DNA Quantitation by Real Time PCR: Advanced Issues

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1. Brief introduction to Quantitative PCR
Calculation of the Quantity of DNA in a Cell

1. **Molecular Weight of a DNA Basepair = 618 g/mol**
   
   - $A = 313 \text{ g/mol}$; $T: 304 \text{ g/mol}$; $A-T$ base pairs = 617 g/mol
   - $G = 329 \text{ g/mol}$; $C: 289 \text{ g/mol}$; $G-C$ base pairs = 618 g/mol

2. **Molecular Weight of DNA = $1.85 \times 10^{12} \text{ g/mol}$**

   There are 3 billion base pairs in a haploid cell, $\sim 3 \times 10^9 \text{ bp}$
   
   $(\sim 3 \times 10^9 \text{ bp}) \times (618 \text{ g/mol/bp}) = 1.85 \times 10^{12} \text{ g/mol}$

3. **Quantity of DNA in a Haploid Cell = 3 picograms**

   1 mole = $6.02 \times 10^{23} \text{ molecules}$
   
   $(1.85 \times 10^{12} \text{ g/mol}) \times (1 \text{ mole}/6.02 \times 10^{23} \text{ molecules})$
   
   $= 3.08 \times 10^{-12} \text{ g} = 3.08 \text{ picograms (pg)}$

   A diploid human cell contains $\sim 6$ pg genomic DNA

4. **One ng of DNA contains the DNA from 167 diploid cells**

   1 ng genomic DNA ($1000 \text{ pg}$)/$6\text{pg/cell} = \sim 333 \text{ copies of each locus}$

   (2 per 167 diploid genomes)
So What’s the bottom line?

• You need to sample sufficient cells to avoid stochastic effects –
  – 167 cells = 1ng total DNA
  – 1pg of DNA is 1/6 of a cell
  – 100 pg is 17 cells
  – Amplification of low levels of DNA risks artifacts – allele drop in, allele drop out, severe peak imbalance

• You also can also overload the system-
  – Peaks heights become nonlinear
  – Pull-up occurs
  – Stutter goes up
  – Noise increases
  – Low level alleles – not relevant to case become visible
Why do you want to be in the DNA quantitation “sweet spot”?

Higher quality data which results in easier data interpretation
- No allele dropout
- Peaks on-scale with no pull-up from dye bleedthrough
- No split peaks from partial adenylation

• STR kits, especially those amplifying more loci, are optimized for a narrow range of input DNA
  - Overall peak balance is predicated on input levels
  - Temperatures and concentrations of primers critical in proper multiplex amplifications
Impact of DNA Amount into PCR

Reason that DNA Quantitation is Important Prior to Multiplex Amplification

- **Too much DNA**
  - Off-scale peaks
  - Split peaks (+/-A)
  - Locus-to-locus imbalance

- **Too little DNA**
  - Heterozygote peak imbalance
  - Allele drop-out
  - Locus-to-locus imbalance

**Stochastic effect when amplifying low levels of DNA produces allele dropout**

Generally 0.5 – 2.0 ng DNA template is best for STR kits

DNA Size (bp)

<table>
<thead>
<tr>
<th>Template Level</th>
<th>DNA Size (bp)</th>
<th>Relative Fluorescence (RFUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pg template</td>
<td>120-140 bp</td>
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<td>5 pg template</td>
<td>120-140 bp</td>
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<tr>
<td>2 ng template</td>
<td>120-140 bp</td>
<td>6000</td>
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<tr>
<td>2 ng template</td>
<td>120-140 bp</td>
<td>2000</td>
</tr>
</tbody>
</table>

Stochastic effect when amplifying low levels of DNA produces allele dropout
Issue: Need Human-Specific DNA Quantitation

• All sources of DNA are extracted when biological evidence from a crime scene is processed to isolate the DNA present.

• Thus, non-human DNA such as bacterial, fungal, plant, or animal material may also be present in the total DNA recovered from the sample along with the relevant human DNA of interest.

• For this reason, the DNA Advisory Board (DAB) Standard 9.3 requires human-specific DNA quantitation so that appropriate levels of human DNA can be included in the subsequent PCR amplification.

• Multiplex STR typing works best with a fairly narrow range of human DNA – typically 0.5 to 2.0 ng of input DNA works best with commercial STR kits.
How was it done in the past?

- **UV/vis** – monitor DNA at 260/280. The ratio for a pure sample is 1.8, less if protein or impurity is present.
- **Problem: Non specific** – dyes, inhibitors interfere, no quality info.
- **Yield gel** – Separate genomic DNA on an agarose gel stain with ethidium bromide.
- **Problem: Non human specific, hard to estimate quantity if degraded**.
- **Fluorescence** – Add an intercalating dye like Sybr Green to a DNA sample. Only DNA provides a fluorescence enhancement.
- **Problem: Non human specific – no quality information**.
- **Slot blot** – ssDNA is bound to a nylon membrane and probed with colorimetric reaction.
- **Problem: subjective, time consuming, poor sensitivity**.
- **Aluquant** – attach bioluminescent probe to DNA.
- **Problem: poor sensitivity**.
Big issue – sensitivity and dynamic range

Quantiblot-ECL  40 pg - 2.0 ng

ACES 2.0  40 pg - 4.0 ng
(ACES tended to work better on degraded DNA)

Real Time PCR  (1-23) pg - 16 ng
(depending on method)

RTPCR has lower detection limit
and larger dynamic range
Quantitative PCR

• 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?
Advantages of QPCR

• The ability to monitor the progress of the PCR reaction as it occurs in real time
• The ability to precisely measure the amount of amplicon at each cycle
• An increased dynamic range of detection
• The combination of amplification and detection in a single tube, which eliminates post-PCR manipulations
Forensic advantages of qPCR

• An automatable procedure for determination of DNA quantity
• The estimate of DNA quantity is the amount of amplifiable DNA – and is directly related to the process of STR typing
• Internal controls and melt curves permit detection of PCR inhibition
• The capability to detect both autosomal and Y DNA template
• Extreme sensitivity is possible with multicopy loci
Disadvantages of QPCR

- The precision is not much better than that of slot blots
- Internal controls do not always respond to inhibition
- Concentration of DNA template mixture may vary between qPCR reaction and STR reaction affecting inhibitor concentration, esp. at low and high concentrations
  - i.e. a 20uL reaction mixture contains 2 uL of template for real time while a 25uL STR reaction mixture contains up to 10uL of DNA template.
- Reagents are expensive and require a complex series of events
- Improper pipetting of standards will cause large errors in precision.
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

• First paper on qPCR:

• Warning: RT-PCR also means reverse transcriptase PCR which is used when working with RNA
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

![Graph showing the stages of a PCR reaction: baseline, exponential, and plateau.](graph.png)
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
So can you use regular PCR for quantitation?

• Methods:

1. End point detection – measure amount of product produced after a set number of cycles
   problem: limiting reagent effects, sequence effects

2. 

3. End point detection with modified internal standard - measure amount of product produced while simultaneously amplifying an internal standard with very similar sequence
   problem: cost of developing proper internal standard, increased sample manipulation
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. If you stopped this reaction at 33 cycles, how much variation in quantity?
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 will be proportional to initial template.

http://env1.gist.ac.kr/joint_unugist/file/g_class11_real_time_pcr_vt.pdf
Real Time PCR

- Quantitation of DNA is 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.

http://www.med.sc.edu:85/pcr/realtime-home.htm
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
Effect of Amplification Efficiency

\[ y = x(1+e)^n \]

**Case 1:** \( e = 0.9 \)
\[ y = 100 \times (1+0.9)^{30} \]
\[ y = 2.3 \times 10^{10} \]

**Case 2:** \( e = 0.8 \)
\[ y = 100 \times (1+0.8)^{30} \]
\[ y = 4.6 \times 10^{9} \]

**Result:** A difference of 0.1 in amplification efficiencies created a 5-fold difference in the final ratio of PCR products.
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

C_t

5.0 ng
1.3 ng
0.31 ng
0.078 ng
0.0 ng (reagent blank)
The output data is plotted on a log scale and the fractional number of cycles required to reach $C_t$ is measured.
Standard curve

Plot the cycle # at threshold CT vs log of concentration

Concentration = \(10^{(-0.297 \times CT + 4.528)}\)
Why the semilog plot?

• Since efficiency is measured as
  Product concentration \( Y = X (1 + E_c)^n \)

\[
\log(Y) = \log(X) + n \log(1+E) \\
\text{and } \log(X) = \log(Y) - n \log(1+E) \\
\log(1/x) = n \log(1+E) - \log(Y)
\]

and thus the log of the template conc. is inversely proportional to the number of cycles and the slope is proportional to the amplification efficiency.

This intercept \( \log y = \# \) of cycles required to amplify one copy of template
Amplification Efficiency

100%  
10-fold = 3.3 cycles

80%  
10-fold = 3.9 cycles

Threshold

Cycle number

Log DNA

http://env1.gist.ac.kr/joint_unugist/file/g_class11_real_time_pcr_vt.pdf
### Error in the $C_T$ Value

Be aware that relatively small changes in $C_T$ result in large variations in estimated concentration.

<table>
<thead>
<tr>
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<td>19.7</td>
<td>78.80</td>
<td>18.77</td>
<td>-14.79</td>
</tr>
</tbody>
</table>

± 0.1 $C_T$  

± 0.3 $C_T$
Regression Analysis

Goal is to produce the best fit line through a field of points. To do this minimize the sum of the squares of the residuals (+/- deviations) from the line.

$$RSS = RSS (\beta_0, \beta_1) = \sum_{i=1}^{n} \varepsilon_i^2 = \sum_{i=1}^{n} (y_i - \beta_0 - \beta_1 x_i)^2.$$  

RSS is the residual sum of squares.
Correlation Coefficient $R^2$

The correlation coefficient is a measure of how straight the line is. It compares two hypotheses:
1: there is no correlation – null hypothesis; 2: there is a correlation.

$$r^2 = 1 - \frac{SS_{reg}}{SS_{tot}} = 1 - \frac{0.86}{4.91} = 0.83$$

http://www.curvefit.com/linear_regression.htm
What does this mean for Quantifiler? Or Duo?

R2 value is above 0.99  All is good

R2 value is between 0.99 and 0.98
  There is a wrong value entered
  There is a pipetting issue with sample loading or serial dilution
  There is a problem with the 23pg standard

R2 value is lower than 0.98  There is
  There is a wrong value entered
  There is a pipetting issue with sample loading or serial dilution
  There is major problem with the 23pg standard

Note that for duo, at 23pg the male amplicon contains 7 DNA copies and the autosomal DNA contains 14 copies. At these copy numbers (especially for the male DNA there are major stochastic issues)
Regression Analysis

Best fit line

Alternate possibilities given the error

Notice 2 things:
1. The ability to define the mean value improves at the center of the regression line
2. The error in the measurement is very high at the ends.
3. The error is unknown above and below the endpoints.

3/15/2011

http://www.curvefit.com/linear_regression.htm
Sensitivity and precision for single locus Taqman probe

The figure illustrates the problem when analyzing samples below 20 pg (qPCR/single locus probes.) Reproducibility suffers from stochastic effects on amplification. Note that the general standard for low copy DNA is <100pg
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. The more dsDNA the more fluorescence

• Fret Probes
  – When probes bind fluorescence increases. The more dsDNA produced, the more fluorescence

• Plexor
  – Primers are labeled with isomeric base +fluor. Complementary base contains quencher. The more dsDNA produced, the lower the fluorescence becomes.
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
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
Figure 1. SYBR Green principle. Principle of SYBR Green-based detection of PCR products in real-time PCR.
Calibration curve for Multilocus ALU qPCR

Note reproducibility of curve
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.

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

Seen by others (JFS 45:1307, 2000)
Taqman

Taqman is a highly specific real time procedure that evolved to measure gene expression

1. Primers are designed to target a particular location on the genome. Prime binding sites are invariant,

2. A 20-30 bp probe is designed to target a specific variable region within the amplicon. (deletion, SNP, etc)

3. The probe contains two dyes, a reporter and a quencher, thus its fluorescence is low. Probe binds during annealing step.

4. Exonuclease activity of Taq during extension destroys the probe and releases the reporting dye from the quencher

5. Fluorescence increases as probes are destroyed during each amplification cycle.
Region to be probed
(if mutation is not present, primers will amplify but probe will not bind.)

Figure 2. TaqMan probe principle. **A** Both the TaqMan probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the quencher to the fluorophore strongly reduces the fluorescence emitted by the fluorophore. **B** During the PCR extension step, Taq DNA polymerase extends the primer. When the enzyme reaches the probe, its 5' → 3' exonuclease activity cleaves the fluorophore from the probe. The fluorescent signal from the free fluorophore is measured. The signal is proportional to the amount of accumulated PCR product.
Molecular beacons

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

– 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

\[ T_{\text{anneal}} < T_m < T_{\text{ext}} \]

Above \( T_m \) loop structure reforms and probe leaves template
Fret Probes

Fret probes work in an opposite manner to Taqman and Molecular Beacons. Fret stands for fluorescence energy transfer.

1. Primers are designed to target a particular location on the genome. Prime binding sites are invariant,

2. Two probes are designed facing each other on a strand. The 5’ and 3’ ends of the probes face each other and are labeled with energy transfer dyes.

3. If the two probes lay next to each other the two dyes interact and fluorescence enhancement is seen during the annealing step.

4. At extension, the probes melt off and fluorescence enhancement is gone.
Figure 3. FRET probe principle. A When not bound to the target sequence, no fluorescent signal from the acceptor fluorophore is detected. B During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor and acceptor fluorophores into close proximity, allowing energy transfer between the fluorophores and resulting in a fluorescent signal from the acceptor fluorophore that is detected. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence. C During the extension step of PCR, the probes are displaced from the target sequence and the acceptor fluorophore is no longer able to generate a fluorescent signal.
1. Primers are designed to target a particular location on the genome. Primers are contain methyl cytocine at the 5’ end.

2. During the amplification step a complimentary (iso-dGTP) binds specifically to the methyl cytocine

3. As a result, the initial fluorescence signal from the unincorporated primers decreases as they are incorporated into the accumulating dsDNA strands.

4. Since the quenching step occurs in a primer and not a probe molecule, plexor permits the application of melt curve analysis in multiple channels.
Figure 1: Base pairing between isoguanine (iso-dG) and 5’-methylisocytosine (iso-dC)
Figure 2: Quenching of the fluorescent signal by dabcyl during product accumulation
Note: only one of the two primers is labeled with the reporter dye.
Amplification Curves

Plexor™ vs. SYBR Green

- **Plexor**
  - fluorescence $\downarrow$ as amplification $\uparrow$

- **SYBR Green**
  - fluorescence $\uparrow$ as amplification $\uparrow$

http://www.promega.com/plexorhy/system.htm

http://www.capitalbio.com/life_sciences/bioanalysis_kit/capitalbio__real-time__qpcr__universal__kits
Melting Curves

If the real time PCR detection system is based on Sybr Green or Plexor, fluorescence will be a function of the amount of dsDNA.

If the temperature is increases the two strands melt and the fluorescence is altered.

The dsDNA melting temperature is a function of length, GC content and conformation.

The shape of the curve can be used to detect SNPs, primer dimers, and inhibitor binding.
The melt curve analysis can be generated by allowing the product to form dsDNA from 60°C-95°C.

Melting temperature ($T_m$) - the temperature at which amplicon disassociation occurs
- Characterizes amplicon homogeneity
- Inhibition

Figure 4: Melt curves with derived $T_m$
Difference plot generated by Applied Biosystems’ HRM Software. A 96 bp-long fragment of the human Keratin 23 gene containing a G>A variant was amplified from DNA samples and melted on the 7500 Fast System. Three replicates of each genotype are shown, wild-type homozygote A/A (green), variant homozygote G/G (red), and heterozygote G/A (blue).
D) SNP ANALYSIS by QPCR melt curves

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

Homozygote alleles in yellow and blue
Heterozygote alleles in red and green
Effects of LCN and inhibition with different detection methods

• SYBR Green
  – Multilocus probes (Alu), one color
  – If no sample, amplification of contaminants occurs at high cycle #
  – If inhibition, no result or poor efficiency curve
  – Melt curve reveals inhibition if inhibitor binds DNA

• Probes (Taqman, Mol. beacons)
  – Multiplex targeted probes – Quant Y, nuclear DNA, int. std
  – If no sample, internal control amplifies but sample doesn’t.
  – If inhibition, poor amplification of internal control may be seen

• Plexor
  – Multiplex targeted primers – nuclear, Y, int. std.
  – No sample, internal control amplifies
  – Inhibition is revealed by changes in internal control
  – Melt may also be affected by inhibition. (no sample no melt curve)
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
Use of internal positive control to detect humic acid inhibition

Figure 3. Humic acid titration in Flexor® HY reactions. DNA samples were amplified in the presence of 0ng (black), 200ng (orange), 300ng (blue), 400ng (red) and 600ng (green) of humic acid. Amplification curves are shown for Flexor® HY autosomal (Panel A), Y (Panel B) and IPC (Panel C) analyses of a 1ng/ul sample.
Table 5-3  Interpreting IPC amplification results

<table>
<thead>
<tr>
<th>Duo Human (VIC® dye) and/or Duo Male (FAM™ Dye)</th>
<th>Duo IPC (NED™ Dye)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification</td>
<td>Amplification</td>
<td>Negative result - no human DNA detected</td>
</tr>
<tr>
<td>No amplification</td>
<td>No amplification</td>
<td>Invalid result</td>
</tr>
<tr>
<td>Amplification (low C\textsubscript{T} and high ΔR\textsubscript{n})</td>
<td>No amplification or C\textsubscript{T} higher than 31</td>
<td>IPC result inconclusive</td>
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<tr>
<td>Amplification (high C\textsubscript{T} and low ΔR\textsubscript{n})</td>
<td>No amplification or C\textsubscript{T} higher than 31</td>
<td>PCR inhibition</td>
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</table>

Note: Positive amplification occurs when the C\textsubscript{T} value for the detector is <40. Because samples contain unknown amounts of DNA, a large range of C\textsubscript{T} values is possible. The IPC system template DNA is added to the reaction at a fixed concentration, therefore, the NED C\textsubscript{T} should range between 28 and 31, with a variation of 1 C\textsubscript{T} across the standard curve samples.
Single vs Multilocus Targets

• Multilocus Primer binding
  – Primers bind at multiple locations throughout the genome – sensitive
  – Syber green requires no special kit – Inexpensive
  – Plexor permits multiplexing based on 20 Y and 10 autosomal DNA loci

• Single Locus 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, Plexor) vs direct correspondence between copy number and [DNA] (Taqman)

• Also the probe method is more specific – both primer and probe must bind to get signal.
Quality of data depends on technique!

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold</td>
<td>0.0175</td>
</tr>
<tr>
<td>Left Threshold</td>
<td>10.000</td>
</tr>
<tr>
<td>Standard Curve Imported</td>
<td>No</td>
</tr>
<tr>
<td>Standard Curve (1)</td>
<td>( \text{conc} = 10^{0.198\cdot CT + 2.796} )</td>
</tr>
<tr>
<td>Standard Curve (2)</td>
<td>( \text{CT} = -5.057\cdot \log(\text{conc}) + 14.140 )</td>
</tr>
<tr>
<td>Reaction efficiency (*)</td>
<td>0.57672 (* = 10^{-1/m} - 1)</td>
</tr>
<tr>
<td>M</td>
<td>-5.0568</td>
</tr>
<tr>
<td>B</td>
<td>14.14006</td>
</tr>
<tr>
<td>R Value</td>
<td>1.000</td>
</tr>
<tr>
<td>R^2 Value</td>
<td>1.000</td>
</tr>
<tr>
<td>Start normalising from cycle</td>
<td>1</td>
</tr>
<tr>
<td>Noise Slope Correction</td>
<td>Yes</td>
</tr>
<tr>
<td>Reaction Efficiency Threshold</td>
<td>Disabled</td>
</tr>
<tr>
<td>Normalisation Method</td>
<td>Dynamic Tube Normalisation</td>
</tr>
<tr>
<td>Digital Filter</td>
<td>Light</td>
</tr>
<tr>
<td>No Template Control Threshold</td>
<td>10%</td>
</tr>
<tr>
<td>Sample Page</td>
<td>Page 1</td>
</tr>
</tbody>
</table>

R-Value: Perfect 1.000 !!

Rayna and Sarah
Reproducible pipetting is critical

• Set up experiment so you are never pipetting less than 2 uL. (dilute stds with appropriate amounts of TE prior to analysis)
• Make sure that the sample is well mixed prior to amplification
• Be aware of the potential for DNA loss due to degradation and adsorption on tube walls.
• Tips for Improving Pipetting Technique
  • Operator technique has a major impact on pipetting performance.
  • **Prewet the pipette tip**—Aspirate and expel an amount of the sample liquid at least three times before taking a sample for delivery. Evaporation in the tip can cause a significant loss of sample before delivery. Prewetting increases humidity in the tip, thus reducing the amount of variation in sample evaporation.
  • **Work at temperature equilibrium**—Allow liquids and equipment to equilibrate to ambient temperature. The volume delivered varies with air pressure, relative humidity, and vapor pressure of the sample; all of which are temperature dependent.
  • **Examine the pipette tip before dispensing**—Wipe the tip carefully and only if there is liquid on the outside. Otherwise, sample liquid may be wicked from the tip.
  • **Use standard-mode pipetting**—For all but viscous samples, standard-mode (also called forward-mode) pipetting yields better accuracy and precision than reverse-mode pipetting. In reverse-mode, the plunger is depressed completely (e.g., past the first stop) to aspirate the sample.
  • **Pause after aspiration**—Pause with the tip in the liquid for one to two seconds after aspirating the sample. This is important because the liquid in the tip bounces slightly when the plunger stops.
  • **Lift the pipette straight out**—Do not touch the tip to the sides of the container. Surface tension causes the sample to vary if the exit angle varies, particularly for small volumes.
  • **Minimize handling of the pipette and tip**—Set the pipette down between deliveries. Body heat transferred to equipment during handling disrupts temperature equilibrium.
  • **Immerse the tip properly**—Immerse the tip 2-5mm below the meniscus and well clear of the container walls and bottom during sample aspiration, otherwise volume is affected.
  • **Use the correct pipette tip**—Securely attach a high-quality tip designed for use with the pipette and appropriate for the size of the container.
  • **Use consistent plunger pressure and speed**—Depress and release the plunger smoothly. Pipettes are precision instruments and give more consistent results when operated with care.
RT-qPCR Instruments Available

• Corbett Research Rotorgene
  – Phenix Research, Hayward, CA
• ABI 7300 or 7500 Sequence Detection Systems
  – Applied Biosystems Foster City, CA
• BioRad iCycler iQ Real-Time Detection System
• Plus many more
ABI 7500

Thermal Cycling System:
- Peltier-based system

Block Format:
- 96-well block

Sample Ramp Rate
- Standard Mode: +/-1.6°C/sec
- 9600 Emulation Mode: +0.8 and -1.6°C/sec
- 2.5°C/sec

Peak Block Ramp Rate
- 4°C-100°C

Temperature Range
- +/-0.25°C (35°C to 95°C)
- +/-0.50°C, 30 seconds after clock start.

Temperature Accuracy
- +/-0.25°C (35°C to 95°C)

Temperature Uniformity
- +/-0.50°C, 30 seconds after clock start.

Optical System Tungsten-halogen lamp excitation source.
- Five-excitation filters, five-emission filters, CCD

7000 System Optical Schematic

- Lamp
- Excitation Filter
- Fold Mirror
- Fresnel Lens
- Well Lenses
- Dichroic Mirror
- Multi-Element Lens
- Filter Wheel
- CCD Camera
- 96-Well Plate

http://env1.gist.ac.kr/joint_unugist/file/g_class11_real_time_pcr_vt.pdf
Figure 4. Emission maximum of selected reporter dyes. The emission maximum (nm) of selected reporter dyes are displayed in parentheses. Emission maximum may vary depending on buffer conditions. Other dyes with similar wavelengths may not be suitable for multiplex assays due to low fluorescence and/or stability.
Rotogene Q

Thermal Cycling System:
- Hot Air based system
- Rotory
- Peak Block Ramp Rate: 15°C/sec
- Temperature Range: Ambient -100°C
- Temperature Accuracy: +/-0.25°C (35°C to 95°C)
- Temperature resolution: +/-0.02°C
- Optical System: Tungsten-halogen lamp excitation source.
  - Five-excitation filters, five-emission filters, CCD

3/15/2011
Qiagen Rotogene 6000
REAGENT COSTs

• 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)

Due to convenience and the presence of the internal control DNA, most labs will utilize kit based systems like Plexor HY or Quantifiler Duo.

http://www.cstl.nist.gov/biotech/strbase/DNAquant.htm
Advanced Issues with QPCR

• Multiplexing capability – currently A, Y, and IPC
  – But, as many as 5 different dyes can be simultaneously detected
    • This might permit mtDNA, and a degradation assay to be added
    • Multiple RNA markers for tissue typing could be developed
  • Melt curve analysis can be used
    – Prescreen DNA for the presence of alleles
    – Detect inhibition
    – Tissue type by detecting methylation markers
How to use Y quantification in casework

• Use it as a presumptive test for the presence of male DNA. - find and amplify the most probabtive samples

• Use it for an estimate of amplification success. If Y ratio is above 10% then Autosomal STR analysis may provide a useful result.

• Use it to estimate the amount of DNA template to get a useful Y STR result.
What does ABI say?

**Improved Workflow**

IPC $C_T$ value can indicate the presence of inhibitors; additional purification of the extract may be necessary.

If the mixture ratio of male to female DNA is high (i.e., > 1:10*), autosomal STR analysis will regularly detect both male and female components.

If an incomplete profile is obtained due to a degraded template or the presence of inhibitors, MiniFiler™ kit analysis may produce additional results.

If the mixture ratio of male to female DNA is low (i.e., < 1:10*), the male component is unlikely to be detected with autosomal STRs and analysis with the Yfiler kit would be the best choice.

Certain types of samples (e.g., very low quantity and inhibited) may be best served by direct analysis with the MiniFiler™ kit to increase the chance of obtaining a result before the sample is exhausted.
Calculation of Male to Female DNA Ratio

The Quantifiler® Duo kit provides the quantity of human and human male DNA in biological samples. From these values, one can calculate the ratio of male and female DNA using the following equation:

\[
\frac{\text{Male DNA}}{\text{Female DNA Ratio}} = \frac{\text{Male DNA}}{\text{Male DNA}} \div \frac{(\text{Human DNA} - \text{Male DNA})}{\text{Male DNA}}
\]

or

\[
\text{Male DNA:Female DNA Ratio} = 1 : (\text{Human DNA} - \text{Male DNA})/\text{Male DNA}
\]

All quantities in the above equations are ng/μL. This ratio determines the extent of the mixture, which is useful for making the choice of STR analysis method: autosomal STRs or Y-STRs.
Utilizing Quantifiler duo as an aid to evidence processing

- Duo gives the quantity of human autosomal DNA permitting proper dilution of sample prior to amplification.
- Duo permits the quantification of Y DNA, and reveals its presence.
- The internal control sequence permits the determination of the presence of certain inhibitors.
- The ratio of male to autosomal DNA permits the determination of the presence of a M/F mixture and the likelihood of success of mixture interpretation. It also indicates if a sample should be processed for Y STRs.
Examine this sample, One can conclude that an approximate 1:1 mixture exists.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Male DNA (SRY) Quantity (ng/μl)</th>
<th>Human DNA (RPPH1) Quantity (ng/μl)</th>
<th>Male:Female DNA Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male Reference</td>
<td>0.228</td>
<td>0.236</td>
<td>1:0.04</td>
</tr>
<tr>
<td>Evidence Sample</td>
<td>0.229</td>
<td>0.507</td>
<td>1:1.21</td>
</tr>
<tr>
<td>Female Reference</td>
<td>ND</td>
<td>0.217</td>
<td>0:1</td>
</tr>
</tbody>
</table>

ND = Not detected
Analysis of Male & Female DNA Mixtures (I)

Mixture Study I

<table>
<thead>
<tr>
<th>Ratio (male:female)</th>
<th>Expected Male : Female DNA ratio</th>
<th>SRY Quantity ng/µL</th>
<th>RPPH1 Quantity ng/µL</th>
<th>Measured Male : Female DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>1:0</td>
<td>0.228</td>
<td>0.236</td>
<td>1:0.04</td>
</tr>
<tr>
<td>1:1</td>
<td>1:1</td>
<td>0.229</td>
<td>0.507</td>
<td>1:1.21</td>
</tr>
<tr>
<td>1:5</td>
<td>1:5</td>
<td>0.240</td>
<td>1.410</td>
<td>1:4.88</td>
</tr>
<tr>
<td>1:10</td>
<td>1:10</td>
<td>0.280</td>
<td>3.030</td>
<td>1:9.82</td>
</tr>
<tr>
<td>1:20</td>
<td>1:20</td>
<td>0.235</td>
<td>4.070</td>
<td>1:16.32</td>
</tr>
<tr>
<td>0:1</td>
<td>Female</td>
<td>0.217</td>
<td></td>
<td>-----</td>
</tr>
</tbody>
</table>

Male DNA added at a constant concentration of 0.2 ng/µl
1:1 mixture of Male/Female DNA Notice how balance within loci varies due to peak balance and peak overlap/stutter issues.
What About Plexor?

• Plexor offers pretty much the same information
• Is male DNA there
• How much Male DNA is present
• The male/Female ratio

• But there is a concern: multicopy analysis may mean that the A/Y ratio will be a bit less accurate.

• However, accuracy does not seem to be a high point of either assay since both are based on the linearity of a semilog plot.

• Conventional wisdom: Plexor - sensitivity, Duo - accuracy

• Ultimate question in any case is how well does it amp in your laboratory!
QPCR and Inhibition

• There is a limit to how quantification reaction can detect inhibitors with the IPC
• Only 2μL DNA is added to a 25μL real time reaction
• Up to 10μL added to 25μL STR reaction (5x more)
• If extract was further concentrated to 10μL, a much higher concentration of inhibitor in STR reaction may exist
QPCR and Inhibition

• Inhibition can be detected by poor amplification of IPC
• For non taqman methods, melt curves may also be used

Effect of Phenol on Plexor melt curves
Hematin Inhibited Samples
Correlation with Identifiler® profiles

IPC can be used effectively for inhibition detection

Results with quantifiler
FIG. 4—Inhibitor study: $C_T$ values for RPPH1, SRY, and IPC targets for inhibited samples containing 0.5 ng/μL DNA and humic acid at final concentrations of 0, 1.0, 2.0, 3.0, 3.75, 7.5, 11.25, 15, and 30 ng/μL in the qPCR.
Table 5-3  Interpreting IPC amplification results

<table>
<thead>
<tr>
<th>Duo Human (VIC® dye) and/or Duo Male (FAM™ Dye)</th>
<th>Duo IPC (NED™ Dye)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No amplification</td>
<td>Amplification</td>
<td>Negative result - no human DNA detected</td>
</tr>
<tr>
<td>No amplification</td>
<td>No amplification</td>
<td>Invalid result</td>
</tr>
<tr>
<td>Amplification (low $C_T$ and high $\Delta R_n$)</td>
<td>No amplification or $C_T$ higher than 31</td>
<td>IPC result inconclusive</td>
</tr>
<tr>
<td>Amplification (high $C_T$ and low $\Delta R_n$)</td>
<td>No amplification or $C_T$ higher than 31</td>
<td>PCR inhibition</td>
</tr>
</tbody>
</table>

**Note:** Positive amplification occurs when the $C_T$ value for the detector is <40. Because samples contain unknown amounts of DNA, a large range of $C_T$ values is possible. The IPC system template DNA is added to the reaction at a fixed concentration, therefore, the NED $C_T$ should range between 28 and 31, with a variation of 1 $C_T$ across the standard curve samples.
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, dynamic range, and automation

• Main issues are quantifying effects of inhibition and degradation on the quality of results.
Thank you