TruSight Whole Genome

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Package Insert

FOR IN VITRO DIAGNOSTIC USE.

Intended Use

TruSight™ Whole Genome is a qualitative *in vitro* diagnostic device intended for whole genome sequencing and detection of single nucleotide variants, insertion/deletions, copy number variants, runs of homozygosity, short tandem repeat expansions, and mitochondrial variations in human genomic DNA extracted from blood.

TruSight Whole Genome includes the TruSight Whole Genome Dx Library Prep with UD Indexes and the TruSight Whole Genome Analysis Application Software. The device is intended to be used with compatible downstream germline applications to develop *in vitro* diagnostic assays, and by qualified laboratory personnel and assay developers.

TruSight Whole Genome is intended to be used on the NovaSeq™ 6000Dx Instrument.

Summary and Explanation

TruSight Whole Genome is a next-generation sequencing assay that uses tagmentation-based PCR-free library preparation, starting from genomic DNA (gDNA) extracted from peripheral whole human blood, and sequencing and primary analysis on the Illumina® NovaSeq 6000Dx Instrument.

Secondary analysis is performed with the TruSight Whole Genome Analysis Application software on the included and required Illumina DRAGEN Server for NovaSeq 6000Dx and includes demultiplexing, alignment to the GrCh38/hg38 human reference genome, and variant calling, as well as annotation and application of the quality control (QC) metric specifications in Table 1 to ensure analytical performance. The assay outputs include run and sample QC reports, and genome variant call format (VCF) files for use with compatible downstream tertiary analysis and reporting software.

TruSight Whole Genome broadly assesses genomic variants across the coding and noncoding regions of the human genome. Variant assessment includes detection of small variants, copy number variants (CNVs), runs of homozygosity (ROH), and short tandem repeat (STR) expansions. Additionally, TruSight Whole Genome detects the absence of the SMN1 c.840C allele (NM_000344.3:c.840C>T), which could indicate SMN1 gene deletion or SMN1/SMN2 gene conversion.[1,2] Biallelic loss of the SMN1 c.840C allele is responsible for approximately 95% of spinal muscular atrophy (SMA) cases.[3]

Table 2 provides information about the variant types validated with TruSight Whole Genome.

Table 1 TruSight Whole Genome Quality Metric Specifications

Output Type	Metric	Specification
Sequencing Run QC	Total % ≥ Q30	≥85.0

Output Type	Metric	Specification
FASTQ QC	Yield per sample (bps)	≥90,000,000,000
Sample Library QC	Average autosomal coverage	≥35.0
	Percent of autosomes with coverage greater than 20X	≥93.94
	Normalized coverage at 60% to 79% GC bins	0.82 ≤ x ≤ 1.13
	Normalized coverage at 20% to 39% GC bins	0.97 ≤ x ≤ 1.06
	Average mitochondrial coverage	≥ 500.0
	Percent Q30 bases	≥ 85.0
	Estimated sample contamination	≤ 0.005

Table 2 Detected Variants Validated with TruSight Whole Genome

Variant type	Validated variant detection
Small variants	Single nucleotide variants (SNVs), short insertions/deletions (1–31 bp)
Copy number variants (CNVs)	≥10 kb gains and losses
Runs of homozygosity (ROH)	≥500 kb
Mitochondrial SNVs	% heteroplasmy if ≥ 4.75%
Short tandem repeat (STR) expansions	Targeted loci (AFF2, AR, ATN1, ATXN1, ATXN10, ATXN2, ATXN3, ATXN7, ATXN8OS, C9ORF72, CACNA1A, CBL, CNBP, CSTB, DIP2B, DMPK, FMR1, FXN, GLS, HTT, JPH3, NIPA1, NOP56, NOTCH2NL, PABPN1, PHOX2B, PPP2R2B, and TBP)
SMN1 variant	NM_000344.3:c.840C/T

Principles of the Procedure

TruSight Whole Genome is intended for the preparation of PCR-free libraries to produce human whole-genome sequencing data. The assay begins with preparing libraries from quantified genomic DNA extracted from peripheral human whole blood, includes sequencing and analysis on the NovaSeq 6000Dx Instrument using the TruSight Whole Genome Analysis Application, and ends with variant calling and annotation.

The TruSight Whole Genome assay procedure consists of the following steps:

- **Batch Planning and Run Creation**—It is strongly recommended to plan the batch and runs prior to starting library preparation. Up to 24 sample libraries may be prepared in a library preparation batch. Based on the number of samples, different flow cell configurations may be used (6-plex on S2 and 16-plex on S4). The

Library Tube ID, sample names and corresponding indexing is recorded during run planning and run creation. For more information on run creation, refer to TruSight Whole Genome Analysis Application Guide (document # 200049931). Follow the planned batch during execution of the library preparation workflow.

- **Preparation for Protocol**—Some reagents are frozen and must be brought to room temperature. Due to the short workflow, it is possible to complete prep and start sequencing on the same day. Thus, sequencing consumables for planned runs may also be thawed during this step. Quantified genomic DNA samples are thawed and diluted for optimized DNA input.
- **Library Preparation**
  - **Tagment Genomic DNA**—Uses Bead-Linked Transposomes PCR-Free (BLT-PF) to tagment the DNA input. During tagmentation, gDNA is fragmented, tagged with adapters, and immobilized on the surface of magnetic BLT-PF beads.
  - **Post Tagmentation Cleanup**—Cleans up the adapter-tagged DNA on BLT-PF and removes stop buffer to prepare for Ligate Indexes.
  - **Ligate Indexes**—Adds unique dual indexes to libraries to enable multiplexing. Performs gap extension and elutes single-stranded DNA libraries off beads.
  - **Size-Selection and Clean Up Libraries**—A bead purification procedure with double-sided size-selection removes fragments too small and too large to target a median fragment length of approximately 450 bp, range ~360 to 550 bp.
  - **Pool and Denature Libraries**—The self-normalization feature of BLT-PF enables pooling by volume without qPCR or other normalization. The specified volume of each library is pooled according to the plan for each run, and denatured with 0.2N NaOH (diluted HP3). The denatured pool is then transferred to the NovaSeq 6000Dx Library Tube with the ID that corresponds to the planned run.
- **Sequencing and Analysis**—Consumables in the S2 and/or S4 configuration are loaded onto the NovaSeq 6000Dx Instrument, including the associated NovaSeq 6000Dx Library Tube(s) with pooled libraries. Upon loading, the Library Tube ID is scanned and, if entered during run planning, is used to select the corresponding planned run. Otherwise, the associated planned run must be manually selected.
  Pooled libraries are clustered onto a flow cell, and then sequenced using sequencing by synthesis (SBS) chemistry on the NovaSeq 6000Dx. SBS chemistry uses a reversible terminator method to detect fluorescently labeled single nucleotide bases as they are incorporated into growing DNA strands.
  The Real-Time Analysis (RTA) software performs primary analysis that includes base calling and assigning a quality score to each base call. Primary analysis data is automatically transferred to the Illumina DRAGEN Server.
  Demultiplexing and DRAGEN analysis is automatically performed using the TruSight Whole Genome Analysis Application. As part of this analysis, each run and sample library are reviewed for validity using analytical metrics described in *Quality Controls* on page 31, and results are provided in consolidated and individual sample reports. For valid sample libraries, annotated genome Variant Call Format (VCF) files are generated. For more information on the analysis workflow, refer to the TruSight Whole Genome Analysis Application Guide (document # 200049931).

Limitations of the Procedure

- For *in vitro* diagnostic use.
- TruSight Whole Genome is compatible with genomic DNA derived from human peripheral whole blood.
- The assay does not include reagents for DNA extraction or quantitation. The analytical testing results, including *Interfering Substances* on page 35, have been obtained with whole blood using representative DNA extraction kits and DNA quantitation kits. All diagnostic tests developed for use with TruSight Whole Genome require full validation for all aspects of performance with DNA extraction and DNA quantitation kits of choice.
- The assay has been configured and tested for the sample plexity and index sets indicated in the following table.

Library Prep Batch Size	Plexity	Run Configuration	Indexing
6, 12, 18, or 24 samples	6-plex	1-4 S2 runs	S2 Set 1 to 4
16 samples	16-plex	1 S4 run	S4 Set 1 or 2
22 samples	16-plex + 6-plex	1 S4 run + 1 S2 run	S4 Set 1 or 2, S2 Set 1 to 4 (not used for S4)

- The assay does not enforce positive sample tracking. While the summary ploidy QC result reported by the software may optionally be used to identify sample swaps, it will not identify Males swapped for Males or Females swapped for Females.
- The assay only provides validation up to genome VCF files output. All diagnostic tests developed for use with TruSight Whole Genome require full validation for all aspects of performance with downstream applications of choice.
- The assay does not report variant calls for samples that fail quality control.
- The assay defines high confidence tiers only for SNVs and insertions/deletions 1–5 bp due to strict criteria used for defining a genomic context as high confidence for a given variant type in *Small Variants Confidence Tier Determination* on page 37.
- The assay is designed to evaluate CNVs across the entire reportable genome, regardless of genomic context, and excludes regions with features that reflect limitations of the reference genome, such as centromeres, telomeres, and common CNVs segregating in populations.
- The assay performance was not assessed for copy number variants below 10 kb.
- The assay does not report translocations, inversions, or balanced rearrangements.
- The assay performance was not assessed for mitochondrial DNA (mtDNA) insertions or deletions.
- The assay only reports results for STR loci listed in Table 2. When the true STR expansion lengths exceed approximately 135 bp, the observed length will often be an underestimate of the true length due to technical limitations of short reads, with this effect being even more pronounced for FMR1. Once the true STR length exceeds the median fragment length (~330 bp), the STR length estimate plateaus.

- The assay does not report SMN1 or SMN2 copy number.
- The assay does not make claims on the pathogenicity of the detected variants.

Product Components

TruSight Whole Genome consists of the following:

- TruSight Whole Genome Dx Library Prep with UD Indexes, 24 sample (catalog # 20093209) and
- TruSight Whole Genome Analysis Application (catalog # 20106190, installed by trained Illumina personnel)

Reagents

Reagents Provided

TruSight Whole Genome Dx Library Prep Box 1, PN 20072256

Reagent Name	Quantity	Fill Volume	Active Ingredients	Storage Temperature
Bead-Linked Transposomes PCR-Free (BLT-PF)	1	460 µl	Streptavidin Magnetic Beads linked with transposomes in buffered aqueous solution.	-25°C to -15°C
Extension Ligation Mix (ELM)	1	1.6 ml	Ligase, DNA Polymerase, and dNTPs in buffer aqueous solution.	-25°C to -15°C
2N NaOH (HP3)	1	400 µl	2N sodium hydroxide (NaOH) solution.	-25°C to -15°C
Tagmentation Buffer 1 (TB1)	1	290 µl	Buffered aqueous solution containing magnesium salt, and dimethylformamide.	-25°C to -15°C

TruSight Whole Genome Dx Library Prep Box 2, PN 20072257

Reagent Name	Quantity	Fill Volume	Active Ingredients	Storage Temperature
Tagmentation Wash Buffer 2 (TWB2)	1	41 ml	Buffered aqueous solution containing detergent and salt.	15°C to 30°C
Resuspension Buffer (RSB)	1	20 ml	Buffered aqueous solution.	15°C to 30°C
Cleanup Beads (CB)	1	10 ml	Solid-phase paramagnetic beads in buffered aqueous solution.	15°C to 30°C
Stop Tagment Buffer 2 (ST2)	1	1.4 ml	Detergent solution in water.	15°C to 30°C
Neutralization Buffer (NB)	1	450 µl	Tris-HCl solution.	15°C to 30°C

TruSight Whole Genome Dx 32 Unique Dual Indexes, PN 20072258

Reagent Name	Quantity	Fill Volume	Active Ingredients	Storage Temperature
UDI PCR-Free (32 Indexes)	1	37 µl	Unique dual (UD) index adapters arranged in plate.	-25°C to -15°C

Consumables Required, Not Provided

- Ethanol 100% (200 proof), molecular biology grade
- Certified RNase/DNase-free water
- NovaSeq 6000Dx S2 Reagent Kit (300 cycles) (catalog # 20046931)
- NovaSeq 6000Dx S4 Reagent Kit (300 cycles) (catalog # 20046933)
- NovaSeq 6000Dx S2 Buffer Cartridge (catalog # 20062292)
- NovaSeq 6000Dx S4 Buffer Cartridge (catalog # 20062293)
- NovaSeq 6000Dx Library Tube (catalog # 20062290)
- NovaSeq 6000Dx Library Tube, 24 Pack (catalog # 20062291)

Storage and Handling

- Room temperature is defined as 15°C to 30°C.

- If any of the packaging or contents of the TruSight Whole Genome Dx Library Prep components are damaged or compromised, contact Illumina Customer Service.
- Reagents are stable when stored as indicated until the specified expiration date on the kit labels. For storage conditions, refer to *Reagents Provided* on page 5. Store the assay components at their specified temperature and do not use expired reagents. Do not interchange components from different kit lots. Kit lots are identified on the box labels.
- Changes in the physical appearance of the reagents can indicate deterioration of the materials. If changes in the physical appearance occur (eg, obvious changes in reagent color or cloudiness), do not use the reagents. If precipitation is observed for ST2, heat at 37°C for 10 minutes, and then vortex until precipitate dissolves.
- Stability of the TruSight Whole Genome Dx Library Prep has been evaluated and performance demonstrated for up to four uses of the frozen tubes when frozen between uses.

Equipment and Materials

Equipment Required, Not Provided

Verify the calibration status of equipment before starting the assay.

Equipment	Supplier
Vortexer capable of 3000 rpm, flat bottom or cup	General lab supplier
Microsample incubator calibrated to ensure temperature accuracy of ± 2°C	SciGene, catalog # 1057-30-O (or equivalent)
Microsample incubator insert for 96-well MIDI plates	Illumina, catalog # BD-60-601
Microcentrifuge	General lab supplier
96-well microplate centrifuge	General lab supplier
Plate shaker with the following specifications: • Can shake at 1800 rpm • Mixing orbit constant 2 mm • Mixing accuracy of ± 25 rpm	VWR, catalog # 1808-0506 (or equivalent)
Sealing wedge or roller	General lab supplier

Equipment	Supplier
Magnetic stand with the following specifications: • Designed for paramagnetic bead precipitation / separation • Magnets on the side of the stand, not the bottom • For 96-well MIDI plates	Thermo Fisher Scientific, catalog # AM10027 (or equivalent)
NovaSeq 6000Dx Instrument	Illumina, catalog # 20068232
Precision pipettes (single-channel): • 10 µl • 20 µl • 200 µl • 1000 µl Precision pipettes (8-channel): • 20 µl • 200 µl Make sure pipettes are calibrated regularly and are accurate within 5% of the stated volume	General lab supplier
Pipette Aid	General lab supplier

Materials Required, Not Provided

Make sure that you have the required materials before starting the protocol.

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

Materials	Supplier
5 ml serological pipettes	General lab supplier
10 ml serological pipettes	General lab supplier
Adhesive seals for 96-well plates with the following specifications: • Peelable, optically clear polyester • Strong adhesive that withstands multiple temperature changes from -40°C to 110°C • DNase/RNase free	General lab supplier
Microcentrifuge tubes, nuclease-free (1.5, 1.7, or 2.0 ml, unless specified as 0.5 ml)	General lab supplier

Materials	Supplier
Nuclease-free reagent reservoirs, 50 ml or equivalent (PVC, disposable trough)	General lab supplier
15 ml conical tubes	General lab supplier
50 ml conical tubes	General lab supplier
20 µl aerosol resistant pipette tips	General lab supplier
200 µl aerosol resistant pipette tips	General lab supplier
1000 µl aerosol resistant pipette tips	General lab supplier
96-well storage plates, 0.8 ml (MIDI plate)	Thermo Fisher Scientific, part # AB-0859 (or equivalent)
96-well PCR plates, 0.2 ml (polypropylene, free from RNAse/DNAse, low bind)	General lab supplier
Ice bucket and ice	Not applicable
Quantified genomic DNA samples	Not applicable

Specimen Collection, Transport, and Storage

CAUTION

Handle all specimens as if they are potentially infectious agents.

- Follow safety procedures, including use of PPE, when collecting, transporting, storing, and processing human blood samples.
- Transportation of whole blood must comply with country, federal, state, and local regulations for the transport of etiologic agents.
- Collect 2–5 ml peripheral whole blood in EDTA tubes and store at 2°C to 8°C for up to five weeks before extraction.
- No adverse effect on assay performance was observed with whole blood specimens with elevated bilirubin, hemoglobin, triglycerides, biotin, or EDTA present. Refer to Interfering Substances.
- TruSight Whole Genome is compatible with commercially available extraction kits and protocols that are appropriate for use in Next Generation Sequencing (NGS). Refer to *DNA Extraction Method Evaluation* on page 35.
- TruSight Whole Genome is compatible with DNA eluted in a Tris buffered solution containing ≤ 10 mM EDTA, such as 10 mM Tris, 1 mM EDTA pH 8.0 (TE).
- Elution and storage of DNA in TE is recommended. For stability, avoid storage in water.

DNA Input Recommendations

- Before beginning the TruSight Whole Genome assay, quantify the genomic DNA extracted from whole blood using any fluorometric quantification method that uses nucleic acid binding dyes. It is recommended that gDNA for samples intended for a particular library preparation batch and sequencing run are quantified together to eliminate batch-to-batch variability when possible, or process controls are used to ensure ≤ 25% DNA quantitation batch-to-batch variability.
- Avoid pipetting small sample volumes (< 2 µl) to ensure accurate DNA quantitation and input.
- The TruSight Whole Genome Dx Library Prep requires sufficient DNA to saturate the BLT-PF beads for effective self-normalization of library yields and optimal performance. Due to the variation of results from different quantification methods, the following table provides the recommended DNA input for three quantitation methods to ensure optimal assay performance. The use of other quantitation methods may require optimization. Refer to *DNA Input Sensitivity* on page 35.

Quant Method	Target DNA Input (ng)	Minimum DNA Stock Concentration
Quant-iT PicoGreen dsDNA Assay Kit	280	11.2 ng/µl
Qubit dsDNA Broad-Range (BR) Assay Kit	280	11.2 ng/µl
AccuClear Ultra High Sensitivity dsDNA Quantitation Kit	350	14 ng/µl

Proficiency Recommendations

Operator proficiency and successful assay implementation may be assessed by performing the complete workflow once according to the instructions for use. This workflow can be performed with either a single library prep of 6 samples and sequencing run using an S2 flow cell or a single library prep of 16 samples and sequencing run using an S4 flow cell. Success is indicated by passing the run and library QC metrics recorded in the Consolidated Report output by the TruSight Whole Genome Analysis Application software. Refer to TruSight Whole Genome Analysis Application Guide (document # 200049931).

Illumina recommends the inclusion of genomic DNA samples extracted from peripheral whole blood that meet the qualification criteria of DNA stock concentration and volume to demonstrate successful assay integration with upstream laboratory processes such as sample collection and storage, and DNA extraction and quantitation procedures. Commercially available genomic DNA reference samples derived from a single human donor such as NA24385/HG002 (National Institute of Standards and Technology Genome in a Bottle Consortium) may also be used.

If issues arise, consult the *Troubleshooting* on page 65 section for recommended actions and contact Illumina Technical Support.

Warnings and Precautions

- **Some components of this assay contain 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 Safety Data Sheets (SDS), visit support.illumina.com/sds.html.
- Immediately report any serious incidents related to this product to Illumina and the Competent Authorities of the member states in which the user and the patient are established.
- Handle all specimens as if they are known to be infectious.
- Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- This assay contains polyethylene glycol. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact.
- This assay contains sodium hydroxide. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact.
- The library preparation procedures require an RNase/DNase-free environment. Thoroughly decontaminate work areas with an RNase/DNase-inhibiting cleaner.
- Use nuclease-free microcentrifuge tubes, plates, pipette tips, and reservoirs.
- Use calibrated equipment throughout the assay. Make sure to calibrate equipment to the speeds, temperatures, and volumes specified in this protocol.
- Use precision pipettes to ensure accurate reagent and sample delivery. Calibrate regularly according to manufacturer specifications.
- Make sure to use equipment specified for the assay and to set programs as directed.
- Stated temperatures for the microsample incubator indicate set reaction temperature, not necessarily the temperature of the equipment.
- Do not interchange kit components from different TruSight Whole Genome Dx Library Prep lots. Lots are identified on the box label.
- Proper laboratory practices are required to prevent nucleases and PCR products from contaminating reagents, instrumentation, samples, and libraries. Nuclease and PCR product contamination can cause inaccurate and unreliable results.
- Proper plate type is required for optimal assay performance and storage. Make sure to follow plate transfer instructions in the *Instructions for Use* on page 15.
- Cross-contamination or sample loss can occur if plate seals are not carefully applied or removed (refer to *Handling Library Prep Plates* on page 13).

- Failure to follow the procedures as outlined can result in erroneous results, or a significant reduction in library quality.
- Store the assay reagents or components at the specified temperature.
- Do not store reagents in a frost-free storage unit.
- Do not use reagents that have been stored improperly.
- Do not use any components beyond their stated expiration date.
- Prepare 0.2N NaOH (diluted HP3) fresh on day of use and discard the remaining volume after use.
- Prepare fresh 80% ethanol with RNase/DNase-free water on day of use. Ethanol can absorb water from the air, which might impact results. Dispose of 80% ethanol after use in accordance with local, state, and/or federal regulations. Use molecular biology grade ethanol.

Procedural Notes

Tips and Techniques

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers with a multichannel pipette, change tips between ***each well***.
- Carefully seal and unseal plates on a benchtop to prevent sample cross-contamination.
- To avoid contamination, each index well is single-use.
- Use indicated trough volumes and do not pour remaining volume from trough back into stock tubes as this may cause contamination. There is sufficient volume to support the workflow.
- Do not pool together libraries from different preps.

Pipetting Accuracy

Use the following guidelines when using multichannel pipettes:

- Make sure barrier tips are well-fitting and appropriate for the multichannel pipette brand and model.
- Affix tips with a rolling motion to make sure all tips attach equally well.
- Aspirate with equal volume levels of liquid across all tips.
- Pipette viscous solutions (BLT-PF,CB,ELM,TWB2) slowly.
- After dispensing, make sure that liquid dispensed from every tip.

Avoiding Foaming

- Pipette slowly and invert to mix. Do not vortex ELM and TWB2.

Handling Index Plates

- Pierce foil seal only for indexes that will be used.

- Handle plate by edges and avoid touching foil seal with anything other than clean pipette tips.
- Do not reuse wells that have been pierced.
- Dispose of unused volume (~30 µl) after use from pierced wells of index plate and place seal over pierced wells to avoid cross-contamination.
- Do not place seal over unused wells as this interferes with piercing.

Handling Library Prep Plates

- Always seal the plate before storing, shaking, incubating, or centrifuging.
- To seal the plate, apply the adhesive cover to the plate with a sealing wedge or roller.
- Make sure the edges and wells are completely sealed to reduce risk of cross-contamination and evaporation.
- Always seal plates with a new adhesive plate seal. Do not reuse seals.
- Place the plate on a flat surface before carefully removing the seal.
- If not otherwise specified, steps can be performed with the plate on or off the magnet.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of the source plate to the corresponding well of the destination plate.

Troughs

- Reagent troughs may be used where indicated. Use the following guidelines:
  - Prepare trough with CB after vortexing. It is not necessary to return CB to tube and vortex before the second bead addition step.
  - Label TWB2 and RSB troughs to avoid confusion.
  - Dispose of reagents when indicated or at the end of the workflow.
- Use recommended volume. Recommended volumes include 1 ml overage for trough dead volume.
- RSB and TWB2 are packaged in similar tubes. Carefully read each label before use.

Centrifugation

- Centrifuge only at indicated steps in the procedure to consolidate liquid or beads in the bottom of the well to prevent sample loss.

Handling Beads

- Do not freeze Cleanup Beads (CB).
- When washing beads:
  - Use Magnetic Stand-96 for all MIDI plates.
  - Dispense liquid so that no beads remain adhered to the side of the well.
  - Keep the plate on the magnetic stand.

- Always add reagents to middle or bottom of well without disturbing the bead pellet. Do not add reagents to the top of the well.
- Pipette bead suspensions slowly.
- Vortex the beads until they are well dispersed. The color of the liquid must appear homogeneous. Vortex when specified in the protocol to ensure beads are resuspended at time of use.
- If beads do not resuspend, shake again.
- If beads are aspirated into pipette tips when they are not intended to be, dispense reactions back to the plate on the magnetic stand, and wait until the liquid is clear (2 minutes).
- Store upright to ensure beads are submerged in the buffer when returning to storage after use.

Controls

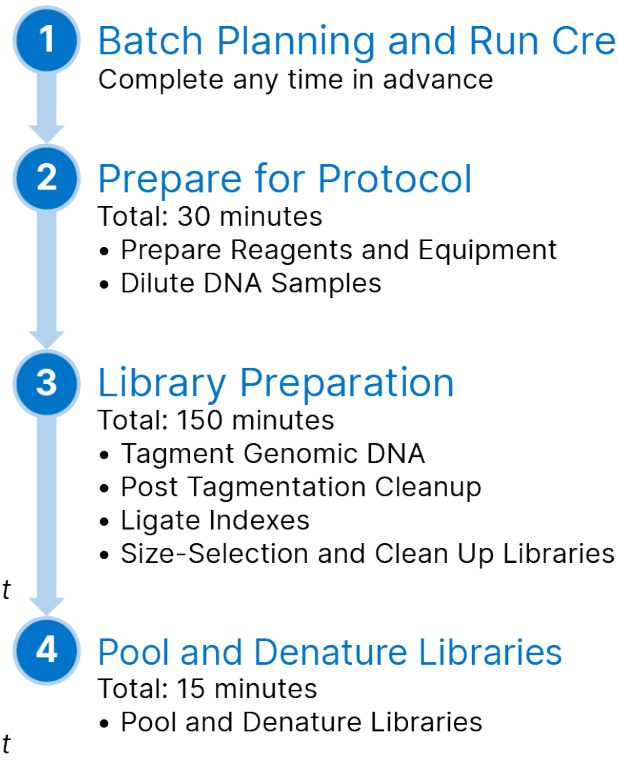
TruSight Whole Genome uses analytical controls built into the TruSight Whole Genome Analysis Application software for data qualification and does not require use of external batch controls. Refer to *Quality Controls* on page 31 for more information on metric specifications.

Instructions for Use

TruSight Whole Genome Dx Library Prep Workflow

The following diagram illustrates the TruSight Whole Genome Dx Library Prep workflow. Safe stopping points are marked between steps.

If stopping, return remaining reagents in original tubes to their storage temperature indicated in *Reagents Provided* on page 5. If continuing, move to the next section in the protocol with the prepared reagents.



Batch Planning and Run Creation

Plan the number of sample libraries for the batch, and indexing and pooling for sequencing runs.

TruSight Whole Genome has been evaluated and performance demonstrated for four sets of indexes for the S2 flow cell (Figure 1, Table 4) and two sets of indexes for the S4 flow cell (Figure 2, Table 5). The software enforces use of specified index sets. Do not mix and match between specified index sets.

Sequencing plexity outside of these recommendations is not supported.

S2 index and S4 index sets together support library prep batch sizes of 6, 12, 16, 18, 22, and 24 samples. Use the compatible index sets listed in Table 3 for each library prep batch size.

CAUTION

Arrange samples in the plate using an orientation that matches the planned indexing, ie rows A to H for a 16-plex, or rows A to F for a 6-plex. Add indexes using a multichannel pipette to avoid skipping a well or adding two sets of indexes to a single sample, which can cause no results or false results, respectively.

Table 3 Index Set Options for Library Prep Batch

Library Prep Batch Size	Index Set	Flow Cell Configurations
6 samples	S2 index set 1, 2, 3, or 4 (choose any 1 set)	S2 x 1
12 samples	S2 index set 1, 2, 3, or 4 (choose any 2 sets)	S2 x 2
18 samples	S2 index set 1, 2, 3, or 4 (choose any 3 sets)	S2 x 3
24 samples	S2 index set 1, 2, 3, and 4	S2 x 4
16 samples	S4 index set 1 or 2	S4 x 1
22 samples	S4 index set 1 + S2 index set 3 or 4	S4 x 1 and S2 x 1
	S4 index set 2 + S2 index set 1 or 2	

Figure 1 Index plate layout showing four index sets for S2 flow cell sequencing

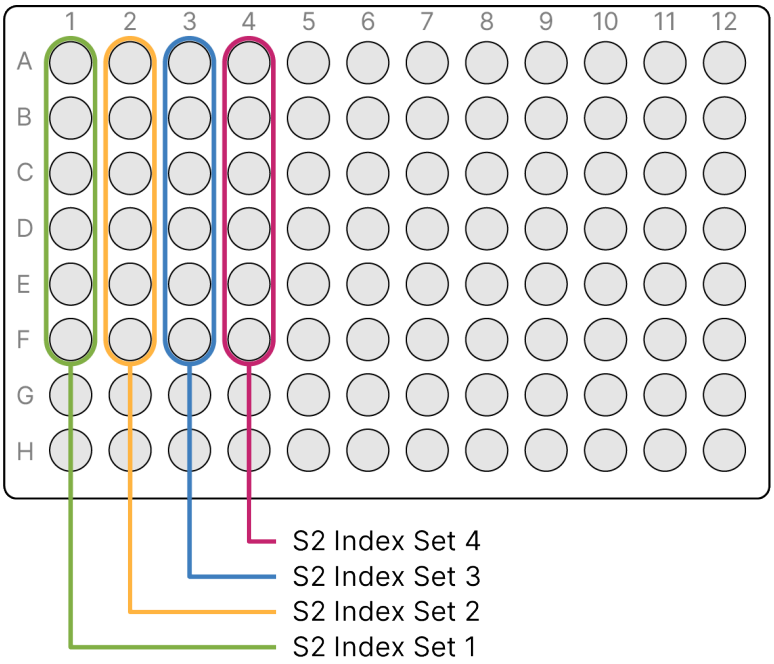


Table 4 S2 Index Sets for S2 flow cell

	S2 Index Set 1 (Green)	S2 Index Set 2 (Yellow)	S2 Index Set 3 (Blue)	S2 Index Set 4 (Magenta)
	1	2	3	4
A	UDP0037	UDP0065	UDP0081	UDP0089
B	UDP0038	UDP0066	UDP0082	UDP0090
C	UDP0039	UDP0067	UDP0083	UDP0091
D	UDP0040	UDP0068	UDP0084	UDP0092
E	UDP0041	UDP0069	UDP0085	UDP0093
F	UDP0042	UDP0070	UDP0086	UDP0094

Figure 2 Index plate layout showing two index sets for S4 flow cell sequencing

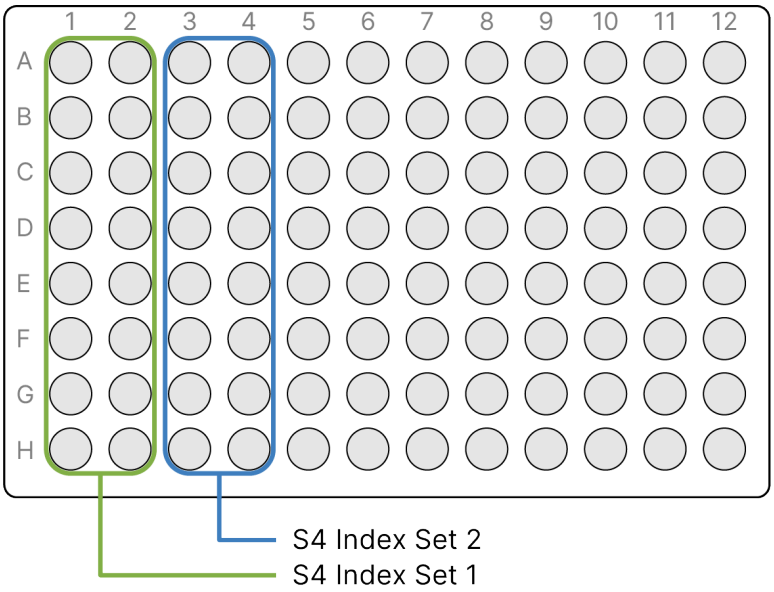


Table 5 S4 Index Sets for S4 flow cell

	S4 Index Set 1 (Green)		S4 Index Set 2 (Blue)	
	1	2	3	4
A	UDP0037	UDP0065	UDP0081	UDP0089
B	UDP0038	UDP0066	UDP0082	UDP0090
C	UDP0039	UDP0067	UDP0083	UDP0091
D	UDP0040	UDP0068	UDP0084	UDP0092
E	UDP0041	UDP0069	UDP0085	UDP0093
F	UDP0042	UDP0070	UDP0086	UDP0094
G	UDP0043	UDP0071	UDP0087	UDP0095
H	UDP0044	UDP0072	UDP0088	UDP0096

Record unique batch name and sample data, including Sample ID, associated index plate well ID (refer to *Appendix A* on page 77), library plate, library plate well ID, and library tube ID (if known). This information is entered during run creation.

For instructions on how to use the application to Create Runs, refer to TruSight Whole Genome Analysis Application Guide (document # 200049931). Record the Run Name to use during consumables loading.

⚠ CAUTION

Make sure that the indexes and associated samples used during library prep match those recorded and used to Create Run. Discrepancies may cause the reporting of incorrect results or no results.

Prepare for Protocol

Prepare Reagents and Equipment

If planning to sequence same day, thaw sequencing consumables in advance. Refer to NovaSeq 6000Dx Instrument Product Documentation (document # 200010105) for detailed instructions.

1. Preheat microsample incubator with MIDI plate insert to 47°C.
2. Remove the following reagents from the box and thaw as follows.

Table 6 -25°C to -15°C Storage

Reagent	Box Name	Thaw Instructions
BLT-PF	TruSight Whole Genome Dx Library Prep 1	Thaw at room temperature for 30 minutes.
ELM	TruSight Whole Genome Dx Library Prep 1	Thaw at room temperature for 30 minutes. Then keep on ice until needed.
HP3	TruSight Whole Genome Dx Library Prep 1	Thaw at room temperature for 30 minutes.
TB1	TruSight Whole Genome Dx Library Prep 1	Thaw at room temperature for 30 minutes.
UD Indexes	TruSight Whole Genome Dx 32 Unique Dual Indexes	Thaw at room temperature for 30 minutes.

Table 7 15°C to 30°C Storage

Reagent	Box Name	Thaw Instructions
CB	TruSight Whole Genome Dx Library Prep 2	Use at room temperature.
RSB	TruSight Whole Genome Dx Library Prep 2	Use at room temperature.
ST2	TruSight Whole Genome Dx Library Prep 2	Use at room temperature.
TWB2	TruSight Whole Genome Dx Library Prep 2	Use at room temperature.
NB	TruSight Whole Genome Dx Library Prep 2	Use at room temperature.

CAUTION

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.

Prepare DNA Samples

Prepare the following consumables.

- Quantified gDNA samples:
  a. Bring to room temperature.
  b. Centrifuge briefly to collect droplets.
  c. Pulse vortex or pipette to mix, and then centrifuge briefly.
- RSB—Vortex or invert to mix. Keep at room temperature.
  – RSB and TWB2 are packaged in similar tubes. Carefully read each label before use.

Procedure

Depending on the DNA input, which varies based on the DNA quantitation method used, calculate the volumes required to prepare diluted DNA samples. Formulas are provided below for the three DNA quantitation methods tested. Refer to *DNA Input Recommendations* on page 10 and *Appendix B* on page 80 for more information.

Calculations assume a minimum pipetting volume of 2.0 µl and include 10% overage. Rounding should be performed at the last steps, after calculations are completed, using the required number of decimals to ensure accurate pipetting.

Option 1: 280 ng DNA Input for Quant and Qubit Broad Range Quantitation Methods

The minimum DNA stock concentration of sample is 11.2 ng/µl. Samples < 11.2 ng/µl are more likely to fail library QC after sequencing. Depending on the concentration of the DNA stock, use one of the below equations to perform calculations.

1. For DNA stock concentration 11.2 to 154.0 ng/µl, calculate the volume of DNA stock and RSB needed using a total volume of diluted DNA of 27.5 µl (25 µl plus 10% overage) as constant:
   a. Calculate the volume of DNA stock:

$$DNA\ stock\ volume\ (\mu l) = \frac{(Input\ DNA\ target\ (ng) + 10\%\ overage)}{DNA\ stock\ concentration\ (ng/\mu l)}$$
$$= 280\ ng \times 1.1\ /\ DNA\ stock\ concentration\ (ng/\mu l)$$
$$= 308\ ng\ /\ DNA\ stock\ concentration\ (ng/\mu l)$$

b. Calculate the volume of RSB stock:

$$RSB\ volume\ (\mu l) = Total\ volume\ of\ diluted\ DNA\ (\mu l) - calculated\ DNA\ stock\ volume\ (\mu l)$$
$$= 27.5\ (\mu l) - calculated\ DNA\ stock\ volume\ (\mu l)$$

c. Verify calculations: Confirm the calculated DNA stock volume (µl) + the calculated volume of RSB (µl) = 27.5 µl, the total volume of diluted DNA (a constant, 25 µl plus 10% overage).

2. Alternatively, for DNA stock concentrations > 154.0 ng/µl, calculate the total volume of diluted DNA and RSB needed using the DNA stock volume 2.0 µl and target diluted DNA stock concentration 11.2 ng/µl as constants.

a. Calculate the total volume of diluted DNA:

$$Total\ volume\ of\ diluted\ DNA\ (\mu l) = \frac{DNA\ stock\ concentration\ (ng/\mu l) \times volume\ of\ DNA\ stock\ (\mu l)}{Target\ diluted\ DNA\ stock\ concentration}$$
$$= DNA\ stock\ concentration\ (ng/\mu l) \times 2.0\ \mu l\ /11.2\ ng/\mu l$$

b. Calculate the volume of RSB:

$$RSB\ volume\ (\mu l) = Calculated\ total\ volume\ of\ diluted\ DNA\ (\mu l) - DNA\ stock\ volume\ (\mu l)$$
$$= Calculated\ total\ volume\ of\ diluted\ DNA\ (\mu l) - 2.0\ \mu l$$

c. Verify calculations: Confirm the calculated total volume diluted DNA (µl) - the calculated volume of RSB (µl) = 2.0 µl, the DNA stock volume (a constant).

Proceed to Step 3 below.

Option 2: 350 ng DNA Input for Accuclear Ultra High Sensitivity Quantitation Method

The minimum DNA stock concentration of sample is 14.0 ng/µl. Samples < 14.0 ng/µl are more likely to fail library QC after sequencing. Depending on the concentration of the DNA stock, use one of the below equations to perform calculations.

1. For DNA stock concentration 14.0 to 192.5 ng/µl, calculate the volume of DNA stock and RSB needed using a total volume of diluted DNA of 27.5 µl (25 µl plus 10% overage) as constant:

a. Calculate the volume of DNA stock:

$$DNA\ stock\ volume\ (\mu l) = \frac{(Input\ DNA\ target\ (ng) + 10\%\ overage)}{DNA\ stock\ concentration\ (ng/\mu l)}$$
$$= 350\ ng \times 1.1\ /\ DNA\ stock\ concentration\ (ng/\mu l)$$
$$= 385\ ng\ /\ DNA\ stock\ concentration\ (ng/\mu l)$$

b. Calculate the volume of RSB stock:

$$RSB\ volume\ (\mu l) = Total\ volume\ of\ diluted\ DNA\ (\mu l) - calculated\ DNA\ stock\ volume\ (\mu l)$$
$$= 27.5\ (\mu l) - calculated\ DNA\ stock\ volume\ (\mu l)$$

c. Verify calculations: Confirm the calculated DNA stock volume (µl) + the calculated volume of RSB (µl) = 27.5 µl, the total volume of diluted DNA (a constant, 25 µl plus 10% overage).

2. Alternatively, for DNA stock concentrations > 192.5 ng/µl, calculate the total volume of diluted DNA and RSB needed using the DNA stock volume 2.0 µl as a constant.
   a. Calculate the total volume of diluted DNA:

   $$Total\ volume\ of\ diluted\ DNA\ (\mu l) = \frac{DNA\ stock\ concentration\ (ng/\mu l) \times 2.0\ \mu l}{14.0\ ng/\mu l}$$

   b. Calculate the volume of RSB:

   $$RSB\ volume\ (\mu l) = Total\ volume\ of\ diluted\ DNA\ (\mu l) - DNA\ stock\ volume\ (\mu l)$$
   $$= Total\ volume\ (\mu l) - 2.0\ \mu l$$

   c. Verify calculations: Confirm the calculated total volume diluted DNA (µl) - the calculated volume of RSB (µl) = 2.0 µl, the DNA stock volume (a constant).

3. Label a new 0.5 ml microcentrifuge tube for each diluted sample.
4. Add volume of RSB calculated above to the respective tube for each diluted sample.
5. Add volume of DNA stock calculated above to the respective tube for each diluted sample.
6. Pulse vortex, and then centrifuge briefly.

Library Preparation

Use the preparation steps in this section to prepare reagents in advance.

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

Preparation

Prepare the following consumables:

- BLT-PF (Bead-Linked Transposomes PCR-Free)—Vortex to mix. If using multiple tubes, vortex to mix and then combine.
- TB1 (Tagmentation Buffer 1):
  a. Vortex to mix.
  b. Centrifuge briefly.
- ST2 (Stop Tagment Buffer 2):
  a. Inspect for precipitation. If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates dissolve.
  b. Vortex thoroughly, and then centrifuge briefly.
- ELM (Extension Ligation Mix):
  a. Invert to mix. Do not vortex.
  b. Store on ice until use.
- HP3 (2N NaOH):
  a. Vortex, and then centrifuge briefly.

b. Keep at room temperature.

- NB (Neutralization Buffer):
  a. Vortex, and then centrifuge briefly.
  b. Keep at room temperature.
- CB (Cleanup Beads):
  a. Vortex 1 minute.
  b. Invert 2–5 times, and then vortex thoroughly to resuspend.
- Index adapters (UDI PCR-Free (32 Indexes)):
  a. Vortex, and then centrifuge briefly.
  b. Keep at room temperature.
- TWB2 (Tagmentation Wash Buffer 2):
  a. Label the tube cap TWB2.
  b. Invert thoroughly to mix.
- In a microcentrifuge tube labeled 0.2N NaOH, combine the following volumes to prepare 0.2N NaOH according to the planned batch size. Vortex to mix.

NOTE If planning to pool and denature libraries on the same day, prepare additional 0.2N NaOH. Refer to *Preparation* on page 29.

Reagent	6 Samples (µl)	12 Samples (µl)	16 Samples (µl)	18 Samples (µl)	22 Samples (µl)	24 Samples (µl)
HP3	30	60	80	90	110	120
RSB	270	540	720	810	990	1080

- In a 15 ml conical tube, combine the following volumes to prepare 80% EtOH according to the planned batch size. Overage for trough usage is included. Vortex to mix.

Reagent	6 Samples (ml)	12 Samples (ml)	16 Samples (ml)	18 Samples (ml)	22 Samples (ml)	24 Samples (ml)
100% ethanol, pure (200 proof)	4	8	8	12	12	12
Nuclease-free water	1	2	2	3	3	3

CAUTION

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.

Tagment Genomic DNA

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

Consumables

- 96-well MIDI plate
- BLT-PF (Bead-Linked Transposomes PCR-Free)
- Tagmentation Buffer 1 (TB1)
- ST2 (Stop Tagment Buffer 2)

Procedure

1. Confirm microsample incubator with MIDI plate insert is preheated to 47°C.
2. Label a new 96-well MIDI plate LP1 (Library Plate 1).
3. Designate and record sample well IDs for tagmentation of diluted DNA samples and reagents.
4. Transfer 25 µl diluted sample DNA to each well.
5. Add 10 µl TB1 to each well.
6. Vortex BLT-PF vigorously for 1 minute to resuspend. Do not centrifuge. Repeat as necessary.
7. Add 15 µl BLT-PF to each well.
8. Seal and shake LP1 at 1800 rpm for 1 minute.
9. Incubate LP1 in preheated microsample incubator at 47°C for 8 minutes.

NOTE Light condensation on the plate seal is expected. Do not centrifuge.

10. Remove seal and add 10 µl ST2 to each well.
11. Seal and shake LP1 at 1800 rpm for 1 minute then proceed to the next step.

Post Tagmentation Cleanup

The following steps wash away unbound DNA and performs buffer exchange to prepare for the next step.

Consumables

- TWB2 (Tagmentation Wash Buffer 2)
- Trough

About Reagents

- Pipette TWB2 slowly to minimize foaming.
- RSB and TWB2 are packaged in similar tubes. Carefully read each label before use.

Procedure

1. Remove seal and place LP1 on the magnetic stand and wait until the liquid is clear (2 minutes).
2. Prepare TWB2 trough with volumes according to the following table and clearly label the trough TWB2. Volumes include 1 ml overage for trough dead volume. Keep trough for later steps.

Reagent	6 Samples (µl)	12 Samples (µl)	16 Samples (µl)	18 Samples (µl)	22 Samples (µl)	24 Samples (µl)
TWB2	3700	6400	8200	9100	10900	11800

3. With LP1 on the magnetic stand, use a multichannel pipette set to 60 µl to remove and discard supernatant from each well without disturbing the bead pellet.
4. Using a multichannel pipette, add 150 µl TWB2 to each well.
5. Seal and shake LP1 at 1800 rpm for 1 minute.
6. Remove seal and place LP1 on the magnetic stand and wait until the liquid is clear (2 minutes).
7. Return BLT-PF to frozen storage during incubation then proceed to the next step.

Ligate Indexes

In this section users ligate the unique dual index adapters to each sample according to indexing planned during *Batch Planning and Run Creation* on page 15.

Consumables

- ELM (Extension Ligation Mix)
- Index adapters (UDI PCR-Free (32 Indexes))
- TWB2 (Tagmentation Wash Buffer 2) trough
- 0.2N NaOH (Diluted HP3)

About Reagents

- The index plate wells cannot be reused.
- Aspirate and dispense ELM slowly due to the viscosity of the solution.
- RSB and TWB2 are packaged in similar tubes. Carefully read each label before use.

Procedure

1. Keep LP1 on the magnetic stand and complete the following steps:
   a. Use a multichannel pipette set to 150 µl to remove and discard supernatant from each well.
   b. Without disturbing the bead pellet, use a 20 µl pipette to remove and discard residual TWB2 from each well.
   c. Add 45 µl ELM to each well.
   d. Pierce the foil seal on the index adapter plate for each of the planned index wells using a P200 multichannel pipette and new pipette tips. To avoid contamination, use a new pipette tip for each well.
   e. Add 5 µl index adapters to the corresponding sample wells of the LP1 according to indexes selected during batch planning using a P-10 or P-20 multichannel pipette.
2. Seal and shake LP1 at 1800 rpm for 1 minute.
3. Incubate LP1 in preheated microsample incubator at 47°C for 8 minutes.

   NOTE Light condensation on the plate seal is expected. Do not centrifuge.

4. Return ELM to frozen storage during incubation.
5. Remove seal and place LP1 on the magnetic stand and wait until the liquid is clear (2 minutes).
6. With LP1 on the magnetic stand, use a multichannel pipette set to 50 µl to remove and discard supernatant from each well without disturbing the bead pellet.
7. Wash beads as follows.
   a. Add 150 µl TWB2 onto the beads in each well using a multichannel pipette.
   b. Seal and shake LP1 at 1800 rpm for 1 minute.
   c. Remove seal and place LP1 on the magnetic stand and wait until the liquid is clear (2 minutes).
   d. With LP1 on the magnetic stand, use a multichannel pipette set to 150 µl to remove and discard supernatant from each well without disturbing the bead pellet.
8. Wash beads a **second** time.
9. With LP1 on the magnetic stand, use a multichannel pipette set to 20 µl to remove and discard residual TWB2 from each well without disturbing the bead pellet.
10. Add 45 µl of previously prepared 0.2N NaOH to each well.
11. Seal and shake LP1 at 1800 rpm for 1 minute then proceed to the next section.

Size-Selection and Clean Up Libraries

This step uses a double-sided size-selection of libraries. In the first step, Cleanup Beads are added to the eluted libraries and BLT-PF beads. Then, supernatant containing the eluted single-stranded library is transferred to a new plate while fragments that are too large remain behind. In the second step, Cleanup Beads are added to the transferred libraries and fragments that are too small are removed. Then, libraries are eluted and transferred to the final library plate (FLP).

Consumables

- 96-well MIDI plate
- Troughs (3)
- PCR plate
- CB (Cleanup Beads)
- RSB (Resuspension Buffer)
- Freshly prepared 80% ethanol (80% EtOH)

Preparation

1. Vortex CB, and then invert until fully resuspended.
2. Prepare CB trough with volumes according to the following table and label trough CB. Volumes are sufficient for both addition steps and include 1 ml overage in the trough for trough dead volume. There is no need to mix between CB addition steps. The beads will remain dispersed for the duration of the procedure.

Reagent	6 Samples (µl)	12 Samples (µl)	16 Samples (µl)	18 Samples (µl)	22 Samples (µl)	24 Samples (µl)
CB	1480	1960	2280	2440	2760	2920

Procedure

1. Remove seal and add 40 µl CB to wells of the LP1 MIDI plate containing BLT-PF and 0.2N NaOH.
2. Seal and shake LP1 at 1800 rpm for 1 minute.
3. Incubate LP1 off magnetic stand at room temperature for 2 minutes.
4. Remove seal and place LP1 on the magnetic stand and wait until the liquid is clear (5 minutes).
5. While the plate incubates, label a new 96-well MIDI plate LP2.
6. *Transfer* 80 µl supernatant from LP1 while on the magnetic stand to the corresponding wells of LP2 using a multichannel pipette.
7. Add 40 µl CB to each well in the LP2 MIDI plate.
8. Seal and shake LP2 at 1800 rpm for 1 minute.
9. Discard the LP1 MIDI plate.
10. Incubate LP2 off magnetic stand at room temperature for 2 minutes.
11. Remove seal and place LP2 on the magnetic stand and wait until the liquid is clear (5 minutes).
12. With LP2 on the magnetic stand, use a multichannel pipette set to 120 µl to remove and discard supernatant from each well without disturbing the bead pellet.
13. Pour previously prepared 80% EtOH into a labeled trough and wash beads with LP2 on magnet as follows.
    a. Add 180 µl 80% EtOH using a multichannel pipette.

b. Wait 30 seconds.

c. Use a multichannel pipette set to 180 µl to remove and discard supernatant from each well without disturbing the bead pellet.

14. Wash beads a **second** time.
15. With LP2 on the magnetic stand, use a multichannel pipette set to 20 µl to remove and discard residual EtOH from each well without disturbing the bead pellet.
16. Keep LP2 on the magnetic stand for 4 minutes to air dry.
17. Discard unused 80% EtOH and trough.
18. Prepare RSB trough with volumes according to the following table and label trough RSB. Volumes include 1 ml overage for trough dead volume.

Reagent	6 Samples (µl)	12 Samples (µl)	16 Samples (µl)	18 Samples (µl)	22 Samples (µl)	24 Samples (µl)
RSB	1390	1780	2040	2170	2430	2560

19. Add 65 µl RSB onto the beads in each well.
20. Seal and shake LP2 at 1800 rpm for 1 minute.
21. Incubate LP2 at room temperature for 2 minutes.
22. Remove seal and place LP2 on the magnetic stand and wait until the liquid is clear (2 minutes).
23. Label a new PCR plate FLP (Final Library Plate) and with the batch name used when creating the run.
24. *Transfer* 60 µl supernatant from LP2 while on the magnetic stand to the corresponding wells of FLP using a multichannel pipette.

CAUTION

Supernatant contains final library and will be used during the pool and denature step. Do not discard.

25. Discard all troughs along with unused reagents in troughs.
26. Discard LP2 MIDI plate.

SAFE STOPPING POINT

If stopping, seal the final library plate (FLP) with Microseal B and store at -25°C to -15°C for up to 14 days.

Pool and Denature Libraries

In this section users create pools planned in *Batch Planning and Run Creation* on page 15 and dilute and denature.

Consumables

- HP3 (2N NaOH), or 0.2N NaOH if prepared on same day—Vortex, and then centrifuge briefly.

- NB (Neutralization Buffer)—Vortex, and then centrifuge briefly.
- RSB (Resuspension Buffer)—Vortex or invert to mix.
- Microcentrifuge tubes (1 for reagent preparation and 1 for each planned library pool)
- NovaSeq 6000Dx Library Tube (PN 20062290 or PN 20062291) (1 tube for each planned library pool)

Preparation

1. Combine the following volumes in a microcentrifuge tube to prepare 0.2N NaOH. Label tube 0.2N NaOH. If additional 0.2N NaOH was prepared during Library Prep and protocol is performed on the same day, skip this step.
   To prevent small pipetting errors, extra volume is prepared.

Reagent	Volume for Each S2 Flow Cell (µl)	Volume for Each S4 Flow Cell (µl)
HP3	5	10
RSB	45	90

2. Vortex, and then centrifuge briefly.

Procedure

1. If the FLP plate was stored frozen, prepare as follows. Otherwise, move to step 2.
   FLP plate:
   a. Thaw at room temperature for 30 minutes.
   b. Centrifuge at 1000 × g for 1 minute.
   c. Remove seal from FLP.
   d. Pipette mix 5 to 10 times using a multichannel pipette set to 30 µl.
   e. Seal and centrifuge at 1000 × g for 1 minute.
2. Select one of the following options to pool, denature, and dilute the libraries for each set of 6 or 16 samples planned for sequencing.
   Option 1 Sequence 6 libraries on S2 flow cell.
   a. For each library pool, label a new microcentrifuge tube with the pool name, for example pooled libraries (PL) 1, 2, 3, etc.
   b. Remove seal and transfer 25 µl of each DNA library barcoded from a given S2 index set from the FLP plate to the PL tube for each corresponding planned run according to the sequencing pools planned during *Batch Planning and Run Creation* on page 15. For example, combine libraries prepared using S2 Index Set 1 into the PL tube.
   c. Apply adhesive plate seal to the FLP plate and return to storage.
   d. Add 37 µl 0.2N NaOH to each PL tube.
   e. Vortex each PL tube to mix. Centrifuge briefly.

f. Incubate each PL tube at room temperature for 8 minutes.

g. Add 38 µl of NB to each PL tube.

h. Vortex each PL tube to mix. Centrifuge briefly.

i. Transfer 225 µl of denatured, diluted library to a clean NovaSeq 6000Dx Library Tube.

CAUTION

If previously specified, the NovaSeq 6000Dx Library Tube ID will be used to identify and associate the planned run. Make sure the Library Tube ID into which the pool is transferred is the same Library Tube ID specified in Create Run, or an incorrect association of sample results may occur. If Library Tube ID is specified in planned run, confirm the correct tube is used. If not previously specified, record the Library Tube ID used and revise the planned run, otherwise the associated planned run(s) will need to be manually selected when loading the instrument using the run name.

Option 2 Sequence 16 libraries on S4 flow cell.

a. Label a new microcentrifuge tube with the pool name, for example pooled libraries (PL) 1, 2, 3, etc.

b. Remove seal and transfer 18 µl of each DNA library from the FLP plate to the PL tube according to the sequencing pool planned during *Batch Planning and Run Creation* on page 15. For example, combine libraries using S4 Index Set 1 into the PL tube.

c. Apply adhesive plate seal to the FLP plate and return to storage.

d. Add 22 µl of RSB to the PL tube.

e. Add 77 µl 0.2N NaOH to the PL tube.

f. Vortex PL tube to mix. Centrifuge briefly.

g. Incubate PL tube at room temperature for 8 minutes.

h. Add 78 µl of NB buffer to PL tube.

i. Vortex PL tube to mix. Centrifuge briefly.

j. Transfer 465 µl of denatured, diluted library to a clean NovaSeq 6000Dx Library Tube.

CAUTION

If previously specified, the NovaSeq 6000Dx Library Tube ID will be used to identify and associate the planned run. Make sure the Library Tube ID into which the pool is transferred is the same Library Tube ID specified in Create Run, or an incorrect association of sample results may occur. If Library Tube ID is specified in planned run, confirm the correct tube is used. If not previously specified, record the Library Tube ID used and revise the planned run, otherwise the associated planned run(s) will need to be manually selected when loading the instrument using the run name.

3. Proceed directly to sequencing if planning to start the run the same day.

SAFE STOPPING POINT

If stopping, cap the NovaSeq 6000Dx Library Tube and store at -25°C to -15°C for up to 30 days.

Prepare for Sequencing

1. Follow preparation instructions in the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105) for consumables in the kit planned for sequencing.
2. If NovaSeq 6000Dx Library Tube containing pooled library was stored frozen, prepare as follows. If proceeding directly from previous section, move to 3.
   a. Thaw at room temperature for 30 minutes.
   b. Remove cap and gently pipette mix five times using a P1000 pipette set to 300 µl for the S4 flow cell library pool or a P200 pipette set to 145 µl for the S2 flow cell library pool.
   c. Cap NovaSeq 6000Dx Library Tube and shake any droplets to the bottom by hand. Do not vortex or centrifuge.
3. Load consumables. Refer to NovaSeq 6000Dx Instrument Product Documentation (document # 200010105) for details.

Interpretation Of Results

TruSight Whole Genome is designed to sequence the human whole genome. Variants are reported for samples that pass analytical quality controls (QC) for use with downstream tertiary analysis germline applications.

- A sequencing, FASTQ, or sample quality result is considered valid only if the quality metric meets or exceeds the defined specification. If the quality metric is below the defined specification, the performance will be reported as FAIL and the sample must be repeated. For information on the quality metric specifications used to determine sample validity, refer to *Quality Controls* on page 31.
- Samples that pass all quality thresholds are expected to provide variant calling performance described in the accuracy study (refer to *Accuracy* on page 41).
- Small variants are annotated with high, intermediate, or low confidence based on each variant type's expected performance (refer to *Small Variants Confidence Tier Determination* on page 37).
- Interpretation of all variant information must be validated by the laboratory using the analysis output files provided. Refer to TruSight Whole Genome Analysis Application Guide (document # 200049931) for a description of information provided in the output files.

Quality Controls

Sequencing run and sample validity are determined automatically using analytical controls and are reported by the TruSight Whole Genome Analysis Application (refer to Table 8 for additional details about the quality control metric specifications). TruSight Whole Genome does not require the use of external positive controls.

- The QC results are reported in a consolidated report, for all samples in a run, and in individual sample QC reports. The reports are output by the software to the analysis folder. Refer to the TruSight Whole Genome Analysis Application Guide (document # 200049931) for location of the analysis folder and the run folder.

- Failure of sequencing run quality control specification invalidates the sequencing run and halts additional analysis.
- Failure of any sample FASTQ or library specification invalidates the sample library and prevents the output of the associated CRAM or VCF files.
- Additional quality control measures may apply in accordance with local, state, and/or federal regulations or accreditation requirements.

F or more information on repeating sequencing runs or library preparation, refer to *Troubleshooting* on page 65.

Table 8 TruSight Whole Genome Quality Control Metric Specification Descriptions

	Metric	Specification	Description
Sequencing Run QC	Total % ≥ Q30	≥ 85	Measure of base quality at the run level. Minimum specification is set because too low %Q30 runs will not pass Q30 bases in Sample Library QC.
FASTQ QC	Yield per sample (bps)	≥ 90,000,000,000	Minimum is set to be equivalent to ~26x average autosomal coverage to triage samples that will not pass library QC to reduce analysis time.

	Metric	Specification	Description
Sample Library QC	Average autosomal coverage	≥ 35	Average coverage across the autosomes. Minimum specification is set to ensure analytical performance.
	Percent of autosomes with coverage greater than 20X	≥ 93.94	Measure of coverage uniformity that detects issues not necessarily related to GC bias. Minimum specification is set to ensure analytical performance.
	Normalized coverage at 60% to 79% GC bins	0.82 ≤ x ≤ 1.13	Measure of coverage uniformity that detects GC bias, specifically a loss of coverage in areas of the genome with higher %GC and lower %AT base composition. Minimum and maximum specifications are set to ensure analytical performance.
	Normalized coverage at 20% to 39% GC bins	0.97 ≤ x ≤ 1.06	Measure of coverage uniformity that detects GC bias, specifically a loss of coverage in areas of the genome with lower %GC and higher %AT base composition. Minimum and maximum specifications are set to ensure analytical performance.
	Average mitochondrial coverage	≥ 500	Coverage of the mitochondrial chromosome. Minimum specification is set to ensure mitochondrial SNV limit of detection.
	Percent Q30 bases	≥ 85	Measure of base quality. Minimum specification is set to ensure analytical performance.
	Estimated sample contamination	≤ 0.005	Detects contaminating reads from other samples. Maximum specification is set to ensure mitochondrial SNV limit of detection (the variant type with highest sensitivity to contamination).

Performance Characteristics

The following validation studies were performed using the TruSight Whole Genome workflow outlined in the *Instructions for Use* on page 15, and were designed to ensure assay robustness against common sources of variation and to provide recommendations for consistent performance. These studies used the analytical QC metric specifications outlined in Table 8 as the benchmark for successful assay performance and as a prerequisite for establishing analytical variant calling performance.

Cross-Contamination

The cross-contamination study evaluated incorrect Index Read detection due to well-to-well contamination during sample library preparation and run-to-run contamination between consecutive sequencing runs. 24 blood samples were used to evaluate cross-contamination. 24 libraries in total were each prepared by two operators using the S2 configuration index sets 1–4, and pooled libraries were sequenced in order of index set on one NovaSeq 6000Dx Instrument. 16 libraries each were prepared by two operators using the S4 configuration index sets 1 and 2 in two replicates, and pooled libraries with alternating index sets were sequenced on the same NovaSeq 6000Dx.

To evaluate cross-contamination, correct index reads were compared to index reads from adjacent wells for well-to-well contamination and previous sequencing run for run-to-run contamination. The amount of run-to-run contamination was ≤ 0.003178% for S2 and ≤ 0.002487% for S4 runs. To evaluate sample-to-sample contamination, the sample library QC metric for estimated sample contamination was used. The amount of sample-to-sample contamination was 0.001, the lowest value reported out by the analysis software. These results indicate there is low risk for contamination within the library preparation and sequencing workflows.

In-Use and Intermediate Stability

Library preparation reagents were assessed for stability during kit use, including multiple freeze-thaw events and open-tube stability.

For freeze-thaw cycle testing, the frozen components were subjected to five freeze-thaw events to support one event for unpacking and four events for kit usage. For in-use stability, the volume required to prepare six sample libraries was removed at each of three freeze-thaw cycles to simulate volume depletion during use, and components were stored for an additional 31 days prior to testing. Upon testing with gDNA extracted from six blood donors, all data passed assay analytical control metrics. These results indicate frozen library preparation reagents may be used with up to four freeze-thaw cycles and 30-days in-use stability.

Intermediate stability was assessed for the individual libraries and the pooled and denatured libraries. All data passed assay analytical control metrics indicating up to 14-days stability for the individual libraries and up to 30-days stability for the pooled and denatured libraries when stored frozen (-25°C to -15°C) as described in the safe stopping points.

Blood Specimen Collection and Storage

Blood collection tube compatibility and specimen storage was examined using four donors and blood drawn into EDTA collection tubes from three different suppliers. Genomic DNA (gDNA) was extracted from each upon arrival for time-zero and then again after blood was kept for 16, 33, and 43 days of storage at 2°C to 8°C. The extracted gDNA was stored frozen (-25°C to -15°C) in the elution buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) and then quantified and used for library preparation and sequencing. All data passed assay analytical control metrics indicating assay compatibility with three different EDTA blood collection tubes and blood stored up to five weeks at 2°C to 8°C.

DNA Extraction Method Evaluation

Three commercially available extraction kits were evaluated for assay performance. Two kits used magnetic beads, one with and one without solid phase and cellulose-based binding, and one kit used a silica membrane based nucleic acid purification method using spin columns (Table 9).

The evaluation was performed by two operators with one lot of extraction reagents per method and whole blood collected in EDTA tubes from four putatively healthy donors. Each blood sample was extracted four separate times according to the manufacturer's instructions across non-consecutive days for 16 total observations per kit. The extracted gDNA was used to prepare libraries for sequencing and analysis.

All observations (16/16) for each extraction method passed the assay analytical control metrics. Assay performance was not affected by choice of sample gDNA extraction method. Analytical accuracy and reproducibility studies used gDNA extracted with Kit 3 (silica filter column isolation with spin columns).

Table 9 Extraction Methods Tested for TruSight Whole Genome Performance

Kit	Extraction Method
1	Magnetic bead extraction with solid phase reversible immobilization (SPRI)
2	Magnetic bead extraction with mobile solid phase and cellulose-based binding
3	Silica filter column isolation with spin columns

DNA Input Sensitivity

The amount of gDNA input recommended for testing per sample is 280 ng or 350 ng depending on DNA quantification methods listed in *DNA Input Recommendations* on page 10.

To determine performance across a range of gDNA input concentrations, the amount of DNA used in the assay was tested at levels ranging ±28.6% of the recommended input. The results demonstrated that -25% of the recommended gDNA input is a lower limit for the assay. The assay functions appropriately with gDNA input up to +28.6% of the recommended input.

The characterization of three distinct quantification methods demonstrated that different methods have different levels of variability and may produce dissimilar results. If using a method other than those listed in *DNA Input Recommendations* on page 10, the target gDNA input may need to be optimized. It is recommended that gDNA for samples intended for a particular library preparation batch and sequencing run are quantified together to eliminate batch-to-batch variability when possible, or process controls are used to ensure ≤ 25% gDNA quantitation batch-to-batch variability.

Interfering Substances

This study evaluated performance with both endogenous and exogenous substances associated with human blood and blood collection tubes. Bilirubin, hemoglobin, and triglycerides were selected for evaluation to simulate icteric, hemolyzed and lipemic samples, respectively. Biotin and EDTA were selected for evaluation

due to presence in blood and blood collection tubes (BCTs), and for potential impact on the assay chemistry. Substances were spiked into the donor blood samples prior to extraction either directly or after dissolving in solvent. Test concentration and details of the spike-in for each substance is provided in the following table.

Table 10 Interfering Substances Tested for TruSight Whole Genome Performance

Substance	Test Concentration	Solvent used in Spike Solution	% Spike added to Blood
Bilirubin (unconjugated)	40 mg/dL (0.4 mg/ml)[1]	DMSO	4 %
Hemoglobin	1000 mg/dl (10 mg/ml)[1]	N/A – Dissolved in blood	N/A – Dissolved in blood
Triglycerides	1500 mg/dl (15 mg/ml)[1]	100% Ethanol	4 %
Biotin	0.00351 mg/ml[2]	Water	4%
EDTA	5.4 mg/ml[3]	Water	3 %

[1] The concentrations were chosen to be the highest observed concentrations according to the "Supplemental Tables for Interference Testing in Clinical Chemistry, CLSI EP37-ED1:2018".
[2] The concentration was chosen to be three times the "Highest Drug Concentration Under Therapeutic Treatment" stated in "Supplemental Tables for Interference Testing in Clinical Chemistry, CLSI EP37-ED1:2018".
[3] The concentration was chosen based on the EDTA concentration which varies in blood collection tubes ranging up to 1.8 mg/ml and to simulate a short fill event a blood draw of 33% the nominal BCT volume leading to 3x higher EDTA concentration in blood corresponding to 5.4 mg/ml.

Blood from four donors was used in testing. For each interfering substance, an aliquot of whole blood from each donor was spiked with the interferent and then split among four gDNA extraction replicates. A control was processed similarly without addition of substances. The paired test and control conditions were processed for each donor within the same extraction event, and the extracted gDNA was then processed within a single library prep and sequencing event. There was no impact to assay performance and no evidence for interference in response to any of the substances tested.

Sample Indexing Equivalency

TruSight Whole Genome provides a choice of four 6-plex index sets for S2 runs or two 16-plex index sets for S4 sequencing run configurations. The assay was shown to provide equivalent performance when libraries are sequenced on either the NovaSeq 6000Dx S2 or S4 sequencing run configurations. Additionally, both the S2 and S4 run configurations were shown to achieve > 95% of sample libraries with a minimum of 35.0x coverage when tested with the prescribed index sets. Thus, different index sets and pooling used to sequence on the S2 and S4 flow cells can be used interchangeably to provide scalability to accommodate fluctuations in sample throughput and provide flexibility in laboratory processes.

Analytical Performance

Initial characterization studies were conducted to determine the confidence tier thresholds for small variants, the limit of blank/limit of detection for mitochondrial SNVs, and the size thresholds for accurate detection of STR expansions when using the TruSight Whole Genome workflow. Samples representing the variant classes assessed by TruSight Whole Genome were included in the evaluation of analytical accuracy and repeatability, including within-laboratory precision and external reproducibility. Analytical performance is reported for sequencing runs and samples that passed all quality controls, except for the contrived mixture samples used to assess mitochondrial SNVs at or near the limit of detection which failed the contamination metric. Results for each of these studies are described in sections below.

Initial Characterization Studies

Small Variants Confidence Tier Determination

For this study, a logistic regression model was trained on highly reproducible and poorly reproducible variant sites from 96 replicates of NA12878 to define thresholds for high, medium, and low confidence tiers.

High confidence bases for a given variant type are those in which predicted within-lab reproducibility meets or exceeds 99% for a given score threshold and the percentage of non-N bases that satisfy that criterion exceeds 30%. If a small variant type does not have a score threshold that meets these criteria, that variant type will not have a high confidence tier. Intermediate confidence bases are those in which predicted within-lab reproducibility meets or exceeds 95% for a given score threshold and variant type. Low confidence bases are those in which predicted within-lab reproducibility is below 95% for a given score threshold and variant type. Variant calls for a particular variant type with a high or intermediate confidence tier include the majority of the %non-N bases (ie, excluding gaps) (refer to Table 6) and demonstrate high performance when assessed against small variant truth sets and in extensive assessments of within-lab precision of NA12878 replicates.

Variant type	Confidence tier	% non-N bases
SNV	High	89.14%
	Intermediate	3.30%
	Low	7.56%
Short deletions (1-5 bp)	High	90.88%
	Intermediate	2.45%
	Low	6.67%
Intermediate deletions (6-15 bp)	Intermediate	86.94%
	Low	13.06%
Long deletions (≥ 16bp)	Intermediate	85.42%
	Low	14.58%

Variant type	Confidence tier	% non-N bases
Short insertions (1-5bp)	High	88.94%
	Intermediate	4.61%
	Low	6.45%
Intermediate insertions (6-15bp)	Intermediate	89.37%
	Low	10.63%
Long insertions (≥ 16bp)	Intermediate	48.92%
	Low	50.63%

Mitochondrial SNV Limit of Blank / Limit of Detection Determination

Limit of Blank (LoB) and Limit of Detection (LoD) studies were conducted for mitochondrial SNVs. For the mitochondrial SNV study, LoB was assessed using loci known to have no variant (ie reference call). LoD is defined as the mtDNA SNV variant allele frequency for which the detection rate of that variant is 95%.

To determine LoB and LoD for the detection of heteroplasmic mtSNVs, thoroughly characterized gDNA samples from two different blood donors were mixed in a titration study to five dilution levels with 20 replicates per dilution level. The dilution levels were designed to target mtSNV variant percentages (1.2 - 6% VAF) to mimic various levels of mitochondrial heteroplasmy. Mixed gDNA samples were processed and reads were down-sampled to achieve 500x average mitochondrial coverage. A total of 42 contrived “heteroplasmic” sites were used in downstream evaluation. A regression analysis was used to estimate the required mixing ratios to target 1x LoD and 2x LoD for a subset of mtSNVs.

Positions at which gDNA from both blood samples have reference allele genotypes were evaluated for mtSNV calls that passed filter with a non-reference allele. The false positive rate was calculated as 0.8%, consistent with a zero-LoB assumption according to the "Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures, CLSI EP17-A2-ED1:2012”. Each of the 42 positions were analyzed independently using probit regression. The LoD value was defined as the expected VAF value corresponding to the 95% (C95) detection rate. The overall reported LoD value, defined as the 95th percentile of the LoD values from the truth sites, was 4.75% VAF. The mean of the distribution of absolute differences between observed and expected VAF for all observations was calculated to be 0.83% with an upper 95% confidence limit of 0.86% VAF.

STR Expansion Threshold Determination

Due to technical limitations of spanning STRs that exceed sequencing read length (~135 bp), the observed STR length with TruSight Whole Genome will often be an underestimate of true length. Once the true STR length exceeds the median fragment length (~330 bp), the STR length estimate plateaus. For this reason, TruSight Whole Genome assesses a targeted set of loci for which the assay can accurately discriminate STRs with observed lengths within normal variation from those with lengths greater than observed in a putatively healthy population (“expanded”) (refer to Table 2 for list of loci assessed by TruSight Whole Genome).

To ensure aggregate Negative Percent Agreement (NPA) of 95% across all STR sites assessed by TruSight Whole Genome, per-loci thresholds for calling an expanded STR at that site were set to achieve an average 99.94% NPA per site. To account for the inherent variability in the STR size estimates within a putatively healthy population, thresholds were set based on the distribution of independently observed STR lengths in the putatively healthy 1000 Genomes Project data set (2,504 samples from various populations processed with DRAGEN 3.7.5 and ExpansionHunter 4.0.2).[4]

To confirm the thresholds established using the 1000 Genomes Project data set, extracted gDNA from 16 cell line reference samples (Center for Disease Control's Genetic Testing Reference Material (Get-RM) Program) with a variety of independently estimated STR sizes were processed with TruSight Whole Genome. 10 library replicates for each of the 16 samples were prepared and tested by six operators for a total of 960 observations, and STR sizes were independently estimated for each replicate. The observed sample level false positive rate across all targeted loci was 0.35%.

The limit of detection (LoD) was estimated for the 28 targeted STR loci with the cell lines tested based on the allele sizes observed with TruSight Whole Genome and the allele sizes expected based on prior independent characterization (Table 11). For select loci, a limit of detection was determined for more than one STR at the same site for a total of 35 STRs. LoD is the estimated size at which the expected STR expansion is detected for 95% of alleles based on a probit model with the confirmed thresholds for distinguishing normal and expanded STR sizes. The data across all sites with known allele sizes was pooled together to get LoD estimates for every site based on the site-specific threshold for an expanded STR. The FMR1 repeat length was systematically underestimated compared to other STRs and required a custom model to properly estimate LoD.

Confirmed site-specific thresholds for expanded STRs, estimated expected and observed LoD for targeted sites, and the disease threshold based on available literature (for illustrative purposes only) of targeted STR sites are provided in Table 11. For STR expansions longer than the threshold dictated by the read length and for which the expected length cannot be directly observed, an observed length approximates an average length that would be observed over several sequencing runs. For STR expansions shorter than the threshold dictated by the read length, the expected and observed lengths are the same.

Table 11 Estimated Detection Capability Summary for TruSight Whole Genome Targeted STR Sites

Target Locus[a]	Expanded STR Threshold (bp) based on 1000 Genomes Project data set	Estimated LoD (expected length, bp)	Estimated LoD (observed length, bp)	Disease Threshold (true length, bp)[b]
AFF2	168	266	221	600[5]
AR	114	115	115	114[6]
ATN1	90	92	92	135[7,8]
ATXN1	114	115	115	114[7,8]
ATXN10	200	298	233	3995[7,8]
ATXN2	102	102	102	105[7,8]
ATXN3	135	189	182	180[7,8]

Target Locus[a]	Expanded STR Threshold (bp) based on 1000 Genomes Project data set	Estimated LoD (expected length, bp)	Estimated LoD (observed length, bp)	Disease Threshold (true length, bp)[b]
ATXN7	60	60	60	111[7,8]
ATXN7_GCC	93	101	101	N/A
ATXN8OS	200	298	233	237[7,8]
ATXN8OS_CTA	90	92	92	N/A
C9ORF72[c]	200	298	233	360[9,10]
CACNA1A	57	57	57	60[7,8]
CBL	171	281	227	243[5]
CNBP	192	308	237	300[5,11]
CNBP_CA	102	102	102	N/A
CNBP_CAGA	68	80	80	N/A
CSTB	200	298	233	348[12,13]
DIP2B	200	298	233	N/A
DMPK	122	132	142	150[14]
FMR1	175	433	212	600[d,15]
FXN	102	102	102	198[6,16]
FXN_A	200	298	233	N/A
GLS	111	115	115	270[17]
HTT	108	115	115	120[18]
HTT_CCG	42	42	42	N/A
JPH3	99	101	101	123[19]
NIPA1	33	33	33	N/A
NOP56	84	84	84	3900[20,21]
NOP56_CGCCTG	24	24	24	N/A
NOTCH2NL	129	175	174	213[22,23]
PABPN1	27	27	27	N/A
PHOX2B	60	60	60	75[5,24]
PPP2R2B	87	90	90	198[7,8]
TBP	129	175	174	135[7,8]

[a] Loci with alternate STRs are annotated by LOCI_<ALTERNATE_REPEAT> (eg ATXN7_GCC).

[b] Disease thresholds provided for illustrative purposes only based on published literature; N/A (Not Applicable) in this column indicates that the STR may not be associated with a published pathogenic expansion.
[c] 100% of replicates of NA23378 detected an STR expansion in C9ORF72, suggestive of a previously uncharacterized expansion at that site in that sample. This cell line sample was excluded from the analysis.
[d] Intermediate expansions may also be associated with a phenotype.

This study demonstrated similar precision and accuracy profiles for STR size estimates across different targeted loci, with limit of detection for STR expansions being largely driven by the selected threshold (based on size distribution in the 1000 Genomes Project population) rather than by differences in detection capability across sites. All estimated LoD values in the expected length scale were greater than the lengths seen in putatively healthy populations and lower than many published disease thresholds, making the associated STR expansion calling thresholds useful for marking the repeat at a particular locus as potentially expanded. Thresholds reported here were used to assess accuracy of STR expansion detection.

Accuracy

Analytical accuracy was determined by comparing TruSight Whole Genome variant calls to results obtained using alternative methods. Reference methods were chosen based on considerable difference compared to TruSight Whole Genome, which uses Nextera™ bead-linked library preparation, 2-dye sequencing chemistry on the NovaSeq 6000Dx, and DRAGEN 3.9.5 for variant calling. A representative approach to validation of TruSight Whole Genome was conducted with samples representing variants across all the variant classes included in the output of the assay. A total of 459 unique samples passing analytical QC were used to evaluate accuracy of TruSight Whole Genome. Samples were tested across three lots of library preparation reagents and consumables, four lots of S4 sequencing kits, eight operators, five NovaSeq 6000Dx Instruments, and two internal sites. 31 independent library pools were prepared and sequenced.

The following table provides definitions of metrics calculated in accuracy studies.

Term	Definition
Lower Confidence Level (LCL)	One-sided 95% lower confidence limit using the Wilson method.
Negative Percent Agreement (NPA)[1]	Percent of negative sites as defined by the reference method which are concordantly identified as negative with TruSight Whole Genome.
Positive Percent Agreement (PPA)[2]	Percentage of variants called in the reference method which are concordantly called with TruSight Whole Genome.
Technical Positive Predictive Value (TPPV)[3]	Percentage of variants called with TruSight Whole Genome which are concordantly called in the reference method.

[1] For STR expansion detection accuracy and SMN1 allele detection accuracy, NPA = True Negative / (True Negative + False Positive).
[2] For STR expansion detection accuracy and SMN1 allele detection accuracy, PPA = True Positive / (True Positive + False Negative).
[3] For STR expansion detection accuracy and SMN1 allele detection accuracy, TPPV = True Positive / (True Positive + False Positive).

Small Variant Accuracy

Accuracy of small variant calling was assessed using genomic DNA extracted from peripheral whole blood of 195 putatively healthy donors. TruSight Whole Genome variant calls were compared to variant calls from a clinically validated whole-genome sequencing test performed at the Illumina Laboratory Services (ILS) CLIA Laboratory as the reference method. The reference method whole-genome sequencing workflow uses a ligation-based TruSeq™ PCR-free library preparation, 4-dye sequencing chemistry on the HiSeq™ Sequencing System, and DRAGEN 3.8.4 for variant calling. Insertions and deletions > 31 bp in size were not characterized in this study because they were not validated in the reference method.

A summary of accuracy for all small variant calls is shown in Table 12 and Table 13.

Table 12 TruSight Whole Genome Assay Accuracy for Small Variants Stratified by Confidence Tier and Size (Putatively Healthy Blood Samples)

Variant Sub-Type	Confidence Tier	Reference Method Concordant Calls	Reference Method Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
SNVs	High	261,728,580	1,573,877	261,603,149	208,639	99.4% (99.4%)	99.9% (99.9%)
	Intermediate	6,677,589	421,718	6,519,811	151,128	94.1% (94.0%)	97.7% (97.7%)
	Low	6,864,840	3,251,709	6,649,756	2,151,388	67.9% (67.8%)	75.6% (75.5%)
Short deletion (1-5 bp)	High	11,978,745	201,783	12,246,922	67,277	98.3% (98.3%)	99.5% (99.5%)
	Intermediate	2,875,258	45,290	3,050,170	47,593	98.4% (98.4%)	98.5% (98.5%)
	Low	1,802,544	228,582	1,966,974	221,449	88.7% (88.7%)	89.9% (89.8%)
Medium deletion (6-15 bp)	Intermediate	858,673	20,079	860,493	18,361	97.7% (97.7%)	97.9% (97.9%)
	Low	145,618	28,300	157,398	41,824	83.7% (83.6%)	79.0% (78.9%)
Long deletion (16-31 bp)	Intermediate	344,168	14,334	336,976	31,165	96.0% (95.9%)	91.5% (91.5%)
	Low	54,444	23,438	53,835	47,272	69.9% (69.6%)	53.2% (53.0%)

Variant Sub-Type	Confidence Tier	Reference Method Concordant Calls	Reference Method Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
Short insertion (1-5 bp)	High	11,212,366	164,651	11,380,307	49,776	98.6% (98.5%)	99.6% (99.6%)
	Intermediate	1,015,324	41,890	988,512	36,051	96.0% (96.0%)	96.5% (96.5%)
	Low	639,663	198,700	576,797	180,458	76.3% (76.2%)	76.2% (76.1%)
Medium insertion (6-15 bp)	Intermediate	790,968	18,163	798,572	17,111	97.8% (97.7%)	97.9% (97.9%)
	Low	76,105	24,188	88,389	35,819	75.9% (75.7%)	71.2% (71.0%)
Long insertion (16-31 bp)	Intermediate	159,927	3,135	159,432	8,639	98.1% (98.0%)	94.9% (94.8%)
	Low	102,552	22,199	103,892	55,724	82.2% (82.0%)	65.1% (64.9%)

Table 13 Summary of TruSight Whole Genome NPA of Small Variant Calls Stratified by Confidence Tier

Confidence Tier	Concordant Negative Calls	Reference Method Exclusive Negative Calls	NPA (LCL)
High	202,276,243,790	127,465,816	99.9% (99.9%)
Intermediate	3,307,740,675	77,650,177	97.7% (97.7%)
Low	3,653,569,580	439,038,662	89.3% (89.3%)

A supplemental accuracy study was performed to evaluate small variant detection with commercially available reference cell line DNA samples (Coriell Institute for Medical Research) with well-characterized call sets generated by the Genome in a Bottle (GIAB) Consortium. For this study, the GIAB call sets were used as the reference method. The truth set in these samples includes insertions and deletions greater than 31 bp, so larger insertions and deletions were included in this assessment. These samples included HG001-005 and NA24695 with the results shown in aggregate in Table 14.

Table 14 TruSight Whole Genome Assay Accuracy for Small Variants Stratified by Confidence Tier and Size (Well-Characterized Cell Line Samples)

Variant Sub-Type	Confidence Tier	GIAB Concordant Calls	GIAB Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
SNVs	High	21,431,369	2,552	21,439,303	3,954	>99.9% (> 99.9%)	>99.9% (> 99.9%)
	Intermediate	908,172	1,259	910,058	2,175	99.9% (99.9%)	99.8% (99.8%)
	Low	720,717	59,691	722,180	28,721	92.4% (92.3%)	96.2% (96.1%)
Short deletion (1-5 bp)	High	1,080,383	690	1,090,370	730	99.9% (99.9%)	99.9% (99.9%)
	Intermediate	423,547	788	437,019	606	99.8% (99.8%)	99.9% (99.9%)
	Low	263,828	2,624	281,217	2,088	99.0% (99.0%)	99.3% (99.2%)
Medium deletion (6-15 bp)	Intermediate	142,671	238	144,997	167	99.8% (99.8%)	99.9% (99.9%)
	Low	86,174	812	91,710	546	99.1% (99.0%)	99.4% (99.4%)
Long deletion (≥ 16 bp)	Intermediate	34,414	315	34,580	55	99.1% (99.0%)	99.8% (99.8%)
	Low	9,985	393	10,212	106	96.2% (95.9%)	99.0% (98.8%)
Short insertion (1-5 bp)	High	927,288	221	925,787	271	>99.9% (> 99.9%)	>99.9% (> 99.9%)
	Intermediate	158,346	294	137,081	250	99.8% (99.8%)	99.8% (99.8%)
	Low	93,857	2,402	75,687	1,427	97.5% (97.4%)	98.1% (98.1%)

Variant Sub-Type	Confidence Tier	GIAB Concordant Calls	GIAB Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
Medium insertion (6-15 bp)	Intermediate	91,117	116	89,054	60	99.9% (99.9%)	99.9% (99.9%)
	Low	37,925	745	36,670	406	98.1% (98.0%)	98.9% (98.8%)
Long insertion (≥ 16 bp)	Intermediate	11,081	46	11,110	17	99.6% (99.5%)	99.8% (99.8%)
	Low	14,086	607	14,312	262	95.9% (95.6%)	98.2% (98.0%)

Copy Number Variant Accuracy

Accuracy of CNV calling was assessed using the same reference method and putatively healthy blood donor samples (195) that were used to assess small variant calling accuracy. Each CNV is considered as detected in the call set if at least 50% of that CNV is covered by the union of CNV calls of the same type (GAIN/LOSS) in the matched call set. TruSight Whole Genome defines a set of genomic regions that are excluded from CNV calling based on an assessment of sample data from 1000 Genomes and 77 putatively healthy blood donors using metrics related to coverage depth outliers, coverage variance outliers, and gaps in coverage to ascertain regions of the genome that are non-reportable for CNV. CNV calling was only evaluated over genomic regions that were common to both the reference method and TruSight Whole Genome. A summary of accuracy for all CNV calls is shown in Table 15 and Table 16.

Table 15 TruSight Whole Genome Assay Accuracy for CNVs Stratified by Size and Type

Size	Type	Reference Method Concordant Calls	Reference Method Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
10-25 kbp	GAIN	443	98	342	56	81.89% (79.01%)	85.93% (82.82%)
	LOSS	4,162	457	4,155	679	90.11% (89.36%)	85.95% (85.11%)
25-50 kbp	GAIN	355	117	370	76	75.21% (71.81%)	82.96% (79.83%)
	LOSS	1,587	16	1,622	7	99.00% (98.50%)	99.57% (99.21%)

Size	Type	Reference Method Concordant Calls	Reference Method Exclusive Calls	Assay Concordant Calls	Assay Exclusive Calls	PPA (LCL)	TPPV (LCL)
50–100 kbp	GAIN	228	0	187	20	>99.99% (98.83%)	90.34% (86.42%)
	LOSS	723	5	697	6	99.31% (98.60%)	99.15% (98.36%)
≥100 kbp	GAIN	371	1	335	5	99.73% (98.80%)	98.53% (97.01%)
	LOSS	541	23	569	1	95.92% (94.32%)	99.82% (99.22%)
Overall (All CNVs ≥ 10 kbp)	GAIN	1,397	216	1,234	157	86.61% (85.15%)	88.71% (87.24%)
	LOSS	7,013	501	7,043	693	93.33% (92.84%)	91.04% (90.49%)

Table 16 Summary of TruSight Whole Genome NPA of CNV Calls

Size	Type	Concordant Negative Calls	Reference Method Exclusive Negative Calls	Assay Exclusive Calls	NPA (LCL)
Overall (All CNVs ≥ 10 kbp)	GAIN	548,478,033,220	5,701,311	6,400,382	> 99.99% (> 99.99%)
	LOSS	548,591,794,675	11,719,913	8,543,877	> 99.99% (> 99.99%)

Runs of Homozygosity Accuracy

Technical Positive Predictive Value (TPPV) for ROH calls was assessed using the same reference method and putatively healthy blood donor samples (195) that were used in the small variant and CNV accuracy assessments. ROH events were determined by identifying regions in the genome containing a sequence of homozygous SNV calls lacking heterozygous SNVs or long gaps without variants. Such seed regions were then extended to the left and right and assessed for surrounding homozygous calls or the presence of heterozygous SNVs. ROH events detected by TruSight Whole Genome were compared to SNV calls from the reference method. A summary of TPPV for ROH calls is shown in Table 17.

Table 17 TruSight Whole Genome Accuracy for ROH Events Stratified by Size

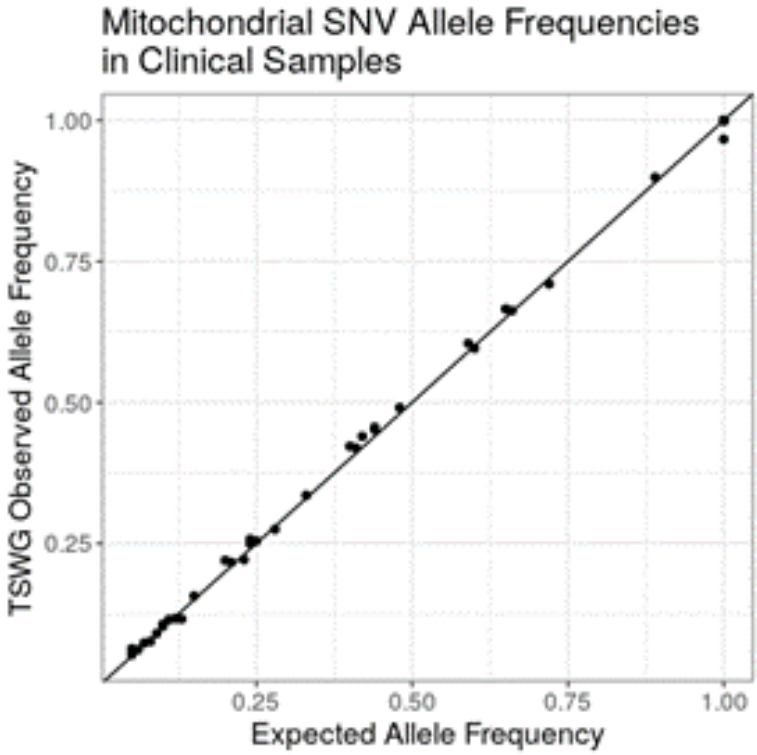
Size	TPPV Mean	TPPV LCL
10-25 kbp	81.44%	80.77%
25-50 kbp	82.14%	81.82%
50–100 kbp	81.77%	81.55%
100–500 kbp	82.19%	81.98%
≥ 10 kbp	82.07%	81.94%
≥ 500 kbp	85.47%	84.66%

Positive Percent Agreement (PPA) for ROH detection was determined in externally sourced clinical samples by comparing TruSight Whole Genome calls to ROH calls from orthogonal methods including chromosomal microarray and PCR-based assessment. An ROH event was considered detected if at least 50% of the region reported as ROH by the orthogonal method overlapped the union of ROH events called by TruSight Whole Genome. The PPA between the TruSight Whole Genome Assay and orthogonal methods was 34/34 (100%) for all expected ROH events (≥ 4 Mb).

Heteroplasmic Mitochondrial SNV Accuracy

Accuracy of mtSNV calling was assessed in 41 previously banked clinical samples sourced from external sites. Each clinical sample contained a previously reported mtSNV at a defined site and with a defined degree of heteroplasmy based on mtDNA Targeted Known Analysis with Heteroplasmy (MITOP). Allele frequencies estimated by TruSight Whole Genome were highly correlated to the expected frequencies as predicted by MITOP. All expected mtDNA SNVs were detected, resulting in a PPA of 100% (41/41).

Figure 3 TruSight Whole Genome Observed Mitochondrial SNV Allele Frequencies vs Expected Allele Frequencies



An additional mtSNV accuracy study was performed using the same 195 blood samples and reference method described in the small variant and CNV accuracy studies. The negative reference set was defined as confident non-variant calls (PASS filter) and the positive reference set was defined as mtSNV calls with an allele frequency > 2.5%. Positions with either a non-pass filter or non-SNV variant call were excluded. A summary of accuracy for mtSNVs is shown in Table 18.

Table 18 TruSight Whole Genome Accuracy of mtDNA SNV Calls

Accuracy Metric	Reference Method Concordant Positive	Reference Method Exclusive Positive	Assay Exclusive Positive	Reference Method Concordant Negative	Reference Method Exclusive Negative	Assay Exclusive Negative	Accuracy Metric Value (LCL)
PPA	6875	0	N/A	N/A	N/A	N/A	>99.99% (99.96%)
TPPV	6875	N/A	6	N/A	N/A	N/A	99.91% (99.83%)
NPA	N/A	N/A	N/A	3171049	24268	20564	99.24% (99.23%)

STR Expansion Detection Accuracy

Accuracy of STR expansion detection was based on 160 total samples prepared by extraction of gDNA from clinically affected individuals with expansions in specific sites confirmed by PCR / Repeat-Primed (RP)-PCR or Southern Blot performed in a CLIA laboratory setting. The thresholds determined in Table 11 were used to define STR status of an allele at a specific locus as normal (estimated STR size less than or equal to the threshold) or expanded (greater than the threshold).

PPA was computed using only clinically confirmed samples, NPA was computed using only individual putatively healthy blood samples, and TPPV was computed across both sample groups. For alleles where a clinically confirmed sample was not available, PPA could not be calculated. Additionally, for alleles where a clinically confirmed sample was not available and there were no false positive calls, TPPV could not be calculated. NPA was calculated for all STR expansions. The number of clinical samples tested for a given STR expansion and accuracy metrics are provided in Table 19.

Table 19 TruSight Whole Genome Accuracy Metrics for STR Expansions

STR Expansion	Clinical Samples Tested	PPA	TPPV	NPA
AFF2	0	N/A	N/A	>99.99%
AR	8	>99.99%	>99.99%	>99.99%
ATN1	4	>99.99%	>99.99%	>99.99%
ATXN1	7	66.67%	>99.99%	>99.99%
ATXN10	0	N/A	N/A	>99.99%
ATXN2	5	80.00%	>99.99%	>99.99%
ATXN3	9	>99.99%	90.00%	99.74%
ATXN7	2	>99.99%	>99.99%	>99.99%
ATXN7_GCC	0	N/A	N/A	>99.99%
ATXN8OS	0	N/A	0.00%	99.74%
ATXN8OS_CTA	0	N/A	N/A	>99.99%
C9ORF72	21	>99.99%	>99.99%	>99.99%
CACNA1A	5	>99.99%	83.33%	99.74%
CBL	0	N/A	N/A	>99.99%
CNBP	0	N/A	N/A	>99.99%
CNBP_CA	0	N/A	N/A	>99.99%
CNBP_CAGA	0	N/A	N/A	>99.99%
CSTB	0	N/A	0.00%	99.74%
DIP2B	0	N/A	0.00%	99.74%
DMPK	42	>99.99%	>99.99%	>99.99%

STR Expansion	Clinical Samples Tested	PPA	TPPV	NPA
FMR1	47	> 99.9%	>99.99%	>99.99%
FXN	0	N/A	0.00%	99.74%
FXN_A	0	N/A	N/A	>99.99%
GLS	0	N/A	N/A	>99.99%
HTT	10	>99.99%	83.33%	99.49%
HTT_CCG	0	N/A	N/A	>99.99%
JPH3	0	N/A	N/A	>99.99%
NIPA1	0	N/A	N/A	>99.99%
NOP56	0	N/A	N/A	>99.99%
NOP56_CGCCTG	0	N/A	N/A	>99.99%
NOTCH2NL	0	N/A	N/A	>99.99%
PABPN1	0	N/A	N/A	>99.99%
PHOX2B	0	N/A	N/A	>99.99%
PPP2R2B	0	N/A	N/A	>99.99%
TBP	0	N/A	N/A	>99.99%
ALL	**160**	**98.12%**	**92.35%**	**99.94%**

The assessment of overall PPA of STR expansion detection across all loci represents a good approximation of the locus specific PPA using the available clinical samples. Assessment of PPA specifically for the FMR1 locus can serve as a lower bound for PPA of loci that were not directly profiled due to its large threshold for STR size abnormality.

SMN1 Allele Detection Accuracy

The accuracy of detection of the absence of the C allele in SMN1 (NM_000344.3:c.840C) was assessed in 26 clinical samples from cases with diagnosis of Spinal Muscular Atrophy (SMA) and homozygous loss of exon 7 in SMN1 confirmed by digital droplet PCR or MLPA. The accuracy of identifying the presence of the SMN1 c.840C allele was assessed in putatively healthy individual blood samples. Each sample was assigned a single statistical metric (True Positive (TP), False Positive (FP), False Negative (FN), or True Negative (TN)) based on the detected presence (negative SMA status) or absence (positive SMA status) of the C allele at the c.840 position of SMN1 gene compared to the expected status. PPA, TPPV, and NPA estimates were made across both the positive and negative sample set (refer to Table 20).

Table 20 Accuracy Metrics for Detection of Absence of SMN1 c.840C Alleles

Accuracy Metric	TP	FP	TN	FN	Accuracy Metric Value
PPA	26	N/A	N/A	0	>99.99%
TPPV	26	0	N/A	N/A	>99.99%
NPA	N/A	0	195	N/A	>99.99%

Repeatability

Within-laboratory Precision

Within-laboratory precision was evaluated using extracted gDNA with a variety of known variants across the genome. These included mtSNVs near and well above LoD, samples containing the SMN1 c.840C allele and samples with FMR and HTT1 repeat expansions at lengths near and well above LoD. Samples were tested using nine unique conditions designed with three operators, three library prep reagent lots, three sequencing consumables lots, and three sequencing instruments.

Each sample was run in duplicate on the same run to assess within-run variation and each test case was tested twice for two runs per condition for between-run variation. Each sample was assessed using 36 observations and the design afforded 18 degrees of freedom for assessing repeatability. The list of panel members, sample type, and assessed variants per each panel member is shown in Table 21. Samples 1-4 and 9-12 were derived from both males and females of self-identified Caucasian, African, and Asian ancestry to provide a diverse sample set.

Table 21 Sample Composition of Panel Used for Within-Laboratory Precision Study

Panel	Sample #	Sample Type	Variants
A	1	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	2	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	3	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	4	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	5	Contrived mixture of gDNA from blood	Mitochondrial SNVs at Low LoD level
	6	Contrived cell line NA20241[1]	STR expanded in FMR1 loci at Low LoD level
	7	Contrived cell line NA20208	STR expanded in HTT loci at Low LoD level
	8	Contrived cell line NA23686	Absence of SMN1 c.840C
B	9	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	10	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	11	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	12	gDNA from blood	Small variants, CNV, ROH, STR not expanded, presence of SMN1 c.840C
	13	Contrived mixture of gDNA from blood	mtSNVs at High LoD level
	14	Contrived cell line NA07862	STR expanded in FMR1 loci at High LoD level
	15	Contrived cell line NA20253	STR expanded in HTT loci at High LoD level
	16	Contrived cell line NA03814	Absence of SMN1 c.840C

High LoD level: Variant allele frequency approximately at 2.0x – 4.0x LoD.
Low LoD level: Variant allele frequency approximately at 1.0x – 1.5x LoD.
[1] Results for NA20241 were not reported in final numbers as it was determined to be significantly below 1.0x LoD and thus did not meet sample requirements.

In the qualitative assessment, metrics of reproducibility are reported treating the variants as qualitative entities (variant present or variant not present). Different definitions of positive or negative calls and different qualitative metrics were assessed and reported for each variant type (Table 22). When assessing reproducibility of small variant, CNV, and ROH calls, the variant calls made in a characterization run replicate was used for each sample that served as the comparator point for all other replicates of that sample in the study.

Table 22 Summary of Qualitative Assessment of Reproducibility for Each Variant Type

Variant Type	Positive	Negative	Type of Comparison	Qualitative Metrics
Small variants	Variant call passing filters	Homozygous reference call passing filters	Concordance with call set from initial characterization runs	Average Positive Agreement (APA) and Average Negative Agreement (ANA)
CNVs	CNV call passing filters	Genomic positions not overlapping a passing copy number variant call	Concordance with call set from initial characterization runs	APA and ANA
ROH	ROH call	Genomic positions not overlapping an ROH call	Concordance with call set from initial characterization runs	APA and ANA
STR expansion	Sample with STR expansion in at least one targeted locus	Sample without expansions in any of the targeted loci	Concordance with sample status defined by characterization of sample by orthogonal assay	Percent Positive calls (PPC) and Percent Negative Calls (PNC)
SMN1 c.840C detection	Sample without the C allele at c.840 position of SMN1 (SMA positive)	Sample containing at least one copy of the C allele at c.840 position of SMN1 (SMA negative)	Concordance with sample status defined by characterization of sample by orthogonal assay	PPC and PNC
mtSNV	Mitochondrial SNV call passing filters	Non-variant position in mitochondrial chromosome passing filters	Concordance with variant and non-variant calls made in undiluted samples	PPC and PNC

The quantitative assessment of the different variant types involved an evaluation of variability of either quantitative metrics that underpin the qualitative calls or, in the case of small variants, of the agreement metrics relative to a reference call set. This study performed both an assessment of total variability in quantitative metrics across replicates as well as the contribution of different factors included in the study to the variability in

such quantitative metrics through Variance Components Analysis. Table 23 summarizes the quantitative metrics used in the analysis of each variant type as well as the factors that were assessed for contribution to variability in the quantitative metric.

Table 23 Summary of Quantitative Metrics Used in Assessment of Precision for the Difference Variant Types

Variant Type	Quantitative Metrics	Factors Assessed for Contribution to Variability
Small variants	APA and ANA	Operator, library prep kit lot, instrument, sequencing consumables lot, variant subtype, genomic context
CNVs	Normalized depth of coverage over CNV region	Operator, library prep kit lot, instrument, sequencing consumables lot, variant subtype, variant length
ROH	ROH score over ROH region	Operator, library prep kit lot, instrument, sequencing consumables lot, variant subtype, variant length
STR expansion	STR size estimate	Operator, library prep kit lot, instrument, sequencing consumables lot, STR site, STR length
SMN1 c.840C detection	Log-likelihood ratio for the presence of the reference allele (C) at the targeted position	Operator, library prep kit lot, instrument, sequencing consumables lot, SMA status
Mitochondrial SNV	Variant allele frequency	Operator, library prep kit lot, instrument, sequencing consumables lot, variant position, expected variant allele frequency

The results for the variance components analysis are presented in Table 24. For small variants, the majority of variance was attributed to residual error and not explained by the assay-related factors included in the design, including sequencing kit lot, sequencing instrument, library prep kit lot, operator, and run to run. The one exception was observed for SNVs in intermediate confidence regions for which the majority of variance was attributed to the sequencing kit lot. In general, a higher amount of variance was attributed to assay related factors for small variants in low confidence regions of the genome. For all other variant types, the majority of variance was attributed to residual error and not assay-related factors. This study demonstrates that for most of the small variant subtypes, filtering for high and intermediate confidence regions in the genome may be used to increase repeatability and decrease the variability of the assay. *External Reproducibility* on page 59 provides a comprehensive analysis of the assay reproducibility.

Table 24 Results of the Variance Components Analysis Study

Metric	Variant Subtypes	Confidence Tier	Residual	Sequencing kit lot	Run-to-run	Instrument	Library Prep kit lot	Operator
APA	Short deletion (1-5 bp)	High	79.36%	17.52%	0.00%	0.00%	3.13%	0.00%
		Intermediate	76.97%	18.59%	1.53%	0.00%	2.91%	0.00%
		Low	67.85%	24.87%	4.4%	0.00%	2.88%	0.00%
	Medium deletion (6-15 bp)	Intermediate	61.17%	29.06%	7.42%	0.00%	2.35%	0.00%
		Low	59.33%	31.76%	6.38%	0.17%	2.35%	0.00%
	Long deletion (16-31 bp)	Intermediate	52.93%	33.72%	11.67%	0.17%	1.51%	0.00%
		Low	49.10%	37.01%	11.08%	1.42%	1.39%	0.00%
	Short insertion (1-5 bp)	High	89.93%	7.32%	1.76%	0.00%	0.99%	0.00%
		Intermediate	74.52%	19.96%	3.44%	0.00%	2.08%	0.00%
		Low	60.64%	29.72%	8.49%	0.00%	1.15%	0.00%
	Medium insertion (6-15 bp)	Intermediate	81.76%	15.78%	0.00%	0.00%	2.41%	0.06%
		Low	51.28%	35.07%	12.07%	0.00%	1.58%	0.00%
	Long insertion (16-31 bp)	Intermediate	87.59%	9.83%	1.18%	0.00%	1.40%	0.00%
		Low	52.47%	35.32%	10.14%	0.23%	1.85%	0.00%
	SNV	High	78.01%	17.45%	0.00%	0.13%	1.23%	3.17%
		Intermediate	79.71%	16.95%	0.77%	0.20%	1.29%	1.09%
		Low	56.63%	36.08%	6.97%	0.22%	0.00%	0.09%
ANA	SNV	High	55.07%	21.84%	21.07%	1.80%	0.21%	0.00%
		Intermediate	28.53%	49.08%	20.11%	1.27%	1.00%	0.00%
		Low	51.78%	36.04%	9.76%	2.42%	0.00%	0.00%

Metric	Variant Subtypes	Confidence Tier	Residual	Sequencing kit lot	Run-to-run	Instrument	Library Prep kit lot	Operator
Depth	CNV GAIN (10 kbp, 25 kbp)	N/A	73.28%	2.87%	0.00%	0.00%	1.01%	0.00%
	CNV GAIN (25 kbp, 50 kbp)	N/A	72.99%	5.25%	0.00%	0.00%	0.00%	0.56%
	CNV GAIN (50 kbp, 100 kbp)	N/A	66.40%	5.16%	0.00%	0.00%	0.00%	0.00%
	CNV GAIN (100 kbp, 500 kbp)	N/A	43.51%	14.92%	14.01%	0.20%	0.00%	15.72%
	CNV LOSS (10 kbp, 25 kbp)	N/A	83.41%	0.00%	0.00%	0.00%	0.00%	0.00%
	CNV LOSS (25 kbp, 50 kbp)	N/A	84.67%	1.20%	0.00%	0.00%	0.00%	0.00%
	CNV LOSS (50 kbp, 100 kbp)	N/A	84.16%	2.43%	0.00%	0.00%	0.00%	0.00%
	CNV LOSS (100 kbp, 500 kbp)	N/A	81.25%	5.22%	0.00%	0.00%	0.00%	0.55%

Metric	Variant Subtypes	Confidence Tier	Residual	Sequencing kit lot	Run-to-run	Instrument	Library Prep kit lot	Operator
Score over region	ROH (1 kbp, 10 kbp)	N/A	74.32%	1.65%	0.00%	0.00%	0.00%	0.52%
	ROH (10 kbp, 25 kbp)	N/A	84.78%	0.00%	0.00%	0.00%	0.00%	0.00%
	ROH (25 kbp, 50 kbp)	N/A	84.92%	0.00%	0.00%	0.00%	0.00%	0.00%
	ROH (50 kbp,100 kbp)	N/A	85.63%	0.00%	0.00%	0.00%	0.00%	0.00%
	ROH (100 kbp, 500 kbp)	N/A	85.76%	0.00%	0.00%	0.00%	0.00%	0.00%
	ROH ≥ 500 kbp	N/A	84.81%	0.00%	0.00%	0.00%	0.00%	0.00%

Metric	Variant Subtypes	Confidence Tier	Residual	Sequencing kit lot	Run-to-run	Instrument	Library Prep kit lot	Operator
Size estimate for STR loci[1]	AFF2	N/A	99.43%	0.00%	0.00%	0.00%	0.00%	0.00%
	ATXN7	N/A	100%	0.00%	0.00%	0.00%	0.00%	0.00%
	ATXN7_GCC	N/A	99.43%	0.57%	0.00%	0.00%	0.00%	0.00%
	CNBP	N/A	100%	0.00%	0.00%	0.00%	0.00%	0.00%
	CNBP_CA	N/A	95.45%	4.55%	0.00%	0.00%	0.00%	0.00%
	CSTB	N/A	96.45%	0.87%	2.57%	0.00%	0.00%	0.11%
	DIP2B	N/A	100%	0.00%	0.00%	0.00%	0.00%	0.00%
	FMR1	N/A	71.02%	10.06%	0.00%	17.33%	0.64%	0.95%
	FXN_A	N/A	94.52%	1.37%	0.00%	1.37%	1.37%	1.37%
	HTT	N/A	82.23%	0.00%	11.99%	3.81%	0.00%	1.97%
	HTT_CCG	N/A	99.43%	0.00%	0.00%	0.00%	0.00%	0.00%
	NOTCH2NL	N/A	99.43%	0.00%	0.00%	0.29%	0.29%	0.00%
	TBP	N/A	90.91%	0.00%	0.00%	0.00%	0.00%	0.00%
Log likelihood ratio	c.840C in NA03814	N/A	65.71%	18.98%	0.00%	0.00%	0.00%	15.32%
	c.840C in NA23686	N/A	87.64%	0.00%	0.00%	5.90%	0.00%	6.46%
VAF	mtSNVs near LOD	N/A	83.13%	0.37%	0.00%	0.00%	0.00%	0.05%

[1] Variance components analysis was not performed for loci for which no variance was observed.

External Reproducibility

External reproducibility was determined using a single lot of library prep and sequencing reagents at three external trial sites with two operators at each site. The same samples used in the *Within-laboratory Precision* on page 51 study (Table 21) were used in the reproducibility study with one exception: the sample NA20241 was replaced with NA20239 to evaluate the FMR1 loci STR expansion at low LoD. In total, 16 unique samples were tested as two sub-panels of eight unique samples each (Panel A and Panel B) by each operator at each site. Three sequencing runs were performed for duplicate libraries of each sub-panel for a total of 36 sequencing runs per unique sample.

The sample pass rate across 576 sample libraries with valid sequencing runs, defined as the number of samples passing sample library QC metrics on the first attempt, was 99.1% (571/576; 95% CI: 98.0%, 99.6%). All test results are based on initial testing.

Reproducibility of SNVs, insertions, deletions, CNVs, and ROH was assessed by comparing data to a reference call set based on usual performance across three characterization runs (Table 25 and Table 26). Reproducibility of STR expansions, the absence of the SMN1 c.840C allele, and mtSNVs was assessed by comparing data to known status (Table 27).

Table 25 Reproducibility of TruSight Whole Genome for SNVs, CNVs, and ROH

Variant type — stratification	Concordant Positive Calls[1] / Positive Calls[2]			Average Positive Agreement (%) (95% CI)[3]
	Site 1	Site 2	Site 3	
Small variants (high confidence)				
SNVs	687,996,150 / 688,770,402	666,509,635 / 667,253,493	688,001,697 / 688,766,887	99.9 (99.9-99.9)
Insertions — 1-5 bp	34,087,135 / 34,137,298	33,025,772 / 33,073,087	34,089,204 / 34,137,792	99.9 (99.9-99.9)
Deletions — 1-5 bp	44,096,186 / 44,255,442	42,733,935 / 42,883,089	44,102,515 / 44,256,695	99.6 (99.6-99.6)
Small variants (intermediate confidence)				
SNVs	42,238,226 / 42,737,228	40,920,370 / 41,391,560	42,236,751 / 42,725,827	98.8 (98.8-98.9)
Insertions — 1-5 bp	11,075,073 / 11,204,210	10,734,488 / 10,855,790	11,080,468 / 11,204,818	98.9 (98.9-99.9)
Insertions — 6-15 bp	4,307,181 / 4,339,975	4,173,626 / 4,205,261	4,308,408 / 4,340,277	99.3 (99.2-99.3)
Insertions — ≥ 16 bp	611,952 / 632,214	593,114 / 612,877	612,222 / 632,498	96.8 (96.8-96.8)
Deletions — 1-5 bp	24,571,502 / 24,851,492	23,814,655 / 24,076,930	24,586,095 / 24,855,041	98.9 (98.9-98.9)
Deletions — 6-15 bp	8,737,319 / 8,900,796	8,473,410 / 8,624,403	8,746,773 / 8,902,016	98.2 (98.2-98.2)
Deletions — ≥ 16 bp	3,590,282 / 3,779,907	3,481,192 / 3,662,448	3,594,420 / 3,780,659	95.0 (95.0-95.0)
Small variants (low confidence)				
SNVs	78,507,103 / 96,859,682	76,365,789 / 94,066,720	78,863,977 / 97,058,652	81.2 (81.2-81.2)

Variant type — stratification	Concordant Positive Calls[1] / Positive Calls[2]			Average Positive Agreement (%) (95% CI)[3]
	Site 1	Site 2	Site 3	
Insertions — 1-5 bp	17,312,805 / 19,370,351	16,859,987 / 18,807,745	17,406,355 / 19,418,516	89.6 (89.5-89.6)
Insertions — 6-15 bp	5,543,985 / 6,529,886	5,404,652 / 6,338,556	5,584,241 / 6,550,066	85.1 (85.1-85.2)
Insertions — ≥ 16 bp	3,284,197 / 4,275,286	3,205,165 / 4,158,315	3,314,025 / 4,298,399	77.0 (77.0-77.0)
Deletions — 1-5 bp	31,659,416 / 34,194,748	30,751,952 / 33,158,757	31,746,379 / 34,226,245	92.7 (92.7-92.7)
Deletions — 6-15 bp	9,189,220 / 9,987,568	8,928,794 / 9,684,179	9,217,516 / 9,995,101	92.1 (92.1-92.2)
Deletions — ≥ 16 bp	3,335,400 / 3,909,364	3,241,968 / 3,791,331	3,346,219 / 3,912,857	85.4 (85.4-85.5)
CNVs — gains ≥ 10 kbp	7,883 / 8,275	7,664 / 8,012	7,916 / 8,282	95.5 (95.2-95.8)
CNVs — losses ≥ 10 kbp	11,517 / 12,089	11,248 / 11,777	11,516 / 12,113	95.3 (95.1-95.5)
ROH — ≥ 500 kbp	6,641 / 6,765	6,519 / 6,663	6,616 / 6,756	98.0 (97.8-98.2)

[1] Total number of concordant positive calls = Query Concordant Positive (QCP) + Reference Concordant Positive (RCP).
[2] Total number of positive calls = Query Concordant Positive (QCP) + Query Exclusive Positive (QEP) + Reference Concordant Positive (RCP) + Reference Exclusive Positive (REP).
[3] 2-sided 95% confidence interval calculated via the Wilson Score method.

Table 26 Reproducibility of TruSight Whole Genome for ANA of SNVs, CNVs, and ROH

Variant type — stratification	Concordant Negative Calls[1] / Negative Calls[2]			Average Negative Agreement (%) (95% CI)[3]
	Site 1	Site 2	Site 3	
Small variants (high confidence)	486,282,620,918 / 486,388,081,375	470,948,205,740 / 471,054,131,230	486,285,759,770 / 486,389,857,817	>99.9 (>99.9->99.9)
Small variants (intermediate confidence)	17,249,915,828 / 17,427,817,811	16,699,106,194 / 16,874,794,553	17,253,834,878 / 17,429,035,482	99.0 (99.0-99.0)
Small variants (low confidence)	24,072,615,254 / 25,608,493,410	23,454,103,344 / 24,947,163,687	24,180,801,788 / 25,695,956,102	94.0 (94.0-94.0)
CNVs — gains ≥ 10 kbp	592,486,270,144 / 592,500,222,476	573,973,293,084 / 573,985,772,396	592,487,297,632 / 592,500,614,241	>99.9 (>99.9->99.9)
CNVs — losses ≥ 10 kbp	592,548,802,882 / 592,559,825,216	574,030,570,254 / 574,041,311,257	592,547,683,360 / 592,559,141,007	>99.9 (>99.9->99.9)
ROH — ≥ 500 kbp	542,968,586,606 / 547,402,885,905	525,724,060,526 / 530,011,754,808	543,014,319,116 / 547,444,495,449	99.2 (99.2-99.2)

[1] Total number of concordant negative calls = 2 × Concordant Negative (CN).
[2] Total number of negative calls = 2 × Concordant Negative (CN) + Reference Exclusive Negative (REN) + Query Exclusive Negative (QEN).
[3] 2-sided 95% confidence interval calculated via the Wilson Score method.

Table 27 Reproducibility of TruSight Whole Genome for STRs, SMN1, and mtSNVs

Variant type – stratification	Total Expected Positive Calls	Positive Calls			Total Expected Negative Calls	Negative Calls			Percent Positive Calls (95% CI)[1]	Percent Negative Calls (95% CI)[1]
		Site 1	Site 2	Site 3		Site 1	Site 2	Site 3		
STR expansions - High level of detection (2x-4x LOD)										
STR expansions - FMR1	35	12	11	12	N/A	N/A	N/A	N/A	100 (90.1-100)	N/A
STR expansions - HTT	36	12	12	12	N/A	N/A	N/A	N/A	100 (90.4-100)	N/A
STR expansions – FMR1 and HTT combined	71	24	23	24	N/A	N/A	N/A	N/A	100 (94.9-100)	N/A
STR expansions - Low level of detection (1x-1.5x LOD)										
STR expansions - FMR1	36	11	10	11	N/A	N/A	N/A	N/A	88.9 (74.7-95.6)	N/A
STR expansions - HTT	36	12	12	12	N/A	N/A	N/A	N/A	100 (90.4-100)	N/A
STR expansions – FMR1 and HTT combined	72	23	22	23	N/A	N/A	N/A	N/A	94.4 (86.6-97.8)	N/A

Variant type – stratification	Total Expected Positive Calls	Positive Calls			Total Expected Negative Calls	Negative Calls			Percent Positive Calls (95% CI)[1]	Percent Negative Calls (95% CI)[1]
		Site 1	Site 2	Site 3		Site 1	Site 2	Site 3		
STR expansions – 28 main target STR loci combined	N/A	N/A	N/A	N/A	285	96	93	96	N/A	100 (98.7-100)
Absence of SMN1 c.840C	71	24	24	23	285	96	93	96	100 (94.9-100)	100 (98.7-100)
mtSNVs – high level (2x-4x LOD)	1080	360	360	360	457,524	152,491	152,489	152,484	100 (99.6-100)	>99.9 (>99.9->99.9)
mtSNVs – low level (1x-1.5x LOD)	1080	360	359	360	457,524	152,481	152,489	152,483	99.9 (99.5-99.9)	>99.9 (>99.9->99.9)

[1] Two-sided 95% confidence interval calculated via the Wilson Score method.

Troubleshooting

Use the following table to troubleshoot issues in the workflow. If a sequencing run or library preparation for a sample fails two times, additional troubleshooting may be necessary. Contact Illumina Technical Support.

Issue Type	Observation	Possible Cause	Recommended Action
Run Creation Issue	The associated Planned Run cannot be manually selected in NovaSeq 6000Dx Control Software after loading consumables	Incorrect Library tube ID was specified during run planning	Refer to Run Revision in TruSight Whole Genome Analysis Application Guide (document # 200049931).

Issue Type	Observation	Possible Cause	Recommended Action
Sequencing Issue	Sequencing failure status in Illumina Run Manager	The sequencing run was aborted or failed to complete due to NovaSeq 6000Dx or sequencing consumable handling issue	Refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). After addressing the issue, library may be repooled and resequenced up to one time (due to volume).
		Run completed but failed to cluster. Possible NovaSeq 6000Dx issue, sequencing consumable handling issue, or catastrophic library preparation failure due to reagent handling issue or operator error (eg skipped a step or discarded instead of transferred supernatant during size-selection)	Assess individual library yields in FLP by qPCR for ≥ 0.94 nM (assume 450 bp insert size) to rule in/out library prep versus sequencing-related issues. If library preparation issues are ruled out and a sequencing-related issue is suspected, refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). If a library preparation issue is suspected, review *Tips and Techniques* on page 12 and *Instructions for Use* on page 15 before repeating library preparation and sequencing. If there are repeated failures, contact Illumina Technical Support.

Issue Type	Observation	Possible Cause	Recommended Action
Sequencing data fails to transfer to server	Sequencing file transfer for analysis failure status in Illumina Run Manager	Network connectivity issue, or instrument or server power disruption occurred during run data transfer	Check for power disruption or loss of instrument network connectivity. Wait for the system to be idle (sequencing to complete), then go to Instrument Settings, IVD SETTINGS to confirm connection to the specified Output Location using the Browse function. If further troubleshooting is required, refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). If after resolving connection or power issues, file transfer does not restart and complete, contact Illumina Technical Support.
Analysis fails to start	Analysis not started status in Illumina Run Manager although Sequencing file transfer for analysis completed	Pairing or connection between instrument and DRAGEN Server for NovaSeq 6000Dx lost or DRAGEN license expired.	Wait for the system to be idle (sequencing to complete), then go to DRAGEN to confirm the DRAGEN license is valid. If the license has expired contact Illumina. If the license is valid, select **Run Self-Test**. If test fails, or if the option to run a self-test is unavailable, log into Instrument to check for an error related to server pairing. Refer to the System Configuration section of the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). Analysis should automatically start after the issue is resolved. Exit the page and navigate to the Active Runs tab to confirm the analysis is in progress. If the issue persists, contact Illumina.

Issue Type	Observation	Possible Cause	Recommended Action
Analysis becomes stuck	Analysis in progress status in Illumina Run Manager for much longer than expected	Network connectivity or instrument or server power may have been disrupted during analysis causing analysis to become stuck	Cancel analysis and check for power disruption or loss of instrument network connectivity. Wait for the system to be idle (sequencing to complete), then go to Instrument Settings (IVD SETTINGS) and confirm connectivity to the specified Output Location. If further troubleshooting is required, refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). After addressing the issue, Requeue analysis with no changes. Refer to TruSight Whole Genome Analysis Application Guide (document # 200049931).

Issue Type	Observation	Possible Cause	Recommended Action
Analysis files fail to transfer	Analysis file transfer to storage failed status in Illumina Run Manager	Network connectivity issue or instrument or server power disruption occurred during analysis file transfer	Cancel analysis and check for power disruption or loss of instrument network connectivity. Wait for the system to be idle (sequencing to complete), then go to Instrument Settings (IVD SETTINGS) and confirm connectivity to the specified Output Location. If further troubleshooting is required, refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). After addressing the issue, Requeue analysis with no changes. Refer to TruSight Whole Genome Analysis Application Guide (document # 200049931).
Analysis fails on requeue	Analysis failed after requeue	If requeuing analysis, the original run may have been deleted or archived and is no longer in location specified for external storage location	Check original run is still in the external storage location. If archived, recover from archive and then requeue analysis again.
Sequencing QC fails	Summary Sequencing QC Result FAIL in Consolidated Report	“Total % >=Q30” below analytical specification due to mishandling of sequencing consumables (failure to thaw completely or invert to mix after thawing)	Refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). After addressing the issue, library may be repooled and resequenced up to one time (due to volume).

Issue Type	Observation	Possible Cause	Recommended Action
FASTQ QC fails for all samples	Summary FASTQ QC Result and Summary Sample Library QC FAIL, with individual library QC metric results reported as ND, for all samples in Consolidated Report with Summary Sequencing QC Result PASS	Index Adapter Kit specified during Create Run is not aligned with that which was used during library preparation	View samples to review the index information used in analysis in IRM. If a correction is needed, refer to Requeue Analysis in TruSight Whole Genome Analysis Application Guide (document # 200049931).
FASTQ QC fails for one or more samples in absence of low run yield; Non-Indexed Total Yield (GB) ≥ 2800 GB on S4 or ≥ 1000 GB on S2	Summary FASTQ QC Result and Summary Sample Library QC FAIL, with individual library QC metric results reported as ND, for one or more but not all samples in Consolidated Report without low run yield	Errors of use during library prep or pooling	Assess remaining volume(s) in the final library plate (FLP) to confirm error of use of omitting samples from pooled libraries. Volume allows operator to repool and resequence up to one time. Alternatively, requeue failed samples in next library prep batch and run after reviewing the *Instructions for Use* on page 15. Optionally, assess individual library yields in FLP by qPCR for ≥ 0.94 nM (assume 450 bp insert size) to rule in/out library prep-related issues. Requeue failed samples in next library prep batch and run after reviewing the *Instructions for Use* on page 15. It is not recommended to pool libraries across library preparation batches due to batch-to-batch fluctuations in yields which may result in higher %CV and a higher incidence of failing “Average autosomal coverage”.

Issue Type	Observation	Possible Cause	Recommended Action
FASTQ QC fails for some not all samples with low run yield; Non-Indexed Total Yield (GB) low, < 2800 GB on S4 or <1000 GB on S2	Summary FASTQ QC Result and Summary Sample Library QC FAIL, with individual library QC metric results reported as ND, for one or more but not all samples in Consolidated Report with low run yield	May indicate a library prep or sequencing-related issue	Assess individual library yields in FLP by qPCR for ≥ 0.94 nM (assume 450 bp insert size) to rule in/out library prep- versus sequencing-related issues. If suspected sequencing issue, refer to the NovaSeq 6000Dx Instrument Product Documentation (document # 200010105). After addressing the issue, libraries may be repooled and resequenced up to one time (due to limited volume). If a library preparation issue is suspected, review *Tips and Techniques* on page 12 and *Instructions for Use* on page 15 before repeating library preparation and sequencing. If there are repeated failures, contact Illumina Technical Support.

Issue Type	Observation	Possible Cause	Recommended Action
Library QC fails due to low coverage	Summary Sample Library QC Result FAIL for one or more samples in Consolidated Report due to Average autosomal coverage, and/or Percent of autosome with coverage greater than 20X, and/or Average mitochondrial coverage over genome not passing analytical specification	Sample quality or library preparation issue(s)	Perform re-quantification with process controls to rule out issues related to DNA input. Review review *Tips and Techniques* on page 12 and *Instructions for Use* on page 15 before requeuing failed sample(s) in next library prep batch and run. If there are repeated failure(s) for the same sample(s), this may indicate sample quality issue(s). If failure is observed again but with different samples, this may indicate a library preparation-related issue related to operator, reagent, consumable or equipment. If the issue persists, contact Illumina Technical Support.
Library QC fails based on GC bias	Summary Sample Library QC Result FAIL for one or more samples in Consolidated Report due to Normalized coverage at 60% to 79% GC bins and/or Normalized coverage at 20% to 39% GC bins not passing analytical specification	Excessive ELM carry over or skipped wash causing GC bias in coverage	Review *Tips and Techniques* on page 12 and *Instructions for Use* on page 15 before requeuing failed sample(s) in next library prep batch and run.

Issue Type	Observation	Possible Cause	Recommended Action
Library QC fails based on contamination for one or more but not all samples in run	Summary Sample Library QC Result FAIL for one or more but not all samples in Consolidated Report due to Estimated sample contamination not passing analytical specification	Contaminated sample(s) or did not change tips during sample or library preparation	Review *Tips and Techniques* on page 12 and *Instructions for Use* on page 15 before requeuing failed sample(s) in next library prep batch and run. If there are repeated failure(s) for the same sample(s), sample DNA may be contaminated.
Library QC fails based on contamination for all samples in run	Summary Sample Library QC Result is reported as FAIL for all samples in Consolidated Report due to Estimated sample contamination not passing analytical specification	Contaminated reagent or did not change tips during sample dilution or library preparation	Review *Tips and Techniques* on page 12 for avoiding contamination. Requeue failed samples in next library prep batch and run using fresh sample dilutions and library preparation kit.
ND Summary Ploidy Result	Summary Ploidy Result reported as ND (not determined) in Consolidated Report	Sex was listed as Unknown during Create Run	Confirm "Provided sex chromosome ploidy" in Consolidated Report was "Unknown". It is recommended to list Sex as "Male" or "Female" in sample data when known during Create Run.
		DRAGEN reported a sex ploidy result other than XX or XY such as X0 or XXY	Review "Ploidy estimation" output by DRAGEN in the Consolidated Report.

Issue Type	Observation	Possible Cause	Recommended Action
DISCORDANT Summary Ploidy Result	Summary Ploidy Result reported as DISCORDANT in Consolidated Report.	Potential sample swap issue	Review to confirm sample data entered during Create Run was correct. If incorrect, requeue analysis with changes. If correct, and a sample swap issue is suspected, then it is recommended to requeue the DISCORDANT sample(s) in next library prep batch and run to avoid the reporting of wrong results. Sample software does not enforce failure for a sample with a DISCORDANT Summary Ploidy Result.

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Appendix A

S4 Index Set 1

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A01	UDP0037	TGTAATCGAC	GATCACCGCG
B01	UDP0038	GTGCAGACAG	TACCATCCGT
C01	UDP0039	CAATCGGCTG	GCTGTAGGAA
D01	UDP0040	TATGTAGTCA	CGCACTAATG
E01	UDP0041	ACTCGGCAAT	GACAACTGAA
F01	UDP0042	GTCTAATGGC	AGTGGTCAGG
G01	UDP0043	CCATCTCGCC	TTCTATGGTT
H01	UDP0044	CTGCGAGCCA	AATCCGGCCA
A02	UDP0065	TAATGTGTCT	GTAAGGCATA
B02	UDP0066	ATACCAACGC	AATTGCTGCG
C02	UDP0067	AGGATGTGCT	TTACAATTCC
D02	UDP0068	CACGGAACAA	AACCTAGCAC
E02	UDP0069	TGGAGTACTT	TCTGTGTGGA
F02	UDP0070	GTATTGACGT	GGAATTCCAA
G02	UDP0071	CTTGTACACC	AAGCGCGCTT
H02	UDP0072	ACACAGGTGG	TGAGCGTTGT

S4 Index Set 2

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A03	UDP0081	TGTCGCTGGT	TCGTCTGACT
B03	UDP0082	ACCGTTACAA	CTCATAGCGA
C03	UDP0083	TATGCCTTAC	AGACACATTA
D03	UDP0084	ACAAGTGGAC	GCGCGATGTT
E03	UDP0085	TGGTACCTAA	CATGAGTACT
F03	UDP0086	TTGGAATTCC	ACGTCAATAC

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
G03	UDP0087	CCTCTACATG	GATACCTCCT
H03	UDP0088	GGAGCGTGTA	ATCCGTAAGT
A04	UDP0089	GTCCGTAAGC	CGTGTATCTT
B04	UDP0090	ACTTCAAGCG	GAACCATGAA
C04	UDP0091	TCAGAAGGCG	GGCCATCATA
D04	UDP0092	GCGTTGGTAT	ACATACTTCC
E04	UDP0093	ACATATCCAG	TATGTGCAAT
F04	UDP0094	TCATAGATTG	GATTAAGGTG
G04	UDP0095	GTATTCCACC	ATGTAGACAA
H04	UDP0096	CCTCCGTCCA	CACATCGGTG

S2 Index Set 1

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A01	UDP0037	TGTAATCGAC	GATCACCGCG
B01	UDP0038	GTGCAGACAG	TACCATCCGT
C01	UDP0039	CAATCGGCTG	GCTGTAGGAA
D01	UDP0040	TATGTAGTCA	CGCACTAATG
E01	UDP0041	ACTCGGCAAT	GACAACTGAA
F01	UDP0042	GTCTAATGGC	AGTGGTCAGG

S2 Index Set 2

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A02	UDP0065	TAATGTGTCT	GTAAGGCATA
B02	UDP0066	ATACCAACGC	AATTGCTGCG
C02	UDP0067	AGGATGTGCT	TTACAATTCC
D02	UDP0068	CACGGAACAA	AACCTAGCAC
E02	UDP0069	TGGAGTACTT	TCTGTGTGGA
F02	UDP0070	GTATTGACGT	GGAATTCCAA

S2 Index Set 3

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A03	UDP0081	TGTCGCTGGT	TCGTCTGACT
B03	UDP0082	ACCGTTACAA	CTCATAGCGA
C03	UDP0083	TATGCCTTAC	AGACACATTA
D03	UDP0084	ACAAGTGGAC	GCGCGATGTT
E03	UDP0085	TGGTACCTAA	CATGAGTACT
F03	UDP0086	TTGGAATTCC	ACGTCAATAC

S2 Index Set 4

Index Plate Well ID	Index Name	i7 Bases	i5 Bases
A04	UDP0089	GTCCGTAAGC	CGTGTATCTT
B04	UDP0090	ACTTCAAGCG	GAACCATGAA
C04	UDP0091	TCAGAAGGCG	GGCCATCATA
D04	UDP0092	GCGTTGGTAT	ACATACTTCC
E04	UDP0093	ACATATCCAG	TATGTGCAAT
F04	UDP0094	TCATAGATTG	GATTAAGGTG

Appendix B

Additional calculations for Option 1: 280 ng DNA Input for Quant and Qubit Broad Range Quantitation Methods

Calculation of the concentration limits for the DNA stock concentration of 11.2 to 154.0 ng/µl:

The minimum concentration is based on 280.0 ng DNA input / 25.0 µl volume = 11.2 ng/µl.

Based on a minimum pipetting volume of 2.0 µl, the maximum concentration is 280 ng*1.1 (10% overage) / 2.0 µl = 154.0 ng/µl, in a total volume of 27.5 µl.

Example calculations with 280.0 ng DNA input

Worked example for DNA stock concentration = 95.0 ng/µl:

- DNA stock volume (µl) = 280.0 ng x 1.1/95.0 ng/µl = 3.242 µl, rounds to 3.24 µl for accurate pipetting with P-10.
- Total volume of diluted DNA is fixed at 27.5 µl.
- RSB volume (µl) =27.5 µl - 3.24 µl = 24.26 µl, rounds to 24.3 µl for accurate pipetting with P-200.

Worked example for DNA stock concentration = 308.0 ng/µl:

- DNA stock volume (µl) is fixed at 2.0 µl
- Total volume of diluted DNA (µl) = 308.0 ng/µl x 2.0 µl/11.2 ng/µl =55.0 µl
- RSB volume (µl) = 55.0 µl - 2.0 µl = 53.0 µl

Additional calculations for Option 2: 350 ng DNA Input for Accuclear Ultra High Sensitivity Quantitation Method

Calculation of the concentration limits for DNA stock concentrations of 14.0 to 192.5 ng/µl:

The minimum concentration is based on 350.0 ng DNA input / 25.0 µl volume = 14.0 ng/µl.

Based on a minimum pipetting volume of 2.0 µl, the maximum concentration is 350 ng*1.1 (10% overage) / 2.0 µl = 192.5 ng/µl.

Example calculations with 350.0 ng DNA input

Worked example for DNA stock concentration = 118.75 ng/µl:

- DNA stock volume (µl) = 350.0 ng x 1.1/118.75 ng/µl = 3.242µl, rounds to 3.24 µl for accurate pipetting with P-10
- Total volume of diluted DNA is fixed at 27.5 µl.
- RSB volume (µl) =27.5 µl - 3.24 µl = 24.26 µl, rounds to 24.3 µl for accurate pipetting with P-200.

Worked example for DNA stock concentration = 308.0 ng/µl:

- DNA stock volume (µl) is fixed at 2.0 µl
- Total volume of diluted DNA (µl) = 308.0 ng/µl x 2.0 µl/14.0 ng/µl = 44.0 µl
- RSB volume (µl) = 44.0 µl - 2.0 µl = 42.0 µl

Revision History

Document	Date	Description of Change
Document # 200050132 v00.1	May 2024	Corrected input volume for Accuclear Ultra High Sensitivity Quantitation Method.
Document # 200050132 v00	April 2024	Initial release.

Patents and Trademarks

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Product Labeling

For a complete reference of symbols that appear on product packaging and labeling, refer to the symbol key at support.illumina.com on the *Documentation* tab for your kit.