The NIST Experience with Identifiler® Plus

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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 3500*xl* 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

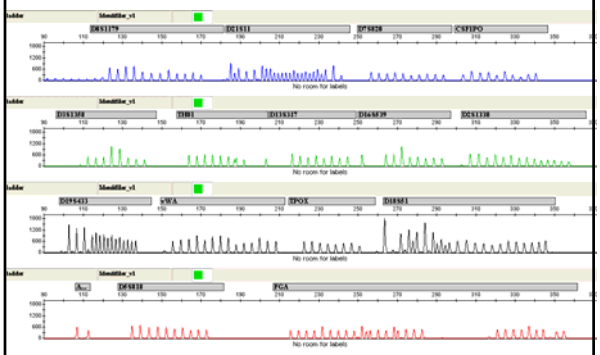
Initial incubation step	Cycle (28 or 29 cycles[1])		Final extension	Final hold
	Denature	Anneal/Extend		
HOLD	CYCLE		HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 3 min	60 °C 10 min	4 °C ∞

Identifiler — **This step is eliminated**

Initial Incubation Step	Denature	Anneal	Extend	Final Extension	Final Step
HOLD	CYCLE (28 cycles)			HOLD	HOLD
95 °C 11 min	94 °C 1 min	59 °C 1 min	72 °C 1 min	60 °C 60 min	4–25 °C (forever)

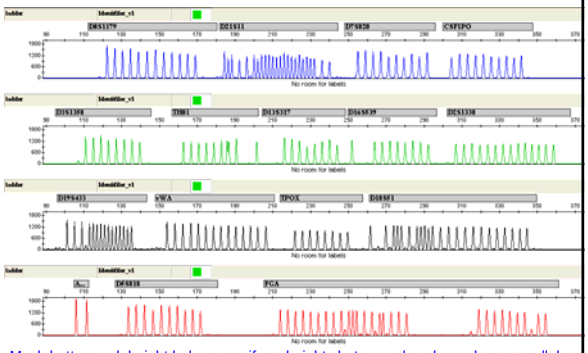
Identifiler Plus is about 1 hour shorter than Identifiler

Identifiler Allelic Ladder



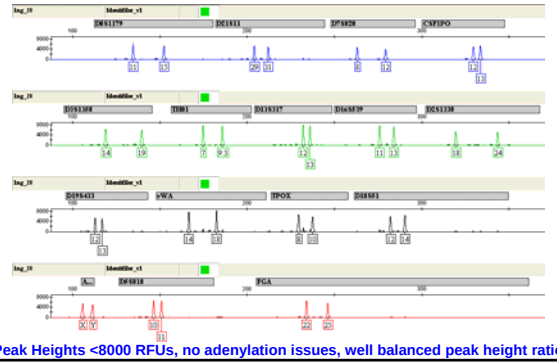
Peak height imbalance, peak heights between dye channels are imbalanced

Identifiler Plus Allelic Ladder



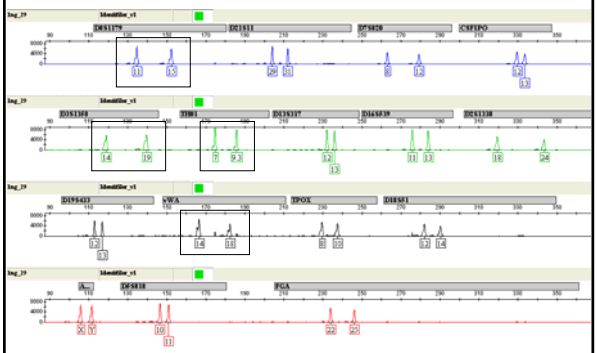
Much better peak height balance, uniform heights between dye channels, same alleles

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



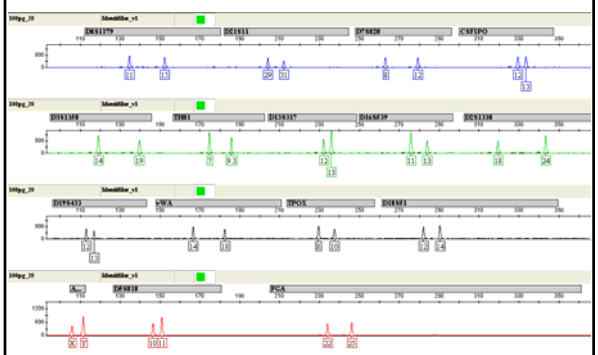
Peak Heights <8000 RFUs, no adenylation issues, well balanced peak height ratios

Identifiler Plus with 1 ng, 29 cycles



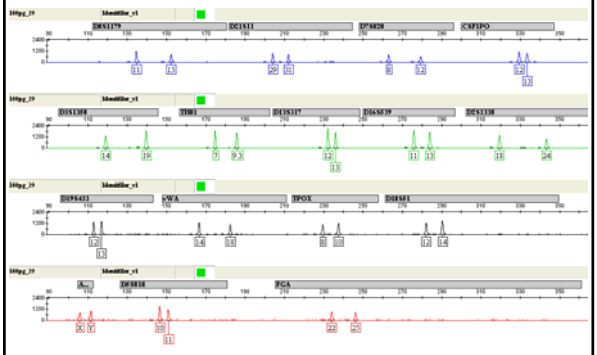
Peak Heights <9000 RFUs, incomplete adenylation, bleed through

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

Gill, P. (2001) *Croatian Med. J.* 42(3): 229-232

- 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

LT-DNA profiles should be interpreted with careful guidelines

Stochastic (Random) Effects with LT-DNA

When Combined with Higher Sensitivity Techniques

Loss of True Signal (***False Negative***)

Heterozygote Peak Imbalance

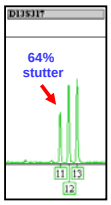
Identifiler, 30 pg DNA, 31 cycles

Allelic **Drop-out**

Identifiler, 30 pg DNA, 31 cycles

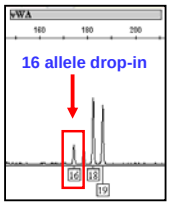
Gain of False Signal (***False Positive***)

Higher Stutter



Identifiler, 10 pg DNA, 31 cycles

Allelic Drop-in

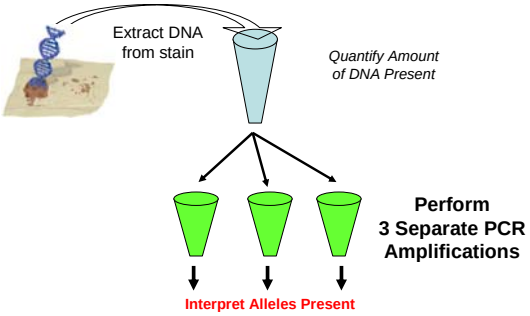


Identifiler, 10 pg DNA, 31 cycles

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



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

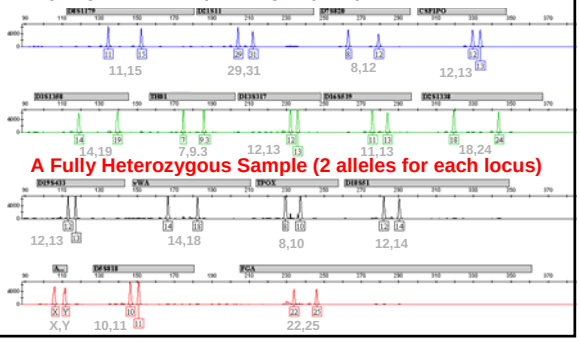
NIST Example LT-DNA Data with Identifiler Plus

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)

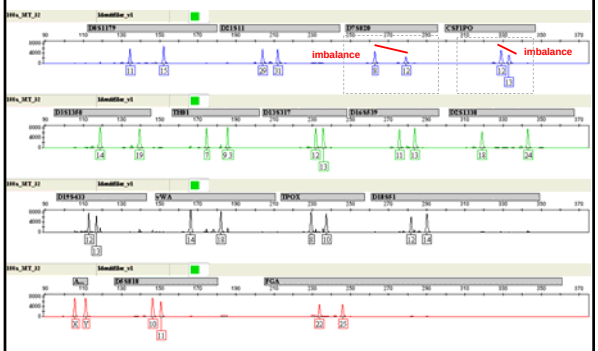
Identifiler Plus (½ Reaction)
1 ng @ 28 cycles

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



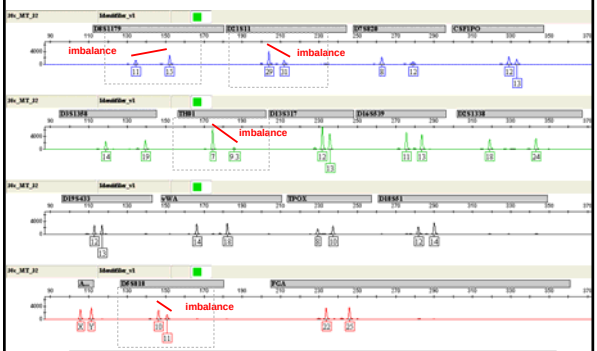
A Fully Heterozygous Sample (2 alleles for each locus)

Identifiler Plus, 100 pg @ 32 cycles, ½ Reaction



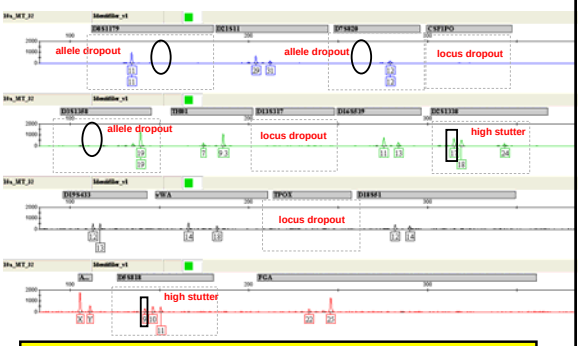
***No drop-out, slight peak height imbalance, full profiles in all replicates**

Identifiler Plus, 30 pg @ 32 cycles, ½ Reaction



***No allelic drop-out in replicates, significant peak height imbalance**

Identifiler Plus, 10 pg @ 32 cycles, ½ Reaction

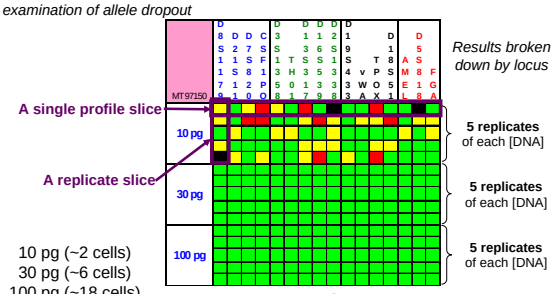


*Significant allelic drop-out in replicates, high stutter and allelic drop-in

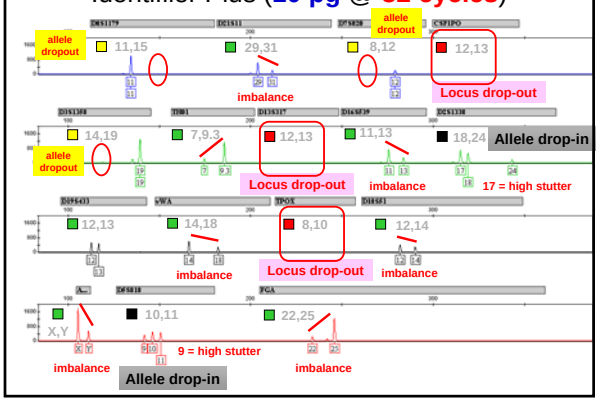
Sensitivity Comparison

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

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



Identifiler Plus (10 pg @ 32 cycles)

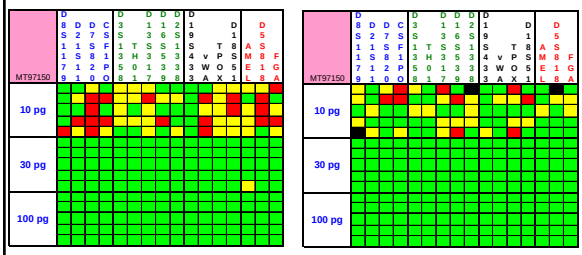


Impact of Three More PCR Cycles

Identifiler Plus

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

*Full type = both alleles above 50 RFU (does not account for peak imbalance)

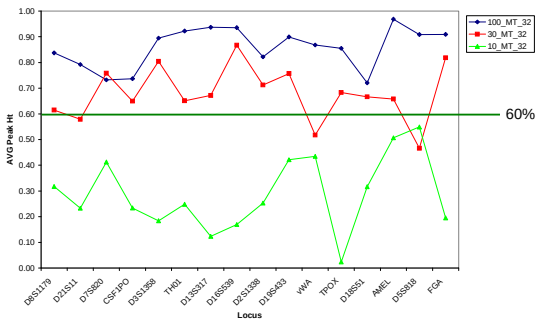


29 Cycles — 32 Cycles

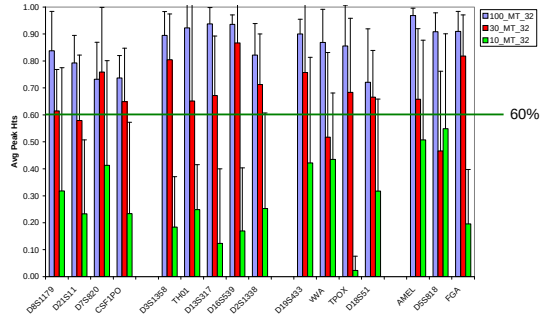
33% vs. 53% full profiles

38% improvement with 3 extra cycles

Peak Height Ratio: Identifiler Plus, 32 cyc

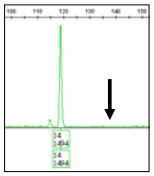


Peak Height Ratio: Identifiler Plus, 32 cyc

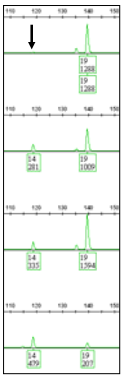


D3S1358 replicates with 3 extra cycles

False Homozygote with 1494 RFUs, well above any stochastic thresholds



Allele "19" drop-out



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

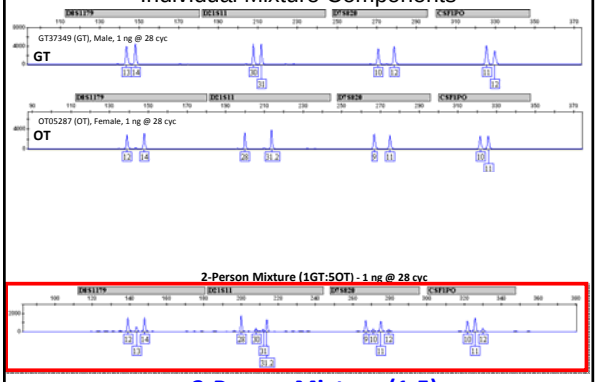
Identifiler Plus, 32 cycles, 10 pg DNA

Examination of LT-DNA Mixtures with Identifiler Plus

LT-DNA Mixture Samples

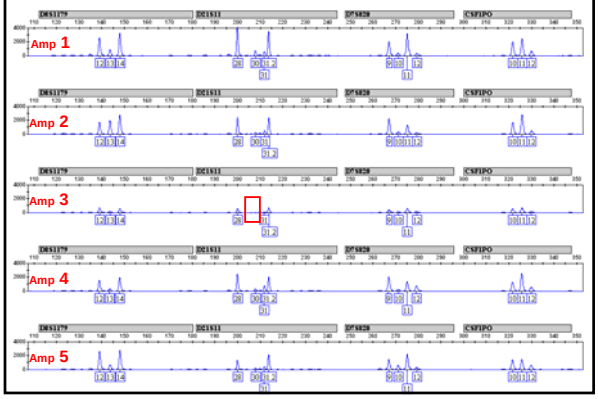
- 2 samples (male and female) were mixed together at 1:3 and 1:5 – 1 ng (1:3 and 1:5) or 100 pg (1:5) or 50 pg (1:3) total DNA
- 3 person mixture (2 males and female) were mixed together at 1:2:3 – 1 ng or 100 pg total DNA
- Identifiler Plus (28 and 31 cycles) was tested (half reactions)
- 5 replicates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

Individual Mixture Components



2-Person Mixture (1:5)

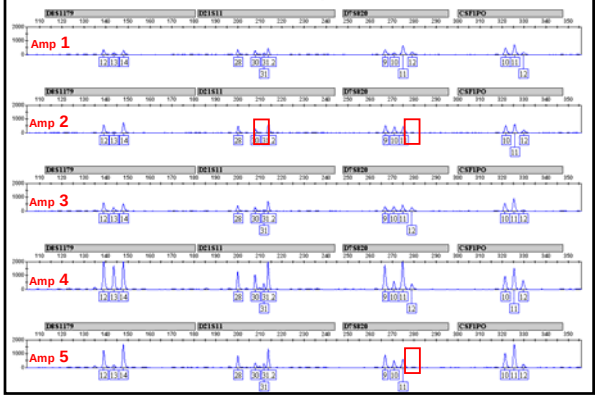
GT:OT, 1:5, 100 pg @ 31 cyc



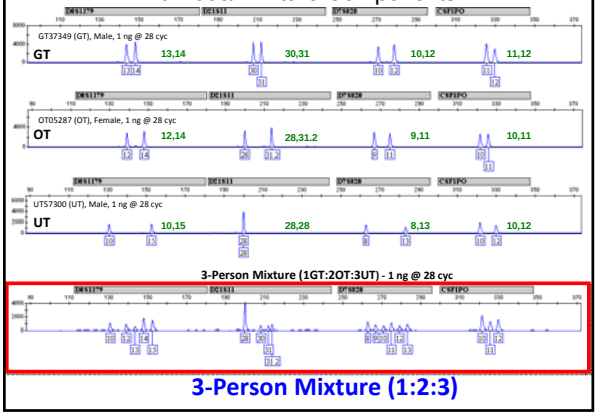
Individual Mixture Components

2-Person Mixture (1:3)

GT:OT, 1:3, 50 pg @ 31 cyc



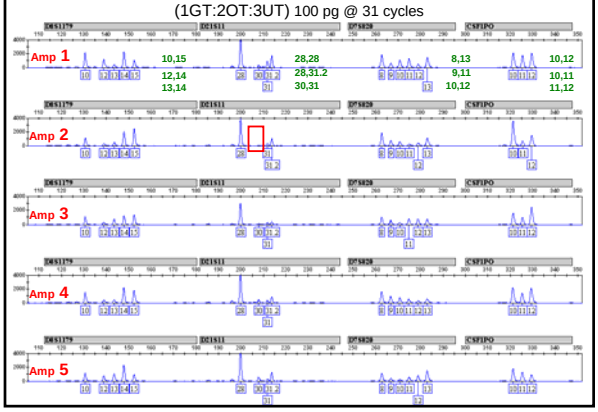
Individual Mixture Components



3-Person Mixture (1:2:3)

Replicate Results from 3-Person Mixture

(1GT:2OT:3UT) 100 pg @ 31 cycles



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.

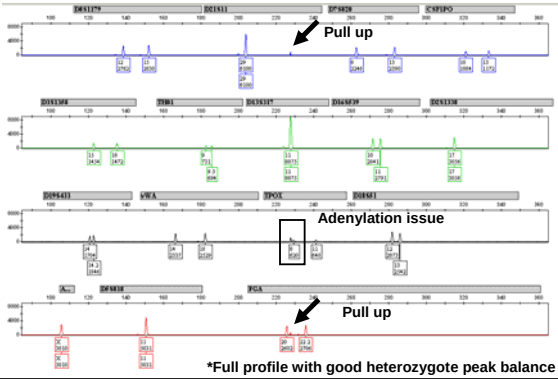
Examination of direct PCR with Identifiler Plus

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

Data provided by Erica Butts

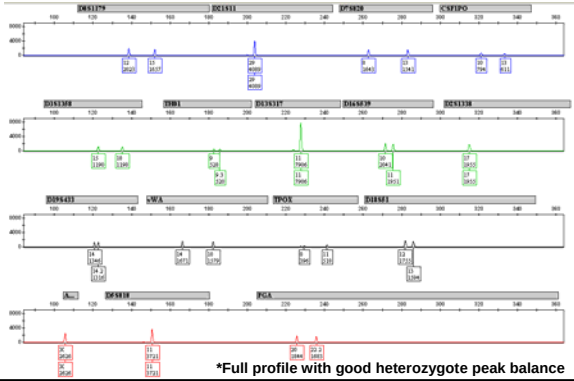
903 Blood Punch, Sample 1



*Full profile with good heterozygote peak balance

Data provided by Erica Butts

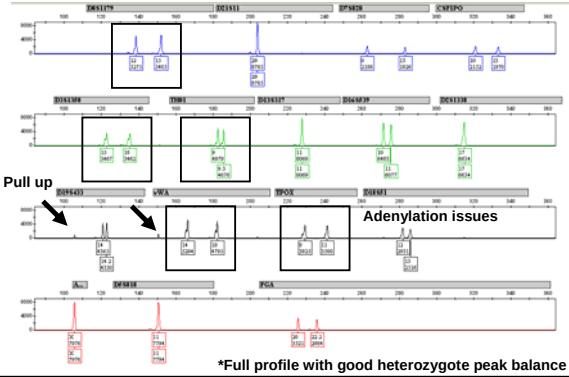
903 Blood Punch, Sample 2



*Full profile with good heterozygote peak balance

Data provided by Erica Butts

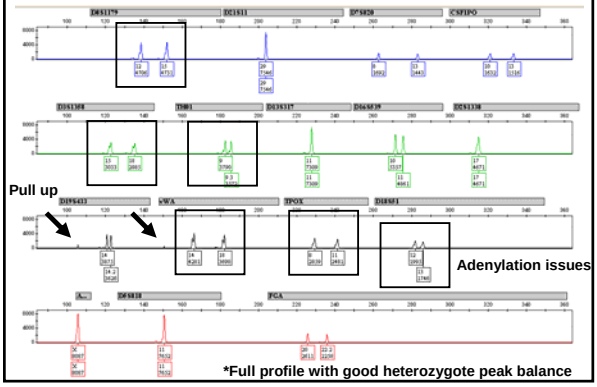
FTA Blood Punch, Sample 1



*Full profile with good heterozygote peak balance

Data provided by Erica Butts

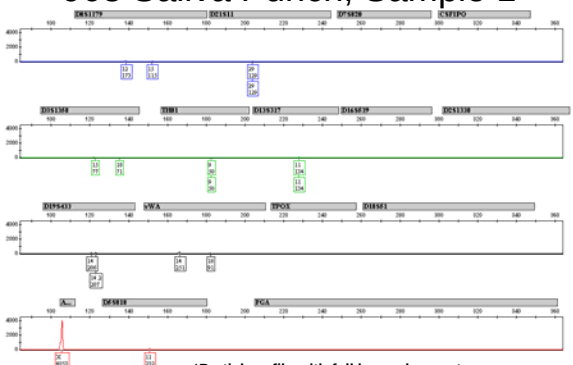
FTA Blood Punch, Sample 2



*Full profile with good heterozygote peak balance

Data provided by Erica Butts

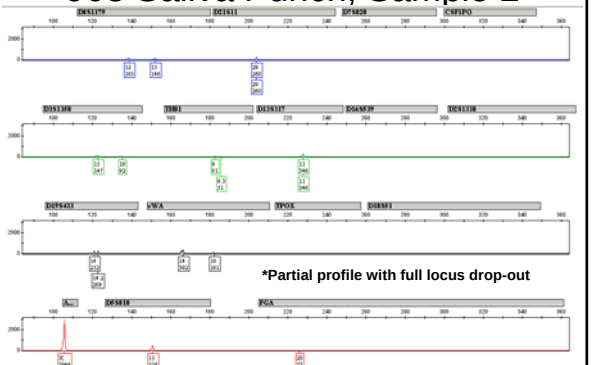
903 Saliva Punch, Sample 1



*Partial profile with full locus drop-out

Data provided by Erica Butts

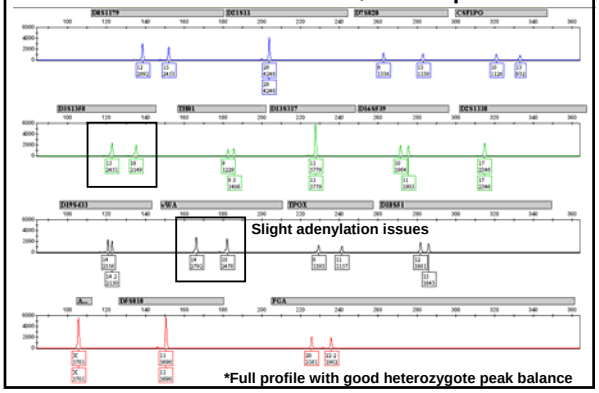
903 Saliva Punch, Sample 2



*Partial profile with full locus drop-out

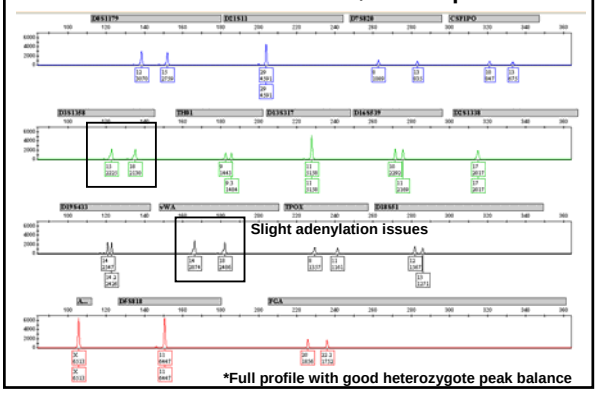
Data provided by Erica Butts

FTA Saliva Punch, Sample 1



Data provided by Erica Butts

FTA Saliva Punch, Sample 2



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.

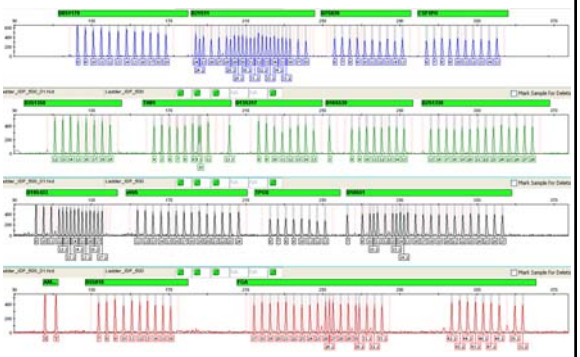
Identifiler Plus data on the 3500*xl* Genetic Analyzer (@ AFDIL)

3500*xl* – 24 Capillary Array

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
- 3500*xl* data compared to 3130*xl* data
  - Default injection of 1.2 kV, 24 sec (3500*xl*)
  - Low injection of 1.2 kV, 10 sec (3500*xl*)
  - Default injection of 3 kV, 10 sec (3130*xl*)

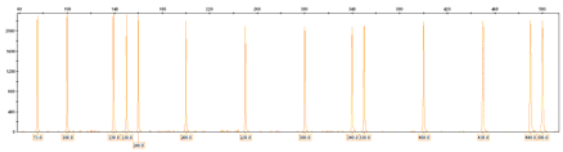
Identifiler Plus Ladder 3500*xl*



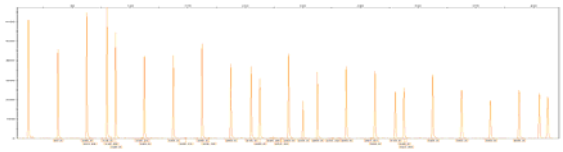
Excellent peak height balance, uniform peak heights between dye channels

Different Size Standards (3500*xl*)

3500*xl* – GS 500

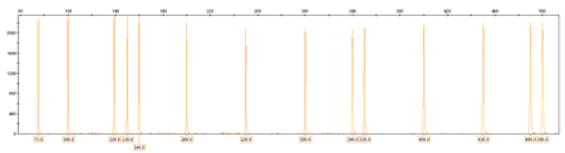


3500*xl* – GS 600 v2

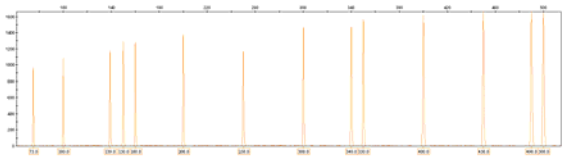


Size Standards (3500*xl* vs 3130*xl*)

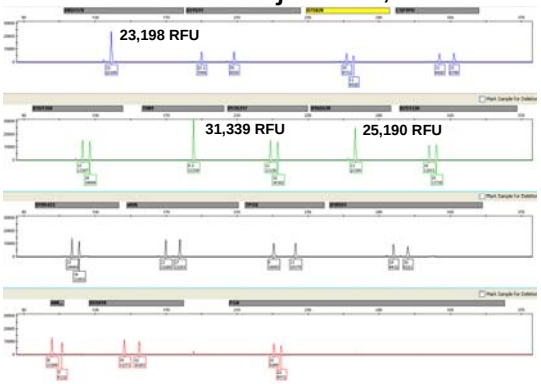
3500*xl* – GS 500



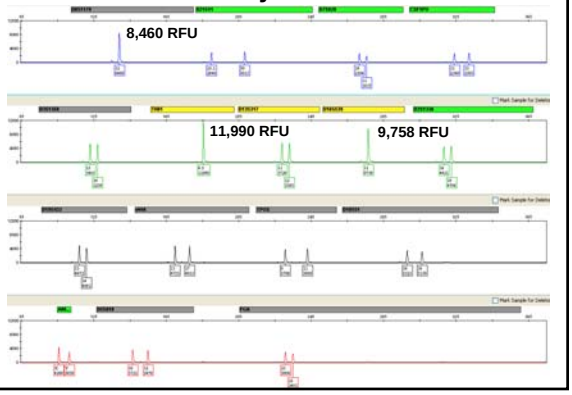
3130*xl* – GS 500



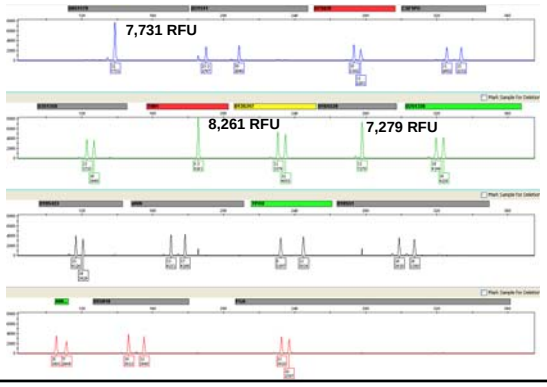
3500*xl* Default Injection, GS 500



3500*xl* Low Injection, GS 500

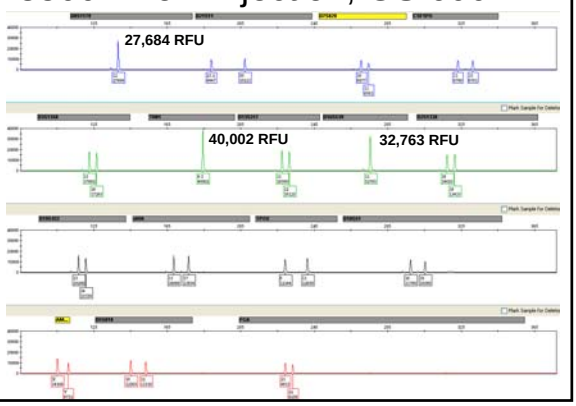


3130*xl* Default Injection, GS 500



3500xl Default Injection, GS 600 v2

3500xl Low Injection, GS 600 v2



Summary of Data Observed

- The RFU scale for the 3500*xl* is different than the 3130*xl* (30000 RFU vs 8000 RFU).
- The 3500*xl* instrument is more sensitive than the 3130*xl* – can adjust the injection time and voltage.
- Identifiler Plus profiles on the 3500*xl* 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 3500*xl* are well balanced including good inter- and intra-locus balance as well as between dye channels.

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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.

NIST Team for This Work

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