Digital PCR Seminar

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dPCR involves performing PCR with end-point data collection in a large number of separate reaction chambers, or partitions. Results are obtained by counting the number of partitions in which the amplified target sequence is detected (regarded as positive) and the number of partitions in which there is no amplification (regarded as negative). Absolute quantification of the mean number of target sequences per partition is achieved by applying a Poisson correction to the fraction of the positive partitions. This compensates for the fact that more than one copy of template may be present in some partitions.

“Absolute quantification” used in dPCR refers to an estimate derived from the count of the proportion of positive partitions relative to the total number of partitions and their known volume.

Unlike qPCR, in which the quantification cycle (Cq) depends on variable features such as the instrument, fluorescent reporter dye, and assay efficiency, dPCR relies on a simple count of the number of successful amplification reactions. The counting of positive partitions in an ideal dPCR is definitive and does not require a calibration curve to convert Cq to copy number; knowing the partition number and volume is sufficient.
Digital PCR has many applications, including the detection and quantification of:

• low-level pathogens
• rare genetic sequences
• copy number variations associated with chromosomal rearrangements or gene/chromosomal dosage
• relative gene expression in tissues or single cells
• cDNA concentrations
• New Digital PCR Application for Allele-Specific Copy Number Analysis

dPCR was initially developed to investigate minority target measurement, for which rare variants are measured in the presence of large numbers of wild-type sequences. Detection and quantification of rare mutations can provide a useful tool in several scenarios such as the diagnosis and staging of cancer.

The ability to measure extremely low concentrations of specific DNA sequences, independent of a standard curve, with high precision, in a complex background, is unique to dPCR.
Experimental Workflow on the QuantStudio™ 3D Digital PCR System

- Instrument reads one chip at a time
- Less than a minute to read one chip
- Factory calibrated to detect FAM™, VIC® and ROX® dyes; also compatible with SYBR Green I assays (detects SYBR on the FAM channel and can detect HEX on the VIC channel). ROX is the reference dye in MM so can only singleplex or duplex.
- Intuitive touch screen operation
- Upload results to cloud for analysis
- Instrument stores results for last ~600 chips (max)
- 20,000 reaction wells per chip
- Minimal sample loss
- One sample per chip
- Simple and consistent loading
- Sealed consumable minimizes contamination
- Each chip identified by unique 2D barcode
- Fixed reaction volume minimizes upfront sample manipulation
• Total reaction volume loaded on chip is ~14.5 µl

• Reaction volume is an important determinant of sensitivity.

• Partition volume is 755 pL

• Chip surface has hydrophobic coating to enable isolation of independent reactions.

• dPCR only measures the copies that are amplifiable.

• As such there are sequence and sample specific factors to consider prior to your experiment

Factors that affect template amplification

- Sequence damage or DNA integrity
- Assay inhibition or poor sensitivity of assay (primer design)
- Molecular dropout due to linked targets or secondary structure (RE can help, multiple primer design may be necessary)
- Chemical modifications (e.g. formalin crosslinking)
- Denaturation state (single vs double stranded)
- For RNA templates, reverse transcription efficiency
Preparing DNA Samples

• DNA Quality
  • Use an optimized DNA extraction protocol, preferably ending in water
  • salting-out procedures and crude lysates are not recommended
  • Make sure DNA extracts do not contain PCR inhibitors
  • A\textsubscript{260/230} and A\textsubscript{260/280} ratios should be between 1.7 and 1.9
  • ~2.0 for RNA
  • Make sure DNA is not degraded
    • E.g. as visualized on an agarose gel

• DNA Quantity
  • Thermo recommends the following methods of quantitation:
    • nucleic acid quantitation using the Qubit® 3.0
    • Spectrophotometry (OD260) for quality ratios
  • The volume of sample added to a digital PCR reaction depends on the
    • Concentration of genomic or complementary DNA (gDNA or cDNA) present in each sample
    • Number of copies of the target sequence present in the genome or total RNA of your samples.
  • If target copy number is unknown, qPCR data can be used if available.
  • Note-assays should be tested with regular or qPCR to verify if possible.
• Goal is to dilute the samples so that each partition will contain, on average, ~0.6 to ~1.59 copies of the target sequence.

• **Example for human gDNA templates**
  • Human genomic DNA has 3.3 pg/copy of a given gene (E. coli 0.004 pg/copy)
  • Each partition is 755 pL
  • To determine an appropriate copy number per chip:

  • **Low end of range:**
    0.6 copies / 755 pL x 20,000 partitions = 12,000 copies total ->
    ~795 copies/µL or 2.62 ng/µL in the dPCR reaction mix.

  • **High end of range:** 1.59 copies / 755 pL x 20,000 partitions = 31,800 copies total needed ->
    ~2105 copies/µL or 6.94 ng/µL in the dPCR master mix.

(I don’t follow these numbers – easier to calculate total DNA needed – 31,800 copies * 3.3pg/copy = 104.9ng in
14.5µl so 104.5/29 half µls = 3.62 so add this to total needed = 104.9 + 3.62 = 108.5ng total in 15µl)

*Highest precision (meaning Poisson stats calculations) is achieved at 1.59 copies per partition*
Prepare the digital PCR reactions

**Required items**
- TaqMan® Assay(s) ($140-170/200 rxns)
- QuantStudio™ 3D Digital PCR Master Mix ($140/200 rxns)
- Pipettes and tips, P10 to P1000
- Reaction tubes
- Molecular grade water
- Microcentrifuge
- Vortex
- Gloves, marker pen, lint-free wipes
- Chips (comes with all loading consumables) - $52/12 ($884/200)
- $5-6 per sample

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume (μL)</th>
<th>Stock</th>
<th>Final</th>
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<tbody>
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<td>QuantStudio™ 3D Digital PCR Master Mix, 2X</td>
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<td>1X</td>
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<tr>
<td>TaqMan® Assay, 20X (primer/probe mix)</td>
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<td>20X</td>
<td>1X</td>
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<td>23 ng/μL*</td>
<td>2.3 ng/μL*</td>
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<tr>
<td>Water</td>
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<tr>
<td>Total volume (sample/1 chip)</td>
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<td>-</td>
<td>-</td>
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</table>

*Just an example, it will depend on the application*
QuantStudio™ 3D Workflow with Chip Loader

1. Chip & rxn mix equilibrate to room temp (~15’)
2. Place chip and lid on loader & load 14.5 μL of rxn mix
3. Add several drops of immersion fluid after reaction is loaded
4. Apply lid
5. Inject immersion fluid
6. Apply fold-back adhesive tape over fill port
7. Cycle chip on ProFlex® PCR System
8. Read chip on QuantStudio™ 3D Digital PCR Instrument

11° Front Tilt
Wait for chips to reach room temperature.
• Set the destination for the imaging data
• You may, for example, use a USB memory stick to collect the run data

• Open the chip tray and load the Digital PCR 20K Chip face-up into the bay. Confirm that the Digital PCR 20K Chip is correctly aligned within the chip tray, then close it.

Enter a prefix or regret will ensue. You have about 20 seconds.
• FAM™ and VIC® data will be shown after analysis is complete
• After reviewing the results of the run, touch Done
• Further analysis using cloud- or server-based AnalysisSuite software is recommended

• You may run the imaged Digital PCR 20K Chip again for up to 1 hour after thermal cycling.
• If you read multiple Chips in rapid succession, touch the scroll buttons to review the results of the previously imaged chips.
• The Instrument retains a copy of the analyzed data for the imaged Chip that you can access from the Run History screen

Note: The results will remain in the QuantStudio™ 3D Instrument cache for up to ~600 readings. After ~600 chips, the instrument removes the oldest data file in the cache to store each new reading
Analyze your data in AnalysisSuite in the cloud

- Alternatively, just go to Thermofisher.com and search “AnalysisSuite”
- All users must create a new login, or use a pre-existing (validated) account username and password (previous Life Technologies accounts would have been transferred to Thermofisher.com and can still be used)
- Case-sensitive
- Make sure to use Google Chrome

- Once logged in, you are taken to this page where you can look at and add to old projects or create a new one.

<table>
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<tr>
<th>Project</th>
<th># of Imported Chips</th>
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<tr>
<td>50 100 ng 2015-12-3</td>
<td>3</td>
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</table>

Either open an existing project, or at bottom of screen, can import a project/create a new one.
The software is organized into 6 "tabs", as shown below:

- **Overview** of project specifics (samples, targets, dilutions, # chips etc.)
- **Import into or remove data from Project**
- **Designate reporters used; define assay, samples, targets, dilutions**
- **View and assess data quality**
- **Chart form and graphical view of data**
- **Export data for further analysis**

If performing rare allele detection, check this box and designate which reporter represents the rare target.

By clicking on "Actions", you can import/export the 'Define Chips' table, or assign settings to >1 chips simultaneously.
Review Data Tab

• Data is also viewable in Scatter plot form (default view for rare allele experiments)

  Look for good cluster separation

  Can use lasso tool to manually select data points and adjust calls

User can adjust plot settings

FAM

FAM + VIC

No Amp

VIC

• Double-clicking chip will expand it

• Default view is to “Color by Call” (i.e. FAM +ve, VIC +ve, FAM & VIC +ve, or No Amp)

• Look for random distribution of positive partitions

• Moving cursor over chip image reveals a “magnifier”

• Clicking “Actions” reveals the ability to “Color by Quality”

# of wells that were filled with dPCR reaction

18,488

# of wells that passed the quality threshold cutoff

17,029

magnifier
As the sample becomes more concentrated, the chance of more than 1 molecule being present within a positive partition increases. However, the distribution of molecules throughout the partitions approximates a Poisson distribution and a Poisson correction is applied.

Note how precision skyrockets with few data points. Can’t be helped with rare events.

Filter can be applied to any column with a funnel icon.
Under “Show Settings”, you can change the confidence level (90, 95, or 99% - error bars grow with higher confidence desired), precision desired (wide range, 10% standard), and algorithm used (Poisson or Poisson plus).
Export creates a .csv file. All data are exported in a fixed order and format regardless of the sorts and column organization in the tables below.

- Project data as CSV
- Chip data as XML

Export data as .csv, further analysis in 3rd party graphing program
Export well fluorescence values (for advanced users)

- All data are exported in a **fixed format** that does not reflect column sorting performed in AnalysisSuite™ prior to export.
### Quantification Result

<table>
<thead>
<tr>
<th>Assay</th>
<th>Target</th>
<th>Sample</th>
<th>Ctg</th>
<th>Copy/Inclusion</th>
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<td>133.7%</td>
</tr>
</tbody>
</table>

### Assay

- **FAM**: Fluorescein Amine Maleimide
- **VIC**: VIC: 6-carboxy-4-(N,N-diethylamino)quinoline-3-sulfonic acid

### Data

- **Copy/Inclusion**: The ratio of the copied to the included sequence
- **Ctg**: Copy/Inclusion value
- **Comment**: Additional comments or notes related to the data