ctDNA Evaluation Set: Insights

Demonstration of Compatibility with Capture-Probe Workflows

Summary

The Sequins™ ctDNA Evaluation Set is a robust spike-in reference material designed to evaluate the performance of Sequins technology within capture-based ctDNA workflows. Using both Twist and IDT hybrid-capture systems, this study showed that Sequins can be efficiently captured with corresponding custom probes and delivered consistent quantitative performance across workflows. The data demonstrated strong linearity between expected and observed variant allele frequencies (VAFs), comparable sensitivity across platforms, and no interference with sample target capture. Taken together, these results show that the ctDNA Evaluation Set provides a practical means for laboratories to test whether the Sequins mirrored control design, molecular ladder, and associated analyses work effectively in their own workflow, while informing the subsequent development of bespoke Sequins control sets for routine assay monitoring, validation, and quality control.

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.

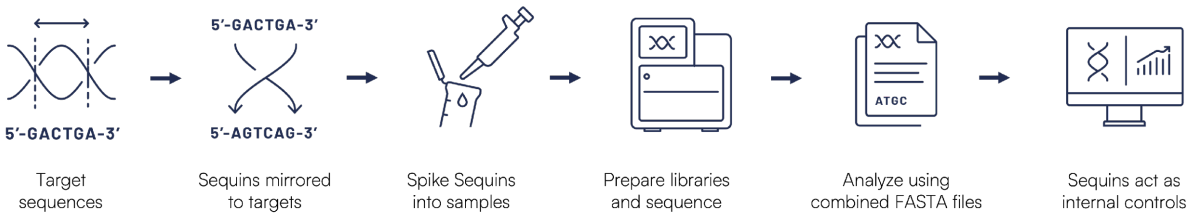

Figure 1: Schematic showing the design and use of Sequins in an NGS workflow.

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, where 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.

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, we summarize data from two studies which demonstrated the use of the ctDNA Evaluation Set with Twist and IDT capture probes, respectively. Together, these studies showed that Sequins molecules can be captured efficiently and reproducibly in probe-based workflows.

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

Compatibility with Hybrid-Capture Ecosystems

The performance of the Sequins ctDNA Evaluation Set was assessed in two distinct hybrid-capture workflows: (1) using ssDNA probe sets and reagents from IDT (Integrated DNA Technologies, USA) and (2) using dsDNA probe sets and reagents from Twist (Twist Bioscience, USA) (Table 1).

Methodology

Twist-based capture

The Evaluation Set was spiked into Twist cfDNA Pan-Cancer Reference Standard v2 samples at two input levels: 0.035% (7pg) and 0.07% (14pg) relative to total DNA input across four sample VAF levels, namely wild-type (0%), 0.1%, 1%, and 5% (20ng input each). Libraries were prepared using the Twist cfDNA Library Prep Kit, followed by hybrid-capture with the Twist Standard Hyb v2 and Wash Kit. Sample and Evaluation Set variants were co-captured using the Twist Control Panel A against Twist cfDNA Pan-Cancer Reference Standard v2 and Twist Custom Panel (design ID: TE-92032694) against the Evaluation Set.

IDT-based capture

The Evaluation Set was spiked into normal male human cell-free DNA (50ng) at five input levels ranging from 0.0016% (0.8pg) to 1% (500pg). Libraries were prepared using the IDT xGen cfDNA & FFPE DNA Library Prep v2 Kit with hybrid-capture using the IDT xGen Hybridization and Wash Reagents v3 Kit. Sample and Evaluation Set variants were co-captured using the IDT xGen Human ID Hyb probe set together with a custom IDT-designed probe set targeting the Evaluation Set.

Table 1: Summary of Twist and IDT panels used for hybrid-capture.

Parameter	Twist	IDT
Panel name	Control panel A	Human ID Hyb
Number of targets in the panel	398	76
Panel Target size (kb)	119.4	20
Control Target size (kb)	4.9	4.9
Number of probes in the Evaluation Set probe set	64	218
Evaluation Set probe tiling density	2x-4x	2x-10x
Probe type	dsDNA	ssDNA
Library Prep and Hybridization Capture Kit	Twist cfDNA Library Prep Kit and Twist Standard Hyb v2 and Wash Kit	xGen cfDNA&FFPE DNA Library Kit, xGen Hybridization and Wash v3 Kit
Sequencing platform	NovaSeq X Plus	NextSeq 2000

Bioinformatics analysis

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. Coverage profiles, insert size distributions, and spike-in titration behavior (Figures 3, 4 & 6) were evaluated using UMI-deduplicated reads. VAF ladders and detection sensitivity (Figures 2 & 5) were assessed using calibrated duplex consensus reads, where Evaluation Set regions were downsampled to a defined coverage level using *sequintools calibrate* to enable direct comparisons. Variant calling was conducted using Mutect2, with targeted sites specified for ladder evaluation and unspecified for sensitivity analyses.

Results

The Sequins ctDNA Evaluation Set is compatible with Twist and IDT hybrid-capture workflows

The captured Evaluation Set ladder demonstrated strong linearity in the Twist and IDT workflows, with an observed R^2 of 0.98 for both and slopes of 0.99 and 1.05, respectively (Figure 2A-B). This indicated a consistent quantitative response, despite differences in workflow chemistry and capture design. The sensitivity curves were likewise comparable between the two workflows (Figure 2C), supporting comparable detection performance across the evaluated VAF range under the same analysis pipeline.

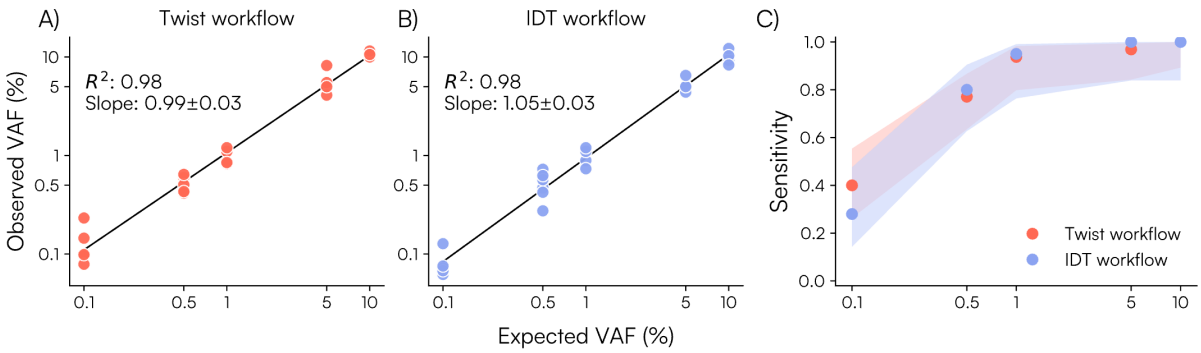

Figure 2: Performance of the Sequins ctDNA Evaluation Set across capture-based workflows. (A-B) Evaluation Set ladders showing observed versus expected variant allele frequency (VAF) after calibration to equivalent coverage for the Twist (A) and IDT (B) workflows. The slopes were close to 1 and R^2 values were 0.98, demonstrating a strong linear relationship between observed and expected frequencies, indicating accurate and consistent variant quantification across both workflows. **(C)** Pooled detection sensitivity as a function of expected VAF from calibrated data for Twist and IDT workflows, with 95% Wilson confidence intervals. Observed sensitivity was comparable between workflows, demonstrating similar downstream calling performance.

To assess whether inclusion of the Evaluation Set co-capture probe set affected the capture efficiency of the sample panel probe set, the sample targets were captured either alone (Human ID) or in combination with Evaluation Set probe set (Human ID + Sequins) using the IDT workflow. No interference with the IDT Human ID Hyb Panel was observed (Figure 3). Coverage uniformity across sample targets remained consistent regardless of whether targets were captured alone or co-captured with the Evaluation Set over a range of spike-in levels (Figure 3). Similarly, the insert size distribution remained unchanged following co-capture with the Evaluation Set (Figure 4).

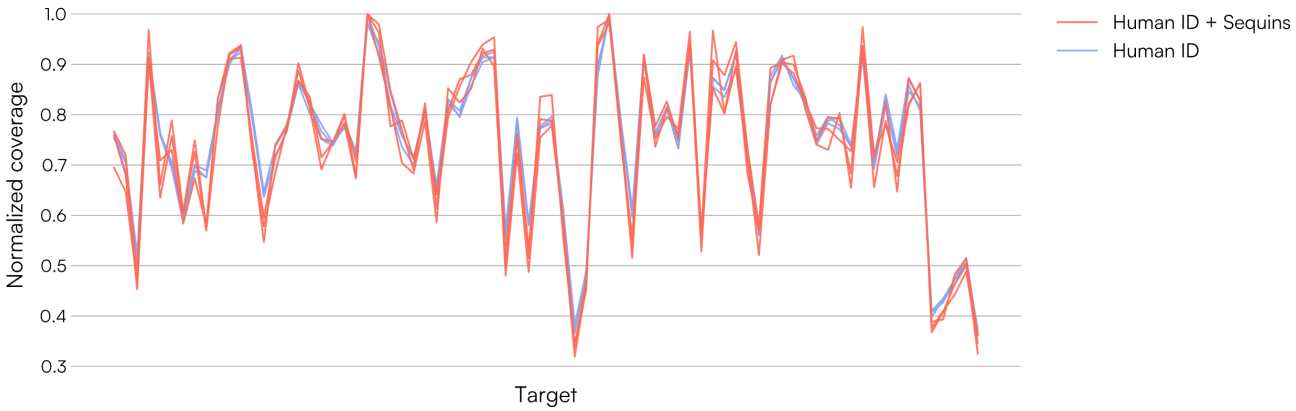

Figure 3: Combined mean coverage for each target in the IDT Human ID Hyb Panel when captured with and without Sequins. Coverage of each target remained consistent when the Sequins were also captured.

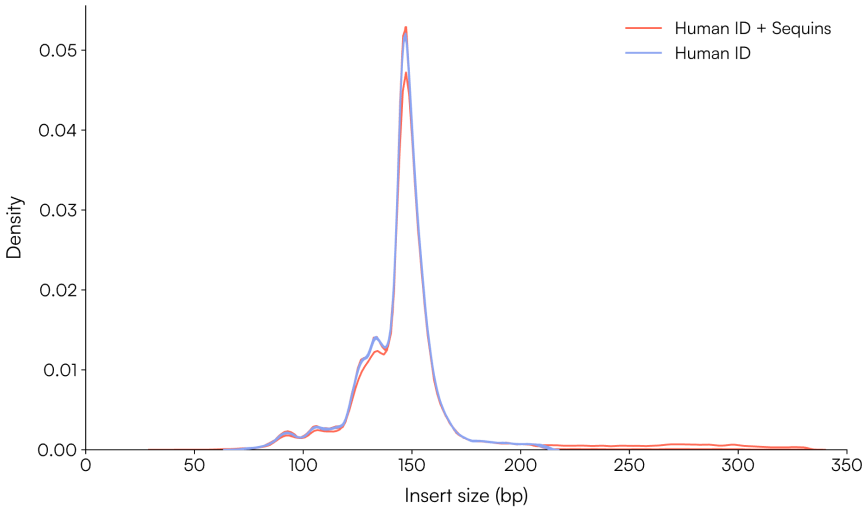

Figure 4: Insert size distribution of the IDT Human ID Hyb Panel when captured with and without the ctDNA Evaluation Set. The distribution of insert sizes was unaffected by the addition of the Evaluation Set with the peaks at ~150bp and ~130bp maintained.

Collectively, these results establish broad chemistry compatibility for the Sequins ctDNA Evaluation Set and demonstrate that accurate, linear quantification of ctDNA variants is achievable across a range of hybrid-capture workflows.

When included in every sample, the molecular ladder contained with the ctDNA Evaluation Set provides a quantitative response profile that can be evaluated sample-to-sample and trended run-to-run to support longitudinal assessment of assay performance. Associated metrics, such as slope, linearity, and consistency across VAF tiers, can be used to monitor analytical stability and detect changes related to routine workflow variation.

Sensitivity Benchmarking with the Sequins ctDNA Evaluation Set

In the Twist hybrid-capture workflow, the 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 a robust approach 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 5, 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 5). 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 or laboratory workflows.

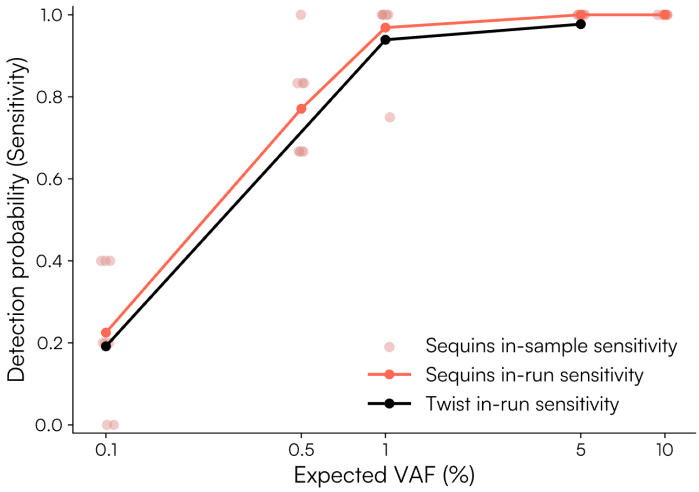

Figure 5: Variant detection probability as a function of expected VAF. Detection probability increases with VAF for both ctDNA Evaluation Set (Sequins) and Twist cfDNA Pan-Cancer Reference Standard v2, with Sequins and Twist in-run sensitivities closely tracking each other after calibration of the ctDNA 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.

When spiked into every sample, the ctDNA Evaluation Set provides multiple VAF tiers in a single control, enabling run-specific LOD95 estimation as a function of aggregate detection rate across tiers. This avoids the need to run multiple separate low-VAF reference samples in every run, while still delivering the sensitivity information needed for routine performance monitoring. Custom-designed Sets could incorporate additional variants within each tier and be optimized to user requirements, enabling more robust LOD95 estimation, including on a per-sample basis.

Optimal ctDNA Evaluation Set Spike-in Level

Reads mapping to the Evaluation Set and the captured sample naturally compete for sequencing output. It is, therefore, important to balance the Evaluation Set spike-in level so that sufficient control coverage is achieved without disproportionally increasing its share of reads relative to the sample. Performance data showed the

Evaluation Set footprint can be predictably reduced by lowering the spike-in amount while maintaining optimal performance (Figure 6). Decreased Evaluation Set spike-in concentrations resulted in a corresponding increase in the proportion of reads aligning to the sample with a concomitant decrease in Evaluation Set coverage. Importantly, this reduction in spike-in concentration did not affect the performance of the molecular ladder, with the linearity and slope maintained across the dilution series. However, below genome equivalence, where Evaluation Set copy number fell below sample copy number, control coverage dropped below sample coverage. At 0.0016% it no longer provided the coverage excess needed for calibration (Figure 6B).

For accurate sensitivity calibration, the Evaluation Set should therefore be spiked in at a level that maintains control coverage slightly above sample coverage. As this relationship will vary with incumbent panel size and complexity, spike in levels should be optimized for each target panel.

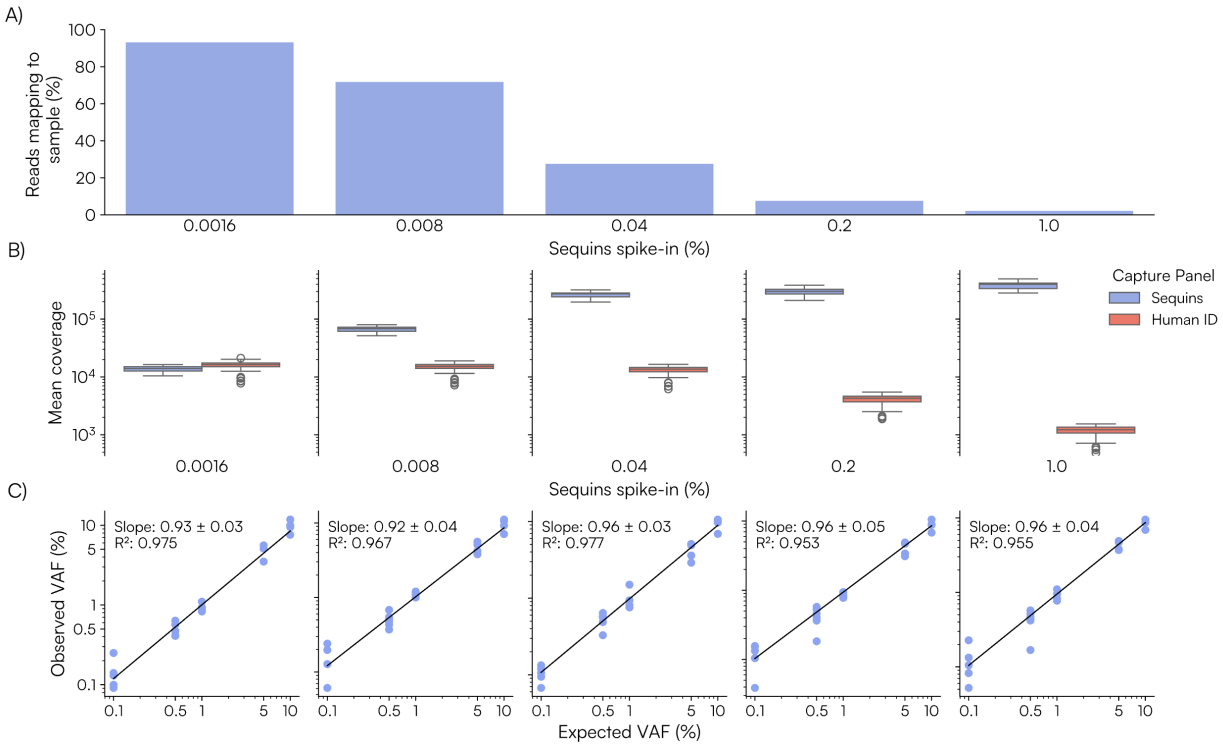

Figure 6: Titration of the Sequins ctDNA Evaluation Set spiked into cfDNA co-captured with the IDT Human ID Hyb Panel. (A) The percentage of reads mapping to the human sample (blue bars) increased with decreasing Sequins spike-in concentration (from 1% to 0.0016%). **(B)** The coverage distribution for the ctDNA Evaluation Set (blue) and human sample (red) demonstrated that Sequins coverage decreased with decreasing spike-in amounts. **(C)** The molecular ladder showed a linear relationship between observed and expected variant allele frequencies (VAFs) across all spike-in concentrations, with slopes close to 1 and high R^2 values.

Conclusion

This study demonstrates that the Sequins ctDNA Evaluation Set, comprising a synthetic molecular ladder, provides a practical means of evaluating compatibility between Sequins technology and capture-based ctDNA workflows. The Evaluation Set is intended to help laboratories assess whether the Sequins mirrored design, ladder structure, and associated analytical approach function effectively within their own workflow, thereby informing the subsequent design of bespoke Sequins control sets for routine assay monitoring, validation, and quality control.

Our results showed that the Evaluation Set:

- Performed consistently across Twist and IDT hybrid-capture workflows, demonstrating compatibility across different capture chemistries
- Showed strong concordance with sample sensitivity across the tested VAF range, supporting its value as a workflow assessment tool
- Benefited from run-level aggregation of replicate ladder measurements, which improved the precision of sensitivity estimates
- Allowed spike-in levels to be adjusted to optimize sequencing resource allocation while maintaining ladder linearity

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.