TruSeq™ Custom Amplicon Kit Dx

FOR IN VITRO DIAGNOSTIC USE

Catalog # 20005718: 1-4 uses, up to 96 Libraries

Intended Use

The Illumina TruSeq Custom Amplicon Kit Dx is a set of reagents and consumables used to prepare sample libraries from DNA extracted from peripheral whole blood and formalin-fixed, paraffin-embedded (FFPE) tissue. User-supplied analyte specific reagents are required for the preparation of libraries targeting specific genomic regions of interest. The generated sample libraries are intended for use on Illumina's high-throughput DNA sequence analyzers.

Principles of Procedure

The Illumina TruSeq Custom Amplicon Kit Dx is intended for manually preparing libraries used for the sequencing of DNA from peripheral whole blood specimens and formalin-fixed, paraffin-embedded (FFPE) tissue. Using the reagents provided in the TruSeq Custom Amplicon Kit Dx, genomic DNA is processed through the library preparation steps, which specifically amplify the intended genomic regions of each sample using analyte specific oligonucleotides, while also adding the indexes and flow cell capture sequences to the amplified products. DNA from whole blood specimens follows the germline workflow, while DNA from FFPE tissue follows the somatic workflow. The resulting sample libraries are ready for sequencing on an Illumina high-throughput DNA sequence analyzer and analysis from instrument software modules that correspond to germline or somatic workflows.

All reagents are provided except the analyte specific oligonucleotides, which are user designed.

Library preparation consists of 4 key steps: Hybridization, Extension-Ligation, PCR Amplification, and Library Normalization.

Library Preparation

- **Hybridization**—Hybridizes a pool of upstream and downstream oligonucleotides specific to the regions of interest to the sample genomic DNA. At the end of this process, a three-step wash procedure with a filter capable of size selection removes unbound oligonucleotides from the genomic DNA.
- **Extension-Ligation**—Connects the hybridized upstream and downstream oligonucleotides. A DNA polymerase extends from the upstream oligonucleotides through the targeted region, followed by ligation to the 5′ end of the downstream oligonucleotide using a DNA ligase. The result is the formation of products that contain the oligonucleotides specific to the regions of interest flanked by sequences required for amplification.
- **PCR Amplification**—Amplifies the extension-ligation products using primers that add index sequences for sample multiplexing and flow cell capture sequences required for cluster generation on an Illumina sequencer. At the end of this process, a PCR clean-up procedure purifies the PCR products (referred to as a library).
- **Library Normalization**—Normalizes the quantity of each library to ensure more equal library representation in the final pooled library. At the end of this process, the pooled library is loaded onto an Illumina sequencer for sequencing using sequencing by synthesis (SBS) chemistry.

Limitations of the Procedure

1. For *in vitro* diagnostic use.
2. Indel (insertions, deletions, and combinations thereof) content of length greater than 25 bp is not aligned by the assay software. Consequently, indels of length greater than 25 bp are not detectable by the assay software.

3 The system has been validated for the detection of single nucleotide variants (SNVs) and up to 25 bp deletions and 24 bp insertions when used with the Germline and Somatic Variant Modules. For somatic calling, at a variant frequency of 0.05, 25 bp deletions and 18 bp insertions were tested.

4 Amplicon reads with extreme variant content may not be aligned by the assay software, resulting in the region being reported as wild type. Such extreme content includes:
   - Reads containing more than three indels
   - Reads of length at least 30bp with SNV content greater than 4% of the total amplicon target length (excluding probe regions)
   - Reads of length less than 30bp with SNV content greater than 10% of the total amplicon length (including probe regions)

5 Large variants (include multi-nucleotide variants, large insertions, deletions, or combinations thereof) may be reported as separate smaller variants in the output VCF.

6 Deletion variants may 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 insertions and deletions 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 (2) 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, consequently, the calls made during sequencing. For example:
   - A variant in phase with a variant in the primer region may 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 may cause a false positive call at the end of the read adjacent to the primer.

9 Indels may 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.

11 Copy number variants or structural variants, such as fusions or translocations, have not been validated.

12 Germline-specific limitations
   - The Germline Variant Module is designed to deliver qualitative results for germline variant calling (eg, homozygous, heterozygous, wild type).
   - When used with the Germline Variant Module, the minimal coverage per amplicon needed for accurate variant calling is 150x. 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 9 repeats in the reference are required for a variant to be filtered, and only repeats with length up to 5 bp are considered (R5x9).

13 Somatic-specific limitations
   - The Somatic Variant Module 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 minimal coverage per amplicon needed for accurate variant calling is 450x per oligonucleotide pool. The number of samples and the total number of bases targeted affect coverage. GC-content and other genomic content can affect coverage.

- Variants in certain repetitive context are filtered out in the VCF files. The RMxN repeat filter is used to filter variants in all or part of the variant sequence is present repeatedly in the reference genome adjacent to the variant position. For somatic variant calling, at least 6 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 TruSeq Custom Amplicon Kit Dx consists of the following:

- TruSeq Custom Amplicon Kit Dx (Catalog # 20005718)

Reagents

Reagents Provided

The Illumina TruSeq Custom Amplicon Kit Dx has been configured to process up to 96 libraries in a single use (96 samples for germline workflow and 40 samples for somatic workflow [2 libraries are required per sample]). The kit will also support four library preparation uses with 24 libraries per use for the germline workflow and 20 libraries per use for the somatic workflow.

See the following tables for a complete list of reagents provided in this kit.

TruSeq Custom Amplicon Kit Dx, Box 1

Table 1 Box 1A Pre-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Hybridization Buffer	1 tube	4.32 ml	Buffered aqueous solution containing salts and formamide	-25°C to -15°C
Extension-Ligation Mix	1 tube	4.8 ml	Buffered aqueous solution containing proprietary blend of DNA polymerases, DNA ligase, and dNTPs	-25°C to -15°C
Index Primers A (A501) - H (A508)	1 tube per primer	192 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
Index Primers 1 (A701) - 12 (A712)	1 tube per primer	128 µl	PCR primers with index sequences and sequencing adapters	-25°C to -15°C
PCR Polymerase	1 tube	56 µl	Proprietary DNA polymerase	-25°C to -15°C
PCR Master Mix	1 tube	2.8 ml	Buffered aqueous solution containing salts and dNTPs	-25°C to -15°C

Table 2 Box 1B Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Library Normalization Diluent	1 tube	4.6 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide	-25°C to -15°C
Library Dilution Buffer	1 tube	4.5 ml	Buffered aqueous solution	-25°C to -15°C
PhiX Internal Control	1 tube	10 µl	Buffered aqueous solution containing PhiX genomic DNA	-25°C to -15°C

TruSeq Custom Amplicon Kit Dx, Box 2

Table 3 Pre-Amp Reagents

Component	Quantity	Fill Volume	Contents	Storage
Filter Plate	4 plates	N/A	Polypropylene microtiter plate with a modified polyethersulfone membrane	15°C to 30°C

Table 4 Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Elution Buffer	1 tube	4.8 ml	Buffered aqueous solution	15°C to 30°C
Library Storage Buffer	1 tube	3.5 ml	Buffered aqueous solution	15°C to 30°C

NOTE
Box 2 contains pre-amp and post-amp reagents in a single box.

TruSeq Custom Amplicon Kit Dx, Box 3

Table 5 Box 3A Pre-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
Stringent Wash Buffer	1 bottle	24 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	2°C to 8°C
Universal Wash Buffer	1 tube	4.8 ml	Buffered aqueous solution containing salts	2°C to 8°C

Table 6 Box 3B Post-Amp Reagents

Component	Quantity	Fill Volume	Active Ingredients	Storage
PCR Clean-Up Beads	1 tube	5 ml	Buffered aqueous solution containing solid phase paramagnetic beads and polyethylene glycol	2°C to 8°C
Library Normalization Wash	2 tubes	4.8 ml	Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide	2°C to 8°C
Library Beads	1 tube	1.2 ml	Buffered aqueous solution containing solid phase paramagnetic beads	2°C to 8°C

Reagents Required, Not Provided

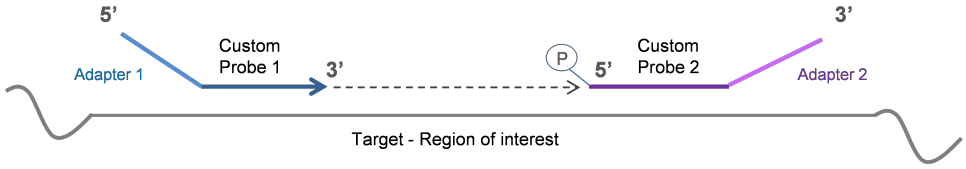
Custom Oligonucleotide Pool

Analyte-specific oligonucleotides are intended to be developed by the user and are not included with the library preparation kit. Figure 1 illustrates the custom oligo design principle. The oligo design should satisfy the following requirements:

- For germline workflow, one pair of custom oligonucleotides should be designed for each amplicon: one Custom Probe 1 (upstream locus-specific oligonucleotide [ULSO]) and one Custom Probe 2 (downstream locus-specific oligonucleotide [DLSO]).
- For somatic workflow, two pairs of custom oligonucleotides should be designed for each amplicon. Each pair consists of one Custom Probe 1 (upstream locus-specific oligonucleotide [ULSO]) and one Custom Probe 2 (downstream locus-specific oligonucleotide [DLSO]). One pair should target the plus strand and the other the minus strand.

- Custom oligonucleotides should surround the region of interest. The region of interest can be between 150 and 250 bp to allow complete sequencing of the fragment with a 2 x 150 cycle sequencing run.
- Both oligonucleotides should hybridize to the same DNA strand.
- Custom oligonucleotides should contain Illumina-specific adapters to allow addition of indices and sequencing adapters by PCR.
  - Adapter 1 (5′- CAACGATCGTCGAAATTCGC-3′) should be located at the 5′ end of the Custom Probe 1 (ULSO).
  - Adapter 2 (5′- AGATCGGAAGAGCGTCGTGTA-3′) should be located at the 3′ end of the Custom Probe 2 (DLSO).
- Custom Probe 2 (DLSO) is phosphorylated at the 5′ end to support ligation step after Custom Probe 1 (ULSO) extension.

Figure 1 Oligo Design for the TruSeq Custom Amplicon Kit Dx



- The following oligonucleotides design parameters are recommended:
  - Length range from 22 to 30 nucleotides (gene-specific region).
  - Total size of the amplicon from 190 to 290 base pairs, including adapters for germline workflow or 160 to 250 base pairs, including adapters for somatic workflow.
  - Recommended primer GC content can range from 25% to 70%.
  - Recommended Tm range from 55°C to 70°C.
  - Oligonucleotide concentration should be 15 nM per oligo in the custom pool.
  - No additional oligonucleotide purification required after synthesis. De-salting is recommended.
  - Oligonucleotides can be diluted in TE buffer.
  - Number of amplicons per sample can range from 16 to 384.
  - Design oligonucleotides to leave extra bases between the primer end and region of interest to enable detection of insertion and deletions at the extremes of regions of interest (see item 7 of Limitations of the Procedure on page 2).
  - If tiling is necessary to cover an entire region of interest, the region of overlap in the target region between binding sites for the adjacent probe sets should be 1 bp larger than the size of the deletion to be detected. For example, to enable detection of 3 bp deletions, the region of overlap between adjacent probe sets must be > 4 bp. Adjacent probe sets should be designed to alternating strands to avoid interference.

Refer to the *Limitations of the Procedure* for the minimal coverage per amplicon needed for variant calling. The number of samples per run should be calculated based on the minimal coverage required by the sequencing instrument and will depend on the total length and coverage uniformity of the custom oligonucleotide pool(s).

A manifest file should be created for each custom oligonucleotide pool. The manifest is a text file that contains information about targeted genomic regions and is required for the sequencer to run the analysis. Visit the Illumina website to download a template for the manifest file.

Pre-Amp Reagents

- 10 N NaOH (prepare from tablets or use a standard solution)
- TE Buffer
- RNase/DNase-free water

Post-Amp Reagents

- 10 N NaOH (prepare from tablets or use a standard solution)

- Ethanol, 200 proof for molecular biology
- TE Buffer
- RNase/DNase-free water

Storage and Handling

1. Room temperature is defined as 15°C to 30°C.
2. The following reagents are shipped frozen and are stable when stored at -25°C to -15°C until the specified expiration date.
   - Hybridization Buffer
   - Extension-Ligation Mix
   - Index Primers A (A501) - H (A508)
   - Index Primers 1 (A701) - 12 (A712)
   - PCR Polymerase
   - PCR Master Mix
   - Library Normalization Diluent
   - Library Dilution Buffer
   - PhiX Internal Control

   The reagents are stable for a maximum of six freeze/thaw cycles that occur before the specified expiration date.
3. The following reagents are shipped refrigerated and are stable when stored at 2°C to 8°C until the specified expiration date.
   - Stringent Wash Buffer
   - Universal Wash Buffer
   - PCR Clean-Up Beads
   - Library Beads
   - Library Normalization Wash
4. The following reagents are shipped ambient and are stable when stored at room temperature until the specified expiration date:
   - Elution Buffer
   - Filter Plate
   - Library Storage Buffer
5. Changes in the physical appearance of the reagents provided can indicate deterioration of the materials. If changes in the physical appearance occur (eg, obvious changes in reagent color or cloudiness apparent with microbial contamination), do not use the reagents.
6. The Hybridization Buffer, Stringent Wash Buffer, and Library Normalization Diluent reagents might form visible precipitates or crystals. Before use, vortex vigorously, and then visually inspect to ensure that no precipitates are present.
7. Adhere to the following best practices when handling PCR Clean-Up Beads and Library Beads:
   - The beads should never be frozen.
   - Allow the beads to reach room temperature.
   - Immediately prior to use, vortex the beads until well-suspended and color appears homogeneous.
   - Thoroughly mix the sample after the beads are added by pipetting up and down ten times. A shaker can be used to thoroughly mix samples.
   - Incubate the bead/sample mixture at room temperature for the entire duration indicated.
   - Follow instructions when using the magnetic stand. Wait for the solution to clear before aspirating. Keep the plate on the magnetic stand when slowing aspirating the supernatant, taking care not to disturb the separated beads.
8. The PCR amplification plate can remain on the thermal cycler overnight, or it can be stored at either condition listed below. Before storing, seal the plate well.

- 2°C to 8°C for up to two days
- -25°C to -15°C for up to 1 week

9 Do not freeze the Library Beads or mix with the Library Normalization Diluent reagent if not used immediately.
10 The completed library normalization plate (LNP) can be stored at 2°C to 8°C for up to 3 hours, or -25°C to -15°C for up to 1 week.
11 The storage plate (SGP) can be stored at -25°C to -15°C for up to 48 hours.
12 The diluted amplicon library (DAL) can be stored at -25°C to -15°C for up to 84 days.
13 Load diluted amplicon pool onto the reagent cartridge immediately after denaturation.

Equipment and Materials

Equipment and Materials Provided, Sold Separately

1 An Illumina high-throughput DNA sequence analyzer and associated sequencing consumables
2 **TruSeq Index Plate Fixture Kit**, Catalog # FC-130-1005
3 **TruSeq Index Plate Fixture & Collar Kit**, Catalog # FC-130-1007
4 **Index Adapter Replacement Caps**, Catalog # DX-502-1003
5 TruSeq Custom Amplicon Kit Dx Dx - FFPE QC, Catalog # 20006259 (for somatic workflow)

Equipment and Materials Required, Not Provided

Pre-Amp Equipment and Materials

1 **Heat Block**—One heat block for a 96 well plate is required. The heat block must meet the following specifications.
   - Heated lid
   - Temperature Range: Ambient +5°C to 99°C
   - Temperature Regulation: ±0.1°C at 37°C; ±0.4°C at 60°C
2 **Sample Incubator**—One incubator (hybridization oven) is required. The incubator must meet the following specifications.
   - Temperature Range: 10°C to 100°C
   - Temperature Regulation: ±0.2°C
3 **Tabletop Centrifuge**—One tabletop centrifuge (a separate centrifuge is required in the post-amp lab area). The centrifuge must meet the following specifications.
   - Can maintain 20°C
   - Fits a 96-well plate with filter unit
   - Accepts 5 ml tubes
   - Attains speeds of 280 to 2400 × g
4 **Heat Sealer**—Recommended for overnight hybridizations to prevent evaporation at the 40°C incubation.
5 **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the post-amp lab area.) The use of precision pipettes ensures accurate reagent and sample delivery. Single-channel or multi-channel pipettes can be used if they are calibrated regularly and are accurate within 5% of stated volume.
6 **Consumables**—The following consumables are required.
   - 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
   - 96-well storage plates, 0.8 ml (MIDI plates)
   - Solution basin, PVC, DNase, RNase-free (trough)
   - Adhesive aluminum foil seal (withstanding temperature range inclusive of 95°C) or seals compatible with a heat sealer
   - Seal compatible with PCR thermal cycler
   - Aerosol resistant pipette tips

Post-Amp Equipment and Materials

1. **Thermal Cycler**—One thermal cycler is required. The thermal cycler must have a heated lid and meet the following performance specifications:
   - Temperature Control Range: 4°C to 99°C
   - Control Accuracy: ±0.25°C from 35°C to 99°C
2. **Microplate Shaker**—One microplate shaker is required in the post-amp lab area. The plate shaker must meet the following performance specifications:
   - Max Mixing Speed: 3000 rpm
   - Mixing Speed Range: 200 to 3000 rpm
3. **Tabletop Centrifuge**—One tabletop centrifuge is required (a separate centrifuge is required in the pre-amp lab area). The centrifuge must meet the following specifications.
   - Can maintain 20°C
   - Fits a 96-well MIDI plate
   - Accepts 5 ml tubes
   - Attains speeds of 280 to 2400 × g
4. **Heat Block**—One heat block for 1.5 ml to 2 ml tubes is required. The heat block must meet the following specifications.
   - Temperature Range: Ambient +5°C to 99°C
   - Temperature Regulation: ±0.1°C at 37°C; ±0.4°C at 60°C
5. **Magnetic Stand**—One magnetic stand for a 96 well plate is required. Better performance is seen when the magnets are on the side of the stand and not on the bottom.
6. **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the pre-amp lab area.) The use of precision pipettes is required to ensure accurate reagent and sample delivery. Single-channel or multi-channel pipettes can be used if they are calibrated regularly and are accurate within 5% of stated volume.
7. **Gel Electrophoresis Supplies**—Gel electrophoresis supplies and apparatus are required along with an appropriate staining method to visualize PCR products in the gel.
8. **Consumables**—The following consumables are required.
   - 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
   - 96-well storage plates, 0.8 ml (MIDI plates)

     NOTE
     Make sure that the 96-well plate is fit compatible with the magnetic stand.

   - 2–4% TBE Agarose gel
   - 100 bp DNA molecular weight marker
   - DNA loading dye
   - Conical tubes, 15 ml
   - Eppendorf microcentrifuge tubes (screw-top recommended)
   - PCR eight-tube strips
   - Solution basins, PVC, DNase, RNase-free (trough)
   - Adhesive aluminum foil seals
   - Microseal® 'B' (Bio-Rad) or equivalent
   - Aerosol resistant pipette tips

Specimen Collection, Transport, and Storage

Germline Workflow

The following conditions should be met when handling blood and DNA extracted from the blood.

CAUTION
Handle all blood specimens as if they are known to be infectious for HIV, HBV, and other bloodborne pathogens.

1 Whole blood specimens collected in K_2EDTA tubes can be used.
2 Store whole blood specimens for no longer than seven days at room temperature, up to 30 days at 2°C to 8°C, or up to 30 days if frozen at -25°C to -15°C.
3 Transport whole blood for no longer than seven days at room temperature, 30 days at 2°C to 8°C, or 30 days if frozen at -25°C to -15°C. Transportation of whole blood must comply with country, federal, state and local regulations for the transport of etiologic agents.
4 Frozen genomic DNA samples are stable for 6 freeze/thaw cycles.

NOTE
No adverse effect on kit performance was observed with whole blood specimens when elevated bilirubin, cholesterol, hemoglobin, triglyceride, or EDTA was present.

DNA Extraction (Germline Workflow)

Any validated DNA extraction method can be used.

Somatic Workflow

The following conditions should be met when handling tumor tissue and DNA extracted from the tissue.

1 Tumor tissue should be formalin-fixed and paraffin-embedded.
2 Extracted genomic DNA should be kept between 2°C and 8°C for a maximum of 28 days or stored frozen between –25°C to –15°C for a maximum of 161 days.
3 Frozen genomic DNA samples are stable for 2 freeze/thaw cycles.

NOTE
No adverse effect on kit performance was observed with FFPE tissue when Deparaffinization Solution, paraffin wax, xylene, ethanol, Proteinase K, wash solutions, hemoglobin, or necrotic tissue was present.

DNA Extraction (Somatic Workflow)

Illumina recommends column-based DNA extraction kits, using double the amount of Proteinase K, overnight Proteinase K incubations with agitation, and final elutions in at least a 30 µl volume. Bead-based extraction methods and methods using only lysis of crude cell extracts are not recommended for use with these reagents.

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.

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

1 Handle all blood specimens as if they are known to be infectious for Human Immunodeficiency Virus (HIV), Human hepatitis B virus (HBV), and other blood-borne pathogens (universal precautions).
2 Failure to follow the procedures as outlined may result in erroneous results or significant reduction in sample quality.
3 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.
4 Do not use any kit components beyond their stated expiration date on the kit carton label. Do not interchange kit components from different kit lots. Note that kit lots are identified on the kit carton label.

5 Store the kit components at the specified temperature in designated pre-amplification and post-amplification areas.
6 Avoid repeated freeze-thaw cycles of the reagents. Refer to *Procedural Notes* for the number of uses of the kit.
7 To prevent sample or reagent degradation, ensure that all sodium hypochlorite vapors have fully dissipated prior to starting the protocol.
8 Proper laboratory practices and good laboratory hygiene is required to prevent PCR products from contaminating reagents, instrumentation, and genomic DNA samples. PCR contamination may cause inaccurate and unreliable results.
9 To prevent contamination, ensure that pre-amplification and post-amplification areas have dedicated equipment (eg, pipettes, pipette tips, vortexer, and centrifuge).
10 Avoid cross contamination. Use fresh pipette tips between samples and between dispensing reagents. Mix samples with a pipette and centrifuge the plate when indicated. Do not vortex the plates. Using aerosol-resistant tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.
11 Index-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 associated with samples before starting the sequencing run. Mismatches between the sample sheet and plate layout results in loss of positive sample identification and incorrect result reporting.
12 Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air, impacting results.
13 Ensure that all ethanol is removed from the bottom of the wells during wash steps. Residual ethanol may impact results.
14 Adhere to the specified drying time following the magnetic stand step to ensure complete evaporation. Residual ethanol can impact the performance of subsequent reactions.
15 Do not mix the custom oligonucleotide pool and Hybridization Buffer for storage. When combined, the custom oligo pool becomes unstable, even when stored frozen.
16 The use of thermal cyclers with active cooling (eg, Peltier, thermoelectric cooled) is not recommended for the hybridization step. The passive cooling step is critical for proper hybridization.
17 Always add PCR Polymerase to the PCR Master Mix just prior to use. Never store the combined working solution.
18 During the library normalization step, it is extremely critical to completely resuspend the library bead pellet. This is essential for achieving consistent cluster density on the sequencing flow cell.
19 Adhere to the specified incubation times in the library normalization step. Improper incubation can affect library representation and cluster density.
20 Due to the number of plate transfers and subsequent potential for contamination, extreme care should be taken to ensure that well contents remain fully in the well. Do not splash contents.

Acronyms

Table 7 Illumina TruSeq Custom Amplicon Kit Dx Acronyms

Acronym	Definition
AMP	AMplification Plate (library)
CLP	CLean-up Plate
COP	Custom Oligonucleotide Pool
DAL	Diluted Amplicon Library
FPU	Filter Plate Unit

Acronym	Definition
HYB	HYBridization Plate
LNP	Library Normalization Plate
NTC	Negative Template Control
PAL	Pooled Amplicon Library
POS	POsitive Control
SGP	StoraGe Plate

Procedural Notes

1. The kit may be used up to 4 times if fewer than 96 libraries need to be processed. With 4 uses, the germline workflow supports 24 libraries per use and the somatic workflow supports 20 libraries per use if the pipetting techniques described in *Instructions for Use* are followed.
2. Illumina requires that 1 positive control DNA sample and a negative control (NTC or No Template Control) are included in every use, which is defined as a set of samples processed in parallel. The positive control DNA sample should be a well-characterized sample with a known variation in the region of interest.
3. Before beginning the TruSeq Custom Amplicon Kit Dx protocol, extract and quantitate the DNA.
4. For the germline workflow, quantitate the DNA using a spectrophotometer. Verify that the A260/A280 of the DNA sample is > 1.5. Normalize the DNA sample to 5ng/µl. Each sample requires 10 µl of genomic DNA (total of 50 ng).
5. The 50 ng DNA input recommendation for the germline workflow allows for DNA quantity variation; library yield and sequencing performance is driven by this input level.
6. For the somatic workflow, qualify the DNA using Illumina TruSeq Custom Amplicon Dx - FFPE QC. Library yield and sequencing performance is dependent on sample quality as measured by the FFPE QC method.

Sample Throughput

For the Illumina TruSeq Custom Amplicon Kit Dx, the library throughput for a sequencing run can be from 1 to 96 libraries on the MiSeqDx, and 8 to 96 libraries on the NextSeq 550Dx. The somatic workflow requires 2 libraries for each sample.

The indexing primers used during PCR amplification must be chosen based on the desired final sample throughput to ensure each library uses a unique index combination.

Index Primer Sequences

Table 8 Sequences for Index Primers A (A501) – H (A508)

Index Primer	Sequence
Index Primer A (A501)	TGAACCTT
Index Primer B (A502)	TGCTAAGT
Index Primer C (A503)	TGTTCTCT
Index Primer D (A504)	TAAGACAC
Index Primer E (A505)	CTAATCGA
Index Primer F (A506)	CTAGAACA
Index Primer G (A507)	TAAGTTCC
Index Primer H (A508)	TAGACCTA

NOTE
On the NextSeq™ 550Dx, index primers A501-A508 are read as reverse-complement. The reverse-complement sequences should be used when considering index diversity requirements for two-channel sequencing chemistry.

Table 9 Sequences for Index Primers 1 (A701) – 12 (A712)

Index Primer	Sequence
Index Primer 1 (A701)	ATCACGAC
Index Primer 2 (A702)	ACAGTGGT
Index Primer 3 (A703)	CAGATCCA
Index Primer 4 (A704)	ACAAACGG
Index Primer 5 (A705)	ACCCAGCA
Index Primer 6 (A706)	AACCCCTC
Index Primer 7 (A707)	CCCAACCT
Index Primer 8 (A708)	CACCACAC
Index Primer 9 (A709)	GAAACCCA
Index Primer 10 (A710)	TGTGACCA
Index Primer 11 (A711)	AGGGTCAA
Index Primer 12 (A712)	AGGAGTGG

Instructions for Use

Sample Layout

Before performing library preparation, a sequencing run is created with Local Run Manager, the software on the sequencing instrument. The run is populated with samples, and the manifest file is selected. The resulting sample layout

is printed or exported to a file to be used as a reference when preparing libraries from the samples. For detailed instructions, refer to the module-specific reference guide corresponding to the intended workflow and sequencing instrument. Samples can be entered manually, or imported according to the reference guide instructions.

Germline versus Somatic Workflow Instructions

TruSeq Custom Amplicon Kit Dx is intended to manually prepare libraries for sequencing of DNA from peripheral whole blood specimens and formalin-fixed and paraffin-embedded (FFPE) tissue. Using the reagents provided in the TruSeq Custom Amplicon Kit Dx, genomic DNA is processed through the library preparation steps, which specifically amplify the intended genomic regions of each sample using analyte-specific oligonucleotides, while also adding the indexes and flow cell capture sequences to the amplified products. DNA from whole blood follows the germline workflow, while DNA from FFPE tissue follows the somatic workflow.

Resulting sample libraries are ready for sequencing on an Illumina high-throughput DNA sequence analyzer and analysis by instrument software modules (germline or somatic) corresponding to the workflows.

NOTE

Throughout the *Instructions for Use*, where there are differences in instruction for running germline workflow versus somatic workflow, it is called out in the step. These differences are summarized in Table 10.

Table 10 Differences between the Workflows to Analyze Germline versus Somatic Variants

Step	Parameter	Germline Workflow	Somatic Workflow
Preanalytical	Sample Type	DNA from whole blood	DNA from FFPE tissue
Preanalytical	DNA input	50 ng	Based on ΔCq
Preanalytical	Sample QC Method	A260	TSCA Dx - FFPE QC
Hybridization of Oligonucleotide Pool	Hybridization Approach	Single Strand	Dual Strand
Hybridization of Oligonucleotide Pool	Number of Oligonucleotide Pools	1	2
PCR Amplification	Volume of Index Primers	4 µl	9 µl
PCR Amplification	Volume of indexing PCR reaction[HM4]	50 µl	60 µl
PCR Amplification	PCR cycles	28	32
Verify Library Preparation	Library yield	Optional evaluation by gel (CLP products)	Evaluated by gel (AMP products)
PCR Clean-up	Volume of PCR clean-up beads	45 µl	55 µl

Hybridization of Oligonucleotide Pool (Pre-Amp)

Preparation

1. Bring the analyte-specific oligonucleotide pool(s), Hybridization Buffer, genomic DNA samples, and positive control sample to room temperature.
2. Vortex the custom oligo pool(s) and Hybridization Buffer vigorously to make sure that all precipitates have completely dissolved, then briefly centrifuge the oligonucleotide pool tubes to collect liquid. Ensure no precipitates are visible in the Hybridization Buffer.
3. Set a 96-well heat block to 95°C.
4. Pre-heat an incubator to 37°C.
5. Create the sample plate according to the printed plate layout from Local Run Manager.

Procedure

1 Set out a new 96-well PCR plate (hereafter referred to as the **HYB** plate).
2 Choose one of the following workflows (germline or somatic) based on the variant types you are targeting.
   - ***Germline Workflow:***
     - Add 10 µl of sample or control at 5 ng/µl (50 ng total) to the appropriate wells in the **HYB** plate according to the plate layout.
   - ***Somatic Workflow:***
     - Add 10 µl of sample or control diluted in accordance with TruSeq Custom Amplicon Dx - FFPE QC. Samples or controls are added to the plate in two wells for hybridization to both oligonucleotide pools according to the plate layout.
3 Choose one of the following workflows (germline or somatic) based on the variant types you are targeting.
   - ***Germline Workflow:***
     - Add 10 µl of 1X TE Buffer to the no template control (NTC) well. Follow the generated plate layout for correct well selection.
   - ***Somatic Workflow:***
     - Add 10 µl of 1X TE Buffer to the no template control (NTC) wells (2). Follow the generated plate layout for correct well selection.
4 Choose one of the following workflows (germline or somatic) based on the variant types you are targeting.
   - ***Germline Workflow:***
     - Add 5 µl of the custom oligonucleotide pool to all wells containing genomic DNA and NTC according to the plate layout.
   - ***Somatic Workflow:***
     - Add 5 µl of the custom oligonucleotide pool A to wells containing genomic DNA and NTC according to the plate layout.
     - Add 5 µl of the custom oligonucleotide pool B to wells containing genomic DNA and NTC according to the plate layout.

   Wells receiving each pool are mutually exclusive.
5 Add 40 µl of Hybridization Buffer to each sample and NTC in the **HYB** plate. Gently pipette up and down 3–5 times to mix.
6 Seal the **HYB** plate and centrifuge 1000 × g at 20°C for 1 minute.

CAUTION
To limit possible evaporation during the hybridization reaction, use of a heat sealer to seal the HYB plate is strongly recommended for overnight hybridizations. If a heat sealer is not available, seal the HYB plate with an adhesive aluminum foil seal and secure thoroughly with a sealing roller or wedge and proceed to the next step when the temperature reaches 40°C.

7 Place the **HYB** plate in the pre-heated, 96-well heat block at 95°C, close lid, and incubate for 1 minute.
8 Reduce the heat block setting to 40°C and continue incubating until the heat block reaches 40°C (approximately 80 minutes).

   Gradual cooling is critical for proper hybridization; therefore, PCR thermal cyclers with active cooling (eg, Peltier, thermoelectric cooled) are not recommended for this process.

SAFE STOPPING POINT
After the heat block reaches 40°C, the **HYB** plate is stable holding at 40°C for up to 18 hours. Before removing from the heat block, reinforce the foil seal with a sealing roller or wedge.

Removal of Unbound Oligonucleotides

Preparation

1 Bring Extension-Ligation Mix, Stringent Wash Buffer, and Universal Wash Buffer to room temperature, and then vortex briefly.

2 Assemble the filter plate assembly unit (hereafter referred to as the **FPU**) in order from top to bottom: lid, filter plate, adapter collar, and MIDI plate.
3 Pre-wash the filter plate membrane as follows:
   a Add 50 µl of Stringent Wash Buffer to each sample and NTC well.
   b Cover the filter plate with the lid and centrifuge at 2400 × g at 20°C for 5 minutes.

NOTE
Check to verify that all wells of the filter plate are draining completely. If the wash buffer does not drain completely, centrifuge again at 2400 × g at 20°C until all liquid has gone through (an additional 5–10 minutes).

CAUTION
It is critical to control the centrifuge temperature during the washing steps. If the temperature reaches 25°C or higher, the higher temperature may lead to higher stringency in primer binding. In rare cases, if samples have SNVs in primer binding regions, the higher stringency may lead to allele dropout.

Procedure

1 Remove the **HYB** plate from the heat block and centrifuge at 1000 × g at 20°C for 1 minute.
2 Transfer the entire volume (approximately 55 µl) of each sample to the corresponding wells of the filter plate.
3 Cover the filter plate with the lid and centrifuge at 2400 × g at 20°C for 5 minutes.
4 Wash the filter plate as follows:
   a Add 50 µl of Stringent Wash Buffer to each sample and NTC well.
   b Cover the filter plate with the lid and centrifuge at 2400 × g at 20°C for 5 minutes.

NOTE
Check to verify that all wells of the filter plate are draining completely. If the wash buffer does not drain completely, centrifuge again at 2400 × g at 20°C until all liquid has gone through (an additional 5–10 minutes).

5 Repeat the wash as described in the previous step.
6 Discard all the flow-through (containing formamide), then reassemble the **FPU**.
7 Add 45 µl of Universal Wash Buffer to each sample and NTC well of the **FPU**.
8 Cover the filter plate with the lid and centrifuge at 2400 × g at 20°C for 5 minutes.

NOTE
Check to verify that all wells of the filter plate are draining completely. If the wash buffer does not drain completely, centrifuge again at 2400 × g at 20°C until all liquid has gone through (an additional 5–10 minutes).

Extension-Ligation of Bound Oligonucleotides

Procedure

1 Add 45 µl of Extension-Ligation Mix to each sample and NTC well of the filter plate.
2 Seal the filter plate with adhesive aluminum foil, and then cover with the lid.
3 Incubate the **FPU** in the pre-heated 37°C incubator oven for 45 minutes without rotation.
4 While the **FPU** is incubating, prepare the **AMP** (Amplification Plate) as described in the following section.

PCR Amplification

Preparation

1 Prepare fresh 0.05 N NaOH.
2 Determine the index primers to be used according to the printed plate layout from Local Run Manager.
3 Bring PCR Master Mix and the appropriate index primers to room temperature. Vortex each thawed tube to mix, and then briefly centrifuge the tubes to collect the liquid.
4 Set out a new 96-well PCR plate (hereafter referred to as the **AMP** plate).
5 Add index primers to the **AMP** plate based on your workflow:
   — ***Germline Workflow:***

- Add 4 µl of the selected index primers [A (A501) – H (A508)] to the appropriate well in a column of the **AMP** plate.
- Discard the original white caps and apply new white caps.
- Add 4 µl of the selected index primers [1 (A701) – 12 (A712)] to the appropriate row of the **AMP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***
- Discard the original orange caps and apply new orange caps.

— ***Somatic Workflow:***

- Add 9 µl of the selected index primers [A (A501) – H (A508)] to the appropriate well in a column of the **AMP** plate.
- Discard the original white caps and apply new white caps.
- Add 9 µl of the selected index primers [1 (A701) – 12 (A712)] to the appropriate row of the **AMP** plate. ***Tips must be changed after each row to avoid index cross-contamination.***
- Discard the original orange caps and apply new orange caps.

6 Prepare the PCR Master Mix/PCR Polymerase PCR working solution as follows:

a For 96 libraries, add 56 µl of PCR Polymerase to 2.8 ml of PCR Master Mix.
The ratio of PCR Master Mix to PCR Polymerase already includes dead volume.
b Invert the prepared PCR working solution 20 times to mix.
c The PCR working solution is stable at room temperature for 10 minutes.

Procedure

1 Remove the **FPU** from the incubator.
2 Remove the aluminum foil seal. Cover the filter plate with the lid and centrifuge at 2400 × g at 20°C for 2 minutes.
3 Add 25 µl of 0.05 N NaOH to each sample and NTC well on the filter plate. Pipette the NaOH up and down 5–6 times.
4 Cover and incubate the filter plate at room temperature for 5 minutes to elute the libraries.
5 While the filter plate is incubating, transfer 22 µl of the PCR working solution to each well of the **AMP** plate containing index primers.
6 Transfer samples eluted from the filter to the **AMP** plate as follows:

a Taking care not to pierce the filter membrane, gently pipette the samples up and down 5–6 times using a P20 pipette set to 20 µl.
b Transfer 20 µl from the filter plate to the corresponding wells of the **AMP** plate.
c Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR working solution.
d Transfer the remaining reaction wells from the filter plate to the **AMP** plate in a similar manner. ***Tips must be changed after each transfer to avoid index and sample cross-contamination.***

7 Seal the **AMP** plate and secure with a roller or wedge.
8 Centrifuge at 1000 × g at 20°C for 1 minute.
9 Transfer the **AMP** plate to the post-amplification area.
10 Perform PCR based on your workflow using the following thermal cycler program with the heated lid on:

— ***Germline Workflow***:

- 95°C for 3 minutes

Then 28 cycles of:

- 95°C for 30 seconds
- 66°C for 30 seconds
- 72°C for 60 seconds

- 72°C for 5 minutes
- Hold at 10°C

— ***Somatic Workflow***:

- 95°C for 3 minutes

Then 32 cycles of:
- 95°C for 30 seconds
- 66°C for 30 seconds
- 72°C for 60 seconds

- 72°C for 5 minutes
- Hold at 10°C

SAFE STOPPING POINT

If not proceeding immediately to PCR Clean-Up, the **AMP** plate can remain on the thermal cycler overnight, be stored at 2°C to 8°C up to 48 hours, or be stored at -25° to -15°C for up to 1 week.

Verify Library Preparation

Procedure

Verify your library preparation by performing the following steps.

Germline Workflow:

There is no verification of library preparation in the germline workflow.

Somatic Workflow:

1. Combine 5 µl of amplified product with 15 µl of water and DNA loading dye, if necessary.
2. Run on a 2-4% TBE agarose gel with 50-100 bp ladder to confirm the presence and brightness of the library product (product size is panel dependent).
   - Samples that show amplification in one Oligo Pool or both Oligo Pools are considered valid and can be processed through the remainder of the workflow.
   - Samples showing little to no amplification in one Oligo Pool or both Oligo Pools are considered invalid and should not be processed through the remainder of the workflow.
   - If an invalid gel result is observed, library preparation for that sample or samples will need to be repeated starting at *Hybridization of Oligonucleotide Pool (Pre-Amp)*.
   - If bands are not observed on the gel for the repeat run, check sample quality or oligo panel design.
   - If the Blank NTC sample shows amplification in Oligo Pool A and/or B, this indicates contamination.

PCR Clean-Up

Preparation

1. Bring the PCR Clean-Up Beads to room temperature.
2. Prepare fresh 80% ethanol from absolute ethanol.

Procedure

1. Centrifuge the **AMP** plate at 1000 × g at 20°C for 1 minute.
2. Set out a new MIDI plate (hereafter referred to as the **CLP** plate).
3. Invert PCR Clean-Up Beads 10 times. Vortex vigorously and then invert 10 more times. Visually inspect solution to ensure that beads are resuspended.

NOTE

PCR Clean-Up Beads are extremely viscous and require extra care in pipetting. To avoid excessive reagent loss, slowly aspirate and slowly dispense bead volumes, and visually inspect that all beads are dispensed from pipette tips prior to tip ejection. Aspirate the appropriate volume and dispense without pipet-mixing or pre-wetting the pipette tips.

4. Add PCR Clean-Up Beads to the **CLP** plate in the following steps, depending on workflow:
   - ***Germline Workflow:***
     - Slowly add 45 µl of PCR Clean-Up Beads to each well of the **CLP** plate.
     - Transfer the entire PCR product from the **AMP** plate to the **CLP** plate (approximately 50 µl).

- *Somatic Workflow:*
  - Slowly add 55 µl of PCR Clean-Up Beads to each well of the **CLP** plate.
  - Transfer the entire PCR product from the **AMP** plate to the **CLP** plate (approximately 60 µl).

5 Seal the **CLP** plate and shake on a microplate shaker at 1800 rpm for 2 minutes.
6 Incubate at room temperature without shaking for 10 minutes.
7 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
8 With the **CLP** plate on the magnetic stand, carefully remove and discard the supernatant.
9 With the **CLP** plate on the magnetic stand, wash the beads as follows:
   a Add 200 µl of freshly prepared 80% ethanol to each sample well.
   b Incubate the plate on the magnetic stand for a minimum of 30 seconds or until the supernatant is clear.
   c Carefully remove and discard the supernatant.
10 Repeat the wash as described in the previous step.
11 Use a P20 multi-channel pipette set to 20 µl to remove excess ethanol.
12 Remove the **CLP** plate from the magnetic stand and air-dry the beads for 5 minutes.
13 Carefully add 30 µl of Elution Buffer to the beads then vortex briefly.

NOTE
Elution Buffer is viscous and requires slow aspiration and dispensing of volumes.

14 Seal the **CLP** plate with Microseal 'B' and a roller or wedge, and then shake on a microplate shaker at 1800 rpm for 5 minutes. After shaking, verify if samples were resuspended.
If a bead pellet is still visible in some wells, use a P200 pipette set to 30 µl to resuspend each individual bead pellet. Visually inspect the tips to see that the beads are dispensed back into wells before ejecting tips. Reseal the **CLP** plate and shake on a microplate shaker at 1800 rpm for an additional 5 minutes.
15 Incubate at room temperature for 2 minutes.
16 Place the **CLP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
17 Set out a new MIDI plate (hereafter referred to as the **LNP** plate).
18 Transfer 20 µl of the supernatant from the **CLP** plate to the **LNP** plate.
19 Carefully transfer 20 µl of supernatant from the **CLP** plate to the **LNP** plate.
20 Seal the **LNP** plate with an adhesive plate seal, and then centrifuge at 1000 × g at 20°C for 1 minute to ensure all the supernatant is at the bottom of the well.
21 [Optional] Transfer the remaining 10 µl of supernatant from the **CLP** plate to a new plate and label the plate with a run name and date. Store this plate at -25°C to -15°C until completion of the sequencing run and data analysis.
The cleaned up PCR products can be used for troubleshooting efforts in the event of sample failures.

SAFE STOPPING POINT
If stopping at this point, seal the **LNP** plate and centrifuge at 1000 × g at 20°C for 1 minute. The plate is stable for up to 3 hours at 2°C to 8°C or -25°C to -15°C for up to a week.

Library Normalization

Preparation

1 Prepare fresh 0.1 N NaOH.
2 Bring Library Normalization Diluent, Library Beads, Library Normalization Wash to room temperature.
3 Remove Library Storage Buffer from room temperature storage and set aside.
4 Vortex Library Normalization Diluent vigorously and ensure that all precipitates have dissolved.
5 Vortex Library Beads vigorously for 1 minute with intermittent inversion until the beads are resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

Procedure

1 Mix Library Normalization Diluent and Library Beads in a fresh 15 ml conical tube (use a fresh 1.5 ml tube if processing < 24 samples) as follows:

a For 96 samples, add 4.4 ml of Library Normalization Diluent.
b Resuspend Library Beads: Vortex Library Beads vigorously for 1 minute with intermittent inversion. Use a P1000 set to 1000 µl to completely resuspend the Library Beads by slowly pipetting up and down at least 10 times until no pellet is found at the bottom of the tube when the tube is inverted.

CAUTION
It is critical to completely resuspend the library bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. Resuspending the beads is essential for achieving consistent cluster density on the flow cell.

CAUTION
Library Beads are extremely viscous and require extra care in pipetting. To avoid excessive reagent loss, slowly aspirate, slowly dispense bead volumes, and visually inspect that all beads are dispensed from pipette tips before tip ejection.

c For 96 libraries, pipette 800 µl of Library Beads to the tube containing Library Normalization Diluent. For fewer libraries, the ratio is 7.2 µl of Library Beads to 37.8 µl of Library Normalization Diluent per library. Include dead volume for pipetting error.
d Mix by inverting the tube 15–20 times.

2 Add 45 µl of the combined Library Normalization Diluent/Library Beads working solution to each well of the **LNP** plate containing libraries.
3 Seal the **LNP** plate with Microseal 'B' and a roller or wedge, and then shake on a microplate shaker at 1800 rpm for 30 minutes.

NOTE
If proceeding with sequencing on the same day, now is a good time to begin thawing the reagent cartridge. Follow the instructions to thaw the reagent cartridge in the applicable instrument package insert.

4 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
5 While the **LNP** plate is on the magnetic stand, remove the seal, and then carefully remove and discard the supernatant.
6 Remove the **LNP** plate from the magnetic stand and wash the beads with Library Normalization Wash as follows:
a Add 45 µl of Library Normalization Wash to the beads in the **LNP** plate.
b Seal the **LNP** plate with Microseal 'B' and a roller or wedge, and then shake on a microplate shaker at 1800 rpm for 5 minutes.
c Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
d Remove and discard all supernatant.
7 Repeat the Library Normalization Wash procedure as described in the previous step.
8 Seal the **LNP** plate with an adhesive plate seal.
9 Centrifuge the **LNP** plate at 1000 × g at 20°C for 30 seconds to collect residual wash buffer.
10 Place the **LNP** plate on the magnetic stand for 2 minutes.
11 Use a P20 multi-channel pipette set to 20 µl to carefully remove excess Library Normalization Wash. Do not disturb the beads.
12 Remove the **LNP** plate from the magnetic stand and add 30 µl of 0.1 N NaOH to each well.
13 Seal the **LNP** plate with Microseal 'B' and a roller or wedge, and then shake on a microplate shaker at 1800 rpm for 5 minutes.
14 During the 5 minute elution, set out a new 96-well PCR plate (hereafter referred to as the **SGP** plate.)
15 Add 30 µl Library Storage Buffer to each well to be used in the **SGP** plate.
16 After the 5-minute elution, ensure all beads in the **LNP** plate are resuspended. If the beads are not completely resuspended, gently pipette those wells up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 minutes.
17 Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
18 Slowly transfer the supernatant (approximately 30 µl) from the **LNP** plate to the **SGP** plate. Gently pipette up and down 5 times to mix. Use new tips with each transfer.

19 Seal the **SGP** plate and then centrifuge at 1,000 × g at 20°C for 1 minute. Proceed immediately to *Library Pooling*. Discard the **LNP** plate.

Prepare for Library Sequencing

Preparation

1 Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C.
2 In an ice bucket, prepare an ice water bath.
3 Remove the Library Dilution Buffer and PhiX Internal Control from -25°C to -15°C storage and thaw.
4 After they are thawed, chill the Library Dilution Buffer and PhiX Internal Control in the ice water bath.
5 Vortex the Library Dilution Buffer, briefly centrifuge, and make sure that all the precipitates have dissolved completely.

Denature and Dilute PhiX Internal Control

PhiX Internal Control is supplied at 10 nM and must be denatured to single-stranded DNA and diluted to 20 pM before use. The following instructions provide 1 ml of denatured 20 pM PhiX Internal Control, which is enough for several DALs (> 20).

1 Prepare 0.1N NaOH.
2 Invert the tube several times to mix.

CAUTION
Using freshly diluted NaOH is essential to completely denature samples for cluster generation on the sequencer.

TIP
If PhiX is prepared the same day as Library Normalization, the same stock of 0.1N NaOH can be used.

3 Combine the following volumes to dilute the PhiX Internal Control library to 2 nM:
   — 2 µl of 10 nM PhiX Internal Control library
   — 8 µl of 1X TE Buffer
4 Combine the following volumes to result in a 1 nM PhiX Internal Control library:
   — 10 µl of 2 nM PhiX Internal Control library
   — 10 µl of 0.1 N NaOH
5 Vortex briefly to mix the 1 nM PhiX Internal Control library solution.
6 Briefly centrifuge the 1 nM PhiX Internal Control library solution to collect contents.
7 Incubate for 5 minutes at room temperature to denature the PhiX Internal Control library solution into single strands of DNA.
8 Add 980 µl of prechilled Library Dilution Buffer to the tube containing denatured PhiX Internal Control library. The final concentration is 20 pM denatured PhiX Internal Control library.

TIP
The denatured 20 pM PhiX Internal Control library can be stored up to 3 weeks at -25°C to -15°C as single-use aliquots.

Library Pooling

1 Vortex the Library Dilution Buffer and make sure that all the precipitates have dissolved completely.
2 Briefly centrifuge to collect contents.
3 Set out a fresh screw top tube (hereafter referred to as the **PAL** [Pooled Amplicon Library] tube).
4 Determine the samples to be pooled for sequencing. A maximum of 96 libraries can be pooled for sequencing.
5 Remove the seal from the **SGP** plate. Transfer 10 µl of each library to be sequenced from the **SGP** plate to a PCR 8-tube strip, changing tips each time.
6 Reseal the **SGP** plate with an adhesive plate seal and store at -25°C to -15°C for up to 48 hours.

TIP
The **SGP** plate can be used to pool fewer samples when the initial sequencing coverage is insufficient.

7 Combine and transfer the contents of the PCR 8-tube strip into the **PAL** tube. Mix the **PAL** tube thoroughly.
8 Set out 3 fresh screw top tubes (hereafter referred to as the **DAL** [Diluted Amplicon Library] tubes).
9 Add 585 µl of Library Dilution Buffer to the **DAL** tubes.
10 Transfer 5 µl of denatured PhiX (20 pM) to each **DAL** tube containing Library Dilution Buffer. Pipette up and down 3–5 times to rinse the tip and to make sure that the transfer is complete.
11 Transfer 10 µl of **PAL** to each **DAL** tube. Pipette up and down 3–5 times to rinse the tip and to make sure that the transfer is complete.
12 Briefly vortex the **DAL** tubes and briefly centrifuge the **DAL** tubes to collect liquid.

TIP
Depending on the kit usage, additional Library Dilution Buffer may be needed from an Illumina sequencing consumables kit for the respective sequencing instrument .

SAFE STOPPING POINT
If not proceeding immediately to sequencing, the **DAL** tubes can be stored at -25°C to -15°C for up to 84 days.

Prepare for Sequencing using MiSeqDx

1 Proceed with one **DAL** tube for sequencing.
2 If the **DAL** tube was stored frozen, thaw completely.
3 Mix the **DAL** tube by vortexing the tube at maximum speed.
4 Briefly centrifuge the **DAL** tube.
5 Incubate the **DAL** tube on a heat block at 96°C for 2 minutes.
6 After the incubation, invert the **DAL** tube 1–2 times to mix, then immediately place in the ice-water bath.
7 Keep the **DAL** tube in the ice-water bath for 5 minutes.

CAUTION
Perform the heat denaturation step immediately before loading the **DAL** tube into a reagent cartridge to ensure efficient template loading on the sequencing flow cell.

Refer to the *MiSeqDx Instrument* package insert for preparing the reagent cartridge, loading sample libraries onto the reagent cartridge, and setting up the sequencing run.

Prepare for Sequencing using NextSeq 550Dx

1 Proceed with one **DAL** tube for sequencing.
2 Set out a fresh screw top tube (hereafter referred to as the **FDT** [Final Dilution Tube]).
3 If the **DAL** tube was stored frozen, thaw completely.
4 Mix the **DAL** tube by vortexing the tube at maximum speed.
5 Briefly centrifuge the **DAL** tube.
6 Transfer an aliquot of the **DAL** to the **FDT**. The **DAL** volume needed to achieve proper cluster density is dependent upon the oligo pool used, and typically ranges between 130 – 160 µl.
7 Bring the **FDT** to a total volume of 1300 µl with Library Dilution Buffer.
8 Mix the **FDT** tube by vortexing the tube at maximum speed.
9 Briefly centrifuge the **FDT** tube.
10 Incubate the **FDT** tube on a heat block at 96°C for 2 minutes.
11 After the incubation, invert the **FDT** tube 1–2 times to mix, then immediately place in the ice-water bath.
12 Keep the **FDT** tube in the ice-water bath for 5 minutes.

CAUTION
Perform the heat denaturation step immediately before loading the **FDT** tube into a reagent cartridge to ensure efficient template loading on the sequencing flow cell.

Refer to the *NextSeq 550Dx Instrument* package insert for preparing the reagent cartridge, loading sample libraries onto the reagent cartridge, and setting up the sequencing run.

Quality Control Procedures

Good laboratory practices mandate that a positive control DNA sample and a negative (no-template) control sample are included in every library preparation use. The positive control DNA sample should be a well-characterized sample with known variants in the region of interest.

For the somatic workflow, all libraries (including libraries for the controls) are examined by gel electrophoresis as previously described.

Performance Characteristics

Germline studies used either the MiSeqDx™ Universal Kit 1.0 (DNA Extraction and Interfering Substances) or the TruSeq Custom Amplicon Kit Dx (DNA input) for library preparation. The 2 kits use identical reagents and have only one workflow difference: the number of polymerase chain reaction (PCR) cycles (28 and 32, respectively). The increased PCR cycles allow for a lower DNA input with the TruSeq Custom Amplicon Kit Dx (50 ng) relative to the MiSeqDx Universal Kit 1.0 (250 ng), as demonstrated in the DNA input study using the TruSeq Custom Amplicon Kit Dx. Each study specifies the library preparation reagents and sequencing consumables used, but all studies reflect the performance characteristics for the TruSeq Custom Amplicon Kit Dx due to the equivalence with the Universal Kit 1.0.

Somatic studies used the TruSeq Custom Amplicon Kit Dx.

Libraries prepared with the MiSeqDx Universal Kit 1.0 used Illumina version 1 sequencing consumables as the readout for performance, whereas as the TruSeq Custom Amplicon Kit Dx used version 3 sequencing consumables as the readout. Sequencing was performed on MiSeqDx instruments. Studies using the two gene or one gene panels as representative mutation panels used assay-specific workflows and analysis modules.

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 are correctly reported by the assay.
   - (# 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 are correctly reported by the assay.
   - (# 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). Two studies specifically include no calls in their "% correct calls" metric, and this inclusion of no calls is noted for the applicable tables.
5. Call rate is calculated as total number of loci passing filters divided by the total number of positions sequenced or reportable. This metric does not consider the agreement of the calls with the reference method.

Sample Carryover

Both the germline and somatic workflows involve library preparation and sequencing of multiple samples plus controls processed all at one time. The Sample Carryover study was conducted to evaluate if false positive results, due to carryover from well to well contamination during a library preparation use or from run to run contamination between

consecutive sequencing runs, impact test results. Somatic variants were used as they can be detected at lower allele frequencies than germline variants.

The samples consisted of 4 genomic DNA samples from cell lines, each containing different panel mutations in a two gene panel. The samples were such that a mutation at a position in one, has a reference (wild type) sequence in the other.

Well to well carryover is defined as a failure mode potentially created by manual processing steps (pipetting, sample mix-up, and so on). To evaluate carryover from one sample well to another, 2 test runs were performed:

- A checkerboard layout of a high input genomic DNA sample containing a mutation in Gene 1 alternating with a sample of low input genomic DNA containing a mutant in Gene 2.
- A checkerboard layout of a high input genomic DNA sample containing a mutation in Gene 2 alternating with a sample of low input genomic DNA containing a mutation in Gene 1.

In each run, a total of 12 replicates were evaluated for false positives (eg, a Gene 1 mutation was reported in a well designated as a Gene 2 mutant sample or vice versa).

Run to run carryover is defined as a failure mode potentially created by residue from a previous sequencing run. To determine if there is carryover between sequencing runs, 2 plates each containing 11 replicates of a single unique sample of high input genomic DNA plus a blank sample were prepared and sequenced consecutively on one MiSeqDx instrument and evaluated for false positives. The first run contained 11 replicates of a Gene 2 mutant sample plus 1 blank. The second run contained 11 replicates of a Gene 1 mutant sample plus 1 blank. The Gene 2 mutant sample library was sequenced first followed by a subsequent sequencing run with the Gene 1 mutant sample library, followed by another repeat sequencing run of the Gene 2 mutant sample libraries. If any Gene 2 mutations are observed in a Gene 1 mutant-only run, and conversely, this observation would indicate carryover.

Zero false positives (0/24, 0%) due to *well to well* carryover were reported. All expected mutations were detected. Zero false positives (0/24, 0%) due to *run to run* carryover were reported. All expected mutations were detected. Zero false positives (0/48, 0%) due to *total* carryover (well to well and run to run carryover combined) were reported.

Germline Performance Characteristics

The DNA input study used a 23 chromosome panel as the representative mutation panel. The other studies used a single gene panel as the representative mutation panel.

DNA Extraction

Three different extraction methods (magnetic bead extraction, alcohol precipitation and silica filter column isolation) were evaluated using K_2EDTA anticoagulated whole blood. The library preparation was completed using the MiSeqDx Universal Kit 1.0. Fourteen (14) unique blood samples were used in the study representing a range of genotypes from a one gene panel. The 3 DNA extraction methods were tested independently by 2 different operators who each performed 3 sequencing runs per extraction method. Each extraction was performed by each operator on different days. The DNA concentration and A260/A280 ratio of the extracted gDNA samples was determined using spectrophotometry. The total sample size for each extraction method in this study was 168 (14 samples x 2 operators/extraction method x 3 runs/operator x 2 replicates/extracted gDNA sample). Results for each method are presented in Table 11.

Table 11 Accuracy, Call Rate, and Sample First Pass Rate by Extraction Method

Extraction Method	Number of samples tested	Call Rate	Accuracy [1]	Sample First Pass Rate[2]
Alcohol Precipitation	168	100%	100%	100%
Silica Filter Column Isolation	168	100%	100%	100%
Magnetic Bead extraction	168	100%	100%	100%

[1]Accuracy - The percent agreement with a reference test method (bidirectional sequencing by Sanger) calculated for those base positions that receive a base call.

[2]Sample First Pass Rate - The number of samples that meet the specified call rate the first time they are processed (ie, without the need for a rerun or additional processing) as a percentage of the total number of samples run during a single MiSeqDx sequencing experiment.

DNA Input

The DNA input range for library preparation (TruSeq Custom Amplicon Kit Dx) was evaluated by performing a serial dilution study using 13 DNA samples and a representative assay designed to query various genes covering 12,588 bases across 23 different chromosomes. MiSeqDx Reagent Kit v3 was used as the sequencing readout.

Each sample was tested in duplicate at 5 DNA input levels ranging from 250 ng to 12 ng (250 ng, 100 ng, 50 ng, 25 ng, and 12 ng). For determination of accuracy, sample genotypes were compared to Platinum Genomes version 2016-01. Results were determined for each input level. PPA for each variant type (deletions, insertions, and SNVs) is presented in Table 1; NPA is presented in Table 13. All input levels had similar accuracy. The recommended DNA input is 50 ng with 25 ng and 100 ng providing a lower and upper limit to meet the accuracy claim.

Table 12 PPA Results for each DNA Input by Variant Type

DNA Input (ng)	Variant Type	Expected Variants	Total TP	Total FN	Variant No Calls	PPA (%)
12	Deletion	552	534	3	15	99.4
25			541	0	11	100
50			542	0	10	100
100			542	0	10	100
250			542	0	10	100
12	Insertion	588	569	0	19	100
25			572	0	16	100
50			572	0	16	100
100			572	0	16	100
250			572	0	16	100
12	SNV	1752	1725	2	25	99.9
25			1739	3	10	99.8
50			1742	0	10	100
100			1740	0	12	100
250			1735	0	17	100

Table 13 NPA for each DNA Input

DNA Input (ng)	Expected Variants	TN	FP	Ref No Calls	NPA (%)
12	2892	307179	0	3935	100
25	2892	309767	0	1347	100
50	2892	309999	0	1115	100
100	2892	309754	0	1360	100
250	2892	308922	0	2192	100

Interfering Substances

To assess the impact of interfering substances on the library preparation, a representative assay designed to query a single gene covering 11,529 bases was evaluated in the presence and absence of potential interferents. The library preparation was completed using the Universal Kit 1.0. Eight (8) whole blood samples representing 8 unique genotypes were used in the study. Four endogenous interfering substances (bilirubin, cholesterol, hemoglobin, and triglyceride) were tested by spiking them into the blood specimens before the DNA was extracted. To assess interference resulting from blood collection (short draw), EDTA was spiked into blood samples at 2 concentrations. The concentration limits for each substance are shown in Table 14. Also, to assess interference resulting from sample preparation, 15% wash buffer was added to 8 purified genomic DNA. The one gene panel was used. A 100% call rate was achieved for all samples tested in addition to 100% reproducibility in genotype calls between samples in the presence and absence of interfering substances.

Table 14 Call Rate for each Test Substance

Test Substance	Total Number of Replicates	Concentration Tested in Blood (Upper Limit)	Concentration Tested in Blood (Lower Limit)	Call rate
Bilirubin	16	684 µmol/L	137 µmol/L	100%
Cholesterol	16	13 mmol/L	2.6 mmol/L	100%
Hemoglobin	16	2 g/L	0.4 g/L	100%
Triglyceride	16	37 mmol/L	7.4 mmol/L	100%
EDTA	16	7 mg/mL	2.8 mg/mL	100%

Somatic Performance Characteristics

The DNA input study used a 26 gene panel as the representative mutation panel. The other studies used a 2 gene panel as the representative mutation panel.

DNA Input

TruSeq Custom Amplicon Dx - FFPE QC was used to evaluate a set of DNA samples extracted from FFPE specimens comprised of 9 different tissues. Per the FFPE QC, a Cq value was measured for each sample and compared to a control to calculate ΔCq values that ranged from -1.2 to 6.4. Samples were diluted 1:8, 1:4, 1:2, or treated as neat in accordance with the kit instructions. Some samples were further diluted (up to 1:64) to increase their ΔCq values. Two samples whose ΔCq values called for 1:8 dilutions were also processed without dilution to test inputs higher than the recommended. All dilutions were processed through library preparation and sequenced. Variants calls from the somatic variant module were compared to bidirectional Sanger sequencing conducted on specific gene targets dependent on the

tissue type. Dilutions were grouped into one of four ΔCq ranges and analyzed for accuracy and no calls (Table 15). The upper limit on input is a ΔCq of 2 which is achieved by iterative dilutions of samples with input <ΔCq of 2 according to kit instructions. The lower limit on input is ΔCq of 4. ΔCq values between 2 and 4 achieve equivalent accuracy. Assays using ΔCq to assess FFPE samples should determine the necessary cutoff to achieve the desired accuracy and precision.

Table 15 Accuracy and No Calls by ΔCq Group

ΔCq group	Variants					Wild Type Positions			
	Expected	TP	FN	No calls	PPA	TN	FP	No calls	NPA
ΔCqs -1.2 and -0.8	1	1	0	0	100	1387	1	0	99.9
ΔCqs 1.5 – 4	19	18	0	1	100	14358	1	78	99.9
ΔCqs ~4	19	18	0	1	100	14333	1	103	99.9
ΔCqs ~5	22	20	2	0	90.9	15878	1	439	99.9

Extraction

An extraction methods study was conducted to evaluate the impact of 3 commercially available extraction kits on the library preparation performance. The kits used columns as their basis for extraction and included reagents for deparaffinization and to partially reverse formalin cross-linking which are specific to FFPE tissue. The methods were modified by doubling the amount of proteinase K and digesting with an overnight incubation with agitation. DNA we eluted in the lowest recommended volume for a given kit or at least 30 µl. Ten (10) samples were tested in duplicate with each extraction kit. All replicates (20/20) tested with each kit met the assay quality control specifications. A two gene representative assay was used. PPA was 100% (16/16) and NPA was 100% (1104/1104) for each kit. Sanger sequencing was used as the reference method.

Interfering Substances

An Interfering Substances study was conducted to evaluate the impact of potentially interfering substances on the performance of the library preparation. Assay performance was evaluated in the presence of exogenous substances, (paraffin wax, xylene, ethanol and Proteinase K, extraction solutions), as well as endogenous substances (necrotic tissue and hemoglobin).

Exogenous Substances

The exogenous substances tested are extraction solutions commonly used during the DNA extraction process and are listed with tested quantities in Table 16. Fifteen (15) colorectal FFPE specimens were tested per interfering substance and compared with untreated controls. The specimens represented wild type samples containing no Gene 1 panel mutations (5/15 specimens), as well as specimens containing prevalent mutations (10/15 specimens). Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run.

Table 16 Substances Tested

Interfering Substance	Actual Amount [µl / 25 µl eluate]
Deparaffinization Solution	1.69×10^{-04}
Paraffin Wax (in Xylene)	2.50×10^{-05}
Xylene	2.50×10^{-05}
Ethanol	1.69×10^{-04}
Proteinase K[1]	3.30×10^{-06}
Wash Solution[2]	6.25×10^{-01}
1X Wash Solution[3]	6.25×10^{-01}
Wash Buffer AW1 [1]	6.25×10^{-02}
Wash Buffer AW2 [1]	6.25×10^{-01}

[1-3]Three commercially available column-based DNA isolation kits.

For all exogenous substances tested, all 15 specimens passed the sample qualification requirement (15/15, 100% Sample QC Pass Rate) and showed a valid result after library preparation and sequencing (15/15, 100% Sample First Pass Rate).

PPA is calculated on a per sample basis. OPA and NPA are calculated on a per mutation basis at the DNA level; there are 56 mutations per sample at the DNA level. All 15 specimens for all 9 exogenous substances showed agreement with the untreated control condition at all mutant (10/10) and non-mutant positions (830/830). None of the potentially interfering substances evaluated at the maximum concentrations expected to be encountered in the process of genomic DNA (gDNA) extraction from FFPE tissue impacts performance of the TruSeq Custom Amplicon Kit Dx.

Endogenous Substances (Hemoglobin)

Fifteen (15) colorectal FFPE samples were each tested in the presence or absence of 2 mg/mL hemoglobin, a CLSI "high" amount of hemoglobin. The specimens represented wild type samples containing no representative panel mutations (5/15 specimens), as well as samples containing prevalent Representative Panel mutations (10/15 specimens). Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run. All 15 specimens passed the sample qualification requirement (15/15, 100% Sample QC Pass Rate) and showed a valid result after library preparation and sequencing (15/15, 100% Sample First Pass Rate). All 15 specimens showed agreement at all mutant (10/10) and non-mutant positions (830/830) with the untreated control condition. The concentration of hemoglobin tested does not affect performance of the TruSeq Custom Amplicon Kit Dx.

Endogenous Substances (Necrotic)

Fifteen (15) colorectal FFPE samples composed of wild type samples containing no panel mutations (10/15 specimens), as well as samples containing prevalent Representative Panel mutations (5/15 specimens) and between 10–80% necrotic tissue as determined by pathology review, were utilized for evaluation of endogenous necrotic specimens. Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run. 14/15 specimens gave a valid result after library preparation and sequencing (93.3% Sample First Pass Rate). The overall percent agreement was 99.9% (783/784) relative to Sanger sequencing. PPA was 100% (4/4) and NPA was 99.87% (779/780). The 1 false positive detected was likely due to a sample mutation frequency below limit of detection of Sanger sequencing. Overall, the TruSeq Custom Amplicon Kit Dx meets performance characteristics with tissue containing 10–80% necrosis.

Revision History

Document #	Date	Description of Change
Document # 1000000029772 v05	August 2021	Updated EU Authorized Representative address. Added Revision History table.

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