APPLICATION NOTE

Simplifying ribosome profiling: RiboLace™ Pro and NEXTFLEX small RNA-Seq kit.

Authors

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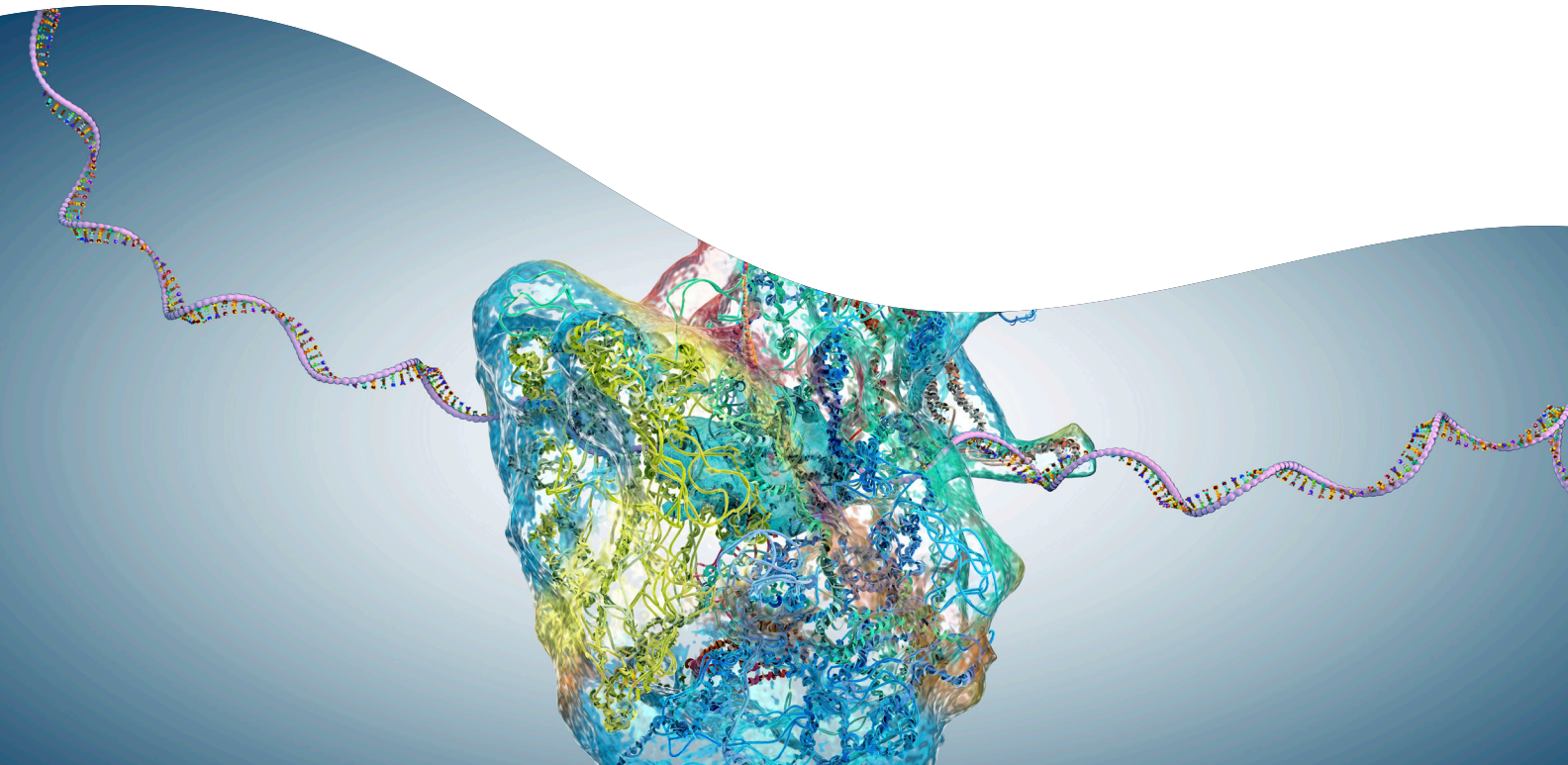
Introduction

Ribosome profiling (Ribo-seq) is a sequencing-based technique that captures ribosome-protected RNA fragments (RPFs) to provide a quantitative snapshot of translation at codon resolution. Ribo-seq enables direct measurement of translational output, identification of translated open reading frames, and detection of ribosome pausing and reading-frame utilization. Since its original description, Ribo-seq has been applied to study translational regulation in diverse biological contexts, including cellular stress responses, development and differentiation, viral infection and cancer[1-4]. These studies demonstrate that changes in protein synthesis are frequently uncoupled from steady-state mRNA abundance and cannot be inferred reliably from RNA-seq alone.

Ribo-seq is technically demanding. Classical workflows rely on polysome fractionation and ultracentrifugation to isolate translating ribosomes, followed by carefully controlled nuclease digestion to generate footprints of the correct size[1]. These steps represent common sources of experimental variability, affecting footprint length distributions, triplet periodicity, and overall data quality. As a result, Ribo-seq implementation often requires substantial protocol optimization and hands-on expertise beyond that typically required for conventional RNA-seq.

RiboLace™ is a technique that selectively enrich actively translating ribosomes using a puromycin-derived reagent, followed by isolation through magnetic beads[5].



This method obviates the need for sucrose gradients and ultracentrifugation, allowing for translational analysis from smaller input amounts compared to conventional polysome-based workflows. The initial publication describing RiboLace™ established that this approach provides ribosome footprints with canonical size distributions, distinct triplet periodicity, and metagene profiles comparable to classical polysome-based Ribo-seq methodologies. Subsequent applications have demonstrated its utility in eukaryotic cell lines and tissues, facilitating the study of translational regulation under defined biological conditions and supporting its adoption as an alternative strategy for isolating actively translating ribosomes[6-9].

In this application note, RPFs obtained from active ribosomes captured via RiboLace™ were prepared for sequencing using the NEXTFLEX™ Small RNA-Seq Kit, which offers an optimized workflow for short RNA inserts[4].

Methods

For this proof of principle, HEK293T cells were used as starting material. Active ribosomes were isolated using RiboLace™ from Immagina Biotechnology, following the manufacturer's instructions[10]. After ribosome pulldown, RNA was extracted and quantified.

In the workflow described in this application note, 1 µg of total extracted RNA was used as input for pre-treatment with T4 PNK (New England Biolabs)[11] to generate 5′ phosphate (5′-P) and 3′ hydroxyl (3′-OH) ends compatible with the downstream NEXTFLEX Small RNA-Seq Kit v4 (Revvity). Following phosphorylation, small RNAs were enriched using the Zymo RNA Clean and Concentrator kit, quantified using a NanoDrop™ spectrophotometer and 100 ng of enriched RNA was used as starting material for library preparation with the NEXTFLEX Small RNA-Seq Kit v4, without the addition of tRNA/YRNA blockers. Libraries were amplified with 16 PCR cycles.

Alternatively, after RNA extraction following the pulldown, RPFs can be size-selected by running the RNA on a 15% TBE-Urea gel. The desired RNA fraction can then be excised and recovered using a gel extraction kit (e.g., PAGExt as suggested in the protocol, Immagina Biotechnology)[12]. In this case, the phosphorylation step can start from as little as 5 ng of purified RNA, followed by downstream library preparation.

Final library size was evaluated using the Agilent 2100 Bioanalyzer™ with the Agilent High Sensitivity DNA Kit, as shown in *Figure 1*. Sequencing was carried out on a NovaSeq™ 6000 platform (single-end, 100 bp), with a target of approximately 100 million reads per sample. Each experiment included two replicates. A distinct peak at 160 nucleotides was observed as expected, and the presence of additional peaks at longer insert sizes did not compromise sequencing quality.

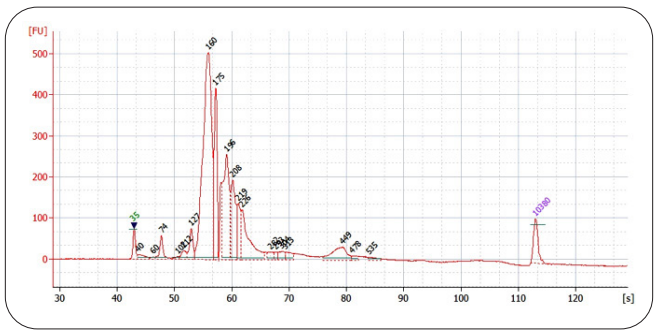


Figure 1: **Library profile.** Typical fragment size distribution of a library ready for sequencing

Results

Alignment statistics

Sequencing data were analyzed using Immagina's comprehensive MARTIAN™ bioinformatics pipeline[13], which includes adapter trimming, unique molecular identifier (UMI) collapsing, genome and transcriptome alignment, and peptidyl-site (p-site) calculation. After excluding reads mapping to rRNA, tRNA, and snRNA *(Table 1A)* and removing PCR duplicates, the proportion of unique- and multi-mapped reads was examined to assess read mappability and the specificity of footprint assignment to transcripts or ORFs (Table 1B). The mapping profiles obtained here are consistent with those observed in previously reported Ribo-seq datasets[14].

Table 1. **Read alignment statistics A.** Number and Percentage of reads aligned on rRNA, tRNA and snRNA databases compared to the total number of trimmed reads *(Raw input (M))*. **B.** Percentage of reads uniquely and multi-mapped to the transcriptome after removal of rRNA-, tRNA-, snRNA-aligned and PCR duplicates removal. Note that the *Transcriptome multi (M)* values reflect the total number of alignments rather than unique reads. A single read may align to multiple transcript loci and is therefore counted once per valid alignment, causing the sum of multi-mapped alignments to exceed the total number of input reads.

A

Sample name	Raw input (M)	rRNA count (M)	rRNA (%)	tRNA count (M)	tRNA (%)	snRNA count (M)	snRNA (%)
A1	98.14	72.26	73.63	0.68	0.7	6.48	6.59
E1	104.85	77.23	73.66	0.69	0.67	7.06	6.74

B

Sample name	Input (M)	Transcriptome unique (M)	Transcriptome unique (%)	Transcriptome multi (M)	Transcriptome multi (%)
A1	18.72	15.37	10.33	133.44	89.67
E1	19.85	16.13	10.24	141.47	89.76

Read length distribution

The distribution of read lengths indicates the quality of the library, showing enrichment for genuine RPFs instead of random RNA fragments. In both replicates, there was a clear peak at the predicted length, which aligns with successful enrichment of actively translating ribosomes (Figure 2).

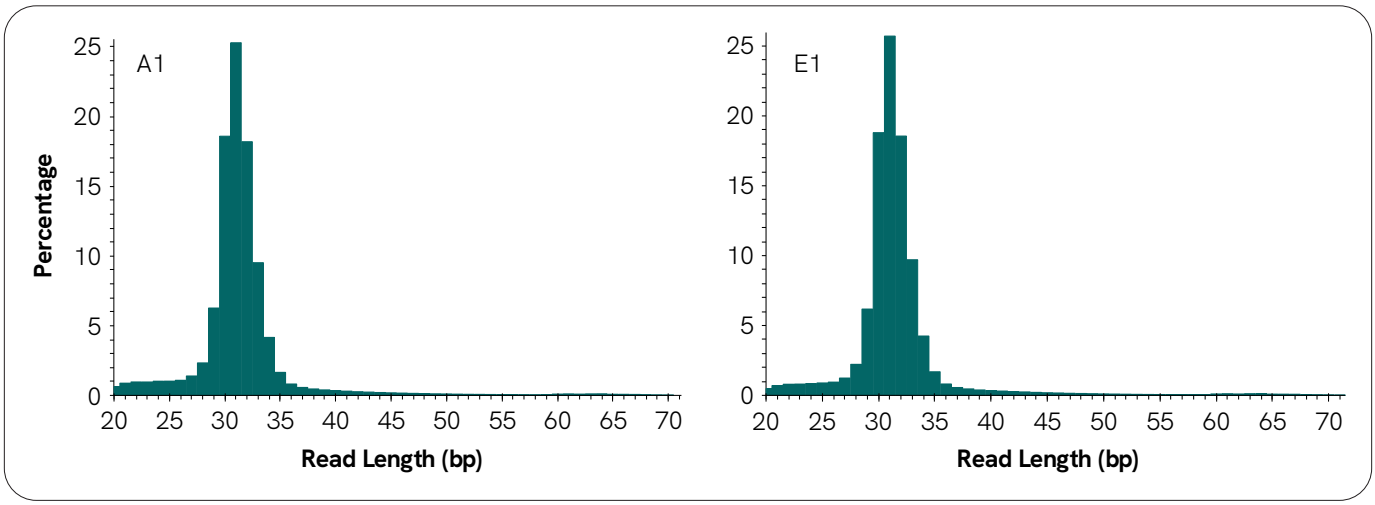


Figure 2: **Read length distribution of replicates.** The plot shows the read length distribution for replicates A1 and E1 relative to total reads. Observed RPF lengths correspond to the typical footprint size of eukaryotic ribosomes (approximately 28–35 nucleotides). A sharp, well-defined peak at a specific length is indicative of high-quality ribosome profiling data.

Read region and frame plot

The *read region and frame* plot provides insights into ribosome occupancy and reading frame distribution along mRNAs. RPFs are mapped to the coding sequence (CDS, green), 5′ untranslated region (5′UTR, yellow), or 3′ untranslated region (3′UTR, red). Higher ribosome density within the CDS indicates active translation, while lower density suggests reduced translational activity.

Within each region, RPFs are further classified by reading frame (frame 0, 1, or 2). In properly translating mRNAs, the majority of RPFs align to frame 0, corresponding to the canonical coding frame. In the example shown, 82.1% of P-sites map to the CDS with a strong enrichment in frame 0 (Figure 3), consistent with high-quality Ribo-seq datasets[14].

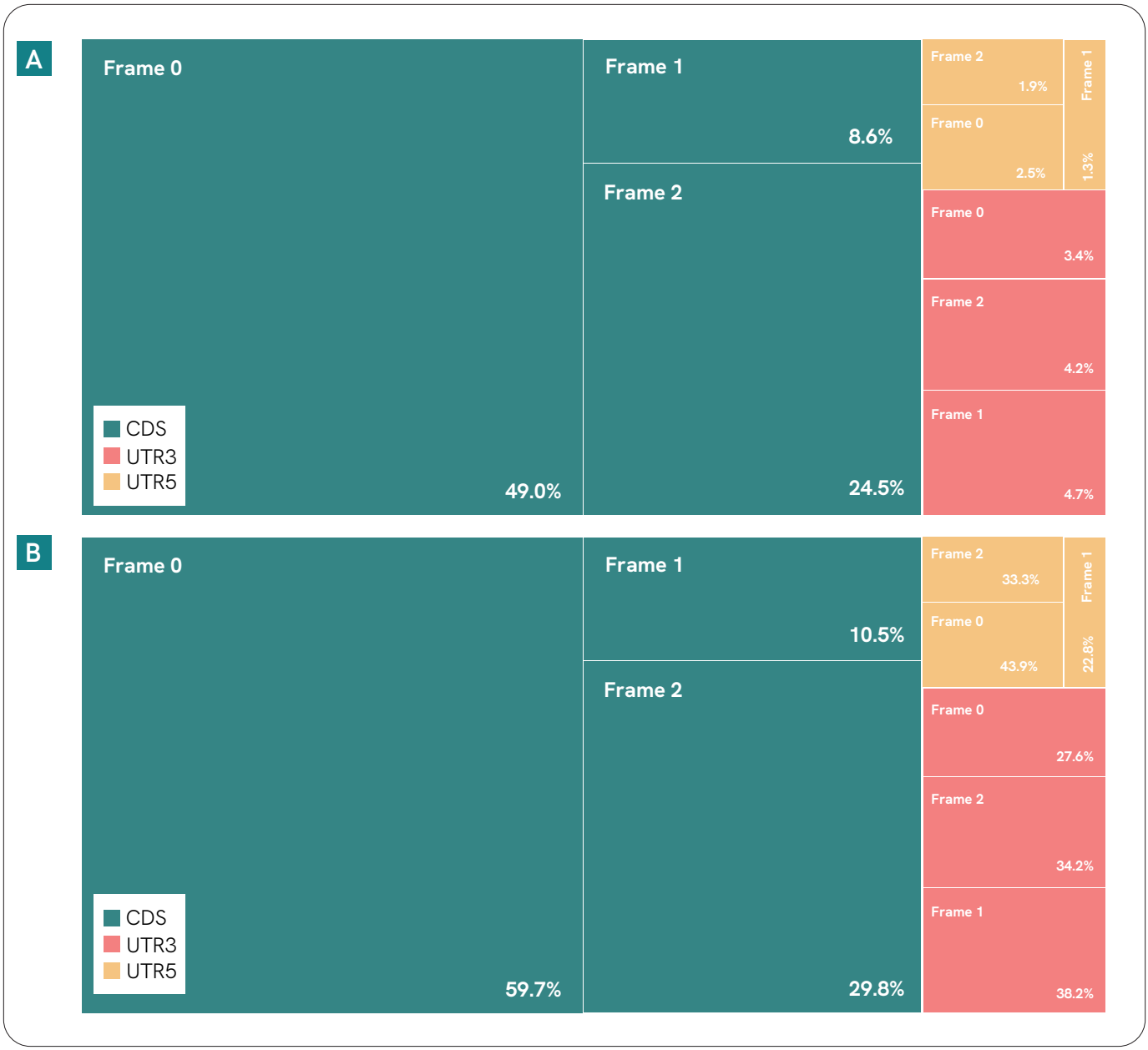


Figure 3: **P-site distribution across transcripts regions and reading frames.** The read region and frame plot shows the percentage of P-sites mapping to the 5′ UTR, coding sequence (CDS), and 3′ UTR corresponding to the three possible reading frames. Only replicate A1 is shown. In panel **A** percentages are calculated over the total (CDS + UTRs), whereas in panel **B** percentages are calculated separately within each region (CDS, 5′ UTR and 3′ UTR).

Metagene profile

The metagene profile (metaprofile) summarizes average ribosome occupancy (P-site frequency) across all transcripts to provide an overview of ribosome behavior relative to the translation start and stop sites. P-site positions were aggregated over a ±100 nt window around initiation and termination sites (Figure 4). Sharp, well-defined peaks at characteristic offsets indicate accurate P-site assignment and coordinated ribosome positioning during translation initiation and termination.

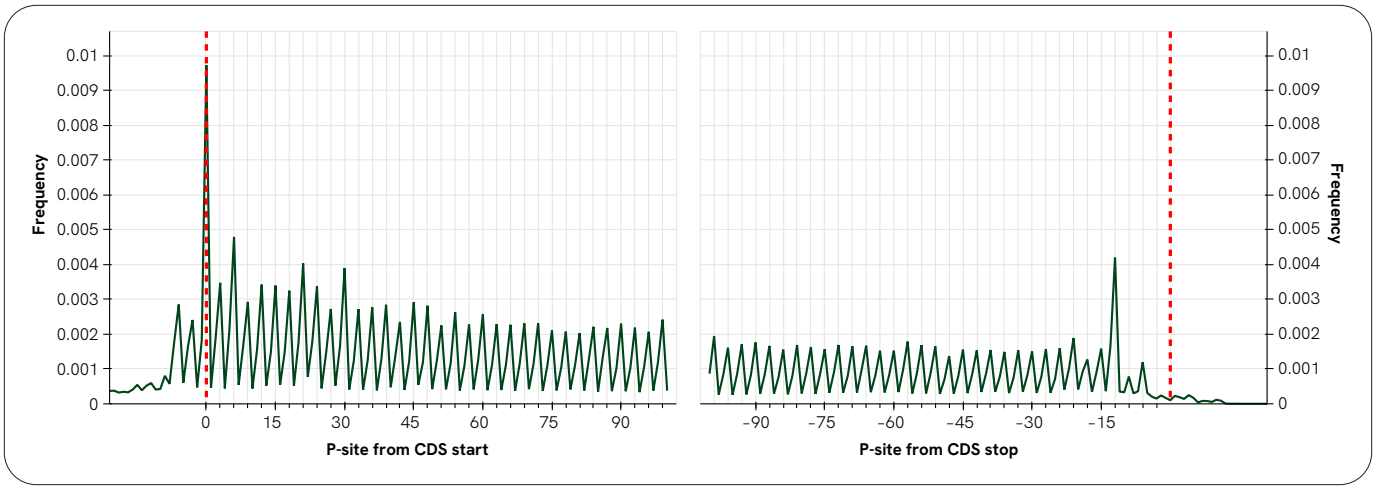


Figure 4: **Metagene profile.** Metagene profile showing P-site positions for all transcripts around translation initiation sites (from 0 to 100 nt) and translation termination sites (from 0 to -100 nt) highlighted with a red line. Only replicate A1 is shown.

Comparison between replicates

A scatter plot comparing normalized ribosome footprint abundance between replicates A1 and E1 is shown, with each point representing an ORF (Figure 5). The high Pearson correlation (r = 0.971) demonstrates strong reproducibility and consistent quantification of ribosome occupancy, underscoring the reliability of the ribosome profiling workflow and analysis.

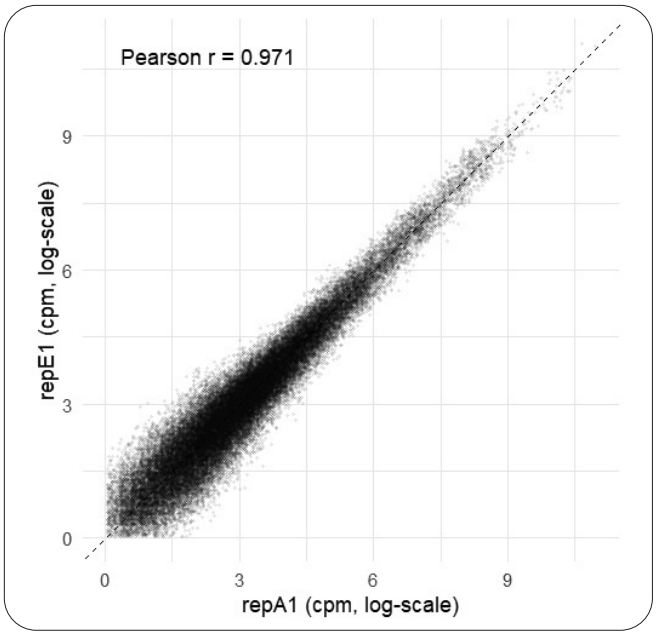


Figure 5: *Replicate comparison.* Scatter plot of log-transformed, depth-normalized counts per million (CPM) for ORFs in two independent Ribo-seq replicates (A1 and E1). Only transcripts detected in both libraries are shown, with each point representing a single ORF.

Conclusion

This application note highlights the effective pairing of RiboLace™ technology with the NEXTFLEX Small RNA-Seq Kit v4 for ribosome profiling. Together, this combination enables efficient isolation of RPFs without the need for fractionation or ultracentrifugation, while producing high-quality Ribo-seq libraries that display expected footprint length distributions, strong coding sequence enrichment, clear three-nucleotide frame periodicity and high reproducibility between replicates. By combining these technologies, researchers can streamline the workflow and obtain robust reproducible translational data from eukaryotic cells or tissues.

References

1. Ingolia, N.T., et al. (2009). Genome-wide analysis *in vivo* of translation with nucleotide resolution using ribosome profiling. Science. 324(5924):218-23. doi: 10.1126/science.1168978.
2. Brar, G.A., et al. (2012). High-resolution view of the yeast meiotic program revealed by ribosome profiling. Science. 335(6068):552-7. doi: 10.1126/science.1215110.
3. Stern-Ginossar, N., et al. (2012). Decoding human cytomegalovirus. Science. 338(6110):1088-93. doi: 10.1126/science.1227919.
4. Raven, A., et al. (2026). Hepatic zonation determines tumorigenic potential of mutant β-catenin. Nature. doi: 10.1038/s41586-025-09733-1.
5. Clamer, M., et al. (2018). Active Ribosome Profiling with RiboLace. Cell Rep. 25(4):1097-1108.e5. doi: 10.1016/j.celrep.2018.09.084.

6. Lauria, F., et al. (2020). SMN-primed ribosomes modulate the translation of transcripts related to spinal muscular atrophy. Nat Cell Biol. 22(10):1239-1251. doi: 10.1038/s41556-020-00577-7.
7. Zou, Z., et al. (2023). FMRP phosphorylation modulates neuronal translation through YTHDF1. Mol Cell. ;83(23):4304-4317.e8. doi: 10.1016/j.molcel.2023.10.028.
8. Hu W, Zeng H, Shi Y, et al. (2022) Single-cell transcriptome and translatome dual-omics reveals potential mechanisms of human oocyte maturation. *Nat Commun*. 2022;13(1):5114. doi:10.1038/s41467-022-32791-2
9. Jaeger AM, Stopfer LE, Ahn R, et al. (2022) Deciphering the immunopeptidome in vivo reveals new tumour antigens. Nature;607(7917):149-155. doi:10.1038/s41586-022-04839-2
10. https://www.immaginabiotech.com/media/ee/3f/06/1740581232/Mod0802051_RiboLace%20Pro_v3.4.pdf
11. https://www.neb.com/en/protocols/non-radioactive-phosphorylation-with-t4-pnk-or-pnk3-phosphatase-minus
12. https://www.immaginabiotech.com/product/pagext
13. https://www.immaginabiotech.com/our-technologies/martian
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