Crystal digital PCR™ for genome editing and high multiplexing mutation detection

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Presentation Outline

• Crystal Digital PCR workflow for multiplexing DNA assays

• Tag copy number assessment in CRISPR-edited cell lines

• 6-color Crystal Digital PCR for cancer monitoring in clinical trials
PARTITIONING

PRINCIPLE
OF DIGITAL PCR

PCR

READING & ANALYSIS

Wild-type DNA

RESULTS
636 cp/µL with 2.2 % uncertainty

POISSON STATISTICS

\[ \frac{N_{pos}}{N_{tot}} \]
**PRINCIPLE OF CRYSTAL DIGITAL PCR™**

**PARTITIONING**

- Wild-type DNA
- GMO DNA 1
- GMO DNA 2
- miRNAs
- etc...

**Droplet crystal:** Self-assembled array of droplets

**PCR**

**READING & ANALYSIS**

- C = 102 cp/µL
- C = 152 cp/µL
- C = 8 cp/µL

2 Parameters for good quantification In dPCR:

- Number of droplets
- Size of the droplets
PERFORM CRYSTAL DIGITAL PCR™ IN 2H30 WITH MINIMUM HANDS-ON TIME

**DESCRIPTION**

- Pipette 25 µL of PCR mix into the Sapphire Chips and seal with cap
- Place Sapphire chip into the Geode and launch the combined partitioning and thermocycling program
- Image Chips using three fluorescent detection channels
- Analyze results using our intuitive Crystal Miner software

**PROCESS TIME**

- 2H30
- 5 min
- 2h10 min
- 10 min
- 5 min

**HANDS-ON TIME**

- 5 min
- 15 s
- 15 s
- 5 min
The Naica™ System Applications
Across Life Sciences & Translational Research

- Oncology Patient Monitoring
- Food & GMO Testing
- Quality Control
- Gene Editing & Epigenetics
- NiPT
- Neurobiology
- Assay Development
- Infectious Diseases
- Environmental Testing
- Hematology
Presentation Outline

- Crystal Digital PCR workflow for multiplexing DNA assays
- Tag copy number assessment in CRISPR-edited cell lines
- 6-color Crystal Digital PCR for cancer monitoring in clinical trials
AIMS of the project

Generate homozygous knock-in clones from human cell lines using CRISPR

▸ Minimize the number of clones selected for in-depth analysis
  ▸ Validate digital PCR (dPCR) with reference genomes as a high-predictive tool for genotype assessment
  ▸ Quantitatively assess the number of on-target and off-target events using dPCR
CRISPR–Cas9 machinery

- CRISPR components to perform locus–specific genome breaks

- Jinek et al., Science 2012
- Mali et al., Science 2013
- Cong et al., Science 2013
- CRISPR 101: A desktop resource, Addgene
- How To Use CRISPR: Your Guide to Successful Genome Engineering, Synthego
For knock-in generation, we exploit the HDR pathway
CRISPR Workflow

- Screening of CRISPR genome-edited knock-ins

- cdPCR was chosen to screen CRISPR edited genomes by assessing the integrated tag copy-number and comparing it with the expected number

Guo et al., Plant Meth. 2018
Gehre et al., preprint https://doi.org/10.1101/635151
Bulletin 6872, Biorad
The Validation Principle

• Southern blot (SB) vs. cdPCR: interrogating the whole genome

- Southern Blot: ~2 weeks
- Crystal Digital PCR: ~3 hours
dPCR validation – assay design

- dPCR assay to assess total SNAP-tag integrations

- Primers of the cdPCR SNAP-assay are within the SNAP tag
- Reference assay on the same chromosome ~10kbp away
dPCR Validation results

- Counting SNAP-tag copy number at TPR locus (triploid in U-2 OS cells)

- Clone 1-C10 resulted in one extra integration
- Clone 3-E07 resulted in ~1 integration – speculatively heterozygous
- Clone 2-G04 resulted in expected copy-number but major rearrangements in SB
- cdPCR SNAP copy-number mostly matches genotype predictions out of SB
dPCR Validation results

- Counting mEGFP copies at the Nup93 locus (triploid in HeLa Kyoto cells)

- Clone 214 resulted in ~2 integrations - not detectable with GFP-sbp
- mEGFP copy-number mostly matches genotype predictions out of SB
dPCR validation – HDR assay design

- Additional dPCR assay to check locus specificity of mEGFP integration

- Forward primer of the dPCR HDR-assay is moved outside of the left homology arm

- Reference assay was adapted to match the new amplicon size (~1.4 kbp)
dPCR Validation results

- Extending dPCR-based quantification of tag insertions at the target locus

Clone 214 resulted to be heterozygous as predicted by dPCR

- HDR assay adds information about the number of specific integrations
Conclusions

- dPCR robust tool to screen CRISPR clones with high predictivity of generated genotypes
- dPCR is a quantitative tool to assess CRISPR strategy efficiency
- HDR-based dPCR assays can be optimized by shortening homology arms of the donor DNA
Presentation Outline

• Crystal Digital PCR workflow for multiplexing DNA assays

• Tag copy number assessment in CRISPR-edited cell lines

• 6-color Crystal Digital PCR for cancer monitoring in clinical trials
Announcing 6-color Crystal Digital PCR™

Workflow

Sapphire Chips → Naica™ Geode → 6-Color Reader → Software
Introducing 6-color Crystal Digital PCR™

Examples of compatible fluorophores:

<table>
<thead>
<tr>
<th>Channel</th>
<th>Fluorophores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FAM</td>
</tr>
<tr>
<td>2</td>
<td>YY®</td>
</tr>
<tr>
<td>3</td>
<td>Atto 550</td>
</tr>
<tr>
<td>4</td>
<td>ROX</td>
</tr>
<tr>
<td>5</td>
<td>Cy®5</td>
</tr>
<tr>
<td>6</td>
<td>Atto 700</td>
</tr>
</tbody>
</table>

Chip Compatibility

- Sapphire chips (36 samples per day per 8h shift)
- 3 chips per run
- Time-to-result: ≤ 3 hours, for 6 channels
6-color Detection Channels: Proof of concept

6-color Lung Cancer Panel for *EGFR* mutation quantification

**Workflow**

1. **Sapphire Chips**
2. **Naica™ Geode**
3. **6-Color Reader**
4. Analysis

*This Photo* by Unknown Author is licensed under [CC BY-NC](https://creativecommons.org/licenses/by-nc/).
6-color Crystal Digital PCR™ quantifies > 90% of known EGFR mutations in a single assay

- Quantifies 19 of the most prevalent TKI sensitizing and resistance EGFR mutations
- Maximize the use of your precious sample
- Minimize time to results

Madic et Jovelet et al., 2018 Oncotarget
6-color Detection of the most prevalent sensitizing and resistance *EGFR* mutations in NSCLC

33 Tumor samples (21 Frozen, 12 FFPE)
- 24 *EGFR* sensitizing anomalies (73%)
- 13 T790M and 5 C797S resistance mutations (54%)
- 9 WT

49 cfDNA samples
- 35 *EGFR* sensitizing anomalies (71%)
- 14 T790M and 3 C797S resistance mutations (35%)
- 14 WT

Madic et Jovelet et al., 2018 Oncotarget
6-color Crystal Digital PCR™ Breast and Rectal cancer assays
Motivation

- **4 year** EU-funded LIMA project led by Philips
- **Goal:** Combine liquid biopsy monitoring & MRI scans to predict and monitor cancer therapy response

Challenge

- **100 patients per trial**
  - Breast: 10 samples per patient - 1000 samples
  - Rectal: 4 samples per patient - 400 samples
- **Reliable, rapid and cost-effective quantification** of at least 6 targets from a single blood sample

Workflow

1. **Sapphire Chips**
2. **Naica™ Geode**
3. **6-Color Reader**

**Image:** HORIZON 2020
# Clinical Trial 6-color Crystal Digital PCR™ Panels

<table>
<thead>
<tr>
<th>Channel</th>
<th>Fluorophores</th>
<th>Target</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FAM</td>
<td><strong>PIK3CA</strong> H1047R</td>
<td><strong>ERBB2</strong> (<em>HER2</em> amp.)</td>
</tr>
<tr>
<td>2</td>
<td>YY</td>
<td><strong>PIK3CA</strong> H1047 WT</td>
<td><strong>PIK3CA</strong> H1047 WT</td>
</tr>
<tr>
<td>3</td>
<td>Atto 550</td>
<td><strong>PIK3CA</strong> E542K</td>
<td><strong>MRM1</strong> (<em>Polysomy 17 Ref.</em>)</td>
</tr>
<tr>
<td>4</td>
<td>ROX</td>
<td><strong>PIK3CA</strong> E545K</td>
<td><strong>PIK3CA</strong> Mut (<strong>H1047R / E542K / E545K</strong>)</td>
</tr>
<tr>
<td>5</td>
<td>Cy®5</td>
<td><strong>PIK3CA</strong> H1047L</td>
<td><strong>TSN</strong> (<em>Amplification Ref.</em>)</td>
</tr>
<tr>
<td>6</td>
<td>Atto 700</td>
<td>PhiX (Int. Ctrl.)</td>
<td>PhiX (Int. Ctrl.)</td>
</tr>
</tbody>
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**RECTAL Cancer Panel**  
patient coverage: 10% - 30%

**BREAST Cancer Panel**  
patient coverage: 25% - 35%

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Multiplex oncology panels display high clinical utility:

- Lung (>90% of known \textit{EGFR} mutations in NSCLC)
- Breast (25–35% patient coverage)
- Rectal (10–30% patient coverage)

Maximize information output of your precious samples while minimizing time to results.
Acknowledgements

- Ellenberg Lab
  - Jan Ellenberg
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  - Beatriz Serrano Solano

- Genomics Core Facility
  - Vladimir Benes

- 6-color development team at Stillla
LEARNING CENTER: www.gene-pi.com

DIGITAL PCR
Learn, train and experiment with cutting-edge tools and methods

What do you want to learn?

LAUNCH IN MARCH 2019:

- 2 tutorials
  - Rare Mutation Detection
  - CNV
- 1 video
- 20 items
- 3 memos
- 2 online statistical tools
  - Poisson Law
  - CNV
THANK YOU FOR YOUR ATTENTION!

ANY QUESTIONS?

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For more information on product and workflow, visit our website at www.stillatechnologies.com