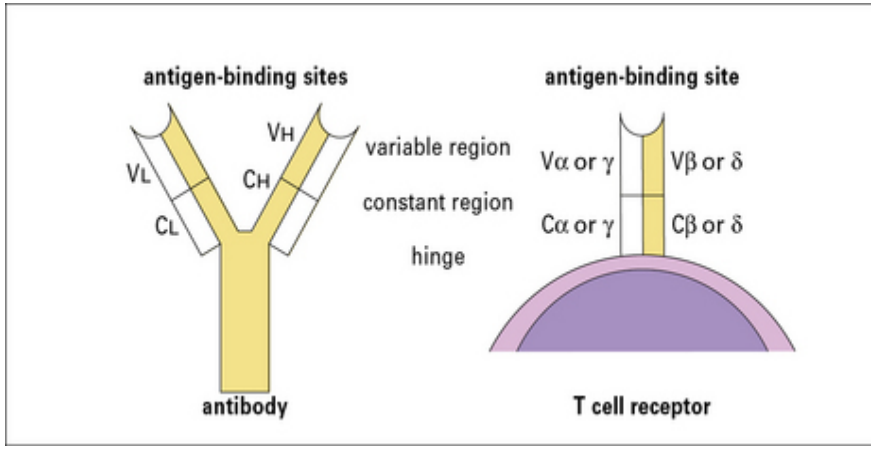
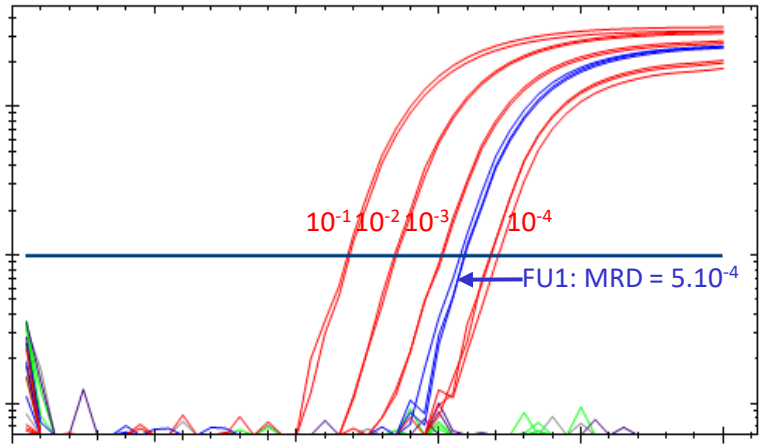
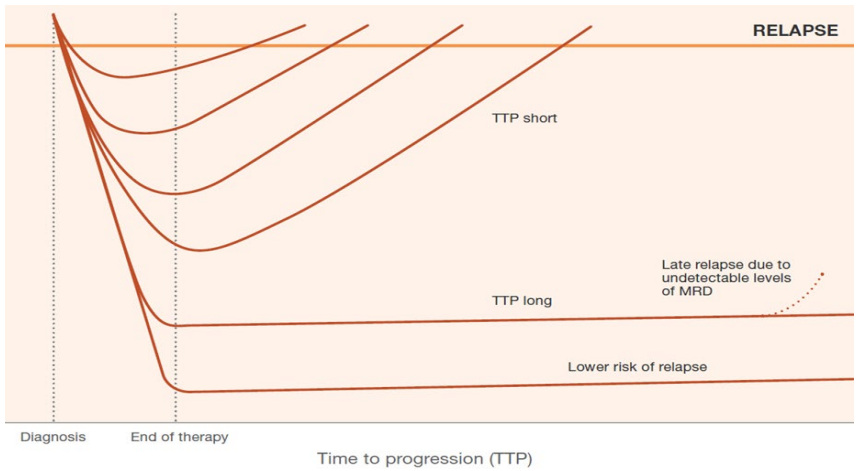






MOLECULAR MRD APPLICATIONS IN HEMATO-ONCOLOGY

Emmanuelle Kabongo Kanjinga en Marleen Bakkus

MB&C workshop 9/2/2024

Universitair Ziekenhuis Brussel

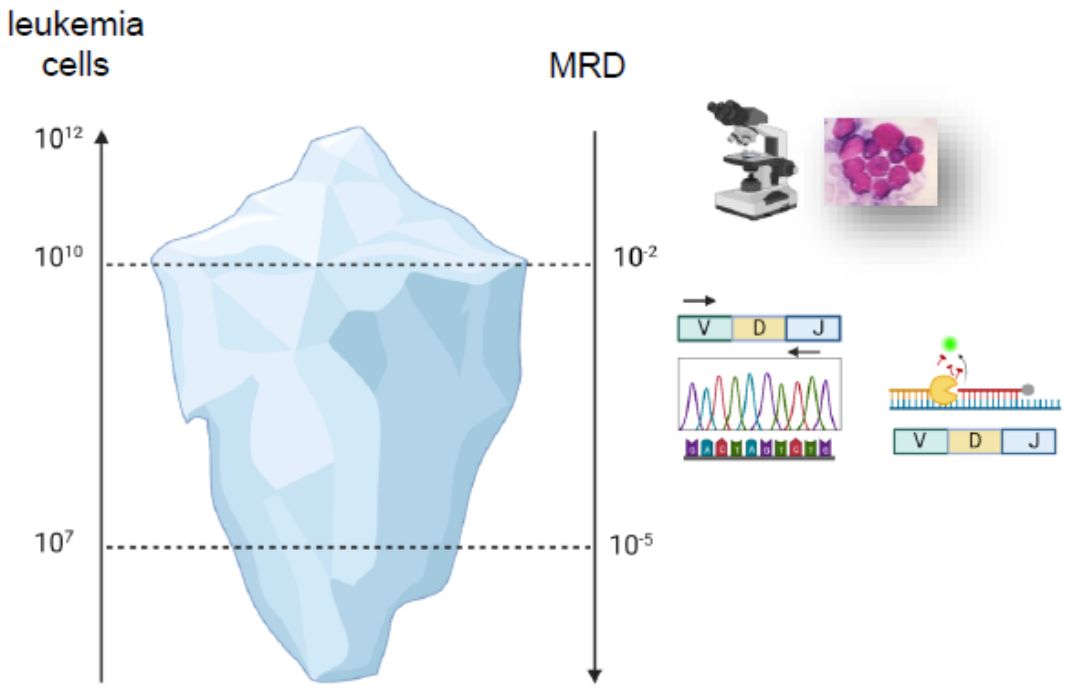
VUB

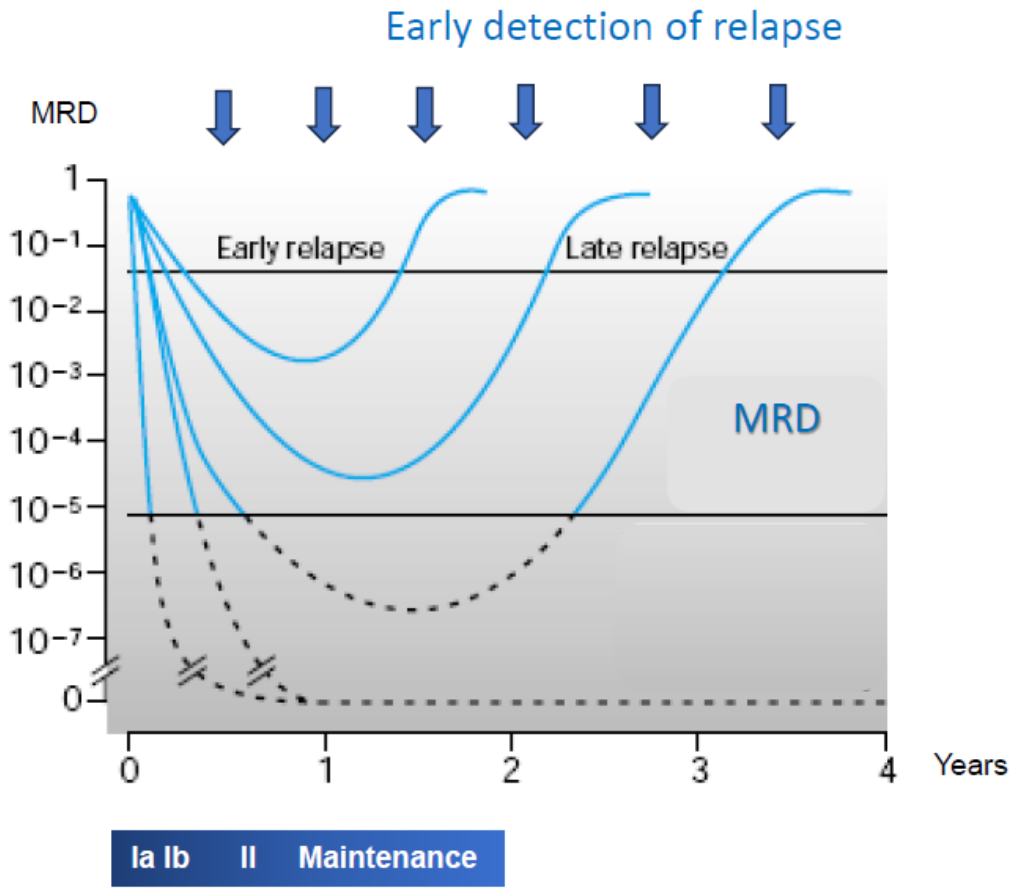
MINIMAL RESIDUAL DISEASE (MRD)

Frame

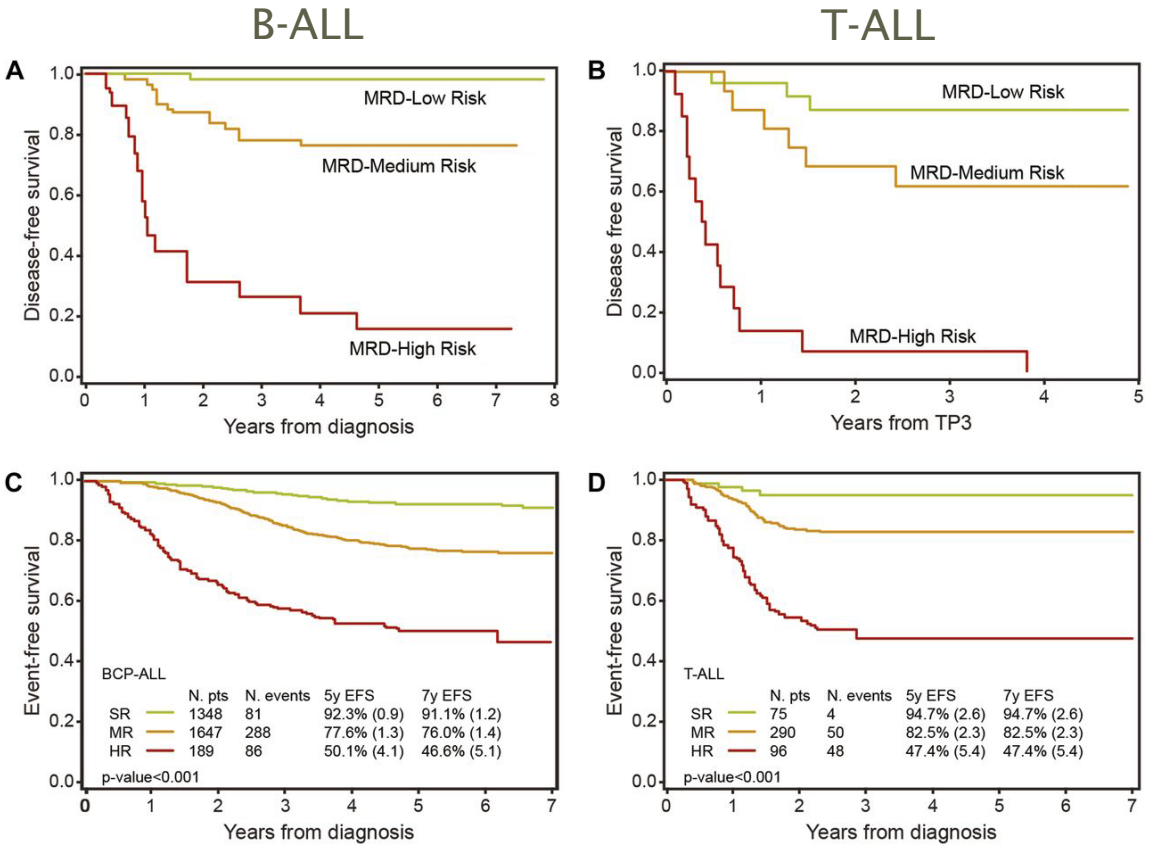
- What is MRD?
- Why important?
- How to measure?
- ALL
- MM
- AML (Emma)

MINIMAL RESIDUAL DISEASE (MRD)





WHY MRD MEASUREMENTS



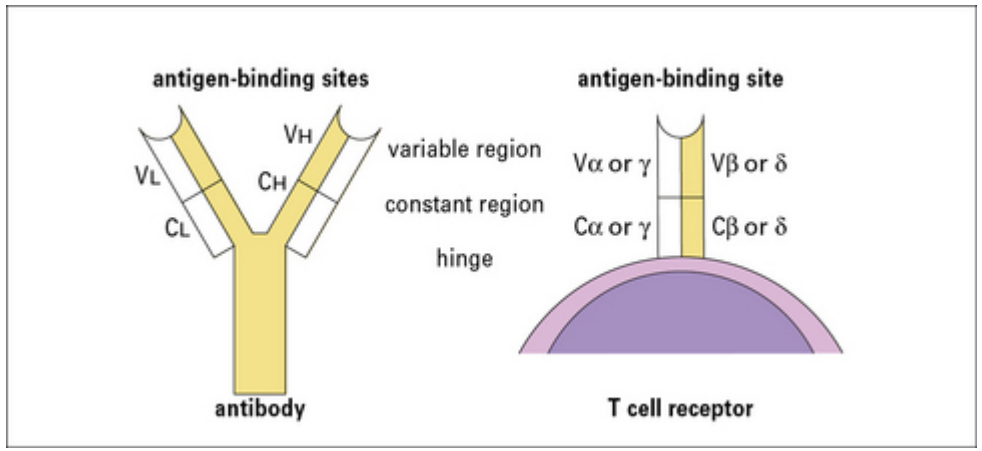
- Independent prognostic factor for relapse-free survival, disease-free survival and overall survival in ALL (Cavé et al, NEJM 1998 and Van Dongen et al, Lancet 1998 and many others thereafter)
- Speed and depth of the molecular response are used to assess treatment response (low risk, intermediate risk and high risk MRD) and guide clinical decisions.
- Monitoring disease burden before SCT
- Recognition of impending relapse
- Potential end-point in clinical trials

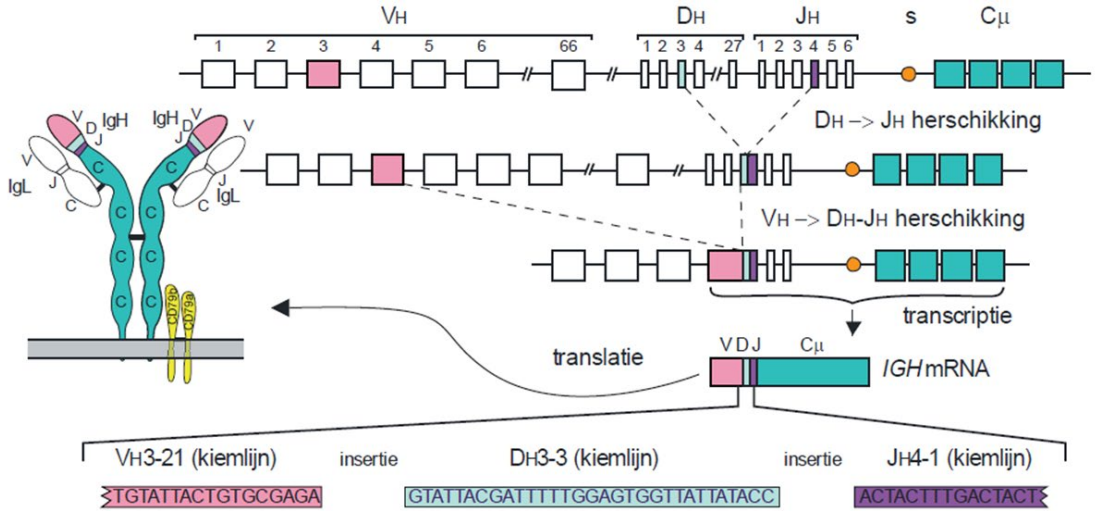
MRD TARGETS

Where is Wally?

TARGETS IN B/T LYMPHOID MALIGNANCIES

Junctional regions of immunoglobuline (Ig) and T-cel receptor (TCR) gene rearrangements





T/B lymphoid malignancies

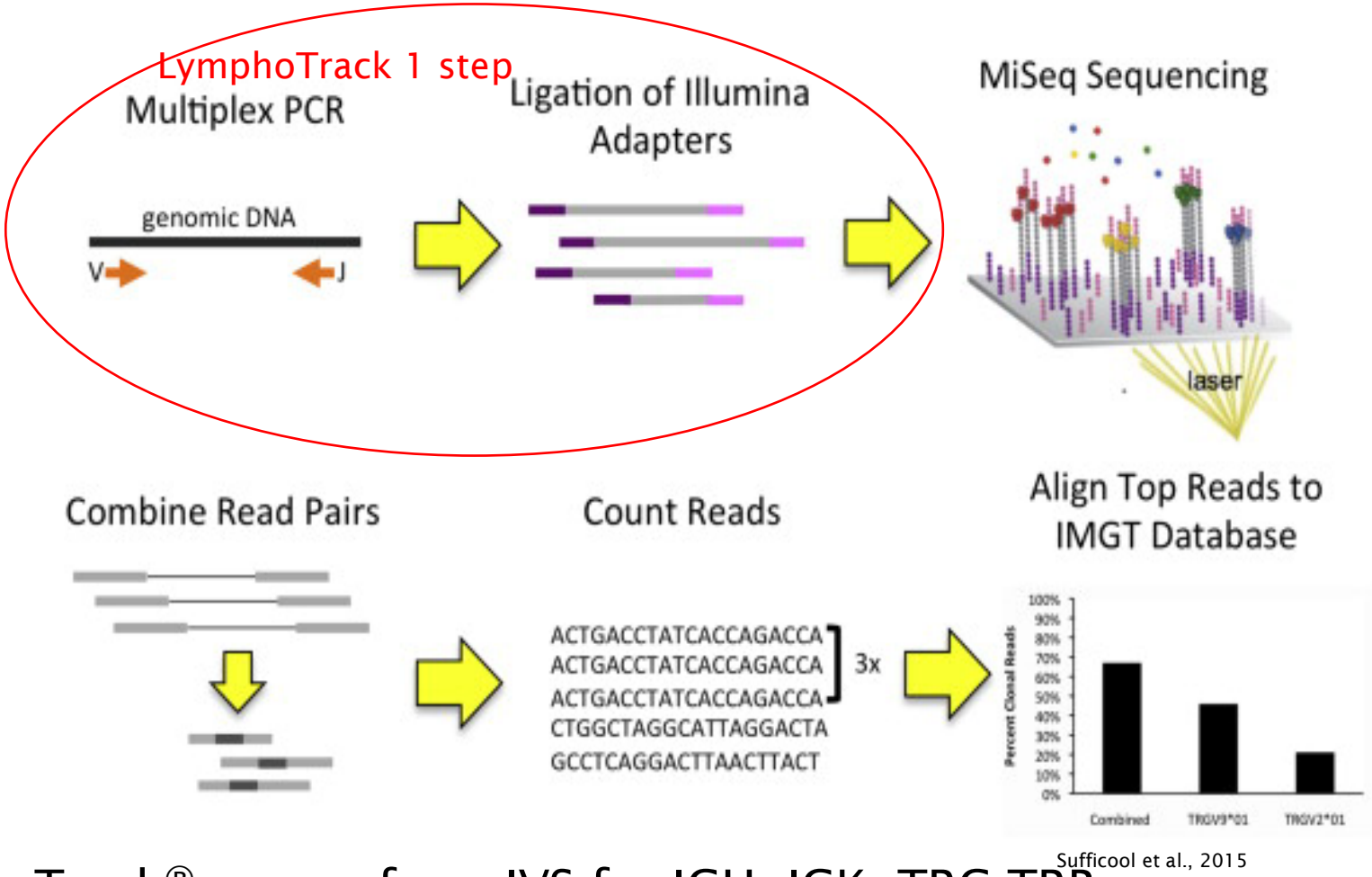
IG/TCR TARGETS

Applicability

Locus	B-lineage ALL	T-lineage ALL	CLL	B-NHL	Multiple Myeloma
IgH, Igκ	>95%	20-25% (~DH-JH)	>95%	~80%	>95%
TCRβ	~35%	~90%			
TCRγ	~55%	~95%			
TCRδ	~40%	~55%			
Vδ2-Jα29	~40-45%				

Ref.: J.J.M. van Dongen en V.H.J. van der Velden: Detection of minimal residual disease in ALL
Van Krieken et al., Leukemia 2006
Pot et al.,Methods Mol. Biol., 2013

NGS FOR IG/TCR-CLONOTYPE DETECTION: HOW?



Sufficool et al., 2015

LymphoTrack® assays from IVS for IGH, IGK, TRG,TRB

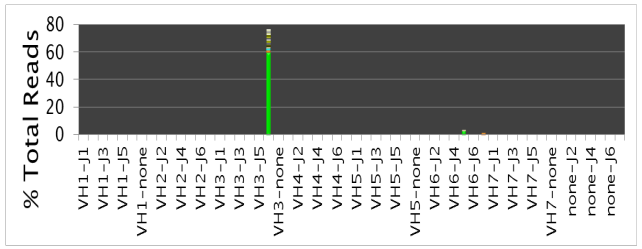
EURO-Clonality assay for IGHinc and TCRD (*Brüggemann et al., Leukemia 2019, 33, 2241*)

IG/TR NGS WORKFLOW

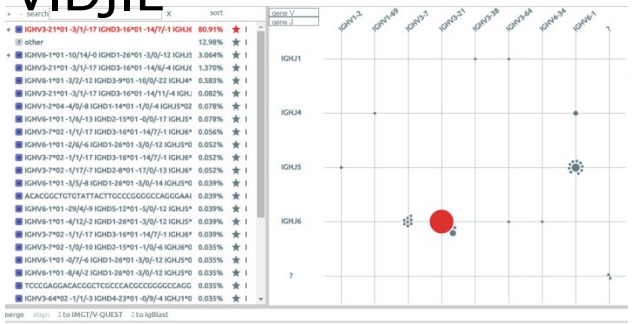
NGS: MiSeq (Illumina)

Analysis

- LymphoTrack (Invivoscribe)



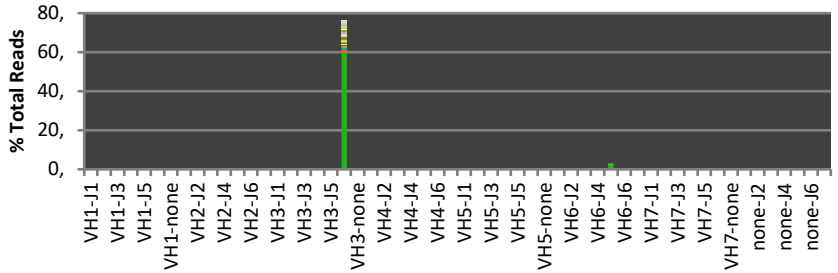
VIDJIL



Mathieu Giraud, Mikaël Salson, et al., “Fast multiclonal clusterization of V(D)J recombinations from high-throughput sequencing”, BMC Genomics 2014, 15:409 http://dx.doi.org/10.1186/1471-2164-15-409

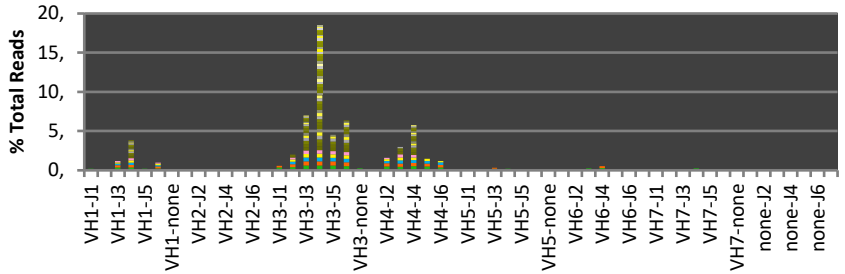
NGS DIAGNOSTIC SAMPLE

Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
1	GCCTCTGGATTCA	281	12878	IGHV3-21_02	IGHJ6_03	59,8058793	59,8058793
2	TGCCATCTCCGGC	280	532	IGHV6-1_02	IGHJ5_02	2,4706265	62,2765058
3	GCCTCTGGATTCA	278	337	IGHV3-21_02	IGHJ6_03	1,5650397	63,8415455
4	GCCTCTGGATTCA	278	244	IGHV3-21_02	IGHJ6_03	1,1331445	64,9746900
5	TGCCATCTCCGGC	255	103	IGHV6-1_02	none	0,4783356	65,4530256
6	GCCTCTGGATTCA	281	91	IGHV3-21_02	IGHJ6_03	0,4226072	65,8756327
7	GCCTCTGGATTCA	280	67	IGHV3-21_02	IGHJ6_03	0,3111503	66,1867831
8	GCCTCTGGATTCA	281	55	IGHV3-21_02	IGHJ6_03	0,2554219	66,4422050
9	GCCTCTGGATTCA	281	46	IGHV3-21_02	IGHJ6_03	0,2136256	66,6558306
10	GCCTCTGGATTCA	281	38	IGHV3-21_02	IGHJ6_03	0,1764733	66,8323039



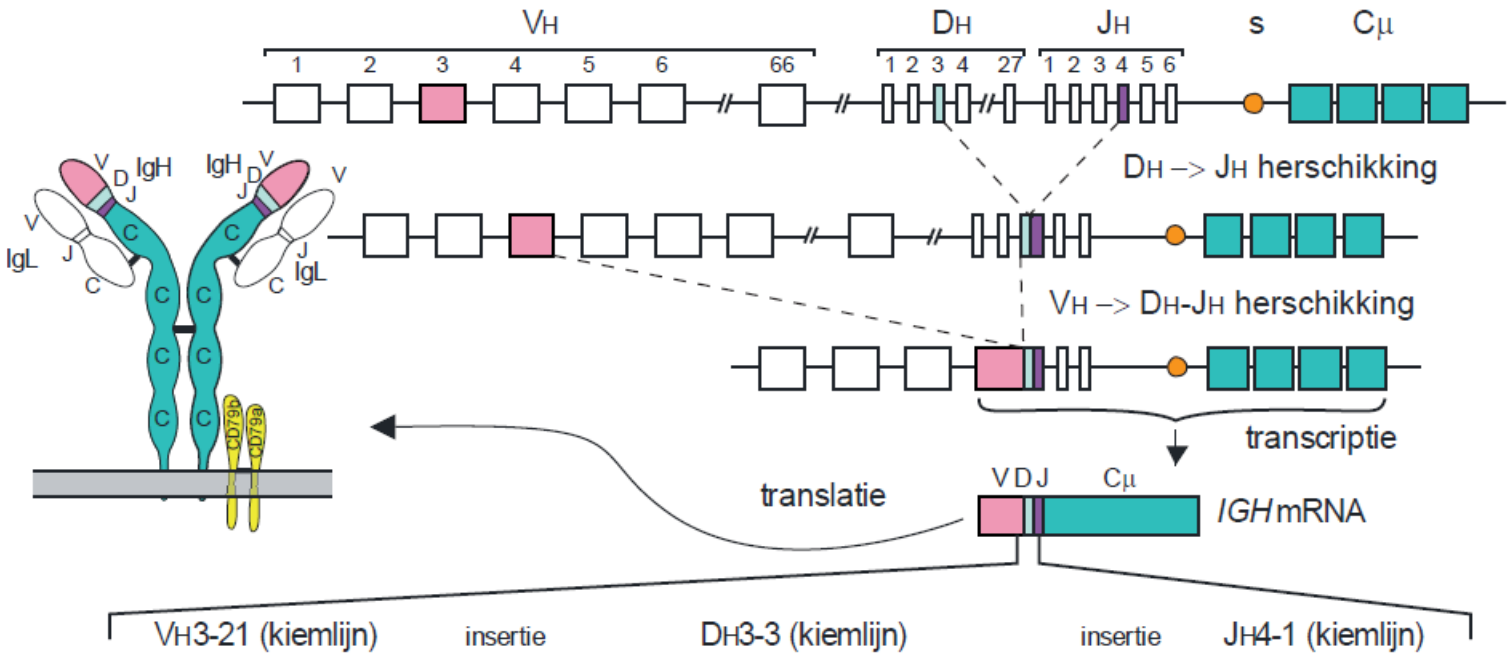
MRD possible

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %
1	GCCTCTGGATTCA	272	81	IGHV3-64_01	IGHJ4_02	0,07	0,07
2	GCCTCTGAATTCA	275	77	IGHV3-11_05	IGHJ6_02	0,07	0,13
3	GCCTCTGGATTCA	269	74	IGHV3-11_01	IGHJ4_02	0,06	0,20
4	GCCTCTGGATTCA	281	73	IGHV3-30_18	IGHJ4_02	0,06	0,26
5	GCCTCTGGATTCA	278	73	IGHV3-9_01	IGHJ6_02	0,06	0,32
6	GCCTCTGGATTCA	290	72	IGHV3-48_03	IGHJ6_02	0,06	0,38
7	GCCTCTGGATTCA	272	72	IGHV3-11_05	IGHJ4_02	0,06	0,44
8	GCGTCTGGATTCA	284	72	IGHV3-33_01	IGHJ4_02	0,06	0,50
9	GCCTCTGGATTCA	281	72	IGHV3-23_04	IGHJ5_02	0,06	0,56
10	GCCTCTGGATTCA	287	72	IGHV3-15_02	IGHJ3_02	0,06	0,63



MRD not possible

ALL: ACUTE LYMPHOBLASTIC LEUKEMIA

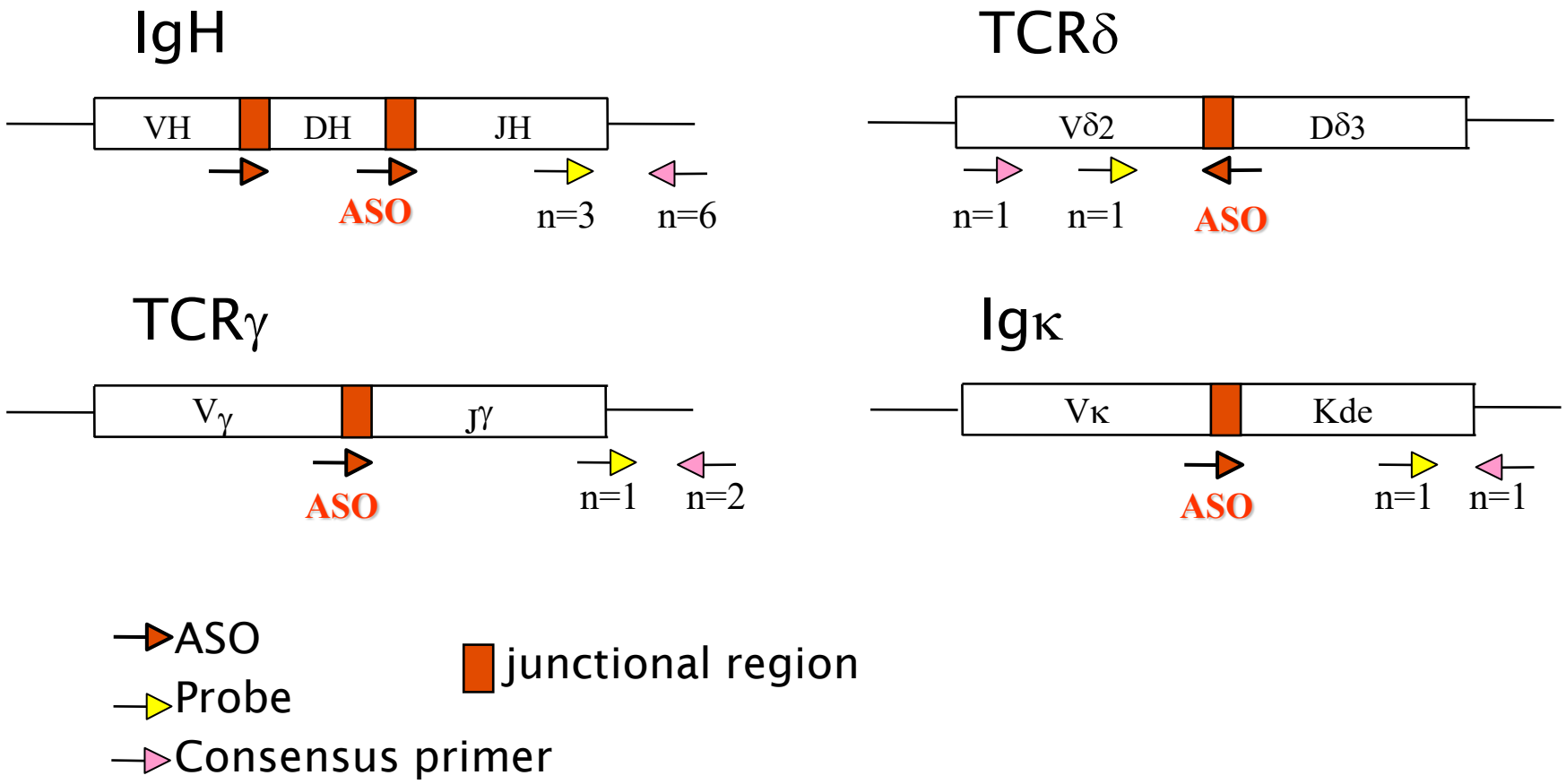


Clonal sequence

TGTATTACTGTGC CCGGACTG TTTTGGAGTGGTTATTATACC GGT ACTACTTTGACTACT

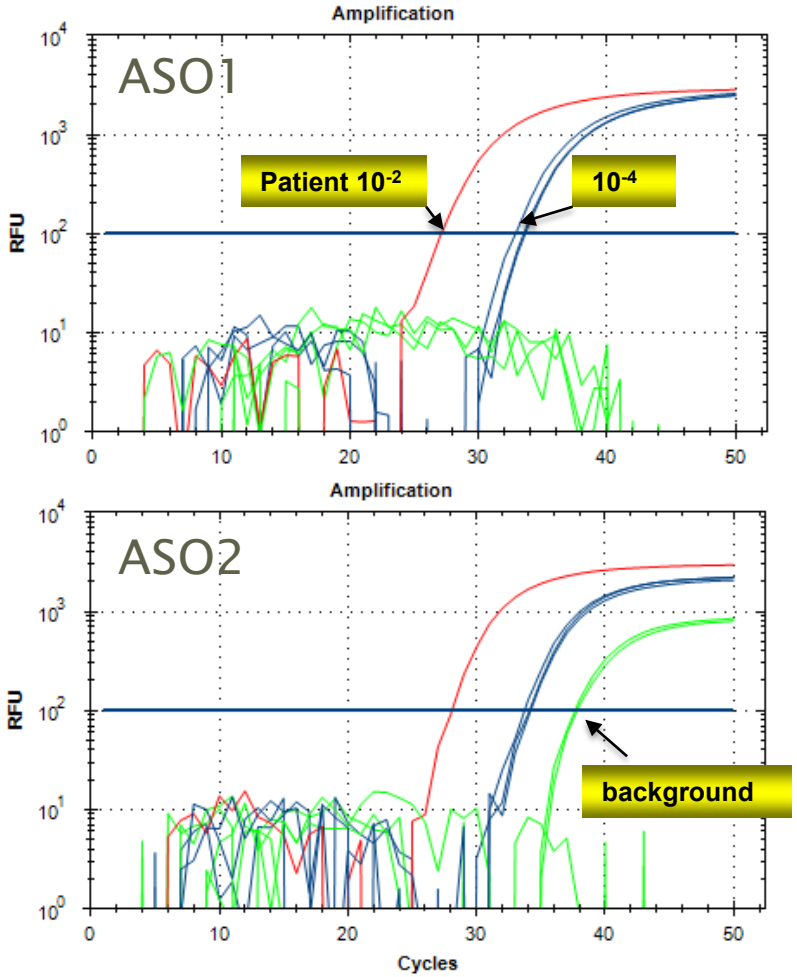
ASO-primer

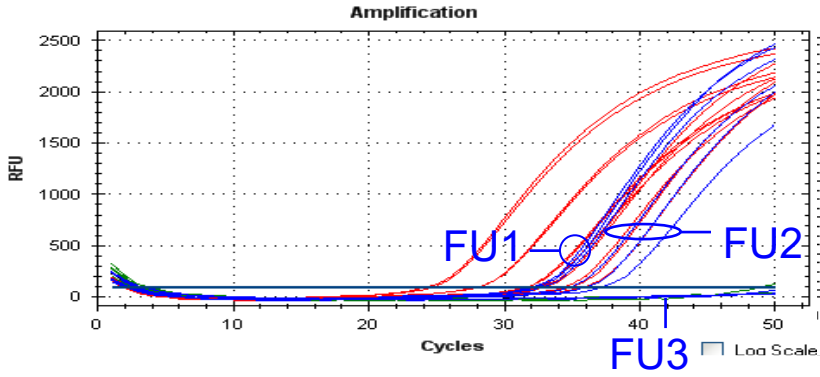
ASO-PRIMERS IN RQ-PCR

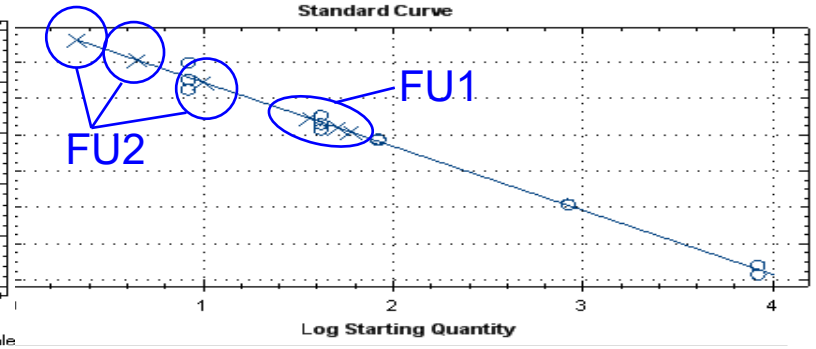


Ref: Verhagen et al., Leukemia 2000, Van der Velden et al. Leukemia 2002

ASO-PCR OPTIMISATION AND MRD TESTING







-- Standard curve: patient Dx DNA dilution in normal DNA

Quantitative range: 10^{-4}
Sensitivity: 10^{-5}

→ FU1: 0.04%

→ FU2: positive, non-quantifiable

→ FU3: negative

EURO-MRD

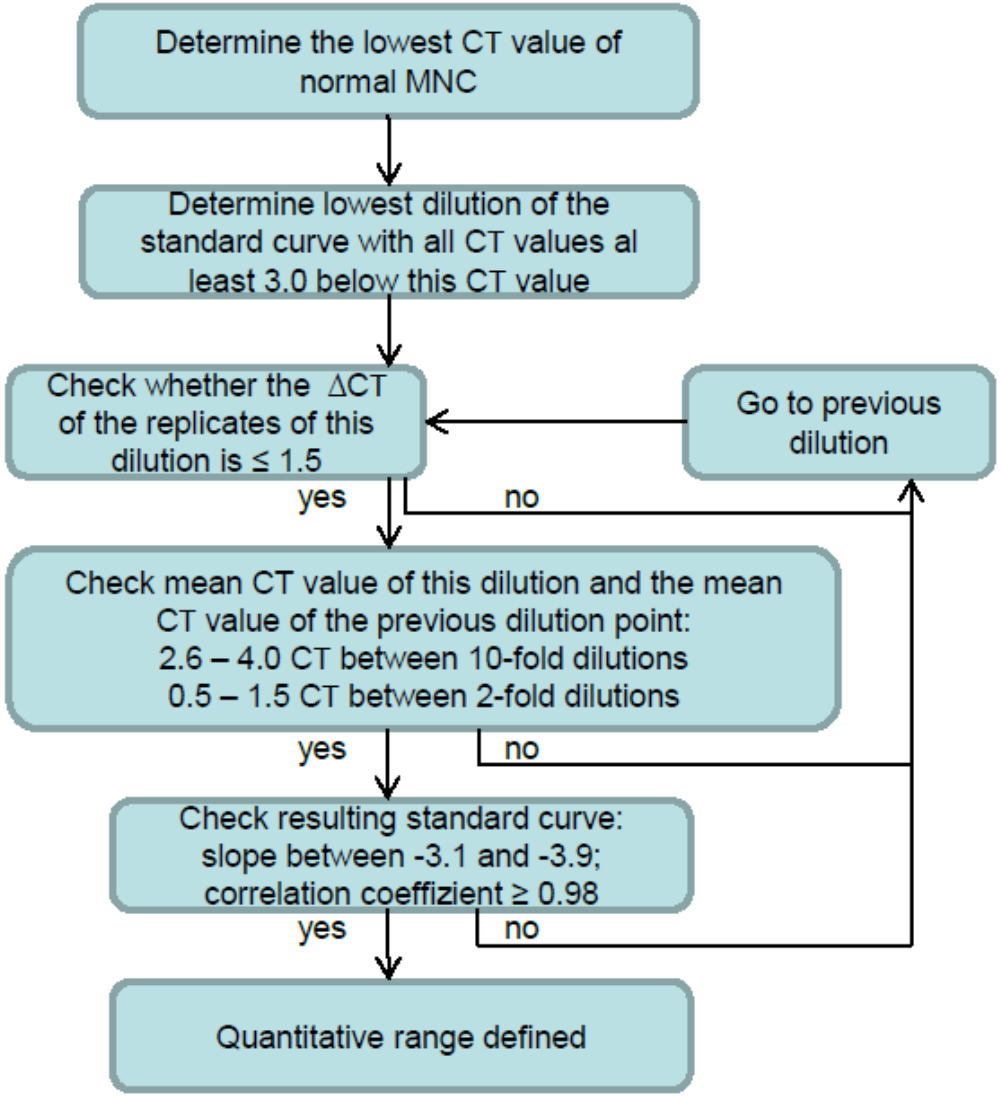
68 laboratories
26 countries

www.euromrd.org

AIMS

1. Organisation of a quality -control program twice a year;
2. Collaborative development and evaluation of new MRD strategies and techniques;
3. Development of guidelines for the interpretation of RQ-PCR based MRD data.

SENSITIVITY AND QUANTITATIVE RANGE (EURO-MRD GUIDELINES)



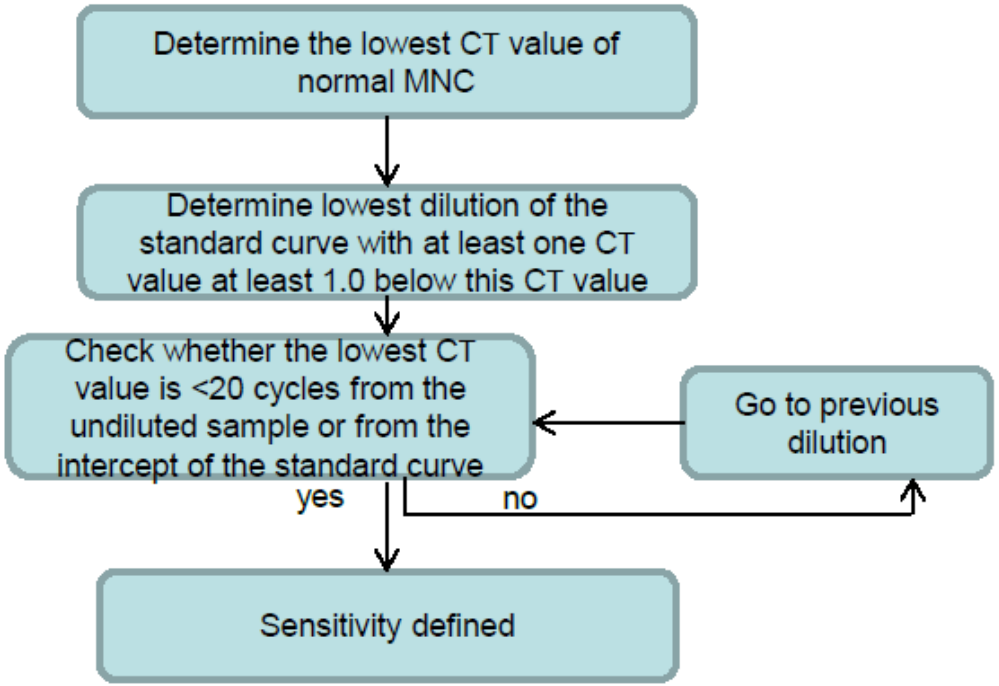
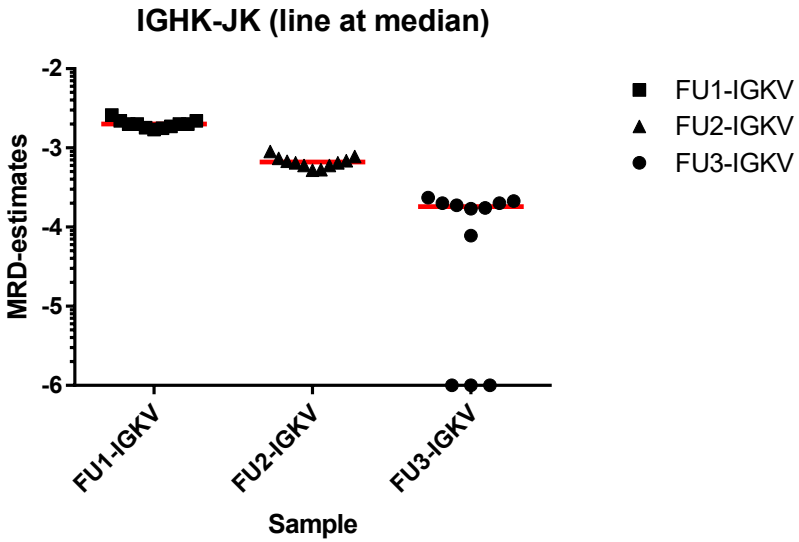
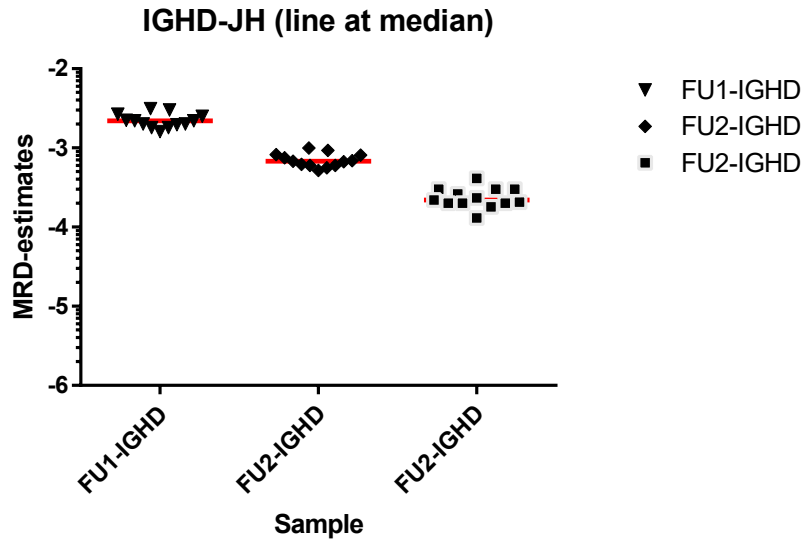


Diagram adapted from V. van der Velden EuroMRD BOOK Chapter 15

STANDARDISATION qASO-PCR EURO-MRD

	IGHV-JH	DD2-DD3	IGHD-JH	IGKV-JK	target values
median FU1	0,002	0,002	0,0022	0,002	0,002
median FU2	0,0006395	0,0006105	0,00068	0,000664	0,0006
median FU3	0,0002	0,000195	0,00022	0,000181	0,0002





ALLTOGETHER

Preventing over- and under- treatment by further refinement of the stratification based on biological characteristics and MRD.

- Definition of a low risk group in which therapy can be safely reduced
- Introduction of more targeted therapy (CAR-T and inotuzumab) may replace more toxic conventional therapy

Green: activated 2020 (NL, Denmark, Finland, Sweden, Belgium, Norway and UK)

Blue: 2022

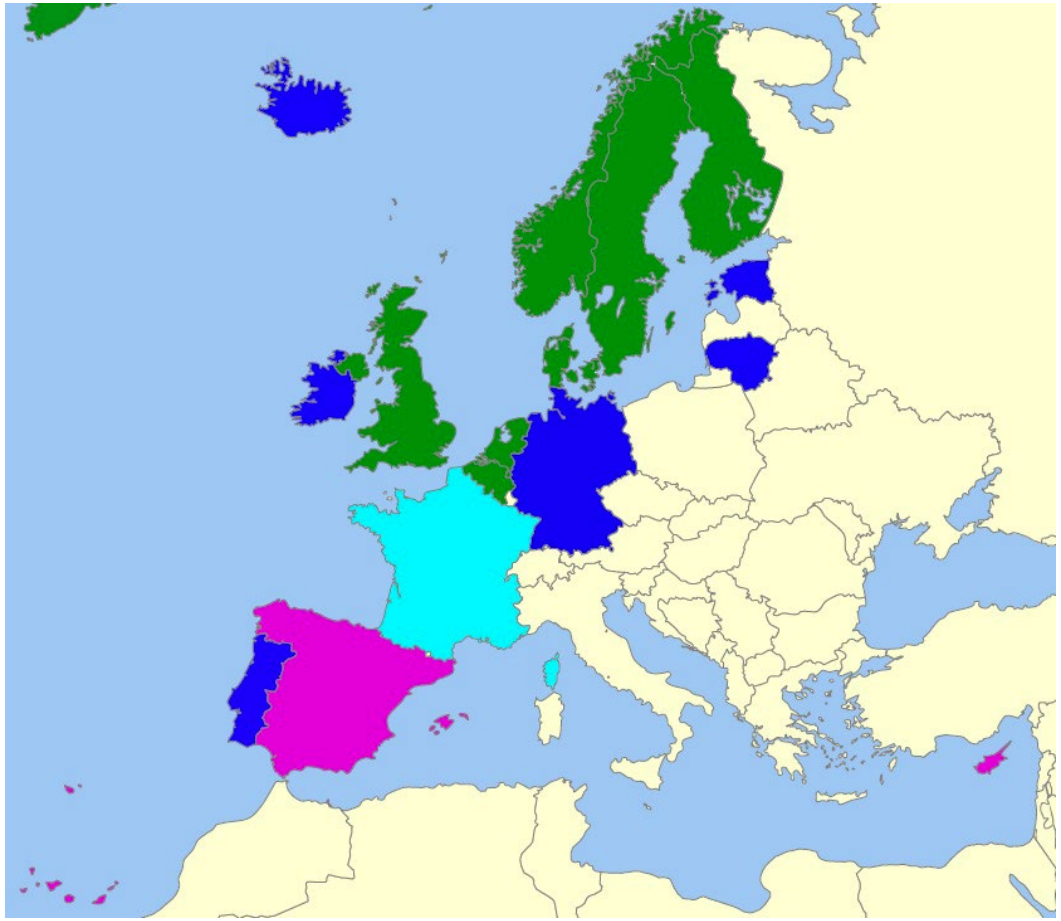
France: end ‘22

Spain: pilot in 2022

1ste pt in NL 13/7/2020

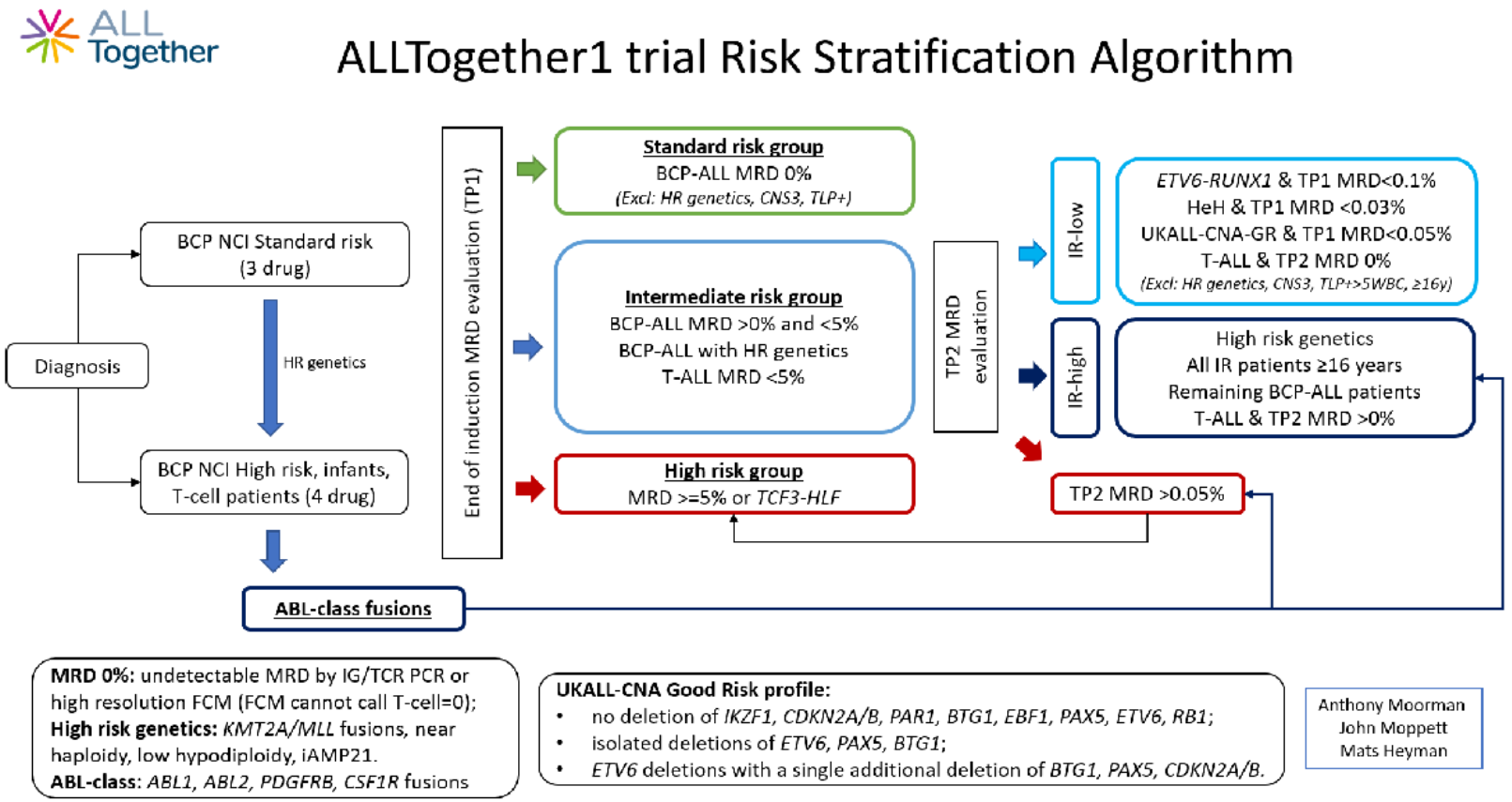
Total: 2208 pt (29/1/2024), 13 countries

Aim: 9100 pt after 7 years



Overview of the risk stratification algorithm for the ALLtogether trial

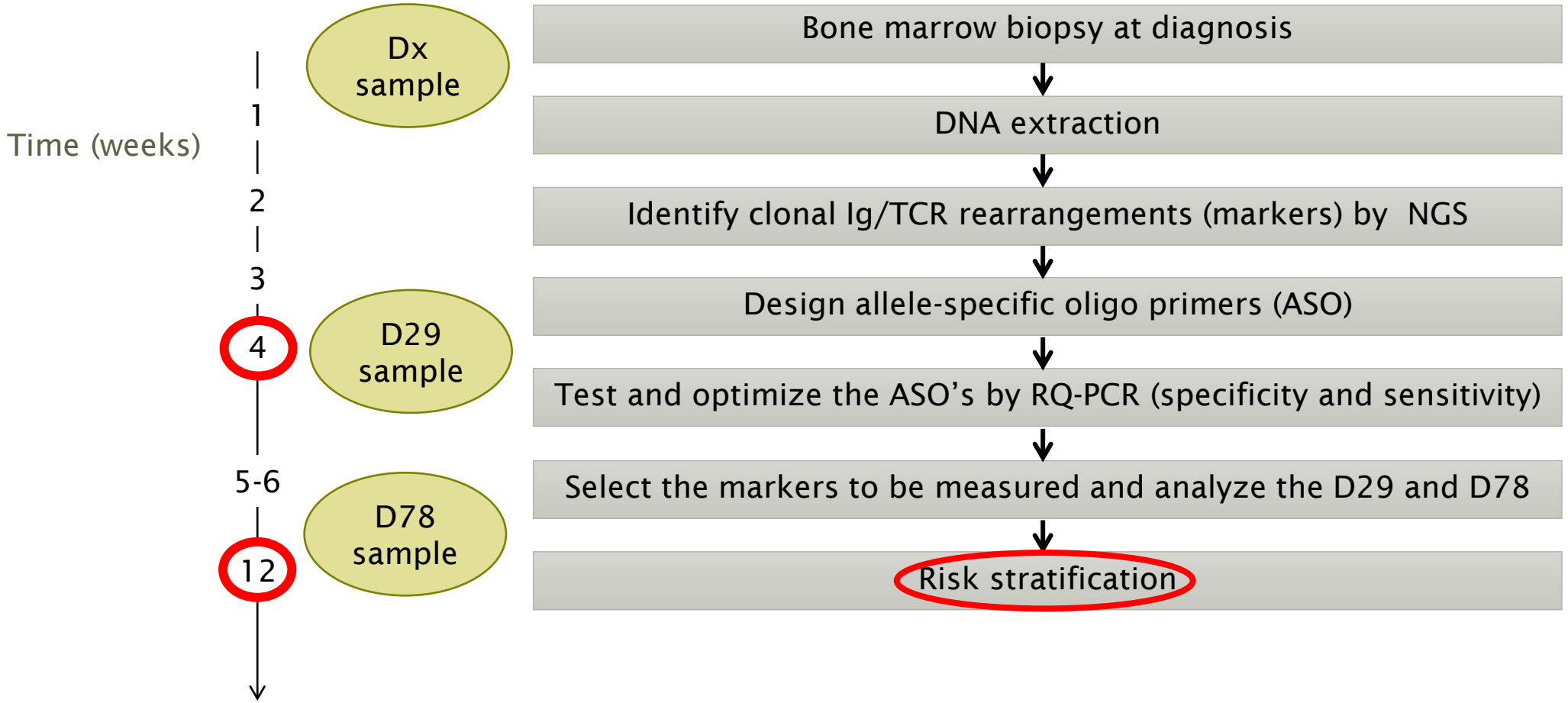
Figure 2: Minimal Residual Disease (MRD) and genetic risk stratification of patients in the ALLTogether1 trial. NB Age (>16 years) and CNS disease status (CNS3) over-ride some of these classification – see protocol for full details



Universitair Ziekenhuis Brussel

VUB

TIME-FRAME



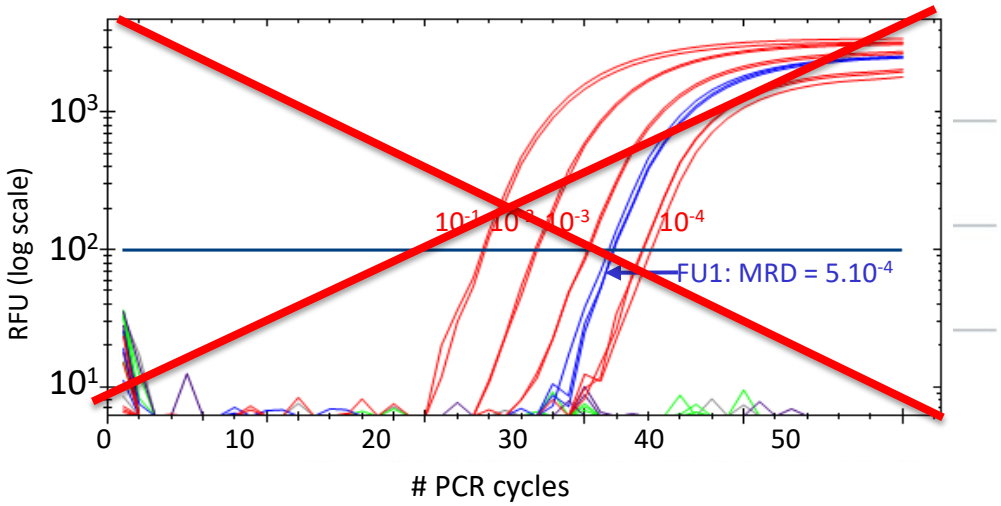
Samples: Dx, d29 (end of induction EOI); d78 (end of consolidation EOC)

IG/TCR-ASO-PCR

- Implemented in childhood ALL clinical trials since the early 1990s and today in standard of care treatment protocols all over the world (with defined time points and thresholds). Now also in adults (HOVON100, HOVON146)
- Golden standard, implemented all over Europe and beyond, very well standardized via the EURO-MRD network
- Advantages: stable matrix (DNA), very sensitive (1^{E}-05), applicability in ALL >90%
- Disadvantages: time-consuming (2-4 wks) and technically very demanding for the design, need of preferential two good ASO's (prevention of false negatives), high cost
- What about NGS?

NGS FOR MRD?

qASO-PCR



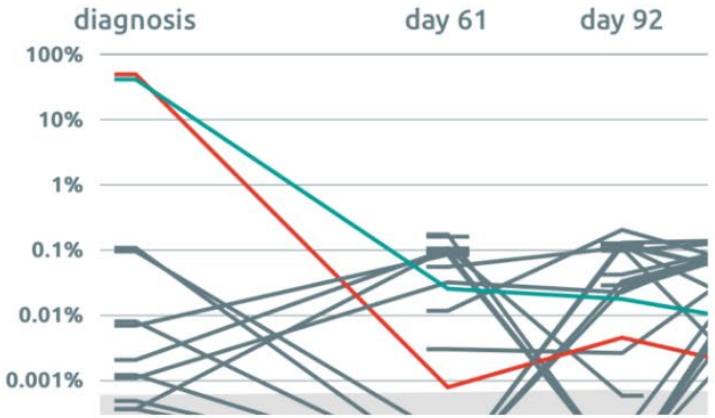
Targets: 1-2

Patient-specific

Sensitivity: 10^{-5}

TAT: 1-2 days

NGS



Targets all rearrangements

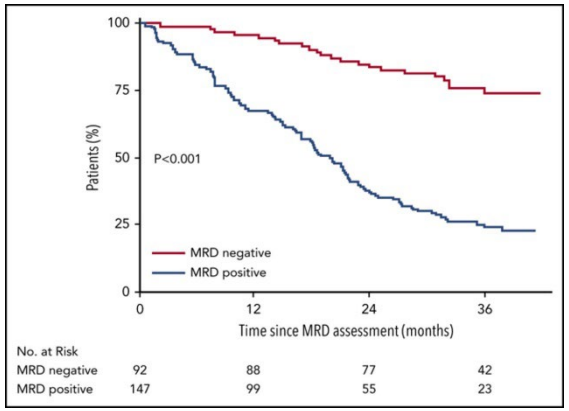
No patient-specific assay

Sensitivity: 10^{-5} (10^{-6})

TAT: 1-2 weeks

MRD IN MM

- Prognostic marker
- Since the 2010s: starting to see MRD negativity in clinical trials using modern 3-drug combination therapies
- (Near) future: MRD testing for clinical decision making in standard clinical practice
- MRD testing: next generation flow cytometry and NGS (not implemented and standardized in many countries)



Aurore Perrot et al. Blood 2018;132:2456-2464

ASO design in MM, minimum 1 with QR $\leq 1^{E}$-04

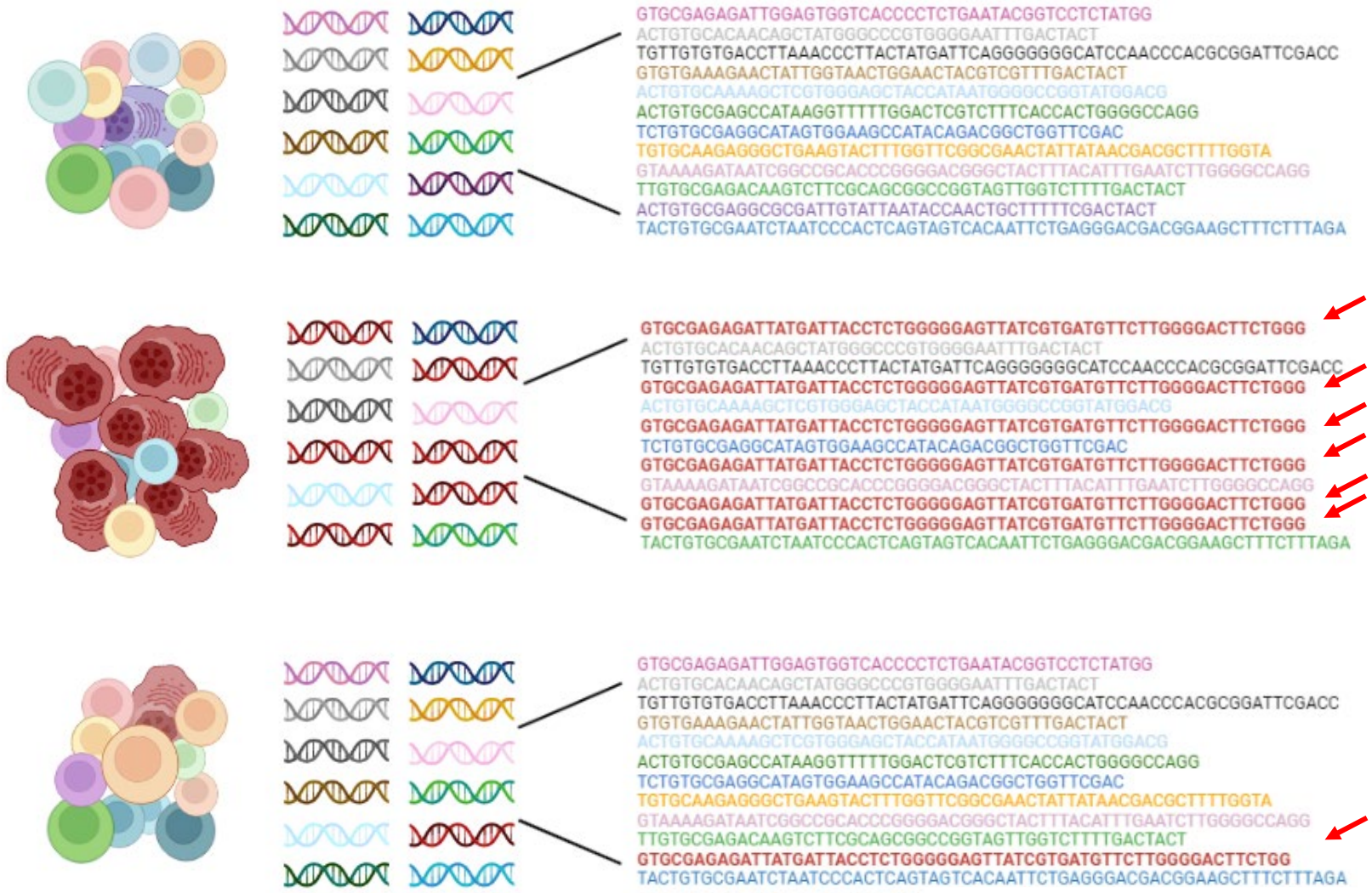
N=14

	ASO+	ASO failure
NGS+/Sanger+	7	**5**
NGS+/Sanger-	2	0

Applicability:
qASO: only 64%, **NGS-MRD: >95%**

MRD IN MM

A diagnostic sample is needed



Normal lymphocytes

Multiple myeloma

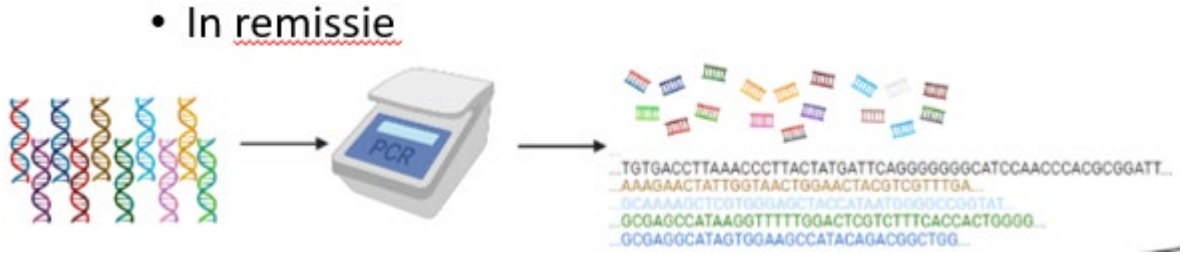
MRD positivity

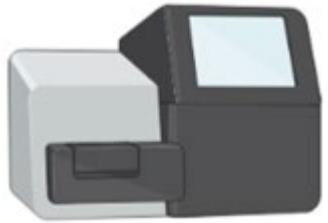
IG-NGS-MRD TEST

Same principle as diagnostic NGS

Difference with diagnoses:

- Only NGS for the clonal target(s)
- More DNA needed (>sensitivity)
  - first pull bone marrow aspirate (EDTA)
  - 3-5 ml
- More sequencing reads needed
  - 250.000
- Calibrators for calculations
  - gBlocks (IGH, IGK)
- Bio-IT tools





NGS-MRD: CALIBRATOR

G-blocks: double stranded, linear, nucleic acids, sequence verified

IGH set:	IGHV1-69*01 (-2/TCAGA/-10) IGHD3-3*01 (-8/CCGA/-7) IGHJ4b
	IGHV3-66*01 (-0/TCTAGGAGGG/-6) IGHD2-15 (-3/GCG/-0) IGHJ6b
	IGHV4-34*01 (-0/C/-2) IGHD5-24 (-2//-0)IGHJ5b
TCRG-set	TRGV5*01 (-5/TCCTCGGG/-11) TRGJ1*01 (=Jg1.3)
	TRGV8*01 (-0/CTT/-0) TRGJP1*01 (=Jg1.1)
	TRGV9*01 (-0/CTCC/-10) TRGJ1*02 (=Jg2.3)
IGK-set	IGK: V3D-15*01 0 / +9 / -2 J4*01
	IGK: V2-30*01 -1 / +7 / -4 KDE
	intron (-1/4/-11) kde

- 10 copies each in 1 µl
- 4 µl DNA plus 1 µl gblock-pool in PCR
- Count calibrator sequences in MRD sample => CCF
- Check for calibrator sequence in neg CTRL sample => contamination

4-11-2018

CALIBRATION FOR MRD TESTING

$$\text{Calibrator correction factor (CCF)} = \frac{\#\text{copies of calibrator}}{\#\text{calibrator reads}}$$

$$\text{MRD} = \frac{\#\text{ clonotype reads x CCF}}{\text{total \# cells (qAlbumin PCR)}}$$

Faham,M et al. Deep-sequencing approach for minimal residual disease detection in acute lymphoblasticleukemia. Blood 2012 120: 5173-5180

Gawad et al. Massive evolution of the immunoglobulin heavy chain locus in children with B precursor acute lymphoblastic leukemia. Blood 2012 120: 4407-4417

VALIDATION NGS-MRD: LINEARITY, SENSITIVITY, CALIBRATION

Dilution
Input 50
Input # c
300.000

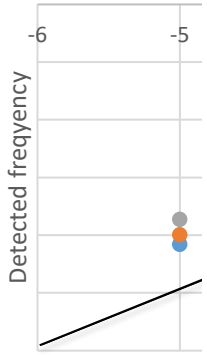
ocks each

The Journal of Molecular Diagnostics, Vol. 23, No. 5, May 2021

ELSEVIER

the Journal of Molecular Diagnostics
jmdjournal.org

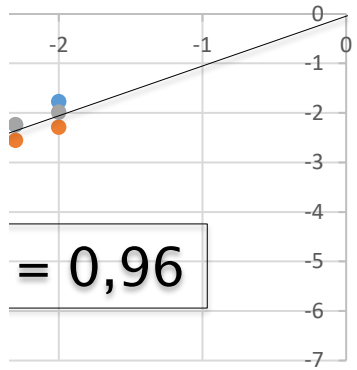
brated)



Validation of a PCR-Based Next-Generation Sequencing Approach for the Detection and Quantification of Minimal Residual Disease in Acute Lymphoblastic Leukemia and Multiple Myeloma Using gBlocks as Calibrators

Check for updates

Jona Van der Straeten,* Wouter De Brouwer,† Emmanuelle Kabongo,* Marie-Françoise Dresse,† Karel Fostier,† Rik Schots,† Ivan Van Riet,† and Marleen Bakkus*

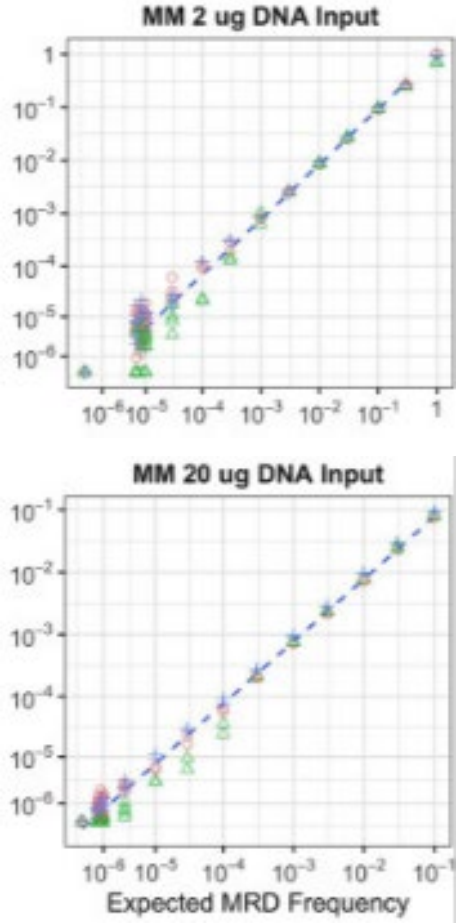


= 0,96

MRD SENSITIVITY = CELL NUMBER

Sensitivity 10-5 → 600 ng ~100,000 cells
3x

Sensitivity 10-6 → 6 µg ~1000,000 cells
3x



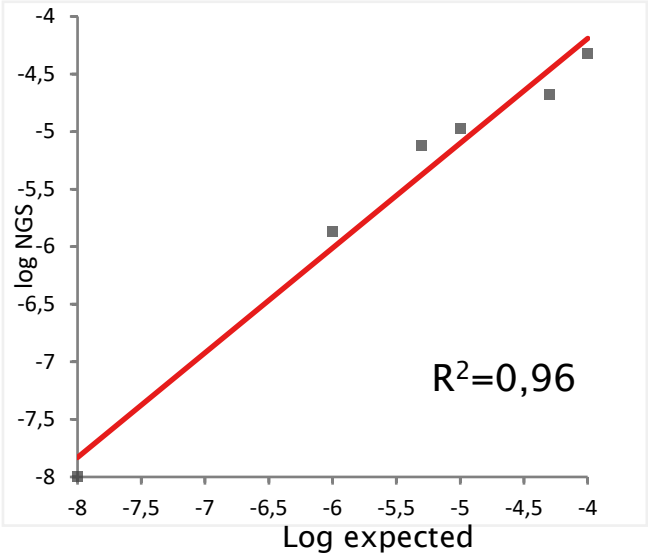
Ching, BMC Cancer 2020

INCREASING DNA INPUT

Concentration of DNA using a speedvac

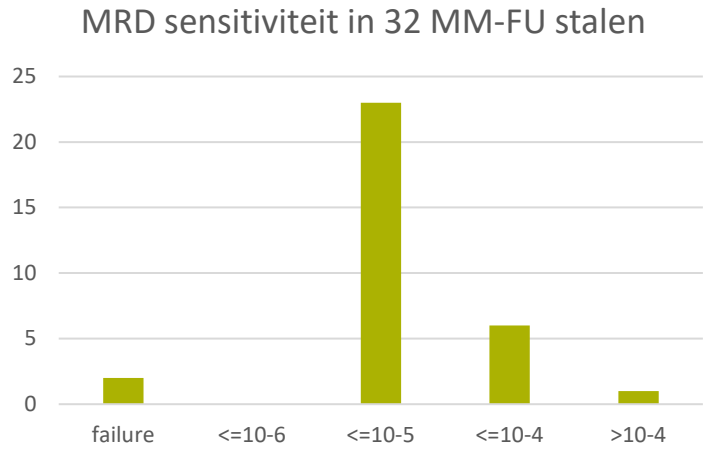
Dilution experiment (patient in healthy control)

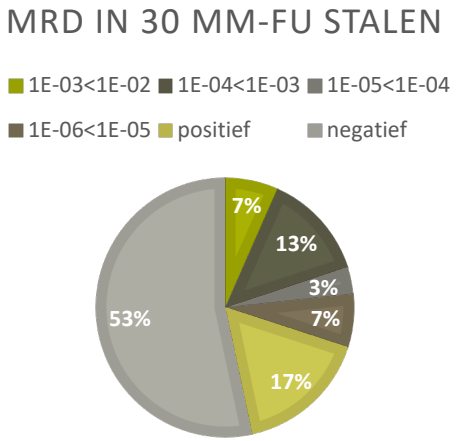
- Input: 7 µg of concentrated DNA
- Requested reads: 1^{E}+06

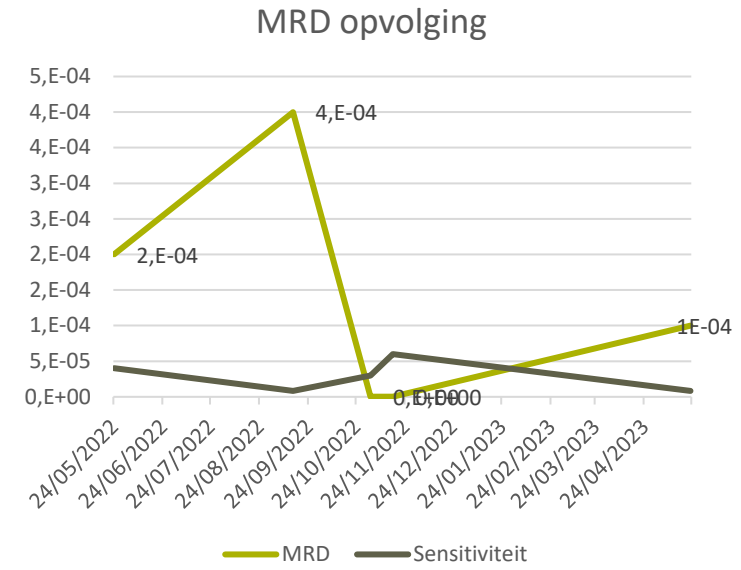


Dilution	1E-04	5E-05	1E-05	5E-06	1E-06	0E+00
Theoretical MRD	-4,00	-4,30	-5,00	-5,30	-6,00	negative
NGS-MRD	-4,32	-4,68	-4,97	-5,13	-5,86	negative

MMOVE: MRD IN MM BM SAMPLES IN CR







Universitair Ziekenhuis Brussel

VUB

FAILURES

Voorspelbaar op basis van de klonale Ig-herschikking?

Failure: 2 stalen met klonale IGK merker, maar finaal niet specifiek genoeg, want kwam ook voor in normale controle DNA.

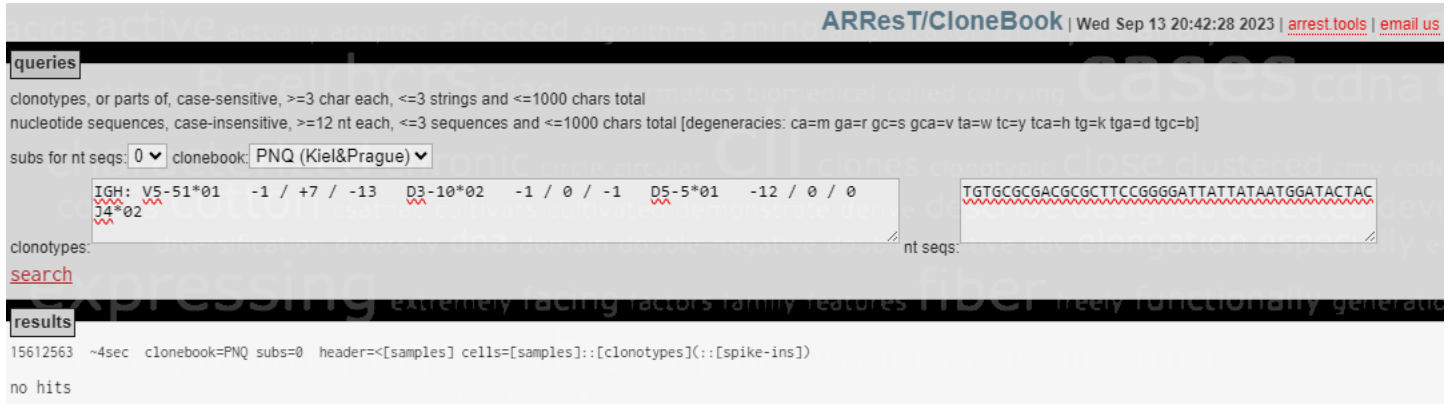
Tool : Clonebook (ARResT/CloneBook @ the BAT cave (infspire.org); initiatief van Kiel & Praag))

Patient 1: IGK: V4-1*01 -1/0/-3 KDE : AGTACTCCTCGCCCTAGTGG: **89::95** (werd 89x gevonden in 1091 verschillende stalen en in 95 clonotypes)

Patient 2: IGK: KIntron -1 / +1 / -2 KDE : CTTTCCTGATAAGCCCTAGTG : **19::882**

In vergelijking met een goede merker:

IGH: V5-51*01 -1 / +7 / -13 D3-10*02 -1 / 0 / -1 D5-5*01 -12 / 0 / 0 J4*02 : **0::0**



CONCLUSIONS

- NGS-MRD: NGS quantitative data correlate very well with the qASO-PCR data (>10^{-5}).
- gBlocks, at 10 copies each, are useful calibrators
- NGS-MRD can be highly sensitive (10^{-5} - 10^{-6}, depending on input DNA): CONCENTRATION of DNA
- IG/TCR-NGS-MRD is especially useful in MM because the qASO-PCR is less applicable due to somatic hypermuations.
- The qASO-PCR remains the golden standard in ALL because shorter TAT and very well standardised via EURO-MRD consortium (ALLTogether protocol TAT <5 days)
- IG/TCR-NGS-MRD: better specificity and sensitivity and gives additional information (IG/TR repertoire) but not (yet) internationally standardised
- Always be critical about 'MRD NEGATIVITY', should be defined for each sample

Kabongo Emmanuelle

MRD ANALYSIS IN AML

Universitair
Ziekenhuis
Brussel

VUB

CLINICAL SIGNIFICANCE

AML 2022 ELN guidelines

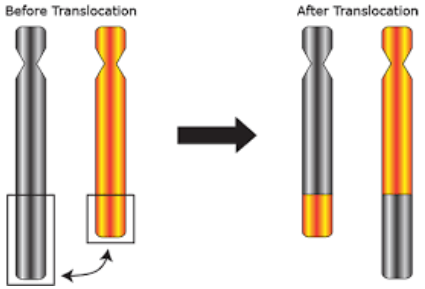
- ± 35% of the leukemia
- Adults (± 60jaar)
- Classification: genetical abnormalites
  - ± 45% CN-AML: **CEBPa** ; **NPM1**; **Flt3;** DNMT3A of NRAS
  - NPM1 or FLT3-ITD: ±1/3 of AML
- NPM1mut + adverse cytogenetic characteristics → poor prognosis
- Flt3-ITD AR no longer used → IR-group

Risk category†	Genetic abnormality
Favorable	• t(8;21)(q22;q22.1)/*RUNX1*::*RUNX1T1*†,‡ • inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB*::*MYH11*†,‡ • Mutated *NPM1*†,§ without *FLT3*-ITD • bZIP in-frame mutated *CEBPA*‖
Intermediate	• Mutated *NPM1*†,§ with *FLT3*-ITD • Wild-type *NPM1* with *FLT3*-ITD (without adverse-risk genetic lesions) • t(9;11)(p21.3;q23.3)/*MLLT3*::*KMT2A*†,¶ • Cytogenetic and/or molecular abnormalities not classified as favorable or adverse
Adverse	• t(6;9)(p23.3;q34.1)/*DEK*::*NUP214* • t(v;11q23.3)/*KMT2A*-rearranged# • t(9;22)(q34.1;q11.2)/*BCR*::*ABL1* • t(8;16)(p11.2;p13.3)/*KAT6A*::*CREBBP* • inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2)/*GATA2, MECOM(EVI1)* • t(3q26.2;v)/*MECOM(EVI1)*-rearranged • −5 or del(5q); −7; −17/abn(17p) • Complex karyotype,** monosomal karyotype†† • Mutated *ASXL1, BCOR, EZH2, RUNX1, SF3B1, SRSF2, STAG2, U2AF1, and/or ZRSR2*‡‡ • Mutated *TP53*[a]

2022 ELN risk classification by genetic markers at Dx
Dohner et al. Blood 2022

TARGETS

Fusion transcripts

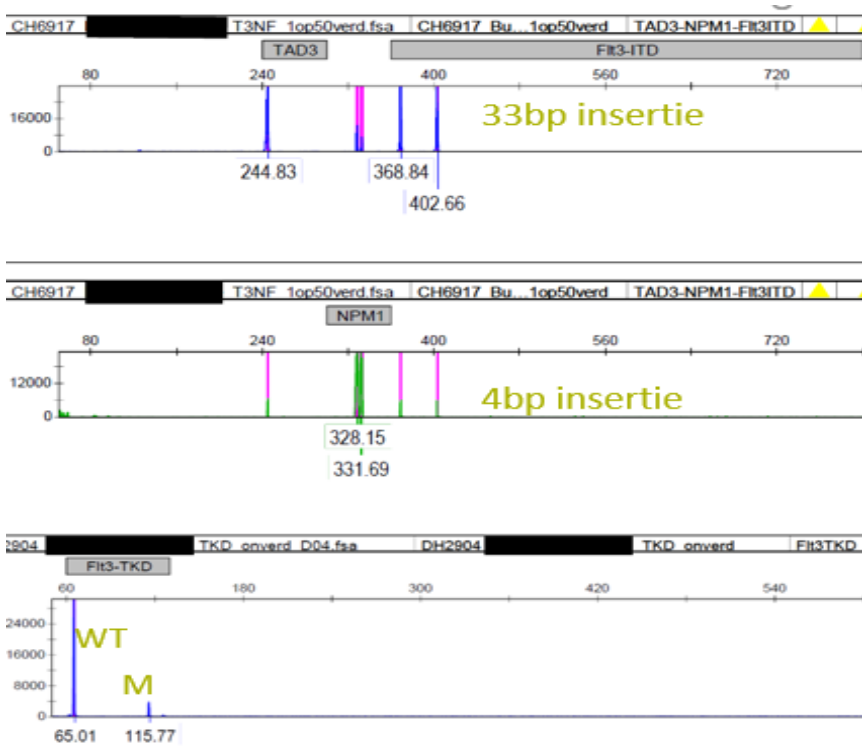


Translocation	Frequency
t(9;22): BCR-ABL1	CML (>90%), ALL (<40%)
t(15;17): PML-RARA	AML (5-10%)
t(8;21): RUNX1-RUNXT1	AML (5-10%)
Inv16: CBF-MYH11	AML (5-10%)
t(12;21): ETV6-RUNX1	B-ALL (children ~15%, adult ~2%)
t(4;11): KMT2A-AFF1	B-ALL (infant 50-85%, children 2-20%, adult ~10%)
KMT2A::XX (>80 partners)	Therapy related (t-)AML
t(1;19): TCF3-PBX1	B-ALL (children 2-6%, adult ~3%)

AML AT UZ BRUSSELS

Diagnosis

- *Sample preparation:*
  - Specimen: B or BM EDTA
- *Tests*:
  - RNA
    - Screening translocations (28)
  - DNA:
    - **multiplex PCR** (CE)
      - CEBPa: b-ZiP domain
      - NPM1: exon 12 → 4bp insertion
      - FLT3-ITD: ±75 - 80%
      - FLT3-TKD: ±3 - 8%
    - **NGS myeloïd panel**
      - Identification lengths / integration site / sequences

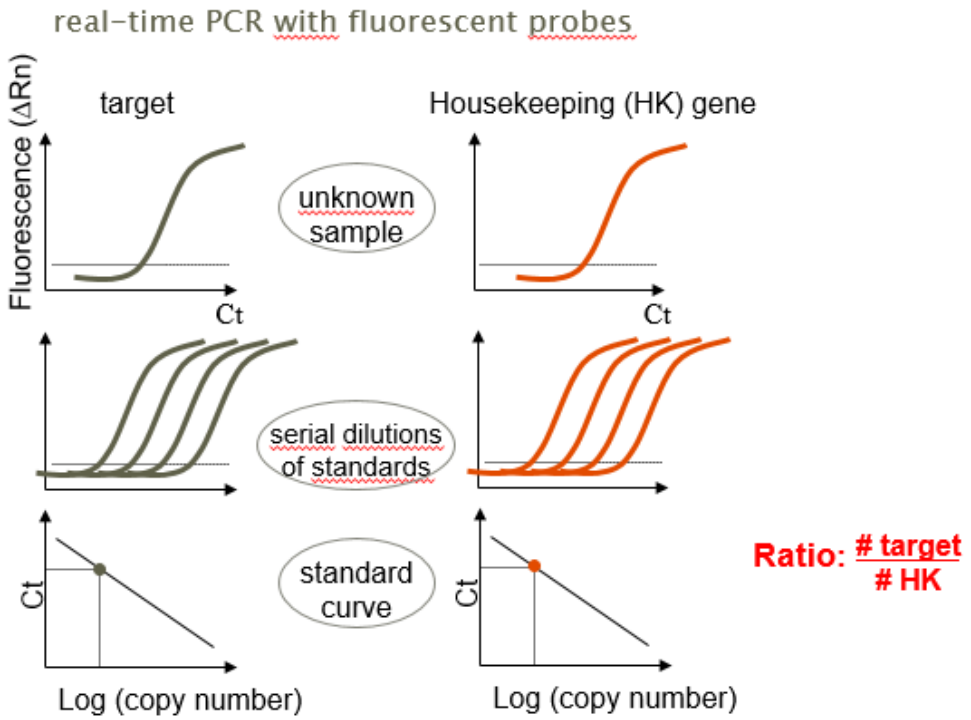


Fragment analysis by capillary electrophoresis (CE):
CEBPa-TAD3; FLT3-ITD and TKD; NPM1

MRD testing

- *Sample preparation :*
  - Specimen: BM EDTA
    (1 log ≠ sensitivity BM vs B)

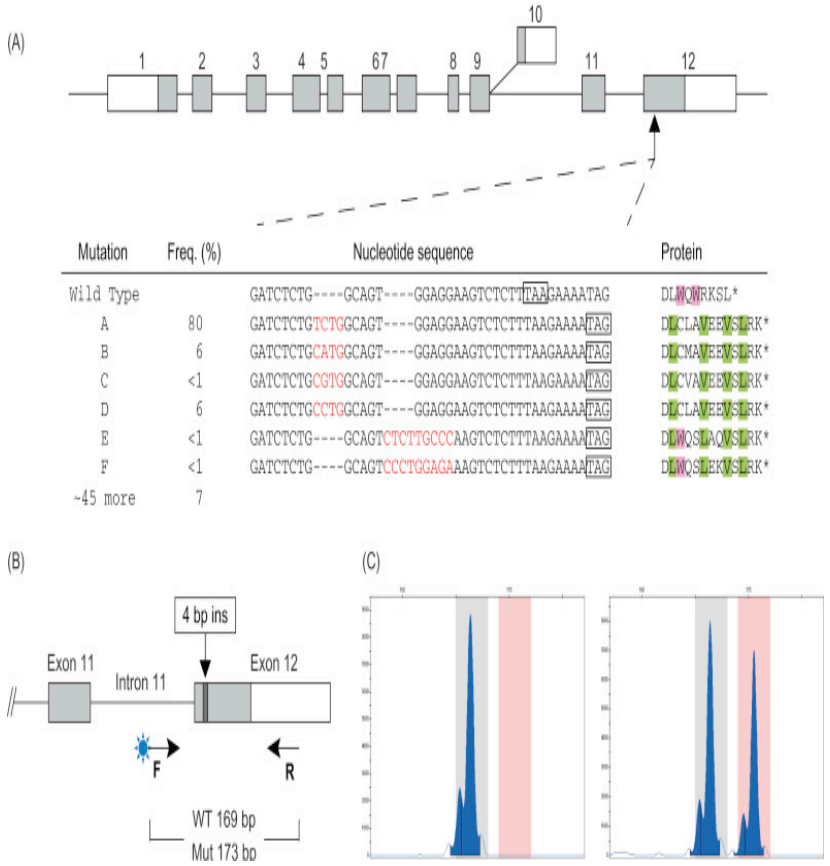
- *Tests:* FU molecular markers
  - RNA
    - **single translocation**: PLM::RARA / CBFb::MYH11 / RUNX1::RUNXT1 /…
    - **RT-PCR**: qNPM1
  - DNA
    - **NGS**: FLT3-ITD (+ NPM1)
    - **CE** : FLT3/NPM1/CEBPa multiplex PCR (10% sensitivity)



Sensitivity: 10^{-4} -10^{-5}

AML TARGETS

NPM1: Nucleophosmin



Blood 2006;107:4011-20.

- Somatic mutations in exon 12 ➔ >50 Suptypes
  - Subtype: A, B, D en R → A: 75 à 80% (TCTG)

AML TARGETS

NPM1: Nucleophosmin (chr 5q35.1)

- Subtype A
- 2µg RNA: cDNA with M-MLV
- NPM1mutA stnd (Ipsogen®)
- Pos. ctrl: OCI-AML3 (10^{-2} and 10^{-4})
- Neg. ctrl: HL60
- Housekeeping gene: ABL1-gen
- % MRD = (#copy NPM1 / #copy ALB)*100

RT-qPCR

b

Genomic Systems	
gNPM mut A-F	5'-AGGCTATTCAAGATCTCTGTCTGG -3'
gNPM-R2	5'-AAGTTCTCACTCTGCATTATAAAAAGGA-3'
gNPM mut B-F	5'-CTATTCAAGATCTCTGCATGGCA-3'
gNPM mut D-F	5'-TATTCAAGATCTCTGCCTGGCA-3'
gNPM mut E-F	5'-TCTCTGGCAGTCCCTCGC-3'
gNPM mut G-F	5'-GGCAGTGCTTCGCCCA-3'
gNPM mut H-F	5'-AGATCTCTGGCAGTGTTTTTCAAAA-3'
gNPM-R1	5'-AAAGGACAGCCAGATATCAACTGTT-3'
g.Probe	5'-TTCCGTCTTATTTCATTTCT-3'
cDNA Systems	
cNPM-F	5'-GAAGAATTGCTTCCGGATGACT-3'
c.Probe	5'-FAM-ACCAAGAGGCTATTCAA-MGB-3'
cNPM mut A-R	5'-CTTCCTCCACTGCCAGACAGA-3'
cNPM mut B-R	5'-TTCCTCCACTGCCATGCAG-3'

Primers/probe conbination for RT-qPCR with cDNA
Gorello et al 2006

AML TARGETS

NPM1: RT-qPCR

NPM1 detection with RT-qPCR → S: depends on ABL-copies

From: European LeukemiaNet 2020 recommendations for treating chronic myeloid leukemia

	MMR	MR[4]	MR[4.5]	MR[5]
Minimum sum of reference gene transcripts	10,000 ABL1[a] 24,000 GUSB[a]	10,000 ABL1 24,000 GUSB	32,000 ABL1 77,000 GUSB	100,000 ABL1 240,000 GUSB
BCR-ABL1 transcript level on the IS[b]	≤0.1%	≤0.01%	≤0.0032%	≤0.001%

[a]Minimal sensitivity for accurate quantification.

[b]International Scale, IS.

AML TARGETS

NPM1: MRD in AML 2022 ELN richtlijnen (ref. Dohner et al. Blood 2022)

Monitoring of MRD (Mol-MRD) by qPCR:

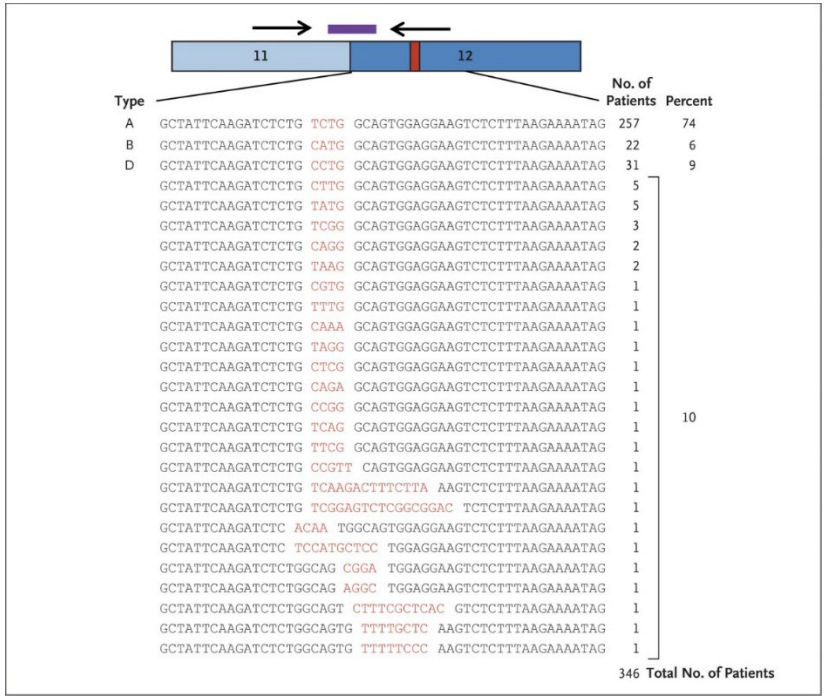
- LOD ≤ 1^{E}-03
- 2-3 replica
- MRD^{+} or MRD^{-}: 40 cycli → threshold dependent
- Complete remission MRD low level (CR_{MRD-LL}) <2% (expression-level)
  - End of consolidation: MRD negative (↓↓ relaps rate)
- MRD^{+} : ≥ 0,1% VAF

ref. Dohner et al. Blood 2022

AML TARGETS

NPM1: Nucleophosmin (chr 5q35.1)

NGS (amplicon-base)



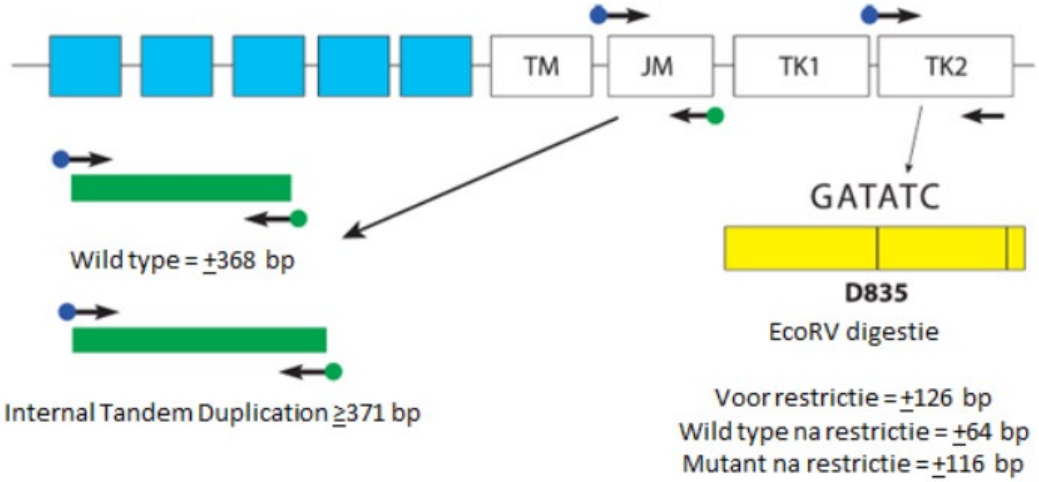
Ivey A et al. N Engl J Med 2016;374:422-433

- Advantage:
  - Amplification and sequencing
  - Subtype independent
  - Multiplex of samples / genes
- Disavantage:
  - expensive
  - Bio-IT for data analyse

AML TARGETS

Flt3: fms related tyrosine kinase

- FLT3-TKD: RE (EcoRV-HF)
- FLT3-ITD: n x (3bp)



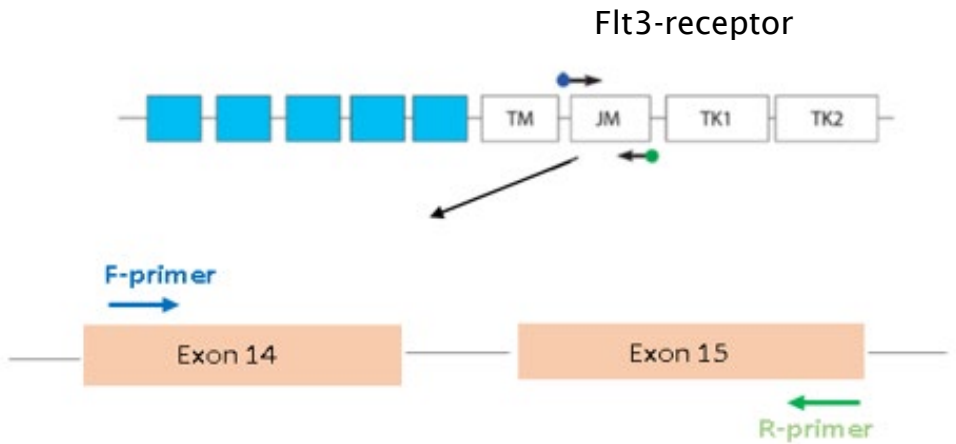
Invivoscribe®

AML TARGETS

FLT3 (chr 13q12)

- ITD: exon 14-15
  - n x 3bp duplication
- Patient specific
  - Evolving at relaps (Blätte et a. Lekemia 2019)
- Pos. ctrl: MV4-11 (10^{-2} and 10^{-4})
- Neg. ctrl: healthy donors (GL POOL)
- DNA quality: Albumine
- % MRD = (# reads mutant / # reads WT+M) * 100

NGS (amplicon-base)



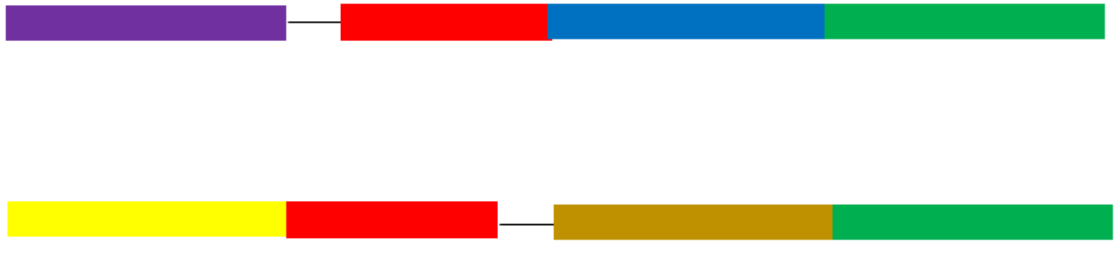
Forward (F) and reverse (R) primers on Flt3 gene

TECHNICS - NGS FOR MRD

PCR amplification➔ one-step

Primers: Thol et al. Genes, Chromosomes & Cancer 2012

- Target sequence: ≠ targets (NPM1 mut : exon 12 + FLT3-ITD: exon 14-15)
- Paired-end reads: read1 forward (5' →3') vs read2 (3'→5') reverse strand
- Index: ≠ samples
- P5/P7: sequencing segments



NGS Forward primer

NGS Reverse primer

primers of NPM1_mut and Flt3-ITD for NGS.

≠ index → Combination of 16 sample per target

TECHNICS - NGS FOR MRD

PCR amplification➔ one-step

BM: DNA isolation

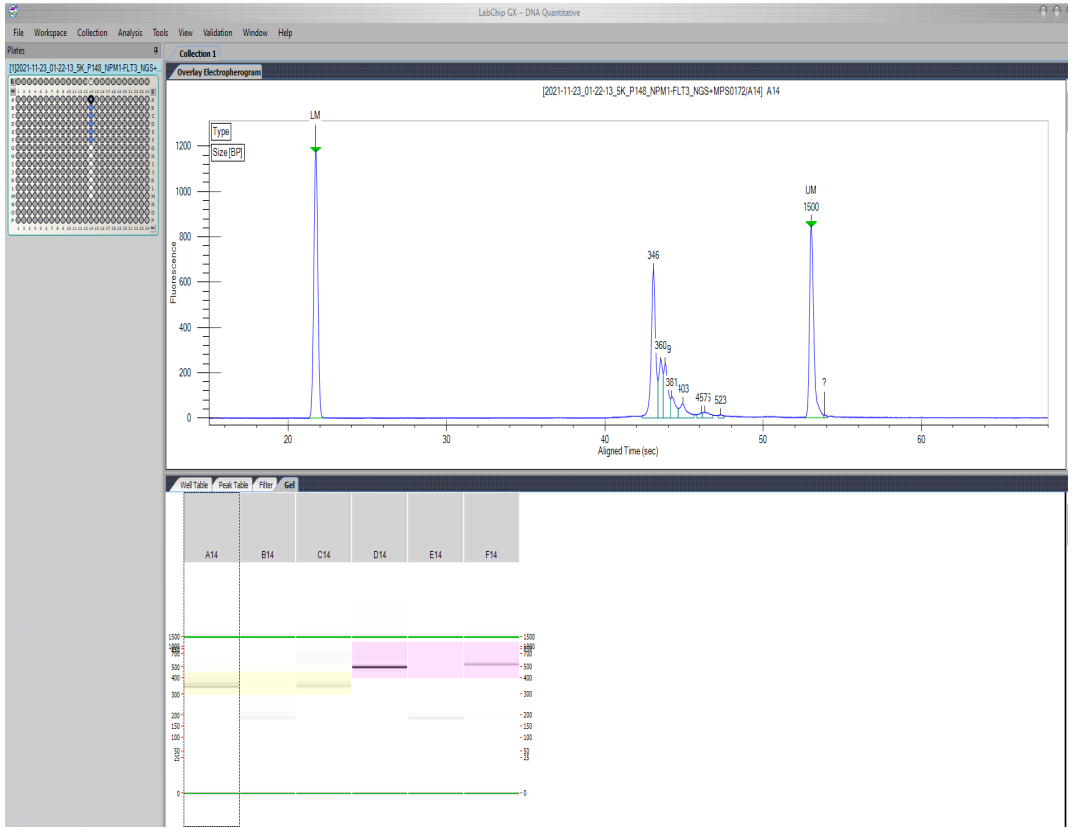
- → mesuring by nanophotometer and albumine quantification
- Input: ≥1 µg DNA or max 10µl
- In duplo
- Pos ctrl:
  - FLT3-ITD: MV4-11 (10^{-4})
  - NPM1: OCI-AML3 (10^{-4})
- Neg cltr: pool of healty donor (GL POOL)
- PCR 25µl reaction:
  - 12,5 µl NEB next HF 2x + 1,25 µl F primer + 1,25 µl R primer + 10 µl DNA/H2O
- PCR-program:
  - 98˚C 2 min. / (98˚C 10 sec. / 64˚C 30 sec. / 72˚C 30 sec.) X 35 / 72˚ 2 min. / 10 ˚C ∞

TECHNICS - NGS FOR MRD

Purification / Quantification / 2nM Library

- Amplicons purification: Agencourt AMPure XP systeem
- Quantification:
  - Qubit: measuring amplicons concentration
  - Labchip Caliper: visualisation and length determination
    - Flt3-ITD: 567bp
    - NPM1: 365bp
- Dilution: each amplicon to 2nM with EBT

- 2nM library : equivalent molarity
- Pooling with other libraries + PhiX (sequencing control)
- Sequencing
- Sample sheet: demultiplexing



LapChip Caliper Electropherogram

TECHNICS - NGS FOR MRD

MiSeq flow cell

- MiSeq sequencing flow cell:
  - V2 vs V3
    - V3 flow cell: 2x300 cycli (56h)
    - 15 vs 25 millions reads
  - 1 millions reads per sample:
    - R1 and R2 combined

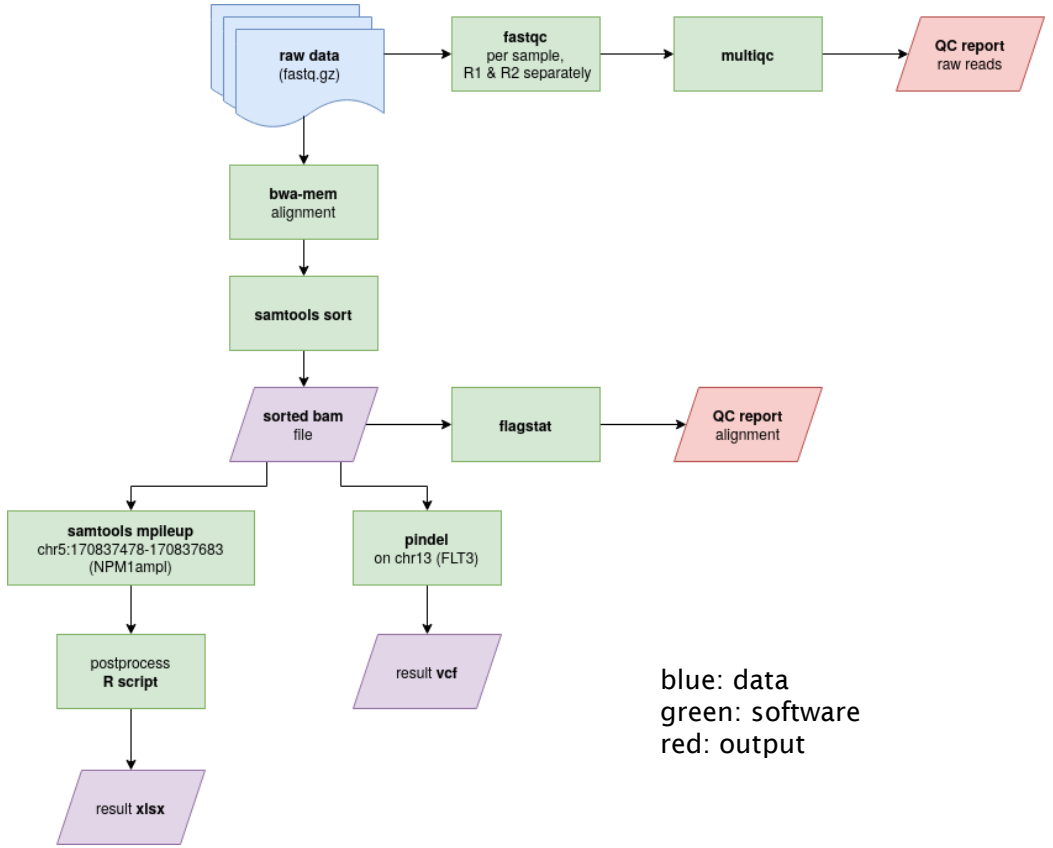
	250000 reads		1000000 reads	
	tot. # reads	# reads Flt3-ITD	tot. # reads	# reads Flt3-ITD
MV4-11 1E-01	87710	4262	489623	19379
MV4-11 1E-02	104244	392	572341	1752
MV4-11 1E-03	104612	48	519512	214
MV4-11 5E-04	55460	0	257381	50
MV4-11 1E-04	115488	0	645726	12
MV4-11 5E-05	97690	0	551780	7
MV4-11 1E-05	97000	0	493391	0

TECHNICS - NGS FOR MRD

Bio-informatica

Raw data ➔ results

1. Comparison to reference genome
2. Samtools sort: file sorting per chromosome
3. Variants calls
   - > Pindel: Flt3-ITD
   - > Samtools mpileup: NPM1
4. QC: raw reads / bam file



QC controle of NGS results Figure from C. Olsen

TECHNICS - NGS FOR MRD

NPM1 results

Samtool Mpileup:

NPM1 subtype A

→ TCTG insertion

→ Positie: 170837543

	A	B	C	D	E	F	G
1	chr	position	REF	ALT	DP	counts	frequency
2	chr5	170837487	A	GT	1000006	2	1,99999E-06
3	chr5	170837491	T	TG	1000010	2	1,99998E-06
4	chr5	170837505	C	CA	1000024	2	1,99995E-06
5	chr5	170837509	A	TT	1000026	7	6,99982E-06
6	chr5	170837513	C	TT	1000028	5713	0,00571284
7	chr5	170837515	T	TC	1000028	4	3,99989E-06
8	chr5	170837515	T	TG	1000028	3	2,99992E-06
9	chr5	170837517	T	TC	1000026	4	3,9999E-06
10	chr5	170837518	T	TC	1000025	2	1,99995E-06
11	chr5	170837519	T	TC	1000024	2	1,99995E-06
12	chr5	170837522	T	TC	1000015	4	3,99994E-06
13	chr5	170837525	T	TC	999376	4	4,0025E-06
14	chr5	170837526	T	TC	997049	79	7,92338E-05
15	chr5	170837530	G	GC	989569	2	2,02108E-06
16	chr5	170837536	T	TA	988420	7	7,08201E-06
17	chr5	170837541	A	AT	987553	20	2,02521E-05
18	chr5	170837542	T	TC	987498	20	2,02532E-05
19	chr5	170837543	C	TCTG	987397	56	5,67148E-05
20	chr5	170837546	T	TG	987267	22	2,22837E-05
21	chr5	170837549	C	GG	987137	8	8,10424E-06
22	chr5	170837550	A	TG	987079	5	5,06545E-06
23	chr5	170837552	T	GG	987025	6	6,07887E-06
24	chr5	170837557	G	GA	986987	6	6,07911E-06
25	chr5	170837558	A	GG	986977	3	3,03958E-06
26	chr5	170837559	A	GT	986966	2	2,02641E-06
27	chr5	170837560	G	AT	986947	2	2,02645E-06
28	chr5	170837561	T	TC	986954	19	1,92512E-05
29	chr5	170837563	T	TC	986960	3	3,03964E-06

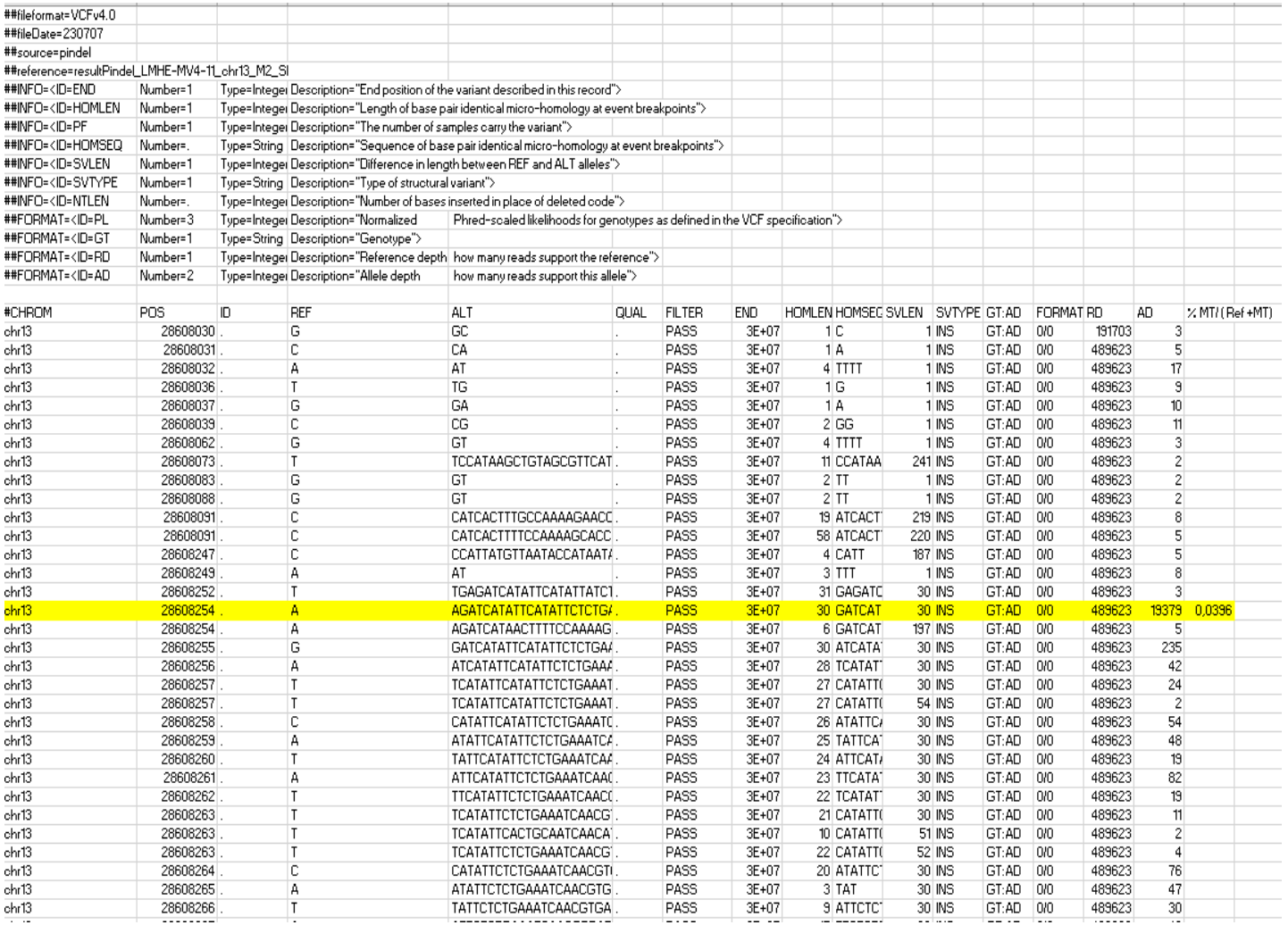
TECHNICS - NGS FOR MRD

FLT3-ITD results

Pindel:

FLT3-ITD

- Position and length
- Pos ctrl:
  - position chr13:28608254
  - Length fragment: 30bp



##fileformat=VCFv4.0																
##fileDate=230707																
##source=pindel																
##reference=resultPindel_LMHE-MV4-11_chr13_M2_SI																
##INFO=<ID=END	Number=1	Type=Integer	Description="End position of the variant described in this record">													
##INFO=<ID=HOMLEN	Number=1	Type=Integer	Description="Length of base pair identical micro-homology at event breakpoints">													
##INFO=<ID=PF	Number=1	Type=Integer	Description="The number of samples carry the variant">													
##INFO=<ID=HOMSEQ	Number=.	Type=String	Description="Sequence of base pair identical micro-homology at event breakpoints">													
##INFO=<ID=SVLEN	Number=1	Type=Integer	Description="Difference in length between REF and ALT alleles">													
##INFO=<ID=SVTYPE	Number=1	Type=String	Description="Type of structural variant">													
##INFO=<ID=NTLEN	Number=.	Type=Integer	Description="Number of bases inserted in place of deleted code">													
##FORMAT=<ID=PL	Number=3	Type=Integer	Description="Normalized	Phred-scaled likelihoods for genotypes as defined in the VCF specification">												
##FORMAT=<ID=GT	Number=1	Type=String	Description="Genotype">													
##FORMAT=<ID=RD	Number=1	Type=Integer	Description="Reference depth	how many reads support the reference">												
##FORMAT=<ID=AD	Number=2	Type=Integer	Description="Allele depth	how many reads support this allele">												
#CHROM	POS	ID	REF	ALT	QUAL	FILTER	END	HOMLEN	HOMSEC	SVLEN	SVTYPE	GT:AD	FORMAT	RD	AD	% MT/ (Ref +MT)
chr13	28608030	.	G	GC	.	PASS	3E+07	1	C	1	INS	GT:AD	0/0	191703	3	
chr13	28608031	.	C	CA	.	PASS	3E+07	1	A	1	INS	GT:AD	0/0	489623	5	
chr13	28608032	.	A	AT	.	PASS	3E+07	4	TTTT	1	INS	GT:AD	0/0	489623	17	
chr13	28608036	.	T	TG	.	PASS	3E+07	1	G	1	INS	GT:AD	0/0	489623	9	
chr13	28608037	.	G	GA	.	PASS	3E+07	1	A	1	INS	GT:AD	0/0	489623	10	
chr13	28608039	.	C	CG	.	PASS	3E+07	2	GG	1	INS	GT:AD	0/0	489623	11	
chr13	28608062	.	G	GT	.	PASS	3E+07	4	TTTT	1	INS	GT:AD	0/0	489623	3	
chr13	28608073	.	T	TCCATAAGCTGTAGCGTTCAT	.	PASS	3E+07	11	CCATAA	241	INS	GT:AD	0/0	489623	2	
chr13	28608083	.	G	GT	.	PASS	3E+07	2	TT	1	INS	GT:AD	0/0	489623	2	
chr13	28608088	.	G	GT	.	PASS	3E+07	2	TT	1	INS	GT:AD	0/0	489623	2	
chr13	28608091	.	C	CATCACTTTGCCAAAAGAACC	.	PASS	3E+07	19	ATCACT	219	INS	GT:AD	0/0	489623	8	
chr13	28608091	.	C	CATCACTTTTCCAAAAGCACC	.	PASS	3E+07	58	ATCACT	220	INS	GT:AD	0/0	489623	5	
chr13	28608247	.	C	CCATTATGTTAATACCATAAT	.	PASS	3E+07	4	CATT	187	INS	GT:AD	0/0	489623	5	
chr13	28608249	.	A	AT	.	PASS	3E+07	3	TTT	1	INS	GT:AD	0/0	489623	8	
chr13	28608252	.	T	TGAGATCATATTCATATTATCT	.	PASS	3E+07	31	GAGATC	30	INS	GT:AD	0/0	489623	3	
chr13	28608254	.	A	AGATCATATTCATATTCTCTGA	.	PASS	3E+07	30	GATCAT	30	INS	GT:AD	0/0	489623	19379	0,0396
chr13	28608254	.	A	AGATCATAACTTTTCCAAAAG	.	PASS	3E+07	6	GATCAT	197	INS	GT:AD	0/0	489623	5	
chr13	28608255	.	G	GATCATATTCATATTCTCTGAA	.	PASS	3E+07	30	ATCATA	30	INS	GT:AD	0/0	489623	235	
chr13	28608256	.	A	ATCATATTCATATTCTCTGAAA	.	PASS	3E+07	28	TCATAT	30	INS	GT:AD	0/0	489623	42	
chr13	28608257	.	T	TCATATTCATATTCTCTGAAAT	.	PASS	3E+07	27	CATATT	30	INS	GT:AD	0/0	489623	24	
chr13	28608257	.	T	TCATATTCATATTCTCTGAAAT	.	PASS	3E+07	27	CATATT	54	INS	GT:AD	0/0	489623	2	
chr13	28608258	.	C	CATATTCATATTCTCTGAAATC	.	PASS	3E+07	26	ATATTC	30	INS	GT:AD	0/0	489623	54	
chr13	28608259	.	A	ATATTCATATTCTCTGAAATCA	.	PASS	3E+07	25	TATTCA	30	INS	GT:AD	0/0	489623	48	
chr13	28608260	.	T	TATTCATATTCTCTGAAATCAA	.	PASS	3E+07	24	ATTCAT	30	INS	GT:AD	0/0	489623	19	
chr13	28608261	.	A	ATTCATATTCTCTGAAATCAAC	.	PASS	3E+07	23	TTCATA	30	INS	GT:AD	0/0	489623	82	
chr13	28608262	.	T	TTCATATTCTCTGAAATCAACG	.	PASS	3E+07	22	TCATAT	30	INS	GT:AD	0/0	489623	19	
chr13	28608263	.	T	TCATATTCTCTGAAATCAACGT	.	PASS	3E+07	21	CATATT	30	INS	GT:AD	0/0	489623	11	
chr13	28608263	.	T	TCATATTCACTGCAATCAACA	.	PASS	3E+07	10	CATATT	51	INS	GT:AD	0/0	489623	2	
chr13	28608263	.	T	TCATATTCTCTGAAATCAACG	.	PASS	3E+07	22	CATATT	52	INS	GT:AD	0/0	489623	4	
chr13	28608264	.	C	CATATTCTCTGAAATCAACGT	.	PASS	3E+07	20	ATATTC	30	INS	GT:AD	0/0	489623	76	
chr13	28608265	.	A	ATATTCTCTGAAATCAACGTG	.	PASS	3E+07	3	TAT	30	INS	GT:AD	0/0	489623	47	
chr13	28608266	.	T	TATTCTCTGAAATCAACGTGA	.	PASS	3E+07	9	ATTCTC	30	INS	GT:AD	0/0	489623	30	

TECHNICS - NGS FOR MRD

Calculation

Samtools (NPM1) and Pindel (Flt3-ITD):

$$\text{MRD (\%)} = \frac{\text{\# reads mutant}}{\text{\# reads WT+M}} * 100$$

sensitivity: sample dependent → # input DNA (cells) in PCR + Albumine

→ S = 2/tot .# cells

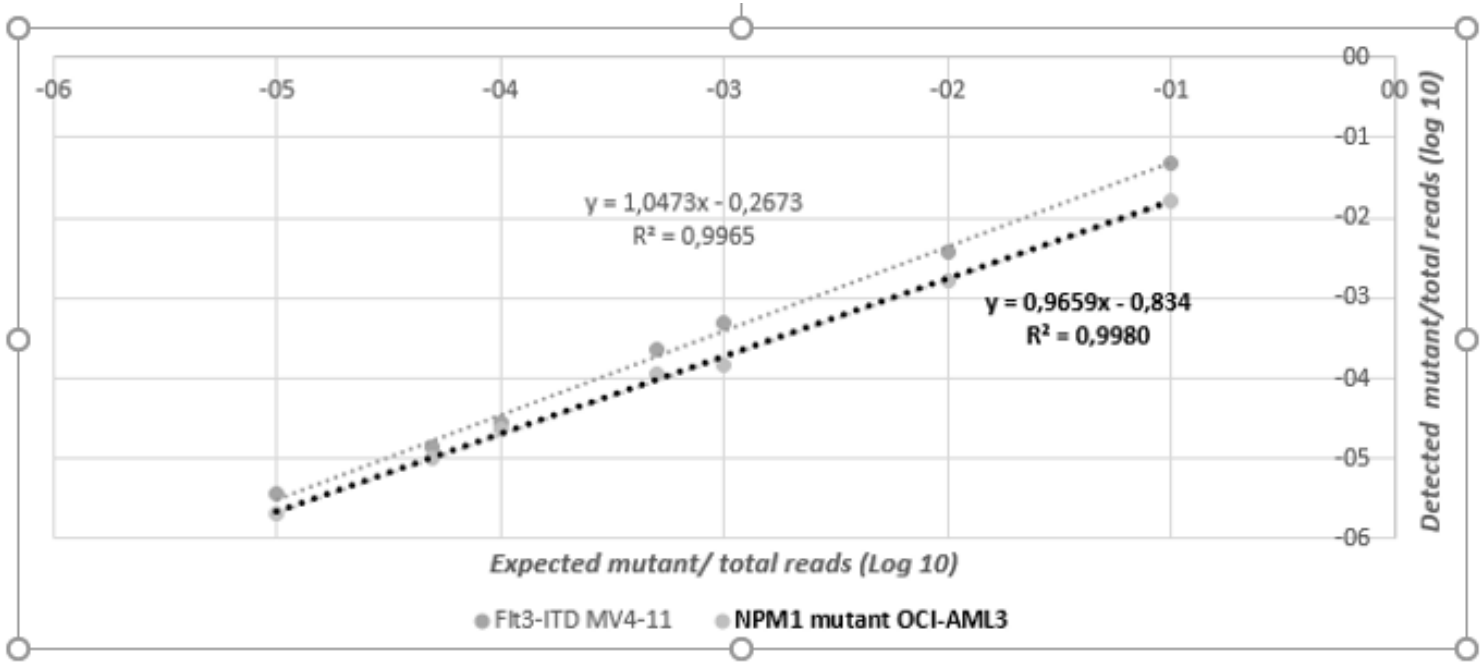
Albumine concentration correction → convertion in # cells (1 cell = 6pg DNA)

TECHNICS - NGS FOR MRD

validation: Linearity

Sensitivity: dilution series of positive cell line in negative controle (GL POOL):

MV4-11 / OCI-AML3 + GL POOL



Linearity of NGS-MRD assay for FLT3-ITD and NPM1 mut A:
Log conversion expected – detected MRD

TECHNICS - NGS FOR MRD

EQA

UK Neqas MRD for AML by Molecular Methods - 222302					
sample	**target**	**MRD_NGS total %**	**MRD (%)qNPM1**	**UK Neqas results (median %)**	**results NGS**
sample 046	NPM1	negative	negative	negative	negative
sample 047	NPM1	0,039	1,5	5,1	positive
sample 048	NPM1	0,012	0,3	1,1	positive
Edu A	FLT3	0,26	/	0,14	positive
Edu B	FLT3	0,033	/	0,028	positive
Edu C	FLT3	negative	/	negative	negative
GL POOL	NPM1	/	/	/	negative
	FLT3	/	/	/	negative

FUTURE

LMHE:

- Bio-informatics standardisation
- ddPCR for NPM1: cheaper and faster

ACKNOWLEDGEMENTS

Lab Molecular Hematology UZ Brussel
Kristel Vannerom
Myriam Ajbar
Leila Daif
Joeri Coignau
Kristien Vander Gucht
Emma Kabongo Kanjinga
Jona Van der Straeten
Marleen Bakkus
Eleni Linskens

Brightcore
Ben Caljon
Toon Janssen

BSPHO
AllTogether

Myeloom centrum Brussel
Ivan van Riet
Wouter De Brouwer
Robbe Heestermans

MMOVE
BHS Multiple Myeloma-werkgroep

Wetenschappelijk fonds Willy Gepts - UZ Brussel

VRAGEN?