qPCR in forensic DNA analysis

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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

Crime

Police investigation

Traces evidence, information

Sampling

DNA analysis and db search

Police, CSI or at forensic lab

Forensic laboratory (SKL)

Match report

Court of law
Workflow in forensic DNA analysis

- Sampling/screening
- DNA extraction/purification
- Quantification (qPCR)
- Multiplex PCR
- Capillary electrophoresis
- DNA profile quality assessment
Sampling
Finding and identifying stains

Protein based tests
Tissue specific enzymes
Reaction => colour change

Light source
Fluorescence from body fluids (e.g. 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)

AGAC AGAC AGAC AGAC AGAC AGAC

Allele: 8 (8 repetitions)

AGAC AGAC AGAC AGAC AGAC AGAC AGAC AGAC

- 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

Amelogenin

<table>
<thead>
<tr>
<th>STR</th>
<th>02_D3S1358</th>
<th>03_TH01</th>
<th>04_D21S11</th>
<th>05_D18S51</th>
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<td>X</td>
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<td>517</td>
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<table>
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<th>STR</th>
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<td>17</td>
<td>153</td>
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</table>
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)

<table>
<thead>
<tr>
<th>STR marker:</th>
<th>D3</th>
<th>vWa</th>
<th>D16</th>
<th>D2</th>
<th>D8</th>
<th>D21</th>
<th>D18</th>
<th>D19</th>
<th>TH01</th>
<th>FGA</th>
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<tbody>
<tr>
<td>DNA profile of suspect:</td>
<td>14/15</td>
<td>17</td>
<td>10/12</td>
<td>20/21</td>
<td>14</td>
<td>14/16</td>
<td>9/10</td>
<td>17/21</td>
<td>7/9</td>
<td>22</td>
</tr>
<tr>
<td>DNA profile from cig. butt found on crime scene</td>
<td>14/15</td>
<td>17</td>
<td>10/12</td>
<td>20/21</td>
<td>14</td>
<td>14/16</td>
<td>9/10</td>
<td>17/21</td>
<td>7/9</td>
<td>22</td>
</tr>
</tbody>
</table>
Reference samples, Sweden

New DNA db law, 1 January 2006
Forensic reference samples

Buccal swab cells transferred to FTA paper
Punch from paper used in PCR
Semi-automated DNA analysis
+ 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 exchanges 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

Fluorescence intensity (Nr of amplicons)

PCR cycle number
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  
\[\text{Cannot saturate reaction}\]
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)

- **Denature**
  - Primer
  - Probe
  - Reporter fluorescence quenched

- **Anneal**
  - Primer
  - Probe
  - Reporter fluorescence quenched

- **Extend**
  - Primer
  - Taq
  - Reporter fluorescence detected

www.nature.com
Determining the quantification cycle (Cq)
Determining the quantification cycle ($C_q$)

Fluorescence intensity

$C_q$: PCR cycle number

$f^\prime\prime\prime(\text{max})$
Determining the quantification cycle (Cq)

- **Fluorescence intensity**
- **PCR cycle number**
- **Threshold**
- **f''(max)**
- **Cq1, Cq2**
- **PCR cycle number**
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

Requirements on IAC?

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

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
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

• Web-based Java software: QPCR
  http://icbi.at/software/qpcr/qpcr.shtml
Pre-PCR processing and PCR inhibition
Applications of diagnostic qPCR

- Food and feed chain
- Archaeology
- Bioterrorism
- Clinical diagnostics
- Environmental studies
- Forensics
PCR in the test tube

- Polymerase
- Primer
- Target DNA
- Nucleotide
- Fluorophore

Mg2+
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
<table>
<thead>
<tr>
<th>PCR Inhibitor</th>
<th>Mechanism</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Calcium ions</td>
<td>Competing with Mg$^{2+}$</td>
<td>Bickley et al. 1996</td>
</tr>
<tr>
<td>EDTA</td>
<td>Chelation of Mg$^{2+}$</td>
<td>Rossen et al. 1992</td>
</tr>
<tr>
<td>IgG</td>
<td>Binds to ssDNA</td>
<td>Abu Al-Soud et al. 2000</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Release of iron ions</td>
<td>Abu Al-Soud, Rådström 2001</td>
</tr>
<tr>
<td>Proteinases</td>
<td>Degr. of Polym.</td>
<td>Powell et al. 1994</td>
</tr>
</tbody>
</table>
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

Articles on PCR vs. Year

- Articles on PCR range from 0 at 1986 to over 25,000 at 2010.
- The number of articles on PCR has significantly increased over the years, particularly from 1998 to 2006.
PCR in the literature

![Graph showing the number of articles on PCR and those dealing with PCR inhibition from 1986 to 2010.](image-url)


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

<table>
<thead>
<tr>
<th>PCR cycle number</th>
<th>DNA copies</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Lag&quot; phase</td>
<td>AE~1.0</td>
</tr>
<tr>
<td>Exp. phase</td>
<td>AE~1.0</td>
</tr>
<tr>
<td>Linear phase</td>
<td>AE&lt;1.0</td>
</tr>
<tr>
<td>Plateau phase</td>
<td>AE = 0</td>
</tr>
</tbody>
</table>

AE: amplification efficiency

Effect of PCR inhibitors

Inhibitor-tolerant polymerase

Affected polymerase
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) Reference method
# Evaluation of alternative DNA polymerases

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Mean assay amplification efficiency</th>
<th>Dynamic range of amplification (log units)</th>
<th>Detection limit (cells/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-X-Act Short</td>
<td>1.12±0.06</td>
<td>3.3</td>
<td>0.16</td>
</tr>
<tr>
<td>ExTaq HS</td>
<td>0.99±0.05</td>
<td>2.6</td>
<td>0.31</td>
</tr>
<tr>
<td>PicoMaxx HF</td>
<td>0.93±0.05</td>
<td>3.3</td>
<td>0.31</td>
</tr>
<tr>
<td>OmniTaq&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95±0.04</td>
<td>2.6</td>
<td>0.63</td>
</tr>
<tr>
<td>Taq</td>
<td>1.26±0.10</td>
<td>2.6</td>
<td>0.63</td>
</tr>
<tr>
<td>KAPA2G Robust&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.08±0.11</td>
<td>2.0</td>
<td>0.63</td>
</tr>
<tr>
<td>AmpliTaq Gold&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.46±0.67</td>
<td>1.3</td>
<td>0.31</td>
</tr>
<tr>
<td>rTth</td>
<td>1.40±0.10</td>
<td>2.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Tth</td>
<td>1.38±0.23</td>
<td>2.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protein engineered polymerase  
<sup>b</sup> Reference method
DNA profile quality assessment
DNA profile quality assessment

Intensity

PH_{1.2}

PH_{1.1}

1.1 1.2

2.1 2.2

3.1 3.2
DNA profile quality assessment

Intensity  Local balance

PH_{1.2}

PH_{1.1}
DNA profile quality assessment

Intensity
Local balance
Global balance

PH_{1.2}
PH_{1.1}

1.1 1.2
2.1 2.2
3.1 3.2
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)

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

Global balance:
Shannon entropy (SH)

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

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

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

AmpliTaq Gold\(^a\)
ExTaq HS\(^b\)
PicoMaxx HF\(^c\)
ExTaq HS/PicoMaxx HF blend\(^d\)
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

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Standard extraction</th>
<th>Standard + dilution 1:2</th>
<th>Standard + column pur.</th>
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</thead>
<tbody>
<tr>
<td>Standard polymerasea</td>
<td>0.05</td>
<td>0.08</td>
<td>1.64</td>
</tr>
<tr>
<td>2x standard polymerase</td>
<td>0.40</td>
<td>1.75</td>
<td>1.69</td>
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<tr>
<td>Alternativ X+Ya</td>
<td>10.85</td>
<td>5.59</td>
<td>2.54</td>
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</table>

Results presented as mean values of quality index
a) AmpliTaq Gold
b) ExTaq HS + PicoMaxx HF
Sample treatment vs. improved analysis

Saliva

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Standard extraction</th>
<th>Standard + dilution 1:2</th>
<th>Standard + column pur.</th>
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</thead>
<tbody>
<tr>
<td>Standard polymerase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32</td>
<td>1.51</td>
<td>1.74</td>
</tr>
<tr>
<td>2x standard polymerase</td>
<td>1.89</td>
<td>1.78</td>
<td>1.93</td>
</tr>
<tr>
<td>Alternativ X+Y&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.48</td>
<td>3.61</td>
<td>4.01</td>
</tr>
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</table>

Results presented as mean values of quality index
a) AmpliTaq Gold
b) ExTaq HS + PicoMaxx HF
Routine analysis of crime scene samples

Saliva

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Complete profiles (%)</th>
<th>Partial profiles (%)</th>
<th>Negative profiles (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard polymerase(^a)</td>
<td>38</td>
<td>47</td>
<td>15</td>
</tr>
<tr>
<td>Alternative X+Y(^b)</td>
<td>87</td>
<td>8</td>
<td>5</td>
</tr>
</tbody>
</table>

DNA concentrations 0.025-0.15 ng/µL

<table>
<thead>
<tr>
<th>a) AmpliTaq Gold</th>
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<tr>
<td>b) ExTaq HS + PicoMaxx HF</td>
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</tbody>
</table>
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

Non-ionic detergents
NP40
Tween 20

Polymers
PEG400

Organic solvents
DMSO
Effect of PCR facilitators and buffer pH

Fluorescence intensity

PCR cycle

Moist snuff extract
Trehalose
Effect of PCR facilitators and buffer pH

- Moist snuff extract
- Trehalose

Fluorescence intensity vs. PCR cycle

- pH 8.3
- pH 8.8
Questions?