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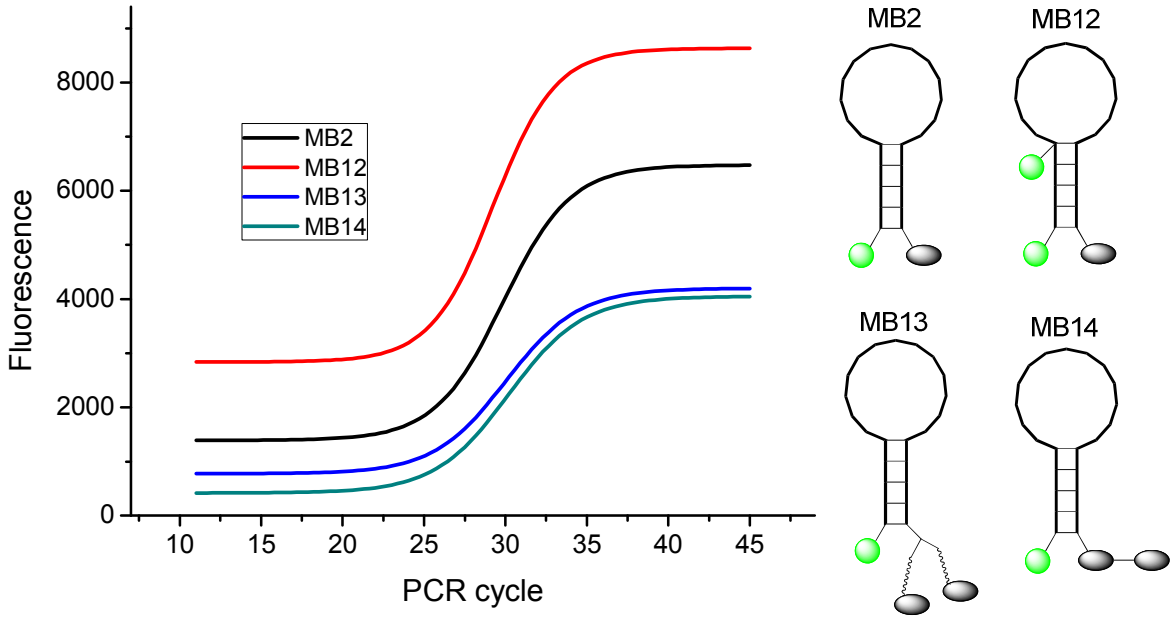
GRAPHICAL ABSTRACT



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PAPER

Design of Molecular beacons: 3′ couple quenchers improve fluorogenic properties of a probe in real-time PCR assay

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Convenient preparation of fluorogenic hairpin DNA probes (Molecular beacons) carrying a pair of FAM fluorophores (located close to 5′-terminus of the probe) or a pair of BHQ1 quenchers on 3′-terminus (with $(BHQ1)_2$ or BHQ1-BHQ1 composition) is reported. These probes were used for the first time in real-time PCR assay and showed considerable improvements in fluorogenic properties (the total fluorescence increase or signal-to-background ratio) in assay conditions *vs* conventional one-FAM-one-BHQ1 Molecular beacon probes as well as *vs* hyrdolyzable one-FAM-one-BHQ1 TaqMan probes. At the same time, such multiple modifications of the probe do not influence its Cq (a fractional PCR cycle used for quantification). The probe MB14 containing BHQ1-BHQ1 pair showed in the PCR fluorescence/background value 9.6 which is more than twice higher in comparison with regular probe MB2 (4.6). This study demonstrates prospects for the design of highly fluorogenic Molecular beacon probes suitable for quantitative real-time PCR and for other potential applications (e.g. intracellular RNA detection, SNP/mutation analysis etc.).

Introduction

Molecular beacons (MB),[1] DNA probes equipped with fluorophore-quencher pair, find diverse applications as diagnostic tools,[2] including hybridization detection in living cells.[3] They are widely used in quantitative real-time PCR (qPCR), a fast and reliable technique for detection of a genetic material. MB probe is usually an oligonucleotide capable to form a secondary structure (stem-loop) and terminated with a fluorescent dye and a quencher. These are attached to a stem part thus adjoining each other and facilitating non-radiative deactivation of fluorophore excited state by various mechanisms.[4]

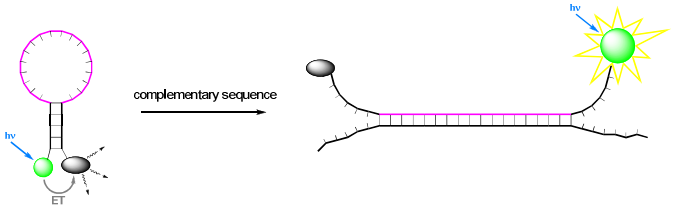

Fig. 1 Molecular beacon principle. Fluorescence of a dye (green) is considerably reduced in the closed (hairpin) form of MB due to energy transfer (ET) to nonfluorescent quencher (grey). Upon hybridization with a target the probe sequence (magenta) forms rather rigid, extended duplex, the distance between dye and quencher increases dramatically thus reducing the quenching.

The probe sequence recognizing a nucleic acid target is located predominantly in the loop. Upon heating or hybridization to a target the stem dissociates moving dye and quencher apart and fluorescence flares up (Fig. 1). The fluorescence intensity correlates with target sequence amount produced in the course of a PCR, in accordance with the PCR efficiency and cycle number.

MB probes are usually prepared in automated solid-phase synthesizers by chemical assembly of oligonucleotide chain in 3′→5′ direction. A non-nucleoside unit containing quencher is used as 3′ starting solid support. A fluorescent dye is introduced to 5′ terminus by means of a modifying phosphoramidite reagent.

There were several attempts to improve efficiency of MB. New types of fluorescent emitters (e.g. quantum dots,[5] silver nanoclusters[6]) and quenchers (e.g. graphite,[7] graphene oxide[8]) were used in MB probes. However, oligonucleotide conjugates with these nanosized dyes are hardly suitable for thermal cycling in PCR.

The increase of dye' number seems to have no prospects because of self-quenching based on energy migration between dye molecules. Noteworthy, a donor – acceptor pair was successfully used in MB as a combined fluorophore in "wavelength-shifting Molecular beacons".[9] An approach with several quenchers appears to be more fruitful: a 5′-bunch of three DABCYL or Eclipse quencher residues (the superquencher) gave dramatic improvement in fluorescence signal-to-background ratio in 3′-dye labeled MB.[10] Later, a 5′ located "linear superquencher" containing three BlackBerry quencher residues showed excellent quenching ability for 5′-pyropheophorbide containing MB.[11] However, these and other[12] techniques are rather complicated and allow to place a quenching cluster in 5′-terminal position *vs* 3'-terminal position in classic MB, thus limiting choice of the dye for 3′-labeling (the dye

must resist the chemistry of repeating cycles of oligonucleotide synthesis or, alternatively, must be attached post-synthetically). Efficient quenching of Cy3 dye with a couple of azobenzene based dyes (methyl red, 4′-methylthioazobenzene-4-carboxylate or 4′-dimethylamino-2-nitroazobenzene-4-carboxylate) in MB stem should also be noted.[13]

Copper catalyzed azide–alkyne cycloaddition reaction (CuAAC, Huisgen–Meldal–Sharpless reaction, 'click chemistry') was reported in 2002[14] and shortly thereafter was applied for the attachment of a fluorescent dye to an oligonucleotide.[15] The technique became a powerful tool for nucleic acid modification.[16] Recently, we used this reaction for the synthesis of fluorogenic TaqMan qPCR probes containing one or two fluorophores and one or two quenchers.[17] Some of these probes were superior to the standard one-dye-one-quencher TaqMan probes. The data evidenced that the new approach could have more prospects on secondary structured MB rather than on linear TaqMan probes. Here we report application the developed techniques[17] to increase fluorogenic ability of MB probes.

Experimental section

Fluorogenic DNA probes

For Molecular beacon design we used Zuker's mfold software[18] on an open access web server http://mfold.rna.albany.edu/?q=mfold. Probes were designed with a hairpin as the only secondary structure.

Molecular Beacon probes were prepared using oligonucleotide synthesis on a BiosSet ASM-800 instrument on a 200 nmol scale using standard manufacturer's protocols. Fluorophore and quencher attachment to oligonucleotides were performed as described earlier[17] using FAM phosphoramidite,[19] BHQ1 phosphoramidite,[20] 5-alkyne dT phosphoramidite,[21] BHQ1 solid support (Glen Research), dialkyne solid support,[17] FAM azide and BHQ1 azide (Primetech LLC; Minsk, Belarus). To introduce BHQ1-BHQ1 linear pair into 3'-end of MB14, a combination of BHQ1 support and BHQ1 phosphoramidite was used; $(BHQ1)_2$ bunch pair in MB13 was attached via 'click' modification in solution with BHQ1 azide an oligonucleotide started from dialkyne support;[17] MB12 was prepared using 'click' in solution with FAM azide.[17]

MBs were purified using 20% PAGE (acrylamide/bis-acrylamide 19:1, 7 M urea) and then by HPLC as described,[17] except the column was thermostated at 45ºC.

Quantitative real-time PCR

Plasmid pTZ-Fat carrying a fragment of *Fusarium avenaceum* elongation factor 1α (template), downstream Fat65F primer 5′-GGT CGC TTA TCT GCA CTC GGA-3′ and upstream primer 5′-GTC ACT CGA GTG GCG GGG TAA G-3′ [17,22] were used in real-time PCR to produce 290 bp fragment containing 26 bp probe sequence (see Results and Discussion). *PCR assay* was performed on DT-96 instrument (DNA Technology, Moscow, Russia). The reaction mixture (35 µL) contained 75 mM Tris-HCl, 20 mM ammonium sulphate, 0.01% Tween-20, 1 mM of each dNTP, 1 µM primers, 0.2 µM MB, 2.5 u. of Taq polymerase and DNA template (plasmid pTZ-Fat, 8 to 8,000,000 copies), pH 8.8[17,22]. The experiments were repeated twice and analyzed by the geometric method (C_q) using DNA Technology software; the background fluorescence of every sample well was taken into account. PCR cycles were: 94°C, 90 s (1 cycle); then 94°C – 10 s, 64°C – 15 s, 67°C – 10 s (45 cycles) (fluorescence is registered at 64°C using FAM detection channel) or 94°C – 10 s, 55°C – 15 s, 64°C – 15 s, 67°C – 10 s (45 cycles) (fluorescence is registered at 55°C using FAM detection channel).

MB melting experiments

MB melting experiments were performed in two repeats on the same instrument (FAM detection channel) with 0.2 µM MB in above PCR buffer without dNTP, primers, Taq polymerase and plasmid template. Melting temperatures were calculated as first derivative maxima.

Results and Discussion

Variations of the stem part in MB structure

The aim of this research was to study the structure–fluorescent properties relationship for several modified MBs in real-time PCR assay conditions. The influence of fluorophore and quencher(s) location toward each other was of particular interest.

The typical MB has a loop of 15–25 nucleotides long and a stem of 4–8 base pairs. Hybridization of MBs to targets[23] and their re-folding[24] have been systematically studied. In real-time PCR assay MB probe is added in the reaction mixture; in the course of the amplification it hybridizes with appearing target fragment, and the fluorescence rises. The excess of MB at the temperature of fluorescence detection (on particular step of a PCR cycle) must be predominantly in a closed, hairpin form, with minimal fluorescence. Naturally, in case of insertion of additional step for fluorescence measurement this must not affect the PCR efficiency.

The 26-nucleotide probe sequence 5′-tcattcgaaacgcattcattaccccg, a part of *Fusarium avenaceum* elongation factor 1α (GeneBank accession number JF278604),[22] was very similar to 27-mer TaqMan probe sequence used in our previous work.[17] The target sequence (containing probe sequence inside) was amplified from the plasmid pTZ-Fat[17,22] as 67–357 nucleotide fragment of the translation elongation factor 1α gene of *Fusarium avenaceum*:
5′-GGTCGCTTATCTGCACTCGGAACCCGCCAAACCTGGCGG
GGTATCACCACGACATCTTGCTAACTCTTGACAGACCGGT
CACTTGATCTACCAGTGCGGTGGTATCGACAAGCGAACCA
TCGAGAAGTTCGAGAAGGTTAGTCAATATCCCTTCGATTA
CGCGCGCTCCCATCGATTCCCACGACTCGCTCCC**TCATTC**
GAAACGCATTCATTACCCCGCTCAAGTCCGAAAATTTTG
CGGTGCGACCGTGATTTTTTTTGGTGGGGTATCTTACCCCG
CCACTCGAGTGAC-3′ (primer positions are underlined; the probe sequence is highlighted).

Fourteen MBs containing above probe sequence and different in the stem part were synthesized and tested in real-time PCR (Table 1, Figs 2, 3, Electronic supplementary material, Table S1, Fig. S1–S3). The MB probes were compared with TaqMan probes P1 and P7 used in our previous study.[17] First, probes MB1–MB8 containing stems of various length (4–8 bp) and composition were prepared. All of them contained one 5′-FAM dye and one 3′-BHQ1 quencher. For fluorescence detection a primer annealing step (64ºC) or an additional PCR cycle step (55ºC) were used (see Experimental part). The probe MB2 with 7 bp in the stem (1a/t+6g/c) showed better fluorogenic ability for both fluorescence detection temperatures. Remarkably, the similar probe MB8 (7 bp in stem, 6g/c+1a/t) containing an a/t pair in the terminal positions showed much worse signal/background ratio together with Tm decrease by 4ºC. However, the increase of stem length to 8 c/g pairs (probe MB7) reduces the overall fluorescence increase, probably due to excessive hairpin stability.

It should be noted that the background fluorescence intensities of MB probes when detected above Tm, is similar to those of linear TaqMan probes P1 and P7. In case the detection temperature is 10ºC below the Tm value, the background fluorescence is reduced at least threefold (e.g. for MB2 at 55ºC). The results demonstrate the contribution of spatial convergence to Förster and non-Förster quenching.

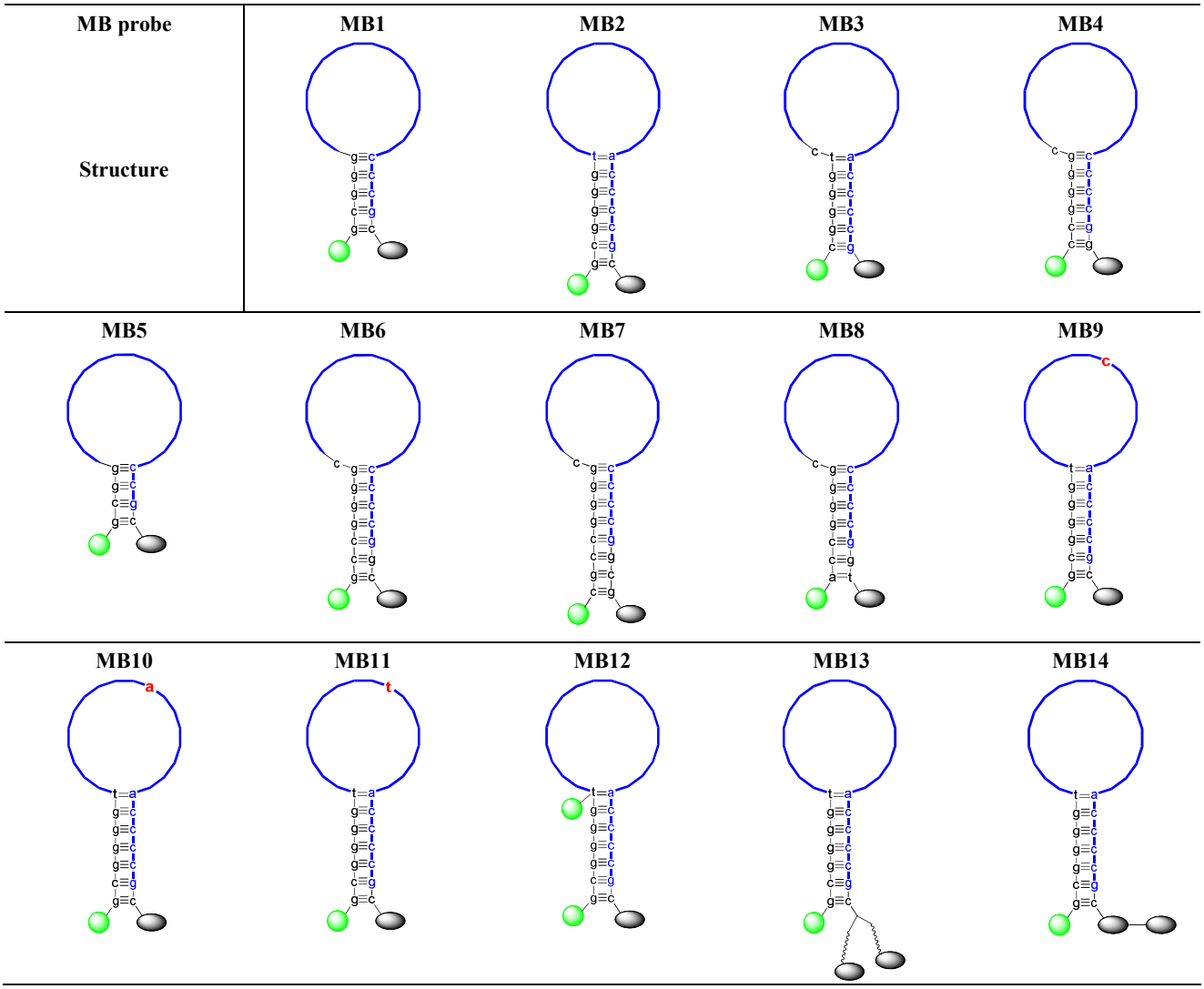

Fig. 2 Schematic structures of MB probes in hairpin form. Non-probe (black), stem, and mismatch (red) nucleosides are shown, other designations:

- 26-mer probe sequence: 5'-**tcattcgaaacgcattcattaccccg**-3'

- FAM

- BHQ1

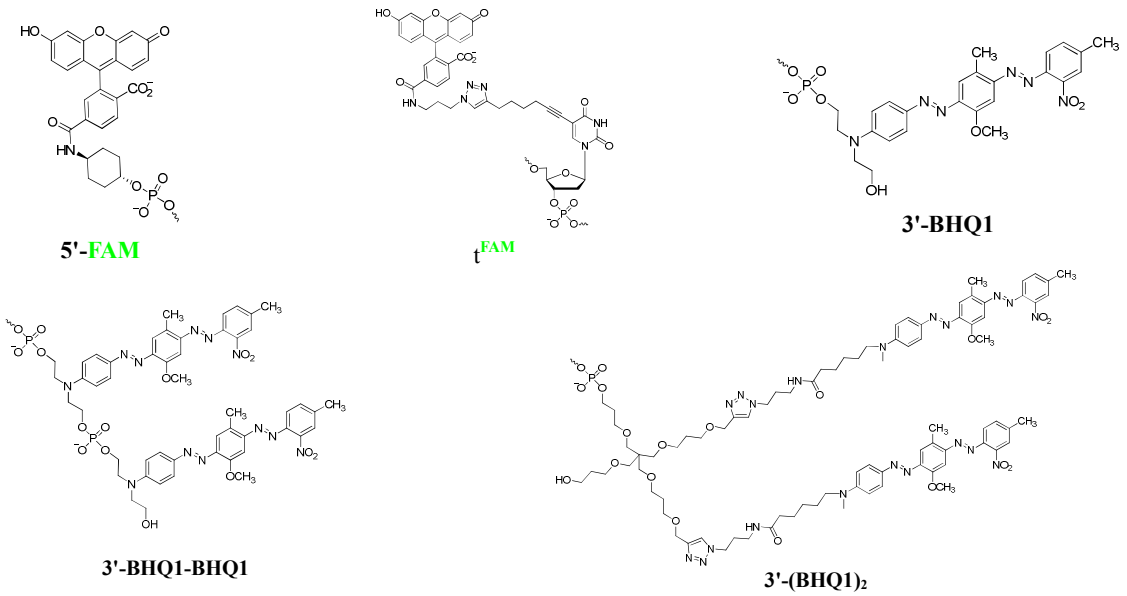

Table 1. Structures, melting temperatures, and fluorescence of MB probes *vs* TaqMan probes.

#	Probe structure, 5'→3' [a]	Fluorescent probe melting [b]		Real-time PCR [c]			
		Tm, °C	I_m/I_0	Relative background fluorescence, I_{0MBi}/I_{0P1}, detected at		Relative fluorescence increase, I_{fMBi}/I_{0MBi}, detected at	
				64ºC	55ºC	64ºC	55ºC
P1	FAM-tcattcgaaacgcattcattaccccgc-**BHQ1**	–		1.00	–	1.56	–
P7	FAM-tcat(t[FAM])cgaaacgcattcattaccccgc-**BHQ1**	–		0.77	–	1.91	–
MB1	FAM-gcgggtcattcgaaacgcattcattaccccgc-**BHQ1**	56.9	5	1.00	0.74	1.48	2.30
MB2	FAM-gcggggtcattcgaaacgcattcattaccccgc-**BHQ1**	67.3	11	0.63	0.29	2.32	4.65
MB3	FAM-cggggtctcattcgaaacgcattcattaccccg-**BHQ1**	49.2	9	1.13	0.97	1.23	1.47
MB4	FAM-ccggggctcattcgaaacgcattcattaccccgg-**BHQ1**	56.8	13	0.83	0.50	1.30	2.05
MB5	FAM-gcggtcattcgaaacgcattcattaccccgc-**BHQ1**	47.7	2.6	1.27	1.21	1.30	1.49
MB6	FAM-gccggggctcattcgaaacgcattcattaccccggc-**BHQ1**	69.8	14	0.68	0.46	1.86	2.79
MB7	FAM-cgccggggctcattcgaaacgcattcattaccccggcg-**BHQ1**	72.8	21	0.35	0.28	2.07	2.84
MB8	FAM-accggggctcattcgaaacgcattcattaccccggt-**BHQ1**	63.4	19	0.89	0.42	1.59	3.23
MB9	FAM-gcggggtcattcgaaaccattcattaccccgc-**BHQ1**	66.3	23	0.67	0.29	– [d]	1.36
MB10	FAM-gcggggtcattcgaaacacattcattaccccgc-**BHQ1**	65.9	21	0.76	0.31	1.10	1.58
MB11	FAM-gcggggtcattcgaaactcattcattaccccgc-**BHQ1**	66.3	24	0.67	0.28	– [d]	1.38
MB12	FAM-gcgggg(t[FAM])cattcgaaacgcattcattaccccgc-**BHQ1**	62.4	3.6	0.97	0.59	1.72	3.04
MB13	FAM-gcggggtcattcgaaacgcattcattaccccgc-(**BHQ1**)$_2$	71.8	12	0.30	0.16	2.99	5.41
MB14	FAM-gcggggtcattcgaaacgcattcattaccccgc-**BHQ1-BHQ1**	71.6	347	0.25	0.09	3.59	9.63

[a] The probe part of MB is underlined, mismatches are highlighted in red, structures of modifications are given below.

[b] – melting temperatures (±0.5°C) of MB probes were determined using heating and cooling in thermocycler (90 ºC→20 ºC→90 ºC, see Experimental part), mean of two repeats, as first derivative maxima (see Electronic supplementary material, Fig. S18). I_m/I_0 – fluorescence intensity ratio of molten (linear/random coil form) and stem-loop (hairpin form) of probes MB1–MB14 (±20%);

[c] – I_{0P1} – starting (background) fluorescence intensity of probe P1; I_{0MBi} – starting (background) fluorescence intensity of probes MB1–MB14; I_{fMBi} – final (plateau) fluorescence intensity of probes MB1–MB14; the values (±20%) were calculated from PCR experiments (conditions see Experimental part), mean of two repeats, sigmoid fit of fluorescence curve (see Electronic supplementary material, Figs S4–S17);

[d] – there was no sigmoidal shape of cycle/fluorescence intensity curve.

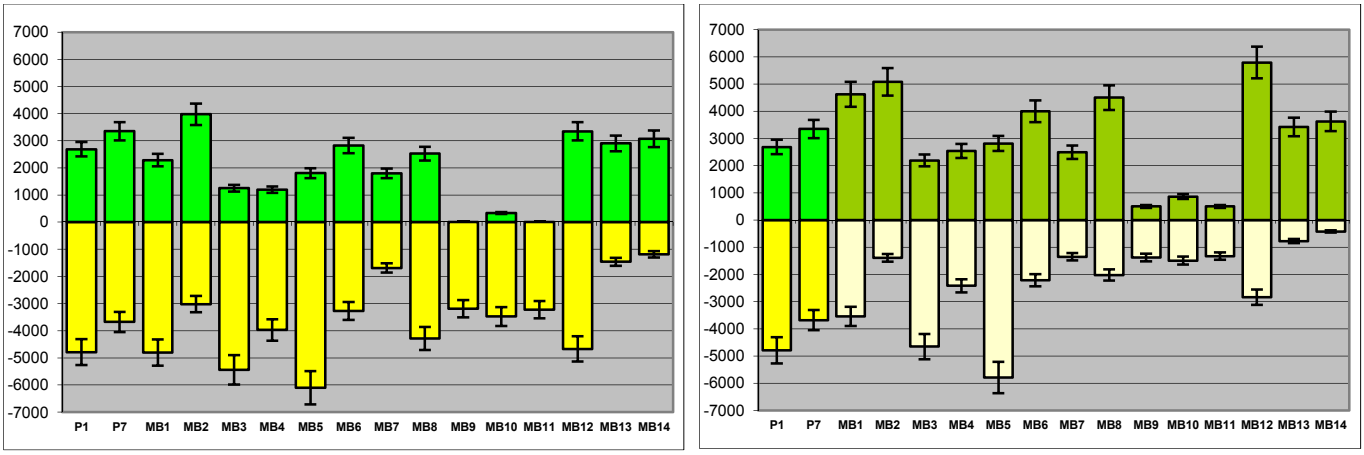

Fig. 3 Relative background fluorescence, detected at 64ºC ( ) and 55ºC ( ); fluorescence increase after 45 cycles, detected at 64ºC ( ) and 55ºC ( ). For convenience of comprehension the background fluorescence values are shown as negative (Y axis labels are arbitrary units). Fluorescence increase (positive bars) is the difference between final and starting plateau levels for sigmoidally fitted fluorescence intensity/PCR cycle curve. Data were collected on FAM channel of a rtPCR cycler (mean values from two repeats, see Experimental part). Error values of data collection are within ±10% (highest limits of possible errors coming from UV/vis measurements of DNA probes, dilution procedures, and fluorescence reading in rtPCR cycler based on multiple repetitive experiments with several probes). Data for P1 and P7 collected using same cycler are given from the reference.[17]

MBs containing one mismatch

Basing on the optimized structure, MB2, we prepared next six modified probes, MB9–MB14. Probes MB9–MB11 contained one mismatch (point mutation, single nucleotide replacement) in the middle part of a probe sequence. Mutations have almost no effect on Tm (corresponding to hairpin/random coil equilibrium), however, reduce dramatically the ability to hybridize with a target.

Detection on 55ºC shows small sigmoid fluorescence increase for all mutant probes. Upon 64ºC detection only MB10 probe with purine→purine (g→a) replacement gives slight fluorescence increase with 1.1 value of signal/background ratio. The results show that one can easily select probe structure and conditions for single nucleotide polymorphism discrimination in real-time PCR.

Effects of MB modifications with two fluorophores or quenchers

Probe MB12 contains two FAMs; interestingly, its background fluorescence is less than one of standard TaqMan P1 and Molecular beacon MB1, but higher than this of modified TaqMan P7 and optimized beacon MB2. Base modification in the stem part of MB reduces Tm by 5ºC. When detected at 55ºC, MB12 showed the highest total fluorescence increase in PCR conditions (Fig. 3) along with moderate signal/background ratio (Table 1).

At last, probes MB13 and MB14 contain two BHQ1 residues as parallel or linear pair, respectively. These modifications add 4–5ºC to the Tm, greatly reduce the background fluorescence, and increase the fluorescence signal/background ratio (up to about 10 for MB14 for detection at 55ºC). Probe MB13 has higher background fluorescence *vs* MB14, probably due to long linker arms for attachment of BHQ1 residues.

Thus, detection at 55ºC gives significantly greater increase in fluorescence as compared with 64ºC detection. On the other hand, higher detection temperature is preferable for the mutation discrimination: in this case, a point mutation containing probes (MB9–MB11) are practically inactive.

Experiments with different numbers of copies of the original target confirm that the introduction of additional modifications (probes MB12–MB14) does not cause the lag in cycles of threshold crossing in comparison with conventional probe MB2 (see Electronic supplementary material, Fig. S20).

It should be noted that signal/background values given in Table 1 correspond the endpoint of PCR; they result from combination of three states (shapes) of MB probe: hairpin, random coil, and hybrid with the probe.[23a] Signal/background values for simple thermal melting of MB probes (hairpin → random coil transformation) are usually much greater, except for probes MB5 and MB12 (Table 1, Electronic supplementary material, Fig. S19), and not always correlate with duplex stability. The most remarkable difference is observed in case of probes with BHQ1 pairs: probes MB13 and MB14 have similar Tm values, however the signal/background ratio upon fluorescence melting is more than two order of magnitude higher for MB14. The latter contains BHQ1 residues on short linkers *vs* extended ones in MB13. The nature of such a dramatic increase of the quenching efficiency remains unclear: is it simply the effect of shorter distance on Förster quenching or the contact quenching is also facilitated by spatial prerequisites? Next, the co-operative action of two BHQ1 residues cannot be excluded, although UV/Vis spectra show no sign of BHQ1 association (BHQ1-BHQ1 in MB14 *vs* $(BHQ1)_2$ in MB13 and BHQ1 in other MBs; data not shown). The result shows the extreme importance of the linker for the quenching efficiency in MB hairpin form and paves the way for further studies.

Hybridization with an excess of complementary target should give similar difference of resulting and starting background fluorescence. Presumably, such probes could be used to detect hybridization in cases where the signal/background ratio is of critical importance for the sensitivity, e.g., for hybridization in living cells.

Conclusion

To conclude, we studied relationships between DNA probe structure and fluorescent properties. Molecular beacon probes containing two FAM fluorophores or two BHQ1 quenchers were used for the first time in real-time PCR. The probe MB14, containing linear quencher pair, has very high signal/background ratio in fluorescent melting. This probe also allows maximizing the fluorogenic effect in qPCR.

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Notes and references

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Electronic Supplementary Information (ESI) available: MS data for MB probes, fluorescence melting and real time PCR plots. See DOI: 10.1039/b000000x/

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