DNA strands with alternating incorporations of LNA and 2'-*O*-(pyren-1-yl)methyluridine: SNP-discriminating RNA detection probes

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SUPPORTING INFORMATION

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Protocol: synthesis, purification and quality control of modified ONs. ONs were synthesized via machine-assisted solid-phase DNA synthesis (0.2 µmol scale; succinyl linked LCAA-CPG support) using extended coupling (4,5-dicyanoimidazole as activator, 15 min, ~98% coupling yield) and oxidation times (45 s) during incorporation of the corresponding phosphoramidites of monomer $\mathbf{Y}^{S1,S2}$ and LNA monomers (A^{Bz}, $^{m}C^{Bz}$, G^{dmf} and T LNA phosphoramidites, Exiqon, Vedbæk, Denmark). Cleavage from solid support and removal of protecting groups was accomplished using 32% aq. ammonia (55 °C, 12 h). ONs were purified via ion-pair reverse phase HPLC (XTerra MS C18 column) using a triethylammonium acetate buffer - water/acetonitrile (v/v) gradient, detritylated (80% aq. AcOH), and precipitated from acetone (-18 °C for 12-16h). The identity of all modified ONs was verified through MALDI-MS/MS analysis recorded in positive ion mode on a quadrupole time-of-flight tandem mass spectrometer equipped with a MALDI source using anthranilic acid as a matrix (Table S1). Purity (>80%) was verified by ion-pair reverse phase HPLC running in analytical mode.

Protocol: thermal denaturation, absorbance and fluorescence studies. ON concentrations were estimated using the following extinction coefficients (OD/µmol): dA (15.2), dC (7.05), dG (12.01), T (8.4), rA (15.4), rC (9.0), rG (13.7), U (10.0), and pyrene (22.4). Strands were thoroughly mixed and denatured by heating to 70-85 °C, followed by cooling to the starting temperature of the experiment. Quartz optical cells with a path length of 1.0 cm were used. Thermal denaturation curves of duplexes (1.0 µM final concentration of each strand) were recorded on a Peltier-controlled UV/VIS spectrophotometer (Varian Cary 100) using a medium salt buffer (100 mM NaCl, 0.1 mM EDTA, and pH 7.0 adjusted with 10 mM Na_2HPO_4 and 5 mM Na_2HPO_4). A temperature ramp of 0.5 °C/min was used in all experiments. Thermal denaturation temperatures (T_m's) were determined as the maximum of the first derivative of

denaturation curves. The experimental temperatures ranged from at least 15 °C below T_m (although not below 3 °C) to at least 20 °C above T_m. Reported T_m-values are averages of at least two experiments within ± 1.0 °C unless otherwise mentioned.

UV-Vis absorption spectra (300-400 nm) of ONs and duplexes with DNA/RNA targets were recorded at 5 °C using the same solutions and instrumentation as above.

Steady-state fluorescence emission spectra of single-stranded probes and the corresponding duplexes with DNA/RNA targets were recorded on a Peltier-controlled fluorimeter (Varian Cary Eclipse) using the same solutions as above (i.e., each strand at 1.0 μM concentration in non-deoxygenated T_m buffer). Spectra were obtained as an average of five scans using an excitation wavelength of λ_{ex} = 350 nm, excitation slit = 5.0 nm, emission slit = 2.5 nm, and a scan speed of 600 nm/min. Spectra were recorded at 5 °C to ascertain maximal duplex formation.

Table S1. MALDI-MS analysis of modified ONs used in this study.[a]

ON	Sequence	Calc. *m/z* [M+H]	Found *m/z* [M+H]
ON1a	5'-GTG **a**T**a** TGC	2810	2810
ON1b	5'-GTG A**Y**A TGC	2970	2970
ON1c	5'-GTG **a****Y****a** TGC	3026	3026
ON2a	3'-CAC T**a**T CAC	2711	2710
ON2b	3'-CAC **Y**A**Y** ACG	3115	3115
ON2c	3'-CAC **Y****a****Y** ACG	3143	3142
ON3a	3'-CA**c** T**a**T **a**CG	2780	2780
ON3b	3'-CAC **Y**A**Y** ACG	3115	3115
ON3c	3'-CA**c** **Y****a****Y** **a**CG	3212	3213
ON4a	3'-CA**c** T**t**T **a**CG	2771	2771
ON4b	3'-CAC **Y**T**Y** ACG	3105	3105
ON4c	3'-CA**c** **Y****t****Y** **a**CG	3203	3202
ON5a	3'-CA**c** T**c**T **a**CG	2770	2770
ON5b	3'-CAC **Y**C**Y** ACG	3091	3091
ON5c	3'-CA**c** **Y****c****Y** **a**CG	3202	3203
ON6a	3'-CA**c** T**g**T **a**CG	2796	2796
ON6b	3'-CAC **Y**G**Y** ACG	3131	3131
ON6c	3'-CA**c** **Y****g****Y** **a**CG	3228	3229
ON7	3'-GC GT**t** **Y****a****Y** **t**TG CG	4487	4487
ON8	3'-GC GT**t** **Y****c****Y** **t**TG CG	4477	4477
ON9	3'-GC GT**t** **Y****t****Y** **t**TG CG	4478	4478
ON10	3'-GC GT**t** **Y****g****Y** **t**TG CG	4503	4503

[a] For structures of **Y** and LNA monomers, see Fig. 1 in the main manuscript.

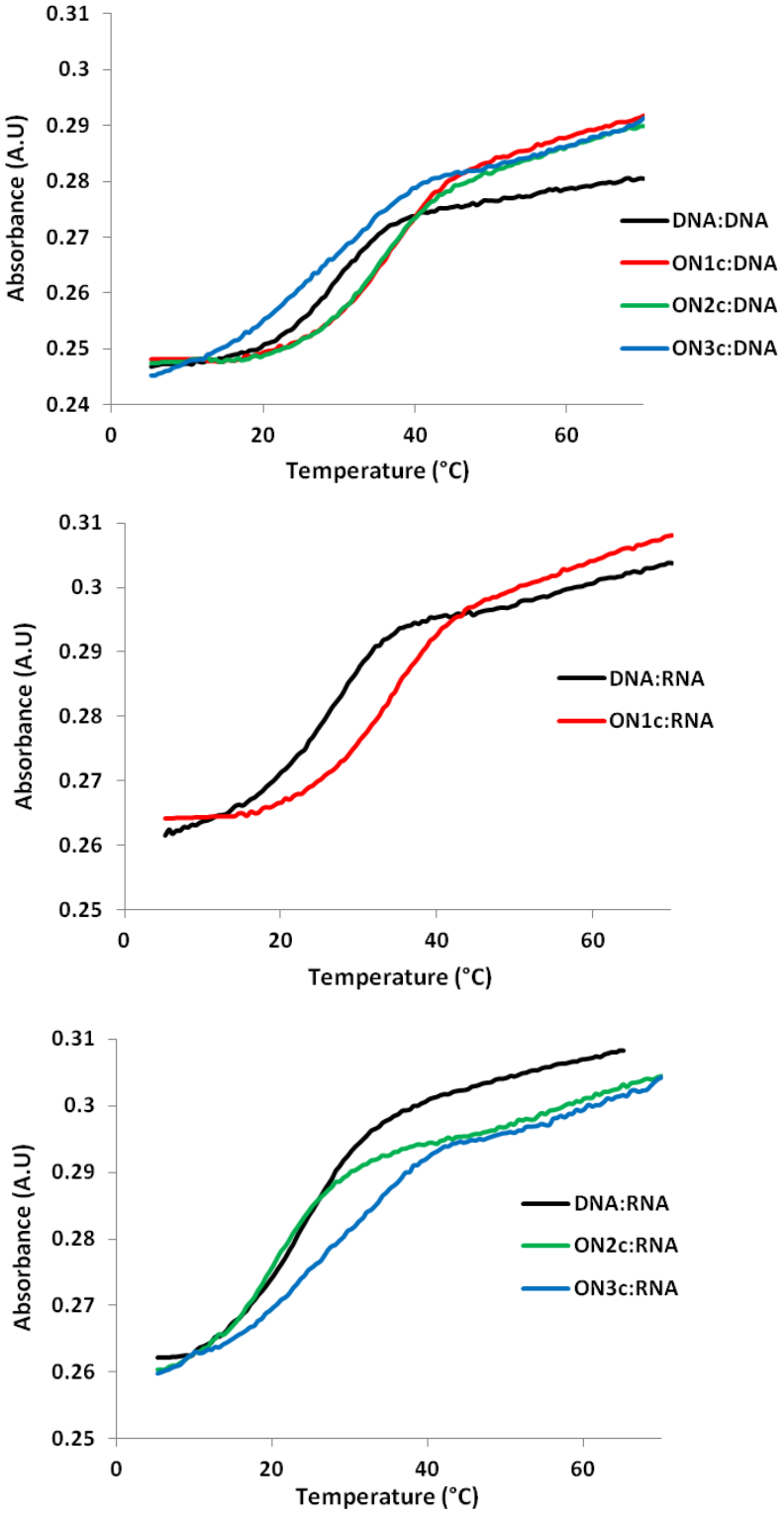

Figure S1. Representative thermal denaturation curves. For experimental conditions, see Table 1.

Discussion of T_m's for duplexes involving reference strands ON3-ON6 (a/b-series).

The nature of the central LNA nucleotide has little relative influence on the thermal denaturation properties of duplexes involving LNA-modified reference strands (compare ΔT_m values for **ON3a**-**ON6a**, Tables 1 & 2). Duplexes involving **Y**-modified reference strands with a central purine are more thermostable than those with a corresponding pyrimidine (compare ΔT_m values for **ON3b**/**ON6b** vs **ON4b**/**ON5b**, Tables 1 & 2). This is in agreement with previous NMR studies, which have shown that the nucleobase of the 3'-flanking nucleotide is stacking with the intercalating pyrene moiety of a **Y**-monomer.[S3]

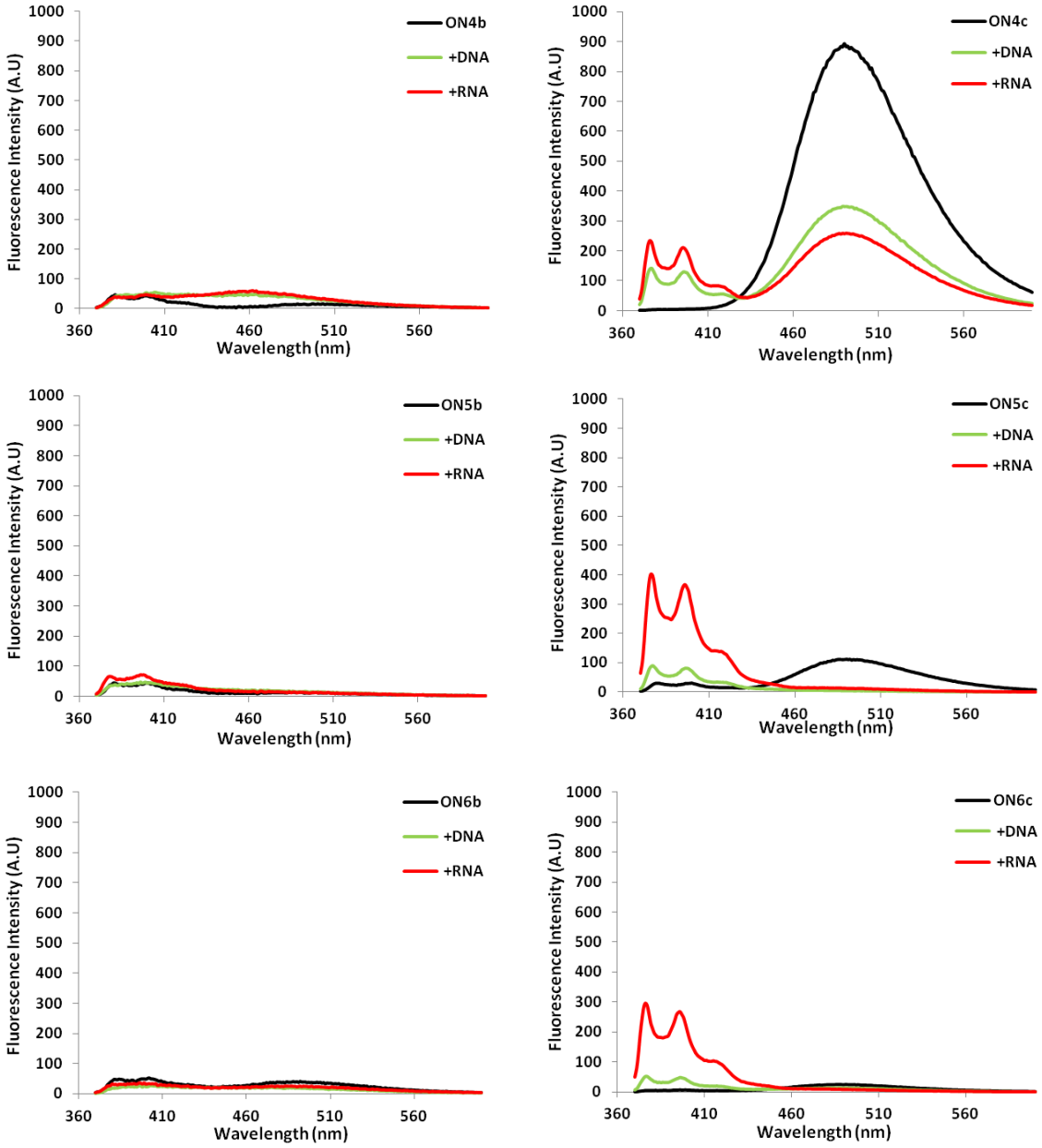

Figure S2. Steady-state fluorescence emission spectra of **ON4-ON6** and corresponding duplexes with DNA/RNA targets. Spectra were recorded at T = 5 °C using λ_{ex} = 350 nm and each strand at 1.0 μM concentration in T_m buffer.

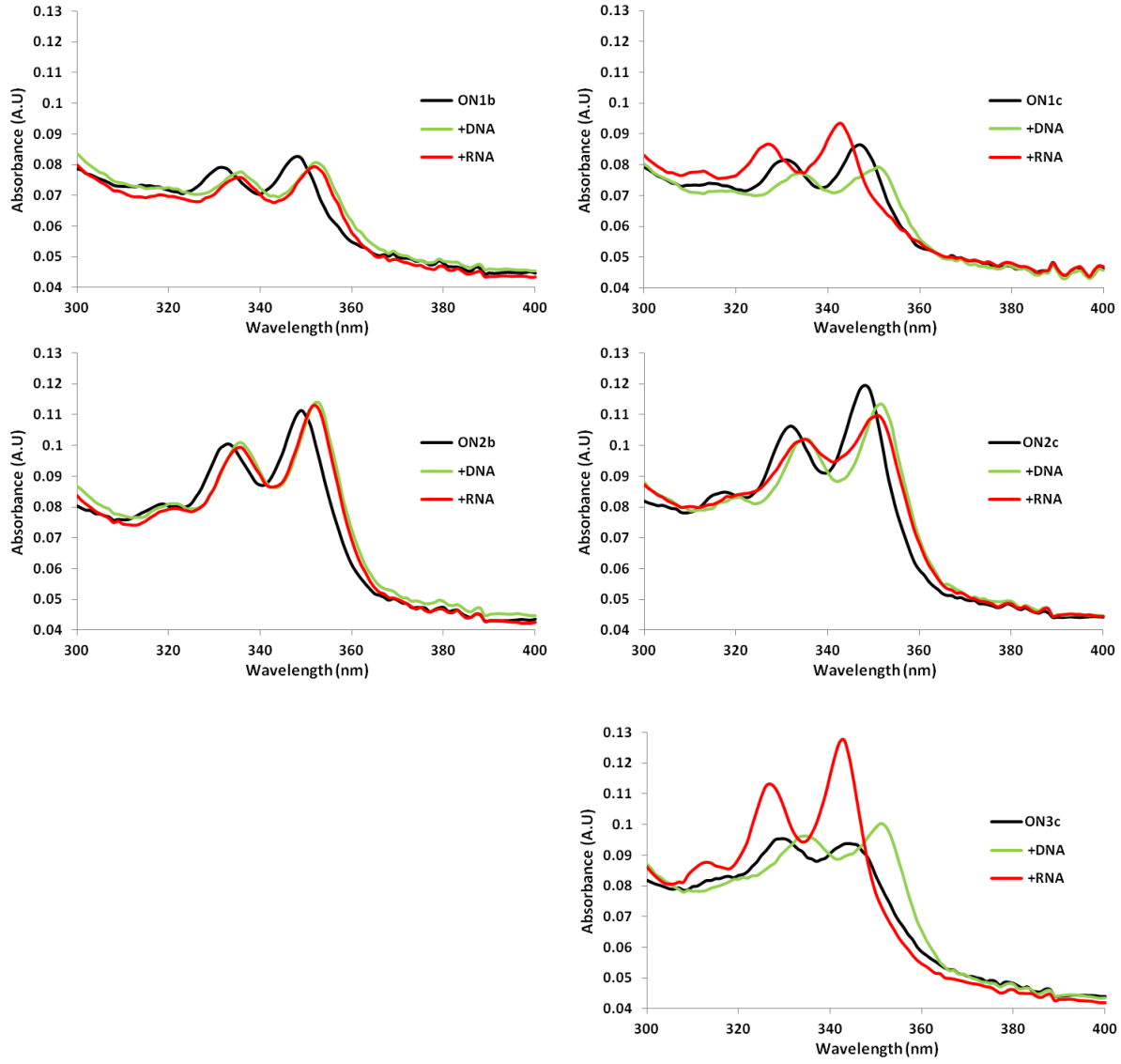

Figure S3. Absorption spectra of **ON1-ON3** and the corresponding duplexes with DNA/RNA targets. Spectra were recorded at T = 5 °C using each strand at 1.0 μM concentration in T_m buffer.

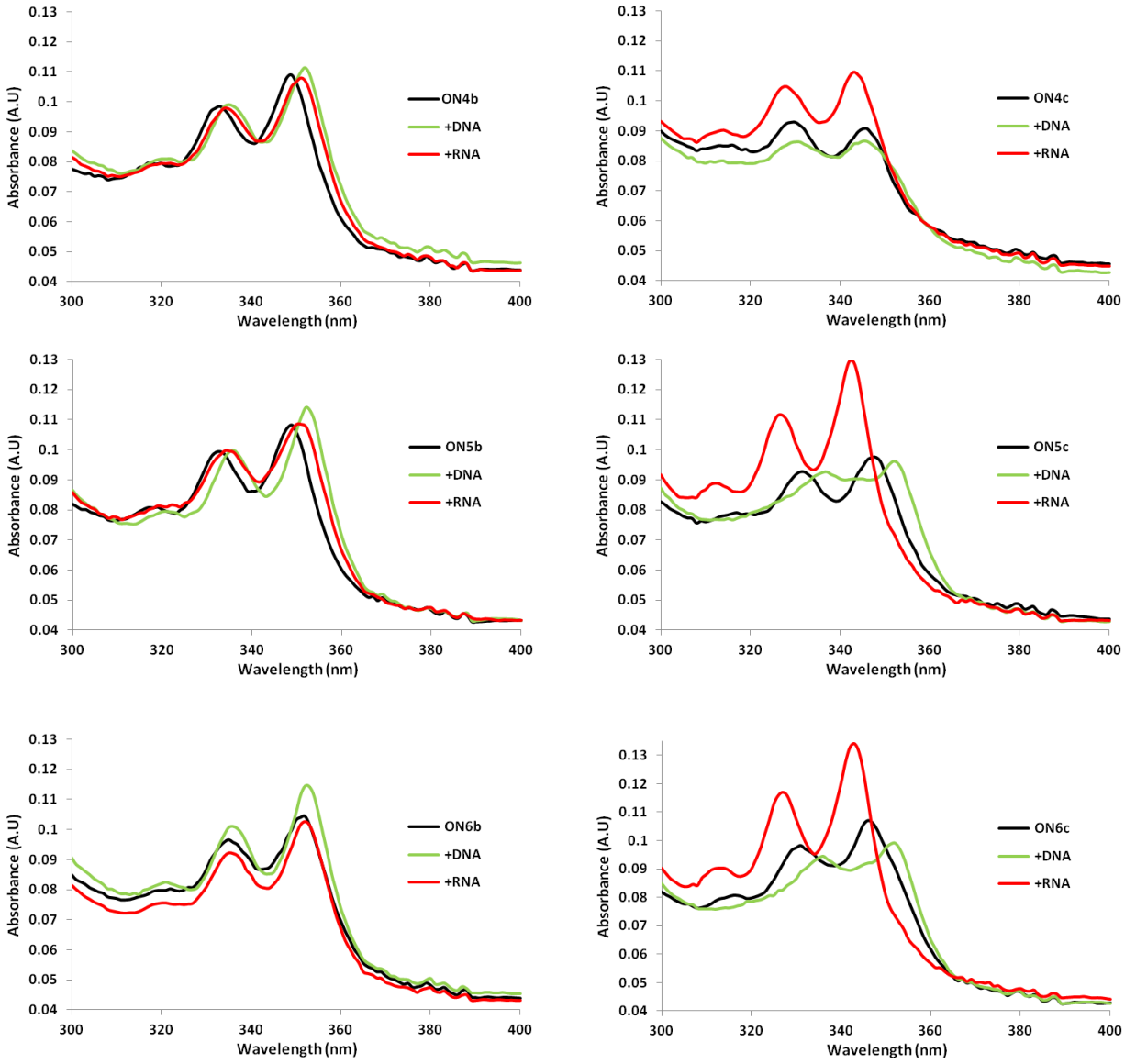

Figure S4. Absorption spectra of **ON4-ON6** and the corresponding duplexes with DNA/RNA targets. Spectra were recorded at T = 5 °C using each strand at 1.0 μM concentration in T_m buffer.

Discussion regarding RNA mismatch discrimination of ON3c-ON6c.

ON3c-ON6c, which feature a central –L**Y**L**Y**L- motif, display similar thermal discrimination of centrally mismatched RNA targets as unmodified reference strands (compare ΔT_m's for **ON3c-ON6c** vs **ON3 ref-ON6 ref**, Table S2).

Duplexes between **ON3c-ON6c** and centrally mismatched RNA targets display significantly stronger excimer and weaker monomer emission than fully base-paired duplexes (Fig. S5). However, due to the low T_m's of the mismatched duplexes (T_m = 11-23.5 °C, Table S2) relative to the experimental temperature of the fluorescence experiments (T = 5 °C), we could not unequivocally establish whether the observed fluorescent discrimination of mismatches was due to different localization of pyrene moieties in mismatched duplexes, denaturation (duplex $\rightarrow$ single strands), or a combination hereof. Subsequent studies with 13-mer probes – for which higher absolute T_m's of mismatched duplexes are observed – suggested that the observed optical discrimination indeed is due to differential positioning of pyrene moieties in mismatched versus matched duplexes (*vide infra*).

Table S2. Thermal denaturation temperatures (T_m's) of duplexes between **ON3-ON6** and complementary or mismatched RNA targets.[a]

			T_m [ΔT_m]/°C			
			5'-GU GA**B**AU GC			
ON	Sequence	**B** =	A	C	G	U
ON3c	3'-CA **cYaYa** CG		11.0 [-13.5]	13.0 [-11.5]	15.0 [-9.5]	**24.5**[b]
ON3 ref	3'-CA CTATA CG		15.5 [-9.0]	16.5 [-8.0]	15.5 [-9.0]	**24.5**
ON4c	3'-CA **cYtYa** CG		**26.5**[b]	16.5 [-10.0]	16.5 [-10.0]	15.5 [-11.0]
ON4 ref	3'-CA CTTTA CG		**28.5**	13.5 [-15.0]	20.5 [-8.0]	12.5 [-16.0]
ON5c	3'-CA **cYcYa** CG		23.5 [-23.0]	21.5 [-25.0]	**46.5**	22.0 [-24.5]
ON5 ref	3'-CA CTCTA CG		11.5 [-26.0]	12.0 [-25.5]	**37.5**	12.5 [-25.0]
ON6c	3'-CA **cYgYa** CG		17.5 [-20.0]	**37.5**	15.5 [-22.0]	18.5 [-19.0]
ON6 ref	3'-CA CTGTA CG		10.5 [-23.0]	**33.5**	15.5 [-18.0]	15.5 [-18.0]

[a] For conditions of thermal denaturation experiments, see Table 1. T_m values of fully matched duplexes are shown in bold. ΔT_m = change in T_m relative to fully matched DNA:RNA duplex.

[b] Broad transition. Error of T_m value estimated at ±3.0 °C.

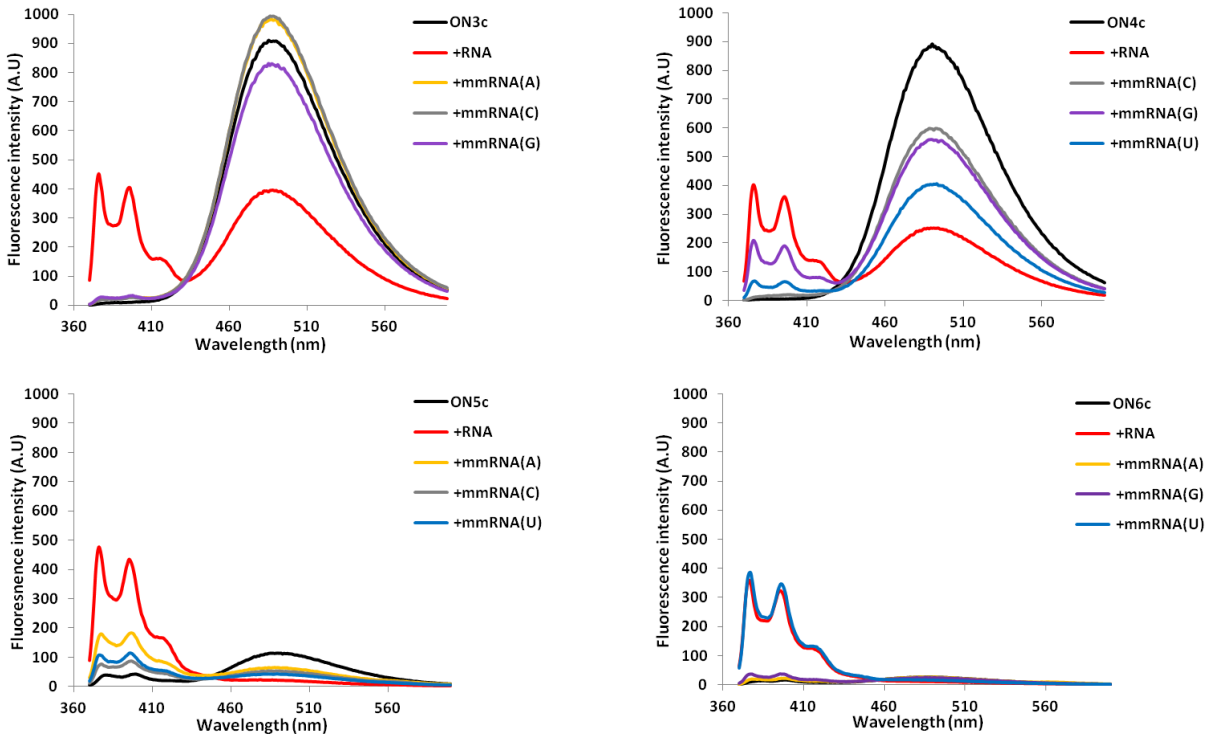

Figure S5. Steady-state fluorescence emission spectra of **ON3c-ON6c** and the corresponding duplexes with complementary or mismatched RNA targets. Mismatched nucleotide in RNA target strand (mmRNA) listed in parenthesis. Spectra were recorded at T = 5 °C using λ_{ex} = 350 nm and each strand at 1.0 μM concentration in T_m buffer.

Table S3. Thermal denaturation temperatures (T_m's) of duplexes between 13-mer **ON7**-**ON10** and complementary DNA.[a]

		T_m [ΔT_m]/°C
ON	Sequence	+DNA
ON7	3'-GCGT **t<u>Y</u>a<u>Y</u>t** TGCG	50.0 [-1.0]
ON8	3'-GCGT **t<u>Y</u>c<u>Y</u>t** TGCG	51.5 [-2.0]
ON9	3'-GCGT **t<u>Y</u>t<u>Y</u>t** TGCG	43.0 [-8.0]
ON10	3'-GCGT **t<u>Y</u>g<u>Y</u>t** TGCG	51.5 [-3.0]

[a] For conditions of thermal denaturation experiments, see Table 1. T_m's of the corresponding reference duplexes are 51.0 °C (**ON7** ref), 53.5 °C (**ON8** ref), 51.0 °C (**ON9** ref), and 54.5 °C (**ON10** ref).

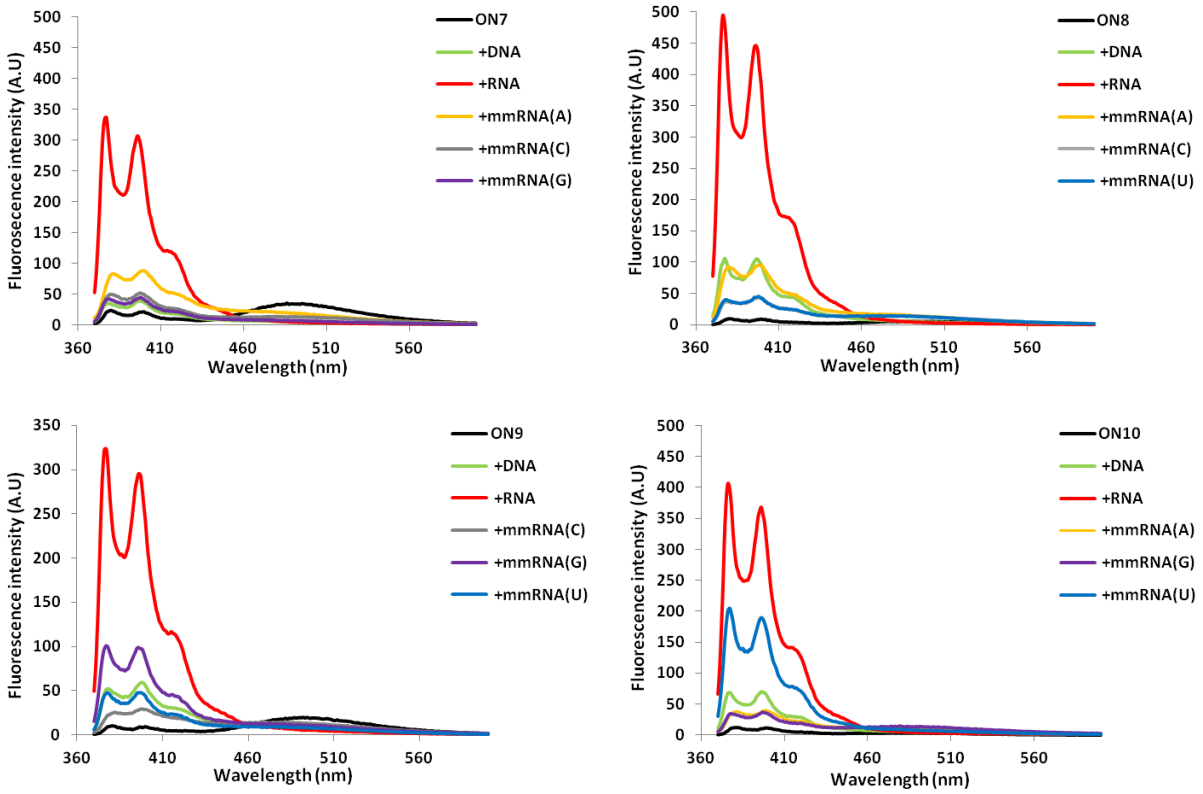

Figure S6. Steady-state fluorescence emission spectra of **ON7-ON10** and the corresponding duplexes with complementary DNA/RNA or centrally mismatched RNA targets (mismatched nucleotide listed in parenthesis). Spectra recorded at T = 5 °C using λ_{ex} = 350 nm and each strand at 1.0 μM concentration in T_m buffer.

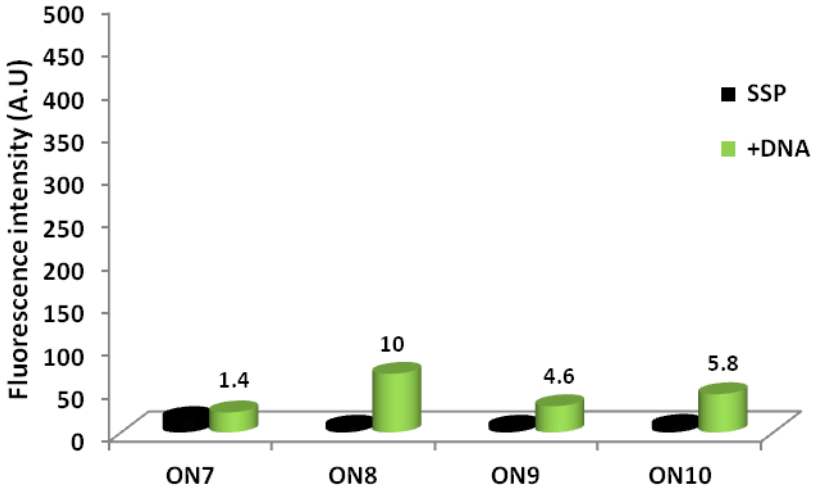

Figure S7. Fluorescence intensity of single-stranded probes (SSP) **ON7-ON10** and corresponding duplexes with complementary DNA. Hybridization-induced increases/decreases – defined as the intensity ratio between a duplex and single-stranded probe (SSP) – are listed above histograms. Intensity recorded at λ_{em} = 376 nm and T = 5 °C using each strand at 1.0 μM concentration. For spectra, see Fig. S6.

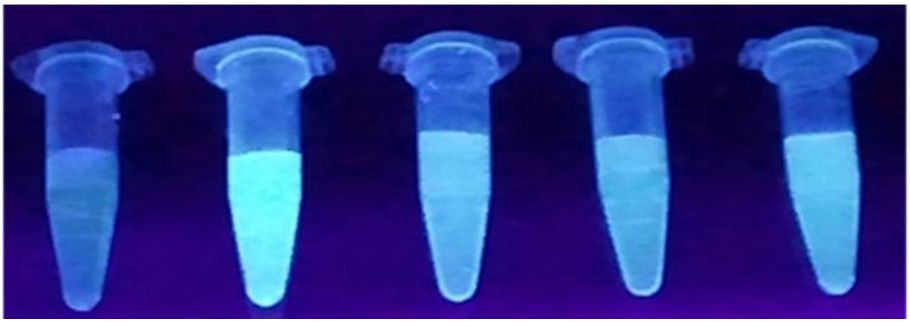

Figure S8. RNA mismatch discrimination using **ON8** (mismatched nucleotide listed in parenthesis). Image was recorded on a MultiDoc-It Imaging System (UVP) equipped with a LM-20E Transilluminator and a digital camera. Excitation setting 365 nm; each oligo used at 5 μM concentration in T_m buffer; T = 5 °C.

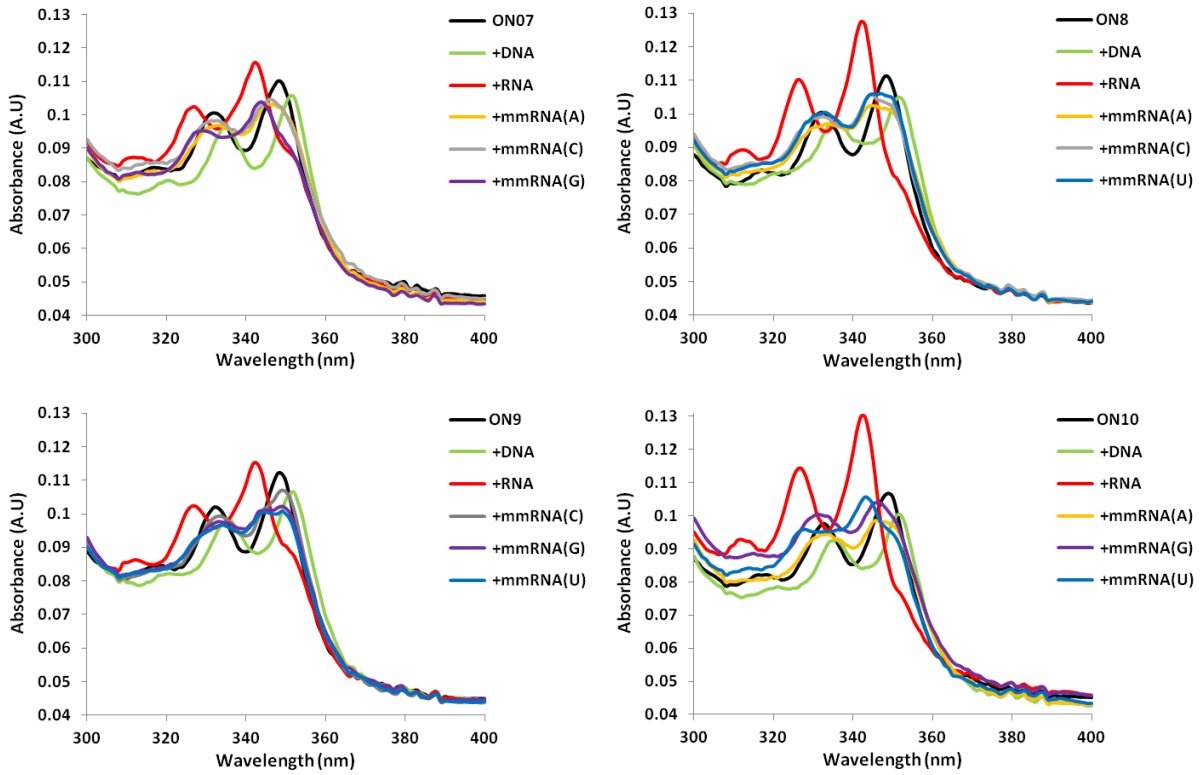

Figure S9. Absorption spectra of **ON7-ON10** and the corresponding duplexes with complementary DNA/RNA or centrally mismatched RNA targets (mismatched nucleotide listed in parenthesis). Spectra recorded at T = 5 °C using each strand at 1.0 μM concentration in T_m buffer.

Table S4. Pyrene absorption maxima for **ON7-ON10** and the corresponding duplexes with DNA targets.[a]

		λ_{max} (nm)	
ON	Sequence	SSP	+DNA
ON7	3'-GC GT **t<u>Y</u>a<u>Y</u>t** TG CG	348	352 [+4]
ON8	3'-GC GT **t<u>Y</u>c<u>Y</u>t** TG CG	348	352 [+4]
ON9	3'-GC GT **t<u>Y</u>t<u>Y</u>t** TG CG	348	352 [+4]
ON10	3'-GC GT **t<u>Y</u>g<u>Y</u>t** TG CG	349	352 [+3]

[a] Conditions as described in footnote of Table 1. Spectra were recorded at T = 5 °C using each strand at 1.0 μM concentration in T_m buffer. SSP denotes single-stranded probe. For spectra, see Fig. S9.

References.

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