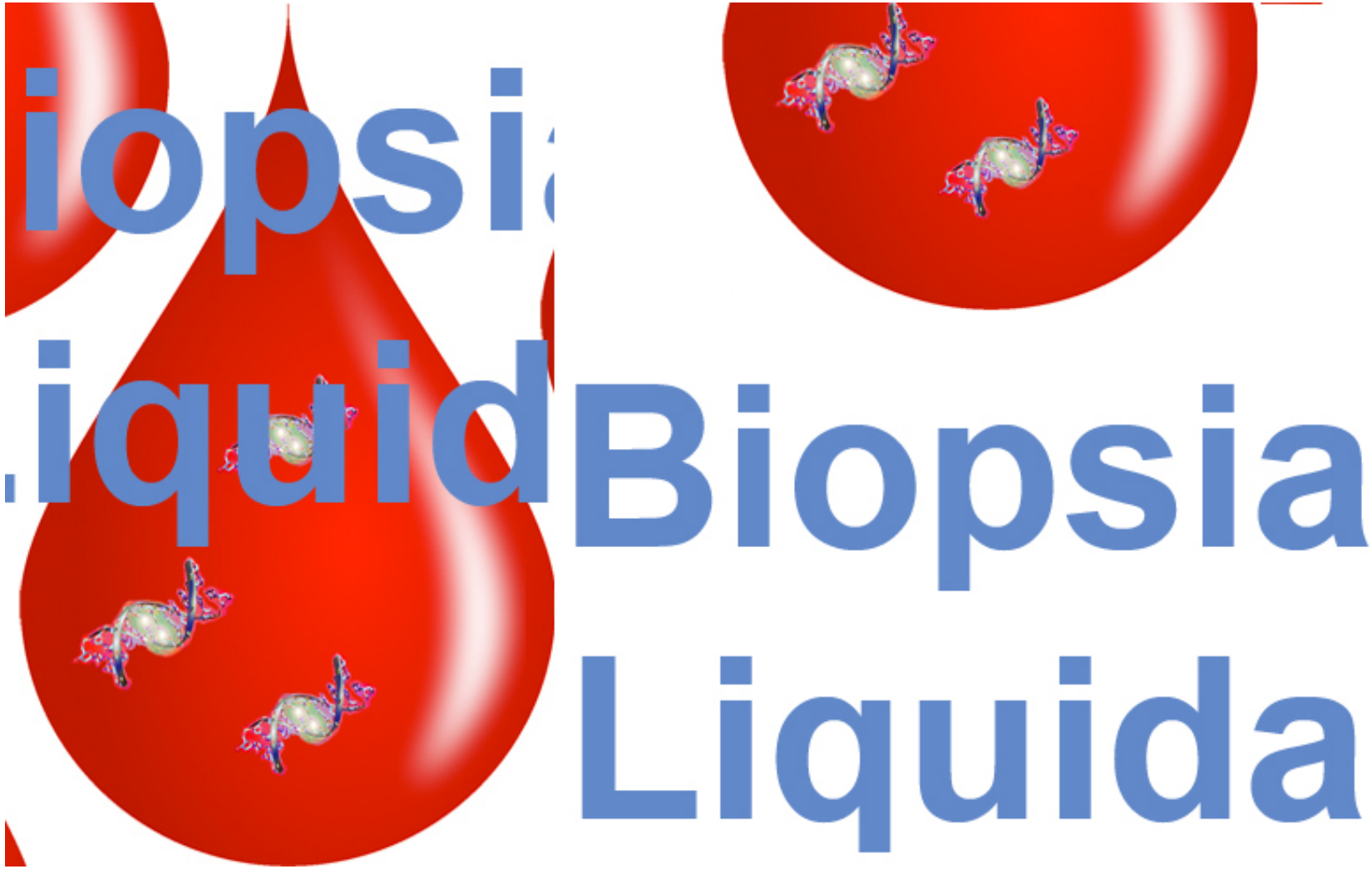
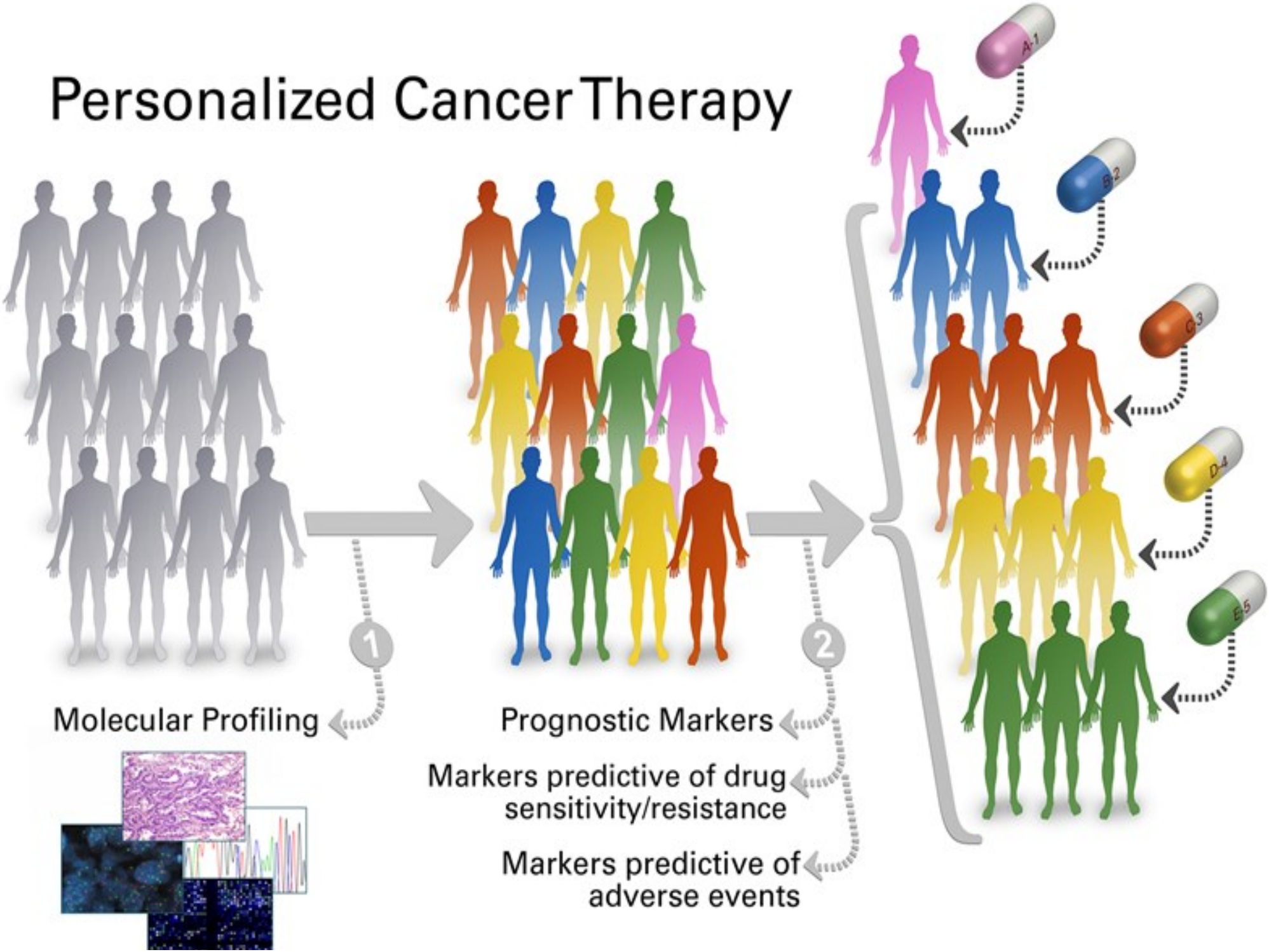

iopsi
iquid
Biopsia
Liquida

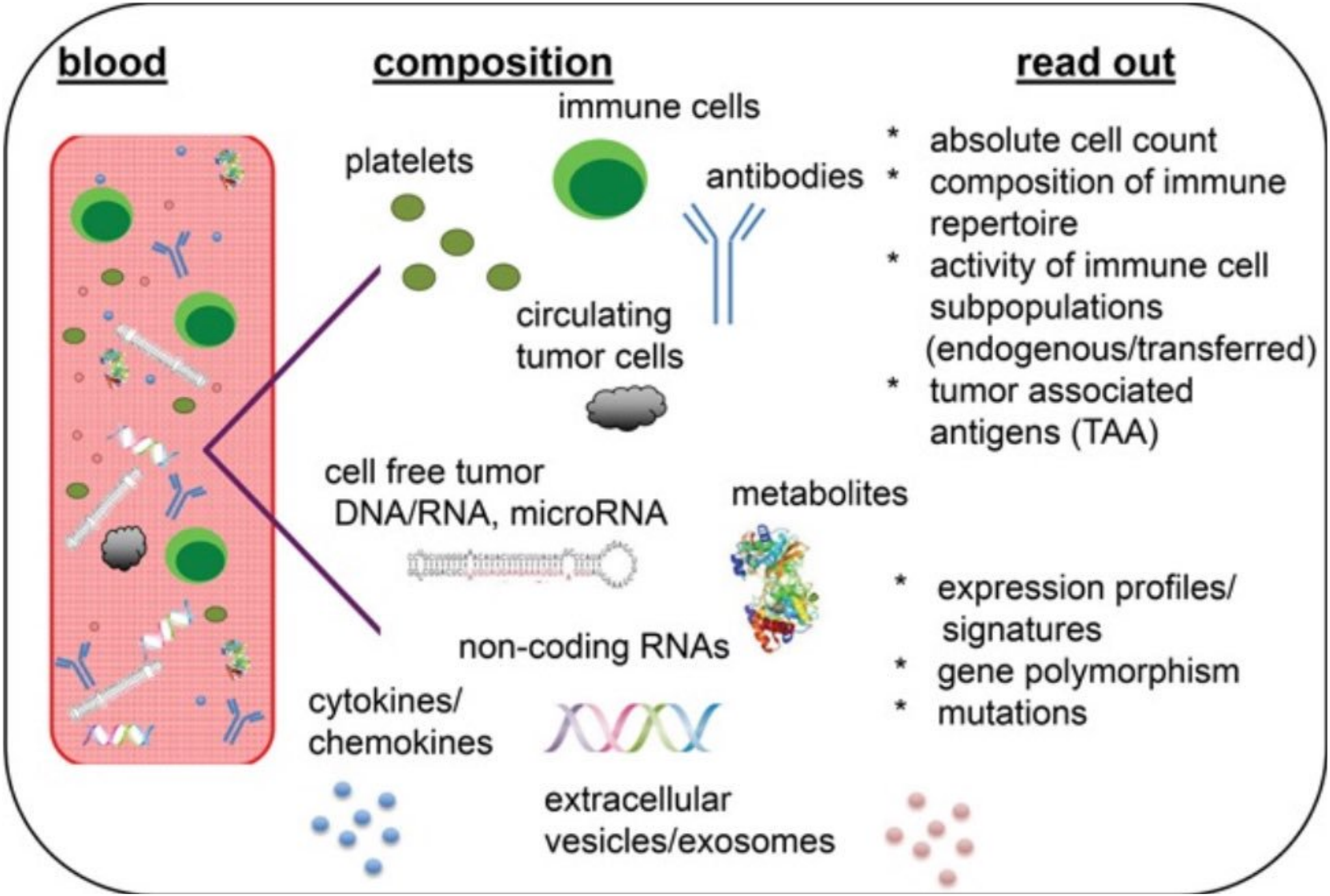
Personalized Cancer Therapy



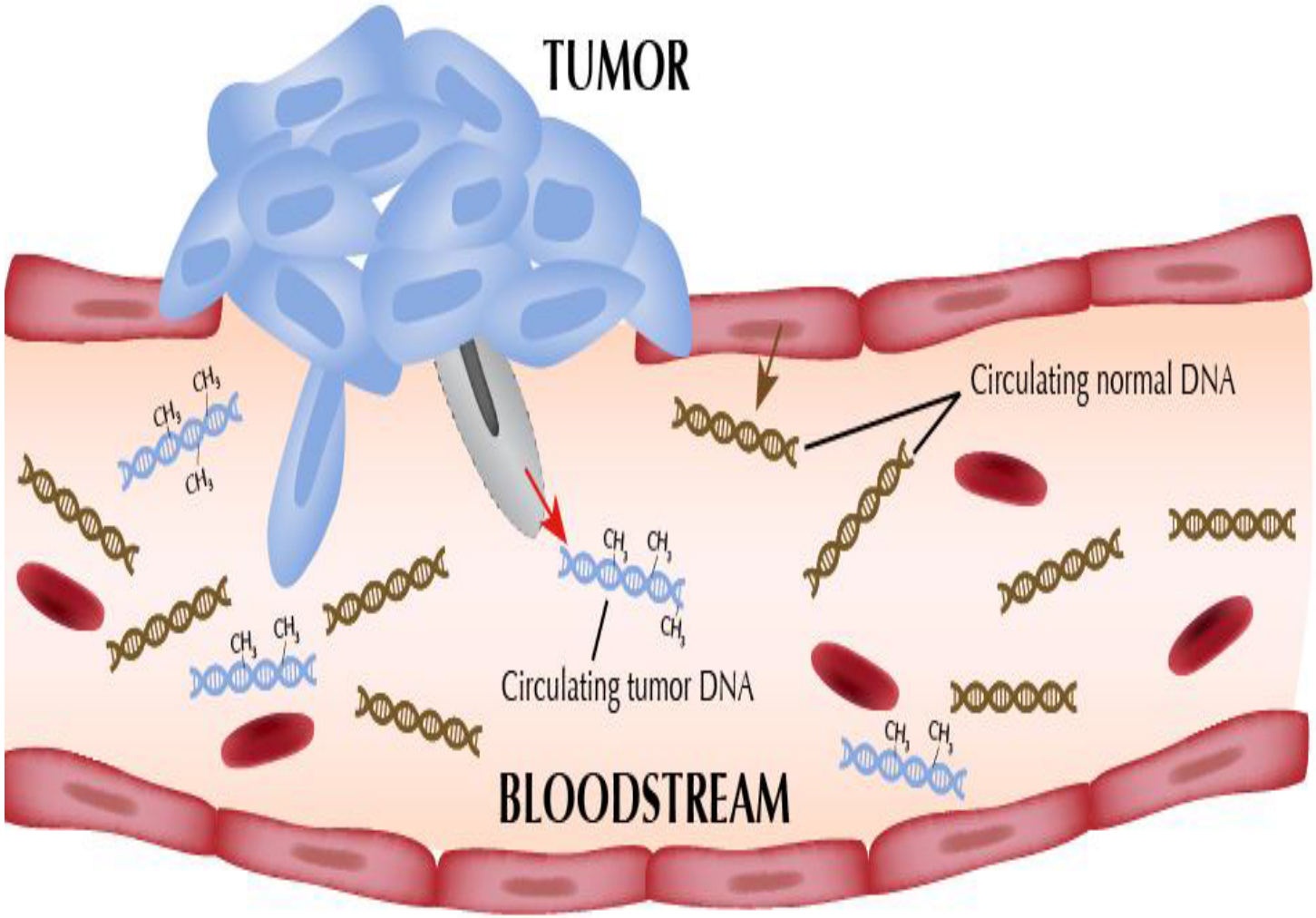
Tumor DNA (FFPE - biopsy)

Circulating tumor cells (CTC in liquid biopsy)

Circulating tumor DNA (ctDNA in liquid biopsy)

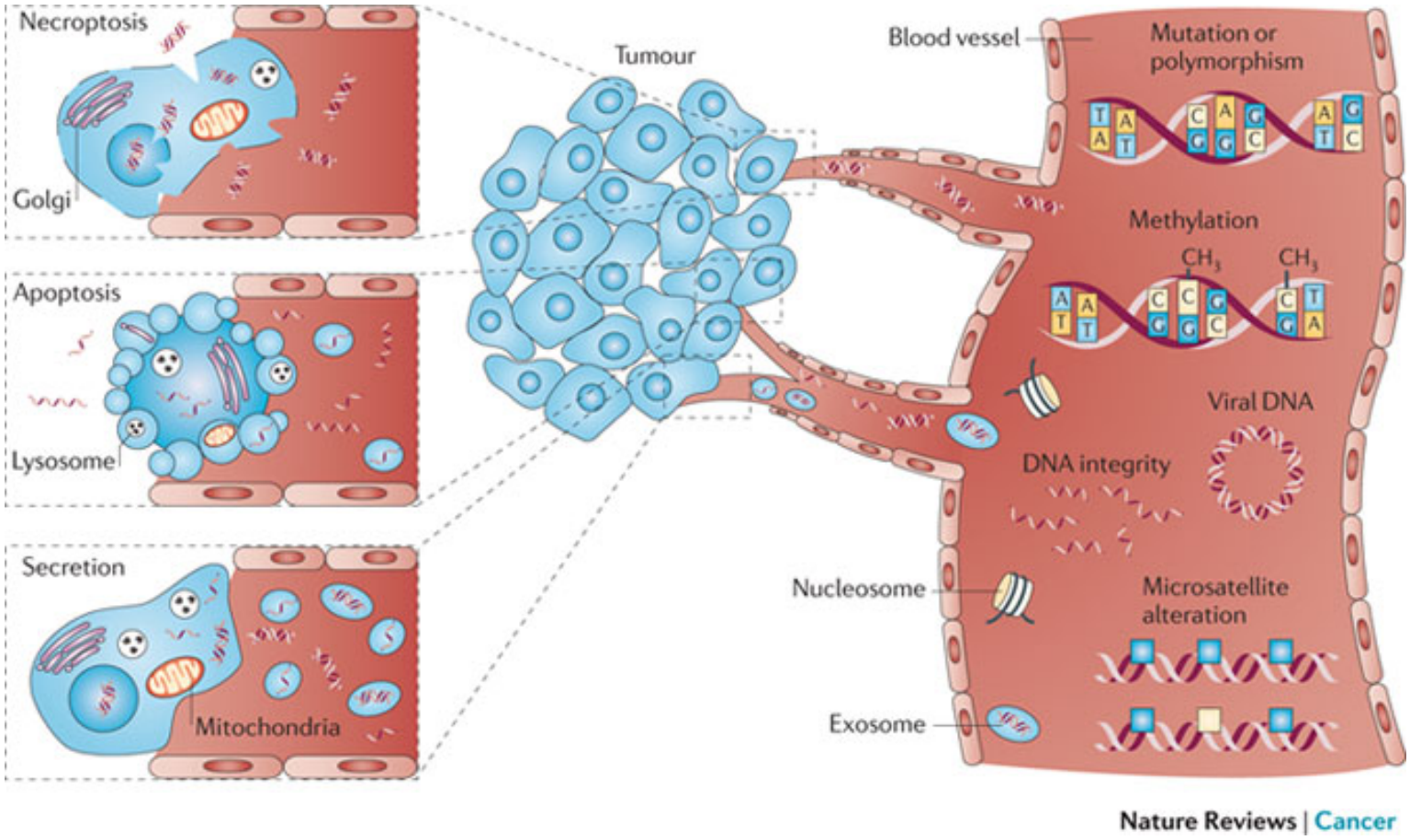

blood
composition
read out
immune cells
platelets
antibodies
circulating
tumor cells
cell free tumor
DNA/RNA, microRNA
metabolites
non-coding RNAs
cytokines/
chemokines
extracellular
vesicles/exosomes
* absolute cell count
* composition of immune repertoire
* activity of immune cell subpopulations (endogenous/transferred)
* tumor associated antigens (TAA)
* expression profiles/ signatures
* gene polymorphism
* mutations

Circulating tumor DNA (ctDNA)

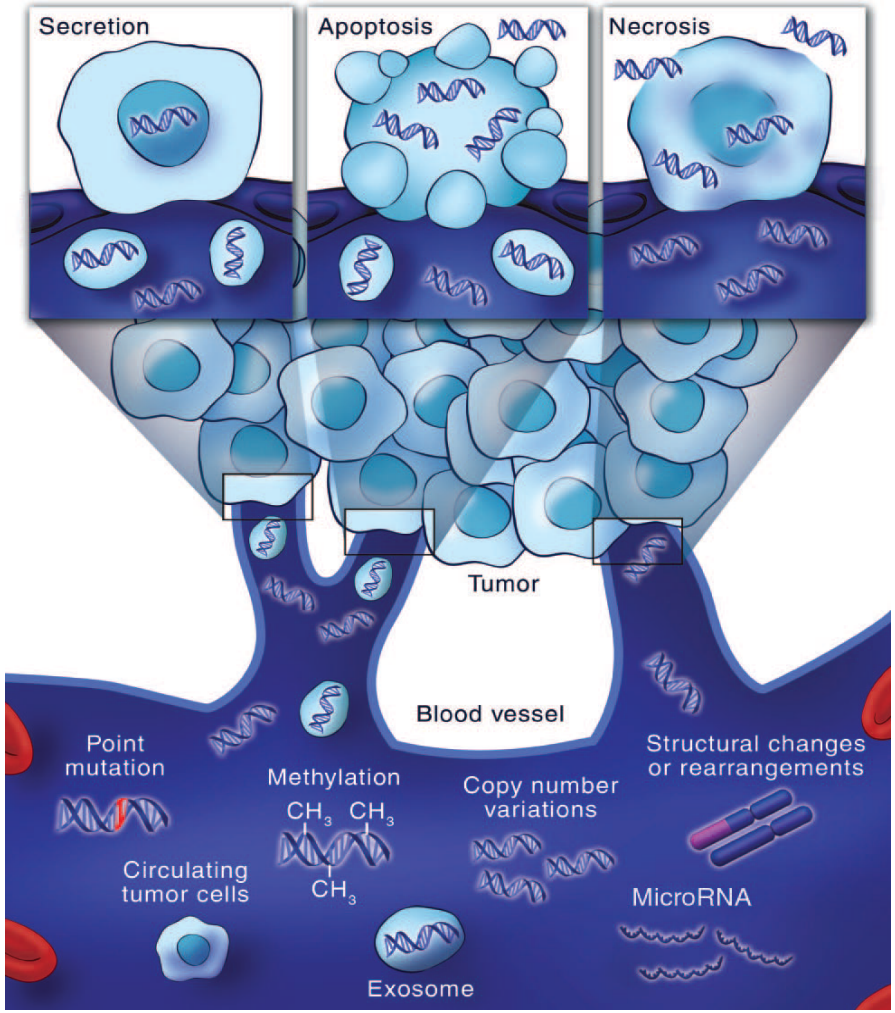


ctDNA

ctDNA from tumor tissue is released through secretion, necrosis and apoptosis, but mainly through apoptosis.

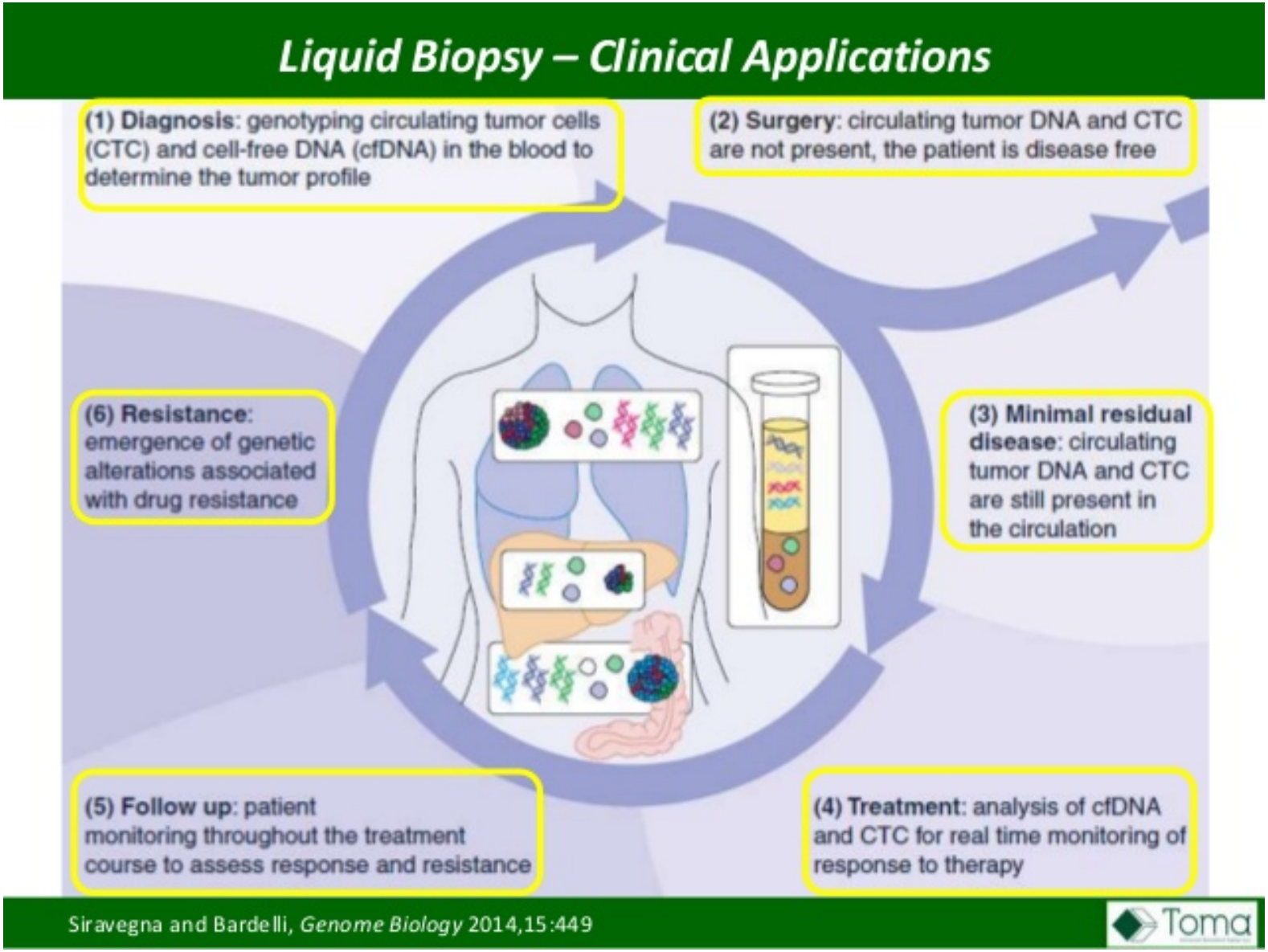


Circulating Tumour DNA (ctDNA)



Diaz and Bardelli, 2014 Journal of Clincial Oncology 32

- ctDNA is tumour DNA that has been shed into the bloodstream
- ctDNA can be present in 0.01% - >90% of the total Cell Free DNA (cfDNA)
- The amount of ctDNA is related to the tumour burden and varies between patients with different clinical presentations

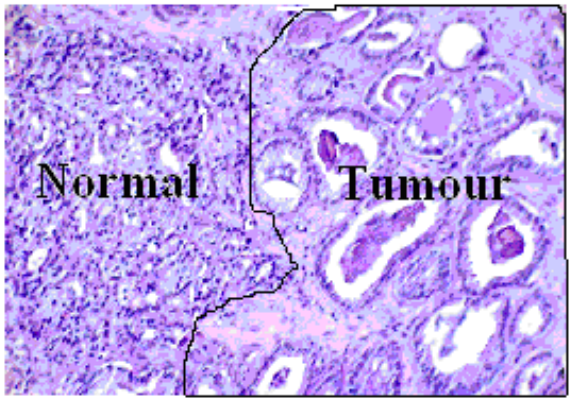


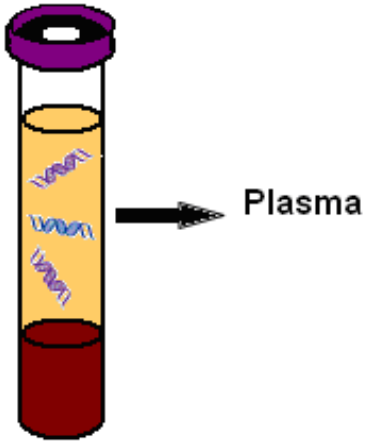
Cell-free DNA (cfDNA) is found in blood plasma at concentrations of 10-100 ng/ml, in fragments of 150 base pairs. When cfDNA comes from tumor cells, it is called ctDNA, and can be identified by point mutations and chromosome rearrangements typically found in cancer genomes.

FFPE versus ctDNA

- **FFPE Samples**
- Tumour DNA extracted from fixed biopsy samples or tumour resections
- Problems with quality of DNA due to fixation
- Mixture of normal and tumour DNA
- Long time to process by histopathologists.
- Macrodissected to enrich tumour content
- Some patients have no tumour sample available
- The sample represents the tumour at one fixed time point

- **ctDNA Samples**
- ctDNA shed directly from tumour
- Extracted from the plasma component of whole blood
- Large fragment sizes possible
- Small quantities extracted ~ 30ng/ 5ml plasma
- Separate out plasma within a few hours of receipt of blood sample.
- Serial samples can be taken at various time points during the patient's treatment





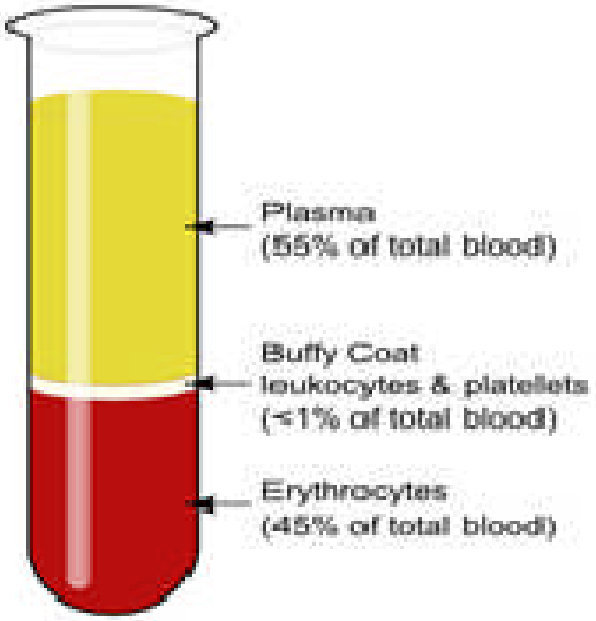
cell-free DNA (cfDNA) testing

- Cell-free DNA (cfDNA) in plasma of healthy individuals : Mandel and Métais (1948)
- A proportion of cfDNA in pregnant women is fetus-derived (cffDNA) : Lo et al. (1997)
- Non-Invasive Prenatal testing (NIPT) : 2012 : start
  2015 : > 1 million tests
  Market : 4 billion USD

- Increased concentrations of cfDNA in the circulation of cancer patients : Leon et al. (1977)
- A proportion of cfDNA is tumor-derived : Stroun et al. (1987)
- Circulating tumor DNA (ctDNA) testing (liquid biopsy) : 2015 : start
  Market : 40 billion USD

ctDNA Collection

- ctDNA has a very short half life ranging from 15 minutes to several hours
- It is stable in plasma at -80ºc
- Blood can be sampled in ETDA tubes but the plasma has to isolated and stored at -80ºc within one hour of collection
- Preservative tubes can be used to stabilise the cfDNA in blood for up to 4 days at room temperature.



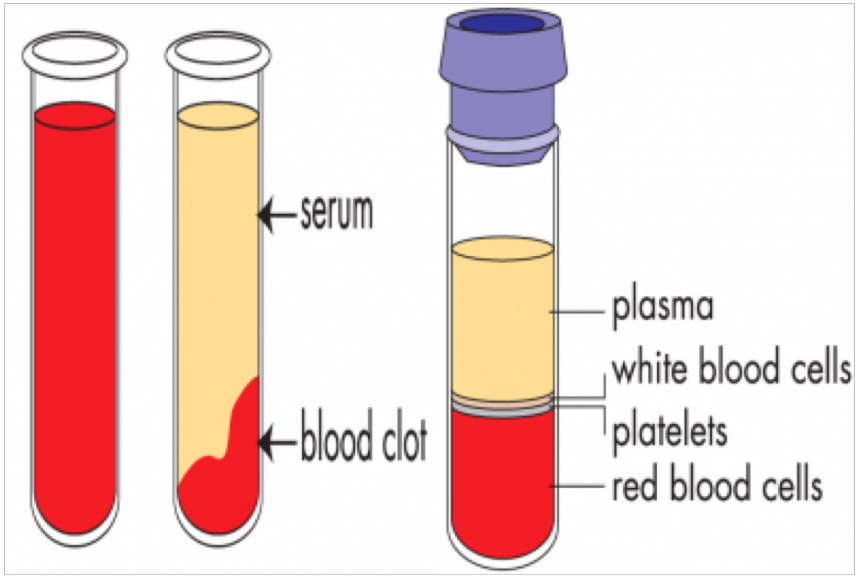
Plasma

✓No coagulazione del sangue

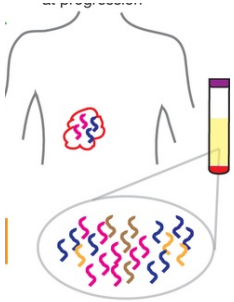
VS

Siero

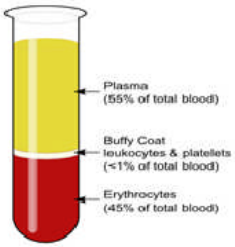
✓Sangue subisce processo di coagulazione



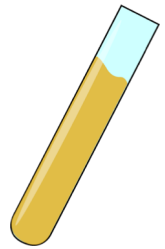
ctDNA Workflow



Blood sample taken in Cell Save preservative tubes

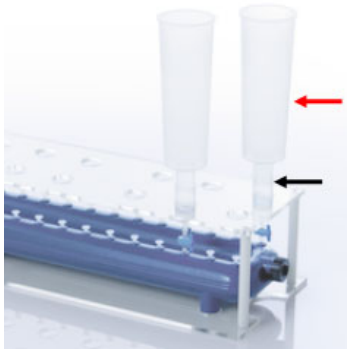


Sample arrives in lab and spun to isolate the plasma



Plasma is stored at -80ºc

Sample is extracted on the same day as the downstream process set up due to ctDNA instability



ctDNA is extracted from the plasma using the QIAamp Circulating Nucleic Acid on the QIAVac system

Set up:
Pyrosequencing
Next-generation sequencing
Quantative PCR
BEAMing
Digital PCR

Problems with ctDNA

- Due to the unstable nature of ctDNA the sample is has to be collected and processed correctly
- Only get **30ng** of **cfDNA** per **5ml** plasma extraction
- The amount of ctDNA is related to the tumour burden and varies between patients
- Difficult to discriminate ctDNA from normal cfDNA
- The technique used must be sensitive enough to pick up the low level variants

Technique	Sensitivity	Optimal Application
Sanger sequencing	> 10%	Tumor tissue
Pyrosequencing	10%	Tumor tissue
Next-generation seqeuncing	2%	Tumor tissue
Quantative PCR	1%	Tumor tissue
ARMS	0.10%	Tumor tissue
BEAMing, PAP, Digital PCR, TAM-Seq	0.01% or lower	ctDNA, rare variants in tumor tissue

Diaz and Bardelli, 2014 Journal of Clincial Oncology 32

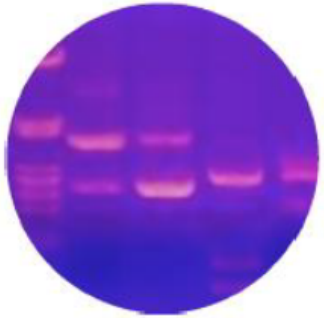
Summary of sensitivity and specificity methods used for cfDNA analysis (Rolfo et al., 2014)

Method	Sensitivity	Specificity
qPCR	high	low
Methylation specific polymerase chain reaction (MS-PCR)	high	high
Sanger sequencing	very low	high
Pyrosequencing	low	low
NGS	low	high
ARMS	low	high
BAEMing (beads, emulsion, amplification, magnetics)	high	high
Digital PCR	high	high
TAM-sequencing	high	high

Droplet Digital PCR (ddPCR)

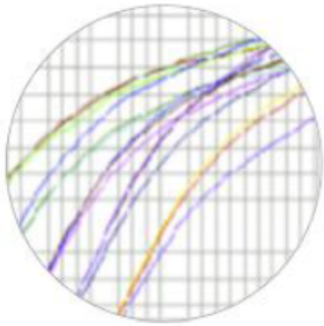
Che cos'è la ddPCR?

1980



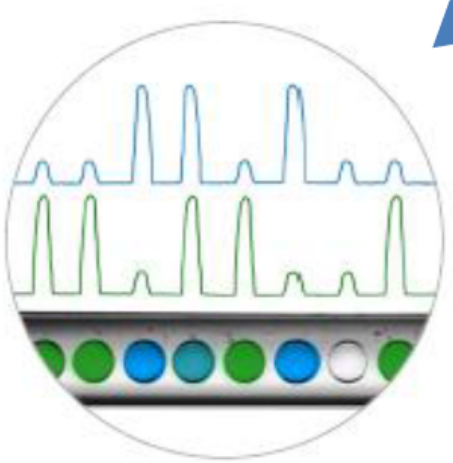
PCR
Qualitative

1990



Real-time PCR
Relative Quantitation

2010

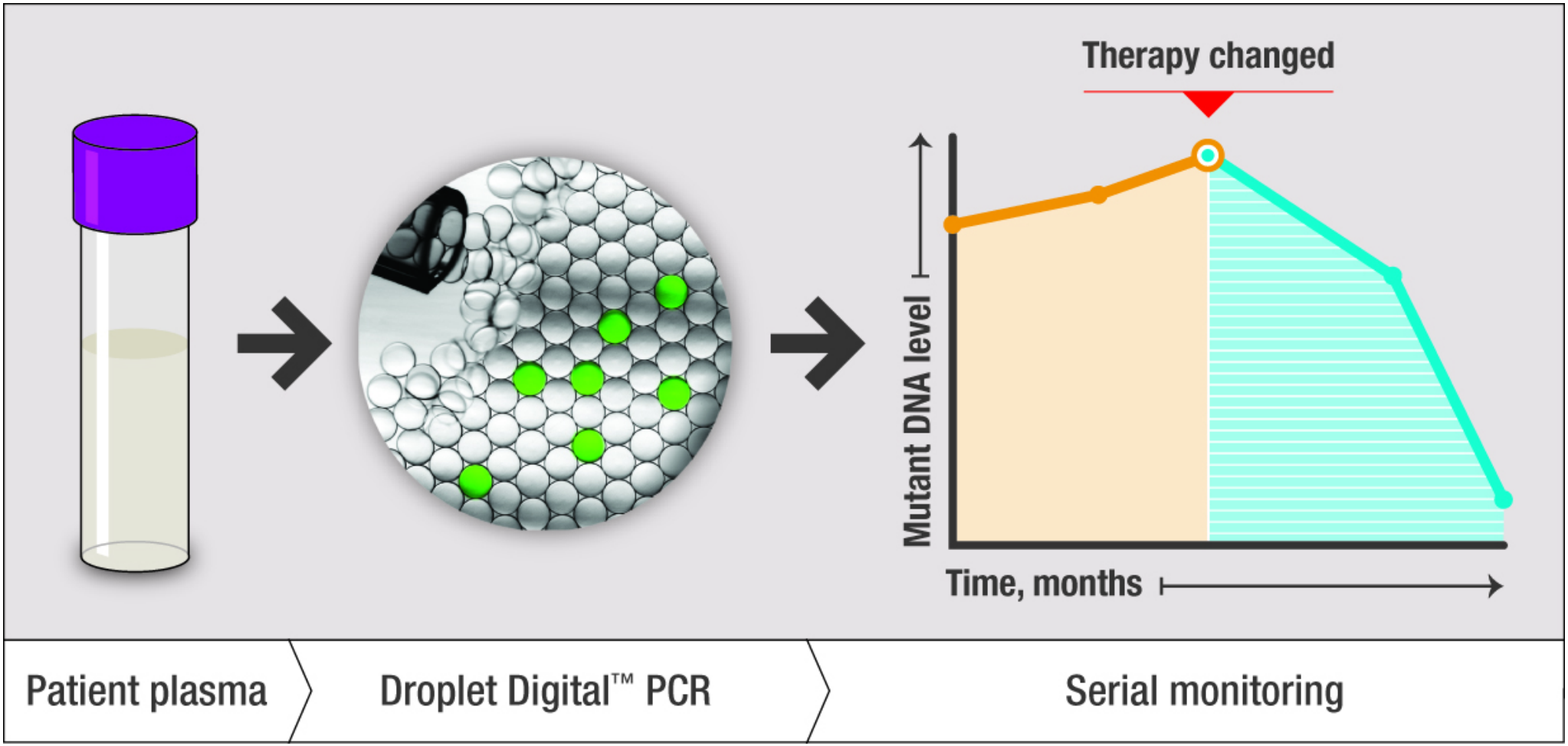


Droplet Digital PCR
Absolute Quantitation

Droplet digital PCR

Accurate absolute quantification of template molecules by separation of target molecules and counting statistics.

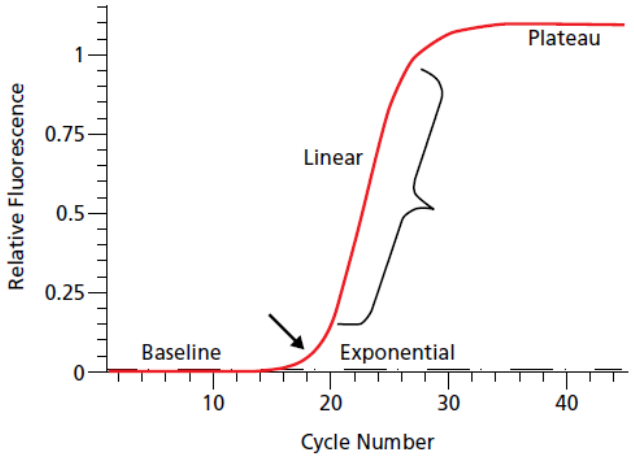
A sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet.


Therapy changed
Mutant DNA level
Time, months
Patient plasma
Droplet Digital™ PCR
Serial monitoring

ddPCR Principle

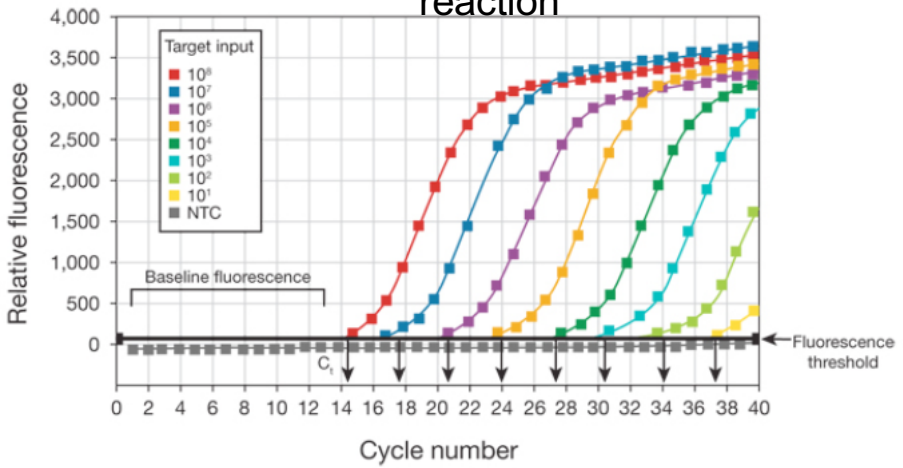
Standard PCR

Quantitation of an end product in a bulk reaction



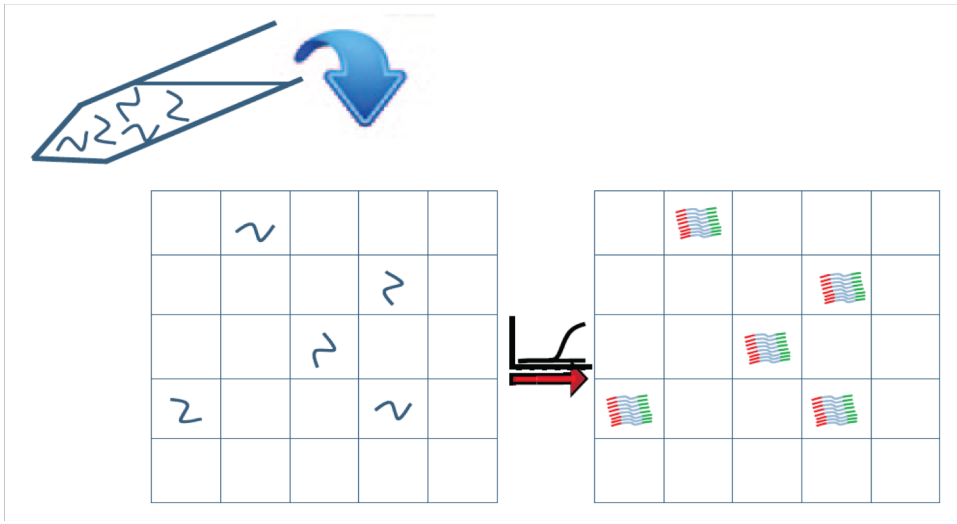
qPCR

Quantitation of product in real time in a bulk reaction



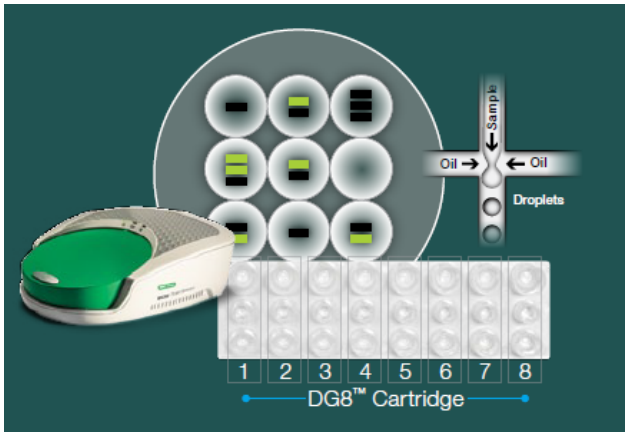
ddPCR

- Bulk reaction separated into smaller partitions.
- Each partition intended to contain zero or one copy of DNA.
- The fraction of positive partitions is fitted to a Poisson distribution to determine the absolute starting copy number of DNA.



ddPCR Principle: Process

Bio-Rad QX200 System



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Absolute Quantification: No need for a standard curve because number of targets in a given volume (i.e. concentration) is counted directly (and adjusted for multiple targets/droplet by **Poisson statistics**)

- **Precision**: The ability to consistently make the same measurement on replicates of a sample
- **Reproducibility**: **The ability of a researcher to obtain the same measurement on the same sample from experiment to experiment in the same lab OR in another lab across the globe**.
- **Sensitivity**: The ability to measure very few copies of a target with precision mostly limited by the technology independent statistics of sampling
- **Accuracy**: The ability to make the correct measurement – this requires validation before this can be claimed, but is then an intrinsic property of the ddPCR assay used.

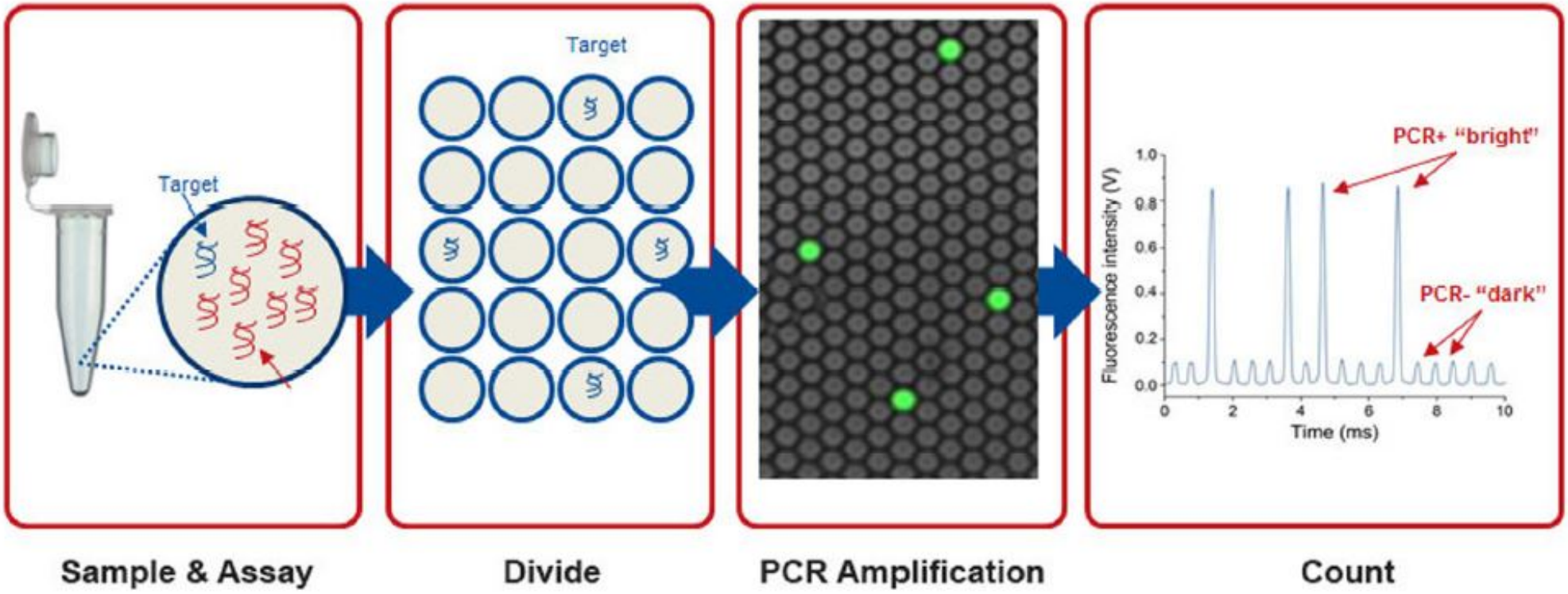
Bio-Rad QX200™ Droplet Digital PCR System

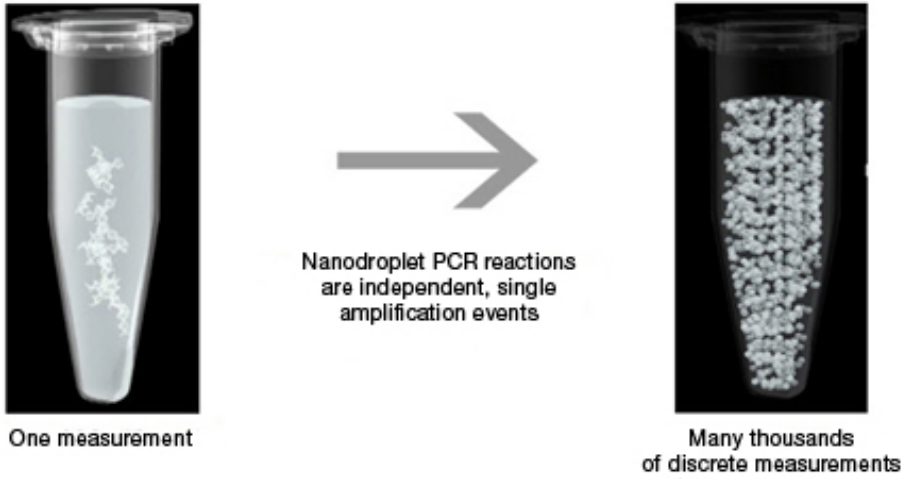
Droplet Reader

Droplet Generator

Reagents

Consumables





•**Removal of PCR bias** — error rates are reduced by removing the amplification efficiency reliance of qPCR, enabling the detection of small (1.2-fold) differences

1

Prepare ddPCR reaction mix

- Combine DNA/RNA sample, primers, and/or probes with one of Bio-Rad's ddPCR supermixes
- Fully validated PrimePCR™ ddPCR assays can be used

2

Generate droplets

- Load the ddPCR reaction mix into the wells of a droplet generator cartridge
- 8 x 20,000 droplets are generated from each run in the QX100™ or QX200 droplet generator
- Target DNA (–) and background DNA (–) are randomly distributed in droplets

3

Perform PCR

- Transfer the droplets to a 96-well PCR plate and seal the plate
- Run the PCR protocol

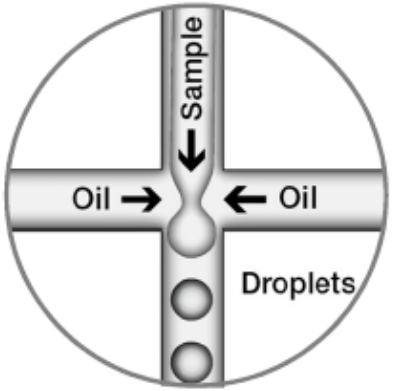
4

Read and analyze results

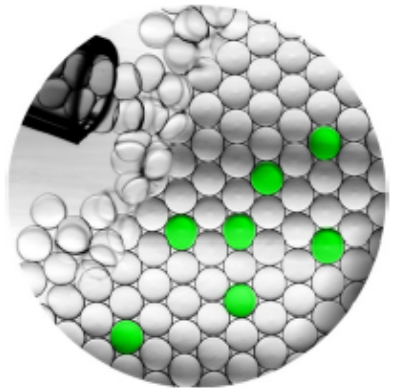
- After PCR, load the 96-well PCR plate into the QX100 or QX200 droplet reader
- Positive and negative droplets in each sample are read
- Analyze concentrations with QuantaSoft™ software

Droplet Digital PCR Workflow

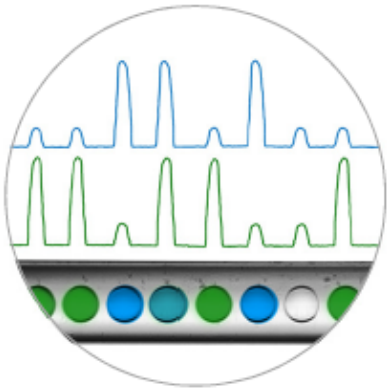
- Partition reagents and sample into thousands of droplets
- Perform PCR on thermal cycler
- Count droplets with a positive PCR product (fluorescent) and a negative PCR product
- Digital readout provides concentration of target DNA



Make Droplets



PCR Droplets



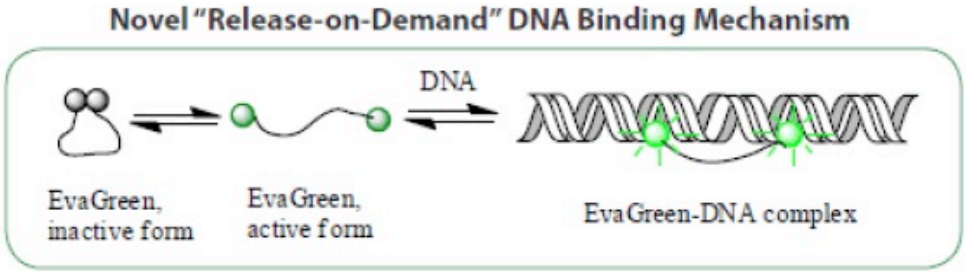
Read Droplets

Results

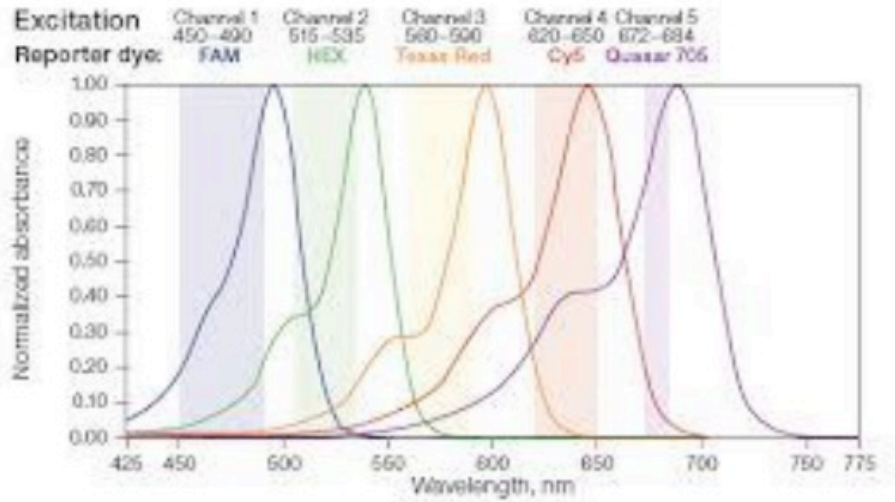
Droplet Digital PCR (ddPCR)

Che molecole può leggere?

EvaGreen ™



Sonde TaqMan ™



- ✓FAM
- ✓HEX/VIC

Droplet Digital PCR (ddPCR)

Principali vantaggi ddPCR

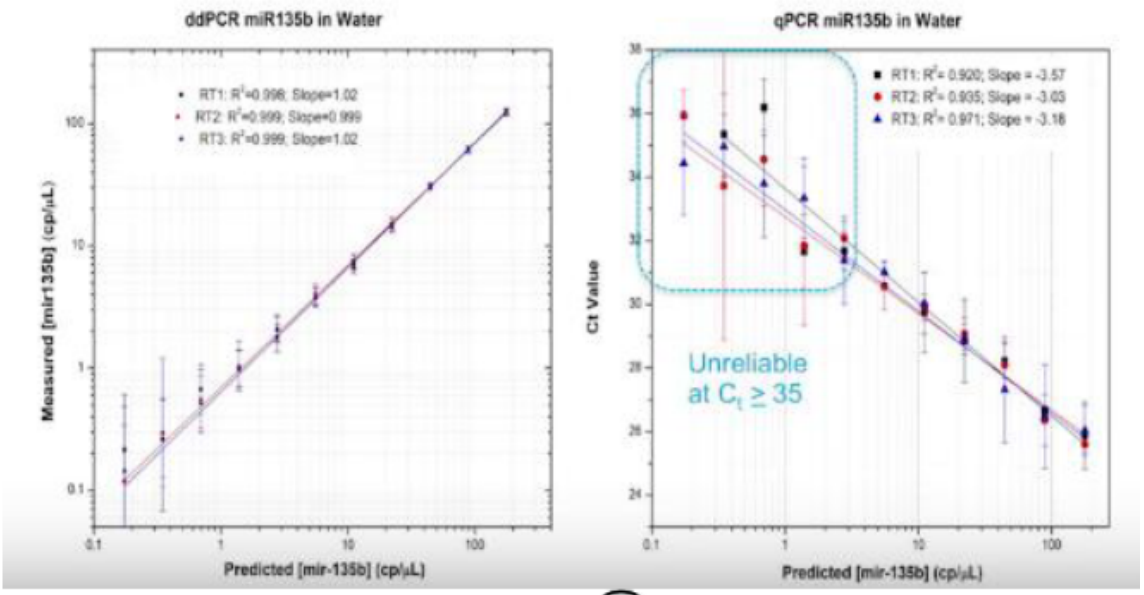
✓Non sono necessari replicati

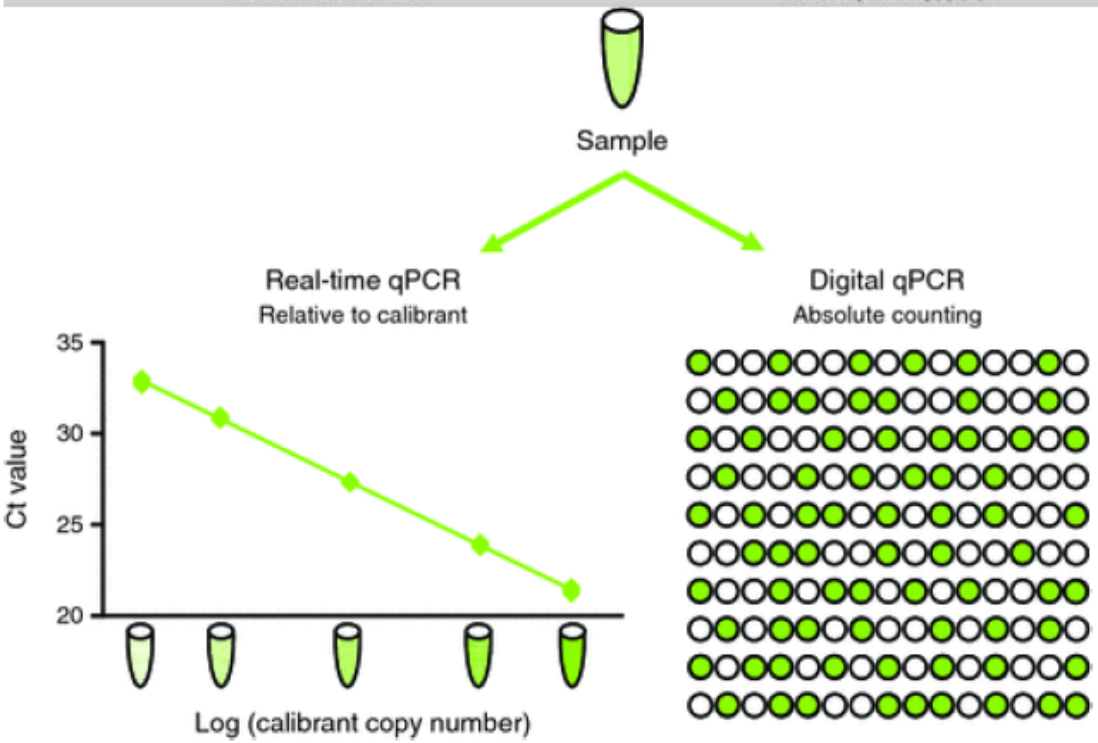
✓Maggiore riproducibilità alle basse concentrazioni

✓Quantificazione assoluta (copie/ul per pozzetto)

✓Meno sensibili agli inibitori della reazione di amplificazione

✓Possibilità di confrontare campioni analizzati in momenti diversi e/o in laboratori diversi





Droplet Digital PCR (ddPCR)

Siméon Denis Poisson
(1781-1840)

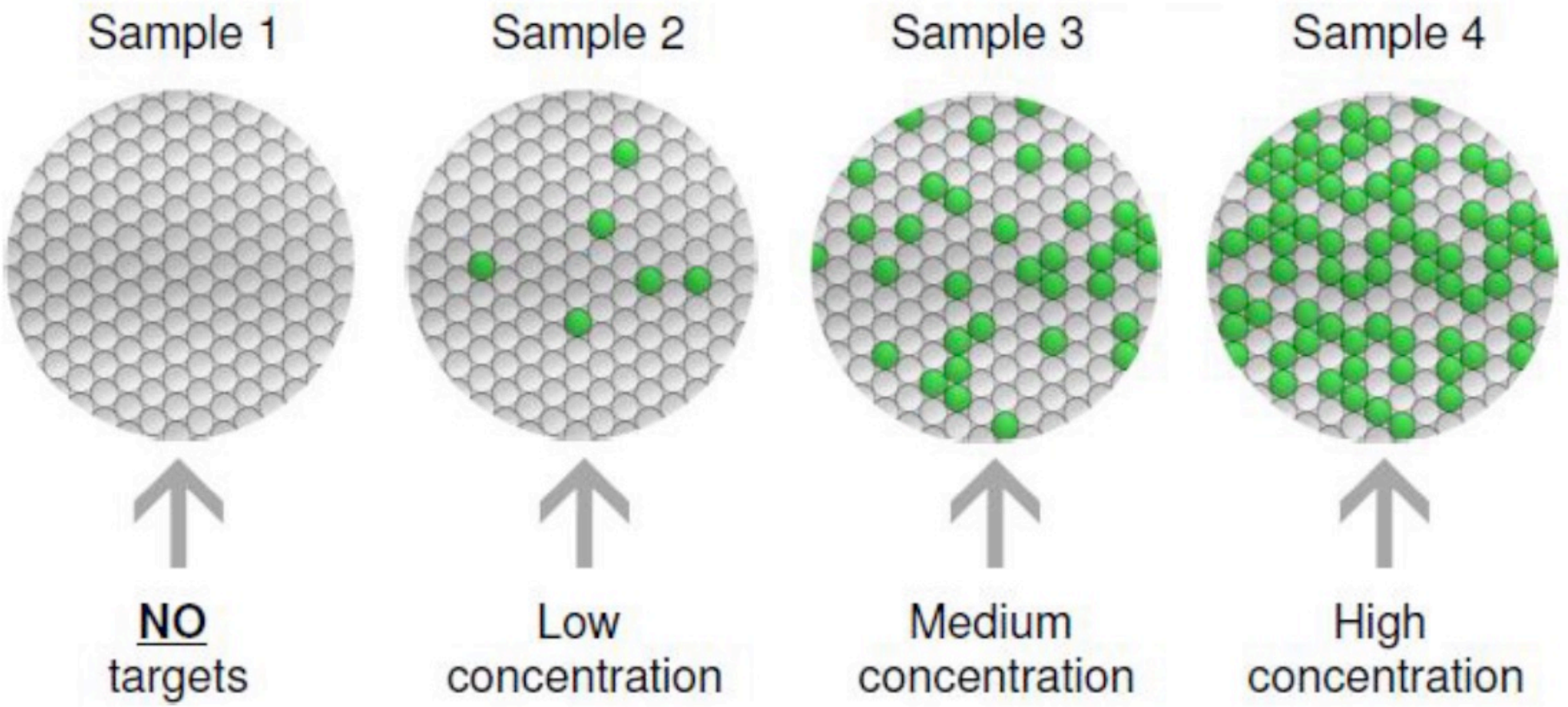
Legge di Poisson (Legge degli eventi rari)

$$-\ln\left(1-\frac{\text{positives}}{\text{total counted}\ (\text{positives} + \text{negatives})}\right)$$

Vi è una distribuzione casuale (di eventi indipendenti) delle molecole di DNA da analizzare nelle gocce: queste molecole vengono ripartite casualmente durante la formazione dell'emulsione del campione. Alcune non conterranno templato, altre 1 sola molecola, altre ancora più di una. Producendo un numero sufficientemente elevato (20.000) di gocce avremo più probabilità che queste conterranno una sola molecola di DNA.

Il numero di gocce contenenti una molecola di DNA/cDNA dipenderà dalla concentrazione di DNA/cDNA del campione di partenza

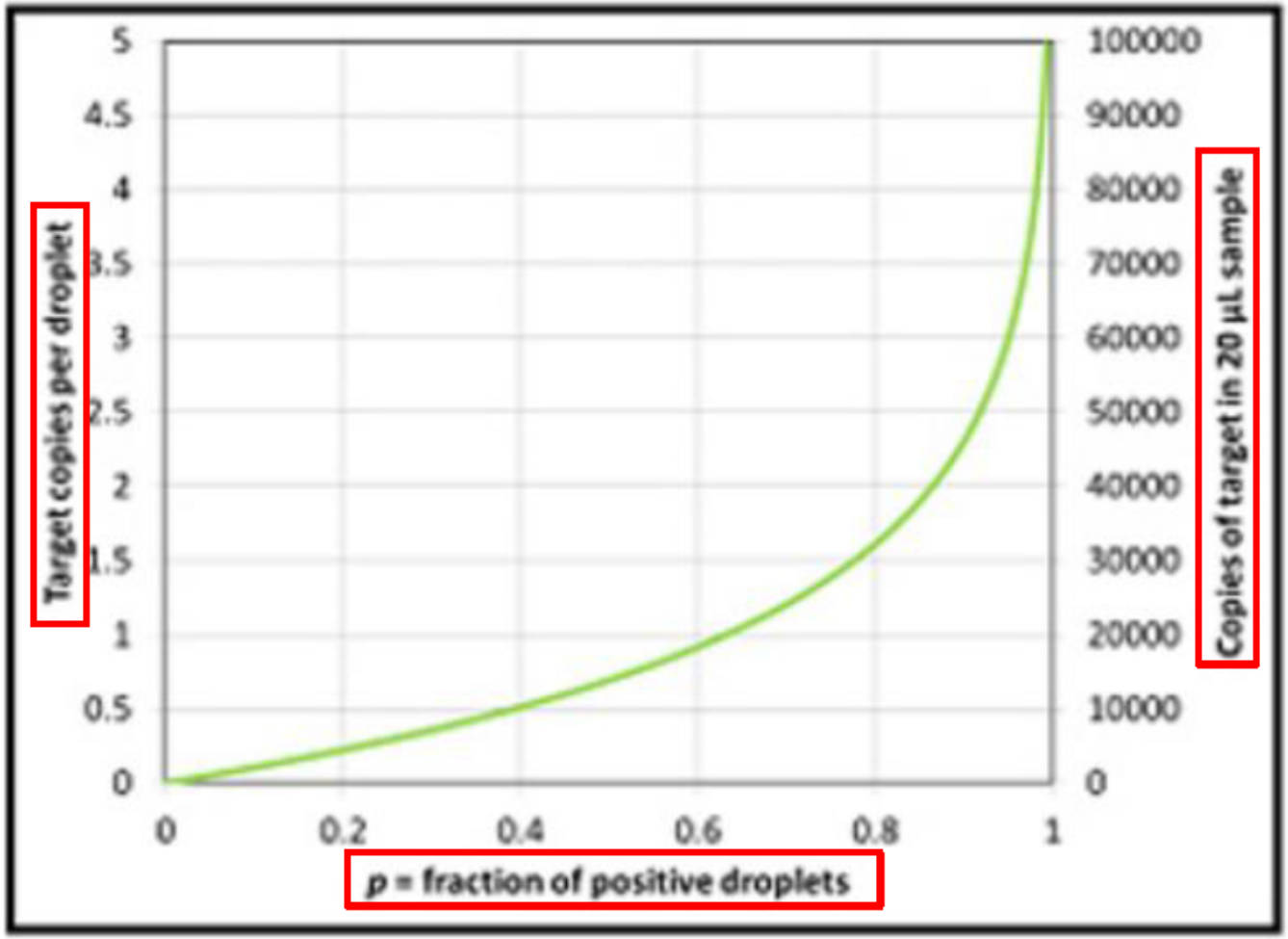
Droplet Digital PCR (ddPCR)



L'equazione di Poisson può essere utilizzata per determinare il numero di molecole di templato che andranno a finire in ogni goccia.

Droplet Digital PCR (ddPCR)

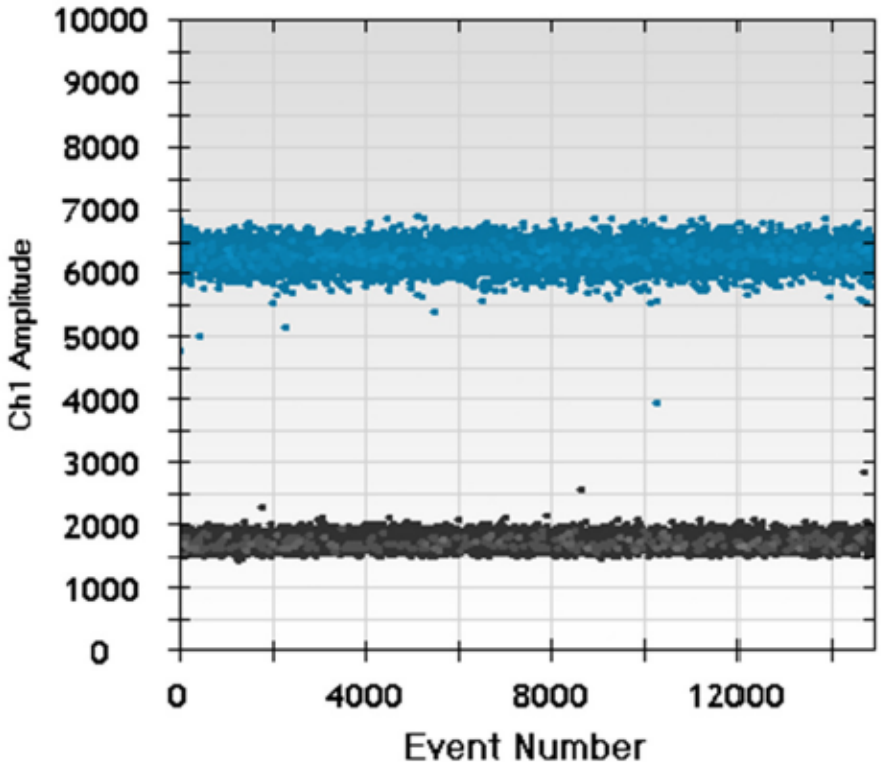
Legge di Poisson (Legge degli eventi rari)



$$p = \frac{\text{positives}}{\text{total counted}\ (\text{positives} + \text{negatives})}$$

Droplet Fluorescence Converted to a Digital Signal

- Positive droplets contain at least one copy of target DNA (cDNA)
- Positive droplets have increased fluorescence vs. negatives
- Quantasoft software measures the number of positive and negative droplets per fluorophore per sample



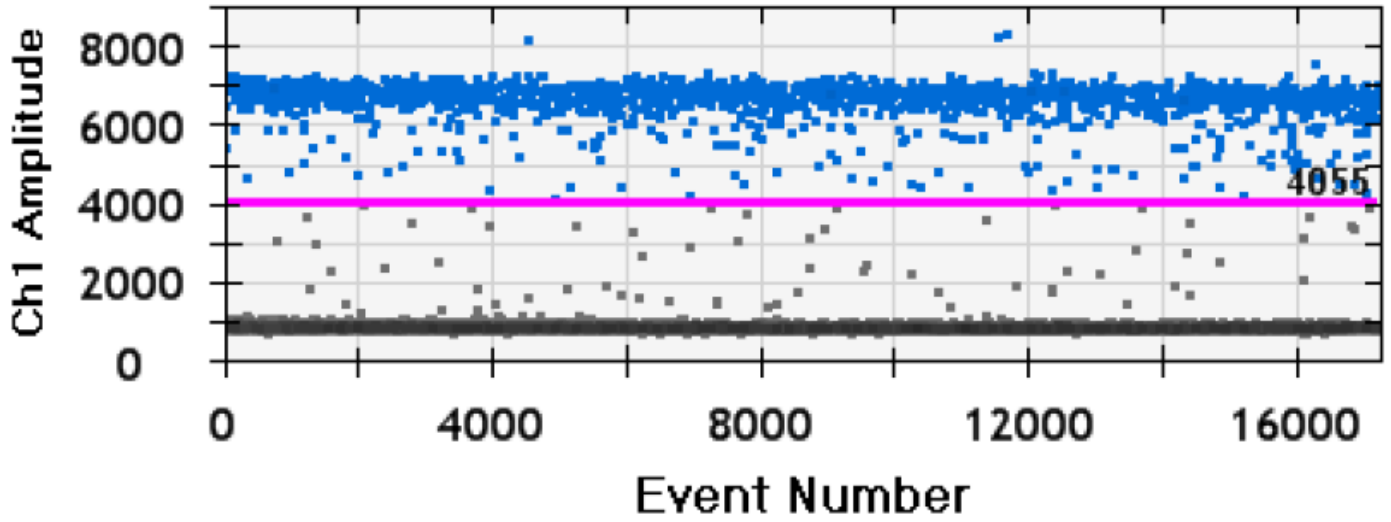
Each positives counted as 1

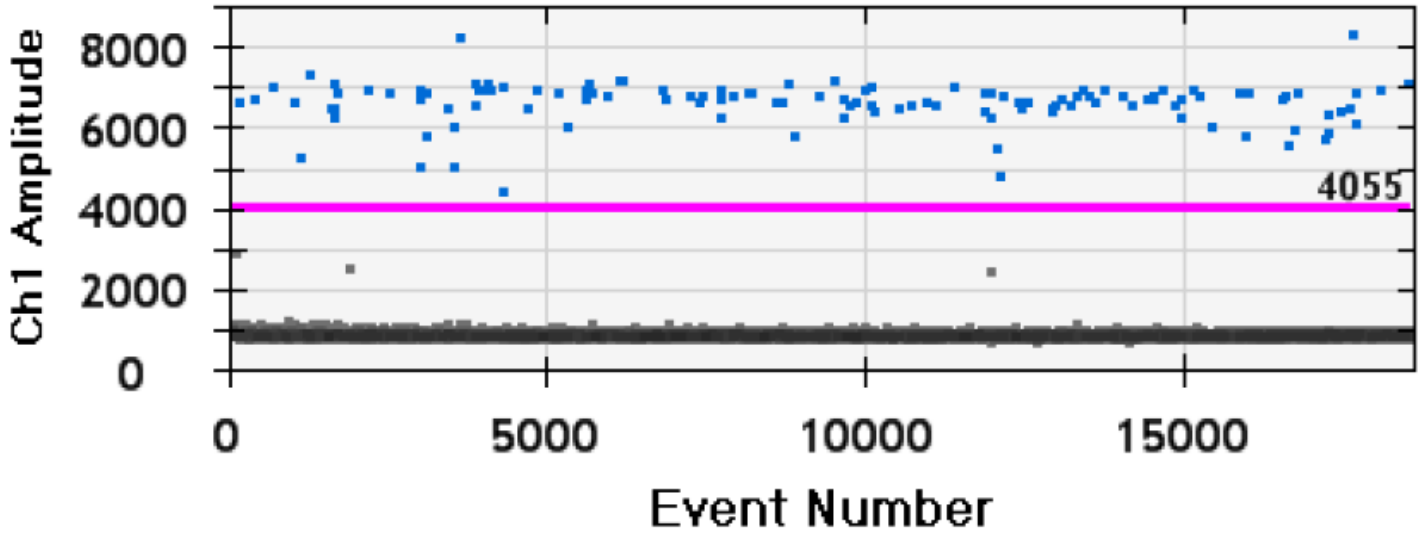
Each negatives counted as 0

Droplet Digital PCR (ddPCR)

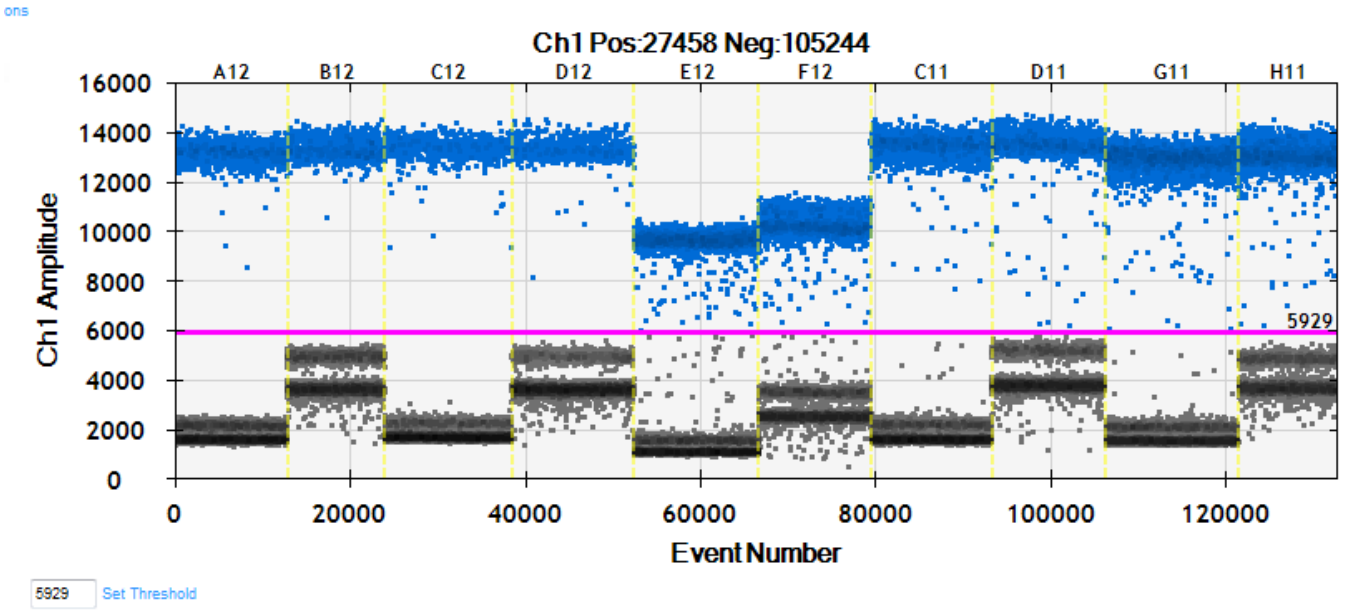
Plot 1D

Analisi dei dati

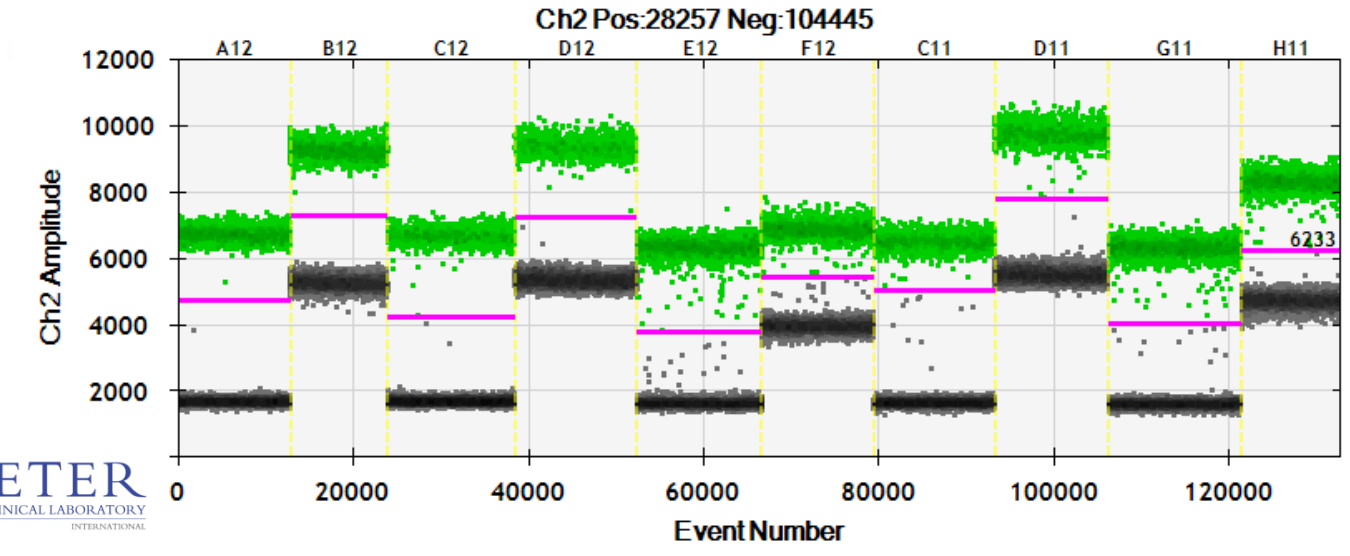




Bio-Rad Quantasoft
1D Amplification



Channel One



Channel Two

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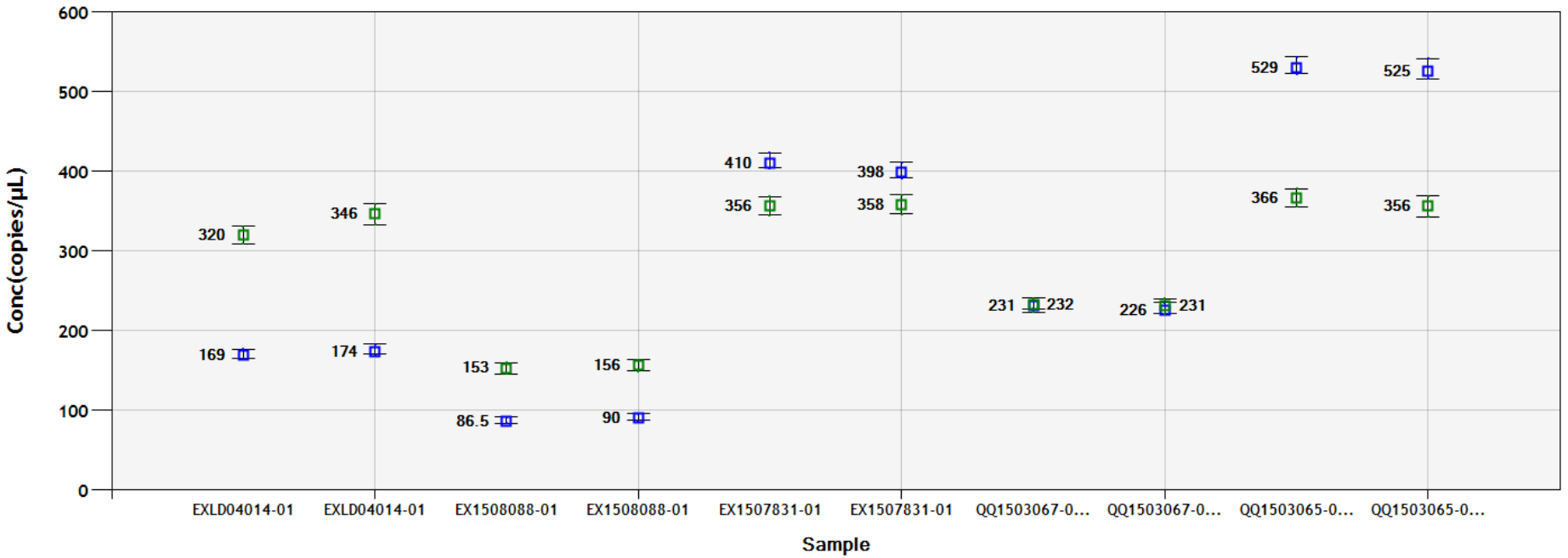
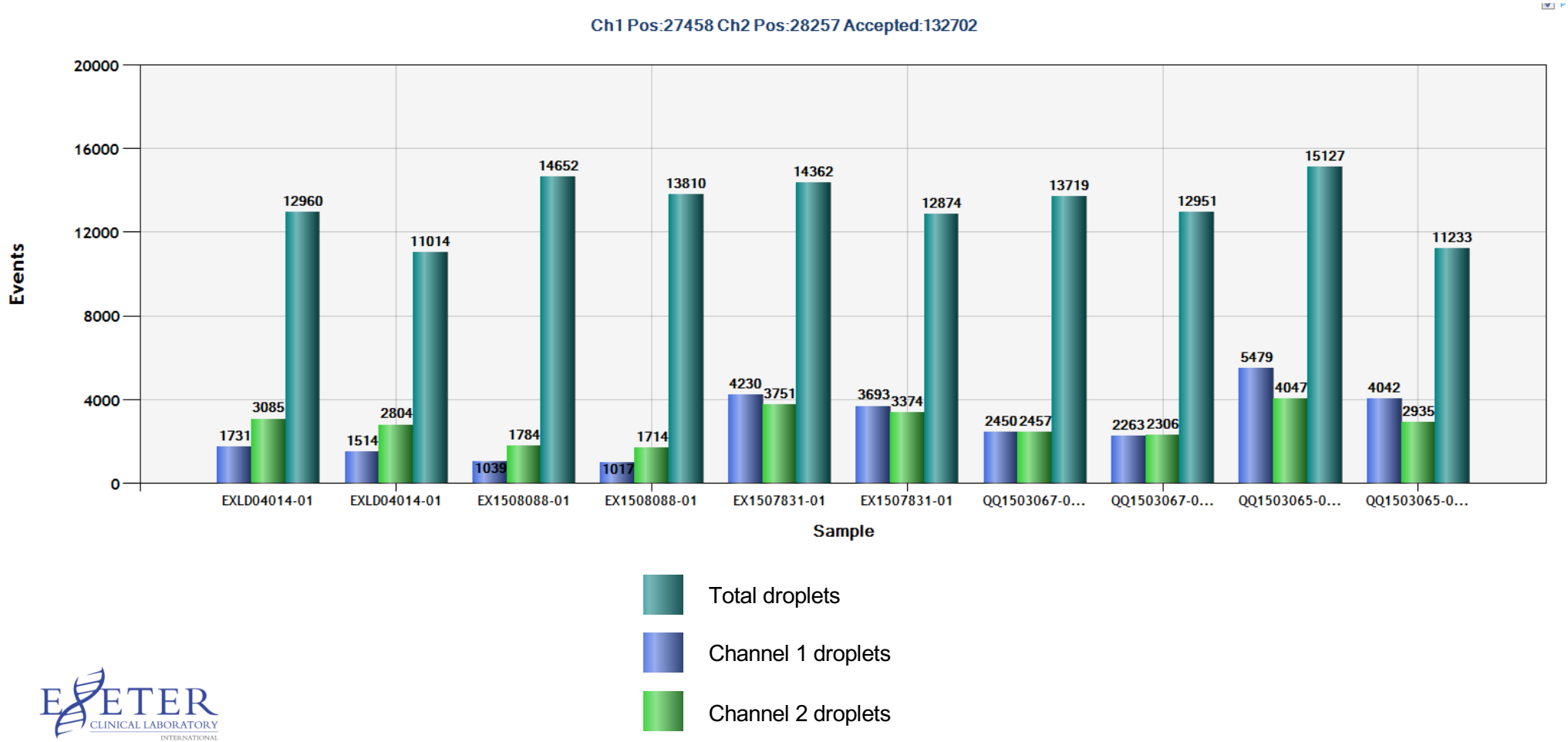
Bio-Rad Quantasoft Concentration



Channel 1 concentration

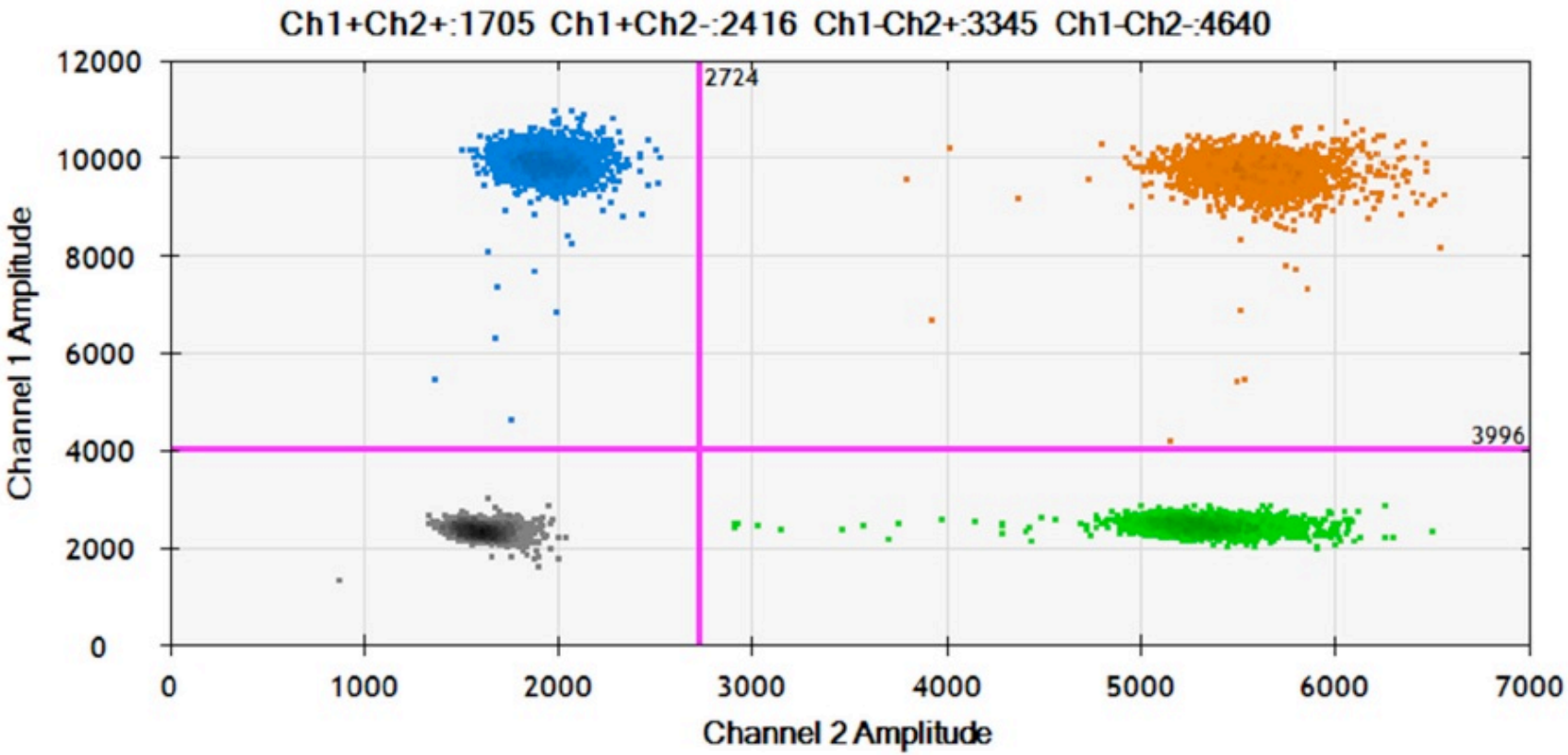
EXETER CLINICAL LABORATORY INTERNATIONAL

Bio-Rad Quantasoft
Events

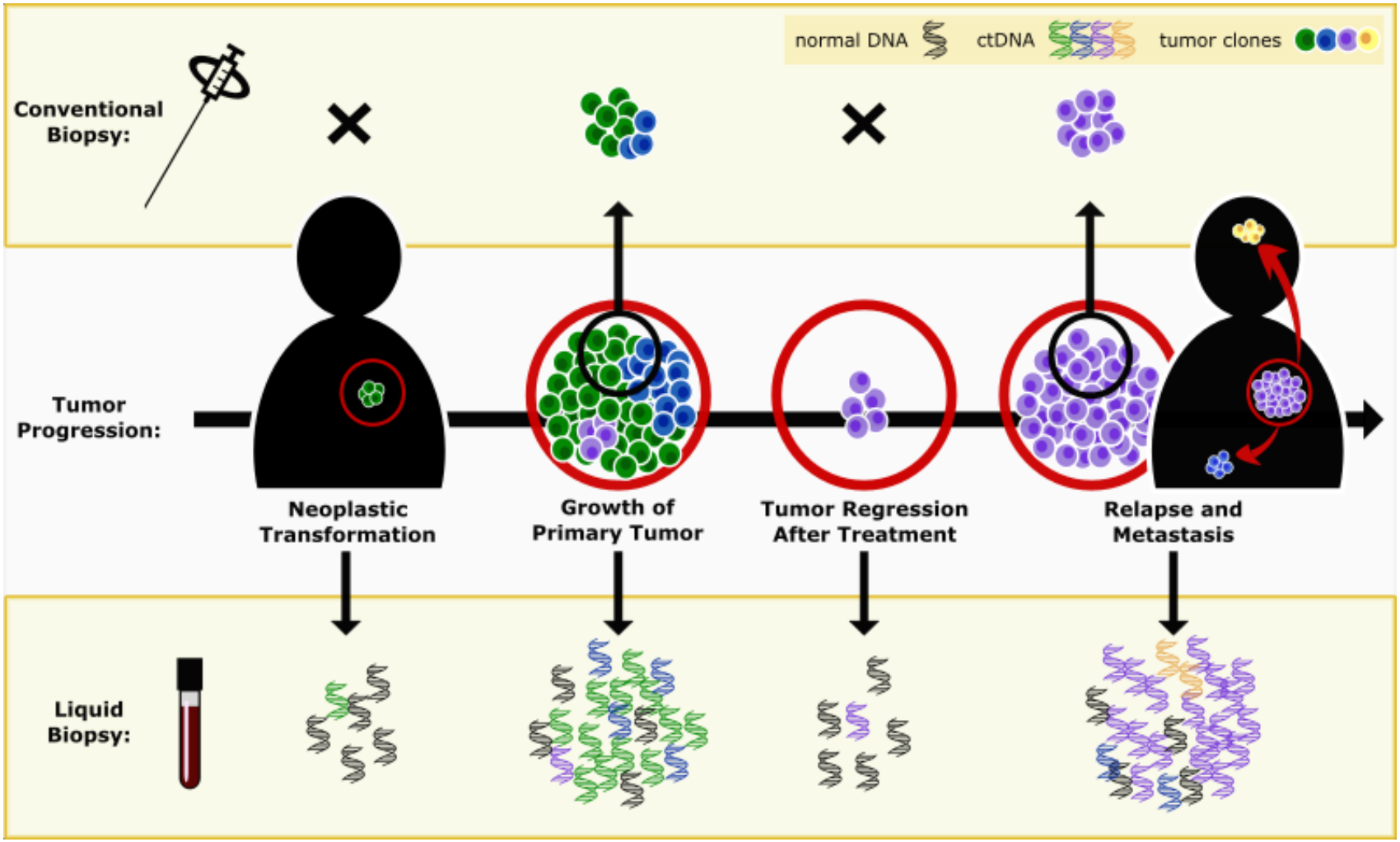


EXETER CLINICAL LABORATORY INTERNATIONAL

Simultaneous Detection of Two Targets

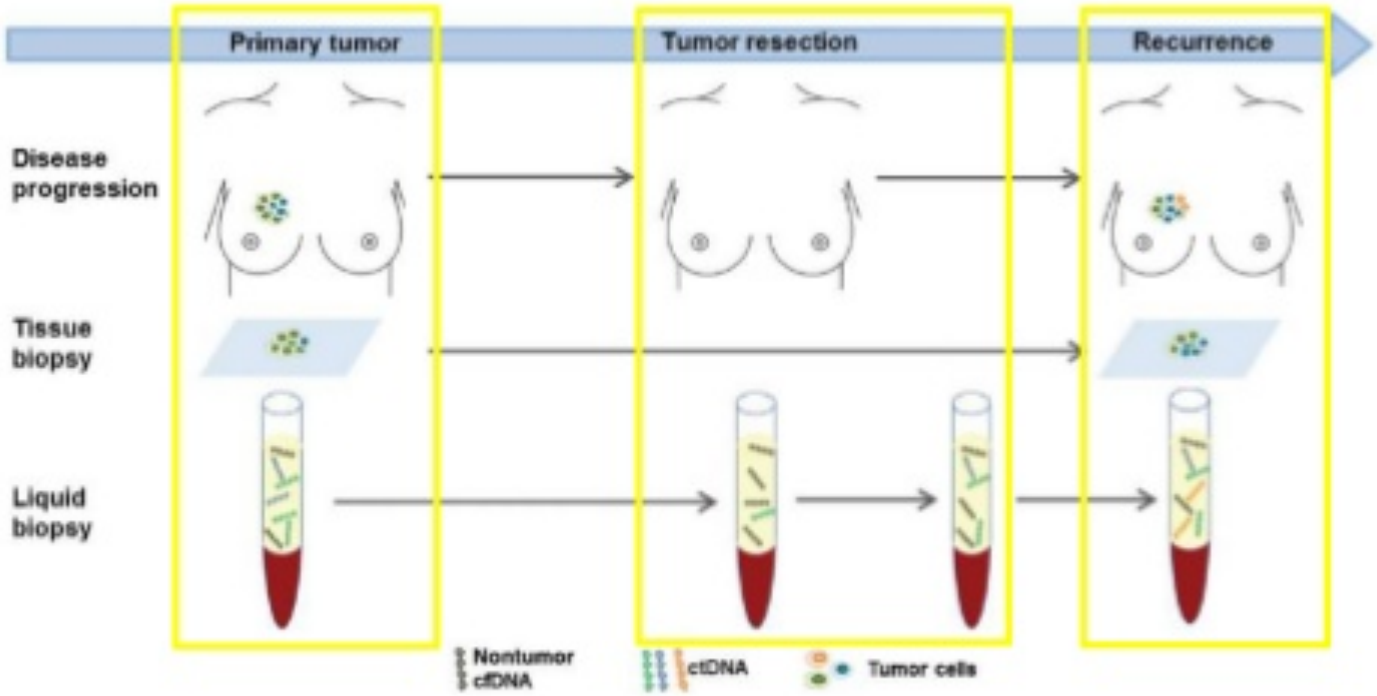


BIO-RAD


normal DNA
ctDNA
tumor clones
Conventional Biopsy:
Tumor Progression:
Neoplastic Transformation
Growth of Primary Tumor
Tumor Regression After Treatment
Relapse and Metastasis
Liquid Biopsy:

Liquid Biopsy – Clinical Applications

The improved sensitivity and specificity of ddPCR present the opportunity of using blood:



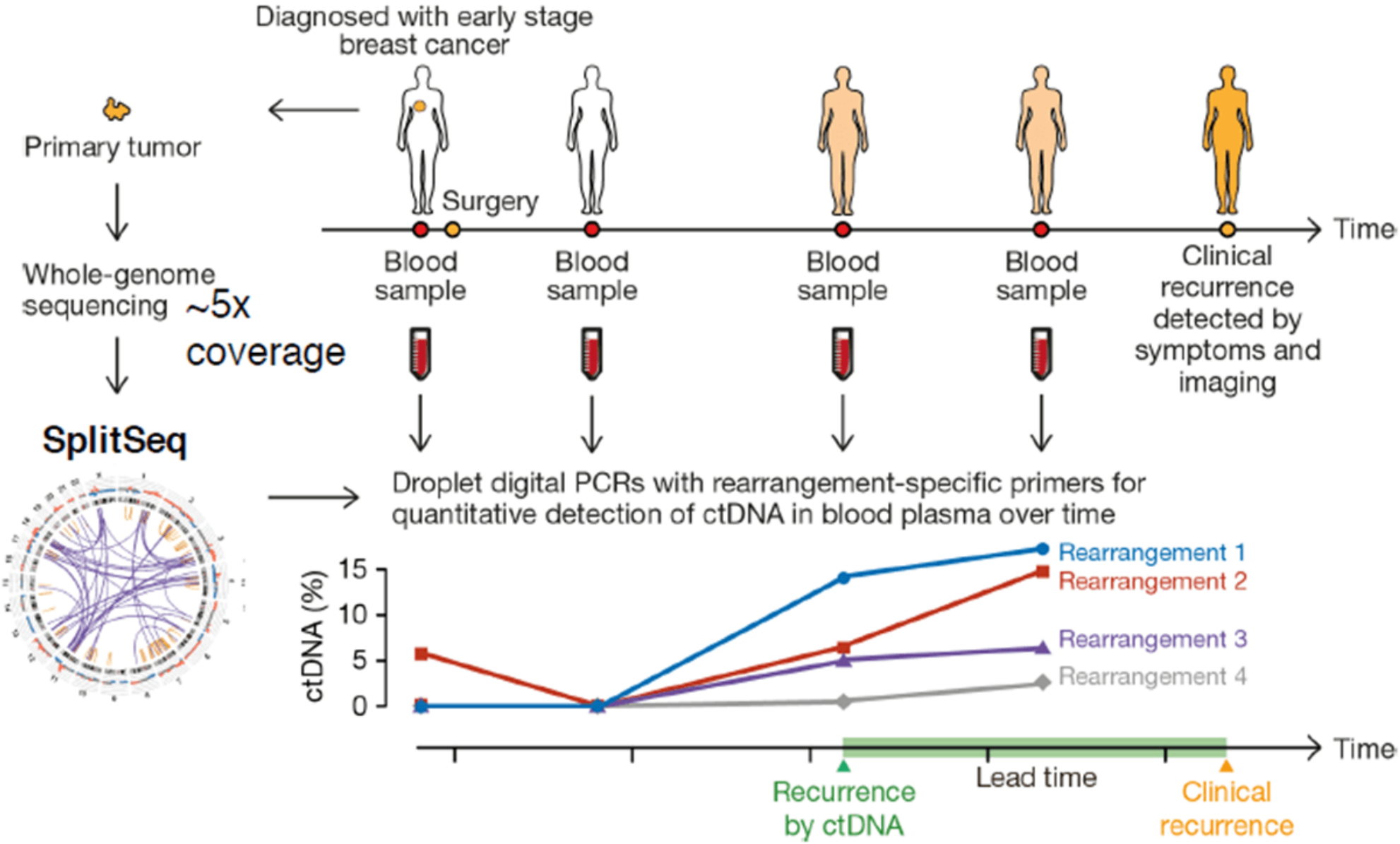
1. For mutations detection in patients with early-stage breast cancer

2. For minimal residual disease may help guide individualized decisions about adjuvant systemic therapies

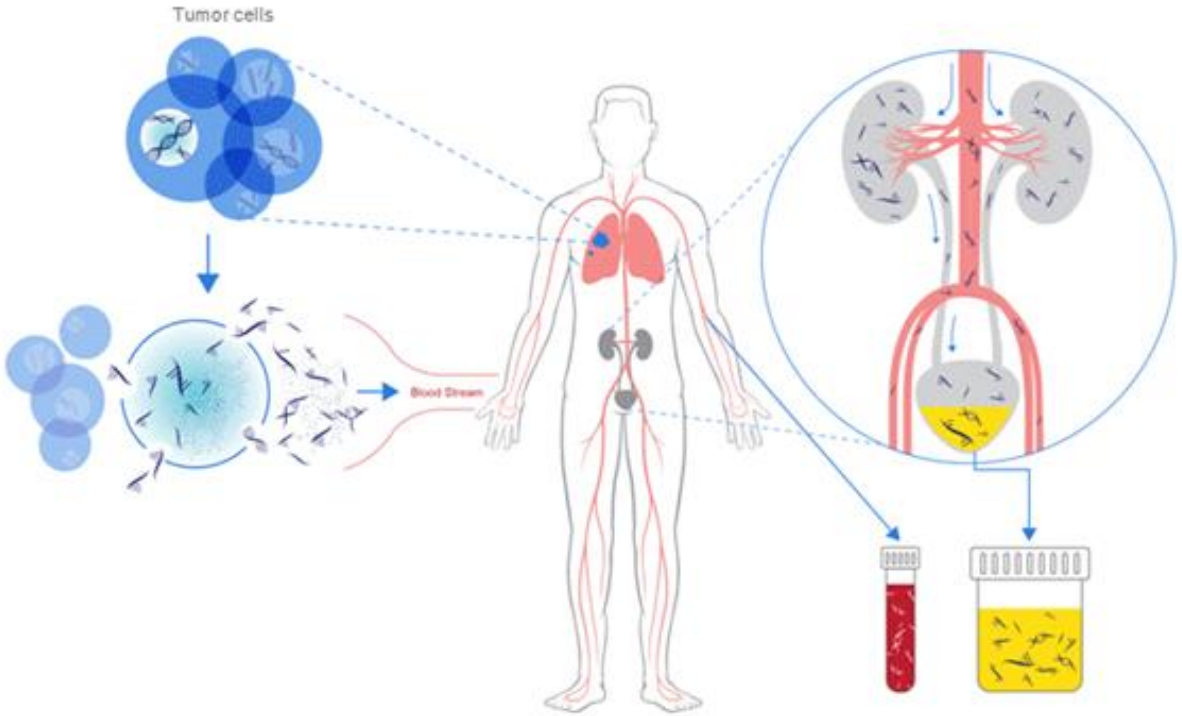
3. For surveillance of patients with a high risk for cancer recurrence

Beaver et al, *Clin Cancer Res*; 20(10) May 15, 2014; Heitzer et al, *Clinical Chemistry* 61:1 (2015)

Toma

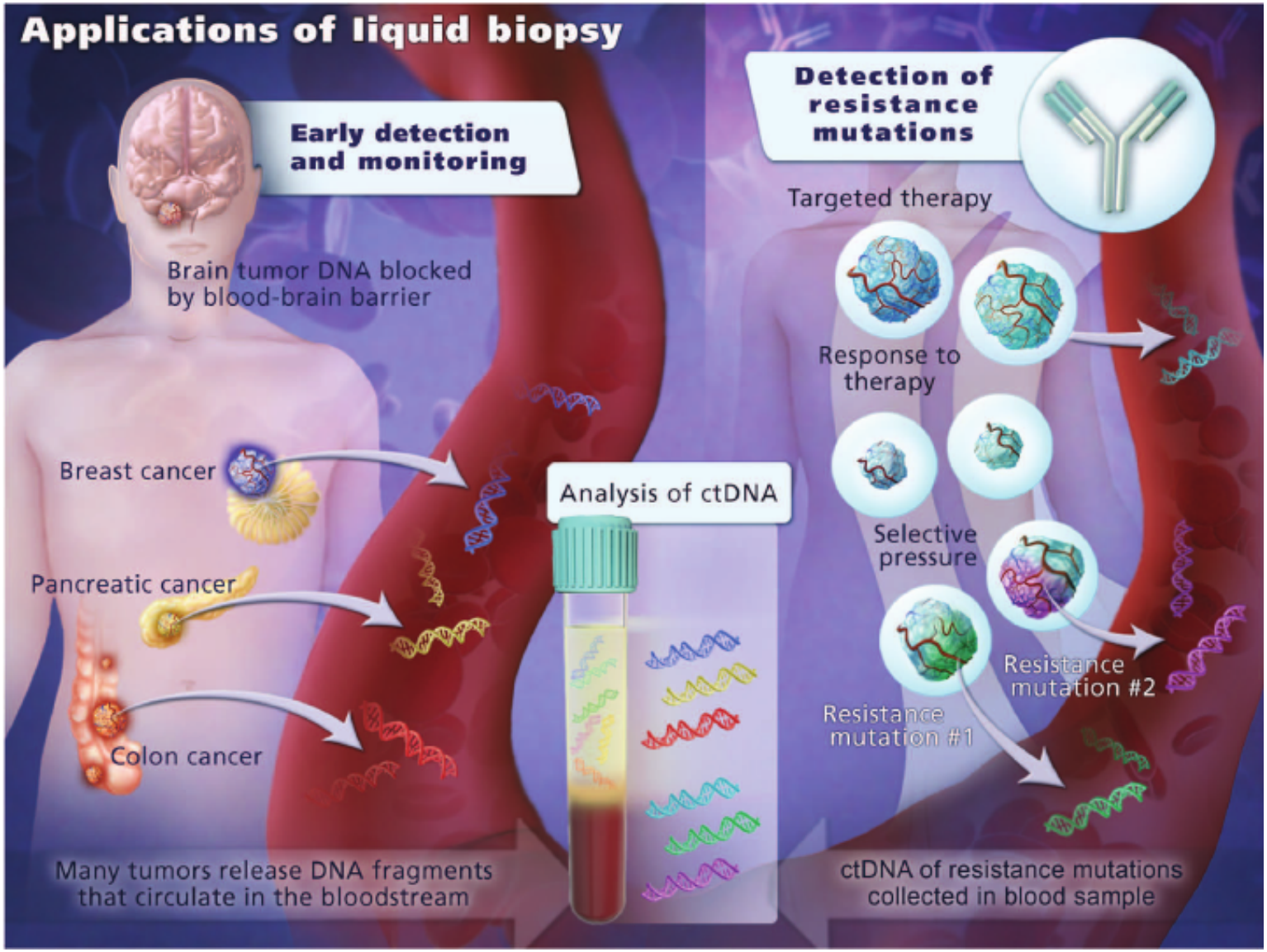

Diagnosed with early stage breast cancer
Primary tumor
Surgery
Time
Whole-genome sequencing
~5x coverage
Blood sample
Blood sample
Blood sample
Blood sample
Clinical recurrence detected by symptoms and imaging
SplitSeq
Droplet digital PCRs with rearrangement-specific primers for quantitative detection of ctDNA in blood plasma over time
ctDNA (%)
15
10
5
0
Rearrangement 1
Rearrangement 2
Rearrangement 3
Rearrangement 4
Time
Lead time
Recurrence by ctDNA
Clinical recurrence

Circulating Tumor DNA (ctDNA)



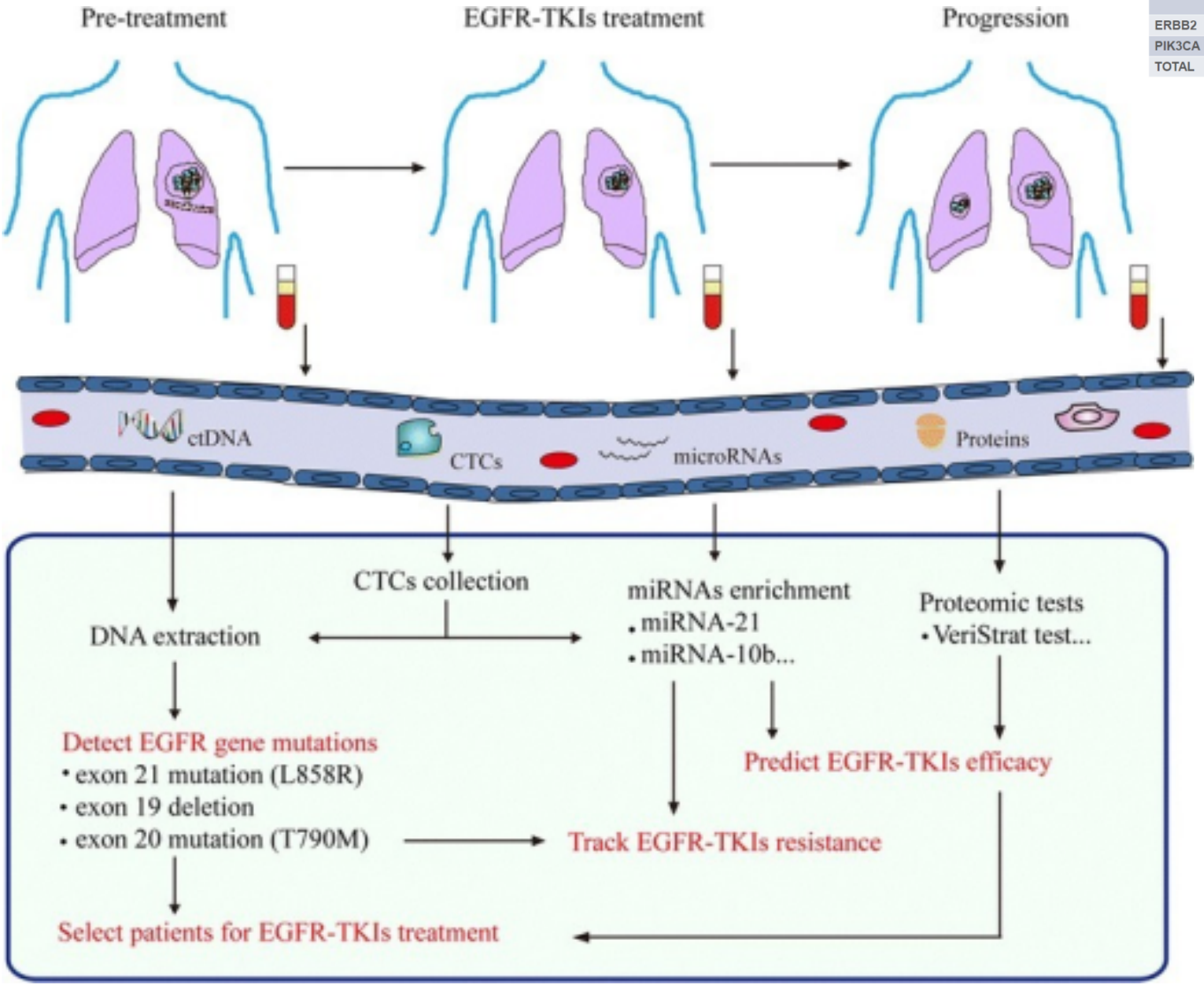
Main Advantages of ctDNA

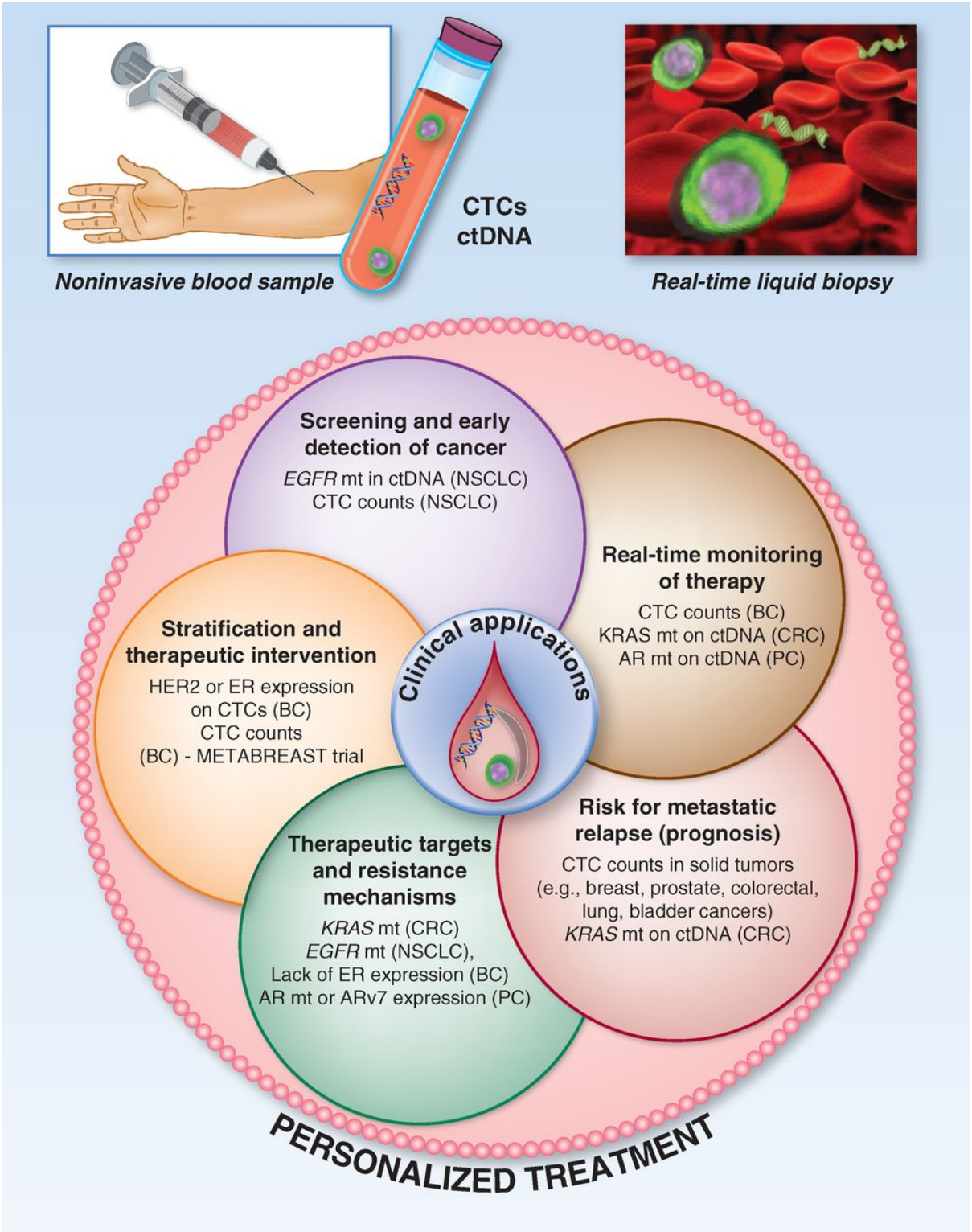
- Captures intratumor heterogeneity
- Systemic overview of cancer
- Frequent sampling options for monitoring applications
- Different analyte options depending on clinical context


Applications of liquid biopsy
Early detection and monitoring
Brain tumor DNA blocked by blood-brain barrier
Breast cancer
Pancreatic cancer
Colon cancer
Many tumors release DNA fragments that circulate in the bloodstream
Analysis of ctDNA
Detection of resistance mutations
Targeted therapy
Response to therapy
Selective pressure
Resistance mutation #1
Resistance mutation #2
ctDNA of resistance mutations collected in blood sample

New UltraSEEK™ Lung Panel

Gene	Coverage (Missense mutations)
BRAF	Codon 469 (exon 11) and codons 594, 600 (exon 15)
EGFR	E709A, E709G, E709K, E709V, G719A, G719D, G719S, G719C, S768I, T790M, L858R, L861Q, L861R, C797S, Exon 19 indels, Exon 20 insertions
KRAS	G12A, G12C, G12D, G12R, G12S, G12V, G13C, G13D, Q61H, Q61K, Q61E, Q61P, Q61R, Q61L
ERBB2	A775_G776insYVMA, G776>VC
PIK3CA	Codons 542, 545 (exon 9), codon 1047 of (exon 20)
TOTAL	5 Genes




CTCs
ctDNA
Noninvasive blood sample
Real-time liquid biopsy
Screening and early detection of cancer
EGFR mt in ctDNA (NSCLC)
CTC counts (NSCLC)
Real-time monitoring of therapy
CTC counts (BC)
KRAS mt on ctDNA (CRC)
AR mt on ctDNA (PC)
Stratification and therapeutic intervention
HER2 or ER expression on CTCs (BC)
CTC counts (BC) - METABREAST trial
Clinical applications
Therapeutic targets and resistance mechanisms
KRAS mt (CRC)
EGFR mt (NSCLC),
Lack of ER expression (BC)
AR mt or ARv7 expression (PC)
Risk for metastatic relapse (prognosis)
CTC counts in solid tumors (e.g., breast, prostate, colorectal, lung, bladder cancers)
KRAS mt on ctDNA (CRC)
PERSONALIZED TREATMENT