Real-Time qPCR Techniques for the Forensic Laboratory

Eric Buel & Janice Nicklas

Vermont Forensic Laboratory

PCR Approach to Human DNA Quantitation

- Human DNA Quantitation with SYBR-Green
- Duplex quantitation assay (male and total Human) with TaqMan
- DNA degradation assessment with Plexor
- Stain donor ID - real-time PCR assay with melting FRET (and quantitation)

Real-Time Instruments

- RG-3000 – 4 color
  - 72 Small tubes
  - 10ul-20ul sample
- RG-6000 – 6 color
  - 72 Small tubes or disposable rotor of 100 "tubes"
  - 10ul-20ul sample
  - Intuitive software
- MX3005P - 5 color
  - 96 well plate format
  - 20ul sample

No ROX used for any of these instruments

Number of published assays

- **Endpoint Analysis (Alu) - not Real-Time PCR**
- **SYBR green assay (Alu)**
- **MGB probe assay (Alu)**
- **Duplex with TaqMan probes (Alu and DYZ5 for male quantitation)**

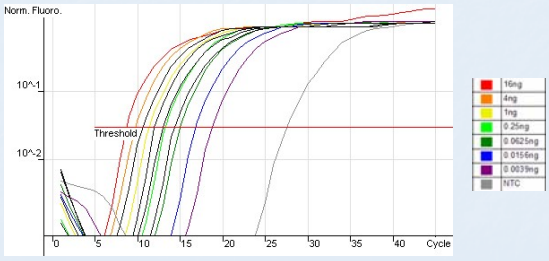
Alu Sequence

- **Family of repetitive elements amplified immensely during primate evolution**
- **500,000 to 1,000,000 copies in the human genome (6-13%)**
- **Consensus sequence is ~280bp in length**
- **Two similar monomers connected by an A rich region**
- **Postulated to be derived from retroposons**
- **Divided into families - J family (oldest - 80 million years), Y family (youngest - 3-4 million years old)**
- **Large number of copies in the human genome make *Alu* an excellent target or marker for human DNA**

SYBR Green Assay

- PCR of *Alu* sequence in the presence of an intercalating dye (SYBR Green)
- As PCR proceeds, more and more product is made and more SYBR Green binds to the PCR product and the fluorescence increases

RESULTS – Amplification plot



Real Time - SYBR ALU PCR Assay

- Optimized/validated for Casework:
  - anneal time and temperature
  - extension time
  - reagents
  - primer concentration
  - animal DNA
  - reproducibility
  - sample types
  - STR results
- J Forensic Sci 48 (5): 936-944 (2003)

Effect of Primer Concentration

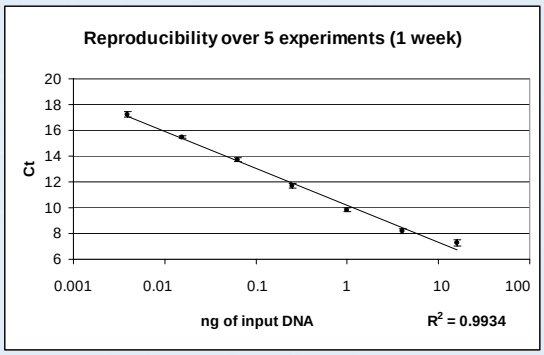
Forward Primer Concentration (pMoles/ul final)	Reverse Primer Concentration (pMoles/ul final)	Ct
0.8	0.8	10.305
0.4	0.8	10.400
0.2	0.8	10.465
0.8	0.4	10.295
0.4	0.4	10.460
0.2	0.4	10.590
0.8	0.2	10.300
0.4	0.2	10.425
0.2	0.2	10.550

Reproducibility

- **Quadruple experiment - same day**
- **Standard curve multiple days**
- **Samples re-run over three days**

Quadruplicate Experiment

Input DNA	mean Ct	stdev	%stdev
16ng	10.05	0.13	1.28
4ng	11.18	0.09	0.80
1ng	12.96	0.08	0.59
0.25ng	15.11	0.17	1.11
0.0625ng	16.93	0.13	0.79
0.0156ng	18.98	0.26	1.39
0.0039ng	20.97	0.20	0.95
0.0010ng	22.90	0.19	0.81
0ng (NTC)	28.06	0.92	3.28



Reproducibility over Time

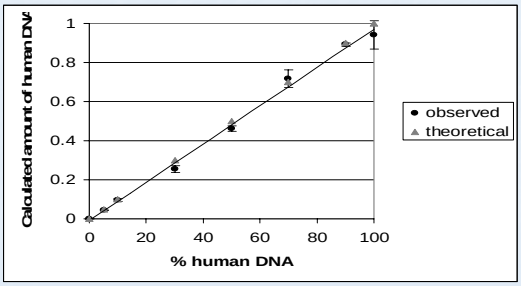
concentration in ng/ul

	Day 1	Day 2	Day 3	Day 4	Day 5	Ave	Stdev	%stdev
databank 1	4.66	4.31	5.20	3.97	4.47	4.52	0.46	10.11
databank 2	4.37	3.83	5.16	3.84	4.10	4.26	0.55	12.88
databank 3	0.90	0.90	1.10	0.94	0.91	0.95	0.08	8.71
standard	0.94	0.96	1.12	1.07	0.99	1.02	0.08	7.72
QB stdA	4.01	3.51	5.16	4.03	4.47	4.24	0.62	14.58
QB cal2	0.15	0.11	0.14	0.12	0.14	0.13	0.01	10.35
blood - 3mo sunlight	1.66	1.52	1.90	1.51	1.45	1.61	0.18	11.27
blood on denim	0.74	0.67	0.63	0.56	0.62	0.64	0.07	10.24

Reactions with Animal DNA

- Thirteen animal species (chimp, baboon, macaque, cat (7), dog (4), deer, cow, chicken, fish, rabbit, rat, mouse, horse, moose, pig, Drosophila) plus bacteria and yeast tested at 10 ng level
  - Primates (chimp, baboon, macaque) amplify well
  - Other animal DNA at the 10 ng input level gave less than 4 pg with most less than 1pg, Typical 1,000 fold difference in amplification efficiency
- Mixing experiment-Rat/Human DNAs

Rat/Human mixture
(constant total of 1 ng)

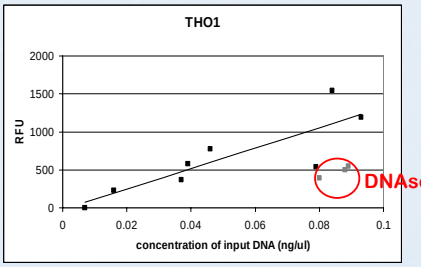


Results on Selected Samples

Sample	Slot blot result (ng/ul)	*Alu* assay result (ng/ul)	THO1 (162-183bp) RFU of smaller allele	D7S820 (256-292bp) RFU of smaller allele
Databank #1	0.44	0.322	2110	715
Databank #3	0.04	0.193	1706	449
Female fraction - G	0.4	0.664	1679	768
Male fraction – G	0.24	0.428	2194	675
Standard - I	0.1	0.121	(2941)[a]	760
Envelope seal #1	0.2	0.262	1238	391
Swab of fingerprint	0.03	0.037	371	(193)[a]
Blood – 3 mo old	0.6	0.888	1388	(653)[a]
Swab of blood/metal	0.5	0.401	1893	883
Blood on denim	1.0	0.245	1237	1060

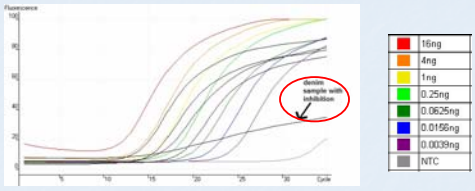
[a] parenthesis are ½ RFU of single peak (homozygote)

STR results for samples with concentrations less than 0.1ng/ul (input less than 1ng)

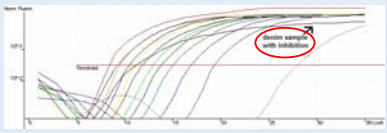


Effect of Inhibitors

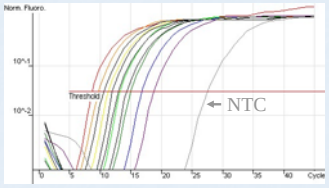
RAW DATA



ANALYZED DATA



No Template Control



If any sample does not cross threshold by 28-30 cycles (like NTC) then inhibitors MUST be present

The NTC always has Ct of about 27-29 cycles due to ambient human DNA in air & water

With Alus present at 1,000's of copies/ cell, the Ct of NTC represents ~1/1000 of a single cell

Seen by others (JFS 45:1307, 2000)

Development vs Internal Validation

- Development
  - Primer concentration - SYBR concentration
  - Anneal temp / time - Elongation time
  - Sensitivity / reproducibility
  - Tests with animal / DNA different samples
  - Stability of Mastermix
  - Inhibitors / BSA
- Validation
  - Comparison to known assay
  - Sensitivity / different samples
  - Reproducibility – duplicates & over time
  - Determine a good range for your STR platform
  - Compile validation results / Write SOP / Train analysts

Use in Our Lab

- Started work on Development in mid 2002
- Started using for All Casework in mid 2003
- Dilute DNA 1/20, run 2ul of sample
- Used for about 220 cases since Jan 2005
- 2ng/ul Ct – 8.34±0.54 (%stdev 6.43%)
- 2ng/ul mean concentration - 2.24±0.26 (%stdev 11.65%)
- Measured ABI 9947 concentration (expected 0.1ng/ul) – 0.11±0.06 (%stdev 58.29%)
- 0.5ng/ul Promega control (only 2007)– 0.70±0.25 (%stdev 36.36%)

Use in Our Lab

- CODIS (Identifiler)
  - If concentration below 0.05ng/ul, re-extract
  - If greater than 1.5ng/ul, dilute down
- Casework
  - If quant low, then re-quant or re-extract or use a better, alternate sample
  - If only the one sample, may try STRs anyway but realizing it probably won't work or give only a partial profile

Ease of Use Real Time - SYBR ALU PCR Assay

- Dilute primer
- Make 2 mastermixes (keeps > 6mo)
  - add SYBR Green to purchased SIGMA ReadyMix
  - mix primer, BSA dilution and H_2O
- Testing evidence- Mix 2 mastermixes, aliquot into tubes, add evidence DNA, PCR in Real-time instrument (just like performing PCR for STRs!!)
- Wait 72 minutes (hands off!!)
- Hit analyze button – Instrument software does the rest
- QA/QC - Clean laser window/weekly, Alcohol treat the balance tubes once/ month, Perform temperature check with Omega temperature device once/ month, Keep log of results with controls

What does it cost?

- **Real time instrument $25K-$100K**
- **Assay cost:**

	price/well
Quantiblot®	**$0.60**
AluQuant™	**$1.00**
Quantifiler	**$2.37**
Alu SYBR Assay	**$0.60**

Advantages of the ALU Assay

- Detects inhibition / no amplification
- Amplicon is a Good Size for predicting STR results (124bp vs 64bp)
- Cheaper ($0.60 vs $2.37)
- More sensitive (1 pg vs 23 pg)

Gender Assay

- **Often of importance to quickly determine gender of sample (victim vs suspect)**
- **Often important to determine presence and concentration of male DNA in sample (vaginal swab)**
- **Develop PCR assay for gender**
- **Multiplex with ALU assay to "kill two birds with one stone"**

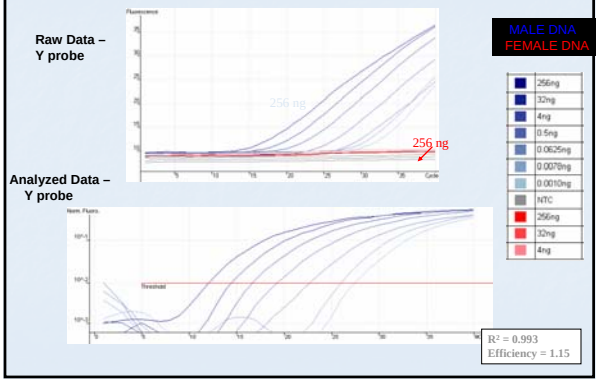
Our Duplex assay

- **Simultaneous determination of total human and male DNA using a duplex real-time PCR assay (J Forensic Sci 51(5):1005-15, 2006)**
- **Real-time PCR (TaqMan-based, MGB probes)**
- **Duplex assay - Male and total human DNA simultaneously**
- **Multicopy targets**
  - ***Alu*** **(total human) – 127bp, range = 128ng to 0.5pg - VIC labeled**
  - **DYZ5 (male, Y specific) – 137bp, range = 128ng to 4pg - FAM labeled**

DYZ5

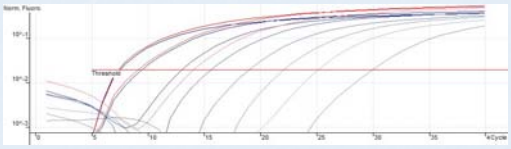
- **Tried other repeated Y sequences - DYZ3 and DYZ1**
- **DYZ5 is a Y-specific repeated sequence**
- **Yp11.2**
- **A repeat of approximately 20,300 bp**
- **The testis-specific protein, Y encoded, genes are part of the DYZ5 repeat unit**
- **There is one array of ~540-800 kb and another minor block of 60 kb on the Y chromosome**
- **Conserved in the great apes but is not present in other mammals**

TaqMan™ GENDER ASSAY

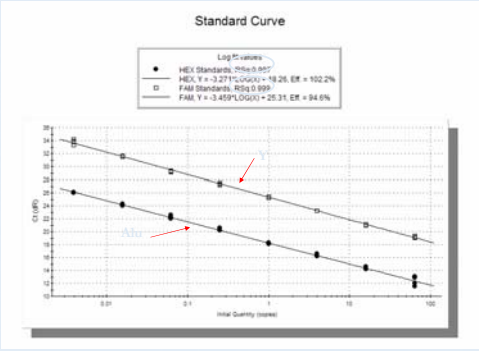


TaqMan™ Total Human Assay

Analyzed Data – ALU probe



$R^2 = 0.997$
Efficiency = 0.99



Comparison to Quantifiler™

	Quantifiler™ Human+Male	Our Assay
%Stdev - total	0.43 to 29.8%	7.6 to 34.7%
%Stdev – male	3.27 to 27.7%	3.6 to 32.4%
Male:Total Ratio Range	0.79 to 2.62 mean-1.18±0.24	0.54 to 1.23 mean-0.83±0.14
Cost	$5.46/sample	$0.80/sample

DNA Degradation Assay

- Can we tell how degraded the DNA in a sample is?
- Will the usual quantitation assays correctly predict input DNA concentration?
- Should we proceed with regular STRs, mini-STRs or mtDNA analysis?

DNA Degradation Assay

- Real-time PCR (Plexor™-based)
- Multicopy target
  - *Alu* (total human)
  - Triplex assay – Three sized PCR products (63bp, 123bp, 246bp) amplified simultaneously
- Determine ratio of 3 size products to determine degradation state
- Also quantitates human DNA

Plexor™ Assay (based on EraGen)

- Forward Primer designed with a 5' iso C with attached Fluorescent dye
- During second round of amplification, an iso G with attached quencher is incorporated into amplicon-base pairs to iso C residue
- Fluorescence is quenched due to proximity of iso C and iso G fluor and quencher
- Monitor the decrease in Fluorescence during PCR

TECHNIQUE
(taken from Plexor™ literature)

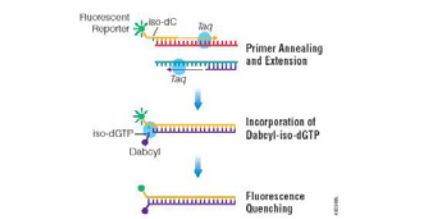


Figure 1. Schematic diagram illustrating the Plexor™ System real-time PCR process.

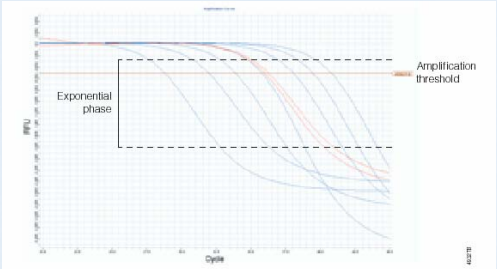
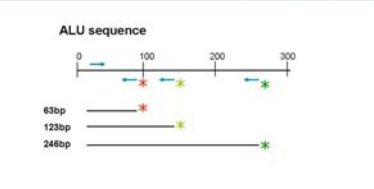


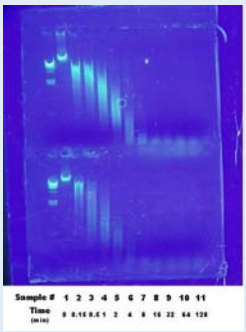
Figure 2. Representative Plexor™ qPCR System amplification curve. The amplification curves show the fluorescence (in relative fluorescence units, RFU) at each cycle of the reaction. The amplification threshold is indicated by a horizontal line across the graph. This line is used to determine the cycle threshold (C_t) for the samples.

Our Assay Design

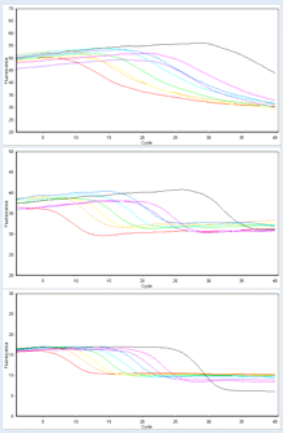


Positions of the one forward (unlabeled) and three reverse (different dye labeled) primers on the *ALU* sequence. Plexor *Alu* primers were custom designed by Promega. PCR was performed using the Plexor QPCR Mastermix in a RG-6000 real-time PCR instrument.

Example of time course of degradation of human DNAs with DNase I



RAW DATA

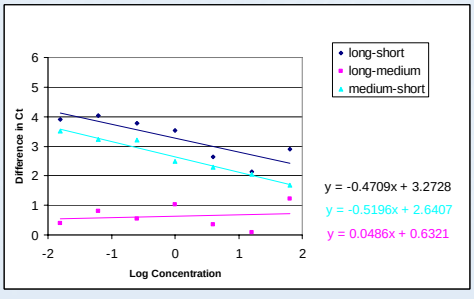


Long Product (FAM)

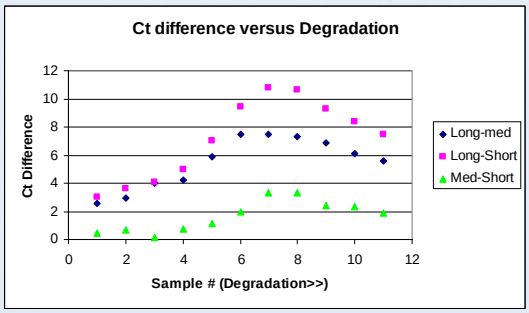
Medium Product (CAL Fluor Orange 560)

Short Product (Quasar 670)

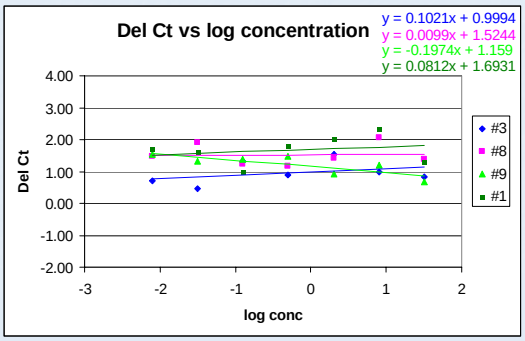
Plot of Ct differences versus input DNA



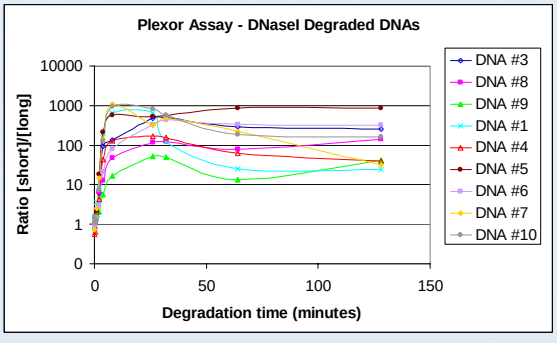
Assay Results on Degraded DNA



Duplex Assay – Del Ct vs Conc



Duplex Assay – Nine Degraded DNAs



Future Directions

- **Try naturally-degraded DNAs (heat, sunlight) and adjucated casework samples**
- **Try using assay results with STRs and mini-STRs**
- **Validate**

Stain Donor Assay- Screening with Real-Time PCR

Can we develop quick, efficient methods to quickly identify a sample as coming from a victim or possible suspect so we can just profile the few relevant crime scene stains and not ALL of them?

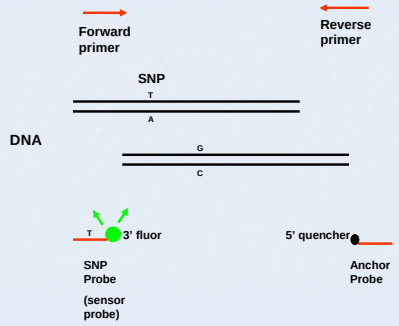
Stain Donor Assay

- **Use SNPs in a Real Time PCR format to give a fast, simple, inexpensive "profile-type" assay**
- **Need not be definitive profile; just a screening assay**
- **Using an assay with four SNPs where both alleles are represented in the general population at about 50% (p=q=0.5) the chance of two random individuals having the same result (same genotype) is only 2%, for six SNPS it is 0.3%, while for eight SNPs it is only 1 in 2500**
- **Multiplex**
- **Dual probe FRET Hybridization assay- with melt curve analysis**

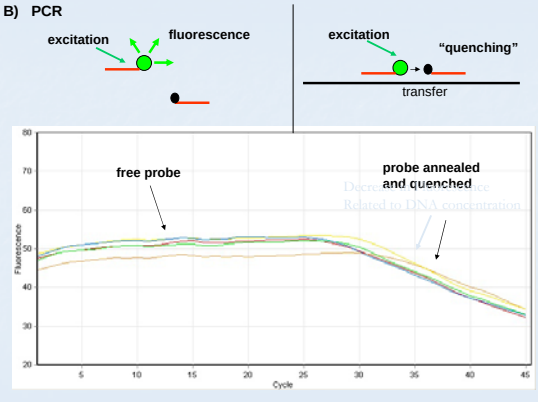
Dual probe FRET hybridization

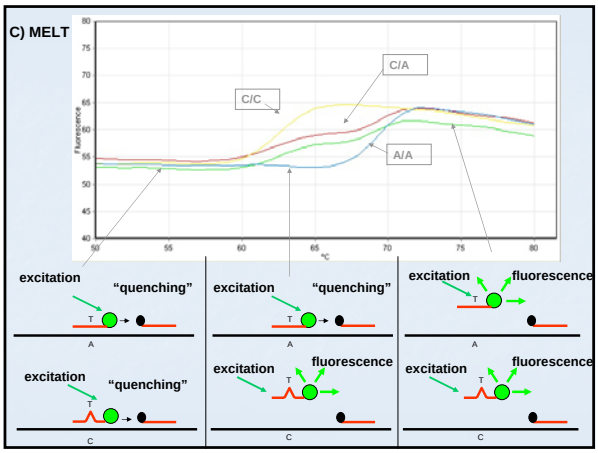
- **SNP probe** (sensor probe)-
  - Perfect complement to one SNP allele
  - One base mismatch to other allele
  - Contains 3' Fluor
  - To multiplex-design different probes for additional SNP sites with different fluorescent dyes
- **Anchor probe**
  - Binds adjacent to SNP probe (SNP 3'-5' anchor)
  - Contains 5' quencher (quenches SNP probe when both are bound to target)
  - Designed to have a higher Tm than Sensor probe

A) COMPONENTS in a dual probe FRET hybridization assay

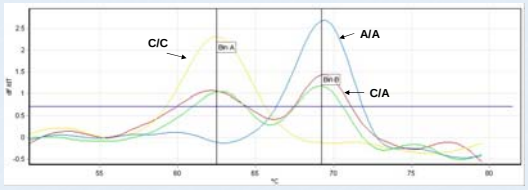


B) PCR





D) ANALYSIS



Melt Curves derived from the change in fluorescence as temperature is increased

Homozygote alleles in yellow and blue
Heterozygote alleles in red and green

Choice of SNPs

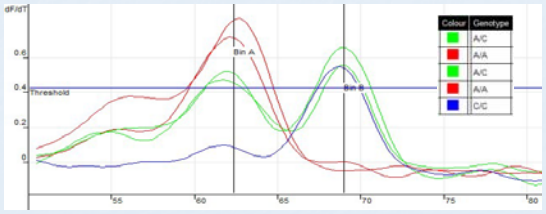
- Variations in allele frequencies between populations were low (low Fst). (SNP useful in any population)
- Found in ALFRED database (http://alfred.med.yale.edu/alfred/)
- Additional criteria were that the SNPs were:
  - non-coding
  - not medically relevant
  - allele frequencies (p and q) close to 0.5
  - located on different chromosomes
- Gender of the individual from whom a sample derived
  - Sequence difference (SD) between ZFX and ZFY
  - Probe contains two differences between the X and Y

Description of Loci in each Assay

SNP/SND	6-plex w gender	6-plex wo gender	4-plex#1	4-plex #2
A2BP1				Quasar 670
ATP13A4	CAL Fluor Red 610	CAL Fluor Red 610	CAL Fluor Red 610	
FLJ43720	CAL Fluor Orange 560	CAL Fluor Orange 560	CAL Fluor Orange 560	
LY9	Biosearch Blue	Biosearch Blue		CAL Fluor Orange 560
PALLD		FAM		FAM
RAB31	Quasar 705	Quasar 705		CAL Fluor Red 610
THSD2	Quasar 670	Quasar 670	Quasar 670	
ZFXY	FAM		FAM	

FLJ Locus Results

(in this assay sensor probe is a perfect match to "C" allele)

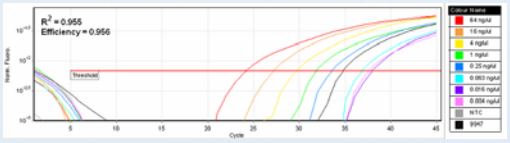


6 channel Rotorgene by Corbett

Quantitation with the FRET assay

Real-time amplification (quantification) results using a standard curve and one sample (9947) on the RG6000.

Results for RAB31 are shown.



Quantitation Results for 6-plex Assay in RG6000

Sample	ZFXY	ATP13A4	THSD2	FLJ43720	Ly9	RAB31	Mean
R^2	0.986	0.977	0.998	0.989	0.974	0.976	0.983
Efficiency	0.977	1.092	0.986	1.040	0.952	1.072	1.020

Individual	ZFXY	ATP 13A4	THSD2	FLJ43720	Ly9	RAB31	Mean	TaqMan *Alu* assay
#2	0.0014	1.16	1.06	0.98	1.71	0.64	0.93	2.48
#3	0.68	0.88	0.99	0.85	0.79	1.07	0.88	2.08
#4	0.12	0.12	0.19	0.18	0.12	0.13	0.14	0.34
#5	5.37	5.33	3.95	5.63	20.22	7.12	7.94	5.89
#6	–	0.05	0.06	0.05	0.13	0.11	0.08	0.11
#7	0.01	1.79	1.33	1.60	1.62	2.92	1.55	1.67
#8	33.60	45.21	88.11	89.22	76.96	144.45	79.59	94.8
#9	32.07	42.55	31.09	47.70	65.83	26.47	40.95	38.2

Why home brew?

- No commercial qPCR kits available at the time
- They are expensive
- We are trying to develop new assays that perform several functions at once
- Most took a year to develop (gender assay longer because of wrong choice of locus)
- Validation generally part of development – lag time is getting analysts to have time to implement
- SYBR and Gender assays easy to implement – buy large quantities of probes/primers from ABI or other vendor (still using same batch of Alu primers for SYBR assay). Buy several vials of one lot of DNA standard. Buy mix in several boxes of same lot
- No harder than commercial kit – still have to QA/QC the lots of those

This work was supported under Award numbers 2000-IJ-CX-K012 and 2005-DA-BX-K003 from the Office of Justice Programs, National Institute of Justice, Department of Justice. Points of view in this document are those of the authors and do not necessarily represent the official position of the U.S. Department of Justice.

THANKS

To NIJ for funding the research shown here today!

Thanks also to the DNA team at the VFL for advice and support:
Margaret Schwartz, Ph.D.
Marcie LaFountain, M.S.
Joseph Abraham, B.S.
Trisha Conti, Ph.D.
Rebekah Herrick, B.A.

For protocols, please feel free to contact us at ebuel@dps.state.vt.us or jnicklas@dps.state.vt.us