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Isolate and sequence ribosome-protected mRNA fragments using size-exclusion chromatography

Investigating translationally active mRNA involves isolating and sequencing RNA directly associated with the ribosome. Traditionally, isolating these complexes involved sucrose gradient ultracentrifugation, which is tedious and technically difficult. ARTseq™ (*a*ctive *R*NA *t*ranslation) Ribosome Profiling Kits provide an alternative method for obtaining ribosome-protected RNA fragments using size-exclusion chromatography (SEC) columns. Both methods produce similar results, yet ARTseq™ offers a more rapid and simple method of enriching ribosome-bound mRNA fragments.

Introduction

Traditional RNA-seq experiments are effective for surveying the transcribed regions of the genome but are not designed to specifically measure mRNA involved in active translation. Recent interest in this area of research has led to the development of ribosome-profiling techniques to directly examine the transcripts associated with the translation machinery. One technique is to isolate and sequence the nuclease-resistant ribosome-protected fragments or footprints (RPFs), which comprise the approximately 30 nucleotides of mRNA bound to the ribosome during translation.

Existing ribosome-profiling protocols in the literature[1] require multiple PAGE purifications and can take 5–7 d from cell lysis to a sequencer-ready library. In contrast, we have streamlined these protocols to develop the ARTseq™ Ribosome Profiling Kits. The ARTseq™ protocol requires only two PAGE purifications and significantly reduces the time and labor to move from cell lysis to a library ready for Illumina sequencing. Besides eliminating half of the PAGE purifications, we also developed the new use of SEC spin columns instead of sucrose gradients or cushions to purify RPFs. These columns do not require the use of specialized equipment, such as an ultracentrifuge, gradient stations or fractionation collectors, and reduce bench time for purifying the ribosome footprints from approximately 1 d to 1.5 h.

Methods overview

The ARTseq™ kits contain needed reagents and protocols to lyse cells, isolate RPFs and convert them into an Illumina-compatible sequencing library. To generate the RPFs, cells are lysed with the included polysome buffer, the lysate is treated with a nuclease and then it is passed through an SEC column. This step is followed by RNA extraction from the monosome fraction. The RNA samples are then treated with Epicentre's Ribo-Zero™ kit to deplete the samples of as much rRNA contamination as possible before PAGE purification of the relatively short (~30 nt) RPFs. Following reverse transcription the cDNA is circularized using Epicentre's CircLigase™ enzyme to create a template for PCR. Indexed PCR primers are used during amplification to permit multiplexing. For a general overview of the protocol, see **Figure 1**.

Sequencing and data analysis of sucrose and SEC column methods

ARTseq™ libraries of HEK293 cells were constructed using sucrose gradient and SEC columns for isolating the monosomes. The sequencing

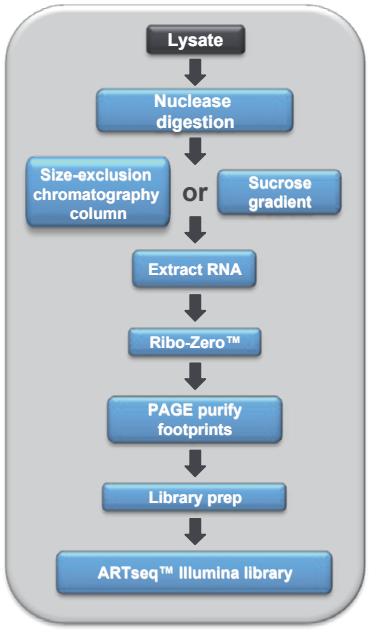

Figure 1 | **ARTseq™ Ribosome Profiling protocol overview.**

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APPLICATION NOTES

Table 1 | Sequencing performance metrics.

Method	rRNA[a]	tRNA[a]	Genome and splice junctions[a]
Size-exclusion chromatography column	30.0%	7.6%	44.2%
Sucrose gradient	44.2%	2.7%	36.6%
Total RNA	2.5%	3.4%	45.0%

[a]Percent of reads aligning to specified areas of the genome.

data was aligned and annotated using existing RNA-seq tools such as TopHat and Cufflinks; data quality was measured using several common RNA-seq metrics, utilizing a nonfootprinted, Ribo-Zero™-treated total RNA library as a reference for comparison (**Table 1**). Sucrose gradient and SEC column techniques produced comparable results. The relative percentage of reads aligning to coding transcripts, as well as the overall levels of expected contaminants such as rRNA and tRNA, are similar for both RPF purification methods (**Table 1** and **Fig. 2a**). In contrast, the nonfootprinted total RNA sample resulted in less sequencing contaminants than the RPF samples, suggesting that ribosome-enriched samples pose a particular challenge when they undergo depletion of short rRNA fragments generated by nuclease treatments. After filtering the data of these expected contaminants, the resulting reads that align to mRNA are of high quality and can be further categorized to highlight the differences between footprinted samples versus more traditional total RNA–type samples.

Characteristics and distribution of aligned reads to the genome and transcript

Because RPF samples are derived from the translationally active fraction of the cell, we expected most of the reads to map to coding regions of the transcriptome rather than to untranslated regions. Indeed, this expectation is demonstrated in **Figure 2a**. Additionally, regardless of whether the sucrose gradient or SEC method was used to isolate the RPFs, the distribution and enrichment of coding sequence over other regions of the transcripts are very similar. We also examined if the type of monosome purification method used affected coverage distribution across transcripts (**Fig. 2b**). Nonfootprinted, total RNA samples showed fairly even coverage across transcripts with very little 5′ or 3′ bias. Conversely, both the sucrose gradient and SEC column samples demonstrated a distinct bias toward the 5′ end of transcripts—a characteristic of footprinted samples due to a combination of the nuclease digestion and treatment with cycloheximide, the drug used to inhibit translation elongation and lock ribosome positions.

Conclusion

Ribosome profiling is a powerful new technique capable of defining the proteome of complex organisms by systematic monitoring of cellular translation processes. ARTseq™, a method to perform ribosome profiling, provides a simple alternative to isolating RPFs by using size-exclusion columns. The SEC column method is rapid, scalable and does not require specialized equipment, unlike sucrose gradient or cushion techniques. With these improvements, ARTseq™ is now the preferred method for investigating translational control.

1. Ingolia, N.T. *et al.* Genome-wide analysis *in vivo* of translation with nucleotide resolution using ribosome profiling. *Science* **324**, 218–223 (2009).

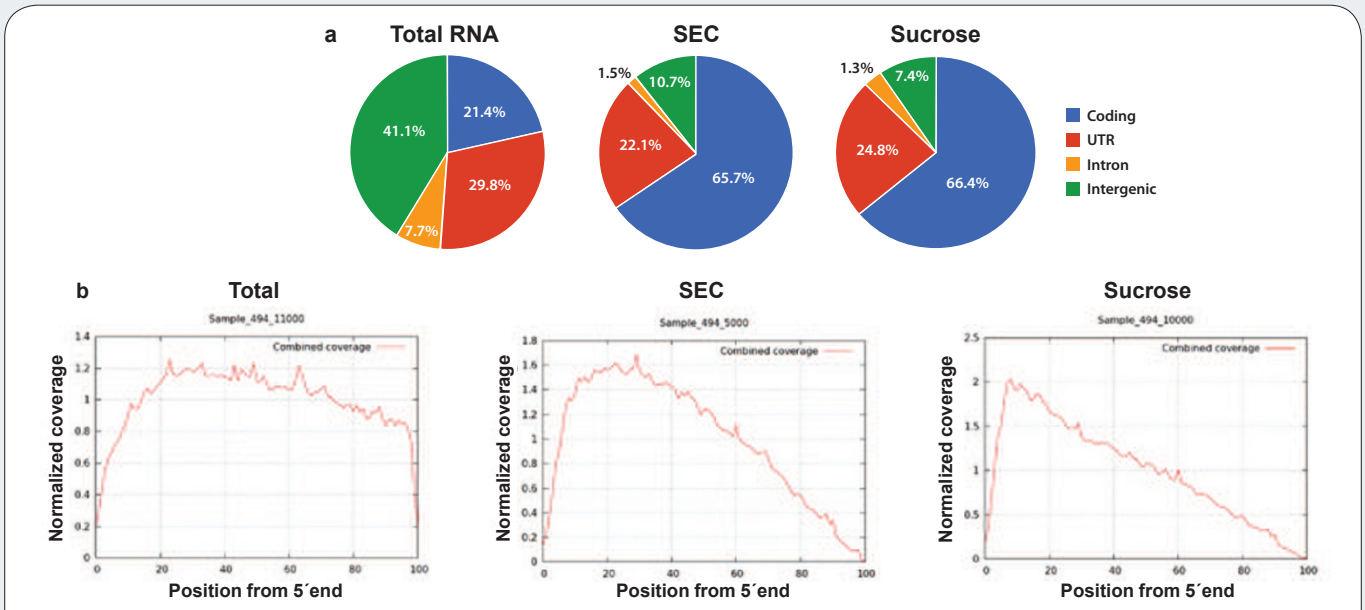

Figure 2 | **Distribution of aligned reads.** (**a**) Percent of reads that align to coding regions, untranslated regions (UTRs), introns or intergenic regions. (**b**) Coverage as a function of distance from the 5′ end of transcripts. The total RNA sample demonstrates a fairly equal distribution of reads from 5′ to 3′. In contrast, samples prepared from footprinted RNA show 5′ bias and very little coverage near transcript 3′ ends, characteristic of ribosome footprints. Libraries from both size-exclusion chromatography (SEC) and sucrose gradient samples demonstrate very similar profiles.