PART I: Assay User Manual
oncoReveal™ CDx

oncoReveal™ CDx Kit 48 Tests P/N: HDA-LC-2001-48

For In Vitro Diagnostic Use
Caution: Federal law restricts this device to sale by or on the order of a physician.

CONTENTS

INTENDED USE/INDICATIONS FOR USE

The oncoReveal™ CDx is a qualitative next generation sequencing based *in vitro* diagnostic test that uses amplicon-based target enrichment technology for detection of single nucleotide variants (SNVs), insertions and deletions in 22 genes using DNA isolated from formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens and using the Illumina MiSeqDx®. The test is intended as a companion diagnostic to identify patients who may benefit from treatment with the targeted therapies listed in Table 1 in accordance with the approved therapeutic product labeling.

Additionally, oncoReveal™ CDx is intended to provide tumor mutation profiling to be used by qualified health care professionals in accordance with professional guidelines in oncology for previously diagnosed cancer patients with solid malignant neoplasms. Genomic findings other than those listed in Table 1 are not prescriptive or conclusive for labeled use of any specific therapeutic product.

Table 1 List of Somatic Variants for Therapeutic Use

Indication	Gene	Variant	Targeted therapy
Colorectal Cancer (CRC)	KRAS	KRAS wild-type (absence of mutations in codons 12 and 13)	ERBITUX® (cetuximab), or VECTIBIX® (panitumumab)
Non-Small Cell Lung Cancer (NSCLC)	*EGFR*	Exon 19 In Frame Deletions and Exon 21 L858R Substition Mutations	EGFR Tyrosine Kinase Inhibitors approved by FDA*

* For the most current information about the therapeutic products in this group, go to: https://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm301431.htm

CONTRAINDICATIONS

There are no known contraindications.

LIMITATIONS

1. The oncoReveal™ CDx has only been validated for CDx use with CRC and NSCLC tumor tissues. Test only the indicated tissue types.
2. The oncoReveal™ CDx has only been validated for pan cancer tumor profiling for solid malignant neoplasms.
3. Genomic findings reported by the oncoReveal™ CDx described as Level 2: Cancer Mutations with Potential Clinical Significance or Level 3: Cancer Mutations with Potential Clinical Significance are not prescriptive or conclusive for labeled use of any specific therapeutic product.
4. The oncoReveal™ CDx has been validated for use with genomic DNA extracted from FFPE tumor tissues. Other sample types or fixation methods have not been evaluated.
5. The oncoReveal™ CDx has not been validated for use with fine needle aspirates (FNA) as a specimen type.
6. Targeted molecular testing can only provide information for the targeted regions. A negative test result cannot rule out the possibility of other mutations with clinical utility outside of the target region. For example, samples with results reported as “No mutation detected” may harbor *KRAS* and *EGFR* variants not reported by the assay.
7. A negative “No mutation detected” result does not rule out the presence of a mutation that may be present but below the limits of detection of this test (see Analytical Sensitivity: Limit of Detection section).
8. A “No Call” result for Level 2 and Level 3 variants are at risk of being false negative results.
9. Positive mutation Call for Level 2 and Level 3 variants may be at risk of being false positive calls since they may be reported when the variant does not meet coverage requirements.
10. The oncoReveal™ CDx is not to be used for diagnosis of any disease.
11. This assay does not interrogate all variants or genes (*NRAS*) that confer resistance to cetuximab and panitumumab.
12. The assay has been validated using samples with a minimum of 30% tumor nuclei in the tissue area to be extracted.
13. The oncoReveal™ CDx is designed to report out somatic variants and is not intended to report germline variants. However, not all rare and novel germline variants, not listed in the germline database(s) may be filtered.
14. Use of this product should be limited to personnel trained in the techniques of Next-Generation Sequencing library preparation and the use of the Illumina MiSeqDx instrument.
15. Only the Illumina MiSeqDx instrument installed with Pillar LC-HS module has been validated for use with the oncoReveal™ CDx.
16. Only the PiVAT software has been validated for use with the oncoReveal™ CDx.

PRINCIPLES OF THE PROCEDURE

OVERVIEW

The oncoReveal™ CDx is an NGS *in vitro* diagnostic test that uses amplicon-based target enrichment technology for detection of SNVs, insertions and deletions in 22 genes using DNA isolated from FFPE tumor tissue specimens and using the Illumina MiSeqDx instrument. In addition to the companion diagnostic (CDx) claims noted in Table 1 of the intended use/indications for use, the oncoReveal™ CDx also reports SNV, insertions in deletion of the 22 genes listed Table 2 to provide tumor mutation profiling to be used by qualified health care professionals in accordance with professional guidelines in oncology for previously diagnosed cancer patients with solid malignant neoplasms.

Table 2 Genes targeted by the oncoReveal™ CDx for the detection of SNVs, insertions and deletions.

AKT1	*CTNNB1*	*ERBB2*	*FGFR1*	*KRAS*	*NOTCH1*	*PTEN*	*TP53*
ALK	*DDR2*	*ERBB4*	*FGFR2*	*MAP2K1*	*NRAS*	*SMAD4*	
BRAF	*EGFR*	*FBXW7*	*FGFR3*	*MET*	*PIK3CA*	*STK11*	

The oncoReveal™ CDx prepares sample DNAs for sequencing by amplifying target regions containing mutational hot spots using the SLIMamp® (stem-loop inhibition mediated amplification) technology. Sequencing uses the Illumina® MiSeqDx® Instrument and genetic variation present in the sample sequence is analyzed, quantified, and reported using Pillar Bioscience's proprietary PiVAT® (Pillar Variant Analysis Toolkit) software.

FFPE DNA EXTRACTION AND QUANTIFICATION

Genomic DNA extracted from each FFPE specimen is quantified using a DNA-based fluorescent dye assay to determine if they meet the minimum required amounts for the test.

LIBRARY PREPARATION

Gene-specific multiplex PCR (GS-PCR) amplification is performed using the sample genomic DNA to enrich hot spots in a single tube workflow. The GS-PCR products are purified and amplified again using primers that add index sequences for cluster generation on the Illumina MiSeqDx instrument. The indexed libraries are subsequently purified, quantified, and normalized for library pooling. The pooled libraries are loaded onto the MiSeqDx instrument for sequencing using a paired-end protocol.

DATA ANALYSIS

The sequencing run is initiated via the Pillar Module which interfaces with the Illumina Local Run Manager (LRM) software. The base calls generated during primary analysis on the MiSeqDx instrument

are then demultiplexed and FASTQ files for each sample are generated. Sequence run data are then manually transferred to the PiVAT software for secondary analysis. Secondary analysis includes alignment, paired-end assembly, variant calling, and report generation. oncoReveal™ CDx is designed to detect and report:

Level 1: CDx variants listed in Table 1 of Indications for Use
Level 2: Cancer Mutations with Evidence of Clinical Significance
Level 3: Cancer Mutations with Potential Clinical Significance

MATERIALS AND REAGENTS

ONCOREVEAL™ CDX KIT

CAUTION: oncoReveal™ CDx kit(s) are to be unpacked and placed at the indicated storage temperatures in Table 3 upon receipt.

Table 3 oncoReveal™ CDx Kit Reagents

Kit Box 1: GS-PCR Reagent	Quantity	Storage
Gene Specific PCR Master Mix	1 tube (red cap)	-25°C to -15°C
LC Oligo Pool	1 tube (yellow cap)	-25°C to -15°C
Positive Control (PosCtrl)	1 tube (clear cap)	-25°C to -15°C
Uracil-DNA glycosylase (UDG)	1 tube (blue cap)	-25°C to -15°C
Kit Box 2: Indexing PCR Reagent	Quantity	Storage
Indexing PCR Master Mix	1 tube (green cap)	-25°C to -15°C
Forward indexing primers (A501-A508)	8 tubes (white caps)	-25°C to -15°C
Reverse indexing primers (A701-A706)	6 tubes (orange caps)	-25°C to -15°C
Kit Box 3: PCR Product Purification Reagent	Quantity	Storage
Purification Beads	1 bottle	2°C to 8°C
Kit Box 4: Index Tube Caps	Quantity	Storage
White caps (for A501-A508 primers)	24 caps	Ambient
Orange caps (for A701-A706 primers)	18 caps	Ambient

MATERIALS AND EQUIPMENT

Table 4 Materials required but not provided in the oncoReveal™ CDx Kit

Material & purpose	Material description/specification(s)
Reagent kit for extraction and purification of DNA from formalin-fixed paraffin-embedded (FFPE) tissues used in clinical diagnostic applications.	See DNA EXTRACTION Column- or bead-based kits for extraction and purification of DNA from FFPE tissues. Proteinase K treatment and final elution volume 25µL are recommended for optimal results with this assay.
Reagent kit for quantification of double-stranded DNA (dsDNA) in biological samples used in clinical diagnostic applications.	The assay should: • accurately measure dsDNA for initial sample concentrations from 0.2 ng/µL to 15 ng/µL.

Material & purpose	Material description/specification(s)
	• be compatible with a variety of biological samples, including purified genomic DNA from FFPE tissues, and PCR products. • contain a fluorescent dsDNA-binding dye, appropriate buffer, and DNA standards for calibration. • be designed for use with a fluorometer instrument.
Reaction vessels intended for use with a fluorometer instrument for the quantification of dsDNA used in clinical diagnostic applications.	The reaction vessels should be compatible for use with dsDNA quantification assay and fluorometer instrument to provide consistent and accurate fluorescence measurements.
PhiX Library Control intended as a control in nucleic acid sequencing workflows used in clinical diagnostic applications.	Library of bacteriophage PhiX DNA fragment at or above 20 pM. The fragments should have an average size of 500 bp and consist of base composition at ~45% GC and ~55% AT. The control should be used as directed by the manufacturer.
MiSeqDx® Reagent Kit v3 (600 cycles)	Illumina/20037124
Ethanol, 200 proof for molecular biology	General lab supplier
Nuclease-free water	General lab supplier
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5	General lab supplier
10 N NaOH or 1 N NaOH	General lab supplier
1.5 mL microcentrifuge tubes	General lab supplier
96-well PCR plates, 0.2 mL	General lab supplier
Microplate sealing film	General lab supplier
Conical tubes, 15 mL	General lab supplier
Conical tubes, 50 mL	General lab supplier
Aerosol filter pipette tips	General lab supplier
Solution basin (trough or reservoir)	General lab supplier

Table 5 Software provided during system setup

Equipment	Source/Part Number
Pillar LC-HS module v2.1 or higher	Pillar Biosciences/SFW-2008
oncoReveal™ CDx PiVAT® Workstation with software version 2.1 or higher	Pillar Biosciences/SFW-2012
Pillar Sample Sheet Tool version 3.2 or higher	Pillar Biosciences/TL-0059

Table 6 Equipment required but not provided

Equipment	Source/Part Number
MiSeqDx Instrument†	Illumina/DX-410-1001
Fluorometer instrument†	A fluorometer instrument compatible for use with DNA quantification kit.
Vortexer	General lab supplier
Magnetic stand intended for use with PCR product purification workflow.	General lab supplier
Microfuge	General lab supplier
Thermal cycler† with heated lid capability	General lab supplier
Single- and multi-channel pipettes†, 0.5 to 1000 µL	General lab supplier
Centrifuge adapted for PCR plates	General lab supplier

† Equipment should be maintained and/or calibrated according to the manufacturer's instructions

Other general lab supplies that are needed to execute the protocol include laboratory gloves, ice, ice buckets, tube racks, etc. For reagents, consumables, and equipment required in both pre- and post-PCR processes, dedicated supplies (including gloves, lab coats, etc.) should be available in both areas.

PRECAUTIONS AND HANDLING REQUIREMENTS

WARNINGS AND PRECAUTIONS

User must adhere to the test procedure and following precautions when using the oncoReveal™ CDx kit.

1. The oncoReveal™ CDx is for *In Vitro* Diagnostic Use only.
2. The assay has been validated with DNA extracted from FFPE tumor tissues.
3. The assay has been validated with a fluorescence-based dsDNA quantification assay for quantification of FFPE extracted DNA and quantification of prepared library. Please see Table 4 for assay specifications.
4. Do not use expired or incorrectly stored reagent components.
5. Refer to Illumina MiSeqDx instrument package insert (Document # 15050260) for additional warnings, precautions, and procedures.
6. All reagents supplied in the oncoReveal™ CDx reagent kit is intended for use with this test. Do not substitute the reagents as this may affect performance.
7. Exercise care when performing calculations and conversion to the correct units of measure.
8. Use caution in workflow with regards to sample entry and pipetting especially during sample dilutions.
9. Use caution throughout the workflow with regards to DNA quantification of FFPE DNA and prepared libraries.
10. Use of poorly maintained and/or uncalibrated equipment may affect assay performance.

GOOD LABORATORY PRACTICES

1. **WORK AREAS**: Supplies should not be moved from one area to another to reduce the risk of contamination from PCR amplicons. Separate storage areas (including refrigerators and freezers) should also be designated for pre- and post-PCR products.
2. **LAB CLEANLINESS**: Clean work areas between use with laboratory cleaning solution (70% alcohol or fresh-made 10% hypochlorite solution) to reduce the possibility of contamination. A periodic cleaning of the floor is also recommended.
3. **FLOOR**: Items that have fallen to the floor are assumed to be contaminated and should be discarded. Gloves should also be changed after handling a contaminated item. If a sample tube or non-consumable item has fallen and remained capped, thoroughly clean the outside with a laboratory cleaning solution before use (70% alcohol or freshly made 10% hypochlorite solution).
4. **MULTICHANNEL PIPETTES**: Use multichannel pipettes for consistency and efficiency when dispensing or transferring reagents and/or samples.

5. **PIPETTE TIPS**: Use aerosol-resistant tips and change tips between each sample to prevent cross-contamination. Discard any tips that may have become contaminated due to contact with gloves, lab bench, tube exteriors, etc.
6. **OPEN CONTAINERS AND LIDS**: To prevent possible contamination from the air, keep tubes closed when not directly in use, avoid reaching over open containers, and cover plates with seals or lint-free laboratory wipes.
7. Preparation of samples for PCR amplification should be conducted in a location physically separated from areas where DNA samples are amplified during library preparation to avoid contamination of unamplified samples with highly enriched and abundant PCR amplification products resulting in potential No Template Control (NTC) failure and cross-contamination.

SPECIMEN HANDLING AND STORAGE

SPECIMEN HANDLING

The oncoReveal™ CDx was validated with DNA extracted from FFPE tumor tissues.

TO PREPARE TISSUE SAMPLES FOR DNA EXTRACTION:

1. All tissues must be formalin fixed and embedded in paraffin according to accepted histological methods.
2. Use FFPE sections with ≥ 30% tumor content by area for processing without macrodissection.
3. For FFPE sections that are less than 30% tumor content by area, enrich tumor content by macrodissecting multiple sections to obtain ≥ 30% tumor content by area.
4. Scrape or trim excess paraffin away from the tissue using a fresh, sterile scalpel.
5. Use serial sections if combining multiple sections for DNA extraction.

CAUTION: Extracted DNA giving a dsDNA quantification of > 4.5 ng/µl can be used for the oncoReveal™ CDx. If extracted DNA does not meet the minimum dsDNA quantification assay requirement, additional sections can be used for extractions, if available.

RECEIPT AND STORAGE OF SAMPLES

It is recommended that FFPE sections in curls or slides format be stored at 15°C to 30°C for up to 30 days prior to DNA extraction.
It is recommended extracted genomic DNA (from FFPE tissues) be stored at -25°C to -15°C for up to 6 months before use.

DNA EXTRACTION

The assay has been validated to work with DNA isolated from FFPE tumor tissues using column- or bead-based DNA extraction kits. Proteinase K treatment and final elution volume of 25 µL volume are recommended for optimal results with this assay.

oncoReveal™ CDx supports extracted DNA samples with quantified dsDNA concentration > 4.5 ng/µl. If extracted DNA samples do not meet the input requirement, extract, and quantify additional tumor tissues, if available. For best results, macro-dissect sections such that tumor content is ≥ 30% and contains < 50% necrotic tissues.

Do not proceed with the testing if FFPE tissue tumor content is < 30%, or FFPE tissue necrotic content is ≥ 50%.

CAUTION: Only solid tumor FFPE sections are to be used in the oncoReveal™ CDx.

TEST PROCEDURE

QUANTIFICATION OF DNA EXTRACTED FROM FFPE TISSUES

NOTE: The oncoReveal™ CDx was validated with DNA extracted from FFPE tissues quantified using a **fluorescence-based dsDNA quantification assay** (see Table 4 for assay specifications). DNA quantification is performed to determine if the DNA is of sufficient quantity for use with the assay.

1. Follow the manufacturer's user guide for dsDNA quantification assay on how to **prepare standards and samples for quantification** and **calculate sample concentration**.
2. Measure concentration of extracted DNA samples and calculate sample concentration in ng/µl.

DNA samples with dsDNA quantity that meet input requirement may proceed to Gene-specific PCR according to the recommendations in Table 7 below.

CAUTION: Ensure measured FFPE DNA concentration is calculated and reported in **ng/µl.**

Table 7 Quantified DNA input requirement and dilution recommendation

DNA conc (ng/µl)	Recommendation
≤4.5	Not supported. Repeat DNA extraction.
4.6 to 12.0	No dilution necessary.
>12.0	Dilute DNA sample to 12.0 ng/µl.

Do not proceed with the testing if minimum requirement for DNA concentration is not met.

3. Qualified extracted DNA samples should be stored on ice if they are processed further within the same day, but they should be frozen at -20°C for extended storage (see NOTE below).

NOTE: The amount of DNA extracted may vary with respect to the total yield, the degree of fragmentation, and the degree of base deamination due to variability in the amount of tissue in FFPE specimens, fixation process and storage length.

If extracted DNA samples do not meet the input requirement, extract, and quantify with more tumor tissues, if available.

STOPPING POINT: Extracted FFPE DNAs may be stored at 2 to 8°C for up to 30 days and at -25°C to -15°C for up to 6 months.

GENE-SPECIFIC PCR AMPLIFICATION

CAUTION: No Template Control (NTC) and Positive Control (PosCtrl) MUST be included for each **"Batch"** of samples (processed on the same plate). Perform GS-PCR Product setup in the **pre-PCR area**.

PREPARATION

1. Determine the GS-PCR plate layout, i.e., well assignment of the samples and controls (NTC and PosCtrl) to be included in the batch.
2. Dilute DNA samples (if necessary) according to recommendation in Table 7.
3. Remove Gene-Specific PCR Master Mix and LC oligo pool from **Kit Box 1** from storage to thaw.
4. Prepare an ice bucket to keep the reagents on ice when in use.
5. Program the GS-PCR cycling profile in Table 9 into the selected thermal cycler.

PROCEDURE

1. Prepare sufficient GS-PCR reaction mix for the batched samples according to the order of addition and indicated volume in Table 8. GS-PCR total reaction volume is 25 µl.

Table 8 GS-PCR reaction mix reagent order of addition and volume per reaction

Reagent	Cap color	1x Volume (µl)
Gene-Specific PCR Master Mix	Red	12.5
LC oligo pool	Yellow	5.0
UDG (5 units/µl)	Blue	1.0

2. Mix GS-PCR reaction mix thoroughly. Centrifuge tube briefly to collect droplets.
3. Transfer 18.5 µl of the GS-PCR reaction mix to each assigned well of the GS-PCR plate.
4. Add 6.5 µl of DNA diluent to the assigned "NTC" well in the GS-PCR plate.
5. Add 6.5 µl of Positive Control to the assigned "PosCtrl" well in the GS-PCR plate.
6. Add 6.5 µl of DNA sample (diluted if necessary) to the assigned sample well in the GS-PCR plate.
7. Seal the GS-PCR plate and mix thoroughly. Centrifuge briefly to collect droplets and remove air bubbles
8. Perform GS-PCR using the following GS-PCR cycling profile (Table 9) with heated lid.

Table 9 GS-PCR cycling profile

No. of cycles	Temperature (°C)	Time (min)
1	37	10
1	95	15
5	95	1
	60	6
18	95	0.5
	72	3
1	8	Hold

9. After the GS-PCR cycling protocol is complete, proceed to GS-PCR Product Purification steps below.

CAUTION: Use care when returning GS-PCR reagents to oncoReveal™ CDx Reagent Kit Box 1 for storage at -25°C to -15°C.

GS-PCR PRODUCT PURIFICATION

NOTE: Perform GS-PCR Product Purification in the post-PCR area.

PREPARATION

1. Bring materials to room temperature. It is critical that PCR purification steps are performed at room temperature.
2. Remove the purification beads in **Kit Box 3** from storage and allow to equilibrate to room temperature for at least 30 min prior to use.
3. Fresh 70% ethanol should be prepared for optimal results.
4. Dispense sufficient 70% ethanol solution, Purification Beads, and water in disposable trough for convenient dispense using a multichannel pipette.

PROCEDURE

1. Centrifuge the GS-PCR plate briefly to collect any droplets adhering to the walls.
2. Remove plate seal and add 25 µl of nuclease-free water to each reaction well.
3. Shake or vortex the Purification Beads well before use. It should appear homogenous and consistent in color.
4. Add 60 µl Purification Beads to each reaction well. Mix beads and sample thoroughly by pipette mixing 10 times. If bubbles form on the bottom of the wells, centrifuge the plate briefly and mix again.
5. Incubate the reactions for 5 minutes at room temperature.
6. Place the plate on a magnetic rack for up to 5 minutes until the solution clears.
7. Leave the plate on the magnetic rack.
8. Carefully remove and discard the supernatant from each well without dislodging the beads from the wall of each well.
9. Add 150 µl of freshly prepared 70% ethanol to each reaction well without disturbing the beads.
10. Incubate the reactions for 30 seconds, and then carefully remove and discard the supernatant from each well.
11. Repeat 70% ethanol wash for a total of two washes. Keep the samples on the magnetic rack and let the beads air dry at room temperature for up to 5 minutes or until residual ethanol has dried.

NOTE: Avoid over-drying the beads (bead ring/pellet appears cracked if over dried) as over-dried beads are difficult to resuspend and may decrease elution efficiency.

12. Remove the samples from the magnetic rack.
13. Resuspend the dried beads in each well by adding 64 µl nuclease-free water and pipette mixing 10 times. If bubbles form on the bottom of the wells, centrifuge the samples briefly and mix again.

14. Incubate the samples at room temperature for at least 5 minutes to elute the product.
15. Purified GS-PCR samples should be stored on ice if they are processed further within the same day, but they should be frozen at -25°C to -15°C for extended storage—see note below.

STOPPING POINT: Purified GS-PCR products may be stored frozen at -25°C to -15°C for up to 60 days.

INDEXING PCR AMPLIFICATION

NOTE: Perform indexing PCR master mix preparation in the pre-PCR area. Add purified GS-PCR product in the post-PCR area.

CAUTION: The oncoReveal™ CDx kit supports the multiplexing of up to 48 libraries per MiSeqDx v3 flow cell in up to 6 batches of varying size. However, careful planning of index-pair use across batches is required to achieve this.

Figure 1 shows the available index-pair positions on a full 48-library MiSeqDx flow cell. It is recommended that sample library batch(es) be mapped onto available positions to ensure pooled libraries from multiple batches do not exceed the 48-library limit per flowcell/run.

		1	2	3	4	5	6
		A701	A702	A703	A704	A705	A706
A	A501	A701 A501	A702 A501	A703 A501	A704 A501	A705 A501	A706 A501
B	A502	A701 A502	A702 A502	A703 A502	A704 A502	A705 A502	A706 A502
C	A503	A701 A503	A702 A503	A703 A503	A704 A503	A705 A503	A706 A503
D	A504	A701 A504	A702 A504	A703 A504	A704 A504	A705 A504	A706 A504
E	A505	A701 A505	A702 A505	A703 A505	A704 A505	A705 A505	A706 A505
F	A506	A701 A506	A702 A506	A703 A506	A704 A506	A705 A506	A706 A506
G	A507	A701 A507	A702 A507	A703 A507	A704 A507	A705 A507	A706 A507
H	A508	A701 A508	A702 A508	A703 A508	A704 A508	A705 A508	A706 A508

Figure 1 Available index-pair positions for a maximum 48-library sequencing run.

PREPARATION

1. Determine the combination of indices to be used and the Indexing-PCR plate layout. The oncoReveal™ CDx kit contains eight 5-series (A501-508) and six 7-series (A701-A706) indices, enough to support the multiplexing of up to 48 libraries onto a single MiSeqDx v3 flow cell.
2. The **Pillar Sample Sheet Tool** is a Microsoft Excel-based tool that must be used to create Sample Sheet to ensure compatibility for data analysis using PiVAT software. The tool may be downloaded from the Pillar Biosciences website or transferred from the oncoReveal™ CDx PiVAT® workstation to a USB drive and then to an Excel equipped workstation. In addition to visualization of the indexing plate layout, the tool provides a variety of checks that may help to avoid downstream errors.
3. Program the Indexing-PCR cycling profile in Table 11 into the selected thermal cycler.
4. Remove reagents from **Kit Box 2** from storage to thaw.

5. Prepare an ice-bucket to keep the reagents on ice when in use.

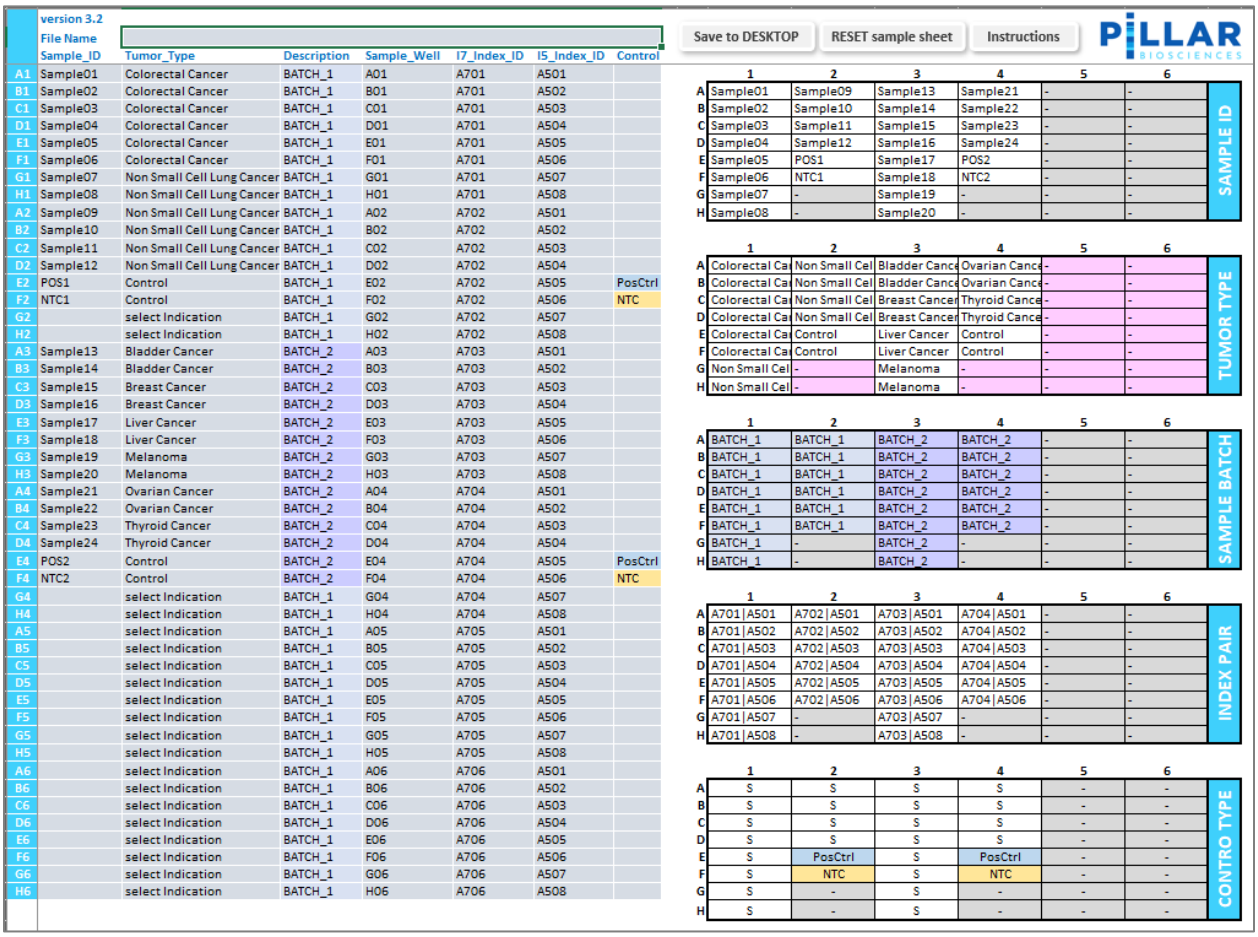


Figure 2 Pillar Sample Sheet Tool

PROCEDURE

1. Obtain a new plate for Indexing-PCR plate setup.
2. For each indexing reaction, add 4 µl of the assigned forward and reverse indexing primer to each sample or control well being used, using the guide above to prevent overlap of index pairs on the MiSeqDx flow cell. Care must be taken to prevent accidental cross contamination of indices. Each well to be used for indexing PCR should now have 8 µl total of index primers.
3. Prepare sufficient Indexing-PCR reaction mix for the samples to be indexed according to the indicated volume in Table 10. Indexing-PCR total reaction volume is 50 µl.

Table 10 Indexing-PCR reaction mix reagent volume per reaction

Reagent	Cap color	1x Volume (µl)
Indexing PCR Master Mix	Green	25.0
Nuclease-free water	N/A	11.0

4. Mix Indexing-PCR reaction mix thoroughly. Centrifuge plate briefly to collect droplets.
5. Add 36 µl of Indexing-PCR reaction mix to each assigned well of the Indexing-PCR plate. Be sure to change tips when moving to new wells to prevent cross-contamination of indices.
6. Place the plate containing the purified GS-PCR product on the magnetic rack to separate the beads from the eluent.
7. Carefully uncover the purified GS-PCR product samples and carefully transfer 6 µl of the GS-PCR product to the corresponding well containing indexing reagents, avoiding the magnetic particles. Small amounts of bead carry-over may occur and will not impact the PCR reaction.
8. Seal the Indexing-PCR and mix thoroughly. Centrifuge briefly to collect droplets and remove air bubbles.
9. Perform Indexing-PCR using the following Indexing-PCR cycling profile (Table 11) with heated lid.

Table 11 Indexing-PCR cycling profile

No. of cycles	Temperature (°C)	Time (min)
1	95	2
5	95	0.5
	66	0.5
	72	1
1	72	5
1	8	Hold

10. After the Indexing-PCR cycling protocol is complete, proceed directly to Indexed Libraries Purification steps below.

CAUTION: Use care when returning Indexing-PCR reagents to oncoReveal™ CDx Reagent Kit Box 2 for storage at -25°C to -15°C.

INDEXED LIBRARIES PURIFICATION

NOTE: Perform Indexed Libraries Purification in the post-PCR area.

PREPARATION

1. Bring materials to room temperature. It is critical that PCR purification steps are performed at room temperature.
2. Remove the purification beads in **Kit Box 3** from storage and allow to equilibrate to room temperature for at least 30 minutes prior to use.
3. Fresh 70% ethanol should be prepared for optimal results.
4. Dispense sufficient 70% ethanol solution, Purification Beads, and water in disposable trough for convenient dispense using a multichannel pipette.

PROCEDURE

1. Centrifuge the Indexing-PCR plate briefly to collect any droplets adhering to the walls.
2. Shake or vortex the Purification Beads well before use. It should appear homogenous and consistent in color.
3. Remove plate seal and add 50 µl of Purification Beads to each reaction well. Mix beads and library thoroughly by pipette mixing 10 times. If bubbles form on the bottom of the wells, centrifuge the plate briefly and mix again.
4. Incubate the reactions for 5 minutes at room temperature.
5. Place the plate on a magnetic rack for up to 5 minutes until the solution clears.
6. Leave the plate on the magnetic rack.
7. Carefully remove and discard the supernatant from each well without dislodging the beads from the wall of each well.
8. Add 150 µl of freshly prepared 70% ethanol to each reaction well without disturbing the beads.
9. Incubate the reactions for 30 seconds, and then carefully remove and discard the supernatant from each well.
10. Repeat 70% ethanol wash for a total of two washes. Keep the samples on the magnetic rack and let the beads air dry at room temperature for up to 5 minutes or until residual ethanol has dried.

NOTE: Avoid over-drying the beads (bead ring/pellet appears cracked if over dried) as over-dried beads are difficult to resuspend and may decrease elution efficiency.

11. Remove the samples from the magnetic rack.
12. Resuspend the dried beads in each well by adding 32 µl nuclease-free water and pipette mixing 10 times. If bubbles form on the bottom of the wells, centrifuge the samples briefly and mix again.
13. Incubate the samples at room temperature for at least 5 minutes to elute the product.

14. Place the plate on a magnetic rack for up to 5 minutes until the solution clears.
15. Transfer 30 µl of clear supernatant (purified indexed libraries) from each well of Indexing-PCR plate to a new plate.
16. Purified indexed libraries should be stored on ice if they are processed further within the same day, but they should be frozen at -20°C for extended storage—see note below.

STOPPING POINT: Purified indexed libraries may be stored frozen at -25°C to -15°C for up to 90 days.

QUANTIFICATION OF INDEXED SAMPLE LIBRARIES

IMPORTANT: The oncoReveal™ CDx was validated with DNA libraries quantified using a **fluorescence-based dsDNA quantification assay** (see Table 4 for assay specifications). DNA quantification is performed to determine if the DNA library is of sufficient yield for sequencing on the MiSeqDx instrument.

1. Follow the manufacturer's user guide for dsDNA quantification assay on how to **prepare standards and samples for quantification** and **calculate sample concentration**.
2. Use a **minimum of 4 µl** per sample library for quantification.
3. Measure concentration of indexed sample libraries and calculate sample concentration in ng/µl.
4. **Convert sample library concentration in ng/µL to nM** by multiplying measured concentration in ng/µl by conversion factor of 5.

$$Conc_{Library}\ in\ nM = Conc_{Library}\ in\ ng/\mu l \times 5$$

PosCtrl and NTC must meet the following library yield check in Table 12 before proceeding to Library Normalization and Pooling.

CAUTION: Ensure measured library concentration is calculated and reported in **nM** for library yield check.

Table 12 Controls library yield check

Control	Library conc (nM)	Recommendation
PosCtrl	≥3.5	Proceed to next step.
	<3.5	Positive Control library yield is low. Repeat library preparation from Indexing PCR amplification or Gene-Specific PCR Amplification.
NTC	≤2.0	Proceed to next step.
	>2.0	No Template Control may be contaminated. Repeat library preparation from Indexing PCR amplification or Gene-Specific PCR Amplification.

Do not proceed with the testing if minimum requirement for PosCtrl or NTC library concentration is not met.

5. Indexed libraries should be stored on ice if they are processed further within the same day, but they should be frozen at -20°C for extended storage (see note below).

STOPPING POINT: Purified indexed libraries may be stored frozen at -25°C to -15°C for up to 90 days.

LIBRARY NORMALIZATION AND POOLING

NOTE: The indexed sample libraries should be normalized to a final concentration of 3.5 to 5.0 nM prior to pooling to generate Library Mix.

PREPARATION

If sample libraries were stored frozen, thaw completely at room temperature. Vortex briefly to mix and centrifuge briefly to collect droplets adhering to the walls.

PROCEDURE

Normalize each sample library based on the calculated concentration in nM according to the recommendations in Table 13 below.

CAUTION: Ensure measured library concentration is calculated and reported in **nM** for library yield check and dilution calculation.

Table 13 Quantified indexed libraries dilution table

Library conc (nM)	Recommendation
<3.5	Not supported.
3.5 to 4.5	No dilution necessary.
>4.5	Dilute to library to 4.0 nM.

Do not proceed with the testing if minimum requirement for sample library concentration is not met.

6. For libraries that require dilution, calculate the volume of diluent (10 mM Tris-Cl with 0.1% Tween-20, pH 8.5 or nuclease-free water) required to **dilute the 4 µl of each library to 4.0 nM** using the formula below.

$$Vol_{Diluent}\ in\ \mu l = \frac{4\ \mu l\ \times\ Conc_{Library}}{4\ nM} - 4\ \mu l$$

7. Obtain a new plate for normalizing libraries.
8. Add the calculated volume of library dilution solution to its corresponding library stock well. The NTC is diluted by the same amount as the least concentrated sample library.
9. Transfer 4 µl of each purified indexed library from the library stock plate to its corresponding library stock well in the normalization plate.
10. After preparing the normalized libraries, seal the plate and vortex to mix thoroughly. Centrifuge the plate briefly to collect droplets.

11. Label a new 1.5 ml microcentrifuge tube for the library mix. Add 4 µl for each sample to be sequenced from the normalized libraries plate to the tube. It is recommended that a multi-channel pipettor be used to combine libraries across columns into a single unused column ("pool" column) followed by manual transfer of all well contents within the "pool" column to the tube.
12. Vortex the solution in the tube to mix thoroughly.
13. The resulting pooled libraries is now the **Library Mix**.
14. Indexed libraries should be stored on ice if they will be processed further within the same day, but they should be frozen at -20°C for extended storage (see note below).

STOPPING POINT: Purified indexed libraries may be stored frozen at -25°C to -15°C for up to 90 days.

QUANTIFICATION OF LIBRARY MIX

IMPORTANT: The oncoReveal™ CDx was validated with DNA libraries quantified using a **fluorescence-based dsDNA quantification assay** (see Table 4 for assay specifications). DNA quantification is performed to determine if the pooled Library Mix has a final concentration of 3.5 to 4.5 nM to prevent over- or under-clustering on the MiSeqDx instrument.

The DNA standard(s) provided in dsDNA quantification assay kit can be used as an independent QC sample to ensure the quantification step is as accurate as possible.

Follow the manufacturer's user guide for dsDNA quantification assay on how to **prepare standards and samples for quantification** and **calculate sample concentration**.

1. Use a minimum of 4 µl of the DNA Standard (provided in dsDNA quantification assay kit) to prepare ONE DNA Standard for quantification.
2. Use a minimum of 4 µl of Library Mix to prepare TWO replicates of Library Mix for quantification.
3. Perform measurements in the following order: DNA Standard, Library Mix replicate 1, replicate 2, and the DNA Standard again.
4. Check the 2 measurement reads of DNA Standard are ±10% of expected. See example in Table 14 below.

Both Standard DNA reads should meet the concentration check (±10% of expected) before proceeding to next steps.

Table 14 DNA standard measurement check.

DNA Standard read	Recommendation
>10% from expected	Repeat preparation and quantification of DNA Standard and Library Mix.
≤10% from expected	Proceed to next step.

5. Calculate Library Mix concentration in ng/µl.
6. Convert Library Mix concentration in ng/µL to nM. Multiply measured concentration in ng/µl by conversion factor of 5.

$$Conc_{Library}\ in\ nM = Conc_{Library}\ in\ ng/\mu l \times 5$$

7. Check the average measurements of two Library Mix replicates are ±10% of each other.
8. If the two reads are not ±10% of each other, repeat check with two additional reads.
9. If repeat reads are not ±10% of each other, repeat preparation and quantification of DNA standard and Library Mix.
10. If the two reads are within ±10%, average of two quantifications will be used to determine if Library Mix may proceed to sequencing according to the recommendations in the Table 15 below.

CAUTION: Ensure Library Mix concentration is calculated and reported in **nM** for Library Mix concentration check and dilution calculation.

Table 15 Library mix (average of 2 replicates) dilution table

Library mix conc (nM)	Recommendation
<3.5	Not supported. Repeat Library Normalization and Pooling.
3.5 to 4.5	No dilution necessary, proceed to Library Mix Denaturation.
>4.5	Dilute to 4.0 nM.

Do not proceed with the testing if minimum requirement for library mix concentration is not met.

11. For Library Mix that require dilution, calculate the volume of diluent (10 mM Tris-Cl with 0.1% Tween-20, pH 8.5 or nuclease-free water) required to dilute the 4 µl of Library Mix to 4.0 nM using the formula below.

$$Vol_{Diluent}\ in\ \mu l = \frac{4\ \mu l\ \times\ Conc_{Library\ Mix}}{4\ nM} - 4\ \mu l$$

12. Add the calculated volume of diluent to Library Mix.
13. Repeat quantification of 4 nM adjusted Library Mix from Step **Error! Reference source not found.**.
14. Place the Library Mix on ice until ready to proceed to denaturation.

LIBRARY MIX DENATURATION

IMPORTANT: The oncoReveal™ CDx was validated with the MiSeqDx Reagent Kit v3 on the Illumina MiSeqDx instrument.

PREPARE THE REAGENT CARTRIDGE

1. Thaw the MiSeqDx Cartridge in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the water line printed on the reagent cartridge. Do not allow the water to exceed the maximum water line.
2. Allow the reagent cartridge to thaw in the room temperature water bath for approximately 1 hour or until thawed.
3. Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.
4. Remove any water using a lint free wipe.

INSPECT THE REAGENT CARTRIDGE

5. Invert the reagent cartridge ten times to mix the thawed reagents, and then inspect that all positions are thawed.
6. Inspect reagents in positions 1, 2, and 4 to make sure that they are fully mixed and free of precipitates.
7. Gently tap the cartridge on the bench to reduce air bubbles in the reagents.
8. Place the reagent cartridge on ice or set aside at 2°C to 8°C (up to 6 hours) until use.

PREPARE DENATURATION REAGENTS

9. Label a new 1.5 ml microcentrifuge tube for the 0.2 N NaOH. Combine 800 µl nuclease-free water with 200 µl 1.0 N NaOH in the tube. Invert the tube several times to mix.
10. The result is 1 mL of 0.2 N NaOH. Use fresh dilution within 12 hours.
11. Remove HT1 from -25°C to -15°C storage and thaw at room temperature. Store at 2°C to 8°C until ready to dilute denatured libraries.

DENATURE LIBRARY MIX

12. Label a new 1.5 ml microcentrifuge tube for the denatured Library Mix.
13. Combine 5 µl of Library Mix and 5 µl of 0.2 N NaOH in the tube.
14. Vortex briefly and then centrifuge at 280 × g for 1 minute to collect droplets.

15. Incubate at room temperature for 5 minutes.

DILUTE DENATURED LIBRARY MIX

16. Add 990 µl prechilled HT1 to the tube of denatured Library Mix.
17. Vortex briefly and then centrifuge briefly.
18. Place the denatured Library Mix on ice until ready to proceed to final dilution.

DENATURE AND DILUTE PHIX CONTROL TO 20 PM

19. Label a new 1.5 ml microcentrifuge tube for the denatured 20 pM PhiX Control.
20. Combine 2 µl of 10 nM PhiX library and 3 µl of 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20 in the tube. The result is 5 µl of 4 nM PhiX library.
21. If not prepared within the last 12 hours, prepare a fresh dilution of 0.2 N NaOH.
22. Add 5 µl of 0.2 N NaOH to the 5 µl of 4 nM PhiX library.
23. Vortex briefly to mix.
24. Centrifuge at 280 × g for 1 minute.
25. Incubate at room temperature for 5 minutes.
26. Add 990 µl prechilled HT1 to the 10 µl of denatured PhiX library. The result is 1 ml of a 20 pM PhiX library.
27. Invert or vortex briefly to mix and then centrifuge at 280 × g for 1 minute to collect droplets. The denatured 20 pM PhiX library can be stored up to 3 weeks at -25°C to -15°C.

COMBINE DENATURED LIBRARY MIX AND PHIX LIBRARY

28. Label a new 1.5 ml microcentrifuge tube for the mixture that will be loaded on the reagent cartridge.
29. Combine 594 µl of the denatured and diluted Library Mix with 6 µl of denatured 20 pM PhiX library.
30. Set aside on ice until ready to load onto the reagent cartridge.

CREATE RUN WITH LOCAL RUN MANAGER

IMPORTANT: The oncoReveal™ CDx was validated with the Illumina MiSeqDx instrument. The "Pillar LC-HS" analysis module is accessible from the Local Run Manager Dashboard.

1. To set up a run, use the Create Run command from the Local Run Manager dashboard and select "Pillar LC-HS" module from the drop-down list. Create Run pages include the following sections:
    - Run Name
    - Samples
2. The run name is the name that identifies the run from sequencing through analysis. A run name can have up to 40 alphanumeric characters. Spaces, underscores, and dashes are allowed.
3. A run description is optional and can have up to 150 alphanumeric characters.
4. Specify samples for the run using as outlined below.

CREATE SAMPLE SHEET USING PILLAR TOOL

5. Download the Pillar Sample Sheet Tool from the Pillar Biosciences website or transferred from the oncoReveal™ CDx PiVAT® workstation to a USB drive and then to an Excel equipped workstation.
6. Enter required information:
    - File Name
    - Sample ID
    - Tumor Type
    - Batch
    - I7 Index
    - I5 Index
    - Controls (NTC and PosCtrl)
7. Select "Save to DESKTOP."
8. Make sure that the Sample Sheets you want to import are available in an accessible network location connected to the instrument or on a USB drive.

IMPORT SAMPLE SHEET INTO ILLUMINA LRM

1. From the Illumina LRM interface, click Import Samples and browse to the location of the Sample Sheet file.
2. Click 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.

LOAD SAMPLE LIBRARIES ONTO CARTRIDGE

IMPORTANT: When the reagent cartridge is fully thawed and ready for use, you are ready to load samples into the cartridge.

1. Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir on the reagent cartridge labeled “Load Sample” in position 17. Do not pierce any other reagent positions. Other reagent positions are pierced automatically during the run.
2. Pipette 600 µl of the denatured Library Mix and PhiX mixture into the Load Samples reservoir. Avoid touching the foil seal.
3. Check for air bubbles in the reservoir after loading sample. If air bubbles are present, gently tap the cartridge on the bench to release the bubbles.
4. Proceed directly to the run setup steps using the MiSeq Operating Software (MOS) interface.

RUN SETUP

IMPORTANT: See the MiSeqDx Instrument Reference Guide for MOS (MiSeq Operating Software for IVD Use) for complete run setup instructions. Refer to the Illumina website for the most current version of the guide.

1. Log in to the MiSeqDx with your Local Run Manager software password.
2. From the Home screen of the MOS software, select Sequence.
3. Select a run from the list, and then select Next.
4. A series of run setup screens open in the following order: Load Flow Cell, Load Reagents, Review, and Pre-Run check.
5. When the Load Flow Cell screen appears, clean and then load the flow cell.
6. Close the flow cell latch and flow cell compartment door.
7. Both the latch and compartment door must be closed before beginning the run. When the flow cell is loaded, the software reads and records the RFID. Confirmation that the RFID was successfully read appears in the lower-right corner of the screen.
8. Follow the software prompts to load the MiSeqDx SBS Solution (PR2) bottle, make sure that the waste bottle is empty, and load the reagent cartridge.
9. When the MiSeqDx SBS Solution (PR2) bottle and reagent cartridge are loaded, the software reads and records the RFID. Confirmation that the RFID was successfully read appears in the lower-right corner of the screen.
10. 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).

PIVAT® ANALYSIS

1. When the run has completed, transfer the run data to the PiVAT computer using USB removable storage medium.
2. See the PiVAT User Manual (UM-0042) for instructions on run data transfer, start analysis and view analysis results.

QUALITY CONTROL

No Template Control (NTC) and Positive Control (PosCtrl) are included for each "Batch" of up to 46 samples (processed on the same plate). Up to 6 batches may be included in a single sequencing run and analyzed through the PiVAT® software. PosCtrl is a cell line DNA containing the CDx variants with expected variant allele frequencies. The PosCtrl must generate expected mutations to be valid. NTC is a reaction setup using DNA diluent or nuclease-free water with no template/DNA input. The NTC should not detect any mutations. If the NTC and/or PosCtrl are invalid, the PiVAT® software will fail the entire batch and no results will be reported for all samples within the batch. See Table 17 in "Results" section for recommended actions.

Table 16 NGS-QC in PiVAT: Run, Sample and Variant Calling Passing Criteria

Category	QC Metrics	Passing Criteria
a. NGS Run level QC		
Run – Invalid if any QC metric(s) fails	PosCtrl	Expected mutations are detected
	PosCtrl	No unexpected mutation(s) detected
	NTC	No mutation detected
	NTC	Maximum coverage < 50x or < 0.5% of median within-run sample coverage
b. NGS Sample level QC		
Sample – Not Valid if any QC metric(s) fail	Sequencing base quality	Bases (with Q Score ≥ Q30) ≥ 75%
	Amplification specificity	Effective On-Target Rate[1] ≥ 70%
	Coverage[2]	Minimum depths of the three amplicons covering CDx mutations ≥ 1000x
c. Variant level QC		
CDx mutation thresholds	Non-C>T|G>A	• Variant coverage ≥ 10x and Total coverage ≥ 1000x • Average variant base Q-score ≥ 30 and VAF ≥ 1%
	C>T|G>A	• Variant coverage ≥ 10x and Total coverage ≥ 1000x • Average variant base Q-score ≥ 30 and VAF ≥ 1.5%
Group 1[3] non-CDx mutation thresholds	Non-C>T|G>A	• Variant coverage ≥ 10x and Total coverage ≥ 1000x • Average variant base Q-score ≥ 30 and VAF ≥ 1%
	C>T|G>A	• Variant coverage ≥ 10x and Total coverage ≥ 1000x • Average variant base Q-score ≥ 30 and VAF ≥ 1.5%

Category	QC Metrics	Passing Criteria
	No Call[4]	Coverage < 1000x
Group 2[3] non-CDx mutation thresholds	Any mutations	• Variant coverage ≥ 10x and Total coverage ≥ 500x • Average variant base Q-score ≥ 30 and VAF ≥ 3.2%
	No Call[4]	Coverage < 500x

[1] Effective On-Target Rate = Mapping rate * On-target rate

[2] Coverage: the coverage after paired-end assembly by PiVAT®. All markers in the assay are bi-directional sequenced with 2x150bp sequencing protocol due to the short amplicon sizes (144-162bp including primers). 1x coverage = 1x forward + 1x reverse of sequencing reads. Only uniquely mapped reads are analyzed.

[3] Group 1 non-CDx mutations include: EGFR G719X, T790M; KRAS A59X, Q61X, K117N, A146X; and BRAF V600E; all other non-CDx mutations in Group 2.

[4] No call: the variants on the amplicon covered less than the threshold is at the risk of being false negative calls.

RESULTS

INTERPRETATION OF RESULTS

All run and sample validation are performed by the oncoReveal™ CDx PiVAT® software. A valid run may include both valid and invalid sample results.

Table 17 Interpretation of PiVAT® Run Summary results

Results	Interpretation	Action
Run PASS	PosCtrl and NTC results within expected range.	None.
Run FAIL; NTC FAIL	NTC result above expected range and/or contaminated.	See Troubleshooting section for recommended resolution(s) for NTC contains amplicons. Repeat sequencing with prepared libraries and PiVAT analysis of entire run. If run invalid on repeat run, repeat entire run starting from Gene-Specific PCR Amplification.
Run FAIL; PosCtrl FAIL	PosCtrl result below expected range and/or contaminated.	See Troubleshooting section for recommended resolution(s) for improper library quantification and cross-contamination. If failure can be attributed to misquantification of sample library or library mix, repeat sequencing of prepared libraries with correct quantification and PiVAT® analysis of entire run. Otherwise, repeat entire run starting from Gene-Specific PCR Amplification.
Run FAIL; PosCtrl FAIL; NTC FAIL	NTC and PosCtrl results outside expected range and/or contaminated.	Repeat entire run starting from Gene-Specific PCR Amplification.

Table 18 Interpretation of PiVAT® Patient Summary results

Results	Interpretation	Action
Sample not valid	Sample result is invalid.	If failure can be attributed to misquantification of the invalid sample library, repeat sequencing of prepared library with correct quantification and PiVAT analysis. Otherwise, repeat testing of invalid sample starting from Gene-Specific PCR Amplification. If the sample remains invalid, extract fresh DNA from additional FFPE if available and repeat testing from Gene-Specific PCR Amplification.
Sample valid	See reportable results below:	
Mutation(s) Detected for Therapeutic Use		
Mutation detected	Mutation detected in targeted *EGFR* and/or *KRAS* region.	See Indications for Use section.
None detected	Mutation not detected in targeted *EGFR* and/or *KRAS* region.	
Mutation(s) with Evidence of Clinical Significance		
Mutation detected	Cancer mutation(s) with evidence of clinical significance detected.	See list of targeted tumor profiling genes and codons in Information About the Assay section of the report.
None detected	Mutation not detected in targeted gene regions.	
Mutation detected	Cancer mutation(s) with potential clinical significance detected.	See list of targeted tumor profiling genes and codons in Information About the Assay section of the report.
None detected	Mutation not detected in targeted gene regions.	
Pertinent No Calls		
No call detected	Mutations in the listed codon(s) are not detected and have insufficient coverage.	See list of targeted tumor profiling genes and codons in Information About the Assay section of the report.
None	All mutations assessed have sufficient coverage.	

SUMMARY OF NON-CLINICAL PERFORMANCE

Note: The studies described below include data performed with oncoReveal™ Dx Lung and Colon Cancer Assay (original PMA/CDx) and oncoReveal™ CDx (PMA Supplement/tumor profiling).

ANALYTICAL SENSITIVITY:

LIMIT OF BLANK

A Limit of Blank (LoB) of zero was determined across 70 independent sample libraries prepared from four FFPE specimens each of normal (non-tumor) colon and normal (non-tumor) lung tissue with 9 replicates per sample spanning low and high DNA input, two reagent lots, and three sequencing analyses. No false positive observations were made for the CDx variants.

A supplemental LoB study was conducted by evaluating additional tumor-matched normal tissues. 16 FFPE specimens from normal tissues for ten cancer types were evaluated: lung, colon, bladder, breast, uterus, kidney, liver, pancreas, skin, and thyroid. Each sample was tested with 4 to18 replicates at the maximum specified DNA input (80 ng), with two reagent lots, two to three replicates over two to three sequencing runs. All 105 replicate measurements yielded valid results. No false positive calls were observed confirming the false positive rate at 80 ng DNA input as zero.

LIMIT OF DETECTION

The limit of detection (LoD) based on positive calls for the oncoReveal™ CDx was estimated to determine the lowest variant allele frequency (VAF) at which 100% of the 20 test replicates produced correct calls. A minimum of five titration levels were tested across 2 reagent lots at 30 ng DNA input. Four clinical NSCLC and CRC specimens containing CDx targets were evaluated and the LoD results are shown in the Table 19 below.

Table 19 Limit of detection of CDx mutations

Gene	Variant	Variant Type	Original LoD Study (VAF%)	2nd LoD Study (VAF%)
KRAS	G13D	SNV	3.3	2.6
KRAS	G12D	SNV	3.4	1.8
EGFR	L858R	SNV	3.0	1.5
EGFR	Exon 19 Del	DelIns (a complex mutation with 19bp deletion and 1bp insertion)	3.7	1.7

Eleven (11) NSCLC and CRC specimens containing 14 tumor profiling variants (13 SNVs and 1 insertion) were evaluated to determine LoD. The LoD for tumor profiling variants were estimated using the hit rate approach where LoD is defined as the VAF detected at ≥95% hit rate. A minimum of 5 titration levels were tested with 20 replicates per level with two reagent lots (10 replicates per lot) using the minimum specified DNA input for the oncoReveal™ CDx, which is 30 ng. LoD was subsequently confirmed using six replicates across 20 samples which included 10 tumor types (specimens from

bladder, breast, renal, colon, liver, skin, lung, pancreatic, thyroid, and uterine/endometrial cancer). LoD of tumor profiling variants are summarized in the Table 20 below.

Table 20 Limit of detection of tumor profiling (non-CDx) variants

Gene	Nucleotide Change	Amino Acid Change	Variant Type	LoD (VAF%)
BRAF	c.1799T>A	p.Val600Glu	SNV	1.4
EGFR	c.2155G>T	p.Gly719Cys	SNV	1.6
EGFR[1]	c.2303G>T	p.Ser768Ile	SNV	4.8
EGFR	c.2314_2319dup	p.Pro772_His773dup	Insertion	2.2
EGFR	c.2369C>T	p.Thr790Met	SNV	3.0
KRAS	c.182A>T	p.Gln61Leu	SNV	2.2
KRAS	c.436G>A	p.Ala146Thr	SNV	2.8
PIK3CA	c.1624G>A	p.Glu542Lys	SNV	4.4
PIK3CA	c.3140A>G	p.His1047Arg	SNV	4.1
SMAD4[1]	c.533C>G	p.Ser178Ter	SNV	3.7
TP53[1]	c.817C>T	p.Arg273Cys	SNV	4.1
TP53	c.818G>A	p.Arg273His	SNV	4.7
TP53[1]	c.880G>T	p.Glu294Ter	SNV	4.5
TP53	c.892G>T	p.Glu298Ter	SNV	4.7

[1] LoD was estimated at hit rate of 95%

TUMOR CONTENT

The minimum tumor fraction required to support the robustness of the oncoReveal™ CDx was evaluated. Four clinical samples with different percentages of initial tumor cell content (30% to 80%) were estimated before the study by an external pathology lab. These were then diluted with DNA extracted from tissue-matched normal FFPE samples resulting in five levels of final tumor content and analyzed with 20 replicates per level using the oncoReveal™ CDx. The data show robustness of oncoReveal™ CDx in samples with tumor content above 10% at 30 ng DNA input. The data supports oncoReveal™ CDx requirement of 30% tumor content.

Table 21 Detection rate of diluted tumor content by variant

Gene Exon	Nucleotide Change	Amino Acid Change	Test Level	Detection Rate	VAF Range	VAF Mean	VAF SD	Diluted Tumor Content (%)
KRAS Exon 2	c.35G>A	p.Gly12Asp	L1	20/20	8.7 - 10.7	9.78	0.47	28.8
			L2	20/20	4.3 - 5.8	4.87	0.39	14.3

Gene Exon	Nucleotide Change	Amino Acid Change	Test Level	Detection Rate	VAF Range	VAF Mean	VAF SD	Diluted Tumor Content (%)
			L3	20/20	2.7 - 4	3.41	0.37	10.0
			L4	20/20	2.1 - 3	2.52	0.24	7.4
			L5	20/20	1.5 - 2.2	1.81	0.19	5.3
KRAS Exon 2	c.38G>A	p.Gly13Asp	L1	20/20	5.4 - 7.3	6.27	0.53	25.0
			L2	20/20	3.8 - 4.7	4.25	0.26	16.9
			L3	20/20	2.7 - 3.8	3.30	0.32	13.1
			L4	20/20	2 - 3.1	2.60	0.34	10.3
			L5	15/20	1.6 - 2	1.71	0.12	6.8
EGFR Exon 19	c.2237_2255delinsT	p.Glu746_Ser752delinsVal	L1	20/20	6.2 - 9.7	7.85	1.05	39.9
			L2	20/20	3.1 - 6.4	4.92	0.86	25.0
			L3	20/20	2.4 - 5.1	3.72	0.65	18.9
			L4	20/20	1.6 - 3.4	2.42	0.42	12.3
			L5	20/20	1 - 2.6	1.66	0.41	8.4
EGFR Exon 21	c.2573T>G	p.Leu858Arg	L1	20/20	7.2 - 9.6	8.32	0.70	18.0
			L2	20/20	4.9 - 7.1	6.05	0.62	13.1
			L3	20/20	1.9 - 4.3	3.02	0.56	6.6
			L4	20/20	2 - 3.7	2.59	0.49	5.6
			L5	20/20	1.2 - 1.9	1.53	0.18	3.3

DNA INPUT

The recommended DNA input range of the oncoReveal™ CDx is 30 ng to 80 ng. The DNA input range was evaluated at 5, 10, 20, 40, 80, and 160 ng in duplicate using DNA extracted from 10 FFPE samples containing reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement. The expected variants (*KRAS* G12X, *KRAS* G13X, *EGFR* Exon 19 deletion and *EGFR* L858R) present in the 10 samples were called correctly at DNA inputs of 5-160 ng. At 5 ng of DNA input, 5 out of 20 samples failed to generate sequencing libraries that meet the library yield requirement of ≥ 3.5 nM. At 10 ng of DNA input, 2 out of 20 samples failed the library yield requirement. Seven samples that failed library yield QC requirement were processed to completion to assess results below minimum DNA input of 30 ng/test. The data showed that 10-80 ng of DNA input for the oncoReveal™ CDx produced accurate results (at the variant level PPA=100.0% [95% CI: 95.4%, 100%] (80/80), NPA=100.0% [95% CI: 99.9%, 100%] (9999/10000); and therefore, supports a DNA input range of 30 ng to 80 ng for the oncoReveal™ CDx.

To assess the robustness of detection insertion variants in the recommended DNA input, five clinical samples representing 3 tumor types, NSCLC, CRC, and melanoma were tested at DNA input outside the recommended DNA input range (13 to 318 ng). Concordance at each DNA input level evaluated was determined against an evNGS comparator. Insertions 1 – 9 bases in length in four genes, *EGFR, ERBB2*, *TP53* and *PTEN* at 2.5 – 77% VAFs were evaluated. All insertion were detected at the DNA inputs evaluated by both oncoReveal™ CDx and the evNGS assay, except for a *PTEN* insertion evaluated at 5.1% VAF and 21 ng DNA input level, which was not detected by the evNGS comparator. The discordance may be explained by the variant being below the LoD of the evNGS comparator.

In addition, a supplemental evaluation was performed to confirm performance at 30 ng DNA input extracted from FFPE of eight additional tumor types (other than CRC and NSCLC). The tissues include melanoma, bladder, breast, endometrial, liver, melanoma, pancreatic, cancer, and thyroid cancers. Eighty-four (84) libraries were prepared with 100% valid results that included a total of 106 positive mutation calls confirmed using evNGS comparator methods for evaluation. These results confirm that the assay produced accurate results across eight additional tumor types using minimum DNA input at 30 ng with PPA 100% and NPA of 99.97%.

ACCURACY

ACCURACY – STUDY 1 (CDx VARIANTS)

Analytical accuracy was performed to demonstrate the concordance between the oncoReveal™ CDx and an externally validated comparator method for the ability of oncoReveal™ CDx to detect reportable SNVs and short and medium deletions for CDx genes *EGFR* and *KRAS*. A total of 263 samples (177 CRC and 86 NSCLC) were tested. Of these samples, 6 yielded invalid results with the validated NGS comparator method and 6 yielded invalid results or did not meet workflow QC with oncoReveal™ CDx. Among the 251 valid samples, 87 positive and 160 negative samples were concordant between the two assays. There were 4 discordant samples between the oncoReveal™ CDx and the comparator assay. The samples included simple SNVs, complex SNV and indels that are targeted by the oncoReveal™ CDx. The results at the variant, sample and bin levels are shown in the tables below.

Table 22 Overall agreement result by CDx variant, sample, and gene

Bin	Test + Comp + TP	Test + Comp – FP	Test - Comp + FN	Test - Comp – TN	Total N	PPA (95%CI)	NPA (95%CI)	PPV (95%CI)	NPV (95%CI)
by Gene									
EGFR CDx variant in NSCLC	18	2	0	7675	7695	100.0% (82.4%, 100.0%)	100.0% (99.9%, 100.0%)	90.0% (69.9%, 97.2%)	100.0% (100%, 100.0%)
KRAS CDx variant in CRC	69	2	0	5199	5270	100.0% (94.7%, 100.0%)	100.0% (99.9%, 100.0%)	97.2% (90.3%, 99.2%)	100.0% (99.9%, 100.0%)
by Sample									

Bin	Test + Comp + TP	Test + Comp – FP	Test - Comp + FN	Test - Comp – TN	Total N	PPA (95%CI)	NPA (95%CI)	PPV (95%CI)	NPV (95%CI)
Sample (*EGFR*)	18	2	0	61	81	100.0% (82.4%, 100.0%)	96.8% (89.1%, 99.1%)	90.0% (69.9%, 97.2%)	100.0% (94.1%, 100.0%)
Sample (*KRAS*)	69	2	0	99	170	100.0% (94.7%, 100.0%)	98.0% (93.1%, 99.5%)	97.2% (90.3%, 99.2%)	100.0% (96.3%, 100.0%)

Note: oncoReveal™ CDx does not have “No Call” in “Valid” samples. Invalid data are excluded from this analysis.

PPA = TP / (TP + FN) x 100%

NPA = TN / (TN + FP) x 100%

PPV = TP / (TP + FP) x 100%

NPV = TN / (TN + FN) x 100%

Comp + = Sample positive for at least one targeted variant when tested with comparator method

Test + = Sample positive for at least one target variant when tested with oncoReveal™ CDx

Agreement of *EGFR* CDx variants in NSCLC and *KRAS* CDx variants in CRC are summarized Table 23 and
Table 24 below, respectively.

Table 23 EGFR CDx variants in NSCLC agreement by variant type and class

Bin	Test + Comp + TP	Test + Comp - FP	Test - Comp + FN	Test - Comp - TN	Total N	PPA (95%CI)	NPA (95%CI)	PPV (95%CI)	NPV (95%CI)
by Variant Type									
SNV	8	0	0	73	81	100.0% (67.6%, 100.0%)	100.0% (95.0%, 100.0%)	100.0% (67.6%, 100.0%)	100.0% (95.0%, 100.0%)
Complex SNV	0	0	0	243	243	N/A	100.0% (98.4%, 100.0%)	N/A	100.0% (98.4%, 100.0%)
Deletion (15-18bp)	10	2	0	7359	7371	100.0% (72.2%, 100.0%)	100.0% (99.9%, 100.0%)	83.3% (55.2%, 95.3%)	100.0% (99.9%, 100.0%)
by Variant Class									
C>T|G>A	0	0	0	0	0	N/A	N/A	N/A	N/A
Non C>T|G>A	18	2	0	7675	7695	100.0% (82.4%, 100.0%)	100.0% (99.9%, 100.0%)	90.0% (69.9%, 97.2%)	100.0% (99.9%, 100.0%)

Table 24 KRAS CDx variants in CRC agreement by variant type and class

Bin	Test + Comp + TP	Test + Comp - FP	Test - Comp + FN	Test - Comp - TN	Total N	PPA (95%CI)	NPA (95%CI)	PPV (95%CI)	NPV (95%CI)
by Variant Type									
SNV	69	2	0	1969	2040	100.0% (94.7%, 100.0%)	99.9% (99.6%, 100.0%)	97.2% 90.3%, 99.2%)	100.0% (99.8%, 100.0%)
Complex SNV	0	0	0	3230	3230	N/A	100.0% (99.9%, 100.0%)	N/A	100.0% (99.9%, 100.0%)
Deletion (15-18bp)	0	0	0	0	0	N/A	N/A	N/A	N/A
by Variant Class									
C>T|G>A	43	1	0	636	680	100.0% (91.8%, 100.0%)	99.8% (99.1%, 100.0%)	97.7% (88.2%, 99.6%)	100.0% (99.4%, 100.0%)
Non C>T|G>A	26	1	0	4563	4590	100.0% (87.1%, 100.0%)	100.0% (99.9%, 100.0%)	96.3% (81.7%, 99.3%)	100.0% (99.9%, 100.0%)

As the accuracy study samples were enrolled by the oncoReveal™ CDx, PPA and NPA values were adjusted using a prevalence of 6.9% for *EGFR* variants and 36.1% for *KRAS* variants in the intended use/indications for use population. The summary of the agreement statistics is shown in Table 25 below.

Table 25 Summary of Agreement Statistics

Binned by Sample	PPV (95%CI)	NPV (95%CI)	Unadjusted		Adjusted	
			PPA (95%CI)	NPA (95%CI)	PPA (95%CI)	NPA (95%CI)
Sample (*EGFR*)	90.0% (69.9%, 97.2%)	100.0% (94.1%, 100.0%)	100.0% (82.4%, 100.0%)	96.8% (89.1%, 99.1%)	100.0% (46.6%, 100.0%)	99.3% (97.7%, 99.8%)
Sample (*KRAS*)	97.2% (90.3%, 99.2%)	100.0% (96.3%, 100.0%)	100.0% (94.7%, 100.0%)	98.0% (93.1%, 99.5%)	100.0% (93.2%, 100.00%)	98.4% (94.6%, 99.6%)

In total, there were 4 samples that were discordant. Among the 4 discordant results, 3 mutations detected by the oncoReveal™ CDx (one for *EGFR* and 2 for *KRAS*) had low VAFs, which were below the assay cut-off for the externally validated NGS assay (evNGS). The remaining discordant variant was a complex *EGFR* Exon 19 deletion, which was detected by oncoReveal™ CDx, while the evNGS did not detect.

To evaluate additional samples with positive calls at the low VAF, DNA from 11 representative positive clinical samples with adequate leftover material from the accuracy study were diluted with normal FFPE

DNAs to create samples with low VAFs. In total 16 low VAF samples were generated and tested with oncoReveal™ CDx. Of these, 5 NSCLC samples that were positive for 4 different EGFR Exon 19 deletions were diluted to 0.6x-2.1x LoD levels, 2 NSCLC clinical samples positive for EGFR L858R mutation were diluted to 0.5x-1.7x LoD levels, and 4 CRC samples positive for KRAS mutations were diluted to 0.6x-2.2x LoD levels. All these sample runs met the sequencing quality metrics criteria. The results of the diluted samples were positive based on the original calls.

ACCURACY – STUDY 2 (TUMOR-PROFILING VARIANTS)

Analytical accuracy was performed to demonstrate the concordance between the oncoReveal™ CDx and two externally validated comparator methods (evNGS, A & B) for the ability of oncoReveal™ CDx to detect reportable SNVs, deletions and insertions for tumor profiling in 22 genes. The concordance analysis was done for overall agreement, by variant types, and per gene.

Comparator Method A

A total of 271 samples represented by 10 tumor types were tested across 15 genes using comparator method A. Of these samples, 9 yielded invalid results with comparator method A (externally validated NGS) and 6 yielded invalid results or did not meet workflow quality control (QC) with oncoReveal™ CDx. Among the 257 valid samples, 181 positive and 65 negative samples were concordant between the two assays. There were 11 discordant samples that consist of 7 Test+/Comp- observation, and 4 samples with a positive concordant call in both assays for one mutation but contained an additional variant(s) called by only one assay. In 3 samples an additional low VAF variant (<3%) was reported by oncoReveal™ CDx and not comparator A and in 1 sample, a low VAF variant (<5%) was reported in comparator A but not oncoReveal™ CDx. The three discordant variants with high VAFs (33%- 75%), two 15bp deletions and one 6-bp insertion, were confirmed positive in a third orthogonal method. All other discordant mutations are expected due to the low allelic fractions and the differences in VAF cut-offs applied by oncoReveal™ CDx and comparator method A. Sample level agreement is shown in Table 26 below.

Table 26 Sample level agreement between oncoReveal™ CDx and Comparator Method A

Total Samples	# Concordant	# Discordant	% Agreement (95% CI)
257	246	11	95.7% (92.5%, 97.6%)

The aggregated results at the variant type level is shown in Table 27 and gene level is shown in Table 28 below. As this accuracy study samples were enrolled by the oncoReveal™ CDx, the PPV and NPV were direct calculations; however, the PPA and NPA values were adjusted using the proportion of positive variants detected by oncoReveal™ CDx.

Table 27 Comparator Method A: Overall variant-level agreement and binned by variant type

Variant Type	# Samples	# Variants	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)	Adjusted PPA (95% CI)	Adjusted NPA (95% CI)
All	257	243636	99.6% (245/246) (97.7%, 99.9%)	99.9% (243380/243390) (99.9%, 99.9%)	96.1% (245/255) (92.9%, 97.9%)	99.9% (243380/243381) (99.9%, 99.9%)	99.0% (94.4%, 99.8%)	99.9% (99.9%, 99.9%)
SNV	257	141864	99.6% (228/229) (97.6%, 99.9%)	99.9% (141629/141635) (99.9%, 99.9%)	97.4% (228/234) (94.5%, 98.8%)	99.9% (141629/141630) (99.9%, 99.9%)	99.3% (95.9%, 99.9%)	99.9% (99.9%, 99.9%)

Variant Type	# Samples	# Variants	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)	Adjusted PPA (95% CI)	Adjusted NPA (95% CI)
MNV	257	31354	100.0% (4/4) (51.0%, 100.0%)	100.0% (31350/31350) (99.9%, 100.0%)	100.0% (4/4) (51.0%, 100.0%)	100.0% (31350/31350) (99.9%, 100.0%)	100.0% (16.5%, 100.0%)	100.0% (99.9%, 100.0%)
Deletion	257	42148	100.0% (11/11) (74.1%, 100.0%)	99.9% (42134/42137) (99.9%, 99.9%)	78.6% (11/14) (52.4%, 92.4%)	100.0% (42134/42134) (99.9%, 100.0%)	100.0% (31.0%, 100.0%)	99.9% (99.9%, 99.9%)
Insertion	257	28270	100.0% (2/2) (34.2%, 100.0%)	99.9% (28267/28268) (99.9%, 99.9%)	66.7% (2/3) (20.8%, 93.9%)	100.0% (28267/28267) (99.9%, 100.0%)	100.0% (6.4%, 100.0%)	99.9% (99.9%, 99.9%)

Table 28 Comparator Method A: Variant-level agreement by gene

Gene	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)
AKT1	100.0% (4/4) (51.0%, 100.0%)	100.0% (767/767) (99.5%, 100.0%)	100.0% (4/4) (51.0%, 100.0%)	100.0% (767/767) (99.5%, 100.0%)
ALK	Not Evaluable	100.0% (8995/8995) (99.9%, 100.0%)	Not Evaluable	100.0% (8995/8995) (99.9%, 100.0%)
BRAF	100.0% (34/34) (89.8%, 100.0%)	99.9% (21296/21297) (99.9%, 99.9%)	97.1% (34/35) (85.5%, 99.5%)	100.0% (21296/21296) (99.9%, 100.0%)
CTNNB1	100.0% (11/11) (74.1%, 100.0%)	100.0% (44964/44964) (99.9%, 100.0%)	100.0% (11/11) (74.1%, 100.0%)	100.0% (44964/44964) (99.9%, 100.0%)
DDR2	Not Evaluable	100.0% (514/514) (99.3%, 100.0%)	Not Evaluable	100.0% (514/514) (99.3%, 100.0%)
EGFR	100.0% (30/30) (88.6%, 100.0%)	99.9% (68586/68589) (99.9%, 99.9%)	90.9% (30/33) (76.4%, 96.9%)	100.0% (68586/68586) (99.9%, 100.0%)
ERBB2	80.0% (4/5) (37.6%, 96.4%)	99.9% (12330/12331) (99.9%, 99.9%)	80.0% (4/5) (37.6%, 96.4%)	99.9% (12330/12331) (99.9%, 99.9%)
ERBB4	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
FBXW7	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
FGFR1	Not Evaluable	100.0% (771/771) (99.5%, 100.0%)	Not Evaluable	100.0% (771/771) (99.5%, 100.0%)
FGFR2	100.0% (4/4) (51.0%, 100.0%)	100.0% (4365/4365) (99.9%, 100.0%)	100.0% (4/4) (51.0%, 100.0%)	100.0% (4365/4365) (99.9%, 100.0%)

Gene	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)
FGFR3	100.0% (5/5) (56.6%, 100.0%)	100.0% (4621/4621) (99.9%, 100.0%)	100.0% (5/5) (56.6%, 100.0%)	100.0% (4621/4621) (99.9%, 100.0%)
KRAS	100.0% (93/93) (96.0%, 100.0%)	99.9% (25603/25607) (99.9%, 99.9%)	95.9% (93/97) (89.9%, 98.4%)	100.0% (25603/25603) (99.9%, 100.0%)
MAP2K1	100.0% (1/1) (20.7%, 100.0%)	99.9% (7194/7195) (99.9%, 99.9%)	50.0% (1/2) (9.5%, 90.5%)	100.0% (7194/7194) (99.9%, 100.0%)
MET	Not Evaluable	100.0% (2827/2827) (99.9%, 100.0%)	Not Evaluable	100.0% (2827/2827) (99.9%, 100.0%)
NOTCH1	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
NRAS	100.0% (10/10) (72.2%, 100.0%)	100.0% (12069/12069) (99.9%, 100.0%)	100.0% (10/10) (72.2%, 100.0%)	100.0% (12069/12069) (99.9%, 100.0%)
PIK3CA	100.0% (49/49) (92.7%, 100.0%)	100.0% (28478/28478) (99.9%, 100.0%)	100.0% (49/49) (92.7%, 100.0%)	100.0% (28478/28478) (99.9%, 100.0%)
PTEN	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
SMAD4	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
STK11	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
TP53	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable

Comparator Method B

A second validated Comparator Method B (evNGS B) was used to include 6 additional genes not targeted by Method A. A total of 213 samples represented by 10 tumor types were tested across 19 genes using comparator method B. Of these samples, 19 yielded invalid results with the externally validated NGS (evNGS B) comparator method B and 15 yielded invalid results or did not meet workflow quality control (QC) with oncoReveal™ CDx. Nine samples failed both assays. Among the 187 valid samples, 158 positive and 10 negative samples were concordant between the two assays. There was a total of 19 discordant samples that consist of 2 Test+Comp- observation, and 17 samples that showed positive concordance in the targeted variant(s) but contained additional positive variant calls in either oncoReveal™ CDx or comparator B. Sample level agreement is shown in Table 29 below.

Table 29 Sample level agreement between oncoReveal™ CDx and Comparator Method B

Total Samples	# Concordant	# Discordant	% Agreement (95% CI)
187	168	19	89.8% (84.7%, 93.4%)

The aggregated results at the variant type level is shown in Table 30Table 27 and gene level is shown in Table 31 below. As this accuracy study samples were enrolled by the oncoReveal™ CDx, the PPV and NPV were direct calculations; however, the PPA and NPA values were adjusted using the proportion of positive variants detected by oncoReveal™ CDx.

Table 30 Comparator B: Overall variant-level agreement and binned by variant type

Variant Type	# Samples	# Variants	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)	Adjusted PPA (95% CI)	Adjusted NPA (95% CI)
All	187	661045	98.6% (345/350) (96.7%, 99.4%)	99.9% (660677/660695) (99.9%, 99.9%)	95.0% (345/363) (92.3%, 96.8%)	99.9% (660677/660682) (99.9%, 99.9%)	94.3% (91.1%, 96.3%)	99.9% (99.9%, 99.9%)
SNV	187	250954	98.7% (308/312) (96.8%, 99.5%)	99.9% (250627/250642) (99.9%, 99.9%)	95.4% (308/323) (92.5%, 97.2%)	99.9% (250627/250631) (99.9%, 99.9%)	94.4% (90.9%, 96.5%)	99.9% (99.9%, 99.9%)
MNV	187	37587	100.0% (6/6) (61.0%, 100.0%)	100.0% (37581/37581) (99.9%, 100.0%)	100.0% (6/6) (61.0%, 100.0%)	100.0% (37581/37581) (99.9%, 100.0%)	100.0% (27.5%, 100.0%)	100.0% (99.9%, 100.0%)
Deletion	187	238051	100.0% (21/21) (84.5%, 100.0%)	99.9% (238028/238030) (99.9%, 99.9%)	91.3% (21/23) (73.2%, 97.6%)	100.0% (238028/238028) (99.9%, 100.0%)	89.3% (66.0%, 96.8%)	100.0% (99.9%, 100.0%)
Insertion	187	134453	90.9% (10/11) (62.3%, 98.4%)	99.9% (134441/134442) (99.9%, 99.9%)	90.9% (10/11) (62.3%, 98.4%)	99.9% (134441/134442) (99.9%, 99.9%)	86.7% (44.1%, 97.6%)	99.9% (99.9%, 99.9%)

Table 31 Comparator Method B: Variant-level agreement by gene

Gene	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)
AKT1	100.0% (4/4) (51.0%, 100.0%)	100.0% (370/370) (99.0%, 100.0%)	100.0% (4/4) (51.0%, 100.0%)	100.0% (370/370) (99.0%, 100.0%)
ALK	100.0% (1/1) (20.7%, 100.0%)	100.0% (6357/6357) (99.9%, 100.0%)	100.0% (1/1) (20.7%, 100.0%)	100.0% (6357/6357) (99.9%, 100.0%)
BRAF	100.0% (37/37) (90.6%, 100.0%)	99.9% (15296/15297) (99.9%, 99.9%)	97.4% (37/38) (86.5%, 99.5%)	100.0% (15296/15296) (99.9%, 100.0%)
CTNNB1	94.7% (18/19) (75.4%, 99.1%)	100.0% (32706/32706) (99.9%, 100.0%)	100.0% (18/18) (82.4%, 100.0%)	99.9% (32706/32707) (99.9%, 99.9%)
DDR2	Not Evaluable	100.0% (561/561) (99.3%, 100.0%)	Not Evaluable	100.0% (561/561) (99.3%, 100.0%)
EGFR	100.0% (22/22) (85.1%, 100.0%)	100.0% (49533/49533) (99.9%, 100.0%)	100.0% (22/22) (85.1%, 100.0%)	100.0% (49533/49533) (99.9%, 100.0%)
ERBB2	100.0% (7/7) (64.6%, 100.0%)	100.0% (8782/8782) (99.9%, 100.0%)	100.0% (7/7) (64.6%, 100.0%)	100.0% (8782/8782) (99.9%, 100.0%)

Gene	PPA (n/N) (95% CI)	NPA (n/N) (95% CI)	PPV (n/N) (95% CI)	NPV (n/N) (95% CI)
ERBB4	100.0% (6/6) (61.0%, 100.0%)	100.0% (3734/3734) (99.9%, 100.0%)	100.0% (6/6) (61.0%, 100.0%)	100.0% (3734/3734) (99.9%, 100.0%)
FBXW7	100.0% (13/13) (77.2%, 100.0%)	100.0% (16443/16443) (99.9%, 100.0%)	100.0% (13/13) (77.2%, 100.0%)	100.0% (16443/16443) (99.9%, 100.0%)
FGFR1	100.0% (1/1) (20.7%, 100.0%)	100.0% (934/934) (99.6%, 100.0%)	100.0% (1/1) (20.7%, 100.0%)	100.0% (934/934) (99.6%, 100.0%)
FGFR2	100.0% (7/7) (64.6%, 100.0%)	100.0% (2985/2985) (99.9%, 100.0%)	100.0% (7/7) (64.6%, 100.0%)	100.0% (2985/2985) (99.9%, 100.0%)
FGFR3	100.0% (3/3) (43.9%, 100.0%)	99.9% (3362/3363) (99.8%, 99.9%)	75.0% (3/4) (30.1%, 95.4%)	100.0% (3362/3362) (99.9%, 100.0%)
KRAS	96.8% (30/31) (83.8%, 99.4%)	99.9% (17919/17921) (99.9%, 99.9%)	93.8% (30/32) (79.9%, 98.3%)	99.9% (17919/17920) (99.9%, 99.9%)
MAP2K1	100.0% (2/2) (34.2%, 100.0%)	100.0% (5608/5608) (99.9%, 100.0%)	100.0% (2/2) (34.2%, 100.0%)	100.0% (5608/5608) (99.9%, 100.0%)
MET	Not Evaluable	100.0% (2431/2431) (99.8%, 100.0%)	Not Evaluable	100.0% (2431/2431) (99.8%, 100.0%)
NOTCH1	Not Evaluable	Not Evaluable	Not Evaluable	Not Evaluable
NRAS	100.0% (21/21) (84.5%, 100.0%)	100.0% (8394/8394) (99.9%, 100.0%)	100.0% (21/21) (84.5%, 100.0%)	100.0% (8394/8394) (99.9%, 100.0%)
PIK3CA	100.0% (57/57) (93.7%, 100.0%)	99.9% (20699/20700) (99.9%, 99.9%)	98.3% (57/58) (90.9%, 99.7%)	100.0% (20699/20699) (99.9%, 100.0%)
PTEN	100.0% (40/40) (91.2%, 100.0%)	99.9% (131789/131795) (99.9%, 99.9%)	87.0% (40/46) (74.3%, 93.9%)	100.0% (131789/131789) (99.9%, 100.0%)
SMAD4	100.0% (10/10) (72.2%, 100.0%)	99.9% (32713/32715) (99.9%, 99.9%)	83.3% (10/12) (55.2%, 95.3%)	100.0% (32713/32713) (99.9%, 100.0%)
STK11	100.0% (6/6) (61.0%, 100.0%)	100.0% (15702/15702) (99.9%, 100.0%)	100.0% (6/6) (61.0%, 100.0%)	100.0% (15702/15702) (99.9%, 100.0%)
TP53	95.2% (60/63) (86.9%, 98.4%)	99.9% (284359/284364) (99.9%, 99.9%)	92.3% (60/65) (83.2%, 96.7%)	99.9% (284359/284362) (99.9%, 99.9%)

REPRODUCIBILITY

MULTI-SITE PRECISION – STUDY 1 (CDX VARIANTS)

The reproducibility of the oncoReveal™ CDx was evaluated using 10 clinical samples with target variants adjusted to a variant allele frequency percent (VAF%) in the range of 1-3x of the currently established LoD using DNA extracted from clinically normal tissue. The sample panel included two (2) FFPE CRC specimens with *KRAS* mutations (Gly12Asp and Gly13Asp), two (2) FFPE NSCLC specimens with *EGFR* mutations (Glu746_Ser752delinsVal and Leu858Arg) and one (1) FFPE CRC specimen negative for CDx variants. Each variant was present at both high and low VAF% levels.

The study was conducted at three sites with 2 operators at each site performing 3 runs on non-consecutive days. One sequencing instrument and 2 reagent lots were used at each site. Each sample was tested in 4 replicates in each run for a total of 36 possible results (3 sites by 3 runs by 4 replicates). The study produced a total of 360 test results.

PPA and NPA values with two-sided 95% confidence intervals were calculated across all tests performed. The observed PPA value for target variants was 100% (98.7%,100%), and NPA was 100% (100%,100%). A variance component analysis was performed for each of the sample/variant level to estimate variability of the assay including site, operator, day (site, operator), replicate and reagent lot. The total standard deviations of VAF% ranged from 0.33% to 0.70%.

Each of the study sites performed a total of 120 tests. The observed PPA value for target variants was 100% (96.2%,100%), and NPA was 100% (100%,100%) at each site. All 4 replicates tested for each sample at both high and low VAF% levels were concordant in each of the 9 runs tested across 3 sites with no false negatives, i.e., 100% concordant (within run precision). An analysis of test performance across study sites, measured as PPA and NPA with 95% confidence intervals, is provided in Table 32 below.

Table 32 Study 1:CDx variants agreement by site and 3-sites combined

Bin	N	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
Overall_3ites	360	288	0	0	45072	100% (98.7%,100.0%)	100% (100.0%,100%)
Site 1	120	96	0	0	15024	100% (96.2%,100%)	100% (100.0%,100%)
Site 2	120	96	0	0	15024	100.0% (96.2%,100%)	100.0% (100.0%,100%)
Site 3	120	96	0	0	15024	100.0% (96.2%,100%)	100.0% (100.0%,100%)

Observed mean VAF% and positive call rates with 95% confidence intervals across sample variants at both high and low VAF% concentration for the 36 replicates were analyzed and are reported below.

Table 33 Study 1: Variant agreement and variant frequency level

VAF Level	Gene/ Exon	Nucleotide Change	Amino Acid Change	N	Mean VAF (%)	Positive Call Rate (%)	95% CI (LB,UB)
High	*KRAS* Exon 2	c.35G>A	p.Gly12Asp	36	6.80	36/36 (100%)	90.4%,100%
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	6.91	36/36 (100%)	90.4%,100%
	EGFR Exon 19	c.2237_2255delinsT	p.Glu746_Ser752delinsVal	36	5.14	36/36 (100%)	90.4%,100%
	EGFR Exon 21	c.2573T>G	p.Leu858Arg	36	8.91	36/36 (100%)	90.4%,100%
Low	*KRAS* Exon 2	c.35G>A	p.Gly12Asp	36	4.69	36/36 (100%)	90.4%,100%
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	3.66	36/36 (100%)	90.4%,100%
	EGFR Exon 19	c.2237_2255delinsT	p.Glu746_Ser752delinsVal	36	3.02	36/36 (100%)	90.4%,100%
	EGFR Exon 21	c.2573T>G	p.Leu858Arg	36	4.96	36/36 (100%)	90.4%,100%

* LoD is based on an originally established VAF% indicated in Table 19.

Lot-to-Lot Precision

A total of 3 manufactured reagent lots were used in the study with 2 reagent lots tested at each site. The calculated PPA and NPA values were identical across reagent lots with mean and two-sided 95% confidence intervals of 100% (96.2%,100%) for PPA and 100% (100%,100%) for NPA. The reagent lot component of the total standard deviation of VAF% ranged from 0.08% to 0.33%.

Thermocycler Variability

A total of 3 make/model of thermo cycler were used in the study. The calculated PPA and NPA values were identical across reagent lots with mean of 100% for PPA and 100% for NPA and with the two-sided 95% confidence intervals as reported in the table below.

Table 34 Precision by thermocycler

Bin	N	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
Eppendorf MasterCycler	120	96	0	0	15024	100% (96.2%, 100%)	100% (100%, 100%)
ABI GeneAmp 9700	40	32	0	0	5008	100% (89.3%, 100%)	100% (99.9%, 100%)

Bin	N	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
Bio-Rad C1000	200	160	0	0	25040	100% (97.7%, 100%)	100% (100.0%, 100%)

MULTI-SITE PRECISION – STUDY 2 (CDX VARIANTS)

A 3-site precision study was conducted using 11 clinical samples with target CDx variants adjusted to a VAF% in the range of 1-1.5x of the LoD defined based on the second LoD study (see Table 19) using DNA extracted from tissue-matched clinically normal FFPE tissue. The sample panel included three (3) FFPE NSCLC specimens with unique *EGFR* Exon 19 deletion variants, three (3) FFPE NSCLC specimens with *EGFR* Exon 21 L858R mutations, three (3) FFPE CRC specimens with unique *KRAS* G12 variants and two (2) FFPE CRC specimens with *KRAS* G13 variants.

The study was conducted at three sites with 2 operators at each site performing 3 runs on non-consecutive days. Each sample was tested in 4 replicates in each run for a total of 36 possible results (9 runs by 4 replicates). The 3-site study performed a total of 396 tests.

Positive and Negative Call Rate values with two-sided 95% confidence intervals were calculated across all tests performed. Observed mean VAF% and positive call rates with 95% confidence intervals across 11 clinical samples with target variants for the 36 replicates is presented in Table 35 below. Positive and negative call rate values for target variants by site and gene are shown in Table 36 and Table 37, respectively. Pos and neg call rate on a site- and gene-level was greater than 98% and 100% for all comparisons.

Table 35 Agreement by specimen at the variant level

Sample ID	Gene Exon	Nucleotide Change	AminoAcid Change	Total Calls	VAF mean	Fold LoD**	Pos_Call_Rate (n/N) (2-sided 95% CI)
1	*EGFR* Exon 19	c.2240_2254del	p.Leu747_Thr751del	36	2.56	1.5	100.0% (36/36) (90.4%, 100.0%)
2	*KRAS* Exon 2	c.35G>A	p.Gly12Asp	36	2.79	1.6	100.0% (36/36) (90.4%, 100.0%)
3	*KRAS* Exon 2	c.34G>T	p.Gly12Cys	36	1.83	1.0	100.0% (36/36) (90.4%, 100.0%)
4	*EGFR* Exon 21	c.2573T>G	p.Leu858Arg	36	2.32	1.5	100.0% (36/36) (90.4%, 100.0%)
5	*EGFR* Exon 19	c.2236_2250del	p.Glu746_Ala750del	35*	2.19	1.3	100.0% (35/35) (90.1%, 100.0%)
6	*KRAS* Exon 2	c.38G>A	p.Gly13Asp	36	3.34	1.3	100.0% (36/36) (90.4%, 100.0%)
7	*EGFR* Exon 21	c.2573T>G	p.Leu858Arg	36	1.78	1.2	97.2% (35/36) (85.8%, 99.5%)

Sample ID	Gene Exon	Nucleotide Change	AminoAcid Change	Total Calls	VAF mean	Fold LoD**	Pos_Call_Rate (n/N) (2-sided 95% CI)
8	*KRAS* Exon 2	c.35G>T	p.Gly12Val	36	1.58	0.9	100.0% (36/36) (90.4%, 100.0%)
9	*EGFR* Exon 21	c.2573T>G	p.Leu858Arg	36	1.60	1.1	94.4% (34/36) (81.9%, 98.5%)
10	*EGFR* Exon 19	c.2235_2249del	p.Glu746_Ala750del	35	1.91	1.1	97.1% (34/35) (85.5%, 99.5%)
11	*KRAS* Exon 2	c.38G>A	p.Gly13Asp	36	2.93	1.1	100.0% (36/36) (90.4%, 100.0%)

* One replicate produced “Not Valid” result. Investigation suggests the library was inadvertently excluded during library pooling and was not sequenced.

** LoD is based on newly defined VAF% as indicated in Table 19.

Table 36 Overall agreement and agreement by site

Site	Positive/ Total Calls	Positive Call Rate (2-sided 95% CI)	Negative/ Total Calls	Negative Call Rate (2-sided 95% CI)
Overall	390/394	99.0% (97.4%, 99.6%)	49250 / 49250	100% (100.0%, 100.0%)
Site 1	130/131	99.2% (95.8%, 99.9%)	16375 / 16375	100% (100.0%, 100.0%)
Site 2	131/132	99.2% (95.8%, 99.9%)	16500 / 16500	100% (100.0%, 100.0%)
Site 3	129/131	98.5% (94.6%, 99.6%)	16375 / 16375	100% (100.0%, 100.0%)

Table 37 Agreement by gene

Gene	Positive/ Total Calls	Positive Call Rate (2-sided 95% CI)	Negative/ Total Calls	Negative Call Rate (2-sided 95% CI)
EGFR	210/214	98.1% (95.3%, 99.3%)	26750 / 26750	100.0% (100.0%, 100.0%)
KRAS	180/180	100.0% (97.9%, 100.0%)	22500 / 22500	100.0% (100.0%, 100.0%)

MULTI-SITE PRECISION – STUDY 3 (NON-CDX TUMOR PROFILING VARIANTS)

A multi-site reproducibility study was performed to support oncoReveal™ CDx performance to detect tumor profiling mutations from different cancer indications. The reproducibility of the oncoReveal™ CDx assay was evaluated using 10 clinical samples with target tumor profiling variants adjusted to %VAF in the range of 1-1.5x LoD. The sample panel included FFPE tissues from six tumor types, including: bladder, colorectal, melanoma, NSCLC, pancreatic, and uterine/endometrial cancers and is summarized in Table 38. The study was conducted at three sites performing 3 runs on non-consecutive days. One sequencing instrument and one reagent lot were used at each site. Each sample was tested

with up to 4 replicates in each run for a total of up to 36 possible results (3 sites by 3 runs by 4 replicates). The study produced a total of 348 test results.

Table 38 Multi-site reproducibility study 31 variants (12 genes) 10 clinical samples.

Sample	Gene	Exon	Nucleotide Change	Amino Acid Change	Variant type	Variant level***	Ratio Mean	Mean VAF	Median	SD	CV %
1	*BRAF*	15	1799T>A	V600E	SNV	T-2	6.76	12.84	12.88	0.53	4.1
1	*FBXW7*	10	1436G>A	R479Q	SNV	T-3	1.97	8.85	8.76	0.67	7.6
1	*PIK3CA*	10	1634A>G	E545G	SNV	T-3	2.82	12.71	12.72	0.49	3.8
1	*PTEN*	1	17_18del	K6RfsTer4	Del	T-3	2.11	9.50	9.44	0.63	6.6
1**	*PTEN*	7	710dup	F238VfsTer5	Ins	T-3	*0.93*	4.18	4.12	0.35	8.4
1	*PTEN*	7	800del	K267RfsTer9	Del	T-3	1.15	5.16	5.11	0.58	11.2
1	*PTEN*	8	968del	N323MfsTer21	Del	T-3	1.54	6.95	6.91	0.38	5.5
1	*TP53*	7	714dup	N239Ter	Ins	T-3	1.39	6.26	6.25	0.54	8.7
2	*FBXW7*	9	1417dup	R473KfsTer4	Ins	T-3	1.52	6.84	6.78	0.68	9.9
2	*NRAS*	3	182A>G	Q61R	SNV	T-3	1.08	4.88	4.83	0.60	12.3
2	*TP53*	5	455del	P152RfsTer18	Del	T-3	2.23	10.02	10.19	1.42	14.1
3	*EGFR*	20	2300_2308 dup	A767_V769dup	Ins	T-2	1.57	2.98	3.03	0.69	23.0
4**	*ERBB2*	20	.2321_232 6dup	A775_G776ins VA	Ins	T-3	*0.94*	4.23	4.24	0.52	12.4
5	*BRAF*	15	1798_1799 delinsAG	V600R	MNV	T-2	1.76	7.93	7.93	0.50	6.3
6	*FGFR3*	9	1118A>G	Y373C	SNV	T-2	1.08	4.86	4.87	0.82	16.8
7	*FGFR3*	9	1118A>G	Y373C	SNV	T-2	1.44	6.49	6.50	0.88	13.5
8	*EGFR*	20	2303_2311 dup	S768_D770dup	Ins	T-3	2.80	5.32	5.39	0.53	10.
8	*FGFR2*	12	1647T>G	N549K	SNV	T-3	1.17	5.27	5.26	0.58	11.

Sample	Gene	Exon	Nucleotide Change	Amino Acid Change	Variant type	Variant level***	Ratio Mean	Mean VAF	Median	SD	CV %
8	*PIK3CA*	10	1637A>G	N546R	SNV	T-3	1.02	4.60	4.61	0.32	6.9
8	*PTEN*	5	313del	C105VfsTer8	Del	T-3	1.03	4.64	4.58	0.41	8.8
8	*PTEN*	8	968del	N323MfsTer21	Del	T-3	1.08	4.85	4.87	0.33	6.8
9	*EGFR*	20	2303_2311 dup	S768_D770dup	Ins	T-3	1.83	3.47	3.58	0.49	14.2
9*	*PIK3CA*	10	1637A>G	Q546R	SNV	T-3	*0.76*	3.19	3.17	0.27	8.0
9*	*PTEN*	5	313del	C105VfsTer8	Del	T-3	*0.72*	3.24	3.18	0.29	9.0
9*	*PTEN*	8	968del	N323MfsTer21	Del	T-3	*0.81*	3.63	3.60	0.36	10.0
9*	*FGFR2*	12	1647T>G	N549Lys	SNV	T-3	*0.82*	3.64	3.60	0.31	8.5
10	*KRAS*	2	34G>T	G12C	SNV	T-1	1.96	3.72	3.68	0.42	11.3
10	*KRAS*	2	35G>A	G12D	SNV	T-1	6.57	17.07	16.91	1.01	5.9
10	*ERBB2*	20	2321_2326 dup	A775_G776ins VA	Ins	T-3	7.46	33.59	34.48	3.27	9.7
10	*SMAD4*	6	778dup	Y260LfsTer4	Ins	T-3	2.54	11.42	11.38	0.74	6.5
10	*TP53*	5	.378dup	S127LfsTer22	Ins	T-3	1.34	6.03	5.99	0.72	11.9

* Mean observed VAF falls in 0.7 – 0.8x LoD were analyzed with inclusion and exclusion.

** Mean observed VAF is > 0.9x LoD and is included for agreement analysis.

*** Variant Level refer to tumor profiling levels 1 through 3

Ins=Insertion, Del=Deletion

C= colorectal, P= Pancreas, L= non-small cell lung cancer, M= Melanoma, BL= Bladder, U= Uterine/Endometrial

Site to Site Reproducibility:

Site to site reproducibility was assessed via positive and negative call rate for each test site (Table 36). Analysis was performed with and without variants with allele frequencies 0.7-0.9x below the LoD of the device. The overall positive agreement across all sites was 96.0% (1044/1088; 94.6-97.0% CI) when assessed using all 31 variants detected in the sample panel and 100% (944/944; 99.6-100.0% CI) when assessed excluding four variants below the LoD (0.7 – 0.9x LoD) of the device. Negative call rate

agreement was 100% when assessed both with (1263916/1263936; 99.998-100.0% CI) and without (1263916/1263936; 99.998-100.0% CI) variants below LoD.

Table 39 Multi-site agreement by site.

SITE	# samples	# libraries	# variants	Positive Call Rate (n/N) (2-sided 95% CI)	Negative Cal Rate (n/N) (2-sided 95% CI)
ALL	10	348	31	96.0% (1044/1088) (94.6%, 97.0%)	100.0% (1263916/1263936) (99.998%, 100.0%)
Site 1	10	120	31	96.0% (357/372) (93.5%, 97.5%)	100.000% (435840/435840) (99.999%, 100.0%)
Site 2	10	108	31	95.6% (329/344) (92.9%, 97.3%)	99.995% (392236/392256) (99.992%, 100.0%)
Site 3	10	120	31	96.2% (358/372) (93.8%, 97.7%)	100.0% (435840/435840) (100.0%, 100.0%)
Excluded 4 variants with allele frequencies between 0.7-0.9x LoD					
ALL	10	348	27	100.0% (944/944) (99.6%, 100.0%)	100.0% (1263916/1263936) (99.998%, 100.0%)
Site 1	10	120	27	100.0% (324/324) (98.8%, 100.0%)	100.000% (435840/435840) (99.999%, 100.0%)
Site 2	10	108	27	100.0% (296/296) (98.7%, 100.0%)	99.995% (392236/392256) (99.992%, 100.0%)
Site 3	10	120	27	100.0% (324/324) (98.8%, 100.0%)	100.0% (435840/435840) (100.0%, 100.0%)

Agreements Per Variant Type:

The positive call rates for SNV, MNV, insertions, and deletions, stratified by variant allele frequency (VAF) relative to the LoD of the device, are summarized in Table 40. Overall positive call rate for variants above LoD (1x - >5x) was 100% for all variant types, including variants 1x-2x above the LoD (592/592; 99.4-100.0% CI). Variants detected by the oncoReveal™ CDx below the LoD of the device had an overall positive call rate of 79.2 (168/212; 73.3-84.2% CI). Number of variants and VAF range for each stratum is reported.

Table 40 Multi-site agreement analysis by variant type.

Mutation type	Mean VAF range	# variants	Positive Call_Rate (n/N) (2-sided 95% CI)	Mean VAF range
All	0.7 – 1x LoD	6	79.2% (168/212) (73.3%, 84.2%)	3.2 – 4.2
	1 – 2x LoD	17	100.0% (592/592) (99.4%, 100.0%)	3.0 – 8.9
	2 – 5x LoD	5	100.0% (176/176) (97.9%, 100.0%)	5.3 – 12.7
	>5x LoD	3	100.0% (108/108) (96.6%, 100.0%)	12.8 – 33.6
SNV	0.7 – 1x LoD	2	70.8% (51/72) (59.5%, 80.1%)	3.2 – 3.6
	1 – 2x LoD	7	100.0% (240/240) (98.4%, 100.0%)	3.7 – 8.9
	2 – 5x LoD	1	100.0% (36/36) (90.4%, 100.0%)	12.7 – 12.7
	>5x LoD	2	100.0% (72/72) (94.9%, 100.0%)	12.8 – 17.1
Insertion	~1x LoD	2	100.0% (68/68) (94.7%, 100.0%)	4.2 – 4.2
	1 – 2x LoD	5	100.0% (180/180) (97.9%, 100.0%)	3.0 – 6.8
	2 – 5x LoD	2	100.0% (68/68) (94.7%, 100.0%)	5.3 – 11.4
	>5x LoD	1	100.0% (36/36) (90.4%, 100.0%)	33.6 – 33.6
Deletion	0.7 – 1x LoD	2	68.1% (49/72) (56.6%, 77.7%)	3.2 – 3.6
	1 – 2x LoD	4	100.0% (136/136) (97.3%, 100.0%)	4.6 – 6.9
	2 – 5x LoD	2	100.0% (72/72) (94.9%, 100.0%)	9.5 – 10.0
MNV	1 – 2x LoD	1	100.0% (36/36) (90.4%, 100.0%)	7.9 – 7.9

Analysis of Source of Variance

Variance due to site, operator, or day of run was assessed using Average Positive Agreement (APA) and Average Negative Agreement (ANA). Results are summarized in Table 41.

Table 41 Inter-site, inter-operator, and inter-day/run analysis of the multi-site reproducibility study.

Pair Name	INTER-SITE		INTER-OPERATOR		INTER-DAY/RUN	
	APA (2-sided 95%CI)	ANA (2-sided 95%CI)	APA (2-sided 95%CI)	ANA (2-sided 95%CI)	APA (2-sided 95%CI)	ANA (2-sided 95%CI)
ALL	99.0% (98.5%, 99.4%)	100.0% (100.0%, 100.0%)	99.5% (99.1%, 99.7%)	100.0% (100.0%, 100.0%)	98.3% (98.0%, 98.6%)	100.0% (100.0%, 100.0%)
SNV	100.0% (98.5%, 100.0%)	100.0% (100.0%, 100.0%)	99.1% (98.3%, 99.5%)	100.0% (100.0%, 100.0%)	97.8% (97.2%, 98.2%)	100.0% (100.0%, 100.0%)
Insertion	100.0% (98.3%, 100.0%)	100.0% (100.0%, 100.0%)	100.0% (99.6%, 100.0%)	100.0% (100.0%, 100.0%)	100.0% (99.9%, 100.0%)	100.0% (100.0%, 100.0%)
Deletion	100.0% (97.7%, 100.0%)	100.0% (100.0%, 100.0%)	99.3% (98.3%, 99.7%)	100.0% (100.0%, 100.0%)	96.7% (95.8%, 97.4%)	100.0% (100.0%, 100.0%)
MNV	100.0% (86.2%, 100.0%)	100.0% (100.0%, 100.0%)	100.0% (96.2%, 100.0%)	100.0% (100.0%, 100.0%)	100.0% (98.7%, 100.0%)	100.0% (100.0%, 100.0%)

Agreement Per Sample

Positive and negative call rates were assessed for each of the 10 samples within the sample panel used in the multi-site reproducibility study. Samples contained between 1-8 variants per sample. One sample contained five total variants, four of which were below the LoD of the device. Positive call rate and negative call rate were assessed with and without including the four variants below LoD. Results are summarized in Table 42.

Table 42 Positive and Negative Call Rates per Sample used in Multi-Site Reproducibility

Sample	# libraries	# variants	Positive Call Rate (n/N) (2-sided 95% CI)	Negative Call Rate (n/N) (2-sided 95% CI)
1	36	8	100.0% (288/288) (98.7%, 100.0%)	100.0% (131580/131580) (99.997%, 100.0%)
2	36	3	100.0% (108/108) (96.6%, 100.0%)	99.998% (131758/131760) (99.994%, 100.0%)

Sample	# libraries	# variants	Positive Call Rate (n/N) (2-sided 95% CI)	Negative Call Rate (n/N) (2-sided 95% CI)
3	36	1	100.0% (36/36) (90.4%, 100.0%)	99.999% (131831/131832) (99.996%, 100.0%)
4	32	1	100.0% (32/32) (89.3%, 100.0%)	100.0% (117184/117184) (100.0%, 100.0%)
5	36	1	100.0% (36/36) (90.4%, 100.0%)	99.998% (131830/131832) (99.994%, 100.0%)
6	36	1	100.0% (36/36) (90.4%, 100.0%)	99.998% (131829/131832) (99.993%, 100.0%)
7	32	1	100.0% (32/32) (89.3%, 100.0%)	99.995% (117178/117184) (99.989%, 100.0%)
8	32	5	100.0% (160/160) (97.7%, 100.0%)	99.998% (117054/117056) (99.994%, 100.0%)
9	36	5	75.6% (136/180) (68.8%, 81.3%)	99.998% (131685/131688) (99.993%, 100.0%)
	36	1[1]	100.0% (36/36) (90.4%, 100.0%)	99.998% (131685/131688) (99.993%, 100.0%)
10	36	5	100.0% (180/180) (97.9%, 100.0%)	99.999% (131687/131688) (99.996%, 100.0%)

[1] 4 variants at 0.7 – 0.9x LoD excluded.

A summary of the panel-wide precision results was presented in Table 43 below. The precision analysis was performed for the 31 variants (as listed in Table 38). A total of 13 SNVs, 8 deletions and 10 insertions were evaluated. The results showed that all mutations have 100% concordance in all replicates except for 4 mutations. These 4 mutations were believed to be discordant because they were below the LoD (0.7 – 0.9x LoD) of the device.

The coefficient of variation (%CV) for the mutation allele frequency was also calculated for all 36 replicates. 20 out 31 samples had %CV ≤10%, 10/31 had between 10 and 14% and one sample had 23%. All runs passed the quality metrics criteria.

Table 43 Panel-Wide Precision Summary Results for All Replicates Tested by the 3-sites

Gene Exon	Mutation (cDNA/Protein Changes)	NC* range	VAF range	VAF mean	VAF median	VAF (SD)	VAF (%CV)	Positive/Total Calls	Positive Call Rate (two-sided 95% CI)
ERBB2 exon20	2321_2326dup A775_G776insVA	1.03 - 1.37	24.16 - 36.8	33.59	34.48	3.27	10%	36/36	100.0% (90.4%, 100.0%)
BRAF exon15	1799T>A V600E	1.22 - 1.58	12.01 - 14.41	12.84	12.88	0.53	4%	36/36	100.0% (90.4%, 100.0%)

Gene Exon	Mutation (cDNA/Protein Changes)	NC* range	VAF range	VAF mean	VAF median	VAF (SD)	VAF (%CV)	Positive/Total Calls	Positive Call Rate (two-sided 95% CI)
KRAS exon2	35G>A G12D	1.03 - 1.19	15.2 - 18.97	17.07	16.91	1.01	6%	36/36	100.0% (90.4%, 100.0%)
PIK3CA exon10	1634A>G E545G	1.5 - 1.8	11.85 - 13.82	12.71	12.72	0.49	4%	36/36	100.0% (90.4%, 100.0%)
EGFR exon20	2303_2311dup S768_D770dup	0.34 - 0.46	4.03 - 6.33	5.32	5.39	0.53	10%	32/32	100.0% (89.3%, 100.0%)
SMAD4 exon6	778dup Y260Lfs*4	1.06 - 1.34	10.01 - 13.11	11.42	11.38	0.74	6%	36/36	100.0% (90.4%, 100.0%)
TP53 exon5	455del P152Rfs*18	0.4 - 0.61	7.39 - 12.55	10.02	10.19	1.42	14%	36/36	100.0% (90.4%, 100.0%)
PTEN exon1	17_18del K6Rfs*4	0.94 - 1.19	8.18 - 10.86	9.50	9.44	0.63	7%	36/36	100.0% (90.4%, 100.0%)
FBXW7 exon10	1436G>A R479Q	1.19 - 1.59	7.88 - 10.44	8.85	8.76	0.67	8%	36/36	100.0% (90.4%, 100.0%)
KRAS exon2	34G>T G12C	1.03 - 1.19	2.54 - 4.5	3.72	3.68	0.42	11%	36/36	100.0% (90.4%, 100.0%)
EGFR exon20	2303_2311dup S768_D770dup	0.36 - 0.45	2.5 - 4.45	3.47	3.58	0.49	14%	36/36	100.0% (90.4%, 100.0%)
BRAF exon15	1798_1799delinsAG V600R	1.39 - 1.8	6.88 - 8.88	7.93	7.93	0.50	6%	36/36	100.0% (90.4%, 100.0%)
EGFR exon20	2300_2308dup A767_V769dup	0.51 - 0.66	1.72 - 4.45	2.98	3.03	0.69	23%	36/36	100.0% (90.4%, 100.0%)
PTEN exon8	968del N323Mfs*21	0.84 - 1.25	6.37 - 7.73	6.95	6.91	0.38	5%	36/36	100.0% (90.4%, 100.0%)
FBXW7 exon9	1417dup R473Kfs*4	0.89 - 1.02	5.17 - 8.11	6.84	6.78	0.68	10%	36/36	100.0% (90.4%, 100.0%)
FGFR3 exon9	1118A>G Y373C	0.19 - 0.28	5.03 - 8.72	6.49	6.50	0.88	13%	32/32	100.0% (89.3%, 100.0%)

Gene Exon	Mutation (cDNA/Protein Changes)	NC* range	VAF range	VAF mean	VAF median	VAF (SD)	VAF (%CV)	Positive/Total Calls	Positive Call Rate (two-sided 95% CI)
TP53 exon7	714dup N239*	0.67 - 0.83	5.06 - 7.48	6.26	6.25	0.54	9%	36/36	100.0% (90.4%, 100.0%)
TP53 exon5	378dup S127Lfs*22	0.53 - 0.79	4.66 - 7.65	6.03	5.99	0.72	12%	36/36	100.0% (90.4%, 100.0%)
FGFR2 exon12	1647T>G N549K	0.61 - 0.78	3.96 - 6.56	5.27	5.26	0.58	11%	32/32	100.0% (89.3%, 100.0%)
PTEN exon7	800del K267Rfs*9	1.01 - 1.3	4.21 - 6.86	5.16	5.11	0.58	11%	36/36	100.0% (90.4%, 100.0%)
NRAS exon3	182A>G Q61R	1.05 - 1.58	3.6 - 6.18	4.88	4.83	0.60	12%	36/36	100.0% (90.4%, 100.0%)
FGFR3 exon9	1118A>G Y373C	0.13 - 0.19	3.55 - 7.16	4.82	4.82	0.82	17%	36/36	100.0% (90.4%, 100.0%)
PTEN exon8	968del N323Mfs*21	0.83 - 1.25	4.21 - 5.41	4.85	4.87	0.33	7%	32/32	100.0% (89.3%, 100.0%)
PTEN exon5	313del C105Vfs*8	1.21 - 1.45	3.79 - 5.77	4.64	4.58	0.41	9%	32/32	100.0% (89.3%, 100.0%)
PIK3CA exon10	1637A>G Q546R	1.5 - 1.73	4.09 - 5.19	4.60	4.61	0.32	7%	32/32	100.0% (89.3%, 100.0%)
ERBB2 exon20	2321_2326dup A775_G776insVA	0.55 - 0.72	3.38 - 5.26	4.23	4.24	0.52	12%	32/32	100.0% (89.3%, 100.0%)
PTEN exon7	710dup F238Vfs*5	1.01 - 1.3	3.64 - 5.34	4.18	4.12	0.35	8%	36/36	100.0% (90.4%, 100.0%)
FGFR2 exon12	1647T>G N549K	0.6 - 0.87	3.2 - 4.31	3.70	3.63	0.26	7%	33/36	91.7% (78.2%, 97.1%)
PTEN exon8	968del N323Mfs*21	0.82 - 1.18	3.2 - 4.49	3.70	3.69	0.33	9%	32/36	88.9% (74.7%, 95.6%)
PIK3CA exon10	1637A>G Q546R	1.44 - 1.73	3.21 - 3.87	3.41	3.37	0.17	5%	18/36	50.0% (34.5%, 65.5%)

Gene Exon	Mutation (cDNA/Protein Changes)	NC* range	VAF range	VAF mean	VAF median	VAF (SD)	VAF (%CV)	Positive/Total Calls	Positive Call Rate (two-sided 95% CI)
PTEN exon5	313del C105Vfs*8	1.18 - 1.52	3.22 - 4.02	3.48	3.46	0.21	6%	17/36	47.2% (32.0%, 63.0%)

* NC= Normalized coverage

LOT-TO-LOT PRECISION – STUDY 4 (NON-CDX TUMOR PROFILING VARIANTS)

Performance of oncoReveal™ CDx was assessed across 3 reagent lots used to test 14 clinical samples at 10 replicates each for a total of 140 libraries. The testing was performed by different operators using different thermocyclers and assayed over five sequencing runs. Lot-to-lot precision as measured by APA across all variants is >98% (Table 45). The samples used to determine lot-to-lot reproducibility are summarized in Table 44 and results from the pairwise APA and ANA analysis between the three lots used in testing are detailed in Table 45.

Table 44 The Cohort of 14 Samples Used in the Lot-to-Lot Reproducibility Study

Sample	Tumor Type	No. of observed variants		
		SNV	Insertion	Deletion
1	Colorectal cancer	1	1	1
2	Colorectal cancer	1	1	0
3	Colorectal cancer	0	0	1
4	Colorectal cancer	4	0	1
5	Colorectal cancer	0	1	0
6	Non-small cell lung cancer	0	1	0
7	Colorectal cancer	2	0	2
8	Colorectal cancer	1	1	0
9	Bladder cancer	1	0	0
10	Kidney cancer	1	0	1
11	Thyroid cancer	1	0	0
12	Uterine/ovarian cancer	1	1	0
13	Uterine/ovarian cancer	1	0	1
14	Pancreatic cancer	2	1	1
		16	7	8

Table 45 Overall lot-to-lot precision and by variant type

Variant Type	Analysis	Between Lot A & B	Between Lot A & C	Between Lot B & C
ALL	APA	98.3% (95.1%, 99.4%)	98.3% (95.1%, 99.4%)	98.9% (95.9%, 99.7%)
	ANA	100.0% (99.997%, 100.0%)	100.0% (99.997%, 100.0%)	100.0% (99.998%, 100.0%)
SNV	APA	96.6% (90.3%, 98.8%)	96.5% (90.1%, 98.8%)	97.6% (91.7%, 99.3%)
	ANA	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)
Insertion	APA	100.0% (91.6%, 100.0%)	100.0% (91.6%, 100.0%)	100.0% (91.6%, 100.0%)
	ANA	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)
Deletion	APA	100.0% (92.6%, 100.0%)	100.0% (92.6%, 100.0%)	100.0% (92.6%, 100.0%)
	ANA	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)	100.0% (99.99%, 100.0%)

Positive and negative call rates were calculated for each of the 14 samples used in lot-to-lot reproducibility testing. Results are summarized in Table 46.

Table 46 Per Sample Analysis of lot-to-lot Reproducibility

Sample	# libraries	Total Unique Variants	Positive Call_Rate (n/N) (2-sided 95% CI)	Negative Call_Rate (n/N) (2-sided 95% CI)
1	10	3	100.0% (30/30) (88.6%, 100.0%)	100.0% (36600/36600) (100.0%, 100.0%)
2	10	2	100.0% (20/20) (83.9%, 100.0%)	100.0% (36610/36610) (100.0%, 100.0%)
3	10	1	100.0% (10/10) (72.2%, 100.0%)	100.0% (36620/36620) (100.0%, 100.0%)
4	10	5	98.0% (49/50) (89.5%, 99.6%)	100.0% (36580/36580) (100.0%, 100.0%)
5	10	1	100.0% (10/10) (72.2%, 100.0%)	100.0% (36620/36620) (100.0%, 100.0%)
6	10	1	100.0% (10/10) (72.2%, 100.0%)	100.0% (36620/36620) (100.0%, 100.0%)
7	10	4	100.0% (40/40) (91.2%, 100.0%)	100.0% (36590/36590) (100.0%, 100.0%)

Sample	# libraries	Total Unique Variants	Positive Call_Rate (n/N) (2-sided 95% CI)	Negative Call_Rate (n/N) (2-sided 95% CI)
8	10	2	100.0% (20/20) (83.9%, 100.0%)	100.0% (36610/36610) (100.0%, 100.0%)
9	10	1	90.0% (9/10) (59.6%, 98.2%)	100.0% (36620/36620) (100.0%, 100.0%)
10	10	2	100.0% (20/20) (83.9%, 100.0%)	100.0% (36610/36610) (100.0%, 100.0%)
11	10	1	100.0% (10/10) (72.2%, 100.0%)	100.0% (36620/36620) (100.0%, 100.0%)
12	10	2	55.0% (11/20) (34.2%, 74.2%)	100.0% (36610/36610) (100.0%, 100.0%)
13	10	2	100.0% (20/20) (83.9%, 100.0%)	100.0% (36610/36610) (100.0%, 100.0%)
14	9	4	80.6% (29/36) (65.0%, 90.2%)	100.0% (32931/32931) (100.0%, 100.0%)

EXTRACTION METHOD EQUIVALENCE

A study evaluating performance of three commercially available FFPE tissue extraction kits was conducted because extraction kits are not included in the oncoReveal™ CDx kit. Four FFPE CRC (including one FFPE CRC negative for CDx variants), one normal colon tissue, four FFPE NSCLC (including one FFPE negative for CDx variants) and one normal lung tissue sample were used in the study. The six tumor specimens that were selected to be CDx variant positive and included reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement. Genomic DNA was extracted using 3 commercially available FFPE extraction kits. Each extracted DNA sample was run in duplicate using the oncoReveal™ CDx. The PPA was 100% (95% CI: 75.8%,100%) (12/12) and NPA was 100% (95% CI: 99.8%,100%) (2508/2508) at the variant level for each of the two commercially available FFPE DNA extraction kits compared to the validated reference kit. The results demonstrate that the 3 methods yield DNA with comparable quality and quantity to generate reliable results when used with oncoReveal™ CDx.

A supplementary study was performed to evaluate eight additional tumor types (excludes CRC and NSCLC) using three commercially available FFPE tissue extraction kit. They include melanoma, bladder, breast, endometrial, liver, melanoma, pancreatic, cancer, and thyroid cancers. Sixteen FFPE samples representing eight tumor types were used for DNA extractions. 100% of samples (16/16) extracted using ReliaPrep passed minimum DNA concentration threshold of 4.6 ng/uL while 93.75% of samples (15/16) extracted using FormaPure produced a minimum of 4.6 ng/uL. These results are equivalent or better than QIAamp (93.75%; 15/16). The 16 samples per method have 23 positive variants between them for a total of up to 46 variants in the libraries prepared in duplicate. The PPA for

DNA extracted with ReliaPrep was 100.0% (46/46) and with FormaPure was 100.0% (44/44) compared to the reference QIAamp of 100.0% (44/44). Together, the data presented shows equivalence between all three extraction methods tested.

GUARDBANDING

The tolerances encompassing the library preparation and sequencing workflow steps were assessed, which correspond to the test's most critical steps that could lead to assay failure. Each workflow steps tested included 3 test conditions: low; nominal as defined by the assay instructions for use; and high. The guard-banding range for each study was designed such that the maximum and minimum test points challenged the system, while still being within operational error range.

Ten FFPE-extracted DNA samples were prepared and analyzed over 4 sequencing runs to assess library preparation workflow steps such as PCR input and thermal cycling temperature offset. The seven tumor specimens were selected to be CDx variant positive and included reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement. The six CDx variant positive tumor specimens were tested for each assay specification tested. One reference standard DNA (HD799: Quantitative Multiplex Formalin Compromised (Moderate) formalin compromised DNA) containing reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement was prepared and analyzed over 5 sequencing runs to assess library sequencing workflow steps such as library concentration and number of libraries per run. The conditions of testing of the assay's most critical steps are shown in Table 47. All studies resulted in zero failures and 100% agreement across conditions as shown in Table 47.

Table 47 Guardbanding variables and agreement results

Process	Variable	Nominal value/ range	Test values	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
GS-PCR	DNA input/test	30 - 80 ng	5	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
			160	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
I-PCR	Purified GS-PCR product input volume/test	6 µL	3	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
			9	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
GS- & I-PCR	Cycling temperatures	Standard profile in	Standard - 1°C	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)

Process	Variable	Nominal value/ range	Test values	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
		User Manual	Standard + 1°C	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
Library pooling	Number of libraries† per sequencing run	12 - 48 libraries/ run	6	4	0	0	500	100% (51.0%, 100%)	100% (99.2%, 100%)
			12	10	0	0	1250	100% (72.2%, 100%)	100% (99.7%, 100%)
			54	52	0	0	6500	100% (93.1%, 100%)	100% (99.9%, 100%)
Library normalization	Library input†† per sequencing run	3.5 - 4.5 nM	1, 2, 3,4, 5 and 6 nM	46	0	0	5750	100% (92.3%, 100%)	100% (99.9%, 100%)

† Sample libraries including PosCtrl and NTC

†† 1 library each tested at 1, 2, 3, 5 and 6nM; 41 libraries tested at 4 nM (reference)

ANALYTICAL SPECIFICITY

INTERFERENCE

To evaluate the potential impact of interfering substances on the performance of the oncoReveal™ CDx, four CRC and four NSCLC FFPE specimens containing reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement were evaluated in the presence of exogenous and endogenous substances. Each specimen was assessed with two replicates, for a total of 16 libraries with the addition of the following eight interfering substances tested at low and high concentrations: Paraffin in xylene (0.000002% and 0.000267%), Proteinase K (0.000004 and 0.000043 mg/mL), lysis buffers Buffer ATL (0.0002% and 0.0019%), and Buffer AL (0.0002% and 0.0021%), extraction wash buffers AW1 (0.06% and 0.33%), and AW2 (5.7% and 16.7%), ethanol (4.0% and 11.9%) and hemoglobin (1 mg/mL and 2 mg/mL). Testing was performed at 1-1.5x LoD for the CDx variants for exogenous interfering substances and near the minimum assay requirement of 30 ng DNA input. The concentrations for exogenous interferents are given relative to the eluted DNA sample, and for hemoglobin, relative to the lysis solution post-deparaffinization. No impact on the performance of the oncoReveal™ CDx was observed for each substance and at each level tested.

Table 48 Agreement analysis (CDx variants) of interfering substances in CRC and NSCLC

Study	Substance	Test value	Test+ Comp +	Test+ Comp -	Test- Comp +	Test- Comp -	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
Endogenous	Hemoglobin	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
Exogenous	Buffer AL	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Buffer ATL	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Buffer AW1	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Buffer AW2	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Ethanol	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Xylene	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
	Proteinase K	Min	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)
		Max	8	0	0	1000	100% (67.6%, 100%)	100% (99.6%, 100%)

Potential impact of interfering substances on the performance of the oncoReveal™ CDx for the tumor profiling indication were evaluated in three tumor types (from melanoma, breast cancer and thyroid cancer). One hundred eighty-one (181) libraries were analyzed with seven exogenous substances and

142 libraries were analyzed with three endogenous substances (refer to Table 49 for the potential interfering substances evaluated and levels tested).

Table 49 Potential Interfering substances tested

Exogenous/ Endogenous	Int Substance	Min/Max	Value
Exogenous	Xylene	Min	0.000002% (v/v)
Exogenous	Xylene	Max	0.000267% (v/v)
Exogenous	Qiagen Proteinase K	Min	0.000004 mg/mL
Exogenous	Qiagen Proteinase K	Max	0.000043 mg/mL
Exogenous	Buffer ATL	Min	0.0002% (v/v)
Exogenous	Buffer ATL	Max	0.0019% (v/v)
Exogenous	Buffer AL	Min	0.0002% (v/v)
Exogenous	Buffer AL	Max	0.0021% (v/v)
Exogenous	Qiagen AW1	Min	0.06% (v/v)
Exogenous	Qiagen AW1	Max	0.33% (v/v)
Exogenous	Qiagen AW2	Min	5.7% (v/v)
Exogenous	Qiagen AW2	Max	16.7% (v/v)
Exogenous	Ethanol	Min	4.0% (v/v)
Exogenous	Ethanol	Max	11.9% (v/v
Exogenous	Control	Control	N/A
Endogenous	Hemoglobin	Min	2% (g/g)
Endogenous	Hemoglobin	Max	4% (g/g)
Endogenous	Melanin	Min	0.009% (g/g)
Endogenous	Melanin	Max	4% (g/g)
Endogenous	Triglycerides	Min	2% (g/g)
Endogenous	Triglycerides	Max	12% (g/g)
Endogenous	Control	Control	N/A

Ten (10) clinical samples representing 11 variants (4 CDx, 7 tumor profiling variants) were evaluated in the study. Agreement analysis of valid samples against no substance controls showed PPA and NPA at 100% for all substances and levels tested. No impact on the performance of the oncoReveal™ CDx assay was observed for each substance and at each level tested.

NECROTIC FRACTION

Retrospective analyses of impact of necrotic tissue content in FFPE samples from clinical validation and analytical accuracy studies are shown below. Samples with <20% necrotic tissue content in analytical accuracy study excluded from retrospective analysis. 274 CRC and 276 NSCLC FFPE specimens with varying quantities of necrosis (1% to 70%) were assessed. All samples >20% necrotic content passed library yield QC, PiVAT® results were valid and concordant with comparator assays. Five discordant results were observed in CRC that were not correlated with high necrotic content. Three discordant calls were observed in NSCLC that were not correlated with high necrotic content. For details on the discordant results, see the footnotes in Table 50. No clear trend in decreasing performance with increasing necrotic fraction in the sample was observed supporting the conclusion that the performance of the oncoReveal™ CDx is robust within the recommended range of necrotic content less than 50%.

Table 50 Summary of assay performance by necrotic content bin

Indication[6]	%Necrotic Bin	# Enrolled/Tested	# lib yield QC fail	# lib yield QC pass	# PiVAT® invalid	# PiVAT® valid	# Included in analysis	# of CDx-	# of CDx+	# CDx- Concordant result [1]	# CDx+ Concordant result [1]
CRC	<=10	116	6	110	0	110	96	62	34	61[2]	32[3,4a]
	11 – 20	11	1	10	0	10	9	6	3	6	3
	21 – 30	4	0	4	0	4	4	1	3	1	3
	31 – 40	1	0	1	0	1	1	1	0	1	0
	41 – 50	1	0	1	0	1	1	1	0	1	0
	>50	1	0	1	0	1	1	0	1	0	1
	Not Available	140	9	131	0	131	114	58	56	58	54[3,4b]
	CRC total	**274**	**16**	**258**	**0**	**258**	**226**	**129**	**97**	**128**	**93**
NSCLC	<=10	187	8	179	0	179	176	120	56	120	56
	11 – 20	15	0	15	0	15	15	12	3	12	3
	21 – 30	7	0	7	0	7	7	4	3	4	3

Indication[6]	%Necrotic Bin	# Enrolled/Tested	# lib yield QC fail	# lib yield QC pass	# PiVAT® invalid	# PiVAT® valid	# Included in analysis	# of CDx-	# of CDx+	# CDx- Concordant result [1]	# CDx+ Concordant result [1]
	31 – 40	0	0	0	0	0	0	0	0	0	0
	41 – 50	1	0	1	0	1	1	1	0	1	0
	>50	1	0	1	0	1	1	0	1	0	1
	Not Available	65	6	59	0	59	59	37	22	34[5]	22
	NSCLC Total	**276**	**14**	**262**	**0**	**262**	**259**	**174**	**85**	**171**	**85**
	CRC + NSCLC	**550**	**550**		**520**		**485**	**485**		**477**	

[1] FDA-approved comparator companion diagnostic (CCD) assay used for concordance analysis of CRC samples in clinical validation studies: *therascreen*® KRAS assay. The CCD assay used for concordant analysis of NSCLC samples in clinical validation studies: **cobas**® EGFR Mutation Test v2. The concordance results above are provided based on the CCD1 and FCD results only. For details on the discordant results, see Table 57 and Table 59.

[2] The replicates of the comparator (CCD1/CCD2 = *KRAS* negative) were discordant with FCD (*KRAS* 13VAL; c.38_39delinsTT). It is inferred that *therascreen* KRAS is not designed to detect complex SNVs, this result may indicate an error by *therascreen* KRAS assay.

[3] The replicates of the comparator (CCD1/CCD2 = *KRAS* 12VAL) were discordant with FCD (*KRAS* 12PHE; c.34_35delinsTT). It is inferred that *therascreen* KRAS is not designed to detect complex SNVs, this result may indicate an error by *therascreen* KRAS assay.

[4] The replicates of the comparator were discordant.

[a] CCD1 = KRAS 12ALA; CCD2 = KRAS 12VAL; FCD = KRAS 12VAL

[b] CCD1 = KRAS 12ARG; CCD2 = KRAS 12CYS; FCD = KRAS 12CYS

[5] A total of three unique clinical specimens with *EGFR* L858R mutation showed discordant results. For all three samples, their CCD1/CCD2 results using cobas were both negative and their oncoReveal™ CDx results were positive with VAF range 1.9% to 4.9%. These results suggest the discordant cases are likely due to difference in detection sensitivity (Limit of Detection: cobas=5%) and the oncoReveal™ CDx results are likely correct.

[6] The impact of necrosis on the performance of oncoReveal™ was also evaluated for the tumor profiling indication by assessing the valid rate of the samples processed in the accuracy study. Of the 312 samples with necrotic tissue content (0 - 60%) available, 284 samples (with 9 insertion variants) passed oncoReveal™ and comparator QC metrics and were included in the concordance analysis. For samples with 0-10% necrosis, the concordance was 92%, For samples with 10-20% necrosis, the concordance was 83%. For this group, there were 4 discordant. One sample had low VAF levels near LoD and one had low quality by the comparator, not the oncoReveal™ assay. The reason for discordance of two out of the 4

samples are likely not due to necrosis but the reason is unknown. For samples with 20-30%, 30-40%, 40-50% and 50-60% necrosis, the concordances were 100%, respectively. Overall concordance was 93%.

CROSS-REACTIVITY

An *in-silico* cross-reactivity analysis was performed to evaluate the specificity of the primers used in the OR/Dx-LCCA. The primers were checked for specificity to the human genome (hg19) and the genomes of representative protozoal, viral, fungal, and bacterial human pathogens. A total of 177 human and 259 pathogen non-target sequences with some similarity to the human genome were identified using *in-silico* PCR and BLAT analysis. These sequences were converted to FASTQ format and processed through the PiVAT software. The test samples produced no on-target reads and no variant calls for any of the non-target sequences while producing the expected variant calls for positive controls included in the analysis. These results demonstrated that the primers are specific for the intended targeted sequences.

CROSS-CONTAMINATION

To assess intra-run cross-contamination, 24 replicates of a positive cell line sample containing *EGFR* L858R at ~50% VAF and 24 replicates of NTC were processed on the same plate in a checkerboard format. No false positive calls (0/24, 0%) were detected in all NTC samples. Therefore, no cross-contamination was observed.

To assess inter-run cross-contamination, a retrospective study utilizing sequencing runs generated as part of validation testing was analyzed. Indices that were used in Sequencing Run 1 and theoretically absent from Sequencing Run 2 (unexpected indices) were identified and enumerated in the output of Sequencing Run 2. Reads from index combinations used in Sequencing Run 1 could arise from run-to-run carryover, or they could arise from within run events, such as PCR errors and index hopping. The fraction of reads associated with unexpected indices across all five Run 2 data sets analyzed was less than 1% (≤0.4%) of the minimum number of reads for any sample within that run, well below the level where the unexpected reads could generate false positive results.

STABILITY

REAGENT KIT SHELF-LIFE STABILITY

Three separately manufactured kit lots including all components of the oncoReveal™ CDx were stored according to the storage conditions specified in product labeling. The stability of the reagents was evaluated by testing at least three (3) reference standard DNA including reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement at specified time points from baseline.

- HD701 - Quantitative Multiplex gDNA Multiplex
- HD803 - Quantitative Multiplex Formalin Compromised (Severe) formalin compromised DNA
- HD799 - Quantitative Multiplex Formalin Compromised (Moderate) formalin compromised DNA

Each of the assay QC metrics were evaluated in addition to final calls. Calls and metrics were confirmed against the calls for the kit at the baseline time (i.e., month 0). The data currently available support at least 13 months of stability for oncoReveal™ CDx kit components for all 3 lots evaluated. The shelf-life stability will continue to be evaluated to extend the shelf-life stability claim.

Table 51 Agreement results for kit reagent shelf-life stability study

Kit Lot	Timepoint (months)	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
Lot 1	2	100% (67.6%, 100%)	100% (99.0%, 100%)
	4	100% (67.6%, 100%)	100% (99.0%, 100%)
	7	100% (67.6%, 100%)	100% (99.0%, 100%)
	9	100% (67.6%, 100%)	100% (99.0%, 100%)
	14	100% (80.6%, 100%)	100% (99.5%, 100%)
	16	100% (80.6%, 100%)	100% (99.5%, 100%)
Lot 2	4	100% (86.2%, 100%)	100% (99.5%, 100%)
	7	100% (86.2%, 100%)	100% (99.5%, 100%)
	10	100% (86.2%, 100%)	100% (99.5%, 100%)
	13	100% (86.2%, 100%)	100% (99.5%, 100%)
	17	100% (86.2%, 100%)	100% (99.5%, 100%)
Lot 3	3.5	100% (86.2%, 100%)	100% (99.5%, 100%)
	6.5	100% (86.2%, 100%)	100% (99.5%, 100%)
	10	100% (86.2%, 100%)	100% (99.5%, 100%)
	13	100% (86.2%, 100%)	100% (99.5%, 100%)
	16	100% (86.2%, 100%)	100% (99.5%, 100%)

The stability of the reagents was further evaluated in an additional study by testing three (3) clinical samples with target CDx variants adjusted to a VAF% in the range of 1-1.5x of the LoD levels based on second LoD study (Table 19) using DNA extracted from tissue-matched clinically normal FFPE tissue. The sample panel included one (1) FFPE NSCLC specimens with *EGFR* Exon 19 deletion variant, one (1) FFPE CRC specimen with *KRAS* G12 variant and one (1) FFPE CRC specimen with *KRAS* G13 variant. Three reagent kit lots aged 17, 18 and 24 months were used as representative assay reagent lots to test the samples in replicates of five with each of the 3 reagent lots for a total of 15 replicates per sample at the minimum DNA input of 30 ng.

Each of the assay QC metrics were evaluated in addition to final calls. The detection rate of each sample across all three lots tested was 100% (15/15) as shown in Table 52 below.

Table 52 Performance of each reagent kit lot across clinical samples

Gene Exon	Nucleotide Change	Mean VAF (%)	Fold LoD*	Total Calls	Lot	Detection rate (%)
KRAS Exon 2	c.35G>A	2.49	1.4	5	1	5/5 (100%)
				5	2	5/5 (100%)
				5	3	5/5 (100%)
KRAS Exon 2	c.38G>A	3.53	1.4	5	1	5/5 (100%)
				5	2	5/5 (100%)
				5	3	5/5 (100%)
EGFR Exon 19	c.2235_2249del	1.94	1.1	5	1	5/5 (100%)
				5	2	5/5 (100%)
				5	3	5/5 (100%)

The stability of the reagents was evaluated in an additional study by testing seven insertion variants adjusted to a VAF% in the range of 1-1.5x of the LoD and at low DNA input (30 ng) with three aged lots to supplement the reagent kit shelf-life stability. Three reagent kit lots aged 19 (lot A), 12 (lot B) and 6 (Lot C) months were used as representative assay reagent lots to test the samples for a total of 10 replicates per sample (Table 53).

Table 53 Performance of Each Reagent Kit lot Across Clinical Samples

Gene	Amino acid Change	Mean VAF (%)	Fold LoD*	Total Calls	Lot	Detection rate (%)
PTEN	p.Phe238ValfsTer5	5.95	1.5	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)
TP53	p.Asn239Ter	5.27	1.2	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)

ERBB2	p.Ala775_Gly776insValAla	5.46	1.2	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)
EGFR	p.Ala767_Val769dup	4.41	2.3	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)
SMAD4	p.Tyr260LeufsTer4	5.64	1.3	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)
EGFR	p.Ser768_Asp770dup	3.12	1.6	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)
FBXW7	p.Arg473LysfsTer4	5.41	1.2	4	A	4/4 (100%)
				3	B	3/3 (100%)
				3	C	3/3 (100%)

REAGENT KIT LOT INTERCHANGEABILITY

The interchangeability of oncoReveal™ CDx kit components was assessed using clinical samples and three independent manufactured lots of reagents. The gene specific PCR and first cleanup steps were performed using reagents from a given reagent kit lot while the subsequent indexing PCR and second cleanup steps were performed using a second reagent kit lot. A total of three unique combinations of GS-PCR + cleanup and Indexing PCR + cleanup using three independent reagent lots were used to demonstrate interchangeability between multiple lots.

The positive and negative call rates were measured by PPA and NPA analysis. The PPA across all three combinations was 98.9% (87/88) with a minimum hit rate of 95.8% (23/24) for a single combination. The NPA was 100.0% (11,000/11,000) across all combinations with a minimum negative hit rate of 100.0% (3,000/3,000) for a single combination. The results demonstrate that components of different lots of oncoReveal™ CDx can be used interchangeability and does not impact results of the assay.

REAGENT KIT TRANSPORT STABILITY

The reagent kit stability studies were performed as one large study that included data points for in-use freeze-thaw stability and transport stability testing under recognized summer and winter profiles for international shipments. The transport stability study was performed to demonstrate that the shipping configurations for all kit components provide adequate thermal and physical protection as packages are transported from the manufacturing site to customers. Three (3) separately manufactured kits and component reagent lots were exposed to simulated transport challenges intended to simulate the longest estimated international shipping times of 72 hours and 144 hours. The simulated transport conditions included both physical and temperature challenges, which include 2 packaging

configurations (one (1) kit per shipping box and four (4) kits per shipping box) and 4 temperature profiles (72-hour summer, 72-hour winter, 144-hour summer and 144-hour winter). The 144-hour profiles correspond to two runs of the 72-hour profiles. The 72-hour international profile is considered to be a worse case than the 48-hour domestic profile, so a domestic profile was not performed.

Temperature challenge was performed at 3, 4, and 8 months to simulate shipping of aged components. After each temperature challenge, kits produced QC metrics and variant calls equivalent to baseline (month 0) QC metrics and variant calls of the control kit in the lot. No individual kit boxes experienced temperatures higher than -15°C. No sign of deterioration or degradation was observed for all labels.

Physical challenge was performed only at 0 months, since the acceptance criteria was visual integrity rather than function (i.e., physical challenge is extremely unlikely to affect the functional integrity of the reagents, and so it was not tested). No packages having undergone simulated transport shipping displayed signs of physical damage which may impede the function of the assay or workstation and monitor.

Each of the kits and components undergoing temperature challenge was functionally tested using at least three (3) reference standard DNA containing reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement to establish transport stability. The data demonstrate that all kit components show acceptable transport stability at the simulated time points.

IN-USE STABILITY

The in-use stability study evaluated both open vial stability and freeze-thaw stability. For each of Lots 1, 2, and 3, representative kits were subjected to at least five (5) freeze-thaw cycles and four (4) uses. Additional testing was performed for Lot 3 to assess at least five (5) freeze-thaw cycles and two (2) uses. The reagents were evaluated by testing at least three (3) reference standard DNA containing reportable CDx SNV and deletion variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement. In-use stability was tested using Lots 1-3 at the baseline (i.e., month 0), 4 and 9 months in a combined study with transport stability (see above).

To perform reagent freeze-thaw cycle, the reagents were removed from freezer storage and placed in a 2°C to 8°C environment overnight (minimum of 12 hours) to simulate use, then returned to freezer storage for a minimum of 12 hours.

Each of the assay QC metrics were evaluated in addition to final calls. The data demonstrate in-use stability for at least 5 freeze-thaw cycles.

Table 54 Agreement results for reagent kit transport and in-use stability study

Kit Lot	Ship Configuration (# kits/shipping box)	Simulated Thermal Profile*	Freeze-thaw cycles**	PPA (2-sided 95% CI)***	NPA (2-sided 95% CI)***
1	4	Summer	3	100% (75.8%, 100%)	100% (99.0%, 100%)
			4	100% (75.8%, 100%)	100% (99.0%, 100%)
			5	100% (75.8%, 100%)	100% (99.0%, 100%)
			6	100% (75.8%, 100%)	100% (99.0%, 100%)
3	4	Summer	3	100% (75.8%, 100%)	100% (99.0%, 100%)
			4	100% (75.8%, 100%)	100% (99.0%, 100%)
			5	100% (75.8%, 100%)	100% (99.0%, 100%)
			6	92% (64.6%, 99%)	100% (99.0%, 100%)
3	1	Summer	5	100% (75.8%, 100%)	100% (99.0%, 100%)
			6	100% (75.8%, 100%)	100% (99.0%, 100%)
3	4	Winter	3	100% (75.8%, 100%)	100% (99.0%, 100%)
			4	100% (75.8%, 100%)	100% (99.0%, 100%)
			5	100% (75.8%, 100%)	100% (99.0%, 100%)
			6	100% (75.8%, 100%)	100% (99.0%, 100%)
2	1	Winter	3	100% (75.8%, 100%)	100% (99.0%, 100%)
			4	100% (75.8%, 100%)	100% (99.0%, 100%)
			5	100% (75.8%, 100%)	100% (99.0%, 100%)

Kit Lot	Ship Configuration (# kits/shipping box)	Simulated Thermal Profile*	Freeze-thaw cycles**	PPA (2-sided 95% CI)***	NPA (2-sided 95% CI)***
			6	100% (75.8%, 100%)	100% (99.0%, 100%)
3	1	Winter	5	92% (64.6%, 99%)	100% (99.0%, 100%)
			6	100% (75.8%, 100%)	100% (99.0%, 100%)

* Summer and winter international profiles per ISTA 7D

** Test kit lots that had undergone simulated thermal challenges were removed from storage and subjected to the indicated freeze-thaw cycles prior to testing.

*** Agreement between test kit lots (temperature challenged and/or freeze-thawed) and control kit lot.

FFPE (SECTION AND BLOCK) AND DNA SAMPLE STABILITY – STUDY 1

Stability of FFPE blocks at ambient temperature was assessed at baseline, 11-month, 13-month, and 14-month time points. FFPE blocks selected for NSCLC and CRC CDx variants were sectioned, extracted, and processed for sequencing within 3 days of each time point. No loss of sample integrity was observed at the 11-month (100% library passing rate) and 13-month time points (100% library passing rate), supporting an 11-month block stability claim.

Stability of FFPE sections (curls) was assessed at baseline, 30 days, and 60 days to support stability at 30 days. At each timepoint, eight FFPE specimens stored at ambient conditions were extracted using QIAGEN QIAamp FFPE extraction kit and processed for sequencing within 3 days. The samples contained *EGFR* (L858R and Ex19 del) and *KRAS* (G12D and G13D) variants. FFPE curls were stable, as measured by PPA/NPA analysis, at both the 30-day and 60-day time points, supporting a claim of a 30-day stability. PPA was 100% (95% CI: 80.6%,100%) (16/16) and NPA was 100% (95% CI: 99.8%,100%) (2000/2000) compared to baseline testing.

Stability of DNA extracted from FFPE clinical samples using QIAGEN QIAamp FFPE extraction kit was assessed after storage at 4°C or -20°C, and after 5 cycles of freeze-thaw. Stability at 4°C was assessed after 60 days, 8 months, and 8.25 months and stability at -20°C was assessed after 3 months, 9 months and 10 months. FFPE derived DNA was stable, as measured by PPA/NPA analysis, at all the time points tested following storage at 4°C. PPA was 100% (95% CI: 80.6%,100%) (16/16) and NPA was 100% (95% CI: 99.8%,100%) (2000/2000) for the timepoints 60 days and 8.25 months compared to baseline testing. For the timepoint 8 month, PPA was 100% (95% CI: 67.6%,100%) (8/8) and NPA was 100% (95% CI: 99.6%,100%) (1000/1000). FFPE derived DNA was stable, as measured by PPA/NPA analysis at all the time points tested following storage at -20°C. The resulting PPAs and NPAs were 100% for both the 9 month and 10 month timepoints, and the lower bounds of the 95% confidence intervals were 94.7% and 100.0% for the PPA and NPA, respectively.

Data presented here supports a claim of DNA storage stability at 8 months at 4°C and 9 months at -20°C. FFPE derived DNA that was subjected to 5 cycles of freeze-thaw was stable, as determined by PPA/NPA analysis using DNA that undergone 1 cycle of freeze-thaw as a control. PPA was 100% (95% CI: 80.6%,100%) (16/16) and NPA was 100% (95% CI: 99.8%,100%) (2000/2000). This data supports a DNA freeze-thaw stability claim of 5 cycles.

FFPE (SECTION AND BLOCK) AND DNA SAMPLE STABILITY – STUDY 2

A study was conducted to test FFPE block stability for CDx variants. 9 clinical specimens (6 FFPE NSCLC blocks, 3 FFPE blocks) representing 9 CDx variants and 12 non-CDx variants were used. Stability of FFPE blocks at ambient temperature was assessed at baseline, 11-month, 13-month, and 14-month time points. FFPE blocks selected were sectioned, extracted, and processed for sequencing within 3 days of each time point. No loss of sample integrity was observed at the 11-month (100% library passing rate) and 13-month time points (100% library passing rate), supporting an 11-month block stability claim.

A study was conducted to test FFPE-extracted DNA stability for CDx variants. To measure the stability of FFPE extracted DNA derived from CRC or NSCLC tissues stored at - 20°C, 11 CRC and NSCLC clinical samples with Table 1 reportable variants at 1-1.5X LoD were tested at four timepoints: baseline, 3 months, 9 months, and 10 months. A total of 172 libraries were prepared with 4 replicates per sample for each timepoint. The resulting PPAs and NPAs were 100% for both the 9- and 10-month timepoints, and the lower bounds of the 95% confidence intervals were 94.7% and 100.0% for the PPA and NPA, respectively. This data supports the claim of FFPE extracted DNA derived from CRC or NSCLC tissues stability for 9 months when stored at - 20°C.

FFPE (SECTION AND BLOCK) AND DNA SAMPLE STABILITY – STUDY 3

A study was designed to test the stability of FFPE blocks, FFPE curls, and extracted FFPE DNA corresponding to eight additional tumor types, including bladder, breast, endometrial, liver, pancreatic, renal, and thyroid cancers, and melanoma. FFPE blocks and FFPE curls were stored at room temperature while extracted DNA was stored at -20°C for the duration of testing. Additionally, extracted DNA was subject to multiple rounds of freeze/thaw cycles to simulate repeat usage of the sample prior to testing using oncoReveal™ CDx. Baseline measurements and two (2) subsequent time points were assessed for each stability claim and sample integrity was measured by PPA and NPA analysis by comparing variants called at each time point to baseline variant calls. Freeze/thaw stability was assessed by PPA and NPA analysis comparing variant calls after one (1) round of freeze/thaw to five (5) rounds of freeze/thaw. A total of 16 unique FFPE samples from eight tumor types were used in each stability study and were minimally tested in duplicate.

For DNA stability, the age of samples tested ranged from 16 to 17 weeks at the first time point (T1) and 21 to 38 weeks at the second time point (T2). The resulting PPA and NPA analyses showed 100% agreement at both T1 and T2 with lower bounds of the 95% CI for both were 92.3% and 100%, respectively, across all tissues tested.

PPA and NPA analysis of DNA samples subject to five (5) rounds of freeze/thaw cycles showed 100% agreement and lower bound of the 95% CI to be 92.3% and 100.0% respectively across all tissues tested. Together, this data supports a stability claim for FFPE DNA isolated from bladder, breast, endometrial, liver, pancreatic, renal, and thyroid cancers, and melanoma of 16 weeks and 5 freeze/thaw cycles.

For FFPE block and FFPE curl stability, samples tested ranged from 16 to 49 weeks at the first time point (T1) and 21 to 51 weeks at the second time point (T2) for both stability experiments. The resulting PPA and NPA analyses for block stability showed 100% agreement at both T1 and T2 and lower bounds of the 95% CI for were 92.0% and 100% respectively for T1 and 92.1% and 100% respectively for T2.

The resulting PPA and NPA analyses for curl stability showed 100% agreement at both T1 and T2 and lower bounds of the 95% CI for both were 92.1% and 100% respectively for T1 and 92.0% and 100% respectively for T2. Taken together, these data support a stability claim for FFPE blocks and FFPE curls derived from bladder, breast, endometrial, liver, pancreatic, renal, and thyroid cancers, and melanoma of 16 weeks.

STABILITY OF ASSAY INTERMEDIATES

The workflow for the oncoReveal™ CDx incorporates several optional stopping points to hold assay intermediates. The stability of the intermediate products was evaluated by incorporating two optional stopping points specified in the assay instructions for use. Ten FFPE-extracted DNA samples were included in this study which contained reportable CDx SNV and indel variant representatives of *EGFR* and *KRAS* genes indicated in Table 1 of the intended use/indications for use statement. Each sample was processed to completion for sequencing at baseline and the resulting intermediates stored. The intermediates were then removed from storage at different time points and processed to completion to assess the impact of storage on assay performance.

The study results support the conclusion that the 60-day hold of Gene-Specific PCR (GS-PCR) products and 90-day hold on indexed libraries at recommended storage condition did not result in a decrease in oncoReveal™ CDx performance.

Table 55 Agreement analysis of assay intermediates stability study

Assay intermediate	Timepoint (days)	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
GS-PCR	0	N/A	N/A	N/A	N/A	N/A	N/A
GS-PCR	35	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
GS-PCR	62	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
GS-PCR	90	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)

Assay intermediate	Timepoint (days)	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
I-PCR	35	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
I-PCR	90	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
I-PCR	233	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
Combo*	GS-PCR 35 + I-PCR 27	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)

* Combination intermediate stability was tested by performing indexing on GS-PCR products that were 35 days old. The resulting indexed libraries were stored for 27 days at -25 to -10°C before being sequenced.

Table 56 Stability of assay intermediates

Assay intermediate(s)	Storage condition	Intermediate stability
GS-PCR products	-25°C to -15°C	60 days
Indexed libraries	-25°C to -15°C	90 days

SUMMARY OF CLINICAL PERFORMANCE

The reasonable assurance of safety and effectiveness for the CDx claims of oncoReveal™ CDx were established through clinical concordance studies using a non-inferiority statistical testing approach. Two clinical concordance studies were conducted to support the CDx claims indicated in Table 1 of the intended use/indications for use statement for *EGFR* Exon 19del/L858R in NSCLC and *KRAS* wild-type (absence of mutation in codons 12 and 13) in CRC. A non-inferiority statistical testing approach was used according to Li (2016)[3]. oncoReveal™ CDx test, considered the follow-on companion diagnostic (FCD), was compared to an FDA-approved CDx test, considered the comparator companion diagnostic (CCD) test for each of the clinical concordance study, using samples representative from the intended use/indications for use population for that specific device.

ONCOREVEAL™ CDX CONCORDANCE STUDY FOR *EGFR* EXON 19 DELETION AND EXON 21 L858R IN NSCLC

The concordance of *EGFR* exon 19 in frame deletions and exon 21 L858R substitution mutations was determined between the oncoReveal™ CDx (FCD) and the approved Roche Molecular Systems' **cobas** v2 EGFR Mutation Test (CCD). As the *EGFR* mutations are relatively infrequent in the intended use/indications for use population, a stratified design was used with a target endpoint of 30 to 50% positives in the study population.

A total of 331 DNA samples extracted from NSCLC FFPE specimens were submitted for testing using two successful replicates of CCD. The first replicate of CCD (CCD1) was used to enroll samples into the study. After exclusion of ineligible or failed samples, 257 samples remained for the concordance analysis. Test outcomes from the 257 samples with valid CCD1, CCD2, and FCD results are tabulated below in Table 57.

Table 57 2x2x2 Matrix for Analysis of Concordance Outcomes for Non-Inferiority (NSCLC Tissue)

	Enrollment CCD+ (CCD1+)			Enrollment CCD- (CCD1-)		
	CCD2+	CCD2-	Total	CCD2+	CCD2-	Total
FCD+	85	0	85	0	3[1]	3
FCD -	0	0	0	0	169	169
Total	85	0	85	0	172	172

[1] A total of three unique clinical specimens with *EGFR* L858R mutation show discordant results. For all three samples, their CCD1/CCD2 results using cobas were both negative and their oncoReveal™ CDx results were positive with VAF range 1.9% to 4.9%. These results suggest the discordant cases are likely due to difference in detection sensitivity (Limit of Detection: cobas=5%) and the oncoReveal™ CDx results are likely correct.

The agreements for the non-inferiority test proposed by Li (2016)[3] using the data from the 2x2x2 contingency table above are shown in the table below. Note that, since a mutation enriched population drawn from the intended use/indications for use population was used for the study, a correction was performed to adjust the observed PPA and NPA based on the prevalence of the *EGFR* mutations in the intended use/indications for use population (see Li (2016)[3] for details).

All the upper bounds of the 95% confidence intervals were determined to be equal or less than 4%, supporting a conclusion that the agreement between the oncoReveal™ CDx and **cobas** EGFR Mutation Test v2 is non-inferior to the agreement between two replicates of CCD by a margin of 4%.

Table 58 Observed and adjusted PPA and NPA for EGFR in NSCLC

Parameter	Agreement (%)
PPA_{C1F}	100.0
PPA_{C1C2}	100.0
NPA_{C1F}	98.3
NPA_{C1C2}	100.0
PPA_{C2F}	100.0
PPA_{C2F} †	100.0
PPA_{C2C1}	100.0
PPA_{C2C1} †	100.0
NPA_{C2F}	98.3
NPA_{C2F} †	98.3
NPA_{C2C1}	100.0
NPA_{C2C1} †	100.0
See Section 4.2, p.361 in Meijuan Li (2016) Statistical Methods for Clinical Validation of Follow-On Companion Diagnostic Devices via an External Concordance Study, Statistics in Biopharmaceutical Research, 8:3, 355-363 for detailed methodology The parameter Pc, the “true” minor allele frequency (MAF) for the mutations of interest as analyzed by the oncoReveal™ CDx, must be estimated experimentally and was estimated to be 0.07 † Adjusted for variant enrichment in study design using the parameter Pc	

ONCOREVEAL™ CDX CONCORDANCE STUDY FOR *KRAS* WILD TYPE (ABSENCE OF MUTATION IN CODON 12 AND 13) IN CRC

The concordance of *KRAS* codon 12 and 13 mutation results was determined between the oncoReveal™ CDx (FCD) and the approved QIAGEN *therascreen* KRAS RGQ PCR (CCD). A stratified design was used with a target endpoint of 30 to 50% *KRAS* positive specimens in the study population.

A total of 374 DNA samples extracted from CRC FFPE specimens were submitted for testing using two successful replicates of CCD. The first replicate of CCD was used to enroll samples into the study. After exclusion of ineligible or failed samples, 219 samples remained for the concordance analysis. Test outcomes from the 219 samples with full CCD1, CCD2, and FCD results are tabulated below in Table 59.

Table 59 2x2x2 Matrix for Analysis of Concordance Outcomes for Non-Inferiority (CRC Tissue)

	Enrollment CCD+ (CCD1+)			Enrollment CCD- (CCD1-)		
	CCD2+	CCD2-	Total	CCD2+	CCD2-	Total
FCD+	87	2[1a,1b]	89	0	1[4]	1
FCD -	2[2]	2[3a, 3b]	4	0	125	125
Total	89	4	93	0	126	126

[1] The replicates of the comparator were discordant.

[a] CCD1 = KRAS 12VAL; CCD2 = KRAS 12ALA; FCD = KRAS 12VAL

[b] CCD1 = KRAS 12ASP; CCD2 = KRAS negative; FCD = KRAS 12ASP

[2] The replicates of the comparator (CCD1/CCD2 = KRAS 12VAL) were discordant with FCD (KRAS 12PHE; c.34_35delinsTT). It is inferred that therascreen KRAS is not designed to detect complex SNVs, this result may indicate an error by therascreen KRAS assay.

[3] The replicates of the comparator were discordant.

[a] CCD1 = KRAS 12ARG; CCD2 = KRAS 12CYS; FCD = KRAS 12CYS

[b] CCD1 = KRAS 12ALA; CCD2 = KRAS 12VAL; FCD = KRAS 12VAL

[4] The replicates of the comparator (CCD1/CCD2 = KRAS negative) were discordant with FCD (KRAS 13VAL; c.38_39delinsTT). It is inferred that therascreen KRAS is not designed to detect complex SNVs, this result may indicate an error by therascreen KRAS assay.

The agreements for the non-inferiority test proposed by Li (2016)[3] using the data from the 2x2x2 contingency table above are shown in the table below. Note that, since a mutation enriched population drawn from the intended use/indications for use population was used for the study, a correction was performed to adjust the observed PPA and NPA based on the natural frequency of the *KRAS* mutations in the intended use/indications for use population (see Li (2016)[3] for details).

All the upper bounds of the 95% confidence intervals were determined to be less than 5%, supporting a conclusion that the agreement between the oncoReveal™ CDx and QIAGEN *therascreen* KRAS RGQ PCR is non-inferior to the agreement between two replicates of CCD by a margin of 5%.

Table 60 Observed and adjusted PPA and NPA for KRAS in CRC

Parameter	Agreement (%)
PPA_{C1F}	95.7
PPA_{C1C2}	95.7
NPA_{C1F}	99.2
NPA_{C1C2}	100.0
PPA_{C2F}	97.8
PPA_{C2F} †	97.8
PPA_{C2C1}	100.0
PPA_{C2C1} †	100.0
NPA_{C2F}	97.7
NPA_{C2F} †	98.0
NPA_{C2C1}	96.9
NPA_{C2C1} †	97.6
Section 4.2, p.361 in Meijuan Li (2016) Statistical Methods for Clinical Validation of Follow-On Companion Diagnostic Devices via an External Concordance Study, Statistics in Biopharmaceutical Research, 8:3, 355-363 *The parameter Pc, the “true” MAF for the mutation of interest as analyzed by the oncoReveal™ CDx, must be estimated experimentally, for the case of KRAS codon 12 and 13 mutations, 0.36 † Adjusted for enrichment	

TROUBLESHOOTING

Issue	Potential Cause	Solution
Sample result is invalid	Improper library quantification	If failure can be attributed to misquantification of the invalid sample library, repeat sequencing of prepared library with correct quantification and PiVAT analysis. Otherwise, repeat testing of invalid sample starting from Gene-Specific PCR Amplification. If the sample remains invalid, extract fresh DNA from additional FFPE if available and repeat testing from Gene-Specific PCR Amplification.
	DNA quantity or quality	
Low yield of gene-specific product	DNA quantity or quality	The recommended input for the assay is 30-80 ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification.
Low indexing efficiency	Improper purification	Incomplete purification or loss of gene-specific product will affect the indexing PCR reaction. The purified product can be checked on an agarose gel to ensure the gene-specific product was not lost.
		The purification bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct purification concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
		Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for indexing amplification.
Low library yield	DNA quantity or quality	The recommended input for the assay is 30-80 ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples.
		Run the product from the gene-specific PCR on agarose gel to check the yield.
		The product can also be checked on an agarose gel after indexing PCR before and after bead purification.
	Improper purification	Incomplete purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure the product was not lost during PCR cleanup.

Issue	Potential Cause	Solution
		The bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct purification concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
	DNA quantification assay kit	Consistent over- or under-quantification of extracted FFPE DNA or libraries. Confirm quantification with a different dsDNA quantification assay.
The libraries over-cluster or under-cluster on the MiSeqDx	Normalization and mix of libraries is not 20 pM	Check the 4 nM Library Mix using dsDNA quantification assay. Dilute the denatured library mix as needed to adjust for the difference in concentration.
	Improper library quantification	Improper library quantification may result in artificially high or low yields, which affects downstream normalization.
		Re-quantify the final libraries and/or the normalized libraries to check for the expected values.
	Improper Purification	Changing the ratio of purification beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering.
		The purification bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct Purification concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
		The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
RUN QC = FAIL and MiSeqDx Clusters Passing Filter <75% and Cluster Density >1600 k/mm^2	Sequencing quality	Repeat quantification of Library Mix and dilute to 4 nM to repeat sequencing run.
No-template control (NTC) contains amplicons	Cross-contamination	Make sure to change tips between samples, and avoid reaching over tubes or plates. When liquid handling, be careful to avoid waving used tips over samples. Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples. Work spaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination.

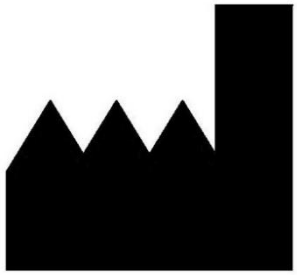
Issue	Potential Cause	Solution
		Periodically clean the work space, floor, equipment, and instrumentation with a laboratory cleaning solution (10% bleach, 70% isopropanol, or 70% ethanol) to break down amplicons on surfaces. Repeat template line wash of the MiSeqDx with sodium hypochlorite solution (NaOCl) according to Illumina Instructions for Use.

REFERENCES

1. MiSeqDx Reagent Kit v3 Package Insert
2. Local Run Manager Software Reference Guide for MiSeqDx
3. Meijuan Li: Statistical Methods for Clinical Validation of Follow-On Companion Diagnostic Devices via an External Concordance Study, Statistics in Biopharmaceutical Research, 8:3, 355-363, 2016
4. Li MM, Datto M, Duncavage EJ, et al: Standards and guidelines for the interpretation and reporting of sequence variants in cancer. A joint consensus recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. J Mol Diagn 19:4-23, 2017

LEGAL NOTICES

COMPANY INFORMATION



Pillar Biosciences, Inc.
9 Strathmore Road
Natick, MA 01760
(800) 514-9307
support@pillarbiosci.com
https://pillarbiosci.com/