RENEW

White Paper

Revolutionizing Liquid Biopsy cfDNA Whole Genome Methylation Sequencing

Summary

Although cell-free DNA (cDNA) has massive potential as a powerful genetic and epigenetic biomarker of disease, cDNA methylation-based liquid biopsies have yet to meaningfully enter clinical testing due to limitations in cDNA biology and available methods to detect and analyze methylation. cfDNA is highly fragmented and nicked, and occurs in low concentrations in circulation. Available methods to detect and analyze DNA methylation introduce significant DNA damage, errors, and biases.

Here, Renew Biotechnologies (Renew) presents a whole genome cDNA application of NESSI-Seq, a novel library preparation technology that enhances specificity, sensitivity, and data quality and leverages Oxford Nanopore Technologies (ONT) to capture the advantages of native sequencing and long reads. NESSI-Seq preserves DNA in its native state, eliminating the need for bisulfite conversion and PCR amplification and enabling accurate methylation and hydroxymethylation analysis. NESSI-Seq technology improves the specificity, sensitivity, and clinical viability of cDNA methylation assays to facilitate clinical applications of cDNA assays across diverse therapeutic areas.

This innovative approach holds promise for advancing personalized medicine and cDNA-based liquid biopsies by providing more reliable and comprehensive genomic and epigenomic data. The potential for tissue-of-origin and copy number variation analyses using wgNESSI-Seq are demonstrated in this white paper, showcasing its broad applicability and impact.

Introduction

In an era of high-throughput omics and precision medicine, treatments and disease management plans are increasingly tailored to patients' molecular profiles. This approach enhances therapy efficacy, reduces adverse effects, and promotes personalized healthcare and longevity. Thus, less invasive technologies aimed at profiling patient biology to guide care are rapidly advancing.

Liquid biopsies, in particular, have gained immense clinical attention for their ability to detect disease biomarkers with minimal invasiveness. These biopsies analyze body fluids—such as blood, urine, saliva, and cerebrospinal fluid-for cell-derived molecules to diagnose and monitor disease.

Challenges With Current Methods

cfDNA Biology

Among common liquid biopsy targets, cfDNA stands out for its ease of isolation, relative stability, scalability, and adaptability across diverse therapeutic areas. However, cfDNA research and liquid biopsies are hindered by several limitations in the biological characteristics of cfDNA molecules, including:

1

Extensive Fragmentation and Nicking
Typically resulting from cellular processes like apoptosis, necrosis, and active release, cfDNA[1] is inherently highly fragmented and nicked[2]. These characteristics reduce read lengths and sequencing efficiency and increase error rates.

2

Low Concentrations
cfDNA typically occurs in low concentrations in body fluids[3]. cfDNA molecules of interest make up a small fraction of total cfDNA, requiring highly sensitive methods for detection and sequencing[4].

3

Nonuniform Fragment Lengths
cDNA fragment lengths can vary significantly, making it difficult to generate sequencing libraries and achieve uniform coverage across regions of interest.

Bisulfite Sequencing

Current protocols for analyzing DNA methylation, such as bisulfite sequencing, introduce significant DNA damage and biases. Although there has been a mild increase in clinical trials involving DNA methylation-based strategies over the last 20 years[5], the widespread application of DNA methylation analysis in clinical settings has been limited by the inherent limitations of bisulfite sequencing. Since its first use in 1992[6], bisulfite sequencing has become the standard for analyzing DNA methylation and involves three primary steps:

1

Bisulfite Conversion
Treatment of DNA with bisulfite chemically converts unmethylated cytosines to uracils, leaving methylated cytosines intact, differentiating methylated from unmethylated sites.

2

PCR Amplification
Post-conversion, PCR amplifies the modified DNA. During this process, uracils are read as thymine, maintaining the distinction between originally methylated and unmethylated cytosines.

3

Sequencing and Bioinformatic Analysis
The amplified DNA is sequenced, and sophisticated bioinformatics tools decode the methylation landscape, identifying the methylation status based on the cytosine and thymine distribution in the sequence data.

Despite its widespread use, bisulfite sequencing presents significant challenges, such as DNA degradation from the harsh chemical conditions required for conversion[7], leading to the loss of up to 90% of the DNA template. One recent study investigating bisulfite conversion efficiency of six commonly used kits demonstrated a recovery rate of 18-50%[8]. The technique is further compromised by incomplete conversion rates and PCR amplification biases that distort quantification and can under or overestimate global methylation[9].

Advantages of Long-Read Sequencing

Long-read, third-generation sequencing (TGS) technologies such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio) provide effective alternatives for sequencing and methylation detection that eliminate the need for bisulfite conversion and PCR amplification. These platforms maintain the native state of DNA, thereby preserving epigenetic modifications like DNA methylation and hydroxymethylation. They produce long reads that address many of the limitations of traditional short-read sequencing technologies and enable accurate epigenetic analyses that reflect underlying biology. Because Renew's technology leverages the Oxford Nanopore Technologies platform, nanopore sequencing will be highlighted in this white paper (Figure 1).

Although initially challenged by higher error rates, TGS technologies have significantly improved over the past decade and have established themselves as clinically viable tools for DNA sequencing[10].

Nanopore sequencing, in particular, is being applied more broadly in clinical settings compared to its competitors due to the small size and portability of the platform, as well as the low cost of setup and the rapid sequencing workflow, enabling real-time insights for clinical outputs[11,12].

Although ONT has primarily been used for long-read sequencing, recent work has shown that Nanopore sequencing can be adapted for shorter reads without additional processing[13].

One recent feasibility study demonstrated the potential for utilizing a shallow Nanopore whole genome approach for cfDNA-based liquid biopsy in cancer[14]. Here, we improve upon this approach by incorporating Renew's proprietary NESSI-Seq technology to improve sensitivity, throughput, and data quality.

Oxford Nanopore Technologies

To achieve native reads and single molecule resolution, ONT sequencing devices pass DNA and RNA molecules through protein ion channels—nanopores—embedded in an electro-resistant membrane to determine base identities and their modifications. As DNA molecules pass through a nanopore, they disrupt the flow of ionic current through the pore. The disruptions in current are decoded with AI base-calling algorithms to determine DNA sequences and their epigenetic modifications in real time.

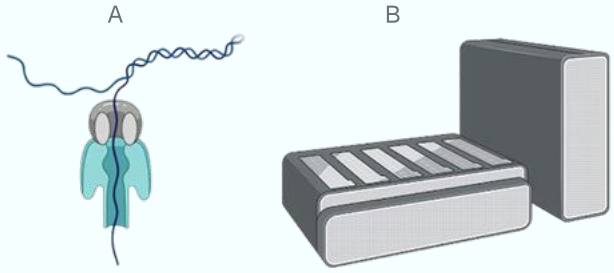

Figure 1. Renew's sequencing technology leverages the Oxford Nanopore Technologies platform. Representative Nanopore (A) and Oxford Nanopore Technologies sequencer (B).

Introducing Whole Genome NESSI-Seq for cfDNA

NESSI-Seq

Renew Biotechnologies (Renew) introduces NESSI-Seq, a proprietary library preparation protocol that enhances specificity, sensitivity, and data quality and enables target enrichment. NESSI-Seq technology leverages the ONT platform to capture the benefits of long reads and native analysis. Improved sensitivity and data quality enable accurate calls and quantification of cDNA, even at low concentrations. Analyzing native DNA molecules via nanopore sequencing enables the direct and simultaneous acquisition of DNA sequence and base modification data. Thus, methylation and hydroxymethylation modifications can be detected without bisulfite conversion or PCR amplification, eliminating their associated biases.

The NESSI-Seq workflow eliminates conversion and amplification steps to simplify library prep and streamline the acquisition of rich cDNA methylation data without additional biases or prep (Figure 2). This method departs from traditional approaches that require deeper sequencing to compensate for PCR and other biases. Instead, NESSI-Seq represents a shift towards the direct analysis of individual DNA molecules, providing a more accurate reflection of biological reality.

By treating each molecule as a significant and informative biomarker, Renew's approach simplifies the process and enhances the reliability of methylation analysis.

Figure 2. Renew's NESSI-Seq technology simplifies library prep and streamlines the acquisition of accurate DNA sequence and methylation data with single-molecule resolution. NESSI-Seq leverages a proprietary library preparation protocol designed to couple directly with Nanopore sequencing. The technology leverages the power of native reads and eliminates biases introduced by bisulfite conversion and amplification, the de facto standard for targeted methylation analysis.

Whole Genome NESSI-Seq for cDNA

Here, we present a low-pass, whole genome cfDNA application of NESSI-Seq for research, clinical diagnostic development, and other genomic and epigenomic-based use cases. This approach offers a cost-effective, comprehensive method for analyzing methylation and hydroxymethylation at any combination of CpGs present within cfDNA samples, providing a clearer and more holistic picture of cfDNA biology. By capturing multiple genomic features at targeted regions in single runs via nanopore sequencing, NESSI-Seq delivers more informative data, which is particularly beneficial for robust forward genetic and epigenetic screens and clinical diagnostics[15].

This method's ability to simultaneously assess a wide array of genomic features enhances its utility for detecting disease progression, identifying biomarkers, and personalizing treatment strategies.

The following case study data support this approach and demonstrate the clinical potential of targeted NESSI-Seq for quantifying low concentrations of cfDNA, assessing DNA's tissue of origin, and analyzing copy number variants. These examples illustrate just a few of NESSI-Seq's expanding applications, underscoring its value for researchers and clinical service providers aiming to advance precision medicine.

Case Studies

Tissue of Origin Analysis

Identifying DNA's cell or tissue of origin via methylation analysis is significant in various clinical settings. In oncology, for instance, identifying tumor-derived DNA's cell-of-origin can help pinpoint the original site of a metastatic tumor and distinguish between cancer subtypes'. In developmental biology, these analyses can be used to track cellular differentiation to understand mechanisms of development and stem cell behavior! In neurology, detecting cell-of-origin from DNA can be used to identify the cellular sources of DNA released during neuron death in neurodegenerative diseases[17,18]. Despite the clinical relevance of DNA methylation profiling for identifying cell or tissue origin, its broader applications have been limited. Challenges include the restricted number of target cDNA loci that can be analyzed and biases inherent in bisulfite sequencing, which have impeded its widespread adoption in clinical practice.

The ability to quantitatively identify DNA's cell of origin by its methylation profiles holds great potential for clinical diagnostics[18,19]. To demonstrate that Renew's wgNESSI-Seq protocol can be used to quantify cDNA at low concentrations via analyzing cell type-specific methylation signatures, regions with Tissue 1-specific methylation patterns were identified by analyzing publicly available methylation data. To confirm these regions as identifiers of Tissue 1-derived DNA, gDNA was extracted from our tissue of interest (Tissue 1) and sequenced via wgNESSI-Seq. Methylation analysis identified 8,457 regions with Tissue 1-specific methylation signatures (average 963 bp per region). Figure 3A showcases one of the 8,457 regions with extensive differential methylation between Tissue 1 and Tissue 2 (not of interest). Tissue 1-derived gDNA was then fragmented to mimic the size profile of cDNA and bioinformatically mixed with blood at varying ratios (Figure 3B).

Bioinformatically targeting the 8,457 regions with Tissue 1-specific methylation signatures following NESSI-Seq, the correlation between predicted and actual values of gDNA fractions revealed an R^2= 0.999 (Figure 3B). Each mixture contained approximately 2000 randomly selected reads. The grey shadow represents the expected 95% Cl based on sampling error with perfect read assignment.

To investigate the consistency of detection with very low inputs of real cfDNA, cfDNA was isolated from Tissue 1 and blood plasma and sequenced via wgNESSI-Seq. The 8,457 regions with Tissue 1-specific methylation patterns were analyzed to identify cfDNA derived from Tissue 1. The cfDNA from Tissue 1 and blood plasma were bioinformatically mixed at various concentrations, and estimates for the proportion of Tissue 1-derived cfDNA in these mixed samples were calculated. The results revealed a high correlation ($R^2 = 0.997$) between expected and observed proportions of Tissue 1-derived cfDNA, even with an average of less than 100 reads per dilution (Figure 3C).

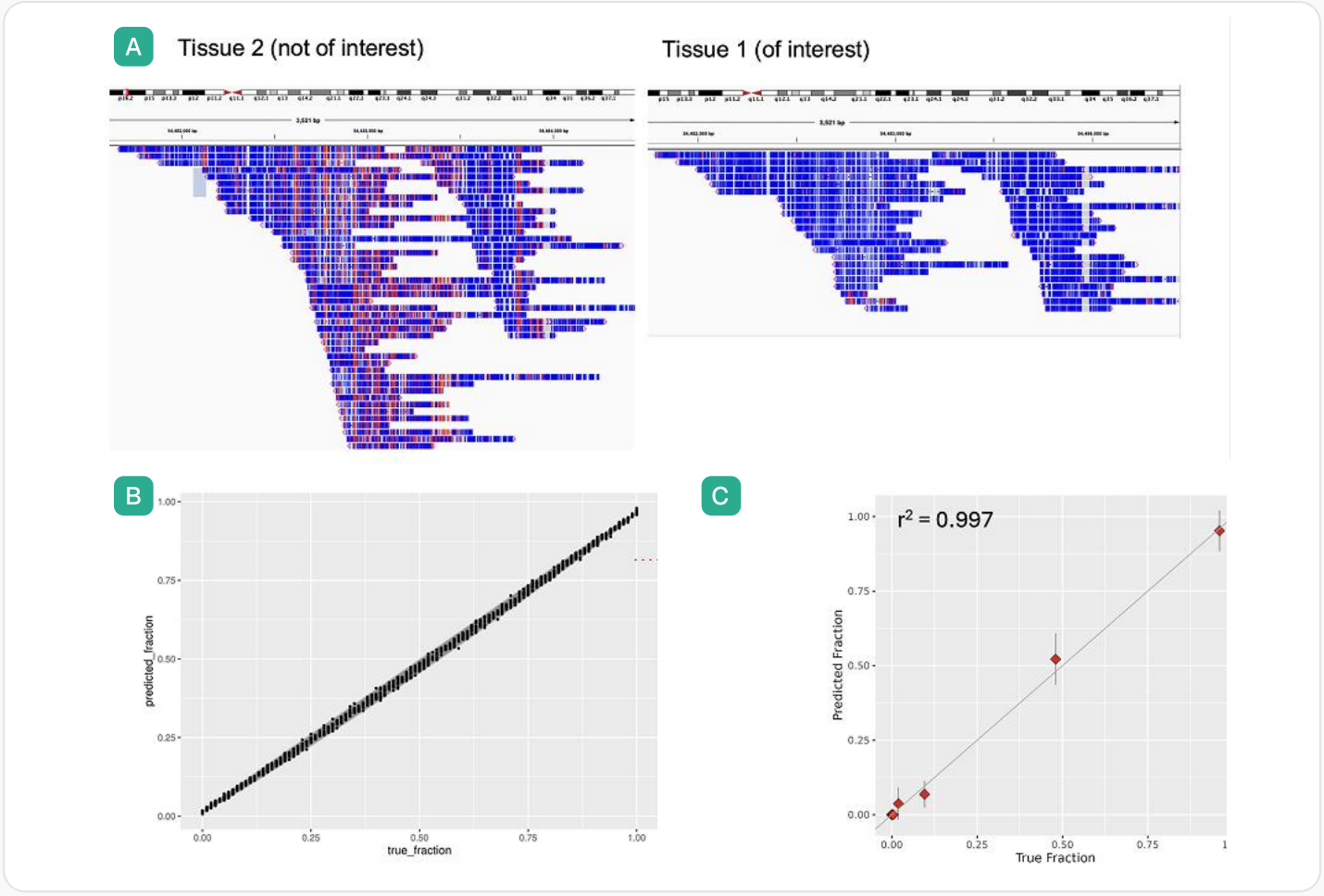

Figure 3. Tissue-specific methylation patterns can be quantitatively identified in tissues of interest with NESSI-Seq. Tissue 1-specific methylation patterns were determined and confirmed with wgNESSI-Seq on gDNA derived from our tissue of interest (Tissue 1) compared to other tissues (Tissue 2) (A). Correlations between predicted and actual fraction values of Tissue 1-derived gDNA were plotted, revealing high accuracy in analyses of 2000 randomly selected reads (B). To assess the quantitative value at low concentrations of cfDNA, cfDNA was isolated from blood plasma and sequenced wgNESSI-seq at different dilutions (C). From an average of less than 100 reads per dilution, wgNESSI-seq for cfDNA revealed an $R^2 = 0.997$.

Copy Number Variation Analysis

Copy-number variation (CNV) can inform the diagnosis of developmental diseases and the risk of developing other conditions[20]. To test whether wgNESSI-Seq can detect changes in copy number, differing amounts of female and male cfDNA were mixed (Figure 4). By analyzing the read depths of the X and Y chromosomes, relative copy numbers were captured to validate the efficacy of wgNESSIseq for CNV detection. wgNESSI-Seq quantitatively distinguished between copy numbers less than 25% apart.

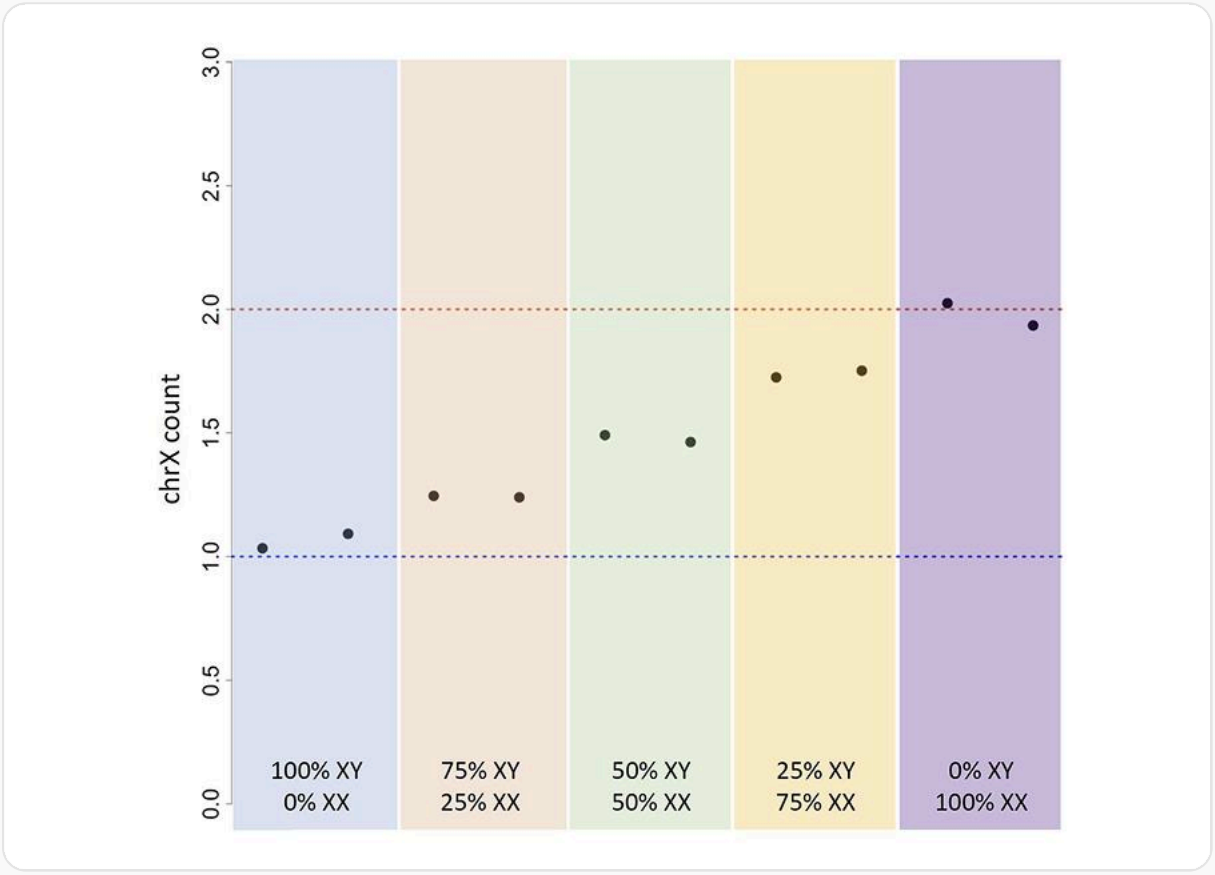

Figure 4. Copy Number Variations can be detected with wgNESSI-Seq. Varying amounts of female and male cfDNA were mixed and sequenced via wgNESSI-Seq. NESSI-Seq accurately differentiated samples with copy number differences of less than 25%.

Looking Forward

As Renew continues to pioneer advancements with NESSI-Seq and explore its rapidly growing use cases, Renew's vision in which genomic and epigenomic data are more accessible, actionable, and integral to personalized medicine becomes increasingly attainable. The integration of NESSI-Seq into research and clinical settings is expected to accelerate, driven by its ability to provide detailed insights into the genetic and epigenetic mechanisms underlying complex diseases. Looking forward, we anticipate several developments:

Broader Accessibility
Efforts will be intensified to make NESSI-Seq and Nanopore technology more accessible to a wider range of laboratories and institutions, enabling broader adoption across various fields, including oncology, genetics, and personalized medicine. This will involve partnerships, training programs, and support systems to ensure seamless integration.

Enhanced Computational Tools
With the increase in data complexity and volume generated by NESSI-Seq, the development of advanced computational tools and algorithms will be crucial. These tools will be designed to handle, analyze, and interpret datasets with larger numbers of targets more efficiently, providing deeper targeted insights. Standardized protocols will be developed to ensure consistent and accurate results across different applications.

Integration with Other Technologies
We foresee a future where NESSI-Seq will be seamlessly integrated with other diagnostic and therapeutic technologies, creating a comprehensive toolkit for medical professionals that enhances patient care through precision medicine. This includes combining NESSI-Seq with imaging technologies, other molecular diagnostics, and personalized treatment plans to provide a holistic approach to patient care.

Regulatory Advancements
As the technology proves its efficacy and safety, regulatory approvals will be sought to formalize its use in clinical diagnostics, ensuring it meets regulatory health standards. Achieving these approvals will allow NESSI-Seq to be adopted more widely in clinical settings, providing reliable and accurate diagnostic information for patient care.

Conclusions

The application of wgNESSI-Seq to cDNA tissue of origin analysis and quantification at low concentrations highlights its potential for cDNA-based liquid biopsy. Leveraging nanopore sequencing enables the simultaneous acquisition of multiple layers of genomic and epigenomic information to accelerate multi-omics approaches in diagnostic medicine.

At its core, the evolution of NESSI-Seq represents a fundamental shift in methylation analysis, transitioning away from depth-dependent methods that can obscure true biological signals to direct analysis of native molecules. By eliminating conversion and amplification steps that introduce synthetic DNA and bias, NESSI-Seq offers an unaltered view of DNA sequence and methylation, enhancing the accuracy and relevance of genetic and epigenetic data. Thus, NESSI-Seq is positioned as a powerful tool to deepen our understanding of the molecular drivers of health and disease, setting the stage for developing more precise diagnostics and targeted therapies.

As we continue to advance NESSI-Seq technology, Renew Biotechnologies is dedicated to expanding its impact in both research and clinical domains, accelerating innovation to improve patient outcomes. Our commitment goes beyond advancing technology; it is about reshaping how we approach, diagnose, and treat disease in an era of precision medicine.

To learn more about Renew's NESSI-Seq technology and how it can improve your cfDNA studies

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