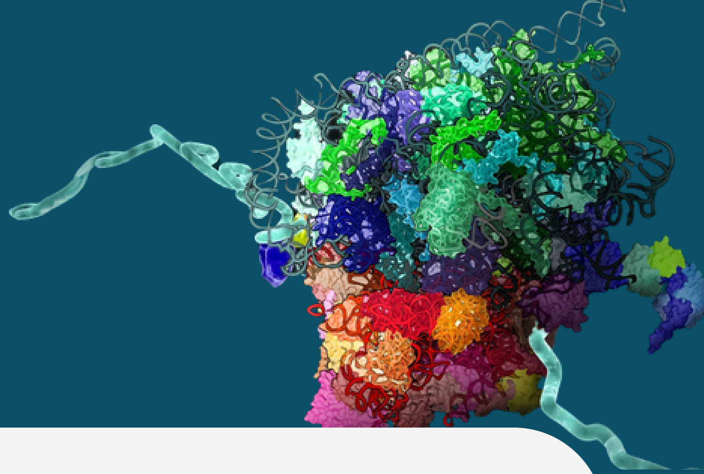

APPLICATION NOTE

nano-tRNAseq

Simultaneously investigate both tRNA abundance and tRNA modifications at unprecedented resolution

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Executive Summary

Transfer RNA (tRNA) modifications and abundance plays a crucial role in gene regulation, disease mechanism, and therapeutic development. Traditional methods face biases and technical limitations, hindering comprehensive analysis. The nano-tRNAseq, a cutting-edge sequencing technology by Immagina Biotechnology, enables direct, single-molecule quantification of full-length tRNA and its modifications using nanopore Direct RNA Sequencing (DRS). This solution provides deeper insights into tRNA biology, overcoming the limitations of conventional techniques. Available as a benchtop kit, nano-tRNAseq offers a scalable platform to explore tRNA dynamics, advancing RNA research and paving the way for new diagnostic and therapeutic breakthroughs.

Beyond codon stability: Understanding the complexities of tRNA abundance and modifications

Cloverleaf shaped tRNA molecules (Figure 1) are fundamental components of the translational machinery, traditionally recognized for their roles in maintaining codon-anticodon pairing and ensuring the fidelity of protein synthesis. However, beyond their classic function, tRNA abundance and chemical modifications play critical roles in regulating translation. Understanding these factors is essential for deciphering the intricate mechanisms of gene expression control.

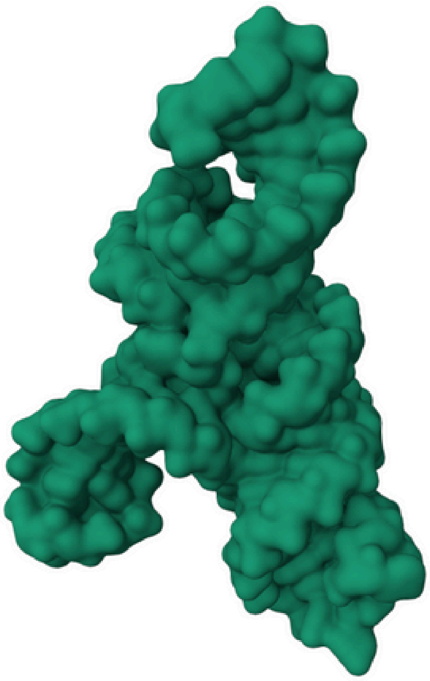

Figure 1: Cloverleaf and 3D model structure of tRNA

tRNA modifications such as methylation, pseudouridylation, thiolation, and acetylation affect tRNA stability, structure, and decoding accuracy [1]. Their specific incorporation along the tRNA sequence introduces an additional layer of complexity, contributing to the vast combinatorial diversity of tRNA species. These modifications help maintain proper folding and structural integrity, [2] ensuring efficient interactions with the ribosome and enhancing translational precision.

Beyond modifications, tRNA abundance is tightly regulated by transcriptional activity, degradation pathways, and cellular stress responses, enabling cells to fine-tune protein synthesis under varying conditions.

Recent research has demonstrated that both tRNA abundance and post-transcriptional modifications are dynamically regulated in response to environmental stimuli, enzymatic activity, and cellular developmental stages.

Dysregulation of tRNA modifications and abundance has been implicated in a wide range of cellular processes, raising fundamental questions about their collective influence on translation:

- **How do they impact codon usage bias, translational efficiency, and translational speed?**
- **How does crosstalk between tRNA modifications and other RNA modifications regulate gene expression?**
- **How do these factors influence tRNA stability and turnover?**

Addressing these questions could unlock the potential of tRNAs as diagnostic biomarkers, therapeutic targets, or even novel RNA-based medicines.

However, studying both tRNA abundance and modifications simultaneously has been challenging due to the lack of effective analytical tools. Novel approaches that enable the concurrent quantification of tRNA abundance and the characterization of biochemical modifications hold promise for uncovering new insights into tRNA biology and their broader implications for human health and disease.

The Significance of tRNA Modifications in Cellular Function and Disease

RNA is the most heavily modified of all RNA types, with over 150 distinct types identified across different organisms.

In humans, at least 43 distinct chemical modifications have been identified, with an average of 13 modifications per cytosolic tRNA molecule [3,4] (Figure2).

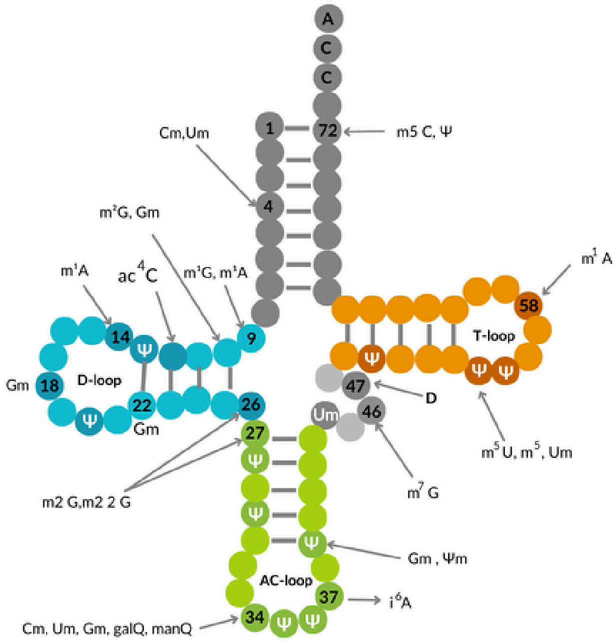

Figure 2: Schematic representation of human cytoplasmic tRNA modifications. Modified nucleotides are numbered and each type of tRNA modification is indicated. The abbreviation of each RNA modification conforms with the RNA modification database MODOMICS6. Um (2'-O-methyluridine), Gm (2'-O-methylguanosine), Cm (2'-O-methylcytidine), m2G (N2-methylguanosine), m1G (1-methylguanosine), m1A (1-methyladenosine), ac4C (N4-acetylcytidine), Ψ (pseudouridine), D (dihydrouridine), m22G (N2,N2-dimethylguanosine), galQ (galactosyl-queuosine), manQ (mannosyl-queuosine), i6A (N6-isopentenyladenosine), m7G (7-methylguanosine).

Even subtle alterations in modification patterns can have profound biological consequences (Table 1). These modifications ensure tRNA stability, proper folding, accurate mRNA decoding, and efficient interaction with ribosomes. They also play important roles in gene expression regulation and cellular stress response [5].

Table 1: A wide range of chemical modifications can occur throughout a tRNA molecule.

Common tRNA chemical modifications	
tRNA Modification	**Impact**
Nucleotide base methylation of adenine, cytosine, uracil, or guanine targeting distinct tRNA species and positions	Maintains tRNA structural integrity and functional efficiency
Ribose methylation	Enhances tRNA stability, improves translational accuracy, and strengthens interactions with ribosomes
Isomerization of uridine into pseudouridine (Ψ)	Enhances tRNA stability, enhances codon-anticodon pairing, and strengthens interactions with ribosomes
Conversion of adenosine-to-inosine	Enables wobble-base-paring and expands codon recognition to enhance translational flexibility
Thiolation to replace oxygen atoms with sulfur atoms	Improves thermal stability and base-pairing
Hyper-modifications in the anticodon	Ensures precise codon recognition, prevents frame shifting, and enhances translational fidelity
Acetylation on bases or the ribose backbone	Enhances structural flexibility and prevents degradation to support efficient codon recognition
Reduction of uridine to dihydrouridine	Increases structural flexibility, helps maintain structural integrity, and optimizes interactions with ribosomes
Addition of an isopentenyl group to adenine or guanine	Enhances codon recognition and translational efficiency, particularly under stress conditions

Aberrant tRNA modification has been associated with a wide variety of human diseases, including the following:

Cancer: Atypical tRNA modifications contribute to translational dysregulation associated with oncogenesis and tumor progression. tRNA modification can also have profound effects on immune-cell function that correlate with cancer immune surveillance [6-9].

Neurodegenerative Disorders: Dysregulated tRNA modification disrupts protein homeostasis in neurons, leading to defects in cell adhesion, migration, and differentiation which impacts brain growth, nervous system development, and neurological disorders [4-6,10].

Metabolic Disorders: Altered translation and dysfunction due to tRNA modifications can disrupt enzyme activity, redox homeostasis, and metabolic pathways that have been associated with obesity, Type 2 diabetes, cardiomyopathies, and lactic acidosis [7,10,11].

Mitochondrial Disorders: Since mitochondria encode for their own tRNAs, any dysregulated tRNA modifications can impair mitochondrial protein synthesis resulting in compromised oxidative phosphorylation, leading to energy deficits [5,7,12].

Immune Disorders: Certain tRNA modifications stabilize codon paring to support increased translation and rapid immune cell proliferation and differentiation. Altered modifications can impair immune responses, leading to weakened defense mechanisms against infections, cancer, and autoimmune conditions [7,13,14].

Some studies suggest that strong correlations between tRNA abundance, dysfunctional modifications, and disease may present opportunities for diagnostic, prognostic, and therapeutic biomarkers [6]. For example, type-specific signatures of tRNA sequence and modifications have emerged as potential predictive biomarkers for some cancers [8,9].

Proof-of-concept studies have already resulted in promising strategies for interventions such as targeting tRNA-modifying enzymes [8,15,16].

Technological advancements are emerging quickly to provide more effective methods for the integrated study of tRNA abundances and modifications. Although each approach has advantages and disadvantages, any of them may provide new insights to advance research into the biological function and dynamics of tRNA abundance, their modifications, and their involvement in human diseases.

Challenges in Analyzing tRNA Abundance and Modifications

The heavily modified nature of post-transcriptional tRNA molecules and their dynamic interaction with the translational machinery pose many challenges to analyzing the role of active tRNAs. Various methods provide some answers, but none offer the comprehensive information needed. Available methods may also be labor-intensive, limited to tRNA isolation, or constrained by sequence targeting requirements [12].

- **tRNA microarrays** can reveal tRNA abundances, but they require conversion of the tRNA into cDNA. Any present tRNA modifications that disrupt Watson-Crick base pairing, which are abundant in tRNAs, will cause premature termination or base pair mismatches that yield truncated reads and incorporation errors [12,16,17].
- **Next-generation sequencing (NGS)** can provide tRNA abundance, but it also requires the conversion of the tRNA molecules into cDNA prior to creating the sequencing library. Therefore, NGS methods cannot detect most tRNA modifications [13,17,18].
- **Nanopore direct RNA sequencing (DRS)** is well-established as a long-read RNA sequencing technology that enables direct sequencing of individual, full-length, native RNA strands. cDNA/PCR sequencing is not required [17,19]. However, commonly used library preparation solutions lead to poor sequencing yields and biases towards longer tRNAs [17,18].

Additional Challenges in Studying tRNA Modifications and Abundance

- **Lack of Standardized and Robust Solutions**

Most tRNA analysis workflows rely on custom-built protocols, leading to reproducibility issues and variability across different studies. A commercially available, standardized, and highly reproducible solution is needed.

- **Difficulty in Capturing Modified tRNAs and Their Regulatory Networks**

tRNA modifications play a central role in translation regulation, but conventional techniques fail to map their interactions with tRNA abundance, preventing a deeper understanding of these regulatory networks.

- **High Sample Input Requirements in Traditional Workflows**

Many current methods require large amounts of RNA input, making it difficult to study tRNAs from rare or limited biological samples such as clinical specimens or small cell populations.

- **Inefficiency in High-Throughput Studies**

Traditional techniques, including LC-MS and microarrays, are labor-intensive and unsuitable for large-scale studies, restricting their application in high-throughput research.

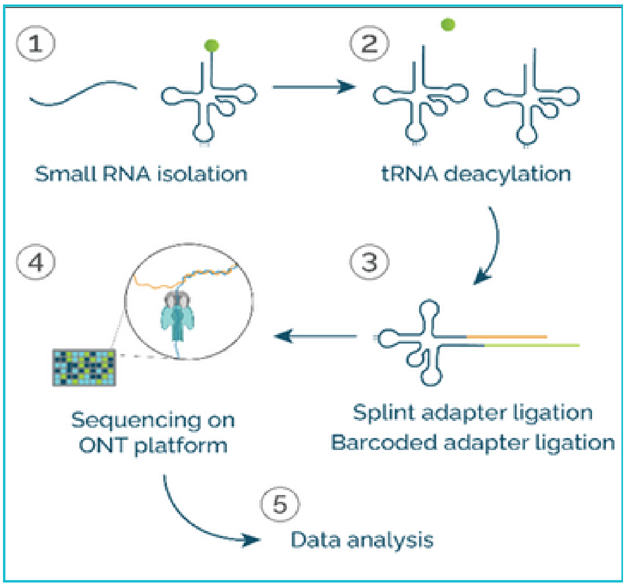

Figure 3: Simple overview of the nano-tRNAseq protocol

nano-tRNA-seq: A Comprehensive Solution for tRNA Analysis

nano-tRNAseq emerges as a groundbreaking technology designed to address the complexities of tRNA analysis. This is a new library preparation technology for nanopore DRS that facilitates the direct sequencing of tRNA molecules, enabling simultaneous quantification of abundance and precise mapping of modification sites [17]. It identifies RNA modifications across the entire length of a transcript and for all isoacceptors.

The nano-tRNAseq library preparation protocol (Figure 3), improves base calling and precise mapping by extending the 5' and 3' ends of mature tRNAs with RNA adapters. The 3' CCA overhang typically present in the mature tRNAs incorporates a double ligation of RNA adapters at both the 5' and 3' ends of the tRNA molecules. This addition improves the proportion of base-called and mapped tRNA molecules.

nano-tRNAseq is designed to analyze tRNA with high precision and sensitivity. This method allows researchers to:

- **Map tRNA isoforms within different cellular environments.**
- **Measure modifications in tRNA molecules, detecting changes in response to cellular conditions.**
- **Investigate interactions between tRNA modifications, shedding light on regulatory pathways.**
- **Track site-specific modifications across various tRNA isoacceptors, helping to understand their role in translation efficiency.**

By offering a deeper look into tRNA biology, nano-tRNAseq provides valuable insights for studies in gene expression, translation regulation, and disease pathology.

Conclusion: Paving the Way for Advanced tRNA Research

Advancing the understanding of tRNA abundance and modifications and their potential therapeutic application requires a concerted effort across translational biology. nano-tRNAseq presents a valuable solution to bridge this gap. This powerful technology is now available in a user-friendly benchtop kit provided exclusively by Immagina Biotechnology. The nano-tRNAseq nanopore DRS library preparation kit enables the direct sequencing of full-length, native tRNA molecules for simultaneous quantitation of tRNA abundances and precise modification mapping at single-transcript resolution. Providing new insights into tRNA biology, nano-tRNAseq has the potential to advance our understanding of tRNA dynamics, ultimately providing new avenues for disease diagnostics, prognostics, and therapeutic development.

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