LPAV-najaarscursus 2019

Melanocytaire laesies met focus op moleculaire pathologie

30 november 2019, Amersfoort

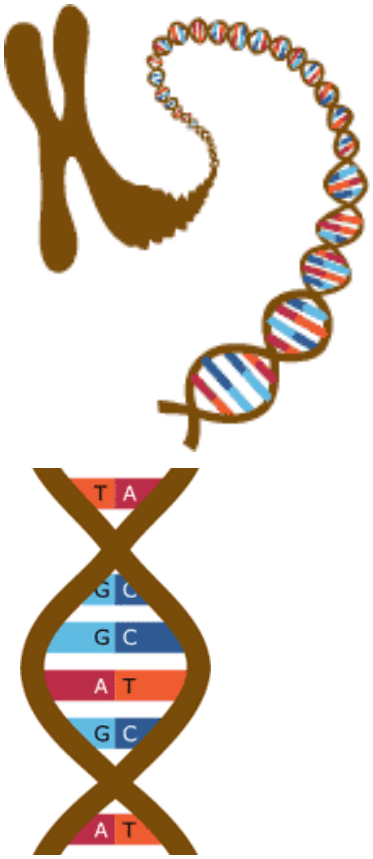
Introductie basisprincipes: moleculaire afwijkingen en detectiemethodes

Dr. Patricia Groenen, KMBP Radboudumc

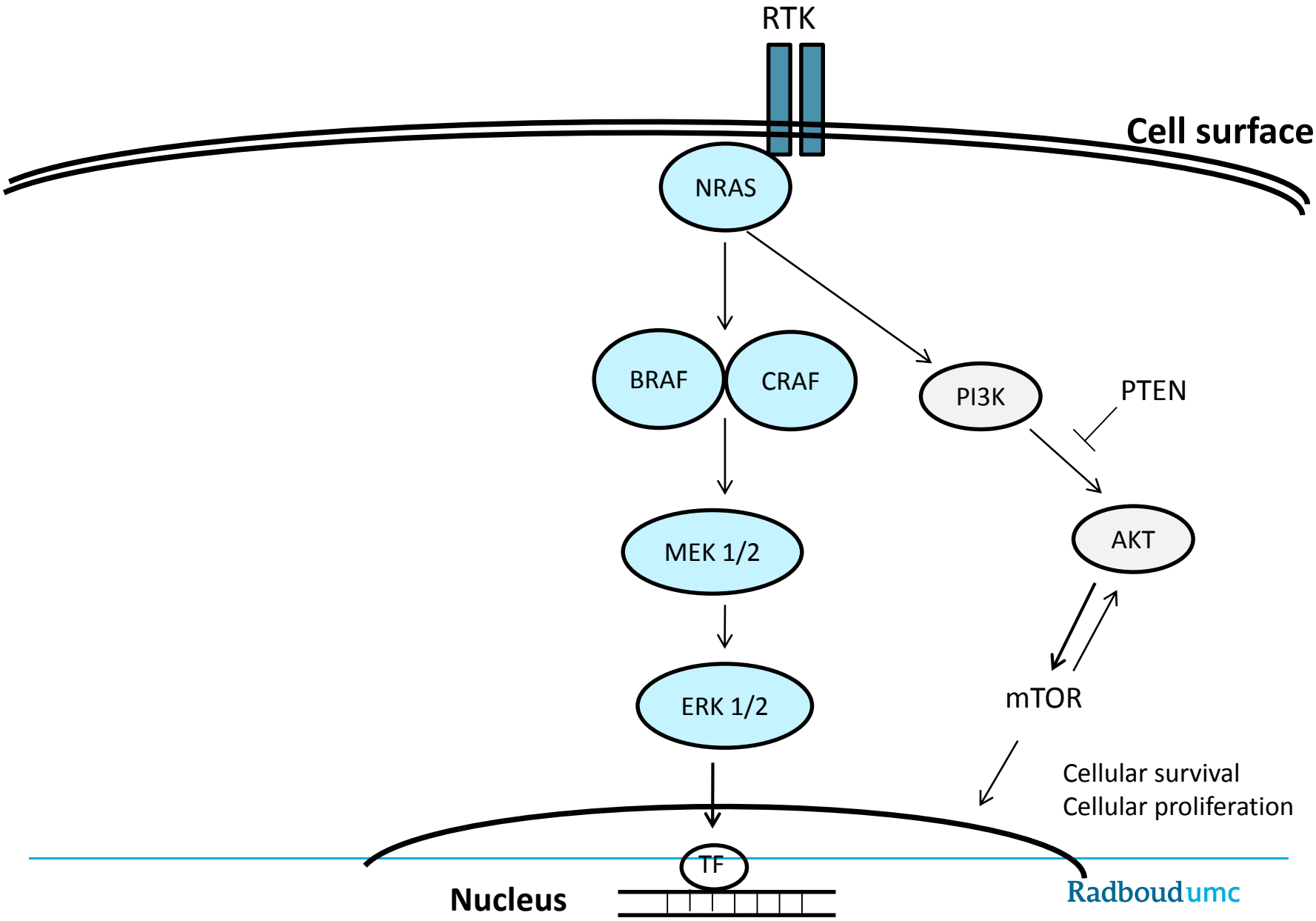
Radboudumc

Topics

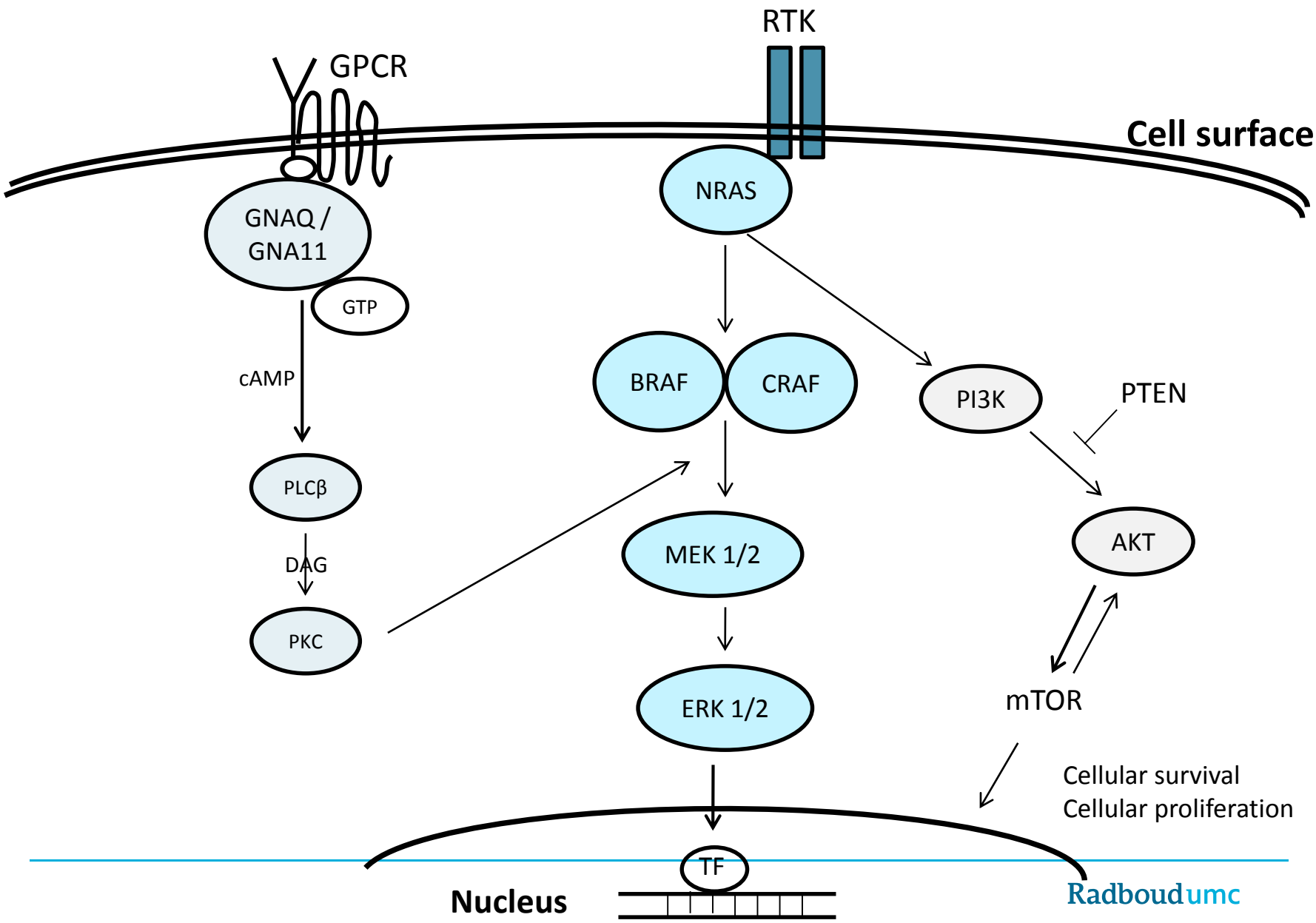
- Melanoma; important mutations
- Mutation analysis
  - by NGS (tissues as source)
  - by ddPCR low level detection
- Melanoma; important Copy number variations
- CNV detection methods
  - > MLPA
  - > array
  - > NGS



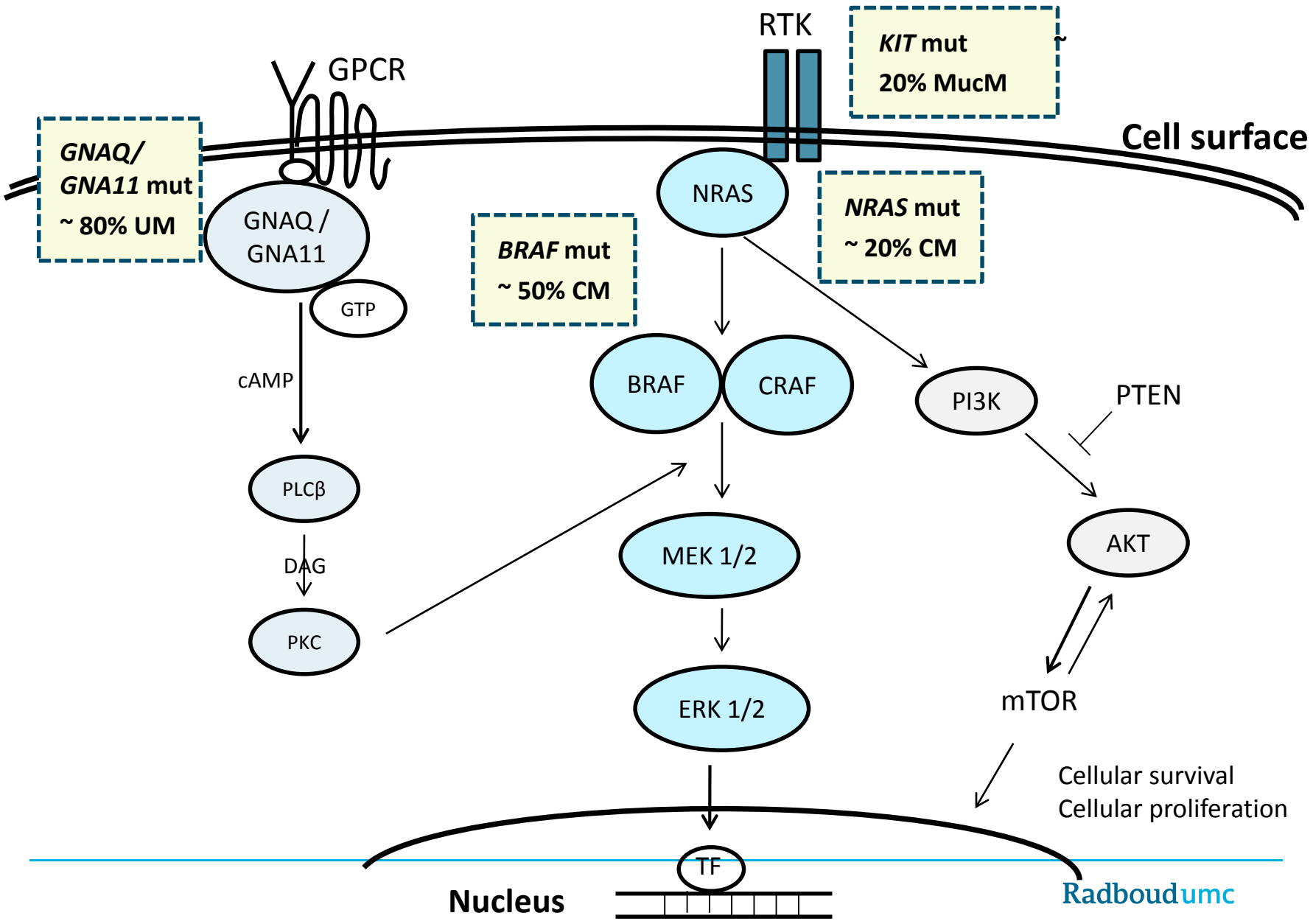
Source: https://educationalgames.nobelprize.org/educational/medicine/dna_double_helix/readmore.html



Source; H. Küsters-vandeVelde, PJTA Groenen, dept. Pathology, Radboudumc



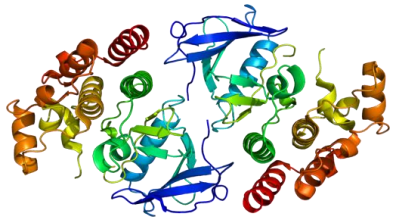
Source; H. Küsters-vandeVelde, PJTA Groenen, dept. Pathology, Radboudumc



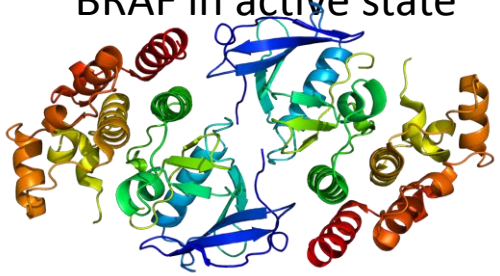
Source; H. Küsters-vandeVelde, PJTA Groenen, dept. Pathology, Radboudumc

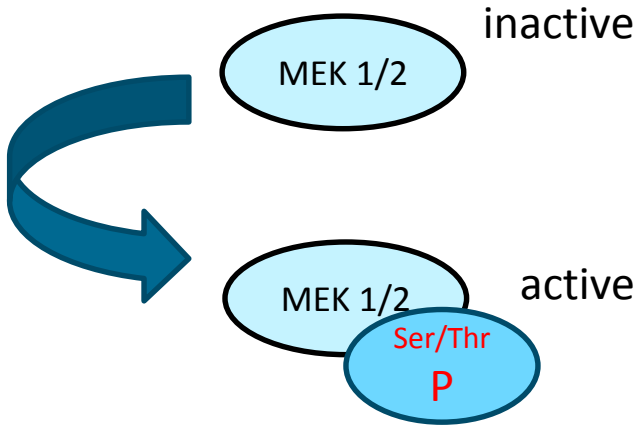
BRAF is a serine-threonine kinase

Growth factor absent
Inactive state

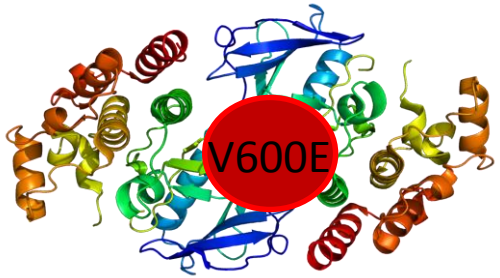


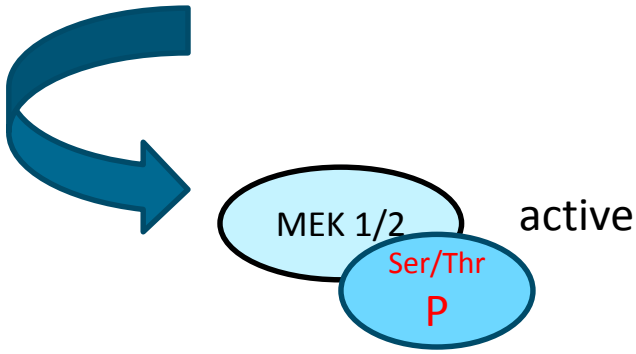
Growth factor present
BRAF in active state





Growth factor absent / present
V600 > BRAF active state





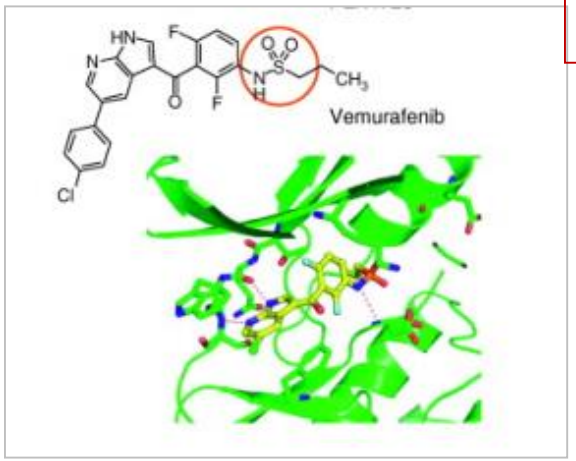
BRAF structure from: https://biopharmconsortium.com/2010/08/28/phase-i-trial-of-rocheplexxikons-plx4032-a-selective-targeted-therapeutic-for-metastatic-melanoma-published-in-the-new-england-journal-of-medicine/

Source; PJTA Groenen, dept. Pathology, Radboudumc

Radboudumc

BRAF inhibition by Vemurafinib, competitive inhibition of the BRAF kinase domain

Only effective for BRAF-V600 mutated melanoma



From: Targeting the RAS pathway in melanoma. Zhenyu Ji et al. Trends in molecular medicine 2012; 18: 27-35

RTK

Cell surface

NRAS

BRAF-i

BRAF

CRAF

V600E

PI3K

PTEN

MEK 1/2

AKT

ERK 1/2

mTOR

Cellular survival
Cellular proliferation

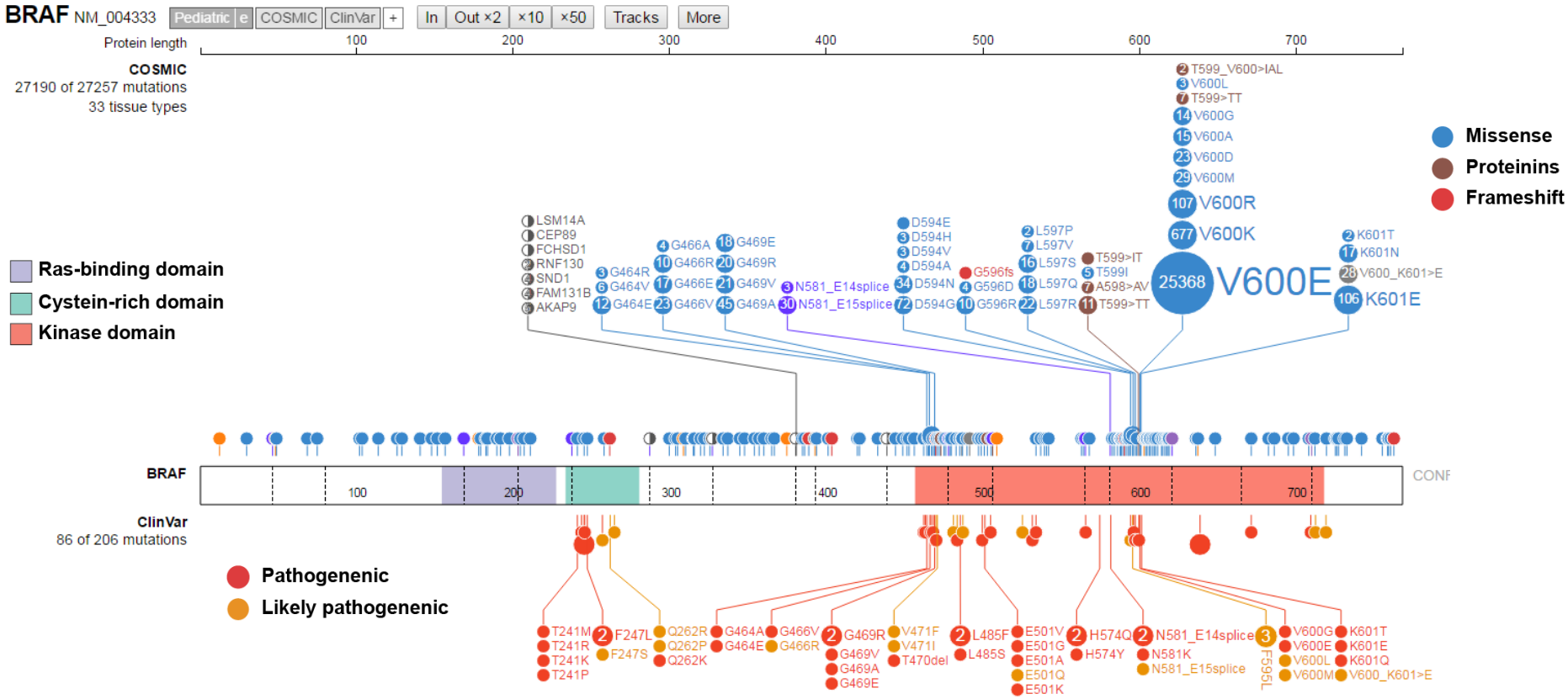
TF

Nucleus

Radboudumc

Source; H. Küsters-vandeVelde, PJTA Groenen, dept. Pathology, Radboudumc

BRAF


BRAF NM_004333
Pediatric
COSMIC
ClinVar
In
Out ×2
×10
×50
Tracks
More
Protein length
100
200
300
400
500
600
700
COSMIC
27190 of 27257 mutations
33 tissue types
Missense
Proteinins
Frameshift
Ras-binding domain
Cystein-rich domain
Kinase domain
V600E
V600K
V600R
K601E
BRAF
ClinVar
86 of 206 mutations
Pathogenenic
Likely pathogenenic

BRAF: activerende mutaties

Table 1 | Classification of cancer-associated BRAF mutants

BRAF mutation		Kinase activity	RAS dependency	Dimer dependency	Sensitivity to vemurafenib
	Wild type	Neutral	Yes	Yes	Insensitive
Class 1	V600E	High	No	No	Sensitive
	V600K	High	No	No	Sensitive
	V600D	High	No	No	Sensitive
	V600R	High	No	No	Sensitive
	V600M	Intermediate	No	No	Sensitive
Class 2	K601E	High	No	Yes	Insensitive
	K601N	High	No	Yes	Insensitive
	K601T	High	No	Yes	Insensitive
	L597Q	High	No	Yes	Insensitive
	L597V	Intermediate	No	Yes	Insensitive
	G469A	High	No	Yes	Insensitive
	G469V	High	No	Yes	Insensitive
	G469R	Intermediate	No	Yes	Insensitive
	G464V	Intermediate	No	Yes	Insensitive
	G464E	Intermediat			

< Previous Article | Next Article >

Klasse 1: V600 mutaties: stabiliserer

Klasse 2: Non-V600 activerende mut

Mutaties in A en P-loop; ge

stabiliseren van de inactiev

Yao et al. Nature (Epub 2017 Aug 2)

BRAF-inhibitors can exert control of disease in BRAF T599I mutated melanoma

a case report

Gallo, Susanna[a]; Coha, Valentina[a]; Caravelli, Daniela[a]; Becco, Paolo[a,e]; Venesio, Tiziana[b]; Zaccagna, Alessandro[c]; Giacone, Elena[c]; Marenco, Federica[c]; Pisacane, Alberto[b]; Racca, Manuela[d]; Gammaitoni, Loretta[a]; Aglietta, Massimo[a,e]; Carnevale-Schianca, Fabrizio[a]

Melanoma Research: April 2018 - Volume 28 - Issue 2 - p 143–146
doi: 10.1097/CMR.0000000000000417

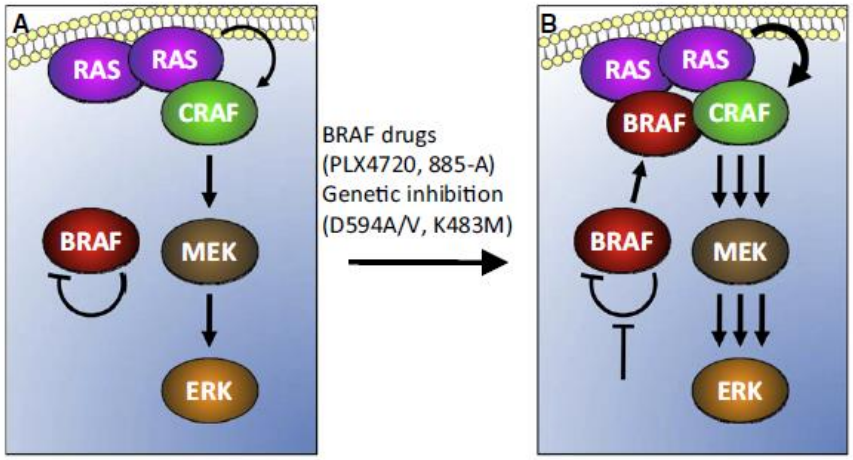
BRAF: mutaties 'verminderde activiteit'

Klasse 3: Verstoorde kinase activiteit (o.a. 'kinase dead' mutaties)

- Melanoma: mutaties gaan meestal samen met activerende RAS of NF1 mutaties
- Colon/long: mutaties gaan meestal samen met activatie van een tyrosine kinase receptor

Table 1 | Classification of cancer-associated BRAF mutants

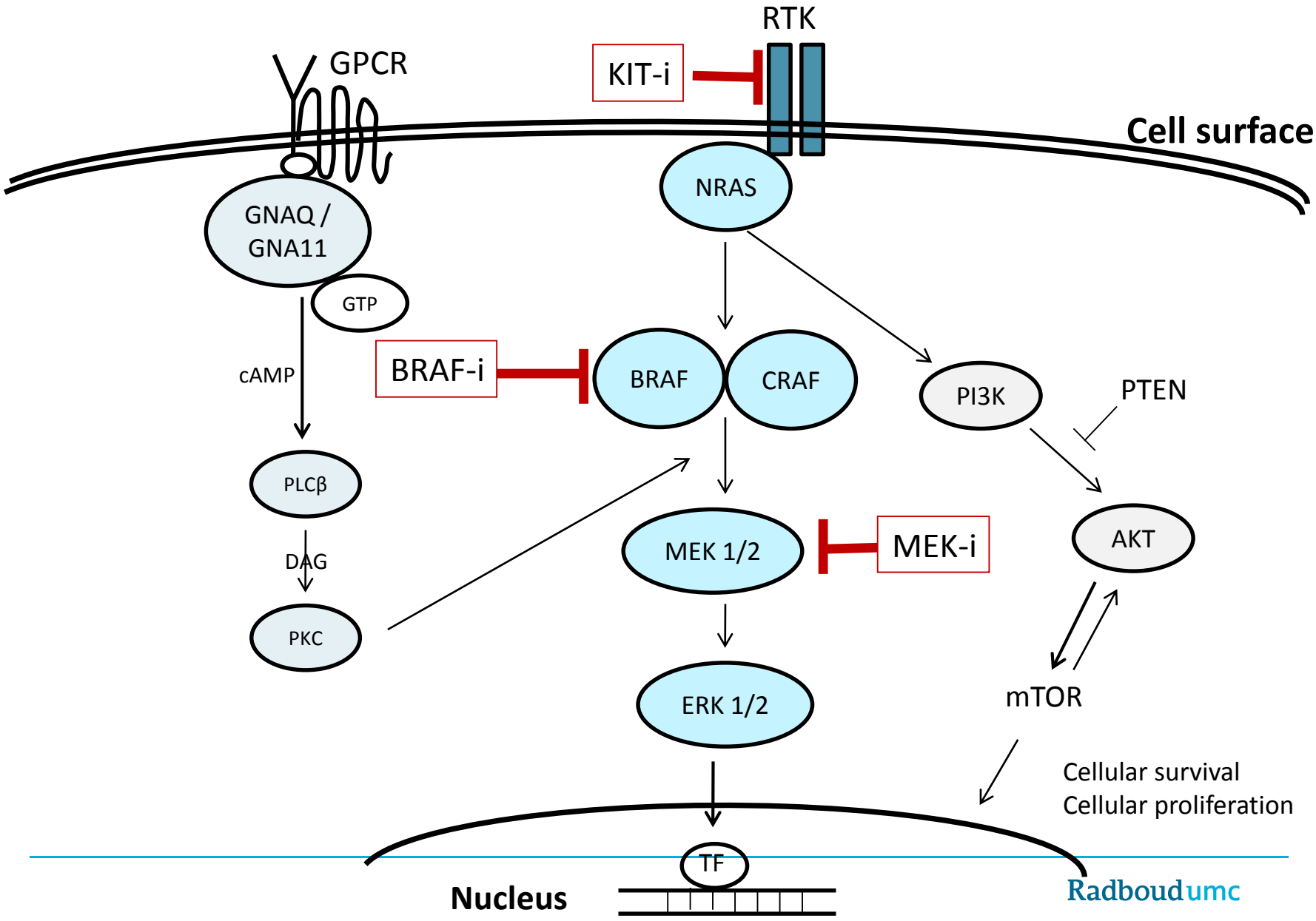
BRAF mutation		Kinase activity	RAS dependency	Dimer dependency	Sensitivity to vemurafenib
	Wild type	Neutral	Yes	Yes	Insensitive
Class 3	D287H	Low	Yes	Yes	Insensitive
	V459L	Low	Yes	Yes	Insensitive
	G466V	Low	Yes	Yes	Insensitive
	G466E	Low	Yes	Yes	Insensitive
	G466A	Low	Yes	Yes	Insensitive
	S467L	Low	Yes	Yes	Insensitive
	G469E	Low	Yes	Yes	Insensitive
	N581S	Low	Yes	Yes	Insensitive
	N581I	Low	Yes	Yes	Insensitive
	D594N	None	Yes	Yes	Insensitive
	D594G	None	Yes	Yes	Insensitive
	D594A	None	Yes	Yes	Insensitive
	D594H	None	Yes	Yes	Insensitive
	F595L	Low	Yes	Yes	Insensitive
	G596D	Low	Yes	Yes	Insensitive



→ amplificatie van RAS-activatie

Radboudumc

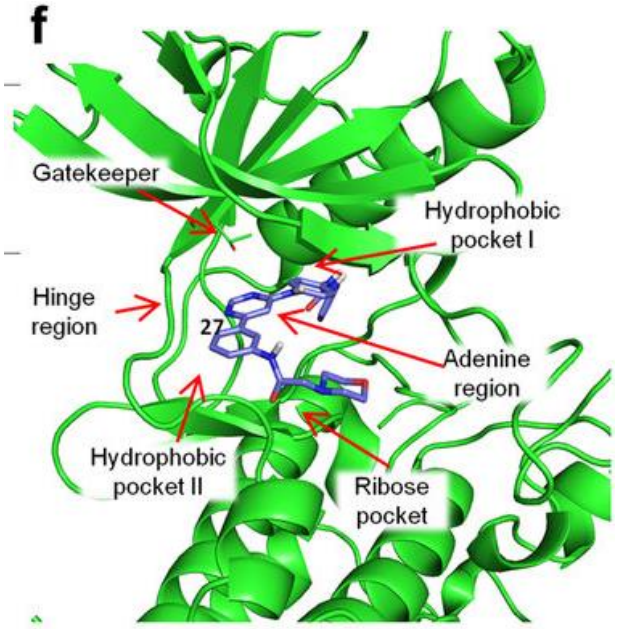
Heidorn et al. Cell (2010) 209-221
Yao et al. Nature (Epub 2017 Aug 2)



Source; H. Küsters-vandeVelde, PJTA Groenen, dept. Pathology, Radboudumc

Relevante mutaties voor predictie therapie respons

- KIT mut ; imatinib



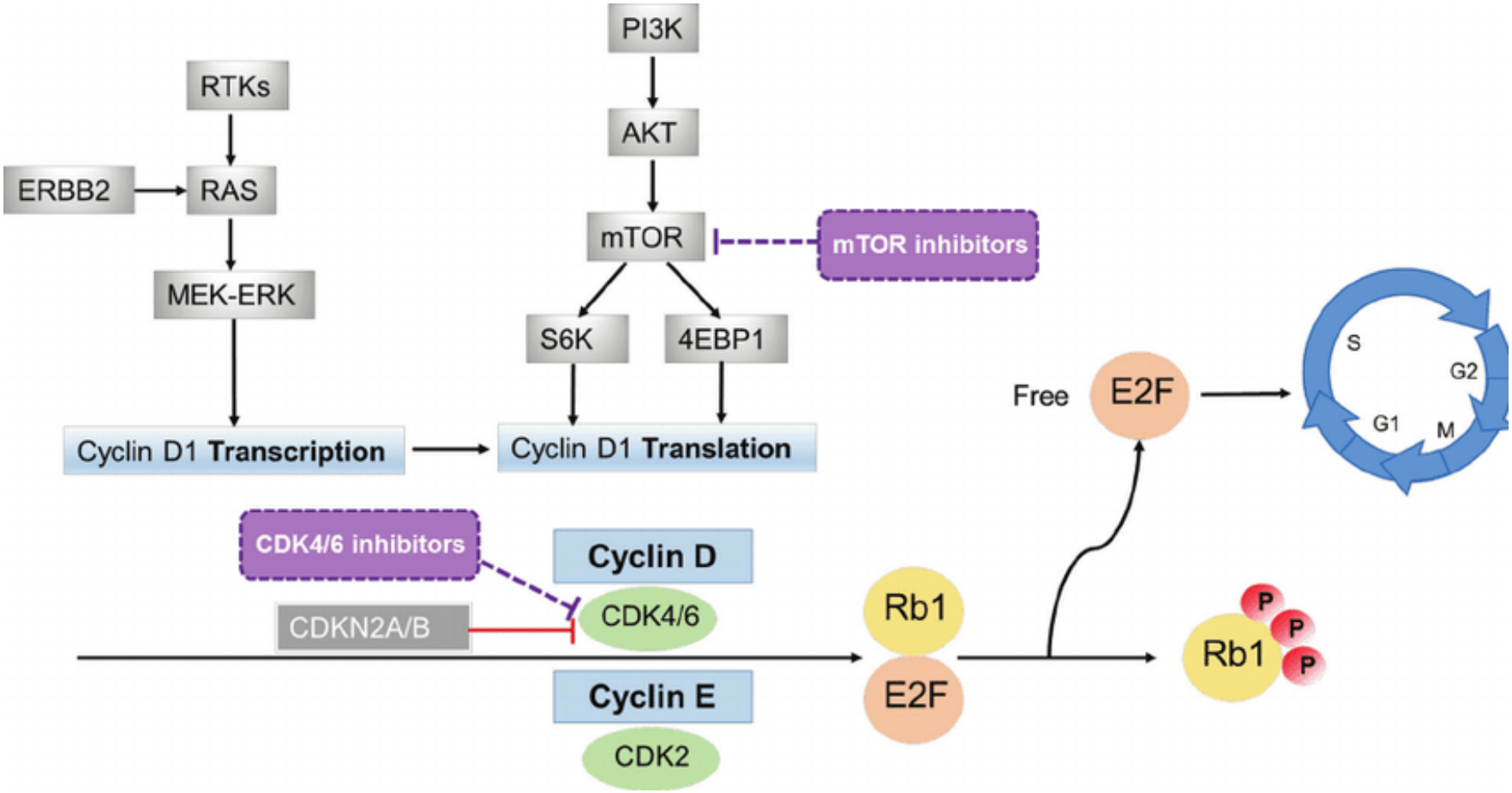
From: Targeting kinases with anilinopyrimidines: discovery of *N*-phenyl-*N*′-[4-(pyrimidin-4-ylamino)phenyl]urea derivatives as selective inhibitors of class III receptor tyrosine kinase subfamily
Valentina Gandin et al., 2015 Nature Scientific RepoRts | 5:16750 | DOI: 10.1038/srep16750

Radboudumc

Relevante mutaties voor predictie therapie respons

- KIT mut ; imatinib

- CDKN2A mut ; palbociclib in DRUP
- CCND1 ampl ; palbociblib in DRUP



Palboclib is a CDK4/6 inhibitor

Radboudumc

Schwaederle, Maria & Daniels, Gregory & Piccioni, David & Fanta, Paul & Schwab, Richard & Shimabukuro, Kelly & Parker, Barbara. (2014).
Cyclin alterations in diverse cancers: Outcome and co-amplification network. Oncotarget. 6. 10.18632/oncotarget.2848.

Relevante mutaties voor predictie therapie respons

- KIT mut ; imatinib

- CDKN2A mut ; palbociclib in DRUP
- CCND1 ampl ; palbociblib in DRUP

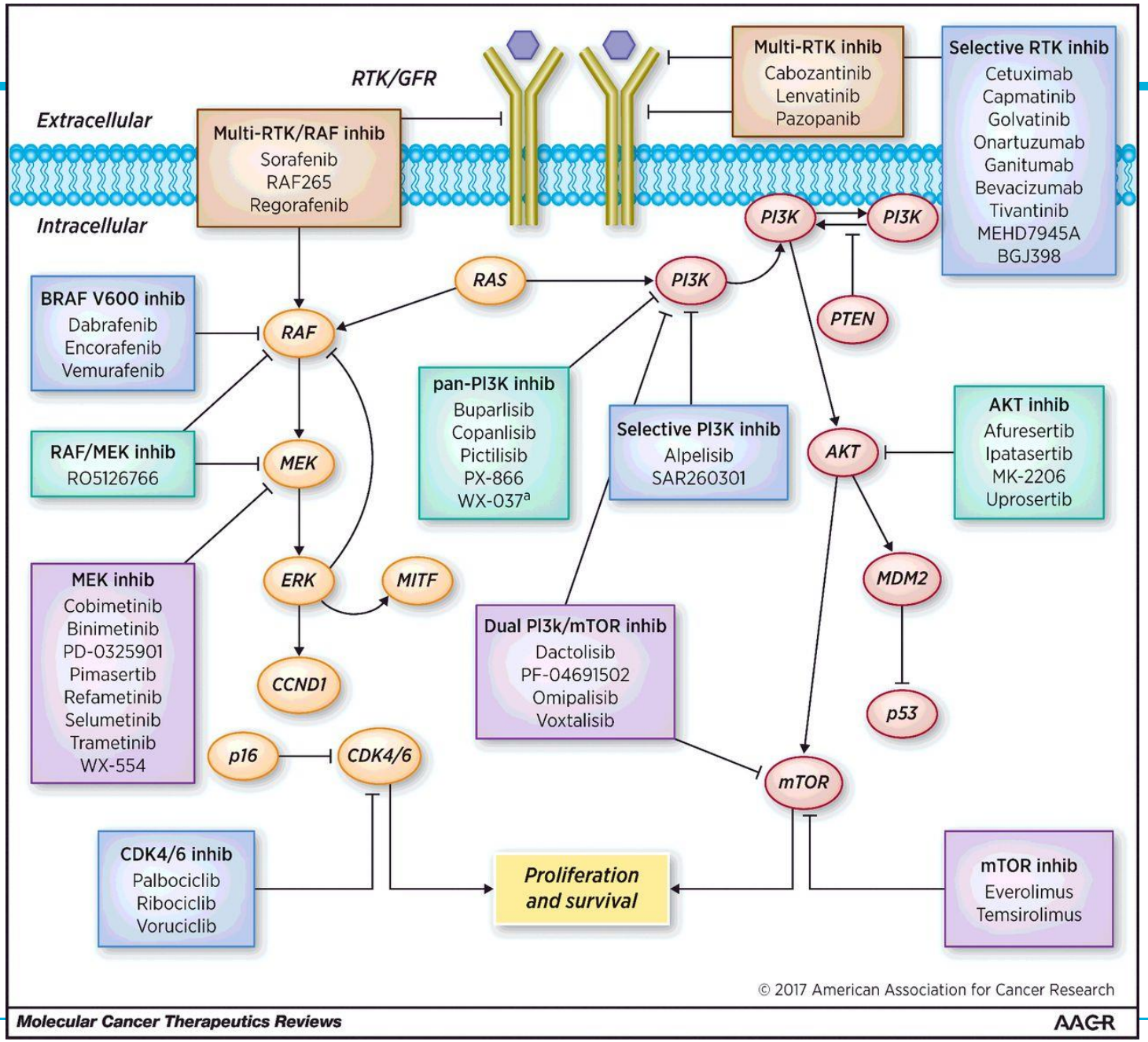
- B2-microglobuline (B2M); frame-shift deletion in exon 1
- Janus kinase 1 (JAK1): Q503* nonsense mutation
- Janus kinase 2 (JAK2): F547 splice-site mutation

Published in final edited form as:
N Engl J Med. 2016 September ; 375(9): 819–829. doi:10.1056/NEJMoa1604958.

Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma

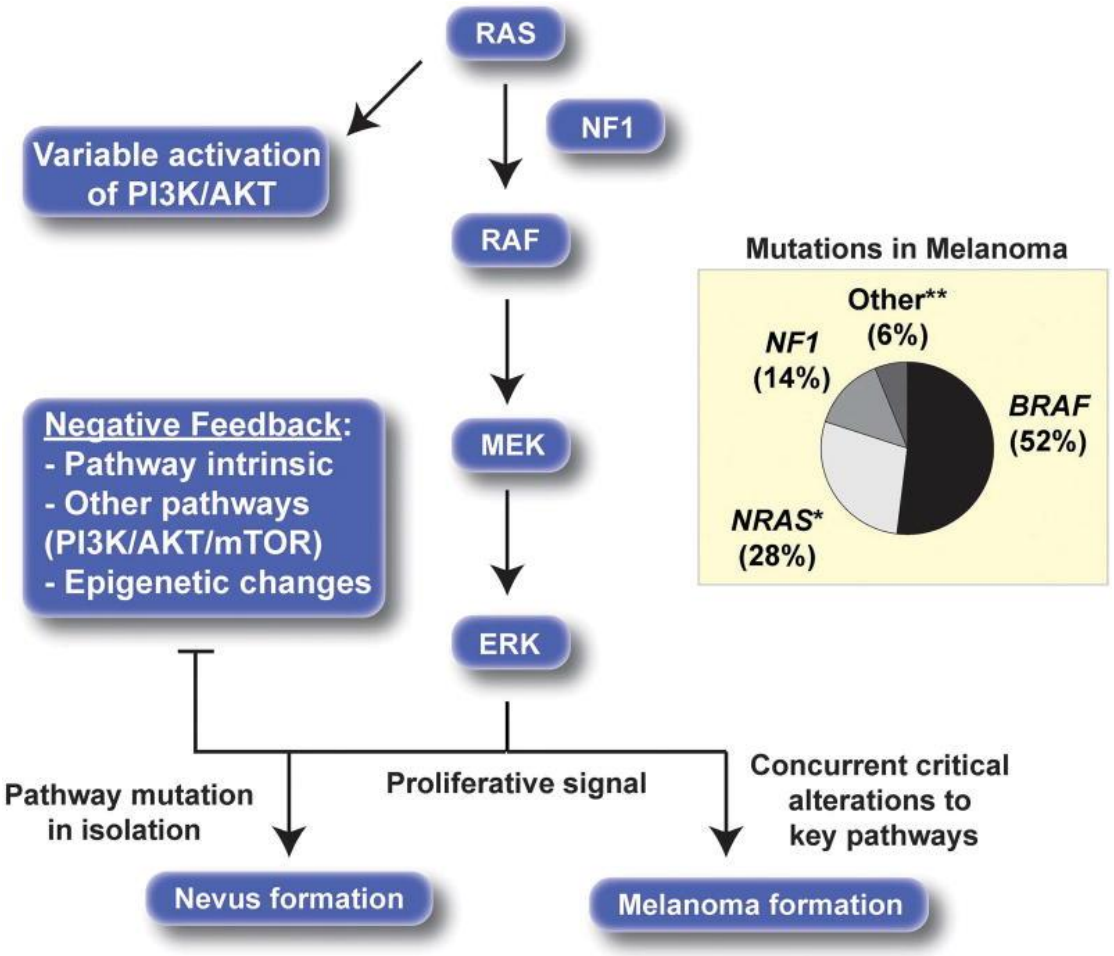
Jesse M. Zaretsky, B.S., Angel Garcia-Diaz, Ph.D., Daniel S. Shin, M.D., Helena Escuin-Ordinas, Ph.D., Willy Hugo, Ph.D., Siwen Hu-Lieskovan, M.D., Ph.D., Davis Y. Torrejon, M.D., Gabriel Abril-Rodriguez, M.Sc., Salemiz Sandoval, Ph.D., Lucas Barthly, M.Sc., Justin Saco, B.S., Blanca Homet Moreno, M.D., Riccardo Mezzadra, M.Sc., Bartosz Chmielowski, M.D., Ph.D., Kathleen Ruchalski, M.D., I. Peter Shintaku, Ph.D., Phillip J. Sanchez, Ph.D., Cristina Puig-Saus, Ph.D., Grace Cherry, R.N., N.P., Elizabeth Seja, B.A., Xiangju Kong, M.Sc., Jia Pang, B.S., Beata Berent-Maoz, Ph.D., Begoña Comin-Anduix, Ph.D., Thomas G. Graeber, Ph.D., Paul C. Tumeh, M.D., Ton N.M. Schumacher, Ph.D., Roger S. Lo, M.D., Ph.D., and Antoni Ribas, M.D., Ph.D.

melanoma



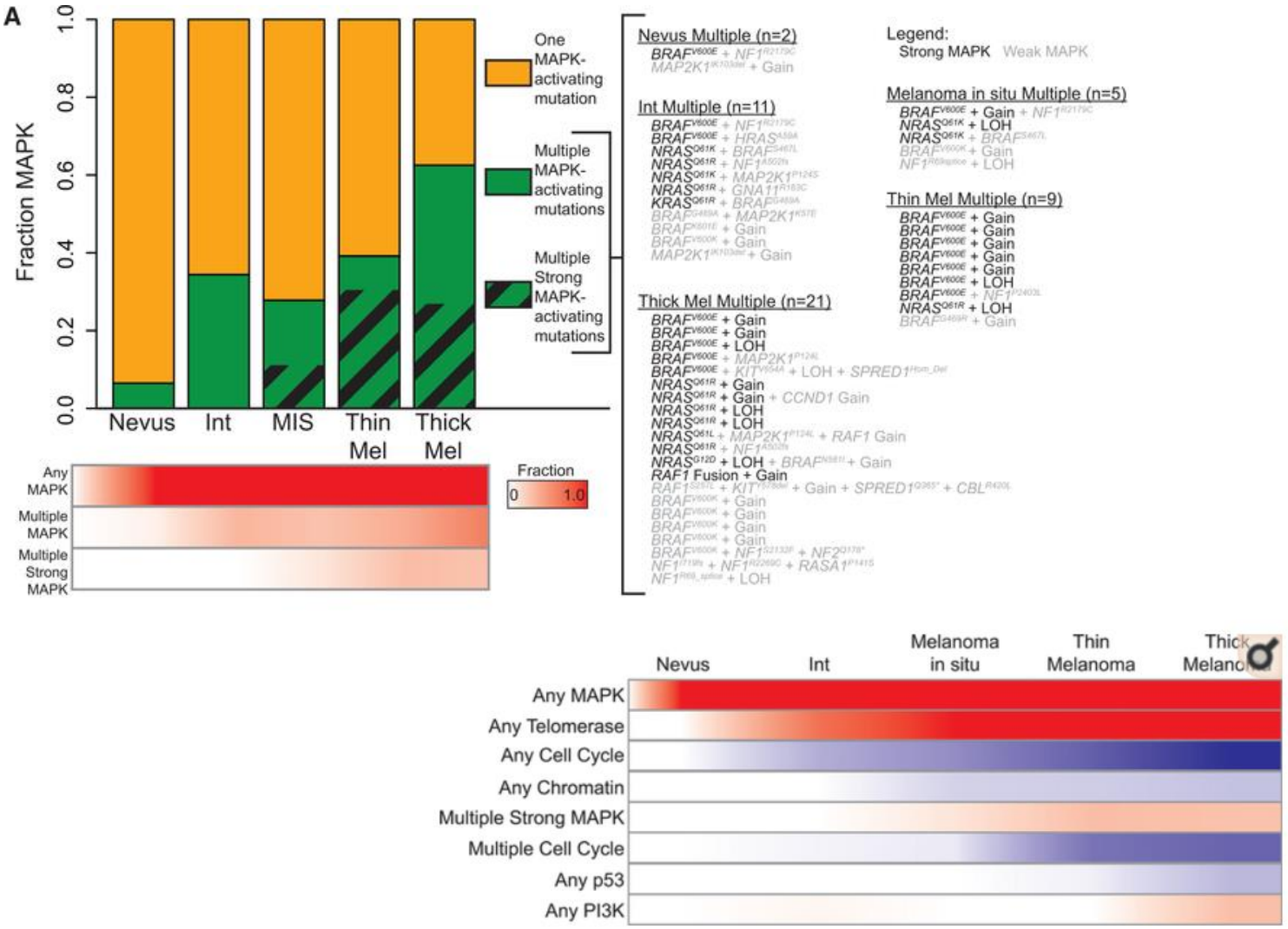
Rational Approaches for Combination Therapy Strategies Targeting the MAP Kinase Pathway in Solid Tumors
Anthony W. Tolcher, Wei Peng and Emiliano Calvo
DOI: 10.1158/1535-7163.MCT-17-0349 Published January 2018

Pathway melanocytic nevi and melanoma



Melanocytic **nevi** and **melanoma**: unraveling a complex relationship.
Damsky WE et al. Oncogene. (2017)

Meer genomische afwijkingen bij melanoom progressie



Somatic Alterations in Key Signaling Pathways that Drive Melanoma Appear at Specific Points in the Melanoma Progression Cascade

Each heatmap reflects the frequency that a given pathway is activated (red) or inactivated (blue) at a specific point in the melanoma progression cascade.

Fig 1 and 7 from: Cancer Cell. 2018 Jul 9;34(1):45-55.e4.**Genomic and Transcriptomic Analysis Reveals Incremental Disruption of Key Signaling Pathways during Melanoma Evolution.**
Shain AH[1], Joseph NM[2], Yu R[3], Benhamida J[2], Liu S[3], Prow T[4], Ruben B[5], North J[6], Pincus L[6], Yeh I[7], Judson R[3], Bastian BC[8]. doi: 10.1016/j.ccell.2018.06.005

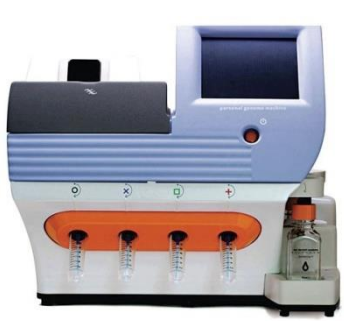
Breakthroughs for molecular analysis

- 1977 Sanger sequencing
- 1983 Polymerase chain reaction
- 2008 Next Generation Sequencing Technologies
- 2010-2019… **Mutation detection by NGS**
  Targeted assays / Whole exome seqeuncing

Sequence analysis

Next Generation Sequencing (NGS)

whole genome sequencing (WGS)	whole exome sequencing (WES)	‘panel’ sequencing	Sanger sequencing



Radboudumc

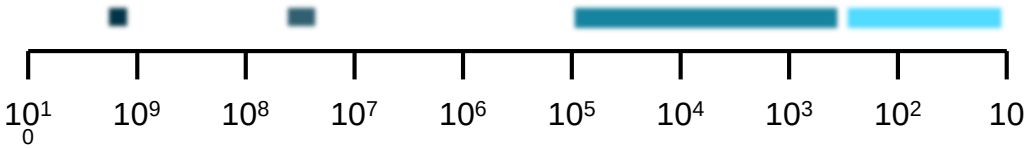
Source: A. Eijkelenboom, dept of Pathology, Radboudumc

Sequence analysis

Next Generation Sequencing (NGS)

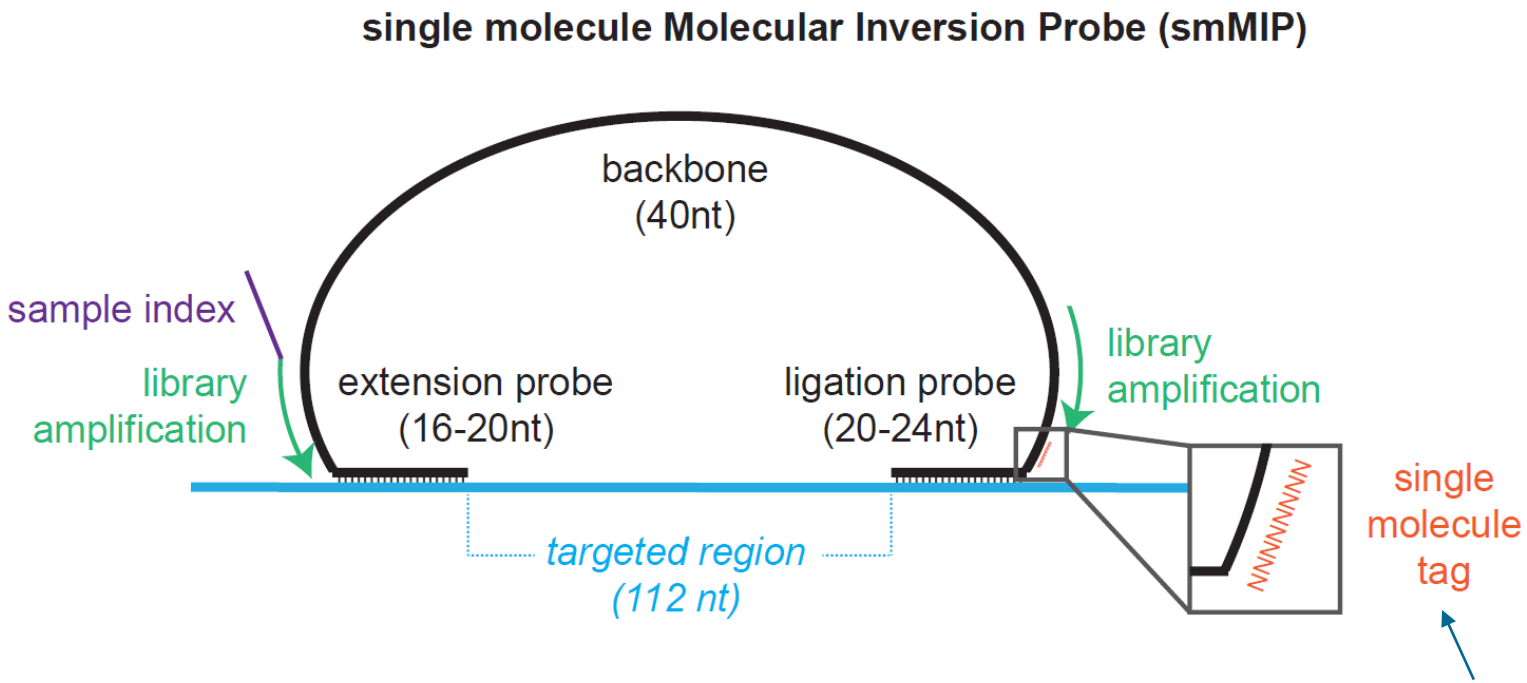
whole genome sequencing (WGS)	whole exome sequencing (WES)	‘panel’ sequencing	Sanger sequencing

sequenced basepairs:



Source: A. Eijkelenboom, dept of Pathology, Radboudumc

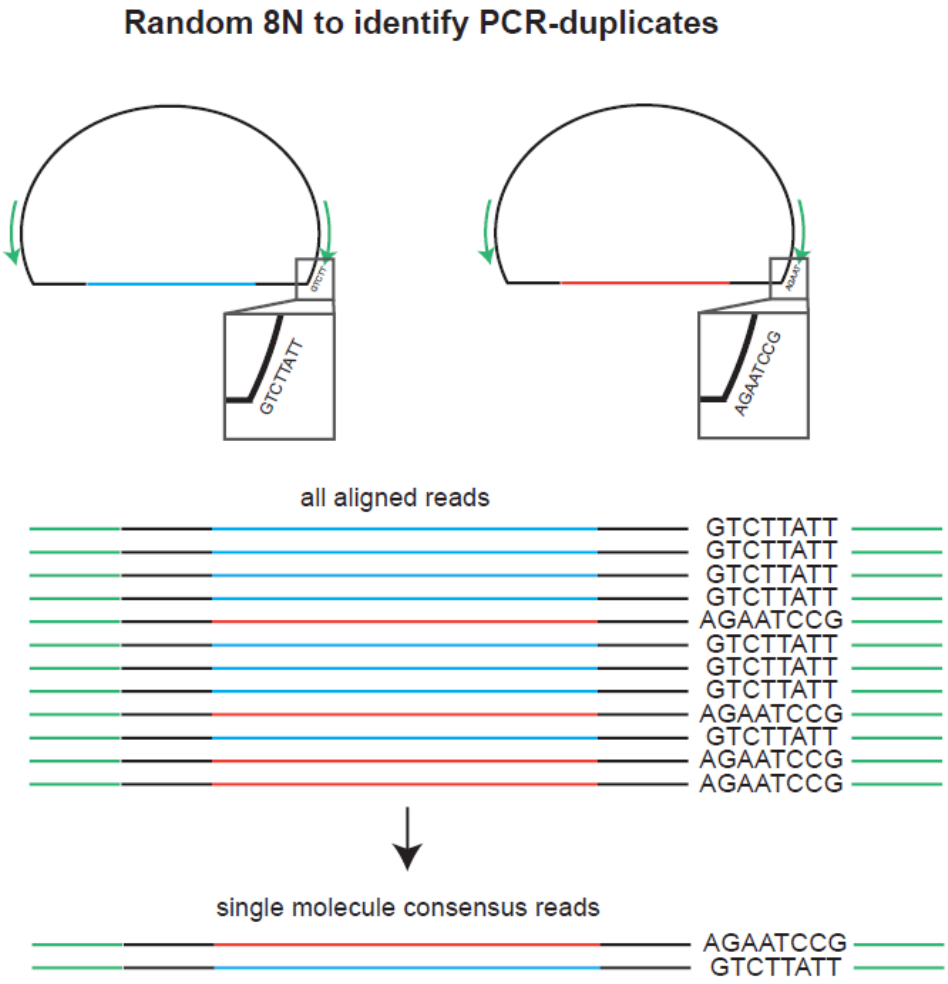
NGS by smMIPs



Unique Molecular Identifier
UMI

Radboudumc

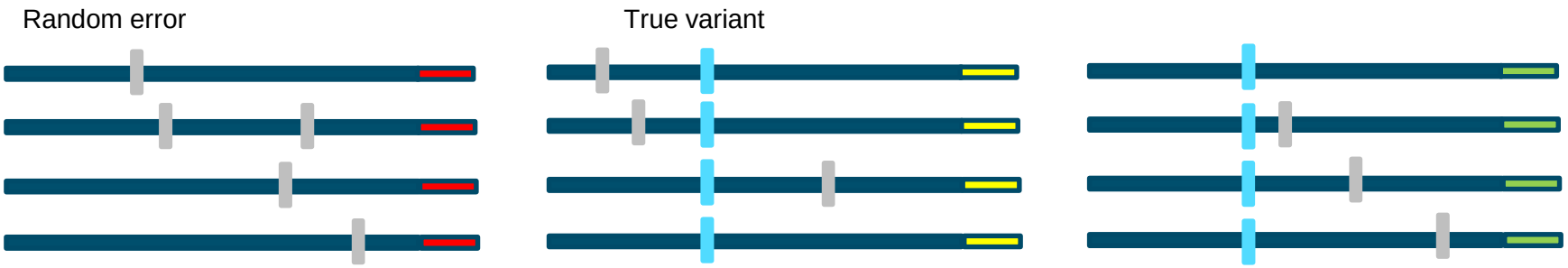
Unique Molecular Identifiers (UMI's)



Assembly of PCR duplicates:

- Known number of genomic template molecules

Unique Molecular Identifiers (UMI's)



Single molecule consensus read

Assembly of PCR duplicates:

- Known number of genomic template molecules
- Reduction of false positive variants due to PCR/sequencing artefacts

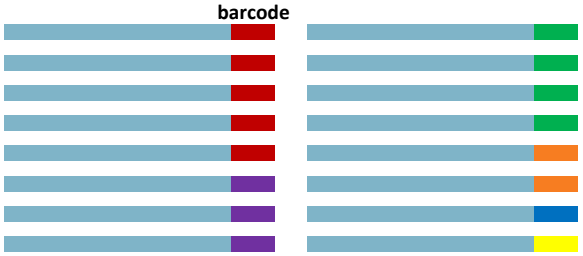
Radboudumc

Source: PJTA Groenen, dept. of Pathology, Radboudumc

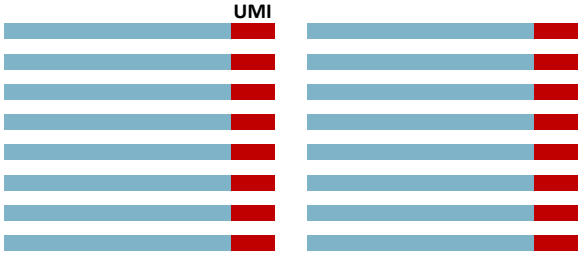
Begrippen by Next Generation Sequencing

Coverage

Aantal sequentie reads per target regio



Coverage: 16 reads
Unieke coverage*: 6 reads



Coverage: 16 reads
Unieke coverage*: 1 read

Variant allel frequentie (VAF)

Percentage sequentie reads met de mutatie

VAF: 50%

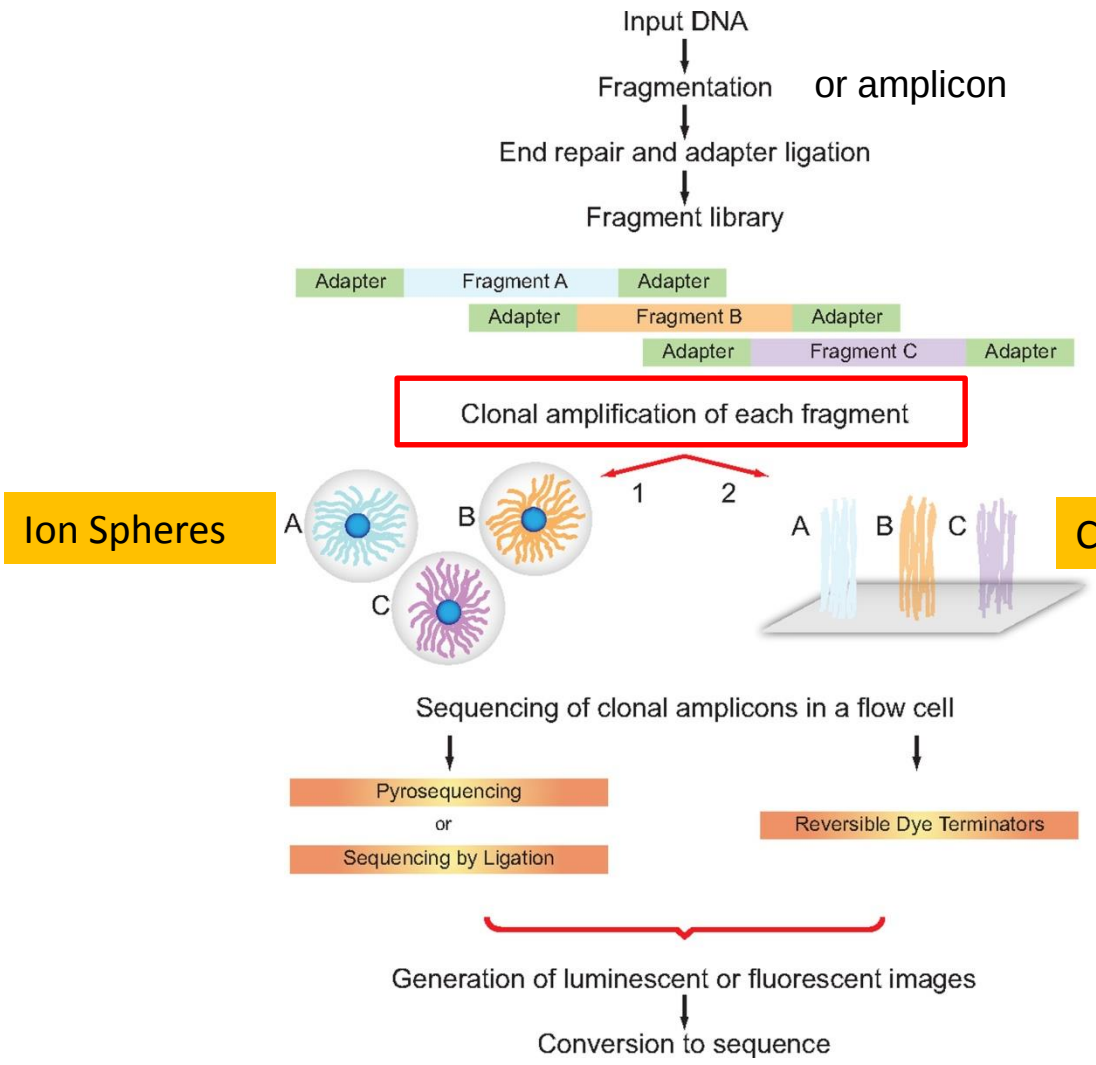
VAF: 10%

<u>Kiembaan varianten:</u>
VAF 50% : 1/2 genkopieën aangedaan
VAF 100% : 2/2 genkopieën aangedaan

*Unieke coverage =aantal unieke DNA moleculen

Radboudumc

Source: L.Kroeze, dept. of Pathology, Radboudumc



Library preparation

Amplification

Ion Spheres

Clusters

Sequencing

Analysis

Radboudumc

NGS (Illumina)

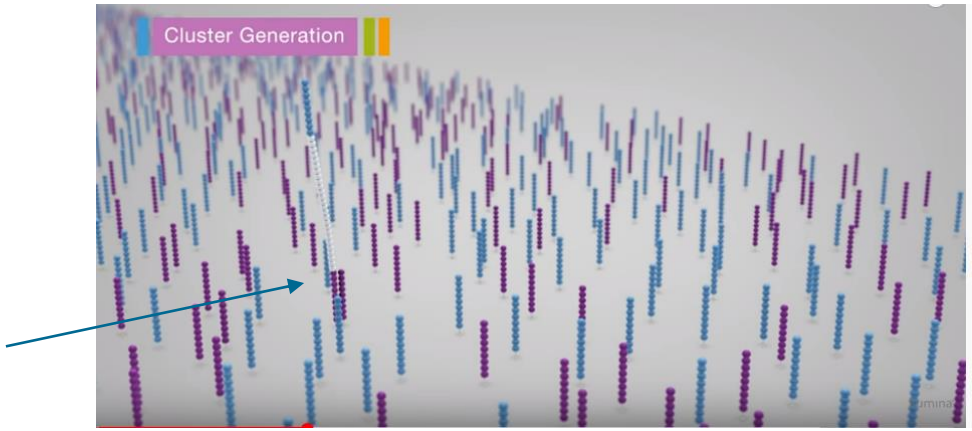
- Library construction

  fragments; attach adaptor sequences

- Cluster generation

  the adaptors are complementory to the two type of oligo's present on the flow cell

- Sequencing

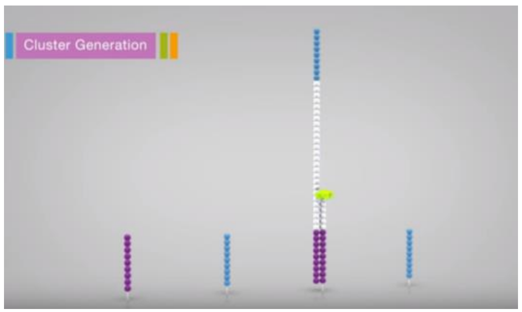


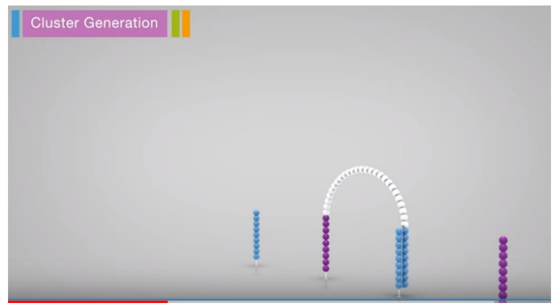
Radboudumc

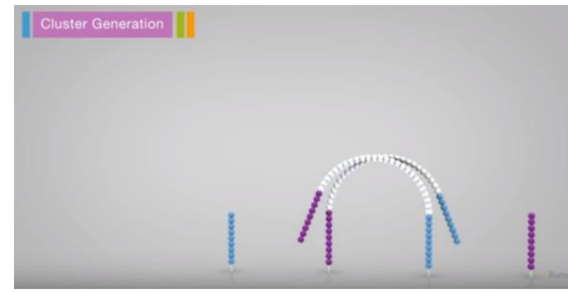
Source figure cluster generatation: Introduction Illumina sequencing by synthesis, screenshots of You-tube

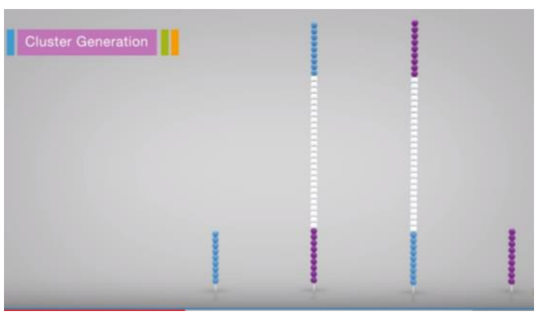
Illumina sequencing by synthesis

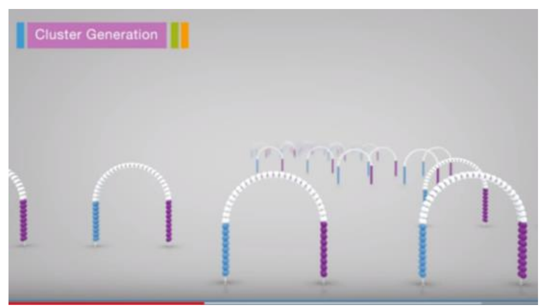
- Cluster generation
  - Add and hybridize the library to the flow cell
  - Bridge amplification
  - Generating clusters from indidual molecules

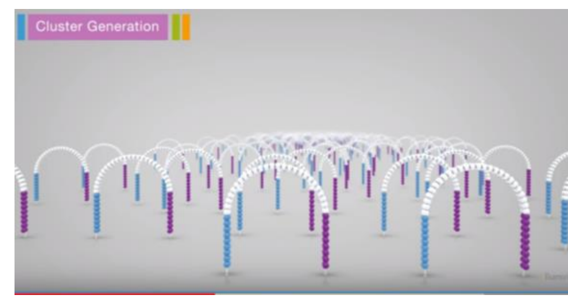




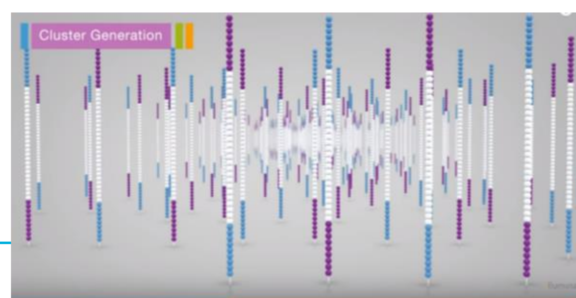








To identify samples after pooling, each sample is uniquely tagged with a sequence index during the sample preparation protocol.



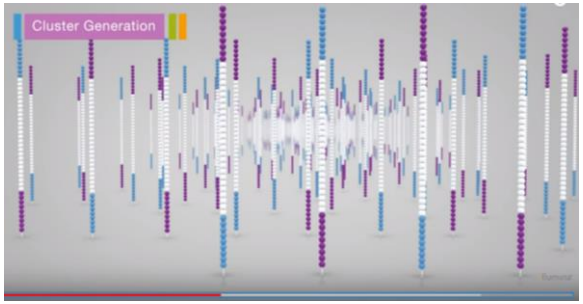
NGS (Illumina)

- Library construction

  fragments; attach adaptor sequences

- Cluster generation

  the adaptors are complementory to the two type of oligo's present on the flow cell



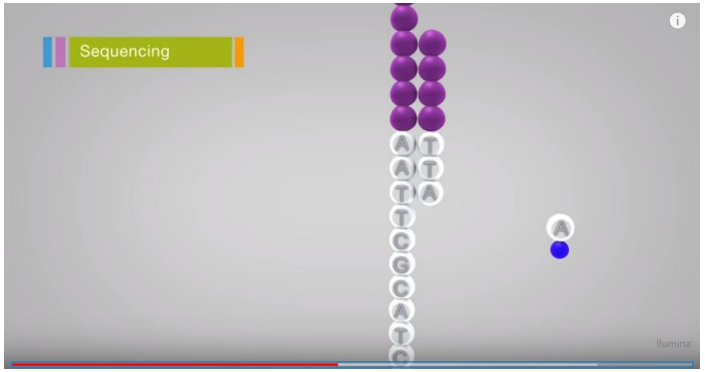
- Sequencing

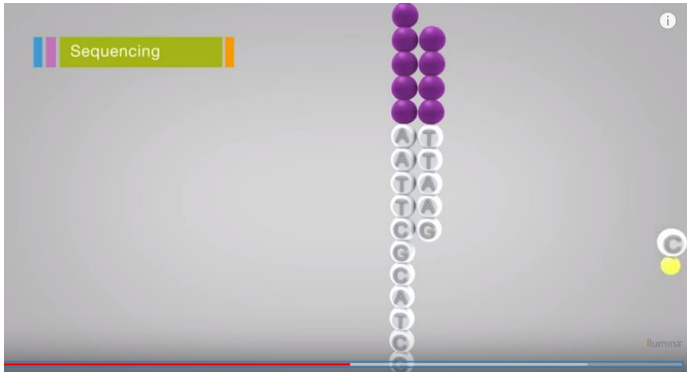
Radboudumc

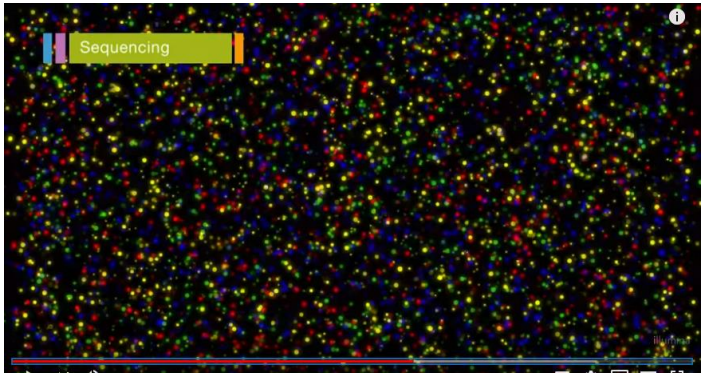
Source figures cluster generatation: Introduction Illumina sequencing by synthesis, screenshots of You-tube
PJTA Groenen, dept. of Pathology, Radboudumc

Illumina sequencing by synthesis

- Sequencing : Add all 4 fluorescently tagged nt
  - A single base is incorporated at a time; a reversible terminator is on every nucleotide to prevent multiple additions in one round, remove terminator and repeat
  - Image 4 colors







Radboudumc

Source figures Seqeuncing: Introduction Illumina sequencing by synthesis, screenshots of You-tube
PJTA Groenen, dept. of Pathology, Radboudumc

Illumina sequencing by synthesis

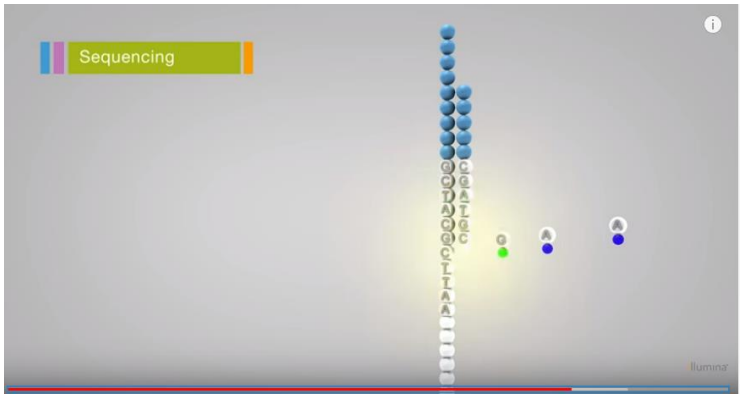
- Sequencing : paired end

- Data analysis

  multiple steps

  Note: the sequencing occurs for millions of clusters at once

  reference genome

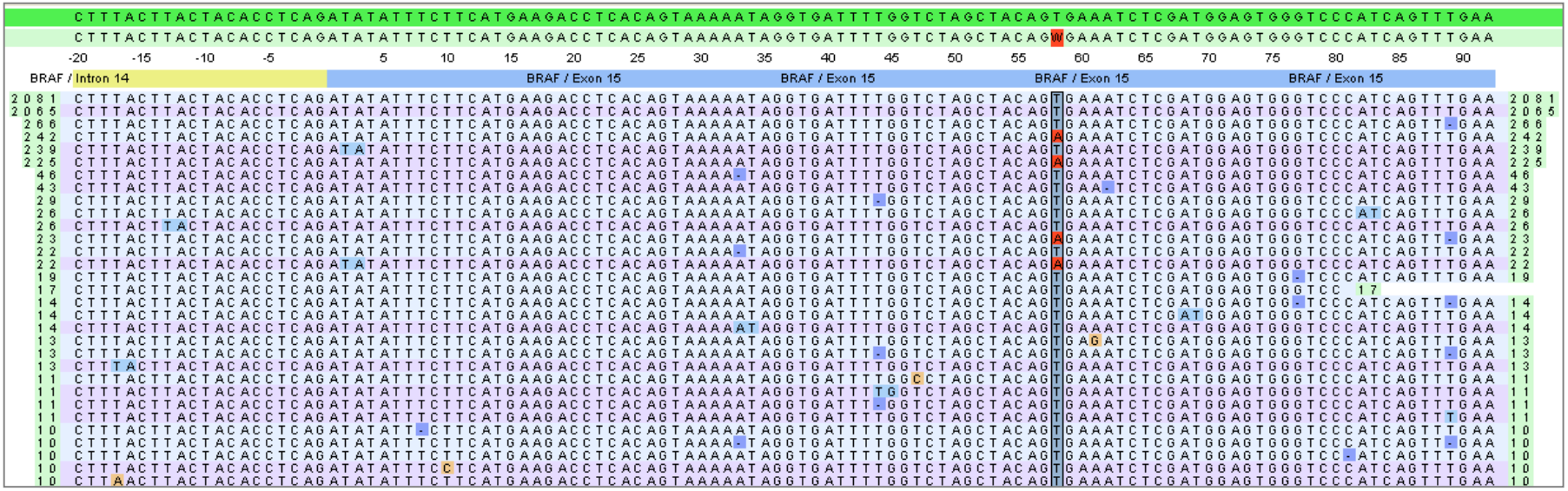


Radboudumc

Source figures Seqeuncing: Introduction Illumina sequencing by synthesis, screenshots of You-tube
PJTA Groenen, dept. of Pathology, Radboudumc

Result *BRAF* mutation analysis

screenshot reads

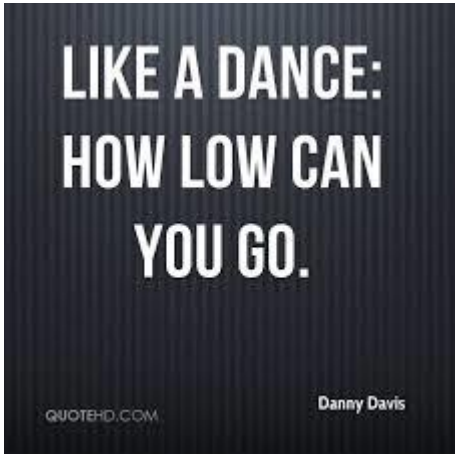


BRAF RefSeq NM_004333.4:
codon 600: mutatie c.1799T>A (p.(Val600Glu) alias p.V600E); 9% mutant allel
NRAS RefSeq NM_002524.3:
codon 12, 13 en 61: geen mutatie
KIT RefSeq NM_000222.2:
Exon 9,11,13,14 en 17: geen mutatie

Radboudumc

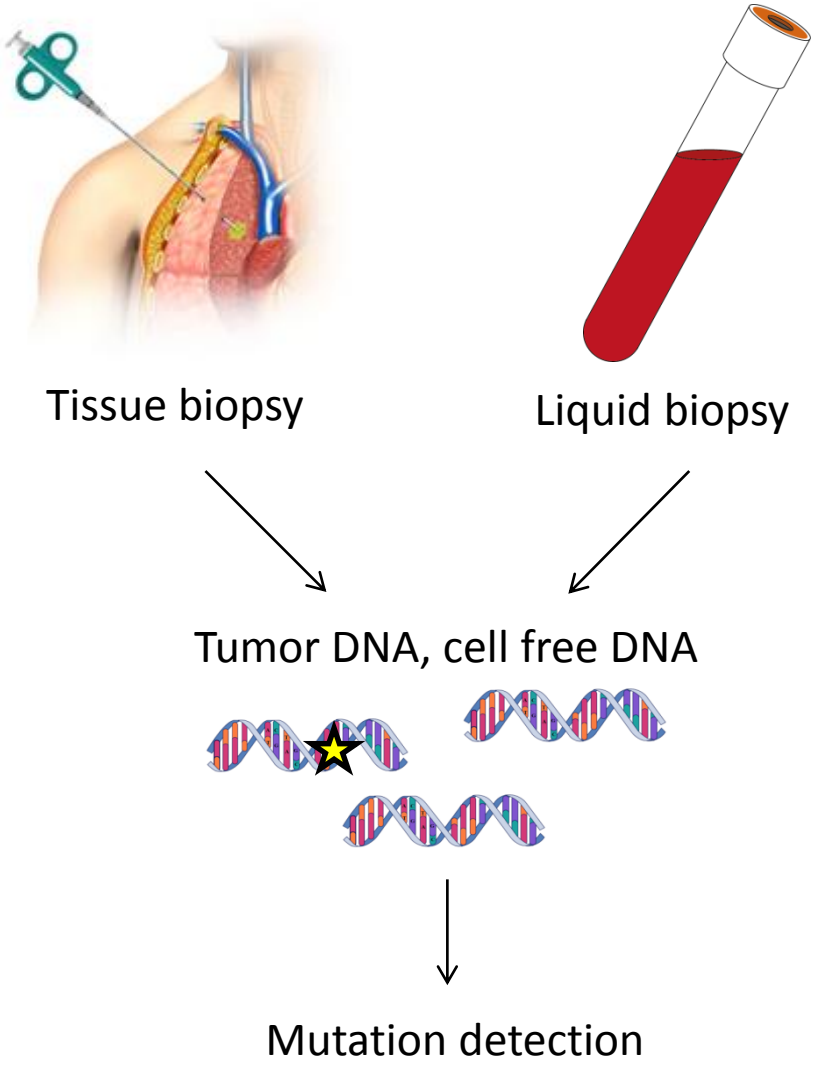
Source: PJTA Groenen, dept. of Pathology, Radboudumc

Low level mutation detection



Liquid Biopsy

- Used for diagnostic and monitoring purposes, residual disease detection and treatment selection
- Prevent sampling bias and invasive or difficult biopsies
- Con: cell free DNA from indeed the specific tumor?



Radboudumc

Source: M. Geerlings & S. Tolmeijer, Laboratory Tumor Genetics, Radboudumc

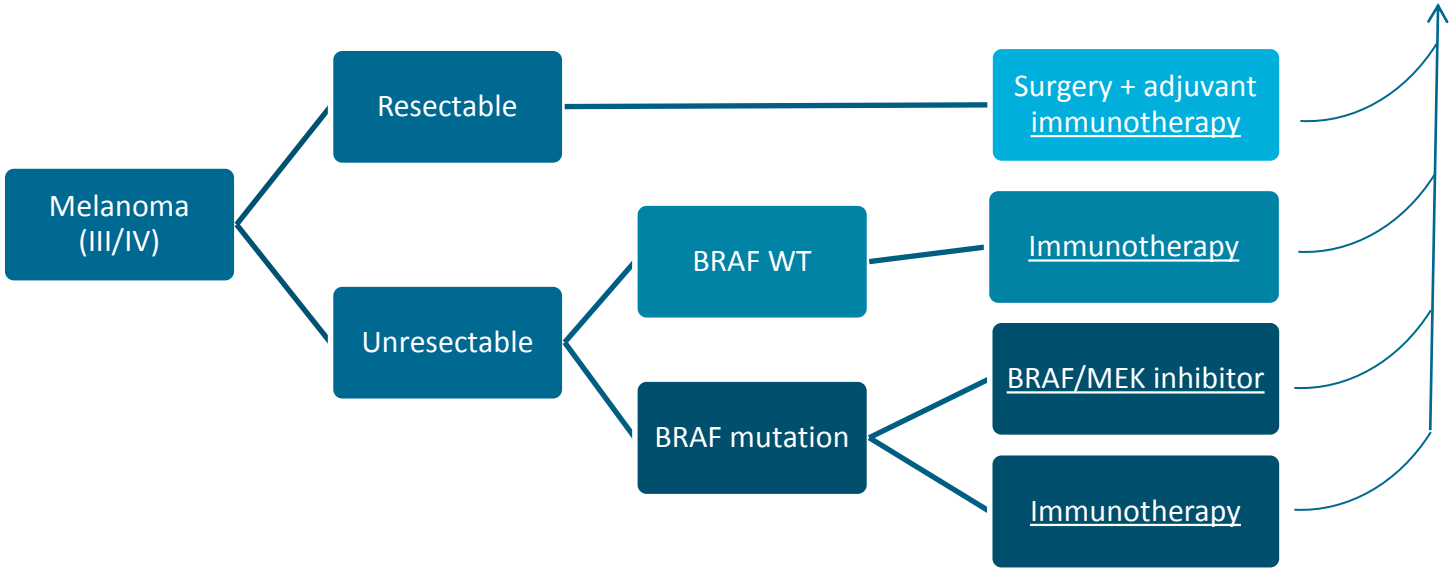
Melanoma

Imaging golden standard → can be inconclusive

There is need for biomarkers for clinical guidance!

- Early stages (I/II): surgery + IFN or radiotherapy
- Later stages (III/IV):

Single or combination regime?
High toxicity
Expensive
Only beneficial for some patient



(1) Intergraal kankercentrum Nederland (IKCN)
(2) ESMO clinical practice guidelines for diagnosis, treatment and follow-up

(1) Eggermont, *et al*. 2018
(2) McArthur, *et al.* 2014

ctDNA suitable biomarker?

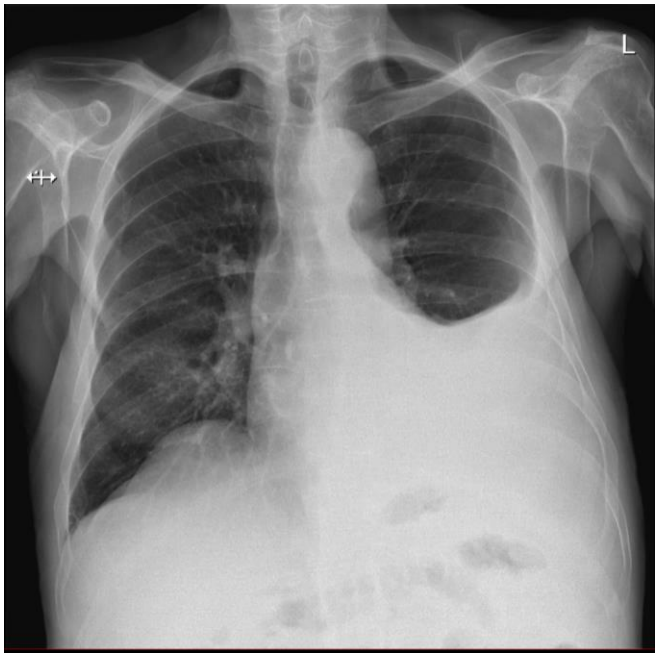
- **High ctDNA %**
- **Associated mutations**
  - 50% BRAF mutation
    - 80-90% BRAF V600E
    - 10-20% BRAF V600K
  - 15-25% NRAS

(1) Santiago-Walker, *et al.* 2015
(2) Sanmamed, *et al.* 2015

(1) Gray, *et al.* 2015
(2) Ashida, *et al.* 2017

Enkele voorbeelden indicatie voor spoed BRAF bepaling

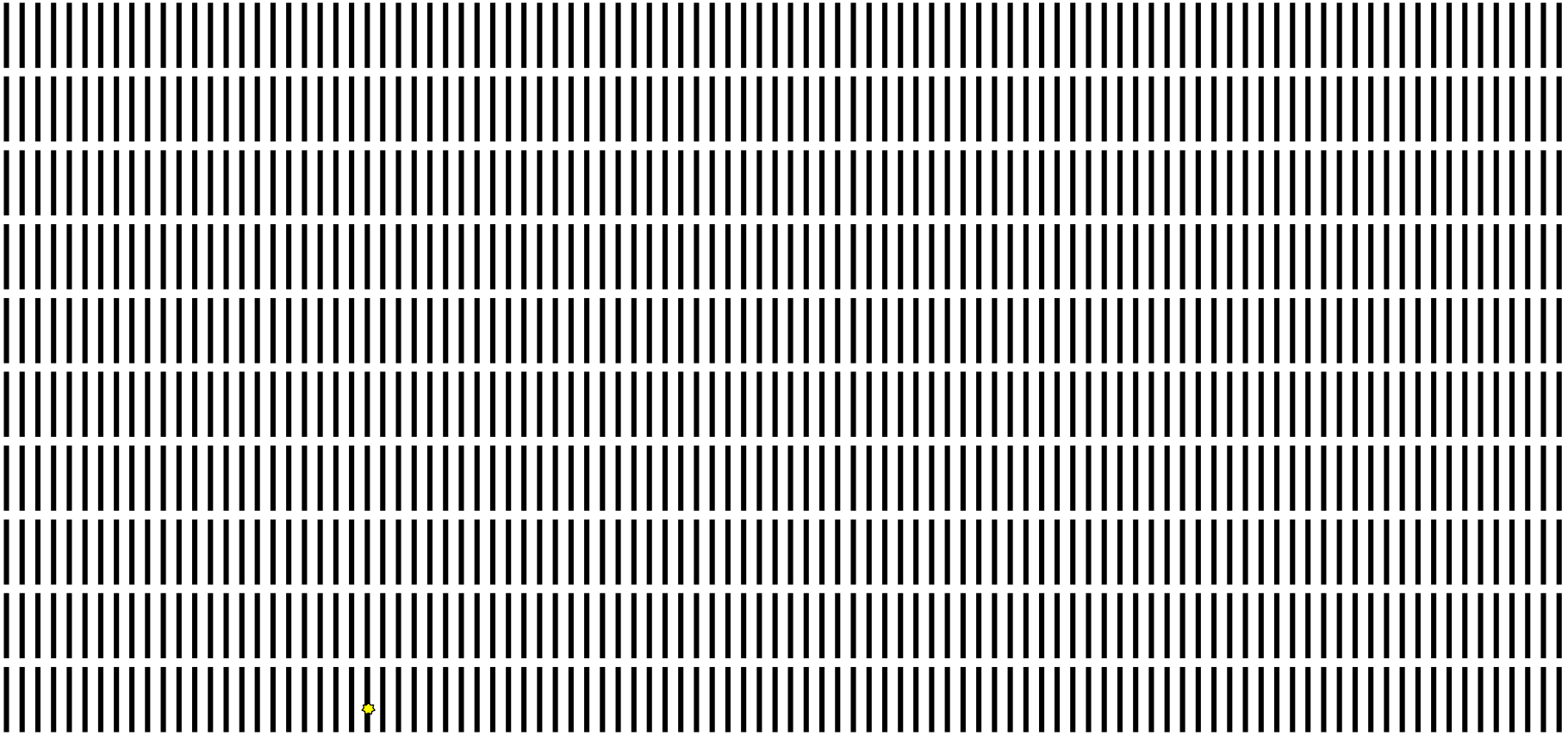
- Dreigende dwarslaesie bij wervelmetastase
- Sufheid, dreigende inklemming bij hersenmetastasen
- Ernstige dyspnoe bij exudatief pleuravocht obv pleurale localisatie melanoom



Radboudumc

Source: A. Amir, dept Pathology, Radboudumc

How can these aberrations be detected?

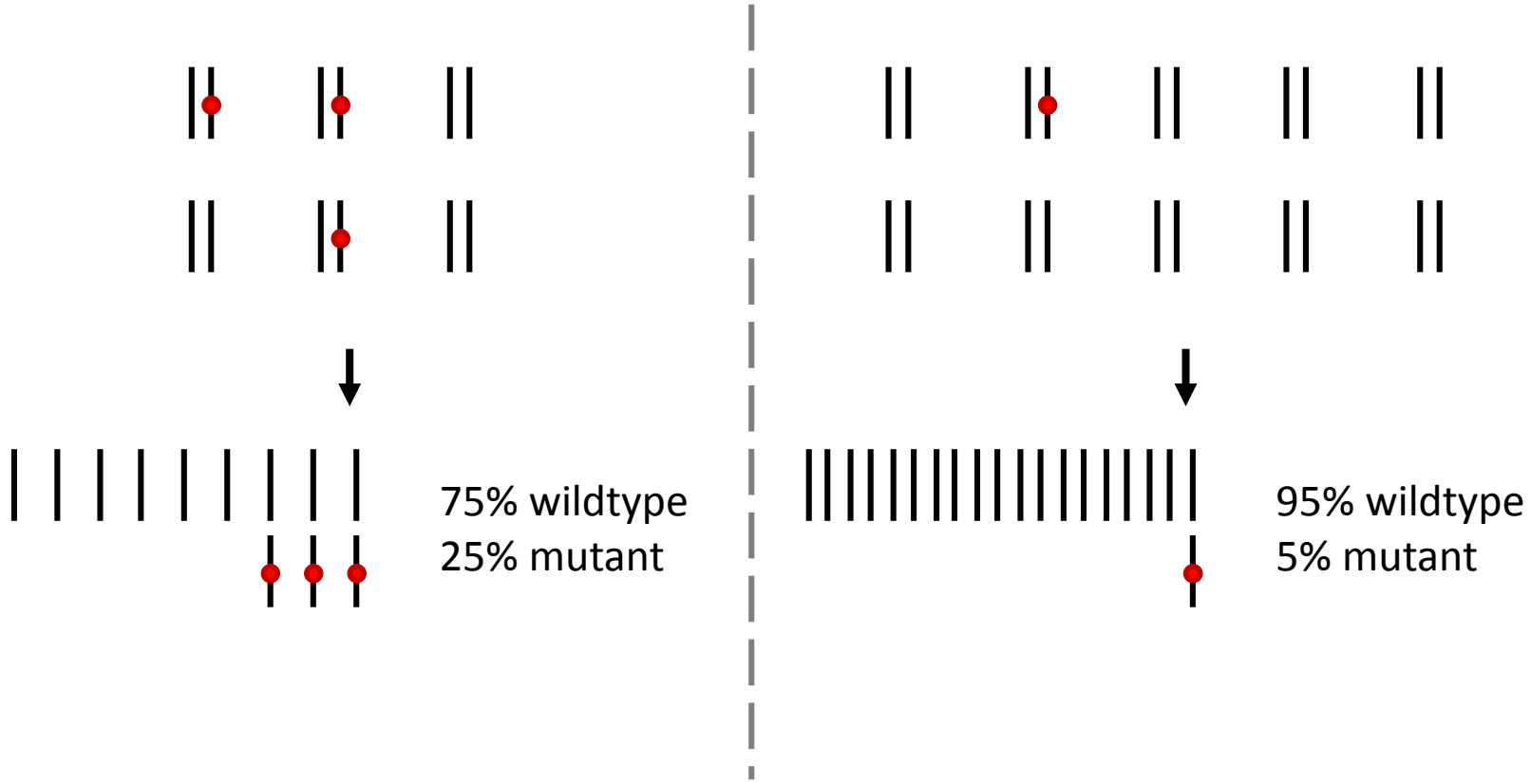


→ Sensitive & specific techniques needed!

Radboudumc

Source: M. Geerlings & S. Tolmeijer, Laboratory Tumor Genetics, Radboudumc

Sensitivity vs tumorload (tissue)

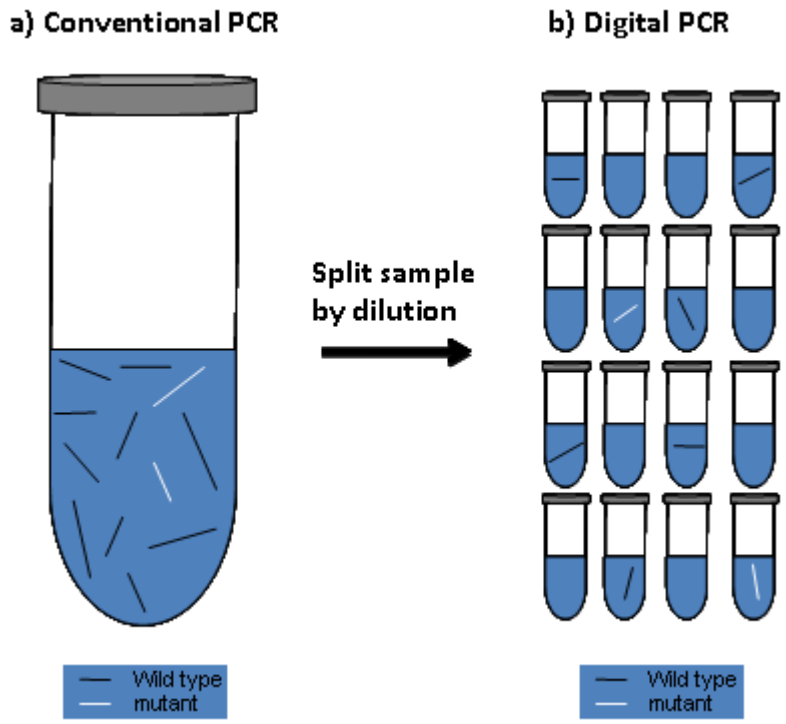


Radboudumc

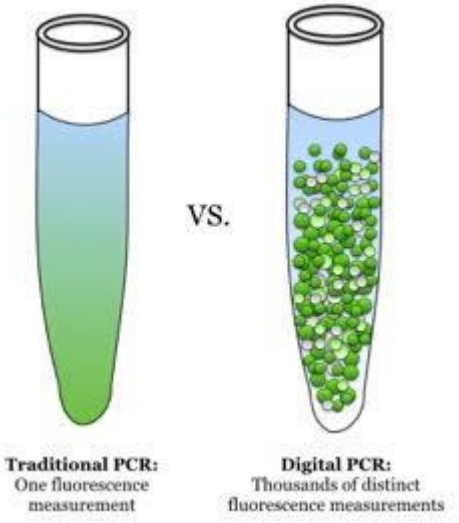
Source: M. Geerlings & S. Tolmeijer, Laboratory Tumor Genetics, Radboudumc

High sensitivity detection

by partitioning the DNA strands and then do the PCR



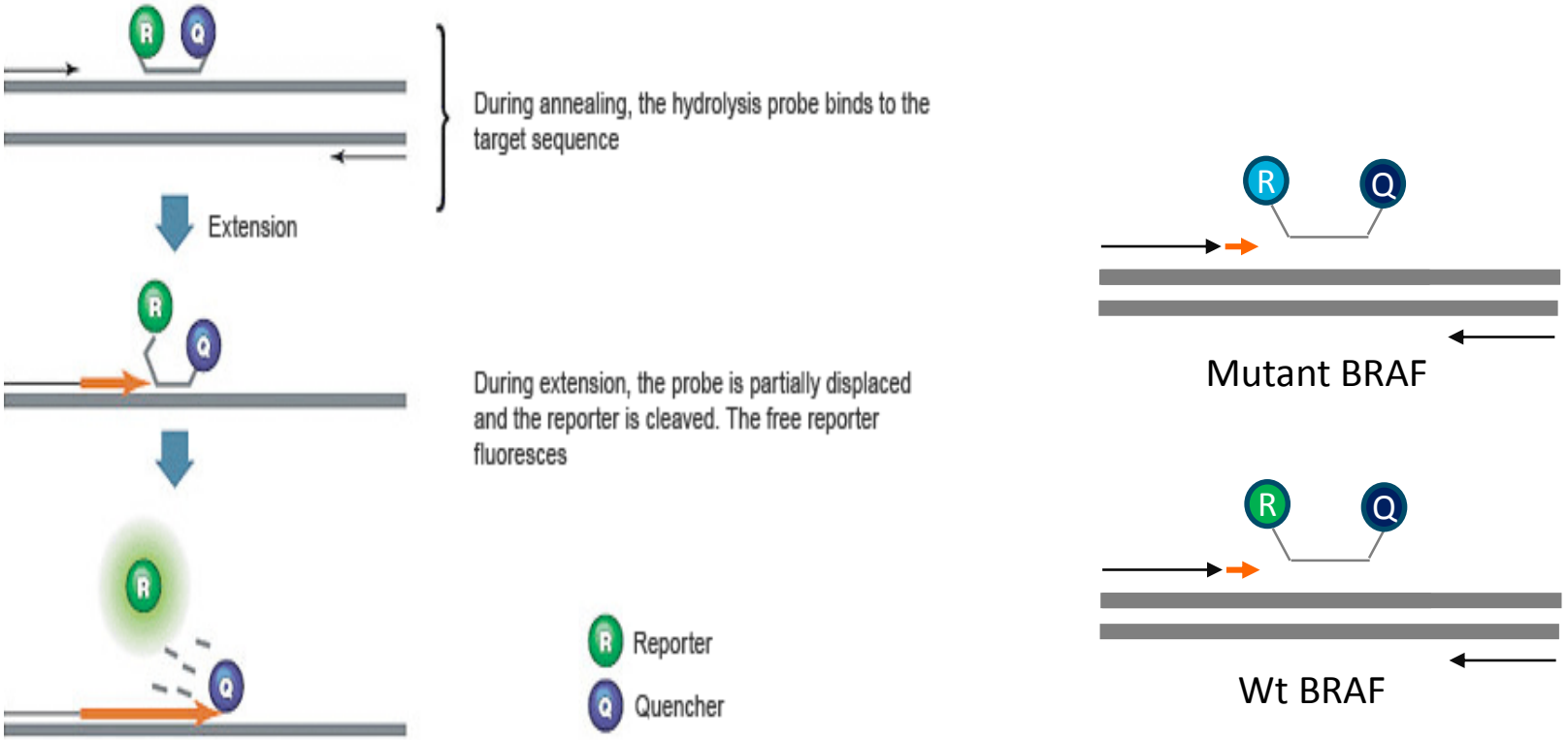
PCR using fluorescent hydrolysis probes



Digital droplet PCR (ddPCR)

http://digital-pcr.gene-quantification.info/
https://en.wikipedia.org

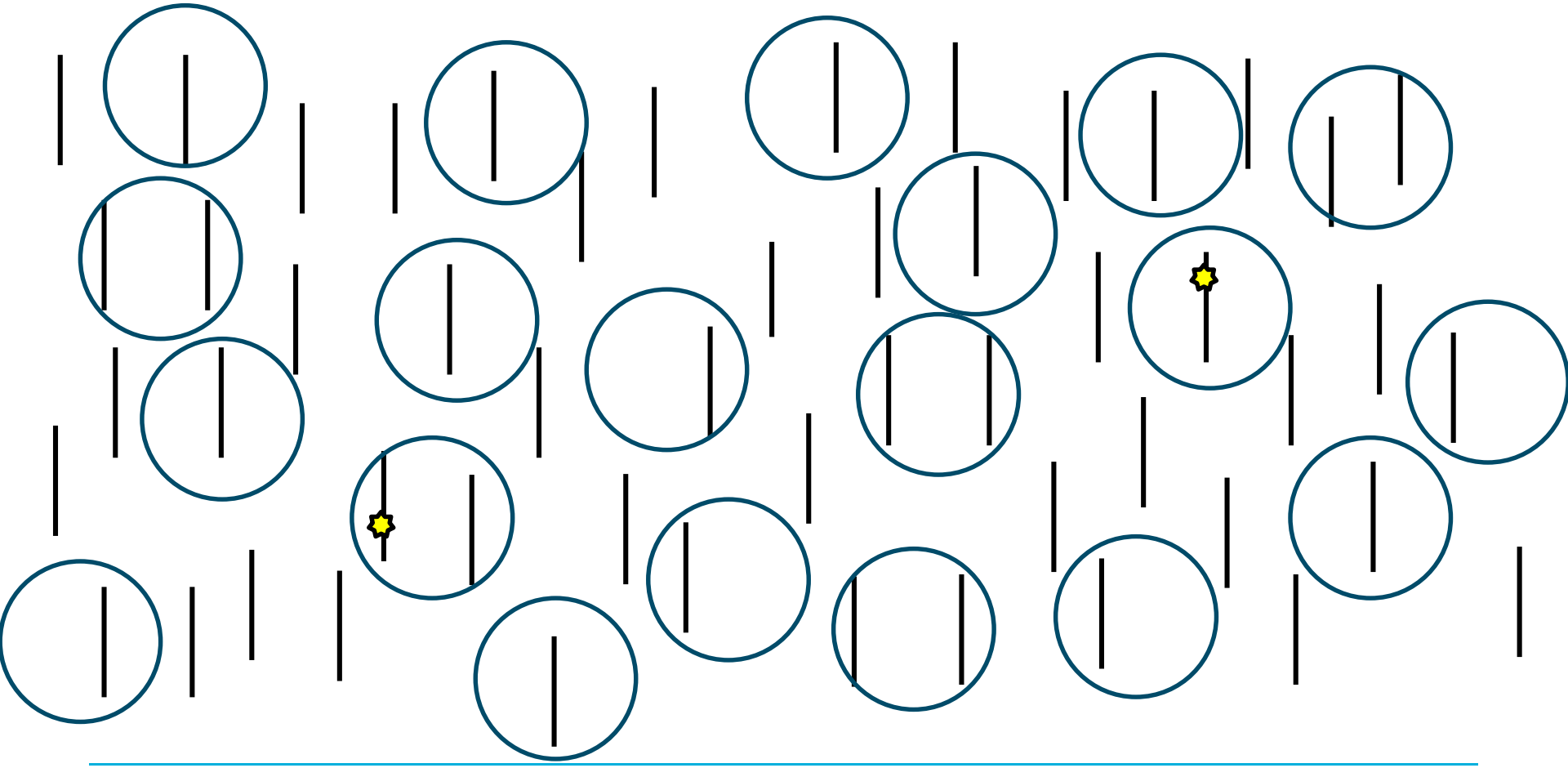
ddPCR uses hydrolysis probes



- Intact probe: the fluorescence of the reporter is quenched due to its proximity to the quencher
- PCR extension: the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.

https://www.bio-rad.com
Source: PJTA Groenen, dept. Of Pathology, Radboudumc

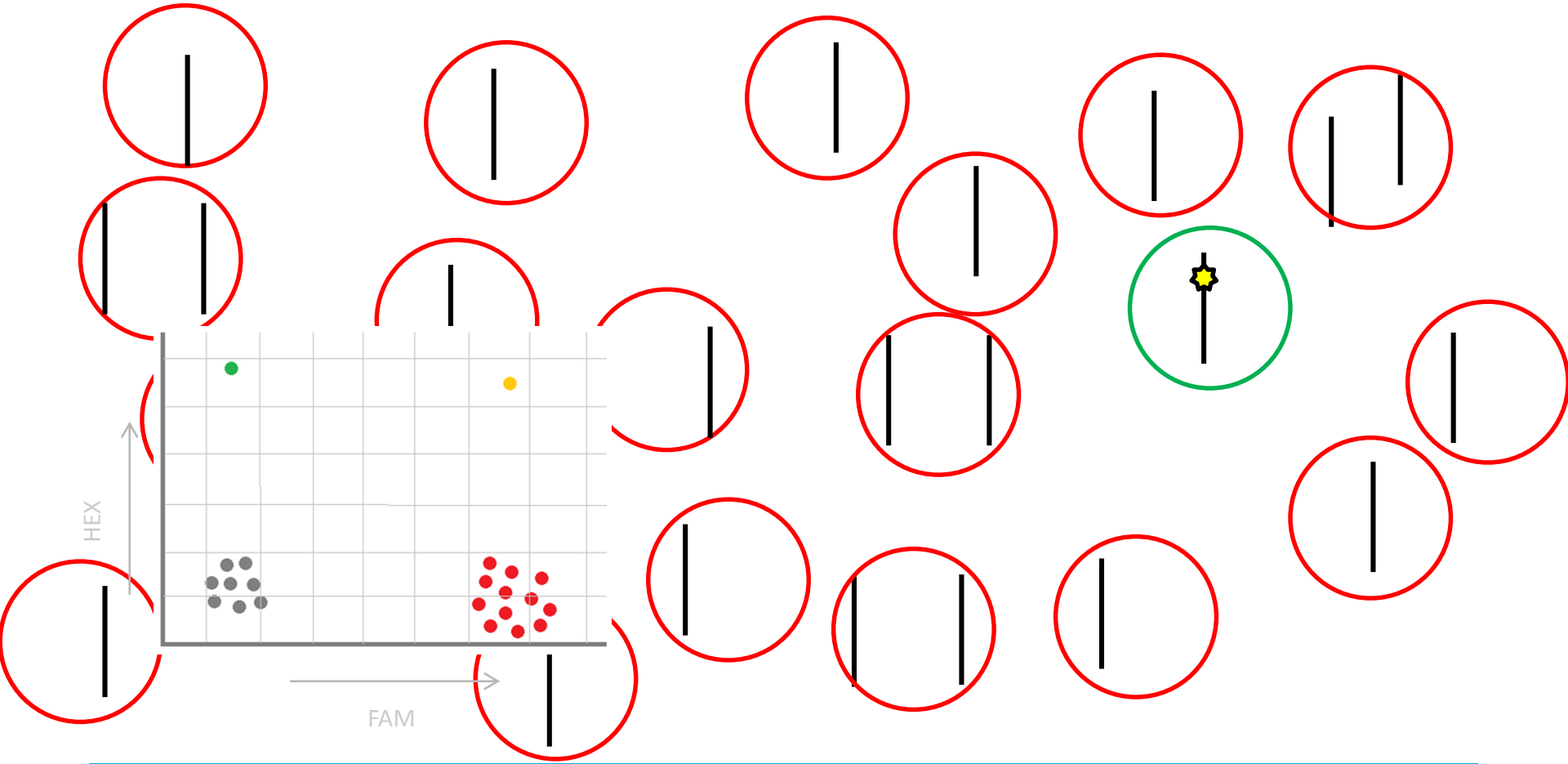
Droplet digital PCR (ddPCR) - BRAF



Radboudumc

Source: M. Geerlings & S. Tolmeijer, Laboratory Tumor Genetics, Radboudumc

Droplet digital PCR (ddPCR)



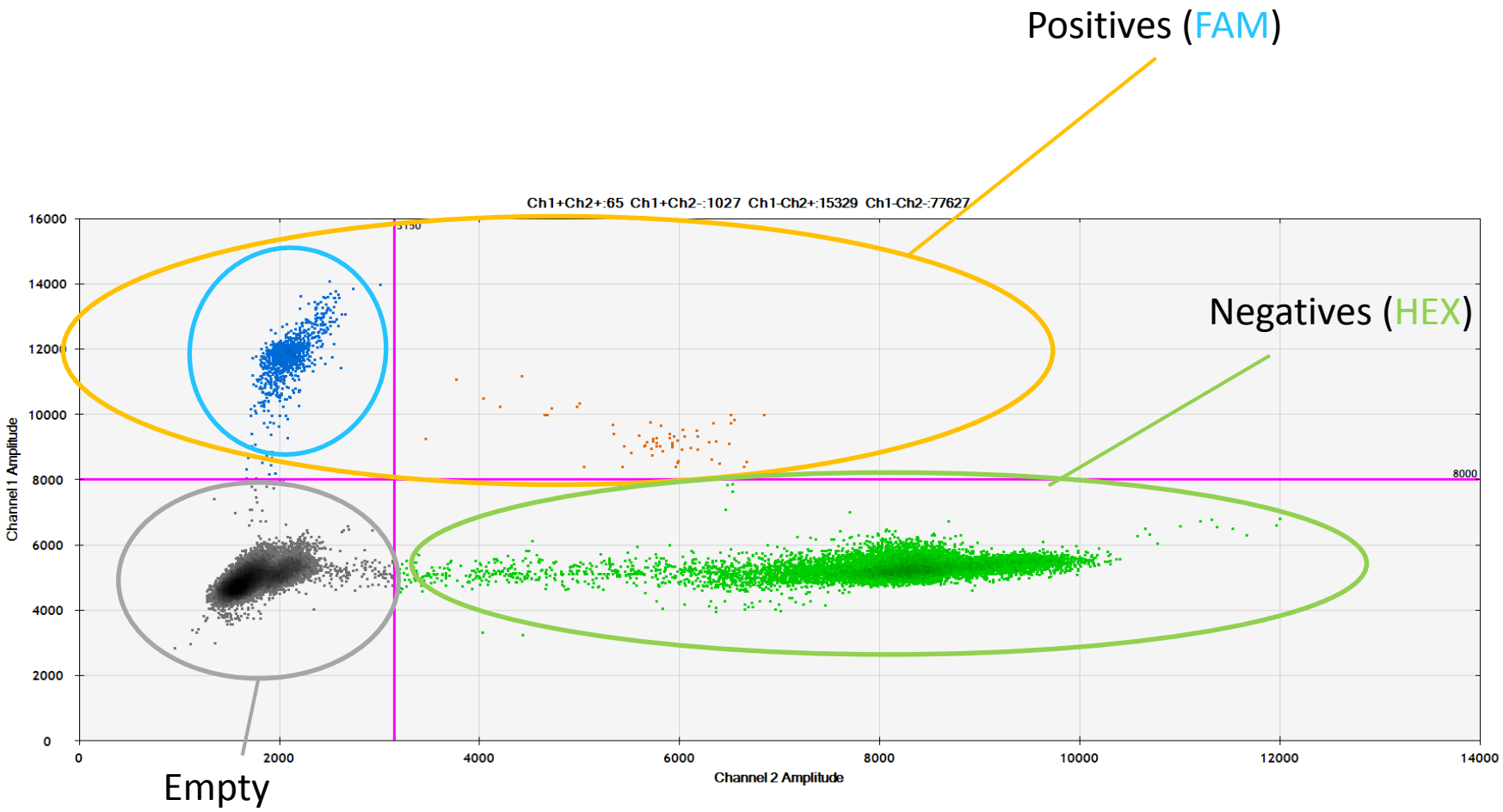
Radboudumc

Source: M. Geerlings & S. Tolmeijer, Laboratory Tumor Genetics, Radboudumc

ddPCR BRAF

Assay	Aminozuurverandering	Mutatie op DNA niveau
ddPCR BRAF 1	BRAF p.V600E	c.1799T>A
	BRAF p.V600K	c.1798_1799delinsAA
	BRAF p.V600R	c.1798_1799delinsAG
ddPCR BRAF 2	BRAF p.V600E ("E2")	c.1799_1800delinsAA
ddPCR BRAF 3	BRAF p.V600D	c.1799_1800delinsAT
ddPCR BRAF 4	BRAF p.V600D	c.1799_1800delinsAC
ddPCR BRAF 5	BRAF p.V600L	c.1798G>C
ddPCR BRAF 6	BRAF p.V600L	c.1798G>T
ddPCR BRAF 7	BRAF p.K601E	c.1801A>G
geen ddPCR	BRAF p.D594G	c.1781A>G

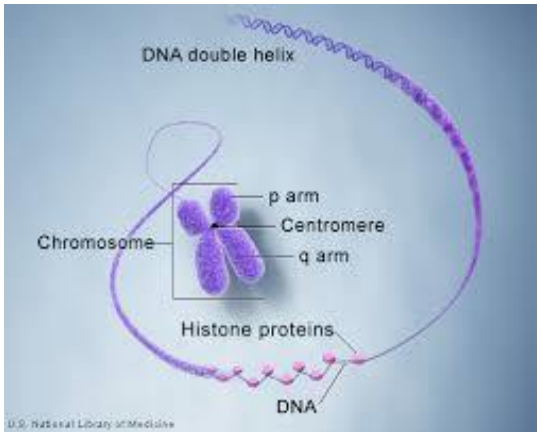
BRAF V600E,R,K ddPCR validation

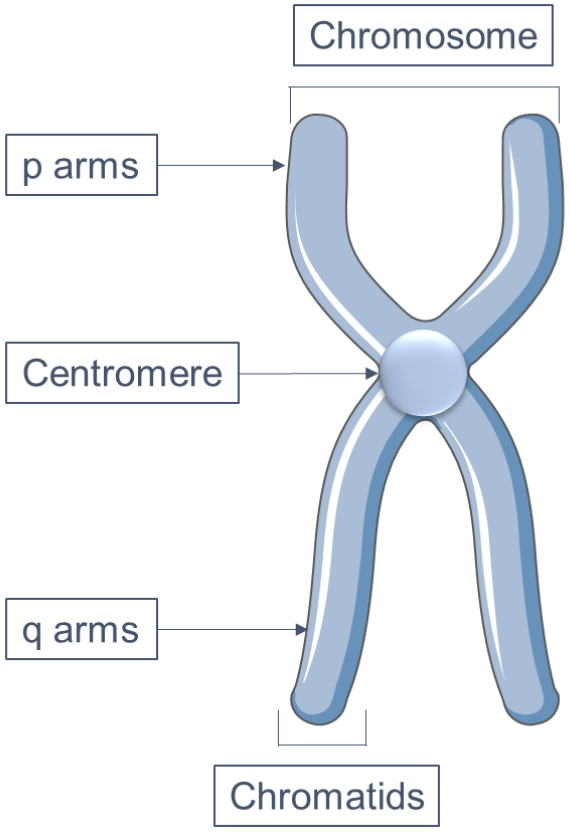


Radboudumc

Source: M. Geerlings & S. Tolmeijer, PJTA Groenen, Laboratory Tumor Genetics, Radboudumc

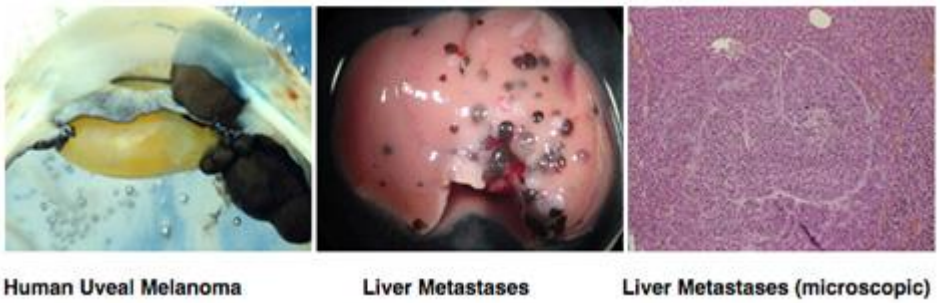
Copy number alterations



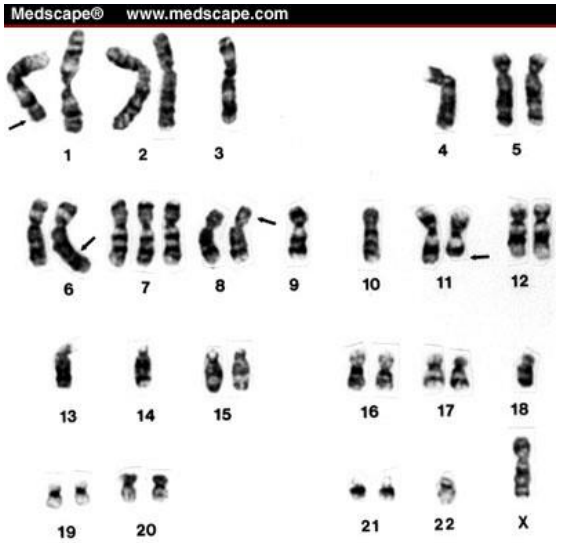


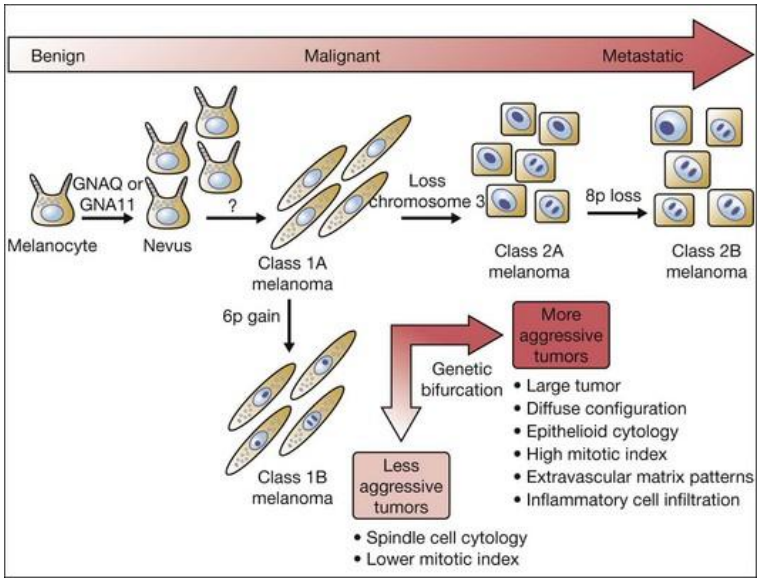
https://ghr.nlm.nih.gov/
https://www.creativebiomart.net

Uveal melanoma

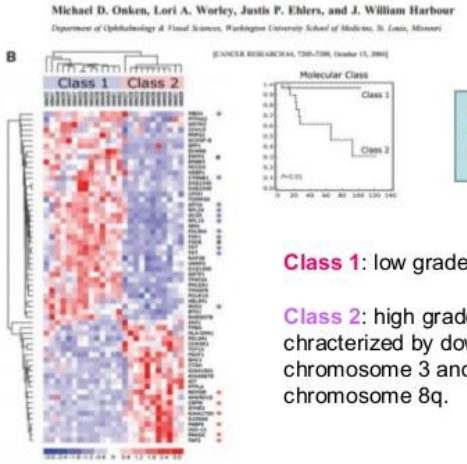


Human Uveal Melanoma | Liver Metastases | Liver Metastases (microscopic)





Uveal melanoma prognosis



Monosomy of **chromosome 3** is the most frequent chromosome aberration in uveal melanoma (50%)

Class 1: low grade tumors with low metastatic risk

Class 2: high grade tumors with metastatic risk chracterized by down-regulation of genes on chromosome 3 and up-regulation of genes on chromosome 8q.

Radboudumc

Diagram on the left: Sodhi A and Merbs S, Book: Retina, Chapter 141 “Molecular Genetics of Choroidal Melanoma”
Heatmap and survival curve on the right: Onken MD et al Cancer Res 2004

Melanoma treatment response and CNVs

Personalized Medicine and Imaging

Clinical Cancer Research

Copy Number Changes Are Associated with Response to Treatment with Carboplatin, Paclitaxel, and Sorafenib in Melanoma

Melissa A. Wilson[1], Fengmin Zhao[2], Sanika Khare[3], Jason Roszik[4], Scott E. Woodman[4], Kurt D'Andrea[3], Bradley Wubbenhorst[3], David L. Rimm[5], John M. Kirkwood[6], Harriet M. Kluger[7], Lynn M. Schuchter[1,8], Sandra J. Lee[2], Keith T. Flaherty[9], and Katherine L. Nathanson[3,8]

Abstract

Purpose: Copy number alterations have been shown to be involved in melanoma pathogenesis. The randomized phase III clinical trial E2603: carboplatin, paclitaxel, ± sorafenib (CP vs. CPS) offers a large collection of tumor samples to evaluate association of somatic mutations, genomic alterations, and clinical outcomes, prior to current FDA-approved therapies.

Experimental Design: Copy number and mutational analysis on 119 pretreatment samples was performed.

Results: CPS therapy was associated with improved progression-free survival (PFS) compared with CP in patients with tumors with *RAF1* (cRAF) gene copy gains (HR, 0.372; $P = 0.025$) or *CCND1* gene copy gains (HR, 0.45; $P = 0.035$). CPS therapy was associated with improved overall survival (OS) compared with CP in patients with tumors with *KRAS* gene copy gains (HR, 0.25; $P = 0.035$). *BRAF* gene copy gain and *MET* amplification were more common in samples with V600K versus V600E mutations ($P < 0.001$), which was validated in The Cancer Genome Atlas (TCGA) dataset.

Conclusions: We observed improved treatment response with CPS in patients with melanoma whose tumors have *RAF1* (cRAF), *KRAS*, or *CCND1* amplification, all of which can be attributed to sorafenib targeting CRAF. These genomic alterations should be incorporated in future studies for evaluation as biomarkers.

Clin Cancer Res; 22(2); 374–82. ©2015 AACR.

Translational Cancer Mechanisms and Therapy

Clinical Cancer Research

Genetic Aberrations in the CDK4 Pathway Are Associated with Innate Resistance to PD-1 Blockade in Chinese Patients with Non-Cutaneous Melanoma

Jiayi Yu[1], Junya Yan[1], Qian Guo[1], Zhihong Chi[1], Bixia Tang[1], Bin Zheng[2], Jinyu Yu[1], Ting Yin[1], Zhiyuan Cheng[1], Xiaowen Wu[1], Huan Yu[1], Jie Dai[1], Xinan Sheng[1], Lu Si[1], Chuanliang Cui[1], Xue Bai[1], Lili Mao[1], Bin Lian[1], Xuan Wang[1], Xieqia Yan[1], Siming Li[1], Li Zhou[1], Keith T. Flaherty[3], Jun Guo[1], and Yan Kong[1]

Clin Cancer Res. 2019 Nov 1;25(21):6511-6523. doi: 10.1158/1078-0432.CCR-19-0475. Epub 2019 Aug 2.

CDK4 gain

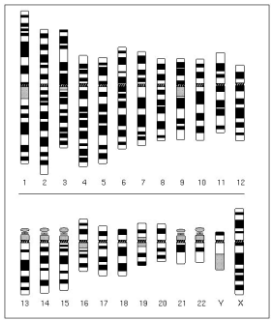
MET/VEGFR blockade with cabozantinib demonstrated no improvement in PFS/ OFS. ? Possible utility for cabozantinib in combination with immunotherapy

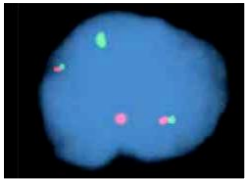
Radboudumc

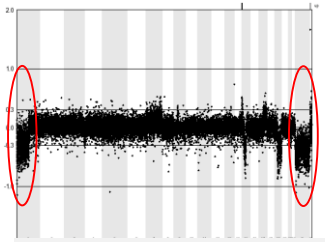
Chromosomal abnormalities

Methods for detection in a historical perspective

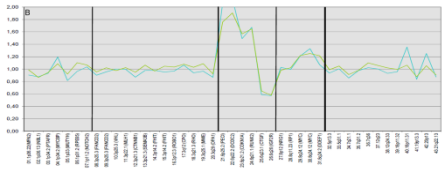
- 1956 Karyotyping: human 46 chromosomes (no 48!)
- 1970 First banded human karyotype
- 1975 Southern blotting using extracted DNA
- 1980-1985 FISH technology
- 1996 Comparative genomic hybridization (CGH)
- 2003 Array-CGH
- 2002 MLPA multiplex ligation dependent probe hybridization
- 2003 > Array technology in different flavors
- 2012 > Next generation sequencing technology for copy number variations





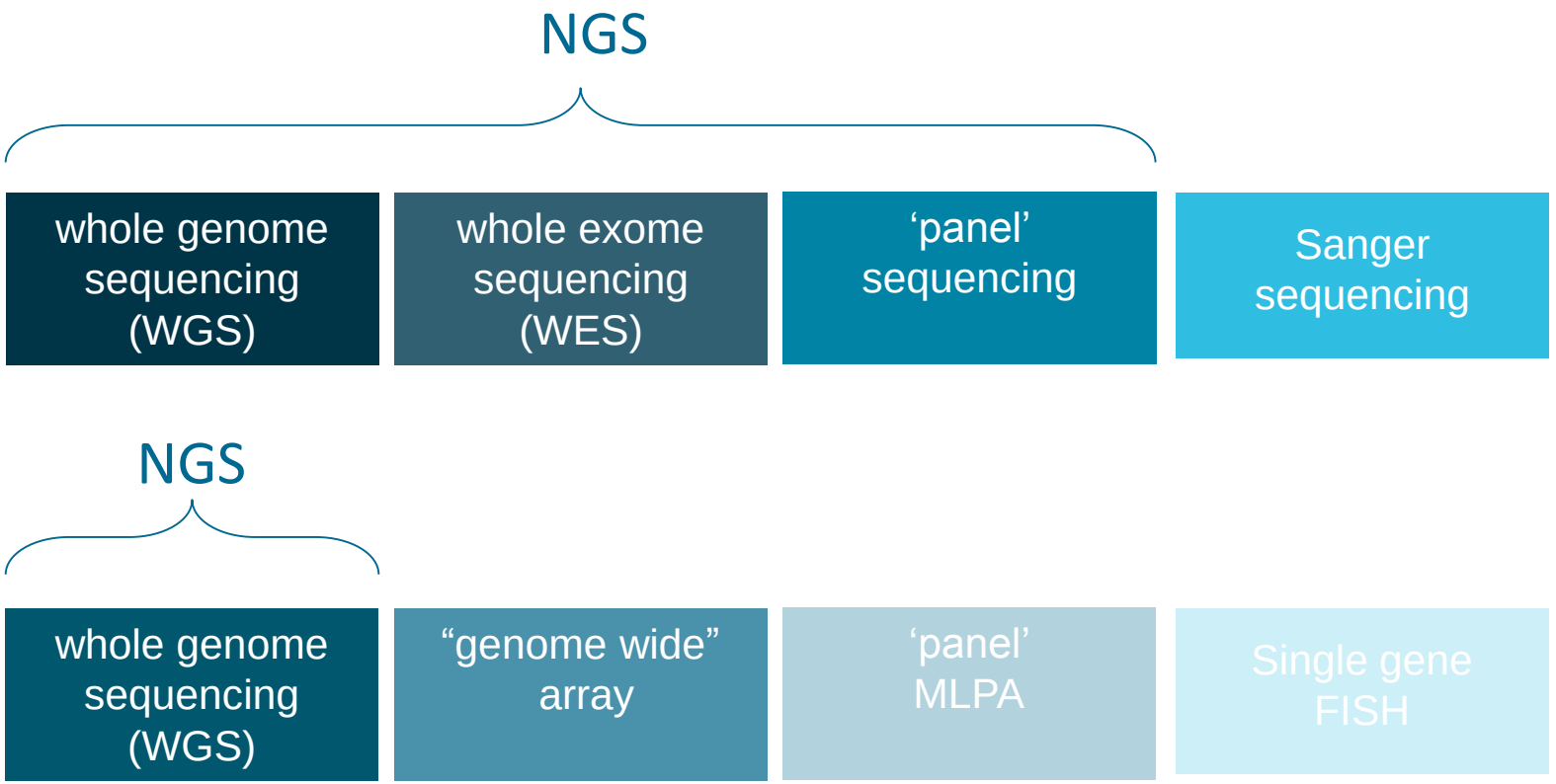


Glioblastoma loss 1p/19q



Melanomatosis (case C), 6pgain, 6q loss

Sequencing and CNV detection

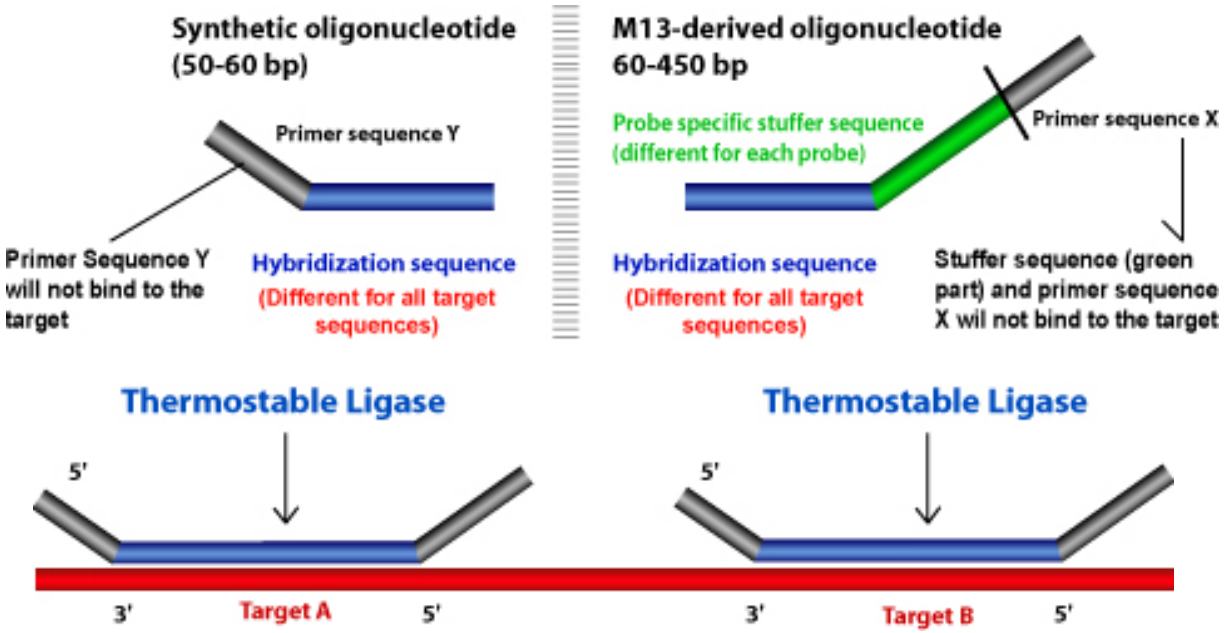


Radboudumc

Source: A. Eijkelenboom, PJTA Groenen, dept of Pathology, Radboudumc

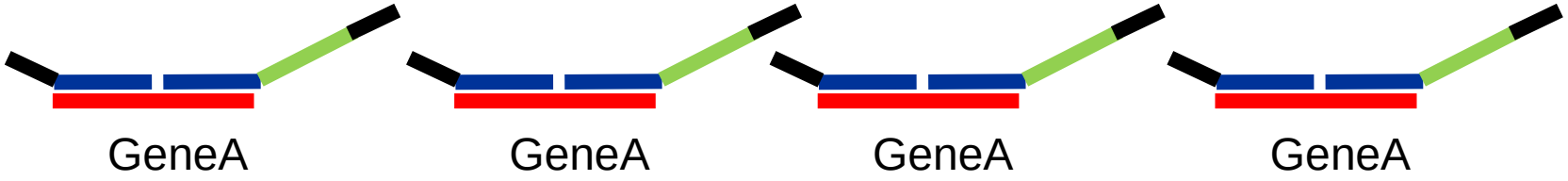
MLPA Multiplex Ligation Dependent Probe Amplification

- **Amplification** of MLPA probes, not of sample DNA
- Probes are composed of synthetic oligonucleotides complementary to the target sequence, primers sequences X and Y that are needed for PCR after probe hybridization and a stuffer sequence which differs in lenght between the different probes in the assay

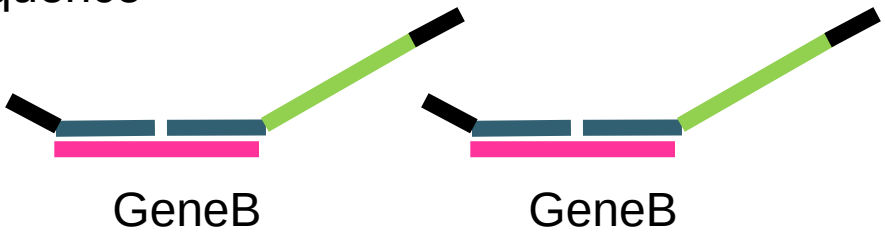


MLPA method

Probe for gene A



Probe B, with a longer stuffer sequence



Known information, from the kit design:

Stuffer length a – corresponds to a specific gene (fragment)

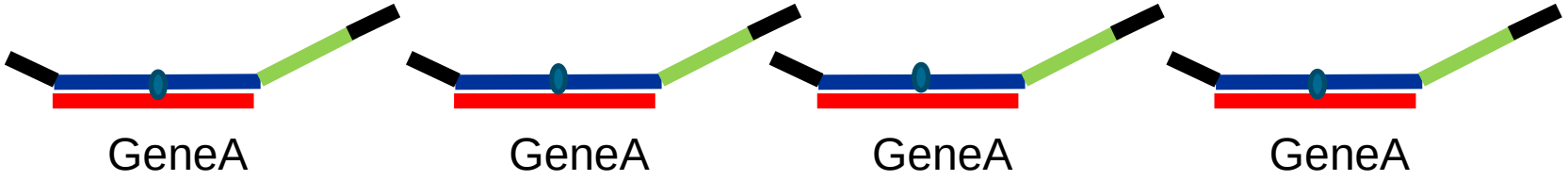
Stuffer length b – another gene segment

etcetera

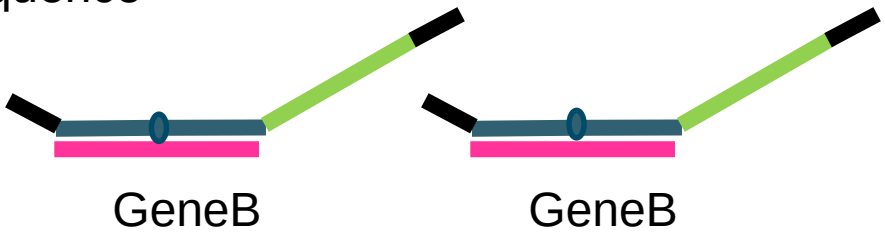
Source: PJTA Groenen, dept. of Pathology, Radboudumc

MLPA: quantitative information

Probe for gene A



Probe B, with a longer stuffer sequence

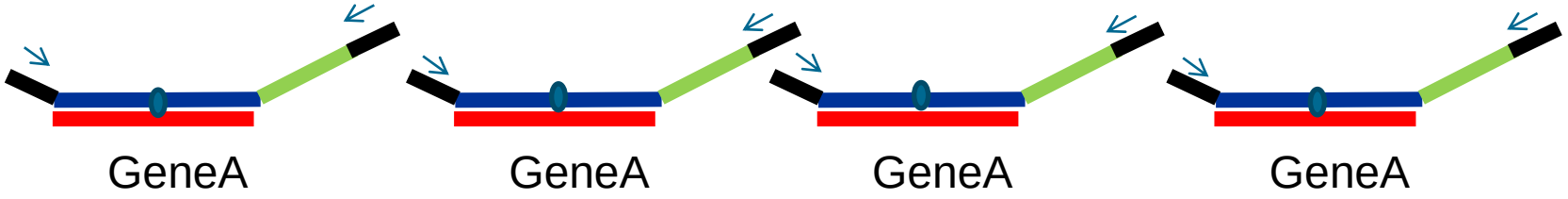


Radboudumc

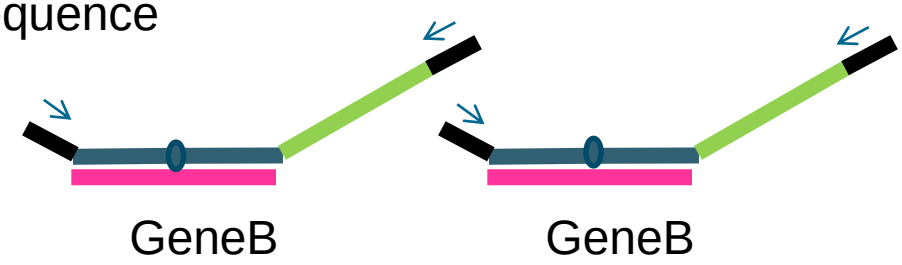
Source: PJTA Groenen, dept. of Pathology, Radboudumc

MLPA: ligation followed by PCR

Probe for gene A



Probe B, with a longer stuffer sequence



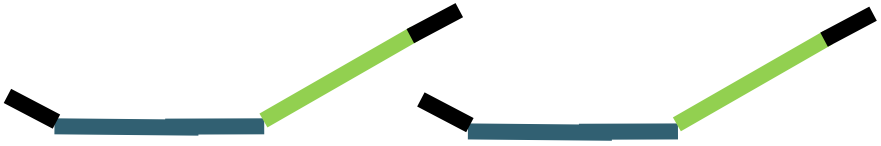
Radboudumc

Source: PJTA Groenen, dept. of Pathology, Radboudumc

MLPA: probes are amplified by PCR

PCR product gene A

PCR product gene B

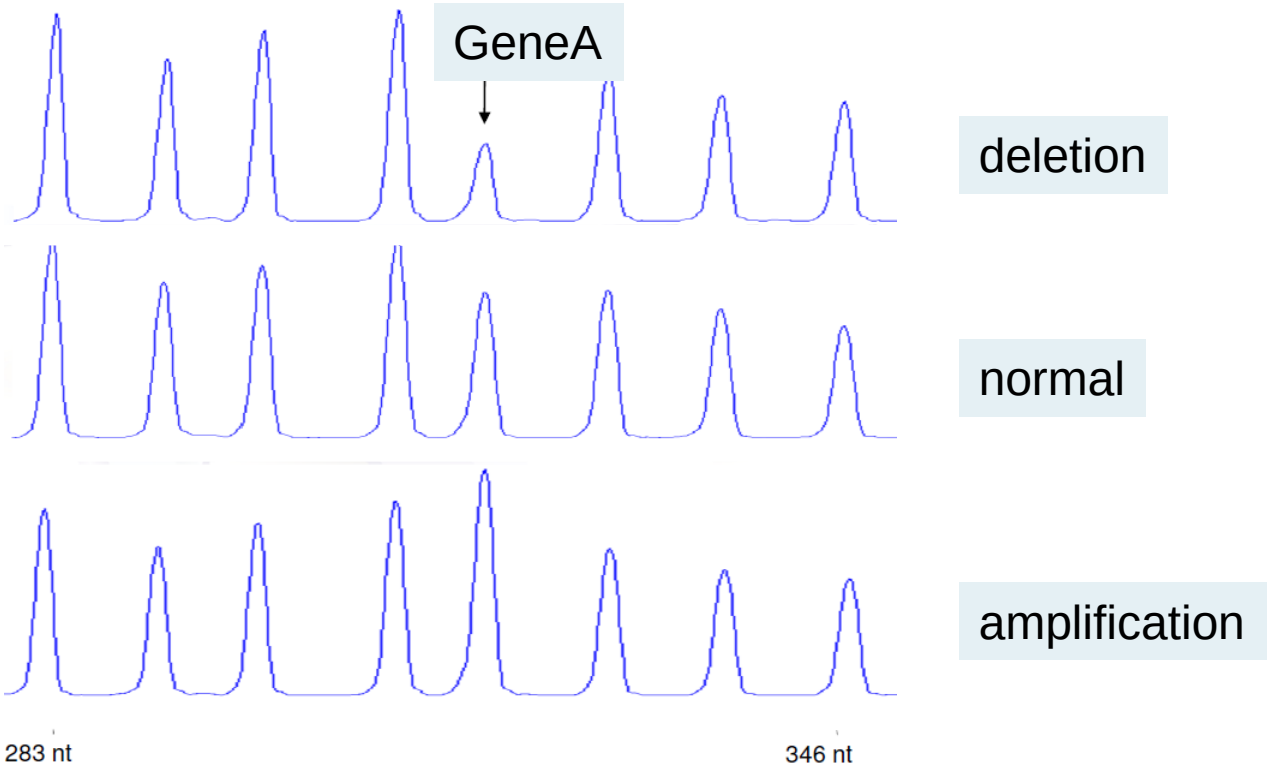


> Differently sized fragments > separation by gelelectrophoresis

Radboudumc

Source: PJTA Groenen, dept. of Pathology, Radboudumc

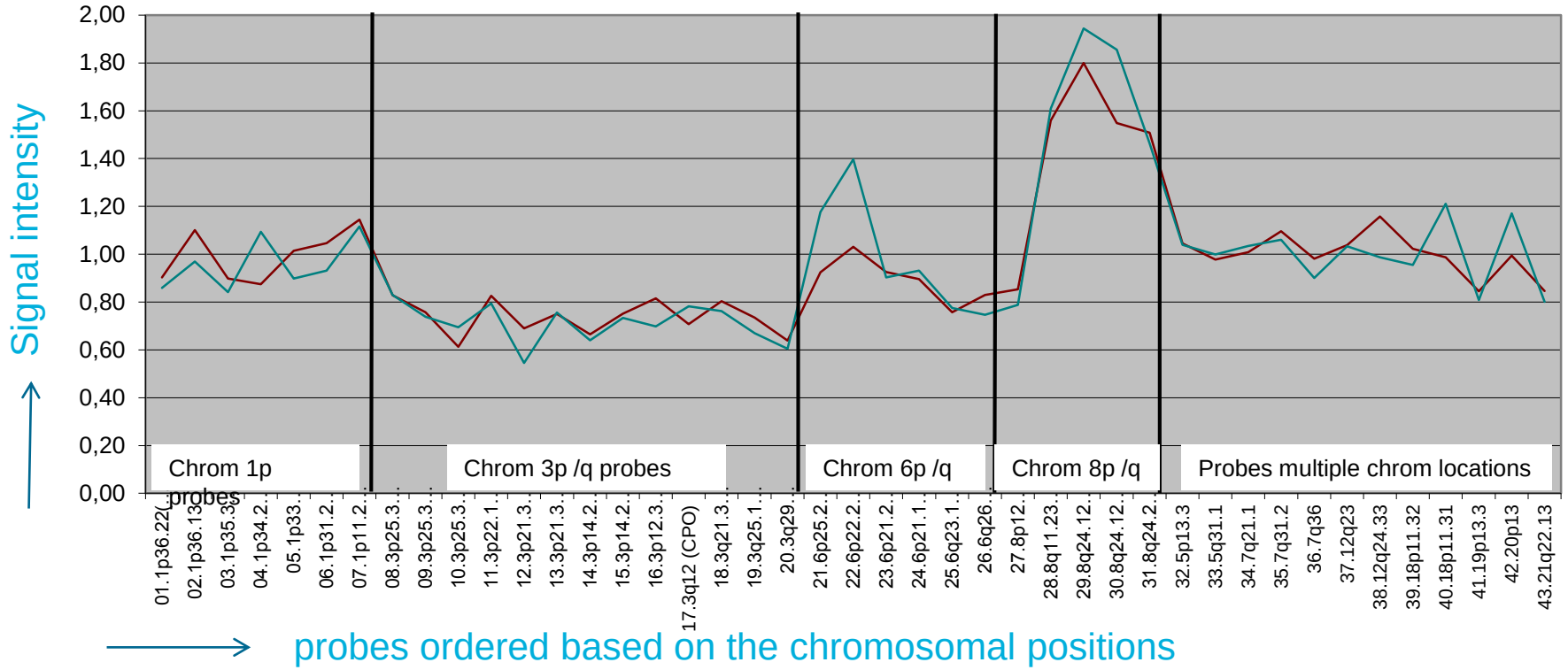
MLPA Result



Peak patterns are generated on a patient sample and a reference DNA-sample.
Relative peak heights are compared
Each gene (segment) is defined by the length of the stuffer!
Differences reflect copy number changes of sequences detected by MLPA
Data peaks are being processed and shown in plots, see an example at the next slide

Source: PJTA Groenen, dept. of Pathology, Radboudumc

MLPA result of a patient sample

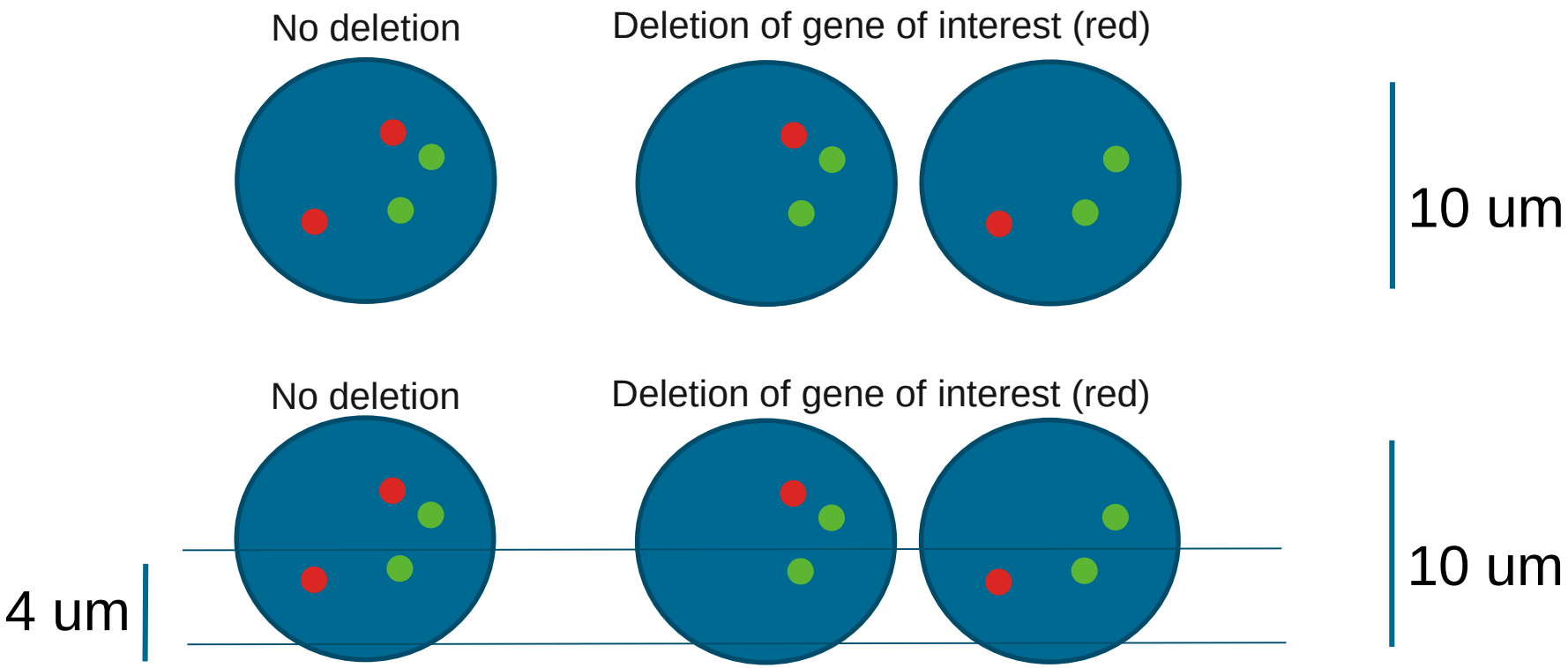


A patient sample is run in duplicate (red and blue lines).
The controle probes, present on multiple chromosomal locations have a signal intenstity of 1, meaning normal.
The sample displays lower signals (around 0.7) of the chrom 3p /q probes, representing monosomy 3.
Also high intensity signals of chrom 8q probes are seen, representing a gain of chrom 8q.

Detection of monosomy 3: FISH ?

FISH and detection deletions

high risk of misevaluation

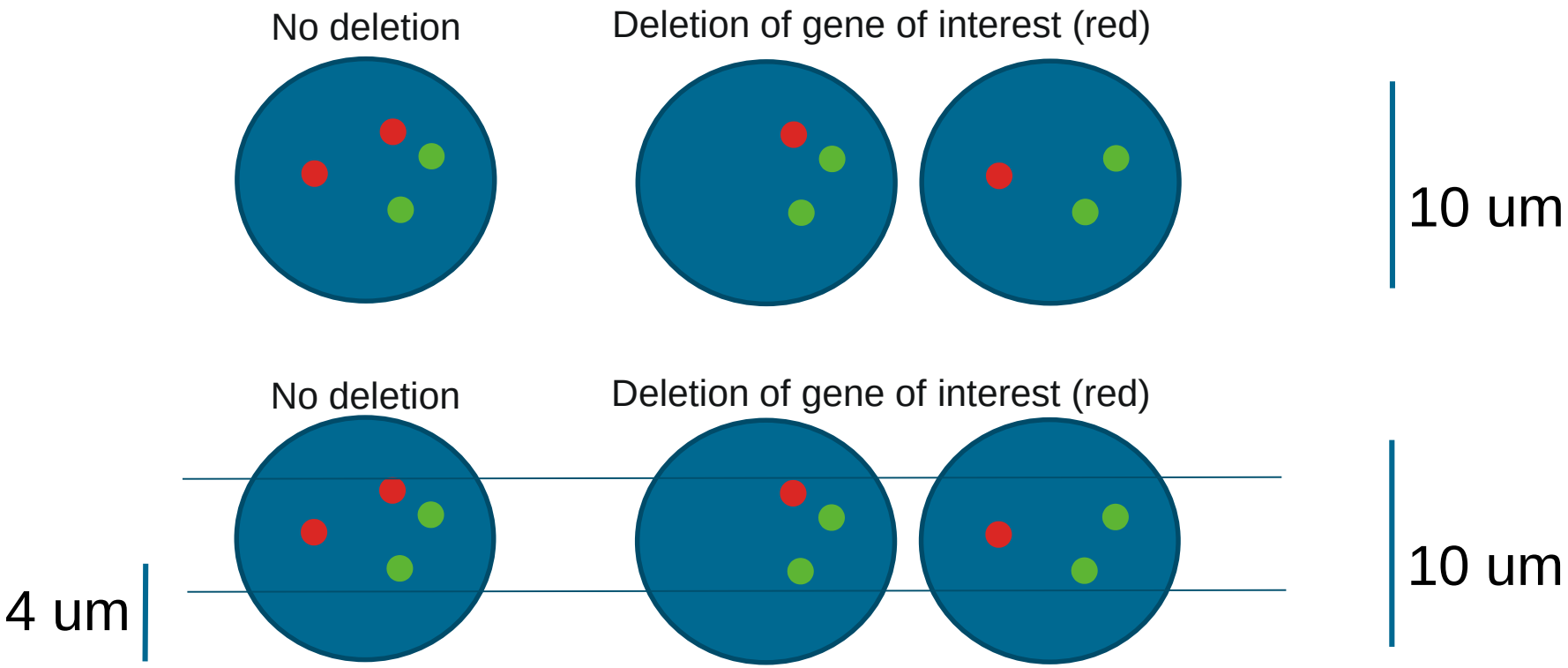


Radboudumc

Source: PJTA Groenen, dept. of Pathology, Radboudumc

FISH and detection deletions

high risk of misevaluation



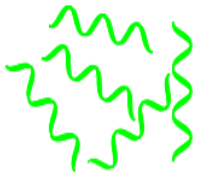
Radboudumc

Source: PJTA Groenen, dept. of Pathology, Radboudumc

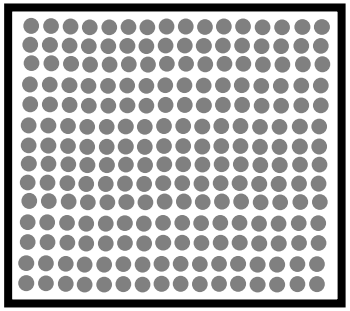
Genomic analysis by array technology

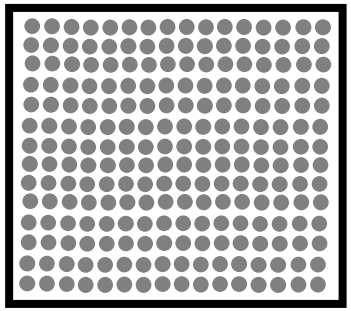
Genome-wide

Tumoral DNA



Reference DNA





Hybridization
to complementory
probes
spotted on a solid
surface

Example of
Affymetrix
gene chip
array

DATA analysis
(tumoral DNA data are corrected for reference DNA values)

When data-sets of reference DNA are available these can be
(in silico) used for the evaluation of the tumoral DNA data.

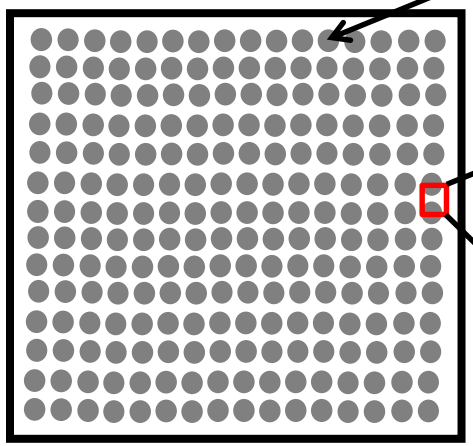
Radboudumc

Source: figures of the Affymetrix website 2015
PJTA Groenen, dept. of Pathology, Radboudumc

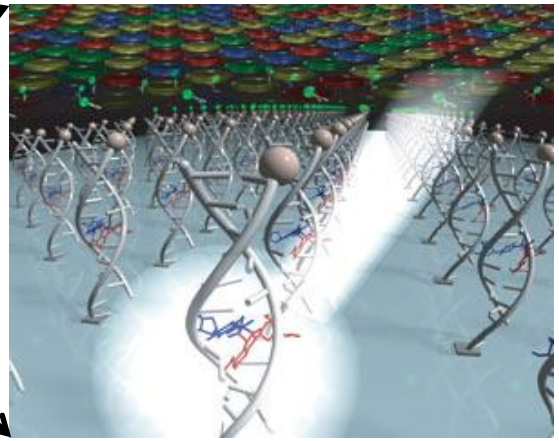
Genomic analysis by array technology

- These are the convergence of DNA hybridization, fluorescence microscopy, and solid surface DNA capture.
- Array: DNA probes immobilized on a solid surface capture

- Fragmented nucleic acid sequences of target, labelled with fluorescent dyes.
- A detection system that records and interprets the hybridization signal.



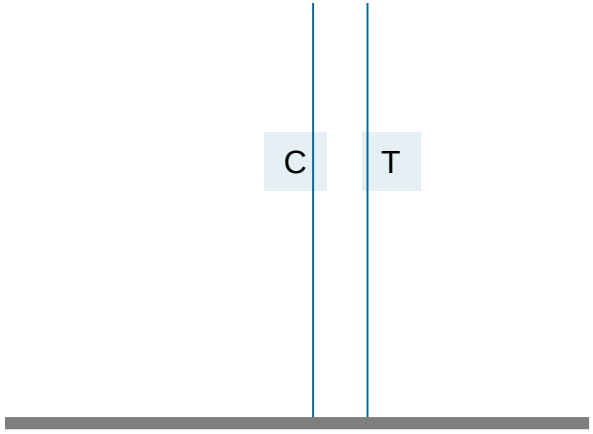
25 nucleotides in lenght



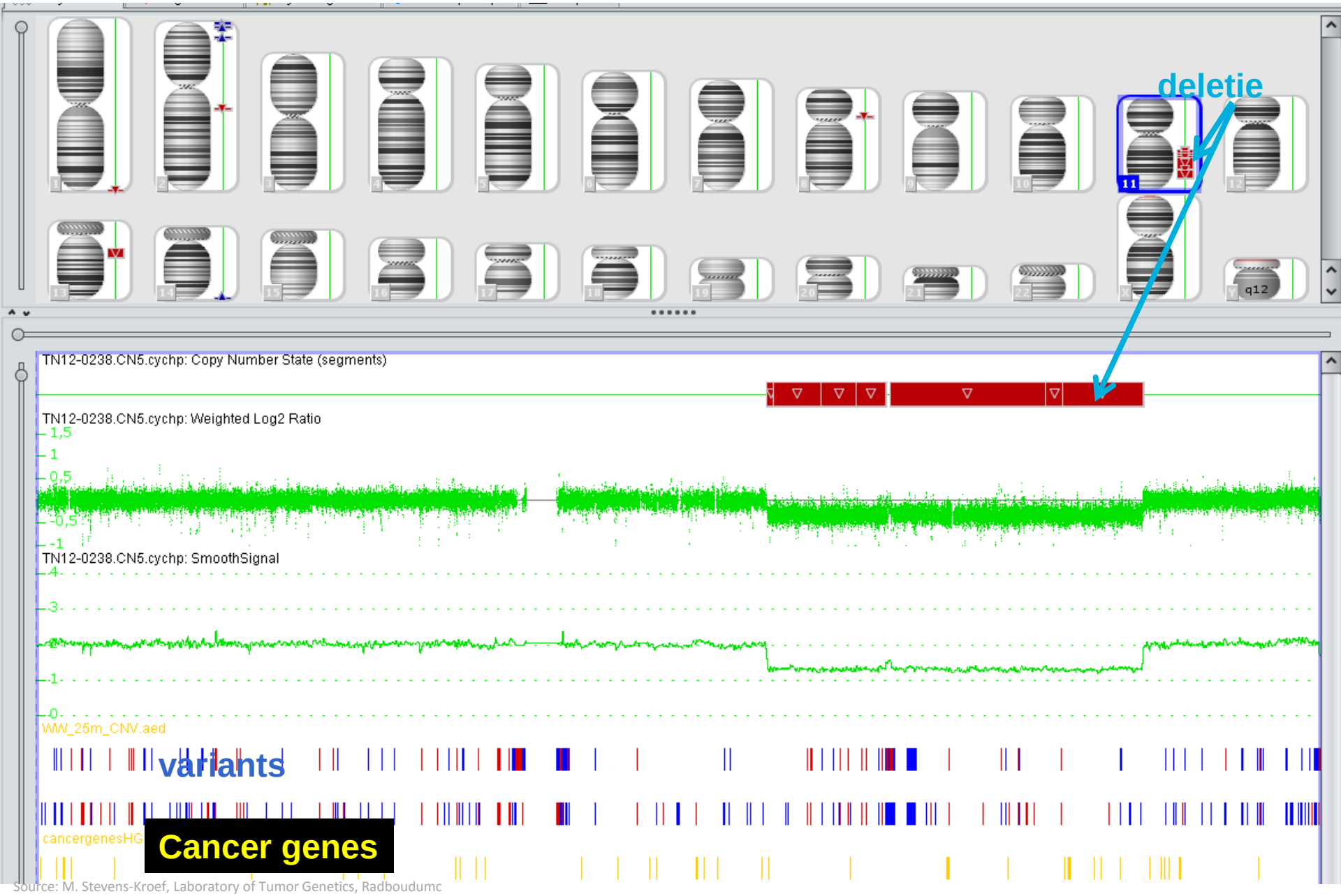
Source: figures of the Affymetrix website 2015

CytoScan array uses SNPs

- With more than 2.6 million copy number markers, CytoScan HD Suite covers all OMIM™ and RefSeq genes.
- Plus approximately 750,000 genotype-able SNPs, which provide high-resolution copy number, accurate breakpoint estimation, and loss of heterozygosity (LOH) detection > allele-specific oligonucleotide (ASO) probes
- Of each SNP's both variants are covered on the array, 25 mers, all nt must bind to provide signal
- Allows detection of copy neutral Loss of Heterozygosity

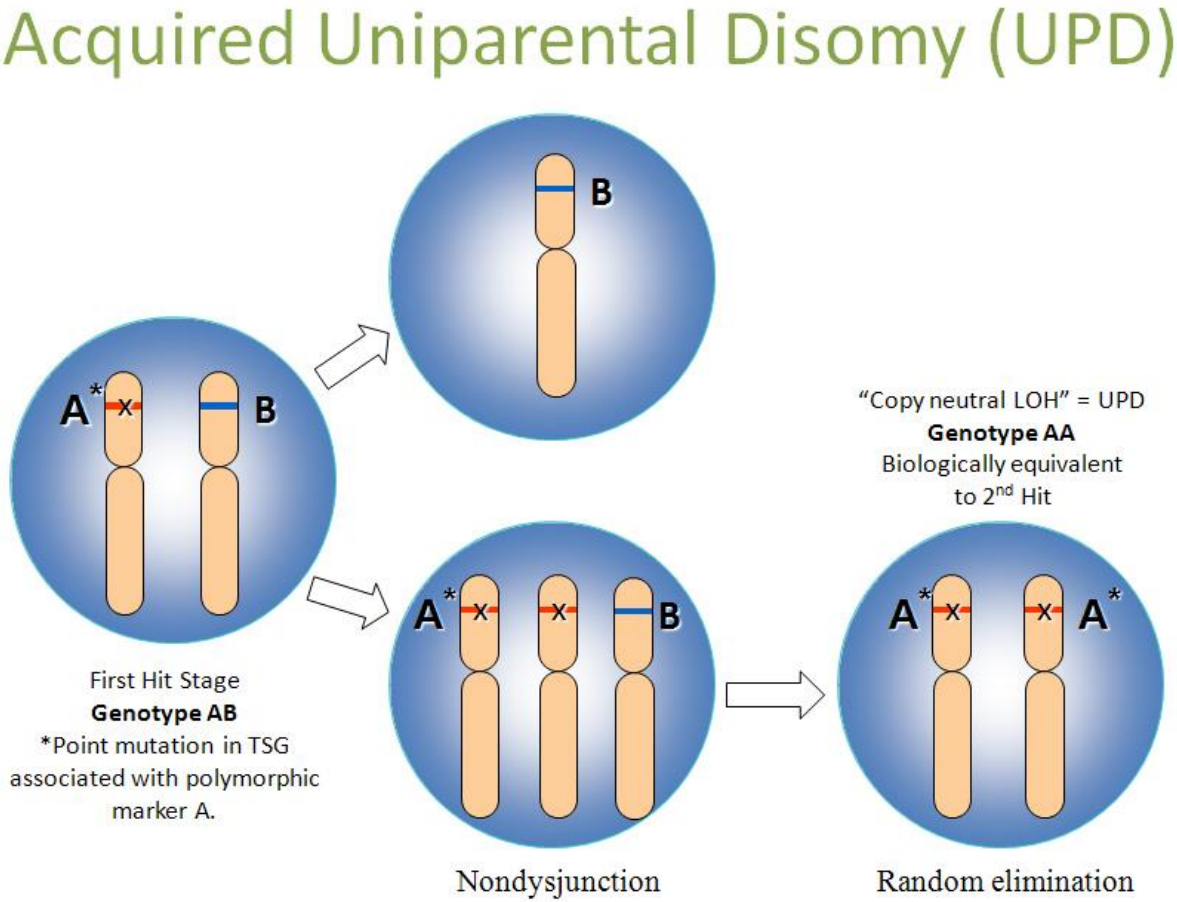


Example Affymetrix CytoScan HD / ChAS



Source: M. Stevens-Kroef, Laboratory of Tumor Genetics, Radboudumc

Copy-neutral LOH, aUPD



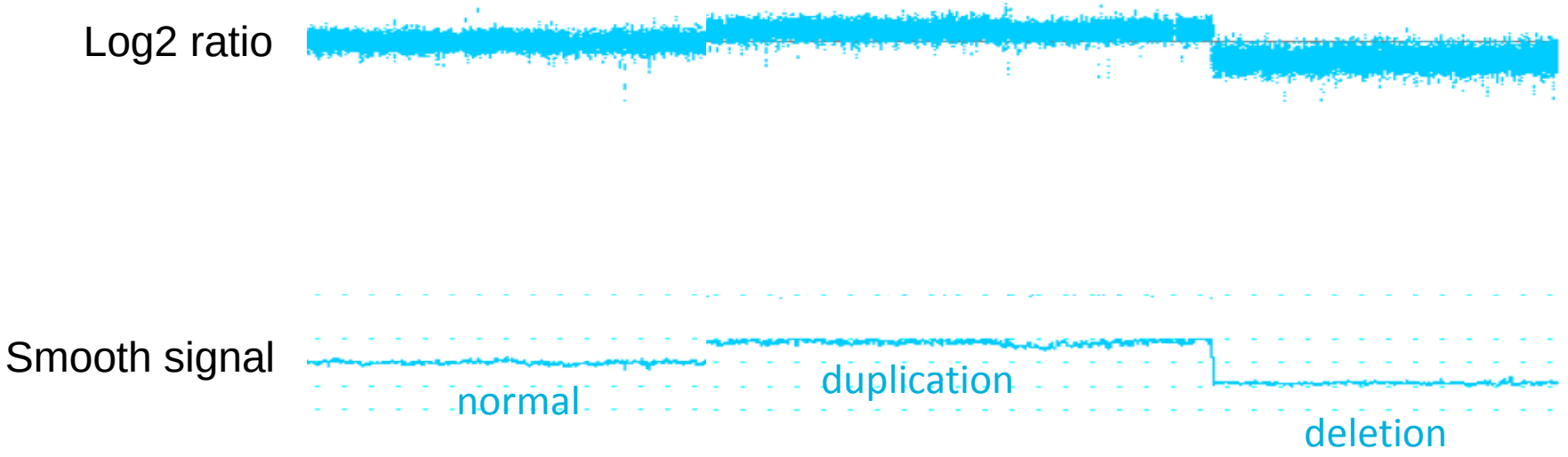
Source: https://en.wikipedia.org/wiki/Loss_of_heterozygosity#/media/File:Copy_neutral_LOH.jpg

SNP detection

- SNP at a position in the genome:
  A and B
- Two alleles
- either BB
  AB
  AA

Source: PJTA Groenen, dept. of Pathology, Radboudumc, and figure adapted from Affymetrix information 2015

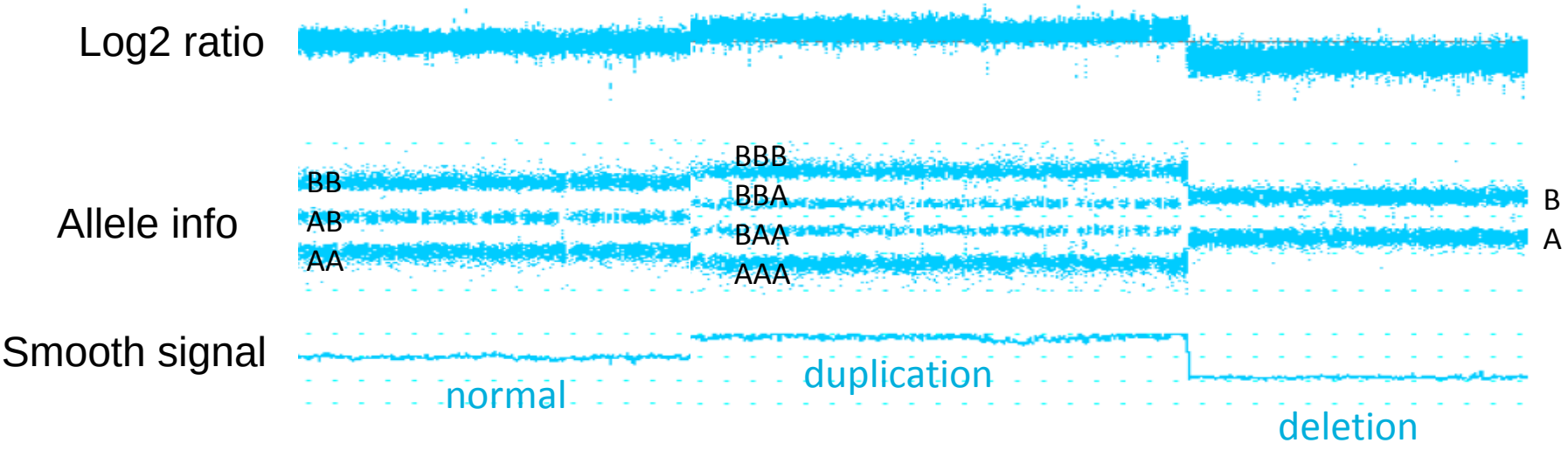
CNV (log2) and SNP allelic ratio by CytoScan array



Radboudumc

Source: M. Stevens-Kroef and PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

CNV (log2) and SNP allelic ratio by CytoScan array



Radboudumc

Source: M. Stevens-Kroef and PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

CNV (log2) and SNP allelic ratio by CytoScan array

Example 2

Log2 ratio

Radboudumc

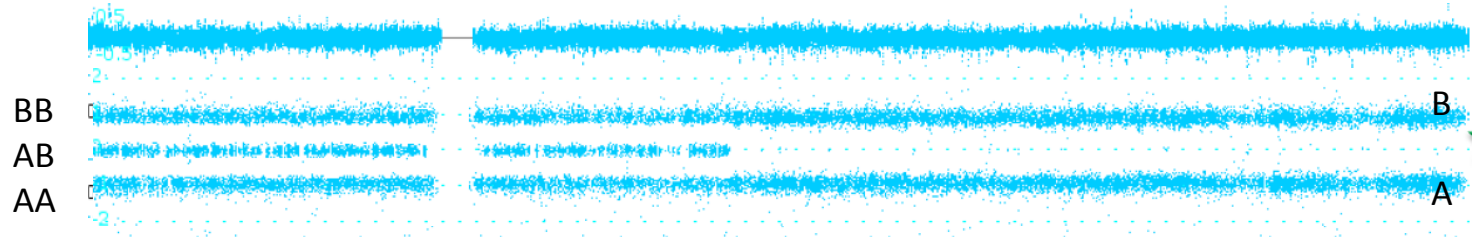
Source: M. Stevens-Kroef and PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

CNV (log2) and SNP allelic ratio by CytoScan array

Example 2

Log2 ratio

Allele info

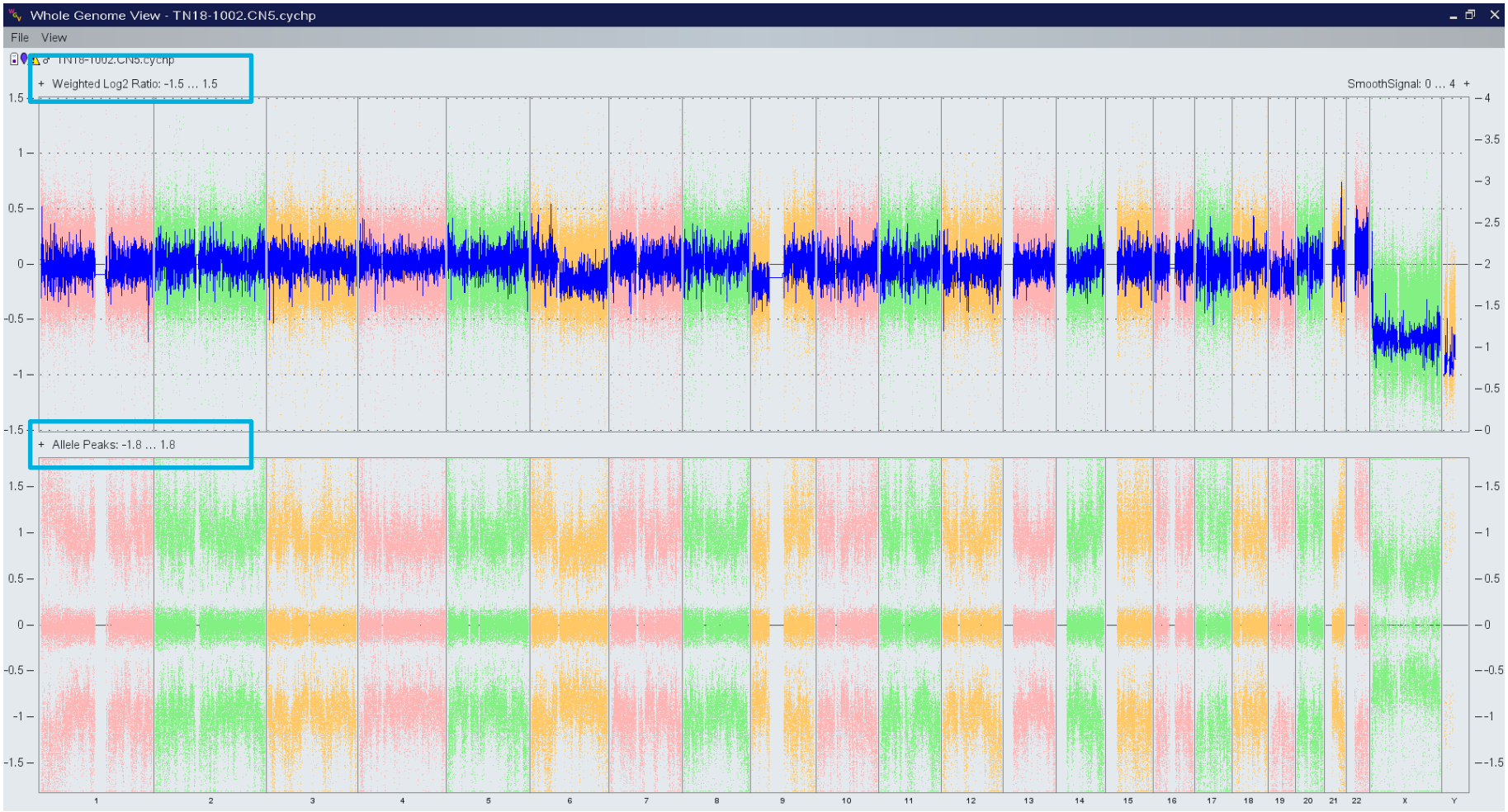


Copy neutral
loss of heterozygosity

Radboudumc

Source: M. Stevens-Kroef and PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

Result CytoScan array (case 1)



Case 1: geen *BRAF, NRAS, HRAS* mutatie

DD: Spitz naevus - spectrum van hooggradige STUMP/melanoom

Radboudumc

Source: M. Stevens-Kroef, PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

Case 1 results and conclusion

Note: FFPE, 20% neoplastische cellen

- Gedetecteerde genetische afwijkingen relevant voor differentiaal diagnose bij melanoom:
- • GEEN verlies 3p
- • GEEN verlies van 3p21 (BAP1)
- • GEEN amplificatie 4q12 (KIT)
- • GEEN amplificatie 7q34 (BRAF)
- • WEL verlies 9p21 (CDKN2A)
- • GEEN gain 11p
- • GEEN COMPLEX KARYOTYPE (>2 CNA >5 Mb)
- Overige afwijkingen: Verlies van 6q

- CONCLUSIE

  Er is geen mutatie aangetoond in BRAF, NRAS, HRAS en CTNNB1.

  SNP array analyse toont verlies van 9p21 (CDKN2A). Er zijn tevens additionele afwijkingen namelijk CNVs van 6q en 9p, aangetoond. Deze array data passen niet meer goed bij een benigne laesie en duiden op een laesie in het spectrum van hooggradige STUMP/melanoom.

Result CytoScan array (case 2)

Radboudumc

Source: M. Stevens-Kroef, PJTA Groenen, Laboratory of Tumor Genetics, Radboudumc

Case 2 results and conclusion

Note: FFPE, 80% neoplastische cellen

Gedetecteerde genetische afwijkingen relevant voor differentiaal diagnose bij melanoom:

- WEL verlies van een deel van 3p
- WEL verlies van 3p21 (BAP1)
- GEEN amplificatie 4q12 (KIT)
- GEEN amplificatie 7q34 (BRAF)
- GEEN (homozygoot) verlies 9p21 (CDKN2A)
- GEEN gain 11p
- GEEN COMPLEX KARYOTYPE (>2 CNA >5 Mb)
- mannelijk profiel

- Overige afwijkingen: Verlies van 18p en Y

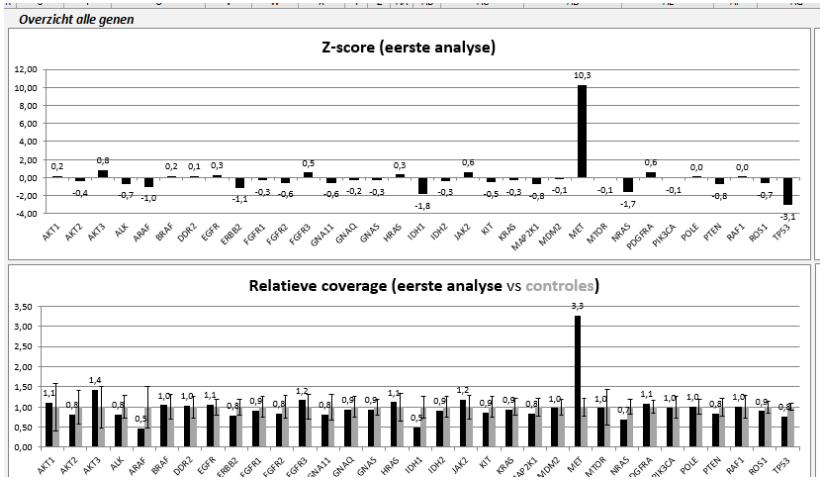
CONCLUSIE

Er is een afwijkend mannelijk profiel waargenomen waarbij er een partiele deletie is van chromosoomarm 3p. Deze partiele 3p deletie omvat het BAP1 gen hetgeen een belangrijk gen is met betrekking tot metastasering van uvea melanoom. Het is vooralsnog onvoldoende beschreven in hoeverre verlies van dit BAP1 gen vergelijkbare prognostische relevantie heeft als monozomie 3 bij uvea melanomen is.

NGS-gebaseerdeCNV detectie

voorbeeld: MET amplificatie (long aanvraag R18-03446)

?	Eerste analyse			
gen	effect	relatieve coverage	z-score	aantal kopieën*
ALK		0,8	-1	0
BRAF		1,0	0	2
EGFR		1,1	0	2
ERBB2 (HER2)		0,8	-1	0
FGFR1		0,9	0	1
FGFR2		0,8	-1	1
FGFR3		1,2	1	3
KIT		0,9	-1	1
KRAS		0,9	0	1
MDM2		1,0	0	2
MET	*amplificatie*	3,3	10	20
PDGFRA		1,1	1	3
PIK3CA		1,0	0	2
PTEN		0,8	-1	1
TP53		0,8	-3	0



NB:

1) uitgaande van het percentage tumorcellen kunnen in dit materiaal amplificaties worden aangetoond van minimaal 18 kopieën
2) bij inclusie van SNPs in NGS is bepaling allelic ratio mogelijk

Relatieve coverage	maat aantal reads
Z-score	maat voor afwijking t.o.v gemiddelde bij normaal verdeling. Z-scores van ± 3 passen niet bij normale verdeling en duiden op een reële afwijking
Aantal kopieën	op basis van relatieve coverage en tumorcel percentage

Radboudumc

Source: PJTA Groenen, dept. Of Pathology, Radboudumc

Learning objectives; tick the boxes…

- Melanoma; important mutations ☐
- Mutation analysis
  - by NGS (tissues as source) ☐
  - by ddPCR low level detection ☐
- Melanoma; important Copy number variations ☐
- CNV detection methods
  - > MLPA ☐
  - > array ☐
  - > NGS ☐