Exploiting extension bias in polymerase chain reaction to improve primer specificity in ensembles of nearly identical DNA templates

Journal Club

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Introduction

- Small biases in amplification efficiency can quantitatively translated into substantial differences in amplicon concentrations
- Potential for exploiting inherent PCR biases (from diverse sources) for sequence discrimination (in genotyping, metagenomic profiling, …) -> incorporating into primer design
- The influence of 3'terminal mismatches on amplification

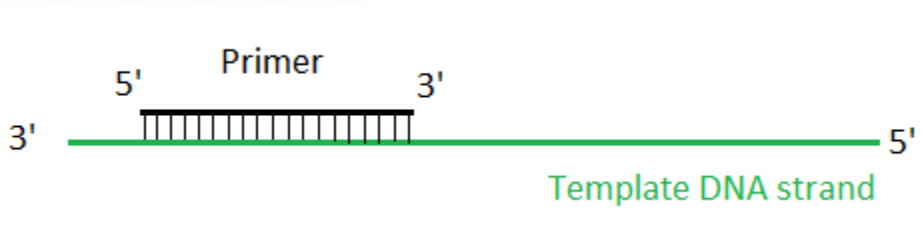
PCR amplification efficiency

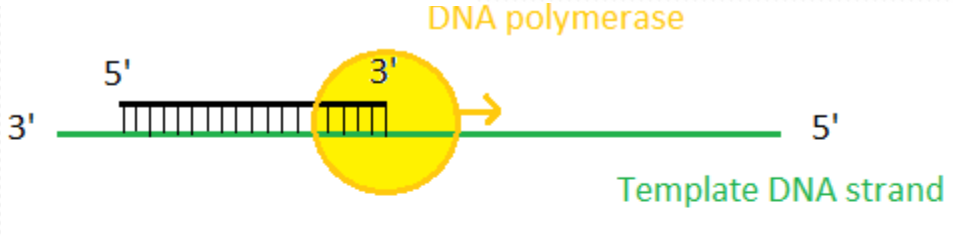
$$Eff_{obs} = \sqrt{Eff_{f\_primer} * Eff_{r\_primer}}$$

Potential amplification efficiency for primer

↓

Hybridization efficiency **x** Elongation efficiency





$$Eff_{hyb} = \left(\frac{P_0 * K_{off}}{1 + P_0 * K_{eff}}\right)$$

$$K_{eff} = \frac{4 * K_{pt} * K_{pd} * P_0}{\left(-1 - K_{pf} + \sqrt{(1 + K_{pf})^2 + 8 * P_0 * K_{pd}}\right) * (1 + K_{tf})}$$

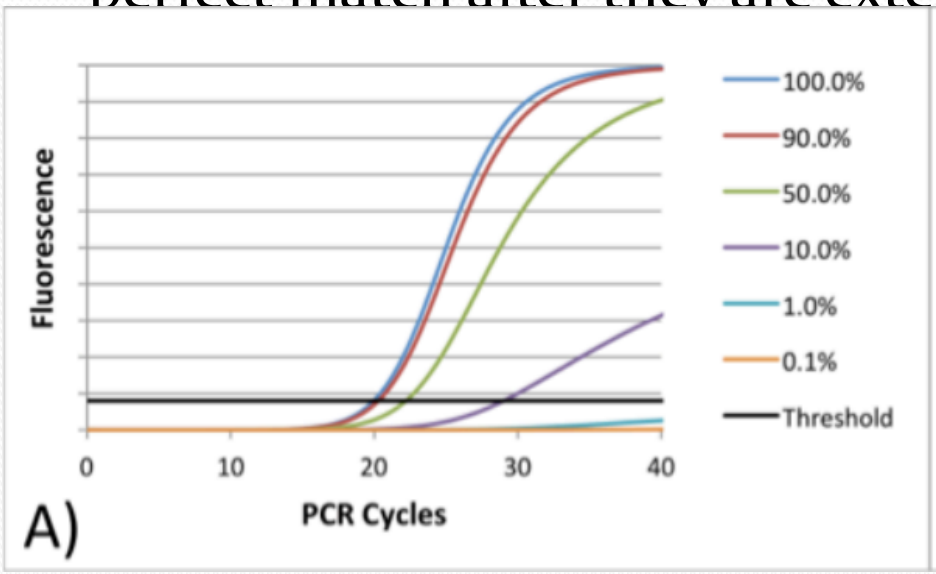
$$[Na^+_{eq}] = [Mon^+] + 3.33\sqrt{[Div^{2+}] - [dNTPs]}$$

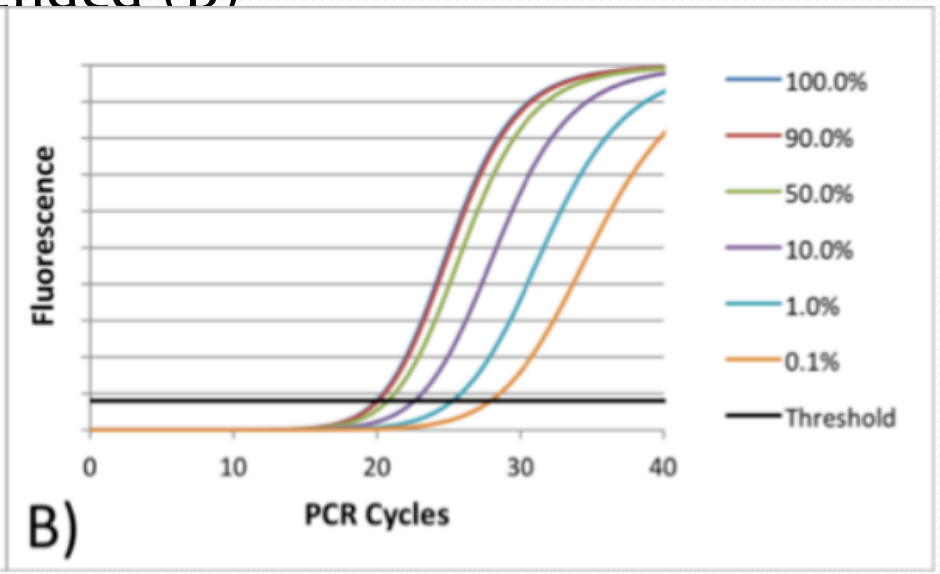
$$K = \exp(-\frac{\Delta G^o}{RT})$$

↓

How to measure elongation efficiency of qPCR?

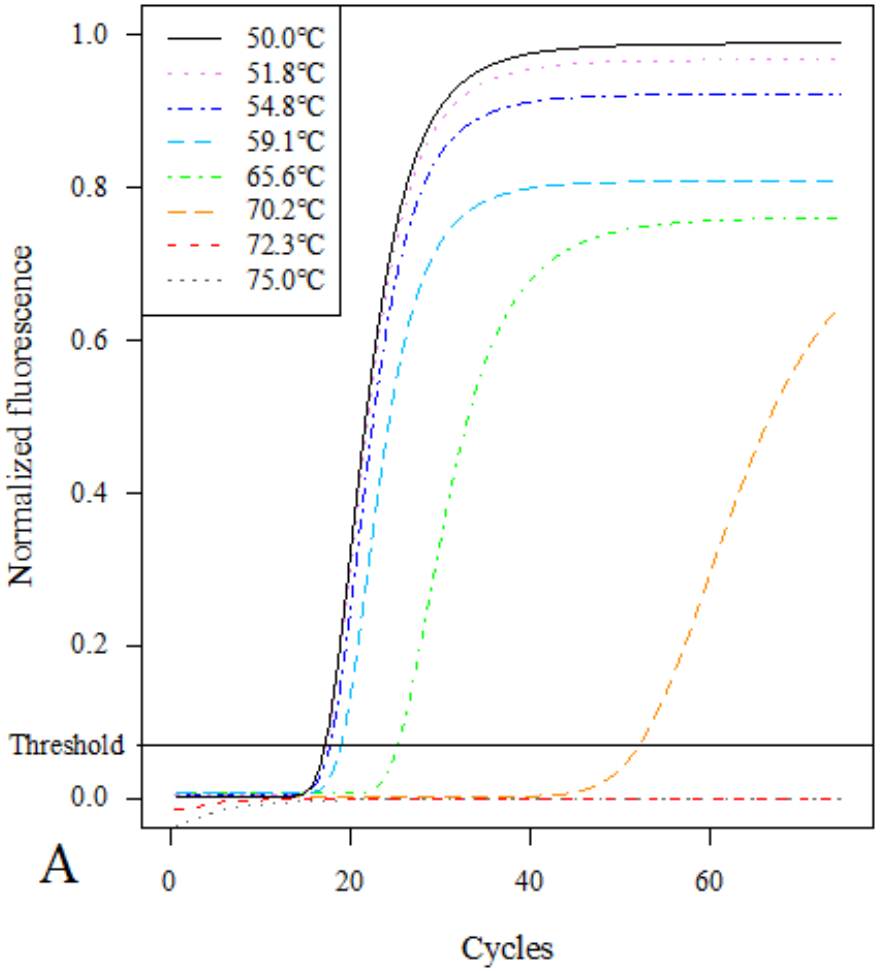
- Decreased hybridization efficiency (Eff_{hyb}) results in a delayed amplification and a decrease in slope of the amplification curve (A).
- Decreased elongation efficiency (Eff_{elong}) only results in delayed amplification because mismatched primers become perfect match after they are extended (B)

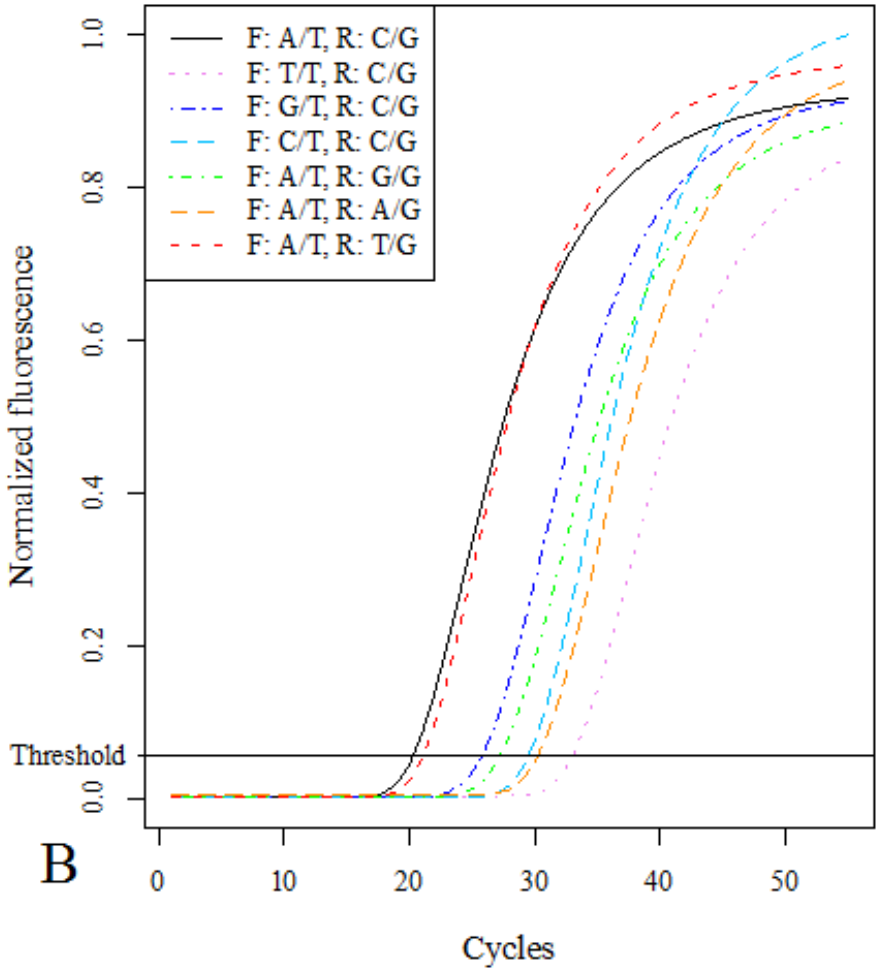




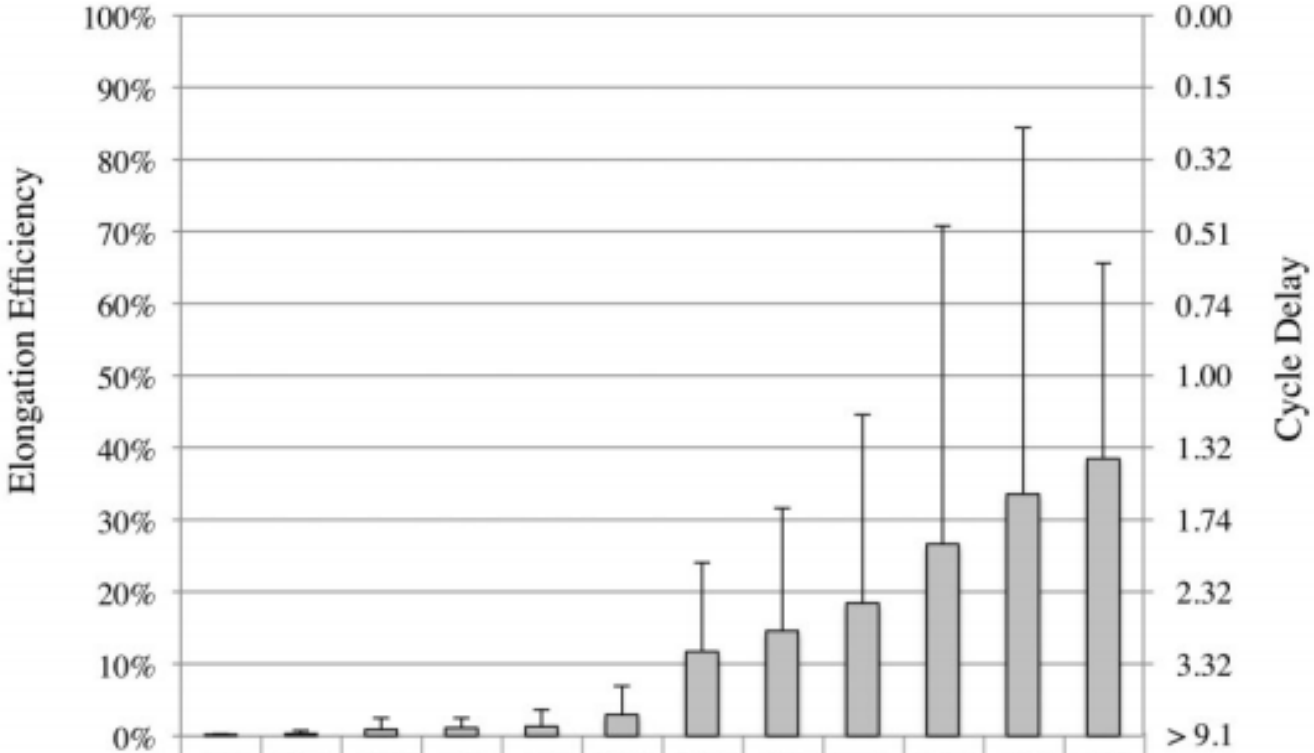
- **$Eff_{elong} = 2^{-\Delta Ct}$ $(Eff_{hyb} = 100\%)$**

Comparison of decreased hybridization efficiency with decreased elongation efficiency



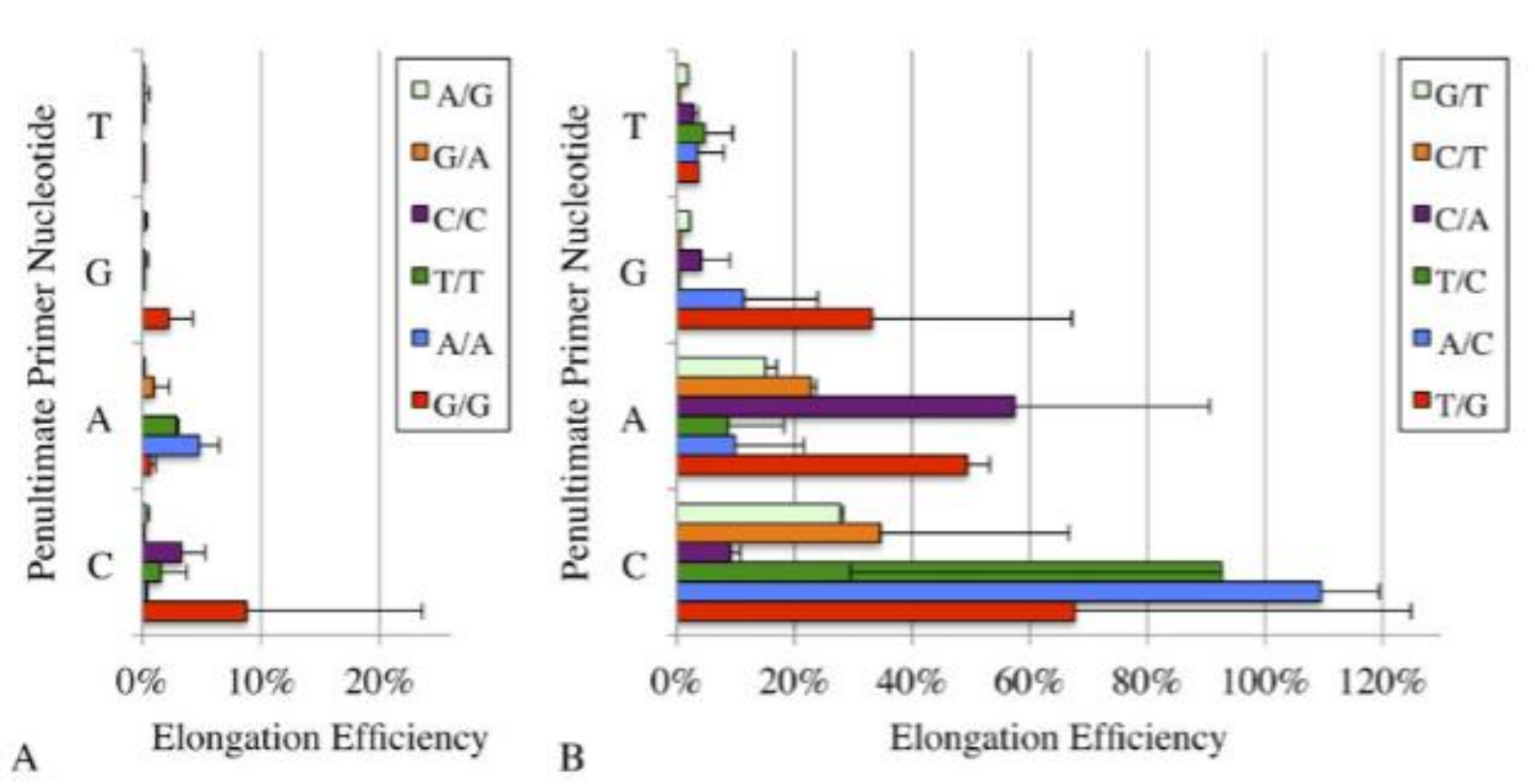


Effect of 3’-terminal mismatch on elongation efficiency

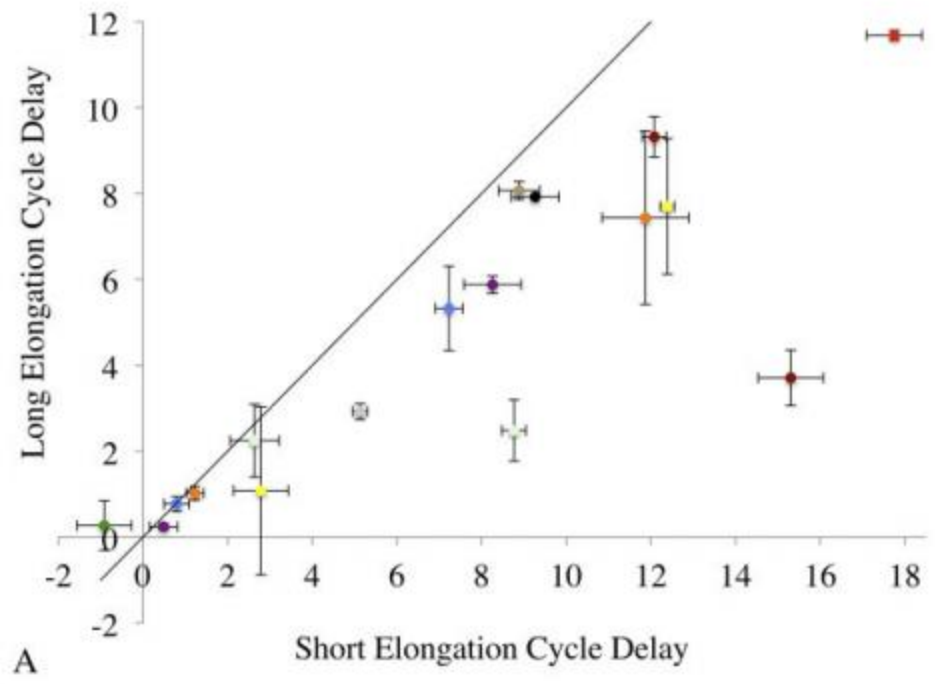


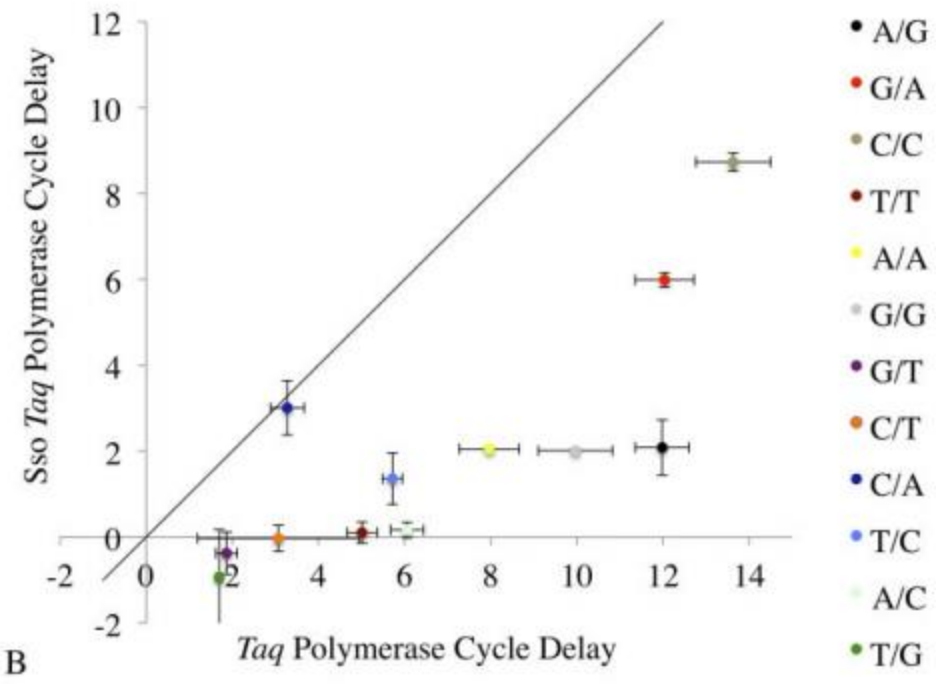
	A/G	G/A	C/C	T/T	A/A	G/G	G/T	C/T	C/A	T/C	A/C	T/G
Ayyadevara *et al.* (2000)	+	+	+	+	+	+	++	+	+	+	+	+
Day *et al.* (1999)	+	++	++	++	+	++	+++	++	++	++	+++	++
Huang *et al.* (1992)	–	–	–	+	+	–	++	+++	++	++	++	++
Kwok *et al.* (1990)	+	+	+	+++	++	+++	+++	+++	+++	+++	+++	+++
Li *et al.* (2004)	+	+	+	+++	+	+	+++	+++	+++	+++	+++	+++
Stadhouders *et al.* (2010)	+	+	+	++	+	+	+++	++	+++	++	+++	+++
Wu *et al.* (2009)		–			–				–			

Dependence of terminal mismatch elongation efficiency on neighbouring nucleotide

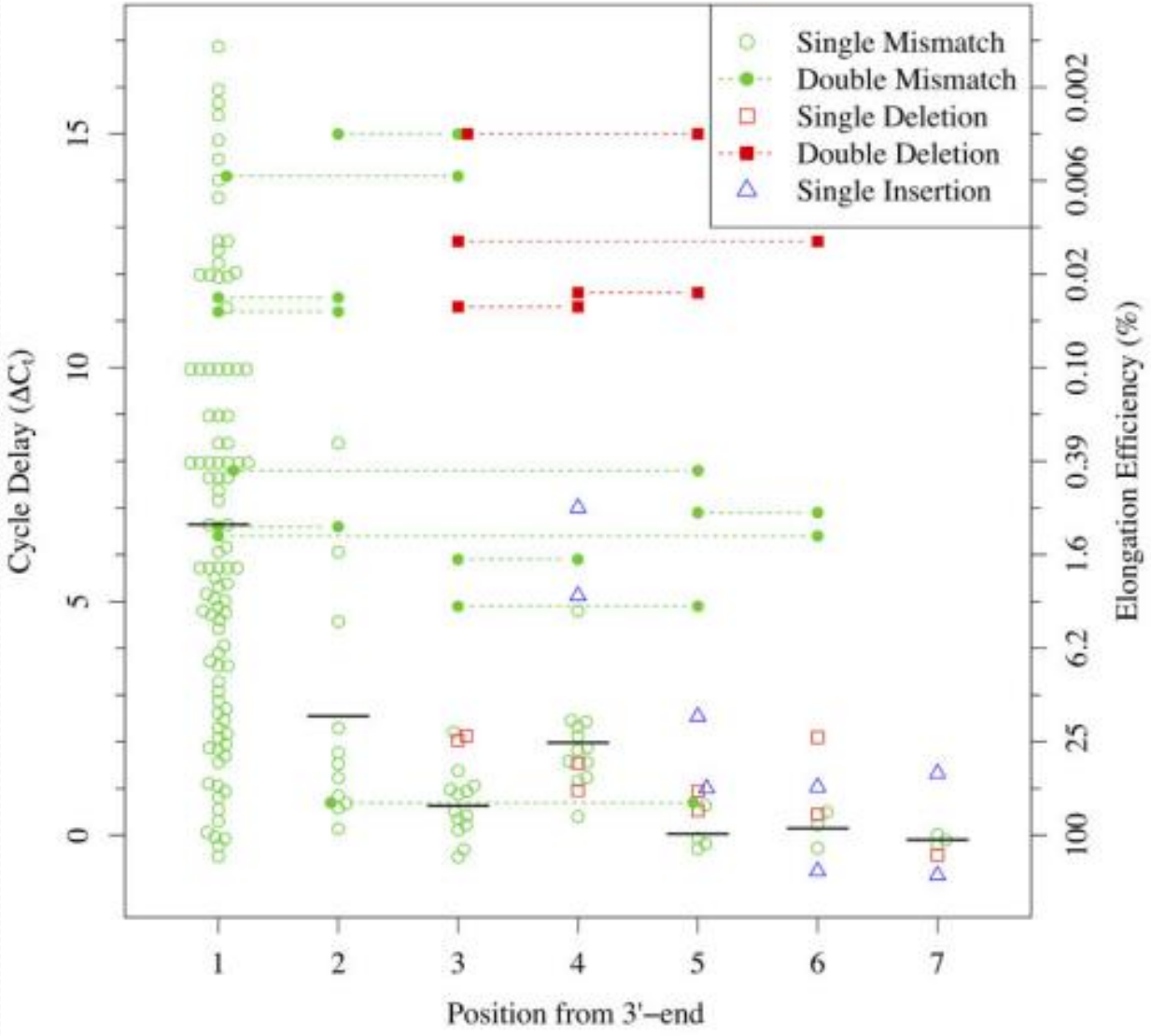


Elongation time (10 s vs 180 s) and type of polymerase affect the elongation efficiency of 3’-terminal mismatches





The effects of mismatches, insertions and deletions near the 3’-terminus on elongation efficiency



4 primer design strategies for discrimination of a single nucleotide difference (SNP):

1. strategy: Primers with mismatch in the centre of F or R primer (negative effect on Eff_{hyb} (but not on Eff_{elong} of nontarget))

-> cycle delays 1,4 - 3,4 nontarget vs target allele

2. strategy: mismatch at the 3'-terminus of F or R primer

-> cycle delays 2,2 – 7,6 (effect on elongation efficiency)

3. strategy: mismatch at the 3'-terminus + additional induced mismatch to both wild type and SNP alleles at the 6th position (interior mismatch slightly decrease Eff_{hyb}, but not Eff_{elong} of target allele, but double mm affect Eff_{elong} of nontarget)

-> cycle delays 11,6 – 12,7 cycles

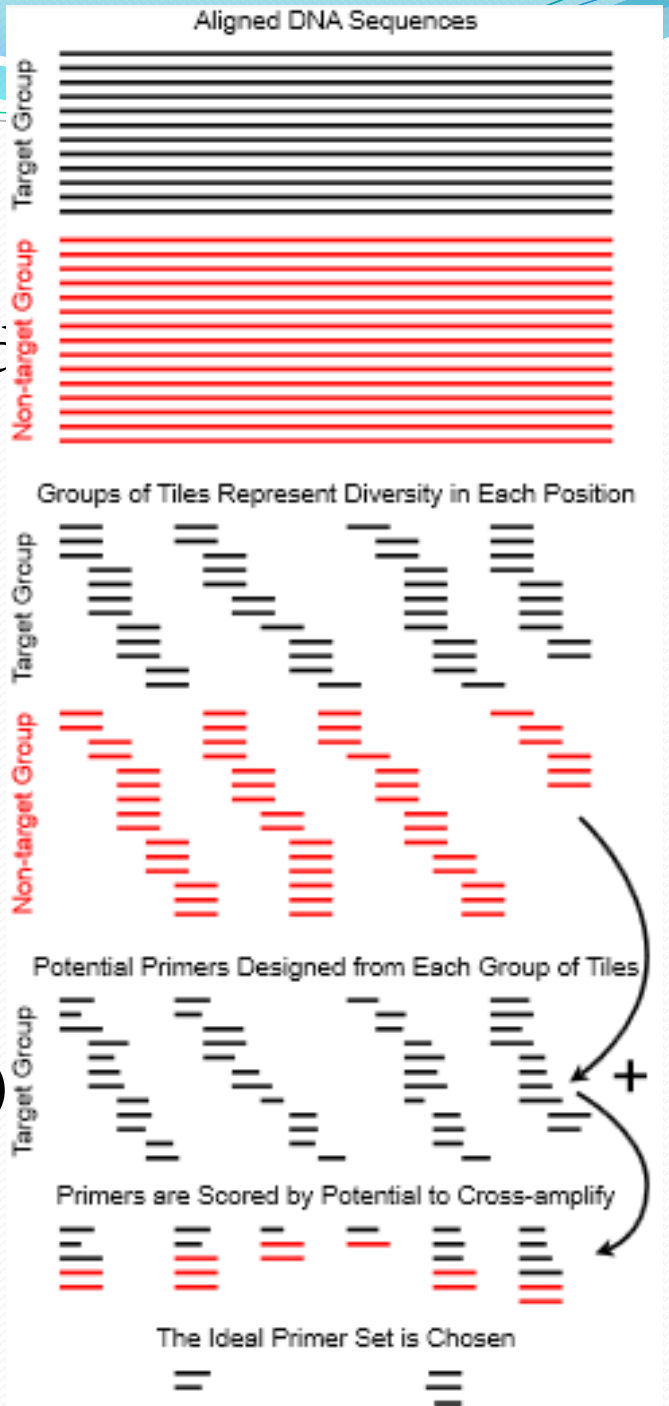
4. strategy: mismatch at the 3'-teminus + additional induced mismatch in the penultimate (2.) position

-> cycle delays 9,0 – 13,7 cycles

Lowering the time given for elongation (30s -> 5s) -> increased cycle delays.

Incorporating of elongation efficiency into primer design: Design Primers (Decipher package)

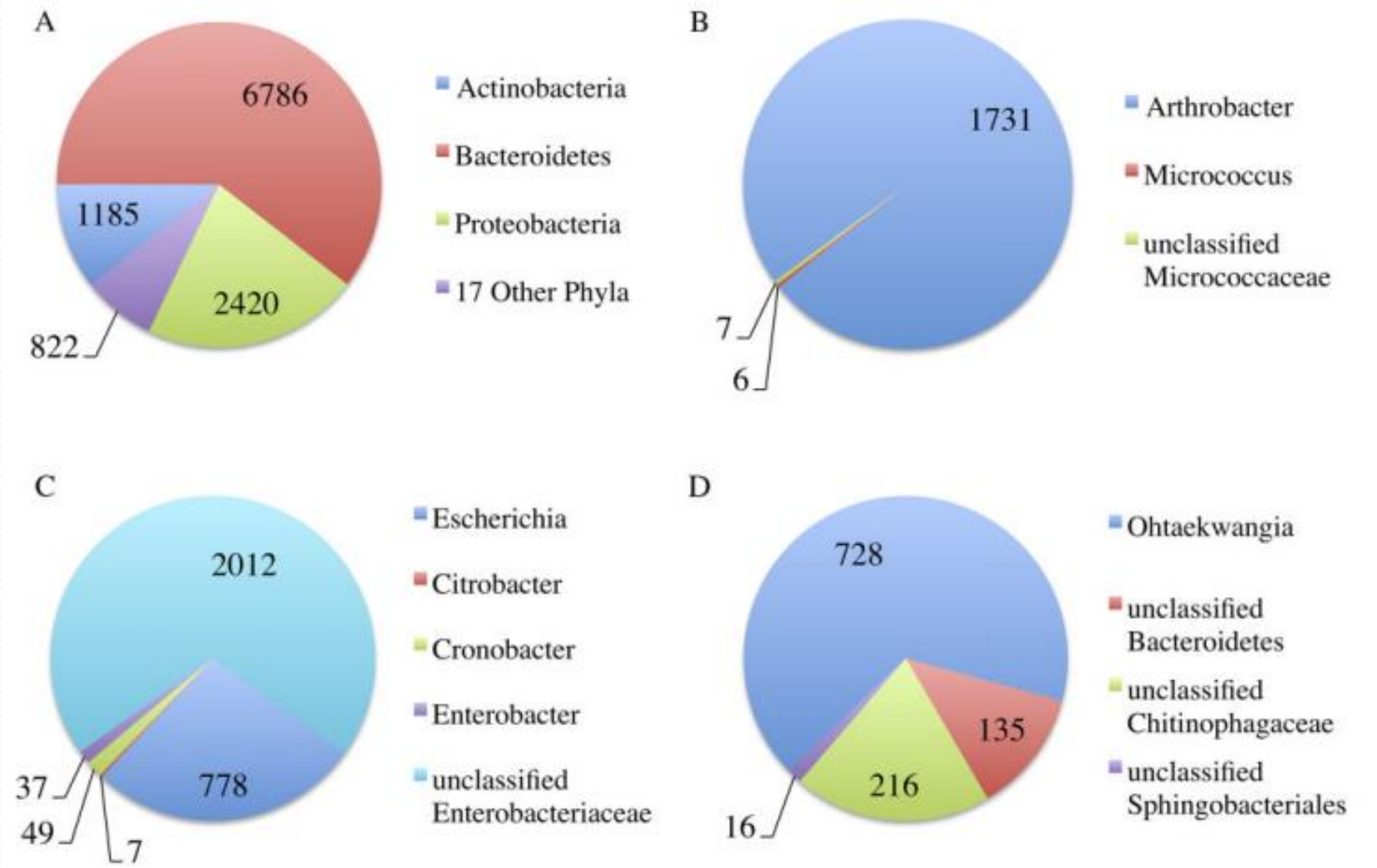
- A file of aligned sequences are classified into target and nontarget group
- A set of overlapping k-mers („tiles“) is formed – possible target sites
- A set of potential primers is designed each tiles (pr. length with minimum Eff_{hyb} at the annealing temp.)
- Candidate primers are scored by their ability to result in a false positive hybridization (amplification efficiency)
- The optimal set of F and R primers chosen to minimize false positive overlap.



Experimental validation of the designed primers (with Design Primers)

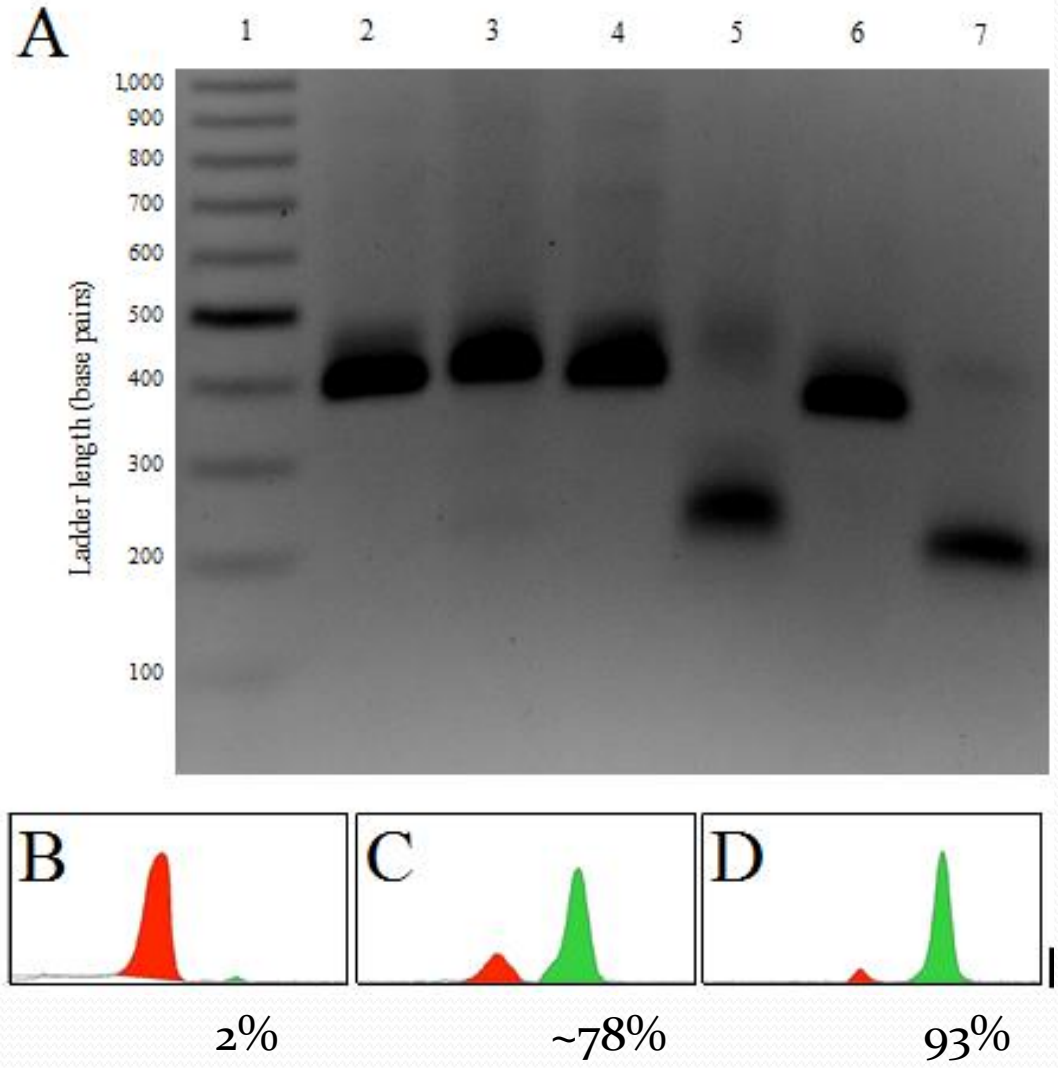
- Design of genus-specific primers with Design Primers (length 17-26, amplicon size 300-1200 nt) for 1834 bacterial and 109 archeal genera (1 696 150 16S rRNA sequences in the Ribosomal Database Project (RDP)
  - With strategy 3: no predicted cross-amplification for 66% of genera and <5 cross-amp. for 85% genera
- DNA extracted from water sample (collected from the Sacramento Delta in California)
- Universal 16S primers and 454 pyrosequencing, RDP classifier -> microbial community composition
- 4 genus specific primers (strategy 2) -> pyrosequencing:
  - Bacteroidetes (60%): Emticicia (20/11379, Ohtaekwangia (16/11379)
  - Proteobacteria (21%): Escherichia (1/11379)
  - Actinobacteria (10%): Arthobacter (1/11379)

Experimental validation of the designed primers (with strategy 2)



Comparison of three different design strategies for targeting *Ohtaekwangia* sequences

Gel runs of PCR products before and after digestion with the restriction enzyme HinfI, which cuts near the center of *Ohtaekwangia* amplicons.
Lane 2-3, B: strategy 1
Lane 4-5, C: strategy 2
Lane 6-7, D: strategy 3



Conclusions

- Accounting for the roles of **sequence context, elongation time and polymerase type** in determining elongation efficiency of terminal mismatches -> rational choices for designing specific primers
- Partitioning amplification efficiency into hybridization and elongation efficiencies enables to determine the **positional effect of mismatches, indels** on elongation efficiency
- Inducing a mismatch to the target template at the 6th position was preferable to the 2nd position where it might affect elongation efficiency of target amplicon
- Extension parameters can be integrated into a primer design algorithm to further increase specificity over hybridization efficiency alone.

Thanks for listening!