ctDNA Evaluation Set

A new era of ctDNA accuracy: powerful calibration built into every sample

Background

Next-generation sequencing (NGS) can be used to identify genetic variation and disease-associated mutations, and it has become a principal tool in biomedical research and clinical testing. However, numerous factors influence the accuracy of variant detection using NGS including PCR amplification biases, capture efficiency, sequencing depth, read length and sequencing errors. To maximize genomic insight, it is imperative that a system of control standards is incorporated to correct for accumulated errors, improve data quality and interpretation.

Introduction to Sequins

Sequins™ (sequencing spike-ins) are synthetic nucleic acid reference controls that directly mirror naturally occurring sequences. Because Sequins retain the same nucleotide composition as the natural sequence, they enable accurate representation of genomic complexity without compromising integrity of the sample and results. Sequins perform equivalently throughout sequencing workflows, providing a true measure of control.

Sequins' innovative design enables production of synthetic mirrored sequences that directly represent almost any genomic feature, in any organism with a reference genome. This includes common and clinically relevant variants and analytically challenging regions of the genome. By combining sequins in precise ratios, quantitative features of genome biology, such as variant allele frequencies or copy-number variation, can also be emulated.

Sequins are simply 'spiked-in' to a sample prior to library preparation and together, progressed through a workflow (Figure 1). Sequins controls can then be distinguished from the native sample in the output library by virtue of their mirrored sequence enabling normalization and comparison between samples, runs, laboratories, chemistries and sequencers.

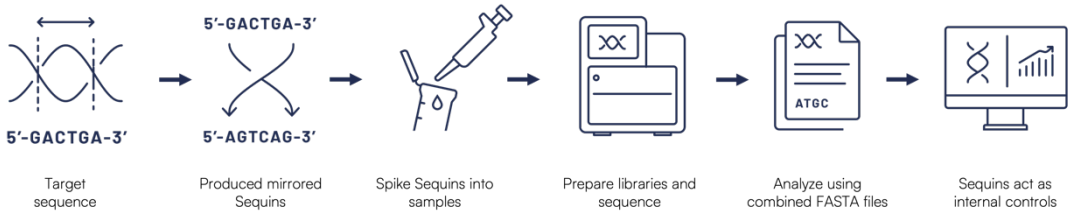

Figure 1. Schematic showing the design and use of Sequins in an NGS workflow.

Sequins for Circulating Tumor DNA

Circulating tumor DNA (ctDNA) sequencing has fast become a powerful method for early detection, screening and recurrence monitoring of cancer. ctDNA testing leverages the ability to detect low concentration DNA fragments shed from cancer cells into the bloodstream. With minimal-invasiveness through blood draws and unbiassed by heterogeneity, "liquid biopsies" for ctDNA can quickly detect cancer-associated actionable variants, reveal information about cancer type and stage and response to therapies. Using highly sensitive techniques, such as next-generation sequencing (NGS), ctDNA is analyzed to identify specific cancer-related mutations, genetic aberrations, and other molecular markers. However, ctDNA is typically present at very low variant allele frequencies (VAFs) making detection accuracy dependent on high sequencing coverage, high fragment depth, and assay calibration.

While ctDNA testing holds great promise, tumor type, mass, stage and background cell-free DNA make it challenging for accurate and reliable variant detection. Sequins internal standards address these challenges by introducing synthetic mirror DNA controls with known variant compositions and concentrations directly into each sample. This allows for precise estimation of VAFs, determination of the limit of detection (LoD), assay normalization, and calibration of variant calls against internal benchmarks.

By integrating Sequins, laboratories can confidently quantify sensitivity, verify precision, and ensure reproducible, comparable ctDNA results across workflows and platforms.

Benefits of Sequins for ctDNA

Quantification and Limit of Detection Calibration	Sequins representing synthetic cancer variants at varying allele frequencies provide a source of truth against which samples can be calibrated enabling confident and reliable identification of low-frequency variants.
Quality Control and Assay Validation	Sequins serve as an internal quality control and are subjected to the same technical influences as the samples they are combined with. This enables evaluation and optimization of key parameters related to sequencing workflows and sample quality.
Standardization Across Labs and Studies	Spike-in controls enable normalization both within and between samples, workflows, and locations to enable unprecedented standardization and interoperability.

The Sequins ctDNA Evaluation Set comprises a molecular ladder developed for the application of assessing variant allele frequency and sensitivity in the context of general cell-free cancer testing. The set targets twenty-two cancer genes with seventeen challenging INDELs and five SNPs at precise variant allele frequencies from 0.1% to 10% and is applicable to a range of cancers. Sequins are pre-mixed in a single tube which are spiked into each sample prior to library preparation and sequencing.

Sequins Evaluation Set content

Gene	ClinVar UID	Variant	VAF %
BRCA1	ClinVar_1076672	c.2219_2220insTAAT (p.Ser741fs)	0.1
WT1	ClinVar_449416	c.1120C>T (p.Arg374Ter)	0.1
SMAD4	ClinVar_24867	c.1587dup (p.His530fs)	0.1
APC	ClinVar_1072211	c.476_488del (p.Tyr158_Tyr159insTer)	0.1
RB1	ClinVar_1073997	c.46_61GCC[2]GCGGAACCCCAGGCACCGCCGCCGCCGCCGCGGAACCCC[1] (p.Pro21fs)	0.1
BRCA2	ClinVar_9342	c.658_659del (p.Val220fs)	0.5
PMS2	ClinVar_1328224	c.741_742insGTGTGTGAAG (p.Ser248fs)	0.5
TSC1	ClinVar_1013340	c.903dup (p.Asn302fs)	0.5
VHL	ClinVar_2218	c.499C>T (p.Arg167Trp)	0.5
MEN1	ClinVar_1070954	c.1375_1391dup (p.Ala467fs)	0.5
MAX	ClinVar_404110	c.211_221del (p.Ile71fs)	0.5
MLH1	ClinVar_89935	c.18_34del (p.Val7fs)	1.0
PTEN	ClinVar_545882	c.865_866insTTCT (p.Lys289fs)	1.0
SDHAF2	ClinVar_532513	c.177dup (p.Asp60Ter)	1.0
TSC2	ClinVar_237966	c.148A>G (p.Met50Val)	1.0
MSH6	ClinVar_1070916	c.3964_3980dup (p.Asn1327delinsLysAsnLeuArgArgTer)	5.0
BMPR1A	ClinVar_529927	c.366_384del (p.Glu123fs)	5.0
TMEM127	ClinVar_126966	c.265_268del (p.Thr89fs)	5.0
MSH2	ClinVar_90711	c.1638_1639dup (p.Asn547fs)	10.0
RET	ClinVar_230926	c.1998G>C (p.Lys666Asn)	10.0
STK11	ClinGen_CA402950689	g.1221314G>A (g.1221314G>A)	10.0
SDHB	ClinVar_428926	c.17_42dup (p.Ala15delinsProSerProTer)	10.0

Sequins Performance

Sequencing data confirmed the accuracy, linearity, and sensitivity of the Sequins ctDNA Evaluation Set over a wide range of variant allele frequencies, validating their effectiveness for calibrating and benchmarking ctDNA assays (Figure 2).

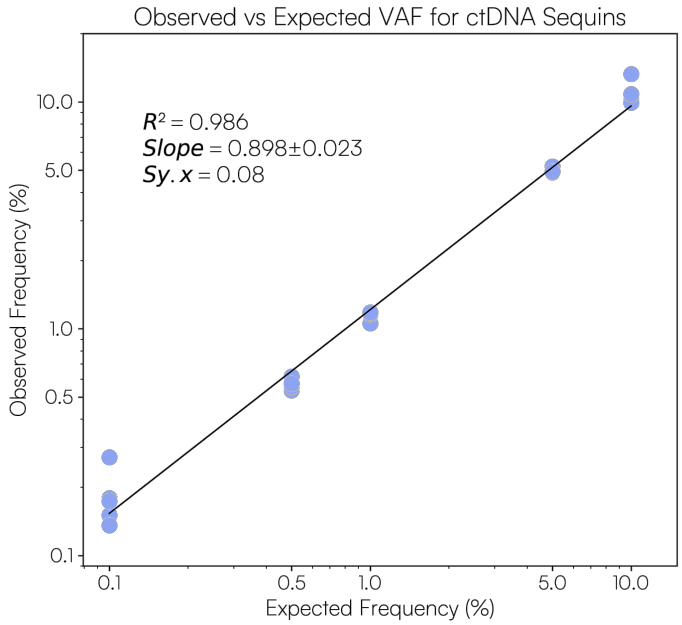

Figure 2. Observed versus expected variant allele frequencies (VAFs) for synthetic Sequin variants. Twenty-two cancer gene variants representing varying allele frequencies (10%, 5%, 1%, 0.5% and 0.1%) were sequenced neat on Illumina NextSeq platform using the IDT xGen™ cfDNA & FFPE Library prep kit v2 MC, where ladder linearity is maintained across all spike-in amounts.

Designed to be run in combination with targeted gene panels, users can source a vendor to design and synthesize capture probes for the Sequins ctDNA Evaluation Set to enable the combined use of Sequins controls and capture panels. More information on optimization of Sequins input amounts and performance can be found in our ctDNA Evaluation Set Technical Note.

Customizable Backbone

The Sequins ctDNA Evaluation Set provides a comprehensive control backbone and can be further customized with additional gene targets through our On-Demand program.

We are releasing the Sequins ctDNA Evaluation Set through our Evaluation Program. Please visit https://sequins.bio to find out more and enquire.

Contact Information

Ordering and Support

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Key Publications

Deveson, I.W., Gong, B., Lai, K. et al. (2021) Evaluating the analytical validity of circulating tumor DNA sequencing assays for precision oncology. *Nat Biotechnol* 39, 1115—1128.

Deveson, I.. Chen, W., Wong. T. et al. (2016) Representing genetic variation with synthetic DNA standards. *Nat Methods*. 13. 784- 791.

Hardwick SA, Deveson IW. Mercer TR. (2017) Reference standards for next-generation sequencing. *Nat Rev Genet*. 2017 Aug:18(8):473-484. doi: 10.1038/nrg.2017.44.

Deveson, I.W., Madala, B.S., Blackburn, J. et al. (2019) Chiral DNA sequences as commutable controls for clinical genomics. *Nat Commun*. 10, 1342.

Blackburn, J., Wong. T., Madala, B.S. et al. (2019) Use of synthetic DNA spike-in controls (sequins) for human genome sequencing. *Nat Protoc*. 14, 2119-2151.