Assay Optimization for ddPCR

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Droplet Digital PCR (ddPCR): Absolute quantification of nucleic acids

BioRad QX200 System:
- QX200 Auto Droplet Generator
- C1000 Deep Well Thermocycler
- QX200 Droplet Reader
  - Probe-based assays
  - EvaGreen-based assays

Suggested Applications:
- Copy Number Variation
- Gene Expression and miRNA analysis
- Rare Sequence Detection
- NGS Library Quantification
- Single Cell Validation
- Genome Edit Detection
Data: the Good, the Bad, and the Ugly

Images produced with BioRad’s QuantaSoft Pro Analysis Software

Great!
Optimized Multiplexed Assays
- Distinct band of positive droplets
- Distinct band of negative droplets
- Minimal “rain”

Needs some work...
Assays are not optimized
- No clear separation of positive and negative droplets
- Positive signal is indistinguishable from negative signal

Start over 😞
No Controls
- Only one band of signal is observed
- Abundance of “rain”
- Lack of NTC and Positive Control
**Reasons for Failed Runs**

- **Samples are underloaded or overloaded**
  - Expected expression levels for target sequence and reference sequence?
  - Wide target input range 5 pg – 500 ng per sample

- **PCR isn’t working**
  - Sample quality issues - the presence of inhibitors
  - RNA projects: Sample degradation? Are we using the best RT kit?
  - DNA projects: Are we using an appropriate restriction enzyme?

- **Design flaw**
  - Assays are not compatible for multiplexing
  - Probes were made incorrectly (wrong dye, missing quencher)
  - RNA projects: Is there DNA contamination?

*Almost always a combination of these issues!*
**Required Controls**

**Controls are run with all optimization tests and sample runs**

**Positive Control** (500 ng minimum) - preferably one sample that is selected by the investigator and expresses both target and reference sequences to verify the assays are working

- Purchased Control DNA or RNA
- Sample from your lab
  - Similar origin
  - Same extraction method as sample set
  - Stored and handled the same as sample set
- gBlock or synthesized oligo construct may be needed for rare sequence detection
  - *additional positive control required for reference sequence*

**No Template Control (NTC)** - Nuclease Free Water

- Verify that there isn’t any cross-contamination
- It provides a baseline of negative signal which helps in setting the threshold in QuantaSoft Analysis software
## Sample Quality Assessment

### DNA Samples

<table>
<thead>
<tr>
<th>Purity (Nanodrop)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>• 260/280 ratio at 1.8-2.0</td>
<td></td>
</tr>
<tr>
<td>• 260/230 ratio at 2.0</td>
<td></td>
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</table>

**Perform sample clean up (Qiagen kit)?**

**Tapestation assessment is not typically done for DNA projects**

<table>
<thead>
<tr>
<th>Concentration</th>
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<tr>
<td>• If sample is clean use Nanodrop measurement</td>
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<tr>
<td>• If sample contains impurities run Qubit assay</td>
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### RNA Samples

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**Perform sample clean up (Qiagen kit)?**

<table>
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<th>Integrity (Tapestation)</th>
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<tbody>
<tr>
<td>• RIN above 6.0</td>
<td></td>
</tr>
<tr>
<td>• Presence of DNA</td>
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**Perform DNase treatment?**

<table>
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Optimizing Sample Input

- Run a dilution series of the Positive Control to determine optimal input
- Test assays separately
- Possible range of input from nanograms ($1 \times 10^{-9}$) to femtograms ($1 \times 10^{-15}$)

Input levels for wells C01 and D01 are the best options for this assay.

Robust, clean signal in every dilution step – good example of a reference assay.

No separation of positive and negative signal despite sample dilution – something else may be wrong.

Sample problem?
PCR problem?
Assay Design issue?
Optimizing Thermocycling Conditions

- Using optimal input for Positive Control, run a temperature gradient to determine most efficient annealing temperature.
- Test assays separately.
- If necessary, number of cycles and length of anneal/extend time can be manipulated to improve signal.

Well E01 is best annealing temp as shown by greatest amount of signal separation and least amount of rain. Well G01 is a NTC.

Multiplex Testing

Example of assay conditions optimized separately that work well when multiplexed. Proceed with sample processing.

Example of assays that were optimized separately but when combined are not working. Assay redesign or run assays in separate wells.
To Summarize...

ddPCR assay optimization can be a very time consuming endeavor but is crucial for the success of every ddPCR project

ddPCR optimization is best achieved by:

• Careful selection of target sequences including a reference sequence
• Careful selection and design of assays
• Choosing a positive control that best models your sample set
• Sending adequate amounts (500 ng+) of the positive control and samples so that all optimizations can be completed and samples processed without interruption
• Quality and quantity assessment of all samples and positive controls prior to testing
• Rigorous optimization testing to determine optimal sample input and best thermocycling conditions
• Inclusion of controls for every test and sample run

Please note that client samples and reagents are always stored safely and appropriately. Any remaining samples will be promptly returned to the client following the completion of the project!
For any ddPCR questions or concerns please contact the Genomics Research Core at:

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