Electronic Supplementary Material (ESI) for Analyst.
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Supporting Information

A microfluidic electrophoretic dual dynamic staining method for the identification and relative quantitation of dsRNA contaminants in mRNA vaccines

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Supplementary Materials Information

The ssRNA ladder (NEB catalog #N0364S) contains fragments of 50 nt, 80 nt, 150 nt, 300 nt, 500 nt, and 1,000 nt, while the dsRNA ladder (NEB catalog #N0363S) contains fragments of 21 bp, 30 bp, 50 bp, 80 bp, 150 bp, 300 bp, and 500 bp. It must be noted that the fragment concentration of these samples, in particular the dsRNA ladder, has not been fully characterized by the provider since difficulties in synthesis can lead to significant batch-to-batch differences. The mRNA and dsRNA samples were custom ordered from Genewiz (Genewiz Genomics Headquarters, South Plainfield, NJ) and have lengths of 4,001 nt and 4,001 bp, respectively (provided below). While the sequences were selected to simulate an mRNA vaccine, it must be noted that the product used in this study contains uridines instead of pseudouridines. Unless specified otherwise, all samples were used at room temperature and were not heated prior to analysis. Lastly, all experiments shown include data from three independent runs/experimental repeats (fresh samples, gel-dye, and chip preparation) with two to three repeats/instrumental repeats (same sample gets analyzed multiple times in the same gel-dye and chip preparation), leading to a total of 6-9 data points per data set presented.

Sequence. mRNA and dsRNA sample sequence.

The custom, long mRNA and dsRNA samples were ordered from Genewiz (Genewiz Genomics Headquarters) and have lengths of 4,001 nt and bp, respectively. The dsRNA is a duplex form of the mRNA sample and as such they share sequences. The sequence for the mRNA and dsRNA 5' strand is provided below. The sequence is believed to have a limited or even negligible effect on the study itself, but it is provided in case it is useful to the reader.

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Figure S1. Assessment of the effect of heat during sample preparation

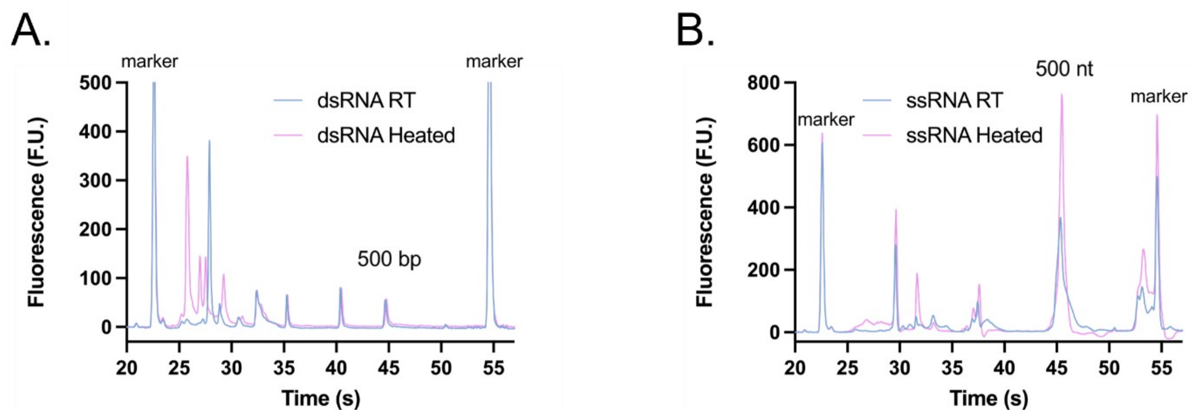

Figure S1: Assessment of the effect of heat during dsRNA and ssRNA sample preparation. Generally, RNA molecules that are single-stranded are heated prior to analysis to prevent their tertiary structure (i.e., hairpins) from interfering with the accurate measurement of their mobility. However, upon analyzing both ssRNA and dsRNA (Figure S1) it was observed that while the use of heat resulted in better defined peaks for the ssRNA sample, it also denatured smaller strands of dsRNA causing the fabrication of dsRNA peaks. Acknowledging that numerous techniques exist for the analysis of mRNA molecules, and that the main focus of this study is to characterize the harmful dsRNA contaminant in mRNA vaccines, it was decided that the conditions would be selected to optimize the detection and characterization of dsRNA instead. This denaturing makes the dsRNA more difficult to characterize, since dsRNA can be present in mRNA vaccines in any length equal to or inferior to the mRNA length. Due to dsRNA being the main focus of this work, it was decided to prioritize all lengths of dsRNA over that of ssRNA fragments. Therefore, heating was excluded from the sample preparation. These experiments were conducted using a 6% gel matrix. In addition, while it is difficult to infer from these electropherogram snapshots, the 500 bp dsRNA peak (the last one of the dsRNA ladder) has a migration time of ~45 s while the 500 nt ssRNA peak (the peak after the peak with double intensity in the ladder, which is 300 nt long) has a migration time of ~53 s. Note that the first and last peak in both electropherograms are a lower (left) and an upper (right) marker used to align the electropherograms. Since the upper marker (right) overlaps with some of the larger ssRNA fragments, it was excluded for the study.

Figure S2. Optimization of stain concentration based peak response

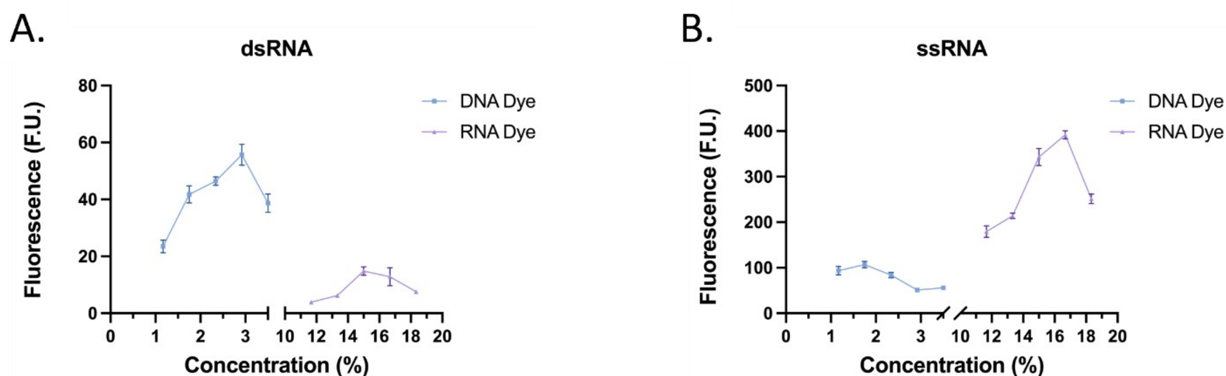

Figure S2: Optimization of the ideal dye concentration to detect and characterize both dsRNA and ssRNA fragments based on the response of the 50 bp or nt fragment of each ladder. The fragment size was selected arbitrarily but is believed to be representative of the behavior across each ladder. These experiments were conducted using a 6% gel matrix. When dye concentrations were changed, DMSO was used to keep the gel matrix to dye volume ratio constant for a given dye type. More specifically, the DNA dye (ThermoFisher/Revvity) was diluted to 1.17%, 1.75%, 2.34%, 2.92% and 3.50% dye concentration while the RNA dye (ThermoFisher/Revvity) was diluted to 11.67%, 13.33%, 15.00%, 16.67%, and 18.33% dye concentration. The percentages are all relative concentrations based on the stock vials provided by Revvity of proprietary composition and concentration and are therefore not indicative of the molarity of the solution. To determine the dye concentration of interest (that produced the desirable outcome), we arbitrarily selected a starting concentration for each dye based on provider recommendations, and then probed the neighboring concentrations until a maximum labeling efficiency was achieved.

Figure S3. Selection of the gel matrix concentration

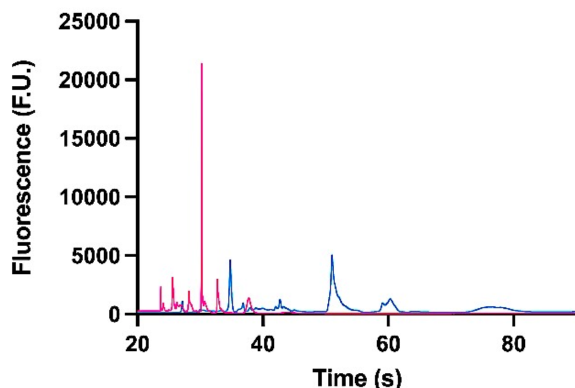

Figure S3: The same ssRNA ladder was analyzed first using 6% gel (teal) and then using a 3% gel (pink) matrix mixture. During these experiments it was noted that likely due, or at least in part, to the significantly slower migration using the 6% gel matrix, the ladder showed a significantly greater spread of the peaks at 6% gel than at 3% gel. For this reason, 3% gel was selected as the matrix for the remainder of the experiments. Although not included here, the dsRNA ladder was also assessed with both gel percentages and the results looked similar, although the absence of a larger fragment such as the 1,000 nt present in the ssRNA ladder made the changes in spread less obvious in the dsRNA ladder.

Table S1. Comparison of dsRNA and ssRNA mobility

Table S1: Comparison of the migration of different fragments in a dsRNA and a ssRNA ladder within a microfluidic platform. These experiments were conducted using a DNA dye and 3% gel matrix.

Fragment size (bp or nt)	dsRNA migration time (s)	dsRNA mobility ([µm/s]/[V/cm])	ssRNA migration time (s)	ssRNA mobility ([µm/s]/[V/cm])
50	24.5 ± 0.2	1.39 ± 0.01	27.9 ± 1.0	1.38 ± 0.05
80	28.5 ± 0.2	1.35 ± 0.01	*	*
150	30.2 ± 0.3	1.27 ± 0.01	31.47 ± 1.0	1.22 ± 0.04
300	34.1 ± 0.3	1.13 ± 0.01	34.5 ± 0.7	1.11 ± 0.02
500	37.2 ± 0.3	1.03 ± 0.01	37.1 ± 0.7	1.03 ± 0.02

* Note that some fragments were excluded from the analysis due to difficulty distinguishing it from other fragments or from the noise.

Table S2. Comparison of dsRNA peak response

Table S2: Comparison of the dsRNA fragment peak areas and heights using different dye types. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix.

	Area		Height	
Fragment size (bp)	**Area DNA dye (F.U. × s)**	**Area RNA dye (F.U. × s)**	**Height DNA dye (F.U.)**	**Height RNA dye (F.U.)**
21	1458.0 ± 22.8	724.2 ± 29.7	7855.5 ± 114.3	4129.8 ± 102.9
30	345.0 ± 27.5	51.3 ± 1.7	1628.6 ± 91.7	299.3 ± 4.8
150	142.9 ± 7.8	46.7 ± 9.8	804.9 ± 37.9	255.8 ± 26.0
300	226.5 ± 14.9	90.9 ± 19.9	967.8 ± 97.8	284.4 ± 22.1
500	346.6 ± 25.0	196.1 ± 22.8	930.7 ± 50.9	226.2 ± 14.2

Table S3. Comparison of ssRNA peak response

Table S3: Comparison of the ssRNA fragment peak areas and heights using different dye types. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix.

	Area		Height	
Fragment size	**Area DNA dye**	**Area RNA dye**	**Height DNA**	**Height RNA**

(nt)	(F.U. × s)	(F.U. × s)	dye (F.U.)	dye (F.U.)
50	772.7 ± 124.8	1883.5 ± 12.9	31341 ± 568.7	10287.0 ± 244.9
150	662.2 ± 128.3	1434.8 ± 23.4	1091.3 ± 191.5	2214.7 ± 58.6
300	2157.5 ± 596.9	6566.3 ± 80.1	5423.5 ± 1918.5	11187.7 ± 451.4
500	1565.7 ± 104.7	2296.8 ± 59.5	4845.2 ± 1319.0	5103.6 ± 226.6
1000	1246.9 ± 66.7	1761.1 ± 60.9	1197.8 ± 122.7	1932.8 ± 68.4

Figure S4. Comparison of ladder peak height response using different stains

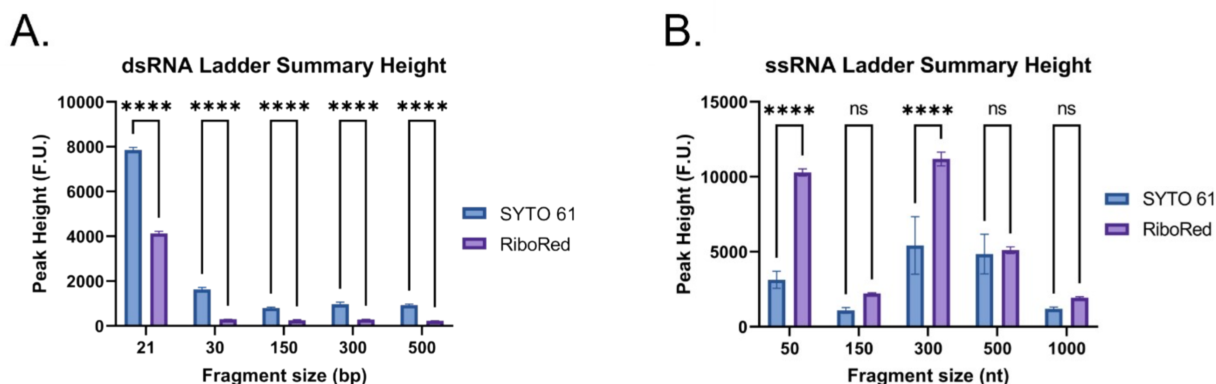

Figure S4: Fluorescent staining differences between a dsRNA and an ssRNA ladder using SYTO 61 (blue) and RiboRed (purple). Peak heights produced by (A) the dsRNA ladder and (B) the ssRNA ladder with each stain. In the bar graphs, "*" indicates the level of significance in the difference (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), while "ns" indicates that there is no statistical difference.

Figure S5. Comparison of pure long dsRNA and mRNA peak height response using different stains

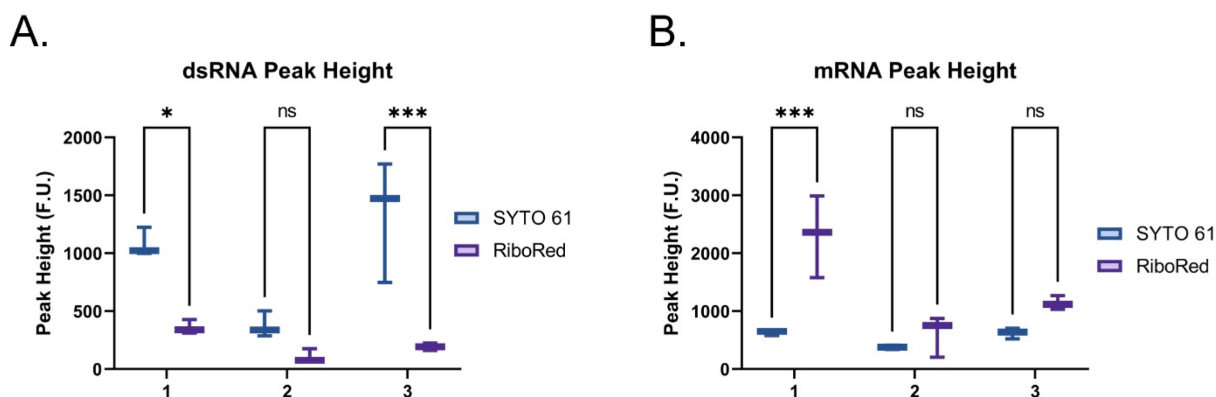

Figure S5: Custom pure (A) dsRNA and (B) mRNA fragment peak height using SYTO 61 (blue) and RiboRed (purple). The data includes results from three independent runs, each with two to three

repeats. In the comparisons, "*" indicates the level of significance in the difference (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), while "ns" indicates that there is no statistical difference.

Table S4. Comparison of a long dsRNA fragment peak response

Table S4: Comparison of a long dsRNA fragment peak areas and heights using different dye types. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix.

	Area		**Height**	
Run #	**Area DNA dye (F.U. × s)**	**Area RNA dye (F.U. × s)**	**Height DNA dye (F.U.)**	**Height RNA dye (F.U.)**
1	641.3 ± 4.9	220.5 ± 23.5	1082.2 ± 124.2	359.4 ± 61.1
2	319.4 ± 53.8	159.4 ± 9.3	376.0 ± 112.7	107.8 ± 59.1
3	387.3 ± 103.5	112.0 ± 4.0	1330.4 ± 526.3	192.7 ± 45.5
Average	**449.3 ± 158.1**	**170.48 ± 47.8**	**929.5 ± 510.1**	**223.4 ± 127.6**

Table S5. Comparison of a long mRNA fragment peak response

Table S5: Comparison of a long mRNA fragment peak areas and heights using different dye types. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix.

	Area		**Height**	
Run #	**Area DNA dye (F.U. × s)**	**Area RNA dye (F.U. × s)**	**Height DNA dye (F.U.)**	**Height RNA dye (F.U.)**
1	4749.1 ± 186.3	11399.7 ± 2077.4	626.3 ± 42.6	2310.0 ± 706.8
2	3220.6 ± 141.7	3777.0 ± 632.6	375.3 ± 36.7	611.4 ± 356.0
3	5082.6 ± 469.0	7536.2 ± 576.6	622.2 ± 93.9	1140.4 ± 119.6
Average	**4350.8 ± 899.0**	**7571.0 ± 3486.8**	**541.3 ± 136.0**	**1354.0 ± 852.5**

Figure S6. Comparison of mixed long dsRNA and mRNA peak height response using different stains

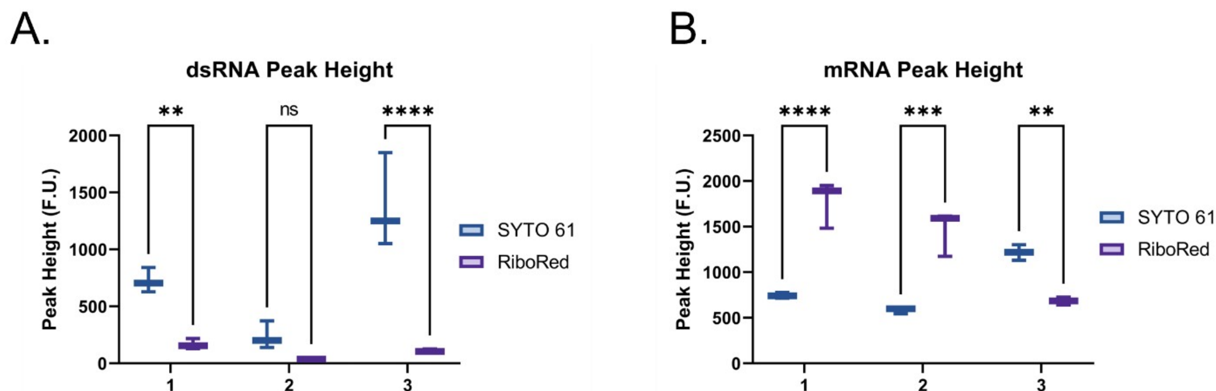

Figure S6: Custom mixed (A) dsRNA and (B) mRNA fragment peak height using SYTO 61 (blue) and RiboRed (purple). The data includes results from three independent runs, each with two to three repeats. In the comparisons, "*" indicates the level of significance in the difference (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), while "ns" indicates that there is no statistical difference.

Table S6. Comparison of a long dsRNA fragment peak response when mixed with mRNA

Table S6: Comparison of a long dsRNA fragment peak areas and heights using different dye types when mixed with mRNA. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix. Here, the dsRNA was used at a concentration of 2.60 ng/µL, while the mRNA was used at a concentration of 5.90 ng/µL based on triplicate nanodrop readings.

	Area		**Height**	
Run #	**Area DNA dye (F.U. × s)**	**Area RNA dye (F.U. × s)**	**Height DNA dye (F.U.)**	**Height RNA dye (F.U.)**
1	445.5 ± 20.8	83.6 ± 20.3	724.2 ± 108.5	166.7 ± 46.2
2	206.4 ± 34.2	27.9 ± 9.6	237.2 ± 120.9	38.6 ± 11.2
3	381.0 ± 4.5	50.1 ± 15.1	1383.1 ± 415.8	110.0 ± 14.3
Average	**344.3 ± 109.0**	**53.9 ± 27.8**	**781.5 ± 545.8**	**105.1 ± 495.0**

Table S7. Comparison of a long mRNA fragment peak response when mixed with dsRNA

Table S7: Comparison of a long mRNA fragment peak areas and heights using different dye types when mixed with dsRNA. These experiments were conducted using a DNA dye at 1.2%, an RNA dye at 15.0%, when applicable, and a 3% gel matrix. Here, the dsRNA was used at a concentration of 2.60 ng/µL, while the mRNA was used at a concentration of 5.90 ng/µL based on triplicate nanodrop readings.

	Area		Height	
Run #	**Area DNA dye (F.U. × s)**	**Area RNA dye (F.U. × s)**	**Height DNA dye (F.U.)**	**Height RNA dye (F.U.)**
1	4034.8 ± 37.6	7971.1 ± 700.0	743.6 ± 30.7	1775.7 ± 256.5
2	4328.0 ± 237.2	7120.1 ± 765.7	583.8 ± 35.7	1460.1 ± 248.2
3	5082.8 ± 144.5	8244.2 ± 425.7	1216.6 ± 85.7	684.4 ± 59.8
Average	**4405.7 ± 460.4**	**7778.5 ± 756.4**	**848.0 ± 289.2**	**1384.6 ± 495.0**

Figure S7. RiboRed and SYTO 61 peak area ratio for dsRNA and mRNA

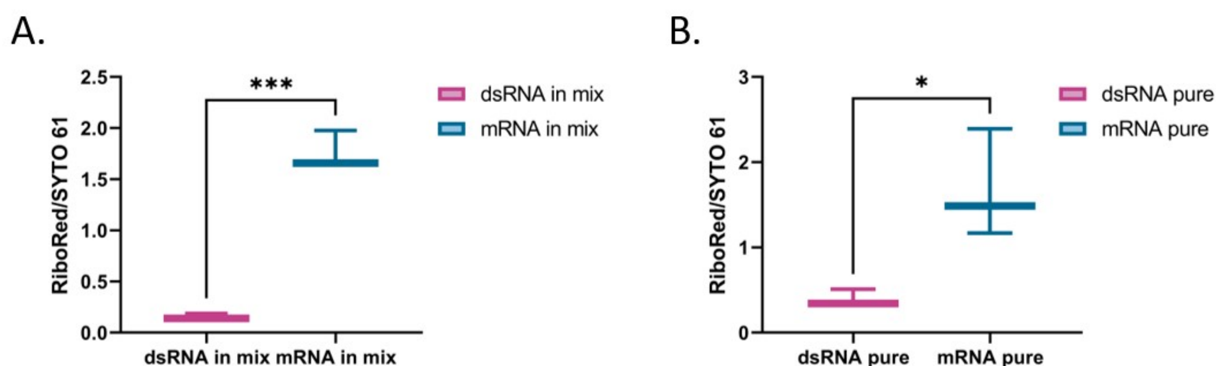

Figure S7: Peak area ratios between RiboRed and SYTO 61 for dsRNA (pink) and mRNA (teal) when analyzed (A) mixed and (B) individually. These results include data from three independent runs, each with two to three repeats. Here, it is important to note that the difference in response is exacerbated when comparing the pure to the in-mix dsRNA and mRNA responses. While the exact mechanism behind this response remains unknown, we believe a key factor in this difference is that when comparing the areas yielded by the "pure" RNA molecules, four different electropherograms have to be combined and compared to each other (one per stain type, two per molecule) since the samples are run separately whereas the "in mix" samples only require the combination of two. This can significantly decrease the error associated to the combination of electropherograms since each dsRNA and mRNA peak for each stain type is now being extracted from the same run, which means that the conditions (i.e., conductivity) in which each peak was produced is completely matched across molecule types. In the comparisons, "*" indicates the level of significance in the difference (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001), while "ns" indicates that there is no statistical difference.

Figure S8. Interaction between RiboRed and SYTO 61 with dsRNA and mRNA

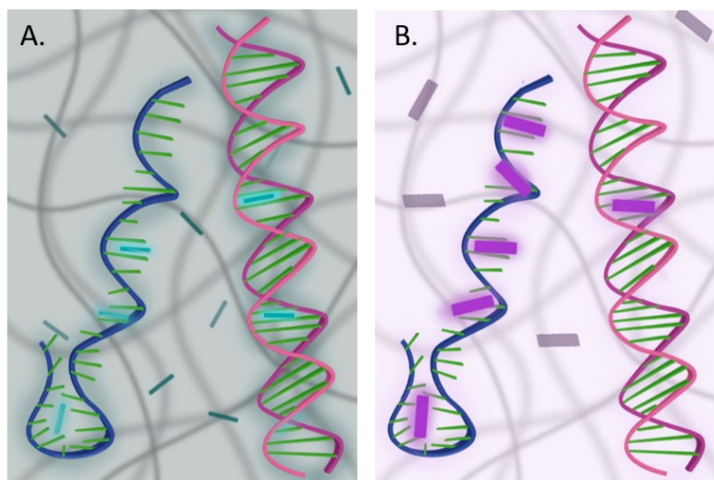

Figure S8: Interaction between mRNA (blue) and dsRNA (pink) with (A) SYTO 61 and (B) RiboRed. It is important to note that while dsRNA will label better with SYTO 61 than with RiboRed and the opposite for mRNA, our results suggest that despite this trend, mRNA will still label better than dsRNA with both stains. We hoped to highlight this by showing dsRNA bound to less fluorophores than mRNA even in the optimal labeling conditions for dsRNA.

Figure S9. Linearity in dsRNA and mRNA response

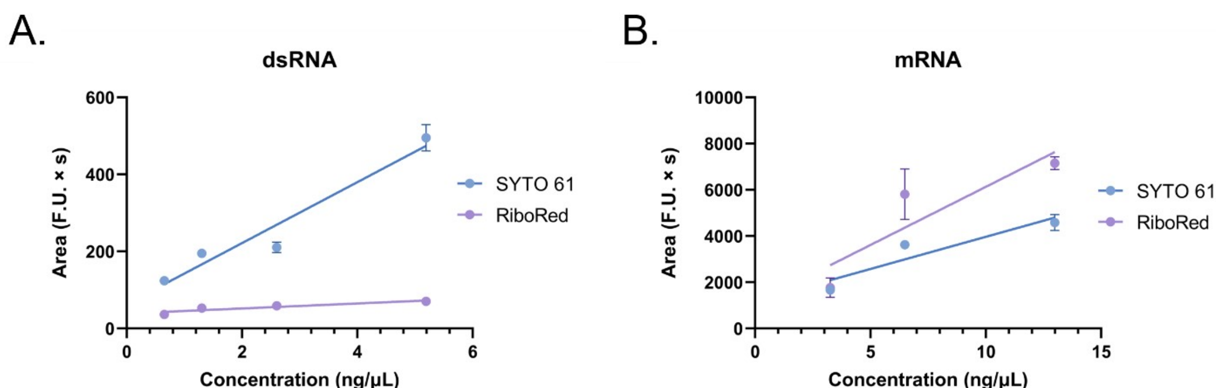

Figure S9: Assessment of the linearity in the response of (A) dsRNA and (B) mRNA as their concentrations increased using SYTO 61 (blue) and RiboRed (purple). (A) The line of best fit for the dsRNA sample using SYTO 61 was y=79.05x+63.87 with an R^2=0.93 while that of RiboRed was y=6.49x+39.21 with an R^2=0.75. (B) The line of best fit for the mRNA sample using SYTO 61 was y=278.2x+1189 with an R^2=0.84 while that of RiboRed was y=503.0x+1103 with an R^2=0.75. While all samples showed a roughly linear relation between area and concentration, it was interesting to note that for both dsRNA and mRNA, RiboRed yielded the lowest R^2 value of 0.75 for both molecules, while SYTO 61 yielded the highest, with values of 0.93 and 0.84, respectively. Another interesting trend that was observed during these experiments is that for both molecule types, the slope of the line of best fit was highest with the most efficient stain, SYTO 61 in the case of dsRNA and RiboRed in the case of mRNA. Based on the equations for the lines of best fit yielded for each molecule by both fluorescent stains (Figure 7), in the case of dsRNA the lines

would converge when the dsRNA concentration is -0.34 ng/μL while the mRNA lines would converge when the mRNA concentration is 0.38 ng/μL.

Table S8. Assessment of dsRNA and mRNA peak area ratios at varying concentrations of dsRNA.

Table S8. Assessment of dsRNA and mRNA peak area ratios (RNA dye/DNA dye) at varying concentrations of dsRNA so that it represented 5.0%, 7.5% and 10.0% of the total mRNA concentration.

dsRNA percentage (%)	dsRNA concentration (ng/μL)	dsRNA peak area ratio	mRNA concentration (ng/μL)	mRNA peak area ratio
5.0	0.65	0.07 ± 0.02	12.99	2.11 ± 0.44
7.5	0.97	0.10 ± 0.00	12.99	2.21 ± 0.34
10.0	1.30	0.11 ± 0.01	12.99	1.75 ± 0.22
Average	**-**	**0.09 ± 0.02**	**-**	**2.03 ± 0.37**