Development of Protocols for Rapid Amplification of STR Typing Kits: The Use of ‘Non-Standard’ Thermal Cycler

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National Institute of Standards and Technology

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Rapid PCR

Applications
• Faster sample-to-answer
• Increase throughput
• Integrated protocols for forensics and biometrics
• Single source reference samples = 1 ng of DNA

Initial Questions
• Robustness
• Concordance
• Sensitivity
• PCR artifacts
• Stutter, peak height balance
• Locus-to-locus balance

Develop a PCR protocols for typing multiplex STR kits in less than 2 hours

Recent Literature on Integrated Typing Systems and Rapid PCR
• Development of a fast PCR protocol enabling rapid generation of AmpFlSTR Identifiler profiles for genotyping of human DNA data and rapid testing. Foster and Laurin Investig Genet. (2012) 12:4
• A protocol for direct and rapid multiplex PCR amplification on forensic samples. Verheij et al. (2012) Forensic Sci Genet. 8:72–79
• The development of mini-ampliconic STR loci for rapid analysis of forensic DNA samples on a microfluidic system. Aboul et al. (2012) Electrophoresis 31:2672–9

Final version can be found at http://www.cstl.nist.gov/biotech/strbase/NISTpub.html#Presentations
Commercial STR Kits
Thermal Cycling Times

<table>
<thead>
<tr>
<th>Year</th>
<th>Run on a 9700 thermal cycler</th>
<th>Hot start</th>
<th>Time per cycle</th>
<th>Cycles Post soak</th>
<th>Total time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997/98</td>
<td>Profiler Plus/Cofiler</td>
<td>11 min</td>
<td>3 min</td>
<td>28</td>
<td>60 min</td>
</tr>
<tr>
<td>1999</td>
<td>SGM Plus</td>
<td>11 min</td>
<td>3 min</td>
<td>28</td>
<td>45 min</td>
</tr>
<tr>
<td>2000</td>
<td>PowerPlex 16</td>
<td>12 min</td>
<td>3 min</td>
<td>55</td>
<td>30 min</td>
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<tr>
<td>2001</td>
<td>Identifiler</td>
<td>11 min</td>
<td>3 min</td>
<td>28</td>
<td>60 min</td>
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<tr>
<td>2003</td>
<td>PowerPlex Y</td>
<td>12 min</td>
<td>1 min</td>
<td>45 s</td>
<td>30 min</td>
</tr>
<tr>
<td>2004</td>
<td>PowerPlex X5</td>
<td>11 min</td>
<td>3 min</td>
<td>30</td>
<td>80 min</td>
</tr>
<tr>
<td>2007</td>
<td>PowerPlex X5S</td>
<td>2 min</td>
<td>4 min</td>
<td>30</td>
<td>45 min</td>
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<tr>
<td>2007</td>
<td>Identifiler</td>
<td>11 min</td>
<td>3 min</td>
<td>20 s</td>
<td>30 min</td>
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<tr>
<td>2009</td>
<td>PowerPlex 16HS</td>
<td>2 min</td>
<td>4 min</td>
<td>30</td>
<td>45 min</td>
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<td>SGM</td>
<td>11 min</td>
<td>3 min</td>
<td>20 s</td>
<td>30 min</td>
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<tr>
<td>2009</td>
<td>Identifiler Direct</td>
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<td>3 min</td>
<td>28</td>
<td>35 min</td>
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<tr>
<td>2010</td>
<td>Identifiler Plus</td>
<td>11 min</td>
<td>3 min</td>
<td>20 s</td>
<td>30 min</td>
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<td>2011</td>
<td>PowerPlex 380</td>
<td>2 min</td>
<td>1 min</td>
<td>30 s</td>
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<tr>
<td>2012</td>
<td>SGMExpress (direct)</td>
<td>1 min</td>
<td>48 s</td>
<td>26</td>
<td>5 min</td>
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</table>

Thermal Cyclers

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Cycler</th>
<th>Tube Vol (µL)</th>
<th># samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Biosystems</td>
<td>GeneAmp PCR System 9700</td>
<td>0.2 mL</td>
<td>96</td>
</tr>
<tr>
<td>Eppendorf</td>
<td>Mastercyler Pro S</td>
<td>0.2 mL</td>
<td>96</td>
</tr>
<tr>
<td>Qiagen</td>
<td>Rotor-Gene Q</td>
<td>0.1 mL</td>
<td>72</td>
</tr>
<tr>
<td>Cepheid</td>
<td>SmartCycler</td>
<td>25 µL</td>
<td>16</td>
</tr>
<tr>
<td>Stock</td>
<td>Piko</td>
<td>50 µL</td>
<td>8</td>
</tr>
<tr>
<td>Thermo Scientific – Finnzymes</td>
<td>Piko</td>
<td>20 µL</td>
<td>96</td>
</tr>
<tr>
<td>Analytik Jena</td>
<td>SpeedCycler</td>
<td>20 µL</td>
<td>96</td>
</tr>
<tr>
<td>Ahram</td>
<td>Palm PCR</td>
<td>20 µL</td>
<td>12</td>
</tr>
</tbody>
</table>

• We currently have these 8 thermal cyclers in house are developing rapid PCR protocols
• Varying characteristics of heating/cooling and tube (reaction vessel)
• Rotor-Gene Q and SmartCycler are real-time PCR instruments

PCR Thermal Cycling Profile
Identifiler STR kit
28 cycle PCR

<table>
<thead>
<tr>
<th>95°C</th>
<th>10 min</th>
<th>95°C</th>
<th>1 min</th>
<th>58°C</th>
<th>10 s</th>
<th>72°C</th>
<th>1 min</th>
<th>60°C</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1 min</td>
<td>58°C</td>
<td>10 s</td>
<td>72°C</td>
<td>1 min</td>
<td>72°C</td>
<td>1 min</td>
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<td></td>
</tr>
</tbody>
</table>

36 min on Gene Amp 9700

Demonstration of rapid multiple PCR amplification involving 16 genetic loci Vallone et al.
## PCR Thermal Cycling Profile

**Identifiler STR kit**

- **28 cycles of PCR**

<table>
<thead>
<tr>
<th>95°C</th>
<th>95°C</th>
<th>58°C</th>
<th>72°C</th>
<th>72°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>5 s</td>
<td>10 s</td>
<td>10 s</td>
<td>1 min</td>
</tr>
</tbody>
</table>

**36 min on Gene Amp 9700**


<table>
<thead>
<tr>
<th>95°C</th>
<th>95°C</th>
<th>61°C</th>
<th>72°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>5 s</td>
<td>15 s</td>
<td>1 min</td>
</tr>
</tbody>
</table>

**2-step PCR**

**31 min on Gene Amp 9700**


<table>
<thead>
<tr>
<th>95°C</th>
<th>95°C</th>
<th>61°C</th>
<th>72°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>5 s</td>
<td>15 s</td>
<td>1 min</td>
</tr>
</tbody>
</table>

**Reduced PCR artifacts**

### Rapid PCR Protocols: Thermal Cyclers

#### GeneAmp 9700 (Applied Biosystems)

- Heating rate: 4°C/s
- Heating mechanism: Thermal block (Al)
- Tube format: 0.2 mL
- 96 reactions per instrument run
- 28 cycles; 3-step (36 min), 2-step (31 min)

#### Mastercycler Pro S (Eppendorf)

- Heating rate: 6°C/s
- Heating mechanism: Thermal block (Ag)
- Tube format: 0.2 mL
- 96 reactions per instrument run
- 28 cycles; 3-step (19 min), 2-step (16.5 min)

#### SmartCycler (Cepheid)

- Heating rate: 10°C/s
- Heating mechanism: heating plates and air circulating fan
- Tube format: proprietary 25 μl tubes
- 16 reactions per instrument run
- 28 cycles; 3-step (21.8 min), 2-step (18.2 min)

#### Rotor-Gene Q (Qiagen)

- Heating rate: 15°C/s
- Heating mechanism: Air chamber (spinning rotor)
- Tube format: 0.1 mL
- 72 tube reactions per instrument run
- 28 cycles; 3-step (36 min), 2-step (32 min)
Rapid Advancement: Thermal Cyclers

**Philisa (Streck)**
- Heating rate: 15°C/s (cooling 12°C/s)
- Heating mechanism: Thermal block (Ag)
- Tube format: proprietary 50 µL tubes
- 8 reactions per instrument run
- 28 cycles; 3-step (17 min); 2-step (14 min)
- Requires the use of gel loading tips to load PCR product into CE setup plate due to tube design

**Piko (Thermo Scientific)**
- Heating rate: 5°C/s
- Heating mechanism: Thermal block
- Tube format: 20 µL plate
- 96 reactions per instrument run
- 28 cycles; 3-step (30.5 min); 2-step (25.5 min)

**Palm PCR (Ahram)**
- Heating rate: 7 speeds
- Heating mechanism: Thermal block
- Tube format: 20 µL tubes
- 12 reactions per instrument run
- 28 cycles; 17-37 min (only select T<sub>r</sub> and ramp)
- Hand held and battery operated for portability
Rapid Advancement: Thermal Cyclers

**SpeedCycler² (Analytik Jena)**

- Heating rate: 15°C/s (cooling 10°C/s)
- Low Profile Rapid (LPR) block
- Heating mechanism: Thermal block (Ag block, Au coated)
- Tube format: 20 µL (LPR) or 0.2 mL tube
- 96 reactions per instrument run
  - 28 cycles; 3-step (22 min); 2-step (18.5 min) – LPR times

**Difficulty obtaining tubes/plates**

No data collected

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**Effective heating/cooling rate**

<table>
<thead>
<tr>
<th>min</th>
<th>Cycler</th>
<th>Effective Heating/Cooling deg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>GeneAmp 9700</td>
<td>1.6</td>
</tr>
<tr>
<td>19</td>
<td>Mastercycler Pro S</td>
<td>6.8</td>
</tr>
<tr>
<td>36</td>
<td>Rotor-Gene Q</td>
<td>1.6</td>
</tr>
<tr>
<td>22</td>
<td>SmartCycler</td>
<td>4.4</td>
</tr>
<tr>
<td>17</td>
<td>Phelix</td>
<td>10.9</td>
</tr>
<tr>
<td>30</td>
<td>Piko</td>
<td>2.2</td>
</tr>
<tr>
<td>22</td>
<td>SpeedCycler²</td>
<td>4.4</td>
</tr>
<tr>
<td>17</td>
<td>Palm PCR</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Rates for heating/cooling were estimated from the total cycling time.

---

**Comparative Throughput (Cycling)**

<table>
<thead>
<tr>
<th>Cycler</th>
<th># samples</th>
<th>3-step</th>
<th>2-step</th>
<th>3-step</th>
<th>2-step</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneAmp PCR System 9700</td>
<td>96</td>
<td>36</td>
<td>32</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td>Mastercycler Pro S</td>
<td>96</td>
<td>19</td>
<td>17</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Rotor-Gene Q</td>
<td>72</td>
<td>36</td>
<td>32</td>
<td>2</td>
<td>72</td>
</tr>
<tr>
<td>SmartCycler</td>
<td>16</td>
<td>22</td>
<td>18</td>
<td>6</td>
<td>122</td>
</tr>
<tr>
<td>Phelix</td>
<td>8</td>
<td>17</td>
<td>14</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Piko</td>
<td>96</td>
<td>30</td>
<td>26</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>SpeedCycler²</td>
<td>96</td>
<td>22</td>
<td>19</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>Palm PCR</td>
<td>12</td>
<td>17</td>
<td>17</td>
<td>8</td>
<td>136</td>
</tr>
</tbody>
</table>

While cycling times may be rapid, the throughput in some cases is reduced from the standard 96-well format.
Experiments

• Performance of each cycler with 2- and 3-step thermal cycling protocols (n = 15 samples)

• Sensitivity study: 1 sample, 7 concentrations in duplicate; compare 2- and 3-step PCR protocols

• 95 samples run on the 9700 (2- versus 3-step comparisons)

• Initial results developing a rapid-direct PCR protocol

DNA Polymerases

• AmpliTaq Gold® is typically used
  – Heat activated (avoid non-specific PCR products)

• SpeedSTAR™ HS DNA Polymerase
  – Extension times of 100 bps are possible (compared to 20 bps for other polymerases)
  – Hot-start formulation is antibody mediated

• Qiagen
  – QIAGEN Fast Cycling PCR Kit

• New England Biolabs/Finnzymes
  – Phusion and Phire DNA Polymerases

• KAPA Biosystems
  – KAPA2G Fast PCR Kits

• Biotium
  – Cheetah™ Taq

• Fermentas
  – PyroStart Master Mix

• EMD Millipore
  – KOD DNA Polymerase

Rapid PCR Conditions

• 1 X Takara PCR mastermix, 1 U SpeedStar polymerase
  – Premix Ex Taq™ (Perfect Real Time)

• 10 μL total reaction in a thin walled tube (8-strip) or proprietary tube

• 2 μL of Identifier PCR primer mix

• ~1 ng of template DNA
AB 9700 3-Step

1 ng of DNA template

36 min
n = 15
Het balance >0.85 for all loci

AB 9700 2-Step

1 ng of DNA template

31 min
n = 15
Het balance >0.85 for all loci

MasterCycler Pro 3-Step

1 ng of DNA template

19 min
n = 15
Het balance >0.85 for all loci
MasterCycler Pro 2-Step

1 ng of DNA template

17 min
n = 15
Het balance >0.80 for all loci

SmartCycler 3-Step

1 ng of DNA template

22 min
n = 15
Het balance >0.85 for all loci

SmartCycler 2-Step

1 ng of DNA template

18 min
n = 15
Het balance >0.78 for all loci
RotorGene 3-Step

1 ng of DNA template

36 min
n = 15
Het balance >0.80 for all loci

RotorGene 2-Step

1 ng of DNA template

32 min
n = 15
Het balance >0.84 for all loci

Piko 3-Step

1 ng of DNA template

30 min
n = 15
Het balance >0.83 for all loci
Piko 2-Step

1 ng of DNA template

26 min

n = 15

Het balance >0.76 for all loci

Philisa 3-Step

1 ng of DNA template

17 min

n = 15

Het balance >0.85 for all loci

Philisa 2-Step

1 ng of DNA template

14 min

n = 15

Het balance >0.75 for all loci
Sensitivity Rotor-Gene

2-step (32 min)

3-step (36 min)

Sensitivity Piko

2-step (28 min)

3-step (30 min)

Sensitivity Philisa

2-step (14 min)

3-step (17 min)
RFU comparison for 9700 data
2-step versus 3-step

Peak Height Balance and Stutter
9700 data 2-step versus 3-step

Intra-color balance
9700 data 2-step versus 3-step
PCR Artifacts in the TH01 amplicon range

3-step PCR protocol

160 bp 168 bp 184 bp

PCR Artifacts (3-step only)

<table>
<thead>
<tr>
<th>Artifacts Observed</th>
<th>D16 @ 207 bp</th>
<th>D1 @ 131 bp</th>
<th>D8 @ 121 bp</th>
<th>D8 @ 174 bp</th>
<th>TH01 @ 166 bp</th>
<th>TH01 @ 184 bp</th>
<th>TPOX @ 219 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>STBS</td>
<td>35</td>
<td>14</td>
<td>28</td>
<td>59</td>
<td>83</td>
<td>77</td>
<td>33</td>
</tr>
<tr>
<td>Smart Cycler</td>
<td>4</td>
<td>10</td>
<td>2</td>
<td>19</td>
<td>32</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>Master Cycler Pro</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>8</td>
<td>1</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Rotor-Gene</td>
<td>6</td>
<td>7</td>
<td>11</td>
<td>25</td>
<td>40</td>
<td>22</td>
<td>114</td>
</tr>
<tr>
<td>Streck Phiila</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Total # Artifacts 302 80 7 114 2

N = 95 samples

- TH01 @ 184 is often covered/disguised by the base of the 9.3 allele peak
- 50 RFU threshold used when identifying artifacts

Rapid STR profiling in a lab setting

NIST presentation at the 2012 MAAFS Meeting

"Rapid DNA Testing Approaches for Reference Samples"

Example Rapid Work Flow in Lab Setting

single source reference samples

DNA extraction → Rapid PCR → CE separation and detection

- ZyGEM liquid-based extraction
- Rapid Identifier (9700 & Philisa cycler)
- Separations on an 8 capillary 3500

Direct PCR → CE separation and detection

- PP18D (9700)
- Separations on an 8 capillary 3500

8 unique samples were typed in parallel

ZyGEM → 9700 (2-step) → 3500

Total time from swab to answer: 1 hour 57 min 8 sec
Includes set up times

ZyGEM → Streck (2-step) → 3500

Total time from swab to answer: 1 hour 39 min 48 sec
Includes set up times
Direct Rapid PCR

- Can a direct rapid PCR protocol be developed?
- Overcome inhibitors in blood/FTA paper using rapid polymerases or additives
- Initial testing of protocols for typing blood from 903 and FTA paper substrates
Direct Rapid (Best result from FTA)

Blood (1.2 mm)  
9700 (31 min) 2-step 
SpeedStar polymerase mix 
Promega Direct Amp Reagent for FTA

Summary and Future Work

• Successful protocols developed for 6/7 cyclers tested
  – 14 min PCR on Philisa cycler
• Continue work on Palm PCR and SpeedCycler
• Under the stated conditions sensitivity is around 250-500 pg of template DNA
• Further understanding of DNA polymerases and PCR enhancers in new commercial mastermixes
• 2-step PCR protocol:
  – Faster
  – Similar sensitivity compared to 3-step
  – Comparable RFUs; peak height balance and stutter
  – Fewer PCR artifacts
• Complete STR profiling in < 2 h (swab-to-answer)

Thank you for your attention!

Questions?
Peter.Vallone@nist.gov (1-301-975-4872)

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Dave Duewer – Data analysis software (stutter, peak height ratios, multiplex balance)

Outside funding agencies:
FBI – Evaluation of Forensic DNA Typing as a Biometric Tool
NIJ – Interagency Agreement with the Office of Law Enforcement Standards