

# RIBO NUCLEASE



Application  
note.

ALL-IN-ONE  
RiboLace™  
Gelfree kit

# OPTIMIZATION



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**June 2025**

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# *Executive Summary*

In this application note, we present the latest advancements of Immagina's ribosome profiling technology (RiboLace™ gel-free solution). By optimizing nuclease (NUX) digestion and transitioning to a gel-free workflow, Immagina's protocol significantly enhances data quality, reproducibility, and workflow efficiency. Our results demonstrate high RPFs length precision, signal-to-noise ratio, and superior mapping fidelity compared to traditional gel-based methods. Together, these improvements position RiboLace™ gel-free as a streamlined, reliable, and highly accessible solution for modern ribosome profiling studies.

## *Simple and efficient ribosome profiling*

Ribo-Seq is a powerful tool for dissecting translational regulation (for an overview of the methodology refer to the following link: <https://www.immaginabiotech.com/services/ribosome-profiling>). Conventional ribosome profiling (Ribo-seq) protocols typically rely on gel-based size-selection and separation of ribosome-protected RNA fragments (RPFs) and often necessitate ultracentrifugation to isolate ribosome-RPF complexes. While these procedures have proven effective, they remain labor-intensive, time-consuming, and require specialized equipment. Moreover, traditional protocols do not distinguish between actively translating RNA and RNA bound to inactive ribosomes, limiting the accuracy of the results [1,2] in detecting true signature of protein synthesis.

Immagina's RiboLace™ technology (<https://www.immaginabiotech.com/our-technologies/ribolace>) selectively capture actively translating ribosomes through a bead-based pull-down method, thus eliminating the requirement for antibodies or genetic tags [3]. Despite these improvements, RiboLace™ first version (before 2023, Cat #RL00S-04, #RL00P-12, #RS0XL-12) relies on gel-based size selection, which remains a significant bottleneck due to workflow complexity, time demands, and limited accessibility.

To overcome these challenges, we developed a gel-free version of the RiboLace™ protocol (Cat #GF001-12, #MX001-36). This innovative approach replaces gel purification with biochemical selection methods to enrich for RPFs, significantly simplifying the workflow and enhancing accessibility. Additionally, the gel-free approach uniquely facilitates the detection of disomes, footprints indicative of two ribosomes positioned closely together, without necessitating complex procedures. When coupled with our unique RiboSeq LaceSeq™ library preparation kit (LaceSeq™, <https://www.immaginabiotech.com/our-technologies/LaceSeq™>, Cat #LS001-12), this streamlined method simultaneously profiles monosomes and disomes distribution along RNAs, offering substantial workflow efficiency and accuracy.

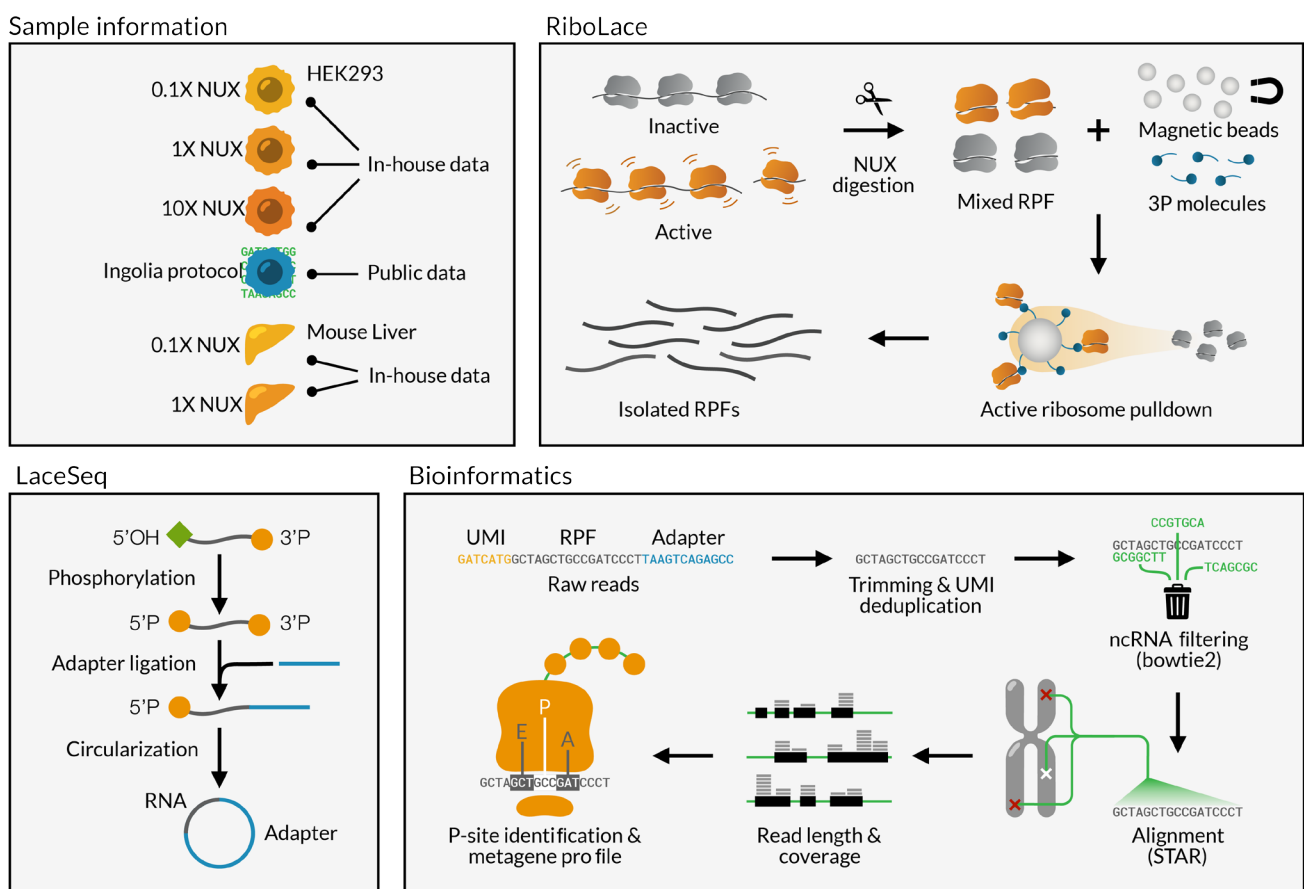
## *Importance of NUX optimization*

The precise control of nuclease (NUX) concentration during RNA digestion is crucial for generating homogeneous size distribution of high-quality RPFs. This optimization is especially critical in gel-free workflows, where purification relies primarily on biochemical selectivity rather than physical separation methods. RiboLace™ protocol relies on a careful optimization of NUX concentration which is essential to achieve optimal RNA digestion without compromising the integrity and specificity of the RPFs population. The NUX concentration provided in the kit is broadly suitable for a wide range of biological samples, from cultured cells to tissue lysates. However, when working with challenging samples, performing a lab-specific NUX optimization step, as outlined in the protocol, is highly recommended.

## Data analysis

To systematically assess the impact of varying NUX concentrations on RiboLace™ performance, we analyzed samples from the HEK293 cell line treated with three distinct NUX concentrations: 0.1X (mild-digesting condition), 1X (protocol recommendation), and 10X (over-digesting condition). Moreover, we also wanted to check performance on tissue samples and thereby included 2 mouse liver tissue samples, utilizing either the 0.1X and 1X NUX concentrations, respectively. Samples were processed following the standard protocol guidelines, subjected to next-generation sequencing (NGS), and analyzed using our comprehensive MARTIAN™ bioinformatics pipeline (<https://www.immaginabiotech.com/our-technologies/martian>), including adapter trimming, unique molecular identifier (UMI) collapsing, genome/transcriptome alignment, and peptidyl-site (p-site) calculation.

For benchmarking purposes, we included a previously published HEK293 dataset processed with the conventional ribosome profiling method, long considered the standard in the field, originally developed by Ingolia et al. [4]. Data processing for this external control was conducted consistently, using the same bioinformatics pipeline to facilitate direct comparisons (Fig. 1).



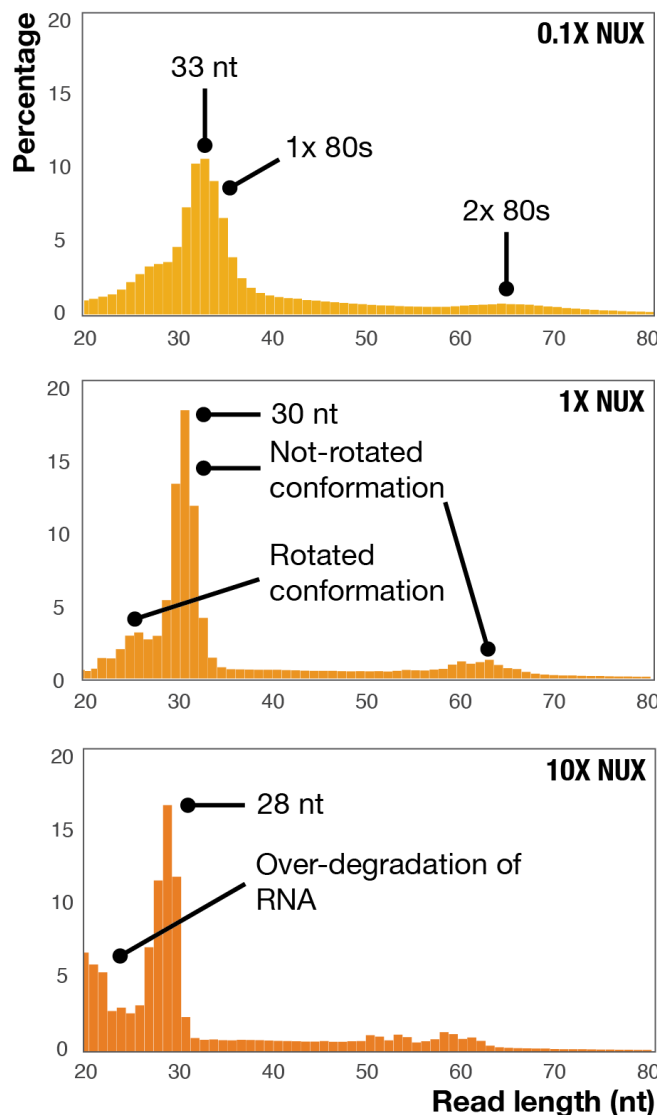
**Figure 1 | Technology overview.** HEK293 cells and murine liver tissue samples were subjected to the Immagina RiboLace™ protocol at varying concentrations of nuclease. Public ribo-seq data obtained from HEK293 cells and using the standard Ingolia et al. protocol was included for comparative purposes. Isolated RPFs are prepared for sequencing using the Immagina LaceSeq™ protocol. Resulting reads are consistently processed through the in-house developed MARTIAN™ bioinformatic pipeline.

## Increasing NUX concentration changes the RPF length distribution

Comparison between the recommended NUX concentration (1X NUX) and mild digestion conditions (0.1X NUX) revealed that 1X NUX led to more efficient digestion, especially at the edges of ribosome footprints, and resulted in a noticeable reduction of background noise. The average fragment length shifted from approximately 33 nt (0.1X NUX) to around 30 nt (1X NUX) (Fig. 2, Top & Center). In parallel, we observed a shift in the 2x80S peak, corresponding to the well-characterized disome conformation [5], suggesting more accurate and consistent RNA fragmentation.

It is important to note that increasing the NUX amount without carefully monitoring its activity may lead to over-digestion, causing the disome peak to disappear and resulting in a higher proportion of very small fragments (<25 nucleotides), thus increasing contamination from over-digested RNAs (Fig. 2, Bottom; Table 1).

We also observed a minor decrease in the number of uniquely mapped reads onto the transcriptome: 13.2%, 11.8%, and 11.6% for the 0.1X, 1X, and 10X samples, respectively (Table 2). This slight decline is most likely due to the general read distribution shifting towards shorter fragment lengths, thereby reducing the likelihood that individual reads map uniquely to the transcriptome. Due to the observed over-digestion, the 10X NUX sample was excluded from further analyses at this stage.



**Figure 2 | Shifts in read distribution with increased NUX.** Histograms of the read length distributions of RPFs with varying NUX concentrations. Note the large peak for monosomes and the smaller peak at approximately 2x the length for disomes.

Sample	Cell line	Raw reads (M)	Pass trimming (M)	% rRNA <sup>a</sup>
0.1X NUX	HEK293	19.9	13.6	57.6
1X NUX	HEK293	26.6	22.2	76.8
10X NUX	HEK293	21.7	17.7	85.7
Ingolia protocol (Ingolia et al.)	HEK293	72.1	65.5	14.5 <sup>b</sup>

**Table 1 | Trimming and filtering statistics.**

<sup>a</sup> This percentage is based on the reads that pass the trimming step.

<sup>b</sup> The selected data sets utilize a rRNA depletion step, hence the lower rRNA counts.

Sample	Input reads (M)	Transcriptome unique (%)	Transcriptome multi (%)
0.1X NUX	4.9	13.2	86.8
1X NUX	4.6	11.9	88.1
10X NUX	2.4	11.6	88.4
Ingolia protocol (Ingolia et al.)	39.1	3.7	96.3

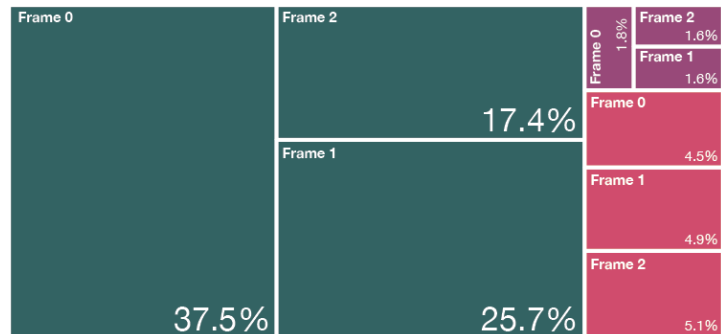
**Table 2 | Alignment statistics.** Input reads for alignment are obtained from reads that pass trimming, rRNA, tRNA and snRNA filtering.

## Increasing NUX improves mapping quality and signal to noise ratio

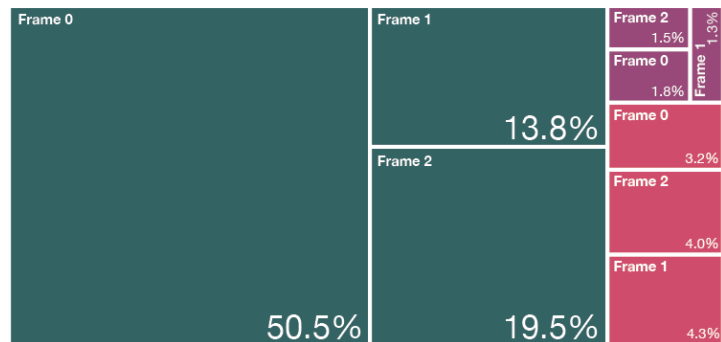
As RPFs are primarily isolated from ribosomes engaged in active translation, two quality control metrics are of particular importance. First, the majority of reads should originate from the coding sequence (CDS) region where translation actively occurs, rather than from the 5' or 3' untranslated regions (5' UTR and 3' UTR). Second, since ribosomes translate one codon at a time, moving forward in three-nucleotide "steps," the calculated p-site positions should predominantly lie in translation frame 0 across transcripts.

Our analysis showed that, even under mild-digesting conditions (0.1X NUX), the RPFs mapping results were comparable to that of the conventional Ingolia et al. protocol, despite the latter utilizing a gel extraction step to preferentially isolate RPFs (Fig. 3, Top & Bottom). The majority of calculated p-sites were localized within the CDS, and most CDS reads aligned to the correct frame. Using the recommended NUX concentration (1X NUX) we observed a substantial improvement in frame 0 CDS reads, increasing from 37.5% (0.1X NUX) to 50.5% (1X NUX; Fig. 3, Top & Center). Simultaneously, the proportion of reads mapping to the 5' and 3' UTRs decreased from 19.5% to 16.8%.

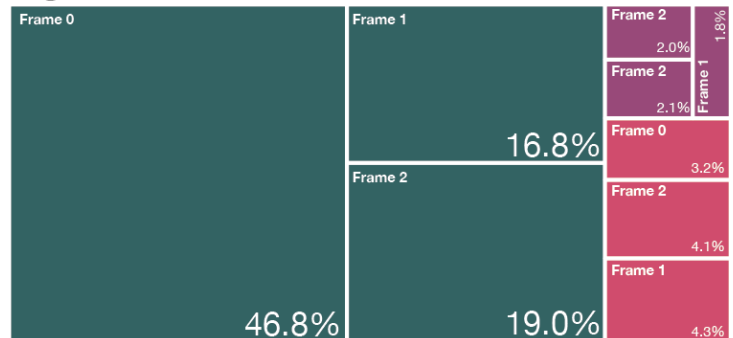
### 0.1X NUX



### 1X NUX



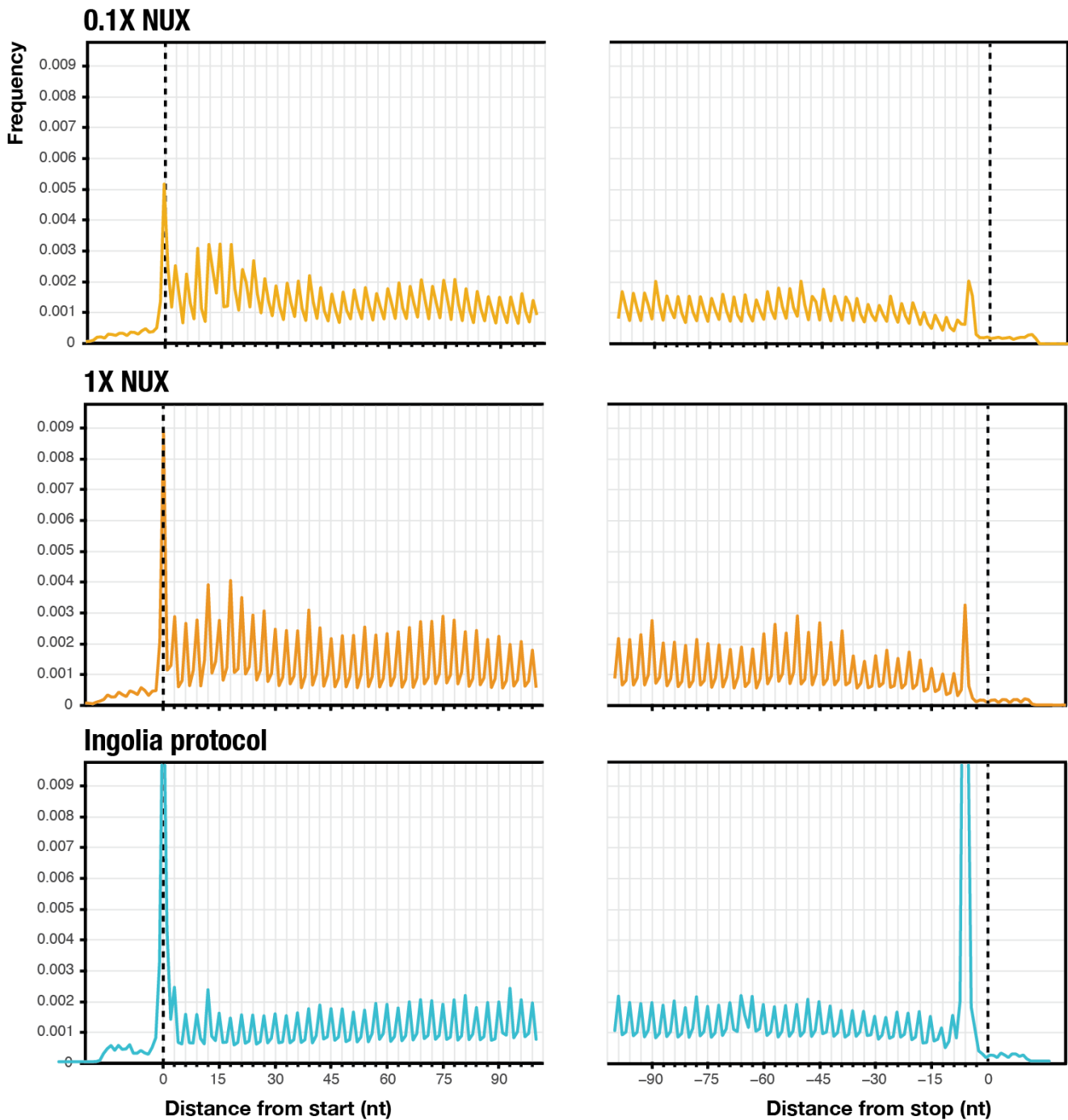
### Ingolia protocol



■ CDS ■ 3' UTR ■ 5' UTR

**Figure 3 | Region and frame of mapped RPF reads.** Treemap plots of the percentage of p-sites lying in each frame and transcript region across protocols.

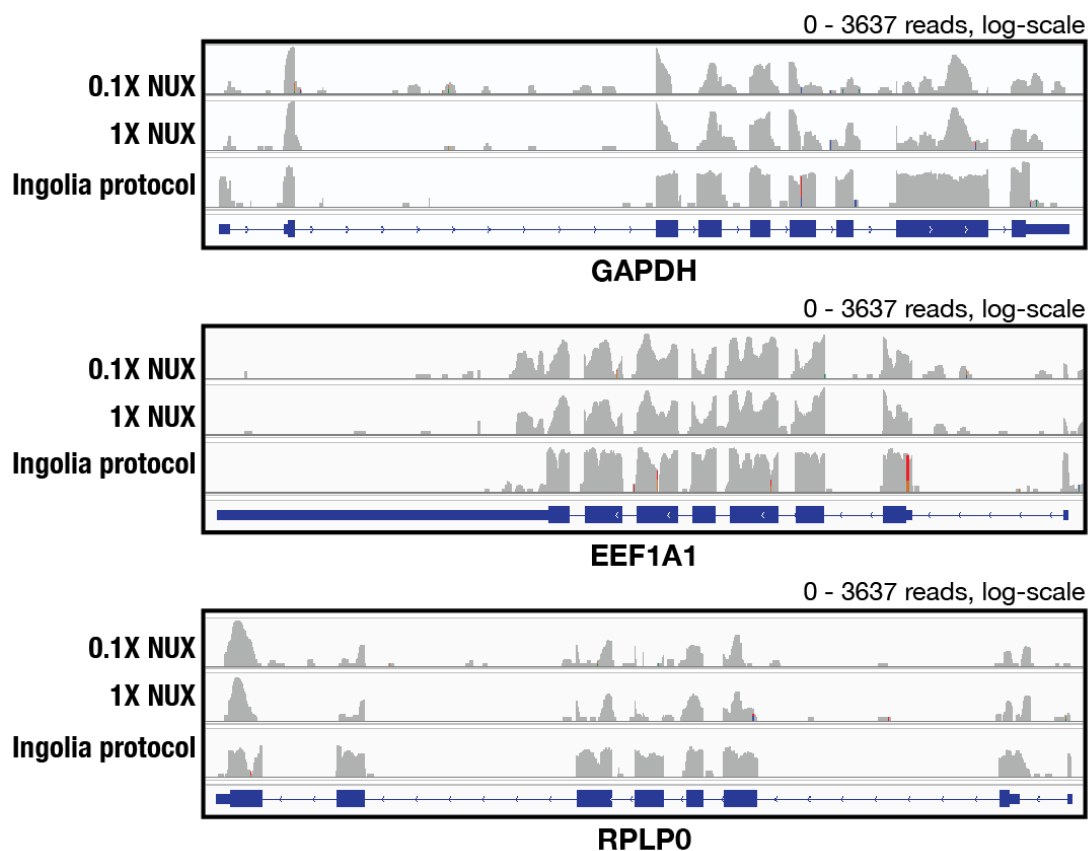
We further utilized the calculated p-site information to generate metagene plots, in which the frequency of p-sites is plotted across transcript coordinates. As expected, an ideal metagene plot should exhibit a sharp three-nucleotide periodicity, with clear frame 0, 1 and 2 stops. Indeed, metagene plots generated from samples processed with 1X NUX concentrations demonstrated a clearer three-nucleotide periodicity compared to both 0.1 NUX condition and the Ingolia et al. protocol (Fig. 4).



**Figure 4 | Metagene plots.** Each plot shows the frequency of p-sites for all reads across all individual transcript coordinates.

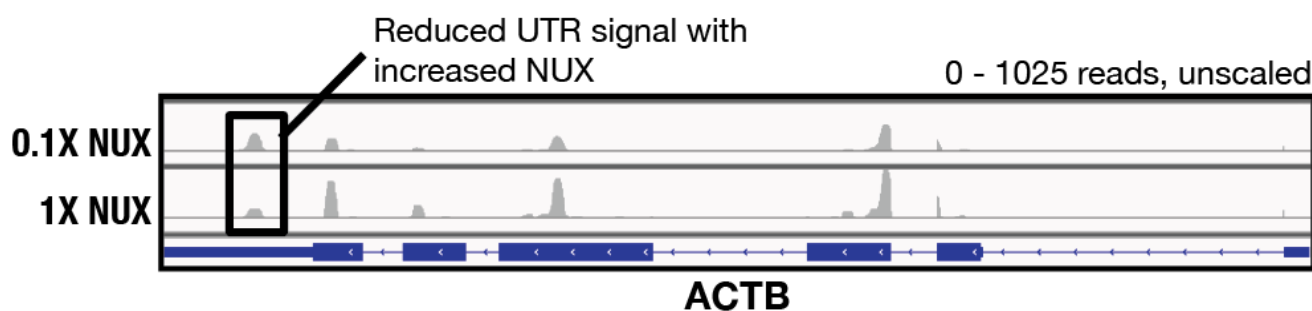
## *RiboLace™ Gel-Free: faster, clearer, and more insightful profiling*

To further investigate, we examined the coverage of RPF-derived reads over three individual housekeeping genes as examples, using the Integrative Genome Viewer (IGV, <https://igv.org/>). For GAPDH, RPLP0, and EEF1A1, the overall coverage profiles were similar to those obtained using the Ingolia protocol, despite the external origin of the control dataset and potential batch effects such as differences in cell culture and handling conditions (Fig. 5). Discrepancies at individual positions may still result from batch effects or, alternatively, from the different chemistry employed in our kit. Since RiboLace™ specifically targets actively translating ribosomes, we observe coverage where translation is truly occurring, rather than in regions where ribosomes are stalled or translation is inefficient, as often seen with conventional protocols that do not distinguish between active and inactive ribosomes. Indeed, our kit demonstrated increased resolution in identifying these translation bottlenecks compared to the flatter coverage distribution observed with the Ingolia protocol.



**Figure 5 | Housekeeping gene coverage plots.** The log-normalized coverage of reads on the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) and ribosomal protein lateral stalk subunit P0 (RPLP0) &, across NUX and protocols. Generated using IGV.

Finally, occasional UTR peaks were observed in both our data and that generated with the Ingolia protocol (Fig. 5). Some of these are actually coming from unconventional ORFs while other peaks might be unexpected; their presence can be attributed to the absence of size-selection steps. Importantly, using the recommended NUX concentration (1X NUX) substantially enhances data quality (Fig. 6, Fig. 3).

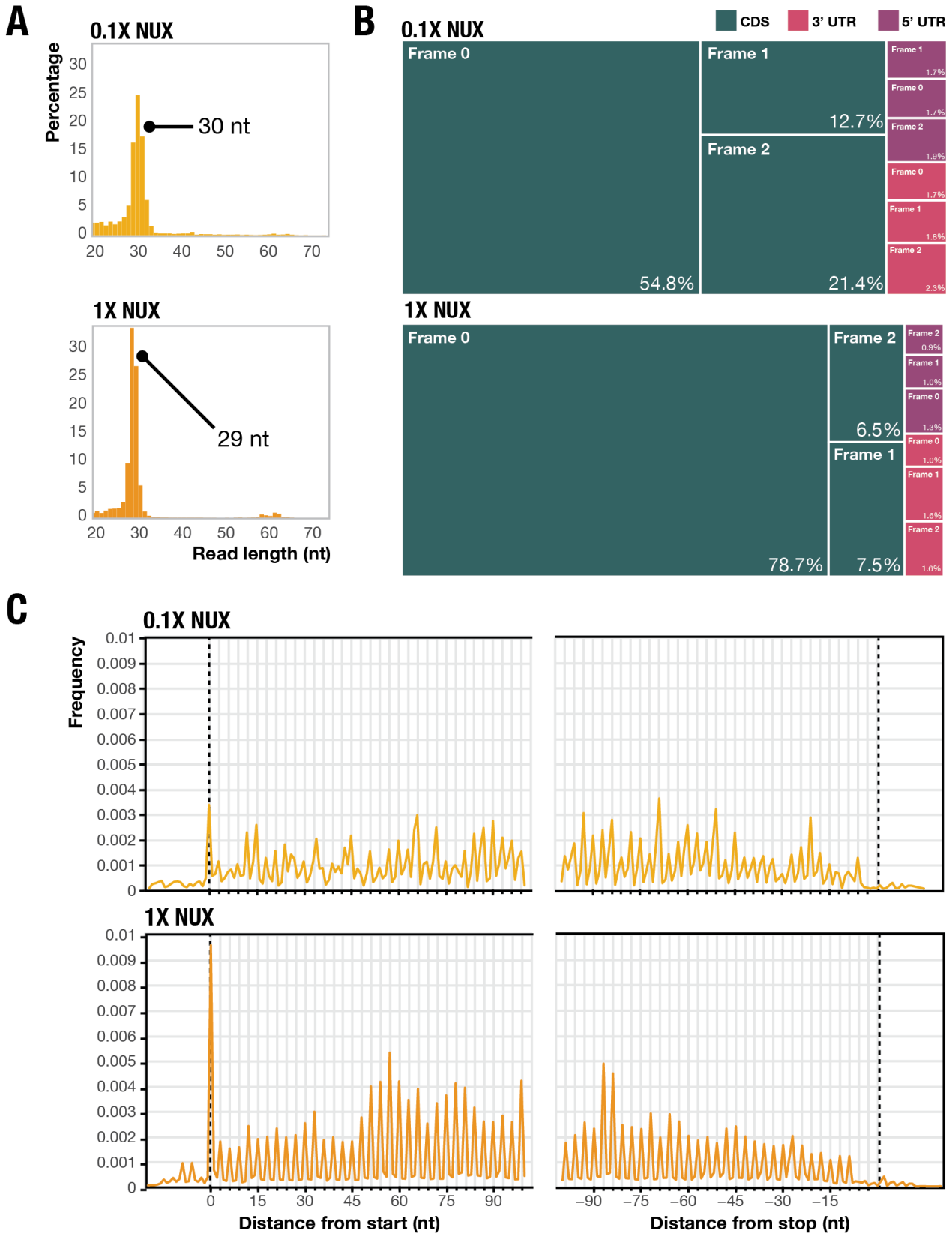


**Figure 6 | Lower UTR signals with standard NUX concentration.** Raw coverage of ACTB for the different NUX concentrations. Generated using IGV.

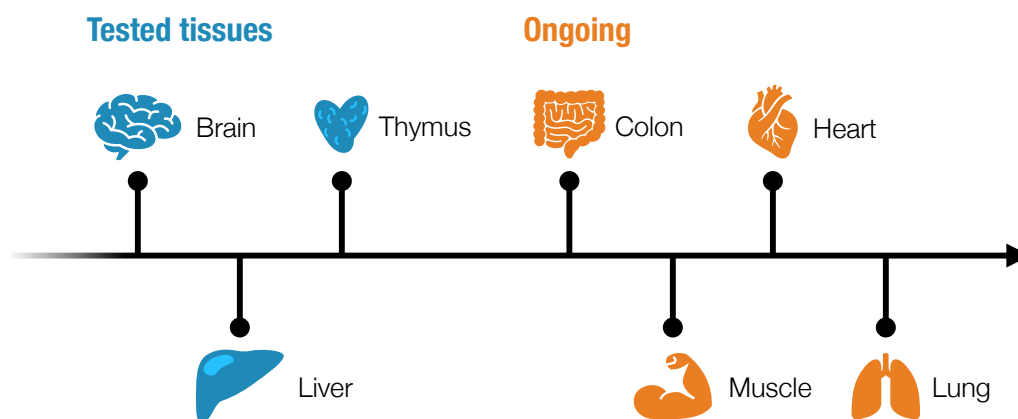
## *The current RiboLace™ protocol increases performance on tissue samples*

Analysis of the two murine liver samples underscores the importance of properly tuning NUX concentration when working with heterogeneous tissue samples (Fig. 7). While using 0.1X NUX is acceptable for tested cultured cells, resulting only in slightly increased background, liver samples clearly benefit from the recommended 1X concentration. Using the recommended NUX concentration, we observed a sharper and more defined read length distribution, along with a minor shift of the 80S footprint peak from 30 to 29 nt compared to 0.1X (Fig. 7A). More importantly, the proportion of reads in frame 0 within coding sequences jumped from 54.8% to 78.7% (Fig. 7B), producing a markedly cleaner metagene profile with distinct 3-nt periodicity and minimal background signal (Fig. 7C). Given the heterogeneity of tissue samples, users may also find it beneficial to fine-tune NUX concentrations in their own lab settings to optimize results.

At Immagina, we are currently in the process of evaluating the performance of the gel-free RiboLace™ kit on multiple tissue types. As of the publication of this application note, we have observed excellent performance on liver (Fig. 7), brain and thymus samples and collections for colon, lung, muscle and heart tissue are in progress (Fig. 8).



**Figure 7 | Performance of Immagina RiboLace™ on tissue samples.** Two murine liver tissue samples were ran through the RiboLace™ protocol with 0.1X and 1X NUX concentration and processed through the MARTIAN™ bioinformatics pipeline. **(A)** Read length distributions. **(B)** Treemap plots of the percentage of p-sites within each transcript region. **(C)** Metagene plots.



**Figure 8 | Completed and ongoing tissue RiboLace™ analyses at Immagina.**

## *Conclusions and take-home message*

In this application note, we demonstrated that the gel-free RiboLace™ protocol significantly enhances the quality, efficiency, and reproducibility of ribosome profiling experiments. By using the recommended digestion conditions, the protocol enhances cleavage precision at ribosome footprint boundaries compared to mild-digesting conditions, shifting the fragment size distribution toward the expected RPF length and minimizing background from partially digested RNA. This leads to clearer metagene periodicity and greater enrichment of coding sequence reads, setting a new standard by outperforming conventional protocols in key ribosome profiling metrics. It is worth noting that using a lower concentration (e.g., 0.1X NUX) can reduce the proportion of rRNA reads, which may be advantageous for specific studies focused on translation efficiency. This flexibility allows users to tailor digestion conditions according to their experimental needs.

Importantly, this data show that the protocol's recommended NUX concentration performs reliably not only with standard cell lines but also works effectively on more complex biological samples such as tissues, which often present greater heterogeneity and experimental challenges. This broad applicability further highlights the robustness of the RiboLace™ protocol across diverse sample types.

When performing ribo-seq experiments, it is equally crucial to emphasize that robust bioinformatic filtering remains a core determinant of data quality (<https://www.immaginabiotech.com/our-technologies/martian>, for more details: <https://github.com/ImmagineBiotechnology/Documents/blob/main/Martian.md>). Selecting appropriate read lengths that correspond to bona fide ribosome-protected fragments and ensuring accurate p-site assignment are essential steps in minimizing artifacts and maximizing signal-to-noise ratio. Proper computational processing—including adapter trimming, UMI collapsing, frame analysis, and careful selection of RPF lengths—can greatly enhance the quality of downstream analyses, regardless of the wet-lab protocol employed.

Together, the RiboLace™ gel-free protocol offers a highly accessible, efficient, and reliable solution for ribosome profiling. These improvements ensure greater flexibility for a wide range of research needs while maintaining consistency and comparability with established protocols.

## *References*

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