UNIKLINIK KÖLN

29th January 2018
BRCA Tumour Testing
Masterclass - Jerusalem

Review of methodologies for tumor testing; highlighting experience from University Hospital Cologne

29.01.2018 | **Carina Heydt**

Agenda

- How to conduct *BRCA* tumour testing:
  - Preparation of tissue for testing (Macro-dissection, DNA extraction & quantification)
  - Review of available methodology
  - Demonstration of commercial kits and equipment
  - Sequencing
  - Factors which may influence your choice of methodology/kit/equipment
  - Ensuring a good quality test/ result

Is it possible to use your existing germline process for sporadic tumour material?

Germline vs. somatic mutations

Germline mutation

- Present in all body cells
- heterozygous
- Can be inherited
- Cause cancer family syndrome
- >50% Allele frequency of mutation

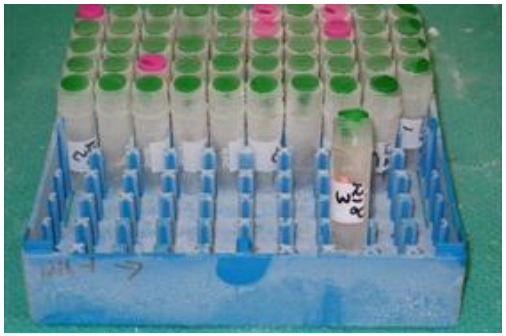
Somatic mutation

- Occur in tumour tissue only
- Cannot be inherited
- Allele frequency of mutation depends on percent of tumour cells (>10%) and normal cells
- Mutation detection depends on quality of tumour material (FFPE)

http://www.selectscience.net

http://www.minalevidds.com

Standard in Pathology



http://www.braintumour.ca

How is the tissue prepared for DNA extraction?

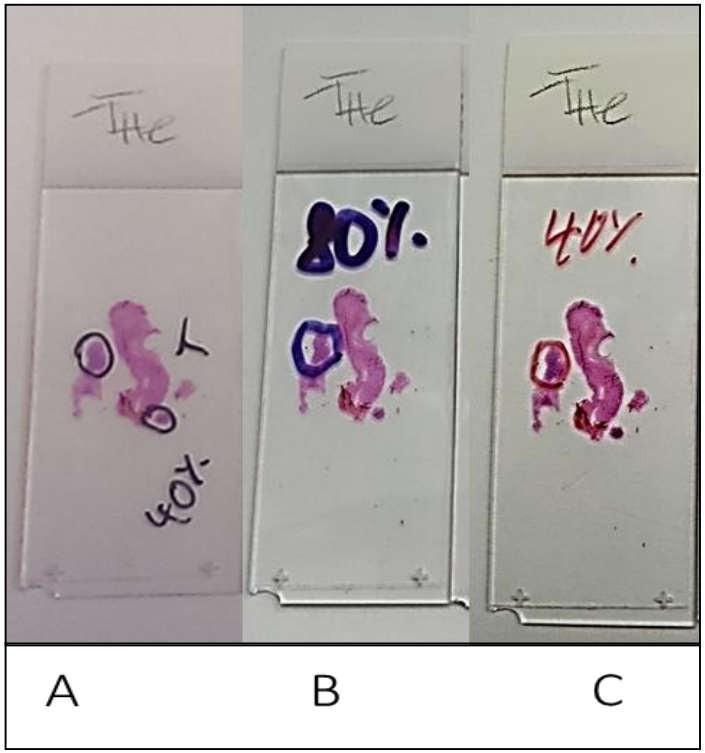
Histological check by pathologist

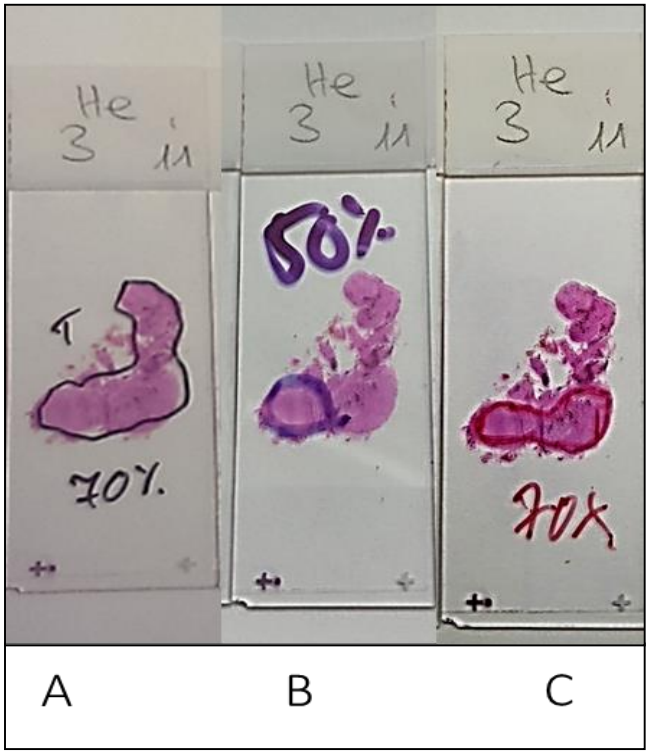
- Buffered formalin is essential

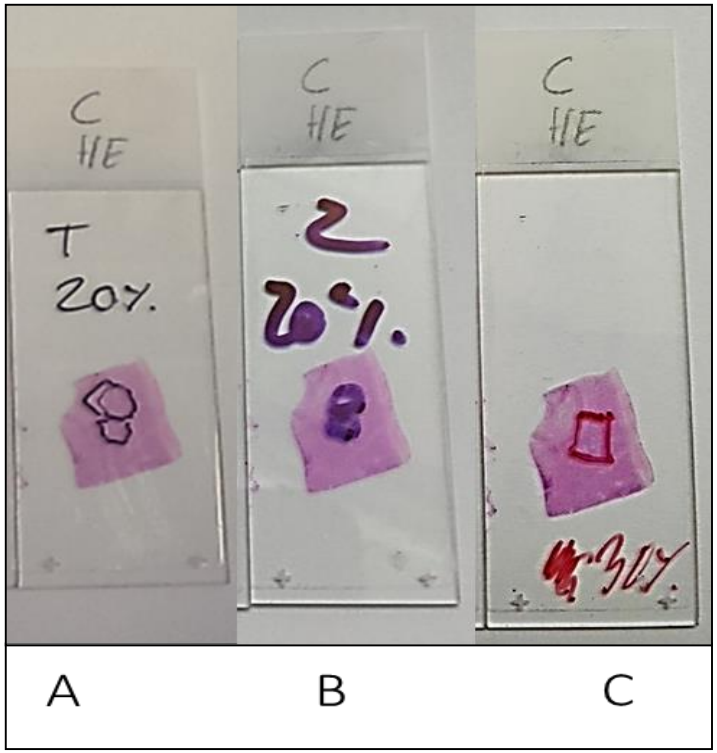
- Examining haematoxylin and eosin (HE) stained slide
- Highlighting the tumour area precisely
- Little as possible normal cells should be included
- Somatic mutations occur in tumour tissue only
- Pen should be indelible
- Estimating of tumour cell content (Ratio normal/tumour cells)
- At least 10-20% depending on method used

Microscopic examination

Tumour area and tumour cell percentage estimated by three different pathologists

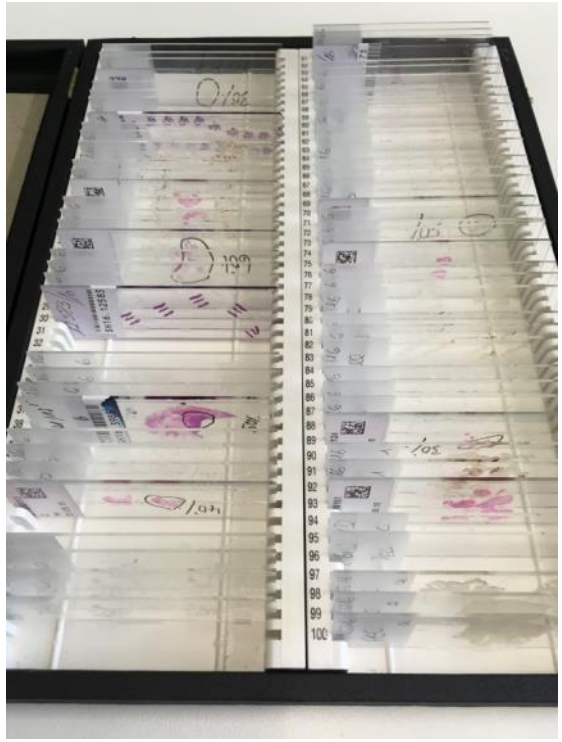




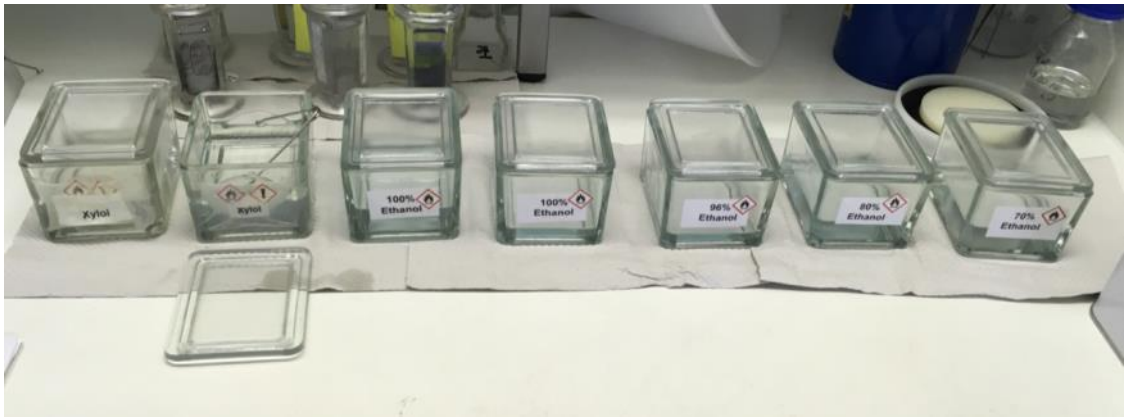


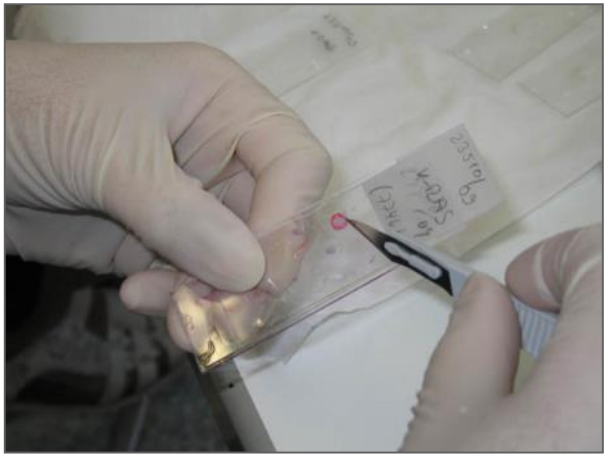
Section cutting for macrodissection

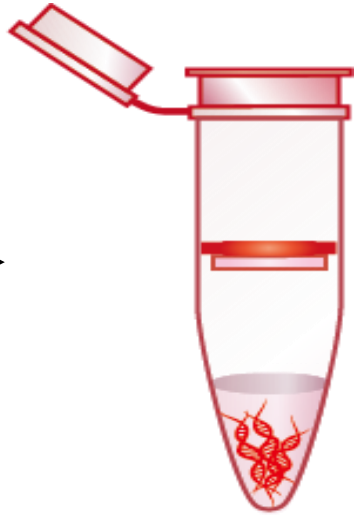
- Three to nine 10 µm thick slides depending on size of tumour area
- Avoid cross contamination between samples
- Use fresh blade for each tissue block
- Clean water bath



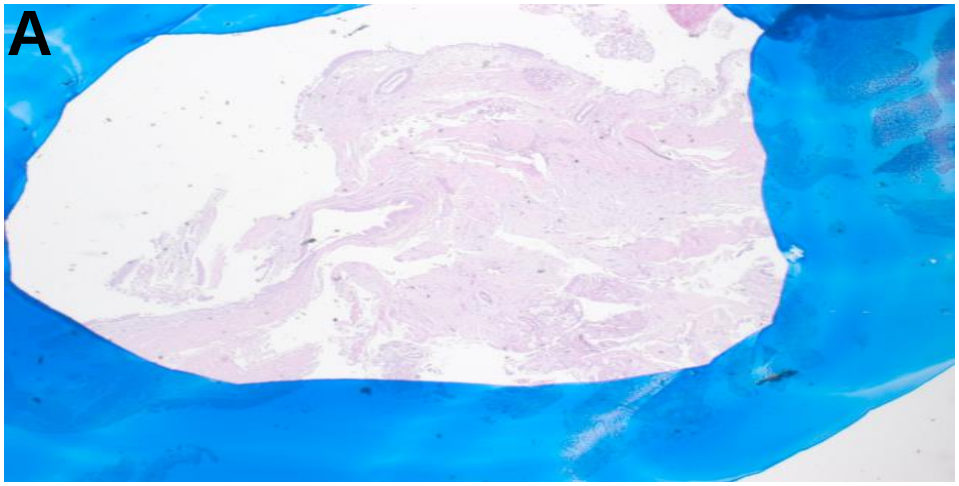
Deparaffinization and macrodissection of tumour area



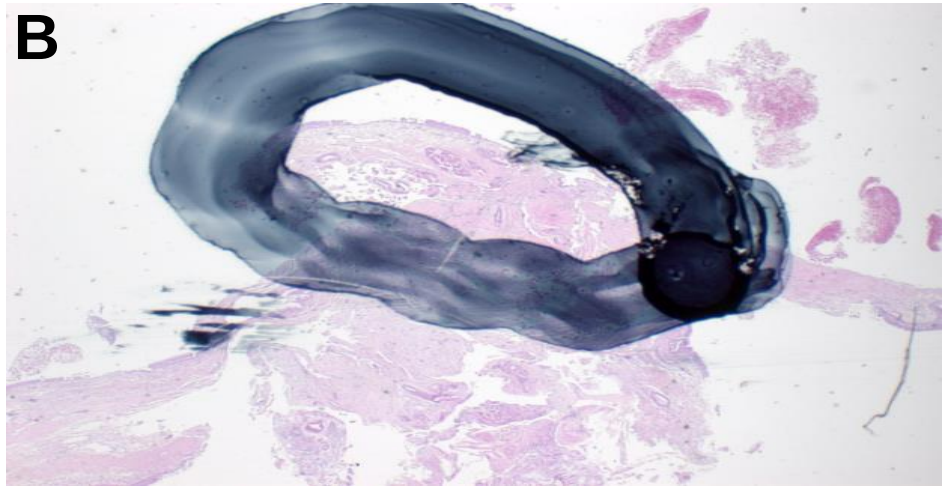




Importance of exact macrodissection



Institute B



Institute C

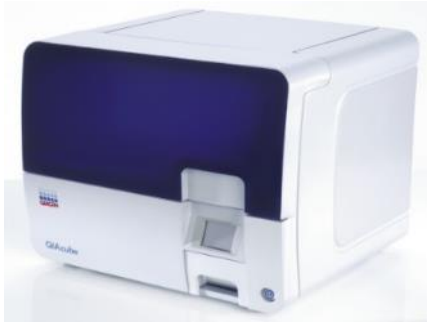
Platform	EGFR mutation status	allele frequency %	DNA conc. (ng/µl)
Institute B	19: c.2236_2250del p.E746_A750del	14	17,32
Institute C	19: c.2236_2250del p.E746_A750del	54	3,78

Fassunke et al., Int J Mol Med, 2015

How is the DNA extraction step conducted?

Options for DNA extraction methods

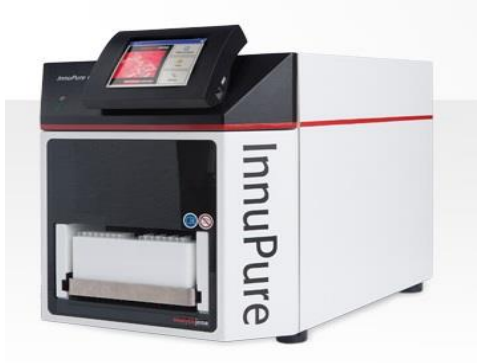
Automated systems



QIAcube (Qiagen)

QIAsymphony SP (Qiagen)

Maxwell 16 (Promega)



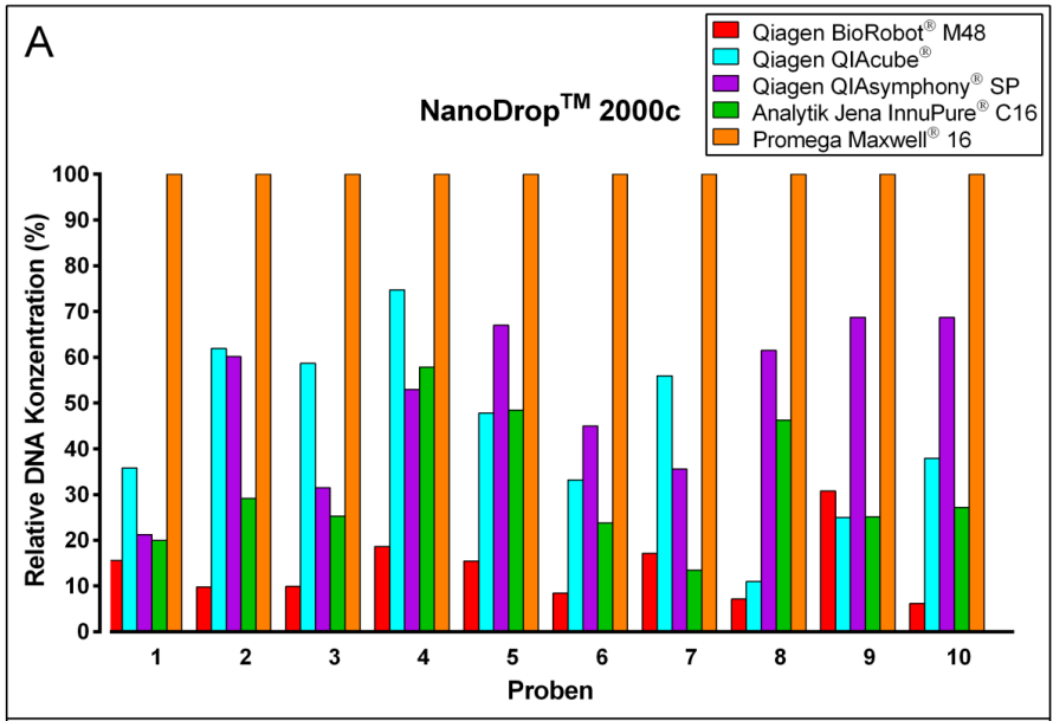
InnuPure C16 (Analytik Jena)

Manual kits

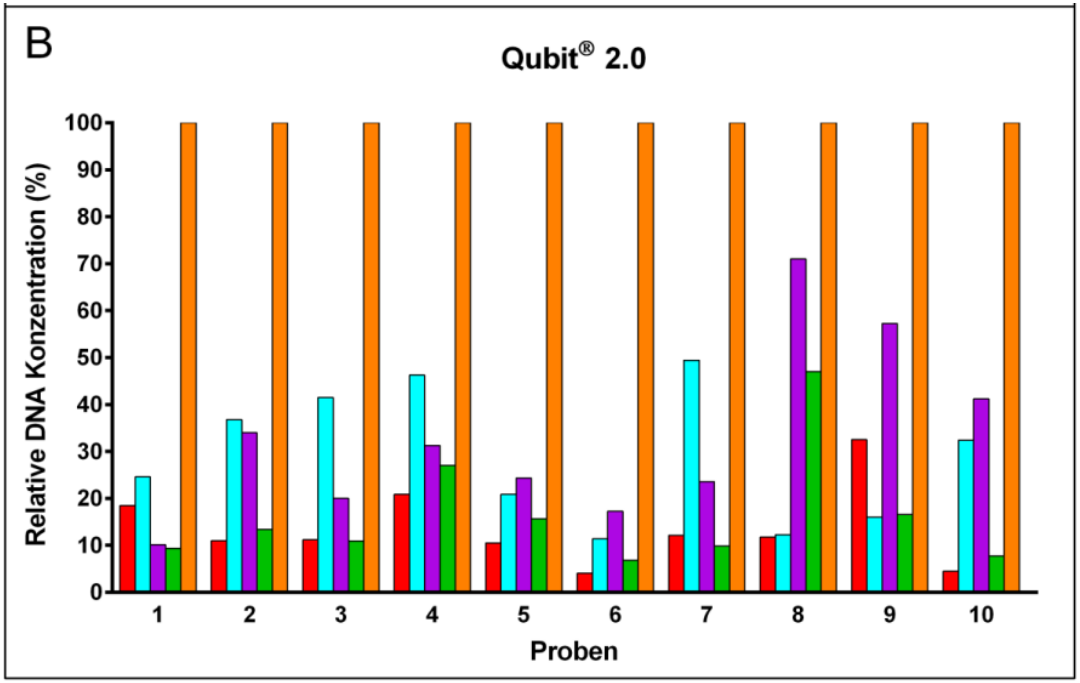
- QIAamp DNA FFPE Tissue Kit (Qiagen)
- RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific)
- ReliaPrep™ FFPE gDNA Miniprep System (Promega)
- Custom FFPE DNA extraction methods

and many more

Options for DNA extraction methods



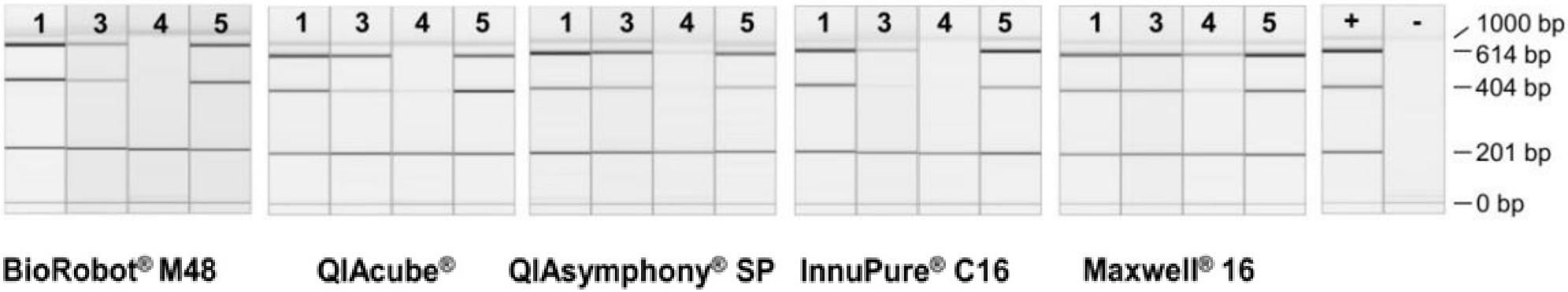
- automated DNA extraction on different devices leads to different yields
- important for the extraction of small biopsies (up to 75% of cases in lung cancer diagnostics)



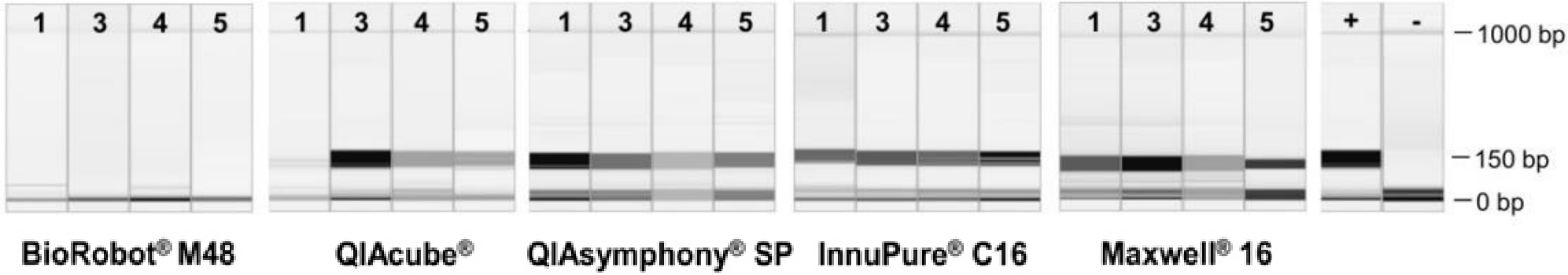
Heydt et al., Plos ONE, 2014

Options for DNA extraction methods

Performance of single-plex PCR:

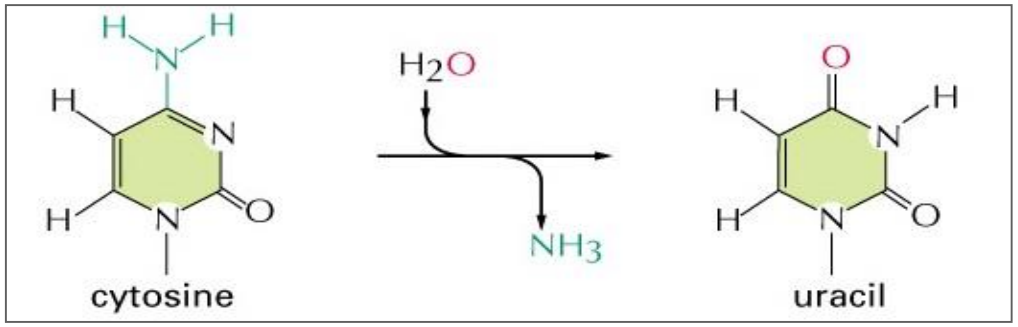


Performance of multi-plex PCR:

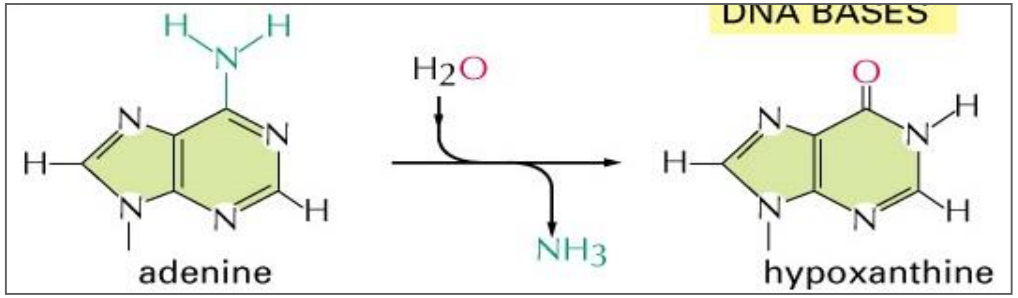


Heydt et al., Plos ONE, 2014

Fixation artefacts



⇒ transition **G>A**



⇒ transition **T>C**

- formalin fixation leads to DNA artefacts by deamination
- Deamination of cytosine leads to uracil, deamination of adenine to hypoxanthine
- Wrong bases are incorporated during PCR
- Look like point mutation
- All types of transition may occur: C>T, G>A, A>G, T>C

Key issues with formalin-fixed paraffin-embedded (FFPE) DNA extraction

- Buffered formalin is essential
- Precise highlighting of tumour area on HE stained slide by pathologist
  - Varying tumour cell content, for NGS >10%
  - Mutations in tumour cells only
- Exact macrodissection of tumour area with scalpel
- You have to avoid cross-contamination during section cutting with a microtome and during macrodissection with other samples
- Complete deparaffinization and lysation
- Use the system that give the highest DNA quality and quantity and best results in downstream applications
- Avoid too high salt concentration in DNA solution
- You have to keep in mind that:
  - Formalin leads to artefacts by deamination (G<>A and C<>T artefacts)
  - FFPE DNA is mostly highly fragmented
  - Varying quality of FFPE material depending on tissue degradation and appropriate formalin fixation

Is there a minimum amount of DNA needed to continue to the next step?

DNA quantification methods

- **UV absorbance**
  - NanoDrop® (Thermo Fisher Scientific)

- **Fluorescent dye-based quantification**
  - Qubit® 2.0/3.0 Fluorometer (Thermo Fisher Scientific)
  - Quantus™ Fluorometer(Promega)
  - Quant-iT™ PicoGreen® dsDNA Assay (Thermo Fisher Scientific)

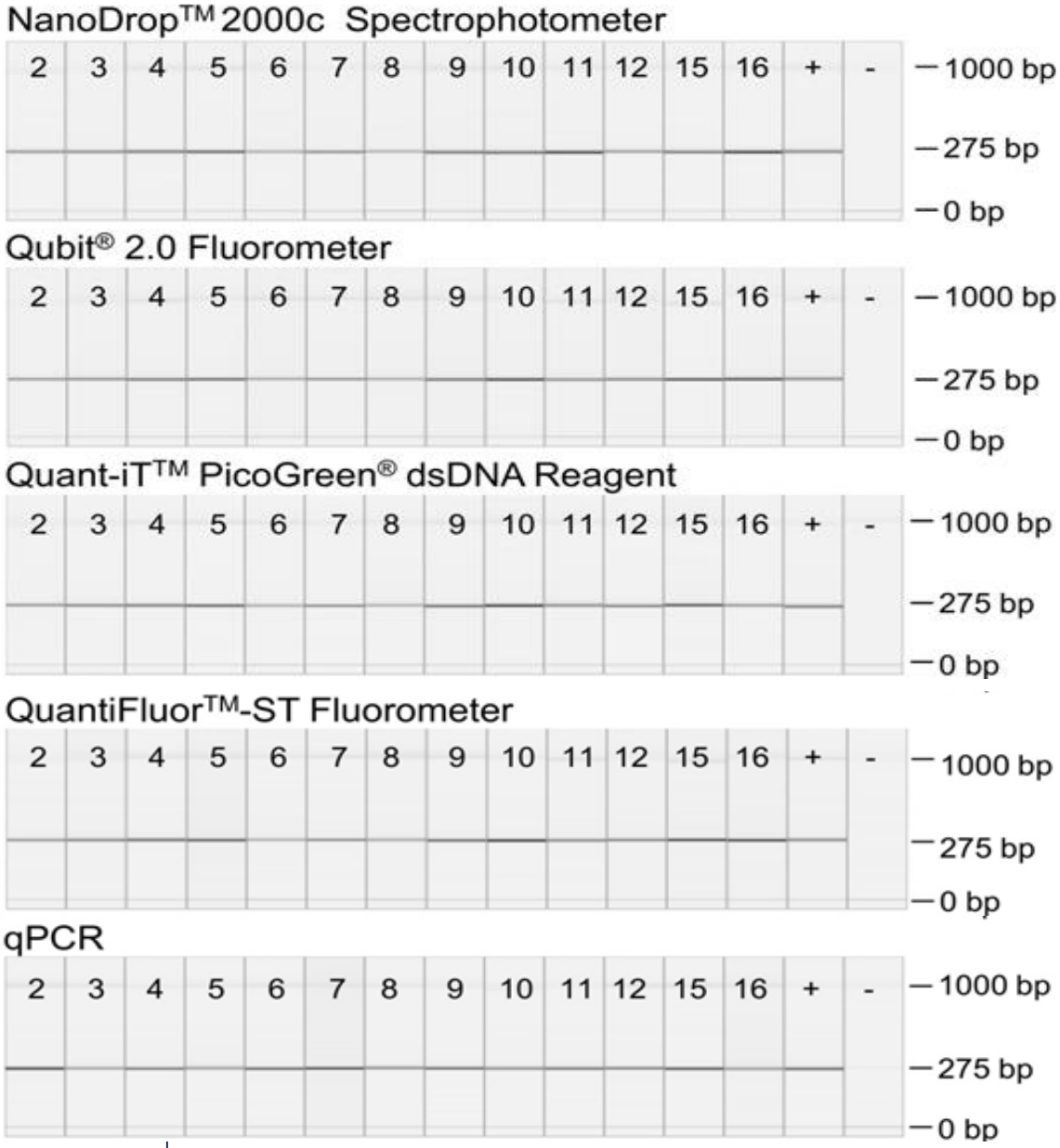
DNA quantification methods

- **qPCR (custom primers)**
  - SsoFast™ EvaGreen® Supermix (BioRad)
  - GoTaq® qPCR Master Mix (Promega)

- **qPCR (commercially available)**
  - GeneRead DNA QuantiMIZE Kit (Qiagen)
  - KAPA hgDNA Quantification and QC Kit (KapaBiosystems)
  - TruSeq FFPE DNA Library Prep QC Kit (Illumina)
  - Agilent NGS FFPE QC Kit (Agilent)

Manufacturers of FFPE tumour testing assays mostly advice you which DNA quantification you should use with their assay.

Comparison of quantification methods



- 20 ng DNA extracted with the Maxwell system and determined with each of the measurement techniques of were amplified

⇒ no difference in amplification

⇒ the same is true for multiplex PCR

What are the factors involved in choosing a tumour *BRCA* assay?

Parallel sequencing – next generation sequencing (NGS)

- Whole genome = too much information
  - difficult regions, e.g. repetitive regions, cannot be avoided
  - costs are high, not suited for bench-top systems
  - extensive bioinformatics needed for evaluation and data storage

HQ DNA

- Whole exome = exons and adjacing splice-sites
  - no complete coverage of all gene regions
  - some fusions can‘t be detected

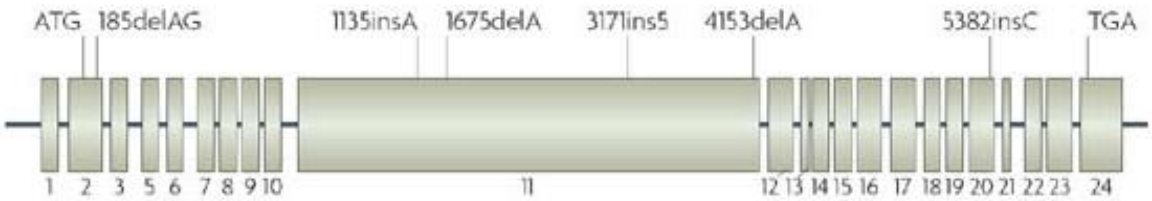
HQ and HQ FFPE DNA

- Targeted sequencing = gene panel
  - defined target regions
  - PCR-based or hybridization-based enrichment
  - detection of gene fusions and copy number changes is possible

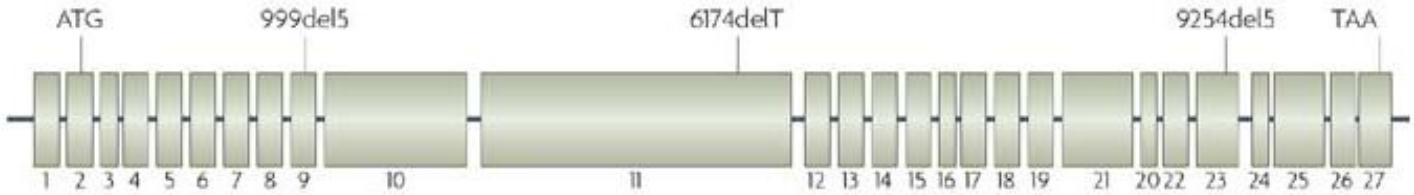
HQ and FFPE DNA

BRCA tumour testing

*BRCA*1:
24 Exons



BRCA2:
27 Exons



Nature Reviews | Cancer

- Two large genes
- All positions are relevant for tumour testing

PCR-based vs. Hybridization-based

PCR-based (Qiagen)

Hybridization-based (Agilent)

DNA-Extraction

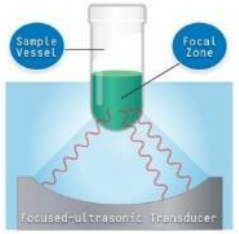
DNA-Quantification

4 x ≤10 ng

Multiplex PCR
Fragments: 125 – 175 bp

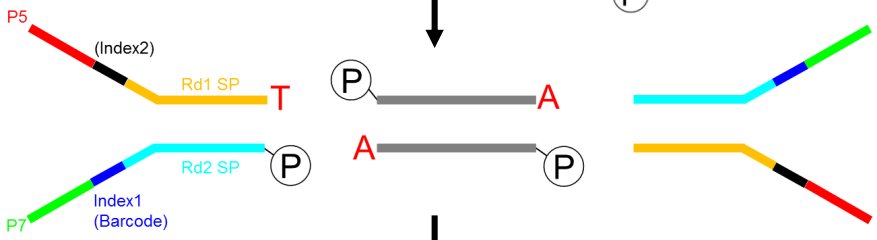
> 50 ng

Sonification
Fragments: ~150 bp



End-Repair

Adenylation



Adapter ligation

2. PCR (1. PCR): ~10 Cycles

MiSeq™ System (Illumina)

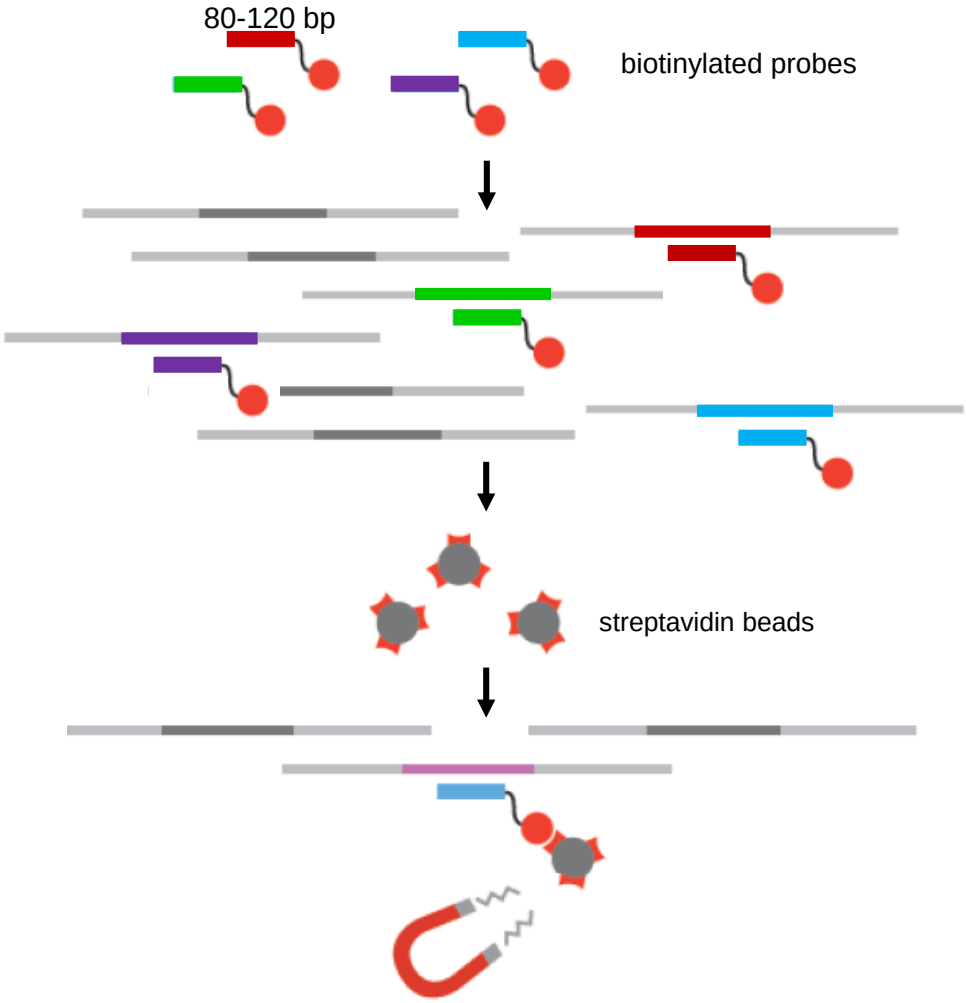
Library-Quantification

Fragments: 250 - 300 bp

Enrichment

PCR-based vs. Hybridization-based

Hybridization of biotinylated probes to targeted regions.
Enrichment using streptavidin beads.



http://www.illumina.com/index-d.html


Target fragments


2. PCR (12-14 Cycles)

Quantification

Fragments: 250 - 300 bp

NextSeq 500 System (Illumina)

PCR-based vs. Hybridization-based

PCR-based

- Less chemicals required
- No DNA fragmentation required
- Faster and cheaper
- More robust and easy
- Date interpretation easier
- Assays for FFPE Material available
- Low DNA input

- Multiplexing is limited
- Polymerase read errors
- Amplification of FFPE fixation artefacts
- Duplicate reads
- Can not detect fusion events
- Detection of copy number changes might be difficult

Hybridization-based

- Easier to add new targets
- 1 panel for all entities
- Detection of gene fusions and complex events
- Fixation artefacts are avoided
- Less rounds of amplification
- Better duplicate filtering

- Expensive and complex procedure and data interpretation
- Not specifically designed for FFPE material
- Higher DNA input (>50 ng)
- 20% of samples have <50 ng of DNA
- Strong bias with LQ FFPE DNA
- Use of larger Benchtop-Sequencer like NextSeq 500

PCR-based vs. Hybridization-based

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- Use of larger Benchtop-Sequencer like NextSeq 500

Usable with FFPE material

- SureSelect XT (HS) (Agilent)
- TruSight Exome (Illumina)
- TST170 (Illumina)

and more

Available PCR-based *BRCA* assays for FFPE material

Commercial Kits

- Ion AmpliSeq™ *BRCA1* and *BRCA2* Panel or Oncomine BRCA Research Assay (Thermo Fisher Scientific)
- BRCA tumor MASTR plus Dx (Multiplicom - Agilent)
- TruSeq Amplicon - *BRCA1* and *BRCA2* (Illumina)
- Archer VariantPlex BRCA assays (Archerdx)
- GeneRead DNAseq Targeted Panel V2 - Human *BRCA1* and *BRCA2* Panel (Qiagen)
- QIAseq Targeted DNA Panel (V3 Chemistry) - Human *BRCA1* and *BRCA2* Panel (Qiagen)

Lab Developed Assays

- Targeted sequencing using smMIP (molecular inversion probes)[1]
- Other lab developed NGS sequencing assays

How is the GeneRead library from Qiagen prepared and constructed?

GeneRead DNAseq Targeted Panel V2 - Human *BRCA1* and *BRCA2* Panel (Qiagen)

- **Multiplex PCR:**
  - GeneRead DNAseq Targeted Panel V2 - Human *BRCA1* and *BRCA2* Panel (NGHS-102X)
  - GeneRead DNAseq Panel PCR Reagent V2

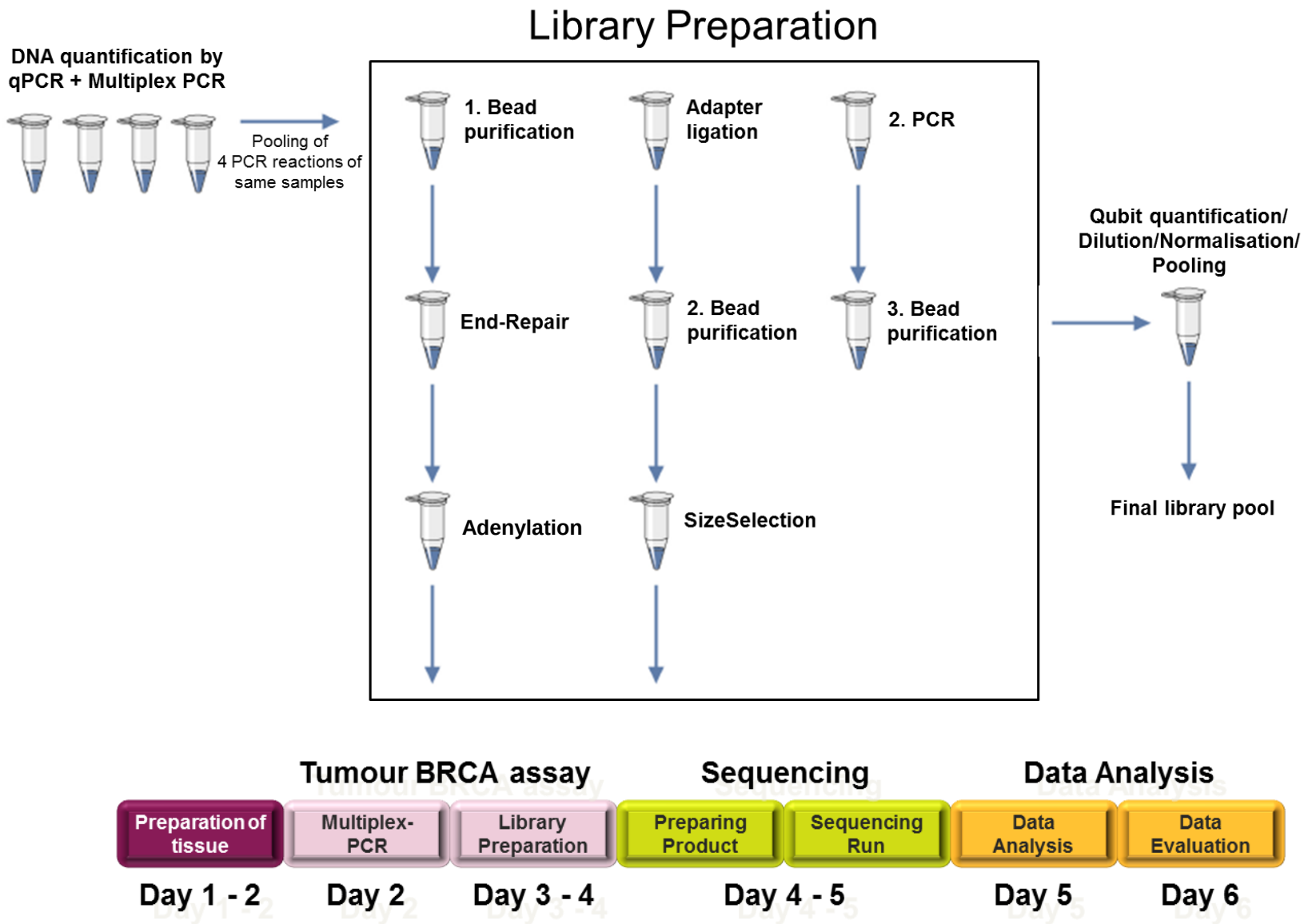
- **Library Prep:**
  - GeneRead DNA Library I Core Kit (for Illumina)
  - GeneRead DNA I Amp Kit (for Illumina)
  - Nextflex-96™ DNA Barcodes (96 Barcodes for Illumina - BIOO Scientific)

Or

GeneRead DNA Library I Kit (96) including 96 Barcodes for Illumina (Qiagen)

Or

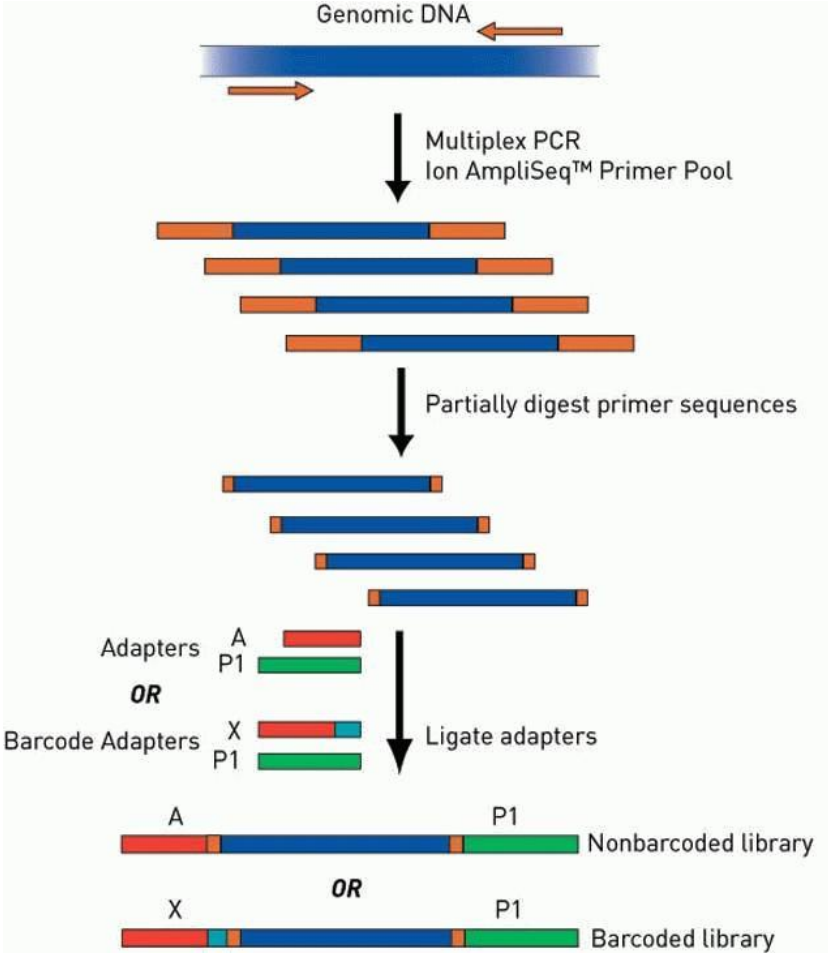
QIAseq 1-Step Amplicon Library Kit (96) including 96 Barcodes for Illumina (Qiagen)


Library Preparation
DNA quantification by qPCR + Multiplex PCR
Pooling of 4 PCR reactions of same samples
1. Bead purification
Adapter ligation
2. PCR
End-Repair
2. Bead purification
3. Bead purification
Adenylation
SizeSelection
Qubit quantification/ Dilution/Normalisation/ Pooling
Final library pool
Tumour BRCA assay
Sequencing
Data Analysis
Preparation of tissue
Multiplex-PCR
Library Preparation
Preparing Product
Sequencing Run
Data Analysis
Data Evaluation
Day 1 - 2
Day 2
Day 3 - 4
Day 4 - 5
Day 5
Day 6

How are other PCR-based libraries prepared and constructed?

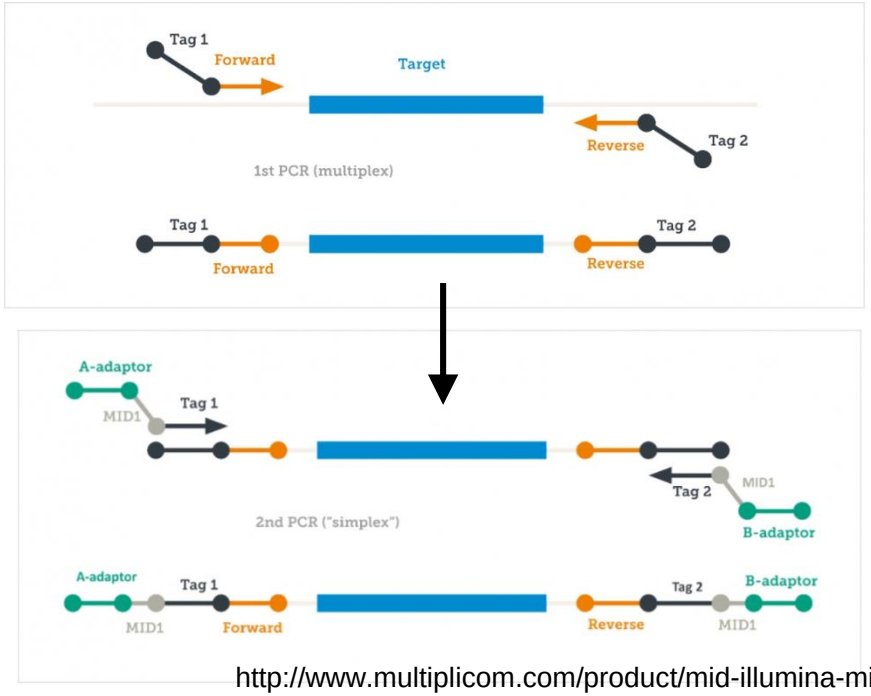
Thermo Fisher Scientific and Multiplicom BRCA Assays

Thermo Fisher Scientific



https://www.thermofisher.com/

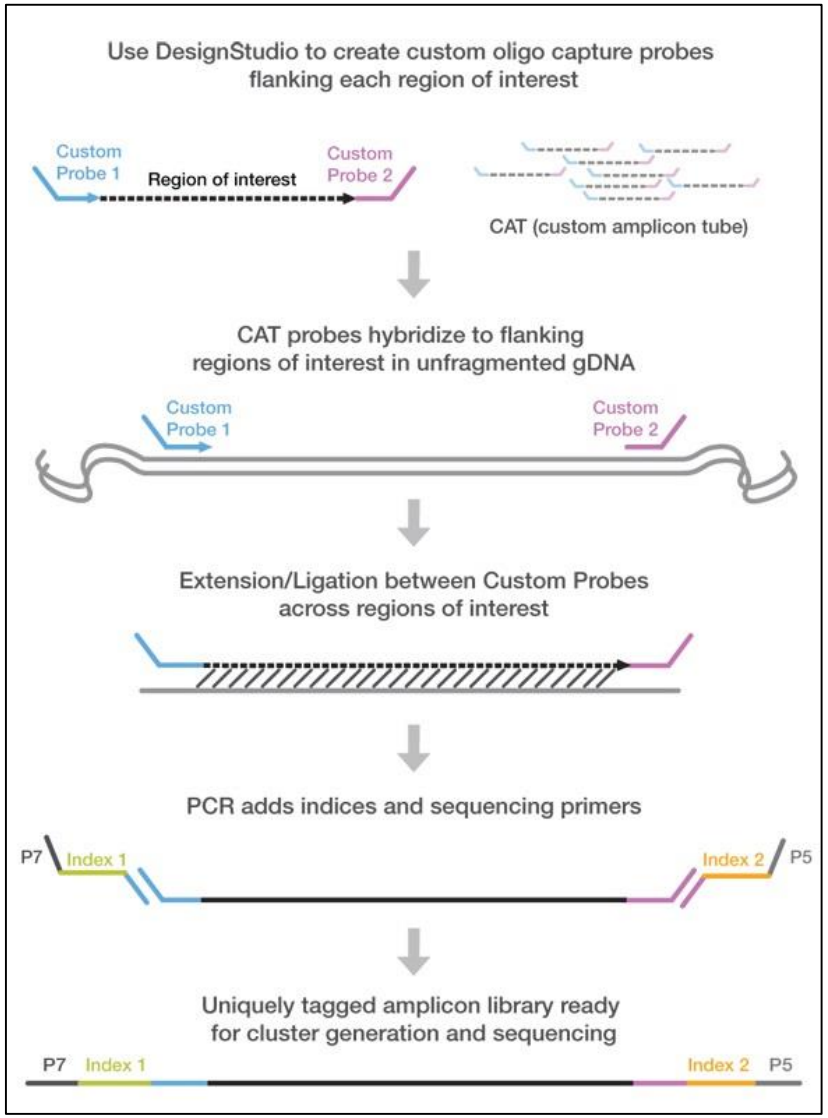
Multiplicom (Agilent)

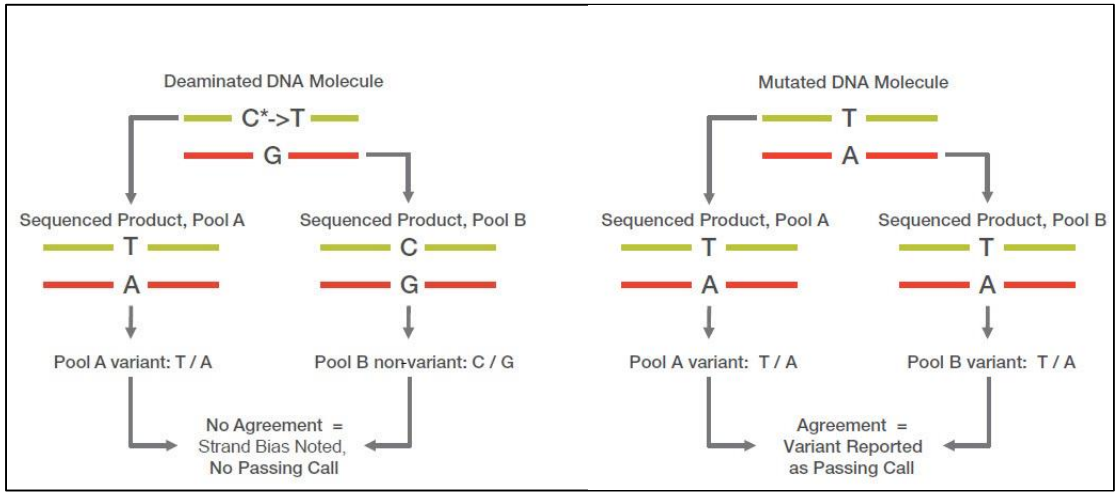


http://www.multiplicom.com/product/mid-illumina-miseqr

- Multiplex PCR of target regions
- Removal of PCR duplicates not possible
- Fixation artefacts cannot be distinguished from real mutations
- No Molecular Barcodes

TruSeq Amplicon - Cancer Panel (Illumina)



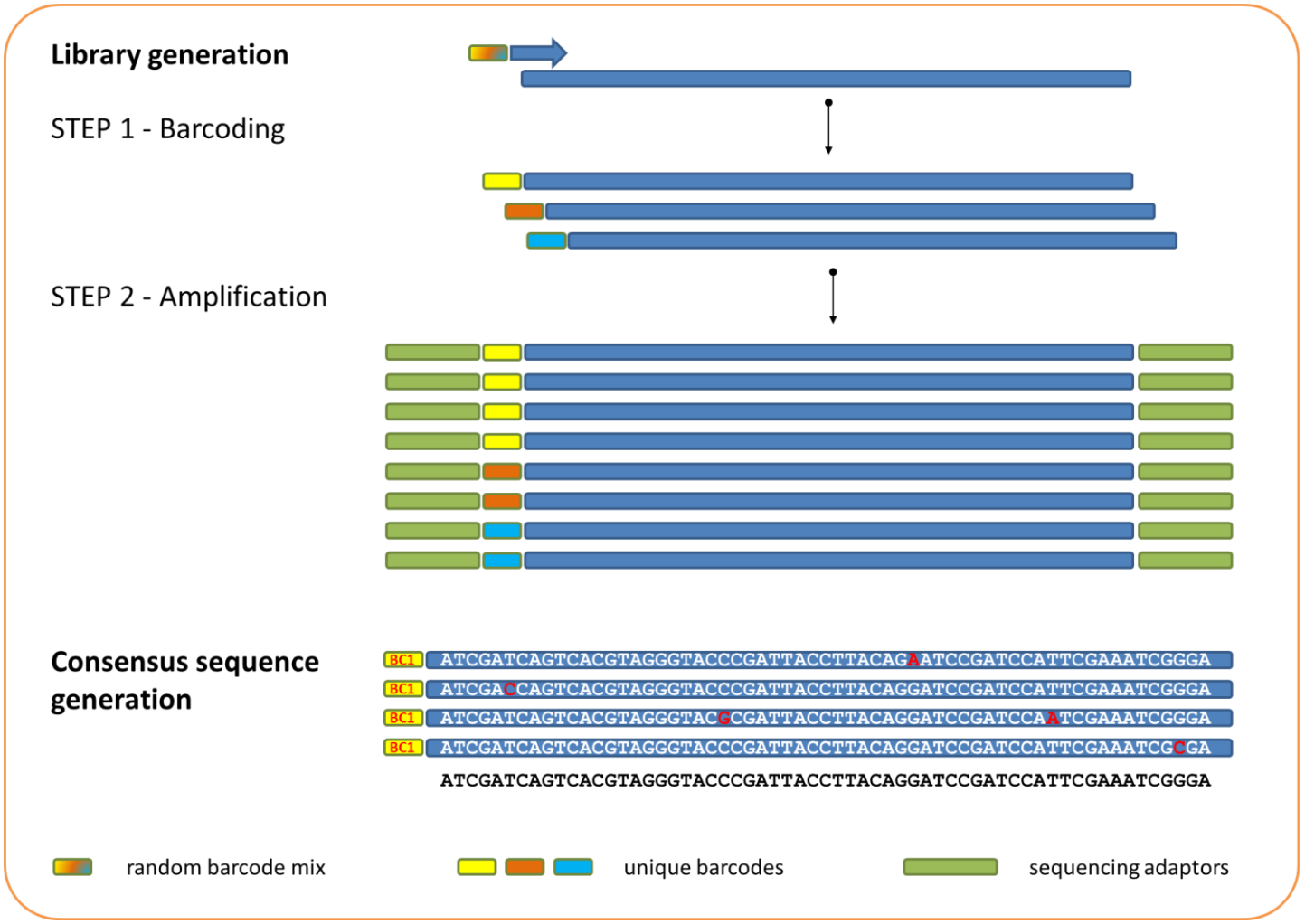


- Hybridization of Cancer Panel oligonucleotide probes
- Dual strand sequencing allows easy differentiation of damage artifacts from FFPE treatment from true somatic mutations.
- No molecular barcodes

https://www.illumina.com/

Molecular barcodes

→ Low frequency variant detection, removal of PCR duplicates → unique reads

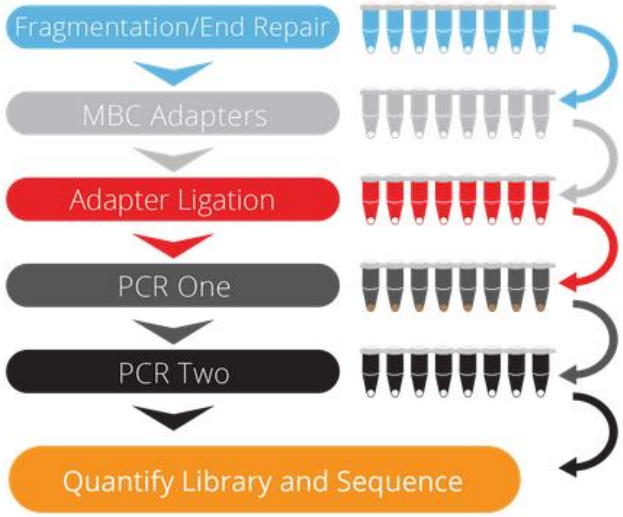


http://www.imgm.com/images/imgm/ScientificOverview/MGx-Overview_Bild-LEA-Seq.png

- HaloPlex – Agilent
- MBC Adapter – Archer
- QiaSeq Targeted Panels – Qiagen

Archer Variant Plex and QiaSeq Targeted Panel (Qiagen)

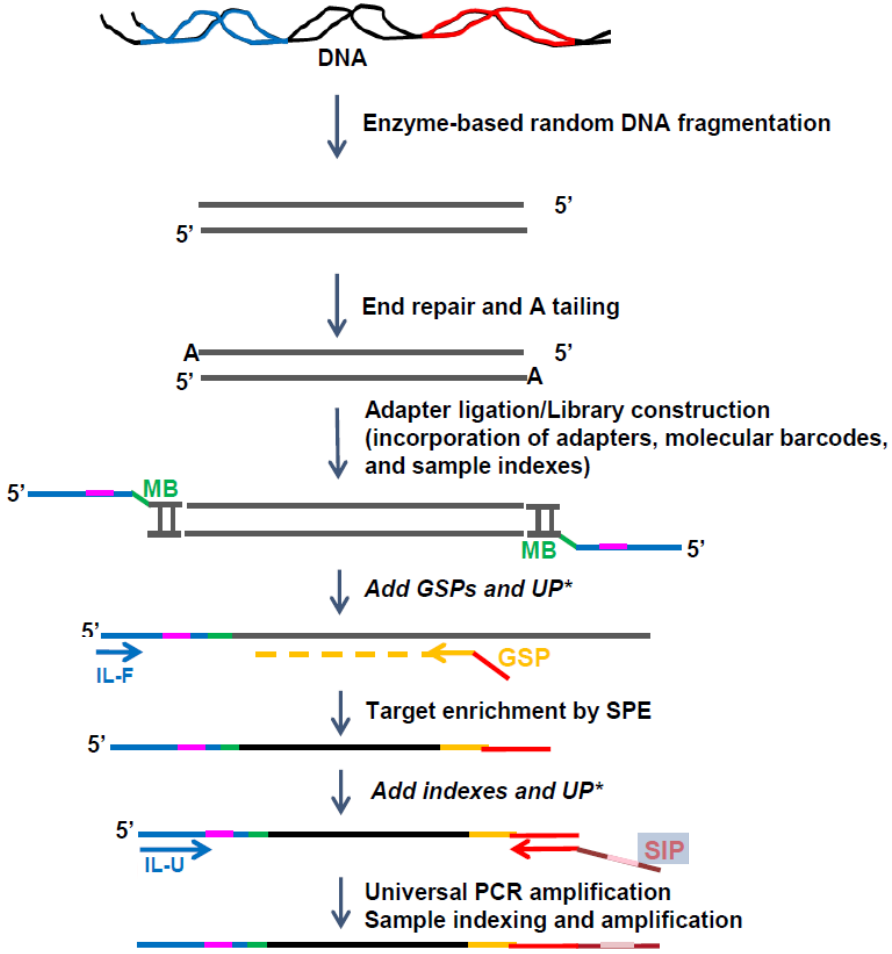
Archer Variant Plex



http://archerdx.com/home/workflow

- Single Primer extension
- Molecular Barcodes
- Removal of PCR duplicates and fixation artefacts

QIAseq (Qiagen)



http://www.qiagen.com

Which devices and methods can be used for the different quality checkpoints during your library preparation?

Checking the correct fragment size of multiplex PCR and library

2100 Bioanalyzer Instruments (Agilent)

4200 TapeStation strument (Agilent)

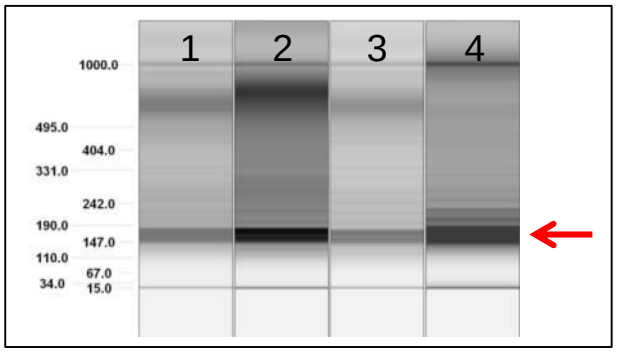
Or conventional agarose gels

QIAxcel Advanced System (Qiagen)

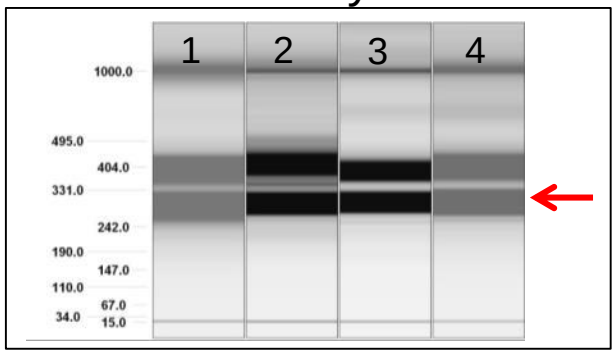
Fragment Analyzer™
(Advanced Analytical Technologies)

Multiplex PCR and library

Has the PCR worked?



Has the library worked?



Multiplex-PCR

Fragments: 125 – 175 bp

Pooling of 4 PCR reactions of same samples

End-Repair

Adenylation

Adapter ligation

2. PCR

P5
(Index2)
Rd1 SP
Rd2 SP
Index1
(Barcode)
P7

Library quantification (Qubit)

Fragmente: 250 - 300 bp

Library quantification methods

- **Fluorescent dye-based quantification**
  - Qubit® 2.0/3.0 Fluorometer (Thermo Fisher Scientific)
  - Quantus™ Fluorometer(Promega)
  - Quant-iT™ PicoGreen® dsDNA Assay (Thermo Fisher Scientific)

- **qPCR (custom primers)**
  - SsoFastTM EvaGreen® Supermix (BioRad)
  - GoTaq® qPCR Master Mix (Promega)

- **qPCR (commercially available)**
  - KAPA Library Quantification Kit (Roche)
  - QIAseq Library Quant Array (Qiagen)
  - NEBNext® Library Quant Kit for Illumina® (NEB)
  - qPCR NGS Library Quantification Kit (Agilent)

What are the options for performing sequencing?

Sequencing Instruments

MiSeq™ System (Illumina)

NextSeq™ System (Illumina)

Ion PGM™ System (Thermo Fisher Scientific)

MiniSeq™ System (Illumina)

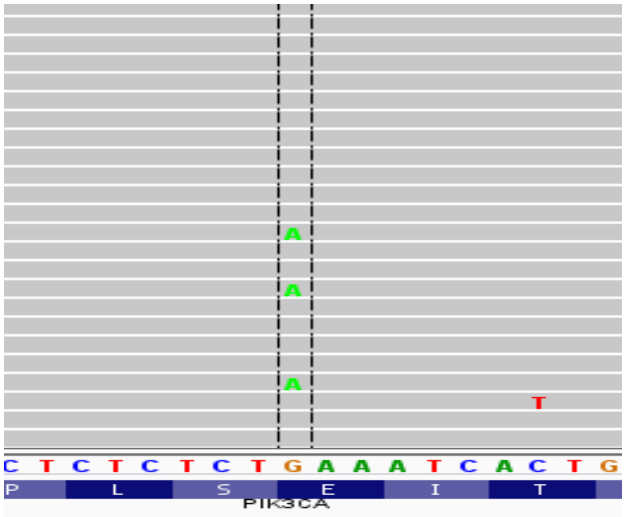
GeneReader NGS System (Qiagen)

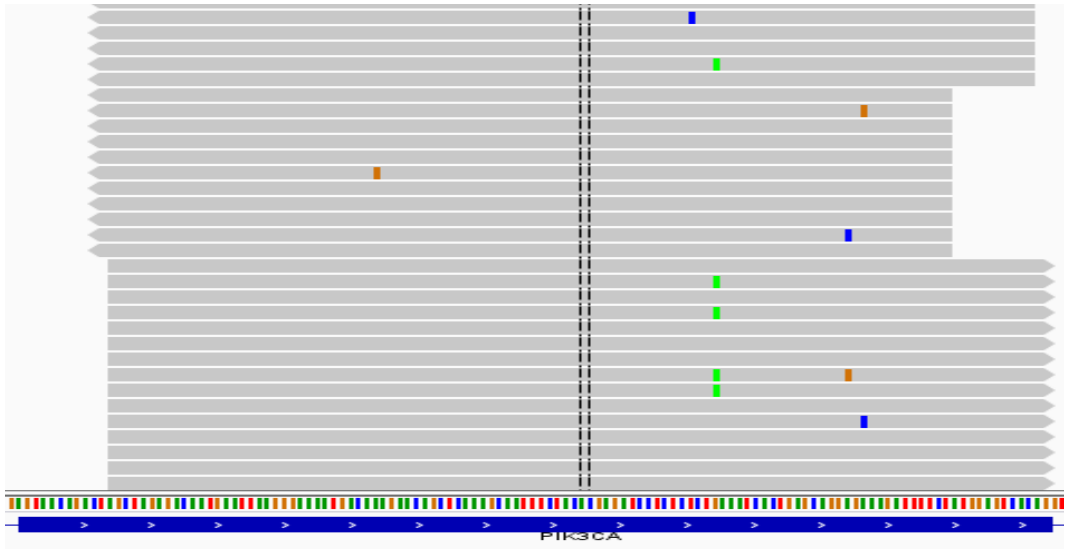
Ion S5™ System (Thermo Fisher Scientific)

Sequencing Platforms - Performance

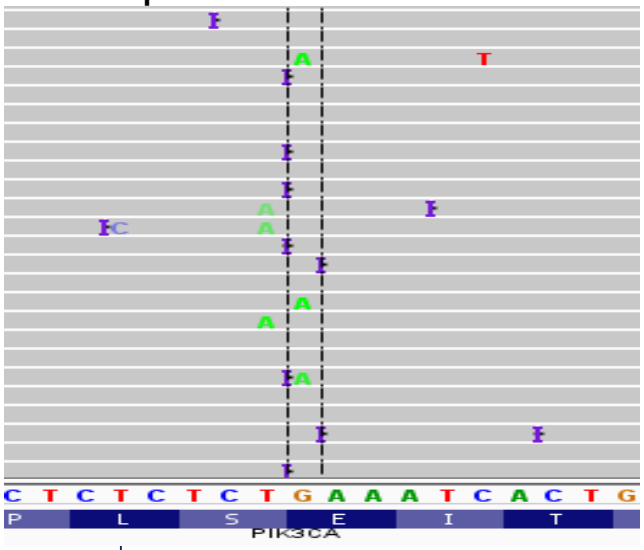
Comparison of two different platforms in 2012/13: sequencing of **homopolymeric** regions

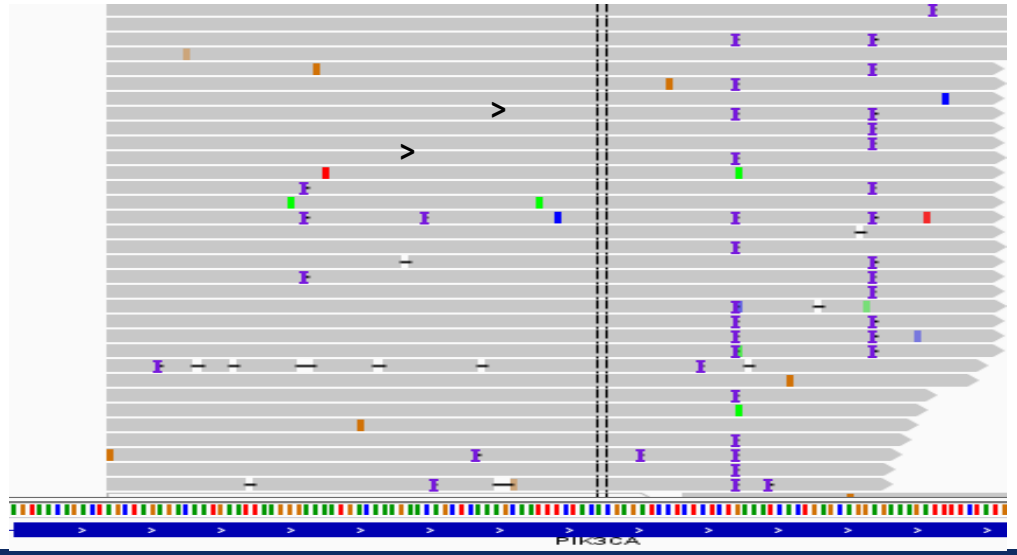
MiSeq





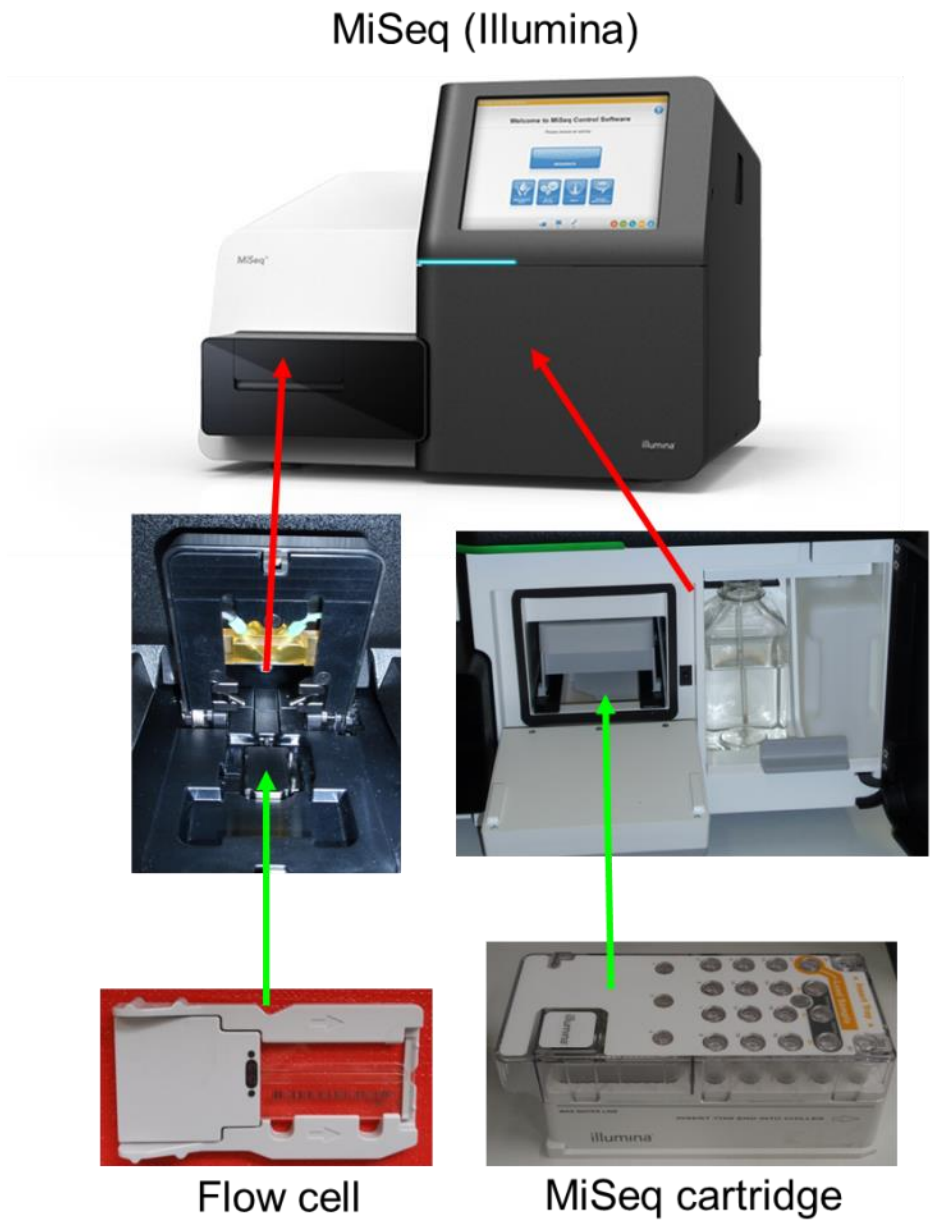
Other platform





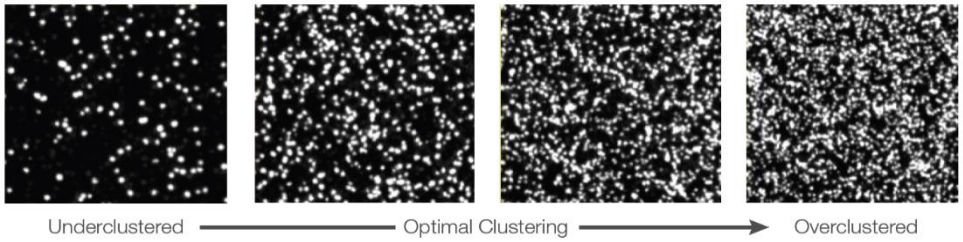
MiSeq Sequencing

- Preparing libraries for sequencing
- Normalisation
- Pooling
- Denaturation with NaOH
- Dilution to 8 - 12 pM
- Adding PhiX control spike-in
- Loading onto the MiSeq cartridge



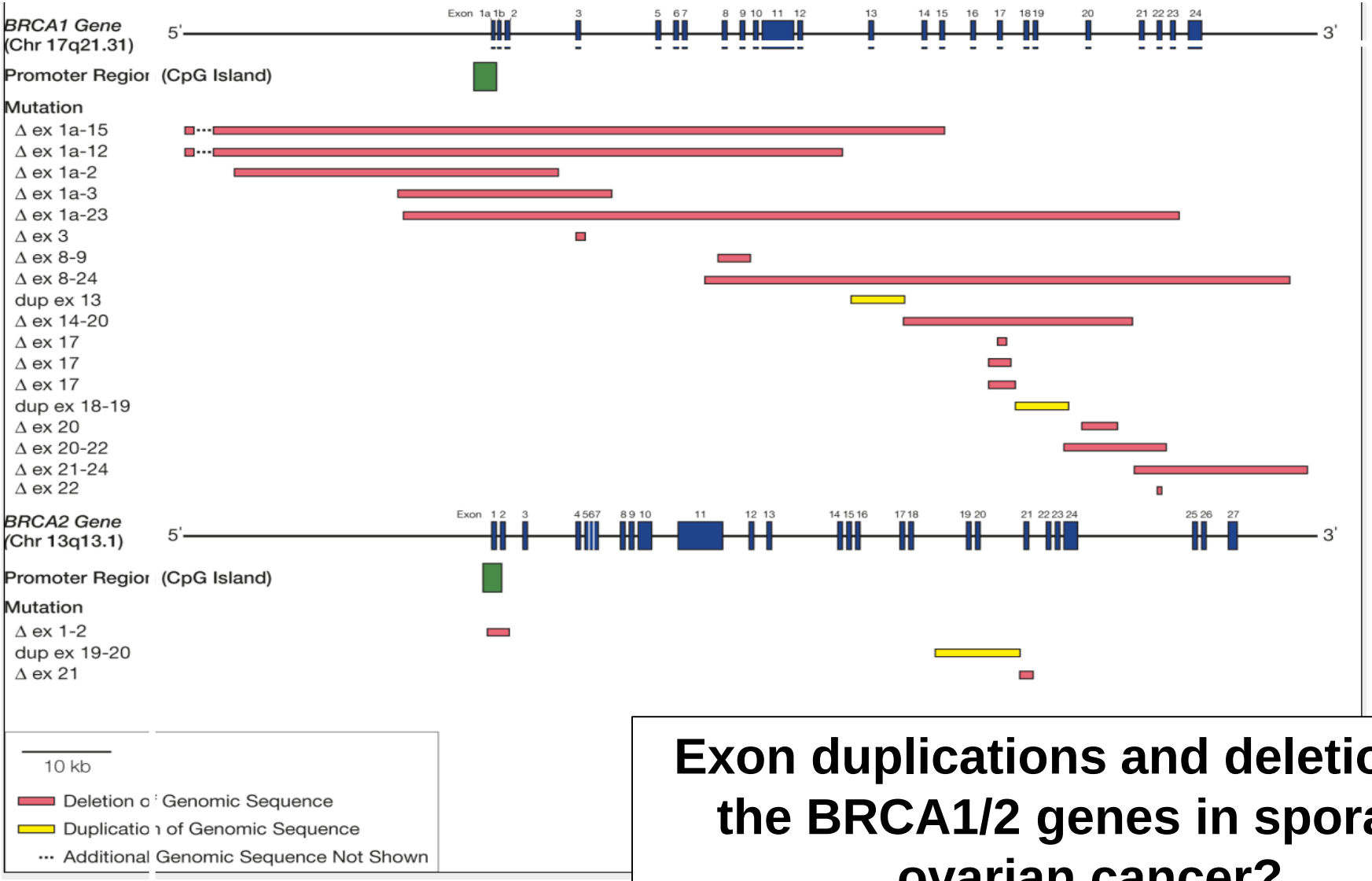
Quality Control during each Individual Run

- **Evaluation of sample quality/quantity**
  - DNA concentration (Qubit/qPCR)
  - Fragment size (PCR/Library)
- **Separation of pre- and post working areas**
- **Within-run controls**
  - Negative control (without DNA)
  - Negative control (with extraction reagents, when lot- no. changes)
  - Change of barcodes between runs
- **Control of run parameters**
  - Quality of base calling (Q30 Score)
  - Cluster density (CD)
  - Cluster passing filter (CPF)
  - Coverage (≥ 200)
  - Allele frequency (≥ 5% for mutation calling)



Can you detect exon duplications and deletions of the BRCA1/2 genes in sporadic ovarian cancer samples?

Inherited genomic rearrangements of *BRCA1* and *BRCA2*



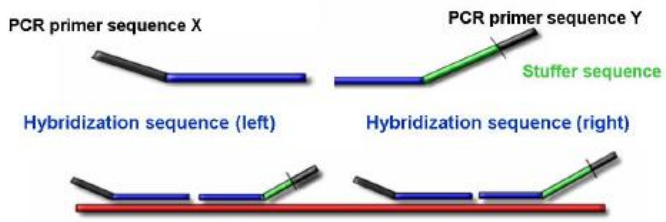
Walsh et al. JAMA 2006

Exon duplications and deletions of the BRCA1/2 genes in sporadic ovarian cancer?

Detection of copy number changes and exon duplications and deletions by MLPA (MRC-Holland)

MLPA technology

1. Denaturation and Hybridization

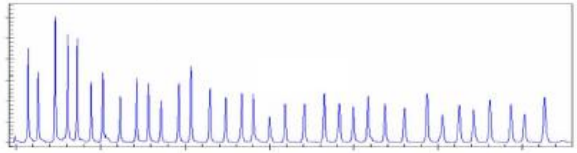


2. Ligation

3. PCR with universal primers X and Y

exponential amplification of ligated probes only

4. Fragment analysis



MRC-Holland MLPA®

8

MLPA results (Coffalyser software)

Statement from MCR-Holland

Is it possible to use DNA extracted from paraffin coupes for MLPA?

Yes. You can download a protocol for DNA extraction from formaldehyde-treated, paraffin-embedded tissues (FFPE) here. We have been notified that the extraction of DNA from paraffin by other methods did not always result in satisfactory results.

Blood

details | fragment analysis | comparative analysis

	sample name	sample type	FMRS	X	Y	gender	analy...	CAS	PSLP	FSLP	RSQ	RPQ
1	P002 310 151014	sample				female						
2	P002 315 363093	sample				female						
3	P002 316 363094	sample				female						
4	P002 317 007077	sample				female						
5	P002 319 228018	sample				female						
6	P002 320 173012	sample				female						
7	P002 329 363099	sample				female						
8	P002 333 232027	sample				female						
9	P002 334 031006	sample				female						
10	P002 336 363095	sample				female						
11	P002 339 363101	sample				female						
12	P002 343 075007	sample				female						
13	P002 344 151015	sample				female						
14	P002 345 151016	sample				female						
15	P002 346 151018	sample				female						

FFPE

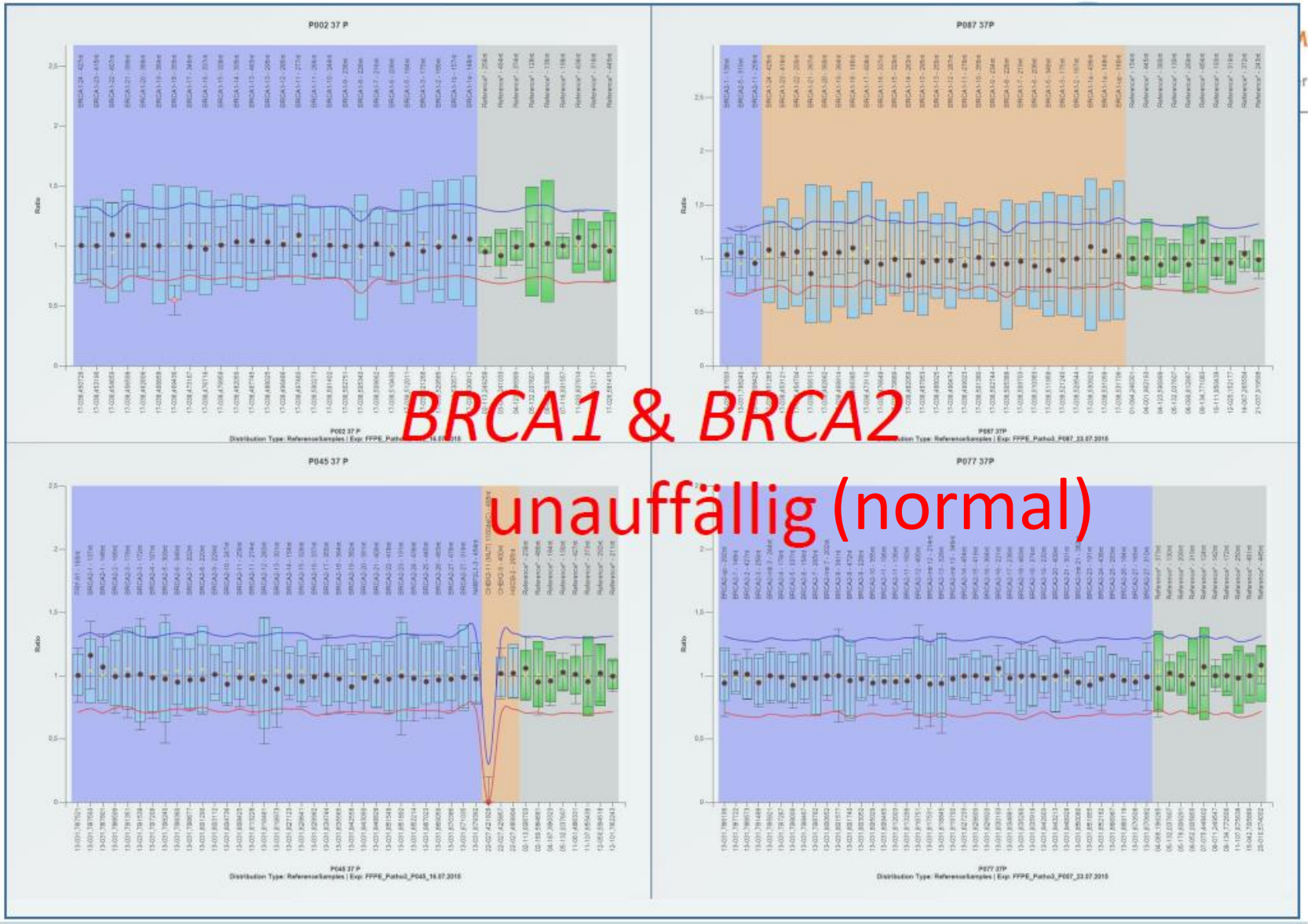
details | fragment analysis | comparative analysis

	sample name	sample type	FMRS	X	Y	gender	analy...	CAS	PSLP	FSLP	RSQ	RPQ
1	P087 M11.37 1d alles	sample				female						
2	P087 M14.38 2 OT	sample				female						
3	P087 M92P	sample				female						
4	P087 M110.15 Tu	sample				female						
5	P087 M110P	sample				female						
6	P087 M173P	sample				female						
7	P087 M291P	sample				female						
8	P087 M371P	sample				female						
9	P087 O12P	sample				female						
10	P087 O21P	sample				female						
11	P087 O71P	sample				female						
12	P087 O136P	sample				female						
13	P087 O162P	sample				female						

- **High quality:** acceptable without reviewing.
- **Low quality:** failed samples → unsuitable, REJECT. *Coffalyser.Net gives information to solve root cause*
- **Intermediate-quality:** view data and recommendations to improve results.

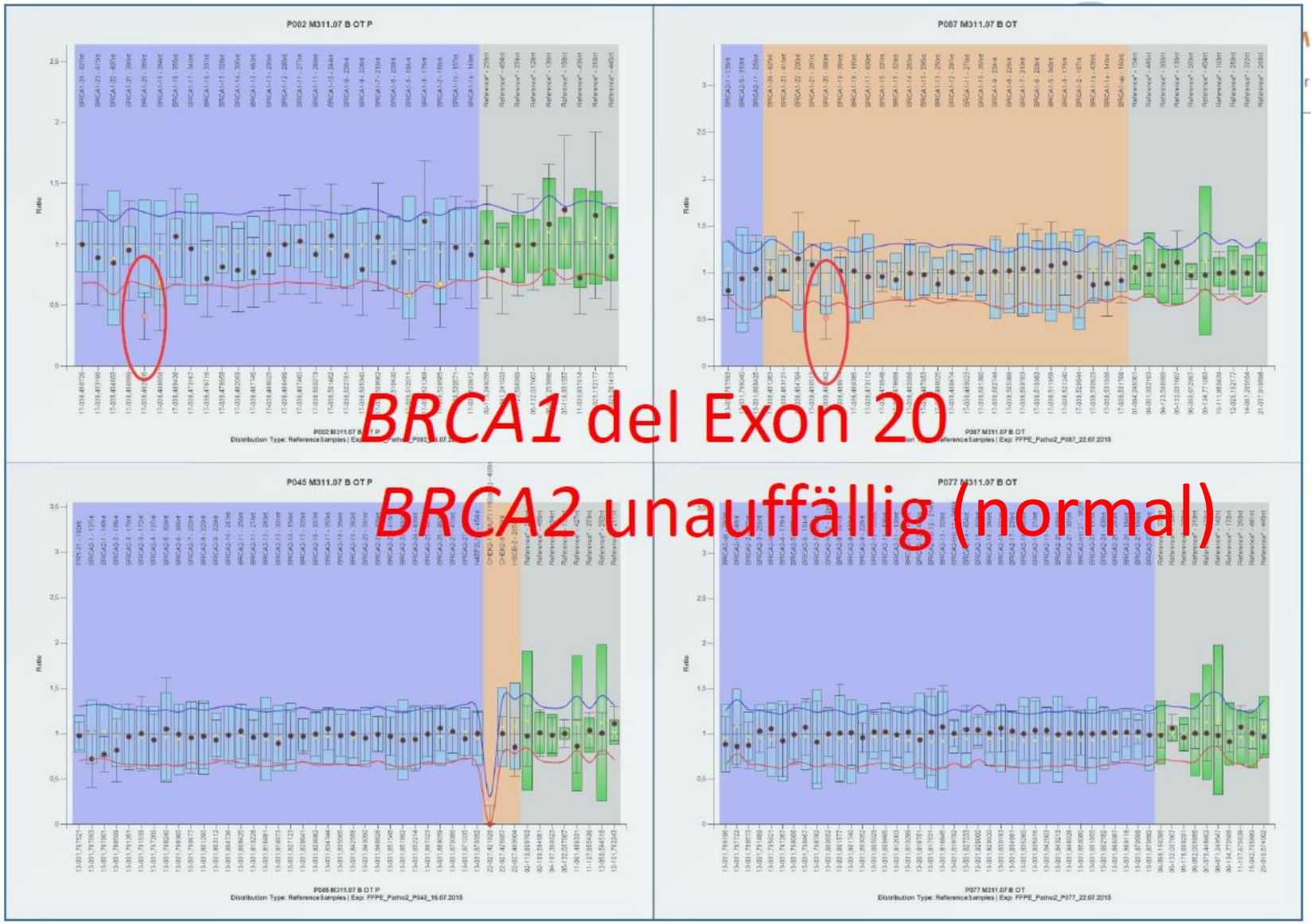
- Preliminary signal sloping (PSLP)
- Final signal sloping (FSLP)
- Reference sample quality (RSQ)
- Reference probe quality (RPQ)
- Coffalyser analysis score (CAS)

MLPA results (Coffalyser software)

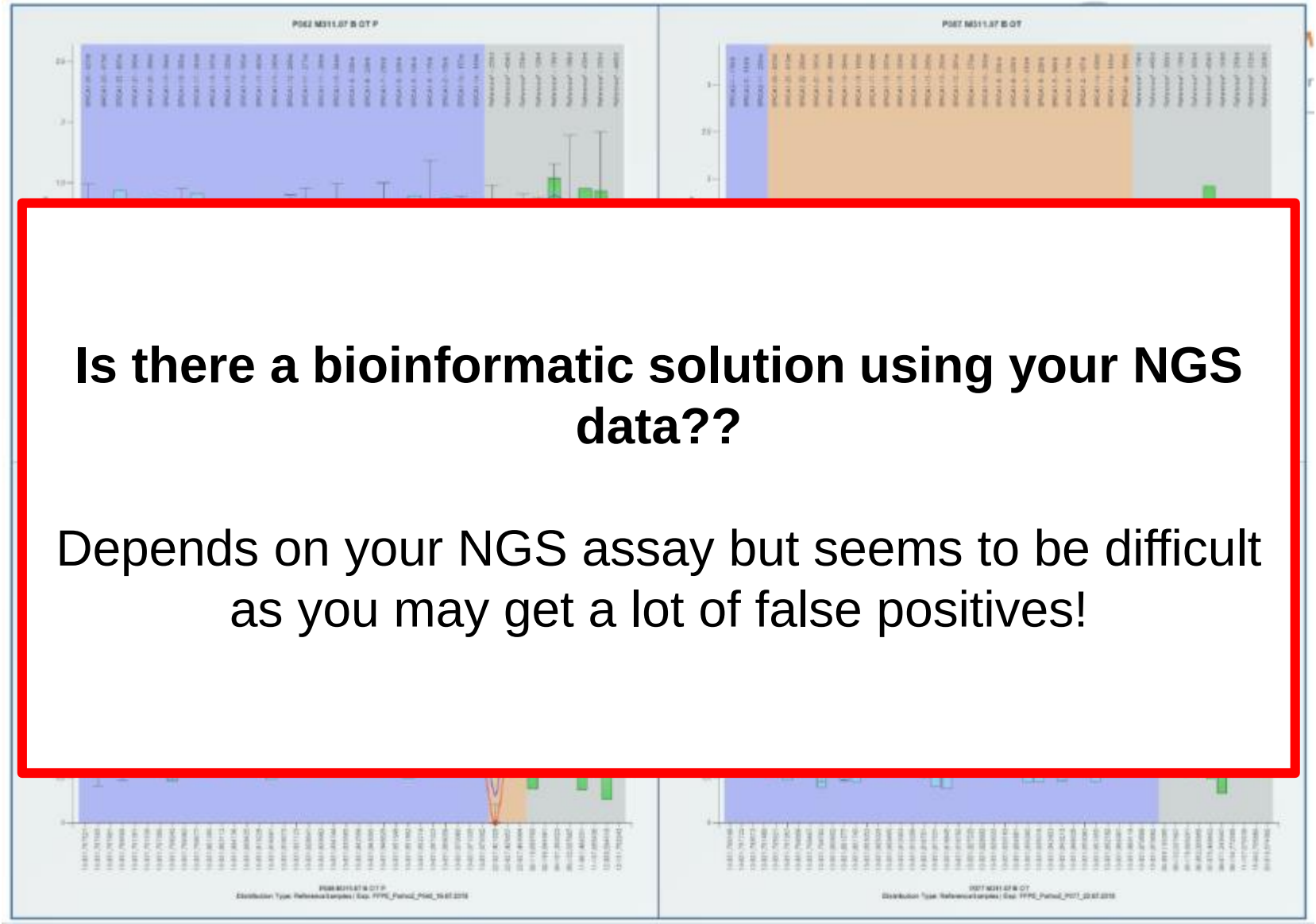


MLPA results (Coffalyser software)

MLPA results (Coffalyser software)



MLPA results (Coffalyser software)



Is there a bioinformatic solution using your NGS data??

Depends on your NGS assay but seems to be difficult as you may get a lot of false positives!

What are the most important pieces of advise you can give to someone starting *BRCA* tumour testing?

Summary and Top Tips!

- Correct estimation of tumour cell content and highlighting of the tumour area
- Precise macrodissection with new scalpel for each case → avoid cross contamination
- Choose a DNA extraction method with good DNA quality and quantity that works well in downstream applications
- Negative control (no DNA) running alongside samples through library preparation and sequencing
- Correct handling of beads during purification steps
- Barcodes should be changed between runs
- The amplicon coverage > 200x
- Allelic fraction of mutation >5%
- Validating the whole process from DNA extraction to data interpretation as a whole

It is important to choose 1 method, with which you have good experience. Validate the chosen method and then use only this validated method!

Thank you for your attention – Questions?

Institute of Pathology, Cologne

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