Investigation of the effects of sample degradation and inhibition in forensic DNA typing

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Large Multiplex Kits provide Efficient and Rapid Analysis of Convicted Offender Samples

Jane Doe
231657

But what about degraded DNA?

Such samples present a special challenge

Skeletal material being prepped for extraction
DNA Degradation

1. polymer hydrolyzes
   (nucleic acids break apart)

2. Pyrimidine dimers
   (bases X-link)

3. Chemical oxidation
   (bases become unreadable)
DNA Degradation

Note loss of intensity of larger alleles
Figure 6: The comparison of a standard blood DNA sample with an oxidized blood DNA sample. Samples were amplified via Power Plex 16 STR kit. The products from blood DNA, blood DNA oxidized with 1% H$_2$O$_2$ for 18h and blood DNA oxidized with 0.5% NaClO in 200pg DNA.
Miniplex 1 vs Powerplex 16
Allele Dropout: Standard DNA kit
Promega Powerplex 16

Size Range

- **100 bp**: D3 (100%), TH01 (97%), D21 (76%), D18 (23%), Penta E (16%)
- **200 bp**: D5 (81%), D13 (61%), D7 (58%), D16 (39%), CSF (23%), Penta D (16%)
- **300 bp**: A (87%), vWA (90%), D8 (74%), TPOX (61%), FGA (35%)
Allele Dropout: Miniplex Loci

Big Miniplex

Miniplex 2

Miniplex 4

D5S818 94%
D8S1179 97%
D16S539 94%

TH01 100%
CSF1PO 100%
D21S11 90%
TPOX 90%
D7S820 68%

FGA

vWA 100%
D18S51 97%
D13S317 91%
Discrepant samples between STR multiplexes

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<thead>
<tr>
<th>Locus</th>
<th>Origin</th>
<th>PowerPlex 16</th>
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<tbody>
<tr>
<td>1</td>
<td>D13S17</td>
<td>African American</td>
</tr>
<tr>
<td>2</td>
<td>D13S17</td>
<td>Hispanic</td>
</tr>
<tr>
<td>3</td>
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<td>Hispanic</td>
</tr>
<tr>
<td>5</td>
<td>D13S17</td>
<td>Hispanic</td>
</tr>
<tr>
<td>6</td>
<td>D5S818</td>
<td>African American</td>
</tr>
<tr>
<td>7</td>
<td>vWA</td>
<td>African American</td>
</tr>
<tr>
<td>8</td>
<td>vWA</td>
<td>African American</td>
</tr>
<tr>
<td>9</td>
<td>vWA</td>
<td>African American</td>
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<td>vWA</td>
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<td>15</td>
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<td>African American</td>
</tr>
<tr>
<td>16</td>
<td>vWA</td>
<td>Caucasian</td>
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</table>
Sequencing Results

D13S317 Primers and Mutations

Redundant Sequence Results in PP16 Allele being 4 bases shorter instead of resulting in loss of primer binding!
Sequencing Results (cont.)
Application of MiniSTRs in bone/bone reassociation Yugoslavia


Fig. 1. An example of a bone-bone re-association composed of eight sets of anthropologically associated/articulated partial remains recovered from two secondary mass graves. Each set of remains, represented within the black lines, were sampled. PowerPlex 16 profiles of the torso and right lower leg were matched with family reference samples resulting in a personal identity for the victim. Miniplex typing of the other sets of remains provided matches to the PowerPlex 16 profiles, allowing for a virtually complete skeleton to be reassociated and returned to the family.

Fig. 2. Proportion of reportable alleles recovered from either PP16 or the ICN short-amplicon multiplexes obtained from a series of five highly challenging bone samples. (a) Results for MP1, (b) results for MP2 and (c) results for MP3.
ABI MiniSTRs

Standard analysis reveals only a partial profile and the case hits a brick wall.

Profile is uploaded to a DNA database.

MiniSTR technology from Applied Biosystems helps identify the criminal.

MiniFiler™ Kit detects more loci and provides a hot lead.

Investigator collects ligature at crime scene.

Criminalist attempts to obtain a DNA profile with standard STR technology.

Turn cold cases into hot leads.
Amplification results for a sample degraded with Dnase 1 that was quantified using Duo. Both Identifiler and minifiler were used. The data show that in spite of the degradation of larger sized alleles, lower alleles amplified properly with Identifiler (1ng template). Larger alleles were recovered with Minifiler (0.25ng template).

Figure 7. Identifiler® kit profile obtained for a sample degraded with 0.05 units of DNase I.

Figure 8. Minifiler™ kit profile obtained for a sample degraded with 0.05 units of DNase I.

A comparison of Identifiler and Minifiler for a degraded DNA sample

Arthur J. Eisenberg, Ph.D., Xavier G. Aranda, M.S., and John V. Planz, Ph.D. Department of Pathology and Human Identification, University of North Texas Health Science Center, Forensic News, (October 2006)
Non-Traditional Samples

- The obvious application for Mini-STRs are samples which are degraded, difficult or of low copy
- We have performed experiments to examine their sensitivity and
  - Capability to amplify DNA from bone and hair
  - Capability to amplify DNA from highly degraded samples
  - Big question: How to determine quality and quantity of low level DNA?

- Answer QPCR?
Work in FIU Laboratory - with assistance of Vermont Crime Lab

- Development of miniplex STRs for degraded DNA typical sizes 60-120 bp.

- Slot blot works poorly on these samples

- So develop a series of different primers to selectively amplify degraded DNA
Determination of DNA Quality by qPCR

*Alu*Ya5 Primers – Nicklas and Buel

Primer design
Real Time PCR – 82 bp Alu
Real Time PCR – 124 bp Alu
Real Time PCR – 201 bp Alu
Quantitation of DNase I degraded DNA using 3 primer sets

An example of the quantitation results obtained with a degraded DNA sample. Error bars represent 95% confidence interval.
Multiplexed Degradation Assay

Simultaneous analysis of a 190 bp THO1 and 67 bp CSF loci with a 77 bp IPC

Application of a multiplexed single locus probe system
Using template DNA degraded with DNsasel for various times
## Interpreting the qPCR Degradation Ratio

<table>
<thead>
<tr>
<th>Degradation Ratio</th>
<th>STR Implications</th>
</tr>
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<tbody>
<tr>
<td>1 – 3</td>
<td>none</td>
</tr>
<tr>
<td>3 – 5</td>
<td>&quot;wedge&quot; effect, possible cross-dye pull-up</td>
</tr>
<tr>
<td>&gt;5 (&gt;10 ⇒ artifacts expected to be significant)</td>
<td>increasing &quot;wedge&quot; effect, pull-up, dropped-out alleles at larger loci, off-scale peaks, called stutter peaks, -A shouldering</td>
</tr>
</tbody>
</table>
Recovery of DNA from degraded Samples
UT Forensic Anthropology Center

Implications for Mass Disasters

And Questions about Recovery of Ancient DNA
Sample Extract Yields for Brush Covered Samples

DNA Concentrations Using Small and Large Alu Primers on a Brush Covered Sample

<table>
<thead>
<tr>
<th>Time of Placement</th>
<th>Concentration of DNA (ng/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>250</td>
</tr>
<tr>
<td>1</td>
<td>150</td>
</tr>
</tbody>
</table>

- **Small Primer**
- **Large Primer**
Sample Extract Yields for Full Burial Samples

DNA Concentrations Using Small and Large Alu Primers on a Burial Sample

Concentration of DNA (ng/µL)

Time of Placement

Small Primer
Large Primer
Effect of sample age on recovery of DNA – UT Forensic Anthropology Center
Application to Telogen Hairs found at Crime Scenes

- Generally hair samples are poor samples for nuclear DNA typing
- Telogen hair contains little tissue
- DNA is poorly incorporated in hair
- Hair contains melanin, a PCR inhibitor
- mtDNA is the usual method due to these problems
Nuclear DNA Extracted from Telogen Hairs

Average Extracted DNA, Individuals

- <10 pg/μL: 72%
- 11 to 25 pg/μL: 18%
- >25 pg/μL: 10%
Virtual Yield Gel for Hair Samples using Three Different Sized ALU Amplicons
Amplified Sample - Miniplex

100 pg DNA control, 12.5 µL reaction volume, 0.5 µL BSA added, 32 cycles

5 µL Hair extract, 12.5 µL reaction volume, 0.5 µL BSA added, 32 cycles
Amplified Sample – Powerplex
(Similar results obtained with Identifiler)

100 pg DNA control, 12.5 µL reaction volume, 32 cycles

5 µL Hair extract, 12.5 µL reaction volume, 32 cycles
Results for telogen hairs

• <100 pg – Mini 2 (D5, D8, D16)
  – 60% 2 loci (D5 and D16)
  – 80% at least 1 locus (D16)

• 100-500 pg Mini 2 + Mini 4 (vWA, D18, D13)
  – 70% 3 or more loci
  – 30% 1 loci or less

• >500 pg all 3 sets
  – 70% 4 loci or more
  – 40% 6 loci or more

Sometimes you can run telogen hairs
Conclusions

• MiniSTRs are for degraded DNA
• Validation data reveals a robust and sensitive multiplex amplification
• Virtual yield gel using qPCR helpful for proper analytical results
• Stochasitic effects still occur for samples under 125pg
• Improved results are possible for bone and telogen hair
• Degradation is still a problem
Inhibition and degradation

Which way to turn?
The Problem of Degradation vs Inhibition in DNA typing

Degraded DNA Sample

Ski slope effect

Powerplex 16 9947A Positive Control 0.250 ng/ 12.5 ul

Bone Sample 2003.5.6 0.250 ng/ 12.5 ul

Humic Acid Inhibited DNA Sample

Less predictable effects

TH01 TPOX CSF1PO FGA D7S820

RFU

 Degraded DNA Sample

Humic Acid Inhibited DNA Sample
PCR Inhibition: Observations

- Inhibitors act in many ways – The most worrisome are those which co-extract with DNA
- These inhibitors produce various effects on data including peak balance problems, locus specific dropout, enhanced stutter, and poor sensitivity
- Mechanisms may vary with type of inhibitor and sequence of amplicon
- It is important to understand concentration effects and mechanisms so that inhibition cannot be confused with degradation, dropout and mixture effects
The Issue:

- With increasing interest in the forensic community in the interpretation of compromised samples and mixtures, we need to be able to better interpret electropherograms in court
  - We need to determine the relative effects of DNA degradation and inhibition on peak height ratios.
  - We need to understand the combinatorial effects of different inhibitors
  - We need to understand the environmental aspects of degradation and soil inhibition
  - We need to explore the interpretation of low level mixtures in the presence of a major contributors
MiniSTR Amplification w/ increasing Humic Acid

Humic Acid: Mini 2 and Mini 4

Inhibitor Concentration (ng /25 µl)

D5 Most Affected

Ratio I/I₀

D5S818
D8S1179
D16S539
vWA
D18S51
D13S317

Inhibitor Concentration (ng /25 µl)
MiniSTR Amplification w/ increasing calcium

D5 Least Affected

Ratio I/I0

Inhibitor Concentration (μM)

D5S818
D8S1179
D16S539
vWA
D18S51
D13S317
Three potential mechanisms exist for PCR inhibition

1) Blocking of primer binding
2) Binding of the inhibitor to the polymerase.
3) Binding of the inhibitor to the DNA template.
PCR Inhibitors - consequences

- In the PCR process, the enzyme moves along the DNA strand, adding complementary bases.

- If inhibitors are present the PCR process fails - why?

- In our initial studies, the failure seems to be both a function of sequence and amplicon size.

- Mechanisms for inhibition appears to vary with size and sequence.
PCR Inhibition: Observations

• Inhibitors act in many ways – The most worrisome are those which co-extract with DNA
• These inhibitors produce various effects on data including- peak balance problems, locus specific dropout, enhanced stutter, and poor sensitivity
• Mechanisms appear to vary with type of inhibitor and sequence of amplicon
• It is important to understand concentration effects and mechanisms so that inhibition cannot be confused with degradation, dropout and mixture effects
Tests for PCR inhibition using Realtime PCR with high resolution melt

• Compare inhibition for a single locus (TH01) with primers of various lengths and melting temperatures
• Determine the effect of length and sequence on PCR inhibition
• Examine melt temperatures of amplicons in inhibited samples
• Classify inhibitors by effect on PCR
• Determine mechanism of different PCR inhibitors
Experimental Design

- **Locus** – HUMTH01 STR
- **DNA** – Homozygous 9.3 allele
- **Primers**
  - 3 lengths (~100, 200, 300 bp)
  - 3 Tm (58, 60, 62° C)
  - Amplified product (one product)
- **qPCR conditions**
  - Standard conditions for Alu quantification (Nicklas et al. 2003)
    - No BSA
    - Lower Primer Concentration
    - Reduced Taq
    - Annealing temperature appropriate for Tm (5 °C < Tm)
- **Inhibitors**
  - Calcium, humic acid, hematin, collagen, melanin, tannic acid
qPCR Calcium Inhibition

- No shift in take off cycle
- No change in melting curve
- Efficiency of amplification affected
- No difference for size or Tm

Conclusion: Taq Inhibitor
Inhibition of PP16 with CaHPO₄

CaHPO₄ 1.5 mM

CaHPO₄ 2 mM

CaHPO₄ 2.7 mM

D16, TPOX

D18, CSF, FGA PD, PE
qPCR Humic Acid Inhibition

- Shift in take off cycle
- Change in melting curve
- No efficiency of amplification change
- Size effects on melt curve

**Conclusion:** Sequence specific Inhibitor
Inhibition of PP16 with Humic Acid

Control Male 500pg

Humic Acid 16ng/ul

Humic Acid 24ng/ul

Am D3
d18 CSF PE PD
Inhibition of Identifiler w/ Humic Acid (1ng template DNA)

D3, FGA, D7, CSF, D16, D2, D18,
qPCR Melanin Inhibition

- Large shift in take off cycle
- Change in melting curve
- No change in efficiency of amplification

Conclusion: Sequence specific inhibitor
qPCR Collagen Inhibition

- Minimal shift in take off cycle
- Change in melting curve
- Change in efficiency of amplification

Conclusion: Taq inhibitor through binding DNA
Inhibition of pp16 with Collagen

- Collagen 16ng/μL
- Collagen 24ng/μL
qPCR Tannic Acid Inhibition

- small shift in take off cycle
- No Change in melting curve
- No Change in efficiency of amplification

Conclusion: Inhibition affecting the availability of template
Size Effects

• Inhibition tests examined both concentration and amplicon size of THO1 target – 100, 200, 300bp

• Results indicate Ct effects are reduced for smaller amplicons for many inhibitors. This indicates a potential advantage for these types of inhibitors with MiniSTRs and difficulty in detecting inhibitors via standard sized qPCR amplicons. 300bp shows the effect best.
Effect of amplicon length on inhibition (increasing Heme)

Larger amplicons tend to show DNA inhibitor binding effects. Small amplicons show changes in efficiency; amplifications show loss of product.
Effect of Amplicon length on Inhibition: Increasing Melanin

Larger amplicons tend to show DNA inhibitor binding effects. Small amplicons not effectned. Loss of product evident.
Important point for these results

• Just as MiniSTRs may be useful in amplifying DNA that is affected by inhibitors, short amplicons may be unable to pick up inhibitors that only affect large STRs.

• Inhibitors that bind Taq will always be detected regardless of amplicon size.
Transition metal ions

Metal cations present in degraded samples represent a different type of inhibition.

Zn$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ form DNA-metal ion complexes, termed M-DNA. at pH conditions above 8,

These cations produce severe effects in CE injection and analysis.

Types of Inhibitors

• Length dependent
  – Largest amplicon least affected by inhibition
    • Collagen
    • Affects signal – possible dye quencher
    • Possible binding to DNA

• Tm/Sequence dependent kinetics
  – Humic Acid - 60 Tm least affected
  – Tannic Acid* – 58 Tm least affected

• Change in melt curve/DNA binding
  – Humic acid, collagen
  – Larger amplicons with melanin and hematin
Types of inhibitors 2

• Taq inhibition
  – Calcium
• Length dependent/primer extension
  – Smallest amplicon least affected by inhibition
    • Hematin
    • Melanin
    • Tannic Acid
• DNA intercalation
  – Metalized DNA (transition metals Zn, Co)
  – Certain dyes
Clean-Up of PCR Inhibitors

• Bovine serum albumin (BSA)
  - Relieves PCR inhibition by making enzyme more efficient and binding certain inhibitory compounds

• Low-melting temperature agarose/sephadex/filtration
  – Relieves PCR inhibition by capturing large polymers like DNA, releasing smaller inhibitory compounds

• Electrophoretic Purification
  – Inhibitors move at different rates under applied fields

• Addition of higher concentrations of Taq polymerase
  – Overwhelm inhibitors that bind to taq

• Dilution of Sample
  – DNA still amplifies, inhibitors are less concentrated and bind to taq and/or other reaction components

• Destruction of inhibitors w/ NaOH
Conclusions

• Inhibitors can produce different profiles than degraded DNA. The effect depends on the type of the inhibitor.

• Taq inhibitors like calcium tend to affect amplification efficiency and affect longer alleles first.

• DNA Inhibitors like humic acid tend to reduce the amount of available DNA and are more sequence specific.

• All inhibitors appear to have a sized dependency and should amplify more efficiently with miniSTRs.

• However, DNA binding inhibitors may still cause loss of certain loci due to sequence specific effects.

• Short real time PCR probes will not pick up inhibition as well as longer ones.
Thank you
Acknowledgements

• Jan Nicklas and Eric Buel
• Jiri Drabek
• Denise Chung, Kerry Opel
• Nancy Tatarek, Lee Meadows Jantz
• John Butler, Yin Shen
• George Duncan

• Major support provided by
• The National Institute of Justice