qPCR in forensic DNA analysis

Johannes Hedman

Researcher, Applied Microbiology, Lund University

Specialist, Swedish National Laboratory of Forensic Science

Forensic science

”Every contact leaves a trace”

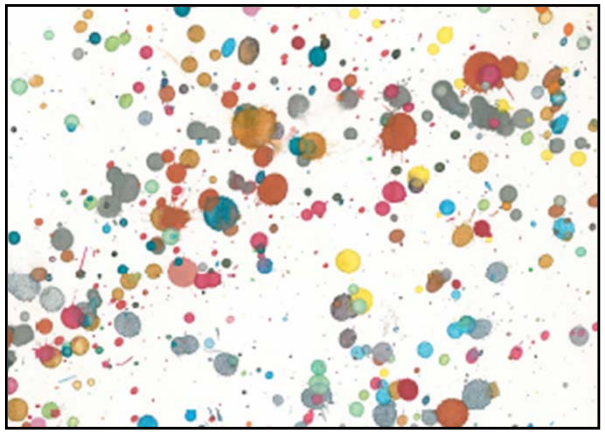
Edmond Locard (1877-1966)

Forensic science in Sweden:

Harry Söderman (1902-1956)

What could serve as biological evidence from a crime scene?

Anything!



All tissue types

Foods

Cans and bottles

Clothes

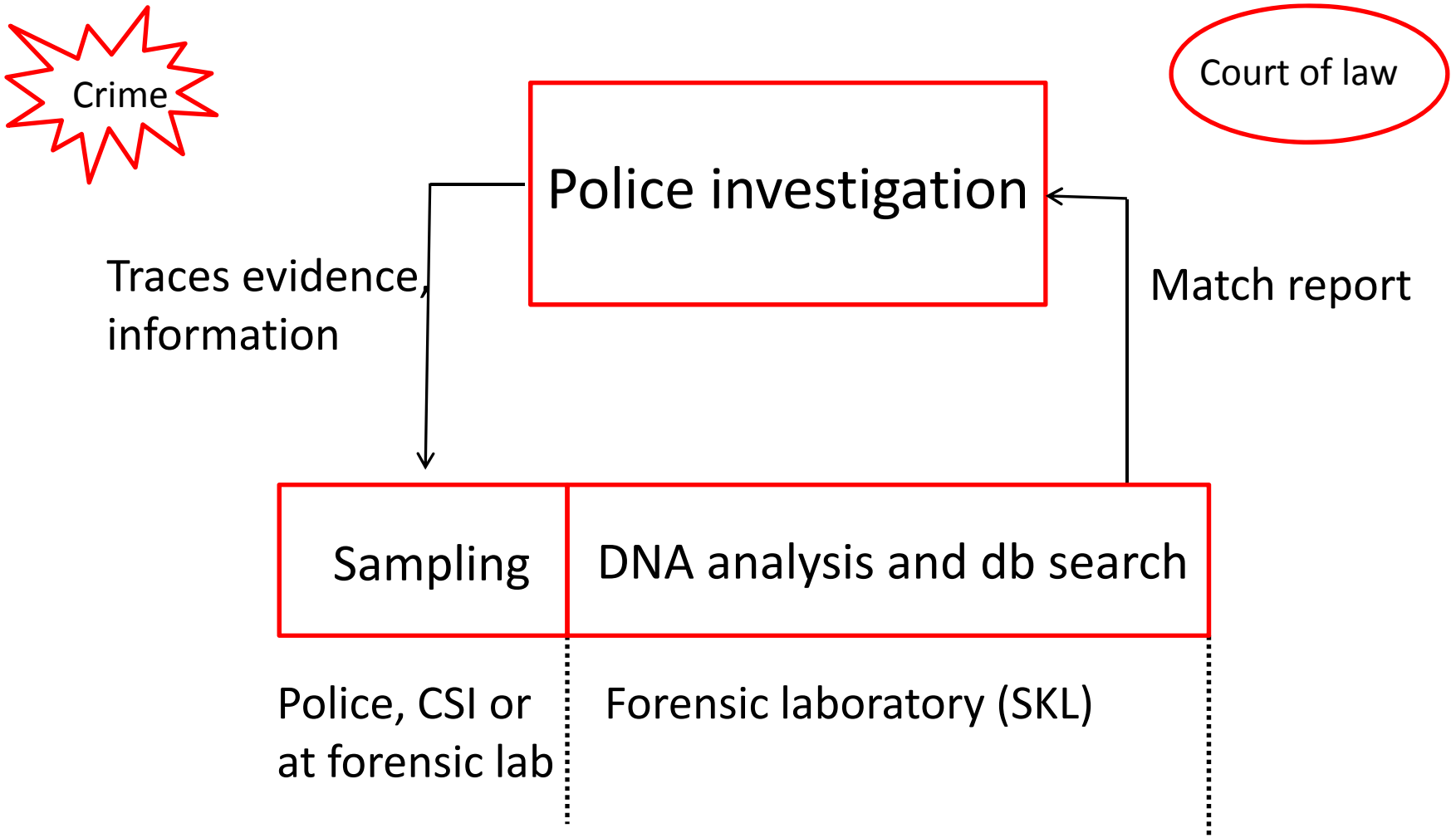
Tobacco products

Weapons and cartridges

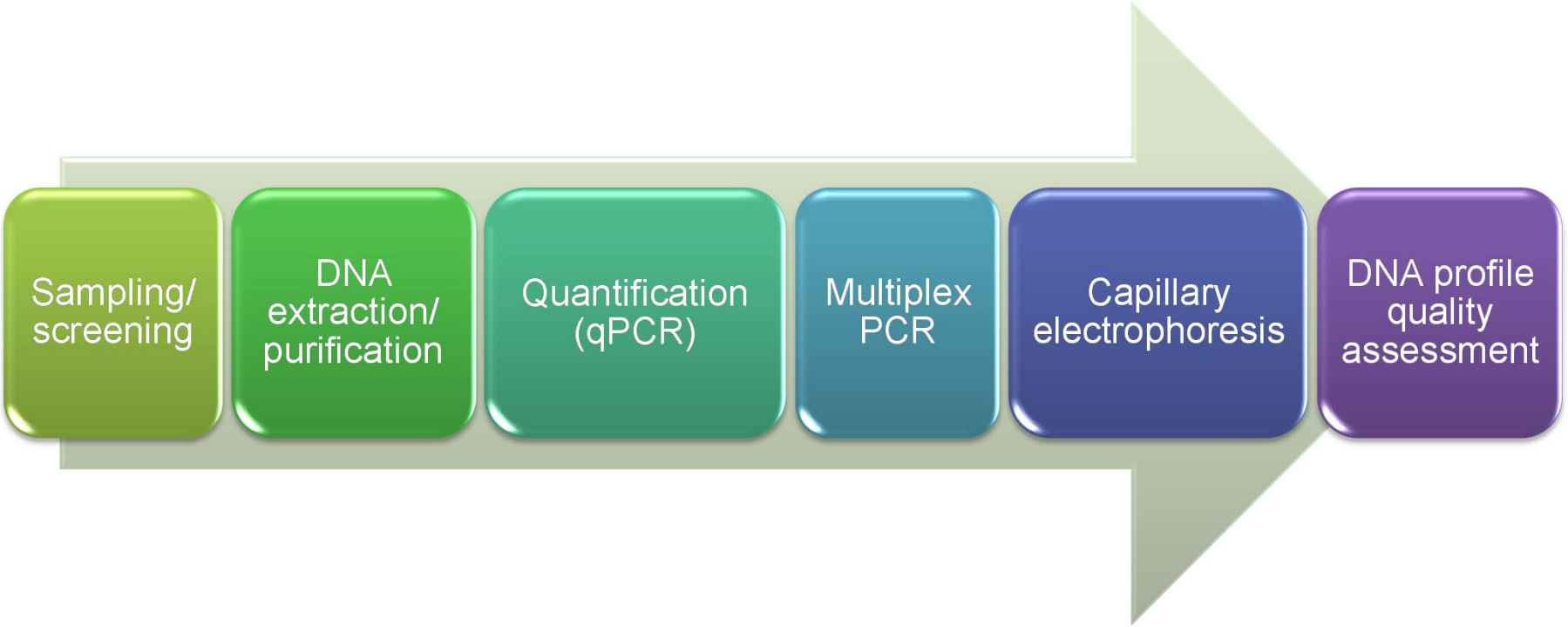
Challenges

- Heterogeneous samples
- Low amounts of cells/DNA of varying quality
- Impurities (PCR inhibitors)
- DNA mixtures

Forensic DNA analysis



Workflow in forensic DNA analysis



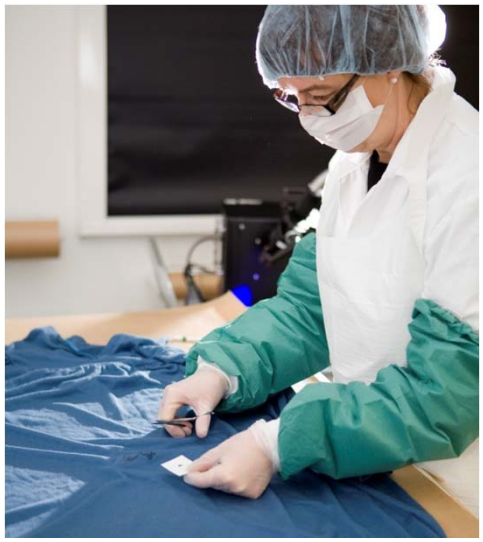
Sampling

Finding and identifying stains

Protein based tests

Tissue specific enzymes

Reaction=> colour change



Light source

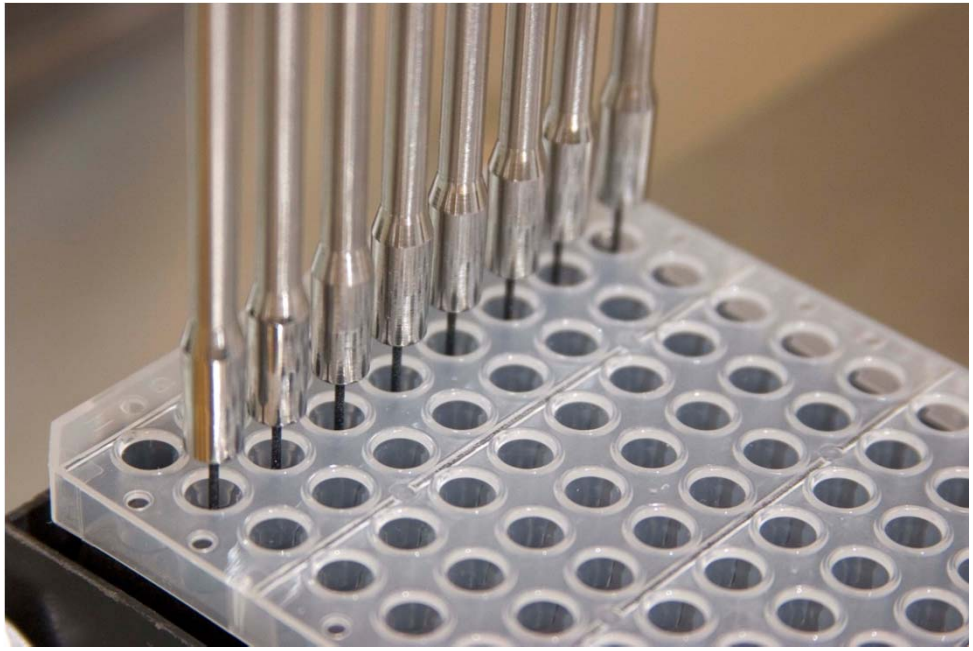
Fluorescense from body fluids (eg. proteins)

Sampling

Swabbing, cutting, tapeing

DNA extraction/purification

Manual and automated methods



qPCR in forensic DNA analysis

- Quantification used for normalisation of DNA profiling PCR (Short tandem repeats, STR)
- Control of amplifiability (IAC)
- Indication of DNA degradation
- Commercial kits using hydrolysis probes (TaqMan)

Short tandem repeats (STR)

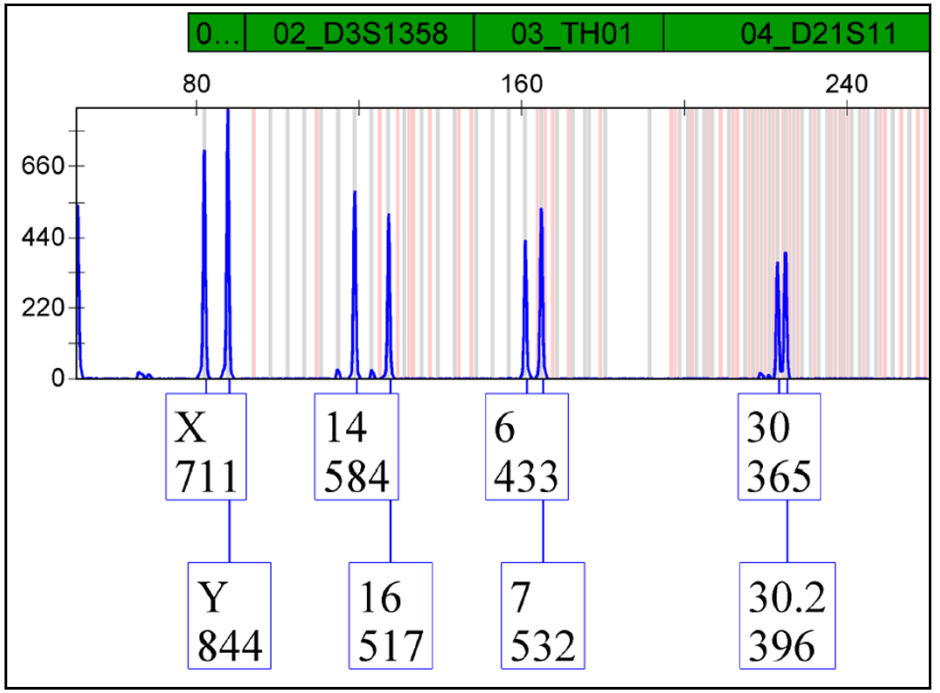
Allele: 6 (six repetitions)

Allele: 8 (8 repetitions)

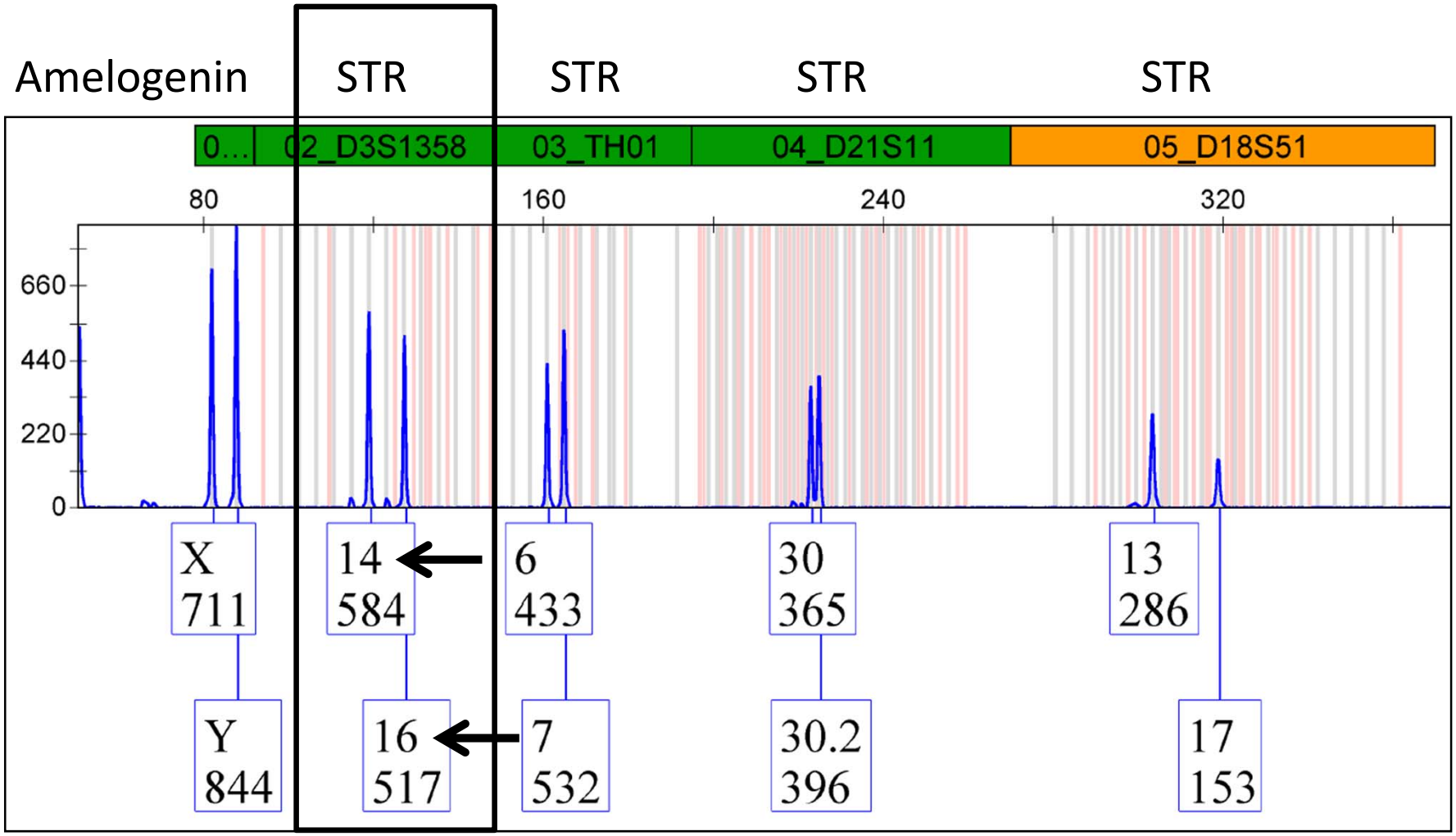
- Standard forensic DNA profiling: 15 tri/tetranucleotide STRs
- Multiplex PCR (parallel amplification and detection)

DNA profile generation

Capillary gel electrophoresis and software

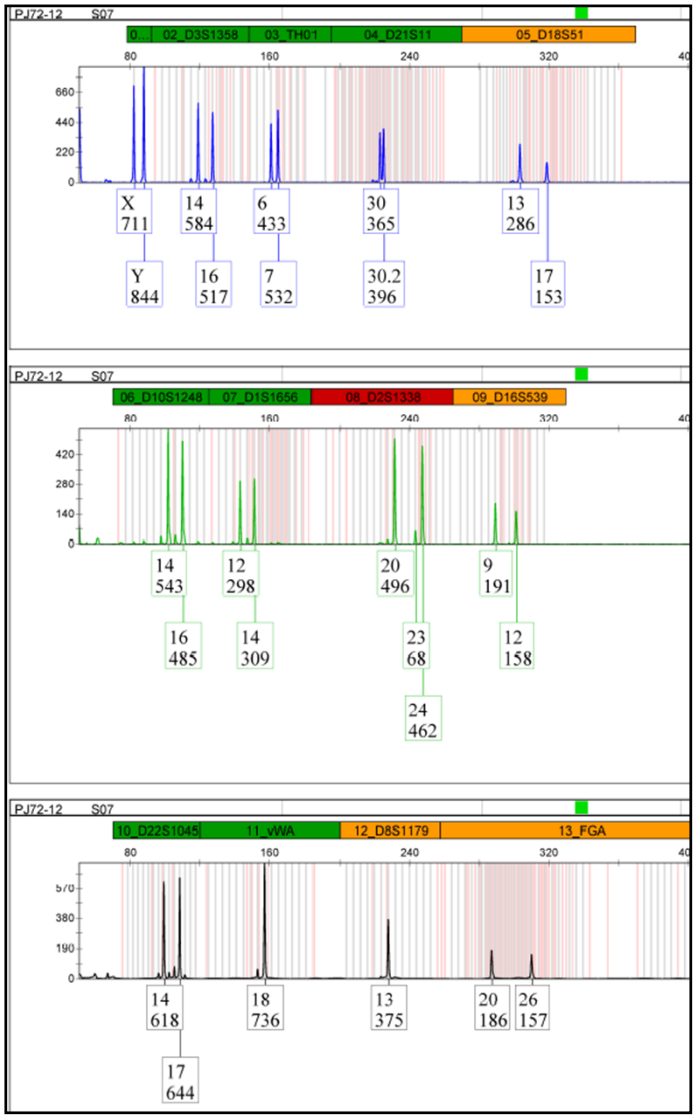


Short tandem repeat (STR) profile



Forensic DNA profiling

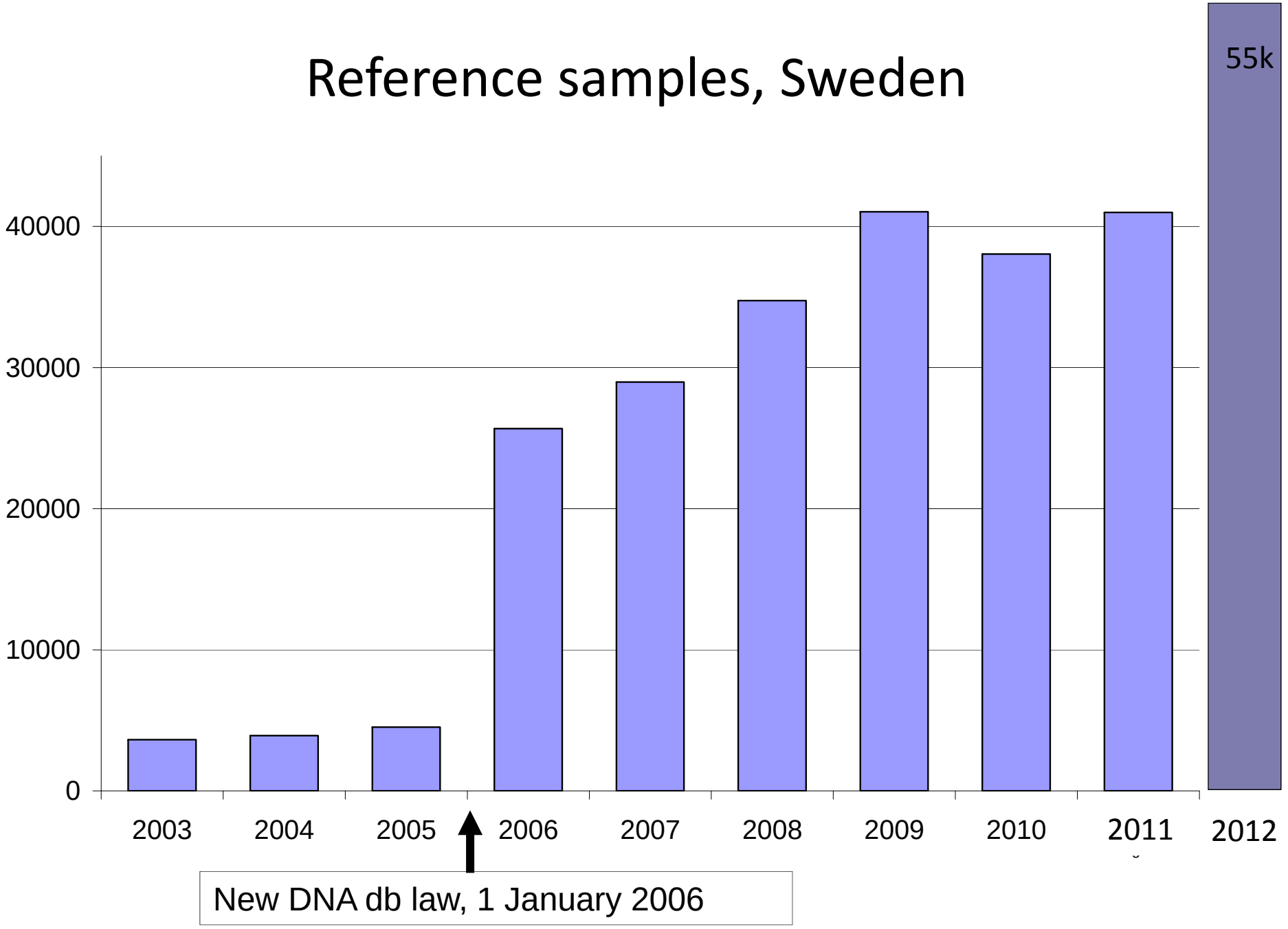
- Complete profiles from ca 150-200 pg DNA (ca 25-30 human cells)
- Separation by fragment size and fluorophore
- One base-pair resolution
- 96-well plate format
- Automated processes



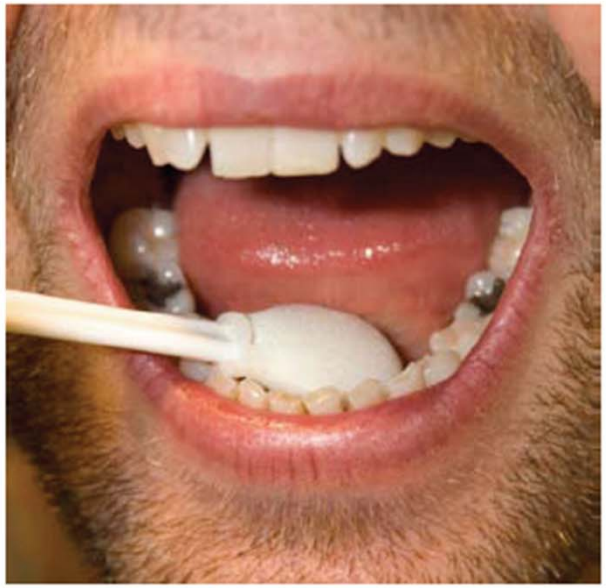
Comparison of DNA profiles
(suspect vs crime scene sample)

STR marker:	D3	vWa	D16	D2	D8	D21	D18	D19	TH01	FGA
DNA profile of suspect:	14/15	17	10/12	20/21	14	14/16	9/10	17/21	7/9	22
DNA profile from cig. butt found on crime scene	14/15	17	10/12	20/21	14	14/16	9/10	17/21	7/9	22

Reference samples, Sweden



Forensic reference samples



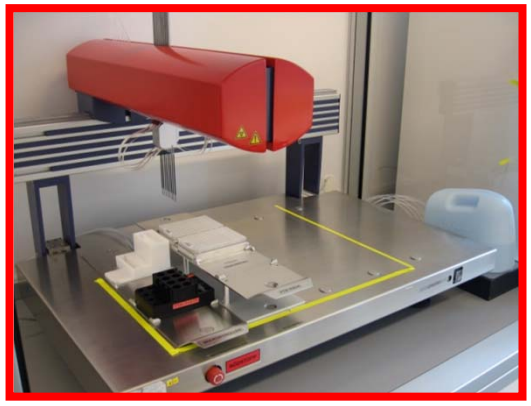
Vänster Bild 1: Provtagningspinnen gnuggas mot insidan av kinden.

Höger Bild 2: Skumgummit dras under tungan och saliv samlas upp.

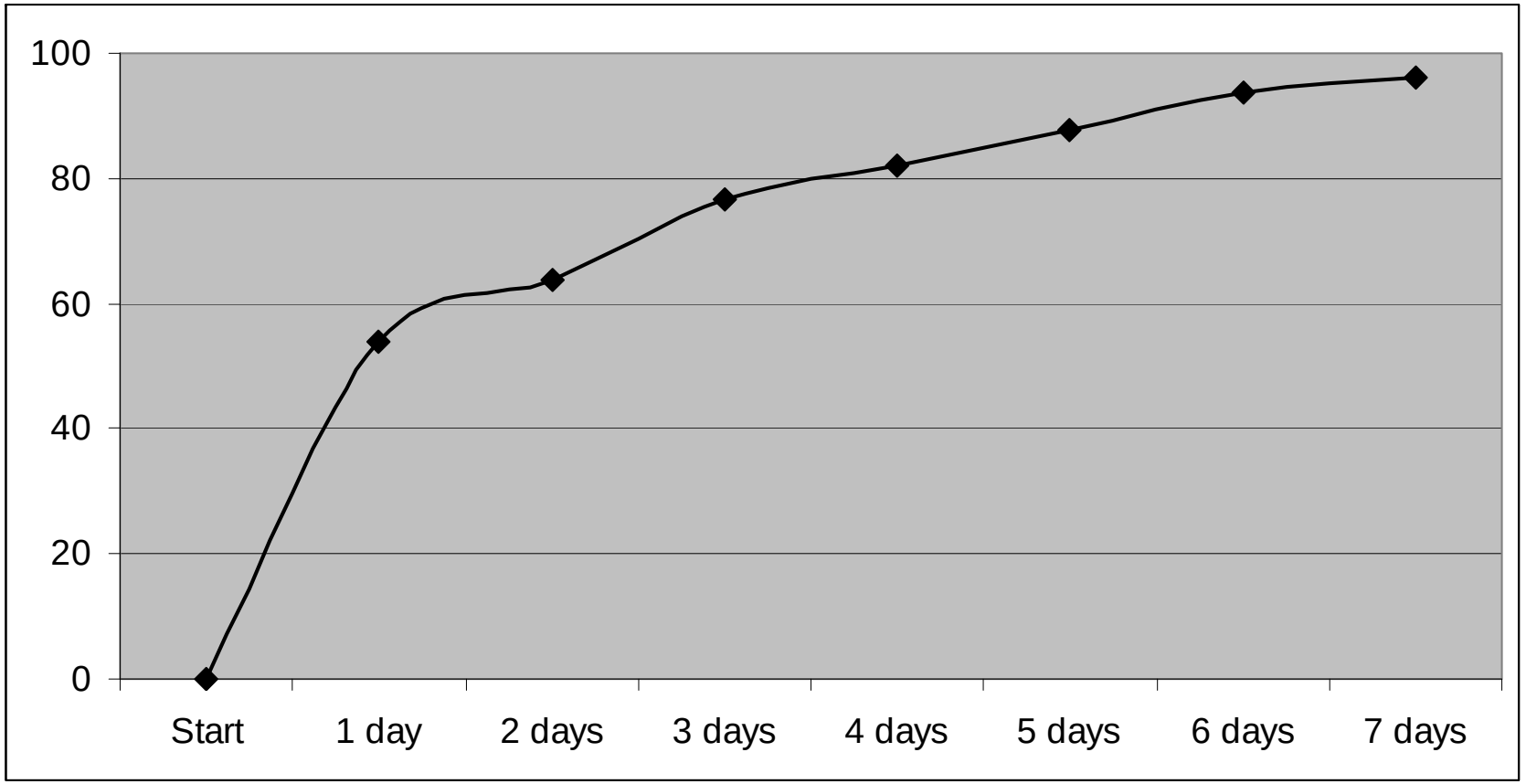
Buccal swab cells transfered to FTA paper

Punch from paper used in PCR

Semi-automated DNA analysis



Approved reference sample profiles (%)



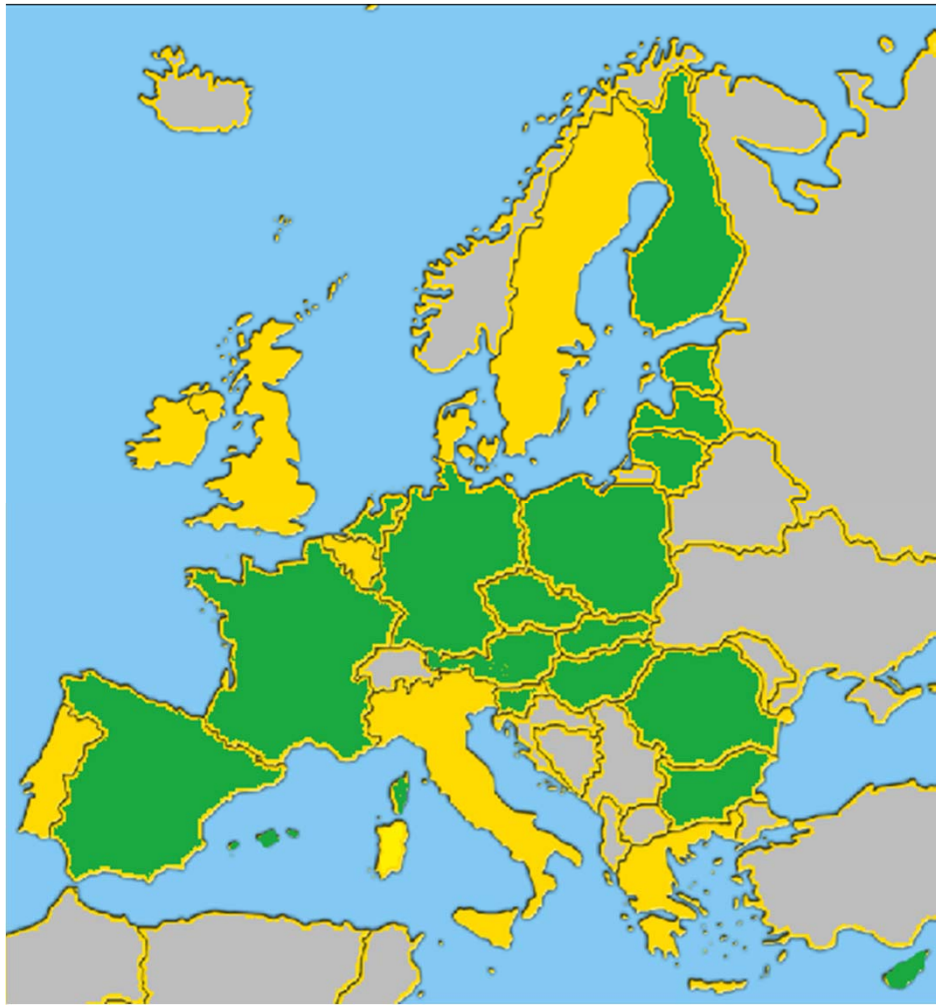
+ 1 day: Profile searched against national DNA db,
hit reports generated, suspect profiles loaded onto DNA db

National DNA databases

Sverige:	ca 130 000 persons (1.4%)
Storbritannien:	ca 6 million (9%)
USA:	ca 12 million (3.5%)
Kina:	ca 16 million (1%)
UAE:	Aim: 100%

Exchange of DNA profile information: Prüm treaty

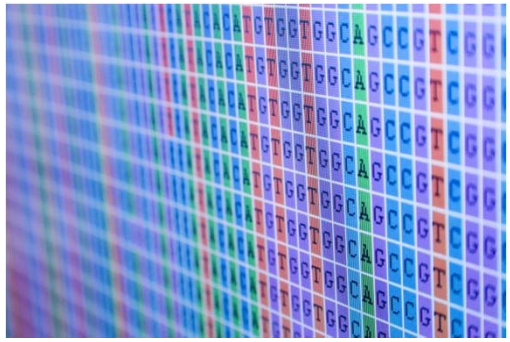
Operational countries



Sweden exhanges with:
Netherlands
Finland
Poland (today 8 oct!)

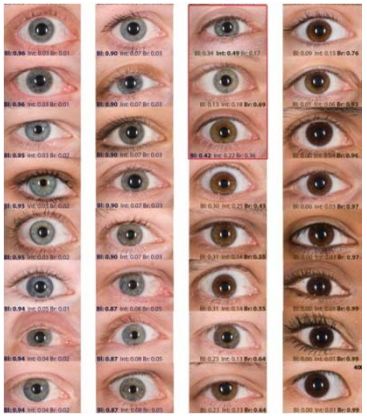
Soon:
Lithuania
Slovakia

Coming methods



”Next generation sequencing”

Eg complex mixtures



Visible characteristics

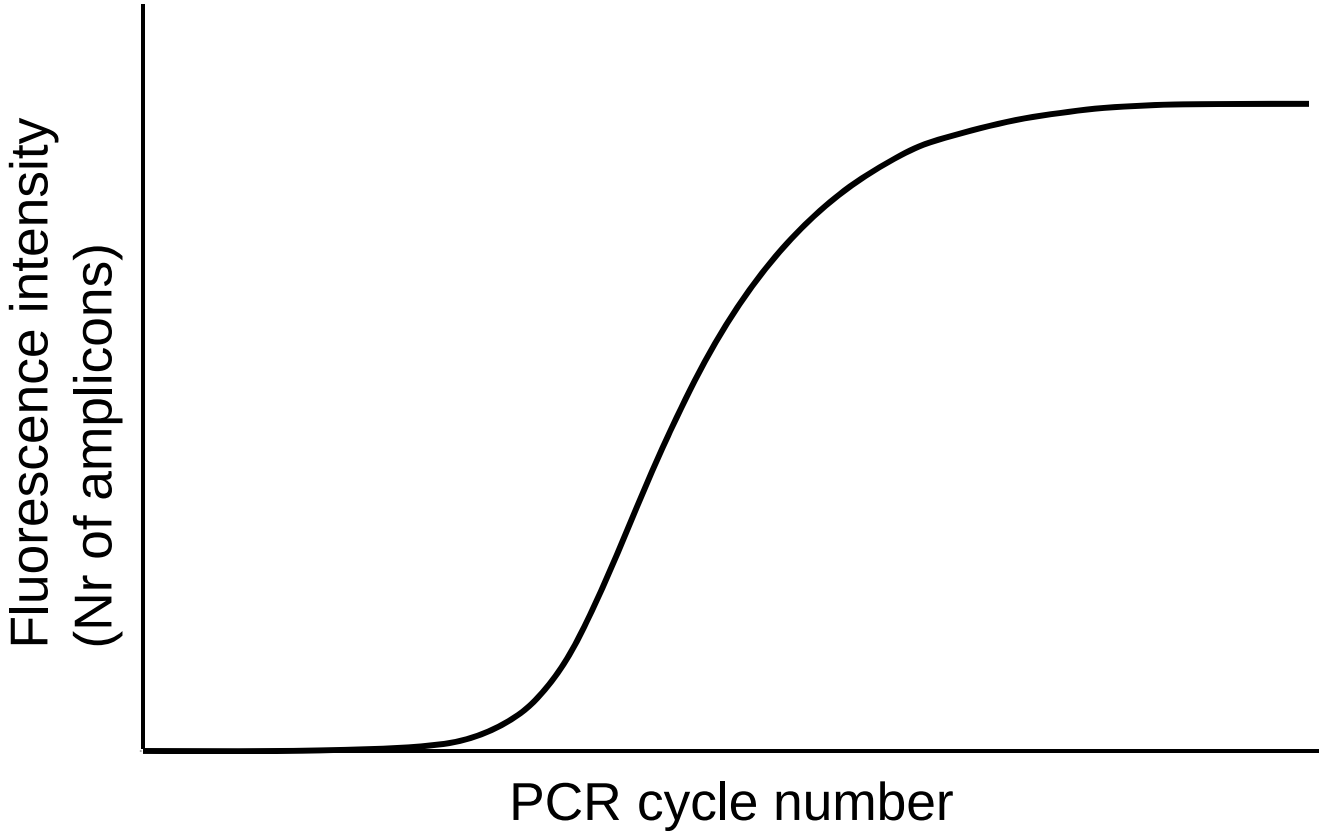
Hair colour, eye colour etc

Quick analysis

”Lab-on-a-truck” rather than
”lab on-a-chip”

qPCR: Kinetics and quality control

Monitor amplification



qPCR detection principles

Fluorescence detection during amplification

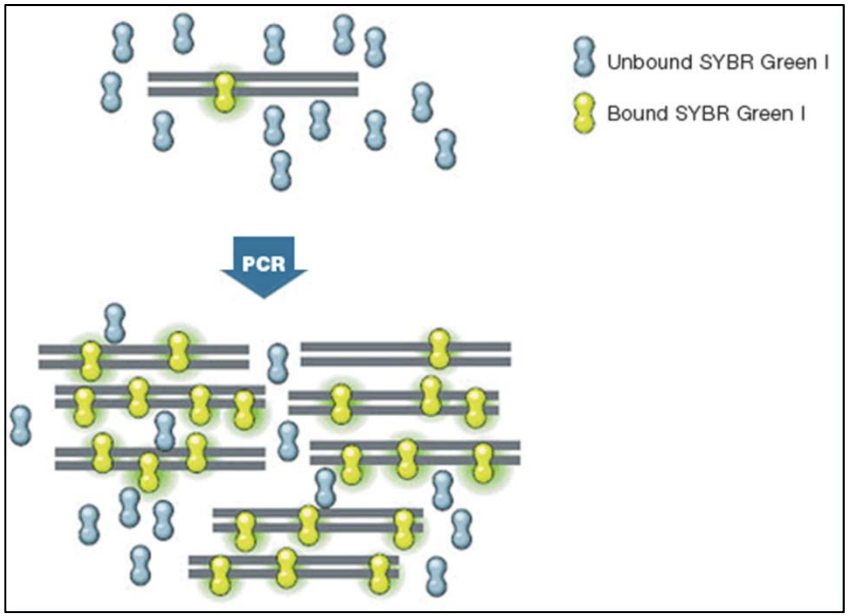
- Dyes binding to dsDNA
- Labelled probes
- Labelled primers

SYBR Green dye

Most commonly used dye

Excitation max: 497 nm
Emittance max: 520 nm

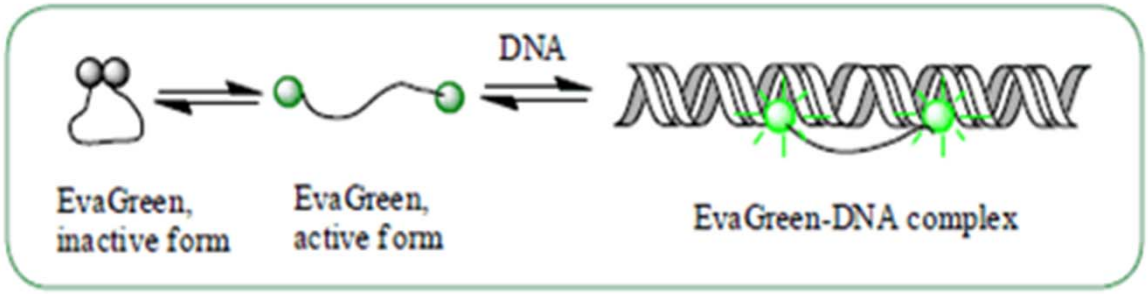
Strong fluorescence increase when bound to dsDNA



SYBR Green disturbs PCR at high concentrations, due to strong binding to dsDNA (intercalation) and inhibition of DNA polymerase

⇨ Cannot saturate reaction

EvaGreen dye



Excitation max: ca 500 nm
Emittance max: ca 530 nm

Strong fluorescence increase when bound to dsDNA

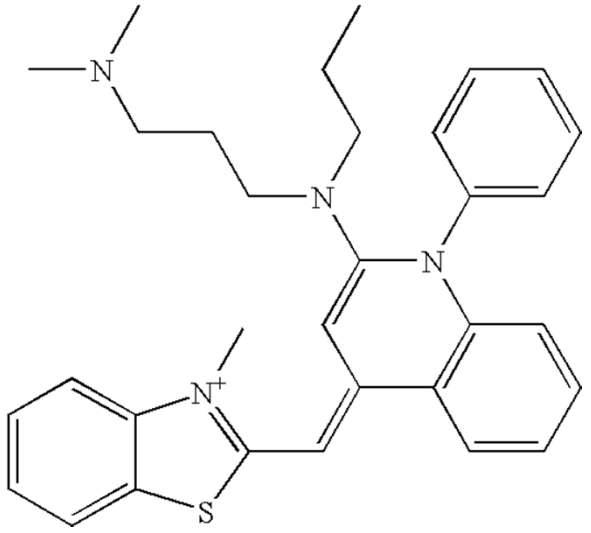
Lower affinity for dsDNA compared to SYBR Green

Less PCR inhibitory ⟹ possible to add ca 3 times more dye and (maybe) reach saturation

Molecular structures of SYBR Green and EvaGreen

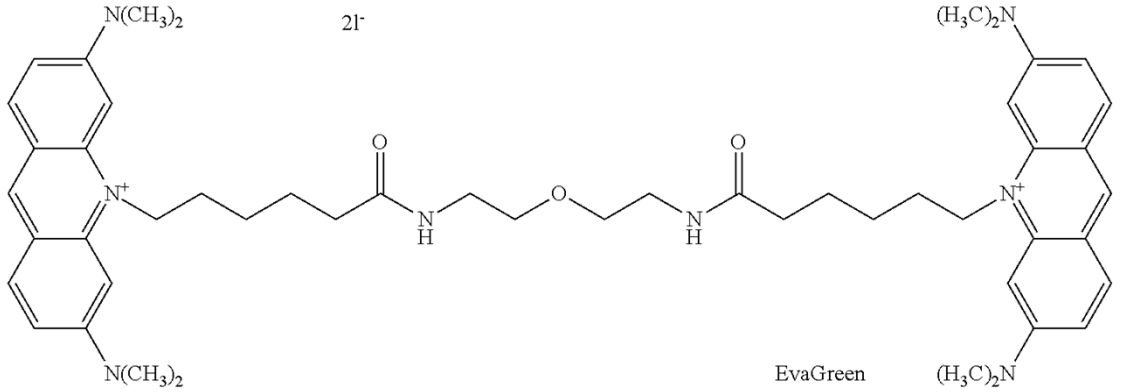
SYBR Green I

- asymmetrical cyanine dye



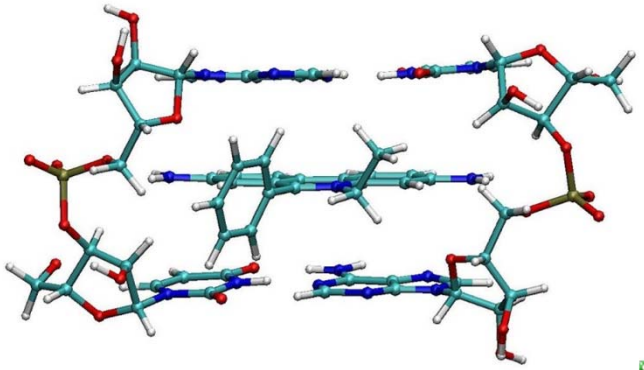
EvaGreen

- symmetrical cyanine dye

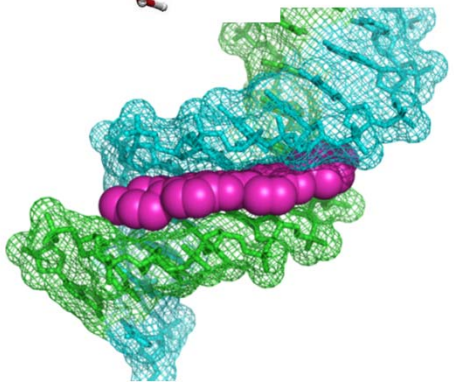


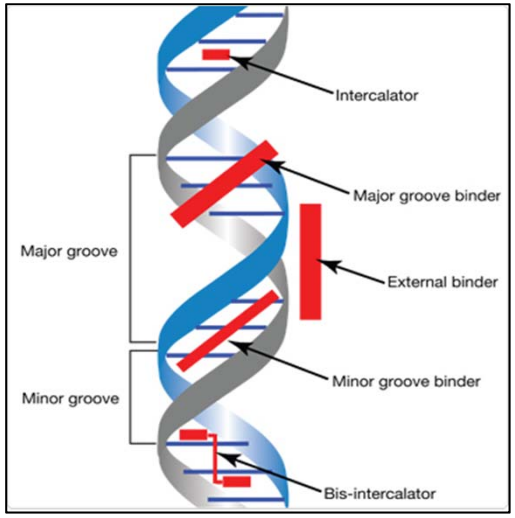
Intercalation vs minor groove binding

Intercalation



Minor groove binding

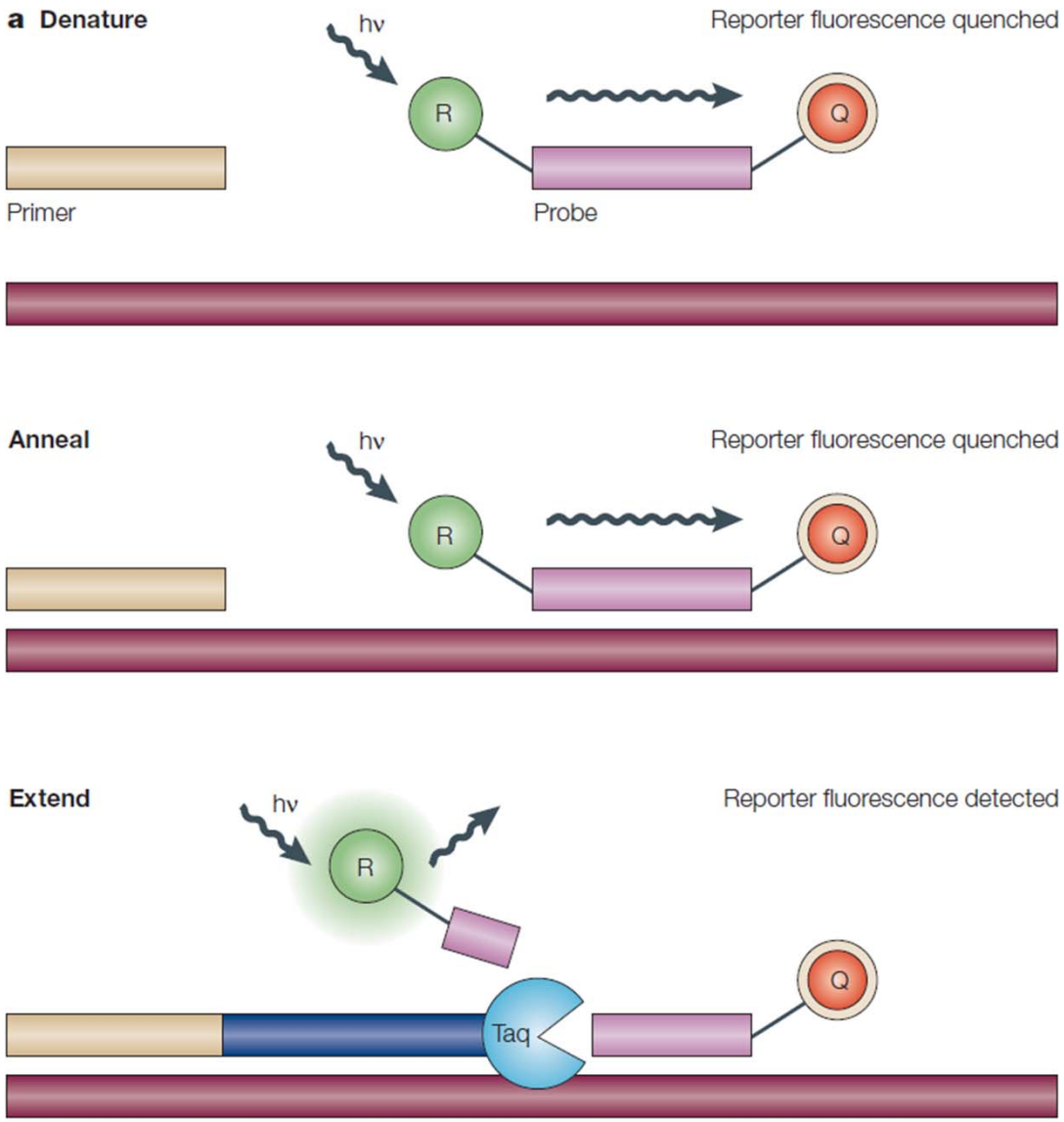




qPCR dyes probably bind dsDNA in more than one fashion

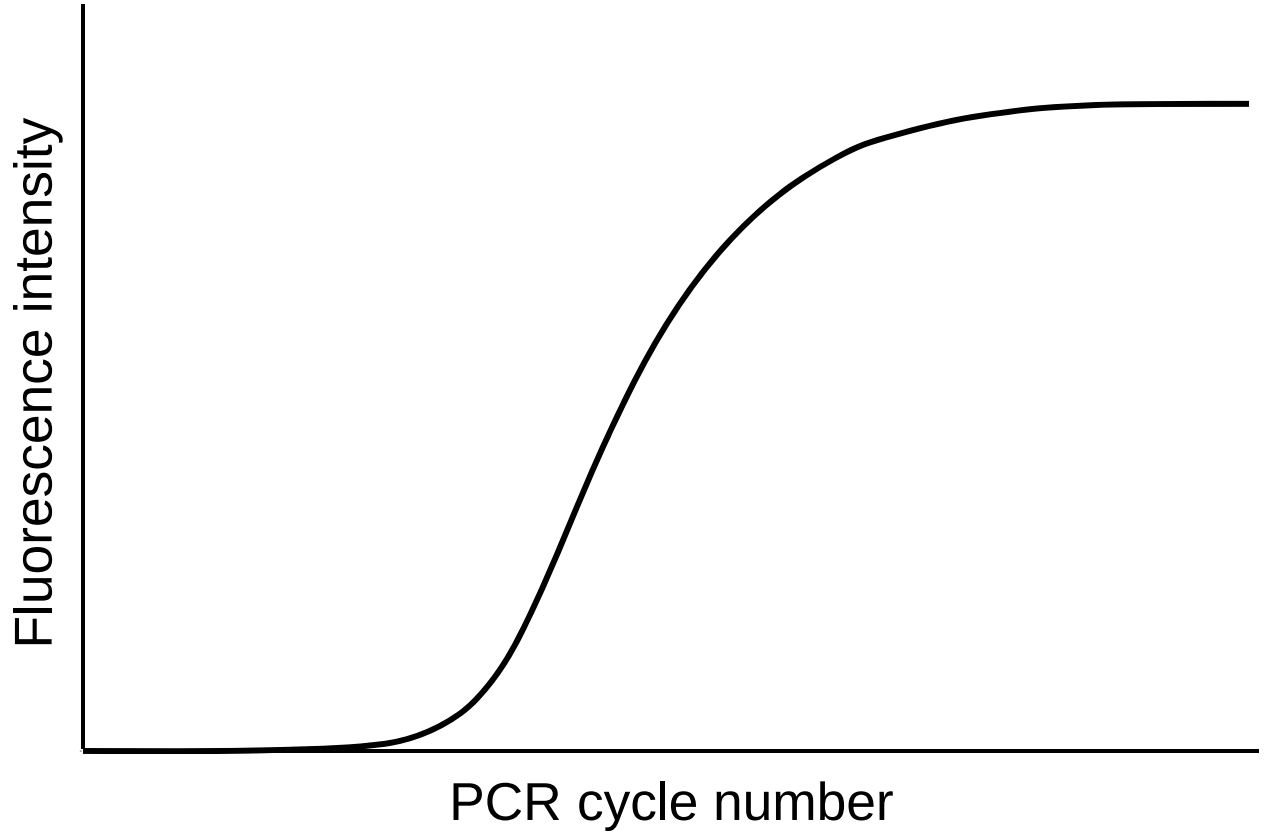
Hydrolysis probe

(TaqMan)



www.nature.com

Determining the quantification cycle (Cq)



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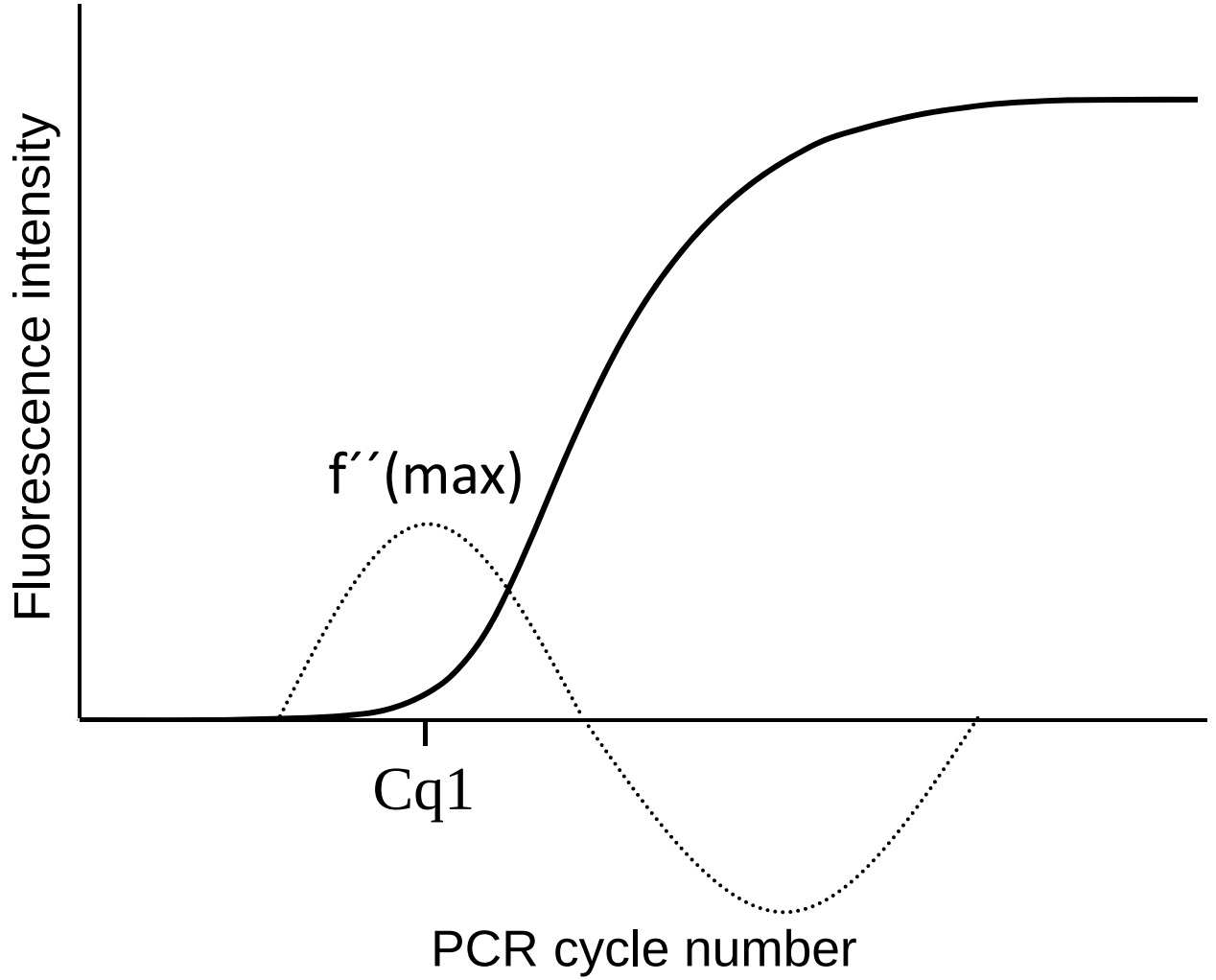

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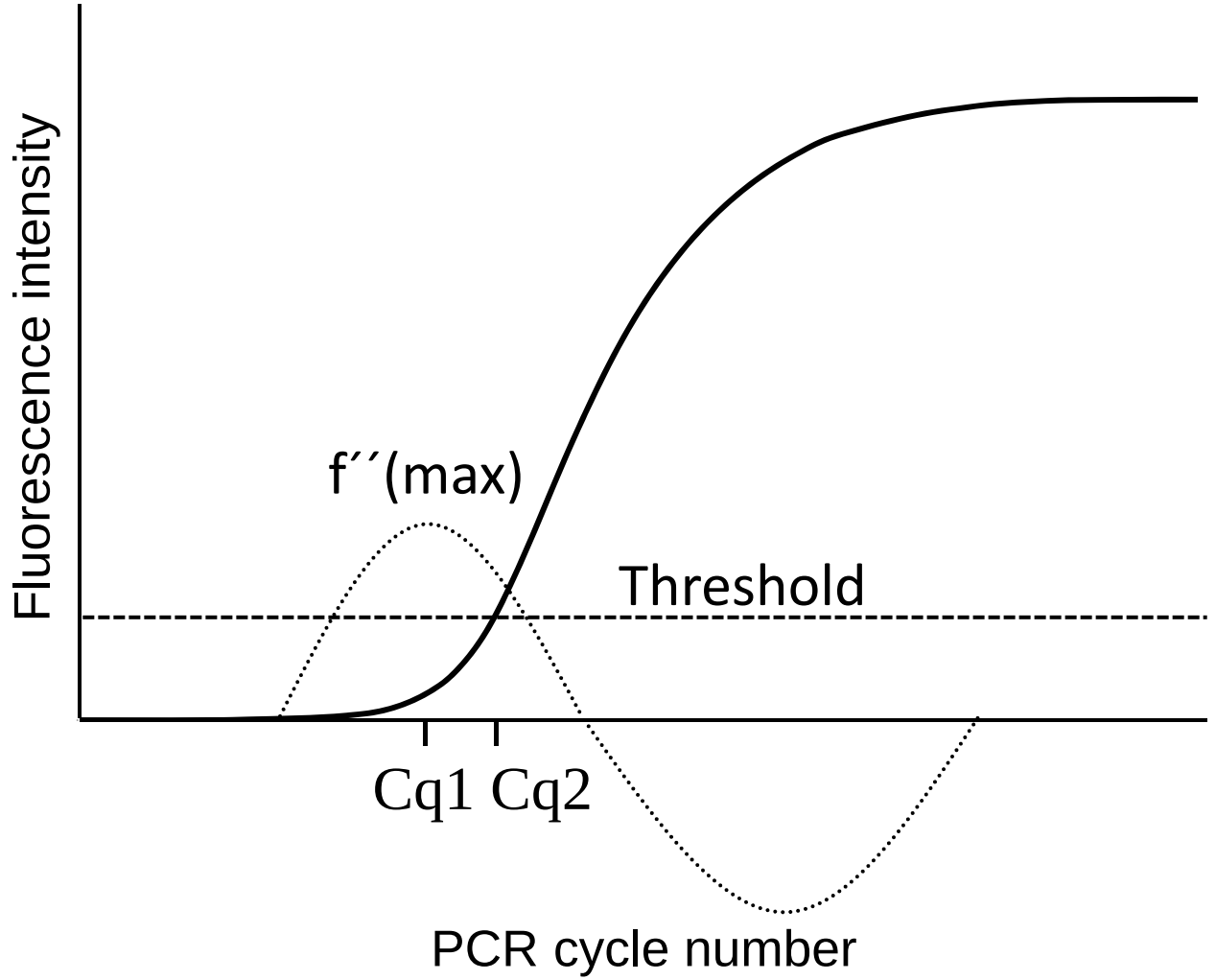


Quality control in qPCR

- PCR control or process control
- Internal or external control

Quality control in qPCR

- Internal Amplification Control (IAC)
- Kinetic Outlier Detection (KOD)

Internal Amplification Control (IAC)

- ”Alien” DNA added in known amount present in reaction
- Monitors PCR success (controlling inhibition, avoiding false negatives)
- Strongly recommended in diagnostic qPCR[1]

Requirements on IAC?

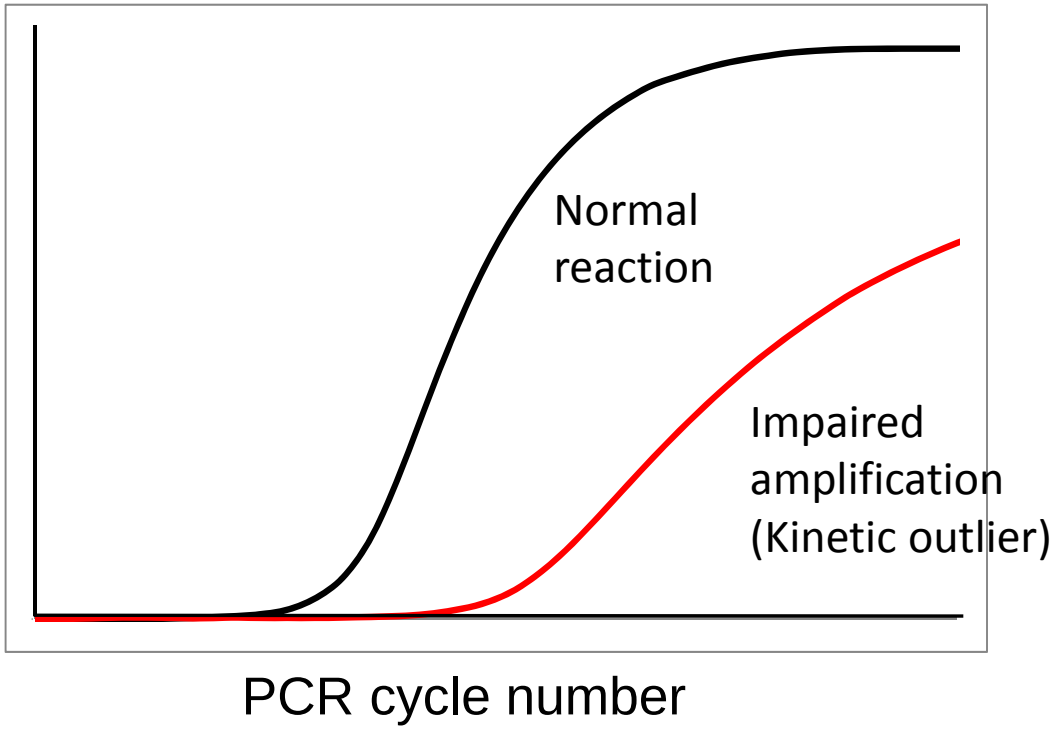
1) Hoorfar, J., N. Cook, et al. (2003). "Making internal amplification control mandatory for diagnostic PCR." J Clin Microbiol **41**(12): 5835-5835.

IAC requirements

- Preferably same primers as target, to ensure similar inhibitory effects
- Low amount, not to compete with target amplification
- Same length or longer than target
- Should be more easily affected by inhibitors compared to target

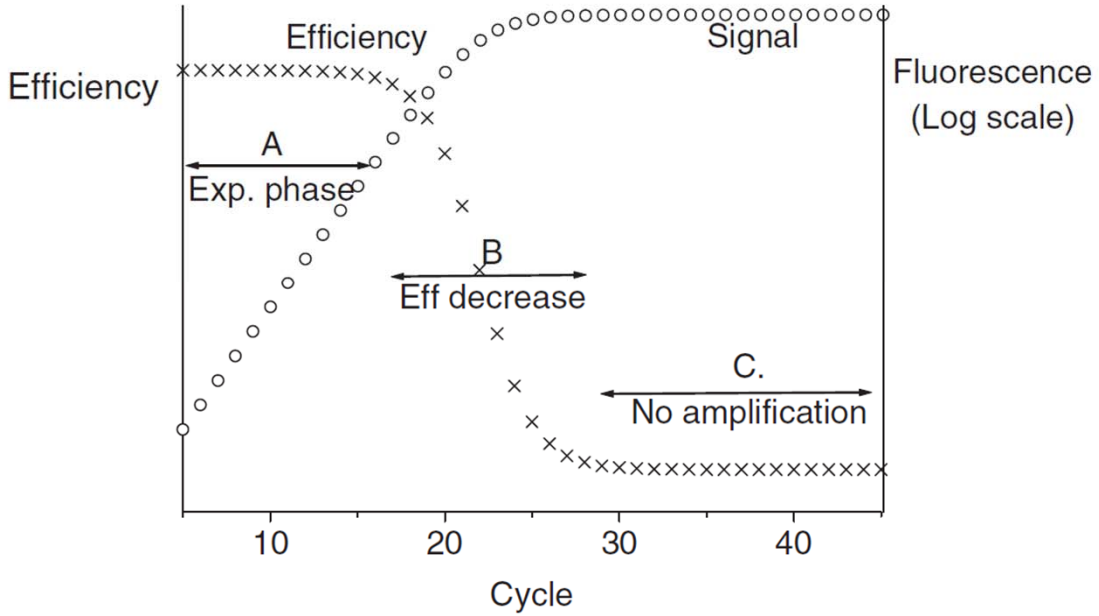
Kinetic Outlier Detection (KOD)

- Determine quality of reaction from target amplification curve



Kinetic Outlier Detection (KOD)

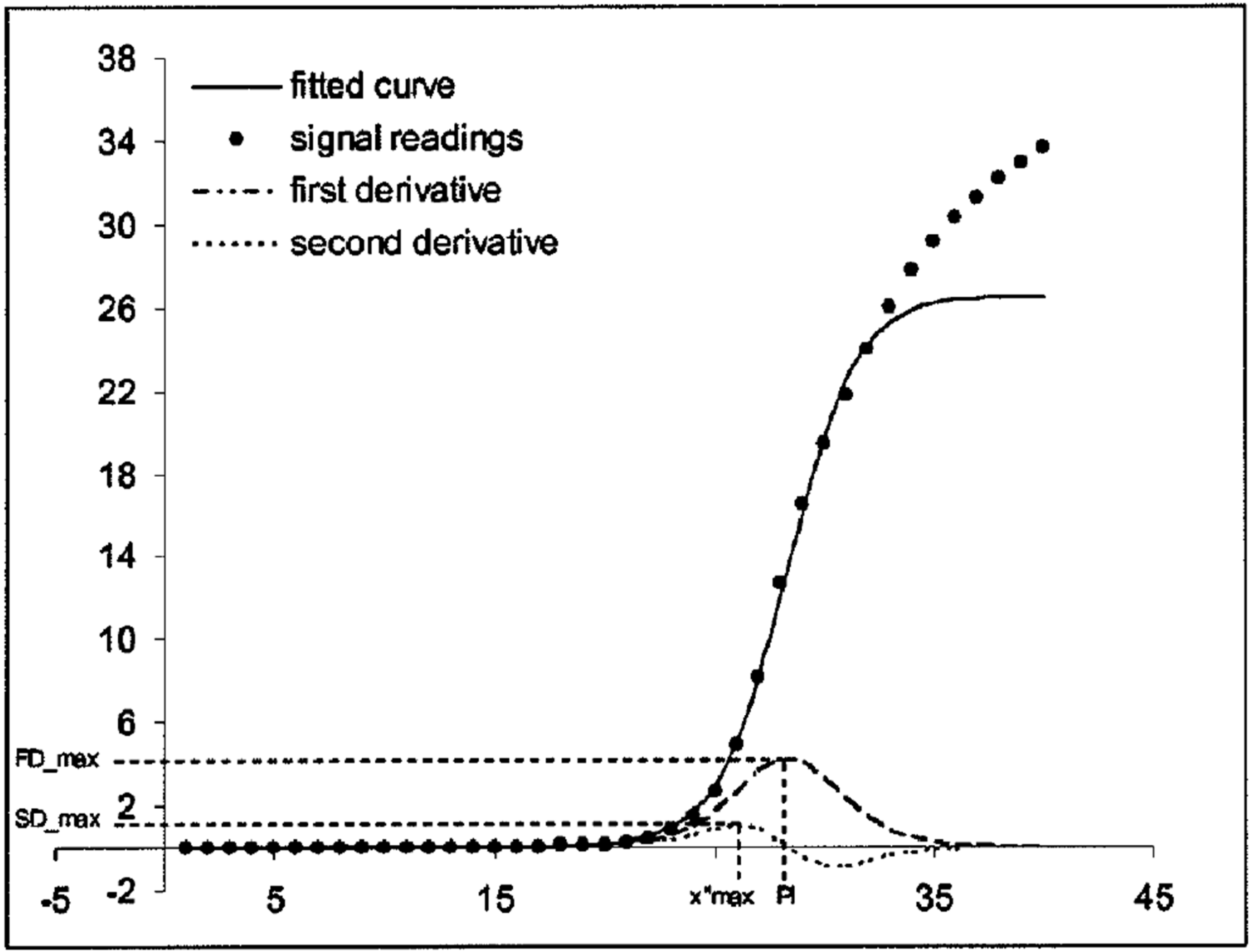
- Univariate: Calculation of amplification efficiency from mathematical model of curve
  - Large variation, several different methods



Bar T, Kubista M, Tichopad A: **Validation of kinetics similarity in qPCR**. *Nucleic Acids Res* 2011, **40**:1395-1406.

Kinetic Outlier Detection (KOD)

- Multivariate: Combining two measures for amplification quality
    - More robust, supposedly better discrimination between pure and affected reactions
- Maxima of first and second derivative of mathematical model fitted to curve



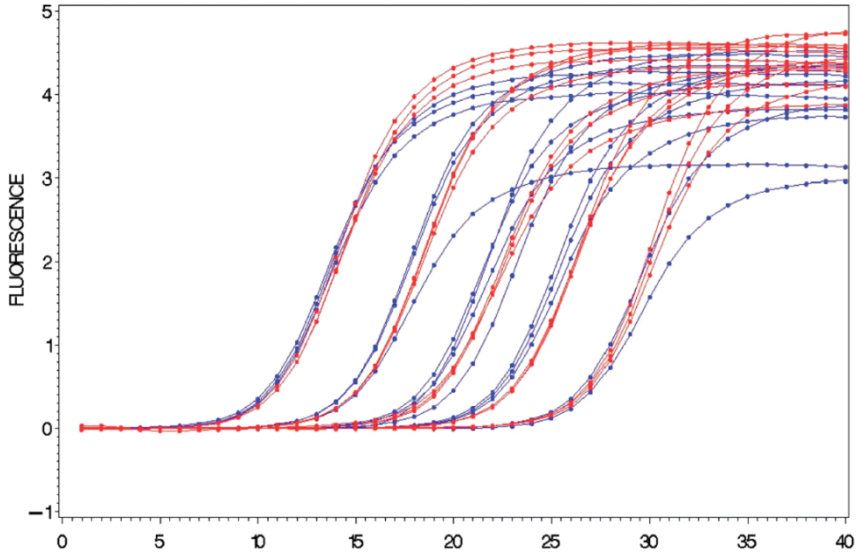
Tichopad A, Bar T: **Assessment of reaction kinetics compatibility between polymerase chain reactions**. US Patent Application 20090176232.

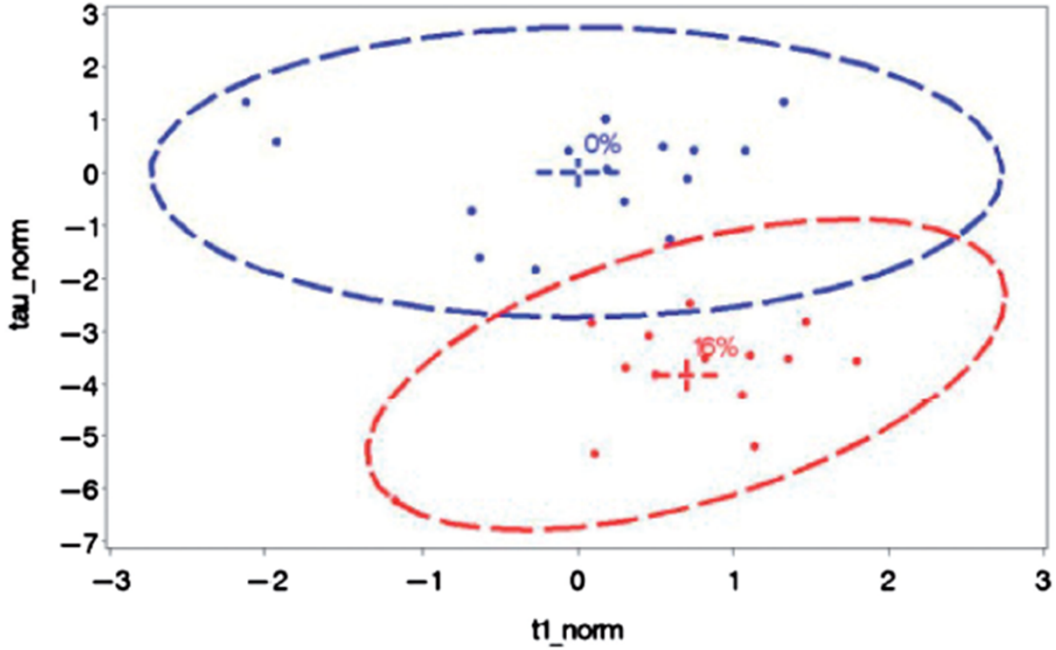
Multivariate KOD

Red: Pure reactions

Blue: Tannic acid added (2 ng)

95% confidence intervals





Free softwares for qPCR data handling

- R package: qpcR

http://cran.r-project.org/web/packages/qpcR/index.html

Ritz C, Spiess AN: **qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis**. *Bioinformatics* 2008, **24**(13):1549-1551.

- Web-based Java software: QPCR

http://icbi.at/software/qpcr/qpcr.shtml

Pabinger S, Thallinger GG, Snajder R, Eichhorn H, Rader R, Trajanoski Z: **QPCR: Application for real-time PCR data management and analysis**. *BMC Bioinformatics* 2009, **10**:268.

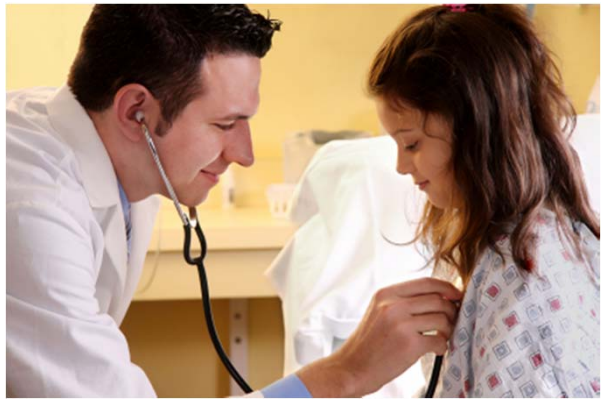
Pre-PCR processing and PCR inhibition

Applications of diagnostic qPCR

Food and feed chain

Archaeology

Bioterrorism

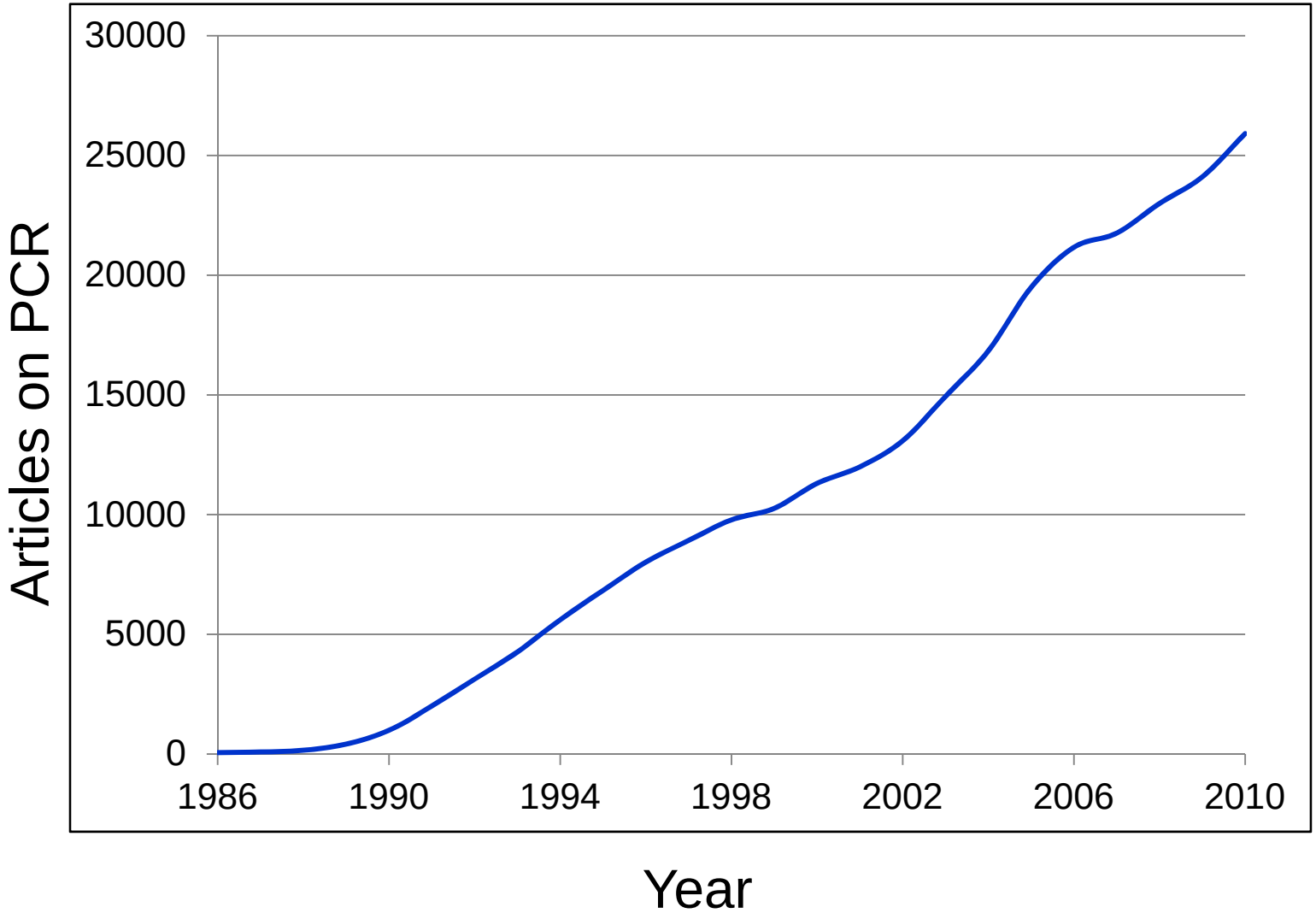


Clinical diagnostics

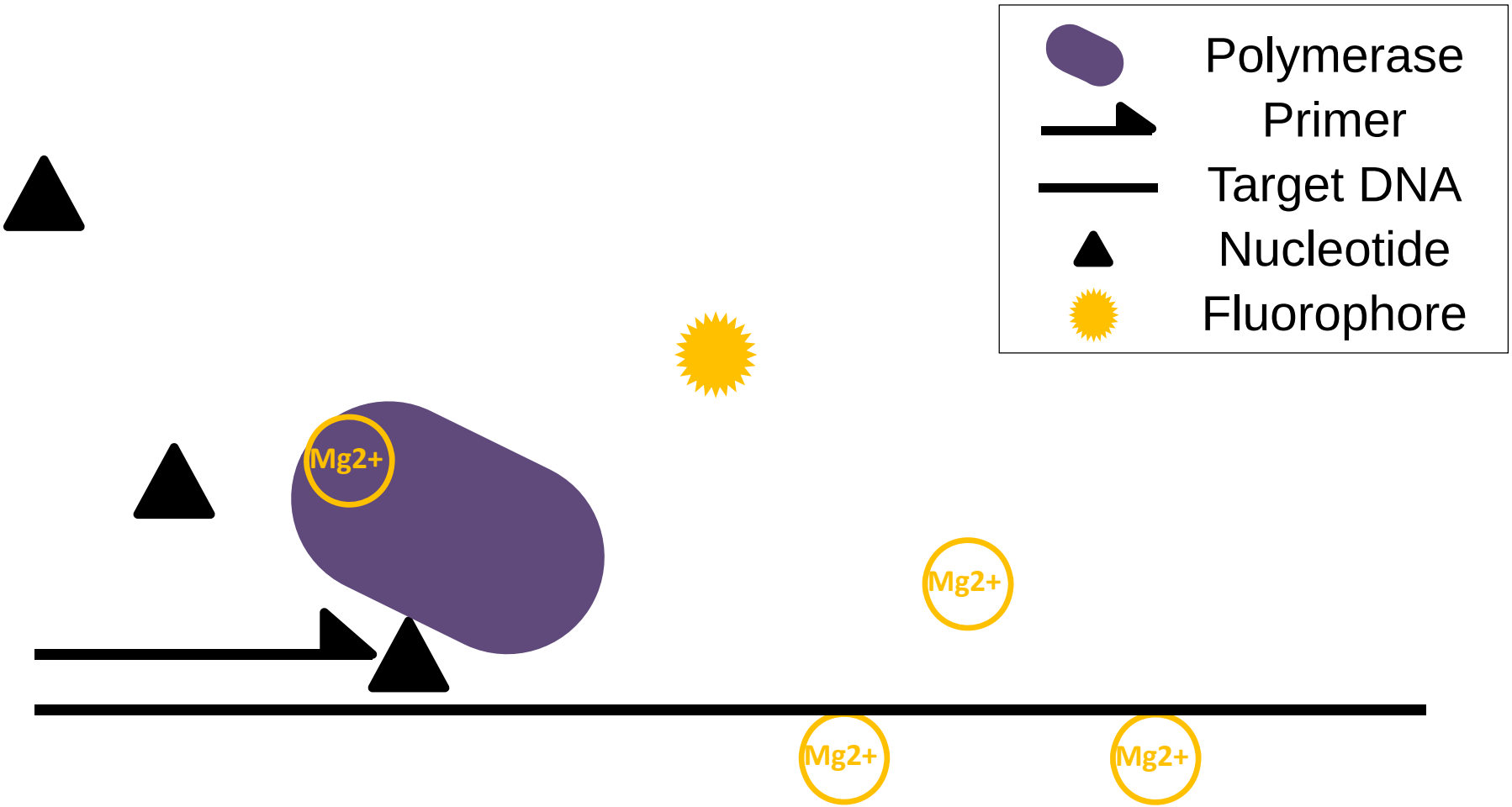
Environmental studies

Forensics

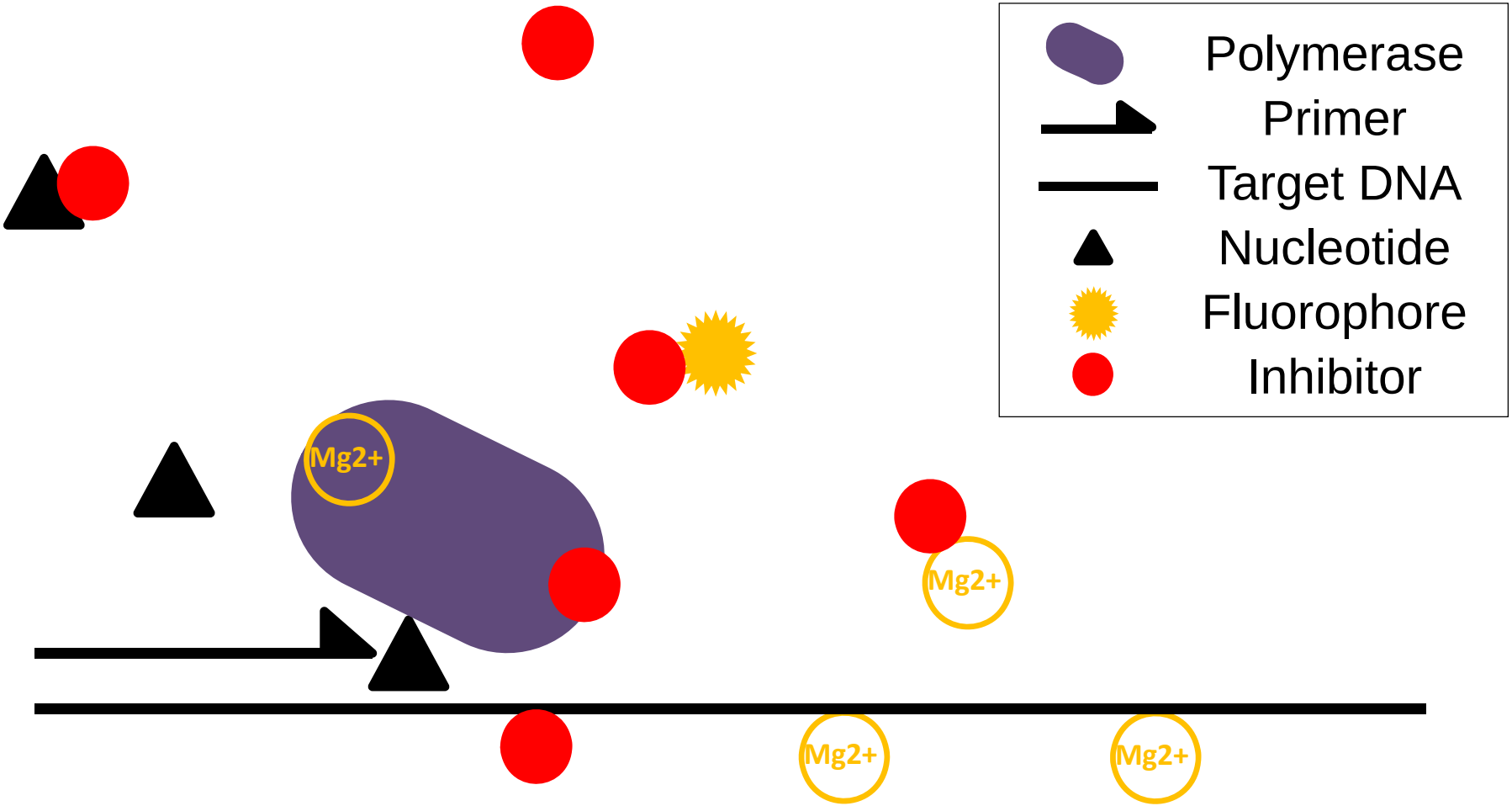
PCR in the literature



PCR in the test tube



PCR in the test tube



PCR inhibitors may act by:

(i) inactivating the thermostable DNA polymerase
(ii) disturbing the ion composition of the reaction
(iii) capturing nucleic acids

Specific qPCR inhibitors:

(iv) interfering with fluorogenic probes or DNA-intercalating dyes
(v) some compounds may generate background fluorescence or quench the excitation light from the fluorogenic molecules

PCR Inhibitor	Mechanism	Ref.
Calcium ions	Competing with Mg^{2+}	Bickley et al. 1996
EDTA	Chelation of Mg2+	Rossen et al. 1992
IgG	Binds to ssDNA	Abu Al-Soud et al. 2000
Lactoferrin	Release of iron ions	Abu Al-Soud, Rådström 2001
Phenol	Denatur. of Polym.	Katcher, Schwartz 1994
Polysaccharides	Binding to Polym.	Monteiro et al. 1997
Proteinases	Degr. of Polym.	Powell et al. 1994
Humic acids	Binds DNA, binds/reacts with polymerase, quenches fluorescence	

Hedman J, Knutsson R, Ansell R, Rådström P, Rasmusson B (2013). Pre-PCR processing in bioterrorism preparedness: improved diagnostic capabilities for laboratory response networks. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 11:87-101

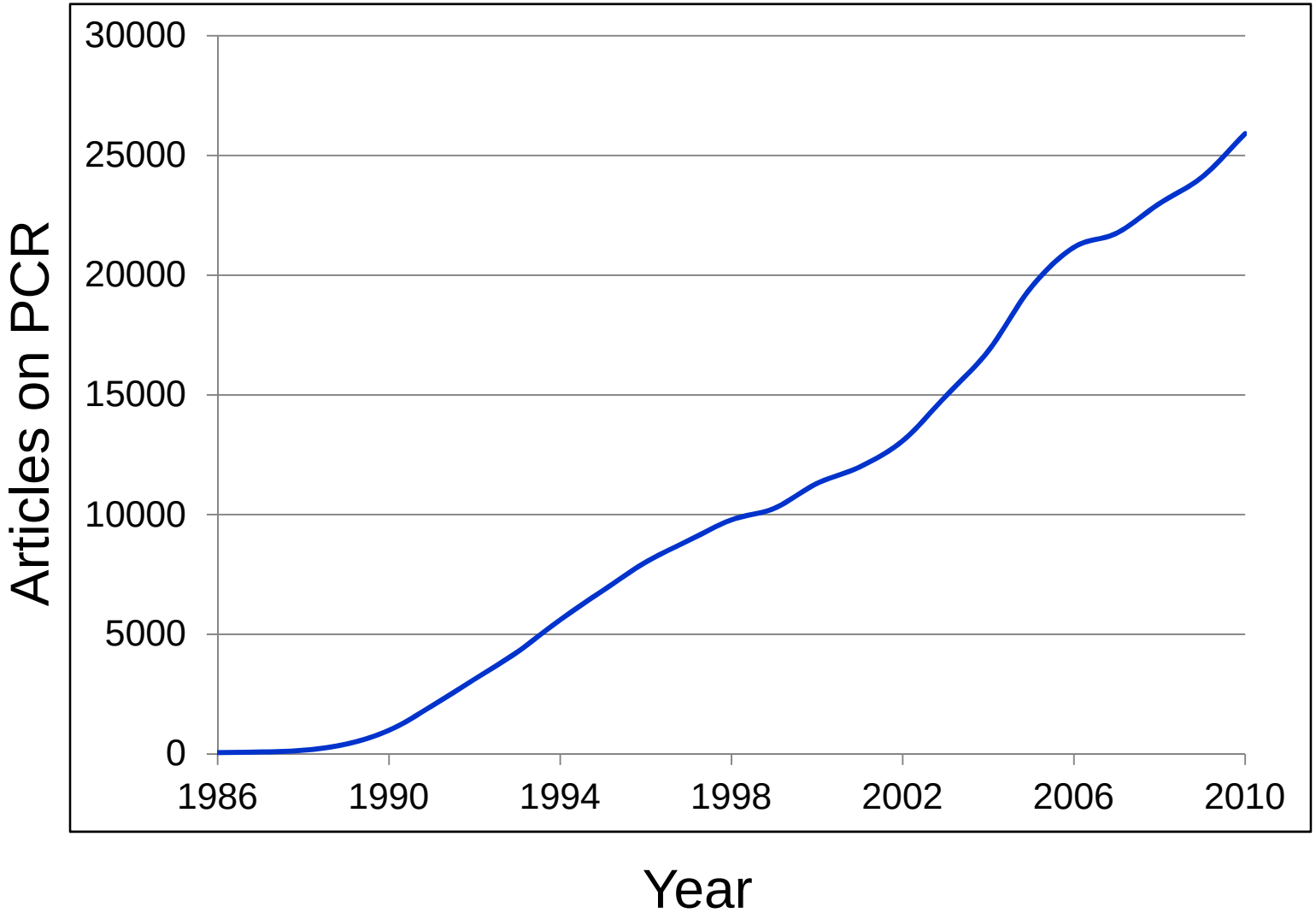
Effect of PCR inhibitors

(i) inhibitors can dramatically affect the detection limit, accuracy and precision

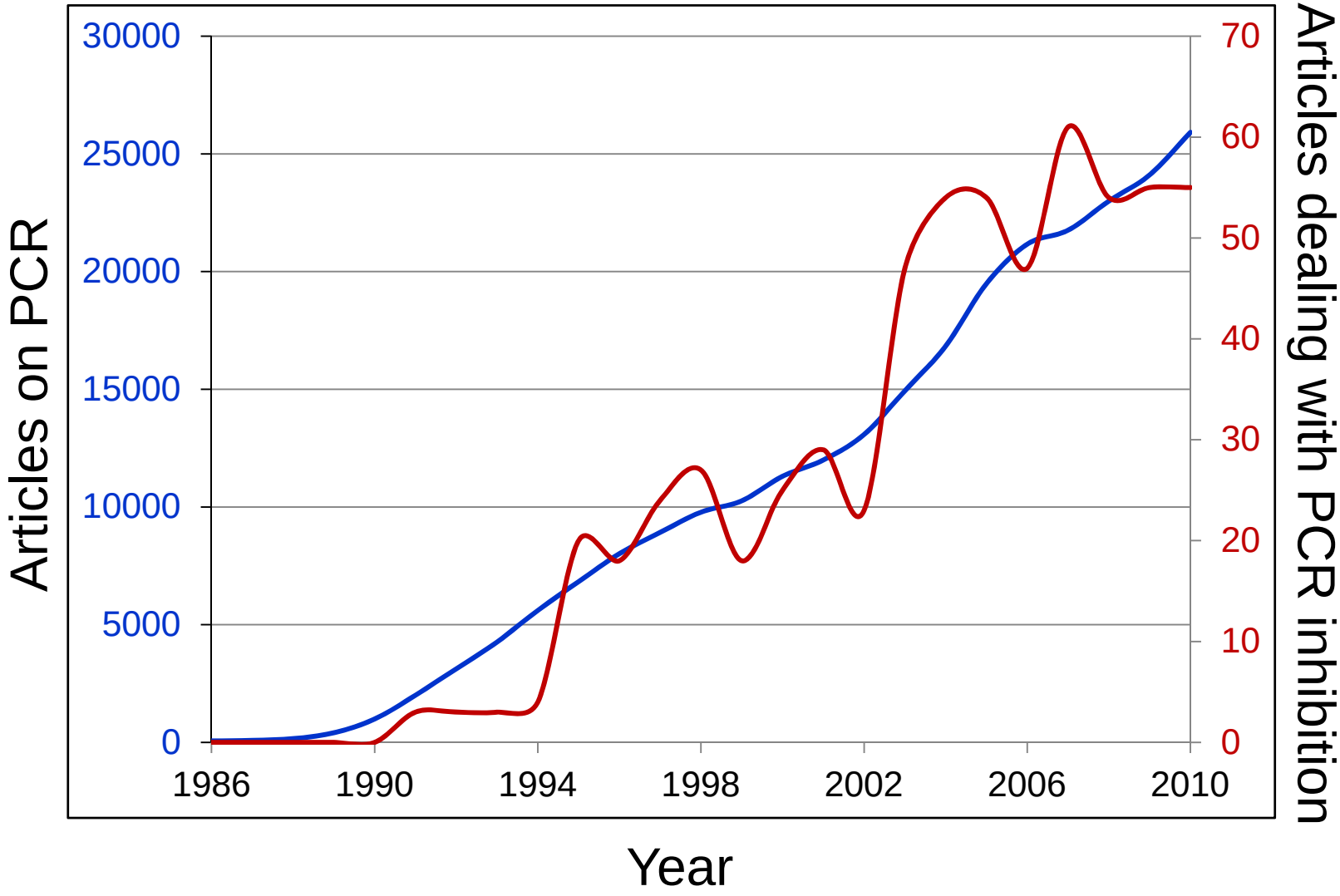
(ii) change the amplification efficiency/kinetics and thus generate ambiguous data in qPCR

(iii) cause failed amplification

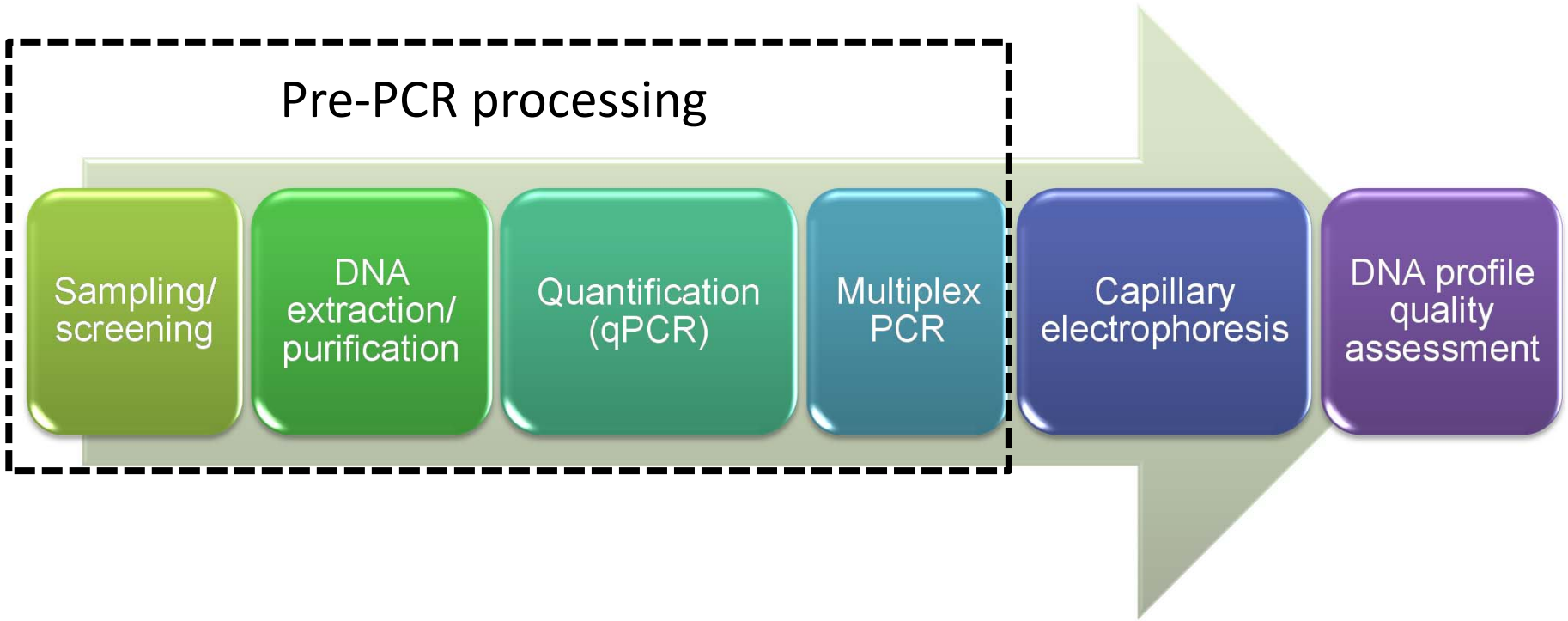
PCR in the literature



PCR in the literature



Diagnostic PCR



Hedman J, Lövenklev M, Wolffs P, Löfström C, Knutsson R, Rådström P (2013). Pre-PCR processing strategies. In: PCR Technology, Current innovations (3rd ed.), ed. Nolan, T. CRC Press, Boca Raton, USA. 3-17

Hedman J, Rådström P (2013). Overcoming inhibition in real-time diagnostic PCR, Methods Mol Biol 943:17-48

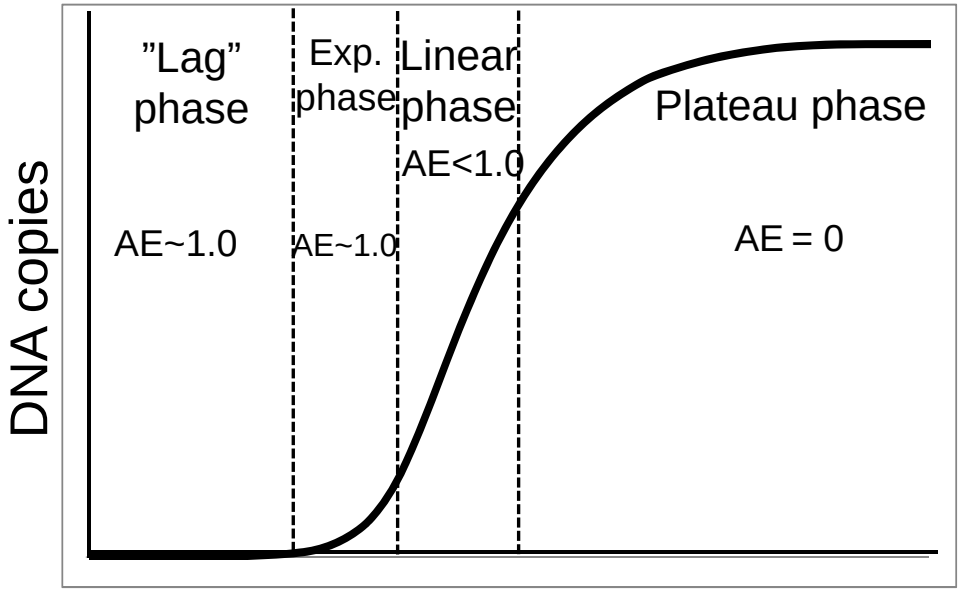
Hedman J, Knutsson R, Ansell R, Rådström P, Rasmusson B (2013). Pre-PCR processing in bioterrorism preparedness: improved diagnostic capabilities for laboratory response networks. Biosecurity and Bioterrorism: Biodefense Strategy, Practice, and Science 11:87-101

Goals of Pre-PCR processing

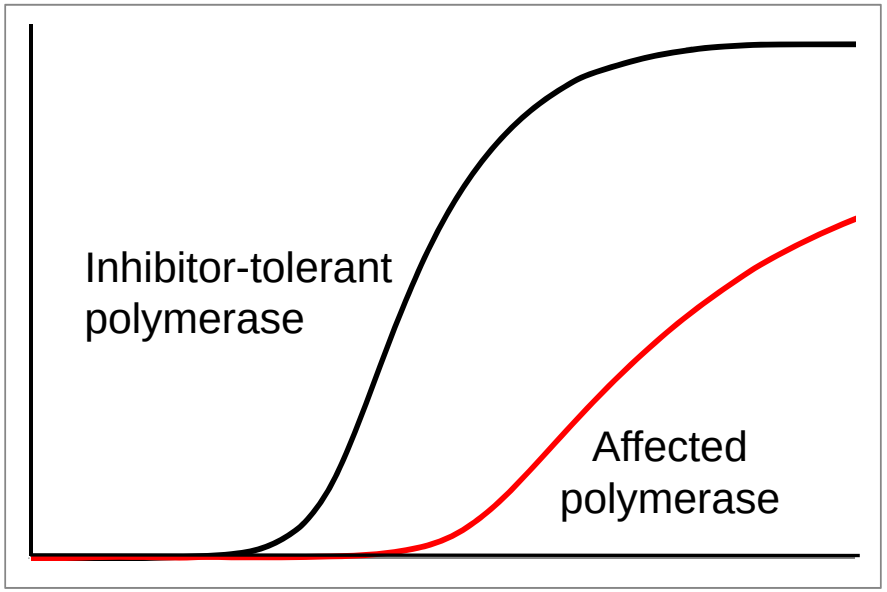
- Minimise effect of PCR inhibitors
- Maximise amount of target
- Heterogeneous to homogeneous
- Allow precise quantification

Pre-PCR processing:
Customising the DNA polymerase-buffer system

Kinetics of PCR

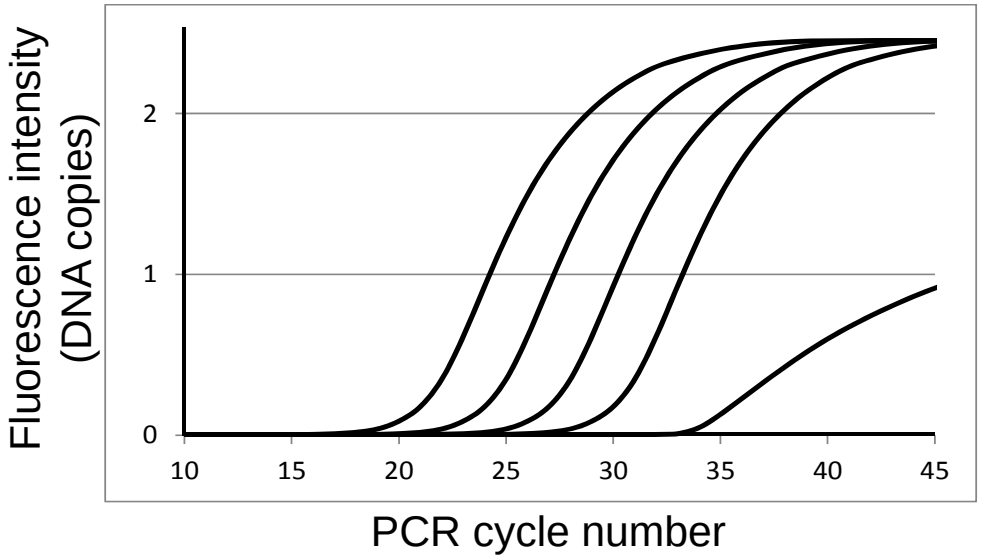


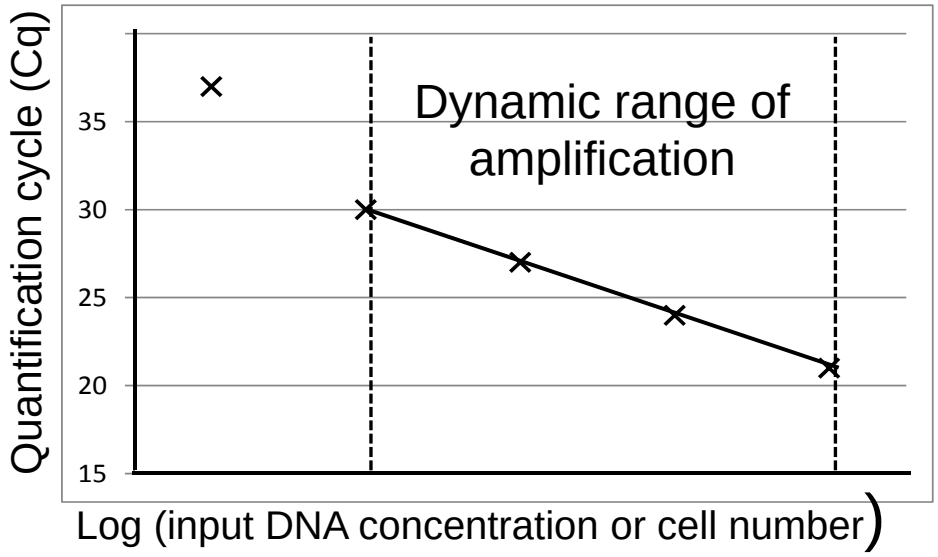
Effect of PCR inhibitors



AE: amplification efficiency

Evaluation of alternative DNA polymerases

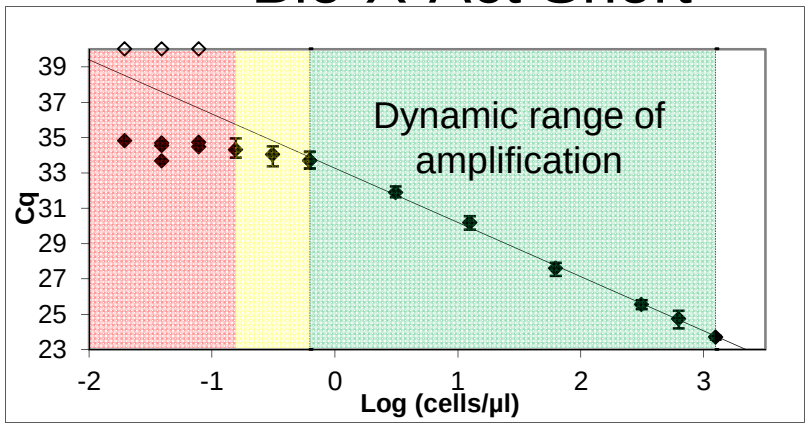




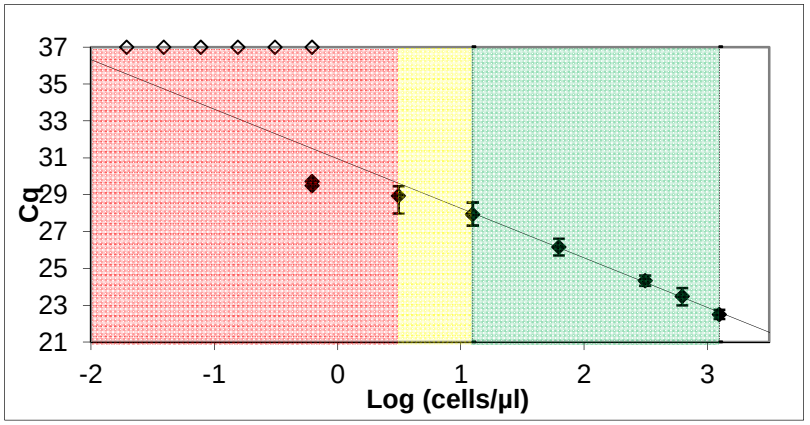
Model system
qPCR
Hydrolysis (TaqMan) probe
Singleplex (one target)
Amplicon: 156 bp
Standardised mock crime scene samples: dilution series of saliva
Screening of 15 DNA polymerases

Evaluation of alternative DNA polymerases

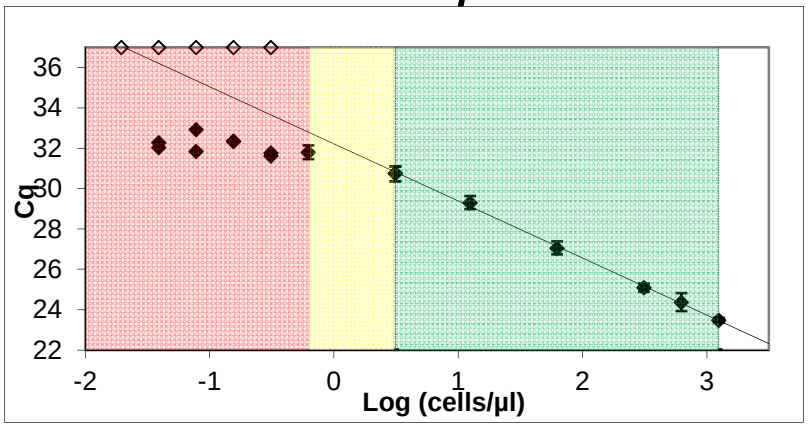
Bio-X-Act Short



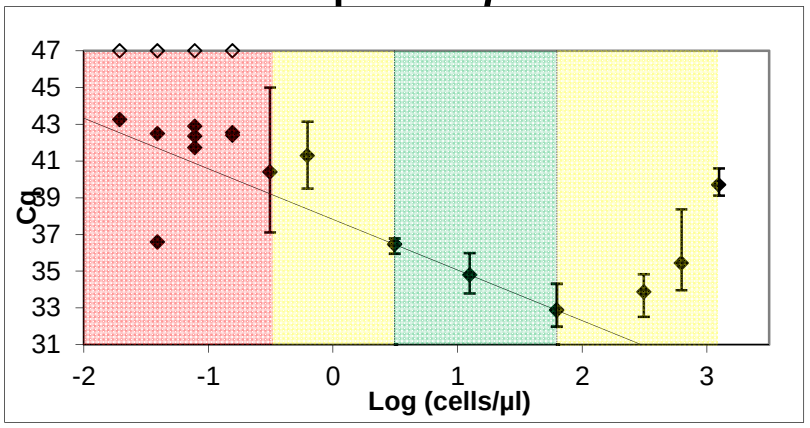
Tth



Taq



Ampli*Taq* Gold[a]



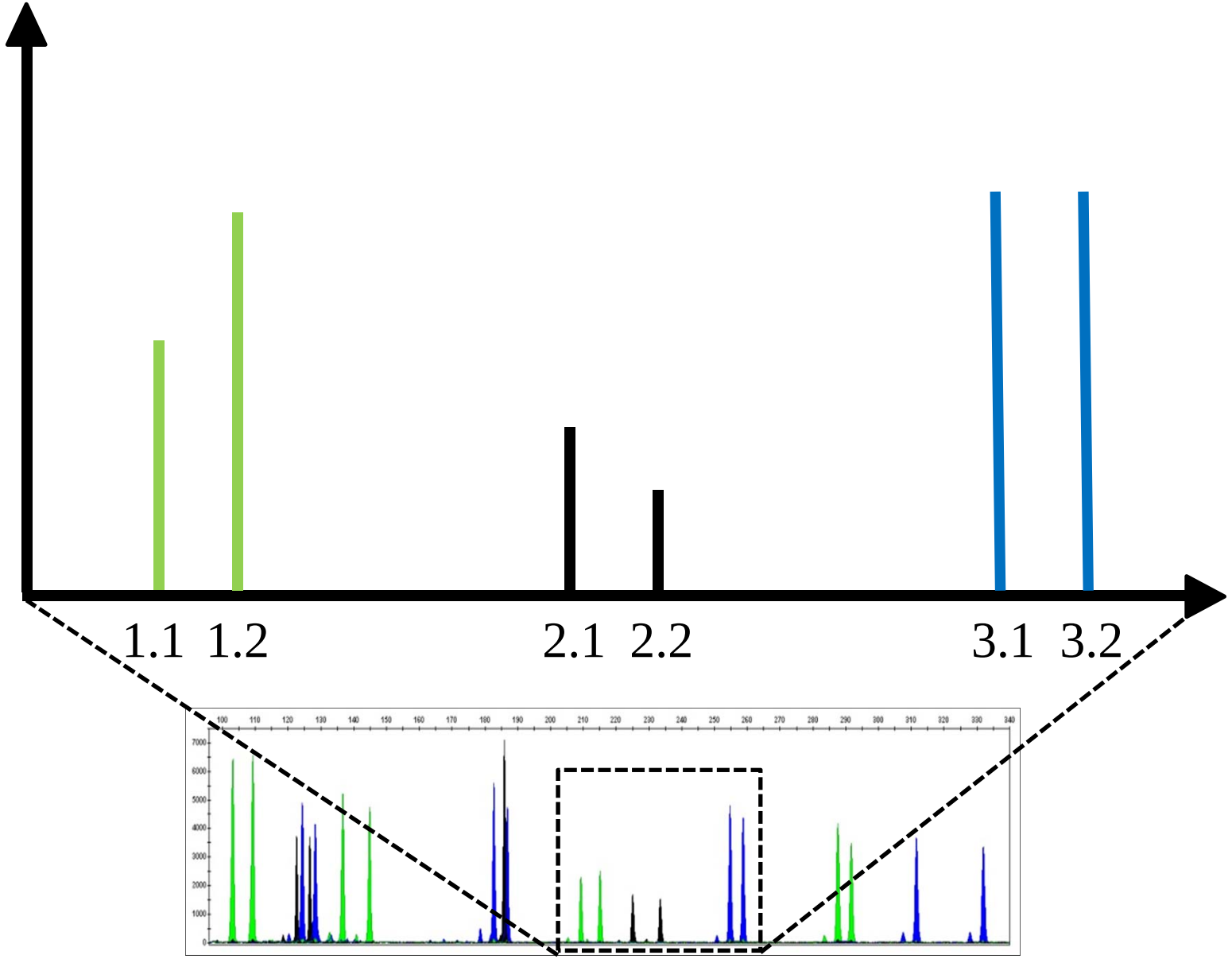
a) Reference method

Evaluation of alternative DNA polymerases

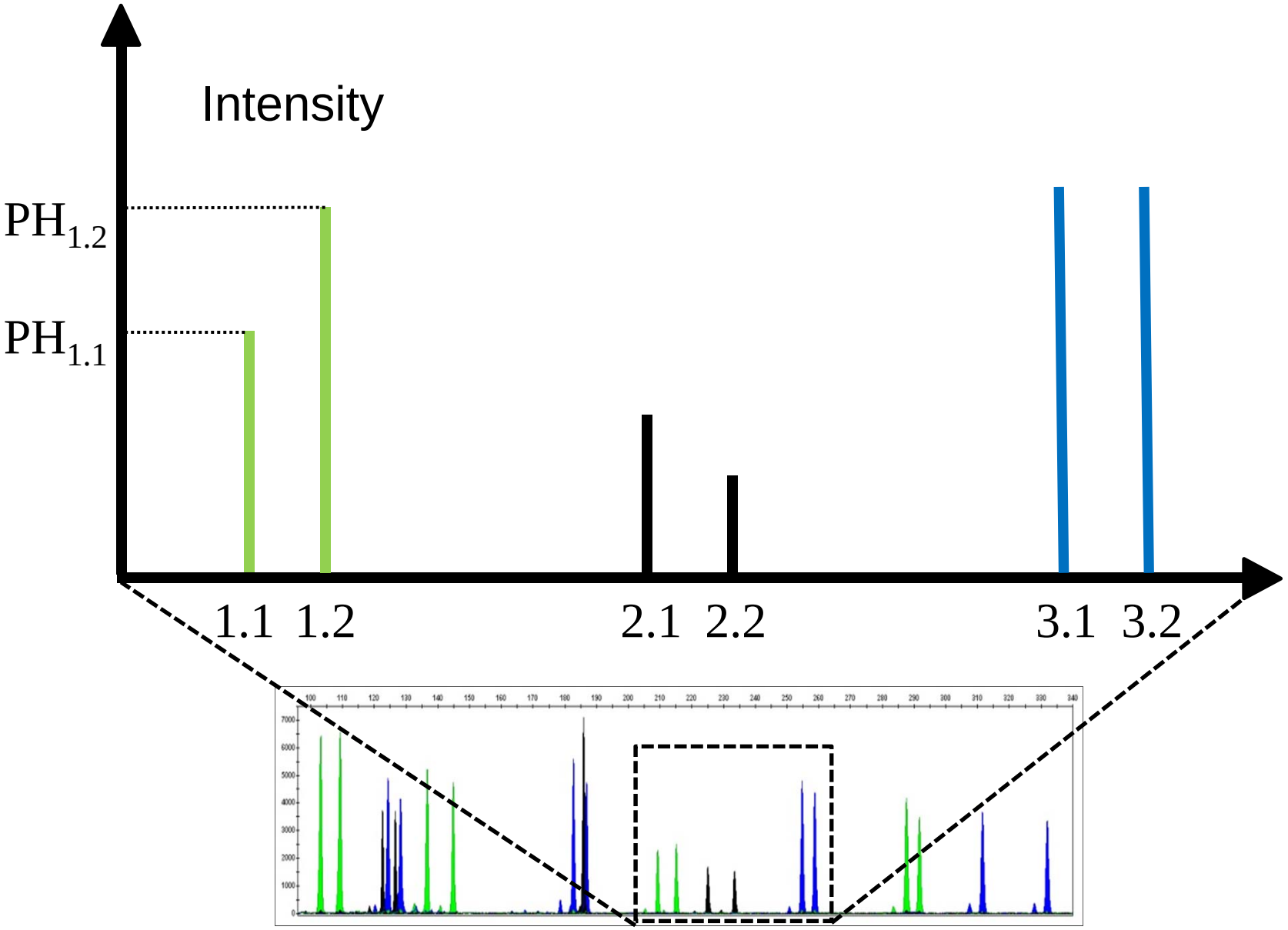
DNA polymerase	Mean assay amplification efficiency	Dynamic range of amplification (log units)	Detection limit (cells/µL)
Bio-X-Act Short	1.12±0.06	3.3	0.16
Ex*Taq* HS	0.99±0.05	2.6	0.31
PicoMaxx HF	0.93±0.05	3.3	0.31
Omni*Taq*[a]	0.95±0.04	2.6	0.63
Taq	1.26±0.10	2.6	0.63
KAPA2G Robust[a]	1.08±0.11	2.0	0.63
Ampli*Taq* Gold[b]	1.46±0.67	1.3	0.31
r*Tth*	1.40±0.10	2.0	3.1
Tth	1.38±0.23	2.0	3.1

a) Protein engineered polymerase
b) Reference method

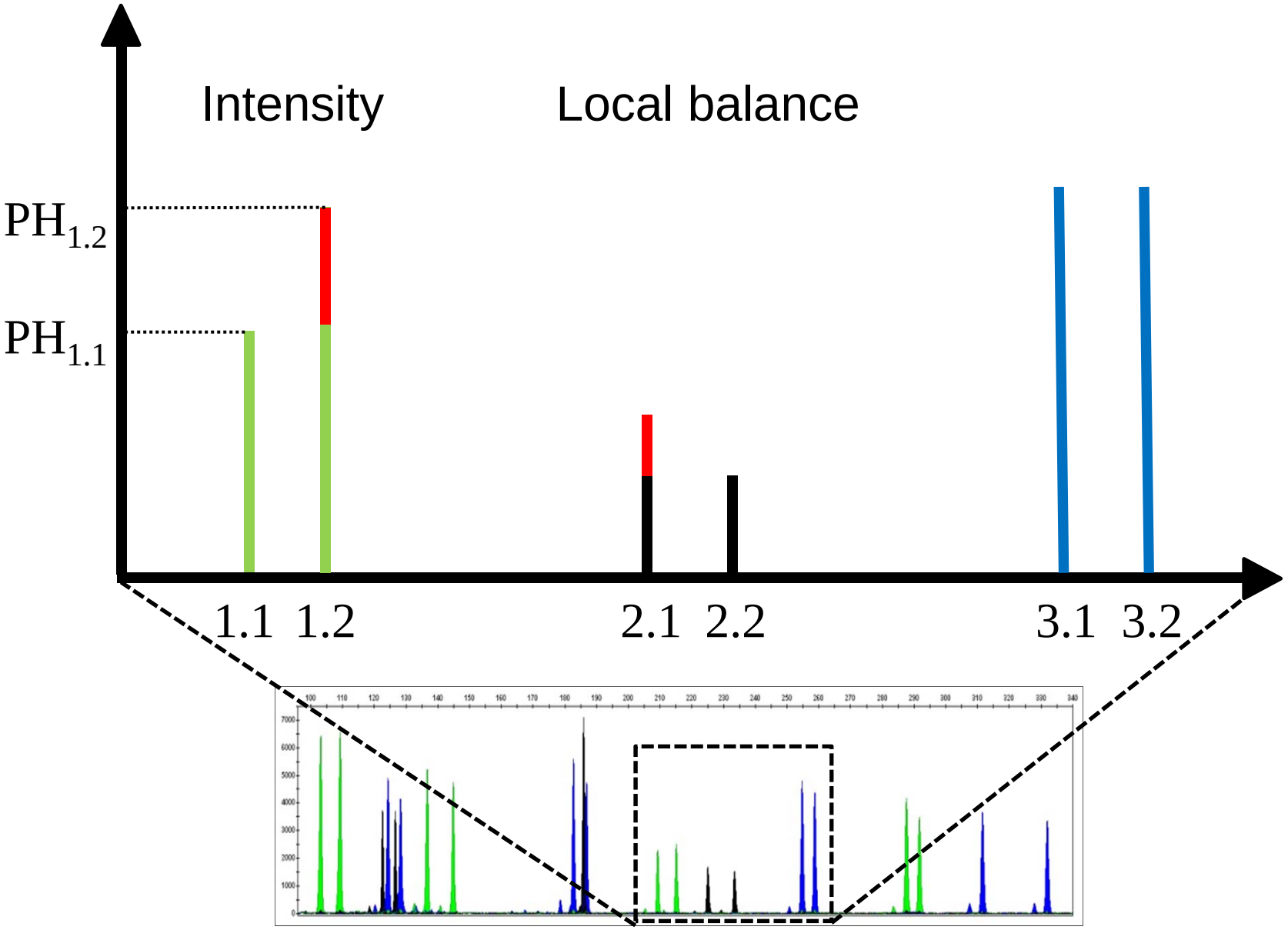
DNA profile quality assessment



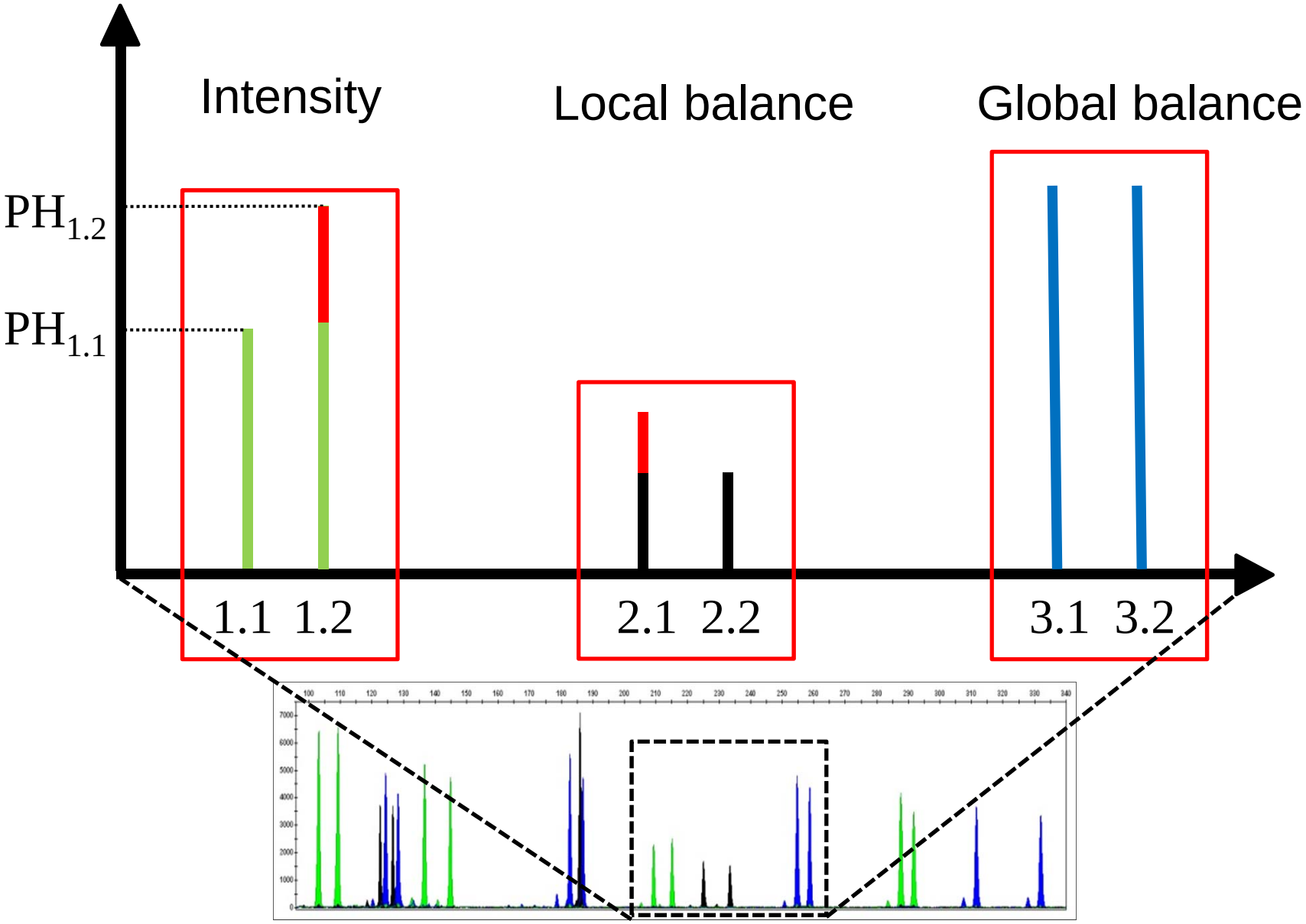
DNA profile quality assessment



DNA profile quality assessment



DNA profile quality assessment



Forensic DNA Profile Index (FI)

Intensity:
Total sum of peak heights (TPH)

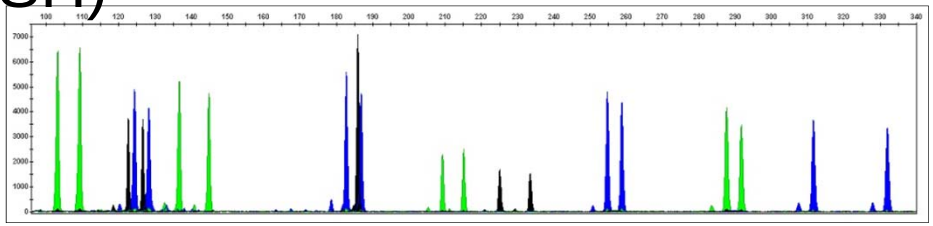
$$TPH = \sum_{i=1}^{M} PH_i; \quad PH_i = PH_{i.1} + PH_{i.2}$$

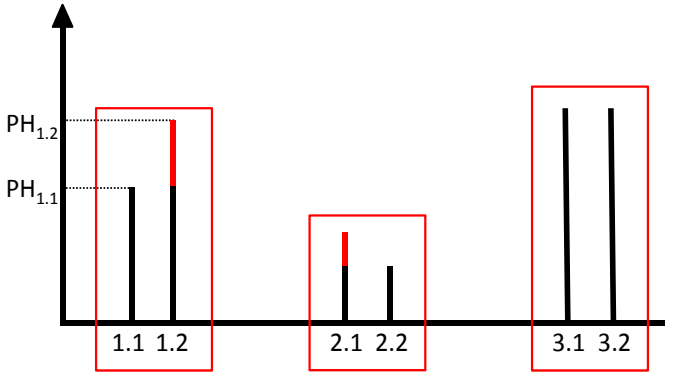
Local balance:
Mean local balance (MLB)

$$MLB = \frac{1}{M} \sum_{i=1}^{M} LB_i; \quad LB_i = \frac{PH_{i.\min}}{PH_{i.\max}}$$

Global balance:
Shannon entropy (SH)

$$SH = -\sum_{i=1}^{M} p_i \cdot \ln(p_i); \quad p_i = \frac{PH_i}{TPH}$$





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Global balance:
Shannon entropy (SH)

$$SH = -\sum_{i=1}^{M} p_i \cdot \ln(p_i); \quad p_i = \frac{PH_i}{TPH}$$

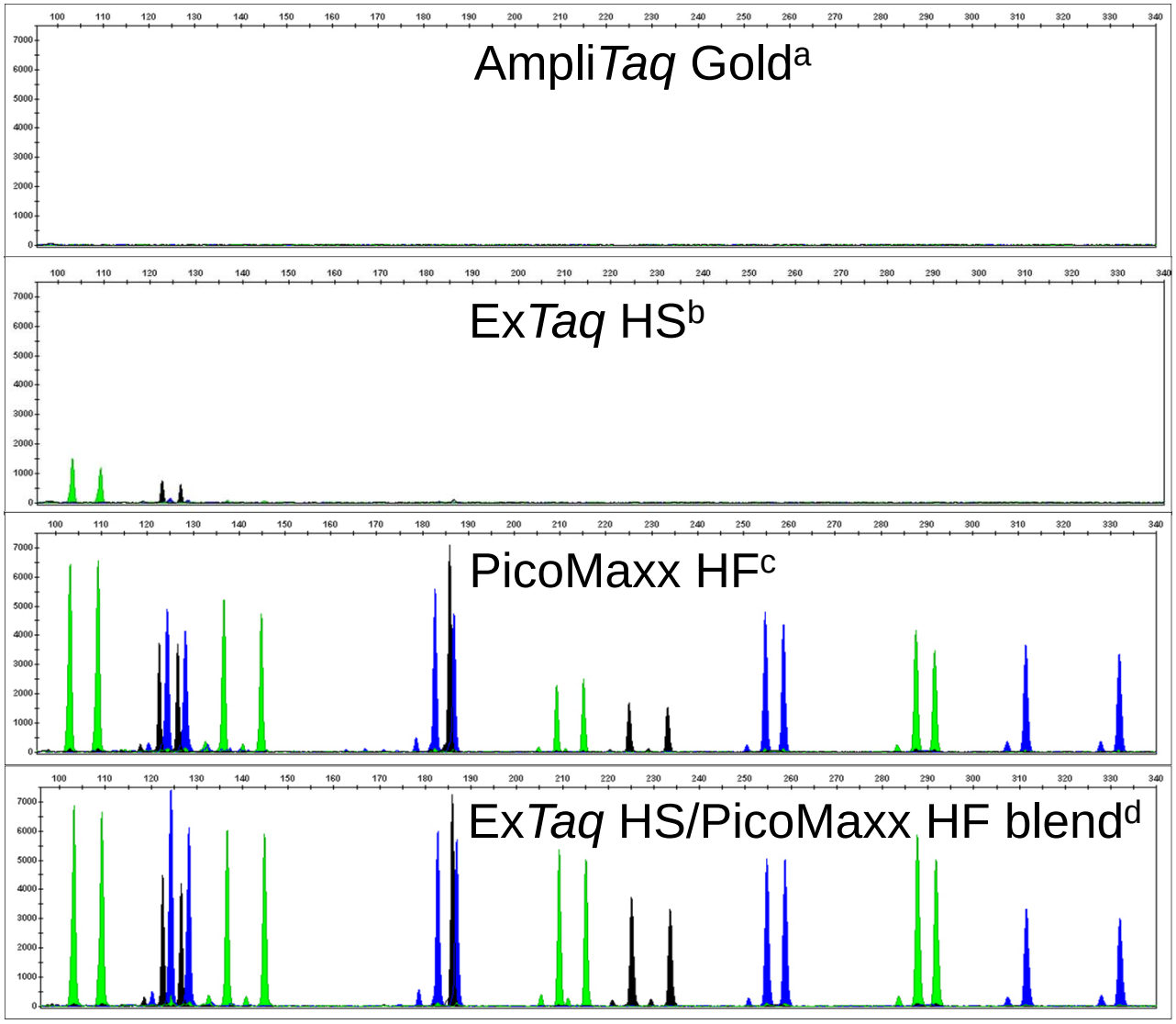
$$FI = c_1 \cdot a_1 \cdot tph + c_2 \cdot a_2 \cdot mlb + c_3 \cdot a_3 \cdot sh + K$$

Alternative DNA polymerases in forensic analysis

DNA 0.19 ng/µL

FI values:

a) 0.05
b) 0.50
c) 10.85
d) 13.85

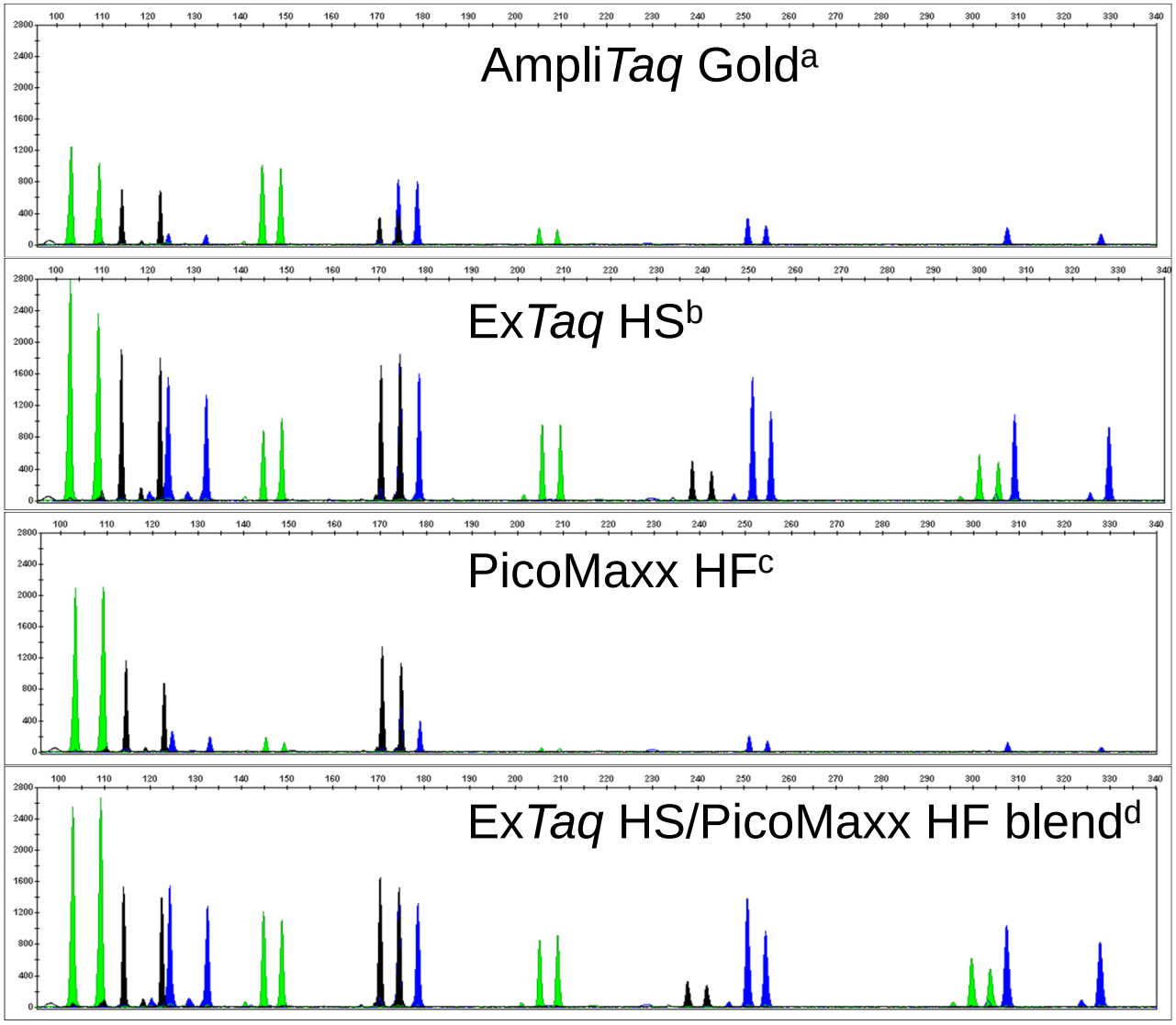


Alternative DNA polymerases in forensic analysis

DNA 0.21 ng/µL

FI values:

a) 1.51
b) 3.85
c) 1.36
d) 3.55

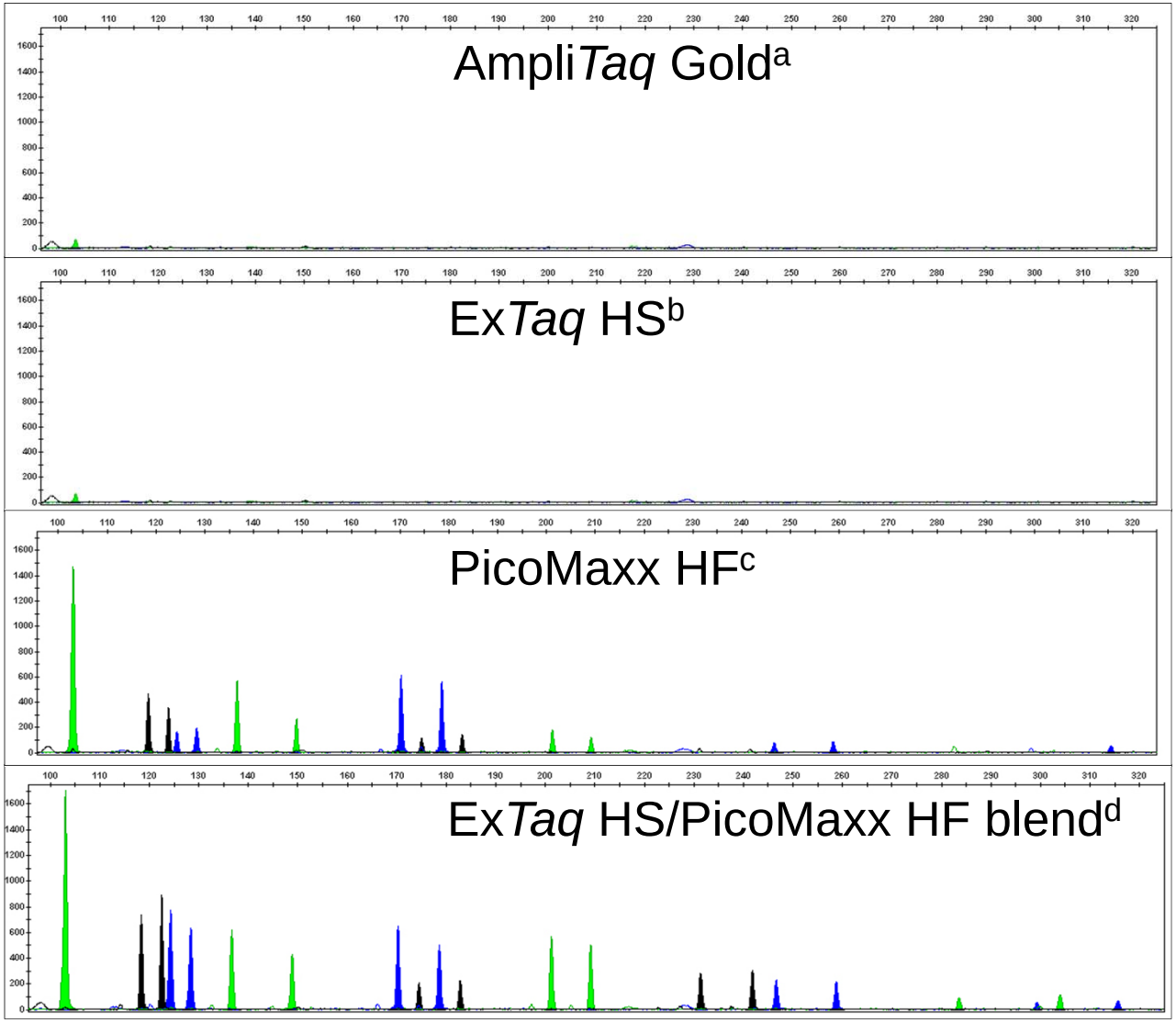


Alternative DNA polymerases in forensic analysis

DNA 0.13 ng/µL

FI values:

a) 0.05
b) 0.05
c) 1.17
d) 2.04



Sample treatment vs. improved analysis

Blood

DNA polymerase	Standard extraction	Standard + dilution 1:2	Standard + column pur.
Standard polymeras[a]	0.05	0.08	1.64
2x standard polymerase	0.40	1.75	1.69
Alternativ X+Y[b]	10.85	5.59	2.54

Results presented as mean values of quality index

a) AmpliTaq Gold
b) ExTaq HS + PicoMaxx HF

Sample treatment vs. improved analysis

Saliva

DNA polymerase	Standard extraction	Standard + dilution 1:2	Standard + column pur.
Standard polymeras[a]	0.32	1.51	1.74
2x standard polymerase	1.89	1.78	1.93
Alternativ X+Y[b]	6.48	3.61	4.01

Results presented as mean values of quality index

a) AmpliTaq Gold
b) ExTaq HS + PicoMaxx HF

Routine analysis of crime scene samples

Saliva

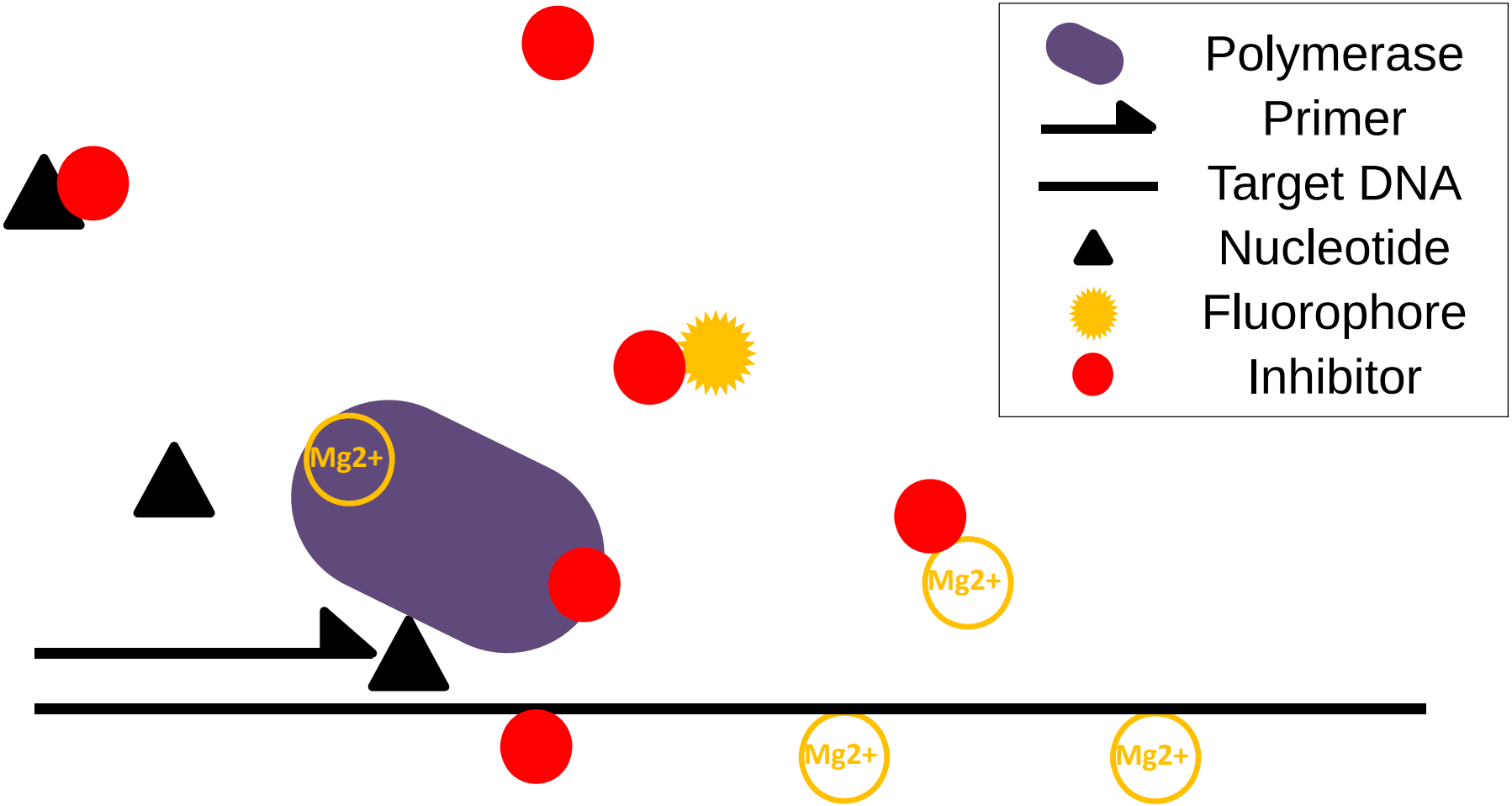
DNA polymerase	Complete profiles (%)	Partial profiles (%)	Negative profiles (%)
Standard polymerase[a]	38	47	15
Alternative X+Y[b]	87	8	5

DNA concentrations 0.025-0.15 ng/µL

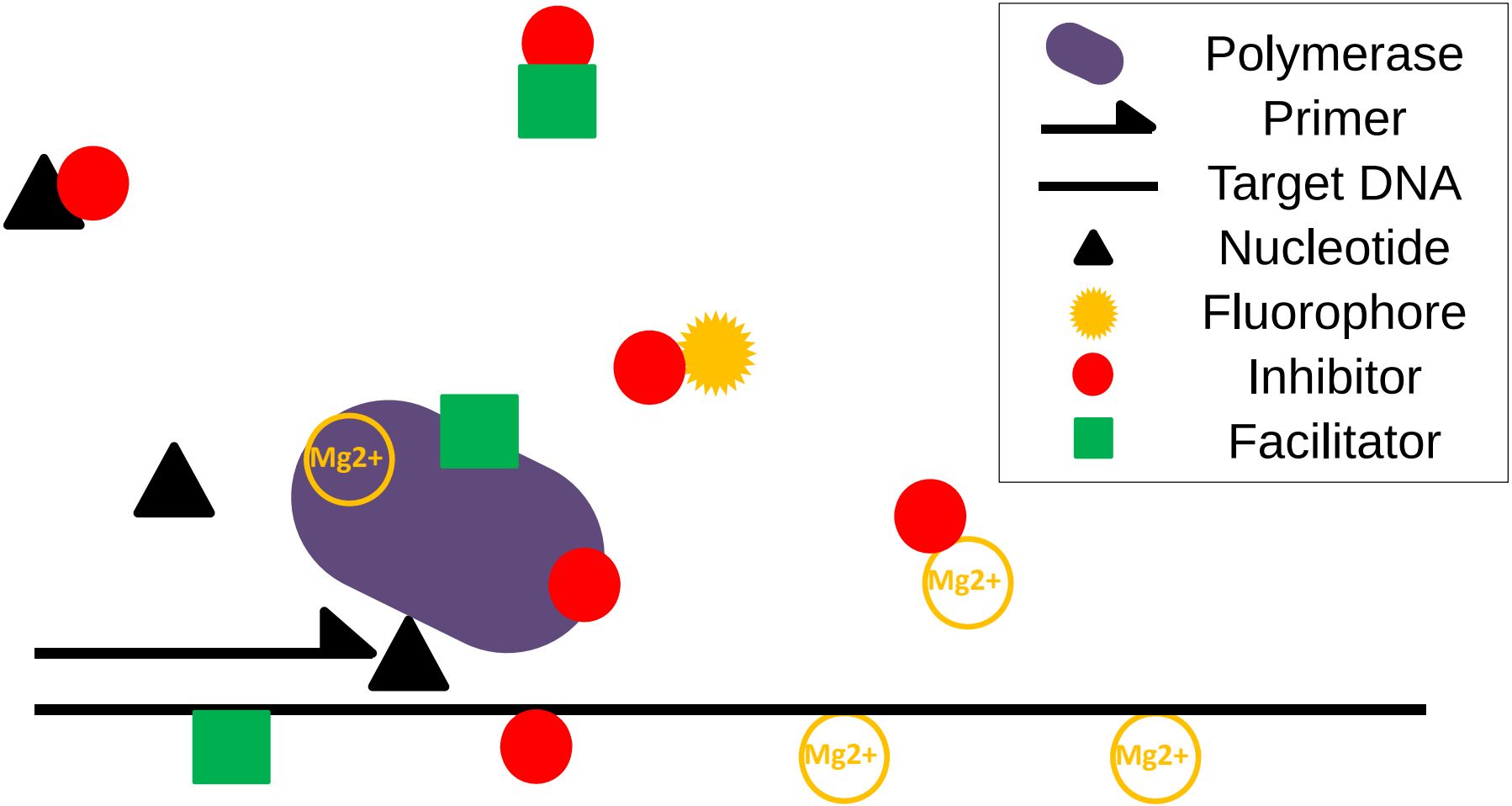
a) Ampli*Taq* Gold

b) ExTaq HS + PicoMaxx HF

Alternative way of relieving inhibition



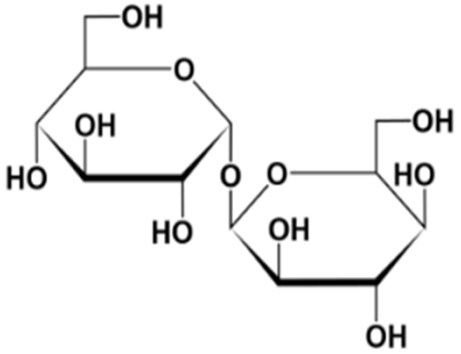
Alternative way of relieving inhibition



PCR facilitators

Proteins
Bovine serum albumin (BSA)
T4 gene 32 protein (gp32)

Biologically compatible solutes
Betaine
L-carnitine
Sorbitol
Trehalose



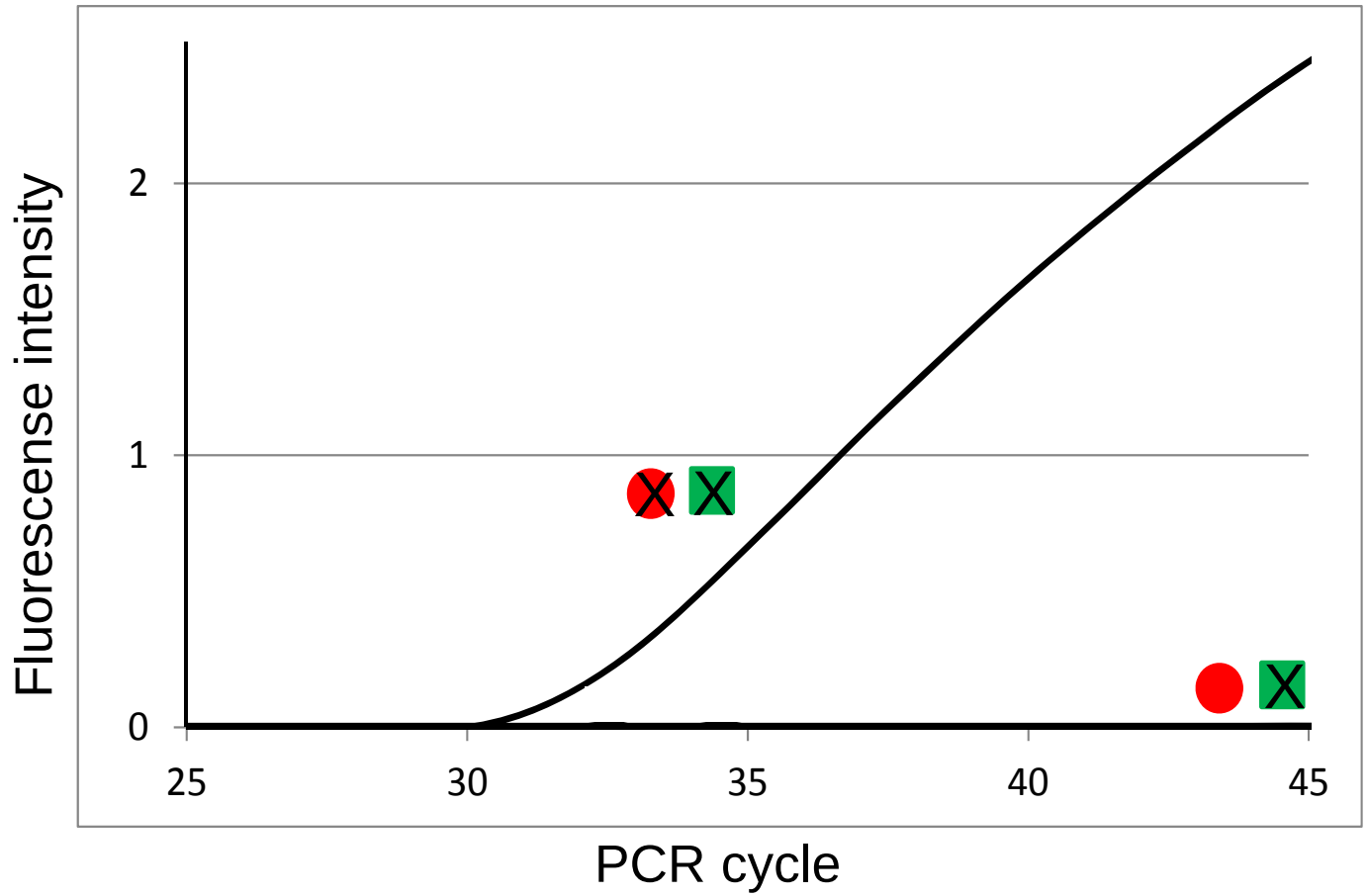
Trehalose

Non-ionic detergents
NP40
Tween 20

Polymers
PEG400

Organic solvents
DMSO

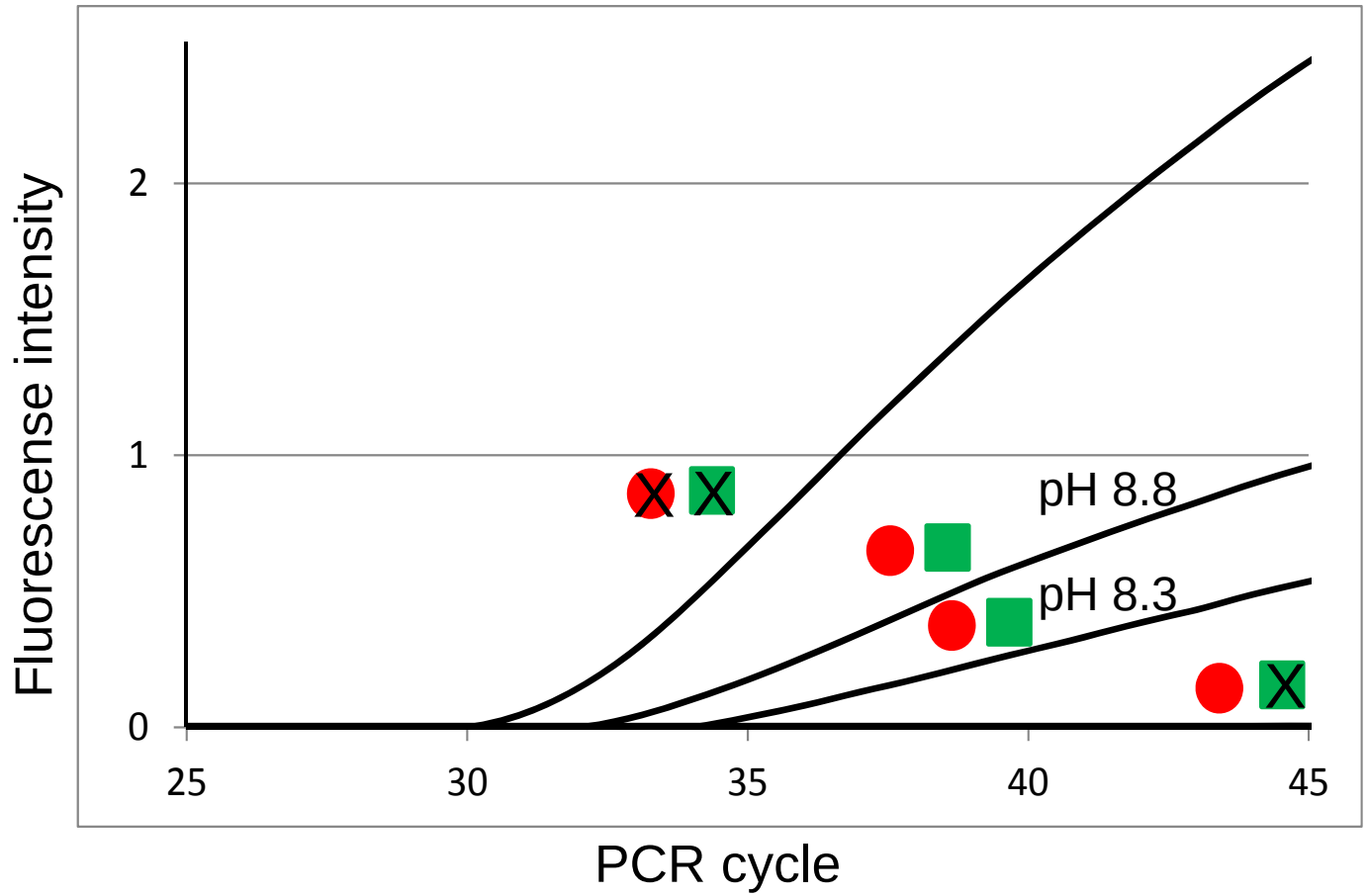
Effect of PCR facilitators and buffer pH



Moist snuff extract

Trehalose

Effect of PCR facilitators and buffer pH



Moist snuff extract

Trehalose

Questions?