Translating Droplet Digital PCR into Clinical Use

Christopher Campbell
West Midlands Regional Genetics Laboratory
• Introduction to digital PCR

• Applications in the West Midlands Regional Genetics Laboratory

• Future directions of the technology
Digital PCR

• Partition a sample into many individual real-time PCR reactions.

Fixed number of wells
Up to 200K, Fluidigm

Variable number/size droplets
Scalable sensitivity

• A portion of these reactions contain the target molecule while others do not
• Positive reactions are counted
• Fraction of negative reactions is used to deduce the absolute number of target molecules in the sample
**PCR**
Qualitative

**Real-time PCR**
Relative Quantification

**Droplet Digital PCR**
Absolute Quantification

<table>
<thead>
<tr>
<th>Precision</th>
<th>Poor precision</th>
<th>Precision dependent on many variables</th>
<th>Desired precision can be achieved by increasing total number of PCR replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>Low sensitivity</td>
<td>Detection is capable down to a 2-fold change</td>
<td>Linear response to the number of copies present to allow for small fold change differences to be detected</td>
</tr>
</tbody>
</table>
Key advantages of Droplet Digital PCR

• Absolute quantification means no standard curve or ΔΔCq

• Cost effective when compared with quantitative PCR

• Highly flexibility
  – High throughput with single well measurements for 96 samples
  – High sensitivity, dependent on the number of droplets
  – High precision
Two Droplet Digital PCR platforms

**QX100**

- 20K droplets/20ul PCR reaction
- 0.9nL droplets
- Biorad ddPCR Supermix
- (~£3 per reaction)

**RainDrop**

- 5-10M droplets/25ul PCR reaction
- 0.5pL droplets
- Open system
- (~£19 per reaction)
QX100 Process

- Partition reagents (multiplex/uniplex) and sample into 20,000 droplets
- Perform PCR on thermal cycler
- Count positive fluorescent and negative droplets
- Diplex on the QX100 using 2 channels (FAM and VIC)
- Digital readout provides concentration of target DNA
Quantification

- Positive droplets contain at least one copy of target DNA (or cDNA)
- Positive droplets have increased fluorescence vs. negatives
- Measure positive and negative droplets per fluorophore per sample
Current JAK2 V617F mutation analysis strategy

- Somatic JAK2 V617F mutation is found in several chronic myeloproliferative neoplasms
  - Polycythemia vera (97%), essential thrombocythemia (57%), and chronic idiopathic myelofibrosis (50%).
- ~2000 samples per year
- TAT 14 days

Current two tier strategy

1. Allele Specific Oligonucleotide Assay (~£3)
2. Quantitative PCR analysis (~£30)
Allele Specific Oligonucleotide Assay

Positive

Ratio: 0.58

Within the normal range

Cross hybridisation

Ratio: 0.01

199.58
Quantitative JAK2 PCR

• Ipsogen real time PCR JAK2 MutaQuant assay for:
  - ‘Equivocal’ results by ASO (between 1.5% and 5.0%)
  - Post transplant patients (20)
  - Monitoring for positive patients

• Sensitivity to detect JAK2 V617F down to 0.2%

• Relatively few patients; turn around times may be up to 2 months
JAK2 V617F ddPCR assay using QX100

- Assay designed by Quantalife

- Duplex assay using hydrolysis probes specific for JAK2 wild type (VIC) and JAK2 V617F mutation (FAM)
Optimisation

- Gradient PCR

- Reduce DNA viscosity by:
  - Digestion
  - Shearing
  - **Dilution**

- Simple translation of Taqman qPCR to ddPCR format
Validation

Normal controls

- JAK2 positive cut off
- Negative JAK2 (cross hybridisation)
- Negative droplets

Comparison to validated control JAK2 V617F

<table>
<thead>
<tr>
<th></th>
<th>COST EQA</th>
<th>WMRGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.3%</td>
<td></td>
</tr>
</tbody>
</table>

JAK2 V617F
Poisson statistics

- QX100 generates a maximum of 20,000 droplets
- Increased starting concentration means that some droplets may contain more than 1 copy of target
- Positive and negative reactions are counted and fit to a Poisson distribution
- Estimate the absolute copies of template molecules present in the sample volume.
Poisson statistics

Reducing the number of template molecules per well decreases sensitivity, but increases accuracy.

Patient with 40% JAK2 V617F

Final concentration template 1.25ng/ul

Final concentration template 10ng/ul
Comparison:

JAK2 ddPCR and current ASO assay

ASO systematically overestimates JAK2 V617F mutation load

Comparison:

JAK2 ddPCR and Ipsogen MutaQuant qPCR for low level JAK2 V617F mutation load

Good correlation
Routine service provision

- Quantitative JAK2 V617F service available shortly

- Mutation detection reliably below 1% using 1 well of QX100
  - 1% clinically relevant mutation load, Best Practice Guidelines *(Bench et al. 2013)*

- Increased sensitivity for low level/post transplant JAK2 V617F using Raindance platform.
  - Improve turnaround times
  - Improve sensitivity
  - Clinical relevance; measuring allele burden in post transplant MPN predicts outcome and risk of relapse. *(Lange T. et al, Haematologica 2013)*
JAK2 V617F Assay on RainDrop

<table>
<thead>
<tr>
<th>Lane</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay VIC/FAM</td>
<td>JAK2 WT/V617F</td>
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<td>JAK2 WT/V617F</td>
</tr>
<tr>
<td>Template</td>
<td>NTC</td>
<td>WT</td>
<td>Low Pos</td>
<td>High Pos</td>
</tr>
<tr>
<td>ng Input</td>
<td>0</td>
<td>280</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>ul Input</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td># Droplets</td>
<td>3084391</td>
<td>2765093</td>
<td>2248946</td>
<td>1614098</td>
</tr>
<tr>
<td># Neg</td>
<td>3084295</td>
<td>2702127</td>
<td>2244551</td>
<td>1609802</td>
</tr>
<tr>
<td># WT</td>
<td>22</td>
<td>62691</td>
<td>3967</td>
<td>2791</td>
</tr>
<tr>
<td># V617F</td>
<td>0</td>
<td>0</td>
<td>56</td>
<td>1440</td>
</tr>
<tr>
<td>% WT</td>
<td>0.001</td>
<td>2.267</td>
<td>0.176</td>
<td>0.173</td>
</tr>
<tr>
<td>% V617F</td>
<td>0.000</td>
<td>0.000</td>
<td>0.002</td>
<td>0.089</td>
</tr>
<tr>
<td>% V617F/WT</td>
<td>0.0</td>
<td>0.0</td>
<td>1.4</td>
<td>34.0</td>
</tr>
</tbody>
</table>

lower limit of detection of 1 in more than 1,000,000.
KIT $D816V$ assay

- Uniplex assay
- Majority of patients with systemic mastocytosis carry the somatic KIT gene mutation $D816V$
  - Low level in blood/bone marrow
  - Enriched CD117+ fraction
Near future applications of the technology

BCR-ABL1 gene fusion analysis
- Duplex assay trailed through Raindance Technologies
- Clinical relevance of greater sensitivity (CMR5)
- Absolute quantification may eventually negate the need for the International Scale
- Currently undergoing optimisation
- Aim to transfer further common leukaemia fusions onto this platform
Multiplexing Assays

- Single molecule endpoint PCR enables higher-plex assay

Multiplex with color **QX100**

- Target 1
- Target 2
- PCR+
- PCR -
- PCR+

Multiplex with intensity and colour **RainDrop**

- Target 1
- Target 2
- PCR+
- PCR -
- PCR+

Different intensity for different targets

FAM Intensity

VIC Intensity

Target3  Target4  Target5

PCR (-)  Target1  Target2

FAM Intensity

VIC Intensity
Development of non-invasive prenatal diagnosis at WMRGL

Conventional or real-time PCR
• Detection of Y chromosome (SRY)
• Paternally-inherited mutations (absent in maternal genome)

Sensitive droplet digital PCR (or targeted NGS deep sequencing)
Enables small fold changes in allelic balance to be detected
As above plus:-
• Measurement of Foetal fraction
• Adds Maternally-inherited mutations (e.g. DMD)

Advantages of NIPD
• Negates miscarriage risk associated with invasive diagnosis.
• Early detection
Future applications of droplet digital PCR

Minimal residual disease monitoring in leukaemia

• Currently validating a 54 gene myeloid panel at WMRGL
  – Raindance target panel enrichment
  – Sequencing using MiSeq

• Application
  – Prognostic information
  – Clinically actionable mutations
  – Identification of multiple mutation targets in each patient for minimal residual disease monitoring by droplet digital PCR
Future applications of droplet digital PCR

Detection and quantification of cell free tumour DNA (and RNA)

- Non invasive (blood, urine)
- Detection of actionable mutations without biopsy
  - e.g. KRAS and EGFR in colorectal cancer
- Quantification for patient management
  - e.g. Assessing the response to surgery or other intervention through presence/level/absence of circulating biomarkers
- Surveillance for early detection of cancer recurrence
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