Label-free selective DNA detection with high mismatch recognition by PNA beacons and ion exchange HPLC†

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Two 11mer peptide nucleic acid (PNA) beacons were synthesized and tested for the detection of full-matched or single mismatched DNA. Fluorescent measurements carried out in solution showed only partial discrimination of the mismatched sequence, while using anion-exchange HPLC, in combination with fluorimetric detection, allowed DNA analysis to be performed with high sensitivity and extremely high sequence selectivity. Up to >90 : 1 signal discrimination in the presence of one single mismatched base was observed. The analysis was tested on both short and long DNA oligomers. Detection of DNA obtained from PCR amplification was also performed allowing the selective detection of the target sequence in complex mixtures. Label free detection of the DNA with high sequence selectivity is therefore possible using the present approach.

Introduction

Genome-based technologies rely on the possibility of selectively recognizing DNA sequences of particular interest. The quest for new and selective methods and technologies for the detection of specific DNA tracts is gaining more and more importance in diagnostics, from biomedical to more large scale items such as food and feed.[1]

One very important class of probes is represented by the so called molecular beacons (MB), which are composed of a sequence specific oligonucleotide coupled with a fluorophore and a quencher (or a quenching surface) at each end, held together by a zipper DNA sequence made of complementary antiparallel tracts; this structure allows a "switch-on" of fluorescence to be produced upon interaction with the target DNA sequence.[2] A variety of applications to DNA or RNA detection have been proposed using MB probes;[3] detection of single point mutations can be achieved by MB through careful design of the sequence and selection of the detection temperature,[4a,b] or by means of more elaborate strategies.[4c,d]

Peptide nucleic acids (PNAs) are oligonucleotide mimics based on a polyamide backbone, superior to oligonucleotide probes in the recognition of single base mutations.[5,6] PNA-beacons and the related "light up probes" have been recently described,[7] displaying the advantages of higher selectivity and simpler design.[8,9] PNA beacons lacking of the stem tract were designed, and were shown to be able to undergo a "switch on" in fluorescence upon binding to complementary DNA, being quenched in their free form, probably due to the hydrophobic interaction of fluorophore and quencher in aqueous solution.[9]

One of the major limitations in the use of PNA and other MB in diagnostics is represented by the fluorescence background of the free (uncomplexed) probe, which interferes with the signal obtained from the analyte sequence, especially for DNA amplified by complex biological samples.

In previous works, we have demonstrated that ion-exchange (IE) HPLC can be used to directly visualize the PNA:DNA interaction, since the duplex shows retention times different from those of PNA and ss- or dsDNA.[10] When the DNA to be analyzed is labelled with fluorescent groups, the chromatographic profile is simpler, but interfering peaks of primers and of nonspecific amplified DNA can be present. We report here the combined use of PNA-beacon and IE-HPLC analysis for the label-free detection of DNA, taking advantage on one side of the separation of the free probe from the complex, and on the other side of the very specific signal generated by the PNA beacon, which avoids the presence of non-specific peaks.

Results and discussion

Design of the PNA beacons

The PNA beacons and DNA sequences used in the present study are listed in Chart 1.

The beacons were synthesized using the same design described earlier,[9a] without including a zipper stem of complementary sequences, and using dabcyl as a quencher and carboxyfluorescein as fluorophore. The beacon **1** showed a relatively low T_m of the PNA:DNA duplex (Table 1), while beacon **2** has the same sequence of a PNA previously utilized as a probe for the detection of Roundup Ready soybean in HPLC, with a higher PNA:DNA melting temperature.[10]

Fluorimetric detection

Both beacons in their free state showed similar low background fluorescence with $F(\mathbf{1})/F(\mathbf{2}) = 1.02$ under the same conditions

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Table 1 Comparison between the fluorescence response and peak area for beacon **1** and **2** with oligonucleotides in the presence of different DNA oligonucleotides. Standard deviations are in parentheses

				Fluorimetric F/F_0[c] (520 nm)		
PNA	DNA	T_m/°C[a]	c/μM[b]	25 °C	35 °C	HPLC area[d]
1	None	—	1	1.0(0.1)	1.0(0.1)	1.7(0.6)
1	**3-G**	48	0.1	1.8(0.1)	1.5(0.1)	7.2(1.5)
1	**3-G**	48	1	8.6(0.3)	5.5(0.1)	100.0(7.7)
1	**3-C**	32	1	1.3(0.1)	1.2(0.1)	1.7(0.4)
1	**3-A**	30	1	1.5(0.1)	1.4(0.1)	2.6(1.1)
1	**3-T**	31	1	1.2(0.1)	1.2(0.1)	2.0(0.4)
1	**5**		1	1.0(0.1)	1.0(0.1)	1.3(0.8)
2	None	—	0.1	1.0(0.1)	1.0(0.1)	1.6(0.4)
2	**4-A**	65	0.1	7.2(0.5)	7.0(0.2)	100.0(4.4)
2	**4-C**	54	0.1	5.2(0.4)	4.2(0.1)	1.0(0.4)
2	**4-T**	53	0.1	5.0(0.4)	3.9(0.1)	1.1(0.3)
2	**4-G**	55	0.1	6.4(0.6)	5.1(0.3)	1.1(0.8)
2	**6**		0.1	1.4(0.1)	1.1(0.1)	1.6(0.4)
2	**79-A**	—	0.1	6.9(0.5)	6.5(0.2)	82.6(8.3)
2	**79-T**	—	0.1	1.8(0.1)	1.5(0.1)	2.1(0.2)

[a] PNA:DNA melting temperature $c = 5$ μM of each strand. [b] Concentration of the beacon and of DNA used in the measurement (in strand). [c] Fluorescence intensity normalized to the value of the free beacon. [d] Area of the PNA:DNA peak normalized to that of the full matched, measured at 25 °C for PNA **1**, and at 35 °C for PNA **2**.

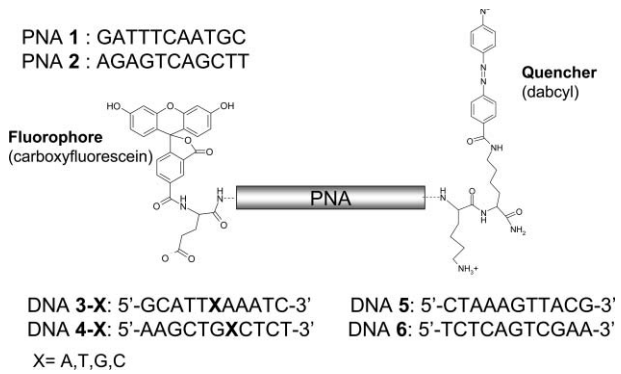

Chart 1

(either at 1 μM or 0.1 μM concentration). Hybridization of the two beacons with fully complementary or single mismatched oligonucleotides gave rise to a switch-on of the beacon fluorescence, which was sequence-selective and depended on the beacon affinity for the DNA. The PNA **1** showed a very weak fluorescence enhancement at 0.1 μM concentration upon addition of the full match DNA **3-G**, while at a higher concentration (1 μM), a significant enhancement was observed (Table 1). In the presence of mismatched DNA the fluorescence increase was much lower, but not negligible.

Using PNA **2** at 0.1 μM concentration, the addition of complementary DNA **4-A** induced a significant increase in fluorescence intensity, in agreement with the T_m of PNA:DNA being higher than that of PNA **1**. Since both beacons showed similar background fluorescence the different increase is due to incomplete hybridization, which corresponds to different stability of the PNA:DNA duplex and hence a different dissociation constant. It was therefore possible to use this PNA beacon at a lower concentration than PNA **1**. However, since the mismatched sequences also have a higher T_m, the fluorescence increase obtained with mismatched DNA **4-(C, T, G)** was significant.

By increasing the temperature from 25 °C to 35 °C for the PNA **1** the full match response was decreased and selectivity was not increased, while for the PNA **2** a higher selectivity was observed since the PNA **2**:DNA **4-A** showed an almost identical fluorescence increase, while the response obtained with mismatched sequences decreased in all cases. However, still a >3 fold increase in fluorescence was observed for all the mismatched DNA **4-(C, T, G)**.

For PNA **2** a slightly lower increase was observed upon addition of a longer 79mer DNA target (DNA **79-A**) which contains the target sequence **4-A** and is identical to a PCR fragment previously tested for Roundup Ready soybean.[10] The presence of a single mismatch (T instead of A, DNA **79-T**) gave rise to a very low increase, suggesting a possible competition of secondary structures formed in the ssDNA target (such as partial hybridization of complementary tracts), which competes with PNA hybridization.

Addition of complementary parallel DNA sequences (DNA **5** for PNA **1** and DNA **6** for PNA **2**) induced only a very low or no fluorescence increase, with only a 1.4 increase at 25 °C for the PNA **2**:DNA **6** case, which was reduced to 1.1 by increasing the temperature to 35 °C.

Therefore, using fluorimetric measurements it was possible to discriminate between full match and mismatched DNA at 1 μM concentration of **1** and 0.1 μM for **2**, but with only partial mismatch recognition; the possibility of detection of a single mismatch with high selectivity strongly depends on the sequence used and on the measuring conditions.

IE-HPLC detection of oligoncleotides

Using IE-HPLC analysis with fluorescence detection allowed high selectivities to be obtained (Fig. 1) for both PNA **1** and **2**. The target DNA sequences were mixed with the beacon and injected into the HPLC system using an anion exchange column (TSK-gel NPR); the elution was carried out with a NaCl gradient, as reported in the Experimental section.

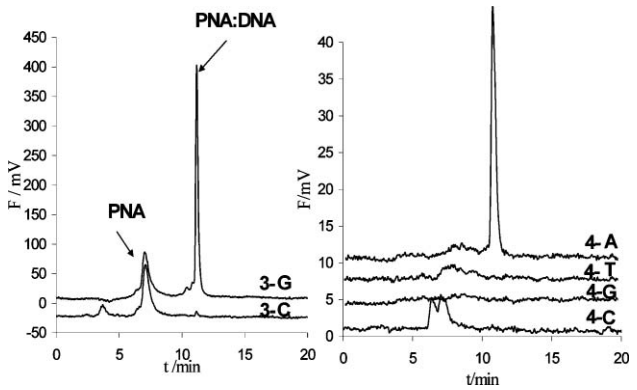

Fig. 1 IE HPLC profiles obtained using the beacons **1** (1 μM, left panel) and **2** (0.1 μM, right panel) with 1 : 1 fully matched complementary oligonucleotides (**3-G** for beacon **1** and **4-A** for beacon **2**) or with a single mismatched oligonucleotide (lower traces, mismatched base indicated). Column: TSK-gel DNA NPR (4.6 mm ID × 7.5 cm); eluents: A = Tris 0.02 M, pH = 9.0, B = NaCl 1 M in eluent A. Linear gradient: from 100% A to 100% B in 20 min; flow rate: 0.5 mL min^{-1}. $T = 25$ °C for left panel and 35 °C for right panel. Fluorescence detector $\lambda_{ex} = 497$ nm, $\lambda_{em} = 520$ nm.

In the fluorescence detection mode, only the residual beacon (broad peak at lower retention time, visible in Fig. 1a) and the PNA:DNA complex were observed, the latter being enhanced by the "switch on" of the fluorescence due to complexation.

Therefore, an excess of beacon can be used to reveal the DNA without overlap of the corresponding peak with that of the complex. According to their DNA affinity, different concentrations of the two PNA were used (1 μM for PNA **1** and 0.1 μM for PNA **2**).

For PNA **2**, at 25 °C a linear increase of the fluorescence response was observed by increasing the DNA concentration in the range 1–100 nM, with a detection limit of 2 nM (*i.e.* of 20 fmol of injected DNA). Furthermore, the chromatographic system has a significant denaturing effect for the mismatched PNA:DNA duplexes, allowing the detection of the full matched DNA with complete selectivity (Fig. 1 and Table 1).

Best results in terms of single-mismatch recognition were observed at 25 °C for beacon **1** and at 35 °C for beacon **2**, according to the different melting temperatures of the PNA:DNA duplexes.

In particular, for the PNA **2** at 35 °C (and 0.1 μM concentration), a high signal increase was observed with DNA **4-A** (a 91 fold increase compared to 1.1 value of the background), while no signal significantly different from background was detected using the mismatched oligonucleotides, giving rise to almost perfect sequence selectivity. Similar results were obtained for the beacon **1** at 25 °C and at 1 μM concentration (Table 1), but with a lower signal to noise ratio.

Upon injection of a mixture of beacon **2** and of the 79mer synthetic oligonucleotide (DNA **79-A**), a peak with increased retention time was observed (Fig. 2, left). In the presence of a single nucleotide mismatch (T instead of A, DNA **79-T**) no peak was observed in the fluorescence channel (while the corresponding peak was detected by UV, as shown in Fig. 2, right panel, trace b). Though the mismatch considered in this study has no biological significance, the present results suggest that selectivity can be very high also in the case of PCR products.

These results are remarkable if compared with data obtained using fluorescence detection by us and by others at similar temperatures.[7b,9a] For example, using fluorimetric detection a full-match to mismatch ratio of 1.5 at 25 °C and 5 at 37 °C was observed.[9a] Furthermore, the present method avoids the use of enzymatic reactions, which were reported to improve the full-match to mismatch signal, but required additional treatments.[9d]

IE-HPLC detection of PCR products

The beacon **2** was subsequently tested against the same **79-A** DNA obtained as a PCR product from RR soybean flour and with a 201bp PCR product not containing the sequence (tract of peanut DNA, complete sequences available in the Electronic Supplementary Information). In both cases, unbalanced PCR with an excess of one primer was carried out, in order to obtain the single-stranded target DNA. The beacon was added after PCR completion, in order to avoid possible inhibition of amplification

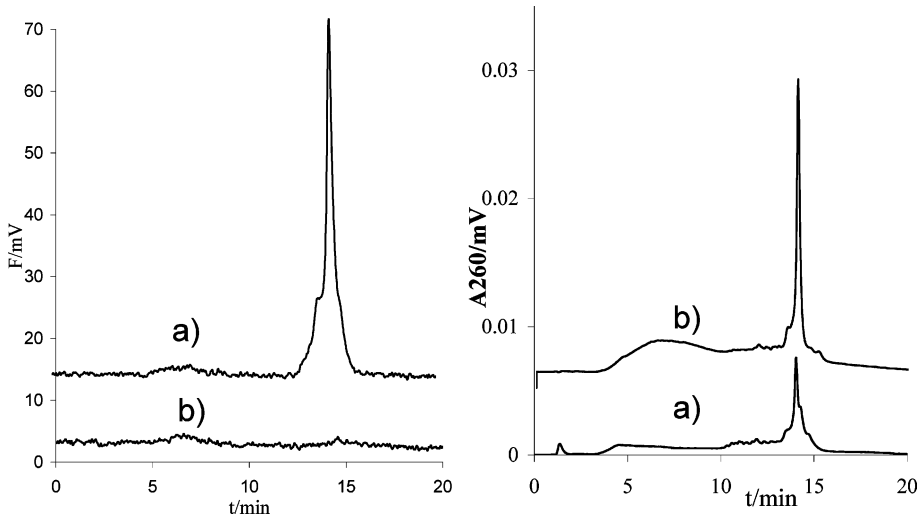

Fig. 2 IE HPLC profiles obtained by fluorescence (left panel) and by UV (right panel) using the beacon **2**; a) beacon **2** (0.1 μM) + full match synthetic 79-mer (**79-A**); b) beacon **2** (0.1 μM) + synthetic **79-T**. $T = 25$ °C. All other conditions were as reported in Fig. 1.

due to PNA binding. Using fluorimetric detection, only an increase to $F/F_0 = 3.8$ was observed for the target PCR amplicon, while the non-specific PCR sample gave rise to an increase to $F/F_0 = 1.2$. The use of more elaborate measurements such as fluorescence anisotropy or time-resolved fluorescence did not allow the signal selectivity to be substantially increased (results not shown).

In contrast, in the HPLC system, two peaks were observed by injecting the mixture of beacon **2** and the PCR product (obtained from RR soy): one corresponding to the retention time of the PNA:DNA duplex formed by the synthetic **79-A** DNA and one corresponding to the beacon (Fig. 3, left panel, d).

No interfering peak was observed upon addition of either a blank sample or the 201nt nonspecific PCR product, although an enhancement of the intensity of the broad beacon peak was observed, due to a nonspecific interaction with the components of the PCR reaction (Fig. 3, left panel, c).

It is interesting to compare the simple chromatogram obtained in the fluorescence detection mode with the UV chromatograms of the same samples (Fig. 3, right panel); in the latter, all the reagents present in the PCR reaction are visible, with a series of intense peaks between 5 and 8 min, (mononucleotide triphosphates or their hydrolysis products, primers, additives *etc.*), while both aspecific and specific PCR products were observed as small peaks between 10 and 15 min. Only a very small peak (not even visible in Fig. 3) of the PNA:DNA duplex could be detected in the case of the matched sample.

The use of PNA beacons therefore allows enhancement of the signal generated by the PNA:DNA hybrid, selective detection of the target DNA in the presence of other components, and discrimination of specific *versus* nonspecific PCR in a label-free DNA detection scheme.

Conclusions

In conclusion, the present results allow us to propose a sensitive and selective method for the detection of specific DNA sequences by means of the simple and widely used technique of anion exchange HPLC with fluorescence detection.

Detection of single point mutations of clinical interest or SNP identification can be envisaged. Even more selective responses could be obtained using this strategy in combination with modified PNA backbones which allow the sequence selectivity to be increased.[11,12]

Since new trends in analytical techniques involving microfluidic devices[13] and high throughput HPLC systems, allow multiple analysis to be performed in a parallel way, the present method can be useful in the development of new advanced, highly specific diagnostic tools.

Experimental

Reagents

PNA monomers were from Applera (Milan, Italy); MBHA-Rink amide resin was from Novabiochem (Inalco spa, Milan, Italy); *O*-(benzotriazolyl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-diisopropylethylamine (DIEA) were from Aldrich (Milan, Italy) and *N*-methylpyrrolidone (NMP) was from Advanced Biotech Italia srl (Seveso, Italy).

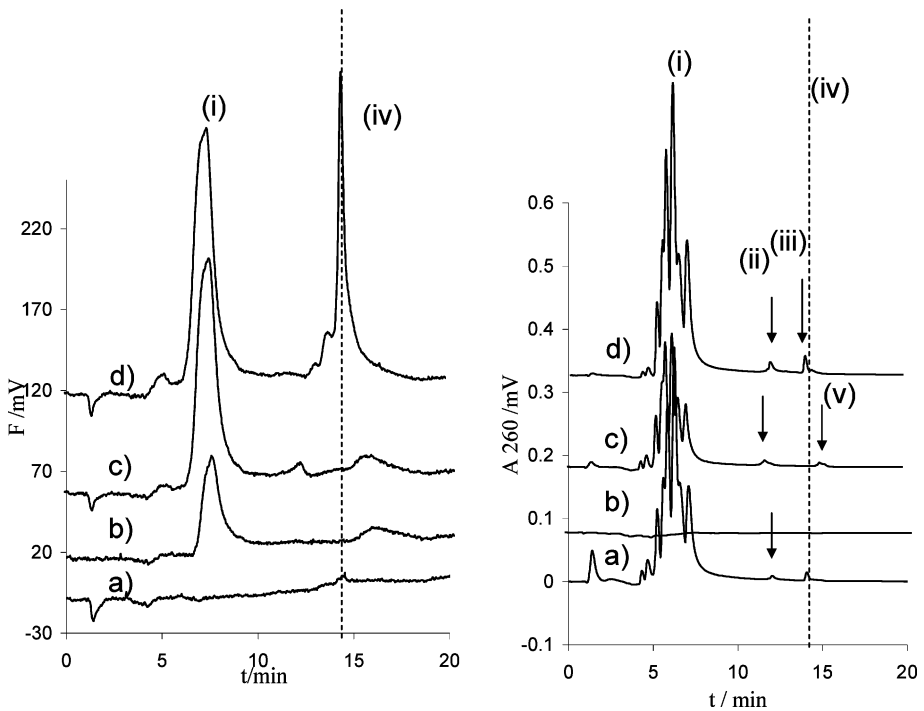

Fig. 3 IE HPLC profiles obtained using the beacon **2**. Left panel fluorescence detector; right panel: UV detection at 260 nm; a) PCR product alone; b) beacon **2** (1 μM) alone; c) beacon **2** (1 μM) + nonspecific PCR product; d) beacon **2** (1 μM) + specific PCR product. Attribution: i) beacon and components of PCR reaction; ii) aspecific PCR products (primer-dimers); iii) 79-mer RR PCR product; iv) PNA beacon–DNA hybrid; v) 201bp nonspecific PCR product (negative control). $T = 25$ °C. All other conditions were as reported in Fig. 1.

All solvents used for HPLC were of chromatographic grade. Doubly-distilled water was produced by Millipore Alpha-Q purification module.

Oligonucleotides used for melting temperature measurement were purchased from Thermoelectron (Ulm, Germany), and their purity was checked by ion-exchange HPLC. Standard soybean flour of known GMO content (certified material) were obtained from FLUKA (Milan, Italy).

Beacon synthesis, purification and characterization

The synthesis was performed on an ABI 433A peptide synthesizer with software modified to run the PNA synthetic steps (scale: 5 micromol), using Fmoc chemistry and standard protocols with HBTU–DIEA coupling. A brief description of various steps is reported hereinafter.

An MBHA-Rink amide resin (29 mg, 0.64 mmol active sites per g) was downloaded to 0.17 mmol g^{-1} with Fmoc- Dabcyl-Lys-OH. To this a second lysine unit (Fmoc(Boc)Lys) was coupled using a 4-fold excess and HBTU–DIEA.

The PNA oligomer was subsequently synthesized, after Fmoc-deprotection, according to the procedures described in reference 5.

After completion of the PNA part, an Fmoc(OtBu)Glu-OH residue was coupled at the N-terminal PNA monomer and deprotected. 125 μL of a 0.4 M solution of carboxyfluorescein in NMP were pre-mixed with 125 μl of a 0.4 M solution DHBtOH in NMP and 7.8 μl of DIC (50 μmol). This solution was then added to the resin and stirred overnight. After the coupling, the resin was drained and washed with NMP (10 times). A qualitative Kaiser test (negative) was performed. The resin was then washed with DCM (10 times) and dried under vacuum.

The PNAs were cleaved from resin by using TFA–*m*-cresol (9 : 1); the cleaving solution was filtered on sintered glass, the filtrate was concentrated under a nitrogen stream, and the crude products were precipitated by addition of Et_2O. The mixture was cooled at −20 °C for 2 hours, followed by centrifugation (5 min at 5000 rpm, twice) and removal of solvent by pipetting to afford the crude PNA products as a red precipitate. Residual ether was removed under a stream of nitrogen.

The crude PNAs were purified on RP HPLC with UV detection at 260 nm. A semi-preparative C18 (5 μm, 250 × 10 mm, Jupiter Phenomenex, 300 A) column was utilized, eluting with 0.1% TFA in water (eluent A) and 0.1% TFA in water–acetonitrile 60 : 40 (eluent B); elution gradient: from 100% A to 100% B in 50 min, flow: 4 ml min^{-1}.

The PNA beacons were characterized by MS-ESI (Micromass ZMD) which gave positive ions consistent with the final products.

PNA beacon **1**. Yield (after purification): 5.8%. calculated MW: 3989.0. ESI-MS. *m/z* 798.8 (MH_5^{5+}), 665.8 (MH_6^{6+}); 570.9 (MH_7^{7+}) found: 798.8, 665.7, 570.7

PNA beacon **2**. Yield (after purification): 6.1%. calculated MW 4014.0 (MW), ESI-MS. *m/z* 803.8 (MH_5^{5+}), 670.0 (MH_6^{6+}); 574.4 (MH_7^{7+}) found: 803.6, 669.9, 574.4.

UV melting analysis

The PNA beacons were dissolved in water and their concentrations (290 μM for PNA **1** and 306 μM for PNA **2**) were determined by UV absorption at 260 nm on an UV/Vis Lambda Bio 20 spectrophotometer (Perkin Elmer) using the following molar absorptivities:154133 L mol^{-1} cm^{-1} for PNA **1** and 155533 L mol^{-1} cm^{-1} for PNA **2**, calculated according to their base composition as indicated in reference 5*c*) and molar absorptivity of the carboxyfluorescein and dabcyl units.

For thermal melting measurements, solutions of 1 : 1 DNA–PNA were prepared in pH = 7.0 sodium phosphate buffer (100 mM NaCl, 10 mM phosphate, 0.1 mM EDTA). Concentrations were 5 μM (in strand) for each component. Thermal denaturation profiles (Abs *vs.* *T*) of the hybrids were measured at 260 nm with an UV/Vis Lambda Bio 20 Spetrophotometer equipped with a Peltier Temperature Programmer PTP6 which is interfaced to a personal computer. A temperature ramp of 1 °C min^{-1} from 25 °C to 90 °C was used; UV absorbance was recorded at 260 nm every 0.5 °C. The melting temperature (T_m) was determined from the maximum of the first derivative of the melting curves.

Fluorescence measurements

Fluorescence measurements were performed on a Perkin Elmer Luminescence Spectrometer LS 55. All solutions were prepared in Tris buffer (0.25 mM $MgCl_2$, 10 mM Tris, pH = 8.0).

Concentrations (in strand) were 0.1 or 1 μM for each component). All samples were excited at 497 nm and the emission was monitored at 520 nm. Triplicate measurements were performed for each composition.

HPLC measurements

All experiments were carried out using an Alliance 2690 Separation Module HPLC system (Waters), equipped with a temperature controller, dual λ absorbance detector 2487 (Waters) and scanning fluorescent detector 474 (Waters). Anion-exchange chromatographic measurements were carried out using the column TSK-gel DNA-NPR 4.6 mm ID × 7.5 cm. Binary linear gradient was used: eluent A = Tris 0.02M pH 9; B = Tris 0.02M, NaCl 1M pH 9. flow: 0.5 mL min^{-1}. Linear gradient: from 100% A to 100% B in 20 min; Fluorescence detector λ_{ex} = 497 nm, λ_{em} = 520 nm. The chromatograms obtained by the fluorescence detector were corrected by subtraction of the baseline obtained with injection of water in order to compensate for the baseline drift.

For the evaluation of the detection limit of beacon **2**, the synthetic full match oligomer **4-A** was used. Samples of five different concentrations (in triplicate) ranging from 0 to 100 nmol L^{-1} were injected (10 μL injection).

DNA extraction and PCR amplification

DNA extraction from Soybean flour and asymmetric unbalanced PCR amplification was carried out using Wizard Plus Minipreps System (Promega Italia, Milan, Italy) as described previously in reference 10*b*. The PCR reactions were carried out in a PCR-sprint Thermal Cycler (Thermo Hybaid, Basingstoke, UK) using the following conditions: 1 cycle of DNA denaturation and Blue Taq activation at 95 °C for 5 min; 40 cycles consisting of DNA denaturation at 95 °C for 50 s, primer annealing at 60 °C for 50 s and elongation at 72 °C for 50 s; one step of final elongation at

72 °C for 5 min. The PCR products were immediately analyzed by 2% agarose gel in 0.5× TBE or stored at −20 °C until use. In the unbalanced PCR a first amplification was performed as described above, then a small amount (2–5 μL) of the reaction mixture was amplified in the second run using a fresh mastermix solution of the same composition except that a 10 fold excess of the primer on the target sequence was used.

Acknowledgements

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