Capillary Electrophoresis in DNA Analysis

Real-time PCR and miniSTRs

NEAFS Workshop
Mystic, CT
September 29-30, 2004

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Outline for Workshop

- Introductions
- STR Analysis
- Introduction to CE and ABI 310
- Data Interpretation
- Additional Topics – Real-time PCR and miniSTRs
- Higher Throughput Approaches
- Troubleshooting the ABI 310 (Participant Roundtable)
- Additional Topics – Y-STRs, validation, accuracy
- Review and Test

Why is Accurate DNA Quantitation Important in Forensic DNA Testing?

- Limited amount of DNA available
  - Usually cannot perform multiple tests for quantity
  - Want to preserve DNA for STR testing
- Optimal signal from multiplex STR reactions is only in a tight concentration range (usually 0.5-2 ng)
  - Too much DNA leads to split peaks, off-scale peaks, and bleed through between dye colors
  - Too little DNA leads to loss of loci or alleles due to stochastic effects

Calculation of DNA Quantities in Genomic DNA

Important values for calculations:
1 bp = 618 g/mol A: 313 g/mol; T: 304 g/mol; A-T base pairs = 617 g/mol
G: 329 g/mol; C: 289 g/mol; G-C base pairs = 618 g/mol

1 genome copy = ~3 x 10^9 bp = 23 chromosomes (one member of each pair)

1 mole = 6.02 x 10^{23} molecules

Standard DNA typing protocols with PCR amplification of STR markers typically ask for 1 ng of DNA template. **How many actual copies of each STR locus exist in 1 ng?**

1 genome copy = (~3 x 10^9 bp) x (618 g/mol/bp) = 1.85 x 10^{12} g/mol

= (1.85 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)

= 3.08 x 10^{-12} g = **3.08 picograms (pg)**

Since a diploid human cell contains two copies of each chromosome, then

each diploid human cell contains ~6 pg genomic DNA

∴ 1 ng genomic DNA (1000 pg) = ~333 copies of each locus **(2 per 167 diploid genomes)**

Butler, J.M. (2001) *Forensic DNA Typing*, Box 3.1, ©Academic Press

Introduction

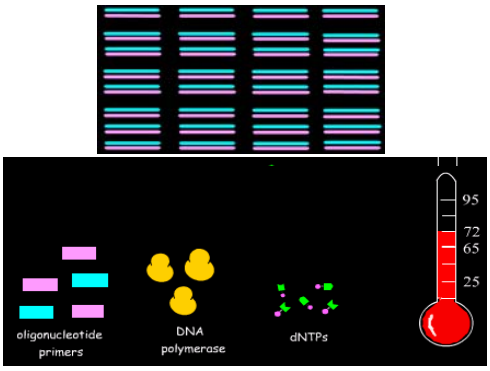
- What is rtPCR or qPCR?
- How does it work?
- How does it compare to traditional methods of Human DNA quantitation?
- What techniques are available?
- What systems are available?

History

- RtPCR is a very recently developed technique
  - Developed by Higuchi in 1993
  - Used a modified thermal cycler with a UV detector and a CCD camera
  - Ethidium bromide was used as intercalating reporter As [dsDNA] increased fluorescence increased
- Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. "Kinetic PCR analysis: real-time monitoring of DNA amplification reactions" Biotechnology (N Y). 1993 Sep;11(9):1026-30

Figure 3. Mechanism of PCR

D. Voet, J. Voet, and C. Pratt, Fundamentals of Biochemistry, 1999



PCR amplification

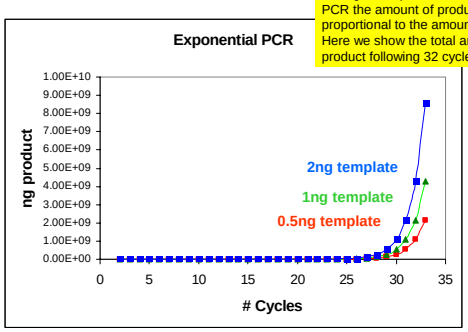
- Theoretically the quantity of PCR template T doubles with each cycle.
- After 2 cycles the quantity of product is 2T
- After N cycles the quantity of product is

$$P = (2)^n T$$

  - Thus there is a exponential relationship between the original quantity of product and the amount of template

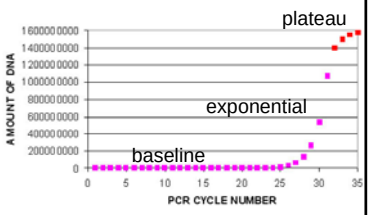
PCR Product Amount is Proportional to the Amount of Input DNA Template

During the exponential expansion of the PCR the amount of product produced is proportional to the amount of template. Here we show the total amount of product following 32 cycles.



What is qPCR?

- To use PCR as a Quantitative technique, the reaction must be clearly defined
- In fact there are several stages to a PCR reaction
  - Baseline stage
  - Exponential stage
  - Plateau stage

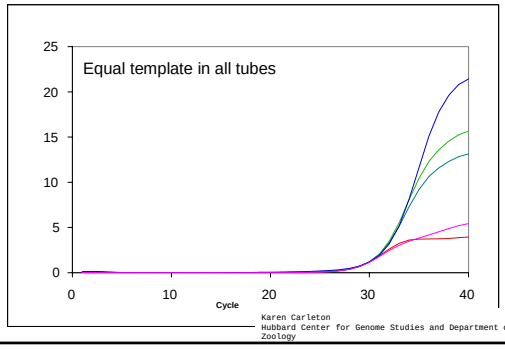


PCR plateaus

- PCR product can not double forever
  - Limited by
  - Amount of primer
  - Taq polymerase activity
  - Reannealing of product strands
- Reach plateau
  - No more increase in product
- End point detection
  - Run for fixed # cycles and then quantify on agarose gels

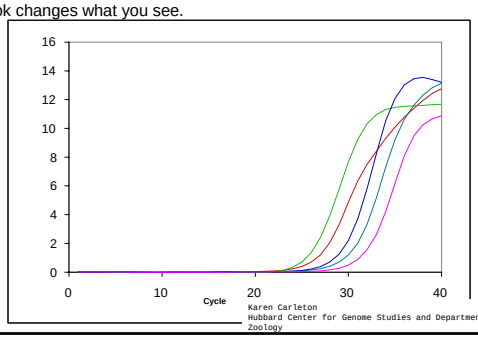
Problem #1: End point plateau does not depend on T

Even if same amount of template, different tubes will reach different PCR plateaus



Problem #2: For endpoint detection, how many cycles should you do?

Different wells reach plateau at different cycle numbers. When you look changes what you see.



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Hubbard Center for Genome Studies and Department of Zoology

Issues for quantitation by non RT-PCR methods

- In spite of its use in mixture resolution, PCR is not technically a quantitative technique
- The time and rate at which plateau appears varies with temperature, tube position, inhibitors, matrix
- Once plateau appears, increase in product concentration is non linear
- Standards can be added but they must have the same primer binding sites and similar sequence to target

Solution

- Use data when still in exponential phase
  - PCR product proportional to initial template
- Need to look at PCR product each cycle
  - Use fluorescent detection, where fluorescence is proportional to PCR product
- Use real time PCR machine which records fluorescence for each well at each cycle

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Quantitation using the PCR Reaction

- PCR proceeds exponentially doubling each cycle:

$$Y_n = Y_{n+1}(1+E_c)$$

Where E_c is the efficiency (E_c = 1 for a perfect amplification) and Y_n is the yield of product for a particular cycle

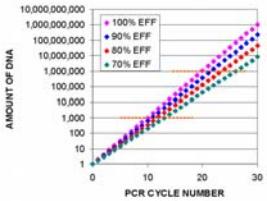
- During the exponential stage of the reaction

E_c is relatively constant and the reaction yield Y is a function of the quantity of input DNA, X

$$Y = X (1+ E_c)^n$$

Effect of efficiency on [DNA]

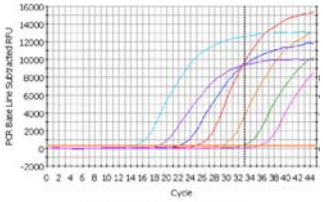
- E_c is a function of:
  - Hybridization efficiency
  - Quantity of reactants/target DNA
  - Temperature



http://www.med.sc.edu:85/pcr/realtime-home.htm

Real Time PCR

- Quantitation of DNA is a based on the number of cycles required to reach a threshold intensity, C_t.
- The greater the amount of starting DNA, the sooner this threshold value is reached.



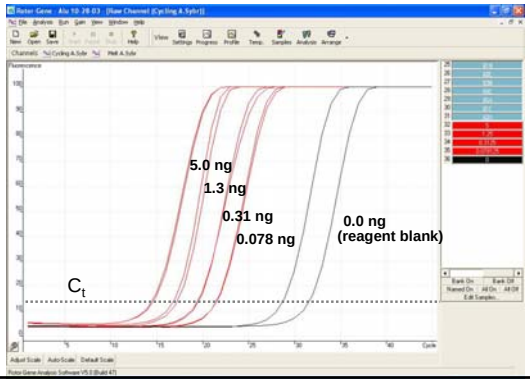
C_t

http://www.med.sc.edu:85/pcr/realtime-home.htm

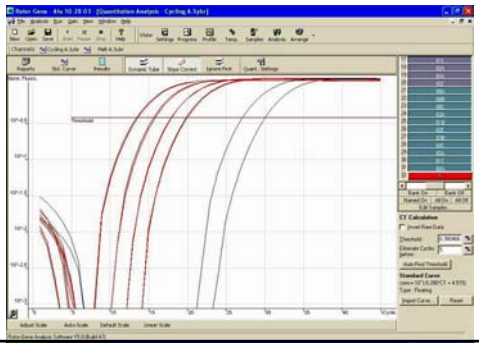
Quantitation using C_t

- The log of DNA template concentration vs C_t is plotted using a series of stds yielding a calibration curve
- The unknown is then run and the number of cycles required to reach threshold, C_t is compared to the calibration curve.

Development of a standard curve

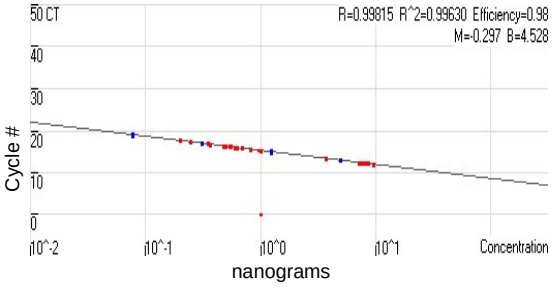


The output data is plotted on a log scale and the fractional # cycles required to reach C_t is measured



Standard curve

Plot the cycle # at threshold CT vs concentration



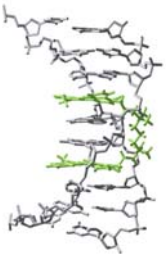
Concentration = 10^(-0.297*CT+ 4.528)

Detection Methods

- Fluorescent intercalating dye - SYBR Green
  - Fluorescence increases with concentration of dsDNA
- Taqman probes
  - Fluorescence increases as quenched probe is digested
- Molecular beacons
  - Fluorescence increases as quenched probe hybridizes to template

SYBR green product detection

- Easy
  - Fluorescence only with dsDNA
  - Use with existing PCR primers
- Generic,
  - Detects all double stranded products, including primer dimers
  - However, can be very specific with proper primer design
- Singleplexed
  - Multiple probes cannot be used



dsDNA Intercalation

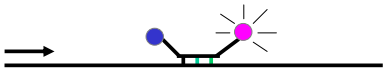
http://www.probes.com/handbook/figures/1557.html

Molecular beacons

– Consist of ssDNA with an internal complementary sequence that keeps reporter and quencher dyes close → No fluorescence

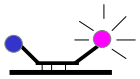
Molecular beacon 

– Following denaturation, beacon anneals to template, separating both dyes and yielding fluorescence proportional to PCR product concentration



Molecular Beacons

- Improved specificity and multiplexing
  - Non-specific amplification will not produce a signal
  - Can multiplex several probes (quantify nuclear, Y, int std.)
- Can be tricky to design
  - Loop portion – binds to DNA template
  - Stem portion – must be complementary to other stem
  - Probe must denature from template below 72º so Taq polymerase does not chew it up during extension step



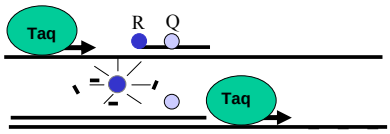
$T_{anneal} < T_m < T_{ext}$

Above T_m loop structure reforms and probe leaves template

Taqman

Probe also binds to PCR product during extension but is always quenched

- 5'-3' exonuclease activity of Taq polymerase digests probe and frees reporter dye from quencher
- Free dye accumulates with PCR product



Probes vs SYBR Green

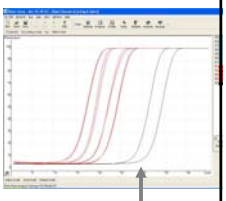
- SYBR Green
  - Singleplex probes (Alu)
  - If no sample, amplification of contaminants occurs at high cycle #
  - If inhibition, no result or poor efficiency curve
- Probes (Taqman, Mol. beacons)
  - Multiplex targeted probes – Quant Y, nuclear DNA, int. std
  - Inhibition and no sample can yield no result (if single locus probe)
  - to check for inhibition, an internal std. is used
- Choice: Simplicity (SYBR green) vs Multiplexing (probes)

Single vs Multilocus Targets

- SYBR Green – Multilocus Probe
  - Alu inserts occur at multiple locations throughout the genome - sensitive
  - If no sample, amplification of contaminants occurs at high cycle #
  - Syber green requires no special kit –Inexpensive
- Probes (Taqman, Mol. beacons)
  - Single location in genome
  - an internal std. is used to check for amplification and correct for changes in efficiency
  - Lower sensitivity due to noise at low copy number
- Choice: Sensitivity (SYBR green) vs Internal Standard Precision (probes)

Effects of Inhibitors on Alu Assay

- Use Alu sequence, present at 1,000's of copies/cell
  - Assay is sensitive to ambient human DNA in air and water
  - Normal Reagent blanks have a Ct at about 27-29 cycles
- If inhibitors are present – no amplification occurs or efficiency is altered
  - Thus low level ambient DNA serves as an internal control for inhibitors
- For non Alu based RtPCR, an internal standard is required to detect inhibition



Slot blot versus real time PCR




Slot Blot

- 2 days of rinses, incubations, pipettings, washes, exposures, and developments
- Semiquantitation by manual comparison or through scanner
- Quantity obtained may not reflect final result due to variations in PCR efficiency

- 2 hours setup and run time **RtPCR**
- Automated quantitation
- Quantity obtains reflects amplifiable result

Big issue – sensitivity and dynamic range

Quantiblot-ECL 40 pg - 2.0 ng

ACES 2.0 40 pg - 4.0 ng
No longer available
(ACES tended to work better on degraded DNA)

Real Time PCR 1.0 pg - 16 ng

RTPCR has lower detection limit and larger dynamic range


Comparison Studies Slot blot vs RtPCR

	Reference	RTi-PCR	Quantiblot
1	5	5.38	6.25
2	1.25	1.14	0.56
3	0.3125	0.29	0.56
4	0.078125	0.08	0.12
5	5	4.92	8.75
6	1.25	1.32	0.63
7	0.3125	0.30	0.81
8	0.078125	0.09	0.23

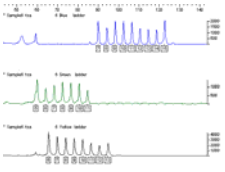
Calibration studies in our lab with experimental primers

sample	rtPCR	slot blot	Tho1 Allele
blood on stick	0.32	0.50	1880
blood on metal	0.40	0.50	1890
blood on concrete	0.40	0.50	1860
blood on leaves	0.08	0.20	1540
blood on cardboard	0.27	0.24	1450
blood on cloth	0.04	0.05	577
blood on denim	0.25	1.00	1240

Validation work of Jan Nicklas and Eric Buel
Nicklas, J.; Buel, E., J. Forens. Sci. 2003, 48(5) pp. 936-944

Work in OhioU/FIU Laboratory

- Development of miniplex STRs for degraded DNA typical sizes 60-120 bp.
- Slot blot works poorly on these samples
- Current assay has 124bp Alu product – reduction to 84bp testing in progress

RT-PCR Instruments Cited

- Corbett Research Rotorgene
  - Phenix Research, Hayward, CA
- ABI 7000 Sequence Detection System
- ABI 7700 (discontinued)
- ABI 7900HT Sequence Detection System
  - Applied Biosystems Foster City, CA

Real-Time PCR Efforts

- Marie Allen – nuclear and mtDNA assay (BioTechniques 2002, 33(2): 402-411)
- Eric Buel – Alu system (JFS 2003, 48(5):936-944)
- Centre for Forensic Sciences – nuclear; TH01 flanking region (JFS 2003, 48(5):1041-1046)
- John Hartmann – Alu system (SWGDAM Jan 2003)
- CA-DOJ – TH01 assay (NIJ DNA Grantees June 2003)

- SYBR Green assay – human-specific with right PCR
- Quantifiler kit (ABI) – separate nuclear and Y assays

NIST Lessons Learned from Real Time-PCR Assays

We are using ABI 7000 (some work also with Roche LightCycler)

- Results are RELATIVE to standards used
- Single source and mixed source samples with same UV concentrations differ with RT-PCR assays
- Need to keep instrument clean to avoid background fluorescence problems
- Assay reagent costs:
  - Quantifiler: $2.46/sample (only permits 2 µL/sample)
  - SYBR Green: $0.80/sample (up to 10 µL/sample)
  - QuantiBlot: $0.54/sample (5 µL/sample)

Information from Quantifiler Kit Manual

Table 6-10 Comparison with A_{260} and Quantiblot kit

Sample	Sex	A_{260} Result (ng/µL)	QB[a] Result (ng/µL)	Quantifiler Human Kit			Quantifiler Y Kit		
				Result (ng/µL)	% Diff. from A_{260}	% Diff. from QB	Result (ng/µL)	% Diff. from A_{260}	% Diff. from QB
1	M	17.5	20	6.69	61.7	66.6	10.13	41.9	49.4
2	M	15.4	20	14.3	7.1	28.5	16.78	9.0	16.1
3	M	13.9	30	15.48	11.4	48.4	14.30	2.9	52.3
4	M	11.4							37.8
5	M	10.3							45.0
6	M	13.9							32.2
7	M	11.5							69.3

The different methods produced similar quantification results.

Table 6-11 Average differences from A_{260} and Quantiblot kit

Method	Average Difference (%)	
	Quantifiler Human Kit	Quantifiler Y Kit
A_{260}	16.9	15.1
Quantiblot	42.0	35.5

Quantifiler Kits User's Manual 6-29

Conclusions

- RTPCR is a homogeneous PCR based method for human specific quantification
  - Is easily automated, provides electronic storage of data
  - SYBR green or targeted probes can be used
- Results give quantity of amplifiable DNA – not necessarily overall quantity
  - Inhibition can be detected
  - Multiplexing can be used
- Big advantages are speed and dynamic range

Acknowledgements

- Jan Nicklas and Eric Buel - Vermont Crime Laboratory
- Jiri Drabek
- Denise Chung, Kerry Opel
- Nancy Tatarek
- John Butler, Yin Shen

- Major support provided by
- The National Institute of Justice
- The OU Provost's Undergraduate Research Fund
- Ohio University Research Incentive Fund

Chemistry & Biochemistry

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http://dna-9.int-med.uiowa.edu/realtime.htm
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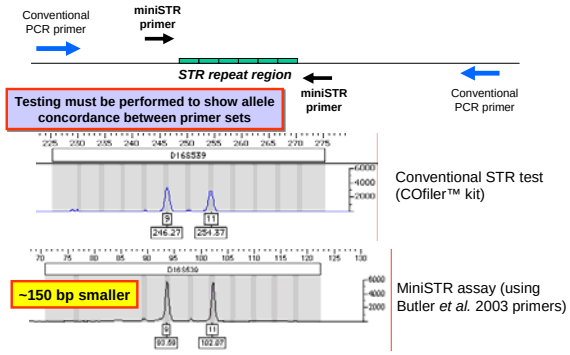
miniSTRs

STR Size Reduction
Through Moving Primer Positions
Closer to the Repeat Region

A miniSTR is a reduced size STR amplicon that enables higher recovery of information from degraded DNA samples



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 7.2, ©Elsevier Science/Academic Press

miniSTR Work

- miniSTRs (*a.k.a. BodePlexes*) are being used successfully in WTC effort
- Collaboration between John Butler and Bruce McCord (NIJ-funded) to further develop reduced size STR amplicons
- Mike Coble (NRC postdoc) at NIST is developing new miniSTR loci that are unlinked to CODIS loci

J. Forensic Sci. 2003 48(5): 1054-1064

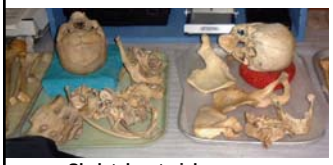
John M. Butler,[1] Ph.D.; Yin Shen,[2,3] Ph.D.; and Bruce R. McCord Ph.D.[2]

The Development of Reduced Size STR Amplicons as Tools for Analysis of Degraded DNA*

Describes new primer sequences for all CODIS loci and initial assays developed

Large Multiplex Kits provide Efficient and Rapid Analysis of Convicted Offender Samples

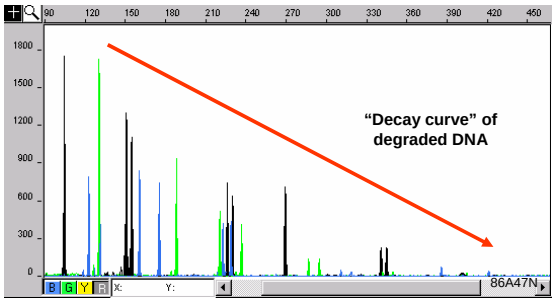
But what about degraded DNA ?



Skeletal material being preped for extraction

Such samples present a special challenge

PowerPlex 16 Result on Aged Blood Stain
(15 years at room temperature storage)



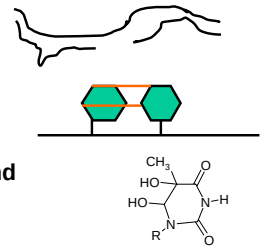
NIST

The Miniplex Approach to Degraded DNA

1. Redesign primers to make each STR amplicon as short as possible.
2. Avoid overlap by having only 1 STR locus in each dye lane.
3. Provide an alternative to mtDNA for degraded DNA template.
4. Develop of specialized STR systems for degraded DNA.

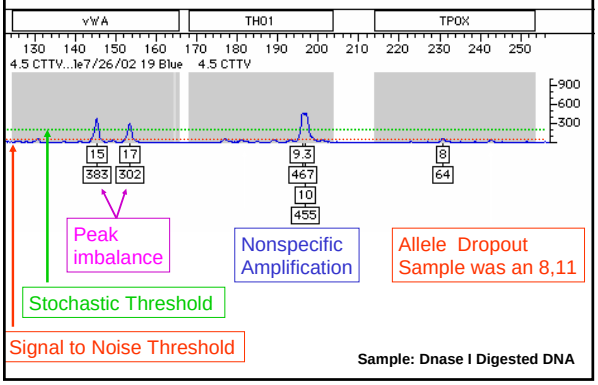
DNA Degradation

1. Strand breakage
2. Pyrimidine dimers
3. Chemical oxidation and hydrolysis
4. Bacterial degradation and metal contamination



Thymine glycol

The Problem with Degraded DNA



Sample: Dnase I Digested DNA

Degraded DNA

1. Fragmentation due to the environment
2. The presence of PCR inhibitors

Result

1. Poor amplification efficiency
2. Peak imbalance and allele dropout

Current Miniplex Loci

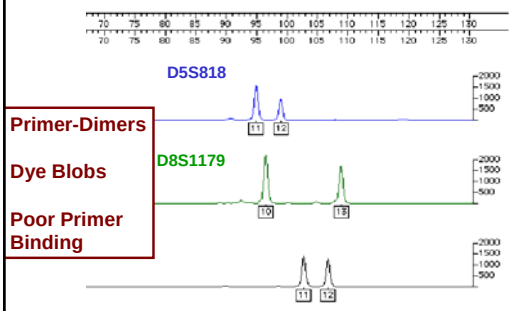
and reduction in size relative to ABI kits

Dye Label:	Blue	Green	Yellow
Miniplex 1	TH01	CSF1P0	TPOX
	-105	-191	-148
Miniplex 2	D5S818	D8S1179	D16S539
	-53	-37	-152
Miniplex 3	FGA	D21S11	D7S820
	- 71	-33	-117
Miniplex 4	vWA	D18S51	D13S317
	-64	-151	-105
Miniplex 5	Penta D	Penta E	D2S1338
	-282	-299	-198

Miniplex Primer Sets

		FAM	VIC	NED
Big Miniplex	Miniplex 1	TH01	CSF1PO	TPOX
	Miniplex 3	FGA	D21S11	D7S820
Miniplex 2		D5S818	D8S1179	D16S539
Miniplex 4		vWA	D18S51	D13S317

MINI 2 PROFILE - BLOOD



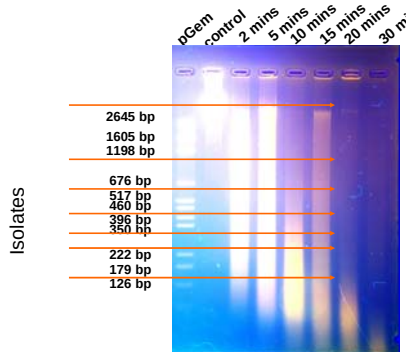
The Big Question:

How does it work with degraded DNA?

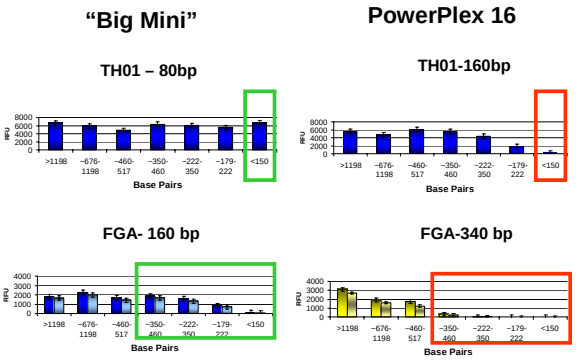
Approach: Examine the effect of template size on DNA amplification

1) Extract large quantities of DNA from liquid blood
2) Digest with DNaseI
3) Cut sections at different size ranges and amplify
4) Compare with a commercial multiplex kit

DNA Degraded With DNase I

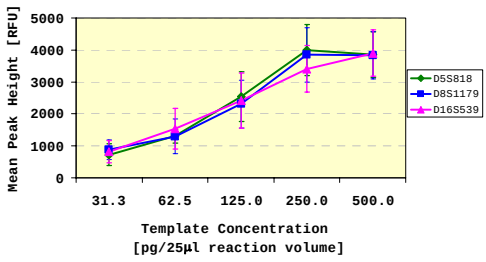


Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) A study on the effects of degradation and template concentration on the efficiency of the STR miniplex primer sets. *J. Forensic Sci.* 49(4): 733-740.

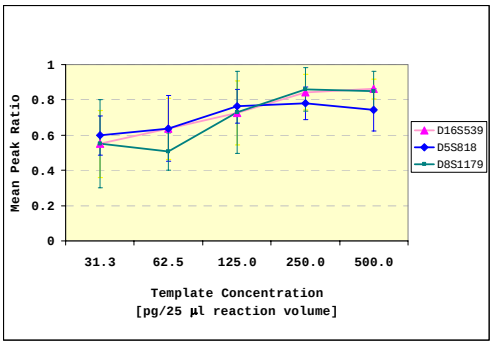


Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) A study on the effects of degradation and template concentration on the efficiency of the STR miniplex primer sets. *J. Forensic Sci.* 49(4): 733-740.

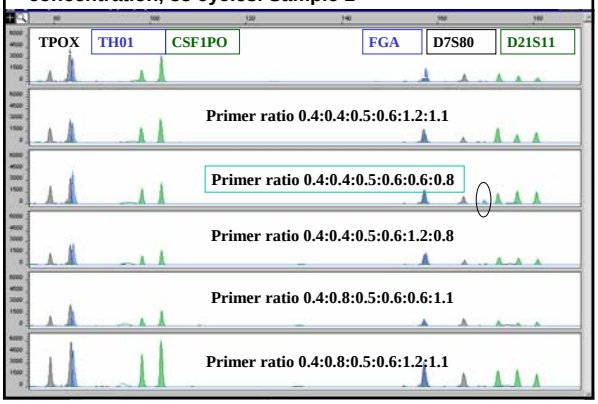
Mini 2: Sensitivity



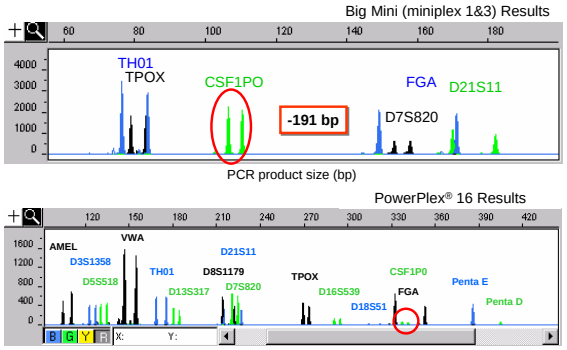
Mini 2: Peak Balance



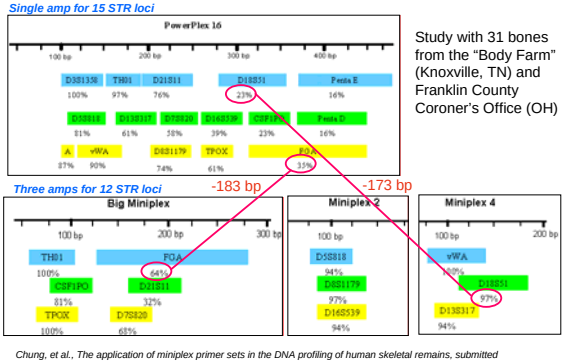
Big Mini Primer Set, Cell line K562, 0.1ng/25 uL concentration, 33 cycles. Sample 1



Comparison between MiniSTR and Commercial Kit Results Degraded DNA Template - NIST



Comparison of PCR Amplification Success Rates with Commercial Kit vs. miniSTR Assays



Study with 31 bones from the "Body Farm" (Knoxville, TN) and Franklin County Coroner's Office (OH)

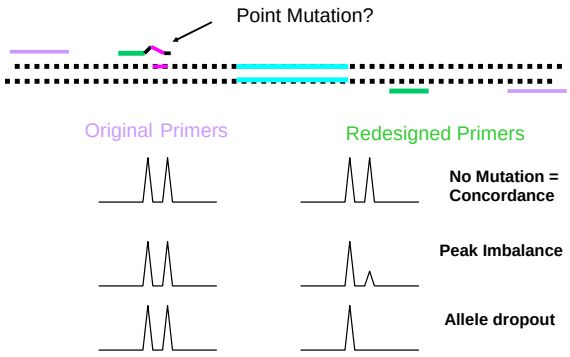
Chung, et al., The application of miniplex primer sets in the DNA profiling of human skeletal remains, submitted

Results

Samples from the Anthropological Research Facility			
Primer Set	**Samples tested**	**Full Profile**	**Partial Profile**
Miniplex 2	25	23 (92%)	2 (8%)
Miniplex 4	25	22 (88%)	3 (12%)
Big Miniplex	25	6 (24%)	19 (76%)
Miniplex 1		20 (80%)	5 (20%)
Miniplex 3		7 (28%)	18 (72%)
PowerPlex 16	25	3 (12%)	22 (88%)
Samples from the Franklin County Coroner's Office			
Primer Set	**Samples tested**	**Full Profile**	**Partial Profile**
Miniplex 2	6	6 (100%)	0
Miniplex 4	6	6 (100%)	0
Big Miniplex	6	3 50%)	3 (50%)
Miniplex 1		5 (83%)	1 (17%)
Miniplex 3		3 (50%)	3 (50%)
PowerPlex 16	6	2 (33%)	4 (67%)

Chung, et al., The application of miniplex primer sets in the DNA profiling of human skeletal remains, submitted

Primer Design- Concordance?



Results of Allele Concordance between Miniplex Primers and Commercial STR Kit Primers

Drabek, J., Chung, D.T., Butler, J.M., McCord, B.R. (2004) Concordance study between miniplex STR assays and a commercial STR typing kit, *J. Forensic Sci.* 49(4): 859-860.

- 532 U.S. population samples (Caucasians, African Americans, and Hispanics) examined with four miniplexes (including all CODIS loci except D3S1358)
- Results compared to Identifiler STR kit (6,384 genotypes compared)
- 15 discrepancies (0.23%) – most of which occur in 2 loci (D13S317 and VWA) and involve deletions between the two primer sites
- Representative samples from each discrepant locus have been sequenced to locate the polymorphic nucleotides causing the allele dropout

Allele Discordance Resulting from Mutations in Miniplex Primer Binding Site

	Locus	Origin	Miniplex	Identifiler	PP16	Likely Cause
1	D13S317	AA	**11**,13	10,13	10,13	deletion outside of allele 11
2	D13S317	H	**9**,14	8,14	8,14	deletion outside of allele 9
3	D13S317	AA	**10**,11	9,11	9,11	deletion outside of allele 10
4	D13S317	H	**10**,11	9,11	9,11	deletion outside of allele 10
5	D13S317	H	**10**,14	9,14	9,14	deletion outside of allele 10
6	D5S818	AA	11,**11**	11,12	11,12	primer binding site mutation
7	vWA	AA	**16**,16	12,16	12,16	primer binding site mutation
8	vWA	AA	**18**,18	13,18	13,18	primer binding site mutation
9	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
10	vWA	AA	**15**,15	14,15	14,15	primer binding site mutation
11	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
12	vWA	AA	**17**,17	14,17	14,17	primer binding site mutation
13	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
14	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation
15	vWA	AA	**19**,19	14,19	14,19	primer binding site mutation

Proposed mechanism

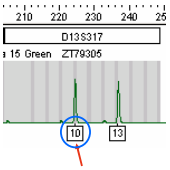
Deletion outside Miniplex primers-limited region (samples 1-5)

Mutation/polymorphism in Miniplex primer binding region (samples 6 - 15)

Examination of Concordance:

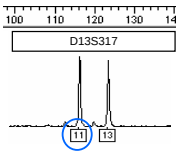
African American sample ZT79305

NIST Identifiler data



Really "11-1" allele

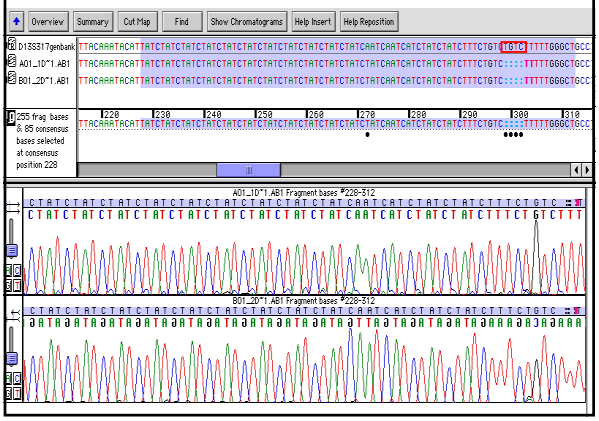
Ohio U miniSTR data



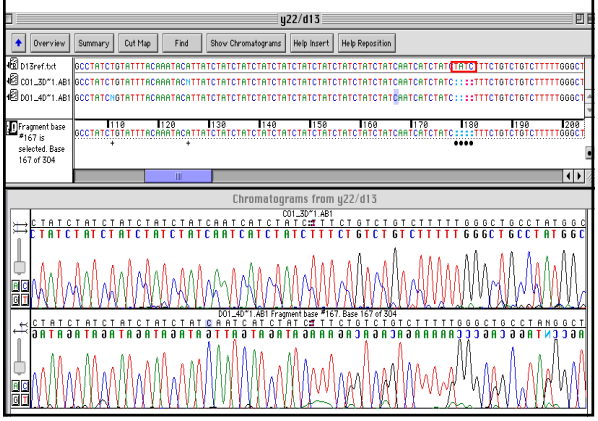
This problem has been seen multiple times by NYC OCME review of WTC BodePlex data

A deletion *outside* the miniSTR primers causes the kit produced allele to appear one repeat smaller…

ZT79305: D13S317 TGTC deletion



Y22: D13S317 TATC deletion

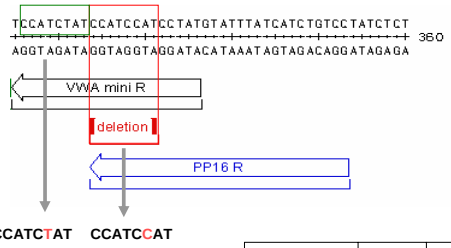


D13S317 Primers and Mutations

PT83871: VWA CCATCCAT deletion

VWA Primers and Mutations

VWA Mutation Region



CCATCTAT **CCATCCAT**

- Allele drop out with Miniplex primer
- PPlex 16 primers still bind because there is only one base pair difference in the adjacent sequence
- ABI reported this as a C-T polymorphism (Lazaruk et al. 2001)
- There is actually 2 repeats (8 bp) more than the actual allele called in commercial kits

Sample	Miniplex	PP16
PT83871 (AA)	**16**,16	12,16
PT84216 (AA)	**18**,18	13,18
MT95095 (AA)	**19**,19	14,19

New miniSTR (non-CODIS) Loci Under Investigation

STR Locus	Sequence Motif	Allele Range	Size Range (bp)	Observed Heterozygosity
D1S1677	$(GGAA)_n$	9-18	81-117	0.75
D2S441	$(TCTA)_n$	9-17	78-110	0.76
D4S2364	$(GAAT)(GGAT)(GAAT)_n$	8-12	67-83	0.53
D10S1248	$(GGAA)_n$	10-20	83-123	0.78
D14S1434	$(GATA)_n(GACA)_n$	13-20	70-98	0.68
D22S1045	$(TAA)_n$	5-16	76-109	0.77

Coble, M.D. and Butler, J.M. (Jan 2005) *J. Forensic Sci., in press*