Assay User Manual

ONCO/Reveal™ Dx Lung and Colon Cancer Assay

ONCO/Reveal™ Dx Lung and Colon Cancer Assay Kit 48 Tests P/N: HDA-LC-2002-48

For In Vitro Diagnostic Use

Contents

INTENDED USE

The ONCO/Reveal Dx Lung and Colon Cancer Assay (O/RDx-LCCA) is a next generation sequencing based test for detection of somatic mutations in DNA derived from formalin-fixed paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) tumor tissue. The test is intended to be used to select patients with NSCLC or CRC that may benefit from treatment with the targeted therapies listed in Table 1 in accordance with the approved therapeutic product labeling. The O/RDx-LCCA is intended to be used on the Illumina MiSeqDx® instrument.

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	Iressa® (gefitinib), Gilotrif® (afatinib), Tarceva® (erlotinib), or Vizimpro® (dacomitinib)

Genomic findings other than those listed in Table 1 may be used by qualified health care professionals in accordance with professional guidelines and are not prescriptive or conclusive for labeled use of any specific therapeutic product. The test is not indicated to be used for standalone diagnostic purposes, screening, monitoring, risk assessment, or prognosis.

Analytical performance using CRC and NSCLC specimens were established for gene variants for which there is evidence of clinical significance, listed in Table 2. All variants fall into Tier 1: Variants with strong clinical significance or Tier 2: Variants with potential clinical significance according to ASCO, AMP and CAP.

Table 2 List of Variants with Established Analytical Performance Only

Gene	Variant ID	Cancer	Nucleotide Change
EGFR	T790M	NSCLC	c.2369C>T
EGFR	G719A	NSCLC	c.2156G>C
EGFR	G719C	NSCLC	c.2154_2155delinsTT ; c.2155G>T
EGFR	G719D	NSCLC	c.2156G>A
EGFR	G719S	NSCLC	c.2155G>A
EGFR	Exon 20 In-frame Insertions	NSCLC	Multiple
BRAF	V600E	NSCLC	c.1799T>A ; c.1799_1800delinsAA
KRAS	Exon 2 Mutation	NSCLC	Multiple

Gene	Variant ID	Cancer	Nucleotide Change
KRAS	A59E	CRC	c.176C>A
KRAS	A59G	CRC	c.176C>G
KRAS	A59T	CRC	c.175G>A
KRAS	A59S	CRC	c.175G>T
KRAS	Q61E	CRC	c.181C>G
KRAS	Q61H	CRC	c.183A>C ; c.183A>T
KRAS	Q61K	CRC	c.180_181delinsAA ; c.180_181inv ; c.181C>A
KRAS	Q61L	CRC	c.182A>T ; c.182_183delinsTC ; c.182_183delinsTG ; c.182_183inv
KRAS	Q61R	CRC	c.182A>G ; c.182_183delinsGC ; c.182_183delinsGT
KRAS	K117N	CRC	c.351A>C ; c.351A>T
KRAS	A146T	CRC	c.436G>A
KRAS	A146P	CRC	c.436G>C
KRAS	A146V	CRC	c.437C>T
BRAF	V600E	CRC	c.1799T>A ; c.1799_1800delinsAA

PRINCIPLES OF THE PROCEDURE

OVERVIEW

The ONCO/Reveal Dx Lung & Colon Cancer Assay 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 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 is 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.

MATERIALS AND REAGENTS

ONCO/REVEAL DX LUNG AND COLON CANCER ASSAY KIT

CAUTION: ONCO/Reveal Dx Lung and Colon Cancer Assay kit(s) are to be unpacked and placed at the indicated storage temperatures in Table 3 upon receipt.

Table 3 Assay Kit Reagents

Kit Box 1: GS-PCR Reagent	Quantity	Storage
Gene Specific PCR Master Mix	1 tube (red cap)	-15°C to -25°C
LC Oligo Pool	1 tube (yellow cap)	-15°C to -25°C
Positive Control (PosCtrl)	1 tube (clear cap)	-15°C to -25°C
Uracil-DNA glycosylase (UDG)	1 tube (blue cap)	-15°C to -25°C
Kit Box 2: Indexing PCR Reagent	**Quantity**	**Storage**
Indexing PCR Master Mix	1 tube (green cap)	-15° to -25°C
Forward indexing primers (A501-A508)	8 tubes (white caps)	-15° to -25°C
Reverse indexing primers (A701-A706)	6 tubes (orange caps)	-15° to -25°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 Assay Kit

Material	Source/Part Number
DNA extraction and purification reagents	See DNA EXTRACTION
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific/Q32851 or Q32854
Qubit™ Assay Tubes	Thermo Fisher Scientific/Q32856
PhiX Control v3, 10 nM	Illumina/FC-110-3001
MiSeqDx® Reagent Kit v3 (600 cycles)	Illumina/20012552
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

Material	Source/Part Number
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 Equipment and software required but not provided

Equipment	Source/Part Number
MiSeqDx® Instrument†	Illumina/DX-410-1001
Pillar LC-HS module	Pillar Biosciences/SW-0001
ONCO/Reveal Dx Lung and Colon Cancer Assay PiVAT Software	Pillar Biosciences/SFW-2005
Qubit Fluorometer†	Thermofisher Scientific
Vortexer	General lab supplier
Magnetic stand for 96 wells	Life Technologies/12331D or 12027, Beckman Coulter/# A32782 or equivalent
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 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 ONCO/Reveal Dx Lung and Colon Cancer Assay kit.

1. The ONCO/Reveal Dx Lung and Colon Cancer Assay is for *In Vitro* Diagnostic Use only.
2. The assay has been validated with DNA extracted from NSCLC and CRC FFPE tissues.
3. The assay has been validated with the Qubit™ dsDNA HS Assay Kit for quantification of FFPE extracted DNA and quantification of prepared library.
4. Do no 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 ONCO/Reveal Dx Lung and Colon Cancer Assay reagent kit are 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 fresh-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 ONCO/Reveal Dx Lung and Colon Cancer Assay was validated with DNA extracted from FFPE tumor tissues from CRC and NSCLC patients.

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 sections with ≥ 30% tumor content by area for processing without macrodissection.
3. For 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 Qubit dsDNA quantification of ≥ 1.5 ng/µl can be used for the ONCO/Reveal Dx Lung and Colon Cancer Assay. If extracted DNA do not meet the minimum Qubit dsDNA quantification 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 15 to -25 °C for up to 30 days before use.

DNA EXTRACTION

The assay has been validated to work with DNA isolated from FFPE NSCLC and CRC tissue samples. Column-based DNA extraction kits with Proteinase K treatment with agitation and final elution with 25 µL volume per section are recommended for DNA extractions intended for use with this assay.

ONCO/Reveal Dx Lung and Colon Cancer Assay supports extracted DNA samples with quantified dsDNA concentration > 1.5 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.

CAUTION: Only CRC or NSCLC FFPE sections are to be used in the ONCO/Reveal Dx Lung and Colon Cancer Assay.

TEST PROCEDURE

QUANTIFICATION OF DNA EXTRACTED FROM FFPE TISSUES

NOTE: The ONCO/Reveal Dx Lung and Colon Cancer Assay was validated with DNA extracted from FFPE tissues quantified using the **Qubit™ dsDNA HS Assay Kit**. DNA quantification is performed to determine if the DNA is of sufficient quantity for use with the assay.

1. Follow Qubit™ manufacturer's user guide for dsDNA HS Assay Kit on how to **prepare standards and samples**, **reading standards and samples**, and **calculate sample concentration**.
2. Measure concentration of extracted DNA samples and calculate sample concentration in ng/µl.
3. DNA samples with Qubit dsDNA quantity that meet input requirement may proceed to Gene-specific PCR according to the recommendations in the Table 6 below.

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

Table 6 Quantified DNA input requirement and dilution recommendation

DNA conc (ng/µl)	Recommendation
<1.5	Not supported. Repeat DNA extraction.
1.5 to 12.0	No dilution necessary.
>12.0	Dilute DNA sample to 12.0 ng/µl.

4. Qualified extracted DNA samples 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).

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 -15°C to -25°C for up to 30 days.

GENE-SPECIFIC PCR AMPLIFICATION

CAUTION: No Template Control (NTC) 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 6.
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 8 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 7. GS-PCR total reaction volume is 25 µl.

Table 7 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 8) with heated lid.

Table 8 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 completes, proceed to GS-PCR Product Purification steps below.

CAUTION: Use care when returning GS-PCR reagents to O/RDx-LCCA Reagent Kit Box 1 for storage at -15°C to -25°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 will be processed further within the same day, but they should be frozen at -15°C to -25°C for extended storage—see note below.

STOPPING POINT: Purified GS-PCR products may be stored frozen at -15°C to -25°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 ONCO/Reveal Dx Lung and Colon Cancer Assay 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.

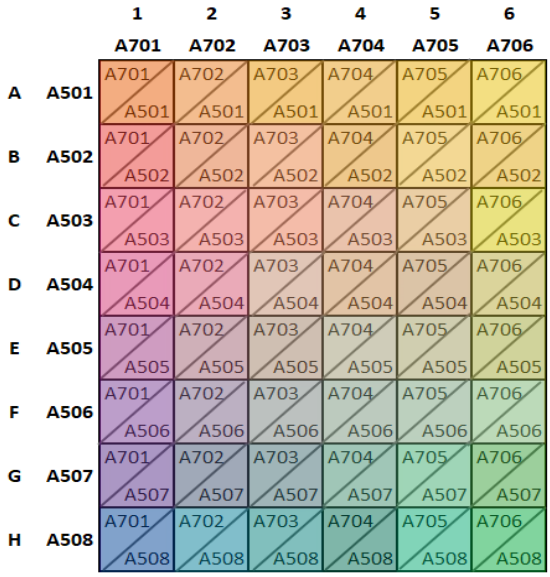

Figure 1 Available index-pair positions for a maximum 48-library sequencing run.

Preparation

1. Determine the combination of indices to be used and the Indexing-PCR plate layout. The ONCO/Reveal Dx Lung and Colon Cancer 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 may be used as an aid in the batching process. The tool may be downloaded from the Pillar Biosciences website or transferred from the O/RDx-LCCA IVD 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 10 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.

FILE NAME | Save to DESKTOP | RESET sample sheet | Instructions | PILLAR BIOSCIENCES

	Sample_ID	Description	Sample_Well	I7_Index_ID	I5_Index_ID	Control
A1	UOM64-10ng	BATCH_1	A1	A701	A501	
B1	UOM64-20ng	BATCH_1	B1	A701	A502	
C1	UOM64-40ng	BATCH_1	C1	A701	A503	
D1	608-10ng	BATCH_1	D1	A701	A504	
E1	608-20ng	BATCH_1	E1	A701	A505	
F1	608-40ng	BATCH_1	F1	A701	A506	
G1	632	BATCH_1	G1	A701	A507	
H1	UOM23	BATCH_1	H1	A701	A508	
A2	UOM74-10ng	BATCH_1	A2	A702	A501	
B2	UOM74-20ng	BATCH_1	B2	A702	A502	
C2	NTC1	BATCH_1	C2	A702	A503	NTC
D2	613-10ng	BATCH_1	D2	A702	A504	
E2	613-20ng	BATCH_1	E2	A702	A505	
F2	613-40ng	BATCH_1	F2	A702	A506	
G2	HzMod1	BATCH_1	G2	A702	A507	PosCtrl
H2	UOM37	BATCH_1	H2	A702	A508	
A3	UOM80-10ng	BATCH_1	A3	A703	A501	
B3	UOM80-20ng	BATCH_1	B3	A703	A502	
C3	UOM80-40ng	BATCH_1	C3	A703	A503	
D3	626-10ng	BATCH_1	D3	A703	A504	
E3	626-20ng	BATCH_1	E3	A703	A505	
F3	626-40ng	BATCH_1	F3	A703	A506	
G3	616	BATCH_2	G3	A703	A507	
H3	UOM62	BATCH_2	H3	A703	A508	
A4	UOM81-10ng	BATCH_2	A4	A704	A501	
B4	NTC2	BATCH_2	B4	A704	A502	NTC
C4	UOM81-40ng	BATCH_2	C4	A704	A503	
D4	628-10ng	BATCH_2	D4	A704	A504	
E4	628-20ng	BATCH_2	E4	A704	A505	
F4	628-40ng	BATCH_2	F4	A704	A506	
G4	UOM69	BATCH_2	G4	A704	A507	
H4	hzMod2	BATCH_2	H4	A704	A508	PosCtrl
A5	UOM91-10ng	BATCH_2	A5	A705	A501	
B5	UOM91-20ng	BATCH_2	B5	A705	A502	
C5	UOM91-40ng	BATCH_2	C5	A705	A503	
D5	629-10ng	BATCH_2	D5	A705	A504	
E5	629-20ng	BATCH_2	E5	A705	A505	
F5	629-40ng	BATCH_2	F5	A705	A506	
G5	UOM65	BATCH_3	G5	A705	A507	
H5	hzSev	BATCH_3	H5	A705	A508	
A6	UOM92-10ng	BATCH_3	A6	A706	A501	
B6	UOM92-20ng	BATCH_3	B6	A706	A502	
C6	UOM92-40ng	BATCH_3	C6	A706	A503	
D6	648-10ng	BATCH_3	D6	A706	A504	
E6	648-20ng	BATCH_3	E6	A706	A505	
F6	648-40ng	BATCH_3	F6	A706	A506	
G6	HzMod3	BATCH_3	G6	A706	A507	PosCtrl
H6	NTC3	BATCH_3	H6	A706	A508	NTC

SAMPLE ID

	1	2	3	4	5	6
A	UOM64-10ng	UOM74-10ng	UOM80-10ng	UOM81-10ng	UOM91-10ng	UOM92-10ng
B	UOM64-20ng	UOM74-20ng	UOM80-20ng	NTC2	UOM91-20ng	UOM92-20ng
C	UOM64-40ng	NTC1	UOM80-40ng	UOM81-40ng	UOM91-40ng	UOM92-40ng
D	608-10ng	613-10ng	626-10ng	628-10ng	629-10ng	648-10ng
E	608-20ng	613-20ng	626-20ng	628-20ng	629-20ng	648-20ng
F	608-40ng	613-40ng	626-40ng	628-40ng	629-40ng	648-40ng
G	632	HzMod1	616	UOM69	UOM65	HzMod3
H	UOM23	UOM37	UOM62	hzMod2	hzSev	NTC3

SAMPLE BATCHING

	1	2	3	4	5	6
A	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
B	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
C	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
D	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
E	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
F	BATCH_1	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3
G	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3	BATCH_3
H	BATCH_1	BATCH_1	BATCH_2	BATCH_2	BATCH_3	BATCH_3

INDEX PAIR

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

CONTROL TYPE

	1	2	3	4	5	6
A	S	S	S	S	S	S
B	S	S	S	NTC	S	S
C	S	NTC	S	S	S	S
D	S	S	S	S	S	S
E	S	S	S	S	S	S
F	S	S	S	S	S	S
G	S	PosCtrl	S	S	S	PosCtrl
H	S	S	S	PosCtrl	S	NTC

Figure 2 Pillar_sample_sheet_tool

Procedure

1. Obtain a new plate for Indexing-PCR plate setup.
2. For each indexing reaction, add 4 ul 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 ul total of index primers.
3. Prepare sufficient Indexing-PCR reaction mix for the samples to be indexed according to the indicated volume in Table 9. Indexing-PCR total reaction volume is 50 µl.

Table 9 Indexing-PCR reaction mix reagent volume per reaction

Reagent	Cap color	1x Volume (µl)
Indexing PCR Master Mix	Green	25.0
Nucelase-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 10) with heated lid.

Table 10 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 completes, proceed directly to Indexed Libraries Purification steps below.

CAUTION: Use care when returning Indexing-PCR reagents to O/RDx-LCCA Reagent Kit Box 2 for storage at -15°C to -25°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 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 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 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 -20°C for up to 90 days.

QUANTIFICATION OF INDEXED SAMPLE LIBRARIES

IMPORTANT: The ONCO/Reveal Dx Lung and Colon Cancer Assay was validated with DNA libraries quantified using the **Qubit™ dsDNA HS Assay Kit**. DNA quantification is performed to determine if the DNA library is of sufficient yield for sequencing on the MiSeqDx® instrument.

1. Follow Qubit™ manufacturer's user guide for dsDNA HS Assay Kit on how to **prepare standards and samples**, **reading standards and samples**, and **calculate sample concentration**.
2. Use a **minimum of 4 µl** per sample library to prepare Qubit sample.
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.

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

5. PosCtrl and NTC must meet the following library yield check in Table 11 before proceeding to Library Normalization and Pooling.

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

Table 11 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 Amplication 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 Amplication or Gene-Specific PCR Amplification.

6. 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 -20°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

1. 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

1. Normalize each sample library based on the calculated concentration in nM according to the recommendations in Table 12 below.

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

Table 12 Quantified indexed libraries dilution table

Library conc (nM)	Recommendation
<3.5	Not supported.
3.5 to 5.0	No dilution necessary.
>5.0	Dilute to library to 4.0 nM.

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

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

3. Obtain a new plate for normalizing libraries.
4. 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.
5. Transfer 4 µl of each purified indexed library from the library stock plate to its corresponding library stock well in the normalization plate.
6. After preparing the normalized libraries, seal the plate and vortex to mix thoroughly. Centrifuge the plate briefly to collect droplets.
7. 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.
8. Vortex the solution in the tube to mix thoroughly.
9. The resulting pooled libraries is now the **Library Mix**.
10. 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 -20°C for up to 90 days.

QUANTIFICATION OF LIBRARY MIX

IMPORTANT: The ONCO/Reveal Dx Lung and Colon Cancer Assay was validated with DNA libraries quantified using the **Qubit™ dsDNA HS Assay Kit**. DNA quantification is performed to determine if the pooled Library Mix has a final concentration 3.5 to 4.5 nM to prevent over- or under-clustering on the MiSeqDx® instrument.

Qubit dsDNA HS Standard #2 is used as an independent Library QC sample to ensure the quantification step is as accurate as possible.

Preparation

1. Follow Qubit™ manufacturer's user guide for dsDNA HS Assay Kit on how to **prepare standards and samples**, **reading standards and samples**, and **calculate sample concentration**.
2. Prepare sufficient Qubit working solution for a minimum of 5 samples.
3. Use 4 µl of Qubit dsDNA HS Standard #2 to prepare Library QC sample.
4. Use 4 µl of Library Mix to prepare Qubit sample.

Procedure

1. Add 4 µl of Qubit dsDNA HS Standard #2 to 196 µl Qubit working solution to prepare 200 µl **Library QC sample.**
2. Add 4 µl of Library Mix to 196 µl Qubit working solution to prepare 200 µl **Library Mix sample**.
3. Measure concentration of the prepared Qubit samples: Library QC, Library Mix and the Library QC again.
4. Qubit read refers to the direct measurement of the Qubit sample (not calculated stock sample concentration) as it appears on the Qubit fluorometer.
5. Both Library QC Qubit reads (in ng/mL) must meet the following concentration check in Table 13 before proceeding to next steps.

CAUTION: Ensure Qubit measured Library QC concentration is calculated and reported in **ng/mL** for Qubit read check.

Table 13 Library QC Qubit read check

Library QC Qubit read (ng/mL)	Recommendation
<180	Repeat preparation and quantification of Library QC and Library Mix.
180 to 220	Proceed to next step.
>220	Repeat preparation and quantification of Library QC and Library Mix.

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

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

8. Library mix may proceed to sequencing according to the recommendations in the Table 14 below.

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

Table 14 Library mix dilution table

Library mix conc (nM)	Recommendation
<3.0	Not supported. Repeat Library Normalization and Pooling.
3.0 to 4.5	No dilution necessary, proceed to Library Mix Denaturation.
>4.5	Dilute to 4.0 nM.

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

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

10. Add the calculated volume of diluent to Library Mix.
11. Repeat Qubit quantification of 4 nM adjusted Library Mix from Step 2.
12. Place the Library Mix on ice until ready to proceed to denaturation.

LIBRARY MIX DENATURATION

IMPORTANT: The ONCO/Reveal Dx Lung and Colon Cancer Assay was validated with the MiSeqDx® Reagent Kit v3 on the Illumina MiSeqDx® instrument.

Prepare the Reagent Cartridge

13. 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.
14. Allow the reagent cartridge to thaw in the room temperature water bath for approximately 1 hour or until thawed.
15. 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.
16. Remove any water using a lint free wipe.

Inspect the Reagent Cartridge

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

Prepare Denaturation Reagents

21. 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.
22. The result is 1 mL of 0.2 N NaOH. Use fresh dilution within 12 hours.
23. Remove HT1 from -15°C to -25°C storage and thaw at room temperature. Store at 2°C to 8°C until ready to dilute denatured libraries.

Denature Library Mix

24. Label a new 1.5 ml microcentrifuge tube for the denatured Library Mix.
25. Combine 5 µl of Library Mix and 5 µl of 0.2 N NaOH in the tube.
26. Vortex briefly and then centrifuge for 1 minute.
27. Incubate at room temperature for 5 minutes.

Dilute Denatured Library Mix

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

Denature and Dilute PhiX Control to 20 pM

31. Label a new 1.5 ml microcentrifuge tube for the denatured 20 pM PhiX Control.
32. 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.
33. If not prepared within the last 12 hours, prepare a fresh dilution of 0.2 N NaOH.
34. Add 5 µl of 0.2 N NaOH to the 5 µl of 4 nM PhiX library.

35. Vortex briefly to mix.
36. Centrifuge at 280 × g for 1 minute.
37. Incubate at room temperature for 5 minutes.
38. Add 990 µl prechilled HT1 to the 10 µl of denatured PhiX library. The result is 1 ml of a 20 pM PhiX library.
39. Invert to mix. The denatured 20 pM PhiX library can be stored up to 3 weeks at -15°C to -25°C.

Combine Denatured Library Mix and PhiX Library

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

CREATE RUN WITH LOCAL RUN MANAGER

IMPORTANT: The ONCO/Reveal Dx Lung and Colon Cancer Assay 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 the following options:

Enter Samples Manually

1. Use the blank table on the Create Run screen.
2. Select the number of samples and index set from the drop-down list.
3. Enter a unique sample library name. Use alphanumeric characters, dashes, or underscores.
4. For positive or no template control samples, right-click and select the control type.
5. [Optional] Select the Description tab and enter a library batch identifier. Use alphanumeric characters, dashes, or underscores. Ensure each batch has its corresponding positive and no template controls.
6. Select the Index 1 (i7) tab and select an Index 1 adapter from the drop-down list.
7. Select the Index 2 (i5) tab and select an Index 2 adapter from the drop-down list.
8. Choose an option to view, print, or save the plate layout as a reference for preparing libraries:
    - Select the Print icon to display the plate layout. Select Print to print the plate layout.
    - Select Export to export sample information to an external file.
9. Select Save Run.

Import Samples

1. Click Import Samples and browse to the location of the sample information file. There are three types of files you can import.
    - Select Template on the Create Run screen to make a new plate layout. The template file contains the correct column headings for import. Enter sample information in each column for the samples in the run. Delete example information in unused cells, and then save the file.
    - Use a file of sample information that was exported from the analysis module using the Export feature.
    - Download the Pillar_sample_sheet_tool from the Pillar Biosciences website or transferred from the PiVAT workstation to make a new plate layout. Enter required information and select "Save to DESKTOP".
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 v2 (document # 1000000021961) for complete run setup instructions.

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. A 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. A 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-0038) for instructions on run data transfer, start analysis and view analysis results.

QUALITY CONTROL

No Template Control (NTC) and Positive (PosCtrl) are included for each "Batch" of up to 46 samples (processed on the same plate). If the NTC and/or PosCtrl is invalid, the entire run is invalid. See Table 15 in "Results" section for recommended actions.

RESULTS

INTERPRETATION OF RESULTS

All run and sample validation are performed by the ONCO/Reveal Dx Lung and Colon Cancer Assay PiVAT software. A valid run may include both valid and invalid sample results.

Table 15 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 16 Interpretation of PiVAT Patient Summary results

Results	Interpretation	Action
Variant(s) detected	Mutation detected in targeted EGFR and/or KRAS region.	See Intended Use section.
No variant detected	Mutation not detected in targeted EGFR and/or KRAS region.	See Intended Use section.
Invalid	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.

SUMMARY OF NON-CLINICAL PERFORMANCE

ANALYTICAL SENSITIVITY: LIMIT OF BLANK

A Limit of Blank (LoB) of zero was determined across 72 independent sample libraries prepared from four FFPE specimens each of normal colon and normal lung tissue with 4-6 replicates per sample spanning low and high DNA input, two reagent lots, and three sequencing analyses. No false positive observations were made for the clinically and analytically relevant genes and no samples or libraries were excluded from the analysis. It was confirmed that each LoB sample was negative for somatic variants included in the LoD analysis.

ANALYTICAL SENSITIVITY: LIMIT OF DETECTION

The limit of detection (LoD) based on positive calls for the ONCO/Reveal Lung & Colon Cancer Assay was estimated to determine the lowest variant allele frequency (VAF) at which at least 95% of the test replicates produced correct calls. A total of 10 clinical NSCLC and CRC specimens were evaluated and these included samples with SNVs, deletions ranging up to 18 nucleotides, and insertions of up to 6 nucleotides and SNVs, including variants near homopolymer blocks of 5 or more bases and in regions with 39% to 65% GC content. Five titration levels were tested and each level was tested with 10 replicates per sample for each of the two reagent lots.
The LoD is based on the highest VAF% with 95% or more correct calls observed for the variant being tested and claimed LoD for each variant was calculated as the average VAF% across replicates for that level level. Measured LoDs varied between 1.4% and 3.0% for SNV variants, 1.7% for the DelIns variant, and 2.2% for the Insertion variant. The sample set contained two different FFPE specimens containing the same variant.

Table 17 Summary of O/RDxLCCA variant limit of detection

Gene	Variant	Variant Category	Estimated VAF%
KRAS	G13D	SNV	2.8
KRAS	G12D	SNV	1.9
EGFR	L858R	SNV	1.6
EGFR	Exon 19 Del	DelIn	1.7
BRAF	V660E	SNV	1.4
EGFR	G719C	SNV	2.4
EGFR	Exon 20 Ins	Insertion	2.2
EGFR	T790M	SNV	3.0
KRAS	Q61L	SNV	2.2
KRAS	A146T	SNV	2.9

ACCURACY

Concordance of the O/RDx-LCCA was assessed for the ability to detect SNVs, short and medium indels deletions with one externally validated comparator method (Oncomine Focus Assay). A total of 208 samples (84 CRC and 124 NSCLC) were tested of which 6 yield invalid results with the validated NGS comparator method and 2 yield invalid results with O/RDxLCCA. Among the 200 valid samples, 168 (84.0%) were identified as being positive for at least one variant with the comparator method. The samples included simple SNVs, short and medium indels that are targeted by the O/RDxLCCA. The results at the variant, sample and bin levels are shown in the tables below. Concurrence of the data from the clinical concordance study is also considered a demonstration of analytical accuracy.

Table 18 Overall agreement result by variant, sample and bin type.

	OFA+	O/RDx LCCA+	PPA	95%_LB_CI	95%_UB_CI	OFA-	O/RDx LCCA-	NPA	95%_LB_CI	95%_UB_CI
Variant	228	219	96.1%	92.7%	97.9%	60372	60366	100.0%	100.0%	100.0%
Sample	168*	167	99.4%	96.7%	99.9%	32	27	84.4%	68.2%	93.1%
SNV	208	199	95.7%	92.0%	97.7%	20192	20187	100.0%	99.9%	100.0%
Short indel (<6 nt)	5	5	100.0%	56.6%	100.0%	10595	10595	100.0%	100.0%	100.0%
Medium indel (6-50 nt)	15	15	100.0%	79.6%	100.0%	29585	29584	100.0%	100.0%	100.0%

Note: Invalid data are excluded from this analysis

* 160/168 have exact variant match within-sample; 8 have at least one variant match. PPA = TP/TP+FN x 100%

NPA = TN/TN+FP x 100%

OFA+ = Sample positive for at least one targeted variant when tested with OFA Pillar+ = Sample positive for at least one target variant when tested with O/RDxLCCA

Table 19 Agreement results by gene.

Gene	OFA+	O/RDx LCCA+	PPA	95%_LB_CI	95%_UB_CI	OFA-	O/RDx LCCA-	NPA	95%_LB_CI	95%_UB_CI
AKT1	4	3	75.0%	30.1%	95.4%	196	196	100.0%	98.1%	100.0%
ALK	1	0	0.0%	0.0%	79.3%	599	599	100.0%	99.4%	100.0%
BRAF	26	26	100.0%	87.1%	100.0%	1374	1373	99.9%	99.6%	100.0%
CTNNB1	4	4	100.0%	51.0%	100.0%	1196	1196	100.0%	99.7%	100.0%
EGFR	39	39	100.0%	91.0%	100.0%	36761	36760	99.997%	99.985%	100.0%
ERBB2	2	1	50.0%	9.5%	90.5%	998	998	100.0%	99.6%	100.0%
FGFR2	0	0	N/A	N/A	N/A	800	800	100.0%	99.5%	100.0%
FGFR3	4	3	75.0%	30.1%	95.4%	1796	1796	100.0%	99.8%	100.0%
KRAS	109	108	99.1%	95.0%	99.8%	10691	10688	99.97%	99.92%	99.99%
MET	7	7	100.0%	64.6%	100.0%	593	593	100.0%	99.4%	100.0%
NRAS	3	3	100.0%	43.9%	100.0%	997	997	100.0%	99.6%	100.0%
PIK3CA	28	24	85.7%	68.5%	94.3%	3972	3971	100.0%	99.9%	100.0%
MAP2K1	1	1	100.0%	20.7%	100.0%	399	399	100.0%	99.0%	100.0%

REPRODUCIBILITY

Site-to-site Reproducibility

The reproducibility of the O/RDx-LCCA was evaluated using a total of 10-member sample panel: 2 FFPE CRC specimens with KRAS mutations (Gly12Asp and Gly13Asp), 1 FFPE CRC specimen with BRAF mutation (Val600Glu) and 2 FFPE NSCLC specimens with EGFR mutations (Glu746_Ser752delinsVal and Leu858Arg) were prepared at high and low variant frequency 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 reagents lots were used at each site. Each panel member was tested in 4 replicates in each run for total of 36 possible results (3 sites X 3 runs X 4 replicates).
The positive call rates, with two-sided 95% confidence intervals were calculated. The positive call rate for DNA variants was 100% (90.4%,100%) for all samples tested. 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) and replicate. The total standard deviations of VAF ranged from 0.33% to 0.70%.

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 reagent lot component of the total standard deviation of VAF ranged from 0.07% to 0.33%.

Thermocycler variability

A total of 3 make/model of thermo cycler were used in the study. The thermocycler component of the total standard deviation of VAF ranged from 0.05% to 0.39%.

Table 20 Positive call rate by sample/variant 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	6.81	36/36 (100%)	90.4%,100%
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	6.92	36/36 (100%)	90.4%,100%
	BRAF Exon 15	c.1799T>A	p.Val600Glu	36	8.33	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	8.92	36/36 (100%)	90.4%,100%
Low	KRAS Exon 2	c.35G>A	p.Gly12Asp	36	4.70	36/36 (100%)	90.4%,100%
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	3.66	36/36 (100%)	90.4%,100%
	BRAF Exon 15	c.1799T>A	p.Val600Glu	36	4.98	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	4.97	36/36 (100%)	90.4%,100%

Table 21 Variance component estimates by sample/variant level

VAF Level	Gene/Exon	Nucleotide Change	Amino Acid Change	N	Mean VAF (%)	Site SD% (%CV)	Operator SD% (%CV)	Run (Site/Operator) SD%	Replicate SD% (%CV)	Total SD% (%CV)
High	KRAS Exon 2	c.35G>A	p.Gly12Asp	36	6.8	0.00 (0.00)	0.00 (0.00)	0.23 (0.03)	0.15 (0.02)	0.48 (7.06)
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	6.9	0.00 (0.00)	0.00 (0.00)	0.15 (0.02)	0.10 (0.01)	0.49 (7.13)
	BRAF Exon 15	c.1799T>A	p.Val600Glu	36	8.3	0.00 (0.00)	0.00 (0.00)	0.12 (0.01)	0.16 (0.02)	0.56 (6.69)
	EGFR Exon 19	c.2237_2255 delinsT	p.Glu746_Ser 752delinsVal	36	5.1	0.00 (0.00)	0.00 (0.00)	0.29 (0.06)	0.14 (0.03)	0.70 (13.59)
	EGFR Exon 21	c.2573T>G	p.Leu858Arg	36	8.9	0.00 (0.00)	0.00 (0.00)	0.17 (0.02)	0.18 (0.02)	0.67 (7.50)
Low	KRAS Exon 2	c.35G>A	p.Gly12Asp	36	4.7	0.00 (0.00)	0.00 (0.00)	0.10 (0.02)	0.09 (0.02)	0.40 (8.52)
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	3.7	0.00 (0.00)	0.00 (0.00)	0.08 (0.02)	0.07 (0.02)	0.33 (8.94)
	BRAF Exon 15	c.1799T>A	p.Val600Glu	36	5.0	0.00 (0.00)	0.00 (0.00)	0.09 (0.02)	0.09 (0.02)	0.37 (7.52)
	EGFR Exon 19	c.2237_2255 delinsT	p.Glu746_Ser 752delinsVal	36	3.0	0.00 (0.00)	0.00 (0.00)	0.18 (0.06)	0.14 (0.05)	0.64 (21.11)
	EGFR Exon 21	c.2573T>G	p.Leu858Arg	36	5.0	0.00 (0.00)	0.00 (0.00)	0.28 (0.06)	0.11 (0.02)	0.59 (11.95)

DNA INPUT

The standard DNA input range of the ONCO/Reveal Dx Lung and Colon Cancer Assay is between 10 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 15 FFPE samples containing representative SNVs, insertions and deletions. The expected variants (KRAS G12D, KRAS G12S, KRAS G12V, KRAS G13D, KRAS A146T, BRAF V600E, EGFR Exon 19 deletion, EGFR L858R, EGFR Exon 20 insertion, and EGFR G719S) present in all 15 samples were called correctly at DNA inputs of 5-160 ng. At 5 ng of DNA input, 8 out of 30 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 30 samples failed the library yield requirement. This study confirms that 10-80 ng of DNA input for the O/RDx-LCCA Assay produced accurate results (PPA=100.0% (126/126), NPA=100.0% (27013/27014).

TUMOR CONTENT

The minimum tumor fraction required to support the robustness of the ONCO/Reveal Lung & Colon Cancer Assay was evaluated. Ten clinical samples with different percentages of initial tumor cell content (30% to 85%) were estimated before the study by an external pathology lab. These were then diluted to five levels and analyzed with 20 replicates per level using the ONCO/Reveal Lung & Colon Cancer Assay. The data show that the assay is robust down 9.8% tumor content whereby all samples yield 100% detection of expected variants. The data supports ONCO/Reveal Lung & Colon Cancer Assay requirement of 30% tumor content.

ANALYTICAL SPECIFICITY

Interference

To evaluate the potential impact of interfering substances on the performance of the O/RDxLCCA process, this study evaluated three colorectal and three non-small cell lung cancer FFPE specimens in the presence of exogenous and endogenous substances. Each specimen was assessed with two replicates, for a total of 12 libraries with the addition of the following interfering substances: Paraffin in xylene (0.0001% and 0.0002%), Proteinase K (0.04 and 0.08 mg/mL), extraction wash buffer (1% and 2.5%), ethanol (0.8% and 1.6%) and hemoglobin (1 mg/mL and 2 mg/mL. 285 CRC and NSCLC FFPE specimens with varying quantities of necrosis (1% to 50%) were assessed. 4 discordant results were observed in CRC that were not correlated to high necrotic content. No clear trend in decreasing performance with increasing tumor fraction in the sample was observed supporting the conclusion that the performance of the ONCO/Reveal Dx Lung and Colon Cancer Assay is robust within the recommended range of necrotic content less than 50%

Cross-Reactivity

An *in silico* cross-reactivity analysis was performed to evaluate the specificity of the primers used in the OR/Dx-LCC Assay. The primers were checked for specificity to the human genome 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

24 replicates of a positive cell line sample containing EGFR L858R ~50% and BRAF V600E ~67%) and 24 replicates of NTC were processed on the same plate in a checkerboard format. No false positive (0/24, 0%) was observed in all NTC samples. The positive samples were detected at a VAF range of 50% to 68%. No cross-contamination was observed.

GUARDBANDING

The tolerances encompassing the library preparation and sequencing workflow steps were assessed corresponding to the test's most critical steps which 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. 10 FFPE 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. One reference standard DNA was prepared and analyzed over 5 sequencing runs to assess library sequencing workflow steps such as library concentration and number of libraries per run. All studies resulted in zero failures and 100% agreement across conditions.

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 O/RDxLCCA kit. Five FFPE CRC and NSCLC tissue samples were used in the study of which eight are variant positive and 2 wild types. Genomic DNA was extracted using each of the 3 commercially available FFPE extraction kits. Each extracted DNA was run in duplicate using the O/RDxLCCA. The positive percent agreement was 100% (16/16) and negative percent agreement was 100% (4584/4584) for each of the three FFPE DNA extraction kits. The results demonstrate that the 3 methods yield DNA with comparable quality and quantity to generate reliable results.If the sample remain invalid, repeat library preparation, sequencing and analysis starting from DNA extracted with additional tumor tissues.

SUMMARY OF CLINICAL PERFORMANCE

Two clinical concordance studies were conducted to support the companion diagnostic (CDx) claims indicated in Table 1 of the intended use statement. A non-inferiority statistical testing approach was used according to Li (2016). O/RDx-LCC test or follow-on companion diagnostic (FCD) was compared to an approved CDx test or companion diagnostic (CCD) using samples representative of the intended use population for that specific device.

EGFR EXON 19 DELETION AND EXON 21 L858R RESULTS (NSCLC)

The EGFR diagnostic results from the FCD were compared against those obtained from the approved Roche cobas® v2 EGFR Mutation Test (CCD). A total of 305 samples were tested for the full analysis. After exclusion of ineligible or failed samples, 257 samples remained for analysis. All the upper bounds of the 95% confidence intervals are less than 4%, supporting a conclusion that the agreement between ONCO/Reveal Lung and Colon Cancer Assay and cobas® EGFR Mutation Test v2 is non-inferior to the agreement between two replicates of CCD by a margin of 4%.

Table 22 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+	91	0	91	0	3*	3
FCD -	0	0	0	0	163	163
Total	91	0	91	0	166	166

** For the NSCLC arm of the study, there were 3 discordant calls where both replicates of the comparator were concordant (no mutation detected) and the ONCO/Reveal Dx-LCCA call across all 3 samples was identical (EGFR L858R) with VAF reported between 1.9% and 4.9%. The samples were tested with an externally validated method (Oncomine Focus Assay) and showed recovery of one discordant call at VAF 3.7% (matched with 4.9% of O/RDx-LCCA). The discordant is likely attributed to the higher Limit of Detection of the cobas EGFR L858R of 5%.*

KRAS CODONS 12 AND 13 RESULTS (CRC)

The KRAS diagnostic results from the FCD were compared against those obtained from the approved Qiagen therascreen KRAS RGQ PCR test (CCD). A total of 300 samples were tested for the full analysis. After exclusion of ineligible or failed samples, 189 samples remained for analysis. All the upper bounds of the 95% confidence intervals are less than 6%, supporting a conclusion that the agreement between FCD and CCD is non-inferior to the agreement between two replicates of CCD by a margin of 6%.

Table 23 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+	79	2*	81	0	1***	1
FCD -	2**	2*	4	0	103	103
Total	81	4	85	0	104	104

** In four of the cases, the replicates of the comparator were discordant and by definition the ONCO/Reveal Dx-LCCA call would be discordant as well.*
*** In two of the cases, the comparator call was concordant (KRAS 12Val) between replicates and the ONCO/Reveal Dx-LCCA call was a KRAS Gly12Phe (c.34_35delinsTT) deletion-insertion mutation for both discordant samples. It is suspected that therascreen® KRAS is not designed to detect deletion-insertion events, this result may indicate an error by therascreen® KRAS Assay.*
**** In one of the cases, the comparator call was concordant (no mutation) between replicates and the ONCO/Reveal Dx-LCCA call was a KRAS Gly13Val (c.38_39delinsTT) deletion-insertion mutation for both discordant samples. It is suspected that therascreen® KRAS is not designed to detect deletion-insertion events, this result may indicate an error by therascreen® KRAS Assay.*

LIMITATIONS

1. The ONCO/Reveal Dx Lung and Colon Cancer Assay has been validated for use with CRC and NSCLC tumor tissues. Test only the indicated tissue types.
2. The ONCO/Reveal Dx Lung and Colon Cancer Assay has been validated with DNA extracted from NSCLC and CRC FFPE tissues.
3. 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.
4. Only the Illumina MiSeqDx® instrument installed with Pillar LC-HS module has been validated for use with this assay.
5. Only the PiVAT® software has been validated for use with this assay.
6. Quantification of FFPE extracted DNA and prepared libraries in this assay has been validated with Qubit™ dsDNA HS Assay Kit.
7. The ONCO/Reveal Dx Lung and Colon Cancer Assay only determines the presence or absence of the KRAS, EGFR and BRAF mutations listed in Table 1 and Table 2 of the Intended Use.
8. Samples with results reported as “No variant detected” may harbor KRAS, EGFR and BRAF variants not detected by the assay.
9. KRAS mutations can be detected in NSCLC tissues. Therapy with Erbitux® or Vectibix® is only indicated for KRAS mutations in CRC tissues.
10. The ONCO/Reveal Dx Lung and Colon Cancer Assay is not to be used for diagnosis of any disease.

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of gene-specific product	DNA quantity or quality	The recommended input for the assay is 10-80ng 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 10-80ng 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. 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.
The libraries over-cluster or under-cluster on	Normalization and mix of libraries is not 20 pM	Check the 4 nM Library Mix using Qubit. Dilute the denatured library mix as needed to adjust for the difference in concentration.

Issue	Potential Cause	Solution
the MiSeqDx	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.
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. 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. 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

PiVAT®, SLIMamp®, Pillar®, ONCO/Reveal™ are trademarks of Pillar Biosciences, Inc.
Illumina™, MiSeqDx® are trademarks of Illumina, Inc.
Qubit® is a trademark of Thermo Fisher Scientific.

COMPANY INFORMATION

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

Authorized Representative: QAdvis EAR AB
Authorized Representative Address: Ideon Science Park, Scheelevagen 17, SE-223 70, Lund, Sweden