Laboratory Aspects of Biomarker Studies

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Disclosures

- Member, Indoc Research (NFP)
- Research partner with ThermoFisher
- Research program with Astra Zeneca
- Consultant to Labceutics, AZ

Objectives

- Understand the pre-analytic, analytic and post- analytic phases of biomarker testing
- Understand the impact of some key parameters of biomarker measurement

bi·o·mark·er

/ˈbīōˌmärkər/

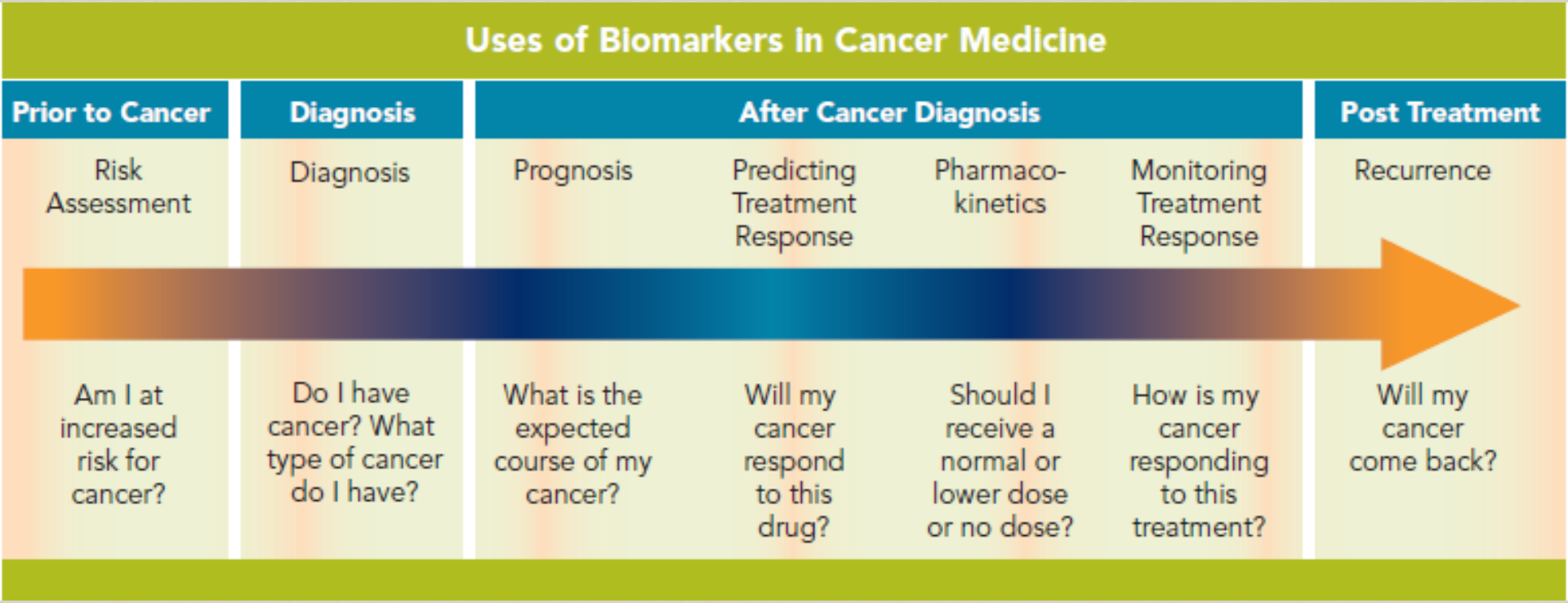
noun

a measurable substance in an organism whose presence is indicative of some phenomenon such as disease, infection, or environmental exposure.
"a biomarker that may predict aggressive disease recurrence in liver transplant recipients"

Molecular Biomarkers. A **biomarker** is a characteristic that can be objectively measured as an indicator of normal biological processes, pathogenic processes or a pharmacological response to a therapeutic intervention.

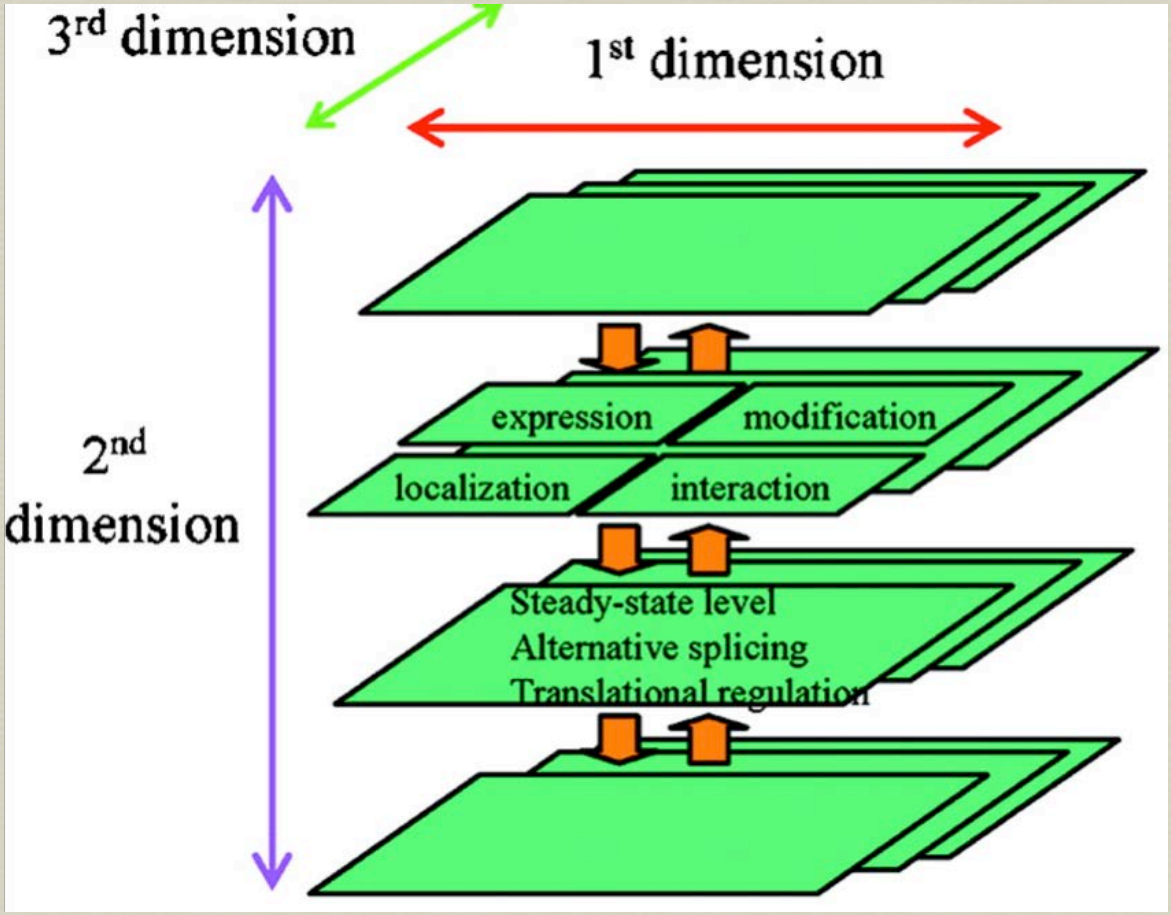
Journal of Molecular Biomarkers & Diagnosis - OMICS Group
www.omicsonline.org/**molecular-biomarkers**-diagnosis.php

What kind of question?



Source: Biomarkers in Cancer- An Introductory Guide for Advocates, Research Advocacy Network, 2010.

What kind of biomarker?



Other
- lipids
- metabolites
- physiological

Protein
- where
- which version
- functionality

RNA
- which type
- where
- when

DNA
- single nucleotide changes
- copy number changes
- methylation

Delineating biomarker studies

- pre analytical
  - sample quality, sample prep, sample suitability
- analytical
  - quality issues around library prep, amplification, sequencing run
- post analytical
  - base calling and alignment
  - clinical interpretation

Pre-analytic

Specimen

- What type of specimen?
- Is there sufficient material of interest?
- Is it a tissue type that is validated for the assay?
- Is it a sample type that is validated for the assay?

Biomarker

- What type of biomarker does the assay need?
- Is the quantity sufficient?
- Is the quality sufficient?

Analytic

Template preparation

- Is the assay targeted or global?
- Is there sufficient representation of molecules?

Generation of data

- Did the assay generate appropriate data?
- Did the assay achieve sufficient sensitivity?
- Was the quality sufficient to analyze?

Post- analytic

Analysis

- Are all QC metrics met?
- Are there variants of interest?
- Are they clinically relevant

Molecular Diagnostic Pre-Analytic Phase

Tumor Sample

FFPE Processing

Extraction

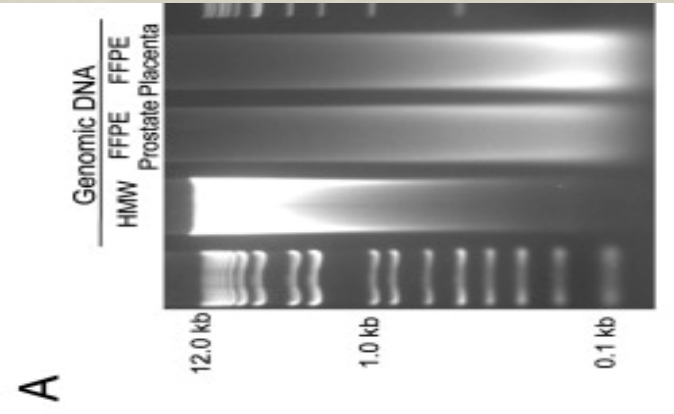
Storage

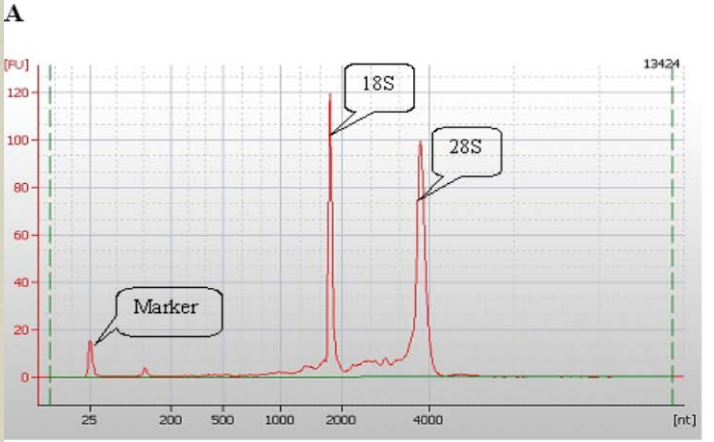
Quantification/Quality assessment

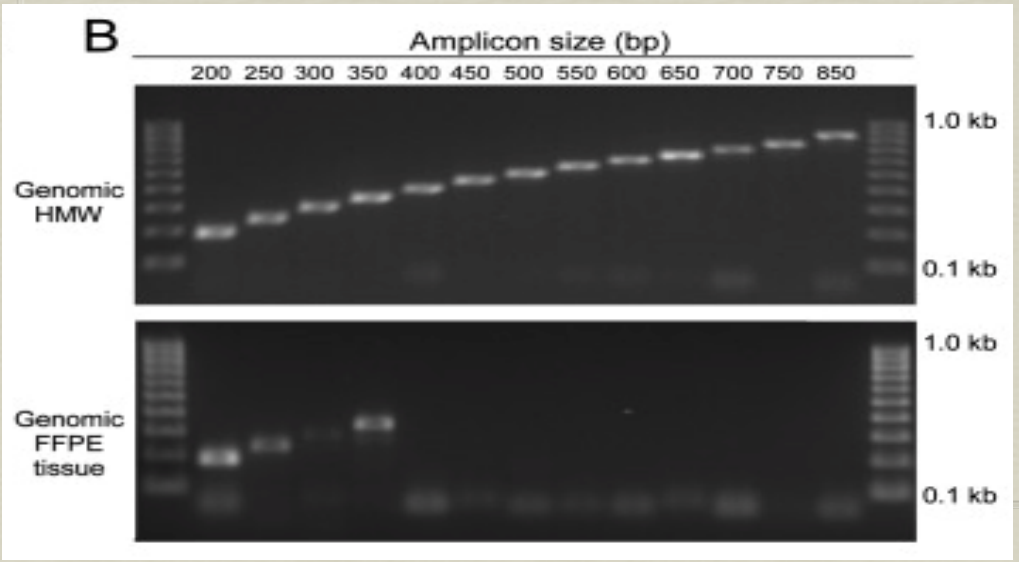
Sample considerations

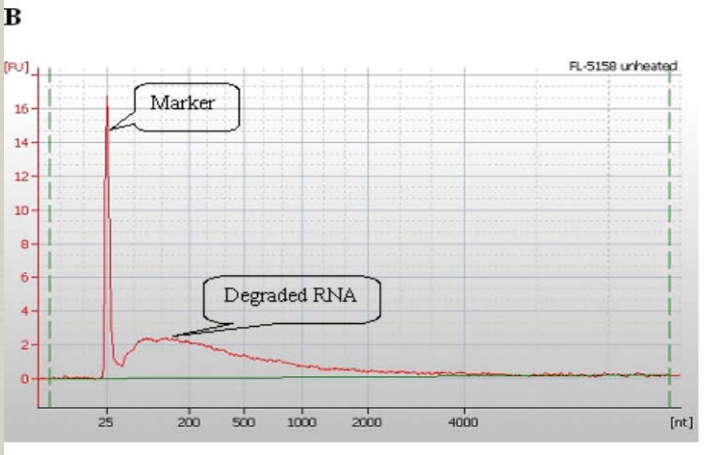
- The type of sample you have access to makes a difference in what you can measure
- The collection of the sample can influence the behaviour of a biomarker
- The processing of a sample will influence the behaviour of a biomarker
- Biomarkers don't necessarily behave the same in different sample types

Tissue Treatment and Effects









Deitrich et al, PLoS One, 2013

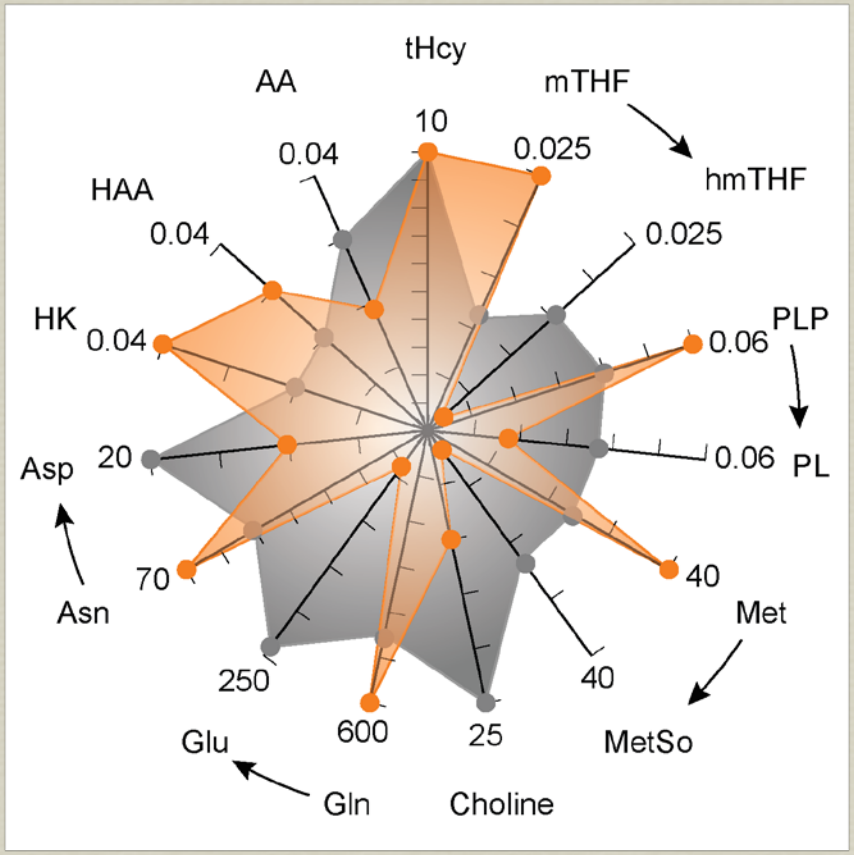
Effect of fixation on biomarkers

Table 1. FFPE-Related Preanalytical Factors Categorized by the Extent Each Has Been Investigated in the Literature for Potential Effects on DNA, RNA, Protein, and Morphology Analytes

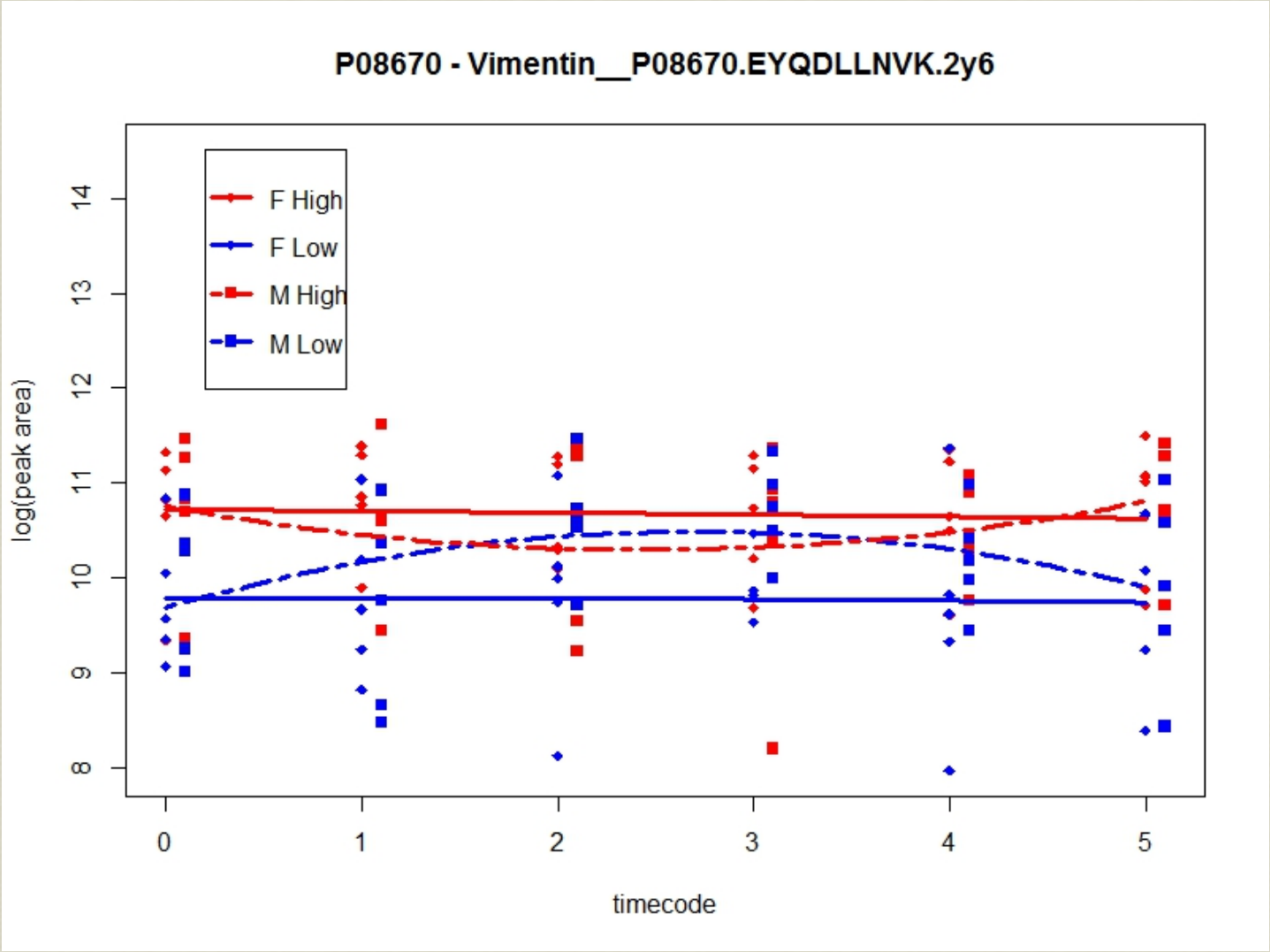
Comprehensive (All 4 Analytes Have Been Evaluated)	Incomplete (Some but Not All Analytes Have Been Evaluated)	Unexplored (No Analytes Have Been Evaluated)
Cold ischemia	Postmortem interval	Pathology ink
Decalcification	Warm ischemia time	Fixative age
Fixation duration	Specimen size	Commercial versus in-house fixative
Duration of paraffin block storage	Prefixation handling	Use of recycled formalin
	Fixative buffer	Movement during fixation
	Tissue to fixative ratio	Light exposure during fixation
	Fixation temperature	Fixation container
	Fixative delivery method	Fixation alone or with other biospecimens
	Dehydration reagent and conditions	Postfixation wash solution and conditions
	Clearing reagent and conditions	Reagents and conditions of interim alcohol storage
	Paraffin embedding reagent and conditions	Use of recycled dehydration and clearing reagents
	FFPE block size or section thickness	Automated versus manual processing
	Type of slide or adhesive	Use of recycled paraffin for impregnation and embedding
	Slide drying duration and temperature	Embedding conditions
	Storage duration of slide-mounted FFPE sections	Slide pretreatment
		Equipment and conditions of sectioning and section transfer

Taken from Bass et al, (2014)Archives of Pathology & Laboratory Medicine: Vol. 138, No. 11, pp. 1520-1530.

Stability of dynamic markers

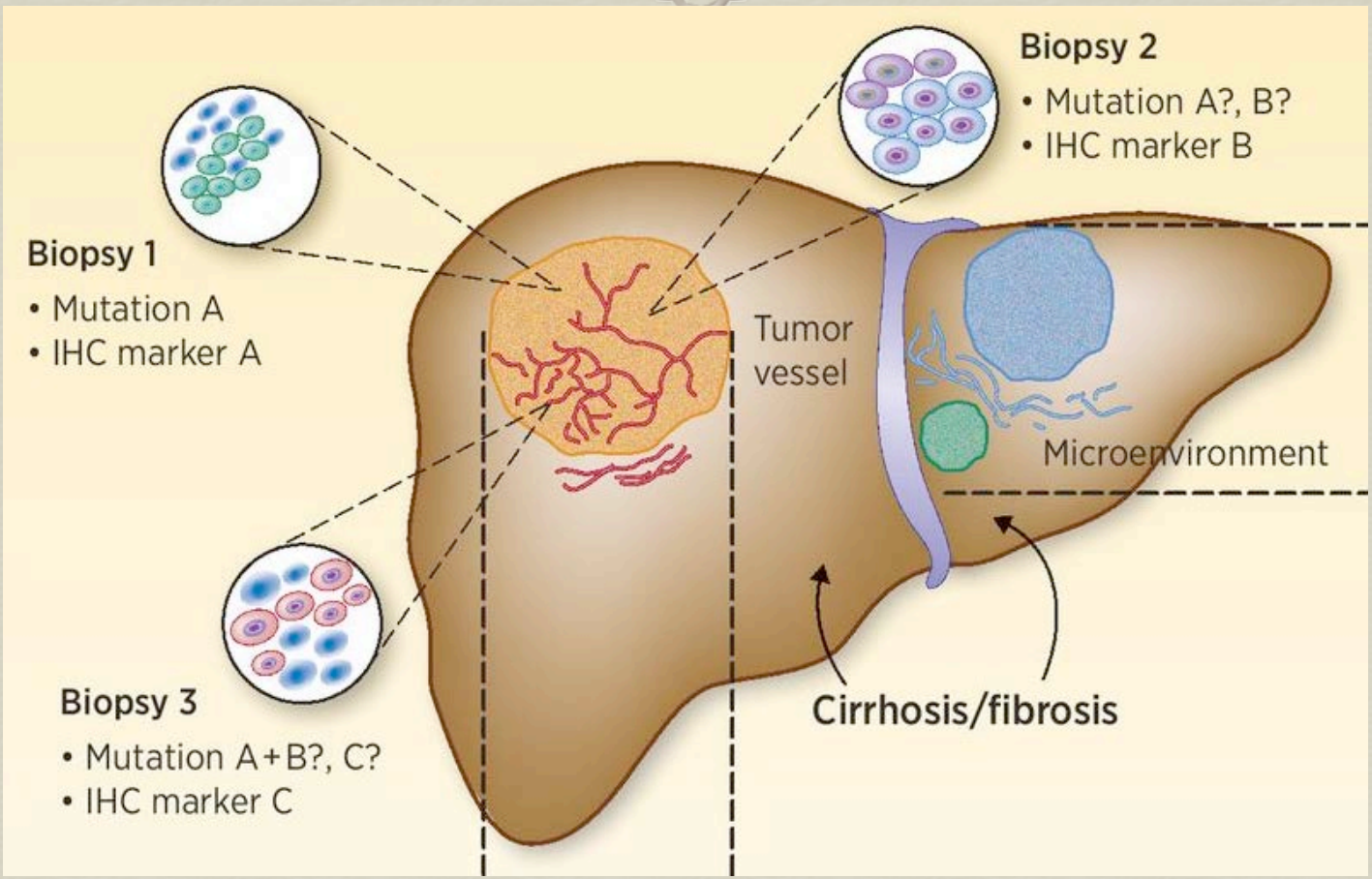


Biomarker profile of selected metabolites in fresh (orange) and improperly stored (grey) serum/plasma. Arrows indicate conversions, and concentrations are given in µmol/L.

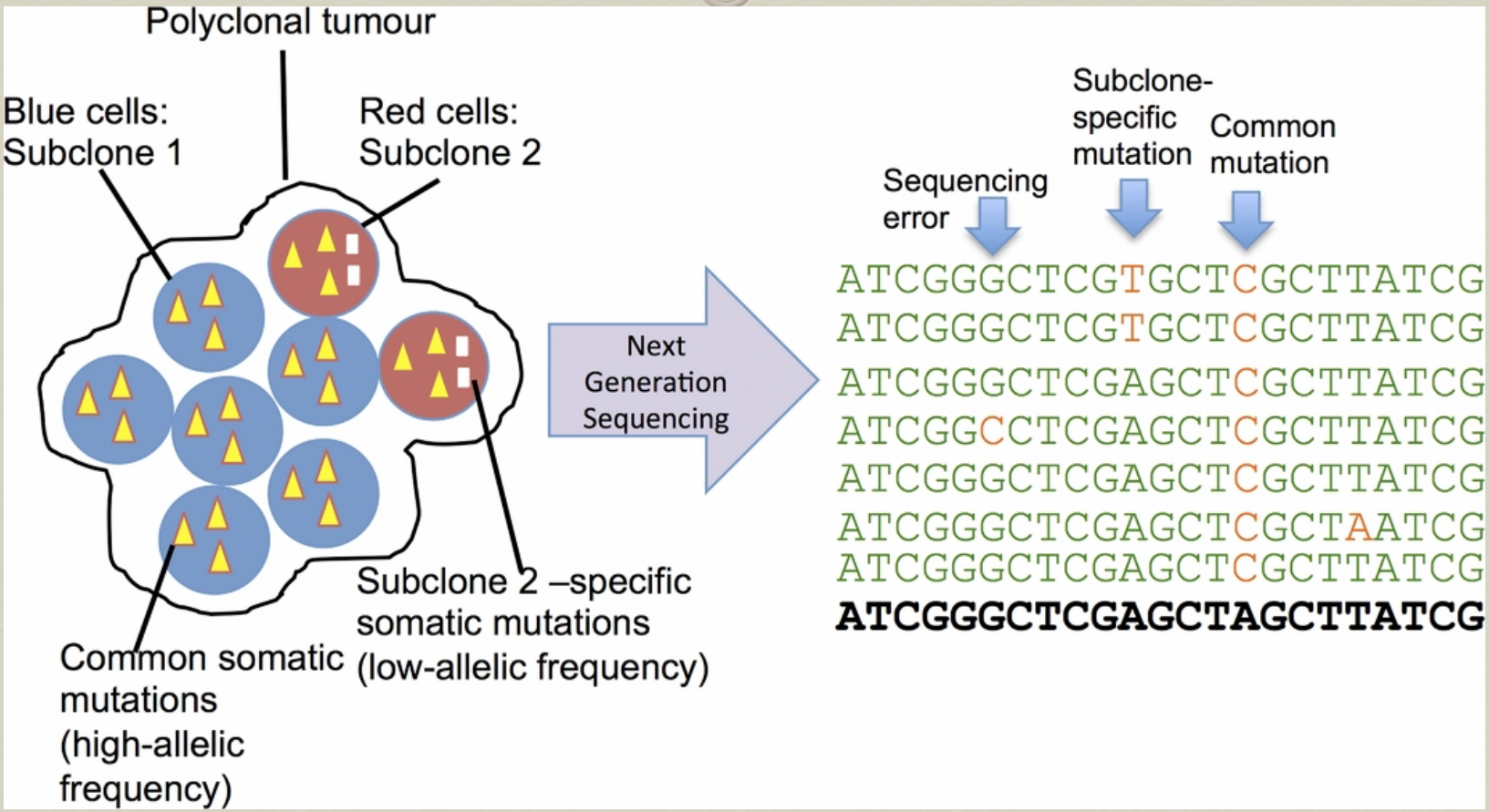

P08670 - Vimentin__P08670.EYQDLLNVK.2y6
F High
F Low
M High
M Low
log(peak area)
8
9
10
11
12
13
14
0
1
2
3
4
5
timecode

Tumor Heterogeneity

You can miss a biomarker, even if it is there



Even if you sample in the optimal area…



, Sutton, Graham 2013, Hum Mutn 34:1432-1438. Stead

Limit of detection

For high input:

$$\frac{4\ reads\ with\ mutation\ of\ interest}{\#\ reads} = Limit\ of\ detection$$

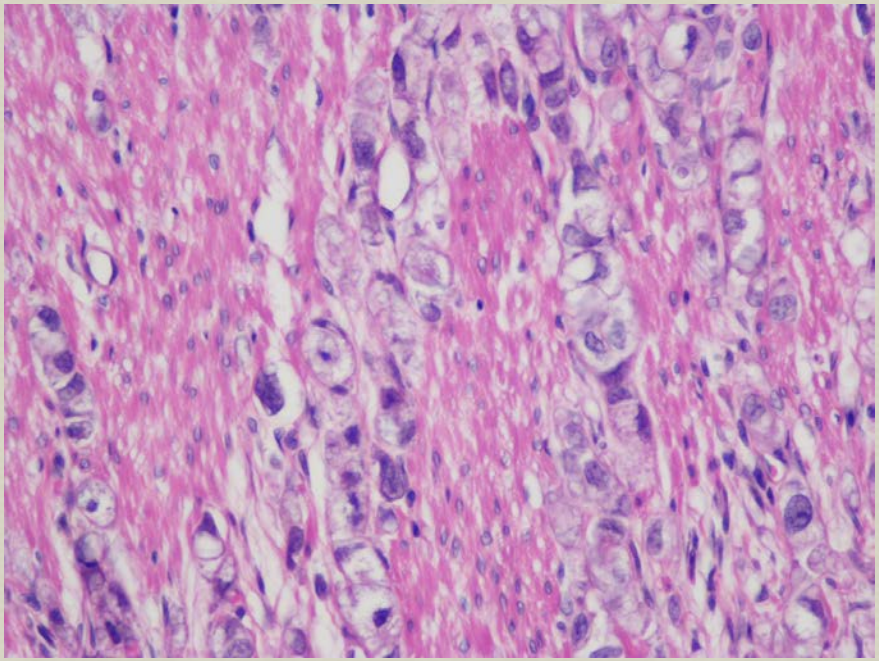
$$\frac{4\ reads\ with\ mutation\ of\ interest}{\#\ reads} = 0.01\%\ desired$$

$$\#\ reads = 40{,}000x!$$

For Research Use Only

Estimating tumour %

- Roughly
  - 100 tumor nuclei
  - 200 normal nuclei
  - Tumor cellularity 33%

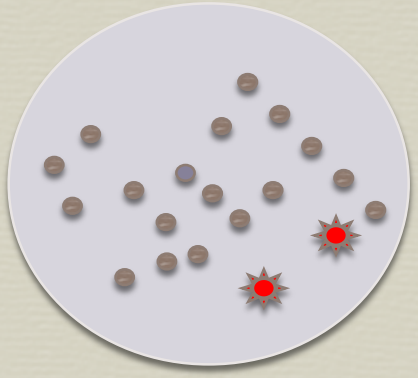


How accurate is this?

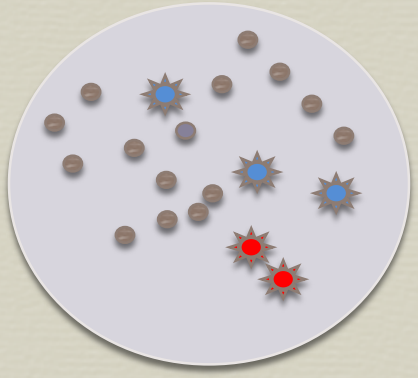
- Tumors are not always diploid
  - If tetra-ploid: 2x 2N DNA content
    - 50% tumor DNA and 50% normal DNA
  - If octo-ploid: 4x 2N DNA content
    - 66% tumor DNA and 33% normal DNA
  - If hypodiploid (34 chromosomes, 0.75x 2N DNA content in tumor)
    - 27% tumor DNA 63% normal DNA

- Tumour nuclei are different sizes compared to normal
  - Tumor nuclei at least 3x longer in one dimension than normal (and more variable in shape)
    - Normal nuclei 5um diameter spheres (these are muscle nuclei so I realize they are probably tapered in the Z dimension, but for math sphere is easier)
      - ~65 um^3 volume
    - Tumor nuclei 15um diameter spheres
      - ~1767 um^3 volume (27 x larger)

Relationship of variant frequency to % tumour cells



10% tumour cells
100% of the tumour cells heterozygous
Therefore, the variant is present at 5%



25% tumour cells
40% of the tumour cells heterozygous
Therefore, the variant is present at 5%

Analytical considerations

- have to do with the parameters of the assay itself
- how it is run, who runs it, when, what quality metrics are available
- are machines calibrated
- are controls included
- are limits of detection known
- are pipelines locked and applied

Analytical validity

Sensitivity
How often is test positive when biomarker is present?

Specificity
How often is test negative when biomarker is not present?

Robustness
How repeatable and reproducible is assay within and between labs?

Stability
What is the impact of sample parameters on test result?

Clinical validity

Sensitivity
How often is the biomarker present when disease or outcome is present?

Specificity
How often is the biomarker not present when disease or outcome is not present?

PPV
What is the probability that someone with a positive test will have the disease or outcome?

NPP
What is the probability that someone with a negative test will not have the disease or outcome?

Clinical utility

The likelihood that the test result informs clinical decision making and improves outcome

Analytical pitfalls

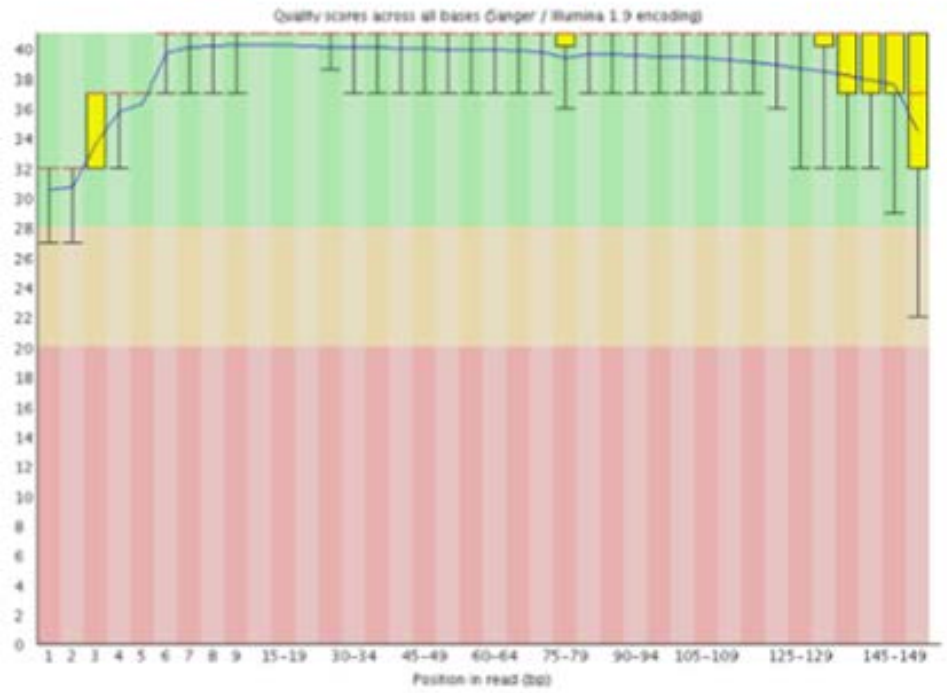
Most of the analytic pitfalls need to be identified and dealt with by the lab

As end users of the data, you will not likely need to deal with these factors

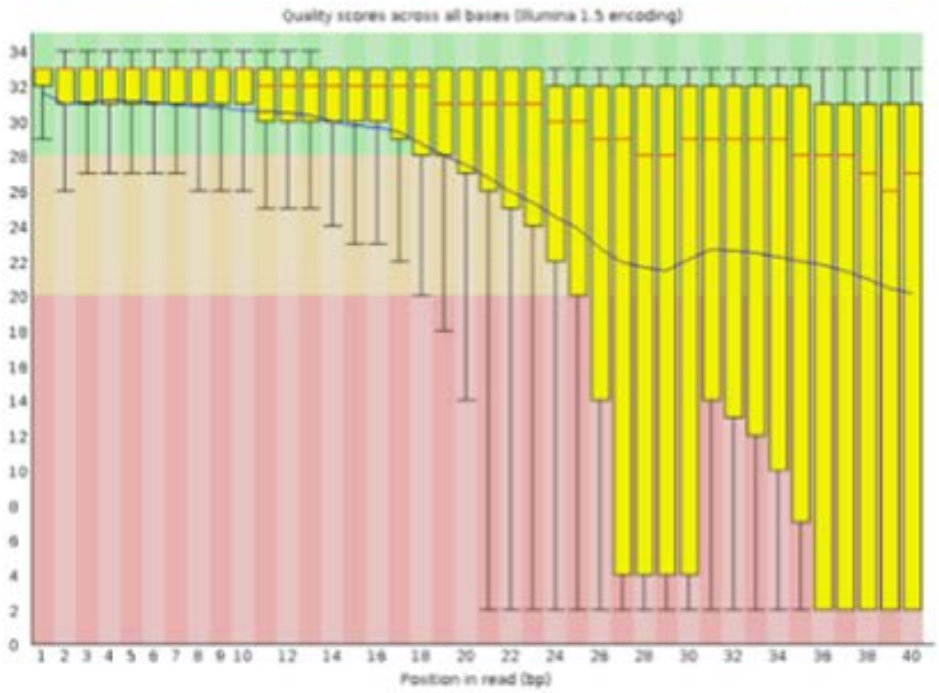
But a well informed end user will be aware that these factors exist

FastQC: Per base sequence quality

Good data



Bad data

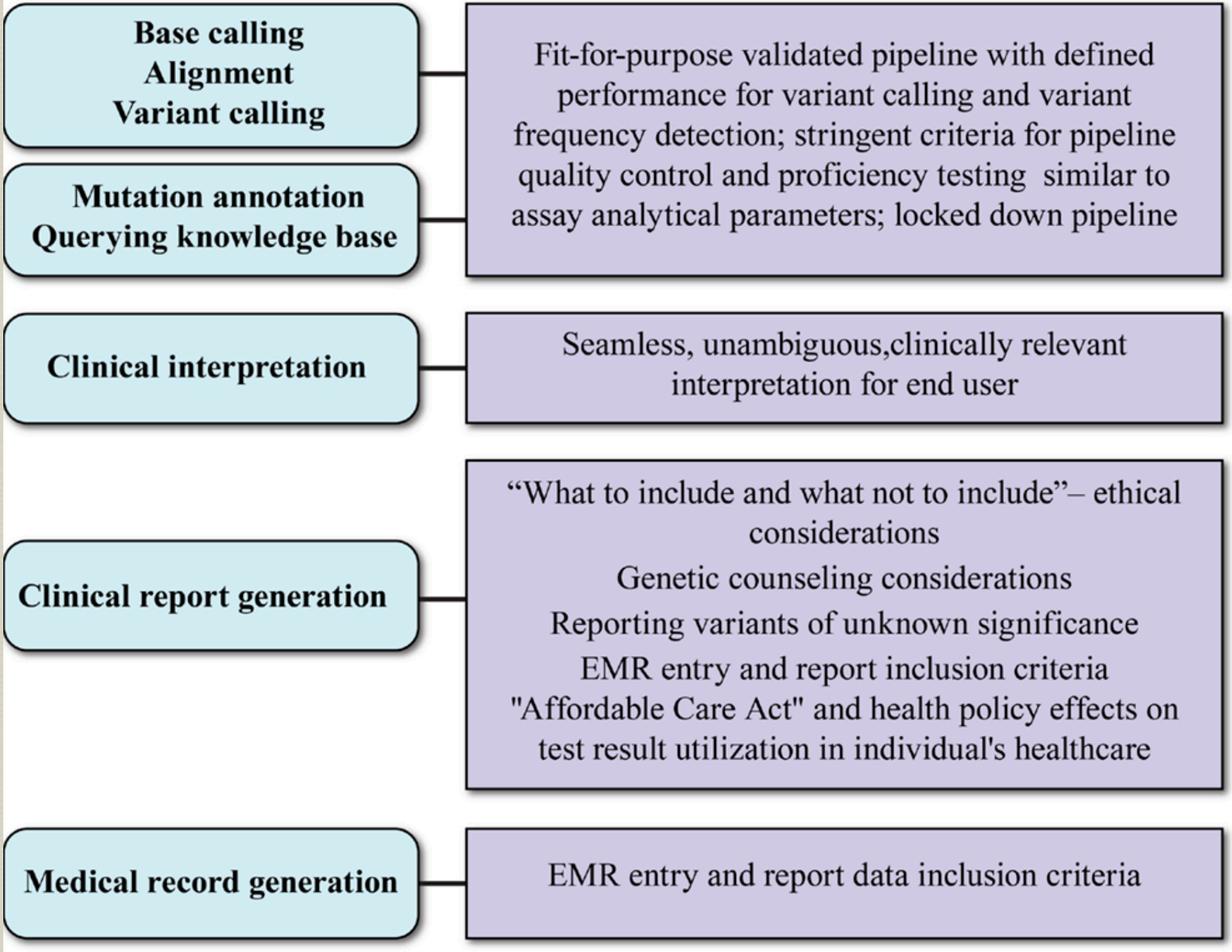


Relationship Between Sequencing Quality Score and Base Call Accuracy

Quality Score	Probability of Incorrect Base Call	Inferred Base Call Accuracy
10 (Q10)	1 in 10	90%
20 (Q20)	1 in 100	99%
30 (Q30)	1 in 1000	99.9%

Post analytical considerations

- Are QC metrics established and did the assay meet these?
- How confident can you be in the variant calling?
- What is the difference between variant calling (biomarker identification) and variant interpretation (clinical interpretation)?
- Defining when a biomarker is present or absent is part of the locked pipeline for the assay
- Interpreting what the presence or absence or behaviour of a biomarker means in the clinical context is different


Base calling
Alignment
Variant calling
Mutation annotation
Querying knowledge base
Fit-for-purpose validated pipeline with defined performance for variant calling and variant frequency detection; stringent criteria for pipeline quality control and proficiency testing similar to assay analytical parameters; locked down pipeline
Clinical interpretation
Seamless, unambiguous,clinically relevant interpretation for end user
Clinical report generation
“What to include and what not to include”– ethical considerations
Genetic counseling considerations
Reporting variants of unknown significance
EMR entry and report inclusion criteria
"Affordable Care Act" and health policy effects on test result utilization in individual's healthcare
Medical record generation
EMR entry and report data inclusion criteria

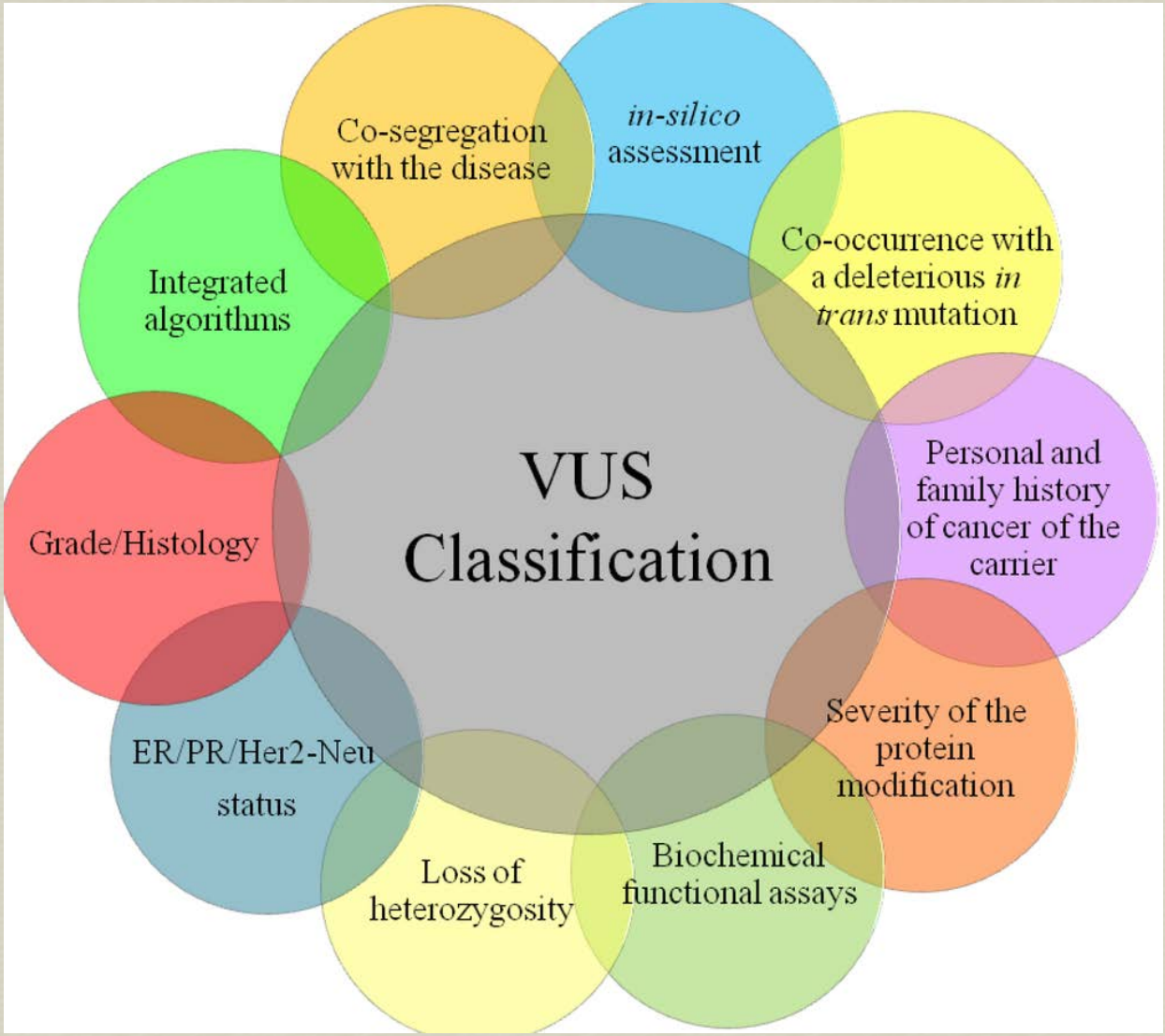
Variant interpretation

- Biomarker of interest is BRCA2- looking for variants in the coding sequence
- identify a variant that is c.9976A>T which results in a stop codon being inserted at codon 3326
- c.9976A>T, p.Lys3326*
- Because protein truncation is a mechanism of loss of BRCA2 activity, one might consider this to be a pathogenic finding

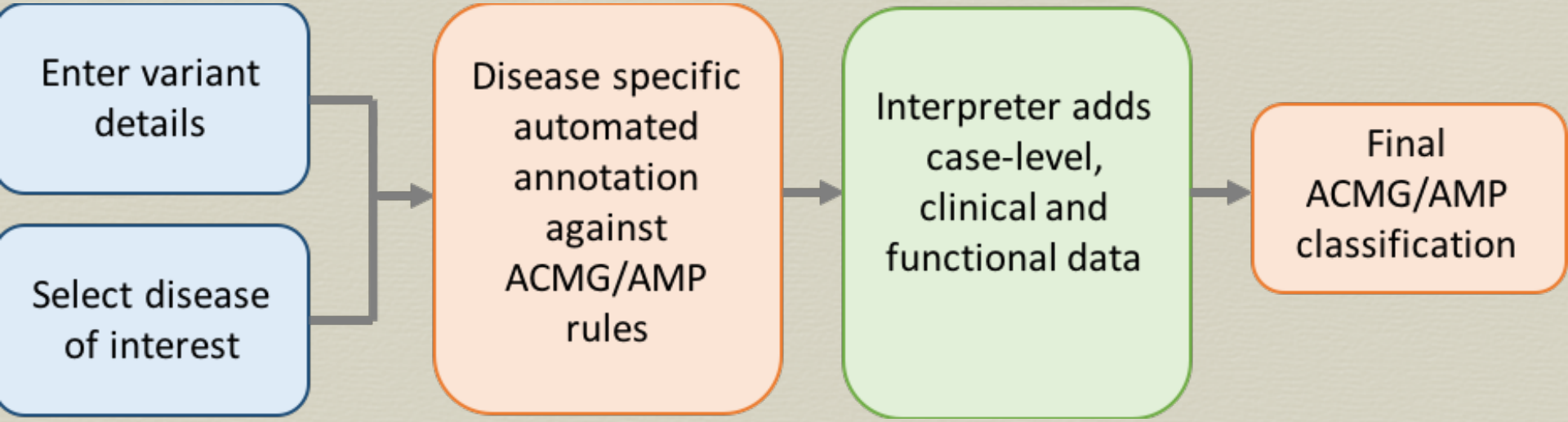
Characteristics of this variant

- occurs at the end of the protein, in a region with little functional impact
- occurs in the general population at frequencies well above the frequency of hereditary breast cancer
- all major interpretation sites list it as benign
- BUT there is limited evidence that it actually confers an increased risk of pancreatic cancer

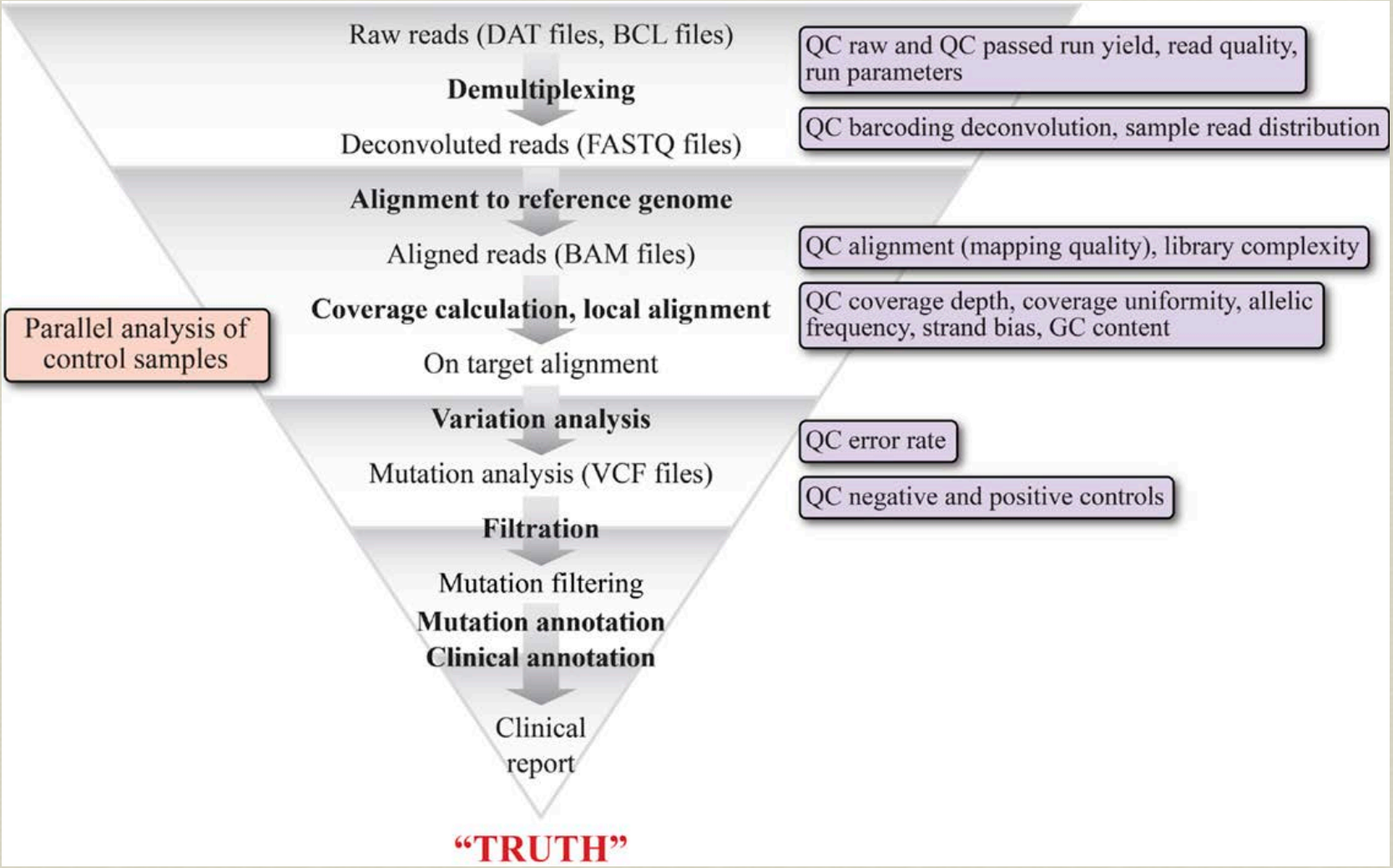
Variant interpretation



Tools for standardization of interpretation



Before you get it…



Front. Oncol., 17 April 2014 | http://dx.doi.org/10.3389/fonc.2014.00078

Conclusions

- What kind of question are you asking?
- What kind of biomarker will you measure?
- What type of sample will you have access to?
- How will your sample have been handled?
- How will your measurements be made?
- How reliable is your biomarker measurement?