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Ribofilio: A tool to estimate ribosomes drop-off rate in Saccharomyces Cerevisiae

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ABSTRACT

The premature ribosome drop-off phenomenon plays crucial roles in translational accuracy. However, the quantification of ribosome drop-off in Saccharomyces cerevisiae is sill under study. We provide 'Ribofilio' a python tool that rely on a high sensitive binning strategy to estimate the ribosome drop-off rate in Saccharomyces cerevisiae. We show that a significant rate of ribosome drop-off is both measurable and quantifiable. Moreover, we show that the drop-off rate is variable, depending on stress conditions, gene length, and gene ontology.

Ribofilio is available on conda and github: https://github.com/SherineAwad/ribofilio.git.
A tutorial to ribosomal profiling protocol usig ribofilio is found here: https://ribofilio.readthedocs.io/en/latest/protocol.html

INTRODUCTION

Translation of messenger RNA (mRNA) into proteins is a complex process. Ribosomes play a pivotal role in ensuring that mRNA is decoded accurately and rapidly. (7, 15). However, this translation machinery is prone to errors. One of the possible errors occurs when the ribosome fails to complete a full-length synthes process of a protein. This leads to a premature termination of the translation process. (13, 16). There are various mechanisms known to intervene in the translation abortion. Some of these mechanisms exert their strength when the cell faces stressing conditions that hinder mRNA translation, e.g. amino acid starvation. (17). In bacteria, the tmRNA-SmpB complex (8, 9) , RF3 (19) , ArfA (3) and ArfB (2) are main abortion-mediating factors known to help rescue stalling ribosomes and eventually lead to premature termination of protein synthesis. Moreover, a proofreading mechanism interrupts the synthesis of miscoded polypeptides in a translation abandonment process. (18)

There is also the possibility of unspecific events which can interrupt the elongation of the nascent peptide commonly known as processivity errors. (4, 10, 11). This includes a false stop codon causing a premature termination, (1, 5).

In addition, a drop-off of ribosomes can be triggered by , local depletion of ternary complexes. (20)

All these translation abandoment factors and processivity errors prevent the ribosome from reaching the final stop codon. We will use the term 'ribosomes drop-off rate' to denote all the events that entail the premature detachment of the ribosomes from the mRNA template, irrespective of the mechanism that caused this event.

Ribosomes drop-off doesn't necessarily occur in stressing conditions.(6, 10). The frequency of ribosomes drop-off rate

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is expected to be at a basal value when the cell is not facing any stressing conditions.

Chiarugi et al. in (16) provides a quantitative method for ribosomes drop-off rate in E.Coli. We are extending their approach in Saccharomyces cerevisiae, and studying the effect of gene length and gene ontology categories on the drop-off rate.

MATERIALS AND METHODS

Ribofilio: A tool for estimating drop-off rate

We developed 'ribofilio' an open source python tool to estimate the drop-off rate of ribosome. Ribofilio is found on our github repository `https://github.com/SherineAwad/ribofilio/`. Ribofilio takes as input the genome transcripts, the footprint reads in bed format and if mRNA reads is provided, ribofilio will normalize the drop-off rate using the mRNA reads. Ribofilio runs on a full list of genes, a subset of genes, or even on a single gene. We also provide a tutorial to how to estimate drop off rate of ribosomes using ribofilio starting from fastq reads: `https://ribofilio.readthedocs.io/en/latest/protocol.html`

A snakemake reproducible pipeline is found in our github repository `https://github.com/SherineAwad/ribofilio/pipeline`. All scripts are found in our `https://github.com/SherineAwad/ribofilio/pipeline/scripts/`. In addition, a user can run our pipeline using '−−use-conda' mode to pull the same tools' versions we used.

Data Preparation

We used ten datasets from GSE91068 and GSE134152 from GEO see Table 1. We used trim_galore to trim adapters and quality filter the reads. We used trim_galore's default parameters and -a was set to the corresponding adapters shown in Table 2 supplementary text. We then aligned the reads to yeast transcripts Saccharomyces_cerevisiae.R64-1-1.cdna.all.fa using bowtie2 (12) and generated a bed file using 'bedtools bamtobed' command (14). See Table 1 in the supplementary text for the unique alignments percentages for all the datasets. For the sake of reproducibility, we provide a Makefile to pull the datasets and the yeast genome we used: `https://github.com/SherineAwad/ribofilio/tree/master/data/Makefile`.

Gene Ontology and Gene Length Subsets

We downloaded the gene ontology from biomart using our script 'getGO.R'. We used 'gcluster.py' script to cluster genes based on their length: [0-500],]500-1000],]1000-2000] ,]2000, 3000],]3000-4000],]4000-5000], and]5000-∞]. All scripts are found in `https://github.com/SherineAwad/ribofilio/pipeline/scripts/`.

Computing the average number of RPFs per ORF: a binning strategy

Here we explain the core of ribofilio. For each dataset reported in Table 1, we created a nucleotide vector positions where position i reports the number of ORFs that their 3 end is at position i. We normalized each in positions vector with the number of genes that their length covers position i.

$$positions_i = \frac{totalRNAseqreadsforORF_i}{Li}$$

where Li is the number of genes which their length reach position i. Then grouped all positions into bins of size BINSIZE. This results in the Bin vector composed by cells (i):

$$BIN_{(j)} = \frac{\sum_j positions_i}{BINSIZE}$$

We use the RPF reads to generate the RPF Bin vector and similarly use their RNA-seq reads to generate the RNA Bin vector. To normalize the amount of RPFs with the abundance of the corresponding RNA-seq reads, we divided the value in each cell of the RPF Bin vector by their corresponding RNA Bin (i) given by:

$$NRPF_{(i)} = \frac{RPF_i}{RNA_i}$$

Estimation of Drop-off rate and its standard error

$$Y = Ae^{-RX}$$

We estimate the relationship between average number of RPFs per bin Y normalized by its corresponding RNA and the bin number X to obtain an estimation of the drop-off rate r per codon. Following similar theoretical considerations as in XXX, we tested a hypothesis that the dependence of Y on X follows an exponential decay as in equation (**??**)

where X is the bin number =1,2, .. and A is the intercept which has no interest in this scope, and R represents the drop-off

rate is the probability per bin that a ribosome prematurely detaches from the mRNA template. The drop-off rate per codon r reflects that the drop-off rate can occur anywhere inside each bin. Consider r the drop-off rate per codon, then the probability that the ribosome does not drop off within a bin of lc codon is $(1-r)^{lc}$. Hence, the probability that the ribosome drop-off rate R drops off anywhere within the bin is $1-(1-r)^{lc}$ and the drop-off per codon r is $1-(1-R)^{lc}$.

RESULTS

We examined the drop-off rate of three control datasets D1, D2, and D3. See Table 2 and Figure 1 (a), Figure 2 (a), and Figure 3 (a) for the drop-off rates of datasets D1, D2, and D3 respectively. The three control datasets show a straight line slope with RMSE $<$0.05 with a pvalue $<$0.01. This validates our hypothesis that ribosomes drop off at an exponential decay rate. However, the regression fits introduces some noise reflected by a low Rsquare $<$70 for the three control datasets. Hence, there could be hidden factors affecting the drop-off rate. To examine these possible factors, we investigated the effect of environmental conditions, gene ontology, and genes length on the drop-off rate.

Drop-off rate is variable based on environmental conditions

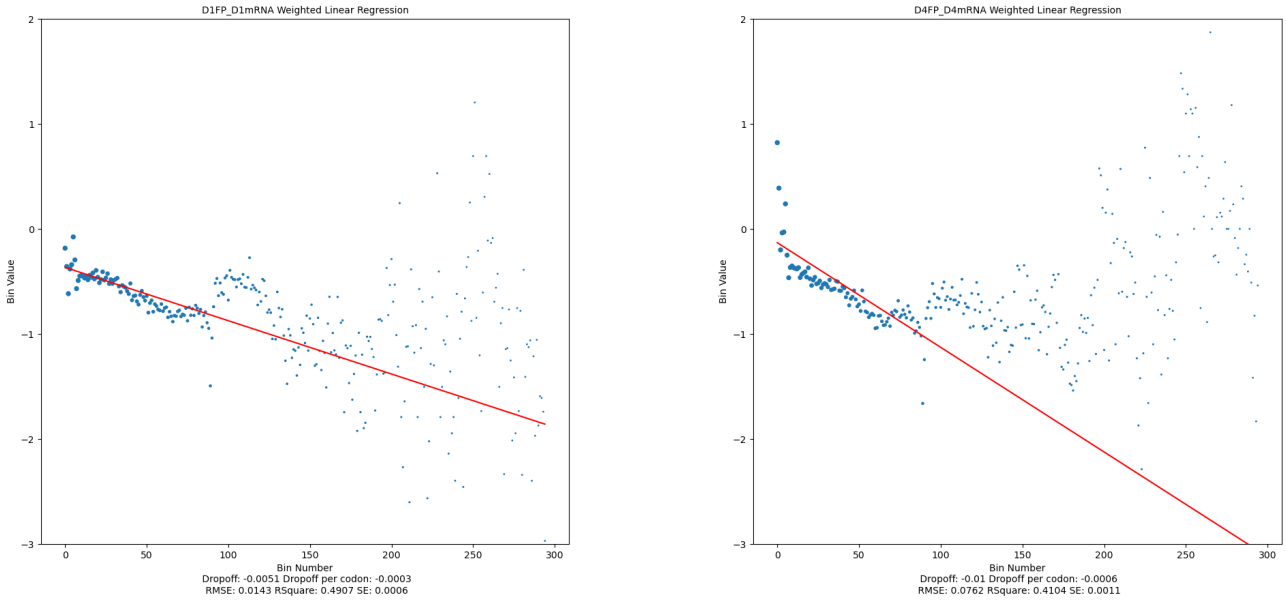
To examine any possible factor that affect drop-off rate, we studied the drop-off rate of treatment datasets (D4, D5, and D6) of the control datasets (D1, D2, and D3). The RMSE is $<$0.5 for D4, D5, and D6, the low Rsquare $<$70 (see Table 3). To examine whether the treatment condition changes the drop-off rate, we compared the drop-off rate of the control dataset and its corresponding treatment dataset. Table 3 in the Supplementary test shows the drop-off rate and the corresponding fitting statistics of treatment datasets D4, D5, and D6 when compared to the control datasets D1, D2, and D3 respectively. Although there is a significant change of the drop-off rate under the treatment condition (see Table 3 Supplementary text), however, this effect is not the main factor affecting the drop-off rate (See 3).

We also examined the drop-off of yeast under both rich and starvation conditions. Table 4 shows the drop-off rate for under rich conditions (datasets D7, and D8) and under starvation conditions (datasets D9 and D10). Table 4 in the supplementary text shows there is no significant change in drop off rate between drop-off rate under rich conditions (D7 and D8) and under starved conditions (D9 and D10). Although the RMSE for D7, D8, D9, and D10 are low, the low Rsquare shows that there are other factors affecting the drop off rate rather than the rich and starvation conditions. (See table 4).

Drop-off rate is variable based on gene ontology category

To further investigate the possible factors that affect the drop-off rate, we run ‘ribofilio‘ using subset mode ‘-s‘ on different gene ontology terms. See subsection Gene Ontology and Gene Length Subsets and see Table 5 in the supplementary text for the details of each GO category description and set size.

Table 5 shows the significant GO term per each control dataset provided that the GO term shows 1) a significant pvalue $<$0.01; 2) a significant pvalue $<$0.01 when compared to its corresponding control dataset. 3) Rsquare $>$68%; and 4) RMSE $<$5. See Table 6, 7, and 8 in supplementary text for all GO ontology drop-off rate and regression fitting statistics for D1, D2, and D3. See Table 11, 12, and 13 in the supplementary



(a) **(b)**

Figure 1. Weighted Linear Regression plot for vector NRPF for control dataset D1 and its corresponding treatment condition dataset D4. The x-axis is the bin number and the y-axis is the vector NRPF in Log exponential. The red line corresponds to the drop-off rate r. **(a)** D1 **(b)** D4

text for a significance t-test comparison between each GO subgroup and its corresponding control dataset.

The response to stress GO (GO:0006950) with set size equals 33 is commonly significant among the three control datasets D1, D2, and D3. This shows that although some GO term could affect the drop-off rate of ribosomes, we still need to further investigate other possible factors that affect the drop-off rate of ribosomes.

Gene Length affects drop-off rate

To examine whether gene length could affect the drop-off rate, we run ‘ribofilio‘ using subset mode ‘-s‘ on different gene length windows (<500,]500, 1000],]1000, 2000],]2000, 3000],]3000, 4000],]4000, 5000], and >5000). See subsection Gene Ontology and Gene Length Subsets . Table 6 shows the detailed values of drop-off rate, drop-off rate per codon and their corresponding regression fittings for different gene lengths. We notice that the drop-off rate is decreasing as the gene length increase.

For all control datasets, and for gene lengths >500, the drop-off rate has Rsquare >68 and RMSE is <0.5. This shows the drop-off rate for gene lengths >500 has a very good straight line fit. This indicates not only the drop-off rate follows an exponential decay, but also the drop-off rate for gene length >500 don’t have the noise previously introduced.

Also, for all control datasets, D1, D2, and D3, the drop-off rate of gene length greater than 500, the drop-off rate has a significant pvalue <0.01 when compared to a slope of zero.

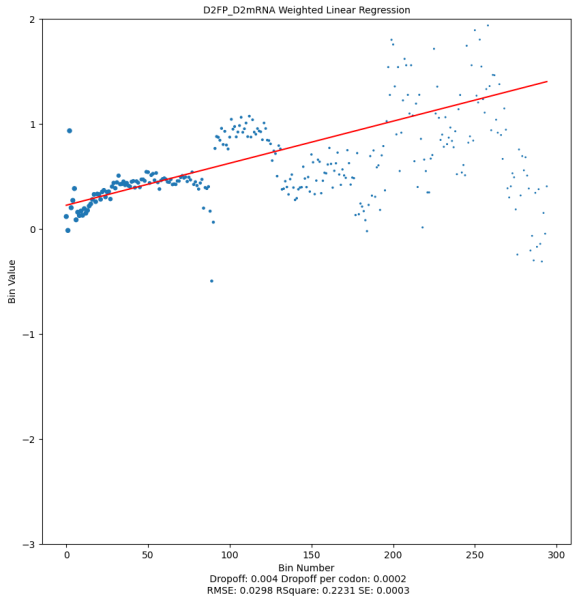
Table 14 in the supplementary test shows the significance stats of gene length subsets drop-off rate when compared to the control datasets D1, D2, and D3. The drop-off rate of all gene lengths >500 has a significant pvalue <0.01 when compared to the control datasets.

This shows that the gene length is a significant factor that affect the ribosomes’ drop-off rate.

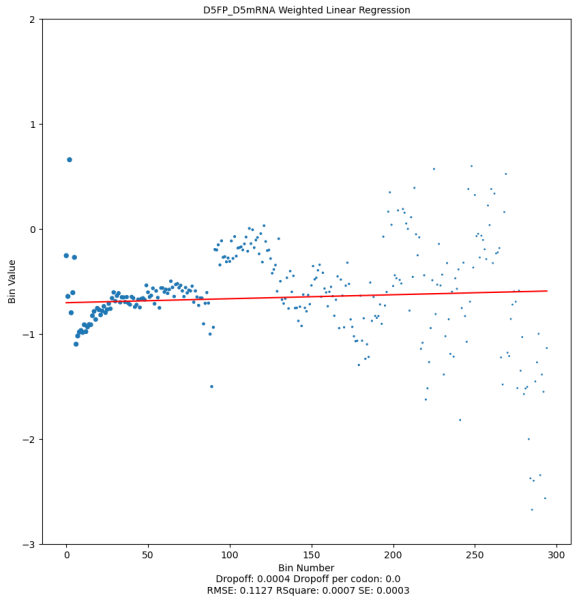
Gene Length affects drop-off rate on control vs treatment datasets Table 18 in supplementary text shows the effect of gene length on drop-off rate of control dataset vs their corresponding treatment conditions.

DISCUSSION AND CONCLUSION

The ribosome drop-off rate is measurable and quantifiable. Our method is robust against the binsize choice. We have



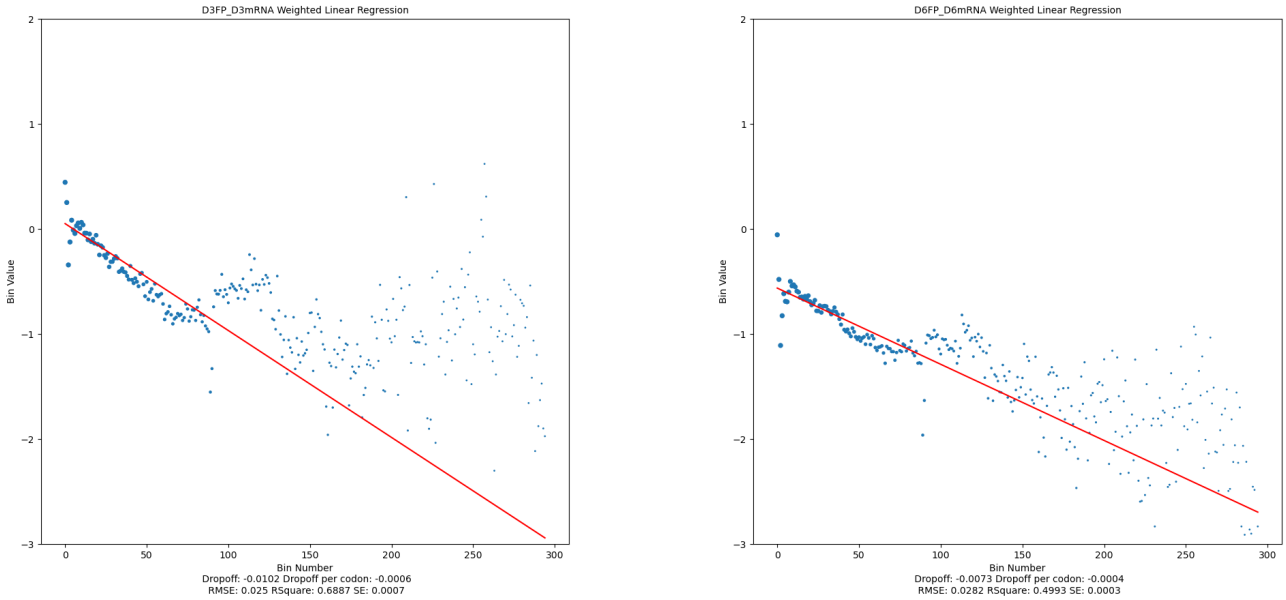
(a)



(b)

Figure 2. Weighted Linear Regression plot for vector NRPF for control dataset D2 and its corresponding treatment condition dataset D5. The x-axis is the bin number and the y-axis is the vector NRPF in Log exponential. The red line corresponds to the drop-off rate r. **(a)** D2 **(b)** D5

changed the binsize from 25, 50, and 100. The drop-off per rate per codon is constant. We have shown that the magnitude of ribosome drop-off is highly variable and dependent on case-specific factors, including experimental conditions and specific gene ontology categories. Furthermore, the drop-off rate increases as the gene length increases. The drop-off rate is close to random when gene length is less than 500. We can conclude that the gene length is a main factor affecting the drop-off rate.



(a) **(b)**

Figure 3. Weighted Linear Regression plot for vector NRPF for control dataset D3 and its corresponding treatment condition dataset D6. The x-axis is the bin number and the y-axis is the vector NRPF in Log exponential. The red line corresponds to the drop-off rate r. **(a)** D3 **(b)** D6

Table 1. Datasets Description. Column 1 is Dataset ID. Column 2 is GSE series ID. Column 3 is the GSM ID of the footprint. Column 4 is the GSM ID of the corresponding mRNA. Column 5 is Condition details about the dataset.

Dataset	Series	Footprint	mRNA	Details
D1	GSE91068	GSM2420488	GSM2420486	Synthetic Defined
D2	GSE134152	(GSM3938053 GSM3938054)	GSM3938051	Synthetic Defined (protocol 1)
D3	GSE134152	GSM39380599	GSM3938057	Synthetic Defined (protocol 2)
D4	GSE91068	GSM2420489	GSM2420487	Methionine Restriction
D5	GSE134152	(GSM3938055, GSM3938056)	GSM3938052	Glucose Restriction (protocol 1)
D6	GSE134152	GSM3938060	GSM3938058	Glucose Restriction (protocol 2)
D7	GSE13750	GSM346111	GSM346117	Rich (1)
D8	GSE13750	GSM346114	GSM346118	Rich (2)
D9	GSE13750	GSM346115	GSM346120	Starved (1)
D10	GSE13750	GSM346116	GSM346122	Starved (2)

Table 2. Drop-off rate for Control datasets D1, D2, and D3. Column 1: Dataset ID (see Table 1 for a description of each dataset). Column 2: Drop-off rate (R). Column 3: Drop-off rate per codon (r). Column 4: RMSE. Column 6: R square Column 7: Standard Error Estimate (SE).Column 8: Confidence Interval 95%. Column 9: Pvalue: Null Hypothesis that the drop-off rate is not different from a slope of zero

Dataset	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
D1	-0.0051	-0.0003	0.0143	0.4907	0.0006	R±0.0011	<0.00001
D2	0.004	0.0002	0.0298	0.2231	0.0003	R±0.0006	<0.00001
D3	-0.0102	-0.0006	0.025	0.6887	0.0007	R±0.0013	<0.00001

Table 3. Drop-off rate for Treatment datasets D4, D5, and D6. Column 1: Dataset ID (see Table 1 for a description of each dataset). Column 2: Drop-off rate (R). Column 3: Drop-off rate per codon (r). Column 4: RMSE. Column 6: R square Column 7: Standard Error Estimate (SE).Column 8: Confidence Interval 95%. Column 9: Pvalue: Null Hypothesis that the drop-off rate is not different from a slope of zero

Dataset	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
D4	-0.01	-0.0006	0.0762	0.4104	0.0011	R±0.0021	<0.00001
D5	0.0004	0.0	0.1127	0.0007	0.0003	R±0.0007	0.133
D6	-0.0073	-0.0004	0.0282	0.4993	0.0003	R±0.0006	<0.00001

Table 4. Drop-off rate per codon for Treatment datasets D7, D8, D9, and D10. Column 1: Dataset ID (see Table 1 for a description of each dataset). Column 2: Drop-off rate (R). Column 3: Drop-off rate per codon (r). Column 4: RMSE. Column 6: R square Column 7: Standard Error Estimate (SE).Column 8: Confidence Interval 95%. Column 9: Pvalue: Null Hypothesis that the drop-off rate is not different from a slope of zero

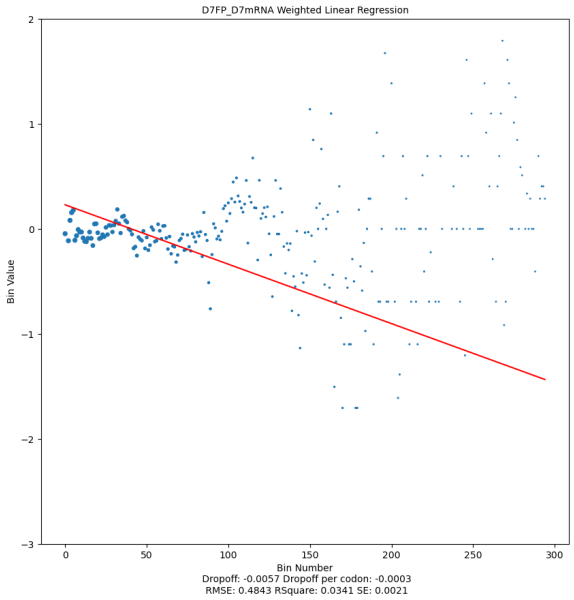
Dataset	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
D7	-0.0057	-0.0003	0.4843	0.0341	0.0021	R±0.0041	0.0038
D8	-0.0053	-0.0003	0.3147	0.0462	0.0019	R±0.0038	0.0029
D9	-0.0079	-0.0005	0.5336	0.0594	0.0027	R±0.0053	0.0016
D10	-0.0077	-0.0005	0.4188	0.0709	0.0027	R± 0.0052	0.002

Table 5. Drop-off rate per codon for dataset D1 per GO subsets: Column 1: GO ID (see Supplementary Table 01 for the respective GO names). Column 2: Drop-off rate (R). Column 3: Drop-off rate per codon (r). Column 4: RMSE. Column 6: R square Column 7: Standard Error Estimate (SE).Column 8: Confidence Interval 95%. Column 9: Pvalue: Null Hypothesis that the drop-off rate is not different from a slope of zero

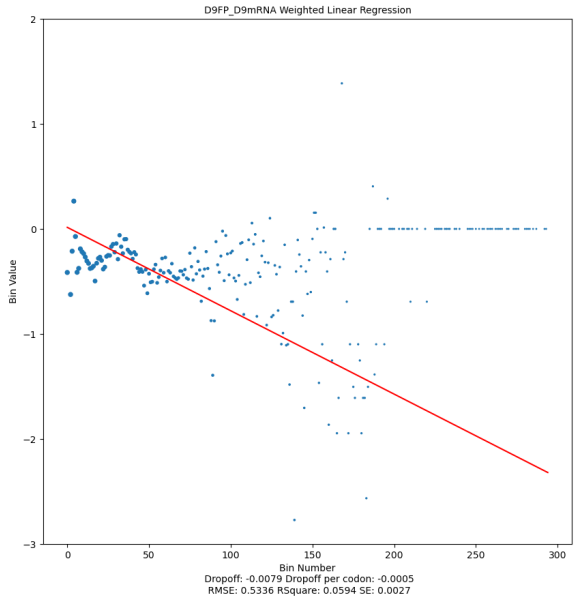
D1									
GO	Size	Description	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
GO:0006950	33	Response to Stress	-0.2064	-0.0113	3.2499	0.7287	0.021	R±0.0418	<0.00001
GO:0022857	111	Transmembrane Transporter Activity	-0.04	-0.0024	0.0601	0.7682	0.0042	R±0.0084	<0.00001
D2									
GO:0006950	33	Response to Stress	-0.2265	-0.0123	3.5607	0.7471	0.0207	0.0412	<0.00001
D3									
GO:0006950	33	Response to Stress	-0.2166	-0.0118	4.4808	0.6821	0.0241	R±0.0482	<0.00001
GO:0022857	111	Transmembrane Transporter Activity	-0.0568	-0.0033	0.0888	0.8188	0.009	R±0.018	<0.00001
GO:0030684	2	Preribosome	-0.0594	-0.0035	0.0909	0.6985	0.0078	R±0.016	<0.00001
GO:0044249	6	Cellular biosynthetic process	-0.0747	-0.0043	0.0864	0.7758	0.008	R± 0.0164	<0.00001
GO:0009651	20	Response to Salt Stress	-0.0467	-0.0027	0.2279	0.7495	0.0035	R±0.0069	<0.00001

Table 6. Drop-off rate per codon for dataset D1 and dataset D3 per Gene Length subsets: Column 1: Gene Length subgroup. Column 2: Drop-off rate (R). Column 3: Drop-off rate per codon (r). Column 4: RMSE. Column 5: Standard Error Estimate (SE).Column 6: Confidence Interval 95%. Column 7: Pvalue: Null Hypothesis that the drop-off rate is not different from a slope of zero

D1							
Gene Length	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
<500	0.0275	0.0017	0.0628	0.0663	0.0362	R±0.0834	0.2341
]500-1000]	-0.0607	-0.0035	0.0454	0.6601	0.0111	R±0.0234	<0.00001
]1000-2000]	-0.0375	-0.0022	0.0356	0.7754	0.0031	R±0.0063	<0.00001
]2000-3000]	-0.0281	-0.0017	0.0302	0.8478	0.0013	R±0.0026	<0.00001
]3000-4000]	-0.0265	-0.0016	0.0249	0.9196	0.0008	R±0.0016	<0.00001
]4000-5000]	-0.0228	-0.0014	0.0354	0.9088	0.0007	R±0.0014	<0.00001
>5000	-0.0133	-0.0008	0.0784	0.8355	0.0007	R±0.0013	<0.00001
D2							
Gene Length	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
<500	-0.0409	-0.0024	0.175	0.0531	0.0452	R±0.1043	0.1964
]500-1000]	-0.0284	-0.0017	0.0186	0.5091	0.0089	R±0.0187	0.0025
]1000-2000]	-0.02	-0.0012	0.0151	0.6989	0.0019	R±0.0039	<0.00001
]2000-3000]	-0.019	-0.0011	0.0097	0.8873	0.0009	R±0.0018	<0.00001
]3000-4000]	-0.019	-0.0011	0.0127	0.92	0.0007	R±0.0013	<0.00001
]4000-5000]	-0.0179	-0.0011	0.0363	0.8568	0.0007	R±0.0014	<0.00001
>5000	-0.0093	-0.0006	0.0488	0.7989	0.0005	R±0.0009	<0.00001
D3							
Gene Length	Drop-off (R)	drop-off per codon (r)	RMSE	Rsquare	SE	CI	Pvalue
<500	-0.0304	-0.0018	0.0586	0.0846	0.0274	R±0.0631	0.1499
]500-1000]	-0.1104	-0.0063	0.0272	0.9144	0.007	R±0.0147	<0.00001
]1000-2000]	-0.0589	-0.0034	0.0368	0.8914	0.0032	R±0.0065	<0.00001
]2000-3000]	-0.0378	-0.0022	0.0402	0.8831	0.0016	R±0.0031	<0.00001
]3000-4000]	-0.0328	-0.0019	0.0436	0.9089	0.0011	R±0.0023	<0.00001
]4000-5000]	-0.0268	-0.0016	0.0477	0.9113	0.0009	R±0.0017	<0.00001
>5000	-0.0139	-0.0008	0.0818	0.8407	0.0006	R±0.0012	<0.00001

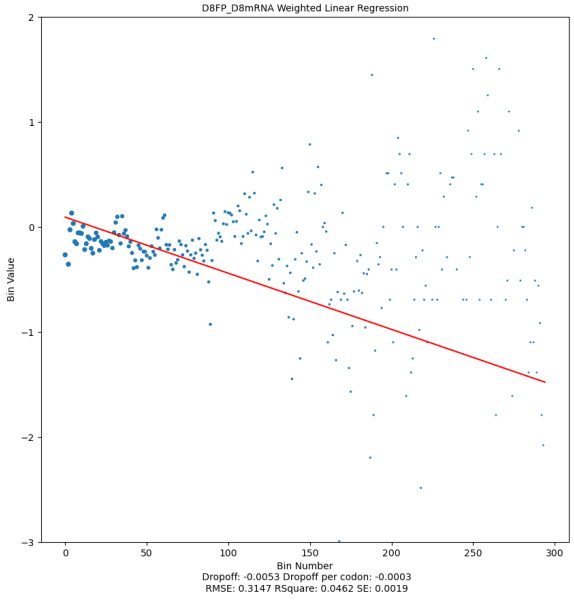


(a)



(b)

Figure 4. Weighted Linear Regression plot for vector NRPF for rich (1) dataset D7 and its corresponding starved (1) condition dataset D9. The x-axis is the bin number and the y-axis is the vector NRPF in Log exponential. The red line corresponds to the drop-off rate r. **(a)** D7 **(b)** D9



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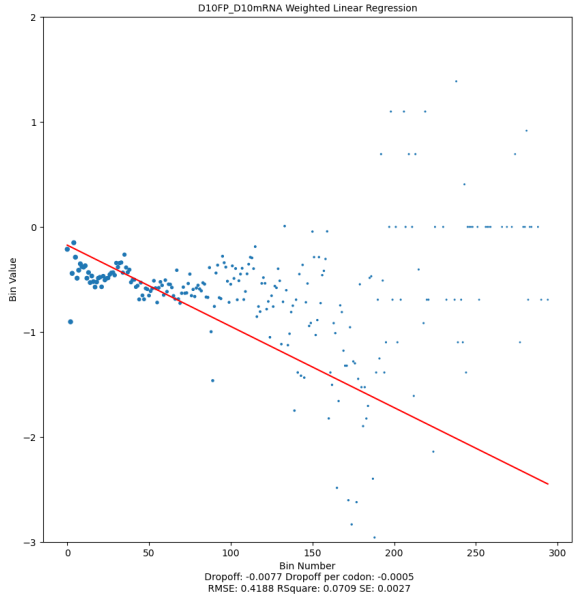

(b)

Figure 5. Weighted Linear Regression plot for vector NRPF for rich (1) dataset D8 and its corresponding starved (1) condition dataset D10. The x-axis is the bin number and the y-axis is the vector NRPF in Log exponential. The red line corresponds to the drop-off rate r. **(a)** D8 **(b)** D10

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Conflict of interest statement. None declared.

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