

# Literature Review

The claims within these publications have not been verified or validated by Invivoscribe

# Clonality Testing

Gel & Capillary Electrophoresis



# EuroClonality Design



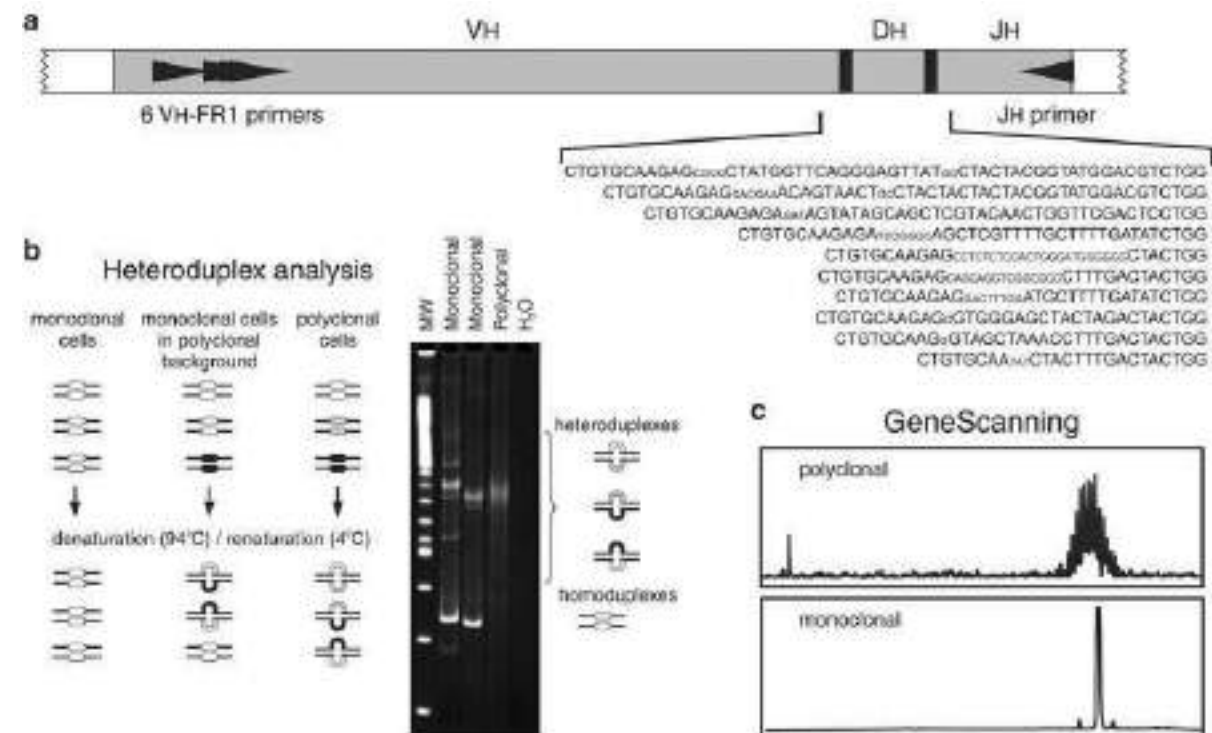
BIOMED-2/EuroClonality description of primer and assay design

Descriptions of *IGH*, *IGK*, *IGL*, *TCRB*, *TCRG*, and *TCRD* loci

Good resource for justification of Biomed-2 designs

**Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936.**

van Dongen JJ<sup>1</sup>, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuurink E, García-Sanz R, van Krieken JH, Droese J, González D, Bastard C, White HE, Spaargaren M, González M, Parreira A, Smith JL, Morgan GJ, Kneba M, Macintyre EA.



# EuroClonality Interpretation



Describes selection of targets

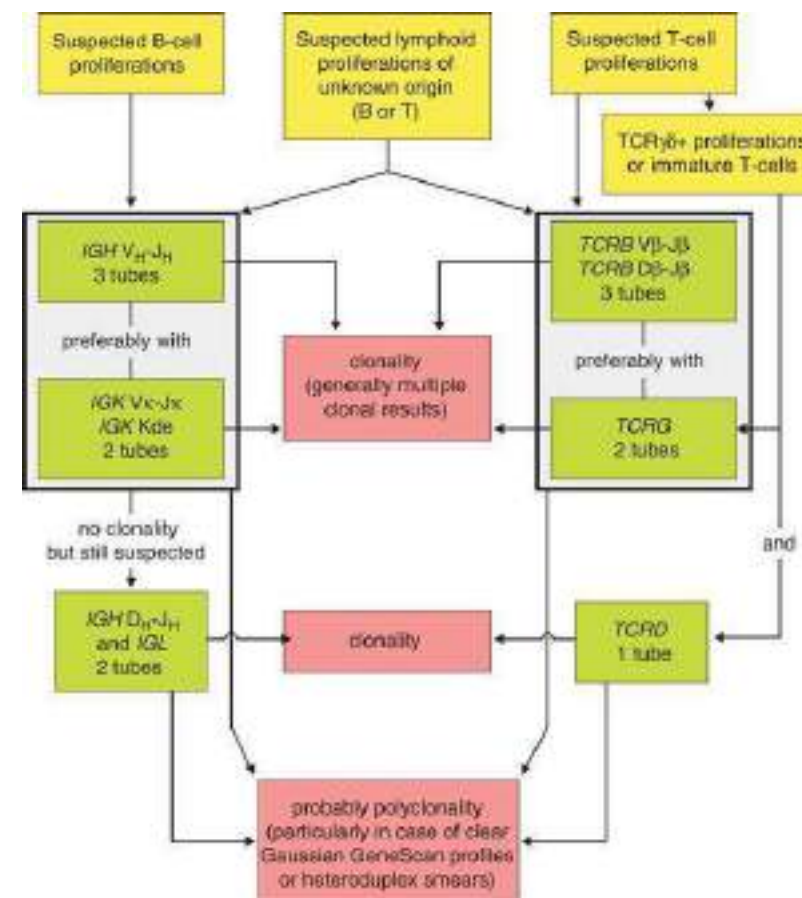
Discusses very specific technical and biological pitfalls of molecular clonality testing

Lists immunobiological conditions such as pseudoclonality

Provides a uniform system for technical description

## EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations.

Langerak AW<sup>1</sup>, Groenen PJ, Brüggemann M, Beldjord K, Bellan C, Bonello L, Boone E, Carter GI, Catherwood M, Davi F, Delfau-Larue MH, Diss T, Evans PA, Gameiro P, Garcia Sanz R, Gonzalez D, Grand D, Håkansson A, Hummel M, Liu H, Lombardia L, Macintyre EA, Milner BJ, Montes-Moreno S, Schuurin E, Spaargaren M, Hodges E, van Dongen JJ.



# Multiplexing Increases Detection Rates



Testing complimentary targets **improves confidence**

Majority of mature B-cell malignancies can be identified by the use of 3 *IGH* tubes and 2 *IGK* tubes

A positive result in any one tube is sufficient for clonality determination

**Significantly improved PCR-based clonality testing in B-cell malignancies by use of multiple immunoglobulin gene targets. Report of the BIOMED-2 Concerted Action BHM4-CT98-3936.**

Evans PA<sup>1</sup>, Pott Ch, Groenen PJ, Salles G, Davi E, Berger F, Garcia JF, van Krieken JH, Pals S, Kluin P, Schuurung E, Spaargaren M, Boone E, González D, Martínez B, Villuendas R, Gameiro P, Diss TC, Mills K, Morgan GJ, Carter GI, Milner BJ, Pearson D, Hummel M, Jung W, Ott M, Canioni D, Beldjord K, Bastard C, Delfau-Larue MH, van Dongen JJ, Molina TJ, Cabecadas J.

	<i>IGH</i> FR1	<i>IGH</i> FR2	<i>IGH</i> FR3	<i>IGH</i> (FR1,2,3)	<i>IGK</i> (Vk-Jk, Kde)	Total ( <i>IGH</i> , <i>IGK</i> )
MCL (n=54)	100%	98%	96%	100%	100%	100%
B-CLL/SLL (n=56)	95%	91%	93%	100%	100%	100%
FL (n=109)	73%	76%	52%	84%	84%	100%
MZL (n=41)	73%	85%	68%	88%	83%	97%
DLBCL (n=109)	68%	61%	50%	79%	80%	96%
TOTAL (n=369)	79%	78%	66%	88%	88%	98%

# Multiplexing Increases Detection Rates



Testing complimentary targets  
**improves confidence**

Majority of T-cell malignancies can be identified by the use of 3 *TCRB* tubes and 2 *TCRG* tubes

A positive result in any one tube is sufficient for clonality determination

**Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies Report of the BIOMED-2 Concerted Action BHM4 CT98-3936.**

[Brüggemann M<sup>1</sup>](#), [White H](#), [Gaulard P](#), [Garcia-Sanz R](#), [Gameiro P](#), [Oeschger S](#), [Jasani B](#), [Ott M](#), [Delsol G](#), [Orfao A](#), [Tiemann M](#), [Herbst H](#), [Langerak AW](#), [Spaargaren M](#), [Moreau E](#), [Groenen PJ](#), [Sambade C](#), [Feroni L](#), [Carter GI](#), [Hummel M](#), [Bastard C](#), [Davi F](#), [Delfau-Larue MH](#), [Kneba M](#), [van Dongen JJ](#), [Beldjord K](#), [Molina TJ](#).

	<i>TRB</i>	<i>TRG</i>	<i>TRB+TRG</i>
<b>T-PLL</b>	100%	94%	100%
<b>T-LGL</b>	96%	96%	100%
<b>PTCL-U</b>	98%	94%	100%
<b>AITL</b>	89%	92%	95%
<b>ALCL</b>	74%	74%	79%*
<b>Total</b>	91%	89%	94%*

\*Approximately 20–25% of ALCL are known to have no TCR gene rearrangements and are defined as null ALCL  
**J.J.M. van Krieken et al. *Leukemia*. 2007 21:201-206.**

51 T-ALL cases tested with IdentiClone® TCRG, TCRB, and TCRD assays

74.5% of T-ALL cases had clonality detected in at least one TCR locus

Clonality detected in **93.8%** of childhood cases (<12 years)

## T-Cell Receptor Rearrangements Determined Using Fragment Analysis in Patients With T-Acute Lymphoblastic Leukemia.

Kim H<sup>1</sup>, Kim IS<sup>#2</sup>, Chang CL<sup>#3</sup>, Kong SY<sup>4</sup>, Lim YT<sup>5</sup>, Kong SG<sup>6</sup>, Cho EH<sup>7</sup>, Lee EY<sup>1</sup>, Shin HJ<sup>8</sup>, Park HJ<sup>9</sup>, Eom HS<sup>10</sup>, Lee H<sup>10</sup>.

Frequency of TCR clonality detected using fragment analysis at initial diagnosis according to three age groups of T-ALL patients

	Total (N=51)	Children (18 yr; N=26)	Adolescents (12–18 yr; N=9)	Adults (>18 yr; N=26)
None, N (%)	13 (25.5)	1 (7.7)	4 (30.8)	8 (61.5)
TCRβ, N (%)	2 (3.9)	1 (50.0)		1 (50.0)
TCRγ, N (%)	11 (21.6)	5 (45.5)		6 (54.5)
TCRδ, N (%)	5 (9.8)	2 (40.0)		3 (60.0)
TCRβ+TCRγ, N (%)	11 (21.6)	6 (54.5)	3 (27.3)	2 (18.2)
TCRβ+TCRδ, N (%)	0 (0.0)			
TCRγ+TCRδ, N (%)	4 (7.8)			4 (100)
TCRβ+TCRγ+TCRδ, N (%)	5 (9.8)	1 (20.0)	2 (40.0)	2 (40.0)
At least one TCR	38 (74.5)	15 (93.8)	5 (55.6)	18 (69.2)

# TCRG 2.0 Comparison

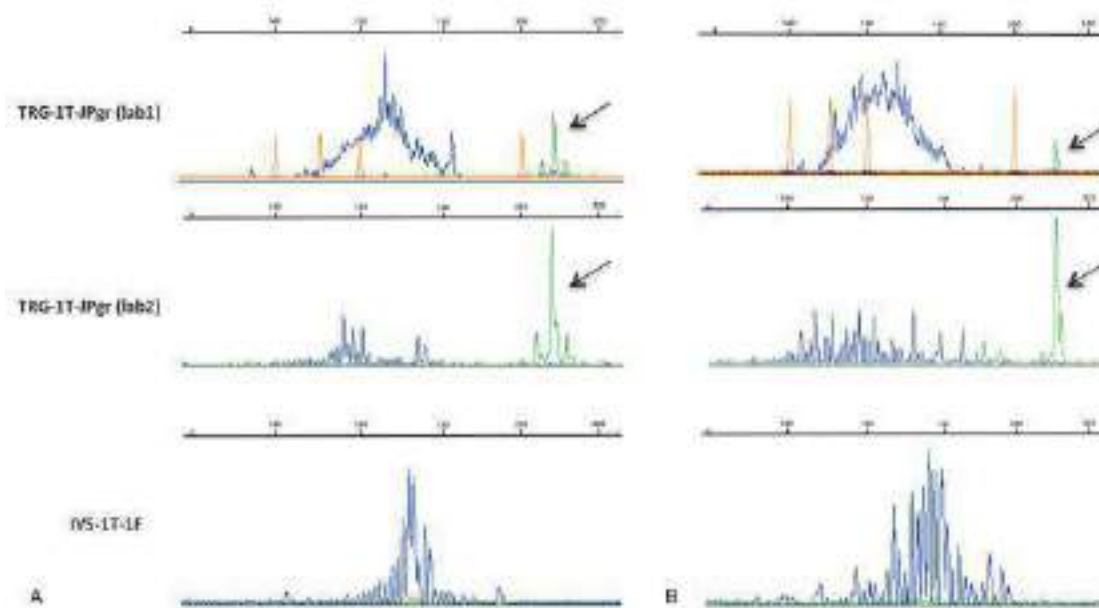
Direct comparison of EuroClonality 2-tube design, EuroClonality 1-tube design, and Invivoscribe design

Invivoscribe 1-tube assay:

- reduced DNA input
- simplified interpretation
- reduced false positives
- FFPE samples may amplify better due to shorter amplicon size
- availability of an interpretation algorithm

A New and Simple TRG Multiplex PCR Assay for Assessment of T-cell Clonality: A Comparative Study from the EuroClonality Consortium

[Marine Armand](#),<sup>1</sup> [Coralie Derrieux](#),<sup>2</sup> [Kheira Beldjord](#),<sup>3</sup> [Tamara Wabeke](#),<sup>4</sup> [Dido Lenze](#),<sup>5</sup> [Elke Boone](#),<sup>6</sup> [Monika Bruggemann](#),<sup>7</sup> [Paul A.S. Evans](#),<sup>8</sup> [Paula Gameiro](#),<sup>9</sup> [Michael Hummel](#),<sup>5</sup> [Patrick Villarese](#),<sup>2</sup> [Patricia J.T.A. Groenen](#),<sup>10</sup> [Anton W. Langerak](#),<sup>4</sup> [Elizabeth A. Macintyre](#),<sup>2</sup> and [Frederic Davi](#)<sup>1</sup>





# Clonality Testing

NGS



# Evolution of Clonality Testing



“All of these studies highlight that the emergence of NGS has opened the door to a **new era in diagnostic medicine**, bringing the vision of ‘personalized medicine’ closer to reality.”

“Hence, this technology becomes available for healthcare applications, physicians and patients will increasingly demand **refined diagnosis and treatment strategies** tailored to the clinical needs of an individual patient.”

The evolution of clonality testing in the diagnosis and monitoring of hematological malignancies

[Anna Gezzola](#), [Claudia Mannu](#), [Mauro Rossi](#), [Maria Antonella Laginestra](#), [Maria Rosaria Sapienza](#), [Fabio Fuligni](#), [Maryam Etebari](#), [Federica Melle](#), [Elena Sabatini](#), [Claudio Agostinelli](#), [Francesco Bacchi](#), [Carlo Alberto Sagramoso Sacchetti](#), [Stefano Aldo Pilati](#), and [Pier Paolo Piccaluga](#)<sup>§</sup>

	Time	Material load	Sensitivity	Specificity
Southern blotting	Days	10,000–20,000 ng	Low/ intermediate	Very high
Polymerase chain reaction	Hours	100–500 ng	Very high	high
Next-generation sequencing	Hours	20 ng	High? <sup>*</sup>	High? <sup>*</sup>
*To be validated.				

# Clonality in Mycosis Fungoides



Most common cutaneous neoplasm;  
diagnosis is often challenging

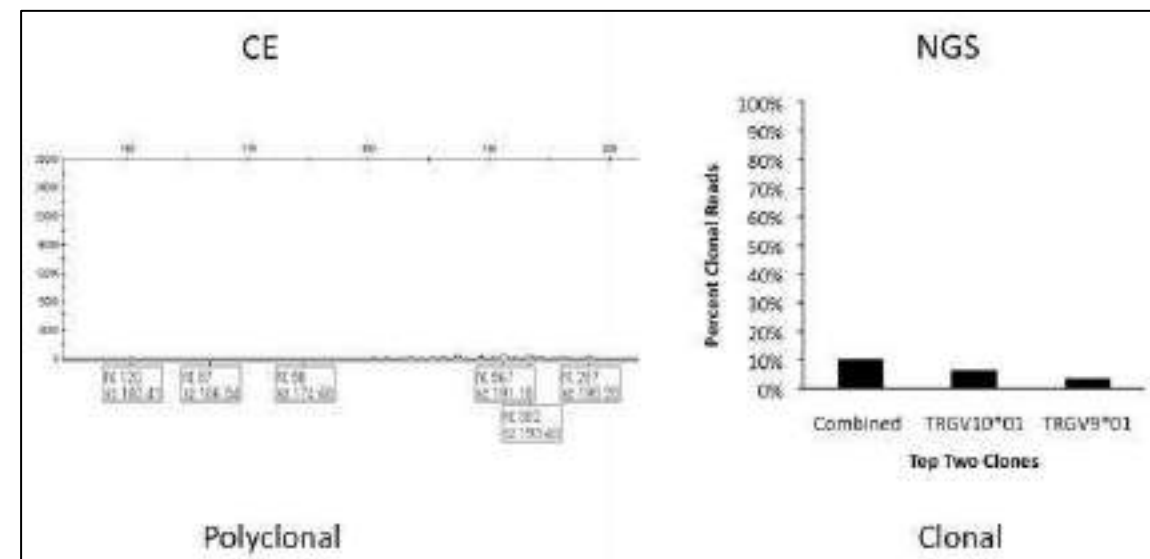
Sought to determine if NGS-based  
detection has increased sensitivity  
for T-cell clonality

35 FFPE tested by CE & NGS

- 44% of samples showed clonal rearrangement by CE
- **85% of samples showed clonal rearrangement by NGS**

**T-cell clonality assessment by next-generation sequencing improves detection sensitivity in mycosis fungoides.**

Sufficool KE<sup>1</sup>, Lockwood CM<sup>1</sup>, Abel HJ<sup>1</sup>, Hagemann IS<sup>1</sup>, Schumacher JA<sup>2</sup>, Kelley TW<sup>3</sup>, Duncavage EJ<sup>4</sup>.





# LymphoTrack<sup>®</sup> Assays

Publications

# Establishment of Routine IGH Testing

MSKCC validation of LymphoTrack<sup>®</sup> IGH assays for clinical use

>1000 samples tested over 1.5 years

Direct comparison to capillary electrophoresis assays

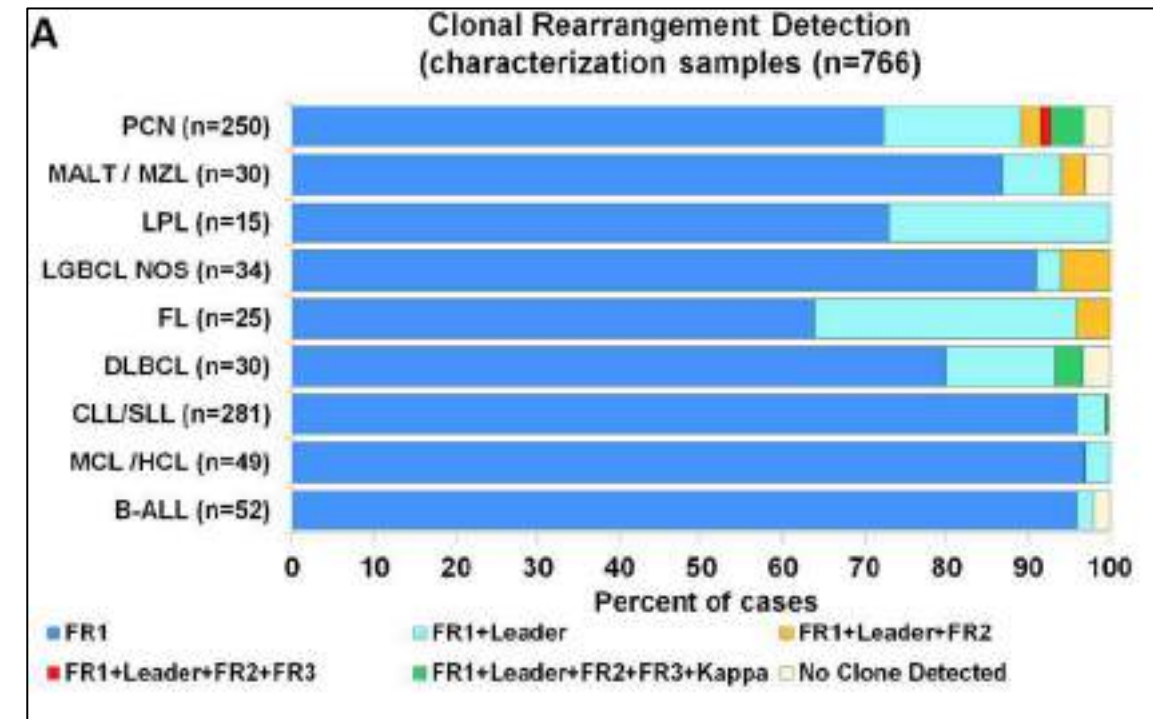
- 96% Concordance
- **NGS shows superior performance**

>97% detection

Studies: DNA Input, LOD, Precision

Establishment of Immunoglobulin Heavy (IGH) Chain Clonality Testing by Next-Generation Sequencing for Routine Characterization of B-Cell and Plasma Cell Neoplasms

Maria E. Arcila,<sup>\*\*</sup> Wayne Yu,<sup>†</sup> Mustafa Syed,<sup>†</sup> Hannah Kim,<sup>†</sup> Lidia Maciag,<sup>†</sup> JinJuan Yao,<sup>†</sup> Caleb Ho,<sup>†</sup> Kseniya Petrova,<sup>†</sup> Christine Mung,<sup>†</sup> Paulo Salazar,<sup>†</sup> Ivelise Rijo,<sup>†</sup> Tessara Baldi,<sup>†</sup> Ahmet Zehir,<sup>†</sup> Ola Landgren,<sup>†</sup> Jae Park,<sup>†</sup> Mikhail Roshal,<sup>†</sup> Ahmet Dogan,<sup>†</sup> and Khedoudja Nafa<sup>†</sup>



# Establishment of Routine TRG Testing

Aims to **reduce the subjectivity** present in fragment analysis

Provides a laboratory specific interpretation algorithm for LymphoTrack<sup>®</sup> TRG

Shows **significant added value** of NGS

- **92% Sensitivity** of NGS in fresh samples
- Analysis of **FFPE** was more successful in NGS (34/36 cases) than capillary electrophoresis (16/36 cases)

Evaluation of next-generation sequencing-based clonality analysis of T-cell receptor gamma gene rearrangements based on a new interpretation algorithm.

Nollet F<sup>1</sup>, Vanhouteghem K<sup>1</sup>, Vermeire S<sup>1</sup>, Maelbrancke E<sup>1</sup>, Emmerechts J<sup>1</sup>, Devos H<sup>1</sup>, Cauwelier B<sup>1</sup>.

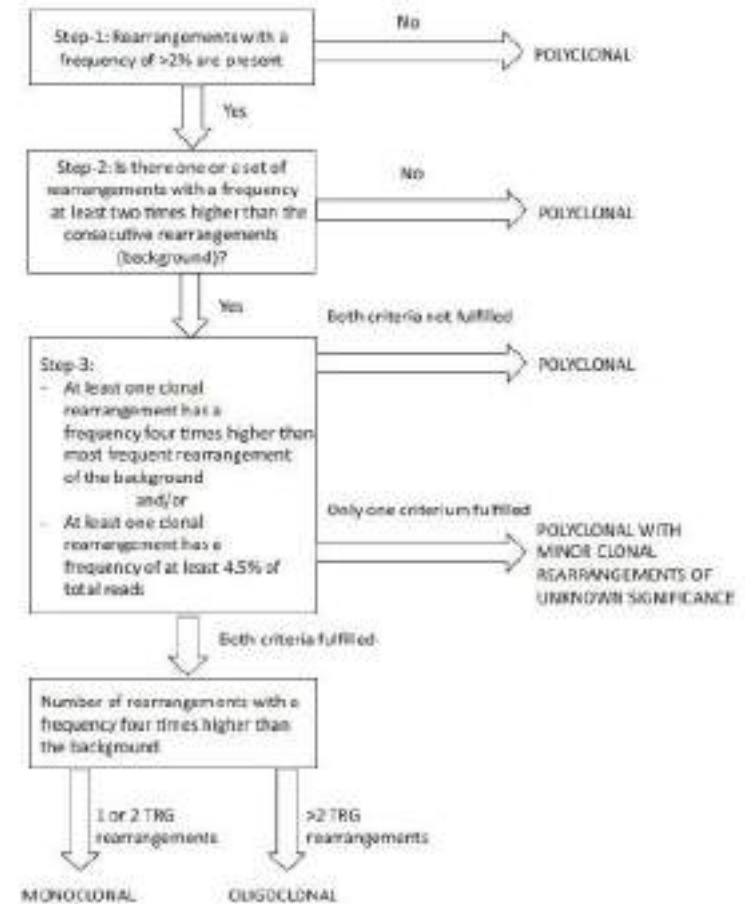


FIGURE 1 Interpretation algorithm for each LymphoTrack Dx TRG assay reaction

# Case Study – TRG Clonality

Describes **41 cases in detail**

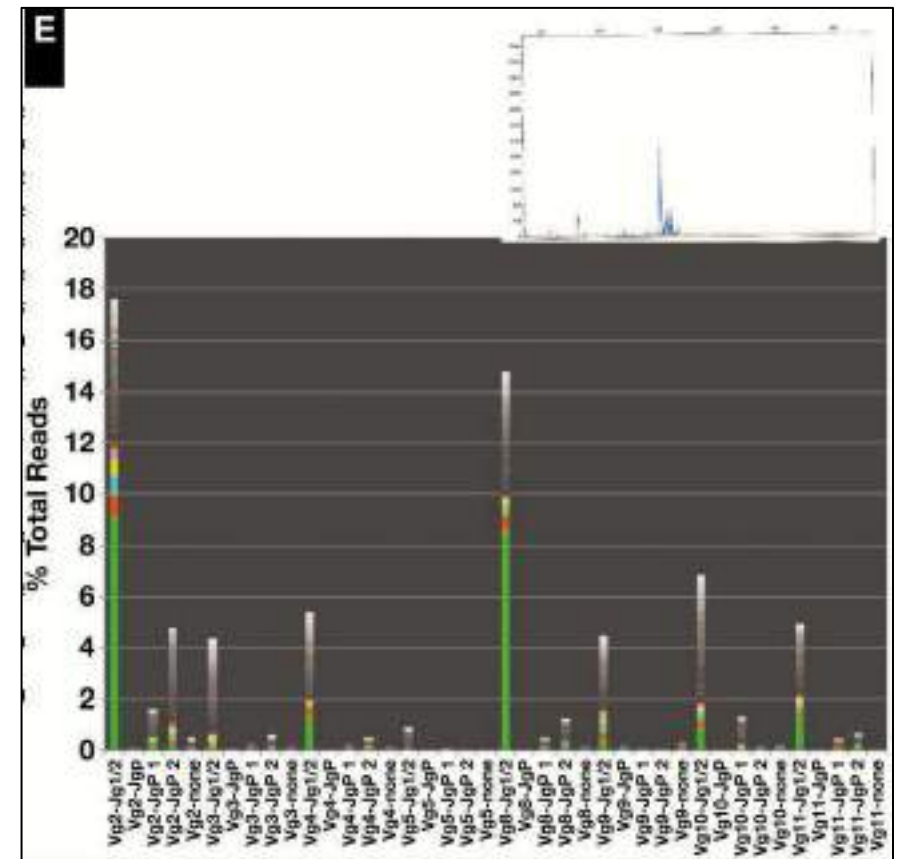
Provides the laboratory's strategy for interpretation of NGS results

Direct comparison to capillary electrophoresis assays

- **NGS had greater sensitivity**
- NGS identified small clones including and minimum clonal percentages within all T cells

The Value of T-Cell Receptor  $\gamma$  (TRG) Clonality Evaluation by Next-Generation Sequencing in Clinical Hematolymphoid Tissues.

[Kansal R<sup>1</sup>](#), [Grody WW<sup>1</sup>](#), [Zhou J<sup>1</sup>](#), [Dong L<sup>1</sup>](#), [Li X<sup>1</sup>](#).



# Case Study – B- and T-Cell Clonality

B-cell lymphoma evolved into a T-cell lymphoma

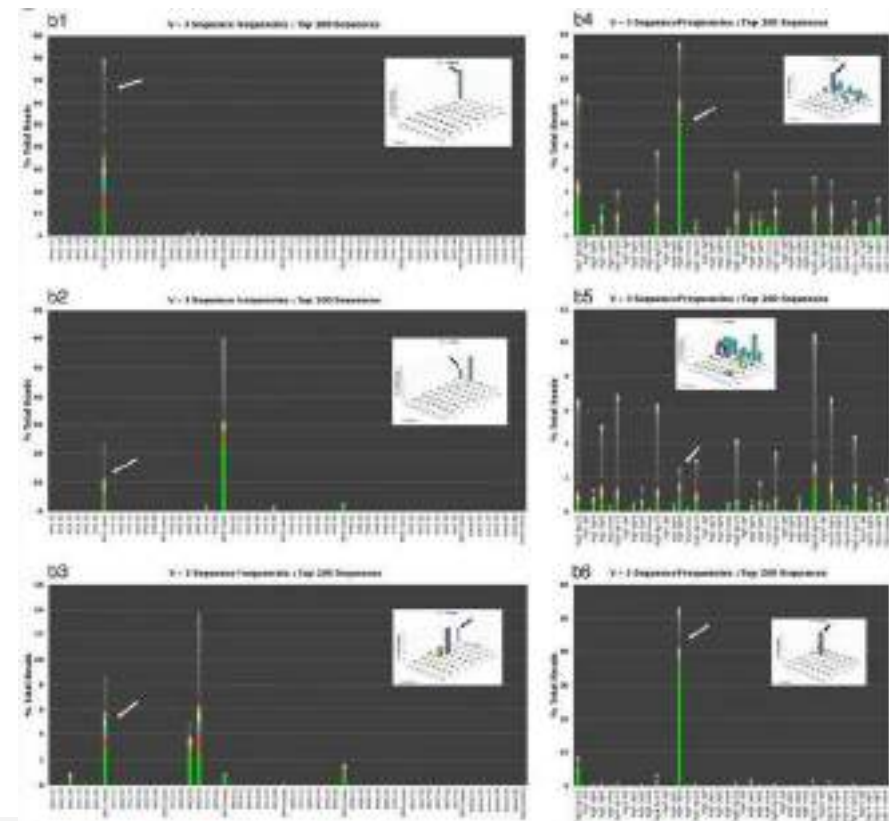
Complex morphology and difficulty classifying two relapses

**NGS detected** both B- and T cell clones in primary sample and both relapses

“The *IGH* and *TRG* genes were monoclonally rearranged in the primary and both relapses. The monoclonal rearrangement of the *IGH* gene was detected only by deep sequencing.”

Molecular genetic data favoring a sequential clonal transformation of a large B cell lymphoma into an anaplastic large T cell lymphoma, ALK-negative

[Tomas Vanecek](#), [Kimberly Walker](#), [Linden L. Watson](#), [Arundhati Rao](#), [Debby Rampisela](#) & [Ludvik R. Donner](#)





# Case Study – Repertoire Analysis

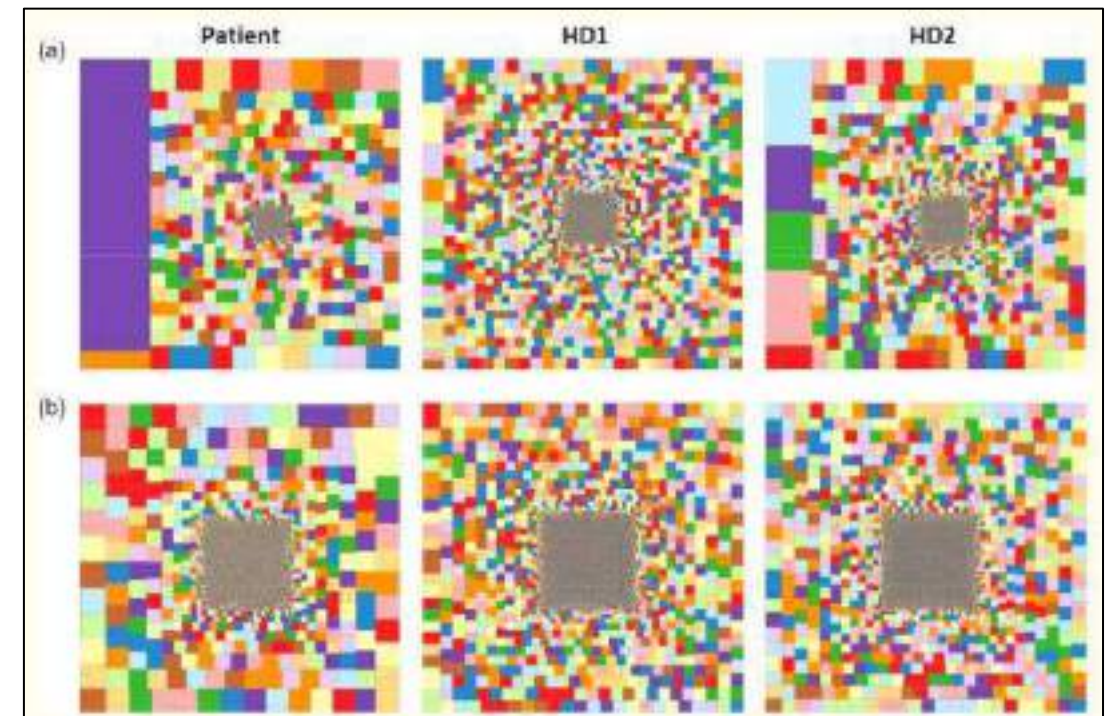
Patient with RALD, an autoimmune lymphoproliferative syndrome

Performed **repertoire analysis** using **LymphoTrack<sup>®</sup>** assays

“Our results demonstrate, at least for our reported RALD patient, how peripheral T and B clonal expansions reciprocally limit lymphocyte production and restrict the lymphocyte receptor repertoire in this disease”

T and B cell clonal expansion in Ras-associated lymphoproliferative disease (RALD) as revealed by next-generation sequencing

[S. Levy-Mendelovich](#), <sup>1,2,3,4,†</sup> [A. Lev](#), <sup>1,3,†</sup> [E. Rechavi](#), <sup>1,3</sup> [O. Barel](#), <sup>3,5</sup> [H. Golan](#), <sup>2,3</sup> [B. Bielorai](#), <sup>2,3</sup> [Y. Neumann](#), <sup>2,3</sup> [A. J. Simon](#), <sup>1,3,5,6</sup> and [B. Somech](#)<sup>1</sup>



# Repertoire & SHM Analysis

22 HCV+ patients and 7 healthy controls

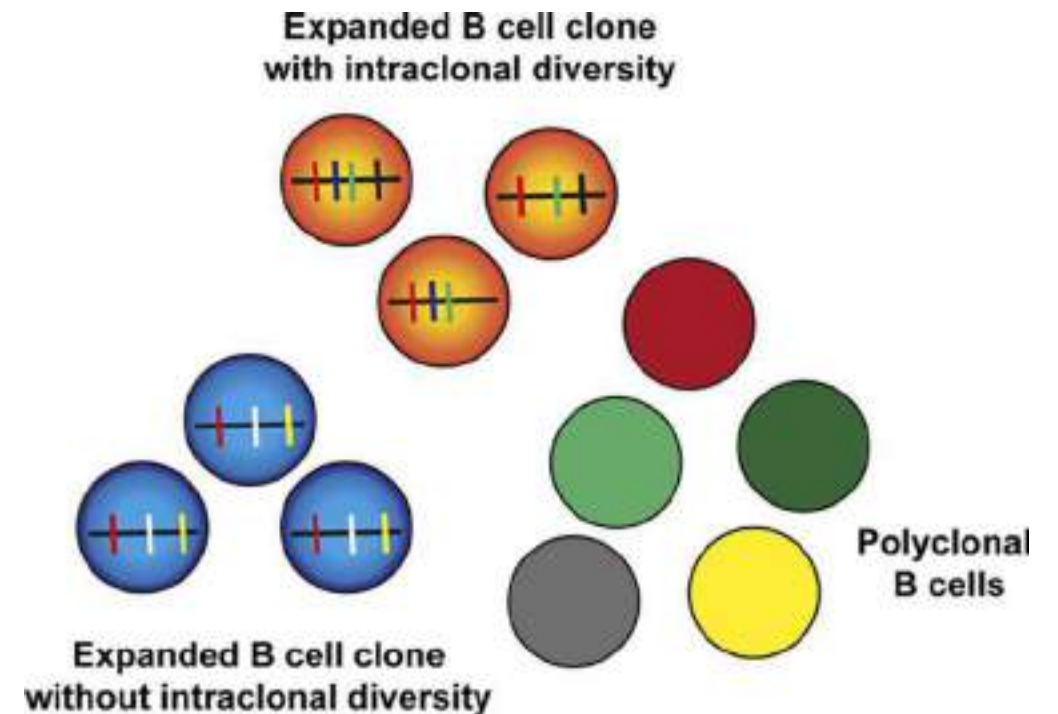
Very thorough **repertoire and somatic hypermutation analysis**

Found increased usage of several *IGHV* genes

Many large, expanded B cell clones are consistently found, mainly among IgM+ memory B cells

**Biased IGH VDJ gene repertoire and clonal expansions in B cells of chronically hepatitis C virus-infected individuals.**

Tucci FA<sup>1</sup>, Kitanovski S<sup>2</sup>, Johansson P<sup>1,3</sup>, Klein-Hitpass L<sup>1</sup>, Kahraman A<sup>4</sup>, Dürig J<sup>3</sup>, Hoffmann D<sup>2,5</sup>, Küppers R<sup>1,5</sup>.



# Somatic Hypermurmutation (SHM) Literature



# SHM Status can be Prognostic in CLL



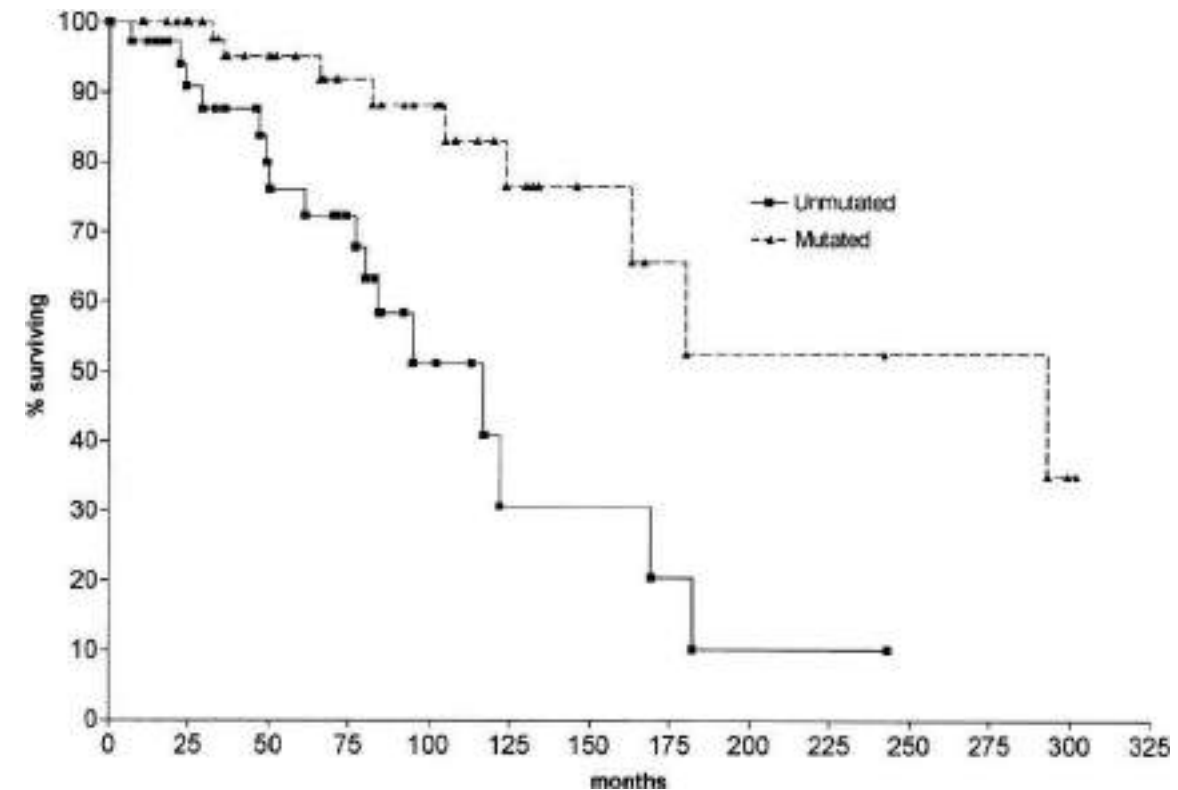
Early paper on CLL cells and *IGHV* mutational status

Sequenced **84 patients**

“Median survival for stage A patients with unmutated V(H) genes was 95 months compared with 293 months for patients whose tumors had mutated V(H) genes”

Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia.

Hamblin TJ<sup>1</sup>, Davis Z, Gardiner A, Oscier DG, Stevenson FK.



# SHM Status is also Predictive



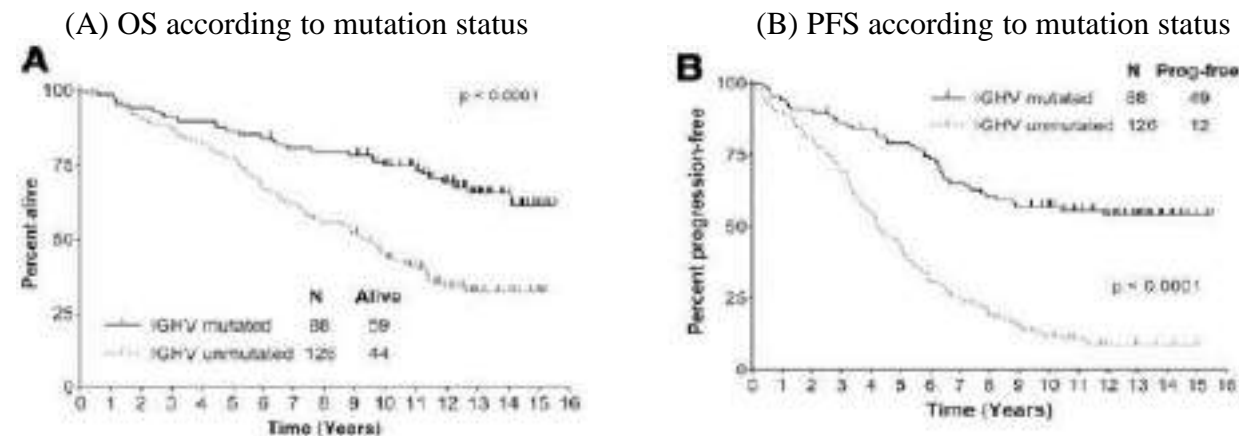
M-CLL patients compared to UM-CLL patients who received the same treatment:

- Prolonged response
- Delayed progression
- Significant improvement in survival overall

Determining the SHM status is not only prognostic, but also predictive.

## Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in *IGHV*-mutated chronic lymphocytic leukemia

Philip A. Thompson,<sup>1\*</sup> Constantine S. Tam,<sup>2\*</sup> Susan M. O'Brien,<sup>1</sup> William G. Wierda,<sup>1</sup> Francesco Stingo,<sup>3</sup> William Plunkett,<sup>4</sup> Susan C. Smith,<sup>1</sup> Hagop M. Kantarjian,<sup>1</sup> Emil J. Freireich,<sup>1</sup> and Michael J. Keating<sup>1</sup>



	OS (%)	PFS (%)
<i>IGHV</i> -M	65.5	53.9
<i>IGHV</i> -UM	32.2	8.9

# SHM in Mantle Cell Lymphoma



Identified *IGH* rearrangements in all **55 FFPE samples** (used an FR1 primer)

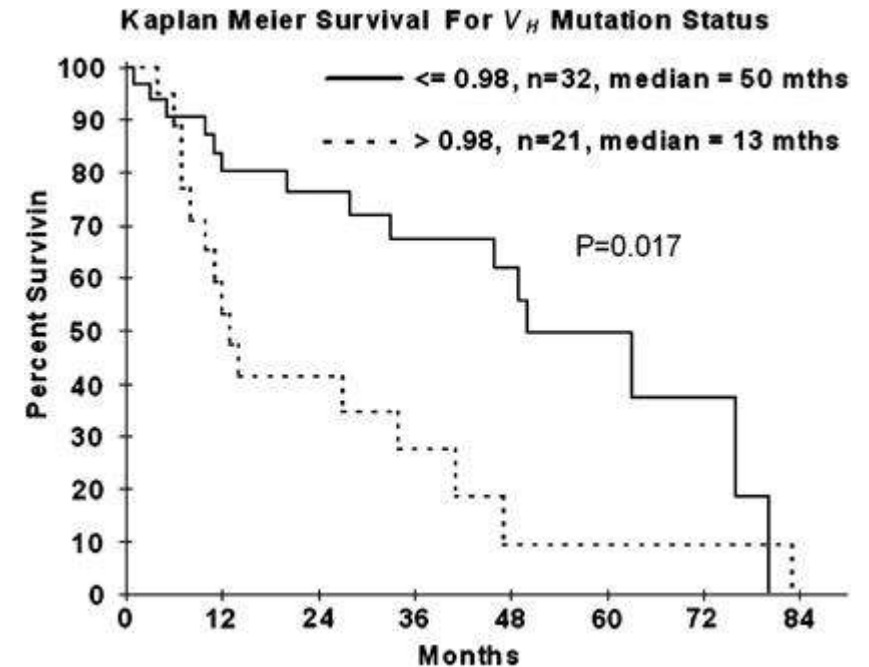
Identified a bias in *VH* gene utilization (*VH1-69*, *VH4-59*, and *VH3-74*) and relationship to gene and mutational status

Significant correlation between the degree of mutation rate and overall survival.

Continues to use **2% mutation** rate threshold

**Immunoglobulin *VH* somatic hypermutation in mantle cell lymphoma: mutated genotype correlates with better clinical outcome.**

[Lai R<sup>1</sup>](#), [Lefresne SV](#), [Franko B](#), [Hui D](#), [Mirza I](#), [Mansoor A](#), [Amin HM](#), [Ma Y](#).



	Hazard ratio	P-value
Mutated vs un-mutated	3.71	0.01
Performance status (<1 vs $\geq 1$ )	1.64	0.45
International prognostic index	1.05	0.93

# Should *IGHV* be Tested at Diagnosis?



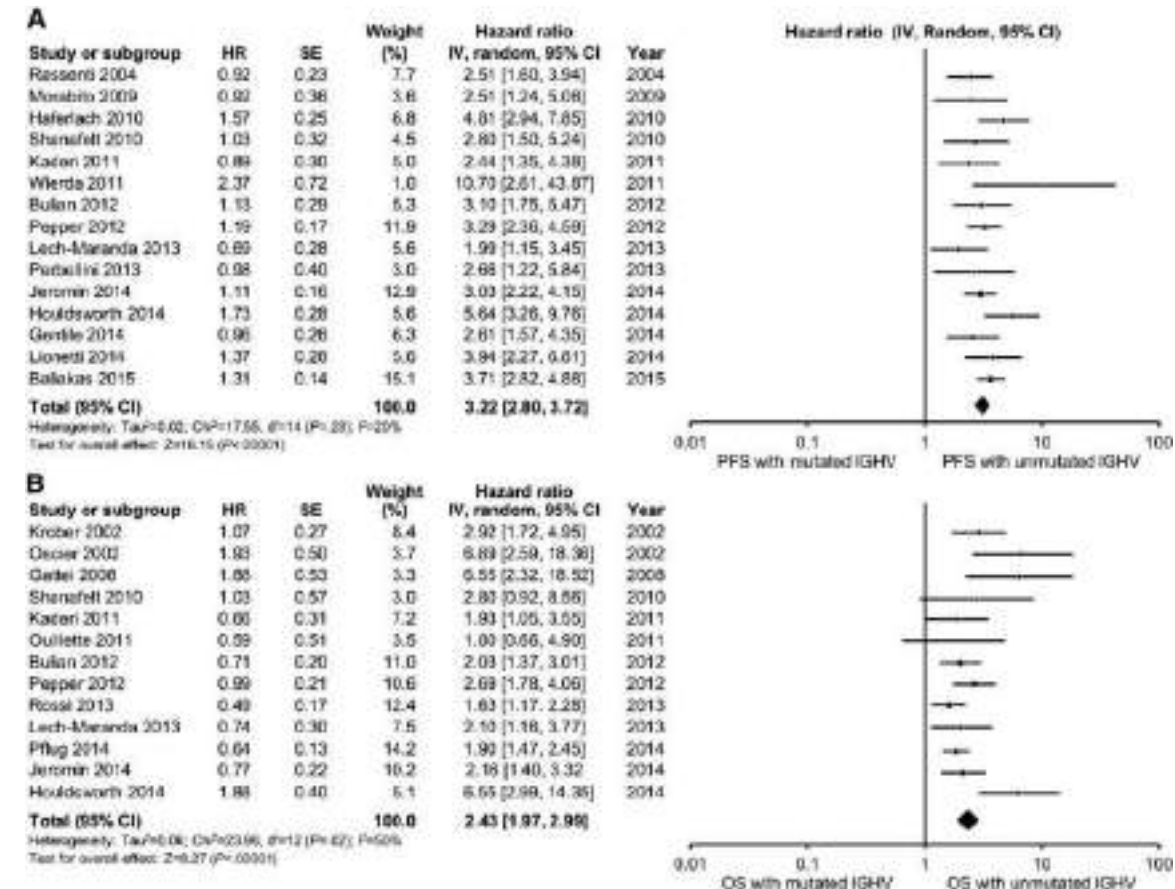
## Review of 31 different studies

Unmutated *IGHV* genes had significantly reduced progression-free survival and overall survival

**Yes!** – *IGHV* mutation testing is recommended as a standard clinical test for all newly diagnosed patients with CLL

Should *IGHV* status and FISH testing be performed in all CLL patients at diagnosis? A systematic review and meta-analysis.

Parikh SA<sup>1</sup>, Strati P<sup>1</sup>, Tsang M<sup>1</sup>, West CP<sup>2</sup>, Shanafelt TD<sup>1</sup>.



# SHM in the Era of Next Gen Sequencing



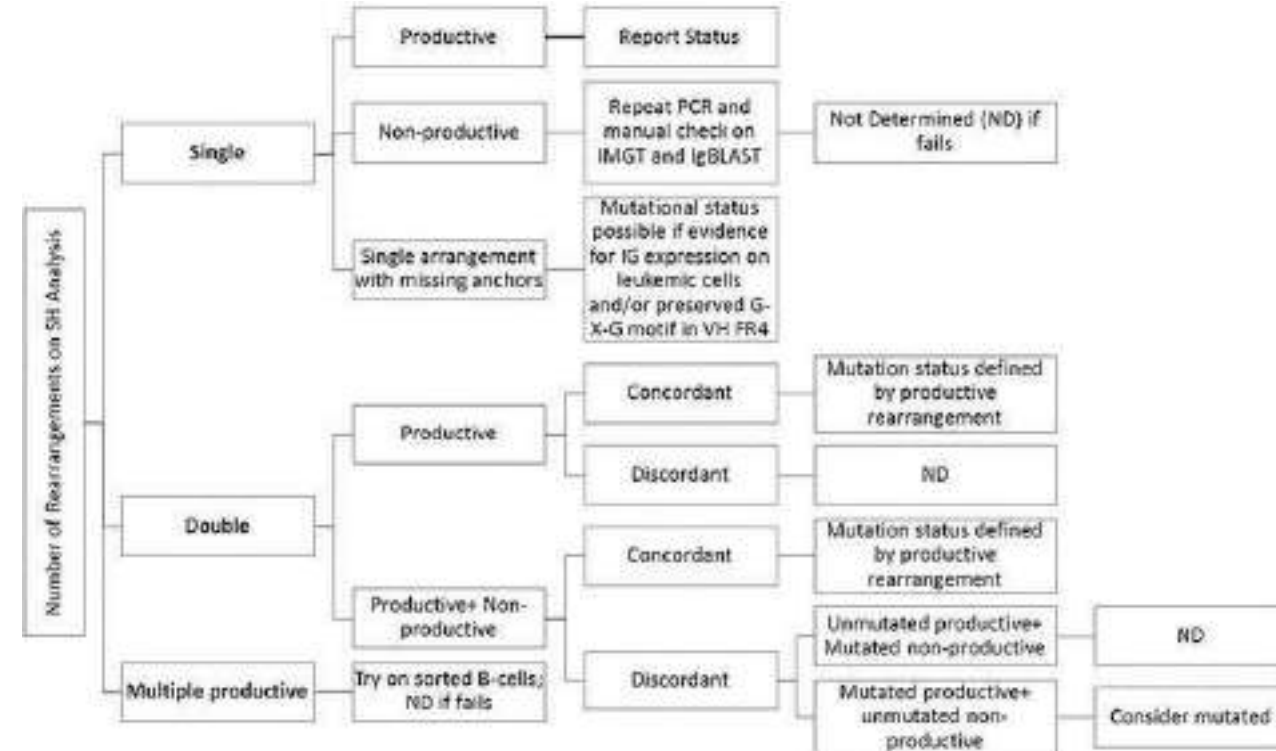
Review paper detailing the evolution of somatic hypermutation testing in CLL

Summarizes many considerations such as primers, platform, controls, informatics, and interpretation

Provides example interpretation complex case studies

Evaluation of Somatic Hypermutation Status in Chronic Lymphocytic Leukemia (CLL) in the Era of Next Generation Sequencing

[Sanjeev Kumar Gupta](#)<sup>1</sup>, [David S. Viswanatha](#)<sup>2,\*</sup> and [Keyur P. Patel](#)<sup>3,\*</sup>







Provides technical considerations and reporting guidelines

“Determining the SHM level is therefore important, not only for general assessment of the disease course in CLL, but also for **guiding treatment decisions**: put simply, it is not only a **prognostic test**, but also a **predictive test** for the use of certain therapies”

## Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations

R. Rosenquist<sup>1,2</sup>, P. Ghia<sup>3</sup>, A. Hadzidimitriou<sup>4</sup>, L-A. Sutton<sup>1,2</sup>, A. Agathangelidis<sup>3</sup>, P. Baliakas<sup>1</sup>, N. Darzentas<sup>5</sup>, V. Giudicelli<sup>6</sup>, M-P. Lefranc<sup>6</sup>, A. W. Langerak<sup>7</sup>, C. Belessi<sup>8</sup>, F. Davi<sup>9</sup>, K. Stamatopoulos<sup>1-4</sup>

**Table 1.** Technical considerations for determination of the IGHV somatic hypermutation status of clonotypic IGHV-IGHD-IGHJ gene rearrangements in CLL

Item	Recommendations	Remarks
<i>Material</i>		
Anticoagulants	EDTA (or CPT)	
Cells/tissue	Blood, bone marrow, tissue biopsy	Purification of B cells usually not necessary unless low fraction of leukemic cells
Nucleic acid	gDNA or cDNA	cDNA useful when mutations within the IGHJ gene impair amplification
<i>Production of template for sequencing</i>		
Primers	5': leader 3': IGHJ or IGHC	VH FR1, VH FR2 and VH FR3 primers are not acceptable IGHC primers (on cDNA) useful when mutations within IGHJ gene impair amplification
Amplification	Multiplex PCR	individual PCR reactions (for each 5' primer) may be useful when more than one rearrangement found
Detection of IGH rearrangement	GeneScan or PAGE electrophoresis	Agarose gel electrophoresis strongly discouraged (lack of resolution)
Cloning	Not necessary	Except in rare circumstances (more than one rearrangement not isolated by simplex PCR)
<i>Sequencing</i>		
Methodology	Direct, both strands	Both strands mandatory for high-quality sequence
Sequence alignment	IMGIT/V-QUEST (www.imgt.org)	Adjustable parameters: (1) search for insertions/deletions; (2) number of accepted D genes
IGHV identity (%)	Automatic or adjusted	Adjusted: use option 'search for insertions/deletions' when low % identity
Stereotypic subset identification	ARRest/AssignSubsets (bat.infospire.org/arrest/ericll.org/pages/services/tool)	Applicable for the current 19 major BcR stereotyped subsets in CLL <sup>9</sup>

Abbreviations: BcR, B-cell receptor; cDNA, complementary DNA; CLL, chronic lymphocytic leukemia; CPT, citrate/pyridoxal 5'-phosphate/Tris; EDTA, ethylenediaminetetraacetic acid; gDNA, genomic DNA; PAGE, polyacrylamide gel electrophoresis. <sup>9</sup>Agathangelidis and colleagues.<sup>7</sup>

# Improving Disease Stratification in CLL

2 studies, 497 total samples

24.4% of CLL samples express **multiple productive** clonally unrelated *IGHV* rearrangements

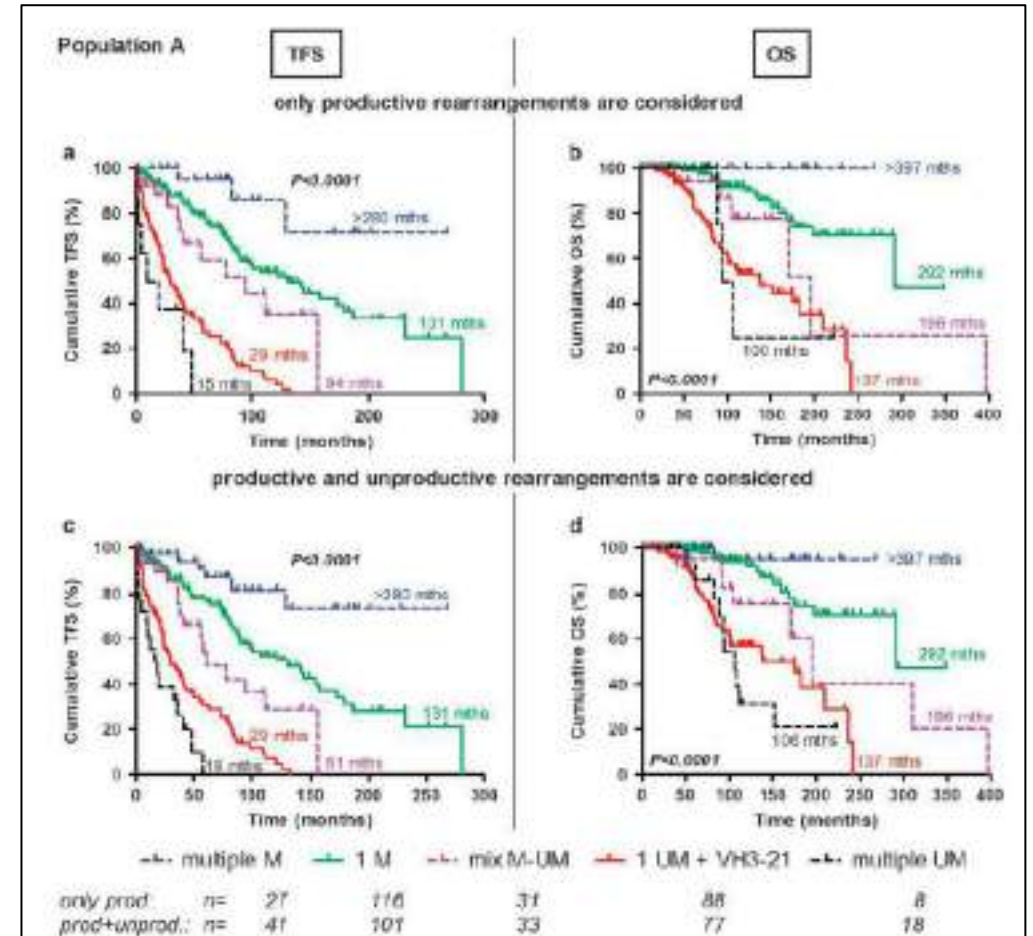
Proposes 5 new subsets for **improved stratification** for identifying outcomes

Demonstrates **exclusion of PCR bias**

**Improved prognostication** for 92 out of 270 subjects

**Targeted deep sequencing reveals clinically relevant subclonal *IgHV* rearrangements in chronic lymphocytic leukemia.**

Stamatopoulos B<sup>1,2,3</sup>, Timbs A<sup>1</sup>, Bruce D<sup>1</sup>, Smith T<sup>4</sup>, Clifford R<sup>1,3</sup>, Robbe P<sup>1,3</sup>, Burns A<sup>1,3</sup>, Vavoulis DV<sup>3</sup>, Lopez L<sup>5</sup>, Antoniou P<sup>3</sup>, Mason J<sup>1</sup>, Dreau H<sup>1</sup>, Schuh A<sup>1,6</sup>.



# Differential Protein Expression

Analyzed **26 CLL patients** and 8 healthy controls

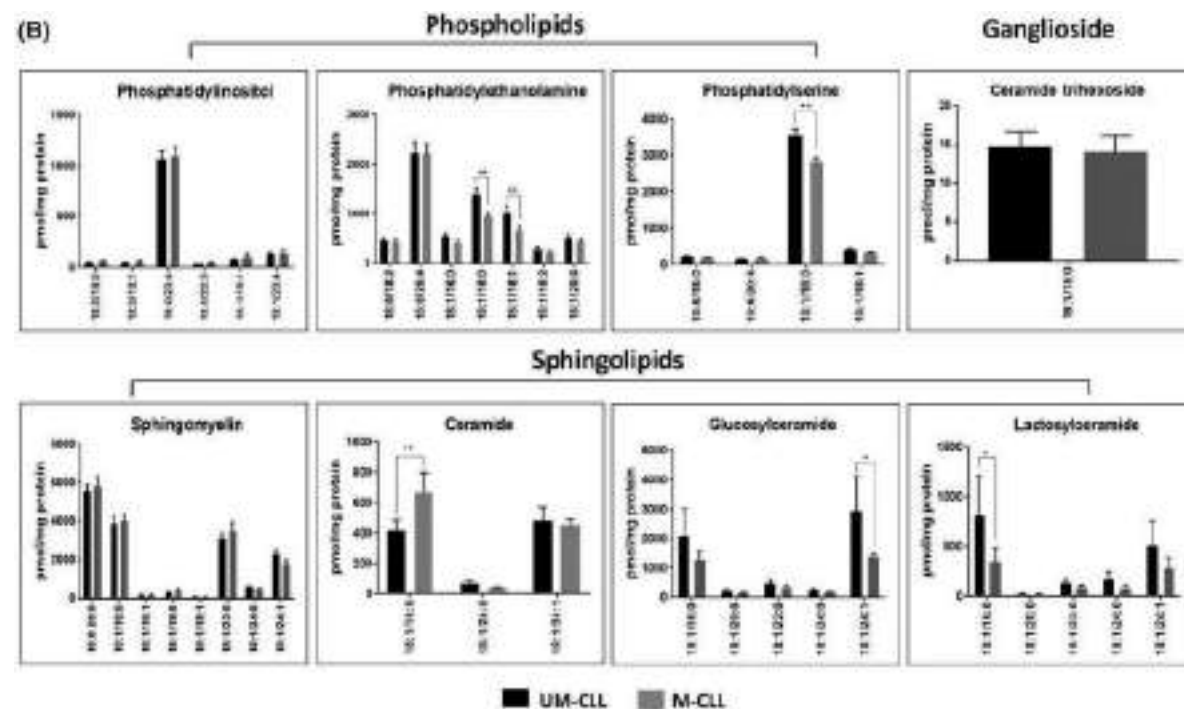
Used LymphoTrack<sup>®</sup> to evaluate somatic hypermutation

349 proteins were differentially expressed between normal/CLL cells

189 proteins were differentially expressed between Mutated and UnMutated CLL

## Altered Expression of Metabolic Pathways in CLL Detected by Unlabelled Quantitative Mass Spectrometry Analysis

Lauren A Thurgood<sup>1</sup>, Eveline S Dwyer<sup>1</sup>, Karen M Lower<sup>1</sup>, Tim K Chataway<sup>2</sup>, Bryone J Kuss<sup>1,3</sup>



# MRD Literature



# NGS MRD Review

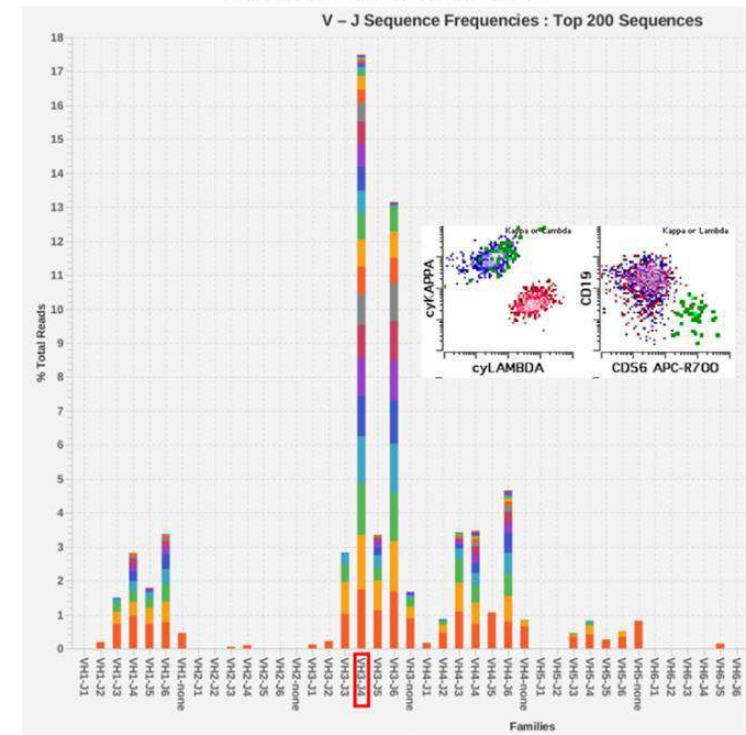
Review paper which provides a good case for updating Dx technology for detection of MRD in myeloma patients, comparing MCF, ASO-PCR, F-PCR and NGS

NGS and MCF provide a **high level of sensitivity**, enabling prognostic significance in stratifying patients into different levels of MRD

**Minimal residual disease detection of myeloma using sequencing of immunoglobulin heavy chain gene VDJ regions.**

Ho C<sup>1</sup>, Arcila ME<sup>2</sup>.

**Results from MRD detection in post-treatment marrow sample of a myeloma patient, including 10-color MFC and NGS**



# NGS MRD Review



Review paper that details some of the intricacies of testing, including selecting a sensitivity

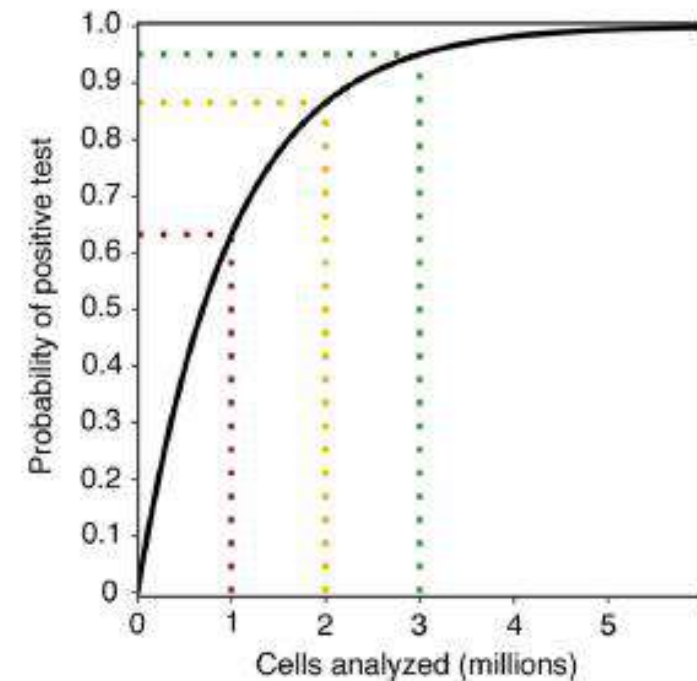
Provides background on why cell equivalents are important

“Modern NGS-based MRD assays are applicable to >95 % of patients, as long as a tumor sample of adequate quality can be obtained for baseline characterization”

Monitoring minimal residual disease in the bone marrow using next generation sequencing

[Even H. Rustad](#), MD, PhD<sup>1</sup> and [Eileen M. Boyle](#), MD, MSc<sup>2</sup>

**Limit of detection of MRD test**



# NGS MRD vs. qPCR



210 samples from **76 patients**

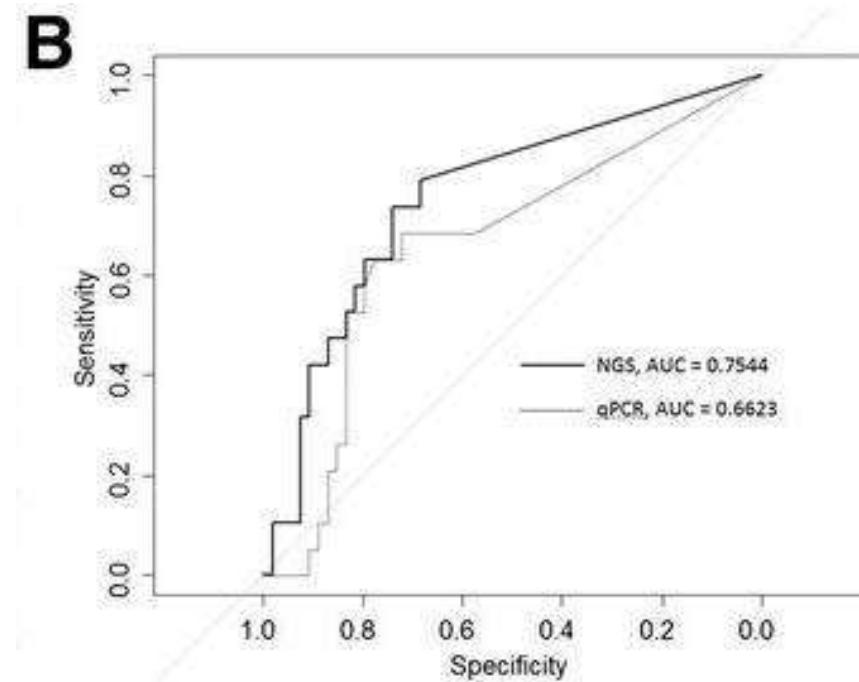
NGS provides a more complete picture than qPCR by assessing normal immune repertoire

At both day 33 & 78 relapse was significantly higher for those with reduced diversity

“NGS-MRD positivity at day 33 provided a more accurate prediction of relapse than qPCR-MRD positivity”

The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL

[Michaela Kotrova](#), [Katerina Muzikova](#), [Ester Mejstrikova](#), [Michaela Novakova](#), [Violeta Bakardjeva-Mihaylova](#), [Karel Fiser](#), [Jan Stuchly](#), [Mathieu Giraud](#), [Mikael Saison](#), [Christiane Pott](#), [Monika Bruggemann](#), [Marc Fullgrabe](#), [Jan Stary](#), [Jan Trka](#)<sup>OR</sup> and [Eva Fronkova](#)



# NGS MRD vs. Flow Cytometry



For Internal Use only

56 patients with B-cell ALL

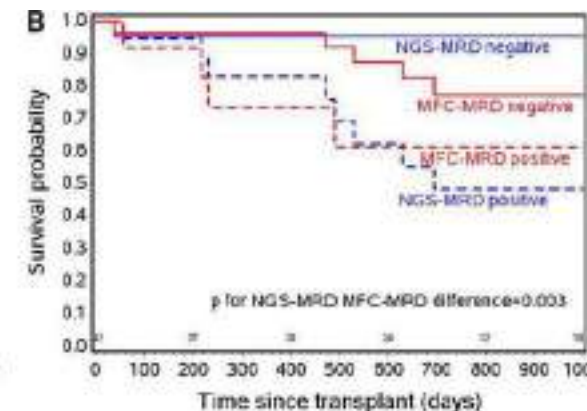
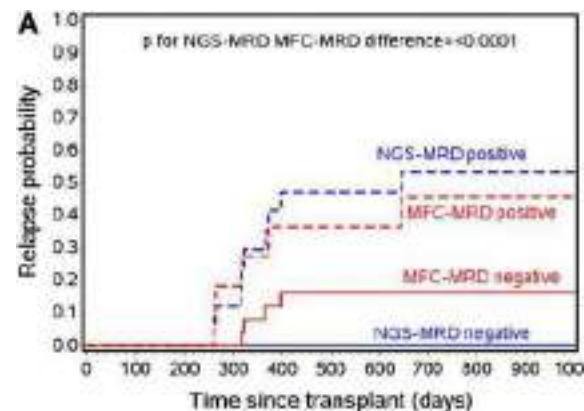
Direct comparison of 6 color flow cytometry to Next Generation Sequencing

7 relapses among 11 samples that were Flow-negative, but NGS-positive

No relapse from Flow-positive, NGS-negative samples

IgH-V(D)J NGS-MRD measurement pre- and early post-allotransplant defines very low- and very high-risk ALL patients.

Pulsipher MA<sup>1</sup>, Carlson C<sup>2</sup>, Langholz B<sup>3</sup>, Wall DA<sup>4</sup>, Schultz KR<sup>5</sup>, Bunin N<sup>6</sup>, Kirsch I<sup>7</sup>, Gastier-Foster JM<sup>8</sup>, Borowitz M<sup>9</sup>, Desmarais C<sup>7</sup>, Williamson D<sup>7</sup>, Kalos M<sup>10</sup>, Grupp SA<sup>11</sup>.





# LymphoTrack<sup>®</sup> Assay Performance

Performance of LymphoTrack<sup>®</sup> Assays in dilution experiments

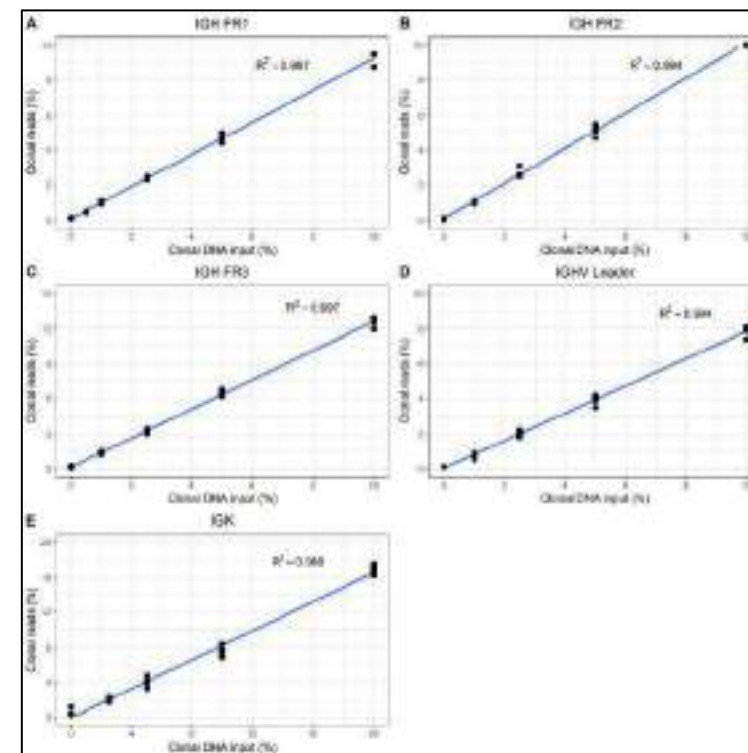
**Demonstrates linear performance** of all LymphoTrack<sup>®</sup> assays

At 2.5% dilution, the expected clonal sequence is detected above polyclonal background

**Clonality detection rates of 95% or higher** were achieved for multiple myeloma in this study

Baseline identification of clonal V(D)J sequences for DNA-based minimal residual disease detection in multiple myeloma

Even H. Rustad<sup>1,2</sup>, Malin Hultcrantz<sup>1</sup>, Venkata D. Yellapantula<sup>3</sup>, Theresia Akhlaghi<sup>1</sup>, Caleb Ho<sup>4</sup>, Maria E. Arcila<sup>4</sup>, Mikhail Roshal<sup>4</sup>, Akshar Patel<sup>5</sup>, Denise Chen<sup>6</sup>, Sean M. Devlin<sup>3</sup>, Austin Jacobsen<sup>7</sup>, Ying Huang<sup>7</sup>, Jeffrey E. Miller<sup>7</sup>, Elli Papaemmanuil<sup>3</sup>, Ola Landgren<sup>1\*</sup>



# LymphoTrack<sup>®</sup> MRD vs. ASO-PCR

This study establishes a standardized experimental design to track MRD

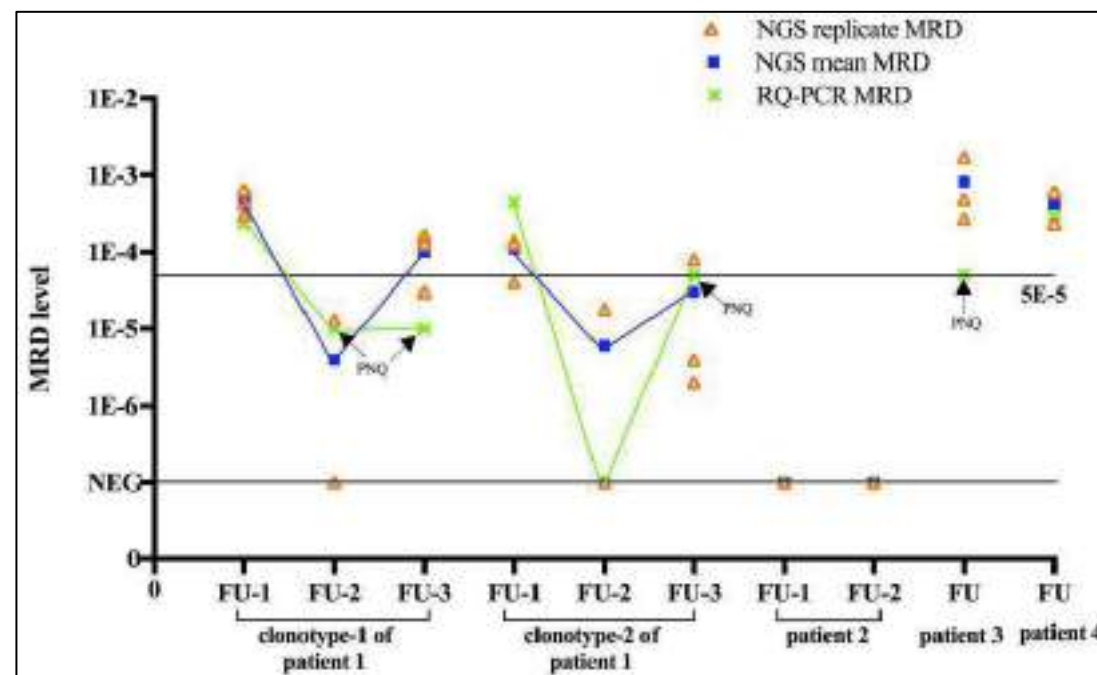
Verifies a sensitivity of  $10^{-5}$

NGS demonstrates **improved sensitivity** and **provides quantification** of MRD in cases with 'positive-but-not-quantifiable' ASO-PCR results

Standardized Minimal Residual Disease Detection by Next-Generation Sequencing in Multiple Myeloma

Qiumei Yao,<sup>1</sup> Yinlei Bai,<sup>2</sup> Alberto Orfao,<sup>3</sup> and Chor Sang Chim<sup>1,\*</sup>

Collaborative study involving institutions from Hong Kong, China and Spain



# LymphoTrack<sup>®</sup> MRD vs Flow Cytometry

This study demonstrates that **NGS performs better** than MCF

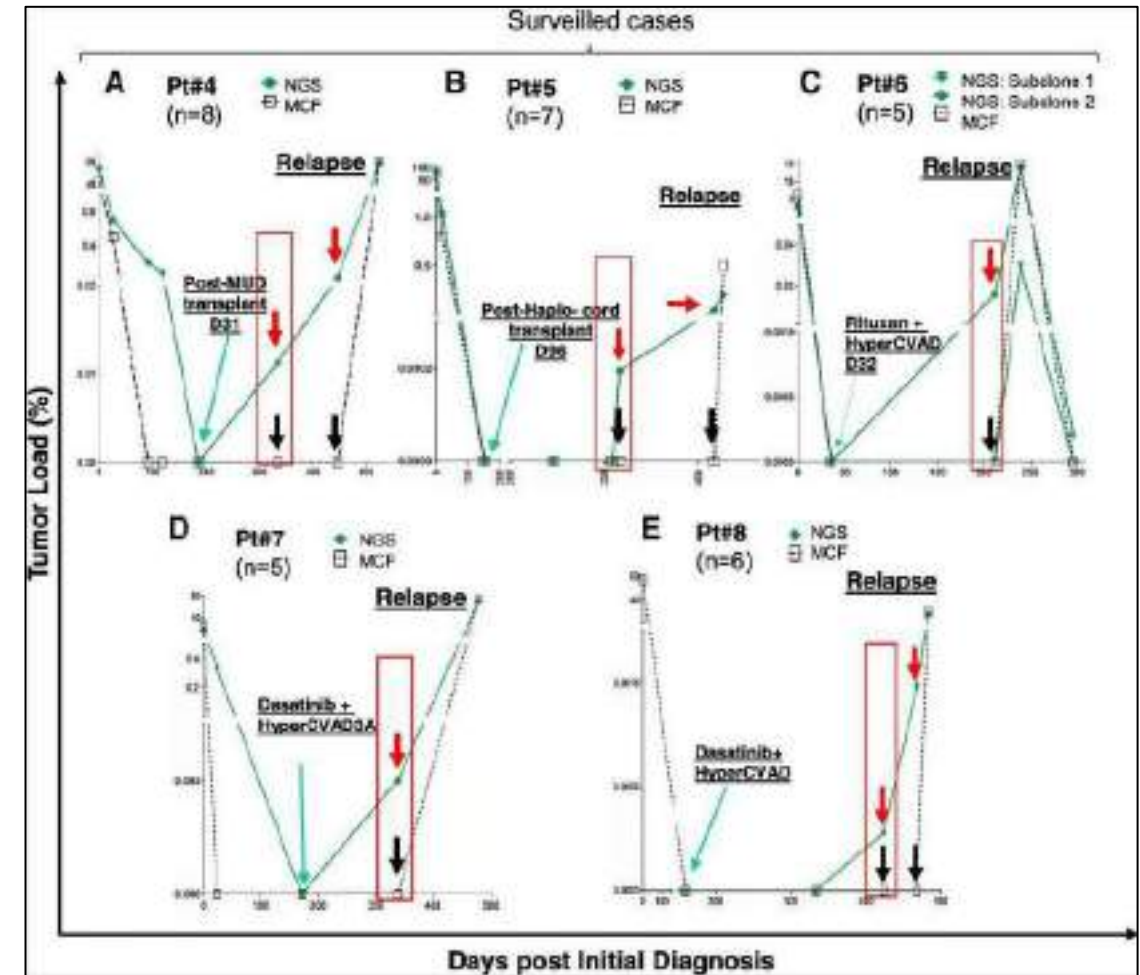
- NGS enables better risk stratification and earlier preemptive therapies against impending relapse, thus potentially improving outcome for B-ALL patients.

**122 B-ALL samples** from 30 subjects

MRD could be detected as early as **25.6 weeks prior to relapse**

Simple deep sequencing-based post-remission MRD surveillance predicts clinical relapse in B-ALL.

Cheng S<sup>1</sup>, Inghirami G<sup>1</sup>, Cheng S<sup>2</sup>, Tam W<sup>3</sup>.



# Case Study – B-ALL MRD

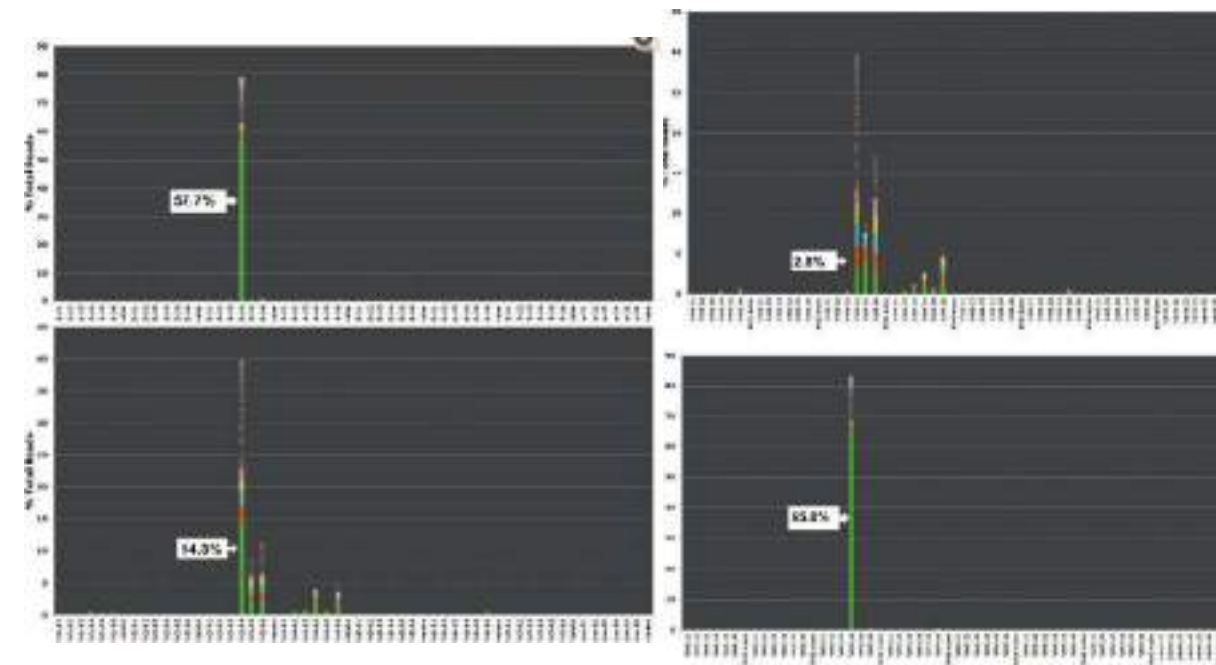
8 B-ALL patients with up to 3 timepoints each tracked with multiple methods

Has a great table that gives a **direct comparison** of cytogenetics, flow cytometry, FISH, molecular testing, fragment analysis, and LymphoTrack<sup>®</sup>

“At 10 months after initial diagnosis, which was just before full-blown relapse, BCR/ABL1 was negative but **IGH NGS showed the presence of initial clones**”

## Detection of Immunoglobulin Heavy Chain Gene Clonality by Next-Generation Sequencing for Minimal Residual Disease Monitoring in B-Lymphoblastic Leukemia.

Shin S<sup>1,2</sup>, Hwang IS<sup>3</sup>, Kim J<sup>1</sup>, Lee KA<sup>1</sup>, Lee ST<sup>4</sup>, Choi JR<sup>5</sup>.



# Case Study – B-ALL MRD

Table 1. Patient demographics and results of bone marrow analysis and clonality tests

Patient ID	Sex	Age (yr)	Sample		Blasts in BM (%)	Leukemic cells in flow cytometry (%)	Cytogenetics	Abnormal cells by FISH (%)	Molecular analysis	Fragment analysis	N of reads and type of clone detected by NGS				
			ID	Time <sup>1</sup>							Clone 1 (% type)	Clone 2 (% type)	Clone 3 (% type)	% of total clones	Total N of reads
1	M	4	1-1*	Diagnosis	92.5	96.9	Not interpretable	53.6	Negative <sup>2</sup>	Positive	44,001 (27.3, VH1-J4)	9,877 (6.0, VH1-J4)	9,760 (6.0, VHS-J4)	39.3	160,971
			1-2*	10 days	4.0	Negative	ND	4.0	ND	Positive	27,547 (11.6)	9,019 (3.8)	4,846 (2.1)	17.5	236,250
			1-3*	30 days	1.5	Negative	46,XY (20)	Negative	ND	Negative	0	0	0	0	78,806
2	M	3	2-1*	Diagnosis	87.0	88.2	Not interpretable	70.1	Negative <sup>2</sup>	Positive	119,153 (23.0, VHS-J4)	114,278 (22.0, VH2-J4)	-	45.0	518,728
			2-2*	2 weeks	NA	Negative	ND	ND	ND	Negative	3,212 (8.7)	12,824 (2.6)	-	3.2	489,937
			2-3*	30 days	0.7	Negative	46,XY (20)	Negative	ND	Negative	0	0	-	0	298,570
3	F	2	3-1*	Diagnosis	91.4	77.0	Not interpretable	62.8	Negative <sup>2</sup>	Positive	8,350 (23.7, VH6-J5)	3,741 (10.6, VH6-J5)	-	34.3	35,182
			3-2*	2 weeks	6.8	1.0	ND	11.3	ND	Positive	7,585 (10.5)	3,569 (4.9)	-	15.4	72,494
			3-3*	30 days	1.0	Negative	46,XX (20)	Negative	ND	Negative	515 (8.5)	247 (0.2)	-	0.7	100,618
4	F	4	4-1*	Diagnosis	87.8	75.3	Not interpretable	75.3	Negative <sup>2</sup>	Positive	232,951 (54.8, VH1-J5)	41,075 (9.7, VH1-J5)	-	64.5	425,184
			4-2*	10 days	NA	Negative	ND	Negative	ND	Positive	51,167 (11.8)	8,317 (1.9)	-	13.7	434,643
			4-3*	30 days	0.9	Negative	46,XX (20)	Negative	ND	Negative	0	0	-	0	214,809
5	M	8	5-1*	Diagnosis	96.8	53.5	Not interpretable	65.9	Negative <sup>2</sup>	Positive	207,993 (53.8, VHS-J6)	39,502 (10.2, VH3-J6)	-	64.0	386,933
			5-2*	2 weeks	0	Negative	46,XY(20)	Negative	ND	Negative	468 (8.1)	0	-	0.1	339,695
6	M	3	6-1*	Diagnosis	87.0	93.6	Not interpretable	76.8	Negative <sup>2</sup>	Positive	172,072 (34.3, VHS-J4)	23,264 (7.3, VHS-J4)	-	61.6	316,634
			6-2*	2 weeks	0	Negative	ND	Negative	ND	Negative	9,290 (2.0)	1,208 (0.3)	-	2.3	462,911
7	M	7	7-1*	Diagnosis	92.0	90.8	45,XY,der(7)t(9;10)(q10;q10)(20)	96.3	Negative <sup>2</sup>	Positive	2,429 (25.5, VH4-J6)	-	-	25.5	9,513
			7-2*	30 days	1.0	Negative	ND	Negative	ND	Negative	0	-	-	0	7,422
8	M	15	8-1*	Diagnosis	93.0	88.2	52,XY,+8,t(9;22)(q34;q11.2),+der(22)t(9;22),+mar1,+mar2,+mar3,+mar4 [1]/46,XY[17]	80.6	3.25*	Positive	8,565 (57.7, VHS-J4)	-	-	57.7	14,825
			8-2*	30 days	0	Negative	ND	ND	3.94 × 10 <sup>-18</sup>	Negative	3,501 (14.3)	-	-	14.3	24,427
			8-3*	10 months	0.2	Negative	ND	Negative	Negative <sup>2</sup>	Negative	400 (2.8)	-	-	2.8	14,099
			8-4*	Relapse**	87.0	71.8	52,XY,+8,t(9;22)(q34;q11.2),+der(22)t(9;22),+mar1,+mar2,+mar3,+mar4 [25]/46,XY[11]	89.1	4.36*	Positive	25,456 (65.8)	-	-	65.8	53,881

\*Eighteen samples from Patients 1-7 were tested by using the Ion Torrent PGM (Thermo Fisher Scientific). Four samples from Patient 8 were tested by using MiSeq (Illumina). <sup>1</sup>Days, weeks, or months after induction chemotherapy. <sup>2</sup>Five probe sets were tested by using the IG/IMYC/CEP8 bi-color dual fusion probe, BCR/ABL dual color dual fusion translocation probe, CDW28 spectrum orange/CEP9 spectrum green probe, TEL/MML2 dual color extra signal probe, and MLL break-apart arrangement probe (Vysis, Downers Grove, IL, USA) at initial diagnosis. Follow-up tests were done by using the probe with abnormal results at initial diagnosis. <sup>3</sup>Molecular analysis was done by using HemaVision kit (DNA technology, Aarhus, Denmark). <sup>4</sup>BCR/ABL1 transcript frequency (relative ratio of BCR/ABL1 to GAPDH) was quantified by using the LightCycler 9.2.2 Quantification (Roche Molecular Systems, Branchburg, NJ, USA) kit. <sup>5</sup>Patent B relapsed at one year after diagnosis. Abbreviations: NGS, next-generation sequencing; BM, bone marrow; ND, not done; NA, not available due to dry tap.

# LymphoTrack<sup>®</sup> MRD vs Flow Cytometry

106 **multiple myeloma** patients, 3 months after transplantation

## Strong **correlation of NGS and MFC**

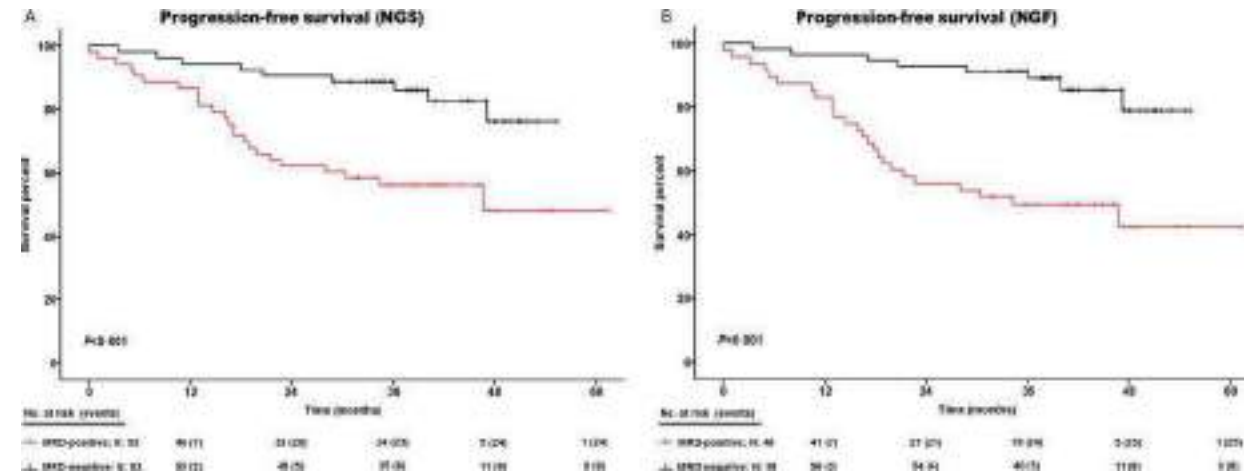
“interpretation of results is usually more difficult for **NGF, requiring high expertise**, while LymphoTrack’s solution is more user-friendly and semi-automated.”

Results support the use of **NGS to detect and evaluate MRD in MM** patients.

These findings **reinforce the use of MRD assessment as an endpoint in MM clinical trials** and underline the need of standardization and quality assessment in future studies for all MRD approaches in MM.

Comparison of next-generation sequencing (NGS) and next-generation flow (NGF) for minimal residual disease (MRD) assessment in multiple myeloma

[Alejandro Medina](#),<sup>1</sup> [Noemi Puig](#),<sup>1</sup> [Juan Flores-Montero](#),<sup>2</sup> [Cristina Jimenez](#),<sup>1</sup> [M.-Eugenia Sarasquete](#),<sup>1</sup> [María García-Alvarez](#),<sup>1</sup> [Isabel Prieto-Conde](#),<sup>1</sup> [Carmen Chillón](#),<sup>1</sup> [Miguel Alcoceba](#),<sup>1</sup> [Norma C. Gutierrez](#),<sup>1</sup> [Albert Oriol](#),<sup>3</sup> [Laura Rosinol](#),<sup>4</sup> [Joan Bladè](#),<sup>4</sup> [Mercedes Gironella](#),<sup>5</sup> [Miguel T. Hernandez](#),<sup>6</sup> [Veronica Gonzalez-Calle](#),<sup>1</sup> [Maria-Teresa Cedena](#),<sup>7</sup> [Bruno Paiva](#),<sup>8</sup> [Jesus F. San-Miguel](#),<sup>8</sup> [Juan-Jose Lahuerta](#),<sup>7</sup> [Maria-Victoria Mateos](#),<sup>1</sup> [Joaquin Martinez-Lopez](#),<sup>7</sup> [Alberto Orfao](#),<sup>2</sup> [Marcos Gonzalez](#),<sup>1</sup> and [Ramon Garcia-Sanz](#)<sup>1</sup>



# LymphoTrack<sup>®</sup> Immune Repertoire

**Repertoire analysis** is an exciting research application of LymphoTrack.

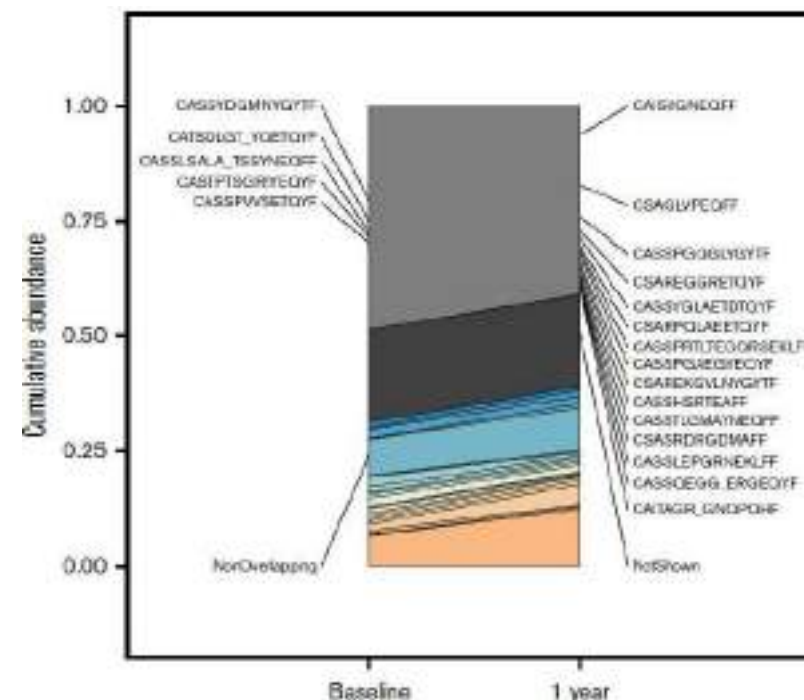
Studying the diversity of receptor sequences may lead to **new insights** into the **immune system**.

Study of the **long-term immune recovery** in MCL patients treated with venetoclax and ibrutinib

Demonstrated the usefulness in **comparing flow cytometry results with NGS** as it helps correctly identify changes in T-cell repertoire.

Immune recovery in patients with mantle cell lymphoma receiving long-term ibrutinib and venetoclax combination therapy

[Joanne E. Davis](#),<sup>1,2</sup> [Sasanka M. Handunnetti](#),<sup>2,3</sup> [Mandy Ludford-Menting](#),<sup>1,2</sup>  
[Chia Sharpe](#),<sup>1,2</sup> [Piers Blombery](#),<sup>2,3,4</sup> [Mary Ann Anderson](#),<sup>2,3,5</sup> [Andrew W. Roberts](#),<sup>2,3,5</sup>  
[John F. Seymour](#),<sup>2,3</sup> [Constantine S. Tam](#),<sup>2,3</sup> [David S. Ritchie](#),<sup>1,2,3</sup> and [Rachel M. Koldej](#)  
1,2



# LeukoStrat<sup>®</sup> Assays

*FLT3* References



# Current *FLT3* Treatment Info



Approximately **1 in 3** AML patients has a *FLT3* mutation (20-25% ITD, 5-10% TKD)

Comprehensive review of current and possible future **strategies for management of AML**

Mentions LeukoStrat<sup>®</sup> CDx *FLT3* Mutation Assay and US FDA approval for midostaurin

Emerging treatment paradigms with *FLT3* inhibitors in acute myeloid leukemia

Nicholas J. Short, Hagop Kantarjian, Farhad Ravandi, and Naval Daver

Table 1. Characteristics of *FLT3* inhibitors currently in clinical development.

<i>FLT3</i> inhibitor	Non- <i>FLT3</i> targets	<i>FLT3</i> -TKD mutation activity	Single-agent CRc rates in R/R <i>FLT3</i> -mutated AML	Dose	Major toxicities	Approval status
Sorafenib	c-KIT, PDGFR, RAF, VEGFR	No	<10%	400 mg bid	Rash, hemorrhage, myelosuppression	Available off-label (US FDA approved for hepatocellular, renal cell, and differentiated thyroid cancer)
Midostaurin	c-KIT, PKC, PDGFR, VEGFR	Yes	<10%	50 mg bid	GI toxicity, myelosuppression	US FDA and EMA approved for adults with newly diagnosed <i>FLT3</i> -mutated AML in combination with intensive chemotherapy (improves overall survival versus chemotherapy alone)
Guzartinib	c-KIT, PDGFR, RET	No	24-47%	30-60 mg daily	QTc prolongation, myelosuppression	US FDA approval sought for use in relapsed/refractory setting (improves overall survival versus chemotherapy)
Crenolanib	PDGFR	Yes	17-39%	100 mg tid	GI toxicity	Drug development plan is focused on chemotherapy-based combination
Gilteritinib	AML	Yes	37-41%	120 mg daily	Elevated transaminases, diarrhea	US FDA approved for adults with relapsed/refractory <i>FLT3</i> -mutated AML (full data not yet released)

Clinical trial with 717 total patients

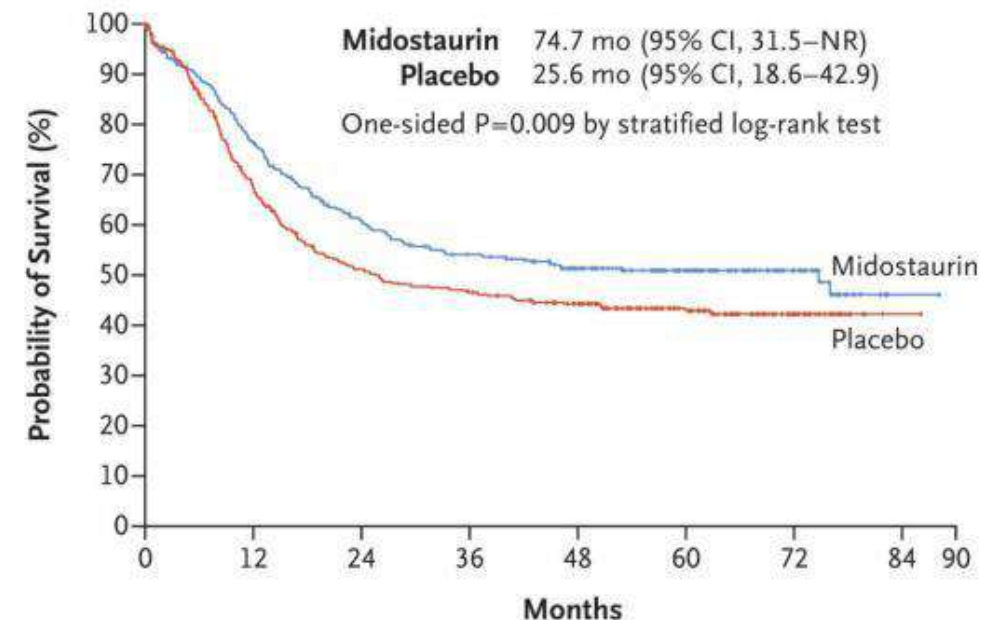
Basis for FDA premarket approval of **LeukoStrat® CDx FLT3** Mutation Assay as a **companion diagnostic to midostaurin**

“The addition of the multitargeted kinase inhibitor **midostaurin** to standard chemotherapy **significantly prolonged overall and event-free survival** among patients with AML and a *FLT3* mutation.”

## Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation.

Stone RM<sup>1</sup>, Mandrekar SJ<sup>1</sup>, Sanford BL<sup>1</sup>, Laumann K<sup>1</sup>, Geyer S<sup>1</sup>, Bloomfield CD<sup>1</sup>, Thiede C<sup>1</sup>, Prior TW<sup>1</sup>, Döhner H<sup>1</sup>, Marcucci G<sup>1</sup>, Lo-Coco F<sup>1</sup>, Klisovic RB<sup>1</sup>, Wei A<sup>1</sup>, Sierra J<sup>1</sup>, Sanz MA<sup>1</sup>, Brandwein JM<sup>1</sup>, de Witte T<sup>1</sup>, Niedewieser D<sup>1</sup>, Appelbaum FR<sup>1</sup>, Medeiros BC<sup>1</sup>, Tallman MS<sup>1</sup>, Krauter J<sup>1</sup>, Schlenk RF<sup>1</sup>, Ganser A<sup>1</sup>, Serve H<sup>1</sup>, Ehninger G<sup>1</sup>, Amadori S<sup>1</sup>, Larson RA<sup>1</sup>, Döhner H<sup>1</sup>.

### Median Overall Survival



### No. at Risk

Midostaurin	360	269	208	181	151	97	37	1
Placebo	357	221	163	147	129	80	30	1

# Cross-Validation of *FLT3* Assays

**RATIFY trial** performed testing at 9 reference labs across 6 countries

50 samples tested in 3 replicates

Found **considerable intra- and interlab variability**. 89 of 379 (24%) of values were wrongly assigned above/below a cutoff of 0.7

## ***FLT3*mutation Assay Laboratory Cross Validation: Results from the CALGB 10603/Ratify Trial in Patients with Newly Diagnosed *FLT3*-Mutated Acute Myeloid Leukemia (AML)**

Christian Thiede, MD, Thomas W. Prior, MD, Serena Lavorgna, PhD, Jürgen Krauter, MD, Eva Barragán, Josep Narededeu, MD PhD, Joop H. Jansen, Andrew H. Wei, MBBS, PhD, Weiqiang Zhao, MD PhD, Xiaohong Li, Celine Faltaud, PhD, Eva Tiede, PhD, Richard A. Larson, MD, Clara D. Bloomfield, MD, Hartmut Döhner, MD, Gerhard Ehninger, MD, Richard M. Stone, MD, Konstanza Döhner, MD

“Conclusions: This first report of round robin testing for *FLT3* mut shows that using a standardized protocol, the **qualitative assessment of *FLT3* mut is feasible** with high accuracy.

However the assessment of *FLT3*-AR clearly shows a considerable variability, which can be reduced by using a triplicate analysis... **Further standardization of *FLT3* testing appears highly warranted.**”

# Ratio of *FLT3*-ITD for Transplant Decisions



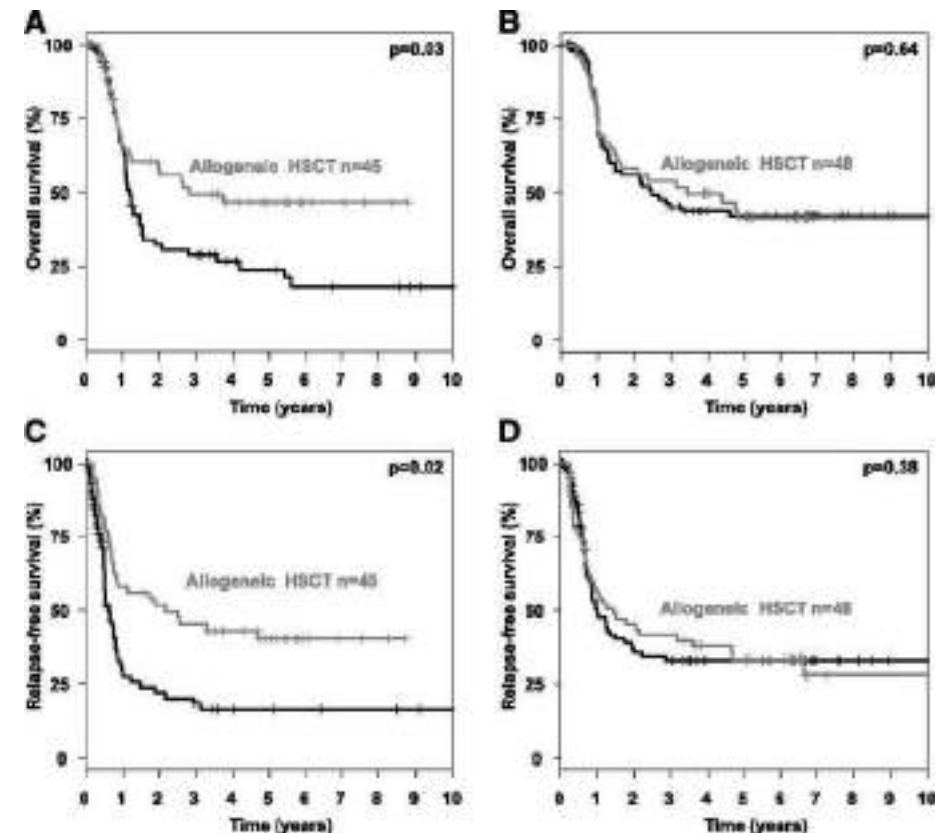
323 patients with *FLT3*-ITD positive AML

Uses a ratio threshold of 0.50 to determine ITD-high or ITD-low samples

“Multivariable analyses revealed a **high allelic ratio** as a **predictive factor** for the beneficial effect of allogeneic HSCT”

**Differential impact of allelic ratio and insertion site in *FLT3*-ITD-positive AML with respect to allogeneic transplantation.**

Schlenk RF<sup>1</sup>, Kayser S<sup>1</sup>, Bullinger L<sup>1</sup>, Kobbe G<sup>2</sup>, Casper J<sup>3</sup>, Ringhoffer M<sup>4</sup>, Held G<sup>5</sup>, Brossart P<sup>6</sup>, Lübbert M<sup>7</sup>, Salih HR<sup>8</sup>, Kindler T<sup>9</sup>, Horst HA<sup>10</sup>, Wulf G<sup>11</sup>, Nachbaur D<sup>12</sup>, Götze K<sup>13</sup>, Lamparter A<sup>1</sup>, Paschka P<sup>1</sup>, Gaidzik VI<sup>1</sup>, Teleanu V<sup>1</sup>, Späth D<sup>1</sup>, Benner A<sup>14</sup>, Krauter J<sup>15</sup>, Ganser A<sup>15</sup>, Döhner H<sup>1</sup>, Döhner K<sup>1</sup>; German-Austrian AML Study Group.



# Data for Gilteritinib in R/R AML

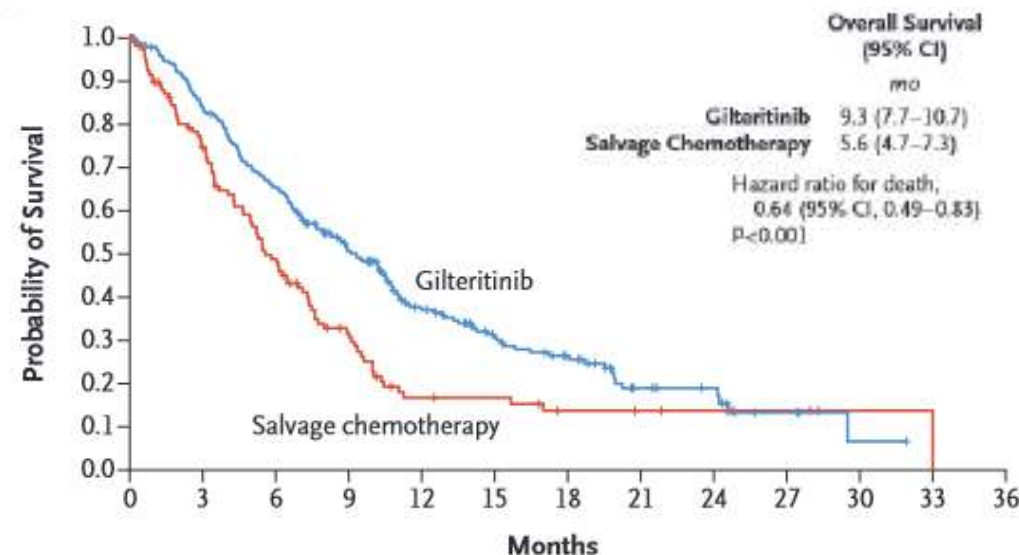
“Gilteritinib resulted in significantly longer survival and higher percentages of patients with remission than salvage chemotherapy among patients with relapsed or refractory **FLT3-mutated AML**” (ADMIRAL clinical trial number NCT02421939)

- FDA Approved (US)
- MHLW Approved (Japan)
- CE-Marked (EU)

## Gilteritinib or Chemotherapy for Relapsed or Refractory FLT3-Mutated AML

A.E. Perl, G. Martinelli, J.E. Cortes, A. Neubauer, E. Berman, S. Paolini, P. Montesinos, M.R. Baer, R.A. Larson, C. Ustun, F. Fabbiano, H.P. Erba, A. Di Stasi, R. Stuart, R. Olin, M. Kasner, F. Ciceri, W.-C. Chou, N. Podoltsev, C. Recher, H. Yokoyama, N. Hosono, S.-S. Yoon, J.-H. Lee, T. Pardee, A.T. Fathi, C. Liu, N. Hasabou, X. Liu, E. Bahceci, and M.J. Lewis

Overall Survival among Patients with FLT3-Mutated Relapsed or Refractory AML Treated with Gilteritinib or Salvage Chemotherapy (Intention-to-Treat Population).



No. at Risk	0	3	6	9	12	15	18	21	24	27	30	33	36
Gilteritinib	247	206	157	106	64	44	31	14	11	4	1	0	0
Salvage chemotherapy	124	84	52	29	13	12	8	7	5	3	1	0	0

CI: confidence interval

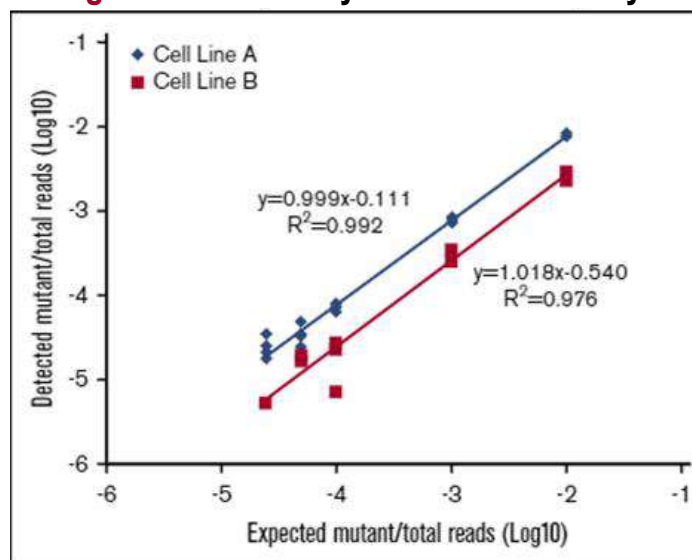
Demonstrates higher specificity and **2 orders of magnitude increased sensitivity** than currently available PCR or NGS-based *FLT3*-ITD assays

Supports use of guiding therapy decisions for patients with AML.

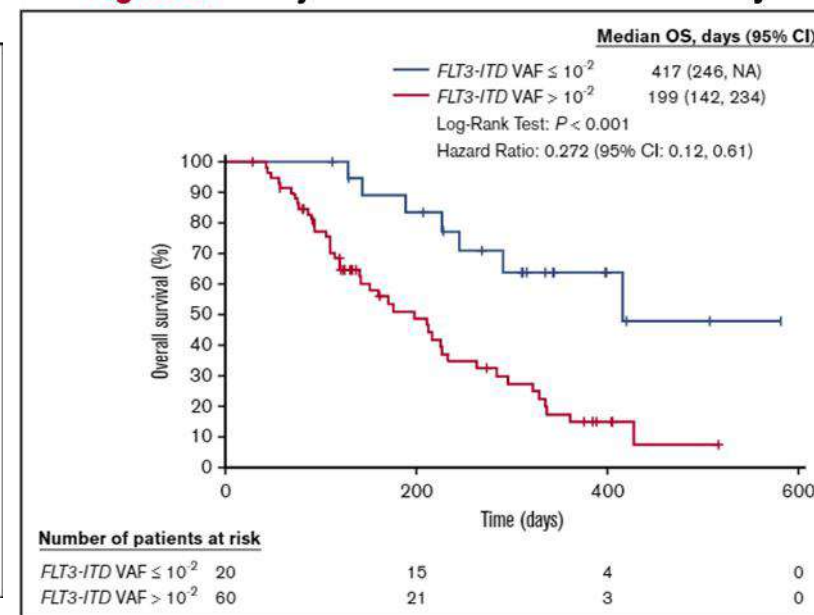
**A next-generation sequencing-based assay for minimal residual disease assessment in AML patients with *FLT3*-ITD mutations.**

Levis MJ<sup>1</sup>, Perl AE<sup>2</sup>, Altman JK<sup>3</sup>, Gocke CD<sup>1</sup>, Bahceci E<sup>4</sup>, Hill J<sup>4</sup>, Liu C<sup>4</sup>, Xie Z<sup>5</sup>, Carson AR<sup>5</sup>, McClain V<sup>5</sup>, Stenzel TT<sup>5</sup>, Miller JE<sup>5</sup>.

**Figure 1. Linearity of NGS-MRD assay**



**Figure 2. Subject OS in the CHRYSALIS study**



# References

External Posters/Abstracts



# Multiple Myeloma MRD

In this study 110 patients, diagnosis and follow up (220 samples) were tested

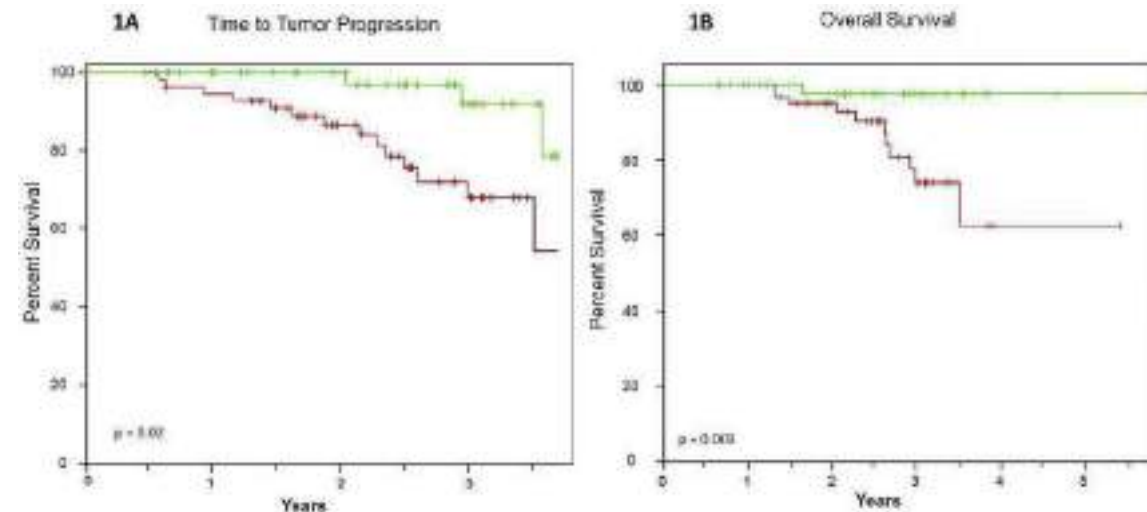
## High correlation of NGS and MFC

14/18 patients who experienced progression were MRD positive with LymphoTrack<sup>®</sup> by NGS

“The **applicability** of the LymphoTrack<sup>®</sup> was **very high in this study (95.5%)**, similar to that reported in other studies for MFC and different NGS approaches”

## New Alternatives for the Evaluation of Minimal Residual Disease (MRD) Detection By Next Generation Sequencing in Multiple Myeloma

Alejandro Medina, Cristina Jiménez, Noemi Puig, Beatriz Sanchez-Vega, Juan Flores-Montero, Marcos Conzález, María Jose Calasanz, Rosa M. Ayala, María E Sarasquete, Bruno Palva, María Teresa Cedena, Marta Fernández-Mercado, Inmaculada Rapado, Laura Rosinol, Enrique M. Ocio, Albert Oriol, Miguel Alcoceba, Miguel T Hernández, Rafael Martínez Martínez, María-Victoria Mateos, Juan-José Lahuerta, Joan Bladé, Alberto Orfao, Jesus F. San Miguel, Joaquin Martínez-López, and Ramón García-Sanz





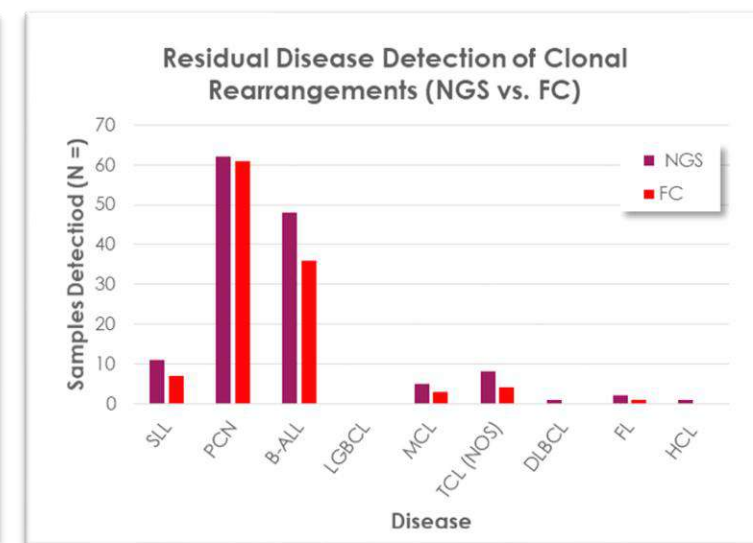
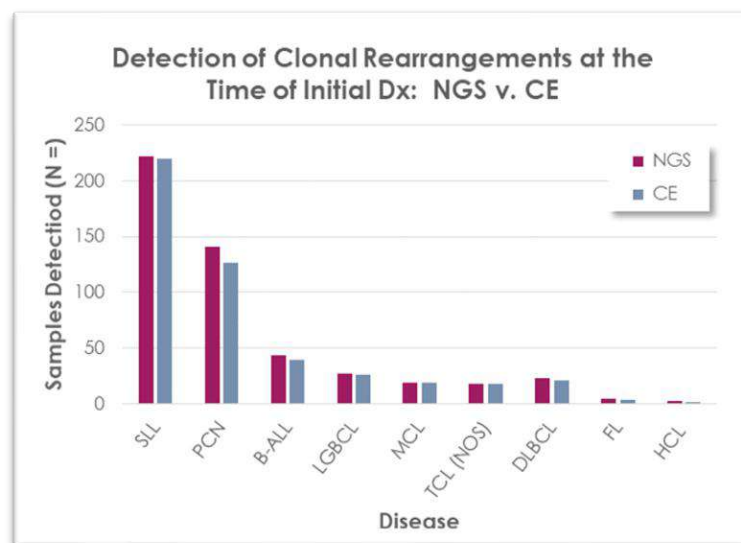
# LymphoTrack<sup>®</sup> vs. CE and FC

Laboratory reports that LymphoTrack<sup>®</sup> detected clonal rearrangements in **94% of Dx cases vs. 89% by CE.**

**NGS** was equivalent to FC for detection of plasma cell neoplasms (PCN) but **showed advantages in disease monitoring** for B-ALL, B- and T-cell lymphomas.

## Next Generation Sequencing (NGS) Based IGH and TCR Clonality Assays Provide Excellent Specificity and Sensitivity for Routine Clonal Characterization and Monitoring of Lymphoproliferative Disorders

Maria E. Arcila, Mustafa Syed, MS, Wayne Yu, B.S, Hannah Kim, B.S, JinYuan Yao, MD, Caleb Ho, MD, Kseniya Petrova-Drus, Mikhail Roshal, MDPH, Jae H. Park, MD, Ola Landgren, MD PhD, Ahmet Dogan, MD PhD, Khedoudja Nafa, PhD



# Development of Clonal Populations

Case study of 9 patients with B-ALL after treatment for MM

In one patient, LymphoTrack<sup>®</sup> was used to verify that B-ALL and MM clones did not share common *IGH* rearrangements

NGS can be considered as a tool to characterize development of clonal lymphoid populations during therapy

## Clinical and Pathological Features of B-Cell Acute Lymphoblastic Leukemia Following Maintenance Treatment with Lenalidomide for Multiple Myeloma

Mark Elaine Geyer, MD, Brian C. Shaffer, MD, Heather Landau, MD, Hani Hassoun, MD, Christopher Famulare, MS, Mikhail Roshal, MD PhD, Ahmet Dogan, MD PhD, Maria E. Arcila, Jae H. Park, MD

**Table 1: Disease characteristics of patients developing B-ALL following lenalidomide treatment.**

ID #	Age at B-ALL Dx (years)	B-ALL Cytogenetics at Dx	B-ALL Molecular Features at Dx	B-ALL Induction	Post-Remission B-ALL Therapy	Disease status following post-remission therapy or pre-ALL/CLL	ALL/CLL Type	Relapse of B-ALL?	B-ALL Dx to Last F/U (months)	Last F/U
1	66.1	FISH: <i>IGH</i> rearrangement, loss of signal for 2 MLL	Unknown	VPOCy	Dauno, AraC, then All-AT	MRD+ CR	10/10 MUD	Y	41.1	Alive in MRD- CR
2	66.2	Karyotype: -7, 10, 12i	Unknown	VPOCy	All-AT	MRD- CR	10/10 MRD	Y	32.7	Alive in MRD- CR
3	60.4	Normal	Unknown	VPOCy	Dauno, AraC, then All-AT	CR	CD34-Selected, 10/10 MRD	Y	7.1	Died in CR from complications of GVHD, septic shock
4	61.3	FISH: del(13q)q14.1, del(13q)q31, single copy of TP53, CEP 17	RBI G120* (subclonal), CREBBP loss, CBL L380P	VPOCy	POMP	MRD- CR	None	Y	37.1	Developed MDS. Died without known evidence of B-ALL
5	64.1	Normal	FORX1-CRLF2 fusion, IGH0 R149C, SH2B3 E573K	VPDA	POMP, then All-AT	MRD- CR	10/10 MUD	Y	10.9	Alive in MRD- CR
6	63.7	FISH: Complex	Loss of PZF2 exons 2-3, RBI E320* (subclonal), RBI PS68K*5, TP53 Q255 (subclonal), TP53 H91F (subclonal), TP53 K132* (subclonal), TP53 P196T	HyperCVAD	HyperCVAD, POMF, then All-AT	MRD- CR	10/10 MRD	Y	10.3	Alive in MRD- CR
7	68.9	Unknown	Unknown	Augmented HyperCVAD	Binatumumab	MRD- CR	None	Y	14.3	Alive in MRD- CR
8	52.2	Unknown	Unknown	VP	NE	NE	NE	NE	4.1	NE
9	64.0	Karyotype: unusual band pattern of 16q	IGH-CRLF2 rearrangement, CRLF2 F232C, TET2 Q88P, GRIK2A T116M	Len discontinuation alone	Len discontinuation alone	MRD+ CR	None	Y	7.0	Alive with MRD on abiraterone alone

Legend: B=Diagnosis, VPOCy=vincristine, prednisone, doxorubicin, cyclophosphamide, VPDA=vincristine, prednisone, daunorubicin, pegaspargase, HyperCVAD=hyperfractionated cyclophosphamide, ifosfamide, doxorubicin, and decarbazine, Dauno=daunorubicin, AraC=cytarabine, Forx=forxane, Vinorel=vinorelbine, methotrexate, mercaptopurine, Len=lenalidomide, MRD=minimal residual disease, CR=complete response, All-AT=alemtuzumab, 10/10 MRD=10/10 HLA-matched relapse/intermittent, NE=not evaluable

<https://ashpublications.org/blood/article/130/Supplement%201/1285/116383>

# TRG Sequencing in AITL

## High Throughput T Cell Receptor Sequencing Augments Diagnosis and Response Assessment in Patients with Angioimmunoblastic T Cell Lymphoma

Reinhard Marks, MD, Sylvia Kock, Claudia Wehr, Jürgen Finke, MD, Martin Werner, MD, Justus Duyster, Paul Fisch, MD

Laboratory analysed TRG repertoire in several different tissues using LymphoTrack<sup>®</sup> TRG assays

Detected T-cell clonality in all 6 cases of AITL

In some cases, lymphoma-specific TCR sequences could be detected up to one year before diagnosis

Observed sustained detection of sequences in bone marrow in apparently successful treated patients with refractory clinical symptoms

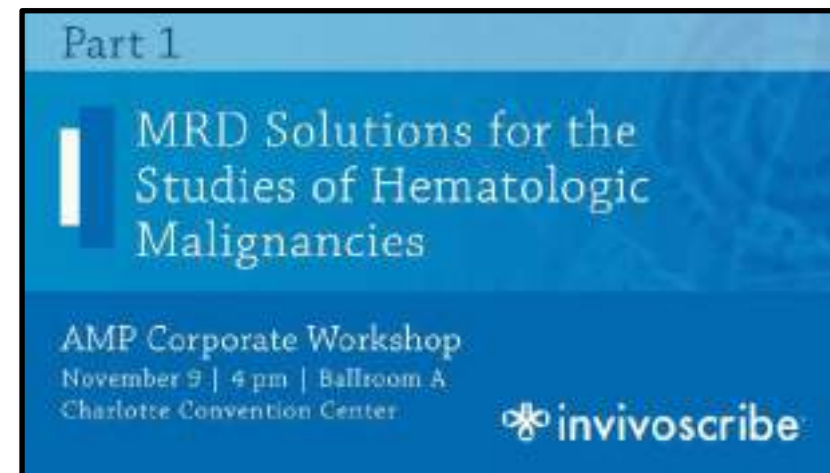
# References

Videos





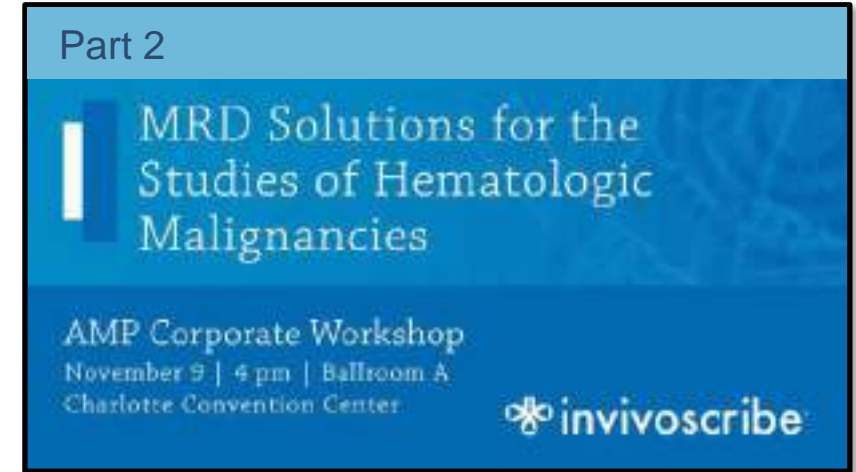
- Highlights of talk by Dr. Maria Arcila  
Deputy Chief of Molecular Diagnostic Service & Medical Director of Molecular Hematopathology at Memorial Sloan Kettering Cancer Center
- Her lab's approach to studies of minimal residual disease (MRD) with the use the NGS-based LymphoTrack<sup>®</sup> IGH & TRG Assays.
- Comparisons between flow and NGS methods
- Cases from multiple disease states



<https://www.youtube.com/watch?v=FPmxBU0NfZk>



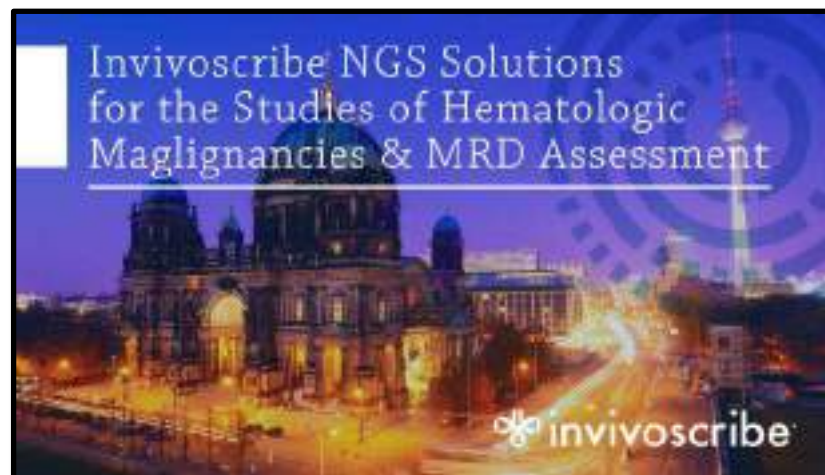
- Highlights of talk by Dr. Brad Patay  
Chief Medical Officer, Invivoscribe, Inc.
- Measurable (“minimal”) Residual Disease (MRD): *FLT3* Internal Tandem Duplication (ITD)
- The speaker discusses the value of MRD testing in acute myeloid leukemia patients and the *FLT3* ITD MRD next-generation sequencing testing service offered by LabPMM, Invivoscribe's clinical laboratories.



<https://www.youtube.com/watch?v=zPT4dSOcN2E&t=6s>



- Highlights of talk by Dr. Riccardo Bomben  
Group leader at the Onco-Hematology Clinic (Aviano, Italy)
- Discusses his lab's approach to study CLL and *IGHV* SHM
- Compares traditional Sanger Sequencing following ERIC guidelines to the results obtained with the LymphoTrack<sup>®</sup> Assays



<https://www.youtube.com/watch?v=Ytzck01ovfg>



## NGS Based Clonality Testing - Assessing Clonality Status, Somatic Hypermutation and Monitoring Minimum Residual Disease (MRD)

- Dr. Maria Arcila

Deputy Chief of Molecular Diagnostic Service & Medical Director of Molecular Hematopathology at Memorial Sloan Kettering Cancer Center



<https://vimeo.com/317371393>





## Analysis of Human BCR Rearrangement Repertoires in Health and Disease

- Bettina Budeus  
Group Molecular Genetics, Essen University Hospital, Essen, Germany

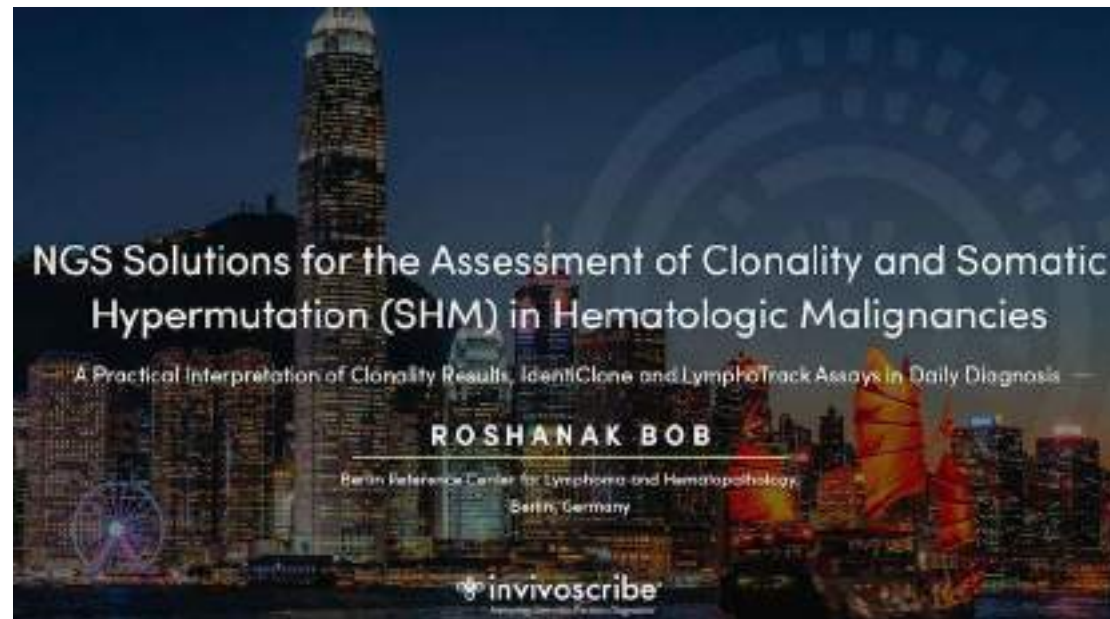


<https://vimeo.com/340315124>



## A Practical Interpretation of Clonality Results, IdentiClone® and LymphoTrack® Assays in Daily Diagnosis

- Roshanak Bob  
Berlin Reference Center for Lymphoma and Hematopathology, Berlin, Germany



<https://vimeo.com/340323164>



## Analysis of *IGHV* Mutational Status, T-Cell Clonality and MRD by NGS

- Oliver Giles Best

CLL Research Laboratory, Northern Blood Research Centre, Kolling Institute of Medical Research, University of Sydney, Royal North Shore Hospital, St. Leonards, Sydney, Australia



<https://vimeo.com/341472372>



## Case Studies of *IGHV* Analysis and NGS Mutation Detection in Lymphoid Malignancy

- Piers Blombery  
Peter MacCallum Cancer Centre, Melbourne, Australia



<https://vimeo.com/341475803>



## Invivoscribe LeukoStrat<sup>®</sup> CDx *FLT3* Mutation Assay

- Dr. Bradley Patay  
Invivoscribe, CMO, San Diego, USA



<https://vimeo.com/347596491>



## Minimal Residual Disease Detection of B-cell Lymphoid and Plasma Cell Neoplasms using an NGS-Based Clonal Rearrangement Assay

- Dr. Caleb Ho  
Assistant Attending Pathologist at Memorial Sloan Kettering Cancer Center
- Dr. Ryan Schmidt  
Assistant Professor of Clinical Pathology / Assistant Director at University of Southern California / Children's Hospital Los Angeles



<https://vimeo.com/349961430>



## Clonality Assessment and MRD Analysis in ALL and MM Using the LymphoTrack® Dx Assays

- Marleen Bakkus  
Laboratory for Molecular Hematology, UZ Brussel, University Hospital Brussels, Brussel, Belgium



<https://vimeo.com/355617155>

## Analysis of Human BCR Rearrangement Repertoires in Health & Disease

- Marc Seifert  
Institute of Cell Biology (Tumor Research), AG Immunology and Lymphomagenetics, Essen University Hospital, Essen, Germany



<https://vimeo.com/356038625>





## B- and T-Cell Clonality Analysis and CLL IGHV Mutational Status – an Overview

- Fiona Quinn  
Cancer Molecular Diagnostics Dept, St. James's Hospital, Dublin, Ireland

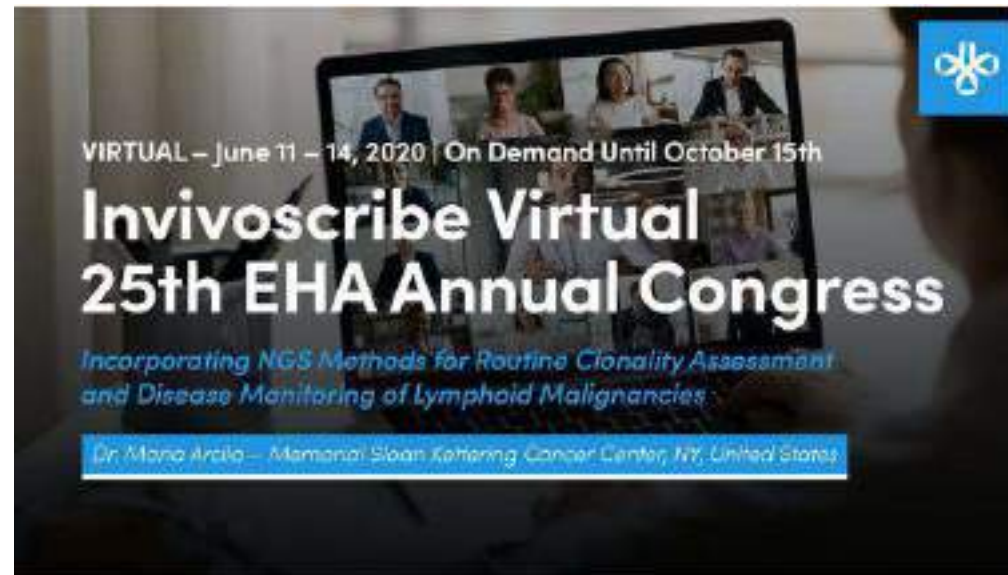


<https://vimeo.com/356284961>



## Incorporating NGS Methods for Routine Clonality Assessment and Disease Monitoring of Lymphoid Malignancies

- Dr. Maria Arcila  
Memorial Sloan Kettering Cancer Center, NY, United States



<https://vimeo.com/428587272>



## Clonality Analysis with NGS is the New Frontier in MRD Detection

- Prof. Sara Galimberti  
University of Pisa, Department of Clinical and Experimental Medicine, Italy

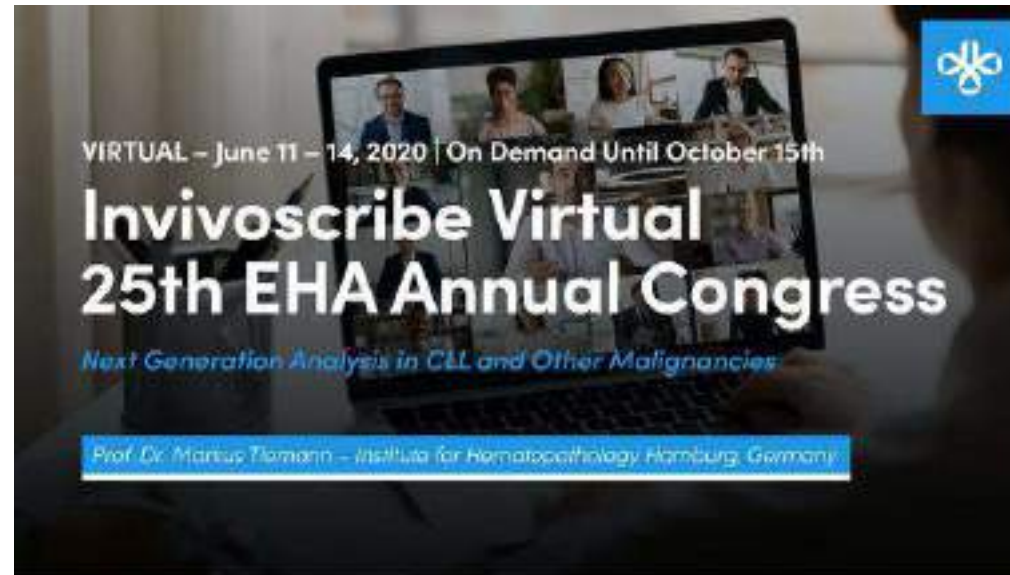


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## Next Generation Analysis in CLL and Other Malignancies

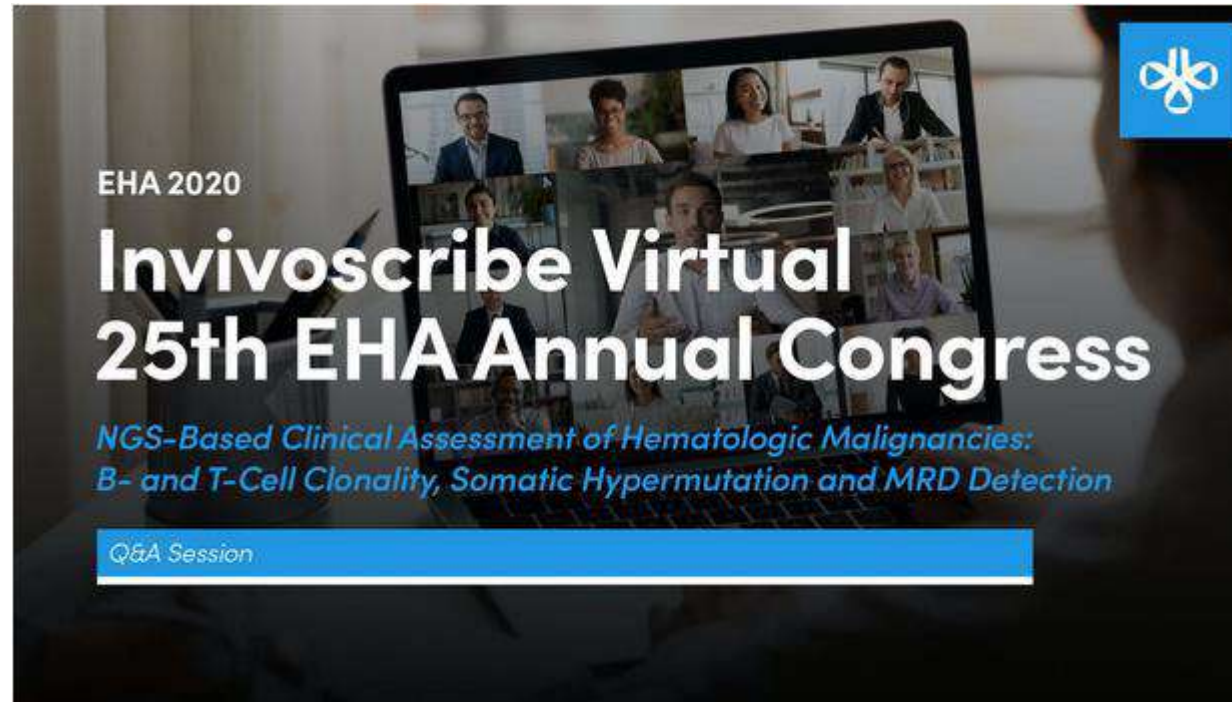
- Prof. Dr. Markus Tiemann  
Institute for Hematopathology Hamburg, Germany



<https://vimeo.com/428594643>



## Q&A session

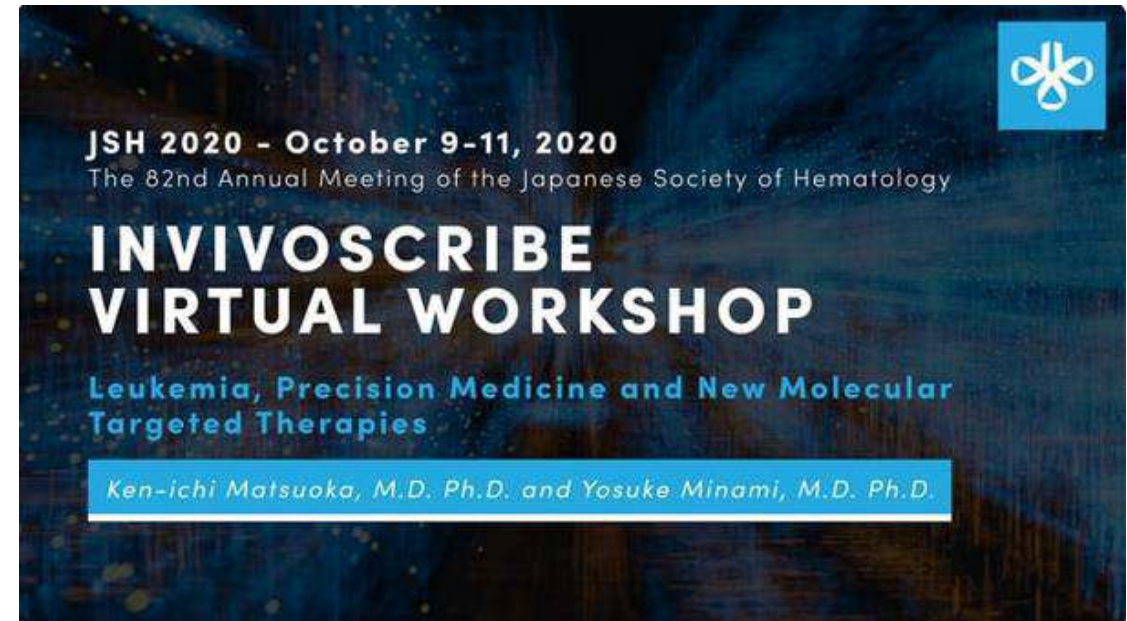


<https://vimeo.com/430153275>



## Leukemia, Precision Medicine and New Molecular Targeted Therapies

- Yosuke Minami, M.D., Ph.D.  
Department of Hematology, National Cancer Center Hospital East
- Ken-ichi Matsuoka, M.D. Ph.D.  
Department of Hematology and Oncology, Okayama University

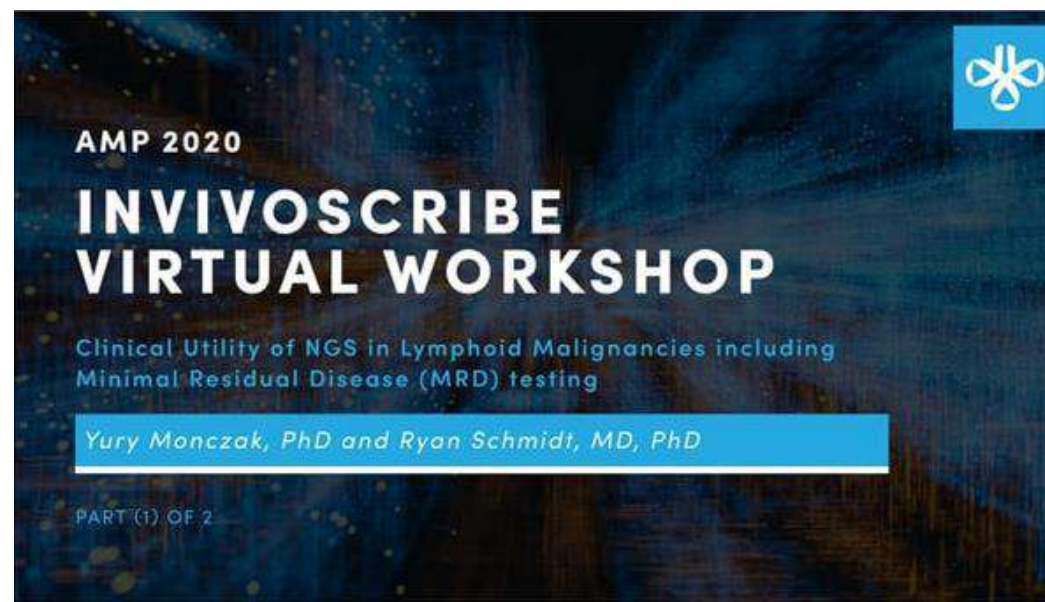


<https://vimeo.com/481393310>



## Clinical Utility of NGS in Lymphoid Malignancies including Minimal Residual Disease (MRD) testing

- Yury Monczak, Ph.D.  
McGill University Health Center /  
Jewish General Hospital
- Ryan J. Schmidt, MD, Ph.D.  
Children's Hospital Los Angeles / USC  
Keck School of Medicine

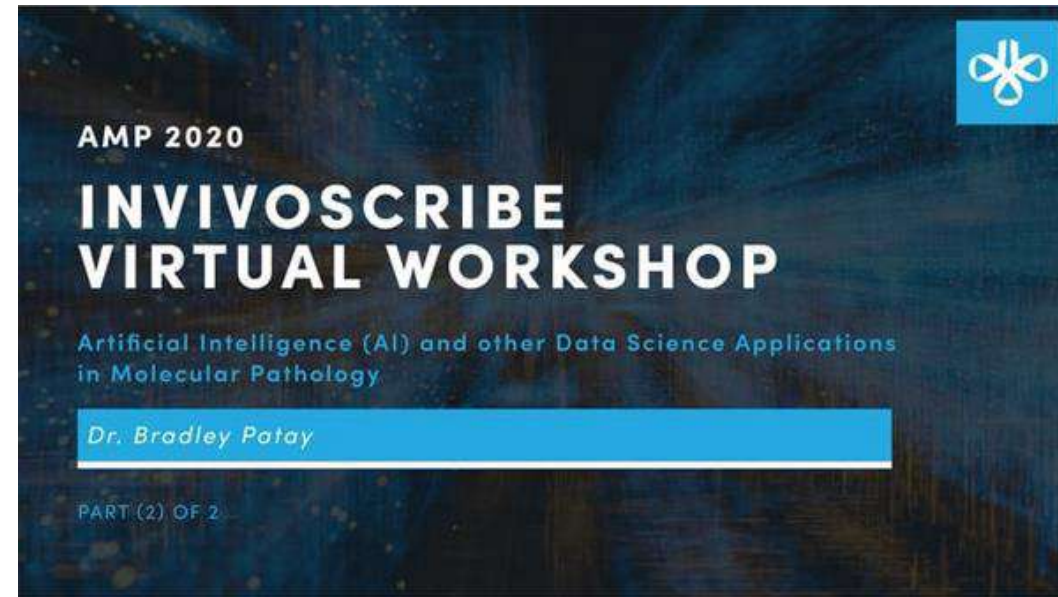


<https://vimeo.com/480863214>



## Clinical Utility of NGS in Lymphoid Malignancies including Minimal Residual Disease (MRD) testing

- Dr. Bradley Patay  
Chief Medical Officer,  
Invivoscribe, Inc.



<https://vimeo.com/480855946>



# References

Invivoscribe Posters





## DEVELOPMENT OF AN NGS ASSAY FOR *IGK* THAT CAN BE COMBINED WITH *IGH* FOR IDENTIFYING CLONAL POPULATIONS IN LYMPHOID MALIGNANCIES

Niels C. Adams<sup>1</sup>, Gillian M. Pawlowsky<sup>1</sup>, Ying Huang<sup>2</sup>, Kasey Hutt<sup>1</sup>, Michael Klass<sup>2</sup>, Jeffrey Miller<sup>2</sup>

<sup>1</sup>LabPMM GmbH, Munich, Germany  
<sup>2</sup>Invivoscribe Technologies, Inc., San Diego, CA, United States

### Introduction

Previously we developed next-generation sequencing assays for the detection and characterization of clonality in B cells, targeting the *IGH* locus with single-step PCR approach followed by sequencing and analysis. These *IGH* assays were shown to be both sensitive and robust in a number of different laboratories and have demonstrated utility for tracking minimal disease (MD) in cancer patients that can be used for the identification and tracking of clonality as *IGH* rearrangements are stable markers that are retained even in heavily treated or relapsed B cell malignancies such as non-Hodgkin's Lymphoma and having a variety of B cell clones.

The immunoglobulin kappa (IGK) in the *IGK* locus (IGK) prior to rearrangement within *IGK*. Accordingly, *IGK* is an ideal marker for tracking minimal disease (MD) through MD.

This poster describes the development of an assay for *IGK* that can be combined with *IGH* that will increase the chance of identifying clonal populations in lymphoid malignancies such as non-Hodgkin's Lymphoma and having a variety of B cell clones.

### Methods

- Genomic DNA from peripheral blood, bone marrow, and/or aspirates were tested for *IGK* gene rearrangements.
- Optimized V and J regions were targeted at *IGH* and *IGK* gene segments that are rearranged in lymphoid cells as well as the *IGH* rearrangement in that procedure (this selection).
- Multiple PCR master mixes simultaneously amplified *IGK* J gene rearrangements and incorporated sample identification into amplicons, which facilitated combining and sequencing library generation (testing up to 12 samples and controls in each run).
- Amplicon products were purified, quantified, the concentrations adjusted, pooled, and the normalized libraries were loaded into the NextSeq 500. The matched restriction PCR libraries were sequenced using Ion-Seq (Illumina) for variant calling (sequencing 600 bp).

FIGURE 1: ASSAY DESIGN

Single-label single-step PCR with three rearrangement-specific primers:



- Identical PCR programs to existing lymphoma assays (*IGH*, *IGK* V1 & J1) in the same cycle.
- Assays can be combined with other assays using the same index.
- Analysis program separates *IGK* data from other assays.

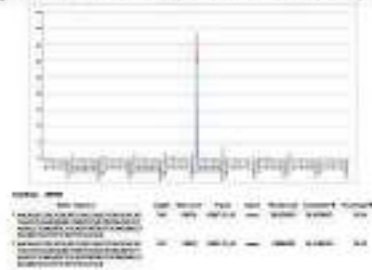
FIGURE 2: ASSAY WORKFLOW



From DNA to clonal sequence results in < 24 hours.

FIGURE 3: CLINICAL DATA

Clinical samples that are not detectable as clonal by *IGH* can be detected by clonality by *IGK*.



### Results

- Data generated with the lymphoid *IGK* assay and bioinformatics package reproducibly identified clonality and DNA sequences of *IGH* V1 & J1 as well as V1 & J1 and IGH J10 gene rearrangements.
- Accumulated data outputs include frequency distribution and V/J gene usage.
- Detecting cell lines solely derived from polyclonal B cell DNA confirmed the linearity of V1 & J1 and J10 run-to-run variation of the assay.

FIGURE 4: ASSAY CHARACTERISTICS

- Polyclonal templates have low initial frequency (< 1:1000).
- Sequences present at 2.5% are easily identifiable.
- Heteroplasmic regions convert to two sequences being reported.
- Software solution for consolidation of sequencing data (indexing).
- Assay has high linearity ( $R^2 > 0.99$ ).
- Little variation between runs and operators ( $R^2 > 0.98$ ).

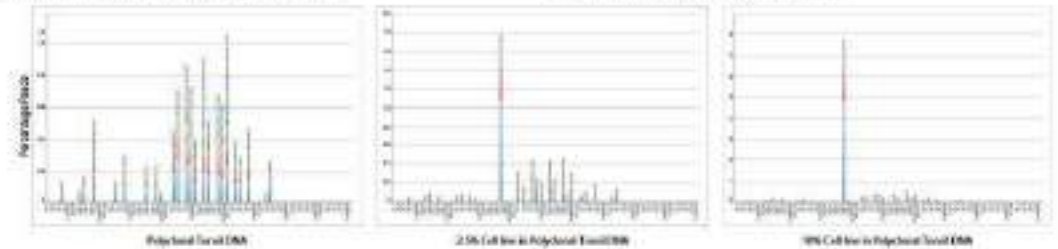
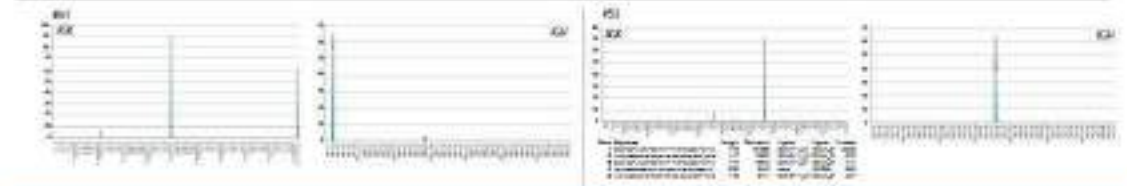


FIGURE 5: CLONAL *IGH* IN CLINICAL SAMPLES



### Conclusions

We have previously developed PCR and NGS assays for the *IGH* locus.

We have added an NGS clonality assay for *IGK* that identifies clonal *IGH* V1 & J1 as well as V1 & J1 and IGH J10 gene rearrangements and associated specific rearranged DNA sequences to the repertoire that can refer to used in follow-up to further characterize samples that are difficult to characterize by *IGH* alone.

Alternatively, a more time and cost efficient approach would be to run both assays at the same time on samples to be run simultaneously.

These assays are being developed to detect and monitor lymphoproliferative disease.

A further advantage is that the assays identify clonal lymphocyte populations by their unique DNA sequences that can subsequently be used to track disease in the clinical setting.



## CLINICAL ASSESSMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) SAMPLES FOR SOMATIC HYPERMUTATION STATUS BY NEXT-GENERATION SEQUENCING AND SANGER SEQUENCING

María Arida<sup>1</sup>, Ying Huang<sup>2\*</sup>, Kasey Hutt<sup>1</sup>, Jeff Panganiban<sup>1</sup>, Tessara Baldi<sup>1</sup>, Khedoudja Nafa<sup>1</sup>, Jordan Thornes<sup>1</sup>, Jeffrey E. Miller<sup>2</sup> and Michael Klass<sup>2</sup>

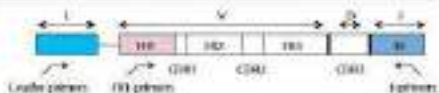
<sup>1</sup>Diagnostic Molecular Pathology Laboratory, Memorial Sloan-Kettering Cancer Center, New York, United States; <sup>2</sup>Invivoscribe Technologies, Inc., San Diego, United States

### Introduction

- Somatic hypermutation (SHM) is an important process to increase the affinity of immunoglobulin molecules. The presence of > 2% SHMs is an important prognostic factor for CLL samples.
- The current method used to determine SHM status requires two steps of PCR amplification followed by (1) deep bisulfite conversion to convert clonality, followed by a Sanger sequencing step. This multistep approach is labor intensive and time consuming.
- Next generation sequencing (NGS) based assays for PCR SHM assays (MSeq<sup>®</sup> and PGM<sup>®</sup>) have been developed to address these limitations.
- The clinical performance of LymphoTrack<sup>®</sup> Dx/Assay assays revealed SHM status was assessed with 100% sensitivity, blinded CLL samples (7.1% and 33.3%) in comparison with the traditional PCR/Sanger method.

### Materials and Methods

FIGURE 1. PROBE DESIGN



LymphoTrack<sup>®</sup> Dx SHM assays have been developed for both the MSeq and PGM NGS platforms. Both assays and NGS platforms were used in this study. The MSeq SHM Assay amplifies two master mixes. One amplifies genomic DNA between the upstream constant (C) region and the downstream joining (J) region of the V region. The other amplifies from the transcribed (T) to the J region. Amplification products from V/J primers, from the transcribed (T) region, and from P/J primers are compared against a reference of the (T) region to the downstream joining (J) region. The PGM Assay only amplifies V/J primers. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on either the MSeq or PGM platforms. The K121185 and K21185 assays for MSeq use 24 nucleotides allowing analysis of 12 patient samples. K21185 assays for PGM uses 32 nucleotides allowing analysis of 16 patient samples. Turnaround time for the PGM analysis of 12 patient samples in either platforms is 1 day. Analysis for the K121185 is independent to be increased sequencing length of the amplicon.

FIGURE 2. WORKFLOW



Genomic DNA (200ng) was amplified with consensus primers using a single multiplex PCR. Amplicons were purified using the Agencourt AMPure XP PCR Purification system. Before pooling of the libraries, Amplicon libraries were quantified before being loaded onto the MSeq or PGM. MSeq data was generated using the MSeq v3.1 (Illumina) HiSeq 2500 cycle for 150 on the MSeq v3.1 (Illumina) HiSeq 2500 cycle for the leader assay. PGM data was generated using the Ion PGM Template CE2 400 kit and Sequencing 400 kit. K121185 was not analyzed on the PGM due to length limitations of the PGM platform.

FIGURE 3. LYMPHOTRACK Dx SHM SOFTWARE



Seq ID	Sequence	Depth	Seq. Count	Y-axis	X-axis	Y-axis	X-axis	Y-axis	X-axis	Y-axis	X-axis
1	CTCCGCGGCTCA...	288	104887	100%	100%	100%	100%	100%	100%	100%	100%
2	CTCCGCGGCTCA...	287	1047	100%	100%	100%	100%	100%	100%	100%	100%
3	CTCCGCGGCTCA...	286	1046	100%	100%	100%	100%	100%	100%	100%	100%
4	CTCCGCGGCTCA...	285	1045	100%	100%	100%	100%	100%	100%	100%	100%
5	CTCCGCGGCTCA...	284	1044	100%	100%	100%	100%	100%	100%	100%	100%
6	CTCCGCGGCTCA...	283	1043	100%	100%	100%	100%	100%	100%	100%	100%
7	CTCCGCGGCTCA...	282	1042	100%	100%	100%	100%	100%	100%	100%	100%
8	CTCCGCGGCTCA...	281	1041	100%	100%	100%	100%	100%	100%	100%	100%
9	CTCCGCGGCTCA...	280	1040	100%	100%	100%	100%	100%	100%	100%	100%
10	CTCCGCGGCTCA...	279	1039	100%	100%	100%	100%	100%	100%	100%	100%

Atypical MSeq K21185 reads from a 200 sample showed evidence for clonality with one sequence at 61.6% of the total reads and mutation rate at 1.2%. Only the top 10 sequences are presented here. PGM data from either MSeq or PGM can be analyzed by LymphoTrack<sup>®</sup> Dx software running on a Windows PC. LymphoTrack<sup>®</sup> Dx Software generates frequency distributions, DNA sequences, V/J assignment and usage, and SHM status. The SHM status is determined two ways: 1) alignment statistics obtained from the BLAST algorithm for the top 10 reads and the calculation of insertions and gaps to the V-gene reference sequence; 2) the mutation rate is calculated as the sum of insertions and gaps divided by the V-gene length.

### Results

- The standard PCR/Sanger method was able to detect 47 out of 50 samples with 30.3-90 samples exhibiting SHM rate > 2.0 and 11 CLL samples exhibiting SHM rate < 2.0. These samples were not available and were excluded for comparison in Table 1.
- The LymphoTrack Dx K121185 Assay - MSeq was able to detect 58 out of 60 samples with 33.3-90 samples exhibiting SHM rate > 2.0 and 16 CLL samples exhibiting SHM rate < 2.0.
- The LymphoTrack Dx K21185 Assay - PGM was able to detect 58 out of 60 samples with 33.3-90 samples exhibiting SHM rate > 2.0 and 16 CLL samples exhibiting SHM rate < 2.0.

TABLE 1. SENSITIVITY, SPECIFICITY AND PREDICTIVE VALUE OF THE SHM LYMPHOTRACK Dx ASSAYS FOR DETERMINING SHM STATUS

Test	SHM	SHM (%)	SHM (%)	SHM (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
MSeq K121185	58/60 (96.7%)	5	0	17 (28.3%) and 11 (18.3%)	100	100	100	100
MSeq K21185	58/60 (96.7%)	0	0	17 (28.3%) and 11 (18.3%)	100	100	100	100
PGM K21185	58/60 (96.7%)	0	0	17 (28.3%) and 11 (18.3%)	100	100	100	100

SHM status (SHM) for MSeq: 100% (SHM) > 2.0%, 0% (SHM) < 2.0%. SHM status for PGM: 100% (SHM) > 2.0%, 0% (SHM) < 2.0%. SHM status for Sanger: 47% (SHM) > 2.0%, 11% (SHM) < 2.0%. SHM status for MSeq: 100% (SHM) > 2.0%, 16% (SHM) < 2.0%. SHM status for PGM: 100% (SHM) > 2.0%, 16% (SHM) < 2.0%.

FIGURE 4. SHM CORRELATION



- A comprehensive NGS assay has been developed for both MSeq and PGM platforms that identifies clonal SHM V region rearrangements, associated specific V region DNA sequences and determines the SHM status in CLL specimens as well as the method of expression with the determination of the sequence being in frame, the absence of stop codons, and the degree of coverage.
- These NGS assays have demonstrated excellent clinical concordance for determining SHM status in CLL specimens as compared to the standard PCR/Sanger sequencing method.

### Conclusions

- A comprehensive NGS assay has been developed for both MSeq and PGM platforms that identifies clonal SHM V region rearrangements, associated specific V region DNA sequences and determines the SHM status in CLL specimens as well as the method of expression with the determination of the sequence being in frame, the absence of stop codons, and the degree of coverage.
- These NGS assays have demonstrated excellent clinical concordance for determining SHM status in CLL specimens as compared to the standard PCR/Sanger sequencing method.





## Validation and clinical implementation of next generation sequencing for routine IGH and TCRG clonality assessment

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H44

### Introduction

IGH and TCR gamma clonality testing is an important component in the diagnosis of lymphoproliferative disorders. Capillary electrophoresis (CE) of multiplexed PCR products is a common method of analysis. Although robust, simple and highly reproducible, it does not provide a full characterization of clonal sequences and lacks sensitivity and specificity to track clones in subsequent samples. We describe our clinical validation and implementation of an NGS based assay for initial clonal characterization and minimal residual disease assessment through patient specific clone tracking.

### Methods

Blood, bone marrow and FFPE tissue samples submitted for routine clonality assessment were selected. DNA was extracted and tested using both standard CE and LymphoTrack<sup>®</sup> IGH + TCRG - MiSeq assays (Invivoscribe). Positive, negative and no template controls were run with all assays. Sensitivity and LOD were assessed based on dilution studies for detection of initial clonal population and subsequently for tracking of a pre-characterized clone at minimal residual disease levels.

### Results

A total of 100 samples were analyzed including 120 clonal (defined by initial CE testing, 82 IGH, 34 TCRG) and 34 non-clonal samples with 84% concordance between the 2 methods. Discordant results (clonal by CE, non clonal by NGS) were attributable to pseudo-clonality in the post treatment setting. Based on a minimum input of 50ng of high quality DNA, analytical sensitivity was 5% for diagnostic samples (un-characterized clone) with good inter and intra-assay reproducibility. Further dilution studies to establish LOD for tracking a previously characterized clone showed accurate detection at 1x10<sup>-5</sup> with 1-2ug DNA input. The mean number of reads per sample was approximately 500,000.

### Conclusion

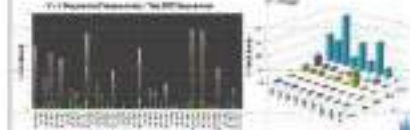
Assessment of clonality by NGS methods provides significant improvement over existing clonality assays using fragment analysis by CE. Sensitivity for detection of a diagnostic clone is similar to the CE assays but provides full characterization of the clone to enable tracking in subsequent samples at the MRD level. NGS testing readily resolved pseudo-clonality calls in post-treatment samples by differentiating clonal products of same size but different sequences interpreted as clonal by the CE method. However, the LymphoTrack<sup>®</sup> assays remain expensive and with higher TAT compared to CE.

### Acknowledgements

The authors would like to thank the Diagnostic Molecular Pathology Laboratory members and Invivoscribe for their technical support.

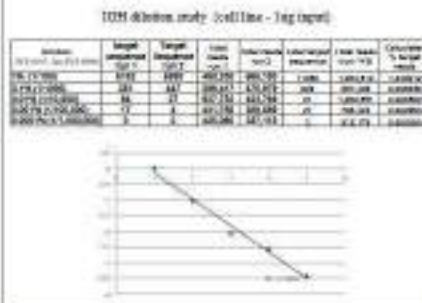
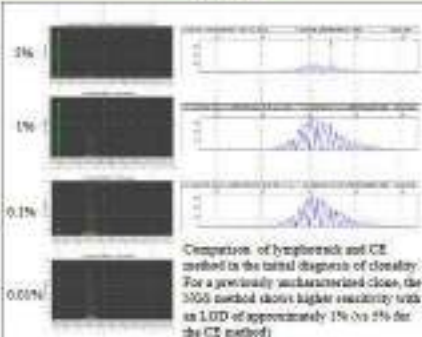
### Results

Lymphotrack read summary for polyclonal sample

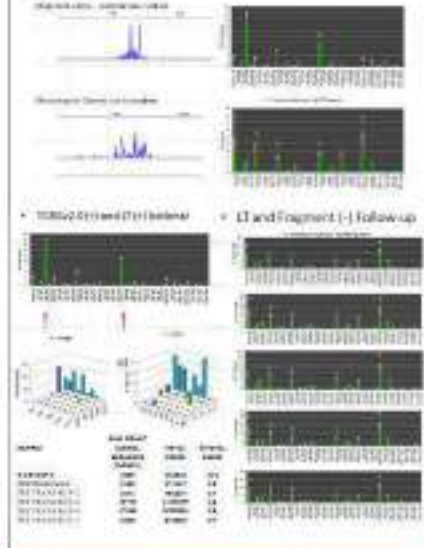


Lymphotrack read summary for clonal sample (diffuse large B-cell)

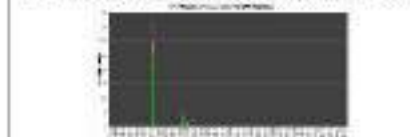
Region	V	J	Reads	Identity
IGHV1-2	1	1	1000000	99%
IGHV1-2	1	1	1000000	99%
IGHV1-2	1	1	1000000	99%
IGHV1-2	1	1	1000000	99%
IGHV1-2	1	1	1000000	99%



MRD case study #1  
57 yr male with growing subcutaneous nodule in the arm diagnosed as typical T cell proliferation seen consistent with angioimmunoblastic T cell lymphoma. Bone marrow obtained for evaluation of disease, pre-treatment.



MRD case study #1 - 51 yo male with newly diagnosed plasma cell neoplasm



Diagnosis sample  
Bone marrow: Plasma cell myeloma, involving 80-90% marrow cellularly/Small monoclonal B-cell population detected by flow cytometry



Post treatment sample  
Bone marrow: Microbiology: no evidence. Flow cytometry: Minute abnormal plasma cell population detected by flow cytometry (0.009%)  
Lymphotrack: 0.12% (74452220, 96% identity)

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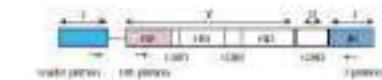
## Development of LymphoTrack<sup>®</sup> Bioinformatics Methods: Clonality Testing, Somatic Hypermutation and Minimal Residual Disease

Kasey Hutt, Jeff Panganiban, Austin Jacobsen, Jordan Thomas, Jeffrey E Miller, and Michael Klass  
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### Introduction

- Clonality assessment is an important diagnostic indicator for the malignant transformation of lymphoid cells.
- Minimal Residual Disease (MRD) is a key prognostic indicator for determining efficacy of treatment.
- IMPROVED REPLICATION YIELDS is a naturally occurring process to increase B cell receptor diversity, and serve as a positive prognostic factor in chronic lymphocytic leukemia (CLL).
- Next Generation Sequencing (NGS) assays for monitoring these disease states are exciting the cutting edge of diagnostic methods, but require the development of novel bioinformatics tools to confidently analyze the resulting data in a single assay. We have developed the LymphoTrack<sup>®</sup> bioinformatics tools to analyze IGH, IGH and IGH-gene on the Illumina<sup>®</sup> MiSeq<sup>®</sup> and ThermoFisher<sup>®</sup> IonTorrent<sup>®</sup> platforms, for the purpose of assessing clonality, IGHV SHM and MRD.

### Materials and Method



**FIGURE 1. PROBE DESIGN**  
 LymphoTrack<sup>®</sup> assays have been developed for both Illumina<sup>®</sup> and IonTorrent<sup>®</sup> platforms, for the IGH, IGH and IGH-gene. Each assay employs 4 separate reaction mixtures with respective upstream regions for clonality with the downstream regions targeted to the IGH-V, D, and J segments, allowing for parallel analysis of 12 samples (plus controls) on a 96-well assay on 1) IonTorrent, allowing for parallel analysis of 12 samples (plus controls).



**FIGURE 2. BIOINFORMATICS PIPELINE**  
 LymphoTrack<sup>®</sup> bioinformatics analysis begins on MiSeq<sup>®</sup> with indexing of paired reads (read 1 and read 2) at the start of the run. The resulting sequencing data is then filtered for index reads, read quality, and sequencing quality. The resulting reads are then aligned to the reference sequence database, and sequence statistics are calculated. Downstream calculations for clonality and SHM require the identification of a clonal read, and for the case of SHM the amount of DNA being assayed. MRD results are reported for each sample, and into a single matrix for the clonal read, which is then a confidence score is assigned to these results based on the variability of read, which is based on the amount of DNA assayed, or the read depth achieved for that sample. The confidence is based on theoretical sampling using a binomial distribution.



**FIGURE 3. LYMPHOTRACK<sup>®</sup> SOFTWARE OUTPUT**  
 A typical MiSeq<sup>®</sup> (CLL) result from a large patient sample showing evidence for clonality with over 99% sensitivity (97% of total reads) and mutation rate of 0%. Top 10 coverage tracks are presented in the table. IGH-gene data from other MiSeq<sup>®</sup> or IonTorrent<sup>®</sup> can be analyzed by LymphoTrack<sup>®</sup> software using an Illumina<sup>®</sup> V2. The LymphoTrack<sup>®</sup> software generates frequency distributions, clonality, SHM, and MRD data. An additional module allows for the tracking of clonality for MRD purposes.

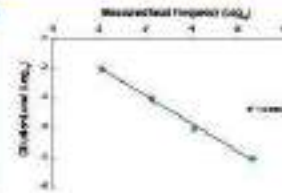
### Results



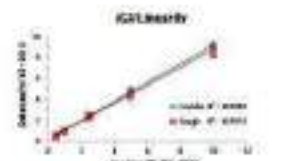
**FIGURE 4. SHM CORRELATION**  
 LymphoTrack<sup>®</sup> new calculations for SHM and MRD closely match results generated by single sequencing and SHM mutation rate calculations.



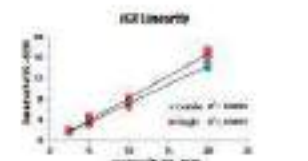
**FIGURE 5. MRD OUTPUT EXAMPLE**  
 Example output for MRD results that include the read count and frequency, as well as confidence scores for various levels of detection. Additional output will include response rates for assays.



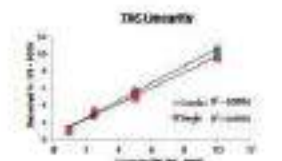
**FIGURE 6. MRD CLONALITY CORRELATION**  
 Clonality assessment and SHM using low coverage between different levels and measured read frequency.



**FIGURE 7. MRD CLONALITY CAPABILITY**  
 LymphoTrack<sup>®</sup> software is able to analyze samples stored with different target loci (IGH, IGH and IGH-gene) even when clonality is similar without a significant change in signal.



**FIGURE 8. MRD CLONALITY CAPABILITY**  
 LymphoTrack<sup>®</sup> software is able to analyze samples stored with different target loci (IGH, IGH and IGH-gene) even when clonality is similar without a significant change in signal.



**FIGURE 9. MRD CLONALITY CAPABILITY**  
 LymphoTrack<sup>®</sup> software is able to analyze samples stored with different target loci (IGH, IGH and IGH-gene) even when clonality is similar without a significant change in signal.

### Conclusions

- LymphoTrack<sup>®</sup> bioinformatics analysis accommodates a wide variety of important diagnostic and prognostic indicators. The thoughtful presentation of results across an equally sophisticated determination of clonality, SHM, and MRD.
- LymphoTrack<sup>®</sup> bioinformatics analysis also provides the resources necessary to drive deeper into the data by providing full sequence information and 1) coverage, allowing for an unprecedented level of granularity previously unattainable using typical sequencing data.

LymphoTrack<sup>®</sup> Assays are for research use only (RUO). Not for clinical diagnostic purposes. LymphoTrack<sup>®</sup> is a brand of CLL-RD diagnostic products, not available for sale or use with both America.



Memorial Sloan Kettering  
Cancer Center

## Assessment of Immunoglobulin Heavy Variable Gene Usage and Somatic Hypermutation Status in Splenic Marginal Zone Lymphomas Using Next Generation Sequencing

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### INTRODUCTION

Splenic marginal zone lymphoma (SMZL) is a rare indolent B-cell neoplasm involving the spleen, bone marrow (BM) and, frequently, blood. Its distinction from similar indolent B-cell malignancies may often be challenging, particularly when diagnosis must be based on the BM findings alone without the support of spleen histology. Prior studies have shown that SMZL exhibit specific immunoglobulin heavy variable gene (IGHV) gene biases which are distinct from other entities and thus ancillary testing could be potentially utilized to aid in the diagnosis or further stratifying this disease. This assessment is, however, often not feasible in the clinical setting as current methods are laborious and not performed in most laboratories. In this study, we explore the utility of next generation sequencing (NGS) for the clinical characterization of IGHV in a cohort of SMZL and compare it to other subtypes of marginal zone lymphomas (MZL) reported in the literature.

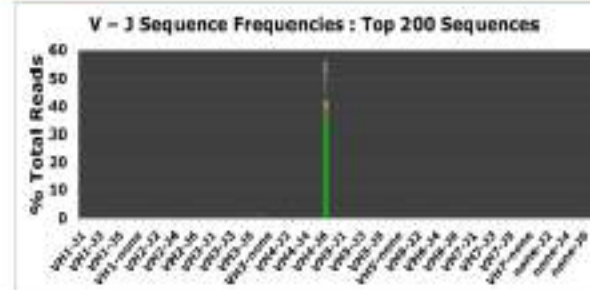
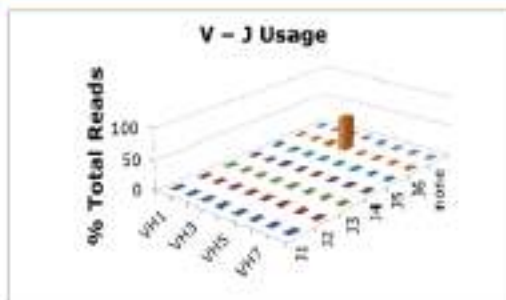
### METHODS

BM samples from patients with an established diagnosis of SMZL and submitted for routine clonality assessment were selected for the study. After establishing the presence of IGH clonality by capillary electrophoresis, the samples were analyzed using an NGS assay targeting IGH-FR1 (LymphoTrack, Invivoscribe) and sequenced by Illumina MiSeq. Data was analyzed using the LymphoTrack IGH-FR1 and Somatic Hypermutation (SHM) software. Clinical and ancillary laboratory data were collected from the electronic medical records.

### RESULTS

A total of 20 BM samples were available for analysis (1 sample failed analysis due to low DNA input). Patients included 8 women and 12 men with a median age at diagnosis of 68 years (range: 47 to 87). IGHV families most frequently rearranged were IGHV3 (11/20, 55%) and IGHV4 (6/20, 30%). The IGHV genes most frequently rearranged were IGHV4-34 (4/20, 20%) followed by IGHV3-23 (2/20, 10%), IGHV3-30 (2/20, 10%), IGHV3-33 (2/20, 10%), IGHV3-73 (2/20, 10%) and IGHV1-3 (2/20, 10%). Using a 98% identity cut-off value, 12/20 cases (60%) showed SHM. Review of the literature showed a similar pattern of IGHV usage to other subtypes of MZL.

### CASE 10: IGHV4-34/J6 USAGE



### SUMMARY OF CASES

Case No.	Sex	Age	IGHV Gene Usage	IGHJ Gene Usage	Somatic Hypermutation Status
1	F	57	V5-51	J4	Unmutated
2	M	65	V3-33	J4	Hypermutated
3	F	80	V3-31	J4	Hypermutated
4	M	63	V4-34	J4	Hypermutated
5	M	47	V3-33	J1	Hypermutated
6	F	70	V4-34	J1	Hypermutated
7	M	71	V4-61	J4	Unmutated
8	M	78	V3-73	J6	Unmutated
9	M	65	V5-9	J1	Hypermutated
10	M	54	V4-34	J6	Hypermutated
11	M	66	V4-34	J4	Hypermutated
12	F	70	V3-33	J4	Hypermutated
13	M	75	V4-34	J4	Unmutated
14	F	62	V1-3	J4	Unmutated
15	F	71	V1-3	J6	Hypermutated
16	F	73	V3-73	J6	Hypermutated
17	M	52	V3-33	J4	Hypermutated
18	M	47	V1-3	J4	Unmutated
19	M	87	V3-33	J6	Unmutated
20	M	77	V3-43	J1	Unmutated

### RESULTS

- We confirm that SMZL have a biased IGHV gene usage, which is in keeping with prior literature.
- This usage, however, has significant overlap with other subtypes of MZL considered in the differential diagnosis and therefore does not provide a means of discrimination for diagnostic purposes. This finding however, suggests that the pathogenesis of SMZL may involve epitopes or an antigenic trigger common to other indolent lymphomas.
- Whether particular molecular characteristics of the IG receptors might be associated with clinical outcome, genetic or phenotypic features is an area that deserves further study.

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## Next Generation Sequencing Demonstrates Clinical Utility and Increased Sensitivity in Detection of IgVH Somatic Hypermutation in Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL)



**MOLECULAR PATHOLOGY**  
LABORATORY NETWORK, INC.

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### Introduction

Chronic Lymphocytic Leukemia/Small lymphocytic lymphoma (CLL/SLL) is the most common leukemia diagnosed among adults in Western countries and is associated with heterogeneous clinical outcomes. IgVH somatic hypermutation (SHM) status is a primary component of the CLL International Prognostic Index (CLL-IPI) working group formulation for disease risk stratification.<sup>1</sup> Unmutated IgVH has been established as a strong and independent predictor of adverse clinical prognosis and reduced overall survival.

Clinical laboratory evaluation of IgVH SHM status traditionally involves RT-PCR followed by Sanger Sequencing using RNA extracted from patient peripheral blood or bone marrow aspirate samples, however RNA lability places significant burden on the submitting physician to ensure specimen transit time is minimized. Furthermore, Sanger Sequencing is time and labor intensive, and sensitivity of IgVH SHM detection may be limited for low abundance CLL/SLL clones. ZAP-70 expression by flow cytometry has therefore been widely utilized as a surrogate marker for IgVH SHM status. Positive ZAP-70 expression is usually associated with IgVH unmutated disease and an unfavorable clinical course.<sup>2</sup> Unfortunately, standardization for this marker is known to be poor; interpretation of the flow cytometry last mode data may be highly subjective, contributing to significant variability in clinical reporting.<sup>3</sup> Significant discordance between ZAP-70 expression patterns and expected IgVH SHM results may also be seen and has been attributed to pre-analytic sample processing factors in some studies.<sup>4</sup>

Herein we report on the clinical utility of a next generation sequencing (NGS) based approach to IgVH SHM testing using DNA derived from CLL/SLL patient samples.

### Materials and Methods

**NGS:** 400 untreated IgVH sequences obtained were prepared using denaturing bead primers, PfuI digestion, and patient sample DNA. Indexed libraries were prepared and sequenced on the Ion Torrent PGM<sup>®</sup> instrument, with generated FASTQ files analyzed by LyngbyTech<sup>®</sup> PGM Software (Danish ZIN, A/S). A 4-fold difference between first and third most abundant (TM) sequences defined clonal read data,<sup>5</sup> and IgVH somatic hypermutation status was revealed in accordance with recommended CIP reporting guidelines.<sup>6</sup>

**Sanger sequencing (SS):** Samples were previously tested at an outside surgical reference laboratory. Patient sample DNA was extracted and sequenced for clonal IgVH followed by PCR amplification using VH1 primers and JH primers. Identified clonal VH sequences were sequenced against a known database for homology. IgVH homology was defined as follows: 100% = unmutated, 97%-99% = borderline, <97% = mutated. CLL clones were required to comprise at least 50% of total analyzed B-cells.

**ZAP-70:** Flow cytometry was performed on a Beckman Coulter Navios instrument utilizing ZAP-70 Clone 8J2 (Dorland). 1 of all 100 cells served as internal control sub-population. CD5+CD20+ B-out ZAP-70 results were defined as follows: >25% = positive, <25% = borderline, <20% = negative.



Case #	Age	Sex	Diagnosis	ZAP-70	SHM Status	NGS Sensitivity	SS Sensitivity	Flow Cytometry
00001	65	M	CLL	+	U	100%	100%	+
00002	62	F	CLL	+	M	100%	100%	+
00003	68	M	CLL	+	M	100%	100%	+
00004	71	F	CLL	+	M	100%	100%	+
00005	69	M	CLL	+	M	100%	100%	+
00006	71	F	CLL	+	M	100%	100%	+
00007	67	M	CLL	+	M	100%	100%	+
00008	64	F	CLL	+	M	100%	100%	+
00009	66	M	CLL	+	M	100%	100%	+
00010	63	F	CLL	+	M	100%	100%	+
00011	68	M	CLL	+	M	100%	100%	+
00012	65	F	CLL	+	M	100%	100%	+
00013	67	M	CLL	+	M	100%	100%	+
00014	69	F	CLL	+	M	100%	100%	+
00015	66	M	CLL	+	M	100%	100%	+
00016	64	F	CLL	+	M	100%	100%	+
00017	68	M	CLL	+	M	100%	100%	+
00018	65	F	CLL	+	M	100%	100%	+
00019	67	M	CLL	+	M	100%	100%	+
00020	69	F	CLL	+	M	100%	100%	+
00021	66	M	CLL	+	M	100%	100%	+
00022	64	F	CLL	+	M	100%	100%	+
00023	68	M	CLL	+	M	100%	100%	+
00024	65	F	CLL	+	M	100%	100%	+
00025	67	M	CLL	+	M	100%	100%	+
00026	69	F	CLL	+	M	100%	100%	+
00027	66	M	CLL	+	M	100%	100%	+
00028	64	F	CLL	+	M	100%	100%	+
00029	68	M	CLL	+	M	100%	100%	+
00030	65	F	CLL	+	M	100%	100%	+

### Results

- SS-PCR failed to detect a clonal population in 5/25 cases evaluated (20%), whereas, NGS detected clonal populations in all 25 cases studied.
- VJ-2H gene utilization was detected by NGS in 1 of the 5 cases for which SS-PCR failed to detect a B-cell clone.
- Accuracy: NGS demonstrated 100% concordance with SS-PCR among evaluable cases where SS-PCR did not fail to detect a clonal population.
- NGS limit of detection: ~1% clonal B-cells (data not shown)
- NGS Precision: 100% (data not shown)
- Strength of agreement between NGS and ZAP-70 assays was 0.84 (Good), whereas, SS-PCR and ZAP-70 assays showed moderate agreement (0.585 (Kappa statistic)).

### Conclusions

- Evaluation of IgVH SHM status using NGS is feasible for routine clinical testing in the private reference laboratory setting and confers significant advantages over traditional methodologies.
- NGS may show improved sensitivity in detection of clonal populations compared to Sanger Sequencing.
- DNA is an acceptable specimen substrate for NGS IgVH SHM detection, limiting practical constraints typically associated with RNA based testing.
- NGS significantly streamlines laboratory workflow:
  - Three day clinical reporting is feasible.
  - Away hands on time is minimized (<5 hours).
  - Batch multiplexing of patient samples facilitates significant reductions in cost and labor.
- NGS sample input requirements are minimized. Clinically valid NGS results may be obtained using 1ml of peripheral blood or bone marrow, or 0.025 µg DNA.
- NGS data output is automated and non-subjective, and may include relative frequencies of clonal IgVH reads, percentage homology to most closely matched germline IgVH reference sequences, and VH gene utilization profiles of patient read data.

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## A Single Institution Experience Using Next Generation Sequencing (NGS) for Analysis of Immunoglobulin Heavy Chain Variable Region (IGVH) Somatic Hypermutation in Chronic Lymphocytic Leukemia

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### Introduction

Somatic hypermutation (SHM) status of the immunoglobulin heavy chain variable region (IGHV) gene is an important prognostic marker in B-cell chronic lymphocytic leukemia (CLL). SHM, defined as a mutation load ≥2% (compared to germ line sequence), is associated with a better prognosis, whereas an unmutated status (mutation <2%) is associated with adverse prognosis. SHM status is commonly detected by PCR amplification of the noncoding CLL IGHV-JH region followed by Sanger sequencing, but this process is relatively labor intensive. Recently, Next Generation Sequencing (NGS) technology has been used to detect clonal IGHV rearrangements and to determine SHM status in CLL patients. NGS represents a significant improvement over Sanger sequencing by simultaneously detecting and characterizing IGHV rearrangements using a single multiplex PCR library, thereby allowing direct identification of the SHM status in CLL patients. This study presents our experience using NGS for the detection of IGHV SHM status in CLL.

### Materials and Methods

Sequences were analyzed using Sequencher software version 5.0 (Gene Codes Corporation, Ann Arbor, MI) and the consensus coreg sequence was analyzed using IMGT/IG-CLST software (IMGT, Montpellier, France).

#### Next Generation Sequencing

Multiplex leader PCR was performed including adapter ligation and sample indexing using the LymphoTrack IGHV Leader Somatic Hypermutation Assay Panel for MiSeq (Invivoscribe, San Diego, CA). A check gel was run to assess the quality and presence of clonal PCR product using the QIAzol DNA High Resolution Kit. If no band or an indistinct band was present, FR1 multiplex PCR was performed using the LymphoTrack IGHV FR1 Panel for MiSeq (Invivoscribe, San Diego, CA). PCR product libraries were purified using the Agencourt AMPure XP Purification kit (Beckman Coulter, Inc., CA) and subjected to next generation sequencing (NGS) on the MiSeq platform (Illumina, San Diego, CA), after normalization and sample pooling. NGS generated data was evaluated for sequence quality and converted to FASTQ files. After de-multiplexing, individual patient samples were analyzed and graphically displayed using the Invivoscribe LymphoTrack IGHV SHM Software for MiSeq (Invivoscribe, San Diego, CA). A minimum total read (coverage) depth of 100,000X and a clonal IGHV fraction of at least 10% was required to adequately evaluate clonal rearrangements for SHM status. Recurrent patterns of clonal gene rearrangements and potential artifacts were determined. In some cases, additional assessment using IMGT/IG-CLST was performed.

### Materials and Methods

#### Patient Samples and RNA Preparation

Peripheral blood and bone marrow aspirate samples with at least 2% clonal B cells were obtained and RNA was isolated using the RNeasy Blood and Tissue Spin Kit (Qiagen Research Corp., Irvine, CA) for peripheral blood and miRNeasy Spin Kit (Qiagen USA, Valencia, CA) for bone marrow samples. Reverse transcription was performed using the NuScribe Reverse Transcriptase and Random primer cDNA synthesis kit (Life Technologies, Grand Island, NY).

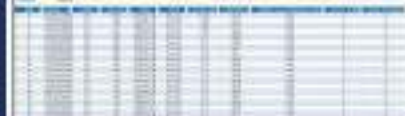
#### Sanger Sequencing

Leader PCR was performed with 7 individual PCR primer sets for each patient (7 consensus V-family primers and JH primer). A rapid capillary gel electrophoresis step was used to assess the quality and presence of clonal bands using the QIAzol DNA High Resolution Kit (Qiagen USA, Valencia, CA). If a distinct clonal leader band was not present, FR1 PCR was then pursued using 8 individual PCR primer sets (8 consensus V-family primers and 1 JH primer) and assessed for the presence of a clonal band, following which the positive PCR product was treated with exoGAP-IT (Affymetrix, Santa Clara, CA) to remove excess nucleotides and primers. The purified PCR product was sequenced using the BigDye Terminator 4.1 version 1.1 (Life Technologies, Carlsbad, CA) per manufacturer's instructions. Sequencing reactions were purified using Sephadex Performa DTR Ultra 16 well plates (Sigma, St. Louis, MO) and analyzed by capillary electrophoresis on the ABI 3130XL (Applied Biosystems, Foster City, CA).

### Results

- 12 normal donor samples and 48 CLL samples with known IGHV status (by Sanger sequencing) were evaluated using the NGS methodology.
- All normal samples were negative for clonal gene rearrangements.
- 348 CLL samples were excluded from the study due to Sanger sequencing or NGS failure.
- Concordance with Sanger method was observed in 41/43 of the samples (95.3%) using NGS.
- Additional findings: a) Different VH gene designation between Sanger and NGS informatics; b) Different SHM status between NGS and Sanger methods; c) Occasional clonal rearrangements variability detected between platforms.
- Patterns of artifact and intraclonal heterogeneity were identified.
- Successful PCR and NGS required a clonal B-cell population of at least 5%.

### Results



### Results



### Discussion

- Discordant findings were present in 24% (24%) of comparison samples; additional minor discrepancies identified in 2 cases.
- Causes of discordant results:
  - NGS versus Sanger differences:
    - VH non-nature (JGOT vs. JGLAST)
    - Differing SHM status (M vs. U), possibly from suboptimal Sanger reads and sequence sorting with increased ambiguous bases.
    - NGS result negative and Sanger result positive for clonal IGHV; differences in multiple versus single primer reaction PCR set up; or low clonal B-cell percentage.
  - NGS leader versus FR1:
    - Leader result negative and FR1 positive; leader primer site mutation or PCR efficiency.
  - Other: "N" base artifact (sequencing platform/thermal/flow complexity region) vs. true intraclonal heterogeneity.

### Conclusions

- NGS methodology is accurate and acceptable for clinical use in the determination of somatic hypermutation status in CLL. Results obtained in our experience are highly concordant (41/43, 95.3%) in comparison to the current standard of PCR and Sanger Sequencing.
- Advantages of NGS include direct determination of the IGHV rearrangement and percent of somatic hypermutation; however, a clear understanding of the various rearrangement patterns, technical or informatics limitations, potential analytic artifacts and adequate quality parameters is central to ensuring high quality results. Additional needs of NGS in this situation include the ability to assess multiple samples simultaneously and to potentially enable easier minimal residual disease monitoring.

### References

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- Scanlon P, Bahler D. Clinical Laboratory Analysis of Immunoglobulin Heavy Chain Variable Region Genes for Chronic Lymphocytic Leukemia Prognosis. *JMO* 2010; 12(2): 244-248.
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## Development of a Comprehensive IGH NGS Assay for Detecting Suspected B-Cell Clonality

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### Abstracts

**Background:** Non-PCR based targeted high-throughput heavy chain gene sequencing (L, 3, and 3', 5', F1, F2 and F3) are the current gold standard for B-cell clonality testing in suspected B-cell proliferations (lymphoma, non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM)). Next-generation sequencing (NGS) based approaches for testing immune receptor genes have been suggested to improve sensitivity and to identify the specific V-D-J sequences required for clonality testing. Here we report the development of a comprehensive NGS-based assay which allows detection of suspected B-cell clonality in a single multiplexed assay.

**Methods:** The LymphoTrack™ IGH (F1/F2/F3) assay targets the F1/F2, F2/3 and F3/5' regions. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on the Illumina® platform. For each IGH V, a set of 24 primer mixes are provided, each designed with a different index, allowing analysis of 24 patient samples plus positive and negative controls. Single-plex multiplexed PCR was followed by amplicon purification using the AMPure™ XP PCR system. Purified amplicon amounts of amplicons from different samples and mix were pooled to form a library. The normalized and quantification was achieved using the Illumina® HiSeq 2500 system. LymphoTrack™ assay output from the HiSeq™ were analyzed using Invivoscribe's proprietary bioinformatics software, which analyzes and identifies sequences by both index and V, to generate frequency distributions of V-D-J rearrangements and to determine the somatic hypermutation (SHM) rate of the IGH region based on the sequence of F1/F2 amplicons.

**Results:** The ability to detect F1, F2, and F3 regions was verified by testing different known V-D-J rearrangements and real DNA. Cell line DNA, serially diluted into serial dilutions, demonstrated the limit of detection (LOD) in 10x a factor using 50 ng DNA input. The assay achieved excellent linearity (R<sup>2</sup> > 0.99) and reproducibility (< 2% CV) demonstrated by 2 operators, 2 libraries and 2 sets of PCR master mixes. The primary testing of patient DNA from patients in CLL, DLBCL, mantle cell lymphoma, and follicular lymphoma demonstrated that the clonality detection mapped to one V, could be detected by another V, thus increasing the overall detection rate.

**Conclusions:** A comprehensive NGS-based IGH assay has been developed for the Illumina® HiSeq™ platform that identifies clonal IGH V-D-J rearrangements and V-D-J sequences. This NGS-based assay has demonstrated high sensitivity, F1, F2 and F3 regions to increase the false-negative rate due to somatic hypermutation in primer binding sites of immunoglobulin V<sub>H</sub> gene segments.

### Materials and Methods

The LymphoTrack™ IGH (F1/F2/F3) assay targets the F1/F2, F2/3 and F3/5' regions. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on the Illumina® platform.

For each IGH V, a set of 24 primer mixes are provided, each designed with a different index, allowing analysis of 24 patient samples plus positive and negative controls.

Single-plex multiplexed PCR was followed by amplicon purification using the AMPure™ XP PCR system. Purified amplicon amounts of amplicons from different samples and mix were pooled to form a library. The normalized and quantification was achieved using the Illumina® HiSeq 2500 system. LymphoTrack™ assay output from the HiSeq™ were analyzed using Invivoscribe's proprietary bioinformatics software, which analyzes and identifies sequences by both index and V, to generate frequency distributions of V-D-J rearrangements and to determine the somatic hypermutation (SHM) rate of the IGH V region based on the sequence of F1/F2 amplicons.

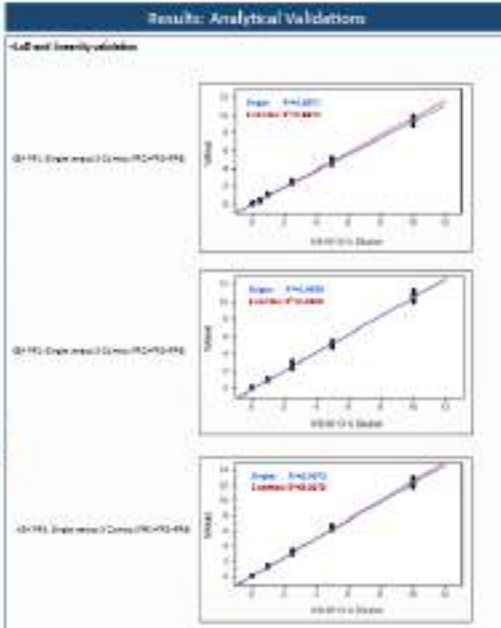
The analysis pipeline consists of over 200 (V-D-J) and a cluster series of 10K, 5K, 2.5K and 1.5K cell line DNA (V-D-J) clusters into serial dilutions.

For each IGH V, LOD and linearity were evaluated in both single V and a combined V-D-J library.

Precision and reproducibility were calculated for each V. A set of combined V-D-J data points were generated from the HiSeq™ V-D-J (200 reads x 2 runs x 2 DNA) for each V.

24x from different DNA samples was tested by both the index-based detection of V-D-J clonality assay and the NGS-based LymphoTrack™ IGH (F1/F2/F3) assay.

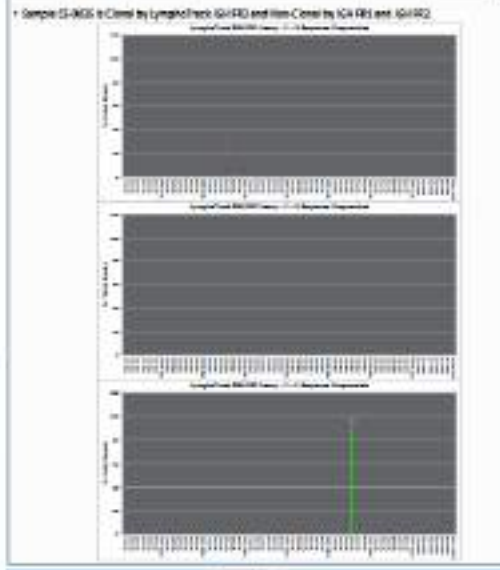
The bioinformatics program was used to perform the data analysis.



### Results: Clinical Study

100% concordance between the read-based IGH assay and the lymphoTrack IGH (F1/F2/F3) assay


Sample	Accepted Ref	Sample Type	Method Used for Clonality Detection	LymphoTrack IGH V1	LymphoTrack IGH F1	LymphoTrack IGH F2
1	F02	PL	Clonal	Clonal	Clonal	Clonal
2	F02	HL	Clonal	Clonal	Clonal	Clonal
3	F04	PL	Clonal	Clonal	Clonal	Clonal
4	F02	PL	Clonal	Clonal	Clonal	Clonal
5	F02	HL	Clonal	Clonal	Clonal	Clonal
6	F02	PL	Clonal	Clonal	Clonal	Clonal
7	F02	PL	Clonal	Clonal	Clonal	Clonal
8	F02	HL	Clonal	Clonal	Non-Clonal	Non-Clonal
9	F02	HL	Non-Clonal	Non-Clonal	Non-Clonal	Non-Clonal
10	CP-DLN	PL	Non-Clonal	Non-Clonal	Non-Clonal	Non-Clonal



### Conclusions

A comprehensive NGS-based IGH assay has been developed for the Illumina® HiSeq™ platform that identifies clonal IGH V-D-J rearrangements and SHM sequences. The LymphoTrack™ assay has demonstrated that combining F1, F2 and F3 helps to decrease the false-negative rate due to somatic hypermutation in primer binding sites of the immunoglobulin V<sub>H</sub> gene segments.





## Next-Generation Sequencing of *NPM1* for Minimal Residual Disease Monitoring in Leukemia Patients

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### Introduction

The nucleophosmin (*NPM1*) gene is an important target for acute myeloid leukemia (AML) stratification. *NPM1* is one of the most commonly mutated genes in AML, with mutations seen in roughly 30% of patients at diagnosis, and in approximately 80% of adult symptomatically relapsed AML patients. Importantly, *NPM1* mutations generally confer a slightly more favorable outcome in AML patients and can mitigate poor prognosis when they occur concurrently with activating mutations in FLT3. As a commonly mutated gene with prognostic value in AML, *NPM1* is an appropriate biomarker for minimal residual disease (MRD) monitoring. MRD detection has proven to be valuable in the clinical management of patients with AML. *NPM1* is of particular importance, as it was recently shown that the presence of *NPM1* mutants after chemotherapy were associated with a greater risk of relapse. Thus, the development of a sensitive and reliable assay to detect *NPM1* mutations at low frequencies represents a significant advancement in guiding treatment for AML patients.

### Materials

**LOD & linearity:**

- A panel of samples was created consisting of self-ligating DNA (SV-0046) containing a known 8946 bp insertion mutation (CGM17558) diluted into DNA from a cell line known to be negative for *NPM1* mutations (SV-0082). The panel contains samples at dilutions that range from  $1.0 \times 10^1$  to  $5.0 \times 10^4$ .

**Sensitivity & specificity:**

- Using the same panel as above, we were able to calculate the sensitivity and specificity of this *NPM1* MRD assay at various input mutation frequencies, including an and above the assay's LOD of  $5 \times 10^4$ .

**Detection of mutations in clinical samples:**

- We tested 5 clinical samples for *NPM1* mutations and assessed the assay's concordance with a CAP/CLIA validated capillary electrophoresis (CE) assay.
- Since no clinical samples were available with *NPM1* mutations at MRD levels, we diluted from positive clinical samples as much as 1 in 1,000 to test detection of mutations closer to the assay's LOD.

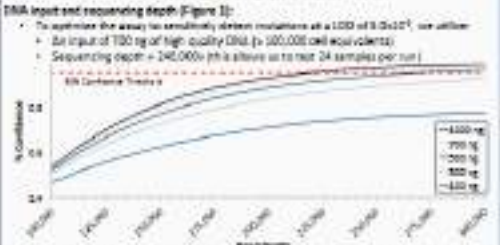
### Methods

***NPM1* target:**

- This next-generation sequencing (NGS) MRD assay targets exon 11 of the *NPM1* gene using a single, optimized PCR amplification that was developed to overcome inherent challenges caused by repetitive sequence across this locus (Figure 1).

**DNA input and sequencing depth (Figure 1):**

- To optimize the assay for sensitivity, detection mutations at a LOD of  $5 \times 10^4$ , we utilized:
  - An input of 100 ng of high quality DNA (p 100,000 cell equivalents)
  - Sequencing depth = 240,000x per sample (p 1.5 million reads per run, 24 samples per run)



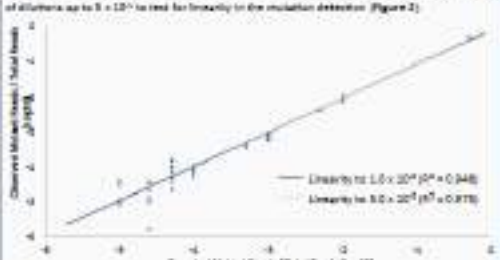
**Figure 1:** Confidence for detecting 5:5 mutation reads in a sample at  $5 \times 10^4$  allele frequency. Data for this figure was calculated using a statistical model that does not incorporate any PCR bias. An input of 700 ng of DNA would require a 2,200,000x per sample detection of a mutation at  $5 \times 10^4$ . Decreased input DNA amount would require additional read depth to maintain a 95% confidence for detecting a mutation at this frequency.

**Data analysis:**

- All generated data was analyzed by the proprietary *NPM1* MRD Data Analysis Tool software developed by Invivoscribe.

### Results: Limit of Detection and Linearity

To establish the limit of detection (LOD) and linearity of *NPM1* variant detection, we used a panel of samples consisting of a cell line containing a known *NPM1* 8 bp insertion mutation (SV-0038; CGM17558) diluted into a background cell line containing no-type *NPM1* exon 11 (SV-0082). We tested our ability to detect mutations at a LOD as low as  $5 \times 10^4$  and used a range of dilutions up to  $5 \times 10^4$  to test for linearity in the mutation detection (Figure 2).



**Figure 2:** Linearity of *NPM1* MRD assay. This figure examines linearity for the detection of the CGM17558 8 bp insertion mutation using dilutions of a positive control (SV-0038).

**Table 1:** Results of the *NPM1* MRD assay at decreasing input mutation frequencies

Expected Mutation Frequency	Number of Tests (N)	% of Tests Positive / No Type Expected Mutation	Mean Observed Mutation Frequency
$5.0 \times 10^1$	2	100%	$4.9 \times 10^1$
$1.0 \times 10^2$	2	100%	$8.4 \times 10^1$
$5.0 \times 10^2$	2	100%	$2.0 \times 10^2$
$1.0 \times 10^3$	4	100%	$7.5 \times 10^2$
$5.0 \times 10^3$	2	100%	$5.9 \times 10^3$
$1.0 \times 10^4$	4	100%	$7.8 \times 10^3$
$5.0 \times 10^4$	20	100%	$7.2 \times 10^4$
$1.5 \times 10^5$	7	71.4%	$1.8 \times 10^5$
$1.0 \times 10^6$	8	87.5%	$1.8 \times 10^6$
LOD	11	0%	LOD

\* A positive call requires the detection of a minimum of 5 NGS reads containing a mutation.

**Limit of Detection:**  
The *NPM1* 8 bp insertion mutation was detected at every dilution level, down to  $5 \times 10^4$ . However, the lowest dilution level where 100% of the tests were positive for the expected mutation (100% sensitivity).  
We established an optimal final LOD of  $5 \times 10^4$  for this *NPM1* MRD assay.

**Linearity:**  
The data suggests strong linearity for the detection of mutations by the *NPM1* MRD assay.  
The data has an  $R^2$  value of 0.948.  
If we only include data down to the LOD of  $5 \times 10^4$ , the  $R^2$  increases to 0.975, suggesting slightly superior linearity in the assay's detection range.

**Precision and Reproducibility:**  
The assay shows exceptional precision and reproducibility both at and above the assay's  $5 \times 10^4$  LOD (data not shown).

### Results: Sensitivity and Specificity

To determine the sensitivity and specificity at different input mutation frequencies, we used the data that was generated for LOD and linearity. We determine that the assay has 100% sensitivity and 100% specificity for mutations at or above a LOD of  $5 \times 10^4$  (Table 2 and Figure 3).

Expected Mutation Frequency	Number of Tests (N)	True Positives	True Negatives	True-Positives / True-Tested	True-Negatives / True-Tested
$5.0 \times 10^1$	2	2	0	1	100%
$1.0 \times 10^2$	2	2	0	1	100%
$5.0 \times 10^2$	2	2	0	1	100%
$1.0 \times 10^3$	4	4	0	1	100%
$5.0 \times 10^3$	2	2	0	1	100%
$1.0 \times 10^4$	4	4	0	1	100%
$5.0 \times 10^4$	20	20	0	1	100%
$1.5 \times 10^5$	7	7	0	1	100%
$1.0 \times 10^6$	8	7	0	1	100%
LOD	11	10	0	100%	11

Observed Mutation	Expected Mutation		PPV - 100%	NPV - 100%
	Positive	Negative		
Positive	10	0	100%	100%
Negative	0	11	100%	100%

Sensitivity = 100%, Specificity = 100%

**Figure 3:** Sensitivity and Specificity Table. This figure shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for this study's mutation detection at or above the LOD of  $5 \times 10^4$ .

In the established LOD of  $5 \times 10^4$ , this assay has 100% sensitivity and specificity for the detection of a 4 bp insertion mutation in *NPM1*. At lower detection limits, our sensitivity and NPV drop, but specificity is 100% remain at 100% due to the lack of false positives in this study.

### Results: Concordance with Clinical Samples

We tested 8 clinical samples from AML patients. Of these, 2 had previously been found to be positive for an *NPM1* mutation using a CAP/CLIA capillary electrophoresis (CE) method.

- The *NPM1* MRD assays' results match the expected results from the CE assay.
- We tested the 2 positive samples and again confirmed the mutation at lower frequencies.

**Table 3:** Results from clinical samples tested using the *NPM1* MRD assay

Sample	Dilution	CE result	Observed Mutation	Observed Frequency
AML-04	None	Positive	4 bp insertion (TGT)	$2.2 \times 10^1$
	1:100		4 bp insertion (TGT)	$9.2 \times 10^1$
	1:1,000		4 bp insertion (TGT)	$2.7 \times 10^2$
AML-11	None	Positive	4 bp insertion (TGT)	$3.4 \times 10^1$
	1:10		4 bp insertion (TGT)	$5.2 \times 10^1$
	1:1,000		4 bp insertion (TGT)	$2.3 \times 10^2$
AML-03	None	Not Detected	Not Detected	0
AML-07	None	Not Detected	Not Detected	0
AML-08	None	Not Detected	Not Detected	0


In addition, we also tested DNA from 6 normal controls and found no false positive mutations.

### Conclusions

The *NPM1* MRD NGS assay is a highly specific test that can detect *NPM1* mutations with a sensitivity at least two orders of magnitude greater than current commercially available assays.

- Strong linearity at a 100% sensitivity and specificity at and above a LOD of  $5 \times 10^4$ .
- Assay is concordant with CAP/CLIA-CE assays for clinical samples with no false positives in normal controls.

This assay provides a reliable tool to assess MRD in AML patients.





## Next-Generation Sequencing of *FLT3/ITD* for Minimal Residual Disease Monitoring in Leukemia Patients

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### Introduction

Minimal residual disease (MRD) detection in patients with leukemia has proven to be useful in the clinical management of disease and can facilitate the development of new therapies. Mutations in the related tyrosine kinase 3 (*FLT3*) gene are the most common mutations found in acute myeloid leukemia (AML) and are characterized by an aggressive phenotype with a high prevalence of relapse. Internal tandem duplication (ITD) mutations within the juxta-membrane domain are the most common mutations in the *FLT3* gene. The development of a sensitive and specific assay for *FLT3/ITD* mutations represents a significant advancement in guiding treatment decisions.

### Results: LOD, LOB and Linearity

As shown in Table 2, the limit of detection (LOD) for cell line A (30bp ITD) and cell line B (228 bp ITD) were determined to be at  $1.5 \times 10^4$  and  $1.5 \times 10^5$ , respectively. This was concordant with the fact that ITDs of a certain sequence and at longer lengths are harder to detect by PCR-based assays. There was no ITD detected in the negative cell line (cell line C) indicating that the limit of blank (LOB) is zero. There was no amplification (ITD positive or negative) detected in the no template control (NTC) samples.

Table 1. Determination of the LOD and LOB of the assay

Cell Line	ITD Size (bp)	Expected Frequency	Positive Cn	Positive	Negative	% Positive*
A	30	$1.5 \times 10^4$	10	10	0	100.0%
		$3.0 \times 10^4$	20	20	0	100.0%
		$4.5 \times 10^4$	30	30	0	100.0%
B	228	$1.5 \times 10^5$	10	10	0	91.0%
C	N/A	NA	NA	0	NA	NA
NTC	N/A	NA	NA	0	0	0.0%

\* a positive call requires the detection of a minimum of 100 reads containing a FLT3/ITD mutation.

### Results: Clinical Sample Testing

A total of 18 clinical samples tested to be negative for *FLT3/ITD* by the standard PCR assay previously were re-tested by the MRD assay. The investigator conducting the MRD assay was blind to any information regarding the presence or absence of *FLT3/ITD* mutation, its length, or the mutant-to-wild type allelic ratio. The summary of clinical sample testing by standard PCR assay and the MRD assay is shown in Table 4. The MRD assay correctly detected the ITD size in 9 clinical samples. Six patients without detectable *FLT3/ITD* by the MRD assay are disease free.

Table 4. Summary of clinical sample testing by standard PCR assay and the MRD assay

Sample Number	Standard PCR assay		FL3/ITD MRD assay of full-length allele		
	Detectable FLT3/ITD	Allelic Ratio	Detectable FLT3/ITD	Allelic Ratio	Pathologic Outcome
1	NA	NA	NA	$1.1 \times 10^4$	On Treatment
2	NA	NA	NA	$1.2 \times 10^4$	Unstable
3	NA	NA	NA	$1.1 \times 10^4$	NA
4	NA	NA	NA	$1.0 \times 10^4$	On Treatment
5	NA	NA	NA	$1.0 \times 10^4$	On Treatment
6	NA	NA	NA	$1.0 \times 10^4$	On Treatment
7	NA	NA	NA	$1.0 \times 10^4$	On Treatment
8	NA	NA	NA	$1.0 \times 10^4$	On Treatment
9	NA	NA	NA	$1.0 \times 10^4$	On Treatment
10	NA	NA	NA	NA	On Treatment
11	NA	NA	NA	NA	On Treatment
12	NA	NA	NA	NA	On Treatment
13	NA	NA	NA	NA	On Treatment
14	NA	NA	NA	NA	On Treatment
15	NA	NA	NA	NA	On Treatment
16	NA	NA	NA	NA	On Treatment
17	NA	NA	NA	NA	On Treatment
18	NA	NA	NA	NA	On Treatment

### Materials and Methods

The next-generation sequencing (NGS) MRD assay was designed to target exons 14 and 15 of the *FLT3* gene with a single PCR amplification. Amplions from up to 24 samples were purified, pooled and sequenced before being analyzed using proprietary software developed by Invivoscribe. Validation was carried out by spiking in fixed amounts of mutant DNA into wild-type DNA to establish a sensitivity equivalent to detection of at least one ITD-containing cell out of 20,000. The DNA input of the assay was 700 ng ( $3.5 \times 10^6$  cell equivalent). The assay was applied to bone marrow DNA from patients with *FLT3/ITD* AML.

Barcode crossover is defined as errors introduced into the DNA barcode from oligo synthesis, PCR, or sequencing causing one barcode sequence to become another barcode sequence. These errors are introduced at  $1 \times 10^{-6}$  frequency. MiSeq instrumentation also has up to a 1% rate of run to run DNA crossover which presents a problem for MRD level detection. We have designed our assay with a proprietary approach to reduce the background errors below our assay detection limit.

### Results: Sensitivity and Specificity

The sensitivity/specificity and precision/reproducibility of the *FLT3/ITD* MRD assay were demonstrated by testing DNA from two cell lines diluted into a background DNA from a wild-type *FLT3* cell line. The validation was carried out with different operators and instruments and conducted on different days. Validation data for samples with read frequency of  $1 \times 10^4$  and  $5 \times 10^4$  (LOD) of the assay is shown in Table 1. The results show excellent sensitivity/specificity and precision/reproducibility.

Table 1. Summary of sensitivity and specificity

Cell Line	ITD Size (bp)	Expected Frequency	Positive Cn	Positive	Negative	Specificity	Sensitivity
A	30	$1.5 \times 10^4$	10	10	0	100%	100%
		$3.0 \times 10^4$	20	20	0	100%	100%
		$4.5 \times 10^4$	30	30	0	100%	100%
B	228	$1.5 \times 10^5$	10	10	0	100%	91%
C	N/A	NA	NA	0	NA	NA	NA

NTC: DNA positive; PC: total positive; TPC: true positive; FPC: false positive

### Results: Linearity of the Assay

DNA from two cell lines with known ITD (30 bp and 228 bp, respectively) were serially diluted into a background DNA from a wild-type *FLT3* cell line and tested with the *FLT3/ITD* MRD assay. Input DNA quantity was 700 ng per dilution point. The experimental data was presented in Table 2 and plotted in Figure 1. As shown in Figure 1, the linearity of the assay is excellent in the range of  $10^4 - 10^6$ .

Table 2. Determination of the linearity of the assay

Known Frequency	Observed Frequency	Relative Error
$1.5 \times 10^4$	$1.5 \times 10^4$	0.0%
	$1.8 \times 10^4$	21.3%
	$2.1 \times 10^4$	40.0%
$1.5 \times 10^5$	$1.5 \times 10^5$	0.0%
	$1.8 \times 10^5$	20.0%
	$2.1 \times 10^5$	40.0%
$1.5 \times 10^6$	$1.5 \times 10^6$	0.0%
	$1.8 \times 10^6$	20.0%
	$2.1 \times 10^6$	40.0%
$1.5 \times 10^7$	$1.5 \times 10^7$	0.0%
	$1.8 \times 10^7$	20.0%
	$2.1 \times 10^7$	40.0%
$1.5 \times 10^8$	$1.5 \times 10^8$	0.0%
	$1.8 \times 10^8$	20.0%
	$2.1 \times 10^8$	40.0%

Figure 1. Linearity of the *FLT3/ITD* MRD assay

### Conclusions

The *FLT3/ITD* MRD assay is a highly-specific test, developed with the accompanying bioinformatics software under full ISO1485 design criteria, which is at least two orders of magnitude more sensitive than current commercially available assays. In addition, the chemistry and bioinformatics software reliably picked up even the larger ITDs missed entirely by other commercial assays. Importantly, the results of clinical samples tested by this MRD assay showed 100% concordance with clinical outcomes. This assay provides a reliable tool to assess MRD in *FLT3* AML patients. The *FLT3/ITD* MRD test is currently being offered through Invivoscribe's international clinical laboratories for personalized molecular medicine (iLabPM).



## LOD and Sensitivity of MyHEME: A Comprehensive Targeted Sequencing Strategy

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### Abstract

**Background:** Next-generation sequencing (NGS) of heterogeneous and/or lymphatic neoplasm genomes provides for recurrent coverage, with the ability to design and use targeted assays to predict outcome and response, and to identify patients responsive to treatment more thoroughly using more predictive combinations of mutations. It is of critical importance that the NGS platform chosen, the analytical pipeline used and the unit of analysis chosen are all highly consistent for future adoption in clinical practice. To maximize the increased depth and resolution of data for the comprehensive characterization of patient and neoplasm genomes, we sequenced both whole patient samples and selected cell lines using a novel, highly targeted strategy involving DNA and RNA. Using selected cell lines, we utilize maximum gene coverage, long read length, and higher sequencing depth to accurately assess variants, both at heterozygous sites in both hemizygous chromosomes and for finding homozygous disease (LOD). Furthermore, understanding mutations in the context of gene structure can help guide choice for personalized treatment. We compare the limit of detection that allows for complex characterization of the molecular changes, with high sensitivity and specificity, that allowing clinical outcome discovery.

**Methods:** To maximize DNA coverage, we designed coding exons (CDS) genes and selected genomic intergenic regions with known genomic gene flanks (IG) genes comprising the MyHEME™ gene panel. We designed targeted assays for the "Custom" MyHEME™ platform to do coverage of exons of 100bp for all DNA and patient samples. Using a custom bioinformatics pipeline, we performed through mutation detection analysis to identify single nucleotide variants (SNVs), indels and rearrangements. In addition, we designed a new frequency to investigate possible mutations, low and complex, using targeted assays. We were able to determine limit of detection rates, sensitivity and specificity.

**Results:** Our analysis of targeted sequencing results from cell lines identified the published genomic variants with a MyHEME targeted gene. Overall, our assay enables detection of variants at 100% in many cases. These variants were more fully characterized for their precise genomic coordinates and nearby sequence context.

**Conclusions:** Our data demonstrate that by specifically targeting gene genes using the MyHEME™ gene panel, we can comprehensively detect mutations for SNVs, IN, and indels. Further, the data demonstrate and patients with these heterozygous conditions, our results show the assay can comprehensively characterize the clinical genome of patients, covering not only primary tumors, but secondary tumors that are present in our data. 5% of the sequenced samples.

### MyHEME DNA Results: Limit of Detection and Linearity

To determine the limit of detection (LOD) and linearity of DNA variant detection using MyHEME, we use cell lines samples composed of 4 cell lines. For cell lines were diluted into a 4<sup>th</sup> cell line (background) at the following dilutions: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%. The LOD frequency is 10% of the dilution, as we are testing LOD as low as 1.0%.

**Figure 1. Linearity of a) 4 heterozygous SNVs, b) 4 heterozygous INs and c) 3 heterozygous and 1 heterozygous indel.**

### MyHEME DNA & RNA Results: Translocations and Gene Fusions

**MyHEME DNA results:** variant targets to detect structural variants that occur within low frequency regions in 100% of cell lines. In our analysis, we detected the following variants:

- 100% (100/100) (100/100) (100/100) translocation
- 100% (100/100) (100/100) (100/100) translocation
- These cell lines were sequenced 5 times at 1.0%, 2.0%, 3.0%, 5% and 10% allele frequencies.

**Table 1. LOD detection of translocations.**

Translocation	Genes	Detection	Filter Strategies
10-22	ABL1-BCR	5	0
10-21	ABL1-BCR	5	0

**MyHEME RNA results:** variant targets to detect gene fusions that occur within any of 576 genes.

- We identified 6 different cell lines containing a fusion gene fully:

  - 11 (11/11) (11/11)
  - 12 (12/12) (12/12) - 1200 (12/12)
  - 13 (13/13) (13/13) - 1200 (13/13)
  - 14 (14/14) (14/14) - 1200 (14/14)
  - 15 (15/15) (15/15) (15/15)
  - 16 (16/16) (16/16) - 1200
  - 17 (17/17) (17/17)
  - 18 (18/18) (18/18)

- We use 2 different RNA fusion finding programs to improve sensitivity and specificity for the detection of gene fusions

- All fusions were validated with their respective fusion assays.

**Table 2. Evaluation of gene fusion detection and sensitivity using 3 gene fusion detection methods**

Program	Total Fusions	Screened Fusions	Sensitivity
A	20	6	100%
B	20	6	100%
C	20	6	100%
Combined	6	6	100%
2.0%	20	6	100%

**Figure 4. Venn diagram illustrating the overlap of gene fusion detection from a different programs.**

**Table 3. Evaluation of Sensitivity and Specificity of High-confidence a) coding and b) non-coding variants in 100/100 MyHEME samples**

	Cells		Genes	
	Coding (n=1,432)	Non-Coding (n=1,568)	Coding (n=1,432)	Non-Coding (n=1,568)
Sensitivity	99.2%	99.2%	99.2%	99.2%
Specificity	99.2%	99.2%	99.2%	99.2%

### Materials and Methods

**Target lists for MyHEME**

- DNA lists: Targets the coding sequences of 576 genes
- RNA lists: Targets the transcripts of 576 genes

**Analysis methods**

- 4ug of DNA or 4ug of RNA is used as input before hybridizing to the MyHEME™ cells
- Captured to get 150 bp DNA sequences of the "Custom" platform
- Customized downstream pipeline identifies and characterizes SNVs, INs, and INs

**Software used to evaluate quality metrics:**

- HT-Seq human reference genome (hg19) (aka "Genome in a Bottle")
- When confidence metrics are used to evaluate the positive (TP) and true negative (TN)
- Larger confidence confidence regions validated as positive or false positives or true negatives
- Used to calculate false positives (FP) and true negatives (TN)
- Customized pipeline containing pipeline of 4 cell lines at different allele frequencies
- Used to analyze LOD, reproducibility and linearity of variant detection
- 100 cell lines with known gene fusions used to evaluate the ability to detect fusions in mixed data

### MyHEME DNA Results: Sensitivity and Specificity

To evaluate MyHEME DNA variant detection sensitivity and specificity, we sequenced the next human reference genome (HG38). The DNA composition sequenced the "Custom" platform. It was to further pipeline to generate an "Integrated" gene fusion dataset consisting:

- A set of 576 MyHEME high-confidence variants. Of these variants, there are:
  - 100 high-confidence coding variants (100/100) and 20,000/20,000 non-coding variants
  - 20,000 high-confidence non-coding variants (20,000/20,000) with high-confidence targets
  - High-confidence variants were used as gold-standard true positives for sensitivity analysis
- High-confidence regions containing 1,432/1,432 bp with high confidence coding and non-coding variants (100/100) reference sets. Of these lists:
  - 4,576,786 of these genes overlap with MyHEME coding targets and 2,262,436 genes overlap with MyHEME non-coding targets
  - Non-coding sites were used as gold-standard true negatives for specificity analysis

### Conclusions

Using MyHEME DNA combined with 1) the HT-Seq RNA MyHEME, 2) custom pipeline containing detection of SNVs and INs, and 3) cell lines with known gene fusions, we discovered:

- Variant Sensitivity at 100%
- Sensitivity rate highest for SNVs (99.2%)
- Variant Specificity at 100% for SNVs and 99.2% for INs
- Using an LOD of 1%, our assay specificity for both SNVs and INs is 99.2%
- Limit of Detection when used 1% allele frequency for 100% of the coding bases of targeted genes
- In addition, as much as 85% of the coding bases of the targeted genes could have an LOD of at least 1.5% with sensitivity 100% of the coding bases used. (100/100)
- Significant sensitivity for detection of SNVs and INs, including pathogenic mutations such as R1476C
- We are able to detect structural variants using both DNA and RNA
- Our data demonstrate that MyHEME is a highly sensitive, accurate and reproducible assay that can comprehensively characterize mutations within samples from a variety of biological organisms.

### DNA Target Gene Coverage and Sequencing Depth

We analyzed target coverage (Figure 1) and sequencing depth (Figure 2) across the coding sequence from 576 genes. These analyses are based on data from 15 samples, including 5 runs of 100/100 and 5 runs of 4 different coverage samples to test the platform.

**Figure 1. MyHEME DNA Target Coverage**

**Figure 2. MyHEME DNA Sequencing Depth**



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## Detection of Clonal Immunoglobulin and T-cell Receptor Gene Rearrangements in Acute Myeloid Leukemia

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### Introduction

Acute myeloid leukemia (AML) carries a high mortality rate and economic burden. Elucidating the heterogeneity of AML will aid in understanding the hematopoietic stem cell (HSC) self-renewal and differentiation. Though AML is classified as a myeloid neoplasm, we were interested in determining the prevalence of clonal rearrangements within the immunoglobulin heavy (IGH) and light (IGK) chains, as well as the T-cell receptor gamma (TRG) loci in AML patient samples.

### Materials and Methods

- DNA was extracted from a random sampling of 200 AML, acute mixed patient residual peripheral blood (PB) or bone marrow (BM) specimens using Qiagen Blood Mini Kit.
- DNA was quantified with NanoDrop and normalized to 10 ng/µl.
- Each DNA sample (20 ng DNA) was tested with 6 different PCR master mixes (M1) from the Invivoscribe Assay Kit: IgH Tubes A, B, C, which target the framework (FR) 1, 2, and 3 regions, respectively; IgH Tube 4, IgK Tube 5, and IgK Tube 6; and IgH Tube 7 TRG 3.0. Amplicon products were analyzed using the ABI 3800 XL instrument. Based on the fluorescent signals, clonal (positive) or polyclonal (negative) were assessed.

PCR for IGH

PCR for IGK

PCR for TRG

Tube A (FR1)   Tube B (FR2)   Tube C (FR3)   Tube 4   Tube 5   Tube 6   Tube 7

ABI 3800 XL

### Results

The clonal (Igh), polyclonal (Ikg) and rearranged (Ika) rates detected by different PCR M1 for 200 AML samples. Combining multiple PCR M1 increased positive detection rate.

Results	monoclonal Igh			monoclonal Ikg			polyclonal Igh		polyclonal Ikg	
	Tube A (FR1)	Tube B (FR2)	Tube C (FR3)	Tube 4	Tube 5	Tube 6	FR1 Overall	FR2 Overall	FR3 Overall	TRG 3.0
Igh	23 (12%)	34 (17%)	28 (14%)	27 (13%)	12 (6%)	22 (11%)	32 (16%)	37 (18%)	37 (18%)	39 (20%)
Ikg	22 (11%)	31 (15%)	30 (15%)	27 (13%)	12 (6%)	22 (11%)	32 (16%)	37 (18%)	37 (18%)	39 (20%)
Ika	36 (18%)	40 (20%)	35 (17%)	34 (17%)	14 (7%)	12 (6%)	42 (21%)	35 (17%)	35 (17%)	35 (17%)
Total	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)	200 (100%)

<sup>1</sup> Not amplified

The assessment positive rates for different PCR M1 were listed as follows:

Assessment Positive Rate			
Igh (FR1+FR2+FR3)	Igh (Tube 4 + Tube 5)	Ikg (FR1+FR2+FR3)	Ika (FR1+FR2+FR3)
8/28 (29%)	1/15 (7%)	1/35 (3%)	1/39 (3%)

Using the 88 positive (clonal) samples, the evaluation rate by specific target combination:

### Results

Sample 134 is detected as positive by 4 PCR M1 (Igh FR1, Igh Tube 4 and 5, and TRG)

Sample 138 is detected as positive by all 6 PCR M1 (Igh FR1, FR2 and FR3, Igh Tube 4 and 5, and TRG)

### Conclusions

- 200 AML samples were tested for clonal rearrangements within the immunoglobulin heavy (IGH) and light (IGK) chains, and the chain (IGK), T-cell receptor gamma (TRG) loci.
- Approximately 80% of AML samples demonstrated at least one clonal IGH or IGK gene rearrangement.
- While it is unclear if it is the malignant myeloid cells or companion lymphoid cells that harbor these somatic gene rearrangements, the relatively high percentage of clonal rearrangements, as a result potential for monitoring in AML, makes this an area worthy of further investigation.



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Memorial Sloan Kettering Cancer Center

## Minimal Residual Disease Detection of Lymphoid and Plasma Cell Neoplasms Using a Next-Generation Sequencing (NGS)-Based Assay

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### Introduction

Lymphoid and plasma cell neoplasms are characterized by clonally-derived T-cell receptor (TCR) or immunoglobulin (Ig) rearrangements. Across clinical laboratories, this is generally determined with standardized multiplex polymerase chain reaction (PCR) assays, in which V-J or D-J products are amplified by fragment size or capillary electrophoresis (CE). However, this approach has relatively low sensitivity and does not provide the specific clonal sequence information required for tracking a clone at low level or in minimal residual disease (MRD) setting. In this study, we assessed the performance of a NGS-based assay, LymphoTrack<sup>TM</sup> (Invivoscribe, San Diego, CA) for detection of low level and MRD using various lymphoid and plasma cell neoplasms in comparison to CE and flow cytometry (FC) assays.

### Material and Methods

DNA was extracted from bone marrow, blood, and formalin-fixed paraffin-embedded tissue from all patients with diagnostic and post-therapy (PT) samples. For clonal rearrangement, PCR primers flanking the most conserved framework region 3 (FR3) in VJ and conserved 2M regions were used. For clonal TCR rearrangement, primers flanking the TRG conserved V1 and J1 regions were used. The amplified products were sequenced on the Illumina MiSeq platform, and analyzed with the proprietary LymphoTrack<sup>TM</sup> analysis software, which provided the quantitation and V-J gene family usage of all unique sequences. With the aid of an in-house developed software, MRD-lymphoclones, the patient-specific diagnostic clonal sequences were used to detect minimal disease involvement in subsequent samples, and compared to concurrent CE and/or color flow cytometry (FC) results available at MSKCC.

### Results

	Diagnostic Samples	Post-Therapy Samples
Acute Lymphoblastic Leukemia	11	14
Mature B-cell Neoplasms	10	20
Mature T-cell Neoplasms	9	11
Plasma Cell Neoplasms	12	10
Total	42	55

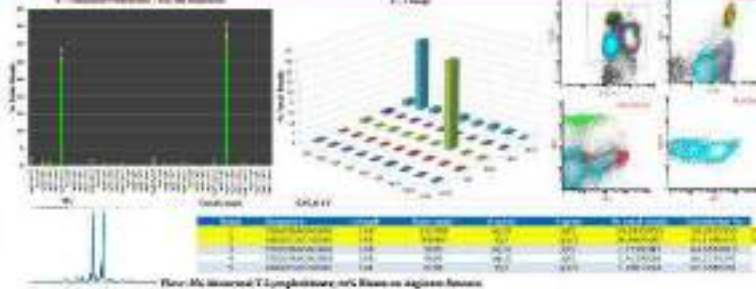
Table 1: Lymphoid and Plasma Cell Neoplasms cases used for Minimal Disease Detection by LymphoTrack<sup>TM</sup>

Table 2: Summary of Total Sequencing Reads and Percentage of Reads Supporting Residual Disease

	Total Sequencing Reads	% of Reads Supporting Residual Disease
Overall	10,634	0.0002
Highly	3,261,901	16.24%
Medium	3,290,090	1.02%

### Results

#### A. Diagnostic Sample with two distinct clones with TRG Primers



#### B. 4 months Post 2<sup>nd</sup> Allogeneic Stem Cell Transplant



Fig. 1. Minimal Disease Detection in a Patient with Relapsed T-Cellular Leukemia/Lymphoma, using TRG-Primers on LymphoTrack<sup>TM</sup>. The patient shows evidence of subsequent relapse by PET scan, and is currently on palliative therapy.

#### C. 6 Months Post 2<sup>nd</sup> Allogeneic Stem Cell Transplant



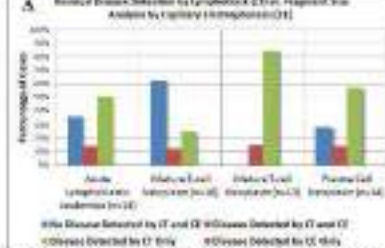
#### D. 8 Months Post 2<sup>nd</sup> Allogeneic Stem Cell Transplant



Flow Cytometry: Absent T cells Morphologic Remission

### Results

#### A. Minimal Disease Detection by LymphoTrack (LT) vs. Fragment Size Analysis by Capillary Electrophoresis (CE)



#### B. Minimal Disease Detection by LymphoTrack (LT) vs. 30 Color Flow Cytometry (FC)

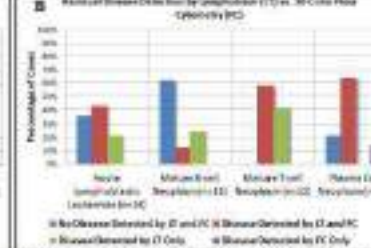


Fig. 2. Comparison of Minimal Disease Detection by LymphoTrack (LT) vs. (A) Capillary Electrophoresis (CE) and (B) Flow Cytometry (FC).

- In 2/14 Plasma Cell Neoplasm (PC) samples, FC detected suspicious atypical plasma cells in 100% and 0.00% of total WBC, but no clonal sequence detected by LT.
  - In one sample, total sequencing reads were inadequate for MRD detection (65,960 total reads).
  - In the other sample, two subsequent samples from the same patient showed no evidence of disease by all detection methods.
- In 12 PC samples from 10 patients, LT detected residual disease, while neither FC or CE detected disease.
  - 10 patients showed subsequent other evidence of persistent recurrent disease, with median follow-up time of 3 months.
- In 18 FC samples from 17 patients, there is no evidence of residual disease by all detection methods.
  - 16/17 patients showed no subsequent evidence of disease, with median follow-up time of 2.7 months.

### Conclusion

Compared to capillary electrophoresis and flow cytometry, LymphoTrack<sup>TM</sup> provides comparable or better MRD detection sensitivity of lymphoid neoplasms, and with increased diagnostic sensitivity by utilizing primer-specific clonal sequences for MRD detection.

### Acknowledgements

The authors would like to thank the diagnostic molecular pathology laboratory members and Invivoscribe for their technical support.

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AMP Annual Meeting, November 10-12, 2016, Charlotte, NC



H33

## Analysis and Characterization of Hematologic Cancers using a Comprehensive NGS Panel Comprised of DNA and RNA Baits Targeting 704 Genes

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### ABSTRACT

Background: Comprehensive genomic characterization of hematologic malignancies is essential for diagnosis, prognosis, and treatment. Next-generation sequencing (NGS) panels are used to identify mutations in DNA and RNA. However, the complexity of the human genome and the high cost of NGS have limited the number of genes that can be analyzed in a single assay. We have developed a comprehensive NGS panel targeting 704 genes, including both DNA and RNA, to enable the detection of mutations in a wide range of hematologic malignancies. This panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants.

### Materials & Methods

This panel was designed to detect mutations in DNA and RNA. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants.

### MYRDAC DNA Results: Coverage and Sequencing Depth

We evaluated DNA coverage (Figure 1) and sequencing depth (Figure 2) across the coding regions of the panel. Mean coverage was 100x and sequencing depth was 100x. Mean coverage was 100x and sequencing depth was 100x. Mean coverage was 100x and sequencing depth was 100x.

### MYRDAC DNA Results: Limit of Detection and Sensitivity

To evaluate the limit of detection (LOD) and sensitivity of the panel, we performed a series of experiments. The LOD was 100x and sensitivity was 100%. The LOD was 100x and sensitivity was 100%. The LOD was 100x and sensitivity was 100%.

### MYRDAC DNA Results: Sensitivity and Specificity

To evaluate the sensitivity and specificity of the panel, we performed a series of experiments. The sensitivity was 100% and specificity was 100%. The sensitivity was 100% and specificity was 100%. The sensitivity was 100% and specificity was 100%.

Gene	Sensitivity		Specificity	
	100x	1000x	100x	1000x
TP53	100%	100%	100%	100%
BCL2	100%	100%	100%	100%
CD20	100%	100%	100%	100%
IGH	100%	100%	100%	100%

### MYRDAC DNA & RNA Results: Translocations and Gene Fusions

We evaluated DNA and RNA results for translocations and gene fusions. The panel is designed to detect translocations and gene fusions. The panel is designed to detect translocations and gene fusions. The panel is designed to detect translocations and gene fusions.

Translocation	Gene	Method	RNA Results
t(8;21)	MLL2-KIT	RNA	+
t(11;22)	SLF6-19K1	RNA	+

### Conclusions

This panel is designed to detect mutations in DNA and RNA. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants. The panel is designed to detect mutations in DNA and RNA, including point mutations, insertions, deletions, and structural variants.

Improving Lives with Precision Diagnostics

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## Assessment of Minimal Residual Disease in Patients with Acute Myeloid Leukemia by Monitoring FLT3 and NPM1 Mutations

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### Introduction

Surrogate understanding of the impact of mutations in acute myeloid leukemia (AML) with minimal residual disease (MRD) is needed to identify patients who will not achieve remission. Existing studies suggest that the presence of transcription mutations at minimal disease levels indicate system susceptibilities to further leukemic relapse and therefore, it is important to quantify the presence or non-presence of these various mutations throughout the course of the disease. The most commonly mutated genes downstream of FLT3 in AML, are mutations in the FLT3 internal tyrosine kinase (ITK) and the nucleophosmin (NPM1) gene; the acquisition of transcriptional mutations, typically upstream genes that occur from early leukemias at the time of hematopoietic stem cell development resulting in the presence of MRD.

### RESULTS: Low, and linearity of observed leukemias

Minimal residual disease (MRD) levels were assessed in 100 patients with acute myeloid leukemia (AML) and FLT3 and NPM1 mutations in AML. The sensitivity, specificity, and linearity of these assays were assessed by comparing the observed (single assay) to the expected (single assay) results.

For the FLT3/ITK assay, 100% sensitivity and 100% specificity for detection of MRD were observed. For the NPM1 assay, 100% sensitivity and 100% specificity for detection of MRD were observed. The observed leukemias were assessed in Table 1 and Table 2.

Figure 1. Linearity of the FLT3/ITK assay in single and double format.

Figure 2. Linearity of the NPM1 assay in single and double format.

Table 1. Summary of observed leukemias by mutation type and the assay used.

Patient ID	FLT3/ITK		NPM1	
	Observed	Expected	Observed	Expected
001	1000	1000	1000	1000
002	1000	1000	1000	1000
003	1000	1000	1000	1000
004	1000	1000	1000	1000
005	1000	1000	1000	1000
006	1000	1000	1000	1000
007	1000	1000	1000	1000
008	1000	1000	1000	1000
009	1000	1000	1000	1000
010	1000	1000	1000	1000

Table 2. Summary of observed leukemias by mutation type and the assay used.

Patient ID	FLT3/ITK		NPM1	
	Observed	Expected	Observed	Expected
011	1000	1000	1000	1000
012	1000	1000	1000	1000
013	1000	1000	1000	1000
014	1000	1000	1000	1000
015	1000	1000	1000	1000
016	1000	1000	1000	1000
017	1000	1000	1000	1000
018	1000	1000	1000	1000
019	1000	1000	1000	1000
020	1000	1000	1000	1000

### Materials and Methods

The FLT3/ITK and NPM1 MRD assays were designed as they would be used in the clinical setting. The FLT3/ITK assay targets exons 19 and 20 of the FLT3 gene, while the NPM1 assay targets exon 12 of the NPM1 gene. The assays were designed to be sensitive and specific for the detection of MRD. The assays were designed to be sensitive and specific for the detection of MRD. The assays were designed to be sensitive and specific for the detection of MRD.

### RESULTS: Clinical sample testing

MRD levels were assessed in 100 patients with acute myeloid leukemia (AML) and FLT3 and NPM1 mutations in AML. The sensitivity, specificity, and linearity of these assays were assessed by comparing the observed (single assay) to the expected (single assay) results.

For the FLT3/ITK assay, 100% sensitivity and 100% specificity for detection of MRD were observed. For the NPM1 assay, 100% sensitivity and 100% specificity for detection of MRD were observed. The observed leukemias were assessed in Table 1 and Table 2.

Figure 1. Linearity of the FLT3/ITK assay in single and double format.

Figure 2. Linearity of the NPM1 assay in single and double format.

Table 1. Summary of observed leukemias by mutation type and the assay used.

Patient ID	FLT3/ITK		NPM1	
	Observed	Expected	Observed	Expected
021	1000	1000	1000	1000
022	1000	1000	1000	1000
023	1000	1000	1000	1000
024	1000	1000	1000	1000
025	1000	1000	1000	1000
026	1000	1000	1000	1000
027	1000	1000	1000	1000
028	1000	1000	1000	1000
029	1000	1000	1000	1000
030	1000	1000	1000	1000

Table 2. Summary of observed leukemias by mutation type and the assay used.

Patient ID	FLT3/ITK		NPM1	
	Observed	Expected	Observed	Expected
031	1000	1000	1000	1000
032	1000	1000	1000	1000
033	1000	1000	1000	1000
034	1000	1000	1000	1000
035	1000	1000	1000	1000
036	1000	1000	1000	1000
037	1000	1000	1000	1000
038	1000	1000	1000	1000
039	1000	1000	1000	1000
040	1000	1000	1000	1000



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#09

## Detecting B-Cell Clonality in Clinical Samples using a Comprehensive NGS LymphoTrack Dx<sup>®</sup> IGH FR1/2/3 Assay

Ying Huang<sup>1</sup>, Xiang Yang<sup>1</sup>, Jeff Hengstenberg<sup>1</sup>, Kaitie Jacobson<sup>1</sup>, Nico Wang<sup>1</sup>, Qun Duong<sup>1</sup>, Arnold M. Bann<sup>1</sup>, Jeffrey C. Alden<sup>1</sup>, and Tom Stamer<sup>1</sup>  
<sup>1</sup>Invivoscribe Technologies, San Diego, USA, Nikolaus Demmel, Deutschland GmbH, Hildesheim, Germany

**Abstract**

Immunoglobulin heavy chain (IGH) rearrangement analysis is a critical tool for the diagnosis and prognosis of B-cell lymphomas. The detection of clonal IGH rearrangements is essential for the diagnosis and prognosis of B-cell lymphomas. The detection of clonal IGH rearrangements is essential for the diagnosis and prognosis of B-cell lymphomas. The detection of clonal IGH rearrangements is essential for the diagnosis and prognosis of B-cell lymphomas.

**Materials and Methods**

The LymphoTrack Dx<sup>®</sup> IGH FR1/2/3 Assay for the detection of clonal IGH rearrangements is a comprehensive NGS assay that provides high sensitivity and specificity for the detection of clonal IGH rearrangements. The assay is based on a comprehensive NGS assay that provides high sensitivity and specificity for the detection of clonal IGH rearrangements.

**IGH<sup>H</sup> Assay Results: Sensitivity, Specificity, Precision and Reproducibility**

Level	Sample Size (n)	Se (%)	Sp (%)	PPV (%)	NPV (%)
High	32	98	99	97	99
Medium	49	98	98	97	98
Low	18	98	98	97	98
All	99	98	98	97	98

**Results: Clinical Study between KINOMEQ<sup>®</sup> and the IGH<sup>H</sup> Assay**

Sample Size (n)	Se (%)	Sp (%)	PPV (%)	NPV (%)
High	98	99	97	99
Medium	98	98	97	98
Low	98	98	97	98
All	98	98	97	98

**Conclusions**

The LymphoTrack Dx<sup>®</sup> IGH FR1/2/3 Assay is a comprehensive NGS assay that provides high sensitivity and specificity for the detection of clonal IGH rearrangements. The assay is based on a comprehensive NGS assay that provides high sensitivity and specificity for the detection of clonal IGH rearrangements.



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H03

## Small Customizable Next-Generation Sequencing Based Target-Capture Panels in a Clinical Environment Can Detect Variant Mutations at Frequencies as Low as 0.5%

Lisa M. Chamberlain, Dina Yu, Andrew S. Carson, Bradley Pines, Valerie McClain, Ognjen Sija, Wood Young, Jeffrey C. Miller and Tom Smedley  
Invivoscribe Technologies, Inc., San Diego, CA

### Introduction

In clinical trials, time and cost are critical. Small, customizable next-generation sequencing (NGS) panels can detect a wide range of variant mutations. The Invivoscribe Next-Generation Sequencing (NGS) based target-capture panels are designed to detect variant mutations at frequencies as low as 0.5%.

### Materials and Methods

Library Preparation: 100 ng of genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK). Library Preparation: 100 ng of genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK). Library Preparation: 100 ng of genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Crawley, UK).

### Results: LoD, LeI, and Linearity

To evaluate the LoD and Linearity of our small, customizable next-generation sequencing (NGS) panels, we performed a series of experiments. The results are shown in Figure 1. The LoD and Linearity of our small, customizable next-generation sequencing (NGS) panels are shown in Figure 1.

### Results: Precision and Reproducibility

Variant detection was performed for 10 replicates on 100 ng of genomic DNA using the Invivoscribe Next-Generation Sequencing (NGS) based target-capture panels. The results are shown in Figure 2. The precision and reproducibility of our small, customizable next-generation sequencing (NGS) panels are shown in Figure 2.

Variant	Frequency	100 ng	50 ng	25 ng	10 ng
BRCA1	0.5%	100%	100%	100%	100%
BRCA2	0.5%	100%	100%	100%	100%
TP53	0.5%	100%	100%	100%	100%
EGFR	0.5%	100%	100%	100%	100%

### Conclusions

Small, customizable next-generation sequencing (NGS) based target-capture panels are an effective tool for detecting variant mutations in a clinical environment. The results of our experiments demonstrate that our small, customizable next-generation sequencing (NGS) panels can detect variant mutations at frequencies as low as 0.5%.



Personalized Molecular Medicine®

## Analysis and Characterization of Hematologic Cancers using a Comprehensive NGS Panel Comprised of DNA and RNA Baits Targeting 704 Genes


*Timothy Stenzel, Andrew R. Carson, Bradley A. Patay, Valerie McClain, Zhiyi Xie, and Jeffrey E. Miller*

Timothy Stenzel, MD, PhD

2017 EHA Conference

Friday June 23<sup>rd</sup>





## DETECTION OF CLONALITY IN CLINICAL SPECIMENS FROM SUSPECTED B-CELL MALIGNANCIES USING COMPREHENSIVE IGH (FR1/2/3) LYMPHOTRACK® MISEQ® AND PGM® ASSAYS

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E847

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### Background

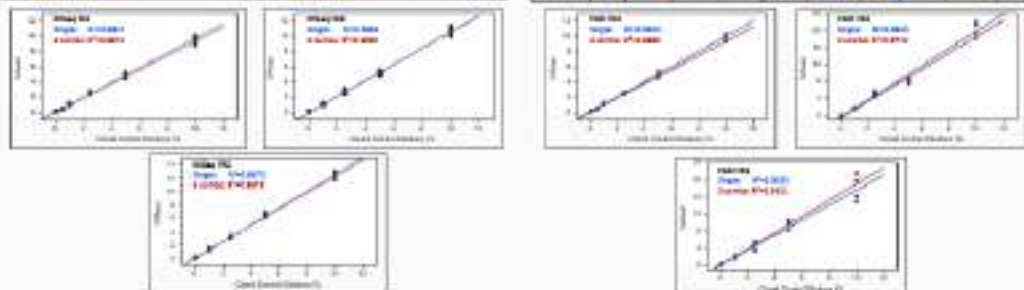
PCR-based capillary electrophoresis (PCR-CE) methods targeting immunoglobulin heavy chain (IGH) framework 1, 2, & 3 (FR1, FR2, FR3), and joining regions (J) are historically the gold standard for clonality testing in suspected B-cell malignancies. Recently, next-generation sequencing (NGS) based approaches for immunoglobulin genes have been developed that improve sensitivity and identify the specific V-(D)-J DNA sequences required to track clones in follow-up testing. We developed comprehensive LymphoTrack® IGH (FR1, FR2, & FR3) Assays for both the Illumina® MiSeq® and ThermoFisher Scientific® Ion PGM™ platforms, which detect the vast majority of rearrangements in a single NGS run. In this pilot study, we compared the performance of both LymphoTrack® IGH MiSeq and PGM Assays to the IdentClone® IGH PCR-CE assay by testing in over 30 anonymized, blinded clinical samples.

### Results: LoD, Linearity, Precision and Reproducibility

Clonal Control (fm)	MiSeq® IGH IGH			MiSeq® IGH FR2			MiSeq® IGH FR3			
	LoD (%)	Mean % Match	CV%	LoD (%)	Mean % Match	CV%	LoD (%)	Mean % Match	CV%	
10	22	285	2.08	17.5	243	22.7%	9.0	304	12.89	6.1
5	46	285	2.50	15.4	243	7.70	9.2	304	7.94	2.4
1.5	46	285	1.20	14.5	243	3.30	9.2	304	2.90	3.0
1	46	285	0.20	24.3	243	1.40	42.8	464	1.81	14.4
0	46	285	0.00	14.8	243	0.28	28.9	464	0.27	18.4


  

Clonal Control (fm)	Ion PGM™ IGH IGH			Ion PGM™ IGH FR2			Ion PGM™ IGH FR3			
	LoD (%)	Mean % Match	CV%	LoD (%)	Mean % Match	CV%	LoD (%)	Mean % Match	CV%	
10	22	285	25.86	4.7	348	18.81	8.9	124	27.89	28.8
5	21	280	2.40	8.7	246	2.80	18.0	124	40.70	8.8
1	24	285	0.31	22.4	348	1.81	14.3	124	1.24	22.1
0	24	1878	0.071	87.3	1878	0.22	45.8	1878	0.08	37.3

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### Materials and Methods



- The LymphoTrack® IGH FR1/2/3 Assays for the MiSeq® and Ion PGM™ were manufactured under cGMP standards and QC tested under a QSR-compliant regulatory system prior to use.
- Limit of detection (LoD), linearity, precision and reproducibility (PVR) were validated using clonal control DNA diluted in wild-type polyclonal (total) DNA.
- DNA from a variety of samples (21 from peripheral blood, 1 from bone marrow aspirates, and 57 from FFPE) were extracted using common extraction methods by collaborators. 33 samples were tested by all assays except that PGM tested additional 5 samples for total 68 samples.
- Libraries were prepared with amplicons generated by the LymphoTrack® IGH FR1/2/3 Assay optimized for each NGS platform.
- Libraries were either sequenced for each FR (individually) or for all FRs (IGH FR1/2/3) combined.
- LymphoTrack® Software - MiSeq® and LymphoTrack® Software - PGM™ analyzed FASTQ data from the MiSeq® and the Ion PGM™, respectively.
- When comparing testing results, only samples that met the specimen and data acceptance criteria for both methods were evaluated.
- All statistical analyses were performed in JMP®.

### Results: Clinical Study between IGH MiSeq® and Ion PGM™ Assays

	MiSeq® IGH Assays				Ion PGM™ IGH Assays				IdentClone IGH Assays			
	FR1	FR2	FR3	FR1/2/3	FR1	FR2	FR3	FR1/2/3	Tube A (FR1)	Tube B (FR2)	Tube C (FR3)	Tube A/B/C (FR1/2/3)
Clonal (N)	28/38 (73%)	26/38 (70%)	21/38 (57%)	28/38 (73%)	27/34 (79%)	28/38 (73%)	13/30 (43%)	13/30 (43%)	28/38 (73%)	21/38 (57%)	13/30 (43%)	28/38 (73%)
Non-Clonal (N)	87/38 (87%)	41/38 (70%)	34/38 (82%)	82/38 (84%)	81/34 (85%)	27/38 (70%)	22/30 (73%)	22/30 (73%)	21/38 (57%)	21/38 (57%)	14/30 (47%)	20/38 (53%)

MiSeq® FR1/2/3	IdentClone IGH Tube A/B/C	
	Clonal	Non-Clonal
Clonal	24	2
Non-Clonal	1	18

Ion PGM® FR1/2/3	IdentClone IGH Tube A/B/C	
	Clonal	Non-Clonal
Clonal	22	0
Non-Clonal	1	18

Ion PGM® FR1/2/3	MiSeq® FR1/2/3	
	Clonal	Non-Clonal
Clonal	22	0
Non-Clonal	0	18

	MiSeq® FR1/2/3 vs IdentClone Tube A/B/C	Ion PGM® FR1/2/3 vs IdentClone Tube A/B/C	Ion PGM® FR1/2/3 vs MiSeq® FR1/2/3
Concordance (%)	95.5	97.8	100
Sensitivity (%)	98.0	98.7	100
Specificity (%)	94.7	100	100
PPV (%)	98.0	100	100
NPV (%)	94.7	94.7	100

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### Conclusions

A comprehensive NGS-based LymphoTrack® IGH FR1/2/3 Assay was developed for both the Illumina® MiSeq® and ThermoFisher Scientific® Ion PGM™ platforms. These assays identify clonal IGH V-(D)-J rearrangements and the specific clonal DNA sequences, critical for determining the BMM rate and tracking residual disease. Excellent concordance was demonstrated between these assays.



## USING NEXT-GENERATION SEQUENCING TO DETECT CLONAL TRG AND TRB GENE REARRANGEMENTS

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### Background

T-cell malignancies arise from transformation and clonal expansion of a single cell. During T-cell development, the T cell receptor genes (TRG) occur rearrange prior to the T cell receptor beta (TRB) locus. Combined, TRG and TRB can identify the vast majority of T-cell rearrangements. Historically, these clonal rearrangements are often identified by regular electrophoresis (GE) methods which provide size estimation information, but not the sequence needed for tracking individual disease during the course of treatment. Recently, next-generation sequencing (NGS)-based approaches for immune receptor genes have been developed to improve the sensitivity of clonal detection and identify the specific V(D)J DNA sequences required as each cancer is being up treated. We have developed and validated LymphoTrack<sup>®</sup> TRG & TRB assays specifically for the Illumina<sup>®</sup> MiSeq<sup>®</sup> platform.

### Material and Methods

1. Schematic illustration of the TRG gene.

2. The workflow for the LymphoTrack<sup>®</sup> TRG & TRB assays - MiSeq<sup>®</sup>.

3. The LymphoTrack<sup>®</sup> TRG and TRB assays for the MiSeq<sup>®</sup> were manufactured under cGMP conditions and QC tested under a QSP-compliant regulatory system prior to use.

4. Limit of detection (LOD), linearity, precision and reproducibility (R%) were tested using serial dilutions of spiked-in wild-type (WT) DNA (1000 copies).

5. 22 cell line DNA samples were tested with the TRG assay.

6. DNA from 48 FFPE samples were extracted using common extraction methods by collectionists. All samples were tested with the TRG and TRB assays.

7. Libraries were prepared with amplicons generated by PCR using proprietary multiplex master mixes with the universal primers, tagging at TRG and TRB V, D, and J exon regions, synthesized with Illumina specific adapters and 24 base oligonucleotide (OL) barcodes (OLB).

8. Libraries were either sequenced for TRG and TRB individually or for TRG + TRB combined.

9. LymphoTrack<sup>®</sup> software - MiSeq<sup>®</sup> analysis was done from the MiSeq.

10. All data and analysis were performed in R.

### Results: TRG + TRB Combo Assay

### Results: Top 5 Sequences % for Normal PB Samples

Sample	TRG	TRB	% TRG	% TRB	% TRG+TRB
HL60	1000	1000	50%	50%	100%
HL60	1000	1000	50%	50%	100%
HL60	1000	1000	50%	50%	100%
HL60	1000	1000	50%	50%	100%
HL60	1000	1000	50%	50%	100%

### Results: Clinical Study

TRG Assay	TRB Assay	TRG+TRB Assay
1000	1000	1000
100	100	100
10	10	10
1	1	1

Sample	TRG	TRB	TRG+TRB	TRG	TRB	TRG+TRB
Sample_001	1000	1000	1000	1000	1000	1000
Sample_002	1000	1000	1000	1000	1000	1000
Sample_003	1000	1000	1000	1000	1000	1000
Sample_004	1000	1000	1000	1000	1000	1000
Sample_005	1000	1000	1000	1000	1000	1000

### Results: LOD, LOB and Linearity

Copy Number	TRG	TRB	TRG+TRB
1000	1000	1000	1000
100	100	100	100
10	10	10	10
1	1	1	1

### Results: Precision and Reproducibility

### Conclusions

- The LymphoTrack<sup>®</sup> TRG Assay - MiSeq<sup>®</sup> was able to consistently detect all known TRG clonal rearrangements from cell line DNA.
- Excellent linearity (R<sup>2</sup>>0.90), sensitivity of detection (2.0%), and reproducibility (<20% CV) were demonstrated with serial dilutions of control cell line DNA.
- Concordance between the LymphoTrack<sup>®</sup> TRG and GE assays was 95% and between the LymphoTrack<sup>®</sup> TRG and TRB assays was 94%.



## Use of a Small Targeted Next-Generation Sequencing Panel for Monitoring Minimal Residual Disease in Acute Myeloid Leukemia

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PL97

### Background

Background: Many personalized therapies for acute myeloid leukemia (AML) have been developed targeting specific biomarkers. The efficacy of these therapies are maximized when the need to determine successful patient response prior to patient response results arrives. Minimal residual disease (MRD) monitoring can help determine effective treatments and predict potential relapse, which are now possible with next generation sequencing. The most recent target single or small numbers of bases data, which are less extension of residual cells, demonstrating. Thus, full coverage better of a sample may require testing with multiple MRD assays, which can be impractical in a clinical setting. The MyMRD assay (MyMRD as a service by LabPMML) is a target capture based assay for MRD characterization of the entire AML clonal population regardless of any prior therapy and can inform the molecular residual status of a patient's malignancy. This targeted assay can identify the mutations in order to assess the value of therapy in 90% of all AML patients, as well as common events in therapy profile responses (TPM) and translocated cytogenetic (MDS).

### Results: LOD and Linearity

Results: The assay shows strong linearity (R<sup>2</sup> > 0.99) over the range of tested variant allele frequencies (VAF) (0.01-0.25). Overall, an LOD of 0.2% was maintained for 95% of the targeted sites in the assay. At this level, the assay shows average across read frequency (AF%) for example 0.2% and the 750-752 insertion deletion (ID) + 0.2% with 2% bias over 100. Across several sites which is known to be difficult and highly repetitive, the detection sensitivity is between 0.2% LOD with a 1% higher CI, due to the complexity of the region. Lower LODs for particular sites of interest were also obtained. For example 0.2% for sites like 752-752 and 752-752 variants.

### Results: Combined Sensitivity and Specificity

Results: The overall sensitivity and specificity is excellent (99%) at the lowest LOD of 0.2%. The specificity remains stable for both DNA and transcripts measured at lower LODs of 0.1%.

VAF	Sensitivity		Specificity	
	DNA	Transcript	DNA	Transcript
0.2%	99.9%	99.9%	99.9%	99.9%
0.1%	99.9%	99.9%	99.9%	99.9%
0.05%	99.9%	99.9%	99.9%	99.9%

### Methods

**Library Preparation:** Whole genome libraries were made from 300 ng of DNA extracted from 100 cells or 1000 AML samples. Our library preparation protocol includes the addition of unique adapters on each sample allowing the multiplexing of up to 96 samples per sequencing run.

**Hybridization:** Whole genome libraries were hybridized with hybrid probes targeting mutation hotspots in a total of 22 genes associated with AML, including AML1, CEBPA, DNMT3A, DNMT3B, DNMT3C, DNMT3L, DNMT3O, DNMT3A2, DNMT3A1, DNMT3B1, DNMT3B2, DNMT3B3, DNMT3B4, DNMT3B5, DNMT3B6, DNMT3B7, DNMT3B8, DNMT3B9, DNMT3B10, DNMT3B11, DNMT3B12, DNMT3B13, DNMT3B14, DNMT3B15, DNMT3B16, DNMT3B17, DNMT3B18, DNMT3B19, DNMT3B20, DNMT3B21, DNMT3B22, DNMT3B23, DNMT3B24, DNMT3B25, DNMT3B26, DNMT3B27, DNMT3B28, DNMT3B29, DNMT3B30, DNMT3B31, DNMT3B32, DNMT3B33, DNMT3B34, DNMT3B35, DNMT3B36, DNMT3B37, DNMT3B38, DNMT3B39, DNMT3B40, DNMT3B41, DNMT3B42, DNMT3B43, DNMT3B44, DNMT3B45, DNMT3B46, DNMT3B47, DNMT3B48, DNMT3B49, DNMT3B50, DNMT3B51, DNMT3B52, DNMT3B53, DNMT3B54, DNMT3B55, DNMT3B56, DNMT3B57, DNMT3B58, DNMT3B59, DNMT3B60, DNMT3B61, DNMT3B62, DNMT3B63, DNMT3B64, DNMT3B65, DNMT3B66, DNMT3B67, DNMT3B68, DNMT3B69, DNMT3B70, DNMT3B71, DNMT3B72, DNMT3B73, DNMT3B74, DNMT3B75, DNMT3B76, DNMT3B77, DNMT3B78, DNMT3B79, DNMT3B80, DNMT3B81, DNMT3B82, DNMT3B83, DNMT3B84, DNMT3B85, DNMT3B86, DNMT3B87, DNMT3B88, DNMT3B89, DNMT3B90, DNMT3B91, DNMT3B92, DNMT3B93, DNMT3B94, DNMT3B95, DNMT3B96, DNMT3B97, DNMT3B98, DNMT3B99, DNMT3B100.

**Sequencing and Analysis:** The MyMRD target amplified libraries were sequenced with the MiSeq<sup>®</sup> platform and analyzed using a proprietary pipeline (MyMRD) implemented on Illumina BaseSpace.

**The MyMRD Assay Workflow:**

**LOD, LOD and Linearity Evaluation:** Certified and one sample were created from 1000 cells lines diluted into a wide range of the background from 0.1% down to 0.01% (0.1% down to 0.01% dilution). The LOD was evaluated at 0.1%, 0.05%, 0.02%, 0.01% and 0.005%. Evaluation of the assay was performed using a target site (DNMT3A G1555A) and a control site (DNMT3A G1555A). The LOD was evaluated at 0.1%, 0.05%, 0.02%, 0.01% and 0.005%. The LOD was evaluated at 0.1%, 0.05%, 0.02%, 0.01% and 0.005%. The LOD was evaluated at 0.1%, 0.05%, 0.02%, 0.01% and 0.005%.

**Clinical Sample Evaluation:** Samples post therapy (post therapy) were tested with the MyMRD assay. The MyMRD assay superior sensitivity of the MyMRD assay, allowing 0.2% - 0.1% LOD at the 0.2% position allows samples with low residual disease (MRD) to be detected. The MyMRD assay is a target capture based assay for MRD characterization of the entire AML clonal population regardless of any prior therapy and can inform the molecular residual status of a patient's malignancy. This targeted assay can identify the mutations in order to assess the value of therapy in 90% of all AML patients, as well as common events in therapy profile responses (TPM) and translocated cytogenetic (MDS).

### Results: Depth of Coverage

Results: The overall read length of detection (LOD) of the MyMRD assay is maintained using whole genome data. Coverage and linearity are maintained across a range of variant allele frequencies (VAF). The minimum 95% coverage for detecting a mutation is a target of 0.2%, which is maintained at 0.2% read coverage. The depth of coverage per sample can be varied by multiplexing between 8 and 12 samples per MiSeq run, depending upon the desired LOD as a function of the number of reads.

• Over 95% of using targets meet or exceed depth for 95% confidence to detect a mutation at 0.2%  
 • Multiplexing 8-12 samples minimum depth is 0.2% or 0.1%



## Small Customizable NGS Based Target Capture Panels Detect Variants in Clinical Specimens at Frequencies as Low as 0.5%

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### Abstract

**Background:** The use of large scale hybridization panels in early stages of disease detection is a major challenge for clinicians. However, as therapeutic targets and further characterizing target panels, genes for every chromosome are being sequenced in the clinical diagnostic space. Therefore, clinical labs are being challenged with the use of smaller hybridization panels for their own diagnostic variant detection by employing specific genomic regions of interest with greater sensitivity than larger gene panels and greater resolution over their workflow on NGS-based assays. Modifications of laboratory methods for small scale panels allow for the maintenance of high analysis quality with lower targeted genes. Our small panels (~400 kb) focus on 4 genes, allowing for high multiplexing of samples in a sequencing, and reduced cost per sample without compromising accuracy.

**Goal:** To assess the sensitivity, linearity, and agreement of small NGS target capture panels with other assays.

**Methods:** Two separate next generation sequencing target capture assays were developed with hybridization methods. One panel contained 4 genes, including five variants in exon 2 of *PCSK9* and several variants in *CELSR3* (PC-4). Another panel made, hybridized with only one gene sequence. Testing sets created out by splitting in-house amounts of in-house DNA into two sets. DNA to determine the linearity and sensitivity of the assays. Sequencing libraries were generated by capturing with both from either one or both panels. Sequencing data was analyzed using proprietary software developed by Invivoscribe, SignaMx, clinical samples were tested for PCR mutations by the other panel, amplicon based read only, and capillary electrophoresis (CE) assay.

**Results:** Data from 33 real time PCR assays using both panels, confirming variants previously identified using other methods. 3 validation sets run on the 4 gene paneling a series of 100000 samples generated in real time containing between 0.2% and 20% variant allele frequencies for expected variants. PCR validation indicates that these small panels detect all expected mutations down to 0.2% variant allele frequencies. Assay linearity for PCR by detection from 0.2% to 10.2% for PCR by detection from 0.2% to 10% is consistent (0.986 and 0.988, respectively). Average sequencing coverage was high, ranging from 1,343 to 7,444. Comparison of PCR analysis of the other panel to amplicon based read only, and CE, PCR-ITD showed a strong linear relationship between calculated VAFs and detected VAFs.

**Conclusions:** Small hybridization panels are cost effective in detecting low frequency variants from clinical samples of genetic data using the less than individual PCR based biological assays would require. Performance across assays is consistent on clinical samples. These smaller assays facilitate the most pertinent genes for a targeted therapy, and lower the potential to greatly assist in understanding the molecular backgrounds of responders, hyperresponders, and non-responders.

### Materials and Methods

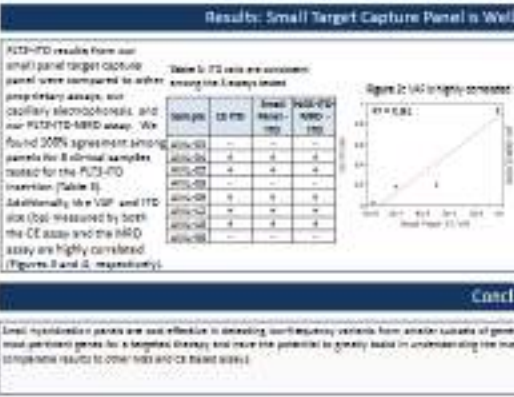
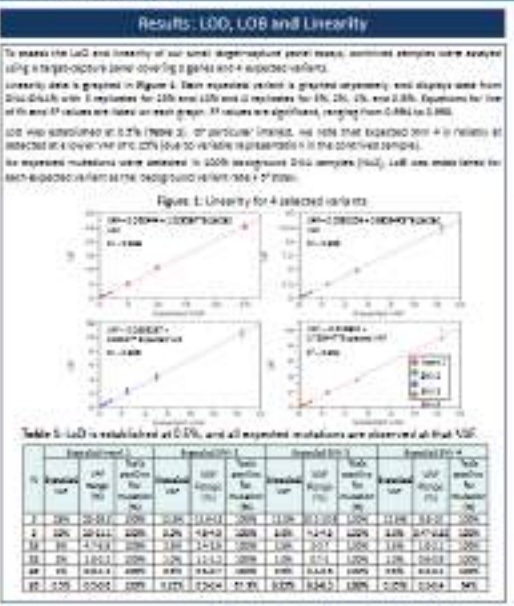
**Library Preparation, Hybridization, Capture, and Sequencing:** Whole genome libraries were prepared and hybridized to panels.

**Controlled Samples:** 8 real time PCR assays were used to generate controlled mixes of DNA with 4 expected variants against background DNA. Control mixes containing 20%, 10%, 5%, 2%, 1%, and 0.5% variant DNA were added to background DNA (DNA-0.0%). Prior to addition, input set lines had expected mutations with variant allele frequencies (VAF) of 4 (mutation 1), 2.5 (SNV 2), 0.5 (SNV 3), 0.5 (SNV 4).

**LoD, LoQ, and Linearity:** 3 replicates of 20% and 10% DNA-0.0% solutions, 2 replicates of 5%, 2%, 1%, and 0.5% DNA-0.0% solutions, and 2 replicates of background DNA were sequenced.

**Precision and Reproducibility:** 24 replicates of 2%, 1%, and 0.5% DNA-0.0% duplicate-derived samples were run through the 4-gene assay by 2 operators on 2 different days of 2 different instruments.

**Comparison of Small Scale Hybridization Panel Results to Capillary Electrophoresis and PCR-ITD:** MMR results DNA from 4 subjects had been previously analyzed by other methods were selected using our small-scale hybridization assay. All 4 DNA samples had previously been analyzed using our capillary electrophoresis (CE) PCR-ITD assay and PCR-ITD-ARD assay. High quality results were compared for agreement.



### Conclusions

Small hybridization panels are cost effective in detecting low frequency variants from smaller subsets of genes while using the less DNA than individual PCR-based diagnostic assays. Small hybridization assays focus on the most pertinent genes for a targeted therapy and have the potential to greatly assist in understanding the molecular backgrounds of therapeutic monitoring. These panels detect assays have high sensitivity and are highly complementary results to other NGS and CE based assays.





## Detection of Clonal TRG and TRB Gene Rearrangements Using Next-Generation Sequencing

TT45

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### Background

T-cell malignancies arise from translocation and clonal expansion of a single cell. During B-cell development, the T cell receptor genes (TRG/locus) rearrange prior to T cell receptor beta (TRB) locus. Combined, TRG and TRB assays can identify the vast majority of T-cell rearrangements in T-cell-lesions with an some focal rearrangements (e.g., B-ALL). Historically, clonal rearrangements have been identified using capillary electrophoresis (CE) methods, which provide size distribution information, but not the sequence needed for clonal gene rearrangement (MIR) throughout the course of treatment. Recently, we have developed next-generation sequencing (NGS) assays for immune receptor genes that increase both the sensitivity of clonality detection and identify the specific V(D)J DNA sequences required to track disease in follow-up MRD testing. Here we present data from the LymphoTrack<sup>®</sup> TRG and TRB Clonality Assays developed for the Illumina<sup>®</sup> MiSeq<sup>®</sup> platform and compare results with the IdentClone<sup>®</sup> CE-based assay.

### Results: TRG/TRB LoD, LoB and Linearity

### Results: TRG/TRB Positive Clinical Sample

### Material and Methods

Schematic illustration of the TRG gene and schematic illustration of the TRB gene. Workflow for the LymphoTrack<sup>®</sup> TRG & TRB Assay - MiSeq<sup>®</sup> is shown, including PCR, Purify Amplicons, Quantify Amplicons, Read Library, Read MiSeq<sup>®</sup>, and Analyze Data.

The LymphoTrack<sup>®</sup> TRG and TRB Assays for the MiSeq<sup>®</sup> were cDNA manufactured, QC tested, and analyzed using LymphoTrack<sup>®</sup> bioinformatics software developed under full design control consistent with our ISO 13485 compliance regulatory system.

Limit of detection (LoD), linearity, precision and reproducibility (RVR) were tested using clonal control DNA at various cell concentrations (1000, 100, 10, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001) for TRG assay.

DNA from 60 formalin-fixed paraffin-embedded (FFPE) samples were extracted using common extraction methods by collaborators. All samples were tested with TRG and TRB Assays.

Control cell line was used for MRD detection at 10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10<sup>1</sup> initial copy with an internal control spiked in to reactions at approx. 1000 cell equivalents.

Oligos were prepared with amplicons generated by PCR using proprietary multiplex primer mixes with the consensus primer targeting all TRG and TRB (V, D, and J) gene families, synthesized with MiSeq<sup>®</sup> specific adapters and 24 bp sequences optimized for the MiSeq platform.

Libraries were either sequenced for TRB and TRG individually or for TRB + TRG combined.

LymphoTrack<sup>®</sup> Software - MiSeq<sup>®</sup> was used to analyze FASTQ data generated from the MiSeq<sup>®</sup>.

When comparing testing results, only samples that met the specimen and data acceptance criteria for both methods were evaluated.

All statistical analyses were performed in JMP.

### Results: TRG/TRB Precision and Reproducibility

Clonal Control Subclone (n)	TRG MiSeq <sup>®</sup>			TRB MiSeq <sup>®</sup>		
	True Pos	Mean % Reads	CV%	True Pos	Mean % Reads	CV%
10	147	6.66	18.4	200	5.163	26.7
5	147	6.50	12.0	200	6.26	18.4
2.5	147	2.49	11.7	200	5.25	28.6
1	147	0.94	13.8	200	2.84	20.0
0	nd/nd	0.02	32.5	nd/nd	0.27	48.8

### Results: Clinical Study between TRB MiSeq<sup>®</sup> and IdentClone<sup>®</sup> Assays

TRB MiSeq <sup>®</sup>	TRB IdentClone <sup>®</sup>	
	Clonal	Non-Clonal
Clonal	26	0
Non-Clonal	0	28

TRB MiSeq <sup>®</sup> vs. TRB IdentClone <sup>®</sup> (n=54)	
Concordance (%)	88.9
Sensitivity (%)	75.0
Specificity (%)	100.0
PPV (%)	100.0
NPV (%)	75.0

### Results: TRG/TRB MRD Detection

### Results: Clinical Study Comparing TRG MiSeq<sup>®</sup> and IdentClone<sup>®</sup> Assays

TRG MiSeq <sup>®</sup>	TRB IdentClone <sup>®</sup>	
	Clonal	Non-Clonal
Clonal	34	1
Non-Clonal	0	39

TRG MiSeq <sup>®</sup> vs. TRB IdentClone <sup>®</sup> (n=73)	
Concordance (%)	89.2
Sensitivity (%)	82.5
Specificity (%)	82.1
PPV (%)	100.0
NPV (%)	84.6

### Results: Clinical Study for LymphoTrack<sup>®</sup> TRG + TRB Assays - MiSeq<sup>®</sup>

	TRB MiSeq <sup>®</sup>		TRG MiSeq <sup>®</sup>		TRG + TRB MiSeq <sup>®</sup>	
	Clonal	Non-Clonal	Clonal	Non-Clonal	Clonal	Non-Clonal
Clonal	18/10 (65%)	0/0 (0%)	16/10 (62%)	0/0 (0%)	34/10 (80%)	0/0 (0%)
Non-Clonal	0/10 (0%)	42/10 (84%)	0/10 (0%)	32/10 (64%)	0/10 (0%)	32/10 (64%)

TRG + TRB MiSeq <sup>®</sup> vs. IdentClone <sup>®</sup>	
Concordance (%)	84.2
Sensitivity (%)	77.0
Specificity (%)	100.0
PPV (%)	100.0
NPV (%)	77.0

### Conclusion

The LymphoTrack<sup>®</sup> MiSeq<sup>®</sup> TRG assay demonstrated excellent linearity (R<sup>2</sup> 0.98), sensitivity (at 10<sup>1</sup> cell 2.5%), and reproducibility (±18%CV) testing serial dilutions of clonal cell line DNA.

Concordance between the LymphoTrack<sup>®</sup> TRG - MiSeq<sup>®</sup> and IdentClone<sup>®</sup> TRG assays was 88.9%.

Concordance between the LymphoTrack<sup>®</sup> TRB - MiSeq<sup>®</sup> and IdentClone<sup>®</sup> TRB assays was 75.0%.

Concordance between the combined TRB+TRG LymphoTrack<sup>®</sup> MiSeq<sup>®</sup> and IdentClone<sup>®</sup> assays was 84.2%.

Excellent sensitivity (≥10.0%) for clonal cell line, reproducibility (±20% CV) for control cell line, and clonal detection for MRD at 10<sup>1</sup> sensitivity was demonstrated for LymphoTrack<sup>®</sup> MiSeq<sup>®</sup> TRG and TRB assays.



## Multiple Highly Concordant Assays Facilitate Analyses of Clinical Samples at Different Scales and Sensitivities

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### Introduction

The uniform detection of clinically relevant mutations is critical to drug and diagnostic development. Technologies such as next-generation sequencing (NGS) have improved sensitivity of detection, but to date there is no "catch-all" method that addresses both the broad discovery phase of development and the more focused approach of target validation. We have developed several methods of variant detection to address this need for varied sensitivities and scales, utilizing technologies ranging from capillary electrophoresis (CE) to next-generation sequencing. For example, Invivoscribe's proprietary NGS, large panel-based assays (such as Mylytic<sup>®</sup> and MyKML<sup>®</sup>) and NGS medium panel-based assays (MyMRD<sup>®</sup>) can be useful to early investigational stages of drug and treatments, while highly targeted amplicon-based assays (FUT3-ITD) can more specifically hone in on clinically relevant biomarkers in later stages of development. Here we demonstrate the ability to reliably detect clinically actionable APM1, FUT3-ITD, and FUT3-TKD variants at varying frequencies in a set of clinical samples across multiple Invivoscribe-developed assays including CE single gene analysis, NGS amplicon assays, and NGS multi-plex medium scale targeted multi-variant panels.

### Materials & Methods

Eight clinical AML samples from peripheral blood and bone marrow were tested at various dilutions using multiple Invivoscribe-developed CE, NGS amplicon, and NGS-panel assays. Several dilutions of these samples were tested to demonstrate sensitivity of the NGS assays. An additional 30+ AML samples were run on the CE and NGS-amplicon assays for FUT3 variants in this study, and small-scale NGS panels are defined as a few genes, and medium-scale panels are 20+ genes. The DNA input for the assays ranged from 50-500 ng of high-quality genomic DNA. In NGS amplicon assays, regions containing variants were amplified by PCR. Up to 30 samples were pooled, pooled, and sequenced. Target capture libraries were generated for NGS panel assays and sequenced. The Illumina MiSeq<sup>®</sup> platform was used for all NGS assays in this study. Sequence data was analyzed using proprietary Invivoscribe MyInformatics<sup>®</sup> software, which produced variant read frequencies (VRF) defined as variant reads divided by total reads.

Legend	
CE	Capillary Electrophoresis
SP	Small Panel
MP	Medium Panel
LP	Large Panel
+	Variant detected
-	Variant not detected
NA	Sample not tested

### Results: Small Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS assays to identify a hair base pair insertion in APM1. Of the eight AML samples, five were positive for this insertion. The CE-based assay detected mutations in 2 out of 4 samples tested (Table 1), while the NGS assays identified APM1 insertions in five samples.

Table 1. Variant: APM1

Sample Name	Dilution	CE Data	NGS Amplicon	Medium Scale NGS Panel
		Call	Variant VRF (%)	Variant VRF (%)
AML-01	1		NA	NA
AML-01	1	+	NA	NA
AML-01	1/10		NA	NA
AML-01	1/100		NA	NA
AML-01	1/1000		NA	NA
AML-01	1		NA	NA
AML-01	1	+	NA	NA
AML-01	1/10		NA	NA
AML-01	1/100		NA	NA
AML-01	1/1000		NA	NA
AML-01	1		NA	NA
AML-01	1	+	NA	NA
AML-01	1/10		NA	NA
AML-01	1/100		NA	NA
AML-01	1/1000		NA	NA
AML-01	1		NA	NA
AML-01	1	+	NA	NA

### Results: Large Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS methodologies to identify internal tandem duplications (ITDs) in FUT3. Of the eight clinical samples examined, five were positive for the FUT3-ITD; all three assays detected FUT3-ITD at similar frequencies (Table 2). Following dilution of the clinical samples, low frequency mutations are no longer observable in the less sensitive CE assays. However, using the more sensitive NGS assays, variants are still detected at frequencies of 10<sup>-4</sup> and 10<sup>-5</sup>.

Table 2. Variant: FUT3-ITD

Sample Name	Dilution	CE Data		NGS Amplicon		NGS Panel	
		Call	Variant VRF (%)	Variant VRF (%)	Variant VRF (%)	Variant VRF (%)	
AML-01	1		NA	NA	NA	NA	
AML-01	1	+	100	100	100	100	
AML-01	1/10		NA	NA	NA	NA	
AML-01	1/100		NA	NA	NA	NA	
AML-01	1/1000		NA	NA	NA	NA	
AML-01	1		NA	NA	NA	NA	
AML-01	1	+	100	100	100	100	
AML-01	1/10		NA	NA	NA	NA	
AML-01	1/100		NA	NA	NA	NA	
AML-01	1/1000		NA	NA	NA	NA	
AML-01	1		NA	NA	NA	NA	
AML-01	1	+	100	100	100	100	
AML-01	1/10		NA	NA	NA	NA	
AML-01	1/100		NA	NA	NA	NA	
AML-01	1/1000		NA	NA	NA	NA	
AML-01	1		NA	NA	NA	NA	
AML-01	1	+	100	100	100	100	

Measured allele frequencies are highly similar between the assays, with R<sup>2</sup> values of linear fit ranging from 0.77 to 0.98 (Figure 1). The lowest R<sup>2</sup> values are observed when comparing results in amplicon-based versus hybridization-based assays due to the difference in amplification bias between the two methods. A study conducted with an additional 31 AML samples indicated excellent linear fit between the CE-based assay and the NGS-amplicon based assay, with an R<sup>2</sup> value of 0.98 (Figure 2).

Figure 3. Concordance between methods (R<sup>2</sup>)

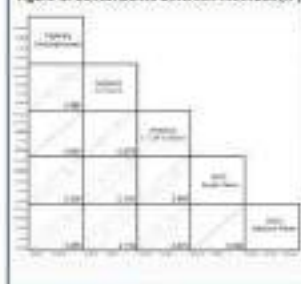
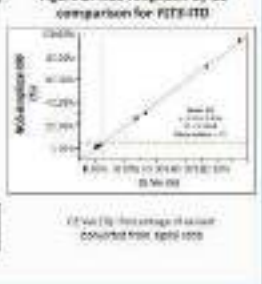


Figure 2. NGS-Amplicon by CE comparison for FUT3-ITD



### Results: Single Nucleotide Variant Detection

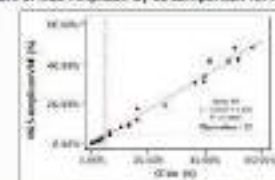
We compared performance across the three assays of a single nucleotide variant in the tyrosine kinase domain of FUT3 (FUT3-TKD). Two clinical samples were positive for the mutation (as determined by CE) and were detected by all three assays (Table 3). As with the other variants, NGS-panel assays detected lower frequency variants that were not detected by the CE assay at frequencies of 10<sup>-4</sup> and 10<sup>-5</sup>.

Table 3. Variant: FUT3-TKD

Sample Name	Dilution	CE Data		NGS Amplicon		NGS Panel	
		Call	Variant VRF (%)	Variant VRF (%)	Variant VRF (%)		
AML-01	1		NA	NA	NA		
AML-01	1	+	100	100	100		
AML-01	1/10		NA	NA	NA		
AML-01	1/100		NA	NA	NA		
AML-01	1/1000		NA	NA	NA		
AML-01	1		NA	NA	NA		
AML-01	1	+	100	100	100		
AML-01	1/10		NA	NA	NA		
AML-01	1/100		NA	NA	NA		
AML-01	1/1000		NA	NA	NA		
AML-01	1		NA	NA	NA		
AML-01	1	+	100	100	100		
AML-01	1/10		NA	NA	NA		
AML-01	1/100		NA	NA	NA		
AML-01	1/1000		NA	NA	NA		
AML-01	1		NA	NA	NA		
AML-01	1	+	100	100	100		

Additionally, 30 clinical AML samples with varying TKD variant allele frequencies (20-58%), as detected by the CE-based assay were tested using the NGS-amplicon based FUT3-TKD assay. The NGS assay correlates with excellent linearity when compared with the CE assay (Figure 3).

Figure 3. NGS-Amplicon by CE comparison for FUT3-TKD



### Conclusions

The ever-changing landscape of drug and molecular diagnostic development requires the diligent application of both cost-effective and scale-appropriate assays. Invivoscribe's assays offer excellent detection and frequency concordance of clinically actionable variants in the set of AML clinical samples, demonstrating reliable variant analysis across assays of different methodologies (CE and NGS) and sensitivities. These assays could be used interchangeably as required for different stages of drug development or clinical treatments. Coupling flexibility of scale with the associated differences in cost and specimen requirements with reliability of detection is of critical importance for the evaluation and incorporation of patient outcomes and personalized medicine.





## Validation of the LeukoStrat® CDx FLT3 Mutation Assay: Used to Detect both Internal Tandem Duplication (ITD) and Tyrosine Kinase Domain (TKD) Mutations and Response to Midostaurin in 1058 Patients with AML

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<sup>1</sup>Invivoscribe, San Diego, USA; <sup>2</sup>LabPMM, LLC, San Diego, CA; <sup>3</sup>LabPMM GmbH, Martinsried, Germany; and <sup>4</sup>LabPMM GK, Kawasaki, Kanagawa, Japan

### Abstract

**Introduction:** Acute myeloid leukemia (AML) in general has a poor prognosis, but AML patients with mutations in the tyrosine kinase kinase 3 (FLT3) have a particularly poor prognosis. An ITD and TKD FLT3 mutation result in constitutive autoactivation and activation of FLT3 - an important driver mutation in AML. The LeukoStrat® CDx assay mutation assay (LSDx) accurately identifies the FLT3 coding regions of the FLT3 gene to detect internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations to identify patients for whom midostaurin treatment might be beneficial. It is the only commercially available digital real-time PCR assay to detect FLT3 ITD and TKD mutations.

**Methods:** Myeloid leukemic cells were isolated from peripheral blood or bone marrow aspirate by a proprietary method. DNA was extracted and amplified by PCR. PCR amplicons were enzymatically digested and along with the H16 amplicon were analyzed by capillary electrophoresis on a validated ABI 3130XL instrument (Applied Biosystems). The assay measured the mutant:WT ratio digital using the "LeukoStrat" analysis software. ACD Mutation Analysis Software (AMAS) developed by Invivoscribe calculated the signal ratio (SR) for both ITD and TKD and reported "Positive" or "Negative" for each sample result or "Not" for invalid results. Invalid results were generated when fluid did not meet quality criteria for analysis with AMAS. Whole-ATD mutations were detected. The results were reported as positive if the mutant:WT ratio signal ratio (SR) met or exceeded the clinical cut-off of 0.05. A validated Roche 454 Next Generation DNA sequencing method (454 sequencing) was used for comparison in which DNA was amplified and the amplicons were sequenced, purified, quantified, sequenced and analyzed. Separately tested were collected from AML patients during the on-year treatment with the drug and from residual clinical samples tested at LabPMM in San Diego.

**Results:** The clinical performance of the CDx was evaluated using data from 1058 AML patient specimens from the ALLTRIAL trial. The ALLTRIAL trial used laboratory developed tests as clinical trial assays (CTAs), covering patients for enrollment at 4 different sites. The CDx vs CTAs concordance for FLT3 status was 91.2% for positive percent agreement (PPA), 92.5% negative percent agreement (NPA) and 92.5% for overall percent agreement (OPA). Further, the hazard ratio for the CDx vs CTAs population was 0.97 versus 0.77 for patients stratified using the CDx assay. CDx agreement to 454 sequencing for PPA and NPA was 91.0% and 95.0% respectively based on 156 patient results. Peripheral blood and bone marrow concordance for PPA and NPA was 90.4% indicating both specimen types can be used for patient diagnosis (peripheral blood). Additionally, clinical samples stored at 3 independent clinical LabPMM laboratories also in 3 countries showed 91.1% positive and negative percent agreement, and high concordance for signal ratios.

**Conclusion:** Overall, there was high concordance between the CDx, the Roche 454, and 454 sequencing with regard to ITD and TKD gene mutation detection. In addition, analysis of patients stratified as positive by the CDx led to a statistically significant increase in response to midostaurin relative to the CDx + CTAs based drug efficacy comparison with the CDx used for patient enrollment (HR 0.97 vs 0.77). The high reproducibility between the 5 LabPMM clinical laboratories, practices worldwide, and the CDx is a highly reproducible and reliable international harmonized tool for patient enrollment on multiple continents. It is currently being used to monitor a clinical phase III trial in patients with AML.

### Materials

- All reagents for the LeukoStrat® CDx FLT3 Mutation Assay are manufactured by either QIAGEN and/or Invivoscribe.
- AMAS is a computer program used to analyze the data generated by the LeukoStrat® CDx.
- AMAS was developed from 156 patient results collected during the ALLTRIAL trial.
- Statistical analysis was performed using SAS or other statistical software.

### Methods



QIAGEN and/or Invivoscribe are trademarks of QIAGEN, Inc.  
 Clinical trial analysis for the CDx FLT3 Mutation Assay was performed by LabPMM in San Diego, CA.  
 ACD Mutation Analysis Software for 454 Next Generation Sequencing was provided by SeqWright in Houston, TX.  
 The statistical program SAS was used to report the statistical analysis.

### Results

**Agreement Table Between CDx and CTAs Based on CDx Results**

Measure of Agreement	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	95% CI (%)	Percent Agreement (N)	95% CI (%)
PPA	91.2% (494/541)	(88.0%, 90.2%)	92.5% (494/532)	(88.4%, 96.5%)
NPA	92.5% (240/259)	(87.1%, 96.9%)	92.5% (240/259)	(88.0%, 96.9%)
OPA	90.7% (734/810)	(87.4%, 93.0%)	92.5% (1028/1100)	(88.1%, 96.2%)

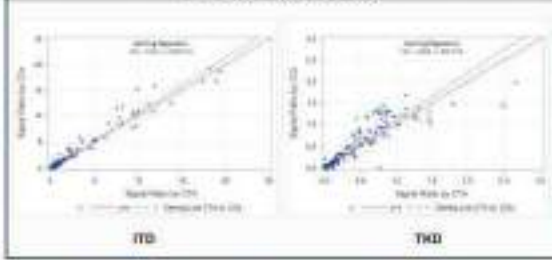
<sup>1</sup> The 95% CI was calculated using the exact Clopper-Pearson method. Invalid means that a sample was tested in the CDx assay but failed to return a valid result.

### Agreement Table Between CDx and 454 Sequencing

Measure of Agreement	Without CDx Invalid		With CDx Invalid	
	Percent Agreement (N)	95% CI (%)	Percent Agreement (N)	95% CI (%)
PPA	91.0% (147/161)	(87.0%, 94.9%)	92.0% (147/160)	(88.0%, 96.0%)
NPA	95.0% (230/242)	(91.0%, 98.0%)	95.0% (230/242)	(91.0%, 98.0%)
OPA	93.0% (377/403)	(89.0%, 96.0%)	93.5% (577/616)	(89.5%, 96.5%)

<sup>1</sup> The 95% CI was calculated using the exact Clopper-Pearson method. Invalid means that a sample was tested in the CDx assay but failed to return a valid result.

### Deming Regression Analysis of ITD and TKD Signal Ratios Between CDx and CTAs in the Overall Population (Samples with extremely high signal ratios were not displayed in order to focus on the spread of values around the Deming line.)

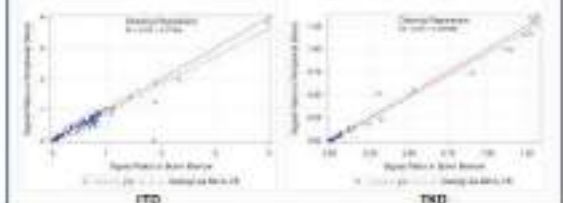


### Agreement Table Between Peripheral Blood and Bone Marrow in 33 FLT3 Positive Patients and 91 FLT3 Negative Patients

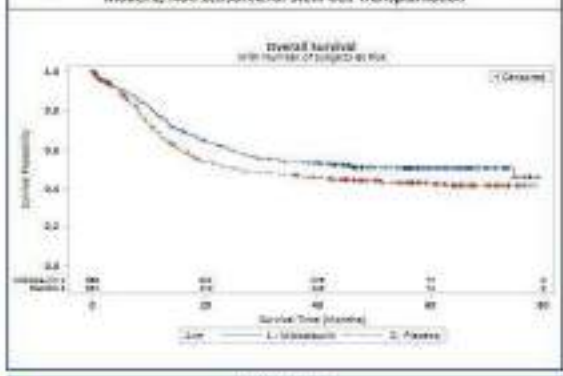
Measure of Agreement	Percent Agreement	95% CI (%)
ITD	95.2%	(89.2%, 100.0%)
TKD	95.2%	(89.2%, 100.0%)

<sup>1</sup> The 95% CI was calculated using a two-proportion binomial testing method.

### Deming Regression Analysis of ITD and TKD Signal Ratios Measured by the CDx Assay Between Peripheral Blood and Bone Marrow in the Overall Population (Samples with extremely high signal ratios were not displayed in order to focus on the spread of values around the Deming line.)



### Kaplan-Meier Curve for Overall Survival in the (CDx+, CDx-) Population - Model 2; Non-censored for Stem Cell Transplantation



### Conclusions

The study showed a high degree of concordance between the CDx and the clinical trial assay with superior predictive for midostaurin response by (CDx+, CDx-) patients. This assay is the only internationally harmonized PCR signal ratio assay whose performance has been defined and is being used in more than a dozen large scale international clinical trials.

AMP, November 16-18 2017, Salt Lake City, USA



## Clonality Detection Using Next-Generation Sequencing and Capillary Electrophoresis Methods in Suspect Lymphoproliferative Samples

H45

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<sup>1</sup>Invivoscribe, San Diego, USA, <sup>2</sup>Institute for Pathodiagnostik, Berlin, Germany

### Introduction

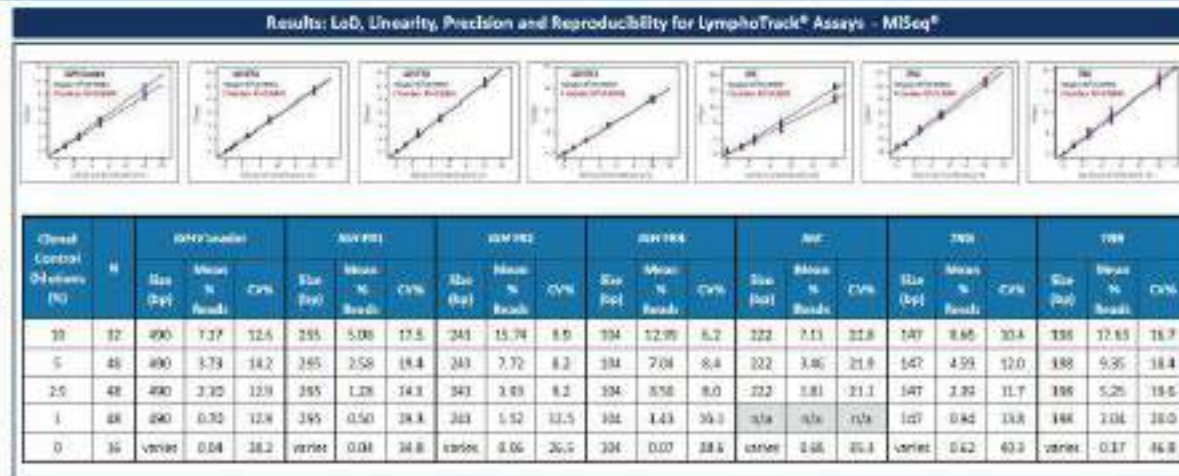
Assays used to detect clonal rearrangements within the immunoglobulin (Ig) and T-cell receptor (TCR) genes have long been used to assist in diagnosis of lymphoproliferative disease. Capillary electrophoresis (CE) based methods remain the gold standard in the majority of laboratories as they are cost effective high throughput assays. However, recently deep sequencing or next-generation sequencing (NGS)-based assays are gaining traction as they complement and supplement data from CE assays, providing both the prevalence of unique clonal Ig/TCR rearrangements as well as identifying the tumor-specific V-J DNA sequences necessary to track clonal sequence in highly sensitive residual disease testing. Here we report test results from 59 clinical samples using LymphoTrack<sup>®</sup> assays with accompanying bioinformatics using the MiSeq<sup>®</sup> and PGM<sup>™</sup> NGS platforms, as well as IdentClone<sup>®</sup> assays on the ThermoFisher<sup>®</sup> 3500 capillary platform.

### Materials and Methods

- LymphoTrack<sup>®</sup> NGS-based Assays for the MiSeq<sup>®</sup> (24 indices) and Ion PGM<sup>™</sup> (12 indices), and IdentClone<sup>®</sup> CE-based Assays were manufactured under cGMP standards and QC tested under a DSIB-compliant regulatory system prior to use.
- Limit of detection (LoD), linearity, precision and reproducibility (RPR) of the LymphoTrack<sup>®</sup> Assays - MiSeq<sup>®</sup> and PGM<sup>™</sup> were validated using clonal control DNA diluted in wild-type polyclonal (total) DNA. Only data from MiSeq<sup>®</sup> are presented here.
- DNA from a variety of samples (peripheral blood, bone marrow aspirates, and formalin-fixed paraffin-embedded (FFPE) were extracted using common extraction methods and tested by the LymphoTrack<sup>®</sup> Assays and IdentClone<sup>®</sup> Assays.
- Single step PCR amplification of 50 ng DNA input was followed by pooling of equimolar amounts of purified amplicons. These were then loaded on the sequencing machine. NGS libraries generated from each target locus were either sequenced alone or in combination with other targets.
- LymphoTrack<sup>®</sup> Software - MiSeq<sup>®</sup>, or LymphoTrack<sup>®</sup> Software - PGM<sup>™</sup> analyzed FASTQ data from the MiSeq<sup>®</sup> and the Ion PGM<sup>™</sup>, respectively.
- When comparing testing results, only samples that met the specimen and data acceptance criteria for both methods were evaluated.
- All statistical analyses were performed in JMP<sup>®</sup>.

### Conclusions

This study demonstrated that the comprehensive NGS LymphoTrack<sup>®</sup> Assays can be utilized for routine Ig/TCR clonality detection. Furthermore, the NGS assays can identify clonal V-J rearrangements and provide the clonal DNA sequences of the tumor-specific clonotypes required to perform follow up MRD testing in order to detect and track residual disease. Combining Ig/TCR assays within one NGS run can improve the overall clonality detection rate, reduce turnaround times in busy labs, and reduce the cost of NGS-based testing.



### Results: Clinical Study between LymphoTrack<sup>®</sup> - MiSeq<sup>®</sup>, LymphoTrack<sup>®</sup> - Ion PGM<sup>™</sup>, and IdentClone<sup>®</sup> Assays

	LymphoTrack <sup>®</sup> Assays - MiSeq <sup>®</sup>				LymphoTrack <sup>®</sup> Assays - PGM <sup>™</sup>		IdentClone <sup>®</sup> Assays			
	IGH/TRU2/3	IGH	TRG	TRB	IGH/TRU2/3	IGH	IGH/TCR A/B/C	IGH/TCR A/B	TRG 2.8	TRG/TCR A/B/C
Clonal (n)	25/24 (41%)	30/24 (54%)	14/20 (70%)	16/20 (80%)	25/24 (46%)	31/23 (78%)	26/24 (108%)	22/24 (92%)	25/24 (104%)	24/20 (120%)
Non-Clonal (n)	32/24 (54%)	35/20 (81%)	41/20 (105%)	41/20 (105%)	22/24 (92%)	23/23 (100%)	28/24 (117%)	32/24 (133%)	25/24 (104%)	28/20 (140%)

Assay	No. of Samples Met Criteria for Both Methods	No. of Samples with Indicated Result				Concordance (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		CI+/NGS <sup>+</sup>	CI+/NGS <sup>-</sup>	CI-/NGS <sup>+</sup>	CI-/NGS <sup>-</sup>					
MiSeq IGH/TRU2/3	44	24	1	1	38	95.5	99.0	94.7	96.0	94.3
MiSeq IGH	55	18	3	0	21	98.2	95.0	100	100	97.2
MiSeq TRG	52	14	2	1	14	94.1	87.5	97.1	93.1	94.4
MiSeq TRB	44	18	6	0	20	86.4	75.0	100	100	78.9
PGM IGH/TRU2/3	45	22	1	0	18	87.8	95.7	100	100	94.7
PGM IGH	49	15	0	0	14	100	100	100	100	100

<sup>+</sup>CI, IdentClone<sup>®</sup> Assays, capillary electrophoresis; NGS, LymphoTrack<sup>®</sup> Assays - MiSeq<sup>®</sup>; PPV, positive predictive value; NPV, negative predictive value.





**invivoscribe** #102

## Performance Evaluation of LymphoTrack® Clonality Assays on Ion PGM™ and Ion 55™ Platforms

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<sup>1</sup>Invivoscribe, Inc. and <sup>2</sup>LabProtek, LLC, San Diego, CA; <sup>3</sup>LabPMM, GmbH, Homburg, Germany

**Abstract**

The LymphoTrack® clonality assay is a highly sensitive and specific method for identifying clonal B-cell populations in peripheral blood. The assay is based on the detection of clonal rearrangements in immunoglobulin genes using a multiplexed, bead-based, flow cytometry-based assay. The assay is performed on the Ion PGM™ and Ion 55™ platforms. The assay is highly sensitive and specific, and is able to detect clonal populations at very low concentrations. The assay is also highly reproducible and can be used for the detection of clonal populations in a wide range of samples. The assay is a highly sensitive and specific method for identifying clonal B-cell populations in peripheral blood. The assay is based on the detection of clonal rearrangements in immunoglobulin genes using a multiplexed, bead-based, flow cytometry-based assay. The assay is performed on the Ion PGM™ and Ion 55™ platforms. The assay is highly sensitive and specific, and is able to detect clonal populations at very low concentrations. The assay is also highly reproducible and can be used for the detection of clonal populations in a wide range of samples.

**Methods and Materials**

The LymphoTrack® clonality assay was performed on the Ion PGM™ and Ion 55™ platforms. The assay is based on the detection of clonal rearrangements in immunoglobulin genes using a multiplexed, bead-based, flow cytometry-based assay. The assay is performed on the Ion PGM™ and Ion 55™ platforms. The assay is highly sensitive and specific, and is able to detect clonal populations at very low concentrations. The assay is also highly reproducible and can be used for the detection of clonal populations in a wide range of samples.

**Results: Clonality and OT2/55 Comparison**

**Results: Single and 3 targets as clonality**

**Results: OT2/PGM and clonality for clinical samples\***

Sample	OT2/PGM	Clonality	OT2/55	Clonality	OT2/55	Clonality	OT2/55	Clonality
1	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
5	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100
7	100	100	100	100	100	100	100	100
8	100	100	100	100	100	100	100	100
9	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	100
11	100	100	100	100	100	100	100	100
12	100	100	100	100	100	100	100	100
13	100	100	100	100	100	100	100	100
14	100	100	100	100	100	100	100	100
15	100	100	100	100	100	100	100	100
16	100	100	100	100	100	100	100	100
17	100	100	100	100	100	100	100	100
18	100	100	100	100	100	100	100	100
19	100	100	100	100	100	100	100	100
20	100	100	100	100	100	100	100	100

**Run Time and Cost Comparison**

Platform	Run Time	Cost
Ion PGM™	10 min	\$100
Ion 55™	10 min	\$100

**Conclusions**

The LymphoTrack® clonality assay is a highly sensitive and specific method for identifying clonal B-cell populations in peripheral blood. The assay is based on the detection of clonal rearrangements in immunoglobulin genes using a multiplexed, bead-based, flow cytometry-based assay. The assay is performed on the Ion PGM™ and Ion 55™ platforms. The assay is highly sensitive and specific, and is able to detect clonal populations at very low concentrations. The assay is also highly reproducible and can be used for the detection of clonal populations in a wide range of samples.

AMP Europe 2018, April 30 - May 2, Rotterdam, Netherlands





**CELL-FREE DNA MONITORING OF MINIMAL RESIDUAL DISEASE IN AML USING A TARGETED NGS GENE PANEL**

P5994

**invivoscribe**

Shiyi Xie, Lisa Chamberlain\*, Andrew Carlson, Valerie McClain, Ogeon Kiva, Bradley Patsy, Martin Blankford, Timothy Stenzel, Jeffrey Miller  
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### Background

We have developed a sensitive 1000 gene panel (MyMRD<sup>®</sup> Research Use Only), which identifies pathways in acute myeloid leukemia (AML) masking remission status. This panel targets mutation hotspots in 23 genes associated with AML, it identifies driver mutations that cause relapse in >90% of all AML patients, as well as common drivers in other myeloid neoplasms (MPPN) and myeloid/plastic syndromes (MPS). We have leveraged data from literature that suggest cell-free DNA (cfDNA) isolated from blood plasma of cancer patients contains non-fragmented DNA fragments with a molecular profile similar to that of bone-marrow tumor cells, and that DNA obtained from plasma provided a more accurate assessment of disease burden than using circulating leukocytes, and results correlated with disease burden. Therefore we set out to investigate whether the MyMRD<sup>®</sup> assay, originally developed for genomic DNA analysis, could be applied to cfDNA to assess mutations at a level comparable to testing of genomic DNA and developed the MyMRD<sup>®</sup> cfDNA assay for characterization and residual disease monitoring of targeted variants.

### Results: LOD and Linearity

A linearity panel was generated by diluting DNA from 8 cell lines containing known variants into a background of genome in a k562 (NA12878) DNA from 20% to 0.2%. Covered samples were then diluted to generate fragments of approximately 300 bp to mimic cfDNA. Samples were sequenced to an average depth of 307X. Expected variant allele frequency (VAF) is plotted against observed variant read frequency (VRF) in Figure 3. R<sup>2</sup> values and linear fit equations are listed in Table 2.

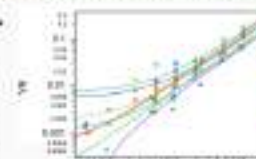


Figure 3: Linearity plot showing VRF vs Observed VAF for various cell lines and variants. The plot shows a strong positive linear correlation between observed and expected variant allele frequencies.

### Results: Linearity of SeraCare Samples

Linearity data for SeraCare samples is presented in Figure 4 and Table 5. VAFs from samples with expected VAFs of 0.125%, 0.25%, 0.5%, 1%, 2%, and 2% are plotted for all targeted variants. Linearity is highly dependent on the particular variant, with R<sup>2</sup> values ranging from 0.888 to 0.988.

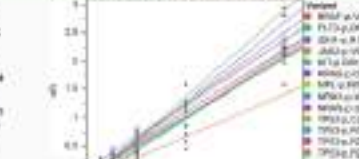


Figure 4: Linearity plot for SeraCare samples showing VRF vs Observed VAF. The plot shows a strong positive linear correlation between observed and expected variant allele frequencies for various variants.

### Methods

Cell-free DNA was extracted from fresh, frozen, and synthetic plasma using the Qiasymphony GigaPrep kit (Qiagen, Hilden, Germany). To assess the limitations of testing cfDNA, DNA fragments with size profiles similar to cfDNA were generated using cell free DNA for minor readability studies during assay development. Genomic DNA was fragmented by sonication and the DNA fragments were also selected using bead based methods (final mean size ~200bp). Whole genome libraries, generated from cfDNA and DNA fragments, were hybridized with MyMRD<sup>®</sup> probes to generate targeted sequencing libraries.

Sequenced libraries were sequenced using Illumina platforms. Sequencing data was analyzed using proprietary InvivoSeq by Informatics<sup>™</sup> software.

### Results: cfDNA Preparation

DNA from plasma, synthetic plasma, and thawed cell line DNA was analyzed for concentration and size. DNA concentrations from extracted synthetic plasma were significantly higher than DNA concentrations from extracted healthy donor plasma (p < 0.001, Figure 1). Additionally, we have found that cfDNA from clinical AML samples is significantly higher in concentration than cfDNA from healthy donor plasma (data not shown). DNA from these sources have a narrow average size range, but are significantly different. Thawed cell line DNA had the smallest average size, followed by healthy donor cfDNA, followed by synthetic plasma DNA (p < 0.05 and < 0.001, respectively, Figure 2). Data is summarized in Table 3, which also includes sample biotinyler traces of cfDNA preparations.

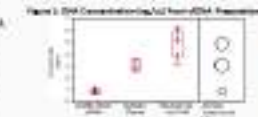


Figure 1: Box plot of DNA concentration for different samples. The plot shows that synthetic plasma has a significantly higher DNA concentration compared to healthy donor plasma.

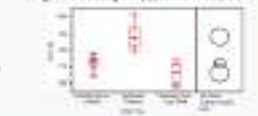


Figure 2: Box plot of DNA size for different samples. The plot shows that synthetic plasma DNA has a significantly larger size compared to healthy donor cfDNA and thawed cell line DNA.

Preparation	Concentration (ng/μl)	Average Size (bp)	Molecular Weight (kbp)
Healthy Donor	~20	~150	~0.1
Healthy Donor cfDNA	~100	~120	~0.1
Synthetic Plasma	~170	~200	~0.1

### Results: Variant Detection of SeraCare samples

Synthetic plasma was purchased from SeraCare (Milford, MA, USA) and DNA was extracted using the same methods developed for human plasma. Samples were tested with the MyMRD<sup>®</sup> cfDNA assay. VAF data is presented in Table 4. While most variants from these samples are not detected in the wild type sample, some KRAS, UTR1, and some TP53 variant all exhibit background reads indicating that residual coverage will affect the potential LOD for this assay, particularly for certain variants.

Variant	Observed VAF	Expected VAF	R <sup>2</sup>	Linear Fit Equation
KRAS	~0.125%	~0.125%	~0.95	y = 0.95x + 0.01
UTR1	~0.25%	~0.25%	~0.92	y = 0.92x + 0.02
TP53	~0.5%	~0.5%	~0.88	y = 0.88x + 0.03

### Results: Depth of Coverage of Synthetic Plasma Samples

Coverage data of sequencing reads for synthetic plasma samples is presented in Figure 5 and Table 6. These samples were sequenced much deeper than those utilized for LOD studies and employed proprietary molecular barcodes to generate libraries to correct for background coverage depth after removing duplicates (Pindel). Overall coverage data indicates the potential for LOD values of 0.1% for 90% of bases, however background reads in wildtype samples (Table 4) demonstrate that this LOD is not global across the panel.

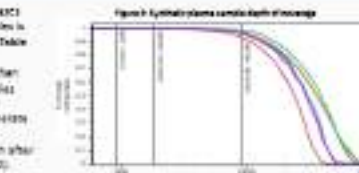


Figure 5: Coverage plot for synthetic plasma samples showing depth vs distance. The plot shows that synthetic plasma samples are sequenced much deeper than those utilized for LOD studies.

Sample	Depth (x)	Coverage (%)	Distance (kb)
Sample 1	~1000	~90%	~0.1
Sample 2	~2000	~95%	~0.1
Sample 3	~3000	~98%	~0.1

### Conclusions

The MyMRD<sup>®</sup> gene panel is a sensitive, reliable assay that provides monitoring of residual disease using cfDNA. The assay is shown to detect clinically important driver variants and has excellent linearity and LOD for targeted variant sites. This assay can potentially replace invasive BM sampling and provide an alternative route for longitudinal genetic monitoring of patients receiving targeted therapy. Additionally, higher sensitivity can be obtained through deeper sequencing, but is limited by background noise at certain sites.

EUROPEAN HEMATOLOGY ASSOCIATION, JUNE 16-17, 2018, STOCKHOLM, SWEDEN - INVIVOSCRIBE BOOTH #112

Improving Lives with Precision Diagnostics<sup>™</sup>

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## Comparison of LymphoTrack® Assays - MiSeq® and Flow Cytometry for Clonality and Minimum Residual Disease Assessment in Multiple Myeloma

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### Background

Multiple myeloma (MM), characterized by presence of clonal plasma cells (PC) in bone marrow (BM), accounts for about 10% of all hematological malignancies. Multiparameter flow cytometry (MFC) is a standard tool used to detect and monitor MM in patients. PCR-based NGS methods have been developed to identify patient-specific gene rearrangements (clonotype) within the immunoglobulin (Ig) loci, and recently international organizations such as IMiG, IMWG, and COMO have included NGS as an recommended tool for MRD assessment in MM. We developed NGS-based LymphoTrack Assays and bioinformatics software to detect and track clonal rearrangements in B cell malignancies using the Illumina MiSeq platform. Here we compare the ability of the LymphoTrack IGH FRL, IGH FR2, IGH FR3, and IGH Assays to identify and track patient specific clonotypes with detection and monitoring by MFC on 301 anonymized, paired diagnostic and MRD MM specimens.

### Results: Diagnostic Samples by LymphoTrack Assays - MiSeq

Assay	Clonal (%)	Non-Clonal (%)	Indefinite (%)
IGH FRL	84/100 (84.0%)	15/100 (15.0%)	2/100 (2.0%)
IGH FR2	66/100 (66.0%)	21/100 (21.0%)	13/100 (13.0%)
IGH FR3	61/100 (61.0%)	30/100 (30.0%)	9/100 (9.0%)
IGH Assay	87/100 (87.0%)	14/100 (14.0%)	0/100 (0.0%)

### Results: MRD Samples by LymphoTrack IGH FRL Assay - MiSeq

MRD Samples (including 170mg DNA)	Flow Cytometry		
LymphoTrack IGH FRL Assay	Positive	Detected	Not Detected
	Detected	24	4
	Not Detected	3	17

Agreement: **85.4%**  
 Sensitivity: **88.0%**  
 Specificity: **81.0%**

### Methods

- Schematic illustration of the IGH gene
- Schematic illustration of the IGH FRL gene
- The workflow for the LymphoTrack IGH FRL, IGH FR2, IGH FR3, and IGH Assays - MiSeq
- The LymphoTrack IGH FRL, IGH FR2, IGH FR3, and IGH Assays for the MiSeq were manufactured using cGMP standards per an ISO 13485 certified ODM. Each assay consists of a one-step PCR Master Mix with 24 different indices to allow the simultaneous testing of multiple samples and targets on the same MiSeq flow cell.
- A complementary bioinformatics software package (LymphoTrack De Software - MiSeq & LymphoTrack MRD Software (MFC)) were developed and validated under ISO15189 flags control.
- 301 paired (diagnostic and MRD) BM samples from MM subjects were tested by MFC utilizing 8-color direct immunofluorescence to diagnose.
- Genomic DNA was extracted from the same BM samples, anonymized, and blocked for binding with the LymphoTrack IGH FRL, IGH FR2, IGH FR3 and IGH Assays - MiSeq.
- DNA from diagnostic samples were tested using 50ng of DNA for each LymphoTrack assay (IGH FRL, IGH FR2, IGH FR3 and IGH Assay) to identify sample specific clonotype, which was then tracked using a single assay in MRD samples. Libraries from diagnostic samples were enriched with 4 targets combined together.
- DNA from 88 MRD samples, which paired diagnostic samples were IGH FR3 positive, were tested using 100ng of DNA (or highest amount available) to the LymphoTrack IGH FRL assays.
- LymphoQuant™ control DNA from clonal cells was added to each PCR reaction at 1,000 cell equivalency when testing these MRD samples to allow the estimation of cell equivalent within each MRD sample tested.
- MiSeq FASTQ files from diagnostic and MRD samples were analyzed by the LymphoTrack De Software - MiSeq and LymphoTrack MRD Software (MFC) respectively.

### Results: MRD Samples by LymphoTrack IGH FRL Assay - MiSeq

### Conclusions

- The combination of LymphoTrack IGH FRL, IGH FR2, IGH FR3 and IGH Assays - MiSeq was able to detect clonotype sequences in 100% of diagnostic samples from MM subjects.
- The LymphoTrack IGH FRL Assay - MiSeq, by itself, achieved 85.4% agreement with MFC in detecting paired MRD samples from MM subjects.
- With the LymphoTrack De Assays, the same reagents and workflow were utilized for both initial clonality testing and for tracking of clonal populations in MM samples.
- Unlike MFC assays, the LymphoTrack Assays and accompanying bioinformatics software can be submitted for approval to regulatory authorities worldwide.





## Detection of Clonal Rearrangements in Multiple Myeloma Samples using LymphoTrack® Assays H045

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### Introduction

Multiple myeloma (MM) is a malignancy of plasma cells. Currently, multiparameter flow cytometry (MFC) is the tool most often used to detect and monitor MM in patients. However, MFC requires fresh specimens and is difficult to standardize between test centers. Since genomic DNA is stable and the assays, platforms, and the accompanying software can be easily standardized between test centers, we developed next-generation sequencing (NGS)-based assays with bioinformatics software to detect the clonal Ig rearrangements associated with MM and track Minimal Residual Disease (MRD). Here we report the results of a pilot study of clonal rearrangement detection and tracking by testing 155 anonymized/blinded MM specimens using LymphoTrack Assays. These assays identify B-cell populations by targeting the IGHV leader, IGH FR1, FR2, FR3, and IGH. Thirty (30) follow-up samples were also tested to demonstrate MRD utility.

### Materials and Methods

- Five (5) LymphoTrack® Assays (IGHV Leader, IGH FR1, IGH FR2, IGH FR3 and IGH) for the MiSeq<sup>®</sup>, each with available with 24 indices, were designed, developed, and manufactured per cGMP, then QC tested and validated under a QSR-compliant regulatory system.
- 50 ng of genomic DNA (only ~7,700 cell equivalents) from 155 MM bone marrow (BM) baseline specimens were procured, anonymized and blinded prior to testing with the four LymphoTrack Assays (IGH FR1, FR2, FR3 and IGH) using the MiSeq<sup>®</sup> platform.
- Libraries generated from all 4 assays were purified, harmonized, pooled, and sequenced in a single MiSeq run. Fourteen (14) samples that tested negative by the above 4 assays were reflex tested with the 5<sup>th</sup> LymphoTrack Assay, IGHV Leader.
- The most prominent clonal rearrangement sequence identified by one of the LymphoTrack Assays was then tracked using the single assay.
- 200 ng DNA (~30,000 cells), typically from "last pull" aspirates with low tumor cell content were tested from 30 subsequent residual follow up specimens.
- In order to generate an estimation of lymphoid cell equivalents within each specimen LymphoQuant<sup>™</sup> internal control (IC; 100 cell equivalents) was added to each PCR reaction in follow up specimens.
- LymphoTrack<sup>™</sup> software (MiSeq<sup>®</sup>) and LymphoTrack MRD software were used to analyze the sequencing results from baseline and follow up samples, respectively.

### Conclusions

- Despite testing baseline specimens which were generally from "last pull" aspirates with low tumor cell content (50ng ~ 7,700 cell equivalents), LymphoTrack Assays were shown to detect clonality in about 80% of MM baseline research specimens.
- Despite testing only 200 ng DNA (~ 30,000 cells), typically from "last pull" aspirates with low tumor cell content, the same LymphoTrack Assays and testing procedure were able to track the clonal sequences in 87% of MM follow up specimens.
- The presence of LymphoQuant IC within each PCR reaction allowed the % clonal cells within each specimen to be estimated.
- LymphoTrack Assays can be potentially useful tools to identify and monitor disease status in MM samples at both diagnostic and subsequent time points throughout the course of treatment.
- Unlike MFC, the LymphoTrack Assays and accompanying bioinformatics software can be easily standardized between laboratories and submitted for approval to regulatory authorities worldwide.

### Results: Using LymphoTrack® Assays - MiSeq® to Detect Clonality in Baseline MM Samples

Total # (MM)	IGH FR1	IGH FR2	IGH FR3	IGH	Overall (IGH FR1/FR2/FR3 + IGH)
C (Clonal)	88/155 (56.8%)	81/155 (52.0%)	80/152 (48.8%)	104/152 (54.9%)	152/163 (78.8%)
NC (Non-clonal)	83/155 (53.2%)	70/155 (45.2%)	70/153 (45.8%)	75/153 (49.0%)	54/255 (21.2%)
I (Invalid)	22/155 (14.0%)	3/155 (1.9%)	2/153 (1.3%)	5/153 (3.3%)	7/155 (4.5%)

### Results: Using LymphoTrack® Assays - MiSeq® to Track Sequences in Follow up MM Samples

Follow up sample	Assay	MRD Results	% clonal cells in sample	LymphoQuant frequency
Sample1_TF1	FR2	Detected	0.144	1.81E-01
Sample1_TF2	FR2	Detected	1.87%	2.81E-01
Sample1_TF3	FR2	Detected	1.42%	2.89E-01
Sample1_TF4	FR2	Detected	0.081	1.14E-01
Sample2_TF1	FR2	Detected	0.004	1.21E-01
Sample2_TF2	FR2	Detected	0.003	1.14E-01
Sample3_TF1	IGH	Detected	0.001	2.81E-01
Sample3_TF2	IGH	Detected	0.001	1.71E-01
Sample3_TF3	FR3	Not Detected	0.000	7.31E-01
Sample3_TF4	IGH	Detected	1.717	1.17E-01
Sample5_TF1	IGH	Detected	0.003	1.44E-01
Sample5_TF2	IGH	Detected	0.001	2.15E-01
Sample5_TF3	IGH	Detected	0.003	7.39E-01
Sample5_TF4	IGH	Detected	0.004	9.63E-01
Sample5_TF5	IGH	Detected	0.010	1.05E-01
Sample5_TF6	IGH	Detected	0.002	1.22E-01
Sample7_TF1	FR3	Detected	2.77%	8.30E-01
Sample7_TF2	FR3	Detected	0.144	1.39E-01
Sample7_TF3	FR3	Detected	0.001	4.14E-01
Sample7_TF4	FR3	Detected	0.044	9.97E-01
Sample7_TF5	FR3	Detected	0.070	1.77E-01
Sample8_TF4	FR3	Detected	0.001	1.55E-01
Sample8_TF5	FR3	Detected	0.001	1.38E-01
Sample8_TF6	FR3	Detected	24.22%	7.04E-01
Sample9_TF1	FR2	Detected	1.130	1.10E-01
Sample9_TF2	FR2	Detected	0.003	2.87E-01
Sample10_TF1	FR3	Not Detected	0.000	2.81E-01
Sample10_TF2	FR3	Detected	0.001	1.31E-01
Sample10_TF3	FR3	Not Detected	0.000	8.33E-01
Sample12_TF1	FR3	Not Detected	0.000	2.46E-01

Overall detecting samples: 20/30 (67%)



AMP, November 1-3 2018, San Antonio, TX, USA



## Minimal Residual Disease in AML can be Monitored Utilizing Cell-Free DNA

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H029

### Introduction

Cell-free DNA (cfDNA) isolated from plasma can be a source of tumor DNA and has immense potential for use in tracking disease states. Though our MyMRD assay was developed for use with genomic DNA from whole blood, here we demonstrate its utility using cfDNA as the specimen type. At least one study has demonstrated that measuring the molecular disease burden in Myeloid leukemia patient specimens in plasma is higher compared with measuring with [1] and that malignant circulating cells were observed in bone marrow (bone) [2]. Additionally, cfDNA can also contain mutations not observed in peripheral blood, bone, or bone marrow [3]. Thus, cfDNA has great potential for use in AML and other malignancies.

There are several advantages to a plasma vehicle blood for up to 72 hours prior to fixation into plasma, increasing the feasibility of using cfDNA as a specimen type. Additionally, to generate whole-genome libraries from cfDNA, while maintaining the integrity of the cfDNA. The optimal and subsequent hybridization of these whole genome libraries with our MyMRD probes to generate targeted libraries for sequencing also maintains cfDNA, can range. The ability to track mutations in cfDNA via at least 5.7x molecular load is demonstrated, including high utility of the methylation (M) in hematological malignancies.

### Results

#### II: Plasma preservation in two different preservation tube types

A comparison of two plasma preservation tubes was performed (Cedar 4 and Biorad 6), with plasma being processed at 2 hours and 48 hours, for significant differences in tubes was observed, indicating that either preservation tube can be used for this timeframe prior to plasma processing (Figure 2, Table 2).

Figure 2: cfDNA from preservation tubes is stable for up to 48 hours post draw in multiple tube types

Table 2: cfDNA from preservation tubes, is stable for up to 48 hours post draw in multiple tube types

Tube	Time	Mean	SD	Min	Max
Cedar 4	2h	12	12	0	60
	48h	4.21	0.39	0.25	6.32
Biorad 6	2h	8.68	0.21	0.12	9.34
	48h	11.86	11.45	0.16	61.33

### Results

#### V. MyMRD cfDNA Assay can detect WAF as low as 0.1%

Plasmas generated from cfDNA of 7 clinical samples were sequenced on MiSeq (Figure 3, blue lines), a high-coverage from cfDNA of 28 healthy donors were sequenced on NextSeq (Figure 3, red lines). First of 25ng cfDNA was used for library preparation with the addition of one sample (one) with only 20ng cfDNA available. The read coverage decreased significantly for this sample when comparing with samples with 25ng cfDNA input (Figure 3). The average read coverage for the cfDNA samples run on the NextSeq is summarized in Table 4. The required read coverage for successful library and sequenced as previous described in the figure 3. The MyMRD cfDNA assay result shows a WAF of 0.1% over 90% of the targeted regions, and a WAF of 0.1% over 87% of larger regions (Table 4). If the 20ng sample is included, that number drops to 85%. The data indicates that, with 25ng cfDNA input, the MyMRD cfDNA Assay could detect WAF as low as 0.1% in the majority of the targeted regions.

Figure 3: Sequencing depth for cfDNA samples using low sequencing vs high sequencing depth

Table 4: Read coverage for cfDNA samples with high sequencing depth

Sequenced ID	Read Depth (copies)	Percentage of Targeted Regions with Read Depth		
		100x	20x	50x
0.1%	9111	93.9%	18.6%	30.0%
0.2%	9993	95.9%	18.3%	4.0%
0.5%	8825	97.0%	1.8%	1.0%
1%	823	98.9%	0.2%	0.0%
2.5%	94	100.0%	0.0%	0.0%
5%	23	100.0%	0.0%	0.0%

### Materials & Methods

cfDNA collection, plasma processing (fractionation of plasma from blood), and extraction of cfDNA from plasma were optimized prior to processing samples from patients and healthy donors. All cfDNA samples were processed within 2 hours of blood draw with the samples stored in plasma preservation tubes were stored for indicated time periods prior to plasma processing. cfDNA concentrations were assessed using fluorometric methods. DNA fragments with sizes similar to cfDNA were generated for most libraries and library studies. Genomic DNA was fragmented by sonication and extracted for a more at 0.1-100ng. Whole genome libraries generated from cfDNA and genomic DNA, were hybridized with MyMRD probes. Targeted libraries were amplified using Illumina adapters. Sequencing data was analyzed using Invivoscribe's MyMRD software.

### Results

#### I: Plasma preservation for up to 72 hours

cfDNA is easily contaminated by cellular DNA, thus typical methods require fractionation of whole blood from EDTA tubes into plasma within 2 hours of blood draw. A 2-hour time limit on plasma processing is difficult to accomplish in the real world, thus we worked with plasma preservation tubes from Biorad A to explore their ability to maintain cfDNA over time. Whole blood from 10 healthy donors was drawn into 4 tubes, one EDTA and 3 Biorad A plasma preservation tubes. Plasma processed from blood stored in Biorad A tubes maintained a full plasma interface for the duration of the experiment. However, plasma at later time points was visually abnormal to have genomic DNA contamination to plasma from EDTA tubes processed within 2 hours of blood draw (Figure 1). This hemolysis effect (seen as a red tint in plasma at 48h plasma) is not observed in the subject and is not expected to affect cfDNA concentrations or future read frequencies.

cfDNA concentrations from plasma derived from Cedar 4 (2h) and Biorad 6 tubes at multiple time points (2, 24, 48 hours) exhibit no significant increase over time, indicating that the tubes are maintaining cellular structures and helping cfDNA and genomic DNA stay intact (Figure 2, Table 2).

Figure 2: cfDNA from preservation tubes is stable for up to 48 hours post draw

Table 2: cfDNA from preservation tubes, is stable for up to 48 hours post draw

Tube	Time	Mean	SD	Min	Max
Cedar 4	2h	12	12	0	60
	48h	4.21	0.39	0.25	6.32
Biorad 6	2h	8.68	0.21	0.12	9.34
	48h	11.86	11.45	0.16	61.33

#### III: Sequencing Library Generation from cfDNA

cfDNA extracted from plasma displayed a typical normal peak at ~150 to and following (n) and (n)- nucleotides (n) (ng) in WAF. Whole-genome libraries were generated using 30-50 ng of cfDNA input (Figure 4), these libraries exhibit similar structure to the reference DNA. Libraries were then hybridized to MyMRD probes. An example of a typical trace of a cfDNA final library is shown below in Figure 4. As seen in whole-genome libraries, mononucleotides, n- and (n)- nucleotides peaks were retained from cfDNA extracted through leaf from genomic.

Figure 4: cfDNA extracted from plasma, whole genomic libraries, and final libraries all exhibit typical characteristic peaks of cfDNA

Figure 4: cfDNA extracted from plasma, whole genomic libraries, and final libraries all exhibit typical characteristic peaks of cfDNA

Figure 4: cfDNA extracted from plasma, whole genomic libraries, and final libraries all exhibit typical characteristic peaks of cfDNA

#### IV. Excellent Linearity of the MyMRD cfDNA Assay

A library panel was generated by diluting cfDNA from 1 cell line containing known variants into a background of genome at a 0.01% (1:10,000) ratio from 0.1% to 0.01% (100-fold dilution) were then sequenced 30 genomic fragments of approximately 100 bp to mimic cfDNA. Samples were assessed to an average depth of 1173. Representative variants with expected variant allele frequency (VAF) is plotted against observed variant read frequency (VRF) in Figure 5. R values are listed in Table 3.

Figure 5: Expected variants demonstrate excellent linearity using cell free DNA stored in cfDNA vials

Table 3: Expected variants demonstrate excellent linearity using cell free DNA stored in cfDNA vials

Gene	Variant Type	R
DNMT3A G246A	SNP	0.976
FLT3L G484A	SNP	0.990
FLT3L G484A	indel	0.984
NRAS G12S	indel	0.981
TP53 G94C	indel	0.999

### Conclusions

The MyMRD cfDNA assay was designed to detect at least one clinically actionable driver variant in 95% of all AML patients for purposes for real-time clinical studies. Plasma containing cfDNA can be processed for up to 72 hours, providing the possibility for clinical application. Higher sensitivity as low as 0.1% WAF can be achieved through deeper sequencing with error correction utilizing whole-molecule libraries and family formation. This assay can potentially replace invasive BM sampling and provide an alternative assessment for monitoring of patients making logistical changes. The MyMRD assay is currently available for research use only (RUO) and will be available as a CDx/CDP regulated assay to LOPMM LLC starting in 2019.

### References and Acknowledgements

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P032

## Error Rate Normalization to Establish Position-Specific LoB & LoD in Next Generation Sequencing Assays

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### Introduction

Next generation sequencing (NGS) continues to be the method of choice for high throughput genotyping. This assay enables a uniform error rate per base (ERPB) level of detection (LoB) and limits of detection (LoD) for every individual due to "background" errors and errors due to PCR, sequencing and alignment errors. Also, the process of aligning sequencing artifacts (indels, structural variants) is often complicated for clinical applications. Next generation sequencing (NGS) assays are often used for clinical applications. The ability to establish a LoB and LoD for every individual is essential for clinical applications. The ability to establish a LoB and LoD for every individual is essential for clinical applications. The ability to establish a LoB and LoD for every individual is essential for clinical applications.

### Materials and Methods

Sequenced data from clinical assays was used for this analysis. The analysis was performed using Invivoscribe's internal software. The analysis was performed using Invivoscribe's internal software. The analysis was performed using Invivoscribe's internal software. The analysis was performed using Invivoscribe's internal software.

### RESULTS

**Figure 1: Error Rate Normalization by Site**

Figure 1. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

**Figure 2: Error Rate Normalization by Site**

Figure 2. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

### RESULTS

**Figure 3: Error Rate Normalization by Site**

Figure 3. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

**Figure 4: Error Rate Normalization by Site**

Figure 4. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

### RESULTS

**Figure 5: Error Rate Normalization by Site**

Figure 5. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

**Figure 6: Error Rate Normalization by Site**

Figure 6. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

### RESULTS

**Figure 7: Error Rate Normalization by Site**

Figure 7. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

**Figure 8: Error Rate Normalization by Site**

Figure 8. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

### RESULTS

**Figure 9: Error Rate Normalization by Site**

Figure 9. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

**Figure 10: Error Rate Normalization by Site**

Figure 10. The plot shows the error rate (ERPB) for each site (A, C, G, T) across different samples. The error rate is normalized to a consistent level across all sites, demonstrating the effectiveness of the normalization process.

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P118

## LymphoTrack® Low Positive Control and LymphoQuant® Internal Control for MiSeq® and Ion S5/PGM™ LymphoTrack Assays

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### Introduction

LymphoTrack assays with the associated lymphoTrack software have been demonstrated to be highly accurate and precise for quantifying lymphocyte subsets. However, the internal control (LQIC) used in these assays is not a true lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled. The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled. The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled.

### Materials and Methods

This study was designed to evaluate the performance of the LQIC in the LymphoTrack assays. The LQIC was compared to the lymphocyte subsets and the results were compared to the LymphoTrack software. The LQIC was compared to the lymphocyte subsets and the results were compared to the LymphoTrack software. The LQIC was compared to the lymphocyte subsets and the results were compared to the LymphoTrack software.

### Principle of LymphoQuant Internal Control (LQIC)

The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled. The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled.

### Results: Inter and Intra Assay Variability for LQIC

Assay	Run	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
LymphoTrack	1	100	1.5	100	1.5	100	1.5	100	1.5
	2	100	1.5	100	1.5	100	1.5	100	1.5
	3	100	1.5	100	1.5	100	1.5	100	1.5
	4	100	1.5	100	1.5	100	1.5	100	1.5
LymphoQuant	1	100	1.5	100	1.5	100	1.5	100	1.5
	2	100	1.5	100	1.5	100	1.5	100	1.5
	3	100	1.5	100	1.5	100	1.5	100	1.5
	4	100	1.5	100	1.5	100	1.5	100	1.5

### Results: Comparison LQIC Results between MiSeq® and Ion S5™

Assay	Mean	CV%	Mean	CV%
LymphoTrack	100	1.5	100	1.5
LymphoQuant	100	1.5	100	1.5

### Conclusions

The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled. The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled.

### Results: Comparison of Expected Cell Counts for Control Samples (27 to 30%)

### Results: Comparison LQIC Results between MiSeq® and Ion S5™

Assay	Mean	CV%
LymphoTrack	100	1.5
LymphoQuant	100	1.5

### Conclusions

The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled. The LQIC is a non-lymphocyte subset and therefore does not behave like a lymphocyte subset. This can lead to inaccurate results if the LQIC is not properly controlled.



**invivoscribe** P020

## Comparing Minimal Residual Disease Detection in Multiple Myeloma using NGS-Based LymphoTrack® Assays and Flow Cytometry

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### Introduction

Multiple myeloma (MM), an advanced hematologic malignancy, is characterized by the presence of MM plasma cells in bone marrow (BM). A low disease burden (minimal residual disease (MRD)) is a prognostic and useful marker and disease-free survival (DFS) and progression-free survival (PFS) are important clinical endpoints. With widespread availability of MRD detection assays (MRD-NAAC and MRD-FC), MRD detection is becoming an important clinical endpoint. However, MRD detection by NGS-based assays (LymphoTrack) and flow cytometry (FC) are not directly comparable. The objective of this study was to compare the sensitivity of LymphoTrack and FC for MRD detection in MM patients.

### Materials and Methods

The LymphoTrack™ 24-plex assay (LTA) for 24 MM-related immunoglobulin (Ig) heavy chain (HC) and light chain (LC) genes was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection.

The sensitivity of the LymphoTrack™ 24-plex assay (LTA) and FC for MRD detection in MM patients was compared. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection.

251 paired BM samples from 104 MM patients were tested by LTA and FC. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection. The LTA assay was used to detect MRD in MM patients. The LTA assay was compared to FC for MRD detection.

### Results: MRD Samples by LymphoTrack Assays – MSeq®

Sample ID	FC	LTA	FC	LTA
Sample 1	1000	1000	1000	1000
Sample 2	1000	1000	1000	1000
Sample 3	1000	1000	1000	1000
Sample 4	1000	1000	1000	1000
Sample 5	1000	1000	1000	1000

### Results: Baseline Samples by LymphoTrack Assays – MSeq®

Sample ID	FC	LTA	FC	LTA
Sample 1	1000	1000	1000	1000
Sample 2	1000	1000	1000	1000
Sample 3	1000	1000	1000	1000
Sample 4	1000	1000	1000	1000
Sample 5	1000	1000	1000	1000

### MRD Detection Comparing DNA Input Levels by LymphoTrack and Flow Cytometry

### Conclusions

- LymphoTrack assays were able to detect MRD at lower concentrations than FC in MM patients.
- LymphoTrack 24-plex assay achieved 91.8% agreement with FC in detecting MRD. Despite using only 1/10th of the amount of DNA used by FC.
- LymphoTrack assays were able to detect MRD at lower concentrations than FC in MM patients.
- LymphoTrack assays were able to detect MRD at lower concentrations than FC in MM patients.

AMP Global May 16-18 2019 Hong Kong



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P032

## Validation of an NGS based assay for monitoring FLT3 ITD and TKD variants in AML subjects

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### Background

FLT3 ITD and TKD are recurrent genetic alterations in AML. FLT3 ITD is associated with poor prognosis and is a prognostic marker. TKD is associated with poor prognosis and is a prognostic marker. The presence of these variants in AML subjects is associated with poor prognosis and is a prognostic marker. The presence of these variants in AML subjects is associated with poor prognosis and is a prognostic marker.

### Materials and Methods

The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects.

### Results: LOD, LUL, and Linearity

The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects.

Variant	Allele	Concentration	LOD	LUL	Linearity
FLT3 ITD	G	100%	100%	100%	100%
		50%	100%	100%	100%
		25%	100%	100%	100%
		10%	100%	100%	100%
TKD	G	100%	100%	100%	100%
		50%	100%	100%	100%
		25%	100%	100%	100%
		10%	100%	100%	100%

### Results: Precision and Reproducibility

The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects.

### Table 1: LOD, LUL, and Linearity

Variant	Allele	Concentration	LOD	LUL	Linearity
FLT3 ITD	G	100%	100%	100%	100%
		50%	100%	100%	100%
		25%	100%	100%	100%
		10%	100%	100%	100%
TKD	G	100%	100%	100%	100%
		50%	100%	100%	100%
		25%	100%	100%	100%
		10%	100%	100%	100%

### Table 2: Precision and Reproducibility

Variant	Allele	Concentration	Precision	Reproducibility
FLT3 ITD	G	100%	100%	100%
		50%	100%	100%
		25%	100%	100%
		10%	100%	100%
TKD	G	100%	100%	100%
		50%	100%	100%
		25%	100%	100%
		10%	100%	100%

### Clinical Correlation

The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects.

### Conclusions

The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects. The assay was validated using 100 samples from 100 AML subjects.

AMP Global, May 16-18 2019, Hong Kong



## A Next-Generation Sequencing Based Analysis of Clonality across 39 Subjects Treated for Lymphoproliferative Disorders Reveals Matching Clones in the Diverse IGH Locus

9339

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### Introduction

The detection of clonality of the immunoglobulin heavy chain (IGH) locus is the basis for early detection of B-cell malignancies. The genetic diversity of the IGH locus has been analyzed in vitro (1), showing that the management and the location of the junctions of the IGH locus are highly diverse across healthy and affected individuals (2). This is supported by the complementary sequencing upon a PCR step which is the most variable region of the IGH locus (3). The routine genetic examinations generated from these techniques and various previous studies (4,5) have shown that the IGH locus is highly diverse in healthy and affected individuals. Here we present an analysis of the diversity of the IGH locus in subjects treated for various lymphoproliferative disorders using a Next-Generation Sequencing (NGS) based assay.

### Methods

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) of 39 subjects treated for various lymphoproliferative disorders. The IGH locus was sequenced using a NGS based assay. The resulting data was analyzed using a bioinformatics pipeline to identify clonal populations. The results were compared to a reference database of IGH sequences from healthy individuals.

### Results: Proportion of Overlapping Clones to Total Clones Between Subjects

Figure 1: Heatmap of overlap ratios between the sequences of any two given subjects within a sequencing run in between two different sequencing runs. The color scale represents the proportion of overlapping clones between subjects, with darker colors indicating a higher proportion of overlapping clones. The diagonal represents the proportion of overlapping clones within a single sequencing run.

### Results: Hierarchical Clustering

Figure 2: Hierarchical clustering of subjects based on the proportion of overlapping clones. The dendrogram shows the relationship between subjects, with subjects that have a higher proportion of overlapping clones clustering together. The subjects are ordered by their proportion of overlapping clones, with the most similar subjects at the top and the least similar at the bottom.

### Results: Proportions of Overlapping Clones Based on Full Average and Diverse Types

Figure 3: Heatmap of overlap ratios based on full average and diverse types. The color scale represents the proportion of overlapping clones between subjects, with darker colors indicating a higher proportion of overlapping clones. The diagonal represents the proportion of overlapping clones within a single sequencing run.

### Results: Subunit Motifs Found in the CDR3 Region of Clones Shared between AB-Tons

Table 1: Top 6 motifs found in 247 clones of clones shared by all runs based on the major IGH CDR3 motifs found in the sequences of 61 subjects by deconvolution at all 28130.

Motif	Number of clones		CDR3 motifs	
	Number	Percentage	Number	Percentage
AGG	24	9.6%	19	7.6%
AGC	4	1.6%	4	1.6%
AGT	7	2.8%	7	2.8%
AGG	14	5.4%	14	5.4%
AGC	4	1.6%	4	1.6%

Background: These are 1379 unique clones sequenced across 39 subjects. The high variability of the IGH CDR3 region is well known to be important in the immune response to a wide range of antigens. The CDR3 region of the IGH locus is the most diverse region of the IGH locus and is the site of the B-cell receptor. The CDR3 region is highly diverse and is the site of the B-cell receptor. The CDR3 region is highly diverse and is the site of the B-cell receptor.

### References

1. ...
2. ...
3. ...
4. ...
5. ...



## Comparing DNA Extraction Methods for the LymphoTrack® IVD TRG Assay

TT054

Maggie Kaminsky, Brandon Givens, Edgar Vigil, Veronika Atkinson, Emily Finnegan, Ying Huang and Jeffrey E. Miller  
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### Introduction

The LymphoTrack® IVD TRG Assay is an investigational sequencing-based assay being evaluated to identify clonal TRG rearrangements, the associated V<sub>H</sub> region (DNA sequence) and to provide the frequency distribution of the V<sub>H</sub> segment utilization. The assay uses microbeads from peripheral blood (PB) to determine the presence or absence of T cell clonality. This is accomplished using individual microbeads with unique barcodes (family-specific) and clonal V<sub>H</sub> regions of the TRG gene and associated J<sub>H</sub> region sequences and a set of 24 individual barcodes. Addition of high quality, inhibitor-free, family-associated DNA from peripheral blood samples to capture the successful PCR product by sequencing in order to provide users with variety of DNA isolation methods. 3 reproducibly available TRG assays have been evaluated for use with the LymphoTrack® IVD TRG Assay.

### Materials and Methods

The LymphoTrack® IVD TRG Assay is being evaluated for identification of clonality in TRG gene rearrangements in individuals with common T cell clonality. The assay family includes T cell receptor genes (TRG) gene locus. The PCR products are targeted to target TRG genes (delta 1, 2, 3, and 4), and the joining (J) gene regions where gene rearrangement occurs during T cell development (Figure 1).

Figure 1: Schematic diagram of the human T cell receptor gamma gene

The sample type for the LymphoTrack® IVD TRG Assay is PB obtained from peripheral whole blood. DNA is amplified via PCR, PCR products are quantified, pooled, and loaded into a Microfluidic cartridge which contains all of the reagents required for library generation and sequencing by synthesis. The sequencing data is compared to the Microfluidic instrument (MGI DGT) file. Invivoscribe's LymphoTrack® software is utilized to perform automated data extraction, analysis, and generate reports from the raw MGI file for clonality determination using the LymphoTrack® IVD TRG Assay - MGI file (Figure 2) below represents an example summary.

Figure 2: LymphoTrack IVD TRG Assay - MGI file processing summary

Three commercially available DNA extraction methods were evaluated in this study: (1) conventional magnetic beads using QIAzol Lysis Reagent, RNeasy Lysis Buffer, and RNeasy Spin Column (QIAzol method), (2) spin column using QIAzol Lysis Buffer and RNeasy Spin Column (QIAzol method), (3) spin column using QIAzol Lysis Buffer and RNeasy Spin Column (QIAzol method). Each method was tested using 100 µg of total RNA from 100 µg of peripheral blood samples (PB) PB1-PB5, by 2 operators. Each PB sample was tested by the LymphoTrack® IVD TRG Assay in duplicate (PB1-PB5) and sequenced together as a single Microfluidic instrument. The study design is illustrated in Figure 3.

Figure 3: DNA Extraction Methods Study

Total RNA yield, measured by LymphoTrack® IVD TRG Assay (TRG) and total RNA yield, measured by conventional magnetic bead extraction methods.

### Results

DNA yield and analysis concentration. DNA yield was quantified using Qubit dsDNA-BS Assay kit. Amplifier concentration determined by the Agilent Bioanalyzer using DNA2000 kit. Data summarized in Table 1.

Table 1: Efficiency with respect to DNA Yield Average Amplifier Concentration per Extraction Method

Extraction Method	Extraction Yield (µg)	Cells/µg per one Extraction	DNA Yield after Extraction (DNA µg)	Extraction Efficiency (%)	Avg. amplifier concentration (µg/µg DNA)
Silica column	290	1,000 (1)	240	83.1	23.7
Precipitation	300	1,120 (1)	220	73.3	28.9
Magnetic bead	290	1,000 (1)	220	75.9	26.2

Separating results (PB1-PB5). Each extraction method consistently detected expected TRG V<sub>H</sub> rearrangements associated with V<sub>H</sub> region DNA sequences. The distribution of V<sub>H</sub> regions with clonal bands (the colored circles) in rearrangements and negative PB samples was similar between the 3 extraction methods as shown in Figure 4.

Clonal Low Positive/Control Samples

Clonal Negative PB Samples

Figure 4: Distribution of V<sub>H</sub> bands for the 2 DNA extraction methods (low positive, control samples and healthy PB samples)

All samples showed 100% concordance with expected clonal results data summarized in Table 2 below.

Table 2: Clonal Detection Sensitivity Study

Extraction Method	Sample Type	Concordance	V <sub>H</sub> Method Concordance	Clonality Concordance
Silica column	Magnetic Bead	100% (100/100)	100% (100/100)	100% (100/100)
	Silica Column	100% (100/100)		
Precipitation	Magnetic Bead	100% (100/100)	100% (100/100)	
	Silica Column	100% (100/100)		
Magnetic bead	Magnetic Bead	100% (100/100)	100% (100/100)	
	Silica Column	100% (100/100)		

### Discussion and Conclusions

All 3 evaluated DNA extraction methods provided enough DNA (DNA µg) for the LymphoTrack® IVD TRG Assay. The precipitation method (PF) provided the highest total DNA yield while the silica column method produced the least amount of DNA. Data is presented in Table 1.

The generated PCR product yields were comparable among these 3 extraction methods. Average amplifier concentrations are presented in Table 1. No PCR inhibition was observed for any of the extraction methods.

The TRG clonality calls generated using the LymphoTrack® IVD TRG Assay - MGI file were 100% concordant for all control samples processed using all 3 DNA extraction methods (Table 2).

Separate components analysis was used to estimate the sensitivity of clonality calls to the extraction method, operator and control error for low positive control samples. The difference among extraction methods contributed to less than 1% of total calls.

The variability in clonality bands for positive samples (i.e. frequency of detected TRG rearrangements) was small with overall CDR3 ranging from 4-16 bp to 24-30 bp. All 3 methods showed similar results.

Components results were generated using 100 µg of peripheral blood with the LymphoTrack® IVD TRG Assay.

This study demonstrated that LymphoTrack® IVD TRG Assay performance is independent of DNA isolation method.





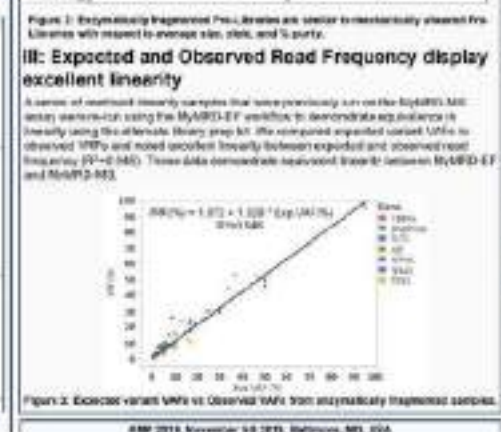
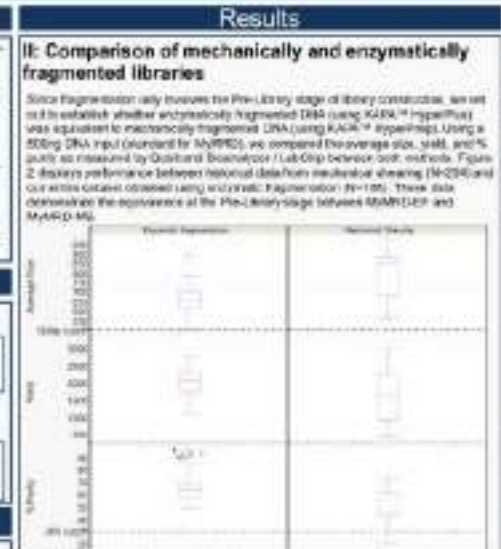
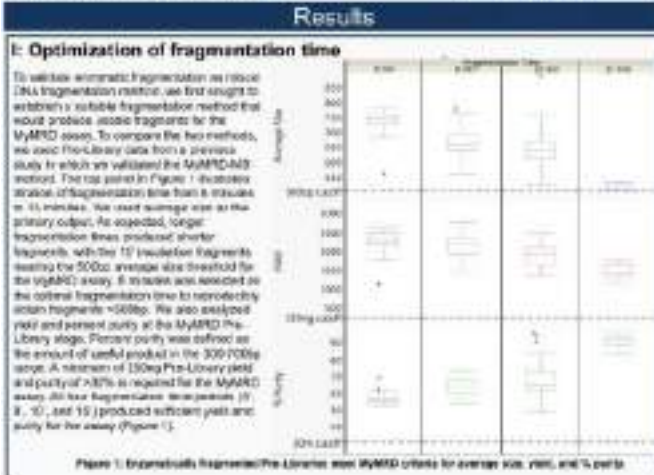
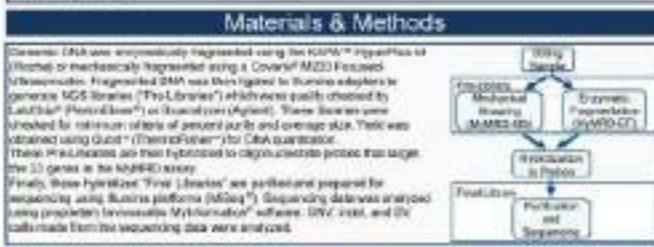
## Evaluation of An Alternative Fragmentation Method in High Throughput NGS Sample Testing of Minimal Residual Disease in Hematological Malignancies

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### Introduction

Invivoscribe® initiates pilot studies with leading pharmaceutical companies testing large numbers of samples using target capture-based next generation sequencing (NGS) to assess such as MyMRC® and MyMRC-IF® to identify clinically actionable variants. The Invivoscribe MyMRC assay is a multi-gene targeted NGS panel that covers 20 gene targets. Mechanical shearing is the standard method of DNA fragmentation for the assay, which requires specialized equipment and can be time consuming. Enzymatic fragmentation of DNA does not require specialized equipment, and can easily be scaled to automation. While mechanical fragmentation has long been the standard for fragmenting DNA in target capture-based NGS workflows, alternative methods such as enzymatic fragmentation can reduce sample-to-sample time and dramatically increase throughput. We compared enzymatic fragmentation using the KAPA® HyperPrep kit to our standard mechanical shearing protocol (KAPA® HyperPrep kit) in the context of the MyMRC assay. In this presentation, the two methods will be referred to as MyMRC-ME (Mechanical Shearing, KAPA® HyperPrep) and MyMRC-EF (Enzymatic Fragmentation, KAPA® HyperPrep).

The present poster compares performance between mechanical fragmentation and mechanical shearing in both wet lab and dry lab, average size and size distribution (range, median, mean, or SNV/indel rate or detection in LUD) assays. We also present data on the detection of structural variants (SVs) as MyMRC-EF was also superior in the detection of SVs from MyMRC-ME.



### Results

#### IV: Limit of Detection: SNVs, Indels, SVs

Gene	Variant	Type	Expected VAF	Observed VAF
FLT3	c.2002C>T p.G239T	SNV	0.33%	0.34%
	c.2008A>G p.G236A	SNV	0.33%	0.33%
	c.2009G>T p.D235F	SNV	0.33%	0.34%
	c.2005G>T p.D235F	SNV	0.33%	0.34%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%
DNMT3A	c.480 G>A p.Y157H	SNV	0.20%	0.20%
	c.480 G>A p.Y157H	SNV	0.20%	0.20%

Previous studies using the MyMRC-ME method revealed the limit of detection (LOD) for indels and SVs to be 0.34% and 0.17%, respectively. These studies defined LOD as the lowest VAF for which the variant is detected in all replicates (N=3). To confirm LOD using enzymatic fragmentation, 4 replicates of enriched samples from the MyMRC-ME study were re-run using the MyMRC-EF workflow. Detection of variants in the MyMRC panel with varying expected VAFs for indels and SVs are listed in Table 1 below. All expected indels and SVs were detected down to 0.33% and 0.17%, respectively. These data establish equivalence between MyMRC-ME and MyMRC-EF in indel and SVV detection.


#### Table 1: SV detection using the MyMRC-EF workflow. MyMRC-EF failed to detect SVs previously detected by MyMRC-ME.

Gene	Variant	Detection	Expected VAF
DNMT3A	c.480 G>A p.Y157H	Detected	0.20%
	c.480 G>A p.Y157H	Detected	0.20%
DNMT3A	c.480 G>A p.Y157H	Detected	0.20%
	c.480 G>A p.Y157H	Detected	0.20%
DNMT3A	c.480 G>A p.Y157H	Detected	0.20%
	c.480 G>A p.Y157H	Detected	0.20%

### Conclusions

With the notable exception of insertion artifacts, enzymatic fragmentation using the KAPA® HyperPrep kit with the MyMRC assay produced libraries comparable to those from mechanically fragmented genomic DNA. Both methods detected the same set of SNVs and indels, resulting in equivalence in linearity and concordance in SNV/indel detection. We observed reduced sensitivity for structural variant detection in enzymatically fragmented samples, we were unable to detect SVs in enzymatically fragmented samples that were detected using MyMRC with mechanical shearing. Incorporating enzymatic fragmentation to current and future assays could provide a cost-effective way to process large numbers of samples and ensure a consistent workflow of NGS libraries, however, mechanical shearing is still required for specimens requiring structural variant detection at low prevalence.






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## TRG Clonality Interrogation by CE and NGS: Bridging the Gap Between Classical and Leading Edge Technologies

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Improving Lives with Precision Diagnostics

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### INTRODUCTION

TRG clonality is a powerful tool in adult B-cell lymphoproliferative disorders and is commonly assessed using PCR-based capillary electrophoresis (CE) assays. CE assays were popularized by their speed and low cost, but suffer from disadvantages such as subjective analysis and slow turnaround when using CE results for tracking. Next-generation sequencing (NGS) offers solutions to these disadvantages by producing less subjective results and almost immediate sequence information that is ready to use for tracking. While it is understandable for labs to be hesitant about switching to newer, unfamiliar technologies, we present the following data to show the concordance between CE and NGS in a format familiar to the current generation.

### OBJECTIVES

First, to present TRG clonality results for 385 clinical samples using both CE and NGS.  
 Second, to transform the NGS data into an in-silico, electropherogram format (called NGS-E), to enable more direct comparison to CE outputs.  
 Third, to present a simple heuristic to demonstrate a key analysis advantage of NGS data. Namely, identifying highly complex peaks from what would be considered clonal peaks in CE, revealing false positives in CE.

### METHODS

385 clinical samples representing a variety of aspect T-cell lymphoproliferative disorders routinely tested using TRG assays were tested using both the LymphoTrack® TRG Dx assay (in development) on the Illumina® MiSeq® platform, and the CE-based IdentiClone® assay for TRG clonality. For NGS results, the %Total Reads (i.e. the percentage of reads an amplicon takes up in the sequencing step) was used as the metric for calling positives and negatives. For CE gels, a standard Relative Peak-Ratio (RRR) calculation was used as a metric for calling positives and negatives.

The results from the NGS-based assay were converted, in-silico, to NGS-E plots, by taking the sizes of the sequenced amplicons and plotting them on a histogram. On CE-based assays, electropherograms are the standard output. The two different groups were then compared (NGS-E vs CE). The NGS-E plots had an important difference: each size column often contained multiple different sequences. For any single column, an information-based metric for variability, Shannon entropy [Figure 2], is utilized to define columns as having many different sequences (high entropy, see Results, Figure 2) or few sequences (low entropy, see Results, Figure 3).

$$H(X) = - \sum_{i=1}^n (p_i) \log_2(p_i)$$

To ensure fair comparisons between samples and size columns that had different numbers of participating amplicons, each size column was initialized with 45 participating amplicons (representing the highest number of participating amplicons across all samples/size), and each initialized amplicon was given a minimum %Total Reads value of 0.0001225. The probability value used for the entropy calculation was derived from the fraction of %Total reads each amplicon contributed to its size column. For a size column with only initialized amplicons (i.e. no actual data, only initialized data), each of the 45 participants contribute an equal probability to the entropy calculation, and the negative sum is maximized (high entropy). For a size column with a few, high-signal amplicons, these signal amplicons take up the vast majority of the entropy calculation, and the negative sum is minimized (low entropy).

### RESULTS: NGS-E VERSUS CE PLOTS

385 clinical samples were run on both NGS and CE assays. Examples of concordant results between these two assays are shown in Figure 2 and 3 for negative and positive TRG clonality, respectively. An example of a discordant result between NGS and CE is shown in Figure 4. As seen in the example, when multiple amplicons have the same size, the CE assay is not able to differentiate amplicons and the CE electropherograms look positive for TRG clonality. Alternatively, the NGS-E plot can delineate amplicons within size bins and show that this sample should be negative for TRG clonality.

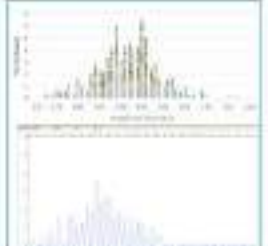
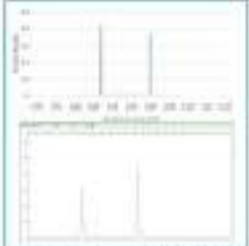
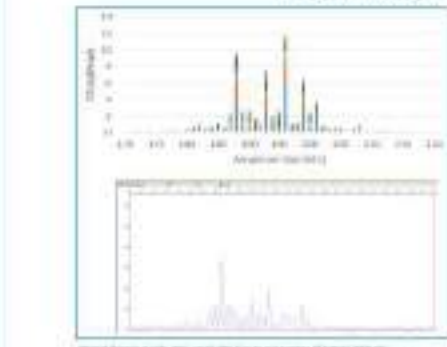




Figure 2: Example of a TRG assay (NGS-E) plot showing CE and NGS concordant negative results (blue).

Figure 3: Example of a TRG assay (NGS-E) plot showing CE and NGS concordant positive results (green).

Figure 4: Example of a TRG assay (NGS-E) plot showing CE and NGS discordant results. The CE assay is positive (yellow) but the NGS-E assay is negative (blue).

### RESULTS: ENTROPY PLOT

For NGS results, the entropy calculation of the peak chosen for CE analysis was used for downstream comparison analysis. Of the 385 clinical samples, 296 (76.6%), average entropy 2.74, blue dots in Figure 5) were concordant negatives between the NGS and CE assays, and 90 (23.3%), average entropy 51.2, green dots in Figure 5) were concordant positives; the remaining 42 (10.8%) were discordant. Of the discordant samples, 16 were NGS+ (red dots, Figure 5), while 26 were CE+ (yellow dots in Figure 5). 16 of these 42 discordant samples were near the cut-off. However, an interesting pattern appeared for the discordant, CE+ samples. If the samples are ranked by the entropy value of the target peak from the NGS-E plot, 14 of the 26 were highly complex peaks (average entropy of 202.1, yellow dots with blue squares, Figure 5) that were correctly distinguished in the NGS data as being polyclonal. These 14 represent mixed clonality calls that were clear from the NGS data, comparison of the CE and NGS-E shows visually how this discordance could happen (example shown in Figure 4). Interestingly, none of the 16 NGS+ discordant samples could be distinguished by peak entropy. If we assume the 14 CE+ discordant samples are false positives, the NGS data was able to reduce the false positive rate by 54% (from 5.7% to 3.2%).

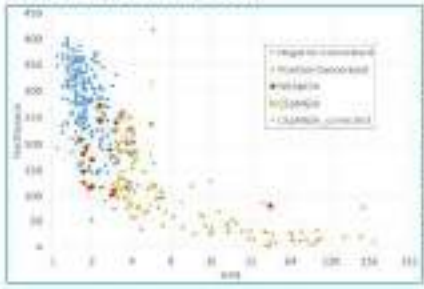


Figure 5: Entropy plot showing the relationship between CE and NGS results based on entropy values. The plot shows CE+ (yellow dots) and CE- (blue dots) results. The legend indicates: NGS+ (green dots), NGS- (blue dots), CE+ (yellow dots), and CE- (blue dots).

### CONCLUSIONS

A significant benefit in switching from CE to NGS for clonality testing is the assurance that the NGS assay gives more accurate results to offset the higher cost and longer time needed to generate data and results. However, it can be argued that the advantages of sequencing should be sufficient to offset the cost and time, the subjective nature of CE analysis and the slow turnaround time to obtaining sequence data from CE may nullify the NGS cost and time. In addition, NGS-E plots, which have the benefit of displaying the results in a manner familiar to those accustomed to CE results. In combination with a simple metric for complexity (which gives more accuracy and reduced false positives by 54% in our dataset), adds to the advantages of NGS over CE for clonality testing. These NGS-E plots can act as a bridge that the CE-centric lab can use to become more accustomed to NGS data, as they address potential concerns about converting to NGS-based testing.

### CONTACT INFORMATION

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