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WES Library Prep Workshop for Students on Illumina NextSeq 550Dx V.3

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We use this protocol and it's working

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Disclaimer

This protocol is based on the guidelines provided in the Illumina DNA Prep with Exome 2.5 Enrichment manual. For detailed instructions, reagent specifications, and troubleshooting, please refer to the official Illumina manual. Adjustments and optimizations may be necessary depending on specific laboratory conditions and experimental needs.

Abstract

This protocol outlines the key wet-lab steps for preparing genomic DNA for Whole Exome Sequencing (WES) using the Illumina NextSeq 550Dx. The protocol is specifically designed for medical students participating in a workshop, focusing on mastering the practical techniques of library preparation, exome capture, and DNA sequencing.

Guidelines

This protocol outlines the key wet-lab steps for preparing genomic DNA for Whole Exome Sequencing (WES) using the Illumina NextSeq 550Dx. The protocol is specifically designed for medical students participating in a workshop, focusing on mastering the practical techniques of library preparation, exome capture, and DNA sequencing.

Materials

1. Ethanol, Pure (Sigma-Aldrich, Germany, Cat. # E7023)
2. 2-Propanol (Sigma-Aldrich, Germany, Cat. # I8912)
3. Phosphate Buffered Saline (GibcoTM- Thermo Fisher Scientific, UK, Cat. # 10010023)
4. Ultrapure™ DNase/RNase Free Distilled Water (Invitrogen, USA Cat. # 10977-015)
5. NextSeq 500/550 High Output Kit v2.5 (300 Cycles)(Illuminna, USA Cat. # 20024908)
6. Illumina DNA Prep with Exome 2.5 Enrichment, (S) Tagmentation set B(96 Samples, 12 plex) (Illuminna, USA Cat. # 20077595)
7. Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 indexes, 96 Samples) (Illuminna, USA Cat. # 20091654).
8. Qubit™ dsDNA HS (High Sensitivity) Assay Kit (Invitrogen, USA, Cat. # Q32851)
9. Agencourt® AMPure XP beads (Beckman Coulter, USA, Cat. # A63880

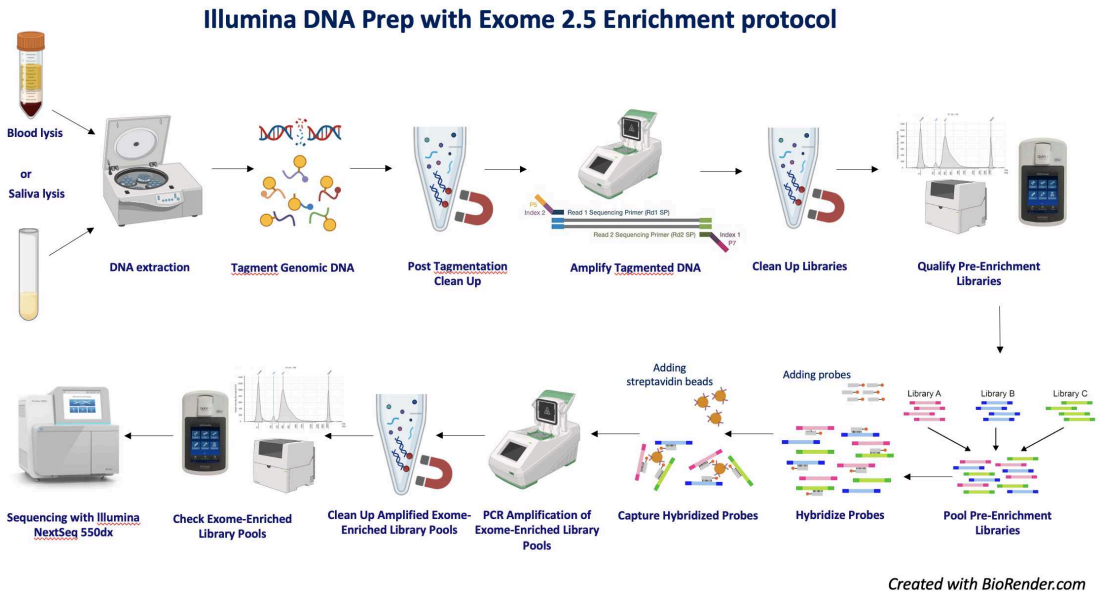
Safety warnings

Chemical Safety Warning: Take proper precautions, and wear appropriate PPE when handling potentially hazardous chemicals. Ensure that chemicals, spent containers, and unused contents are disposed of in accordance with governmental safety standards.

Protocol Overview

1 This protocol outlines the key wet-lab steps for preparing genomic DNA for Whole Exome Sequencing (WES) using the Illumina NextSeq 550Dx. The protocol is specifically designed for medical students participating in a workshop, focusing on mastering the practical techniques of library preparation, exome capture, and DNA sequencing.

2



Learning Objectives

3 Understand the core steps in Whole Exome Sequencing (WES) library preparation.
Learn how to perform targeted enrichment of exonic regions using capture probes.
Prepare and load sequencing-ready libraries on the NextSeq 550Dx.
Gain hands-on experience with DNA quantification, fragmentation, and clean-up techniques.

Materials Needed

4 Completing the Illumina DNA Prep with Exome 2.5 Enrichment protocol requires library prep and enrichment reagents, the exome panel, clean up/size selection beads, and index adapters. In order to carry out the WES workflow effectively, the following materials are required:

4.1 **Reagents**

1. Ethanol, Pure (Sigma-Aldrich, Germany, Cat. # E7023)
2. 2-Propanol (Sigma-Aldrich, Germany, Cat. # I8912)
3. Phosphate Buffered Saline (GibcoTM- Thermo Fisher Scientific, UK, Cat. # 10010023)
4. Ultrapure™ DNase/RNase Free Distilled Water (Invitrogen, USA Cat. # 10977-015)
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8. Qubit™ dsDNA HS (High Sensitivity) Assay Kit (Invitrogen, USA, Cat. # Q32851)
9. Agencourt® AMPure XP beads (Beckman Coulter, USA, Cat. # A63880)

4.2 **Equipment**

Equipment	
NextSeq 550Dx	NAME
Illumina	BRAND
20005715	SKU
https://www.illumina.com/systems/ivd-instruments/nextseq-dx.html	LINK

Equipment

new equipment	NAME
Qubit 2.0 Fluorometer instrument	BRAND
Q33226	SKU
with Qubit RNA HS Assays	SPECIFICATIONS

Equipment

C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module	NAME
Thermal Cycler	TYPE
BioRad	BRAND
1851197	SKU
https://www.bio-rad.com/en-us/sku/1851197-c1000-touch-thermal-cycler-with-96-ndash-deep-well-reaction-module?ID=1851197	LINK

Equipment

Magnetic Stand-96	NAME
ThermoFisher Scientific	BRAND
AM10027	SKU
https://www.thermofisher.com/order/catalog/product/AM10027	LINK

Equipment

4200 TapeStation System	NAME
Electrophoresis tool for DNA and RNA sample quality control.	TYPE
TapeStation Instruments	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263	LINK

4.3 **Recipes**

- Qubit working solution: dilute Qubit dsDNA HS Reagent 1:200 in Qubit dsDNA HS buffer. For n samples, prepare n × 200 µl working solution.
- 80% ethanol: add 2 ml absolute ethanol into 8 ml distilled water.
- 0.2 M NaOH: weigh 0.04 g of NaOH pellet and dissolve it into 5 ml distilled water.

4.4 **Glossary**

Enrichment Bead-Linked Transposomes (Enrichment BLT, eBLT)
Tagmentation Wash Buffer (TWB)
TB1 (Tagmentation Buffer 1)
TAG program (Tagmentation program)
ST2 (Stop Tagment Buffer 2)
TWB (Tagmentation Wash Buffer)
EPM (Enhanced PCR Mix)
IPB (Illumina Purification Beads)
RSB (Resuspension Buffer)
EtOH (Freshly prepared 80% ethanol)
EHB2 (Enrich Hyb Buffer 2)
NHB2 (Hyb Buffer 2 + IDT NXT Blockers)
EE1 (Enrichment Elution Buffer 1)
EEW (Enhanced Enrichment Wash)
ET2 (Elute Target Buffer 2)
HP3 (2 N NaOH)
SMB3 (Streptavidin Magnetic Beads)
EPM (Enhanced PCR Mix)
PPC (PCR Primer Cocktail)

Step-by-Step Protocol with Objectives

10s

5

By following this protocol, students will gain a comprehensive understanding of the experimental workflow involved in WES, which is a crucial tool for identifying genetic variants in disease-related genes.

Note

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

5.1 **Purpose of the Tagment Genomic DNA Step:**

1. **Fragmentation and Tagging**: the input DNA is fragmented by eBLT, which simultaneously adds adapter sequences to the ends of the DNA fragments. This dual action enables efficient preparation for library construction and subsequent enrichment.
2. **Preparation for Sequencing:** by tagging the DNA with adapters, the fragments are ready for amplification and sequencing, ensuring compatibility with the sequencing platform.

5.2 Prepare the following consumables:

Item	Storage	Instructions
eBLT (yellow cap)	2°C to 8° C	Bring to room temperature by incubating at room temperature for 10 minutes. Vortex to mix. Do not centrifuge before pipetting.
TB1	-25°C to -15°C	Bring to room temperature. Vortex to mix.

5.3 Add 2-30 µL DNA to each well of a 96-well PCR plate so that the total input amount is 50-1000 ng

Note

If the DNA volume is < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.

5.4 Vortex eBLT (yellow cap) vigorously for 00:00:10 to resuspend. Repeat as necessary. 10s

5.5 Combine 11.5 µL eBLT and 11.5 µL TB1 to prepare the Tagmentation Master Mix, vortex the Tagmentation Master Mix thoroughly to resuspend and transfer

20 µL Tagmentation Master Mix to each well of the plate or tube.

Note

Multiply each volume by the number of samples being processed. These volumes produce 23 µl Tagmentation Master Mix per sample, which includes extra volume for accurate pipetting.

5.6 Using a 200 µl multichannel pipette set to 40 µL, pipette 10 times to mix Tagmentation Master Mix and gDNA sample, and then seal the plate. Alternatively, seal the plate and shake at 1600 rpm for 00:01:00 1m

6 **PCR - the TAG Program**

6.1 Place on the preprogrammed thermal cycler and run the TAG program. Choose the preheat lid option and set to 100 °C. Set the reaction volume to 50 µL. 55 °C for 00:05:00 and hold at 10 °C. 5m

Note

Wait until the TAG program has reached the 10°C hold temperature before removing the plate and proceeding.

6.2 **Purpose of the Post Tagmentation Clean Up Step:**

1. Washing the Adapter-Tagged DNA: After the DNA is tagmented (fragmented and tagged with adapters), the next step is to thoroughly wash the tagged DNA using **Tagmentation Wash Buffer (TWB)**.
This removes any excess reagents and contaminants, ensuring the DNA is clean and ready for amplification.
2. Magnetic Bead Separation: Magnetic beads are used to immobilize the DNA during the washing steps. A **magnetic stand** is employed to hold the beads in place while the washing buffer is added and excess supernatant is removed. This helps isolate the DNA without losing it during the washing process.

3. Resuspension and Washing: Multiple washing steps are performed to ensure the DNA is properly cleaned. **TWB** is added directly to the beads in three separate washes. During each wash, the
beads are thoroughly resuspended either by pipetting or shaking to ensure that all DNA is exposed to the wash buffer.
4. Preventing Overdrying: A key aspect of this step is to ensure that the beads do not overdry. **TWB**
remains in the wells until the next step (Amplify Tagmented DNA), which prevents the beads from drying out and keeps the DNA in optimal condition for amplification.

6.3 Prepare the following consumables:

Item	Storage	Instructions
ST2 (red cap)	15° to 30° C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until the precipitates are dissolved.
TWB	15° to 30° C	Vortex to mix.

6.4 Add 10 µL ST2 (red cap) to each well of the plate and slowly pipette each well 10 times to resuspend the beads using a 200 µl pipette set to 50 µL, seal and then incubate at Room temperature for 00:05:00. 5m

> Note
>
> Let the 96-well PCR plate stand at room temperature for 2 minutes before procediing with this step!

6.5 **Wash as follows for < 48 samples:** 3m
Place the plate on the magnetic stand and wait until the liquid is clear (00:03:00 aprox), then remove and discard supernatant with a pipette set to 60 µL.

6.6 Remove from the magnetic stand and use a deliberately slow pipetting technique to add 100 µL TWB directly onto the beads, and pipette until the beads are fully resuspended

Note

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

6.7 Place the plate on the magnetic stand and wait until the liquid is clear (00:03:00 aprox.). 3m

6.8 Remove and discard supernatant with a pipette set to 100 µL .

6.9 *Repeat steps two times for a total of three washes.* 5.6

6.10 3m

Pipette each well slowly to resuspend the beads. Seal the plate and place on the magnetic stand until the liquid is clear (~ 00:03:00). Keep on the magnetic stand until step **4** of the Procedure section in ***Amplify Tagmented DNA***. go to step #6.16 to see (blod text).

Note

TWB remains in the wells to prevent overdrying of the beads.

6.11 **C. Amplify Tagmented DNA**

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds prepaired 10 base pair Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for library clustering on the flow cells.

Reagents:

1. EPM (Enhanced PCR Mix)
2. Index adapter plate
3. Nuclease-free water

6.12 **Purpose of the Amplify Tagmented DNA Step**

Amplification: The tagmented DNA is amplified to increase the amount of DNA for sequencing.
This ensures that sufficient material is available for further processing.
Addition of Index Adapters: Prepaired 10 base pair Index 1 (i7) and Index 2 (i5) adapters are added during
amplification, which are essential for distinguishing individual samples during sequencing.
Library Clustering Sequences: The necessary sequences for library clustering on the sequencing flow cells are incorporated during PCR, preparing the libraries for sequencing.

6.13 Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge briefly.
Index adapter plate	-25°C to -15°C	Thaw at room temperature, then keep on ice.

6.14 eBLT PCR program: 9m

Choose the preheat lid option and set to 100 °C. Set the reaction volume to 50 µL. 72 °C for 00:03:00. 98 °C for 00:03:00. (X) cycles of: (98°C for 20 seconds, 60°C for 30 seconds, 72°C for 1 minute) and 72 °C for 00:03:00. Hold at 10 °C.

	A	B
	72°C incubation, 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	

Note

The total running time is ~38 minutes for 9 cycles and ~46 minutes for 12 cycles.

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

6.15 For each sample, combine EPM (23 µL) with Nuclease-free water (23 µL) to prepare the PCR Master Mix, vortex, and then centrifuge the PCR Master Mix at 280 x g for 00:00:10 . 10s

6.16 With the plate on the magnetic stand, remove and discard supernatant with a pipette set to 100 µL , then **remove from the magnetic stand**, and immediately add 40 µL PCR Master Mix directly onto the beads in each sample well and pipette 10 times to mix until the beads are fully resuspended.

6.17 Seal the sample plate and centrifuge at 280 x g for 00:00:10 . Centrifuge the index adapter plate at 1000 × g for 00:01:00 . 1m 10s

6.18 **Prepare the index adapter plate**: [< 96 samples] Pierce the foil seal on the index adapter plate with a new pipette tip for each well. Pierce only the number of samples being processed. [96 samples] Align a new Low DNA binding 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.

6.19 Using a new pipette tip, add 10 µL prepaired Index 1 (i7) and Index 2 (i5) index adapters to each well, and then using a pipette set to 40 µL , pipette 10 times to mix,

and then seal the plate with Microseal 'B'.

6.20 Centrifuge at 280 x g for 00:00:30 and place on the preprogrammed thermal cycler and run the eBLT PCR program. 30s

Note

SAFE STOPPING POINT
if you are stopping, store at -25 °C to -15 °C for up to 30 days.

6.21 **D. Clean Up Libraries:** step in the Illumina DNA Prep with Exome 2.5 Enrichment protocol involves purifying the amplified and indexed libraries using a double-sided bead purification procedure. This process removes excess primers, nucleotides, and other contaminants, ensuring high-quality libraries ready for sequencing This step uses a double-sided bead purification procedure to purify the amplified and indexed libraries.

Reagents:
1. IPB (Illumina Purification Beads)
2. RSB (Resuspension Buffer)
3. EtOH (Freshly prepared 80% ethanol)
4. Nuclease-free water

Note

About Reagents: IPB must be at room temperature before use. Vortex before each use. Vortex frequently to make sure the beads are evenly distributed and aspirate and dispense slowly due to the viscosity of the solution.

6.22 Prepare the following consumables:

Item	Storage	Instructions
IPB	15°C to 30°C	Resuspend IPB beads.
RSB	2°C to 8°C	Bring to room temperature. Vortex to mix.

Note

For each sample, prepare 400 µl fresh 80% EtOH from absolute ethanol. Including an overage of 20% is recommended.

6.23 Use a plate shaker to shake the 96-well PCR plate at 1800 rpm for 00:01:00 and then place the plate on the magnetic stand and wait until the liquid is clear (00:01:00 aprox.). 2m

6.24 Transfer 45 µL supernatant from each well of the PCR plate to the corresponding well of a new MIDI plate and resuspend Illumina Purification Beads.

6.25 Add 77 µL nuclease-free water to each well-containing supernatant and add 88 µL IPB to each well-containing supernatant. Pipette each well 10 times to mix. Seal the plate and incubate at room temperature for 00:05:00 . 5m

6.26 Place on the magnetic stand and wait until the liquid is clear (~ 00:05:00). 5m

6.27 During incubation, vortex the IPB, and then add 20 µL to each well of a new MIDI plate.

6.28 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, and then pipette each well of the MIDI plate 10 times to mix.

Note

Discard the first plate.

6.29 Incubate at room temperature for 00:05:00, and place on the magnetic stand and wait until the liquid is clear (~ 00:05:00), and then without disturbing the beads, remove and discard all supernatant. 10m

6.30 ***Wash two times as follows:*** 30s
a. with the plate on the magnetic stand, add 200 µL fresh [M] 80 % (v/v) EtOH without mixing.
b. wait for 00:00:30.
c. without disturbing the beads, remove and discard supernatant.

6.31 Use a 20 µL pipette to remove and discard residual EtOH, and air-dry on the magnetic stand for 00:05:00, and then remove from the magnetic stand. 5m

6.32 Add 17 µL RSB to the beads, and seal the plate, and then use a plate shaker at 1800 rpm for 00:02:00. 2m

6.33 Incubate at room temperature for 00:02:00, place the plate on the magnetic stand and wait until the liquid is clear (~ 00:02:00). 4m

6.34 Transfer 15 µL supernatant to a new 96-well PCR plate.

Note

SAFE STOPPING POINT

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

6.35 **E. Qualify Pre-Enrichment Libraries**
It is recommended to check the quality or to qualify pre-enrichment libraries before proceeding to enrichment.
If you elect not to check pre-enrichment libraries, perform the following procedure instead to

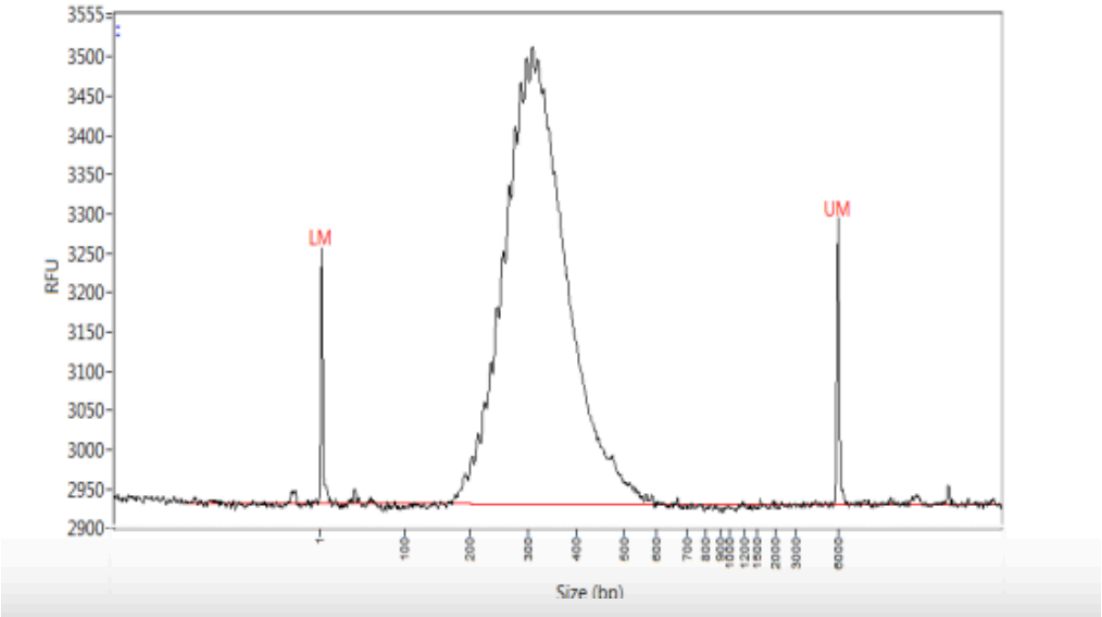
reserve samples for potential troubleshooting later.

Note

Qualify Pre-Enrichment Libraries: Pre-enrichment libraries can be qualified individually, one library at a time, or as a pool before enrichment. Perform the following procedure to qualify pre-enrichment libraries.

6.36 Analyze 1 µl library or pooled libraries using the Tape Station using a

Example Fragment Analyzer Trace



6.37 **F. Pool Pre-Enrichment Libraries**

This step combines DNA libraries with unique indexes into one pool of up to 12 libraries. Fewer pre-enrichment libraries may be pooled, but you may need to perform additional optimization. If using fewer pre-enrichment libraries, you cannot process the full 96 samples through enrichment, as only eight enrichment reactions are supported with this kit.

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

Recommended Pooling Methods

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

* If starting with ≥ 50 ng DNA input, the pre-enrichment library yields were normalized during tagmentation, which uses eBLT. This normalization enables you to pool equal volumes of each
pre-enrichment library in a final pool volume ≤ 30 µl (target 250–500 ng per sample).

6.38 **Quantify Pre-Enrichment Libraries**: After pre-enrichment library quantification, all sample input types can be pooled by mass to achieve optimal library balance and a similar number of sequencing reads per library.
Determine library concentration (ng/µl) by proceeding as follows:
1. Quantify 1 µL of each pre-enrichment library using the Qubit dsDNA BR Assay Kit to determine library concentration (ng/µl). Expect the following pre-enrichment library yield based on sample type and input.

Sample Input Type (ng)	Pre-enrichment Library Yield (ng)
10–49 gDNA	≥ 100
50–1000 gDNA, blood, saliva	≥ 500

Concentration results may differ depending on the quantification method used. The Qubit dsDNA BR Assay is recommended, but validation will be needed when using an alternative method.

6.39 **Poll by Volume**:
When the input is 50–1000 ng gDNA, quantifying and normalizing individual libraries generated in the same experiment is not required.
For a standard 12-plex pool: Combine 2.5 µL of each pre-enrichment library in a 1.7 µL l microcentrifuge tube to generate a 12-plex pool at a total final pool volume of 30 µL .
When preparing a pool of lower plexity (<12 pre-enrichment libraries per pool): combine 2.5 µL of each pre-enrichment library in a 1.7 mL microcentrifuge tube, then

add RSB to bring the total final pool volume up to 30 µL .

Pool pre-enrichment libraries based on the sample volumes in the following table:

Library Pool Plexity	Each Pre-Enrichment Library Volume (µl)	Total Volume (µl)
12-plex	2.5	30

Note

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.

6.40 **G. Hybridize Probes:** This step facilitates the binding of the target regions of DNA within the pre-enrichment library to the exome capture probes, as well as optional mitochondrial genome capture probes. This step binds target regions of DNA within the pre-enrichment library with the exome capture probes, and optional mitochondrial genome or custom capture probes.

Reagents:
1. EHB2 (Enrich Hyb Buffer 2)
2. NHB2 (Hyb Buffer 2 + IDT NXT Blockers) (blue cap)
3. Twist BioScience for Illumina Exome 2.5 Panel (green cap)
4. Nuclease-free water

Note

NHB2 precipitates and separates during storage. Follow the NHB2 preparation instructions before first use.

6.41 Save the IEE (Illumina Exome Enrichment)-HYB program on the Bio-Rad C1000 Touch Thermal Cycler with 96–Deep Well Reaction Module.

6.42 Create the Master Mix and add the following volumes to each well of a new PCR plate or 8-tube strip: Pre-enrichment library pool (30 µl), Twist BioScience for Illumina Exome 2.5 Panel (4 µl), Nuclease-free water (X µl), NHB2 (blue cap) (50 µl) and EHB2 (10 µl).

6.43 Place the sample plate or tubes on the preprogrammed thermal cycler and run the IEE (Illumina Exome Enrichment)-HYB program.

> Note
>
> Proceed immediately to the next procedure when the IEE (Illumina Exome Enrichment)-HYB program hold temperature time ends.

6.44 **H. Capture Hybridized Probes**

This step uses Streptavidin Magnetic Beads (SMB3) to capture the Exome 2.5 and optional mitochondrial probes hybridized to the target regions of interest within the libraries. The captured target regions are then prepared for further processing.

Reagents:

1. EE1 (Enrichment Elution Buffer 1)
2. EEW (Enhanced Enrichment Wash) (amber cap)
3. ET2 (Elute Target Buffer 2)
4. HP3 (2 N NaOH)
5. SMB3 (Streptavidin Magnetic Beads)

6.45 Prepare the following consumables:

Item	Storage	Instructions
EE1	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge briefly before use.
EEW (amber tube)	-25°C to -15°C	Let stand for 2 hours to bring to room temperature. Vortex three times for 30 seconds each. The reagent is heated during the procedure.
ET2	2°C to 8°C	Bring to room temperature. Vortex to mix. Centrifuge briefly before use.
HP3	-25°C to -15°C	Thaw at room temperature. Vortex to mix. Centrifuge briefly before use.
SMB3	2°C to 8°C	Let stand for 2 hours to bring to room temperature. Vortex to mix before use.

Preheat a microheating system with a MIDI heat block insert to incubate the sample plate to 62 °C. An optional second microheating system can be used to preheat EEW.

6.46 1. CAPTURE 30s

Centrifuge the sample plate or tubes of pooled enriched libraries at 280 x g for 00:00:30, and transfer pool from each well of the PCR plate or 8-tube strip to the corresponding well of a new MIDI plate or 1.7 ml microcentrifuge tube.

6.47 Add 250 µL SMB3 to each well or tube, and then mix thoroughly as follow: [**Tube**] Cap the tube and vortex at high speed three times for 00:00:10 each. Place the sample plate or tube on the MIDI heat block insert on the microheating system. Close the lid, and incubate for 00:15:00 at 62 °C. 15m 10s

Note

Proceed to the next step while the pooled libraries incubate.

6.48 Preheat EEW (amber tube) by laying the tube on its side on the MIDI heat block insert on the microheating system to 62 °C.

Note

Alternatively, 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***.

6.49 Immediately centrifuge the plate or tube at 280 x g for 00:00:30. Immediately place on a magnetic stand and wait until the liquid is clear (~ 00:02:00), and using a pipette set to 350 µL, remove and discard all supernatant from each well or from the tube. 2m 30s

6.50 2. WASH

6.51 Remove from the magnetic stand. Add 200 µl preheated EEW (amber tube) to each well or microcentrifuge tube, and then mix thoroughly as follows. [**tube**] Cap the tube and vortex at high speed three times for 00:00:10 s each. 10s

Return unused EEW to the microheating system and keep heated.

6.52 Place the sample plate or tube on the MIDI heat block insert on the microheating system. Close the lid, and incubate for 00:05:00 at 62 °C . 7m 3s

[tube] Centrifuge briefly for 00:00:03 , and immediately place the plate or microcentrifuge tube on a magnetic stand and wait until the liquid is clear (~ 00:02:00).

6.53 5.515-5.53 Using a pipette set to 200 µl, remove and discard all supernatant from each well or from the tube.

Note

Repeat steps 5.51-5.53 two additional times for a total of three washes.

6.54 3. TRANSFER WASH

6.55 Remove the plate or tube from the magnetic stand. Add 200 µL preheated EEW (amber tube) to each well or to the tube, and then mix thoroughly as follows. Cap the tube, and then vortex at high speed three times for 00:00:10 each 10s

6.56 Transfer 200 µL resuspended bead solution to a new MIDI plate or to a new 1.7 µL microcentrifuge tube.

Note

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

6.57 Place the sample plate or tube on the MIDI heat block insert on the microheating system. Close the lid, and incubate for 00:05:00 at 62 °C. Centrifuge briefly for 00:00:03. 5m 3s

6.58 Immediately place on a magnetic stand and wait until the liquid is clear (~ 00:02:00). Using a pipette set to 200 µL remove and discard all supernatant from each well or from the tube. 2m

6.59 Centrifuge the plate or the tube at 280 x g for 00:00:30. Place on a magnetic stand for 00:00:10. 40s

6.60 Use a 20 µl pipette to remove and discard residual liquid from each well or from the tube.

Note

Immediately proceed to Elute to prevent excessive drying of the beads and library yield loss.

6.61 4. ELUTE

6.62 Combine the following volumes to prepare an Elution Master Mix. Multiply each volume by the number of samples being processed EE1 (28.5 µL) + HP3 (1.5 µL), and vortex, and then centrifuge the master mix at 280 x g for 00:00:10. 10s

6.63 Remove the sample plate or tube from the magnetic stand, and add 23 µL Elution Master Mix to each well or to the tube, and then mix thoroughly as follows: cap the tube, and then vortex at high speed three times for 00:00:10 each, and ncubate the plate or the tube at room temperature for 00:02:00. 2m 10s

6.64 Centrifuge at 280 × g for 00:00:30, and place on a magnetic stand and wait until the liquid is clear (~ 00:02:00). 2m 30s

6.65 Transfer 21 µL supernatant from the MIDI plate or 1.7 mL microcentrifuge tube to the corresponding well of a new 96-well PCR plate or to a new 8-tube strip, and add 4 µL ET2 to each well or to the tube containing 21 µL supernatant.
Set pipette to 20 µL and slowly pipette each well or the tube 10 times to mix.

6.66 Centrifuge the sample plate or the tube at 280 x g for 00:00:30 . 30s

6.67 **I. Amplify Exome-Enriched Library Pools**
This step uses PCR to amplify the enriched whole exome library pools.
Reagents:
1. EPM (Enhanced PCR Mix)
2. PPC (PCR Primer Cocktail)

6.68 Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge briefly.
PPC	-25°C to -15°C	Thaw on ice. Invert to mix, then centrifuge briefly.

Save the AMP program on the thermal cycler.

6.69 Add 5 µL PPC to each well or tube, and add 20 µL EPM to each well or tube and mix thoroughly as follows, pipette 10 times to mix, and then cap the 8-tube strip.

6.70 Centrifuge the plate or 8-tube strip at 280 x g for 00:00:30 s. 30s

6.71 Place on the preprogrammed thermal cycler and run the AMP program.

Note

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 at 10°C.

6.72 **J. Clean Up Amplified Exome-Enriched Library Pools:** This step uses Illumina Purification Beads (IPB) to purify the amplified exome-enriched library pools and remove unwanted products.

Reagents:
1. IPB (Illumina Purification Beads)
2. RSB (Resuspension Buffer)
3. Freshly prepared 80% ethanol (EtOH)
Prepare the following consumables:

Item	Storage	Instructions
IPB	15°C to 30°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	2°C to 8°C	Bring to room temperature for 30 minutes. Vortex to mix.

For each sample, prepare 400 µl fresh [M] 80 % (v/v) EtOH from absolute ethanol. Including an overage of 20% is recommended.

6.73 Centrifuge the PCR samples at 280 x g for 00:00:30, and transfer 45 µL from each well of the PCR plate or 8-tube strip to the corresponding well of a new MIDI plate or 1.7 mL microcentrifuge tube. 30s

6.74 Add 40.5 µL IPB to each well or tube, and then mix thoroughly as follows. Cap the tube, and then vortex at high speed for 00:00:10.
Repeat two times and incubate the plate or the tube Room temperature for 00:05:00. 5m 10s

6.75 Centrifuge at 280 x g for 00:01:00, and place on a magnetic stand and wait until the liquid is clear (~ 00:05:00. 6m

6.76 Using a pipette set to 85 µL, remove and discard all supernatant from each well or tube.

6.77 ***Wash two times as follows***: 30s
1. With the plate on the magnetic stand, add 200 µL fresh [M] 80 % (v/v) EtOH without mixing.
2. Wait for 00:00:30.
3. Without disturbing the beads, remove and discard supernatant.

6.78 Use a 20 µL pipette to remove and discard residual EtOH from each well or from the tube.

6.79 Air-dry on the magnetic stand for 00:05:00 . 5m

6.80 Remove from the magnetic stand and add 32 µL RSB each well or to the tube. Cap the tube, and then vortex at high speed three times for 00:00:10 . Repeat two times 10s

6.81 Incubate the plate or the tube at room temperature for 00:05:00 , and then Centrifuge at 280 x g for 00:00:30 . 5m 30s

6.82 Place on a magnetic stand and wait until the liquid is clear (~ 00:02:00). 2m

6.83 Transfer 30 µL supernatant from each well of the PCR plate or 8-tube strip to the corresponding well of a new 96-well PCR plate or 1.7 mL microcentrifuge tube.

Note

SAFE STOPPING POINT

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

6.84 Check Exome-Enriched Library Pools

6.85 Perform the following to check the concentration and quality of each exome-enriched library pool.
Run 1 µl enriched libraries using the Qubit dsDNA BR Assay Kit to quantify library concentration. Run 1 µl pooled library or the individual libraries on the Agilent Technology

2100 Bioanalyzer using a High Sensitivity DNA kit.

Note

Expect a mean fragment size ~350 bp and distribution of DNA fragments with a size range from ~200 bp to ~1000 bp.

6.86 Dilute Libraries to the Starting Concentration

Note

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

6.87 Calculate the molarity value of the library or pooled libraries using the following formula.

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

6.88 **The Library Prep End**

Protocol references

This protocol is based on the guidelines provided in the Illumina DNA Prep with Exome 2.5 Enrichment manual. For detailed instructions, reagent specifications, and troubleshooting, please refer to the official Illumina manual. Adjustments and optimizations may be necessary depending on specific laboratory conditions and experimental needs.