xGen™ Pre-Hybridization Capture Normalase™ Module

REVISION HISTORY

Version	Release date	Description of changes
1	November 2023	Initital release.

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OVERVIEW

xGen™ Pre-Hybridization Capture Normalase Module is an enzymatic normalization method for generating equimolar pools and balanced sample representation for hybridization capture. This method streamlines library normalization by eliminating individual library quantification and pooling of variable volumes. Instead, equal volumes are pooled and taken through the Normalase chemistry. The resulting pool has sample read depth with a CV ≤ 10%, which is more uniform than library pools generated using qPCR quantification and fluorometric assays.

The xGen™ Pre-Hybridization Capture Normalase technology is designed for library preparation workflows and sample types that produce consistent amplified library yields following Normalase PCR. The xGen™ Pre-Hybridization Capture Normalase workflow can select a broad range of library inputs into hybridization capture (100–500 ng), supports multiple insert sizes (150–350 bp), and multiplexing of 4–24 libraries per pool. A 3x minimum yield threshold determined by the desired output quantity must be met for each library. Following normalization, if a reduced pooling volume (<24 µL per library) is preferred, see Appendix A for performing the Pre-Hyb Normalase II step.

xGEN PRE-HYBRIDIZATION CAPTURE NORMALASE WORKFLOW

This protocol has the following steps:

- **Normalase PCR** performs amplification using Normalase primers to a 3x minimum yield based on desired output quantity and conditions libraries for downstream chemistry.
- **The Pre-Hyb Normalase I step** enzymatically selects a user-defined library fraction.
- **Equal Volume Library pooling** creates a multiplex pool of libraries that will be co-hybridized.
- **The Pre-Hyb Normalase II step** enzymatically generates an equimolar library pool.
- **2.0X ratio SPRI purification** concentrates each pool and maximizes recovery of ssDNA library. After eluting in at least 20 µL nuclease-free water, adding xGen Universal Blockers, and drying down each pool, proceed with **Hybridization Capture**.

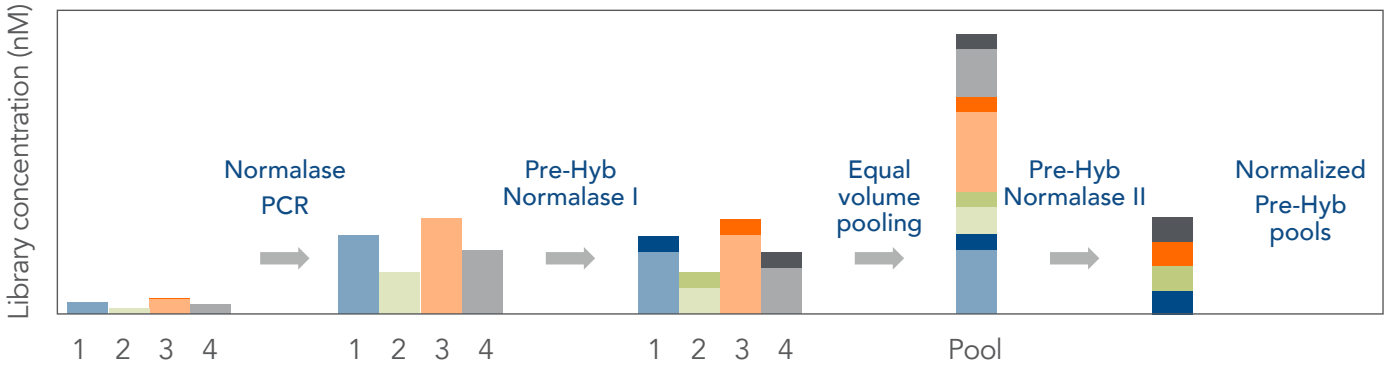

Figure 1. The xGen Pre-Hyb Normalase Module generates an equimolar library pool for downstream hybridization capture.

Workflow options

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

xGen Pre-Hybridization Capture 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 xGen Pre-Hybridization Capture Normalase Module) to index, amplify, and condition libraries for downstream chemistry. 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 that support hybridization capture supplied in xGen Library Prep Kits
- KAPA® HiFi HotStart ReadyMix (Roche)

Contact us for questions regarding compatibility with the xGen Pre-Hybridization Capture Normalase workflow.

xGEN PRE-HYBRIDIZATION CAPTURE NORMALASE MODULE WORKFLOW

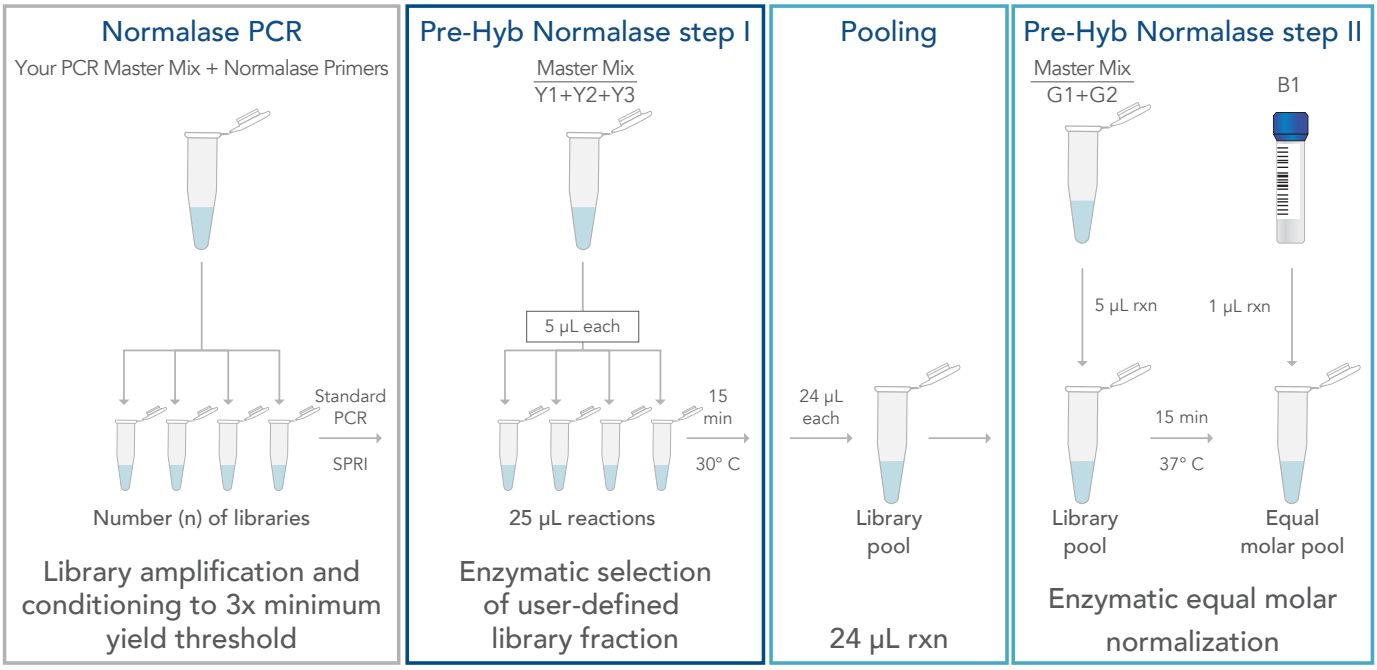

Figure 2. xGen Pre-Hyb Normalase workflow

The xGen Pre-Hybridization Capture Normalase workflow consists of four main steps:

1. **Normalase PCR:** This step replaces primers in conventional library amplification with Normalase primers to amplify to a 3x minimum yield threshold and condition libraries for downstream chemistry. Even if library yields have met the 3x minimum yield threshold prior to amplification, a minimum of 3 cycles is required to condition libraries.
2. **Pre-Hyb Normalase I reaction:** Following Normalase PCR and purification, Pre-Hyb Normalase I Master Mix is added to each amplified library and incubated at 30°C for 15 minutes. During this step, the Normalase enzyme selects a user-defined library fraction. Dilution of Reagent Y2 Pre-Hyb achieves a broad range of selected library quantities for a range of insert sizes.

   Tip: For best results, process all samples that will be pooled together for co-hybridization with the same Pre-Hyb Normalase I Master Mix during this step.
3. **Equal volume pooling:** During this step, 24 µL of each reaction is pooled into a single tube. If your workflow requires a reduced pooling volume (<24 µL per library) see Appendix A for performing the Pre-Hyb Normalase II step.
4. **Pre-Hyb Normalase II:** The library pool is incubated with **Pre-Hyb** Normalase II Master Mix followed by Reagent B1 for inactivation, to produce a final equimolar library pool. A **2.0x ratio SPRI purification** is performed to concentrate each pool and maximize recovery of ssDNA library. After eluting in at least 20 µL nuclease-free water, adding xGen Universal Blockers, and drying down each pool, proceed with **Hybridization Capture**.

Specifications

Reliable results are obtained using libraries with uniform size distributions 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 by adapter ligation with full-length, indexed adapters, an xGen Pre-Hyb Normalase Module is required for compatibility with the xGen Pre-Hyb Normalase workflow. For those workflows, Reagent R5 primers are used in place of standard amplification primers.

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

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

xGen Pre-Hyb Normalase Module—Kit contents

Workflow steps	Component	Volume (µL) 96 rxns	Storage
Normalase PCR	• Reagent R5	528	–20°C
Pre-Hyb Normalase I	• Buffer Y1	454	
	• Reagent Y2 Pre-Hyb	37	
	• Enzyme Y3	52	
Pre-Hyb Normalase II	• Buffer G1*	506	
	• Enzyme G2	21	
	• Reagent B1*	105	
Additional reagent	Low EDTA TE	2 x 1200	Room temperature

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

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 Pre-Hybridization Capture Normalase Module	10017913
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 and xGen DNA Library Kit MC UNI, for example.

Consumables from other suppliers

Item	Supplier	Catalog number
HiFi Polymerase compatible with hybridization capture if not supplied with the library prep kit: Kapa™ HiFi™ HotStart Readymix	Roche	KK2602
Magnetic beads for the cleanup steps: SPRISelect™ beads or AMPure XP beads (or other beads recommended by your xGen Library Prep Kit	Beckman Coulter	B23317/B23318/B23319/ A63880/A63881/A63882
0.2 mL PCR tubes or 96-well plates	Various	Varies
1.5 mL microcentrifuge tubes	Various	Varies
5 to 15 mL centrifuge 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	MSR812/MSP750/ MSR06/MSR2X15
Permagen® 0.2 mL PCR strip or plate magnet. For greater multiplexing of samples, a magnetic rack for 1.5 mL or 15 mL tubes may be needed	Various	Varies
Microcentrifuge	Various	Varies
Vortex machine	Various	Varies
Pipettes ranging from 1 to 1000 µL capacity	Various	Varies
Programmable thermal cycler	Various	Varies
Heat block that adjusts to 37°C (if a high pooling volume is required)	Various	Varies

IMPORTANT GUIDELINES

Meeting the 3X minimum threshold

To ensure that all library yields meet the 3x minimum yield threshold based on your desired output quantity, accurate quantification of input material for library prep is important to determine the required number of PCR cycles to reach the minimum threshold. See the table below for PCR cycling recommendations.

Important: Achieving the minimum threshold for every library is required to achieve the expected results. Libraries that do not meet this yield threshold will be under-represented in your hybridization capture pool 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 yield 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 meet minimum threshold, a minimum of 3 cycles is still required to condition the libraries for downstream chemistry.

When indexing by 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 xGen Pre-Hyb Normalase steps. If using kits from other suppliers, be familiar with the number of cycles required to achieve the 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 B of this protocol.

xGen Pre-Hyb Normalase PCR cycling recommendations below were determined using 200 bp fragmentation of Qubit quantified, high quality NA12878 DNA with xGen DNA EZ. Following stubby adapter ligation, Normalase PCR using Normalase Indexing primers was performed. For each recommended minimum cycle number, library yields required to reach the 3x minimum yield threshold can be achieved. When using reduced quality samples, additional cycles may be required to achieve the minimum threshold.

DNA Input into xGen DNA EZ	Minimum recommended PCR cycles to reach ≥ 300 ng for generating 100 ng per library for hybridization capture	Minimum recommended PCR cycles to reach ≥ 1500 ng for generating 500 ng per library for hybridization capture
≥ 100 ng	7	9
25 ng	9	11
10 ng	10	12
1 ng	13	15

Scaling for Desired xGen Pre-Hyb Normalase Output

This protocol allows you to scale your library quantity based on your library insert size. Examples below are with 200 bp inserts, if using an insert size other than 200 bp, refer to the online calculator. This calculator can also be used to scale the 3x Minimum Yield Threshold and calculate Master Mix and total workflow volumes. See the table below for examples for selecting varying output for 200 bp insert size. For the best results, use the lowest Minimum Threshold possible to minimize the number of required PCR cycles.

Workflow steps		Component		Volume (µL) 96 rxns	Per library output
nM	ng/µL	nM	ng/µL		
340*	75	113	25	neat	500 ng
170	37.5	56	12.5	Dilute 2x	250 ng
85	18.75	28	6.25	Dilute 4x	125 ng

* Achieved using high quality genomic DNA with xGen DNA EZ libraries.

Note: Selected library fraction is the final quantity of library following Normalase Pre-Hyb steps and concentration of library pools by 2.0x SPRI.

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, Pre-Hyb Normalase I, and Pre-Hyb 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 microcentrifuge to collect the contents prior to opening. Attempting to pipette enzymes at –20°C may lead to reagent shortage.
- 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 ready to 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 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 to discuss automating the xGen Pre-Hyb Normalase workflow with your automated liquid handling system.

PROTOCOL

Perform Normalase PCR

When indexing by Adapter Ligation

Assemble the 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.

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 using standard PCR reagents, as indicated in your xGen Library Kit protocol, but also add 1 µL Reagent R6. Thoroughly mix samples by moderate vortexing and pulse spin to collect contents.
2. Add 2 µL each of i5 and i7 to make a unique CDI primer pair for 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 xGen DNA EZ, modify the thermocycler program with a 5-minute final extension step as shown below.

xGen DNA EZ PCR Cycling Conditions	
Refer to the table under Normalase PCR recommendations above for recommended cycle number	Heated lid at 105°C
	98°C for 45 sec
	98°C for 15 sec 60°C for 30 sec 72°C for 30 sec
	Final extension of 72°C for 5 minutes
	Hold at 4°C

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 recommended by your xGen Library Prep Kit to achieve consistent results.

2. Add beads to each sample according to the ratio recommended in the xGen Library Prep 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 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.
7. Repeat the previous step for a second wash with the 80% 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.
9. Remove samples from the magnetic rack. Add 22 µL of Low EDTA TE buffer and resuspend the pellet. Mix well until homogenous by pipetting up and down.
10. Incubate samples for 2 minutes at room temperature.
11. Place samples on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
12. Carefully transfer 20 µL of the sample to a new 0.2 mL PCR tube, without carry-over of any beads.

    Safe Stop: Samples can be stored at –4°C for up to 24 hours or at –20°C long-term.

(Optional) Quantification

If you wish to confirm that library yields have achieved the minimum threshold, perform a library quantification (i.e., fluorometric, electrophoretic chip, or qPCR) before proceeding with the Pre-Hyb Normalase I step.

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

Based on the desired scalable workflow option, use Reagent Y2 Pre-Hyb neat or perform a specified dilution using Low EDTA TE according to the online calculator, then proceed using the protocol below. For example, for a two-fold dilution, mix an equal volume of Reagent Pre-Hyb Y2 and Low EDTA TE, being careful to ensure accurate pipetting volumes.

Perform the Pre-Hyb Normalase I step

1. Preset a thermal cycler program as listed below.

Pre-Hyb Normalase I Program
15 min at 30°C with open lid or lid heating OFF

2. Prepare the Pre-Hyb Normalase I Master Mix as described in the tables below (top: 200 and 350 bp insert size, bottom: 150 bp insert size). 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 pulse-spin to collect contents.

   For 200 and 350 bp insert size, prepare the Pre-Hyb Normalase I Master Mix as described in the table below.

Reagent	Volume per library (µL)	12 Libraries (µL)	24 Libraries (µL)
• Buffer Y1	4.2	50.4	100.8
• Reagent Y2 Pre-Hyb (neat or diluted)	0.3	3.6	7.2
• Enzyme Y3	0.5	6	12
Total volume	5	60	120

   For 150 bp insert size, prepare the Pre-Hyb Normalase I Master Mix as described in the table below.

Reagent	Volume per library (µL)	12 Libraries (µL)	24 Libraries (µL)
• Buffer Y1	4.15	49.8	99.6
• Reagent Y2 Pre-Hyb (neat or diluted)	0.35	4.2	8.4
• Enzyme Y3	0.5	6	12
Total volume	5	60	120

Important: The Pre-Hyb Normalase I Master Mix should be built for a minimum of 12 reactions to ensure pipetting accuracy. For best results, process all samples that will be pooled together for co-hybridization with the same Pre-Hyb Normalase I Master Mix.

3. Using a calibrated P10 pipette, add 5 µL of Pre-Hyb Normalase I Master Mix to each 20 µL library eluate at room temperature. Thoroughly mix by moderate vortexing for 5 seconds.
4. Spin down the libraries 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 Pre-Hyb Normalase I step.

Perform equal volume library pooling

Important: Before beginning equal volume pooling, see the following recommendations.

- xGen Pre-Hyb Normalase supports 4–24 libraries per pool. If you require >24 libraries per pool, contact NGS Applications support for recommendations.
- Consider your desired number of reads for each library and only pool those libraries together that have the same required depth. For example, samples 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 compatible insert sizes that can be co-sequenced together 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 Pre-Hyb Normalase I thermal cycler program, generate a library pool by placing 24 µL of each individual library into an appropriately sized tube. Ensure that the tube used for pooling has sufficient remaining volume for Pre-Hyb Normalase II step, Human Cot DNA, as well as a 2.0X SPRI volume. For example, a 12-plex pool at 24 µL pooling volume per sample equals 288 µL, plus sufficient remaining volume for additional reagents. If your workflow requires a reduced pooling volume (<24 µL per library) see Appendix A for instruction on performing the Pre-Hyb Normalase II step.

   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 Pre-Hyb Normalase II step.

Perform the Pre-Hyb Normalase II step

1. Set a heat block at 37°C

Pre-Hyb Normalase II Program
15 min at 37°C

2. Prepare Pre-Hyb Normalase II Master Mix based on the number of samples per pool. For example, a 24 µL pooling volume requires 5 µL Pre-Hyb Normalase II Master Mix per library and so for a 12-plex pool, add 60 µL Pre-Hyb Normalase II Master Mix to the 288 µL pool for a Pre-Hyb Normalase II reaction volume of 348 µL.

Reagents	Per 24 µL Library Volume (µL)
• Buffer G1	4.8
• Enzyme G2	0.2
Total Volume per Library	5

3. Add Pre-Hyb Normalase II Master Mix to the library pool.
4. Mix well by vortexing for 5 seconds and spin down the library pool in a microcentrifuge.
5. Place the library pools into a 37°C heat block. Proceed directly to Pre-Hyb Normalase inactivation.

Perform Pre-Hyb Normalase inactivation

1. Add Reagent B1 to stop the Pre-Hyb Normalase II reaction as specified below. For example, a 12-plex pool requires 1 µL of Reagent B1 per library and so for a 12-plex pool, add 12 µL Reagent B1 to the 348 µL pool for a final reaction volume of 360 µL. Mix well by vortexing, spin down, and incubate at room temperature for 5 minutes.

Reagent	Per 24 µL Library Volume (µL)
• Reagent B1 (per library)	1

Perform 2.0x Ratio SPRI purification

For maximum recovery of ssDNA library, perform a 2.0x SPRI purification to concentrate each pool.

1. Your final libraries are each at a specified quantity (ng) within the pool and ready for the addition of 7.5 µL xGen Human Cot DNA/COT-1 Human DNA (xGen hybridization capture protocol Appendix A).
2. Ensure the magnetic beads are at room temperature and vortex beads to homogenize the suspension before use.
3. Mix the sample with beads by moderate vortexing for 5 seconds and briefly centrifuge down.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
6. Remove and discard the supernatant without disturbing the pellet (less than 5 µL may be left behind).
7. Add freshly prepared 80% ethanol solution to the sample while it is still on the magnetic rack. **Use a volume that is large enough to submerge the beads.** Incubate for 30 seconds and then carefully remove the ethanol solution.
8. Repeat the previous step for a second wash with the 80% solution.
9. Gently 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.
10. Remove samples from the magnetic rack. Add 21 µL of **water** and re-suspend the pellet. Mix well by pipetting up and down until homogenous.
11. Incubate sample tubes at room temperature for **5 minutes**.
12. Place the samples back on the magnetic rack until the solution clears and a pellet is formed (~2 minutes).
13. Carefully **transfer all** of the eluate into a new 0.2 mL PCR tube without carry-over of any beads.
14. Add 2 µL of xGen Universal Blocker based on your library adapter.
15. Dry down the eluate for hybridization capture as recommended in the xGen Hybridization Capture protocol.

Safe Stop: xGen Pre-Hyb Normalase pools can be stored at stored at –4°C for up to 24 hours or at –20°C long-term.

APPENDIX A: REDUCED POOLING VOLUME RECOMMENDATIONS

This appendix supports an alternate 12µL library pooling option. This option reduces the overall Normalase II volume and provides the option to repeat hybridization capture with the library remainder.

This pooling option requires a 6x minimum yield threshold (relative to final output) since only half the volume is carried through the workflow, and therefore only supports selected outputs of 100–250ng per library. (e.g. for final output of 100 ng per library, a minimum yield threshold of 600 ng should be met).

Important: Perform Pre-Hyb Normalase I to select twice the desired output in ng (e.g. select 200 ng for a final 100 ng output when using the reduced pooling volume) and during equal volume pooling, add 12 µL of each Pre-Hyb Normalase I treated library.

Perform the Pre-Hyb Normalase II step

1. Set a heat block at 37°C.

Pre-Hyb Normalase II Program
15 min at 37°C

2. Prepare Pre-Hyb Normalase II Master Mix based on the per sample pooling volume and number of samples per pool.

Reagents	Per 12 µL Library Volume (µL)
• Buffer G1	2.4
• Enzyme G2	0.1
Total Volume per Library	2.5

3. Add Pre-Hyb Normalase II Master Mix to the library pool based on the per sample pooling volume.
4. Mix well by vortexing for 5 seconds and spin down the library pool in a microcentrifuge.
5. Place the library pools into a 37°C heat block.

Perform Pre-Hyb Normalase inactivation

1. Add Reagent B1 to stop the Pre-Hyb Normalase II reaction as specified below based on the per sample pooling volume, mix well by vortexing, spin down, and incubate at room temperature for 5 minutes.

Reagents	Per 12 µL Library Volume (µL)
• Reagent B1 (per library)	0.5

2. Proceed to Perform 2.0x Ratio SPRI purification.

APPENDIX B: INDEXED ADAPTER SEQUENCES

The full-length adapter sequences used for xGen Pre-Hyb Normalase workflow are listed below. The indicated text shows the location of the index sequences, which are 8 bases for CDI and 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 accompanying Index Sequences Master List for index sequences for preparing your Illumina sequencing sample sheet on the instrument of your choice.

APPENDIX C: TROUBLESHOOTING

Issue	Possible cause	Suggested remedy
Variation of read counts (CV > 10%) between libraries within a Normalase pool	Some libraries did not meet the 3x minimum threshold in 20 µL library volume.	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 Pre-Hyb 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 Pre-Hyb Normalase I Master Mix preparation.	For best results, do not pool libraries processed with different Pre-Hyb Normalase I Master Mix preparations.
Some libraries were significantly under-represented in the sequence data	These libraries may not have met the minimum yield threshold in 20 µL library volume.	Quantify affected libraries to determine library concentration and review whether the number of PCR was appropriate for your sample quality and quantity.
qPCR quantification of pools following the Pre-Hyb Normalase II step indicated no library yield present	Library preparation, amplification, or Pre-Hyb Normalase I failure	Contact us for specific troubleshooting recommendations.
Fluorometric methods such as BioAnalyzer or Qubit indicated no library.	Normalized Pre-Hyb pools are single stranded and will not be detectable.	Quantify pools using a validated qPCR assay.

xGen™ Pre-Hybridization Capture Normalase™ Module

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

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