SWIFT PROTOCOL

Swift Normalase® Amplicon Panels (SNAP)

Single-tube Multiplex PCR NGS Library Prep with a High-throughput Workflow

Protocol for Cat. Nos.:

Panels:

Oncology:
562G1-96, 56G Oncology Panel v2
PC8G1-96, 57G Pan-Cancer Profiling Panel
520G1-96, BRCA1 and BRCA2 Panel
570G1-96, BRCA1, BRCA2, and PALB2 Panel
510G1-96, EGFR Pathway Panel
530G1-96, Comprehensive TP53 Panel
LG8G1-96, Lung Cancer Panel
CR8G1-96, Colorectal Cancer Panel
LN8G1-96, Lynch Syndrome Panel
MY8G1-96, Myeloid Panel

Disease Susceptibility:
550G1-96, CFTR Panel
ACE2G1-96, ACE2 Panel

Sample Tracking:
500G1-96, Sample_ID Panel

Core Kits:
SN-5X296, Swift Normalase Amplicon Panels (SNAP) Core Kit (96 rxns)
SN-5X384, Swift Normalase Amplicon Panels (SNAP) Core Kit (4x96 rxns Bundle)

Indexing Primers:
SN-5S1A96, SNAP Set 1A Combinatorial Dual Indexing Primers (96-plex, 96 rxns)
SN-5S1B96, SNAP Set 1B Combinatorial Dual Indexing Primers (96-plex, 96 rxns)
SN-5S2A96, SNAP Set 2A Combinatorial Dual Indexing Primers (96-plex, 96 rxns)
SN-5S2B96, SNAP Set 2B Combinatorial Dual Indexing Primers (96-plex, 96 rxns)
SN-5S0384, SNAP Set S1AB-S2AB Combinatorial Dual Indexing Primers (384-plex, 4x96 rxns Bundle)

SN91096-1-PLATE, SNAP Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU001-SU096)
SN91096-2-PLATE, SNAP Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU097-SU192)
SN91096-3-PLATE, SNAP Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU193-SU288)
SN91096-4-PLATE, SNAP Unique Dual Indexing Primer Plate (96-plex, 96 rxns, SU289-SU384)
SN91384-PLATES, SNAP Unique Dual Indexing Primer Plates (384-plex, 4x96 rxns Bundle)

Visit swiftbiosci.com/protocols for updates.

Swift BIOSCIENCES™

www.swiftbiosci.com

Table of Contents

Support

For technical support, please contact Swift at TechSupport@swiftbio.com, or by phone: 734.330.2568 (9:00 am – 5:00 pm ET, Monday through Friday).

Product Information

SNAP Kits offer a robust NGS workflow that provides optimal coverage and NGS data quality on Illumina® sequencing platforms. These kits leverage patented multiplex PCR technology, enabling library construction from a variety of sample types, including formalin-fixed, paraffin-embedded (FFPE) and circulating, cell-free DNA (cfDNA) using tiled, overlapping primer pairs within a single multiplexed pool.

See Appendix A for panel specific information on panel content and size as well as multiplexed sequencing recommendations. See individual panel data sheets for details regarding target design and coverage.

Product Feature	Specification
Input Material	10-25 ng DNA
Time	2 hours DNA-to-Library 3 hours DNA-to-Normalized-Library-Pool
Components Provided	• Target-specific multiplex primer pool • PCR and library prep reagents • Swift Normalase • Combinatorial and Unique Dual indexing primers Note: kits do not include magnetic beads
Multiplexing Capability	Up to 384 CDI or 394 UDI Inquire for custom indexing
Recommended Depth	500X coverage (germline variant detection); 5000X coverage (somatic variant detection down to 1% allele frequency)
Performance	>90% on target >90% coverage uniformity (at >20% of the mean)

Applications and Sample Types

SNAP Panels may be used for the following applications and sample types.

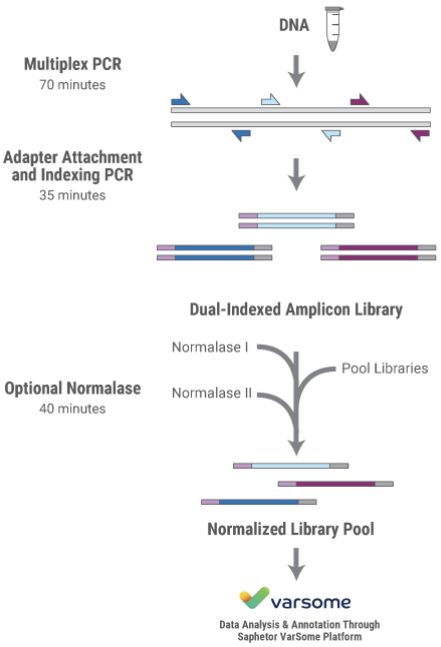
- Applications: Variant discovery, Disease disposition, Oncology, Sample tracking

Sample Types: gDNA from Fresh Frozen, FFPE, cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), blood

SNAP Workflow

Swift Normalase Amplicon Panels (SNAP) utilize multiple overlapping amplicons in a single tube, using a rapid, 2-hour workflow to prepare ready-to-sequence libraries. The PCR1+PCR2 workflow generates robust libraries, even from low input quantities. The libraries may be quantified with conventional methods such as Qubit® or Agilent Bioanalyzer and normalized by manual pooling, or normalized enzymatically with the included Swift Normalase reagents.

This protocol includes instructions for a Multiplex PCR step to enrich target sequences, an Indexing PCR step to amplify and add combinatorial or unique dual indexed adapter sequences, and an optional downstream Normalase step to produce an equal molar library pool.



Kit Contents

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

Protocol Stage	Component	96 rxns	Storage
Multiplex PCR (Pre-PCR box)	Reagent G1*	2x 106 µl	-20 °C
	Reagent G2	317 µl	
	Enzyme G3	1584 µl	
	Pre-PCR TE	1200 µl	
Indexing PCR (Post-PCR box)	Reagent I1	348 µl	
	Enzyme I2	53 µl	
	Enzyme I3	15 µl	
	Enzyme I4	2640 µl	
	Post-PCR TE	4161 µl	
Indexing Box**	SNAP CDI D50X	26 µl each	
	SNAP CDI S7XX	15 µl each	
Indexing Plate**	SNAP UDI (pre-mixed pairs)	12 µl per well	
Normalase (Post-PCR box)	Buffer S1	454 µl	
	Reagent S2	21 µl	
	Enzyme S3	53 µl	
	Buffer N1	101 µl	
	Enzyme N2	10 µl	
	Reagent X1	21 µl	
Additional reagents	PEG NaCl	20 mL	Room Temp

*Reagent G1 is included in the Multiplex Primer pool. Additional Pre-PCR and Post-PCR box reagents and PEG NaCl are included in Cat. No. SN-5X296.

**Indexing boxes are Cat. Nos. SN-5S1A96, SN-5S1B96, SN-5S2A96 and SN-5S2B96. Indexing plates are Cat. Nos. SN91384-PLATE, SN91096-1-PLATE, SN91096-2-PLATE, SN91096-3-PLATE, SN91096-4-PLATE.

Storage and Usage Recommendations

Upon receipt, store the kit at -20 °C with the exception of the PEG solution, which is stored at room temperature. Separate the Multiplex PCR Reagents (keep in pre-PCR area) and Indexing and Normalase Reagents (keep in post-PCR area). 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. Attempting to pipette enzymes at -20 °C may result in a shortage of enzyme reagents. After thawing reagents, briefly vortex (except the enzymes) to mix them well. Enzyme G3 is the only enzyme that may be vortexed. Spin all tubes in a microfuge to collect contents prior to opening. Always add reagents to the master mix in the specified order as stated throughout the Protocol. The indexing primers (SNAP CD and UD Indexes) are the only reagents that are added individually to each sample.

Materials and Equipment Not Included

- SPRIselect beads (Beckman Coulter, Cat. No. B23317/B23318/B23319) or Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880/A63881/A63882)
- Permagen Magnetic Separator (Cat. No. MSR812), Agencourt SPRIPlate or similar magnetic rack for magnetic bead clean-ups
- Qubit, Nanodrop, or similar input DNA quantification assay, optionally Swift Alu primers for qPCR
- Electrophoretic-, fluorometric- or qPCR-based library quantification assay for Illumina libraries
- Microcentrifuge
- Programmable thermocycler operating within manufacturer's specifications
- 0.2 mL PCR tubes or 96-well plate
- Aerosol-resistant tips and pipettes ranging from 1-1000 µL
- 200-proof/absolute ethanol (molecular biology grade) and nuclease-free water for preparation of 80% ethanol

  Pipette tips (i.e. 8-channel or 12-channel), 8-tube strips, unskirted 96 well plate or plate puncher for pre-piercing the foil seal if using the UD indexing plates.

Tips and Techniques

- Assemble all reagent master mixes and reactions ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Calculate the total volume of the master mixes and prepare them in advance to ensure the magnetic beads do not over-dry during size selection steps while awaiting completion of master mix assembly.

- Neglecting to store master mixes and reagents on ice prior to incubations reduces performance of this product.

- To avoid cross-contamination: physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed, including appropriate reagent boxes for Multiplex PCR and Indexing PCR. Clean both the pre-PCR and post-PCR 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 and dispose of pipette tips and other disposables in sealed plastic bags. Move samples to post-PCR area before opening tubes. This workflow, like any amplicon enrichment technology, poses a risk of contamination of surfaces and other samples following the amplification step. Please use caution when opening your sample tubes following the Multiplex PCR step.

Input Material Considerations

The starting material should be quantified with the appropriate assay (qPCR-based for FFPE and cfDNA to determine usable DNA content and sample integrity, Qubit for high quality DNA from whole blood, fresh frozen, or cultured cells). Most panels are designed with amplicon size of 140 bp to ensure compatibility with both FFPE and cfDNA samples.

For qPCR-based determination of sample quantity and integrity, Alu primers are available from Swift at www.swiftbiosci.com Input DNA quantification primers Cat. No. 90396.

https://swiftbiosci.com/wp-content/uploads/2019/02/17-1480-Input-DNA-Protocol-web.pdf

The optimal coverage uniformity, sensitivity, and specificity of this technology are achieved with input amounts in the 10-25 ng range. Between 25-100 ng, coverage uniformity may be mildly reduced while sensitivity and specificity are preserved. Using less than 10 ng may reduce specificity of the assay, reduce library yields and reduce sensitivity of variant calling for low frequency alleles. Consider the following example allele frequencies and detection limits. As shown below it is recommended to use at least 10 ng input for 1% allele detection limit to ensure sufficient copy number of the allele of interest. If less than 10 ng is available, expect a lower detection limit, for example at 1 ng input a 10% limit of detection is expected. When using damaged samples, ensure that the usable amplifiable input is qualified by a qPCR assay as mentioned above.

Sample Quantity	Human Genome Equivalents	Example Allele frequency	Example Allele copies	Likelihood of detection
10 ng	3000	10%	300	✓
10 ng	3000	1%	30	✓
1 ng	300	10%	30	✓
1 ng	300	1%	3	X

Prepare SNAP Libraries

Multiplex PCR Step

1. Load the Multiplex PCR program and allow the block to reach 98 °C before loading samples (confirm lid heating is turned ON and is set to reach 105 °C).

Pre-Program Thermocycler

Multiplex PCR Thermocycler Program	**Lid heating ON**		
	30 sec	98 °C	
	10 sec	98 °C	**4 cycles**
	5 min	* °C	
	1 min	* °C	
	10 sec	98 °C	**18 cycles**
	1 min	64 °C	
	1 min	65 °C	
	Hold	4 °C	

*** NOTE:** Temperature at this step is panel specific. Please use the following panel specific annealing temperature specified below:

Panel	Annealing Temperature (5min)	Extension Temperature (1min)
56G Oncology v2 **BRCA1 and BRCA2** **BRCA1 and BRCA2 and PALB2** **CFTR** **Comprehensive TP53** **Sample_ID** **EGFR Pathway** **ACE2**	**63 ºC**	**65 ºC**
57G Pan-Cancer Profiling	**65 ºC**	**65 ºC**
Lung Cancer **Myeloid** **Colorectal Cancer** **Lynch Syndrome**	**66 ºC**	**66 ºC**

2. Gently nutate Enzyme G3 at Room Temperature for 5 minutes, or until all solutes appear to be in solution. Place back on ice for remainder of use.
3. Load 10 µl of DNA sample directly into each PCR tube.

4. **Keep all tubes on ice during assembly of the master-mix and the reaction until placed in thermocycler.**

Panel-Specific Multiplex PCR Reaction Mix

Before mixing, calculate the total volume of the master mix based on the number of reactions required with appropriate overage for pipetting. Vortex components G1 and G2 and pulse-spin tubes to collect contents. Make the Multiplex PCR Reaction Mix. Keep prepared master mix on ice until ready to use.

Component	Volume (1 Reaction)
• Reagent G1*	2 µl
• Reagent G2	3 µl
• Enzyme G3	15 µl
Reaction Mix	**20 µl**

**Reagent G1 is the panel-specific set of multiplex primers.*

5. Mix the master mix well and then add 20 µl of the Multiplex PCR Reaction Mix to each 10 µl input DNA sample on ice. Mix well, then place in the thermocycler and run the program.

IMPORTANT!
Move samples to post-PCR area before opening tubes.

6. Near the completion of the thermocycler run, prepare the Indexing Reaction Mix (below) in the post-PCR area. **Assemble this reaction mix on ice and keep cold until adding it to samples in the Indexing Step**. All components except indexes may be master-mixed when running multiple samples in parallel.

Indexing PCR Step

Before mixing, calculate the total volume of the master mix based on the number of reactions of choice, with appropriate overage for pipetting. We recommend preparing at least 10 reactions at any one time to maintain a volume of Enzyme I3 that can be accurately pipetted. Keep prepared master mix on ice.

Component	Volume (1 Reaction)
• Reagent I1	3.3 µl
• Enzyme I2	0.5 µl
• Enzyme I3	0.1 µl
• Enzyme I4	25 µl
Reaction Mix	**28.9 µl**

Keep prepared master mix on ice during Size Selection and Clean-Up Step 1.

Size Selection and Clean-Up Step 1

7. Ensure beads and samples are at room temperature. Briefly vortex beads to homogenize before use.
8. Add 30 µl (ratio: 1.0) of magnetic beads to each 30 µl sample. Mix by vortexing. Pulse-spin the samples in a microfuge. Ensure no bead-sample suspension droplets are left on the sides of the tube.
9. Incubate the samples for 5 minutes at room temperature off the magnet.
10. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (≈ 5 minutes).
11. While leaving your sample on the magnet, remove and discard the supernatant without disturbing the pellet (approximately 5 µl may be left behind). Leave tubes on the magnet.
12. Add 180 µl of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds, and then carefully remove the ethanol solution.
13. Repeat, for a second wash with the ethanol solution.
14. Pulse-spin the samples in a microfuge, place back onto the magnet and remove any residual ethanol solution from the bottom of the tube with a small volume tip.
15. Resuspend each bead pellet in 17.4 µl Post-PCR TE Buffer. Proceed to the Indexing PCR Step.

IMPORTANT!

Continue working in the post-PCR area. Keep samples at room temperature. At no time should 'with bead' samples be stored on ice, as this affects binding to magnetic beads.

Indexing PCR Thermocycler Program	**Lid heating ON (105 °C)**		
	20 min	37 °C	
	30 sec	98 °C	
	10 sec	98 °C	**5 cycles***
	30 sec	60 °C	
	1 min	66 °C	
	Hold	4 °C	

***The PCR cycle number can be increased for samples that may give low yields.**

16. Load the Indexing PCR program and allow the block to reach 37 °C before loading samples (confirm lid heating is turned ON and is set to reach 105 °C).
17. Add a unique combination of 2 µl SNAP CD Index D50X + 1.7 µl SNAP CD Index S7XX to each sample

    OR

    Add 3.7 µl of a pre-mixed SNAP UD indexing primer pair to each sample if using the single use plates.

    (see page 19 for UDI plate usage guidelines).
18. Add 28.9 µl of the cold Indexing PCR Reaction Mix to each sample and mix thoroughly (total volume 50 µl).

19. Place in the thermocycler and run the program.

Size Selection and Clean-Up Step 2

20. Ensure PEG NaCl solution is at room temperature. Briefly vortex the PEG NaCl solution to homogenize before use.
21. Add 42.5 µl (ratio: 0.85) of PEG NaCl solution to each 50 µl sample. Mix by vortexing. Ensure no bead-sample suspension droplets are left on the sides of the tube.
22. Incubate the samples for 5 minutes at room temperature off the magnet.
23. Pulse-spin the samples in a microfuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (≈ 5 minutes).
24. While leaving your sample on the magnet, remove and discard the supernatant without disturbing the pellet (approximately 5 µl may be left behind). Leave tubes on the magnet.
25. Add 180 µl of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Use care not to disturb the pellet. Incubate for 30 seconds and then carefully remove the ethanol solution.
26. Repeat, for a second wash with the 80% ethanol solution.
27. Pulse-spin the samples in a microfuge, place back onto the magnet and remove any residual ethanol solution from the bottom of the tube with a small volume tip.
28. Proceed immediately to add 20 µl of Post-PCR TE buffer and resuspend the pellet, mixing well by pipetting up and down until homogenous. Incubate at room temperature for 2 minutes off the magnet. Then place the sample back on the magnet and transfer the clean 20 µl library eluate to a fresh tube. Ensure that eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, pipette eluate into a new tube, place on magnet, and transfer eluate again.

Note: Libraries are now completed and ready to sequence. Please proceed with quantifying and pooling libraries, using either conventional fluorometric (i.e. Qubit) or electrophoretic (i.e. Bioanalyzer) methods, or proceed to Normalase below (reagents are included).

Library Quantification

Accurate library quantification is essential to properly load the sequencing instrument. Libraries can be quantified using fluorometric-, electrophoretic-, or qPCR-based methods and normalized manually. Alternatively, libraries can be enzymatically normalized following the Normalase protocol below. Note, for optimal normalization using Normalase, a minimum of 12 nM yield is needed per sample. If library yields are below 12 nM, increase the number of PCR cycles to pass the 12 nM threshold or switch to the 6 nM threshold Normalase protocol described below.

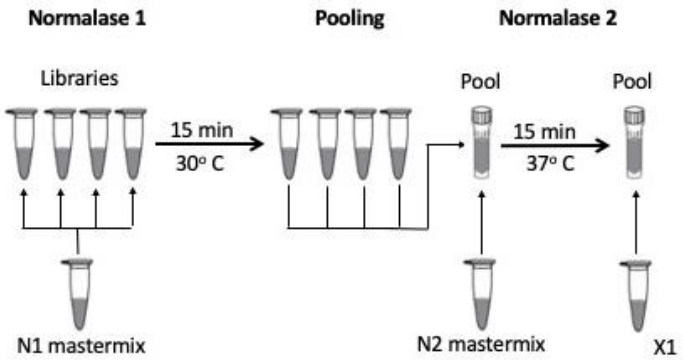
Introduction to Normalase

This guide provides instructions for optional enzymatic normalization of multiplexed SNAP next generation sequencing (NGS) libraries for equimolar pools and balanced sample representation in sequencing. The protocol is designed for SNAP libraries that produce consistent amplified library yields of ≥12 nM following Indexing PCR, and it generates an equal molar library pool. Most samples processed with the SNAP protocol produce amplified library yields of 12 nM or greater; however, if there's concern that all samples won't reach 12 nM, adjusting Normalase chemistry to only require a minimum of 6 nM can alternatively be performed.

A simple calculator for converting between ng/µL and nM is available. Please use a finished library size of 285bp (for use in the Base Pair Length column).

The workflow consists of three steps for libraries amplified to a minimum yield of 12 nM during the adapter attachment and Indexing PCR Step:

1. **Normalase I** to enzymatically select a 4 nM (or 2 nM if using ≥6 nM option) library fraction
2. **Library Pooling** of samples for multiplexed sequencing
3. **Normalase II** to enzymatically generate an equimolar library pool



Workflow schematic: *Normalase I Mastermix is added to samples and incubated at 30ºC for 15 minutes. Sample pooling is then performed and then the Normalase II Mastermix is added to the pool and incubated at 37ºC for 15 minutes. Reagent X1 inactivates the reaction and a final equal molar pool is produced.*

Notes Regarding Normalase Specification

The Normalase product specification is defined by cluster density of the 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 ≤ 15% within a pool. Across Illumina platforms, library types, and insert sizes, the optimization of loading concentration may be required to achieve the optimal number of reads supported by the flow cell of choice.

Normalase I: Enzymatic Selection

If you are concerned that the 12 nM threshold has not been met after Indexing PCR:

- Spot check library yields using either fluorometric methods (ie: Qubit) or electrophoretic methods (ie: Bioanalyzer).
- A Normalase workflow modification can be performed that requires a 6 nM threshold to obtain a 2 nM Normalase pool (see below).

1) Pre-set a thermocycler program as listed below.

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

Prepare the Normalase I Master Mix as 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. For libraries with lower yields ≥ 6 nM, or for a final pool of 2 nM (instead of 4 nM), please use

half of the specified volume of Reagent S2 and add an equal volume of TE, thus reducing concentration by two-fold, then proceed as written.

Reagent	Per Library	24 Libraries	96 Libraries
• Buffer S1	4.3 µl	103.2 µl	412.8 µl
• Reagent S2	0.2 µl	4.8 µl	19.2 µl
• Enzyme S3	0.5 µl	12 µl	48 µl
Total Volume	**5 µl**	**120 µl**	**480 µl**

IMPORTANT!

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

2) Using a calibrated P10 pipette, 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.

3) Spin down the sample tube in a microfuge. Place in the thermocycler and run the program.

Safe Stopping Point

Libraries can be stored at -20 °C post-Normalase I.

Equal Volume Library Pooling

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

NOTE: If you are pooling < 5 libraries, contact tech support at TechSupport@swiftbio.com for low-plex pooling recommendations.

NOTE: If pooling 5 µl per sample does not generate a normalized pool of sufficient volume for instrument loading, contact tech support at TechSupport@swiftbio.com for high sample volume pooling recommendations.

IMPORTANT!

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 100,000 reads can be pooled together whereas samples requiring 1 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.

1. Following the Normalase I incubation, generate a library pool (or pools) by 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 libraries as the volume will exceed the PCR tube maximum volume.

To ensure even pooling, use of a calibrated P10 pipette will produce the best results.

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

Normalase II: Enzymatic Normalization

1. Pre-set a thermocycler program as listed below. Alternatively, if using a 1.5 mL screw cap microfuge tube, set a heat block at 37 °C.

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

2. 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	24 Libraries	96 Libraries
• Buffer N1	0.96 µl	23.04 µl	92.16 µl
• Enzyme N2	0.04 µl	0.96 µl	3.84 µl
Total Volume	**1 µl**	**24 µl**	**96 µl**

*It is recommended to prepare Normalase II master mix for 24 samples even if you are processing less than 24 samples in order to avoid pipetting extremely low volumes; for best results use a calibrated P2 pipet for adding Enzyme N2. 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.

3. Add 1 µl of Normalase II Master Mix multiplied by the total number of libraries within each prepared pool.

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

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

Normalase Inactivation

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

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

2. Add 0.2 µl of Reagent X1 multiplied by the total number of libraries within each prepared pool, see examples below:

Reagent	Per Library	24-Plex Pool	96-Plex Pool
• Reagent X1	0.2 µl	4.8 µl	19.2 µl

3. 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 not to incubate the samples longer than 2 minutes.

4. Your final multiplexed library pools are now equimolar. Proceed to qPCR quantification of your Normalase pool and sequencing. It is not necessary to perform an additional purification step.

Quantification and Calibration of Normalase Pools

To ensure optimal sequencing results, 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 fragment analysis. If you do not have a qPCR assay, validate a commercially available kit by calibrating your qPCR results and sequencer loading concentrations before proceeding (for example KAPA Library Quantification Kit, Cat. No. KK4828).

The Normalase 4 nM formulation may not conform to your qPCR assay quantification due to the lack of precision across different qPCR assays and laboratory practices. Using your validated qPCR assay that reproducibly predicts an optimal number of reads on your sequencing instrument, load your final pool based on your qPCR results. 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 have chosen the 6 nM to 2 nM option but require a higher pool concentration for your sequencer, perform a 2.0X SPRI to concentrate pool and then proceed to qPCR quantification and loading.

Sequencing Recommendations

SNAP libraries may be sequenced using paired-end sequencing on Illumina instruments. We strongly recommend using 2 x 150 paired-end reads. The depth of coverage required will depend on the application (see page 3). Be sure to use either 8 (CD) or 10 (UD) sequencing cycles for each index read.

See Appendix A for panel specific information on content and size as well as multiplexed sequencing recommendations.

Due to the complexity of the libraries, no PhiX spike-in is required on MiSeq or MiniSeq instruments. The NextSeq550 may be sensitive to low complexity and PhiX or another suitable high-complexity library spike-in may be required. Contact Illumina technical support for further information regarding sequencing instrument compatibility with low-complexity sequences.

Please refer to the latest version of Illumina Experiment Manager for detailed instructions on how to set up a sample sheet. Be sure to select the appropriate workflow parameters as noted below:

- Read Type: “Paired End”
- Cycle Read 1: “151”, Cycles Read 2: “151”

Make sure the “Use Adapter Trimming” and “Use Adapter Trimming Read 2” are sel**ected** when setting up the sequencing run. Failure to trim adapter sequences will result in incorrect primer trimming and will lead to inaccurate variant calling. Alternatively, perform adapter trimming by Trimmomatic during data analysis. For more information, see below and consult our Bioinformatics Resources page at swiftbiosci.com/biofx.

Sequencing Data Analysis

Dockerized Data Analysis Workflow

For customers using a SNAP Kit who are comfortable with command line tools and want a ready-to-use variant calling analysis workflow they can run on a Linux or MacOS machine, Swift offers a full variant calling workflow with all tools and reference files pre-installed and configured in a Docker image available for download. For customers without command line tools, VarSome Platform may be used for data analysis on selected panels. Please refer to the data sheet for more details.

Features

- One easy install: Docker image file can be downloaded and loaded onto a Linux or MacOS server running the Docker daemon
- Easy-to-run analysis workflow: includes a Bash run script which handles all Docker commands, requiring only the input FASTQ files and the panel master file as arguments

Requirements

- Linux or MacOS with Docker installed
- At least 8GB of RAM (>= 32GB recommended)

Analysis Overview

1. Adapter trimming and filtering out of reads < 30 bases long (Trimmomatic)
2. Alignment (BWA MEM)
3. Primer trimming (Primerclip)
4. On-target and coverage metrics calculation and reporting
5. Variant calling (GATK Haplotype Caller and/or lofreq)

Follow this Download Link for the analysis workflow: https://ws.onehub.com/folders/apetm9gs

For more detailed information about primer trimming, please review the Primerclip Technical Note at: https://swiftbiosci.com/wp-content/uploads/2019/11/TEC-005-PRIMERCLIP-A-TOOL-FOR-TRIMMING-PRIMER-SEQUENCES-USING-COMMAND-LINE-OR-GALAXY-Rev-1.pdf

Indexed Adapter Sequences

The full-length adapter sequences are below, where the underlined text indicates the location of the index sequences which are 8b for CDI and 10b for UDI. These sequences represent the adapter sequences following completion of the **Indexing PCR** step.

Index 1 (i7) Adapters:
5' – GATCGGAAGAGCACACGTCTGAACTCCAGTCACXXXXXXXX(XX)ATCTCGTATGCCGTCTTCTGCTTG – 3'

Index 2 (i5) Adapters:
5' – AATGATACGGCGACCACCGAGATCTACACYYYYYYYY(YY)ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3'

Please refer to the accompanying Swift Index Master List available at https://swiftbiosci.com/protocols/ for index sequences for preparing your Illumina sequencing sample sheet on your instrument of choice.

Primer Sequences

For reference, the primer sequences are below; these primers include full-length Illumina adapter and index sequences.

i7 primer: Replace 8 (CDI) or 10 (UDI) **X's** with the **REVERSE COMPLEMENT** of the specified i7 index sequence in the Swift Index Master List:
5' – CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX(XX)**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 3'

i5 primer: Replace 8 (CDI) or 10 (UDI) **Y's** with the specified Forward Strand Workflow i5 index sequence in the Swift Index Master List:
5' – AATGATACGGCGACCACCGAGATCTACAC**YYYYYYYY(YY)**ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3'

Please contact techsupport@swiftbio.com if you would like assistance confirming compatibility of your own primers with the SNAP workflow, or your local sales representative or distributor to inquire about the purchase of custom Swift Normalase Indexing Primers that use your own index sequences.

Appendix A

Multiplex Sequencing Recommendations Table

Panels:	Number of Amplicons	Average Amplicon Size (bp)	Total Target Size (kb)	Number of Multiplexing on MiSeq® v2 standard	
				Germline 500X average depth	Somatic 5000X average depth
Oncology Panels					
562G1-96, 56G Oncology Panel v2	263	138	1.5	220	22
PC8G1-96, 57G Pan-Cancer Profiling Panel	285	139	25.9	200	20
520G1-96, BRCA1 and BRCA2 Panel	246	148	22.9	240	24
570G1-96, BRCA1, BRCA2, and PALB2 Panel	302	149	28.9	190	19
510G1-96, EGFR Pathway Panel	17	136	1.5	3520	352
530G1-96, Comprehensive TP53 Panel	21	140	1.8	2850	285
LG8G1-96, Lung Cancer Panel	208	144	18	280	28
CR8G1-96, Colorectal Cancer Panel	186	143	17	320	32
LN8G1-96, Lynch Syndrome Panel	180	140	15	330	33
MY8G1-96, Myeloid Panel	478	142	42	120	12
Disease Susceptibility Panels					
550G1-96, CFTR Panel	87	143	10.01	384	38
ACE2G1-96, ACE2 Panel	41	150	4.0	1463	146
Sample Tracking Panel					
500G1-96, Sample_ID Panel	104	145	N/A – SNPs only	90	N/A

Appendix B

Swift 384 UDI Plate Specifications and Dimensions

This product is dispensed in a 96-well plate. Physical specifications are below.

Plate Dimension	Low-Profile 96-Well Skirted Plates
Length at base plane	127.76 mm
Width at base plane	85.48 mm
Height overall	16.06 mm
Well depth	14.81 mm
Well diameter at opening	5.46 mm
Well diameter at bottom of conical section	2.64 mm
Well volume	200 µl
Well spacing	9.00 mm
Well angle	17.5°
Well offset	
Left edge to well A1	14.38 mm
Top edge to well A1	11.24 mm
Left edge to H12	113.38 mm
Top edge to H12	74.24 mm

Plate Usage Guidelines

Prior to piercing the foil and pipetting out the necessary indexes, please be sure to thaw the plate at room temperature, vortex briefly and centrifuge for one minute to spin down the primer reagents to the bottom of the plate wells.

Carefully pre-pierce the foil seal for the intended well(s) prior to pipetting the primer mix out of the plate to add to your reaction(s). Pre-piercing the foil avoids accidental clogging of pipette tips used for liquid pipetting as well as the introduction of foil into the reaction. In addition, pre-piercing the foil reduces the resistance of multi-channel pipettors which can result in undesired movement of the plate that may cause cross-contamination of reagents. The foil may be pre-pierced with pipette tips (i.e. 8-channel or 12-channel), 8-tube strips, an unskirted 96 well plate or a plate puncher.

During the **Indexing PCR** step, use 3.7 µl of a unique indexing primer pair (SU001-SU384 UDIs) to amplify and index each library, where the UDI primer pair must be added individually to each sample.

Libraries made with uniquely indexed adapters may be pooled prior to cluster generation, subjected to Normalase chemistry, and co-sequenced on the same Illumina flow cell.

Plate Layout

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	SU001	SU009	SU017	SU025	SU033	SU041	SU049	SU057	SU065	SU073	SU081	SU089
B	SU002	SU010	SU018	SU026	SU034	SU042	SU050	SU058	SU066	SU074	SU082	SU090
C	SU003	SU011	SU019	SU027	SU035	SU043	SU051	SU059	SU067	SU075	SU083	SU091
D	SU004	SU012	SU020	SU028	SU036	SU044	SU052	SU060	SU068	SU076	SU084	SU092
E	SU005	SU013	SU021	SU029	SU037	SU045	SU053	SU061	SU069	SU077	SU085	SU093
F	SU006	SU014	SU022	SU030	SU038	SU046	SU054	SU062	SU070	SU078	SU086	SU094
G	SU007	SU015	SU023	SU031	SU039	SU047	SU055	SU063	SU071	SU079	SU087	SU095
H	SU008	SU016	SU024	SU032	SU040	SU048	SU056	SU064	SU072	SU080	SU088	SU096

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	SU097	SU105	SU113	SU121	SU129	SU137	SU145	SU153	SU161	SU169	SU177	SU185
B	SU098	SU106	SU114	SU122	SU130	SU138	SU146	SU154	SU162	SU170	SU178	SU186
C	SU099	SU107	SU115	SU123	SU131	SU139	SU147	SU155	SU163	SU171	SU179	SU187
D	SU100	SU108	SU116	SU124	SU132	SU140	SU148	SU156	SU164	SU172	SU180	SU188
E	SU101	SU109	SU117	SU125	SU133	SU141	SU149	SU157	SU165	SU173	SU181	SU189
F	SU102	SU110	SU118	SU126	SU134	SU142	SU150	SU158	SU166	SU174	SU182	SU190
G	SU103	SU111	SU119	SU127	SU135	SU143	SU151	SU159	SU167	SU175	SU183	SU191
H	SU104	SU112	SU120	SU128	SU136	SU144	SU152	SU160	SU168	SU176	SU184	SU192

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	SU193	SU201	SU209	SU217	SU225	SU233	SU241	SU249	SU257	SU265	SU273	SU281
B	SU194	SU202	SU210	SU218	SU226	SU234	SU242	SU250	SU258	SU266	SU274	SU282
C	SU195	SU203	SU211	SU219	SU227	SU235	SU243	SU251	SU259	SU267	SU275	SU283
D	SU196	SU204	SU212	SU220	SU228	SU236	SU244	SU252	SU260	SU268	SU276	SU284
E	SU197	SU205	SU213	SU221	SU229	SU237	SU245	SU253	SU261	SU269	SU277	SU285
F	SU198	SU206	SU214	SU222	SU230	SU238	SU246	SU254	SU262	SU270	SU278	SU286
G	SU199	SU207	SU215	SU223	SU231	SU239	SU247	SU255	SU263	SU271	SU279	SU287
H	SU200	SU208	SU216	SU224	SU232	SU240	SU248	SU256	SU264	SU272	SU280	SU288

Plate 4

	1	2	3	4	5	6	7	8	9	10	11	12
A	SU289	SU297	SU305	SU313	SU321	SU329	SU337	SU345	SU353	SU361	SU369	SU377
B	SU290	SU298	SU306	SU314	SU322	SU330	SU338	SU346	SU354	SU362	SU370	SU378
C	SU291	SU299	SU307	SU315	SU323	SU331	SU339	SU347	SU355	SU363	SU371	SU379
D	SU292	SU300	SU308	SU316	SU324	SU332	SU340	SU348	SU356	SU364	SU372	SU380
E	SU293	SU301	SU309	SU317	SU325	SU333	SU341	SU349	SU357	SU365	SU373	SU381
F	SU294	SU302	SU310	SU318	SU326	SU334	SU342	SU350	SU358	SU366	SU374	SU382
G	SU295	SU303	SU311	SU319	SU327	SU335	SU343	SU351	SU359	SU367	SU375	SU383
H	SU296	SU304	SU312	SU320	SU328	SU336	SU344	SU352	SU360	SU368	SU376	SU384

Revision History

Document #	Revision	Date	Description of Change
PRT-031	Version 1	08/21/2020	Initial release.
PRT-031	Version 2	10/28/2020	Addition of SNAP 384 UDI Plates and new index information strategy

General Warranty

Swift Biosciences, Inc. (“Swift”) warrants that its products meet Swift’s specifications at the time of delivery. Any sample or model used in connection with Swift’s product literature is for illustrative purposes only and does not constitute a warranty that the products will conform to the sample or model. To the maximum extent permitted by applicable law, Swift hereby expressly disclaims, and the buyer hereby expressly waives, any warranty regarding results obtained through the use of the products including, without limitation, any claim of inaccurate, invalid, or incomplete results. All other warranties, representations, terms and conditions (statutory, express, implied or otherwise) as to quality, condition, description, merchantability, fitness for purpose, or non-infringement (except for the implied warranty of title) are hereby expressly excluded. All warranty claims on products must be made in writing within ninety (90) days of receipt of the products. Swift’s sole liability and the buyer’s exclusive remedy for a breach of this warranty is limited to replacement or refund at the sole option of Swift. The warranties identified in this paragraph are Swift’s sole and exclusive warranties with respect to the products and are in lieu of all other warranties, statutory, express or implied, all of which other warranties are expressly disclaimed, including without limitation any implied warranty of merchantability, fitness for a particular purpose, non-infringement, or regarding results obtained through the use of any product (including, without limitation, any claim of inaccurate, invalid or incomplete results), whether arising from a statute or otherwise in law or from a course of performance, dealing or usage of trade.

Limitation of Liability

Swift Biosciences, Inc. (“Swift”) shall have no liability under the warranties cited above with respect to any defect in the products arising from: (i) specifications or materials supplied by the buyer; (ii) willful damage or negligence of the buyer or its employees or agents; (iii) abnormal working conditions at the buyer’s premises; (iv) failure to follow Swift’s use restrictions or instructions (whether oral or in writing); (v) misuse or alteration of the products without Swift’s approval; or (vi) if the buyer is in breach of its payment obligations in regards to purchasing the products. To the fullest extent allowed by law, in no event shall Swift be liable, whether in contract, tort, strict liability, negligence, warranty, or under any statute or on any other basis for any special, incidental, indirect, exemplary, punitive, multiple or consequential damages sustained by the buyer or any other person or entity arising out of or caused by product, Swift’s performance or failure to perform its obligations relating to the purchase of product or performance of services, Swift’s breach of these terms, the possession or use of any product, or the performance by Swift of any services, whether or not foreseeable and whether or not Swift is advised of the possibility of such damages, including without limitation damages arising from or related to loss of use, loss of data, downtime, procurement of substitute products or services, or for loss of revenue, profits, goodwill, or business or other financial loss. The total liability of Swift arising under or in connection with the purchase of the products, including for any breach of contractual obligations and/or any misrepresentation, misstatement or tortious act or omission (including without limitation, negligence and liability for infringement of any third party intellectual property rights) shall be limited to damages in an amount equal to the amount paid to Swift under the purchase agreement. The exclusion of liability shall apply only to the extent not prohibited by applicable law.

Notice to Purchaser: Limited License

This product is for research use only and is licensed to the user under Swift Biosciences intellectual property only for the purchaser’s internal purposes. Not for use in diagnostic procedures.

Swift Biosciences, Inc.
674 S. Wagner Road • Ann Arbor, MI 48103 • 734.330.2568 • www.swiftbiosci.com