Supplemental Information

Direct Immunodetection of Global A-to-I RNA Editing Activity with a Chemiluminescent Bioassay

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RNA Oligoribonucleotides

Oligonucleotide control strands used in this study were custom designed and purchased from Integrated DNA Technologies.

RNA A:	5'	GUGCCUUUAUGC**A**GCAAGGAUGCG 3'
RNA I:	5'	GUGCCUUUAUGC**I**GCAAGGAUGCG 3'

RNA Denaturation and 3' Fluorescent Labeling

250 pmol (~2 µg) of RNA A or RNA I oligo was first combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then directly combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 0.2 mM Cy5 Hydrazide (Lumiprobe) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 7.5% DMSO. Reactions were incubated at 37 ºC for 2 hours and then ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified using a NanoDrop spectrophotometer (Thermo Fisher). To validate covalent denaturation and labeling, 2 pmol each of unmodified RNA, glyoxalated RNA, and dual glyoxalated and 3' end labeled RNA were analyzed by 20% denaturing PAGE and stained with 1X SYBR gold (Thermo Fisher). The gel was then imaged with a GE Amersham Typhoon RGB scanner.

RNA 3' Biotinylation and Streptavidin Plate Immobilization

To test immobilization and binding capacity of streptavidin-coated plates (Fig. S2), 5 µg of RNA A was first fluorescently labeled with Cyanine 5 (Cy5) using the Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit (Mirus Bio). 5 µg RNA was combined with 5 µL Label IT Tracker reagent in 50 µL 1X Tracker buffer and incubated at 37 ºC for 1 hour. The oligonucleotide was then ethanol precipitated and analyzed by UV/Vis spectrophotometry to measure degree of labeling, confirming approximately 2-3 dye molecules per strand. Next, 250 pmol (~2 µg) of Cy5-labeled RNA A oligo was then combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then directly combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 25 mM biotin-dPEG®$_4$-hydrazide (Sigma Aldrich) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 10% DMSO. Negative control RNA reaction (-biotin) contained no biotin hydrazide. Reactions were incubated at 37 ºC for 2 hours, ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified using a NanoDrop spectrophotometer (Thermo Fisher). Next, various dilutions of -/+ biotin RNA A were prepared in 1X binding/wash buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.01% Tween 20, pH 7.4). Each well of a Pierce™ Streptavidin Coated Plate, White, 96-Well (Thermo Fisher) was then washed two times with 200 µL of 1X binding/wash buffer. In triplicate wells, 100 µL of each RNA dilution was then added to each well and incubated for 1 hour with shaking at room temperature. Plate was then washed two times with 200 µL of 1X binding/wash buffer, and 100 µL of buffer was

added to each well. Fluorescence was measured with a BioTek Cytation 5 plate reader using an excitation and emission wavelengths of 650 nm and 670 nm. Net relative fluorescence units (RFU, arbitrary units) were calculated by subtracting appropriate blank wells (buffer only, no RNA).

EndoVLISA Optimization

To optimize EndoVLISA performance (Figs. S3-9), RNA A and RNA I stocks were first glyoxal denatured and 3' biotinylated as described above. For each EndoVLISA probing combination, 0, 0.1 and 2 pmol of RNA I and 2 pmol RNA A was added to duplicate wells of a white streptavidin-coated plate in 100 µL 1X binding/wash buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.01% Tween 20, pH 7.4) and incubated for 1 hour at room temperature with shaking. Plate was then washed two times with 200 µL of 1X binding/wash buffer, and each well and then received 100 µL of an EndoV-MBP (New England Biolabs) solution in 1X buffer (1:500, 1:1000, or 1:2000). Plates were incubated for 1 hour at room temperature with shaking, followed by 2x200 µL washes with 1X binding/wash buffer. Each well then received 100 µL of an anti-MBP primary mouse monoclonal antibody (New England Biolabs) solution in 1X buffer (1:1000, 1:2000, 1:4000, or 1:8000). After incubating for 1 hour at room temperature with shaking, plates were again washed two times with 200 µL 1X binding/wash buffer, and then 100 µL of a Goat anti-Mouse IgG (heavy + light chain) polyclonal HRP-conjugated secondary antibody (Thermo Fisher) solution in 1X buffer (1:10,000, 1:20,000 or 1:40,000) was added to appropriate wells. Plates were incubated at room temperature for 1 hour with shaking, followed by 2x200 µL washes with 1X binding/wash buffer. Each well was then emptied, and 100 µL of SuperSignal™ ELISA Pico Chemiluminescent Substrate working solution was added followed by shaking for 1 minute at room temperature. Light signal was then measured using a BioTek Cytation 5 platereader using a luminescence detection fiber optic filter (gain = 100). Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Pearson *r* *p*-value was computed in GraphPad Prism 9 using on-target RNA I signals from 0, 0.1 and 2 pmol RNA signals. Fold-selectivity was calculated as the net chemiluminescent signal of RNA I divided by RNA A (2 pmol/well). To acquire an image of chemiluminescent EndoVLISA detection (Fig. 2a), different amounts of RNA A or I as indicated was first immobilized into a 96-well streptavidin coated plate, followed by immunodetection with different reagent combinations (EndoV, 1º antibody, 2º HRP) as described above. Plate was then incubated with 100 µL Pico ELISA chemiluminescent substrate for 1 minute with shaking. Reactions were then transferred to a clear 96-well plate and chemiluminescent signal was captured using a GE Amersham Typhoon RGB scanner. Heatmap image was obtained using the Typhoon acquisition software and reflects relative luminescent intensities. Bar graph denotes net RLU values obtained from BioTek plate reader measurement.

EndoVLISA Linearity and Sensitivity

To assess EndoVLISA performance using optimal conditions identified from initial screen (Fig. S10), decreasing amounts of biotinylated, glyoxal-denatured RNA I strand (0 – 2 pmol) were immobilized in white streptavidin-coated 96-well plates and then probed with the indicated detection reagent combinations (EndoV 1:1000, 1º antibody 1:1000, 2º HRP 1:20,000, ELISA Pico substrate) following the EndoVLISA procedure described earlier. Standard curve was then plotted in both linear and $\log_2$ scale using GraphPad Prism 9. Linear regression (R^2), pearson (*r*) correlation and *p*-value were computed in Prism. This same basic procedure was repeated when testing the SuperSignal™ West Atto chemiluminescent substrate (Thermo Fisher Scientific) and different amounts of 2º HRP (Figs. S11, S12).

In vitro mRNA production

A plasmid encoding GFP was purchased from AddGene (pET28 GFP, 60733). 1 µg of purified plasmid was then linearized by digestion with BamHI-HF (New England Biolabs) for 30 minutes at 37 ºC. Cut plasmid was then isolated using the Monarch® PCR Purification Kit (New England Biolabs). mRNA was then generated *in vitro* using the HiScribe™ T7 Quick High Yield RNA Synthesis Kit at 37 ºC overnight, and mRNA was purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and quantified using a NanoDrop spectrophotometer.

EndoVLISA Selectivity with mRNA

To assess EndoVLISA performance in the presence of off-target mRNA (Fig. 2b, S13), decreasing amounts of RNA I strand (0 – 1 pmol) were mixed in triplicate with 1 µg of *in vitro* transcribed mRNA. These were then directly combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 25 mM biotin-dPEG®$_4$-hydrazide (Sigma Aldrich) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 10% DMSO. Reactions were incubated at 37 ºC for 2 hours, ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified using a NanoDrop spectrophotometer. Each sample was then immobilized in white streptavidin-coated 96-well plates and then subjected to EndoVLISA detection (EndoV 1:1000, 1º antibody 1:1000, 2º HRP 1:40,000, SuperSignal™ West Atto substrate) following the general procedure described earlier. Standard curve was then plotted in GraphPad Prism 9, and linear regression (R^2), pearson (r) correlation and p-value were also computed in Prism.

Liquid Chromatography and Mass Spectrometry (LC/MS)

Ribonucleosides inosine, guanosine, adenosine, cytidine and uridine were purchased from Sigma Aldrich. Pseudouridine was purchased from Santa Cruz Biotechnology, and N^6-methyladenosine (m^6A) was purchased from MedChemExpress. Standard stock concentrations (1 mM) were prepared in pure nuclease-free water and diluted in water where necessary. Reversed-phase LC was performed on an attached Agilent 1260 Infinity II system using a 3 µM, 4.6 mm X 75 mm Atlantis T3, 100Å C18 column (Waters). All samples were analyzed using a linear mobile phase gradient from 0% to 20% acetonitrile in water + 0.1% formic acid over 10 minutes. All mass spectra were obtained using an Agilent 6320 time-of-flight (TOF) electrospray ionization (ESI) LC/MS instrument in positive ionization mode.

Cell Line Maintenance and Transfection

HEK293T cells (ATCC CRL-3216) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and maintained at 37 ºC, 5% CO_2. Cells were grown in T75 Nunc™ tissue culture-treated flasks. When ~50% confluent, cells were then transfected with increasing amounts of plasmid encoding a GFP-tagged ADAR1 enzyme (AddGene, pmGFP-ADAR1-p110, #117928). Transfection was performed directly in flasks with using Lipofectamine 3000 (Thermo Fisher Scientific). Indicated amounts of plasmid (Fig. 3a) were first combined with 50 µL lipofectamine 3000 reagent and 80 µL P3000 solution in 1.5 mL Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific). Transfection mixtures were added directly to each

flask, and after ~48 h incubation at 37 ºC, 5% CO_2, cells were harvested by trypsinization and washed twice in cold 1X phosphate buffered saline.

Western Blotting

Whole cell lysates were first collected by adding 1mL of M-PER lysis reagent (Thermo Fisher Scientific) for each 100 mg (~100µL) of wet cell pellet. After pipetting to homogenize the pellet, mixture was incubated with end-over-end rotation for 10 minutes at room temperature. Cell debris was then removed by centrifugation at ~14,000 × *g* for 15 minutes, after which the supernatant was collected and protein content was estimated using Pierce™ 660nm Protein Assay Reagent (Thermo Fisher Scientific). To verify GFP-ADAR expression, 20 µl of this lysate was loaded into 384-well black plates in triplicate and measured using a BioTek Cytation 5 plate reader using excitation at 488 nm and emission at 509 nm (Fig. S19). For western blotting analysis, 20 µg of whole 293T cell lysate from each transfection group was mixed with 1X reducing sample buffer (Thermo Fisher) and heated to 95 ºC for 5 minutes. Each sample was then resolved on a 4-20% Tris Glycine polyacrylamide gel (Thermo Fisher) along with a PAGERuler Plus Prestained Protein ladder (Thermo Fisher). After electrophoresis, the gel was removed from the cassette and briefly washed with diH_2O, after which it was transferred to a 0.2 µm nitrocellulose membrane (Invitrogen) for 2 hours at 25 V. The membrane was briefly washed with diH_2O, after which it was submerged for 1 hour in 1X blocking buffer comprised of a 5% non-fat dry milk solution (Kroger) in 1X TBST (Thermo Fisher, 25 mM Tris, 150 mM NaCl, 0.5% Tween 20, pH 7.4). The membrane was cut into two pieces and then probed with an anti-ADAR1 rabbit monoclonal antibody (Cell Signaling Technologies D7E2M) at a 1:1000 dilution and an anti-actin mouse monoclonal antibody (Thermo Fisher Scientific MA1-140) at a 1:5000 dilution in 1X blocking buffer for 1 hour with gentle agitation. After washing the blots 3x5 minutes in 1X TBST, membranes were probed with a Goat anti-Rabbit IgG Alexa Fluor 647-conjugated secondary antibody (Thermo Fisher A21244) at a 1:1000 dilution and a Goat anti-Mouse IgG Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher A11001) at a 1:1000 dilution in 1X blocking buffer for 1 hour protected from light. Membranes were washed 3x5 minutes in 1X TBST, followed by imaging of the blot using a GE Amersham Typhoon RGB scanner. Densitometric analysis was performed using ImageJ (Fiji).

293T mRNA Isolation, Processing and EndoVLISA detection

mRNA was collected from each treated flask by first isolating total RNA using the Monarch® Total RNA Miniprep Kit (New England Biolabs). Isolated material was then processed using two rounds of Oligo dT_{25} purification with the Magnetic mRNA Isolation Kit (New England Biolabs). After eluting in nuclease-free water, RNA concentration was estimated using a NanoDrop spectrophotometer. mRNA purity and content was validated using an Agilent 2100 Bioanalyzer (Fig. S19). Isolated mRNA was diluted to 10 ng/µL in nuclease-free water, and 10 ng of this material was analyzed using the Agilent RNA 6000 Pico assay kit. In separate reactions, 1 µg of collected mRNA was combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then directly combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 25 mM biotin-dPEG®$_4$-hydrazide (Sigma Aldrich) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 10% DMSO. Reactions were incubated at 37 ºC for 2 hours, ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified

using a NanoDrop spectrophotometer. Each sample was then immobilized in white streptavidin-coated 96-well plates and then subjected to EndoVLISA detection (EndoV 1:1000, 1º antibody 1:1000, 2º HRP 1:40,000, SuperSignal™ West Atto substrate) following the general procedure described earlier. A standard curve comprising 0-2 pmol of RNA I mixed with 1 µg *in vitro* transcribed mRNA was also processed and detected in parallel and used for estimating inosine content in treated mRNA samples (Fig. S20). Standard curve was plotted in GraphPad Prism 9, and linear regression was used to correlate sample concentration.

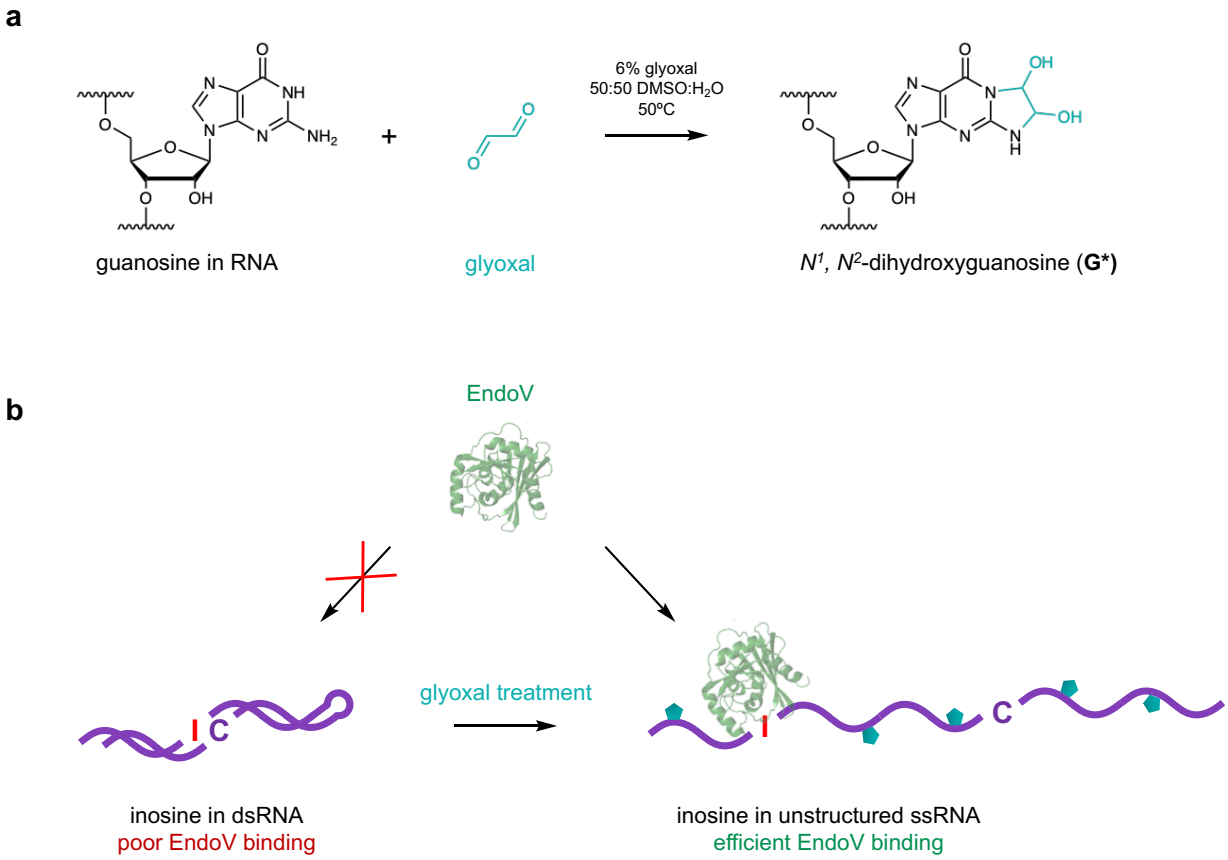
RNA-seq and Alu Indexing

In triplicate, 50 ng of isolated mRNA from each 293T transfection group was used to prepare sequencing libraries with the SMARTer® Stranded Total RNA Sample Prep Kit - Low Input Mammalian 24 reactions, (Takara Bio); standard 8-bp i5 and i7 Illumina index barcodes and adapters were added to each library. All libraries were then sequenced using a NextSeq 500/550 High Output Kit v2.5 300 Cycles (Illumina) to produce paired end 150-bp reads (approximately 15M reads per sample). Raw FASTQ data were first trimmed to remove the first 3 bp using Trimmomatic[1] with parameter HEADCROP:3. QC was performed on data to check read quality (PHRED33) using FastQC [https://www.bioinformatics.babraham.ac.uk/projects/fastqc/] and MultiQC[2] before and after data trimming. Reads were then aligned to the human reference genome hg38 via STAR 2.5.2[3] with additional parameter --outFilterMatchNminOverLread 0.95 as this parameter is optimal for the detection of A-to-I editing[4]. Resulting .bam files were sorted, and had duplicates marked and removed using Samtools 1.3[5] and PicardTools 2.0.1 [http://broadinstitute.github.io/picard/] respectively. The RNA editing indexer package by Roth et al.[2,6] was used as written on the tool GitHub page to process an entire directory of samples using default settings to calculate the Alu Editing Index (AE) for each sample. Raw and processed data files can be accessed via the the US National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under accession number GSE171545.

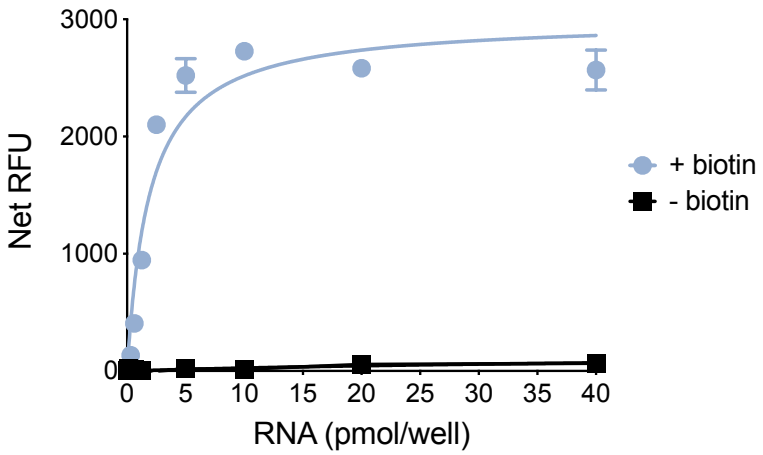
1) Bolger, A. M.; Lohse, M.; Usadel, B., Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **2014,** *30* (15), 2114-2120.
2) Ewels, P, Magnusson, M, and Kaller, M. MultiQC: Summarize analysis results for multiple tools and samples in a single report. Bioinformatics (2016). doi: 10.1093/bioinformatics/btw354.
3) Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635.
4) Roth, S.H., Levanon, E.Y. & Eisenberg, E. Genome-wide quantification of ADAR adenosine-to-inosine RNA editing activity. *Nat Methods* **16,** 1131–1138 (2019). https://doi.org/10.1038/s41592-019-0610-9.
5) Heng Li, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, Richard Durbin, 1000 Genome Project Data Processing Subgroup, The Sequence Alignment/Map format and SAMtools, *Bioinformatics*, Volume 25, Issue 16, 15 August 2009, Pages 2078–2079, https://doi.org/10.1093/bioinformatics/btp352.
6) https://github.com/a2iEditing/RNAEditingIndexer

Human Tissue mRNA and Processing

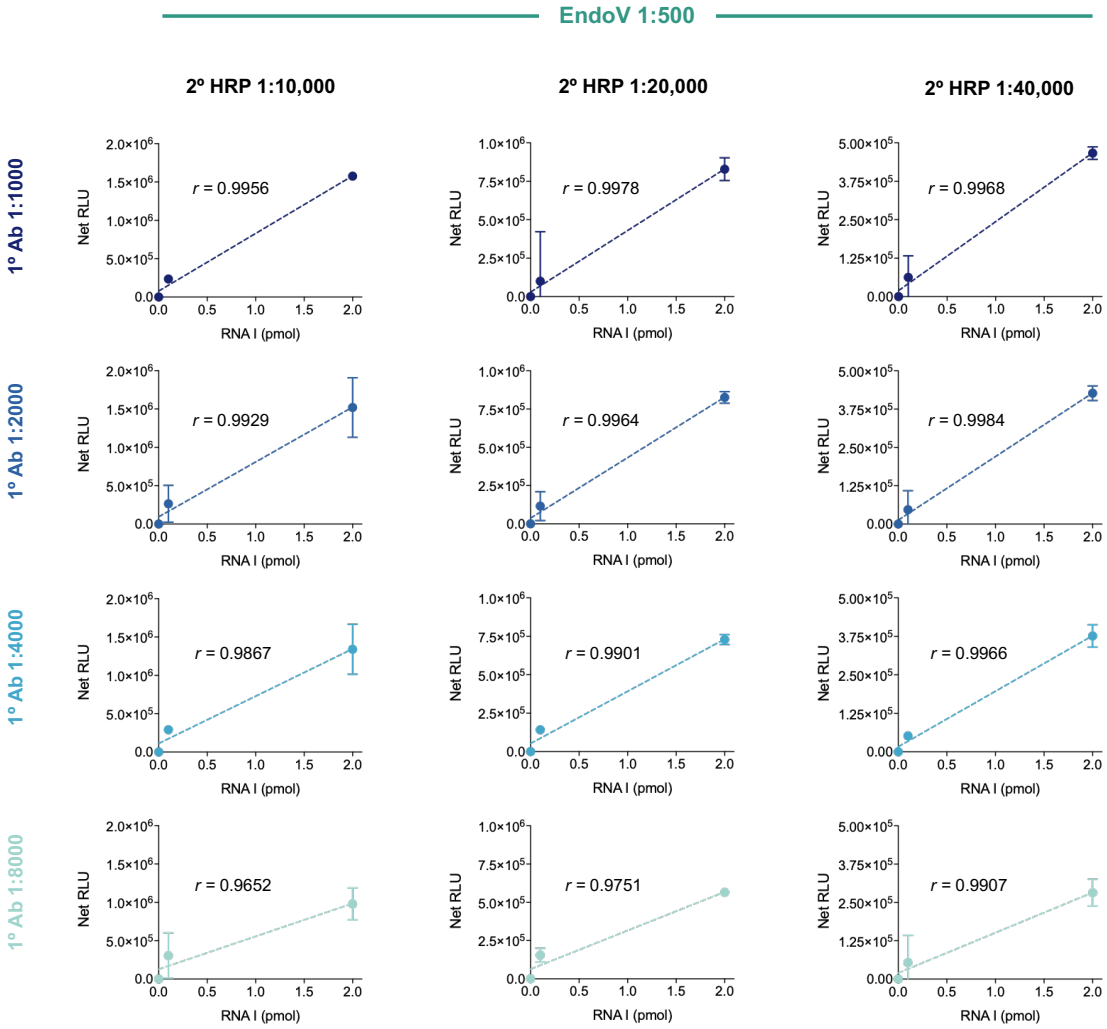
Purified mRNA from human aorta, brain, breast (mammary gland), kidney, and skeletal muscle were obtained from Takara Bio (Clontech). In triplicate reactions, In separate reactions, 500 ng of collected mRNA was combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then directly combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 25 mM biotin-dPEG®$_4$-hydrazide (Sigma Aldrich) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 10% DMSO. Reactions were incubated at 37 ºC for 2 hours, ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified using a NanoDrop spectrophotometer. Each sample was then immobilized in white streptavidin-coated 96-well plates and then subjected to EndoVLISA detection (EndoV 1:1000, 1º antibody 1:1000, 2º antibody-HRP 1:40,000, SuperSignal™ West Atto substrate) following the general procedure described earlier. A standard curve comprising 0-2 pmol of RNA I mixed with 500 ng *in vitro* transcribed mRNA was also processed and detected in parallel and used for estimating inosine content in mRNA samples (Fig. S22). Standard curve was plotted in GraphPad Prism 9, and linear regression was used to correlate sample concentration. For EndoVLISA detection in cancer, normal total RNA from breast (mammary gland), kidney, lung, and liver was purchased from Takara Bio (Clontech). Tumor total RNA from breast and kidney cancer biopsies were also purchased from Takara, while liver and lung total RNA was obtained from BioChain. 250 µg of each sample was subjected to two rounds of Oligo dT_{25} purification with the Magnetic mRNA Isolation Kit (New England Biolabs). After eluting in nuclease-free water, RNA concentration was estimated using a NanoDrop spectrophotometer. In separate reactions, 500 ng of collected mRNA was combined with 62.5 µM $NaIO_4$ (Thermo Fisher Scientific) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and incubated in the dark for 90 minutes at 25 ºC. RNA was then purified using the Monarch® RNA Cleanup Kit (New England Biolabs) and eluted in 35 µl nuclease-free water. This was then directly combined with 50 µl DMSO and 14.5 µL of a 40% glyoxal solution (Sigma Aldrich) and incubated for 2 hours at 50 ºC. Reactions were ethanol precipitated and reconstituted in 30 µL of nuclease-free water. Then was then directly combined with 25 mM biotin-dPEG®$_4$-hydrazide (Sigma Aldrich) in a total volume of 40 µL of 10 mM sodium acetate buffer, pH 5.6 and 10% DMSO. Reactions were incubated at 37 ºC for 2 hours, ethanol precipitated, reconstituted in 30 µL of nuclease-free water, and then quantified using a NanoDrop spectrophotometer. Each sample was then immobilized in white streptavidin-coated 96-well plates and then subjected to EndoVLISA detection (EndoV 1:1000, 1º antibody 1:1000, 2º HRP 1:40,000, SuperSignal™ West Atto substrate) following the general procedure described earlier.



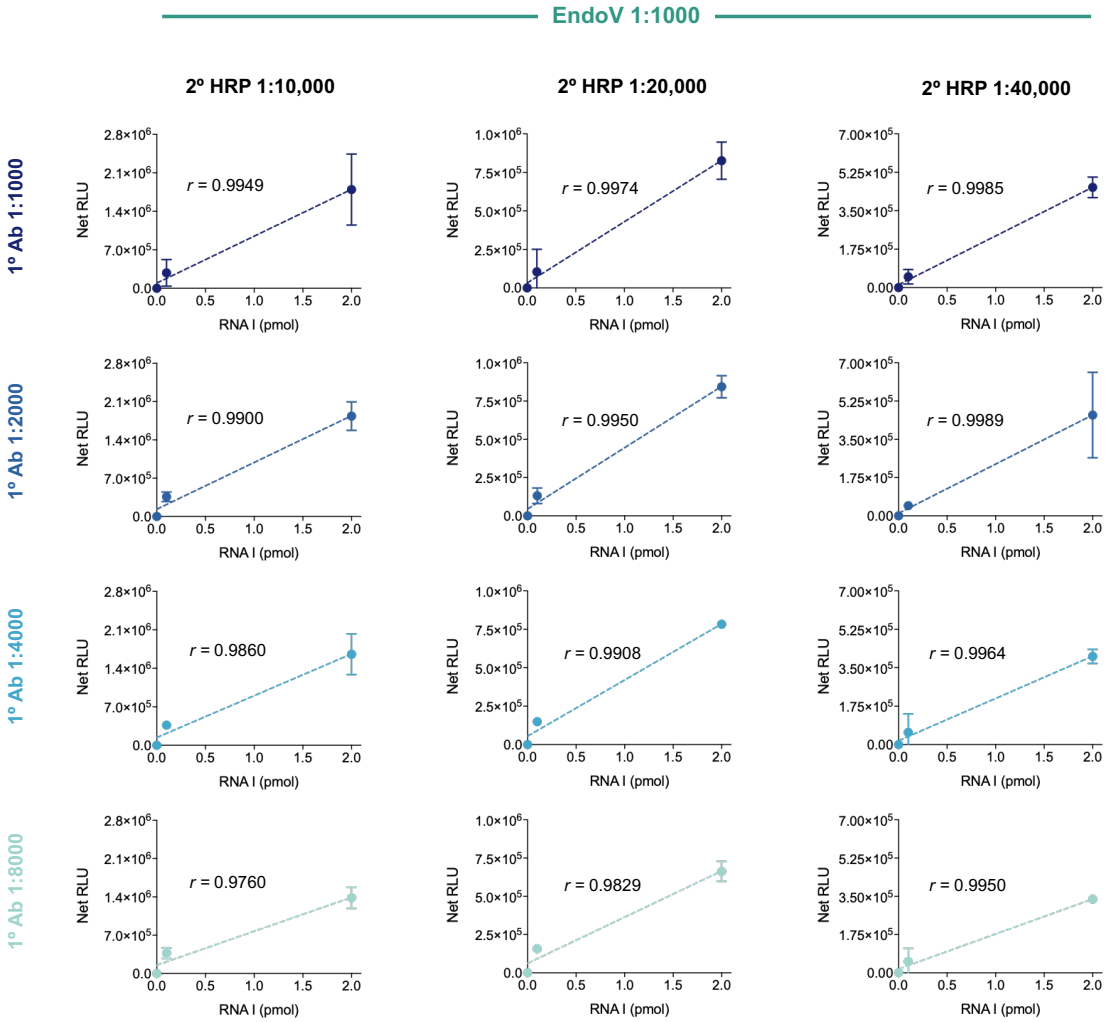
Supplementary Figure 1. Glyoxal denaturation eliminates secondary structure in RNA and enhances EndoV binding. a) Schematic and reaction conditions for covalent glyoxal addition onto the Watson–Crick–Franklin face of guanosine residues, forming a N^1,N^2-dihydroxyguanosine adduct. b) EndoV binds poorly to inosine residing in highly structured dsRNA. Glyoxal potently disrupts RNA secondary structure and does not react with inosine, enabling efficient EndoV binding.



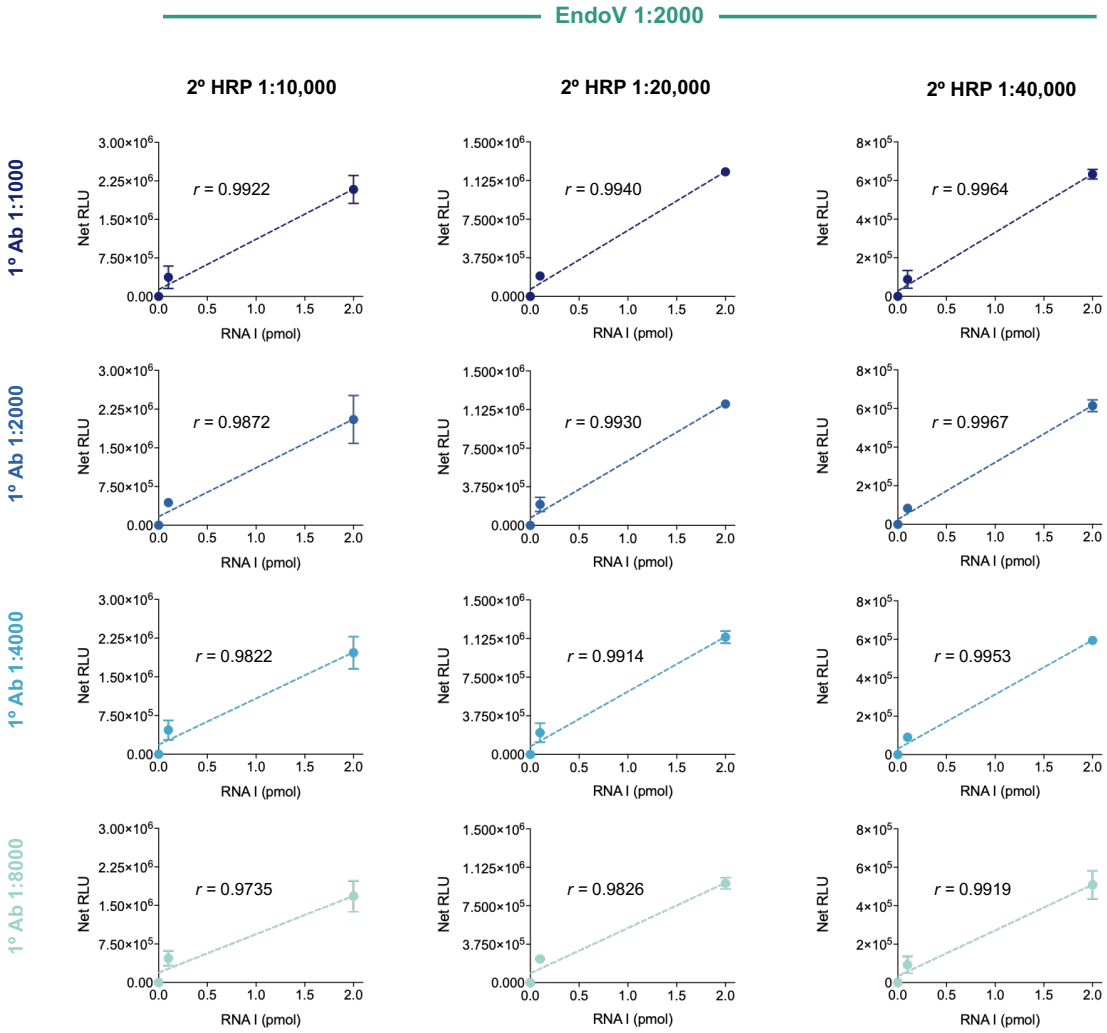
Supplementary Figure 2. Estimated RNA immobilization capacity onto streptavidin-coated plates. A 24 nt RNA I strand was Cy5 labeled, glyoxal denatured, and treated with and without biotin hydrazide labeling. Increasing amounts (0 – 40 pmol) of strand were loaded to each well of a 96-well streptavidin coated plate and incubated at room temperature for 1 hour with gentle shaking. Each well was then washed three times and fluorescence was measured using a BioTek Cytation 5 plate reader. Values represent net relative fluorescence units (RFU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA). Values represent mean with standard deviation (n = 3).



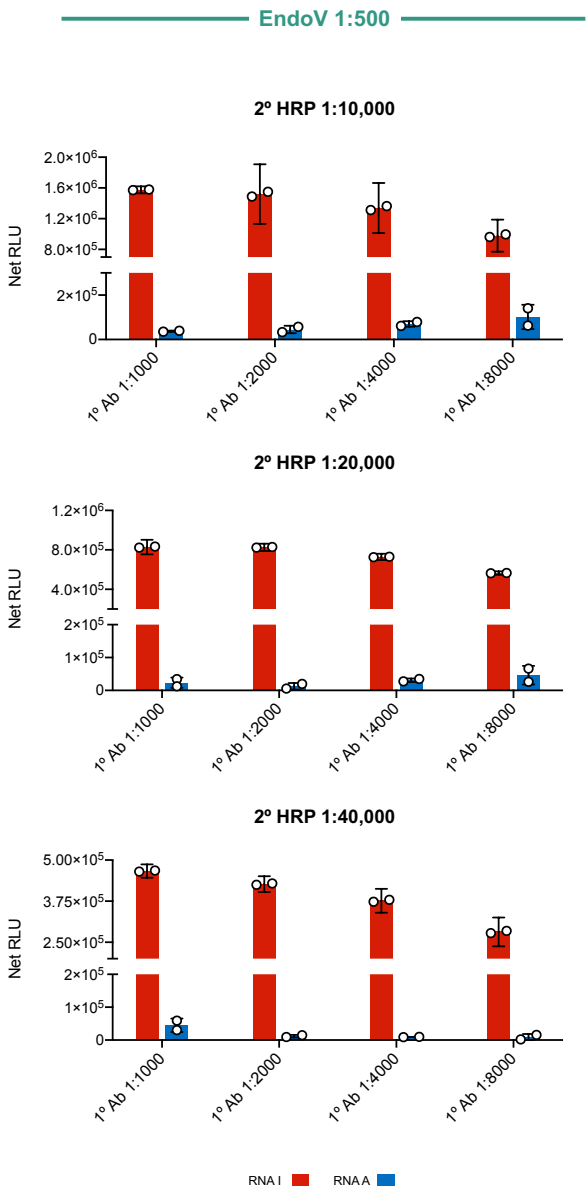
Supplementary Figure 3. Conditional screen to optimize EndoVLISA linearity using EndoV-MBP at 1:500. In duplicate, different amounts of biotinylated, glyoxal-denatured RNA I strand (0, 0.1 and 2 pmol) were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:500 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 2) and error bars denote 95% confidence intervals. Dashed line indicates simple linear regression estimation, and pearson correlation coefficients (*r*) were calculated in GraphPad Prism.



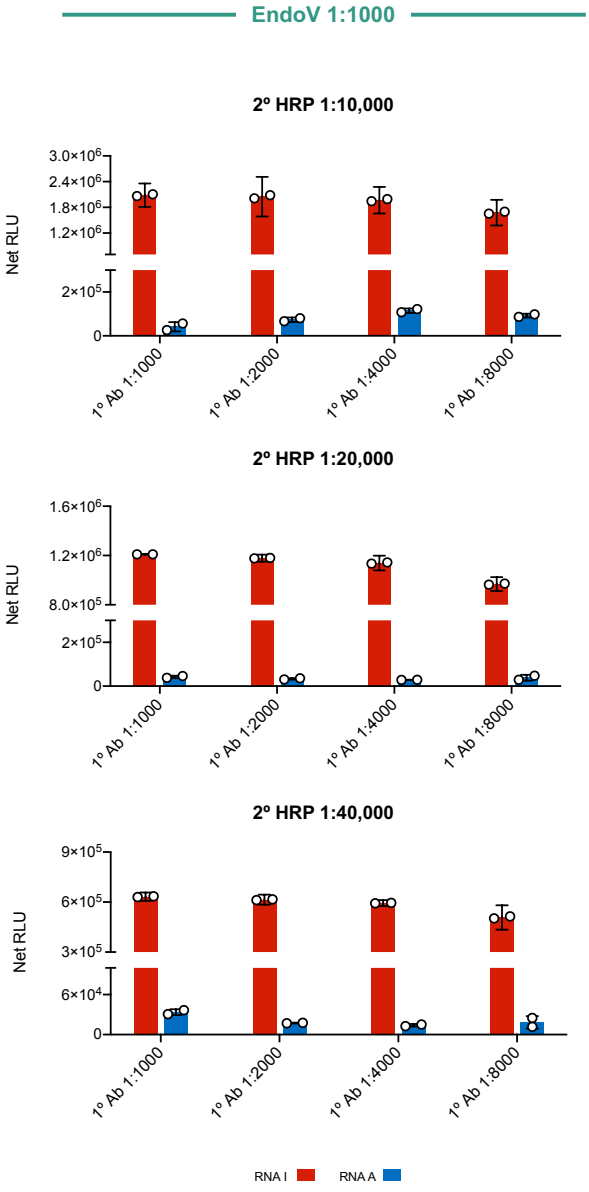
Supplementary Figure 4. Conditional screen to optimize EndoVLISA linearity using EndoV-MBP at 1:1000. In duplicate, different amounts of biotinylated, glyoxal-denatured RNA I strand (0, 0.1 and 2 pmol) were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:1000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 2) and error bars denote 95% confidence intervals. Dashed line indicates simple linear regression estimation, and pearson correlation coefficients (*r*) were calculated in GraphPad Prism.



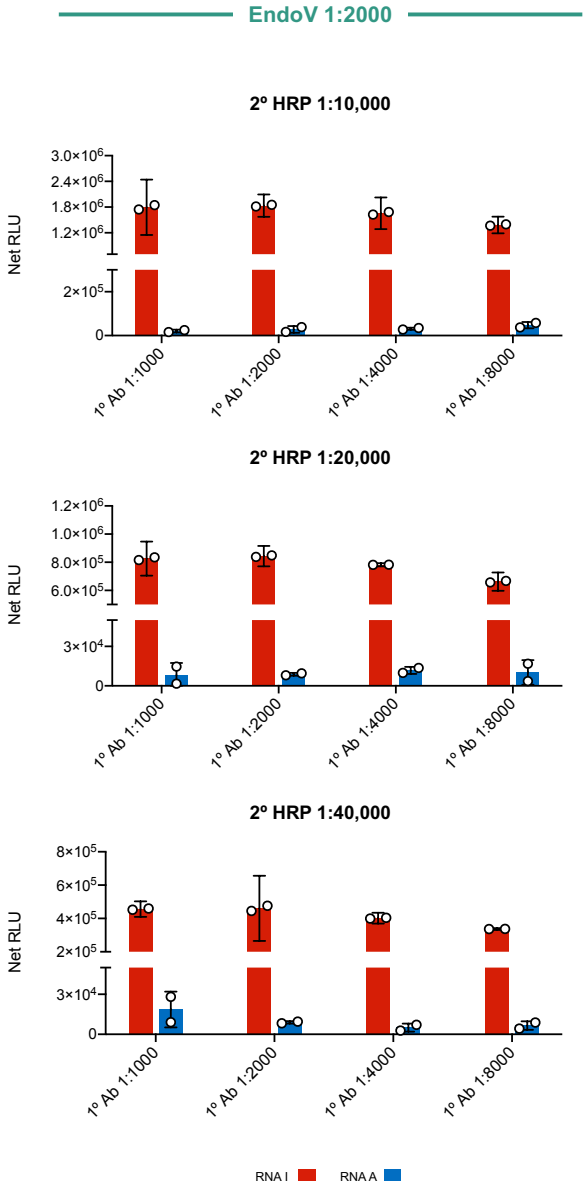
Supplementary Figure 5. Conditional screen to optimize EndoVLISA linearity using EndoV-MBP at 1:2000. In duplicate, different amounts of biotinylated, glyoxal-denatured RNA I strand (0, 0.1 and 2 pmol) were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:2000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 2) and error bars denote 95% confidence intervals. Dashed line indicates simple linear regression estimation, and pearson correlation coefficients (*r*) were calculated in GraphPad Prism.



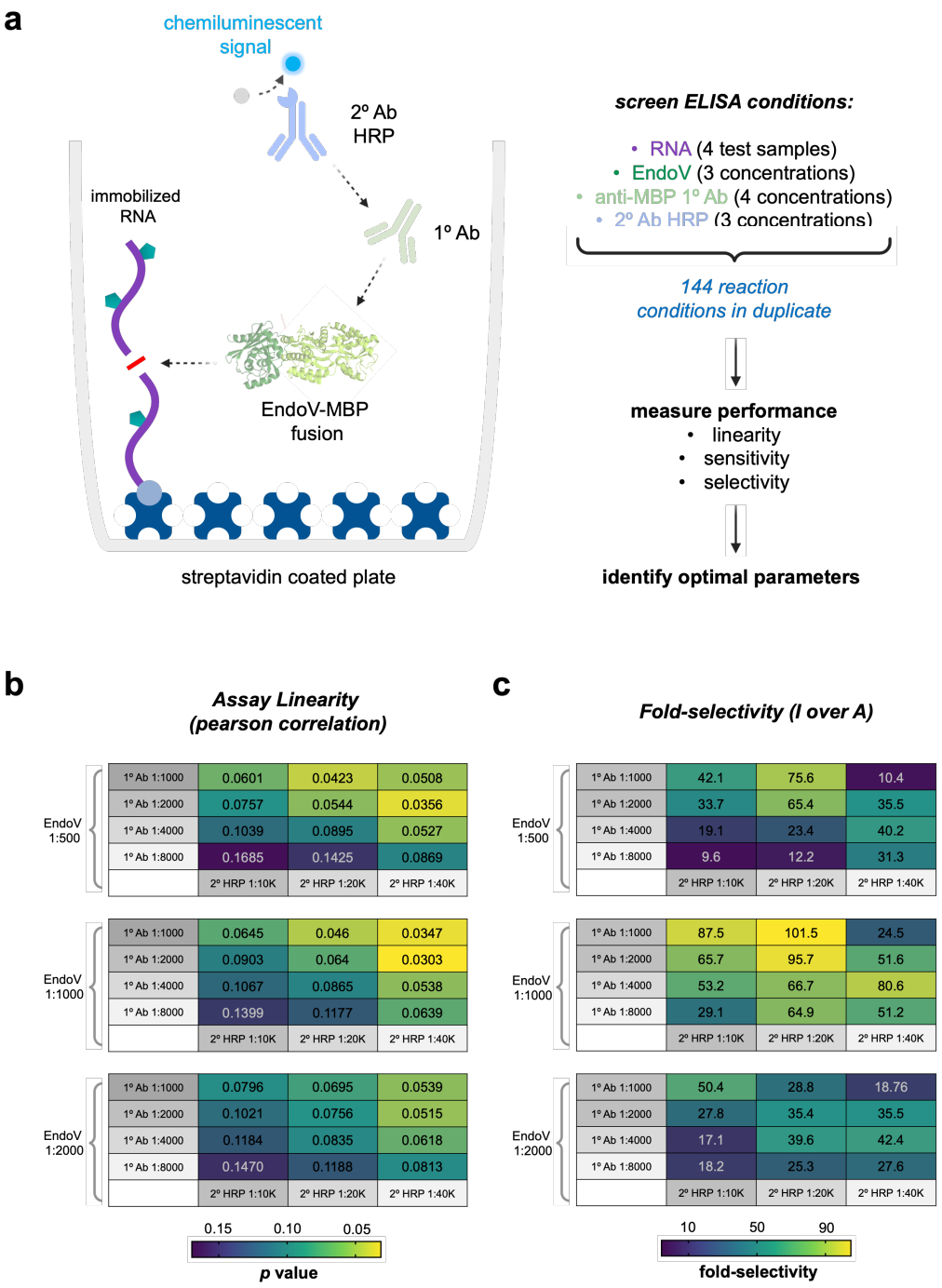
Supplementary Figure 6. Conditional screen to optimize EndoVLISA selectivity using EndoV-MBP at 1:500. In duplicate, 2 pmol of biotinylated, glyoxal-denatured RNA A (blue) or RNA I (red) strand were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:500 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 2) and error bars denote 95% confidence intervals.



Supplementary Figure 7. Conditional screen to optimize EndoVLISA selectivity using EndoV-MBP at 1:1000. In duplicate, 2 pmol of biotinylated, glyoxal-denatured RNA A (blue) or RNA I (red) strand were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:1000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Datapoints represent mean (n = 2) and error bars denote 95% confidence intervals.



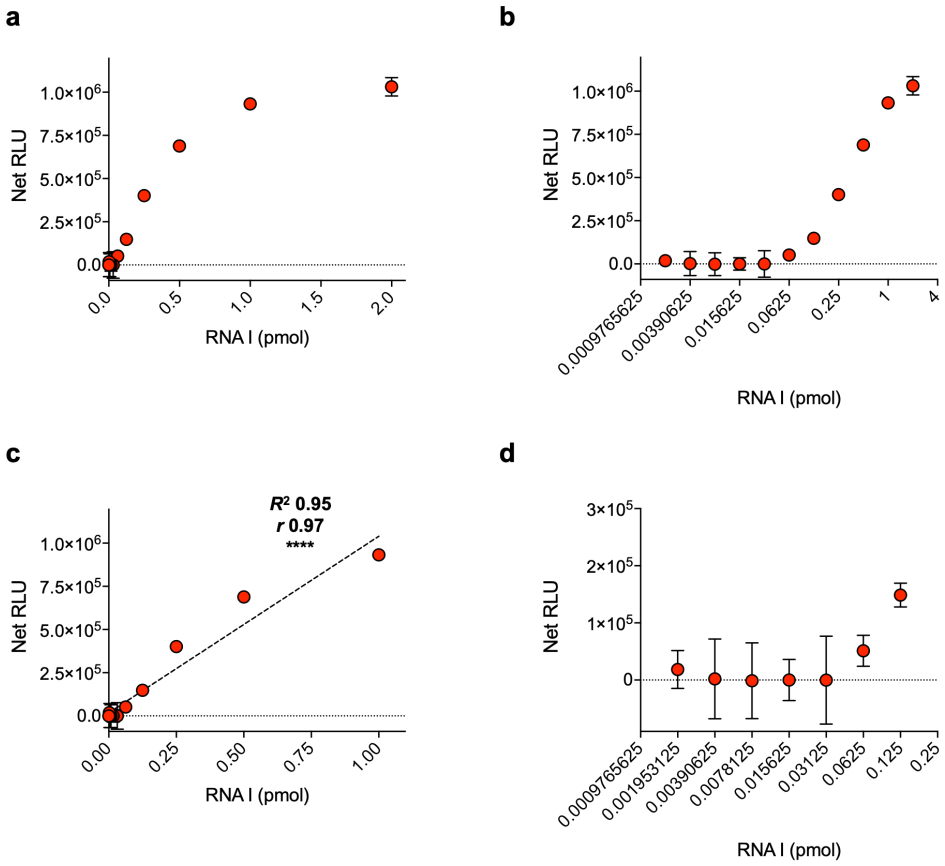
Supplementary Figure 8. Conditional screen to optimize EndoVLISA selectivity using EndoV-MBP at 1:2000. In duplicate, 2 pmol of biotinylated, glyoxal-denatured RNA A (blue) or RNA I (red) strand were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:2000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Different combinations of MBP-targeting 1º antibody (1:1000 – 1:8000) and 2º HRP-conjugated antibody (1:10,000 – 1:40,000) were then used to probe the plate. SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 2) and error bars denote 95% confidence intervals.



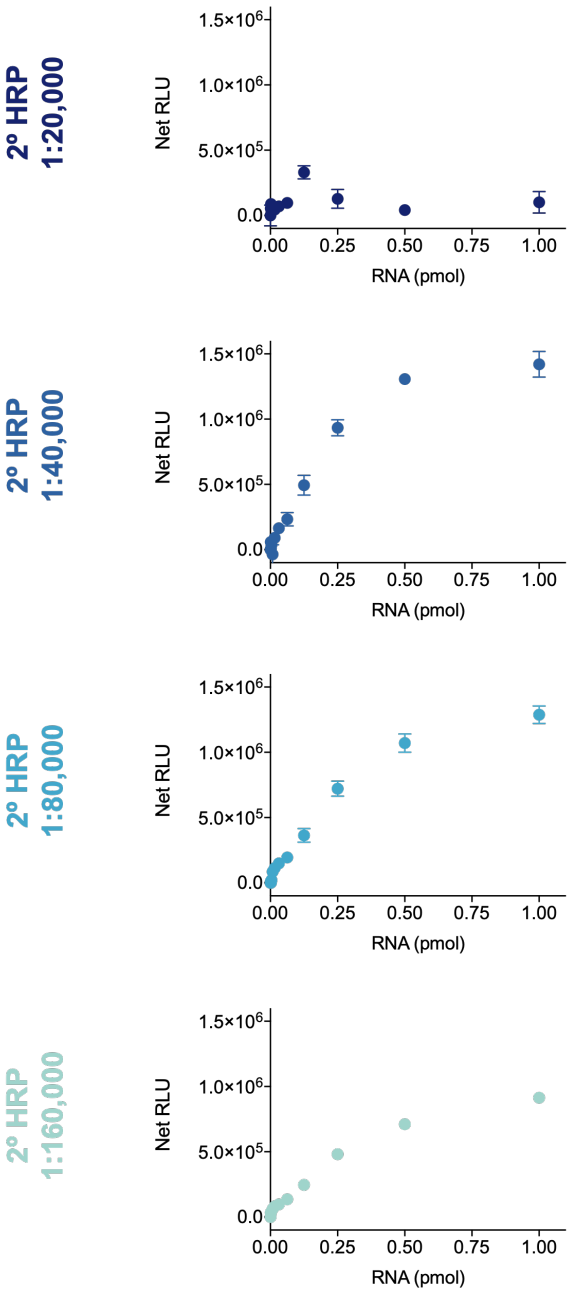
Supplementary Figure 9. Summary of optimizing EndoVLISA performance. a) Schematic of EndoVLISA optimization and main functional components – (1) RNA, (2) eEndoV-MBP fusion protein, (3) anti-MBP primary antibody, (4) goat anti-mouse HRP-conjugated secondary antibody, and (5) chemiluminescent substrate. b,c) Results of optimization screen. In duplicate, various combinations of different components were tested with RNA I and A, and both linearity and selectivity were measured. b) Pearson *r* p-value was computed in GraphPad Prism using on-target RNA I signals from 0, 0.1 and 2 pmol RNA signals. c) Fold-selectivity was calculated as the signal of RNA I divided by RNA A (2 pmol/well).

EndoV	1º Ab	2º HRP	Linearity (*p* value)	Fold-selectivity
1:1000	1:1000	1:20K	0.046	101.467
1:1000	1:4000	1:40K	0.0538	80.568
1:500	1:1000	1:20K	0.0423	75.596
1:1000	1:2000	1:40K	0.0303	51.554
1:500	1:2000	1:40K	0.0356	35.459
1:1000	1:1000	1:40K	0.0347	24.508

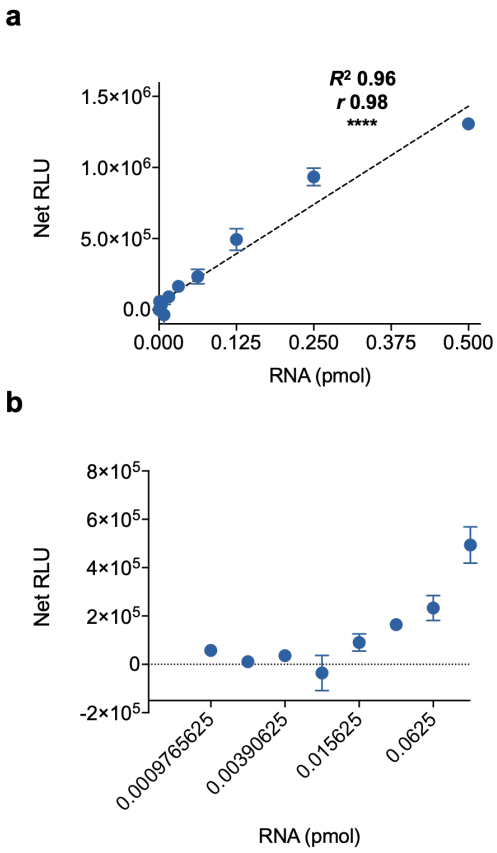
Table S1. Summary of top-performing EndoVLISA component combinations.



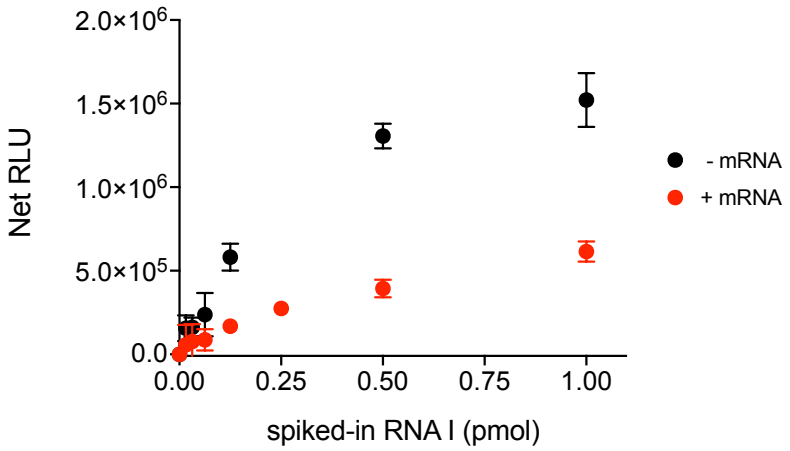
Supplementary Figure 10. Linearity and sensitivity in detecting inosine. Decreasing amounts of biotinylated, glyoxal-denatured RNA I strand were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:1000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Wells were then probed with MBP-targeting 1º antibody (1:1000) and 2º HRP-conjugated antibody (1:20,000), and then SuperSignal™ ELISA Pico chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Full RNA I standard curve in a) linear and b) log_2 scale. c) Partial standard curve (RNA I <1 pmol) in linear scale. Linear regression (black dashed line), R2 and pearson (*r*) correlation (**** denotes $p < 0.0001$) were computed in Prism. d) Partial standard curve (RNA I < 0.125 pmol) in log_2 scale to estimate limit of detection above blank. All values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting blank wells (no RNA). Data points represent mean (n = 3) and error bars denote 95% confidence intervals.



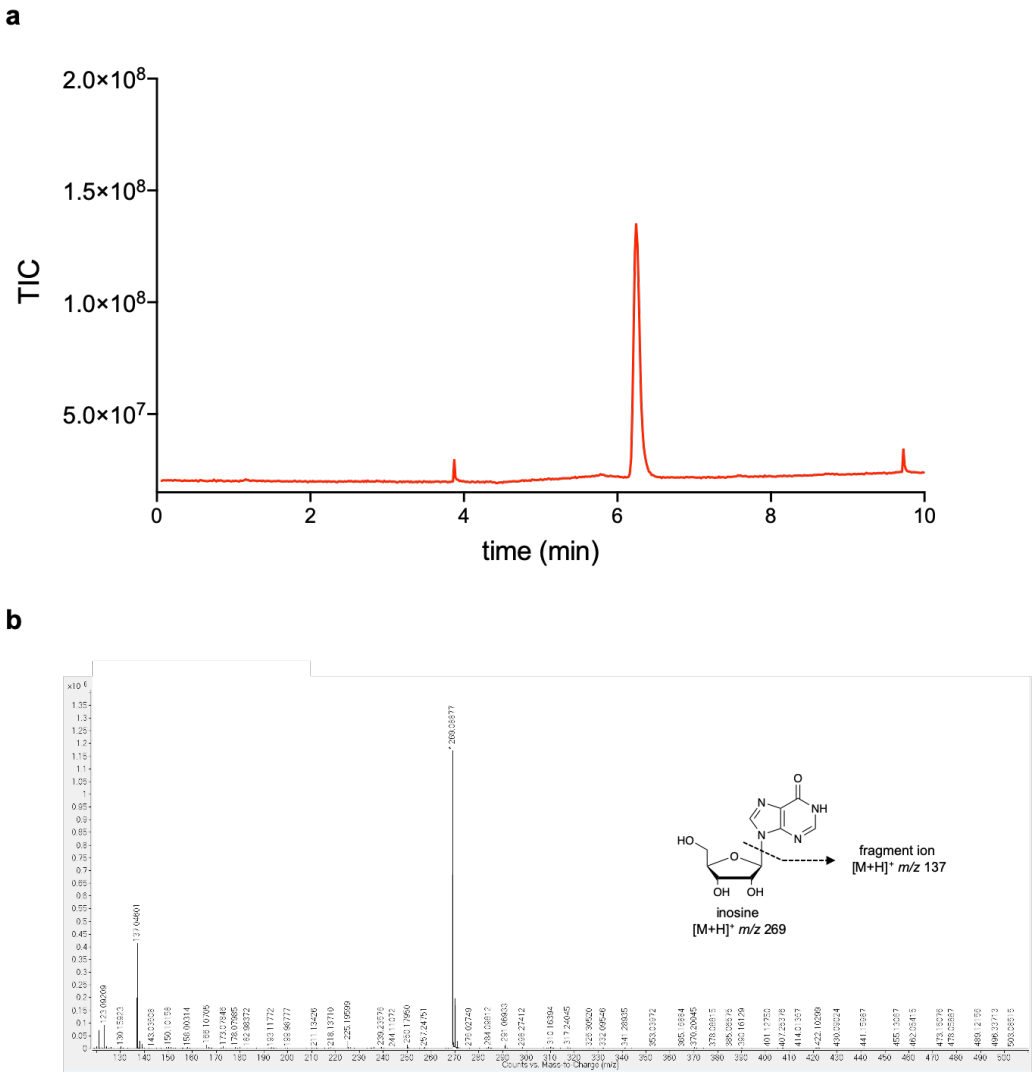
Supplementary Figure 11. Testing 2º antibody-HRP concentrations with a high-sensitivity SuperSignal™ West Atto chemiluminescent substrate. Decreasing amounts of biotinylated, glyoxal-denatured RNA I strand were immobilized in white streptavidin-coated 96-well plates and then incubated with a solution of EndoV-MBP fusion protein at 1:1000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Wells were then probed with MBP-targeting 1º antibody (1:1000) and different amounts of 2º HRP-conjugated antibody (1:20,000-160,000), and then SuperSignal™ West Atto chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Datapoints represent mean (n = 3) and error bars denote 95% confidence intervals.



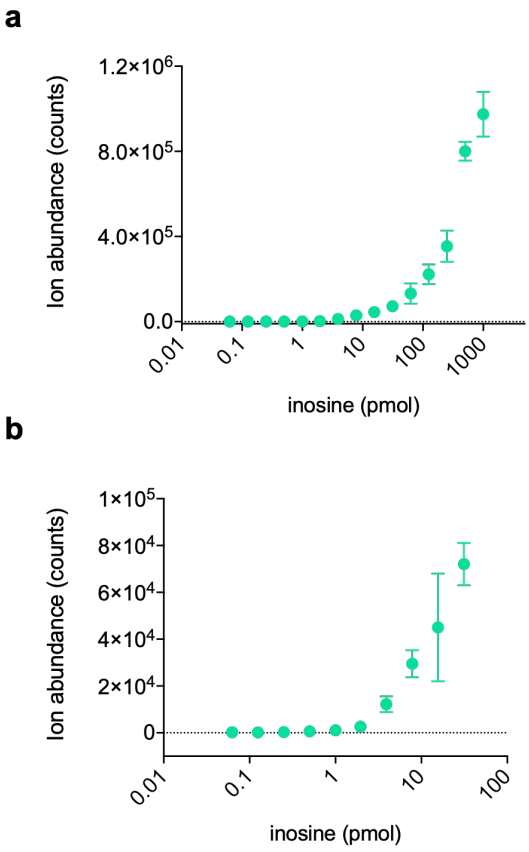
Supplementary Figure 12. Linearity and sensitivity of EndoVLISA with 1:40,000 2º antibody-HRP and high-sensitivity SuperSignal™ West Atto chemiluminescent substrate. a) Full RNA I standard curve in linear scale. Linear regression (black dashed line), R^2 and pearson (r) correlation (**** denotes $p < 0.0001$) were computed in Prism. b) Partial standard curve (RNA I < 0.125 pmol) in $\log_2$ scale to estimate limit of detection above blank. All values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting blank wells (no RNA). Data points represent mean (n = 3) and error bars denote 95% confidence intervals.



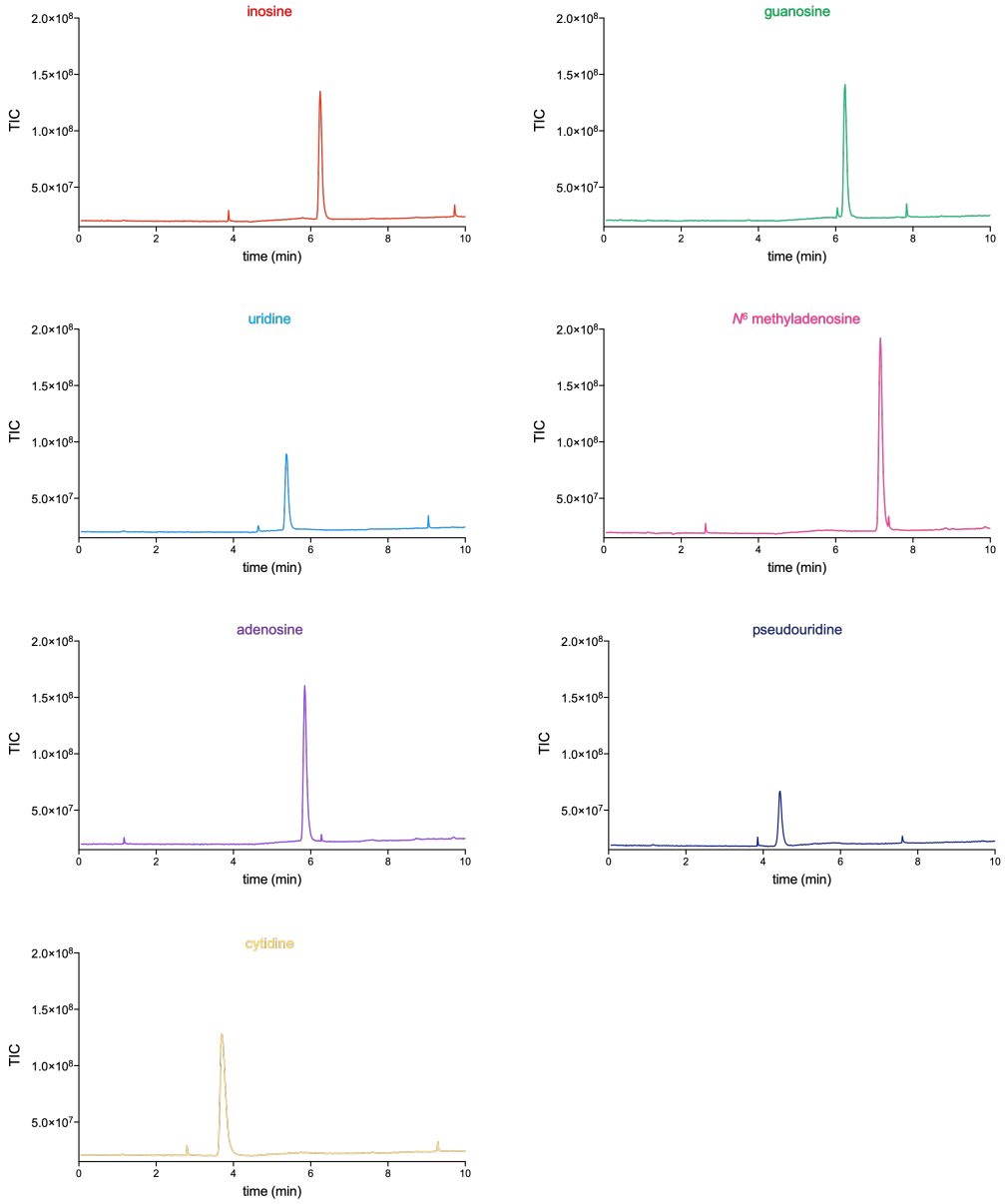
Supplementary Figure 13. EndoVLISA performance in the presence of mRNA. Decreasing amounts RNA I strand was mixed with 1 µg *in vitro* transcribed mRNA and then glyoxal denatured and biotinylated. Samples were then immobilized in white streptavidin-coated 96-well plates and incubated with a solution of EndoV-MBP fusion protein at 1:1000 in 1X buffer (20 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 0.05% Tw 20, pH 7.5). Wells were then probed with MBP-targeting 1º antibody (1:1000) and 2º HRP-conjugated antibody (1:40,000), and then SuperSignal™ West Atto chemiluminescent substrate (Thermo Fisher Scientific) was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent mean (n = 3) and error bars denote 95% confidence intervals.



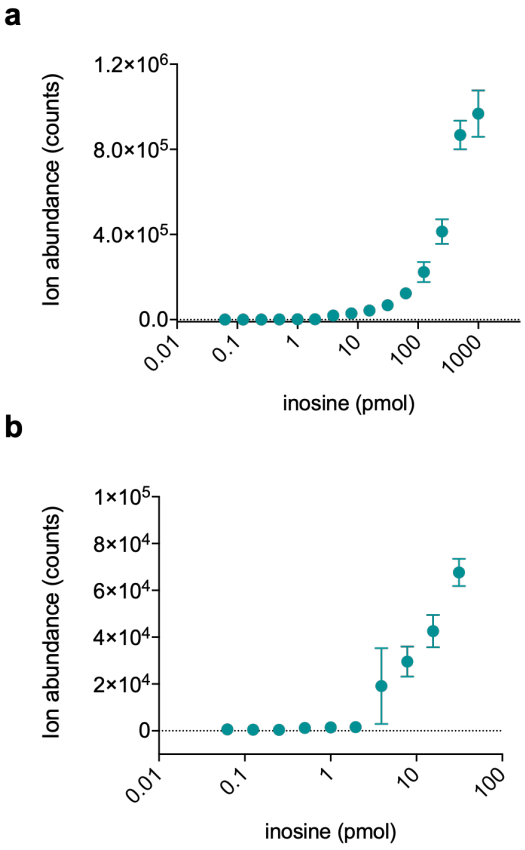
Supplementary Figure 14. Inosine detection using LC/MS. a) Chromatography trace of inosine. 1 nmol inosine in water was separated using an Atlantis T3 column and total ion count (TIC) monitored over time. b) Mass spectrum of separated inosine (6.2 – 6.3 min retention time), with both the parent and fragment ion detected. Y axis denotes TIC values.



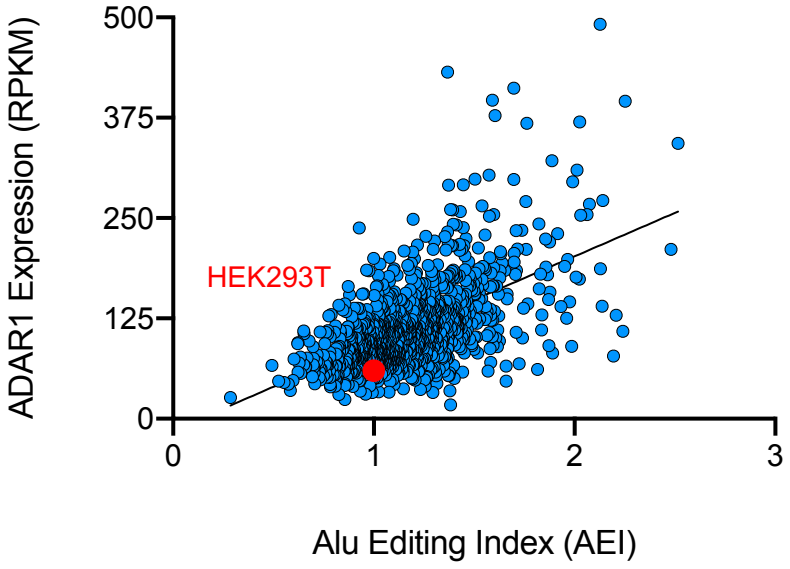
Supplementary Figure 15. Inosine detection sensitivity using LC/MS. Decreasing amounts of inosine ribonucleoside was injected and analyzed by LC/MS. a) Full and b) partial (inosine < 100 pmol) standard curve in $\log_{10}$ scale. All values represent ion abundance counts from raw spectra. Data points represent mean (n = 3) and error bars denote 95% confidence intervals. Mass spectrum of inosine (6.2 – 6.3 min retention time) was used to quantify abundance of the parent ion (*m/z* 269).



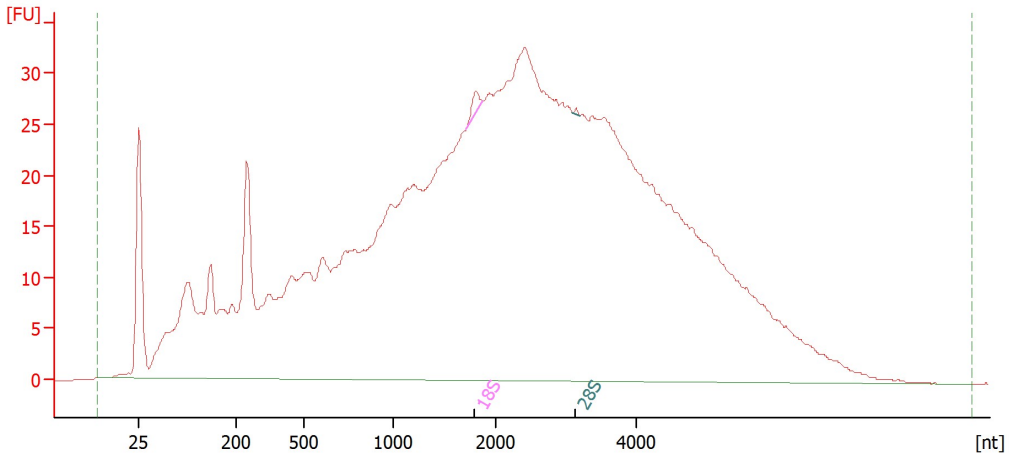
Supplementary Figure 16. Chromatographic separation of ribonucleosides using LC/MS. 1 nmol of each ribonucleoside in water was separated using an Atlantis T3 column and total ion count (TIC) monitored over time.



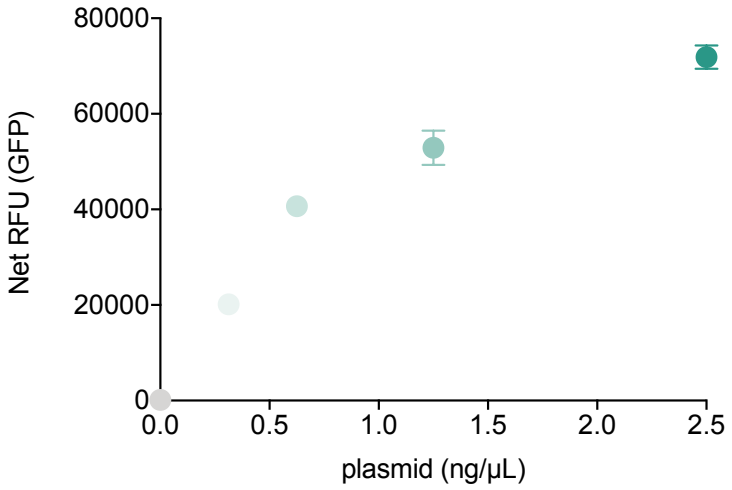
Supplementary Figure 17. Inosine detection sensitivity in complex mixtures using LC/MS. Decreasing amounts of inosine ribonucleoside was spiked into 1 µg of an equimolar mixture of A, U, C, G, m^6A and PsU and then injected and analyzed by LC/MS. a) Full and b) partial (inosine < 100 pmol) standard curve in $\log_{10}$ scale. All values represent ion abundance counts from raw spectra. Data points represent mean (n = 3) and error bars denote 95% confidence intervals. Mass spectrum of inosine (6.2 – 6.3 min retention time) was used to quantify abundance of the parent ion (*m/z* 269).



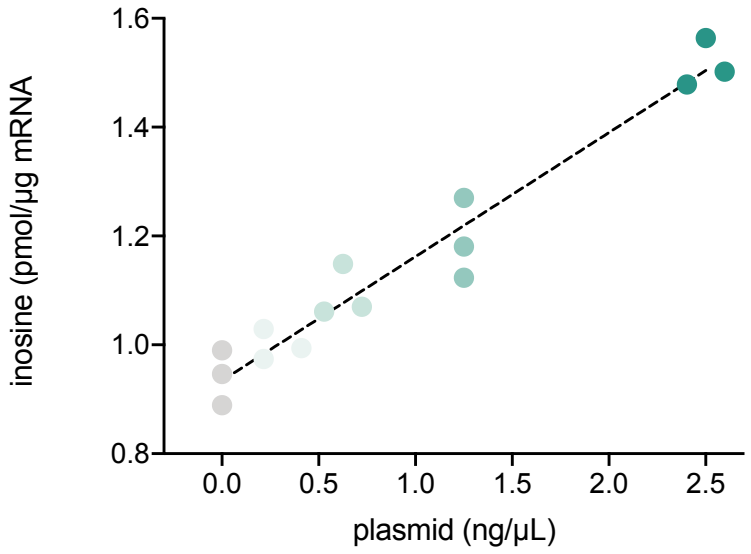
Supplementary Figure 18. Overall editing activity in cell lines. Global A-to-I rates in 1147 different cell lines were estimated from RNA-seq datasets using the Alu Editing Index (AEI) and correlated with ADAR1 expression levels (reads per kilobase of transcript, per million mapped reads, RPKM). Reproduced in GraphPad Prism using data in supplementary table 4 from ref 65. Red dot indicates position of HEK293T cell line.



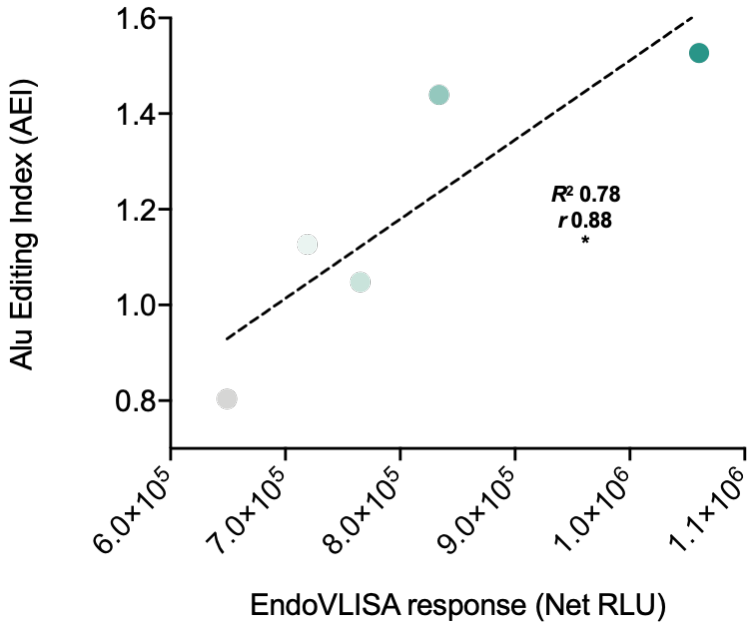
Supplementary Figure 19. Bioanalyzer validation of isolated mRNA purity. Representative electropherogram of isolated polyA+ RNA following two rounds of oligo(dT)$_{25}$, illustrating both high integrity of mRNA and minimal contamination from ribosomal RNA (rRNA) and transfer RNA (tRNA). A 10 ng/µL solution of isolated mRNA was prepared in nuclease free water, and 10 ng of this material was analyzed using the Agilent RNA 6000 pico assay kit.



Supplementary Figure 20. Increased ADAR1 expression in transfected HEK293T cells. Whole cell lysate was collected from HEK293T cells treated with increasing amounts of a GFP-ADAR1p110 plasmid. In triplicate, 20 µl of lysate was loaded into 384-well black plates and measured using a BioTek Cytation 5 plate reader using excitation at 488 nm and emission at 509 nm. Values represent mean (n = 3 wells) and error bars denote S.D. Net relative fluorescence units (RFU, arbitrary units) is calculated by subtracting blank values (lysis buffer).



Supplementary Figure 21. Quantifying inosine levels in HEK293Tcells overexpressing ADAR1. mRNA was isolated from HEK293T cells treated with increasing amounts of a GFP-ADAR1p110 plasmid. mRNA was then biotinylated, glyoxal-denatured, and then 1 µg was immobilized in each well of a white streptavidin-coated 96-well plate. Wells were then probed with a 1:1000 EndoV-MBP solution followed by MBP-targeting 1º antibody (1:1000) and 2º HRP-conjugated antibody (1:40,000). SuperSignal™ West Atto chemiluminescent substrate was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Inosine (pmol/µg mRNA) was calculated based off a standard curve containing known concentrations of RNA I control strand (0-2 pmol) mixed with 1 µg *in vitro* transcribed mRNA. Values represent net relative luminescent units (RLU, arbitrary units) calculated by subtracting appropriate blank wells (no RNA) from each set. Data points represent individual values from each well (n = 3 for each treatment group).



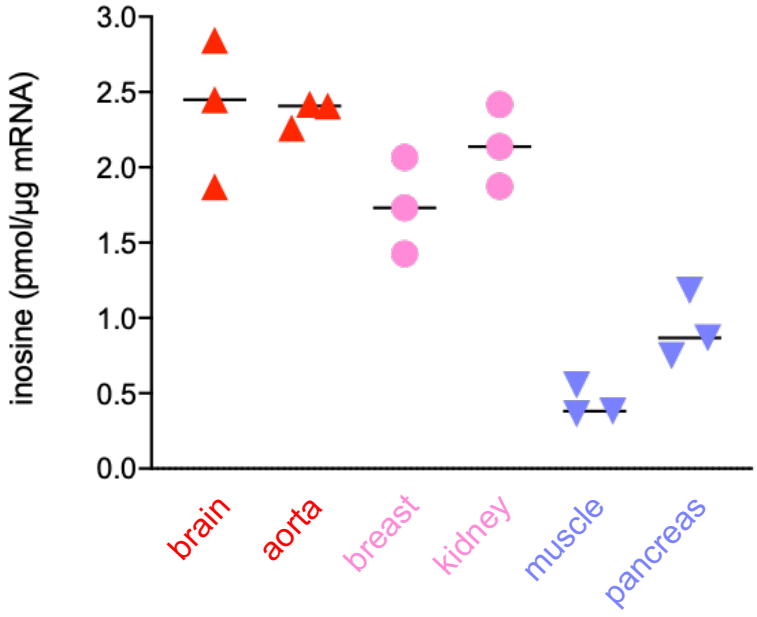
Supplementary Figure 22. Comparing RNA-seq (Alu editing index, AEI) and EndoVLISA in detecting increased RNA editing signatures. Average AEI and EndoVLISA signal (net relative luminescent units, Net RLU) were plotted for each transfection amount (0, 0.31, 0.62, 1.25, 2.5 ng/μL plasmid). Values represent means from triplicate experiments. Linear regression (black dashed line), R^2 and pearson (r) correlation (* denotes $p < 0.05$) were computed in Prism.

Component	Product (vendor and SKU)	Unit Price ($)	Price per sample ($)
library preparation kit	SMARTer® Stranded Total RNA Sample Prep Kit - Low Input Mammalian 24 reactions, (Takara Bio 634861)	$2895.00	$120.63
Sequencing flow cell	NextSeq 500/550 High Output Kit v2.5 300 Cycles (Illumina 20024908)	$4867.00	$304.19*
			Total $419.15

Table S2. Components needed for RNA-seq analysis of A-to-I editing. *Price per sample is variable depending on multiplexing in RNA-seq on the same flow cell (16 samples were used in this study).

Component	Product (vendor and SKU)	Unit Price ($)	Price per sample* ($)
Biotin label	Biotin-dPEG$_4$-hydrazide, 50 mg (Sigma Aldrich QBD10219)	$257.00	$0.05
Plate	Streptavidin 96-well white (5 pack, Thermo Fisher 15218)	$212.00	$0.44
EndoV	Recombinant EndoV-MBP (New England Biolabs, M0305S)	$76.00	$0.30
1º antibody	Anti-MBP Monoclonal Antibody (New England Biolabs, E8032S)	$181.00	$0.72
2º HRP	Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	$204.00	$0.08
Substrate	SuperSignal™ West Atto Ultimate Sensitivity Substrate (100 mL	$400.00	$0.40
			Total $2.00

Table S3. Components needed for EndoVLISA detection of A-to-I editing.*Calculated based on necessary amounts for processing 1 µg of mRNA and performing EndoVLISA detection in one-well of a 96-well plate.



Supplementary Figure 23. Quantifying inosine levels in mRNA isolated from human tissue. Purified mRNA from the indicated tissues was purchased from Takara Bio and then biotinylated and glyoxal-denatured. 500 ng was immobilized in each well of a white streptavidin-coated 96-well plate, and wells were then probed with a 1:1000 EndoV-MBP solution followed by MBP-targeting 1º antibody (1:1000) and 2º HRP-conjugated antibody (1:40,000). SuperSignal™ West Atto chemiluminescent substrate was then added to each well and allowed to incubate with shaking for 1 minute, followed by luminescent measurement using a BioTek Cytation 5 plate reader. Inosine (pmol/µg mRNA) was calculated based off a standard curve containing known concentrations of RNA I control strand (0-2 pmol) mixed with 500 ng *in vitro* transcribed mRNA. Data points represent individual values from each well (n = 3 for each sample).