Illumina DNA Prep with Enrichment

Reference Guide

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Overview

This guide explains how to prepare up to 384 uniquely dual-indexed paired-end libraries from DNA using the Illumina DNA Prep with Enrichment (IDPE) workflow.

The IDPE workflow:

- Uses tagmentation, an enzymatic reaction, to fragment DNA and add adapter sequences in 15 minutes.
- Uses sample normalization at inputs ≥ 50 ng.
- Can prepare libraries directly from whole blood or saliva samples when using an extraction protocol.
- Is compatible with extracted formalin-fixed paraffin-embedded (FFPE) samples, ΔCq ≤ 5 recommended for optimal performance.

Illumina DNA Prep with Enrichment Workflow

The IDPE workflow uses a bead-based transposome complex to tagment genomic DNA. This complex fragments DNA and then tags the DNA with adapter sequences in one step. After it is saturated with input DNA, the bead-based transposome complex fragments a set number of DNA molecules. This fragmentation provides flexibility to use a wide DNA input range to generate normalized pre-enriched libraries of consistent tight fragment size distribution. Following tagmentation, a limited-cycle PCR adds additional adapter sequences to the ends of DNA fragments. This step enables compatibility across all Illumina sequencing systems. A subsequent target enrichment workflow is then applied. Enrichment is performed with either individually prepared libraries (1-plex) or pooled libraries (up to 12-plex). Following pooling, the double-stranded DNA libraries are denatured and biotinylated oligonucleotide probes are hybridized to the denatured library fragments. After hybridization, Streptavidin Magnetic Beads 4 (SMB4) capture the targeted library fragments within the regions of interest. The captured and indexed libraries are eluted from the beads and further amplified before sequencing.

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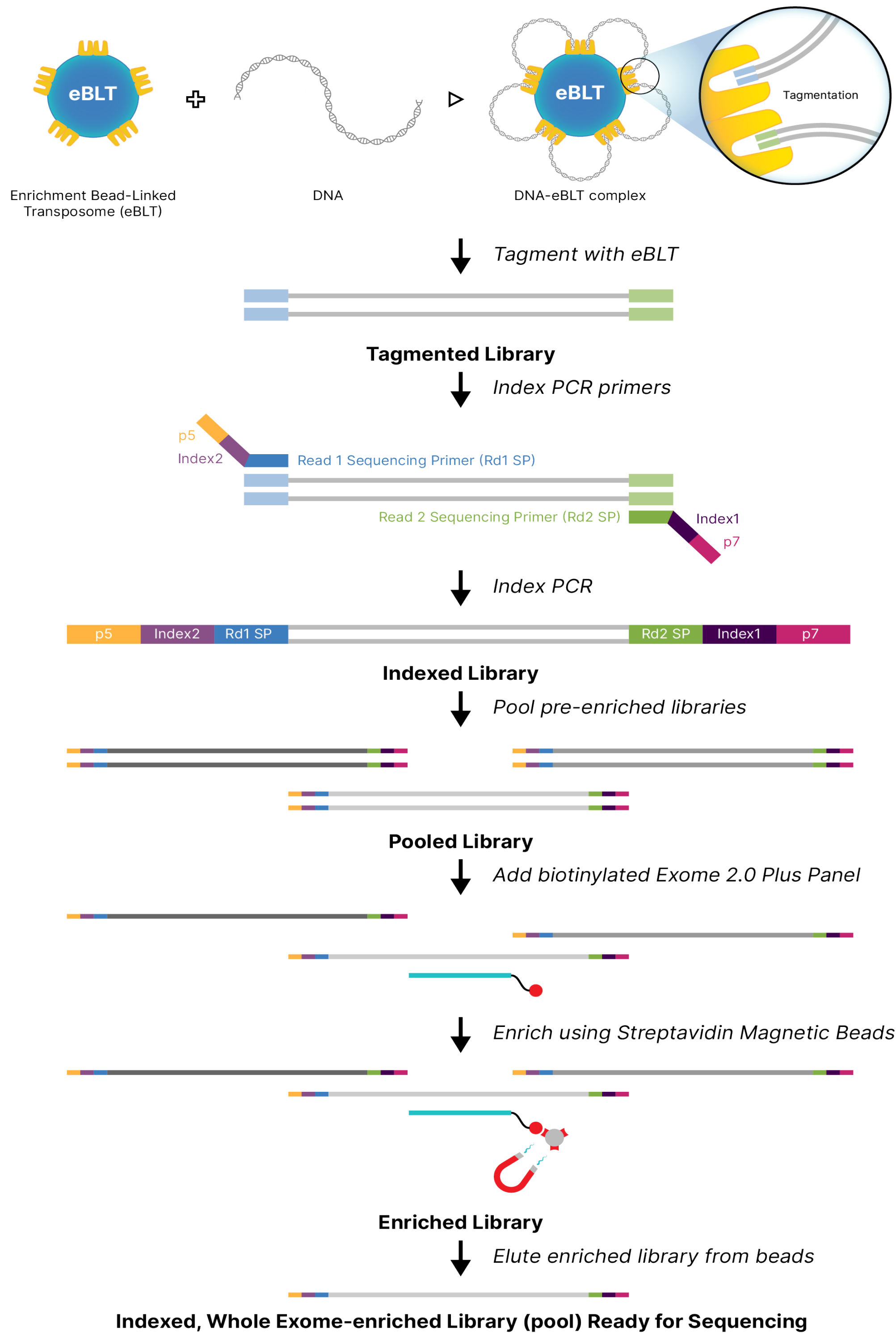

DNA Input Recommendations

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The Illumina DNA Prep with Enrichment protocol is compatible with high-quality, double-stranded genomic DNA (gDNA) inputs of 10–1000 ng. For human gDNA samples and other large complex genomes, the recommended minimum gDNA input is 50 ng.

The Illumina DNA Prep with Enrichment workflow is compatible with blood, saliva, or FFPE samples when using the following protocols and reagent kits:

- Illumina Blood Lysis Protocol (blood) with the Flex Lysis Reagent Kit
- Illumina Saliva Lysis Protocol (saliva)
- QIAGEN AllPrep DNA/RNA FFPE Kit for extraction of FFPE samples (FFPE)
- Infinium FFPE QC Kit for qualification (FFPE)

The recommended number of PCR cycles for the EBLT PCR program is adjusted based on sample input concentration and quality. For more information, refer to *Amplify Tagmented DNA* on page 28.

Table 1 Sample Input Recommendations

Sample Input Type	Quantification of Input DNA Required	Required DNA Input Quality	Normalized Pre-Enriched Library Yield
10–49 ng genomic DNA	Yes	A260/280 ratio of 1.8–2.0 and A260/230 ratio of 2.0–2.2	No
50–1000 ng genomic DNA	No	A260/280 ratio of 1.8–2.0 and A260/230 ratio of 2.0–2.2	Yes
50–1000 ng extracted FFPE	Yes	Δ Cq value ≤ 5	No
Saliva	No	Not applicable	Yes
Blood	No	Not applicable	Yes

Assess gDNA Purity

Use one or more of the following strategies to assess gDNA purity. Make sure that the initial gDNA sample does not contain any organic contaminants, such as phenol and ethanol.

The input DNA must also contain less than 1 mM EDTA. These substances can interfere with the tagmentation reaction and result in assay failure.

- UV absorbance is a common method used for assessing the purity of a gDNA sample. The ratio of absorbance at 260 nm to 280 nm provides an indication of sample purity. This protocol is optimized for gDNA with A260/280 ratios of 1.8–2.0, which indicates a gDNA sample with high purity.

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- For a secondary indication of sample purity, use an A260/230 ratio. Target an A260/230 ratio of 2.0–2.2. Values outside this range indicate the presence of contaminants. For a complete list of contaminants, including sources, avoidance, and effects on the library preparation, refer to the Illumina support site.
- Dilute the starting material in 10 mM Tris-HCl, pH 7.5–8.5. Incomplete tagmentation caused by contaminants can cause library preparation failure, poor clustering, or low quality sequencing results.

Enrichment Panel Recommendations

Supplemental enrichment panels can be designed to add or boost coverage of Illumina Custom Enrichment v2 panels. (Supplemental enrichment panels for Illumina Custom Enrichment Panels are not supported for Illumina DNA Prep with Enrichment.)

The Illumina DNA Prep with Enrichment workflow is compatible with the following fixed and customizable biotinylated oligo enrichment panels:

- TruSight Hereditary Cancer – Enrichment Oligos
- Illumina Exome Panel (CEX)
- Illumina Custom Enrichment Panel v2
- Illumina Custom Enrichment Panel

DesignStudio Assay Design Tool supports assay designs for human hg19 and hg38 genome assemblies. Non-human custom enrichment panel designs are supported through Illumina Technical Support. The Illumina DNA Prep with Enrichment workflow is compatible with the following assays:

- Illumina Custom Enrichment Panel v2
- Illumina Custom Enrichment Panel

If using third-party biotinylated DNA probes (fixed or custom panels), make sure they meet the requirements in *Third-Party Panel Requirements* on page 7.

FFPE Tissue Sample Input Recommendations

To obtain reliable, reproducible results from formalin-fixed paraffin-embedded (FFPE) DNA samples, accurately assess DNA quality to determine the required number of PCR cycles in this protocol. For more information, refer to *DNA Input Recommendations* on page 2.

Use FFPE DNA quality criteria to determine the appropriate input for successful library preparation. For FFPE samples with ΔCq value of ≤ 5, the recommended DNA input is 50–1000 ng. IDPE is not recommended for poor quality FFPE samples with ΔCq > 5. Using samples with ΔCq > 5 is possible, but it might increase chances of library preparation failure or decrease assay performance.

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FFPE Extraction

Use a nucleic acid isolation method that produces high recovery yields, minimizes sample consumption, and preserves sample integrity. The QIAGEN AllPrep DNA/RNA FFPE Kit provides high yield of nucleic acids compared to other extraction methods tested for this assay.

FFPE DNA Qualification

For optimal performance, assess DNA sample quality using the Infinium FFPE QC Kit with KAPA qPCR Master Mix (Universal) and Primer Premix. Perform this assessment on the Bio-Rad CFX96 Touch Real-Time PCR Detection System or equivalent instrument. For more information on FFPE DNA qualification, refer to the *Infinium HD FFPE QC Assay Protocol (document # 15020981)*.

FFPE Reference Samples (Optional)

Use characterized reference materials such as Horizon HD799 (DNA) as a positive control when performing the protocol. You can also use qualified FFPE materials from cell line derived xenografts as reference samples. Use a fluorometric-based method to quantify reference materials before use.

Running a positive control reference sample or no template control consumes reagents and reduces the total number of unknown samples that can be processed.

Blood and Saliva Input Recommendations

The IDPE protocol is compatible with fresh whole blood (requires the Flex Lysis Reagent Kit) and saliva sample inputs. For information about protocols specific to blood and saliva, refer to *Blood Lysis (Optional)* on page 20 or *Saliva Lysis (Optional)* on page 22.

When starting with 10 µl liquid whole blood in EDTA tubes or 30 µl saliva in Oragene tubes, expect normalization of pre-enriched libraries equal to results observed when using 50–1000 ng gDNA input. Blood and saliva are heterogeneous sample types. Therefore the ability of IDPE to generate normalized libraries depends on the total amount of DNA obtained from the lysed sample. The following factors can adversely affect normalization of library independent of kit performance:

- Viscosity of the saliva samples
- Blood sample age
- Sample storage conditions
- Underlying medical conditions affecting white blood cell counts

Consumables & Equipment

The IDPE protocol requires the following Illumina-supplied and user-supplied consumables and equipment.

The protocols have been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Make sure that you have the required consumables and equipment before starting the protocol.

Product Contents

Completing the Illumina DNA Prep with Enrichment protocol requires library prep and enrichment reagents, an enrichment probe panel, and index adapters. The number of index adapters required depends on the number of samples to be uniquely indexed for your experiment. Depending on the sample input type and sequencing requirements, the protocol might require additional, optional consumables.

Component	Kit Options	Illumina Catalog #
Library prep with enrichment reagents[1]	Illumina DNA Prep with Enrichment - (S) Tagmentation, 16 Samples	20025523
	Illumina DNA Prep with Enrichment - (S) Tagmentation, 96 Samples	20025524
[Optional] Library prep only reagents Use for enrichment plexities between 1-plex and 12-plex. Refer to *Non-Standard Enrichment Plexity Reagents* on page 8	Illumina DNA Prep - (S) Tagmentation, 16 Samples	20025519
	Illumina DNA Prep - (S) Tagmentation, 96 Samples	20025520

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Component	Kit Options	Illumina Catalog #
Enrichment probe panel Alternatively, use a third-party probe panel that meets specifications. Refer to *Third-Party Panel Requirements* on page 7.	TruSight Hereditary Cancer (8 Enrichment Reactions)	20029551
	Illumina Custom Enrichment Panel v2 (32 µl, 120-bp)	20073953
	Illumina Custom Enrichment Panel v2 (384 µl, 120-bp)	20073952
	Illumina Custom Enrichment Panel v2 (1536 µl, 120-bp)	20111339
	Illumina Custom Enrichment Panel (8 Enrichment Reactions)	20025371
	Illumina Exome Panel (CEX)	20020183
Index adapters Refer to *Illumina DNA/RNA Unique Dual Indexes*.	Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)	20091654
	Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)	20091656
	Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)	20091658
	Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)	20091660
[Optional] Blood lysis[3]	Flex Lysis Reagent Kit (96 samples)	20018706
[Optional] FFPE QC[2]	Infinium FFPE QC Kit (384 reactions)	WG-321-1001

[1] Illumina DNA Prep with Enrichment Reagents must be ordered with Illumina DNA Prep - (S) Tagmentation Library Prep Reagents.
[2] Required when starting the protocol from extracted FFPE.
[3] Required when starting the protocol from fresh whole blood samples.

Third-Party Panel Requirements

If using third-party biotinylated DNA probes (fixed or custom panels), make sure they meet the following specifications:

- 80 bp or 120 bp probe length
- Between 500 to 675,000 probes
- Single- or double- stranded
- Total probe input of ≥ 3 pmols for enrichment at plexities from 1-plex to 12-plex

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Non-Standard Enrichment Plexity Reagents

To run enrichment plexities between 1-plex and 12-plex, additional pre-enriched library prep reagents are required. Additional enrichment probe panel reagents can also be required depending on the number of enrichment reactions.

The following table provides information on additional library prep reagents needed for nonstandard enrichment plexities based on enrichment plexity, number of samples, and number of required reactions using the 16 sample configuration.

Table 2 Reagents for Non-Standard Plexities

Enrichment Plexity	Number of Samples	Enrichment Reactions Required	Quantity of Catalog #20025523 (Library Prep and Enrichment)	Quantity of Catalog # 20025519 (Library Prep Only)
3	24	8	1	1
3	48	16	1	2
4	24	6	1	1
4	48	12	1	2
6	24	4	1	1
6	48	8	1	2
8	24	3	1	1
8	48	6	1	2

The number of enrichment reactions provided for each enrichment probe panel must meet the number of enrichment reactions required for your desired enrichment plexity and number of samples.

To determine the number of enrichment probe panel reagents that are needed, divide the number of enrichment reactions required by the enrichment reactions listed per enrichment probe panel and round up to the nearest whole number.

Illumina DNA Prep - (S) Tagmentation Contents

Illumina DNA/RNA Prep -Tagmentation Buffers, Store at 15°C to 30°C*

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
1	4	ST2	Stop Tagment Buffer 2
1	1	TWB	Tagmentation Wash Buffer

*Shipped at 2°C to 8°C.

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Illumina DNA Prep -Tagmentation (S) Beads, Store at 2°C to 8°C*

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
1	4	EBLT	Enrichment Bead-Linked Transposomes
1	2	RSB	Resuspension Buffer

*Store the EBLT stock tube upright so the beads are always submerged in the buffer.

Illumina Purification Beads are not included in this kit and must be purchased separately.

Illumina DNA/RNA Prep - Tagmentation PCR Reagents, Store at -25°C to -15°C*

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
1	4	TB1	Tagmentation Buffer 1
2	4	EPM	Enhanced PCR Mix

*Shipped at 2°C to 8°C.

Illumina DNA Prep with Enrichment - (S) Tagmentation Contents

Illumina DNA Fast Hyb - Enrichment Beads + Buffers, Store at 2°C to 8°C

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
4	2	SMB4	Streptavidin Magnetic Beads 4
1	1	RSB	Resuspension Buffer
1	1	EHB2	Enrichment Hyb Buffer 2
1	1	ET2	Elute Target Buffer 2

Illumina Purification Beads are not included in this kit and must be purchased separately.

Illumina DNA Fast Hyb - Enrichment PCR + Buffers, Store at -25°C to -15°C*

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
1	1	EE1	Enrichment Elution Buffer 1

Tube Quantity		Acronym	Reagent Name
16 Samples	96 Samples		
4	4	EEW	Enhanced Enrichment Wash
1	1	PPC	PCR Primer Cocktail
1	1	HP3	2 N NaOH
2	1	NHB2	Hyb Buffer 2 + IDT NXT Blockers
2	1	EPM	Enhanced PCR Mix

*Shipped at 2°C to 8°C.

Illumina DNA/RNA UD Indexes

For index adapter sequences, refer to *Illumina Adapter Sequences*.

Store at -25°C to -15°C

Description
Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples)
Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples)
Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 Indexes, 96 Samples)
Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 Indexes, 96 Samples)

[Optional] Flex Lysis Reagent Kit*

Quantity	Acronym	Reagent Name	Storage Temperature
4	BLB	Blood Lysis Buffer	Room temperature
4	PK1	Proteinase K	-25°C to -15°C

*Shipped at -25°C to -15°C. Promptly store reagents at the indicated tube temperature to ensure proper performance.

[Optional] Infinium FFPE QC Kit

Store at -25°C to -15°C.

Quantity	Acronym	Reagent Name
1	QCP	QC Primer Reagent
1	QCT	QC Template Reagent

User-Supplied Consumables & Equipment

Make sure that you have the required consumables and equipment before starting the protocol.

Some items are required only for specific workflows. These items are specified in separate tables.

The protocol has been optimized and validated using the items listed. Comparable performance is not guaranteed when using alternate consumables and equipment.

Consumables

Consumable	Supplier
Microcentrifuge tubes, 1.7 ml	General lab supplier
Pipette tips, 10 µl	General lab supplier
Pipette tips, 20 µl	General lab supplier
Pipette tips, 200 µl	General lab supplier
Pipette tips, 1000 µl	General lab supplier
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Nuclease-free water	General lab supplier
Conical centrifuge tubes (15 ml or 50 ml)	General lab supplier
Eppendorf twin.tec 96-well LoBind PCR plate, skirted (or similar)	Eppendorf, catalog # 0030129512
Hard-Shell 96-well PCR plates	Bio-Rad, catalog # HSP-9601
Microseal 'B' adhesive seals	Bio-Rad, catalog # MSB-1001
Microseal 'F' foil seals	Bio-Rad, catalog # MSF-1001
RNase/DNase-free 8-tube strips and caps	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR **[Optional]** catalog # 89094-658
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, product # E7023
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058

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Consumable	Supplier
One of the following kits, depending on quantification method: • [Bioanalyzer] Agilent High Sensitivity DNA Kit (2) • [Fragment Analyzer] High Sensitivity NGS Fragment Analysis Kit • [Bioanalyzer] Agilent DNA 1000 Kit (2)	One of the following suppliers, depending on instrument: • Agilent, catalog # 5067–4626* • Advanced Analytical, catalog # DNF-474-0500 • Agilent, catalog # 5067-1504
[TapeStation] (pre-enriched): • Agilent D5000 Screen Tape • Agilent D5000 Reagent	• 5067–5588 • 5067–5589
[TapeStation] (post-enriched): • Agilent D1000 Screen Tape • AgilentD1000 Reagent	• 5067–5582 • 5067–5583
Qubit Assay Tubes	Thermo Fisher Scientific, catalog # Q32856
One of the following kits for quantification: • Qubit dsDNA BR Assay Kit • KAPA Library Quantification Kit – Illumina qPCR Instrument / Reference Dye Universal	• Thermo Fisher Scientific, catalog # Q32850 or Q32853 • Roche, catalog # 07960140001, KAPA code KK4824
Tris-HCl 10 mM, pH 8.5	General lab supplier

* End of life announced. Refer to vendor site for more information.

Consumables for Plate Workflow

Consumable	Supplier
96-well 0.8 ml polypropylene deep-well storage plate (MIDI plate)	Thermo Fisher Scientific, part # AB-0859
Adhesive seal roller	General lab supplier
Hard-Shell 96-well PCR plates	Bio-Rad, part # HSP-9601
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
Microseal 'F' foil seals	Bio-Rad, part # MSF-1001

Consumables for Tube Workflow

Consumable	Supplier
RNase/DNase-free 8-tube strips and caps	General lab supplier
1.7 ml microcentrifuge tubes	General lab supplier

Consumables for Blood and Saliva Input

Consumable	Supplier
Illumina Purification Beads	Illumina, 1 x 100 ml, catalog # 20060057 Illumina, 4 x 100 ml, catalog # 20060058
[Dried Blood]Dried Blood Spots (DBS) card	GE Healthcare, catalog # 10534320
[Blood] Flex Lysis Reagent Kit	Illumina, catalog # 20018706
[Blood] EDTA blood collection tubes	Becton Dickinson
[Saliva] Oragene DNA collection kit for saliva	Genotek, catalog # OGR-500 or OGD-510

Consumables for FFPE Input

Consumable	Supplier
KAPA Library Quantification Kit – Illumina qPCR Instrument / Reference Dye Universal	Roche, catalog # 07960140001, KAPA code KK4824
QIAGEN AllPrep DNA/RNA FFPE Kit	QIAGEN, catalog # 80234
Infinium FFPE QC Kit	Illumina, catalog # WG-321-1001
qPCR plates compatible with your sequencing system	General lab supplier

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Equipment

Equipment	Supplier
Pipettes, multichannel, 10 µl	General lab supplier
Pipettes, multichannel, 20 µl	General lab supplier
Pipettes, multichannel, 200 µl	General lab supplier
Pipettes, single channel, 10 µl	General lab supplier
Pipettes, single channel, 20 µl	General lab supplier
Pipettes, single channel, 200 µl	General lab supplier
Pipettes, single channel, 1000 µl	General lab supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM 10027
Microcentrifuge	General lab supplier
Heat block for 96 well plate • Compatible with the Illumina MIDI heat block insert • Heated lid • Temperature range: Ambient +5°C to 99°C • Temperature regulation: ±0.1°C	General lab supplier
Illumina MIDI heat block insert	Illumina, catalog # BD-60-601
Qubit Fluorometer 4.0	Thermo Fisher Scientific, catalog # Q33238 or Q33327
Vortexer	General lab supplier
One of the following analyzers: • Fragment Analyzer • 2100 Bioanalyzer Desktop System • 4150 TapeStation System • 4200 TapeStation System	Agilent Technologies catalog #: • Refer to web product pages for catalog # • G2939BA* or G2940CA • G2992AA • G2991BA
[Saliva] Water bath or air incubator capable of reaching 50°C	As recommended by DNA Genotek, refer to Genotek's product pages.
[FFPE] Bio-Rad CFX96 Touch Real-Time PCR Detection System or similar qPCR system for FFPE Qualification	Bio-Rad, part # 1855196*
[Optional] Vacuum concentrator Note: Use when concentrating a pooled library.	General lab supplier

* No longer available for purchase.

Equipment for Tube Workflow

Equipment	Supplier
MagneSphere Technology Magnetic Separation Stands (12 position, 1.5 ml)	Promega, catalog # Z5342

Equipment for Plate Workflow

Equipment	Supplier
Magnetic Stand-96	Thermo Fisher Scientific, catalog # AM10027
High-Speed Microplate Shaker	BioShake iQ High-Speed Thermal Mixer • Q Instruments, model # 1808-0506 BioShake XP High-Speed Thermal Mixer • Q Instruments, model # 1808-0505
Microplate centrifuge	General lab supplier

Thermal Cyclers

The following table lists the recommended settings for the thermal cycler. If your lab has a thermal cycler that is not listed, validate the thermal cycler before performing the protocol.

Thermal Cycler	Supplier/Description
Thermal cycler with the following specifications: • Compatible with semi-skirted 96-well PCR plates • Heated lid • Average ramp rate: 2°C/sec to -2.5°C/sec • Maximum ramp rate: 2.5°C to 4°C • Maximum reaction volume: 100 µl • Minimum temperature control range: 4°C to 100°C • Minimum temperature accuracy: ±0.5°C • Minimum temperature uniformity: ±0.5°C	General lab supplier
MJ Research DNA Engine Tetrad	MJ Research part # MJ-T2-4
Eppendorf Mastercycler Pro S (Gradient S, Simulated Tube)	Eppendorf part # 41102404

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Protocol

This section describes the Illumina DNA Prep with Enrichment protocol.

- Review the planned complete sequencing workflow, from sample through analysis, to ensure compatibility of products and experiment parameters.
- Before proceeding, confirm kit contents and make sure that you have the required components, equipment, and consumables. This protocol requires library prep and enrichment reagents, an enrichment probe panel, and index adapters. Enrichment probe panels and index adapters are sold separately. Refer to *Product Contents* on page 6.
  - Third-party biotinylated probes must meet specific requirements. Refer to *Third-Party Panel Requirements* on page 7 to make sure that your third-party probes meet the requirements.
- Follow the protocol in the order shown, using the specified volumes and incubation parameters.

Supported Enrichment Plexities

Illumina DNA Prep with Enrichment reagents are configured and tested at 1-plex and 12-plex enrichment plexity. Although other enrichment plexities are possible, some plexities require additional pre-enrichment library prep and enrichment probe panel reagents.

Obtaining suitable enrichment yields for nonstandard enrichment plexities might require additional optimization. Optimal results are not guaranteed. For information on reagents required for other enrichment plexities, refer to *Non-Standard Enrichment Plexity Reagents* on page 8.

- **Enrichment plexity**—The number of pre-enriched libraries (1–11 optional, 12 recommended) pooled together in one enrichment reaction for hybridization with the enrichment probe panels. For example, combining 12 pre-enriched libraries together creates a 12-plex enrichment pool.
- **Enrichment reaction**—The number of unique enrichment reaction preparations, regardless of the number of pre-enriched libraries pooled per reaction. For example, a single enrichment reaction can prepare a 1-plex or 12-plex enrichment pool.

To calculate the total number of post-enriched libraries, multiply the enrichment plexity per reaction by the number of enrichment reactions. For example, a single enrichment reaction of a 12-plex enrichment pool produces a pool of 12 post-enriched libraries.

When pooling pre-enriched libraries, Illumina DNA Prep with Enrichment reagents support the number of enrichment reactions and plexity indicated.

Table 3 Supported Enrichment Plexities

Illumina DNA Prep with Enrichment Reagents	Enrichment Reactions	Enrichment Plexity
16-sample kit	16 reactions	1-plex
96-sample kit	8 reactions	12-plex

Illumina DNA Prep with Enrichment Workflow

The following diagram illustrates this Illumina DNA Prep with Enrichment workflow.

- Safe stopping points are marked between steps
- Time estimates are based on processing 12 samples at 12-plex enrichment.

Tips and Techniques

Safe Stopping Point

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

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Avoiding Cross-Contamination

- When adding or transferring samples or reagent master mixes, change tips between ***each sample***.
- When adding index adapters with a multichannel pipette, change tips between ***each row*** or ***each column***. If using a single channel pipette, change tips between each sample.
- Remove unused index adapter tubes or plates from the working area.
- Remove partially used index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate with the adhesive seal using a rubber roller to cover the plate before the following steps in the protocol:
  - Shaking steps
  - Thermal cycling steps
  - Centrifuge steps
- Microseal 'B' adhesive seals are effective at -40°C to 110°C and suitable for skirted or semiskirted PCR plates. Use Microseal 'B' seals for thermal cycling or short-term storage.
- Microseal ‘F’ foil seals are effective at temperatures down to -70°C and are suitable for storing the 96-well plates containing the final libraries long term.

Handling Enrichment Bead-Linked Transposomes (Enrichment BLT, EBLT)

- Store the EBLT stock tube upright in the refrigerator so that the beads are always submerged in the buffer.
- Vortex the EBLT stock tube thoroughly until the beads are resuspended before use. To avoid resettling the beads, centrifugation before pipetting is not recommended.
- If beads are adhered to the side or top of a 96-well plate, centrifuge at 280 × g for 3 seconds, and then pipette to resuspend.
- When washing beads:
  - Use the appropriate magnetic stand for the plate.
  - Keep the plate on the magnetic stand until the instructions specify to remove it.
  - Do not agitate the plate while it is on the magnetic stand.
  - Do not disturb the bead pellet.
  - If beads are aspirated into pipette tips, dispense back into the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
  - Dispense Tagmentation Wash Buffer (TWB) directly onto the beads.

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- If liquid becomes adhered to the side or top of the tube or well, centrifuge at 280 × g for 3 seconds to pull volume into liquid.

Preparing Illumina DNA/RNA Unique Dual (UD) Indexes Plate

- If using a NextSeq 500 system, the read lengths must be modified to accommodate 10 base pair indexes. Visit the compatible products page on the Illumina support site.

Blood Lysis (Optional)

Use this protocol when performing the Illumina DNA Prep with Enrichment workflow using blood sample inputs with the Flex Lysis Reagent Kit. This protocol has been validated using fresh whole blood collected in EDTA collection tubes. Store the blood at 4°C and process it within 3 days. The use of frozen blood has not been validated and cannot be recommended.

This protocol is expected to generate > 100 ng of DNA output at the end of the blood lysis step.

Blood is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of blood samples. During the lysis protocol, make sure that the entire blood sample is fully lysed (brown in color following the heat incubation step) before proceeding to subsequent steps. A fully lysed sample makes sure that any blood borne pathogens are eliminated and the sample is no longer biohazardous.

Consumables

- BLB (Blood Lysis Buffer)
- IPB (Illumina Purification Beads)
- PK1 (Proteinase K)
- EtOH (Freshly prepared 80% ethanol)
- EDTA collection tubes (for blood sample collection)
- Nuclease-free water
- 96-well PCR plate

About Reagents

- IPB
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

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Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
BLB	Room temperature	If frozen, thaw at room temperature. If precipitates are observed, heat at 37°C for 10 minutes and vortex until resuspended.
IPB	Room temperature	Use at room temperature.
PK1	-25°C to -15°C	Place on ice until needed.

2. Prepare fresh 80% EtOH from absolute ethanol.
3. Save the following BLP program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - 56°C for 10 minutes

Procedure

1. Combine the following volumes to prepare the Lysis Master Mix. Multiply each volume by the number of samples being processed.
   - BLB (7 µl)
   - PK1 (2 µl)
   - Nuclease-free water (31 µl)

   Reagent overage is included in the volume to ensure accurate pipetting.
2. Vortex and centrifuge the Lysis Master Mix.
3. Invert the EDTA tube 10 times to mix.
4. Transfer 10 µl blood from the tube to one well of a 96-well PCR plate.
5. Add 40 µl Lysis Master Mix to each sample.
6. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
7. Add 20 µl IPB to the well.
8. Using a pipette set to 50 µl, slowly pipette 10 times to mix, and then seal.
9. Place on the preprogrammed thermal cycler and run the BLP program.
10. Place on a magnetic stand and wait 5 minutes.
    The dark brown color of the blood from the lysis reaction keeps the liquid from becoming clear.

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The beads migrate after 5 minutes.

11. Without disturbing the beads, remove and discard supernatant.
12. If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and then wait until the liquid is clear (~2 minutes).
13. Add 150 µl fresh 80% EtOH to the well.
14. Incubate on the magnetic stand for 30 seconds.
15. Pipette to remove and discard the EtOH.
16. Use a 20 µl pipette to remove and discard all residual EtOH.
17. Remove the plate from the magnetic stand.
18. Add 30 µl nuclease-free water and pipette to resuspend.
19. If you are not stopping, proceed immediately to step 3 of *Tagment Genomic DNA* on page 24.

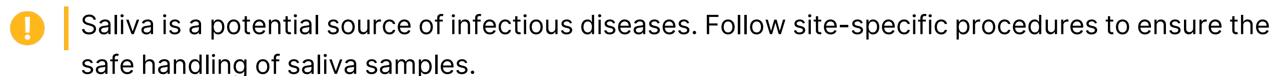
SAFE STOPPING POINT

Seal the plate with a Microseal 'B' adhesive seal and store the plate at 2°C to 8°C for up to 3 days.

Saliva Lysis (Optional)

Use this protocol when performing the Illumina DNA Prep with Enrichment workflow using saliva sample inputs. This protocol is validated for saliva collected only in Oragene DNA saliva collection tubes. The saliva is mixed with the Oragene Dx solution contained in the collection tube, making it stable at room temperature.

This protocol is expected to generate > 100 ng of DNA output at the end of the saliva lysis step.



Saliva is a potential source of infectious diseases. Follow site-specific procedures to ensure the safe handling of saliva samples.

Consumables

- IPB (Illumina Purification Beads)
- 96-well PCR plate
- EtOH (Freshly prepared 80% ethanol)
- Nuclease-free water
- Oragene DNA collection tubes (for saliva sample collection)

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.

- Resuspend frequently to make sure the beads are evenly distributed.
- Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following consumables.

Item	Storage	Instructions
IPB	Room temperature	Use at room temperature.
Saliva samples in Oragene DNA collection tubes	Room temperature	For information on sample preparation and storage, refer to the DNA Genotek website.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. For each sample, add 20 µl nuclease-free water to one well of a 96-well PCR plate.
2. Vortex the heat-treated Oragene DNA collection tube.
3. Transfer 30 µl saliva sample from the tube to the well containing nuclease-free water.
4. Slowly pipette to mix.
   For viscous samples, use a wide-bore pipette tip for more accurate pipetting.
5. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
6. Add 20 µl IPB to the well.
7. Using a pipette set to 50 µl, slowly pipette 10 times to mix.
8. Incubate at room temperature for 5 minutes.
9. Place on a magnetic stand and wait 5 minutes.
10. Without disturbing the beads, remove and discard supernatant.
11. If beads are aspirated into pipette tips, dispense back to the plate on the magnetic stand, and wait until the liquid is clear (~2 minutes).
12. Add 150 µl fresh 80% EtOH to the well.
13. Incubate on the magnetic stand for 30 seconds.
14. Use a 20 µl pipette to remove and discard all residual EtOH.
15. Remove the plate from the magnetic stand.

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16. Add 30 µl nuclease-free water and pipette to resuspend.
17. Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
18. If you are not stopping, proceed immediately to step 3 of *Tagment Genomic DNA* on page 24.

SAFE STOPPING POINT

Seal the plate with a Microseal 'B' adhesive seal and store at 2°C to 8°C for up to 3 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 4 15°C to 30°C Storage

Reagent	Box Name	Instructions
ST2	Illumina DNA/RNA Prep -Tagmentation Buffers	Use at room temperature.
TWB	Illumina DNA/RNA Prep -Tagmentation Buffers	Use at room temperature.

Table 5 2°C to 8°C Storage

Reagent	Box Name	Instructions
EBLT	Illumina DNA Prep - Tagmentation (S) Beads	Bring to room temperature.

Table 6 -25°C to -15°C Storage

Reagent	Box Name	Instructions
EPM	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Thaw on ice.
Index adapter plate	Illumina DNA/RNA UD Indexes	Thaw at room temperature.
TB1	Illumina DNA/RNA Prep - Tagmentation PCR Reagents	Bring to room temperature.

Tagment Genomic DNA

This step uses the Enrichment Bead-Linked Transposomes (EBLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

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Consumables

- EBLT (Enrichment Bead-Linked Transposomes)
- TB1 (Tagmentation Buffer 1)
- Nuclease-free water
- 8-tube strip
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Pipette tips
  - 20 µl
  - 200 µl

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, refer to the SDS at support.illumina.com/sds.html.

About Reagents

- EBLT
  - Must be stored upright so that the beads are always submerged in the buffer.
  - Do not use EBLT that has been stored below 2°C.

Preparation

1. Prepare the following consumables:
   - EBLT—Vortex to mix. Do not centrifuge before pipetting.
   - TB1—Vortex to mix.
2. Save the following TAG program on the thermal cycler:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 55°C for 5 minutes
   - Hold at 10°C

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Procedure

1. Add 2–30 µl DNA to each well of a 96-well PCR plate so that the total input amount is 10–1000 ng.
2. If DNA volume is < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
3. Vortex EBLT for 10 seconds to resuspend. Repeat as necessary.
4. For each sample, combine the following volumes to prepare the Tagmentation Master Mix. Multiply each volume by the number of samples being processed.
   - EBLT (11.5 µl)
   - TB1 (11.5 µl)

   These volumes produce 23 µl Tagmentation Master Mix per sample, which includes extra volume to ensure accurate pipetting.
5. Vortex the Tagmentation Master Mix for 10 seconds to resuspend.
6. Divide the Tagmentation Master Mix volume equally into an 8-tube strip.
7. Using a multichannel pipette, transfer 20 µl Tagmentation Master Mix from the 8-tube strip to each well of the plate containing a sample.
   Use fresh tips for each sample column.
8. Discard the 8-tube strip after the Tagmentation Master Mix has been dispensed.
9. Using a multichannel pipette set to 40 µl, pipette each sample 10 times to resuspend, and then seal the plate. Alternatively, seal the plate and shake at 1600 rpm for 1 minute.
10. Place on the preprogrammed thermal cycler and run the TAG program.
11. Wait until the TAG program has reached the 10°C hold temperature before removing the plate and proceeding.

Post Tagmentation Clean Up

This step washes the adapter-tagged DNA on the EBLT before PCR amplification.

Consumables

- ST2 (Stop Tagment Buffer 2)
- TWB (Tagmentation Wash Buffer)
- 8-tube strip
- Microseal 'B' adhesive seal
- Pipette tips
  - 20 µl
  - 200 µl

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About Reagents

- TWB
  - Pipette slowly to minimize foaming.
  - A deliberately slow pipetting technique minimizes the potential of foaming to avoid incorrect volume aspiration and incomplete mixing.

Preparation

1. Prepare the following consumables:
   - ST2—If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved.
   - TWB—Use at room temperature. Vortex to mix.

Procedure

1. Let the 96-well PCR plate stand at room temperature for 2 minutes.
2. Add 10 µl ST2 to each well of the plate. If you are using a multichannel pipette, pipette ST2 into an 8-tube strip, and then transfer the 10 µl volumes.
3. Using a 200 µl pipette set to 50 µl, slowly pipette each well 10 times to resuspend the beads, and then seal. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute. Repeat as needed to resuspend the beads.
4. Make sure that the plate is sealed and incubate at room temperature for 5 minutes.
5. Centrifuge the samples for approximately 2 seconds, place them on a magnetic stand, and then wait until the liquid is clear (~3 minutes).
6. **[≤ 48 samples]** Wash as follows.
   a. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.
   b. Remove from the magnetic stand.
   c. Use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
   d. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
   e. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
   f. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
   g. Repeat steps b–e for a **second** wash.
7. **[> 48 samples]** Wash as follows.
   a. Perform steps b and step c in one or two column increments until all columns have been processed.
   b. Using a 200 µl multichannel pipette set to 60 µl, remove and discard supernatant.

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c. Immediately add 100 µl TWB directly onto the beads using a deliberately slow pipetting technique.
d. Remove from the magnetic stand.
e. Pipette slowly until beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
f. Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
g. Perform step h and step i in one or two column increments until all columns have been processed.
h. Using a 200 µl multichannel pipette set to 100 µl, remove and discard supernatant.
i. Immediately add 100 µl TWB directly onto the beads using a deliberately slow pipetting technique.
j. Repeat steps d–i for a **second** wash.
k. Repeat steps d–i for a **third** wash.

8. Pipette each well slowly to resuspend the beads. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
9. Seal the plate and place on the magnetic stand until the liquid is clear (~3 minutes).
10. Keep on the magnetic stand until step 4 of the *Procedure* section in *Amplify Tagmented DNA* on page 28.
    The TWB remains in the wells to prevent drying of the beads.

Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds pre-paired 10 base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation. To confirm the indexes of libraries being pooled for enrichment have the appropriate color balance, refer to the *Index Adapters Pooling Guide*.

For a list of compatible index adapters for use with this protocol, refer to *Product Contents* on page 6.

Consumables

- EPM (Enhanced PCR Mix)
- Microcentrifuge tubes, 1.7 ml
- Pipette tips
  - 20 µl
  - 200 µl
- Eppendorf Lo Bind PCR Plate
- Microseal 'B' adhesive seal
- Nuclease-free water

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- Index adapter plate (A, B, C, or D plates)

About Reagents

- Index adapter plates
  - A well can contain > 10 µl index adapters.
  - Do not add samples to the index adapter plate.
  - Each well of the index plate is single use only.

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, then centrifuge briefly.
   - Index adapter plates
     - Use 10 base pair index codes that differ from other indexes, such as other Illumina index adapters which use eight base pair index codes. Confirm that the sequencing system is configured for 10 base pair index codes. If using a NextSeq 500 system, the read lengths must be modified to accommodate 10 base pair indexes.
     - Vortex to mix.
     - Centrifuge at 1000 × g for 1 minute to settle liquid away from the seal.
2. Save the following EBLT PCR program on a thermal cycler using the appropriate number of PCR cycles indicated in the table:
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 72°C for 3 minutes
   - 98°C for 3 minutes
   - (X) cycles of:
     - 98°C for 20 seconds
     - 60°C for 30 seconds
     - 72°C for 1 minute
   - 72°C for 3 minutes
   - Hold at 10°C

   Total running time is ~38 minutes for 9 cycles, and ~46 minutes for 12 cycles.

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Sample Input Type	Number of PCR Cycles (X)
10–49 ng genomic DNA	12
50–1000 ng genomic DNA	9
Saliva	9
Blood	9
50–1000 ng extracted FFPE	12

Procedure

1. For each sample, combine the following volumes to prepare the PCR Master Mix. Multiply each volume by the number of samples being processed. Reagent overage is included in the volume to ensure accurate pipetting.
   - EPM (23 µl)
   - Nuclease-free water (23 µl)
2. Vortex, and then centrifuge the PCR Master Mix at 280 × g for 10 seconds.
3. With the plate on the magnetic stand, use a 200 µl multichannel pipette set to 100 µl to remove and discard supernatant.
   Foam that remains on the well walls does not adversely affect the library.
4. Remove from the magnetic stand.
5. Immediately add 40 µl PCR Master Mix directly onto the beads in each sample well.
6. Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
7. Seal the sample plate and centrifuge at 280 × g for 3 seconds.
8. Centrifuge the index adapter plate at 1000 × g for 1 minute.
9. Prepare the index adapter plate.
   - **[< 96 samples]** Pierce the foil seal on the index adapter plate with a new pipette tip for each well for only the number of samples being processed.
   - **[96 samples]** Align a new Eppendorf PCR plate above the index adapter plate and press down to puncture the foil seal. Discard the Eppendorf PCR plate used to puncture the foil seal.
10. Using a new pipette tip, add 10 µl pre-paired Index 1 (i7) and Index 2 (i5) index adapters to each well.
11. Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
12. Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
13. Place on the preprogrammed thermal cycler and run the EBLT PCR program.

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SAFE STOPPING POINT

If you are stopping, store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 7 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	IPB	Bring to room temperature.

Table 8 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Prep - Tagmentation (S) Beads	Bring to room temperature.

Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified and indexed libraries.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- Nuclease-free water
- 96-well 0.8 ml Polypropylene Deepwell Storage Plate (MIDI plate) (2)
- 96-well PCR plate
- Microseal 'B' adhesive seal
- Microseal 'F' foil seal

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Aspirate and dispense slowly due to the viscosity of the solution.

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Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Use a plate shaker to shake the 96-well PCR plate at 1800 rpm for 1 minute.
2. Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
3. Transfer 45 µl supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate.
4. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
5. For gDNA, blood, or saliva, perform the following steps.
   a. Add 77 µl nuclease-free water to each well-containing supernatant.
   b. Add 88 µl IPB to each well-containing supernatant.
   c. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   d. Seal the plate and incubate at room temperature for 5 minutes.
   e. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
   f. During incubation, thoroughly vortex and resuspend the IPB, and then add 20 µl to each well of a *new* MIDI plate.
   g. Remove seal and transfer 200 µl supernatant from each well of the first plate to the corresponding well of the new MIDI plate containing 20 µl IPB.
   h. Pipette each well in the MIDI plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1800 rpm for 1 minute.
   i. Discard the first plate.
6. For extracted FFPE, perform the following steps.
   a. Add 81 µl IPB to each well of the MIDI plate containing supernatant.
   b. Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
7. Incubate the sealed MIDI plate at room temperature for 5 minutes.
8. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

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9. Without disturbing the beads, remove and discard supernatant.
10. Wash beads as follows.
    a. With the plate on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
    b. Incubate for 30 seconds.
    c. Without disturbing the beads, remove and discard supernatant.
11. Wash beads a **second** time.
12. Use a 20 µl pipette to remove and discard residual EtOH.
13. Air-dry on the magnetic stand for 5 minutes.
14. Remove from the magnetic stand.
15. Add 17 µl RSB to the beads.
16. Seal the plate, and then use a plate shaker at 1800 rpm for 2 minutes.
17. Incubate at room temperature for 2 minutes.
18. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
19. Transfer 15 µl supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Qualify Pre-Enriched Libraries

It is recommended to check the quality or qualify pre-enriched libraries before proceeding to enrichment.

If you elect not to check pre-enriched libraries, perform the following procedure instead to reserve samples for potential troubleshooting later.

Store for Potential Troubleshooting.

1. Transfer 1 µl of each pre-enriched library to a new 96-well PCR plate.
2. Add 4 µl RSB to each pre-enriched library.
3. Seal the plate with Microseal 'F' foil seal.
4. Store at -25°C to -15°C for up to 30 days for future troubleshooting if necessary.

Qualify Pre-Enriched Libraries.

Pre-enriched libraries can be qualified individually (one library at a time) or as a pool before enrichment.

Assess quality of 1 µl library or pooled libraries using one of the following methods.

- Add 1 µl RSB to the 1 µl library or pooled libraries, and then analyze the 2 µl volume using the Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit.
- Analyze 1 µl library or pooled libraries using the Agilent Technology 2100 Bioanalyzer using a DNA 1000 kit.

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- Analyze 1 µl library or pooled libraries using the Agilent 4200 TapeStation with the Agilent D1000 Screen Tape.

Expect the mean fragment size to be between 300 bp and 400 bp and a distribution of DNA fragment size range of 150–1500 bp as shown in the figures below.

For FFPE samples, the mean fragment size can be as low as 250 bp.

Figure 1 Fragment Analyzer Trace: Example

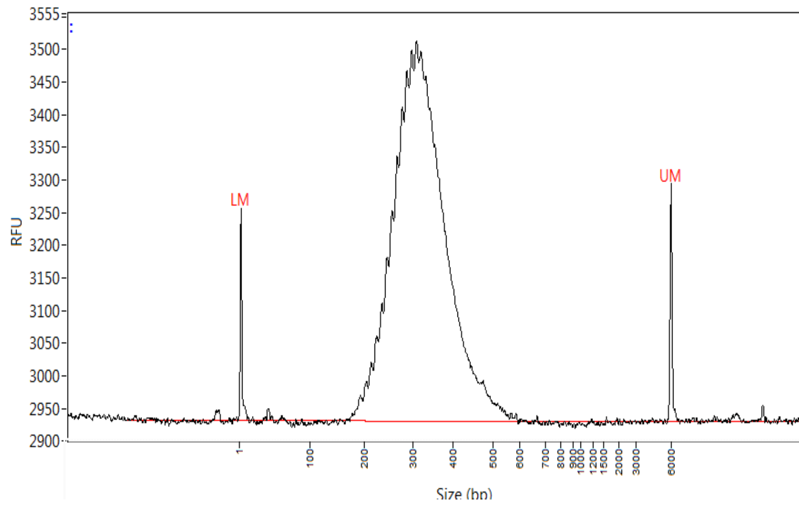
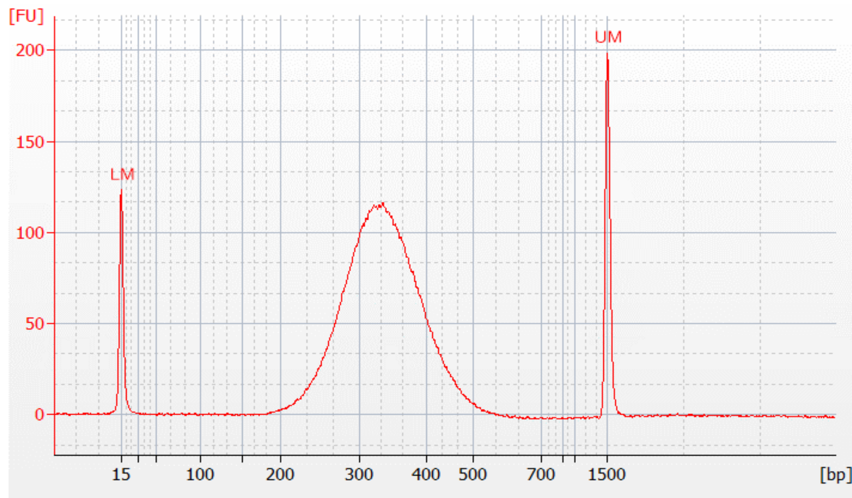


Figure 2 Bioanalyzer Trace: Example



TapeStation Trace: Example

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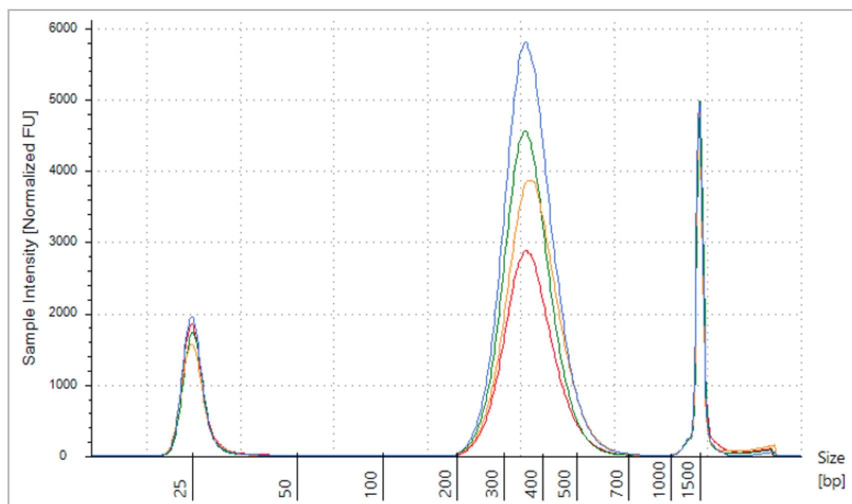

Pool Pre-Enriched Libraries

This step combines DNA libraries with unique indexes into one pool of up to 12 libraries.

Pooling Methods

You can pool by volume or mass. Use the following table to determine the appropriate method for your input.

Sample Input	Pooling Method
10–49 ng gDNA	Mass or volume*
50–1000 ng gDNA	Volume
50–1000 ng extracted FFPE	Mass or volume*
Saliva	Volume
Blood	Volume

* For volume, use 1-plex enrichment.

- One-plex enrichment does not require pooling pre-enriched libraries. However, adding RSB might be necessary.
- After pre-enriched library quantification, all sample input types can be pooled by mass to achieve optimal library balance and similar number of sequencing reads per library.
- The final yield of pre-enriched libraries generated in separate experimental preparations can vary. Therefore, pooling by mass is recommended to achieve optimal library balance when pooling samples from multiple experimental preparations.
- Use 1-plex enrichment for the following situations:
  - 10–49 ng gDNA when using the volume method.

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- 50–1000 ng extracted FFPE when using the volume method.
- Low minor allele frequency detection for germline or somatic variant calling.

Pool by Volume

When the input is 50–1000 ng gDNA, quantifying and normalizing individual libraries generated in the same experiment is not required.

To achieve optimal performance, only pool pre-enriched library samples prepared by the same user, reagent lot, and index adapter plate.

1. Using the sample tracking method you chose in *Protocol* on page 16, record the indexes for the libraries you plan to pool in this step.
2. Pool pre-enriched libraries based on the sample volumes in the following table.

Library Pool Plexity	Each Pre-Enriched Library Volume (µl)	Total Volume (µl)
1-plex	14	30 (with 16 RSB)
12-plex	2.5	30

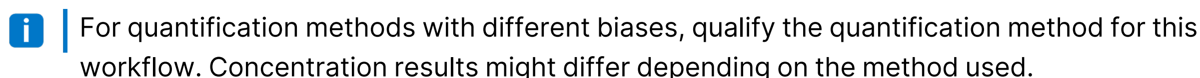
Pool by Mass

To achieve optimal sample balancing when starting with input of 10-49 ng gDNA or 50–1000 ng extracted FFPE samples, pool your libraries at equal concentration after dsDNA quantification.

Quantify Pre-Enriched Libraries

- Quantify 1 µl of the pre-enriched libraries using the Qubit dsDNA BR Assay Kit to determine library concentration (ng/µl).
  Expect the following library yield based on sample type and input.
  The expected library yield is ≥ 100 ng.

Sample Input Type (ng)	Pre-Enriched Library Yield (ng)
10–49 gDNA	≥ 100
50–1000 gDNA, blood, saliva	≥ 250



For quantification methods with different biases, qualify the quantification method for this workflow. Concentration results might differ depending on the method used.

Pool Pre-Enriched Libraries at Equal Concentration

For the following sample input types and applications, a minimum of 500 ng per pre-enriched library for 1-plex enrichment is tested and recommended. Other enrichment plexities might be possible. Optimal results are not guaranteed.

- Degraded samples, such as FFPE

- Somatic variant calling
- Low minor allele frequency detection

When pooling by mass for samples derived from high-quality DNA, as little as 100 ng per pre-enriched library can be used for enrichment. Additional optimization might be required to obtain suitable enrichment yield when pooling pre-enriched libraries < 250 ng. Optimal results are not guaranteed.

If proceeding with < 500 ng per pre-enriched library, decrease the mass of each library to equal concentration based on your sample quantification results. Confirm the total DNA library mass (ng) remains 500–6000 ng. For example, if you have 250 ng per pre-enriched library, you can proceed with 12-plex enrichment with 3000 ng of total DNA library mass and proceed with step 2.

1. Prepare each pre-enriched library. Dilute with RSB as needed.
2. Using the sample tracking method you chose in *Protocol* on page 16, record the indexes for the libraries you plan to pool in this step.
3. Combine each library in a 1.5 ml microcentrifuge tube into the plexities shown in the following table.

Library Pool Plexity	Each Pre-Enriched Library Sample (ng)	Total Mass (ng)
1-plex	500	500
12-plex	500	6000

4. Perform one of the following based on the total volume of the pooled pre-enriched libraries:
   - If pre-enriched library volume = 30 µl, proceed to *Hybridize Probes* on page 42.
   - If pre-enriched library volume < 30 µl, add RSB to reach 30 µl total volume.
   - If pre-enriched library volume > 30 µl, use one of the following methods to concentrate the pooled sample:
     - **Bead-based method**—Follow the instructions in *Concentrate Pooled Libraries (Optional)* on page 37.
     - **Vacuum concentrator**—Use a no heat setting and a medium drying rate.

SAFE STOPPING POINT

If you are stopping, cap the 1.5 ml microcentrifuge tube and store at -25°C to -15°C for up to 30 days.

Concentrate Pooled Libraries (Optional)

If the total volume of the pooled pre-enriched libraries is > 30 µl, the pool of pre-enriched libraries must be concentrated to a final volume of 30 µl. Use this bead-based method to achieve a final volume of 30 µl. For more information, refer to *Pool Pre-Enriched Libraries* on page 35.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- EtOH (Freshly prepared 80% ethanol)
- **[Plate]** Microseal 'B' adhesive seals
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 1.7 ml microcentrifuge tubes
- One of the following magnets:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml).

About Reagents

- IPB
  - Must be at room temperature before use.
  - Vortex to resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. Prepare the following reagents.

Item	Storage	Instructions
IPB	15°C to 30°C	Use at room temperature.
RSB	2°C to 8°C	Bring to room temperature for > 30 minutes. Vortex to mix.

2. Prepare fresh 80% EtOH from absolute ethanol.

Procedure

1. Centrifuge the sample tube at 280 × g for 1 minute.
2. Transfer samples to the corresponding well of a new MIDI plate or a new 1.7 ml microcentrifuge tube.

   If the pool volume is ≥ 178 µl, use a 1.7 ml microcentrifuge tube to prevent MIDI plate wells from overflowing.

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3. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
4. Add 1.8x pool volume of IPB to each well or to the microcentrifuge tube, and then mix thoroughly as follows.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or the tube at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 1 minute.
7. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
8. Remove and discard all supernatant from each well or from the tube.
9. Wash as follows.
   a. Keep on the magnetic stand, add 200 µl freshly prepared 80% EtOH to each well or to the tube.
   b. Wait 30 seconds.
   c. Using a pipette set to 200 µl, remove and discard all supernatant from each well or from the tube.
10. Wash a **second** time.
11. Use a 20 µl pipette to remove and discard residual 80% EtOH.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand and add 32 µl RSB to each well or to the tube.
14. Mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
15. Incubate the sample plate or the tube at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 1 minute.
17. Place on a magnetic stand and wait until the liquid is clear (~5 minutes).
18. Transfer 30 µl supernatant to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
19. Resume the protocol at *Hybridize Probes* on page 42.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, or cap the tube, and store at -25°C to -15°C for up to 30 days.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 9 2°C to 8°C Storage

Reagent	Box Name	Instructions
EHB2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.
ET2	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends.
RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.
SMB4	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

Table 10 -25°C to -15°C Storage

Reagent	Box Name	Instructions
Enrichment Probe Panel (one of the following):		
• TruSight Cancer	Not applicable	Bring to room temperature.
• TruSight Hereditary Cancer	Not applicable	Bring to room temperature.
• Illumina Exome Panel (CEX)	Not applicable	Bring to room temperature.
• Illumina Custom Enrichment Panel	Not applicable	Bring to room temperature.
• Illumina Custom Enrichment Panel v2	Not applicable	Bring to room temperature.

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Reagent	Box Name	Instructions
• Third-party panels (that meet requirements in *Third-Party Panel Requirements* on page 7)	Not applicable	Use the vendor-recommended probe volume per reaction for the third party enrichment assay. Add RSB, if needed, for a total volume of 10 µl per enrichment reaction, regardless of the enrichment plexity.
EE1	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.
EEW (amber tube)	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.
EPM	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.
HP3	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.
NHB2	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

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Reagent	Box Name	Instructions
PPC	Illumina DNA Fast Hyb - Enrichment PCR + Buffers	If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, thaw at room temperature. Otherwise, bring to room temperature for 30 minutes before the NF-HYB thermal cycler program ends, and vortex to resuspend.

Hybridize Probes

This step binds targeted regions of the DNA with the capture probes.

This protocol requires half the probe volume as previous Illumina enrichment protocols.

Illumina DNA Prep with Enrichment reagents are compatible with both Illumina and third-party enrichment DNA oligonucleotide panels. For information the required specifications for third-party panels, refer to *Third-Party Panel Requirements* on page 7.

Probe Panel Hybridization and Wash Temperatures

Recommended temperatures for hybridization and post-hybridization wash steps vary according to the panel, sample type, and application.

Panel / Sample Type	Recommended Temperature for Hybridization / Post-Hybridization Wash
FFPE samples	58°C
Somatic variant calling	58°C
TruSight Hereditary Cancer[1]	58°C
Illumina Exome Panel (CEX)	58°C
All other panels[2]	62°C

[1]For the TruSight Hereditary Cancer panel, changing the wash and hybridization temperatures (58°C or 62°C) during target enrichment may impact sensitivity, variant allele frequency calling, and capture of CNV regions (refer to User-definable parameters in the Illumina DNA Prep with Enrichment workflow and Analyze germline CNVs with TruSight™ Hereditary Cancer Panel on the Illumina support site). The hybridization and wash temperatures may impact other aspects of performance, such as padded unique read enrichment and AT dropout.

[2]Illumina DNA Prep with Enrichment reagents and protocol are highly optimized to provide the most robust on-target enrichment solution. However, in some use cases and with some panels, users may want to adjust select parameters to improve coverage of challenging sequences, such as targeted

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sequencing of GC- and AT-rich content or to address poor-quality samples, such as FFPE tissue. Any adjustments to the optimized protocols should be carefully considered as they can impact performance and available support options.

Consumables

- One of the following enrichment probe panels:
  - TruSight Cancer
  - TruSight Hereditary Cancer
  - Illumina Exome Panel (CEX)
  - Illumina Custom Enrichment Panel – 80-mer, single-stranded oligos.
  - Illumina Custom Enrichment Panel v2 – 120-bp, double-stranded oligos.
  - Third-party panels (that meet requirements in *Third-Party Panel Requirements* on page 7).
- EHB2 (Enrichment Hyb Buffer 2)
- NHB2 (Hyb Buffer 2 + IDT NXT Blockers)
- RSB(Resuspension Buffer)
- One of the following containers:
  - **[Plate]** 96-well PCR plate
  - **[Tube]** 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps

About Reagents

- NHB2 precipitates and separates during storage. Follow the NHB2 preparation instructions before first use.
- Enrichment probe panel refers to the chosen enrichment oligonucleotide panel from Illumina or a third-party vendor to run with this workflow.

Preparation

1. Choose your appropriate enrichment probe panel, and then prepare the following consumables:
   - Enrichment Probe Panel (one of the following):
     - TruSight Cancer—Vortex to mix. Use a total probe volume of 10 µl per hybridization reaction.

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  - TruSight Hereditary Cancer—Vortex to mix. Use a total probe volume of 10 µl per hybridization reaction.
  - Illumina Exome Panel (CEX)—Vortex to mix. Use a total probe volume of 10 µl per hybridization reaction.
  - Illumina Custom Enrichment Panel—Vortex to mix. Use a total probe volume of 10 µl per hybridization reaction.
  - Illumina Custom Enrichment Panel v2—Vortex to mix. Add 4 µl Illumina Custom Enrichment Panel v2, to 6 µl RSB, for a total volume of 10 µl per enrichment reaction to use with this protocol per hybridization reaction.
  - Third-party panel (that meets requirements in *Third-Party Panel Requirements* on page 7)—Use the vendor-recommended probe volume per reaction for the third party enrichment assay. Add RSB, if needed, for a total volume of 10 µl per enrichment reaction, regardless of the enrichment plexity.
- EHB2:
  a. Vortex to mix.
  b. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
- NHB2:
  a. When at room temperature, preheat to 50°C on a microheating system for 5 minutes.
  b. Vortex at maximum speed 3 times for 10 seconds each to resuspend.
  c. Centrifuge briefly.
  d. Pipette up and down from the bottom of the tube. If crystals and cloudiness are observed, repeat vortex, or pipette up and down to mix well until the solution is clear.
     Use while warm to prevent precipitate from reforming.
- RSB—Vortex to mix.
- SMB4—If you are proceeding to the next procedure immediately after the 90 minute hold in the NF-HYB program, bring to room temperature. If you are extending the hold time, bring to room temperature at least 30 minutes before the NF-HYB program ends.

2. Save the following NF-HYB program on the thermal cyclerusing the appropriate number of cycles, which are listed in the Table 11 table.
   - Choose the preheat lid option and set to 100°C
   - Reaction volume is 50 µl
   - 95°C for 5 minutes
   - X cycles of 1 minute each, starting at 94°C for the first cycle, then decreasing 2°C per cycle
   - Hold for 90 minutes at the applicable temperature (refer to *Probe Panel Hybridization and Wash Temperatures* on page 42).

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If optimizing percent duplicates for third-party panels is needed, increasing the 90 minute hold time to 2.5–16 hours might improve performance. Refer to example data sets with increased hybridization times in BaseSpace Sequence Hub Public Data.

- [Optional] Extend the hold to a maximum of 24 hours.

Total running time is ~115 minutes.

Table 11 Cycle Number per Sample or Panel

Sample and Panel Type	Number of Cycles (X)
FFPE (regardless of panel type)	18
Illumina Exome Panel (CEX) (regardless of sample type)	18
Somatic variant calling	18
TruSight Hereditary Cancer	16
All other samples and panels	16

Procedure

1. Add the following volumes to each well of a new PCR plate or 8-tube strip *in the order listed*. Creating a master mix of NHB2, and EHB2, negatively impacts enrichment performance.

Table 12 Illumina Fixed and Custom Enrichment Panel

Item	Volume
Pre-enriched library sample or pool	30 µl
NHB2	50 µl
Custom enrichment probe panel	10 µl
EHB2	10 µl
Total volume	100 µl

Table 13 Illumina Custom Enrichment Panel v2

Item	Volume
Pre-enriched library sample or pool	30 µl
NHB2	50 µl
Custom enrichment probe panel	4 µl
Optional: Supplemental Probe Panel (in place of RSB)	4 µl
EHB2	10 µl
RSB	2-6 µl
Total volume	100 µl

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2. Using a pipette set to 90 µl, pipette each well 10 times to mix.
3. Centrifuge as follows.
   - [Plate] Seal the plate with Microseal 'B' and centrifuge at 280 × g for 30 seconds.
   - [Tube] Cap the tubes and centrifuge at 280 × g for 30 seconds.
4. Place the sample plate or tube on the preprogrammed thermal cycler and run the NF-HYB program.
5. Proceed immediately to the next procedure before the NF-HYB program hold temperature time ends.

Do not allow the hybridization reactions to cool. Precipitation occurs if the temperature of the hybridization reaction falls below room temperature.

Capture Hybridized Probes

This step uses magnetic beads to capture probes hybridized to the target regions of interest within the libraries. Heated washes remove nonspecific binding from the beads. The enriched library is then eluted from the beads.

Consumables

- EE1 (Enrichment Elution Buffer 1)
- EEW (Enhanced Enrichment Wash)
- ET2 (Elute Target Buffer 2)
- HP3 (2N NaOH)
- SMB4 (Streptavidin Magnetic Beads 4)
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 1.7 ml microcentrifuge tubes and 8-tube strip
- One of the following seals:
  - **[Plate]** Microseal 'B' adhesive seal
  - **[Tube]** 8-tube strip caps
- One of the following magnetic stands:
  - **[Plate]** Magnetic Stand-96
  - **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml)

About Reagents

- EEW

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  - Can be cloudy after reaching room temperature.
  - Can appear yellow.
  - Heat before use as instructed.
- SMB4
  - Make sure to use SMB4 and not Illumina Purification Beads for this procedure.
  - Must be at room temperature before use.

Preparation

1. Prepare the following consumables:
   - EE1—Pipette to mix. Centrifuge briefly before use.
   - EEW—Vortex three times for 30 seconds each. The reagent is heated during the procedure.
   - ET2—Vortex to mix. Centrifuge briefly before use.
   - HP3—Vortex to mix. Centrifuge briefly before use.
   - SMB4—Vortex to resuspend. If precipitate or the bead pellet is present, make sure to reach room temperature, pipette up and down to release the pellet, and then vortex to resuspend.
2. Preheat a minimum of one microheating system with a MIDI heat block insert to incubate the sample plate to the applicable temperature (refer to *Probe Panel Hybridization and Wash Temperatures* on page 42). An optional second microheating system can be used to preheat EEW.

Procedure

Capture

1. Centrifuge the sample plate or tube at 280 × g for 30 seconds.
2. Using a pipette set to 100 µl, transfer each sample from the 96-well PCR plate or from the 8-strip tube, to the corresponding well of a new MIDI plate or to a new 1.7 ml microcentrifuge tube.
3. Vortex SMB4 to resuspend, and then add 250 µl to each well or tube, and then mix thoroughly as follows:
   - **[Plate]** Seal the plate and shake at 1200 rpm for 4 minutes.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 15 minutes at the applicable temperature (refer to *Probe Panel Hybridization and Wash Temperatures* on page 42).
   Proceed to step 5 while the sample incubates.

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5. Preheat EEW (amber tube) by laying the tube on its side on the MIDI heat block insert on the second microheating system to the following temperature. If a second microheating system is not available, lay EEW on top of the MIDI plate or next to the 1.7 ml microcentrifuge tube on the MIDI heat block insert during the incubation in step 4. Keep EEW heated until step 2 of the *Wash on page 48*.
6. Centrifuge the sample plate or tube at 280 × g for 30 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Using a pipette set to 350 µl, remove and discard all supernatant from each well or tube.

Wash

1. Remove from the magnetic stand.
2. Add 200 µl preheated EEW (amber tube) to each well or microcentrifuge tube, and then mix thoroughly as follows.
    - **[Plate]** Seal and shake at 1800 rpm for 4 minutes. If splashing occurs, reduce the speed to 1600 rpm.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
3. Return unused EEW to the microheating system and keep heated.
4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 5 minutes at the applicable temperature (refer to *Probe Panel Hybridization and Wash Temperatures* on page 42).
5. Centrifuge at 280 × g for 3 seconds.
6. Immediately place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.
8. Repeat steps 1–7 two additional times for a total of three washes.

Transfer Wash

1. Remove the plate or tube from the magnetic stand.
2. Add 200 µl preheated EEW (amber tube) to each well or microcentrifuge tube, and then mix thoroughly as follows.
    - **[Plate]** Seal and shake at 1800 rpm for 4 minutes. If splashing occurs, reduce the speed to 1600 rpm.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each. Do not centrifuge.
3. Transfer 200 µl to a new MIDI plate or a new tube.

Transferring the reagent minimizes carryover of residual reagents that can inhibit downstream PCR.

4. Place the sample plate or tube on the MIDI heat block insert on the microheating system, close the lid, and incubate for 5 minutes at the applicable temperature (refer to *Probe Panel Hybridization and Wash Temperatures* on page 42).
5. Centrifuge at 280 × g for 3 seconds.
6. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Using a pipette set to 200 µl, remove and discard all supernatant from each well or tube.
8. Centrifuge the plate or the tube at 280 × g for 30 seconds.
9. Place on a magnetic stand for 10 seconds.
10. Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.
11. Immediately proceed to *Elute* on page 49 to prevent excessive drying of the beads and library yield loss.

Elute

1. Combine the following volumes to prepare an Elution Master Mix. Multiply each volume by the number of samples being processed.
   Additional reagent is included in the volume to ensure accurate pipetting due to the potential of reagent foaming.
   - EE1 (28.5 µl)
   - HP3 (1.5 µl)
2. Vortex, and then centrifuge the master mix at 280 × g for 10 seconds.
3. Remove the sample plate or tube from the magnetic stand.
4. Add 23 µl Elution Master Mix to each well or tube, and then mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1800 rpm for 2 minutes.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 2 minutes.
6. Centrifuge at 280 × g for 30 seconds.
7. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
8. Transfer 21 µl supernatant from the MIDI plate or 1.5 ml microcentrifuge tube to the corresponding well of a new 96-well PCR plate or a new 8-tube strip.
9. Add 4 µl ET2 to each well or to the tube containing 21 µl supernatant.
10. Set pipette to 20 µl and slowly pipette each well or the tube 10 times to mix.
11. Centrifuge the sample plate or the tube at 280 × g for 30 seconds.

Amplify Enriched Library

This step uses PCR to amplify the targeted IDPE library.

Consumables

- EPM (Enhanced PCR Mix)
- PPC (PCR Primer Cocktail)
- **[Plate]** Microseal 'B' adhesive seal
- **[Tube]** 8-tube strip caps

Preparation

1. Prepare the following consumables:
   - EPM—Invert to mix, then centrifuge briefly.
   - PPC—Invert to mix, then centrifuge briefly.
2. Save the following AMP program on the thermal cycler using the appropriate number of PCR cycles, which are listed in the following table.
   - Choose the preheat lid option and set to 100°C
   - Set the reaction volume to 50 µl
   - 98°C for 30 seconds
   - (X) cycles of:
     - 98°C for 10 seconds
     - 60°C for 30 seconds
     - 72°C for 30 seconds
   - 72°C for 5 minutes
   - Hold at 10°C

   Total running time is ~35 minutes.

Panel Type	(X) Cycles
Illumina Exome Panel (CEX)	10
All other panels	12[1, 2, 3]

[1] Can be adjusted up to 15 cycles for small third-party panels through subsequent optimization.
[2] Can be adjusted up to 17 cycles for third-party panels that only have 500 probes.
[3] Increasing the number of PCR cycles might result in a higher duplicate rate and smaller fragment sizes for FFPE samples.

Procedure

1. Add 5 µl PPC to each well or tube.

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2. Add 20 µl EPM to each well or tube and mix thoroughly as follows.
   - **[Plate]** Seal plate and shake at 1200 rpm for 1 minute.
   - **[Tube]** Pipette 10 times to mix, and then cap the 8-tube strip.
3. Centrifuge the plate or 8-tube strip at 280 × g for 30 seconds.
4. Place on the preprogrammed thermal cycler and run the AMP program.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to two days. Alternatively, leave on the thermal cycler for up to 24 hours.

Prepare for Protocol

1. Remove reagents from storage.
2. Remove the reagents from the box and prepare as follows.

Table 14 15°C to 30°C Storage

Reagent	Box Name	Instructions
IPB	IPB	Bring to room temperature.

Table 15 2°C to 8°C Storage

Reagent	Box Name	Instructions
RSB	Illumina DNA Fast Hyb - Enrichment Beads + Buffers	Bring to room temperature.

Clean Up Amplified Enriched Library

This step uses IPB to purify the enriched library pools and remove unwanted products.

Consumables

- IPB (Illumina Purification Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (EtOH)
- **[Plate]** Microseal 'B' adhesive seals
- One of the following containers:
  - **[Plate]** 96-well MIDI plate and 96-well PCR plate
  - **[Tube]** 1.7ml tube
- One of the following magnetic stands:

- **[Plate]** Magnetic Stand-96
- **[Tube]** MagneSphere Technology Magnetic Separation Stands (12 position, 1.7 ml)

About Reagents

- IPB
  - Must be at room temperature before use.
  - Resuspend before each use.
  - Resuspend frequently to make sure the beads are evenly distributed.
  - Aspirate and dispense slowly due to the viscosity of the solution.

Preparation

1. RSB—Vortex to mix.
2. For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

Procedure

1. Centrifuge the PCR samples at 280 × g for 30 seconds.
2. Transfer 50 µl supernatant from each well of the PCR plate or from the 8-tube strip, to the corresponding well of a new MIDI plate or a new 1.7 ml microcentrifuge tube.
3. Resuspend IPB as follows.
   a. To mix, invert the bottle manually for 2 minutes, at a rate of 1 inversion per second. While inverting, rotate the bottle 90 degrees every 30 seconds.
   b. If beads are still adhered to the walls of the container, invert the bottle manually for an additional 1 minute.
4. Add 45 µl IPB to each well or tube, and then mix thoroughly as follows.
   - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
   - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
5. Incubate the plate or tube at room temperature for 5 minutes.
6. Centrifuge at 280 × g for 1 minute.
7. Place on a magnetic stand and wait until liquid is clear (~5 minutes).
8. Using a pipette set to 95 µl, remove and discard all supernatant from each well or tube.
9. Wash beads as follows.
   a. Keep on the magnetic stand, add 200 µl fresh 80% EtOH without mixing.
   b. Wait for 30 seconds.
   c. Without disturbing the beads, remove and discard supernatant.

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10. Wash beads a **second** time.
11. Use a 20 µl pipette to remove and discard residual EtOH from each well or tube.
12. Air-dry on the magnetic stand for 5 minutes.
13. Remove from the magnetic stand and add 32 µl RSB to each well or tube.
14. Mix thoroughly as follows.
    - **[Plate]** Seal the plate and shake at 1800 rpm for 1 minute.
    - **[Tube]** Cap the tube, and then vortex at high speed three times for 10 seconds each.
15. Incubate the plate or tube at room temperature for 5 minutes.
16. Centrifuge at 280 × g for 30 seconds.
17. Place on a magnetic stand and wait until liquid is clear (~2 minutes).
18. Transfer 30 µl supernatant from the 96-well PCR plate or from the 8-tube strip, to the corresponding well of a new 96-well PCR plate or a new 1.7 ml microcentrifuge tube.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive seal or Microseal 'F' foil seal, or cap the tube, and store at -25°C to -15°C for up to 7 days.

Check Enriched Libraries

Perform the following to check the concentration and quality of the enriched libraries.

1. Quantify 1 µl enriched libraries to determine library concentration. Follow the manufacturer's recommendations.
   Expect post-enrichment library yields of ≥ 3 ng/µl. Library yields might be less for small panels.

   Total probe molarity proportionally impacts the post-enrichment library yield. Probe panels from third-party vendors might produce proportionally lower enriched library yields. However, sequencing metrics are still expected to meet specification.

2. Run 1 µl pooled library or the individual libraries on a Bioanalyzer using a high sensitivity DNA kit. Expect a mean fragment size ~350 bp and distribution of DNA fragments with a size range from ~200 bp to ~1000 bp.
3. **[Optional]** Add 1 µl RSB to 1 µl pooled or individual libraries, then run 1 µl of the pooled volume on the TapeStation using theAgilent D5000 Screen Tape and Reagents.

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Figure 3 Bioanalyzer Trace: Example 1

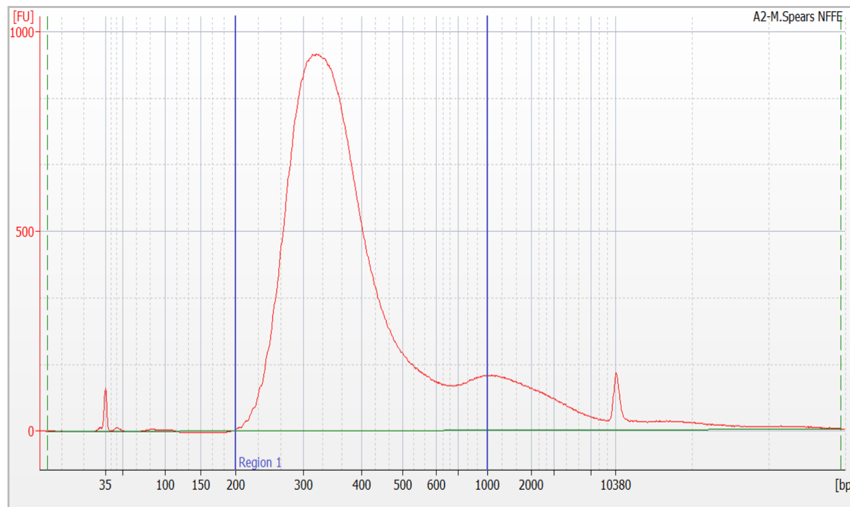
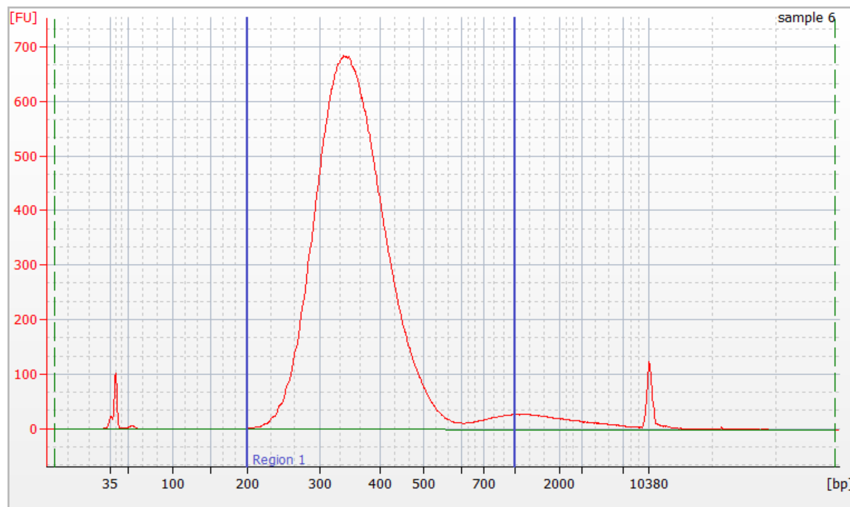


Figure 4 Bioanalyzer Trace: Example 2



TapeStation Trace: Example

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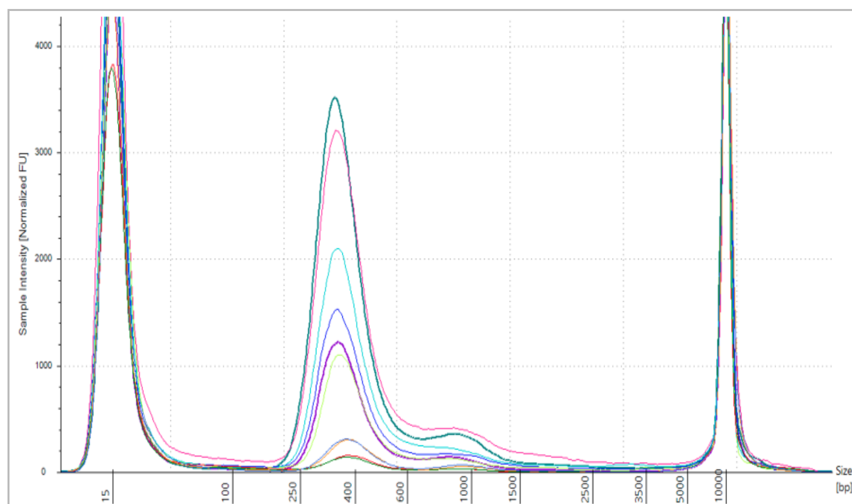

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, regardless of the enrichment probe panel you are using, Illumina recommends setting up a paired-end run with 101 cycles per read (2 × 101) and 10 cycles per Index Read. If you want optional and additional overlapped reads/raw coverage, you can sequence up to 2 × 126 or 2 × 151.

1. Calculate the molarity value of the library or pooled libraries using the following formula.
   - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
   - For all other qualification methods, use 350 bp as the average library size.

$$\frac{\text{concentration in ng}/\mu\text{l}}{660\ \text{g/mol} \times \text{average library size in bp}} \times 10^6 = \text{Molarity (nM)}$$

2. Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
iSeq 100 (v1 reagents)	2	100
iSeq 100 (v2 reagents)	2	75
MiniSeq	2	1.7–1.8
MiSeq (v3 reagents)	4	10–12
MiSeq i100*	0.6	60
NextSeq 550 and NextSeq 500	2	1.4–1.5
NextSeq 1000 and NextSeq 2000	2	1000

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Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
NovaSeq 6000	0.875–0.925	175–185
NovaSeq X	2	150

* Denaturation performed onboard. Refer to the system guide.

3. Dilute libraries using RSB:
   - **Libraries quantified as a multiplexed library pool—**Dilute the pool to the starting concentration for your system.
   - **Libraries quantified individually—**Dilute each library to the starting concentration for your system.
     Add 10 µl each diluted library to a tube to create a multiplexed library pool.
4. Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
   - For the iSeq 100 Sequencing System, refer to the system guide for dilution instructions (libraries are automatically denatured).
   - Refer to the Illumina Denature and Dilute protocol generator and the Illumina support site for pool and denature instructions.
   - The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.

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Resources & References

The support pages on the Illumina website provide software, training resources, product compatibility information, and the following documentation. Always check support pages for the latest versions.

Additional Resources

Resource	Description
Index Adapters Pooling Guide	Provides pooling guidelines and dual-index strategies for using the 10-base pair Illumina DNA/RNA UD Indexes.
Illumina Adapter Sequences	Provides the nucleotide sequences that comprise Illumina oligonucleotides used in Illumina sequencing technologies.
Infinium HD FFPE QC Assay Protocol (document # 15020981)	Provides the protocol to assess DNA input quality for FFPE samples.

Revision History

Document	Date	Description of Change
# 1000000048041 v10	October 2025	Changed SMB3 to SMB4. Updated SMB4 thaw and preparation instructions. Updated thermal cyclers. Added optional Concentrate Pooled Libraries section. Added MiSeq i100 and NovaSeq X.
# 1000000048041 v09	June 2024	Corrected protocol details. Updated equipment. Updated layout and format. Removed reference to Custom Protocol Selector. Removed IPB bead resuspension, TWB handling, and Index preparation sections from tips and techniques, and added each to their appropriate protocol steps. Moved text out of Tips and Techniques, and into relevant protocol steps. Added tape station choices.

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Document	Date	Description of Change
# 1000000048041 v08	March 2024	Converted to HTML format. Corrected additional resource documentation and moved to resource and reference section. Removed list of Acronyms. Removed option for additional reagents. Removed symbol definition table for HTML conversion. Reorganized sections to be consistent with all library prep documentation. Added custom enrichment probe panel options. Added references to DesignStudio assay designer tool. Added enrichment probe panels. Added probe panel hybridization and wash temperature recommendations. Added HiSeq X, NextSeq 1000/2000, and NovaSeq X instruments. Updated Illumina-supplied index adapters.
# 1000000048041 v07	August 2021	Add IPB bead resuspension section to tips and techniques. Clarified the average library size for qualification method other than Bioanalyzer. Replaced AMPure XP with IPB Bead.
# 1000000048041 v06	April 2021	Changed SMB to SMB3.
# 1000000048041 v05	June 2020	Added dilution information for the NextSeq 2000 Sequencing System. Added information for IDT for Illumina Nextera Indexes.
# 1000000048041 v04	April 2020	Changed product name from Nextera Flex for Enrichment to Illumina DNA Prep with Enrichment. Renamed index and reagent kits. Updated catalog numbers for UD Index kits sets C and D. Removed obsolete UD Indexes set A-D kit option.

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Document	Date	Description of Change
# 1000000048041 v03	November 2019	Added Illumina catalog # 20029551 for the TruSight Hereditary Cancer panel. Added TruSight Hereditary Cancer (TSCH) to the acronyms list. Added a chemical warning with a link to the safety data sheet (SDS) to the tagment genomic DNA step. Replaced the reagent Hyb Buffer + IDT NXT Blockers (NHB1) with Hyb Buffer 2 + IDT NXT Blockers (NHB2). Clarified that low minor allele frequency detection when using one-plex enrichment is for germline or somatic variant calling. Removed inaccurate references to Nextera XT v2 and Nextera XT indexes.
# 1000000048041 v02	May 2019	Added information on IDT for Illumina Nextera UD Indexes. Updated language and relocated information previously in Pool Pre-Enriched Libraries to new sections in protocol introduction. Updated documentation introduction to provide more concise information on the Nextera Flex for Enrichment workflow. Updated the Additional Resources section to provide new language and removed extraneous resources. Updated language in Prepare for Pooling to provide more consistent organization.

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Document	Date	Description of Change
# 1000000048041 v01	January 2019	Added a recommended method to quantify FFPE reference materials. Added a hybridization temperature for somatic variant calling. Added symbol descriptions for packaging and consumables. Updated and relocated the recommended gDNA input quantification to the gDNA input < 50 ng section. Updated the sample input recommendations for genomic DNA and extracted FFPE. Updated the final loading concentration recommendations for MiSeq and NovaSeq.
# 1000000048041 v00	October 2018	Initial release.

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