Developing a Rapid Multiplex PCR Amplification Protocol

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Outline

• Rapid PCR
• Conditions and Parameters
• miniSTR 3plex
• Commercial Kits
• Larger Custom Multiplexes
• Current Work
• Conclusions
Things That Are Rapid

PCR?

Typical STR Workflow

Sample Extraction ~2 h
Quantitation ~1.5 h
PCR ~3 h
CE Run ~1.5 h
Data Review ?

How can the time needed for cycling be reduced?

What happens when we alter cycling parameters?
How will existing commercial kits work?
How will different polymerases perform?
How robust will the results be?
Can we develop novel assays and further the understanding/limits of rapid multiplex PCR?

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Applications for Rapid PCR

• Integrated devices (‘Lab on a Chip’)

• **Screening** at a point of interest (airport, border, crime scene, intelligence community)

• Rapid STR typing ‘in the field’
  – Potential for situations/cases when a quick result is needed
  – Provide initial screening information

• Decrease overall time required for STR typing

• Do not necessarily have to use CODIS 13+ loci (fewer loci or alternative loci?)

Growing Interest in DNA for Biometrics

Efforts towards Portable/Mobile DNA Devices

• NEC (Japan)
  – Poster at Promega meeting in Hollywood, CA (Oct 1-4, 2007)
  – Press release on October 15, 2007

• Network Biosystems (based on Dan Ehrlich’s work at Whitehead)
  – http://www.netbio.com

• Mathies group at UC-Berkeley and Microchip Biotech
  – Publications… in *Analytical Chemistry*, *FSI Genetics*, etc.
  – http://www.microchipbiotech.com

• Landers group at UVA and MicroLab Diagnostics
  – Publications… *Proc Natl Acad Sci USA* 2006; 103:19272-19277
  – http://www.microlabdiagnostics.com

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
In the months ahead, we will also challenge the private sector to speed up DNA fingerprinting so that when DNA is left behind, officers can identify suspects more quickly and avoid wrongful arrests. And to do this, we will establish a six-figure prize for anyone who can invent a device tailored to the NYPD that analyzes DNA right at the crime scene. It's just one more way we are trying to bring private sector innovation into the public sector.

**Rapid PCR**

- What do we mean by rapid PCR?
  - Rapid hot start polymerases (save ~10min)
  - Shortening cycling hold times (5 sec vs 1 min)
  - Utilizing existing thermal cycling technology (AB 9700)
    - Eliminating 1 °C/sec ramp rate (9600 emulation)
    - Utilize the 9700 4 °C/sec ramp rate
  - Using commercial polymerases that are ‘faster’

Goal: cycling in less than 45 minutes
Trying simple things first…
Thermal Cyclers

- **RapidCycler 2 Instrument**
  - Ramp rate: 10°C/sec
  - [Website](http://www.idahotech.com)

- **AB 9800**
  - Ramp rate: 6°C/sec
  - [Website](http://www.appliedbiosystems.com)

- **Eppendorf Mastercycler ep**
  - Ramp rate: 4°C/sec
  - [Website](http://www.eppendorfnan.com)

- **AB 9700**
  - Ramp rate: 4°C/sec
  - [Website](http://www.appliedbiosystems.com)

Thermal Cycling

- **Hot Start**
  - 10 min vs 1 min
- **Cycling**
  - 3 min vs 25 sec
- **Soak for +A**
  - 60 min vs 1 min

- **Rapid Thermal Cycling Conditions used**
  - ~3 h
  - 36 min
  - Max Ramp Rate 4°C/sec
  - Ramp rate 1°C/sec

- **Sample 95.0 Cycle 1 of 28**
Thermal Cycling

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Trad</th>
<th>Rapid</th>
<th>Difference (min)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Start</td>
<td>Min</td>
<td>10</td>
<td>1</td>
<td>9.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Hold</td>
<td>Sec</td>
<td>60</td>
<td>5/10</td>
<td>72.3</td>
<td>50.6</td>
</tr>
<tr>
<td>Soak</td>
<td>Min</td>
<td>60</td>
<td>1</td>
<td>59.0</td>
<td>41.2</td>
</tr>
<tr>
<td>Ramp rate</td>
<td>(deg/sec)</td>
<td>1</td>
<td>4</td>
<td>22.4</td>
<td>15.7</td>
</tr>
<tr>
<td>Cycles</td>
<td></td>
<td>28</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>2:58:41</td>
<td>0:35:38</td>
<td>2:23:03</td>
<td></td>
</tr>
</tbody>
</table>

Parameter Purpose
- Hot Start: Primer Dimer, non-specific amplification
- Hold: Denature, annealing, elongation, inter and intra locus balance
- Soak: Full adenylation of PCR products

Evaluate robustness and reproducibility

Comparison of Thermal Cycling Profiles

Rapid cycling is completed by the ~4th cycle of the standard cycling profile

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Initial Work/Assumptions

- Using common materials/conditions
  - AB 9700 (10 μL volume)
  - Standard plastics
  - Commercial Polymerases
  - Final primer concentration ~0.2 μM
  - ~250 μM dNTPs, 2 mM Mg++
  - 5 color dye chemistry for labeling primers
  - Separation on AB 3130
  - Not sample limited (>500 pg of DNA)

Loci for Testing

- 26 autosomal loci characterized in our laboratory
  - Small 3plex panels
  - Larger 26plex

- Available in kits - 13 CODIS +

- Existing commercial STR typing kits are not optimized for rapid PCR

- Challenge for miniaturized/integrated STR typing platforms – since they have to use a commercial kits or develop their own...


http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Polymerases

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>Vendor</th>
<th>MasterMix</th>
<th>Hot Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqGold</td>
<td>Applied Biosystems</td>
<td>no</td>
<td>10 min</td>
</tr>
<tr>
<td>GeneAmp</td>
<td>Applied Biosystems</td>
<td>yes (2x)</td>
<td>1 min</td>
</tr>
<tr>
<td>SpeedSTAR</td>
<td>Takara</td>
<td>no</td>
<td>1 min</td>
</tr>
<tr>
<td>PyroStart</td>
<td>Fermentas</td>
<td>yes (2x)</td>
<td>1 min</td>
</tr>
<tr>
<td>Qiagen Fast Cycling</td>
<td>Qiagen</td>
<td>yes (2x)</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Brief survey of ‘fast’ commercial polymerases

Initial Testing with miniSTR 3plex

- A simple 3plex in 3 dye colors (FAM, VIC, NED)

- MiniSTR loci
  - D2S441, D10S1248 and D22S1045
  - ‘European loci’
    - Amplicon size range 65-140 bp

- These loci were previously tested under standard cycling conditions in a miniSTR multiplex in our lab
miniSTR 3plex Standard Cycling

750 pg template, 28 cycles, TaqGold, ~3 hour cycling time

Initial Rapid Testing Results

D2S441 D10S1248 D22S1045

TaqGold

Qiagen

SpeedSTAR

PyroStart

GeneAmp

w/o 9800 and specific plastics

D22 and D10 not detected
Rapid PCR 28 cycles in 36 minutes

750 pg template, 28 cycles, PyroStart, 36 minute cycling time

miniSTR 3plex Concordance

- 3plex run on a plate of samples (n = 95)
- Concordance was checked with genotypes obtained with Standard Cycling and TaqGold
- 2/285 (0.7%) of the genotype calls were discordant
- Both cases due to D10S1248 heterozygote imbalance
Peak Height Ratio
Imbalance for D10S1248

Rapid Cycling
PyroStart
PHR = 0.14

Standard Cycling
TaqGold
PHR = 0.91

18 allele was successfully amplified with addition of SpeedSTAR polymerase

Standard Cycling did not solve the problem

18 allele was successfully amplified with addition of SpeedSTAR polymerase
Dr. Peter M. Vallone  
Vermont Green Mountain Conference  
August 6th, 2008

- 2 samples were typed as 'homozygous'
- 16 samples with lowest PHR values were amplified with extra polymerase
- Balance was improved with extra SpeedStar polymerase

**Peak Height Ratio for 16 Samples**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>TaqGold</th>
<th>Pyro</th>
<th>Pyro+SS</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT94859</td>
<td>0.70</td>
<td>0.67</td>
<td>0.94</td>
<td>14,19</td>
</tr>
<tr>
<td>PT84230</td>
<td>0.68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT84243</td>
<td>0.63</td>
<td>0.28</td>
<td>0.73</td>
<td>14,17</td>
</tr>
<tr>
<td>OTO5890</td>
<td>0.66</td>
<td>0.30</td>
<td>0.69</td>
<td>14,17</td>
</tr>
<tr>
<td>WT51354</td>
<td>0.67</td>
<td>0.33</td>
<td>0.97</td>
<td>14,17</td>
</tr>
<tr>
<td>UT57303</td>
<td>0.70</td>
<td>0.37</td>
<td>0.79</td>
<td>13,16</td>
</tr>
<tr>
<td>MT97172</td>
<td>0.70</td>
<td>0.37</td>
<td>0.87</td>
<td>13,16</td>
</tr>
<tr>
<td>WT51342</td>
<td>0.71</td>
<td>0.40</td>
<td>0.99</td>
<td>13,16</td>
</tr>
<tr>
<td>WT51355</td>
<td>0.73</td>
<td>0.41</td>
<td>0.88</td>
<td>13,16</td>
</tr>
<tr>
<td>ZT80865</td>
<td>0.74</td>
<td>0.41</td>
<td>0.91</td>
<td>13,16</td>
</tr>
<tr>
<td>UT57310</td>
<td>0.75</td>
<td>0.42</td>
<td>0.88</td>
<td>14,16</td>
</tr>
<tr>
<td>PT84242</td>
<td>0.78</td>
<td>0.42</td>
<td>0.95</td>
<td>12,16</td>
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<tr>
<td>PT84241</td>
<td>0.78</td>
<td>0.46</td>
<td>0.77</td>
<td>13,16</td>
</tr>
<tr>
<td>GT37862</td>
<td>0.78</td>
<td>0.47</td>
<td>0.87</td>
<td>13,16</td>
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<tr>
<td>WT51362</td>
<td>0.78</td>
<td>0.50</td>
<td>0.85</td>
<td>14,16</td>
</tr>
<tr>
<td>ZT80863</td>
<td>0.81</td>
<td>0.51</td>
<td>0.90</td>
<td>12,15</td>
</tr>
</tbody>
</table>

**Peak Height Ratios for 3plex Loci**

<table>
<thead>
<tr>
<th>Locus</th>
<th>PyroStart – Rapid cycling</th>
<th>TagGold – Standard cycling</th>
<th>PyroStart + 1 U SpeedSTAR – Rapid cycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>D10S1248</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2S441</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D22S1045</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Samples with larger allele spreads for D10 exhibited greater imbalance e.g. 14,16 better balance than 14,19

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
D10S1248 PHR Imbalance

- Imbalance is not solely related to amplicon size
- Improved with additional SpeedStar polymerase
- Not an artifact of rapid cycling conditions
- Other reasons
  - Repeat motif?
  - Primer T_m?
  - Sequence region for SNPs?

Sensitivity of miniSTR 3plex

- 500 pg: Low signal
- 250 pg
- 100 pg
- 50 pg

28 cycles, PyroStart polymerase, 36min
Testing Commercials Kits

- Tested PP16, COfiler, Profiler Plus ID, Identifiler, and Minifiler primer mixes
  - 10 μL volume
  - 2 μL primer mix
  - PyroStart + 1 U SpeedSTAR polymerase
  - 1 ng of template DNA
  - 28 cycles (rapid cycling parameters)
  - 36 min

PowerPlex 16 Rapid Cycling

Some potential heterozygote imbalance issues?
Evaluation…

- The large 16 loci kits can be successfully amplified with a rapid thermal cycling protocol
- Additional polymerase is needed

- Which loci show imbalance?
- Limited by poorly adenylating loci?
- Can not alter primer concentrations/sequence
- Further work to be performed: sensitivity, stutter, drop out, etc

Further Evaluation of Our Loci

- We currently have an autosomal 26plex assay working in our lab
- Amplified (25/26) each locus in singleplex under rapid cycling conditions
- Evaluate each locus for signal, adenylation and artifacts
- Rank and test candidate loci in a rapid multiplex
26plex Presented at AAFS 2008

1 ng, 28 cycles, Standard Cycling


Singleplex Evaluation

>5000 RFUs

artifacts

4/25 Het imbalance?

3/25 below 1000 RFUs

-A

17 of the 25 loci pass the initial singleplex screen
Testing 4 Multiplexes

• After singleplex evaluation 4 multiplexes were tested (empirical balancing)
  – 17plex
  – 14plex
  – 12plex
  – 7plex
  \{ Subset of the 17plex \}

• Run under rapid cycling conditions
• 1 ng DNA, 28 cycles, PyroStart + 1 U SpeedSTAR
12plex

14plex

D4 – A issues

D9 Het imbalance?
Rapid Multiplex Concordance

- Results for the rapid multiplexes were compared with previously run assays (Standard cycling – TaqGold)
- N = 16 samples
- D4S2364 adenylation issues/artifacts
- D9S2157 severe het imbalance – allele drop out in 2 samples (13,13 vs 13,14) and (7,7 vs 7,11)
- Further evidence that heterozygote imbalance does not directly track with amplicon size

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Conclusions

• A large multiplex (17plex) can be amplified in **36 min** on an AB 9700 cycler
• Evaluate for adenylation efficiency and heterozygote imbalance
• Larger multiplexes require additional polymerase to obtain complete profiles

• More rigorous testing of our larger multiplexes
• Test other/faster thermal cycling platforms
• Commercial primer mixes can be used – still needs further investigation

Acknowledgements

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GWU

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