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Supporting Information

Phenoxy Radical Reactivity of Nucleic Acids: Practical Implications for Biotinylation

Brandon Wilbanks, Brian Garcia, Shane Byrne, Peter Dedon, and L. James Maher, III*

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1.0 Materials and methods

1.1 In vitro radical biotinylation assay

Oligodeoxyribonucleotides and oligoribonucleotides were synthesized and HPLC-purified by Integrated DNA Technologies (Coralville, IA). Radical biotinylation reactions (40 µL) contained the indicated oligonucleotide (2.5 µM), 10 mM potassium phosphate, pH 6, horseradish peroxidase (HRP, 12.5 µM, Thermo Scientific # 31409), freshly prepared H_2O_2 (500 µM, Sigma # 216763), and freshly prepared biotin tyramide (100 µM, ApexBio # A8011). For NTP competition reactions, ATP, UTP, CTP, or GTP (New England Biolabs # N0450S) was added at a final concentration of 1 mM along with oligonucleotides. Oligonucleotide duplexes were generated by combining 25 µM of each oligonucleotide in phosphate-buffered saline, heating for 5 min at 90 °C, and cooling to room temperature in a water bath at room temperature for 1 h. Duplex DNA was added to biotinylation reactions at 2.5 µM following hybridization. For experiments with heme, HRP was substituted with heme (Sigma #51280) added at a final concentration of 5 µM. Reactions were then incubated at 45 ºC (unless indicated otherwise) for 1.5 h with agitation at 1000 rpm (Benchmark Scientific, model H5000-HC).

Reactions were then quenched by addition of 40 µL 2× quenching buffer (20 mM sodium ascorbate, 10 mM sodium azide) and mixed thoroughly. Quenched reactions were combined with 2 µL proteinase K solution and 18 µL buffer ATL (Qiagen DNEasy Blood and Tissue Extraction kit # 69504) and incubated at 56 ºC for 1 h with agitation at 1000 rpm. All gel images and reported quantification include proteinase K digestion unless noted otherwise. Reactions were then extracted with

phenol:chloroform:isoamyl alcohol (25:24:1, 80 µL, Invitrogen # 15593031) and aqueous phases combined with 0.1 volume 3 M sodium acetate, 1 µl glycogen (Thermo Scientific # R0561) and 2.5 volumes ice-cold 100% ethanol. This mixture was chilled on dry ice for 15 min and subjected to centrifugation for 20 min at 16,000 × g. Samples were washed with 150 µL ice-cold 70% ethanol followed by centrifugation for 10 min at 16,000 × g and air drying. Pellets were resuspended in water (15 µL), and either streptavidin (5 µL of 182 µM stock, Invitrogen # 434301) or water (5 µL) was added with incubation for 30 min at 37 ºC with agitation at 1000 rpm. Samples were then analyzed by electrophoresis. For analysis under native conditions, samples were supplemented with 0.1 volume loading buffer and subjected to electrophoresis at 22.5 V/cm for 30 min through native 10% polyacrylamide (19:1 acrylamide:bisacrylamide) gels. For analysis under denaturing conditions, samples were supplemented with formamide (20 µL, Sigma #F9037), heated to 50˚C for 3 min, and cooled on ice before electrophoresis through 10% polyacrylamide (19:1 acrylamide:bisacrylamide,) gels containing 7.5 M urea.

Gels were imaged on a Typhoon fluorometric imager using the FAM channel. Gels were then stained with 1× SYBR Gold dye (Invitrogen # S11494) in 0.5× TBE buffer for 20 min. Second images were obtained on the Typhoon imager using the SYBR Gold channel.

1.2 Mass spectrometry

Nuclease P1 was purchased from US Biological Life Sciences and reconstituted according to the manufacturer's protocols. Calf intestinal alkaline phosphatase was purchased from Sigma. Spin filters (10 kDa molecular weight cut off) were purchased from VWR.

Each oligonucleotide (100 pmol, 78 µL) was incubated with nuclease P1 (1.5 U, 3 µL) in 30 mM ammonium acetate pH 5.3 and 0.5 mM $ZnCl_2$ (90 µL total reaction volume) for 2 h at 55 °C. The reaction mixture was diluted with Tris-HCl (10 mM final concentration, pH 8.0, 9 µL) and incubated with calf intestinal alkaline phosphatase (51 U, 3 µL) for 2 h at 37 °C. Enzymes were removed by passing the mixture through a VWR 10 kDa spin filter with centrifugation at 12,000 g for 12 min at 4 °C). The solution was lyophilized to dryness and resuspended in H_2O (50 µL). Hydrolyzed oligonucleotides (~20

pmol per 10 μL injection) were analyzed on a Dionex Ultimate 3000 UHPLC system equipped with a Synergi Fusion RP column (2.5 μm particle size, 100 Å pore size, 100 mm length, 2 mm inner diameter). The HPLC was coupled to a Thermo Fisher Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. The column was eluted at 0.35 mL/min at 35 °C with a linear gradient of 3-9% acetonitrile in 97% solvent A (5 mM ammonium acetate pH 5.3) over 15 min. The column was rinsed with 95% acetonitrile in solvent A for 1 min, and then the initial conditions were regenerated by rinsing the column with 97% solvent A for 3 min. High resolution mass spectra were obtained by hybrid quadrupole-Orbitrap mass spectrometry with the following parameters: sheath gas flow rate, 50 L/min; aux gas flow rate, 15 L/min; sweep gas flow rate, 3 L/min; spray voltage, 4.20 kV; and capillary temperature, 275 °C. Collision-induced dissociation was achieved using a collision energy of 40 V.

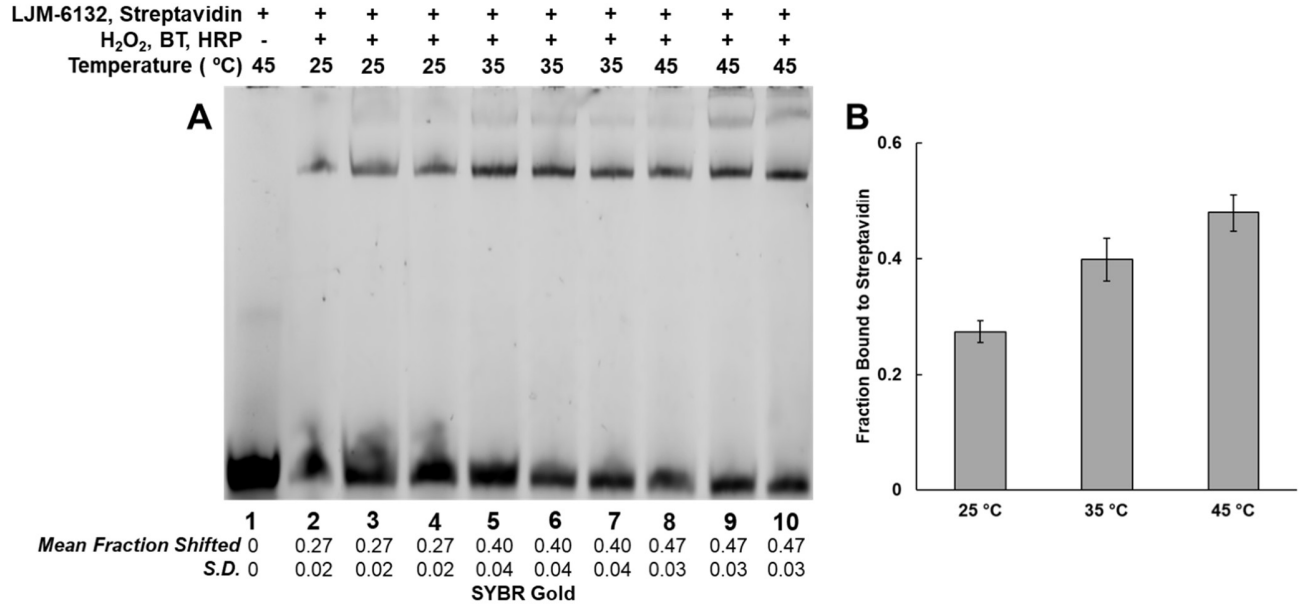
The hydrolyzed RNA oligonucleotide containing BT adducts was analyzed by LC-MS/MS on an Agilent 1290 series HPLC system equipped with a Synergi Fusion RP column (2.5 μm particle size, 100 Å pore size, 100 mm length, 2 mm inner diameter) and a DAD. The HPLC was coupled to an Agilent 6490 triple quadrupole mass spectrometer. The column was eluted at 0.35 mL/min at 35 °C with a linear gradient of 3-9% acetonitrile in 97% solvent A (5 mM ammonium acetate pH 5.3) over 15 min. The column was rinsed with 95% acetonitrile in solvent A for 1 min, and then the initial conditions were regenerated by rinsing the column with 97% solvent A for 3 min. Canonical ribonucleosides and the BT-guanosine adduct were identified and quantified by tandem quadrupole mass spectrometry with electrospray ionization operated with the following parameters: N_2 temperature, 200 °C; N_2 flow rate, 14 L/min; nebulizer pressure, 20 p.s.i.; capillary voltage, 1800 V; and fragmentor voltage, 380 V. For product identification, the mass spectrometer was operated in positive ion multiple reaction monitoring mode using the conditions tabulated below.

Species	Precursor Ion Mass	Product Ion Mass	Collision Energy (V)	Cell Accelerator Voltage (V)
BT-guanosine adduct	645.2	513.2	40	5
rA	268.1	136.1	12	1
rC	244.1	112.1	16	1
rG	284.1	152.1	4	3
rU	245.1	127.1	12	1

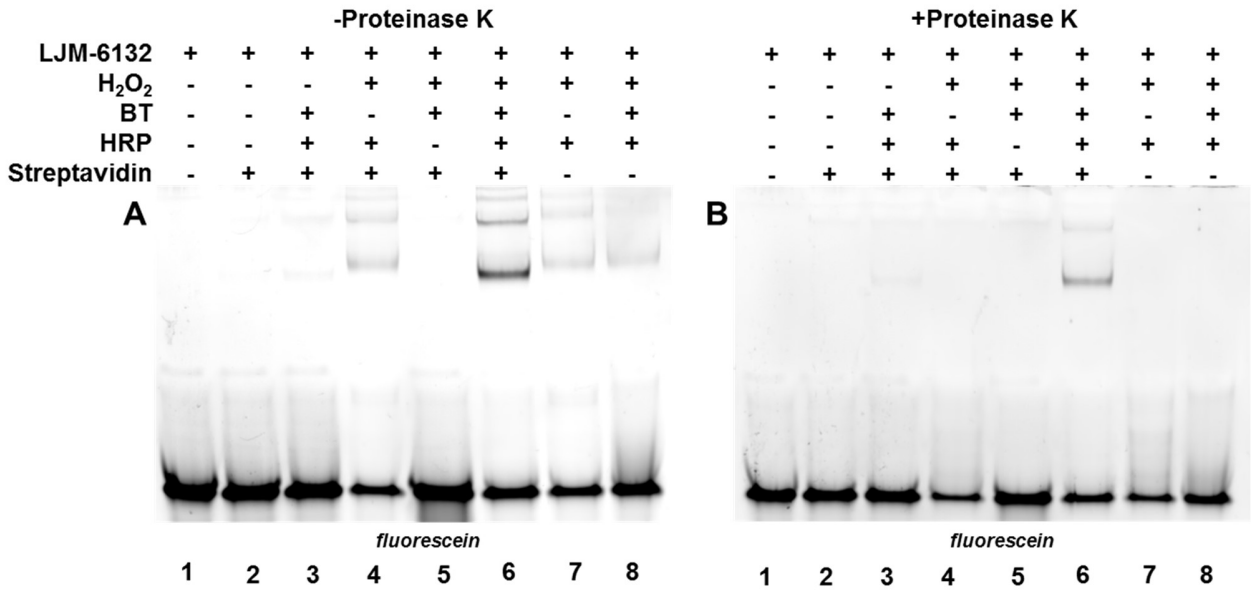
1.3 DNA depurination assay

DNA depurination for control reactions was induced by treatment of 50 pmol deoxyriboligonucleotide with 25 µL ice cold formic acid for 15 min. DNAs were then combined with 200 µL DMS stop buffer (50 mM sodium cacodylate, 1 mM EDTA) and mixed. Samples were then supplemented with 0.1 volume 3 M NaOAc and 750 µL ice cold ethanol. Samples were then incubated on dry ice for 15 min, thawed to room temperature, and subjected to centrifugation at 16,000 × g for 20 min. Supernatant was discarded and DNA pellets were subjected to a second precipitation from ethanol. Recovered pellets from formic acid-treated control reactions and experimental phenoxy radical biotinylation reactions were dissolved in 100 µL 0.1 M piperidine, heated for 3 min at 90˚ C, and allowed to cool to room temperature, frozen on dry ice, and lyophilized. Samples were dissolved in 20 µL formamide, heated for 5 min at 90˚ C, and cooled on ice before electrophoresis through 10% polyacrylamide (19:1 acrylamide:bisacrylamide) gels containing 7.5 M urea.. Gel images were obtained on a Typhoon imager using the FAM channel.

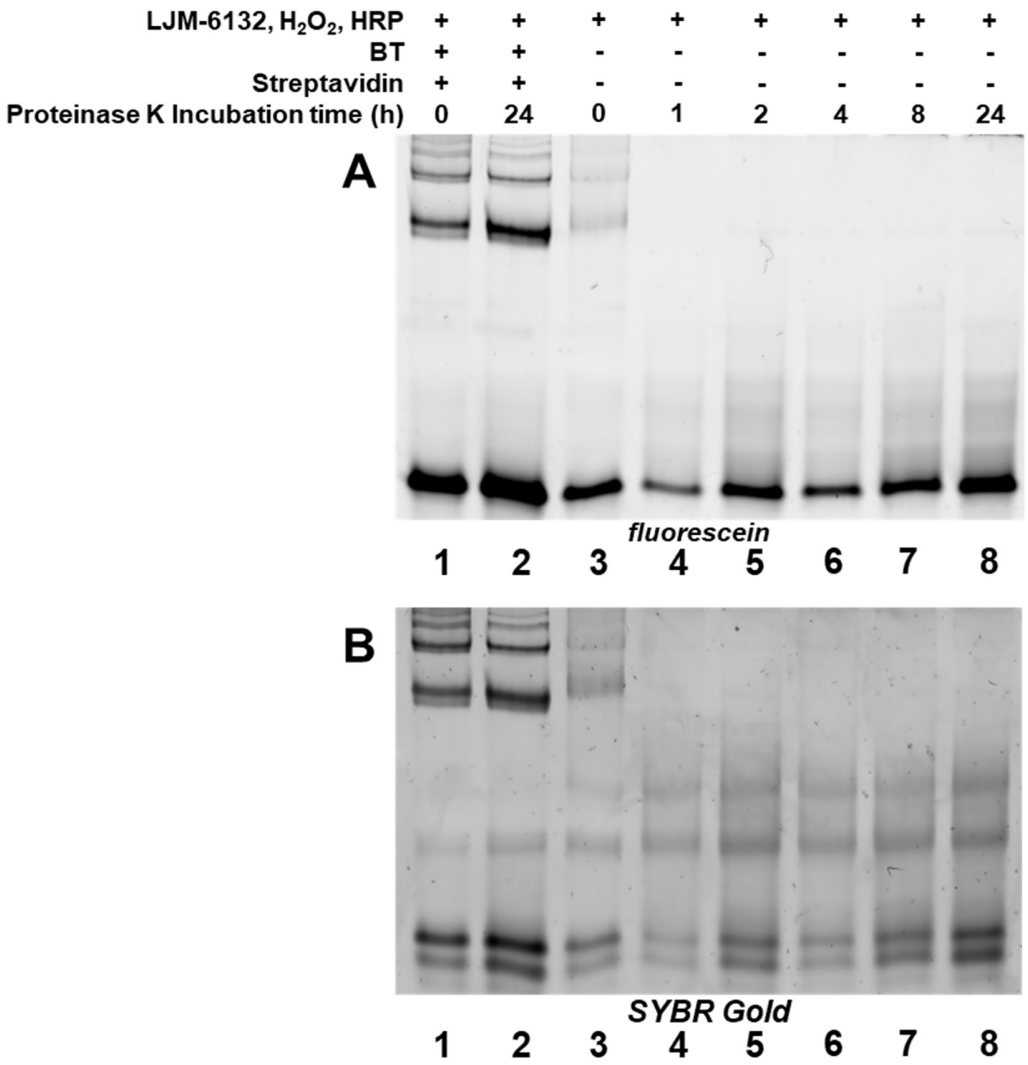
2.0 Supporting Figures



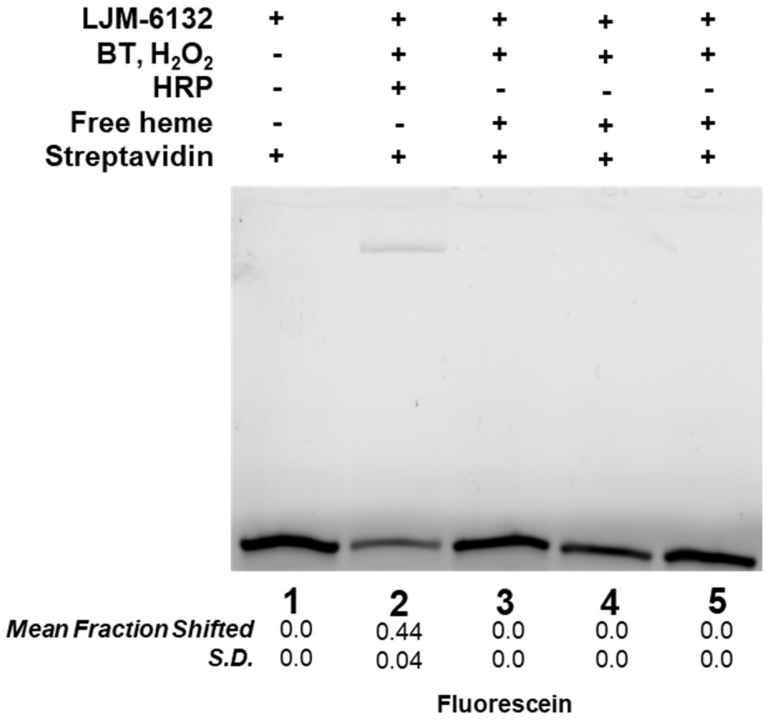
Supplemental Figure S1. Temperature optimization of in vitro biotinylation conditions. A) In vitro biotinylation of LJM-6132 at 25 °C (lanes 2-4), 35 °C (lanes 5-7), and 45 °C (lanes 8-10) visualized by fluorescein filter in native polyacrylamide gel. B) Estimated fraction of LJM-6132 bound to streptavidin quantified by band intensity imaged by FAM. Statistics are calculated from independent experiments performed in triplicate; S.D. is standard deviation.



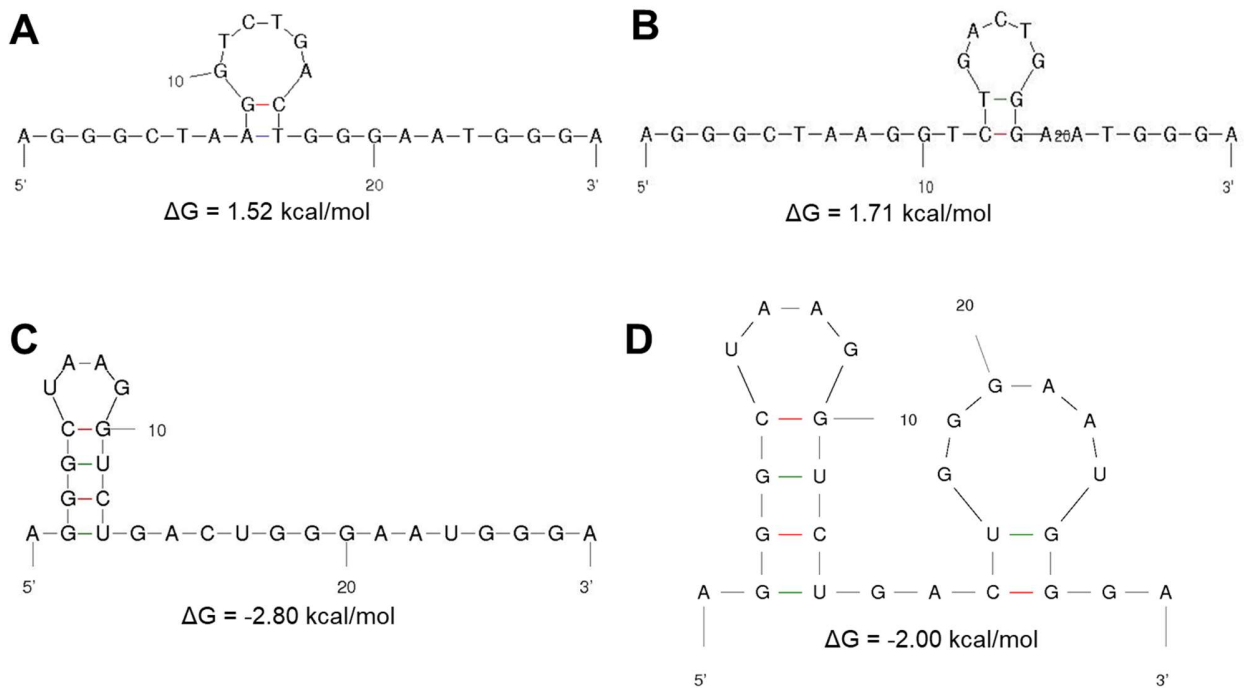
Supplemental Figure S2. Proteinase K depletion of streptavidin-independent fluorescent complexes. Native gel electrophoresis showing that proteinase K digestion for 60 min depletes shifted complexes attributed to reaction of HRP with fluorescent LJM-6132 (lane 5). Fluorescence visualization was with a fluorescein filter. Note: streptavidin addition occurs after proteinase K treatment and subsequent purification.



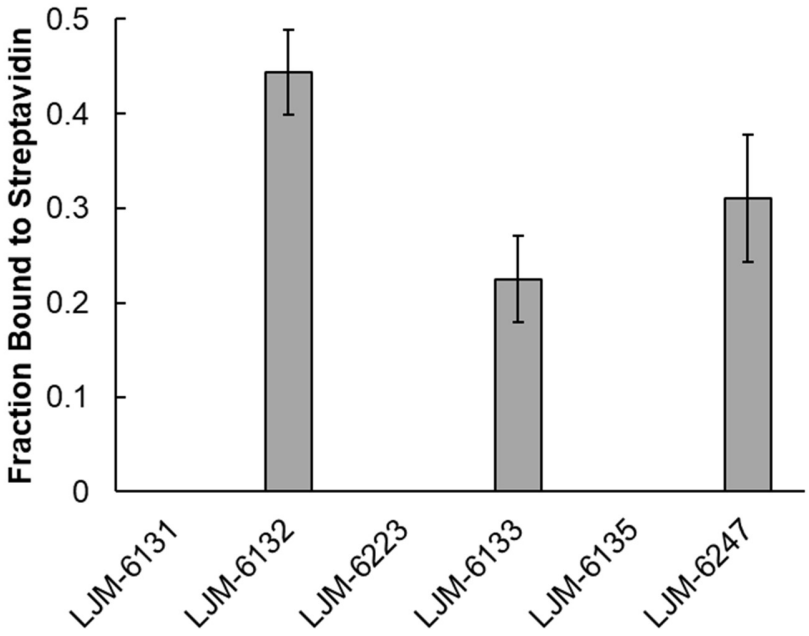
Supplemental Figure S3. Proteinase K depletion of streptavidin-independent fluorescent complexes. Streptavidin detection by native gel electrophoresis of oligodeoxyribonucleotide biotinylation catalyzed by HRP in the presence of BT and H_2O_2 (lane 1) is not affected by prior 24-h proteinase K treatment (lane 2). In contrast, for reactions performed without BT and not detected by streptavidin addition (lanes 3-8), proteinase K treatment for 0, 1, 2, 4, 8, or 24 h (lanes 3-8) depletes shifted complexes attributed to reaction of HRP with fluorescent LJM-6132, but not species attributed to LJM-6132 autoreaction (lanes 4-8). The latter species are not fluorescent.



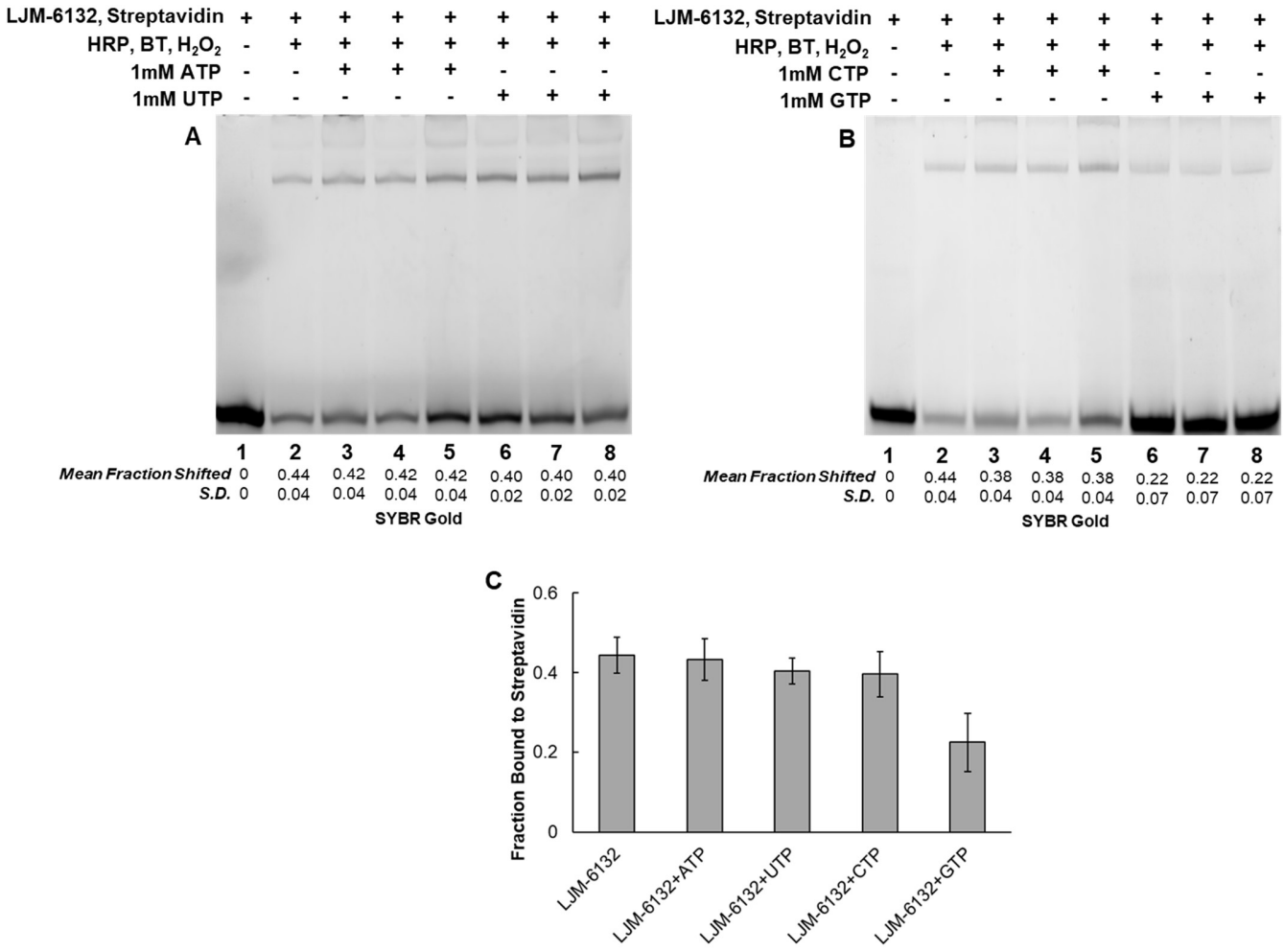
Supplemental Figure S4. LJM-6132 is not biotinylated by free heme. Substitution of HRP with 5 μM free heme in the standard biotinylation reaction (lanes 3-5) eliminates streptavidin-specific gel shift. Statistics are calculated from independent experiments performed in triplicate; S.D. is standard deviation.



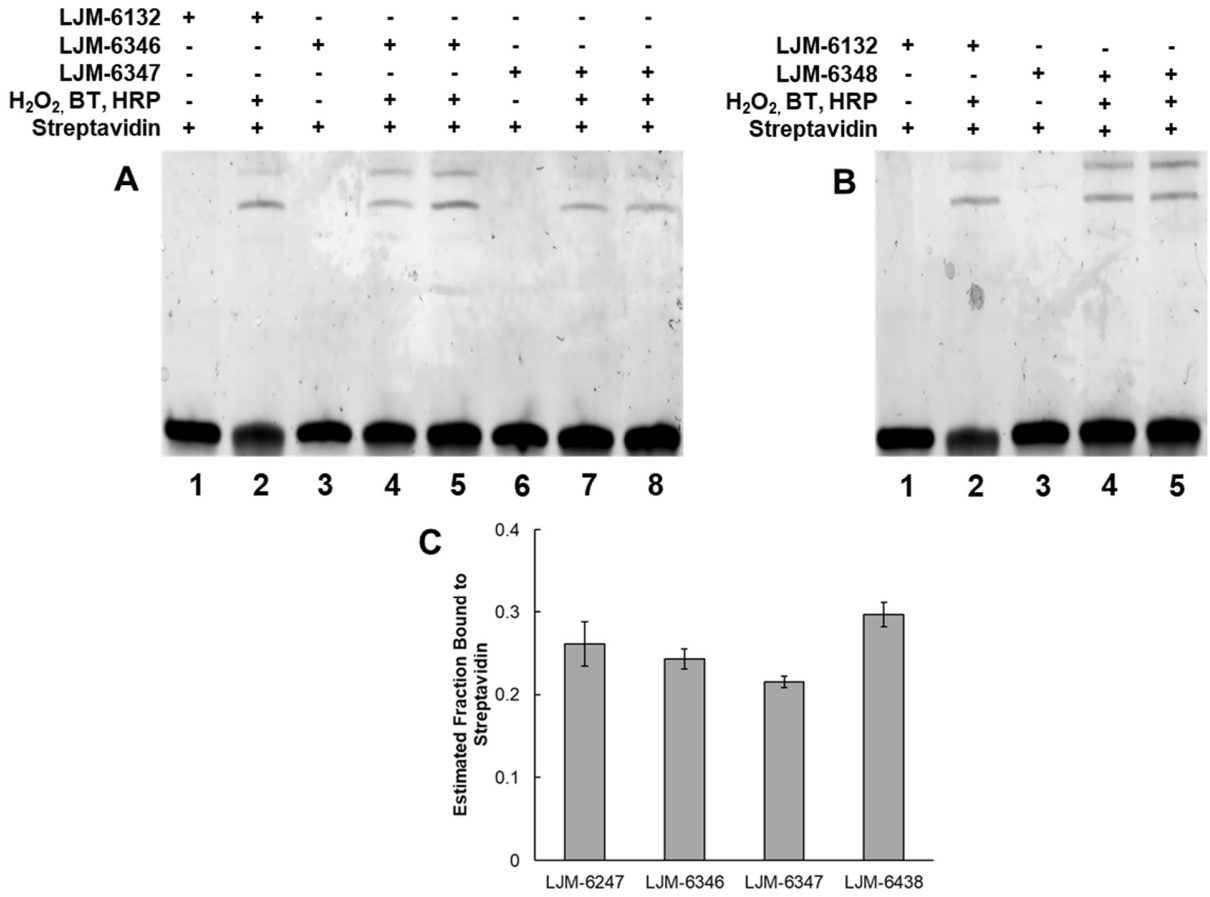
Supplemental Figure S5. Mfold structure predictions of G-rich oligonucleotides of interest. The two most stable predicted structures for LJM-6131 (A, B) and LJM-6133 (C, D) do not indicate the presence of highly stable secondary structures involving conventional base pairs.



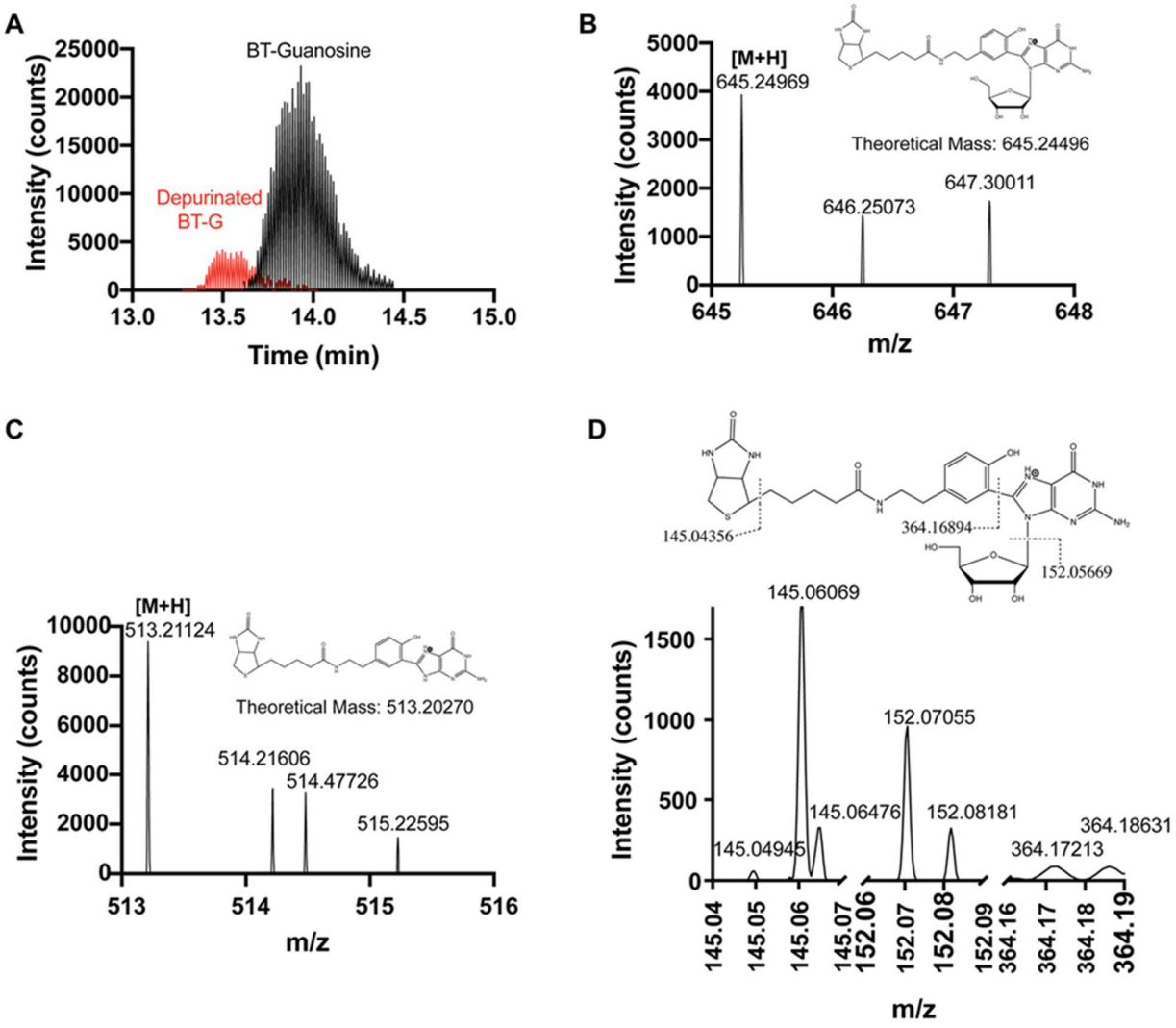
Supplemental Figure S6. Summary of quantification of oligonucleotide fraction bound to streptavidin for oligonucleotides of interest. Statistics are calculated from independent experiments performed in triplicate; S.D. is standard deviation.



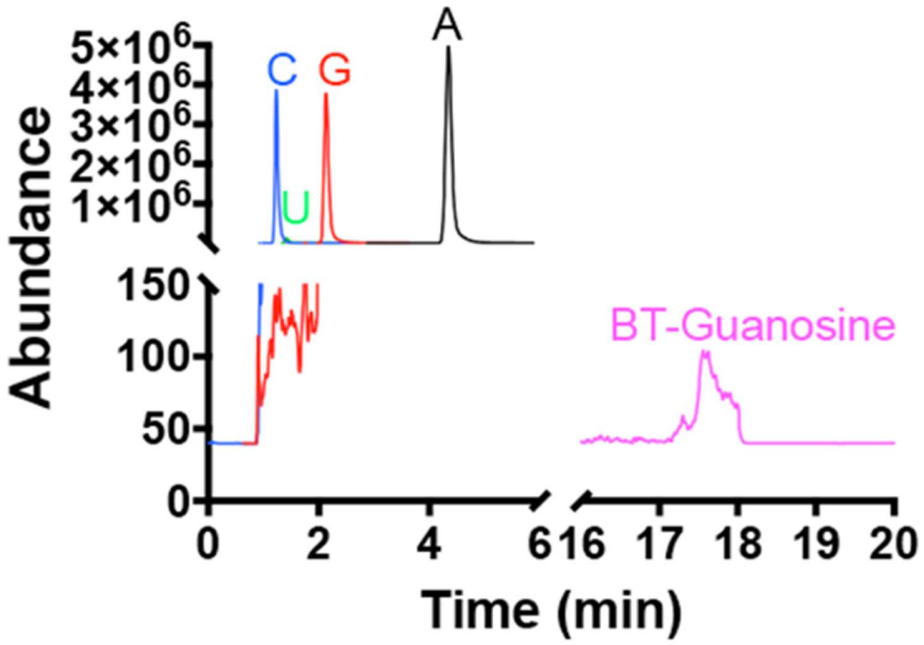
Supplemental Figure S7. Quenching of LJM-6132 biotinylation by NTP competition. Incubation of 1 mM excess NTP with the standard biotinylation reaction reveals that A) ATP, UTP, and B) CTP are not effective competitors, suggesting that they are not biotinylated. In contrast, excess GTP (B, lanes 6-8) reduces the total fraction of LJM-6132 shifted by streptavidin. C) Summary of quantified gel shifts. Statistics are calculated from independent experiments performed in triplicate; S.D. is standard deviation.



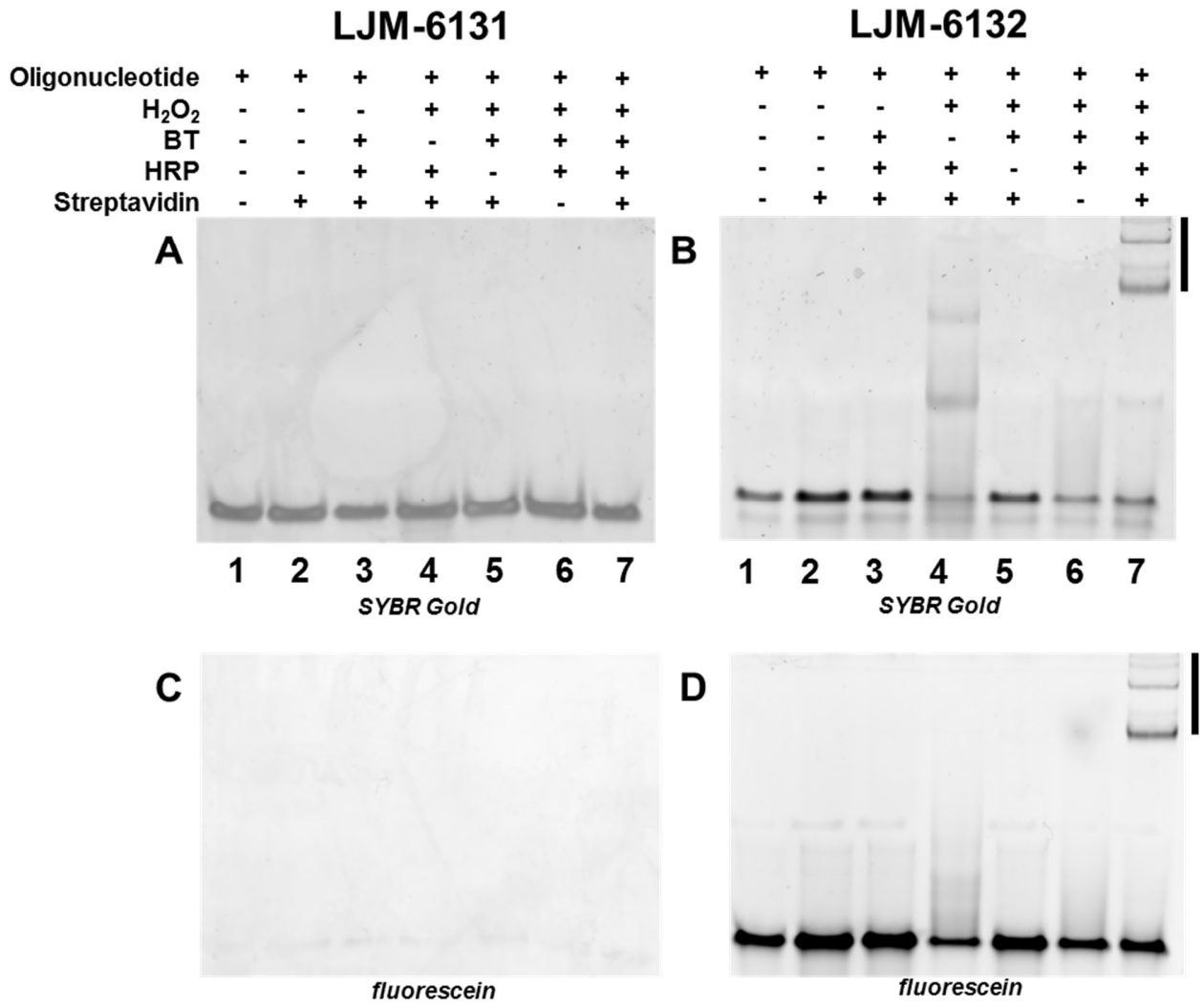
Supplemental Figure S8. LJM-6247 variants are similarly biotinylated. LJM-6247 variants LJM-6346, LJM-6347, and LJM-6348, contain G residues in identical positions as LJM-6247 with all other positions in the sequence randomly substituted with non-G residues. Native gel electrophoresis gel shifts of A) LJM-6346, LJM-6347, and B) LJM-6348 indicate similar degrees of biotinylation for all tested oligonucleotides. C) Quantification of estimated fraction of each oligonucleotide biotinylated and shifted by streptavidin in panels A and B. Results are visualized by SYBR Gold staining. Statistics are calculated from independent experiments performed in triplicate; S.D. is standard deviation.



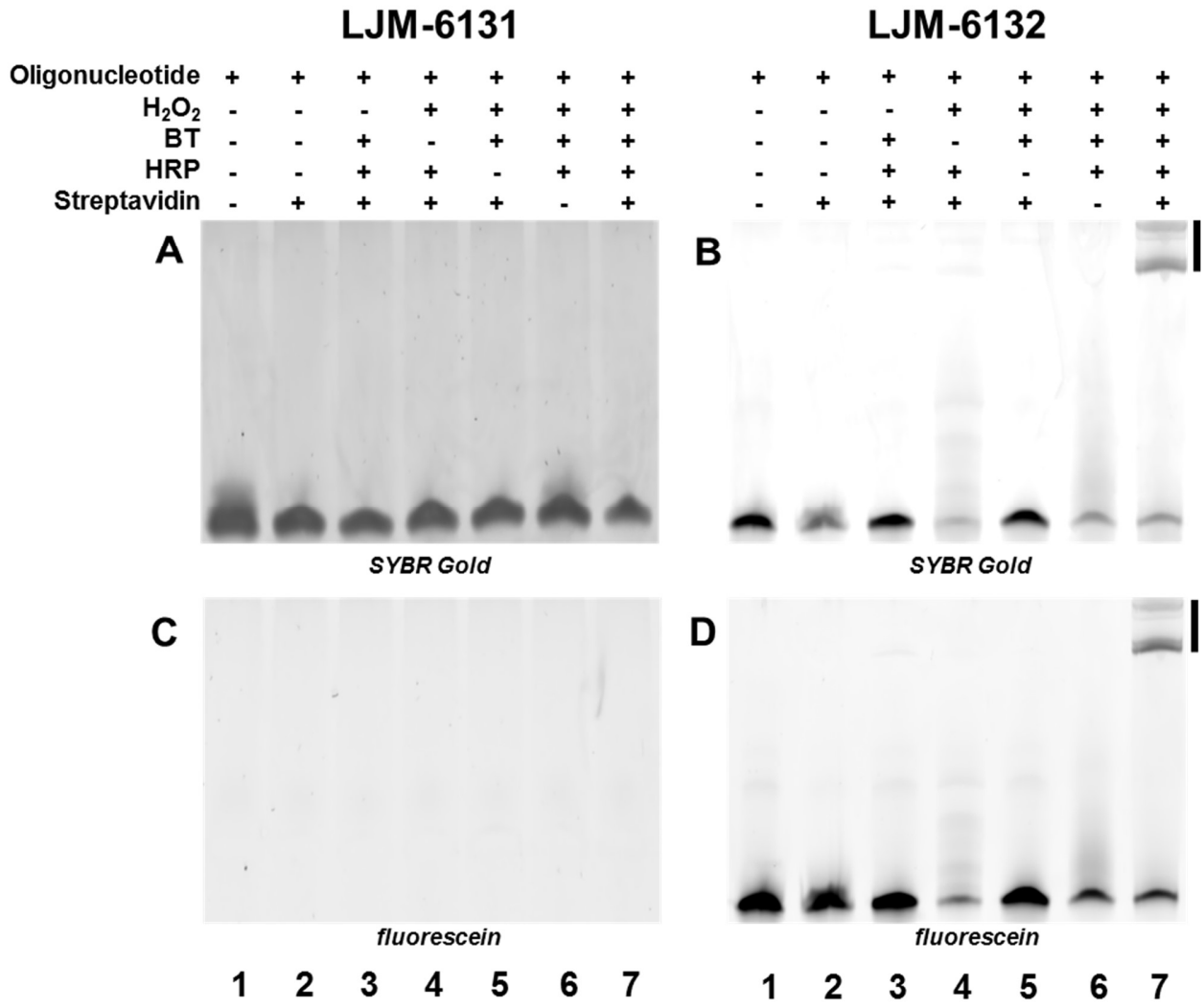
Supplemental Figure S9. Analysis of the BT-guanosine and depurinated BT-guanine adducts in hydrolyzed RNA by chromatography-coupled high-resolution Orbitrap mass spectrometry. (**A**) Extracted ion chromatograms of biotin tyramide-guanosine and depurinated biotin tyramide-guanine adducts present in the RNA hydrolysis mixture. (**B, C**) Mass spectra for the (**B**) biotin tyramide-guanosine adduct and (**C**) depurinated biotin tyramide- guanine adduct provide exact masses that compare favorably with the theoretical masses calculated for each adduct. The additional signals in the mass spectrum for each adduct represent isotopomers (**B**, 646.25073; **C**, 514.21606, 515.22595) and possible contaminants (**B**, 647.30011; **C**, 514.47726). (**D**) Collision-induced dissociation (CID) analysis of the biotin tyramide-guanosine adduct confirms the adduct structure. The inset shows theoretical exact masses correlating with fragments detected by CID.



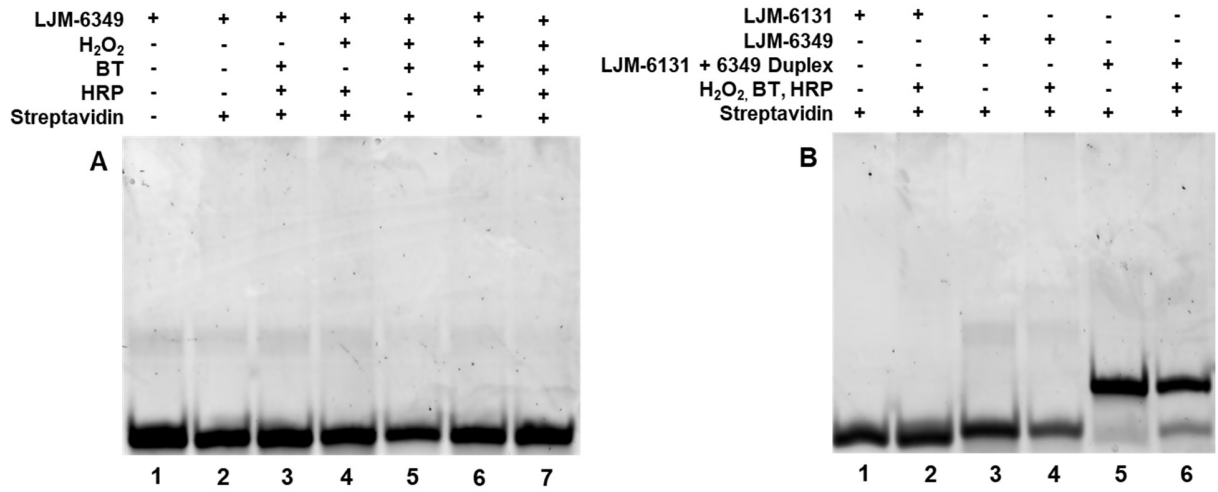
Supplemental Figure S10. Analysis of the BT-guanosine adduct in hydrolyzed RNA by chromatography-coupled triple quadrupole mass spectrometry. Multiple reaction monitoring for the loss of the ribose sugar revealed the four canonical ribonucleosides and the BT-guanosine adduct. The following mass fragmentation transitions were monitored (A: 268.1→136, C: 244.1→112.1, G: 284.1→152.1, U: 245.1→113.1, BT-guanosine: 645.2→513.2). The long retention time of the BT-guanosine adduct is consistent with its significant hydrophobicity. The difference in retention time of the adduct in Figure S9 and Figure S10 is due to minor variations in the chromatography system linked to the Orbitrap and triple quadrupole mass spectrometers.



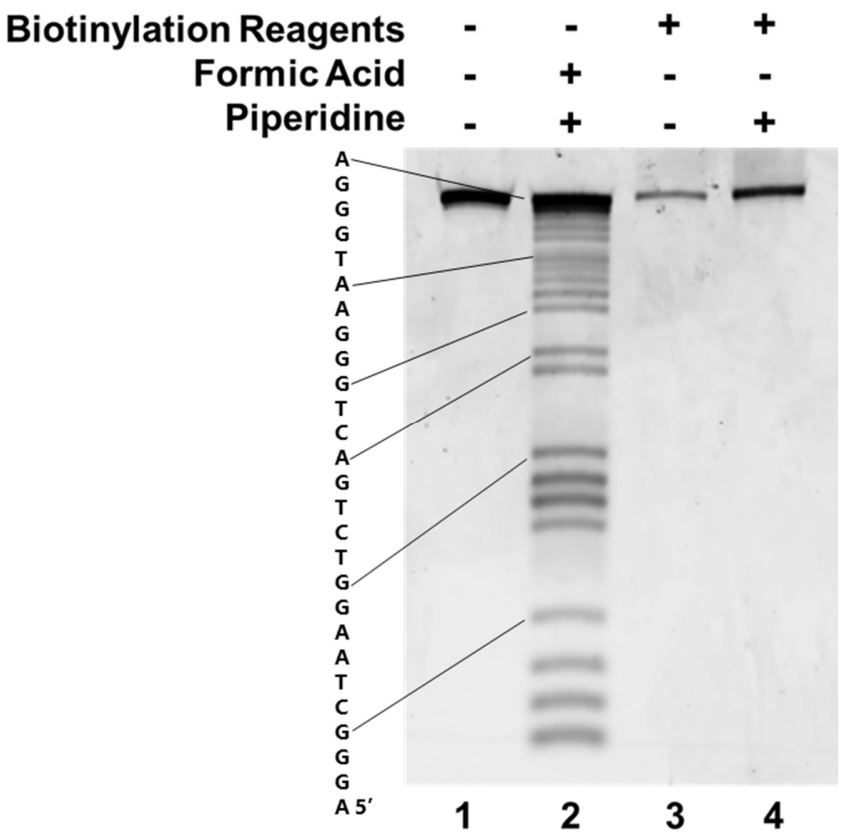
Supplemental Figure S11. Oligodeoxyribonucleotide 5' FAM modification is necessary and sufficient for radical biotinylation catalyzed by HRP in the presence of H_2O_2 and biotin tyramide. Biotinylation of the indicated DNA oligonucleotides is detected by a streptavidin-dependent gel mobility shift under native conditions, followed by staining with SYBR Gold (A, B) or fluorescence visualization with a fluorescein filter (C, D).



Supplemental Figure S12. Oligodeoxyribonucleotide 5' FAM modification is necessary and sufficient for radical biotinylation catalyzed by HRP in the presence of H_2O_2 and biotin tyramide. Biotinylation of the indicated DNA oligonucleotides is detected by a streptavidin-dependent gel mobility shift under denaturing conditions, followed by staining with SYBR Gold (A, B) or fluorescence visualization with a fluorescein filter (C, D).



Supplemental Figure S13. Streptavidin detection of LJM-6349 and LJM-6349 + LJM-6131 duplex biotinylation. A) LJM-6131 reverse complement LJM-6349 is an unmodified oligodeoxyribonucleotide that is not biotinylated under standard assay conditions. B) Hybridized LJM-6131 + LJM-6349 forms a duplex (shifted band, lanes 5 and 6) that is not biotinylated under standard assay conditions. Biotinylation was monitored by native polyacrylamide gel electrophoresis with imaging by SYBR Gold filters.



Supplemental Figure S14. Depurination analysis. DNA LJM-6132 alone (lane 1), treated with formic acid to induce depurination (lane 2), or with biotinylation reagents (lanes 3 and 4), followed by hot piperidine (lanes 2 and 4) to cleave apurinic sites. Resulting material is visualized by fluorescein fluorescence after denaturing polyacrylamide gel electrophoresis.