Development of Protocols for Rapid Amplification of STR Typing Kits: The Use of 'Non-Standard' Thermal Cyclers

Peter M. Vallone and Erica Butts
Applied Genetics Group
National Institute of Standards and Technology

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final version can be found at
http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm#Presentations

Rapid PCR

Applications

- Faster sample-to-answer
- Increase throughput
- Integrated platforms for forensics and biometrics
- Single source reference samples = 1 ng of DNA

Initial Questions

- Robustness
- Concordance
- Sensitivity
- PCR artifacts
- Stutter, peak height balance
- Locus-to-locus balance

Develop a PCR protocols for typing multiplex STR kits in less than 2 hours

Recent Literature on Integrated Typing Systems and Rapid PCR

- Development of a fast PCR protocol enabling rapid generation of AmpFℓSTR® Identifiler® profiles for genotyping of human DNA Foster and Laurin Investig Genet. (2012) Mar 3:6
- Optimization and validation of a fast amplification protocol for AmpFISTR® Profiler Plus® for rapid forensic human identification Laurin and Frégeau Forensic Sci Int Genet. (2012) 6:47-57
- A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples Verheij et al. (2012) Forensic Sci Int Genet. 6:167-75
- The development of mini pentameric STR loci for rapid analysis of forensic DNA samples on a microfluidic system. Aboud et al. (2010) Electrophoresis 31:2672-9
- Integrated Microfluidic System for Rapid Forensic DNA Analysis: Sample Collection to DNA Profile Hopwood et al. *Anal. Chem.* (2010) *Anal. Chem. 82: 6991–6999*
- An integrated microfluidic device for DNA purification and PCR amplification of STR fragments Bienvenue et al. Forensic Sci Int Genet. (2010) 4:178–186
- Fast Multiplexed Polymerase Chain Reaction for Conventional and Microfluidic Short Tandem Repeat Analysis Giese et al. J Forensic Sci, (2009) 54:1287-1296
- Demonstration of rapid multiplex PCR amplification involving 16 genetic loci Vallone et al. Forensic Sci Int Genet. 2008 3:42-45
- Real-time forensic DNA analysis at a crime scene using a portable microchip analyzer Liu et al. Forensic Sci Int Genet (2008) 2:301–309

Commercial STR Kits

Thermal Cycling Times

Thermal Cycling Times for Current STR Typing Kits

Year	Run on a 9700 thermal cycler	Hot start	Time per cycle	Cycles	Post soak	Total time
1997/98	Profiler Plus/Cofiler	11 min	3 min	28	60 min	2:52
1999	SGM Plus	11 min	3 min	28	45 min	2:53
2000	PowerPlex 16	12 min	1 min 45 s	32	30 min	3:00
2001	Identifiler	11 min	3 min	28	60 min	2:58
2003	PowerPlex Y	12 min	1 min 45 s	32	30 min	3:18
2004	Yfiler	11 min	3 min	30	80 min	2:45
2007	PowerPlex S5	2 min	4 min	30	45 min	3:21
2007	minifiler	11 min	3 min 20 s	30	45 min	3:16
2009	ESI 16, 17 ESX 16,17	2 min	4 min	30	45 min	3:22
2009	PowerPlex 16 HS	2 min	1 min 45 s	32	30 min	2:42
2009	NGM	11 min	3 min 20 s	29	10 min	2:33
2009	Identifler Direct	11 min	3 min	26	25 min	2:34
2010	Idenfiler Plus	11 min	3 min 20 s	28	10 min	2:18
2011	PowerPlex 18D	2 min	1 min 10s	27	20 min	1:25
2012	NGM Express (direct)	1 min	48 s	26	5 min	0:45

Thermal Cyclers

Manufacturer	Cycler	Tube Vol (µL)	# samples
Applied Biosystems	GeneAmp PCR System 9700	0.2 mL	96
Eppendorf	Mastercycler Pro S	0.2 mL	96
Qiagen	Rotor-Gene Q	0.1 mL	72
Cepheid	SmartCycler	25 µL	16
Streck	Philisa	50 µL	8
Thermo Scientific – Finnzymes	Piko	20 µL	96
Analytik Jena	SpeedCycler[2]	20 µL	96
Ahram	Palm PCR	20 µL	12

- We currently have these 8 thermal cyclers in house are developing rapid PCR protocols
- Varying characteristics of heating/cooling and tube (reaction vessel)
- Rotor-Gene Q and SmartCycler are real-time PCR instruments

PCR Thermal Cycling Profile

Identifiler STR kit

28 cycle PCR

Standard cycling parameters

≈ 3 h

95°C 10 min | 95°C 1 min | 59°C 1 min | 72°C 1 min | 60°C 60 min

95°C 1 min | 95°C 5 s | 58°C 10 s | 72°C 10 s | 72°C 1 min

36 min on Gene Amp 9700

Demonstration of rapid multiplex PCR amplification involving 16 genetic loci Vallone et al. *Forensic Sci Int Genet. 2008 3:42-45*

PCR Thermal Cycling Profile

Identifiler STR kit

28 cycles of PCR

95°C 1 min | 95°C 5 s | 58°C 10 s | 72°C 10 s | 72°C 1 min

36 min on Gene Amp 9700

Demonstration of rapid multiplex PCR amplification involving 16 genetic loci Vallone et al. *Forensic Sci Int Genet. 2008 3:42-45*

95°C 1 min | 95°C 5 s | 61°C 15 s | 72°C 1 min

2-step PCR
31 min on Gene Amp 9700

Reduced PCR artifacts

Development of a fast PCR protocol enabling rapid generation of AmpFtSTR® Identifiler® profiles for genotyping of human DNA Foster and Laurin Investig Genet. (2012) 3:6

Rapid PCR Protocols: Thermal Cyclers

GeneAmp 9700 (Applied Biosystems)

Heating rate: 4°C/s
Heating mechanism: Thermal block (Al)
Tube format: 0.2 mL
96 reactions per instrument run
28 cycles; 3-step (36 min); 2-step (31 min)

Mastercycler Pro S (Eppendorf)

Heating rate: 6°C/s
Heating mechanism: Thermal block (Ag)
Tube format: 0.2 mL
96 reactions per instrument run
28 cycles; 3-step (19 min); 2-step (16.5 min)

Rapid PCR Protocols: Thermal Cyclers

SmartCycler (Cepheid)

Heating rate: 10°C/s
Heating mechanism: heating plates and air circulating fan
Tube format: proprietary 25 µL tubes
16 reactions per instrument run
28 cycles; 3-step (21.8 min); 2-step (18.2 min)

Rotor-Gene Q (Qiagen)

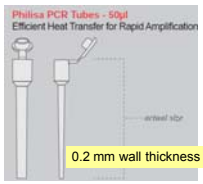
Heating rate: 15°C/s
Heating mechanism: Air chamber (spinning rotor)
Tube format: 0.1 mL
72 tube reactions per instrument run
28 cycles; 3-step (36 min); 2-step (32 min)

Rapid Advancement: Thermal Cyclers

Philisa (Streck)

Runs off of a netbook

Heating rate: 15°C/s (cooling 12°C/s)
Heating mechanism: Thermal block (Ag)
Tube format: proprietary 50 µL tubes
8 reactions per instrument run
28 cycles; 3-step (17 min); 2-step (14 min)



0.2 mm wall thickness

Requires the use of gel loading tips to load PCR product into CE setup plate due to tube design

Rapid Advancement: Thermal Cyclers

Piko (Thermo Scientific)

Heating rate: 5°C/s
Heating mechanism: Thermal block
Tube format: 20 µL plate
96 reactions per instrument run
28 cycles; 3-step (30.5 min); 2-step (25.5 min)

Rapid Advancement: Thermal Cyclers

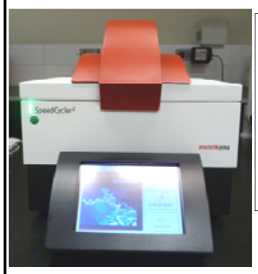
Palm PCR (Ahram)

Heating rate: 7 speeds
Heating mechanism: Thermal block
Tube format: 20 µL tubes
12 reactions per instrument run
28 cycles; 17-37 min (only select T_a and ramp)

Hand held and battery operated for portability

Rapid Advancement: Thermal Cyclers

SpeedCycler² (Analytik Jena)



Heating rate: 15°C/s (cooling 10°C/s)
Low Profile Rapid (LPR) block
Heating mechanism: Thermal block (Ag block, Au coated)
Tube format: 20 µL (LPR) or 0.2 mL tube
96 reactions per instrument run
28 cycles; 3-step (22 min); 2-step (18.5 min) – LPR times

Difficulty obtaining tubes/plates
No data collected

Effective heating/cooling rate

min	Cycler	Effective Heating/Cooling deg/s
36	GeneAmp 9700	1.6
19	Mastercycler Pro S	6.8
36	Rotor-Gene Q	1.6
22	SmartCycler	4.4
17	Philisa	10.9
30	Piko	2.2
22	SpeedCycler²	4.4
17	Palm PCR	10.9

Rates for heating/cooling were estimated from the total cycling time

Comparative Throughput (Cycling)

		3 step	2 step		3 step	2 step
Cycler	# samples	Fastest Cycling Time (min)	Fastest Cycling Time (min)	Runs to complete 96 samples	Total min	Total min
GeneAmp PCR System 9700	96	36	31	1	36	31
Mastercycler Pro S	96	19	17	1	19	17
Rotor-Gene Q	72	36	32	2	72	64
SmartCycler	16	22	18	6	132	108
Philisa	8	17	14	12	204	168
Piko	96	30	26	1	30	26
SpeedCycler²	96	22	19	1	22	19
Palm PCR	12	17	17	8	136	136

While cycling times may be rapid, the throughput in some cases is reduced from the standard 96-well format

Experiments

- Performance of each cycler with 2- and 3-step thermal cycling protocols (n = 15 samples)
- Sensitivity study: 1 sample, 7 concentrations in duplicate; compare 2- and 3-step PCR protocols
- 95 samples run on the 9700 (2- versus 3-step comparisons)
- Initial results developing a rapid-direct PCR protocol

DNA Polymerases

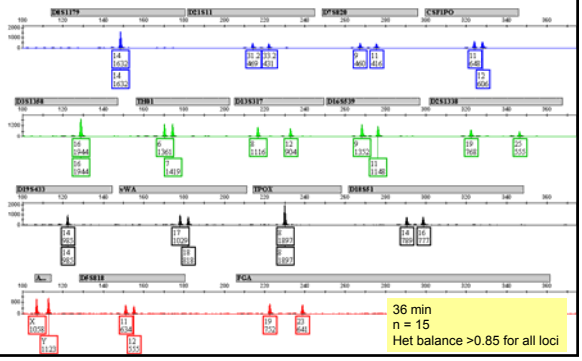
- AmpliTaq Gold® is typically used
  - Heat activated (avoid non-specific PCR products)
- SpeedSTAR™ HS DNA Polymerase
  - Extension times of 100 bp/s are possible (compared to 20 bp/s for other polymerases)
  - Hot-start formulation is antibody mediated
- Qiagen
  - QIAGEN Fast Cycling PCR Kit
- New England Biolabs/Finnzymes
  - Phusion and Phire DNA Polymerases
- KAPA Biosystems
  - KAPA2G Fast PCR Kits
- Biotium
  - Cheetah™ Taq
- Fermentas
  - ~~PyroStart Master Mix~~
- EMD Millipore
  - KOD DNA Polymerse

Rapid PCR Conditions

- 1 X Takara PCR mastermix, 1 U SpeedStar polymerase
  - *Premix Ex Taq*™ (Perfect Real Time)
- 10 μL total reaction in a thin walled tube (8-strip) or proprietary tube
- 2 μL of Identifiler PCR primer mix
- ~1 ng of template DNA

1 ng of DNA template

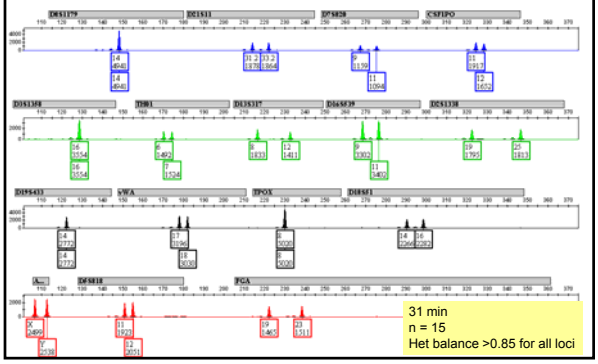
AB 9700 3-Step



36 min
n = 15
Het balance >0.85 for all loci

1 ng of DNA template

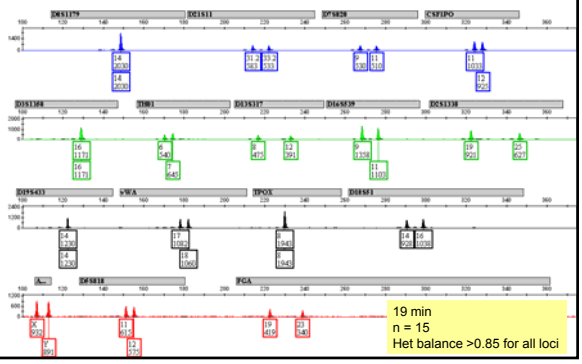
AB 9700 2-Step



31 min
n = 15
Het balance >0.85 for all loci

1 ng of DNA template

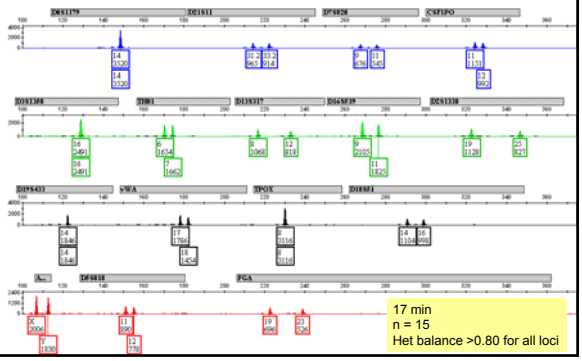
MasterCycler Pro 3-Step



19 min
n = 15
Het balance >0.85 for all loci

1 ng of DNA template

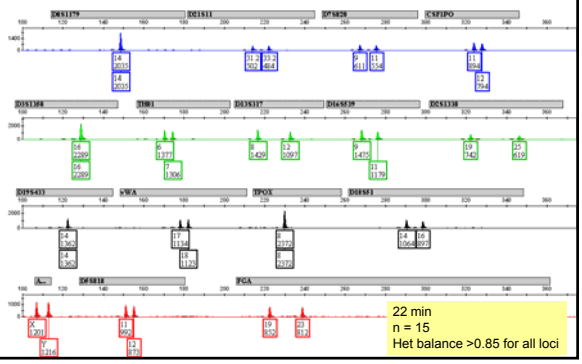
MasterCycler Pro 2-Step



17 min
n = 15
Het balance >0.80 for all loci

1 ng of DNA template

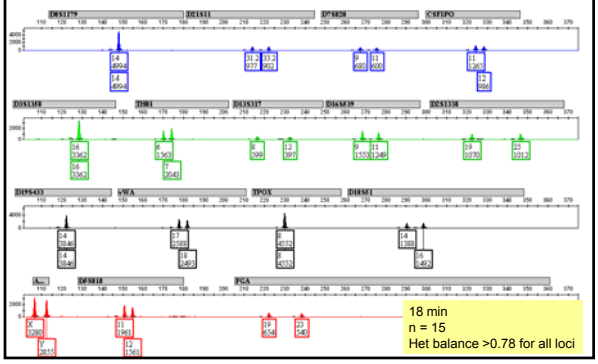
SmartCycler 3-Step



22 min
n = 15
Het balance >0.85 for all loci

1 ng of DNA template

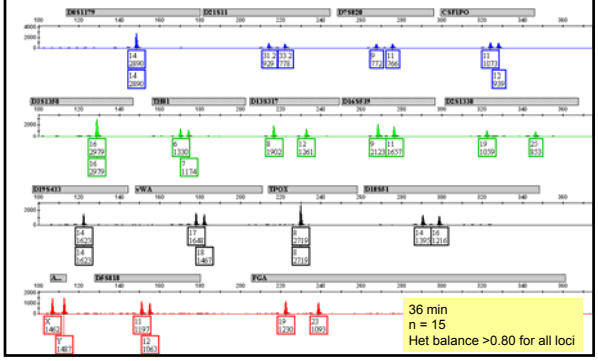
SmartCycler 2-Step



18 min
n = 15
Het balance >0.78 for all loci

1 ng of DNA template

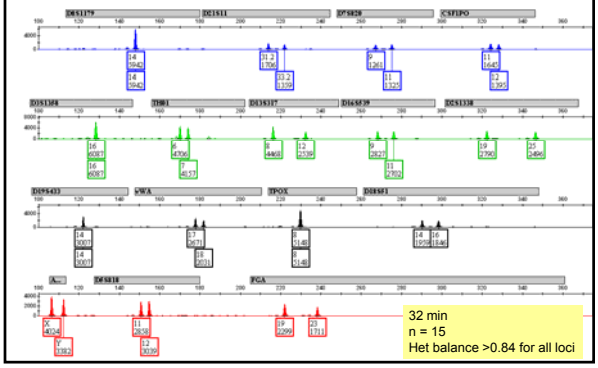
RotorGene 3-Step



36 min
n = 15
Het balance >0.80 for all loci

1 ng of DNA template

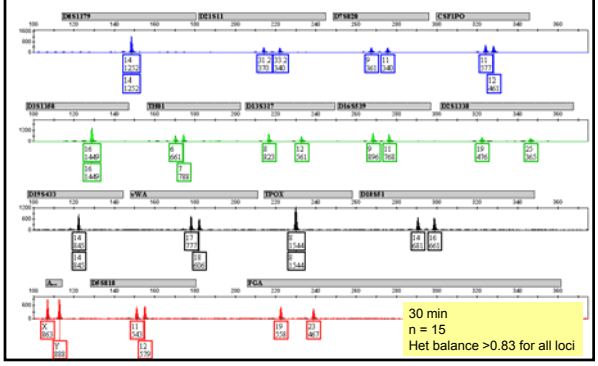
RotorGene 2-Step



32 min
n = 15
Het balance >0.84 for all loci

1 ng of DNA template

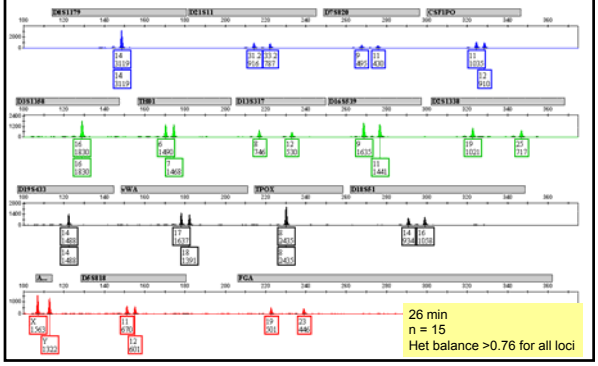
Piko 3-Step



30 min
n = 15
Het balance >0.83 for all loci

1 ng of DNA template

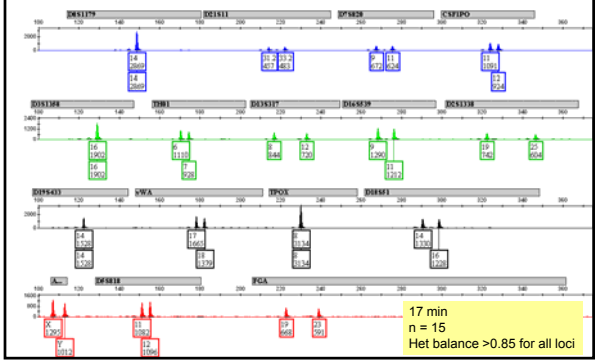
Piko 2-Step



26 min
n = 15
Het balance >0.76 for all loci

1 ng of DNA template

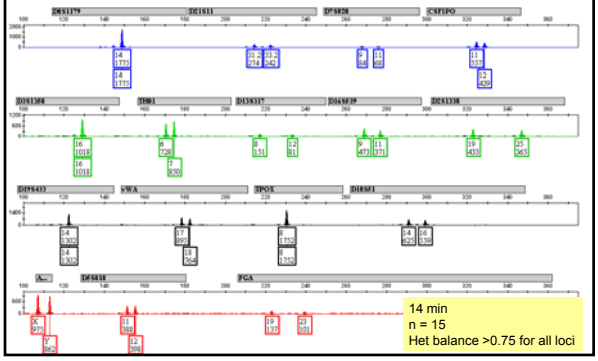
Philisa 3-Step



17 min
n = 15
Het balance >0.85 for all loci

1 ng of DNA template

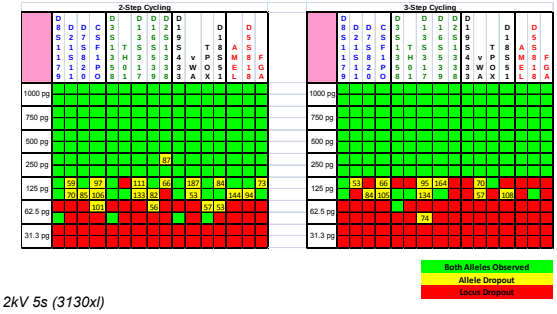
Philisa 2-Step



14 min
n = 15
Het balance >0.75 for all loci

Sensitivity 9700

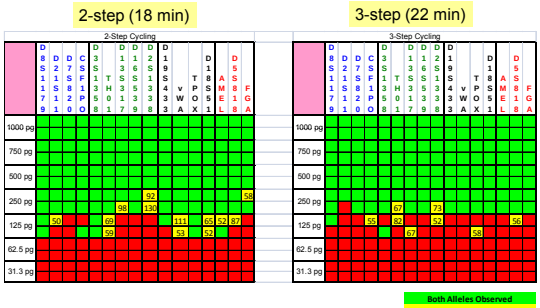
2-step (31 min) 3-step (36 min)



2kV 5s (3130xl)

Sensitivity SmartCycler

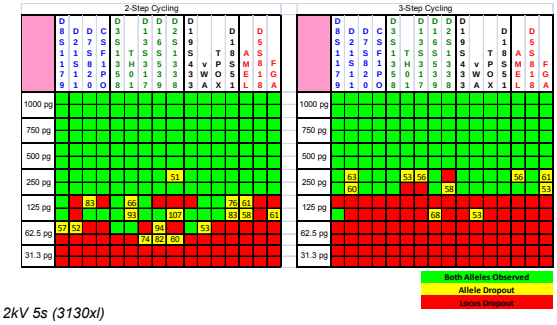
2-step (18 min) 3-step (22 min)



2kV 5s (3130xl)

Sensitivity Mastercycler Pro

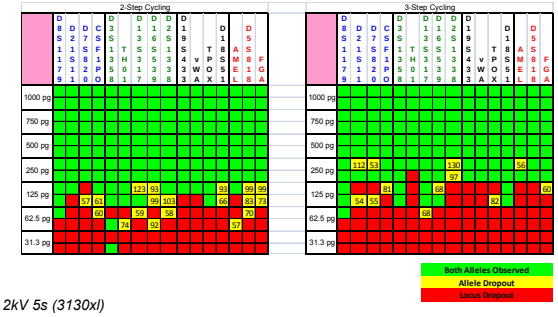
2-step (17 min) 3-step (19 min)



2kV 5s (3130xl)

Sensitivity Rotor-Gene

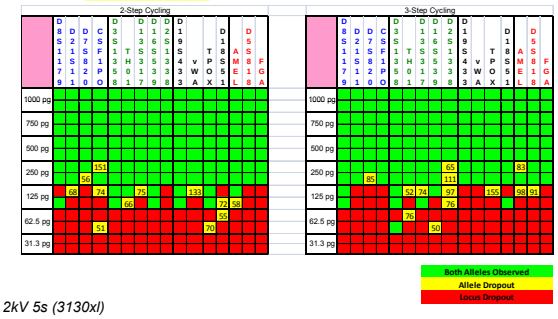
2-step (32 min) 3-step (36 min)



2kV 5s (3130xl)

Sensitivity Piko

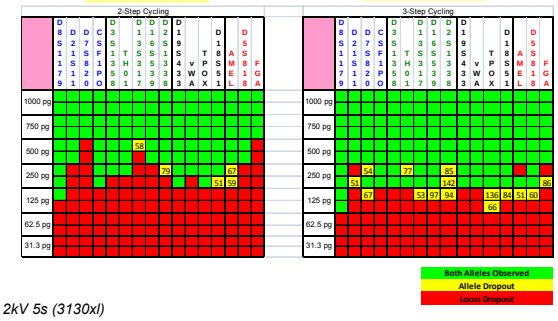
2-step (26 min) 3-step (30 min)



2kV 5s (3130xl)

Sensitivity Philisa

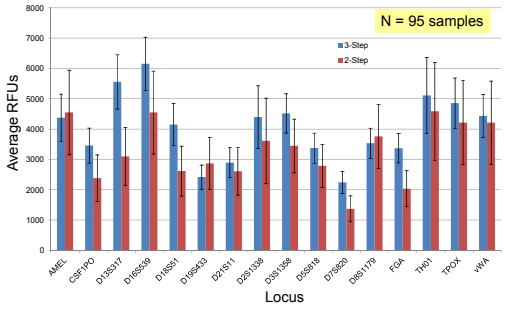
2-step (14 min) 3-step (17 min)



2kV 5s (3130xl)

RFU comparison for 9700 data

2-step versus 3-step



Peak Height Balance and Stutter

9700 data 2-step versus 3-step

N = 95 samples

n		Median PHR 2-step	Median PHR 3-step
95	AMEL	0.937	0.935
78	CSF1PO	0.871	0.904
71	D13S317	0.873	0.917
76	D16S539	0.875	0.909
82	D18S51	0.887	0.899
72	D19S433	0.914	0.929
71	D21S11	0.893	0.894
85	D2S1338	0.900	0.889
78	D3S1358	0.863	0.900
63	D5S818	0.850	0.897
78	D7S820	0.903	0.907
73	D8S1179	0.910	0.905
80	FGA	0.884	0.908
60	TH01	0.940	0.910
62	TPOX	0.919	0.930
77	vWA	0.880	0.893

error = 0.05 - 0.09

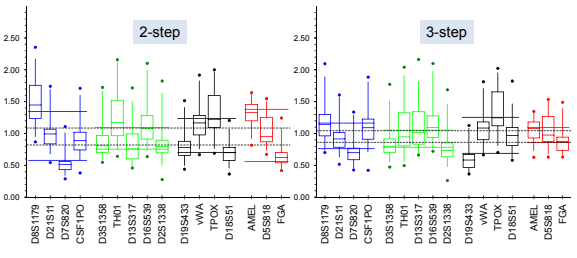
% stutter

n	Locus	2-step Median	2-step MADe	3 step Median	3 step MADe
99	TH01	2.2	1.1	2.1	1.0
115	TPOX	3.2	1.5	3.0	1.4
109	D7S820	5.4	2.2	5.0	2.3
96	D13S317	5.6	2.0	5.3	1.8
75	CSF1PO	6.3	1.9	5.5	1.5
107	D16S539	7.1	2.5	6.8	2.2
92	D5S818	8.0	1.6	7.2	1.1
93	D8S1179	8.4	1.6	7.5	1.6
115	vWA	8.8	2.1	8.1	2.0
133	D21S11	9.2	1.6	8.6	1.4
130	D18S51	9.3	2.8	8.6	2.2
129	FGA	9.5	1.5	8.7	2.4
101	D19S433	9.6	2.4	8.7	1.5
85	D3S1358	10.6	2.1	9.4	1.9
112	D2S1338	10.9	2.3	10.8	2.1

Intra-color balance

9700 data 2-step versus 3-step

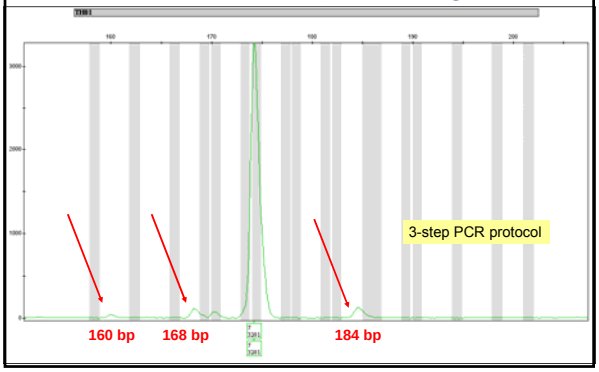
N = 95 samples



Y-axis = Locus RFU / Dye Group Average

Plots created by Dave Duewer's (NIST) excel-based software

PCR Artifacts in the TH01 amplicon range



PCR Artifacts (3-step only)

Artifacts Observed	9700	Smart Cycler	Master Cycler Pro	Rotor-Gene	Streck Philisa
D16 @ 287 bp	35	4	1	6	0
D8 @ 121 bp	6	0	1	3	0
D8 @ 174 bp	14	10	1	7	0
TH01 @ 160 bp	28	2	1	11	0
TH01 @ 168 bp	83	32	1	40	0
TH01 @ 184 bp	59	19	0	25	0
TPOX @ 219 bp	77	13	2	22	2
Total # Artifacts	302	80	7	114	2

N = 95 samples

- TH01 @ 184 is often covered/disguised by the base of the 9.3 allele peak
- 50 RFU threshold used when identifying artifacts

Rapid STR profiling in a lab setting

NIST presentation at the 2012 MAAFS Meeting

"Rapid DNA Testing Approaches for Reference Samples"
http://www.cstl.nist.gov/strbase/pub_pres/Butts-MAAFS2012-rapid-DNA-testing.pdf

Example Rapid Work Flow in Lab Setting

single source reference samples

DNA extraction → Rapid PCR → CE separation and detection

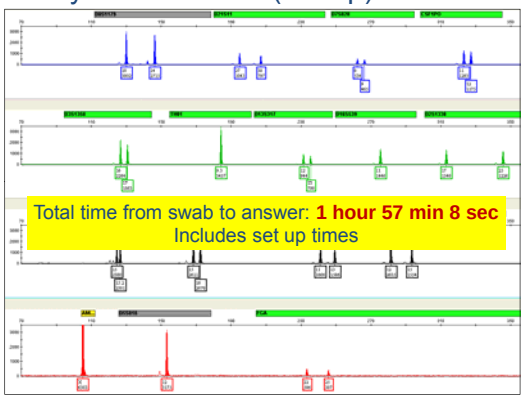
- ZyGEM liquid-based extraction
- Rapid Identifiler (9700 & Philisa cyclers)
- Separations on an 8 capillary 3500

Direct PCR → CE separation and detection

- PP18D (9700)
- Separations on an 8 capillary 3500

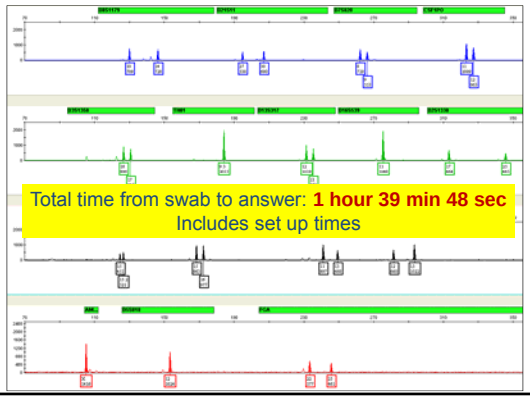
8 unique samples were typed in parallel

ZyGEM → 9700 (2-step) → 3500



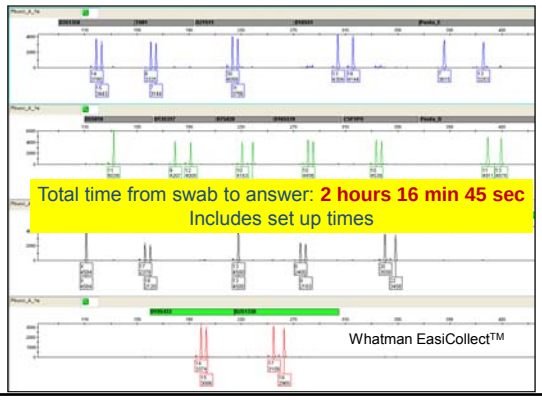
Total time from swab to answer: **1 hour 57 min 8 sec**
Includes set up times

ZyGEM → Streck (2-step) → 3500



Total time from swab to answer: **1 hour 39 min 48 sec**
Includes set up times

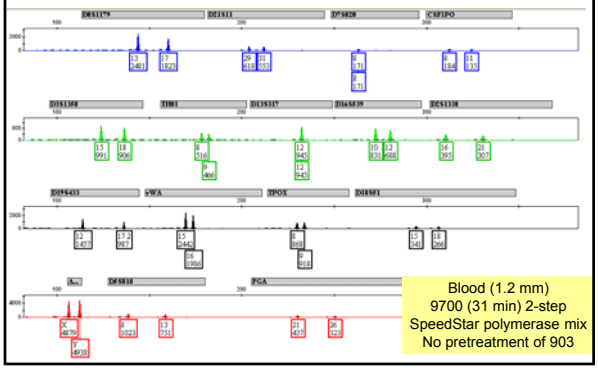
PP18D → 9700 → 3500



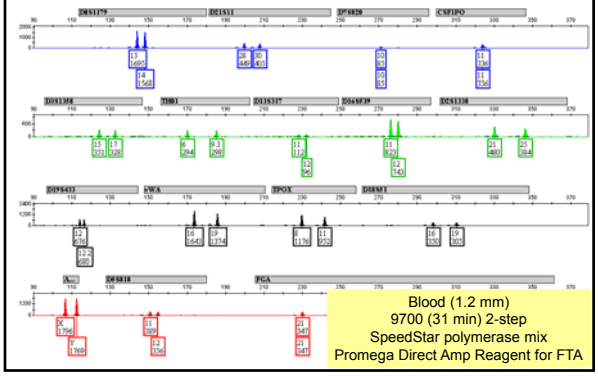
Direct Rapid PCR

- Can a direct rapid PCR protocol be developed?
- Overcome inhibitors in blood/FTA paper using rapid polymerases or additives
- Initial testing of protocols for typing blood from 903 and FTA paper substrates

Direct Rapid (Best result from 903)



Direct Rapid (Best result from FTA)



Blood (1.2 mm)
9700 (31 min) 2-step
SpeedStar polymerase mix
Promega Direct Amp Reagent for FTA

Summary and Future Work

- Successful protocols developed for 6/7 cyclers tested
  - 14 min PCR on Philisa cycler
- Continue work on Palm PCR and SpeedCycler^2
- Under the stated conditions sensitivity is around 250-500 pg of template DNA
- Further understanding of DNA polymerases and PCR enhancers in new commercial mastermixes
- 2-step PCR protocol:
  - Faster
  - Similar sensitivity compared to 3-step
  - Comparable RFUs; peak height balance and stutter
  - Fewer PCR artifacts
- Complete STR profiling in < 2 h (swab-to-answer)

Thank you for your attention!

Questions?

Peter.Vallone@nist.gov (1-301-975-4872)

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