The NIST Experience with Identifiler® Plus

Becky Hill
National Institute of Standards and Technology

Applied Biosystems HID University
McLean, VA
August 17, 2010

Outline of Topics to Discuss

• Background information on Identifiler Plus and the major differences with Identifiler
• Low Template (LT) DNA samples
  – Challenges and limitations with LT-DNA testing
  – Approaches to genotyping low template DNA
  – NIST LT-DNA data and Peak Height Ratios (PHR) with Identifiler Plus
  – LT-DNA mixture samples using Identifiler Plus
• Direct PCR results with Identifiler Plus
• Identifiler Plus data from the 3500xl Genetic Analyzer
• Summary and Conclusions

Identifiler Plus PCR Amplification Kit

Similarities with Identifiler

• Primer sequences and concentrations are the same
• Amplicon sizes are the same (<360 bp)
• Same amount of alleles in allelic ladder
• Same dye set (G5)
• 25 µL reaction volume
• Same species specificity and precision

Differences with Identifiler

• Master Mix and Primer Mix only – no separate Taq/enzyme to add
• 2 recommended cycling protocols
  – 28 cycles: 1 ng optimum input DNA, full profiles with 125 pg
  – 29 cycles: <500 pg input DNA, extra sensitivity for <125 pg
• Shorter thermal cycling conditions (decreased by ~ 1 hour)
  – One denaturation step (20 sec @ 94ºC)
  – One annealing/extending step (3 min @ 59ºC)
  – Much shorter final extension (10 min @ 60ºC)
• Cleaner baseline and improved heterozygote peak balance
• Optimized to overcome inhibition

Thermal Cycling Conditions

Identifiler Plus is about 1 hour shorter than Identifiler
Identifiler Allelic Ladder

Peak height imbalance, peak heights between dye channels are imbalanced

Identifiler Plus Allelic Ladder

Much better peak height balance, uniform heights between dye channels, same alleles

Identifiler Plus with 1 ng, 28 cycles (Standard Protocol)

Peak Heights <8000 RFUs, no adenylation issues, well balanced peak height ratios

Identifiler Plus with 1 ng, 29 cycles

Peak Heights <9000 RFUs, incomplete adenylation, bleed through

MT97150, 100 pg, 28 cycles

Peak Heights <1500 RFUs, no adenylation issues, slight peak height imbalance

MT97150, 100 pg, 29 cycles

Peak Heights <2500 RFUs, no adenylation issues, slight peak height imbalance
Low Template (LT) DNA Samples

Some Definitions of Low Template (LT) DNA
- Working with <100-200 pg genomic DNA
- Considered to be data below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)
- Enhancing the sensitivity of detection (increasing PCR cycles, PCR product clean-up, increasing CE injection/voltage)
- Having too few copies of DNA template to ensure reliable PCR amplification (allelic or full locus drop-out)
- Can often be the minor component of mixture samples consisting of low level DNA template amounts

Challenges of LT-DNA Testing
- Increased chance for contamination (want a sterile lab environment to reduce staff contamination)
- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
  - Heterozygote peak imbalance
  - Allele drop-out
  - Allele drop-in
  - Increased stutter products

Stochastic (Random) Effects with LT-DNA
- Loss of True Signal (False Negative)
- Gain of False Signal (False Positive)

Suggestions for Optimal Results with LT-DNA
- Typically at least 2 – 3 PCR amplifications from the same DNA extract are performed to obtain consensus profiles
- An allele cannot be scored (considered real) unless it is present at least twice in replicate samples
- Extremely sterile environment is required for PCR setup to avoid contamination from laboratory personnel or other sources

Typical LT-DNA Analysis Procedure
- Extract DNA from stain
- Quantify Amount of DNA Present
- Perform 3 Separate PCR Amplifications
- Interpreting Alleles Present
- Develop a Consensus Profile (based on replicate consistent results)
Signal Enhancement Techniques

- Additional PCR cycles
- More sensitive kits (Identifiler Plus)
- Microcon cleanup to remove salts that interfere with electrokinetic injection
- Lower PCR volume (concentrates amplicon)
- Increase TaqGold/enzyme concentration
- Longer CE injection times and voltage
  - 10 s @ 3 kV = 30
  - 5 s @ 2 kV = 10

NIST Example LT-DNA Data with Identifiler Plus

Experimental Design to Study LT-DNA Issues

- Pristine DNA Samples
  - 2 single-source samples
  - heterozygous for all loci tested (permits peak height ratio studies)
- Low DNA Template Amounts
  - Dilutions made after DNA quantitation against NIST SRM 2372
  - 100 pg, 30 pg, and 10 pg (1 ng tested for comparison purposes)
- Replicates
  - 5 separate PCR reactions for each sample
- STR Multiplex Kits
  - Identifiler Plus (half-reactions)
- Increased Cycle Number
  - Identifiler Plus (29 cycles and 32 cycles; 28 for 1 ng)

Identifier Plus (½ Reaction)
1 ng @ 28 cycles

High signal, balanced peak heights (>0.80), no artifacts, low stutter

A Fully Heterozygous Sample (2 alleles for each locus)

Identifiler Plus, 100 pg @ 32 cycles, ½ Reaction

*No drop-out, slight peak height imbalance, full profiles in all replicates

Identifiler Plus, 30 pg @ 32 cycles, ½ Reaction

*No allelic drop-out in replicates, significant peak height imbalance
The NIST Experience with Identifiler Plus

Sensitivity Comparison
Tested sample is heterozygous (possesses 2 alleles) at every locus, which permits an examination of allele dropout

Impact of Three More PCR Cycles

Peak Height Ratio: Identifiler Plus, 32 cyc

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Becky Hill – ABI HID University
The NIST Experience with Identifiler Plus

August 17, 2010

D3S1358 replicates with 3 extra cycles

False Homozygote with 1494 RFUs, well above any stochastic thresholds

*Any combination of 3/5 replicates gives the correct genotype (14,19)

D3S1358 replicates with 3 extra cycles

Allele “19” drop-out

Identifiler Plus, 32 cycles, 10 pg DNA

Examination of LT-DNA Mixtures with Identifiler Plus

LT-DNA Mixture Samples

- 2 samples (male and female) were mixed together at 1:3 and 1:5 – 1 ng (1:3 and 1:5) or 100 pg (1:5) or 50 pg (1:3) total DNA
- 3 person mixture (2 males and female) were mixed together at 1:2:3 – 1 ng or 100 pg total DNA
- Identifiler Plus (28 and 31 cycles) was tested (half reactions)
- 5 replicates with 3 extra cycles

Individual Mixture Components

GT:OT, 1:5, 100 pg @ 31 cyc

2-Person Mixture (1:5)

Individual Mixture Components

2-Person Mixture (1:3)

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
**Summary of Data Observed**

- The results with pristine full heterozygous samples demonstrate that replicate testing can produce reliable information with single source samples at low levels of DNA when consensus profiles are created.

- With 3 extra cycles, there was better recovery at 10 pg of DNA using Identifiler Plus including less allelic and full locus drop-out. However, there is a greater potential for allele drop-in or high stutter.

- Variability of peak heights in replicates was observed with LT-DNA mixtures using Identifiler Plus.

- More minor contributor peaks were called with 3 extra cycles using Identifiler Plus.

**Direct PCR with Identifiler Plus**

**Experimental Design**

- Full reactions (25 µL total volume)
- 1.2mm punches
  - Buccal and blood samples
    - 903 and FTA paper
    - Two samples for each condition
- Punch added directly to Master Mix
- Manufacturer thermal cycling protocol
  - 28 cycles
Becky Hill – ABI HID University
The NIST Experience with Identifiler Plus

August 17, 2010

Data provided by Erica Butts

903 Blood Punch, Sample 1

*Full profile with good heterozygote peak balance

Adenylation issue

Pull up

Data provided by Erica Butts

903 Blood Punch, Sample 2

*Full profile with good heterozygote peak balance

Data provided by Erica Butts

FTA Blood Punch, Sample 1

*Full profile with good heterozygote peak balance

Adenylation issue

Pull up

Data provided by Erica Butts

FTA Blood Punch, Sample 2

*Full profile with good heterozygote peak balance

Adenylation issue

Pull up

Data provided by Erica Butts

903 Saliva Punch, Sample 1

*Partial profile with full locus drop-out

Data provided by Erica Butts

903 Saliva Punch, Sample 2

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Summary of Data Observed

- Full profiles were obtained from FTA blood, FTA saliva, and 903 blood punches for both samples using Identifiler Plus with direct PCR.

- Partial profiles were obtained from 903 saliva samples - this could be due to sampling issues.

- There were some adenylation issues with FTA blood and saliva samples – this could be remedied with lower injection or less PCR cycles.

- FTA blood, FTA saliva, and 903 blood profiles had excellent heterozygote and locus-to-locus peak height balance.

Identifier Plus data on the 3500xl Genetic Analyzer (@ AFDIL)

Experimental Design

- Identifiler Plus kit was used with full reactions (25 µL total volume)
  - 0.5 ng DNA, 28 cycles
    - GS 500 LIZ size standard
    - GS 600 LIZ v2 size standard (normalization)
  - 23 population samples + 1 Allelic Ladder
- 3500xl data compared to 3130xl data
  - Default injection of 1.2 kV, 24 sec (3500xl)
  - Low injection of 1.2 kV, 10 sec (3500xl)
  - Default injection of 3 kV, 10 sec (3130xl)
Excellent peak height balance, uniform peak heights between dye channels

Identifiler Plus Ladder 3500x/l

Different Size Standards (3500x/l)

Size Standards (3500x/l vs 3130x/l)

3500x/l Default Injection, GS 500

3500x/l Low Injection, GS 500

3130x/l Default Injection, GS 500

http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm
Summary of Data Observed

- The RFU scale for the 3500xl is different than the 3130xl (30000 RFU vs 8000 RFU).
- The 3500xl instrument is more sensitive than the 3130xl – can adjust the injection time and voltage.
- Identifiler Plus profiles on the 3500xl are well balanced (inter- and intra-locus and between dye channels).
- The GS 600 v2 size standard is for the normalization of data between different instruments in the lab; the data is comparable to data using the GS 500 size standard.

Conclusions

- Identifiler Plus is a highly sensitive kit that can result in full profiles down to 30 pg of DNA.
- With 3 extra cycles in LT-DNA mixtures, more minor contributor peaks were called using Identifiler Plus.
- Full profiles were obtained from FTA blood, FTA saliva, and 903 blood punches using Identifiler Plus with direct PCR.
- Identifiler Plus profiles on the 3500xl are well balanced including good inter- and intra-locus balance as well as between dye channels.

Acknowledgments

NIST Funding: Interagency Agreement 2008-DN-R-121 between the National Institute of Justice and NIST Office of Law Enforcement Standards

NIST Disclaimer: Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

A special thanks to Applied Biosystems for providing the kits used in this study

Contact Info: becky.hill@nist.gov, 301-975-4275