TruSight Enrichment DNA Sample Preparation

Experienced User Card

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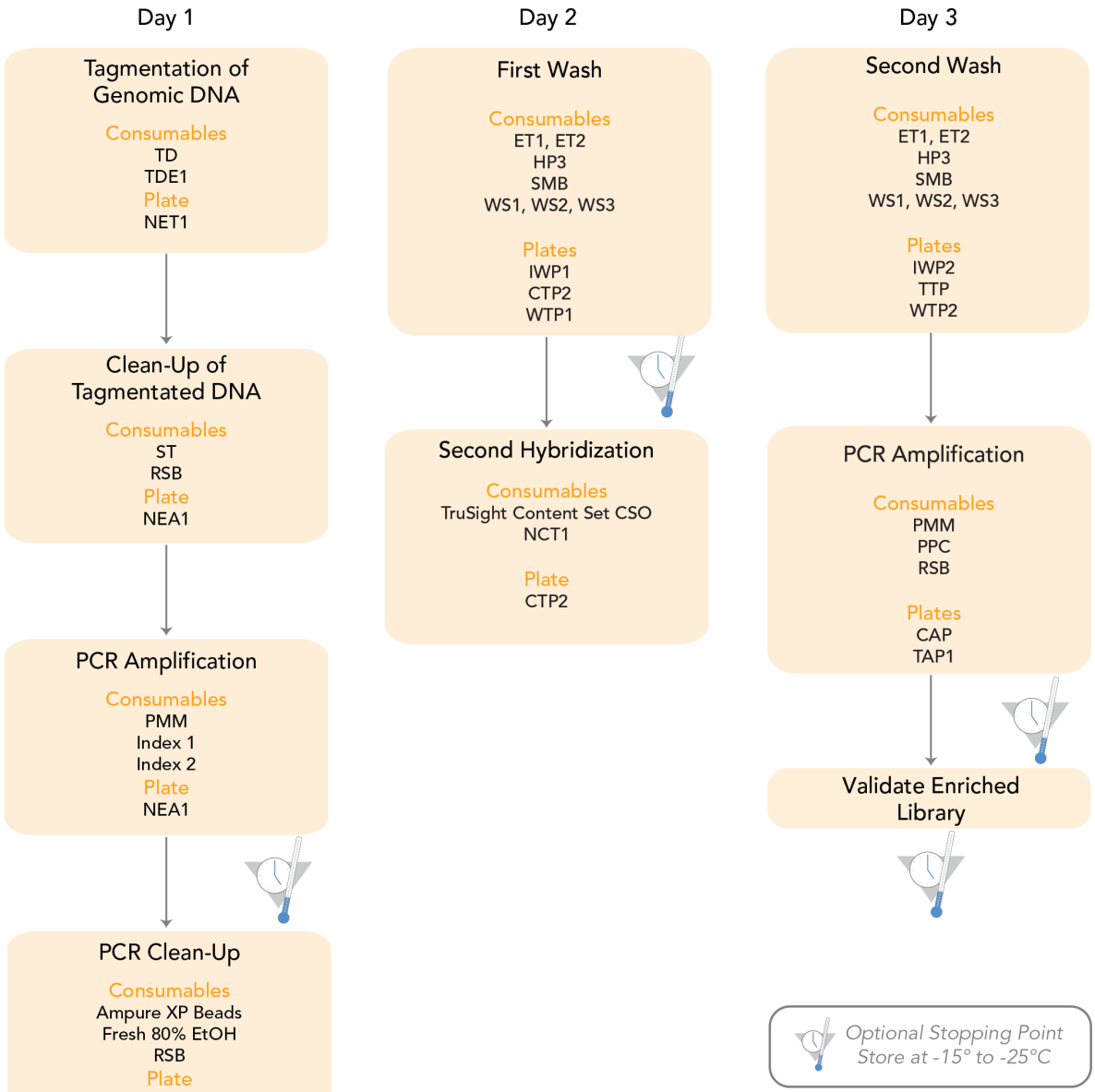

Note regarding biomarker patents and other patents unique to specific uses of products.

Some genomic variants, including some nucleic acid sequences, and their use in specific applications may be protected by patents. Customers are advised to determine whether they are required to obtain licenses from the party that owns or controls such patents in order to use the product in customer's specific application.

Tagmentation of Genomic DNA

During this step genomic DNA is tagmented (tagged and fragmented) by the Nextera transposome. The Nextera transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps.

Consumables

Item	Quantity	Storage	Supplied By
TD (Tagment DNA Buffer)	1 tube	-15° to -25°C	Illumina
TDE1 (Tagment DNA Enzyme)	1 tube	-15° to -25°C	Illumina
96-well hard shell TCY plate	1 plate	Room temperature	User
Genomic DNA (2.5 ng/µl)	50 ng	-15° to -25°C	User
Microseal 'B' adhesive seal		Room temperature	User

Preparation

[_] 1 Remove the TD, TDE1, and genomic DNA from -15° to -25°C storage and thaw on ice.

NOTE
In preparation for the next step of the protocol, ensure ST buffer is at room temperature, and there are no particles or precipitate visible in the solution.

[_] 2 After thawing, ensure all reagents are adequately mixed by gently inverting the tubes 3–5 times, followed by a brief spin in a microcentrifuge.

Procedure

NOTE
Ensure the reaction is assembled in the order described for optimal kit performance. The reaction does not need to be assembled on ice.

[_] 1 Label a new 96-well TCY plate "NET1" (Nextera Enrichment Tagmentation Plate 1) with a smudge resistant pen.

[_] 2 Add 20 µl of genomic DNA at 2.5 ng/µl (50 ng total) to each sample well of the NET1 plate.

[_] 3 Add 25 µl of TD Buffer to the wells containing genomic DNA. Change tips between samples.

NOTE
Calculate the total volume of TD for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NET1 plate.

[_] 4 Add 5 µl of TDE1 to the wells containing genomic DNA and TD Buffer. Change tips between samples.

NOTE
Calculate the total volume of TDE1 for all reactions, and divide among an appropriate number of tubes in an 8-well PCR strip tube. Use a multichannel pipette to dispense into the NET1 plate.

[_] 5 Using a multichannel pipette, gently pipette up and down 10 times to mix. Change tips between samples.

[_] 6 Cover the NET1 plate with Microseal 'B'.

[_] 7 Centrifuge at 280 xg at 20°C for 1 minute.

[_] 8 Place the NET1 plate in a thermocycler and run the following program:

NOTE
Ensure that the thermocycler lid is heated during the incubation.

- 55°C for 5 minutes
- Hold at 10°C

[_] 9 Proceed to *Clean-Up of Tagmented DNA*.

Clean-Up of Tagmented DNA

The tagmented DNA is purified from the Nextera transposome. This step is critical because the Nextera transposome can bind tightly to DNA ends and will interfere with downstream processes if not removed.

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
ST Buffer	1 tube	Room temperature	Illumina
AMPure XP Beads	1 tube	2° to 8°C	User
Freshly Prepared 80% Ethanol (EtOH)			User
96-well hard shell TCY plate	1 plate		User
96-well MIDI plate	1 plate		User

Preparation

[_] 1 Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.

[_] 2 Remove the NET1 plate from the Thermocycler and remove the seal.

[_] 3 Visually inspect the ST buffer to ensure there is no precipitate. If there is precipitate, vortex until all particulates are resuspended.

Procedure

[_] 1 Add 15 µl ST buffer to each well of the NET1 plate with sample.

[_] 2 Gently pipette up and down 10 times to mix.

[_] 3 Incubate at room temperature (20° to 25°C) for 5 minutes.

[_] 4 Centrifuge the NET1 plate at 280 xg at 20°C for 1 minute.

[_] 5 Using a multichannel P200 transfer the entire solution in each well of the NET1 plate to a clean 96-well midi plate labeled "NET2" (Nextera Enrichment Tagmentation Plate 2) with a smudge resistant pen.

[_] 6 Add 52 µl of well-resuspended AMPure XP beads to each well of the NET2 plate with sample and gently pipette up and down 10 times.

[_] 7 Incubate at room temperature for 10 minutes.

[_] 8 Cover the NET2 plate with a seal and centrifuge at 280 xg at 20°C for 1 minute.

[_] 9 Remove the seal and place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

[_] 10 Remove and discard supernatant.

[_] 11 While the NET2 plate is still on the magnet slowly add 200 µl of freshly made 80% ethanol to each sample. Wait 30 seconds.

[_] 12 Remove and discard the ethanol.

[_] 13 Repeat steps 11 and 12. Make sure there is no residual ethanol left in the plate.

[_] 14 While the NET2 plate is still on the magnet stand dry beads at room temperature for 10 minutes.

[_] 15 Remove the NET2 plate from the magnet and add 22.5 µl of RSB buffer.

[_] 16 Pipette mix up and down 10 times to resuspend beads.

[_] 17 Place the NET2 plate on the magnet for 2 minutes until the solution becomes clear.

[_] 18 Label a new 96-well TCY plate "NEA1" (Nextera Enrichment Amplification Plate 1) with a smudge resistant pen.

[_] 19 Transfer 20 µl of supernatant to the new NEA1 plate.

NOTE
(Optional) Check the products of the tagmentation reaction by loading 1 µl of supernatant on a HS Bioanalyzer chip. This should produce a broad distribution of DNA fragments with a size range from ~150 bp – <1 Kb.

PCR Amplification

In this step, the purified tagmented DNA is amplified via a limited-cycle PCR program. The PCR step also adds index 1 (i7) and index 2 (i5) and sequencing, as well as common adapters (P5 and P7) required for cluster generation and sequencing. It is critical to use the full amount of recommended input DNA, as well as to not add extra cycles of PCR cycles to ensure high quality libraries that produce high-quality sequencing results.

Consumables

Item	Quantity	Storage	Supplied By
PMM (PCR Master Mix)	1 tube	-15° to -25°C	Illumina
Index 1 primers (N7XX)	1 tube each index	-15° to -25°C	Illumina
Index 2 primers (E5XX)	1 tube each index	-15° to -25°C	Illumina
TruSeq Index Plate Fixture			Illumina
Microseal 'A' adhesive film			User
96-well hard shell TCY plate	1 plate		User

NOTE
The TruSight Enrichment DNA Sample Preparation kit is designed to work only with Index 2 primers with the "E" prefix. Index 2 primers from other Nextera sample prep kits should not be used.

Procedure

NOTE
For pooling libraries prior to enrichment, it is recommended to pool libraries so all Index 1 (i7) indexes are unique. Choose Index 1 and index 2 primers for PCR accordingly. For further details refer to Appendix A of the *TruSight Enrichment DNA Sample Preparation Guide*.

NOTE
PCR Set up should be done in the NEA1 plate, same plate that your final elution is.

[_] 1 To each well of the NEA1 plate containing sample add:
- 20 µl of PMM
- 5 µl of the Index 1 (i7, N7xx) primer
- 5 µl of the Index 2 (i5, E5xx) primer
- Total volume = 50 µl

[_] 2 Gently pipette mix up and down.

[_] 3 Cover the NEA1 plate with Microseal 'A' and seal with a rubber roller.

[_] 4 Centrifuge at 280 xg at 20°C for 1 minute.

[_] 5 Perform PCR using the following program on a thermal cycler:

NOTE
Ensure that the thermocycler lid is heated during the incubation. Pre-heat lid to 100°C.

- 72°C for 3 minutes
- 98°C for 30 seconds
- 10 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

SAFE STOPPING POINT

If you do not plan to immediately proceed to *PCR Clean-Up* following the completion of PCR, the NEA1 plate can remain on the thermalcycler overnight, or you can store it at 2° to 8°C up to two days.

PCR Clean-Up

This step uses AMPure XP beads to purify the library DNA, and provides a size selection step that removes very short library fragments from the population.

Consumables

Item	Quantity	Storage	Supplied By
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
AMPure XP beads		2° to 8°C	User
80% Ethanol, freshlyprepared			User
96-well MIDI plates	1 plate		User
96-well TCY plates	1 plate		User

Preparation

[_] 1 Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.

Procedure

[_] 1 Remove the NEA1 plate from the thermocycler and centrifuge at 280 xg at 20°C for 1 minute.

[_] 2 Carefully remove the cover so the solution does not splash (if this happens centrifuge again).

[_] 3 Label a new 96-well TCY plate "NEA2" (Nextera Enrichment Amplification Plate 2) with a smudge resistant pen.

[_] 4 Transfer the entire PCR reaction to the NEA2 plate.

[_] 5 Add 45 µl of well-resuspended AMPure XP beads to each well of the NEA2 plate with sample.

[_] 6 Gently pipette up and down 10 times to mix.

[_] 7 Incubate at room temperature for 10 minutes.

[_] 8 Place the NEA2 plate on a magnetic stand for 2 minutes or until the supernatant has cleared.

[_] 9 Carefully remove and discard the supernatant.

[_] 10 While the NEA2 plate is still on the magnet slowly add 200 µl of freshly made 80% ethanol to each sample and wait 30 seconds.

[_] 11 Remove and discard the entire ethanol wash.

[_] 12 Repeat steps 10 and 11.

[_] 13 While the NEA2 plate is still on the magnet stand dry beads at room temperature for 15 minutes.

[_] 14 Remove the NEA2 plate from the magnet and add 40 µl of RSB buffer.

[_] 15 Pipette mix up and down 10 times to resuspend beads.

[_] 16 Place the NEA2 plate on the magnet for 2 minutes until the solution becomes clear.

[_] 17 Label a new 96-well TCY plate "NEL" (Nextera Enrichment Library Plate) with a smudge resistant pen.

[_] 18 Transfer 38 µl of supernatant to the NEL plate.

[_] 19 Quantify the library by either Picogreen or Qubit.

First Hybridization

This process mixes the DNA library with capture probes of targeted regions. The recommended hybridization time makes sure that targeted regions bind to the capture probes thoroughly. It also describes how to combine multiple libraries with different indices into a single pool prior to enrichment.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
NCT1 (Nextera Capture Target Buffer 1)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	1 plate		User
Microseal 'B' adhesive seal			User
PCR grade water			User
500 ng DNA library from NEL Plate			User

Preparation

- Remove the CSO tube from -15° to -25°C storage and thaw at room temperature.
- Remove the NCT1 tube from -15° to -25°C storage and thaw at room temperature.
- Pre-program the thermal cycler as follows:
  - 95°C for 10 minutes
  - 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle
  - 58°C for forever
- Pre-heat the thermal cycler lid to 100°C.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CTP1" (Capture Target Plate 1) with a smudge resistant pen.

Pool Libraries

NOTE
In order to support the number of samples as noted on the kit box, samples will need to be pooled appropriately prior to the enrichment step, as indicated in the user guide.

[_] 1 Reference Table 1 for the amount of DNA libraries to use for enrichment. Illumina recommends using 500 ng of each DNA library, quantified by picogreen or the Qubit Fluorometric Quantitation system. If pooling libraries, combine 500 ng of each DNA library. If the total volume is greater than 40 µl, use a vacuum concentrator without heat to reduce the pooled sample volume to 40 µl.

[_] 2 The recommended pre-enrichment pooling strategy is to pool up to 12 libraries each with a unique Index 1/i7 index. With this pooling approach samples can be sequenced using a

single index read workflow, as described in the HiSeq and GAIIx user guides. If Index1/i7 indexes are not unique, ensure that libraries with different Index 2/i5 indexes are included (e.g. N703/E501 and N703/E502). With this approach, samples can be sequenced using a dual index read workflow, as described in the HiSeq and GAIIx user guides. For further details refer to Appendix A of the *TruSight Enrichment DNA Sample Preparation Guide*.

NOTE
Adding >500 ng library DNA into the enrichment assay (up to 1 ug per sample) may produce greater mean coverage/sample.

Table 1 DNA Libraries for Enrichment

Library Pool Complexity	Total DNA Library Mass (ng)
1-plex	500
2-plex	1000
3-plex	1500
4-plex	2000
5-plex	2500
6-plex	3000
7-plex	3500
8-plex	4000
9-plex	4500
10-plex	5000
11-plex	5500
12-plex	6000

Make CTP1

[_] 1 Thoroughly vortex the NCT1 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

[_] 2 In the order listed below, prepare the reaction mix in each well of the new 300 µl 96-well PCR plate or twin.tech 96-well PCR plate labeled CTP1. Gently pipette the entire volume up and down 5–10 times to mix thoroughly. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
DNA library from NEL plate	40
NCT1	50
TruSight Content Set CSO	10
Total Volume per Sample	**100**

[_] 3 Seal the CTP1 plate with a Microseal 'B' adhesive seal.

Incubate CTP1

[_] 1 Centrifuge the CTP1 plate at 280 xg at 20°C for 1 minute.

[_] 2 Place the sealed CTP1 plate on the pre-programmed thermal cycler. Close the lid and incubate as follows:

- [_] a Choose the pre-heat lid option and set to 100°C.
- [_] b 95°C for 10 minutes.
- [_] c 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
- [_] d 58°C for 16–20 hours.

First Wash

This process uses streptavidin beads to capture probes containing the targeted regions of interest. Three wash steps remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for a second hybridization.

Consumables

Item	Quantity	Storage	Supplied By
HP3 (2N NaOH)	1 tube	-15° to -25°C	Illumina
ET1 (Elute Target Buffer 1)	1 tube	-15° to -25°C	Illumina
ET2 (Elute Target Buffer 2)	1 tube	2° to 8°C	Illumina
SMB (Streptavidin Magnetic Beads)	1 tube	2° to 8°C	Illumina
WS1 (Wash Solution 1)	1 tube	2° to 8°C	Illumina
WS2 (Wash Solution 2)	1 tube	-15° to -25°C	Illumina
WS3 (Wash Solution 3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate			User
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
Microseal 'B' adhesive seal	5		User
PCR grade water			User
PCR tubes			User

Preparation

- Remove the SMB, ET2, WS1, and WS3 tubes from 2° to 8°C storage and let stand at room temperature.
- Remove the ET1, HP3, and WS2 tubes from -15° to -25°C storage and thaw at room temperature.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "IWP1" (Intermediate Wash Plate 1) with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CTP2" (Capture Target Plate 2) with a smudge resistant pen.
- Label a new 96-well MIDI plate "WTP1" (Wash Target Plate 1) with a smudge resistant pen.

Make WTP1

[_] 1 Remove the CTP1 plate from the thermal cycler.

[_] 2 Centrifuge the CTP1 plate at 280 xg at 20°C for 1 minute.

[_] 3 Place the CTP1 plate on a 96-well rack and remove the adhesive seal from the plate.

[_] 4 Transfer the entire contents of each well from the CTP1 plate to the corresponding well of the new 96-well MIDI plate labeled WTP1.

[_] 5 Vortex the SMB tube until the beads are well dispersed, then add 250 µl of well-mixed SMB to the wells of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times until mixed.

[_] 6 Let the WTP1 plate stand at room temperature for 30 minutes.

[_] 7 Centrifuge the WTP1 plate at 280 xg at 20°C for 1 minute.

[_] 8 Remove the adhesive seal from the WTP1 plate.

[_] 9 Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 10 Remove and discard all of the supernatant from each well.

[_] 11 Remove the WTP1 plate from the magnetic stand.

Wash 1 WTP1 and Wash 2 WTP1

Perform WS1 Clean Up and WS2 Clean Up on the WTP1 plate as follows:

WS1 Clean Up

[_] 1 Thoroughly vortex the WS1 tube. Visually make sure that no crystal structures are present.

[_] 2 Add 200 µl WS1 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times to make sure the beads are fully resuspended.

[_] 3 Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Remove the WTP1 plate from the magnetic stand.

WS2 Clean Up

[_] 1 Thoroughly vortex the WS2 tube. Visually make sure that the WS2 is mixed thoroughly.

[_] 2 Add 200 µl WS2 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 3 Place the WTP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Remove the WTP1 plate from the magnetic stand.

[_] 6 Add 200 µl WS2 to each well of the WTP1 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 7 Transfer the entire contents of each well of the WTP1 plate to the corresponding well of the new 96-well PCR plate labeled IWP1.

[_] 8 Seal the IWP1 plate with a Microseal 'B' adhesive seal.

[_] 9 Incubate the IWP1 plate on the thermal cycler at 42°C for 30 minutes with a heated lid set to 100°C.

[_] 10 Place the magnetic stand next to the thermal cycler for immediate access.

[_] 11 Remove the IWP1 plate from the thermal cycler and ***immediately*** place it on the magnetic stand for 2 minutes until the liquid appears clear.

[_] 12 Remove the adhesive seal from the IWP1 plate.

[_] 13 Immediately remove and discard all of the supernatant from each well.

[_] 14 Remove the IWP1 plate from the magnetic stand.

[_] 15 Add 200 µl WS2 to each sample well of the IWP1 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 16 Repeat steps 8–13 once.

Wash 3 WTP1

Perform WS3 Clean Up and Elute Target on the WTP1 plate as follows:

WS3 Clean Up

[_] 1 Remove the IWP1 plate from the magnetic stand.

[_] 2 Add 200 µl WS3 to each well of the IWP1 plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.

[_] 3 Place the IWP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Repeat steps 1–4 once.

[_] 6 To remove any residual WS3, seal the IWP1 plate with a Microseal 'B' adhesive seal.

[_] 7 Briefly centrifuge the IWP1 plate to collect any residual WS3.

[_] 8 Place the IWP1 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 9 Carefully remove the adhesive seal from the IWP1 plate to avoid spilling the contents of the wells.

[_] 10 Remove and discard any residual supernatant from each well.

Elute Target

[_] 1 Mix the following reagents in the order listed in a separate PCR tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
ET1	28.5
HP3	1.5
Total Volume per Sample	**30**

[_] 2 Remove the IWP1 plate from the magnetic stand.

[_] 3 Add 23 µl of the elution pre-mix to each well of the IWP1 plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly.

[_] 4 Seal the IWP1 plate with a Microseal 'B' adhesive seal.

[_] 5 Let the IWP1 plate stand at room temperature for 5 minutes.

[_] 6 Centrifuge the IWP1 plate at 280 xg at room temperature for 1 minute.

[_] 7 Place the IWP1 plate on the magnetic stand for 2 minutes until the liquid appears clear.

[_] 8 Carefully remove the adhesive seal from the IWP1 plate to avoid spilling the contents of the wells.

[_] 9 Transfer 21 µl of supernatant from each well of the IWP1 plate to the corresponding well of the new 96-well PCR plate labeled CTP2.

[_] 10 Add 4 µl ET2 to each well of the CTP2 plate containing samples to neutralize the elution. Gently pipette the entire volume up and down 5–10 times to mix thoroughly.

[_] 11 Seal the CTP2 plate with Microseal 'B' adhesive seal.

[_] 12 Store the remaining reagents as follows:

- [_] a Place the SMB, ET2, WS1, and WS3 tubes in 2° to 8°C storage.
- [_] b Place the ET1, HP3, and WS2 tubes in -15° to -25°C storage.
- [_] c Discard any remaining elution pre-mix.

SAFE STOPPING POINT

If you do not plan to proceed to *Second Hybridization* on page 18 immediately, the protocol can be safely stopped here. If you are stopping, seal the CTP2 plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days. When proceeding, thaw the CTP2 plate on ice.

Second Hybridization

This process mixes the first elution of the DNA library with the capture probes of target regions. The second hybridization make sure that the targeted regions are further enriched.

Consumables

Item	Quantity	Storage	Supplied By
TruSight Content Set CSO (Custom Selected Oligos)	1 tube	-15° to -25°C	Illumina
NCT1 (Nextera Capture Target Buffer 1)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	1 plate		User
Microseal 'B' adhesive seal			User
PCR grade water			User

Preparation

- Remove the CSO tube from -15° to -25°C storage and thaw at room temperature.
- Remove the NCT1 tube from -15° to -25°C storage and thaw at room temperature.
- Pre-program the thermal cycler as follows:

[_] a 95°C for 10 minutes.
[_] b 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
[_] c 58°C for forever.

- Pre-heat the thermal cycler lid to 100°C.
- Ensure CTP2 plate is completely thawed. Centrifuge at 280 xg for 1 minute.

Add CTO/CSO

[_] 1 Thoroughly vortex the NCT1 tube until the solution is completely resuspended. Visually make sure that no crystal structures are present.

[_] 2 In the order listed below, add the following to each well of the CTP2 plate. Gently pipette the entire volume up and down 5-10 times to mix thoroughly. Multiply each volume by the number of samples being prepared.

Reagent	Volume (µl)
NCT1	50
TruSight Content Set CSO	10
PCR Grade Water	15
First elution	25
Total Volume per Sample	**100**

[_] 3 Seal the CTP2 plate with a Microseal 'B' adhesive seal.

Incubate CTP2

[_] 1 Centrifuge the CTP2 plate at 280 xg at 20°C for 1 minute.

[_] 2 Place the sealed CTP2 plate on the pre-programmed thermal cycler. Close the lid and incubate as follows:

- [_] a Choose the pre-heat lid option and set to 100°C.
- [_] b 95°C for 10 minutes.
- [_] c 18 cycles of 93°C for 1 minute, decreasing 2°C per cycle.
- [_] d 58°C for 16–20 hours.

Second Wash

This process uses streptavidin beads to capture probes containing the targeted regions of interest. Three wash steps remove non-specific binding from the beads. The enriched library is then eluted from the beads and prepared for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
HP3 (2N NaOH)	1 tube	-15° to -25°C	Illumina
ET1 (Elute Target Buffer 1)	1 tube	-15° to -25°C	Illumina
ET2 (Elute Target Buffer 2)	1 tube	2° to 8°C	Illumina
SMB (Streptavidin Magnetic Beads)	1 tube	2° to 8°C	Illumina
WS1 (Wash Solution 1)	1 tube	2° to 8°C	Illumina
WS2 (Wash Solution 2)	1 tube	-15° to -25°C	Illumina
WS3 (Wash Solution 3)	1 tube	2° to 8°C	Illumina
96-well MIDI Plate			User
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
Microseal 'B' adhesive seal	5		User
PCR grade water			User
PCR tubes			User

Preparation

- Remove the SMB, ET2, WS1, and WS 3 tubes from 2° to 8°C storage and let stand at room temperature.
- Remove the ET1, HP3, and WS2 tubes from -15° to -25°C storage and thaw at room temperature.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "IWP2" (Intermediate Wash Plate 2) with a smudge resistant pen.
- Label a new 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "TTP" (Temporary Target Plate) with a smudge resistant pen.
- Label a new 96-well MIDI plate "WTP2" (Wash Target Plate 2) with a smudge resistant pen.
- [Optional] Label one new PCR tube per sample “Second Elution for qPCR”.

Make WTP2

[_] 1 Remove the CTP2 plate from the thermal cycler.

[_] 2 Centrifuge the room temperature CTP2 plate at 280 xg at room temperature for 1 minute.

[_] 3 Place the CTP2 plate on a 96-well rack and remove the adhesive seal from the plate.

[_] 4 Transfer the entire contents from each well of the CTP2 plate to the corresponding well of the new 96-well MIDI plate labeled WTP2.

[_] 5 Vortex the SMB tube until the beads are well dispersed, then add 250 µl of well-mixed SMB to the wells of the WTP2 plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 6 Seal the WTP2 plate with a Microseal 'B' adhesive seal.

[_] 7 Let the WTP2 plate stand at room temperature for 30 minutes.

[_] 8 Centrifuge the WTP2 plate at 280 xg at 20°C for 1 minute.

[_] 9 Remove the adhesive seal from the WTP2 plate.

[_] 10 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 11 Remove and discard all of the supernatant from each well.

[_] 12 Remove the WTP2 plate from the magnetic stand.

Wash 1 WTP2 and Wash 2 WTP2

Perform WS1 Clean Up and WS2 Clean Up on the WTP2 plate as follows:

WS1 Clean Up

[_] 1 Thoroughly vortex the WS1 tube. Visually make sure that no crystal structures are present.

[_] 2 Add 200 µl WS1 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times to make sure the beads are fully resuspended.

[_] 3 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Remove the WTP2 plate from the magnetic stand.

WS2 Clean Up

[_] 1 Thoroughly vortex the WS2 tube. Visually make sure that the WS2 is mixed thoroughly.

[_] 2 Add 200 µl WS2 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 3 Place the WTP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Remove the WTP2 plate from the magnetic stand.

[_] 6 Add 200 µl WS2 to each well of the WTP2 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 7 Transfer the entire contents of each well of the WTP2 plate to the corresponding well of the new 96-well PCR plate labeled IWP2.

[_] 8 Seal the IWP2 plate with a Microseal 'B' adhesive seal.

[_] 9 Incubate the IWP2 plate on the thermal cycler at 42°C for 30 minutes with a heated lid set to 100°C.

[_] 10 Place the magnetic stand next to the thermal cycler for immediate access.

[_] 11 Remove the IWP2 plate from the thermal cycler and ***immediately*** place it on the magnetic stand for 2 minutes until the liquid appears clear.

[_] 12 Remove the adhesive seal from the IWP2 plate.

[_] 13 Immediately remove and discard all of the supernatant from each well.

[_] 14 Remove the IWP2 plate from the magnetic stand.

[_] 15 Add 200 µl WS2 to each sample well of the IWP2 plate. Gently pipette the entire volume up and down 10–20 times.

[_] 16 Repeat steps 8–13 once.

Wash 3 WTP2

Perform WS3 Clean Up and Elute Target on the WTP2 plate as follows:

WS3 Clean Up

[_] 1 Remove the IWP2 plate from the magnetic stand.

[_] 2 Add 200 µl WS3 to each well of the IWP2 plate. Gently pipette the entire volume up and down 10–20 times to mix thoroughly.

[_] 3 Place the IWP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 4 Remove and discard all of the supernatant from each well.

[_] 5 Repeat steps 1–4 once.

[_] 6 To remove any residual WS3, seal the IWP2 plate with a Microseal 'B' adhesive seal.

[_] 7 Briefly centrifuge the IWP2 plate to collect any residual WS3.

[_] 8 Place the IWP2 plate on the magnetic stand for 2 minutes at room temperature until the liquid appears clear.

[_] 9 Carefully remove the adhesive seal from the IWP2 plate to avoid spilling the contents of the wells.

[_] 10 Remove and discard any residual supernatant from each well.

Elute Target

[_] 1 Mix the following reagents in the order listed in a separate PCR tube to create the elution pre-mix. Multiply each volume by the number of pooled samples being prepared.

Reagent	Volume (µl)
ET1	28.5
HP3	1.5
Total Volume per Sample	**30**

[_] 2 Remove the IWP2 plate from the magnetic stand.

[_] 3 Add 23 µl of the elution pre-mix to each well of the IWP2 plate. Gently pipette the entire volume of each well up and down 10–20 times to mix thoroughly.

[_] 4 Seal the IWP2 plate with a Microseal 'B' adhesive seal. Use an adhesive seal roller to apply force to the seal and make sure the seal is secured.

[_] 5 Let the IWP2 plate stand at room temperature for 5 minutes.

[_] 6 Centrifuge the IWP2 plate at 280 xg at 20°C for 1 minute.

[_] 7 Place the IWP2 plate on the magnetic stand for 2 minutes until the liquid appears clear.

[_] 8 Carefully remove the adhesive seal from the IWP2 plate to avoid spilling the contents of the wells.

[_] 9 Transfer 21 µl of supernatant from each well of the IWP2 plate to the corresponding well of the new 96-well PCR plate labeled TTP.

[_] 10 Add 4 µl ET2 to each well of the TTP plate containing samples to neutralize the elution. Gently pipette the entire volume of each well up and down 10-20 times to mix thoroughly.

[_] 11 [Optional] The Second Elution for qPCR tube can be used for yield quantification. To do so, dilute 2 µl of supernatant from each well of the TTP plate in 98 µl PCR grade water (1:50 dilution) in a new PCR tube labeled "Second Elution for qPCR". Cap each tube and store at -15° to -25°C.

[_] 12 Store the remaining reagents as follows:

- [_] a Place the SMB, ET2, WS1, and WS3 tubes in 2° to 8°C storage.
- [_] b Place the ET1, HP3, and WS2 tubes in -15° to -25°C storage.
- [_] c Discard any remaining elution pre-mix.

PCR Amplification

This process uses PCR to amplify the enriched DNA library for sequencing.

Consumables

Item	Quantity	Storage	Supplied By
PMM (PCR Master Mix)	1 tube	-15° to -25°C	Illumina
PPC (PCR Primer Cocktail)	1 tube	-15° to -25°C	Illumina
RSB (Resuspension Buffer)	1 tube	-15° to -25°C	Illumina
300 µl 96-well skirtless PCR Plate or twin.tech 96-well PCR Plate	2 plates		User
AMPure XP beads		2° to 8°C	User
80% Ethanol, freshly-prepared			User
Microseal 'B' adhesive seal	3		User

Preparation

- Remove one tube each of PMM and PPC from -15° to -25°C storage and thaw on ice.
- Briefly centrifuge the thawed PPC and PMM tubes for 5 seconds.
- Remove the AMPure XP beads from storage and let stand for at least 30 minutes to bring them to room temperature.
- Pre-program the thermal cycler as follows:
  - Choose the pre-heat lid option and set to 100°C
  - 98°C for 30 seconds
  - 12 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
- Label a 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "CAP" (Cleaned Amplification Plate).
- Label a 300 µl 96-well skirtless PCR plate or twin.tech 96-well PCR plate "TAP1" (Target Amplification Plate 1).

Add PPC

[_] 1 Add the following to each well of the new TAP1 300 µl 96-well PCR plate. Gently pipette the entire volume up and down 10 times to mix thoroughly.

Reagent	Volume (µl)
Second Elution from TTP plate	20
PMM	25
PPC	5
Total Volume per Sample	**50**

[_] 2 Seal the TAP1 plate with a Microseal 'B' adhesive seal.

[_] 3 Centrifuge the TAP1 plate at 280 xg at 20°C for 1 minute.

Amp PCR

[_] 1 Place the sealed TAP1 plate on the pre-programmed thermal cycler. Close the lid and incubate using the pre-programmed settings:

- [_] a Choose the pre-heat lid option and set to 100°C.
- [_] b 98°C for 30 seconds.
- [_] c 12 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 30 seconds
- [_] d 72°C for 5 minutes.
- [_] e Hold at 10°C.

Make CAP

[_] 1 Remove the adhesive seal from the TAP1 plate.

[_] 2 Vortex the AMPure XP Beads until the beads are well dispersed, then add 90 µl of the mixed AMPure XP Beads to each well of the TAP1 plate containing 50 µl of the PCR amplified library. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 3 Incubate the TAP1 plate at room temperature for 15 minutes.

[_] 4 Place the TAP1 plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 5 Using a 200 µl single or multichannel pipette, remove and discard 140 µl of the supernatant from each well of the TAP1 plate.

[_] 6 With the TAP1 plate remaining on the magnetic stand, add 200 µl of freshly prepared 80% EtOH to each well without disturbing the beads.

[_] 7 Incubate the TAP1 plate for at least 30 seconds at room temperature, then remove and discard the supernatant from each well.

[_] 8 Repeat steps 6–7 once for a total of two 80% EtOH washes.

[_] 9 Keep the TAP1 plate on the magnetic stand and allow plate to stand at room temperature for 15 minutes to dry, then remove the plate from the magnetic stand.

[_] 10 Resuspend the dried pellet in each well with 30 µl RSB. Gently pipette the entire volume up and down 10 times to mix thoroughly.

[_] 11 Incubate the TAP1 plate at room temperature for 2 minutes.

[_] 12 Place the TAP1 plate on the magnetic stand at room temperature for 5 minutes or until the liquid appears clear.

[_] 13 Transfer 28 µl of the clear supernatant from each well of the TAP1 plate to the corresponding well of the new 96-well PCR plate labeled CAP.

SAFE STOPPING POINT

If you do not plan to proceed to *Enriched Library Validation* on page 27 immediately, the protocol can be safely stopped here. If you are stopping, seal the CAP plate with a Microseal 'B' adhesive seal and store it at -15° to -25°C for up to seven days.

Enriched Library Validation

Illumina recommends performing the following procedure for quality control analysis on your enriched DNA library and quantification of the DNA library templates.

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantitation of enriched DNA library templates. Quantitate libraries using qPCR as described in the Illumina *Sequencing Library qPCR Quantification Guide*.

Library Pooling Guidelines for the TruSight Enrichment DNA Sample Preparation Kit

For further details refer to Appendix A of the *TruSight Enrichment DNA Sample Preparation Guide*.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 2 Illumina General Contact Information

Illumina Website	www.illumina.com
Email	techsupport@illumina.com

Table 3 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

MSDSs

Material safety data sheets (MSDSs) are available on the Illumina website at www.illumina.com/msds.

Product Documentation

Additional product documentation in PDF is available for download from the Illumina website. Go to www.illumina.com/support, select a product, then click **Documentation & Literature**.

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