Supporting Information

Chemical Profiling of A-to-I RNA Editing Using a Click-Compatible Phenylacrylamide

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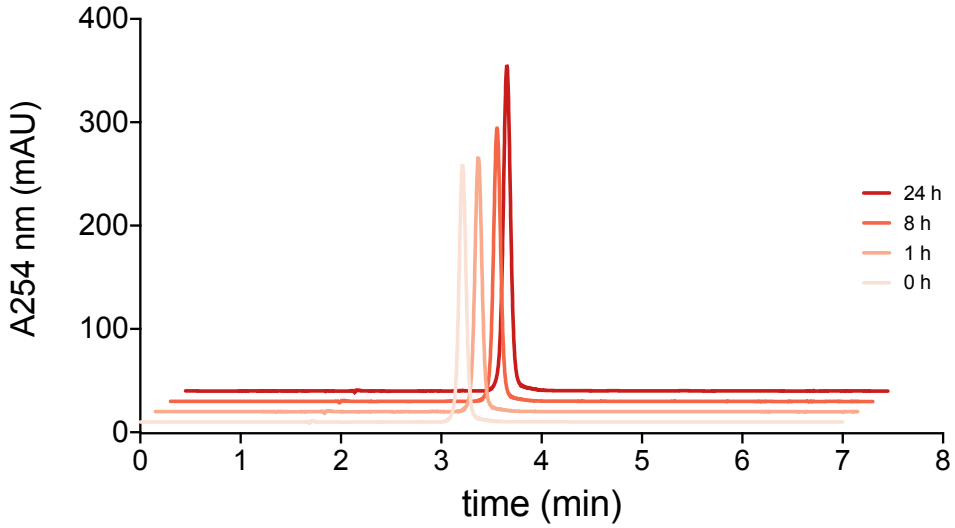
Inosine labeling with acrylamide derivatives and HPLC analysis

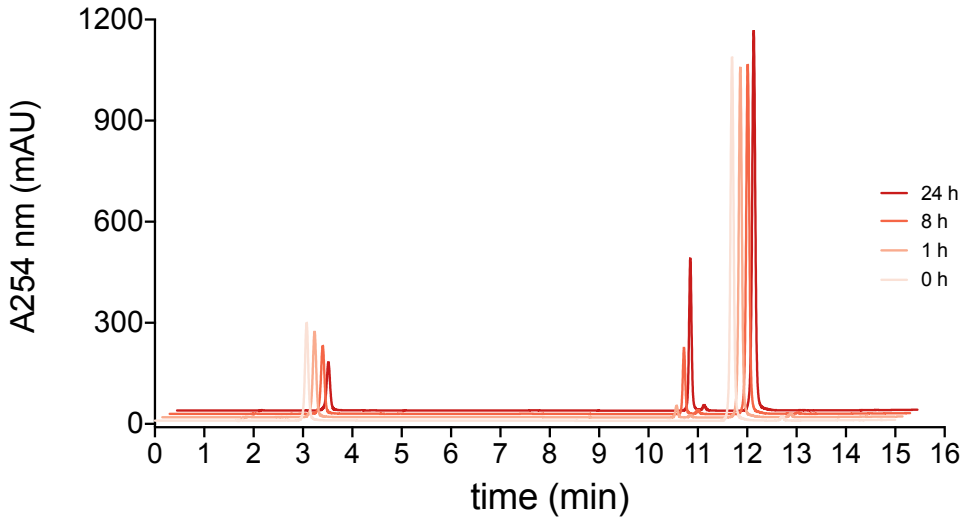
Acrylamide, *N*-phenylacrylamide, and *N*-hydroxyethylacrylamide were purchased from Sigma Aldrich Corporation (St. Louis, MO). mPEG-acrylamide (MW 1000 g/mol) was purchased from Creative PEGWorks (Chapel Hill, NC). Inosine ribonucleoside was purchased from Sigma Aldrich Corporation (St. Louis, MO). Labeling reaction mixtures were comprised of 50 mM inosine and 250 mM of each acrylamide derivative in 50:50 EtOH:1M triethylammonium acetate (TEAA) pH 8.6. Reactions were incubated at 70 °C for the time periods indicated. Reversed-phase HPLC analysis was performed on an Agilent 1260 Infinity II system using a 4 µm, 150 x 4.6 mm Phenomenex Synergi Fusion-RP 80A C18 column. Samples were prepared in a stationary phase solution of 5% acetonitrile in PBS. Acrylamide, mPEG-acrylamide, and *N*-hydroxyethylacrylamide reactions were analyzed using an isocratic mobile phase of 5:95 acetonitrile:water. *N*-phenylacrylamide reactions were analyzed using a linear mobile phase gradient from 5% to 45% acetonitrile in water over 15 minutes. All mobile phases contained 0.1% trifluoracetic acid. Percent conversion in each reaction was defined as the inosine peak area relative to unreacted inosine at the same time point without any reagent.

Figure S1 (Below). Representative HPLC traces of inosine reactivity with different acrylamide derivatives. Reaction times (0-24 h) indicated by included legends.

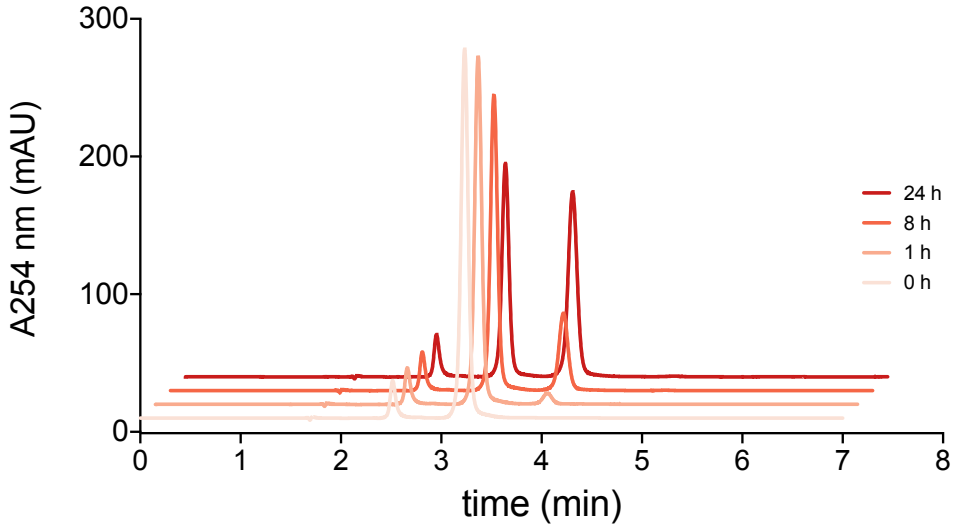
a) Inosine alone



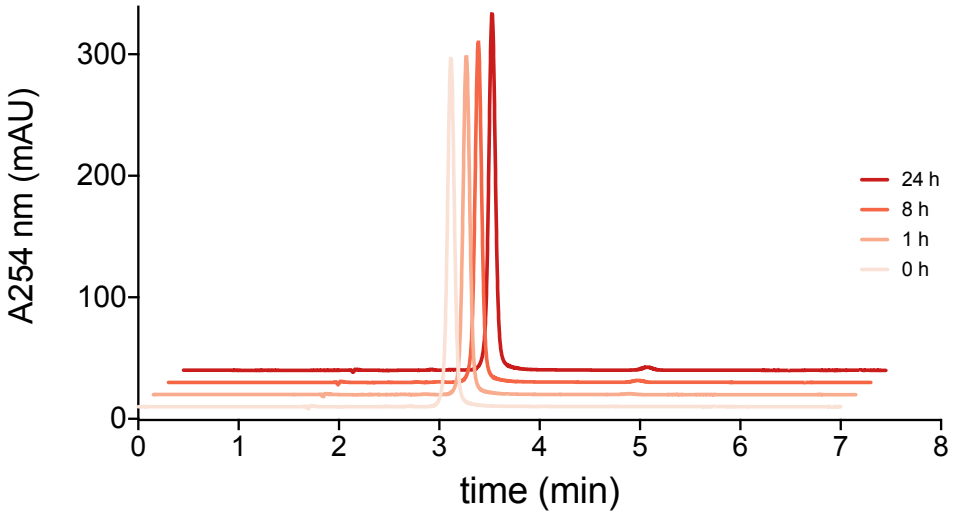
b) Inosine + *N*-phenylacrylamide



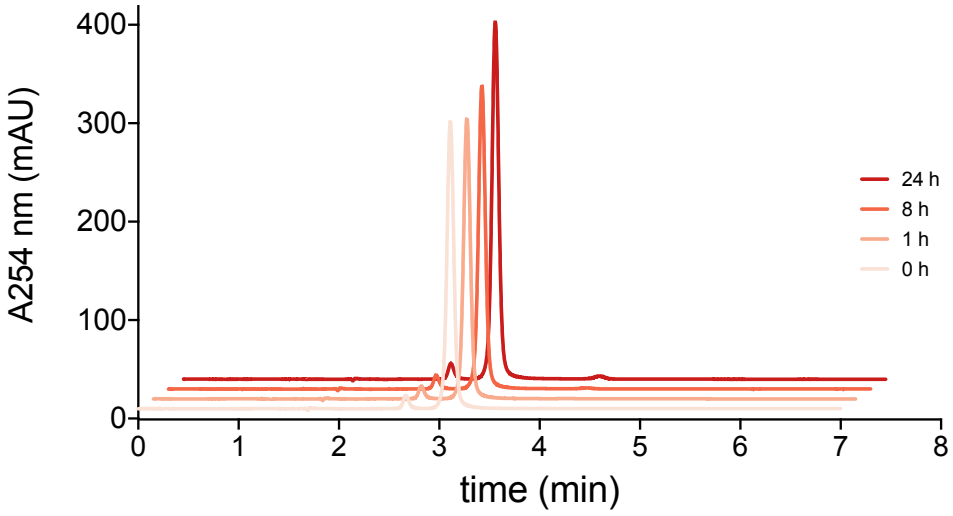
c) Inosine + acrylamide



d) Inosine + mPEG-acrylamide



e) Inosine + *N*-hydroxyethylacrylamide



Synthesis of *N*-(4-ethynylphenyl)acrylamide

Unless otherwise noted, all starting materials were obtained from Sigma Aldrich Corporation (St. Louis, MO) and were used without further purification. Flash column chromatography was carried out using silica gel 60 (230–400 mesh). ^{1}H NMR chemical shifts are expressed in parts per million (δ). Mass spectra were obtained on an Agilent 6230 TOF LC/MS.

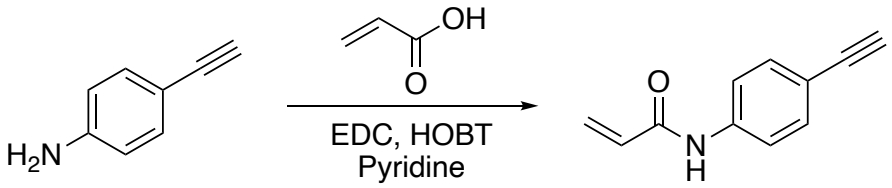


Figure S2. Synthetic scheme for *N*-(4-ethynylphenyl)acrylamide.

To a solution of 4-ethynylaniline (2.0 g, 17.1 mmol), 0.2 eq hydroxybenzotriazole (462.1 mg, 3.42 mmol), and 1.2 eq 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (3.934 g, 20.52 mmol) in anhydrous pyridine (30 mL, 372.4 mmol) under N_2 was added 1.2 eq of acrylic acid (1.41 mL, 20.49 mmol). The reaction was stirred at room temperature overnight. Consumption of 4-ethynylaniline was confirmed by TLC in 1:1 hexanes:ethyl acetate. The crude reaction mixture was diluted in 50 mL ethyl acetate and washed sequentially with water and brine. The aqueous layer was back-extracted twice with ethyl acetate, and the collected organic layer was dried with $MgSO_4$ and filtered. The organic layer was concentrated under reduced pressure and purified by column chromatography (1:1 hexanes:ethyl acetate). The purified product was concentrated under reduced pressure and dried *in vacuo* to yield 1.26 g (42%) of a salmon-colored powder. ^{1}H NMR (400 MHz, DMSO-d6) δ 10.29 (s, 1H), 7.65 (d, *J* = 8.5 Hz, 2H), 7.40 (d, *J* = 8.3 Hz, 2H), 6.40 (dd, *J* = 17.6, 10.0 Hz, 1H), 6.29 – 6.19 (m, 1H), 5.78 – 5.70 (m, 1H), 4.06 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d6) δ 163.73, 139.97, 133.23, 132.85, 132.78, 132.04, 127.87, 119.60, 116.81, 83.96, 80.44. HRMS m/z (ESI) calculated for $C_{11}H_{10}NO$ $(M+H)^+$ 172.0762, found 172.0637

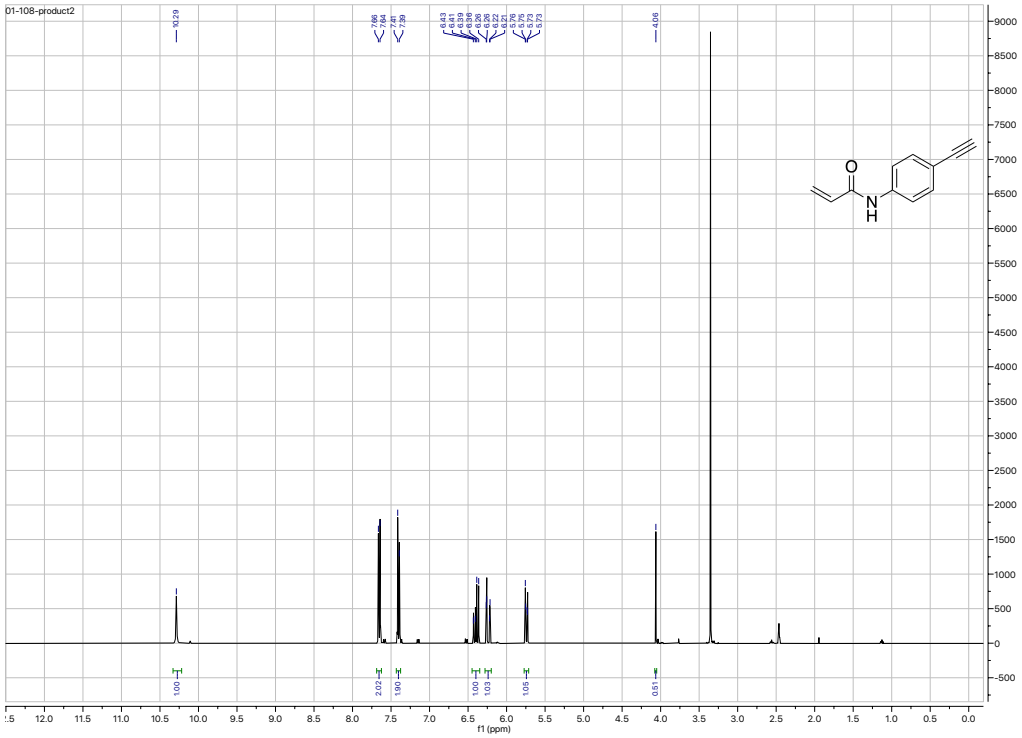


Figure S3. ^{1}H NMR spectrum of EPhAA (400 MHz, DMSO-d6).

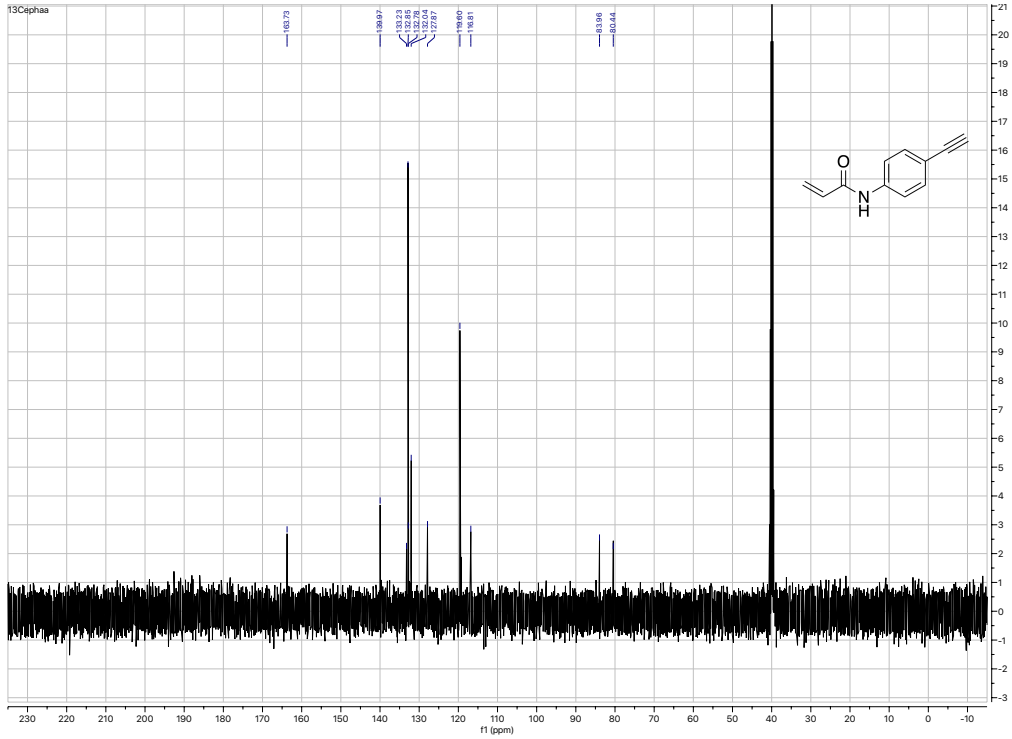


Figure S4. ^{13}C NMR spectrum of EPhAA (400 MHz, DMSO-d6).

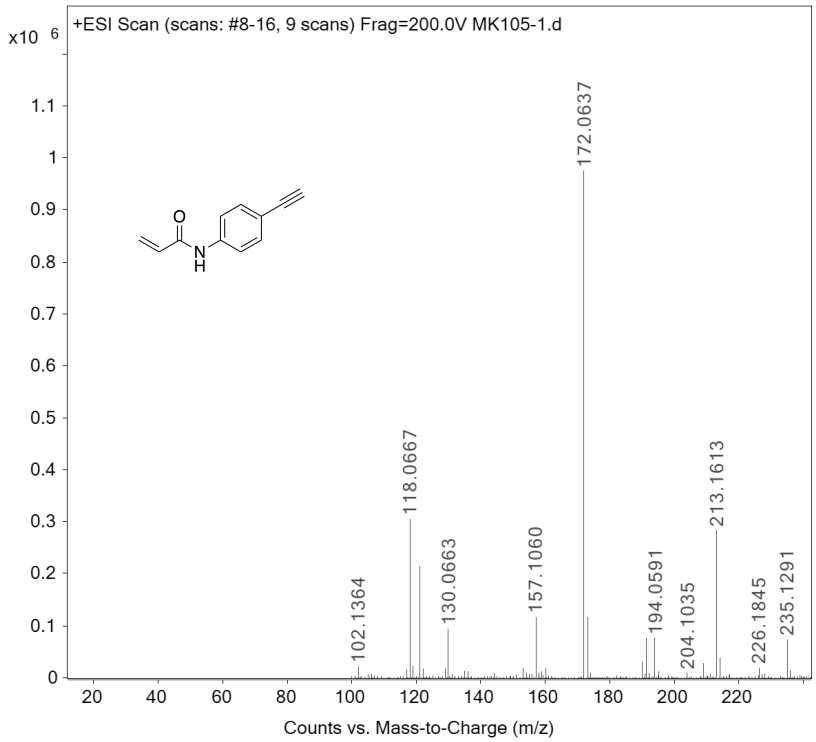


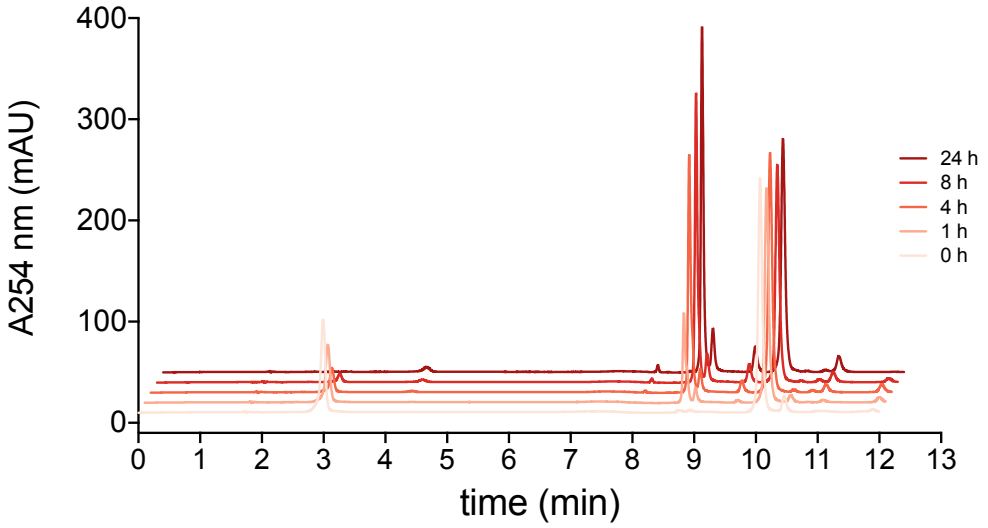
Figure S5. ESI-MS spectrum of EPhAA. Calculated $(M+H)^+$ for $C_{11}H_{10}NO$: 172.0762.

Ribonucleoside labeling and HPLC analysis

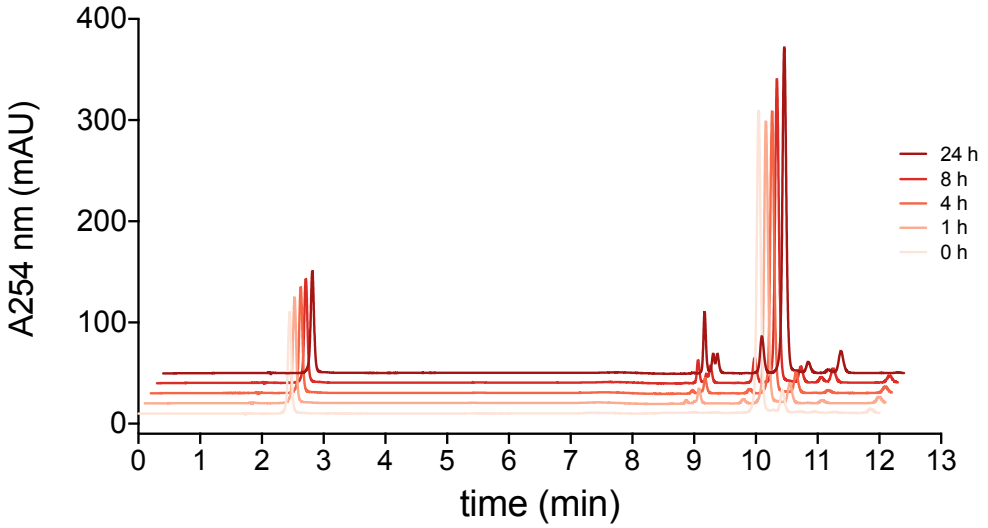
Ribonucleosides inosine, guanosine, adenosine, cytidine and uridine were purchased from Sigma Aldrich Corporation (St. Louis, MO). Pseudouridine (Ψ) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Labeling reaction mixtures were comprised of 50 mM ribonucleoside and 500 mM *N*-(4-ethynylphenyl)acrylamide reagent in 50:50 EtOH:reaction buffer. Phosphate buffered saline (PBS) was used for reactions from pH 6.5-7.5 and 1M triethylammonium acetate (TEAA) for pH 8.0-10.5. Reactions were incubated at 70 °C for the time periods indicated. Reversed-phase HPLC analysis was performed on an Agilent 1260 Infinity II system using a 4 µm, 150 x 4.6 mm Phenomenex Synergi Fusion-RP 80A C18 column. Samples were diluted 1:100 in a stationary phase solution of 5% acetonitrile in PBS. Reactions (1 µL injection) were analyzed using a linear mobile phase gradient from 5% to 45% acetonitrile in water over 15 minutes. All mobile phases contained 0.1% trifluoracetic acid.

Figure S6 (Below). Representative HPLC traces of ribonucleoside reactivity with ethynylphenylacrylamide. Reaction times (0-24 h) indicated by included legends.

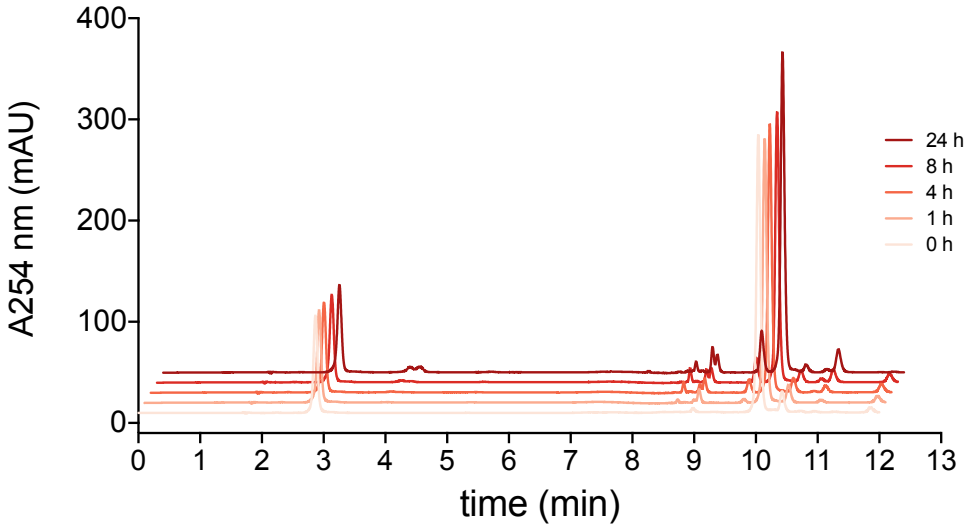
a) inosine



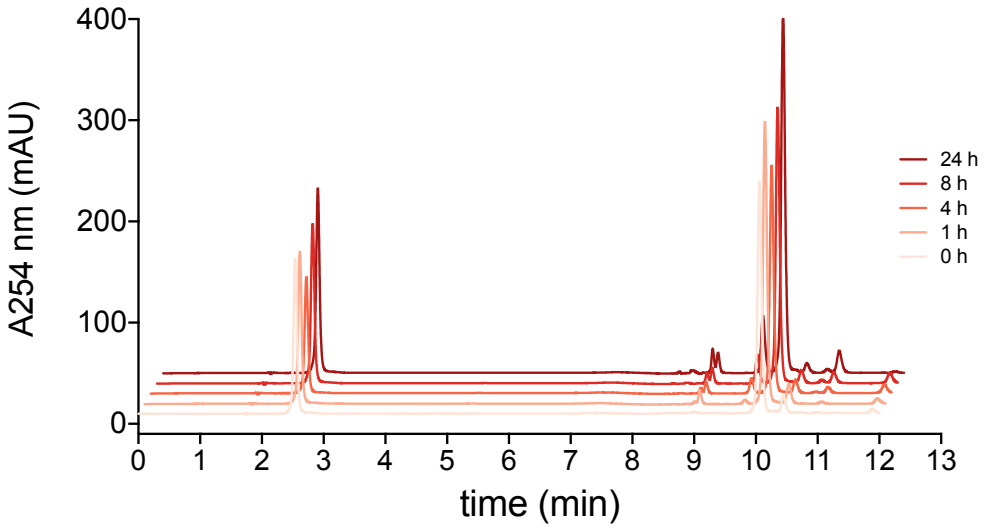
b) uridine



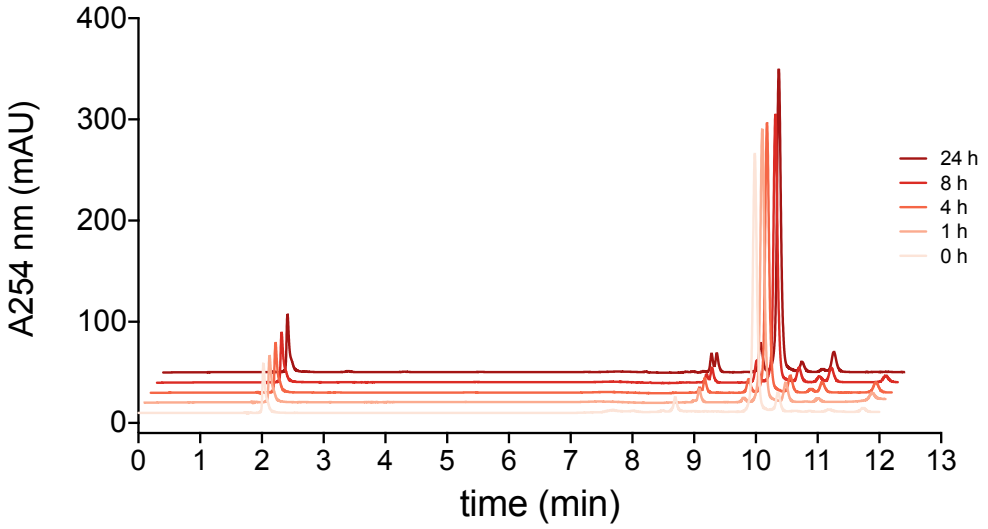
c) guanosine



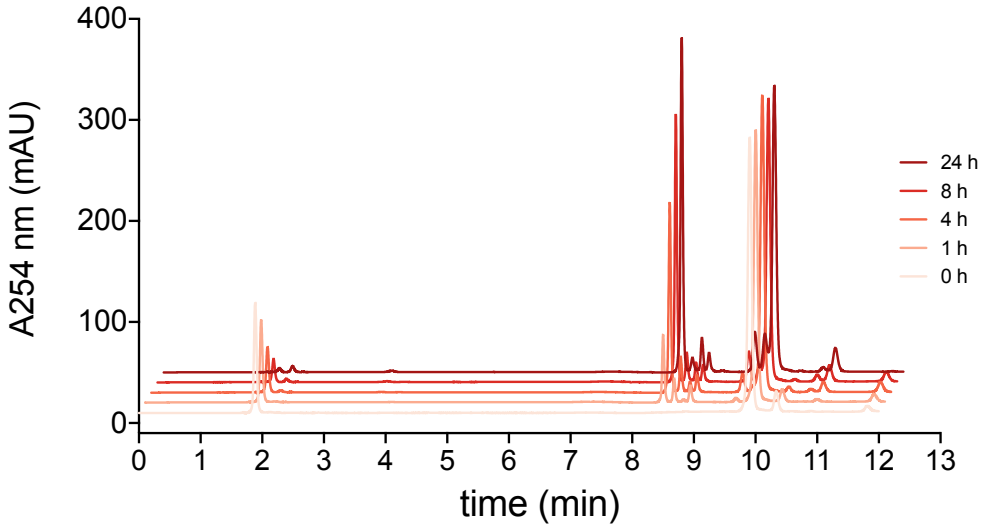
d) adenosine



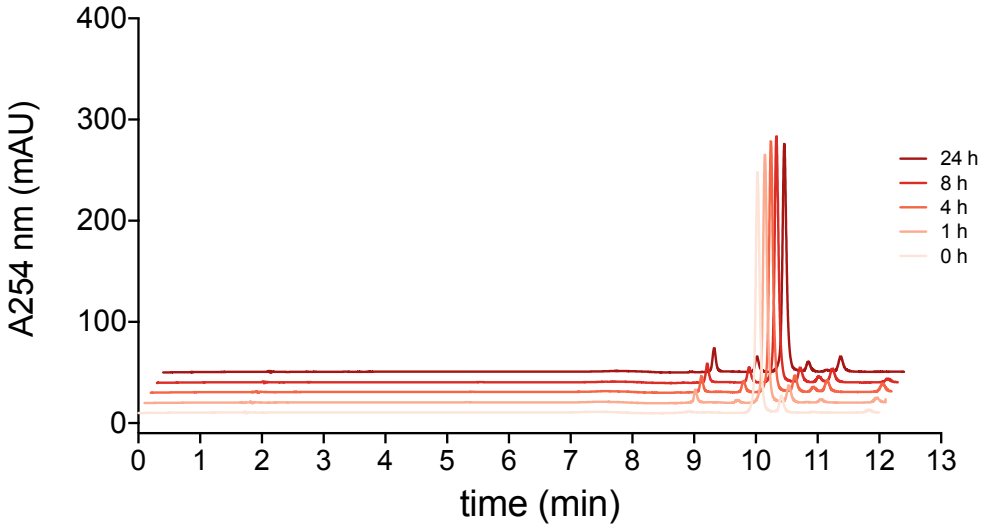
e) cytidine



f) pseudouridine



g) ethynylphenylacrylamide alone



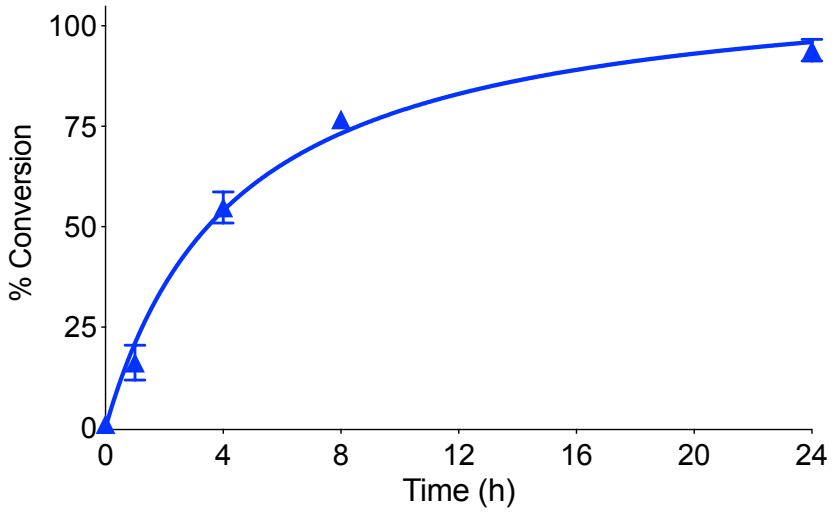
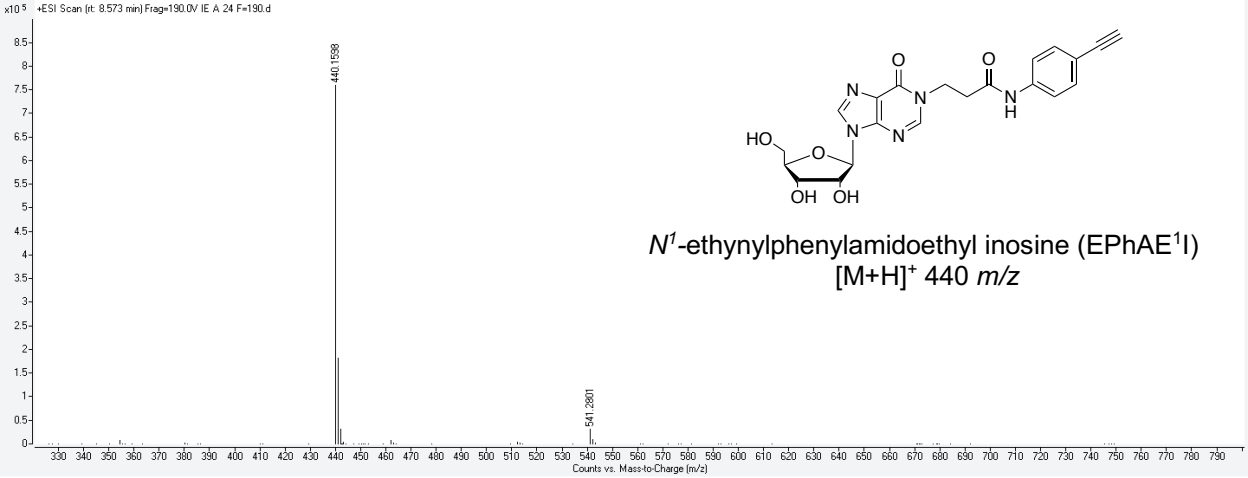


Figure S7. EPhAA reactivity with pseudouridine over 24 hours as monitored by HPLC. Values represent mean with S.D. error bars (n = 3).



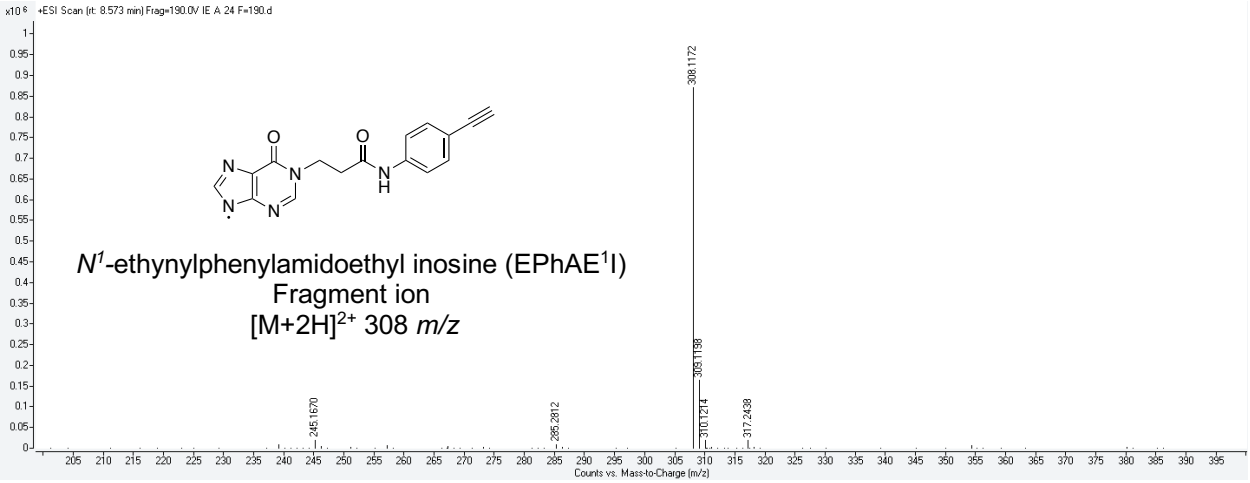
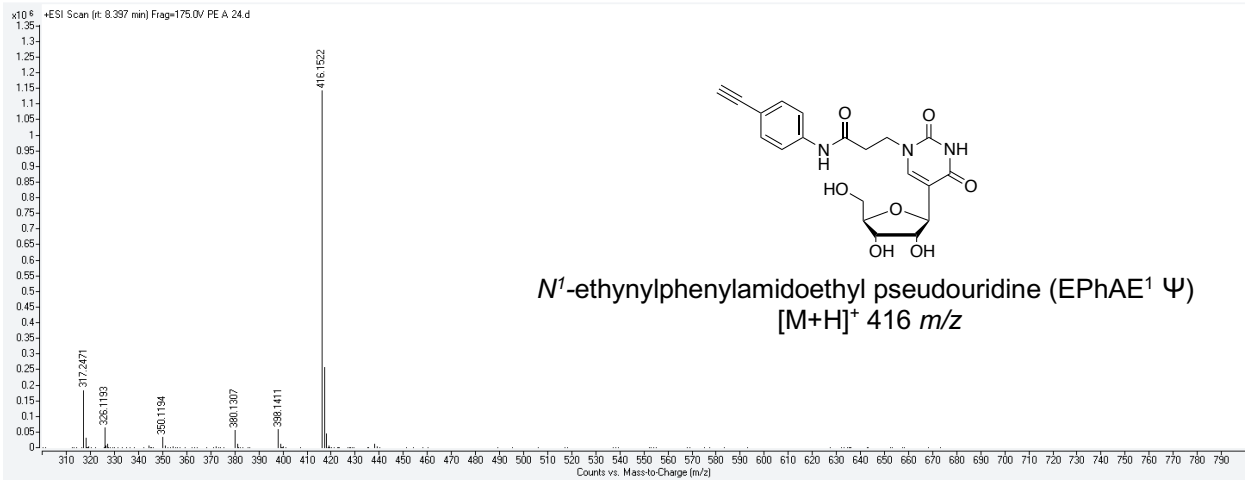


Figure S8. ESI-MS and MS/MS spectra of isolated product fraction for the reaction of inosine and ethynylphenylacrylamide.



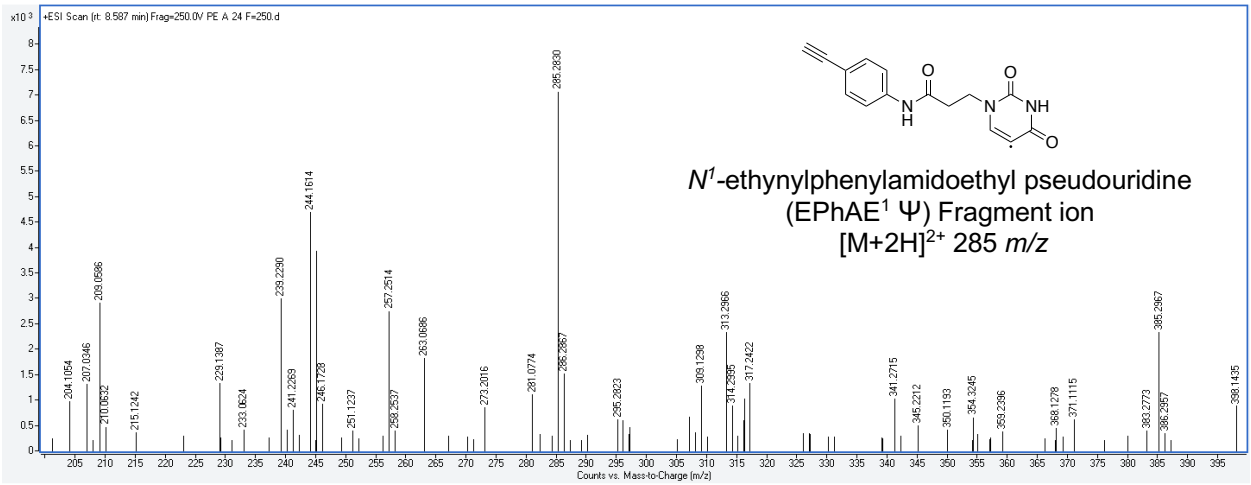


Figure S9. ESI-MS and MS/MS spectra of isolated product fraction for the reaction of pseudouridine and ethynylphenylacrylamide.

DNA oligonucleotide click labeling and PAGE analysis

A FAM labeled DNA strand with an internal alkyne modification (5-octadiynyl deoxyuridine, i5OctdU) was purchased from Integrated DNA Technologies (Skokie, IL) as shown below.

Alkyne DNA 5' FAM/AGCAGCAGGACG/**i5OctdU**/AGCAGAACAGAC 3'

100 pmol of alkyne DNA was CuAAC labeled in a total volume of 100 µL using the Click-&-Go Plus Labeling Kit (Click Chemistry Tools, Scottsdale, AZ) according the manufacturer's protocol. Reactions were given either 1 µL of DMSO (vehicle) or 1 µL of a 5 mM Cy5 picolyl azide (Click Chemistry Tools, Scottsdale, AZ) solution in DMSO. Reactions were incubated for 1 hour at room temperature, after which they were ethanol precipitated and resuspended in 20 µL nuclease-free water. 1 pmol of each sample was resolved on a 10% denaturing polyacrylamide gel and imaged with a GE Amersham Typhoon RGB scanner.

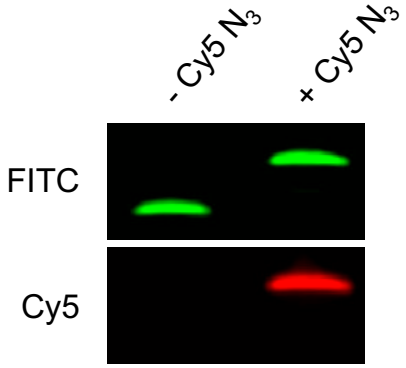


Figure S10. Denaturing PAGE analysis of CuAAC reactions using an alkyne-functionalized DNA with and without Cy5-N_3.

HER1 RNA labeling selectivity

Custom RNA oligonucleotides were purchased from the University of Utah DNA synthesis core facility (Salt Lake City, UT) as shown below.

HER1 RNA A 5’ CCCGCCAACCCCGAGUU**A**GCGGGC 3’

HER1 RNA I 5’ CCCGCCAACCCCGAGUU**I**GCGGGC 3’

In duplicate, 100 pmol of either HER 1 RNA A or HER1 RNA I was added to a 0.1 mL solution of 500 mM *N*-(4-ethynylphenyl)acrylamide in 50:50 EtOH:TEAA buffer, adjusted to pH 8.6, and incubated at 70 °C for the indicated time points. Samples were then ethanol precipitated and resuspended in 20 µL nuclease-free water. RNA was then CuAAC labeled using the Click-&-Go Plus Labeling Kit and 1µL of a 5 mM Cy5 picolyl azide solution in DMSO (Click Chemistry Tools, Scottsdale, AZ). Reactions were incubated for 1 hour at room temperature, after which they were ethanol precipitated and resuspended in 10 µL nuclease-free water. 20 pmol of each sample was resolved on a 10% denaturing polyacrylamide gel, stained with SYBR gold (Thermo Fisher Scientific, Rockford, IL), and imaged with a GE Amersham Typhoon RGB scanner. Densitometric quantification of product bands was performed using ImageJ software. Fold selectivity was defined as the Cy5 lane intensity of RNA I divided by RNA A.

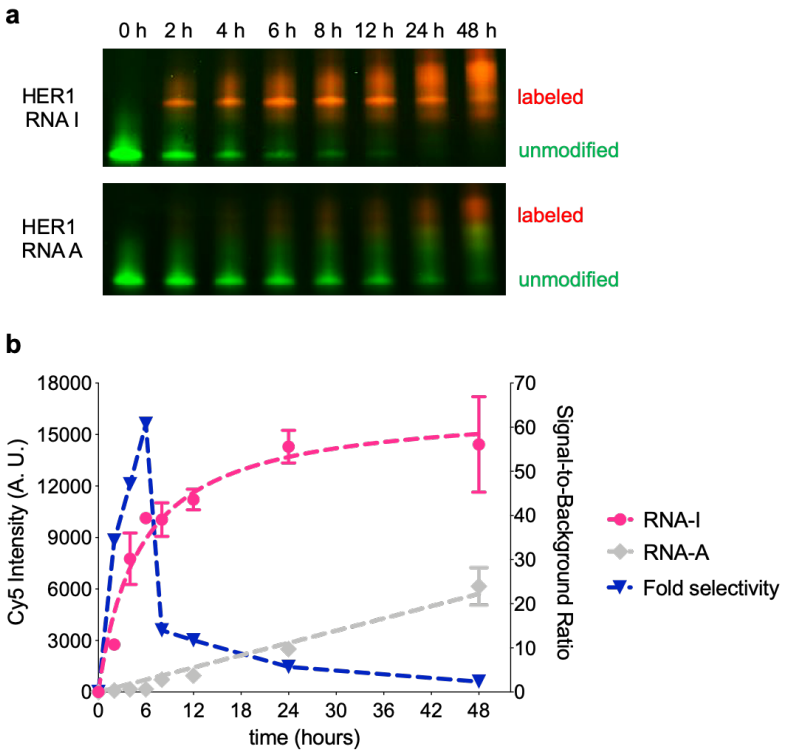


Figure S11. Selectivity for inosine as a function of EPhAA labeling time. a) denaturing PAGE analysis of RNA I and RNA A labeling reactions, showing both Cy5 fluorescence (red) and SYBR gold (green) staining. b) Densitometric Cy5 intensity quantification of RNA I (pink) and RNA A (gray) and fold selectivity (A/I ratio, blue). Values represent mean intensities (arbitrary units, A.U.) with S.D. error bars (n = 2).

HER1 editing rate linearity

In duplicate, varying mixtures of HER1 RNA I and HER1 RNA A were prepared in a 0.1 mL solution of 500 mM *N*-(4-ethynylphenyl)acrylamide in 50:50 EtOH:TEAA buffer, adjusted to pH 8.6, and incubated at 70 °C for 6 hours. Mixtures were defined as follows:

Editing Rate (%)	pmol HER1 RNA A	pmol HER1 RNA I
100	0	100
75	25	75
50	50	50
25	75	25
15	85	15
10	90	10
5	95	5
2.5	97.5	2.5
0	100	0

After labeling, samples were then ethanol precipitated and resuspended in 20 µL nuclease-free water. RNA was then CuAAC labeled using the Click-&-Go Plus Labeling Kit and 1µL of a 5 mM Cy5 picolyl azide solution in DMSO (Click Chemistry Tools, Scottsdale, AZ). Reactions were incubated for 1 hour at room temperature, after which they were ethanol precipitated and resuspended in 10 µL nuclease-free water. 20 pmol of each sample was resolved on a 10% denaturing polyacrylamide gel and imaged with a GE Amersham Typhoon RGB scanner. Densitometric quantification of bands was performed using ImageJ software. Linear regression and Pearson r correlation analysis was performed using GraphPad Prism 8 software.

hADAR1 WT and E1008Q overexpression and purification

hADAR1 wildtype (WT) and hADAR1 E1008Q deaminase domains were generally expressed and purified as previously described (Macbeth and Bass, 2007; Matthews et al., 2016). Mutagenesis of human ADAR1 (hADAR1) deaminase domain was performed using QuickChange XL Site-Directed Mutagenesis (Agilent, Santa Clara, CA) and transformed into XL10-Gold Ultracompetent cells (Agilent, Santa Clara, CA). S. *cerevisiae* BCY123 cells were transformed with a pSc-ADAR construct encoding hADAR1 WT or hADAR1 E1008Q. Cells were streaked on yeast minimal medium minus uracil (Cm-ura) plates. A single colony was used to inoculate a 15 mL Cm-ura starter culture, which was shaken at 300 r.p.m. at 30 °C overnight. The starter culture was used to inoculate 1.5 L yeast growth medium. After cells reached an optical density between 1 - 2, cells were induced with 165 mL of sterile 30% galactose, and protein was expressed for 6 h. Cells were collected by centrifugation and stored at -80 ºC. Cells were lysed in 20 mM Tris-HCl, pH 8.0, 5 % glycerol, 1 mM BME, 750 mM NaCl, 30 mM imidazole, 0.05 % Triton X-100 supplemented with cOmplete EDTA-free protease inhibitor (Sigma Aldrich, St. Louis, MO). Cell lysate was clarified by centrifugation (18,000 rpm, 60 min). Lysate was passed over a 5 mL Ni-NTA column, which was then washed with 50 mL of wash I buffer (20 mM Tris-HCl, pH 8.0, 5 % glycerol, 1 mM BME, 750 mM NaCl, 30 mM imidazole) and 100 mL of wash II buffer (20 mM Tris-HCl, pH 8.0, 5 % glycerol, 1 mM BME, 350 mM NaCl, 30 mM imidazole. Protein was eluted with 20 mM Tris-HCl, pH 8.0, 5 % glycerol, 1 mM BME, 400 mM imidazole and 350 mM NaCl. Fractions containing protein were dialyzed against 50 mM Tris-HCl, pH 8.0, 10 % glycerol, 1 mM DTT, 5 mM EDTA, pH 8.0, 0.01 % NP-40 and 200 mM KCl. Protein concentration was determined through BSA standards visualized by SYPRO Orange (ThermoFisher Scientific) staining on SDS-polyacrylamide gels. Purified protein was stored at -70 ºC in 50 mM Tris-HCl, pH 8.0, 10 % glycerol, 1 mM DTT, 5 mM EDTA, pH 8.0, 0.01 % NP-40 and 200 mM KCl.

In vitro deamination assays

In duplicate for each time point, 100 pmol HER1 RNA A was mixed with 20 pmol of WT or E1008Q hADAR1 enzyme in 20 µL 10 mM Tris HCl pH 7.5, 8.5 mM EDTA, 0.001% Nonidet P-40, 3% glycerol and 40.5 mM potassium glutamate. Each reaction was incubated at 37 °C for the indicated time periods, after which deaminated RNAs were immediately extracted using the Monarch RNA Cleanup Kit (New England Biolabs, Ipswitch, MA). Samples were eluted in 10 µL nuclease-free water and mixed with a 90 µL solution of 500 mM *N*-(4-ethynylphenyl)acrylamide in 50:50 EtOH:TEAA buffer. Reactions were adjusted to pH 8.6 and incubated at 70 °C for 6 hours. After labeling, samples were then ethanol precipitated and resuspended in 20 µL nuclease-free water. RNA was then CuAAC labeled using the Click-&-Go Plus Labeling Kit and 1µL of a 5 mM Cy5 picolyl azide solution in DMSO (Click Chemistry Tools, Scottsdale, AZ). Reactions were incubated for 1 hour at room temperature, after which they were ethanol precipitated and resuspended in 10 µL nuclease-free water. 20 pmol of each sample was resolved on a 10% denaturing polyacrylamide gel and imaged with a GE Amersham Typhoon RGB scanner. Densitometric quantification of bands was performed using ImageJ software. %Editing was calculated using a standard curve of labeling reactions consisting of defined mixtures of HER1 A and I RNA oligos as described earlier. For analysis of initial velocities (below), linear regression was performed using GraphPad Prism 8 software.

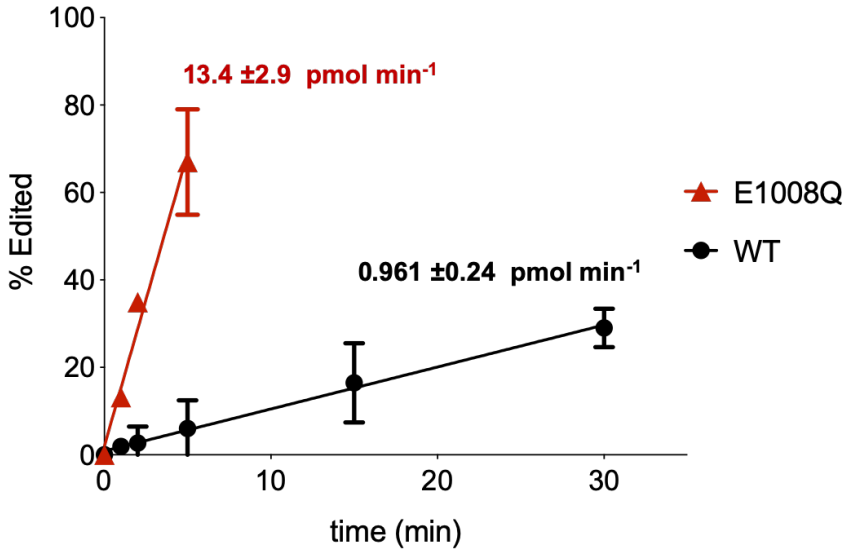


Figure S12. Initial deamination velocities for WT and E1008Q hADAR1 enzymes. Regression analysis for linear portions of each editing curve (Fig. 4). Values represent mean with S.D. error bars. Velocities represent mean with 95% confidence intervals (n = 2).