Single stranded ligation enhances the performance of the duet evoC 6-base assay, enhancing value in low DNA input applications including liquid biopsy

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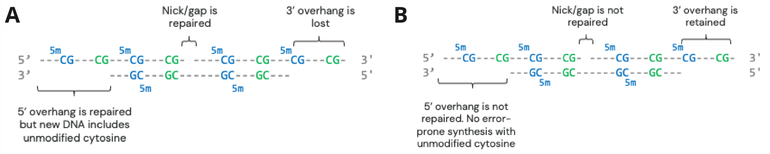
1. Introduction

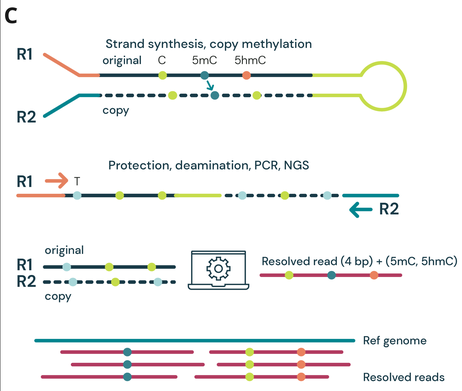
Circulating cell-free DNA (cfDNA) contains rich genetic, epigenetic, and structural information for cancer detection, but its highly fragmented nature, low abundance, and high background make reliable analysis challenging. Conventional double-stranded DNA (dsDNA) workflows rely on end repair and fragment reconstruction, which can lose damaged or ultra-short molecules and introduce artefacts, limiting sensitivity and increasing the limit of detection (LoD), particularly at ultra-low ctDNA fractions.



(A) Double-stranded workflows involving end-repair and A-tailing can result in loss of fragment features though digestion of 3' overhangs, repair of nicks and 5' overhang repair with unmethylated bases where the original fragment may have a 5mC or 5hmC. **(B)** In the single-stranded ligation duet workflow original cfDNA features are retained through no digestion of 3' overhangs or filling of 5' overhangs. Nicks are retained and unmodified bases are not introduced.

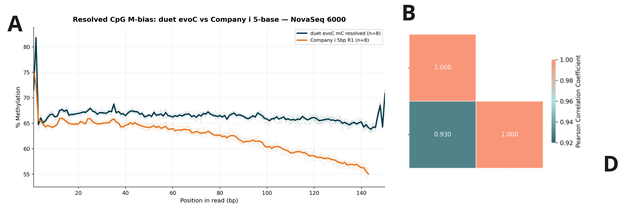
The single-stranded DNA (ssDNA) duet workflow is optimised for cfDNA through:

- Capturing damaged and ultra-short fragments by ligating adapters directly to single-stranded molecules without end repair
- High recovery of unique molecules with higher proportion of shorter fragments known to be enriched in ctDNA
- Complete and accurate genetic data including important C>T variants driving low genetic LoD
- Complete and accurate 5mC and 5hmC calling enabling ultra-low methylation LoD
- Integrated multiomic readouts from the same molecules including genetic variants, independent 5mC and 5hmC, and native fragmentomic features
- Phased multiomic data provides the opportunity to reduce false negatives and improve confidence in detection of tumour-derived signals through a lower LoD for ctDNA detection



(C) Overview of the duet workflow where an hairpin is used to capture an original DNA strand from the cfDNA fragment followed by generation of an unmodified copy strand. A series of enzymatic steps results include copying of only 5mC to the copy strand followed by protection and deamination. The duet construct is PCR amplified before conventional 4-base short read sequencing. The 4-base FASTQ is processed by the duet software and uses a read resolution logic to recapitulate the identity of all 6-bases on the original cfDNA fragment. The resulting aligned BAM contains complete and accurate genetic information, including C>T variants and complete and accurate epigenetic information including both 5mC and 5hmC.

2. The ssDNA duet workflow delivers outstanding analytical performance, enabling ultra-low LoD



E

Assay	LoD95	LoB
duet 6-base	0.563%	0
duet 5-base	0.506%	0

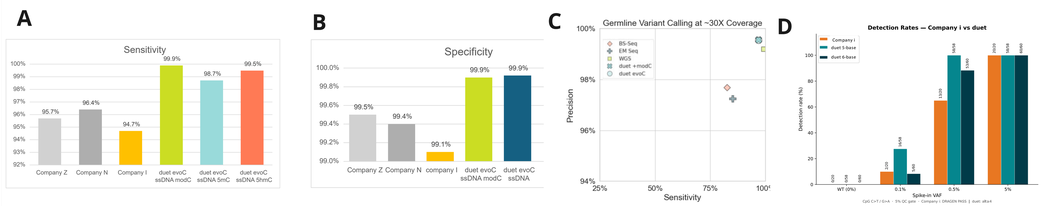
F

	LoD95
Hypermethylation	5.18 ppm
Hypomethylation	14.59 ppm

To achieve ultra-low LoD in cfDNA analysis, high analytical accuracy, reproducibility, and low LoB are essential. The ssDNA duet workflow was assessed for methylation bias across reads, technical reproducibility, sensitivity, specificity, and analytical LoD. The ssDNA approach shows reduced positional methylation bias (**A**), supporting more uniform representation of methylated CpGs across fragments and improved accuracy of 5mC and 5hmC detection, particularly in fragmented cfDNA. Strong Pearson correlations for genomewide per-CpG 5mC were observed between technical replicates even at low coverage (**B**), demonstrating high assay precision and robust methylation calling across CpGs. Using fully methylated or unmethylated controls the ssDNA duet workflow maintained high specificity **(C)** while achieving strong sensitivity **(D)**, indicating effective discrimination of true methylation events with minimal background noise across a range of cfDNA inputs. Finally, low LoDs were achieved for all genetic variants **(F)** and ultra-low LoD for methylation **(E)** using titrated reference standards.

For methylation, LoD95 values were achieved in the low-parts-per-million (ppm) range for both hypermethylated and hypomethylated CpGs, reflecting a low limit of blank and precise methylation calling. Genetic LoD was assessed using reference materials containing defined variant allele frequencies, demonstrating reliable detection of single-nucleotide variants at very low allele fractions. Collectively, these results show that the ssDNA duet workflow delivers outstanding analytical performance across genetics and epigenetics. By combining low bias, high reproducibility, strong sensitivity and specificity, and consistently ultra-low LoD, the duet ssDNA ligation workflow enables confident ctDNA detection in liquid biopsy applications where maximal analytical sensitivity is required including early detection, treatment selection and detection of residual disease.

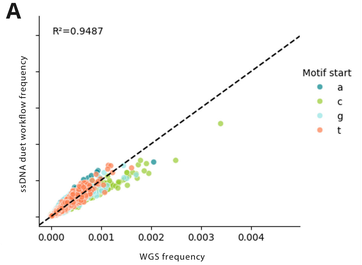
3. duet with single stranded ligation has market leading methylation and genetic accuracy



To benchmark methylation accuracy and variant detection, the ssDNA duet workflow was compared with on-market epigenetic sequencing solutions across methylation sensitivity, methylation specificity, and variant calling performance. The ssDNA duet workflow demonstrated the highest methylation sensitivity **(A)** while simultaneously maintaining superior specificity **(B)**, indicating improved recovery of true methylation signals with minimal false positives. Germline SNP calling performance was equivalent to conventional WGS **(C)**, demonstrating reliable base-calling performance within the ssDNA duet workflow. Focussing on the most common somatic C>T variants, which are often missed by existing epigenetic sequencing approaches, the ssDNA duet workflow's complete genetic output demonstrated improved performance for detecting these substitutions. Together, these data demonstrate that a single-stranded ligation duet workflow enables market-leading methylation accuracy and robust detection of C>T/G>A variants, supporting high-confidence cfDNA analysis in low VAF liquid biopsy contexts.

4. Unlock fragmentomic biomarkers with duet

We demonstrate that a single-stranded duet workflow delivers superior analytical performance for genomic and methylation analysis compared with competing dsDNA approaches. Importantly, the same libraries preserve native fragment information, enabling fragmentomics analysis without additional assays.



We show cfDNA end-motif frequencies that strongly correlate (R^2 =95%) with whole-genome sequencing (WGS) generated from matched clinical cfDNA samples **(A)**. Broader fragmentomic features and classifications are presented in In poster #123 we highlighting the full potential of fragmentomic biomarker that are unlocked by the integrated duet evoC assay including both conventional (e.g. fragment length, nucleosome position) and novel (e.g. 6-base end-motifs).

5. Conclusions

Sensitive cfDNA analysis requires exceptional analytical performance to reliably detect tumour-derived signals at very low allele fractions. The ssDNA duet workflow achieves ultra-low limits of detection by combining high material recovery with outstanding sensitivity and specificity across genomic and epigenetic features.

- Ultra-low limit of detection (LoD) enabled by high material recovery from low-input cfDNA
- Outstanding analytical sensitivity and specificity, supporting confident detection at ultra-low tumour fractions
- Robust detection of genetic variants, 5mC and 5hmC within a single ssDNA assay
- Reduced false positives and false negatives, improving reliability at the limit of detection
- Consistent performance across genomic and epigenetic signals, supporting composite biomarker analysis

6. References

(1)Füllgrabe J. et al. Simultaneous sequencing of genetic and epigenetic bases in DNA. Nat Biotechnol. 2023 Oct;41(10):1457-1464.