TWIST BIOSCIENCE

ctDNA Evaluation Set: Insights

Demonstrating Sequins Compatibility with a Twist-Based ctDNA Hybrid-Capture Workflow

Overview

The Sequins™ ctDNA Evaluation Set was assessed in a Twist-based ctDNA hybrid-capture workflow to evaluate compatibility across library preparation, hybrid-capture, sequencing, and variant analysis. The study used the Twist cfDNA Library Prep Kit and Twist Standard Hyb v2 and Wash Kit, together with a Twist Custom Panel (design ID: TE-92032694) targeting the 23-gene Sequins ctDNA Evaluation Set. The Evaluation Set comprises synthetic mirrored reference and variant alleles formulated as a molecular ladder across five variant allele frequency (VAF) levels: 0.1%, 0.5%, 1%, 5%, and 10%. When spiked into Twist cfDNA Pan-Cancer Reference Standard v2 samples, the Evaluation Set ladder was successfully captured and sequenced, with observed VAFs showing strong concordance with expected VAFs across all tested conditions. These findings support the compatibility of Sequins spike-in controls with a Twist-based ctDNA hybrid-capture workflow and demonstrate their potential as an in-sample companion control for monitoring sensitivity, precision, and quantitative performance.

Background

Circulating tumor DNA (ctDNA) analysis by next-generation sequencing (NGS) is increasingly used in oncology research and precision medicine to detect, monitor, and characterize tumor-derived variants from minimally invasive liquid biopsy samples. However, ctDNA assays are analytically challenging because tumor-derived fragments are often present at low concentrations, resulting in variants observed at very low VAFs within a high background of non-tumor cell-free DNA (cfDNA). Accurate detection of these low-frequency variants can be affected by multiple factors across the workflow, including input quantity and quality of cfDNA, library preparation efficiency, hybrid-capture performance, sequencing depth, error suppression, and downstream bioinformatics analysis.

These challenges create a need for internal reference materials that can be introduced directly into the sample and carried through the same workflow as endogenous ctDNA. Such controls can help laboratories assess whether low-VAF variants are being captured, sequenced, and analyzed as expected within a given assay setting, while also supporting more consistent interpretation of assay sensitivity, precision, and quantitative performance over time. In the context of capture-based ctDNA workflows, this is particularly important because variation in probe design, enrichment efficiency, and sequencing allocation can materially influence analytical performance.

Introduction to Sequins

Sequins (sequencing spike-ins) are synthetic nucleic acid 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 the integrity of the sample and results. Sequins perform equivalently throughout sequencing workflows, providing a true measure of control (Deveson et al. 2019).

Sequins' innovative design enables the 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 VAFs or copy-number variation, can also be emulated (Deveson et al 2021).

Sequins are simply spiked-in to a sample prior to library preparation and progressed together 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 standardization and comparison between samples, runs, laboratories, chemistries, and sequencers.

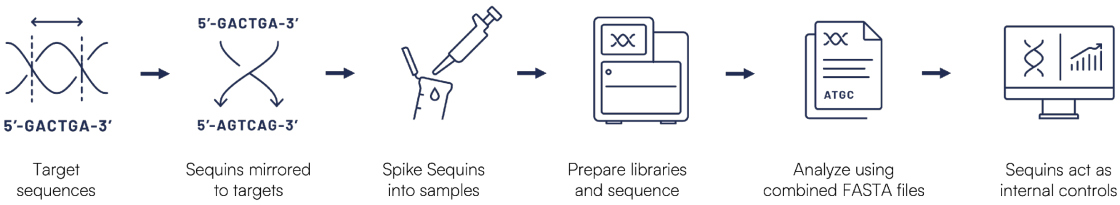

Figure 1: Schematic showing the design and use of Sequins in an NGS workflow.

ctDNA Evaluation Set Overview

Intended Use

The Sequins ctDNA Evaluation Set is a synthetic spike-in control designed for evaluation use in ctDNA assays and is provided for research use only (RUO), enabling laboratories to assess the performance of Sequins technology in their workflows. This includes the mirrored control design, molecular ladder, and associated analyses. The Evaluation Set is intended for initial workflow assessment, to inform the subsequent development of bespoke Sequins control sets tailored to user-specific targets, variants, and VAF requirements, which can then support sample- and run-specific performance assessment, assay development and validation, and routine quality control.

Molecular Ladders

When spiked into every sample, ctDNA molecular ladders provide a framework for generating both sample- and run-specific performance insights within every sequencing run. By including variants at multiple defined allele

frequencies, molecular ladders enable assessment of analytical sensitivity, precision, and quantitative performance across a clinically relevant VAF range. Because the ladder is present in every sample, associated performance metrics such as analytical sensitivity, slope and linearity can be trended over time to support monitoring of analytical consistency and detection of shifts related to routine workflow variation.

Sequins ctDNA Evaluation Set

Sequins controls are designed as matched pairs, with one molecule representing the wild-type allele and the other the variant allele. These molecules are blended at defined ratios to generate the desired VAFs. The ctDNA Evaluation Set is formulated as a molecular ladder spanning 23 cancer genes, with variants represented at allele frequencies of 0.1%, 0.5%, 1%, 5%, and 10% in a single mix (Table 1). The material contained within the Set is fragmented to represent the size profile of cfDNA. Importantly, the patented mirrored design ensures that ladder-specific variants are distinguishable from sample-derived variants during bioinformatics analysis.

Table 1: 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
TP53	ClinVar_1066203	c.934_992dup (p.Ile332fs)	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

Capture-Based Approach

Molecular ladder approaches can be used in capture-based workflows. For Sequins, application in capture-based methods requires corresponding capture probes to account for the mirrored molecular design. In this

technical note, data is presented from a pilot verification study demonstrating the use of the ctDNA Evaluation Set with Twist probes. This showed that Sequins molecules can be co-captured across all target loci consistently in the Twist probe-based workflow.

Further details on target content, variant composition, and sequencing characteristics are provided in the ctDNA Evaluation Set Information Sheet.

Pilot Verification Study

This study was designed to evaluate whether the Sequins ctDNA Evaluation Set could be incorporated into a Twist-based ctDNA workflow without disrupting sample processing, while preserving the expected quantitative performance of the Sequins molecular ladder.

Methodology

To assess workflow compatibility, the Sequins ctDNA Evaluation Set was spiked into Twist cfDNA Pan-Cancer Reference Standard v2 samples representing wild-type, 0.1%, 1%, and 5% VAF levels, using 20ng total DNA input per sample. The Evaluation Set was added at two spike-in levels (0.035% and 0.07%) relative to total DNA input.

Libraries were prepared using the Twist cfDNA Library Prep Kit and enriched using the Twist Standard Hyb v2 and Wash Kit. Two probe sets were used: Twist Control Panel A, targeting 398 variants in the Twist cfDNA Pan-Cancer Reference Standard v2, and a Twist Custom Panel (design ID: TE-92032694) comprising 64 probes targeting the 23-gene ctDNA Evaluation Set. Sequencing was performed on the Illumina NovaSeq X Plus using 2 × 150bp reads.

Sequencing data were processed using a UMI-aware bioinformatics workflow with alignment to the GRCh38 reference genome and the Sequins ctDNA Evaluation Set decoy chromosome. VAF ladder performance (Figure 2) was assessed using duplex consensus reads, and detection sensitivity (Figure 3) using calibrated duplex consensus reads, where Evaluation Set regions were downsampled to match the mean coverage of the Twist target regions within each sample using *sequintools calibrate*. Variant calling was conducted using Mutect2, with targeted sites specified for ladder evaluation and unspecified for sensitivity analyses.

This design enabled direct evaluation of Sequins performance within a Twist-based ctDNA workflow, including capture efficiency, preservation of molecular ladder linearity, and concordance between Sequins-derived and Twist cfDNA Pan-Cancer Reference Standard v2-derived sensitivity estimates.

Key Insights

Sequins ctDNA Evaluation Set is compatible with a Twist hybrid-capture workflow

Across eight tested samples, all Evaluation Set target variants were successfully captured, spanning five VAF tiers from 0.1% to 10% (Figure 2).

Observed VAFs showed strong concordance with expected VAFs across the Evaluation Set molecular ladder, with slopes close to 1 and R^2 values of 0.98-0.99 across samples (Figure 2). This indicated that the expected quantitative relationship between Sequins reference and variant alleles was preserved through the Twist workflow. Importantly, performance was consistent across Twist cfDNA Pan-Cancer Reference Standard v2 backgrounds representing wild-type, 0.1%, 1%, and 5% VAF levels, and across both tested Evaluation Set spike-in concentrations.

Together, these results support compatibility between the Sequins ctDNA Evaluation Set and a Twist-based hybrid-capture workflow. They also demonstrate that Sequins can provide an orthogonal, in-sample molecular ladder for assessing variant detection and quantitative performance in conjunction with an incumbent targeted gene panel capturing variants from Twist cfDNA Pan-Cancer Reference Standard v2.

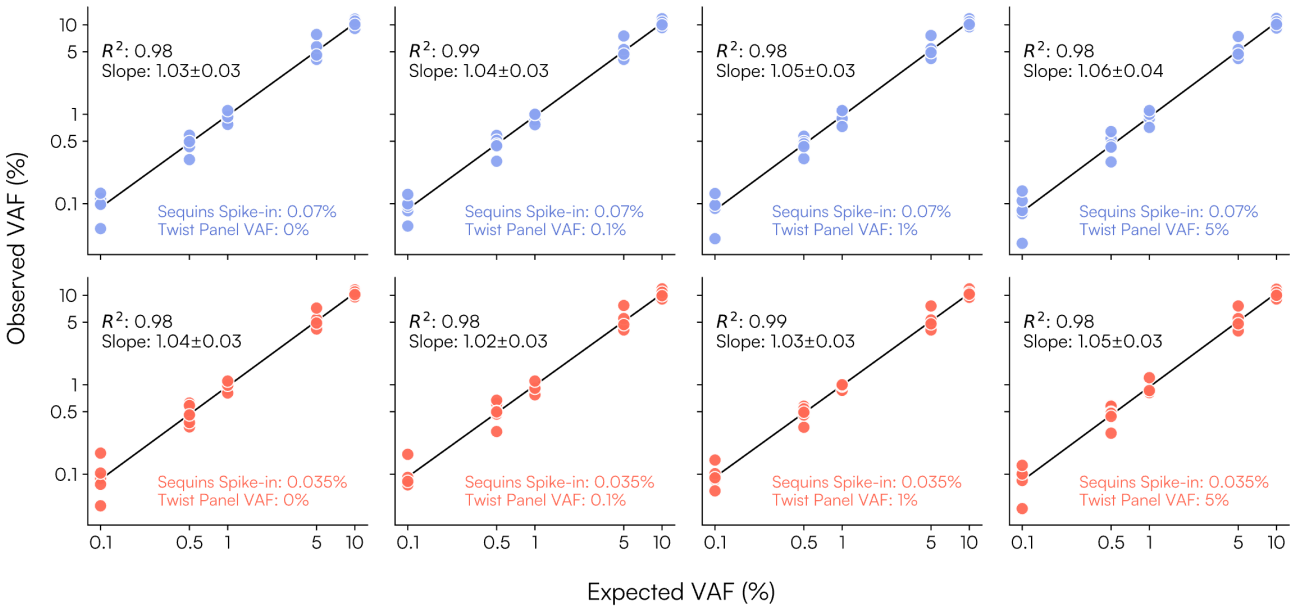

Figure 2: Sequins ctDNA Evaluation Set molecular ladder linearity through a Twist hybrid-capture workflow. Observed versus expected Sequins variant allele frequencies (VAFs) across eight samples processed using Twist cfDNA library preparation and Twist hybridization chemistry. Each panel represents an individual sample tested across two Sequins spike-in concentrations (upper panel at 0.07%; lower panel at 0.035%) and four Twist cfDNA Pan-Cancer Reference Standard v2 backgrounds. Dots represent individual Sequins variants. Slopes close to 1 and R^2 values of 0.98-0.99 demonstrate strong linearity and accurate quantitative recovery of the Sequins molecular ladder through the Twist workflow.

Sensitivity benchmarking alongside Twist ctDNA Reference Material

The Sequins ctDNA Evaluation Set was spiked into Twist cfDNA Pan-Cancer Reference Standard v2 samples representing one of three VAF tiers: 0.1%, 1%, or 5%. Each sample contained a single sample VAF level, while the co-captured Evaluation Set ladder in every sample spanned VAFs from 0.1% to 10%. This design enabled direct comparison between the sensitivity of Twist Control Panel A against Twist cfDNA Pan-Cancer Reference Standard v2 and Twist Custom Panel against the Evaluation Set. After calibrating the Evaluation Set reads to

match the sample's mean effective coverage, sensitivity estimates from the Evaluation Set and sample aligned closely, supporting calibration as an appropriate basis for sensitivity estimation.

As the Evaluation Set ladder contains fewer variants per VAF tier (n=4-6) than the number of variants detected by Twist Control Panel A from the Twist cfDNA Pan-Cancer Reference Standard v2 (n=398), per-sample sensitivity estimates were more variable, particularly at low VAFs, although they continued to reflect overall panel performance (Figure 3, pink circles). Aggregating replicate measurements at the run level markedly reduced this variability, yielding a sensitivity curve that closely matched that of the Twist Control Panel A (Figure 3). A custom-designed Sequins ctDNA ladder which incorporates more variants within each VAF tier (especially at the lowest levels) would provide additional in-sample replicates and thereby further strengthen the robustness of a molecular ladder as a companion control in research and laboratory workflows.

These findings support the use of Sequins as a complementary in-run control within a Twist-based ctDNA workflow. While the Twist cfDNA Pan-Cancer Reference Standard v2 provides broad panel-level performance assessment, Sequins add an internal molecular ladder that travels through the same sample-specific library preparation, capture, sequencing, and analysis workflow.

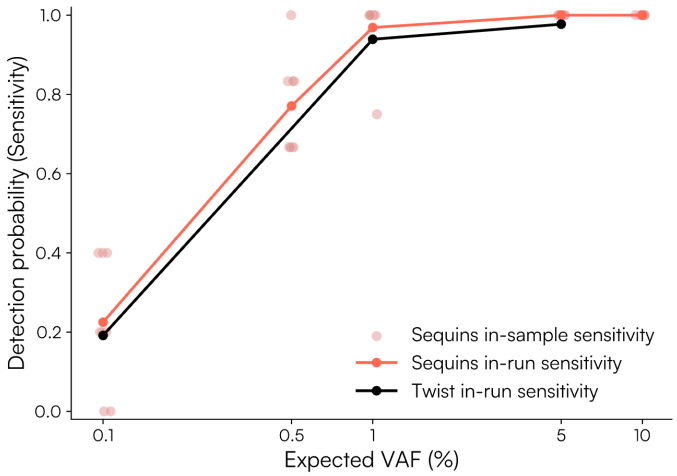

Figure 3: Variant detection probability as a function of expected VAF. Detection probability increases with variant allele frequency (VAF) for both ctDNA Evaluation Set (Sequins) and Twist cfDNA Pan-Cancer Reference Standard v2, with Sequins and Twist in-run sensitivities closely tracking each other after calibration of the ctDNA Evaluation Set coverage to correspond with the mean coverage of the sample. Greater variability in per-sample Sequins sensitivity is observed at lower VAF levels, reflecting stochastic detection behaviour near the limit of detection. Sequins in-sample sensitivity points are horizontally jittered for visibility.

Conclusion

This pilot verification study demonstrated that the Sequins ctDNA Evaluation Set can be successfully incorporated into a Twist-based ctDNA hybrid-capture workflow and, in doing so, provides a practical demonstration of Sequins compatibility with a Twist capture chemistry more broadly. Using Twist cfDNA library preparation, Twist hybridization chemistry, an incumbent targeted panel, and a custom Sequins-specific probe set, the Sequins molecular ladder was successfully captured, sequenced, and quantified across all tested samples. Together, these findings show that Sequins mirrored molecules can be effectively co-captured and analyzed within a Twist-based workflow, while the

ctDNA Evaluation Set serves as an evaluation tool to assess workflow compatibility and inform the subsequent development of bespoke Sequins control sets tailored to specific assay requirements.

The study showed that, under the conditions tested:

- The Sequins ctDNA Evaluation Set was successfully applied alongside an incumbent targeted panel in a Twist-based ctDNA hybrid-capture workflow using a custom Sequins-specific probe set, demonstrating compatibility of Sequins with Twist capture chemistry in this workflow context
- The Sequins molecular ladder maintained strong linearity across expected VAF levels from 0.1% to 10%
- Observed Sequins VAFs showed strong concordance with expected VAFs across Twist cfDNA Pan-Cancer Reference Standard v2 samples
- Sequins-derived sensitivity estimates closely tracked Twist cfDNA Pan-Cancer Reference Standard v2 sensitivity after coverage calibration
- Spike-in levels should be optimized for each panel design, sample input, and desired sequencing depth to achieve sufficient Sequins coverage while preserving sample read allocation[1]

Access to the ctDNA Evaluation Set

The Sequins ctDNA Evaluation Set is available through the Sequins Technology Access Program for laboratories interested in evaluating Sequins in their own ctDNA workflows. The Set is designed for use alongside targeted gene panels with an appropriate Sequins-specific capture probe set and can also serve as a foundation for further customization through the Sequins On-Demand program. To enquire about access, apply via the Sequins website (www.sequins.bio) or contact the team directly at enquiries@sequins.bio.

References

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

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

Disclaimer

Twist and Sequins products are intended for Research Use Only. Not intended for use in diagnostic procedures.

[1] As demonstrated in *ctDNA Evaluation Set: Insights, Demonstrating Sequins Compatibility with IDT-Based ctDNA Hybrid-Capture Workflow* tech note