NextSeq™ 550Dx Instrument

illumina®

Package Insert

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Catalog # 20005715

Intended Use

The NextSeq 550Dx instrument is intended for sequencing of DNA libraries when used with*in vitro* diagnostic assays. The NextSeq 550Dx instrument is to be used with specific registered, certified, or approved *in vitro* diagnostic reagents and analytical software.

Principles of Procedure

The Illumina NextSeq 550Dx instrument is intended for sequencing DNA libraries with *in vitro* diagnostic assays and is intended for use by qualified and trained clinical laboratory personnel trained in the use of in vitro diagnostic procedures performed in a clinical lab. For its input, the NextSeq 550Dx uses libraries generated from DNA where sample indexes and capture sequences are added to amplified targets. Sample libraries are captured on a flow cell and sequenced on the instrument using sequencing by synthesis (SBS) chemistry. 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 image analysis and base calling and assigns a quality score to each base for each sequencing cycle. When primary analysis finishes, secondary analysis can be executed on instrument to process base calls. The NextSeq 550Dx uses different secondary analysis modules depending on the workflow. For the Germline or Somatic Variant Modules, processing includes demultiplexing, FASTQ file generation, alignment, variant calling, and generation of variant call format (VCF and gVCF) files. The VCF and gVCF files contain information about variants found at specific positions in a reference genome.

Dual Boot Configuration

The NextSeq 550Dx includes a dual boot configuration to enable the use of the instrument in either diagnostic (Dx) or research use only (RUO) mode. *In vitro* diagnostic sequencing assays, including the Germline and Somatic Variant Modules, are executed in diagnostic mode. Only IVD sequencing reagents can be used in diagnostic mode. Performance characteristics and limitations of procedure for the NextSeq 550Dx instrument have been established using the Germline and Somatic Variant Modules in diagnostic mode.

Limitations of the Procedure

1. For *in vitro* diagnostic use.
2. The Germline and Somatic Variant Modules, when used with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) or NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles), are capable of delivering:
   - Sequencing output ≥ 90 gigabases (Gb)
   - Read length (in paired end run) 2 x 150 base pairs (bp)
   - Bases equal to or greater than Q30 ≥ 75% at read length of 2 x 150 bp
     Equal or greater than 75% of bases have Phred scale quality scores ≥ 30, indicating base call accuracy greater than 99.9%
3. Reads with indels (insertions, deletions, or combinations) where content length is > 25 bp are not aligned by the assay software. Consequently, indels of length > 25 bp are not detectable by the assay software.
4. The assay software might not align amplicon reads with extreme variant content, resulting in the region being reported as wild-type. Such extreme content includes:
   - Reads containing more than three indels
   - Reads of length at least 30 bp with single nucleotide variant (SNV) content > 4% of the total amplicon target length (excluding probe regions)
   - Reads of length < 30 bp with SNV content > 10% of the total amplicon length (including probe regions)
5. Large variants, including multinucleotide variants (MNVs) and large indels, might be reported as separate smaller variants in the output VCF file.
6. Deletion variants can be filtered or missed when spanning two tiled amplicons if the deletion length is greater than or equal to the overlap between the tiled amplicons.
7. The system cannot detect indels if they occur directly adjacent to a primer and there is no overlapping amplicon. For regions with overlapping amplicons, the assay cannot detect deletions when the region of overlap is smaller than the size of deletion to be detected. For example, if the region of overlap between two adjacent amplicons is two bases, the assay cannot detect any deletions including both of those bases. A single base deletion at either of those bases can be detected.
8. As with any hybridization-based library preparation workflow, underlying polymorphisms, mutations, insertions, or deletions in oligonucleotide-binding regions can affect the alleles being probed and the calls made during sequencing. For example:
   - A variant in phase with a variant in the primer region might not be amplified, resulting in a false negative.
   - Variants in the primer region could prevent the amplification of the reference allele, resulting in an incorrect homozygous variant call.
   - Indel variants in the primer region can cause a false positive call at the end of the read adjacent to the primer.
9. Indels can be filtered due to strand bias if they occur near the end of one read and are soft-clipped during alignment.

10. Small MNVs have not been validated and are only reported in the Somatic Variant Module.
11. Deletions are reported in the VCF at the coordinate of the preceding base per VCF format. Therefore, consider adjacent variants before reporting that an individual base call is a homozygous reference.
12. Germline-specific limitations:
    - The NextSeq 550Dx instrument, using the Local Run Manager Germline Variant Module for NextSeq 550Dx, is designed to deliver qualitative results for germline variant calling (eg, homozygous, heterozygous, wild-type).
    - When used with the Germline Variant Module, the minimum coverage per amplicon needed for accurate variant calling is 150x. As a result, 150 supporting DNA fragments are required, which is equivalent to 300 overlapping paired-end reads. The number of samples and the total number of bases targeted affect coverage. GC-content and other genomic content can affect coverage.
    - Copy number variation can affect whether a variant is identified as homozygous or heterozygous.
    - Variants in certain repetitive context are filtered out in the VCF files. The RMxN repeat filter is used to filter variants if all or part of the variant sequence is present repeatedly in the reference genome adjacent to the variant position. For germline variant calling, at least nine repeats in the reference are required for a variant to be filtered. Only repeats with a length up to 5 bp are considered (R5x9).
    - An indel and an SNV at a single locus can result in only one variant being reported.
13. Somatic-specific limitations.
    - The NextSeq 550Dx instrument, using the Local Run Manager Somatic Variant Module for NextSeq 550Dx, is designed to deliver qualitative results for somatic variant calling (eg presence of a somatic variant with a variant frequency greater than or equal to 0.026 with a limit of detection of 0.05).
    - When used with the Somatic Variant Module, the minimum coverage per amplicon needed for accurate variant calling is 450x per oligonucleotide pool. As a result, 450 supporting DNA fragments are required per oligonucleotide pool, which is equivalent to 900 overlapping paired-end reads. The number of samples and the total number of bases targeted affect coverage. GC-content and other genomic content can affect coverage.
    - For somatic variant calling, at least six repeats in the reference are required for the variant to be filtered, and only repeats with length up to 3 bp are considered (R3x6).
    - The Somatic Variant Module cannot differentiate between germline and somatic variants. The module is designed to detect variants across a range of variant frequencies, but variant frequency cannot be used to differentiate somatic variants from germline variants.
    - Normal tissue in the specimen impacts the detection of variants. The reported limit of detection is based on a variant frequency relative to the total DNA extracted from both tumor and normal tissue.

Product Components

The Illumina NextSeq 550Dx consists of the following:

1. NextSeq 550Dx instrument (Catalog # 20005715)

2. Software components for the NextSeq 550Dx instrument, including the following:

Software Application	Function	Description
NextSeq 550Dx Operating Software (NOS)	Controls instrument operation	The NOS software application manages the operation of the instrument during sequencing and generates images for use by Real-Time Analysis (RTA) software.
Real-time Analysis Software (RTA)	Performs primary analysis	The RTA software application converts the images generated by NOS for each tile per cycle of the sequencing run into base call files, which are inputs for Local Run Manager analysis modules. The RTA software application does not contain a user interface.
Local Run Manager	Interface for module selection	The Local Run Manager software is an on-instrument integrated solution for user management, selecting the appropriate analysis module, and monitoring status.
Somatic Variant Module	Performs secondary analysis	This Local Run Manager analysis module software processes base calls through secondary analysis. The processing includes demultiplexing, FASTQ file generation, alignment, variant calling, and reporting. The variant caller (Pisces) generates VCF files that contain information about variants found at specific positions in a reference genome and includes the measured variant frequency.
Germline Variant Module	Performs secondary analysis	This Local Run Manager analysis module software processes base calls through secondary analysis. The processing includes demultiplexing, FASTQ file generation, alignment, variant calling, and reporting. The variant caller (Pisces) generates VCF files that contain information about variants found at specific positions in a reference genome and identifies each variant as heterozygous or homozygous.

3. **Optional** Illumina DRAGEN Server for NextSeq 550Dx (Catalog # 20086130), including the following software component:

Software Application	Function	Description
Illumina Run Manager	Interface for application module selection	The Illumina Run Manger software is installed on the optional, off-instrument DRAGEN server. Illumina Run Manager enables user management, selection of analysis module, and monitoring sequencing run and analysis status.

The optional Illumina DRAGEN Server for NextSeq 550Dx is only available in select countries. Contact an Illumina representative for regional availability.

Operating Conditions

For more information on operating conditions, see the Environmental Considerations section in the *NextSeq 550Dx Instrument Site Prep Guide (document # 1000000009869)*.

Element	Specification
Temperature	Maintain a lab temperature of 19°C to 25°C (22°C ±3°C). This temperature is the operating temperature of the instrument. During a run, do not allow the ambient temperature to vary more than ±2°C.
Humidity	Maintain a noncondensing relative humidity between 20–80%.

Equipment and Materials

Equipment and Materials Required, Sold Separately

NextSeq 550Dx High Output Reagent Kit v2.5 (75 cycles), Catalog # 20028870

NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles), Catalog # 20028871

Equipment and Materials Required, Not Provided

User-Supplied Consumables for Sequencing Runs

Consumable	Supplier	Purpose
Alcohol wipes, 70% Isopropyl or Ethanol, 70%	VWR, catalog # 95041-714 (or equivalent) General lab supplier	Flow cell cleaning and general purpose
Lab tissue, low-lint	VWR, catalog # 21905-026 (or equivalent)	Flow cell cleaning and general purpose

User-Supplied Consumables for Instrument Maintenance

Consumable	Supplier	Purpose
NaOCl, 5% (sodium hypochlorite)	Sigma-Aldrich, catalog # 239305 (or laboratory-grade equivalent)	Washing the instrument using the manual post-run wash; diluted to 0.12%
Tween 20	Sigma-Aldrich, catalog # P7949	Washing the instrument using manual wash options; diluted to 0.05%
Water, laboratory-grade	General lab supplier	Washing the instrument (manual wash)
Air filter	Illumina, catalog # 20063988	Cleaning the air the instrument takes in for cooling

Guidelines for Laboratory-Grade Water

Always use laboratory-grade water or deionized water to perform instrument procedures. Never use tap water. Use only the following grades of water or equivalents:

- Deionized water
- Illumina PW1
- 18 Megohms (MΩ) water
- Milli-Q water
- Super-Q water
- Molecular biology grade water

Warnings and Precautions

CAUTION

Federal law restricts this device to sale by or on the order of a physician or other practitioner licensed by the law of the State in which he/she practices, to use or order the use of the device.

1. **Some components of reagents provided by Illumina for use with the NextSeq 550Dx instrument 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 additional environmental, health, and safety information, see the Safety Data Sheets (SDS) at support.illumina.com/sds.html.
2. 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.

3. Handle all blood specimens as if they are known to be infectious for human immunodeficiency virus (HIV), human hepatitis B virus (HBV), and other bloodborne pathogen agents (universal precautions).
4. Failure to follow the procedures as outlined can result in erroneous results or significant reduction in sample quality.
5. 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 kit reagents. Wash hands thoroughly after handling specimens and kit reagents.
6. Proper laboratory practices and good laboratory hygiene are required to prevent PCR products from contaminating reagents, instrumentation, and genomic DNA samples. PCR contamination can cause inaccurate and unreliable results.
7. To prevent contamination, make sure that pre-amplification and post-amplification areas have dedicated equipment and consumables (eg, pipettes, pipette tips, heat blocks, vortexers, and centrifuges).
8. Index to sample pairing must match the printed plate layout exactly. Local Run Manager automatically populates the index primers associated with the sample names, when entered in the module. The user is advised to verify the index primers associate with samples before starting the sequencing run. Mismatches between the sample and plate layout results in loss of positive sample identification and incorrect result reporting.
9. Installation of user-supplied antivirus software is strongly recommended to protect the computer against viruses. Consult user manual for instructions on installation.
10. Do not operate the NextSeq 550Dx with any of the panels removed. Operating the instrument with any of the panels removed creates potential exposure to line voltage and DC voltages.
11. Do not touch the flow cell stage in the flow cell compartment. The heater in this compartment operates between 22°C and 95°C and can result in burns.
12. The instrument weighs approximately 185 lbs. and could cause serious injury if dropped or mishandled.

Instructions for Use

The following instructions for use of the NextSeq 550Dx instrument require reagents provided in the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) or NextSeq 550Dx High Output Reagent Kit v2.5 (75 cycles).

Create Run

Create a sequencing run using Local Run Manager or Illumina Run Manager. Instructions for using Local Run Manager are included below and in the NextSeq 550Dx Instrument Reference Guide (document # 1000000009513). For instructions on how to create a run using Illumina Run Manager, refer to the Illumina Run Manager for NextSeq 550Dx Software Guide (document # 200025239).

For instructions on selecting between Local Run Manager or Illumina Run Manager, refer to the Illumina Run Manager for NextSeq 550Dx Software Guide (document # 200025239). For detailed instructions on specific applications, refer to the module or application guide for the specific assay.

The following instructions pertain to using the Local Run Manager Germline and Somatic Variant modules.

Set Parameters

1. Log in to Local Run Manager.
2. Select **Create Run**, and select **Somatic Variant** or **Germline Variant**.
3. Enter a run name that identifies the run from sequencing through analysis.
   Use alphanumeric characters, spaces, underscores, or dashes.
4. [Optional] Enter a run description to help identify the run.
   Use alphanumeric characters, spaces, underscores, or dashes.
5. Select the number of samples and index set from the drop-down list.
   Consider the following information when you make a selection.

- The drop-down list contains numbers of samples with an index set. For example, 24-Set 1 indicates 24 samples to be tested, with indexes from index set 1.
- Index set numbers refer to different sets of i5 and i7 index pairs. Set 1 and Set 2 both provide index diversity. Two index sets are offered to help prevent depletion of a single set.
- Choose the number of samples that is nearest to the number of samples you are testing. If the exact number of samples is not in the list, select the number nearest, but less than the number you are testing. For example, if you want to test 18 samples, select 16 samples.
- Suggested sample wells and index combinations that meet index diversity requirements are highlighted in green.

Import Manifest Files for the Run

1. Make sure that the manifests you want to import are available in an accessible network location or on a USB drive.
2. Select **Import Manifests**.
3. Navigate to the manifest file and select the manifests that you want to add.

NOTE To make manifest files available for all runs using the Germline Variant or Somatic Variant analysis module, add manifests using the Module Settings feature. This feature requires admin user level permissions. For more information, see the *NextSeq 550Dx Instrument Reference Guide (document # 1000000009513)*.

Specify Samples for the Run

Specify samples for the run using one of the options and the directions that follow.

Enter samples manually—Use the blank table on the Create Run screen.

Import samples—Navigate to an external file in a comma-separated values (*.csv) format. A template is available for download on the Create Run screen.

Enter Samples Manually

1. Enter a unique sample name (***Somatic Variant analysis module***) or sample ID (***Germline Variant analysis module***).
   Use alphanumeric characters, dashes, or underscores.
2. [Optional] For positive or negative control samples, right-click and select the control type.
   The control in one sample well auto populates the corresponding well in the other pool with the same control.
3. [Optional] Enter a sample description in the Sample Description field.
   Use alphanumeric characters, dashes, or underscores.
4. Select an Index 1 adapter from the Index 1 (i7) drop-down list.
   When you use suggested sample wells, the software auto-populates i7 and i5 index adapters that meet diversity index requirements. If the exact number of samples you are testing is not in the list, make sure to select index adapters for extra wells.
5. Select an Index 2 adapter from the Index 2 (i5) drop-down list.
6. Select a manifest file from the Manifest drop-down list.
   Samples in Pool A require a different manifest than samples in Pool B.
7. Choose an option to view, print, or save the plate layout as a reference for preparing libraries:
   - Select the **Print** icon to display the plate layout. Select **Print** to print the plate layout.
   - Select **Export** to export sample information to an external file.
8. Select **Save Run**.

Import Samples

1. Select **Import Samples** and browse to the location of the sample information file. There are two types of files you can import.
   - Select **Template** on the Create Run screen to make a new plate layout. The template file contains the correct column headings for import. Enter sample information in each column for the samples in the run. Delete example information in unused cells, and then save the file.
   - Use a file of sample information that was exported from the Germline Variant or Somatic Variant module using the Export feature.
2. Select the **Print** icon to display the plate layout.
3. Select **Print** to print the plate layout as a reference for preparing libraries.
4. Select **Save Run**.

Prepare the Reagent Cartridge

Make sure to follow reagent cartridge directions carefully for successful sequencing.

1. Remove the reagent cartridge from -25°C to -15°C storage.
2. Choose one of the following methods to thaw the reagents. Do not submerge the cartridge. After the cartridge is thawed, dry it before you proceed to the next step.

Temperature	Time to Thaw	Stability Limit
15°C to 30°C water bath	60 minutes	Not to exceed 6 hours
2°C to 8°C	7 hours	Not to exceed 5 days

NOTE If more than one cartridge is thawing in the same water bath, allow for additional thawing time.

3. Invert the cartridge five times to mix reagents.
4. Inspect the bottom of the cartridge to make sure that reagents are thawed and free of precipitates. Confirm that positions 29, 30, 31, and 32 are thawed, as they are the largest and take the longest to thaw.
5. Gently tap on the bench to reduce air bubbles.
   For best results, proceed directly to loading the sample and setting up the run.

Prepare the Flow Cell

1. Remove a new flow cell box from 2°C to 8°C storage.
2. Remove the foil package from the box and set aside at room temperature for 30 minutes.

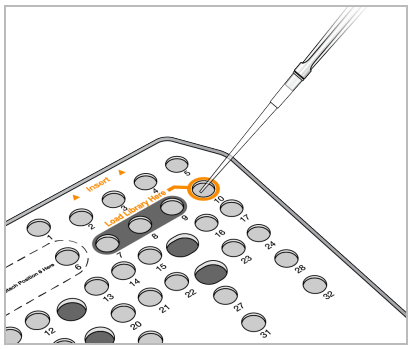
Prepare Libraries for Sequencing

Denature and dilute your libraries to a loading volume of 1.3 ml. In practice, loading concentration can vary depending on library preparation and quantification methods. Dilution of sample libraries depends on the complexity of oligonucleotide pools. For directions on how to prepare sample libraries for sequencing, including library dilution and pooling, see the Instructions for Use section for the applicable library preparation kit. Optimization of cluster density on the NextSeq 550Dx is required.

Load Libraries onto the Reagent Cartridge

1. Clean the foil seal covering reservoir #10 labeled **Load Library Here** using a low-lint tissue.
2. Pierce the seal with a clean 1 ml pipette tip.
3. Load 1.3 ml of prepared libraries into reservoir #10 labeled **Load Library Here**. Avoid touching the foil seal as you dispense the libraries.

Figure 1 Load Libraries



Set Up a Sequencing Run

See the NextSeq 550Dx Instrument Reference Guide (document # 1000000009513) for complete run setup instructions.

1. Log in to the NextSeq 550Dx with your Local Run Manager or Illumina Run Manager software password.
2. From the Home screen of the NOS software, select **Sequence**.
3. Select a run from the list, and then select **Next**.
   A series of run setup screens open in the following order: Load Flow Cell, Load Buffer Cartridge, Load Reagent Cartridge, and Pre-run Check.

   NOTE Runs are only accessible using the same Run Manager used when planning the run. For instruction on how to set the Run Manager Software, refer to Illumina Run Manager for NextSeq 550Dx Software Guide (document # 200025239).

4. When the Load Flow Cell screen appears, clean and then load the flow cell.
   - Remove the flow cell from the foil package.
   - Open the clear plastic clamshell package and remove the flow cell
   - Clean the glass surface of the flow cell with a lint-free alcohol wipe. Dry the glass with a low-lint lab tissue
   - Make sure that the glass surface of the flow cell is clean. If necessary, repeat the cleaning step.
   - Remove the used flow cell from a previous run.
   - Align the flow cell over the alignment pins and place the flow cell on the stage.
5. Select **Load**.
   The door closes automatically, the flow cell ID appears on the screen, and the sensors are checked.
6. Follow the software prompts to empty the spent reagents container, load the NextSeq 550Dx buffer cartridge, and load the NextSeq 550Dx reagent cartridge.
   When the NextSeq 550Dx buffer and reagent cartridges are loaded, the software reads and records the RFID. The buffer and reagent cartridge IDs appear on the screen and the sensors are checked.

7. When the automated pre-run check is complete, select **Start**. (Not required if configured to start automatically.)
8. The Sequencing screen opens when the run begins. This screen provides a visual representation of the run in progress, including intensities and quality scores (Q-scores).

Results

Real-Time Analysis (RTA) is an integrated software that performs image analysis and base calling and assigns a quality score to each base for each sequencing cycle. When primary analysis finishes, the selected application module begins secondary analysis automatically. The secondary analysis processes described here are for Local Run Manager Germline and Somatic Variant Modules on the NextSeq 550Dx instrument.

Demultiplexing

Demultiplexing compares each Index Read sequence to the index sequences specified for the run. No quality values are considered in this step.

Index reads are identified using the following steps:

- Samples are numbered starting from 1 based on the order they are listed for the run.
- Sample number 0 is reserved for clusters that were not assigned to a sample.
- Clusters are assigned to a sample when the index sequence matches exactly or when there is up to a single mismatch per Index Read.

FASTQ File Generation

After demultiplexing, the software generates intermediate analysis files in the FASTQ format, which is a text format used to represent sequences. FASTQ files contain reads for each sample and the associated quality scores. Clusters that did not pass filter are excluded.

Each FASTQ file contains reads for only one sample, and the name of that sample is included in the FASTQ file name. In the Germline and Somatic Variant Modules, eight FASTQ files are generated per sample per oligo pool, four from Read 1 and four from Read 2. This output results in a total of 8 and 16 FASTQ files per sample for Germline and Somatic, respectively. FASTQ files are the primary input for alignment.

Alignment

During the alignment step, the banded Smith-Waterman algorithm aligns clusters from each sample against amplicon sequences specified in the manifest file.

The banded Smith-Waterman algorithm performs semiglobal sequence alignments to determine similar regions between two sequences. Instead of comparing the total sequence, the Smith-Waterman algorithm compares segments of all possible lengths.

Each paired-end read is evaluated in terms of its alignment to the relevant probe sequences for that read.

- Read 1 is evaluated against the reverse complement of the Downstream Locus-Specific Oligos (DLSO).
- Read 2 is evaluated against the Upstream Locus-Specific Oligos (ULSO).
- If the start of a read matches a probe sequence with no more than one mismatch, the full length of the read is aligned against the amplicon target for that sequence.
- If the start of a read matches a probe sequence with no more than three differences (mismatches or shifts due to leading indels), the full length of the read is aligned against the amplicon target for that sequence.
- Indels within the DLSO and ULSO are not observed given the assay chemistry.

Alignments are filtered from alignment results based on mismatch rates over either the region of interest or the full amplicon, depending on the amplicon length. Filtered alignments are written in alignment files as unaligned and are not used in variant calling.

Variant Calling

The variant caller Pisces is designed to make SNV and indel variant calls from libraries prepared for the instrument.

Reports and Additional Output Files

The variant analysis modules produce PDF and tab-delimited (*.txt) reports that display metrics such as sequencing depth and variant counts. The modules also produce output files such as VCF and genome Variant Call Format (gVCF) files for variant-calling applications.

Quality Control Procedures

The NextSeq 550Dx software evaluates each run, sample, and base call against quality control metrics. Positive and negative controls are also recommended in library preparation and need to be evaluated. Evaluate controls as follows:

- **Negative Control (No Template Control) or other negative control**— Must generate the expected result. If the negative control generates a result different from what is expected, then a possible error in sample tracking, incorrect recording of indexing primers, or contamination has occurred.
- **Positive Control Sample**— Must generate the expected result. If the positive control generates a result different from what is expected, then a possible error in sample tracking or incorrect recording of indexing primers has occurred.

Performance Characteristics

Performance characteristics for the NextSeq 550Dx instrument were established using the Germline and Somatic Variant Modules with the TruSeq Custom Amplicon Kit Dx and NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and confirmed using the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). Studies included Sample Indexing, Sample Carryover, DNA Input, Analytical Sensitivity (Limit of Blank / Limit of Detection), Accuracy, Precision, Method Comparison, and Reproducibility.

The analytical studies using the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) were designed to evaluate performance claims previously established with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles). Results demonstrate that reagent kits (v2 and v2.5) have comparable performance using the TruSeq Custom Amplicon Kit Dx. See the *TruSeq Custom Amplicon Kit Dx package insert* for performance characteristics related to pre-analytical factors, such as extraction methods or interfering substances.

Definitions of Calculations Used in Performance Characteristics

1. Positive Percent Agreement (PPA) is calculated as the proportion of loci classified as variants by a reference method that the assay correctly reports.
    - (# variant loci correctly reported by the assay) / (total # of variant loci)

    Variant loci reported by the assay that are concordant with the reference method are true positives (TPs). Variant loci reported as reference calls or as different variant calls by the assay are false negatives (FNs).
2. Negative Percent Agreement (NPA) is calculated as the proportion of loci classified as wild-type by a reference method that the assay correctly reports.
    - (# wild-type loci correctly reported by the assay) / (total # of wild-type loci)

    Wild-type loci reported by the assay that are concordant with the reference method are true negatives (TNs). Wild type loci reported as variants by the assay are false positives (FPs).
3. Overall percent agreement (OPA) is calculated as the proportion of loci correctly reported by the assay relative to a reference method.
    - ((# variant loci correctly reported by the assay) + (# wild-type loci correctly reported by the assay)) / ((total # of variant loci) + (total # of wild-type loci))
4. The calculations of PPA, NPA, and OPA do not include no calls (variant or reference loci not meeting one or more quality filters).
5. Autosomal call rate is calculated as total number of loci passing filters divided by the total number of positions sequenced for chromosomes 1–22; chromosomes X and Y are excluded. This metric does not consider the agreement of the calls with the reference method.

NextSeq 550Dx High Output Reagent Kit v2 (300 Cycles) Performance

Sample Indexing

Sample index primers, added during library preparation, assign a unique sequence to each sample DNA. These unique sequences allow multiple samples to be pooled together into a single sequencing run. Sample indexing is used for both germline and somatic workflows. The purpose of this study was to establish the minimum (8) and maximum (96) number of samples that can be processed in a single sequencing run by the NextSeq 550Dx instrument. Eight unique Platinum Genome samples were tested with 12 different indexing primer combinations per sample. Sample results from four sequencing runs using the Germline Variant Module were compared to Platinum Genomes version 2016-1.0.

For the first set of runs, 96 uniquely indexed sample libraries were tested with a representative assay designed to query a variety of genes covering 12,588 bases per strand across all 23 human chromosomes to verify the ability of the assay to make a genotyping call consistently for a given sample across different indexing primer combinations. For the second set of runs, eight uniquely indexed sample libraries were sequenced in two sequencing runs to verify the minimum number of indexes supported.

For the 96-index runs, PPA for SNVs ranged from 98.7% to 100%, PPA for insertions and deletions was 100% and NPA was 100% for each of the 96 index combinations. The 8-index runs had PPA values of 100% (SNVs, insertions, and deletions) and NPA of 100% for each of the eight index combinations.

Sample Carryover

The NextSeq 550Dx instrument allows for sequencing of multiple samples plus controls in a single sequencing run. A study was conducted to evaluate the extent of sample carryover within a sequencing run (within-run) and between sequencing runs (run-to-run). Two Platinum Genome samples, one male and one female, were tested with a representative assay designed to query various genes covering 12,588 bases (150 amplicons) across 23 different chromosomes, including both sex chromosomes. Libraries were sequenced on the NextSeq 550Dx instrument using the Germline Variant Module. Carryover of male samples into female samples was observed by the presence of Y chromosome amplicon reads in female samples.

Within-run carryover can be introduced during cluster generation, index cycle base calling, and sample demultiplexing. For testing of sample carryover within a sequencing run, a library pool consisting of 46 replicates each of male and female samples plus four no template controls was sequenced one time on the NextSeq 550Dx instrument. Within-run sample carryover was assessed by comparing Y chromosome amplicon coverage of each female replicate to the average Y chromosome amplicon coverage of all male replicates in the pool. The median observed within-run carryover was 0.084%.

For testing of run-to-run sample carryover, two library pools were prepared and sequenced consecutively on one NextSeq 550Dx instrument. The first pool contained 46 replicates of female sample plus two no template controls. The second pool contained 46 replicates of male sample plus two no template controls. Both pools used the same set of index adapters. The female pool was sequenced first followed by a subsequent sequencing run with the male pool, followed by another repeat sequencing run of the female pool. Run-to-run

sample carryover was assessed by comparing Y chromosome amplicon coverage between corresponding replicates of the female pool repeat run and male pool run. The median observed run-to-run carryover was 0.0076%

DNA Input

Blood (Germline)

The blood DNA input range for the TruSeq Custom Amplicon Kit Dx library preparation using the Germline Variant Module workflow was established for the NextSeq 550Dx instrument. This range was evaluated by performing a serial dilution study using 13 Platinum Genome samples with a representative assay designed to query various genes covering 12,588 bases across 23 different chromosomes. The library was sequenced on two NextSeq 550Dx instruments using one lot of NextSeq 550Dx High Output Reagent Kit v2 (300 cycles).

Five samples were tested in duplicate at five DNA input levels ranging from 250 ng to 12 ng (250 ng, 100 ng, 50 ng, 25 ng, and 12 ng). Eight samples were tested in as a single replicate at each of the five DNA input levels. For determination of accuracy, sample genotypes were compared to Platinum Genomes version 2016-1.0. Results were determined for each input level. PPA for each variant type (SNVs, insertions, and deletions) is presented in Table 1; NPA is presented in Table 2. All input levels had similar accuracy. The recommended DNA input for TruSeq Custom Amplicon Kit Dx is 50 ng with 25 ng and 100 ng providing a lower and upper limit to meet performance characteristics.

Table 1 PPA Results for Each DNA Input by Variant Type

DNA Input (ng)	Variant Type	Expected Variants	TP	FN	Variant No Calls	PPA (%)
12	SNV	2412	2381	31	0	98.7
25			2404	8	0	99.7
50			2403	9	0	99.6
100			2412	0	0	100
250			2412	0	0	100
12	Insertion	808	784	3	21	99.6
25			781	5	22	99.4
50			786	2	20	99.8
100			786	0	22	100
250			786	0	22	100

12	Deletion	758	732	12	14	98.4
25			737	7	14	99.1
50			742	2	14	99.7
100			744	0	14	100
250			744	0	14	100

Table 2 NPA for Each DNA Input

DNA Input (ng)	TN	FP	Reference No Calls	NPA (%)
12	430940	4	26	>99.9
25	430936	0	34	100
50	430936	2	32	>99.9
100	430942	0	28	100
250	430942	0	28	100

FFPE (Somatic)

The formalin-fixed paraffin-embedded (FFPE) DNA input range for TruSeq Custom Amplicon Kit Dx library preparation using the Somatic Variant Module workflow was established for the NextSeq 550Dx instrument. The DNA input range was evaluated by performing a serial dilution study using three Platinum Genome samples with a representative assay designed to query various genes covering 12,588 bases across 23 different chromosomes. Platinum Genome cell lines GM12878 and GM12877 were formalin fixed and embedded in paraffin followed by DNA extraction. GM12878 was diluted with GM12877 such that the variant allele frequencies (VAFs) of 79 variants (55 SNVs, 9 insertions, and 15 deletions) were near 0.025, 0.05, or 0.10. In addition, each sample had 91 variants with higher variant frequencies of up to 1.0 VAF. Samples were processed in duplicate at five DNA input levels with mean delta quantitative cycle (dCq) of 2.1, 3.6, 4.6, 6.0, and 7.8 as measured by the TruSeq Custom Amplicon Dx - FFPE QC Kit. Each library was sequenced on two NextSeq 550Dx instruments using two lots of NextSeq 550Dx High Output Reagent Kit v2 (300 cycles). For determination of accuracy, sample variant calls were compared to Platinum Genomes version 2016-1.0. PPA for each variant type (SNVs, insertions, and deletions) is presented in Table 3; NPA is presented in Table 4. The recommended DNA input for variants at 0.05 VAF or above is dCq ≤ 4 with 4.6 providing a lower limit to meet the performance characteristics.

Table 3 PPA Results for Each DNA Input by Variant Type

Mean dCq	Variant Type	Expected Variants	Expected No Calls	Target Dilution VAF					
				0.025		0.05		0.10	
				Variant No Calls	PPA (%)	Variant No Calls	PPA (%)	Variant No Calls	PPA (%)
2.1	SNV	808	Not applicable.	196	100	0	100	0	100
3.6				250	99.3	4	100	0	100
4.6				251	94.6	51	99.2	5	100
6.0				257	65.3	213	91.4	100	100
7.8				254	69.3	185	90.7	100	100
2.1	Insertion	264	8	66	96.5	8	100	8	100
3.6				62	97.0	8	100	8	100
4.6				48	96.3	21	100	8	100
6.0				40	80.4	47	98.2	24	95.8
7.8				57	87.0	56	96.2	31	100
2.1	Deletion	304	16	58	100	16	100	16	100
3.6				80	100	16	100	16	100
4.6				65	95.4	28	100	16	100
6.0				78	74.8	105	94.0	36	100
7.8				76	75.0	79	95.1	57	98.8

Table 4 NPA for Each DNA Input

Mean dCq	Expected Wild Type	Target Dilution VAF					
		0.025		0.05		0.10	
		Reference No Calls	NPA (%)	Reference No Calls	NPA (%)	Reference No Calls	NPA (%)
2.1	93688	344	100	260	100	324	100
3.6		400	100	332	100	380	100
4.6		1308	100	1336	100	784	100
6.0		3900	>99.9	3296	>99.9	2996	100
7.8		3020	>99.9	2880	>99.9	2448	>99.9

Analytical Sensitivity (Limit of Blank [LoB] and Limit of Detection [LoD])

This study was conducted to evaluate the Limit of Blank (LoB) and Limit of Detection (LoD) for the Somatic Variant Module on the NextSeq 550Dx instrument. This was performed using a representative assay designed to query various genes covering 12,588 bases across 23 different chromosomes. Platinum Genome cell lines GM12878 and GM12877 were formalin fixed and embedded in paraffin followed by DNA extraction. GM12878 was diluted with GM12877 such that the variant frequencies of 74 variants (53 SNVs, 7 insertions, and 14 deletions) were 0.05 ± 0.02. GM12877 and diluted GM12878 (GM12878-D) were tested over six consecutive start days with a single instrument, alternating between two lots of NextSeq 550Dx High Output Reagent Kit v2 (300 cycles), for a total of six sequencing runs. This test resulted in 60 replicates for each variant in GM12878-D and 72 replicates for each corresponding wild-type coordinate in GM12877 for each reagent lot. LoB and LoD were calculated using the classical approach stated in CLSI EP17-A2 using the nonparametric option. LoB and LoD were calculated for SNVs, insertions, and deletions separately by pooling the variant frequencies for a given variant type. The Type I error was defined as 0.01, and the Type II error was defined as 0.05.

For the LoB, the pooled variant frequencies were sorted from lowest to highest, and the 99th rank position for each reagent lot for each variant type was calculated (Table 5). The Somatic Variant Module uses a cutoff (the effective LoB) of 0.026 VAF for determining the qualitative detection of variants. The calculated LoB verified that this cutoff results in a Type I error of no more than 0.01.

Table 5 Limit of Blank

Variant Type	Total Observations	LoB Reagent Lot 1 (%)	LoB Reagent Lot 2 (%)
SNV	3816	0.77	0.77
Insertion	504	0.56	0.56
Deletion	1008	1.20	1.20

For the LoD, the percentage of individual mutation frequency for each reagent lot for each variant type falling below the cutoff of 0.026 was calculated Table 6. Because the percentages were less than the Type II error of 5% (0.05), the median of the combined variant frequencies was calculated as the LoD (Table 6). The LoD for each variant type was taken as the larger of the two values calculated for the two reagents lots – 4.97% for SNVs, 5.12% for insertions, and 5.26% for deletions.

Table 6 Limit of Detection

Reagent Lot	Variant Type	Total Observations	# of VAF measurements < 2.6%	% of VAF measurements < 2.6%	Limit of Detection (%)
1	SNV	3180	53	1.7	4.94
	Insertion	420	6	1.4	5.08
	Deletion	840	7	0.8	5.22

2	SNV	3180	51	1.6	4.97
	Insertion	420	5	1.2	5.12
	Deletion	840	7	0.80	5.26

Accuracy

Germline

The following study was conducted to assess the variant calling accuracy of the Germline Variant Module on the NextSeq 550Dx instrument using the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles). 13 unique Platinum Genome samples were tested using a representative assay designed to query a variety of genes covering 12,588 bases (150 amplicons) across 23 different chromosomes. A total of nine runs were performed using three sequencing instruments, three reagent lots, and three operators over five start days. Accuracy was determined for SNVs, insertions, and deletions by comparing the results to a well-characterized composite reference method, Platinum Genomes version 2016-1.0. Confident genomic regions were defined based on this reference method unless specified otherwise.

Table 7 Summary of Germline Agreement

Criteria	Total Observations[1]	Result by Observation[2]	Result by Run[3]
PPA for SNV	819	98.7	>99.9
PPA for insertions	819	95.0	98.9
PPA for deletions	819	100	100
NPA	819	100	100
OPA	819	>99.9	>99.9

[1]Calculated as number of samples per run (91) x number of Runs (9) = 819.
[2]Lowest observed value by sample replicate across all 9 runs.
[3]Lowest value when data from each run are analyzed in aggregate.

Table 8 contains the study data presented with positive and negative percent agreement on a per sample basis, where the variant results are compared to Platinum Genomes version 2016-1.0 for PPA calculations. The three variant types (SNVs, insertions, and deletions) are combined. Because the reference method only provides results for the single nucleotide variants and insertions/deletions, non-variant base results are compared to human genome reference sequence build hg19 for NPA calculations.

Table 8 Germline Agreement per Sample

Sample	Mean Call Rate	Expected Variants[1]	TP	FN	Variant No Calls	TN	FP	PPA	NPA	OPA
NA12877	>99.9	4788	4788	0	0	756762	0	100	100	100
NA12878	>99.9	8505	8379	1	125	751464	0	>99.9	100	>99.9
NA12879	>99.9	6048	5985	5	58	757701	0	99.9	100	>99.9
NA12880	>99.9	6993	6930	0	63	757638	0	100	100	100
NA12881	>99.9	7875	7811	3	61	751653	0	>99.9	100	>99.9
NA12882	>99.9	6300	6174	3	123	754803	0	>99.9	100	>99.9
NA12883	>99.9	7119	7056	0	63	751905	0	100	100	100
NA12884	>99.9	7182	7119	6	57	754146	0	99.9	100	>99.9
NA12885	>99.9	7686	7560	2	124	754173	0	>99.9	100	>99.9
NA12886	>99.9	7245	7182	7	56	752469	0	99.9	100	>99.9
NA12887	>99.9	7119	7119	0	0	750645	0	100	100	100
NA12888	>99.9	6804	6804	0	0	756065	0	100	100	100
NA12893	>99.9	7434	7371	1	62	750015	0	>99.9	100	>99.9

[1] Total number of variants in all sample replicates across 9 runs.

Table 9 contains the study data presented on a per sample basis, where the variant results are compared to the well-characterized composite reference method. Detection is evaluated for each variant type – SNVs, insertions, and deletions – separately. Reference positions are excluded.

Table 9 Germline Agreement per Sample by Variant Type

	SNVs			Insertions			Deletions		
Sample	Expected	TP	FN	Expected	TP	FN	Expected	TP	FN
NA12877	2331	2331	0	1323	1323	0	1134	1134	0
NA12878	5733	5733	0	1260	1197	1	1512	1449	0
NA12879	3591	3591	0	1323	1260	5	1134	1134	0
NA12880	4221	4221	0	1512	1512	0	1260	1197	0
NA12881	4914	4913	1	1512	1449	2	1449	1449	0
NA12882	3717	3717	0	1386	1323	3	1197	1134	0
NA12883	4284	4284	0	1449	1449	0	1386	1323	0
NA12884	4284	4284	0	1575	1512	6	1323	1323	0

	SNVs			Insertions			Deletions		
Sample	**Expected**	**TP**	**FN**	**Expected**	**TP**	**FN**	**Expected**	**TP**	**FN**
NA12885	4725	4725	0	1575	1512	2	1386	1323	0
NA12886	4347	4347	0	1449	1386	7	1449	1449	0
NA12887	4284	4284	0	1323	1323	0	1512	1512	0
NA12888	4158	4158	0	1449	1449	0	1197	1197	0
NA12893	4599	4599	0	1386	1323	1	1449	1449	0

The samples were further analyzed for calling small insertions and deletions (indels). An overall summary is presented in Table 10. There were a total of 71 indels ranging in size from 1–24 bp for insertions and 1–25 bp for deletions.

Table 10 Summary of Germline Indel Detection

Variant Type	Expected Variants	TP	FN	Variant No Calls	PPA
Insertion	18522	18018	27	477	99.9
Deletion	17388	17073	0	315	100

The representative assay consisted of 150 amplicons designed to cover various genomic content. The GC content of the amplicons ranged from 0.19–0.87. Amplicons also had a range of single nucleotide (eg, PolyA, PolyT), dinucleotide, and trinucleotide repeats. Data were compiled on a per amplicon basis (Table 11) to determine the effect of genomic content on percent correct calls. Percent correct calls consists of variant and reference calls and is less than 100% if there are either incorrect or no calls.

Table 11 Germline Amplicon-level Accuracy

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
1	1	36450499	36450591	93	93	Indel	0.22	76167	0	0	100
2	1	109465122	109465200	79	79	Poly A (5), Poly C (5), indel	0.38	64701	0	0	100
3	1	218353867	218353957	91	91	Indel	0.4	74529	0	0	100
4	1	223906657	223906748	92	92	Indel	0.49	75348	0	0	100
5	1	228526602	228526682	81	81	Poly G (5)	0.69	66339	0	0	100
6	1	236372039	236372108	70	70	Poly T (10), indel	0.39	57330	0	0	100
7	1	247812041	247812128	88	88	Poly A (5), CT(3), TAA(3), indel	0.27	72072	0	0	100
8	2	55862774	55862863	90	90	Indel	0.28	73710	0	0	100
9	2	87003930	87004009	80	80	Indel	0.38	65520	0	0	100
10	2	177016721	177016805	85	81	N/A	0.65	66339	0	0	100
11	2	186625727	186625801	75	75	Poly A (8)	0.35	61425	0	0	100
12	2	190323504	190323591	88	88	Poly T (5)	0.42	72072	0	0	100
13	2	200796740	200796826	87	87	Poly T (5), indel	0.31	71253	0	0	100
14	2	212245049	212245139	91	91	Poly T (5), Poly A (6), indel	0.3	74529	0	0	100
15	2	228147052	228147144	93	93	Indel	0.43	76167	0	0	100
16	2	235016350	235016422	73	73	Poly T (5), indel	0.42	59787	0	0	100
17	3	4466229	4466321	93	93	AT(3), indel	0.27	74823	0	1344	98.2
18	3	46620561	46620643	83	83	N/A	0.43	67977	0	0	100
19	3	49851331	49851400	70	70	CT(3), indel	0.49	57330	0	0	100
20	3	189713161	189713248	88	88	Poly A (5), Poly T (5), Poly A (9), TG(3)	0.41	72072	0	0	100
21	3	190106030	190106104	75	74	Indel	0.57	60543	0	63	99.9
22	4	2233667	2233744	78	78	Poly A (6)	0.26	63882	0	0	100

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
23	4	7780541	7780637	97	97	Poly G (6), Poly T (5), Poly A (5)	0.42	79443	0	0	100
24	4	15688604	15688681	78	78	N/A	0.29	63882	0	0	100
25	4	56236521	56236586	66	62	Poly A (5), indel	0.36	50778	0	0	100
26	4	102839244	102839314	71	69	Poly A (5)	0.46	56511	0	0	100
27	4	164446743	164446804	62	62	Poly A (7), indel	0.27	50778	0	0	100
28	5	1882081	1882158	78	75	N/A	0.78	61425	0	0	100
29	5	14769061	14769144	84	84	GT(3), CCA(3)	0.62	68796	0	0	100
30	5	41069808	41069871	64	64	N/A	0.39	52416	0	0	100
31	5	74077114	74077196	83	83	Poly A (6), indel	0.3	67977	0	0	100
32	5	147475343	147475409	67	67	Poly T (5)	0.37	54873	0	0	100
33	5	149323731	149323821	91	91	CT(4), AG(3)	0.55	74529	0	0	100
34	5	155662213	155662287	75	75	Indel	0.43	61425	0	0	100
35	6	6318713	6318814	102	102	Poly G (6)	0.68	83538	0	0	100
36	6	24949983	24950074	92	92	Indel	0.63	75348	0	0	100
37	6	31084900	31084999	100	94	GCT(5), indel	0.61	76608	0	378	99.5
38	6	32147987	32148084	98	98	Poly T (5), TCT(3), CTT(3)	0.55	80262	0	0	100
39	6	32986864	32986958	95	95	Indel	0.53	77805	0	0	100
40	6	33408498	33408583	86	86	Poly C (6)	0.7	70434	0	0	100
41	6	41647401	41647495	95	94	Poly G (5), indel	0.61	76986	0	0	100
42	6	112435865	112435955	91	91	Poly A (5)	0.44	74529	0	0	100
43	7	22202076	22202148	73	73	N/A	0.44	59787	0	0	100
44	7	66276100	66276187	88	88	Indel	0.35	72072	0	0	100
45	7	77365735	77365821	87	87	Poly A (7), AG(4)	0.26	71253	0	0	100
46	7	110939946	110940030	85	85	Indel	0.38	69615	0	0	100
47	7	128533468	128533557	90	90	Poly G (5), indel	0.62	73710	0	0	100
48	7	149503875	149503965	91	91	Poly G (6), Poly C (6), indel	0.71	74529	0	0	100
49	7	154404519	154404599	81	66	N/A	0.31	54054	0	0	100
50	7	156476507	156476599	93	93	Indel	0.35	76167	0	0	100

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
51	8	1817312	1817394	83	83	N/A	0.42	67977	0	0	100
52	8	24811020	24811109	90	89	Poly G (7), CTC(4), indel	0.61	72171	0	720	99.0
53	8	76518625	76518691	67	67	Indel	0.3	54873	0	0	100
54	9	103054909	103055006	98	98	Poly G (6)	0.67	80262	0	0	100
55	9	105586150	105586214	65	65	Indel	0.32	53235	0	0	100
56	9	107620823	107620918	96	96	N/A	0.49	78624	0	0	100
57	9	123769149	123769231	83	83	AT(3)	0.37	67977	0	0	100
58	9	138995345	138995441	97	97	Poly C (6), indel	0.68	79443	0	0	100
59	10	5987120	5987198	79	78	Poly G (5), indel	0.47	63882	0	0	100
60	10	11784629	11784726	98	91	GC(3)	0.87	74529	0	0	100
61	10	27317777	27317855	79	79	Poly T (5)	0.3	64701	0	0	100
62	10	33018351	33018440	90	90	Poly A (5), Poly T (5)	0.2	73710	0	0	100
63	10	45084159	45084253	95	95	Indel	0.35	77805	0	0	100
64	10	55892599	55892687	89	88	AC(11), indel	0.42	71747	0	325	99.5
65	10	101611250	101611329	80	80	N/A	0.49	65520	0	0	100
66	10	118351373	118351453	81	81	N/A	0.51	66339	0	0	100
67	11	8159816	8159912	97	96	N/A	0.45	78624	0	0	100
68	11	30177648	30177717	70	70	Indel	0.46	57330	0	0	100
69	11	47470345	47470444	100	100	N/A	0.65	81900	0	0	100
70	11	59837679	59837740	62	62	Indel	0.37	50778	0	0	100
71	11	64418856	64418957	102	102	N/A	0.59	83538	0	0	100
72	11	93529612	93529684	73	73	Poly A (5)	0.4	59787	0	0	100
73	11	101347052	101347136	85	85	N/A	0.42	69615	0	0	100
74	11	102477336	102477426	91	91	Poly G (6)	0.55	74529	0	0	100
75	11	118406285	118406369	85	85	Indel	0.53	69615	0	0	100
76	11	120357801	120357885	85	85	Poly A (5), CA(3), indel	0.34	69615	0	0	100
77	11	125769313	125769397	85	85	GA(3)	0.52	69615	0	0	100
78	12	2834770	2834853	84	84	Poly C (5), indel	0.52	68796	0	0	100
79	12	26811004	26811096	93	93	Poly A (7), AC(4)	0.33	76167	0	0	100
80	12	30881766	30881846	81	81	N/A	0.49	66339	0	0	100

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
81	12	88474105	88474175	71	71	Poly A (6)	0.35	58149	0	0	100
82	12	120966872	120966966	95	95	Poly G (5)	0.68	77805	0	0	100
83	13	24167504	24167576	73	73	N/A	0.52	59787	0	0	100
84	13	25816961	25817049	89	88	Poly A (5), Poly T (7), Poly A (7), indel	0.22	72072	0	0	100
85	13	44880112	44880200	89	89	Indel	0.49	72891	0	0	100
86	13	77665218	77665294	77	77	Indel	0.39	63063	0	0	100
87	14	31619327	31619393	67	67	GA(3),TA(3)	0.39	54873	0	0	100
88	14	39517884	39517966	83	83	N/A	0.25	67977	0	0	100
89	14	46958962	46959034	73	72	Poly T (5), indel	0.19	58642	0	326	99.4
90	14	58050030	58050110	81	81	Indel	0.38	66339	0	0	100
91	14	82390559	82390649	91	91	Indel	0.35	74529	0	0	100
92	14	92549544	92549609	66	66	Poly A (5)	0.41	54054	0	0	100
93	14	102808496	102808589	94	94	Indel	0.62	76986	0	0	100
94	15	43170751	43170848	98	96	Poly C (5)	0.45	78624	0	0	100
95	15	63446149	63446216	68	68	Indel	0.25	55692	0	0	100
96	15	77879807	77879901	95	93	Poly G (5), indel	0.68	76167	0	0	100
97	15	81625334	81625428	95	95	Poly T (6)	0.43	77805	0	0	100
98	15	85438263	85438334	72	71	Indel	0.65	58149	0	0	100
99	15	89817413	89817503	91	91	N/A	0.36	74529	0	0	100
100	15	89864274	89864343	70	70	Indel	0.56	57330	0	0	100
101	16	1894910	1894972	63	63	N/A	0.27	51597	0	0	100
102	16	28997904	28997998	95	95	Poly C (5)	0.67	77805	0	0	100
103	16	53682908	53682994	87	87	TA(3)	0.41	71253	0	0	100
104	16	57954406	57954509	104	104	Poly C (5)	0.67	85176	0	0	100
105	16	85706375	85706465	91	91	Poly T (5), indel	0.37	74529	0	0	100
106	17	3563920	3564008	89	89	GC(3)	0.64	72891	0	0	100
107	17	3594191	3594277	87	87	Poly C (5), indel	0.67	71247	0	6	100
108	17	3970090	3970180	91	91	Indel	0.46	74529	0	0	100

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
109	17	16084945	16085037	93	93	Indel	0.26	76167	0	0	100
110	17	33998759	33998849	91	89	Poly T (5)	0.54	72891	0	0	100
111	17	39589691	39589774	84	82	Poly A (13), indel (x2)	0.29	66343	27	788	98.8
112	17	41244394	41244484	91	91	Poly A (5)	0.34	74529	0	0	100
113	17	45438866	45438957	92	92	Poly A (7), AT(3), AT(4), AT (4), indel	0.26	75348	0	0	100
114	17	61502432	61502510	79	79	Indel	0.41	64413	0	288	99.6
115	17	64023582	64023667	86	86	Poly T (7)	0.22	70434	0	0	100
116	17	72308237	72308320	84	84	GAG(3)	0.62	68796	0	0	100
117	18	2616456	2616522	67	67	GA(3)	0.31	54873	0	0	100
118	18	6980478	6980568	91	91	N/A	0.37	74529	0	0	100
119	18	9888026	9888094	69	69	Poly A (6), TG(3)	0.43	56511	0	0	100
120	18	38836999	38837073	75	75	Poly A (5), indel	0.37	61425	0	0	100
121	18	47405382	47405462	81	81	CTC(3), indel	0.47	66339	0	0	100
122	18	54815665	54815749	85	85	CT(3), indel	0.45	69615	0	0	100
123	18	59773996	59774060	65	65	N/A	0.48	53235	0	0	100
124	19	625143	625241	99	99	N/A	0.59	81081	0	0	100
125	19	18121418	18121491	74	74	N/A	0.68	60605	1	0	100
126	19	18186574	18186643	70	70	N/A	0.64	57330	0	0	100
127	20	746056	746149	94	94	N/A	0.61	76986	0	0	100
128	20	10633195	10633276	82	82	AC(3)	0.59	67158	0	0	100
129	20	17705633	17705708	76	76	CT(3)	0.58	62244	0	0	100
130	20	21766821	21766890	70	70	GT(3),TG(4), indel	0.46	57330	0	0	100
131	20	25278421	25278521	101	101	Indel	0.63	82719	0	0	100
132	20	50897302	50897368	67	67	Indel	0.36	54873	0	0	100
133	20	62331904	62331994	91	88	Poly G (6)	0.73	72072	0	0	100
134	20	62690860	62690946	87	87	Indel	0.57	71253	0	0	100
135	21	30300823	30300888	66	66	Indel	0.35	54054	0	0	100
136	21	33694176	33694273	98	98	Poly T (6), CA(3)	0.54	80262	0	0	100

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
137	21	36710706	36710792	87	87	GT(3), indel	0.39	71253	0	0	100
138	21	46644924	46644992	69	69	Poly A (6), AG(3), indel	0.32	56439	0	72	99.9
139	21	46705575	46705664	90	90	Poly T (5), Poly A (6)	0.5	73710	0	0	100
140	22	25750774	25750873	100	100	Indel	0.63	81900	0	0	100
141	22	32439233	32439329	97	97	N/A	0.68	79443	0	0	100
142	22	37409844	37409940	97	97	Indel	0.46	79443	0	0	100
143	22	37637596	37637694	99	99	N/A	0.6	81081	0	0	100
144	22	47081347	47081438	92	92	Indel	0.66	75348	0	0	100
145	X	15870424	15870492	69	69	Poly T (5)	0.26	56511	0	0	100
146	X	135288543	135288611	69	69	Poly C (5)	0.62	56511	0	0	100
147	X	135290777	135290847	71	71	N/A	0.52	58149	0	0	100
148	Y	2655397	2655461	65	0	N/A	0.55	0	0	0	N/A
149	Y	2655519	2655609	91	0	N/A	0.48	0	0	0	N/A
150	Y	2655609	2655679	71	0	Poly A (5)	0.37	0	0	0	N/A

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

The sequencing results for sample NA12878 were compared to a highly confident genotype for NA12878, established by the National Institutes of Standards and Technology (NIST) (v.2.19). Out of the 150 amplicons, 92 amplicons were fully contained within the highly confident genomic regions, 41 amplicons had partial overlap, and 17 amplicons had no overlap in the NIST sequence. This outcome resulted in 10,000 coordinates per replicate for comparison. Non-variant base calls were compared to human genome reference sequence build hg19. The accuracy results are shown in Table 12.

Table 12 Germline Agreement of NA12878 Sample with NIST Database

Sample	# Amplicons	Mean Call Rate	TP	FN	TN	FP	PPA	NPA	OPA
NA12878	133	>99.9	6552	1	610470	0	>99.9	100	>99.9

Based on the data provided by this nine-run Germline study, the NextSeq 550Dx instrument can consistently sequence:

- GC content ≥ 19% (all called bases in 819 sequenced amplicons with 19% GC content called correctly with no-call rate of 0.6%)
- GC content ≤ 87% (all called bases in 819 sequenced amplicons with 87% GC content called correctly with zero no-calls)
- PolyA lengths ≤ 9 (all called bases in 819 sequenced amplicons containing a PolyA repeat of nine nucleotides called correctly with zero no calls)
- PolyT lengths ≤ 10 (all called bases in 819 sequenced amplicons containing a PolyT repeat of ten nucleotides called correctly with zero no calls)
- PolyG lengths ≤ 7 (all called bases in 819 sequenced amplicons containing a PolyG repeat of seven nucleotides called correctly with no-call rate of 1.0%)
- PolyC lengths ≤ 6 (all called bases in 2457 sequenced amplicons containing a PolyC repeat of six nucleotides were called correctly with zero no calls)
- Dinucleotide repeat lengths ≤ 11x (all called bases in 819 sequenced amplicons containing an 11x dinucleotide repeat were called correctly with no-call rate of 0.5%)
- Trinucleotide repeat lengths ≤ 5x (all called bases in 819 sequenced amplicons containing a 5x trinucleotide repeat were called correctly with no-call rate of 0.5%)
- Insertion lengths ≤ 24 (66343 out of 66370 called bases in 819 sequenced amplicons containing a 24-nucleotide insertion called correctly with no-call rate of 1.2%; no incorrect calls occurred in region containing 24-nucleotide insertion)
- Deletion lengths ≤ 25 (all called bases in 2457 sequenced amplicons containing a 25-nucleotide deletion called correctly with zero no-calls)

Somatic

The study described here was used to assess variant calling accuracy of the Somatic Variant Module on the NextSeq 550Dx instrument using the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles).

This study used a representative assay designed to query a variety of genes covering 12,588 bases (150 amplicons) across 23 different chromosomes. Platinum Genome DNA was extracted from FFPE treated blocks to generate six unique samples for evaluation in the study.

Sample GM12877 DNA was diluted with sample GM12878 DNA to create GM12877-D5 and GM12877-D7 as a set of unique heterozygous variants with variant frequencies near 5% and 7%. Sample GM12878 DNA was similarly diluted with sample GM12877 DNA to create GM12878-D5 and GM12878-D7. Each of the samples was tested in triplicate except the diluted samples, which were tested in replicates of six. A total of nine runs were performed using three sequencing instruments, three reagent lots, and three operators over five start days. Accuracy was determined for SNVs, insertions, and deletions by comparing results to the well-characterized composite reference method, Platinum Genomes version 2016- 1.0. Confident genomic regions were defined based on this reference method unless otherwise specified.

Table 13 Summary of Somatic Agreement

Criteria	Total Observations[1]	Result by Observation[2]	Result by Run[3]
PPA for SNV	378	98.9	99.9
PPA for insertions	378	96.9	99.9
PPA for deletions	378	97.1	99.9
NPA	378	>99.9	>99.9
OPA	378	>99.9	>99.9

[1]Calculated as number of samples per run (42) x number of Runs (9) = 378.
[2]Lowest observed value by sample replicate across all 9 runs.
[3]Lowest value when data from each run are analyzed in aggregate.

Table 14 contains the study data presented with positive and negative percent agreement on a per sample basis, where the variant results are compared to the well-characterized composite reference method for PPA calculations. The three variant types (SNVs, insertions, and deletions) are combined. Because the reference method only provides results for the single nucleotide variants and insertions/deletions, non-variant base results are compared to human genome reference sequence build hg19 for NPA calculations.

Table 14 Somatic Agreement per Sample

Sample	Mean Call Rate	Expected	TP	FN	Variant No Calls	TN	FP	PPA	NPA	OPA
GM12877	98.7	2052	2025	0	27	318682	15	100	>99.9	>99.9
GM12878	98.8	3645	3564	0	81	317645	0	100	100	100
GM12879	99.8	2592	2538	0	54	323614	2	100	>99.9	>99.9

Sample	Mean Call Rate	Expected	TP	FN	Variant No Calls	TN	FP	PPA	NPA	OPA
GM12884	99.8	3078	3024	0	54	322038	5	100	>99.9	>99.9
GM12885	99.8	3294	3213	0	81	322121	0	100	100	100
GM12888	99.8	2916	2889	0	27	323048	2	100	>99.9	>99.9
GM12877-D5	99.8	9288	8930	0	358	630621	0	100	100	100
GM12877-D7	99.7	9288	9032	0	256	629719	0	100	100	100
GM12878-D5	99.5	9288	8699	42	547	628582	0	99.5	100	>99.9
GM12878-D7	99.7	9288	9108	0	180	629803	0	100	100	100

Table 15 contains the study data presented on a per sample basis, where the variant results are compared to the well-characterized composite reference method. Detection is evaluated for each variant type – SNVs, insertions, and deletions – separately. Reference positions are excluded.

Table 15 Somatic Agreement per Sample by Variant Type

	SNVs			Insertions			Deletions		
Sample	Expected	TP	FN	Expected	TP	FN	Expected	TP	FN
GM12877	999	999	0	567	567	0	486	459	0
GM12878	2457	2457	0	540	513	0	648	594	0
GM12879	1539	1539	0	567	540	0	486	459	0
GM12884	1836	1836	0	675	648	0	567	540	0
GM12885	2025	2025	0	675	648	0	594	540	0
GM12888	1782	1782	0	621	621	0	513	486	0
GM12877-D5	5454	5392	0	1782	1647	0	2052	1891	0
GM12877-D7	5454	5406	0	1782	1728	0	2052	1898	0
GM12878-D5	5454	5192	28	1782	1651	9	2052	1856	5
GM12878-D7	5454	5445	0	1782	1719	0	2052	1944	0

The ten samples were further analyzed for calling small insertions and deletions (indels) (Table 16). There were a total of 71 indels ranging in size from 1–24 bp for insertions and 1–25 bp for deletions.

Table 16 Summary of Somatic Indel Detection

Variant Type	Expected Variants	TP	FN	Variant No Calls	PPA
Insertion	10773	10282	9	482	99.2
Deletion	11502	10667	5	830	>99.9

The 150 amplicons were designed to cover various genomic content. The GC content of the amplicons ranged from 0.19–0.87%. Amplicons also had a range of single nucleotide (e.g. PolyA, PolyT), dinucleotide, and trinucleotide repeats. Data were compiled on a per amplicon basis (Table 17) to determine the effect of genomic content on percent correct calls. Percent correct calls consists of variant and reference calls and is less than 100% if there are either incorrect or no calls.

Table 17 Somatic Amplicon-level Accuracy

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
1	1	36450499	36450591	93	93	Indel	0.22	35066	0	88	99.7
2	1	109465122	109465200	79	79	Poly A (5), Poly C (5), indel	0.38	29827	0	35	99.9
3	1	218353867	218353957	91	91	Indel	0.4	34202	0	283	99.2
4	1	223906657	223906748	92	92	Indel	0.49	34613	0	163	99.5
5	1	228526602	228526682	81	81	Poly G (5)	0.69	30571	0	47	99.8
6	1	236372039	236372108	70	70	Poly T (10), indel	0.39	26452	0	8	100.0
7	1	247812041	247812128	88	88	Poly A (5), CT(3), TAA(3), indel	0.27	33148	0	116	99.7
8	2	55862774	55862863	90	90	Indel	0.28	33928	0	92	99.7
9	2	87003930	87004009	80	80	Indel	0.38	30218	0	22	99.9
10	2	177016721	177016805	85	81	N/A	0.65	30616	0	2	>99.9
11	2	186625727	186625801	75	75	Poly A (8)	0.35	28017	0	499	98.3
12	2	190323504	190323591	88	88	Poly T (5)	0.42	33207	0	57	99.8
13	2	200796740	200796826	87	87	Poly T (5), indel	0.31	32524	9	718	97.8
14	2	212245049	212245139	91	91	Poly T (5), Poly A (6), indel	0.3	33972	0	456	98.7
15	2	228147052	228147144	93	93	N/A	0.43	35051	0	103	99.7
16	2	235016350	235016422	73	73	Poly T (5), indel	0.42	27459	0	136	99.5
17	3	4466229	4466321	93	93	AT(3), indel	0.27	34534	0	620	98.2
18	3	46620561	46620643	83	83	N/A	0.43	31339	0	44	99.9
19	3	49851331	49851400	70	70	CT(3), indel	0.49	26373	0	87	99.7
20	3	189713161	189713248	88	88	Poly A (5), Poly T (5), Poly A (9), TG(3)	0.41	32829	0	857	97.5
21	3	190106030	190106104	75	74	Indel	0.57	27925	0	47	99.8
22	4	2233667	2233744	78	78	Poly A (6)	0.26	29327	4	162	99.4

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
23	4	7780541	7780637	97	97	Poly G (6), Poly T (5), Poly A (5)	0.42	36585	0	117	99.7
24	4	15688604	15688681	78	78	N/A	0.29	29427	0	57	99.8
25	4	56236521	56236586	66	62	Poly A (5), indel	0.36	23356	5	75	99.7
26	4	102839244	102839314	71	69	Poly A (5)	0.46	25942	0	140	99.5
27	4	164446743	164446804	62	62	Poly A (7), indel	0.27	22944	0	560	97.6
28	5	1882081	1882158	78	75	N/A	0.78	28299	0	53	99.8
29	5	14769061	14769144	84	84	GT(3), CCA(3)	0.62	31658	0	94	99.7
30	5	41069808	41069871	64	64	N/A	0.39	24120	0	72	99.7
31	5	74077114	74077196	83	83	Poly A (6), indel	0.3	31297	0	77	99.8
32	5	147475343	147475409	67	67	Poly T (5)	0.37	25277	0	55	99.8
33	5	149323731	149323821	91	91	CT(4), AG(3)	0.55	34308	0	90	99.7
34	5	155662213	155662287	75	75	Indel	0.43	28266	0	163	99.4
35	6	6318713	6318814	102	102	Poly G (6)	0.68	38489	0	67	99.8
36	6	24949983	24950074	92	92	Indel	0.63	34730	0	46	99.9
37	6	31084900	31084999	100	94	GCT(5), indel	0.61	35057	0	483	98.6
38	6	32147987	32148084	98	98	Poly T (5), TCT(3), CTT(3)	0.55	36647	0	406	98.9
39	6	32986864	32986958	95	95	Indel	0.53	35681	0	238	99.3
40	6	33408498	33408583	86	86	Poly C (6)	0.7	32438	0	70	99.8
41	6	41647401	41647495	95	94	Poly G (5), indel	0.61	35441	0	91	99.7
42	6	112435865	112435955	91	91	Poly A (5)	0.44	34354	0	44	99.9
43	7	22202076	22202148	73	73	N/A	0.44	27575	0	28	99.9
44	7	66276100	66276187	88	88	Indel	0.35	33060	0	213	99.4
45	7	77365735	77365821	87	87	Poly A (7), AG(4)	0.26	32423	0	489	98.5
46	7	110939946	110940030	85	85	Indel	0.38	32074	0	56	99.8
47	7	128533468	128533557	90	90	Poly G (5), indel	0.62	33791	0	281	99.2
48	7	149503875	149503965	91	91	Poly G (6), Poly C (6), indel	0.71	34316	0	82	99.8
49	7	154404519	154404599	81	66	N/A	0.31	24901	0	47	99.8
50	7	156476507	156476599	93	93	Indel	0.35	35067	0	87	99.8

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
51	8	1817312	1817394	83	83	N/A	0.42	31365	0	9	>99.9
52	8	24811020	24811109	90	89	Poly G (7), CTC(4), indel	0.61	32781	0	890	97.4
53	8	76518625	76518691	67	67	Indel	0.3	25228	0	146	99.4
54	9	103054909	103055006	98	98	Poly G (6)	0.67	36968	0	76	99.8
55	9	105586150	105586214	65	65	Indel	0.32	24472	0	100	99.6
56	9	107620823	107620918	96	96	N/A	0.49	36203	0	85	99.8
57	9	123769149	123769231	83	83	AT(3)	0.37	31329	0	45	99.9
58	9	138995345	138995441	97	97	Poly C (6), indel	0.68	36472	0	201	99.5
59	10	5987120	5987198	79	78	Poly G (5), indel	0.47	29473	0	11	>99.9
60	10	11784629	11784726	98	91	GC(3)	0.87	34188	0	213	99.4
61	10	27317777	27317855	79	79	Poly T (5)	0.3	29843	0	19	99.9
62	10	33018351	33018440	90	90	Poly A (5), Poly T (5)	0.2	33968	0	68	99.8
63	10	45084159	45084253	95	95	Indel	0.35	35829	0	81	99.8
64	10	55892599	55892687	89	88	AC(11), indel	0.42	32098	88	2048	93.8
65	10	101611250	101611329	80	80	N/A	0.49	30217	0	28	99.9
66	10	118351373	118351453	81	81	N/A	0.51	30531	0	96	99.7
67	11	8159816	8159912	97	96	N/A	0.45	36105	0	192	99.5
68	11	30177648	30177717	70	70	Indel	0.46	26318	0	153	99.4
69	11	47470345	47470444	100	100	N/A	0.65	37785	0	24	99.9
70	11	59837679	59837740	62	62	Indel	0.37	23368	0	68	99.7
71	11	64418856	64418957	102	102	N/A	0.59	38546	0	10	>99.9
72	11	93529612	93529684	73	73	Poly A (5)	0.4	27516	0	78	99.7
73	11	101347052	101347136	85	85	N/A	0.42	32083	0	48	99.9
74	11	102477336	102477426	91	91	Poly G (6)	0.55	34047	0	369	98.9
75	11	118406285	118406369	85	85	Indel	0.53	32065	0	74	99.8
76	11	120357801	120357885	85	85	Poly A (5), CA(3), indel	0.34	32083	0	47	99.9
77	11	125769313	125769397	85	85	GA(3)	0.52	32103	0	27	99.9
78	12	2834770	2834853	84	84	Poly C (5), indel	0.52	31645	16	525	98.3
79	12	26811004	26811096	93	93	Poly A (7), AC(4)	0.33	34824	0	330	99.1
80	12	30881766	30881846	81	81	N/A	0.49	30497	0	121	99.6

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
81	12	88474105	88474175	71	71	Poly A (6)	0.35	26773	0	65	99.8
82	12	120966872	120966966	95	95	Poly G (5)	0.68	35830	9	72	99.8
83	13	24167504	24167576	73	73	N/A	0.52	27498	0	114	99.6
84	13	25816961	25817049	89	88	Poly A (5), Poly T (7), Poly A (7), indel	0.22	32824	0	566	98.3
85	13	44880112	44880200	89	89	Indel	0.49	33574	0	77	99.8
86	13	77665218	77665294	77	77	Indel	0.39	29075	0	31	99.9
87	14	31619327	31619393	67	67	GA(3),TA(3)	0.39	25313	0	13	99.9
88	14	39517884	39517966	83	83	N/A	0.25	31360	0	22	99.9
89	14	46958962	46959034	73	72	Poly T (5), indel	0.19	26499	0	717	97.4
90	14	58050030	58050110	81	81	Indel	0.38	30494	0	133	99.6
91	14	82390559	82390649	91	91	Indel	0.35	34313	0	86	99.7
92	14	92549544	92549609	66	66	Poly A (5)	0.41	24555	0	1527	94.1
93	14	102808496	102808589	94	94	Indel	0.62	35472	0	69	99.8
94	15	43170751	43170848	98	96	Poly C (5)	0.45	36264	0	24	99.9
95	15	63446149	63446216	68	68	Indel	0.25	25667	0	37	99.9
96	15	77879807	77879901	95	93	Poly G (5), indel	0.68	34745	0	432	98.8
97	15	81625334	81625428	95	95	Poly T (6)	0.43	35870	0	40	99.9
98	15	85438263	85438334	72	71	Indel	0.65	26762	0	76	99.7
99	15	89817413	89817503	91	91	N/A	0.36	34286	0	112	99.7
100	15	89864274	89864343	70	70	Indel	0.56	26449	0	11	>99.9
101	16	1894910	1894972	63	63	N/A	0.27	23809	0	5	>99.9
102	16	28997904	28997998	95	95	Poly C (5)	0.67	35860	0	50	99.9
103	16	53682908	53682994	87	87	TA(3)	0.41	32835	0	60	99.8
104	16	57954406	57954509	104	104	Poly C (5)	0.67	39177	0	144	99.6
105	16	85706375	85706465	91	91	Poly T (5), indel	0.37	34075	0	323	99.1
106	17	3563920	3564008	89	89	GC(3)	0.64	33632	0	11	>99.9
107	17	3594191	3594277	87	87	Poly C (5), indel	0.67	32752	0	134	99.6
108	17	3970090	3970180	91	91	Indel	0.46	34343	0	82	99.8

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
109	17	16084945	16085037	93	93	Indel	0.26	35077	0	78	99.8
110	17	33998759	33998849	91	89	Poly T (5)	0.54	33553	0	89	99.7
111	17	39589691	39589774	84	82	Poly A (13), indel (x2)	0.29	30554	53	2296	92.9
112	17	41244394	41244484	91	91	Poly A (5)	0.34	34360	0	38	99.9
113	17	45438866	45438957	92	92	Poly A (7), AT(3), AT(4), AT (4), indel	0.26	34367	0	418	98.8
114	17	61502432	61502510	79	79	Indel	0.41	29751	0	119	99.6
115	17	64023582	64023667	86	86	Poly T (7)	0.22	32176	0	340	99.0
116	17	72308237	72308320	84	84	GAG(3)	0.62	31604	7	141	99.5
117	18	2616456	2616522	67	67	GA(3)	0.31	25273	8	45	99.8
118	18	6980478	6980568	91	91	N/A	0.37	34386	0	12	>99.9
119	18	9888026	9888094	69	69	Poly A (6), TG(3)	0.43	25692	0	399	98.5
120	18	38836999	38837073	75	75	Poly A (5), indel	0.37	27923	0	893	96.9
121	18	47405382	47405462	81	81	CTC(3), indel	0.47	30598	0	20	99.9
122	18	54815665	54815749	85	85	CT(3), indel	0.45	31969	0	161	99.5
123	18	59773996	59774060	65	65	N/A	0.48	24531	0	48	99.8
124	19	625143	625241	99	99	N/A	0.59	37298	0	124	99.7
125	19	18121418	18121491	74	74	N/A	0.68	27881	0	109	99.6
126	19	18186574	18186643	70	70	N/A	0.64	26442	0	26	99.9
127	20	746056	746149	94	94	N/A	0.61	35501	0	31	99.9
128	20	10633195	10633276	82	82	AC(3)	0.59	30951	0	72	99.8
129	20	17705633	17705708	76	76	CT(3)	0.58	28686	0	42	99.9
130	20	21766821	21766890	70	70	GT(3),TG(4), indel	0.46	26372	0	88	99.7
131	20	25278421	25278521	101	101	Indel	0.63	38159	0	20	99.9
132	20	50897302	50897368	67	67	Indel	0.36	25188	0	544	97.9
133	20	62331904	62331994	91	88	Poly G (6)	0.73	32969	0	309	99.1
134	20	62690860	62690946	87	87	Indel	0.57	32818	0	77	99.8
135	21	30300823	30300888	66	66	Indel	0.35	24758	9	181	99.2
136	21	33694176	33694273	98	98	Poly T (6), CA(3)	0.54	36902	0	160	99.6

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

Amplicon	Chromosome	Amplicon Start	Amplicon End	Analyzed Fragment Size	Bases in Confident Regions	Amplicon Genomic Content	GC content	Correct Calls	Incorrect Calls	No Calls	% Correct Calls
137	21	36710706	36710792	87	87	GT(3), indel	0.39	32841	0	48	99.9
138	21	46644924	46644992	69	69	Poly A (6), AG(3), indel	0.32	25939	0	280	98.9
139	21	46705575	46705664	90	90	Poly T (5), Poly A (6)	0.5	33942	0	78	99.8
140	22	25750774	25750873	100	100	Indel	0.63	37733	0	86	99.8
141	22	32439233	32439329	97	97	N/A	0.68	36617	0	49	99.9
142	22	37409844	37409940	97	97	Indel	0.46	36525	0	162	99.6
143	22	37637596	37637694	99	99	N/A	0.6	37398	0	24	99.9
144	22	47081347	47081438	92	92	Indel	0.66	34754	0	22	99.9
145	X	15870424	15870492	69	69	Poly T (5)	0.26	26046	0	36	99.9
146	X	135288543	135288611	69	69	Poly C (5)	0.62	26019	0	63	99.8
147	X	135290777	135290847	71	71	N/A	0.52	26780	0	58	99.8
148	Y	2655397	2655461	65	0	N/A	0.55	0	0	0	NA
149	Y	2655519	2655609	91	0	N/A	0.48	0	0	0	NA
150	Y	2655609	2655679	71	0	Poly A (5)	0.37	0	0	0	NA

FOR IN VITRO DIAGNOSTIC USE. FOR EXPORT ONLY.

The sequencing results for sample GM12878 were compared to a highly confident genotype for NA12878, established by the National Institutes of Standards and Technology (NIST) (v.2.19). Out of the 150 amplicons, 92 amplicons were fully contained within the highly confident genomic regions, 41 amplicons had partial overlap, and 17 amplicons had no overlap in the NIST sequence. This outcome resulted in 10,000 coordinates per replicate for comparison. Non-variant base calls were compared to human genome reference sequence build hg19. The accuracy results are shown in Table 18.

Table 18 Somatic Agreement of GM12878 Sample with NIST Database

Sample	# Amplicons	Mean Call Rate	TP	FN	TN	FP	PPA	NPA	OPA
GM12878	133	98.8	2808	0	258488	0	100	100	100

Based on the data provided by this nine-run Somatic study, the NextSeq 550Dx instrument can consistently sequence:

- GC content ≥ 19% (all called bases in 378 sequenced amplicons with 19% GC content called correctly with no-call rate of 2.6%)
- GC content ≤ 87% (all called bases in 378 sequenced amplicons with 87% GC content called correctly with no-call rate of 0.6%)
- PolyA lengths ≤ 9 (all called bases in 378 sequenced amplicons containing a PolyA repeat of nine nucleotides called correctly with no-call rate of 2.5%)
- PolyT lengths ≤ 10 (all called bases in 378 sequenced amplicons containing a PolyT repeat of ten nucleotides called correctly with no-call rate of less than 0.1%)
- PolyG lengths ≤ 6 (all called bases in 2268 sequenced amplicons containing a PolyG repeat of six nucleotides called correctly with no-call rate of 0.5%)
- PolyC lengths ≤ 6 (all called bases in 756 sequenced amplicons containing a PolyC repeat of six nucleotides called correctly with no-call rate of 0.4%)
- Dinucleotide repeat lengths ≤ 4x (all called bases in 1890 sequenced amplicons containing a 4x dinucleotide repeat were called correctly with no-call rate of 0.9%)
- Trinucleotide repeat lengths ≤ 5x (all called bases in 378 sequenced amplicons containing a 5x trinucleotide repeat were called correctly with no-call rate of 1.4%)
- Insertion lengths ≤ 23 (all called bases in 378 sequenced amplicons containing a 23-nucleotide insertion called correctly with no-call rate of 0.8%)
- Deletion lengths ≤ 25 (all called bases in 1134 sequenced amplicons containing a 25-nucleotide deletion called correctly with no-call rate of 0.7%)

Precision

The NextSeq 550Dx instrument precision was determined by testing 13 unique Platinum Genome samples using three instruments, three reagent lots, and three operators to generate nine sequencing runs over five start days. The representative assay, samples, and reference method are the same as described for the germline accuracy study. Precision contributions were determined by variance component analysis using VAF as the response variable and calculating the standard deviations at the component level for the instrument, reagent lot, operator, and start day (Table 19). The total number of observations used in the analysis for each component of instrument, operator, or reagent lot variability was 699, 176, and 235 for SNVs, insertions, and deletions, respectively.

Table 19 Precision Results for the NextSeq 550Dx Instrument (Standard Deviation)

		Component SD		Total SD	
Component	**Variant Type**	**Max**	**Median**	**Max**	**Median**
Lot	SNV	0.0076	0.0002	0.0833	0.0154
	Insertion	0.0104	0.0000	0.0410	0.0157
	Deletion	0.0046	0.0005	0.0560	0.0187
Instrument	SNV	0.0114	0.0003	0.0840	0.0153
	Insertion	0.0138	0.0009	0.0407	0.0161
	Deletion	0.0079	0.0008	0.0549	0.0187
Operator	SNV	0.0226	0.0008	0.0841	0.0155
	Insertion	0.0344	0.0010	0.0417	0.0164
	Deletion	0.0083	0.0013	0.0547	0.0187
Day	SNV	0.0277	0.0012	0.0825	0.0160
	Insertion	0.0235	0.0012	0.0409	0.0169
	Deletion	0.0271	0.0014	0.0548	0.0188

Method Comparison (Sequencing Platform)

Whole blood and FFPE samples were assessed on the NextSeq 550Dx instrument and MiSeqDx instrument using the TruSeq Custom Amplicon Kit Dx germline and somatic workflows. Variant frequency agreement for blood and FFPE samples were evaluated using multiple representative assays. Figure 2 plots the VAF correlation between the two instruments for one representative assay and Table 20 summarizes this correlation by assay panel. Based on the strong correlation between MiSeqDx instrument and NextSeq 550Dx instrument, performance characteristics related to pre-analytical factors (eg, extraction methods or interfering substances) are determined to be applicable to both instruments. See the TruSeq Custom Amplicon Kit Dx package insert for additional details.

Figure 2 VAF Correlation of MiSeqDx to NextSeq 550Dx Instruments for FFPE (Left) and Blood (Right) Samples Using Assay 1

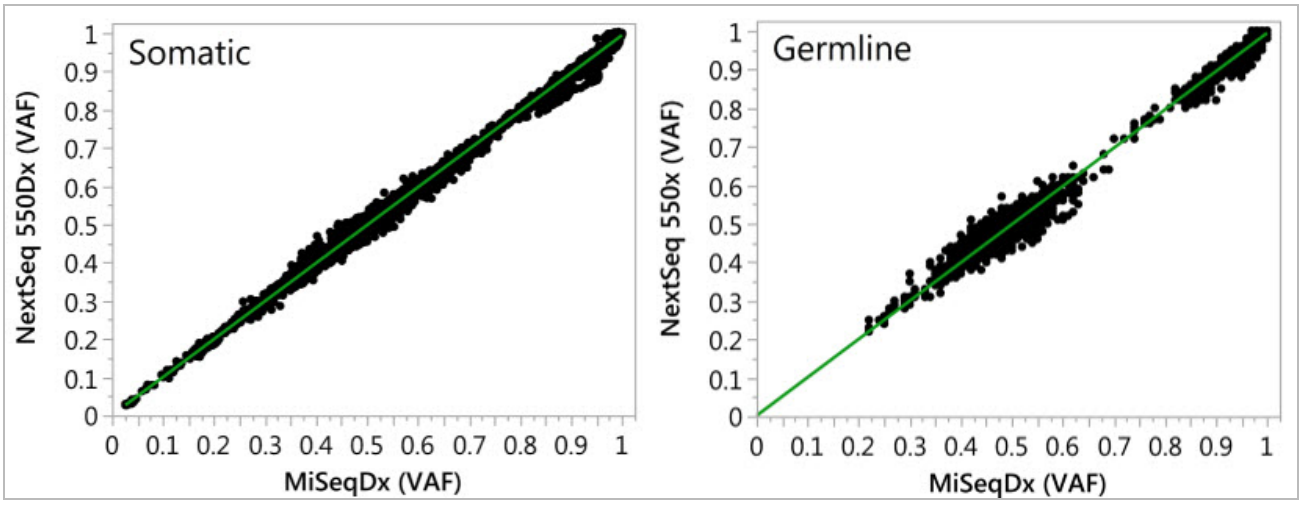


Table 20 Method Comparison Results Using Unique Blood and FFPE Samples

Source of gDNA	Assay (Oligo Panel)	Biological Replicates (Samples)	Technical Replicates (per Sample)	Observations (# of Variants)	Slope	Intercept	Correlation (R^2)
Blood	Assay 1	45	2	8369[1]	0.992	0.002	0.995[2]
Blood	Assay 2	45	2	5457	0.995	0.005	0.981
FFPE	Assay 1	46	2	8319	0.993	0.000	0.997[2]
FFPE	Assay 3	40	1	280	0.969	0.015	0.978

[1]Two data points were removed based on stated limitation for the Germline Variant module.
[2]Coefficient of determination for the VAF plots as illustrated in Figure 2.

Reproducibility

The reproducibility of the NextSeq 550Dx instrument was evaluated using Platinum Genome samples with a representative assay designed to query a variety of genes covering 12,588 bases across 23 different chromosomes using 150 amplicons. Germline testing consisted of seven replicates of 13 samples; somatic testing consisted of six replicates of seven samples at different VAF levels. Samples were prepared using the TruSeq Custom Amplicon Kit Dx.

Testing was performed at three external sites using one lot of NextSeq 550Dx High Output Reagent Kit v2 (300 cycles). A single NextSeq 550Dx instrument was used at each site. Two operators conducted the testing at each site. Each operator performed testing on three nonconsecutive start days for each sample type for a total of 36 runs across the three sites. This testing resulted in 18 runs for each the germline and somatic workflows.

Germline

Germline variants with VAF level of ≥ 0.2 are reported as positive (variant). For expected positive germline variants, the data were evaluated for no call rate and correct positive call rate within each variant type (SNV, insertion, deletion). Table 21 summarizes the observed rates, along with the lower and upper 95% confidence levels (LCL/UCL) calculated using the Wilson Score method, for each variant type.

Table 21 Germline Call Observations for Expected Positive Results by Variant Type

Variant Type	No Call			Correct Positive Call				
	Observed	Total	Percent	Observed	Total	Percent	95% LCL	95% UCL
SNV	16	110,376	0.014	110,349	110,360	99.99	99.98	99.99
Insertions	1026	37,044	2.77	36,018	36,018	100	99.99	100.00
Deletions	648	34,776	1.86	34,128	34,128	100	99.99	100.00

Germline variants with VAF level of < 0.2 are reported as negative (wild-type). For expected negative germline locations, the data were evaluated for no call and correct wild-type call rates. Table 22 summarizes the observed rates, along with the lower and upper 95% confidence levels (LCL/UCL) calculated using the Wilson Score method.

Table 22 Germline Call Observations for Expected Negative Results

Variant Type	No Call			Correct Negative Call				
	Observed	Total	Percent	Observed	Total	Percent	95% LCL	95% UCL
Wild-type	4883	19,600,182	0.025	19,595,299	19,595,299	100	100.00	100.00

Germline variants with VAF level ≥ 0.2 and < 0.7 are called positive heterozygous for the variant, and variants with VAF level ≥ 0.7 are called positive homozygous for the variant. Germline samples with heterozygous variants were used to determine if the inherent variability of the assay would affect the genotype call. The Cx was determined for both cutoffs (0.2 for heterozygous and 0.7 for homozygous genotypes), where x is the proportion of repeated tests that exceed the cutoff. For the lower cutoff of 0.2 VAF, the Cx was ≥ 99.999%, indicating that ≥ 99.999% of heterozygous variants would be called heterozygous. With respect to the upper cutoff of 0.7 VAF, the Cx was ≤ 0.001% thereby indicating that ≤ 0.001% of heterozygous variants would be called homozygous. Table 23 summarizes the results by variant type.

Germline variants with VAF level ≥ 0.2 and < 0.7 are called positive heterozygous for the variant, and variants with VAF level ≥ 0.7 are called positive homozygous for the variant. Germline samples with heterozygous variants were used to determine if the inherent variability of the assay would affect the genotype call. The Cx was determined for both cutoffs (0.2 for heterozygous and 0.7 for homozygous genotypes), where x is the proportion of repeated tests that exceed the cut off. With respect to the lower cutoff of 0.2 VAF, the Cx was ≥ 99.999% thereby indicating that ≥ 99.999% of heterozygous variants would be called heterozygous. For the upper cutoff of 0.7 VAF, the Cx was ≤ 0.001%, indicating that ≤ 0.001% of heterozygous variants would be called homozygous. Table 23 summarizes the results by variant type.

Table 23 Germline Cx Values for Heterozygous Variants

Variant Type	Cutoff At 0.2 VAF	Cutoff At 0.7 VAF
	≥ C99.999%	≤ C0.001%
SNV	94/94	94/94
Insertions	24/24	24/24
Deletions	35/35	35/35
Total	153	153

Somatic

Somatic variants with VAF levels ≥ 0.026 are reported as positive (variant). Observations with VAF levels ≥ 0.01 and < 0.026 were considered as equivocal for the purposes of this analysis (neither positive nor negative, flagged as low variant frequency). To assess the performance, the results were calculated in three ways:

- Best case: Any equivocal result was considered a correct positive call (agreement with the expected results)
- Worst case: Any equivocal result was considered an incorrect call (disagreement with the expected results)
- Exclusion case: Any equivocal result was excluded from the analysis

Three tables, Table 24, Table 25, and Table 26, summarize the call results for the best case, worst case and exclusion case, respectively, along with the lower and upper 95% confidence levels (LCL/UCL) calculated using the Wilson Score method.

Table 24 Somatic Call Observations for Expected Positive Results by Variant Type (Best Case)

Variant type	Correct Positive Call				
	Observed	Total	Percent	95% LCL	95% UCL
SNV	54,346	54,346	100	99.99	100.00
Insertions	18,036	18,036	100	99.98	100.00
Deletions	18,381	18,381	100	99.98	100.00

Table 25 Somatic Call Observations for Expected Positive Results by Variant Type (Worst Case)

Variant Type	Correct Positive Call				
	Observed	Total	Percent	95% LCL	95% UCL
SNV	54,346	54,346	100	99.99	100.00
Insertions	18,000	18,036	99.8	99.72	99.86
Deletions	18,381	18,381	100	99.98	100.00

Table 26 Somatic Call Observations for Expected Positive Results by Variant Type (Equivocal Calls Removed)

Variant Type	Correct Positive Call				
	Observed	Total	Percent	95% LCL	95% UCL
SNV	54,346	54,346	100	99.99	100.00
Insertions	18,000	18,000	100	99.98	100.00
Deletions	18,381	18,381	100	99.98	100.00

Somatic variants with VAF level < 0.01 are reported as negative (wild-type) calls. For expected negative somatic locations, the data were evaluated for no call rate and correct wild-type call rate. Correct wild-type calls were determined by excluding the no calls and subtracting the observed calls that fell into the equivocal zone (VAF levels ≥ 0.01 and < 0.026) as well as the incorrect calls that were above the cutoff (VAF levels ≥ 0.026) from the total. Table 27 summarizes the observed, total, and percentage results for negative somatic locations for no call rate and correct wild-type call rate along with the lower and upper 95% confidence levels (LCL/UCL) calculated using the Wilson Score method.

Table 27 Somatic Call Observations for Expected Negative Results

Variant Type	No Call			Correct Call						
	Observed	Total	Percent	Equivocal	Incorrect	Correct	Total	Percent	95% LCL	95% UCL
Wild-type	36,326	8,909,676	0.408	2254	121	8,870,975	8,873,350	99.97	99.972	99.974

Somatic samples at different VAF levels for the same variant were evaluated to determine the C95 of the assay (within each variant type). To evaluate variability near assay cutoff, samples that had expected VAF levels between 0.02 and 0.07 were used. The C95 was determined for each variant, with the highest C95 for each variant type reported in Table 28.

Table 28 Somatic C95 Summary

Variant Type	N	C95
SNV	74	0.0613
Insertion	24	0.0573
Deletion	33	0.0575

NextSeq 550Dx High Output Reagent Kit v2.5 (300 Cycle) Performance

Overview

The NextSeq 550Dx is supported by two reagent kits: the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). To demonstrate that the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) can meet analytical performance requirements verified and validated with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles), studies were conducted with the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). Two library preparations using the TruSeq Custom Amplicon Kit Dx were performed, one with the Germline workflow and the other with the Somatic workflow. Libraries from each workflow were tested with three lots of NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) using three NextSeq 550Dx instruments. In addition, testing for each workflow included a single run with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles).

Analytical Sensitivity (Limit of Blank [LoB] and Limit of Detection [LoD])

Verification with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) demonstrated that the NextSeq 550Dx instrument could detect variants at 0.05 VAF with a Type II error ≤ 0.05 and that the 0.026 VAF cutoff used by the Somatic Variant Module (effective LoB) supports a Type I error ≤ 0.01. Based on these claims it is expected that a variant at 0.05 VAF is greater than or equal to 0.026 VAF 95% of the time and that a wild-type position is less than 0.026 VAF 99% of the time. To make sure that these claims were met with the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles), repeated measurements were conducted on the NextSeq 550Dx instrument with wild-type samples (LoB samples) and with samples containing variants at 0.05 VAF (LoD Samples) using the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). The proportion of calls above and below the 0.026 cutoff were then compared to the claims established with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles).

Testing included two LoD samples each with a unique set of variants targeted to 0.05 VAF and corresponding LoB samples that were wild-type for targeted variants. For library preparation, LoD and LoB samples were processed in replicates of eight and seven, respectively, using the TruSeq Custom Amplicon Kit Dx. Libraries were initially sequenced using the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) to identify variants/genomic coordinates for LoB/LoD evaluation with the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). All variants with average VAF between 0.045 – 0.055 (LoD Variants) based on the results from the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) were used for LoD analysis (N = 51 variants). For LoB analysis, the 51 corresponding genomic coordinates were assessed.

For evaluation of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles), libraries were sequenced in three runs over three consecutive days using the same instrument and reagent kit lot. This testing amounted to 24 replicates for each of the 51 LoD variants and 21 replicates for each of the corresponding wild-type positions. The proportion of wild-type calls with VAF < 0.026 are provided in Table 29. The proportion of LoD variant calls with VAF greater than or equal to 0.026 are provided in Table 30.

Table 29 Proportion of Calls < 0.026 for Wild-type Positions (LoB Claim Evaluation)

Variant Type	Positions Evaluated	Total Observations	# of VAF Measurements ≥ 2.6%	Proportion < 2.6%	Proportion 95% Confidence Interval
SNV	32	672	0	1	0.994 – 1
Insertion	11	231	0	1	0.984 – 1
Deletion	8	168	0	1	0.978 – 1

Table 30 Proportion of Calls ≥ 0.026 VAF for LoD Variants (LoD Claim Evaluation)

Variant Type	Positions Evaluated	Total Observations	# of VAF Measurements < 2.6%	# of VAF Measurements ≥ 2.6%	Proportion ≥ 2.6%	Proportion 95% Confidence Interval
SNV	32	768	1	767	0.999	0.993 – 1
Insertion	11	264	3	261	0.989	0.967 – 0.996
Deletion	8	192	2	190	0.99	0.963 – 0.997

Accuracy

Germline

The following study was conducted to assess the variant call accuracy with the Germline Variant Module using the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). Twelve unique Platinum Genome samples were tested using a representative assay. A total of 11 runs were performed using three NextSeq 550Dx instruments and three of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).

Accuracy was determined for SNVs, insertions, and deletions by comparing the results to a well-characterized composite reference method, Platinum Genomes version 2016-1.0. Accuracy results from a single sequencing run with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) are provided for reference. A summary of the results is provided inTable 31.

Table 31 Summary of Germline Agreement

Criteria	Total Observations (v2.5)[1]	Result by Observation (v2.5)[2]	Result by Observation (v2)[3]	Result by Run (v2.5)[4]	Result by Run (v2)[4]
PPA for SNV	1056	98.7	98.7	>99.9	>99.9

Criteria	Total Observations (v2.5)[1]	Result by Observation (v2.5)[2]	Result by Observation (v2)[3]	Result by Run (v2.5)[4]	Result by Run (v2)[4]
PPA for insertions	1056	100	100	100	100
PPA for deletions	1056	95.2	95.2	>99.9	>99.9
NPA	1056	100	100	100	100
OPA	1056	>99.9	>99.9	>99.9	>99.9

[1]Calculated as number of samples per run x number of runs (96 samples per run x 11 runs = 1056 observations).
[2]Lowest observed value by sample replicate across all runs (based on 11 runs for NextSeq 550Dx High Output Reagent Kit v2.5).
[3]Lowest observed value by sample replicate across 1 run (96 total observations).
[4] Lowest value when data from each run are analyzed in aggregate.

Somatic

The following study was conducted to assess the variant calling accuracy of the Somatic Variant Module on the NextSeq 550Dx instrument using the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles). Ten Platinum Genome FFPE samples (two with variants diluted down to 0.05 VAF) were tested using a representative assay. A total of 11 runs were performed using three NextSeq 550Dx instruments and three lots of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).

Accuracy was determined for SNVs, insertions, and deletions by comparing the results to a well-characterized composite reference method, Platinum Genomes version 2016-1.0. Accuracy results from a single sequencing run with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) are provided for reference. A summary of the results is provided in Table 32.

Table 32 Summary of Somatic Agreement

Criteria	Total Observations (v2.5)[1]	Result by Observation (v2.5)[2]	Result by Observation (v2)[3]	Result by Run (v2.5)[4]	Result by Run (v2)[4]
PPA for SNV	528	100	100	100	100
PPA for insertions	528	96.9	96.9	> 99.9	> 99.9
PPA for deletions	528	100	100	100	100
NPA	528	> 99.9	> 99.9	> 99.9	> 99.9
OPA	528	> 99.9	> 99.9	> 99.9	> 99.9

[1]Calculated as number of samples per run x number of runs (48 samples per run x 11 runs = 528 observations).
[2]Lowest observed value by sample replicate across all runs (based on 11 runs for NextSeq 550Dx High Output Reagent Kit v2.5).

[3]Lowest observed value by sample replicate across 1 run (96 total observations).
[4]Lowest value when data from each run are analyzed in aggregate.

Precision

Germline

The precision of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) with the Germline Variant Module was evaluated using Platinum Genome samples and a representative assay. Testing consisted of a single library preparation using the TruSeq Custom Amplicon Kit Dx and included 12 samples processed with eight replicates each. Libraries were sequenced with three lots of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) and three NextSeq 550Dx instruments for a total of nine sequencing runs.

Samples with heterozygous variants were used to determine if the inherent variability of the assay would affect the genotype call (N = 153 unique heterozygous variants). The Cx was determined for both Germline Variant Module cutoffs (0.2 for heterozygous and 0.7 for homozygous genotypes), where x is the proportion of repeated tests that exceed the cutoff. For the lower cutoff of 0.2 VAF, the variant with the minimum Cx for the NextSeq 550Dx Reagent Kit v2.5 (300 cycles) was > 99.9%, indicating that > 99.9% of heterozygous variants would be called heterozygous. For the upper cutoff of 0.7 VAF, the variant with the maximum Cx for the NextSeq 550Dx Reagent Kit v2.5 (300 cycles) was < 1.5%, indicating that ≤ 1.5% of heterozygous variants would be called homozygous. Table 33 summarizes the results by variant type. Cx values from the single sequencing run using the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) are provided for reference.

Table 33 Germline Cx Values for Heterozygous Variants

Variant Type	N	Cutoff at 0.2 VAF		Cutoff at 0.7 VAF	
		Min Cx (v2.5)[1]	Min Cx (v2)[2]	Max Cx (v2.5)[1]	Max Cx (v2)[2]
SNV	94	>99.9%	>99.9%	1.5%	1.0%
Insertions	24	100%	100%	0%	<0.1%
Deletions	35	100%	>99.9%	<0.1%	<0.1%

[1]Cx values based on Total Standard Deviation estimates from variance component analysis.
[2]Cx values based on Sample Standard Deviations.

Somatic

The precision of the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) with the Somatic Variant Module was evaluated using Platinum Genome FFPE samples and a representative assay. Testing consisted of a single library preparation using the TruSeq Custom Amplicon Kit Dx and included two samples with eight replicates each. Libraries were sequenced using three lots of NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) and three NextSeq 550Dx instruments for a total of nine sequencing runs.

Somatic variants with expected VAF levels ≤ 0.10 VAF (N = 131 unique variants) were used to evaluate instrument variability near the Somatic Variant Module VAF cutoff (somatic variants with VAF level ≥ 0.026 are called positive for the variant). C95 values were determined for each of the somatic variants. C95 values

represent the VAF at which the probability of being greater than the Somatic Variant Module VAF cutoff is 95%. The highest C95 values by variant type are reported in Table 34. C95 results from a single sequencing run using the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) are provided for reference.

Table 34 Somatic C95 Summary

Variant Type	# of Variants Evaluated	C95 (v2.5)[1]	C95 (v2)[2]
SNV	74	0.064	0.063
Insertions	24	0.062	0.061
Deletions	33	0.060	0.060

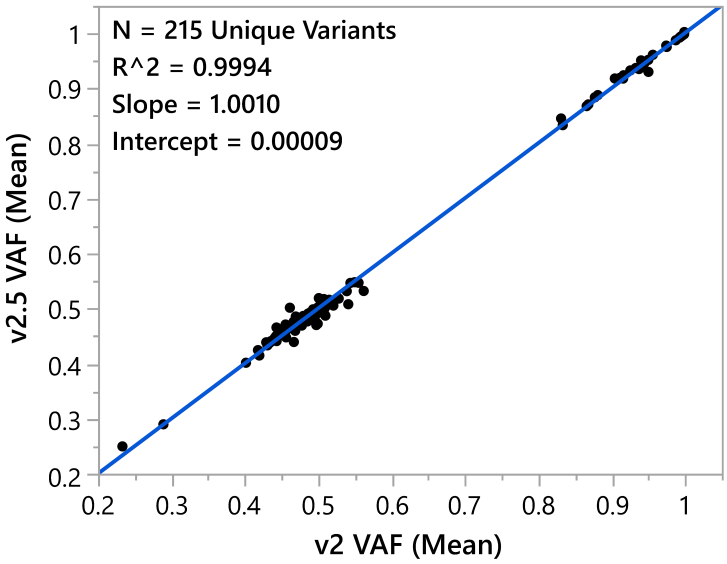
[1]C95 values based on Total Standard Deviation estimates from variance component analysis.
[2]C95 values based on Sample Standard Deviations.

Method Comparison (Reagent Kit)

Germline

The average VAFs from 215 unique variants were evaluated across the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) using results generated from the Germline Variant Module. VAF averages were calculated from 11 sequencing runs (v2.5) and one sequencing run (v2). At least eight replicates were used to calculate the average for each variant. Figure 3 plots the VAF correlation between the two reagent kits. Based on the strong linear VAF correlation and the similarity in results between reagent kits, performance characteristics initially verified and validated with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) with the Germline Variant Module are determined to be applicable to the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).

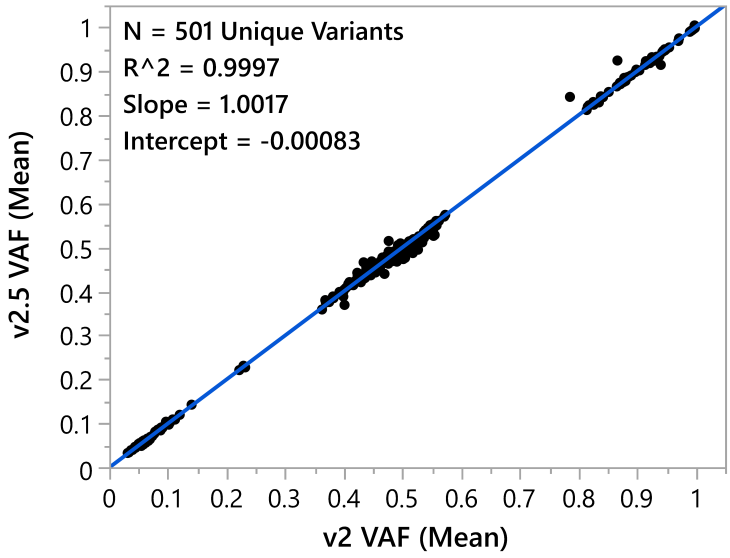
Figure 3 Germline Variant Module Variant Allele Frequency (VAF) correlation between NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).



Somatic

The average VAFs for 501 unique variants were evaluated across the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles) using results generated from the Somatic Variant Module. VAF averages were calculated from 11 sequencing runs (v2.5) and one sequencing run (v2). At least three replicates were used to calculate the average for each unique variant. Figure 4 plots the VAF correlation between the two reagent kits. Based on the VAF correlation and the similarity in results between reagent kits, performance characteristics verified and validated with the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) with the Somatic Variant Module are determined to be applicable to the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).

Figure 4 Somatic Variant Module Variant Allele Frequency (VAF) correlation between the NextSeq 550Dx High Output Reagent Kit v2 (300 cycles) and the NextSeq 550Dx High Output Reagent Kit v2.5 (300 cycles).



Revision History

Document	Date	Description of Change
Document # 200031448 v00	June 2023	Initial Release. Previous document 1000000030326 replaced by this one. Changes from document 1000000030326 v6 to this new document: • Added content to support optional Illumina DRAGEN Server for NextSeq 550Dx. • Updated Air Filter part number. Changes previously made to document 1000000030326: • Updates made to rectify content added inadvertently from source software. • Added Warnings and Precautions statement around reporting serious incidents. • Added a statement to Principles of Procedure specifying intended user. • Removed reference to High Output Reagent Kit v2 (300 cycles). • Added reference to High Output Reagent Kit v2.5 (75 cycles). • Added Revision History table. Updated EU Authorized Representative address.

Patents and Trademarks

This document and its contents are proprietary to Illumina, Inc. and its affiliates ("Illumina"), and are intended solely for the contractual use of its customer in connection with the use of the product(s) described herein and for no other purpose. This document and its contents shall not be used or distributed for any other purpose and/or otherwise communicated, disclosed, or reproduced in any way whatsoever without the prior written consent of Illumina. Illumina does not convey any license under its patent, trademark, copyright, or common-law rights nor similar rights of any third parties by this document.

The instructions in this document must be strictly and explicitly followed by qualified and properly trained personnel in order to ensure the proper and safe use of the product(s) described herein. All of the contents of this document must be fully read and understood prior to using such product(s).

FAILURE TO COMPLETELY READ AND EXPLICITLY FOLLOW ALL OF THE INSTRUCTIONS CONTAINED HEREIN MAY RESULT IN DAMAGE TO THE PRODUCT(S), INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY, AND WILL VOID ANY WARRANTY APPLICABLE TO THE PRODUCT(S).

ILLUMINA DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE PRODUCT(S) DESCRIBED HEREIN (INCLUDING PARTS THEREOF OR SOFTWARE).

© 2023 Illumina, Inc. All rights reserved.

All trademarks are the property of Illumina, Inc. or their respective owners. For specific trademark information, refer to www.illumina.com/company/legal.html.

Contact Information

Illumina, Inc.
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com

CE

IVD

EC REP Illumina Netherlands B.V.
Steenoven 19
5626 DK Eindhoven
The Netherlands

Australian Sponsor
Illumina Australia Pty Ltd
Nursing Association Building
Level 3, 535 Elizabeth Street
Melbourne, VIC 3000
Australia

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.