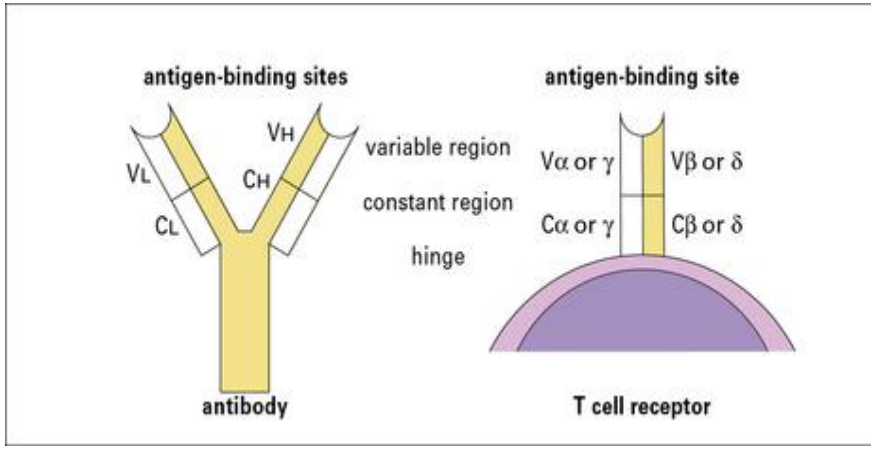
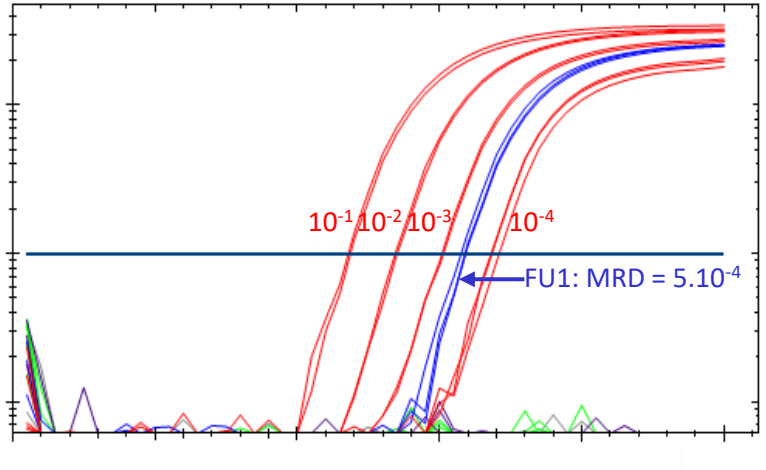
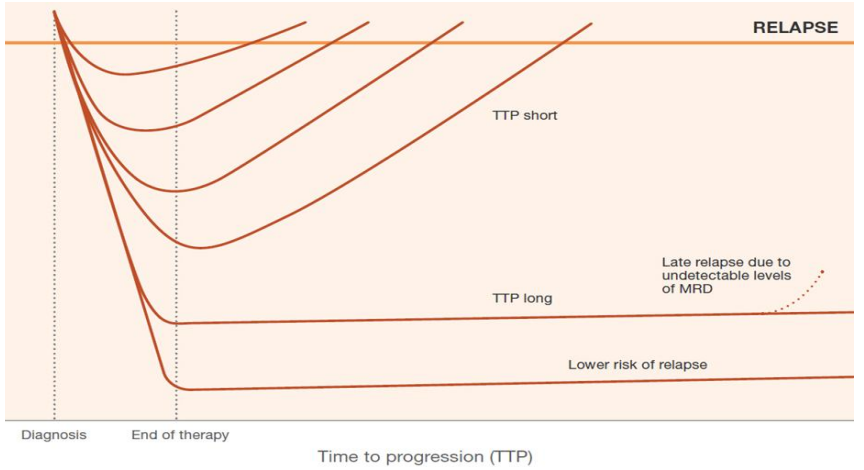






MOLECULAR MRD APPLICATIONS IN HEMATO-ONCOLOGY

Emmanuelle Kabongo Kanjinga, Jona Van der Straeten en Marleen Bakkus

Universitair Ziekenhuis Brussel

VUB

MINIMAL RESIDUAL DISEASE (MRD)

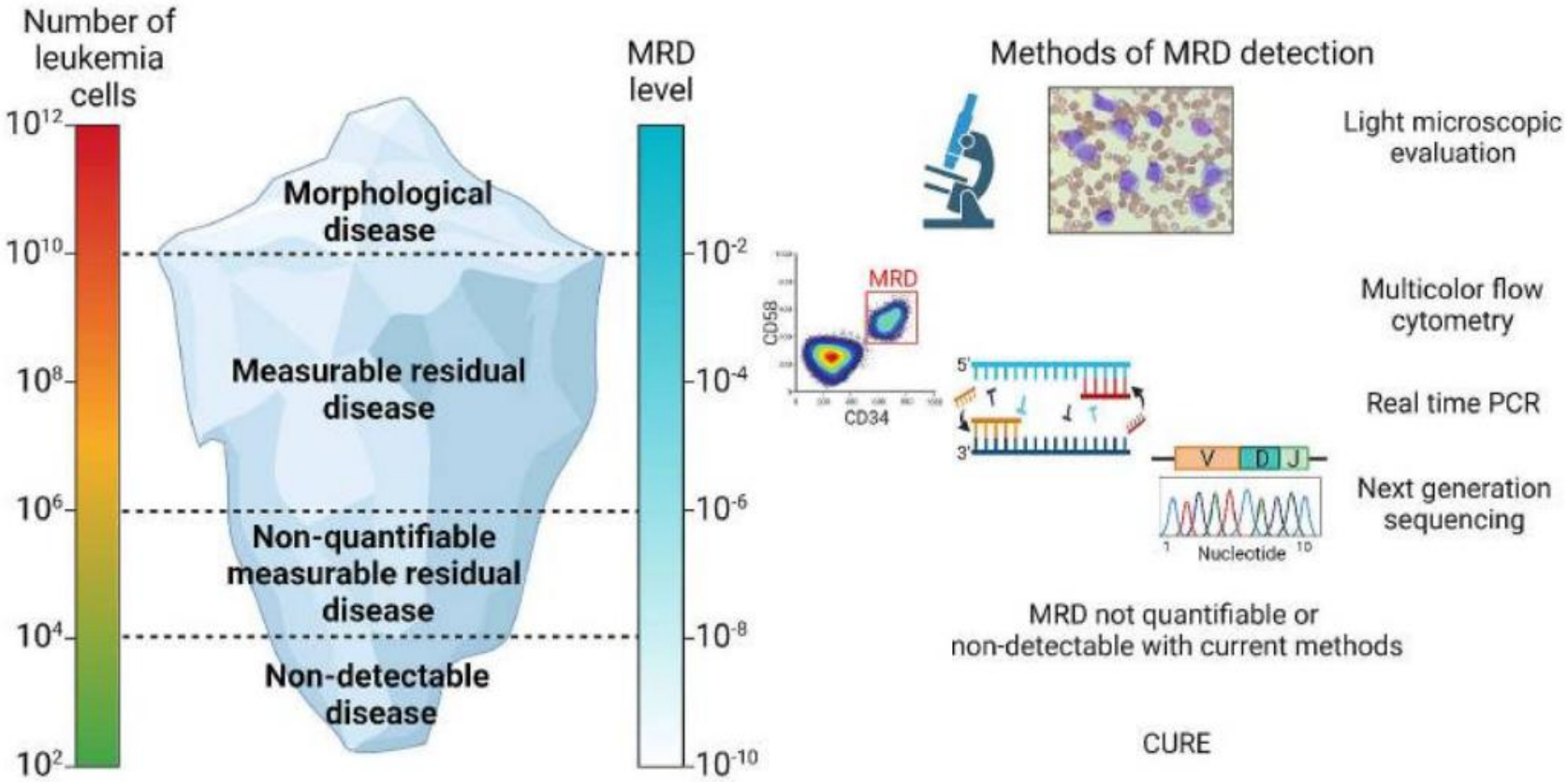
Frame

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

MRD

Minimal/Measurable residual disease

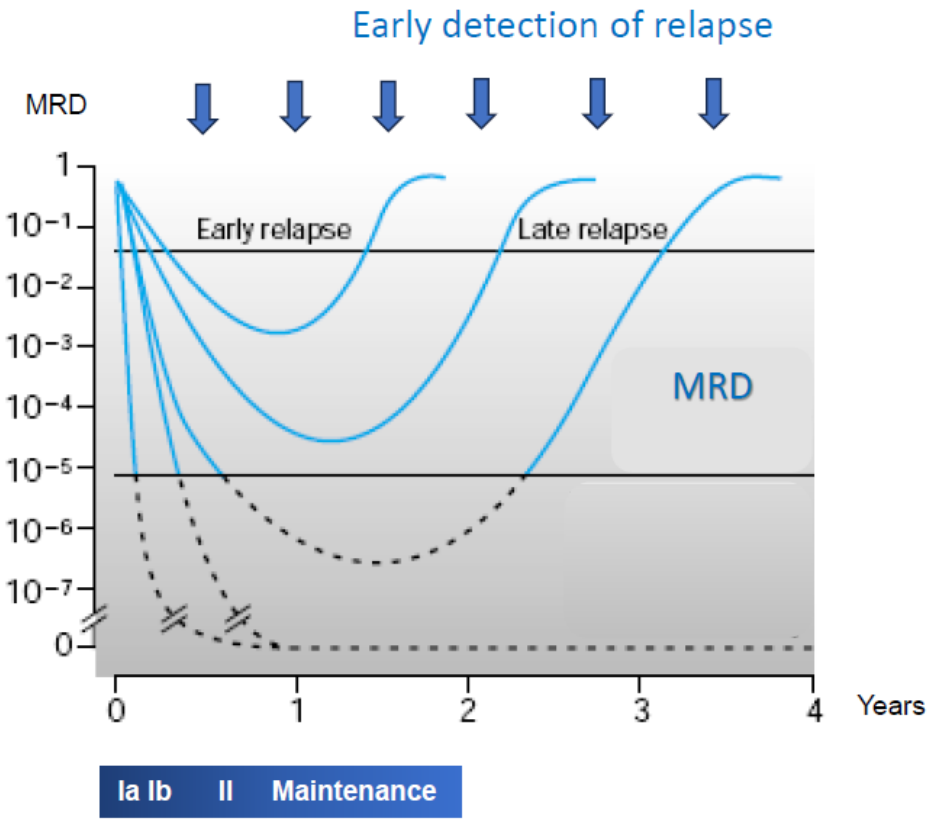
"Persistence of leukemic cells at levels below detection by conventional methods"



Saygin C. et al., Haematologica 2022

MRD DETECTION

Why is it important?



- 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?

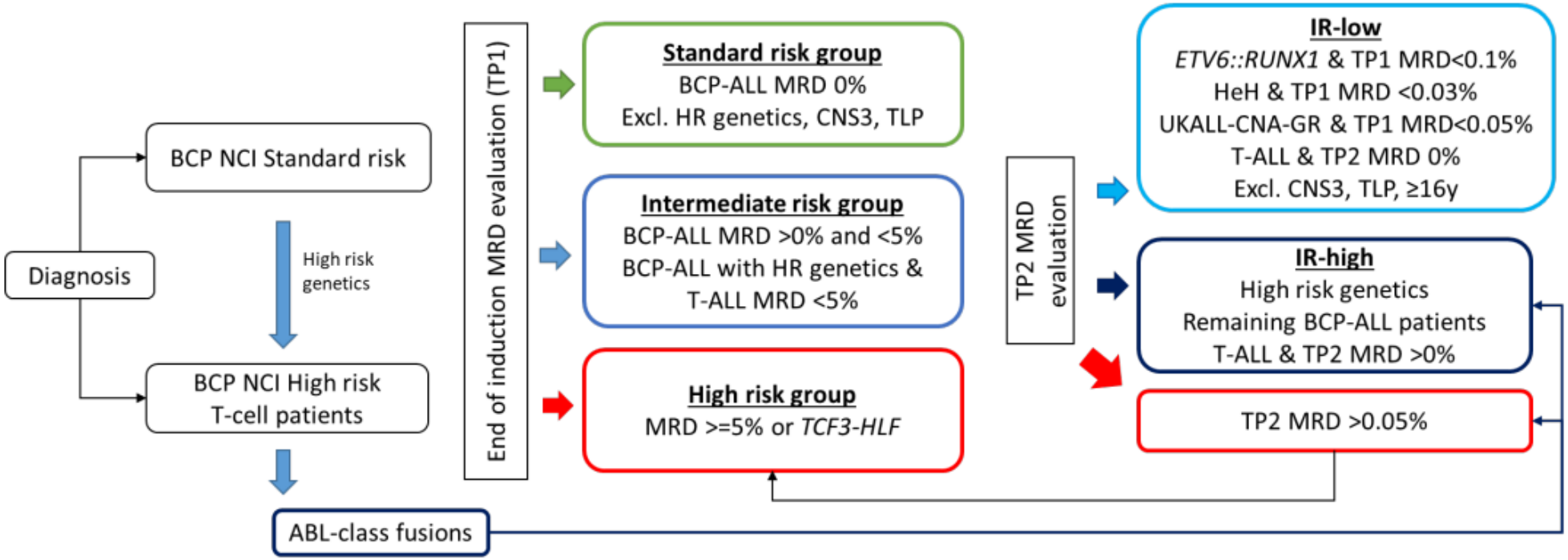
MOLECULAR TARGETS

- Genetic aberrations
  - Translocations
  - Mutations
  - Targets in AML, CML …
  - Used for MRD detection ->Emma

- Ig and TCR rearrangements
  - Applicable in all B- or T-cell derived neoplasia like ALL and MM
  - Used for MRD detection -> Jona

ALLTOGETHER PROTOCOL

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 and Down Syndrome patients have a separate algorithm – see protocol for full details



MRD 0%: undetectable MRD by IG/TCR PCR;
High risk genetics: *KMT2A/MLL* fusions, near haploidy, low hypodiploidy, iAMP21, primary *IG::MYC*
ABL-class: *ABL1, ABL2, PDGFRB, CSF1R* fusions

GR-CNA UKALL profile:
- no deletion of *IKZF1, CDKN2A/B, PAR1, BTG1, EBF1, PAX5, ETV6, RB1*;
- isolated deletions of *ETV6, PAX5, BTG1*;
- *ETV6* deletions with a single additional deletion of *BTG1, PAX5, CDKN2A/B.*

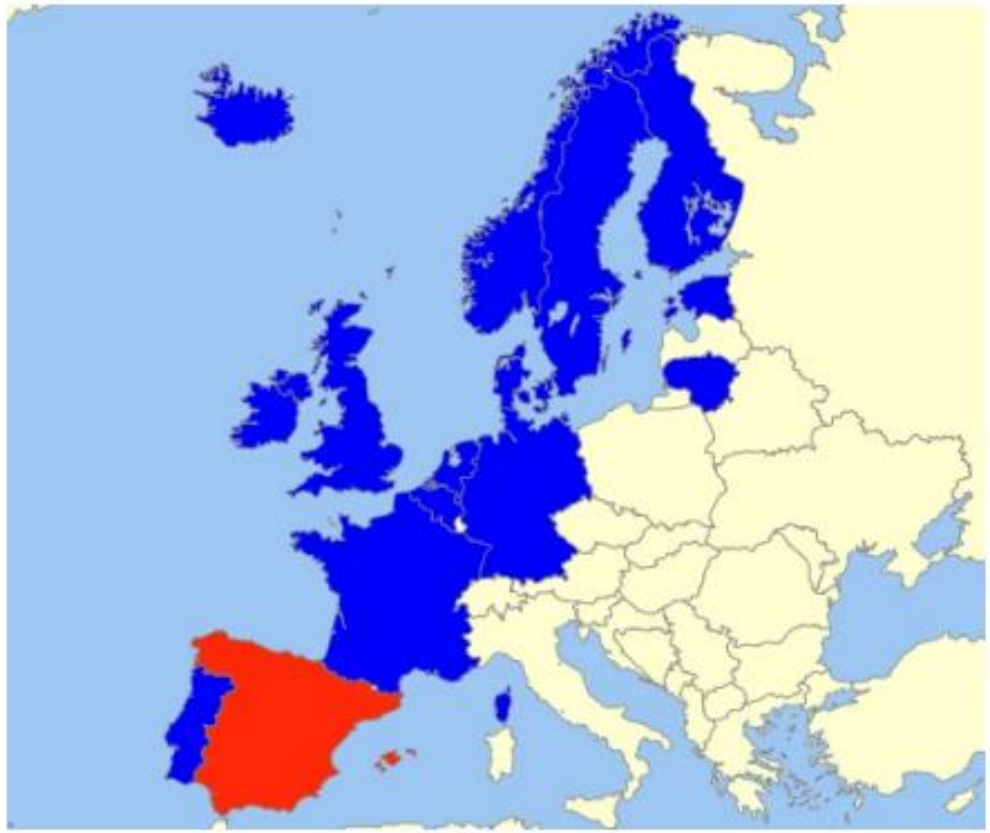
Lab guidelines Summary of the recommendations from the
ALLTogether Genetics group for the genetic screening of patients treated on the ALLTogether1 Protocol
Version 4.0 23 MAR 2023

ALLTOGETHER STUDY

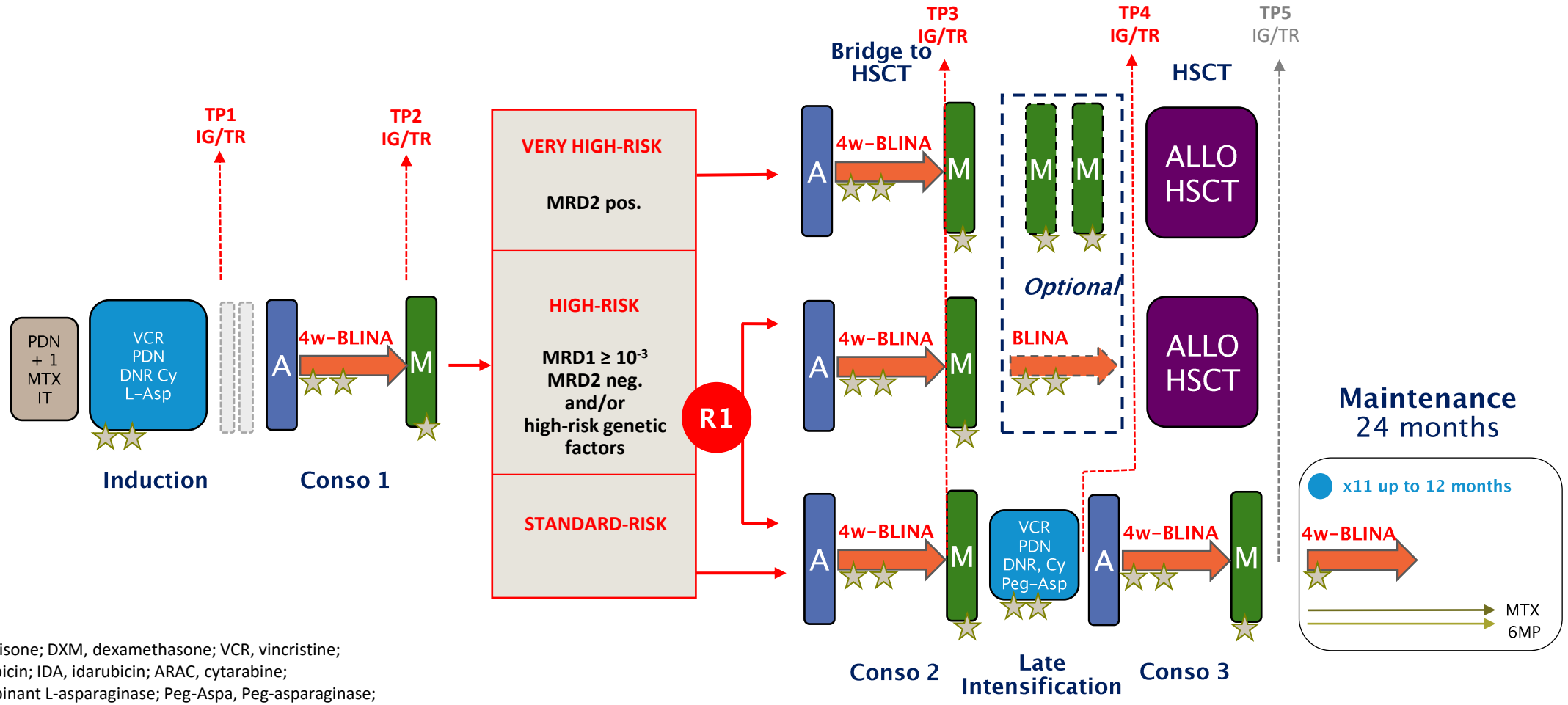
First patient in the study
13th of July 2020; recruited in The Netherlands.

Active countries: **14**
Total number of recruited eligible # patients as of 25 June 2025: **3800**

		Infants (<1 year)	1-9 years	10-15 years	16-24 years	25-45 years	
Country	Sweden	2	277	60	48	31	418
	Denmark	0	140	32	26	16	214
	Norway	1	122	34	20	21	198
	Finland	0	127	35	24	18	204
	Iceland	0	5	1	0	0	6
	Estonia	0	15	2	0	2	19
	Lithuania	0	37	8	4	0	49
	Germany	0	172	35	10	0	217
	Netherlands	1	335	105	36	0	477
	Belgium	6	197	55	14	0	272
	UK	11	640	188	59	9	907
	France	5	491	176	43	0	715
	Portugal	0	42	14	3	0	59
	Ireland	0	34	11	0	0	45
Total		26	2634	756	287	97	**3800**



GRAALL-2024 Ph- B-ALL

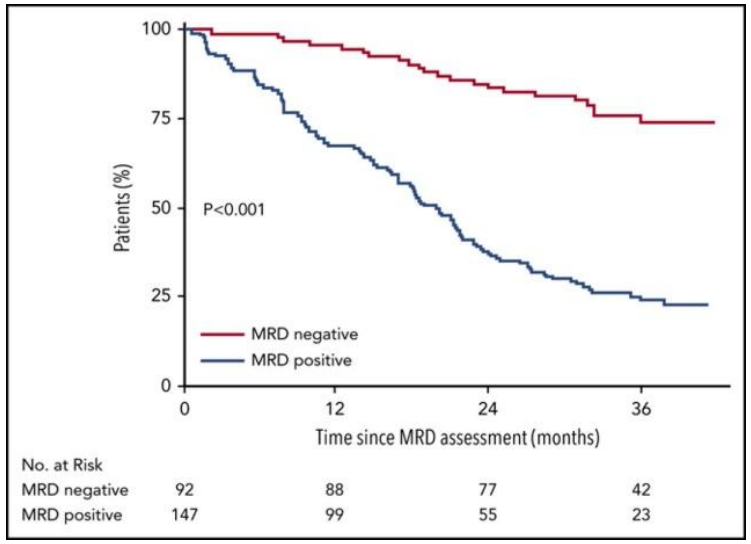


PDN, PO prednisone; DXM, dexamethasone; VCR, vincristine;
DNR, daunorubicin; IDA, idarubicin; ARAC, cytarabine;
L-Aspa, recombinant L-asparaginase; Peg-Aspa, Peg-asparaginase;
MTX, methotrexate; Cy, cyclophosphamide;
6MP, 6-mercaptopurine;
IT, intrathecal; HD, high-dose
triple IT, MTX/ARAC/steroids

VP16, ARAC, 6MP (optional, 1 or 2 cycles)
A HD-ARAC, DXM
M HD-MTX, VCR, 1 triple IT
VCR/PDN reinduction

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

EHA–EMN EVIDENCE–BASED GUIDELINES FOR MRD IN MM

NGF or NGS: sensitivity at least 10–5

Table 1 | Recommendations on examinations at diagnosis, at response assessment, during follow-up and at relapse of MM

Tool	Diagnosis	At response	At follow-up	At relapse
Blood tests				
Blood count and blood smear	Obl	Obl	Obl	Obl
Serum electrophoresis and immunofixation	Obl	Obl	Obl	Obl
Serum free light chain	Obl	Obl to confirm sCR	Obl	Obl
Serum immunoglobulin levels	Obl	Obl	Obl	Obl
Renal and liver function tests	Obl	Obl	Obl	Obl
Calcium	Obl	Obl	Obl	Obl
Lactate dehydrogenase	Obl	Obl	Obl	Obl
Albumin, β_2 microglobulin	Obl	NR	Opt	Obl
Flow cytometry	Opt	NR	NR	Opt
Urine tests				
Urine sample from 24-h urine collection to check for proteinuria and serum free light chain proteinuria	Obl	NR	NR	Obl
Urine electrophoresis and immunofixation	Obl	Obl	NR	Obl
Bone marrow assessments				
Bone marrow cytology and biopsy to confirm plasmacytosis and monoclonality	Obl	Obl to confirm CR or for non-secretory MM	NR	Opt (obl for non-secretory MM)
NGF or NGS to detect clonal plasma cells	Obl	Obl to confirm MRD negativity in patients with CR or sCR	Every 12 months in MRD-negative patients	Opt
Cytogenetics, karyotype and FISH for detection of del17p, t(4;14), t(14;16), t (14;20), 1q gain or amplification, del1p32 and t(11;14), and NGS for *TP53* mutations	Obl	NR	NR	Obl in patients with del17p, del1p32, 1q gain or amplification and *TP53* mutations
Advanced techniques: GEP, NGS	Only in clinical trials	Only in clinical trials	Only in clinical trials	Only in clinical trials
Imaging				
PET–CT or DWI MRI	Obl	Obl to confirm imaging MRD	Every 12 months in MRD-negative patients	Obl (also for detection of paramedullary or extramedullary disease)

At response

confirm MRD negativity in patients with CR or sCR

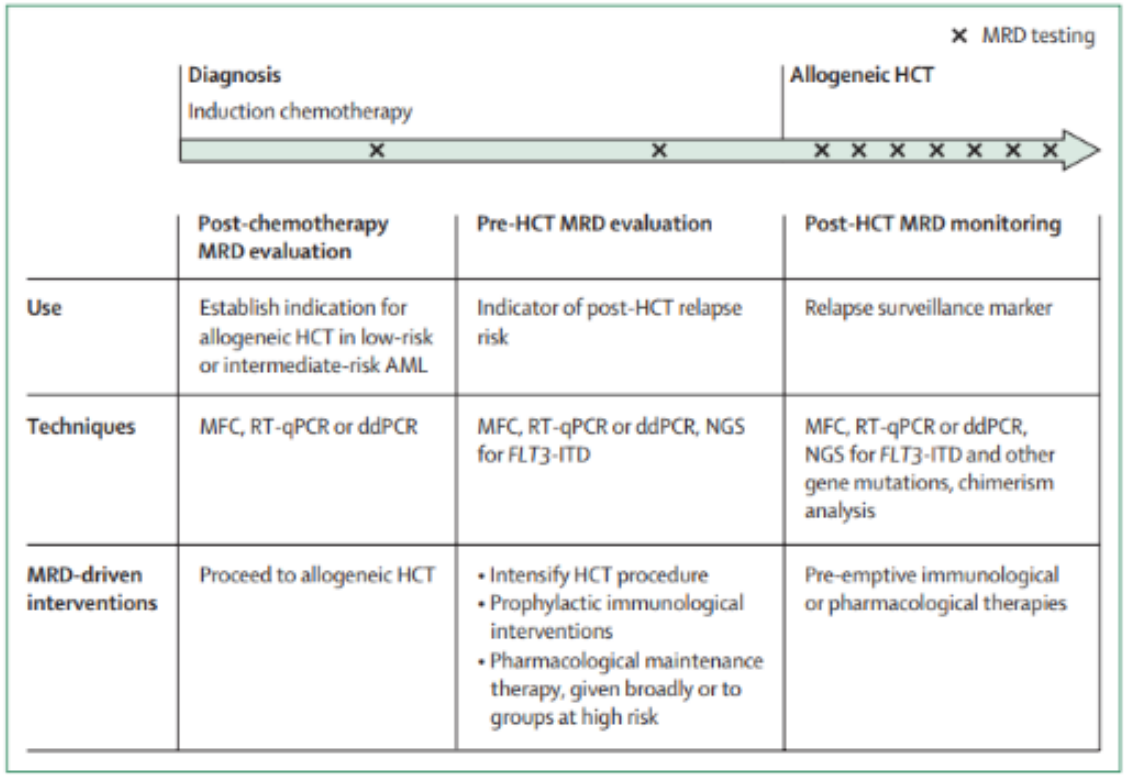
At follow-up

Every 12 months in MRD-negative patients

Nature reviews Clinical Oncology 2025 Dimopoulos et al.

EBMT GUIDELINES ON MRD IN AML TRANSPLANT SETTING

How to assess MRD in AML?



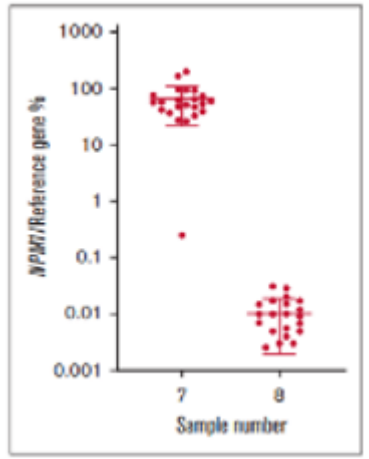
	Post-chemotherapy MRD evaluation	Pre-HCT MRD evaluation	Post-HCT MRD monitoring
Use	Establish indication for allogeneic HCT in low-risk or intermediate-risk AML	Indicator of post-HCT relapse risk	Relapse surveillance marker
Techniques	MFC, RT-qPCR or ddPCR	MFC, RT-qPCR or ddPCR, NGS for *FLT3*-ITD	MFC, RT-qPCR or ddPCR, NGS for *FLT3*-ITD and other gene mutations, chimerism analysis
MRD-driven interventions	Proceed to allogeneic HCT	• Intensify HCT procedure • Prophylactic immunological interventions • Pharmacological maintenance therapy, given broadly or to groups at high risk	Pre-emptive immunological or pharmacological therapies

Lancet oncol. 2025 Sanz et al.

UK NEQAS
Leucocyte Immunophenotyping

Measurable Residual Disease for AML by Molecular Methods (Pilot - Not Accredited)

- NPM1mut
- t(8:21) *RUNX1::RUNX1T1*
- inv(16) *CBFB::MYH11*
- t(15;17) *PML::RARA*
- FLT3-ITD NGS-MRD (6 educational samples)



Scott et al, 2023 Blood advances

CR MRD definitions

Table 5. Definitions for MRD response categories and MRD relapse ELN 2021 ELN 2025

Response category	Abbreviation	Defining criteria
CR with negative MRD	$CR_{MRD}-$	1. Complete morphologic remission and 2. MRD^- in all MRD technologies that were used: a. $FC\text{-}MRD^-$ in BM (if MFC-MRD was used). b. $qPCR\text{-}MRD^-$ in BM (or in PB after cycle 2 for *NPM1*- and CBF-MRD) (if qPCR-MRD was used). c. $NGS\text{-}MRD^-$ in BM (if NGS-MRD was used).
CR with positive MRD	$CR_{MRD}+$	1. Complete morphologic remission, and 2. $MFC\text{-}MRD^+$ in PB and/or BM, or 3. $NGS\text{-}MRD^+$ in PB and/or BM, or 4. $qPCR\text{-}MRD^+$ in PB and/or BM.
CR with molecular MRD detection at low level	CR-MRD-LL	1. Morphologic CR, and 2. Molecular MRD detectable at low level in PB and/or BM (ie, qPCR for *NPM1* ~~<2%~~ or NGS-MRD <0.1%, but above the detection limit of the assay).
MRD relapse	—	1. Conversion of MRD negativity to MRD positivity independent of the MRD technique, or 2. increase in MRD copy numbers ≥1 log_{10} between any 2 positive samples in patients with CR-MRD-LL who are monitored by qPCR. 3. The result of (1) or (2) should be rapidly confirmed in a second consecutive sample, preferably from the BM.

<0.01% in BM, <0.1% in PB

Table 4. ELN 2021 clinical MRD recommendations based on a Delphi poll

D4	NGS-MRD monitoring is useful to refine prognosis in addition to MFC but, to date, there are insufficient data to recommend NGS-MRD as a stand-alone technique	→ FLT3-ITD NGS-MRD

Cloos et al. Blood 2025; *Heuser et al. Blood 2021*

Universitair Ziekenhuis Brussel

VUB

Molecular MRD applications in hemato-oncology: IG/TCR-based MRD

Universitair Ziekenhuis Brussel

VUB

IG/TCR-BASED MRD: OVERVIEW

Techniques and principles

- **Real-time PCR = ASO-PCR**
- **NGS**
- **ASO-PCR versus NGS**

IG/TCR-BASED MRD: TECHNIQUES

1. Real-time PCR:

= ASO-PCR = **a**llele **s**pecific **o**ligonucleotide

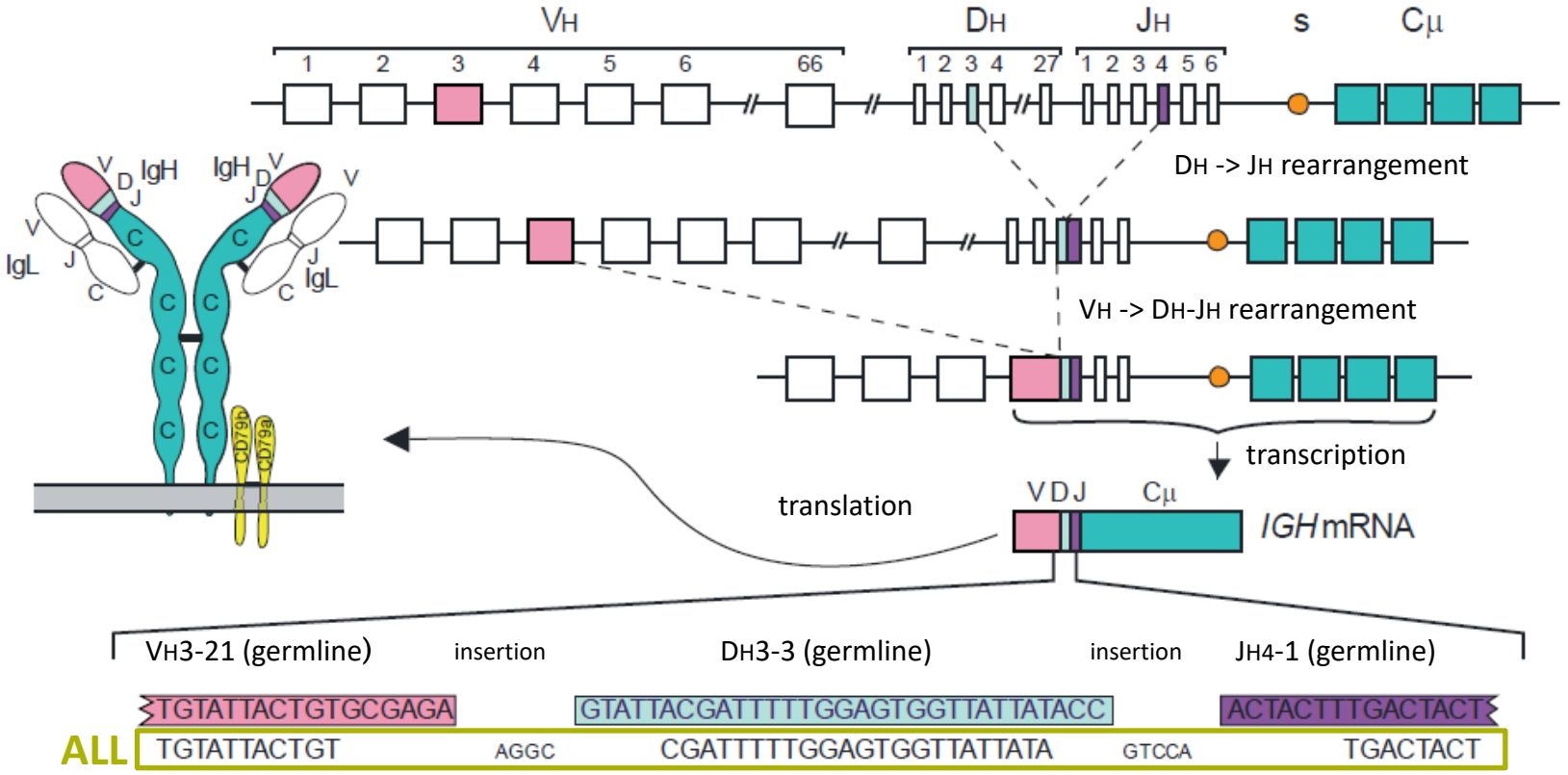
- patient-specific assay → sensitivity 1E-04 – 1E-05
- TAT 1 day
- highly standardized protocol – EuroMRD - accredited
- ALL

2. NGS-MRD:

- sensitivity 1E-05 – 5E-06
- TAT 2 weeks
- implemented and accredited in Belgium – not the standard procedure
- ALL, MM

IG/TCR-BASED MRD: TECHNIQUES

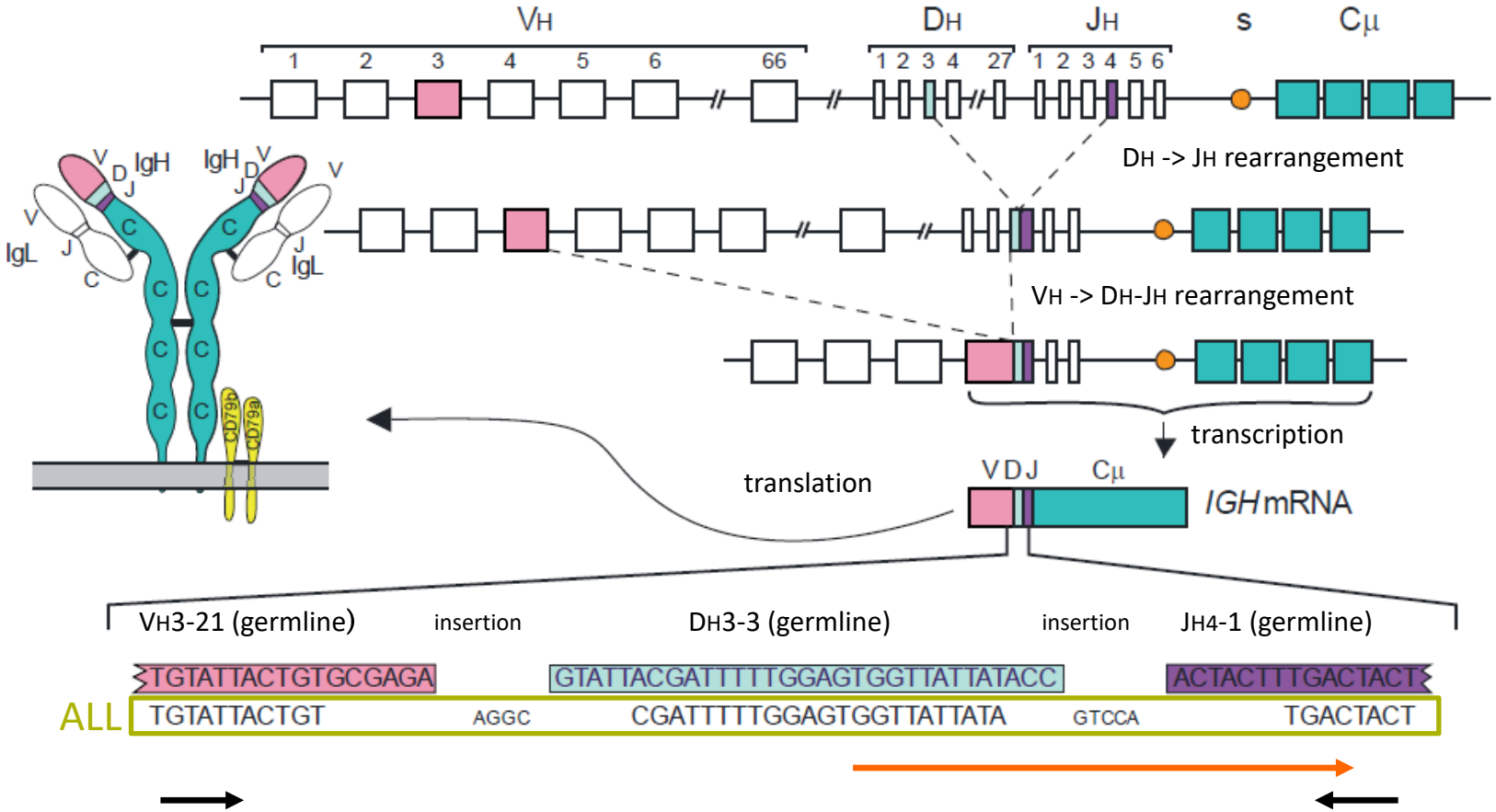
V-D-J recombination



1. **combination of V-D-J**
2. **deletions**
3. **insertions**

IG/TCR-BASED MRD: TECHNIQUES

V-D-J recombination

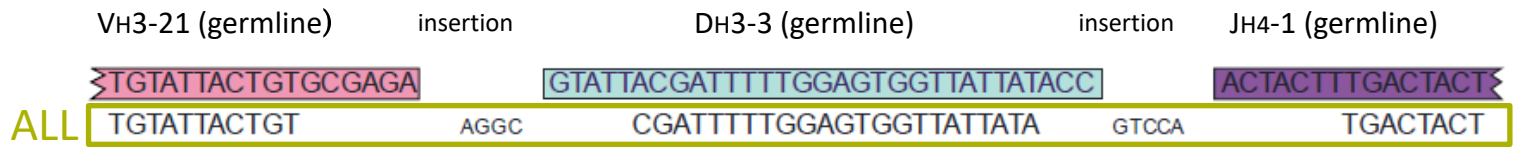


MRD

1. ASO-PCR - **patient specific** primer
2. NGS - **consensus** primer

ASO-PCR: DESIGN

1. **Detection of monoclonal rearrangement(s) – junctional region: PCR-based NGS**



1 run/week:
PCR:
Tuesday/Wednesday **Week 1**
NGS:
over weekend

2. **ASO-design**

Primers recieved by Friday **Week 2**

max. 8 primers
MRD-PCR: 2 primers

3. **ASO-testing**

   A. Real-time PCR - estimation of the quality of the assay (60°C and 5 mM $MgCl_2$)
   B. Temperature gradient
   C. $MgCl_2$ gradient

1-3 days **Week 3**

4. **MRD quantification**

1 day

TAT 1 day ⇔ labour-intensive design

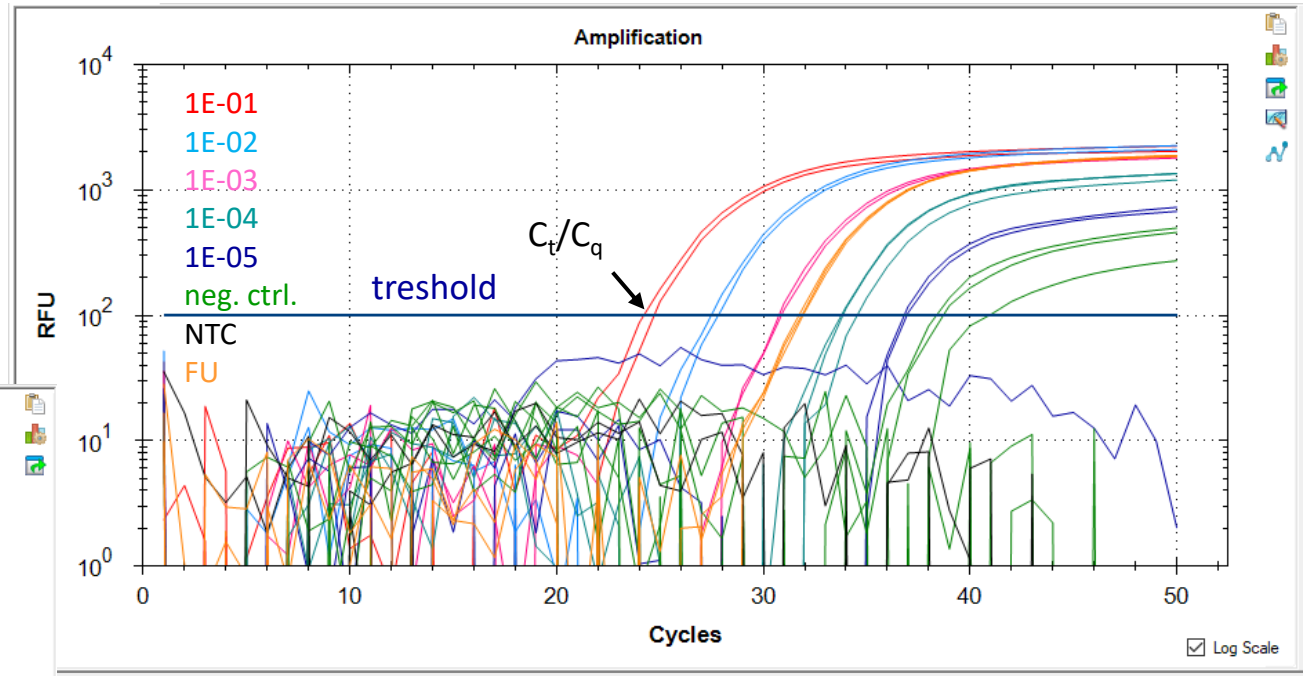
ASO-PCR: SET-UP

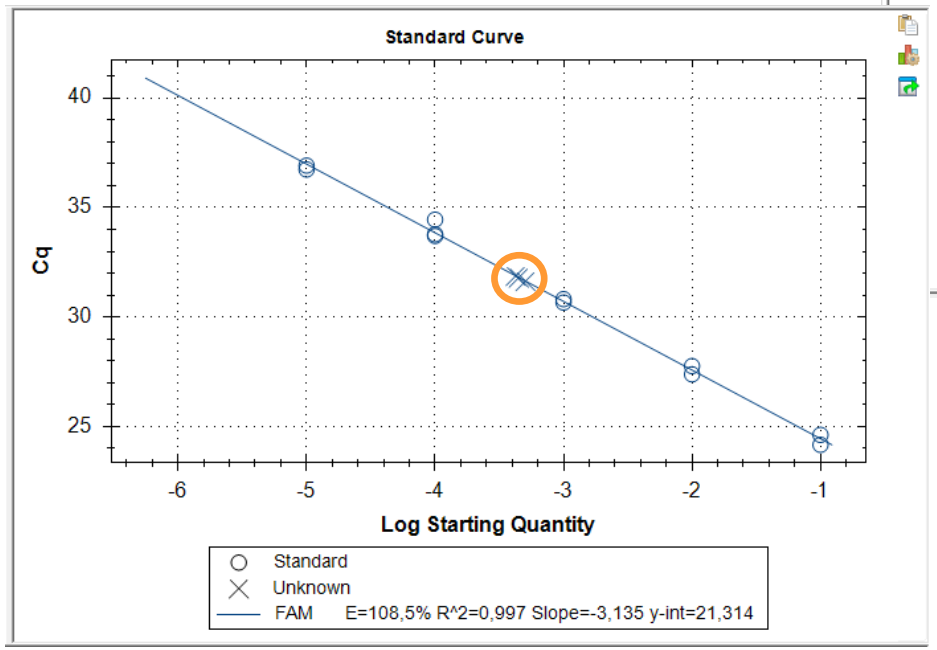
dilution series of diagnostic sample
1E-01 - 1E-05

negative control = GL Pool

no template control (NTC)

follow-up





C_t=cycling treshold = C_q=quantification cycle

C_t FU:
repl. 1: 31.9
repl. 2: 31.8
repl. 3: 31.6
→ MRD FU = 5E-04

ASO-PCR – DATA INTERPRETATION

- **EuroMRD guidelines - 2007**

Leukemia (2007) 21, 604–611

www.nature.com/leu

LEADING ARTICLE

Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data

VHJ van der Velden[1], G Cazzaniga[2], A Schrauder[3], J Hancock[4], P Bader[5], ER Panzer-Grumayer[6], T Flohr[7], R Sutton[8], H Cave[9], HO Madsen[10], JM Cayuela[11], J Trka[12], C Eckert[13], L Foroni[14], U zur Stadt[15], K Beldjord[16], T Raff[17], CE van der Schoot[18] and JJM van Dongen[1], on behalf of the European Study Group on MRD detection in ALL (ESG-MRD-ALL)

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- **Updated EuroMRD guidelines - 2024**

Leukemia www.nature.com/leu

ARTICLE OPEN

Check for updates

MINIMAL RESIDUAL DISEASE

Analysis of measurable residual disease by IG/TR gene rearrangements: quality assurance and updated EuroMRD guidelines

Vincent H. J. van der Velden[1,21] ✉, Isabel Dombrink[2,21], Julia Alten[3], Giovanni Cazzaniga[4,5], Emmanuelle Clappier[6,7], Daniela Drandi[8], Cornelia Eckert[9,10], Eva Fronkova[11], Jeremy Hancock[12], Michaela Kotrova[2], Rebekka Kraemer[2], Mirkka Montonen[13], Heike Pfeifer[14], Christiane Pott[2], Thorsten Raff[2,20], Heiko Trautmann[2], Hélène Cavé[6,15], Beat W. Schäfer[16], Jacques J. M. van Dongen[17,18,19], Jan Trka[11], Monika Brüggemann[2] and EuroMRD Consortium*

Leukemia www.nature.com/leu

LETTER OPEN

Check for updates

MINIMAL RESIDUAL DISEASE

The gray area of RQ-PCR-based measurable residual disease: subdividing the "positive, below quantitative range" category

Michaela Kotrova[1], Eva Fronkova[2], Michael Svaton[2,3,4], Daniela Drandi[5], Felix Schön[1], Patricia Hoogeveen[6], Jeremy Hancock[7], Aneta Skotnicova[2], Anke Schilhabel[1], Cornelia Eckert[8,9], Emmanuelle Clappier[10], Gianni Cazzaniga[11], Beat W. Schäfer[12], Jacques J. M. van Dongen[13], Matthias Ritgen[1], Christiane Pott[1], Vincent H. J. van der Velden[6], Jan Trka[2] and Monika Brüggemann[1] ✉

ASO-PCR – DATA INTERPRETATION

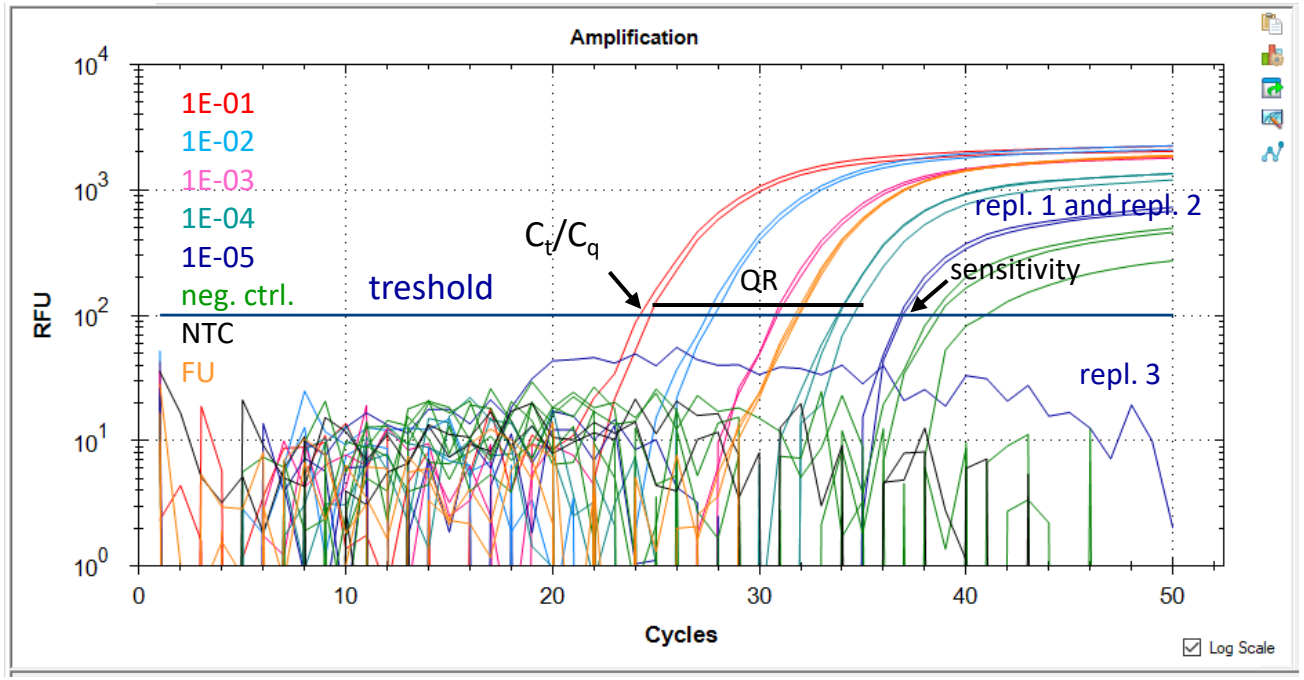
updated EuroMRD guidelines (2024)

-	+	???
MRD negative report: negative	MRD positive AND quantifiable report: MRD value	MRD low positive, <QR (MRD value) report: MRD low positive <1E-04 (4E-05)
		MRD of uncertain significance report: MRD of uncertain significance

ASO-PCR – DATA INTERPRETATION

Quantitative range (QR) and sensitivity

sensitivity. The 'quantitative range' reflects the part of the standard curve in which the MRD levels can be quantified reproducibly and accurately, whereas the 'sensitivity' reflects the lowest MRD level that still can be detected, although not reproducibly and accurately (Figure 1).

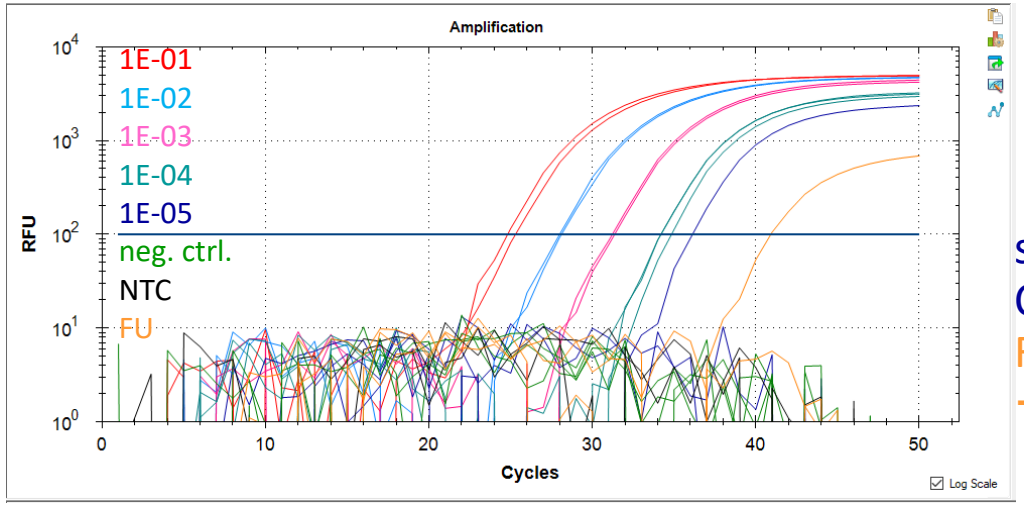


QR = 1E-04
sensitivity = 1E-05

ideal scenario: 2 targets QR = 1E-04
1 target QR 1E-04 and 2nd target QR 5E-04 and sensitivity 1E-04

ASO-PCR – DATA INTERPRETATION

MRD negative:



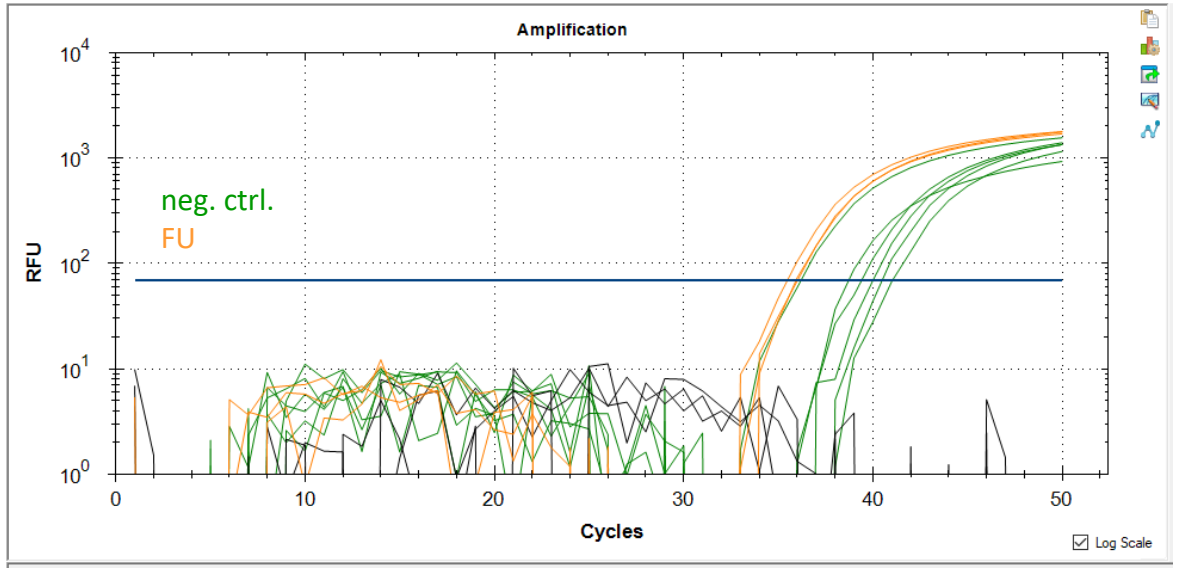
sens. 1E-05
C_t = 36.1
FU – C_t = 40.9
→ MRD neg.

- no amplification of the FU

OR

- all C_t values of the FU are more than 4 C_t above the highest C_t value of the sensitivity

- the lowest C_t value of the FU is whitin 1 C_t from the lowest C_t of the background



lowest C_t background = 36.2
lowest C_t FU = 35.4 → MRD neg.

ASO-PCR – DATA INTERPRETATION

MRD positive:

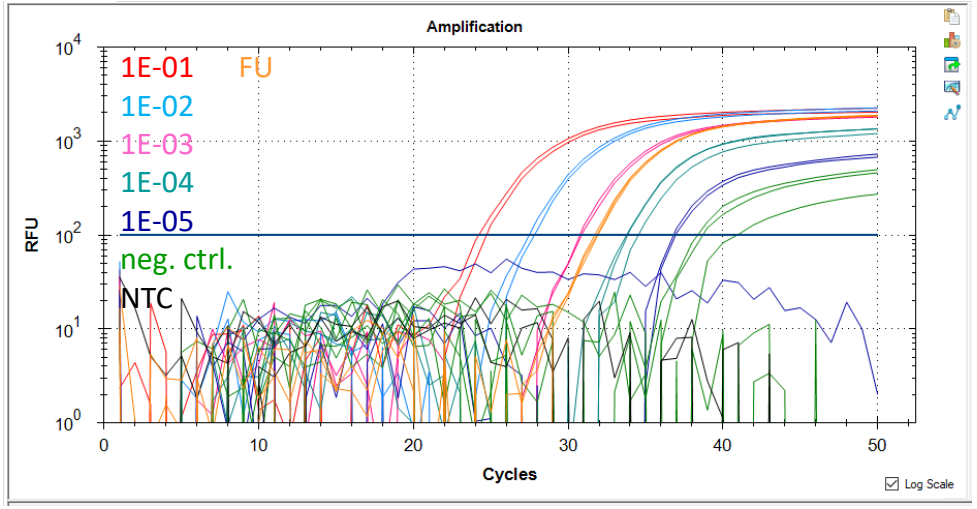
A sample is considered to be MRD positive if

- the C_T value of at least one of the three replicates is $\geqslant$1.0 C_T lower than the *lowest* C_T of background and
- the C_T value of at least one of the three replicates is within 4.0 C_T from the *highest* C_T value of the 'sensitivity' (fulfilling all 'sensitivity' criteria).

MRD positive AND quantifiable:

MRD-positive samples can be quantified if

- the *mean* C_T value of the replicates is lesser than or equal to the *highest* C_T value of the 'quantitative range' and
- the ΔC_T of the replicates is $\leqslant$1.5.



QR = 1E-04 with highest Ct value = 34.5

C_t FU:

repl. 1: 31.9

repl. 2: 31.8 mean C_t = 31.8

repl. 3: 31.6

→ **MRD positive AND quantifiable**

→ MRD = 5E-04

ASO-PCR – DATA INTERPRETATION

MRD positive:

A sample is considered to be MRD positive if

- the C_T value of at least one of the three replicates is $\geqslant$1.0 C_T lower than the *lowest* C_T of background and
- the C_T value of at least one of the three replicates is within 4.0 C_T from the *highest* C_T value of the 'sensitivity' (fulfilling all 'sensitivity' criteria).

MRD positive AND quantifiable:

MRD-positive samples can be quantified if

- the *mean* C_T value of the replicates is lesser than or equal to the *highest* C_T value of the 'quantitative range' and
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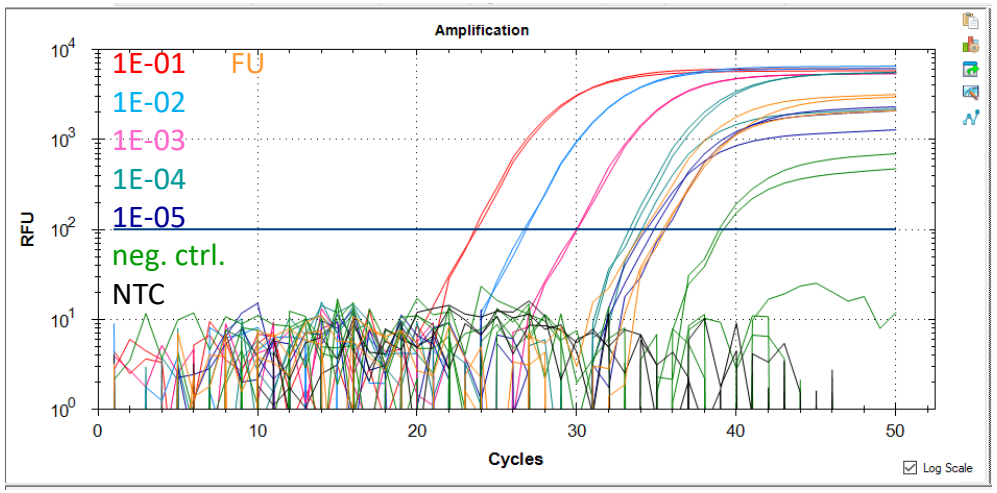


QR = 1E-04 with highest Ct value = 34.0

C_t FU:

repl. 1: 34.2

repl. 2: 35.4 mean C_t = 35.0

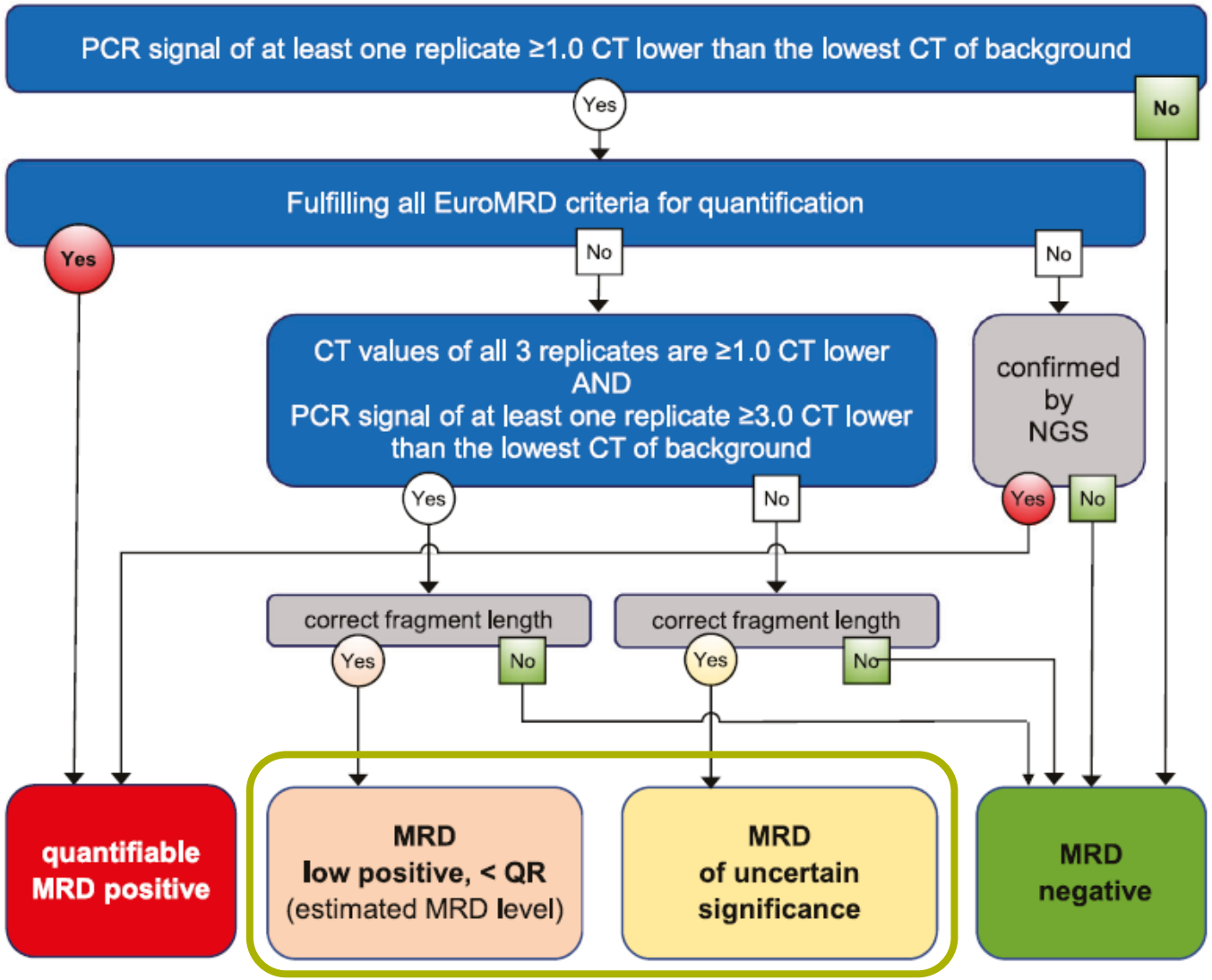
repl. 3: 35.3

→ **MRD positive NOT quantifiable**

→ MRD positive <1E-04

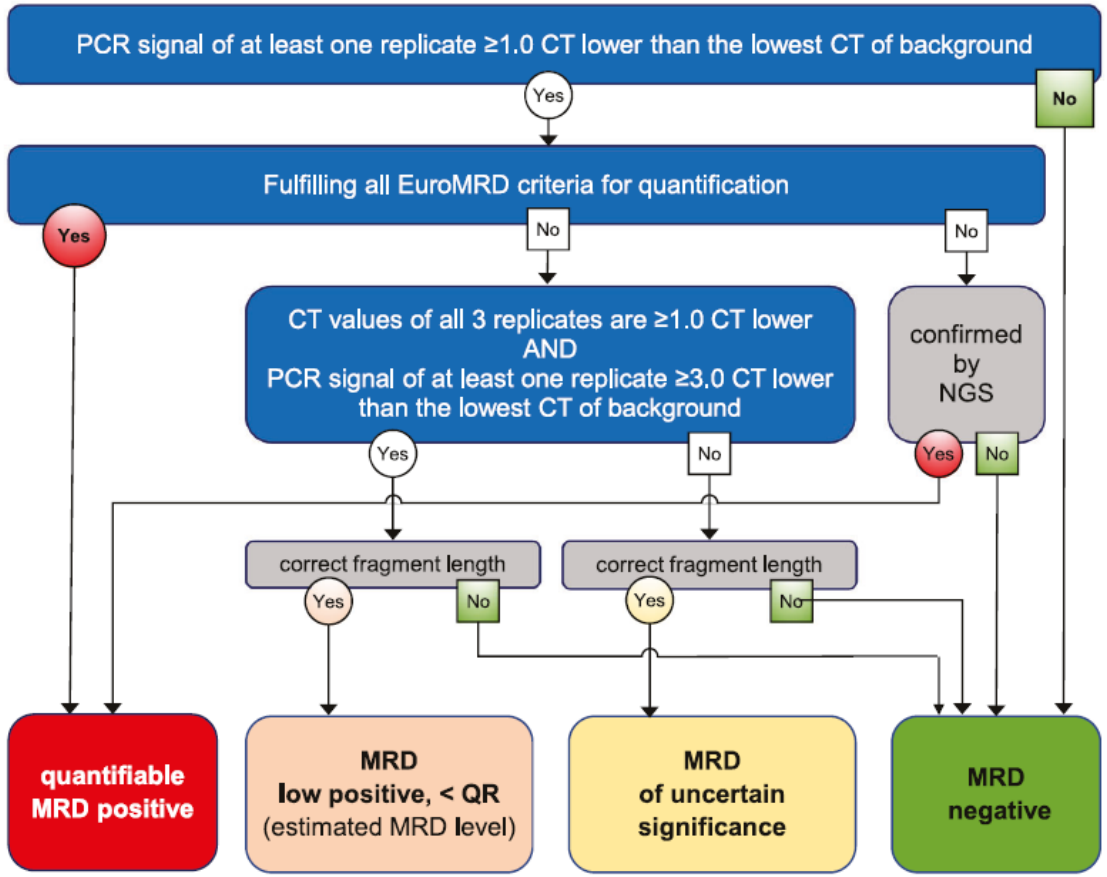
ASO-PCR – DATA INTERPRETATION

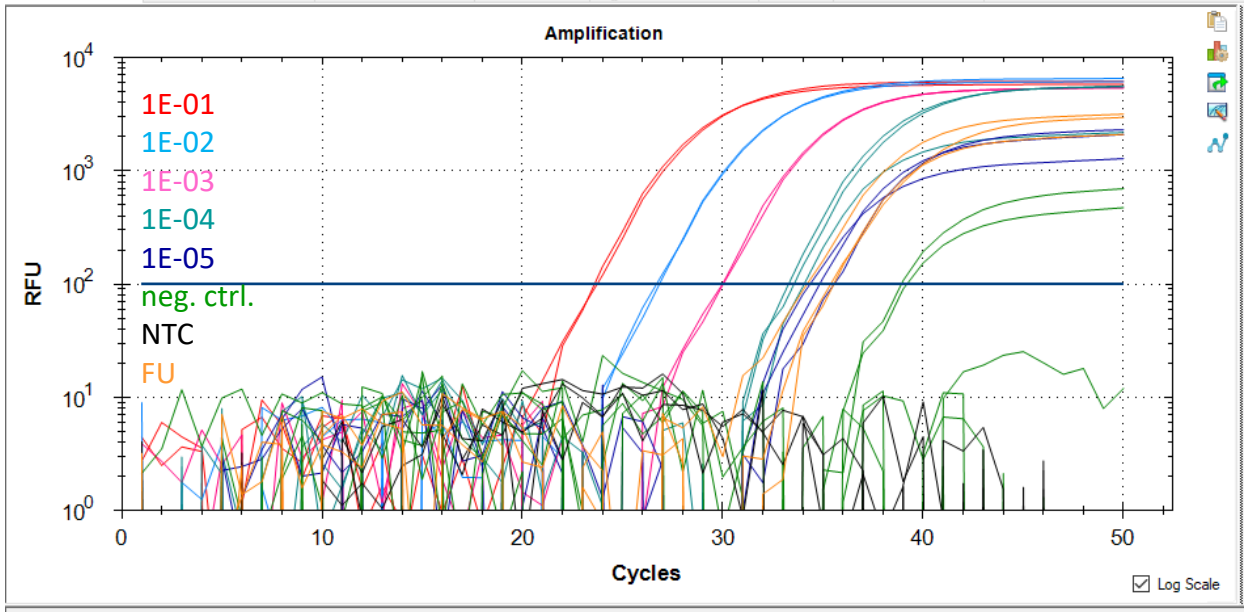
updated EuroMRD guidelines (2024)



ASO-PCR – DATA INTERPRETATION

updated EuroMRD guidelines (2024)





lowest C_t neg. ctrl.: 38.9

QR = 1E-04 with highest Ct value = 34.0

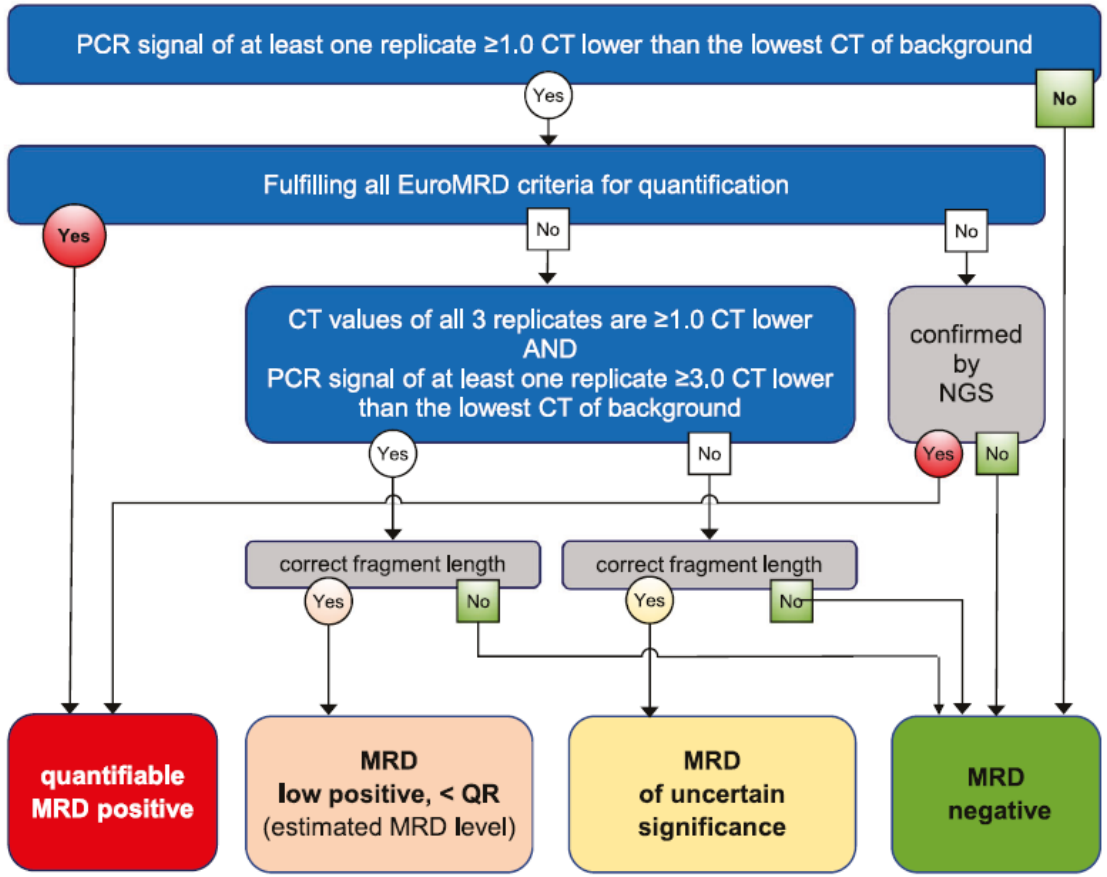
C_t FU:

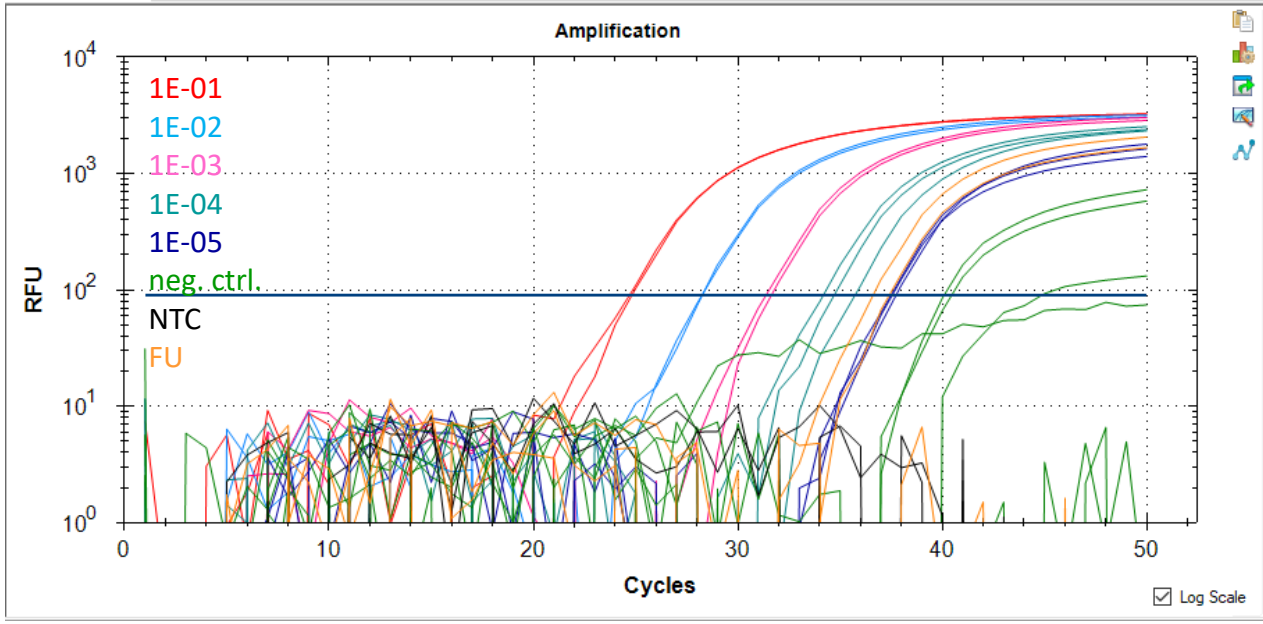
repl. 1: 34.2
repl. 2: 35.4 } mean C_t = 35.0
repl. 3: 35.3

→ **MRD low positive, <1E-04 (4E-05)**

ASO-PCR – DATA INTERPRETATION

updated EuroMRD guidelines (2024)





lowest C_t neg. ctrl.: 40.1

QR = 1E-04 with highest Ct value = 35.7

C_t FU:

repl. 1: /

repl. 2: 36.5

repl. 3: 37.32

→ **MRD of uncertain significance**

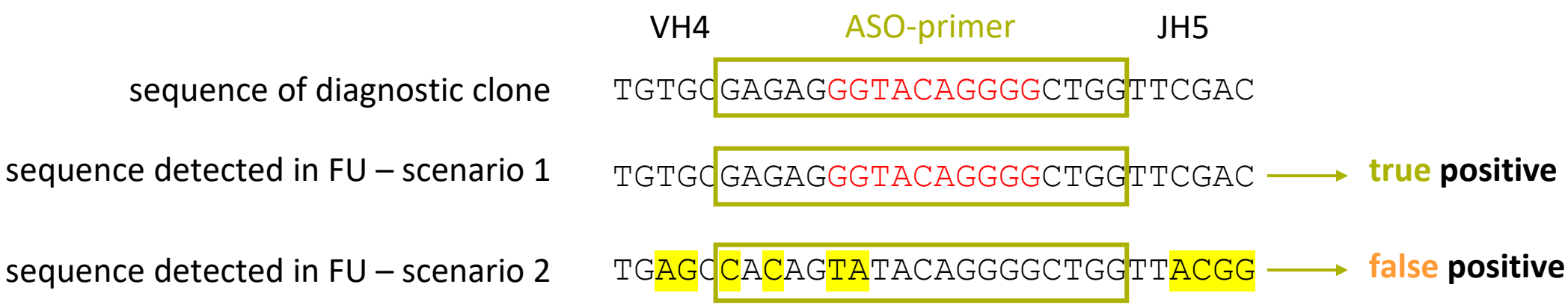
ASO-PCR – DATA INTERPRETATION

updated EuroMRD guidelines (2024)

-	+	???
MRD negative report: negative	MRD positive AND quantifiable report: MRD value	MRD low positive, <QR (MRD value) report: MRD low positive <1E-04 (4E-05)

MRD of uncertain significance
report: MRD of uncertain significance

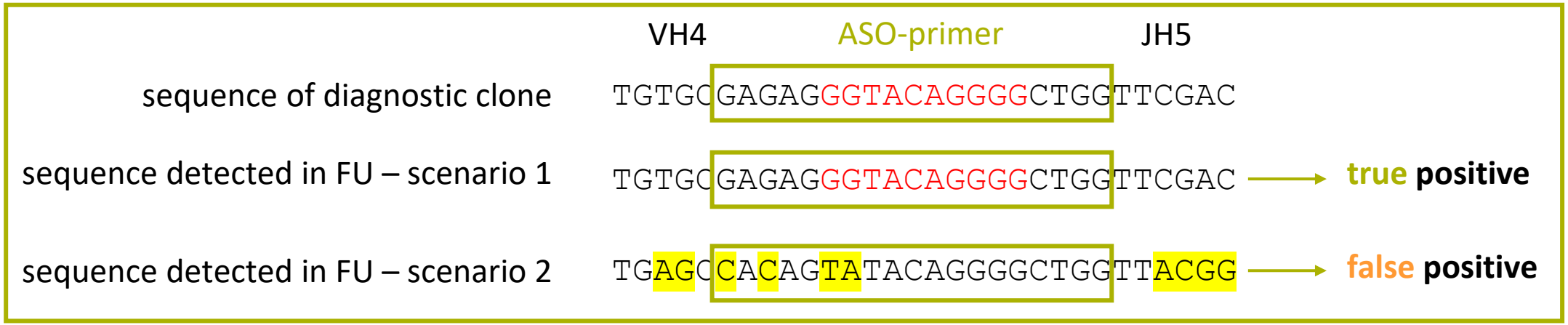
ASO-PCR: MRD of uncertain significance



ASO-PCR

Pros and cons

- ✓ TAT
- ✓ Standardised
- ✓ Sensitivity 1E-04 – 1E-05
- × Diagnostic DNA needed
- × False negativity (subclone – VH-replacement) → **NGS**
- × False positivity

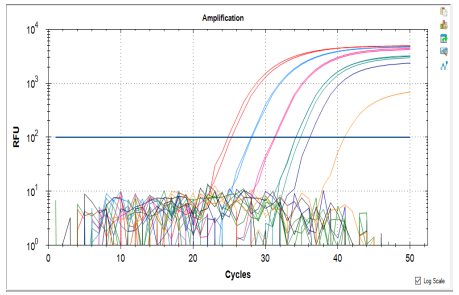


ASO-PCR VERSUS NGS-MRD

ALL – diagnosis: NGS

CGAGAGTGTACCTAACTGGGGATGGGGGTC
CGAGAGTGTACCTAACTGGGGATGGGGGTC → target identification
CGAGAGTGTACCTAACTGGGGATGGGGGTC

ALL – FU: ASO-PCR



result = PCR-product +/-/false positive

ALL – FU: NGS-MRD

	A	B	C	D	E	F	G
1	Z:\Import lists\DNA\Ben\Jona LMOL\251003_M05113_0510_000000000-M36MN\FU_fastq_0337_Fr1\IGH_FR1_ou						
2	Total count	122489					
3	Sample	LMHE-0337-EH0890A-Fr1					
4							
5	Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads
6	1	GCCTCTGGATTCACCGTCAGTAC	305	475	IGHV3-66_01	IGHJ6_02	0,39
7	2	CGCTGTCTATGGTGGGTCCTTCA	279	111	IGHV4-34_02	IGHJ5_02	0,09
8	3	GCGTCTGGATTCACCTTCAGTAG	272	94	IGHV3-33_01	IGHJ4_02	0,08
9	4	GCGTCTGGATTCACCTTCAGTAG	290	89	IGHV3-33_01	IGHJ6_02	0,07
10	5	GCCTCTGGGTTCACCGTCAGTAC	305	76	IGHV3-53_02	IGHJ6_02	0,06
11	6	GCCTCTGGATTCACCTTTAGCAG	309	74	IGHV3-23_04	IGHJ6_02	0,06
12	7	GCCTCTGGATTCACCGTCAGTAC	263	74	IGHV3-66_01	IGHJ4_02	0,06
13	8	GCCTCTGGATTCACCTTCAGTAG	272	71	IGHV3-74_01	IGHJ4_02	0,06

result = sequence +/-

- ✓ diagnostic and FU sample: same NGS workflow

  FU: higher input of DNA and use of calibrator molecules (gBlocks)

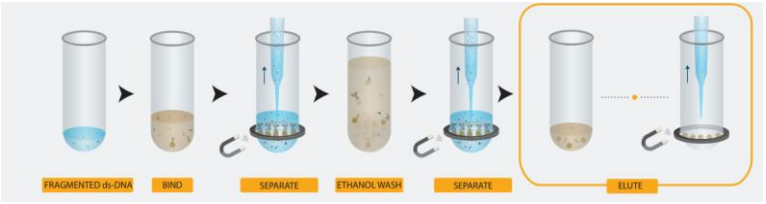
NGS-MRD

Lab set-up

1. IG/TCR PCR

- LymphoTrack – Invivoscribe
- EuroClonality

2. Amplicon purification



- AMPure XP – Beckman Coulter

3. Amplicon quantification

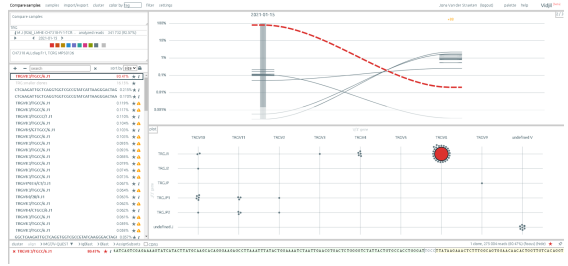
- Qubit – Life Technolgies
- Victor Nivo – Perkin Elmer

4. Library prep

5. NGS run

- MiSeq - Illumina 2x250/2x300 bp

6. Analysis



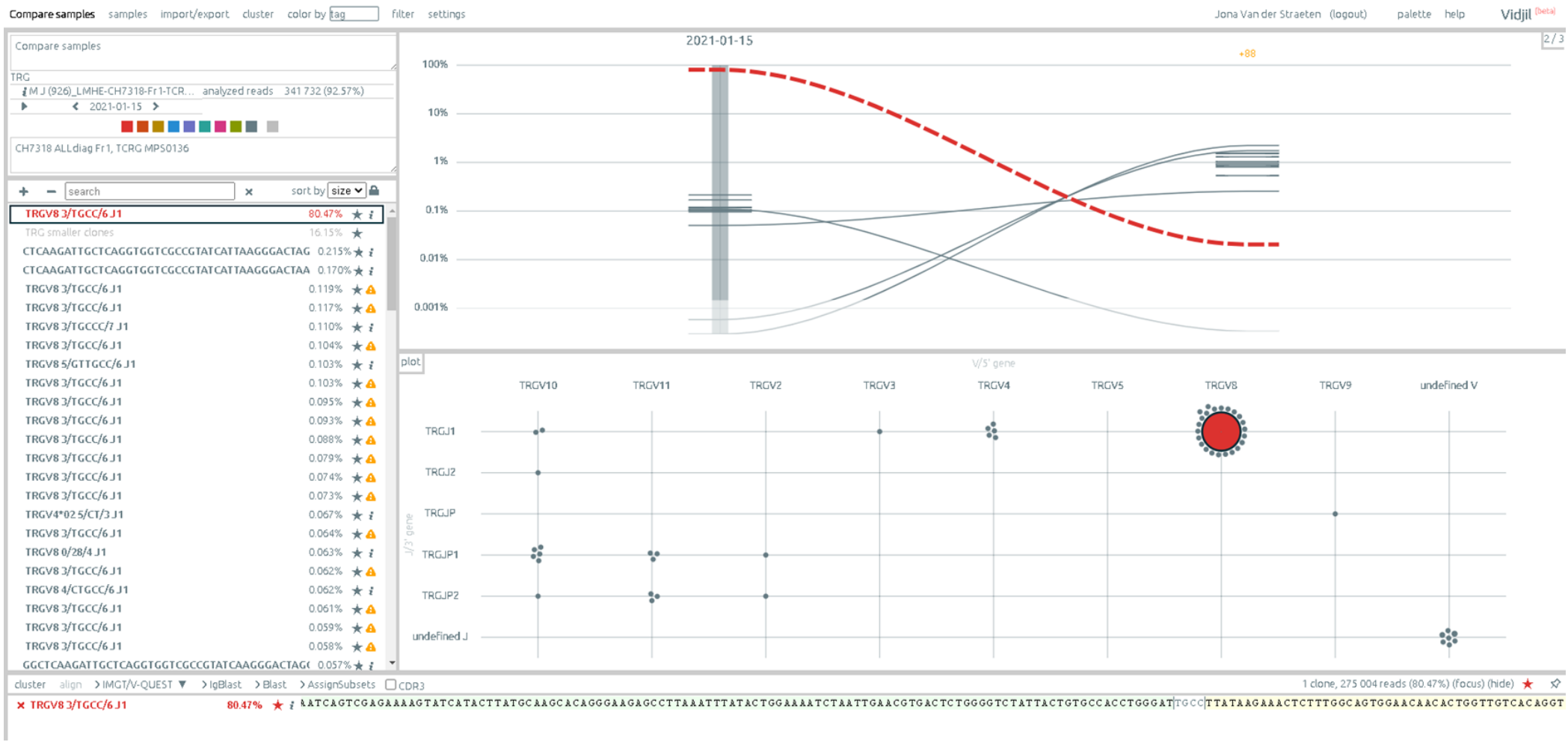
- LymphoTrack software
- VIDJIL

1 run/week → TAT 2 weeks

NGS-MRD

Analysis

VIDJIL



Aurélien Béliard, Marc Duez, Mathieu Giraud, Ryan Herbert, Mikaël Salson, Tatiana Rocher and Florian Thonier – http://www.vidjil.org/

NGS-MRD

Analysis

LymphoTrack

Z:\Import lists\DNA\Ben\Jona LMOL\250228_M05113_0463_000000000-LT6HM\fastq_0311_Fr1\IGH_FR1_output\combined_fastq\LMHE-0311-DH9353-Fr1-IGK_S15_L001_001_combined.fastq_read_summary.tsv

Total count 19772

Sample LMHE-0311-DH9353-Fr1-IGK

MM - diagnosis

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop codon (Y/N)	V-coverage	CDR3
1	TTTCCGGAT	292	2953	IGHV1-24_01	IGHJ6_02	14,935262	14,935262	4,42	Y	Y	100,00	GCAATAGACAGCCTTATGGGTGACCATCTTTCCGGGGAGAACTTCGGTATGGACGTC
2	GCCTCTGGA	296	149	IGHV3-23_04	IGHJ4_02	0,7535909	15,6888529	16,3	Y	N	96,92	GTGGAATTACTACAATGAGGATTACTGCTCTCCACTGGGGCCAGAATTACTGCTACCCAC
3	GCCTCTGGA	131	129	IGHV3-23_04	none	0,6524378	16,3412907	4,85		N	57,71	not found
4	GCCTCTGGA	263										AGAGCTGAGTACCAGCTGCTATTGACTAC
5	GCCTCTGGA	268										
6	GCCTCTGGA	285										
7	GCCTCTGGA	276										
8	GCCTCTGGA	266										
9	GCCTCTGGA	269										
10	GCCTCTGGA	264										

MM - FU

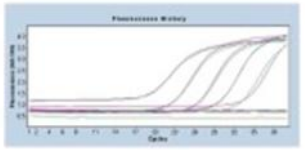
	sequence_name	sequence	sum			
2	zoekseq 1	GTGCAATAGACAGCCTTATGGGTGACCATCTTTCCGGGGAGAACTTCGGTATGGAC	206			
3	gBlock1	CTGTGCGAGATCAGATTTTGGAGTGGTTCCGATGACTACTGG	440			
4	gBlock2	GTGCGAGAGATCTAGGAGGGTTGTAGTGGTGGTAGCTGCTACGCGATTACTAC	2412			
5	gBlock3	GTGCGAGAGGCAGAGATGGCTACAATTACAACTGGTT	1521			
6	UniqueReads					
7	>172146	1757	Acount17	Bcount0	length305	
8	GCCTCTGGATTCACCGTCAGTAGCAACTACATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGTTATTTATAGCGGTGGTAGCACATACTAC					
9	>172150	851	Acount85	Bcount0	length260	
10	GCCTCTGGATTCACCTTCAGTAGCTATAGCATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATACATTAGTAGTAGTAGTAGTACCATATAC					
11	>172148	846	Acount84	Bcount0	length279	
12	CGCTGTCTATGGTGGGTCCTTCAGTGGTTACTACTGGAGCTGGATCCGCCAGCCCCCAGGGAAGGGGCTGGAGTGGATTGGGGAAATCAATCATAGTGGAAGCACCA					
13	>172111	766	Acount76	Bcount0	length268	
14	GCCTCTGGATTCACCTTCAGTAGCTACGACATGCACTGGGTCCGCCAAGCTACAGGAAAAGGTCTGGAGTGGGTCTCAGCTATTGGTACTGCTGGTGACCCATACTAT					
15	>171933	713	Acount71	Bcount0	length260	
16	GCCTCTGGATTCACCTTCAGTAGCTACTGGATGCACTGGGTCCGCCAAGCTCCAGGGAAGGGGCTGGTGTGGGTCTCACGTATTAATAGTGATGGGAGTAGCACAAG					
539	>172112	116	Acount116	Bcount0	length292	
540	TTTCCGGATACACCCTCACTGAATTATCCATGCACTGGGTGCGACAGGCTCCTGGAAAAGGGCTTGAGTGGATGGGAGGTTTTGATTCTGAAGAAGGTGAAACAGTCTACGC					

NGS-MRD

MRD calculation

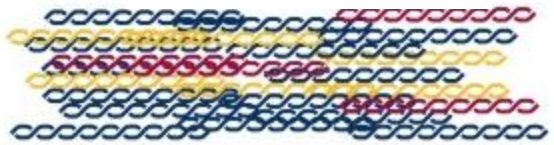
- Non B cell leukocyte
- B-cell
- Leukemic B-cell

Albumin PCR



N_{tot} = total number of leukocytes

Extract DNA



Add known quantity of reference IGH

N_R = number of reference molecules

Amplify IgH molecules

Sequence molecules

S_B = number of B cell reads

S_L = number of leukemic reads

S_R = number of reference reads

N_R = 30x
S_R = 300x

coverage = 10x

$$\rightarrow \text{MRD} = \frac{S_L \times (N_R/S_R)}{N_{tot}}$$

TECHNIQUES: PROS AND CONS

ASO-PCR

- ✓ TAT
- ✓ Standardised
- ✓ Sensitivity 1E-04 – 1E-05
- × Diagnostic DNA needed
- × False negativity (subclone – VH-replacement)
- × False positivity

NGS-MRD

- × TAT
- ✓ Standardised
- !!! ✓ Sensitivity 1E-05 – 5E-06 !!!
- ✓ Less diagnostic DNA needed
- ✓ No false negative/positive → no grey area – no MRD of unknown significance
- × Cost

NGS-MRD

$$\text{sensitivity} = \frac{2}{\text{total number of cells tested}}$$

DNA input 600 ng = 100 000 cells

$$\text{sensitivity} = \frac{2}{100\,000} = 2\text{E-}05$$

testing in duplo = 200 000 cells

$$\text{sensitivity} = \frac{2}{200\,000} = 1\text{E-}05$$

sensitivity 1E-06 → 2 000 000 cells = 1~~2~~ µg DNA

DNA input of 200 000 cells and 10% B-cells

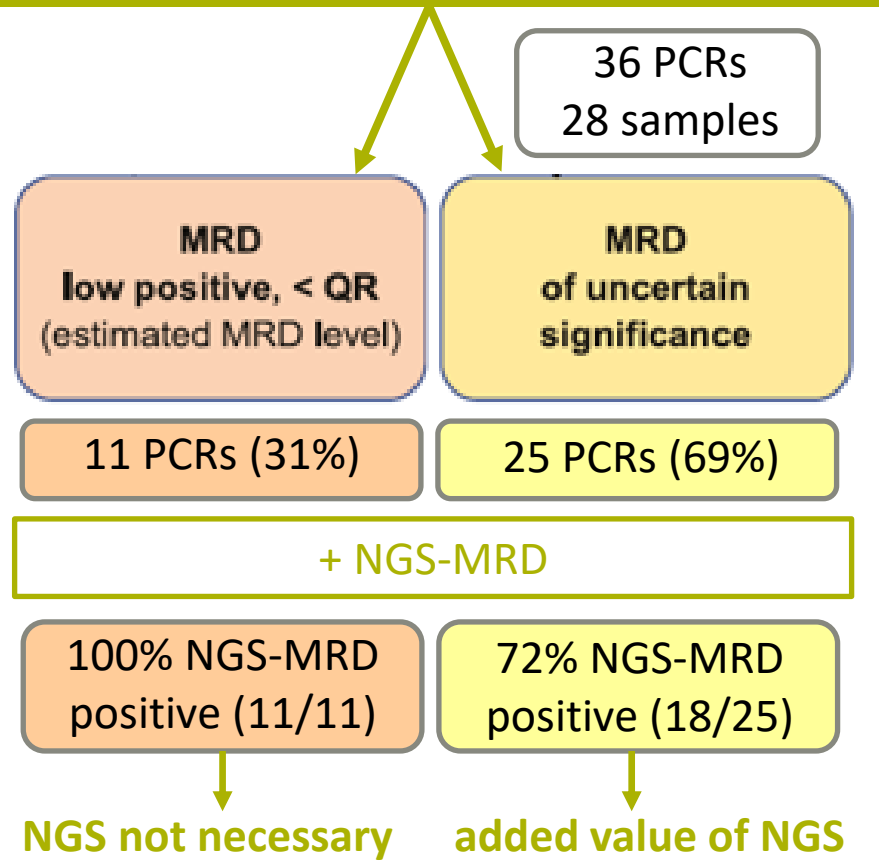
coverage of 10x (gBlocks) → total sequencing reads = 200 000

→ sensitivity of 1E-05

ASO-PCR VERSUS NGS-MRD

time-frame: 02/10/2024 - 06/03/2025

MRD negative report: negative	MRD positive AND quantifiable report: MRD value	MRD positive NOT quantifiable report: positive <QR



ASO-PCR VERSUS NGS-MRD

- MRD low positive, <QR: → **NGS not necessary**
  100% NGS-MRD positive (11/11)

- MRD of uncertain significance: → **added value of NGS**
  72% NGS-MRD positive (18/25)

→ **NGS-MRD: in close consultation with clinician**
→ **re-assign to MRD negative group: overcome unnecessary bone marrow aspiration**

ASO-PCR VERSUS NGS-MRD

MRD of uncertain significance: NGS-MRD in close consultation with clinician

Case:
B-ALL patient – diagnosis 07/03/2017 - relapse II 06/11/2024

technique	sampling date	marker 1	marker 2	overall result
ASO-PCR	6/12/2024	positive	positive, <3E-04	positive, <3E-04
NGS	6/12/2024	positive	positive	positive
ASO-PCR	08/01/2025	negative	negative	negative
ASO-PCR	19/02/2025	negative	negative	negative
ASO-PCR	21/03/2025	negative	negative	negative
ASO-PCR	25/04/2025	negative	negative	negative
ASO-PCR	30/07/2025	positive, <5E-04	negative	negative

clinician:
NGS?
NGS neg.: no BM aspiration needed whitin 6 months

NGS	30/07/2025	negative	negative	negative

ASO-PCR VERSUS NGS-MRD

MRD of uncertain significance: NGS-MRD PSCT

Next-generation sequencing indicates false-positive MRD results and better predicts prognosis after SCT in patients with childhood ALL

M Kotrova[1], VHJ van der Velden[2], JJM van Dongen[2,3], R Formankova[4], P Sedlacek[4], M Brüggemann[5], J Zuna[1], J Stary[4], J Trka[1] and E Fronkova[1]

Minimal residual disease (MRD) monitoring via quantitative PCR (qPCR) detection of Ag receptor gene rearrangements has been the most sensitive method for predicting prognosis and making post-transplant treatment decisions for patients with ALL. Despite the broad clinical usefulness and standardization of this method, we and others have repeatedly reported the possibility of false-positive MRD results caused by massive B-lymphocyte regeneration after stem cell transplantation (SCT). Next-generation sequencing (NGS) enables precise and sensitive detection of multiple Ag receptor rearrangements, thus providing a more specific readout compared to qPCR. We investigated two cohorts of children with ALL who underwent SCT (30 patients and 228 samples). The first cohort consisted of 17 patients who remained in long-term CR after SCT despite having low MRD positivity ($<0.01\%$) at least once during post-SCT monitoring using qPCR. Only one of 27 qPCR-positive samples was confirmed to be positive by NGS. Conversely, 10 of 15 samples with low qPCR-detected MRD positivity from 13 patients who subsequently relapsed were also confirmed to be positive by NGS ($P=0.002$). These data show that NGS has a better specificity in post-SCT ALL management and indicate that treatment interventions aimed at reverting impending relapse should not be based on qPCR only.

Bone Marrow Transplantation (2017) **52,** 962–968; doi:10.1038/bmt.2017.16; published online 27 February 2017

ASO-PCR VERSUS NGS-MRD

MRD of uncertain significance: NGS-MRD PSCT

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30 patients (228 samples) **PSCT**
AND
at least one sample MRD low positive (<1E-04) during PSCT monitoring

<u>17 patients - long term CR after SCT</u>

<u>qPCR:</u> 27 samples MRD low positive (<1E-04) PSCT

<u>NGS:</u> 1/27 NGS-MRD positive (4%)

<u>13 patients - relapsed</u>

<u>qPCR:</u> 15 samples MRD low positive (<1E-04) PSCT

<u>NGS:</u> 10/15 NGS-MRD positive (66%)

→ **Better specificity of NGS in PSCT**

ASO-PCR VERSUS NGS-MRD

MRD of uncertain significance: NGS-MRD PSCT

Patient	Days PSCT	MRD qASO-PCR		MRD NGS	Clinical outcome	
		ASO1	ASO2			
MRD NGS positive					**FU/last consult (days PSCT)**	**Status**
1	139 155	PNQ (3/3) PNQ (3/3)	NA NA	6E-05 4E-04	155	relapse (cutaneous lesion)
2	429	PNQ (1/3)	negative	6E-04	1049	relapse (bone marrow)
3	111 196	PNQ (1/3) negative	PNQ (3/3) PNQ (1/3)	8E-05 1E-05	282	in remission
MRD NGS negative					**Last consult (days PSCT)**	**Status**
4	361 1093	negative PNQ (1/3)	PNQ (3/3) PNQ (2/3)	negative negative	2220	in remission
5	159 244	negative PNQ (1/3)	PNQ (1/3) negative	negative negative	1325	in remission
6	316	PNQ (1/3)	negative	negative	1175	in remission
7	526	PNQ (1/3)	negative	negative	931	in remission
8	62 89	PNQ (1/3) PNQ (1/3)	negative negative	negative negative	89	in remission

IG/TCR-BASED MRD: KEY MESSAGES

- **ASO-PCR**

  = standard molecular technique for MRD in ALL

  positive non-quantifiable ASO-PCR group - two subgroups:

  - MRD low positive, <QR → true positive
  - MRD of uncertain significance → false positivity possible

- **NGS-MRD**

  - in ALL: to exclude ASO-PCR false positivity - in close consultation with clinicians
  - In MM: CR

MOLECULAR MRD APPLICATIONS IN HEMATO-ONCOLOGY

MRD in AML

Universitair Ziekenhuis Brussel

VUB

OVERVIEW

- Introduction
- Molecular Markers for MRD Monitoring in AML
- Techniques for MRD Detection in the Laboratory
- Prognostic Value of MRD
- The Future of MRD Monitoring in AML
- Conclusion / Questions

INTRODUCTION

Acute myeloid leukemia (

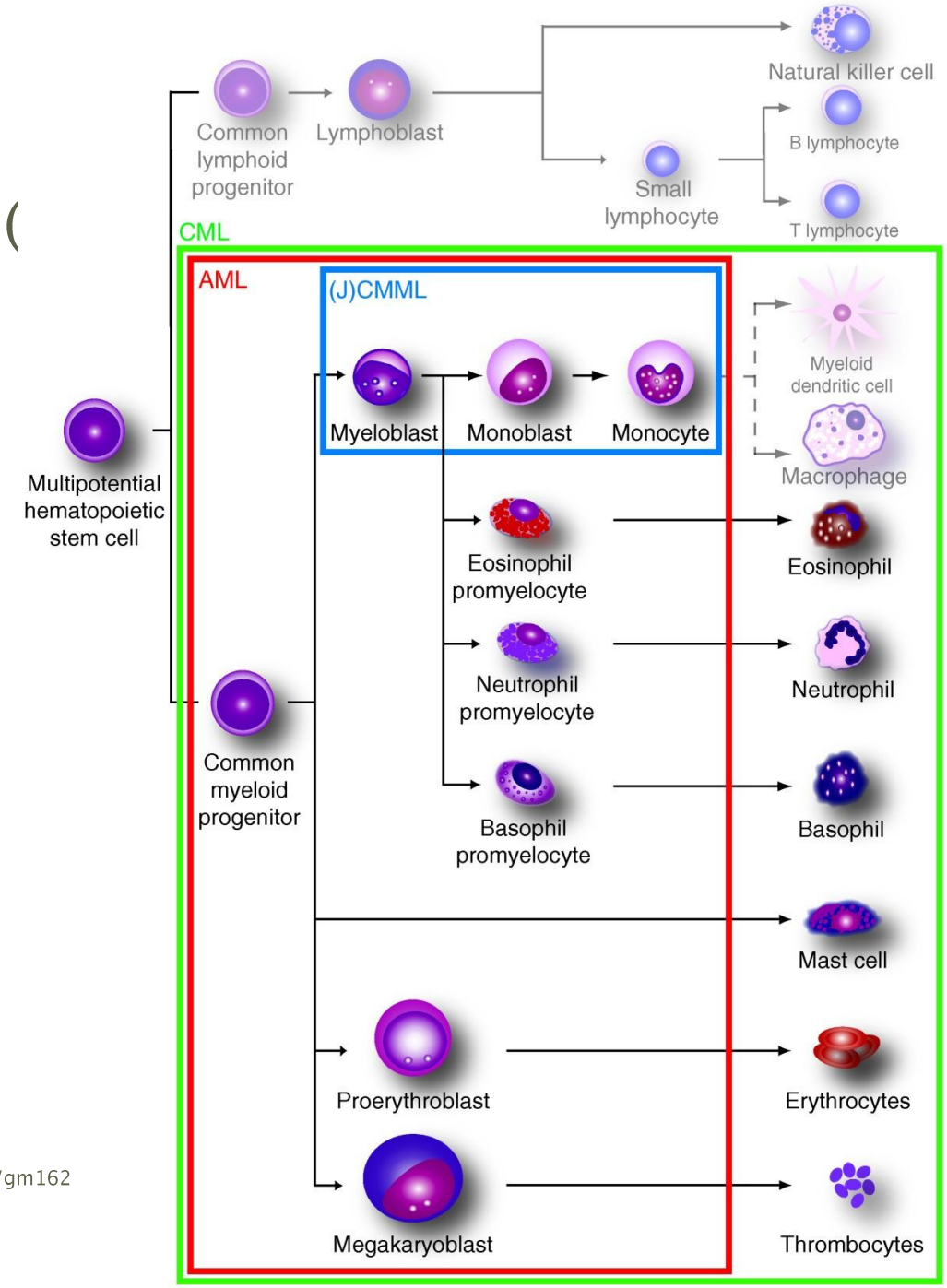


Figure: Hematopoiesis.

Hjelle, S.M., Forthun, R.B., Haaland, I. et al.

Clinical proteomics of myeloid leukemia.

Genome Med 2, 41 (2010). https://doi.org/10.1186/gm162

INTRODUCTION

MRD in AML

Why?

Early MRD detection:

timely intervention and treatment adjustment

Limitations:

- No standardization
- Clonal hematopoiesis mimic MRD (CHIP: e.g. DNMT3A, TET2, ASXL1)

MOLECULAR MARKERS FOR MRD MONITORING IN AML

Fusion transcripts

PML::RARA t(15;17) APL

- 2 major break points:
  bcr1 and bcr3
- Block differentiation and enhance proliferation

RUNX1::RUNX1T1 (ALM-ETO) t(8;21)

- Dominant repressor of RUNX1
- Leukemic self-renewing

CBFB::MYH11 (inv16) t(16;16)

- Block myeloid diffrentiation
- Favorable prognosis

MOLECULAR MARKERS FOR MRD MONITORING IN AML

Mutations

NPM1 mutations

Nucleophosmin

Somatic mutations in exon 12

>50 Suptypes→ A: 75 à 80% (TCTG)

Favorabe prognosis

FLT3 mutations

fms related tyrosine kinase

FLT3-TKD vs FLT3-ITD

Intermediate risk group

MOLECULAR MARKERS FOR MRD MONITORING IN AML

Monitoring at molecular level

Specimen:

- PB / BM

Analyte:

- DNA
- RNA → cDNA

Assay:

- Quantitative PCR (qPCR)
- Digital PCR (dPCR)
- Next generation sequencing (NGS)

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

	RNA	DNA
Advantages	Transcripts (active gene expression)	Not affected by variation in gene expression → more accurate correlation with leukemic cell number
	↑ # copies of leukemic mRNA vs # copies DNA → ↑ expression → ↑ efficiency and sensitivity	↑ stability
Disadvantages	Variation in number of transcript copies per cells	↓ Sensitivity
	Variation in expression level between patients	
	↓ stability	

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Quantitative PCR (qPCR)

- ↑ Input RNA/DNA
- Standard curve
- Housekeeping gene

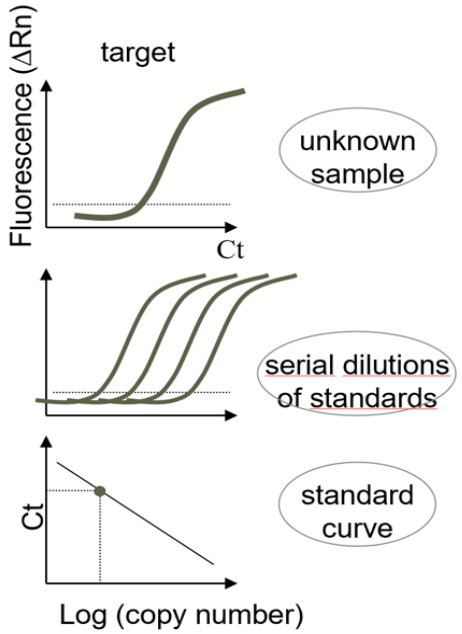


Figure: real-time PCR with fluorescent probes

Digital PCR (dPCR)

- ↑ Input RNA/DNA
- No Standard curve (absolute quantity)
- Partitioning → PCR-reactions
- Housekeeping gene

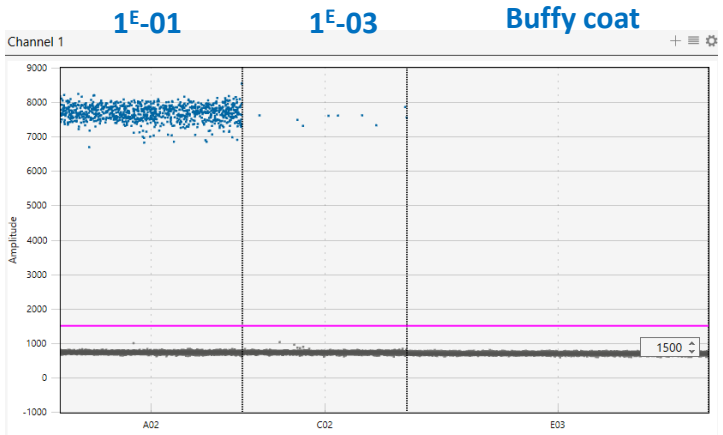


Figure: distribution of samples on ddPCR

Deep sequencing by Next generation sequencing (UHS-NGS)

- ↑ Input DNA/RNA
- No Standard curve (absolute quantity)
- No mutation specific probe
- Housekeeping gene

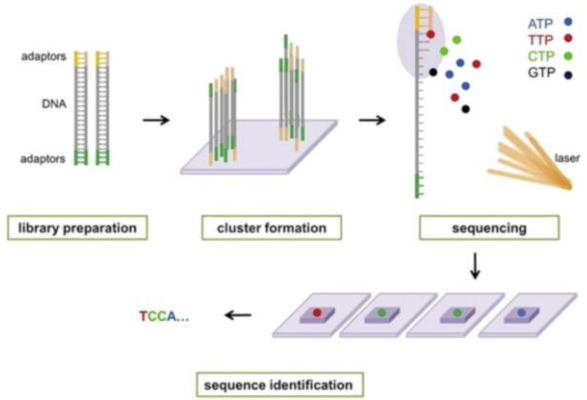


Figure sequencing procedure on flow cell (Zhou, X., Li, Y., 2015. From healthy microflora to disease. Atlas Oral Microbiol. 15–40. https://doi.org/10.1016/B978-0-12-802234-4.00002-1.)

DIGITAL PCR (DPCR)

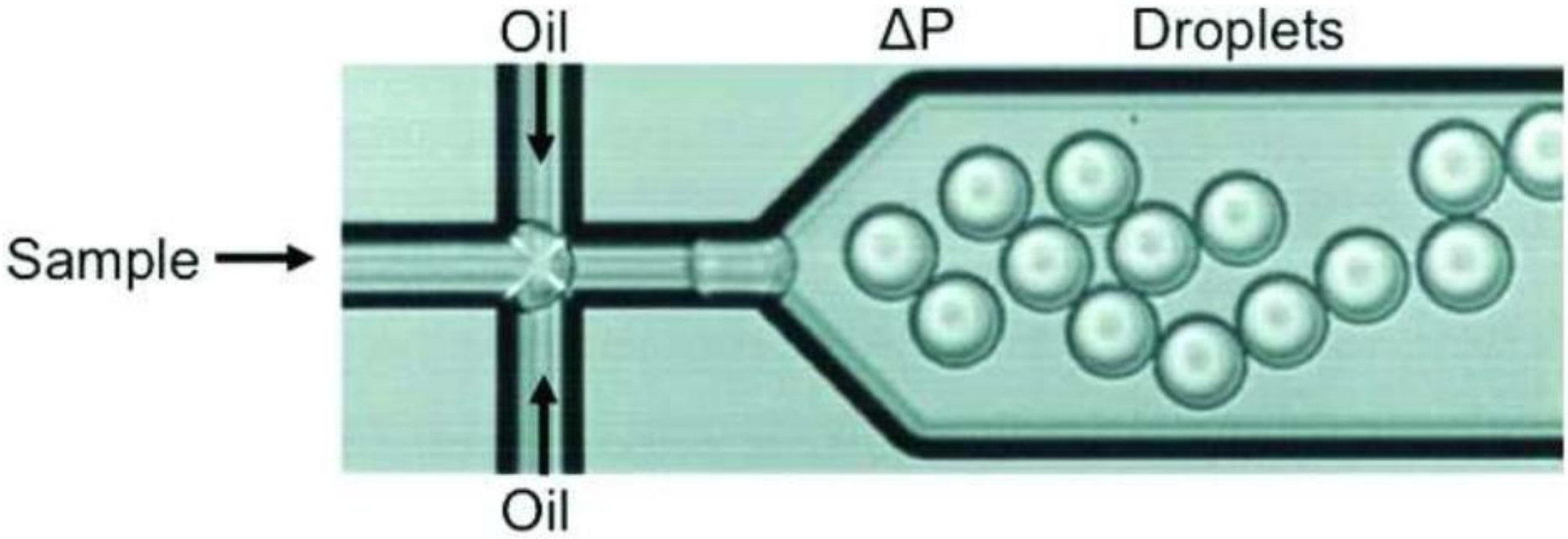
TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital PCR (dPCR)

What?	A PCR technique in which a sample is devided into thousands of nanoliter sized droplets / chambers, allowing absolute quantification without need of standard curve.	Very high sensitivity Absolute quantification Less affected by PCR-efficiency High reproducibility

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital droplet PCR (ddPCR)



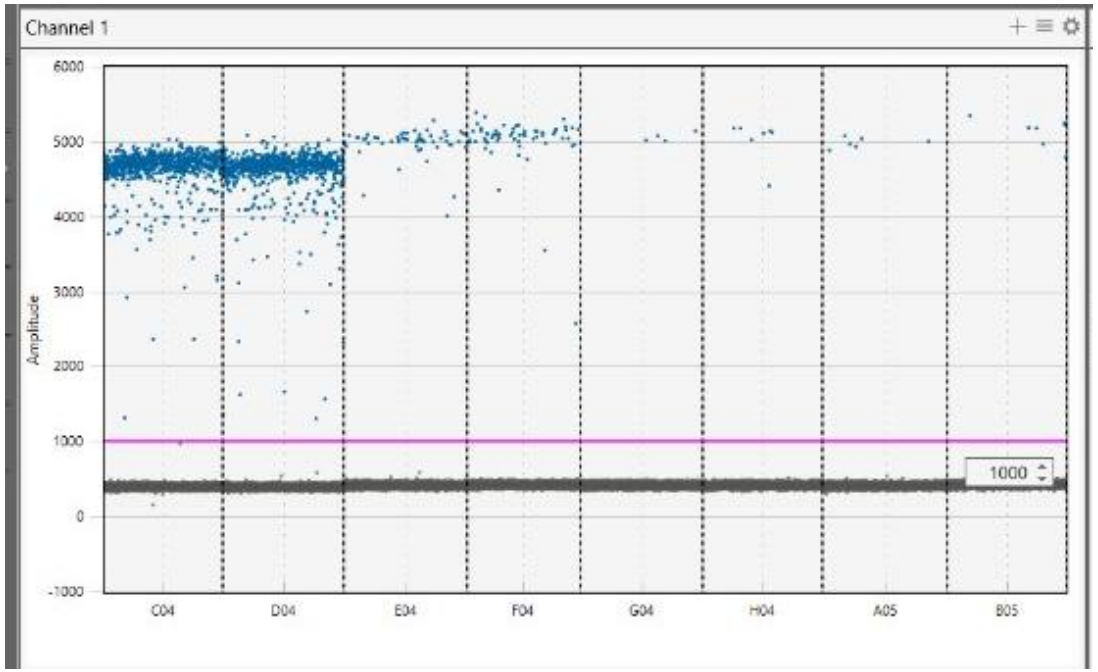
TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital droplet PCR (ddPCR)

Steps	Actions
Sample preparation	• RNA/(c)DNA • PCR mix with primers and fluorescent probe • ↑ replica → ↑ sensitivity • Housekeeping gene
Droplet generator	• Partitioning: 20000 oil droplets • Ideally each droplet contains 0 to max 5 target molecules → each droplet acts as an individual PCR reaction
PCR amplification	Droplets with target become fluorescent
Droplet reading	• Droplets are analyzed one by one in the droplet reader • Each droplet is scored as positive or negative
Data analysis (Poisson statistic)	• Software calculates the absolute number of copies per µL • No standard curve is required

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

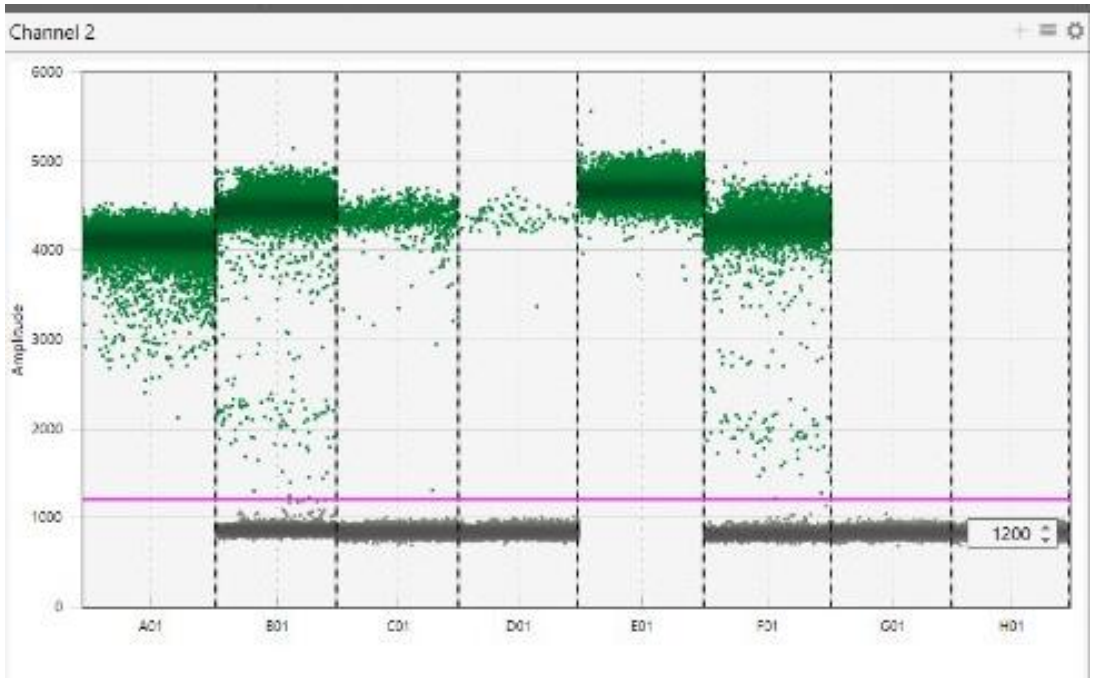
Digital droplet PCR (ddPCR): results software



Well	Sample	Marker	Conc (copies/µl)	Accepted droplets	Positives	negatives
C04	ME-1 1^{E}-02	Inv 16	54,4	19419	822	18597
D04	ME-1 1^{E}-02	Inv 16	53,2	19529	809	18720
E04	ME-1 1^{E}-03	Inv 16	3,67	19894	58	19836
F04	ME-1 1^{E}-03	Inv 16	4,2	18289	61	18228
G04	ME-1 1^{E}-04	Inv 16	0,255	19723	4	19719
H04	ME-1 1^{E}-04	Inv 16	0,455	19350	7	19343
A05	ME-1 1^{E}-04	Inv 16	0,373	20216	6	20210
B05	ME-1 1^{E}-04	Inv 16	0,456	19314	7	19307

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

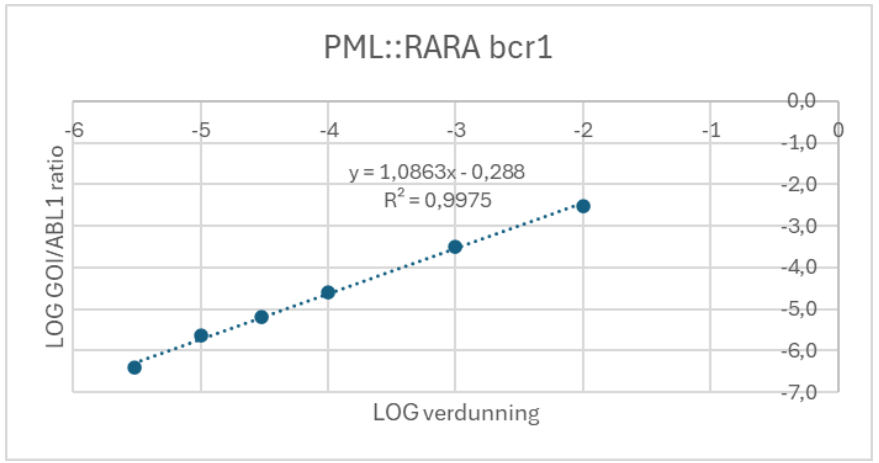
Digital droplet PCR (ddPCR): results software – overexpression

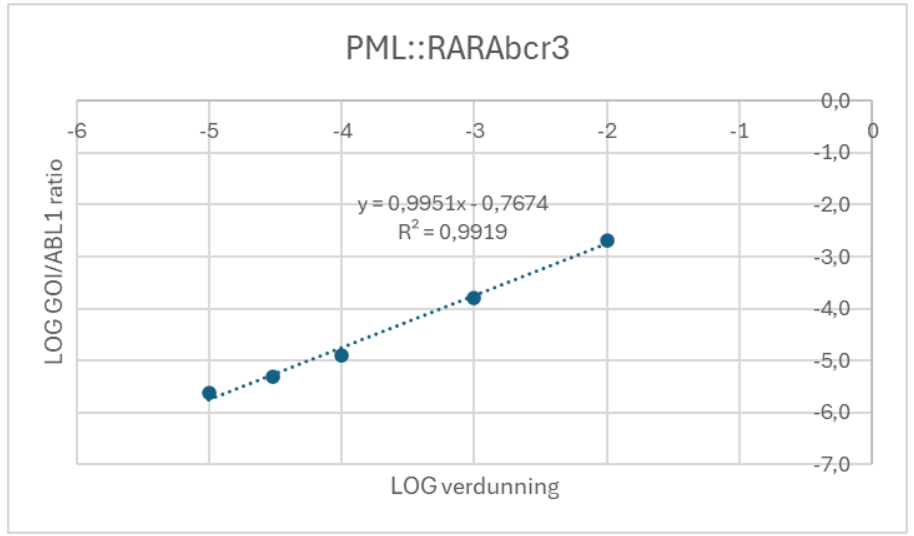


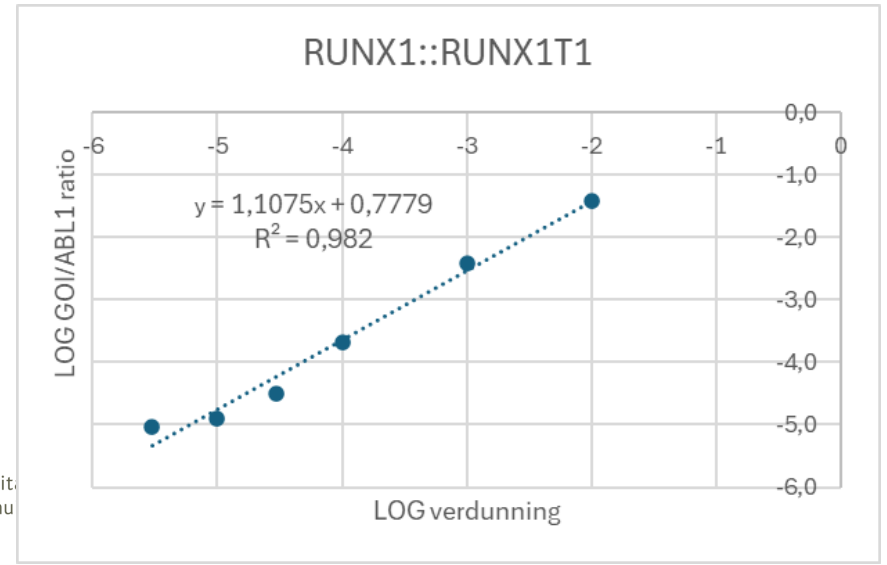
Well	Sample	Marker	Conc (copies/µl)	Accepted droplets	Positives	negatives
A01	HL60 M-MLV	ABL1	1000000	20011	20011	0
B01	HL60 M-MLV 1/10 verd	ABL1	1424	17912	12137	5775
C01	HL60 iScript	ABL1	67,1	18140	942	17199
D01	HL60 iScript 1/10 verd	ABL1	5,83	17960	83	17877
E01	HL60 Vilo	ABL1	1000000	18765	18765	0
F01	HL60 Vilo 1/10 verd	ABL1	1004	18901	10393	8508
G01	MQ	ABL1	0	17986	0	17986
H01	MQ	ABL1	0	17605	0	17605

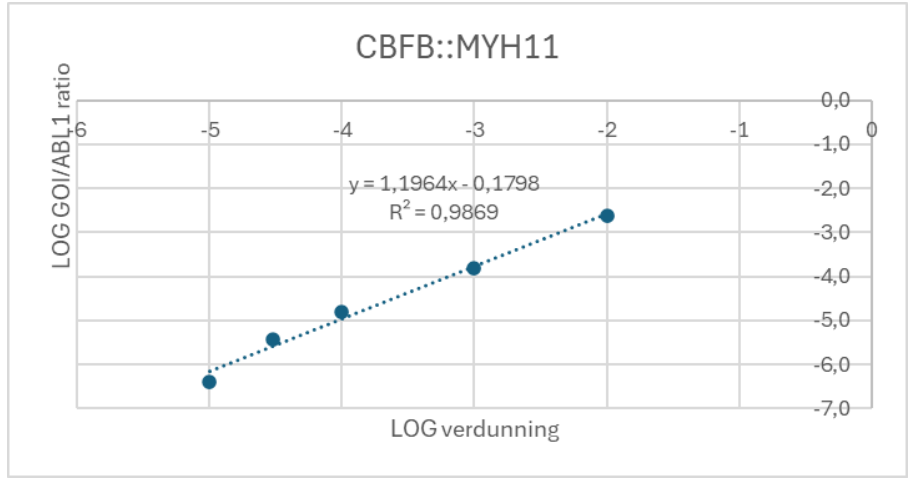
TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital droplet PCR (ddPCR): Validation (Linearity)









TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital droplet PCR (ddPCR): validation (LOD vs LOQ)

Gene of interest (GOI)	Dilution	# Droplets (80µl)	SD (RMS)	LOB	LOD (droplets)	LOD (droplets)
PML::RARA bcr1	1^{E}-05	2,5	0,7	0	1,2	7
PML::RARA bcr3	1^{E}-05	3	0,9	0	1,5	9
RUNX1::RUNX1T1	3^{E}-06	11	1,1	0	1,8	11
CBFB::MYH11	3^{E}-05	5	1,1	0	1,8	11

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Digital droplet PCR (ddPCR): interpretation

Droplet counts	Interpretation	Reporting	Remarks
< 2 positive droplets	Negative	No quantification <LOD	
≥ 2 en < 10 positive droplets	PNQ – positive not quantifiable	Positive < LOQ (estimated value)	Log reduction against Dx
≥ 10 positive droplets	Positive, quantifiable	MRD burden (%) Log reduction against Dx	

NEXT GENERATION SEQUENCING

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Deep sequencing by Next generation sequencing (NGS)

Detection + Quantification → variant allel frequentie (VAF)

Multiple genetic aberrations in a single assay

Beter comprehension + deeper assessment of residual disease.

TECHNIQUES FOR MRD DETECTION

Deep sequencing by Next generation sequencing (NGS)

Agnostic vs targeted NGS-MRD:

- Agnostic (wide) : monitor all clonal mutations in almost all AML patients ➔ low depth of coverage

- Targeted (deep): high coverage depths will increase sensitivity (>20000x)
  - Disadvantage: Missing new clones (false negativity)

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Deep sequencing by Next generation sequencing (NGS)

UZ Brussel: Amplicon-based NGS MRD / One-step PCR

How?	PCR-reaction Library preparation	Sequencing: MiSeq (1 million reads) Bio informatics: variant call (Pindel / Samtool Mpileup)

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Deep sequencing by Next generation sequencing (NGS)

UZ Brussel: Amplicon-based NGS MRD / One-step PCR

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

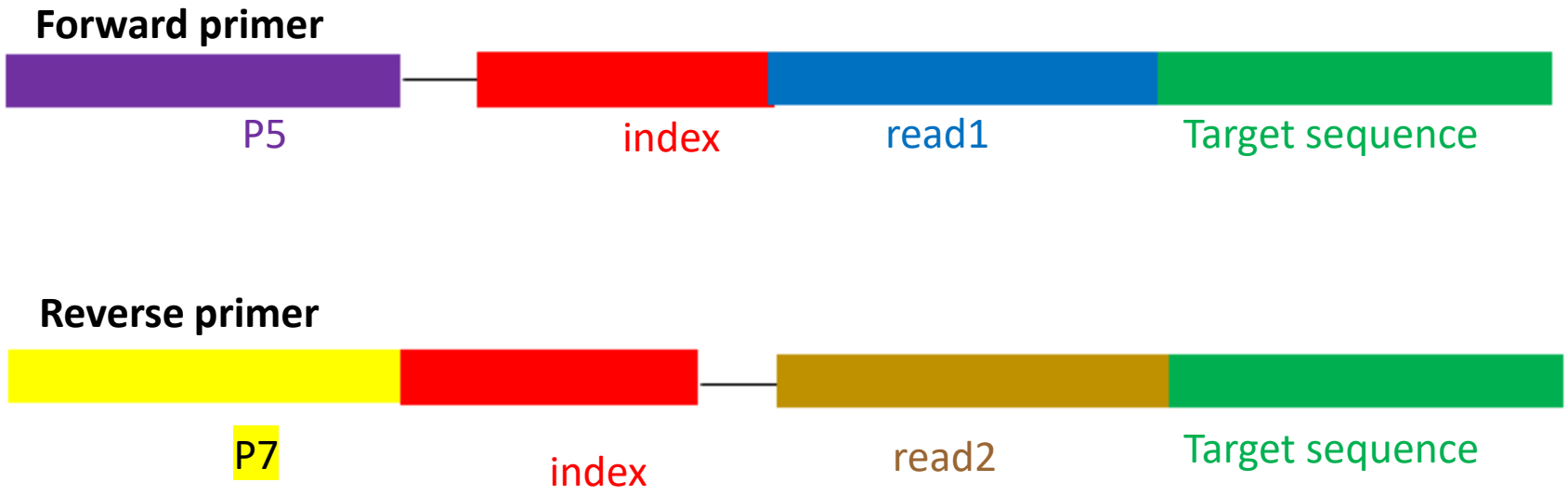


Figure: Schematic representation of NGS primers, forward and reverse primers

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Deep sequencing by Next generation sequencing (NGS)

UZ Brussel: Amplicon-based NGS MRD / One-step PCR

Results

- **MRD calculation**:

$$\text{VAF (\%)} = \frac{\text{\# Reads mutant}}{\text{\# Reads wild type + mutant}} \times 100$$

- **Sensitivity**: sample dependent

\# input DNA with Albumine correction, converted in # cells (for both replicas)

$$\text{Sensitivity} = \frac{2}{\text{Total \# cells}}$$

TECHNIQUES FOR MRD DETECTION IN THE LABORATORY

Deep sequencing by Next generation sequencing (NGS)

ELN AML–MRD 2025:

UHS NGS assay

LOD 1E–04 – 1E–05

UZ brussel: LOD 5E–05

	250.000 reads		500.000 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

PROGNOSTIC VALUE OF MRD

MRD as a predictor of relapse

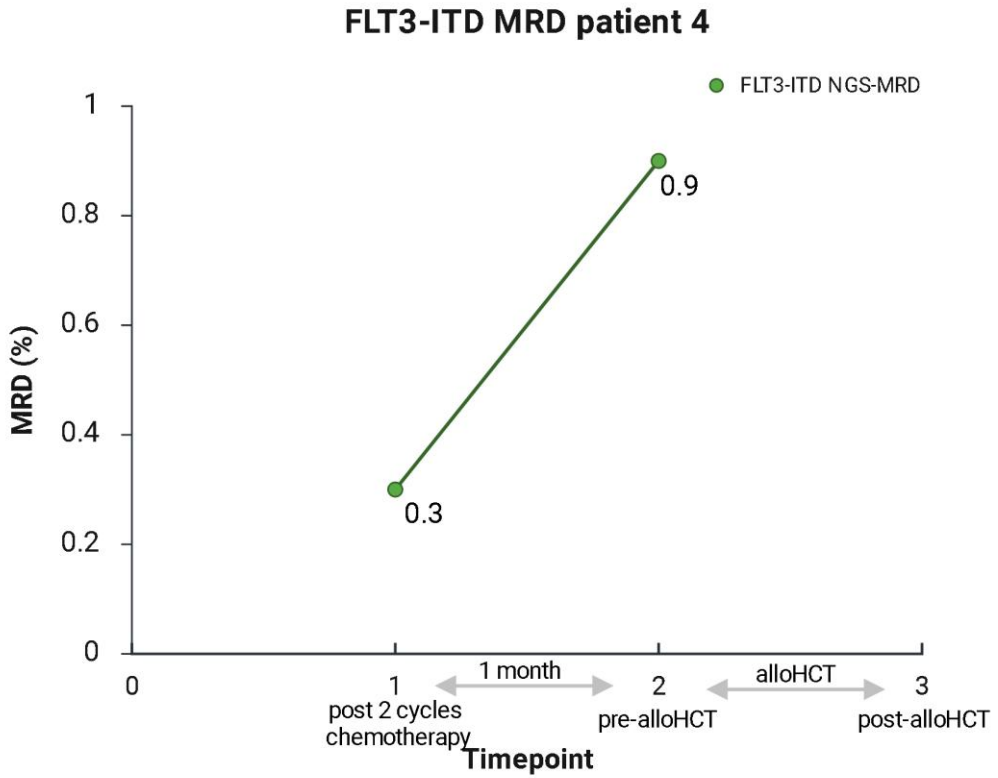
Targeted FLT3-ITD UHS-NGS (FLT3-ITD/mutNPM1 or FLT3-ITD/NPM1 wild type) (LOD measured as VAF (%))				
Time Point	**Tissue**	**VAF (%)**	**MRD burden**	**Qualitative MRD response**
2 cycles of intensive chemotherapy or pre-alloHCT	BM, PB	<LOD	Negative	Optimal
		≥LOD	Positive	High risk of treatment failure
End of Treatment	BM > PB	<LOD	Negative	Optimal
		≥LOD	Positive	High risk of treatment failure
Follow-Up	BM or PB	<LOD	Negative	Optimal
		≥LOD	MRD relapse	MRD relapse

PROGNOSTIC VALUE OF MRD

MRD as a predictor of relapse

- AML with NUP98::NSD1 fusion (t (5;11))
- Induction – consolidation – maintenance (midostaurin)
- alloHCT: matched related donor
- Died due to post-alloHCT complications

Time Point	MRD burden	VAF (%)	Qualitative MRD response
TP1	Positive	0,3	High risk of treatment failure
TP2	Positive	0,9	High risk of treatment failure



PROGNOSTIC VALUE OF MRD

MRD as a predictor of relapse

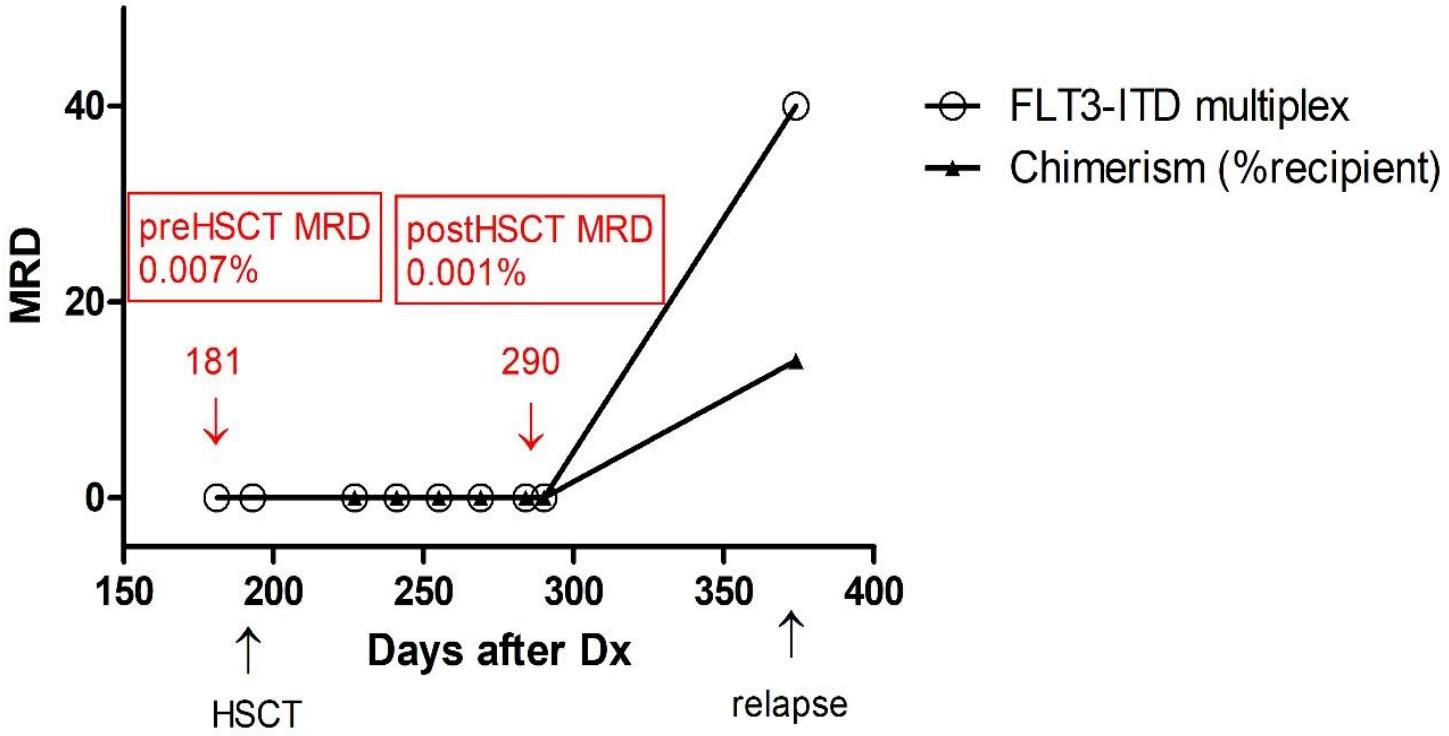


Figure: Evolution of MRD between diagnosis and relapse: comparison between FLT3-ITD NGS-MRD (in red), FLT3-ITD multiplex (black, circle) and chimerism (black, triangle) analysis.
Poster ESHLO meeting 2024

THE FUTURE OF MRD MONITORING IN AML

Emerging MRD Biomarkers	Improvement in the sensitivity of techniques
Genetic biomarkers → loocking at mutations like IDH1/2, WT1, RUNX1, …	Ultra-deep NGS
Epigenetic biomarkers → DNA methylation patterns (stable MRD markers)	Error corrected sequencing
Advanced approaches → RNA based marker (gene expression)	ddPCR on specific mutations
liquid biopsy	Single cell sequencing

CONCLUSION

Summary

MRD monitoring in AML

- Treatment response / Prognostic value
- Further MRD studies for lower-intensity regimens
- EQC in pilot version
- No international standardization

QUESTIONS AND DISCUSSION

ACKNOWLEDGEMENTS

Molecular Hematology Laboratory and HLA – UZ Brussel

- Marleen Bakkus
- Emmanuelle Kabongo
- Eleni Linskens
- Jona Van der Straeten
- Kristel Vannerom
- Myriam Ajbar
- Leila Daif
- Sarah Rossignol
- Kristien Vander Gucht
- Joeri Coignau
- Haaike Colemonts-Vroninks

All the labs and clinical centres for sending samples!!!

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BSPHO
AllTogether

Myeloom centrum UZ Brussel
Ivan Van Riet
Wouter De Brouwer
Robbe Heestermans

MMOVE
BHS multiple myeloma werkgroep

Brightcore
Ben Caljon
Toon Janssen

Wetenschappelijk fonds
Willy Gepts – UZ Brussel

Janssen | Pharmaceutical Companies of Johnson & Johnson

VLK
Vlaamse Liga tegen Kanker
STICHTING VAN OPENBAAR NUT

televie

kinderkankerfonds

Thank you!