# Alternatives to Quantification: Real – Time PCR

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NIST

7th Annual STR MegaPlex and Research Technology Workshop
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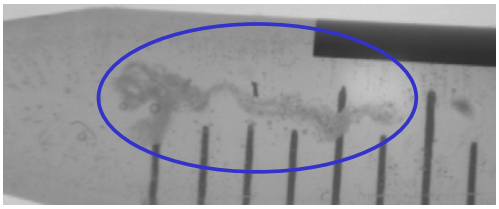
## How do we measure the amount of DNA ?







## Official Disclaimer

Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.



## Spectrophotometric Determination

- 260 nm & 280 nm readings
- 260 nm allows calculation of DNA concentration
- OD =1 ~ 50 ug/mL dsDNA
  ~ 40 ug/mL ssDNA
  ~ 20 ug/mL oligos
- 260 / 280 ratio = 1.8 for DNA



## UV 260/280 :

- Is not Human Specific
  - Does not satisfy FBI QA Document section 9.3
- Requires at least 10 ng/µL for reproducibility (OD 0.2)
- Does not require a [DNA] Standard
- Is influenced by:
  - Salt concentration
  - Residual phenol
  - Residual EtOH
  - Residual Protein

## Pico Green Assay

- Is not Human Specific
  - Does not satisfy FBI QA Document section 9.3
- Requires the sample to be at least 100 pg/µL for reproducibility
  - In a 96 well plate
- Requires <1 h analyst time
- Requires a [DNA] Standard
- Cost ~$0.15 / sample
- Can be made Human Specific by BodeQuant technique



## Quantiblot Assay

- Is Human Specific
  - Does satisfy FBI QA Document section 9.3
- Requires at least 100 pg/µL for reproducibility
- Requires a [DNA] Standard
- Has [DNA] range of 10 ng to 156 pg
  - On a really good day!
- Requires ~ 2 h analyst time.
- Cost $0.40 / sample.

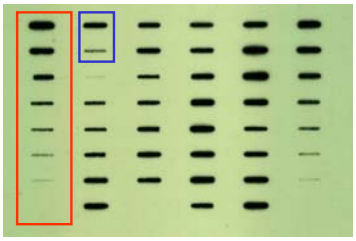


## The Good Days of Quantiblot

All your stds are present!

Cal 1 and Cal 2 look ok!

All your samples are present
&
are within the range of your stds.



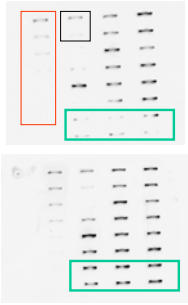


## The Bad Days of Quantiblot

All your stds are NOT present!

Cal 1 and Cal 2 look ok?

All your samples are:
**NOT** present
**NOT** within the range of your stds.
A "duplicate" blot is **different**





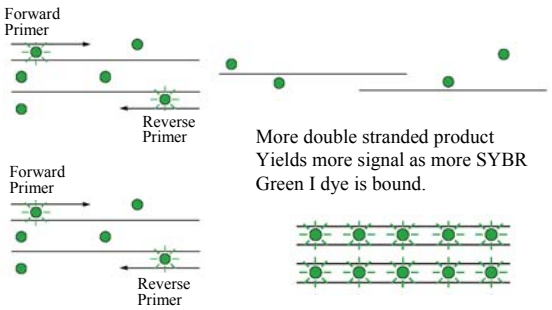
## Real Time – PCR
## Relative DNA Quantification

Methods are based on the PCR process using:
- Thermocyclers
  - that can read fluorescent signals during the PCR process
- SYBR Green I Fluorescence dye or
- Dual labeled Probes (TaqMan Probes)

**<u>A known [DNA] "Standard"</u>**

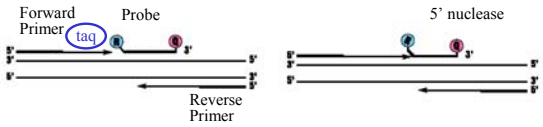


## Steps of a SYBR Green I Assay

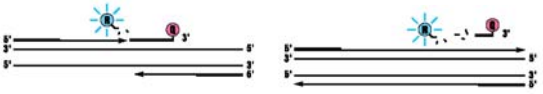


## Steps of a TaqMan Probe Assay

Intact Probe has the fluorescent signal quenched.

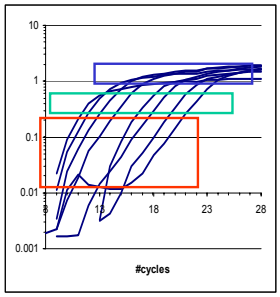



Reporter dye fluoresces after separation from the quencher





## Stages of RT - PCR



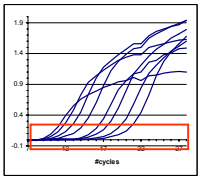

Geometric – Exponential phase
1:1 ratio of signal to product

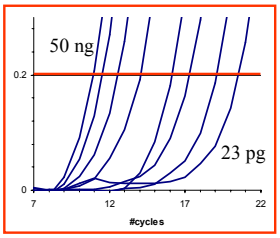
Linear phase
Amplification effiency is continually decreasing resulting in low precision

Plateau
PCR stops - the relative signal remains constant



## Where do you set a Cycle Threshold ?

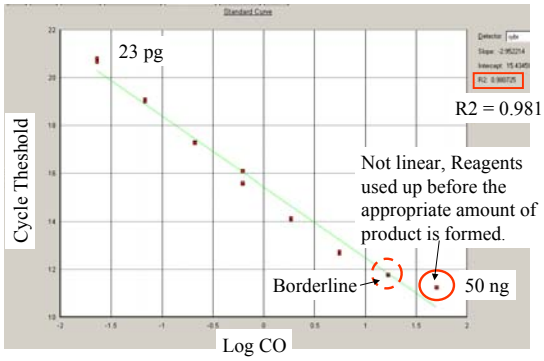



In the Geometric phase above the noise.
Change the placement of the threshold line and you change the *apparent* relative [DNA]

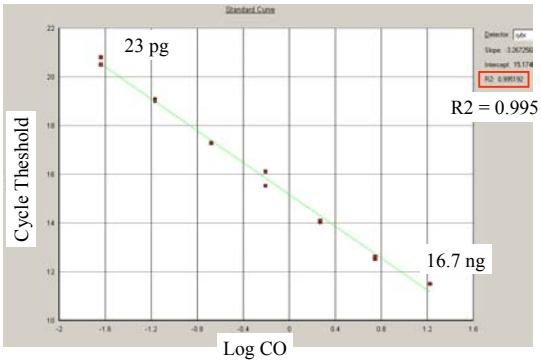


Alu-RT-PCR assay std curve with [DNA] Range of 50 ng to 23 pg





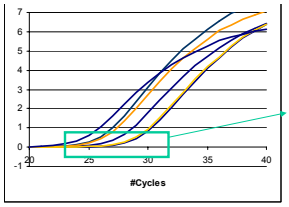

ALU assay std curve with [DNA] Range of 16.7 ng to 23 pg

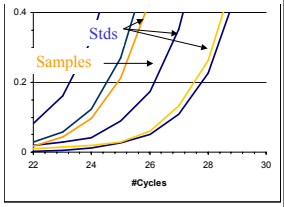





## Good Standards should:

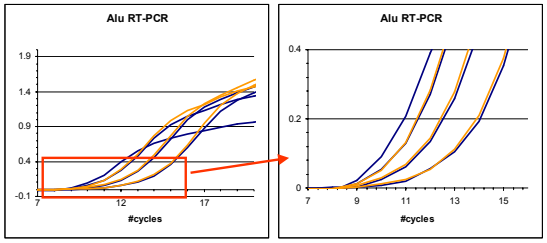
Amplify the same as the samples.
Slopes should be the same and parallel.



## Alu RT-PCR STDS - Samples

Best Linearity between 16 ng to 23 pg






## RT-PCR Choices

Human ID methods SYBR Green-based

- Alu (high copy #)
  - Nicklas & Buel (2003) J. Forensic Sci,, 48 (5):936-944
- TH01
  - Use TH01 monoplex primers with RT-PCR reagents
- Inter-Alu (high copy #)
  - Walker et al. (2003) Anal Biochem 315:122-128



## RT-PCR Choices

Human ID methods Probe based

- CFS-HumRT
  - Richard et al. (2003) J Forensic Sci 48(5):1041-1046
- RB1 (human retinoblastoma susceptibility gene)
  - Andreasson et al. (2002) Biotechniques 33:402-411
- mtDNA (coding region nucletides 8294 to 8436)
  - Andreasson et al. (2002) Biotechniques 33:402-411
- Quantifiler™ Human DNA Quantification Kit
- Quantifiler™ Y Human Male Quantification Kit
  - ABI Quantifiler Kits User's Manual PN4344790



## 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



## $ Cost per sample (20 µL – 25 µL)

| Assay | $ PCR Master Mix | $ Primers | $ TaqMan probe | Total |
|---|---|---|---|---|
| Alu | 0.80* | 0.0025 | NA | $0.8025 |
| TH01 | 0.80* | 0.0025 | NA | $0.8025 |
| CFS-HUMRT | 0.73# | 0.0025 | 0.17 | $0.9025 |
| RB1 | 0.73# | 0.0025 | 0.17 | $0.9025 |
| mtDNA | 0.73# | 0.0025 | 0.17 | $0.9025 |
| Qfiler Human | NA | NA | NA | $2.50 |
| Qfiler Y Male | NA | NA | NA | $2.50 |

* Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA)
# Platinum® Quantitative PCR SuperMix – UDG (Invitrogen, Carlsbad, CA)



## Assay specifications tried at NIST non-probe

| Assay | amplicon | GeneTarget | probe | #Cycles |
|---|---|---|---|---|
| Alu | 124 bp | Alu , Ya5 Subfamily | NA | 28-35 |
| TH01 11p15.5 | ~ 180 bp varies | Human tyrosine hydroxylase gene | NA | 40 |
| CFS-HUMRT 11p15.5 | 62 bp | Human tyrosine hydroxylase gene | * | 40 |

* CFS-HUMRT designed for use with a probe
Tried it in an assay along side TH01 in a plate.

## Results of non-probe TH01 and CFS-HUMRT

16 replicate samples were assayed using each primer set.
Std Curves (10 ng – 21 pg) were run with each primer set.

TH01 = 1.8 ng/µL RSD 15%
CFS-HUMRT = 1.7 ng/µL RSD 18%

±2SD [DNA] Range

TH01 = 1.3 ng/µL – 2.3 ng/µL (1 ng spread)
CFS-HUMRT = 1.1 ng/µL – 2.3 ng/µL (1.2 ng spread)

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## Alu Assay Results

16 replicate samples were assayed using Alu primer set.

Std Curve of 10 ng – 21 pg.

Alu = 2.2 ng/µL RSD 5.8%

±2SD [DNA] Range

Alu = 1.9 ng/µL – 2.4 ng/µL (0.5 ng spread)

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## Assay specifications tried at NIST TaqMan Probes

| Assay | amplicon | GeneTarget | probe | #Cycles |
|---|---|---|---|---|
| CFS-HUMRT 11p15.5 | 62 bp | Human tyrosine hydroxylase gene | 15 bp VIC | 40 |
| RB1 13 | 79 bp | Human retinoblastoma susceptibility gene | 26 bp FAM | 50 |
| mtDNA | 143 bp | tRNA lysine & ATP synthase 8, Coding Region | 29 bp VIC | 50 |
| Qfiler Human 5p15.33 | 62 bp | Human telomase reverse transcriptase (hTERT) | ? FAM | 40 |
| Qfiler Y Male Yp11.3 | 64 or 61 bp | Sex-determining region Y gene (SRY) | ? FAM | 40 |

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## Results for the probe assays

| | |
|---|---|
| CFS-HUMRT | Average RSD 7.1 % |
| RB1 singleplex | Average RSD 5.6 % |
| RB1 multiplex | Average RSD 13 % |
| Qfiler Human | Average RSD 7.7 % |
| Qfiler Y Male | Average RSD 6.8 % |

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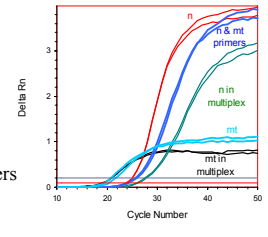
## What About Multiplexing?

This nuclearDNA and mtDNA assay was designed to work as a multiplexed reaction.
When we tried to duplicate the assay, we found changes in the nuclear assay results with the addition of the mtDNA primers and/or probes (the mtDNA primers are added at a much lower concentration than the nDNA).




Do these changes cause problems?

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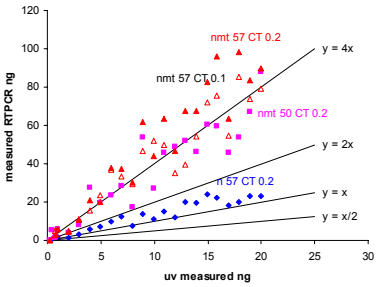
## Effects of the nuclearDNA / mtDNA multiplex

Adding the mtDNA assay to the Nuclear DNA assay resulted in a 4X difference in the [DNA] obtained by the assay.

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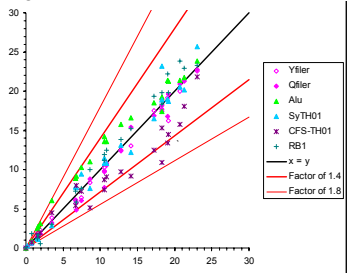
# How do the assays compare?

Following Published Protocols

Series of NIST population samples with a range of [DNA] from 40 pg to 23 ng

The same "Standard" was used for all methods (8 dilutions).

Time for the assay:
Alu-RT-PCR ~ 1.25 h
(fewer cycles required)
The rest ~ 1.75 h




X = median value for all methods
Y = measured value for the method



# 3100 analysis

OK you RT-PCR quantify your samples.
Now you made the appropriate dilutions and amplify!

Samples injected on 3100 after amplification of "1 ng" with PP16 , 30 cycles total.

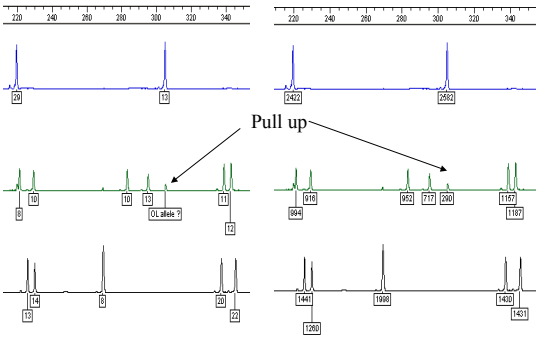
| | D3S1358 | TH01 | D21S11 | D18S51 | Penta E | D5S818 | D13S317 | D7S820 | D16S539 | CSF1PO | Penta D | Amel | vWA | D8S1179 | TPOX | FGA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| avg | 914 | 841 | 1704 | 1897 | 1553 | 1107 | 1702 | 1551 | 1532 | 2091 | 2884 | 1120 | 623 | 1415 | 1217 | 1389 |
| min | 320 | 272 | 453 | 776 | 339 | 356 | 536 | 398 | 443 | 273 | 992 | 577 | 307 | 530 | 508 | 471 |
| max | 1740 | 1948 | 4074 | 3910 | 3917 | 3101 | 4957 | 3814 | 3262 | 4345 | 5917 | 2257 | 1947 | 3624 | 2564 | 3213 |
| median | 824 | 705 | 1406 | 1684 | 1442 | 857 | 1357 | 1382 | 1371 | 2052 | 2786 | 1089 | 566 | 1242 | 1147 | 1360 |

All samples are within acceptable range of peak heights.
One sample had a pull-up issue.



# Pull-up issue

Allele Assignments | Peak Heights






# 310 Analysis

310 data from the same samples.

| | D3S1358 | TH01 | D21S11 | D18S51 | Penta E | D5S818 | D13S317 | D7S820 | D16S539 | CSF1PO | Penta D | Amel | vWA | D8S1179 | TPOX | FGA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| avg | 286 | 290 | 474 | 471 | 354 | 272 | 376 | 325 | 336 | 419 | 504 | 279 | 169 | 292 | 273 | 270 |
| min | 114 | 99 | 113 | 53 | 101 | 81 | 98 | 89 | 48 | 67 | 66 | 172 | 92 | 84 | 129 | 45 |
| max | 510 | 515 | 971 | 771 | 683 | 797 | 1141 | 813 | 741 | 807 | 884 | 452 | 507 | 641 | 595 | 413 |
| median | 283 | 273 | 431 | 519 | 327 | 213 | 299 | 307 | 283 | 453 | 523 | 269 | 154 | 279 | 262 | 285 |

Loss of some loci and peak under 150 rfu for many.

Instrument variability!

Know your entire analysis system!



# Conclusions:

There are several published RT-PCR methods available.

The cost per sample ranges from ~$0.80 to $ 2.50.

Inter – method variability was a factor of 1.8 using the same DNA "standard."

Each method has a working linear range for an approximate [DNA].

But you *still* need to know the final analysis system for it all to work!



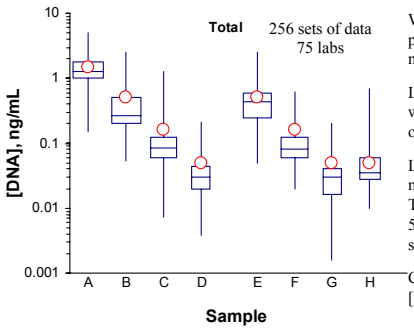
# Preliminary Results NIST QS 04

Consisted of:

8 DNA extracts labeled A – H Shipped Dec 2003 –Jan 2004
Shipped to 84 laboratories for quantification.
Labs asked to use multiple methods / multiple analysts
Last day for submission extended from 15 March to 5 April 2004

As of 23 March 2004:
We have received data from 75 Labs (89%)
Total of 264 sets of data
Participates used 21 different quantification methods

## Preliminary NIST QS 04 Results




Width of the box is proportional to the number data points.

Length of the whiskers is the range of values submitted.

Line in the box is the median value. The box represents 50% of the data submitted.

Circle is the target [DNA].

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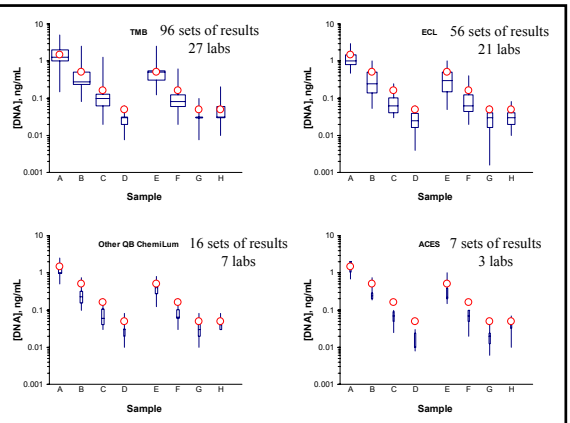


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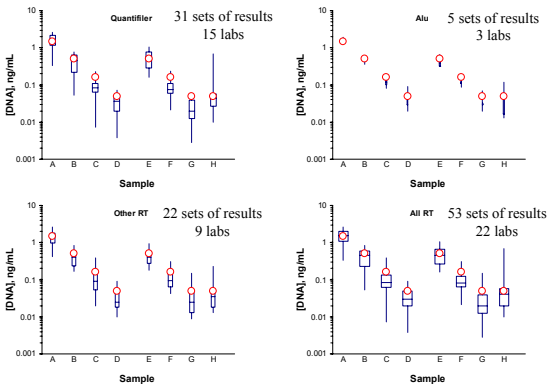


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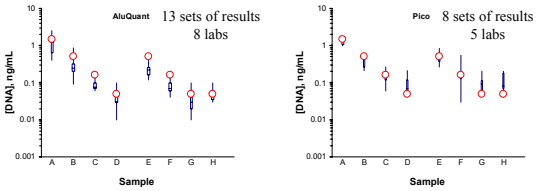


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NIST Project Team:

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Jill Appleby Amy Decker
Mike Coble Dave Duewer

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