SNAQ™-SEQ INTERNAL STANDARDS: QUALITY CONTROL TECHNOLOGY THAT IMPROVES NGS ACCURACY

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A higher standard of accuracy

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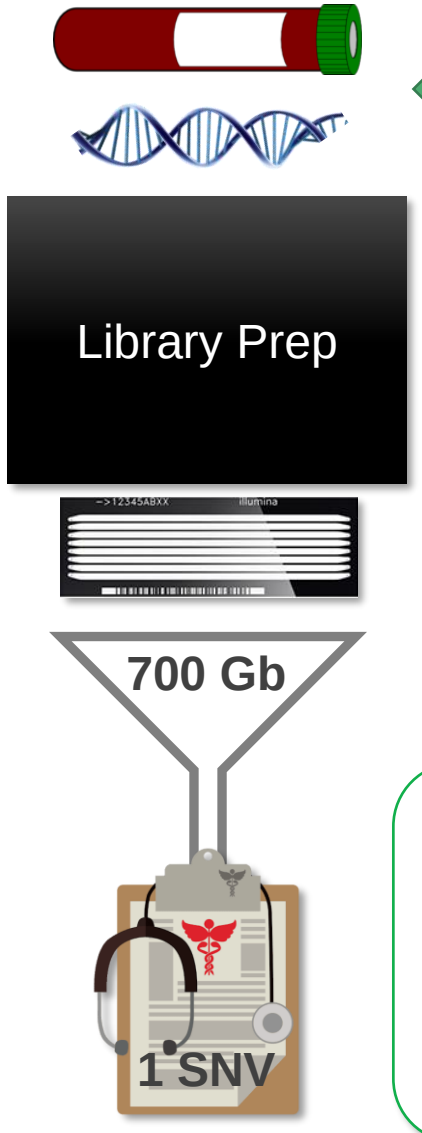
NGS IS A CRITICAL BUT COMPLEX TOOL

- Flexible tool for detecting clinically actionable variants
- Overkill, 0.07% sequence relevance
- Complex testing procedure
  - Microscale fluidics & detection
  - Bioinformatics on Gb sequence
- Modest pass/fail QC procedures
  - Sample input level
  - Insert yields
  - Flowcell metrics
  - PhiX & reference sample
  - Unique Molecular Indices (UMI)



SNAQ-SEQ Internal Standards

140 CONSUMABLES

362 STEPS

52 INSTRUMENT INTERACTIONS

TRILLIONS OF REACTIONS

MANY BIOINFORMATIC STEPS

- **Validation is *not adequate assurance* of tomorrow's performance**
- **Good QC critical for low signal events: ctDNA, exon loss, limits of detection**

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SNAQ-SEQ
LIMIT OF BLANK

- ✓ QC for Variant Calling
- ✓ Increased Accuracy

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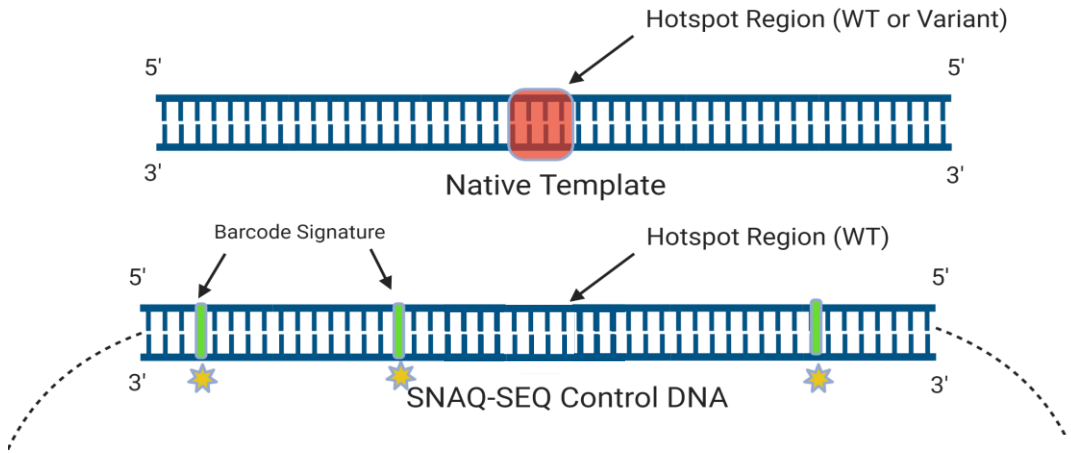
STANDARDIZED **N**UCLEIC **A**CID **Q**UANTIFICATION FOR **SEQ**UENCING (SNAQ-SEQ)



- Based on use of **I**nternal **S**tandards (IS)
  - Designed to clinical ROI
  - Reference sequence manufactured with 10^{-8} error rate
  - Intermittent base changes enable bioinformatic separation
  - Biochemically mimic sample except issues arising from pre-damaged DNA (e.g., FFPE)
  - Added to every sample prior to library prep

- Limit of Blank
  - Ultimate negative control
  - Mimic sample sequencing errors
  - Applications: low VAF
- Accurate quantification
  - Ratio between sample and IS maintained
  - Knowing IS input and ratio enables accurate quantification of input template
  - Applications: CNV, ctDNA/ml plasma, TA

Quality Control for EVERY variant in EVERY sample

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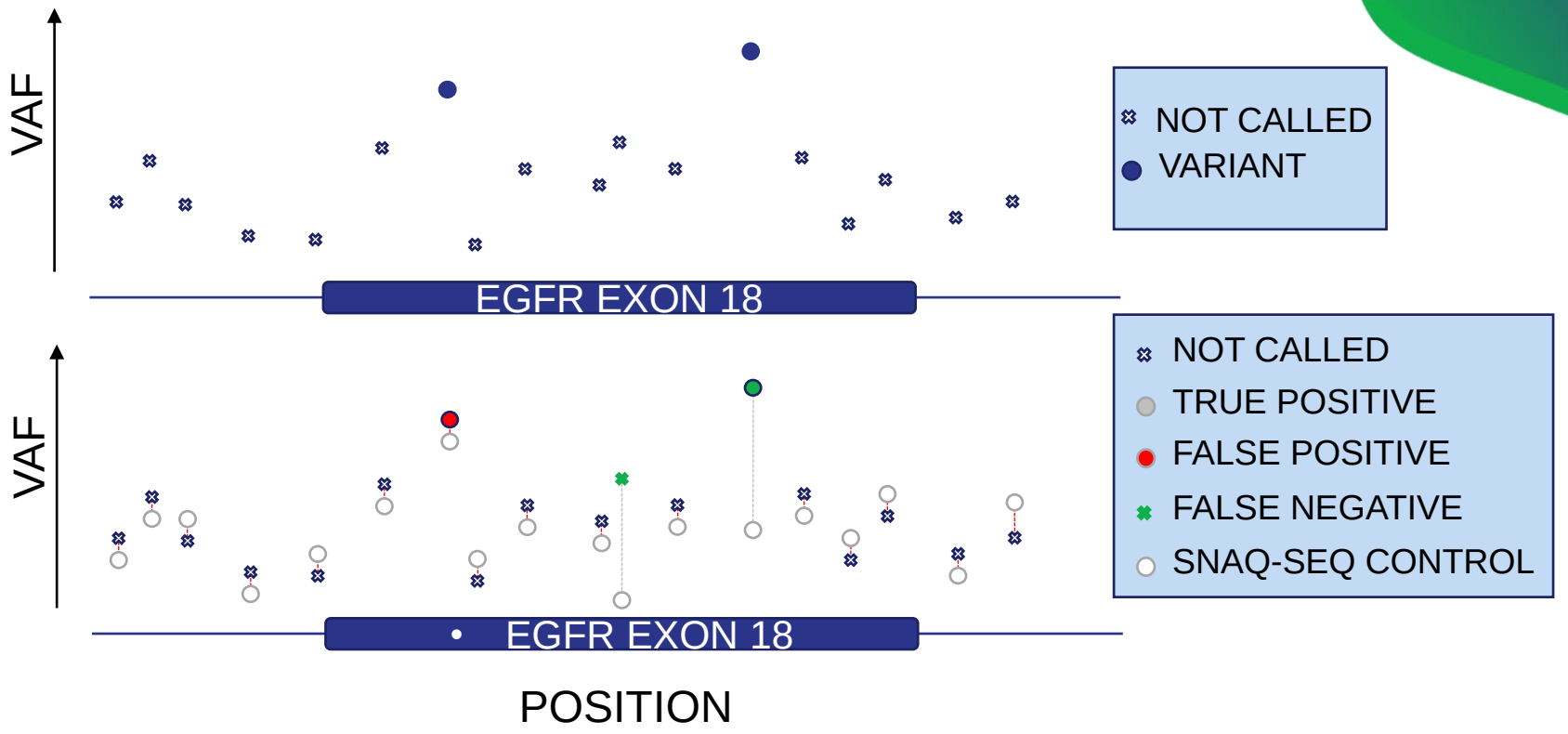
SNAQ-SEQ *LIMIT OF BLANK*: HOW IT WORKS

- Variant calling use probabilistic and heuristics methods
- Current low VAF approaches established during development
  - tumor/normal
  - Panel of normal
  - VAF cutoff
  - UMI
- Variant callers do not handle sample variability or technical method drift
- SNAQ-SEQ determines significant difference between sample and IS variant
- Any variant in the IS indicates a sequencing error



SNAQ-SEQ allows calculation of the significance above background error for *each* variant in *every* sample

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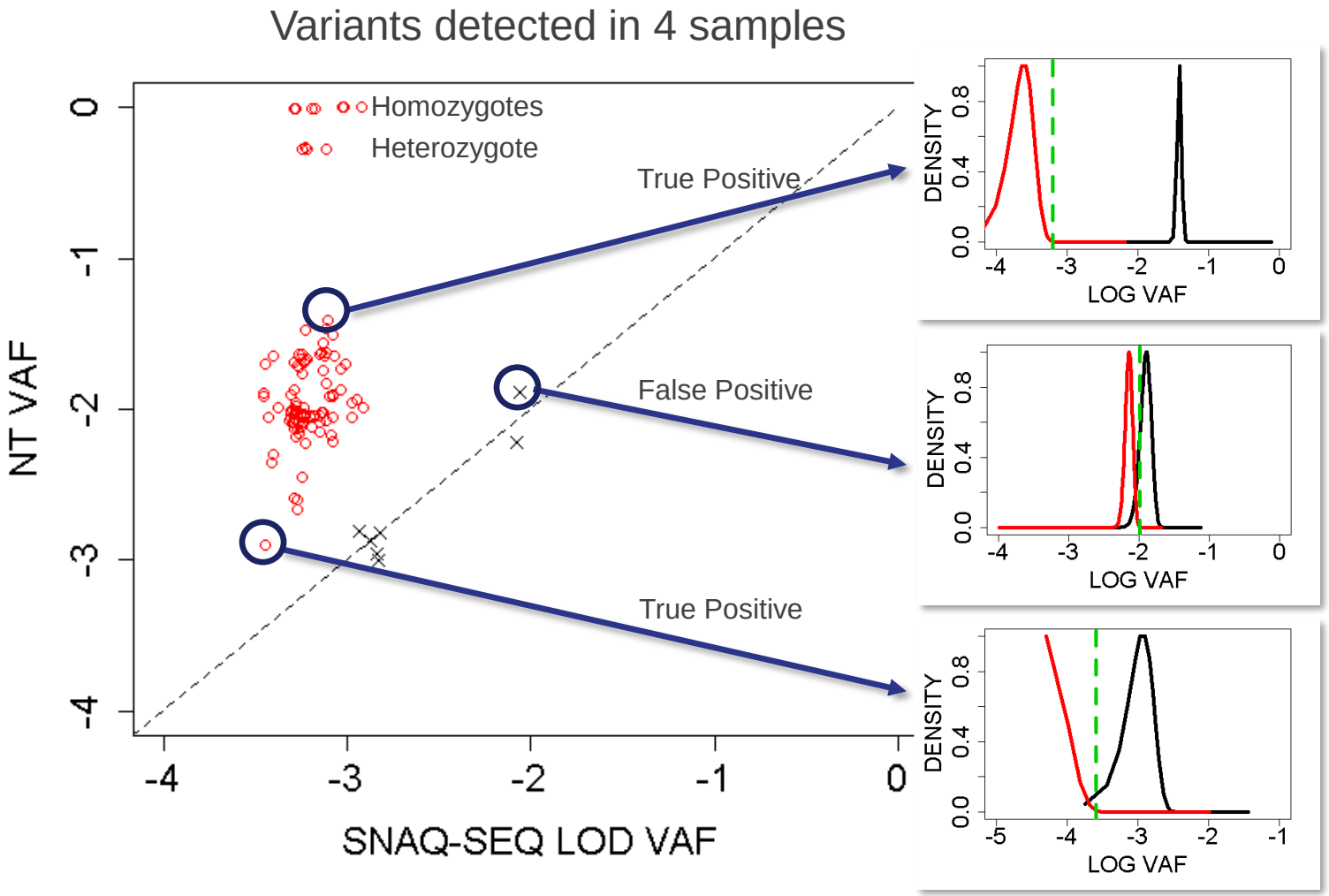
SOMATIC VARIANT DETECTION IN ctDNA



- 25ng contrived ctDNA samples
  - 10 tumor cell line mixture
  - Diluted 5-fold normal genome
  - Fragmented to 150 bp
- Spiked with cfDNA IS
  - 32Kb flanking actionable mutations
  - Fragmented to 150 bp
- Illumina TST-170 library prep with UMI
  - 27 SNV covered by IS
- SNAQ-SEQ analysis of variants
  - Poisson Exact Test used to determine significance
  - Significance cutoff set by Bonferroni adjusted 5% alpha

SNAQ-SEQ eliminates false positive variants

"Assessing synthetic reference sequence internal standards as quality-control for NGS measurement of actionable mutations in circulating tumor DNA" in preparation SEQC2 Workgroup #2 using Accukit™ SEQC2MIX4 Catalog #1154

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SNAQ-SEQ QC EXAMPLE REPORT

GENE	CHROM	POS	REF	OBS	AA_MUT	COSMIC_ID	1:5 ctDNA				1:25 ctDNA			
							1	2	3	4	1	2	3	4
NRAS	chr1	115256529	T	A	p.Q61L	COSM583	0.90%	0.89%	0.62%	0.68%	0.34%			
NRAS	chr1	115258748	C	A	p.G12C	COSM562	0.87%	1.03%	0.85%	0.92%				
NRAS	chr1	115258745	C	A	p.G13C	COSM570								
NRAS	chr1	115258745	C	G	p.G13R	COSM569								
NRAS	chr1	115258745	C	T	p.G13S	COSM571								
MAP2K1	chr15	66729162	C	T	p.P124S	COSM235614	1.02%	0.75%	0.88%	0.90%				
MAP2K1	chr15	66729136	T	C	p.L115P	NA								
TP53	chr17	7577085	C	T	p.E285K	COSM10722	0.45%	0.50%	0.59%	0.36%				
TP53	chr17	7577118	C	A	p.V274F	COSM10769	2.30%	2.05%	2.26%	2.72%	0.48%	0.50%		0.49%
TP53	chr17	7578211	C	T	p.R213Q	COSM10735	2.01%	1.91%	2.06%	1.96%	0.42%	0.30%	0.40%	0.36%
TP53	chr17	7577141	C	A	p.G266V	COSM10958								
TP53	chr17	7577141	C	T	p.G266E	COSM10867								
TP53	chr17	7577550	C	A	p.G244V	COSM43652								
TP53	chr17	7577550	C	T	p.G244D	COSM10883								
TP53	chr17	7578395	G	A	p.H179Y	COSM10768								
TP53	chr17	7578475	G	A	p.P152L	COSM10790								
PIK3CA	chr3	178936091	G	A	p.E545K	COSM763	1.21%	1.25%	1.03%	1.20%				
PIK3CA	chr3	178936091	G	C	p.E545Q	COSM27133								
PIK3CA	chr3	178936092	A	G	p.E545G	COSM764								
PIK3CA	chr3	178936092	A	C	p.E545A	COSM12458								
CTNNB1	chr3	41266125	C	T	p.T41I	NA								
PIK3CA	chr3	178936074	C	G	p.P539R	COSM759								
PIK3CA	chr3	178936082	G	A	p.E542K	COSM760								
PIK3CA	chr3	178936083	A	T	p.E542V	COSM762								
PIK3CA	chr3	178936093	G	T	p.E545D	COSM765								
PIK3CA	chr3	178936094	C	A	p.Q546K	COSM766								
PIK3CA	chr3	178936094	C	G	p.Q546E	COSM6147								
PIK3CA	chr3	178936095	A	G	p.Q546R	COSM12459								
PIK3CA	chr3	178936095	A	C	p.Q546P	COSM767								
EGFR	chr7	55259485	C	T	p.P848L	COSM22943								
MET	chr7	116412043	G	C	p.D1010H	COSM5574327								
MET	chr7	116412043	G	T	p.D1010Y	COSM3182								
FGFR3	chr4	1803568	C	G	p.S249C	COSM715								

- SEQC-2 Workgroup 2 contrived ctDNA samples
- Study concluded not to go below 0.5% VAF
- Report mocks up a 0.5% VAF requirement for a subset of 209 hotspot mutations covered by the IS

✓ Variants passed SNAQ-SEQ QC (green)

✓ Position LOB exceeds 0.5% VAF (orange)

✓ Insufficient coverage for 0.5% VAF (blue)

SNAQ-SEQ provides independent Quality Control for *each hotspot variant in every sample*

"Assessing synthetic reference sequence internal standards as quality-control for NGS measurement of actionable mutations in circulating tumor DNA" *in preparation* SEQC2 Workgroup #2 using Accukit™ SEQC2MIX4 Catalog #1154

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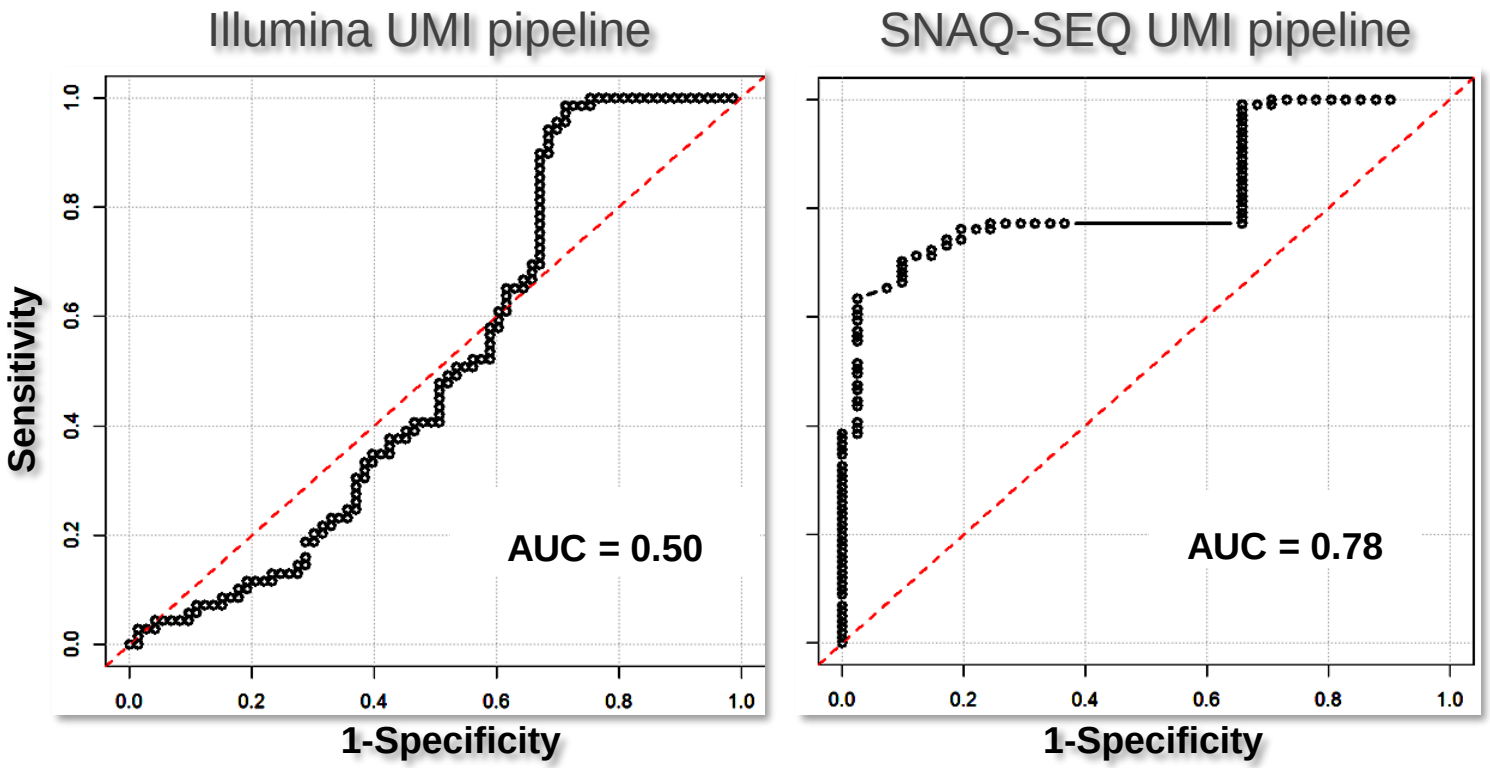
SNAQ-SEQ IMPROVES ACCURACY

- cfDNA samples ranging 0.1% to 0.5% VAF
- Illumina TST-170 specificity varied by VAF cutoff
- SNAQ-SEQ specificity varied by Poisson Exact Test (PET) significance



SNAQ-SEQ significance cutoff performs better than TST-170 VAF

"Assessing synthetic reference sequence internal standards as quality-control for NGS measurement of actionable mutations in circulating tumor DNA" *in preparation* SEQC2 Workgroup #2 using Accukit™ SEQC2MIX4 Catalog #1154

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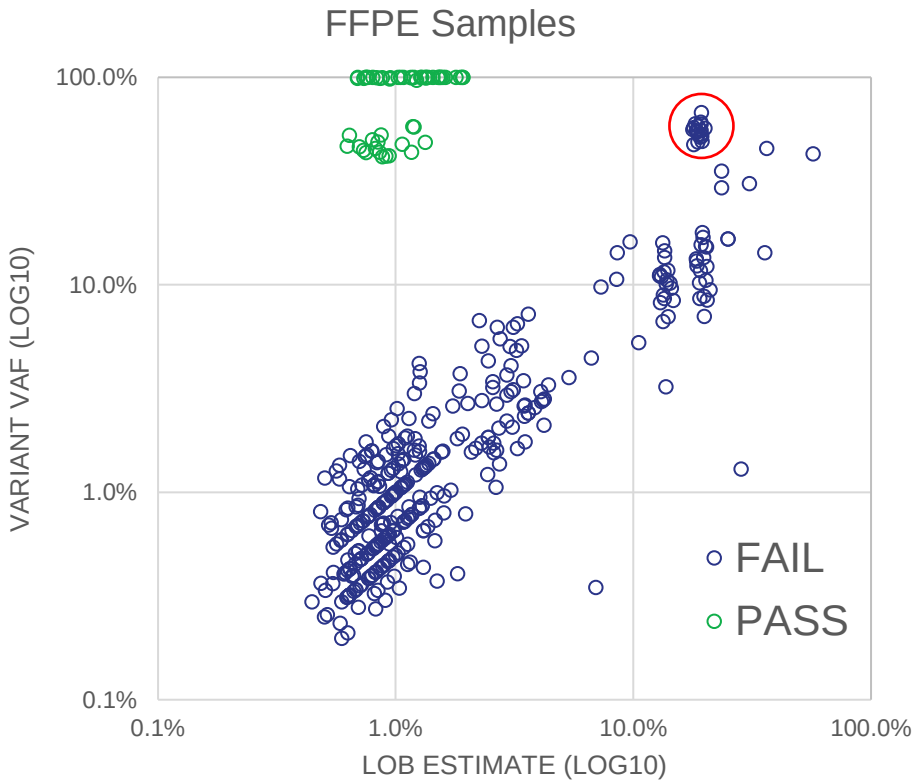
RESCUE OF POOR QUALITY FFPE SAMPLES



- ‘Normal’ FFPE samples were provided to SEQC2 NGS vendors as a simple sample to demonstrate false positive arising from FFPE
- ✓ DNA input was 10% expected which led to high false positive rates among all vendor platforms
  - *Samples were thrown out of study*
- SNAQ-SEQ IS spiked-in at very low levels
- PET cutoff set by eliminate IS false positives
- ✓ SNAQ-SEQ eliminated all false positives but not all variants were rescued due to suboptimal coverage

SNAQ-SEQ “rescues” low input FFPE samples with potential to give a result on ANY sample

Data from: “Identification of key quality control factors that affect targeted NGS variant calling of FFPE processed samples” *in preparation*
Study done with SECQC-2 Targeted Sequencing Workgroup using Accukit™ SEQC2Mix4 Catalog # 1154

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WILL SNAQ-SEQ ENABLE TUMOR VARIANT ABUNDANCE PER PLASMA VOLUME MEASUREMENT?

- Plasma cfDNA varies 2-logs (5 to 1000 ng/ml)[1]
- VAF based monitoring affected by cfDNA levels
- Solution: measure variant/ml plasma using SNAQ-SEQ
- Will addition of SNAQ-SEQ IS into plasma enable quantification of plasma variants?

- Spike 1000 or 10,000 IS in duplicate into 1 ml aliquots drawn from mixture of patient plasma retain
- MagMAX followed by Oncomine Pan-cancer liquid biopsy assay (+torrent server files modified for IS)
- ✓ Expected IS yields covaried with cfDNA (see poster TT33)
- ✓ Variant abundance levels ranged 100-3200 /ml plasma
- ✓ Good reproducibility (%CV < 36%)
- Next: measure ctDNA when varying cfDNA level in plasma

[1]Cancer Biol Ther. 2019; 20(8): 1057–1067

					ALT Counts				IS Coverage				Genome copies/ml plasma				
CHROM	POS	REF	ALT	VAF	10K	10K	1K	1K	10K	10K	1K	1K	10K	10K	1K	1K	%CV
chr17	37879588	A	G	30%	860	971	1059	1047	1992	2182	154	160	2159	2225	3438	3272	28
chr3	178952020	C	T	0.9%	28	30	24	36	1369	1397	120	101	102	107	100	178	17
chr7	55249063	G	A	32%	587	748	763	720	1494	1425	108	145	1965	2625	3532	2483	29
chr7	55259450	C	T	2.9%	95	92	133	126	1728	1951	112	162	275	236	594	389	36

SNAQ-SEQ enables measurement of ctDNA per ml plasma

* Study done in collaboration with Dartmouth Hitchcock Health System using Accukit™ ONCO1LB Catalog #1207

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SNAQ-SEQ COVID-19 SCREENING

- ✓ Evaluate testing efficiency
- ✓ Measure viral load
- ✓ Adjust for reverse transcriptase artifacts as part of variant calling

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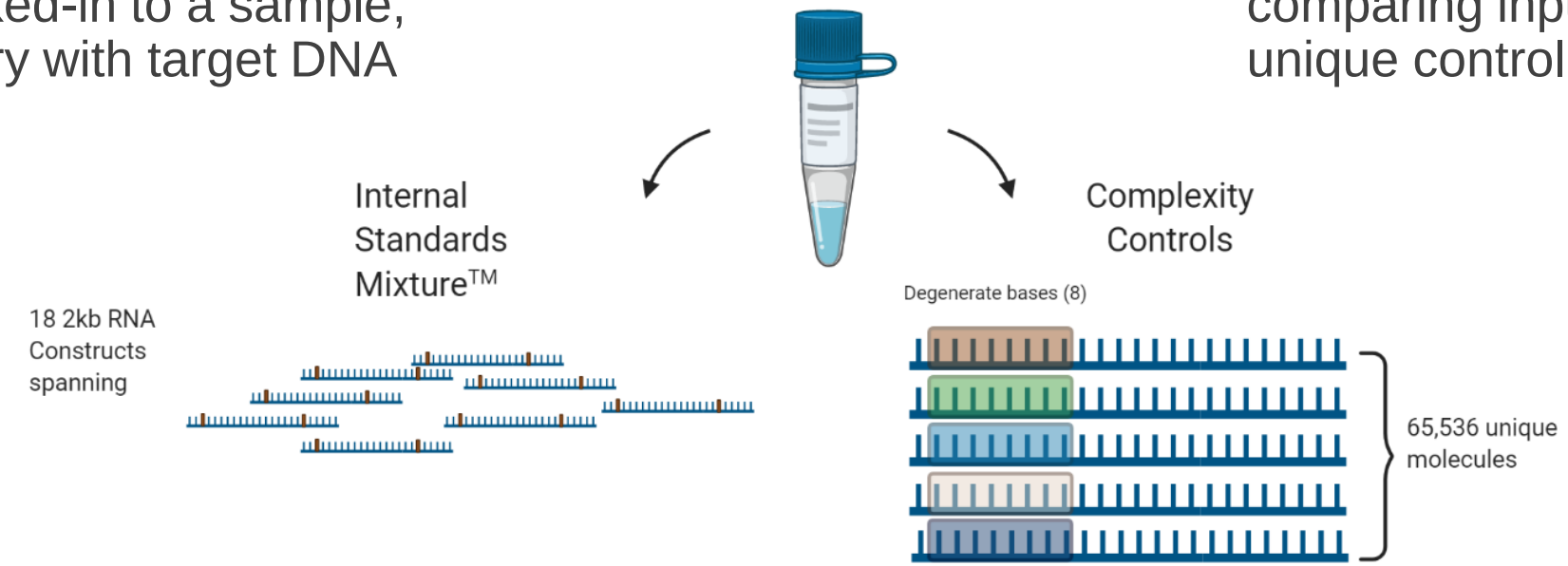
WHAT IS A COMPLEXITY CONTROL?

Complexity Control (CC) is a degenerate sequence that, when spiked-in to a sample, will co-vary with target DNA



✓ CCs provide an estimate of library complexity capture by comparing input vs. detected unique control sequences

✓ Allows estimation of complexity loss due to deduplication

✓ Detects process drift that could impact results *before becoming significant*

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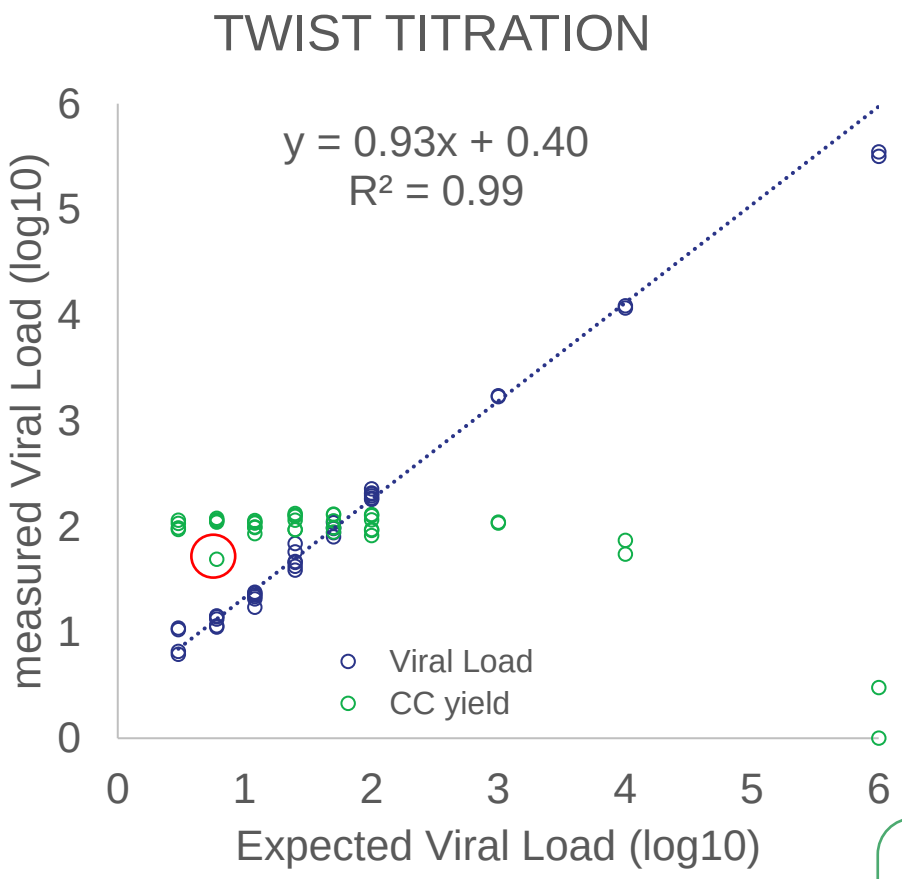
COMPLEXITY CONTROL IN VIRAL TITRATION



- 1000 genome equivalents of Accukit™ SARS-CoV-2 IS added with master mix
- ✓ Indicated amount of TWIST COVID-19 RNA reference material (blue)
- ✓ Complexity capture was 20% (green)
- ✓ One sample showed 2-fold lower CC capture (red circle)
- Above 10^5 viral genome equivalents, the virus copies will outcompete 1000 controls for reads on flow cell

SNAQ-SEQ complexity control indicates template capture efficiency for every sample

Study done in collaboration with the Wellcome Sanger Institute using ARTIC v3 protocol with Accukit™ SARS-CoV-2 (v2, 1000) Catalog #1270

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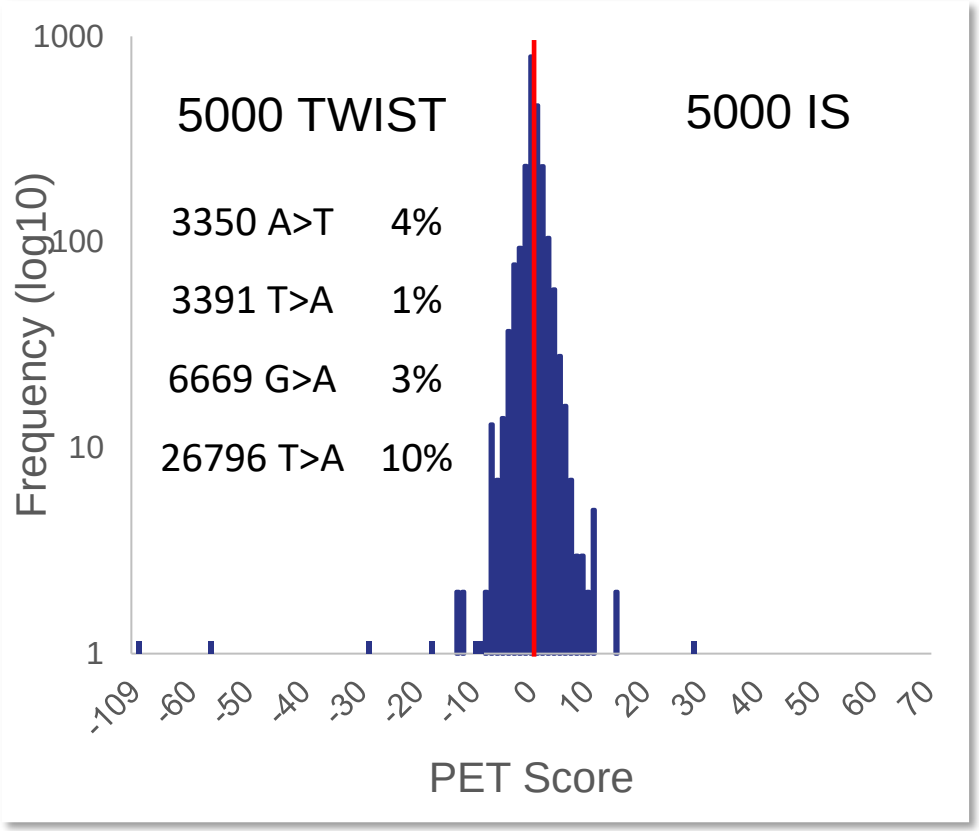
LIMIT OF BLANK ACCOUNTING FOR REVERSE TRANSCRIPTASE ERRORS

- Converting RNA to cDNA as part of ARTIC protocol creates random variants
- TWIST BioScience Synthetic RNA Reference Control compared spiked with AccuGenomics SARS-CoV-2 RNA Internal Standards
- ✓ SNAQ-SEQ RNA IS mirrors the sample RT error in the PET distribution
- ✓ The RT artifacts increase the limit of blank beyond 5% alpha
- User/software would make informed significance cutoff per sample



SNAQ-SEQ captures sequence noise generated from RT and enables low VAF calling in unoptimized pipelines

Study done in collaboration with the Wellcome Sanger Institute using ARTIC v3 protocol with Accukit™ SARS-CoV-2 (v2, 1000) Catalog #1270

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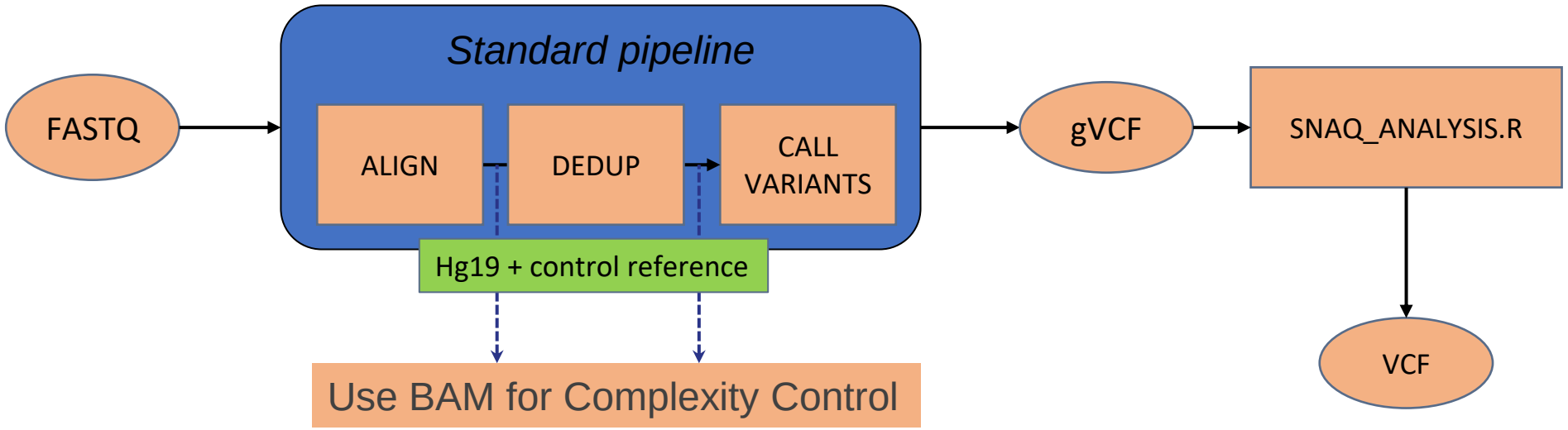
SNAQ-SEQ Analysis Pipeline



SNAQ-SEQ is compatible with any pipeline that accepts reference genomes

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SUMMARY

SNAQ-SEQ technology provides customized solutions for NGS assays across many platforms and delivers:

- ✓ Independent Quality Control for every variant in every sample
- ✓ QC for positives and negatives SNV
- ✓ Capture of RT sequence noise
- ✓ Better variant calling accuracy
- ✓ Potential to rescue poor quality samples

- ✓ Complexity control to measure template capture efficiency
- ✓ Concentration measurements of viral load and plasma ctDNA
- ✓ Compatibility with any pipeline that accepts reference genomes

ACKNOWLEDGMENTS

SEQC-2 Workgroup #2 Team

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ORDERING INFORMATION

Accukit Name	Catalog Number
SEQC2 Mix 4	1154
Accukit™ SARS-CoV-2 RNA (v2, 250)	1269
Accukit™ SARS-CoV-2 RNA (v2, 1000)	1270
Accukit™ BioContaminants	1306
Accukit™ ONCO1LB	1207
Accukit™ ONCO2ST	1208
Accukit™ Inherited Cancer CNV	1263

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