Disclosure of Relevant Financial Relationships

Annette S. Kim, MD, PhD reported the following relevant financial relationship(s) during the content development process for this activity:

***Consultant*, LabCorp, Inc.**

***Research funding,* Multiple Myeloma Research Foundation**

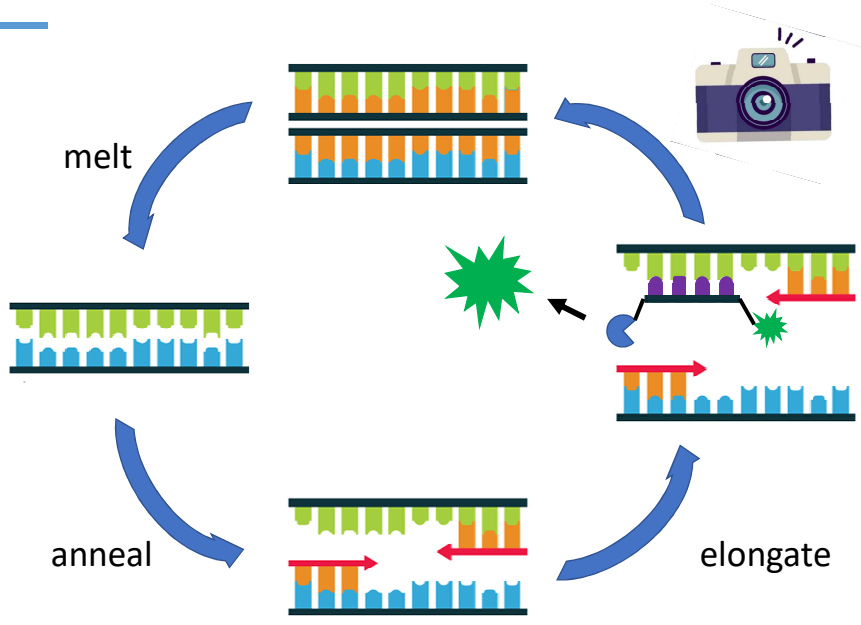
Assumptions and Agenda

Assumption: the basic principles of PCR and NGS as well as concepts of cfDNA/ctDNA have been covered in Day 1.

Agenda

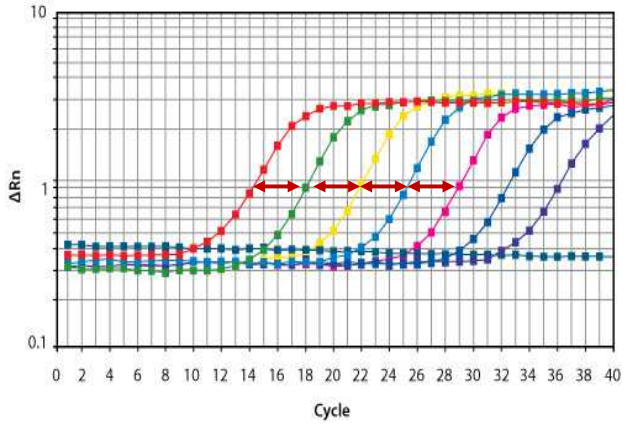
A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

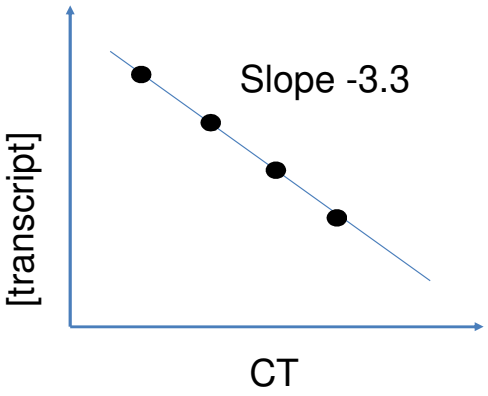
RT-PCR



Calibration Curves

Standard curve of calibrators of 10x dilutions





$2^x = 10$; $x = 3.3$

Each 10x dilution should be 3.3 CT apart.

BCR-ABL1 Math: How big is your denominator?

- ABL1 C_T not matter as much if the sample is positive.

$$\frac{50}{1000} = \frac{500}{10000} = \frac{5000}{100000}$$

- ABL1 C_T matters a lot if the sample is negative… it changes the analytical sensitivity of the assay

$$\frac{0}{100} \neq \frac{0}{10000} \neq \frac{0}{100000}$$

C_T	%IS	MR = log (100/%IS)
24	0.002%	4.7
25	0.004%	4.4
26	0.008%	4.1
27	0.016%	3.8

Molecular Milestones in CML

- Major Molecular Response (MMR): MR3 or 0.1% IS
  - disease progression is uncommon once this level of cytoreduction has been achieved
  - 20-59% of patients achieve MMR within 1 year on imatinib
  - 60-80% of patients achieve MMR within 5 years on imatinib

- Deep Molecular Response (DMR) = at least MR 4 or < 0.01% IS
  - 35-68% of patients achieve DMR within 5 years

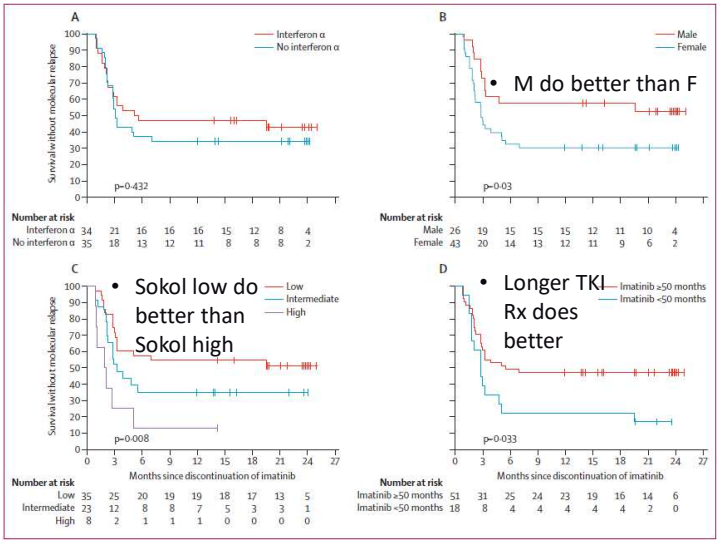
EARLY TREATMENT RESPONSE MILESTONES[i,j]

	BCR-ABL1 (IS)	3 months	6 months	12 months[k]
	>10%[l]	YELLOW	RED	
	>1%–10%	GREEN		YELLOW
	>0.1%–1%	GREEN		LIGHT GREEN
MMR	≤0.1%	GREEN		

Hochhouse *et al.* Leukemia. 2020; 34:966-984.
NCCN Guidelines, CML, 2021.

To STOP or not to STOP…

Stim 1 Trial



- DMR (MR4.5) > 2y and >5 QPCRs during that time
- 38% maintained molecular remission
- 98% of relapses within 8 mo
- All relapse pts responded to imatinib 400mg
- 62 achieved CR again after restarting TKI (median time to CR = 3 mo)

Mahon *et al.* *Lancet Oncol.* 2010; 11:1029-1035.

… That is the question

Table 8 Requirements for tyrosine kinase inhibitor discontinuation.

Mandatory:

- CML in first CP only (data are lacking outside this setting)
- Motivated patient with structured communication
- Access to high quality quantitative PCR using the International Scale (IS) with rapid turn-around of PCR test results
- Patient's agreement to more frequent monitoring after stopping treatment. This means monthly for the first 6 months, every 2 months for months 6–12, and every 3 months thereafter.

Minimal (stop allowed):

- First-line therapy or second-line if intolerance was the only reason for changing TKI
- Typical e13a2 or e14a2 BCR–ABL1 transcripts
- Duration of TKI therapy >5 years (>4 years for 2GTKI)
- Duration of DMR (MR^4 or better) >2 years
- No prior treatment failure

Optimal (stop recommended for consideration):

- Duration of TKI therapy >5 years
- Duration of DMR > 3 years if MR^4
- Duration of DMR > 2 years if $MR^{4.5}$

Table 10. Summary of Limited Longer-Term Follow-up Data from the TKI Discontinuation Trials

Trial	Treatment Prior to Discontinuation	No. of Patients	Depth and Duration of MR Required for Discontinuation	Trigger to Resume TKI Therapy	Median Follow-up	Treatment-free Remission (TFR) Rate
STIM1[213]	Imatinib ± interferon	100	MR5.0 for at least 2 years	Loss of MR5.0	77 months	38% at 60 months
TWISTER[218]	Imatinib ± interferon	40	MR4.5 for at least 2 years	Loss of MR5.0	103 months	45% (molecular relapse-free survival 45% at 8 years)
HOVON[214]	Imatinib + cytarabine	15	MR4.5 for at least 2 years	Loss of MR4.5	36 months	33% at 24 months
A-STIM[215]	Imatinib ± interferon	80	MR5.0 for at least 2 years	Loss of MMR	31 months	61% at 36 months
ISAV study[216]	Imatinib (after failure of interferon or hydroxyurea)	108	CMR for at least 18 months	Loss of MMR	36 months	52% at 36 months
KID study[217]	Imatinib ± interferon	90	MR4.5 for at least 2 years	Loss of MMR	27 months	59% at 24 months
Stop 2G-TKI[219]	Dasatinib/Nilotinib (first- or second-line)	60	MR4.5 for at least 24 months	Loss of MMR	47 months	54% at 48 months
DASFREE[224]	Dasatinib (first- or second-line)	84	MR4.5 for 12 months	Loss of MMR	2 years	46% at 24 months
ENESTFreedom[220]	Nilotinib (first-line)	190	MR4.5 for 12 months	Loss of MMR	96 weeks	49% at 96 weeks
ENESTop study[221]	Nilotinib (second-line)	126	MR4.5 for 12 months	Loss of MMR	96 weeks	53% at 96 weeks
DADI[225]	Dasatinib (first-line)	68	MR4.5 for at least 24 months	Loss of MMR	23 months	55% at 6 months
DADI[222]	Dasatinib (second-line)	63	MR4.0 for at least 12 months	Loss of MR4.0	44 months	44% at 36 months
EURO-SKI[223]	Any TKI	758	MR4.0 for at least 1 year	Loss of MMR	27 months	50% at 24 months

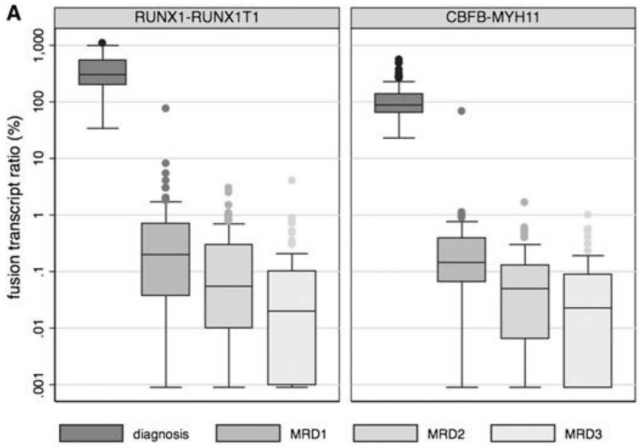
Hochhouse *et al.* *Leukemia*. 2020; 34:966-984.
NCCN Guidelines, CML, 2021.

2021 NCCN Guidelines on APL

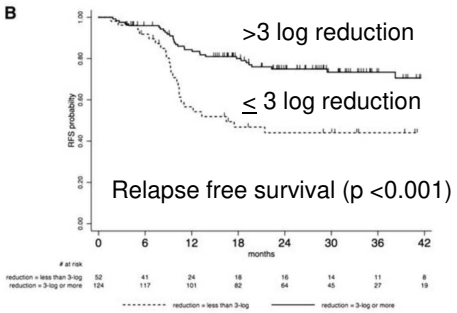
Indication	Testing	Comment
At diagnosis prior to Induction	t(15;17) by **karyotype, FISH, or molecular**	Prove the translocation
	If morphology and clinical history is highly suspicious but testing is negative, consider the possibility of an APL variant	
D10-14 Induction	Recommended to NOT perform any studies	**Morphologic and molecular studies may be misleading**
Post Induction	BM for documentation of morphologic remission	**The presence of measurable cytogenetic or molecular markers does not carry prognostic or therapeutic implications**
Post Consolidation	**PCR on PB** to document mCR **(does not specify quantitative or qualitative)** **(Should do earlier at 3-4 months during consolidation if on ATRA/As2O3)**	**If PCR+, repeat BM and PCR in 2-4 weeks. If still positive = relapse 1. If negative, monitor q 3mo x 2y.**
During Maintenance (PCR- after Consolidation)	For **high risk pts, PCR testing q3mo x 2y** on **PB, in the same lab with same sensitivity** For **low risk pts**, monitoring may not be necessary	***BM is more sensitive, but recommendation for PB.** **If pt becomes PCR+, repeat BM and PCR in 2-4 weeks**
After Relapse Chemotherapy	For cases of documented morphologic remission by BM, PCR on BM to determine molecular status	**Determines consolidation vs clinical trial, auto vs allo SCT decision**

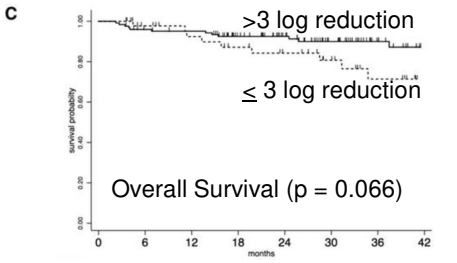
NCCN Guidelines, AML, 2021.

Core Binding Factor Leukemias: t(8;21), inv(16)



- Persistent transcript in remission
- Failure to achieve >3 log reduction is highly predictive of relapse (but not OS)





Jourdan *et al*. *Blood*. 2013; 121(12):2213-2223.
NCCN Guidelines, AML, 2021.

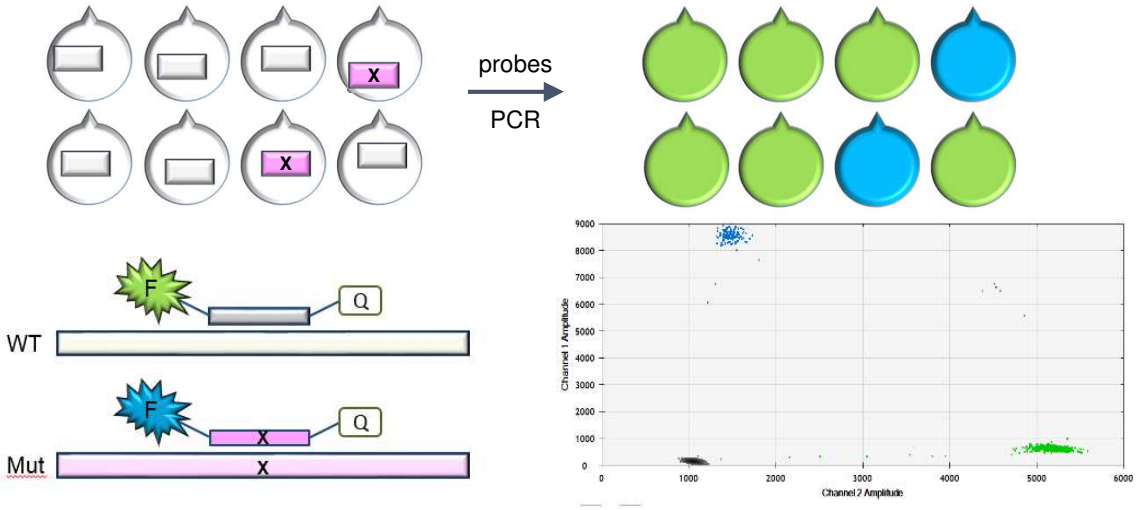
Agenda

Agenda

A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

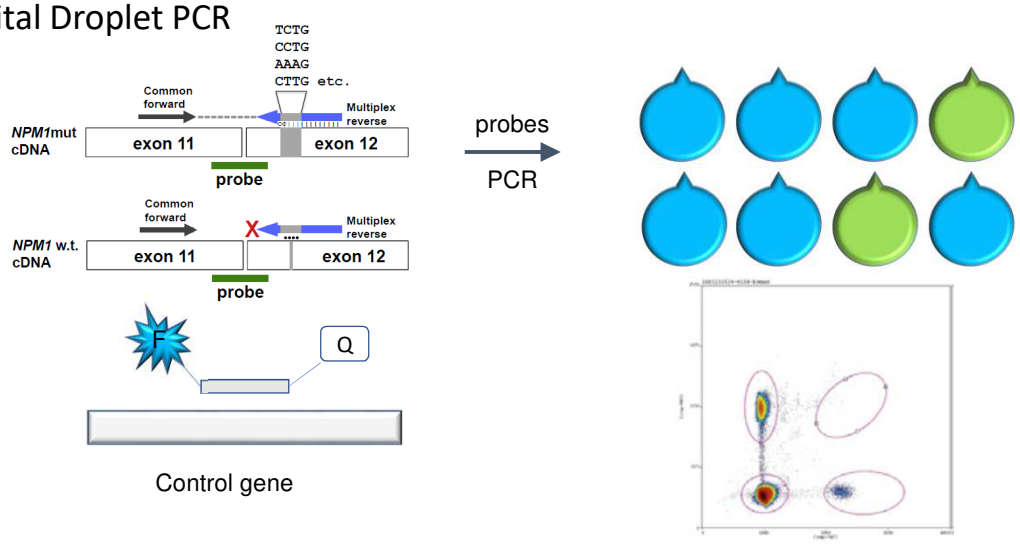
ddPCR

- Digital Droplet PCR



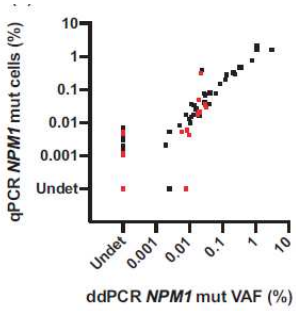
NPM1: Well established marker of AML used for MRD

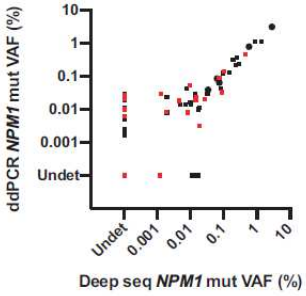
- Digital Droplet PCR



Mencia-Trinchant *et al. JMD.* 2017;19(4):537-548.

NPM1 by ddPCR



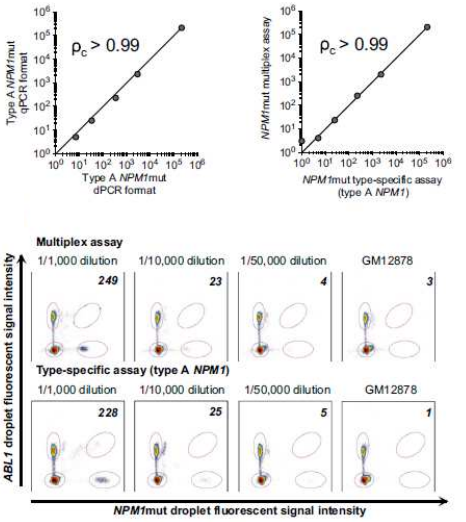


DNA-based MRD method	Sensitivity / PPA (%)	Specificity / NPA (%)	PPV (%)	NPV (%)
BM Detectable leukemic transcripts by RT-qPCR				
qPCR (95% CI)	84.4 (71.2-92.3)	81.8 (61.5-92.7)	90.5 (77.9-96.2)	72.0 (52.4-85.7)
ddPCR (95% CI)	71.1 (56.6-82.3)	100 (93.1-100)	100 (89.3-100)	62.9 (46.3-76.8)
Deep seq (95% CI)	60.0 (45.5-73.0)	100 (93.1-100)	100 (87.6-100)	55.0 (39.8-69.3)
PB Detectable leukemic transcripts by RT-qPCR				
qPCR (95% CI)	92.3 (66.7-98.6)	96.7 (83.3-99.4)	92.3 (66.7-98.6)	96.7 (83.3-99.4)
ddPCR (95% CI)	69.2 (42.4-87.3)	96.7 (83.3-99.4)	90.0 (59.6-98.2)	87.9 (72.7-95.1)
Deep seq (95% CI)	61.5 (35.5-82.3)	96.7 (83.3-99.4)	88.9 (56.5-98.0)	85.3 1(69.9-93.6)

- Typical decision point is at 3 log reduction from baseline.

Type A
LOD 1/10,000

Multiplex
LOD 2.4/10,000



Mencia-Trinchant *et al. JMD*. 2017;19(4):537-548.
Pettersson *et al. Int J Lab Hemat*. 2021;00:1-11.

Agenda

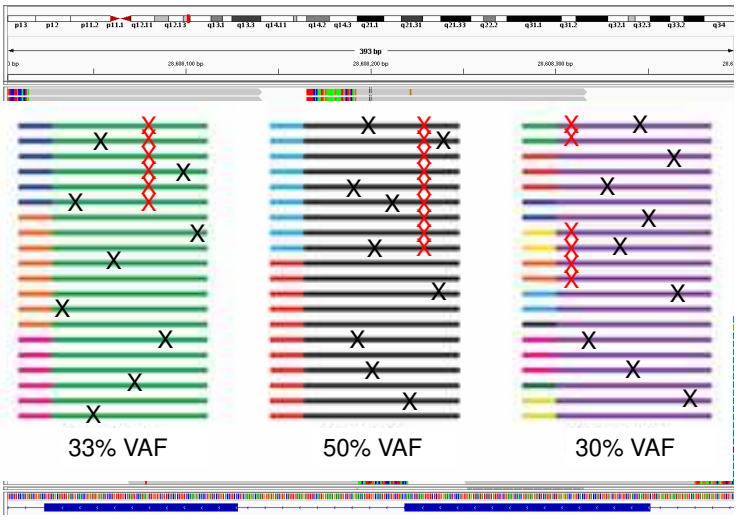
Agenda

A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

Methods to increase analytical sensitivity of NGS

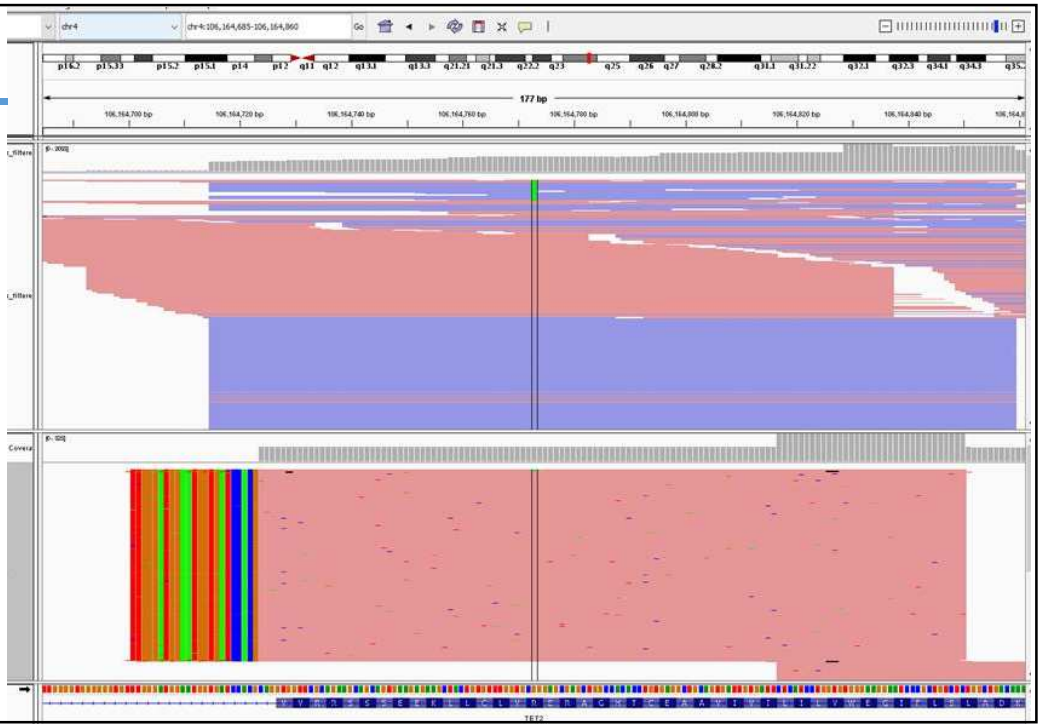
- Just get a LOT of reads (deep and ultra deep sequencing)
- Unique molecular identifiers (UMI, molecular barcodes)
- Error correction on a per nucleotide basis

UMIs



- Noise (individual errors) can be introduced by PCR or by sequencing and can be difficult to separate from a true variant
- If a subset of reads with the same UMI have a change, then it is likely noise.
- If all reads with the same UMI have a change, then it is likely a true variant
- Clinical Utilization: MRD and chimerism

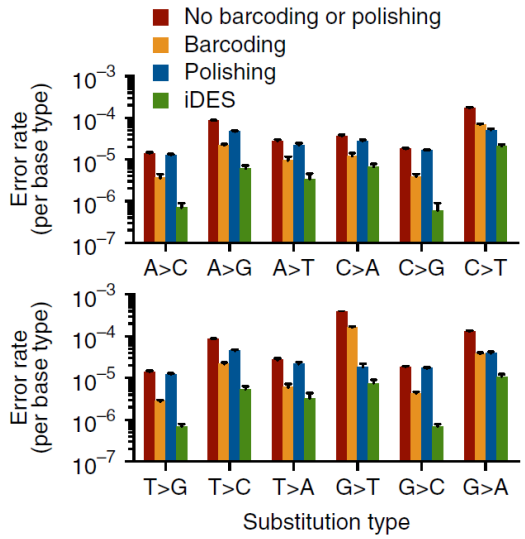
UMIs

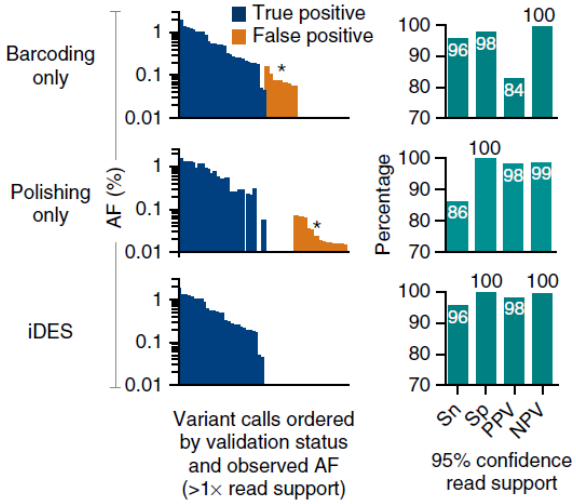


Methods to increase analytical sensitivity of NGS

- Just get a LOT of reads (deep and ultra deep sequencing)
- Unique molecular identifiers (UMI, molecular barcodes)
- Error correction on a per nucleotide basis

Error Correction





Newman *et al. Nature Biotechnol.* 2016;34:547-555.

Agenda

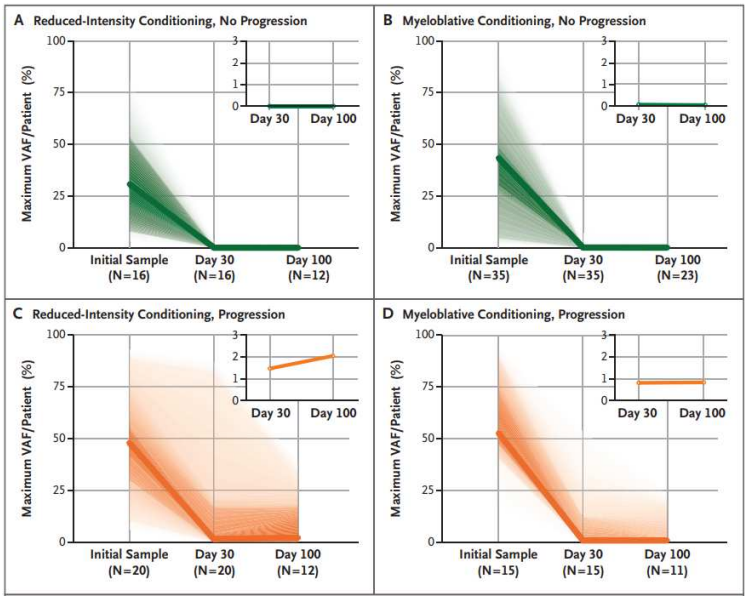
Agenda

A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

MDS MRD

No Progression

Progression

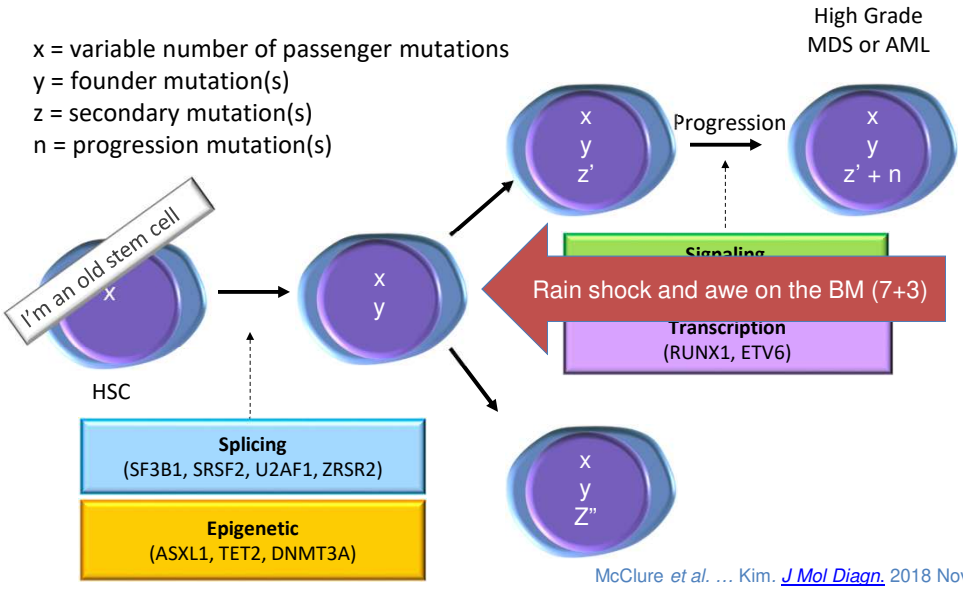


Duncavage et al. *NEJM.*2018;379:1028-1041.

AML MRD

Who are highly dysregulated and have trouble signaling…

x = variable number of passenger mutations
y = founder mutation(s)
z = secondary mutation(s)
n = progression mutation(s)

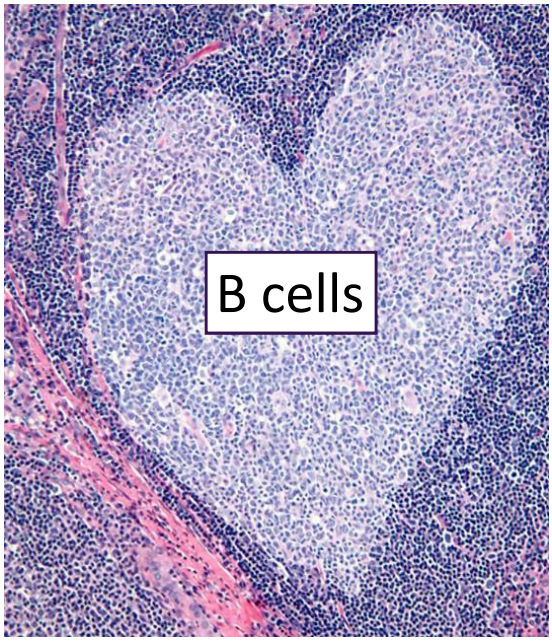


McClure *et al.* … Kim. *J Mol Diagn.* 2018 Nov;20(6):717-737.
Jongen-Lavrencic *et al. NEJM.* 2018:378(13)1189-1199.

Agenda

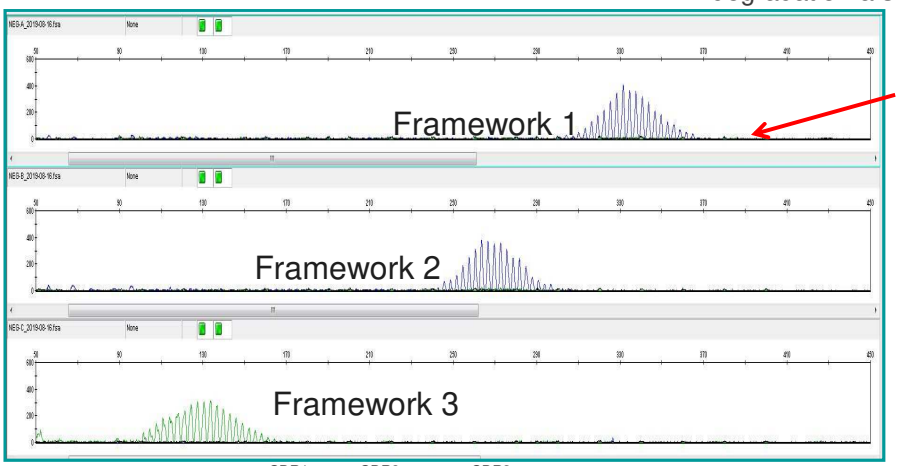
Agenda

A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

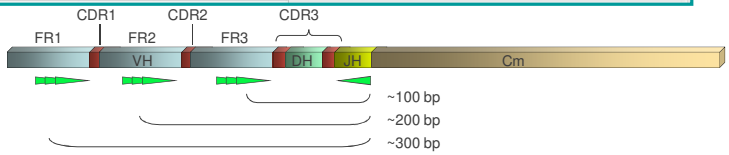


IGH Gene Rearrangements

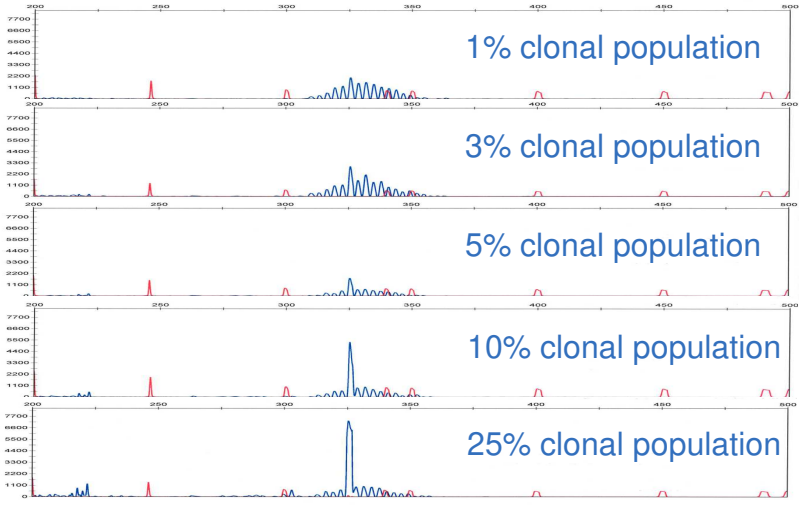
Sizes up to >350 nt, so FFPE degradation a significant concern



3 bp apart (inframe)

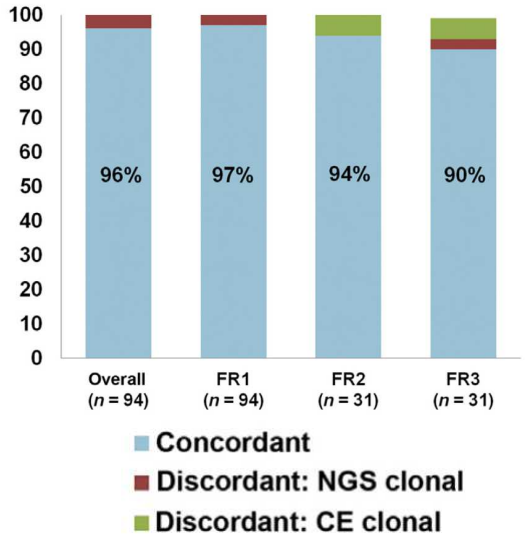


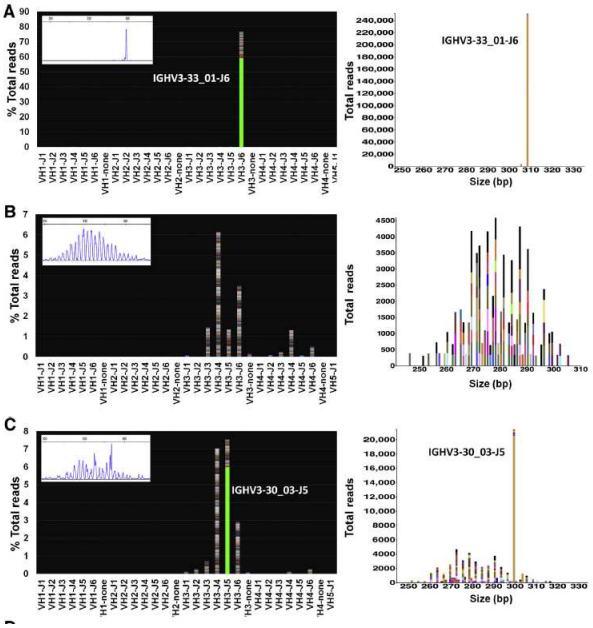
Analytical Sensitivity: how low can you go?



Kim chapter 88 from Wintrobes14th Ed.

NGS vs CE





Arcila *et al. JMD* (2019) 21:330-342.

Clonality rules are still a challenge

MSKCC Clonality Rules

Category 1: Optimal results (>100,000 reads, >50,000 in some cases)

- **dominant sequence(s) >2.5% of total reads: same VJ usage**
- **dominant sequence(s) >10x the polyclonal background**

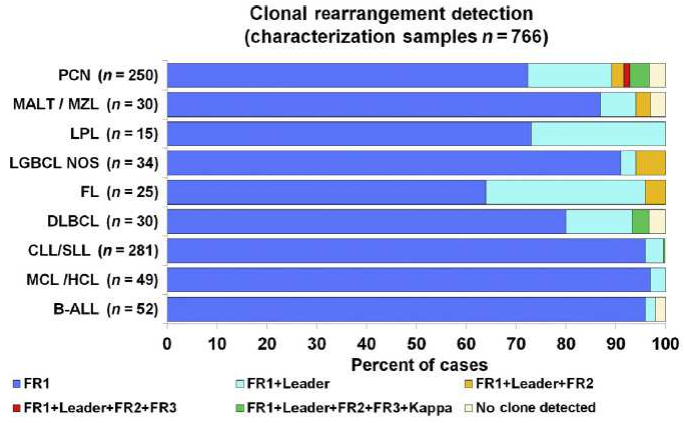
Category 2: Qualified results (30,000-50,000 reads)

- **dominant sequence(s) >5% of total reads: same VJ usage**
- **dominant sequence(s) >20x the polyclonal background**

Category 3: failure (<30,000 reads)

Arcila *et al. JMD* (2019) 21:330-342.

NGS for B cell Neoplasms: Clinical sensitivity



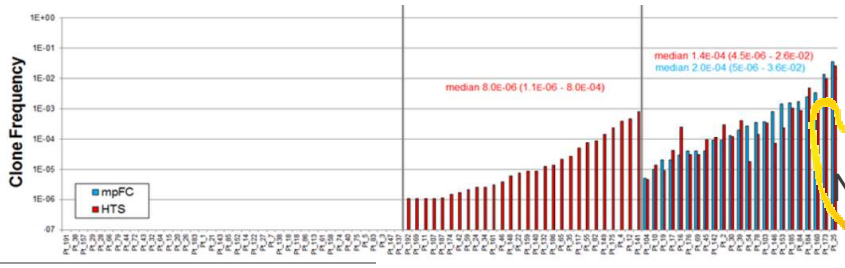
- Analytical LOD: 1% neoplastic cells
- Input DNA as low as 25 ng

Clinical Sensitivity by CE

Method	MCL	CLL	FL	DLBCL	MZL
IGH FR1	100	95-100	30-73	50-68	48-73
IGH FR2	98-100	91-100	30-76	58-61	66-85
IGH FR3	96-100	93-100	13-52	50	62-68
IGH FR1-3	100	100	37-84	79-88	86-88
IGK	75-94	96-100	60-63	58-61	62-68
IGK_{de}	50-75	61-67	57-59	46-58	48-54
all IGK	100	100	80-84	75-80	69-83
IGL	44-75	30-44	21-23	8-28	28-29
IGH FR1-3 and all IGK	100	100	100	96-98	95-100

Arcila *et al. JMD* (2019) 21:330-342.
Kim chapter 88 from Wintrobes14th Ed.

NGS versus Flow Cytometry in B-ALL



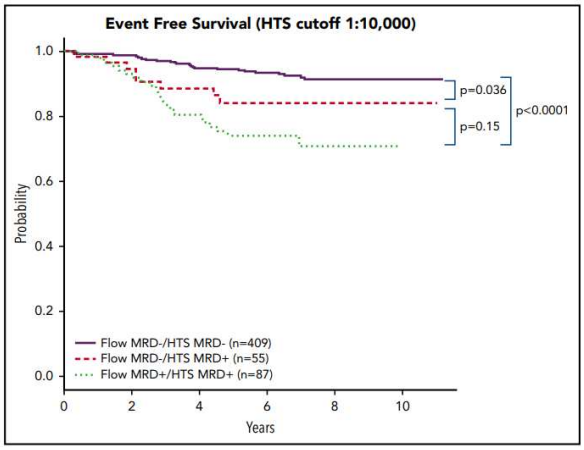
Patient ID	Input cells, n	% MRD		
		Sequencing	Flow cytometry	PCR
9	248 496	0.00004	UD	UD
12	133 669	0.0027	UD	0.0017
25	142 310	0.0106	UD	0.0005
30	120 307	0.0032	UD	0.0076
31	1 109 253	0.0014	UD	UD
41	626 380	0.0005	UD	0.0051
58	171 326	0.0102	UD	0.0120
73	983 289	0.0004	UD	UD
98	162 217	0.0097	UD	0.0038
29	161 142	0.9607	UD	1.2000
36	1 070 242	UD	UD	0.0020

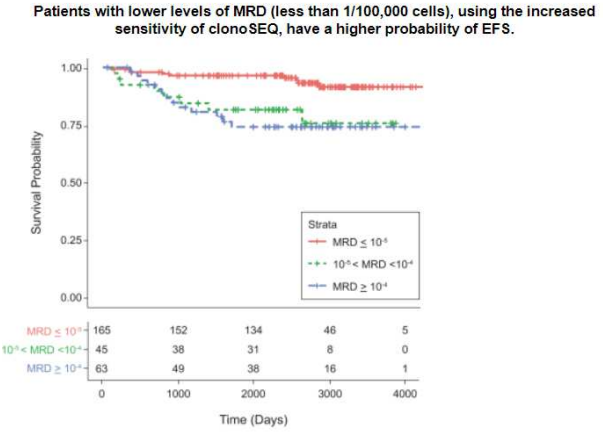
UD indicates undetectable.

90% concordance between NGS and FC
96% concordance between NGS and ASO-PCR

Wu *et al. Clin Cancer Res.* 2014;20:4540-4548.
Farham *et al. Blood.* 2012;120:5173-5180.

More Sensitive is Better!

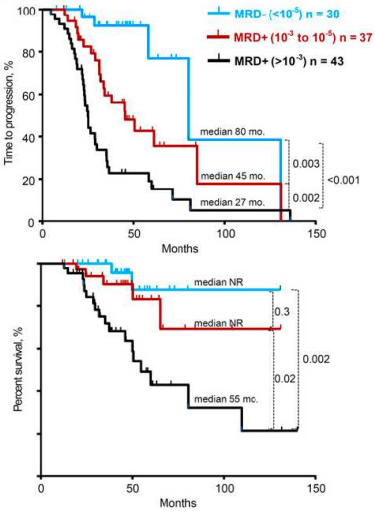




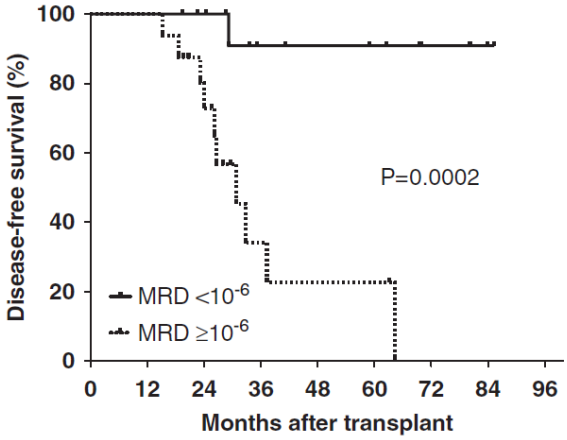
Wood *et al. Blood.* 2018; 131(12):1350-1359.
https://www.sec.gov/Archives/edgar/data/1478320/000119312519161447/d706383ds1.htm

NGS for other B cell Neoplasms

Plasma cell myeloma, on GEM protocols

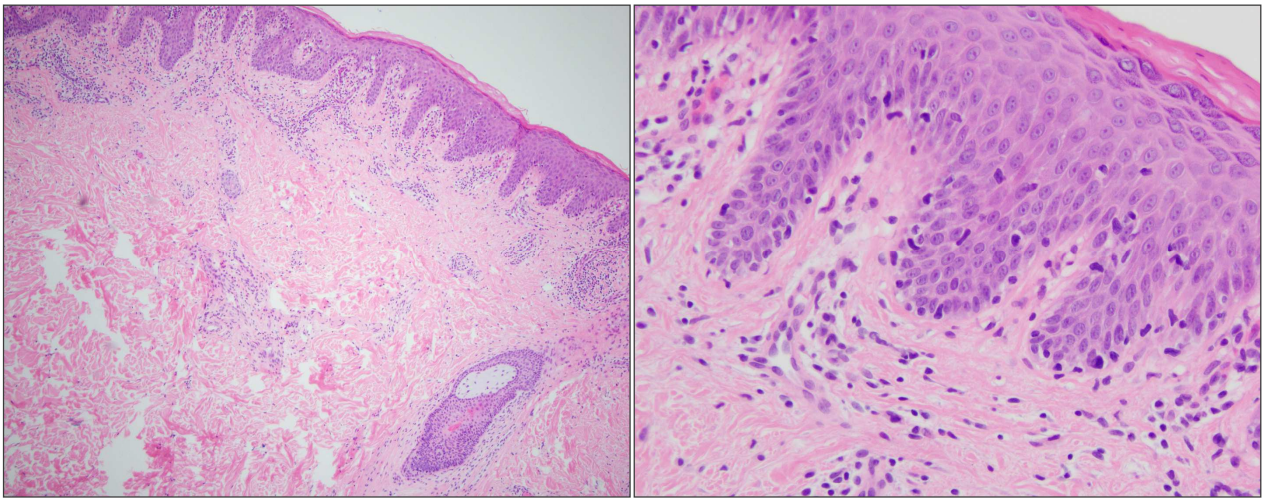


Chronic lymphocytic leukemia, post-SCT relapse



Martinez-Lopez *et al. Blood.* 2014; 123:3073-3079.
Logan et al. Leukemia. 2013; 27:1659-1665.

Mycoses Fungoides



- Tumor burden often very low in ALI vs early patch phase MF
- Biopsy size typically small

TCR NGS? Better Clinical Sensitivity and Quantification for MF

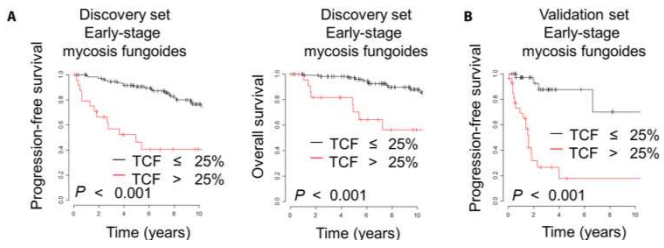
- Compared MF with positive TCRG by CE (n = 15) vs negative TCR by CE (n = 19)

	CE+	CE-
NGS+	13	16
NGS-	2*	3

* Clones found at <5% clone frequency (**analytic sensitivity**)

	CE	NGS
Clinical Sensitivity	**44%**	**85%**

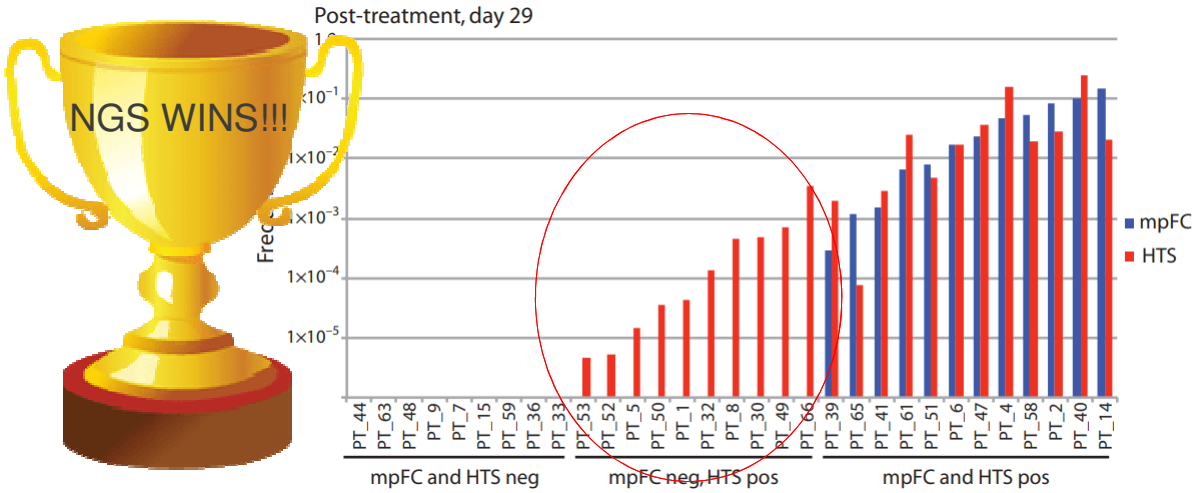
- >25% clone frequency in early stage (stage I A/B, limited to skin) is associated with poor prognosis



Sufficool *et al. J Am Acad Dermatol.* 2015;73:228-236.
Masson *et al. Sci Transl Med.* 2018;10:eaar5894.

NGS for T-ALL MRD

NGS MRD 10-100 fold lower than when both FC and NGS positive



ETP-ALL and near-ETP-ALL: less likely to have a diagnostic complete clonal rearrangement and more likely to be MRD+.

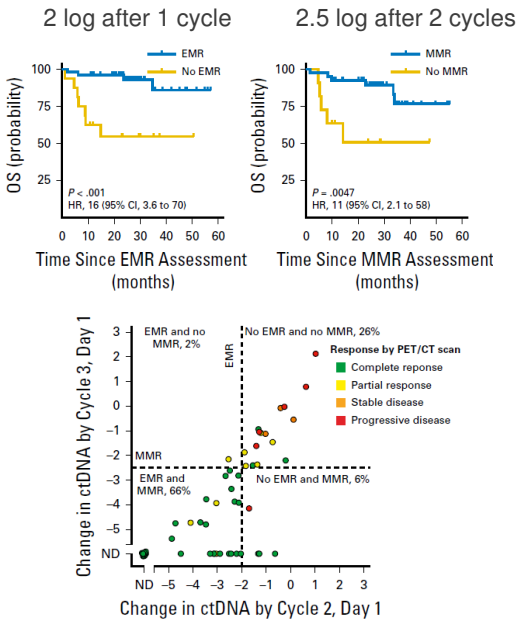
Wu *et al. Sci Transl Med.* 2012;4:134ra63.

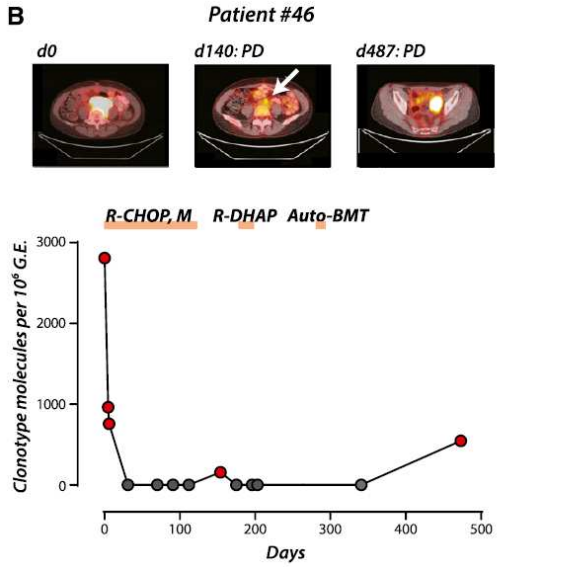
Agenda

Agenda

A. RT-PCR: CML and AML
B. ddPCR : *NPM1* in AML
C. NGS with UMIs and error correction
D. MDS and AML by NGS
E. IGH and TCR by NGS
F. cfDNA for lymphoid neoplasms

cfDNA Monitoring by Gene Rearrangements: DLBCL





Kurtz *et al. Blood.* 2015; 125:3679-3687.
Kurtz *et al. JCO.* 2018; 36:2845-2853.