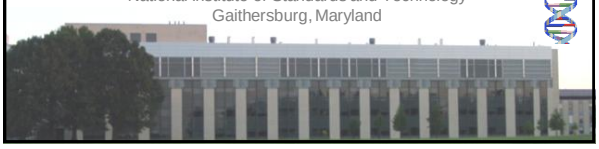
The Copenhagen Forensic Genetic Summer School
Advanced Topics in STR DNA Analysis
June 27-28, 2012

Fundamentals of Capillary Electrophoresis (CE)

Mike Coble and Becky Hill

NIST Applied Genetics Group
National Institute of Standards and Technology
Gaithersburg, Maryland



Pioneers of Capillary Electrophoresis

Stellan Hjertén
Uppsala University

1967
First high voltage CE system (with rotating 3 mm i.d. capillaries)

James Jorgenson
University of North Carolina

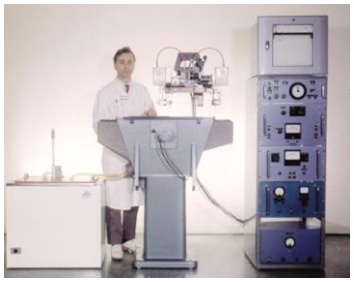
1981
First "modern" CE experiments (with 75 µm i.d. capillaries)

Barry Karger
Northeastern University

1988/90
First DNA separations in a capillary (gel-filled/ sieving polymer)

Stellan Hjertén

Uppsala University (Sweden)



With first fully automated capillary free zone electrophoresis apparatus in 1967

In 2003 at age 75

Received his PhD (1967) under Professor **Arne Tiselius** who had developed moving boundary zone electrophoresis in 1937 **(Noble Prize in 1948)**

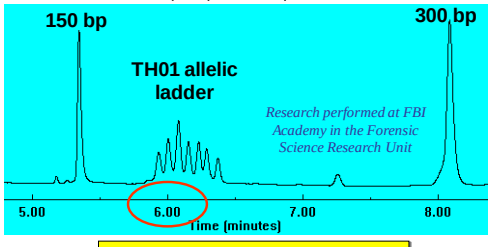
A Brief History of Capillary Electrophoresis

- 1937 – Tiselius develops moving boundary electrophoresis
- 1967 – Hjertén uses rotating 3 mm i.d. tubes for CE
- **1981 – Jorgenson and Lukacs demonstrate first high performance CE separations with 75 µm i.d. capillary**
- 1988 – Karger's group shows DNA separations of single stranded oligonucleotides with gel-filled capillaries
- 1990 – Karger's group shows DNA separations with sieving polymers on DNA restriction fragments
- 1991 – Grossman expands work with sieving polymers
- 1992 – Bruce McCord starts working on PCR product separations with STR allelic ladders

First Rapid STR Typing with Capillary Electrophoresis

Single color detection with dual internal size standards

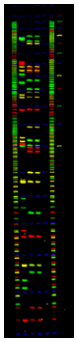
Butler *et al.* (1994) *BioTechniques* 17: 1062-1070



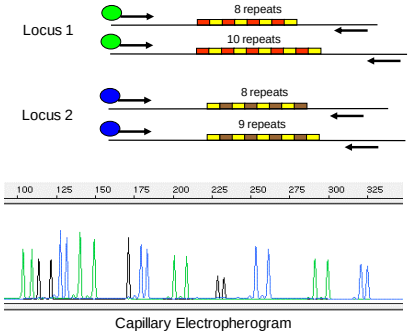
Performed in December 1993

Technology Implementation Takes Time – the FBI did not start running casework samples using STRs and CE until January 1999

STR Allele Separation Can Be Performed by Gel or Capillary Electrophoresis with Detection of Fluorescent Dyes Labeling Each PCR Product



Scanned **Gel Image**



Capillary Electropherogram

Why Use CE for DNA Analysis?

1. **Injection, separation, and detection are automated.**
2. **Rapid separations are possible**
3. **Excellent sensitivity and resolution**
4. **The time at which any band elutes is precisely determined**
5. **Peak information is automatically stored for easy retrieval**

Symbol first used in Oct 1994 at the Promega meeting on a poster by John Butler introducing the use of CE for STR typing

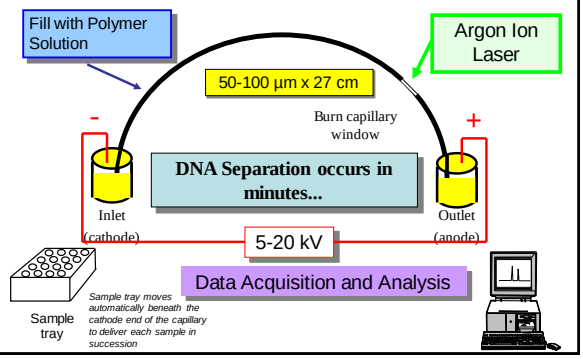
Important Differences Between CE and Gels

- **Room temperature control** is essential for run-to-run precision
  - CE uses sequential rather than simultaneous separations
  - Usually need < 2.0 ºC (must inject allelic ladder regularly)
- **Lower amount of DNA loaded** (injection = nL vs µL) and thus detection sensitivity must be better
- Electrokinetic injection enables **dye artifacts** (blobs) to enter the capillary or microchip CE channel and thus possibly interfere with STR allele interpretation

More Differences between CE and Gels…

- Filling the capillary (or microchip CE channel) is analogous to pouring a gel into a tiny tube…
- Must be more clean around a CE system
  - Because the capillaries (µCE channels) are small, particles of dust or urea crystals can easily plug them
  - Tips of capillary cannot dry out (once buffer solutions have been run through them) for the same reasons
- Bubbles are a BIG problem in CE as they can easily block current flow in the capillary…

Capillary Electrophoresis (CE)



Typical Instruments Used for STR Typing

GeneAmp 9700

Thermal Cycler for PCR Amplification

Capillary electrophoresis instruments for separating and sizing PCR products

single capillary

ABI 310

16-capillary array

ABI 3100

J.M. Butler (2011) *Advanced Topics in Forensic DNA Typing: Methodology*, Table 6.1

Genetic Analyzers from Applied Biosystems

ABI Genetic Analyzer	Years Released for Human ID	Number of Capillaries	Laser	Polymer delivery	Other features
373 (gel system)	1992-2003	--	40 mW Ar+ (488/514 nm)	--	PMTs and color filter wheel for detection
377 (gel system)	1995-2006	--	40 mW Ar+ (488/514 nm)	--	CCD camera
310	1995-	1	10 mW Ar+ (488/514 nm)	syringe	Mac operating system & Windows NT (later)
3100	2000-2005	16	25 mW Ar+ (488/514 nm)	syringe	
3100-Avant	2002-2007	4	25 mW Ar+ (488/514 nm)	syringe	
3130	2003-2011	4	25 mW Ar+ (488/514 nm)	pump	
3130xl	2003-2011	16	25 mW Ar+ (488/514 nm)	pump	
3500	2010-	8	10-25 mW diode (505 nm)	new pump	110V power; **RFID-tagged reagents**; **.hid files**; normalization & 6-dye detection possible
3500xl	2010-	24			
3700	2002-2003	96	25 mW Ar+ (488/514 nm)	cuvette-based	Split beam technology
3730	2005-	48	25 mW Ar+ (488/514 nm)	pump	
3730xl	2005-	96	25 mW Ar+ (488/514 nm)	pump	

Information courtesy of Michelle S. Shepherd, Applied Biosystems, LIFE Technologies.

ABI Genetic Analyzer Usage at NIST

(All instruments were purchased using NIJ funds)

ABI 310 Single capillary

- 1st was purchased in 1996 as Mac (A230, now B233)
- 2nd was purchased in June 2002 as NT (B261)

ABI 3100 → 3130xl 16 capillaries

- 1st purchased in April 2001 as ABI 3100
  - upgraded to 3130xl in Sept 2005
  - Located in a different room (A230, now B219)
- 2nd purchased in June 2002 as ABI 3100
  - Original data collection (v1.0.1) software retained
  - updated to 3130xl in Jan 2007 (B219, now B261)

ABI 3500 8 capillaries

- Purchased Nov 2010 (B233)

DNA Samples Run at NIST

we have **processed >100,000 samples** (from 1996-present)

- **STR kits**
  - Identifiler, PP16, PP16HS, Identifiler Plus, Identifiler Direct, Profiler Plus, Cofiler, SGM Plus, ESI/ESX 17, SE33 monoplex
- **Research & development on new assays**
  - **STRs**: Y-STR 20plex, MeowPlex, miniSTRs, 26plex
  - **SNPs**: SNaPshot assays: mtDNA (one 10plex), Y-SNPs (four 6plexes), Orchid SNPs (twelve 6plexes), ancestry SNPs (two 12plexes), SNPforID (one 29plex), SNPplex (one 48plex)
- **DNA sequencing**
  - Variant allele sequencing

We have a unique breadth and depth of experience with these instruments…

Review Article on STRs and CE

pdf available from http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

Electrophoresis 2004, *25*, 1397–1412

Review

John M. Butler[1]
Eric Buel[2]
Federica Crivellente[3]*
Bruce R. McCord[3]

[1]National Institute of Standards and Technology, Biotechnology Division, Gaithersburg, MD, USA
[2]Vermont Forensic Laboratory, Waterbury, VT, USA
[3]Ohio University, Department of Chemistry, Athens, OH, USA

Forensic DN… using the AB… for STR analy…

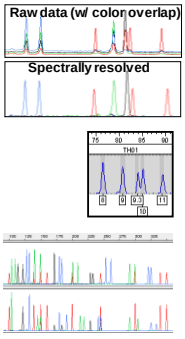
DNA typing with short… applications including… such as the ABI Prism… for many laboratories… ing sample preparati… results using CE syste… ered in the context … throughput and ease …

Contents

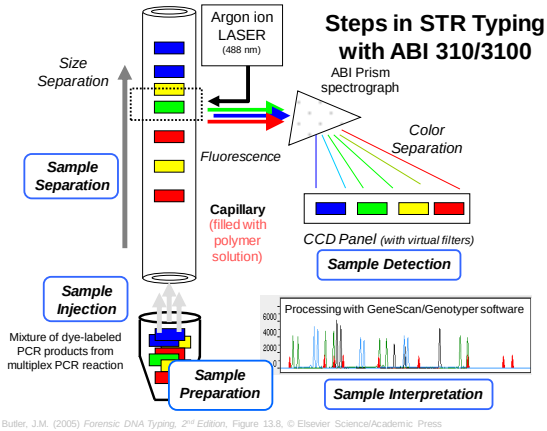
Analytical Requirements for STR Typing

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

- Fluorescent dyes must be **spectrally resolved** in order to distinguish different dye labels on PCR products
- PCR products must be **spatially resolved** – desirable to have single base resolution out to >350 bp in order to distinguish variant alleles
- High **run-to-run precision** – an internal sizing standard is used to calibrate each run in order to compare data over time



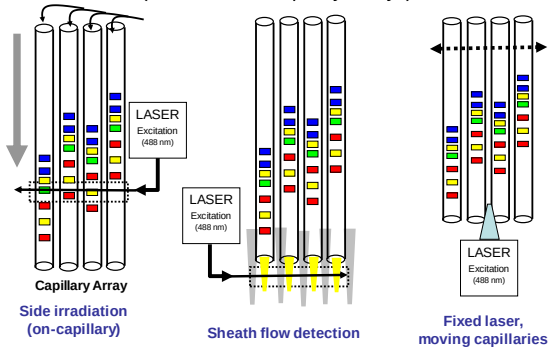
Steps in STR Typing with ABI 310/3100



Butler, J.M. (2005) *Forensic DNA Typing*, 2nd Edition, Figure 13.8, © Elsevier Science/Academic Press

Detection with Multiple Capillaries

(Irradiation for Capillary Arrays)



Side irradiation (on-capillary)

ABI 3100, 3130, 3100*Avant*

Sheath flow detection

ABI 3700

Fixed laser, moving capillaries

MegaBACE

Process Involved in 310/3100 Analysis

- Separation
  - Capillary – 50um fused silica, 43 cm length (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrolidinone
- Injection
  - electrokinetic injection process (formamide, water)
  - importance of sample stacking
- Detection
  - fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

Separation

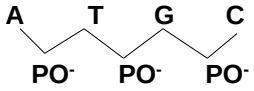
Ohm's Law

- V = IR (where V is voltage, I is current, and R is resistance)
- Current, or the flow of ions, is what matters most in electrophoresis
- CE currents are much lower than gels because of a higher resistance in the narrow capillary
- CE can run a higher voltage because the capillary offers a higher surface area-to-volume ratio and can thus dissipate heat better from the ion flow (current)

DNA and Electrophoresis

"From a practical point of view it is disappointing that electrophoresis cannot be used to fractionate or analyze DNA's on the basis of size" Olivera, Biopolymers 1964, 2, 245

$\mu_{ep} = q/6\pi\eta r$ small ions with high charge move fastest



As size increases so does charge!

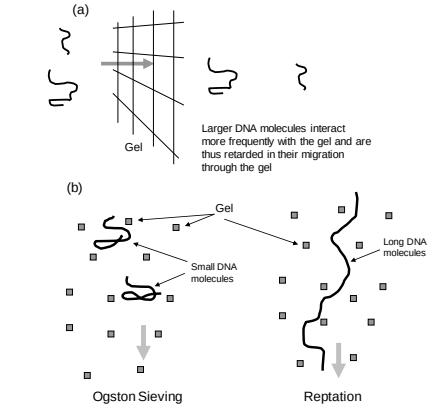


Figure 12.4, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Separation Issues

- Electrophoresis buffer –
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrolidinone for denaturing DNA
  - EDTA for stability and chelating metals
- Polymer solution -- POP-4 (but others work also)
- Capillary wall coating -- dynamic coating with polymer
  - Wall charges are masked by methyl acrylamide
- Run temperature -- 60 °C helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)

What is in POP-4 and Genetic Analyzer Buffer?

Nucleic Acids Research, 1997, Vol. 25, No. 19 3925–3929

Improved single-strand DNA sizing accuracy in capillary electrophoresis

Barnett B. Rosenblum*, Frank Oaks, Steve Menchen and Ben Johnson

PE Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, USA

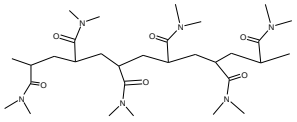
Received May 29, 1997; Revised and Accepted August 6, 1997

See also Wenz *et al.* (1998) *Genome Research* 8: 69-80

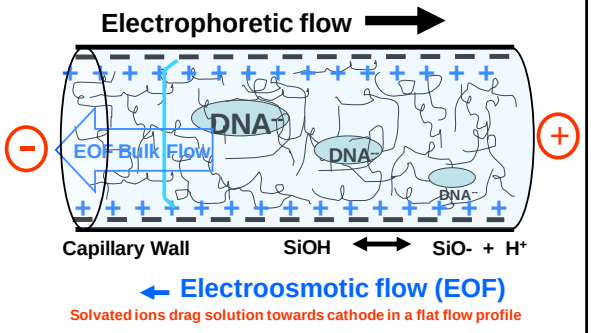
POP-4 (4% poly-dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone)

US Patent 5,552,028 covers POP-4 synthesis

Running buffer contains 100 mM TAPS and 1 mM EDTA (adjusted to pH 8.0 with NaOH) *TAPS = N-Tris-(hydroxymethyl)methyl-3-aminopropane-sulfonic acid*



Capillary Wall Coatings Impact DNA Separations

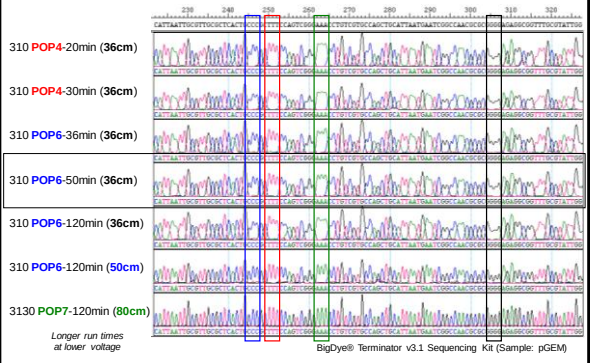


How to Improve Resolution?

1. **Lower Field Strength**
2. **Increase Capillary Length**
3. **Increase Polymer Concentration**
4. **Increase Polymer Length**

All of these come at a cost of longer separation run times

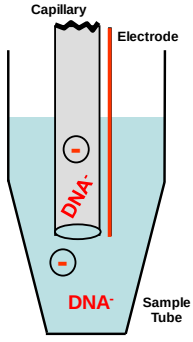
Impact of Capillary Length and Polymer Concentration on DNA Sequencing Resolution



Data collected at NIST by Tomohiro Takamaya (Japanese guest researcher, fall 2007)

Injection

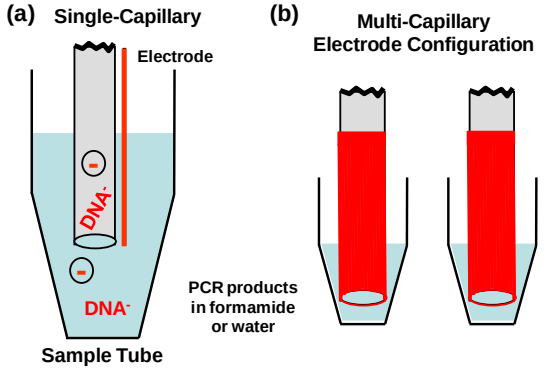
Electrokinetic Injection Process

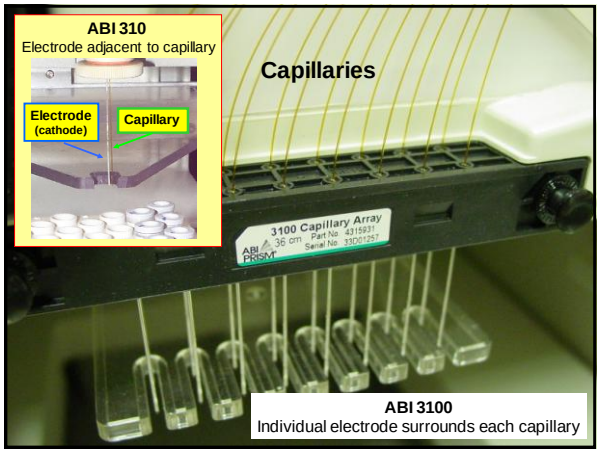


Amount of DNA injected is inversely proportional to the ionic strength of the solution

Salty samples result in poor injections

Capillary and Electrode Configurations





Sample Conductivity Impacts Amount Injected

$$[DNA_{inj}] = \frac{Et(\pi r^2)(\mu_{ep} + \mu_{eof})[DNA_{sample}](\lambda_{buffer})}{\lambda_{sample}}$$

$[DNA_{inj}]$ is the amount of sample injected

E is the electric field applied

t is the injection time

r is the radius of the capillary

μ_{ep} is the mobility of the sample molecules

μ_{eof} is the electroosmotic mobility

$[DNA_{sample}]$ is the concentration of DNA in the sample

λ_{buffer} is the buffer conductivity

λ_{sample} is the sample conductivity

Cl- ions and other buffer ions present in PCR reaction contribute to the sample conductivity and thus will compete with DNA for injection onto the capillary

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

Steps Performed in Standard Module

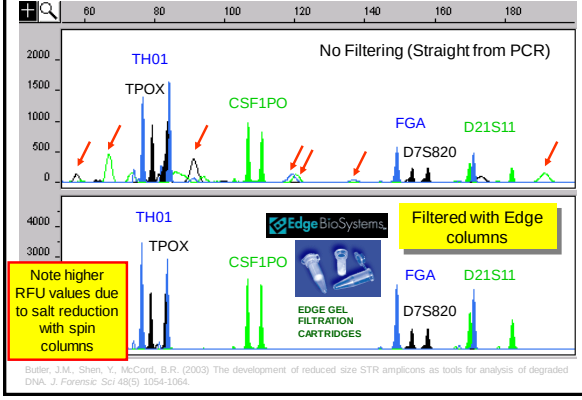
See J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition; Chapter 14

- **Capillary fill** – polymer solution is forced into the capillary by applying a force to the syringe
- **Pre-electrophoresis –** the separation voltage is raised to 10,000 volts and run for 5 minutes;
- **Water wash of capillary – capillary is dipped several times in deionized water to remove buffer salts that would interfere with the injection process**
- **Sample injection** – the autosampler moves to position A1 (or the next sample in the sample set) and is moved up onto the capillary to perform the injection; a voltage is applied to the sample and a few nanoliters of sample are pulled onto the end of the capillary; the default injection is 15 kV (kilovolts) for 5 seconds
- **Water wash of capillary** – capillary is dipped several times in waste water to remove any contaminating solution adhering to the outside of the capillary
- **Water dip** – capillary is dipped in clean water (position 2) several times
- **Electrophoresis** – autosampler moves to inlet buffer vial (position 1) and separation voltage is applied across the capillary; the injected DNA molecules begin separating through the POP-4 polymer solution
- **Detection** – data collection begins; raw data is collected with no spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis

Comments on Sample Preparation

- Use high quality formamide (<100 μS/cm)
- Denaturation with heating and snap cooling is not needed (although most labs still do it…)
- **Post-PCR purification reduces salt levels** and leads to more DNA injected onto the capillary

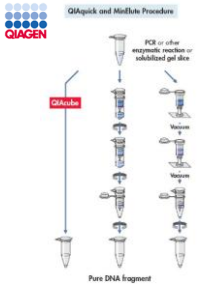
Removal of Dye Artifacts Following PCR Amplification



Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci* 48(5) 1054-1064.

Why MiniElute increases peak heights

- QIAGEN MiniElute **reduces salt levels in samples causing more DNA to be injected**
- **Requires setting a higher stochastic threshold** to account for the increased sensitivity

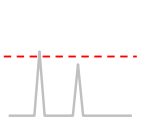


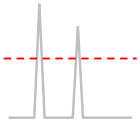
Smith, P.J. and Ballantyne, J. (2007) Simplified low-copy-number DNA analysis by post-PCR purification. *J. Forensic Sci.* 52: 820-829

Stochastic Effects and Thresholds

Regular Injection

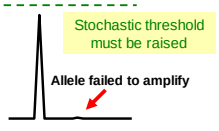
Injection Following Desalting (MiniElute)





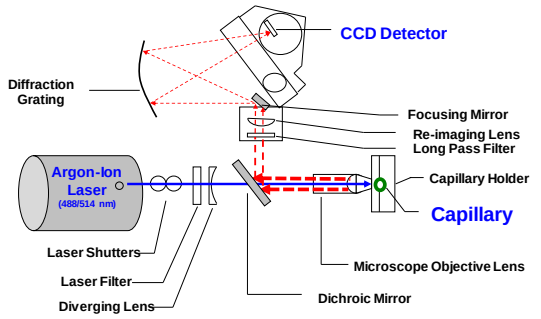
When PCR amplifying low levels of DNA, allele dropout may occur

False homozygote

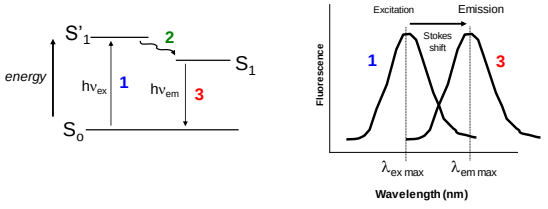


Detection

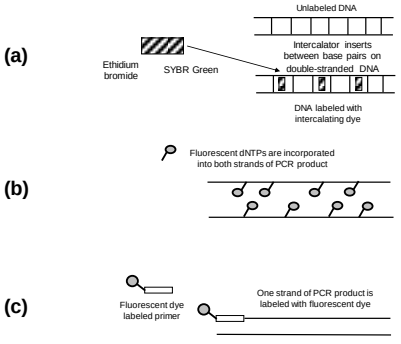
Optics for ABI 310



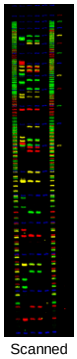
Fluorescence



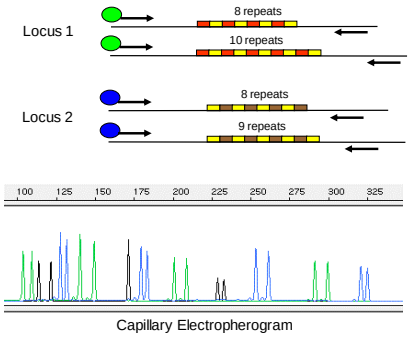
Methods for Fluorescently Labeling DNA



The polymerase chain reaction (PCR) is used to amplify STR regions and label the amplicons with fluorescent dyes using locus-specific primers



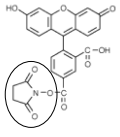
Scanned Gel Image



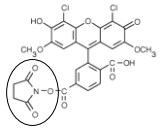
Capillary Electropherogram

ABI Fluorescent Dyes Used in Four-Color Detection

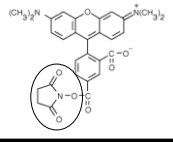
FAM (blue)



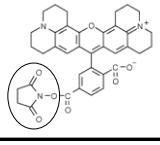
JOE (green)



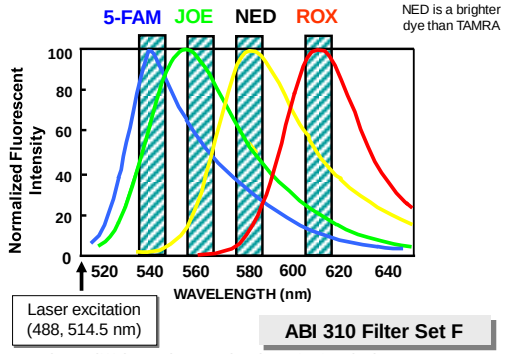
TAMRA (yellow)



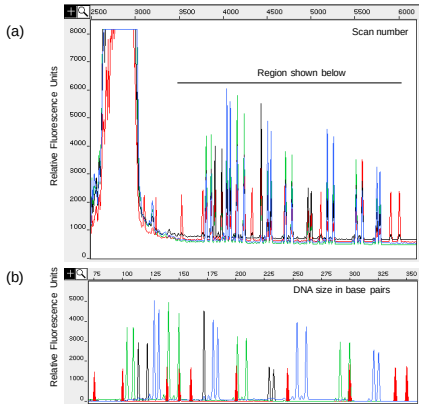
ROX (red)



Fluorescent Emission Spectra for ABI Dyes

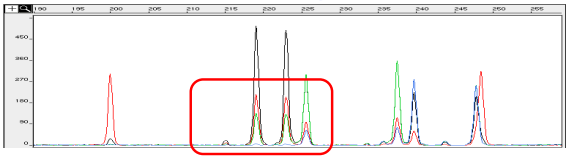


Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.4, ©Academic Press

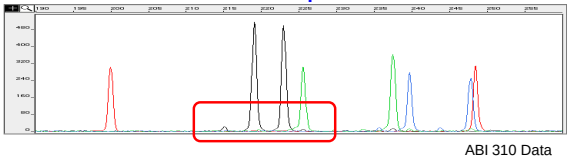


Importance of Spectral Calibration

Before Color Separation



After Color Separation



ABI 310 Data

Matrix with 4 Dyes on ABI 310

$I_{540} = bx_b + gy_b + yz_b + rw_b$ intensity of blue
$I_{560} = bx_g + gy_g + yz_g + rw_g$ intensity of green
$I_{580} = bx_y + gy_y + yz_y + rw_y$ intensity of yellow
$I_{610} = bx_r + gy_r + yz_r + yw_r$ intensity of red

Where
b is the %blue labeled DNA
g is the %green labeled DNA, etc.

x,y,z,w are the numbers in the matrix (sensitivity to each color)

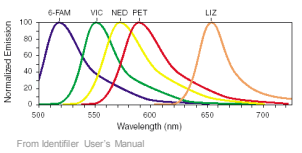
POP4STRMODF

Reactions

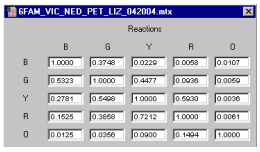
	B	G	Y	R
B	1.0000	0.8502	0.1380	0.0009
G	0.8300	1.0000	0.7622	0.0051
Y	0.6416	0.8324	1.0000	0.1102
R	0.4493	0.6484	0.7851	1.0000

If you solve xyzw for each dye individually
Then you can determine dye contribution for any mixture

5 x 5 matrix for 5-dye analysis on ABI 310

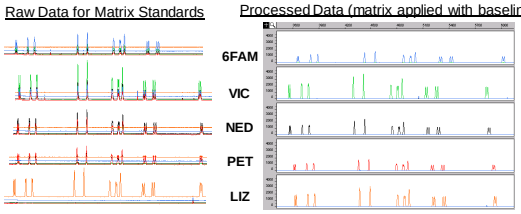


From Identifiler User's Manual



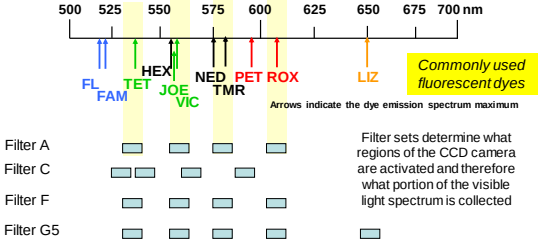
Raw Data for Matrix Standards

Processed Data (matrix applied with baselining)



Virtual Filters Used in ABI 310

Visible spectrum range seen in CCD camera



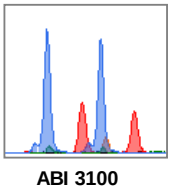
Filter sets determine what regions of the CCD camera are activated and therefore what portion of the visible light spectrum is collected

	Blue	Green	Yellow	Red	Orange	Used with These Kits
Filter A	FL	JOE	TMR	CXR		PowerPlex 16
Filter C	6FAM	TET	HEX	ROX		in-house assays
Filter F	5FAM	JOE	NED	ROX		Profiler Plus
Filter G5	6FAM	VIC	NED	PET	LIZ	Identifiler

Variable Binning Increases Red Peaks

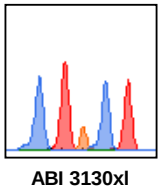
Comparison of Data Collection Versions

(a)



ABI 3100
Data Collection v1.0.1

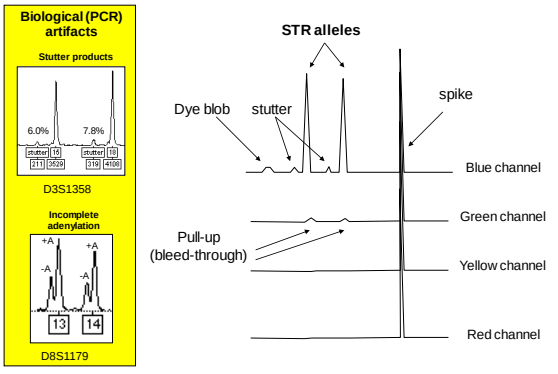
(b)



ABI 3130xl
Data Collection v3.0

The same PCR products examined with different data collection versions. In (a) there is an equal number of pixels of light collected from the CCD camera for the blue-labeled and red-labeled peaks. In (b) the signal increase in the red dye-labeled PCR products is accomplished with 'variable binning' where more pixels of light are collected from the CCD camera in the red-channel to help balance the less sensitive red dye with blue dye-labeled amplicons.

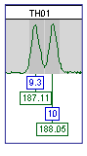
Deciphering Artifacts from the True Alleles



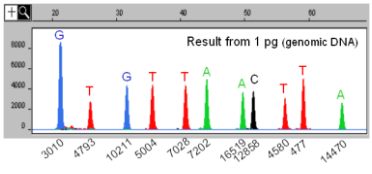
Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 15.4, © Elsevier Science/Academic Press

NIST ABI 3100 Analysis Using POP-6 Polymer

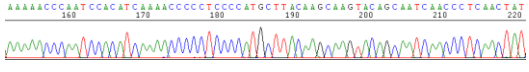
High Resolution STR Typing



SNaPshot SNP Typing
(Coding Region mtSNP 11plex minisequencing assay)



mtDNA Sequencing (HV1)



Maintenance of ABI 310/3100/3130

- Syringe – leaks cause capillary to not fill properly
- Capillary storage & wash – **it dries, it dies!**
- Pump block – cleaning helps insure good fill
- Change the running buffer regularly

YOU MUST BE CLEAN AROUND A CE!

Protocols Used for STR Typing

- Most forensic DNA laboratories follow PCR amplification and CE instrument protocols provided by the manufacturer
- Comments
  - Lower volume reactions may work fine and reduce costs
  - No heat denaturation/snap cooling is required prior to loading samples into ABI 310 or ABI 3100
  - Capillaries do not have to be thrown away after 100 runs
  - POP-4 polymer lasts much longer than 5 days on an ABI 310
  - **Validation does not have to be an overwhelming task**

ABI 3500 Genetic Analyzer

ABI 3500 Genetic Analyzer

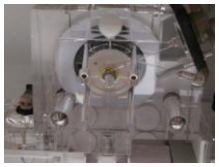
- **3500 (8 capillary)**
- **3500xl (24 capillary)**

New Features of the ABI 3500 CE

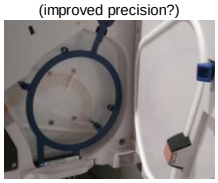
- an improved polymer delivery pump design,
- ready-to-use consumables and containers,
- Radio Frequency Identification (RFID) consumable tracking,
- quality control software features for rapid identification and re-injection of failed samples,
- increased throughput,
- new laser technology,
- reduced power requirements,
- peak height normalization,
- intuitive user software, and integrated primary analysis software,
- improved peak height uniformity across capillaries, runs and instruments
- **6-dye channel capability**

Details of the new ABI 3500

No lower pump block
(fewer air bubbles)



Improved sealing for better temperature control
(improved precision?)



Primary Differences Between 31xx and 3500

31xx Instruments

- Argon ion (Ar+) lasers with 488/514 nm wavelengths for fluorescence excitation
- 220V power requirement
- Optimal signal intensity 1500-3000 RFU
- **Data signal depressed 4-fold during data collection**
- Currently validated and operational in most forensic laboratories (.fsa files)

3500 Instruments

- Single-line 505 nm, solid-state long-life laser
- Smaller footprint
- 110V power requirement
- Optimal signal intensity can approach 20,000-30,000 RFU
- Normalization of instrument-to-instrument signal variability
  - Ability to increase or decrease overall signal
- Requires the use of GeneMapper IDX v1.2 (.hid files)

DNA Community Moving to ABI 3500s

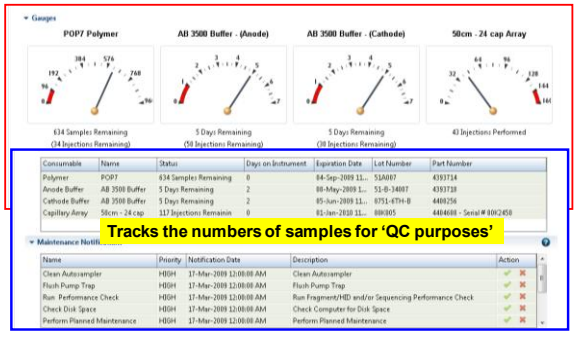
Advantages

- Smaller footprint and 110V power requirement
- Better polymer delivery and temperature control
  - Improved success rates?
- New capabilities
  - between instrument normalization
  - 6-dye detection (bigger kits with more loci)
- Simpler software

Disadvantages

- Up-front cost of new instruments
  - Federal government (NIJ) will likely be expected to foot the bill
- Generates .hid files
  - Requires new analysis software
- Validation down-time
  - New RFU thresholds
- Higher per run cost with RFID tags & limited expiration
  - many labs cannot purchase reagents rapidly throughout the year
- Creating technicians not scientists
  - Plug and play approach leading to loss of understanding for process
  - Less flexible *(impacts research with it)*

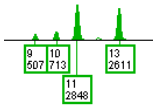
ABI 3500 'Dash Board' Data Collection



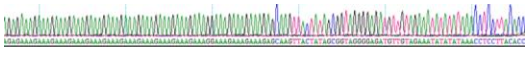
https://www3.appliedbiosystems.com/cms/groups/portal/documents/web_content/cms_064299.jpg

ABI 3500 Generates Excellent Data

STR typing with a 1:7 mixture using **36 cm array and POP4**



DNA sequencing of an SE33 allele using **50 cm array and POP7**



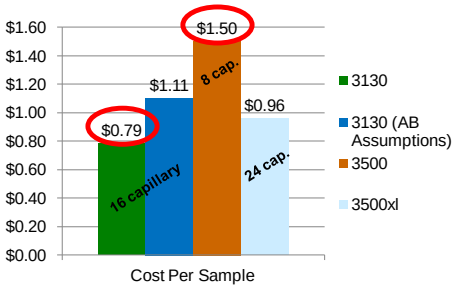
Identifiler Result on ABI 3500xl

Cost for the Forensic DNA Community to Switch from ABI 3100s to 3500s

1. **Instrument up-front cost**
   - Will likely be requested from federal grant funds (NIJ)
2. **New software purchase**
   - Will likely be requested from federal grant funds (NIJ)
   - new .hid file format will not work on current software (GMIDv3.2)
   - 3500 will not create .fsa files with 36cm arrays (HID applications)
3. **Validation time & expense**
   - Relative fluorescent scales are completely different…
4. **Operational cost**
   - ABI claims that the running costs are equivalent to 3130s…

NIST Calculated Cost per Sample for ABI 3130xl vs. 3500 and 3500xl Reagents

Running two plates per day (10 plates per week)



Consumable Costs for the ABI 3500

POP polymer pack with RFID tag

$180 (384 samples)
$455 (960 samples)

Buffer pack with RFID tag

$60 ($25+$35)

8-capillary array

$1200 (160 injections)

"Expires" after 1-week on the instrument

Thus, if you run 1 sample or 960 samples (or 384) in that week, the consumable cost will be the same…

Consumable RFID Tracking Limits

	RFID Hard Stops	Usage Comments From a Research Laboratory Standpoint
Array	None	1. Very easy to change between HID and sequencing 2. Array from validation was stored at least twice and reinstalled on 3500 during validation
Buffer	Expiration Date 7 Days on Instrument # Injections	1. Can no longer use in-house buffer 2. Very easy to change on the instrument (snap-and-go)
Polymer	Expiration Date # Samples # Injections	1. Hard stop with the expiration date has caused us to discard unused polymer we would have otherwise kept on the instrument 2. ~50% of total polymer remains in the pouch after "consumption" 3. Expiration dates have changed purchasing strategy (smaller batches, based on ongoing project needs)

ABI 3500 Validation Studies at NIST


Erica Butts

Main Points:

- The 3500 has proven to be reliable, reproducible and robust in our hands – we have provided feedback to ABI to improve use
- Produces excellent DNA sequencing results
- Signal strength is different compared to ABI 3130xl and requires studies to set analytical and stochastic thresholds
- **Dye-specific analytical thresholds** resulted in less allelic and full locus dropout than applying one analytical threshold to all dyes
- RFID tracking decreases flexibility in our research experience

Presentations/Publications:

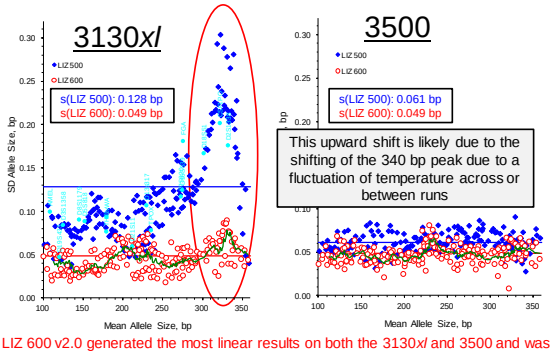
- MAAFS talk (May 2011)
- ABI road show talks (July & Aug 2011)
- ISFG presentation (Sept 2011)
- *Forensic News* (Spring 2012)

http://marketing.appliedbiosystems.com/mk/get/FORENSICNEWS_HIDINACTION#article5

n=16 Allelic Ladders

Validation data from Erica Butts (NIST)

Size Standard Comparison



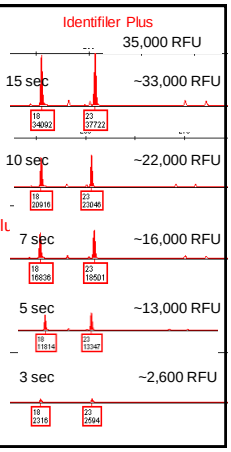
LIZ 600 v2.0 generated the most linear results on both the 3130*xl* and 3500 and was used as the size standard on both instruments for remaining testing

n=20: Identifiler n=15: Identifiler Plus

Injection Parameters

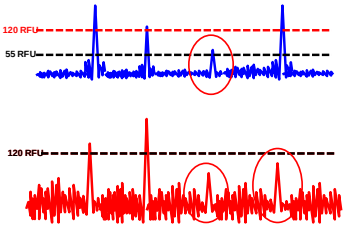
- Injection voltage/time:
  - 1.2 kV for 15 sec
  - 1.2 kV for 10 sec
  - 1.2 kV for 7 sec → Identifiler
  - 1.2 kV for 5 sec → Identifiler Plu
  - 1.2 kV for 3 sec

Standard injection parameters set based on samples with:
1. No pull-up present
2. No drop out present



Validation data from Erica Butts (NIST)

Need for Dye-Specific Thresholds?



Single thresholds for all dye channels assumes all dye channels have the **same** amount of noise

Can cause data to fall below the analytical threshold and not be called

Dye-specific thresholds take into consideration that all dye channels do not have the same level of noise

Can increase the amount of data that is callable

n=84 samples

Analytical Threshold Calculation

Identifiler					
Dye Channel	Average RFU	Stdev	Min RFU	Max RFU	Calculated Noise (RFU)
Blue	9	8.4	1	66	93
Green	13	11.5	3	84	128
Yellow	22	11.6	4	88	138
Red	28	8.8	10	80	116

Identifiler Plus					
Dye Channel	Average RFU	Stdev	Min RFU	Max RFU	Calculated Noise (RFU)
Blue	10	4.6	3	68	55
Green	16	5.6	3	78	72
Yellow	24	7.9	7	63	103
Red	31	8.9	7	81	120

Single Threshold: 140 RFU

Dye-Specific: Rounded to nearest 5 RFU

Single Threshold: 120 RFU

Dye-Specific: Rounded to nearest 5 RFU

- Statistical difference was calculated between dye channels using a z-test
- Statistically each dye channel is different for both Identifiler and Identifiler Plus
  - Must be treated independently

Validation data from Erica Butts (NIST)

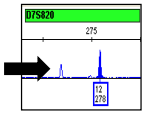
n=560 alleles

Validation data from Erica Butts (NIST)

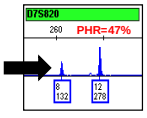
Threshold Comparison

Total of 560 alleles examined (50 pg, 30 pg, and 10 pg) where dropout was observed

Single Threshold (140 RFU)



Dye-Specific Thresholds



14.8% of the total possible allele calls were lost using a single threshold rather than using dye-specific thresholds with **Identifiler**

22.0% of the total possible allele calls were lost using a single threshold rather than using dye-specific thresholds with **Identifiler Plus**

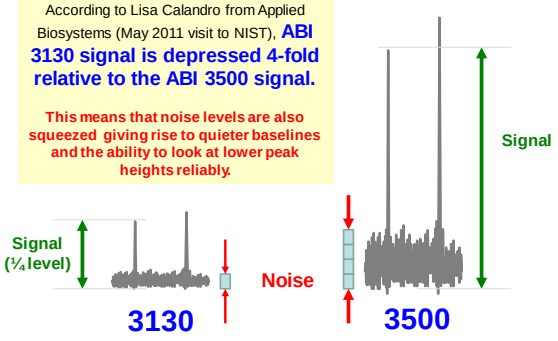
Questions about the ABI 3500

- **Is the 3500 more sensitive** because it shows peaks with higher RFU levels than 3130?
  - Not necessarily → what matters is the signal-to-noise
- **Can we normalize signal across instruments** to generate "equivalent" data between our instruments?
  - I am not aware of anyone using normalization successfully (including Applied Biosystems)
- **Will 6-dye detection be necessary** with the CODIS core loci expansion?

Signal-to-Noise Ratio

According to Lisa Calandro from Applied Biosystems (May 2011 visit to NIST), **ABI 3130 signal is depressed 4-fold relative to the ABI 3500 signal.**

This means that noise levels are also squeezed giving rise to quieter baselines and the ability to look at lower peak heights reliably.



On a relative basis in terms of the signal-to-noise ratio, these data are equivalent

Validation data from Erica Butts (NIST)

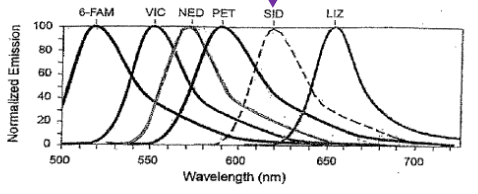
ABI 3500 Validation Considerations

- The 3500 has proven to be reliable, reproducible and robust
  - Out of 498 samples between Identifiler and Identifiler Plus only 5 required reinjection
- Dye-specific analytical thresholds resulted in less allelic and full locus dropout than applying one analytical threshold to all dyes
- Stochastic thresholds are linked to analytical thresholds
  - If the analytical threshold is adjusted, the stochastic threshold should be reevaluated along with expected peak height ratios
    - Requires consideration for overall interpretation workflow which we are still evaluating
- RFID tracking decreases flexibility in our research experience

Applied Biosystems' Sixth Dye?

Patent Application Publication Jun. 4, 2009 Sheet 3 of 3 US 2009/0142764 A1

6th Dye named SID
(emission spectrum falls between PET and LIZ)



US Patent Application (filed October 30, 2008 by Lori Hennessy and Robert Green)
"Methods and Kits for Multiplex Amplification of Short Tandem Repeat Loci"

Potential Issues with 6-dye STR kits

- ABI announced in their Spring 2012 issue of *Forensic News* that a 6-dye STR kit was in development
  - Which would enable another 4-6 loci to be added to a multiplex
- **Most labs now have 3130 or 3130xl instruments**
  - Will all labs have to purchase 3500 instruments?
  - Or will the 3130 or 3730 series instruments be made compatible for 6-dyes?
- Spectral calibration issues and potential bleed through across color channels are untested
- FYI: it appears that information from up to 99 different dyes can be stored in .fsa or .hid files (based on current data file structure schema)

ABI 3500 Open Letter

Status Update on March 2011
Open Letter to Applied Biosystems

Open Letter to Applied Biosystems on Concerns with ABI 3500

- **3/14/11 - emailed ~900 forensic DNA scientists** (SWGDAM, forens-dna, ENFSI, EDNAP) inviting them to sign onto a letter that will be sent to Applied Biosystems expressing concern with ABI 3500
- **Very positive response with 101 who agreed to sign the letter**
- Letter was sent March 31 to the president of ABI and scientists involved with the ABI 3500
- **Community will be notified of ABI's response**

Concerns Expressed in Open Letter

- RFID tags
- New .hid file structure requires new software
- Short shelf life of reagents – would like to see data for expiration times

Hopefully a change will result…

A desire for greater communication with the community – the 3500 FAQ sheet is a good start but does not directly address all of the concerns raised

Brief Timeline of Events

- NIJ requested NIST to explore capabilities, limitations, and cost of ABI 3500 instrument and reagents (May 2010)
- NIST presentations to NIJ (Dec 2010) and SWGDAM (Jan 2011)
- **Open letter support solicited and sent to ABI** (Mar 2011)
- Further discussions between NIST and ABI (Apr-Sept 2011)
- At the Promega ISHI meeting (Oct 2011), ABI announced through a poster at their booth that **polymer and buffer expiration dates will no longer be a hard stop** but only a warning with the future Windows 7 software upgrade

Since May 2011, Erica Butts has presented several validation presentations on our ABI 3500 work – these are available on STRBase

What was learned from the May 11 visit of ABI scientists to NIST…

- RFID over-ride is possible (their R&D lab has instrument that can use "expired" reagents) – they are "considering" making this option available
- New software is required for 3500 .hid or .fsa files due to new file structure
- They do not have ANY data to support short shelf life of 3500 reagents
  - A business decision to set hard stops to keep labs from having failures that lead to ABI having to replace arrays
- ABI 31xx instruments have DEPRESSED signal (i.e., should have a lower analytical threshold)
- **Normalization is not well worked out by ABI or really understood** (although this has been a major selling point for the 3500)
- ABI was shocked that there were concerns with some of the feedback

A Sampling of Feedback Received…

- **People did not just sign the letter but many have an opinion about the issues or concern about ABI customer support (I have received >100 emails – often with some very strong thoughts)**
- "I think that the AB3500 related issues most likely represent the beginning of a sea of problems, against which every independent lab must take arms. **It is not up to the manufacturer of a machine to decide the basic procedures of a lab - it is up to the lab**" (4/29/11)
- "I greatly appreciate your advocacy on behalf of our community. **Hopefully we will be heard**." (4/1/11)

Response from Dr. Robin Cotton
(shared with her permission)

Sent: Saturday, April 30, 2011 10:39 AM

Dear John,

Thank you for the information and the inclusion of the letter from Dr. Klevan. It is clear that **Dr. Klevan does not consider the substantial time and expense which will be required for each forensic laboratory for instrument and software validation.**

The other point which I feel is significant is the need for the additional software purchase. Since he states that the new software is compatible with .fsa files, I think **the company should make a software exchange available at low cost for any lab purchasing the 3500 instrument**. Many commercially available software companies make new versions available at reduced costs to individuals or groups already running an earlier versions. **Because of the increased number of technical changes the 3500 presents, the validation data may be more extensive than was required for previous instrument change-over and thus the validation time and cost to each laboratory will also be increased.**

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It would also be relevant to ask Dr. Klevan to provide figures for the number of current 3500 users without the inclusion of paternity testing laboratories which are all commercial operations. While I am an advocate for private laboratories (both forensic and paternity), these facilities have the option to raise prices and accommodate the need for increased validation time and expense in other ways that do not require federal or other government support.

Additionally, in the Biomedical Forensic Science Masters program here at BU, we feel it is important to teach our students using current instrumentation and techniques. **Introduction of this new instrument will affect many forensic science teaching institutions, both undergraduate and graduate, as well as all current forensic DNA testing laboratories.** These institutions have significantly less access to NIJ funding for large equipment and software than the operating forensic DNA laboratories. Thus **the effect of changes reach into the educational institutions as well.**

Regards,
Robin W. Cotton, Ph.D.
Boston University

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ABI 3500 Open Letter Update

Concerns Expressed in 3/31/11 Open Letter

1. RFID tags
2. New .hid file structure requires new software
3. Short shelf life of reagents – would like to see data for expiration times

At the Promega ISHI meeting (Oct 2011), ABI described data for studies around reagent expiration through a poster at their booth. Sailus, Wheaton, Fisher, Calandro. "Understanding the Consumables on the 3500 Genetic Analyzers in the context of a Human Identification (HID) Laboratory"

They have promised that **polymer and buffer expiration dates will no longer be a hard stop** but only a warning with the future Windows 7 software upgrade (3500 Data Collection v1.3).

Thank you for your attention

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Contact Information

Becky Hill
Research Biologist
becky.hill@nist.gov
301-975-4275

Mike Coble
Research Biologist
michael.coble@nist.gov
301-975-4330

Our team publications and presentations are available at:
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm