PART I: Assay User Manual

oncoReveal™ Dx Lung and Colon Cancer Assay

oncoReveal™ Dx Lung and Colon Cancer Assay 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

INDICATIONS FOR USE

The oncoReveal™ Dx Lung and Colon Cancer Assay (oRDx-LCCA) 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) and deletions in 2 genes from DNA isolated from formalin-fixed paraffin-embedded (FFPE) non-small cell lung cancer (NSCLC) and colorectal cancer (CRC) tumor tissue specimens. The test is intended as a companion diagnostic to identify patients with NSCLC or CRC who may benefit from treatment with the targeted therapies listed in Table 1 in accordance with the approved therapeutic product labeling. The oRDx -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	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

The test is not indicated to be used for standalone diagnostic purposes, screening, monitoring, risk assessment, or prognosis.

PRINCIPLES OF THE PROCEDURE

OVERVIEW

The oRDx-LCCA 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 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. oRDx-LCCA is designed to detect and report somatic variants for *EGFR* Exon 19 deletion, *EGFR* L858R mutation and missense mutations in *KRAS* G12 and *KRAS* G13. Non-targeted variants including germline variants are not reported.

MATERIALS AND REAGENTS

oncoREVEAL DX LUNG AND COLON CANCER ASSAY KIT

CAUTION: oRDx-LCCA kit(s) are to be unpacked and placed at the indicated storage temperatures in Table 2 upon receipt.

Table 2 Assay 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 3 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

Material	Source/Part Number
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 4 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
oncoReveal Dx Lung and Colon Cancer Assay PiVAT Workstation with software version 1.1 or higher	Pillar Biosciences/SFW-2002
Qubit Fluorometer†	Thermo Fisher 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 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 oRDx-LCCA kit.

1. The oRDx-LCCA 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 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 oRDx-LCCA 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 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 oRDx-LCCA 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 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 Qubit dsDNA quantification of > 4.5 ng/µl can be used for the oRDx-LCCA. 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 -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 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.

oRDx-LCCA 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 CRC or NSCLC FFPE sections are to be used in the oRDx-LCCA.

TEST PROCEDURE

QUANTIFICATION OF DNA EXTRACTED FROM FFPE TISSUES

NOTE: The oRDx-LCCA 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 5 below.

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

Table 5 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.

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 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 5.
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 7 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 6. GS-PCR total reaction volume is 25 µl.

Table 6 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 7) with heated lid.

Table 7 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 oRDx-LCCA 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 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 -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.

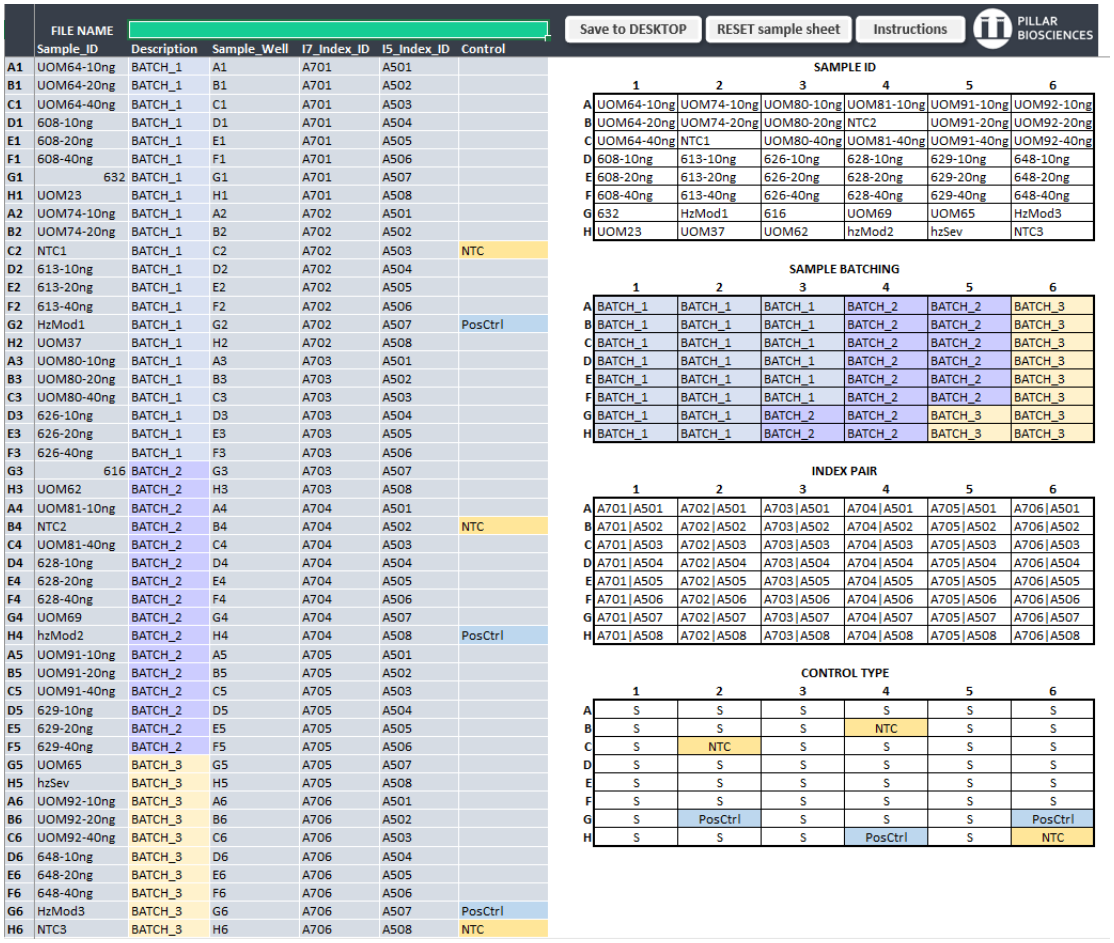
CAUTION: The oRDx-LCCA kit supports the multiplexing of up to 48 libraries per MiSeqDx v3 flow cell in up to 6 batches of varying size. However, careful planning of index-pair use across batches is required to achieve this.
Figure 1 shows the available index-pair positions on a full 48-library MiSeqDx 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 oRDx-LCCA 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 oRDx-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 9 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					
	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

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

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 8. Indexing-PCR total reaction volume is 50 µl.

Table 8 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 9) with heated lid.

Table 9 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 oRDx-LCCA 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 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 INDEXED SAMPLE LIBRARIES

IMPORTANT: The oRDx-LCCA 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 10 before proceeding to Library Normalization and Pooling.

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

Table 10 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 Amplification or Gene-Specific PCR Amplification.

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

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 -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

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 11 below.

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

Table 11 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.

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 -25°C to -15°C for up to 90 days.

QUANTIFICATION OF LIBRARY MIX

IMPORTANT: The oRDx-LCCA 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 quality control (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 12 before proceeding to next steps.

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

Table 12 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.

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

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 13 below.

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

Table 13 Library mix 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.

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 oRDx-LCCA 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 -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

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 -15°C to -25°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 oRDx-LCCA 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 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 as shown in Table 14 below. 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 is invalid, the PiVAT® software will fail the entire batch and no results will be reported for all samples within the batch. See Table 16 in "Results" section for recommended actions.

Table 14 Positive Control (PosCtrl)

Gene	Variant	Expected Allelic Frequency, %
EGFR	ΔE746 - A750	2.00%
EGFR	L858R	3.00%
KRAS	G13D	15.00%
KRAS	G12D	6.00%

Table 15 NGS-QC in PiVAT: Run, Sample and Variant Calling Passing Criteria

Category	QC Metrics	Passing Criteria
Run – FAIL 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
Sample – NOT VALID if any QC metric(s) fail	Sequencing base quality	Bases (with Q Score ≥ Q30) ≥ 75%
	Amplification specificity	Effective On-Target Rate[2] ≥ 70%
	Coverage[1]	Minimum of the amplicon depths[3] ≥ 1000x
Variant reporting threshold	CDx mutations: non-C>T|G>A	Variant coverage >10x and Total coverage ≥ 1000x and Average variant base Q-score ≥ 30 and VAF ≥ 1%
	CDx mutations: C>T|G>A	Variant coverage >10x and Total coverage ≥ 1000x and Average variant base Q-score ≥ 30 and VAF ≥ 1.5%

[1] Coverage: the coverage after paired-end assembly by PiVAT®. All CDx markers in the Pillar 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.

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

[3] Include the 7-key amplicons: three amplicons covering the CDx markers and other four amplicons covering *EGFR*-exon18, *KRAS*-exon3, *KRAS*-exon4 and *BRAF*-V600E.

RESULTS

INTERPRETATION OF RESULTS

All run and sample validation are performed by the oRDx-LCCA PiVAT software. A valid run may include both valid and invalid sample results.

Table 16 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 17 Interpretation of PiVAT Patient Summary results

Results	Interpretation	Action
Mutation detected	Mutation detected in targeted *EGFR* and/or *KRAS* region.	See Intended Use section.
No mutation detected	Mutation not detected in targeted *EGFR* and/or *KRAS* region.	See Intended Use section.
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.

SUMMARY OF NON-CLINICAL PERFORMANCE

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.

Limit of Detection

The limit of detection (LoD) for each positive variant detected by the oRDx-LCCA was estimated using the hit rate approach where LoD is defined as the lowest variant allele frequency (VAF) with 100% hit rate. A total of 4 clinical NSCLC and CRC specimens were evaluated, which included SNVs and a deletion/insertion (DelIns) variant which is a complex mutation with a deletion followed by an insertion. Five titration levels and 2 reagent lots were tested. Each level was tested with 10 replicates per sample at the minimum DNA input (30 ng) for each of the two reagent lots.

The claimed LoD for each variant summarized in Table 18 below in the original study was based on the conservative hit rate approach where the assay produced 100% positive calls. Adequate dilutions were not tested for all samples in the original study to determine the lowest VAF at which at least 95% of the test replicates produce correct calls using the probit approach or the lowest level with 100% hit rate (i.e., worst case scenario, if the minimum requirements for the probit approach are not met). Additional dilutions with one dilution level below 100% hit rate for all samples were tested in a second study to determine the lowest VAF with 100% hit rate. The newly defined LoD for each variant based on the hit rate approach where the assay produced 100% positive calls is summarized in Table 18 below.

Table 18 Summary of oRDx-LCCA variant limit of detection

Gene	Variant	Variant Category	Originally Estimated VAF%	Newly Defined VAF% (Second LoD study)
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 19 bp deletion and 1bp insertion)	3.7	1.7

Tumor Content

The minimum tumor fraction required to support the robustness of the oRDx-LCCA 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 oRDx-LCCA. The data show robustness of oRDx-LCCA in samples with tumor content above 10% at 30 ng DNA input. The data supports oRDx-LCCA requirement of 30% tumor content.

Table 19 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
			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 oRDx-LCCA is between 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 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 oRDx-LCCA 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 oRDx-LCCA.

ACCURACY

Analytical accuracy was performed to demonstrate the concordance between the oRDx-LCCA and an externally validated comparator method for the ability of oRDx-LCCA 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 oRDx-LCCA. Among the 251 valid samples, 87 positive and 160 negative samples were concordant between the two assays. There were 4 discordant samples between the oRDx-LCCA and the comparator assay. The samples included simple SNVs, complex SNV and indels that are targeted by the oRDx-LCCA. The results at the variant, sample and bin levels are shown in the tables below.

Table 20 Overall agreement result by variant, sample and gene

Binned by Gene	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)
EGFR 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 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%)
Binned by Sample	**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: oRDx-LCCA 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 oRDx-LCCA

Agreement of *EGFR* variants in NSCLC and *KRAS* variants in CRC are summarized Table 21 and Table 22 below, respectively.

Table 21 EGFR variants in NSCLC agreement by variant type and class

Binned by Variant Type	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)
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%)
Binned by Variant Class	**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)**
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 22 KRAS variants in CRC agreement by variant type and class

Binned by Variant Type	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)
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
Binned by Variant Class	**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)**
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 oRDx-LCCA, 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 population. The summary of the agreement statistics is shown in the Table 23 below.

Table 23 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 oRDx-LCCA (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 oRDx-LCCA, 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 oRDx-LCCA. 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.

REPRODUCIBILITY

Site-to-Site Reproducibility

The reproducibility of the oRDx-LCCA was evaluated using 10 clinical samples with target variants adjusted to a variant allele frequency percent (VAF%) in the range of 0.8-3x of the LoD originally established based on conservative hit rate approach (see LoD section above) 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 24 below.

Table 24 Overall 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%)	100% 100%,100%
Site 1	120	96	0	0	15024	100% (96.2%, 100%)	100% (100.0%, 100%)
Site 2	120	96	0	0	15024	100% (96.2%, 100%)	100% 100.0%, 100%)
Site 3	120	96	0	0	15024	100% (96.2%, 100%)	100% (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 25 Agreement variant and variant frequency level

VAF Level	Gene/Exon	Nucleotide	Amino Acid Change	N	Mean VAF(%	Fold LoD*	Positive call rate (%)	95% CI (LB,UB)
High	*KRAS* Exon 2	c.35G>A	p.Gly12Asp	36	6.80	2.0	36/36 (100%)	90.4%,100
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	6.91	2.1	36/36 (100%)	90.4%,100
	EGFR Exon	c.2237_2255delin	p.Glu746_Ser752delins	36	5.14	1.4	36/36 (100%)	90.4%,100
	EGFR Exon	c.2573T>G	p.Leu858Arg	36	8.91	3.0	36/36 (100%)	90.4%,100
Low	*KRAS* Exon 2	c.35G>A	p.Gly12Asp	36	4.69	1.4	36/36 (100%)	90.4%,100
	KRAS Exon 2	c.38G>A	p.Gly13Asp	36	3.66	1.1	36/36 (100%)	90.4%,100
	EGFR Exon	c.2237_2255delin	p.Glu746_Ser752delins	36	3.02	0.8	36/36 (100%)	90.4%,100
	EGFR Exon	c.2573T>G	p.Leu858Arg	36	4.96	1.7	36/36 (100%)	90.4%,100

* LoD is based on originally established VAF% as indicated in Table 18.

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 26 Precision by thermocycler

Bin	# tests	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%)
Bio-Rad C1000	200	160	0	0	25040	100% (97.7%, 100%)	100% (100.0%, 100%)

Single-Site Precision Study

An additional single-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 LoD section above) 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 a single site with 2 operators performing 3 runs on non-consecutive days. Each sample was tested in 4 replicates in each run for a total of 12 possible results (3 runs by 4 replicates). The study site performed a total of 132 tests.

PPA and NPA 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 12 replicates is presented in Table 27 below. The observed PPA value for target variants for the site was 99.2% (95.8%, 99.9%) and was 98.6% (92.4%, 99.8%) for *EGFR* and 100% (94.0%, 100%) for *KRAS* on a gene-level as shown in Tables 28 and 29 below. The NPA on a site-level and on a gene-level was 100% for all comparisons.

Table 27 Agreement by specimen at the variant level

Gene Exon	Nucleotide Change	Amino Acid Change	Total Calls	Mean VAF (%)	Fold LoD**	Positive call rate (%)	95% CI (LB, UB)
EGFR Exon 19	c.2240_2254del	p.Leu747_Thr751del	12	2.39	1.4	12/12 (100%)	75.8%, 100%
KRAS Exon 2	c.35G>A	p.Gly12Asp	12	2.80	1.6	12/12 (100%)	75.8%, 100%
KRAS Exon 2	c.34G>T	p.Gly12Cys	12	1.94	1.1	12/12 (100%)	75.8%, 100%

Gene Exon	Nucleotide Change	Amino Acid Change	Total Calls	Mean VAF (%)	Fold LoD**	Positive call rate (%)	95% CI (LB, UB)
EGFR Exon 21	c.2573T>G	p.Leu858Arg	12	2.30	1.5	12/12 (100%)	75.8%, 100%
EGFR Exon 19	c.2236_2250del	p.Glu746_Ala750del	11*	2.23	1.3	11/11 (100%)	74.1%, 100%
KRAS Exon 2	c.38G>A	p.Gly13Asp	12	3.42	1.3	12/12 (100%)	75.8%, 100%
EGFR Exon 21	c.2573T>G	p.Leu858Arg	12	1.73	1.2	12/12 (100%)	75.8%, 100%
KRAS Exon 2	c.35G>T	p.Gly12Val	12	1.54	0.9	12/12 (100%)	75.8%, 100%
EGFR Exon 21	c.2573T>G	p.Leu858Arg	12	1.55	1.0	11/12 (91.7%)	64.6%, 98.5%
EGFR Exon 19	c.2235_2249del	p.Glu746_Ala750del	12	1.89	1.1	12/12 (100%)	75.8%, 100%
KRAS Exon 2	c.38G>A	p.Gly13Asp	12	3.16	1.2	12/12 (100%)	75.8%, 100%

* One replicate produced a 'Not Valid" result. Investigation suggests the library was inadvertently excluded during library pooling and was not sequenced.
** LoD is based on the newly defined VAF% as indicated in Table 18.

Table 28 Overall agreement by site

Site	Positive /Total Calls	PPA (2-sided 95% CI)	Negative/ Total Calls	NPA (2-sided 95% CI)
Site 1	130/131	99.2% (95.8%,99.9%)	16375/16375	100% (100%,100%)

Table 29 Overall agreement by gene

Gene	Positive /Total Calls	PPA (2-sided 95% CI)	Negative/ Total Calls	NPA (2-sided 95% CI)
EGFR	70/71	98.6% (92.4%, 99.8%)	8875 / 8875	100% (100%,100%)
KRAS	60/60	100% (94.0%, 100%)	7500 / 7500	100% (99.9%, 100%)

Adequate number of samples harboring CDx biomarkers/variants at theLoD levels based on the second LoD study (Table 18) were not evaluated in a 3-site reproducibility study. This was due to the onset of the COVID-19 emergency and associated precautionary measures taken by Pillar, specifically an unanticipated reduction in lab supplies and personnel. Therefore, a postmarket 3-site reproducibility study is planned with samples carrying CDx variants and covering different *EGFR* Exon 19 deletions, *EGFR* Exon 21 L858R mutations, and *KRAS* codon 12/13 variants at the newly established LoD levels (Table 18) to supplement the existing studies such that the assay precision is demonstrated to be robust near the true LoD levels of variants that are detected by oRDx-LCCA.

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 oRDx-LCCA 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 samples 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 statement. Genomic DNA was extracted using 3 commercially available FFPE extraction kits. Each extracted DNA sample was run in duplicate using the oRDx-LCCA. 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 oRDx-LCCA.

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 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 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 30. All studies resulted in zero failures and 100% agreement across conditions as shown in Table 30.

Table 30 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%)

Process	Variable	Nominal value/ range	Test values	TP	FP	FN	TN	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
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 User Manual	Standard - 1°C	6	0	0	1254	100% (61.0%, 100%)	100% (99.7%, 100%)
			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 oRDx-LCCA, 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 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 oRDx-LCC assay was observed for each substance and at each level tested.

Table 31 Agreement results of interfering substances study

Study	Substance	Test value	TP	FP	FN	TN	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%)

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 32. No clear trend in decreasing performance with increasing necrotic fraction in the sample was observed supporting the conclusion that the performance of the oRDx-LCCA is robust within the recommended range of necrotic content less than 50%.

Table 32 Summary of assay performance by necrotic content bin

Indication	%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
	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 is provided based on the CCD1 and FCD results only. For details on the discordant results, see Table 39 and Table 41.

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 oRDx-LCCA 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 oRDx-LCCA results are likely correct.

Abbreviations: Lib=sample library

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 were 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 oRDx-LCCA 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 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 oRDx-LCCA kit components for all 3 lots evaluated. The shelf-life stability will continue to be evaluated to extend the shelf-life stability claim.

Table 33 Agreement results for kit reagent shelf-life stability study

KIT LOT	Timepoint (months)	PPA (2-sided 95% CI)	NPA (2-sided 95% CI)
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%)
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%)
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 18) 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 34 below.

Table 34 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%)

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 temperature 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 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 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 35 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%)
			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

The stability of FFPE clinical samples (section and block) was assessed using both retrospective and a real-time analysis.

Retrospective analysis leveraged data from the clinical study and paired observed performance parameters with FFPE block age as determined by the difference between the reported year of specimen collection and the year when specimens were tested. Analysis focused on the QC metrics used throughout the oRDx-LCCA workflow: sample library concentration, PiVAT valid/invalid status, and concordance with results determined by the appropriate comparator assay. Results are summarized in Table 36 below. No significant trend in poorer overall performance with increasing FFPE block age was observed, and robust assay performance was observed for samples over 10 years old. A post-market study will be conducted to prospectively test FFPE block stability for CDx variants.

Table 36 Retrospective analysis of the performance of sample libraries prepared from clinical FFPE specimens of various ages and with known comparator test results

Tumor	FFPE Block Age (years)	# Tested	# Lib Yield QC Fail	# Lib Yield QC Pass	# PiVAT® Invalid	# PiVAT® Valid	# Included in Analysis	# of CCD-	# of CCD+	# CCD- Concordant	# CCD+ Concordant	% FCD Discordant
CRC	0-2	29	0	29	0	29	29	16	13	15	13	3.4%
	3-5	38	0	38	0	38	38	28	10	28	10	0.0%
	6-10	20	0	20	0	20	20	14	6	14	6	0.0%
	11-20	1	0	1	0	1	1	1	0	1	0	0.0%
	>21	2	0	2	0	2	2	1	1	1	1	0.0%

Tumor	FFPE Block Age (years)	# Tested	# Lib Yield QC Fail	# Lib Yield QC Pass	# PiVAT® Invalid	# PiVAT® Valid	# Included in Analysis	# of CCD-	# of CCD+	# CCD- Concordant	# CCD+ Concordant	% FCD Discordant
	Total	90	0	90	0	90	90	60	30	59	30	1.1%
NSCLC	0-2	0	0	0	0	0	0	0	0	0	0	n/a
	3-5	35	0	35	0	35	35	1	34	1	34	0.0%
	6-10	1	0	1	0	1	1	1	0	1	0	0.0%
	11-20	2	0	2	0	2	2	1	1	1	1	0.0%
	>20	0	0	0	0	0	0	0	0	0	0	n/a
	Total	38	0	38	0	38	38	3	35	3	35	0.0%
All	0-5	29	0	29	0	29	29	16	13	15	13	3.4%
	6-10	73	0	73	0	73	73	29	44	29	44	0.0%
	11-15	21	0	21	0	21	21	15	6	15	6	0.0%
	15-20	3	0	3	0	3	3	2	1	2	1	0.0%
	>20	2	0	2	0	2	2	1	1	1	1	0.0%
	Total	128	128		128		128	128		127		0.5%

Abbreviations: Lib=sample library, CCD=FDA-approved comparator companion diagnostic (+/- indicate whether test was positive or negative), FCD=follow-on companion diagnostic (oRDx-LCCA)

Stability of FFPE 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 6 months and 6.5 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. PPA was 100% (95% CI: 80.6%,100%) (16/16) and NPA was 100% (95% CI: 99.8%,100%) (2000/2000) for the timepoints 6 months and 6.5 months compared to baseline testing.

Data presented here supports a claim of DNA storage stability at 8 months at 4°C and 6 months at -20°C. The DNA stability at -20°C will continue to be evaluated to extend the stability claim. 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.

Stability of Assay Intermediates

The workflow for the oRDx-LCCA 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 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 oRDx-LCCA performance.

Table 37 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%)
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 was stored for 27 days at -25 to -10°C before being sequenced.

Table 38 Stability of assay intermediates

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

Indexed libraries	-15°C to -25°C	90 days

SUMMARY OF CLINICAL PERFORMANCE

The reasonable assurance of safety and effectiveness for oRDx-LCCA CDx claims 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 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]. oRDx-LCCA 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 population for that specific device.

oRDX-LCCA 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 oRDx-LCCA (FCD) and the approved Roche Molecular Systems' **cobas** v2 EGFR Mutation Test (CCD). As the *EGFR* mutations are relatively infrequent in the intended 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 39.

Table 39 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 oRDx-LCCA 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 oRDx-LCCA 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 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 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 oRDx-LCCA and **cobas** EGFR Mutation Test v2 is non-inferior to the agreement between two replicates of CCD by a margin of 4%.

Table 40 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 oRDx-LCC Assay, must be estimated experimentally and was estimated to be 0.07 † Adjusted for variant enrichment in study design using the parameter Pc	

oRDX-LCCA 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 oRDx-LCCA (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 41.

Table 41 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 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 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 oRDx-LCCA and QIAGEN *therascreen* KRAS RGQ PCR is non-inferior to the agreement between two replicates of CCD by a margin of 5%.

Table 42 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 oRDx-LCC Assay, must be estimated experimentally, for the case of KRAS codon 12 and 13 mutations, 0.36 † Adjusted for enrichment	

LIMITATIONS

1. The oncoReveal Dx Lung and Colon Cancer Assay has only been validated for use with CRC and NSCLC tumor tissues. Test only the indicated tissue types.
2. The oncoReveal 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 oncoReveal Dx Lung and Colon Cancer Assay only determines the presence or absence of the *KRAS* and *EGFR* mutations listed in Table 1 of the Intended Use.
8. 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.
9. 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 (3.0-3.7%) (see Analytical Sensitivity: Limit of Detection section).
10. This assay does not interrogate all variants or genes (*NRAS*) that confer resistance to cetuximab and panitumumab.
11. The oncoReveal 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 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. 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	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.

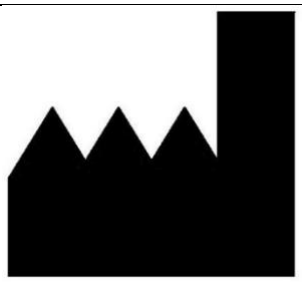
Issue	Potential Cause	Solution
under-cluster on the MiSeqDx	Improper library quantification	Improper library quantification may result in artificially high or low yields, which affects downstream normalization. Re-quantify the final libraries and/or the normalized libraries to check for the expected 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. 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



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