Sequencing and digital PCR: New local services

From advices to data analysis

Toulouse Biotechnology Institute
Bio & Chemical Engineering

www.toulouse-biotechnology-institute.fr

- Outline

- 1- GeT-Biopuces presentation
- 2- Sequencing services
- 3- Digital PCR

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1- Platform presentation

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GeT-Biopuces staff

Marie Ange TESTE

Biologist
Platform manager
IR CNRS

Lidwine TROUILH

Biologist
Sequencing
Microarrays Agilent
Quality manager
IE INSA

Nathalie MARSAUD

Biologist
Sequencing
Digital PCR
IE CDI INSA (80%)

Delphine LABOURDETTE

Bioinformatician
Data analysis,
bioinformatics
informatics manager
IE CDI INSA

Etienne RIFA

Biostatistician-Bioinformatician
Biostatistics,
bioinformatics
developments
IE INRAE (50%)

Jean-Luc PARROU

Biologist
R&D manager
qPCR and dPCR
IR CNRS

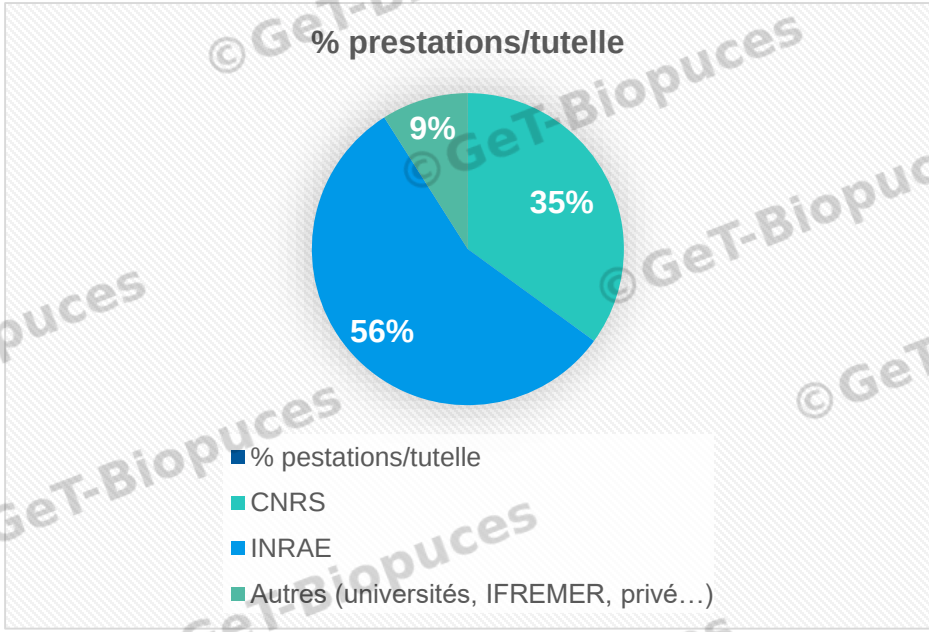
RH

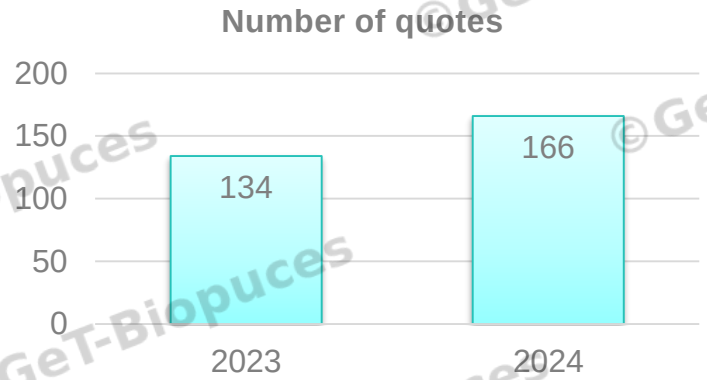
- 2 permanent contracts paid from the platform's own resources

1- Some indicators

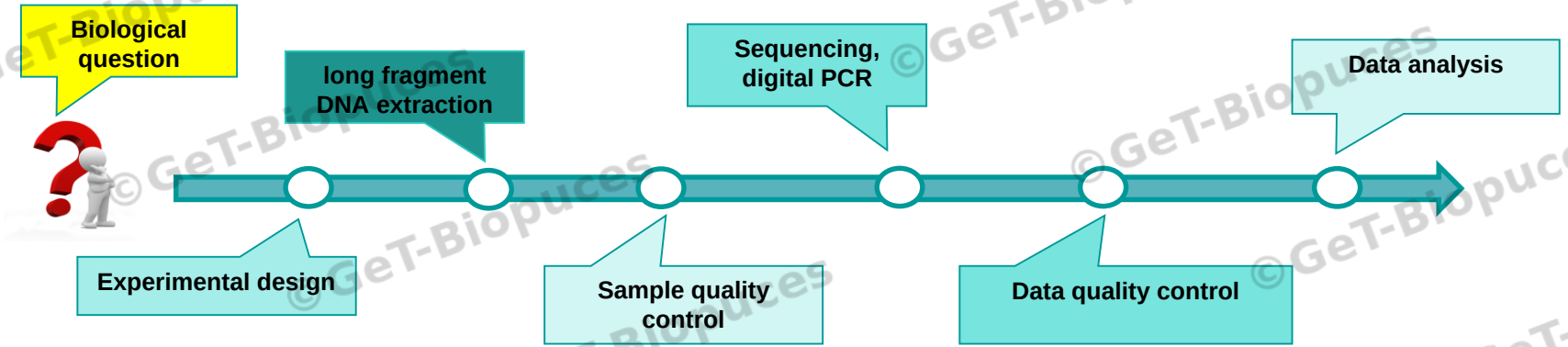
In 2024

- 92% external users / 8% internal users (TBI)
- Promotion activities: 1 poster, 7 platform visits, 7 oral presentations, 2 practical work/courses





2- Expertise and missions



- Advice
- Help with technology selection
- Discussions with the researcher throughout the experiment

Nature of activities : Prestations, collaborations , R&D

Users: Public and private laboratory, local, national and international

biopuces@insa-toulouse.fr

2- Sequencing services

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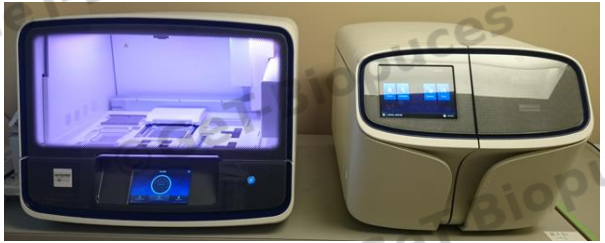
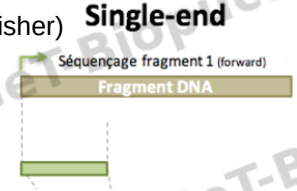
Sequencing with GeT-Biopuces_short reads

- ✓ **Strain resequencing (ADN):**
  - ✓ With a refrence genome
  - ✓ Search for mutations, small insertions/deletions (*approx*.10 bp)
  - ✓ Sequence length of about 200 bp

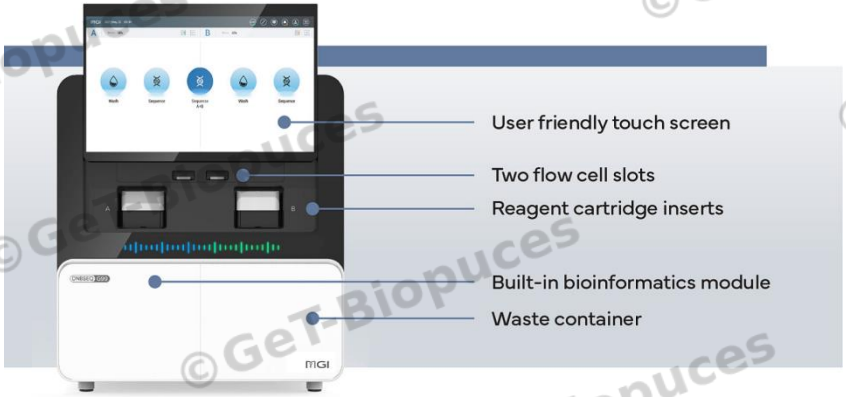


- ✓ **Transcriptomics with RNAseq:**
  - ✓ Transcriptome study
  - ✓ Search for differentially expressed genes between 2 or more conditions
  - ✓ Ribosomal RNA removal or poly-A RNA selection
  - ✓ Sequences of about 80bp

S5 sequencer and the IonChef (ThermoFisher)

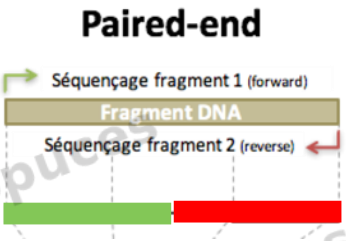


- ✓ **A new sequencer on trial :** the **G99 from MGI**
  - ✓ Prices reduction
  - ✓ Paired-end 2x150bp sequencing

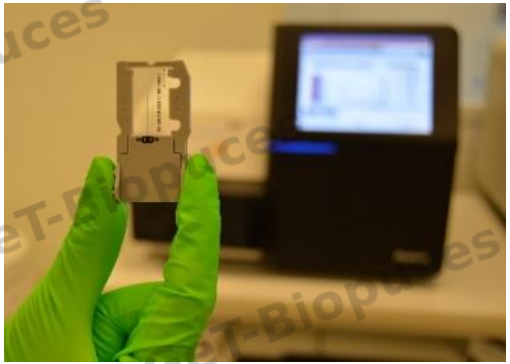


Metagenome sequencing

- ✓ **Metagenomics**
  - ✓ Structure of populations (bacteria, fungi)
  - ✓ PCR on 16S, ITS, 18S
  - ✓ Sequences of 2 X 250bp



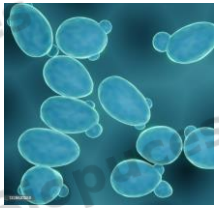
MiSeq sequencer (Illumina)



Long fragment DNA extraction and quality control

✓ **Long fragment extraction**

→ Very good quality
→ Sequencing on MinION (Oxford Nanopore)


- ✓ **Gram+ and Gram- bacteria:** 1.10^9 cells in a 2-mL tube
- ✓ **Yeast:** max 7.10^9 cells in a 50-mL tube
- ✓ **Algae:** from 7.10^7 to 2.10^8 cells in a 2-mL tube

✓ **DNA & RNA libraries**

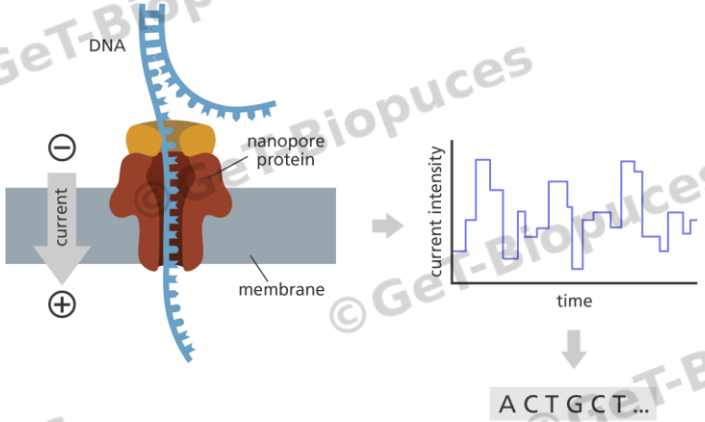
- ✓ **Nanodrop** ND2000: absorbance, single tubes (free access after training)
- ✓ **Spectophotometer** spectrostar (BMG Labtech): absorbance, 96 plate
- ✓ **Qubit Fluorometer** (ThermoFisher): dye-based, single strand (RNA) or double strand (DNA)
- ✓ **Bioanalyzer 2100** (Agilent): assessment of degradation level and quality control of libraries


Long fragment sequencing : genomes

✓ ***De novo* sequencing (DNA)**

- ✓ Sequences length > 1 000bp
- ✓ No reference genome
- ✓ Search for long insertions/deletions
- ✓ Ideal for repeated sequences

MinION sequencer
(Oxford Nanopore)



https://www.yourgenome.org/facts/what-is-oxford-nanopore-technology-ont-sequencing/

Short- *versus* Long-read sequencing :

Exemple of *E.Coli* genome : 4,6 Mb

→ **Short read** seq. : 92 000 fragments of 50 bp
→ **Long read** seq. : 460 fragments of 10 kb …

Genome assembly : faster and better

~ 50-base reads
→ 92,000 "pieces"

~ 10-kb reads
→ 460 "pieces"

Long fragment sequencing : plasmids and fosmids

✓ **Whole plasmids and fosmids sequencing:**

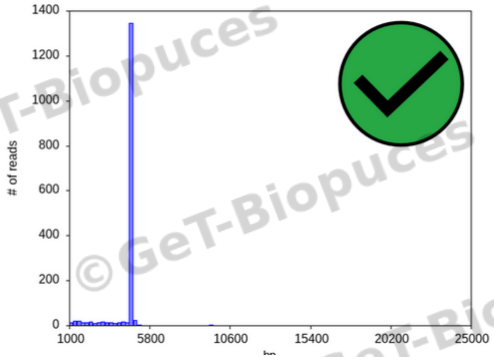
- ✓ No primer design as for Sanger sequencing
- ✓ Whole sequence = insert + backbone
- ✓ Accuracy > 99,99%

→ **New quality control**

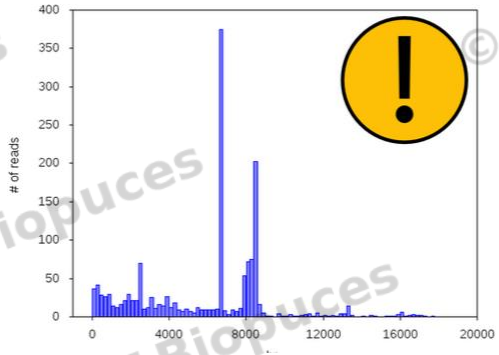
According to a study published in june, 2024
(https://doi.org/10.1101/2024.06.17.596931)

- ✓ Out of 2 500 plasmids studied, about half of them contained errors !!

→ **Sequencing becomes essential**



A histogram with **one dominant peak** typically indicates a clean prep with a single plasmid



A histogram with multiple peaks indicates unexpected products, deletions, recombinations, or <u>concatemers</u>

Data analysis

DNA :

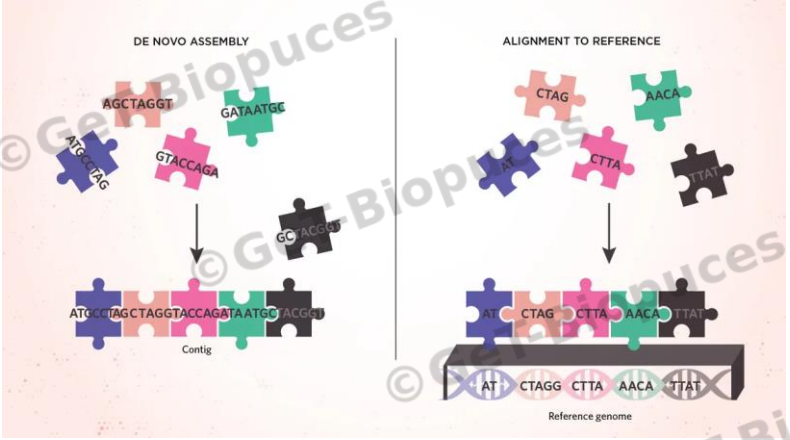
- ✓ Alignment with the reference genome
- ✓ Assembly of new genomes/plasmids
- ✓ Search for mutations
- ✓ Search for insertions/deletions

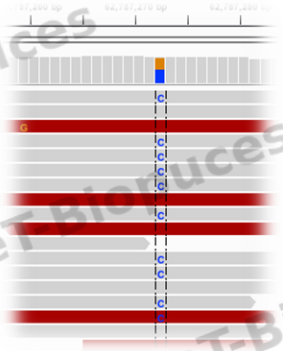
RNA:

- ✓ List of differentially expressed genes
- ✓ Identification of metabolic pathways
- ✓ Single cell RNAseq
- ✓ Spatial transcriptomics

Metagenomics:

- ✓ Identification of microorgamisms families





Few questions on sequencing ?

Digital PCR

Toulouse Biotechnology Institute
Bio & Chemical Engineering

The ‘Digital PCR’ : ready to meet new challenges !

Are there any ?
How many ?! … 16 ?

***Digital* PCR :**
A way to explore fields beyond the limits of qPCR

The ‘Digital PCR’ : ready to meet new challenges !

Outline :

Brief overview of Digital PCR *vs* standard qPCR
The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN
Why so sensitive ?
The mathematics below the absolute counting
Few applications

‘ *Digital* ’ PCR *versus* ‘conventionnal’ and qPCR

Comparison of PCR techniques at a glance

1st generation

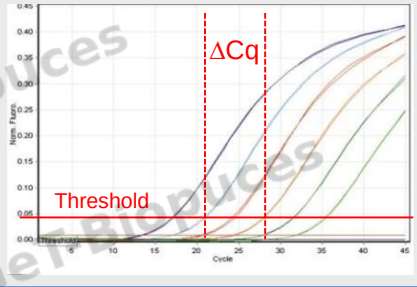
Conventional PCR

Qualitative

- Technically simple
- Low cost
- **End point** detection

2nd generation

Quantitative PCR (qPCR)

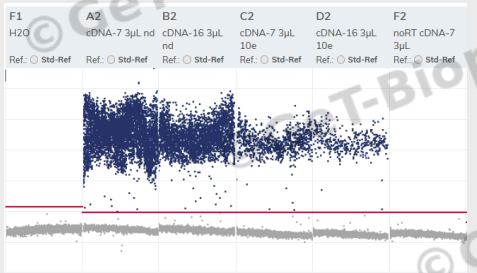


Quantification

- Most often **relative** (**ΔCq**)
- **Wide dynamic range**
- Precision, sensitivity, specificity
- Throughput
- **Real-time** detection

3rd generation

Digital PCR (dPCR)



Absolute Quantification

- No standard curves
- Much **higher precision**
- Better sensitivity
- Low sensitivity to **inhibitors**
- **End point** detection **!**

‘Digital’ PCR **principle :** The nano-scale **partitionning**

Based on microfluidic systems

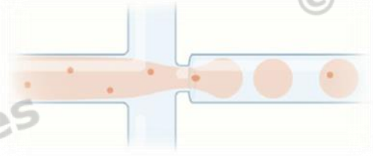
e.g. 20-µL prereaction mix

>> >> >> >> >> >> >> >>

➢ **Droplets …**

e.g. the **ddPCR*** from Bio-Rad

(* trademark name)



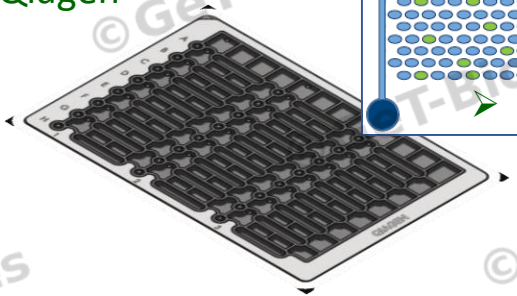
10 to 20 k droplets / sample

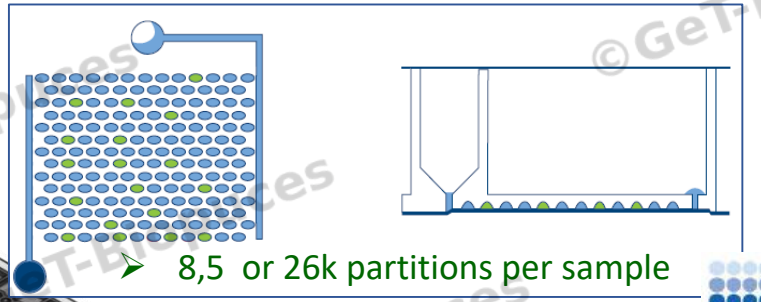
Approx . 20,000 nL-scale elementary reactions .

➢ **Solid, printed systems…**

e.g. the **nanoplates** from Qiagen

➢ From 8 to 96 samples





➢ 8,5 or 26k partitions per sample

The ‘Digital PCR’ : ready to meet new challenges !

Outline :

Brief overview of Digital PCR *vs* standard qPCR

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

Why so sensitive ?

The mathematics below the absolute counting

Few applications

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

You …

Design ! → Prepare the prereaction mix → Load sample and seal nanoplate → Load the nanoplate → *Wait …* → Analyze !

… and the Qiacuity ! → Partitionning → Isolation → Thermocycling → Fluorescence reading

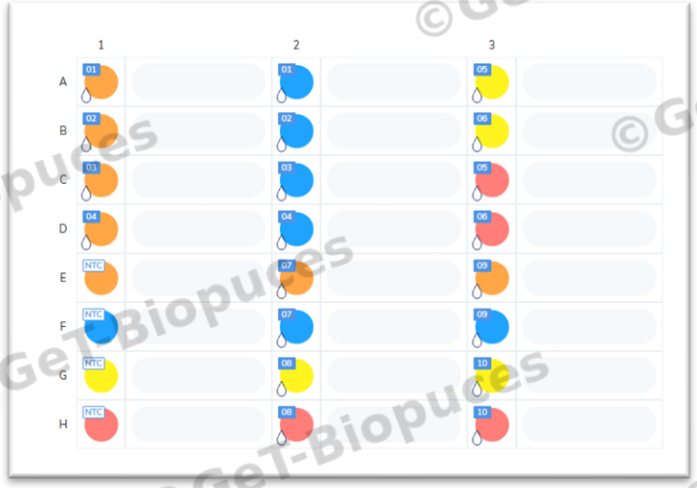
The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

You …

Design ! → Prepare the prereaction mix → Load sample and seal nanoplate → Load the nanoplate → *Wait …* → Analyze !

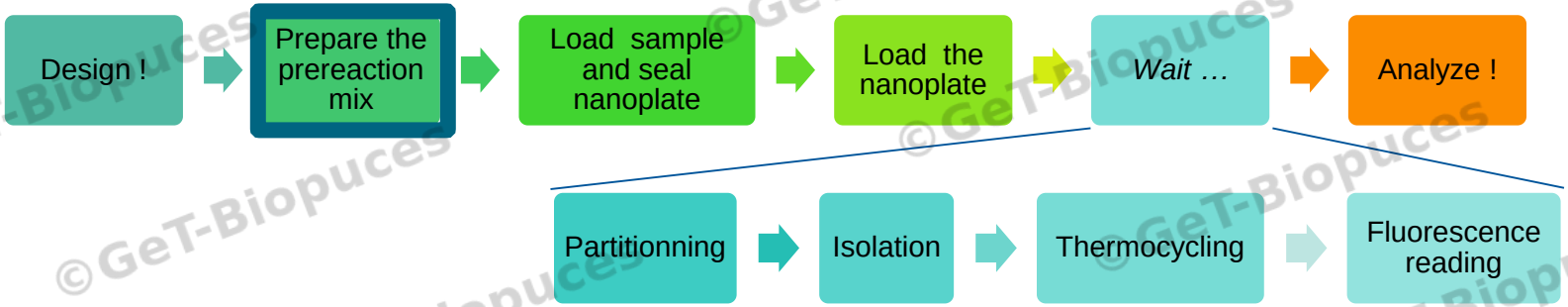
Wait … → Partitionning → Isolation → Thermocycling → Fluorescence reading

Keeping in mind the **(d)MIQE** guidelines, from exp. design to dPCR setup and analysis



- Chose the best Nanoplate — 8 / 24 *samples* / 96 x 8,5 k / 26 k *partitions*
- Simplex reactions, multiplex if possible
- Proper dilutions
- +/- digestion of DNA
- Controls, postive & negative, blanks
- …/…

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN



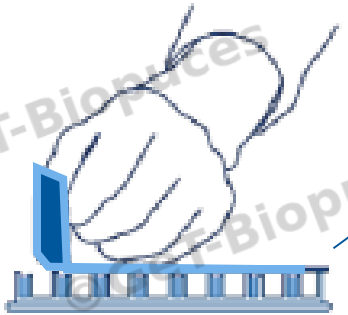
- The commercial Enz mix
  - Intercalating dye
  - probe)
- Your assay(s)
  - primers +/- probes,
  - up to 5 per sample
- Your sample

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

Design ! → Prepare the prereaction mix → Load samples and seal nanoplate → Load the nanoplate → *Wait …* → Analyze !

Wait … → Partitionning → Isolation → Thermocycling → Fluorescence reading

Load … and seal



Rubber seal
applied to the top of the plate

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

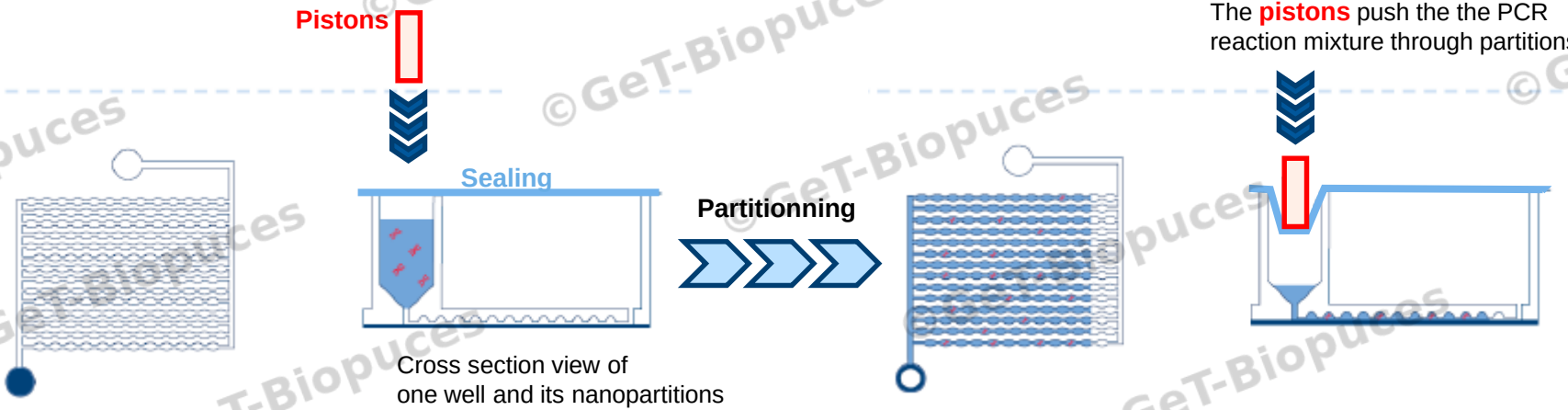
Design ! → Prepare the prereaction mix → Load sample and seal nanoplate → Load the nanoplate → *Wait …* → Analyze !

Wait … → Partitionning → Isolation → Thermocycling → Fluorescence reading

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

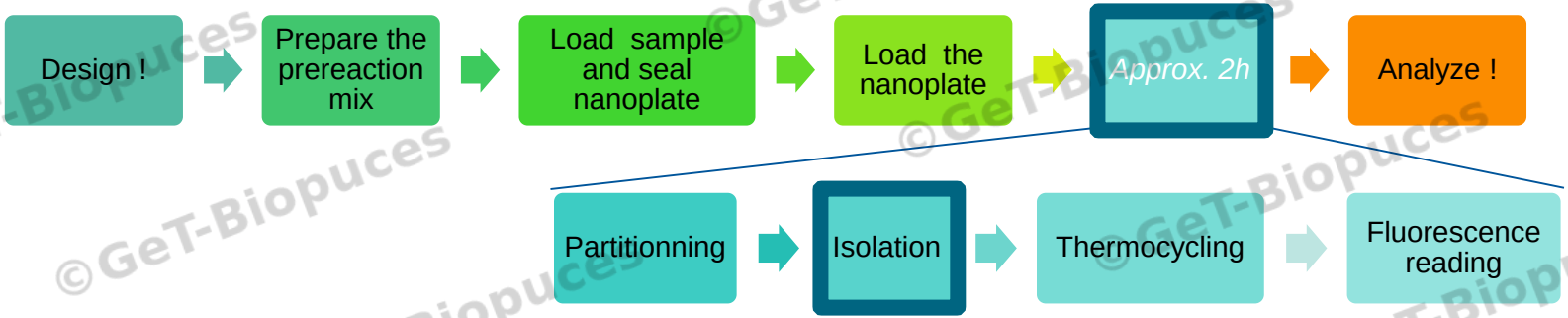
Design ! → Prepare the prereaction mix → Load sample and seal nanoplate → Load the nanoplate → *Approx. 2h* → Analyze !

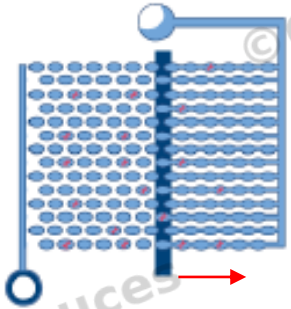
Approx. 2h → Partitionning → Isolation → Thermocycling → Fluorescence reading

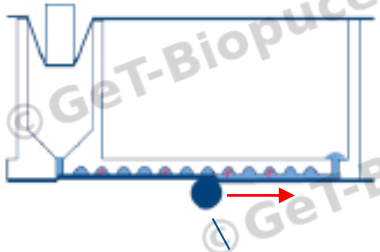


Cross section view of one well and its nanopartitions

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN





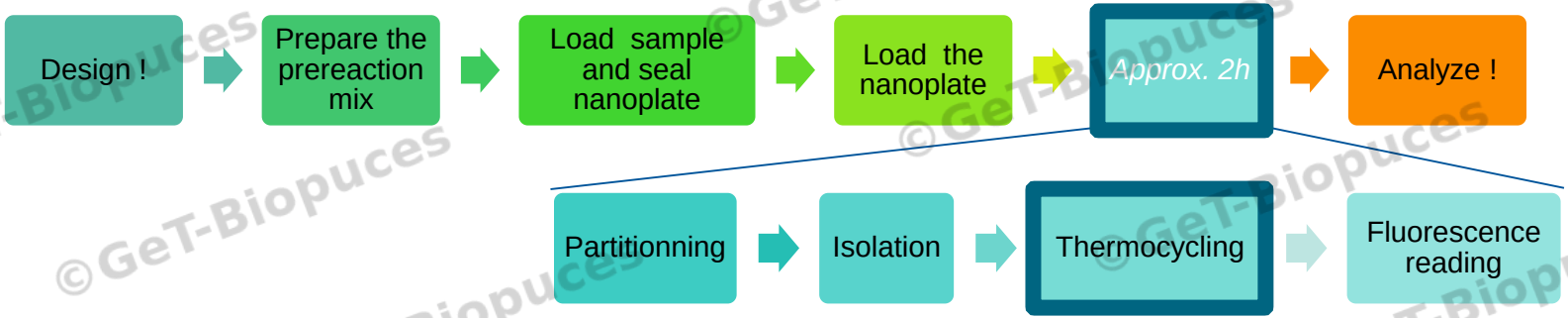


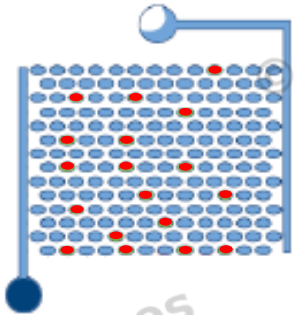
Partition volume :

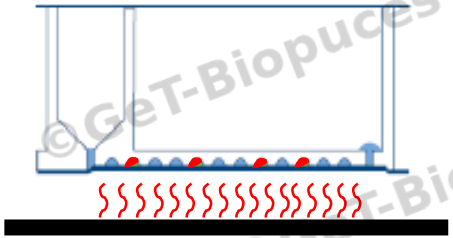
- ✓ **820** pL for **26 k** nanoplates
- ✓ **340** pL for **8,5 k**

The **roller** compreses the bottom seal and **seals partitions individually**

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN



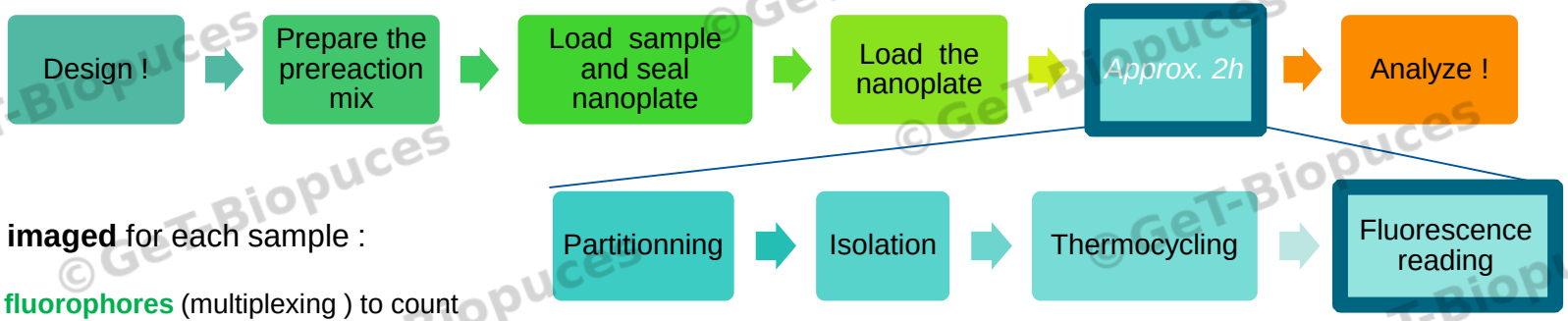




During **thermocycling**,

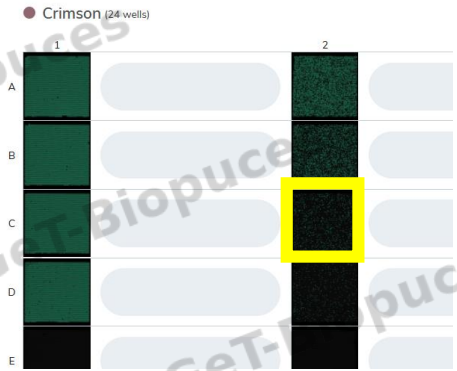
the **target DNA accumulates** in the partitions

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN



The plate is **imaged** for each sample :

- **Up to five fluorophores** (multiplexing) to count the number of **positive**, fluorescent partitions
- **Reference dye** to control the loading >> Total number, **valid** partitions



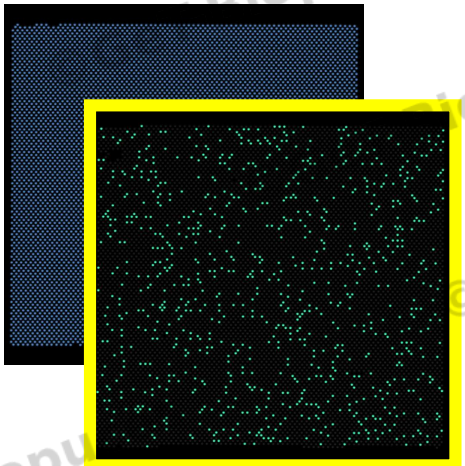
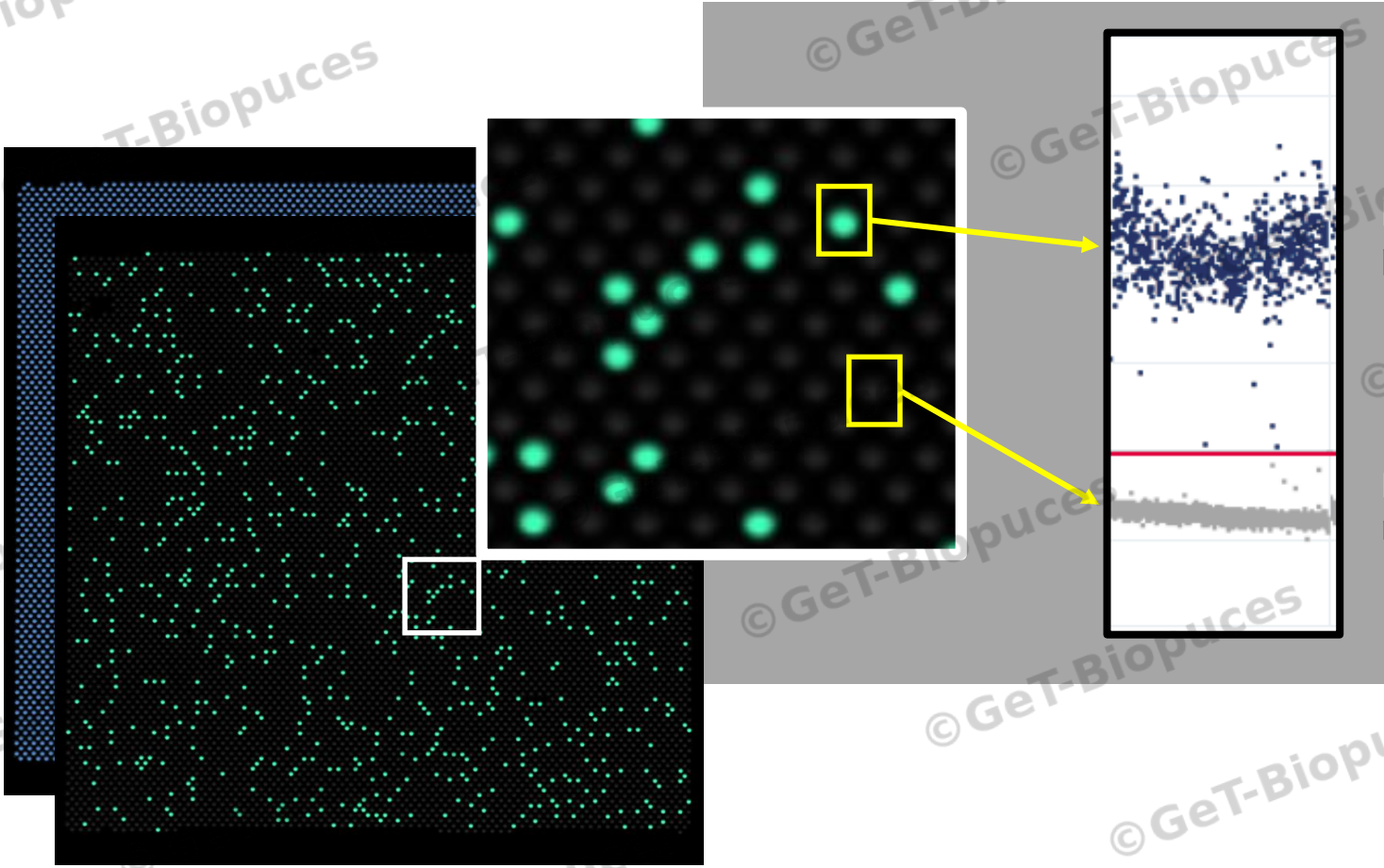


Table 2. Available channels in QIAcuity

Channel	Excitation (nm)	Emission (nm)	Example fluorophores
Green	463–503	519–549	FAM™, EvaGreen®, Atto 488, Alexa Fluor® 488
Yellow	513–534	551–565	HEX™, VIC®
Orange	541–563	582–608	TAMRA™, Atto 550
Red	568–594	613–655	ROX™, Texas Red®
Crimson	588–638	656–694	Cy5®, Quasar 680
Far red	651-690	709-759	Cy5.5. Atto 680
Green / Yellow	463–503	551–565	DY-482XL (LSS G/Y)*
Orange / Red	541–563	613–655	DY-540XL (LSS O/R)*

* For Long Stokes Shift (LSS) dyes, the software provides generic dye names called “LSS” followed by the abbreviation of the used channel combination denoted by the first channel letters. For example, channel combination Green/Yellow is abbreviated as “LSS G/Y”.

That is the ‘ *Digital* ’ PCR : the 0 / 1 analysis of the signal



High-fluorescence,
positive partitions

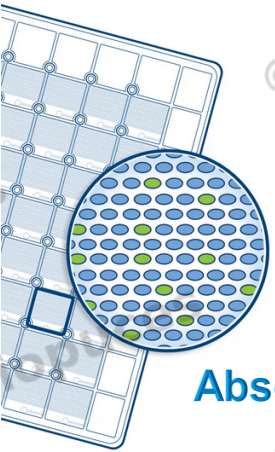
versus

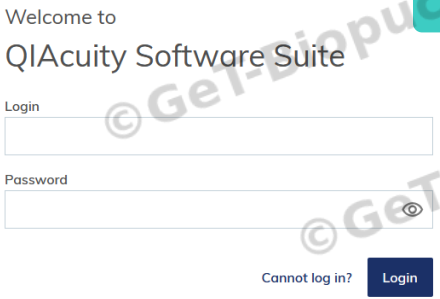
Low-fluorescence,
negative partitions

The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

Design ! → Prepare the prereaction mix → Load sample and seal nanoplate → Load the nanoplate → *Wait for approx. 2 hours* → Analyze !

Partitionning → Isolation → Thermocycling → Fluorescence reading





Absolute counting

Copy Number Variation

Gene expression

Etc .

- Controls
- Threshold positionning
- Bleed through analysis
  (2-D scatterplots and Custom Cross Talk Matrix (CxTM))
- Counting results tables
- Use of dedicated tools and graphs (1-D, 2-D , heatmaps ..)

- Archive and reports
  (DDES, **D**igital PCR **D**ata **E**ssential **S**preadsheet format)

The ‘Digital PCR’ : ready to meet new challenges !

Outline :

Brief overview of Digital PCR *vs* standard qPCR

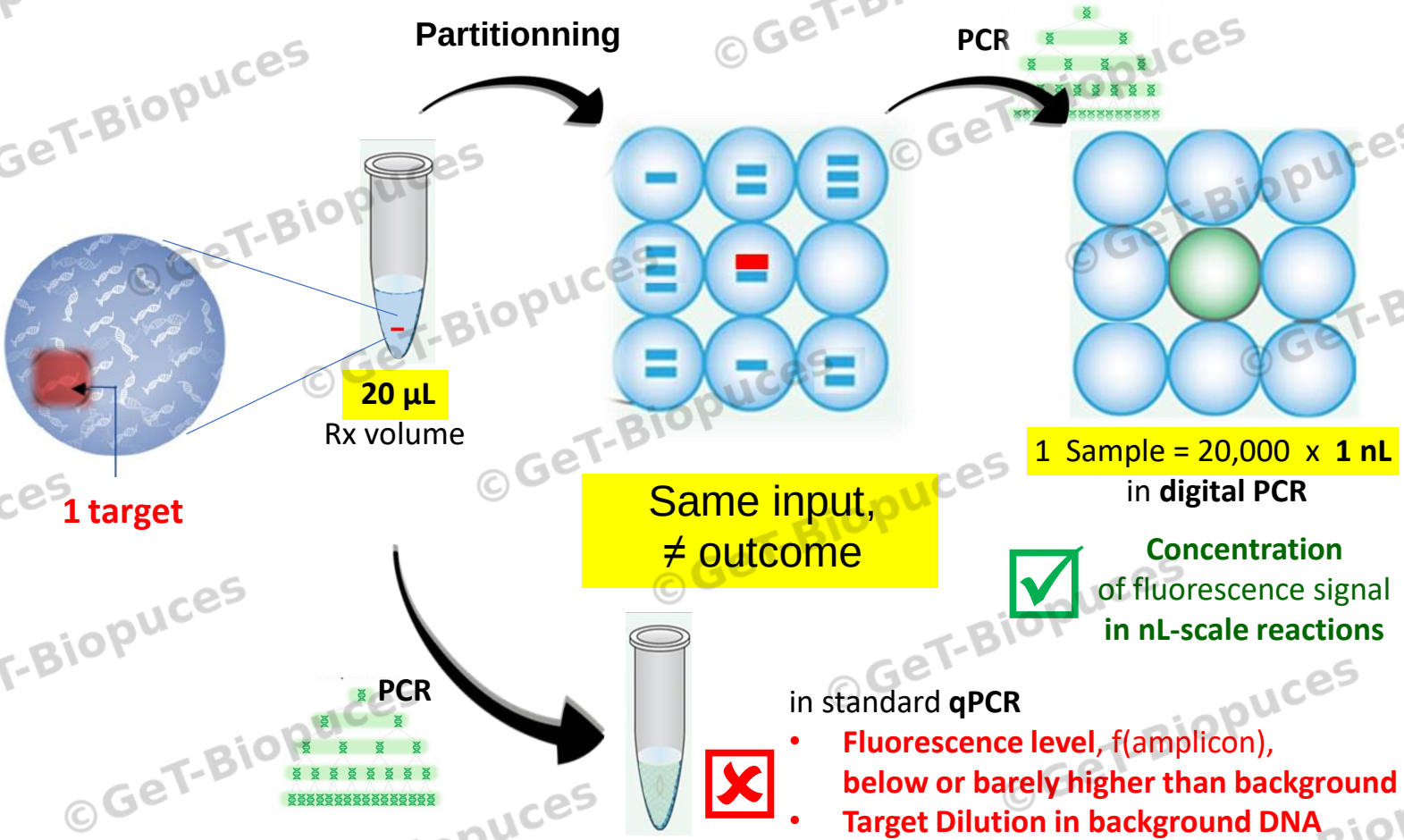
The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN

Why so sensitive ?

The mathematics below the absolute counting

Few applications

Why the digital PCR so sensitive ?



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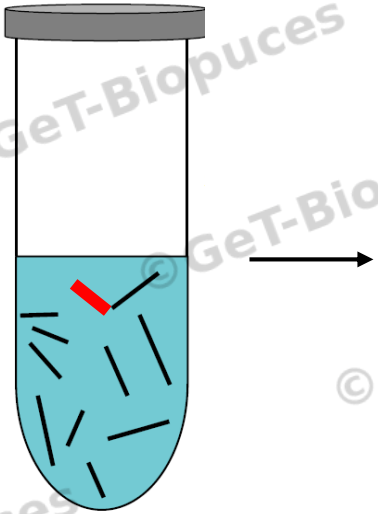
The basics of counting in digital PCR

qPCR reaction

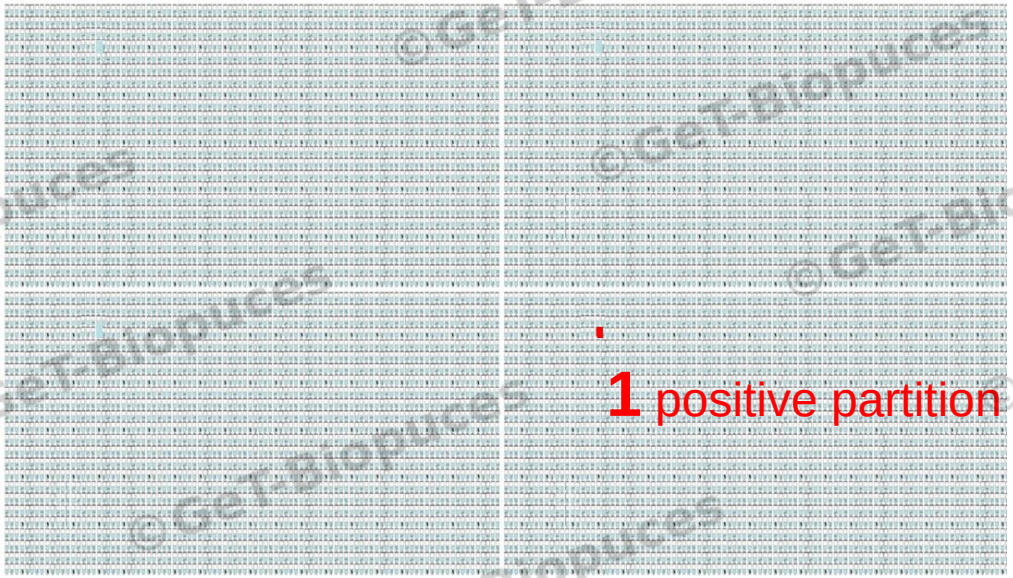
1 x 20 µl



1 target

dPCR

20,000 x 1-nl reactions



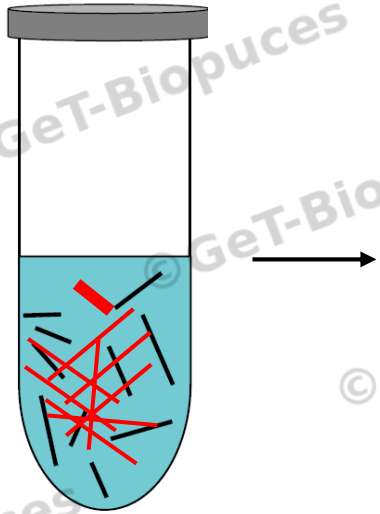
1 positive partition

Theoritically, in **dPCR,** **we can detect (count) 1 single target** from the reaction mix

The basics of counting in digital PCR

qPCR reaction

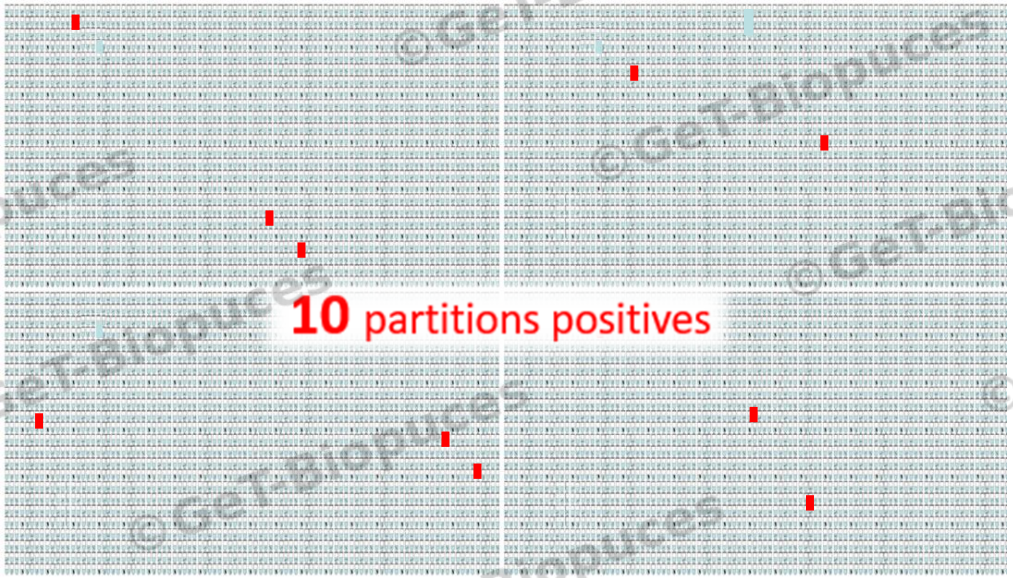
1 x 20 µl



10 targets

dPCR

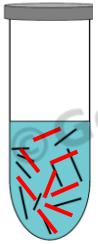
20,000 x 1-nl reactions



And so on and so forth … 100 targets >> 100 positive

…. **WRONG** !

The basics of counting in digital PCR



X targets

[?]

- Random distribution of targets during the partitionning
- **The higher the concentration of targets , the higher the probability to get more than one target per partition.**

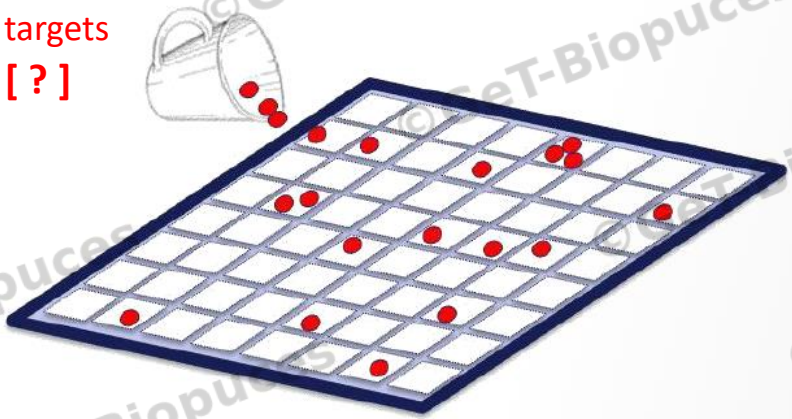
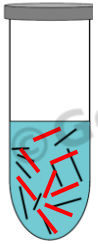


Image credit : Thermofisher

The basics of counting in digital PCR



X targets

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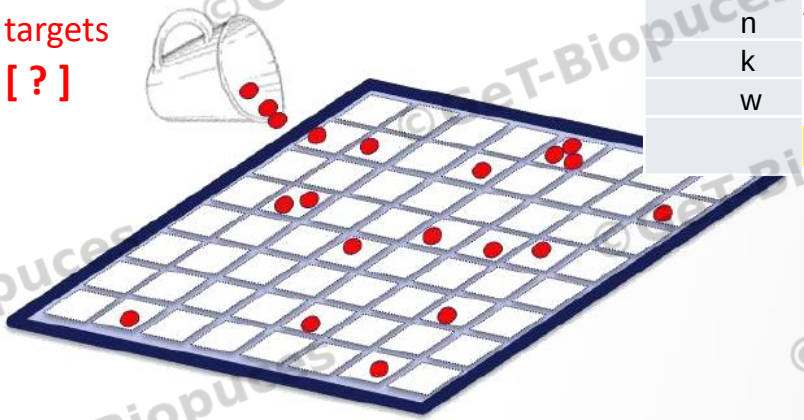
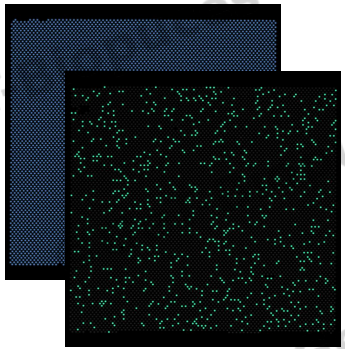
Observed / counted

n	Total, valid partitions	
k	Positives	
w	Négatives	w = n - k

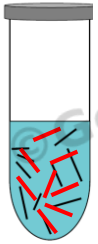
Known partition volume

- ✓ **820** pL for **26 k** nanoplates
- ✓ **340** pL for **8,5 k**





The basics of counting in digital PCR



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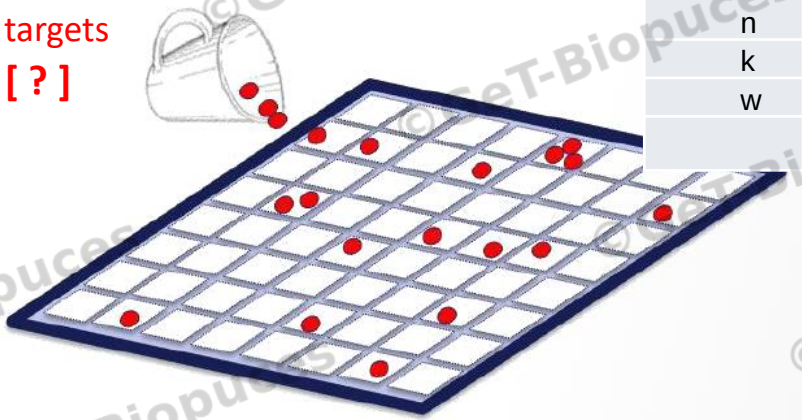


λ, average number of targets per partition :

$$\lambda = -\ln\left(1 - k/n\right)$$

Copies / µL of Rx mix

Estimation of [targets] @ 95 % CI

Siméon Denis Poisson

(1781-1840)

Poisson' law

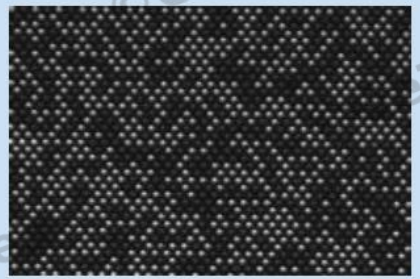
The basics of counting in digital PCR

No target

All partitions are negative

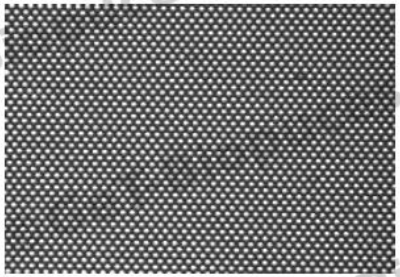
Lower end

Positive **and** negative partitions



Higher end

Positive **and** negative partitions



Over saturated

All partitions are positive

Target copies per partition = -ln(1-p)

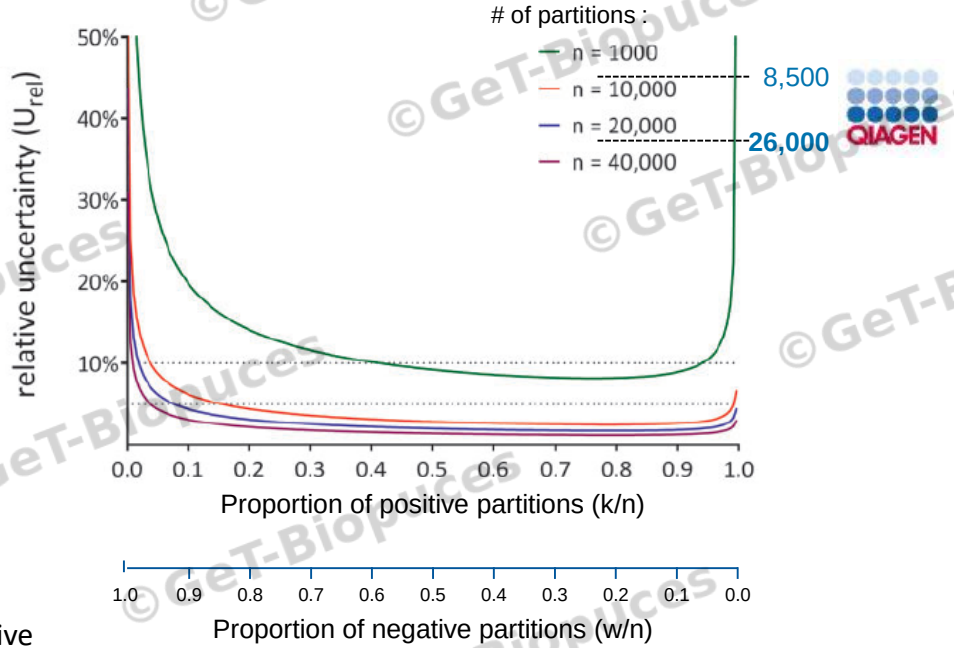
p = fraction of positive partitions

Copies of DNA target/microliter

Poisson' law

The Incertitude in digital PCR (CI @ 95%)

- The higher the number of partitions, the higher the confidence
  - Possibility to fuse wells (‘metawells’)
- Strong degradation of CI (95%) when decreasing partition number
  - Two types of QIAGEN’ nanoplates : 8,500 and 26,000
- The lower the lambda, (or positive partitions number), the higher the error
  → subsampling error ↗



26k nanochip from Qiagen

> *approx.* 200,000 targets analyzed ↔ *approx.* 10 negative

The ‘Digital PCR’ : Applications

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Brief overview of Digital PCR *vs* standard qPCR
The ‘Digital’ PCR workflow with the QIACUITY from QIAGEN
Why so sensitive ?
The mathematics below the absolute counting

Some successful applications on GeT-Biopuces :

- **Copy Number Variation (**alguae and yeast genomes**),**
- **Trace DNA in wastewater** (ARGs, antibiotic resistance genes)
- **(single-cell) gene expression** (human, bacteria)
- **Detection of Bacterial contamination**
- **…**

You are ready to meet new challenges ? Come and discuss with us !

Thank you for your attention !

Questions?

Toulouse Biotechnology Institute
Bio & Chemical Engineering

www.toulouse-biotechnology-institute.fr