Electronic Supplementary Material (ESI) for RSC Advances.

Fluorescence detection of natural RNA using rationally designed "clickable" oligonucleotide probes

A. Okholm,[a] J. Kjems[a] and I. Kira Astakhova[b,]*

[a] Department of Molecular Biology and Genetics, University of Aarhus, C.F. Møllers Allé 3, 8000 Aarhus C, Denmark. Tel: +45 87155494; E-mail: jk@mb.au.dk

[b] Nucleic Acid Center, Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, DK-5230 Odense M, Denmark. Tel: +45 6550 2510; E-mail: ias@sdu.dk

Supplementary Information

Table of contents

I. Materials and methods S2

II. Synthesis and purification of modified oligonucleotides S2

III. Postsynthetic click chemistry S4

IV. MALDI MS and IE HPLC characterization of oligonucleotides (Table S1) S5

V. Thermal denaturation and UV-visible absorbance studies S6

VI. Representative thermal denaturation curves and visible absorbance spectra S7

VII. CD studies and representative spectra S9

VIII. Reference probes and mismatched RNA targets used in this study (Tables S2,S3) S10

IX. Thermal denaturation temperatures for references and "clickable" probes (B) binding complementary and single-mismatched circRNA targets (Figure S3) S12

X. Fluorescence steady-state emission and excitation studies. Determination of quantum yield and limit of target detection values S13

XI. Spectroscopic and photophysical properties of modified probes and duplexes (Tables S4-S7, Figure S4) S14

Materials and methods. Reagents obtained from commercial suppliers were used as received. Reagents and solvents for click chemistry were obtained from commercial suppliers (Fluka, Lumiprobe, Sigma-Aldrich), and were used as received; TBTA[1] was prepared following published procedures. Stock solutions for click chemistry were prepared as described in recent publications.[2] Click reactions were performed in 2 mL reactor tubes under argon and vigorous stirring in a microwave reactor (Emrys Creator, Personal Chemistry), or in 2 mL eppendorf tubes at ambient temperature. Perylene and ozaxine 170 perchlorate were used as standards for emission quantum yield measurements after recrystallization. Photochemical studies were performed using spectroquality methanol and cyclohexane. Other solvents and reagents applied in this study were used as received.

Synthesis and purification of modified oligonucleotides. LNA phosphoramidites (G^L, A^L and C^L) and phosphoramidite **1** were obtained from commercial suppliers (Exiqon and Jena Bioscience, respectively), and were incorporated into synthetic oligonucleotides following manufactures' protocols. Oligonucleotide synthesis was carried out on ÄKTA oligopilot plus instrument (GE Healthcare Life Sciences) in 1 μmol scale using standard manufacturer's protocols. Coupling yields based on the absorbance of the dimethoxytrityl cation released after each coupling were approximately 98−99% for modified monomers $\mathbf{U^P}$, LNA and unmodified DNA phosphoramidites. Cleavage from solid support and removal of nucleobase protecting groups were performed using standard conditions (32% aqueous ammonia for 12 h at 55 ºC). The modified oligonucleotides were purified by DMT-OFF RP-HPLC using the Waters Prep LC 4000 equipped with Xterra MS C18-column (10 μm, 300 mm × 7.8 mm).

---

[1] Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA): T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin ,*Org. Lett.*, 2004, **6**, 2853.

[2] Some reviews on DNA modification with click chemistry: a) A. V. Ustinov, I. A. Stepanova, V. V. Dubnyakova, T. S. Zatsepin, E. V. Nozhevnikova, V. A. Korshun, *Russ. J. Bioorg. Chem.*, 2010, **36**, 401. b) A.H. El-Sagheer, T. Brown, *Chem. Soc. Rev.*, 2010, **39**, 1388. c) F. Amblard, J. H. Cho, R. F. Schinazi, *Chem. Rev*., 2009, **109**, 4207.

Elution was performed starting with an isocratic hold of A-buffer for 2 min followed by a linear gradient to 70 % B-buffer over 40 min at a flow rate of 1.0 mL/min (A-buffer: 0.05 M triethyl ammonium acetate, pH 7.4; B-buffer: 25% buffer A, 75% CH_3CN). RP-purification was followed by precipitation (acetone, -18 ºC, 12 h) and washing with acetone (2 × 0.5 mL). The identity and purity of the products were verified by MALDI-TOF mass spectrometry (Ultraflex II, Bruker) and IE HPLC, respectively. IE HPLC was performed using the Merck Hitachi LaChrom instrument equipped with Dionex DNAPac Pa-100 column (250 mm × 4 mm). Elution was performed starting with an isocratic hold of A- and C-buffers for 2 min followed by a linear gradient to 60% B-buffer over 28 min at a flow rate of 1.0 mL/min (A-buffer: MQ water; B-buffer: 1M $LiClO_4$, C-buffer: 250 mM Tris-Cl, pH 8.0). MALDI-TOF mass-spectrometry analysis was performed using a MALDI-LIFT system on the Ultraflex II TOF/TOF instrument from Bruker and using HPA-matrix (10 mg 3-hydroxypicolinic acid, 50 mM ammonium citrate in 70% aqueous acetonitrile). Unmodified DNA strands were obtained from commercial suppliers and used without further purification.

General method for microwave-assisted CuAAC reactions. Starting oligonucleotide **ON1**–**ON2** (30 nmol) was dissolved in fresh MQ water (325 μL) in a microwave-tube. DMSO (435 μL), 2 M triethylammonium acetate buffer (pH 7.4; 100 μL), azide **3** (40 μL of 10 mM solution in DMSO), ascorbic acid (10 μL of 50 mM freshly prepared stock solution) and Cu(II)-TBTA equimolar complex (50 μL of 10 mM stock solution) were subsequently added. The resulting mixture was tightly closed, mixed on vortex and subjected to microwave conditions (microwave reactor, initial power 60 W (decreased gradually to 15 W after reaching the target temperature), 60 °C, 15 minutes). The reaction was afterwards cooled to room temperature and filtrated twice through Illustra NAP-10 column (GE Healthcare) following manufacture's protocol. The resulting solution was evaporated; the resulting conjugates **P1**−**P2** were analyzed by MALDI TOF mass spectrometry and IE HPLC (Table S2, Figures S1-S2). Yields: 74% (**P1**), 78% (**P2**).

General method for CuAAC reactions with azides 4–5. Starting oligonucleotide **ON1**–**ON2** (30 nmol) was dissolved in fresh MQ water (525 μL) in 2 mL eppendorf tube. DMSO (235 μL), 2 M triethylammonium acetate buffer (pH 7.4; 100 μL), corresponding azide (**4**–**5**; 40 μL of 10 mM solution in DMSO), ascorbic acid (10 μL of 50 mM freshly prepared stock solution) and Cu(II)-TBTA equimolar complex (50 μL of 10 mM stock solution) were subsequently added. The resulting mixture was tightly closed, mixed on vortex and kept at room temperature for 24 h. The reaction was afterwards filtrated twice through Illustra NAP-10 column (GE Healthcare) following manufacture's protocol. The resulting solution was evaporated; the resulting conjugates **P3**−**P6** were analyzed by MALDI TOF mass spectrometry and IE HPLC (Table S2, Figures S1-S2). Yields: 81% (**P3**), 77% (**P4**), 79% (**P5**), 83% (**P6**).

Table S1. IE HPLC retention times and MALDI MS of modified oligonucleotides.

#	Sequence, 5′→3′	Ret. time, min	Found *m/z* $[M-H]^-$	Calc. *m/z* $[M-H]^-$
ON1	d(ACAACAAA^{L}ATC^{L}AC**U**^{P}AG**U**^{P}C**U**^{P}TCC^{L}A)	8.94	7172	7173
ON2	d(GAAGAC^{L}GTG^{L}GA**U**^{P}T**U**^{P}TC**U**^{P}GGA^{L}AGA)	8.94	7382	7382
ON3	d(ACAACAAA^{L}ATC^{L}ACTAGTCTTCC^{L}A)	8.94	7053	7056
ON4	d(GAAGAC^{L}GTG^{L}GATTTTCTGGA^{L}AGA)	8.94	7265	7265
P1	d(ACAACAAA^{L}ATC^{L}AC**M**^{1}AG**M**^{1}C**M**^{1}TCC^{L}A)	10.78	8179	8178
P2	d(GAAGAC^{L}GTG^{L}GA**M**^{1}T**M**^{1}TC**M**^{1}GGA^{L}AGA)	11.96	8392	8387
P3	d(ACAACAAA^{L}ATC^{L}AC**M**^{2}AG**M**^{2}C**M**^{2}TCC^{L}A)	9.87	8540	8542.5
P4	d(GAAGAC^{L}GTG^{L}GA**M**^{2}T**M**^{2}TC**M**^{2}GGA^{L}AGA)	10.19	8751	8751.5
P5	d(ACAACAAA^{L}ATC^{L}AC**M**^{3}AG**M**^{3}C**M**^{3}TCC^{L}A)	10.17	9019	9024
P6	d(GAAGAC^{L}GTG^{L}GA**M**^{3}T**M**^{3}TC**M**^{3}GGA^{L}AGA)	11.23	9230	9233

UV-visible absorbance and thermal denaturation studies were performed in a medium salt phosphate buffer (100 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) on a Beckman Coulter DU800 UV/VIS Spectrophotometer equipped with Beckman Coulter High Performance Temperature Controller. Concentrations of oligonucleotides were calculated using the following extinction coefficients (OD_{260}/μmol): G, 10.5; A, 13.9; T/U/$\mathbf{U^P}$, 7.9; C, 6.6; $\mathbf{M^1}$, 33.2; $\mathbf{M^2}$; 11.8; $\mathbf{M^3}$, 20.0; Reference fluorophores: **iFT**, **6FAM**, 6.5; **5HEX**, 17.2; **AlexaFluor 488**, 21.3.[3] The complementary strands were thoroughly mixed, denatured by heating to 85 °C for 10 min and subsequently cooled overnight to the starting temperature of spectroscopic experiment. Thermal denaturation temperatures (T_m values, ºC) were determined as the maxima of the first derivative of the thermal denaturation curve (A_{260} vs. temperature). Reported T_m values are an average of two measurements within ± 0.5 ºC.

[3] The data for absorbances of **iFT**, **6FAM**, **5HEX** and **AlexaFluor 488** was obtained from commercial suppliers.

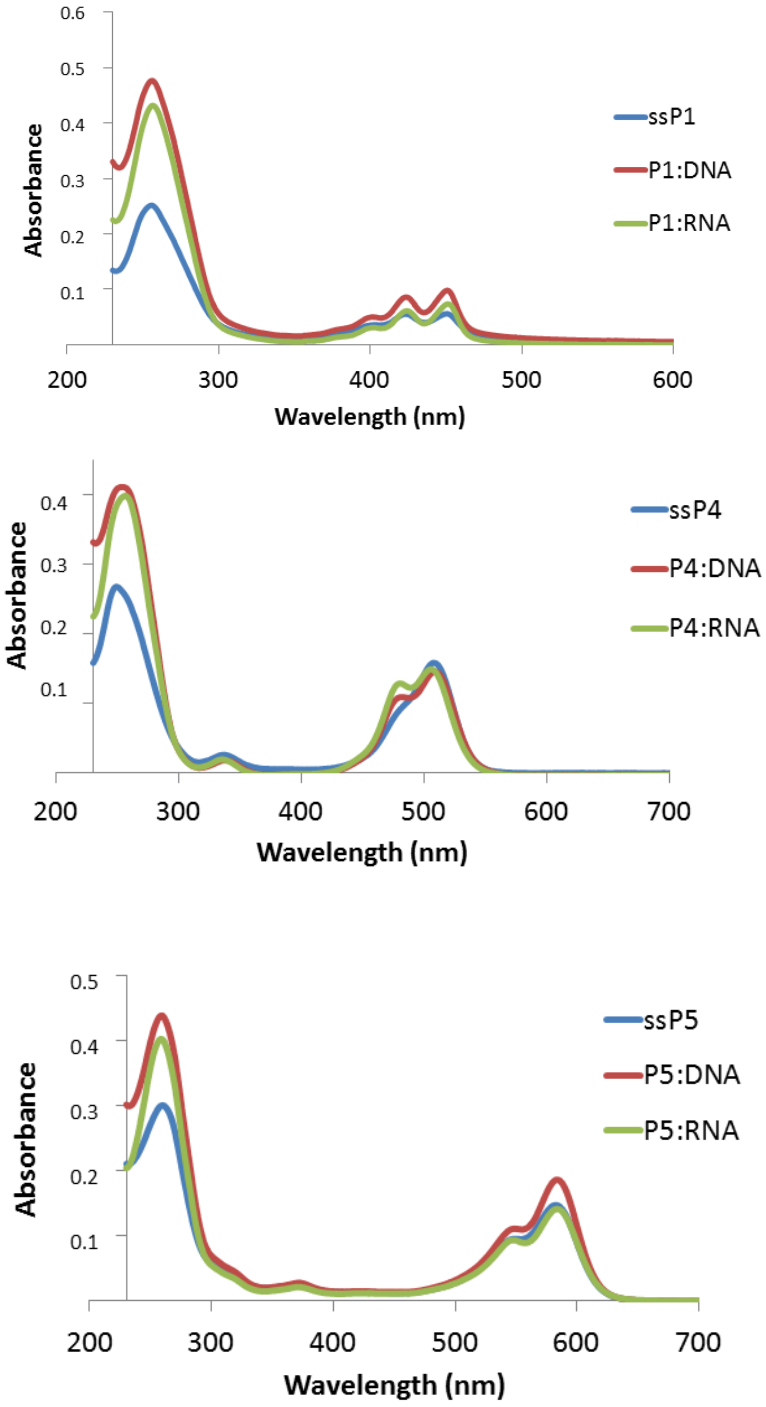


Figure S1. Representative visible absorbance spectra of modified oligonucleotides (not normalized). Spectra were obtained in medium salt buffer at 19 ºC using 1.0 μM concentrations of single-stranded oligonucleotides.

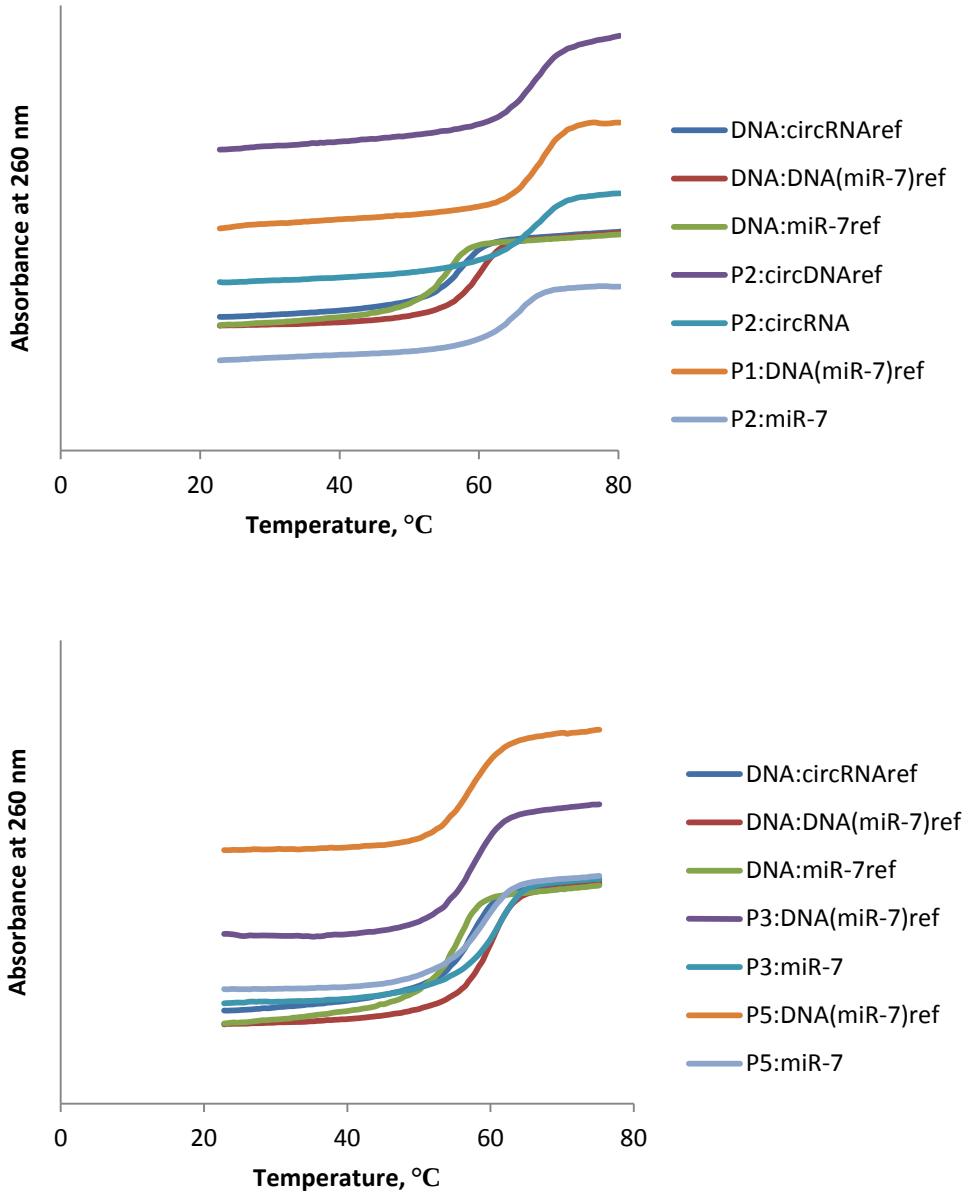


Figure S2. Thermal denaturation curves of duplexes containing monomers $\mathbf{M^1}$–$\mathbf{M^3}$ and unmodified references recorded in a medium salt phosphate buffers using 1.0 μM concentration of oligonucleotides.

CD measurements

CD spectra were recorded on JASCO J-815 CD Spectrometer equipped with CDF 4265/15 temperature controller. Samples for CD measurements were prepared as described in the thermal denaturation studies section except that a concentration of 2.0 μM of both the complementary strands was used. Quartz optical cells with a path-length of 0.5 cm were used.

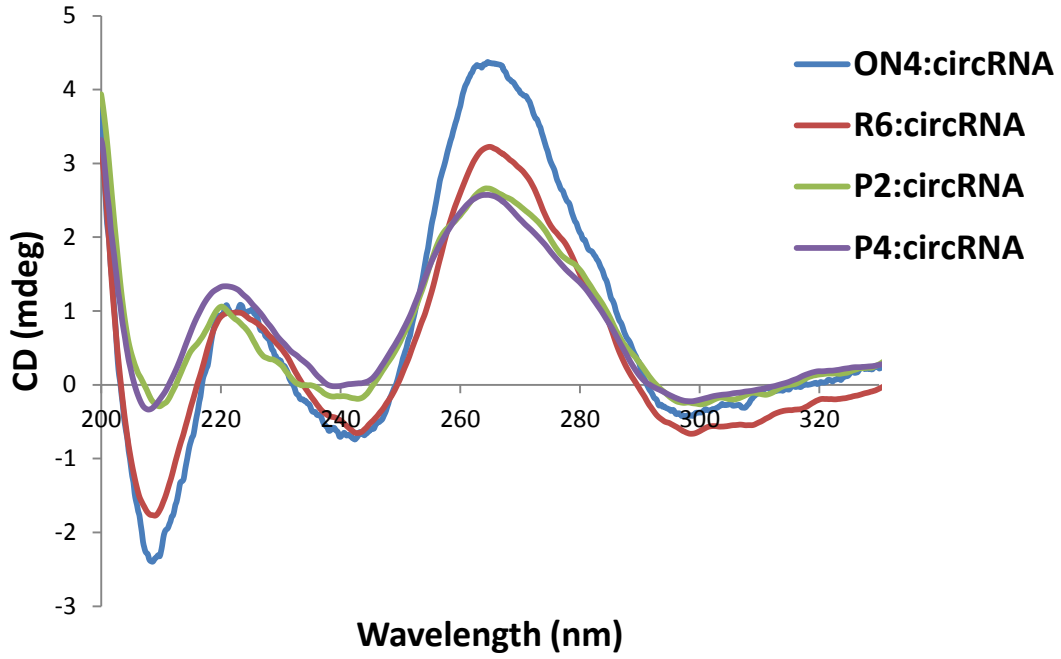


Figure S3. Representative CD spectra of reference oligonucleotides (**ON4** and **R6**) and fluorescent probes **P2** and **P4** against complementary circRNA. The spectra were recorded in medium salt buffer at 19 ºC, using 2.0 μM concentration of complementary strands. Reference strand **R6**: 5'-(6FAM)-d(GAAGAC^{L}GTG^{L}GATTTTCTGGA^{L}AGA), obtained from Exiqon.

Table S2. Reference probes and mismatched miR-7 targets used in this study.*

#	Sequence	Supplier
R1	5'-d(ACAACAAA^{L}ATC^{L}AC(**iFT**)AG(**iFT**)C(**iFT**)TCC^{L}A)	Exiqon
R2	5'-(**6FAM**)-d(ACAACAAA^{L}ATC^{L}ACTAGTCTTCC^{L}A)	Exiqon
R3	5'-(**5HEX**)-d(ACAACAAA^{L}ATC^{L}ACTAGTCTTCC^{L}A)	Exiqon
R4**	5'-d(GAAGAC^{L}GTG^{L}GA(**AF**)T(**AF**)TC(**AF**)GGA^{L}AGA)	Glen Res.**
miR-7	5'-r(UGG AAG ACU AGU GAU UUU GUU GU)	IDT
MT1	5'-r(UGG AAG AC**A** AGU GAU UUU GUU GU)	IDT
MT2	5'-r(UGG AAG AC**G** AGU GAU UUU GUU GU)	IDT
MT3	5'-r(UGG AAG AC**C** AGU GAU UUU GUU GU)	IDT
MT4	5'-r(UGG AAG ACU **U**GU GAU UUU GUU GU)	IDT
MT5	5'-r(UGG AAG ACU **G**GU GAU UUU GUU GU)	IDT
MT6	5'-r(UGG AAG ACU **C**GU GAU UUU GUU GU)	IDT
MT7	5'-r(UGG AAG ACU AGU GAU U**G**U GUU GU)	IDT
MT8	5'-r(UGG AAG ACU AGU GAU U**A**U GUU GU)	IDT

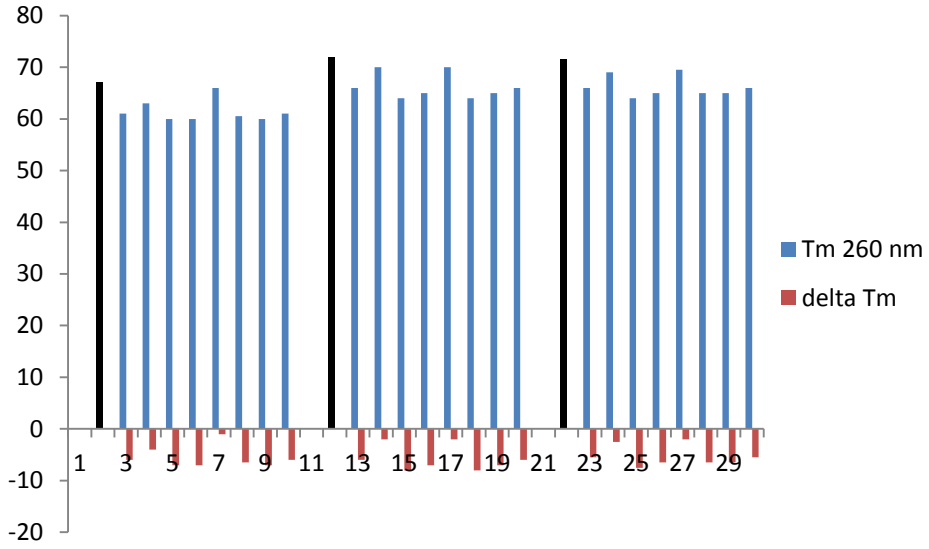
* A mismatched nucleotide is shown in red. ** AF = AlexaFluor 488; the corresponding azide obtained from Glen Research was used in click chemistry reaction with **ON1** according to the similar procedure as described for **4**-**5**. For the chemical structures of the modifications, see web-sources of the corresponding suppliers.

Table S3. Reference probes and mismatched circRNA targets used in this study.*

#	Sequence	Supplier
R5	5'-d(GAAGAC^{L}GTG^{L}GA(**iFT**)T(**iFT**)TC(**iFT**)GGA^{L}AGA)	Exiqon
R6	5'-(**6FAM**)-d(GAAGAC^{L}GTG^{L}GATTTTCTGGA^{L}AGA)	Exiqon
R7	5'-(**5HEX**)-d(GAAGAC^{L}GTG^{L}GATTTTCTGGA^{L}AGA)	Exiqon
R8**	5'-d(GAAGAC^{L}GTG^{L}GA(**AF**)T(**AF**)TC(**AF**)GGA^{L}AGA)	Glen Res.**
circRNA	5'-r(UCU UCC AGA AAA UCC ACG UCU UC)	IDT
MT9	5'-r(UCU UCC AGA **U**AA UCC ACG UCU UC)	IDT
MT10	5'-r(UCU UCC AGA **G**AA UCC ACG UCU UC)	IDT
MT11	5'-r(UCU UCC AGA **C**AA UCC ACG UCU UC)	IDT
MT12	5'-r(UCU UCC AGA A**U**A UCC ACG UCU UC)	IDT
MT13	5'-r(UCU UCC AGA A**G**A UCC ACG UCU UC)	IDT
MT14	5'-r(UCU UCC AGA A**C**A UCC ACG UCU UC)	IDT
MT15	5'-r(UCU UCC AGA AAA UCC A**G**G UCU UC)	IDT
MT16	5'-r(UCU UCC AGA AAA UCC A**U**G UCU UC)	IDT

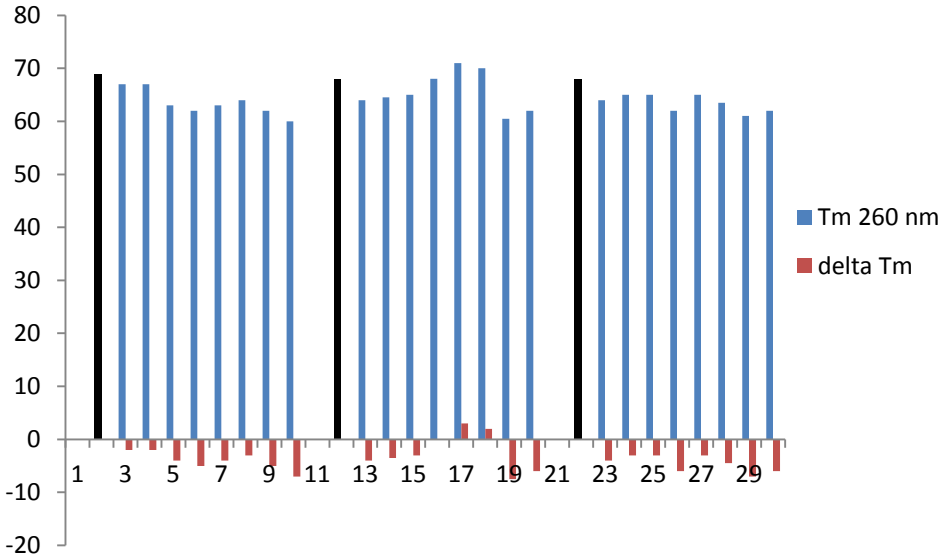
* A mismatched nucleotide is shown in red. ** AF = AlexaFluor 488; the corresponding azide obtained from Glen Research was used in click chemistry reaction with **ON2** according to the similar procedure as described for **4**-**5**. For the chemical structures of the modifications, see web-sources of the corresponding suppliers.

A) **Commercially available reference probes**



Probe:circRNA: **R1** **R2** **R3**

B) **"Clickable" Probes and Reference**



Probe:circRNA: **P2** **P4** **R4**

Figure S3. Thermal denaturation temperatures at 260 nm for references (A) and "clickable" probes (B) binding circRNA targets; ΔT_m is the difference between the T_m values of a fully complementary and a mismatched complex. The first bar in each series corresponds to fully complementary probe:RNA complex with $\Delta T_m = 0$ (shown in black). Reference probes **R1**-**R4** and mismatched RNA targets (**MT1**-**MT8** left to right) are listed in Table S2.

Fluorescence steady-state emission and excitation studies. Determination of quantum yield and limit of target detection values. Fluorescence spectra were obtained using a PerkinElmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller using the following excitation/emission wavelengths: 425/452 nm (**P1**–**P2**) 480/530 nm (**P3**–**P4**), 580/605 nm (**P5**–**P6**), 494/520 nm (**R1**–**R2**, **R5**–**R6**), 533/550 nm (**R3**, **R7**), and 494/519 nm (**R4**,**R8**).

All the measurements were performed at excitation slit of 4.0 nm, emission slit of 2.5 nm, scan speed of 120 nm/min and 0.25-1.0 μM concentrations of the single-stranded probe or the corresponding complementary complex in a medium salt buffer described above. The fluorescence quantum yields (Φ_f) were measured by the relative method[4] using standards of highly diluted solutions of perylene (Φ_f = 0.93)[5] and oxazine 170 perchlorate[6] (Φ_f = 0.58) in cyclohexane and methanol, respectively. The samples used in quantum yield measurements were not degassed; concentrations were 0.25 μM.

Limit of detection (LOD) values were determined by series of dilution experiments following previously described protocol.[7] Relevant fluorescent oligonucleotides (250 nM) and targets at concentrations 500, 250, 100, 50, 20, 10 and 5 nM were mixed in a medium salt phosphate buffer. Upon annealing, a fluorescence signal was measured at λ^{fl} 452 nm (**P1**–**P2**), 530 nm (**P3**–**P4**), and 605 nm (**P5**–**P6**). LOD value of each complex was then defined as a lowest complex concentration such that the fluorescence signal to noice ratio (S/N) relative to the blank solution of a medium salt phosphate buffer was minimum three.

---

[4] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3th. Ed., Springer, Singapore, 2006.

[5] N. Nijegorodov, R. Mabbs, W. S. Downey, *Spectrochim. Acta, Part* A, 2001, **57**, 2673.
[6] .K. Rurack, M. Spieles, *Anal. Chem.* 2011, **83**, 1232.
[7] Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), *Guidance for industry in the manufacture and clinical evaluation of in vitro tests to detect nucleic acid sequences of human immunodeficiency viruses type 1 and 2* (Office of Communication, Training and Manufactures Assistance (HFM-40), Rockville, MD), 1999, pp. 4.

Table S4. Spectroscopic and photophysical properties of modified oligonucleotides and duplexes.[a]

P#	λ^{abs}_{max}, bands I, II (nm)			λ^{fl}_{max} (nm)			Φ_F (19 °C/37 °C)			FB at 19 °C		
	SSP	Duplex with complimentary		*SSP*	Duplex with complimentary		*SSP*	Duplex with complimentary		*SSP*	Duplex with complimentary	
		DNA	*RNA*		*DNA*	*RNA*		*DNA*	*RNA*		*DNA*	*RNA*
P1	423,451	423,450	423,451	457,487	457,487	459,490	0.07/ 0.05	0.31/ 0.22	0.58/ 0.45	3	33	27
P2	423,450	423,450	423,451	457,489	458,488	458,488	0.17/ 0.09	0.27/ 0.24	0.47/ 0.40	9	34	34
P3	507	480,506	480,506	531	525	526	0.39/ 0.51	0.96/ 1.00	0.90/ 0.96	68	138	102
P4	511	482,509	483,506	531	530	528	0.17/ 0.25	0.58/ 0.62	0.90/ 0.99	27	85	133
P5	547,584	549,584	549,584	605	605	605	0.26/ 0.26	0.16/ 0.12	0.23/ 0.23	41	37	32
P6	547,589	546,585	546,585	607	607	607	0.30/ 0.23	0.17/ 0.15	0.18/ 0.18	42	21	30

[a] SSP = single-stranded probe; λ^{abs}_{max}, λ^{fl}_{max} and Φ_F are absorbance, fluorescence maxima and fluorescence quantum yield, respectively. Fluorescence brightness (FB) values were calculated using equation: FB = $\varepsilon_{max} \times \Phi_F$, where ε_{max} is the maximal molar extinction coefficient of the probe and Φ_F is the corresponding fluorescence quantum yield.

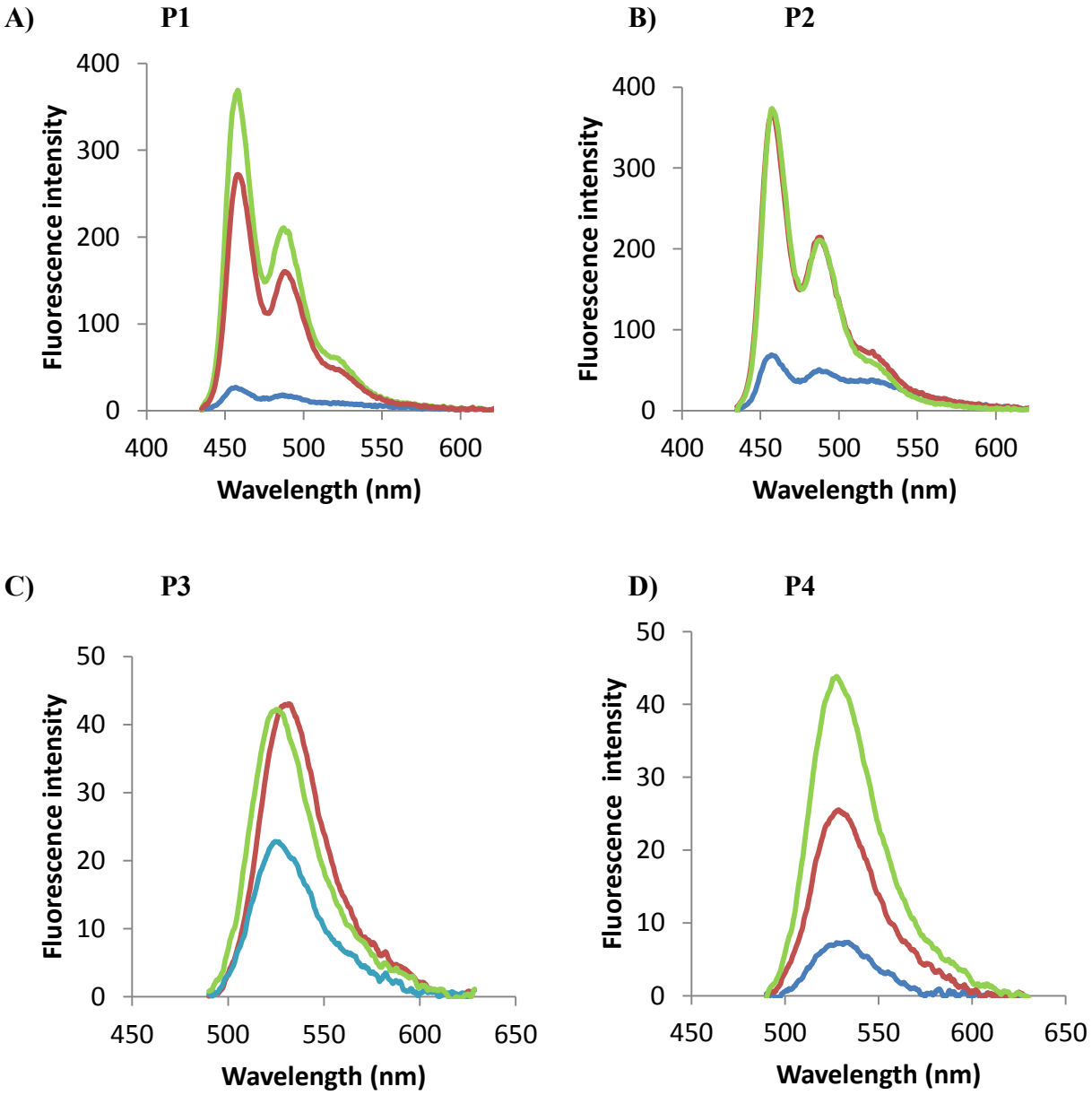


Figure S4. Representative steady-state fluorescence emission spectra of single-stranded probes (blue lines), and their duplexes with complementary DNA and RNA (shown in red and green lines, respectively). Spectra were obtained in a medium salt buffer at 19 ºC using excitation wavelength of 425 nm (A,B) and 480 nm (C,D).

Table S5. Representative discrimination factors for the probes and references detecting miR-7 targets containing single-nucleotide mismatches.[a]

RNA Target	Probe# and *D*						
	P2	**P4**	**P6**	**R1**	**R2**	**R3**	**R4**
MT1	0.1	1.6	0.2	0.6	1.0	1.5	0.8
MT2	0.5	0.4	0.2	1.2	0.9	1.3	0.7
MT3	0.6	0.9	0.4	1.4	0.9	1.4	0.8
MT4	0.3	0.4	0.3	1.2	0.5	0.9	0.8
MT5	0.4	0.5	0.1	1.5	0.9	1.4	0.7
MT6	0.5	0.7	0.1	1.3	0.9	1.2	1.2
MT7	1.0	0.6	0.2	1.2	1.0	1.2	1.2
MT8	0.8	1.5	0.4	1.5	1.0	1.2	1.3

[a] *D* is determined at 19 °C as a ratio of fluorescence intensities at fluorescence maximum of fully complementary double-stranded complex to the corresponding single-mismatched complex.

Table S6. Representative discrimination factors for the probes and references detecting circRNA targets containing single-nucleotide mismatches.[a]

RNA Target	Probe# and *D*						
	P1	**P3**	**P5**	**R5**	**R6**	**R7**	**R8**
MT9	0.3	0.5	0.7	2.1	1.0	1.0	1.2
MT10	0.6	0.9	0.6	0.6	1.1	0.6	1.5
MT11	0.1	0.5	0.5	1.5	1.3	1.0	1.0
MT12	0.2	0.8	0.3	1.3	0.9	1.0	1.1
MT13	0.5	0.5	0.3	0.8	1.2	0.5	1.1
MT14	0.3	0.4	0.5	1.5	0.9	0.7	1.1
MT15	0.2	0.4	0.6	0.9	1.4	1.0	1.1
MT16	0.2	0.7	0.4	0.9	1.1	1.0	1.3

[a] *D* is determined at 19 °C as a ratio of fluorescence intensities at fluorescence maximum of fully complementary double-stranded complex to the corresponding single-mismatched complex.

Table S7. Fluorescence detection of the pure fully complementary circRNA, single-mismatched target **MT13**, and of their mixtures by the probe **P4**.[a]

% Mutant target (mol)	Fluorescence intensity	Quenching of fluorescence in presence of mutation (%)
0	0	0
1,5	1,5	1,8
5,8	5,8	8,8
20	20	24,6
50	50	47
80	80	65
94	94	72
98	98	74
100	100	75

[a] Total concentration of the probe and RNA was 1.0 μM; excitation/emission wavelengths: 480/525 nm.