Swift BIOSCIENCES™

PROTOCOL

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SWIFT NORMALASE KIT

For Enzymatic NGS Library Normalization

Compatible with libraries constructed using full-length, indexed adapters and PCR amplification

Validated with:

- Swift Hot Start High Fidelity Polymerase
- KAPA HiFi HotStart ReadyMix
- NEBNext Ultra II Q5® Master Mix

Protocol for Cat. No. 66096 to be used with indexing kits:

- 2S Set A (Cat. No. 26148)
- 2S Set B (Cat. No. 26248)
- 2S Set A+B (Cat. No. 26396)
- 2S Set A MID (Cat. No. 27148)
- 2S Set B MID (Cat. No. 27248)
- 2S Set A+B MID (Cat. No. 27396)
- 2S Set S1 (Cat. No. 26596)
- 2S Set S2 (Cat. No. 26696)
- 2S Set S3 (Cat. No. 26796)
- 2S Set S4 (Cat. No. 26896)
- 2S Set S1-S4 (Cat. No. 269384)
- 2S Set S1 MID (Cat. No. 27596)
- 2S Set S2 MID (Cat. No. 27696)
- 2S Set S3 MID (Cat. No. 27796)
- 2S Set S4 MID (Cat. No. 27896)
- 2S Set S1-S4 MID (Cat. No. 279384)

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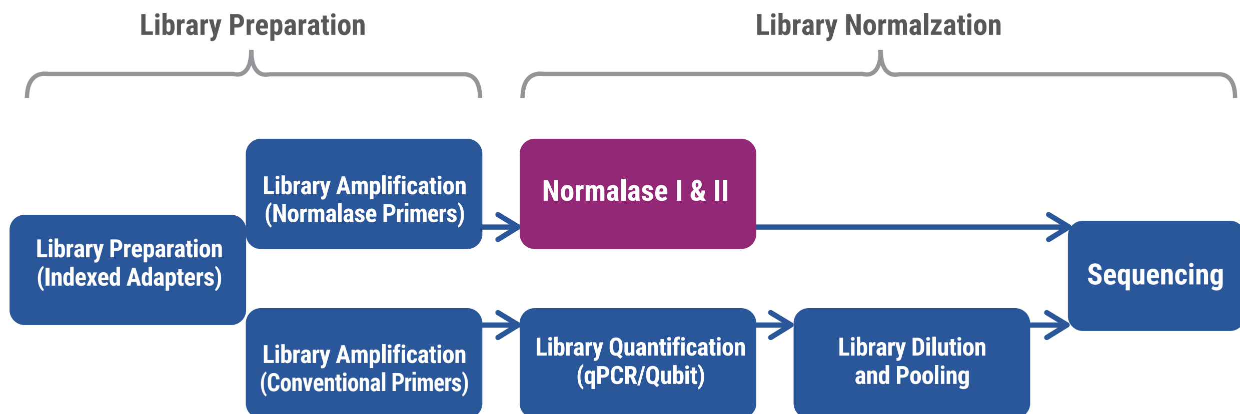
About This Guide

This guide provides instructions for enzymatic normalization of multiplexed next generation sequencing (NGS) libraries for equimolar pooling and balanced sample representation in sequencing. The protocol is designed for workflows that produce consistent amplified library yields of 12 nM or more following Normalase PCR and generates a normalized library pool of 4 nM. This workflow does not introduce more amplification but replaces the primers in conventional library amplification with Normalase primers, allowing the user to maintain their conventional library amplification workflow. The Normalase workflow does not require quantification and is a bead-free normalization method where equal volumes of each library are pooled to simplify high throughput processing.

Even if library yields have met the 12 nM minimum prior to library amplification using Normalase primers, perform-ing a minimum of 3 cycles is required to condition the library for the downstream Normalase enzymology.



IMPORTANT!

Do not begin library construction for Normalase until you read this Protocol. Normalase PCR primers replace your standard primers and are combined with your amplification reagents. Conditions for Normalase library amplification are modified from standard library amplification procedures. Read the entire protocol thoroughly to ensure that you understand all the important information and modifications needed for successful inte-gration of Normalase with your library preparation workflow.

Be aware of your downstream sequencing instrument loading concentration and volume requirements as well as the number of samples to be pooled for multiplexed sequenc-ing. These factors will affect how you pool and complete the Normalase workflow. For example, NovaSeq requires 100-310 µl loading volume at 1.5-3 nM concentration and if multiplexing a lower number of samples, you may need to pool more than 5 µl of each sample to achieve a sufficient loading volume at the required concentration. Refer to Appendix A and B for alternate pooling guides if needed.

The Normalase product specification is defined by cluster density of the final 4 nM Normalase pool when loaded on a MiSeq v2 flow cell at 12 pM to achieve a 1000-1200 K/mm^2 cluster density and CV ≤ 10% within a pool (Swift 2S Turbo Flexible libraries with an average 200 bp insert size). Across other Illumina platforms, library types, and insert sizes, optimization of loading concentration may be required to achieve the desired cluster density.

Product Information

Swift Normalase offers a streamlined solution for equimolar NGS library pooling and uniform multiplexed sequencing without the need for concentration adjustment of individual samples.

The protocol is readily automatable. A 10% overage volume of reagents is supplied in a 96-reaction kit to accommodate automation. Swift Biosciences does not supply automated liquid handling instrumentation or consumables but collaborates with automation solution providers and customers to develop and qualify optimized automated scripts for use of our kits, in combination with liquid handling platforms routinely used in NGS library preparation. Please contact your instrument vendor or TechSupport@swiftbiosci.com if you plan to use the Swift Normalase Kit with your particular automated liquid handling system.

Applications

Swift Normalase is suitable for libraries with the following features:

- Libraries with full-length indexed adapters
- Libraries that have an amplified yield of 12 nM or higher (20 µL volume)
- Libraries prepared for direct sequencing (i.e., whole genome, whole transcriptome)
- Target enriched, post-hybridization libraries with indexed adapters

Best results are obtained using libraries with standard size distribution generated by Covaris shearing or enzy-matic fragmentation (i.e., 150-550 bp inserts) and bead-based size selection.

Libraries with broad or variable size distributions (i.e., transposase-based workflows) that demonstrate size dependent clustering effects that are independent of molarity may have more variable results.

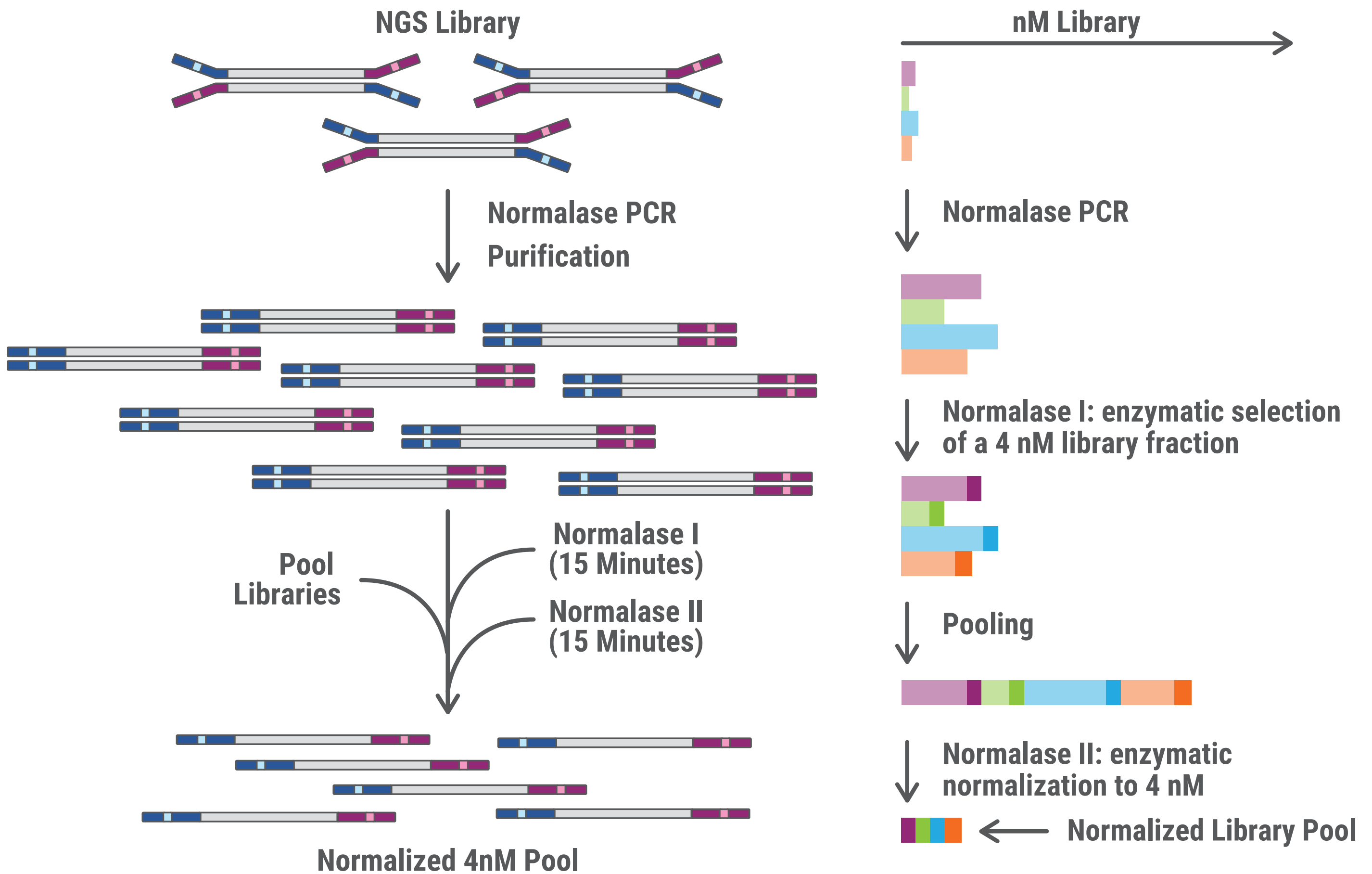
The Swift Normalase Kit is not compatible with these library features:

- PCR-free libraries
- Workflows that require indexing PCR primers to complete library construction
- Libraries prepared for pre-hybridization capture which require greater than 4 nM final library concentrations

Swift Normalase Workflow

This workflow provides an easy solution to generate equimolar NGS library pools for balanced sample representation in multiplexed sequencing. The workflow consists of replacing conventional library amplification with Normalase primers and amplifying to a minimum yield threshold of 12 nM or more (20 µL volume), followed by Normalase I, library pooling, and Normalase II to generate 4 nM library pools.

The bead-based clean-up, post-Normalase PCR is used to remove oligonucleotides and small fragments.



Kit Contents

The Swift Normalase Kit is available with reagents (10% excess volume) for the processing of 96 libraries.

Reagents	Quantity (µl) 96 rxn	Storage (°C)
Reagent R5	528	-20
Buffer Y1	454	-20
Reagent Y2	21	-20
Enzyme Y3	52	-20
Buffer G1*	506	-20
Enzyme G2	21	-20
Reagent B1	105	-20

These reagents are provided at a 5-fold excess plus overage so that library pooling and subsequent steps can be repeated up to 5 times for flexibility in re-pooling and re-sequencing.

Reagents	Quantity (µL)	Storage (°C)
Low EDTA TE	2x1200	RT

IMPORTANT!

Place the enzymes on ice, NOT in a cryo-cooler, for at least 10 minutes to allow enzymes to reach 4 ˚C prior to pipetting.

Material and Equipment Not Included

- Library preparation kit and DNA polymerase for amplification (Swift Hot Start High Fidelity Polymerase reagents provided with the library kit, Kapa HiFi HotStart ReadyMix (KK2602), NEBNext Ultra II Q5 Master Mix (M0544))
- qPCR-based library quantification kit for confirming final library pool concentration
- Optional Qubit® or other fluorometric-based assays for determining individual library concentration prior to Normalase
- Magnetic beads for the clean-up step, e.g., SPRIselect™ beads (Beckman Coulter, Cat. No. B23317/ B23318/ B23319)
- Magnetic rack for the clean-up step, e.g., Invitrogen DynaMag™ or Agencourt® SPRIPlate™
- Microfuge
- Programmable thermocycler
- Heat block that adjusts from 37-95 ºC
- 0.2 mL PCR tubes
- 1.5 mL screw cap microfuge tubes
- Aerosol-resistant, low retention pipettes and tips, 2 to 1000 µL (calibrated P10 pipette preferred for accuracy of 5 µL pipetting during Normalase I and library pooling steps)
- 200-proof/absolute ethanol (molecular biology-grade)
- Nuclease-free water (molecular biology-grade)

Storage and Usage Warning

Upon receipt, store the Swift Normalase Kit at -20 ˚C with the exception of Low EDTA TE solution, which can be stored at room temperature.

The enzymes provided in this kit are temperature sensitive, and appropriate care should be taken during storage and handling. To maximize use of enzyme reagents when ready to use, remove enzyme tubes from -20 ˚C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow enzymes to reach 4 ˚C prior to pipetting. Attempting to pipette enzymes at -20 ˚C may result in a shortage of enzyme reagents.

Tips and Techniques

Avoiding 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.

Post-PCR Clean-Up Step

This protocol has been validated with SPRIselect beads (Beckman Coulter); however, it can be used with Agencourt AMPure® XP (Beckman Coulter). If other beads are used, solutions and conditions for DNA binding may differ.

Consider the information below for performing efficient size selection:

- For the post-Normalase PCR clean-up step, use the same bead ratio as recommended by your library prep supplier post-PCR in order to achieve consistent results and avoid adapter dimers and short library molecules that may otherwise result in your final library.
- However, if the bead ratio recommended by the library prep supplier is greater than a 1.0X ratio (e.g. 1.2X or higher), we recommend performing a 1.0X ratio to ensure efficient removal of unused Normalase primers.

Library Input Considerations

Meeting the Minimum Threshold

In order to ensure that library yields meet 12 nM or higher in the 20 µL eluate follow-ing amplification with Normalase primers, careful quantification of input material for library prep is important to determine the required number of PCR cycles to reach the 12 nM yield.

For high quality samples, it is recommended to determine dsDNA concentration using Qubit, or a similar fluorometric method, as it will accurately represent the double-stranded, adaptable DNA content of your sample.

For low quality human DNA samples, we recommend quantification by qPCR using Alu primer pairs provided in Swift library kits to accurately assess the usable amount of human DNA in the samples and their integrity.

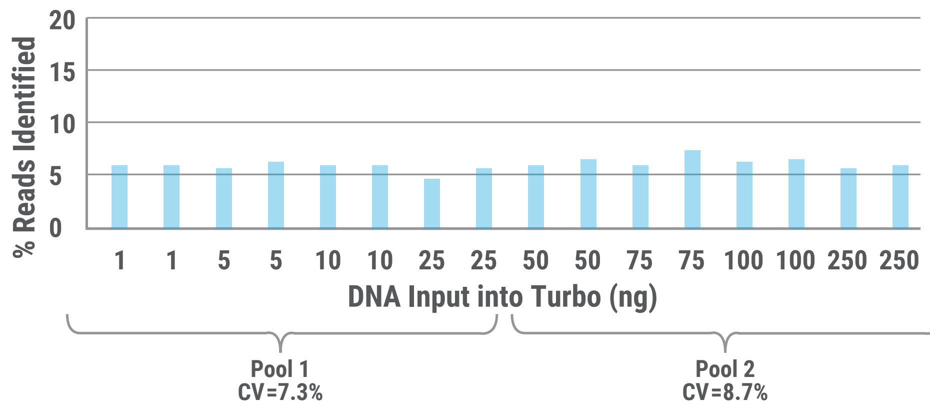
Alternatively, if careful quantification of input material is not possible, determine the lowest usable input quanti-ty that will be used across the sample set and apply the appropriate number of cycles for that input quantity to all samples in order to consistently reach the minimum threshold of 12 nM across the sample set, regardless of varied input quantity or quality.

Use of Normalase primers is required for successful normalization of libraries. Library amplification using Normalase primers (Reagent R5) is required even if your libraries have already met the minimum yield threshold prior to amplification. A minimum of 3 cycles is required to condition the library for the downstream Normalase enzymology.

IMPORTANT!
Library input quantities referenced in this protocol refer to total library yield after PCR amplification with Normalase primers. Amplified library yields must meet the minimum threshold of 12 nM (in 20 µL) in order to obtain a 4 nM normalized yield. Libraries below the minimum threshold will result in normalized yields < 4 nM and will be under-represented in multiplexed sequencing data.

Expected Results

As shown in the plot below, 16 Swift 2S Turbo libraries were prepared using differing inputs of Coriell NA12878 DNA from 1-250 ng that were enzymatically fragmented to 350 bp, ligated to Illumina® unique dual indexed adapters, and followed by the recommended number of PCR cycles for the various inputs indicated in this guide for this library prep. Libraries were prepared by two different individuals and Normalase was performed using two separate pools of 8 libraries each. The pools were then combined into a single pool using an equal volume of 5 µL each, then denatured and loaded at 10 pM onto a MiSeq® v2 flow cell. The overall cluster density was 856 K/mm² which is within the specifications for v2 chemistry, 93% clusters passing filter, and Q30 of 97.5%. Demultiplexing data indicate that individual representation of each library had 7.3% and 8.7% variation within the pools and a 9.5% variation across pools.



Prepare the Reagent Master Mixes and Ethanol

- To create the master mixes, scale reagent volumes as appropriate, using 10% excess volume to compensate for pipetting loss.
- To assemble reagent master mixes for the Normalase PCR, Normalase I, and Normalase II steps, ensure the reagent vials and enzymes are at 4 °C. After thawing reagents, briefly vortex (except the enzymes) to mix them well. Spin tubes in a microfuge 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.

Perform Library Amplification with Normalase Primers

If your conventional library preparation and amplification step yields ≥ 12 nM in 20 µL, simply replace conventional amplification primers with Normalase primers and run amplification with minimum adjustments to the final extension step. If without amplification your library preparation yields ≥ 12 nM, a minimum of 3 cycles using Normalase primers (Reagent R5) is required to condition the library for the downstream Normalase enzymology.

As a general guide, refer to the table below for the recommended number of cycles when using the Swift 2S Turbo Flexible Kit and Accel-NGS® 2S DNA Library Kits. Data in the table below are based on high quality DNA inputs quantified by Qubit. If using lower quality samples or inputs < 1 ng, assess sample quality and quantity by an ALU or other qPCR-based DNA integrity assay to determine the necessary number of PCR cycles to meet ≥ 12 nM minimum yield.

Swift 2S Turbo Flexible DNA Library Kit and Accel-NGS 2S DNA Library Kits

Input Material (ng)	Minimum Normalase PCR Cycles	Average Yield (nM)
≥ 100	3	≥ 12
25	5	
10	7	
1	10	

If using a library preparation kit other than Swift 2S Turbo and Accel-NGS 2S kits, you must be familiar with the number of PCR cycles required to reach at least 12 nM prior to using this kit and these same number of cycles (minimum of 3 cycles) should be used during the Normalase PCR step in this protocol. If your standard conditions produce libraries <12 nM library yield, you must increase the number of PCR cycles to produce at least 12 nM yield in 20 µL volume.

IMPORTANT!
Achieving the minimum threshold of 12 nM is required for optimal performance. Libraries that do not meet this threshold will be less than 4 nM post-Normalase and will be under-represented during cluster generation.

Normalase PCR

1. Set up your library amplification reactions using standard PCR reagents as shown below, except substitute the standard primers with 5 µL of Reagent R5 (the Normalase PCR primers that have a 600 nM final concentration in the reaction).

Swift Hot Start High Fidelity Polymerase PCR rxn	Master Mix	KAPA HiFi HotStart ReadyMix (KK2602) NEBNext Ultra II Q5 Master Mix (M0544) PCR rxn
Sample: 20 µL	Low EDTA TE: 10 µL	Reagent R5: 5 µL
Master Mix: 30 µL	Reagent R2: 4 µL	Sample: 20 µL
Final Volume: 50 µl	Buffer R3: 10 µL	Master Mix: 25 µL
	Enzyme R4: 1 µL	**Final Volume: 50 µl**
	Reagent R5: 5 µL	
	Final Volume: 30 µl	

2. Modify the thermocycler PCR program recommended by the library preparation kit or polymerase of choice with a 5-minute final extension step. Pre-set the thermocycler in accordance with the cycles required for your DNA input quantities based on the library preparation guide with adjusted cycle number if needed to achieve the minimum threshold. Utilize your polymerase of choice with the thermocycler conditions below:

Swift Hot Start High Fidelity Polymerase	KAPA HiFi HotStart ReadyMix	NEBNext Ultra II Q5 Master Mix
Thermocycler Program **Heated Lid at 105 °C** 98 ºC for 30 seconds PCR Cycles: • 98 ºC for 10 seconds • 60 ºC for 30 seconds • 68 ºC for 60 seconds Final extension 68 ºC for 5 minutes Hold at 4 ºC — proceed to clean-up	**Thermocycler Program** **Heated Lid at 105 °C** 98 ºC for 45 seconds PCR Cycles: • 98 ºC for 15 seconds • 60 ºC for 30 seconds • 72 ºC for 30 seconds Final extension 72ºC for 5 minutes Hold at 4 ºC — proceed to clean-up	**Thermocycler Program** **Heated Lid at 105 °C** 98 ºC for 30 seconds PCR Cycles: • 98 ºC for 10 seconds • 60 ºC for 30 seconds • 65 ºC for 60 seconds Final extension 65 ºC for 5 minutes Hold at 4 ºC — proceed to clean-up

Library Purification

3. Ensure the magnetic beads are at room temperature and vortex beads to homogenize the suspension before use.
4. Utilize the same post-PCR bead ratio as recommended by your library prep supplier in order to achieve consistent results and avoid adapter dimers and short library molecules that may otherwise result in your final library. However, if the bead ratio recommended by the library prep supplier is greater than a 1.0X ratio (e.g., 1.2X or higher), we recommend performing a 1.0X ratio (50 µL beads) to ensure efficient removal of unused Normalase primers.
5. Mix the sample with beads by moderate vortexing for 5 seconds.
6. Pulse spin the samples in a tabletop microcentrifuge. Do not centrifuge to excess, as marked by the beads pelleting at the bottom. If this occurs, re-mix your samples and spin again with less force/shorter duration.
7. Incubate the samples for 5 minutes at room temperature.

8. Place the sample on a magnetic rack until the solution clears and a pellet is formed (~ 2 minutes).
9. Remove and discard the supernatant without disturbing the pellet (less than 5 µl may be left behind).
10. 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.
11. Repeat step 10 once more for a second wash with the 80% ethanol solution.
12. Gently spin the samples in a tabletop microfuge and place back on the magnetic rack. Remove any residual ethanol solution from the bottom of the tube using a smaller pipette tip.
13. Remove samples from magnetic rack. Add 22 µl of Low EDTA TE buffer and re-suspend the pellet. Mix well by pipetting up and down until homogenous.
14. Place the sample tubes on a magnetic rack and wait 2 minutes.
15. Carefully transfer 20 µl of the sample to a new 0.2 mL PCR tube without carry over of any beads.

Optional Quantification

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

Safe Stopping Point

For long term storage (i.e., > 2 weeks), libraries can be stored at -20 ˚C post-Normalase PCR purification. If planning to sequence within the next two weeks, proceed to Normalase I.

Normalase I

16. Pre-set a thermocycler program for 15 minutes at 30 °C with open lid or lid heating OFF.
17. Prepare the Normalase I Master Mix (listed 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 prior to use.

	Per Library	24 Libraries	96 Libraries
Buffer Y1	4.3 µl	103.2 µl	412.8 µl
Reagent Y2	0.2 µl	4.8 µl	19.2 µl
Enzyme Y3	0.5 µl	12.0 µl	48.0 µl
Total Volume	**5.0 µl**	**120.0 µl**	**480.0 µl**

IMPORTANT!
The Normalase I Master Mix should be built for a minimum 10 reactions to ensure pipetting accuracy.

18. Using a calibrated P10 pipette, carefully add 5 µl of Normalase I Master Mix into each 20 µl library eluate at room temperature and thoroughly mix by moderate vortexing for 5 seconds.
19. Spin down the sample tube in a microfuge and place in the thermocycler and run the program.

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

Safe Stopping Point

For long term storage (i.e., > 2 weeks), libraries can be stored at -20 ˚C post-Normalase I. If planning to sequence within the next two weeks, proceed to Library Pooling step.

Library Pooling

This step can be repeated up to 5 times to enable various re-pooling combinations as only 5 µl of post-Normalase I library (25 µl volume) is utilized for pooling, and sufficient reagents are provided. Also note that stability of normalized pools is limited with a storage time of two weeks since the resulting normalized pools are single stranded DNA libraries. Therefore, if re-sequencing is required after two weeks, for best results re-pool the librar-ies and repeat Normalase II and inactivation.

Note: If you are pooling less than 5 libraries or post-hybridization capture library pools, see Appendix Section A for low-plex pooling recommendations.

Note: If pooling 5 µl per sample does not generate a final 4 nM pool of sufficient volume for instrument loading, see Appendix Section B for high sample volume pooling recommendations.

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

- Consider your desired number of reads for each sample and only pool those samples together that have the same required 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 sequence depth for each pool.
- Additionally, consider index compatibility as well as insert size. Pool libraries of comparable insert sizes that can be co-sequenced together to avoid size dependent clustering effects that are independent of molarity that can lead to higher variation in sample representation in sequencing data. Also, do not pool libraries with index combinations that have not been validated by the supplier or demultiplexing errors and loss of data may result.

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20. Following the Normalase I incubation, generate a library pool (or pools) by carefully placing 5 µl of each individual library into a 0.2 mL PCR tube if pooling 30 libraries or less (achieves up to a final volume of 186 µl). Alternatively, use a 1.5 mL screw cap microfuge tube, particularly when pooling greater than 30 librar-ies as the volume will exceed the PCR tube maximum volume.
    As mentioned previously to ensure even pooling, use of a calibrated P10 pipette will produce the best results.

21. Thoroughly mix, and spin the library pools in a microfuge, and proceed to Normalase II reaction with the library pools created.

Normalase II

22. Pre-set a thermocycler program for 15 minutes at 37 °C with open lid or lid heating OFF. Alternatively, if using a 1.5 mL screw cap microfuge tube, set a heat block at 37 °C.

23. Pre-mix 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*	Per Library	48 Libraries	96 Libraries
⊙ Buffer G1	0.96 µl	48 µl	96 µl
⊙ Enzyme G2	0.04 µl	2 µl	4 µl
Total Volume	**1.00 µl**	**50 µl**	**100 µl**

**We recommend preparing Normalase II master mix for 48 samples even if you are processing less than 48 samples in order to avoid pipetting extremely low volumes; recommended volumes have been rounded up for convenient pipetting. Although sufficient reagents are supplied for up to 5 repeated Normalase II reactions per sample, repeatedly processing a lower number of samples will result in significant loss of Normalase II reagents.*

24. Add 1 µl of Normalase II Master Mix multiplied by the total number of libraries within each prepared pool, see examples below:

Reagents	Per Library	24-Plex Pool	96-Plex Pool
Normalase II Master Mix	1 µl	24 µl	96 µl

25. Mix well by vortexing for 5 seconds, and spin down the library pools in a microfuge.
26. Place the library pools in the thermocycler and advance the program or place the 1.5 mL screw cap microfuge tubes into the 37˚ C heat block.

Thermocycler Program	Heat Block (1.5 mL screw cap microfuge tube)
15 min at 37 ˚C with open lid or lid heating OFF	15 min at 37 ˚C

Normalase Inactivation

27. Following the Normalase II reaction, pre-set a thermocycler program as listed below.
28. Add 0.2 µl of Reagent B1 multiplied by the total number of libraries within each prepared pool, see examples below:

Reagent	Per Library	24-Plex Pool	96-Plex Pool
Reagent B1	0.2 µl	4.8 µl	19.2 µl

29. Place the library pools in the thermocycler and advance the program or place the 1.5 mL screw cap microfuge tubes into the heat block. If using a 1.5 mL screw cap microfuge tube, set a heat block at 95 °C to incubate your library pools, being careful to not incubate the samples longer than 2 minutes.

Thermocycler Program	Heat Block (1.5 mL screw cap microfuge tube)
Hold at 95 °C 2 min at 95 °C with lid kept at 95 °C Hold at 4 °C	2 min at 95 °C

30. Your final library pools are at 4 nM and are ready for further pooling, dilution, and loading the instrument. It is not necessary to perform an additional purification step.

Final pools contain single stranded DNA and can be stored at - 20 °C for up to two weeks before sequencing. For longer term storage, refer back to the safe stopping point following post-Normalase I, and perform the pooling and subsequent steps as indicated above prior to sequencing.

Recommended Quality Control

To ensure optimal sequencing results we recommend that you perform a qPCR quantification on your final Normalase pool(s). Final library pools are ssDNA and cannot be quantified by fluorometric methods or Bioanalyzer. If you have a validated qPCR assay that reproducibly predicts an optimal number of reads on your sequencing instrument, concentration adjustment of your final pool can be performed based on your qPCR results. If you do not have a validated qPCR assay, we recommend a commercially available kit such as KAPA Library Quantification Kit (Cat. No. KK4828). Follow the manufacturer's protocol assuming a 4 nM concentration and confirm the accuracy of the assay and results before proceeding with concentration adjustment of the final pool(s) based on the qPCR results.

- If qPCR results indicate gross concentration deviation from 4 nM expected normalized pool concentration, indicative of library failure or Normalase processing error, consult the Troubleshooting section of this protocol (Appendix, Section C) or contact Swift technical support at TechSupport@swiftbiosci.com for assistance.
- If qPCR quantification indicated pools were below 4 nM, this may be due to a failure of a subset of samples within a pool. If this is observed, quantify your Normalase I-treated libraries to identify which libraries have failed or are < 12 nM.
- The Normalase product specification is defined by cluster density of the final 4 nM Normalase pool when loaded on a MiSeq v2 flow cell at 12 pM to achieve a 1000-1200 K/mm^2 cluster density and CV ≤ 10% within a pool (Swift 2S Turbo Flexible libraries with an average 200 bp insert size). Across other Illumina platforms, library types, and insert sizes, optimization of loading concentration may be required to achieve the optimal number of reads supported by the flow cell of choice.

If you experience problems with your Swift Normalase Kit, please contact us at TechSupport@swiftbiosci.com, or by phone at 734.330.2568 (9:00 am-5:00 pm ET, Monday-Friday).

Appendix

Section A: Low-plex Pooling Recommendations

Please use the following recommendations for pooling, Normalase II, and Normalase Inactivation if pooling less than 5 libraries or post-hybridization capture library pools. These pooling recommendations are to avoid pipetting errors from dispensing extremely small volumes.

Number of Libraries or Pools	Volume Per Library or Pool	Total Volume	Normalase II MasterMix Volume	Reagent B1 Inactivation Volume
1*	25.00 µl	25 µl	5 µl	1 µl
2	12.50 µl	25 µl		
3	8.30 µl	25 µl		
4	6.25 µl	25 µl		
5	5.00 µl	25 µl		

** Do not proceed with the above recommendations unless sequencing within the next two weeks as remaining library will not be available to repeat the pooling and subsequent steps. Final libraries are single stranded and can be stored at –20 ºC for up to two weeks before sequencing.*

20. 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. As mentioned previously, use of a calibrated pipette will produce best results with this pipetting step to ensure even pooling.

21. Thoroughly mix, and spin the library pools in a microfuge, and proceed to Normalase II reaction with the library pools created.

Normalase II

22. Pre-set a thermocycler program for 15 minutes at 37 °C with open lid or lid heating OFF.

23. Pre-mix 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*	Per Pool	5 Pools
Buffer G1	4.8 µl	24 µl
Enzyme G2	0.2 µl	1 µl
Total Volume	**5.0 µl**	**25 µl**

** We recommend constructing Normalase II master mix for 5 pools even if you are processing less than 5 pools in order to avoid pipetting extremely low volumes.*

Normalase Inactivation

24. Following the Normalase II reaction, pre-set a thermocycler program as listed below.

25. Add 1 µl of Reagent B1 to each pool.

Reagent	Per Pool
Reagent B1	1 µl

26. Place the library pools in the thermocycler and advance the program.

Thermocycler Program
Hold at 95 °C 2 min at 95 °C with lid kept at 95 °C Hold at 4 °C

27. Your final library pools are at 4 nM and are ready for further pooling, dilution, and loading the instrument. It is not necessary to perform an additional purification step.

Final pools contain single stranded DNA and can be stored at - 20 °C for up to two weeks before sequencing. For longer term storage, refer back to the safe stopping point following post-Normalase PCR purification, and perform the pooling and subsequent steps as indicated above prior to sequencing.

For Quality Control recommendations, please refer to page 14.

Section B: High sample volume pooling recommendations

Please use the following recommendations for Sample Pooling, Normalase II, and Normalase Inactivation steps if you require a higher final volume of 4 nM pool than the volume produced by the standard protocol that uses 5 µl per sample. These pooling recommendations can enable you to sequence on NovaSeq or generate higher volumes for loading a single pool over multiple lanes or flow cells.

For example, NovaSeq requires 100-310 µl loading volume at 1.5-3 nM concentration and if multiplexing a lower number of samples, you may need to pool more than 5 µl of each sample to achieve a sufficient loading volume at the required concentration. The table below outlines the flexible options available to support a broad range of final 4 nM pool volumes 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)**	G1	0.96	1.44	1.92	2.88	3.84	4.80
		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)**	B1	0.20	0.30	0.40	0.60	0.80	1.00
D	**Final 4nM 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	111.59	148.80	186.00
		12 samples	74.40	111.60	148.80	223.19	297.60	372.00
		18 samples	111.60	167.40	223.20	334.78	446.40	558.00
		24 samples	148.80	223.20	297.60	446.37	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.**

(Table legend continues on next page.)

D. **Final 4 nM 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 thermocycler, whereas final volumes in gray cells must be prepared in a 1.5 ml screw cap microfuge tube and heat block to accommodate the larger volumes.*

E. **Minimum number of reactions recommended for Normalase II Master Mix assembly.** *We recommend preparing Normalase II master mix for the minimum reaction number shown to avoid pipetting extremely low volumes. Keep in mind that repeatedly processing fewer samples than the recommended master mix volume will result in significant loss of Normalase II reagents.*

20. 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 screw cap microfuge tube, depending on the final desired volume.

    As mentioned previously, use of a calibrated pipette will produce best results with this pipetting step to ensure even pooling.

21. Thoroughly mix, and spin the library pools in a microfuge, and proceed to Normalase II reaction with the library pools created.

Normalase II

22. Pre-set a thermocycler program for 15 minutes at 37 °C with open lid or lid heating OFF. Alternatively, if using a 1.5 mL screw cap microfuge tube, set a heat block at 37°C.

23. Pre-mix Normalase II Master Mix depending on the chosen pooling volume and minimum master mix reaction volume (listed in the table below). The master mix can be stored on ice until use, and then added to pools at room temperature.

Reagents	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 µl	25.92 µl	23.04 µl	28.8 µl	23.04 µl	24.00 µl
Enzyme G1	0.96 µl	1.08 µl	0.96 µl	1.2 µl	0.96 µl	1.00 µl
Total Volume	**24.00 µl**	**27.00 µl**	**24.00 µl**	**30.00 µl**	**24.00 µl**	**25.00 µl**

24. 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, see volumes below:

Reagents	5 µl	7.5 µl	10 µl	15 µl	20 µl	25 µl
Normalase II Master Mix Per Sample	1 µl	1.5 µl	2.00 µl	3.00 µl	4.00 µl	5.00 µl

25. Mix well by vortexing for 5 seconds, and spin down the library pools in a microfuge.

26. Place the library pools in the thermocycler and advance the program or place the 1.5 mL screw cap microfuge tubes into the 37°C heat block.

Thermocycler Program	Heat Block (1.5 mL screw cap microfuge tube)
15 min at 37 ºC with open lid or lid heating OFF	15 min at 37 ºC

Normalase Inactivation

27. Following the Normalase II reaction, pre-set a thermocycler program as listed below.

28. 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, see volumes below:

Reagents	5 µl	7.5 µl	10 µl	15 µl	20 µl	25 µl
⊙ Reagent B1 Per Sample	0.20 µl	0.30 µl	0.40 µl	0.60 µl	0.80 µl	1.00 µl

29. Place the library pools in the thermocycler and advance the program or place the 1.5 mL screw cap microfuge tubes into the heat block. If using a 1.5 mL screw cap microfuge tube, set a heat block at 95°C to incubate your library pools, being careful to not incubate the samples longer than 2 minutes.

Thermocycler Program	Heat Block (1.5 mL screw cap microfuge tube)
Hold at 95 ºC 2 min at 95 ºC with lid kept at 95 ºC Hold at 4 ºC	2 min at 95 ºC

30. Your final library pools are at 4 nM and are ready for further pooling, dilution, and loading the instrument. It is not necessary to perform an additional purification step.

    Final pools contain single stranded DNA and can be stored at -20°C for up to two weeks before sequencing. For longer term storage, refer back to the safe stopping point following post-Normalase I, and perform the pooling and subsequent steps as indicated above prior to sequencing.

    For Quality Control recommendations, please refer to page 14.

Section C: Helpful Information and Troubleshooting

Problem	Possible Cause	Suggested Remedy
Variation of read counts (CV > 10%) between libraries within a Normalase pool	1. Inconsistent pipetting of Normalase I Master Mix or inconsistent pipetting of individual libraries into a pool. 2. Artifact of flow cell loading being outside of Illumina's recommended range	1. Use a P10 pipettor if available and ensure pipettes are maintained and calibrated 2. Adjust loading concentration accordingly
Some libraries were significantly under-represented in the sequence data	These libraries may not have met the minimum threshold of 12 nM in 20 µl library yield	1. Quantify affected libraries to determine if library concentration was less than 12 nM 2. Review whether the number of PCR cycles was appropriate for your sample quality and quantity
Recommended qPCR quantification of pools following Normalase II indicated unexpected yields	See page 14	See page 14
Recommended qPCR quantification of pools following Normalase indicated no library yield present	Library preparation, amplification, or Normalase I failure	Contact TechSupport@swiftbiosci.com for specific troubleshooting recommendations
Recommended qPCR quantification of pools following Normalase indicated significant excess yield of 12 nM or greater	Normalase II failure, selected 4 nM fraction was not enzymatically normalized	Re-pool the post-Normalase I libraries and repeat Normalase II and inactivation
Fluorometric methods such as BioAnalyzer or Qubit indicated no library	Normalase libraries are single stranded and cannot be detected by dsDNA interclating dyes	Perform qPCR quantification
Overall cluster density/ reads passing filter was lower than expected	1. Normalase pools were stored for greater than two weeks 2. Libraries were consistenly less than 12 nM post-library amplification	1. Re-pool the post-Normalase I libraries and repeat Normalase II and inactivation 2. Review whether the number of PCR cycles was appropriate for your sample quality and quantity

If you experience problems with your library prep, please contact us at TechSupport@swiftbiosci.com, or by phone at 734.330.2568 (9:00 am-5:00 pm ET, Monday-Friday).

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