Supporting Information

Oligonucleotide-addressed covalent 3’-terminal derivatization of small RNA strands for enrichment and visualization

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Experimental section

Synthesis of Ado-13-biotin cofactor 6

Analytical TLC was conducted on silica gel plates Silufol 60 F254 (Merck) with detection by UV light or basic potassium permanganate solution. Column flash chromatography was performed on silica gel Merck (60 Å, 230-400 mesh). ^{1}H spectra were recorded on Bruker 400 (400 MHz) spectrometer using solvent as an internal reference. HRMS data were obtained using Agilent 6230 TOF mass spectrometer (ESI). The separation of diastereoisomers was performed with preparative reversed-phase HPLC (column - Agilent Prep-C18, dimensions: 30×150, 10 μm, PN 413910-302). The yields of cofactors were determined according to the data of spectrometer NanoDrop ND-1000 UV/VS.

1-N-Biotinoylprop-2-yn-1-amine **8**. D-Biotin (0.05 g, 0.205 mmol), propargyl amine (0.013 mL, 0.205 mmol), TBTU (0.085 g, 0.265 mmol), and DIPEA (0.073 mL, 0.410 mmol) were reacted in DMF overnight at room temperature. The crude product was concentrated under reduced pressure and purified by Silica gel chromatography using CH_2Cl_2:MeOH (9:1, v/v, R_f = 0.5) as eluent to afford target product (0.057 g, 74%) as a white solid. mp 139–141°C (acetonitrile); **^{1}H NMR (400 MHz, D_2O, δ)**: 4.53-4.50 (m, 1H, C**H**CH_2S), 4.35-4.32 (m, 1H, CH**CH**NH), 3.87 (d, ^3J = 2.4 Hz, 2H, C$\mathbf{H_2}$C≡CH), 3.27-3.22 (m, 1H, C**H**S), 2.91 (dd, ^2J = 13 Hz, ^3J = 5 Hz, 1H, SC$\mathbf{H_a}$H), 2.69 (d, ^2J = 13 Hz, 1H, SCH$\mathbf{H_b}$), 2.57 (t, ^4J = 2.4 Hz, 1H, C≡C**H**), 2.20 (t, ^3J = 7.2 Hz, 2H, $CH_2CH_2CH_2C\mathbf{H_2}CO$), 1.70-1.46 (m, 4H, C$\mathbf{H_2}CH_2C\mathbf{H_2}CH_2CO$), 1.38-1.31 (m, 2H, $CH_2C\mathbf{H_2}CH_2CH_2CO$).

S-Adenosyl-(6-(4-(N-biotinoyl)aminomethyltriazol-1-yl)hex-2-yn-1-yl)-L-homocysteine formate **6**. To a stirred solution of enantiomerically pure cofactor **4** (1.62 mg, 0.0032 mmol) in ammonium formate buffer (0.5 mL, pH 3.5) 3-*N*-biotinoylprop-1-yne **8** (9 mg, 0.032 mmol), $CuSO_4 \times 5H_2O$ (0.79 mg, 0.0032 mmol) and L-ascorbate (1.27 mg, 0.0064 mmol) were added. After 15 min, the mixture was centrifuged. Preparative reversed-phase HPLC was used for purification of target cofactor eluting at a flow rate of 30 ml/min using 20 mmol/L ammonium formate buffer (pH = 3.5) as eluent A and 60% methanol in water as eluent B (the gradient of eluent is shown in table below).

T, min	0	2	10	11	13	14	22
A, %	100	100	60	0	0	100	100
B, %	0	0	40	100	100	0	0

Target cofactor was collected in fractions from 10.0 min till 11.4 min at the interval of 0.4 min. The solvent was evaporated under reduced pressure to afford target product **6** (1.7 mg, 68%) as a white solid. The concentration of cofactor in solution was determined by UV absorption (adenosine extinction - ε_{260} = 15400 L × mol^{-1} × cm^{-1} was used).

^{1}H NMR (400 MHz, D_2O, δ): 8.19 (s, 1H, ArH), 8.16 (s, 1H, ArH), 7.74 (s, 1H, CC**H**NN), 6.00 (d, ^3J = 5.7 Hz, 1H, C1'-H), 4.48-4.44 (m, 2H, NHC**H**CH_2S, C4'-H), 4.34 (t, ^3J = 7.2 Hz, 2H, C$\mathbf{H_2}$NNN), 4.28-4.27 (m, 1H, NHC**H**CHS), 4.24-4.21 (m, 2H, C≡CC$\mathbf{H_2}$S), 3.89-3.87 (m, 2H, SC$\mathbf{H_2}$CH), 3.74 (t, ^3J = 6.6 Hz, 1H, C**H**NH_2), 3.60-3.55 (m, 1H, SC$\mathbf{H_a}$HCH$_2$CH), 3.47-3.40 (m, 1H, SCH$\mathbf{H_b}$CH$_2$CH), 3.12-3.07 (m, 1H, C**H**S), 2.82 (dd, ^2J = 13.2 Hz, ^3J = 5 Hz, 1H, SC$\mathbf{H_a}$HCHNH), 2.61 (d, ^2J = 13.2 Hz, 1H, SCH$\mathbf{H_b}$CHNH), 2.28-2.24 (m, 2H, SCH$_2$C$\mathbf{H_2}$CH), 2.18-2.14 (m, 4H, $CH_2CH_2CH_2C\mathbf{H_2}CO$, $CH_2CH_2C\mathbf{H_2}C$≡C), 1.99-1.96 (m, 2H, $CH_2C\mathbf{H_2}CH_2C$≡C), 1.54-1.34 (m, 4H, C$\mathbf{H_2}CH_2C\mathbf{H_2}CH_2CO$), 1.18-1.12 (m, 2H, $CH_2C\mathbf{H_2}CH_2CH_2CO$),

C2'-**H,** C'3-**H**, CONHC$\mathbf{H_2}$ overlap with residual water signal in D_2O; **HRMS** calcd for $[M]^+$ $C_{33}H_{47}N_{12}O_7S_2$, m/z: 787.3127; found: 787.3126.

Expression and purification of recombinant HEN1

Plasmids pET-His-HEN1 containing the full-length methyltransferase from *Arabidopsis thaliana* with His_6-Tag fused to N-terminus of HEN1 were constructed as described previously.[1] Transformation of *Escherichia coli* BL21(DE3)RIL (Invitrogen) and purification of recombinant protein was performed as described previously.[2]

Preparation of nucleic acids substrates for experiments

All the single-stranded RNA oligonucleotides (**Table S3)** as well as 2'-*O*-methylated RNA oligonucleotide enhanced with LNA used in this study were chemically synthesized and HPLC purified by Metabion. All the single-stranded DNA oligonucleotides (**Tables S3 and S4**) were synthesized by IDT (ones complementary to let-7a and miR-26a) or Metabion (all the rest oligonucleotides used in this study). Cy5 or FAM fluorophore-3'-labeled DNA were synthesized by Metabion, DNA incorporating internal Cy3 fluorophore was produced by IBA. C6-amino labeled DNA oligonucleotide enhanced with LNA was synthesized by Exiqon (product number: 38771-06). Double-stranded substrates were prepared by annealing of corresponding complementary single-stranded oligonucleotides (**Scheme S1**). Mixture of single-stranded oligonucleotides in Annealing buffer (60 mM KCl, 0.2 mM $MgCl_2$, 6 mM HEPES, pH 7.5) was denatured at 85°C for 3 min and re-annealed by decreasing the temperature by -0.6°/min up to 4°C. Total RNA was purified from U2OS cell line using miRNeasy RNA Extraction Kit (QIAGEN) or TRIzol (Invitrogen) reagent according to manufacturer's recommendations. Total RNA from *E. coli* ER1727 was purified using TRIzol (Invitrogen) reagent according to manufacturer's recommendations.

Denaturing polyacrylamide gel electrophoresis analysis

Alkylation reactions were performed using 0.1 µM synthetic double-stranded nucleic acid substrates with 5'-P^{33}-labeled strand of interest and 0.25 µM HEN1 in the presence of 100 µM natural or synthetic cofactors such as AdoMet **1**, Ado-6-amine **2**, Ado-11-amine **3**, Ado-6-azide **4**, Ado-6-ethyne **5**, Ado-13-biotin **6** or Ado-18-biotin **7** as well as in the absence of cofactor (control) in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl Thermo Fisher Ribolock RNase Inhibitor) for 1 h at 37°C. The reactions were quenched manually by addition of Proteinase K to final concentration of 1 mg/ml in Quench buffer (20 mM Tris/HCl, 0.5 mM EDTA, 10 mM NaCl, 1.5% SDS, pH 7.5). Samples modified with AdoMet or Ado-6-azide and its controls without cofactor were additionally treated with sodium periodate as it was described previously.[1] Samples were denatured in 2X RNA Loading Dye (Thermo Fisher) for 5 min at 85°C and resolved on 13% denaturing polyacrylamide gel with 7 M urea under denaturing conditions. Phosphor imaging plates exposed to radioactive gels were scanned by FLA-5100 Image Reader with a red 635 nm laser and IP filter and analyzed using Multi Gauge software (Fujifilm).

Kinetic analysis of RNA/DNA methylation reaction

Methylation reactions under single-turnover conditions were carried out with 0.25 µM of HEN1, 0.1 µM double-stranded nucleic acid substrates with 5'-P^{33}-labeled strand of interest and 20-100 µM AdoMet in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl Thermo Fisher RiboLock RNase Inhibitor) at 37°C. Reactions were quenched with Proteinase K (Thermo Fisher) in the Quench buffer (20 mM Tris/HCl, 0.5 mM EDTA, 10 mM NaCl, 1.5% SDS, pH 7.5) after specified time periods using a Rapid Quench-Flow instrument RQF-3 (KinTek) or manually, then treated with sodium periodate as described previously (Yang et al. 2007) and analyzed on 13% denaturing polyacrylamide gel as described above. At least three technical replicates were analyzed for each time-point. Kinetic parameters were obtained by fitting experimental data to a single-exponential or double-exponential equation using GraFit software.[3]

H^3 incorporation analysis

1 µM of double-stranded substrate with 2-nt overhangs on both 3'-ends (miR173/miR173*, miR173/DNA173-R2:D2, miR173*/DNA173*-R2:D2 or DNA173-R2:D2/DNA173*-R2:D2) was incubated with 0.25 µM HEN1 and 20 µM [methyl-^{3}H] AdoMet in Reaction buffer at 37°C for 30 min. The reactions were quenched with Proteinase K (Thermo Fisher) in the Quench buffer (20 mM Tris/HCl, 0.5 mM EDTA, 10 mM NaCl, 1.5%

SDS, pH 7.5). Samples were spread on 2.3 cm DE-81 filters (Whatman), washed four times with 50 mM Na_3PO_4 (pH 7.0), twice with RNAse-free water, twice with ethanol and once with acetone and air-dried. The incorporation of ^3H-labeled methyl group into substrates was measured using in 3 ml of Rotiszint Eco lipophilic LSC Cocktail (Carl Roth) using a Beckman LS1801 scintillation counter. The background counts (400–600 dpm) were subtracted from the sample counts. Enzymatic activity was estimated by analyzing data from two replicates.

HPLC-MS analysis of modified RNA/DNA products

1 µM of synthetic miR-210/DNA210-R2:D2 or miR-210*/DNA210*-R2:D2 duplexes were incubated with 1 µM HEN1 in the absence of cofactor (control) or in the presence of 100 µM AdoMet, Ado-6-amine, Ado-6-azide, Ado-6-ethyne or Ado-13-biotin in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl RiboLock RNase Inhibitor) for 90 min at 37°C. Reaction mixtures were extracted with an equal volume of chloroform and nucleic acids precipitated with ethanol and 0.1 mg/ml glycogen (Thermo Fisher). Samples were digested with 0.01 u/µl P1 nuclease (Sigma) in Digestion buffer (20 mM NaOAc, pH 5.2, 1 mM $Zn(OAc)_2$) for 40 min at 65°C. Than 0.02 u/µl FastAP (Thermo Fisher) and FastAP buffer up to 1X was added for dephosphorylation, samples were incubated for overnight at 37°C. Mixtures were incubated at 80°C for 10 min and centrifuged at 20 000 x g for 40 min at 4°C. Supernatant was analyzed on a 1290 Infinity HPLC system (Agilent Technologies) using Discovery SH C18 column (Supelco) in 20 mM ammonium formate (pH 3.5) with a gradient to 80% methanol. ESI-MS analysis was performed on a Q-TOF 6520 mass spectrometer in positive ion mode and analyzed using MassHunter Workstation software (Agilent Technologies).

One-step biotin labeling of RNA/DNA hybrids

0.2 µM of corresponding double-stranded substrate was incubated for 1 hour at 37°C with 0.5 µM HEN1 and 200 µM Ado-13-biotin or Ado-18-biotin or AdoMet (control). Reactions were stopped with SDS and Proteinase K as described earlier [4], than samples were precipitated with ethanol and dissolved in water up to 2 µM concentration of the substrate. For extraction of biotinylated RNAs samples were precipitated and streptavidin-coupled magnetic beads Dynabeads MyOne Streptavidin T1 (Thermo Fisher) were used according to manufacturer's recommendations. For detaching of extracted biotinylated miRNA from magnetic beads samples were incubated in 47.5% formamide, 10 mM EDTA at 85°C for 5 minutes. Samples were analyzed on 13% denaturing polyacrylamide gel as described above.

Two-step biotin labeling of RNA/DNA hybrids

0.2 µM of corresponding double-stranded substrate was incubated for 1 hour at 37°C with 0.5 µM HEN1 and 200 µM Ado-6-amine or AdoMet (control). Reactions were stopped with SDS and Proteinase K as described above, than samples were precipitated with ethanol and re-suspended in carbonate buffer (pH 9.5) to 0.4 µM of double-stranded substrate. 20 mg/ml of biotin N-hydroxysuccinimide ester (NHS-biotin, Sigma, M_r = 341.38) or succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin, Thermo Fisher, M_r = 504.65) with detachable biotin group was added for biotinylation and samples were incubated at room temperature for 150 min. For extraction of biotinylated RNAs samples were precipitated and streptavidin-coupled magnetic beads Dynabeads MyOne Streptavidin T1 (Thermo Fisher) were used according to manufacturer's recommendations. To detach the biotin group samples labeled with NHS-SS-biotin were treated with 20% β-mercaptoethanol or 10 mM dithiothreitol at room temperature for 30 min. For detaching of extracted miRNA labeled with NHS-biotin samples were incubated in 47.5% formamide 10 mM EDTA at 85°C for 5 minutes. All the samples were precipitated and analyzed on 13% denaturing polyacrylamide gel as described above.

Two-step fluorescent labeling of RNA/DNA hybrids

0.2 µM of corresponding double-stranded substrate was incubated for 1 hour at 37°C with 0.5 µM HEN1 and 200 µM Ado-6-amine or AdoMet (control). Reactions were stopped with SDS and Proteinase K as described above, samples were precipitated with ethanol and dissolved in the Borate buffer (33.8 mM $Na_2B_4O_7$, 50 mM H_3BO_3, pH 9.5) to a final concentration of 2.5 µM and labeled with 0.1 mg/ml Cy5 NHS ester (GE Healthcare)

for 4 h at room temperature. Excess of Cy5 NHS-ester was removed by RNA Clean and Concentrator-25 columns (Zymo Research). Samples were mixed with equal volume of 66% glycerol and resolved on non-denaturing 12% polyacrylamide gel. After detection of fluorophore by FLA-5100 Image Reader (Fujifilm) using a red 635 nm laser gels were stained with ethidium bromide and RNA was visualized using a blue 473 nm laser.

Selective miRNA methylation in mixture with *E. coli* total RNA

0.1 μM P^{33}-labeled miR173 or miR-210 mixed up with the total RNA pre-extracted from *E. coli* ER1727 (see the Preparation of nucleic acids substrates for experiments section) in ratios of 1:10, 1:50 and 1:100 and 0.12 μM single-stranded corresponding complementary DNA-R2:D2. Obtained mixtures were re-annealed and incubated with 100 μM AdoMet and 0.25 μM HEN1 or without the protein. All the samples were treated with $NaIO_4$, mixed with 2X RNA Loading Dye (Thermo Fisher), denatured for 5 min at 85°C and analyzed on 13% denaturing polyacrylamide gel as described above.

Selective FAM-DNA-directed miRNA alkylation in a mixture with U2OS cell line total RNA

0.1 μM P^{33}-labeled miR173 spike-in premixed with total RNA from human bone osteosarcoma U2OS cells (miR173 is absent in this cell line) in 1:10 ratio and was re-annealed with complementary DNA or complementary DNA containing FAM fluorophore at 3'-terminus. Samples were alkylated for 1 hour at 37ºC in the presence of 0.25 μM HEN1 and 100 μM Ado-11-amine cofactor. Samples were analyzed on 13% denaturing polyacrylamide gel as described above.

Selective DNA-directed miRNA alkylation in a mixture of different miRNAs

Mixtures composed of miR173, miR-26a* and let-7a* (0.1 μM each) with only one of them P^{33}-labeled (miR-26a* or let-7a*) were annealed with 0.12 μM DNA173-R2:D2, DNA26a*-R2:D2 or DNA7a*-R2:D2. Six obtained mixtures were incubated with 100 μM Ado-6-amine and 0.25 μM HEN1 or without the protein. Samples were analyzed on 13% denaturing polyacrylamide gel as described above.

Analysis of RNA/SA-DNA modification

miRNA of interest was annealed with complementary DNA oligonucleotide, containing streptavidin-specific aptamer (SA) [5] on its 3'-terminus. 0.2 μM of corresponding P^{33}-miRNA/SA-DNA substrate was incubated with 0.25 μM of HEN1 and 100 μM of AdoMet, Ado-6-amine or Ado-11-amine or without cofactor (control) in the Reaction buffer for 1 h at 37°C. The reactions were quenched manually by addition of Proteinase K (Thermo Fisher) to final concentration of 1 mg/ml in Quench buffer (20 mM Tris/HCl, 0.5 mM EDTA, 10 mM NaCl, 1.5% SDS, pH 7.5). Samples modified with AdoMet and its controls without cofactor were additionally treated with sodium periodate as it was described previously. All the samples were precipitated and analyzed on 13% denaturing polyacrylamide gel as described above.

Analysis of RNA/SA-DNA extraction

2 μM of single-stranded SA-DNA or double-stranded miRNA/SA-DNA substrate with P^{33}-labeled either RNA or DNA strand were treated with streptavidin-coupled magnetic beads Dynabeads MyOne Streptavidin T1 (Thermo Fisher) according to manufacturer's recommendations. Alternatively, 2 μM P^{33}-labeled miRNA was hybridized with 2 μM of complementary SA-DNA, incubated with 100 μM Ado-6-amine together with 0.5 μM HEN1 or without the protein (control) for 1 hour at 37°C and then treated with streptavidin-coupled magnetic beads. For detaching of extracted miRNA from magnetic beads samples were incubated in 47.5 % formamide, 10 mM EDTA at 85°C for 5 minutes. Input, magnetic beads fraction and supernatant fraction were analyzed on 13% denaturing polyacrylamide gel as described above.

Selective labeling with fluorophore and aptamer-based extraction of individual miRNA

Four mixtures composed of let-7a, miR-26a, miR173 and miR-210 (9 μM each) with only one of them P^{33}-labeled in each mixture were annealed with 18 μM SA210-R2:s0. Then all four mixtures were incubated with

0.4 µM HEN1 and 200 µM Ado-6-azide, 1.5 µM of each miRNA in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl Thermo Fisher RiboLock RNase Inhibitor) at 37°C for 100 minutes. After that samples were treated with magnetic beads according to manufacturer's recommendations and precipitated. Samples were re-dissolved in water up to 5 µM of each RNA and incubated with freshly prepared CuBr : TBTA (3.3 mM : 6.6 mM) DMSO solution and 0.5 mg/ml Cy5.5-alkyne in 50% DMSO for 2 hours at 42°C. Samples were analyzed on 13% denaturing polyacrylamide gel, which was scanned with Fujifilm FLA-5100 gel scanner using far red 670 nm laser with R705 LPFR filter for Cy5.5 detection and red 635 nm laser with IP filter for P^{33}-labeled RNA detection. All obtained gel views were analyzed with MultiGauge (Fujifilm) software.

Förster resonance energy transfer (FRET) assay in solution

0.1 µM of miR173/DNA173-R2:D0 or miR173/DNA173-R2:D0-Cy3 heteroduplex was incubated with 0.25 µM HEN1 and 100 µM Ado-6-amine **2** or without the cofactor in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl Thermo Fisher Ribolock RNase Inhibitor) for 1 h at 37°C. All four reaction mixtures were extracted with an equal volume of chloroform and nucleic acids precipitated with ethanol and 0.1 mg/ml glycogen (Thermo Fisher). Samples were resuspended in Borate buffer (pH 8.6), to a final RNA/DNA concentration of 0.8 µM and incubated with 1 mg/ml Cy5 NHS ester (GE Healthcare) for 3 h at 37°C. To remove the residual Cy5 NHS ester samples were precipitated three times in 96 % ethanol and annealed in Annealing buffer (60 mM KCl, 0.2 mM $MgCl_2$, 6 mM HEPES, pH 7.5) as described in the section about Preparation of the nucleic acids substrates. Then all four samples were diluted to 20 nM in TE buffer (pH 7.5) with 1/100 volume of β-mercaptoethanol. The fluorescence was analyzed with spectrofluorimeter FluoroMax-3 (Jobin Yvon Horiba) at 500 nm excitation and 520-580 nm emission as well as at 590 nm excitation and 610-580 nm emission, 1 nm increment, 0.2 sec integration time and excitation/emission slit of 5 nm. The background fluorescence of RNA/DNA duplex in the buffer was subtracted from RNA/DNA-Cy3, RNA-Cy5/DNA and RNA-Cy5/DNA-Cy3 spectra. The obtained results were processed using GraFit software.

FRET assay on a gel

The alkylation reaction was performed using 0.1 µM of miR173/DNA173-R2:D0 or miR173/DNA173-R2:D0-Cy3 heteroduplexes, 0.25 µM HEN1 and 100 µM Ado-6-azide **4** cofactor in the Reaction buffer (10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.25 mM $MgCl_2$, 0.1 mg/ml BSA, 0.2 u/µl Thermo Fisher Ribolock RNase Inhibitor) for 1 h at 37°C. Additionally, miR173/DNA173-R2:D0-Cy3 heteroduplex was incubated in the absence of cofactor (Cy3 control). After chlorophorm extraction and ethanol precipitation the reaction products were resuspended in water. The click-chemistry labeling was performed using 1.4 µM of RNA/DNA, freshly prepared CuBr : TBTA (3.3 mM : 6.6 mM) DMSO solution and 0.5 mg/ml Cy5.5-alkyne in 50 % DMSO for 2 hours at 42°C. Samples were ethanol-precipitated, dissolved in water and fractionated on 12 % non-denaturing polyacrylamide gel, which was scanned with Fujifilm FLA-5100 gel scanner using green 532 nm laser with O575 LPG filter for Cy3 donor detection, far red 670 nm laser with R705 LPFR filter for Cy5.5 acceptor detection and green 532 nm laser with R705 LPFR filter for FRET detection. The obtained data was analyzed using MultiGauge (Fujifilm) software.

Supporting references

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Tables

Table S1. Comparison of HEN1-mediated methylation rates for miR173 and miR173* strands in natural substrate miR173/miR173* with 2-nt 3'-overhangs on both RNA strands, blunt-ended RNA/RNA substrate miR173/RNA173*-R0:R0s or unnatural RNA/DNA substrates with 2-nt 3'-overhangs miR173/DNA173-R2:D2, DNA173*-R2:D2/miR173*. Observable P^{33}-labeled strand is shown in bold. Corresponding time-course experimental data for unnatural RNA/RNA and RNA/DNA substrates are plotted in **Figures S2** and **S6**. Methylation rates of RNA/RNA substrate are from [4].

Substrate	*Rate, min^{-1} (Amplitude, %)*
miR173*/miR173**	4.5 ± 0.2 (100%)
miR173*/RNA173*-R0:R0*	0.74 ± 0.05 (100%)
miR173*/DNA173*-R2:D2*	27.9 ± 0.8 (100%)
*miR173/**miR173****	35 ± 3 (79 ± 3%) 2.9 ± 1 (21 ± 3%)
*miR173/**RNA173***-R0:R0*	33 ± 3.7 (49 ± 2%) 1.1 ± 0.2 (51 ± 3%)
DNA173-R2:D2/**miR173****	7.6 ± 0.2 (100%)

Table S2. ESI-MS analysis of 2'-*O*-modified 3'-terminal nucleosides of miR-210 and miR-210*.

Substrate	*Synthetic cofactor*	*Compound*	*Formula*	*Theoretical m/z*	*Observed m/z*
<u>miR-210(A)</u>/ *DNA210-R2:D2*	AdoMet **1**	$[Ado\text{-}CH_3 + H]^+$	$[C_{11}H_{15}N_5O_4]^+$	282.1197	282.1196
		$[Ado\text{-}CH_3 + Na]^+$	$[C_{11}H_{14}N_5O_4Na]^+$	304.1016	304.0997
	Ado-6-amine **2**	$[Ado\text{-}6\text{-}NH_2 + H]^+$	$[C_{16}H_{22}N_6O_4]^+$	363.1776	363.1777
	Ado-6-azide **4**	$[Ado\text{-}6\text{-}N_3 + H]^+$	$[C_{16}H_{20}N_8O_4]^+$	389.1681	389.1693
		$[Ado\text{-}6\text{-}N_3 + Na]^+$	$[C_{16}H_{19}N_8O_4Na]^+$	411.1500	411.1492
	Ado-6-ethyne **5**	$[Ado\text{-}6\text{-}C_2 + H]^+$	$[C_{18}H_{20}N_5O_4]^+$	372.1667	372.1680
		$[Ado\text{-}6\text{-}C_2 + Na]^+$	$[C_{18}H_{19}N_5O_4Na]^+$	394.1486	394.1472
	Ado-13-biotin **6**	[Ado-13-Biotin + Na]$^+$	$[C_{29}H_{38}N_{11}O_6SNa]^+$	692.2697	692.2717
		[Ado-13-Biotin + H]$^+$	$[C_{29}H_{39}N_{11}O_6S]^+$	670.2878	670.2886
		[Ado-13-Biotin + 2H]$^{+2}$	$[C_{29}H_{40}N_{11}O_6S]^{+2}$	335.6476	335.6479
<u>miR-210*(G)</u>/ *DNA210*-R2:D2*	AdoMet **1**	$[Guo\text{-}CH_3 + H]^+$	$[C_{11}H_{15}N_5O_5]^+$	298.1146	298.1136
		$[Guo\text{-}CH_3 + Na]^+$	$[C_{11}H_{14}N_5O_5Na]^+$	320.0965	320.0926
		$[Guo\text{-}CH_3 + K]^+$	$[C_{11}H_{14}N_5O_5K]^+$	336.0705	336.0663
		$[Gua + H]^+$	$[C_5H_5N_5O]^+$	152.0567	152.0550
	Ado-6-amine **2**	$[Guo\text{-}6\text{-}NH_2 + H]^+$	$[C_{16}H_{22}N_6O_5]^+$	379.1725	379.1721
		$[Guo\text{-}6\text{-}NH_2 + Na]^+$	$[C_{16}H_{21}N_6O_5Na]^+$	401.1544	401.1513
	Ado-6-azide **4**	$[Guo\text{-}6\text{-}N_3 + H]^+$	$[C_{16}H_{20}N_8O_5]^+$	405.1637	405.1647
		$[Guo\text{-}6\text{-}N_3 + Na]^+$	$[C_{16}H_{19}N_8O_5Na]^+$	427.1449	427.1446
		$[Guo\text{-}6\text{-}N_3 + K]^+$	$[C_{16}H_{19}N_8O_5K]^+$	443.1189	443.1199
	Ado-6-ethyne **5**	$[Guo\text{-}6\text{-}C_2 + H]^+$	$[C_{18}H_{21}N_5O_5]^+$	388.1616	388.1605
		$[Guo\text{-}6\text{-}C_2 + Na]^+$	$[C_{18}H_{20}N_5O_5Na]^+$	410.1435	410.1407
		$[Guo\text{-}6\text{-}C_2 + K]^+$	$[C_{18}H_{20}N_5O_5K]^+$	426.1175	426.1147
		[Gua + H]+	$[C_5H_5N_5O]^+$	152.0567	152.0548
	Ado-13-biotin **6**	[Guo-13-Biotin + H]$^+$	$[C_{29}H_{39}N_{11}O_7S]^+$	686.2828	686.2830
		[Guo-13-Biotin + Na]$^+$	$[C_{29}H_{38}N_{11}O_7SNa]^+$	708.2647	708.2650
		[Guo-13-Biotin + 2H]$^{+2}$	$[C_{29}H_{40}N_{11}O_7S]^{+2}$	343.6451	343.6451

Table S3. Sequences of RNA, DNA, oligonucleotides with LNA and chemically-modified oligonucleotides.

Type	*Name*	*Sequence*
RNA	miR173 [a]	5‘-UUCGCUUGCAGAGAGAAAUCAC-3‘
	miR173* [a]	5‘-GAUUCUCUGUGUAAGCGAAAG-3‘
	miR-26a [b]	5‘-UUCAAGUAAUCCAGGAUAGGCU-3‘
	miR-26a* [b]	5‘-CCUAUUCUUGGUUACUUGCACG-3‘
	miR-210 [b]	5‘-CUGUGCGUGUGACAGCGGCUGA-3‘
	miR-210* [b]	5‘-AGCCCCUGCCCACCGCACACUG-3
	let-7a [b]	5‘-UGAGGUAGUAGGUUGUAUAGUU-3‘
	let-7a* [b]	5‘-CUAUACAAUCUACUGUCUUUCC-3‘
	miR173-U	5‘-UUCGCUUGCAGAGAGAAAUCAU-3‘
	miR173-G	5‘-UUCGCUUGCAGAGAGAAAUCAG-3‘
	miR173-A	5‘-UUCGCUUGCAGAGAGAAAUCAA-3‘
	RNA173*-R0/R0	5‘-GUGAUUUCUCUCUGCAAGCGAA-3‘
	siR173	5’-UUAACGCUUGCAGAGAGAAUCAC-3’
	siR173*	5’- GAUUCUCUCUGCAAGCGUUAAAG-3’
RNA-LNA	RLNA210-R2:D0-CH_3	5‘-AGCC**G**CUG**U**CAC**A**CGCACA**G^{m}** -3‘
DNA-LNA	DLNA7a*-R1:D–1-NH_2 [c]	*5’-GAAAGACAGTAGATTGTAT**A^{NH2}**-3’*
DNA with fluorophores	DNA7a-R2:D2-Cy5	*5’-CTATACAACCTACTACCTCAC**C^{Cy5}**-3‘*
	DNA173-R2:D2-FAM	*5’-GATTTCTCTCTGCAAGCGAAA**G^{FAM}**-3‘*
	DNA173-R2:D0-Cy3	*5’-GAT**T^{Cy3}**TCTCTCTGCAAGCGAA-3‘*
DNA	DNA7a-R0:D0	*5‘-AACTATACAACCTACTACCTCA-3‘*
	DNA7a-R2:D0	*5‘-CTATACAACCTACTACCTCA-3*
	DNA7a-R2:D1	*5‘-CTATACAACCTACTACCTCAC-3‘*
	DNA7a-R2:D2	*5‘-CTATACAACCTACTACCTCACC-3‘*
	DNA7a-R2:D3	*5‘-CTATACAACCTACTACCTCACCA-3‘*
	DNA7a-R2:D5	*5‘-CTATACAACCTACTACCTCACCAGC-3‘*
	DNA7a*-R0:D0	*5‘-GGAAAGACAGTAGATTGTATAG-3‘*
	DNA26a-R0:D0	*5‘-AGCCTATCCTGGATTACTTGAA-3‘*
	DNA26a-R2:D0	*5‘-CCTATCCTGGATTACTTGAA-3‘*
	DNA26a-R2:D1	*5‘-CCTATCCTGGATTACTTGAAC-3‘*
	DNA26a-R2:D2	*5‘-CCTATCCTGGATTACTTGAACG-3‘*
	DNA26a-R2:D3	*5‘-CCTATCCTGGATTACTTGAACGC-3‘*
	DNA26a-R2:D5	*5‘-CCTATCCTGGATTACTTGAACGCAG-3‘*
	DNA26a*-R2:D2	*5‘-TGCAAGTAACCAAGAATAGGCT-3‘*
	DNA173-R0:D0	*5‘-GTGATTTCTCTCTGCAAGCGAA-3‘*
	DNA173-R2:D0	*5‘-GATTTCTCTCTGCAAGCGAA-3‘*
	DNA173-R2:D1	*5‘-GATTTCTCTCTGCAAGCGAAA-3‘*
	DNA173-R2:D2	*5‘-GATTTCTCTCTGCAAGCGAAAG-3‘*
	DNA173-R2:D5	*5‘-GATTTCTCTCTGCAAGCGAAAGGAG-3‘*
	DNA173-R2:D7	*5‘-GATTTCTCTCTGCAAGCGAAAGGATAG-3‘*
	DNA173*-R2:D2	*5‘-TTCGCTTACACAGAGAATCAC-3‘*
	DNA173s-R2:D2	*5‘-GATTCTCTCTGCAAGCGTTAAAG-3‘*
	DNA173s*-R2:D2	*5‘-TTAACGCTTGCAGAGAGAATCAC-3‘*
	DNA210-R2:D2	*5‘-AGCCGCTGTCACACGCACAGTG-3‘*
	DNA210-R0:D0	*5‘-TCAGCCGCTGTCACACGCACAG-3‘*
	DNA210*-R2:D2	*5‘-GTGTGCGGTGGGCAGGGGCTGA-3‘*

[a] – identical to *Arabidopsis thaliana* miRNAs; [b] – identical to *Homo sapiens* miRNAs.

[c] – oligonucleotide is enhanced with LNA (Exiqon; product number: 38771-06).

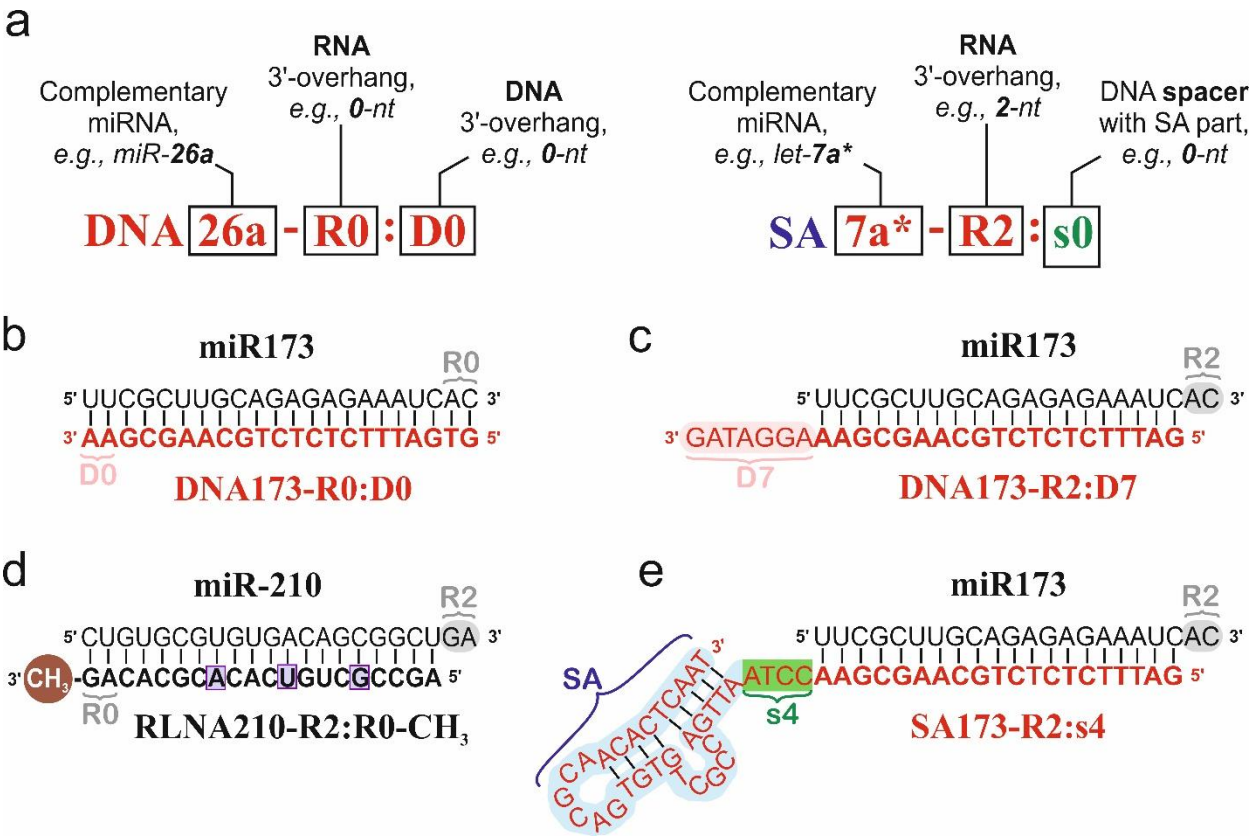
T – DNA nucleotides in italic; U – RNA nucleotides in regular; **N** - Locked nucleotides in squares;

G^{m} – 2’-*O*-methylguanosine; ***A^{NH2}*** – C6-amino labeled deoxyadenosine;

C^{Cy5}*, *G^{FAM} and ***T^{Cy3}*** **–** fluorophore-labeled deoxyribonucleotides.

Table S4. SA-DNA oligonucleotides. Sequences complementary to corresponding miRNAs are shown in bold, streptavidin-specific aptamer [5] depicted in italic.

Name	*Sequence*
Complementary to miR173	
SA173-R0:s0	5‘- GT**GATTTCTCTCTGCAAGCGAA**– – – –*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA173-R2:s0	5‘- **GATTTCTCTCTGCAAGCGAA**– – – –*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA173-R0:s4	5‘- GT**GATTTCTCTCTGCAAGCGAA**CCTA*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA173-R2:s4	5‘- **GATTTCTCTCTGCAAGCGAA**CCTA*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
Complementary to miR-210	
SA210-R0:s0	5‘- TC**AGCCGCTGTCACACGCACAG**– – – –*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA210-R2:s0	5‘- **AGCCGCTGTCACACGCACAG**– – – –*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA210-R0:s4	5‘- TC**AGCCGCTGTCACACGCACAG**CCTA*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘
SA210-R2:s4	5‘- **AGCCGCTGTCACACGCACAG**CCTA*ATTGACCGCTGTGTGACGCAACACTCAAT*-3‘



Scheme S1. Principles of design and entitlements of RNA/DNA heteroduplexes used in this study. a. Explanation of the entitlements system. “DNA” and “RNA” in title represent corresponding type of nucleic acids; “SA” means DNA oligonucleotide containing streptavidin aptamer sequence; “RLNA” and “DLNA” – stand for DNA or RNA oligonucleotide, respectively, enhanced with locked nucleic acid (LNA) nucleotides. **b-e.** Schematic examples of RNA/DNA heteroduplexes. **b.** RNA/DNA duplex with both blunt ends (-R0:D0). **c.** RNA/DNA duplex with 2-nt RNA 3’-overhang and 7-nt DNA 3’-overhang. **d.** miR-210 annealed to complementary RNA, enhanced with three LNA nucleotides and 2’-*O*-CH_3 modification on 3’-terminus, forms 2-nt overhang and one blunt end. **e.** miR173/SA duplex with 2-nt RNA 3’-overhang and 4-nt spacer. RNA sequences are shown in black, DNA – in red, LNA – in purple boxes. Covalent modification is depicted in fuchsia circle (CH_3 – methyl group). RNA overhangs are highlighted in grey; DNA overhangs – in pink. The sequence of 29-nt long streptavidin-specific DNA aptamer (SA) is highlighted in blue, green box depicts spacer sequence between SA and miRNA-complementary part.

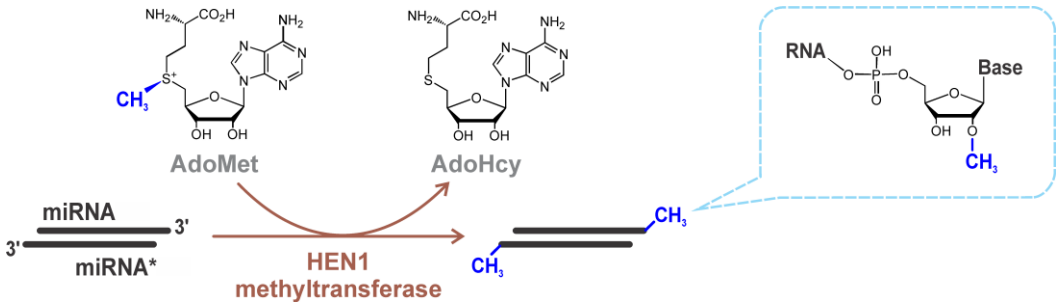


Figure S1. Natural HEN1 methylation reaction. HEN1 methyltransferase from *Arabidopsis thaliana* catalyzes S-adenosyl-L-methionine (AdoMet)-dependent 2'-*O*-methylation of the 3'-terminal nucleotides of miRNA/miRNA* and siRNA/siRNA* duplexes.

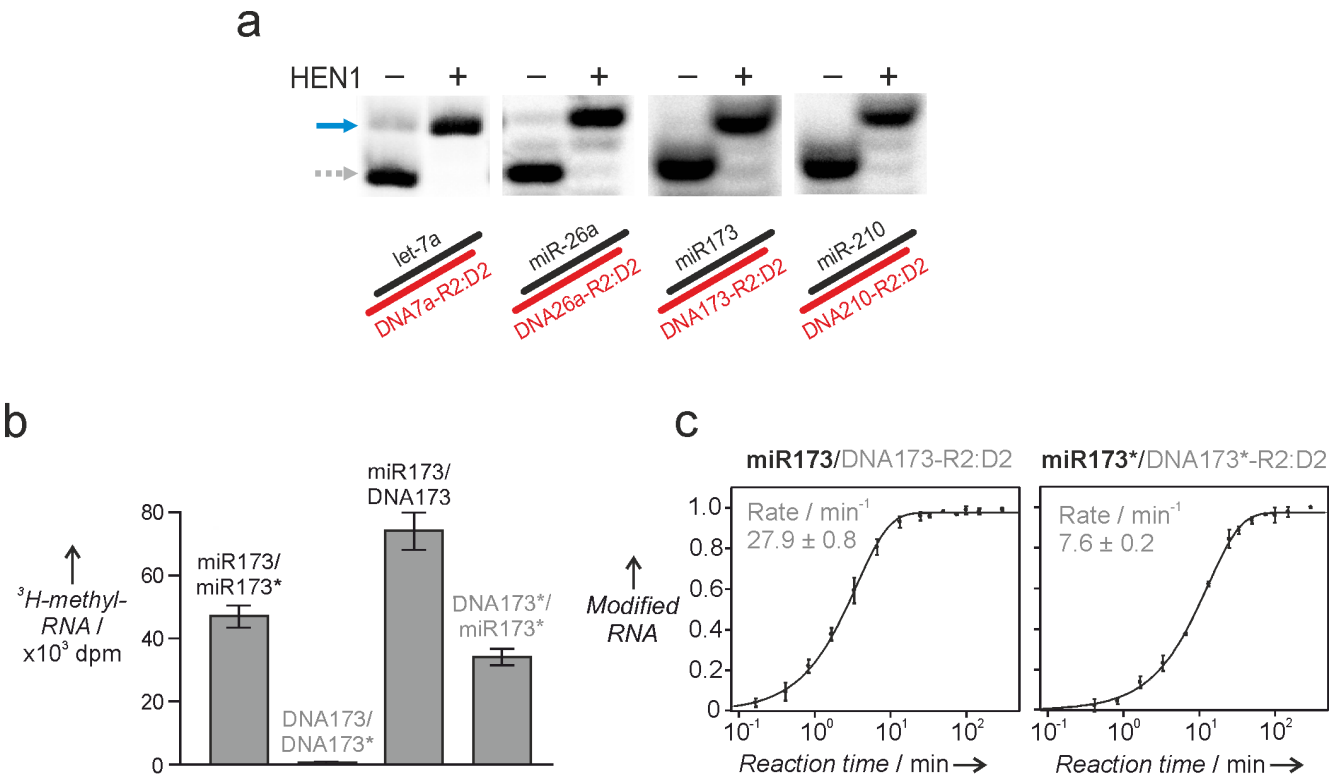


Figure S2. Methylation of RNA/DNA heteroduplexes containing 2-nt RNA 3'-overhangs. a. Methylation of let-7a, miR-26a, miR173, miR-210 miRNAs in heteroduplexes with complementary DNA oligonucleotides (DNA7a-R2:D2, DNA26a-R2:D2, DNA173-R2:D2 or DNA210-R2:D2, respectively). After incubation with HEN1 and AdoMet the samples were treated with $NaIO_4$, which eliminates 3'-terminal nucleoside from the unmodified RNA substrates (grey dashed arrows), increasing the RNA mobility on denaturing polyacrylamide gel. Methylated RNAs (blue solid arrows) are protected from nucleoside elimination. **b.** RNA/RNA and RNA/DNA but not DNA/DNA duplexes with 2-nt overhangs are methylated by HEN1 enzyme. 1 µM substrate was incubated with 0.25 µM HEN1 and 20 µM [methyl-^{3}H] AdoMet at 37°C for 30 min. The incorporation of ^{3}H-labeled methyl groups into substrates was measured using scintillation counter (Beckman). **c.** Methylation kinetics of miR173 or miR173* strand in RNA/DNA duplexes. 0.1 µM 5'-P^{33}-labeled miR173 (left) or miR173* (right) in heteroduplexes incubated with 0.25 µM HEN1 and 20 µM (dashed lines) or 100 µM (solid lines) AdoMet at 37°C. Reactions were stopped by adding SDS and Proteinase K, then samples were treated with sodium periodate and analyzed on denaturing polyacrylamide gel. Methylation rates were calculated using single-exponential equation.

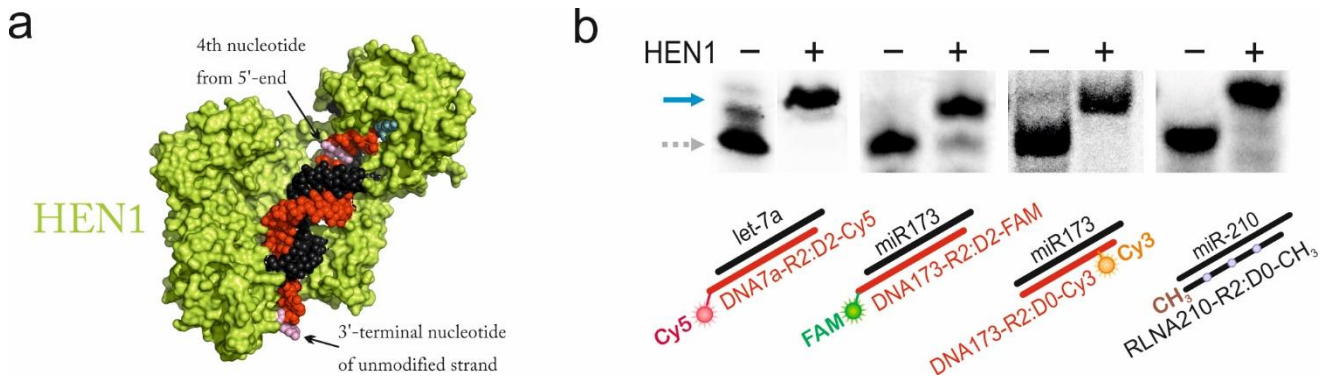


Figure S3. Methylation of miRNAs in heteroduplexes with modified complementary strand. a. Tertiary structure of the HEN1-RNA-AdoHcy complex (3HTX). The relative positions of nucleotides with incorporated reporters: fourth and 3'-terminal nucleotides (pink) of the unmodified RNA strand (space-fill, red). Modified strand of the duplex and AdoHcy are shown as black and blue space-fill, correspondingly, and the protein is coloured in green. **b.** Modification of let-7a in duplex with DNA labeled with Cy5 fluorophore at the 3'-end; miR173 in duplex with 3'-fluorescein (FAM) labeled DNA; miR173 in duplex with DNA containing internal Cy3 attached to 4th position from 5'-terminus; miR-210 in duplex with 3'-methylated RNA with internal LNA nucleotides.

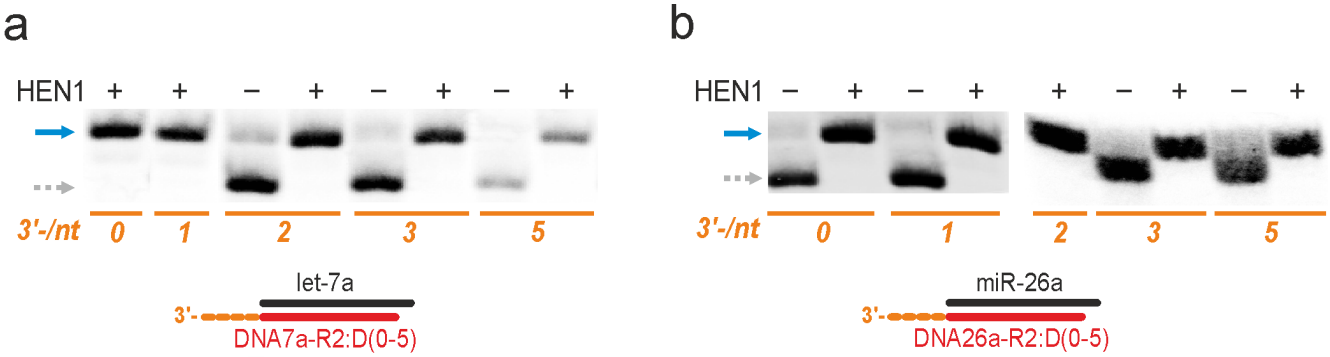


Figure S4. Methylation of RNA/DNA substrates with DNA 3'-overhangs of different length. Methylation of let-7a (**a**) and miR-26a (**b**) in RNA/DNA duplexes with 2-nt RNA strand 3'-overhang and DNA strand possessing 0-5-nt single-strand 3'-extensions (orange dashed lines on a scheme). All studied RNA/DNA substrates are fully methylated. Methylated RNAs are depicted by the solid arrow, non-modified – the dashed arrow.

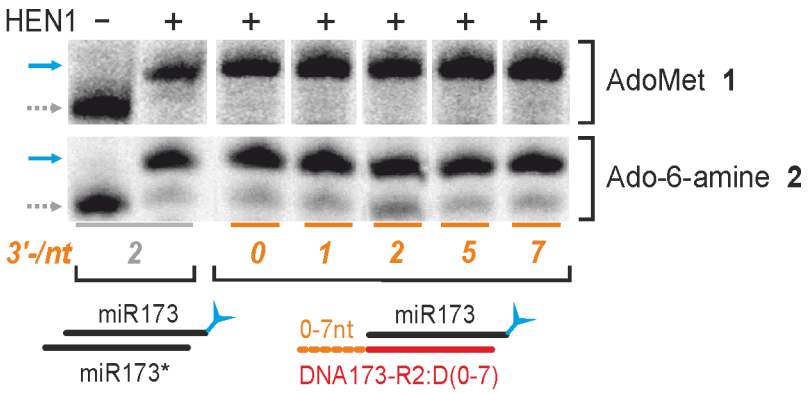


Figure S5. Modification of miR173/DNA173 duplexes with DNA strand possessing 0-7-nucleotide 3'-overhangs. All substrates were fully modified using AdoMet **1** or Ado-6-amine **2**. miR173/miR173* RNA duplex served as control. Solid arrows indicate modified samples, dashed arrows – RNA without modification.

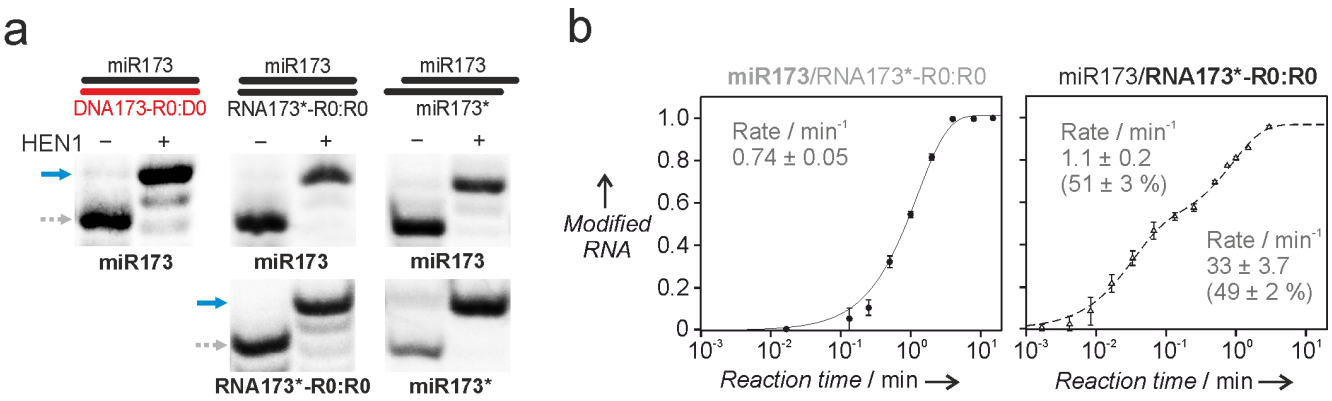


Figure S6. Methylation of blunt-ended substrates. a. Top row: methylation of miR173 strand of RNA/DNA duplex with blunt ends (left), RNA/RNA duplex with blunt ends (center) or natural RNA/RNA duplex with 2-nt overhangs on both 3'-ends (right). Bottom row: methylation of RNA strands, complementary to miR173 in RNA/RNA substrates. Solid arrows depict methylated RNA, dashed arrows – unmodified RNA. **b.** Methylation kinetics of blunt-ended RNA/RNA duplexes. miR173 RNA (left) or complementary RNA173*-R0:R0 strand of duplex (right) were 5'-P^{33}-labeled for visualization. 0.1 µM of each duplex was incubated with 0.25 µM HEN1 and 100 µM AdoMet at 37°C. Reactions were stopped by Proteinase K in SDS-containing buffer. Samples were treated with sodium periodate and analyzed on denaturing polyacrylamide gel. Methylation rates were calculated using single-exponential (left) or double-exponential (right) equations.

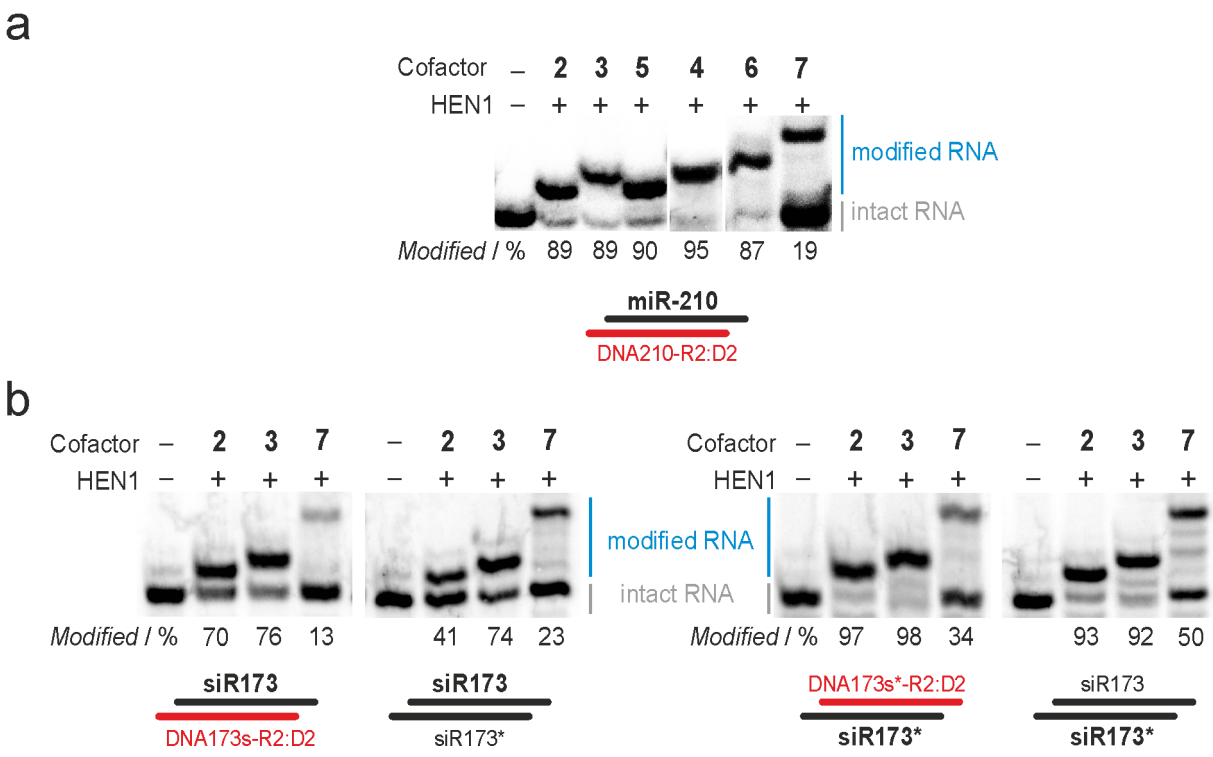


Figure S7. Alkylation of RNA/DNA duplexes with 2-nt overhangs. a. Alkylation of miR-210/DNA210-R2:D2 with (left to right) Ado-6-amine **2**, Ado-11-amine **3**, Ado-6-ethyne **5**, Ado-6-azide **4**, Ado-13-biotin **6** and Ado-18-biotin **7**. The mobility of radiolabeled RNA strands varies according to the size and charge of radicals transferred from different synthetic cofactors. Modified RNA is depicted by blue stripes, intact – grey stripes. **b.** Comparison of alkylation efficiency of siRNA/siRNA* and siRNA/DNA duplexes with 2-nt 3'-overhangs (P^{33}-labeled strand in bold). siR173 (left block) or siR173* (right block) was annealed to fully-complementary DNA (left gel in each block) or fully-complementary RNA (right gel in each block) forming duplexes with 2-nt 3'-overhangs on both the strands. Obtained substrates were incubated with HEN1 and Ado-6-amine **2**, Ado-11-amine **3**, Ado-18-biotin **7** or without protein and cofactor (control).

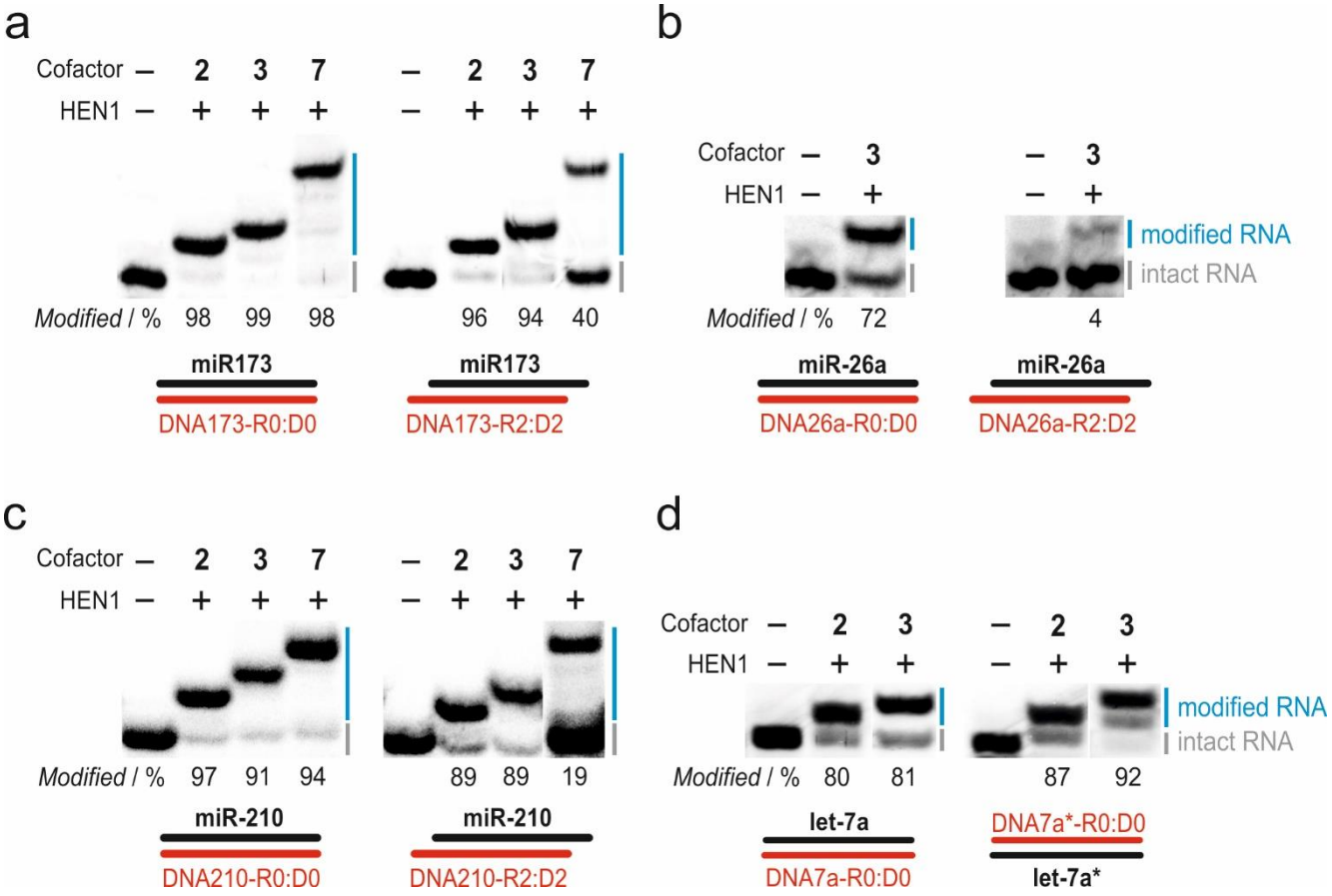


Figure S8. HEN1 alkylates blunt-ended miRNA/DNA substrates. Comparison of alkylation of miR173 (**a**), miR-26a (**b**), miR-210 (**c**) in RNA/DNA heteroduplexes with blunt ends (left) or 2-nt overhangs on 3'-termini (right) with Ado-6-amine **2**, Ado-11-amine **3** and Ado-18-biotin **7**. **d.** Alkylation of blunt-ended let-7a/DNA7a-R0:D0 (left) or let-7a*/DNA7a*-R0:D0 (right) using Ado-6-amine **2** and Ado-11-amine **3**. Modified RNA is highlighted with blue stripes, intact – grey stripes.

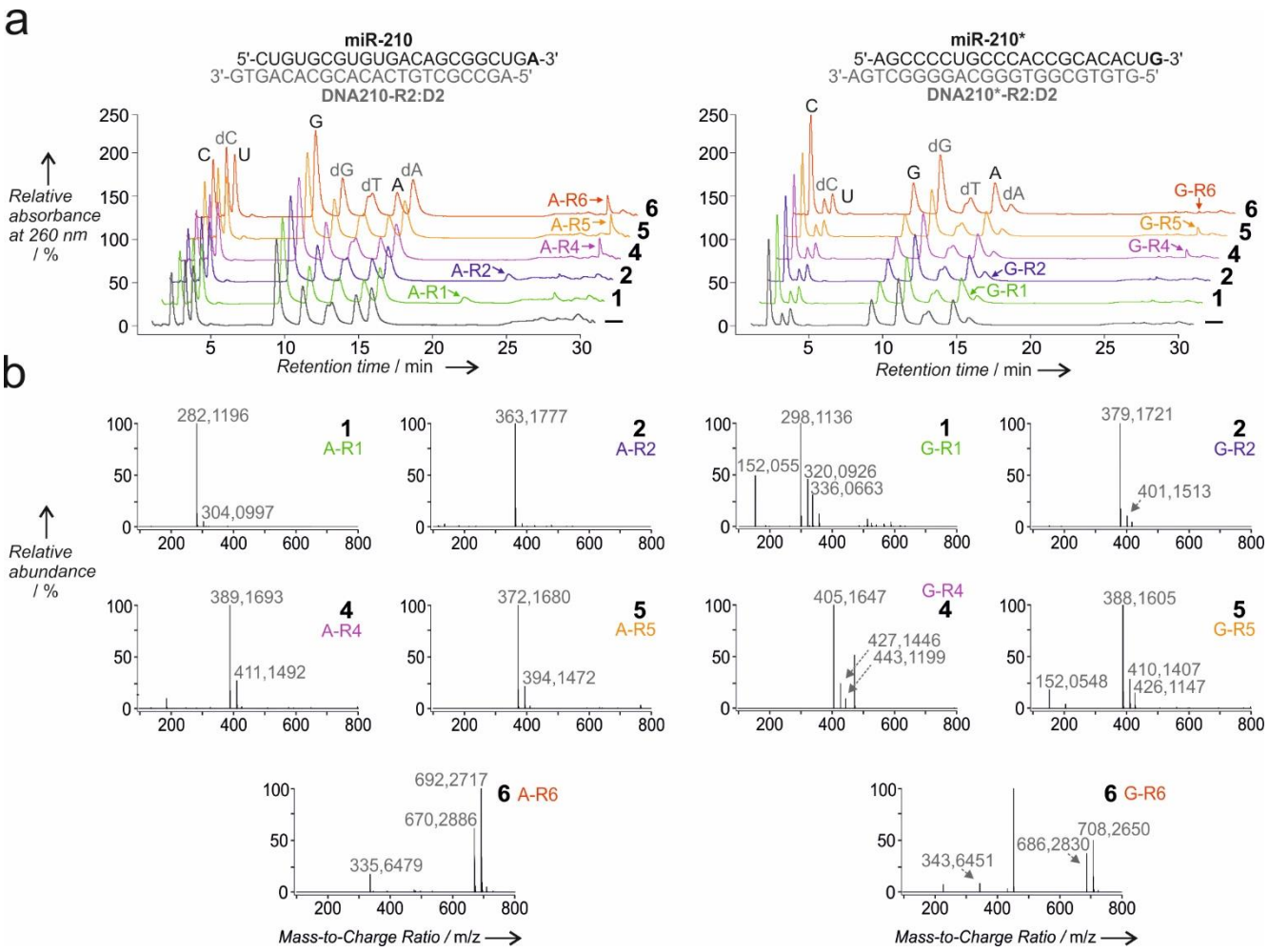


Figure S9. HPLC-MS analysis of the modification of RNA/DNA heteroduplexes. a. Reversed-phase HPLC nucleoside analysis of pre-modified miR-210/DNA210-R2:D2 (left) or miR-210*/DNA210*-R2:D2 (right). 1 μM miRNA/DNA duplexes were incubated with 1.25 μM HEN1 in the absence of cofactor (bottom chromatogram, grey) or presence of 100 μM of cofactor AdoMet **1** (green), Ado-6-amine **2** (blue), Ado-6-azide **4** (purple), Ado-6-ethyne **5** (orange) or Ado-13-biotin **6** (red) for 1 hour 30 min at 37°C. Reaction samples were enzymatically digested with P1 nuclease (Sigma) and dephosphorylated with FastAP (Thermo Fisher), the obtained mixture was analyzed by HPLC-MS methods. HEN1-dependent transalkylation in the presence of cofactors resulted in emerging of alkylated 3'-terminal nucleosides - A for miR-210 or G for miR-210* in addition to four types of non-modified ribonucleoside (C, U, G and A) and four types of deoxyribonucleoside (dC, dG, dT and dA) derivatives. **b.** ESI-MS analysis of miR-210/DNA210-R2:D2 (left) or miR-210*/DNA210*-R2:D2 (right) duplexes pre-modified with **1**, **2**, **4**, **5** or **6** cofactors. Spectrograms represent ions and their relative abundance determined in cofactor-specific peaks of HPLC chromatograms. Experimental mass-to-charge (m/z) ratios corresponding to derivatives of 2'-*O*-modified nucleosides (adenosine derivatives for miR-210 and guanosine derivatives for miR-210*) are labeled with their determined values. List of identified compounds and comparison of their theoretical and experimental m/z values is shown in **Table S2**.

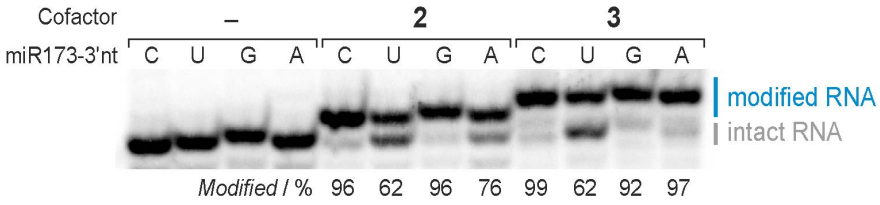


Figure S10. Alkylation of miR173/DNA173-R2:D2 with different nucleotides on 3'-terminus of miR173 strand. P^{33}-labeled wild-type miR173 with 3'-terminal cytosine (C) or variants with changed 3'-terminal nucleoside (miR173-U, miR173-G or miR173-A) were annealed to DNA173-R2:D2 and incubated with HEN1 and Ado-6-amine **2** (center block), Ado-11-amine **3** (right block) or without protein and cofactor (left block). Percentages of modified substrates were quantified using image analysis software Multi Gauge (Fujifilm).

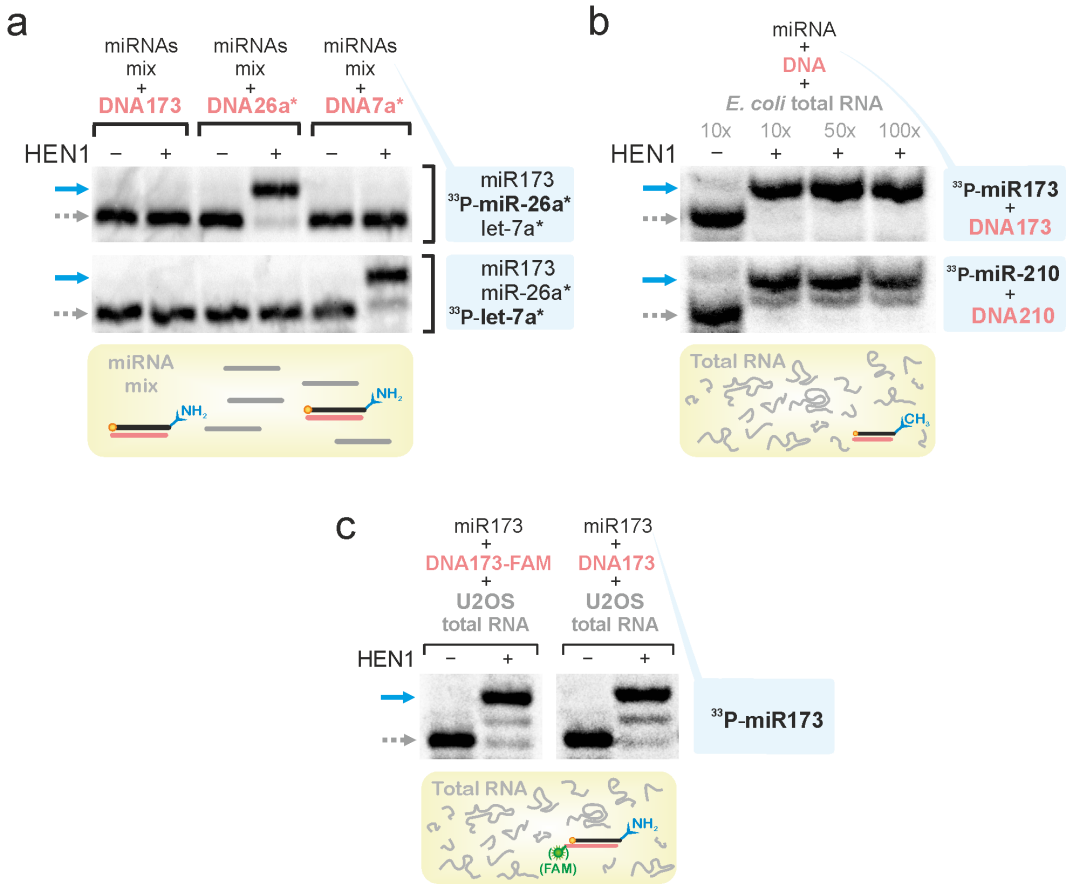


Figure S11. Selectivity of DNA-directed RNA labeling by HEN1. a. Six mixtures each composed of three miRNAs (miR173, miR-26a* and let-7a*) with only one of them P^{33}-labeled (miR-26a* in top gel or let-7a* in bottom gel) and only one DNA (DNA173-R2:D2, DNA26a*-R2:D2 or DNA7a*-R2:D2) were incubated with Ado-6-amine **2** in presence of HEN1 (+) or without it (–). The side chain from cofactor is attached to P^{33}-labeled "target" miR-26a* (top gel) or let-7a* (bottom gel) only when the complementary guide DNA (DNA26a*-R2:D2 or DNA7a*-R2:D2, respectively) is added to the annealing mixture composed of 3 different miRNAs. Solid blue arrows point to the alkylated RNAs, dashed arrows – to unmodified substrates. **b.** 0.1 μM ^{33}P-labeled miR173 (top gel) or miR-210 (bottom gel) premixed with total RNA from *E. coli* strain ER1727 in ratios of 1:10, 1:50 or 1:100 and 0.12 μM corresponding complementary DNA (DNA173-R2:D2 in the top gel or DNA210-R2:D2 in the bottom gel), re-annealed and incubated with AdoMet **1** in presence of HEN1 (+) or without the protein (–). **c.** 0.1 μM spike-in P^{33}-labeled plant miR173 premixed with total RNA from human bone osteosarcoma U2OS cell line in molar ratio of 1:10, was re-annealed with complementary DNA173-R2:D2 containing FAM fluorophore (left block) or standard hydroxyl group (right block) at 3'-terminus. Samples were alkylated for 1 hour at 37 ºC in the presence of 0.25 μM HEN1 and 100 μM Ado-11-amine **3** cofactor.

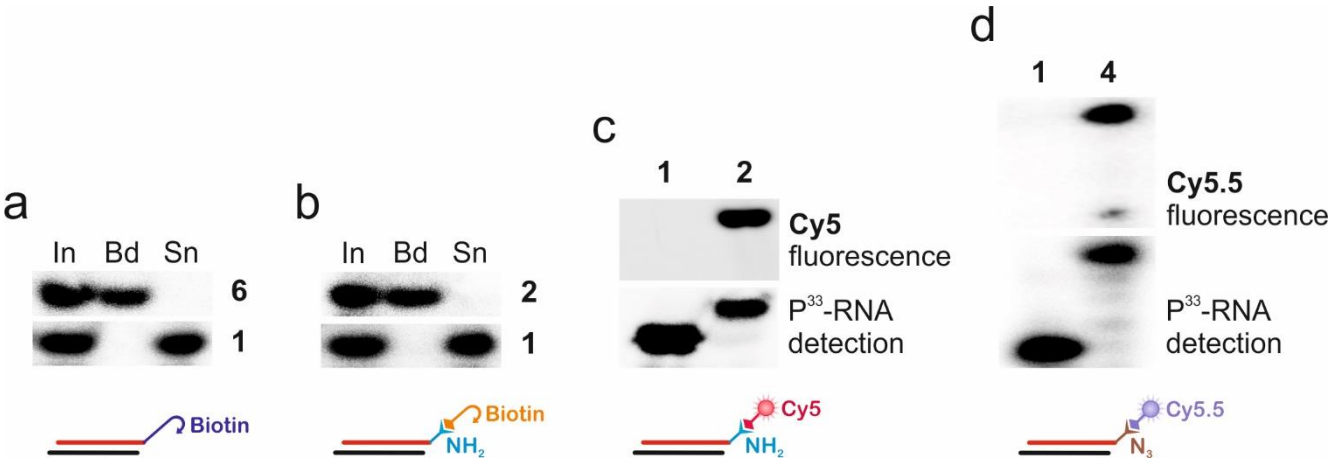


Figure S12. Labeling of miRNA in RNA/DNA duplexes. a. One-step biotinylation: miR-210/DNA210-R2:D2 modified with HEN1 and Ado-13-biotin **6** (top gel) or AdoMet **1** (bottom gel) were incubated with streptavidin-coated magnetic beads. Input (In), magnetic beads (Bd) and supernatant (Sn) fractions were analyzed on denaturing polyacrylamide gel. **b.** Two-step biotinylation: miR-210/DNA210-R2:D2 was incubated with HEN1 and Ado-6-amine **2** (top gel) or AdoMet **1** (bottom gel). The samples were treated with N-hydroxysuccinimide (NHS) ester of biotin and separated using magnetic beads. Input (In), magnetic beads (Bd) and supernatant (Sn) fractions were analyzed on denaturing polyacrylamide gel. **c.** Two-step labeling with Cy5 fluorescent dye: after modification of miR-210/DNA210-R2:D2 in the presence of HEN1 and AdoMet **1** or Ado-6-amine **2**, the samples were treated with Cy5 NHS ester and analyzed on a PAA gel for detection of Cy5 fluorescence using a 635 nm laser (top) and radioactive signal of P^{33}-miR-210 (bottom). **d.** Two-step Cy5.5 fluorophore labeling: miR-210/DNA210-R2:D2 was incubated with HEN1 and AdoMet **1** or Ado-6-azide **4**. After treatment with Cy5.5-ethyne the samples were separated on a denaturing polyacrylamide gel and examined for Cy5.5 fluorescence using a 670 nm laser (top) and radioactive signal of P^{33}-miR-210 (bottom).

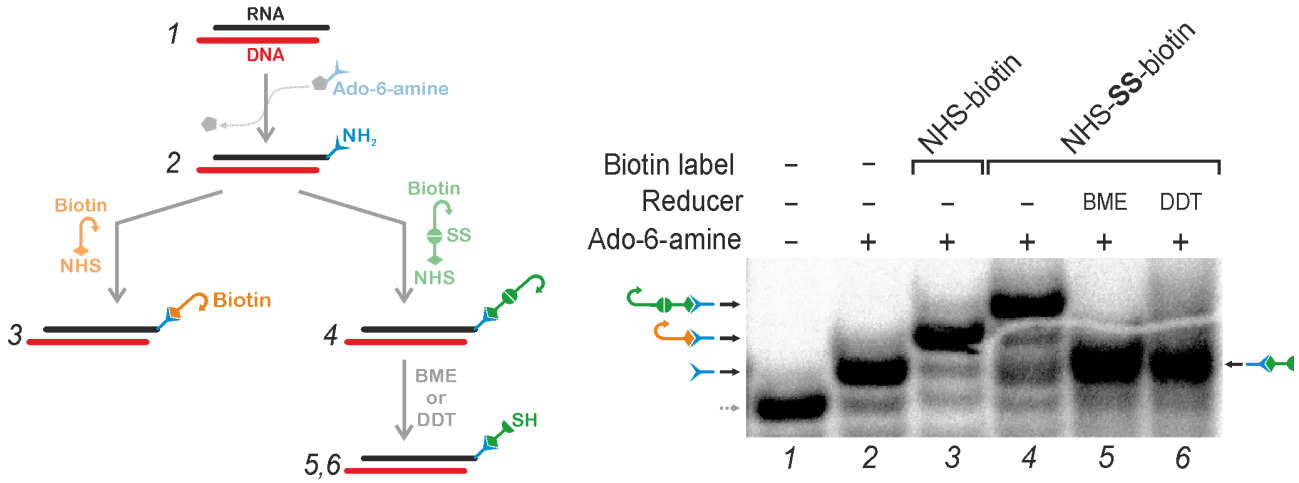


Figure S13. Two-step biotinylation of RNA/DNA substrates. Schematic representation (left) and the results (right) of the experiment. miR-210/DNA210-R2:D2 heteroduplex (sample *1*) was incubated for 1 hour at 37°C with HEN1 and Ado-6-amine **2** (sample *2*). Reactions were quenched with SDS and Proteinase K. After ethanol precipitation samples were dissolved in carbonate buffer (pH 9.5). Biotin N-hydroxysuccinimide ester of biotin (NHS-biotin) was additionally added to sample *3* and succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin) was added to samples *4-6* and incubated at room temperature for 150 min. Subsequently samples *5* and *6* were treated with reducing agents β-mercaptoethanol (BME) or dithiothreitol (DDT) to detach the biotin group. All the samples were precipitated and analyzed on denaturing polyacrylamide gel. Dashed grey arrow shows unmodified RNA, blue Y-shape shows the substrate after first step of labeling with reactive primary amine group, orange or green hook represent corresponding biotin-labeled substrates, and shortened green hook on the right displays NHS-SS-biotin-labeled RNA after the biotin group detaching.

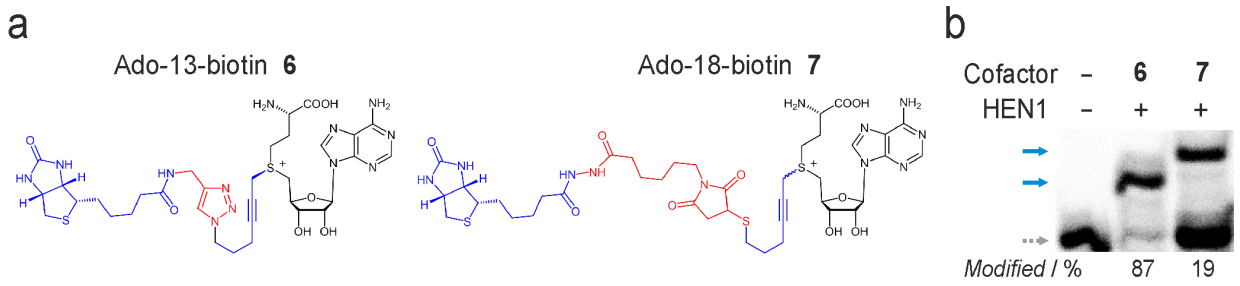


Figure S14. Comparison of synthetic cofactors for one-step biotinylation of RNA/DNA substrates. a. Chemical structures of novel Ado-13-biotin **6**, M_r = 787.3127 (left) and Ado-18-biotin **7**, M_r = 948.3525 (right). Transferable side chains are colored in blue-red, differences in structures of the cofactors are highlighted in red. **b.** 0.2 µM miR-210/DNA210-R2:D2 was incubated for 1 hour at 37°C with 0.5 µM HEN1 and 200 µM cofactor. Reactions were stopped with SDS/Proteinase K and samples analyzed on denaturing polyacrylamide gel. Corresponding biotinylated miR-210 products are marked by solid arrows, unmodified substrate – by dashed arrows.

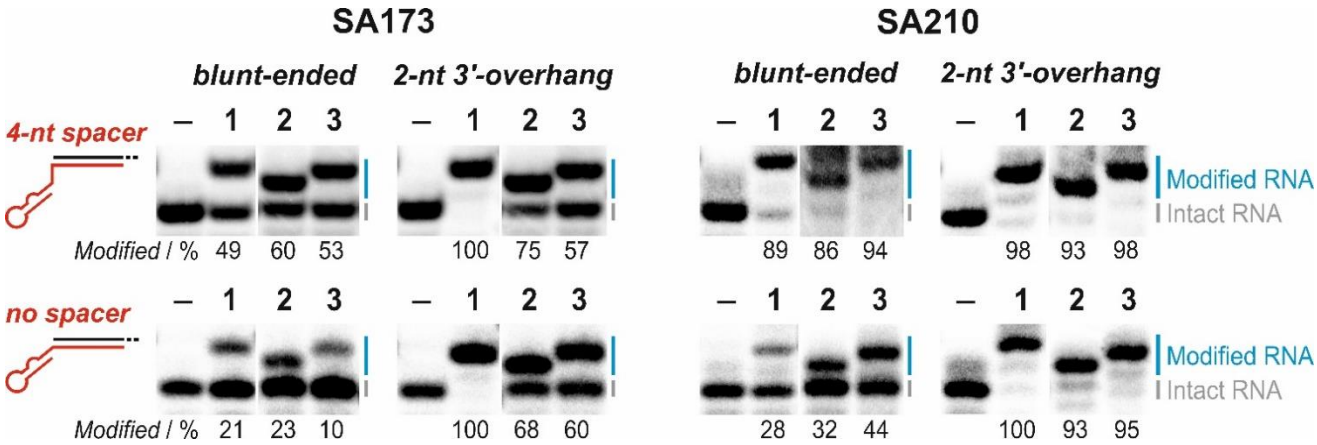


Figure S15. HEN1-catalyzed miRNA modification guided by streptavidin aptamers (SA). miR173 (left side) or miR-210 (right side) were annealed to complementary DNA (SA173 and SA 210, respectively) conjugated to streptavidin-specific aptamer via 4-nt spacer (upper gels) or directly (lower gels). Substrates with blunt-end or 2-nt 3'-overhang the RNA end were incubated with HEN1 and AdoMet **1**, Ado-6-amine **2** or Ado-11-amine **3** or without protein and cofactor (–).

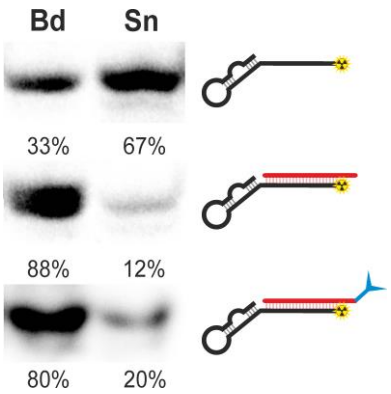


Figure S16. Comparison of streptavidin aptamer SA, SA/miRNA and SA/miRNA$^{\text{-6-azide}}$ extraction using streptavidin-coupled magnetic beads. P^{33}-SA210-R2:s0 (top), P^{33}-SA210-R2:s0/miR-210 (center) and P^{33}-SA210-R2:s0/miR-210 pre-incubated with HEN1 and Ado-6-azide **4** (bottom) were incubated with streptavidin-coupled magnetic beads. Magnetic beads fractions (Bd) and supernatant fractions (Sn) were analyzed on denaturing polyacrylamide gel and percentage of sample in each fraction were calculated using image analysis software Multi Gauge (Fujifilm).

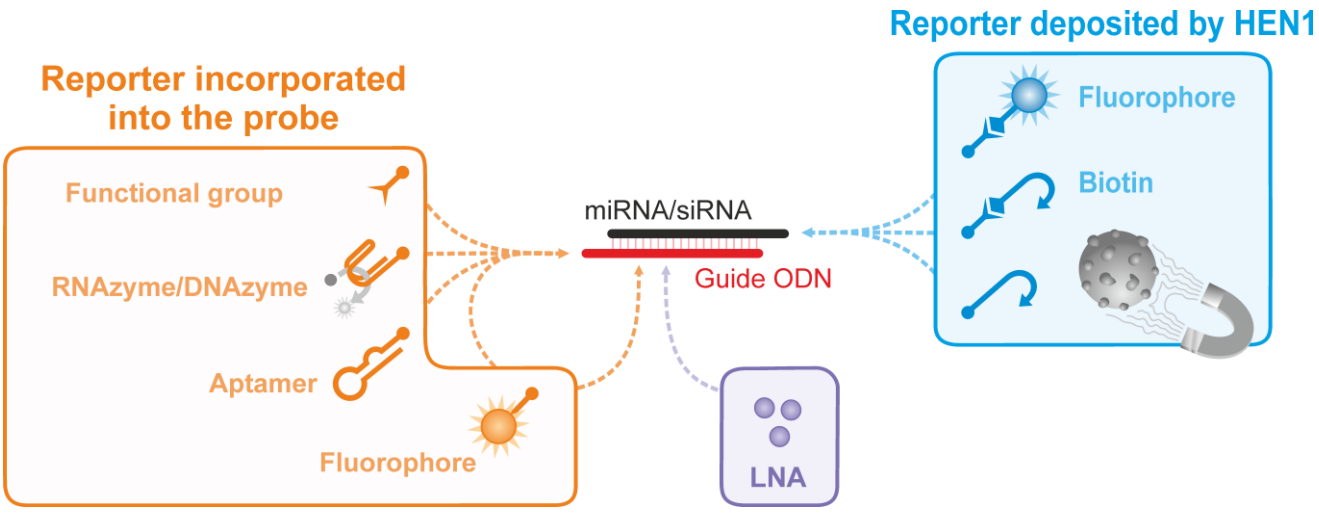


Figure S17. HEN1-mediated oligonucleotide-addressed labeling as a tool for multi-reporter detection. Oligodeoxynucleotide strand (red line) with synthetically incorporated reporter (orange box) guides HEN1-mediated deposit of the second reporter (blue box) onto target small non-coding RNA, such as miRNA or siRNA (black line). LNA (locked nucleic acid) nucleotides incorporated within the sequence could enhance stability of the RNA/DNA duplexes.

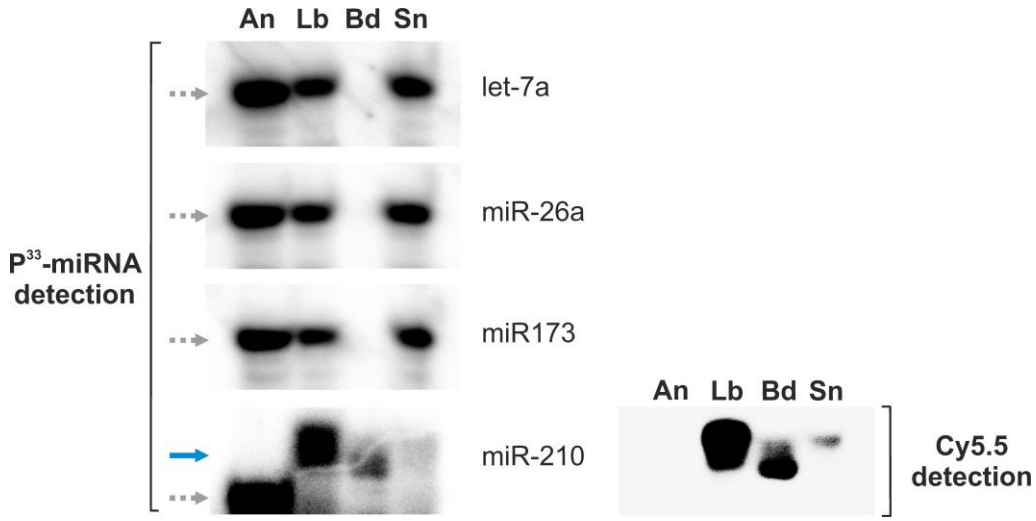


Figure S18. Aptamer mediates selective labeling of target miRNA. Four mixtures containing let-7a, miR-26a, miR173 and miR-210 with only one of miRNAs 5'-P^{33}-labeled in each mixture were annealed to SA210-R2:s0 aptamer, which is complementary to miR-210 ("An" stands for annealing). Samples were incubated with Ado-6-azide **4** and treated with Cy5.5-alkyne fluorophore ("Lb" stands for labeling). Magnetic beads fractions (Bd) separated from supernatant (Sn) were analyzed on denaturing polyacrylamide gel for P^{33} (left four gels) and Cy5.5 (right gel) signal. Only miR-210 is selectively labeled with fluorophore – band shift between unmodified miR-210 (An) and treated sample (Lb) was observed exclusively in P^{33}-miR-210 sample. Solid blue arrow depicts fluorophore-labeled miR-210, dashed arrows – unmodified miRNAs.

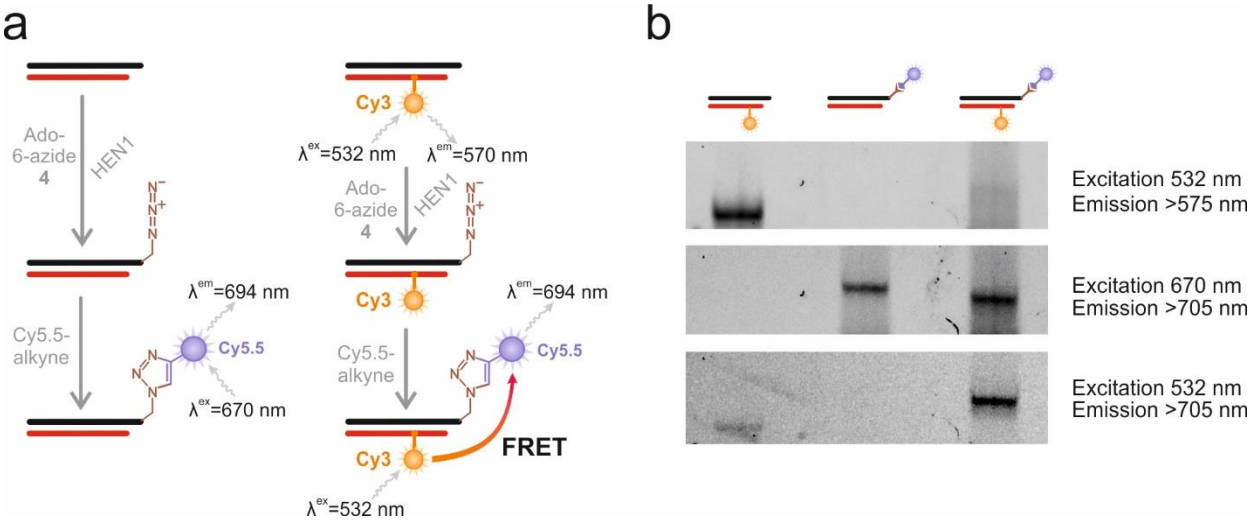


Figure S19. A dual-reporter Förster resonance energy transfer (FRET) assay for selective microRNA detection on a gel. a. A schematic outline of the experiment. A target miR173 strand was annealed with a complementary DNA strand to yield miR173/DNA173-R2:D0 (left, control) or miR173/DNA173-R2:D0-Cy3 (right, assay). The heteroduplexes were modified by HEN1 in the presence of Ado-6-azide **4** cofactor and click-labeled with a Cy5.5-alkyne. **b**. The duplexes were visualized on a non-denaturing 12% polyacrylamide gel and the fluorescence scanned using a green 532 nm laser with a 575 nm cut-off LPG filter (top), a near infra-red 670 nm laser with a 705 nm cut-off LPFR filter (center) or a green 532 nm laser with a 705 nm cut-off LPFR filter (bottom).