ISOFLUX™

Spotlight 59™ Oncology Panel

Targeted NGS Amplicon Library Prep for Illumina Platform

Catalog No. 910-0113 (24 Samples, 96 Reactions)
Catalog No. 910-0114 (4 Samples, 16 Reactions)

Instructions for Use

Document No. 630-0113
Revision C

For Research Use Only
Not intended to treat or diagnose any disease condition

FLUXION

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INTENDED USE

The Spotlight 59™ Panel for Illumina platforms enables the preparation of high quality targeted Next Generation Sequencing (NGS) libraries from a variety of liquid biopsy sample types, including CTCs (circulating tumor cells) enriched from peripheral blood, and circulating, and cell-free DNA (cfDNA). For formalin-fixed, paraffin-embedded tissue (FFPE) samples, the sensitivity/specificity will be lower due to DNA damage, and computational workflow changes are needed for best performance. Please contact Fluxion for more information. Adapters are included for dual indexing and multiplexing up to 24 samples (96 reactions) on a sequencing run.

The kit utilizes Illumina-compatible adapter sequences and has been validated on Illumina platforms only. The table below lists key characteristics and typical performance of available panels using high quality control genomic DNA.

Spotlight 59™ protocol is optimized for 5-25 ng of genomic or cfDNA per reaction. Quantifying the starting genomic material is highly recommended.

Feature	Spotlight 59™ Panel Specification
Packaging Options	24 samples or 4 samples
Input DNA Required	40-100 ng
Time Required	2 hours
Amplicon Size Average	140 bp
CTC/cfDNA Compatible	Yes
Percent On Target	> 95%
Coverage Uniformity (> 20% of Mean)	> 95%

*As quantified by qPCR. Please see section on Quantifying Starting Input Material. 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, Qubit quantification is a reliable indicator of amplifiable content.

Spotlight 59™ contains 277 amplicons with an average size of 140 bp that cover hotspots and contiguous regions of 59 genes. An additional 104 amplicons with an average size of 145 bp that cover exonic SNPs with high minor allele frequency and gender identification targets. These Sample_ID primers have been manufactured as spiked-in to the panel primer pool at a low percentage to account for only 2-4% of total reads. This enables a sequencing depth of 800-20,00X for the germline Sample_ID targets and 20,000X for the main panel targets.

INTRODUCTION

This protocol explains how to prepare up to 24 pooled samples from gDNA (with option of whole genome amplification for samples with limited quantity) or cfDNA for subsequent sequencing.

Before you start

- Upon receipt, store the kit at -20 °C.
- Separate pre-PCR reagent box (Box 1 of 2) from post-PCR reagents (Box 2 of 2) to prevent contamination
- **Please store PEG solution at room temperature.** (PEG solution may freeze during shipping, but extensive testing showed that freezing and thawing cycle does not negatively impact quality of the reagent. If immediately used upon arrival, allow it to reach room temperature before use)

Kit Contents

Kit contains enough reagents for the preparation of either 24 samples or 4 samples, depending on kit size (10% excess volume provided).

Kit	Reagents	96 Reactions	16 Reactions
Multiplex PCR Reagents	Reagents A, B, C, D	53 µl	20 µl
	Reagent G2	320 µl	54 µl
	Enzyme G3	1600 µl	270 µl
	Pre-PCR TE	2400 µl	400 µl
Indexing Reagents	Index D50X	66 µl	22 µl
	Index D7XX	88 µl	44 µl
	Buffer Y1	3274 µl	546 µl
	Enzyme Y2	106 µl	18 µl
	Enzyme Y3	106 µl	18 µl
	Enzyme Y4	212 µl	36 µl
	Post-PCR TE	2400 µl	400 µl

NOTE: This kit includes PEG NaCl Solution (Cat. No. 90148). This kit also contains Alu115 and Alu247 primers for use in input DNA quantification assay by qPCR.

Required Materials Not Supplied

- SPRIselect beads (Beckman Coulter, Cat. No. B23317/B23318/B23319)
- Invitrogen DynaMag™, Agencourt® SPRIPlate® or similar **magnetic rack for magnetic bead clean-ups**
- qPCR-based input DNA quantification assay (for FFPE and cfDNA samples)
- Qubit or similar fluorometric input DNA quantification assay (for high quality samples such as fresh frozen only)
- qPCR-based library quantification assay for Illumina libraries (NEB™, E7630S/L or Kapa Biosystems, KK4824)
- Microcentrifuge
- Programmable thermocycler operating within manufacturer's specifications
- 0.2 ml PCR tubes or 96-well plate
- Aerosol-resistant tips and pipette ranges from 1-1000 µl
- 200-proof/absolute ethanol (molecular biology grade)
- Nuclease-free water (molecular biology grade)
- Sequencing kit for validated instruments (MiSeq, HiSeq or MiniSeq); Note: read lengths must be >=150bp per read, so >=300 cycle kits need to be used.

⚠ Spotlight 59 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.

QUANTIFYING STARTING INPUT MATERIAL

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 Spotlight 59 Amplicon Panel is highly dependent on accurate input quantification. For more information, see section "Quantification and Quality Assessment of Input DNA" in Appendix A in this protocol.

FFPE

Use a qPCR-based assay to quantify starting material with amplicons that are sized to indicate the amplifiable content of the sample. Spotlight 59 Amplicon Panel is designed with amplicons of approximately 140 bp for maximum compatibility with FFPE DNA. Therefore, using a qPCR assay with amplicons in this size range is recommended. Please use one of the following options to quantify:

- Commercially available qPCR-based input quantification kit
- A lab-based qPCR test (see Appendix A for published Alu115-qPCR repeat assay)

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:

Kit	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.

CTCs and Circulating, Cell-Free DNA

Use a qPCR-based assay to quantify starting material with two differently sized amplicons:

(1) a short amplicon to indicate the amplifiable quantity and
(2) a larger amplicon to indicate the molecular weight of the DNA in the sample.

The Spotlight 59™ Amplicon Panel is designed with amplicons of an average size of 140 bp for maximum compatibility with cfDNA.
Therefore, using a qPCR assay with amplicons in this size range is recommended. Please use one of the following options to quantify:

- Commercially available qPCR-based input quantification kit validated for cfDNA assessment
- A lab-based qPCR test with a short amplicon to indicate quantity and a larger amplicon to indicate integrity

Refer to the technical note, "Assessment of Concentration and Integrity of cfDNA" available within the Resource Library on the Accel-Amplicon 56G product page for ALU115/247 qPCR assay details and interpretation of cfDNA quantity and purity (https://swiftbiosci.com/products/accel-amplicon/56g-oncology-panel/).

High Quality Genomic DNA, Whole Blood, and Fresh Frozen Tissue

Use Qubit or a qPCR-based assay to quantify starting material.

Quantify the starting material 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 input 5-10 ng per reaction is also OK but will likely reduce the 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	Yes	Yes
10 ng	3000	1%	30	Yes	Depends on sample quality
1 ng	300	5%	15	Yes	Depends on sample quality
1 ng	300	1%	3	Follows Poisson distribution for presence of copies	Follows Poisson distribution for presence of copies

PROTOCOL

Introduction

This section describes the Spotlight 59 Library Prep protocol. Follow the protocol in the order described using the specified parameters. Before proceeding, verify kit contents and make sure that you have the required equipment and consumables. See Required Materials Not Supplied

Prepare for Pooling

⚠If you plan to pool libraries, record information about your samples before beginning library prep.

Tips and Techniques

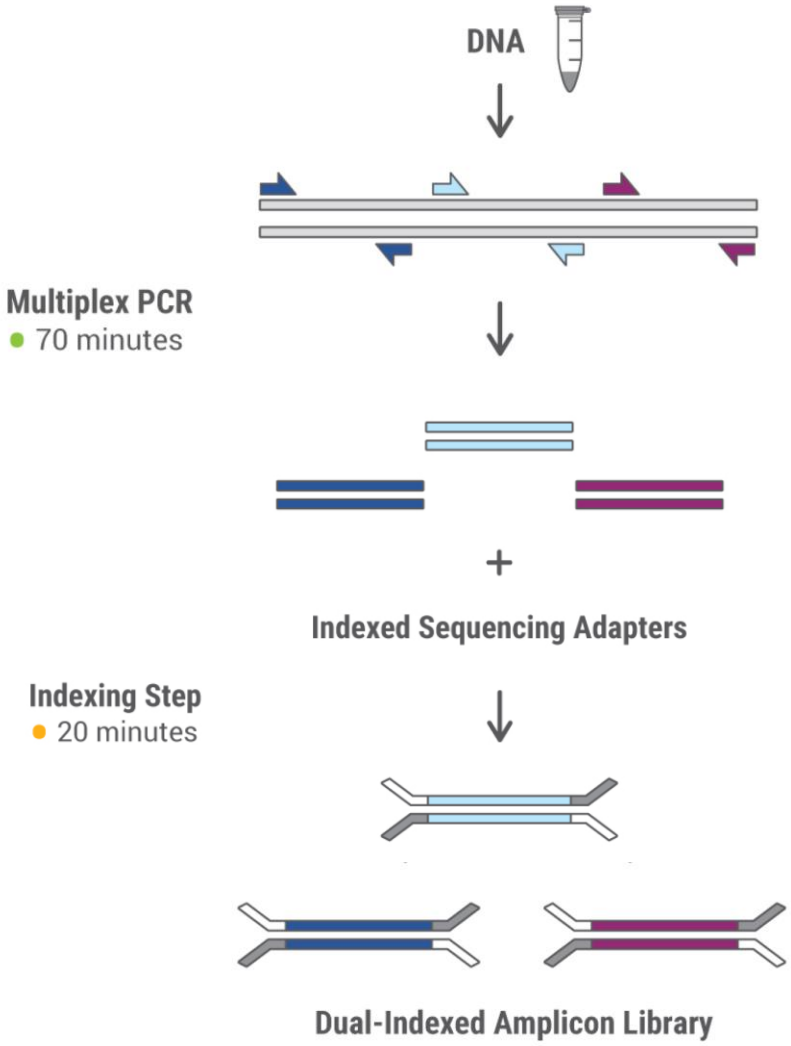
Avoiding Cross-Contamination

- When adding or transferring samples, change tips between each sample.
- When adding adapters or primers, change tips between each sample.
- Remove unused index adapter tubes from the working area.

Handling Beads

- Pipette bead suspension slowly.
- When mixing, mix thoroughly.
- If beads are aspirated into the pipette tips, dispense back to the plate on the magnetic stand and wait until the liquid is clear.
- When washing beads:
    - Use the appropriate magnet for the plate.
    - Dispense liquid so that beads on the side of the wells are wetted.
    - Keep the plate on the magnet until the instructions specify to remove it.
    - Do not agitate the plate while on the magnetic stand. Do not disturb the bead pellet.

PROTOCOL OVERVIEW



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

LIBRARY PREP

For best results, please follow these instructions:

- To maximize efficient use of enzyme reagents, remove enzyme tubes from -20 °C storage and place on ice, NOT in a cryocooler, for at least 10 minutes to allow reagents 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, briefly vortex all reagents except the enzymes in the Indexing Step (Y2, Y3, Y4) and spin down in a microcentrifuge.
- Separate the Multiplex PCR Reagents (keep in pre-PCR area) and Indexing Reagents (keep in post-PCR area).
- Prepare reactions on ice before adding to samples and performing incubations.
- Before starting, prepare a fresh 80% ethanol solution using 200-proof/absolute ethanol and nuclease-free water (approximately 1 ml will be used per sample).
- **Wait 5 min each time you put tubes on a magnetic stand.**

A calculator tool to help scale up your reactions is available by visiting www.swiftbiosci.com on a product page.

Multiplex PCR Step

1. Save the following program on the thermal cycler (confirm lid heating is turned ON).
2. Load 10 µl of sample DNA (adjust with Pre-PCR TE) into each PCR tube.
3. Prepare 4 separate tubes per sample. Label them A, B, C and D.
4. **Assemble on ice.** Components A, B, C or D (For example: Tube A will have reagent A only and not B, C or D) along with G2, and G3 should be gently vortexed first and may be master-mixed when running multiple samples in parallel.

Component	Volume For 1 reaction
Reagent A, B, C or D	2 µl
Reagent G2	3 µl
Enzyme G3	15 µl
Reaction Mix	20 µl
Sample DNA	10 µl
Total	30 µl

Multiplex PCR Thermocycler Program		
30 s	98°C	
10 s 5 min 1 min	98°C 63°C 65°C	4 cycles
10 s 1 min	98°C 64°C	21 cycles
1 min Hold	65°C 4°C	

5. Mix well and then add 20 µl of the Multiplex PCR Reaction Mix to each 10 µl PCR tube. Place in the thermocycler and run the program.

   Treat PCR products with care to avoid contaminating work areas and other samples. 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.
6. **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 SPRI beads.**
7. Make the Indexing Reaction Mix with the following components. **Assemble this reaction mix on ice and keep cold until adding it to samples in the Indexing Step, but leave the samples themselves at room temperature in preparation for SPRI cleanup.** All components may be master-mixed when running multiple samples in parallel.

Components	Volume (1 reaction)
Buffer Y1	31 µl
Enzyme Y2	1 µl
Enzyme Y3	1 µl
Enzyme Y4	2 µl
Reaction Mix	**35 µl**
Multiplex PCR Products and Indexes	**15 µl**
Total	**50 µl**

SPRI Step 1

Ensure beads and samples are at room temperature. Briefly vortex beads to homogenize before use.

1. Add 36 µl of SPRIselect beads to each 30 µl sample (ratio: 1.2).
2. Mix by vortexing. (Ensure no bead-sample suspension droplets are left on the sides of the tube.)
3. Incubate at room temperature for 5 minutes.
4. Briefly spin the samples in a microcentrifuge.
5. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
6. Remove and discard all supernatant from each tube (approximately 5 µl may be left behind).

7. Wash on the magnetic stand 2 times as follows:
    a. Add 200 µl of fresh 80% EtOH to the pellet
    b. Incubate for 30 seconds
    c. Remove the ethanol solution.
8. Briefly spin the samples in a microcentrifuge.
9. Place back onto the magnet.
10. Using a 20 µl pipette, remove residual 80% EtOH from each well.
11. Air-dry the pellet briefly, watching the pellet to avoid cracking or over-drying. Leave tubes on the magnet. Proceed to the Indexing Step for resuspension without delay.

Indexing Step

Continue working in the post-PCR area.

1. Add a unique combination of 5 µl Index D50X + 10 µl Index D7XX to each sample bead pellet.
2. Add 35 µl of the cold Indexing Reaction Mix to each sample and resuspend the pellet (total volume 50 µl).
3. Place in the thermocycler and incubate at 37 °C for 20 minutes (Lid heating OFF).

SPRI Step 2

Ensure PEG NaCl solution is at room temperature. Briefly vortex the PEG NaCl solution to homogenize before use.

1. Add 42.5 µl of PEG NaCl solution to each 50 µl sample (ratio: 0.85).
2. Mix by vortexing. (Ensure no bead-sample suspension droplets are left on the sides of the tube.)
3. Incubate at room temperature for 5 minutes.
4. Briefly spin the samples in a microcentrifuge.
5. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
6. Remove and discard all supernatant from each tube (approximately 5 µl may be left behind).
7. Wash on the magnetic stand 2 times as follows:
    a. Add 200 µl of fresh 80% EtOH to the pellet
    b. Incubate for 30 seconds
    c. Remove the ethanol solution.
8. Briefly spin the samples in a microcentrifuge,
9. Place back onto the magnet.
10. Using a 20 µl pipette, remove residual 80% EtOH from each well.
11. Air-dry the pellet briefly, watching the pellet to avoid cracking or over-drying, while on the magnet.

12. Take tubes off the magnet.
13. Add 20 µl of Post-PCR TE buffer and resuspend the pellet, mixing well by pipetting up and down until homogenous.
14. Incubate at room temperature for 2 minutes off the magnet.
15. Place on a magnetic stand and
16. Transfer 20 µl library eluate to a fresh tube.
17. Ensure that eluate does not contain magnetic beads. If magnetic beads are present, pipette eluate into a new tube, place on magnet, and transfer eluate again.

Library Quantification

Quantify a 1:100,000 dilution of your library in triplicate using a qPCR 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 suboptimal cluster density, impacting sequencing data.

It is not recommended to use a Bioanalyzer for quantifying libraries because:

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

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.

APPENDIX A:

Quantification and Quality Assessment of Input DNA

For NGS library preparation, input quantification by spectrophotometric-based (NanoDrop) or fluorometric-based (Qubit) methods may not provide an accurate assessment of the usable DNA within the sample. Quantification by spectrophotometric-based methods commonly overestimates DNA concentration and is limited to relatively high concentration samples. Quantification by fluorometric-based methods provides accurate DNA concentrations for samples with high quality DNA (e.g., whole blood, fresh frozen samples, cultured cells), but performs poorly with low quality samples and cannot distinguish between cfDNA and high molecular weight cellular gDNA. Therefore, for low quality samples (e.g., FFPE samples) and cfDNA samples, we recommend quantification by a qPCR-method, using both short and long amplicons to accurately determine the concentration and quality of sample DNA [Simbolo M. *et al.* PLoS ONE (2013) 8(6): e62692].

Alu sequences (highly abundant in the human genome) can be used for the sensitive quantification of human genomic DNA. Included in this kit are primers that can be used to amplify **two differently sized amplicons**: short (115 bp; Alu115) and long (247 bp; Alu247) amplicons from genomic Alu repeats. Following input analysis, the appropriate amount of sample DNA can be used as input for NGS library preparation with Spotlight 59.
Spotlight 59 was developed in collaboration with Swift Biosciences, using technology that is similar to other Accel-NGS Library Kits and Accel-Amplicon Panels.
For complete DNA quantification instructions, please refer to “Quantification and Quality Assessment of Human DNA Samples” Protocol available on Swift.com

Sample Sheet – Special Considerations

- Open Illumina Experiment Manager and create a sample sheet.
- On the Instrument selection page, select “MiSeq”.
- In the MiSeq Application Selection page, select category “Other” and select application “FASTQ Only”.
- On the workflow parameter page:
- Enter the Reagent Cartridge barcode.
- Select “TruSeq HT” as the Sample Prep Kit.
- Index Reads: “2”.
- Read Type: “Paired End”.
- Cycles Read 1: “151”, Cycles Read 2: “151”.
- Make sure the “Use Adapter Trimming” and “Use Adapter Trimming Read 2” are selected.

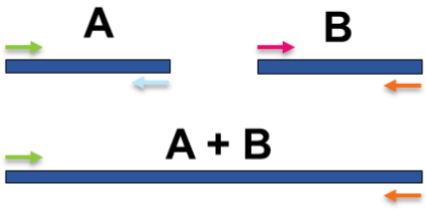
Troubleshooting Common Problems

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.
Incomplete resuspension of beads after ethanol wash during SPRI steps.	Over-drying of beads.	Continue pipetting the liquid over the beads for complete resuspension.
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.
Unusual Bioanalyzer trace.	Secondary structure of adapters and lack of PCR enrichment of the library following the Indexing Step causes migration artifacts on Bioanalyzer.	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 of Spotlight Libraries on Agilent Bioanalyzer or TapeStation" section.)

Library analysis on Agilent Bioanalyzer or TapeStation

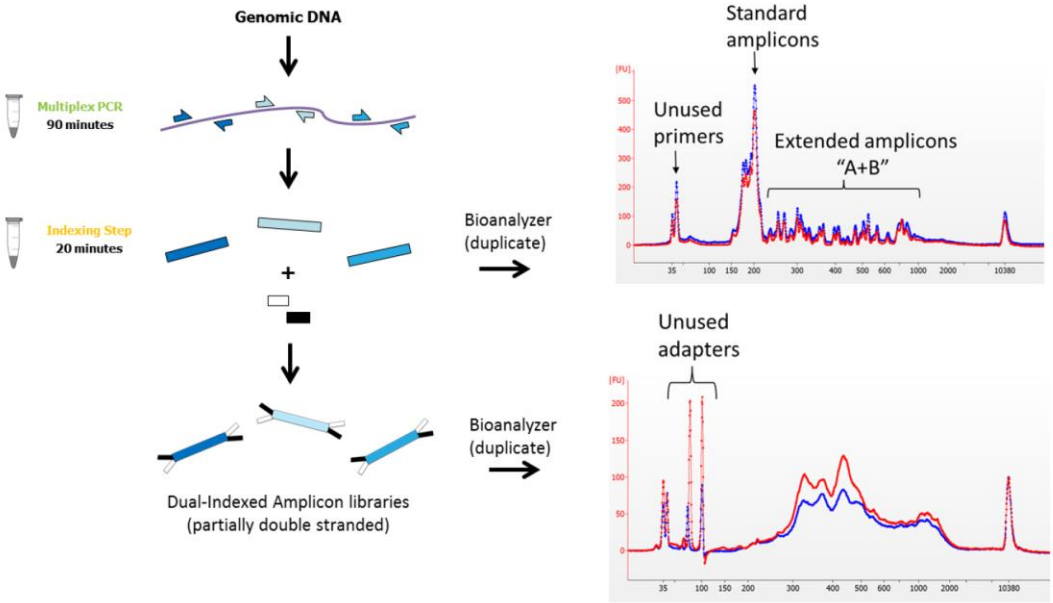
Please note that qPCR-based methods are most accurate for quantifying Spotlight 59 Amplicon libraries. Despite that, this section provides an overview of expected results when using a Bioanalyzer. The secondary structure of Spotlight 59 Amplicon libraries exhibits two features, which should be understood if analyzed using electrophoretic methods such as Agilent Bioanalyzer or TapeStation:

1. 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

2. 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.



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.
*Adapters included in 4 sample/16 reaction kit.

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.
*Adapters included in 4 sample/16 reaction kit.

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