Single-nucleotide polymorphisms and other mismatches reduce performance of quantitative PCR assays

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Problems

- Single nucleotide polymorphisms (SNPs) at primer annealing sites and challenge of finding primers that anneal to SNP-free target regions
- The actual impact of imperfect primer annealing has been largely unknown.
- The effects of the number, type and position of priming mismatches on (q)PCR yield, efficiency and reproducibility has been largely unknown.
- Previously reported results were based on limited numbers of reactions, not all primer positions were assessed and only single master mix was evaluated

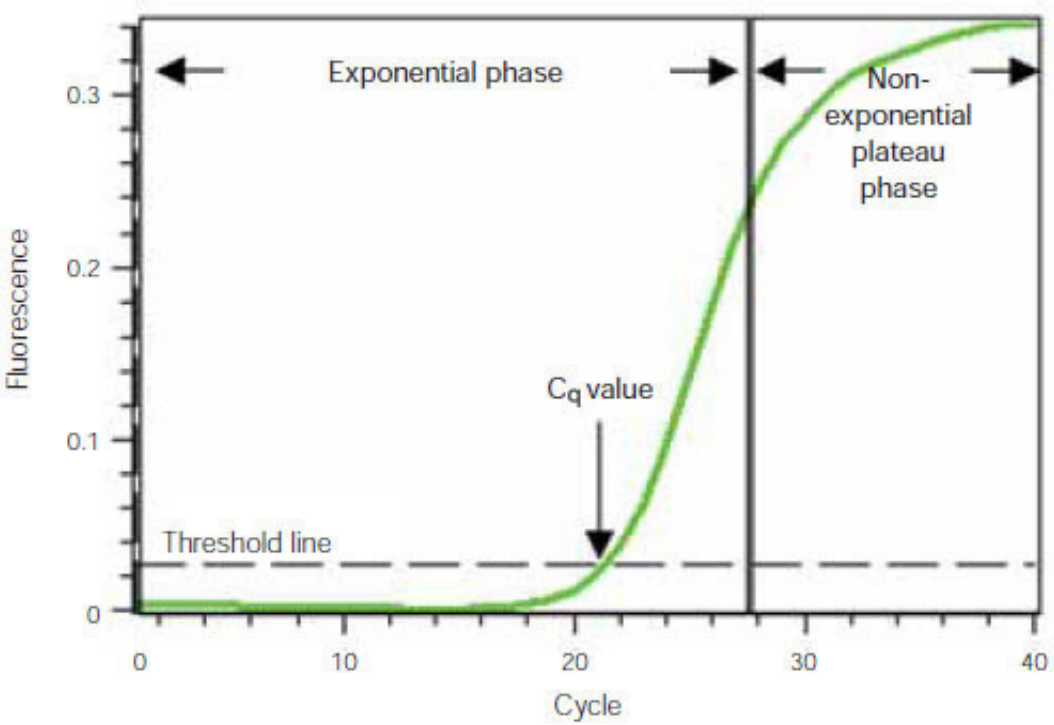
Aim of this research

- To analyse all types of mismatches in the last 5 bases of primer 3’ end in combination with 5 commecially available qPCR master mixes

- To analyse the effects of the number, type and position of mismatches on different qPCR parameters (Cq value, amplification efficiency, PCR yield)

qPCR:

qPCR allows monitoring of the synthesi
using fluorescence.



- **The amount of the fluorescence** re
to the amount of amplified DNA.

- **Cq (quantification cycle)**, Ct (thresh
achives a defined threshold.
  - the cycle at which a stat. signifi
- Cq is inversely correlated to the (log
sample (quantity of target DNA at the start of PCR)

- **Amplification efficiency (/slope) (E):**

$E = 10^{(-1/slope)}$ $X_n = X_o * E_x^n$ $(X_q = X_o * E_x^{Cq})$

  - E_x value ranging from **1** (no amplification) to **2** (perfect doubling).
    Ideally the efficiency (E) of a PCR should be 100% (E=2), meaning that for each cycle the amount of product doubles (**slope**= -3,33…).
  - **n** - the number of PCR cycles,
  - X_n – the amount of the PCR product at cycle n (the fluorescence signal from the target is proportional to the number of target molecules at cycle n).
  - X_o – the amount of the initial target DNA

Materials and methods

- **artificial primers (F and R) and templates** designed using a Perl pipeline, UNAFold, Primer3 software -> 576 primer/template combinations (with a varying number, type, position of mismatches)
- Excluded templates and primers with secondary structures and dimerizing primer pairs

- **qPCR reactions:** 10000 template molecules, Bio-Rad CFX384 instrument, each primer-pair/template combination in triplicate,
  - 5 qPCR master mixes:
    - EvaGreen qPCR Mix Plus (Solis Biodyne, mix A)
    - EvaGreen Supermix (Bio-Rad, mix B)
    - Sso Advanced SYBR Green Supermix (Bio-Rad, mix C)
    - LightCycler 480 SYBR Green Master (Roche, mix D)
    - A custom-made Eurogentec mix (mix E)

- **Cq values** - measured by the threshold method, calculated with CFX Manager software
- **Amplification efficiency** – calculated with the LinRegPCR software package

Materials and methods

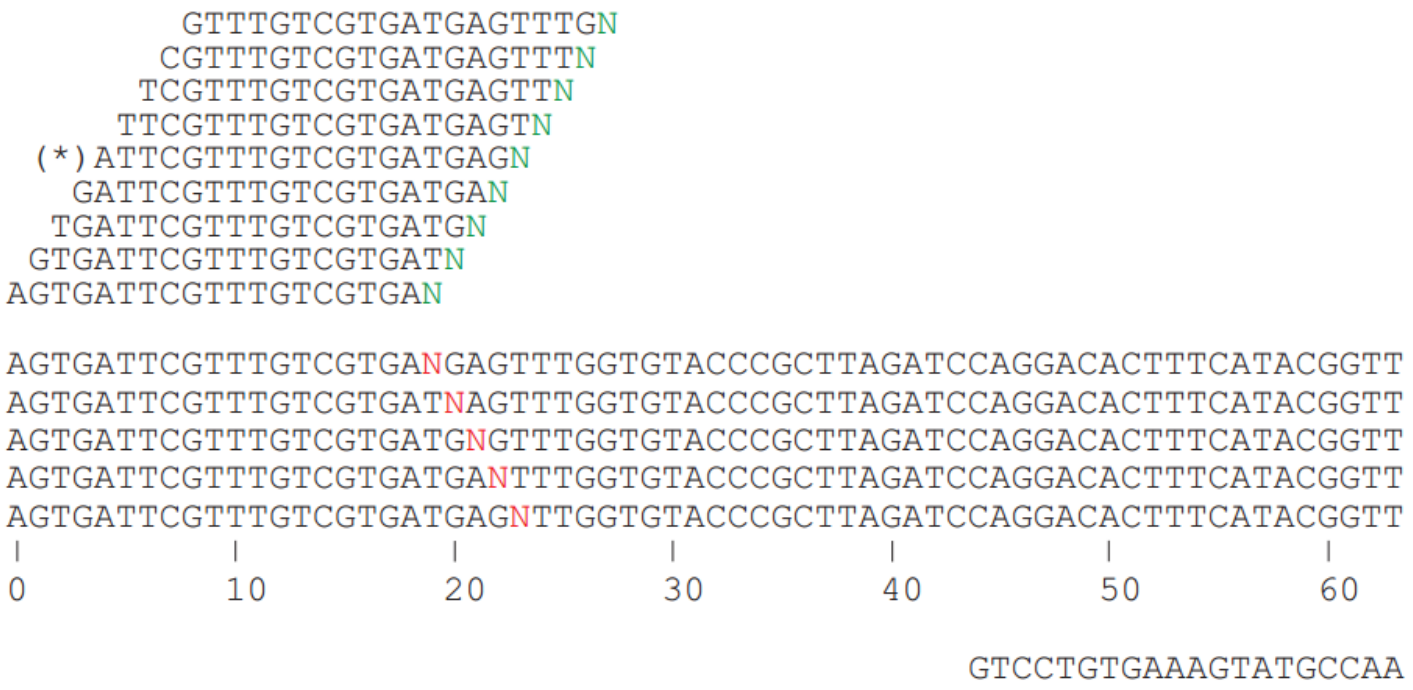


Fig. 1. Experimental setup: 36 tiling forward primers (top sequences), sixteen 64-base templates (middle sequences), and 1 reverse primer (bottom sequence).

All forward primers were derived from the starting forward primer (*). N denotes one of the 4 possible nucleotides (A, C, G, or T).

Materials and methods (mismatch simulations)

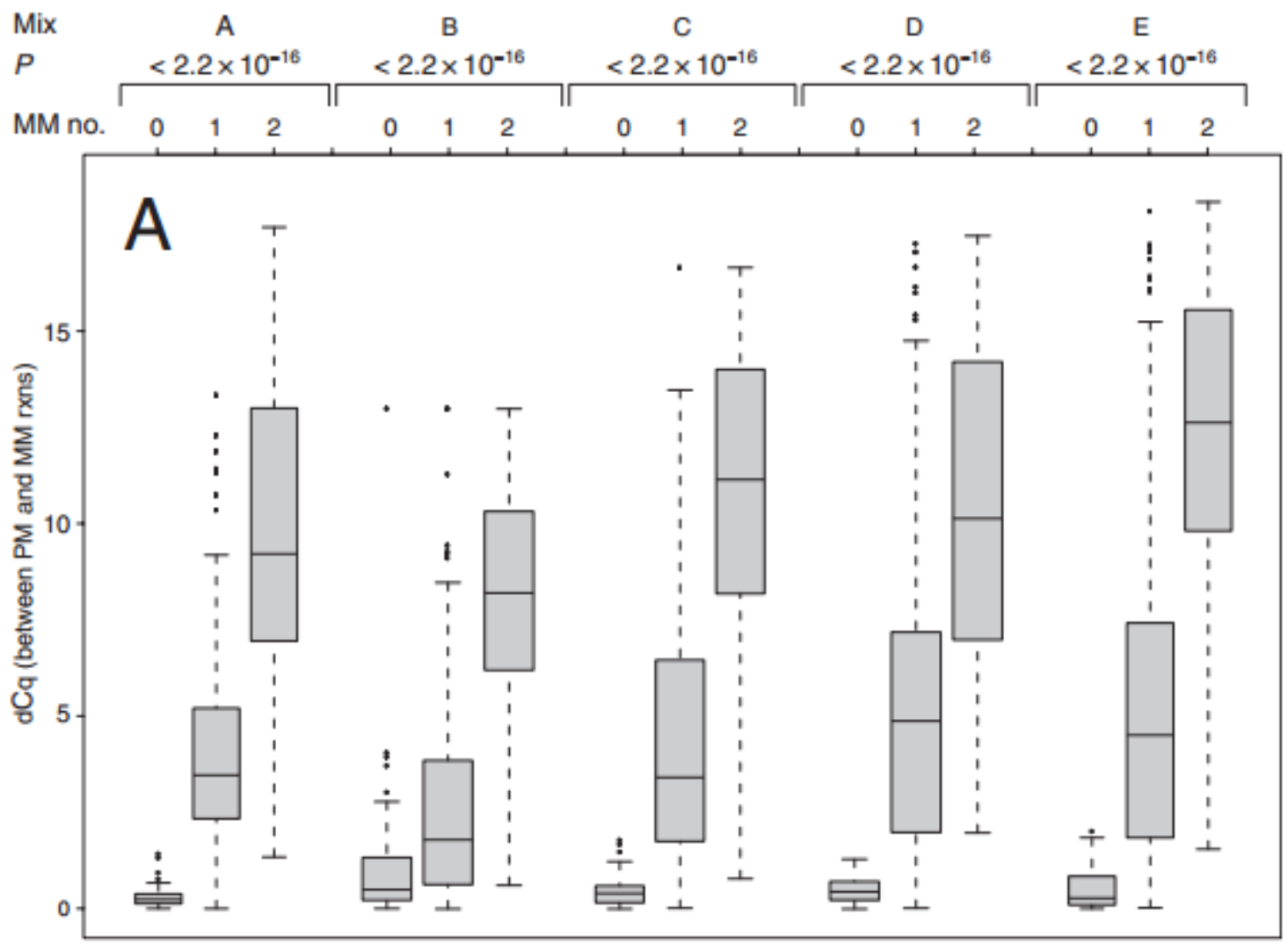
- For 3 20-base forward primer:
  - -> generated all combinations (491 007) with up to 4 mismatches in the 16-base region at their 3' end
  - -> calculated differences in annealing temperature (dTm) and Gibbs free energy (G) for each combination
  - qPCR (SsoAdvanced SYBR Green Supermix)

- 20 F-primers (with 0-4 mismatches), 20 additionally designed R primers (with 0-4 mismatches)
  - -> qPCR (SsoAdvanced SYBR Green Supermix)

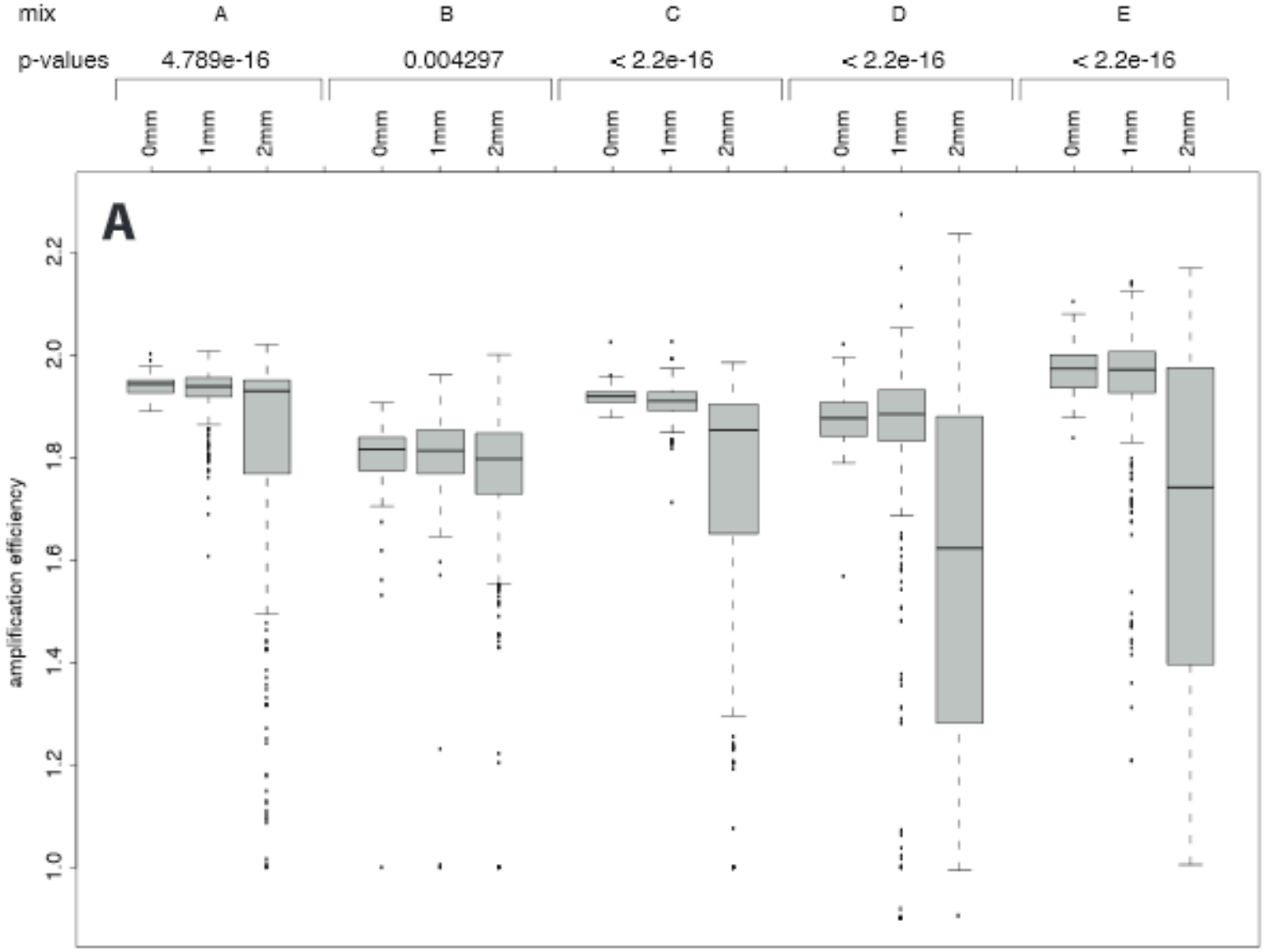
- 21 forward/reverse primer combinations
  - ->qPCR (template molecules per reaction: 20 – 20 000 000)

Results …

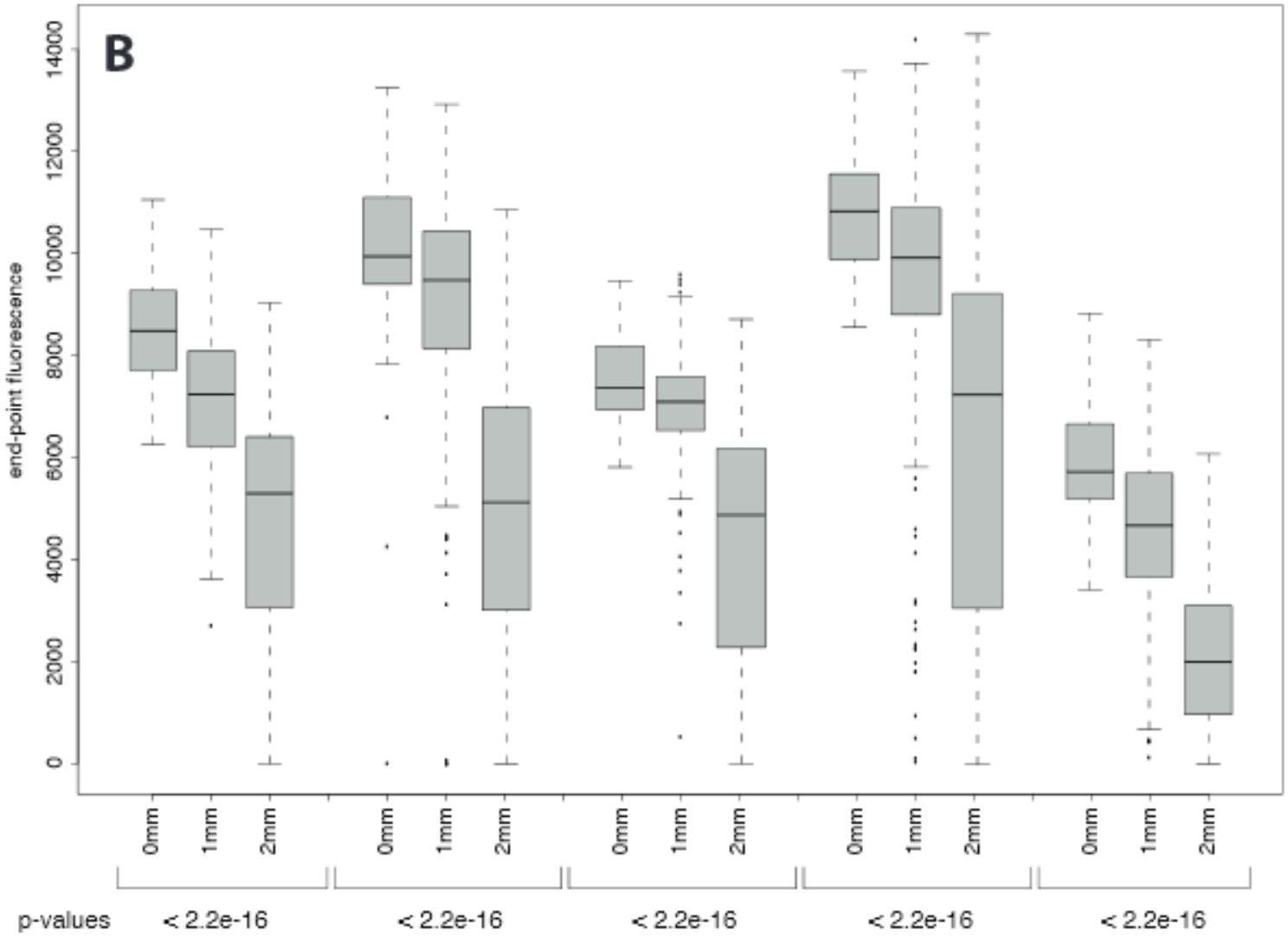
Effect of the number of mismatches (in the forward primer) on dCq value



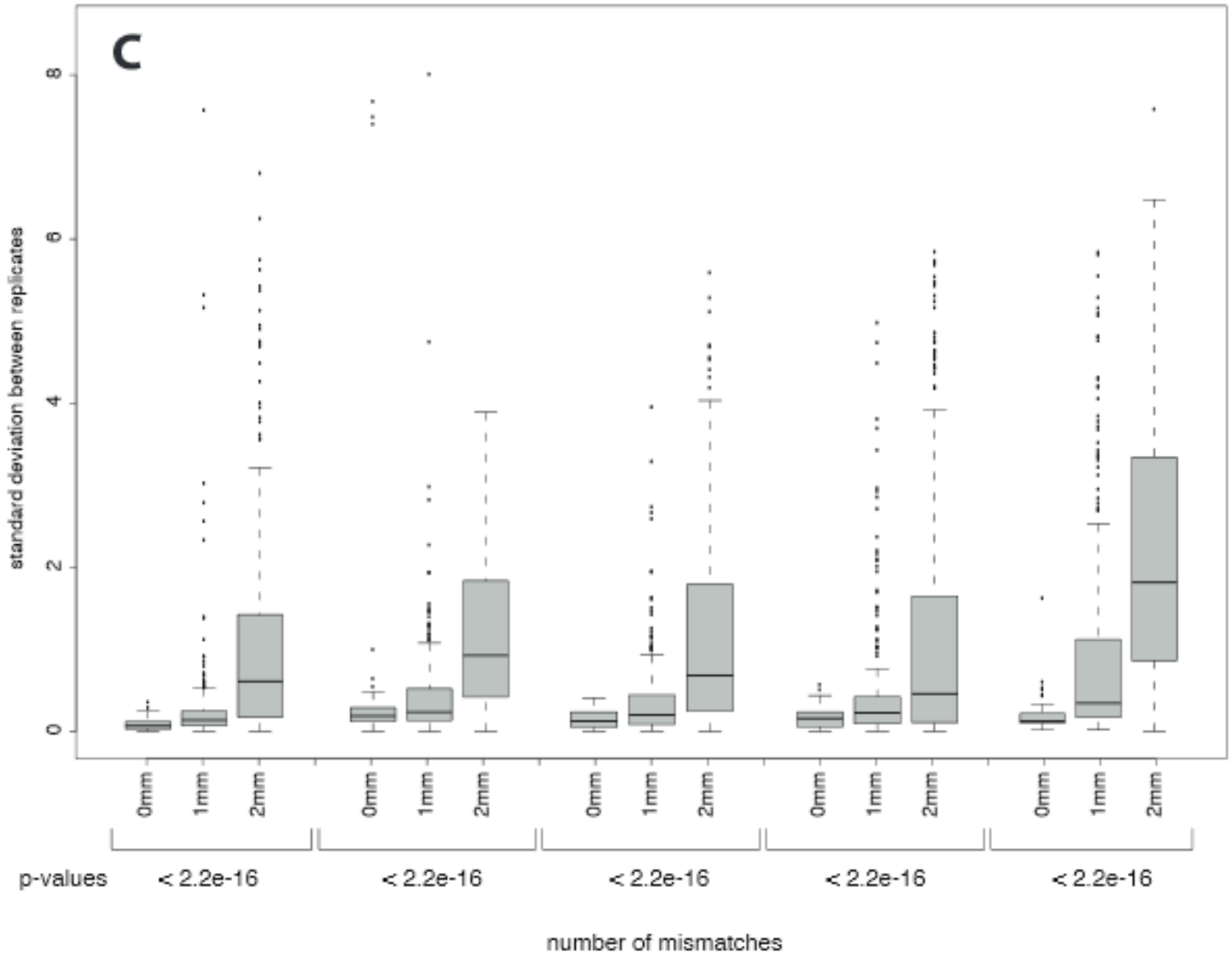
Effect of the number of mismatches (in the forward primer) on amplification efficiency



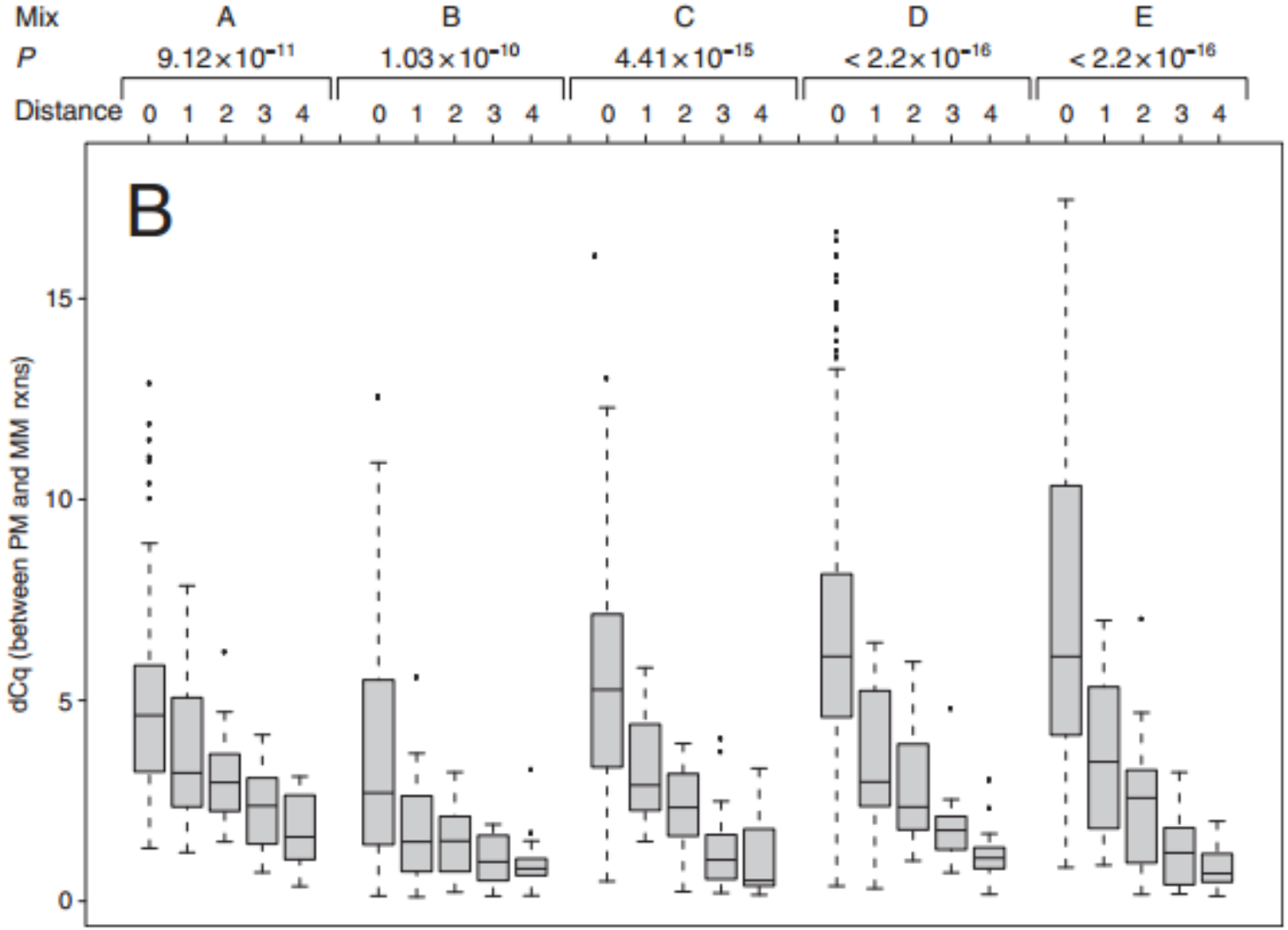
Effect of the number of mismatches (in the F primer) on end-point fluorescence



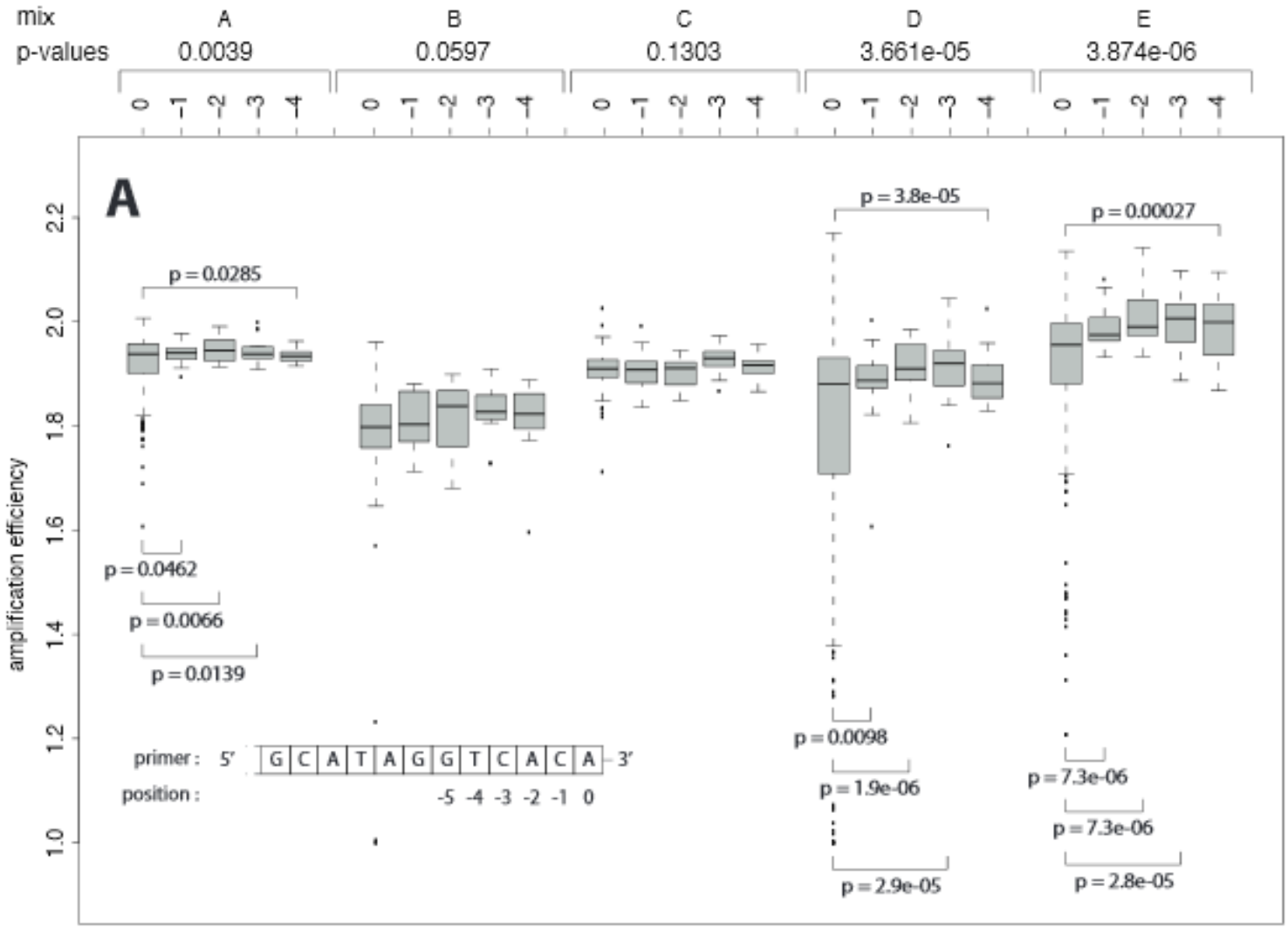
Standard deviation in function of the number of mismatches (in the forward primer)



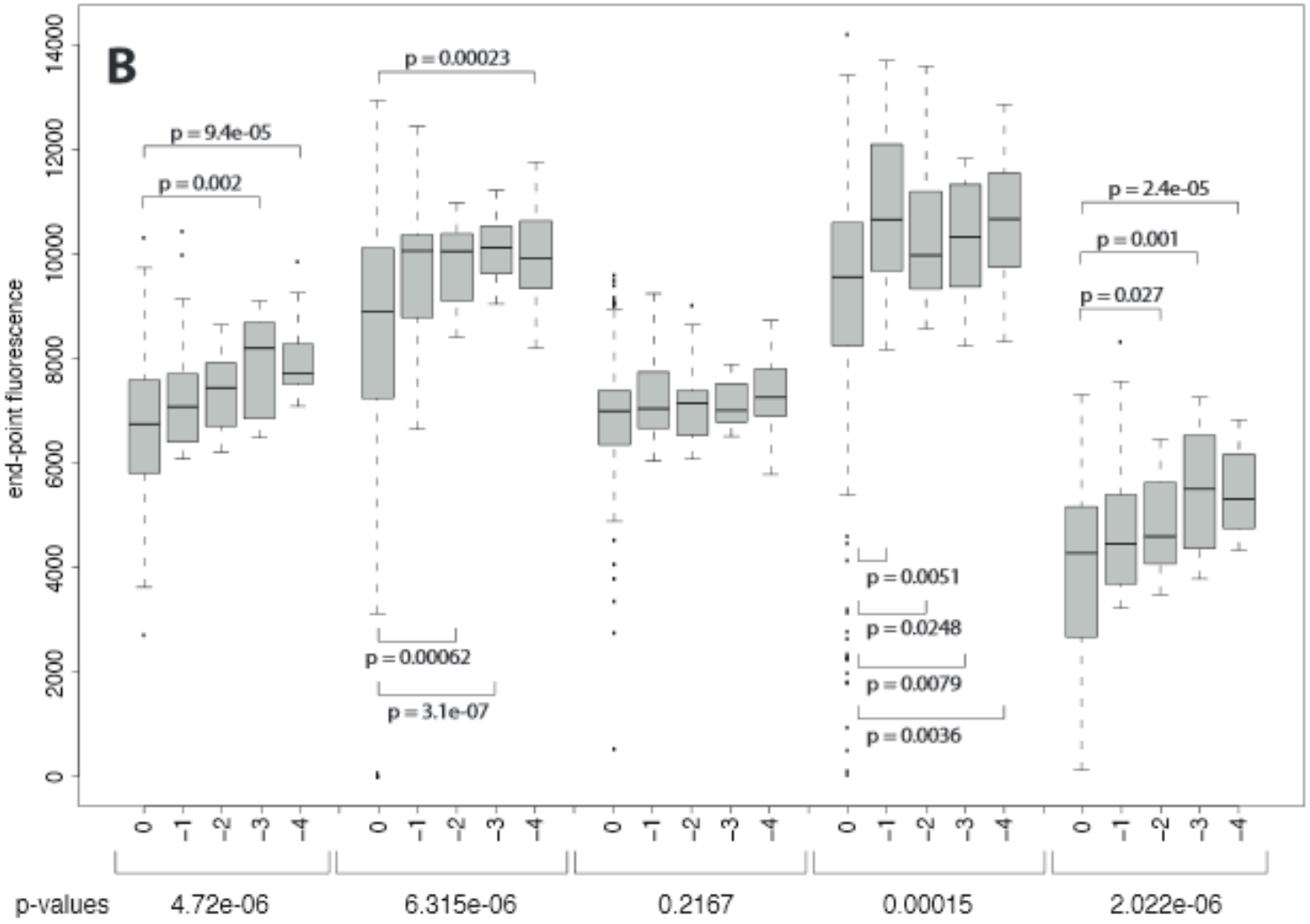
Positional effect of a single mismatch on dCq



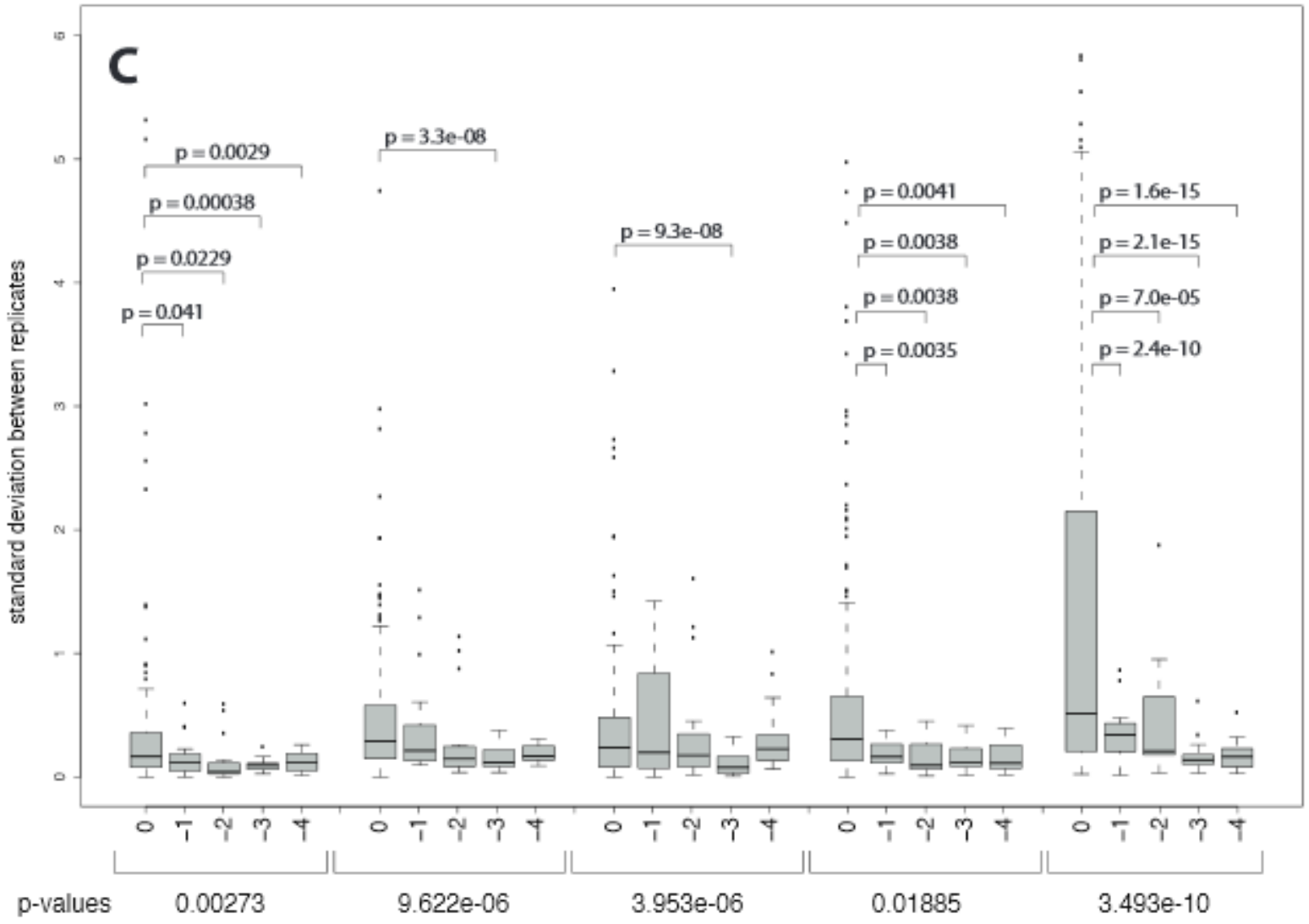
Positional effect of a single mismatch on amplification efficiency



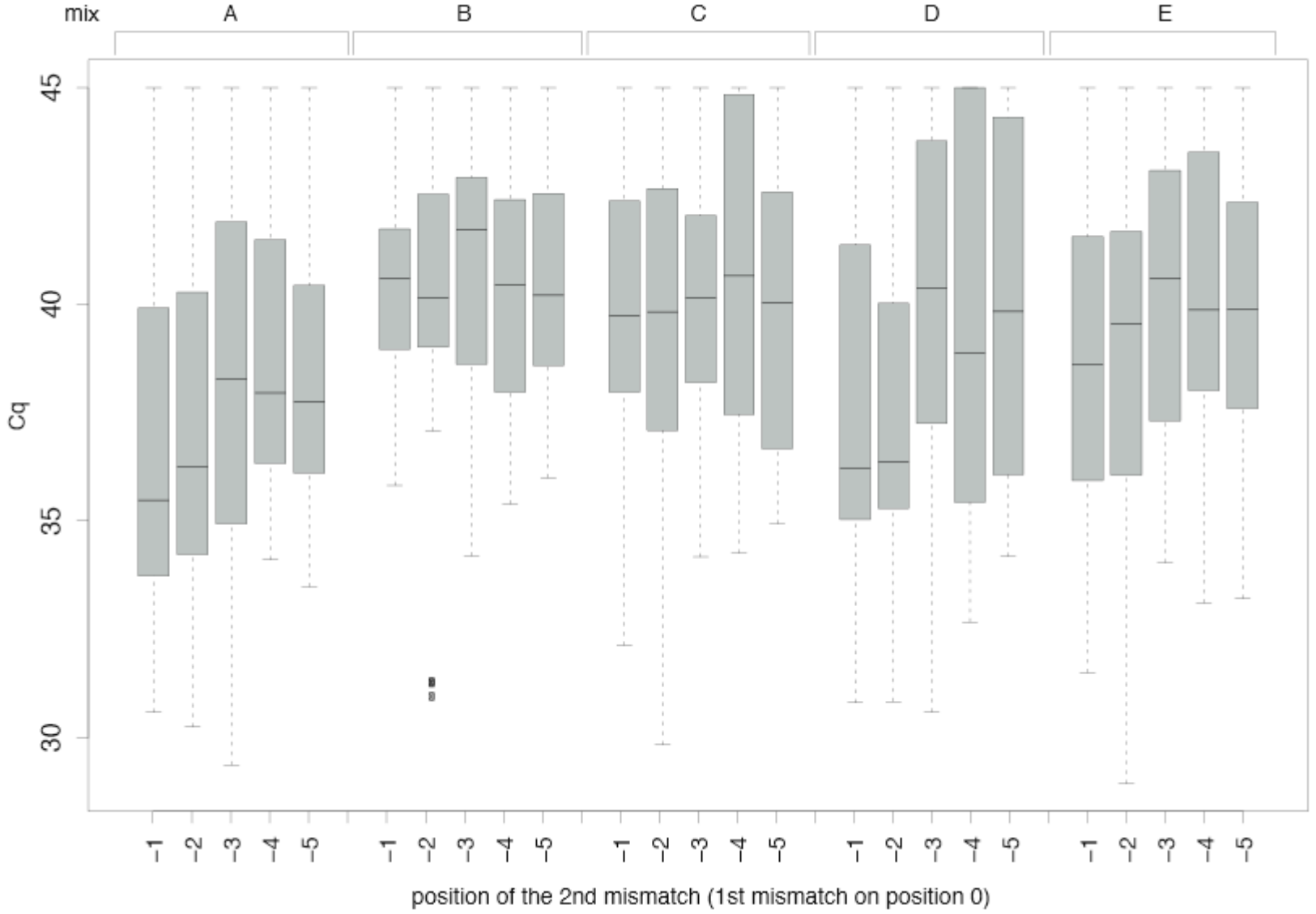
Positional effect of a single mismatch on end-point fluorescence



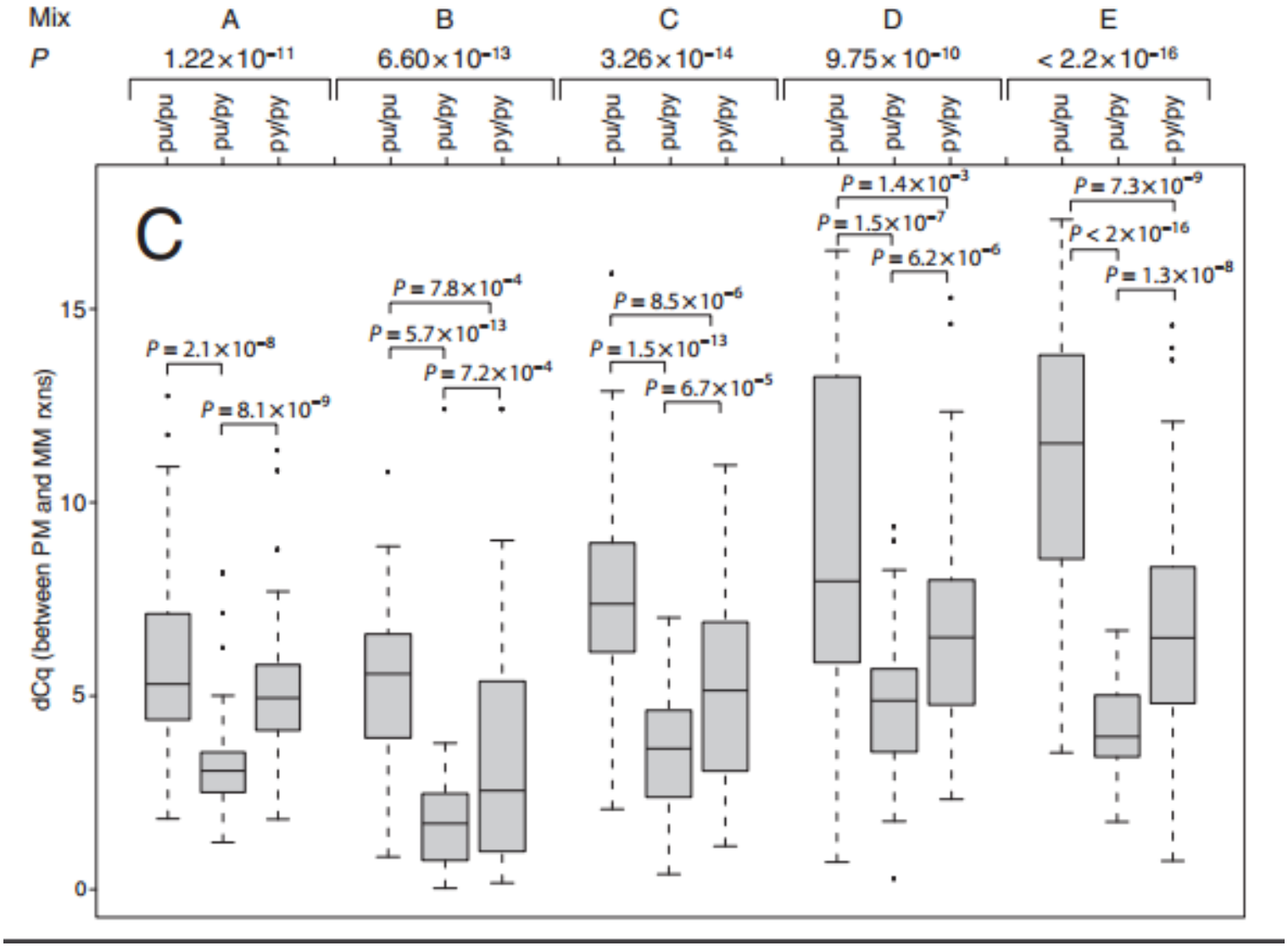
Standard deviation in function of the position of the mismatch



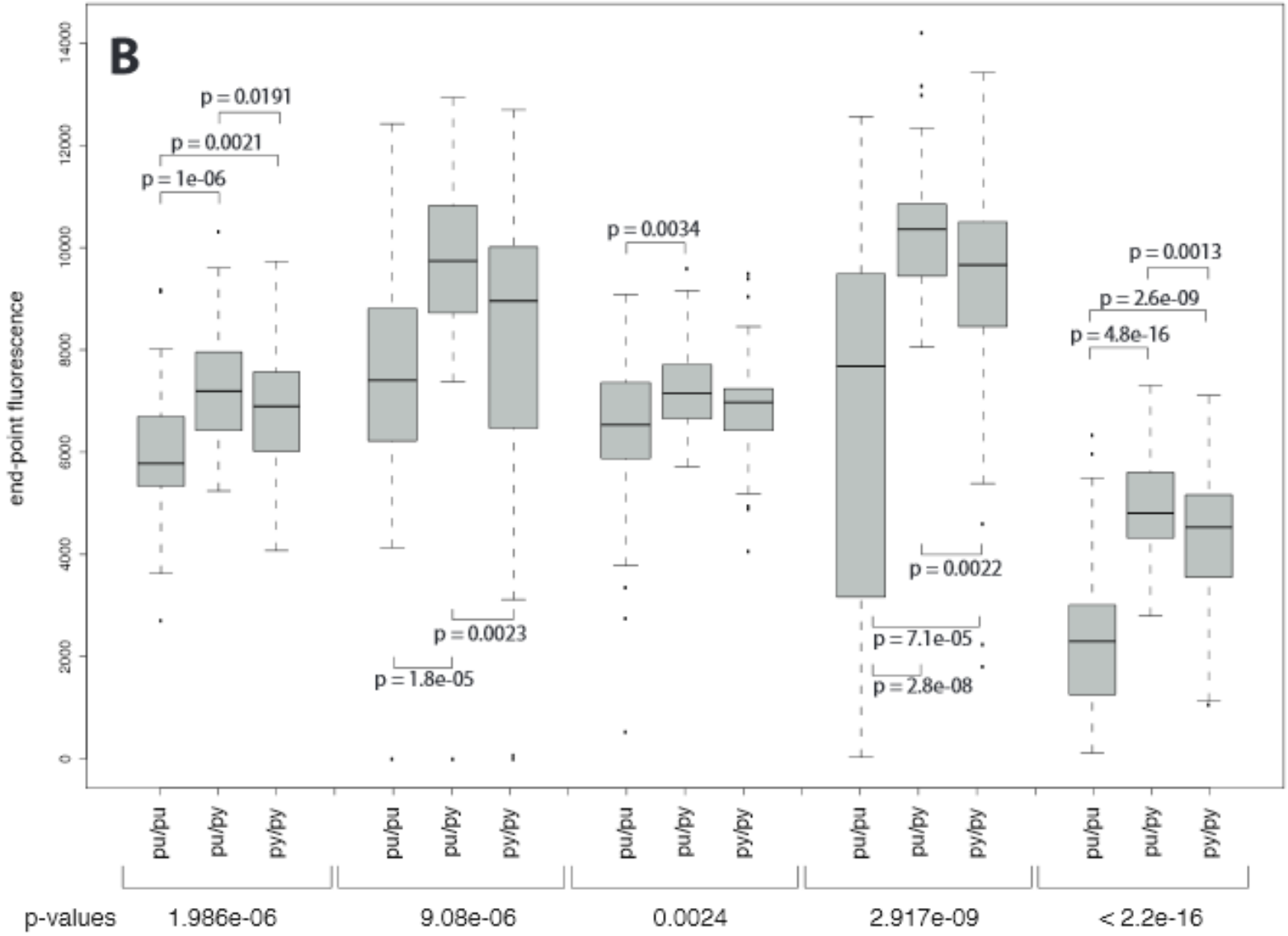
Cq of the mismatch reaction in function of the position of the second mismatch (first is on position 0, mean perfect match Cq=29,1)



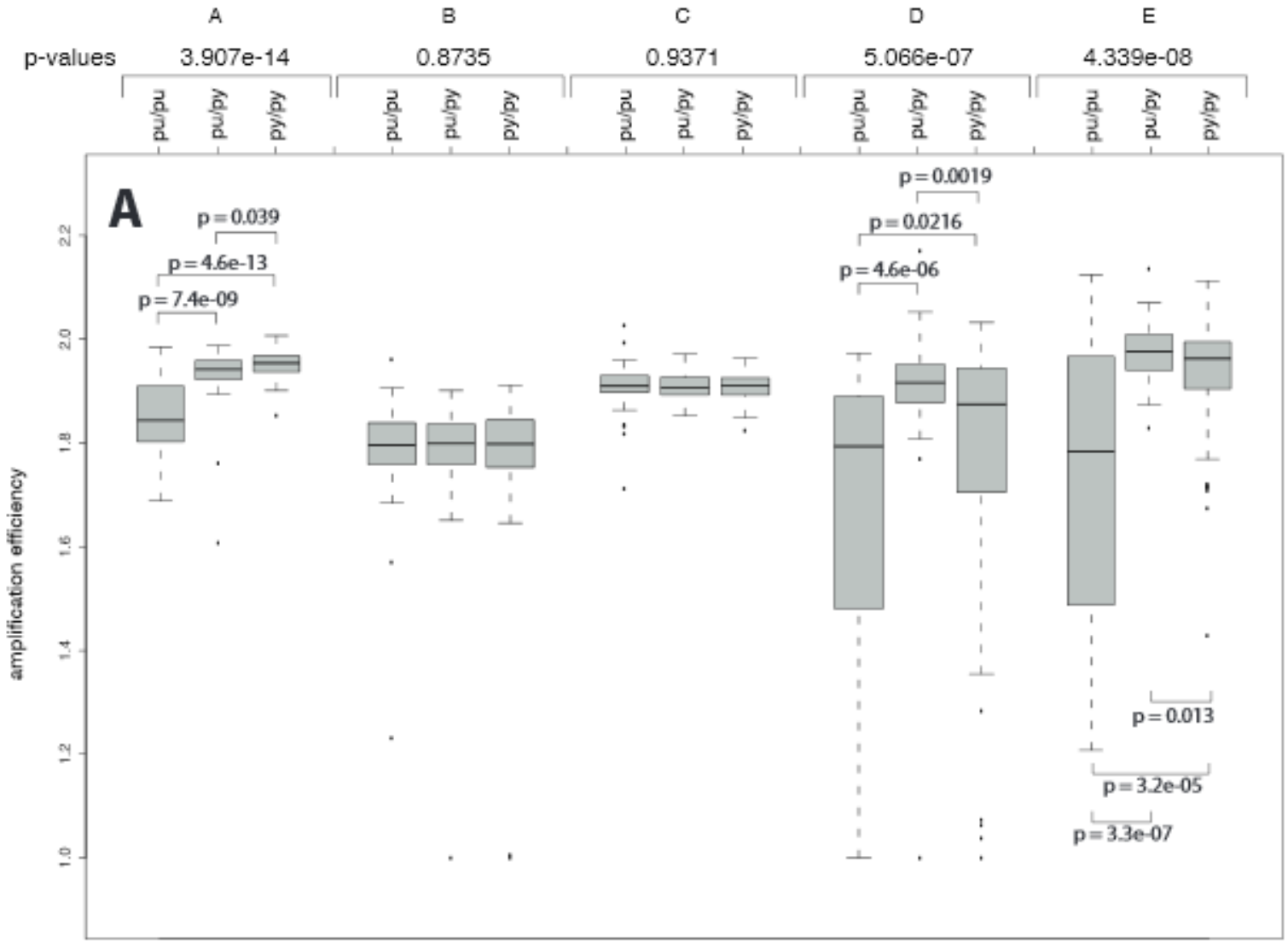
Effect of the type of mismatch at the primer's 3' terminus



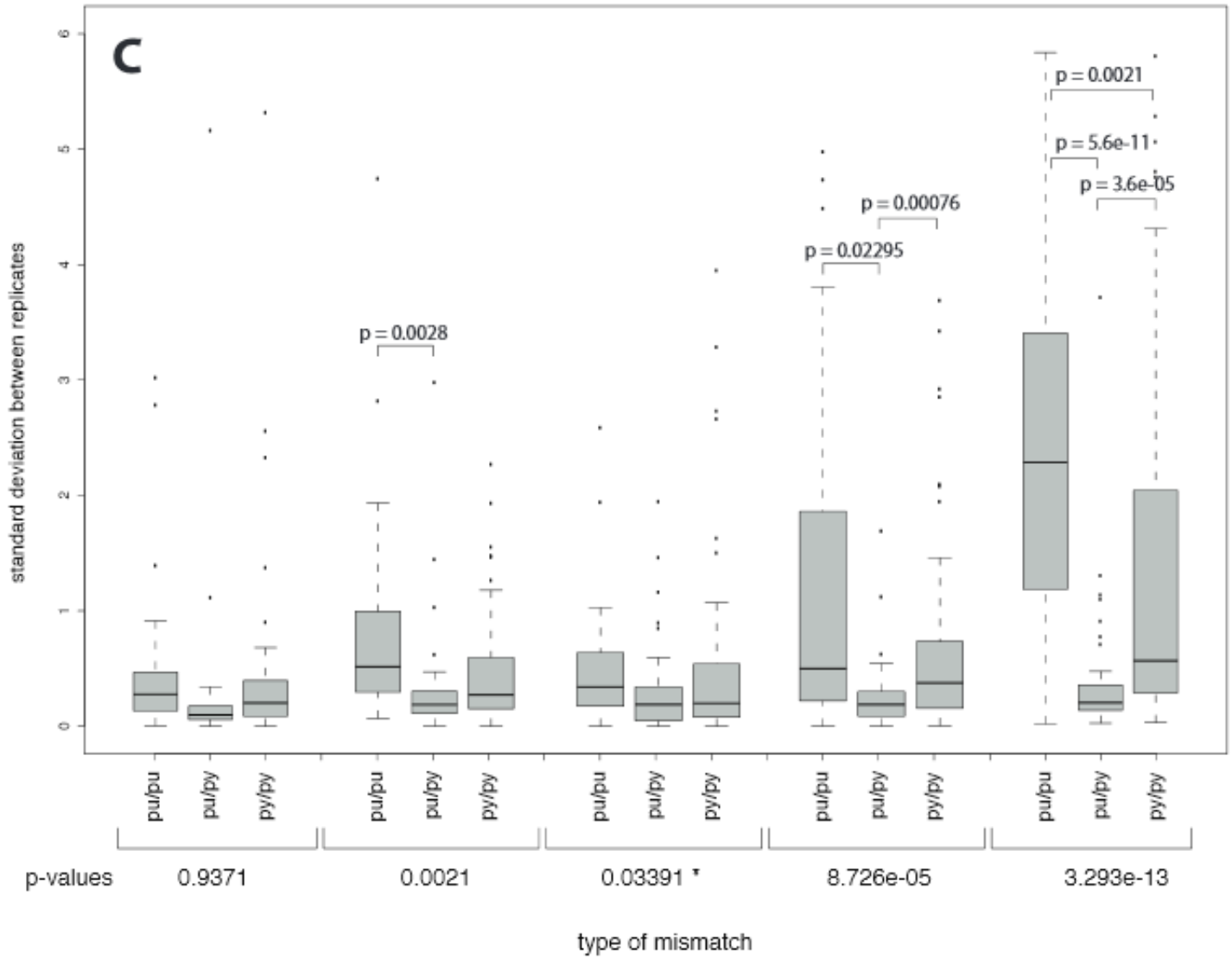
Effect of the type of mismatch at the primer's 3' terminus

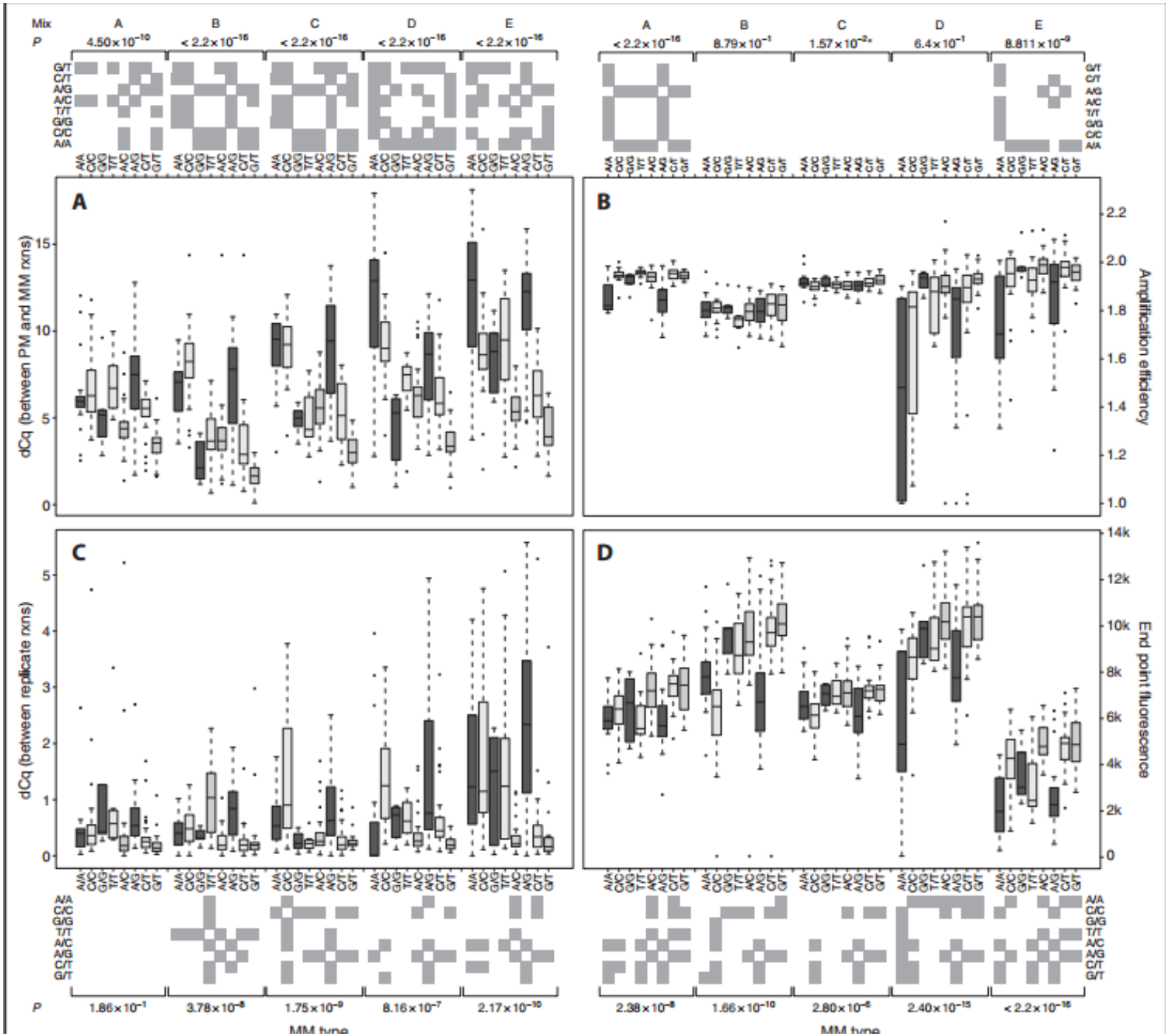


Effect of the type of mismatch at the primer's 3' terminus

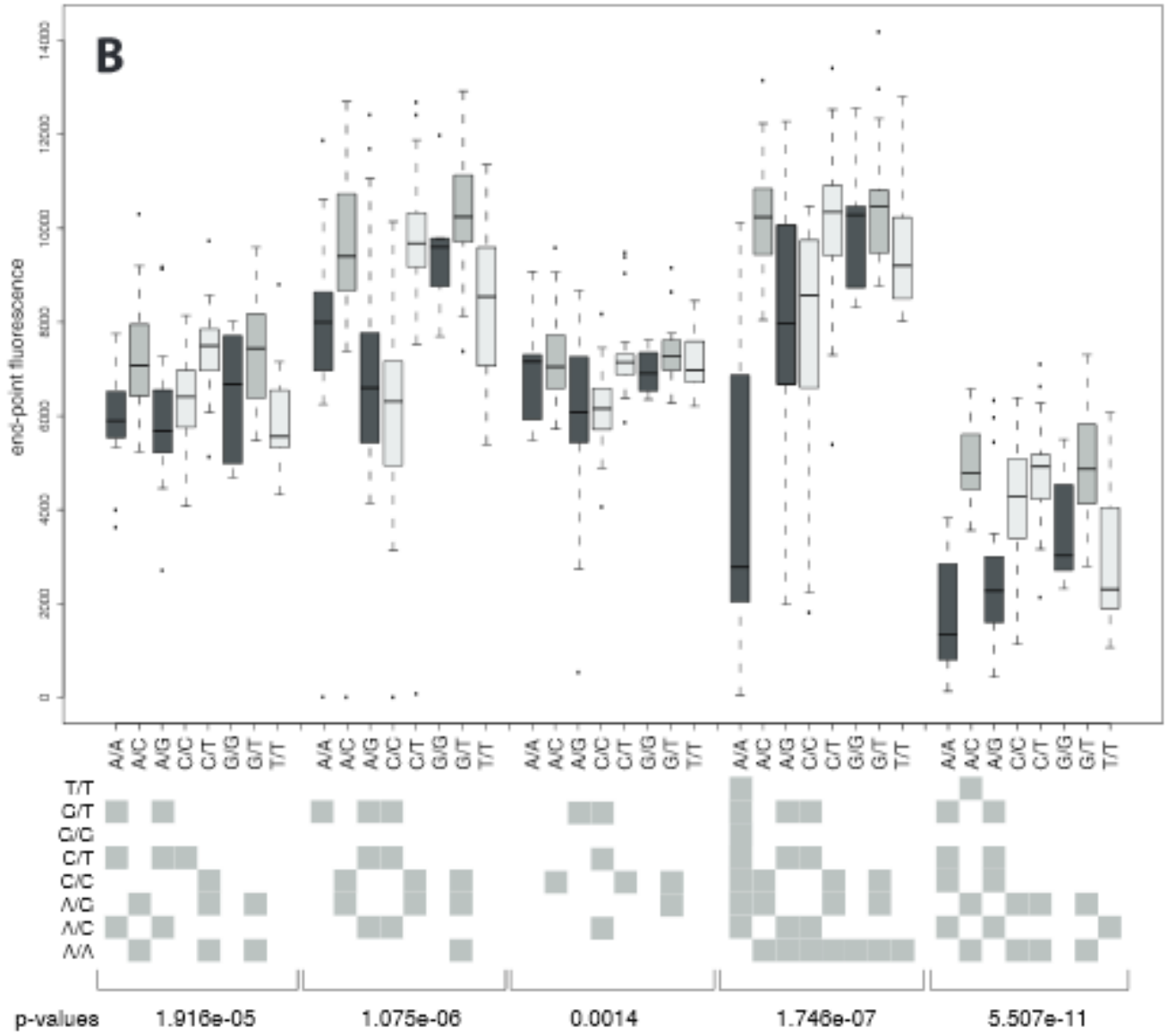


Effect of the type of mismatch at the primer's 3' terminus



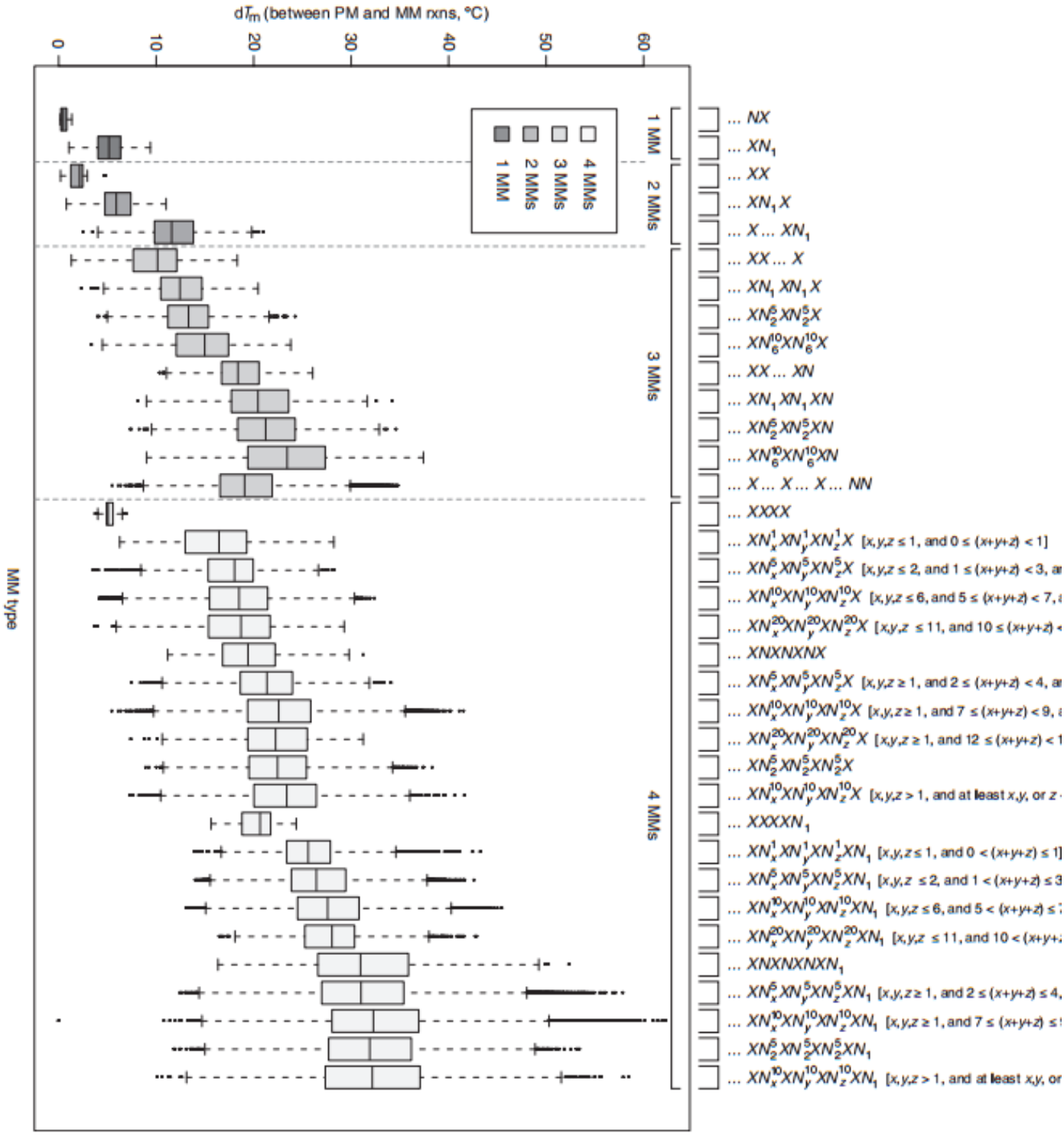

Mix
A
B
C
D
E
P
4.50×10⁻¹⁰
< 2.2×10⁻¹⁶
< 2.2×10⁻¹⁶
< 2.2×10⁻¹⁶
< 2.2×10⁻¹⁶
< 2.2×10⁻¹⁶
8.79×10⁻¹
1.57×10⁻²⁺
6.4×10⁻¹
8.811×10⁻⁹
G/T
C/T
A/G
A/C
T/T
G/G
C/C
A/A
A/A C/C G/G T/T A/C A/G C/T G/T
A
B
C
D
dCq (between PM and MM rxns)
Amplification efficiency
dCq (between replicate rxns)
End point fluorescence
0
5
10
15
1.0
1.2
1.4
1.6
1.8
2.0
2.2
1
2
3
4
2k
4k
6k
8k
10k
12k
14k
1.86×10⁻¹
3.78×10⁻⁸
1.75×10⁻⁹
8.16×10⁻⁷
2.17×10⁻¹⁰
2.38×10⁻⁸
1.66×10⁻¹⁰
2.80×10⁻⁶
2.40×10⁻¹⁵
< 2.2×10⁻¹⁶
MM type
MM type

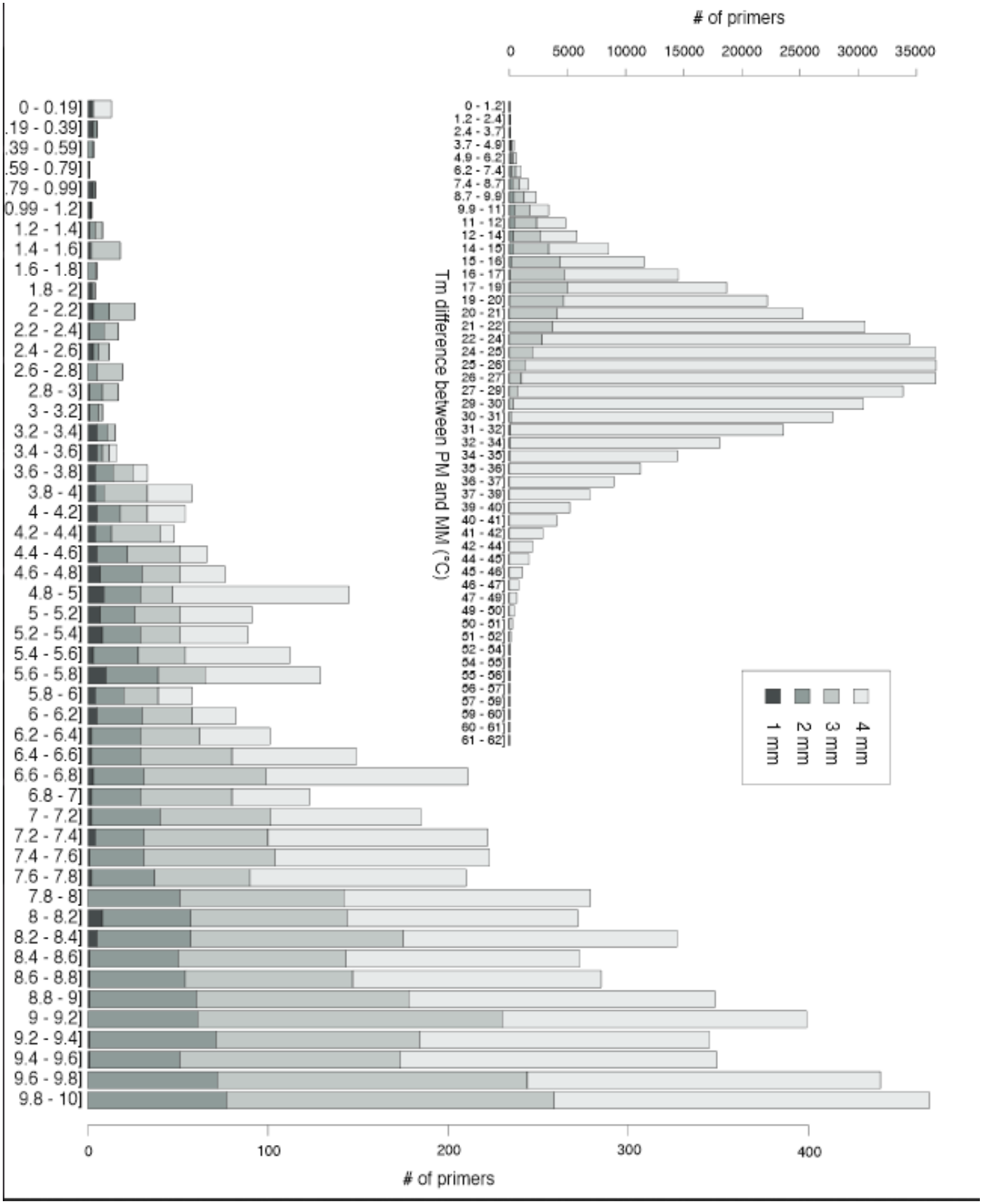
Effect of the type of mismatch at the primer's 3' terminus

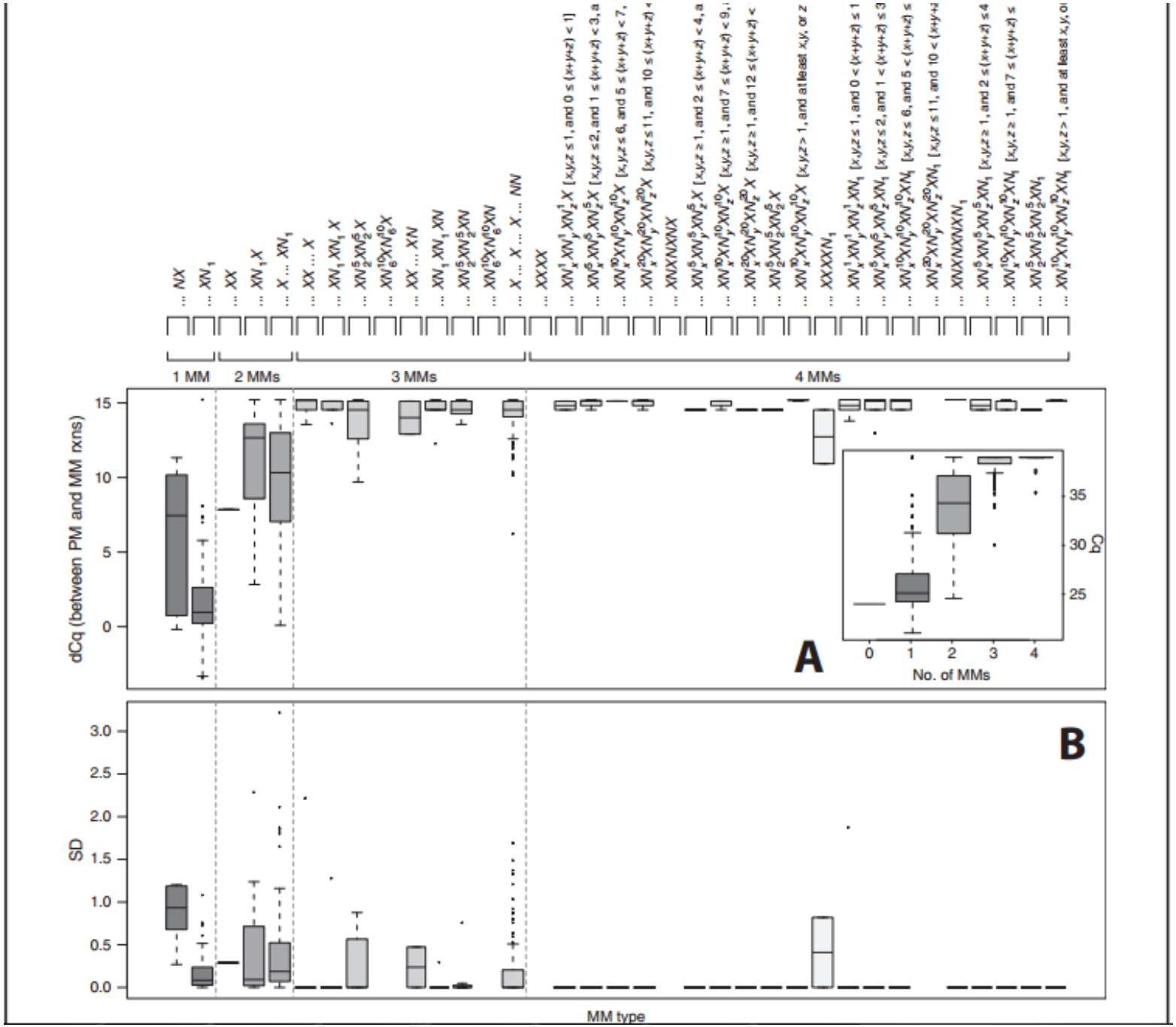


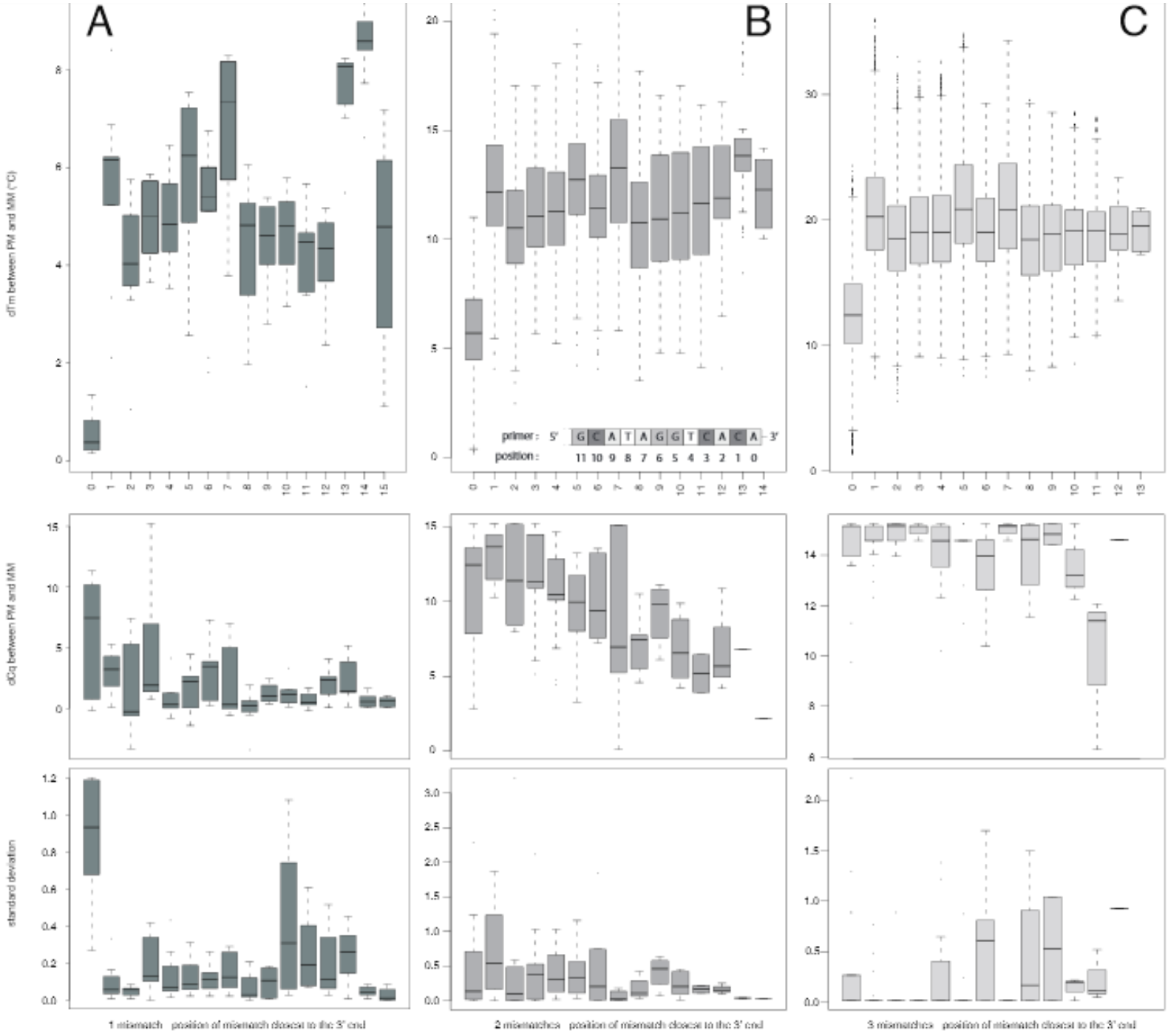
Simulation of the effect of mismatches on annealing temperature

- To predict how many simultaneous mismatches could be tolerated in a qPCR reaction
- To study simulated effect of <=4 concurrent mismatches throughout the 16-base region at the 3’ end of a 20-base primer
- Simulated 491 007 1-4-mismatch combinations
  - calculated dTm
  - 440 randomly selected primers was tested with qPCR

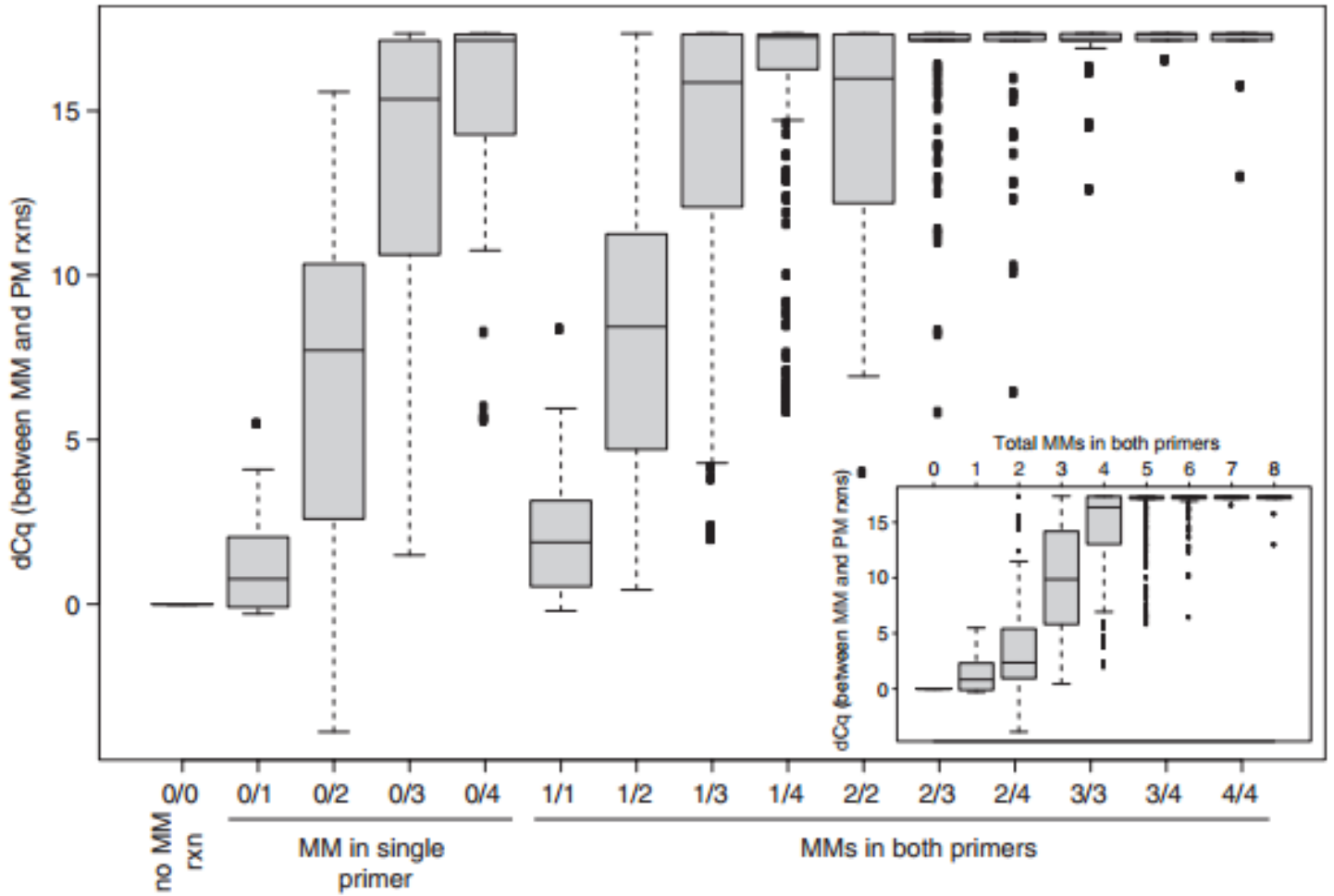

dTm (between PM and MM rxns, °C)
0
10
20
30
40
50
60
1 MM
2 MMs
3 MMs
4 MMs
MM type
... NX
... XN1
... XX
... XN1X
... X... XN1
... XX... X
... XN1XN1X
... XN2 5XN2 5X
... XN6 10XN6 10X
... XX... XN
... XN1XN1XN
... XN2 5XN2 5XN
... XN6 10XN6 10XN
... X... X... X... NN
... XXXX
... XNx 1XNy 1XNz 1X [x,y,z ≤ 1, and 0 ≤ (x+y+z) < 1]
... XNx 5XNy 5XNz 5X [x,y,z ≤ 2, and 1 ≤ (x+y+z) < 3, a
... XNx 10XNy 10XNz 10X [x,y,z ≤ 6, and 5 ≤ (x+y+z) < 7,
... XNx 20XNy 20XNz 20X [x,y,z ≤ 11, and 10 ≤ (x+y+z) <
... XNXNXNX
... XNx 5XNy 5XNz 5X [x,y,z ≥ 1, and 2 ≤ (x+y+z) < 4, a
... XNx 10XNy 10XNz 10X [x,y,z ≥ 1, and 7 ≤ (x+y+z) < 9,
... XNx 20XNy 20XNz 20X [x,y,z ≥ 1, and 12 ≤ (x+y+z) < 1
... XN2 5XN2 5XN2 5X
... XNx 10XNy 10XNz 10X [x,y,z > 1, and at least x,y, or z
... XXXXN1
... XNx 1XNy 1XNz 1XN1 [x,y,z ≤ 1, and 0 < (x+y+z) ≤ 1]
... XNx 5XNy 5XNz 5XN1 [x,y,z ≤ 2, and 1 < (x+y+z) ≤ 3
... XNx 10XNy 10XNz 10XN1 [x,y,z ≤ 6, and 5 < (x+y+z) ≤ 7
... XNx 20XNy 20XNz 20XN1 [x,y,z ≤ 11, and 10 < (x+y+z
... XNXNXNXN1
... XNx 5XNy 5XNz 5XN1 [x,y,z ≥ 1, and 2 ≤ (x+y+z) ≤ 4,
... XNx 10XNy 10XNz 10XN1 [x,y,z ≥ 1, and 7 ≤ (x+y+z) ≤
... XN2 5XN2 5XN2 5XN1
... XNx 10XNy 10XNz 10XN1 [x,y,z > 1, and at least x,y, or


Tm difference between PM and MM (°C)
# of primers
0 - 0.19]
.19 - 0.39]
.39 - 0.59]
.59 - 0.79]
.79 - 0.99]
0.99 - 1.2]
1.2 - 1.4]
1.4 - 1.6]
1.6 - 1.8]
1.8 - 2]
2 - 2.2]
2.2 - 2.4]
2.4 - 2.6]
2.6 - 2.8]
2.8 - 3]
3 - 3.2]
3.2 - 3.4]
3.4 - 3.6]
3.6 - 3.8]
3.8 - 4]
4 - 4.2]
4.2 - 4.4]
4.4 - 4.6]
4.6 - 4.8]
4.8 - 5]
5 - 5.2]
5.2 - 5.4]
5.4 - 5.6]
5.6 - 5.8]
5.8 - 6]
6 - 6.2]
6.2 - 6.4]
6.4 - 6.6]
6.6 - 6.8]
6.8 - 7]
7 - 7.2]
7.2 - 7.4]
7.4 - 7.6]
7.6 - 7.8]
7.8 - 8]
8 - 8.2]
8.2 - 8.4]
8.4 - 8.6]
8.6 - 8.8]
8.8 - 9]
9 - 9.2]
9.2 - 9.4]
9.4 - 9.6]
9.6 - 9.8]
9.8 - 10]
0
100
200
300
400
# of primers
0 - 1.2]
1.2 - 2.4]
2.4 - 3.7]
3.7 - 4.9]
4.9 - 6.2]
6.2 - 7.4]
7.4 - 8.7]
8.7 - 9.9]
9.9 - 11]
11 - 12]
12 - 14]
14 - 15]
15 - 16]
16 - 17]
17 - 19]
19 - 20]
20 - 21]
21 - 22]
22 - 24]
24 - 25]
25 - 26]
26 - 27]
27 - 29]
29 - 30]
30 - 31]
31 - 32]
32 - 34]
34 - 35]
35 - 36]
36 - 37]
37 - 39]
39 - 40]
40 - 41]
41 - 42]
42 - 44]
44 - 45]
45 - 46]
46 - 47]
47 - 49]
49 - 50]
50 - 51]
51 - 52]
52 - 54]
54 - 55]
55 - 56]
56 - 57]
57 - 59]
59 - 60]
60 - 61]
61 - 62]
0
5000
10000
15000
20000
25000
30000
35000
1 mm
2 mm
3 mm
4 mm


1 MM
2 MMs
3 MMs
4 MMs
dCq (between PM and MM rxns)
SD
MM type
No. of MMs
Cq
A
B


A
B
C
dTm between PM and MM (°C)
dCq between PM and MM
standard deviation
primer : 5' G C A T A G G T C A C A - 3'
position : 11 10 9 8 7 6 5 4 3 2 1 0
1 mismatch position of mismatch closest to the 3' end
2 mismatches position of mismatch closest to the 3' end
3 mismatches position of mismatch closest to the 3' end

Effect of mismatches in both primers



Conclusions

- Single mismatches located >5 bp from the 3’ end have a moderate effect on qPCR amplification and can be tolerated.
- The effects of mismatches decrease with distance of a mismatch from the primer’s 3’ end terminus.
- The effect of mismatches at the 3’ terminus is (partly) dependent on the nucleotide composition of the mismatch
- Mismatch-induced inhibition was independent of template concentration for primer pairs harboring a maximum of 1 mismatch per primer, whereas concentration independence decreased at geater dilutions (<=2000 molecules per reaction) in the presence of >1 mismatch in a single primer.

Conclusions

- 4 mismatches in a single primer block amplification almost completely (independent of template concentration and mm position)
- qPCR inhibition with 3 mismatches was also dependent of the mm position in the primer
- 3 mismatches in one of the primers must be combined with at least 2 mismatches in the other primer to block amplification


Thank you for listening!