An Introduction to Digital PCR
Digital PCR Characteristics

- Compared to real-time PCR, digital PCR is more
  - Sensitive in competitive scenarios
  - Precise
  - Specific
- Less affected by PCR inhibitors
  - No need to worry about PCR efficiency
- Multiplexing possible
- Absolute quantitation possible
  - Without a standard sample/curve
- Assay optimisation in some cases could be necessary
  - Follow the standard ‘Taqman® Assay Development Guidelines’
Digital PCR is an analytical technique for absolute quantitation of nucleic acid samples based on PCR amplification of single template molecules.
Analog PCR (qPCR) and Digital PCR:
Absolute Quantitation and Rare Allele Detection

Analog

Rare allele may be difficult to detect in presence of abundant wild-type; standard curve required for quantitation

Digital

Rare allele easily detected and quantified in presence of abundant wild-type, without reference to standards or controls
Digital PCR Uses An Equilibrium Measurement

- Real-time PCR relies upon cycle thresholds (Ct)
  - Ct is an arbitrary unit
- Ct is dependent on:
  - Assay PCR efficiencies
  - Baseline correct methodologies
  - Instrument calibration
- Digital PCR does not rely on cycle thresholds (Ct)
  - Reactions are categorized as positive or negative
  - Absolute quantitation of molecules
  - PCR efficiencies much less likely to influence result

Amplification Plot

Ct=13
Ct=7.8
qPCR versus Digital PCR

• qPCR: relatively easy to set up new methods
  • Quantification Cycle (Cq; also Ct) is an arbitrary unit depending on:
    • Instrument
    • Chemistry
    • Many other factors
  • Experimental standardisation is a challenge

• qPCR $\rightarrow$ Analogue, relative output
  • An ”Absolute Quantitation” compares the ”Unknown” to a ”Standard Sample” using a standard curve

• Digital PCR $\rightarrow$ Binary, digital output
Analog vs. Digital “Molecule Counting”

How many beans in the jar?

If available…

Answer: same # as in reference jar

3420

Unknown ↔ “Known”

Answer: 672 yellow, 912 red…
Key Performance Attributes of Digital PCR

**Absolute Quantification**

- **Sensitivity**: Driven by total volume interrogated
- **Specificity**: Driven by assay & replicates run
- **Precision**: Driven by total replicates run
Sensitivity Explained

Chance of actual number of targets is driven by volume sampled
Specificity Explained

Mutation detection driven by assay and number of replicates (dilution)

dPCR

Equal signal since each target is clonally amplified within its own partition

qPCR

Unequal signal - mutant can’t amplify as wild type uses up most of the reagents, thus getting lost

castPCR also alleviates this
Precision Explained

The ability to consistently obtain the same result demonstrates high precision.

Precision is driven by number of replicates run.

- Precise and accurate
- Precise but not accurate

Statistical Error

Concentration (copies / mL)

- +/- 10%
- +/- 100%

All reactions positive

~1 copy per reaction @peak

- 580 rxns ⇒ ±10%
- 190 rxns ⇒ ±20%
- 60 rxns ⇒ ±50%
Limit of detection – Poisson distribution

How often do we capture at least 1 molecule in each tube?

10µL

10µL

10µL

30 uL / 3 molecules

Answer: ~ 63%

30 uL / 9 molecules

Answer: ~ 95%
Molecule Counting Requires A “Correction” Factor

• **Problem:** Due to random assortment, we cannot be assured that each positive reaction received only a single molecule
  
  • Must have at least one negative reaction (reaction with no molecule)
  
  • Probability of a reaction receiving zero, one, two, three etc. copies is described by the **Poisson model**
  
  • Poisson statistics “corrects” for reactions containing multiple molecules and provides a “probability” that our answer is correct
Accounting for more than one target per partition

The Birthday Paradox

- How many people do we need to have in a room to have a 50% probability of two having the same birthday?

- Similarly, how many molecules are necessary to have a 50% probability of having more than one in a single reaction?
What Is A Poisson Distribution?

- **Poisson distribution** describes the probability of a given number of random events occurring in a fixed interval of time and/or space
  - Probability density function described by solely by $\lambda$
  - Discrete distribution

![Diagram of Poisson distribution](image)

**Probability density function**

- $\lambda = 1$
- $\lambda = 4$
- $\lambda = 10$

![Graph of probability density function](image)
\( \lambda \) – mean copies per partition

- Samples are diluted down to a limiting quantity, such that most individual PCR reactions contain either zero or one target molecule.

- How to find the right dilution?
  - DNA concentration and species known
    - Quantify the DNA using e.g. Spectrophotometry
    - Use this quantity and the known genomic weight to estimate the copy number.
  - DNA/RNA concentration and/or species unknown
    - Necessary to run one or several dilution series to capture the ’digital range’ of the sample.
    - Assay at least 3-4 data points above and below the digital range.
Volume and partitions

- Total volume of partitions
  - Effective reaction size per sample
  - Important for sensitivity

- Partition size
  - 755 pL

- Number of partitions
  - 20,000 per Digital PCR 20K Chip
The Math Behind The Curtain

Your digital PCR platforms calculates this for you.

Poisson Model: 
\[ p(i, \lambda) = e^{-\lambda} \frac{\lambda^i}{i!} \]

For negative reactions, this can be simplified to: 
\[ \lambda = -\ln P \]

Where \( \lambda = \) molecules per rxn and \( P = \) fraction of negative wells


Digital PCR Provides Superior Precision

20,000 Reaction Partitions
Each Measurement Has Confidence Limits

ConfidenceBound\(_{(\text{Lower, Upper})}\) = e\(^{\left[\ln(\text{Upper}) - \ln(\text{Lower})\right] \pm 1.96\sigma_{\text{total}}\} \)

<table>
<thead>
<tr>
<th>% Negative Fraction</th>
<th>0.67%</th>
<th>8%</th>
<th>37%</th>
<th>61%</th>
<th>90%</th>
<th>99%</th>
<th>99.9%</th>
<th>Total Wells</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative Wells</td>
</tr>
<tr>
<td>λ (copies / partition)</td>
<td>5</td>
<td>2.5</td>
<td>1</td>
<td>0.5</td>
<td>0.1</td>
<td>0.01</td>
<td>0.001</td>
<td>-ln(%) Negative</td>
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<tr>
<td>Final concentration (copies / μL)</td>
<td>6,622</td>
<td>3,311</td>
<td>1,324</td>
<td>662</td>
<td>132</td>
<td>13</td>
<td>1.3</td>
<td>λ Partition volume</td>
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<tr>
<td>Upper confidence</td>
<td>5.18</td>
<td>2.57</td>
<td>1.01</td>
<td>0.51</td>
<td>0.11</td>
<td>0.01</td>
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<td>95% Confidence Intervals</td>
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<td>Lower confidence</td>
<td>4.84</td>
<td>2.48</td>
<td>0.98</td>
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<td>0.10</td>
<td>0.01</td>
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“Sweet Spot” Of Digital Precision
Conclusions

Digital PCR...

• Does not necessarily replace real-time PCR but extends the capabilities of existing assays

• “Quantification” of nucleic acid molecules: absolute quantification

• Does not need a reference

• Is appropriate when sensitivity, specificity and/or precision need to be pushed beyond the capabilities of real-time PCR
Questions
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