PROTOCOL

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ACCEL-AMPLICON™ AND ACCEL-AMPLICON PLUS PANELS

Protocol for Cat. Nos.:

Cancer Screening and Profiling:
- Accel-Amplicon 56G Oncology Panel v2 (AL-56248)
- Accel-Amplicon Plus 57G Pan-Cancer Profiling Panel (AP-PC8048)
- Accel-Amplicon Plus BRCA1 and BRCA2 Panel (AP-BR8048)
- Accel-Amplicon Plus BRCA1, BRCA2, and PALB2 Panel (AP-BP8048)
- Accel-Amplicon Plus Colorectal Cancer Panel (AP-CR8048)
- Accel-Amplicon Plus Comprehensive TP53 Panel (AP-TP8048)
- Accel-Amplicon Plus EGFR Pathway Panel (AP-EG8048)
- Accel-Amplicon Plus Lung Cancer Panel (AP-LG8048)
- Accel-Amplicon Plus Lynch Syndrome Panel (AP-LN8048)
- Accel-Amplicon Plus Myeloid Panel (AP-MY8048)
- Accel-Amplicon Plus Breast and Ovarian Cancer Panel* (AP-BO8048)
  **coming soon*

Inherited Disease:
- Accel-Amplicon CFTR Panel (AL-55048)

Sample Tracking:
- Accel-Amplicon Sample_ID Panel (AL-50048)

Build from Scratch:
- Accel-Amplicon Custom NGS Panel

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About This Guide

This guide provides instructions for the preparation of targeted NGS libraries from DNA samples using an Accel-Amplicon panel.

IMPORTANT!
Read the entire Protocol before use, especially the sections pertaining to Product Information, Kit Contents, Materials and Equipment Not Included, and Input Material Considerations.

Applications

Accel-Amplicon panels are suitable for the following targeted sequencing applications. This list is not exhaustive nor meant to restrict applications.

Mutation Type*	Applications	Sample Type
Somatic	• Oncology • Variant discovery	• Fresh frozen DNA • FFPE DNA • Cell-free DNA • Circulating tumor cell DNA • Whole genome amplification products • Peripheral blood lymphocytes (PBL) • Bone marrow
Germline	• Disease predisposition • Sample tracking	• Dried Blood Spot (DBS) • Whole blood • Buccal swab

** Accel-Amplicon panels may be used to interrogate the following types of variants in samples:*
- *base substitutions*
- *small (Copy Number Variation, validation may be required) insertions and deletions occurring in the target sequence*

Product Information

Accel-Amplicon Panels enable the preparation of high quality targeted next-generation sequencing (NGS) libraries from a variety of sample types, including formalin-fixed, paraffin-embedded (FFPE) and circulating, cell-free DNA (cfDNA). Adapters are included for dual indexing and multiplexing up to 96 samples on a sequencing run. The single-tube workflow from DNA to library can be completed within two hours.

The kit provides the primer pool, library preparation reagents, and Illumina®-compatible adapters and has been validated on Illumina platforms. The table below lists key characteristics and typical performance of available panels using high quality control genomic DNA.

Specification	Feature	56G Oncology v2	EGFR Pathway	BRCA1, BRCA2 and PALB2	Sample_ID	CFTR
Input	Input DNA required			10-25 ng		
	Sample types		FFPE, cfDNA, fresh frozen, genomic DNA			Genomic cDNA from whole blood, (DBS), saliva, buccal
Workflow	Time required			2 hours		
	Multiplexing on MiSeq® v2 standard @ 5000X avg. depth	22	96*	19	N/A	68
Design	Number of amplicons	263	17	302	104	87
	Average amplicon size	138 bp	136 bp	149 bp	145 bp	143 bp
	Number of genes covered	56	4	3	Exonic regions with minor allele frequency and gender ID	1
	Total target size	23.6 kb	1.5 kb	28.9 kb	N/A; SNPs only	10.01 kb
Performance	Limit of detection (at 10 ng base substitutions)		1-5%		Germline panel	1-5%
	On target percentage			> 90%		
	Coverage uniformity (at > 20% of mean)			> 90%		

** More may be possible with a custom indexing solution. Please inquire.*

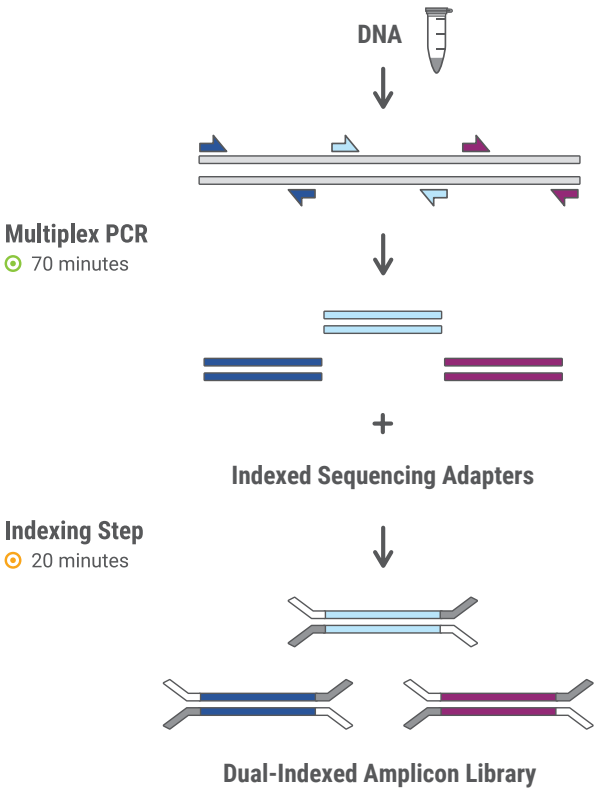
Specification	Feature	57G Pan-Cancer	BRCA1 and BRCA2	Compre-hensive TP53	Lung Cancer	Colorectal Cancer	Lynch Syndrome	Myeloid
Input	Input DNA required	10-25 ng						
	Sample types	FFPE, cfDNA, fresh frozen, genomic DNA						Blood, Bone Marrow, PBL and FFPE
Workflow	Time required	2 hours						
	Multiplexing on MiSeq® v2 standard @ 5000X avg. depth	20	24	96*	28	32	33	12
Design	Number of amplicons	285	246	21	208	186	180	478
	Amplicon size	139 bp	148 bp	140 bp	144 bp	143 bp	140 bp	142 bp
	Number of genes covered	57	2	1	17	16	4	23
	Total target size	25.9 kb	22.9 kb	1.8 kb	18 kb	17 kb	15 kb	42 kb
Performance	Limit of detection (at 10 ng base substitutions)	1-5%						
	On target percentage	> 90%						
	Coverage uniformity (at > 20% of mean)	> 90%						

** More may be possible with a custom indexing solution. Please inquire.*

Accel-Amplicon Panel Workflow

This protocol contains a Multiplex PCR step for the simultaneous production of hundreds of amplicon targets in a single tube and an Indexing step for the addition of dual indexed adapters, enabling multiplexing of up to 96 unique libraries.

Bead-based clean-ups are used to purify the sample by removing unused oligonucleotides and changing buffer composition between steps.



Kit Contents

Accel-Amplicon panels are available in a package size with reagents (10% excess volume) for the preparation of 48 libraries.

Kit	Reagents	Quantity (µl) 48 rxn	Storage (°C)
Multiplex PCR (Pre-PCR)	Reagent G1*	106	-20
	Reagent G2	160	-20
	Enzyme G3	800	-20
	Pre-PCR TE	1200	-20
Indexing Step (Post-PCR)	Index D50X	33 each of D501-D508	-20
	Index D7XX	44 each of D701-D712	-20
	Buffer Y1	1637	-20
	Enzyme Y2	53	-20
	Enzyme Y3	53	-20
	Enzyme Y4	106	-20
	Post-PCR TE	1200	-20

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

Kit	Reagent	Quantity (µl) 48 rxns	Storage (°C)
Additional Components Included	PEG NaCl Solution	20,000	Room Temp
	Alu115 and Alu247 primers	290 of each	-20
	HD701 Control DNA**	8	-20

*** Not included in the Accel-Amplicon CFTR Panel.*

IMPORTANT!
Place the enzymes on ice, NOT in a cryocooler, for at least 10 minutes to allow enzymes to reach 4 ˚C prior to pipetting.

DNA Standard for Accel-Amplicon Panels

All Accel-Amplicon panels (except CFTR) include the Q-Seq HDxTM Reference Standard (HD701) for use as a positive control. The DNA Standard contains precise allelic frequencies for major oncology targets verified using digital PCR (see table below). The DNA Standard is meant to be used as a positive control for variant calling, when verified allelic frequencies are available, or can be run alongside samples to trouble-shoot library yield and performance metrics such as coverage uniformity or on-target.

- **DNA standard concentration:** 5 ng/µl
- **DNA standard volume to be used in the multiplex PCR step:** 2 µl
- **DNA standard total volume provided:** 8 µl

Variants and Expected Allele Frequencies

Gene	AA	CHR	POS	REF	ALT	Expected Allele Frequency (%)
EGFR	G719S	7	55241707	G	A	24.5
PIK3CA	H1047R	3	178952085	A	G	17.5
KRAS	G13D	12	25398281	C	T	15.0
NRAS	Q61K	1	115256530	G	T	12.5
BRAF	V600E	7	140453136	A	T	10.5
KIT	D816V	4	55599321	A	T	10 .0
PIK3CA	E545K	3	178936091	G	A	9.0
KRAS	G12D	12	25398284	C	T	6.0
EGFR	L858R	7	55259515	T	G	3.0
EGFR	ΔE746-A750	7	55242465-55242479	Del15bp		2.0
EGFR	T790M	7	55249071	C	T	1.0
BRCA	A1689fs	13	32913559	delA		33.0

Materials and Equipment Not Included

- Magnetic beads for clean-up steps, e.g., SPRIselect™ beads (Beckman Coulter, Cat. No. B23317/ B23318/B23319) or AMPure® XP Beads (Beckman Coulter, Cat. No. A63880/A63881/ A63882)
- Magnetic rack for clean-up steps, e.g., Invitrogen DynaMag™ or Agencourt® SPRIPlate™
- Library quantification kit (qPCR-based)
- Qubit® or other fluorometric-based assays for determining DNA concentration
- Plastic consumables (0.2 mL PCR tubes, strips, 96-well plates, pipette tips, etc.)
- Centrifuge compatible with format of plastic consumables
- Programmable thermocycler
- Aerosol-resistant, low retention pipettes and tips, 2 to 1000 µL
- 200-proof/absolute ethanol (molecular biology-grade)
- Nuclease-free water (molecular biology-grade)

Storage and Usage Warning

Upon receipt, store the Accel-Amplicon panel kit at -20 ˚C with the exception of PEG solution, which should be stored at room temperature.

Separate the Multiplex PCR Reagents (keep in pre-PCR area) and Indexing 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 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.

After thawing reagents to 4 ˚C, 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 dual indexed adapters are the only reagents that are added individually to each sample.

IMPORTANT!

Assemble all reagent master mixes and reactions ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes, use our **Accel-Amplicon Master Mixing Volume Calculator** 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 yields and performance of this product.

Tips and Techniques

Avoiding 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 Step.
- 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.
- Move samples to post-PCR area before opening tubes.

Accel-Amplicon, like any amplicon enrichment technology, poses a risk of contamination of surfaces and other samples following the amplification step. Please use extreme caution when opening your sample tubes following the Multiplex PCR step. It is highly recommended that separate workspaces and pipettes be maintained for pre-PCR and post-PCR steps. A negative pressure hood should be used for post-PCR steps if available. Clean lab areas using 0.5% sodium hypochlorite (10% bleach) and use specialty barrier pipette tips. Dispose of pipette tips and other disposables in sealed plastic bags.

Size Selection During Clean-up Steps

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

Input Material Considerations

Accel-Amplicon panels enable the preparation of targeted NGS libraries from DNA templates.

The starting material should be quantified with the appropriate assay (qPCR-based for FFPE and cfDNA, Qubit for high quality DNA from whole blood, fresh frozen, or cultured cells) as described in the Quantifying Starting Input Material section.

The optimal coverage uniformity, sensitivity, and specificity of this technology are achieved with qPCR-verified 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 and affect variant calling for low frequency alleles. Consider the following example allele frequencies versus sequencing performance:

Sample Quantity	Human Genome Equivalents (Total Copies)	Example Allele Frequency	Example Allele Equivalents (Copies)	Feasibility of Calling Variant (High Quality DNA)	Feasibility of Calling Variant (FFPE)
10 ng	3000	5%	150	✓	✓
10 ng	3000	1%	30	✓	Depends on sample quality
1 ng	300	5%	15	✓	Depends on sample quality
1 ng	300	1%	3	Follows Poisson distribution for presence of copies	Follows Poisson distribution for presence of copies

Copy Number Variation: Considerations for Experiment Design and Implementation

The Accel-Amplicon Plus panels (listed below) have been validated for Copy Number Variation (CNV) analysis using *ERBB2* amplification confirmed in NIST Genomic DNA Standard for HER2 Measurement (SRM 2373).

- Accel-Amplicon Plus 57G Pan-Cancer Profiling Panel (AP-PC8048)
- Accel-Amplicon Plus Colorectal Cancer Panel (AP-CR8048)
- Accel-Amplicon Plus Breast and Ovarian Cancer Panel* (AP-BO8048)

** Coming soon*

All genes or target regions with at least 10 amplicons can be efficiently assessed for copy number. To increase CNV sensitivity, prepare and a sequence a control DNA, which does not contain an altered copy number at target region of interest, alongside with the test sample. Additionally, a known positive control is recommended for reliable CNV detection.

Prepare the DNA Sample

Input DNA Quantification

IMPORTANT!

Improper quantification of input material can lead to assay failure. Please read this section carefully and quantify the types of input material specified below appropriately to ensure success. The limit of detection, sensitivity, and specificity of Accel-Amplicon Panels is highly dependent on accurate input quantification.

For high quality samples, it is recommended to determine DNA concentration using Qubit, or a similar fluorometric method, as it will accurately represent the double-stranded, amplifiable DNA content of your sample. For cfDNA or low quality DNA samples, we recommend quantification by qPCR using Alu primer pairs provided in this kit to accurately assess the usable amount of DNA in the samples and their integrity. Accel-Amplicon Panels are designed with amplicons of approximately 140 bp for maximum compatibility with FFPE and cfDNA.

High Quality gDNA	Quantify with Qubit or similar fluorometric method
cfDNA, FFPE DNA	Quantify by qPCR with Alu primer pairs (see Appendix, Section A)

- We recommend using between 10-25 ng input DNA per library preparation.
- Input DNA should be re-suspended in 10 µl of Pre-PCR TE buffer.

The following table illustrates how an absorbance-based method (NanoDrop®) and a fluorometric-based method (Qubit) may overestimate FFPE DNA quantity versus the Alu115-qPCR assay:

	NanoDrop (ng/µl)	Qubit (ng/µl)	Alu115-qPCR (ng/µl)
FFPE 1	7.1	2.3	1.5
FFPE 2	26.2	11.4	7.3
FFPE 3	25.2	11.5	10.2
FFPE 4	35.4	15.0	14.7
FFPE 5	59.9	32.4	20.7
FFPE 6	43.0	23.0	16.8
FFPE 7	67.6	35.6	27.4
FFPE 8	76.6	42.2	17.5
FFPE 9	14.1	5.9	3.4
FFPE 10	246.0	84.0	5.8

As shown here, Qubit represents amplifiable DNA content more accurately than NanoDrop, however is not as accurate as the qPCR assay. For sample types with more consistent high quality DNA including whole blood, fresh frozen samples, and cultured cells, quantification by Qubit is a reliable indicator of amplifiable content.

If you have questions related to FFPE or cfDNA sample quality, please contact Swift Technical Support at TechSupport@swiftbiosci.com or tel: 734.330.2568.

Prepare the Reagent Master Mixes and Ethanol

1. To create a master mix, scale reagent volumes as appropriate, using 5% excess volume to compensate for pipetting loss. To calculate the total volume of the master mixes based on the number of reactions of choice, use our Accel-Amplicon Master Mixing Volume Calculator. This tool automatically incorporates 5% excess volume to compensate for pipetting loss.
2. To assemble reagent master mixes for the Multiplex PCR and Indexing steps, ensure the reagent vials are thawed and then stored on ice. After thawing reagents, briefly vortex (except the enzymes) to mix them well. Enzyme G3 is the only enzyme that may be vortexed. If a precipitate is observed in Enzyme G3, warm it gently to room temperature and vortex to dissolve solutes. Spin tubes in a microfuge to collect contents prior to opening. Add reagents in order listed when preparing master mix. Master mixes should be prepared and stored ON ICE until used.

IMPORTANT!

Prepare the reagents in advance to ensure the magnetic beads do not dry out during size selection steps. Always add reagents in specified order. This applies to all reagents except for the indexed adapters that should be added individually to uniquely index each library.

Ensure PEG NaCl solution is at room temperature.

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3. Prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water. Approximately 1 mL of 80% ethanol solution will be used per sample.

Prepare the DNA Libraries

Pre-Program Thermocycler

See table below to pre-program the thermocycler for the Multiplex PCR (Note: panel-specific PCR cycles) and Indexing steps. See insert (supplied in your shipment) for panel-specific information for your Accel-Amplicon Custom NGS Panel. **NOTE:** Cycling conditions and data quality can vary based on input quality and quantity. For samples with <10ng input and/or 10 ng of cfDNA, an additional 1 or 2 PCR cycles is expected to increase yields.

IMPORTANT!
Work in pre-PCR area.

Panel Name	• 56G Oncology v2 • BRCA1 and BRCA2 • BRCA1, BRCA2, and PALB2 • CFTR • Comprehensive TP53 • Sample_ID • EGFR Pathway	• 57G Pan-Cancer Profiling	• Lung Cancer • Myeloid • Colorectal Cancer • Lynch Syndrome
Multiplex PCR Thermocycler Program	**Lid heating ON** 30 sec 98 °C **10 sec** 98 °C **5 min** 63 °C **1 min** 65 °C **4 cycles** **10 sec** 98 °C **1 min** 64 °C **21 cycles** • 56G Oncology v2 • BRCA1 and BRCA2 • BRCA1, BRCA2, and PALB2 **22 cycles** • CFTR • Comprehensive TP53 • Sample_ID **23 cycles** • EGFR Pathway **1 min** 65 °C **Hold** 4 °C	**Lid heating ON** 30 sec 98 °C **10 sec** 98 °C **5 min** 65 °C **1 min** 65 °C **4 cycles** **10 sec** 98 °C **1 min** 64 °C **21 cycles** • 57G Pan-Cancer Profiling **1 min** 65 °C **Hold** 4 °C	**Lid heating ON** 30 sec 98 °C **10 sec** 98 °C **5 min** 66 °C **1 min** 66 °C **4 cycles** **10 sec** 98 °C **1 min** 64 °C **21 cycles** • Lung Cancer • Myeloid **22 cycles** • CFTR • Colorectal Cancer • Lynch Syndrome **1 min** 65 °C **Hold** 4 °C
Indexing Thermocycler Program	**Lid heating OFF** 20 min 37 °C	**Lid heating OFF** 20 min 37 °C	**Lid heating OFF** 20 min 37 °C

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).
2. Load 10 µl of sample DNA (adjust with Pre-PCR TE) into each PCR tube. Keep on ice.
3. **Keep all tubes on ice during assembly of the master-mix and the reaction until placed in thermocycler.** Make the Multiplex PCR Reaction Mix. Components G1, G2, and G3 should be vortexed first and may be master-mixed when running multiple samples in parallel. If any salt precipitant is present in Enzyme G3, allow the vial to reach room temperature and gently vortex to dissolve solids. Place on ice for remainder of use.

Panel-Specific Multiplex PCR Reaction Mix

To calculate the total volume of the master mixes based on the number of reactions of choice, use our Accel-Amplicon Master Mixing Volume Calculator.

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 amplification primers.*

4. Mix the master mix well and then add 20 µl of the Multiplex PCR Reaction Mix to each 10 µl input DNA sample. Place in the thermocycler and run the program.

IMPORTANT!
Move samples to post-PCR area before opening tubes. Keep samples at room temperature. At no time should 'with bead' samples be stored on ice, as this affects binding to magnetic beads.

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

Indexing Step

To calculate the total volume of the master mixes based on the number of reactions of choice, use our Accel-Amplicon Master Mixing Volume Calculator.

Component	Volume (1 Reaction)
Buffer Y1	31 µl
Enzyme Y2	1 µl
Enzyme Y3	1 µl
Enzyme Y4	2 µl
Reaction Mix	**35 µl**

Size Selection and Clean-Up Step 1

6. Ensure beads and samples are at room temperature. Briefly vortex beads to homogenize before use.
7. Add 36 µl (ratio: 1.2) 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.
8. Incubate the samples for 5 minutes at room temperature off the magnet.
9. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (≈ 5 minutes).
10. 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.
11. Add 200 µl of freshly prepared 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.
12. Repeat step 11 once for a second wash with the ethanol solution.
13. 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. Proceed to the Indexing Step.

IMPORTANT!
Continue working in the post-PCR area.

Indexing Step

14. Load the Indexing Thermocycler Program and allow the block to reach 37 °C before loading samples.
15. Add a unique combination of 5 µl Index D50X + 10 µl Index D7XX to each sample bead pellet.

16. Add 35 µl of the cold Indexing Reaction Mix to each sample and resuspend the pellet (total volume 50 µl).
17. Place in the thermocycler and run the program (37 °C for 20 minutes).

Size Selection and Clean-Up Step 2

18. Ensure PEG NaCl solution is at room temperature. Briefly vortex the PEG NaCl solution to homogenize before use.
19. 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.
20. Incubate the samples for 5 minutes at room temperature off the magnet.
21. 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).
22. 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.
23. Add 180 µl of freshly prepared 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.
24. Repeat step 23 once for a second wash with the ethanol solution.
25. 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.
26. 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.

Library Quantification

Quantify a 1:10,000 dilution of the library in triplicate using a qPCR-based assay based upon a library size of 265 bp. Upon calculating library concentration, be sure to adjust for proper library size of the standards in your library quantification kit. Variation in length of DNA in the standards from the kit and your library size may lead to improper estimation of DNA concentration.

Improper library quantification by other methods will lead to uneven pooling and sub-optimal cluster density, impacting sequencing data.

It is not recommended to use an electrophoretic method (such as Agilent Bioanalyzer or TapeStation) for quantifying libraries because:

- As there is no PCR enrichment of the library following the Indexing Step, using an electrophoretic method will not accurately quantify fully adapted library vs. other DNA.
- Library adapters have secondary structure which exhibits migration artifacts when analyzed using an electrophoretic method.

It is not recommended to use a fluorometric method (such as Qubit) for quantifying libraries because:

- As there is no PCR enrichment of the library following the Indexing Step, a fluorometric method will not accurately quantify fully adapted library vs. other DNA.

Sequence the DNA Libraries

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 selected.
Please ensure that adapter trimming is enabled while setting up the sequencing run. Failure to trim adapter sequences will result in incorrect primer trimming and will lead to inaccurate variant calling. To overcome this issue, enable automatic trimming by the sequencer software or perform adapter trimming by Trimmomatic during data analysis. For more information, please consult our Bioinformatics Resources page at swiftbiosci.com/biofx.

MiSeq Loading Recommendations

Recommended Loading Concentration for MiSeq v2 and v3 Reagent Kits

Chemistry	Final Library Pool Concentration	Recommended Loading Concentration
MiSeq Reagent Kit v2	2-4 nM	10-12 pM
MiSeq Reagent Kit v3	4 nM	20 pM

Amplicon libraries can be pooled together to obtain a 2 nM or 4 nM final concentration mix. Denaturation of libraries with freshly diluted 0.2 N NaOH is required before loading on the MiSeq.

- 2 nM library denaturation (supports 10 pM loading)
  - 2 nM library pool (5 µl) + 0.2N NaOH (5 µl).
  - Mix and incubate 5 minutes at room temperature.
  - Add 990 µl of pre-chilled HT1 to obtain a 10 pM denatured library mix, mix well.
  - Load 600 µl in the cartridge.
- 4 nM library denaturation (supports 10 pM-20 pM loading)
  - 4 nM library pool (5 µl) + 0.2N NaOH (5 µl).
  - Mix and incubate 5 minutes at room temperature.
  - Add 990 µl of pre-chilled HT1 to obtain a 20 pM denatured library pool, mix well.
  - Dilute the denatured DNA to load the cartridge:

Final Concentration	10 pM	11 pM	12 pM	20 pM
20 pM Denatured Libraries	300 µl	330 µl	360 µl	600 µl
Pre-chilled HT1	300 µl	270 µl	240 µl	0 µl

  - Load 600 µl of the desired dilution into the cartridge.

When working with other Illumina instruments (i.e. NextSeq, MiniSeq, etc.), please refer to manufacturer's guide for recommendations regarding reagents and loading.

PhiX Spike-In:

Libraries prepared from Accel-Amplicon panels do not require a PhiX spike-in because they inherently have sufficient complexity to yield optimal sequencing data on Illumina platforms.

Bioinformatics Options

Adapter and Primer Trimming

As noted in the earlier section of this manual titled Sequence the DNA Libraries, please ensure that adapter trimming is enabled while setting up the sequencing run. Alternatively, adapter trimming could be performed bioinformatically prior to analysis. In addition, Accel-Amplicon (except

Sample_ID Panel) and Accel-Amplicon Plus Panels are designed with overlapping amplicons to allow for contiguous regions of coverage in a single-tube format. Therefore, synthetic primer sequences will be encountered both at the beginning and end of some reads, which must be trimmed during the first step in data analysis. This can be done using a publicly available tool called Primerclip (http://github.com/swiftbiosciences/primerclip). For more information, please consult our Bioinformatics Resources page at swiftbiosci.com/biofx. Download panel-specific files through our website.

Accel-Amplicon Data Analysis on Genialis Platform

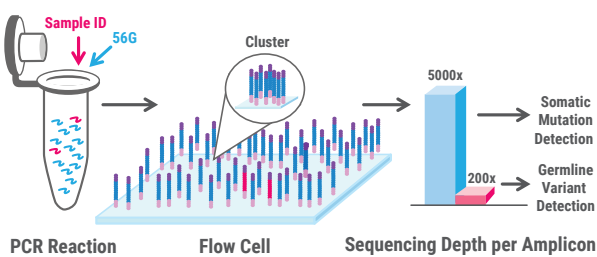
Sequencing data from all standard and custom Accel-Amplicon panels can be analyzed through a web-based software solution supported by Genialis. This data analysis solution has been designed to integrate alignment, trimming, QC and variant calling into one simple pipeline. The Genialis' cloud application also automates an algorithm optimized for accurate variant detection, including low frequency variants. It is fast and easy to use, as it offers data visualization and annotated variants using direct links to publicly available databases such as COSMIC and NCBI.

Please visit genial.is/swiftbio/ to learn more about this service.

Data Analysis Specific to Accel-Amplicon 56G Oncology v2 and Accel-Amplicon 57G Pan-Cancer Profiling Panels:

NOTE: Analysis of the Sample_ID targets included with the panel is optional and will have a minimal impact on your sequencing results if they are left out of the analysis pipeline.

Sample_ID Panel as a Spike-In



Consider the following information in order to achieve the most accurate sequencing results from libraries containing Sample_ID as a spike-in:

- On-target Percentage (% OT): Use the combined BED file (available at www.swiftbiosci.com or by emailing us at TechSupport@swiftbiosci.com) to calculate overall % OT for the entire

panel. Calculating % OT for the entire panel based on a BED file specific to main panel content only or Sample_ID only will result in incorrect assessment of % OT, as the reads from the excluded panel will be incorrectly reported as off-target.

- Coverage Uniformity Percentage (% CU): Use the BED file specific to main panel content or Sample_ID to calculate % CU for each subset of the data. By design, Sample_ID targets in the combined panel have lower coverage than main panel targets because they are for germline variants; therefore, analyzing % CU across the combined panel will inaccurately reflect Sample_ID targets as low coverage dropouts.

Variant Calling

The DNA standard, included in the kit, may be used to verify the variant calling accuracy of your pipeline; as it contains precise allelic frequencies for major oncology targets verified by digital PCR. If additional informatics pipeline advice is needed to use these instructions, please contact Swift Technical Support at TechSupport@swiftbiosci.com or tel: 734.330.2568.

Copy Number Variation Analysis Recommendations

Copy number variation for *ERBB2* (or desired target) can be determined using a publicly available tool called Cnvkit (https://github.com/etal/cnvkit). It is highly recommended to generate a copy number reference from control samples. ERBB2 benchmarking data from positive control samples (NIST HER2 Genomic DNA Standard Reference Material, SRM2373). Sensitivity of CNV detection was maximized using a standard DNA (Coriell NA12878) that was run alongside and with the same panel. Alternatively, please access data from Coriell NA12878 samples sequenced with your respective Accel-Amplicon Plus Panel through our website.

Appendix

Section A: Library Multiplexing Options for MiSeq

Use the following equation to determine possible number of libraries to multiplex per sequencing run:

Level of multiplexing = (number of paired-end reads) / (number of amplicons * intended average read depth)

Panel-specific multiplexing examples are as follows:

MiSeq Reagent Kit	57G Panel Intended Average Depth at 5000x (somatic)	BRCA1 and BRCA2 Intended Average Depth at 1000x (germline)
v2 Nano (300 cycles) (2M paired-end reads)	1	8
v2 Micro (300 cycles) (8M paired-end reads)	5	32
v2 (300 cycles) (30M paired-end reads)	20	96*
v3 (600 cycles) (50M paired-end reads)	34	96*

** Higher level of multiplexing is possible with a custom solution. Please inquire.*

Sample_ID Panel

Number of amplicons = 104

- In order to use the information from the Sample_ID Panel to properly discriminate between samples, it is recommended to sequence the panel to a minimum depth of 200-500X. This corresponds to between 34-90 samples multiplexed on a MiSeq v2 Nano (300 cycles, 2M paired-end reads).
- Compare the target profile across samples of interest to confirm a match. Because each target will exhibit a presence level in each sample of none (~0%), heterozygous (~50%), or homozygous (~100%), examining all 104 targets in combination with gender identification will reveal a unique profile for the sample that can be compared to other samples. For additional information about the targets utilized and their interpretation, please consult the following publications:
    1. Pengelly RJ, Gibson J, Andreoletti G, Collins A, Mattocks CJ, Ennis S. A SNP profiling panel for sample tracking in whole-exome sequencing studies. Genome Med. 2013 Sep 27;5(9):89.
    2. Butler E, Li R. Genetic Markers for Sex Identification in Forensic DNA Analysis. J Forensic Investigation. 2014;2(3): 10.

Section B: Indexed Adapter Sequences

During the Indexing Step in the protocol, you must use a unique combination of Index Adapters to re-suspend and label each library. Libraries made with uniquely indexed adapter combinations may be multiplexed during cluster generation and co-sequenced on the same Illumina flow cell.

CONTENTS: Unique indexed adapters, which should be used where this manual calls for 5 or 10 µl of each Index Primer:

D5 Adapters	Sequence MiSeq, HiSeq 2000/2500	Sequence MiniSeq, NextSeq, HiSeq 3000/4000
D501	TATAGCCT	AGGCTATA
D502	ATAGAGGC	GCCTCTAT
D503	CCTATCCT	AGGATAGG
D504	GGCTCTGA	TCAGAGCC
D505	AGGCGAAG	CTTCGCCT
D506	TAATCTTA	TAAGATTA
D507	CAGGACGT	ACGTCCTG
D508	GTACTGAC	GTCAGTAC

NOTE: Include reverse compliment sequences provided in the table above when using Illumina MiniSeq, NextSeq, or HiSeq 3000/4000 systems.

D7 Adapters	Sequence
D701	ATTACTCG
D702	TCCGGAGA
D703	CGCTCATT
D704	GAGATTCC
D705	ATTCAGAA
D706	GAATTCGT
D707	CTGAAGCT
D708	TAATGCGC
D709	CGGCTATG
D710	TCCGCGAA
D711	TCTCGCGC
D712	AGCGATAG

The number on the product tube label indicates which indexed adapter is provided in the tube. During library prep, make sure to note which indexed adapter combination you are using with your sample and do not use the same indexed adapter combination on two different samples you plan to co-sequence.

Section C: Helpful Information and Troubleshooting

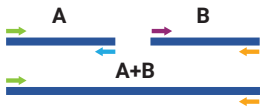
Problem	Possible Cause	Suggested Remedy
Lower than expected yields	Inadequate sample quality and/or quantity, incorrect input quantification method, or incorrect SPRI™ methods.	Use 25 ng of qPCR-quantified input and extend the incubation time for the Indexing Step from 20 minutes to 60 minutes. Perform SPRI carefully.
Unusual electrophoretic trace	Secondary structure of adapters and lack of PCR enrichment of the library following the Indexing Step exhibit characteristics of migration artifacts.	Quantify library with a qPCR-based method; if you need to ascertain amplicon insert size from the sequencing data. (Review full explanation in "Structure of Amplicon Libraries and Migration Behavior" section.)
Precipitates in Enzyme G3	Salt precipitation.	Allow the vial to reach room temperature and gently vortex to dissolve solids. Place on ice for remainder of use.
Lower than expected cluster density	Error in library quantification. Bioanalyzer and Qubit do not accurately quantify fully adapted library vs. other DNA.	Quantify library with a qPCR-based method for flow cell loading calculations.
Incomplete resuspension of beads after ethanol wash during SPRI™ steps	Over-drying of beads.	Continue pipetting the liquid over the beads to break up clumps for complete resuspension.
Shortage of enzyme reagents	Pipetting enzymes at -20 °C instead of 0-4 °C.	Allow enzyme reagents to equilibrate to 0-4 °C for 10 minutes prior to pipetting.
Retention of liquid in pipette tip	Viscous reagents may stick to pipette tip, especially for non-low retention tips.	Pipette up and down several times to ensure all liquid and/or beads are released from the pipette tip.

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).

Section D: Structure of Accel-Amplicon Libraries and Migration Behavior

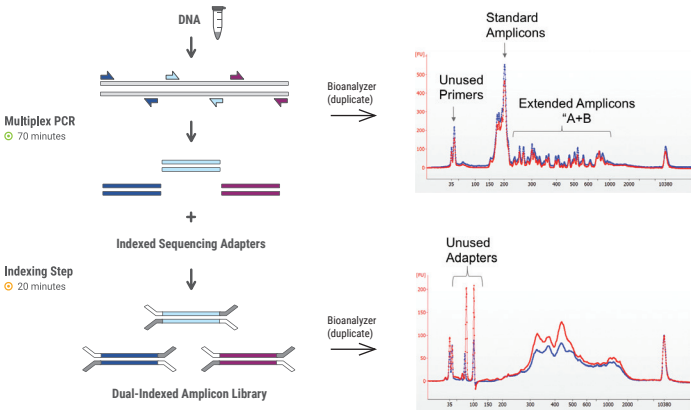
Please note that qPCR-based methods are most accurate for quantifying Accel-Amplicon libraries, however, this section provides an overview of expected results when using an electrophoretic method. The secondary structure of Accel-Amplicon libraries exhibits two features, which should be understood if analyzed using electrophoretic methods such as Agilent Bioanalyzer or TapeStation:

If using high quality DNA, "extended amplicons" can be observed. They are formed from the forward primer and the reverse primer of two adjacent amplicons. Note that these extended amplicons are not formed when using fragmented or cross-linked (FFPE) DNA, or cell-free DNA. Coverage uniformity is not affected by the presence or absence of extended amplicons.



"A+B" is an extended amplicon, which is a contiguous alignable sequence

After indexing, the library is partially single-stranded and the migration is impaired, causing the library to appear large on the Bioanalyzer; therefore, the traces should not be used to accurately determine the size or the quantity of the library.



Section E: Material Safety Data Sheet

Please refer to Accel-Amplicon products material safety data sheet (MSDS) for more information about the potential hazards for each component (reagents, buffers and enzymes) and instructions on safe use.

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Notes

Notes

Swift Biosciences, Inc.
674 S. Wagner Road, Suite 100 • Ann Arbor, MI 48103 • 734.330.2568 • www.swiftbiosci.com