Pillar Biosciences oncoReveal Solid Tumor 22 Gene Panel on Biomek NGeniuS System

App Template Version 1.0.5

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App Template Description

The App Template for Pillar Biosciences oncoReveal Solid Tumor 22 gene panel prepares sample DNAs for sequencing by amplifying target regions containing mutational hot spots of 22 relevant genes using SLIMamp® (stem-loop inhibition mediated amplification) technology. The oncoReveal Solid Tumor 22 gene App Template supports 10-80 ng per PCR reaction for both standard genomic DNA and FFPE DNA. Libraries can be sequenced on Illumina MiSeq or NextSeq.

The App Template allows the user to produce between four and twenty-four libraries in a single continuous batch run. Optionally, users may select from multiple starting and stopping points. The App Template was designed using the Pillar oncoReveal Solid Tumor panel 22 gene kit (HDA-ST-1001-24). Ethanol wash volumes have been reduced to 60µL from 150µL to reduce tip consumption and sample processing time.

In addition to the consumables listed in the oncoReveal Solid Tumor 22 gene assay User Guide (UM-0018), the following consumables are required for a full run:

- 2.0mL Sarstedt Tubes – Skirted Base (Sarstedt P/N: 72.664)
- 5.0mL Sarstedt Tubes – False Bottom with Flat Base (Sarstedt P/N: 60.611.310)
- Axygen® 96-well Polypropylene PCR Microplate, Full Skirt, Clear, Nonsterile (Corning P/N: PCR-96-FS-C)

For research use only. Not for use in diagnostic procedures.

Scoping

Scoping

- Author
  - Pillar scientists with support from Beckman Coulter Life Sciences
- Kit
  - oncoReveal Solid Tumor 22 Gene
    - Version 1.6.0 (User Guide UM-0018)
- Supported
    - DNA Input 10 - 80 ng in 6.5 μL
    - Genomic DNA & FFPE DNA

Scoping

- Kit part number
  - oncoReveal Solid Tumor panel 22 gene kit (HDA-ST-1001-24)

- The following index kit was used for demonstration
  - IDX-AI-1001-96 – Pillar TSCA Index Primers Kit A, 32 Combinations, 96 reactions

App Details

Sections Automated

App Sections
Gene-Specific PCR Sample Prep
Gene-Specific PCR Amplification
Gene-Specific PCR Product Purification
Indexing PCR Sample Prep
Indexing PCR Amplification
Indexing PCR Product Purification

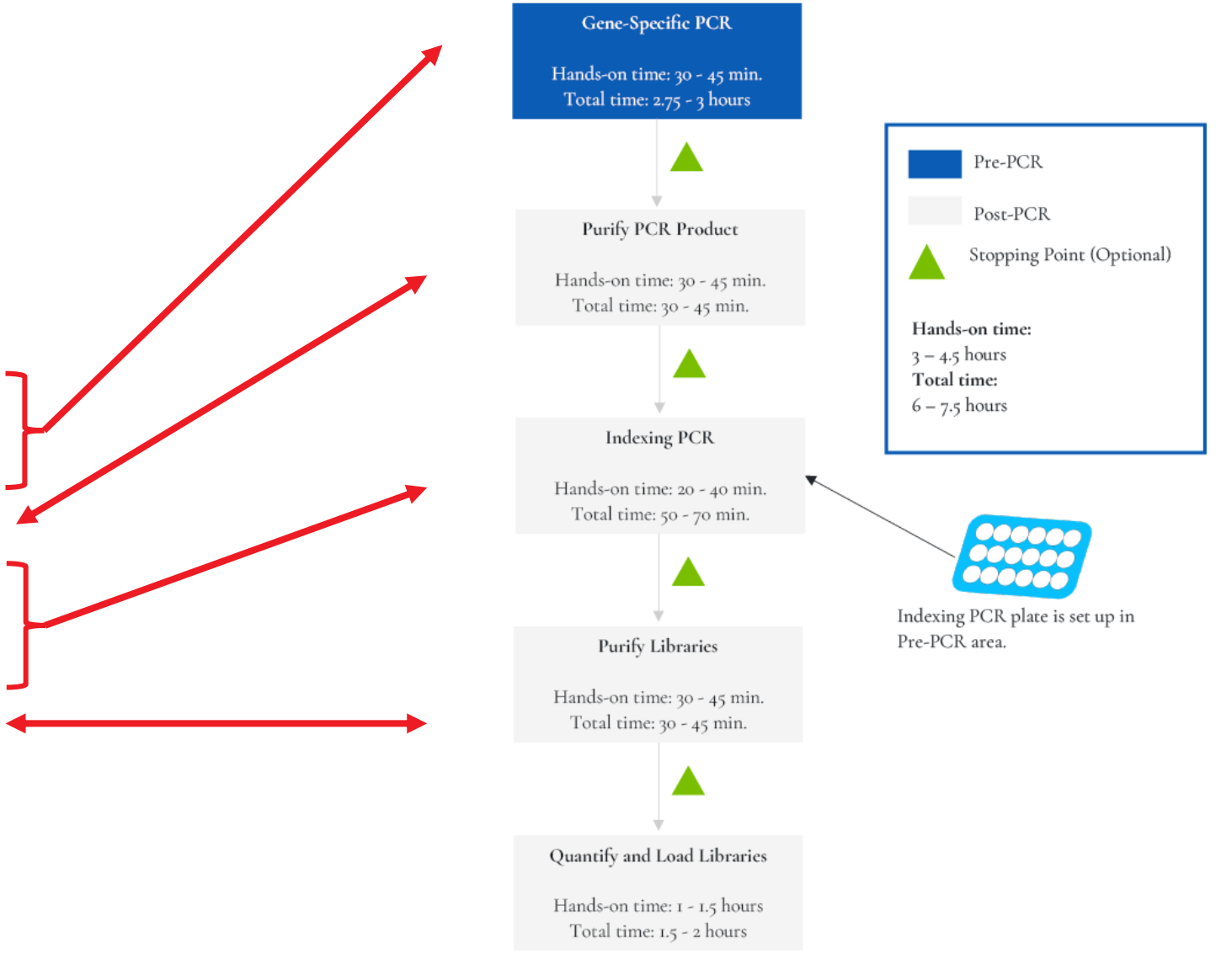


Figure 2. Library preparation workflow for oncoReveal™ Lung and Colon Cancer Panel. The workflow can be completed within one day but contains multiple optional stopping points for users with time constraints.

Section Details

App Sections
Gene-Specific PCR Sample Prep
Gene-Specific PCR Amplification
Gene-Specific PCR Product Purification
Indexing PCR Sample Prep
Indexing PCR Amplification
Indexing PCR Product Purification

PCR steps are each split into two sections.
* Sample Prep adds the reaction mixes to the DNA
* Amplification does the thermal cycling

This allows for the Sample Prep and Amplification to be prepared in pre-PCR / post-PCR settings if desired (and if equipment allows), per the User Guide, although it is not necessary in automation.

If starting at Gene-Specific PCR Amplification, the Gene-Specific PCR Master Mix, LC oligo pool, and UDG must have already been added to the samples.

If starting at Indexing PCR Amplification, the Indexing PCR Master Mix and indices must have already been added to the samples.

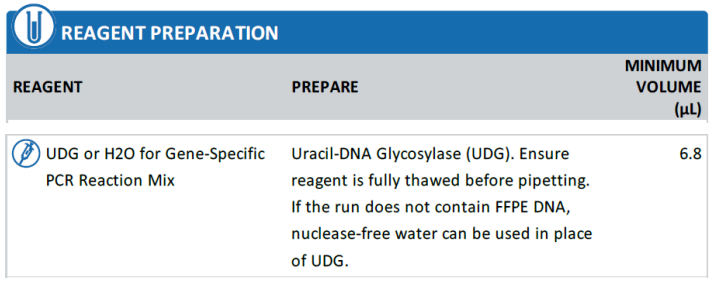
Section Details

App Sections
Gene-Specific PCR Sample Prep
Gene-Specific PCR Amplification
Gene-Specific PCR Product Purification
Indexing PCR Sample Prep
Indexing PCR Amplification
Indexing PCR Product Purification

The App allows for processing both gDNA and FFPE in a single batch run.

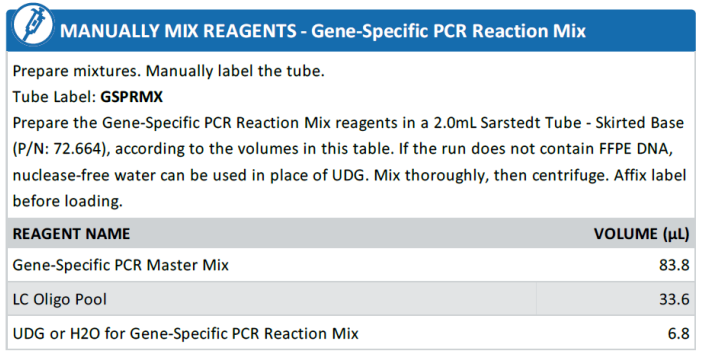
- If *any* sample is FFPE, then Uracil-DNA Glycosylase (UDG) must be included. UDG will not negatively impact performance of gDNA samples. UDG is user-supplied.
- If only gDNA is being processed, the UDG is optional, and can be replaced with H_2O.
- The Gene-Specific PCR *Reaction* Mix is provided to the system as a user-created manual mix.

In the Work Aid's Reagent Preparation and Manually Mix Reagents sections, the above is mentioned.



REAGENT PREPARATION

REAGENT	PREPARE	MINIMUM VOLUME (µL)
UDG or H2O for Gene-Specific PCR Reaction Mix	Uracil-DNA Glycosylase (UDG). Ensure reagent is fully thawed before pipetting. If the run does not contain FFPE DNA, nuclease-free water can be used in place of UDG.	6.8



MANUALLY MIX REAGENTS - Gene-Specific PCR Reaction Mix

Prepare mixtures. Manually label the tube.
Tube Label: **GSPRMX**
Prepare the Gene-Specific PCR Reaction Mix reagents in a 2.0mL Sarstedt Tube - Skirted Base (P/N: 72.664), according to the volumes in this table. If the run does not contain FFPE DNA, nuclease-free water can be used in place of UDG. Mix thoroughly, then centrifuge. Affix label before loading.

REAGENT NAME	VOLUME (µL)
Gene-Specific PCR Master Mix	83.8
LC Oligo Pool	33.6
UDG or H2O for Gene-Specific PCR Reaction Mix	6.8

Section Details

App Sections
Gene-Specific PCR Sample Prep
Gene-Specific PCR Amplification
Gene-Specific PCR Product Purification
Indexing PCR Sample Prep
Indexing PCR Amplification
Indexing PCR Product Purification

The App, as designed by Pillar, does not make use of the Micronic-compatible carousel for introducing indices onto the Biomek NGeniuS system.

- 5 µL of each forward and reverse primer are manually added to an Axygen 96-well PCR Microplate (P/N: PCR-96-FS-C)
- Biomek NGeniuS system will aliquot out of the microplate into a cold storage RV.

In the Work Aid's Reagent Preparation section, the above is mentioned.

REAGENT PREPARATION

REAGENT	PREPARE	MINIMUM VOLUME (µL)
Indices IndexPlate: default	Add 5 µL of the assigned forward and reverse indexing primers to wells of an Axygen 96-well Polypropylene PCR Microplate, Full Skirt, Clear, Nonsterile (P/N: PCR-96-FS-C). It is recommended to match sample and index well positions. Centrifuge before use to ensure there are no bubbles. Wells: A1, B1, C1, D1	10.0

Section Details

App Sections
Gene-Specific PCR Sample Prep
Gene-Specific PCR Amplification
Gene-Specific PCR Product Purification
Indexing PCR Sample Prep
Indexing PCR Amplification
Indexing PCR Product Purification

The Indexing PCR *Reaction* Mix is provided to the system as a user-created manual mix (a dilution of the stock Indexing PCR Master Mix per the User Guide).

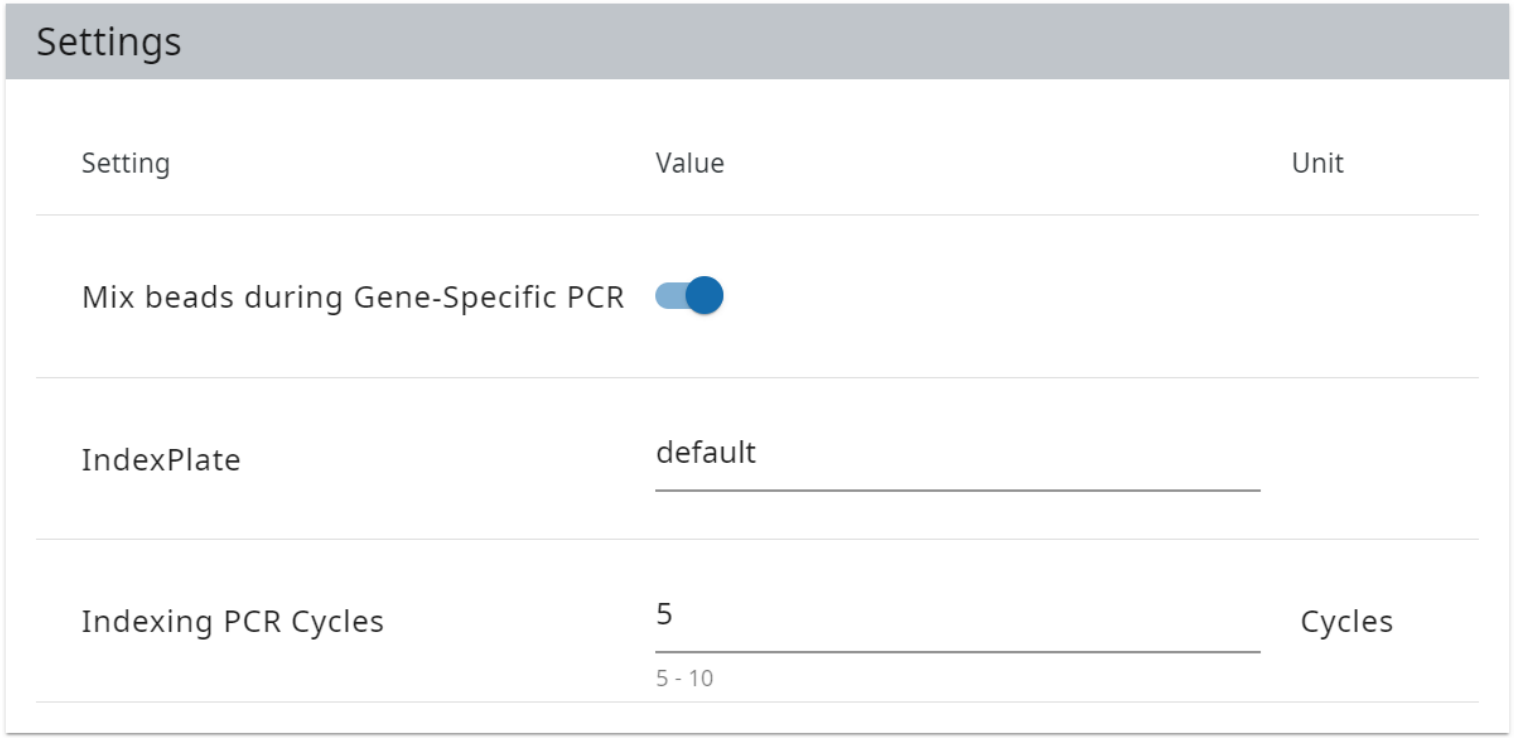
In the Work Aid's Reagent Preparation section, the above is mentioned.

MANUALLY MIX REAGENTS - Indexing PCR Reaction Mix

Prepare mixtures. Manually label the tube.
Tube Label: **IPRMX**
Prepare the Indexing PCR Reaction Mix reagents in a 2.0mL Sarstedt Tube - Skirted Base (P/N: 72.664), according to the volumes in this table. Mix thoroughly, then centrifuge. Affix label before loading.

REAGENT NAME	VOLUME (µL)
Indexing PCR Master Mix	148.7
H2O for Indexing PCR Reaction Mix	65.4

App Settings



Setting	Description
Mix beads during Gene-Specific PCR	When on, uses extra tips and mixes AMPure beads during gene-specific PCR to prevent them from settling. If not selected, mixing will only occur directly before gene-specific product purification.
IndexPlate	Allows the operator to enter in a name for the index plate being used in the batch.
Indexing PCR Cycles	Allows the operator to set the number of indexing PCR cycles performed within a range of 5-10.

Requested Reagent Volumes

Reagent	HDA-ST-1001-24 kit volumes[1]	4 samples volume requested	8 samples volume requested	16 samples volume requested	24 samples volume requested
Gene-Specific PCR Master Mix [2]	480	83.8	133.8	233.8	333.8
LC Oligo Pool [2]	200	33.6	53.6	93.6	133.6
UDG [2,3]	User supplied	6.8	10.8	18.8	26.8
Indexing PCR Master Mix [4]	910	155.6	255.6	455.6	655.6
AMPure XP	User supplied	720	1240	2120	3000
70% EtOH	User supplied	4960	5920	7840	9760

[1] All values in µL, consumed volumes are less than requested due to source labware dead volume requirements
[2] Reagents are combined as a manual mixture with user-supplied UDG or H_20 to create Gene-Specific PCR Reaction Mix
[3] UDG volumes are listed as if at least one FFPE sample was being processed, otherwise UDG is replaced by H_2O
[4] Reagent is combined with H_2O as a manual mixture to create Indexing PCR Reaction Mix

Batch Runs Per Kit

Batch size	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Batches per kit	5	4	4	3	3	3	3	2	2	2	2	2	2	1	1	1	1	1	1	1	1
Samples	20	20	24	21	24	27	30	22	24	26	28	30	32	17	18	19	20	21	22	23	24
Largest batch with leftover volume	0	4	0	6	0	0	0	8	6	4	0	0	0	15	14	13	12	11	10	9	8
Total samples from kit	20	24	24	27	24	27	30	30	30	30	28	30	32	32	32	32	32	32	32	32	32

- The ***Batch size*** can be run ***Batches per kit*** times, leaving enough reagent volume to do one additional batch with ***Largest batch with leftover volume*** samples.
- Run combinations calculated based on reagent vial volumes provided by Pillar.
- 5 µL of each index (A50*X* and A70*X*) is required per sample. The IDX-AI-1001-96 index kit has enough reagent per-index to support 96 samples-worth of Biomek NGeniuS system batch runs.

Estimated Time of Completion

Samples	4	8	16	24
Index Aliquot	00:01	00:01	00:02	00:03
Reagent Aliquot	00:11	00:14	00:17	00:19
Processing	04:12	04:19	04:56	05:35
Total ETC	04:25	04:35	05:16	05:58

Times (hours:minutes) calculated based on 5 indexing PCR cycles, bead mixing, and with all plate-based indices contiguous starting with well A1. Does not include times needed for manual interactions *(e.g.*, reagent thawing, manual pipetting, placing labware into Biomek NGeniuS System, …).

Consumables

		Batch Size (samples)			
Consumable	**Part number**	**4**	**8**	**16**	**24**
RVs	C62705	7	7	7	7
Bulk Reservoirs	C62707	1	1	1	1
Lids	C62706	4	4	4	4
Millitips (boxes)	C59585	8 (1)	8 (1)	8 (1)	8 (1)
Microtips (boxes)	C62712	108 (1)	216 (1)	416 (2)	616 (2)
Seal plate	C70665	1	1	1	1
5.0 mL Sarstedt® vial	60.611*	1	1	1	1
2.0 mL Sarstedt® vial	72.664*	2	2	2	2
Axygen® 96-well PCR Microplate	PCR-96-FS-C*	1	1	1	1
Price Per Sample ($)**	-	37.84	18.92	11.14	7.42

Costs assume using fresh tip boxes. Some clean tips will remain each run, reducing cost of subsequent runs.
* 3rd party part numbers.
** Costs do not include Sarstedt reformat vials, Axygen plate, or empty tip boxes for tip disposal.

Demonstration Data

Experimental Design for Demonstration Run Conditions

Experiment	Sample Throughput	Target Sample Mass (ng)	DNA Type	PCR Cycles	Instrument	Operator(s)
1	4*	80	gDNA + FFPE DNA	5	A	A
2	9*	40	gDNA + FFPE DNA	5	B	B
3	24**	10	gDNA + FFPE DNA	5	A	A

* One negative control (H_2O) per experiment
** Two negative controls (H_2O) per experiment

All experiments used Pillar TSCA Index Primers Kit A

The nM concentration was determined using the following formula:

LibraryConcentration[nM] = LibraryConcentration[ng/µL] x 5.

The ng/µL concentration of the samples was determined using a Qubit dsDNA HS Assay Kit.

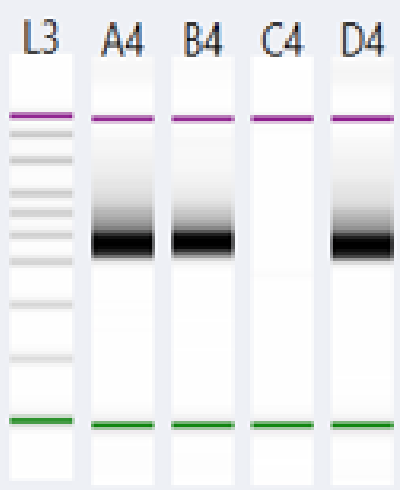
Library Construction Pass Criteria:

- Sample library + positive control library yields >3.5nM
- Non-Template control <2.0nM

Demo run 1, 4 samples, high input

Sample:	gDNA + FFPE DNA
Load concentration (ng/µL):	12.3
Library Prep Input Mass (ng):	80
PCR Amplification Cycles:	5
Version:	1.0.1

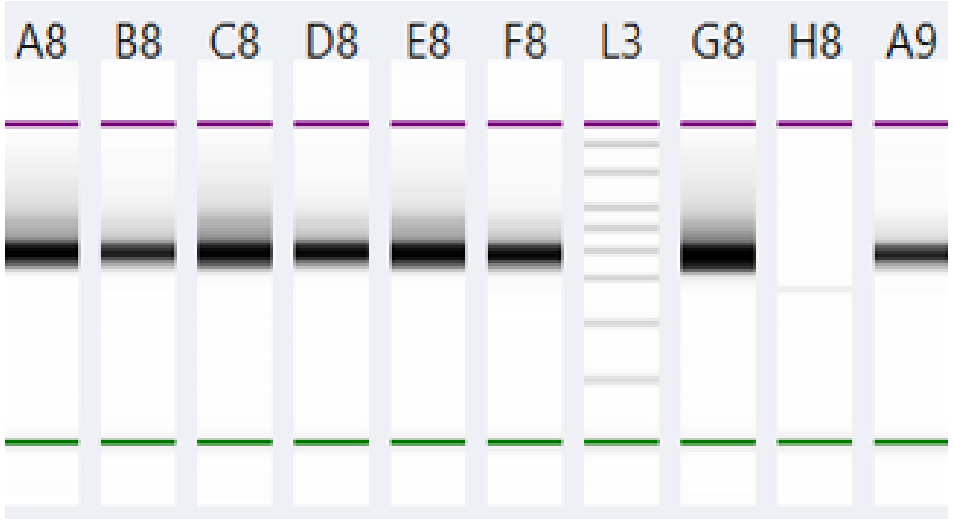
Sample	Stock conc (ng/µL)	Index	Library Yield (nM)
gDNA Sample 1	14.6	501, 706	62.5
FFPE Sample 1	13.3	502, 706	59.5
Negative Control	0	503, 706	0
gDNA Sample 2	14.6	504, 706	70.5



Demo run 2, 9 samples, mid input

Sample:	gDNA + FFPE DNA
Load concentration (ng/μL):	6.2
Library Prep Input Mass (ng):	40
PCR Amplification Cycles:	5
Version:	1.0.1

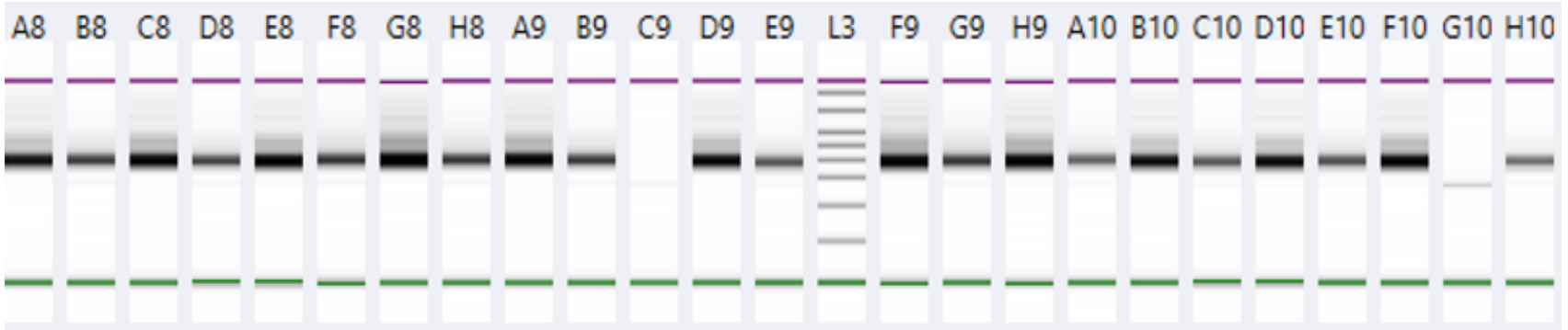
Sample	Stock conc (ng/μL)	Index	Library Yield (nM)
gDNA Sample 1	14.6	505, 706	74
FFPE Sample 1	13.3	506, 706	47.5
gDNA Sample 2	14.6	507, 706	84
FFPE Sample 2	13.3	508, 706	45.75
gDNA Sample 3	14.6	501, 705	70
FFPE Sample 3	13.3	502, 705	55
gDNA Sample 4	14.6	503, 705	88
Negative Control		504, 706	0
FFPE Sample 4	13.3	505, 706	39



Demo run 3, 24 samples, low input

Sample	Stock conc (ng/µL)	Index	Library Yield (nM)
gDNA Sample 1	14.6	506, 705	26.1
FFPE Sample 1	4.5	507, 705	11.05
gDNA Sample 2	14.6	508, 705	26.2
FFPE Sample 2	4.5	501, 704	10.1
gDNA Sample 3	14.6	502, 704	26.7
FFPE Sample 3	4.5	503, 704	11
gDNA Sample 4	14.6	504, 704	31.95
FFPE Sample 4	4.5	505, 704	10.85
gDNA Sample 5	14.6	506, 704	27
FFPE Sample 5	4.5	507, 704	10.3
Negative Control 1	0	508, 704	0.3
gDNA Sample 6	14.6	501, 703	24.2
FFPE Sample 6	4.5	502, 703	9.95
gDNA Sample 7	14.6	503, 703	27.75
FFPE Sample 7	4.5	504, 703	11.35
gDNA Sample 8	14.6	505, 703	26.35
FFPE Sample 8	4.5	506, 703	7.95
gDNA Sample 9	14.6	507, 703	21.95
FFPE Sample 9	4.5	508, 703	9.35
gDNA Sample 10	14.6	501, 702	19.2
FFPE Sample 10	4.5	502, 702	8
gDNA Sample 11	14.6	503, 702	24.35
Negative Control 2	0	504, 702	0.735
FFPE Sample 11	4.5	505, 702	9

Sample:	gDNA + FFPE DNA
Load concentration (ng/µL):	1.55
Library Prep Input Mass (ng):	10
PCR Amplification Cycles:	5
Version:	1.0.1



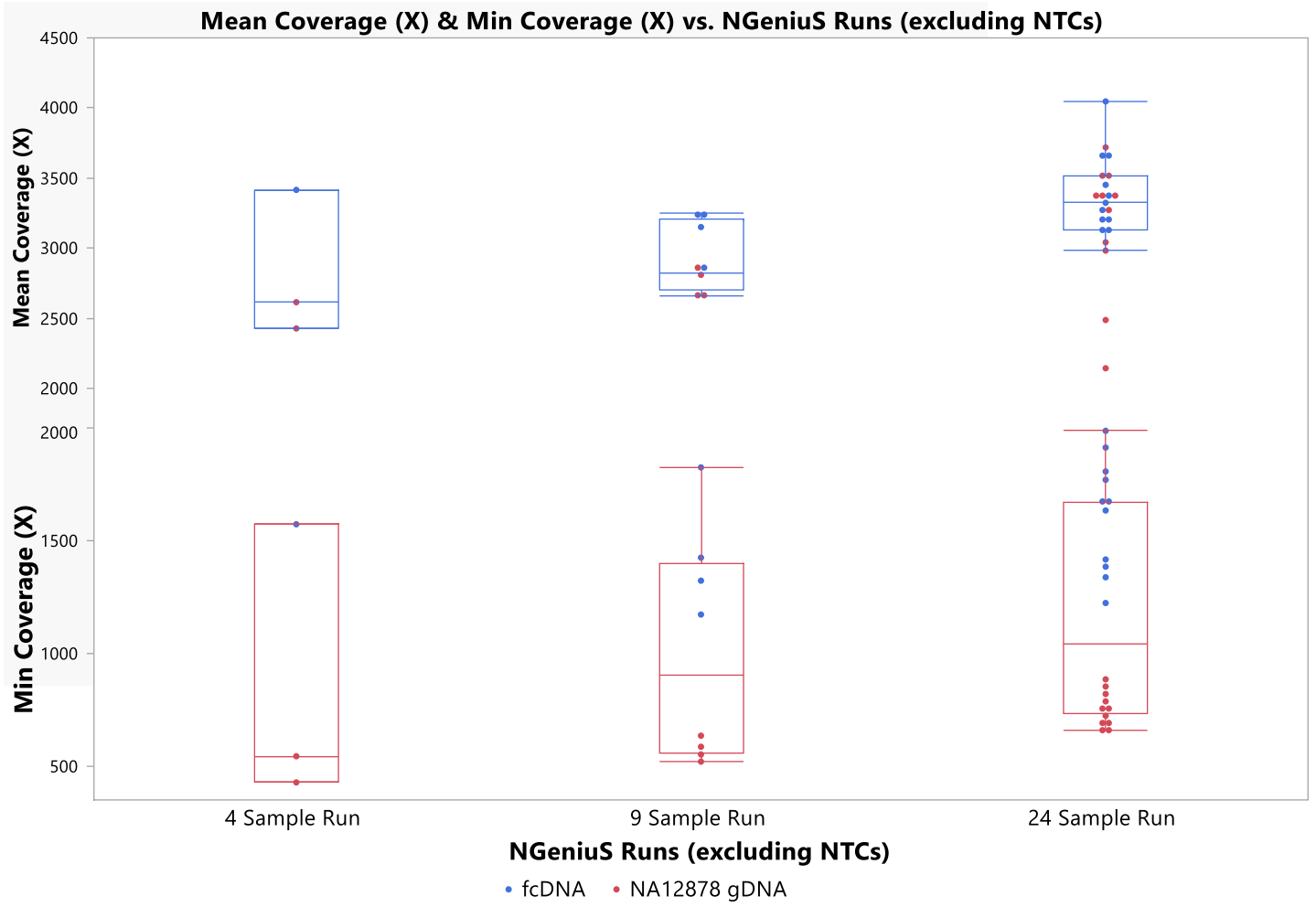
Sequencing and Variant Analysis

Sequencing QC Results - Summary

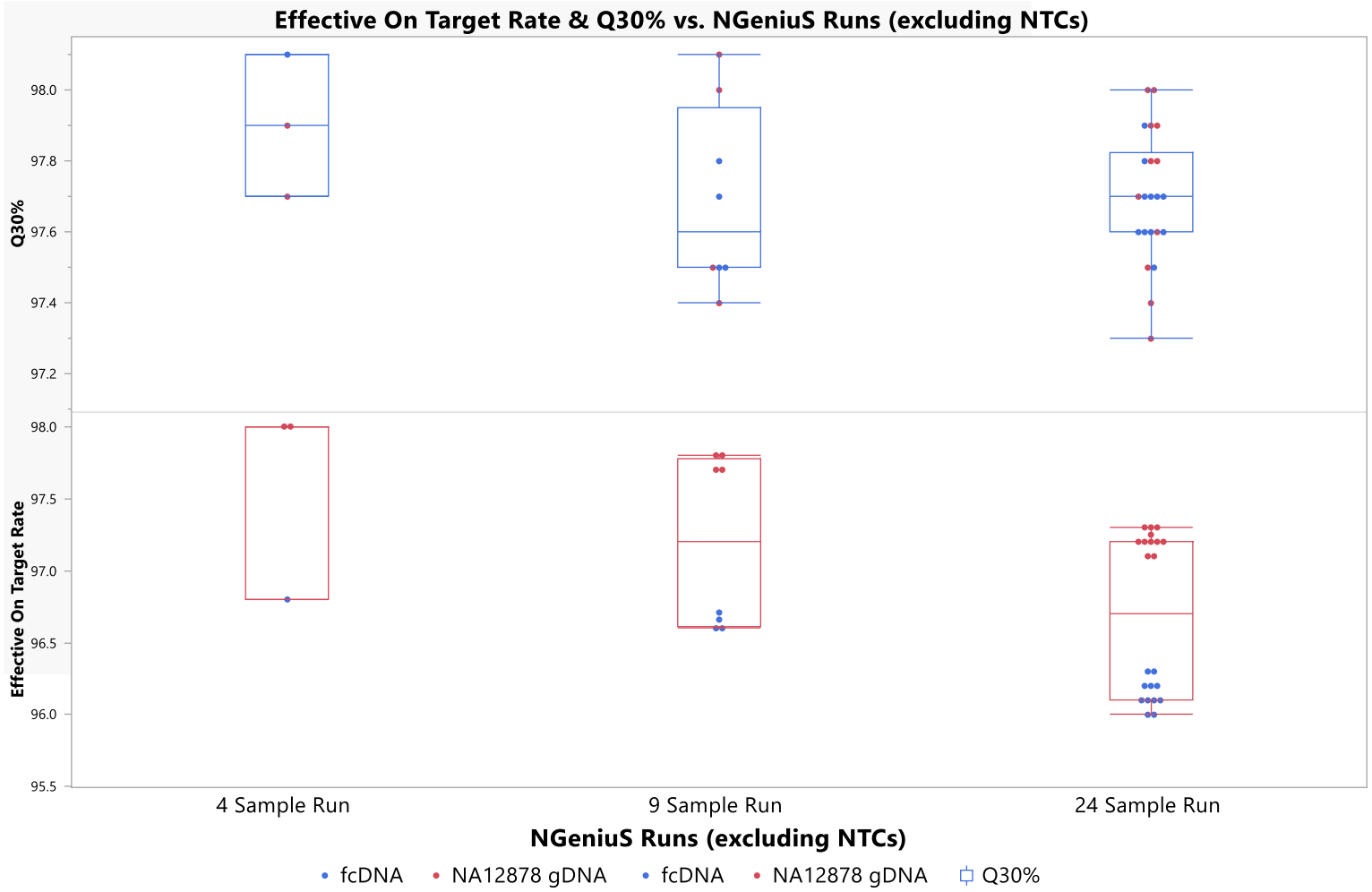
QC Metric	Passing Criteria	Average fcDNA	fcDNA Passing Samples	Average gDNA	gDNA Passing Samples
Mean Coverage (All Amplicons)	≥ 1500X	3330	100% (16/16)	2986	100% (17/17)
Minimum Coverage (Individual Amplicons)	≥ 200X	1569	100% (16/16)	673	100% (17/17)
Overall Q30 (%)	≥ 75%	97.7	100% (16/16)	97.7	100% (17/17)
Effective on Target Rate (%)	≥ 70%	94.5	100% (16/16)	97.0	100% (17/17)

All sequencing runs pooled to 2 nM, then run on NextSeq 1000. (> 110Gb of sequence data per run, ≥ 85% of bases higher than Q30 at 2 × 150 bp)

Sequencing QC Results – By Sample



Sequencing QC Results – By Sample



Variant Call Summary

Gene	Variant	Expected Allele Frequency (%)	Allele Frequency Cutoff (%)	Average Actual Allele Frequency (%), 10 ng DNA Input	Average Actual Allele Frequency (%), 40 ng DNA Input	Average Actual Allele Frequency (%), 80 ng DNA Input	Average Actual Allele Frequency w/ Standard Deviation (%), All Runs	Variant Detection Rate
BRAF	V600E	13.61	1	14.15	15.08	14.71	14.42 ± 1.42	16/16
EGFR	ΔE746-A750	1.84	1	1.90	2.10	1.78	1.94 ± 0.51	16/16
EGFR	L858R	3.65	1	4.35	4.58	4.66	4.43 ± 0.72	16/16
EGFR	T790M	1.31	1.5	1.79	1.30	1.94	1.98 ± 0.38	10/16*
EGFR	G719S	23.4	1.5	24.29	24.82	23.72	24.39 ± 1.21	16/16
KRAS	G13D	15.3	1.5	16.48	16.13	16.37	16.38 ± 1.11	16/16
KRAS	G12D	5.39	1.5	5.48	5.09	6.38	5.43 ± 0.79	16/16
NRAS	Q61K	10.6	1	11.11	11.45	11.78	11.24 ± 1.5	16/16
PIK3CA	H1047R	18.9	3.2	19.03	19.91	19.55	19.28 ± 1.32	16/16
PIK3CA	E545K	7.88	3.2	8.51	8.74	8.95	8.59 ± 0.91	16/16

*The expected allele frequency for EGFR T790M is below the cutoff for this assay. As such, a 100% detection rate is not expected. The Average Actual Allele Frequency is calculated from the 10 variant calls passing the Allele Frequency Cutoff.

Demonstration Summary

Demonstration Summary

- The oncoReveal Solid Tumor 22 Gene App, written by Pillar, on the Biomek NGeniuS Next Generation Library Prep System prepares libraries at input masses between 10 and 80 ng of genomic DNA
- Yield at all tested input masses exceeded 3.5 nM final concentration
- NextSeq 1000 sequencing data of prepared libraries passes Pillar metrics for:
  - Mean coverage
  - Minimum coverage
  - Overall %Q30
  - Effective on target rate
  - Variant Detection rate for genes of interest

General automation considerations

- Please read and understand Biomek NGeniuS System IFU, C43212.
- Spin down index plate before use to make sure indices are at the bottom of wells
- Do not use unsupported index plates
  - Only Axygen® PCR-96-FS-C is supported for this App
  - If the plate geometry is not the same, it could result in an instrument crash
- Make sure foil, if present, of each index well is widely opened to prevent tip-friction binding and lifting of Index Plate
  - Use a *new* P200 or P1000 to pierce and widen *each* well being used
- Avoid bubbles in reagent tubes to ensure accurate liquid level sensing and aliquoting
- The Work Aid requests more volume than what is consumed
  - Dead volume is needed in source tubes to ensure enough is available due to tolerance stack-ups
- Dead volume will be left behind in some storage wells
  - The nature of automation, tolerance stack-ups, and environment necessitates some overage
- Make sure bulk reagents wet the entire length of reservoir
  - Ensures accurate liquid volume sensing
- Prepare samples while Biomek NGeniuS system is aliquoting reagents
  - Avoids sample evaporation while Biomek NGeniuS system is preparing run

oncoReveal Solid Tumor 22 Gene Panel App specific considerations

- Master mixes are manually prepared to reduce dead volumes and maximize kit usage
- Index plate is manually prepared to reduce dead volumes and maximize kit usage
- An excess (~4mL) of H_2O and EtOH is called for in the Work Aid to speed sample processing

App Template Revision Notes

- 1.0.1 Demonstration data obtained.
- 1.0.5 Updated preparation instructions and thermal cycler cooldown if early stopping points are chosen. Chemistry unaffected.