How do we measure the amount of DNA?

Spectrophotometric Determination
- 260 nm & 280 nm readings
- 260 nm allows calculation of DNA concentration
  - OD = 1 ~ 50 µg/mL dsDNA
  - ~ 40 µg/mL ssDNA
  - ~ 20 µg/mL oligos
- 260 / 280 ratio = 1.8 for 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.
**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)

*A known [DNA] “Standard”*

**Steps of a SYBR Green I Assay**

- More double stranded product
  - Yields more signal as more SYBR Green I dye is bound.
**Steps of a TaqMan Probe Assay**

Intact Probe has the fluorescent signal quenched.

- **Forward Primer**
- **Probe**
- **Reverse Primer**

Reporter dye fluoresces after separation from the quencher.

**Steps of RT-PCR**

- **Geometric – Exponential phase**
  - 1:1 ratio of signal to product
- **Linear phase**
  - Amplification efficiency 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**

- 23 pg
- 50 ng

Log CO

R² = 0.981

Not linear, Reagents used up before the appropriate amount of product is formed.

**Good Standards should:**

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

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

- 23 pg
- 16.7 ng

Log CO

R² = 0.995

SMART Meeting (Virginia Beach, VA)
RT-PCR Choices

**Human ID methods SYBR Green-based**

- **Alu (high copy #)**
- **TH01**
  - Use TH01 monoplex primers with RT-PCR reagents
- **Inter-Alu (high copy #)**

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**RT-PCR Choices**

**Human ID methods Probe based**

- **CFS-HumRT**
- **RB1 (human retinoblastoma susceptibility gene)**
- **mtDNA (coding region nucleotides 8294 to 8436)**
- **Quantifiler™ Human DNA Quantification Kit**
- **Quantifiler™ Y Human Male Quantification Kit**
  - ABI Quantifiler Kits User’s Manual PN4344790

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**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

---

### Assay specifications tried at NIST non-probe

<table>
<thead>
<tr>
<th>Assay</th>
<th>amplicon</th>
<th>Gene</th>
<th>Target</th>
<th>probe</th>
<th>#Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu</td>
<td>124 bp</td>
<td>Alu</td>
<td>Ya5 Subfamily</td>
<td>NA</td>
<td>28-35</td>
</tr>
<tr>
<td>TH01</td>
<td>11p15.5</td>
<td>Human tyrosine</td>
<td>hydroxylase gene</td>
<td>NA</td>
<td>40</td>
</tr>
<tr>
<td>CFS-HUMRT</td>
<td>62 bp</td>
<td>Human tyrosine</td>
<td>hydroxylase gene</td>
<td>*</td>
<td>40</td>
</tr>
</tbody>
</table>

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

---

$ Cost per sample (20 µL – 25 µL)

<table>
<thead>
<tr>
<th>Assay</th>
<th>PCR Master Mix</th>
<th>Primers</th>
<th>TaqMan</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alu</td>
<td>0.80*</td>
<td>0.0025</td>
<td>NA</td>
<td>$0.8025</td>
</tr>
<tr>
<td>TH01</td>
<td>0.80*</td>
<td>0.0025</td>
<td>NA</td>
<td>$0.8025</td>
</tr>
<tr>
<td>CFS-HUMRT</td>
<td>0.73*</td>
<td>0.0025</td>
<td>0.17</td>
<td>$0.9025</td>
</tr>
<tr>
<td>RB1</td>
<td>0.73*</td>
<td>0.0025</td>
<td>0.17</td>
<td>$0.9025</td>
</tr>
<tr>
<td>mtDNA</td>
<td>0.73*</td>
<td>0.0025</td>
<td>0.17</td>
<td>$0.9025</td>
</tr>
<tr>
<td>Quantifiler Human</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>$2.50</td>
</tr>
<tr>
<td>Quantifiler Y Male</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>$2.50</td>
</tr>
</tbody>
</table>

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

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)

Assay specifications tried at NIST

<table>
<thead>
<tr>
<th>Assay</th>
<th>amplicon</th>
<th>Gene/Target</th>
<th>probe</th>
<th>#Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFS-HUMRT 1p15.5</td>
<td>62 bp</td>
<td>Human tyrosine hydroxylase gene</td>
<td>15 bp VIC</td>
<td>40</td>
</tr>
<tr>
<td>RB1 13</td>
<td>79 bp</td>
<td>Human retinoblastoma susceptibility gene</td>
<td>26 bp FAM</td>
<td>50</td>
</tr>
<tr>
<td>mtDNA 143 bp</td>
<td>iRNA lysine &amp; ATP synthase β, Coding Region</td>
<td>29 bp VIC</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Qfiler Human 3p15.33</td>
<td>62 bp</td>
<td>Human telomerase reverse transcriptase (hTERT)</td>
<td>? FAM</td>
<td>40</td>
</tr>
<tr>
<td>Qfiler Y Male 1p11.3</td>
<td>64 or 61 bp</td>
<td>Sex-determining region Y gene (SRY)</td>
<td>? FAM</td>
<td>40</td>
</tr>
</tbody>
</table>

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%

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?

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.

SMART Meeting (Virginia Beach, VA)
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

Pull-up issue

Allele Assignments

Peak Heights

Pull up

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!

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.

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

310 Analysis

310 data from the same samples.
Loss of some loci and peak under 150 rfu for many.
Instrument variability!

Know your entire analysis system!

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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John Butler  Pete Vallone
Margaret Kline  Jon Redman
Jill Appleby  Amy Decker
Mike Coble  Dave Duewer

SMART Meeting (Virginia Beach, VA)