Implementation of Digital PCR in a Molecular Diagnostic Laboratory: Evaluation of Minimal Residual Disease and metastatic cancer progression

Benjamin Tournier, PhD
Plateform of Somatic Oncology
INSERM LNC-UMR 1231
University Hospital of Dijon, France
The liquid biopsy in oncology

1. Setting up of the liquid biopsy process at Dijon Hospital
Setting up of the liquid biopsy process at Dijon Hospital

- Global activity of the platform >2000 somatic molecular analyses in solid tumours / year
- Liquid biopsy process set up in 2016
- For metastatic melanoma and lung cancer patients

Number of liquid biopsies performed in 2017

- Melanoma: 160
- Lung Cancer: 124

Diagnosis:
- Melanoma: 72
- Lung Cancer: 15

Follow-up:
- Melanoma: 5
- Lung Cancer: 109

Progression:
- Melanoma: 79
- Lung Cancer: 4

Relapse:
- Melanoma: 4
- Lung Cancer: 0
Setting up of the liquid biopsy process at Dijon Hospital

The liquid biopsy workflow: The blood sampling

- K2 EDTA tubes (BD Vaccutainer®)
- « Cell-free DNA collection tube » (Roche®)

Used for local blood sampling

Used for external blood sampling

- Cell-Free DNA BCT® (Streck)
- PAXgene® Blood DNA Tube (PreAnalitiX, QIAGEN/BD)
- LBgard® Blood Tubes (Biomatrica)
- cf-DNA/cf-RNA Preservative Tubes (NORGEN Biotek corp.)

Setting up of the liquid biopsy process at Dijon Hospital

The liquid biopsy workflow: The pre-analytical steps

- up to 4 hours for K2 EDTA tubes
- up to 7 days for Cell-free DNA collection tubes

1500g 10 min.

-80°C → + 4°C 1 night

RT 1 hour

16 000g 6 min.

cfDNA extraction + Molecular analyses + Storage -20°C
The liquid biopsy workflow: The cfDNA extraction method

- Automated Extraction: QIAsymphony, DSP Circulating DNA Kit
  
  4 mL plasma
  60 µL elution

- Manual Extraction: Macherey-Nagel, NucleoSnap® DNA plasma
  
  Up to 5 mL plasma
  45 µL elution
Setting up of the liquid biopsy process at Dijon Hospital

The liquid biopsy workflow: The cfDNA quantification/qualification

- Qubit ds DNA high Sensivity
- TapeStation 4200, D1000 High Sensivity
Conclusion of critical parameters:

- Plasma minimal volume of 4 mL
- Transport time
- Double centrifugation of plasma (even 3 centrifugation)
- Gentle thawing
1. Setting up of the liquid biopsy process at Dijon Hospital

2. NAICA digital PCR (Stilla Technologies®)
NAICA digital PCR (Stilla Technologies®)

Principle and Workflow

The “Sapphire” chip

Oil loading

Loading of PCR reagents + 14µL of cfDNA

Zoom of a Sapphire chamber during repartition of the reaction mix
The Naica dPCR system: 3 instruments

- Air pump instrument
- Naica Geode:
  - Droplet generator
  - Thermocycler
- Naica Prism3:
  - Fluorescence scanner
The “Sapphire” chip: Generation of droplets & Size calibration
NAICA digital PCR (Stilla Technologies®) Demonstration

Visualization:

BLUE channel (495-515nm)
NAICA digital PCR (Stilla Technologies®) Demonstration

1D graphical representation:

Total number of droplets: 26,363

Number of FAM positive droplets: 5,948

BLUE channel (495-515nm)
NAICA digital PCR (Stilla Technologies®) Demonstration

Visualization:

GREEN channel (560-610nm)

RED channel (655-720nm)
NAICA digital PCR (Stilla Technologies®) Demonstration

1D graphical representation:

GREEN channel (560-610nm)

**Before correction**
7 VIC positive droplets…

… which are actually artefacts

**After correction**
No VIC positive droplets
NAICA digital PCR (Stilla Technologies®) Demonstration

1D graphical representation:

Nature of samples influences the fluorescence intensities…

**Adaptation of fluorescence threshold**
1. Setting up of the liquid biopsy process at Dijon Hospital

2. NAICA digital PCR (Stilla Technologies®)

3. Example of applications
   3.1 \textit{BRAF} testing in Melanoma
Molecular test set up for Melanoma
Testing of *BRAF* V600K/E mutations

Adapted from Punnoose et al., *Clin Cancer Res*, 2012

**Design:**

*BRAF* exon 15

- **Forward primer location:**
- **Reverse probe location:**

**Wild-type probe**
c.1798_1799delinsAA probe p.(V600K)
c.1799T>A probe p.(V600E)

**Amplicon length:** 117bp
Molecular test set up for Melanoma
Testing of BRAF V600K/E mutations

Validation:
1. classical PCR optimization
2. qPCR testing: evaluation of the PCR efficiency
3. Digital PCR testing: evaluation of the noise and determination of the limit of detection (LOD)

Analysis of 25 BRAF wild-type cell-free DNAs:

- **GREEN channel (560-610nm):** 3VIC positive droplets LOD = 6 droplets
- **BLUE channel (495-515nm):** 2Cy5 positive droplets LOD = 6 droplets
- **RED channel (655-720nm):**

Calculations:
1. Corrected mean number of wrong positive droplets ($\mu$ corr.)
2. Limit of blank (LOB)
3. Limit of detection (LOD)
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the BRAF p.(V600E) mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the **BRAF p.(V600E)** mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)

![Graph showing treatment response and DNA concentration over time](image)

**cfDNA concentration (cp/mL)**

**V600E DNA concentration (cp/mL)**
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the **BRAF p.(V600E)** mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the BRAF p.(V600E) mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the **BRAF p.(V600E)** mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)
Example for the MRD checking in a melanoma patient

- Forty-seven year old man diagnosed for an invasive SSM melanoma in 2008
- Detection of the **BRAF p.(V600E)** mutation in a tissue biopsy at diagnosis
- Treatment: Anti-BRAF (TAFINLAR) during 10 months, then radiotherapy, then immunotherapy (NIVOLUMAB)
1. Setting up of the liquid biopsy process at Dijon Hospital

2. NAICA digital PCR (Stilla Technologies®)

3. Example of applications

   3.1 *BRAF* testing in Melanoma

   3.2 *EGFR* testing in NSCLC
Molecular test set up in Lung Cancer
Testing of EGFR deletions of exon 19 + L858R mutation

Design:

**EGFR exon 21**

- Forward primer location
- Reverse primer location
- c.2573T>G probe (p.(Leu858Arg))
- FAM BHQ1
- Cy5 BHQ2

**EGFR exon 19**

- Forward primer location
- Deletion hot-spot probe location (fwd)
- Reference probe location (rvs)
- HEX BHQ1
- FAM BHQ1

Adapted from Oxnard et al., Clin Cancer Res, 2014 and Punnoose et al., Clin Cancer Res, 2012
Molecular test set up in Lung Cancer
Testing of *EGFR* deletions of exon 19 + L858R mutation

1D graphical representation:

Sample: cell-free DNA from patient with *EGFR* Del19 lung ADK

BLUE channel (495-515nm)

GREEN channel (560-610nm)
Molecular test set up in Lung Cancer
Testing of \textit{EGFR} deletions of exon 19 + L858R mutation

2D graphical representation:

Sample: cell-free DNA from patient with \textit{EGFR} Del19 lung ADK

![Graphical Representation](image)

48 FAM+/VIC- droplets → 5,37 mutated copies/µL
Molecular test set up in Lung Cancer
Testing of $EGFR$ deletions of exon 19 + L858R mutation

2D graphical representation:

Sample: Human genomic DNA, $EGFR$ wild-type

5 FAM+/VIC- droplets < to LOB → Negative
Molecular test set up in Lung Cancer
Testing of EGFR L858R/L861Q/T790M mutations

Design:

**EGFR exon 21**

- Wild-type probe (p.Leu858)
  - c.2573T>G probe p.Leu858Arg
  - Forward primer location
  - Reverse primer location

- EGFR exon 21
  - Forward primer location
  - Reverse primer location

**EGFR exon 20**

- c.2369C>T probe p.Thr790Met
  - Forward primer location
  - Reverse primer location

Adapted from Punnoose et al., Clin Cancer Res, 2012
Example for the metastatic cancer progression evaluation in lung cancer

➢ 1. Forty-one year old man at diagnosed for a lung metastatic adenocarcinoma in 2014
➢ 2. Detection of the *EGFR* p.(Leu858Arg) mutation on a tissue biopsy
➢ 3. Treated by an anti-EGFR 2\textsuperscript{nd} generation (GIOTRIF)

27/02/2017: First liquid biopsy:

08/03/2017: Biopsy from a vertebral metastasis: EGFR L858 & T790 wild type but very little tissue sample...

24/07/2017: Second liquid biopsy:
1. Setting up of the liquid biopsy process at Dijon Hospital

2. NAICA digital PCR (Stilla Technologies®)

3. Example of applications
   3.1 BRAF testing in Melanoma
   3.2 EGFR testing in NSCLC

4. Conclusions
Conclusion

Melanoma
- Diagnostic
  - BRAF V600K/E dPCR
  - Follow-up
    - BRAF V600K/E dPCR
    - NGS

NSCLC
- Diagnostic
  - EGFR del. exon 19 + L858R dPCR
  - Progression
    - EGFR L858R/L861Q/T790M dPCR
    - or
      - EGFR del. exon 19 + T790M dPCR
      - MET/RPP30 dPCR
    - NGS
NAICA Digital PCR:

- Fast workflow → possibility to give results in less than 1 day (from blood sampling to dPCR)

- Possibility to multiplex (3 fluorescence channels) → adapted to Diagnosis

- Very sensitive and reliable system → adapted to Follow-up
Plateform of somatic oncology

**Pathology unit:**
Pr. Laurent Martin, MD PhD
Dr. Marie-Hélène Aubriot-Lorton, MD

**Molecular Unit:**
Dr. Bernard Aral, MD PhD
Dr. Caroline Chapusot, PhD
Dr. Benjamin Tournier, PhD
Technical staff:
Manon Aubry
Isabelle Choux
Lyse-Marie Dubois
Celia Pioche
Alicia Remond

**Biobank:**
Dr. Céline Schaeffer, PhD
Laetitia Barbier

**Research team:** Genetic and epigenetic Innovation in Oncology
Pr. Mary Callanan, PhD

**Clinicians**

**Lung Oncology:**
Dr. Anne-Lyse Fanton
Dr. Pascal Foucher
Dr. Ayoubé Zouak

**Dermato-oncology:**
Dr. Bertille Bonniaud
Dr. Sophie Dalac
Dr. Géraldine Jeudy

Thanks for your attention