Supplementary Material (ESI) for Chemical Communications

Increased single nucleotide discrimination in arrayed primer elongation by 4'C modified primer probes

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Electronic Supplementary Information (ESI)

Experimental Section. Synthesis of 4'*C* modified and unmodified primer probes. Oligonucleotides for microarray attachment were synthesized at a scale of 0.2 µmol with a 5' terminal TFA-protected $(CH_2)_6$-NH_2 linker (Link Technologies) using an Applied Biosystems 392 DNA synthesizer and commercially available 2-(cyanoethyl)-phosphoramidites. Unmodified sequences were accessible using standard T or dC^{Bz} columns (ABI LV200 polystyrene, Applied Biosystems). 3' terminal modified sequences were obtained with LCAA-CPG-bound 5'-O-DMT protected T^{Me}, T^{Vin}, T^{MeO}, or $^{5Me}C^{MeO}$ nucleosides respectively. Employed sequences of primer probes in the Farber disease context: Far**T**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-CGT TGG TCC TGA AGG AGG A**T**$^{\mathbf{R}}$)-3'; R = H, Me, Vin, MeO; Far**C**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-CGT TGG TCC TGA AGG AGG A**C**$^{\mathbf{R}}$)-3'; R = H, (5-Me) MeO. Primer probes in the Factor V Leiden disease context: Lei**T**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-CAA GGA CAA AAT ACC TGT ATT CCT **T**$^{\mathbf{R}}$)-3'; R = H, MeO; Lei**C**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-CAA GGA CAA AAT ACC TGT ATT CCT **C**$^{\mathbf{R}}$)-3'; R = H, (5-Me) MeO. Primer probes in the DPyD disease context: DPyD**T**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-GTT TTA GAT GTT AAA TCA CAC TTA **T**$^{\mathbf{R}}$)-3'; R = H, MeO; DPyD**C**$^{\mathbf{R}}$: 5'-d($NH_2(CH_2)_6$-GTT TTA GAT GTT AAA TCA CAC TTA **C**$^{\mathbf{R}}$)-3'; R = H, (5-Me) MeO. After synthesis (trityl-on mode) the solid support-bound oligonucleotides were treated with 10% triethylamine in acetonitrile for 10 min and rinsed hereafter with excess acetonitrile. This procedure cleaves cyanoethyl-protecting groups from phosphate moieties to prevent alkylation of 5' primary amines. The oligonucleotides were cleaved from support and deprotected by treatment with 33% NH_4OH at 55°C for 16 h. After evaporation of NH_4OH the residues were recovered in water and beads were filtered off (Spartan 13/0.45 µm filter units, Schleicher & Schuell). The crude oligonucleotides were purified twice by RP-HPLC with 0.1M triethylammonium acetate buffer (pH 7.0) and lyophilized. The integrity of all oligonucleotides was confirmed by performing ESI-MS.

Preparation of activated glass surfaces. Aminopropyl-silylated glass slides (Genetix) were incubated for 2 h at room temperature in a 10% pyridine/DMF (*v/v*) solution (Fluka) containing 0.2% (*w/v*) 1,4-phenylenediisothiocyanate (PDITC, Fluka). The slides were washed subsequently with DMF and 1,2-dichloroethane, dried with compressed nitrogen and stored desicated until further use.

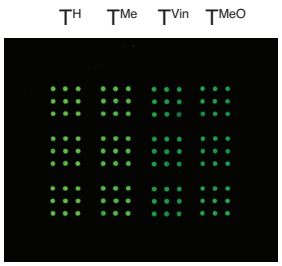
Spotting of amino-modified oligonculeotides to activated derivatized glass slides. Amino-modified oligonucleotides were spotted on PDITC-activated slides at 20 µM in 150 mM sodium phosphate buffer (pH 8.5), arranged each as six replicates with 10 drops per spot (approx. 4 nL) using Nanoplotter 2.0 (GeSiM). In thymidine-modified primer extensions corresponding oligonucleotides were arrayed as three grids, whereas replicates in multiplex primer extensions were arranged as a single grid. Slides were incubated at room temperature for 16 h in a glass petri dish supplied with saturated NaCl solution. Thereafter slides were deactivated by incubation at room temperature for 30 min in a 10% NH_4OH solution, followed by subsequent washing steps in ultrapure water. Slides were then dried with compressed nitrogen and stored at -24° C until further use.

Arrayed primer elongations. To evaluate discrimination efficiency of modified thymidine primer probes in arrayed primer exten-sion, reaction mixtures of 20 µL were prepared containing 1× ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8; New England Biolabs), 200 µM dATP, dGTP, TTP/180 µM dCTP (Fermentas), 20 µM Cy3 dCTP (Amersham Biosciences), 250 nM or 1 µM Farber A/G template (90nt, IBA) respectively, 0.1% BSA (Fluka), 0.5× Self-Seal reagent (MJ Research), and 0.2 U/µL *Vent* (exo-) DNA polymerase (New England Biolabs). LifterSlips (22×22 mm, Erie Scientific) were placed over spotted grid areas and reaction solutions were dispensed under pre-positioned glass coverslips. Primer elongation with PTC-200 DNA Engine thermocycler and 16/16 Twin Tower block (MJ Research) was carried out as follows: Initial denaturation step at 95°C for 3 min, followed by 20 and 40 cycles of denaturation (95°C, 30 sec), annealing (55°C, 35 sec) and extension (72°C, 40 sec). After thermal cycling, arrays were immersed in 0.1× SSC/0.1% SDS to remove cover slips, followed by subsequent washing steps with gentle agitation in 0.1× SSC for 15 min and 5 times with ultrapure water for 5 min each and dried in a compressed nitrogen stream. Employed template sequences: Far**A**: 5'-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT **A**TC CTC CTT CAG GAC CAA CGT ACA GAG)-3', Far**G**: 5'-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT **G**TC CTC CTT CAG GAC CAA CGT ACA GAG)-3'.

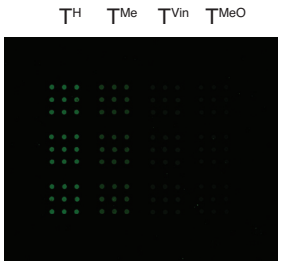
Multiplex arrayed primer elongations. A reaction solution (7 µL) with final concentrations of 1× ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8; New England Biolabs), 200 µM dATP, dGTP, TTP/180 µM dCTP (Fermentas), 20 µM Cy3 dCTP (Amersham Biosciences), 1 µM Farber A/G (90nt), Leiden A/G (98nt), or DPyD A/G (120nt) template (IBA), 0.1% BSA (Fluka), 0.5× Self-Seal reagent (MJ Research), supplemented with 0.2 U/µL Vent (exo-) DNA polymerase (New England Biolabs) respectively was pipetted to designated grid area and covered with a glass cover slip (15×15 mm, Menzel-Gläser). Multiplex primer elongation with PTC 200 slide thermocycler (MJ Research) was carried out according to the aforementioned thermal protocol. After thermal cycling, arrays were immersed in 0.1× SSC/0.1% SDS to remove cover slips, followed by subsequent washing steps with gentle agitation in 0.1× SSC for 15 min and 5 times with ultrapure water for 5 min each and dried in a compressed nitrogen stream. Employed template sequences: Farber disease context: Far**A**: 5'-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT **A**TC CTC CTT CAG GAC CAA CGT ACA GAG)-3', Far**G**: 5'-d(CCG TCA GCT GTG CCG TCG CGC AGC ACG CGC CGC CGT GGA CAG AGG ACT GCA GAA AAT CAA CCT **G**TC CTC CTT CAG GAC CAA CGT ACA GAG)-3'; Factor V Leiden disease context: Lei**A**: 5'-d(GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG C**A**A GGA ATA CAG GTA TTT TGT CCT TG)-3', Lei**G**: 5'-d(GAC ATC ATG AGA GAC ATC GCC TCT GGG CTA ATA GGA CTA CTT CTA ATC TGT AAG AGC AGA TCC CTG GAC AGG C**G**A GGA ATA CAG GTA TTT TGT CCT TG)-3'; DPyD disease context: DPyD**A**: 5'-d(AAA GCT CCT TTC TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA AC**A** TAA GTG TGA TTT AAC ATC TAA AAC)-3', DPyD**G**: 5'-d(AAA GCT CCT TTC TGA ATA TTG AGC TCA TCA GTG AGA AAA CGG CTG CAT ATT GGT GTC AAA GTG TCA CTG AAC TAA AGG CTG ACT TTC CAG ACA AC**G** TAA GTG TGA TTT AAC ATC TAA AAC)-3'.

Data aquisition. Image data were aquired with a GenePix Personal 4100A microarray scanner (Molecular Devices) at a resolution of 5 µm. Fluorescence intensities were analyzed with GenePix Pro 4.1 software (Molecular Devices) by subtracting local background intensities from individual mean intensities to determine normalized fluorescence intensities and match to mismatch signal intensity ratios.

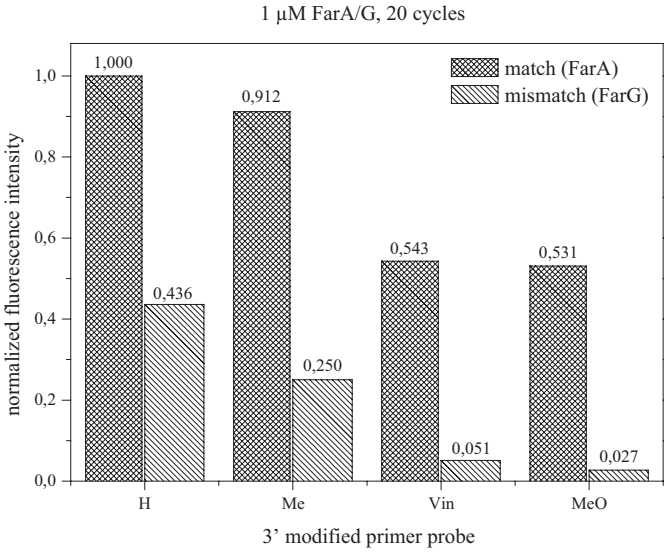
Aquired fluorescence image data in arrayed primer elongations

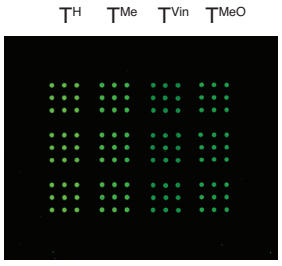


Arrayed primer extension employed with 1 µM FarA template, 20 cycles.

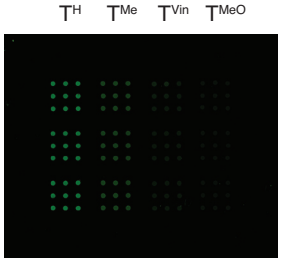


Arrayed primer extension employed with 1 µM FarG template, 20 cycles.

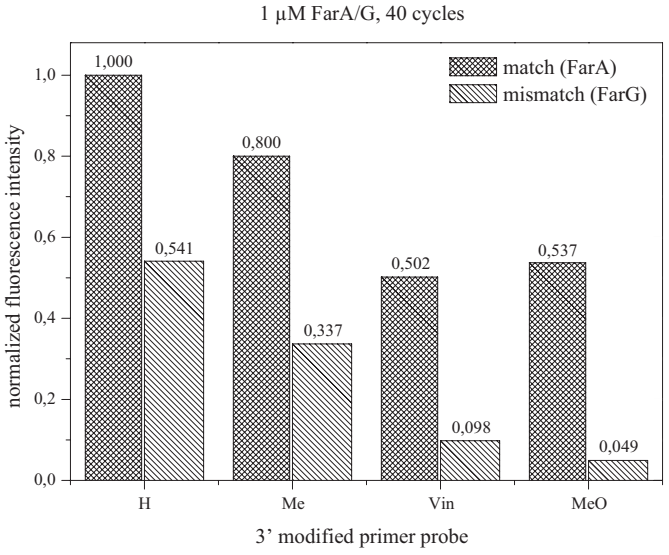




Arrayed primer extension employed with 1 µM FarA template, 40 cycles.

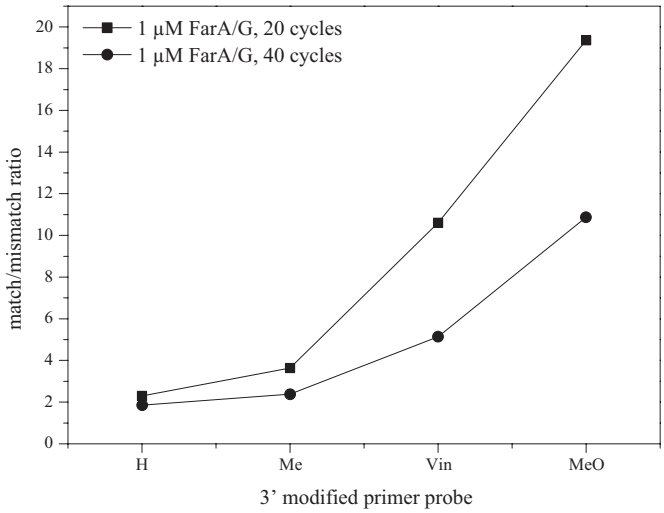


Arrayed primer extension employed with 1 µM FarG template, 40 cycles.

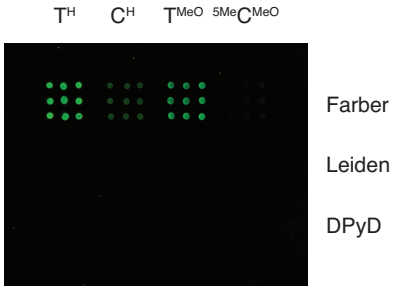


Determination of match to mismatch ratios

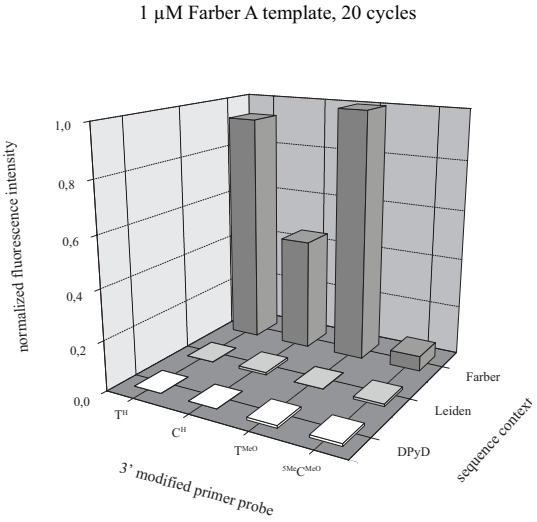
3' modification	1 µM FarA/G 20 cycles	1 µM FarA/G 40 cycles
H	2,30	1,85
Me	3,64	2,38
Vin	10,61	5,14
MeO	19,36	10,87

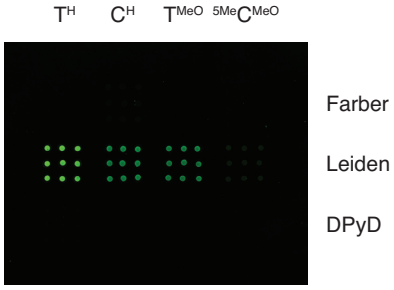


Aquired fluorescence image data in multiplex arrayed primer elongations

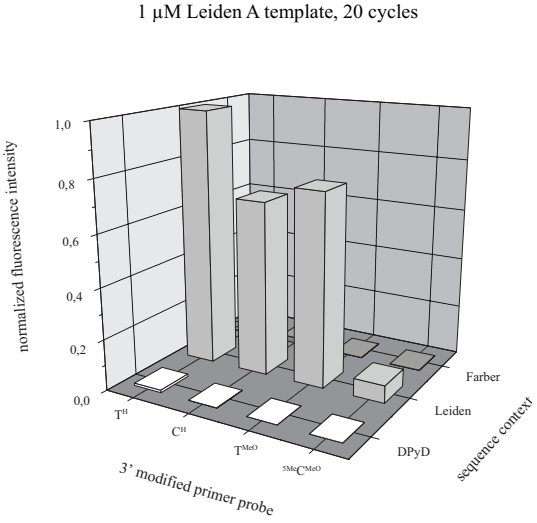


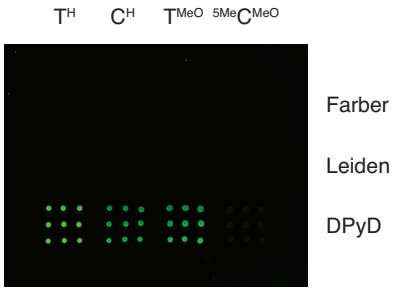
Multiplex arrayed primer extension utilized with 1 µM FarA template, 20 cycles.



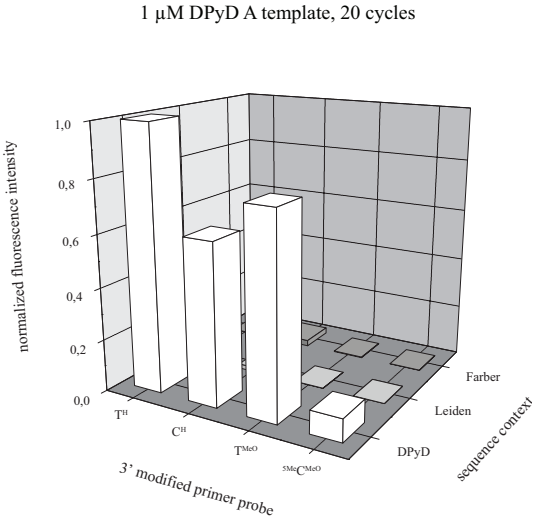


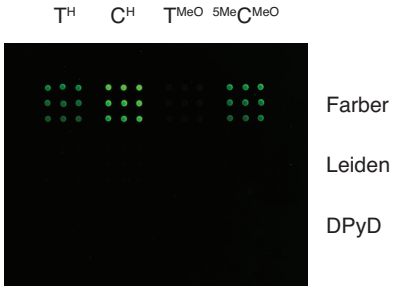
Multiplex arrayed primer extension utilized with 1 µM LeiA template, 20 cycles.



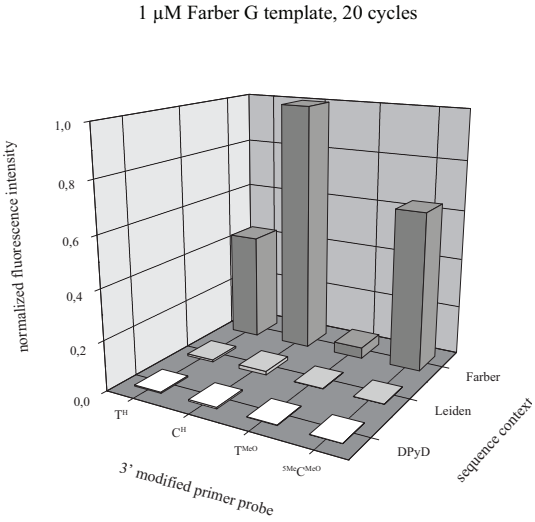


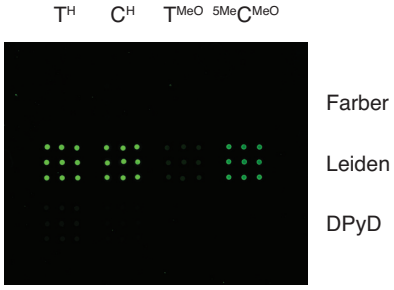
Multiplex arrayed primer extension utilized with 1 µM DPyDA template, 20 cycles.



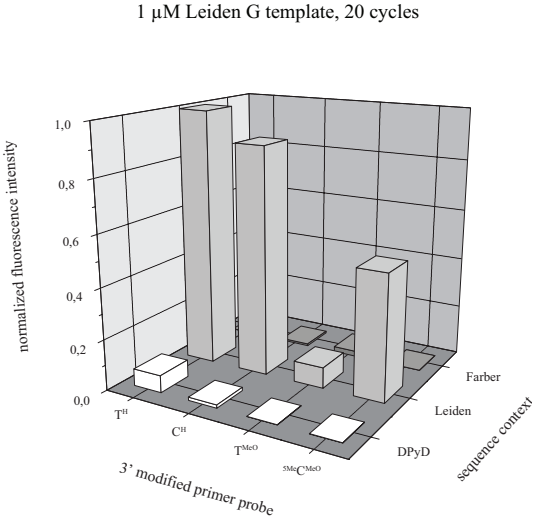


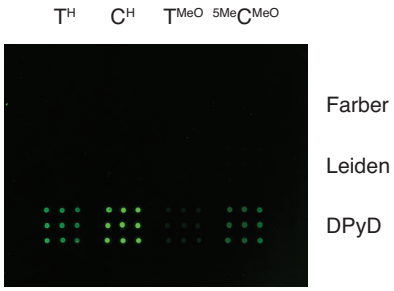
Multiplex arrayed primer extension utilized with 1 μM FarG template, 20 cycles.



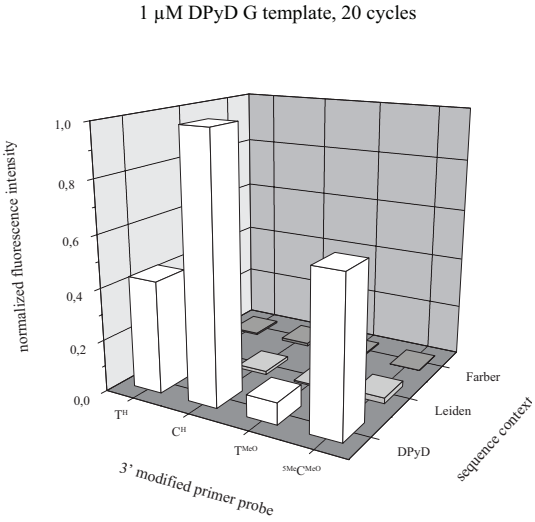


Multiplex arrayed primer extension utilized with 1 μM LeiG template, 20 cycles.





Multiplex arrayed primer extension utilized with 1 μM DPyDG template, 20 cycles.



Determination of match to mismatch rations in multiplex arrayed primer elongations

FarA/G template

Primer sequence	3' Modification	Normalized fluorescence intensity (template)		Match/mismatch ratio (template)	
		(A)	(G)	T/C (A)	C/T (G)
Farber	T^{H}	0,933	0,432	2,056	2,317
	C^{H}	0,454	1,000		
	T^{MeO}	1,000	0,044	17,065	14,603
	$^{5Me}C^{MeO}$	0,059	0,637		
Leiden	T^{H}	-0,002	0,008		
	C^{H}	0,009	0,015		
	T^{MeO}	-0,004	0,002		
	$^{5Me}C^{MeO}$	-0,007	-0,001		
DPyD	T^{H}	-0,003	0,005		
	C^{H}	0,000	0,006		
	T^{MeO}	-0,009	0,002		
	$^{5Me}C^{MeO}$	-0,010	0,001		

LeiA/G template

Primer sequence	3' Modification	Normalized fluorescence intensity (template)		Match/mismatch ratio (template)	
		(A)	(G)	T/C (A)	C/T (G)
Farber	T^{H}	-0,001	0,002		
	C^{H}	0,023	0,007		
	T^{MeO}	-0,001	0,022		
	$^{5Me}C^{MeO}$	-0,001	0,000		
Leiden	T^{H}	1,000	1,000	1,467	0,891
	C^{H}	0,682	0,891		
	T^{MeO}	0,751	0,082	10,900	7,202
	$^{5Me}C^{MeO}$	0,069	0,592		
DPyD	T^{H}	0,010	0,068		
	C^{H}	0,001	0,013		
	T^{MeO}	-0,001	0,001		
	$^{5Me}C^{MeO}$	0,000	0,001		

DPyDA/G template

Primer sequence	3' Modification	Normalized fluorescence intensity (template)		Match/mismatch ratio (template)	
		(A)	(G)	T/C (A)	C/T (G)
Farber	T^{H}	0,001	0,006		
	C^{H}	0,023	0,011		
	T^{MeO}	0,002	0,005		
	$^{5Me}C^{MeO}$	0,001	0,001		
Leiden	T^{H}	0,022	0,010		
	C^{H}	0,017	0,013		
	T^{MeO}	0,001	0,006		
	$^{5Me}C^{MeO}$	0,000	0,022		
DPyD	T^{H}	1,000	0,431	1,626	2,322
	C^{H}	0,615	1,000		
	T^{MeO}	0,764	0,084	8,696	7,045
	$^{5Me}C^{MeO}$	0,088	0,591		