool quantification instructions, see Perform Normalase calibration.

APPENDIX B: HIGH SAMPLE VOLUME POOLING RECOMMENDATIONS

Use the following recommendations for the Sample Pooling, the Normalase II step, and Normalase Inactivation steps when you require a higher volume in your final pool than the volume produced by the standard protocol which uses 5 µL per sample. Contact applicationsupport@idtdna.com for more information.

Note: You may need to pool more than 5 µL of each sample to achieve a sufficient loading volume at the required concentration. This table outlines the options available to support a broad range of final pool volumes—of equivalent molarities—when pooling 24 samples, or less.

A. Per sample pooling volume (µL)*		5	7.5	10	15	20	25
B. Normalase II Master Mix per sample volume (µL)†	• Buffer G1	0.96	1.44	1.92	2.88	3.84	4.80
	• Enzyme G2	0.04	0.06	0.08	0.12	0.16	0.20
	Total	**1.00**	**1.50**	**2.00**	**3.00**	**4.00**	**5.00**
C. Inactivation step per sample volume (µL)	• Reagent B1	0.20	0.30	0.40	0.60	0.80	1.00
D. Final pool volume (µL)	1 sample	6.20	9.30	12.40	18.60	24.80	31.00
	6 samples	37.20	55.80	74.40	111.60	148.80	186.00
	12 samples	74.40	111.60	148.80	223.20	297.60	372.00
	18 samples	111.60	167.40	223.20	334.80	446.40	558.00
	24 samples	148.80	223.20	297.60	446.40	595.20	744.00
E. Minimum number of reactions recommended for Normalase II Master Mix		24	18	12	10	6	5

***A. Alternate per sample pooling volumes.** Be aware that higher pooling volumes will reduce the number of re-pooling iterations available with your remaining sample.

†B. The per sample Normalase II Master Mix reagent volumes. Use these reagent quantities to prepare your Normalase II Master Mix.

C. The per sample Normalase inactivation reagent volumes.

D. Final volumes for pooling 1–24 samples over the range of individual sample volume options of 5–25 µL. These final volumes are the sum of the sample pool volume, Normalase II Master Mix volume, and inactivation volume. Final volumes in white cells can be prepared in a 0.2 mL PCR tube and thermal cycler, whereas final volumes in gray cells must be prepared in a 1.5 mL microcentrifuge tube and heat block to accommodate the larger volumes.

E. Minimum number of reactions recommended for Normalase II Master Mix assembly. Prepare Normalase II Master Mix for the minimum reaction number shown to avoid pipetting extremely low volumes.

Following the Normalase I incubation, generate a library pool (or pools) by carefully placing the specified amount of each individual library (as shown in the table above) into a 0.2 mL PCR tube, or a 1.5 mL microcentrifuge tube, depending on the final desired volume.

Note: Use a calibrated pipette to produce the best results during this pipetting step, ensuring even pooling.

1. Thoroughly mix, pulse-spin the library pools, then proceed to the Normalase II step.

Normalase II step

1. Preset a thermal cycler program for 15 minutes at 37°C with open lid, or lid heating OFF. Alternatively, if using a 1.5 mL microcentrifuge tube, set the heat block at 37°C.
2. Premix Normalase II Master Mix **depending on the chosen pooling volume** and minimum Master Mix reaction volume (listed in this table). The Master Mix can be stored on ice until use, then added to pools at room temperature.

Per sample pooling volume (µL)	5 µL (24 rxn)	7.5 µL (18 rxn)	10 µL (12 rxn)	15 µL (10 rxn)	20 µL (6 rxn)	25 µL (5 rxn)
• Buffer G1	23.04	25.92	23.04	28.8	23.04	24.00
• Enzyme G2	0.96	1.08	0.96	1.2	0.96	1.00
Total volume	24	27	24	30	24	25

3. Add the recommended volume of Normalase II Master Mix, based on your per sample pooling volume multiplied by the total number of samples within each prepared pool:

Per sample pooling volume (µL)	5	7.5	10	15	20	25
Normalase II Master Mix per sample (µL)	1	1.5	2	3	4	5

4. Mix tubes well by vortexing for 5 seconds, then pulse-spin the library pools in a microcentrifuge.
5. Place the library pools in the thermal cycler, or place the 1.5 mL microcentrifuge tubes into the 37°C heat block.

Normalase inactivation

1. Following the Normalase II reaction, preset a thermal cycler program as listed below.
2. Add the recommended volume of Reagent B1, based on your per sample pooling volume, multiplied by the total number of samples within each prepared pool:

Per sample pooling volume (µL)	5	7.5	10	15	20	25
• Reagent B1 (µL)	0.20	0.30	0.40	0.60	0.80	1

3. Place the library pools in the thermal cycler or place the 1.5 mL microcentrifuge tubes into the heat block. If using a 1.5 mL microcentrifuge tube, set the heat block at 95°C to incubate your library pools.

Important: Do not incubate the samples longer than 2 minutes.

Normalase Inactivation Program	Heat block (1.5 mL microcentrifuge tube)
Hold at 95°C	
2 min at 95°C with lid kept at 95°C	2 min at 95°C
Hold at 4°C	

4. Your final multiplexed library pools are now of equivalent molarity. Proceed to the (Optional) Quantification section of your Normalase pool and sequencing.

Safe Stop: Final pools contain single-stranded DNA and can be stored at –20°C for up to four weeks before sequencing. For longer term storage, refer to the safe stop point following the Normalase I step, and perform the pooling and subsequent steps as indicated above before sequencing. For library pool quantification instructions, refer to the Perform Normalase calibration section.

APPENDIX C: INDEXED ADAPTER SEQUENCES

The full-length adapter sequences used for the xGen Normalase workflow are listed below. The indicated text shows the location of the index sequences, which are 8 bases for CDI and 8 or 10 bases for UDI. These sequences represent the adapter sequences following completion of the indexing PCR step (or completion of the adapter ligation step when indexing by ligation).

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 when preparing your Illumina® sequencing sample sheet on your instrument of choice.

APPENDIX D: TROUBLESHOOTING

Issue	Possible Cause	Suggested Remedy
Variation of read counts (CV > 10%) between libraries within a Normalase pool	Some libraries did not meet the minimum threshold of 6 nM or 12 nM in 20 µL library yield.	Quantify affected libraries to determine library concentration and review whether the number of PCR cycles was appropriate for your sample quality and quantity.
	Inconsistent pipetting of Normalase I Master Mix, or inconsistent pipetting of individual libraries into a pool.	Use a P10 pipette, if available, and ensure pipettes are maintained and calibrated.
	Samples being pooled together were processed with a different Normalase I Master Mix preparation.	For best results, do not mix samples processed with different Normalase I Master Mix preparations.
Some libraries were significantly under-represented in the sequence data	These libraries did not meet the minimum threshold of 6 nM or 12 nM in 20 µL library yield.	Quantify affected libraries to determine library concentration and review whether the number of PCR cycles was appropriate for your sample quality and quantity.
qPCR quantification of pools following the Normalase II step indicated yields ≥12 nM	Normalase II failure—selected 4 nM fraction was not enzymatically normalized.	Re-pool the post-Normalase I libraries and repeat Normalase II and inactivation steps.

Appendix D: Troubleshooting (continued)

Issue	Possible Cause	Suggested Remedy
qPCR quantification of pools following Normalase step indicated no library yield	Library preparation, amplification, or Normalase I failure.	Contact applicationsupport@idtdna.com for specific troubleshooting recommendations.
Fluorometric methods such as BioAnalyzer or Qubit indicated no library yield	Normalase pools are single-stranded; therefore, fluorometric methods are not compatible for quantification.	Quantify pools using a qPCR assay.
Overall cluster density/reads passing filter was lower than expected	Normalase pools were stored for greater than four weeks.	Re-pool the post-Normalase I libraries and repeat Normalase II and inactivation steps.
	Libraries were consistently less than 6 nM or 12 nM.	Review whether the number of PCR cycles was appropriate for your sample quality and quantity.
	Failure to add Reagent B1 during inactivation step.	Add Reagent B1, heat inactivate, and re-sequence.

For additional technical support, contact us at applicationsupport@idtdna.com.

xGen™ Normalase™ Module

Technical support: applicationsupport@idtdna.com

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