A Review of PCR Inhibition and It’s Implications for Human Identity Testing

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Introduction

- DNA Extraction and PCR are the two most critical steps in Forensic DNA analysis.
- Many casework samples contain impurities that can co-purify with the DNA and affect PCR based testing.
- A better understanding of what is causing PCR inhibition and the basic mechanism(s) underlying PCR is needed before strategies to overcome inhibition are developed.
PCR Inhibition

• Inhibitors- Substances that interfere or prevent the DNA Amplification process from occurring properly (Bessetti 2007).

• When adequate copies of DNA are present PCR inhibition is the most common cause of PCR failure (Alaeddini 2012).
PCR Inhibitors

- Biological fluids (i.e. blood, semen, saliva) mixed with soil, plant material, or collected off wood, dyed cloth or leather all can contain PCR inhibitors. (Butler 2012)
- PCR Inhibition has been reported in the following forensic based samples, extracted DNA from:
  - Clothing (Larkin 1999)
  - Hair (Eckhart 2000)
Continued

- Skeletal muscle (Belec 1998)
- Blood stains (Akane 1994)
- Urine (Khan 1991)
- Feces (Monteiro 1997)
- Latent fingerprint processing dyes (VonWurmb 2001)
- Bone (Fisher 1993, Sorenson 20030)
- Soil (Braid 2001)
Intrinsic and Extrinsic Chemical Inhibitors

- Collagen Type 1 (tissues) (Scholz 1998)
- Porphyrine Residues, Myoglobin (Muscle) (Boom 1990, Belec 1998)
- Excessive DNA or DNA Template (Paabo 1989, Pillai 1991, Wurm-Schwark 2004)
- Heavy Metals (skeletal remains, soil) (Gaydosh 2012, Gaydosh-Combs 2013, Primorac 2004)
Continued

- Polysaccharides (feces, plant material) (Monteiro 1997)
- Heme (hematin) (blood) (Akane 1994)
- Proteinases (milk) (Powell 1994)
- Urea (urine) (Khan 1991)
- Indigo Dye (blue jeans) (Larkin 1999)
- Melanin (hair) (Eckhart 2000)
Laboratory Based PCR Inhibitors

- SDS, Proteinase K, Phenol
- Proper DNA Extraction protocols and techniques will remove or neutralize these (Newton 1997, Powell 1994, Katcher 1994, Rossen 1992)
Proposed Mechanisms of PCR Inhibition

- Effect of PCR Inhibitors well documented, the mechanism are not at all clear or understood.
- Simulated studies on co-purified inhibitors did not affect all PCR equally (Alaeddine 2012, Huggett 2008)
- Example- Real-Time PCR experiments demonstrated that based on amplicons with variable lengths, melting temperatures, and primer sequences that primers with a higher melting temperature are not as affected by inhibition (Opel 2010)
Mechanisms Continued

- Interfere with cell lysis compromising DNA Extraction (Wilson 1997)
- Degrade or Bind to DNA (Wilson 1997, Combs-Gaydosh 2013)
- Inhibit or effect polymerase activity (Wilson 1997, Akane 1994)
Maillard Reaction

- PCR failure has been attributed to a brown substance that co-purifies with the DNA and not removed by conventional DNA Isolation methods (Alaeddini 2012, Tsai 1991, Watson 2000, Tebbe 1993)
- Substance appears as a blurred, blue fluorescent under UV light on DNA electrophoresis products (Scholz 1998, Hanni 1995)
Continued

- Substance blocks fluorescence detection in real-time PCR (Al-Soud 2000)
- Blocks formation of primer dimers (Fisher 1993)
Humic Compounds and the Maillard Reaction

• Humic compounds are dark-colored, amorphic and stable (Borkovsky 1965)
• Produced through the Maillard Reaction (Rocha 1998)
• Maillard Reaction- biochemical aging process
• Non-enzymatic browning reactions between amines and carbonyl compounds (esp. reducing sugars such as glucose or G-6-P) (Aladenni 2012).
Continued

- Dehydration of the adducts will produce a yellow-brown fluorescent compound that cross-link proteins (Bucala 1984).
- Reaction affected by heating, moisture, pH and sugar type (Abelson 1971).
- Humic Acids (HA) can be extracted with DNA (Tebbe 1993).
- 0.08µg/ml of HA is sufficient to inhibit Taq polymerase (Tebbe 1993).
Continued

- HA have a charge to mass ratio similar to DNA and exhibit structural heterogeneity. This makes hem easier to be co-purified with DNA, though amount and composition might depend on the extraction method employed (Steffan 1988).
Inhibition Properties of HAs

- Physiochemical properties similar to the sugar-phosphate backbone of DNA
- Compete with DNA adsorption sites during DNA purification
- May chelate Mg++
- Has a negative charge like DNA
Properties Continued

- Maillard products may possess DNA breaking activities.
- Entrap DNA making it inaccessible to the DNA polymerase.
- Oxidize to form quinones which covalently bind to and inactivate DNA polymerases (i.e. complete inhibition of TaqMan Real-Time PCR assays have been demonstrated at concentration of 1.4 ng/25 ml reaction. Below that effects amplification efficiencies and quantitation estimates (Hiramoto 1993, Hiramoto 1994, Poinar 1998, Young 1993, Kontanis 2006).
• Some real-time PCR studies suggest a sequence specific of binding to and inactivating a portion of the DNA template (HAs and melanin). Collagen and hematin seem to affect Taq polymerase activity and tannic acid seems to bind to both DNA and Taq (Opel 2010).

• HAs – Uncompetitive Inhibition; binds to the enzyme-substrate complex making it unreactive. ↓Vmax Kmax also shifts Tm to a higher temperature (Sutlovic 2008)
DNA Glycation

- Besides Maillard products co-purifying with DNA, amino groups of nucleic acids can be modified by reducing sugars (Bucala 1985, Ikan 1996).
- Glycation modifies the nitrogenous base of DNA which block PCR.
- PCR Inhibition has been reported to affect ancient DNA (degraded) more than optimal or modern samples. This might be explained by a time dependent DNA glycation (Kemp 2006).
Effects PCR Inhibition on DNA Quantitation


- Small variations in PCR efficiency between samples due inhibitor activity can significantly affect accurate DNA Quantitations (Ramakers 2003)
Continued

- Studies measuring amplification performance using a dilution series suggests that, next to removing the inhibitor, diluting the inhibitor can reduce their negative effect on quantitation (Stahlberg 2003).
Detecting PCR Inhibition

- Gel Electrophoresis (Sambrook 1998)
- Dot Blots (Saiki 1989)
- HPLC (Katz 1990)
- Calorimetric Assays (Mantero 1990)
- Amplification failures of Internal PCR Controls (Imaizumi 2004)
• Use of an Internal Positive Control (IPC) is the method of choice for quantitative real-time PCR in forensic analysis for monitoring PCR inhibition (Kontanis 2006)

• Co-amplifying exogenous DNA (IPC) along with the genomic target in the same reaction (Swango 2006, Swango 2007)

• Used in commercial kits (Green 2005)
Continued

• In the presence of PCR inhibitors, reactions cross the detection threshold (CT) at later cycles due to a decrease in the slope at both the exponential phase curve and the linear phase curve in an amplification plot. As a result of the suppressed amplification samples with inhibitors reach lower detection levels than those w/o (Kotanis 2006).
Not all target sequences are equally susceptible to inhibition, thus the effects on the IPC may not be as predictable as thought (Stahlberg 1997, Hugget 2008, King 2009, Gaydosh-Combs 2013).
Overcoming PCR Inhibition

- General Solutions
- Dilution (Alonso 2001)
- BSA (Oikarinen 2009)
- Heat Soaked PCR (Ruano 1992)
- Hot Start PCR (Kermekchiev 2003)
- Extra Polymerase Enzymes (Eilert 2009)
Extraction Purification Protocols

- Boiling for urine and cervical samples (Van Vleenhoven 1996, Verkooyen 1996)
- Aqueous two-phase sample preparation (Lantz 1994)
- Density Gradient Centrifugation (Lindqvist 1997)
- Enrichment media (bacteria) (Wernars 1991)
- Filtration (bacteria) (DiMichele 1993)
- Proteinase inhibitors (Al-Soud 2000)
• Phytase (fecal) (Thornton 20000)
• Immunological methods (Fluit 1993)
• Magnetic Capture Hybridization (Jacobson 1995, Yankson 2009)
• Electroelution (Chandler 1997, Kallmeyer 2009)
Removal of Humic Acid Compounds

- Has contain phenyl groups (Hedges 1988)
- Polyvinylpolypyrrolidone (PVPP) absorbs phenolic compounds and can be incorporated into purification protocols (Berthelet 1991, Trevors 1992, Young 1993)
- PVPP hydrogen bonds with HAs containing phenol groups to form PVPP-phenolic compounds (Sutlovic 2007)
Adding Extra Extraction Methods

- Amplification success can be enhanced by additional extraction steps or steps to remove PCR inhibitors (Hoff-Olsen 1999)
- Pre-PCR Centricon 100 dialysis (Jung 19910)
- Agarose embedded DNA followed by washing with a lysis solution (difference in size between DNA and smaller PCR inhibitors) (Moreira 1998)
• DNA precipitation with isopropanol or polyethylene glycol 8000 (Hanni 1995, LaMontagne 2002)
• Sephadex G-50 chromatography, Sepharose 4B, Thiopropyl Sepharose 6B (Rogan 1990, Jackson 1997, Shutler 1999)
• Cetyltrimethyl-ammonium bromide (CTAB)- has a hydrophobic group and a (+) charge group. Forms insoluble complexes with cell debris, proteins, polysaccharides and binds to DNA electrostatically (Zheng 2004, Saano 1995, Liu 2007)
Continued

- Bourke et al (Bourke 1999) based a method involving the use of 0.4 mM NaOH and Microcon-100 filter. Inhibitors tend to bind to and stabilize ds-DNA and have a smaller molecular size than DNA. Treating the DNA with 0.4nM NaOH denatures the DNA releasing the inhibitors into solution and then passing it through the Micrcon-100 filter retains the DNA on the membrane but allows smaller molecules to pass it into the solute. The DNA can now be amplified.
• A method replacing the Microcon-100 with the Microcon-30 was developed for use with mt DNA (Kemp 2006).
• Treatment with NaOH is not recommended in Trace or Low Copy Number DNA samples as it has reported to create a significant loss of DNA (Primora 2004).
Diluting the DNA Extract

• Most common method to overcome inhibition is to dilute the inhibitors below their effective concentration (Imaizumi 2005, Shutler 1999, Lalueza 1996). This method though can compromise results in Low Copy Number samples (Ye 2005).
Amplification Facilitators

- BSA
- SSBP T4 gene 32 protein
- Betaine
- 5% DMSO
- 5% Formamide
- 10-100µM Tetramethyiammonium chloride
- 5-15% PEG
• 10-15% Glycerol
• 0.1-2.5% Tween 20
• 7 deaza-dGTP
• Note: Adding an excess concentration of enhancers can sometimes cause PCR inhibition
Polymerase Enzymes

- Adding extra polymerase can sometimes overcome inhibition but it can also cause an increase in non-specific amplification (Fisher 1993, Edwards 2004).
- Using different polymerases with different properties (inhibition and facilitation) might help (Al-Soud 2000).
- i.e. Tth DNA polymerase and some Hot Start polymerases and some T. aquaticus work well with skeletal remains and Has (Eilert 2009)
• Genetically engineered polymerase (Kermekchiev 2009) pfu DNA polymerase, KlenTaq LA and Real Taq DNA polymerases (Matheson 2010) or a blend of inhibitor resistant polymerases – buffer systems (Hedman 2010) have also been reported to overcome some inhibitors
Chaotropic Silica Based Extraction Methods

- May not know specific cause of inhibition so most useful to develop robust extraction methods to remove as many inhibitors as possible (Bourke 1999).

UNTHSC Studies

- Comparative Effects of Hematin and Humic Acid Inhibition of Forensic Multiplex STR Genotyping Systems and Commercial PCR Enhancers
- Metal Ions as Forensically Relevant Inhibitors of PCR
Overview of Experiments

• Aim: determine the best chemistry combination for routine forensic samples subjected to inhibitor treatments

• Evaluate effect of inhibitors on results
  • Forensic multiplex STR genotyping systems
    • Without enhancer treatment
    • Commercially-available PCR enhancers
      STRboost™ (Biomātrica Inc., San Diego, CA)
      Bovine serum albumin (BSA)
Sample Preparation

- Hematin stock solution
  - Porcine hematin (Sigma-Aldrich, St Louis, MO)
  - 0.1 N sodium hydroxide (NaOH) solution (FisherChemical, Fairlawn, NJ)

- Humic Acid stock solution
  - Technical grade humic acid (Sigma-Aldrich, St Louis, MO)
  - TE-4 buffer (10 mM Invitrogen UltraPure™ Tris-HCL, pH 8.0, Invitrogen Corporation, Carlsbad, CA and 0.1 mM GIBCO UltraPure™ EDTA, pH 8.0, GIBCO Products, Grand Island, NY)

- Subsequent dilutions prepared with 0.1 N NaOH solution and TE-4 buffer, respectively

- Final samples: target concentration of inhibitor
  - plus 1 ng/ 7.5 µL of 9947A cell line DNA
Experimental Design

- Two Chemistries: ABI Identifiler and Promega PowerPlex 16 HS
- Two Inhibitors: Varying Concentrations of Hematin and Humic Acid
- Treatments: dH2O, BSA, STRboost (Biometrica)
Analysis & Genotyping

- Analyzed by capillary electrophoresis
  - ABI Prism 310 Genetic Analyzer
    (Applied Biosystems, Foster City, CA)
  - 5 second injection, 30 minute duration
- Genotyping to determine STR profiles
  - GeneMapper® ID v.3.2 software
    (Applied Biosystems, Foster City, CA)
  - 25 rfu allele detection threshold
Methods: Statistical Analysis

• Concordance between duplicate samples
  • Chi-Square Goodness of Fit
  • Paired T-Test hypersensitive to inhibited data

• Results between chemistry combinations
  • Average allele counts, as percent of expected number of alleles for 9947A

• Effect of inhibitor on quality of results
  • Regression analysis
  • Pearson’s correlation coefficients
Results: Chi-Square Test

- Individual allele peak heights between duplicate samples were not significantly different.
- All p-values > 0.999, using the following parameters:
  - PowerPlex® 16 HS
    - \( n \text{ allele pairs} = 25, \text{ df} = 24 \)
  - AmpF/STR® Identifiler®
    - \( n \text{ allele pairs} = 26, \text{ df} = 25 \)
Summary

• Hematin-Treated Samples
  • Failure of AmpF/STR® Identifiler® reactions
    • Subsequent tests suggest NaOH, not hematin
  • PowerPlex® 16 HS System plus STRboost™
    • Enhancer treatment not essential for full profiles

• Humic Acid-Treated Samples
  • AmpF/STR® Identifiler® plus STRboost™
  • PowerPlex® 16 HS System without enhancer
    • BSA effective at the two highest concentrations
Discussion: Biological Context

- Hematin-Treated Samples
  - Realistic: between (#4) 2.5 \(\mu\text{M}\) and (#5) 25 \(\mu\text{M}\)
  - Likely: less than (#2) 0.025 \(\mu\text{M}\)
    (Akane 1994)

- Humic Acid-Treated Samples
  - Realistic: up to (#4) 100 ng/\(\mu\text{L}\)
    (Tebbe & Vahjen 1993)
  - Likely: less than (#1) 3.7 ng/\(\mu\text{L}\)
    (Tsai & Olson 1992)
Background

- **Polymerase chain reaction (PCR) inhibitors**
  - Co-purify with DNA
  - Reduce quality or prevent genotyping results

- **Developmental validation studies**
  - Aim: assess stability, performance threshold
  - No reference to biological relevance
    - Outside the scope of those studies
Broader Problem

- **Ultimate goal:**
  - Improve human identification efforts by preventing carryover of PCR inhibitors during extraction

- **Objectives:**
  - Define tolerance of PCR systems to metals
  - Determine biologically-relevant concentrations of metals for forensic purposes
Overview of DNA Experiments

- **Aim:** determine the effects of metal inhibitors on genetic results obtained from samples amplified using the AmpFLSTR® Identifiler® Plus multiplex system and the PowerPlex® 16 HS System.

- **Evaluate effects of inhibitors, including:**
  - Aluminum (Al)
  - Calcium (Ca)
  - Copper (Cu)
  - Iron (Fe)
  - Nickel (Ni)
  - Lead (Pb)
Amplification, Analysis & Genotyping

- Amplification, duplicate samples
  - AmpFLSTR® Identifiler® Plus  (Applied Biosystems, Foster City, CA)
  - PowerPlex® 16 HS  (Promega Corporation, Madison, WI)
- Fragment analysis by capillary electrophoresis
  - 3130XL Genetic Analyzer  (Applied Biosystems)
  - 10 second injections
- STR profiles, including individual allele peak heights and areas
  - GeneMapper® ID v.3.2 software  (Applied Biosystems)
  - 50 rfu allele detection threshold
Statistical Analyses

- Amplification success
  - Average allele counts, as percent of expected number of alleles for 2800 M DNA

- Effect of inhibitor on quality of results
  - Regression analysis
  - Pearson’s correlation coefficients
Bone Sample Preparation

- Human bone samples
  - Adjudicated casework bone powder (UNTHSC, Center for Human ID)

- Nitric Acid (HNO₃) digestion
  - 100 mg bone, dissolved into 2 mL of pure HNO₃
  - Second dilution into 100 mL of 1% HNO₃
Elemental Analysis

- Inductively Coupled Plasma-Mass Spectrometry
  - Aurora M90 ICP-MS System (Bruker, Bremen, Germany)
  - Triplicate measurements
    - Solution mode
  - Isotopes measured:
    - $^{27}$Al, $^{43}$Ca, $^{63}$Cu, $^{57}$Fe, $^{60}$Ni, and $^{208}$Pb
Average Percent Alleles Obtained from Metal-Treated Samples, Identifiler® Plus

Average Percent 2800M Alleles Obtained Using Identifiler® Plus

Percent Alleles Obtained

Inhibitor Concentration

0.001 0.006 0.03 0.2 1.25 7.5

Al Ca Cu Fe Ni Pb
Average Percent Alleles Obtained from Metal-Treated Samples, PowerPlex® 16 HS

Average Percent 2800M Alleles Obtained Using PowerPlex® 16 HS

- Al
- Ca
- Cu
- Fe
- Ni
- Pb
Inhibition Phenomena: Reduced RFUs

1000 RFU

500 RFU
Inhibition Phenomena: Allelic Drop-Out
Inhibition Phenomena: Preferential Amplification/ Degradation Curve
Inhibition Phenomena:
Electrophoretic Mobility Shift
Inhibition Phenomena:
Electrophoretic Failure
Effect of Fe on Identifiler® Plus Peak Heights

![Graph showing effect of Fe concentration on Identifiler® Plus peak heights.](image-url)
Direction of Future Research

- Determination of co-extracted and co-purified concentrations for each metal from bones
  - Adjudicated casework bone powder
  - Environmental simulation for metal transfer
  - Organic/PCIA and chaotrope/silica-based methods

- Continued collaboration with UNT-Chemistry
  - LA-ICP-MS, allows direct sampling of solid bone