REPORT FINALE - SHORT TERM MOBILITY GABRIELLA VIERO

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Dipartimento di afferenza MATERIALI E DISPOSITIVI

Titolo del programma: Impostazione di una pipeline sperimentale per la mappatura multidimensionale dell'espressione genica attraverso sequenziamento posizionale di frammenti mRNA a risoluzione di singolo nucleotide /

Setting up an experimental pipeline for parallel and multidimensional mapping of gene expression by dual positional sequencing of mRNA fragments at single nucleotide resolution

Sequencing studies of myelodysplastic disorders (MDS) patient identified some common mutations affecting genes encoding RNA binding proteins such as U2AF1. In Halene's lab *in vitro* cellular models of MDS have been obtained by genetically engineering Hel cells, which are able to overexpress the WT and two mutants forms of the protein. The two mutant proteins (U2AF1-S37F and U2AF1-Q157R) have been found in patients and associated to splicing alterations implicated in myeloid malignancies.

At present, possible effects of splicing alterations at the translational level are almost completely unknown. Here we aimed at identifying possible translational defects and ribosomes hot-spots alterations along mRNAs of human polysomes in these cellular models of MDS. To define global translational defects and ribosome occupancy profiles, we performed three types of analysis: classical polysome profiling (POL-Seq), ribosome profiling (RIBO-Seq) active ribosome profiling (Active-RiboSeq). This last technique employs a novel method based on an original puromycin-containing molecule capable of isolating active ribosomes by means of an antibody-free and tag-free technology.

Once sequenced, these data can be helpful to identify transcripts with altered splicing uploaded on polysomes and defects in ribosome positioning along mRNAs in association to defect in binding of RNA binding proteins recurrently mutated in patients with MDS.

PART 1 – POLYSOME PROFILING

First, we searched for the best conditions to obtain reliable polysome profiling analysis in three cell lines in the presence or absence or doxycycline, whose addition to cell cultures induces the overexpression of the exogenous U2AF1-WT or of the two mutants. To do that we applied three protocols for polysomal lysates preparation. The three approaches were aimed at understanding a

convenient cell number, a robust treatment of cells with cycloheximide and an optimal sucrose gradient fractionation procedure.

The first attempt (**Figure 1**) took advantage of the following protocol:

1. Collection of 30 millions cells, centrifugation and medium removal
2. Resuspension of cells from all lines in about 5-6 ml and treatment with cycloheximide (10 ug/ml from a stock of 100 mg/ml in ethanol)
3. Incubation 4 min at 37°C
4. Centrifugation and medium removal
5. Wash with cold PBS in the presence of cycloheximide (10 ug/ml)
6. Collection of pellet and lysis.
7. Measure of absorbance different volumes of lysates keeping as reference the sample with the lowest absorbance, i.e. we load on sucrose the same absorbance for all samples

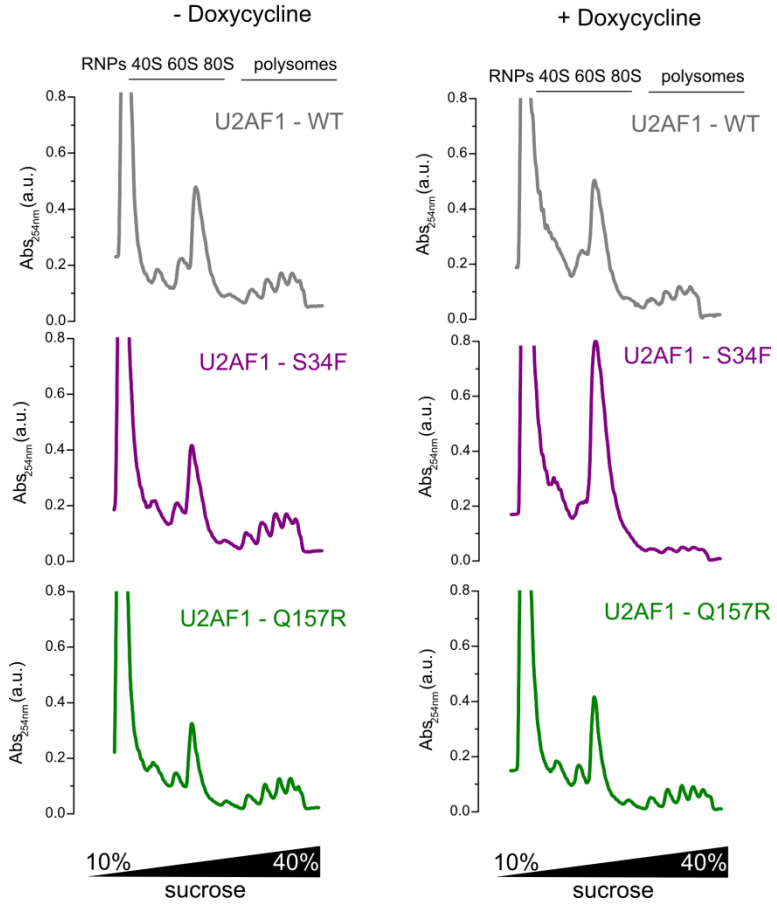

Figure 1. Sucrose gradient absorbance profiles from the first experiment of polysome profiling using Hel cells with and without doxycycline. The treatment with the drug induces the expression of WT and mutants U2AF1. The first peak contains free cytosolic light components (RNPs), and the subsequent peaks include ribosomal subunits (40S and 60S) and monosomes (80S), all associated with non-translating particles. The remaining peaks of the profile represent

polysomes, which sediment with high sucrose concentrations and contain the RNAs associated with ribosomes. The starting amount of cells was 30 millions.

This procedure was very long, and overall the exposure of cells to cycloheximide was much longer (i.e. about 15 minutes) than usual. Therefore the second attempt was aimed at decreasing the cycloheximide exposure.

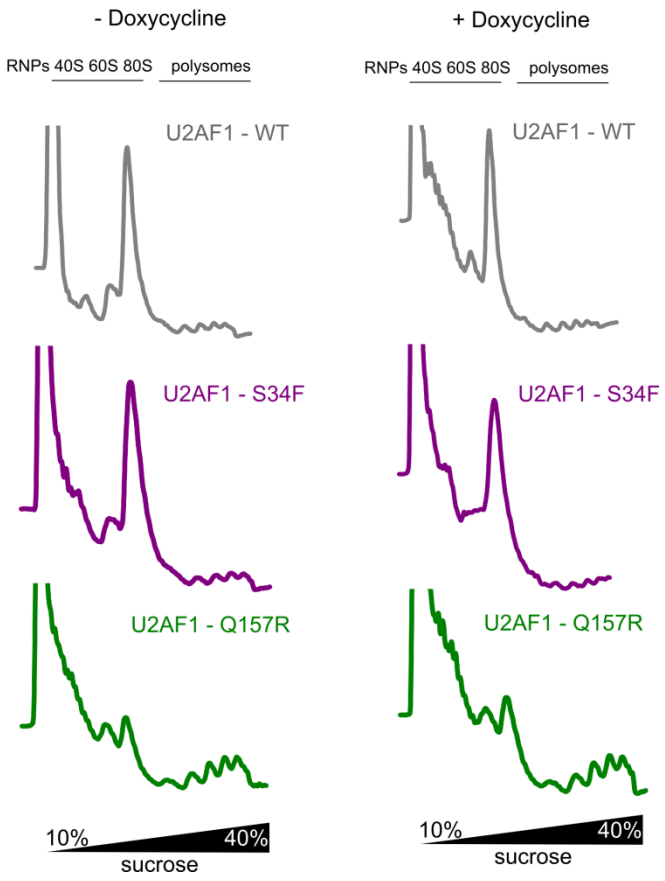

Figure 2. Sucrose gradient absorbance profiles from the second experiment of polysome profiling using Hel cells with and without doxycycline. The lysate obatained in this experiments were used also for ribosome profiling and active-ribosome profiling (see PART 2 of this summary). The starting amount of cells was 30 millions.

The second attempt (Figure 2) was performed using the following protocol:

1. 30 millions cell in different volumes depending on the cell line
2. Treatment with cycloximide (10 ug/ml for a stock of 20 mg/ml in ethanol)
3. Incubation 4 min at 37°C
4. Centrifugation and medium removal
5. Wash with cold PBS in the presence of cycloheximide (10 ug/ml)

6. Collection of pellet and lysis.
7. Measure of absorbance
8. Split the lysates keeping 2 samples apart with 2 Abs of lysates for RIBO-Seq and Active RIBO-seq (see in the second part of the summary these results). The whole remaining lysate was loaded on sucrose fractions.

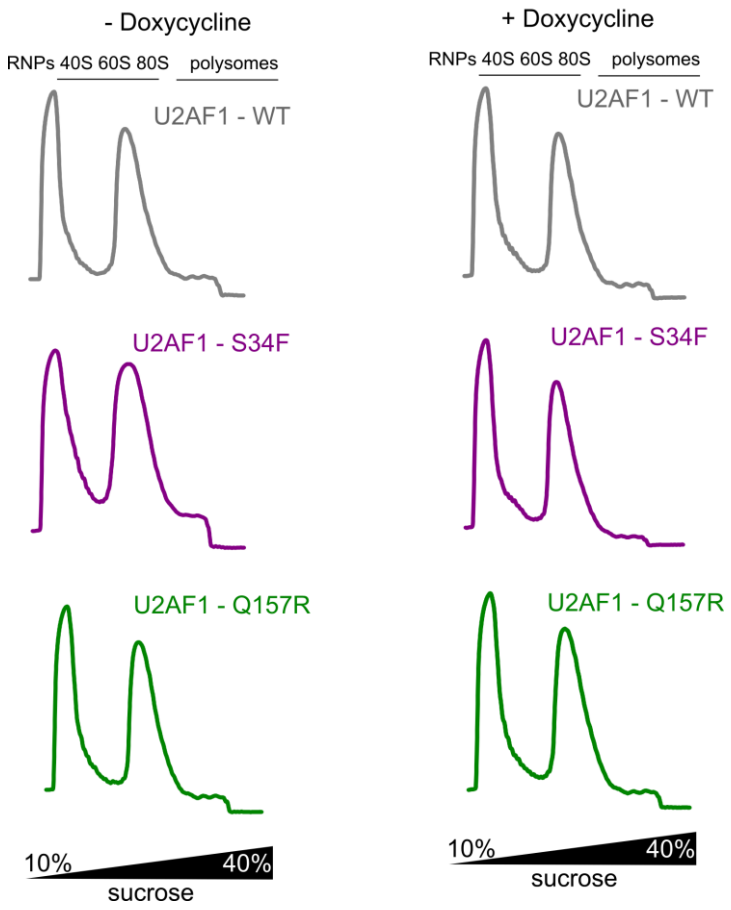

Figure 3. Sucrose gradient absorbance profiles from the second experiment of polysome profiling using HeI cells with and without doxycycline. The starting amount of cells was 50 millions.

In this case we avoided the step with the flasks at RT during counting. We skip this part to decrease the possibility of cold stress that would alter the translational state of cells. In addition, and we skipped the step with the removal of the medium for the same reason. Accoording to this hypothesis and comparing the polysome profiles of the mutant U2AF1-Q157R in Figure 1 and Figure 2, it is possible to appreciate that the ratio between the 80S and the polysomes is more similar to a typical snapshot of cells under active translation.

Nonetheless in all other cases the ratio is going in the opposite direction. We cannot exclude that differences in growth rate between the three cell lines might be a possible of cause of these differences.

The third attempt (Figure 3) was performed using the following protocol:

1. 50 millions cell in different volumes depending on the cell line
2. Treatment with cycloximide (10 ug/ml for a stock of 20 mg/ml in ethanol)
3. Incubation 4 min at 37°C
4. Centrifugation and medium removal
5. Collection of pellet and lysis.
6. Load the whole lysate

In this case we aimed at understanding if the was with PBS in the presence of cycloheximide was the cause of the high 80S peak observed in the previous experiment in four out of six samples. We can conclude that the overall profiles in this case are much worst, most probably because the cell number was exceedingly too high.

Concluding we think that the best conditions are still those used in the first experiment. Nevertheless, if only polysome profiling (and not coupled RIBO-Seq and Active Ribo-seq) are the aim of the future experiments, I strongly suggest: i) to use around 15 millions cells and ii) to use cycloheximide dissolved in water and not ethanol. In fact, I cannot exclude that some differences might have been caused also by the use of cycloheximide stock at concentrations higher that the solubility in ethanol or by the differences in growth rate.

Despite the observed variability in two out of three experiments we observed an intriguing decrease in polysomes in conditions of overexpression of U2AF1-S34F. To quantify this effect we calculated the fraction of ribosomes in polysomes, FRP, as the ration between the area under polysome and the sum of the area under polysomes and the monosome 80S (Figure 4, left panel). This parameter is a good estimation of possible global defects in recruitment of ribosomes in polysomes. We observed that the overexpression of WT and mutants U2AF1 induces a decrease in this parameter with respect to the controls. We cannot exclude that doxycycline can be responsible of this effect. Therefore an additional control of native Hel cells in the presence of doxycycline would be worth doing. The FRP value in the case of the U2AF1-S34F drops to very low levels, suggesting that some the expression of this protein is likely inducing defects not only in splicing but also in translation.

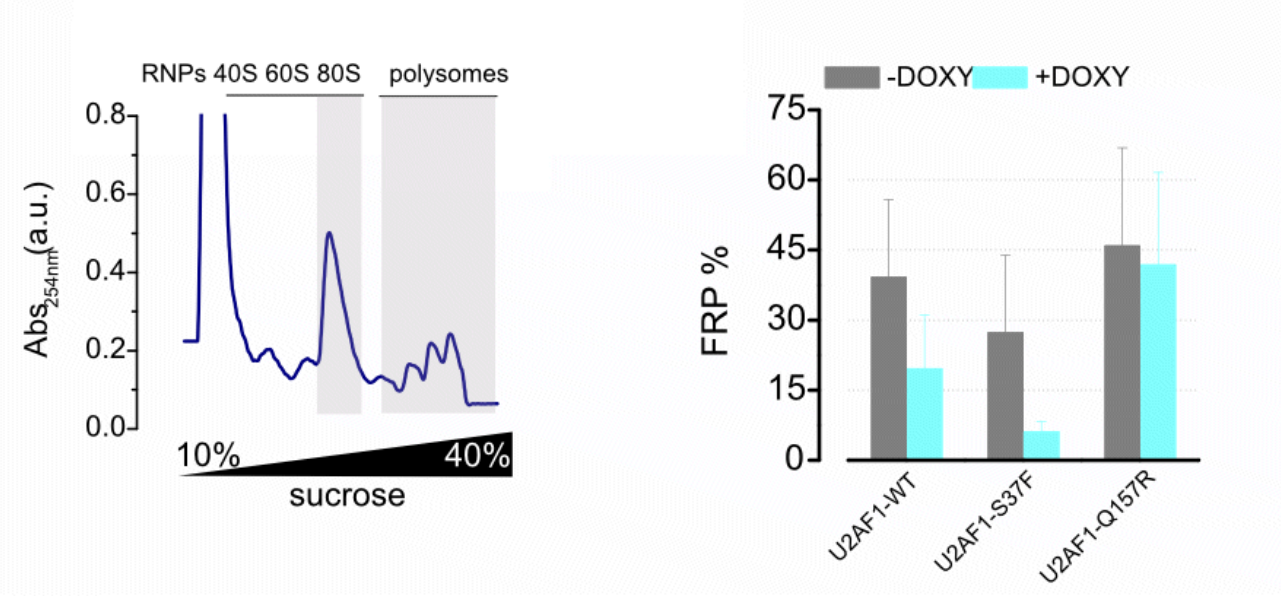

Figure 4. Left panel, definition of 80S and polysome areas to determine the fraction of ribosomes engaged on polysomes (FRP). This value provides an estimate of the translation status/activity of tissues and cells, describing the engagement of ribosomes on RNAs in polysomes and/or the recruitment of mRNAs on polysomes for translation. Right panel, comparison between the fraction of ribosomes in polysomes (FRP) in Hel cell lines with without doxycicline treatment.

These results prompted us to purify the RNA associated to the 80S and polysomes for sequencing and further analysis of splicing alteration in polysomes- and 80S-associated transcripts (Table 1).

Table 1. Characterization of RNA extracted from sucrose gradient for analysis of splicing at the translational level

Sample	Fraction	Poly#	[RNA]/µg/µl	Abs260/Abs280
U2AF1-WT -doxy	80S	Poly2A	1.78	2.0
	polysomes	Poly2A	3.14	1.98
U2AF1-WT +doxy	80S	Poly2D	2.06	1.96
	polysomes	Poly2D	1.46	1.85
U2AF1-S34F +doxy	80S	Poly2E	0.43	1.85
	polysomes	Poly2E	0.98	1.88
U2AF1-Q157R +doxy	80S	Poly2F	0.98	1.98
	polysomes	Poly2F	0.71	1.82

In conclusion this is the protocol for lysate preparation I suggest:

1. Count cells taking the flasks out from the incubator in pairs. As soon as you've counted them, put them back in the incubator and proceed with the next pair of flasks.
2. Put 15 millions of cells (is only polysome profiling is required or 30 millions is you want to proceed in parallel with RIBO-Seq or Active-RIbo-Seq) in a tube.
3. Centrifuge for 5 minutes at room temperature. Remove the supernatant
4. Resuspend cells in about 5-6 ml.
5. Add cycloheximide (10 ug/ml final concentration and use a stock of 10 mg/ml in water)
6. Incubation 4 min at 37°C
7. Centrifugation for 5 minutes at 4 °C and remove medium
8. Wash with cold PBS in the presence of cycloheximide (10 ug/ml)
9. Centrifuge for 5 minutes at 4°C and collect the pellet for lysis using polysome lysis buffer.
10. Measure of absorbance different volumes of lysates keeping as reference the sample with the lowest absorbance, i.e. we load on sucrose the same absorbance for all samples

PART 2 – RIBOSOME PROFILING AND ACTIVE RIBOSOME PROFILING USING RIBO-LACE

To determine the exact distribution of translating ribosomes on mRNAs, Nicholas Ingolia in 2009 developed an assay to analyze the ribosome-RNA interactions occurring in a cell at a given time point, using an endonuclease digestion and sequencing of ribosome protected fragments (Ingolia et al., 2009). In other words, ribosome profiling uses Next Generation Sequencing coupled to the ribosome footprinting technique, that was initially proposed in the seminal work of Wolin and Walter in 1988 (Wolin & Walter, 1988). Despite its many accomplishments, this technique still faces several challenges, the most relevant being that it cannot distinguish between fragments protected either by ribosomes in active translation or by not-active ribosomes. To overcome these limitations, we recently proposed a new variant of ribosome profiling, called Active-Ribo-Seq, which is based on a technology (RiboLace) with a new puromycin-containing molecule. This technique allows to isolate active ribosomes by means of a tag-free pull-down approach.

Here, using two types of ribosome profiling techniques, we aim to define global ribosome occupancy profiles in Hel cells lines expressing an RNA binding protein, U2AF1, in its WT and mutant forms. The goals are to study in these conditions the positions on mRNAs: i) of all ribosomes (RiboSeq) and of ii) active ribosomes (Active-RiboSeq). Working in parallel, we used both techniques with the same cellular lysates employed for the polysome profiling shown in Figure 2. We considered one control cell line (Hel cells inducible for overexpressing U2AF1-WT, in the absence of doxycycline) and the three cell lines induced with doxycycline to overexpress U2AF1-WT and the two mutants U2AF1-S34F and U2AF1-Q157R. For both Ribo-Seq and Active RiboSeq (RiboLace technology), we used a volume of lysate corresponding to 2 units of Abs at 254 nm.

For the isolation of ribosome protected fragments and library preparation, we followed the instructions contained in the manual in the RiboLace kit.

The first step was the treatment of the lysates with an endonuclease, this step was performed following the abovementioned manual instructions. After the digestion, half of each sample was incubated with the beads functionalized with the analog of puromycin, while the remaining lysates were not. After the incubations required to capture active ribosomes in the RiboLace samples, the RNA was extracted and the ribosome protected fragments were isolated after separation in a denaturing UREA 15% polyacrylamide gel (Figure 5).

Following the manual instructions, we isolated and purified the ribosome protected fragments (RPF), treated the purified RPF with PNK to obtained conveniently phosphorylated 5’ ends. Then we proceed with linker ligation and purification of the product by separation in denaturing 15% UREA PAGE (Figure 6).

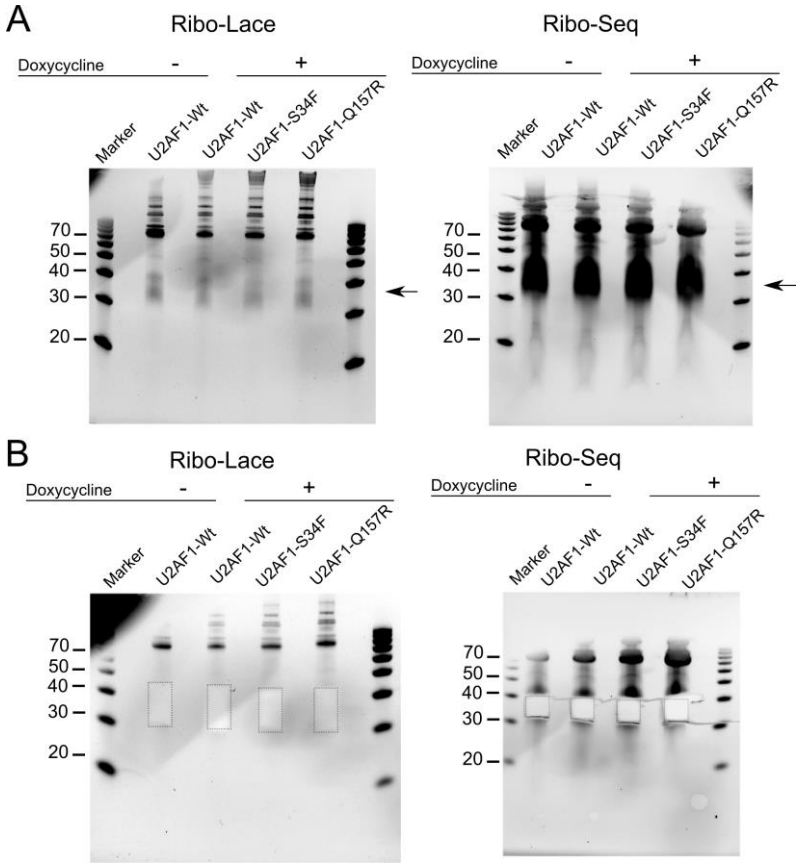

Figure 5. (A) Separation of ribosome protected fragments according to RiboLace (see arrows, left panel) and classical ribosome profiling (right panel) using denaturing 15% PAGE. The ribosome protected fragments appear as faint bands of around 30-40 nt in length. (B) The same gel shown in A after band excision.

The product of the linker addition of the RPF is expected to be at around 55 nt in length. In accordance, we observed these bands and size selected them taking into account the migration of marker and the free linker mix (Figure 6) Then we purified them accordingly to the manual instructions and synthesized the cDNA. Using a TBE-urea PAGE, we then size selected the cDNA to remove the excess of oligonucleotides employed for the retrotranscription (Figure 7). Unfortunately, we observed an unexpected band in the RT lane in the RiboLace samples (left panel Figure 7), which was probably due to a contamination of the well by the the next sample. This contamination might have occurred during sample loading. In fact, the very same RT control sample loaded in the gel with the Ribo-Seq samples, did not shown a band above 100 nt. The bands have been excised (not shown) as in previous cases and the cDNA was purified according to the manufacturer instructions.

Finally we circularized the purified cDNA and stored half of it at -20 C (see Table 2, circDNA). The remaining circDNA was used for amplification and library separation.

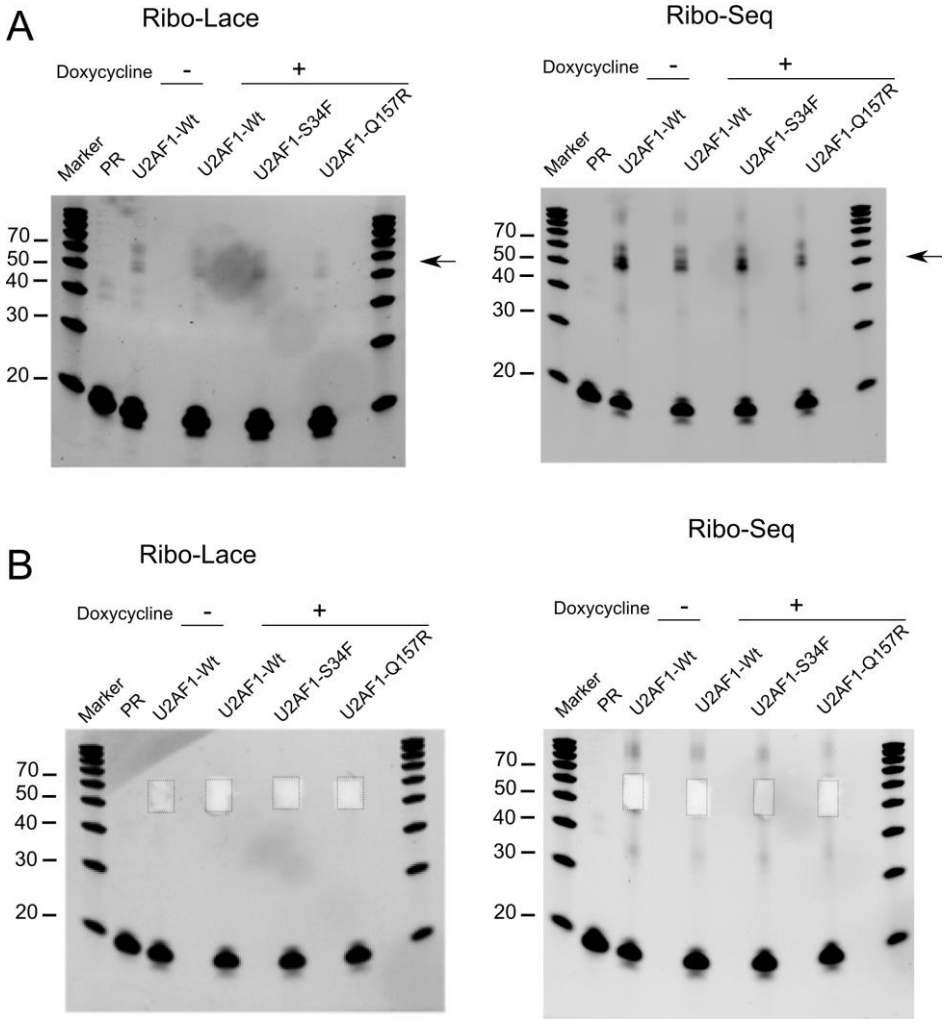

Figure 6. (A) Separation of ribosome protected fragments covalently bound to the linkers according to RiboLace (see arrows, left panel) and classical ribosome profiling (right panel) using denaturing 15% PAGE. The reaction products are visible above the 50 nt length marker. (B) The same gel shown in A after band excision.

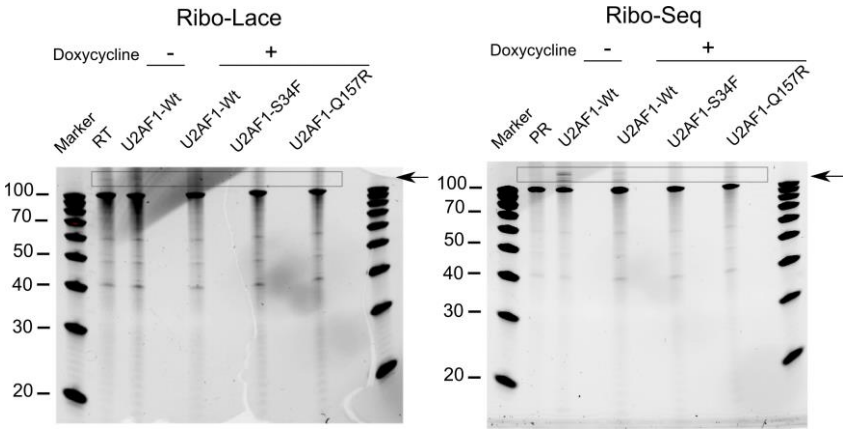

Figure 7. Separation of cDNA obtained from ribosome protected fragments covalently bound to the linkers according to RiboLace (see arrows, left panel) and classical ribosome profiling (right panel) using denaturing 15% PAGE. The reaction products are visible above the 100 nt length marker and are expected to be around 130 nt in length.

During the PCR amplification we added the barcodes provided by the Art-Seq kit according to Table 3. Finally, after the amplification, part of the library was stored at -20°C and part was used to identify the reaction products, expected at around 175 nt in length (Figure 8).

Table 2. Samples produced

Sample	Experiment	Poly#	Purpose	Material
U2AF1-WT -doxy	POL-seq/80S-seq	Poly2A	Splicing analysis	RNA /-80°C
	Ribo-seq	Poly3A	Translation	circDNA/library /-20°C
	Ribo-Lace	Poly3A	Translation	circDNA/library /-20°C
U2AF1-WT +doxy	POL-seq/80S-seq	Poly2D	Splicing analysis	RNA /-80°C
	Ribo-seq	Poly3D	Translation	circDNA/library /-20°C
	Ribo-Lace	Poly3D	Translation	circDNA/library /-20°C
U2AF1-S34F +doxy	POL-seq/80S-seq	Poly2E	Splicing analysis	RNA /-80°C
	Ribo-seq	Poly3E	Translation	circDNA/library /-20°C
	Ribo-Lace	Poly3E	Translation	circDNA/library /-20°C
U2AF1-Q157R +doxy	POL-seq/80S-seq	Poly2F	Splicing analysis	RNA /-80°C
	Ribo-seq	Poly3F	Translation	circDNA/library /-20°C
	Ribo-Lace	Poly3F	Translation	circDNA/library /-20°C

Table 3. Barcodes used for library preparation of ribosome profiling and RiboLace libraries

Sample	Experiment	Poly#	Index Illumina
U2AF1-WT -doxy	Ribo-seq	Poly3A	2
	Ribo-Lace	Poly3A	1
U2AF1-WT +doxy	Ribo-seq	Poly3D	5
	Ribo-Lace	Poly3D	6
U2AF1-S34F +doxy	Ribo-seq	Poly3E	3
	Ribo-Lace	Poly3E	7
U2AF1-Q157R +doxy	Ribo-seq	Poly3F	4
	Ribo-Lace	Poly3F	8

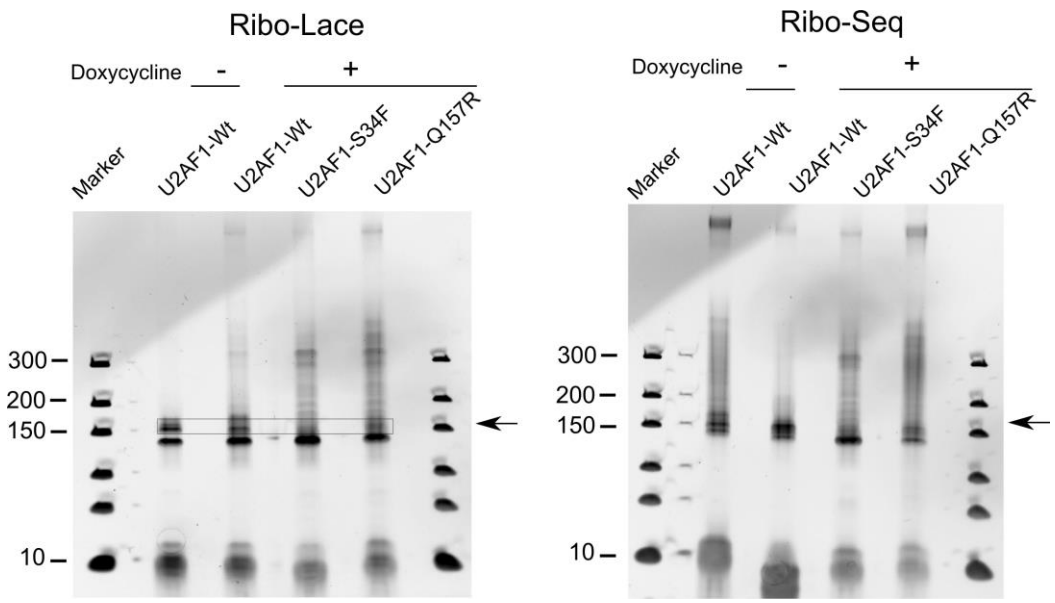

Figure 8 Separation of the libraries using native 8% TBE PAGE. The reaction products are visible above 150 nt.

We did not size select these bands, as we think that the gel should have run for longer time, but there is still circDNA and amplification product to purify and consider them for sequencing..

The summary of all samples produced for eventual sequencing can be found in Table 3.