Rapid PCR Protocols

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Rapid PCR

Applications

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

Develop a PCR protocol for typing the Applied Biosystems Identifiler STR kit in less than 1 hour

Initial Questions

- Robustness
- Sensitivity
- PCR artifacts
- Locus-to-locus balance

- Validation
- Mixtures
- PCR Inhibitors

Common Thermal Cycling Times

Can we reduce PCR cycling times? What are the effects or limitations?

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

DNA Polymerases

- AmpliTaq Gold® is typically used
  - Heat activated (avoid non-specific PCR products)
- SpeedST
  - Extensi ared to 20 bp
  - Still usi
  - Hot-sta

Fermentas
~~PyroStart Master Mix~~

Qiagen
QIAGEN Fast Cycling PCR Kit

New England Biolabs/Finnzymes
Phusion DNA Polymerases

Thermal Cyclers

1. GeneAmp 9700 (Applied Biosystems)
2. Mastercycler Pro S (Eppendorf)
   - Peltier based
3. Rotor-Gene Q (Qiagen)
   - Air heated and cooled
4. SmartCycler (Cepheid)
   - Hot plates for heating, fans for cooling

Intended for real-time PCR

- Cycling for most STR kits is run in the
- '9600 emulation mode' (1°C/s)

1

2

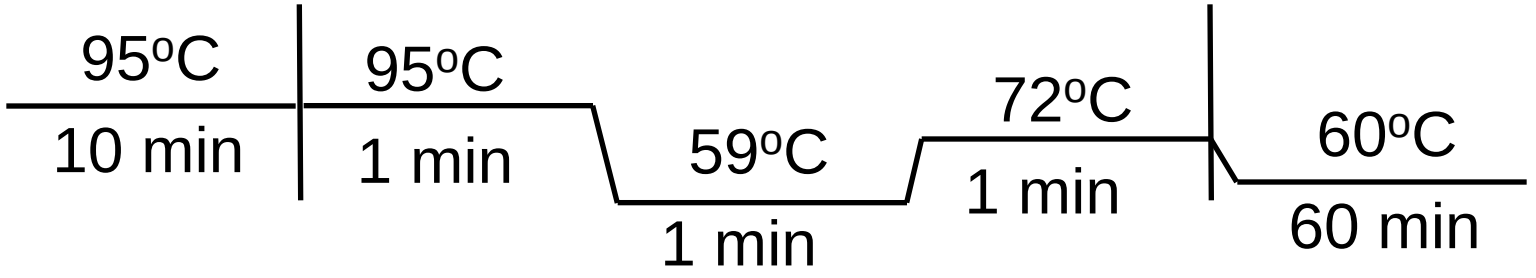
3

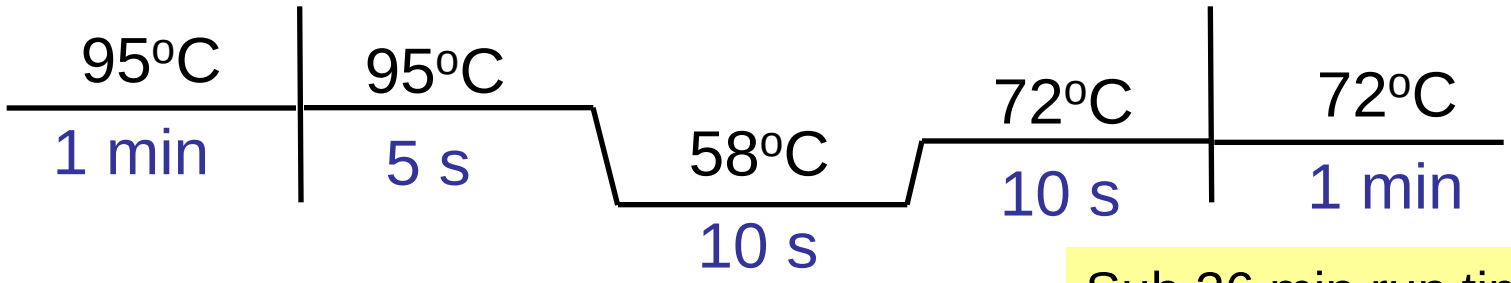
4

PCR Thermal Cycling Profile

Identifiler STR kit

28 cycles of PCR





Sub 36 min run time

Maximum heating/cooling rate of ~2 to 6°C/s (cycler dependent)

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)
- 2 μL of Identifiler PCR primer mix
- ~1 ng of template DNA

- Utilize maximum ramp rate on thermal cyclers
  - GeneAmp 9700 = 1.6°C/s (36 min)
  - Rotor-Gene Q = 1.6°C/s (36 min)
  - SmartCycler = 5.8°C/s (20 min)
  - Mastercycler Pro S = 6.8°C/s (19 min)

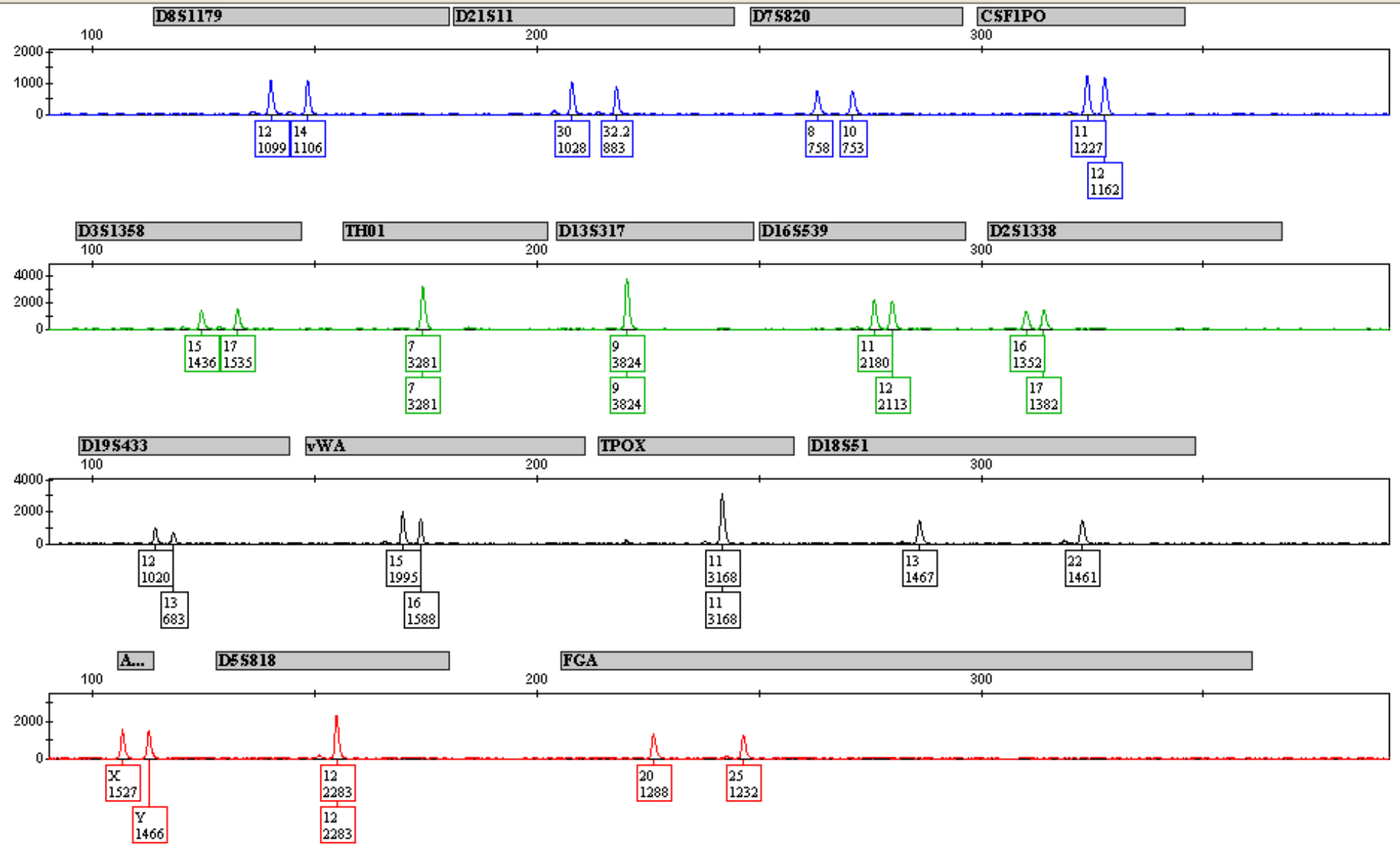
Effective heating/cooling rates

Experimental Design

- Type a set of NIST population samples (n=95)
- Evaluate
  - Full profiles (% success and concordance)
  - Identify artifacts of rapid PCR (adenylation, other)
  - Heterozygote peak height balance
  - Stutter %
  - Signal balance (locus-to-locus) and intensity
  - Sensitivity
- Not inhibitors or mixtures

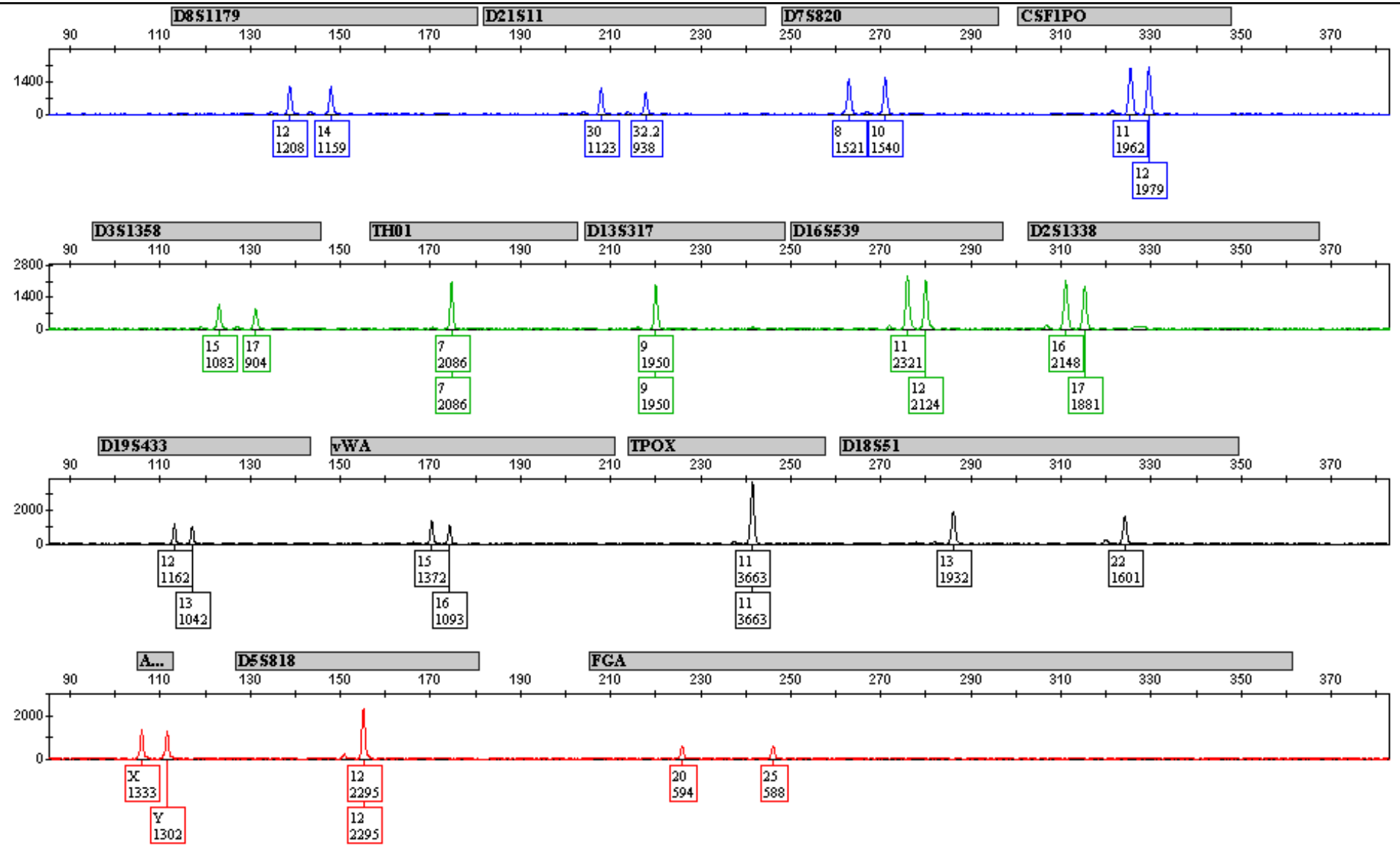
Separated on a 3130xl
Injection = 3kV for 5 s
Allele calling threshold = 50 RFUs

GeneAmp 9700 – 36 min PCR



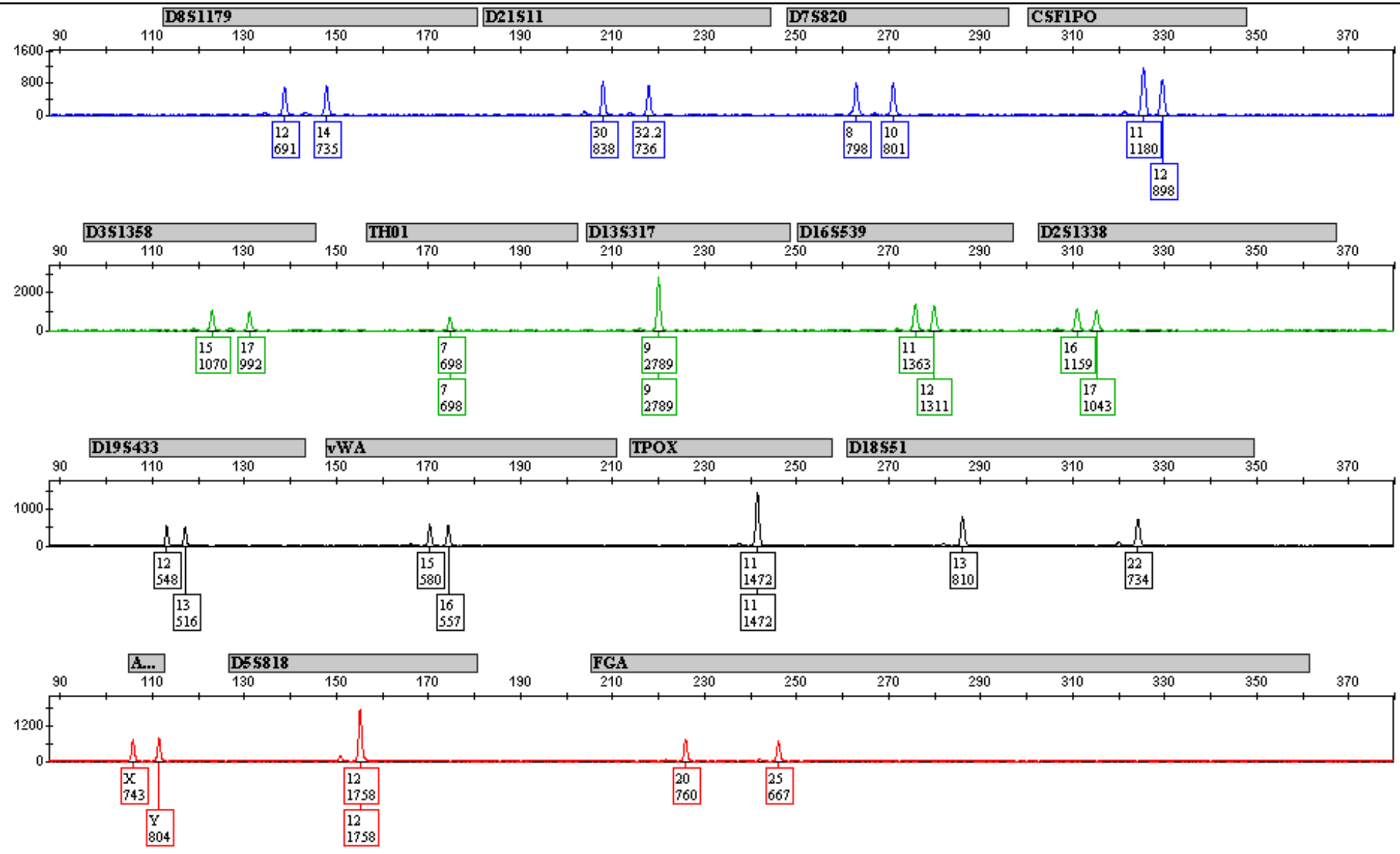
Identifiler STR Profile

Mastercycler Pro S - 19 min PCR



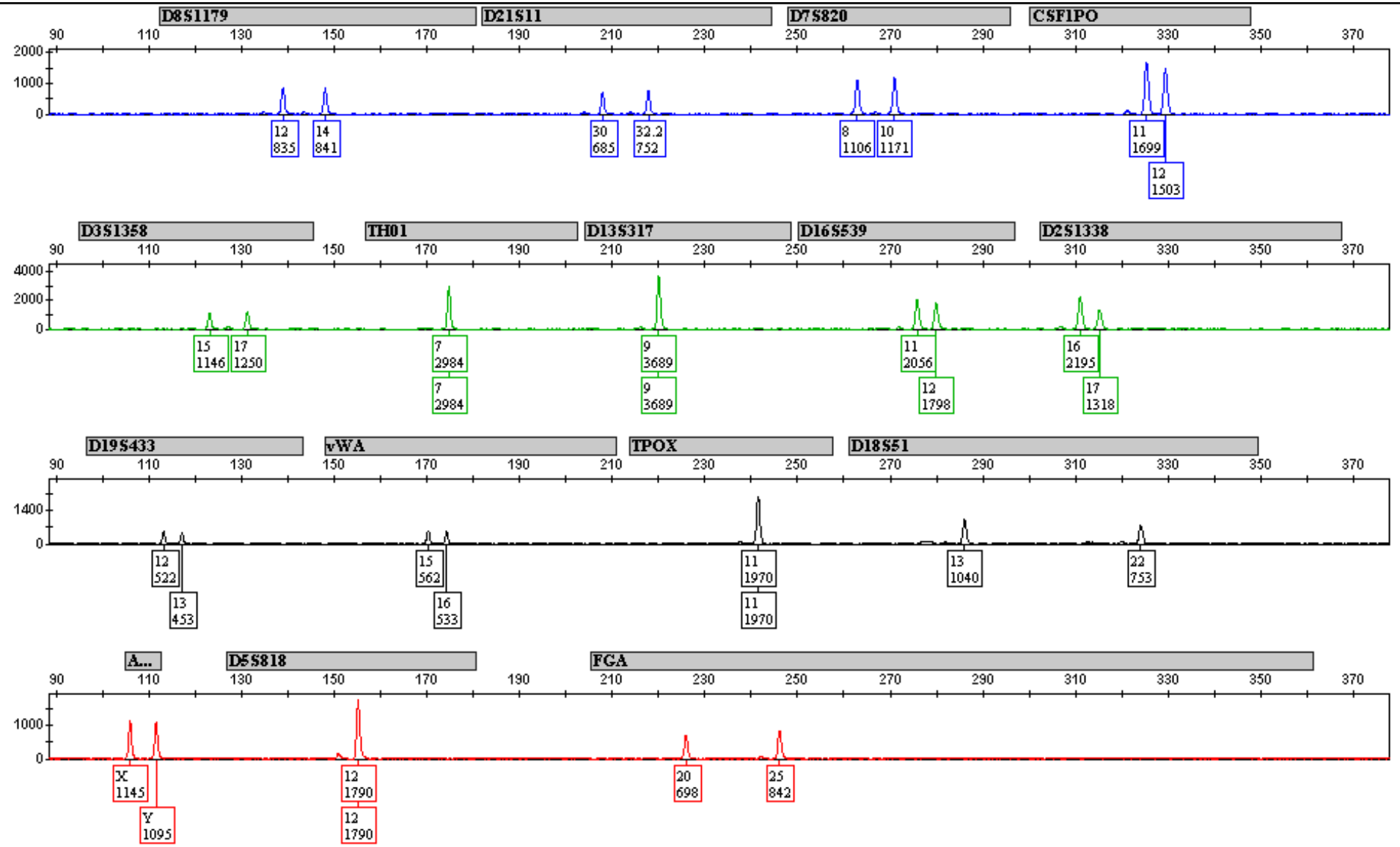
Identifiler STR Profile

Rotor-Gene Q – 36 min PCR



Identifiler STR Profile

SmartCycler – 20 min PCR

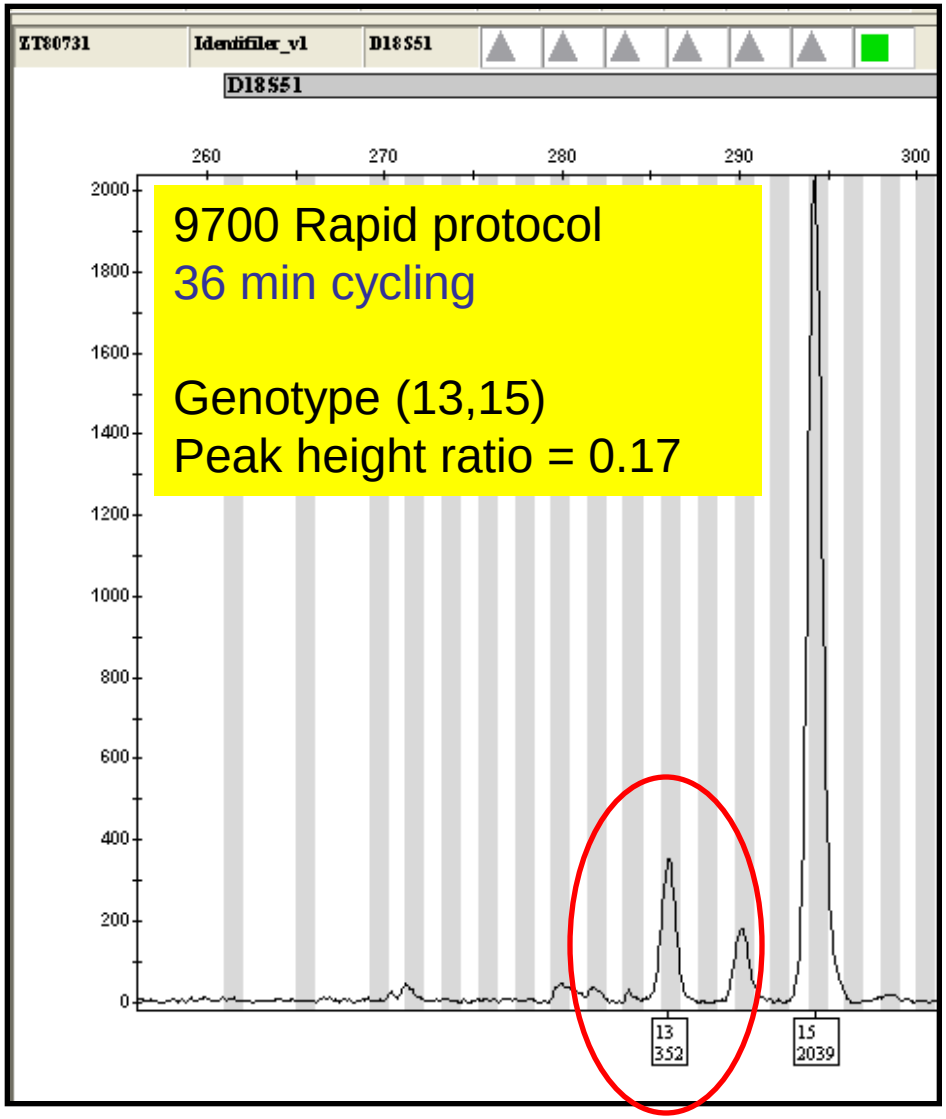


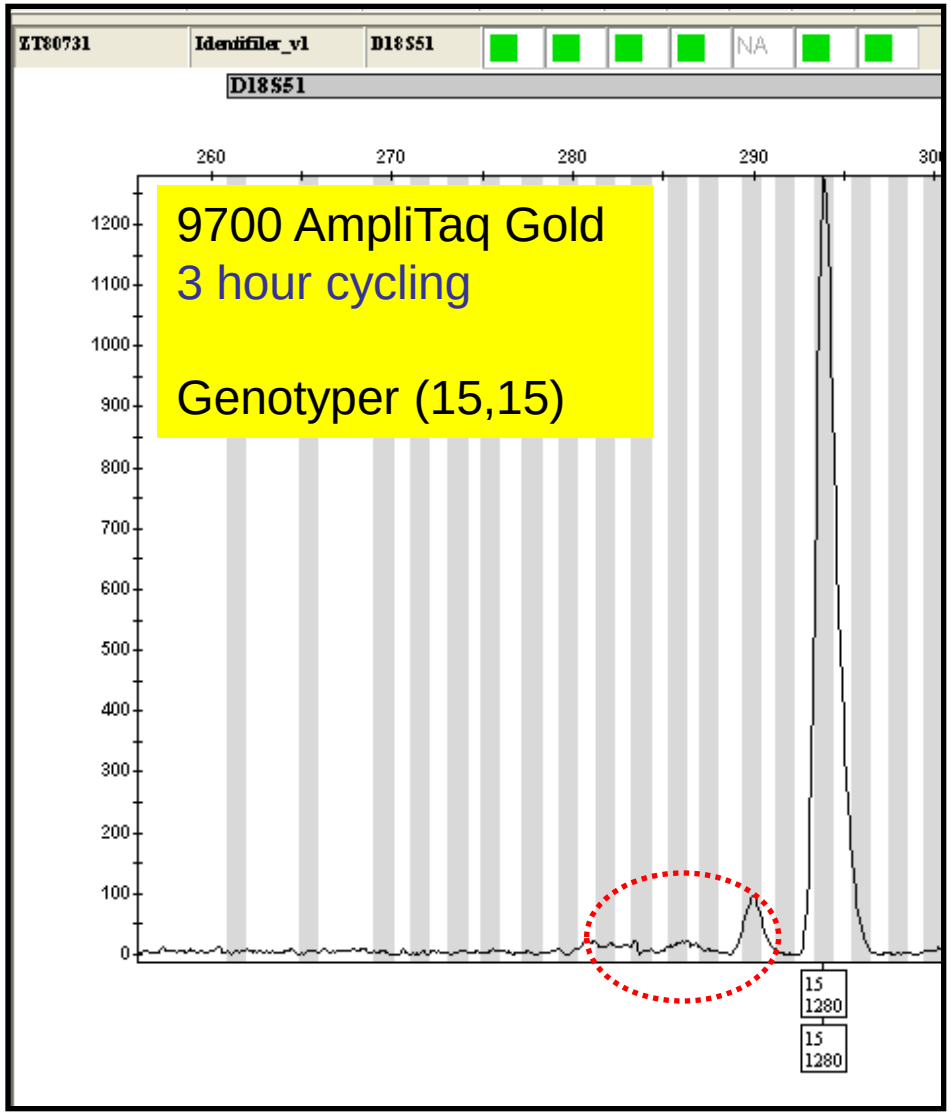
Summary of Initial Results

- All 95 samples were successfully typed on each thermal cycler using the rapid PCR protocol

- One sample gave a discordant genotype

Discordant sample
D18S51 - Identifiler





Discordant sample

- Amplification with PowerPlex 16 indicated a (13,15) genotype

- A SNP under the binding site an Identifiler PCR primer results in the null allele*

**Hill et al., (2007) J. Forensic Sci. 52: 870-873*

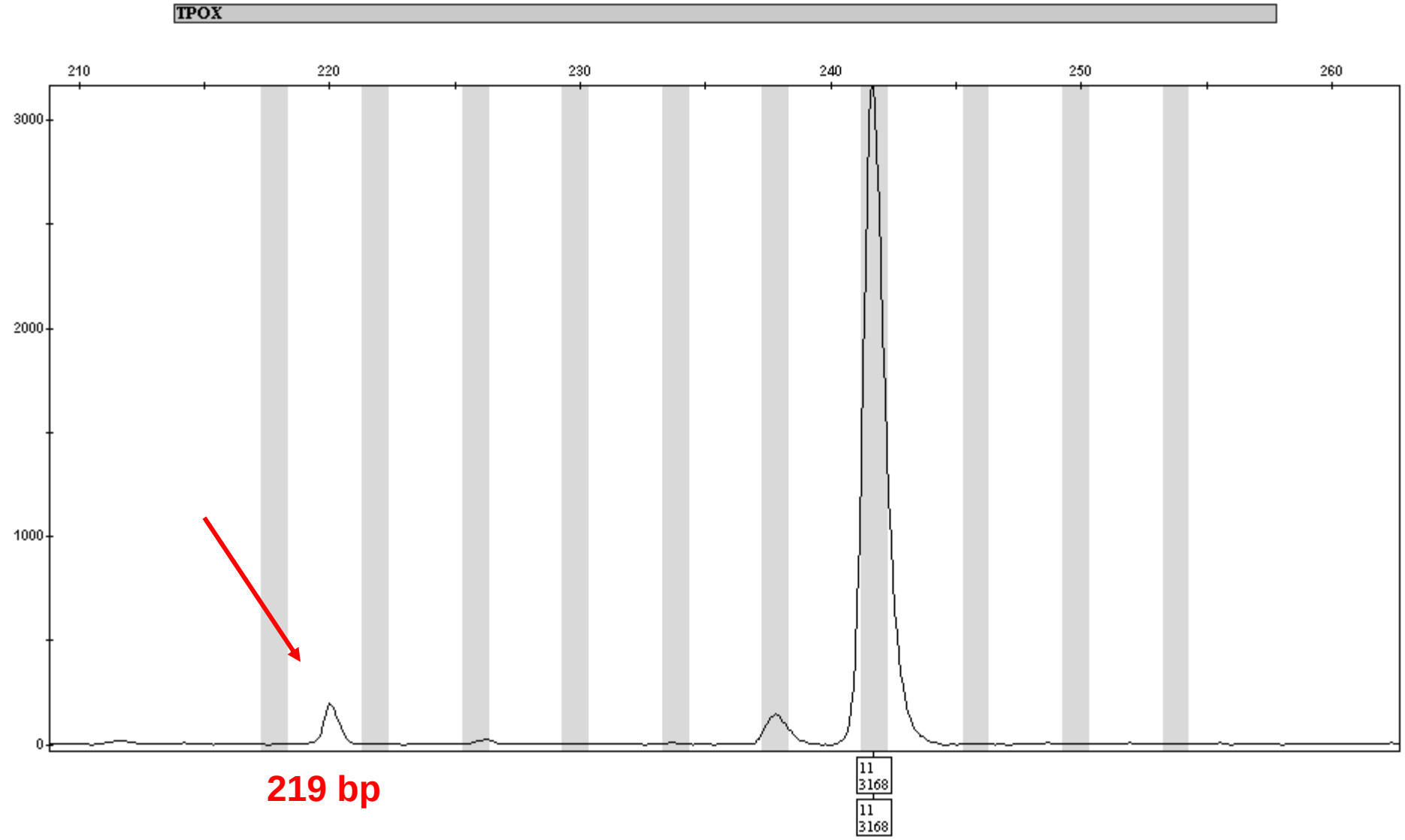
- Still present with a 59^{o}C annealing temperature
  - But with lower signal intensity

Mismatch tolerance

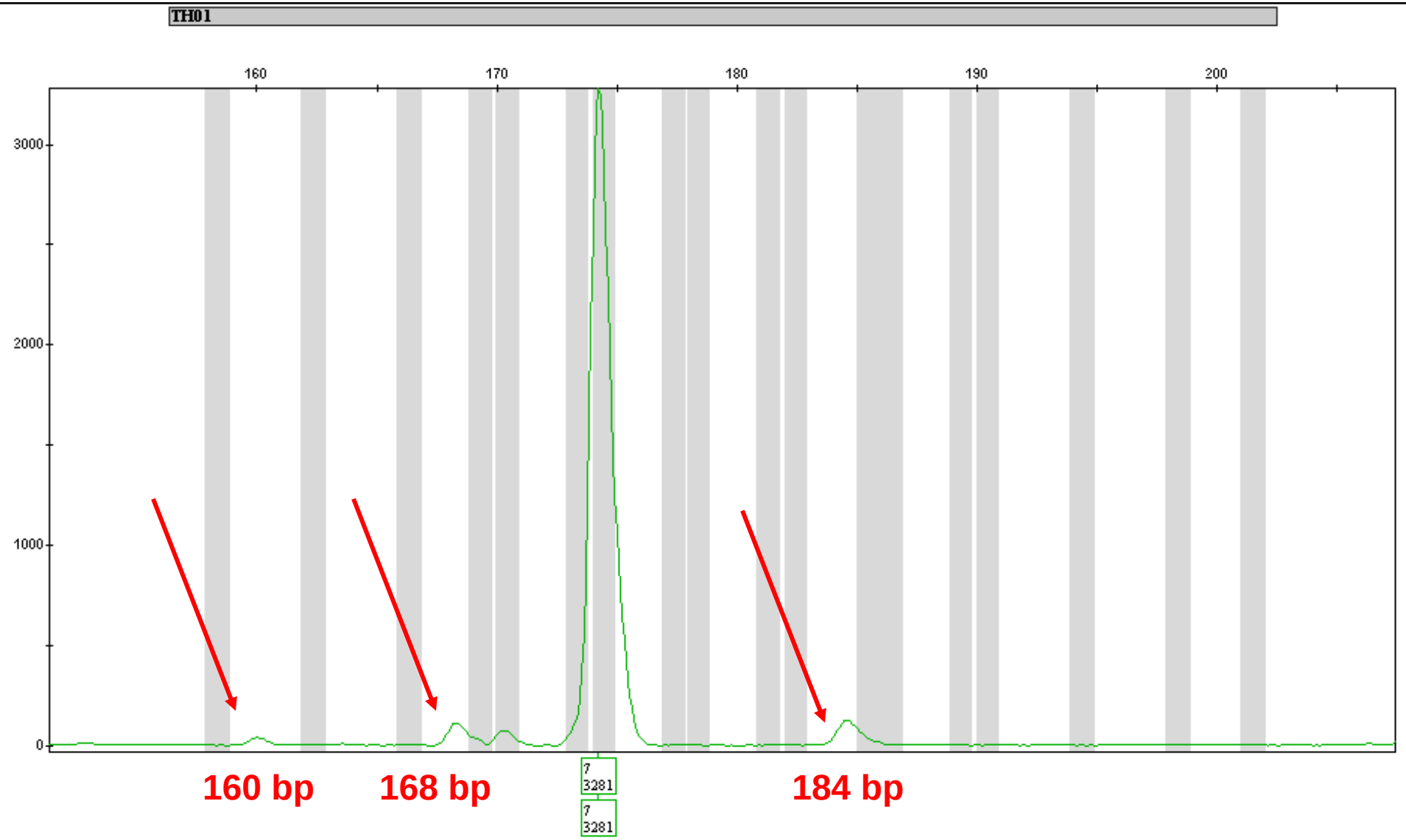
- Higher salt concentration in master mix buffer?
- Inherent characteristic of the DNA polymerase?

Rapid PCR Artifacts

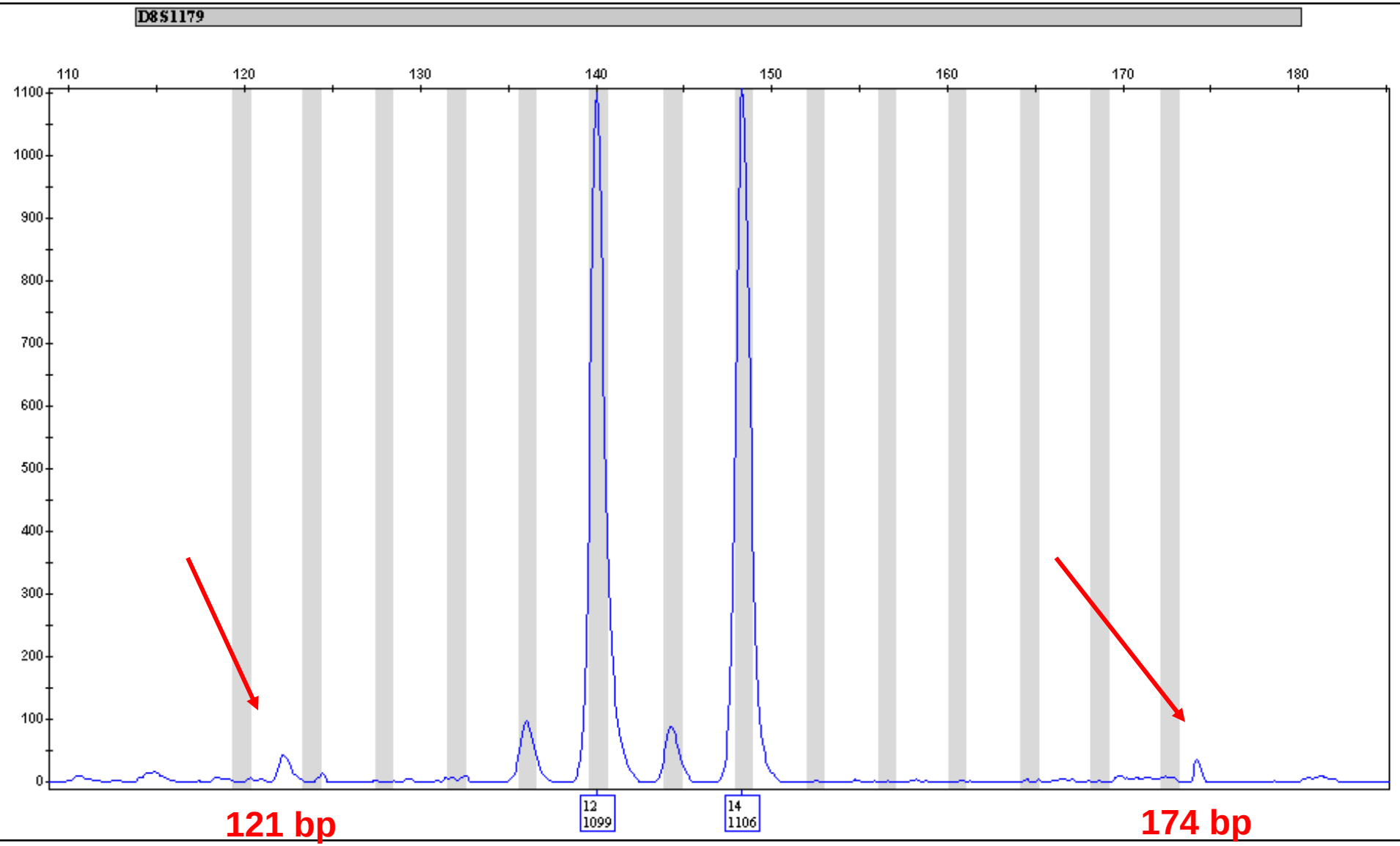
TPOX


TPOX
210
220
230
240
250
260
3000
2000
1000
0
219 bp
11
3168
11
3168

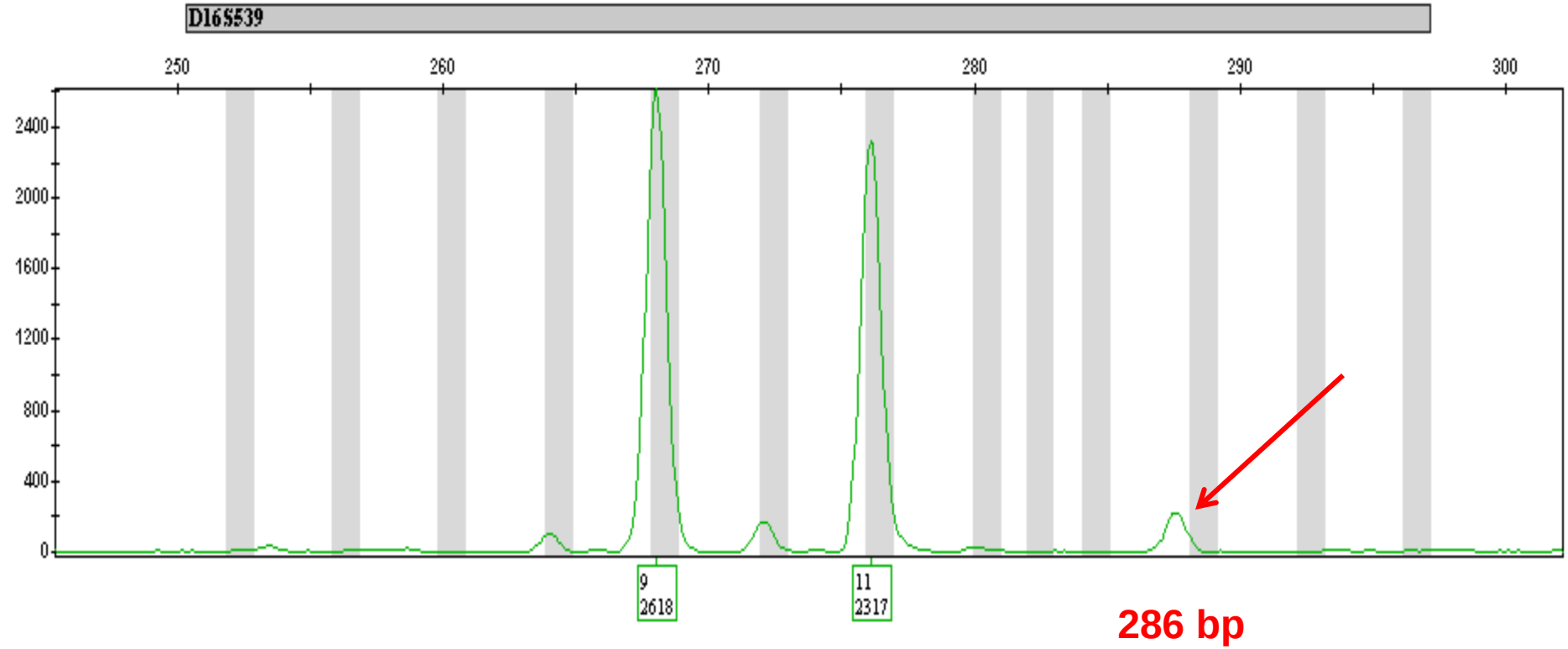
TH01


TH01
160
170
180
190
200
3000
2000
1000
0
160 bp
168 bp
7
3281
7
3281
184 bp

D8S1179


D8S1179
110
120
130
140
150
160
170
180
1100
1000
900
800
700
600
500
400
300
200
100
0
12
1099
14
1106
121 bp
174 bp

D16S539


D16S539
250
260
270
280
290
300
2400
2000
1600
1200
800
400
0
9
2618
11
2317
286 bp

Summary of PCR Artifacts

N = 95

Number of times an artifact was observed

PCR Artifacts Observed	9700	Smart Cycler	Master Cycler Pro	Rotor-Gene
D16S539 (287 bp)	35	4	1	6
D8S1179 (121 bp)	6	0	1	3
D8S1179 (174 bp)	14	10	1	7
TH01 (160 bp)	28	2	1	11
TH01 (168 bp)	83	32	1	40
TH01 (184 bp)	59	19	0	25
TPOX (219 bp)	77	13	2	22

Artifacts only called above 50 RFUs

PCR artifacts did not affect allele calls and exhibited signal intensities similar to stutter peaks

Artifact signal intensities varied based on cycler

Heterozygote peak height ratios

n = 95	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
9700	0.89	0.91	0.90	0.89	0.90	0.90	0.89	0.91	0.88	0.88	0.89	0.90	0.91	0.92	0.90	0.89
SmartCycler	0.90	0.88	0.89	0.87	0.88	0.89	0.88	0.89	0.88	0.89	0.87	0.90	0.86	0.90	0.88	0.89
Mastercycler pro	0.89	0.89	0.89	0.89	0.90	0.90	0.87	0.89	0.88	0.90	0.89	0.89	0.88	0.93	0.90	0.89
Rotor-Gene Q	0.88	0.85	0.86	0.88	0.88	0.90	0.86	0.88	0.88	0.88	0.87	0.89	0.86	0.90	0.88	0.89
9700 Taq Gold	0.87	0.89	0.88	0.84	0.84	0.88	0.83	0.88	0.84	0.87	0.86	0.88	0.84	0.88	0.88	0.86
SD <0.1																

- Average PHR for rapid PCR conditions > 0.85
- Standard deviation per locus < 0.1 (n=95)
- 1 ng of DNA amplified with the rapid PCR protocols exhibited heterozygote peak height balance comparable to traditional kit cycling conditions

Stutter Intensity

N > 80 for all points

	9700 Taq		9700		SmartCy		MasterC		Rotor-G	
Locus	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CSF1PO	3.9	1.8	7.4	4.3	6.8	2.6	7.5	3.3	6.7	2.5
D13S317	4.1	2.7	7.7	5.7	6.7	4.3	7.5	4.2	6.9	5.0
D16S539	4.2	2.2	7.7	3.3	7.7	2.6	8.0	2.3	7.5	3.7
D18S51	7.8	2.7	11.8	5.9	10.6	4.2	12.6	5.5	10.9	4.4
D19S433	7.2	2.4	10.4	3.9	9.6	2.4	10.0	2.5	11.3	4.1
D21S11	5.3	1.5	9.2	1.9	9.6	2.4	10.1	1.9	9.7	2.6
D2S1338	6.1	1.9	12.0	2.8	12.3	3.2	12.7	3.1	12.4	3.2
D3S1358	7.6	4.1	11.7	4.7	10.4	2.3	11.4	3.1	10.4	2.4
D5S818	4.8	3.6	8.8	4.7	8.0	2.6	9.1	3.8	8.3	2.9
D7S820	3.7	1.3	5.9	2.6	5.9	2.0	6.5	2.2	5.8	2.0
D8S1179	5.7	3.6	9.3	4.4	8.5	2.5	8.9	2.2	8.6	2.8
FGA	7.0	3.7	9.9	4.6	10.0	3.8	10.2	3.5	9.6	3.2
TH01	3.0	5.0	5.8	6.5	3.8	3.9	4.8	6.2	5.1	6.6
TPOX	2.6	3.8	4.7	4.1	4.5	3.5	4.1	2.0	4.8	4.0
vWA	6.5	5.0	11.0	4.9	10.5	3.1	10.6	3.6	10.8	4.0

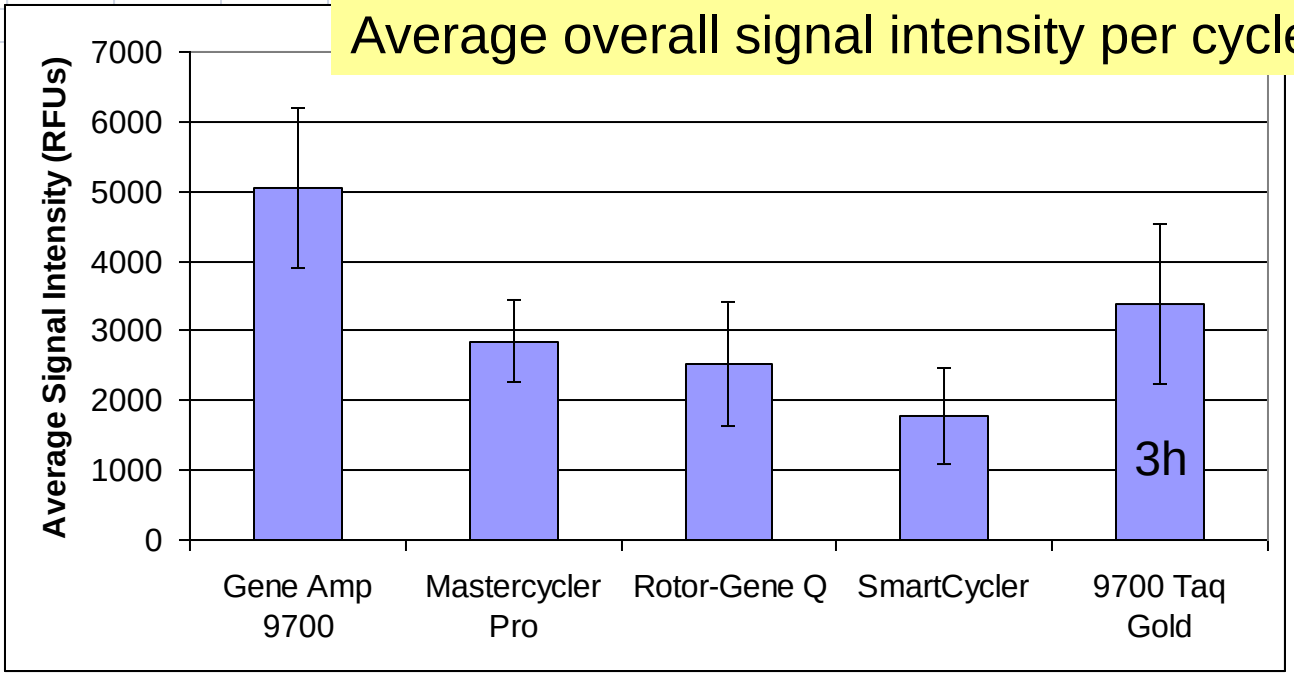
On average, stutter peak intensity for rapid protocol is 30-40 % higher than for PCR standard conditions

Signal Intensity

Average signal intensity per locus (RFUs)

	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
9700	4289	4322	4068	5395	5243	6348	6541	7582	5896	2923	4042	5324	5209	4812	4723	4069
SmartCycler	1377	1036	1603	2133	1712	2625	2564	2409	2514	976	1175	2304	1888	1756	1530	1040
Mastercycler Pro	2488	2461	2783	3948	2005	2175	2081	4150	4431	2286	2807	3321	3927	3042	2466	1260
Rotor-Gene Q	2292	1751	1845	2458	2564	3568	3476	3520	3486	1503	1939	2885	2476	2358	2608	1775
9700 Taq Gold	3554	2958	1601	1840	3221	5166	3309	3501	3228	4631	5155	4241	1804	4146	3699	1952

Average overall signal intensity per cycler (RFUs)



Overall multiplex balance comparable to standard conditions…

Sensitivity Study on 9700

- Titration of highly characterized DNA
  - Stock at 52.44 ng/µL
- 7 concentrations amplified in duplicate
- Injected on the 3130
  - 3 kV for 10 s
- 50 RFU detection cutoff

Standard Injection

9700 36 min	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
1000 pg																
750 pg																
500 pg																
250 pg																
125 pg																
62.5 pg									51							
									180							
31.3 pg						85	71				60			134		56
		61		77		50	75	140						54		

full profile

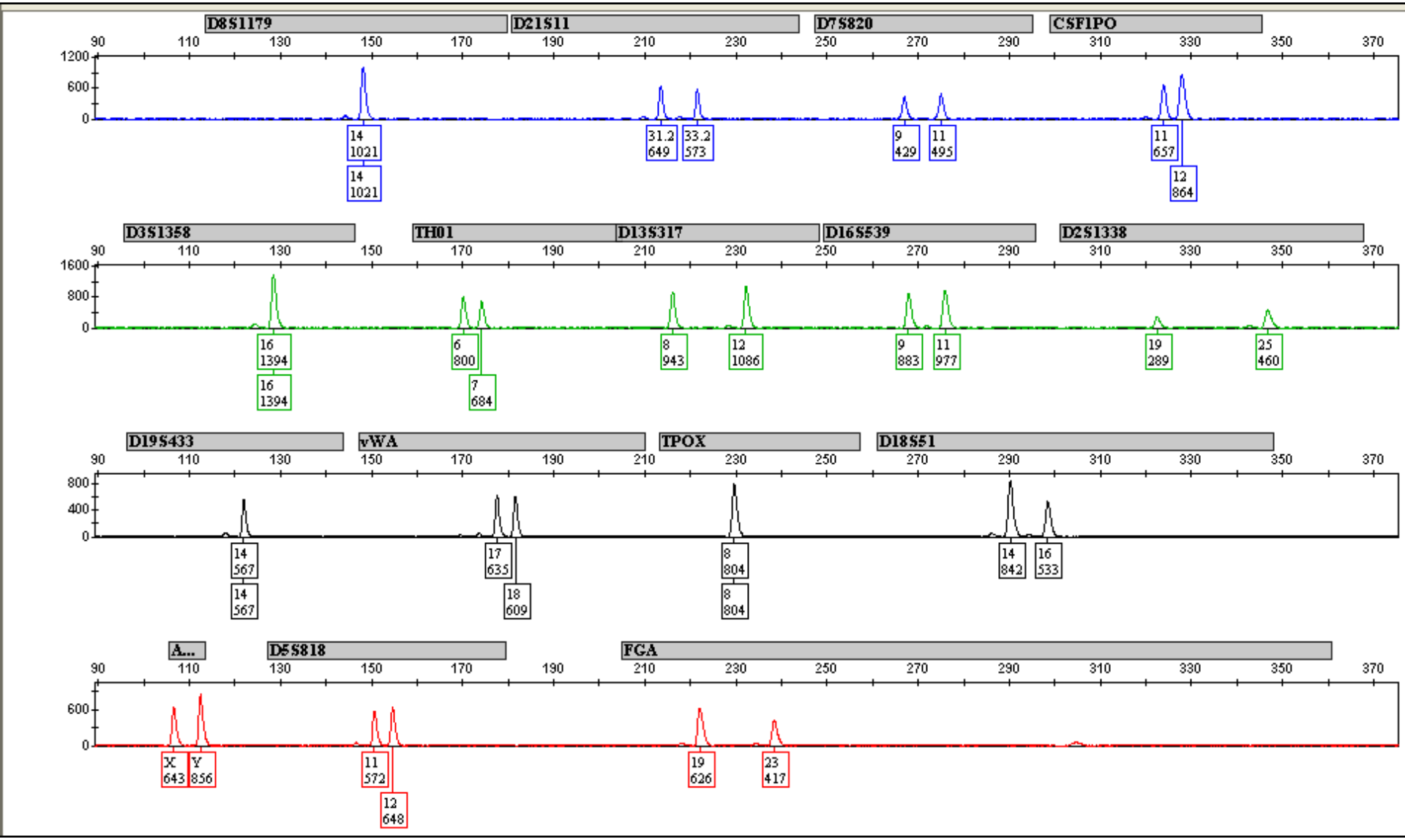
full drop out

one allele

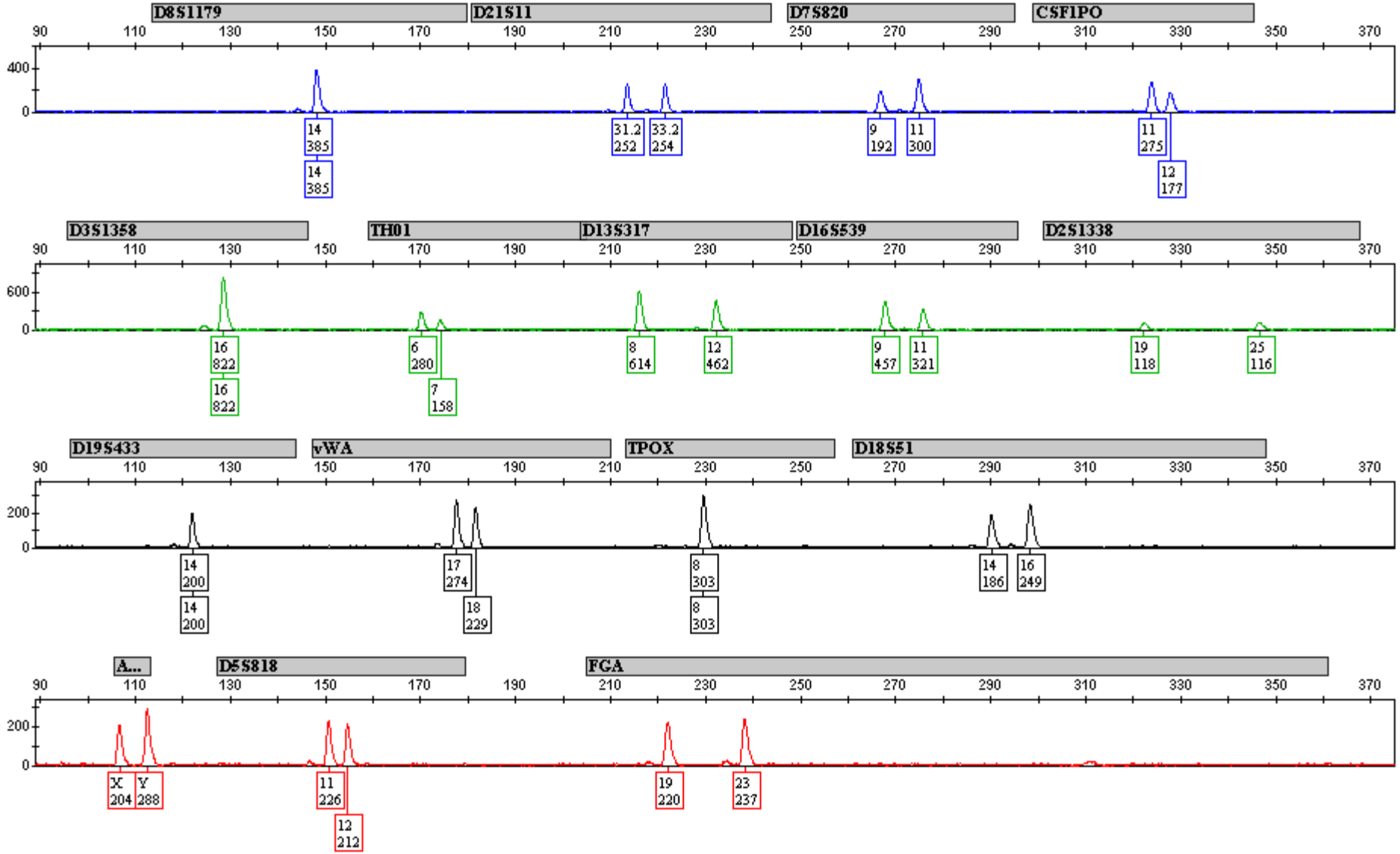
drop in/high stutter

*number corresponds to the height of the peak that is called

Sensitivity Study 250 pg



Sensitivity Study 125 pg



Sensitivity Study 62.5 pg

Peltier Cyclers: 9700 and Mastercycler Pro S

Standard Injection

9700 36 min	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
1000 pg																
750 pg																
500 pg																
250 pg																
125 pg																
62.5 pg									51							
									180							
31.3 pg						85	71				60			134		56
		61		77		50	75	140						54		

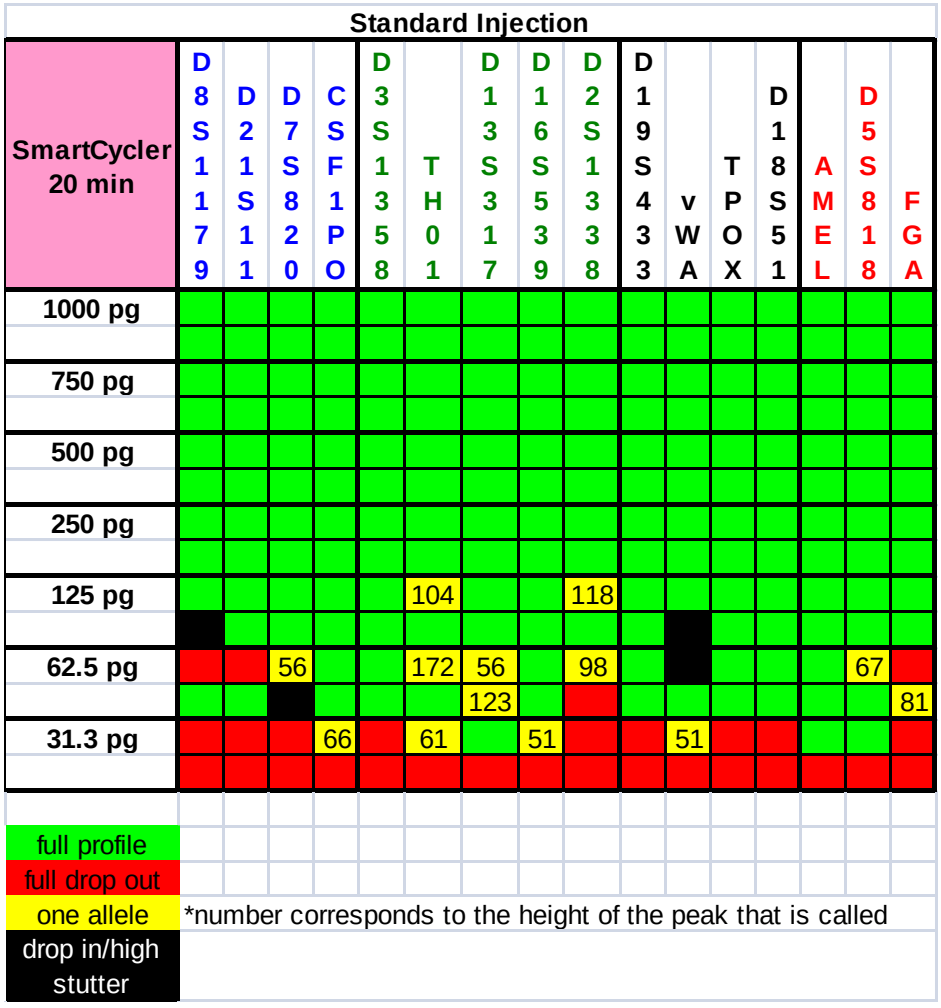
full profile	
full drop out	
one allele	*number corresponds to the height of the peak that is called
drop in/high stutter	

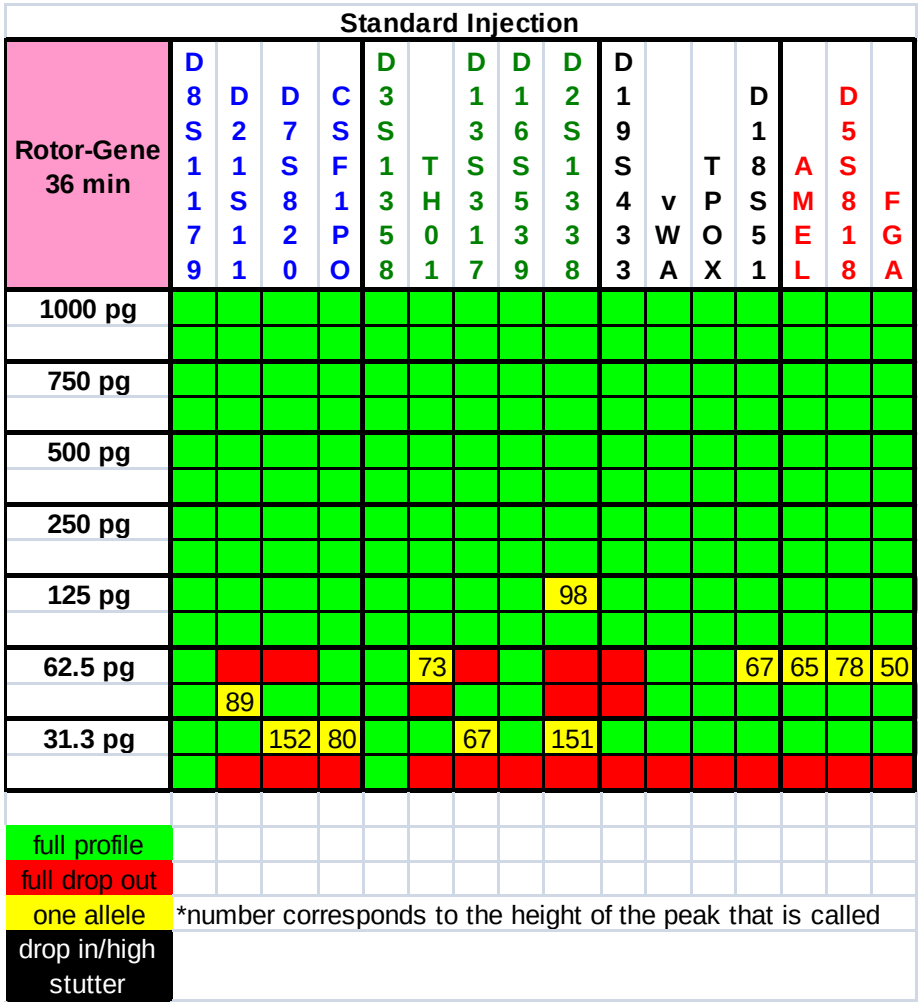
Standard Injection

Mastercycler 19 min	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
1000 pg																
750 pg																
500 pg																
250 pg									59							
125 pg							65		165							
							50						54			
62.5 pg																
		67				63	56	94	127		93					51
31.3 pg																
									64				62			

full profile	
full drop out	
one allele	*number corresponds to the height of the peak that is called
drop in/high stutter	

SmartCycler and Rotor-Gene Q





Changing Volumes and Cycling Times

- Increasing PCR volume (10 µL to 25 µL)
  - Slight decrease in signal intensity of PCR artifacts
  - Heterozygote peak height ratios similar
  - No decrease in signal for stutter peaks
  - Decrease in signal intensity (due to higher volume)

- Effects of increasing cycling hold times for the rapid protocol (36 min, 60 min, 3 hour)
  - Signal intensity of PCR artifacts increased
  - Heterozygote peak height ratios similar
  - No decrease in signal for stutter peaks

Summary of Rapid PCR Protocols

- Rapid PCR protocols can successfully amplify 15 STR loci in 19 to 36 minutes
  - Utility for reference samples, integrated typing systems
- PCR artifacts did not affect allele calls
- Stutter is 30-40% greater
  - Test different ‘fast’ polymerases
  - High stutter may affect DNA mixture interpretation

Summary of Rapid PCR Protocols

- Sensitivity varies by cycler (250 - 500 pg)

- Thermal cycler characteristics affect the quality of an STR profile (faster = fewer artifacts, lower signal, decreased sensitivity)

Thank you for your attention!

Questions?

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Acknowledgements

Dave Duewer – Data analysis software (stutter, peak height ratios, multiplex balance)

Outside funding agencies:

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NIJ – Interagency Agreement with the Office of Law Enforcement Standards