Literature Review

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

invivoscribe®

Improving Lives with Precision Diagnostics®

invivoscribe®
Improving Lives with Precision Diagnostics®

Clonality Testing

Gel & Capillary Electrophoresis

EuroClonality Design

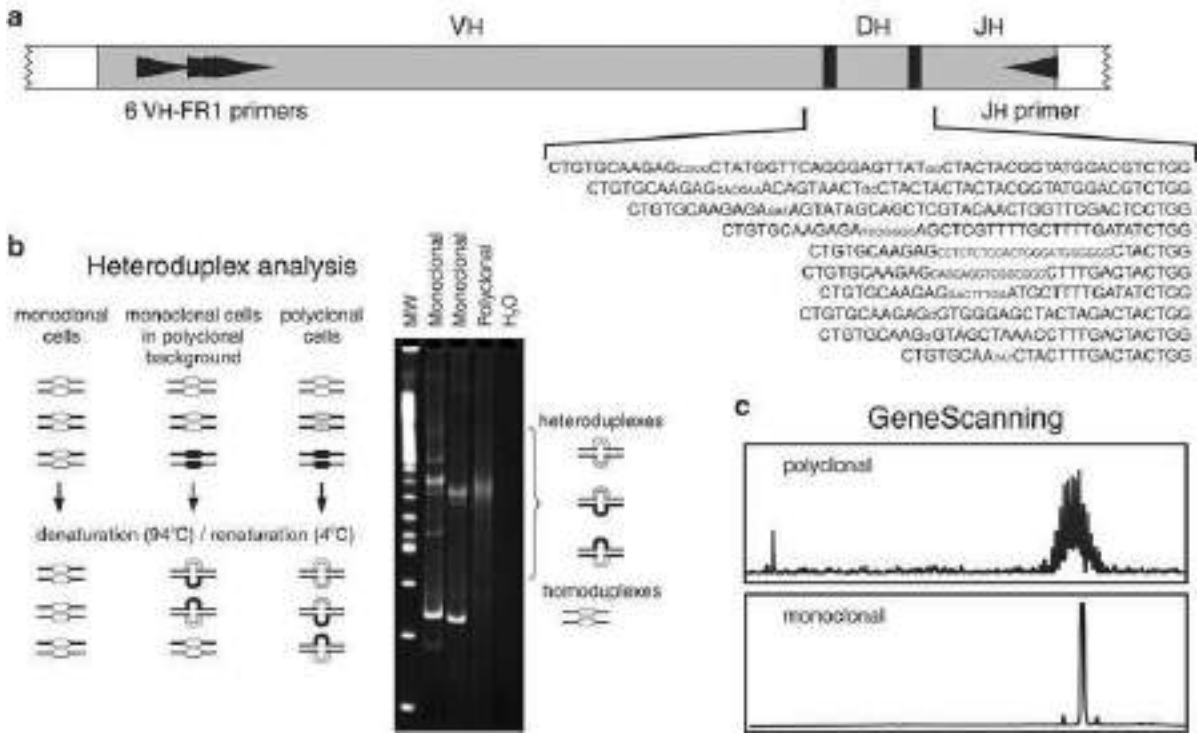
BIOMED-2/EuroClonality description of primer and assay design

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[1], Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, Delabesse E, Davi F, Schuuring 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.



Van Dongen JJ, et al. 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. *Leukemia*. 2003 Dec;17(12):2257-317. https://doi.org/10.1038/sj.leu.2404479

EuroClonality Interpretation

Describes selection of targets

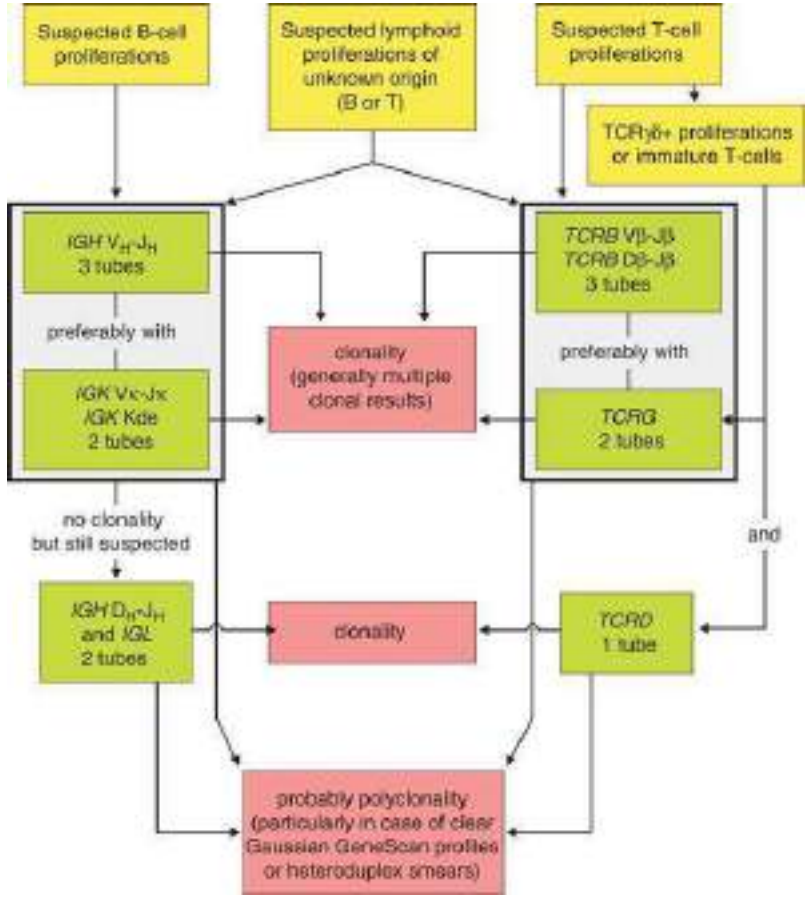
Discusses very specific technical and biological pitfalls of molecular clonality testing

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[1], 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, Schuuring E, Spaargaren M, Hodges E, van Dongen JJ.



Langerak AW, Groenen PJ, Brüggemann M, et al. EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. *Leukemia*. 2012;26(10):2159-2171. doi:10.1038/leu.2012.246

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[1], Pott Ch, Groenen PJ, Salles G, Davi F, Berger F, Garcia JF, van Krieken JH, Pals S, Kluin P, Schuuring E, Spaargaren M, Boone E, González D, Martinez 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, Cabeçadas J.

	IGH FR1	*IGH* FR2	*IGH* FR3	*IGH* (FR1,2,3)	*IGK* (Vk–Jk, Kde)	Total (IGH, IGK)
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%**

Evans, P., Pott, C., Groenen, P. et al. 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. Leukemia 21, 207–214 (2007). https://doi.org/10.1038/sj.leu.2404479

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[1], 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, Foroni L, Carter GI, Hummel M, Bastard C, Davi F, Delfau-Larue MH, Kneba M, van Dongen JJ, Beldjord K, Molina TJ.

	TRB	*TRG*	*TRB+TRG*
T-PLL	100%	94%	**100%**
T-LGL	96%	96%	**100%**
PTCL-U	98%	94%	**100%**
AILT	89%	92%	**95%**
ALCL	74%	74%	**79%***
Total	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.

Brüggemann M, White H, Gaulard P. et al. Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies Report of the BIOMED-2 Concerted Action BHM4 CT98-3936. *Leukemia*. 2007 Feb;21(2):215-21. https://doi.org/10.1038/sj.leu.2404481

IdentiClone® T-Cell Assays in T-ALL

IdentiClone™

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

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)

invivoscribe
Improving Lives with Precision Diagnostics®

Kim H, Kim IS, Chang CL, et al. T-Cell Receptor Rearrangements Determined Using Fragment Analysis in Patients With T-Acute Lymphoblastic Leukemia. *Ann Lab Med*. 2019;39(2):125-132. doi:10.3343/alm.2019.39.2.125

TCRG 2.0 Comparison

IdentiClone™

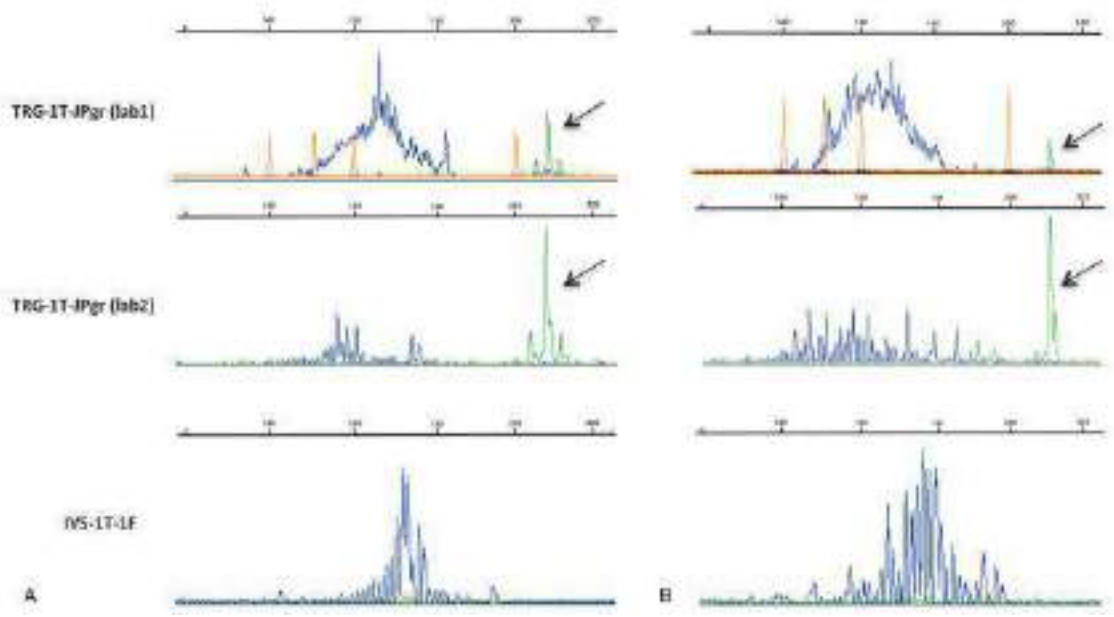
Direct comparison of EuroClonality 2-tube design, EuroClonality 1-tube design, and Invivoscribe design

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



Armand M, Derrieux C, Beldjord K, et al. A New and Simple TRG Multiplex PCR Assay for Assessment of T-cell Clonality: A Comparative Study from the EuroClonality Consortium. Hemasphere. 2019;3(3):e255. Published 2019 Jun 4. doi:10.1097/HS9.0000000000000255

invivoscribe®
Improving Lives with Precision Diagnostics®

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 Gazzola, Claudia Mannu, Maura Rossi, Maria Antonella Laginestra, Maria Rosaria Sapienza, Fabio Fuligni, Maryam Etebari, Federica Melle, Elena Sabattini, Claudio Agostinelli, Francesco Bacci, Carlo Alberto Sagramoso Sacchetti, Stefano Aldo Pileri, and Pier Paolo Piccaluga[sp]

	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?*	High?*

*To be validated.

Gazzola A, Mannu C, Rossi M, et al. The evolution of clonality testing in the diagnosis and monitoring of hematological malignancies [published correction appears in Ther Adv Hematol. 2014 Oct;5(5):181]. Ther Adv Hematol. 2014;5(2):35-47. doi:10.1177/2040620713519729

Clonality in Mycosis Fungoides

Most common cutaneous neoplasm; diagnosis is often challenging

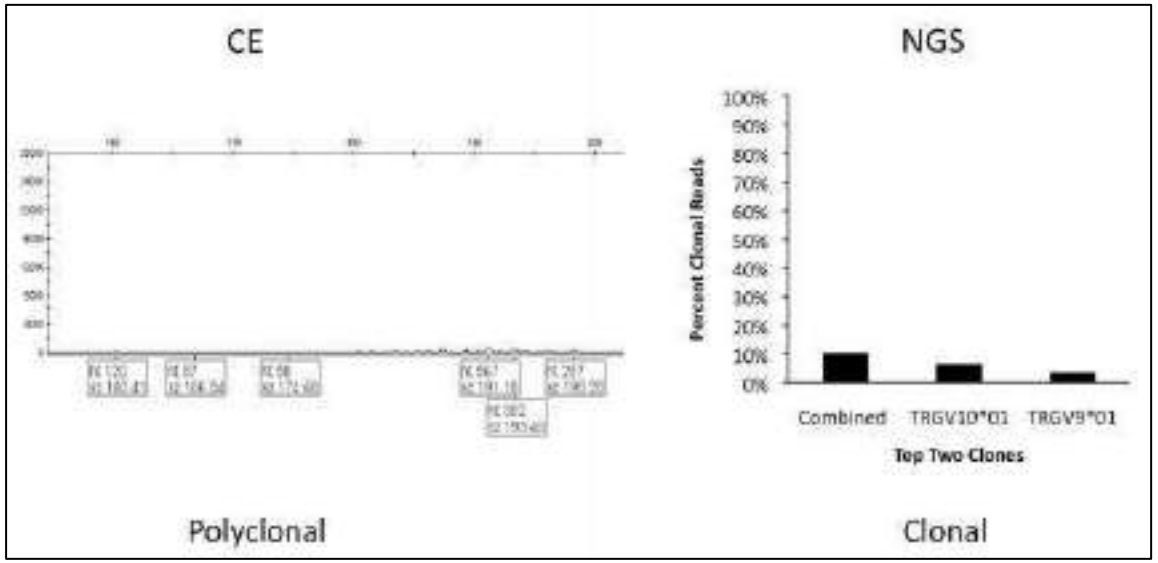
Sought to determine if NGS-based detection has increased sensitivity for T-cell clonality

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[1], Lockwood CM[1], Abel HJ[1], Hagemann IS[1], Schumacher JA[2], Kelley TW[3], Duncavage EJ[4].



Sufficool KE, Lockwood CM, Abel HJ, et al. T-cell clonality assessment by next-generation sequencing improves detection sensitivity in mycosis fungoides. *J Am Acad Dermatol*. 2015 Aug;73(2):228-36.e2. doi: 10.1016/j.jaad.2015.04.030

LymphoTrack® Assays

Publications

invivoscribe®
Improving Lives with Precision Diagnostics®

Establishment of Routine *IGH* Testing

LymphoTrack® Assays

MSKCC validation of LymphoTrack® *IGH* assays for clinical use

>1000 samples tested over 1.5 years

Direct comparison to capillary electrophoresis assays

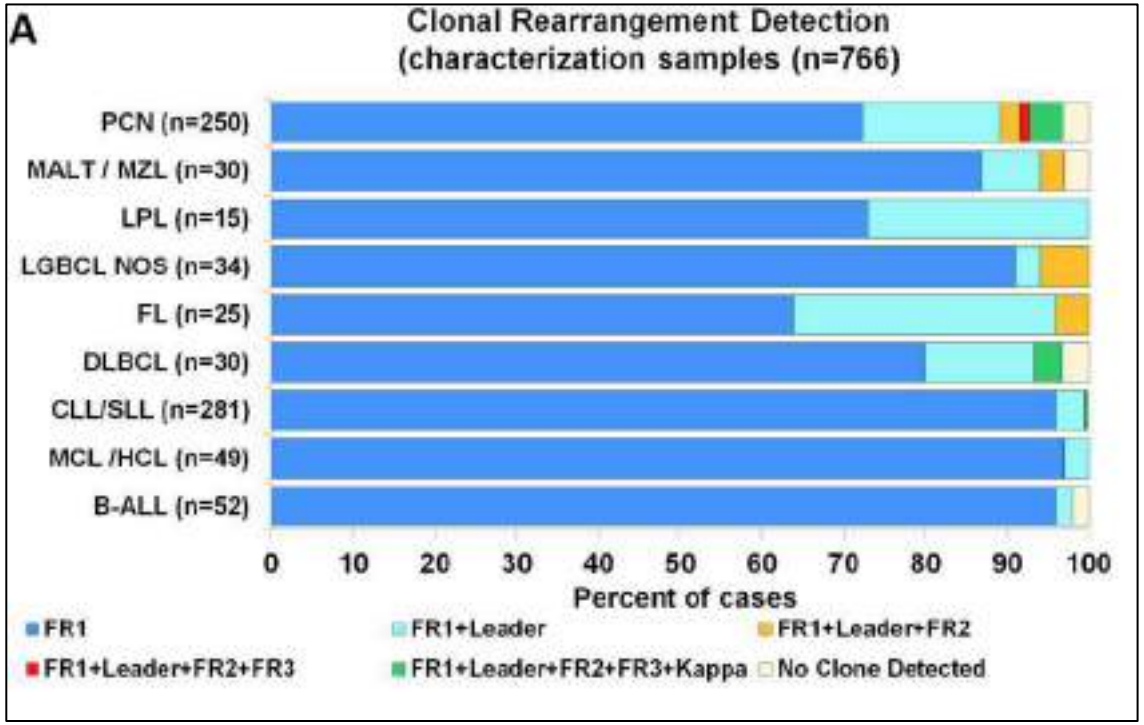
- 96% Concordance
- **NGS shows superior performance**

>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,** Wayne Yu,† Mustafa Syed,† Hannah Kim,† Lidia Maciag,† JinJuan Yao,† Caleb Ho,† Kseniya Petrova,† Christine Moung,† Paulo Salazar,† Ivelise Rijo,† Tessara Baldi,† Ahmet Zehir,† Ola Landgren,† Jae Park,† Mikhail Roshal,† Ahmet Dogan,† and Khedoudja Nafa†



invivoscribe® Improving Lives with Precision Diagnostics®

Arcila ME, Yu W, Syed M, et al. Establishment of Immunoglobulin Heavy (IGH) Chain Clonality Testing by Next-Generation Sequencing for Routine Characterization of B-Cell and Plasma Cell Neoplasms. *J Mol Diagn*. 2019;21(2):330–342. doi:10.1016/j.jmoldx.2018.10.008

Establishment of Routine *TRG* Testing

LymphoTrack® Assays

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

Provides a laboratory specific interpretation algorithm for LymphoTrack® *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[1], Vanhouteghem K[1], Vermeire S[1], Maelbrancke E[1], Emmerechts J[1], Devos H[1], Cauwelier B[1].

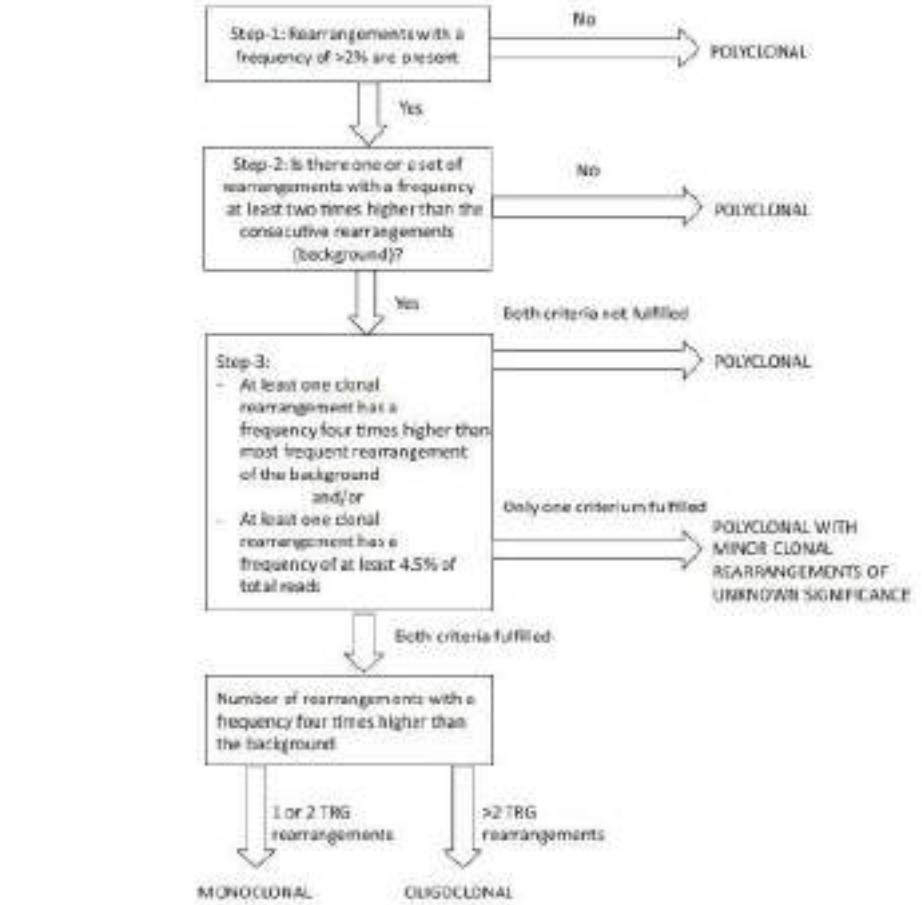


FIGURE 1 Interpretation algorithm for each LymphoTrack Dx TRG assay reaction

Nollet F, Vanhouteghem K, Vermeire S, et al. Evaluation of next-generation sequencing-based clonality analysis of T-cell receptor gamma gene rearrangements based on a new interpretation algorithm. *Int J Lab Hematol*. 2019 Apr;41(2):242-249. doi: 10.1111/ijlh.12954. Epub 2018 Dec 8.

Case Study – *TRG* Clonality

LymphoTrack® Assays

Describes **41 cases in detail**

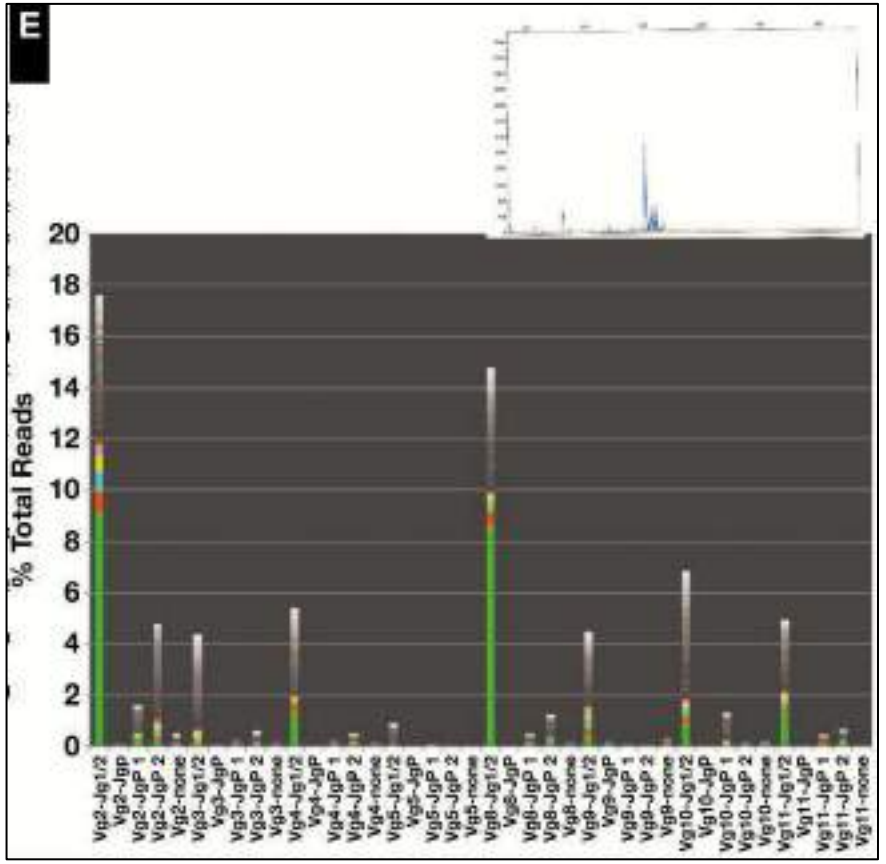
Provides the laboratory's strategy for interpretation of NGS results

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 γ (TRG) Clonality Evaluation by Next-Generation Sequencing in Clinical Hematolymphoid Tissues.

Kansal R[1], Grody WW[1], Zhou J[1], Dong L[1], Li X[1].



Kansal R, Grody WW, Zhou J, et al. The Value of T-Cell Receptor γ (TRG) Clonality Evaluation by Next-Generation Sequencing in Clinical Hematolymphoid Tissues. *Am J Clin Pathol.* 2018 Jul 31;150(3):193-223. doi: 10.1093/ajcp/aqy046.

Case Study – B- and T-Cell Clonality

LymphoTrack® Assays

B-cell lymphoma evolved into a T-cell lymphoma

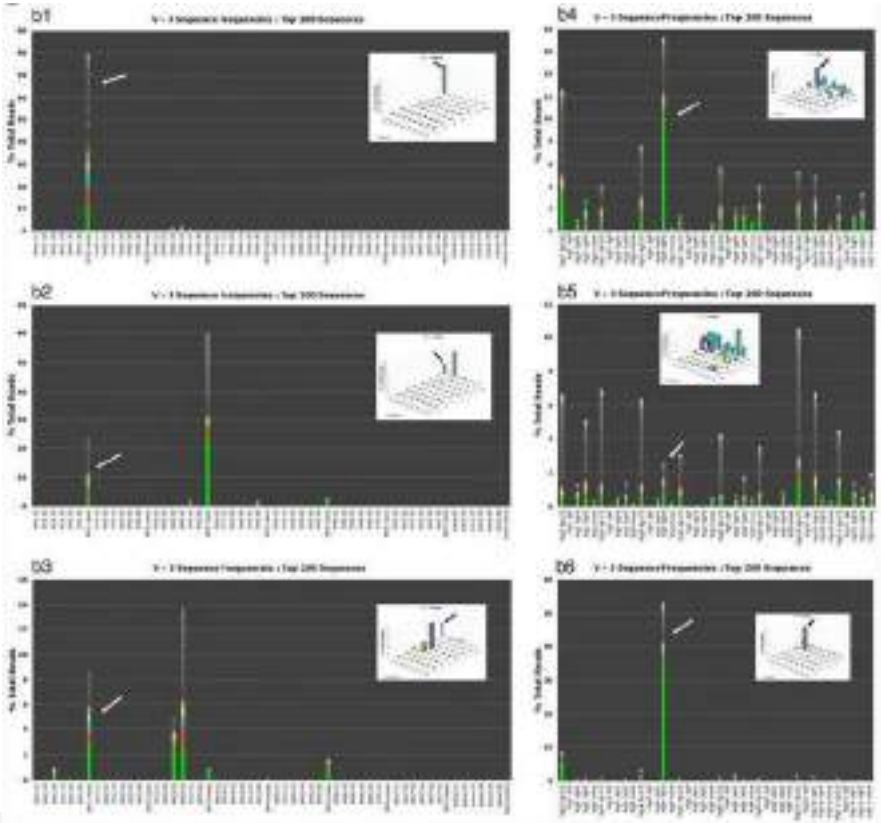
Complex morphology and difficulty classifying two relapses

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



invivoscribe® Improving Lives with Precision Diagnostics®

Vanecek, T., Walker, K., Watson, L.L. et al. Molecular genetic data favoring a sequential clonal transformation of a large B cell lymphoma into an anaplastic large T cell lymphoma, ALK-negative. *J Hematopathol* 8, 243–253 (2015). https://doi.org/10.1007/s12308-015-0245-4

Case Study – Repertoire Analysis

LymphoTrack® Assays

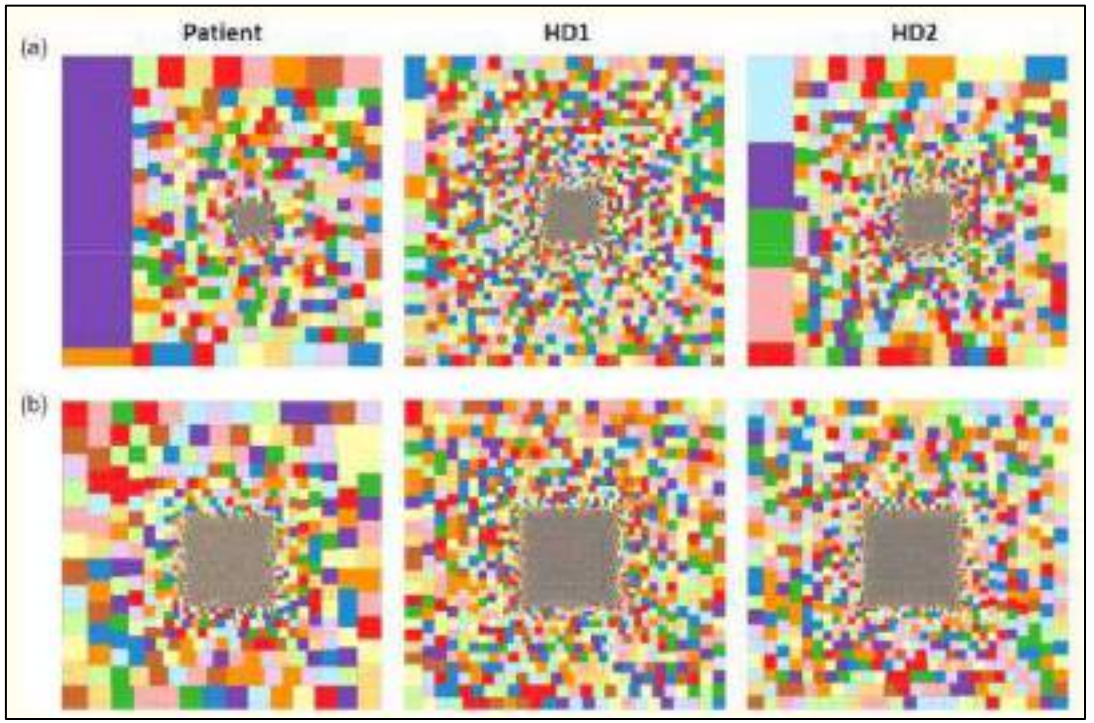
Patient with RALD, an autoimmune lymphoproliferative syndrome

Performed **repertoire analysis using LymphoTrack®** 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,[1,2,3,4,†] A. Lev,[1,3,†] E. Rechavi,[1,3] O. Barel,[3,5] H. Golan,[2,3] B. Bielorai,[2,3] Y. Neumann,[2,3] A. J. Simon,[1,3,5,6] and R. Somech[✉1]



invivoscribe® Improving Lives with Precision Diagnostics®

Levy-Mendelovich S, Lev A, Rechavi E, et al. T and B cell clonal expansion in Ras-associated lymphoproliferative disease (RALD) as revealed by next-generation sequencing. *Clin Exp Immunol*. 2017;189(3):310-317. doi:10.1111/cei.12986

Repertoire & SHM Analysis

LymphoTrack® Assays

22 HCV+ patients and 7 healthy controls

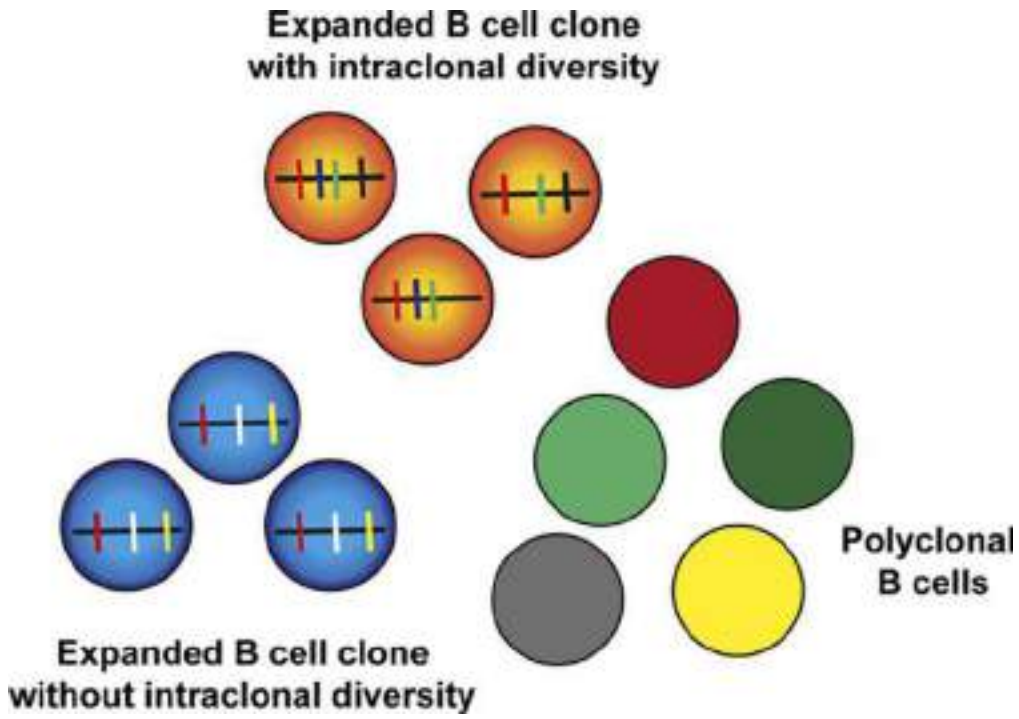
Very thorough **repertoire and somatic hypermutation analysis**

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[1], Kitanovski S[2], Johansson P[1,3], Klein-Hitpass L[1], Kahraman A[4], Dürig J[3], Hoffmann D[2,5], Küppers R[1,5].



Tucci FA, Kitanovski S, Johansson P, et al. Biased IGH VDJ gene repertoire and clonal expansions in B cells of chronically hepatitis C virus-infected individuals. *Blood*. 2018 Feb 1;131(5):546-557. doi: 10.1182/blood-2017-09-805762.

invivoscribe®
Improving Lives with Precision Diagnostics®

Somatic Hypermutation (SHM) Literature

SHM Status can be Prognostic in CLL

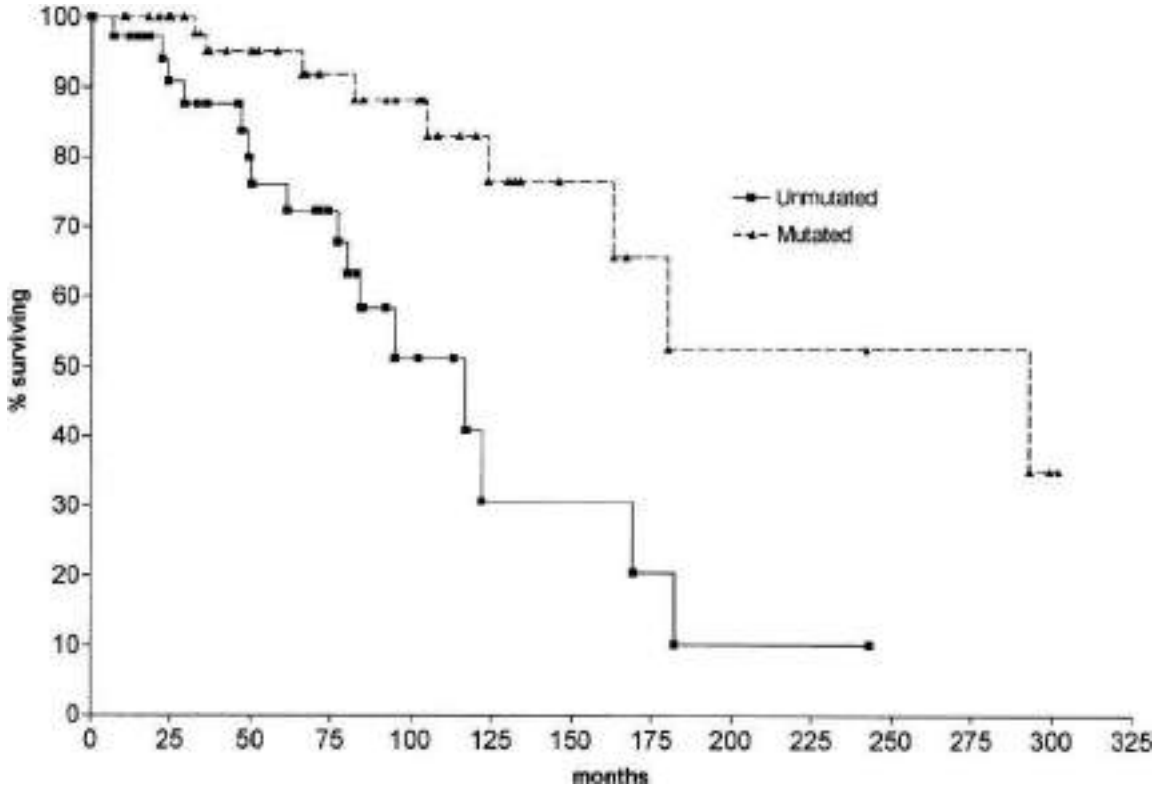
Early paper on CLL cells and *IGHV* mutational status

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[1], Davis Z, Gardiner A, Oscier DG, Stevenson FK.



Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia.. *Blood*. 1999 Sep 15;94(6):1848-54. doi.org/10.1182/blood.V94.6.1848

SHM Status is also Predictive

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

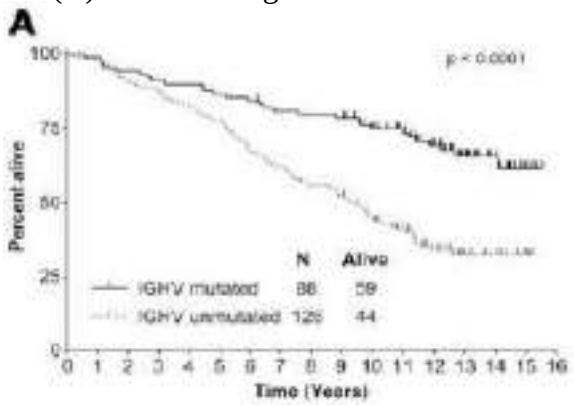
- Prolonged response
- Delayed progression
- Significant improvement in survival overall

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

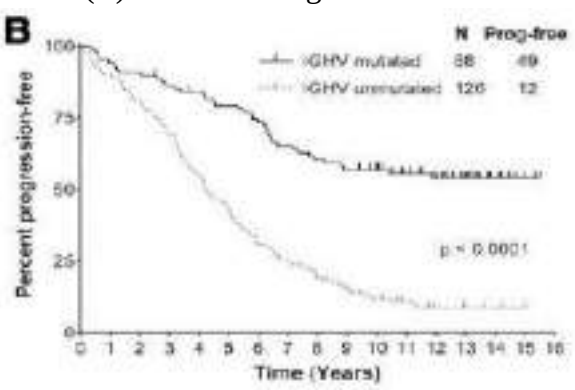
Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in *IGHV*-mutated chronic lymphocytic leukemia

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

(A) OS according to mutation status



(B) PFS according to mutation status



	OS (%)	PFS (%)
IGHV-M	65.5	53.9
IGHV-UM	32.2	8.9

Thompson PA, Tam CS, O'Brien SM, et al. Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in IGHV-mutated chronic lymphocytic leukemia. Blood. 2016;127(3):303-309. doi:10.1182/blood-2015-09-667675

SHM in Mantle Cell Lymphoma

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

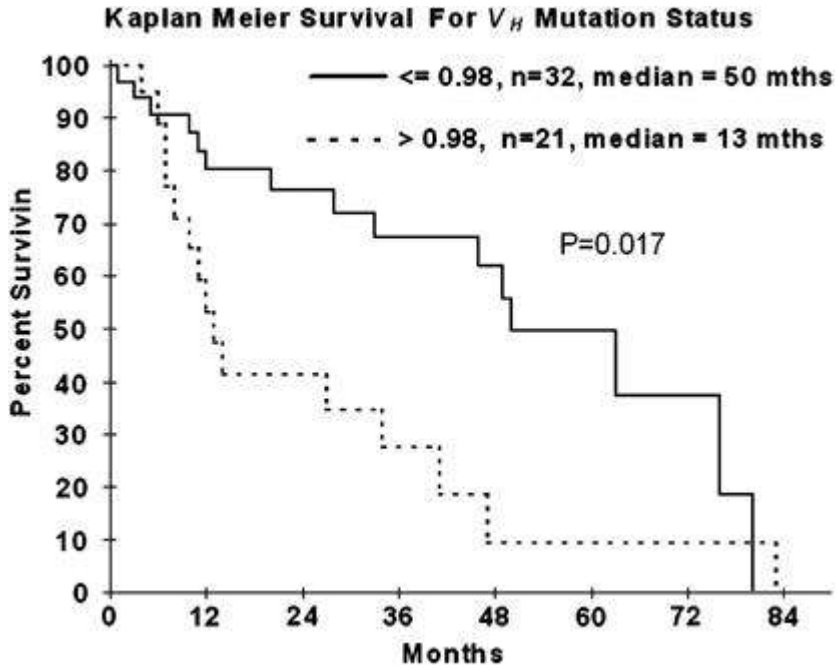
Identified a bias in VH gene utilization (VH1-69, VH4-59, and VH3-74) and relationship to gene and mutational status

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[1], 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* ≥1)	1.64	0.45
International prognostic index	1.05	0.93

Lai R, Lefresne SV, Franko B, et al. Immunoglobulin VH somatic hypermutation in mantle cell lymphoma: mutated genotype correlates with better clinical outcome.. *Mod Pathol.* 2006 Nov;19(11):1498-505. Epub 2006 Sep 15. doi.org/10.1038/modpathol.3800677

Should *IGHV* be Tested at Diagnosis?

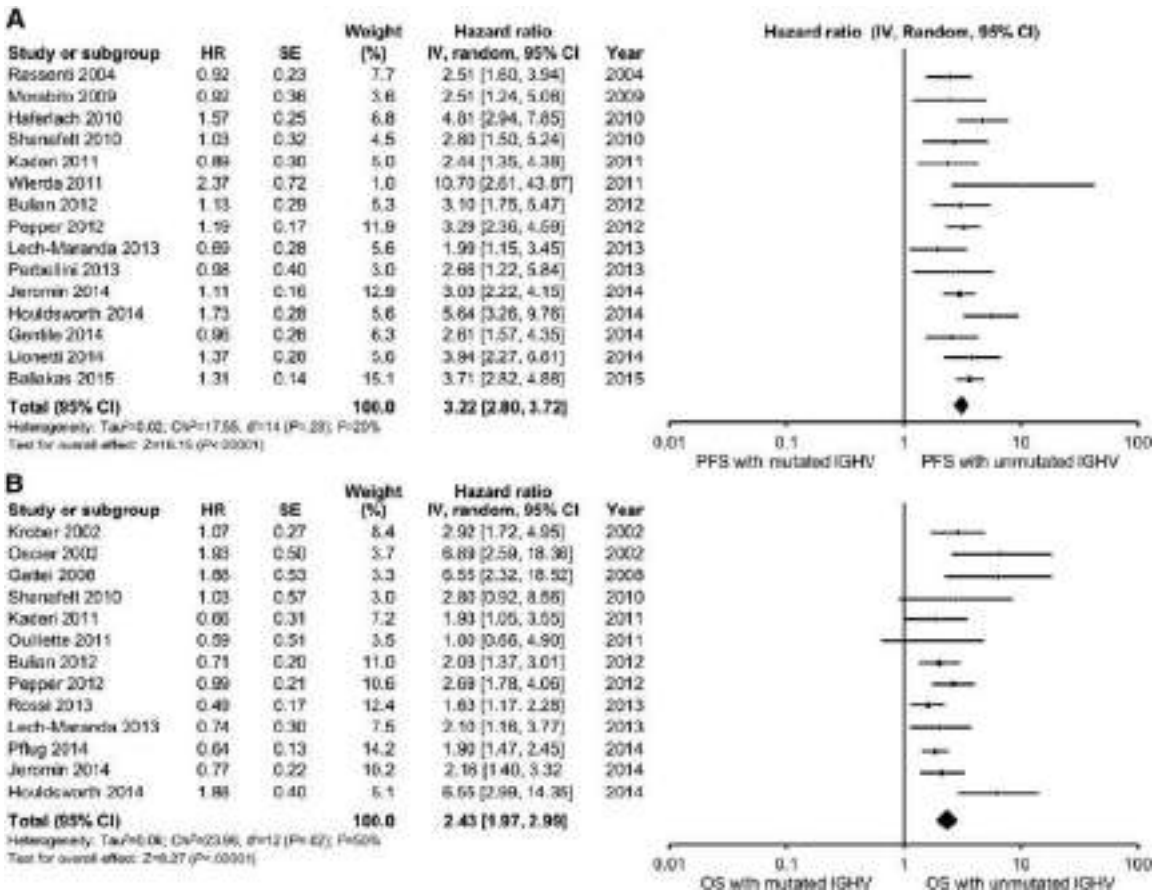
Review of **31 different studies**

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[1], Strati P[1], Tsang M[1], West CP[2], Shanafelt TD[1].



Parikh SA, Strati P, Tsang M, et al. Should IGHV status and FISH testing be performed in all CLL patients at diagnosis? A systematic review and meta-analysis. *Blood*. 2016 Apr 7;127(14):1752-60. doi: 10.1182/blood-2015-10-620864.

SHM in the Era of Next Gen Sequencing

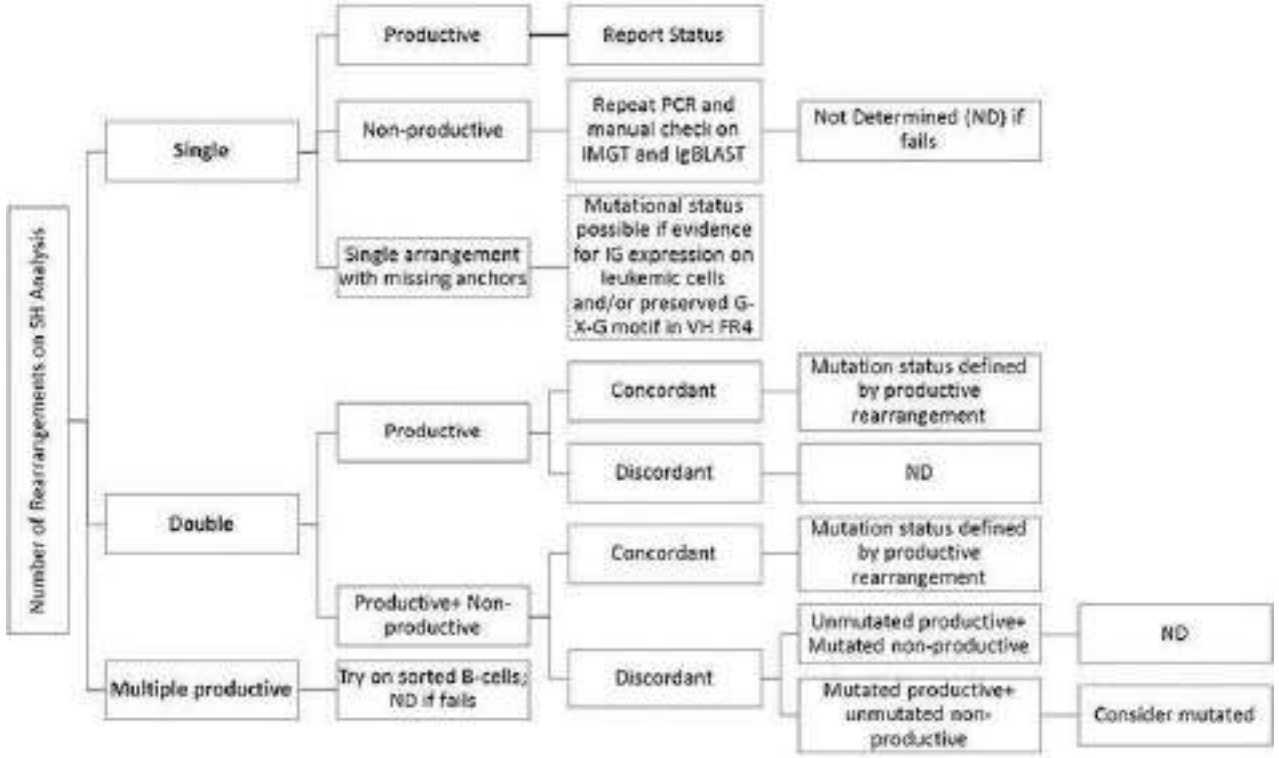
Review paper detailing the evolution of somatic hypermutation testing in CLL

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,[1] David S. Viswanatha,[2,*] and Keyur P. Patel[3,*]



Gupta SK, Viswanatha DS, Patel KP. Evaluation of Somatic Hypermutation Status in Chronic Lymphocytic Leukemia (CLL) in the Era of Next Generation Sequencing. *Front Cell Dev Biol*. 2020;8:357. Published 2020 May 19. doi:10.3389/fcell.2020.00357

ERIC Updated Recommendations

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 [1,2], P Ghia [3], A Hadzidimitriou [4], L-A Sutton [1,2], A Agathangelidis [3], P Baliakas [1], N Darzentas [5], V Giudicelli [6], M-P Lefranc [6], A W Langerak [7], C Belessi [8], F Davi [9], K Stamatopoulos [1,4]

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

Item	*Recommendations*	*Remarks*
Material		
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
Production of template for sequencing		
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)
Sequencing		
Methodology	Direct, both strands	Both strands mandatory for high-quality sequence
Sequence alignment	IMGT/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.infspire.org/arrest/ericll.org/pages/services/tool)	Applicable for the current 19 major BcR stereotyped subsets in CLL[a]

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. [a]Agathangelidis and colleagues.[7]

Rosenquist R, Ghia P, Hadzidimitriou A, et al. Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: updated ERIC recommendations. *Leukemia*. 2017;31(7):1477-1481. doi:10.1038/leu.2017.125

Improving Disease Stratification in CLL

LymphoTrack® Assays

2 studies, 497 total samples

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

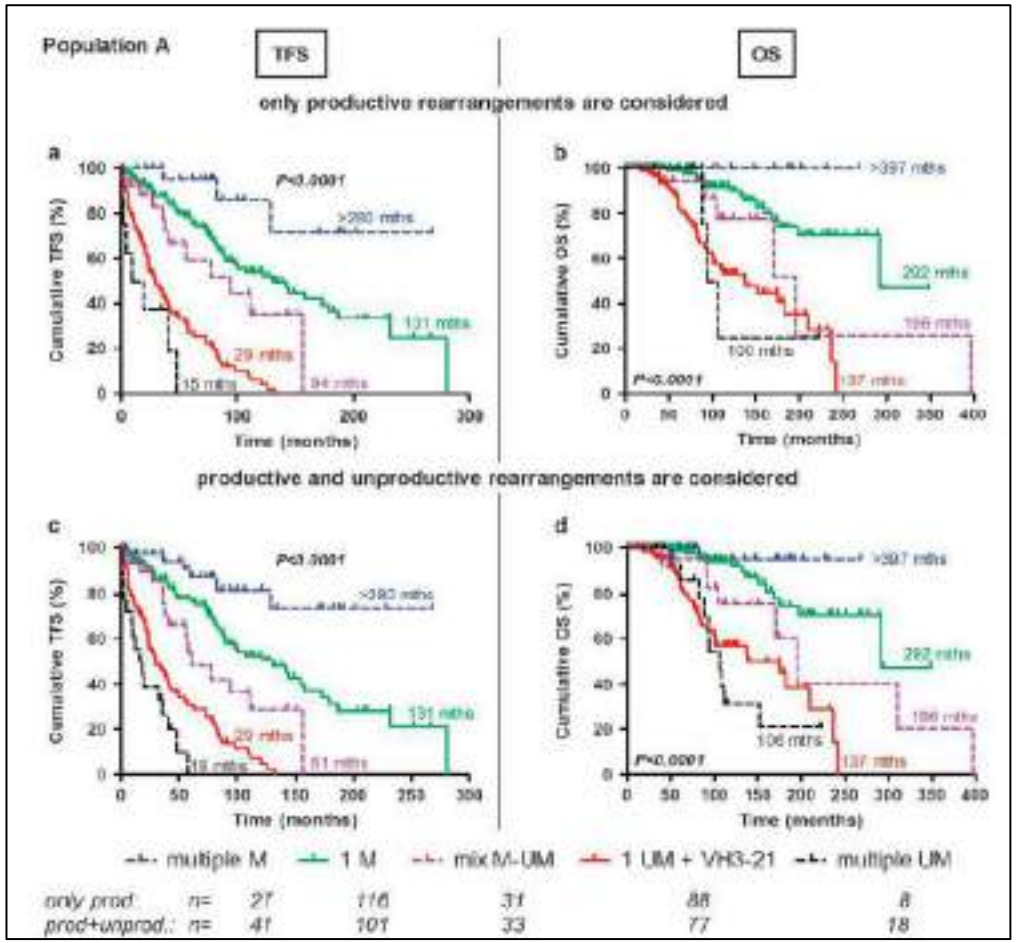
Proposes 5 new subsets for **improved stratification** for identifying outcomes

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[1,2,3], Timbs A[1], Bruce D[1], Smith T[4], Clifford R[1,3], Robbe P[1,3], Burns A[1,3], Vavoulis DV[3], Lopez L[5], Antoniou P[3], Mason J[1], Dreau H[1], Schuh A[1,6].



Stamatopoulos, B., Timbs, A., Bruce, D. et al. Targeted deep sequencing reveals clinically relevant subclonal IgHV rearrangements in chronic lymphocytic leukemia. *Leukemia* 31, 837–845 (2017). doi.org/10.1038/leu.2016.307

Differential Protein Expression

LymphoTrack® Assays

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

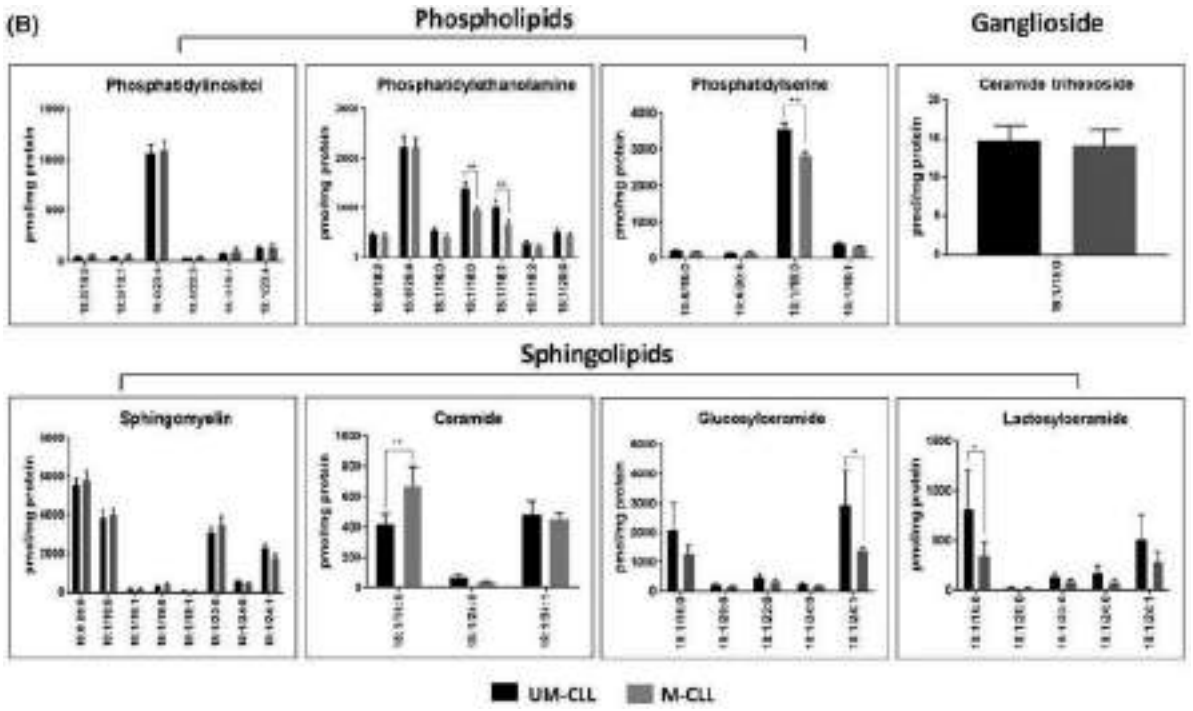
Used LymphoTrack® to evaluate somatic hypermutation

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 [1], Eveline S Dwyer [1], Karen M Lower [1], Tim K Chataway [2], Bryone J Kuss [1 3]



Thurgood LA, Dwyer ES, Lower KM, et al. Altered expression of metabolic pathways in CLL detected by unlabelled quantitative mass spectrometry analysis. *Br J Haematol*. 2019 Apr;185(1):65-78. doi: 10.1111/bjh.15751.

invivoscribe®
Improving Lives with Precision Diagnostics®

MRD Literature

NGS MRD Review

LymphoTrack® Assays

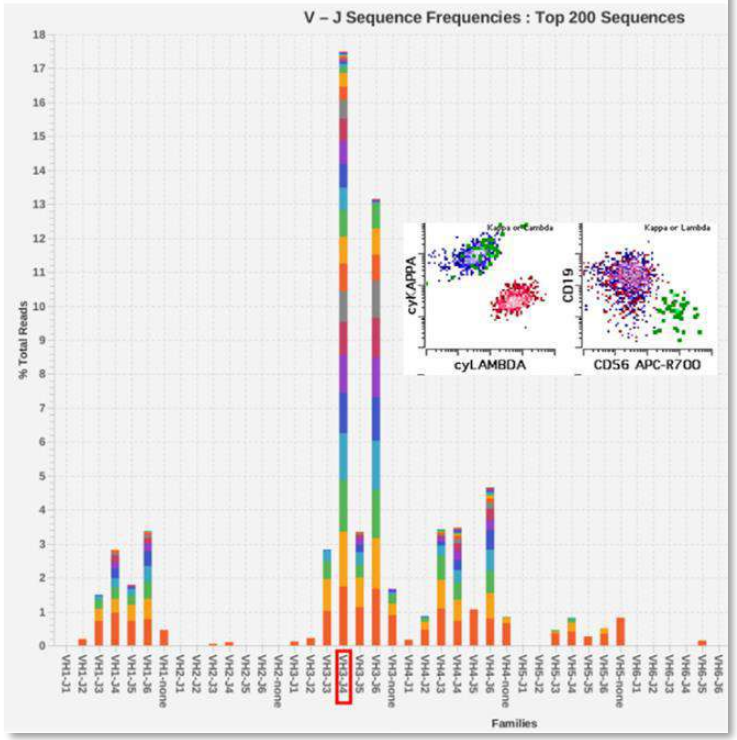
Review paper which provides a good case for updating Dx technology for detection of MRD in myeloma patients, comparing MCF, ASO-PCR, F-PCR and NGS

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[1], Arcila ME[2].

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



invivoscribe® Improving Lives with Precision Diagnostics®

Arcila ME, Ho, C. Minimal residual disease detection of myeloma using sequencing of immunoglobulin heavy chain gene VDJ regions. *Semin Hematol.* 2018 Jan;55(1):13-18. doi: 10.1053/j.seminhematol.2018.02.007.

NGS MRD Review

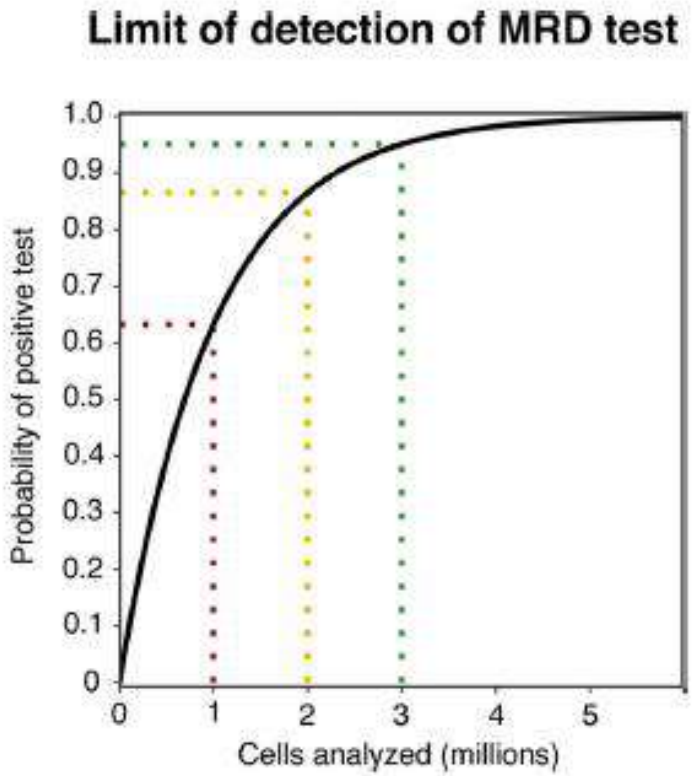
Review paper that details some of the intricacies of testing, including selecting a sensitivity

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[1] and Eileen M. Boyle, MD, MSc[2]



Rustad EH, Boyle EM. Monitoring minimal residual disease in the bone marrow using next generation sequencing. *Best Pract Res Clin Haematol*. 2020;33(1):101149. doi:10.1016/j.beha.2020.101149

NGS MRD vs. qPCR

210 samples from **76 patients**

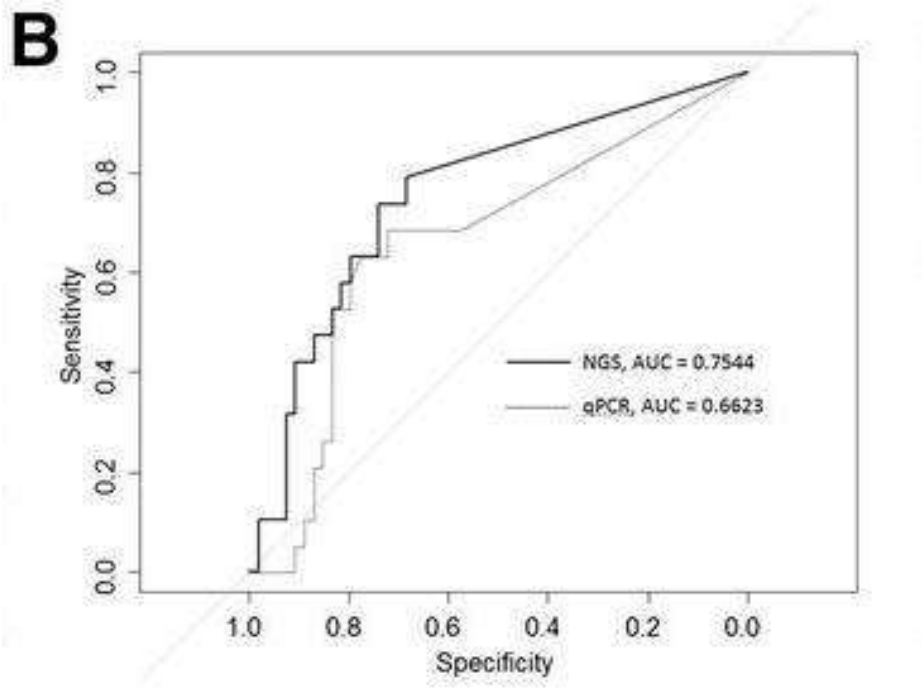
NGS provides a more complete picture than qPCR by assessing normal immune repertoire

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 Bakardjieva-Mihaylova, Karel Fiser, Jan Stuchly, Mathieu Giraud, Mikael Salson, Christiane Pott, Monika Brüggemann, Marc Fuligrabe, Jan Stary, Jan Trka[✉] and Eva Fronkova



Kotrova M, Muzikova K, Mejstrikova E, et al. The predictive strength of next-generation sequencing MRD detection for relapse compared with current methods in childhood ALL. *Blood*. 2015;126(8):1045-1047. doi:10.1182/blood-2015-07-655159

NGS MRD vs. Flow Cytometry

For Internal Use only

56 patients with B-cell ALL

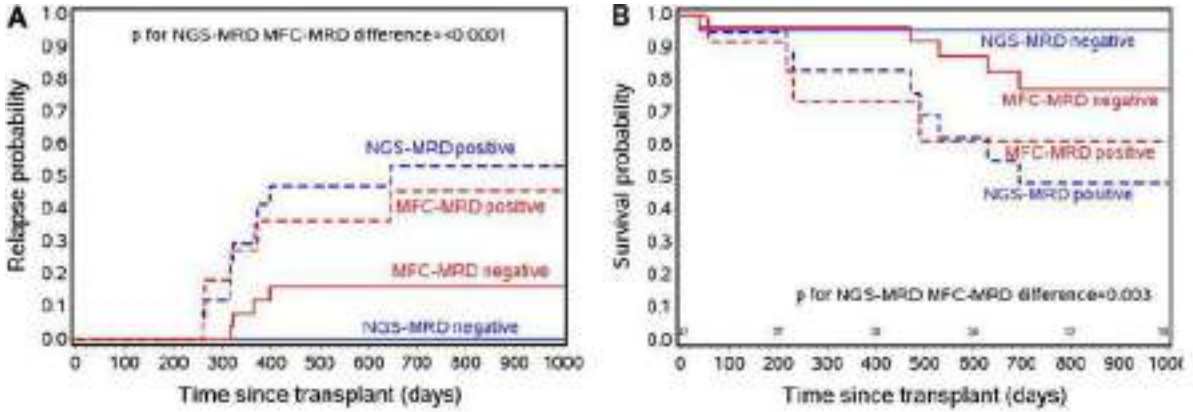
Direct comparison of 6 color flow cytometry to Next Generation Sequencing

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



Pulsipher MA, Carlson C, Langholz B, et al. IgH-V(D)J NGS-MRD measurement pre- and early post-allotransplant defines very low- and very high-risk ALL patients. *Blood*. 2015;125(22):3501-3508. doi:10.1182/blood-2014-12-615757

LymphoTrack® Assay Performance

LymphoTrack® Assays

Performance of LymphoTrack® Assays in dilution experiments

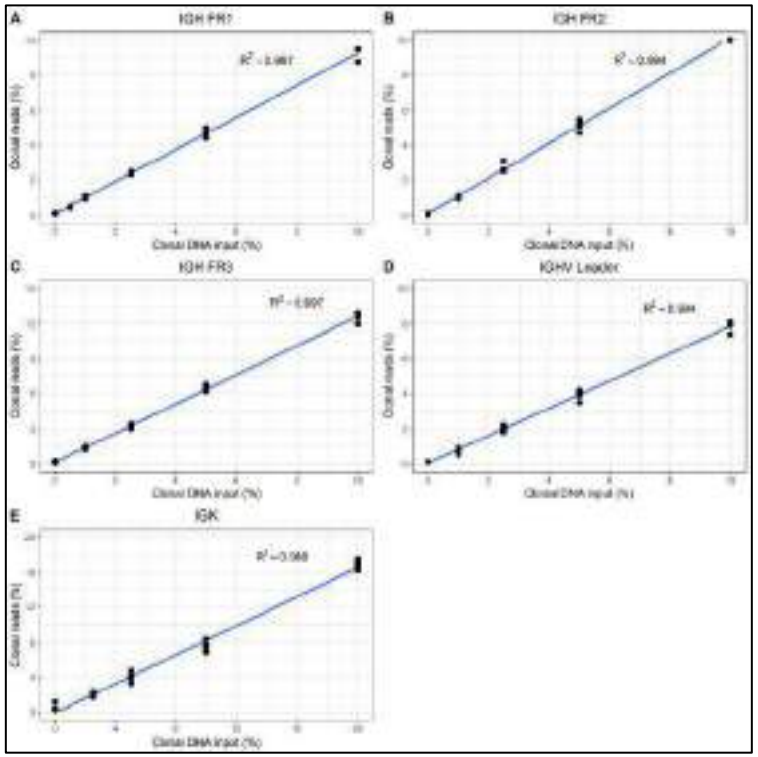
Demonstrates linear performance of all LymphoTrack® assays

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



Rustad EH, Hultcrantz M, Yellapantula VD, et al. Baseline identification of clonal V(D)J sequences for DNA-based minimal residual disease detection in multiple myeloma. PLoS One. 2019;14(3):e0211600. Published 2019 Mar 22. doi:10.1371/journal.pone.0211600

LymphoTrack® MRD vs. ASO-PCR

LymphoTrack® Assays

This study establishes a standardized experimental design to track MRD

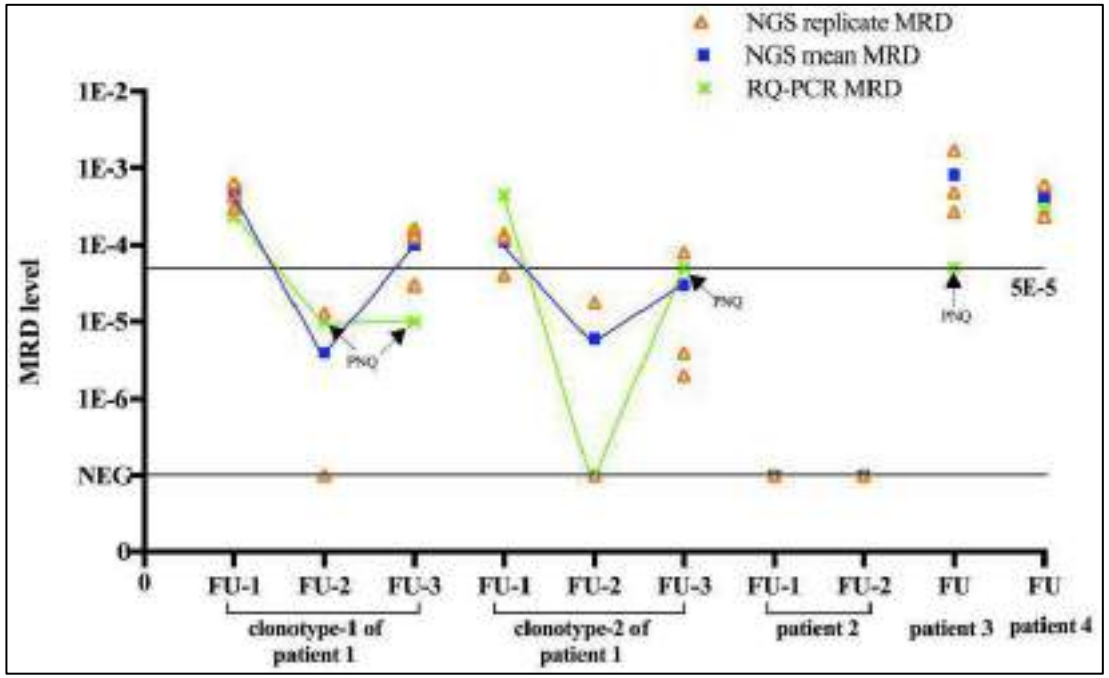
Verifies a sensitivity of **10^{-5}**

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,[1] Yinlei Bai,[2] Alberto Orfao,[3] and Chor Sang Chim[1,*]

Collaborative study involving institutions from Hong Kong, China and Spain



Yao Q, Bai Y, Orfao A, Chim CS. Standardized Minimal Residual Disease Detection by Next-Generation Sequencing in Multiple Myeloma. Front Oncol. 2019;9:449. Published 2019 Jun 6. doi:10.3389/fonc.2019.00449

invivoscribe® Improving Lives with Precision Diagnostics®

LymphoTrack® MRD vs Flow Cytometry

LymphoTrack® Assays

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

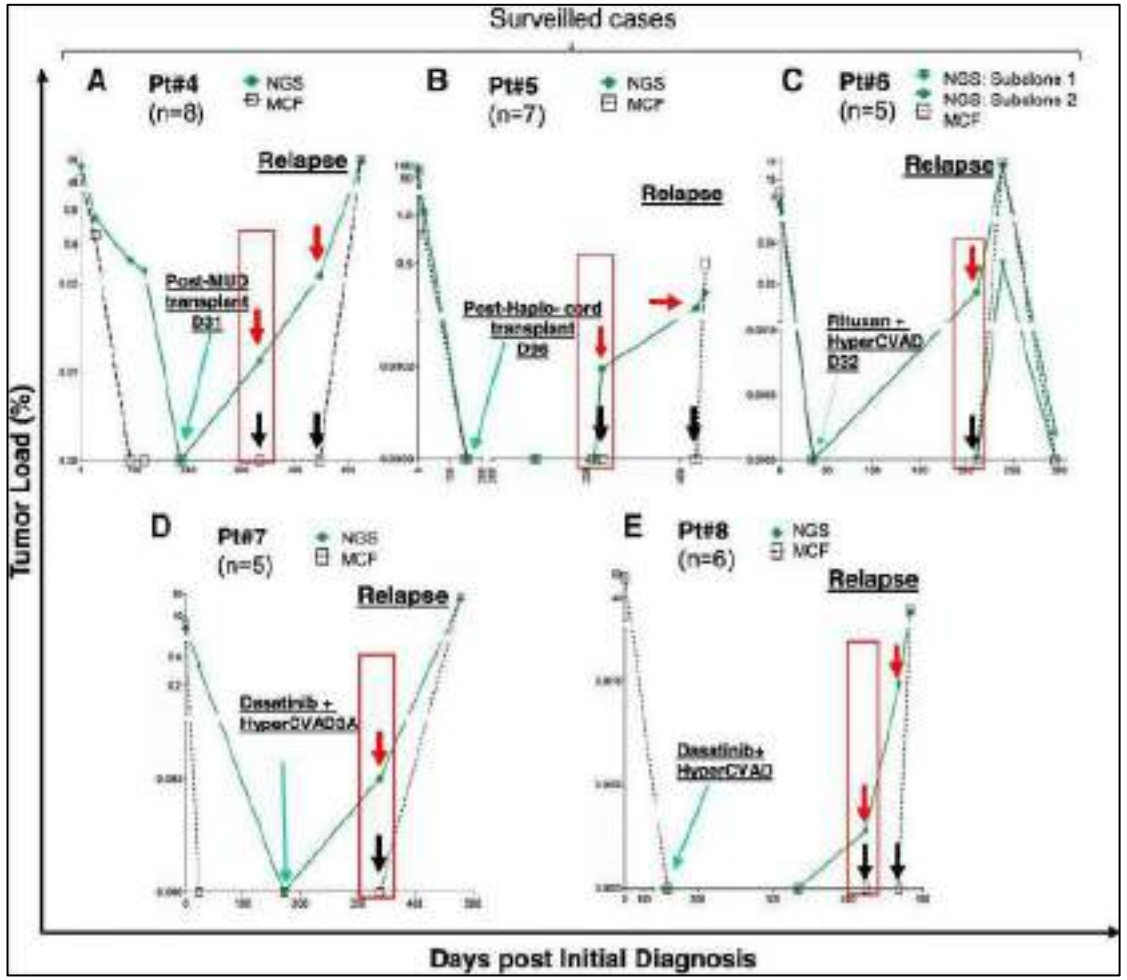
- NGS enables better risk stratification and earlier preemptive therapies against impending relapse, thus potentially improving outcome for B-ALL patients.

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[1], Inghirami G[1], Cheng S[2], Tam W[3].



Cheng S, Inghirami G, Cheng S, Tam W. Simple deep sequencing-based post-remission MRD surveillance predicts clinical relapse in B-ALL. J Hematol Oncol. 2018;11(1):105. Published 2018 Aug 22. doi:10.1186/s13045-018-0652-y

Case Study – B-ALL MRD

LymphoTrack® Assays

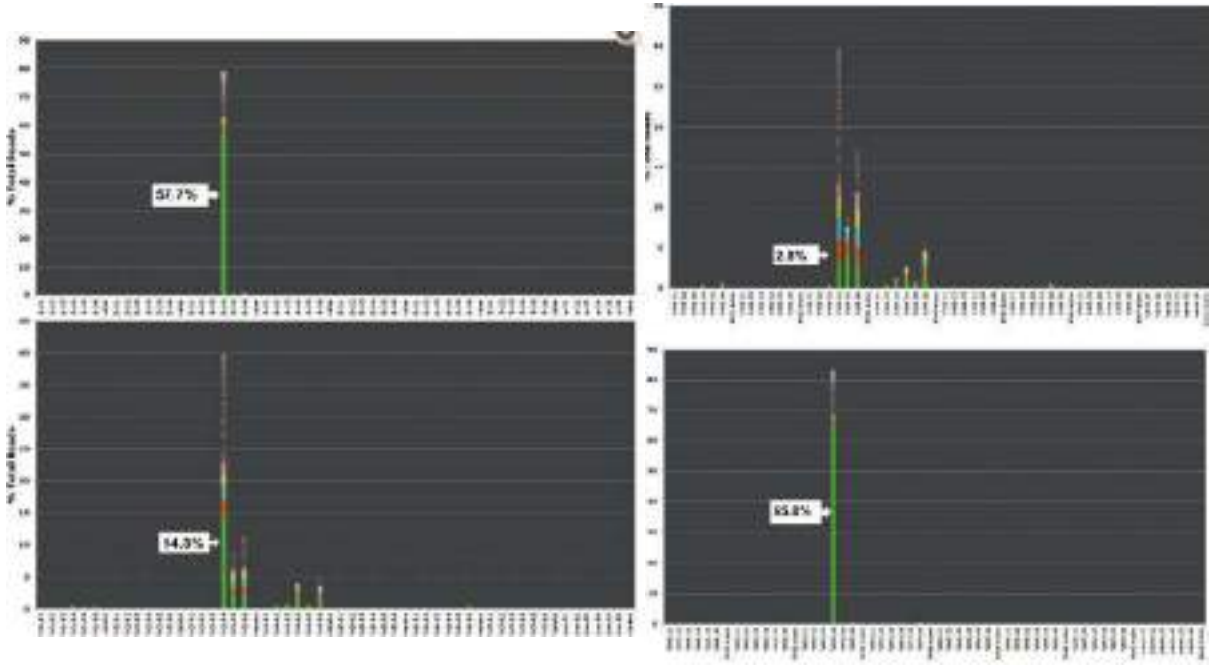
8 B-ALL patients with up to 3 timepoints each tracked with multiple methods

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

"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[1,2], Hwang IS[3], Kim J[1], Lee KA[1], Lee ST[4], Choi JR[5].



Shin S, Hwang IS, Kim J, Lee KA, Lee ST, Choi JR. Detection of Immunoglobulin Heavy Chain Gene Clonality by Next-Generation Sequencing for Minimal Residual Disease Monitoring in B-Lymphoblastic Leukemia. Ann Lab Med. 2017;37(4):331-335. doi:10.3343/alm.2017.37.4.331

Case Study – B-ALL MRD

LymphoTrack® Assays

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

Patient			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	Sex	Age (yr)	ID	Time†							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‡	Positive	44,001 (27.3, VH1-J4)	9,877 (6.0, VH1-J4)	9,765 (6.0, VH3-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‡	Positive	119,153 (23.0, VH3-J4)	114,278 (22.0, VH2-J4)	-	45.0	518,728
			2-2*	2 weeks	NA	Negative	ND	ND	ND	Negative	3,212 (0.7)	12,524 (2.6)		3.2	489,937
			2-3*	30 days	0.7	Negative	46,XY [20]	Negative	ND	Negative	0	0		0	298,579
3	F	2	3-1*	Diagnosis	91.4	77.0	Not interpretable	62.8	Negative‡	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 (0.5)	247 (0.2)		0.7	100,618
4	F	4	4-1*	Diagnosis	87.8	75.3	Not interpretable	75.3	Negative‡	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‡	Positive	207,993 (53.8, VH3-J6)	39,502 (10.2, VH3-J6)	-	64.0	386,903
			5-2*	2 weeks	0	Negative	46,XY [20]	Negative	ND	Negative	468 (0.1)	0		0.1	339,695
6	M	3	6-1*	Diagnosis	87.0	93.6	Not interpretable	76.8	Negative‡	Positive	172,072 (34.3, VH3-J4)	23,264 (7.3, VH3-J4)	-	61.6	316,654
			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;9)(q10;q10)[20]	96.3	Negative‡	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)	89.6	3.25§	Positive	8,565 (57.7, VH3-J4)	-	-	57.7	14,825
			8-2†	30 days	0	Negative	ND	ND	3.10×10^{-5}§	Negative	3,501 (14.3)			14.3	24,427
			8-3†	10 months	0.2	Negative	ND	Negative	Negative§	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[1]	83.1	4.36§	Positive	35,456 (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). ‡Days, weeks, or months after induction chemotherapy. §Five probe sets were tested by using the IGH/MYC/CEP8 tri-color dual fusion probe, BCR/ABL dual color dual fusion translocation probe, CDKN2A spectrum orange/CEP9 spectrum green probe, TEL/AML1 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. ¶Molecular analysis was done by using HemaVision kit (DNA technology, Aarhus, Denmark). #BCR-ABL1 transcript frequency (relative ratio of BCR-ABL1 to GAPDH) was quantified by using the LightCycler t(9;22) Quantification (Roche Molecular Systems, Branchburg, NJ, USA) kit. **Patient 8 relapsed at one year after diagnosis.
Abbreviations: NGS, next-generation sequencing; BM, bone marrow; ND, not done; NA, not available due to dry tap.

Shin S, Hw
Next-Gen
Med. 201

LymphoTrack® MRD vs Flow Cytometry

LymphoTrack® Assays

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

Strong **correlation of NGS and MFC**

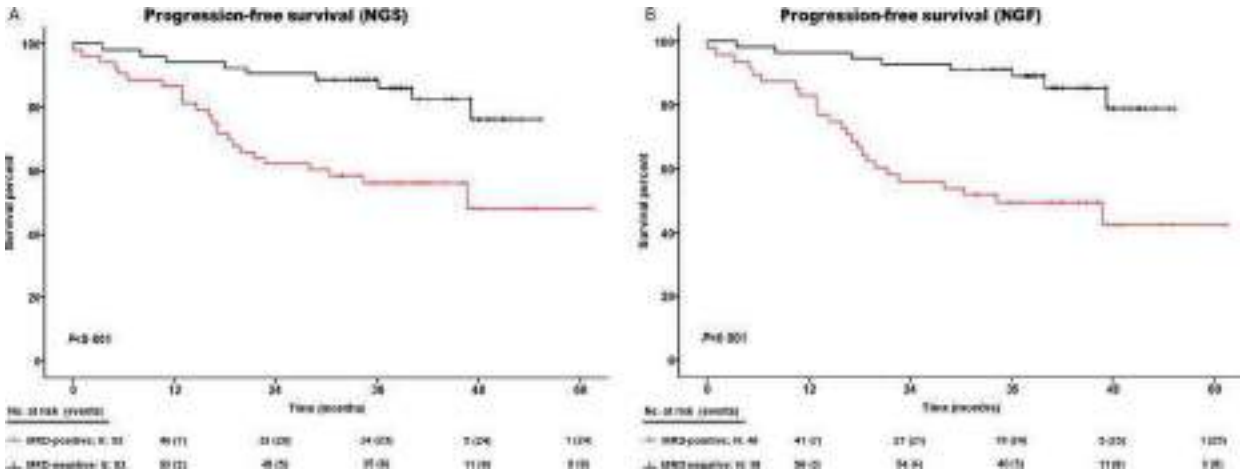
"interpretation of results is usually more difficult for **NGF, requiring high expertise**, while LymphoTrack's solution is more user-friendly and semi-automated."

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



invivoscribe
Improving Lives with Precision Diagnostics®

Medina A, Puig N, Flores-Montero J, et al. Comparison of next-generation sequencing (NGS) and next-generation flow (NGF) for minimal residual disease (MRD) assessment in multiple myeloma. *Blood Cancer J.* 2020;10(10):108. Published 2020 Oct 30. doi:10.1038/s41408-020-00377-0

LymphoTrack® Immune Repertoire

LymphoTrack Assays

Repertoire analysis is an exciting research application of LymphoTrack.

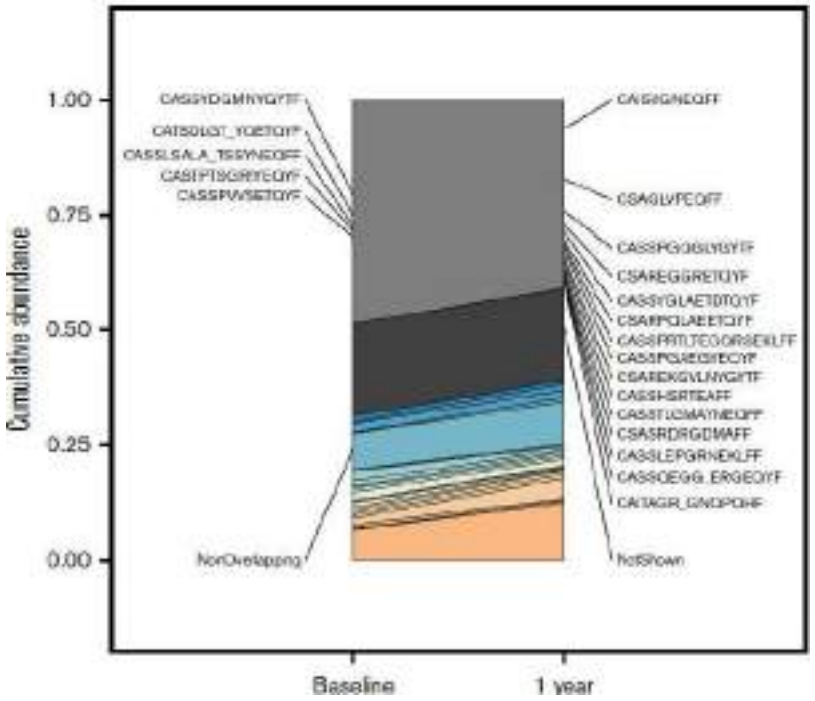
Studying the diversity of receptor sequences may lead to **new insights** into the **immune system**.

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,[1,2] Sasanka M. Handunnetti,[2,3] Mandy Ludford-Menting,[1,2] Chia Sharpe,[1,2] Piers Blombery,[2,3,4] Mary Ann Anderson,[2,3,5] Andrew W. Roberts,[2,3,5] John F. Seymour,[2,3] Constantine S. Tam,[2,3] David S. Ritchie,[1,2,3] and Rachel M. Koldej ⊠[1,2]



Davis JE, Handunnetti SM, Ludford-Menting M, et al. Immune recovery in patients with mantle cell lymphoma receiving long-term ibrutinib and venetoclax combination therapy. *Blood Adv*. 2020;4(19):4849-4859. doi:10.1182/bloodadvances.2020002810

invivoscribe® Improving Lives with Precision Diagnostics®

LeukoStrat® Assays

FLT3 References

invivoscribe®
Improving Lives with Precision Diagnostics®

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® 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.

FLT3 inhibitor	Non-FLT3 targets	FLT3-TKD mutation activity	Single-agent CRc rates in R/R *FLT3*-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 *FLT3*-mutated AML in combination with intensive chemotherapy (improves overall survival versus chemotherapy alone)
Quizartinib	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	AXL	Yes	37–41%	120 mg daily	Elevated transaminases, diarrhea	US FDA approved for adults with relapsed/refractory *FLT3*-mutated AML (full data not yet released)

Short NJ, Kantarjian H, Ravandi F, Daver N. Emerging treatment paradigms with FLT3 inhibitors in acute myeloid leukemia. Ther Adv Hematol. 2019;10:2040620719827310. Published 2019 Feb 15. doi:10.1177/2040620719827310

Midostaurin Trial

LeukoStrat®

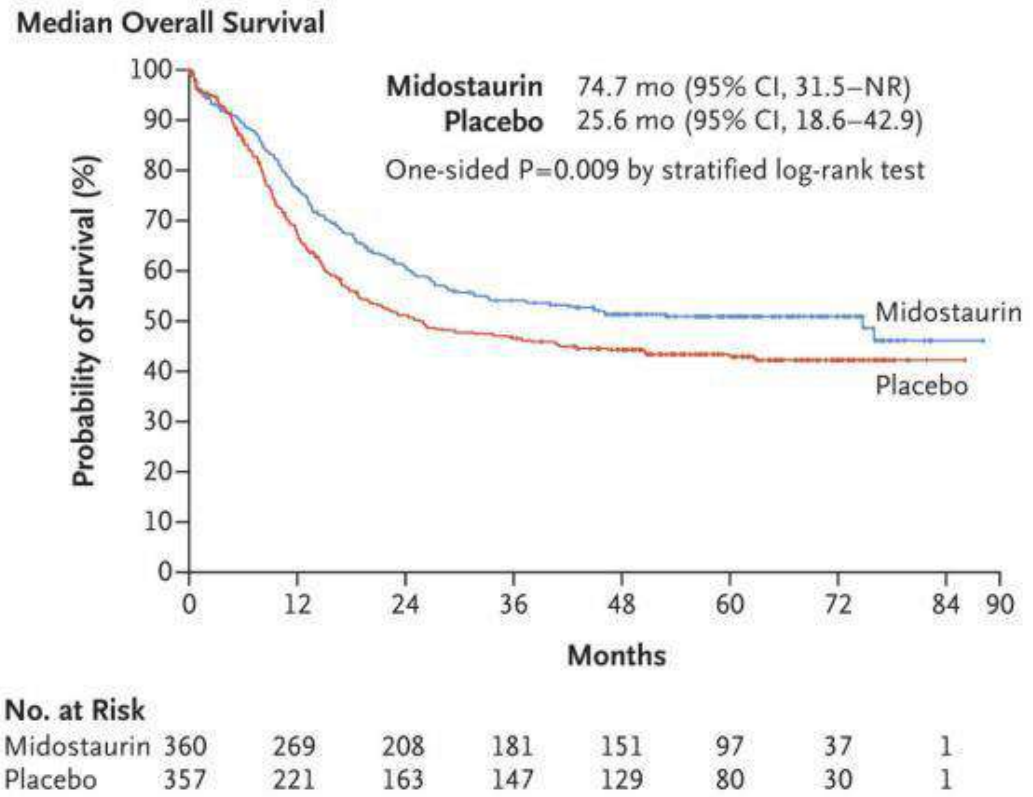
Clinical trial with 717 total patients

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



invivoscribe® Improving Lives with Precision Diagnostics®

Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med*. 2017;377(5):454-464. doi:10.1056/NEJMoa1614359

Cross-Validation of *FLT3* Assays

LeukoStrat®

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 Nomdedeu, MD PhD, Joop H. Jansen, Andrew H Wei, MBBS, PhD, Weiqiang Zhao, MD PhD, Xiaohong Li, Celine Pallaud, PhD, Eve Tiecke, PhD, Richard A. Larson, MD, Clara D. Bloomfield, MD, Hartmut Döhner, MD, Gerhard Ehninger, MD, Richard M. Stone, MD, Konstanze 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."**

invivoscribe® Improving Lives with Precision Diagnostics®

Thiede C, Prior TW, Lavorgna S, et al. FLT3mutation Assay Laboratory Cross Validation: Results from the CALGB 10603/Ratify Trial in Patients with Newly Diagnosed FLT3-Mutated Acute Myeloid Leukemia (AML). *Blood.* 2018 132:2800; doi: https://doi.org/10.1182/blood-2018-99-112127

Ratio of *FLT3*-ITD for Transplant Decisions

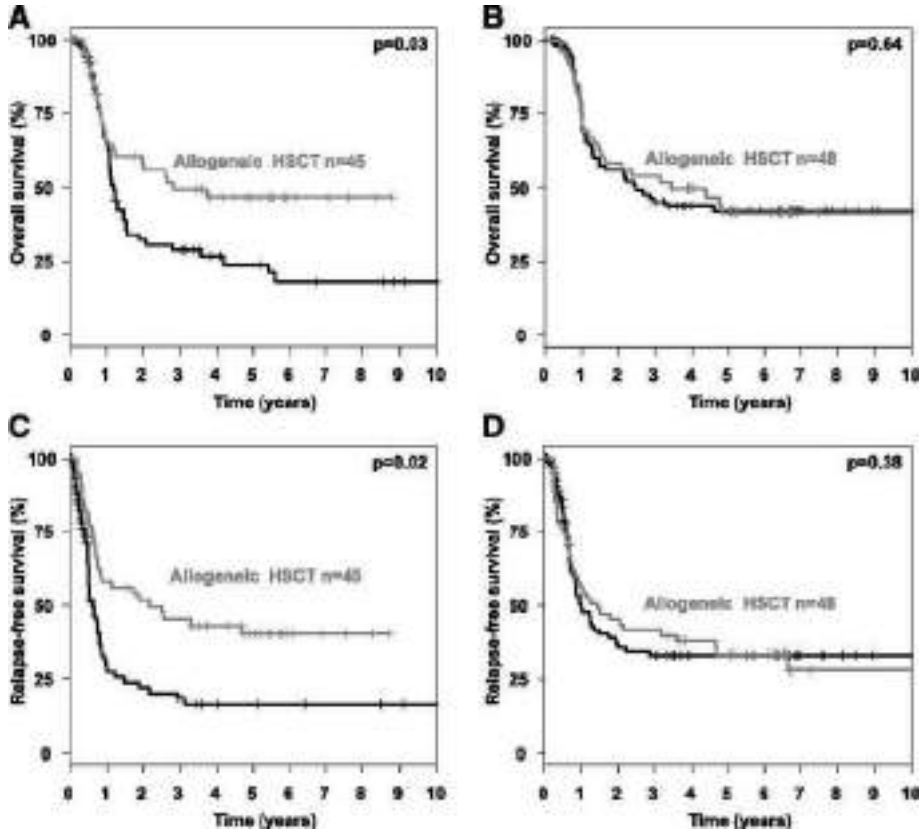
323 patients with *FLT3*-ITD positive AML

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



Schlenk RF, Kayser S, Bullinger L, et al. Differential impact of allelic ratio and insertion site in FLT3-ITD–positive AML with respect to allogeneic transplantation. *Blood*. 2014 Nov 27;124(23):3441-9. doi: 10.1182/blood-2014-05-578070.

Data for Gilteritinib in R/R AML

LeukoStrat®

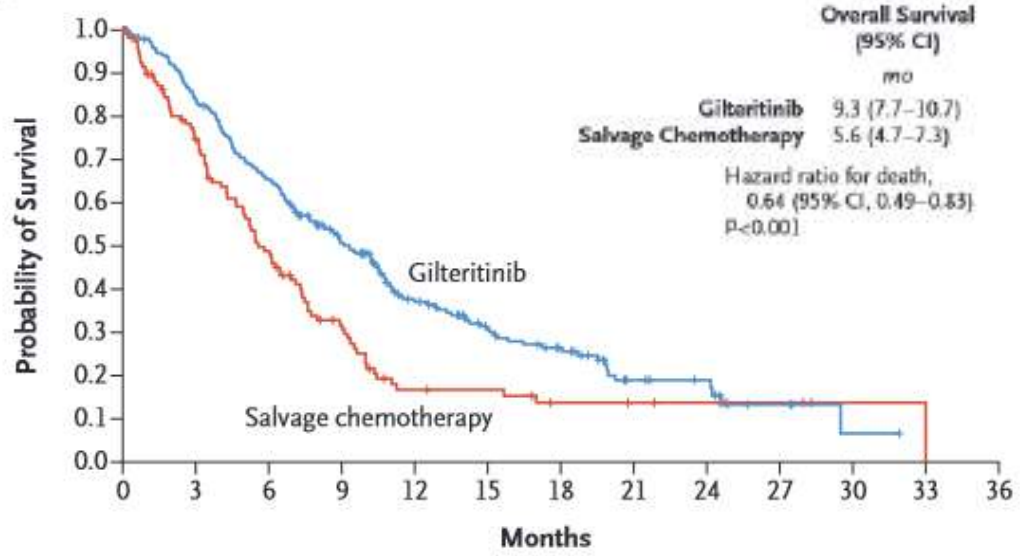
"**Gilteritinib resulted in significantly longer survival and** higher percentages of patients with **remission than salvage chemotherapy** among patients with relapsed or refractory **FLT3-mutated AML**" (ADMIRAL clinical trial number NCT02421939)

- 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. Levis

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



No. at Risk													
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

invivoscribe® Improving Lives with Precision Diagnostics®

Perl AE, Martinelli G, Cortes JE, et al. Abstract CT184: Gilteritinib significantly prolongs overall survival in patients with FLT3-mutated (FLT3mut+) relapsed/refractory (R/R) acute myeloid leukemia (AML): Results from the Phase III ADMIRAL trial. *Cancer Res.* 2019;79(13 Suppl); https://doi.org/10.1158/1538-7445.AM2019-CT184

FLT3-ITD MRD - LabPMM

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[1], Perl AE[2], Altman JK[3], Gocke CD[1], Bahceci E[4], Hill J[4], Liu C[4], Xie Z[5], Carson AR[5], McClain V[5], Stenzel TT[5], Miller JE[5].

Figure 1. Linearity of NGS-MRD assay

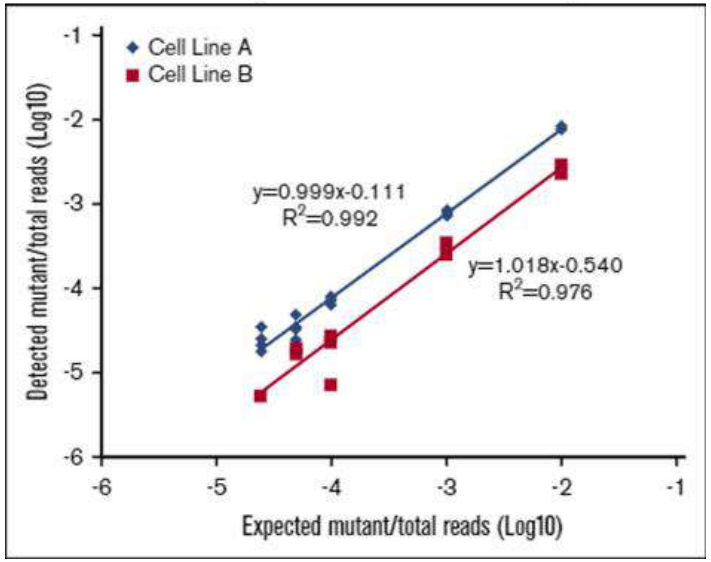
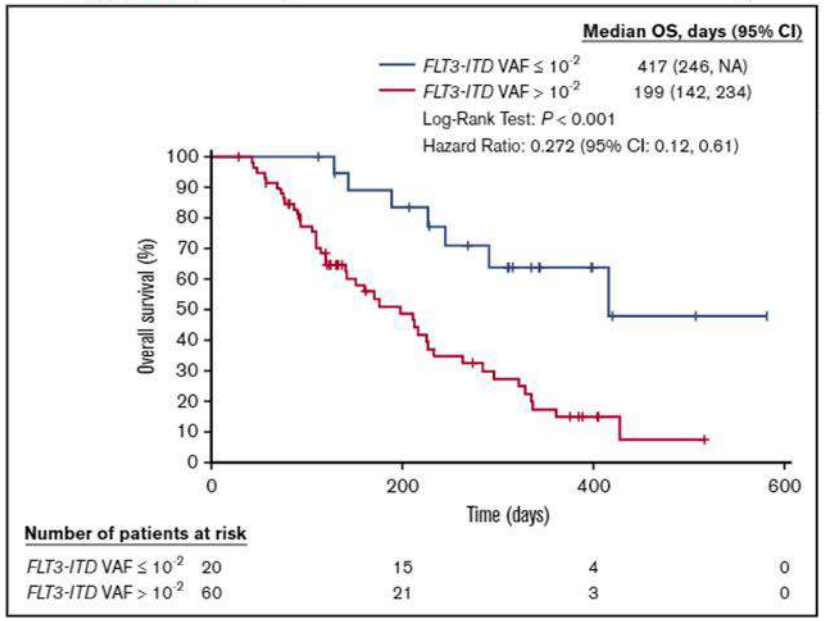


Figure 2. Subject OS in the CHRYSALIS study



Levis MJ, Perl AE, Altman JK, et al. A next-generation sequencing-based assay for minimal residual disease assessment in AML patients with FLT3-ITD mutations. Blood Adv. 2018;2(8):825-831. doi:10.1182/bloodadvances.2018015925

invivoscribe®
Improving Lives with Precision Diagnostics®

References

External Posters/Abstracts

Multiple Myeloma MRD

LymphoTrack® Assays

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

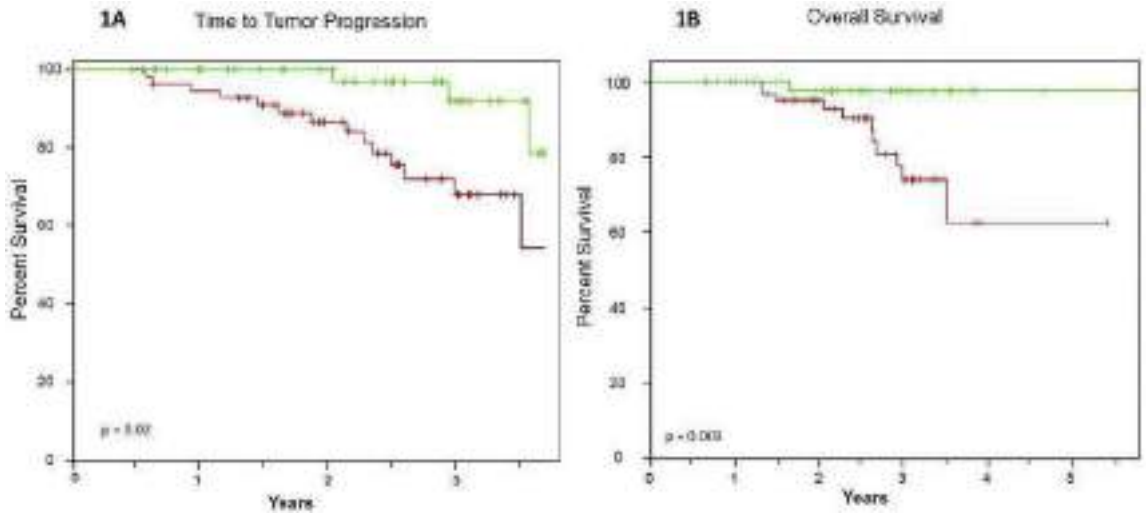
High **correlation of NGS and MFC**

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

"The **applicability** of the LymphoTrack® 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 González, Maria Jose Calasanz, Rosa M. Ayala, Maria E Sarasquete, Bruno Paiva, Maria Teresa Cedena, Marta Fernández-Mercado, Inmaculada Rapado, Laura Rosinol, Enrique M. Ocio, Albert Oriol, Miguel Alcoceba, Miguel T Hernández, Rafael Martinez Martinez, Maria-Victoria Mateos, Juan-José Lahuerta, Joan Bladé, Alberto Orfao, Jesus F. San Miguel, Joaquin Martínez-López, and Ramón García-Sanz



invivoscribe® Improving Lives with Precision Diagnostics®

Medina A, Jimenez C, Puig N, et al. New Alternatives for the Evaluation of Minimal Residual Disease (MRD) Detection By Next Generation Sequencing in Multiple Myeloma. *Blood.* (2017) 130 (Supplement 1): 1783. https://ashpublications.org/blood/article/130/Supplement%201/1783/79532/New-Alternatives-for-the-Evaluation-of-Minimal

LymphoTrack® vs. CE and FC

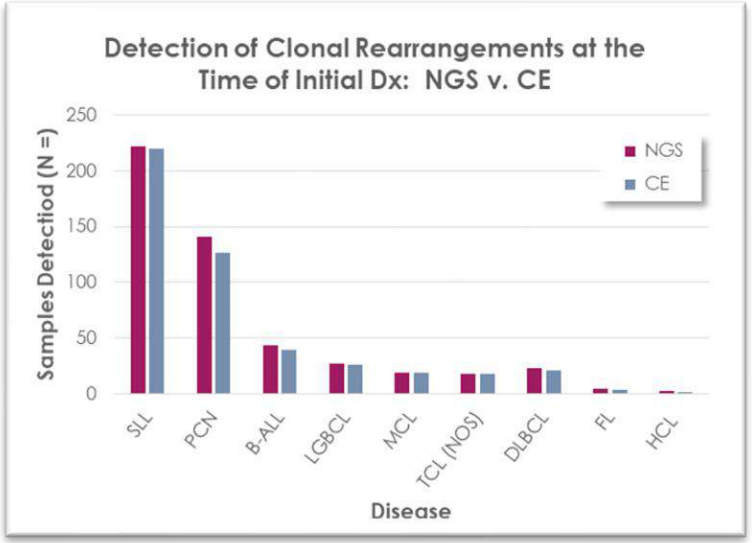
LymphoTrack® Assays

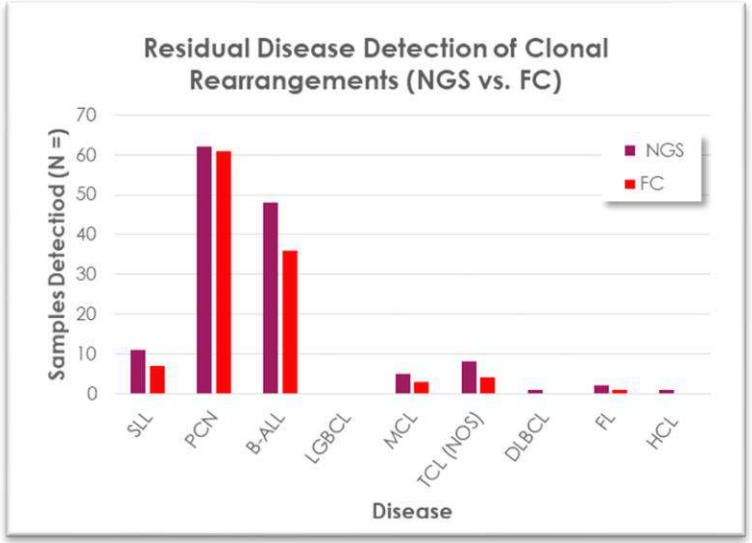
Laboratory reports that LymphoTrack® detected clonal **rearrangements in 94% of Dx cases vs. 89% by CE.**

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, MDPhD, Jae H. Park, MD, Ola Landgren, MD PhD, Ahmet Dogan, MD PhD, Khedoudja Nafa, PhD





Arcilla et al. Next Generation Sequencing (NGS) Based IGH and TCR Clonality Assays Provide Excellent Specificity and Sensitivity for Routine Clonal Characterization and Monitoring of Lymphoproliferative Disorders
622. Lymphoma Biology-Non—Genetic Studies: Poster III | December 7, 2017
http://www.bloodjournal.org/content/130/Suppl_1/4017

Development of Clonal Populations

LymphoTrack® Assays

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

In one patient, LymphoTrack® 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 Blaine Geyer, MD, Brian C. Shaffer, MD, Heather Landau, MD, Hani Hassoun, MD, Christopher Famulare, MS, Mikhail Roshal, MDPhD, 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-AlloHCT	AlloHCT Type	Relapse of B-ALL?	B-ALL Dx to Last F/U (months)	Last Dx
1	66.1	FISH: MLL Rearrangement, loss of signal for 3' MLL	Unknown	VPDCy	Dauno, ARAC, then AlloHCT	MRD+ CR	10/10 MUD	N	41.1	Alive in MRD- CR
2	66.2	Karyotype: -7, t(9;12)	Unknown	VPDCy	AlloHCT	MRD- CR	10/10 MRD	N	38.7	Alive in MRD- CR
3	60.4	Normal	Unknown	VPDCy	Dauno, ARAC, then AlloHCT	CR	CD34-Selected 10/10 MRD	N	7.1	Died in CR from complications of GVHD, septic shock
4	61.3	FISH: del13q14.3, del12p36, single copy of TP53, CEP 17	RB1 G426* (subclonal), CREBBP loss, CBL L380F	VPCy	POMP	MRD- CR	None	N	37.1	Developed MDS. Died without known evidence of B-ALL
5	64.1	Normal	P2RY8-CRLF2 fusion, IDH2 R140Q, SH2B3 E573K	VPDA	POMP, then AlloHCT	MRD- CR	10/10 MUD	N	10.9	Alive in MRD- CR
6	63.7	FISH: Complex	Loss of PLZF exons 2-3, RB1 E323* (subclonal), RB1 P568fs*5, TP53 C242S (subclonal), TP53 I195F (subclonal), TP53 K132* (subclonal), TP53 P139T	HyperCVAD	HyperCVAD, POMP*, then AlloHCT	MRD- CR	10/10 MRD	N	10.3	Alive in MRD- CR
7	68.9	Unknown	Unknown	Augmented HyperCVAD	Blinatumomab	MRD- CR	None	N	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 Q889*, GRIN2A T116M	Len discontinuation alone	Len discontinuation alone	MRD+ CR	None	N	7.0	Alive with MRD on observation alone

Legend: Dx=Diagnosis, VPDCy (or elements thereof)=vincristine/prednisone, doxorubicin, cyclophosphamide, VPDA=vincristine, prednisone, daunorubicin, pegaspargase, HyperCVAD=hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone, Dauno=daunorubicin, ARAC=cytarabine, POMP=prednisone, vincristine, methotrexate, mercaptopurine, Len=lenalidomide, MRD=minimal residual disease, CR=complete response, AlloHCT=allogeneic hematopoietic cell transplantation, 10/10 MRD/MUD=10/10 HLA-matched related/unrelated donor, NE=not evaluable

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

invivoscribe® Improving Lives with Precision Diagnostics®

Blaine Geyer et al. Clinical and Pathological Features of B-Cell Acute Lymphoblastic Leukemia Following Maintenance Treatment with Lenalidomide for Multiple Myeloma 612. Acute Lymphoblastic Leukemia: Clinical Studies: Poster I | December 7, 2017
http://www.bloodjournal.org/content/130/Suppl_1/4017

TRG Sequencing in AITL

LymphoTrack® Assays

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® *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

Marks et al. H 624. Hodgkin Lymphoma and T/NK Cell Lymphoma—Clinical Studies: Poster I | December 7, 2017 High Throughput T Cell Receptor Sequencing Augments Diagnosis and Response Assessment in Patients with Angioimmunoblastic T Cell Lymphoma
https://ashpublications.org/blood/article/130/Supplement%201/1526/79520/High-Throughput-T-Cell-Receptor-Sequencing

invivoscribe®
Improving Lives with Precision Diagnostics®

References

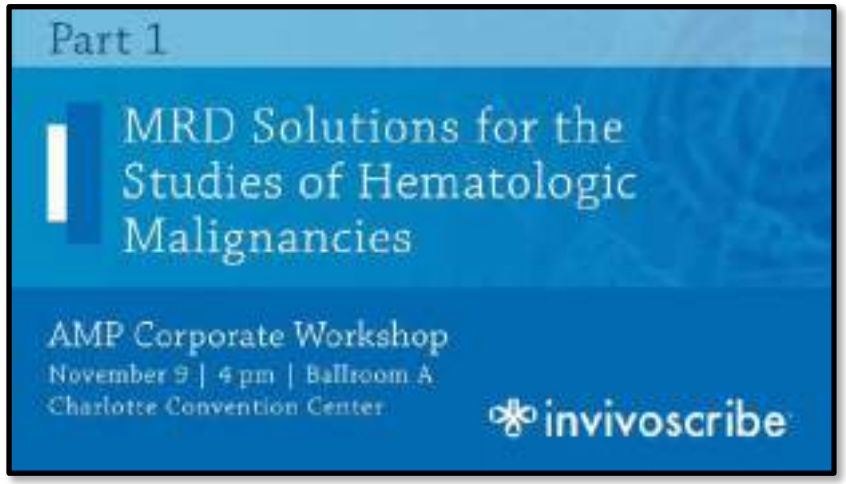
Videos

AMP 2016 Symposium Video 1

- Highlights of talk by Dr. Maria Arcila

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

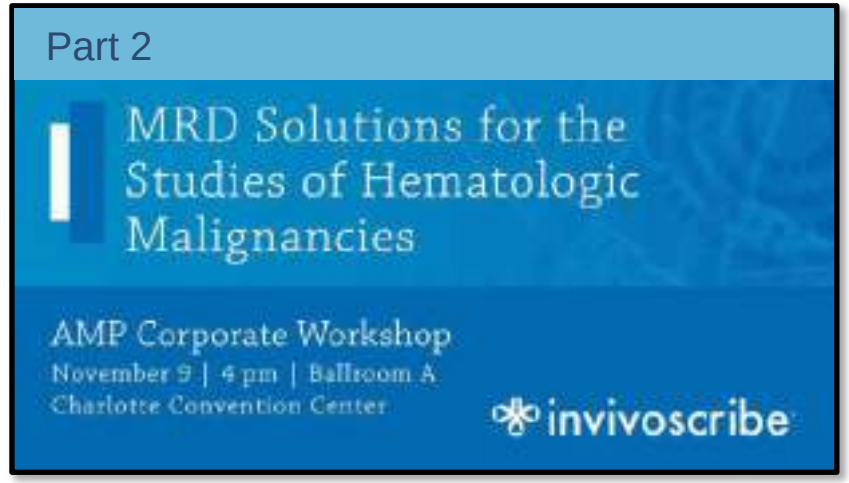
- Her lab's approach to studies of minimal residual disease (MRD) with the use the NGS-based LymphoTrack® *IGH* & *TRG* Assays.
- Comparisons between flow and NGS methods
- Cases from multiple disease states



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

AMP 2016 Symposium Video 2

- 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

AMP Global 2017 Symposium Video

- 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® Assays

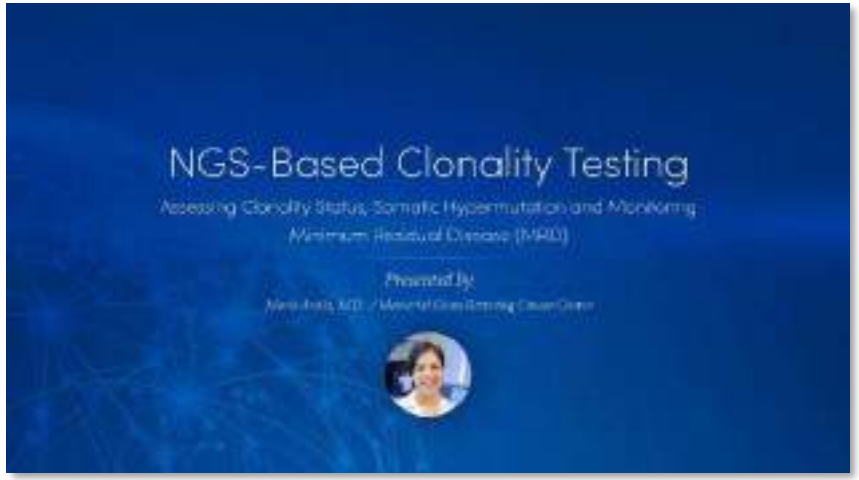
https://www.youtube.com/watch?v=YtzcK01ovfg

Invivoscribe's Webinar

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

AMP Global 2019 Symposium

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

- Bettina Budeus

  Group Molecular Genetics, Essen University Hospital, Essen, Germany

https://vimeo.com/340315124

AMP Global 2019 Symposium

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

AMP Global 2019 Symposium

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

AMP Global 2019 Symposium

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

- Piers Blombery

  Peter MacCallum Cancer Centre, Melbourne, Australia

https://vimeo.com/341475803

ASCO 2019

Invivoscribe LeukoStrat® CDx *FLT3* Mutation Assay

- Dr. Bradley Patay

  Invivoscribe, CMO, San Diego, USA

https://vimeo.com/347596491

Invivoscribe MRD Webinar

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

EHA 2019

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

EHA 2019

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

EHA 2019

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

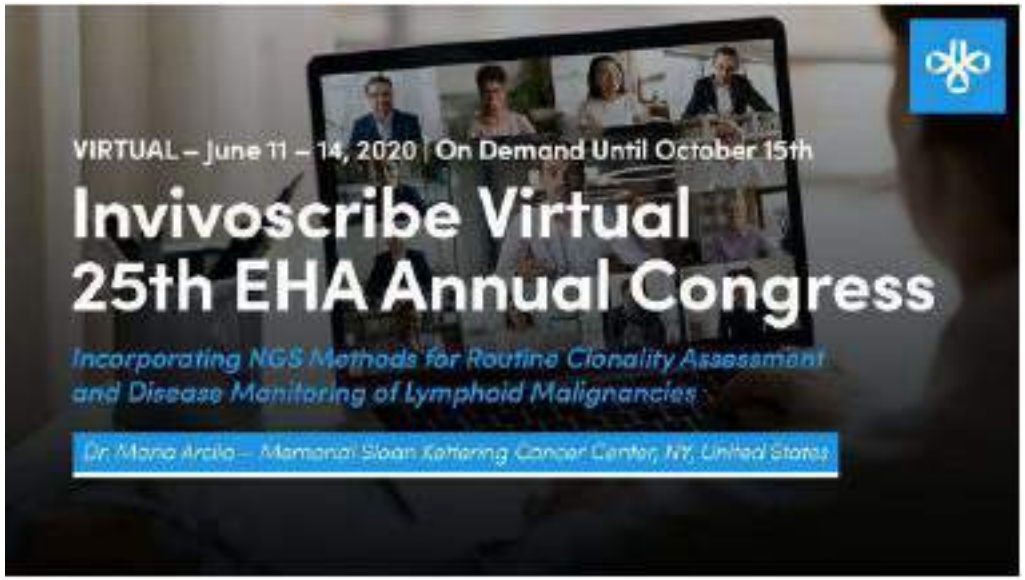
- Fiona Quinn
  Cancer Molecular Diagnostics Dept, St. James's Hospital, Dublin, Ireland

https://vimeo.com/356284961

EHA 2020 Virtual Symposium. Video 1

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

EHA 2020 Virtual Symposium. Video 2

Clonality Analysis with NGS is the New Frontier in MRD Detection

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

https://vimeo.com/428592492

EHA 2020 Virtual Symposium. Video 3

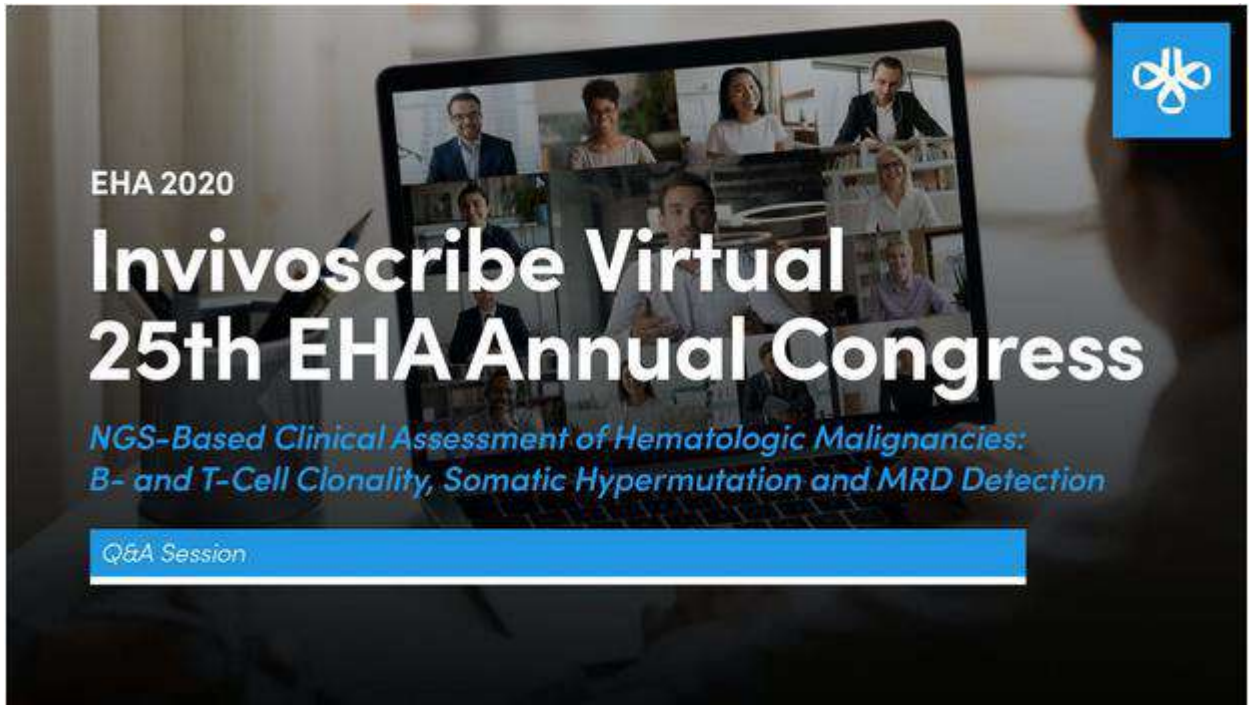
Next Generation Analysis in CLL and Other Malignancies

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

https://vimeo.com/428594643

EHA 2020 Virtual Symposium. Q&A

Q&A session

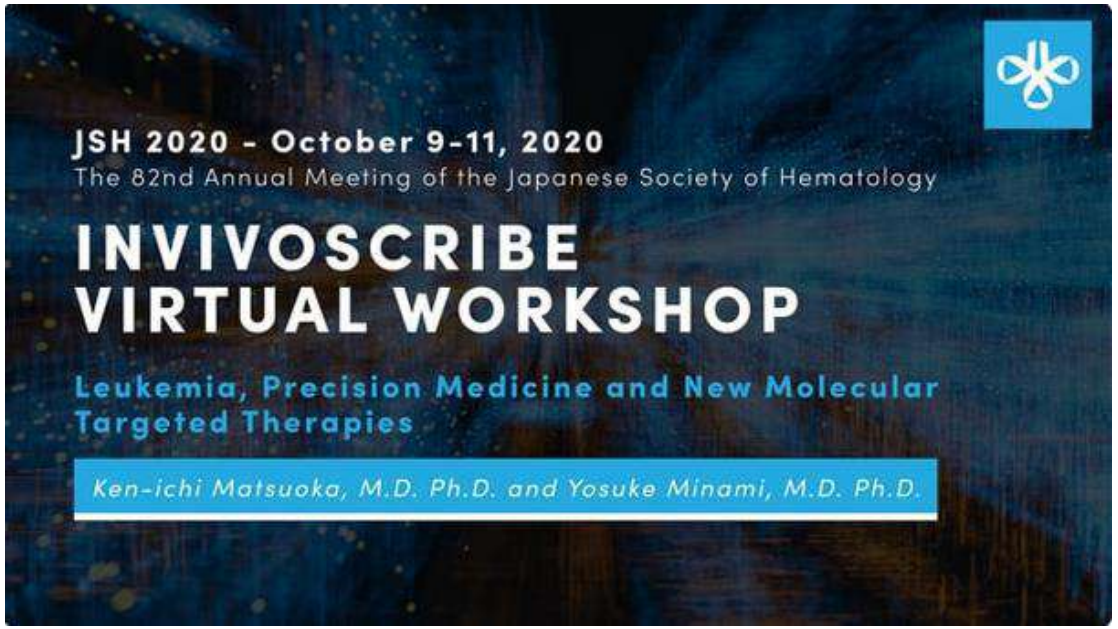


https://vimeo.com/430153275

JSH 2020 Virtual Workshop

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

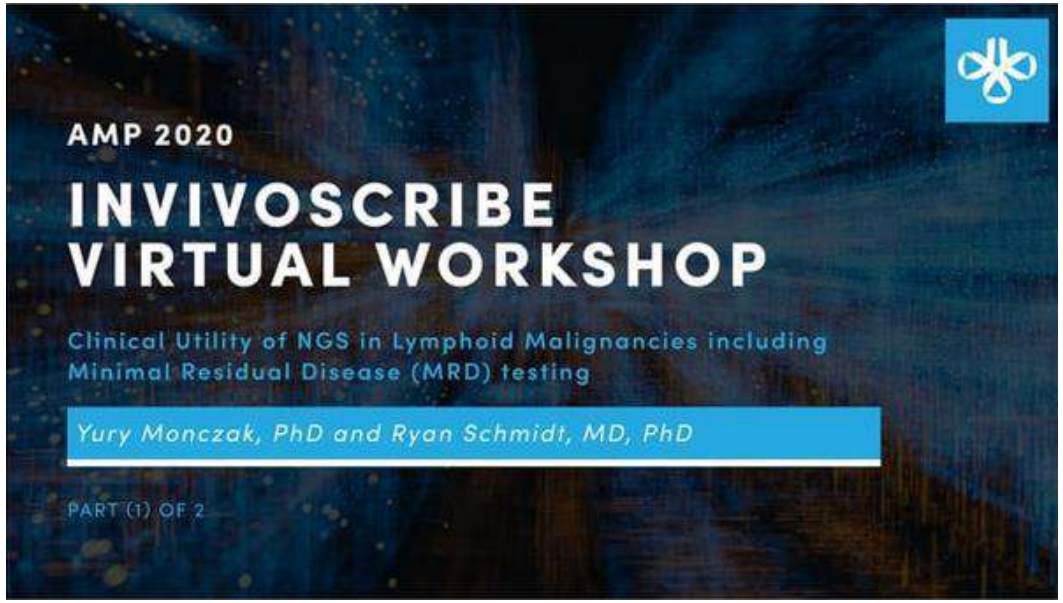


https://vimeo.com/481393310

AMP 2020 Virtual Workshop #1

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

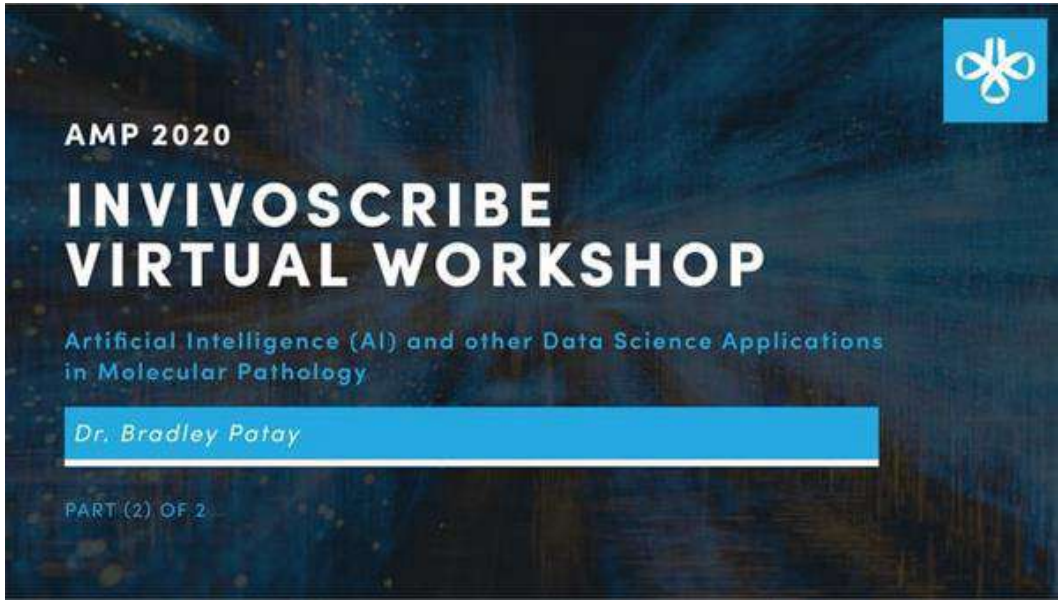


https://vimeo.com/480863214

AMP 2020 Virtual Workshop #2

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

invivoscribe®
Improving Lives with Precision Diagnostics®

References

Invivoscribe Posters

EHA 2015

https://invivoscribe.com/Posters/2015/EHA/EHA_2015_IGK_IGH_P540_HiRes_For_Print.pdf

LabPMM
invivoscribe

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

Niels C. Adams*[1], Gillian M. Pawlowsky[1], Ying Huang[2], Kasey Hutt[2], Michael Klass[2], Jeffrey Miller[1,2]

[1]LabPMM GmbH, Munich, Germany; [2]Invivoscribe Technologies, Inc., San Diego, CA, United States

Introduction

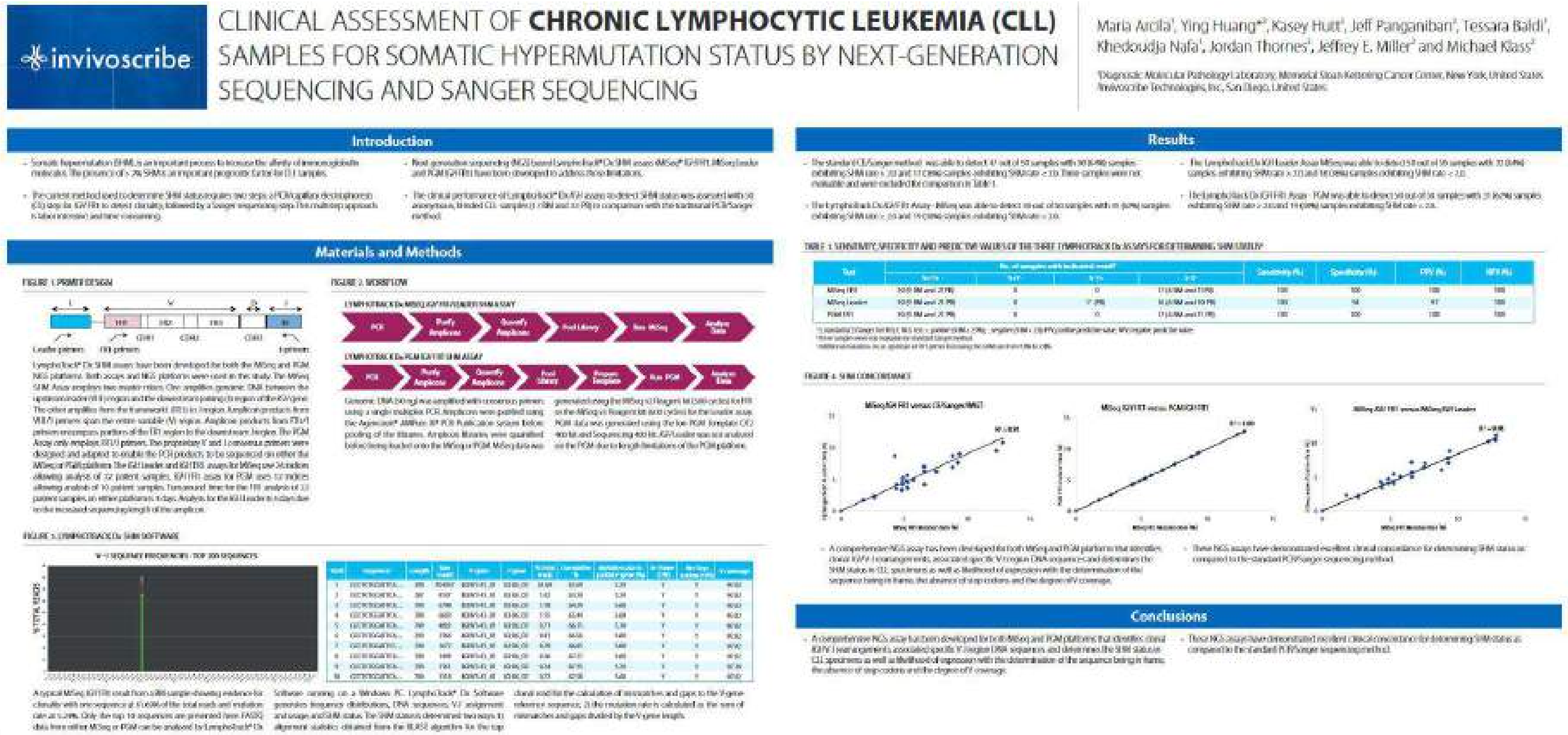
Methods

Results

Conclusions

EHA 2015

https://invivoscribe.com/Posters/2015/EHA/EHA%202015__CLL_P212_HiRes_For_Print_REVISED.pdf



AMP 2015

https://invivoscribe.com/Posters/2015/AMP/NGS_TCRG_Poster_AMP-2015_20151102_MarkE.pdf

Next-Generation Sequencing for Detection of Clonal *TRG* Gene Rearrangements Shows Improved Specificity and Positive Predictive Value Compared to Fragment Analysis Using BIOMED-2 Primers and Capillary Electrophoresis

invivoscribe

Mark D. Ewalt[1,2], Michael Kloss[2], Jeff Pangonilan[2], Ying Huang[2], Tim Stenzel[2], Kasey Hutt[2], Lisa Ma[1], Daniel A. Arber[1], Jason D. Merker[1], Iris Schrijver[1], James L. Zehnder[1]

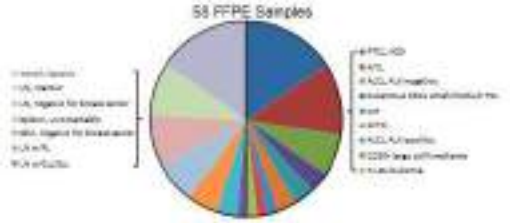
[1]Department of Pathology, Stanford University Medical Center, Stanford, CA USA; [2]Department of Pathology, Cedars-Sinai Medical Center, Los Angeles, CA USA; [3]Invivoscribe® Technologies, Inc., San Diego, CA USA

Introduction

During T cell development, somatic rearrangements of T cell receptor gamma (TRG) genes generate unique V-J rearrangements within each cell. Over-represented *TRG* rearrangements can be identified in the majority of T cell and some B cell malignancies, but are generally not seen in benign reactive processes. PCR-based capillary electrophoresis (PCR-CE) assays are the current gold standard for detecting clonal rearrangements. In this study, we evaluated the Invivoscribe® LymphoTrack® *TRG* clonality assay using the Illumina® MiSeq® to compare the performance of Targeted Next-Generation Sequencing (T-NGS) to the gold standard PCR-CE assay. We also sought to compare the performance of the T-NGS assay in two separate laboratories to assess inter-laboratory reproducibility.

Methods

DNA was isolated from 58 FFPE samples that had previously been evaluated in the Pathology Department at Stanford University Medical Center and evaluated by PCR-CE for TRG gene rearrangement (24 T cell lymphoproliferative disorders, 28 reactive tissues, and 3 lymph nodes involved by B cell lymphoma). T-NGS was performed using the Invivoscribe LymphoTrack *TRG* – MiSeq assay according to the manufacturer's instructions at Stanford University Medical Center and LabPMM®, a subsidiary of Invivoscribe. T-NGS data was analyzed by an Invivoscribe-developed bioinformatics pipeline and results were interpreted using numerical criteria, blinded to results of PCR-CE and histopathologic diagnosis.



Results

Results for the 58 samples were evaluated for sensitivity, specificity, concordance, positive predictive value (PPV), and negative predictive value (NPV). T-NGS analysis was compared between Stanford and LabPMM and showed 91% concordance. 5 cases were discordant; however, 4 of 5 cases identified many of the same clonal sequences with slight differences in frequency.

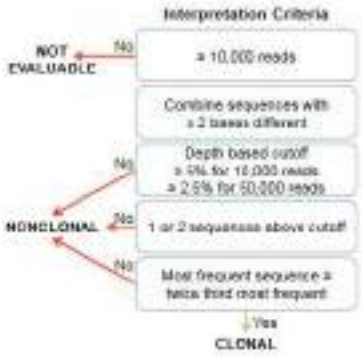
Separately, histopathologic diagnosis was considered the reference and PCR-CE was compared to T-NGS. PCR-CE as compared to T-NGS showed similar sensitivity (88% vs. 79-88%), concordance (88% vs. 90-93%), and NPV (86% vs. 83-90%). In contrast, PCR-CE showed lower specificity (63% vs. 97-100%) and PPV (63% vs. 96-100%).

Results

LabPMM

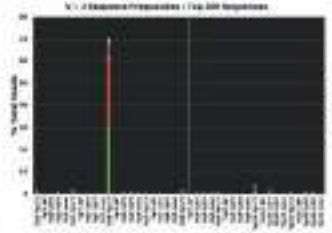
Stanford	Positive	Negative
Positive	19	0
Negative	5	31

Concordance Rate = 91%

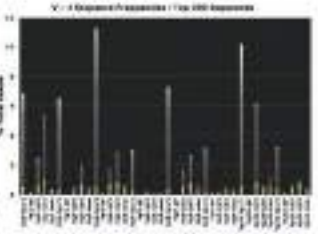


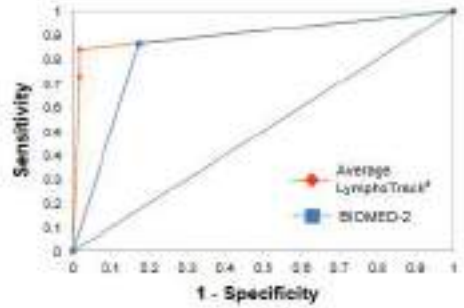
Test	Lab	Sensitivity	Specificity
LymphoTrack®	Stanford	79%	100%
	LabPMM	88%	97%
BIOMED-2		88%	83%

Sample Positive Case



Sample Negative Case





Conclusions

The Invivoscribe® LymphoTrack® *TRG* assay shows good inter-laboratory reproducibility and similar sensitivity, concordance, and NPV to PCR-CE when using histopathologic diagnosis as a reference. In contrast, T-NGS shows a higher specificity and PPV than PCR-CE. In addition, T-NGS offers the potential to follow specific clonal sequences for monitoring of minimal residual disease in T cell malignancies. Given this potential benefit and the superior assay performance demonstrated by our data, T-NGS represents an exciting advance in the diagnosis of clonal lymphoproliferative disorders.

Disclosures

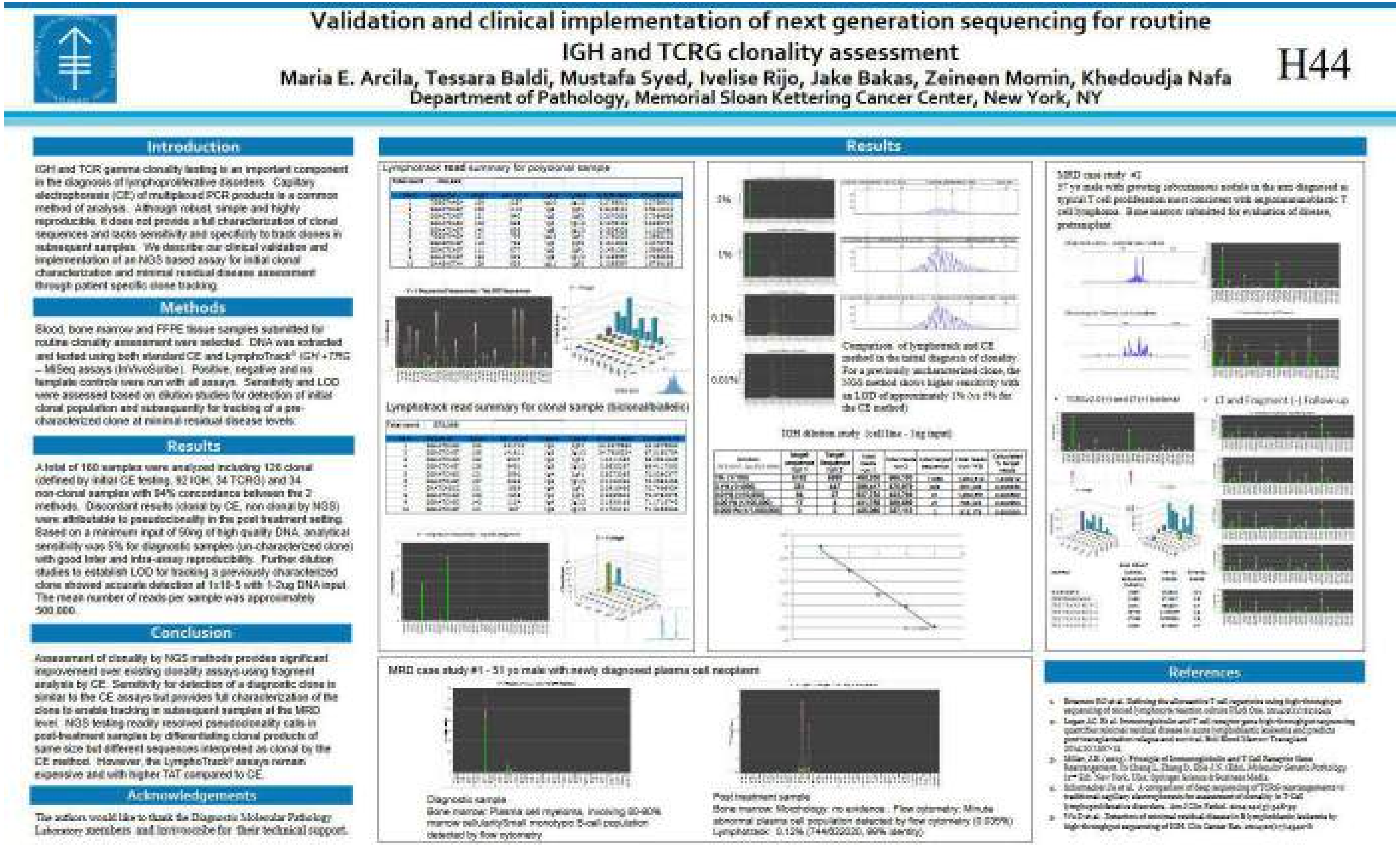
MK, JP, YH, TS, and KH are employees of Invivoscribe®.

ME has received an honorarium and travel expenses from Invivoscribe®.

LymphoTrack® *TRG* Assays are for research use only (RUO). Not for use in diagnostic procedures.

AMP 2015

https://invivoscribe.com/Posters/2015/AMP/Lymphotrack_AMP_2015_Arcila%20Nafa.pdf



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

Maria E. Arcila, Tessara Baldi, Mustafa Syed, Ivelise Rijo, Jake Bakas, Zeineen Momin, Khedoudja Nafa
Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY

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® IGH + TRG – 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 160 samples were analyzed including 126 clonal (defined by initial CE testing, 92 IGH, 34 TCRG) and 34 non-clonal samples with 94% concordance between the 2 methods. Discordant results (clonal by CE, non clonal by NGS) were attributable to pseudoclonality 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 1x10-5 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 pseudoclonality 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® 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 (biclonal/biallelic)

Comparison of lymphotrack and CE method in the initial diagnosis of clonality. For a previously uncharacterized clone, the NGS method shows higher sensitivity with an LOD of approximately 1% vs 5% for the CE method.

IGH dilution study (cell line - 1ug input)

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

Diagnostic sample
Bone marrow: Plasma cell myeloma, involving 80-90% marrow cellularity. Small monotypic B-cell population detected by flow cytometry

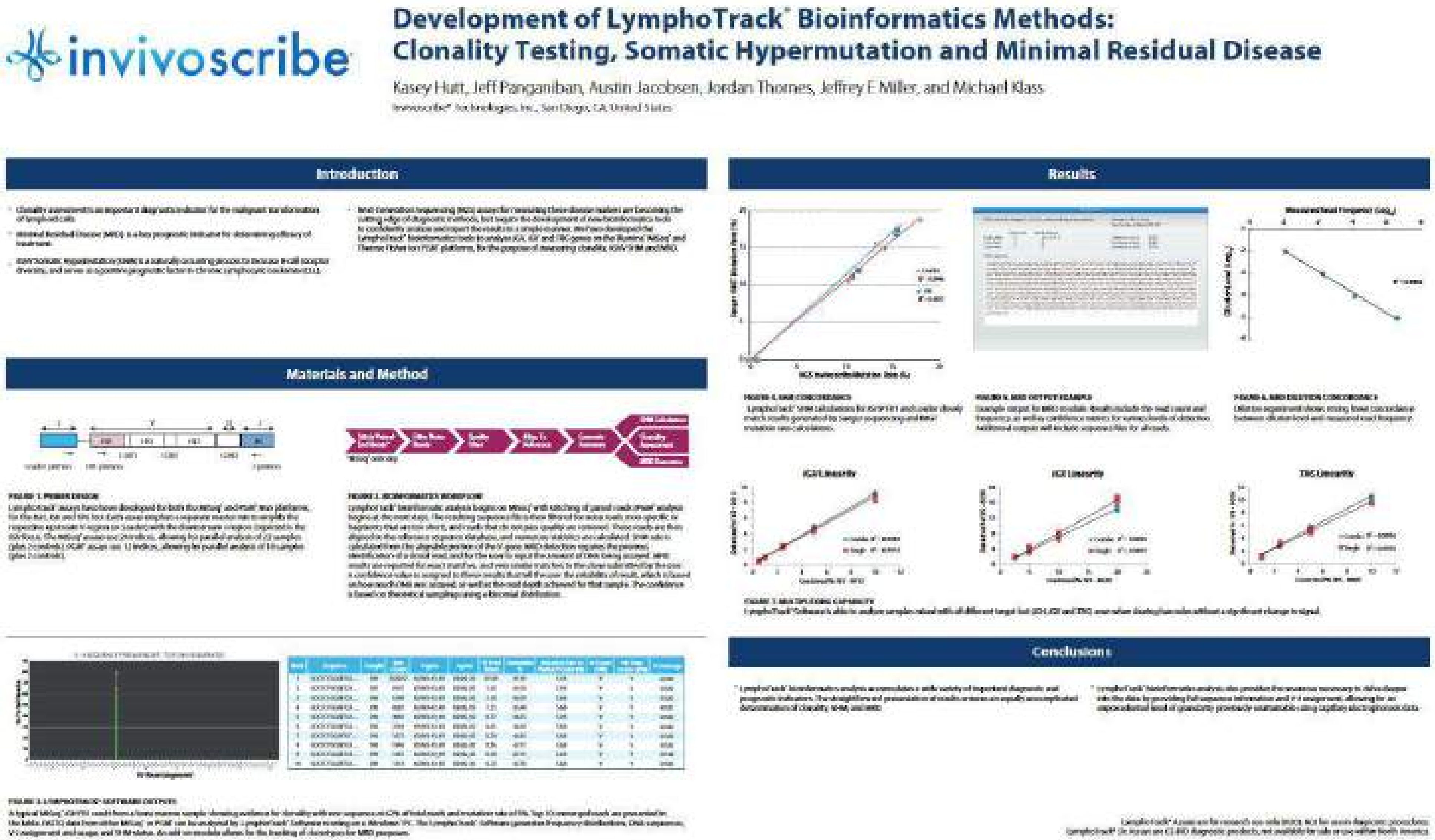
Post treatment sample
Bone marrow: Morphology: no evidence. Flow cytometry: Minute abnormal plasma cell population detected by flow cytometry (0.035%)
Lymphotrack: 0.12%

MRD case study #2

References

AMP 2015

https://invivoscribe.com/Posters/2015/AMP/IVS_Bioinformatics_AMP_2015_20151109_KaseyHutt.pdf



AMP 2016

https://invivoscribe.com/Posters/2016/AMP/MZL%20AMP%202016%202%2011%202016%20Arcila.pdf

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

H59

J. Gomez-Gelvez, C. Ho, W. Yu, M.H. Syed, A. Zehir, T. Baldi, A. Dogan, M. Ladanyi, J. Yao, K. Nafa, and M.E. Arcila
Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, NY

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.

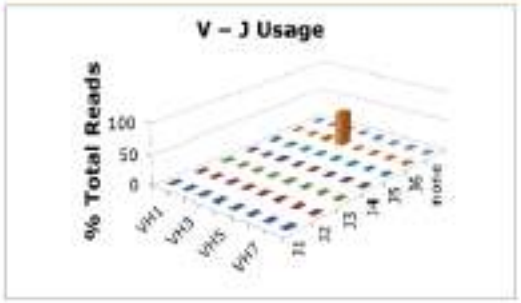
METHODS

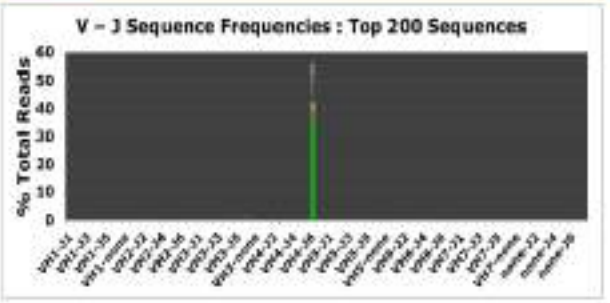
BM samples from patients with an established diagnosis of SMZL and submitted for routine clonality assessment were selected for the study. After establishing the presence of IGH clonality by capillary electrophoresis, the samples were analyzed using an NGS assay targeting IGH-FR1 (Lymphotrack, Invivoscribe) 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	V3-51	J4	Unmutated
2	M	65	V1-23	J4	Hypermutated
3	F	80	V3-21	J4	Hypermutated
4	M	63	V4-34	J4	Hypermutated
5	M	47	V3-23	J1	Hypermutated
6	F	70	V4-38	J2	Hypermutated
7	M	71	V4-61	J4	Unmutated
8	M	78	V3-73	J6	Unmutated
9	M	65	V3-9	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	V3-30	J4	Unmutated
19	M	87	V3-30	J6	Unmutated
20	M	77	V3-43	J1	Unmutated

RESULTS

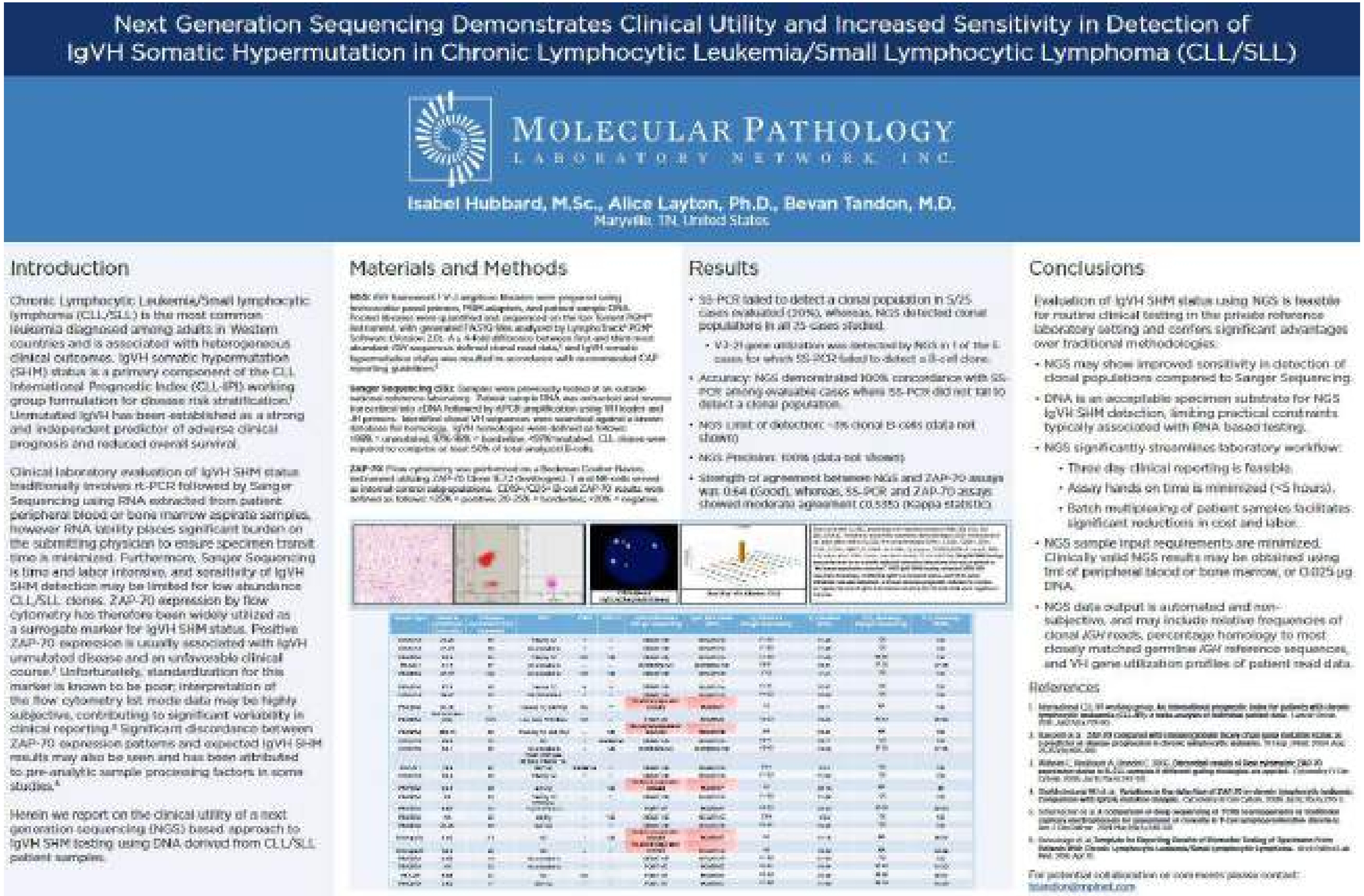
- We confirm that SMZL have a biased IGHV gene usage, which is in keeping with prior literature.
- This usage, however, has significant overlap with other subtypes of MZL considered in the differential diagnosis and therefore does not provide a means of discrimination for diagnostic purposes. This finding 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 the deserves further study.

REFERENCES

1. Arcaini L, Rossi D, Paulli M. Splenic marginal zone lymphoma: from genetics to management. Blood 2008; 111(11):5092-5083.
2. Parry M, Rose-Zerilli MJ, Ljungstrom V, et al. Genetics and Prognostication in Splenic Marginal Zone Lymphoma: Revelations from Deep Sequencing. Clin Cancer Res 2015; 21(18):4174-4183.
3. Bikos V, Darzentas N, Hadzidimitriou A, et al. Over 20% of patients with splenic marginal zone lymphoma express the same immunoglobulin heavy variable gene: ontogenetic implications. Leukemia 2010; 24(7): 1258-1264.
4. Zibellini S, Capello D, Forconi F, et al. Stereotyped Patterns Of B-Cell Receptor In Splenic Marginal Zone Lymphoma. Haematologica 2010; 95(11):1792-1796.
5. Kalpadakis C, Pangalis GA, Dimitriadou E, et al. Mutation analysis of IgVH genes in splenic marginal zone lymphomas: correlation with clinical characteristics and outcome. Anticancer Res 2009; 29(5): 1801-1808.

AMP 2016

https://invivoscribe.com/Posters/2016/AMP/MPLN%20final%20AMP%20poster.pdf



AMP 2016

https://invivoscribe.com/Posters/2016/AMP/IGVH2016AMP%20Mayo.pdf

MAYO CLINIC

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

Kurt Bessonen, Ming Mai MS, Lori Frederick, Rong He MD, David Viswanatha MD
Division of Hematopathology, Dept. of Laboratory Medicine and Pathology
Mayo Clinic, Rochester, Minnesota

Introduction

Somatic hypermutation (SHM) status of the immunoglobulin heavy chain variable region (IGVH) 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 monoclonal CLL IGVH-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 IGVH rearrangements and to determine SHM status in CLL patients. NGS represents a significant improvement over Sanger sequencing by simultaneously detecting and characterizing IGVH 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 IGVH SHM status in CLL.

Materials and Methods

Patient Samples and RNA Preparation

Peripheral blood and bone marrow aspirate samples with at least 5% clonal B cells were obtained and RNA was isolated using the ZR Whole-Blood RNA MiniPrep kit (Zymo Research Corp., Irvine, CA) for peripheral blood and miRNeasy Mini Kit (Qiagen USA, Valencia, CA) for bone marrow samples. Reverse transcription was performed using the Multiscribe 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 QIAxcel 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 (6 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 exoSAP-IT (Affymetrix, Santa Clara, CA) to remove excess nucleotides and primers. The purified PCR product was sequenced using the BigDye Terminator kit version 1.1 (Life Technologies, Carlsbad, CA) per manufacturer's instructions. Sequencing reactions were purified using EdgeBio Performa DTR Ultra 96 well plates (EdgeBio, Gaithersburg, MD) and analyzed by capillary electrophoresis on the ABI 3130xL (Applied Biosystems, Foster City, CA).

Materials and Methods

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

Next Generation Sequencing

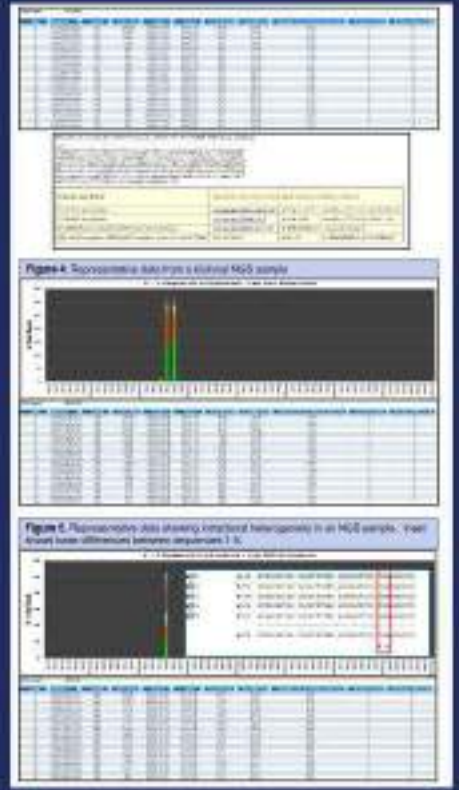
Multiplex leader PCR was performed including adapter ligation and sample indexing using the LymphoTrack IGH/V Leader Somatic Hypermutation Assay Panel for Miseq (Invivoscribe, San Diego, CA). A check gel was run to assess the quality and presence of clonal PCR product using the QIAxcel DNA High Resolution Kit. If no band or an indistinct band was present, FR1 multiplex PCR was performed using the LymphoTrack IGH FR1 Panel for Miseq (Invivoscribe, San Diego, CA). PCR product libraries were purified using the Agencourt AMPure XP Purification kit (Beckman Coulter, Brea, 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 FASTQC files. After de-multiplexing, individual patient samples were analyzed and graphically displayed using the Invivoscribe LymphoTrack IGH/V SHM Software for MiSeq (Invivoscribe, San Diego, CA). A minimum total read (coverage) depth of 100,000X and a clonal IGVH 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/V-QUEST was performed.

Results

- 12 normal donor samples and 46 CLL samples with known IGVH status (by Sanger sequencing) were evaluated using the NGS methodology.
- All normal samples were negative for clonal gene rearrangements.
- 2/46 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 biclonal rearrangements variably 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 2/43 (5%) of comparison samples; additional minor discrepancies identified in 8 cases.
- Causes of discordant results:
  - NGS versus Sanger differences:
    - VH nomenclature (IMGT vs. IgBLAST)
    - Differing SHM status (M vs. UM), possibly from suboptimal Sanger data and sequence contig with increased ambiguous bases
    - NGS result negative and Sanger result positive for clonal IGHV differences in multiplex 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/chemistry/low complexity region) vs. true intraclonal heterogeneity

Conclusions

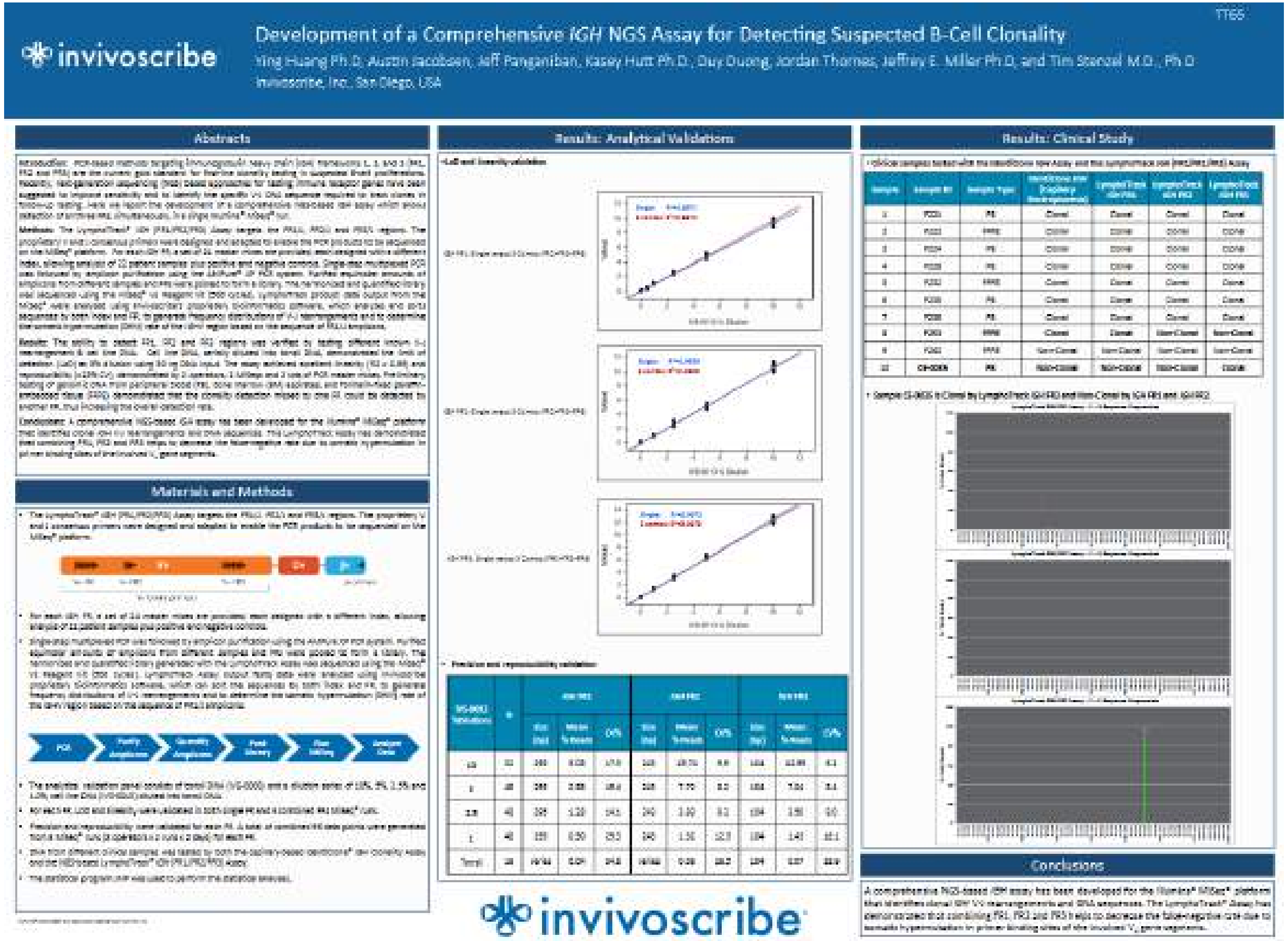
- NGS methodology is accurate and acceptable for clinical use in the determination of somatic hypermutation status in CLL. Results obtained in our experience are highly concordant (41/43, 95.3%) in comparison to the current standard of PCR and Sanger Sequencing.
- Advantages of NGS include direct determination of the IGVH 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 merits of NGS in this situation include the ability to batch multiple samples simultaneously and to potentially enable easier minimal residual disease monitoring.

References

1. Bluethy J, et al., Immunoglobulin transcript sequence and somatic hypermutation computation from unselected RNA-seq reads in chronic lymphocytic leukemia. PNAS 2015; 112: 14: 4322-4327.
2. Scarfone P, Behler D. Clinical Laboratory Analysis of Immunoglobulin Heavy Chain Variable Region Genes for Chronic Lymphocytic Leukemia Prognosis. JMD 2010; 12: 2: 244-249.
3. McClure R, et al., High Throughput Sequencing Using the Ion Torrent Personal Genome Machine for Clinical Evaluation of Somatic Hypermutation Status in Chronic Lymphocytic Leukemia. JMD 2015; 17: 2: 146-154.

AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP2016_PosterTT65_YH_20161107_Dig.pdf



AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP2016_PosterH67_AC_20161107_Dig.pdf

H67

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

Andrew R. Carson Ph.D.[1], Zhiyi Xie Ph.D.[1], Valerie McClain M.S.[1], Jeffrey E. Miller Ph.D.[1,2,3], and Tim Stenzel M.D., Ph.D.[1,2,3]

[1]Invivoscribe, Inc., San Diego, CA, [2]LabPMM LLC, San Diego, CA and [3]LabPMM GmbH, Martinsried, Germany

Introduction

The nucleophosmin (*NPM1*) gene is an important marker for acute myeloid leukemia (AML) stratification. *NPM1* is one of the most commonly mutated genes in AML, with mutations seen in roughly 35% of patients at diagnosis, and in approximately 60% of adult cytogenetically normal (CN) AML patients. Importantly, *NPM1* mutations generally confer a slightly more favorable outcome in AML patients and can mitigate poor prognoses 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 cell line DNA (HS-0048) containing a known *NPM1* 4 bp insertion mutation (COSM17558) diluted into DNA from a cell line known to be negative for *NPM1* mutations (HS-0002). The panel contained samples at dilutions that range from: 1.0×10^{-2} to 5.0×10^{-5}.

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 at and above the assay's LOD claim of 5.0×10^{-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 *NPM1* assay.
- Since no clinical samples were available with *NPM1* mutations at MRD levels, we diluted two positive clinical samples as much as 1: 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 detect mutations at a LOD of 5.0×10^{-4}, we utilize:
  - An input of 700 ng of high quality DNA (≥ 100,000 cell equivalents)
  - Sequencing depth = 240,000× (this allows us to run 24 samples per run)

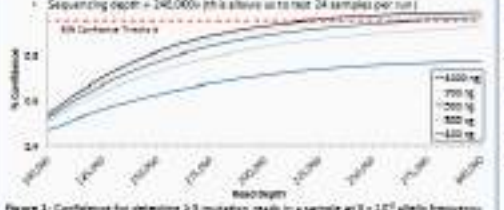


Figure 1: Confidence for detecting ≥ 5 mutation reads in a sample at 5×10^{-4} allelic frequency. Data for this figure was calculated using a statistical model that does not incorporate any PCR bias. An input of 700 ng of DNA would require a ≥ 240,000× for confident detection of a mutation at 5×10^{-4}. Decreased input DNA amount would require additional read depth to maintain ≥ 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* 4 bp insertion mutation (ins-TCTG; COSM17558) diluted into a background cell line containing wild-type *NPM1* exon 11 (HS-0002). We tested our ability to detect mutations at a LOD as low as 1×10^{-4} and used a range of dilutions up to 5×10^{-3} to test for linearity in the mutation detection (Figure 2).

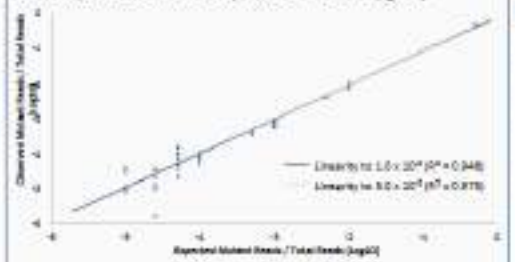


Figure 2: Linearity of *NPM1* MRD assay

This figure examines linearity for the detection of the COSM17558 4 bp insertion mutation via log dilutions of a positive control (HS-0048).

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

Expected Mutation Frequency	Number of Tests (n)	% of Tests Positive* for the Expected Mutation	Mean Observed Mutation Frequency
5.0×10^{-3}	3	100%	4.8×10^{-3}
1.0×10^{-3}	3	100%	8.4×10^{-4}
5.0×10^{-4}	3	100%	3.1×10^{-4}
1.0×10^{-4}	4	100%	7.3×10^{-5}
5.0×10^{-5}	3	100%	5.8×10^{-5}
1.0×10^{-5}	4	100%	7.8×10^{-6}
5.0×10^{-6}	20	100%	7.2×10^{-6}
2.5×10^{-6}	7	71.4%	1.8×10^{-6}
1.0×10^{-6}	8	62.5%	1.8×10^{-6}
0.0	11	0%	0.0

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

Limit of Detection:

The *NPM1* 4 bp insertion mutation was detected at every dilution level, down to 2×10^{-6}. 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 5×10^{-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 5×10^{-4}, the R^2 increases to 0.975, suggesting slightly superior linearity in the assay's detection range.

Precision and Reproducibility:

The assay shows acceptable precision and reproducibility both at and above the assay's 5×10^{-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 5×10^{-4} (Table 2 and Figure 3).

Expected Mutation Frequency	Number of Tests (n)	True Positives	False Positives	True Negatives	False Negatives
5.0×10^{-3}	3	3	0	8	N/A
1.0×10^{-3}	3	3	0	8	N/A
5.0×10^{-4}	3	3	0	8	N/A
1.0×10^{-4}	4	4	0	8	N/A
5.0×10^{-5}	3	3	0	8	N/A
1.0×10^{-5}	4	4	0	8	N/A
5.0×10^{-6}	20	20	0	8	N/A
2.5×10^{-6}	7	5	0	2	N/A
1.0×10^{-6}	8	5	0	3	N/A
0.0	11	N/A	0	N/A	11

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 dimension at or above the LOD of 5×10^{-4}.

At the established LOD of 5×10^{-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 on a PPV remain at 100% due to the lack of false positives in this study.

Results: Concordance with Clinical Samples

We tested 5 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 diluted 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	Detected Mutation	Observed Frequency
AML-01	None	Positive	4 bp Insertion (TCTG)	4.7×10^{-1}
	1 in 10			8.0×10^{-2}
	1 in 100			9.3×10^{-3}
	1 in 1,000			2.7×10^{-3}
AML-02	None	Positive	4 bp Insertion (TCTG)	4.4×10^{-1}
	1 in 10			9.2×10^{-2}
	1 in 100			9.5×10^{-3}
AML-03	None	Not Detected	Not Detected	0.0
AML-04	None	Not Detected	Not Detected	0.0
AML-05	None	Not Detected	Not Detected	0.0

In addition, we also tested DNA from 5 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 and 100% sensitivity and specificity at and above a LOD of 5×10^{-4}.
- Assay is concordant with CAP/CLIA-CE assay for clinical samples with no false positives in normal controls.

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

AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP2016_PosterH66_JX_20161107_Dig.pdf

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

H66

Zhiyi Xie Ph.D[1], Andrew R. Carson Ph.D.[1], Valerie McClain[1], Selena Zhang[1], Wenli Huang[1], Jeffrey E. Miller Ph.D[1], Mark J. Levis M.D., Ph.D[2], Tim Stenzel M.D., Ph.D[1]

[1]Invivoscribe Inc., San Diego, CA, [2]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD

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 fms 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 juxtamembrane 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.

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. Amplicons 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 10,000. The DNA input of the assay was 100 ng (~300,000 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 ~1x10^{-4} frequency. NGS instrumentation also has up to a ~1% rate of run to run DNA carryover which presents a problem for MRD level detection. We have designed our assay with a proprietary approach to reduce the background errors below our 5x10^{-6} 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 1x10^{-4} and 5x10^{-6} (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	Sample Size	TP	FP	TN	FN	Sensitivity	Specificity
A	30	1.0 x 10^{-4}	20	20	0	N/A	0	100.0%	N/A
		5.0 x 10^{-6}	40	40	0	N/A	0	100.0%	N/A
B	108	1.0 x 10^{-4}	20	20	0	N/A	0	100.0%	N/A
C	N/A	0.0	40	N/A	0	40	N/A	N/A	100.0%

TP: true positive; FP: false positive; TN: true negative; FN: false negative

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 (108 bp ITD) were determined to be at 1.0x10^{-4} and 1.0x10^{-4}, 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 amplicon (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	Sample Size	ITD Detection: Positive	ITD Detection: Negative	% Positive*
A	30	0.0 x 10^{0}	40	0	40	0.0%
		5.0 x 10^{-4}	40	40	0	100.0%
		0.0 x 10^{0}	40	0	40	0.0%
B	108	1.0 x 10^{-4}	20	20	0	100.0%
C	N/A	0.0	40	0	40	0.0%
NTC	N/A	0.0	32	0	32	0.0%

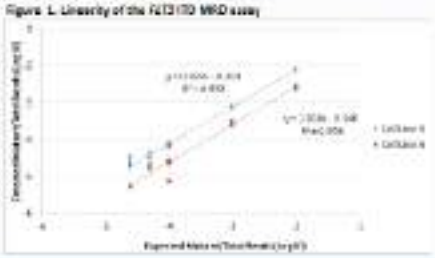
* a positive can require the detection of a minimum of 3 ITD reads containing a *FLT3* ITD mutation.

DNA from two cell lines with known ITD (30 bp and 108 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 100 ng per dilution point. The experimental data was presented in Table 2 and plotted in the 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

Expected Frequency	Detected Frequency: Cell Line A	Detected Frequency: Cell Line B
1.0 x 10^{-3}	8.0 x 10^{-4}	8.0 x 10^{-4}
	7.6 x 10^{-4}	8.7 x 10^{-4}
	8.0 x 10^{-4}	8.0 x 10^{-4}
5.0 x 10^{-4}	4.0 x 10^{-4}	4.0 x 10^{-4}
	4.0 x 10^{-4}	4.0 x 10^{-4}
	3.8 x 10^{-4}	4.0 x 10^{-4}
1.0 x 10^{-4}	7.6 x 10^{-5}	7.1 x 10^{-5}
	8.0 x 10^{-5}	8.0 x 10^{-5}
	7.6 x 10^{-5}	8.0 x 10^{-5}
5.0 x 10^{-5}	4.0 x 10^{-5}	4.0 x 10^{-5}
	4.0 x 10^{-5}	4.0 x 10^{-5}
	3.8 x 10^{-5}	4.0 x 10^{-5}
1.0 x 10^{-5}	8.0 x 10^{-6}	8.0 x 10^{-6}
	8.0 x 10^{-6}	8.0 x 10^{-6}
	7.6 x 10^{-6}	7.1 x 10^{-6}
5.0 x 10^{-6}	4.0 x 10^{-6}	4.0 x 10^{-6}
	4.0 x 10^{-6}	4.0 x 10^{-6}
	3.8 x 10^{-6}	4.0 x 10^{-6}
1.0 x 10^{-6}	8.0 x 10^{-7}	8.0 x 10^{-7}
	8.0 x 10^{-7}	8.0 x 10^{-7}
	7.6 x 10^{-7}	7.1 x 10^{-7}
5.0 x 10^{-7}	4.0 x 10^{-7}	4.0 x 10^{-7}
	4.0 x 10^{-7}	4.0 x 10^{-7}
	3.8 x 10^{-7}	4.0 x 10^{-7}
1.0 x 10^{-7}	0.0	0.0
	0.0	0.0
	0.0	0.0

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



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 NGS 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 *FLT3/ITD* PCR assay: Diagnostic Samples: ITD Size (bp)	Standard *FLT3/ITD* PCR assay: Diagnostic Samples: Allelic Ratio	Standard *FLT3/ITD* PCR assay: Follow-up Samples	NGS MRD assay of follow-up samples: Detected ITD Size (bp)	NGS MRD assay of follow-up samples: Detected ITD Frequency	Follow-up Samples
1	33	0.85%	neg	33	1.2 x 10^{-4}	On treatment
2	33	1.00%	neg	33	1.3 x 10^{-4}	Unavailable
3	na	n/a	neg	na	0.0 x 10^{0}	n/a
4	na	n/a	neg	na	0.0 x 10^{0}	on treatment
5	na	n/a	neg	na	0.0 x 10^{0}	on treatment
6	33	0.9%	neg	33	1.0 x 10^{-4}	Relapsed
7	33 na	0.9% n/a	neg	33 na	1.4 x 10^{-4} 0.0 x 10^{0}	On treatment
8	na	unavailable	neg	n/a	n/a	disease free
9	na	unavailable	neg	no new amplification		disease free
10	78	0.2%	neg	n/a	0.0	Disease free
11	66	unavailable	neg	n/a	0.0	Disease free
12	na	n/a	neg	n/a	na	disease free
13	na	weak	neg	n/a	na	disease free
14		Detected*	neg	34	1.1 x 10^{-4}	Relapsed and died
15		Detected*	neg	33	1.0 x 10^{-4}	Relapsed and died
16		Detected*	neg	n/a	0.0	Disease free

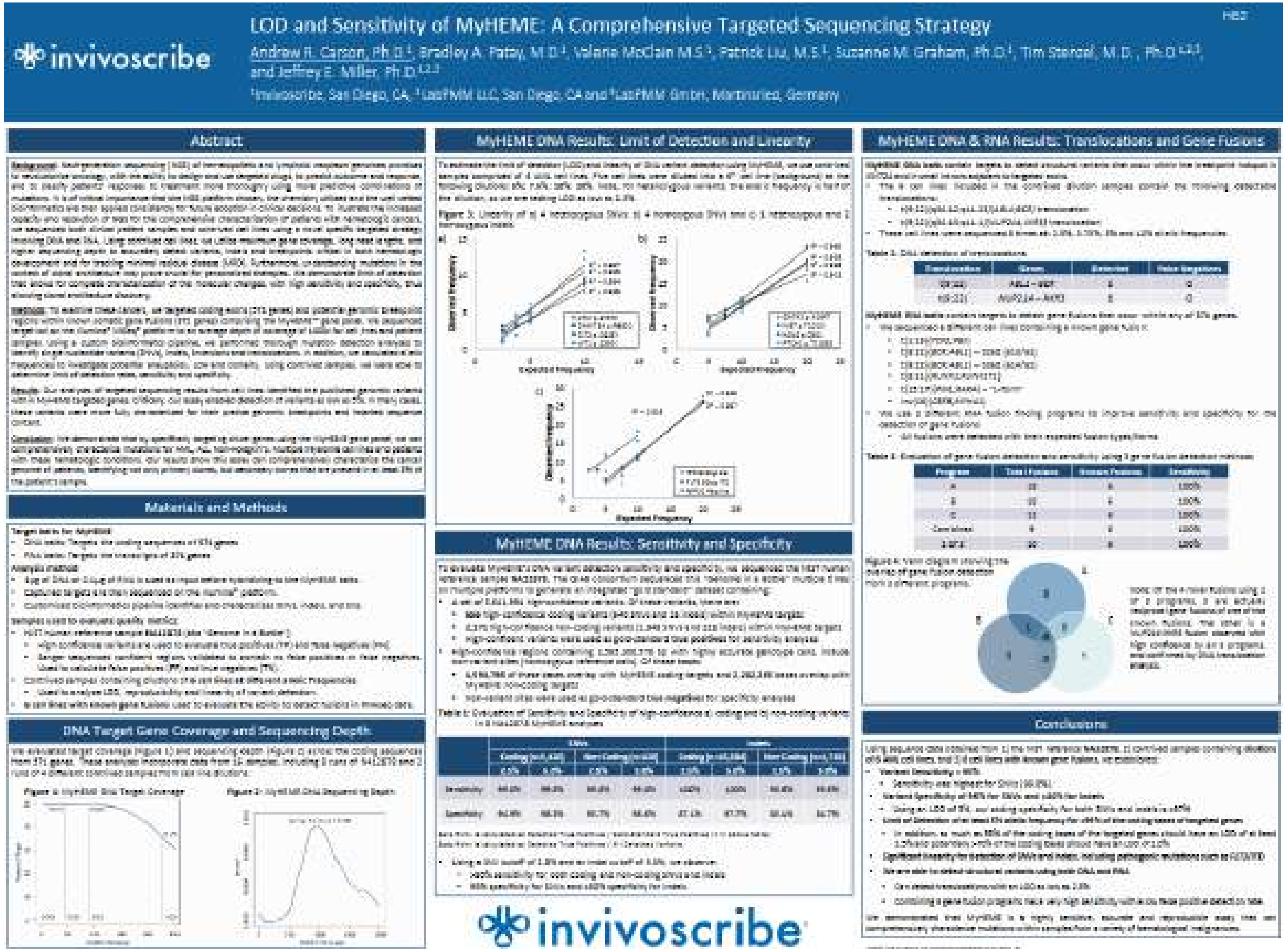
* Does not meet criteria

Conclusions

The *FLT3/ITD* NGS assay is a highly-specific test, developed with the accompanying bioinformatics software under full ISO13485 design control, 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 (LabPMM).

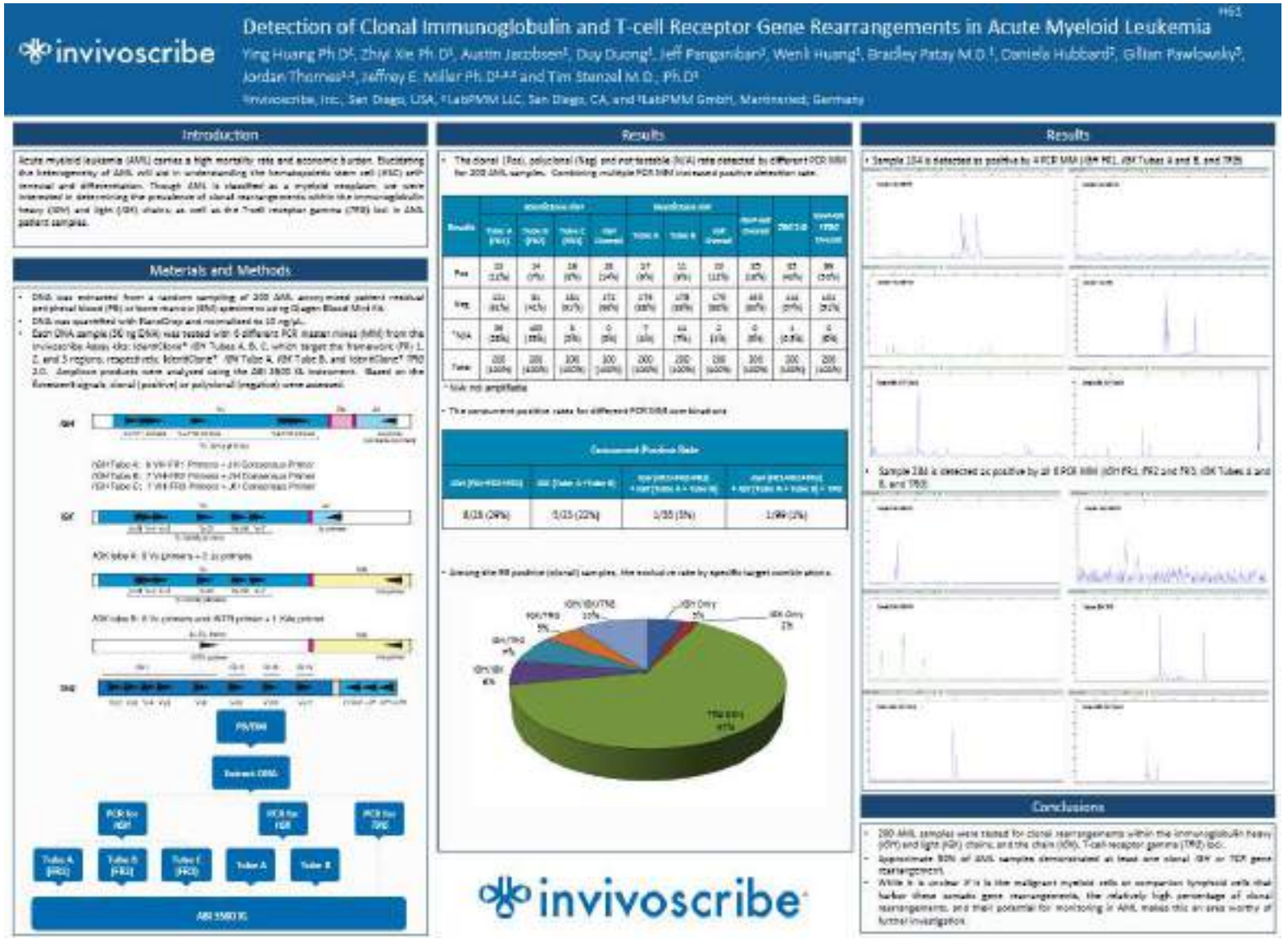
AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP2016_PosterH62_AC_20161114_Dig.pdf



AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP2016_PosterH61_YH_20161107_Dig.pdf



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

Ying Huang Ph.D.[1], Zhiyi Xie Ph.D.[1], Austin Jacobsen[1], Duy Duong[1], Jeff Panganiban[2], Wenli Huang[1], Bradley Patay M.D.[1], Daniela Hubbard[2], Gillian Pawlowsky[2], Jordan Thomas[1,3], Jeffrey E. Miller Ph.D.[1,3,4] and Tim Stenzel M.D., Ph.D.[1]

[1]Invivoscribe, Inc., San Diego, USA, [2]LabPMM LLC, San Diego, CA, and [3]LabPMM GmbH, Mannheim, Germany

Introduction

Materials and Methods

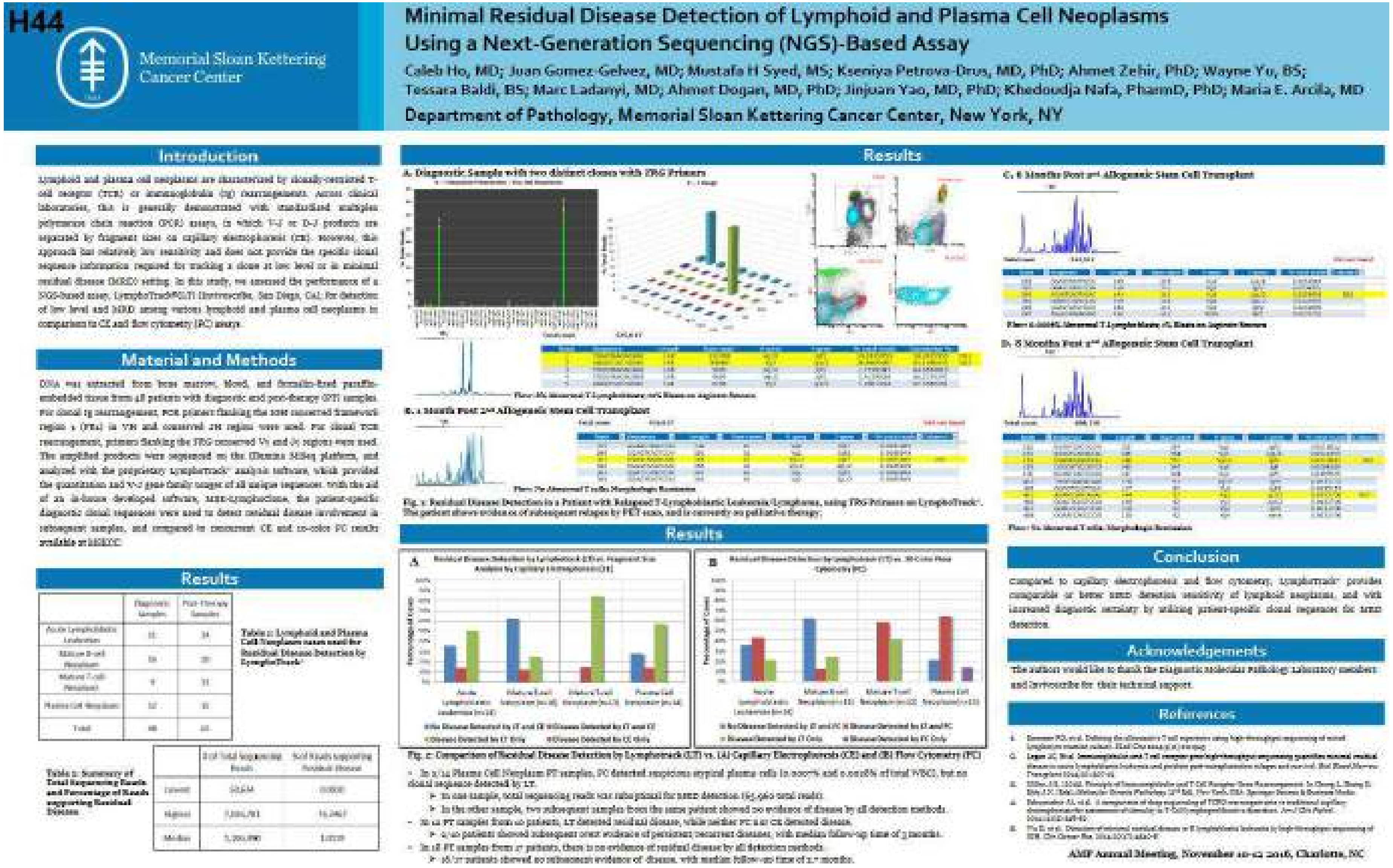
Results

Results

Conclusions

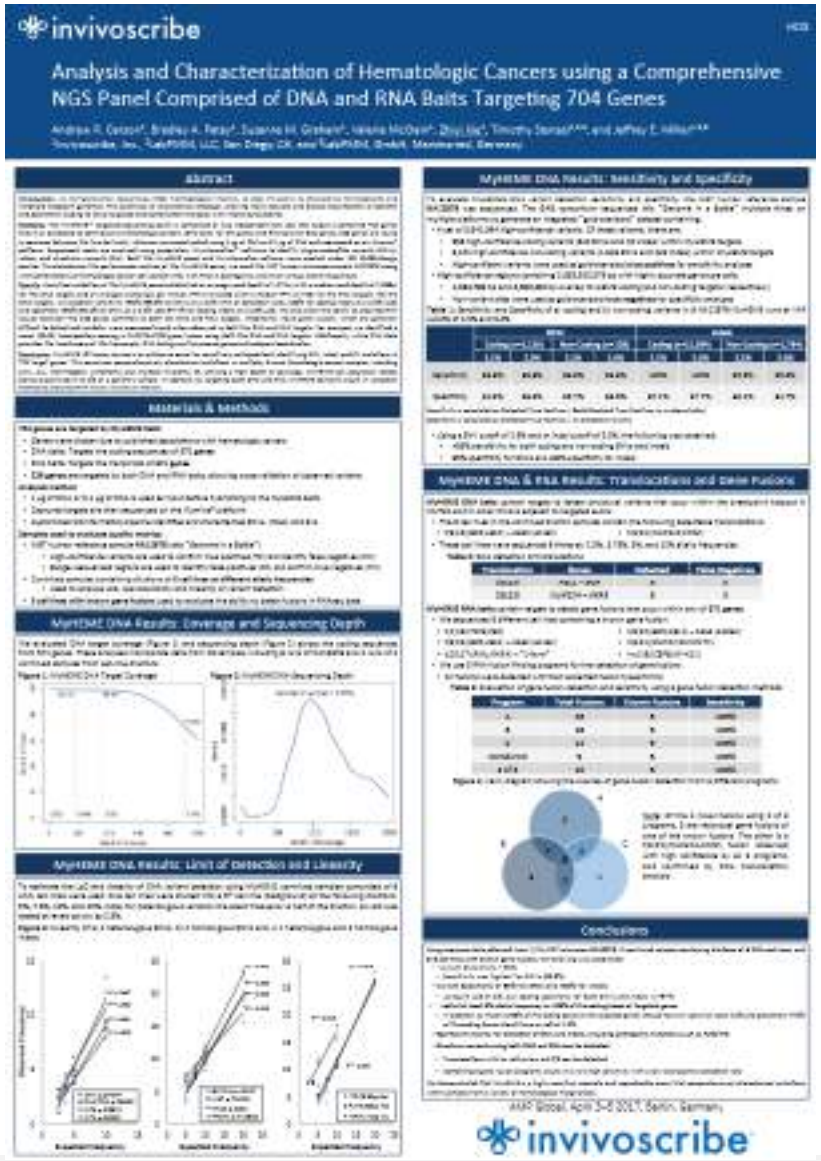
AMP 2016

https://invivoscribe.com/Posters/2016/AMP/AMP%202016%20Lymphotrack%20Poster%20Arcila%20%20FINAL%2011%202%2016.pdf



AMP Global 2017

https://invivoscribe.com/Posters/2017/AMP/MyHEME%20AMPGlobal_20170330_JM_CMW_DC_DJ_ARC_clean.pdf



AMP Global 2017

https://invivoscribe.com/Posters/2017/AMP/H04%20FLT3_NPM1_v3_clean.pdf

invivoscribe

H04

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

Introduction

Material and Methods

Results: LoD, LoB, and Linearity of Contrived Samples

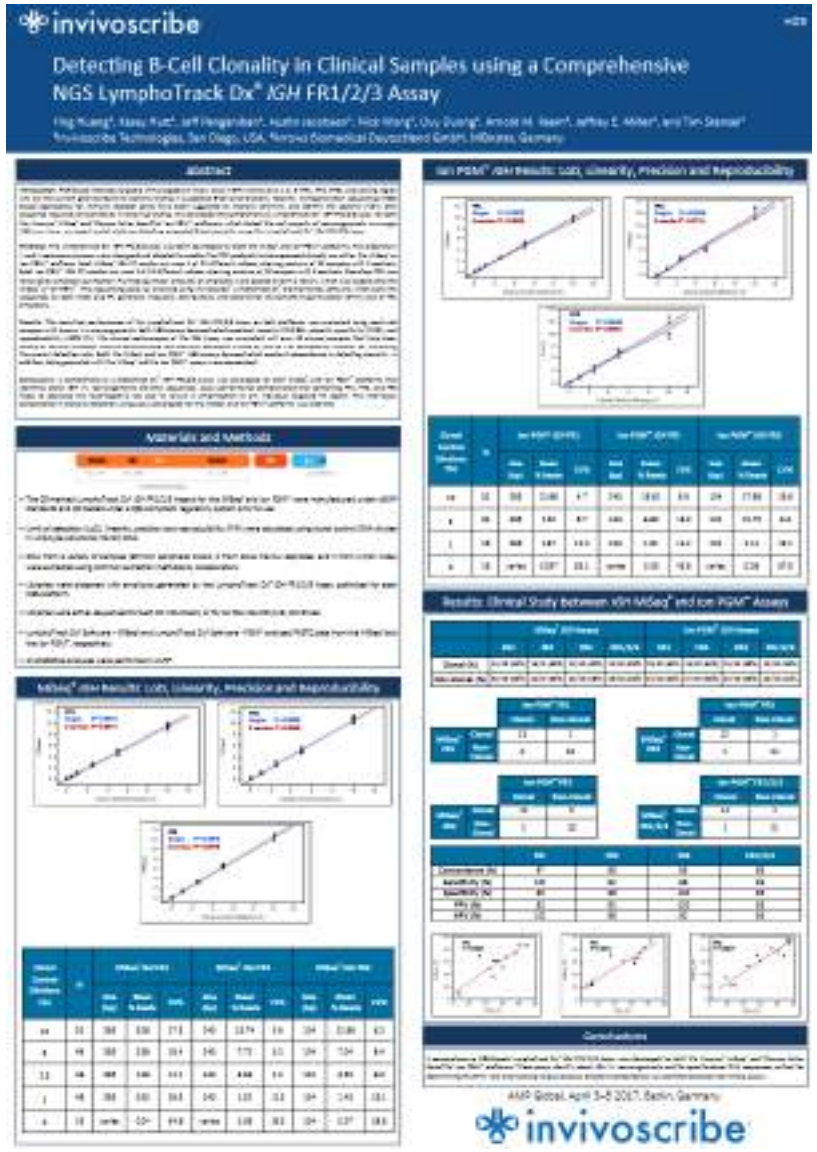
Results: Clinical Sample Testing

Conclusions

invivoscribe

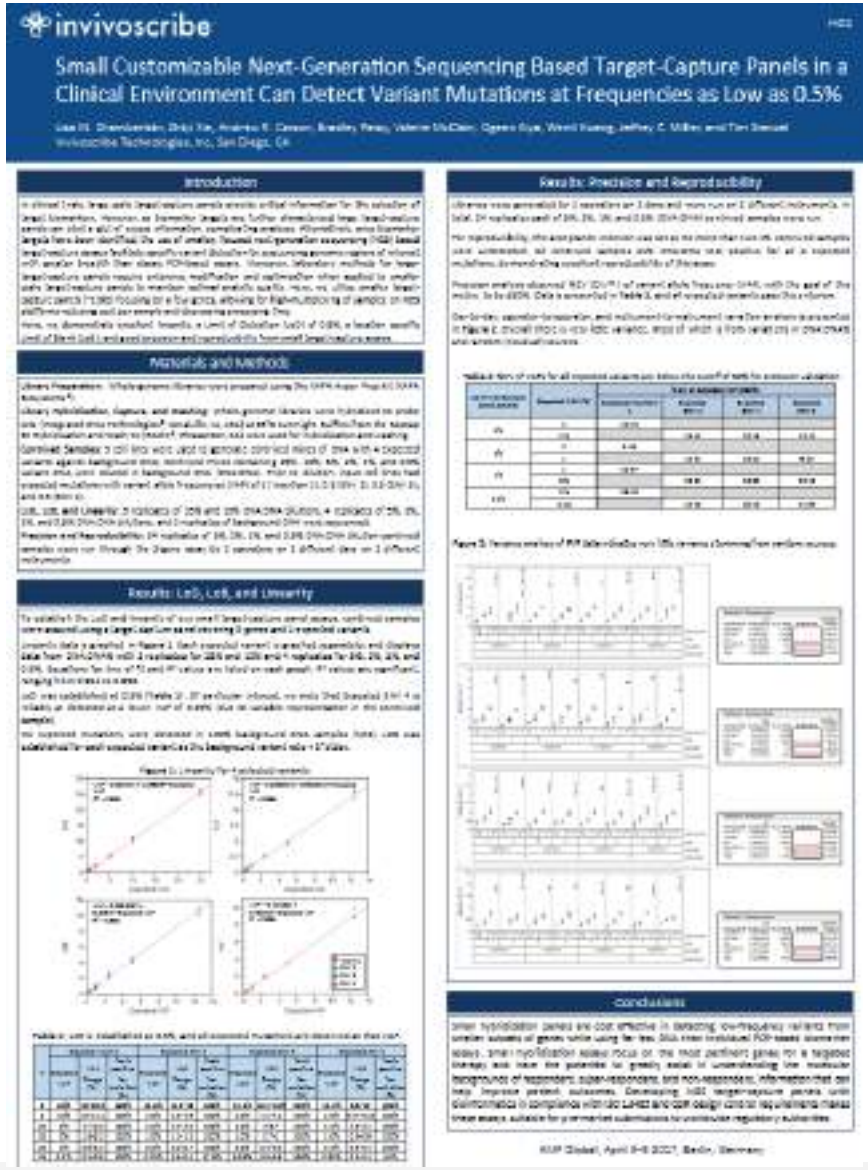
AMP Global 2017

https://invivoscribe.com/Posters/2017/AMP/AMPGlobal2017_H09_IGH_v6b.pdf



AMP Global 2017

https://invivoscribe.com/Posters/2017/AMP/A3G%20poster%20AMPglobal%2003302017_LMC-2.pdf



invivoscribe

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

Introduction

Materials and Methods

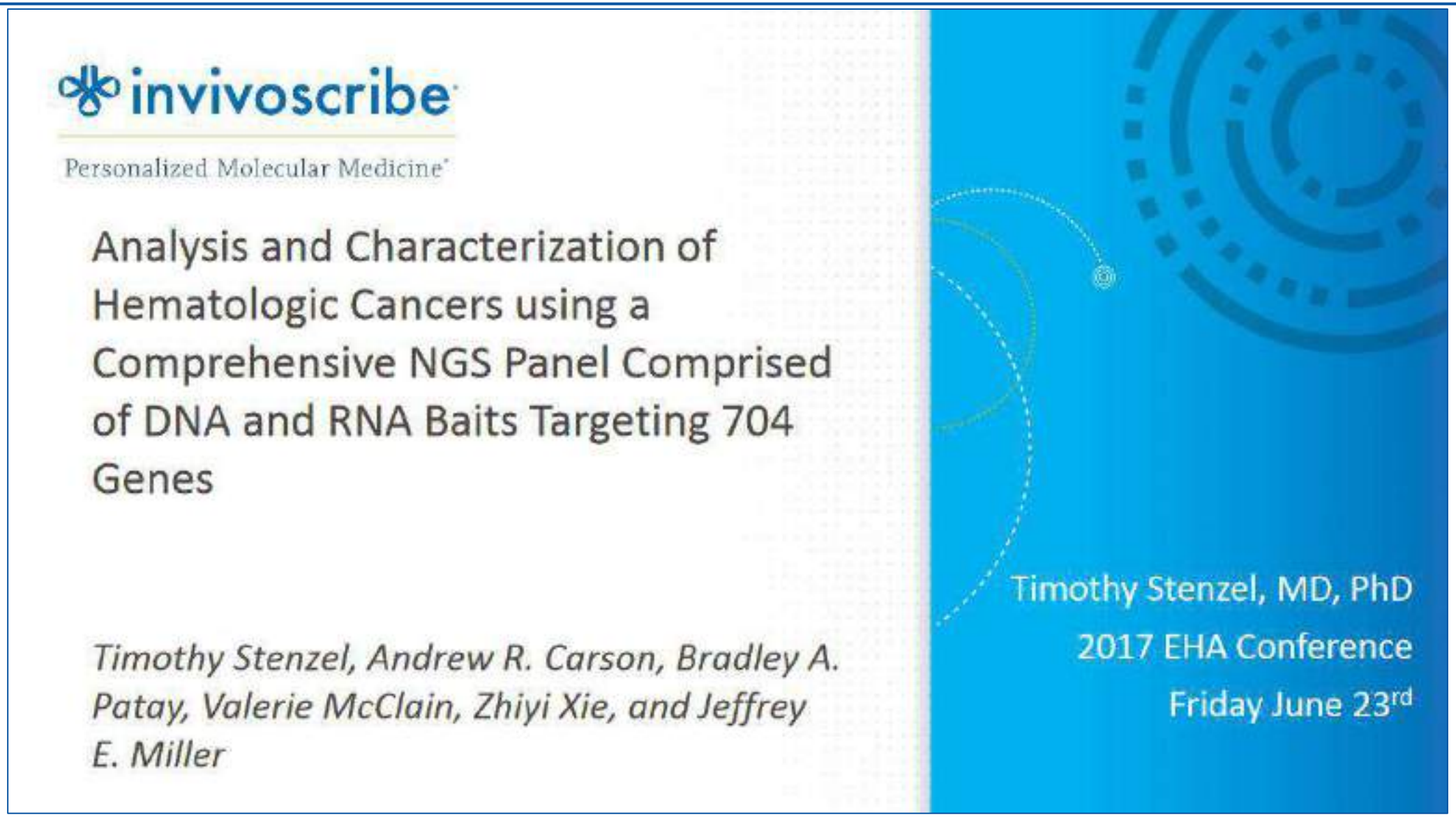
Results: LoD, LoB, and Linearity

Results: Precision and Reproducibility

Conclusions

EHA 2017

https://invivoscribe.com/Posters/2017/EHA/MyHeme_EHA2017_Draft_20170621.ARC_KD.pdf



EHA 2017

https://invivoscribe.com/Posters/2017/EHA/EHA2017_PosterE847_IGH_v3_NW2.pdf

invivoscribe

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

E547

Ying Huang[1], Kasey Hunt[1], Jeff Pongantikorn[1], Austin Jacobsen[1], Nick Wong[1], Roshanak Bobi[1], Stefano A. Pileri[2], Lorca Bernard[2], Elvira Garbrino[2], Tim Stanza[1] and Jeffrey S. Miller[1]

[1]Invivoscribe, San Diego, USA; [2]Institute for Pathodiagnostik Berlin, Germany and [3]Haematopathology Unit at European Institute of Oncology, Milan, Italy

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 immune receptor 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 IdentiClone® IGH PCR-CE assay by testing in over 50 anonymized, blinded clinical samples.

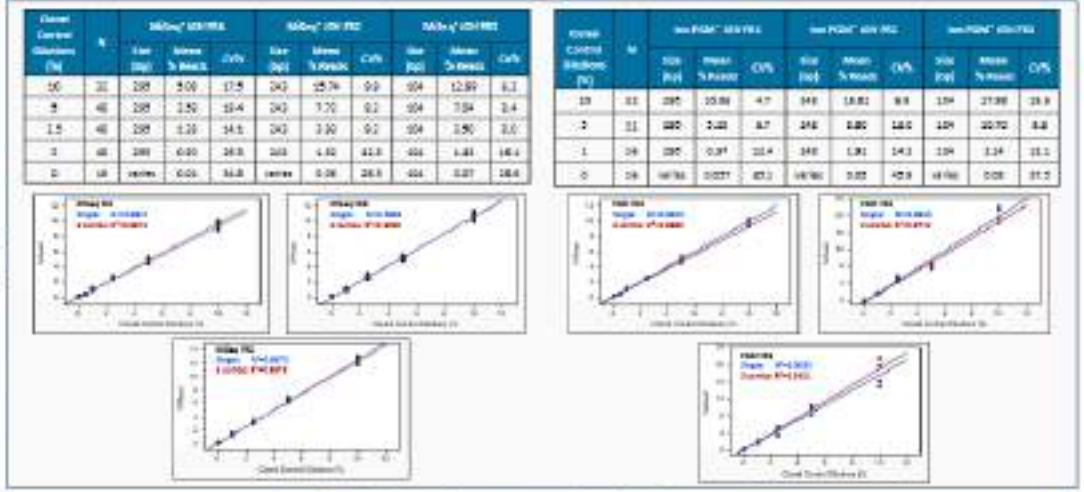
Materials and Methods

- 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 (P/R) were validated using clonal control DNA diluted in wild-type polyclonal (tonsil) DNA.
- DNA from a variety of samples (21 from peripheral blood, 1 from bone marrow aspirates, and 37 from FFPE were extracted using common extraction methods by collaborators. 59 samples were tested by all assays except that FR2 PGM tested additional 5 samples for total 65 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®.

Conclusions

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

Results: LoD, Linearity, Precision and Reproducibility



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

	MiSeq® IGH Assays				Ion PGM™ IGH Assays				IdentiClone 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 (%)	28/59 (51%)	26/59 (27%)	22/59 (77%)	28/59 (51%)	27/54 (62%)	26/88 (27%)	13/100 (38%)	19/100 (38%)	28/59 (51%)	21/59 (38%)	23/100 (38%)	28/59 (28%)
Non-Clonal (%)	31/59 (49%)	41/59 (70%)	46/59 (61%)	31/59 (51%)	31/54 (38%)	27/88 (30%)	22/100 (62%)	22/100 (38%)	21/59 (38%)	21/59 (38%)	24/100 (62%)	20/59 (34%)

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

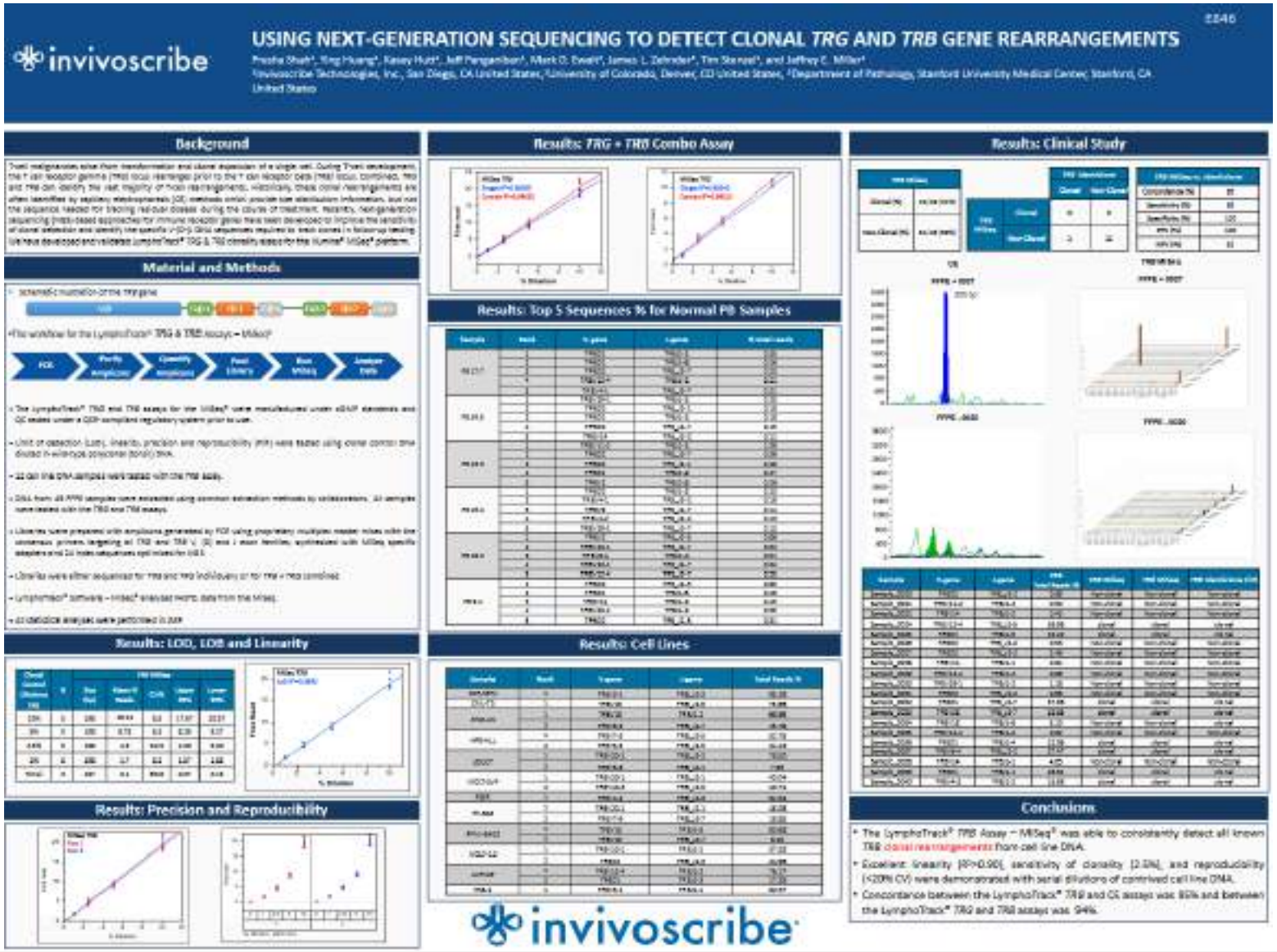
PGM® FR1/2/3	IdentiClone IGH Tube A/B/C: Clonal	IdentiClone IGH Tube A/B/C: Non-Clonal
Clonal	22	0
Non-Clonal	1	18

MiSeq® FR1/2/3	Clonal	Non-Clonal
Clonal	22	0
Non-Clonal	0	22

	MiSeq FR1/2/3 vs. IdentiClone Tube A/B/C	PGM FR1/2/3 vs. IdentiClone Tube A/B/C	PGM FR1/2/3 vs. MiSeq FR1/2/3
Concordance (%)	95.5	97.8	100
Sensitivity (%)	96.0	96.7	100
Specificity (%)	94.7	100	100
PPV (%)	96.0	100	100
NPV (%)	94.7	94.7	100

EHA 2017

https://invivoscribe.com/Posters/2017/EHA/EHA2017_PosterE846_TRB_TRG_Final_DJ.pdf



EHA 2017

https://invivoscribe.com/Posters/2017/EHA/EHA2017_IVSMyMRDPoster_134x90cm_20170615v2_VM_pdf.pdf

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

P197

Valerie McClain[1], Andrew R. Carson[1], Bradley A. Patay[1], Lisa Chamberlain[1], Christopher Chandler[1], Selena Zheng[1], Wenli Huang[1], Ogeen Kiva[1], Daniela Hubbard[1], Daniel Caguioa[1], Zhiyi Xie[1], Jordan Thomas[1], Timothy Stenzel[1], and Jeffrey E. Miller[1,2], [1]Invivoscribe Inc., San Diego, CA, USA, and [2]LabPMM LLC, San Diego, CA USA

Background

Methods

Results: Depth of Coverage

Results: LOD and Linearity

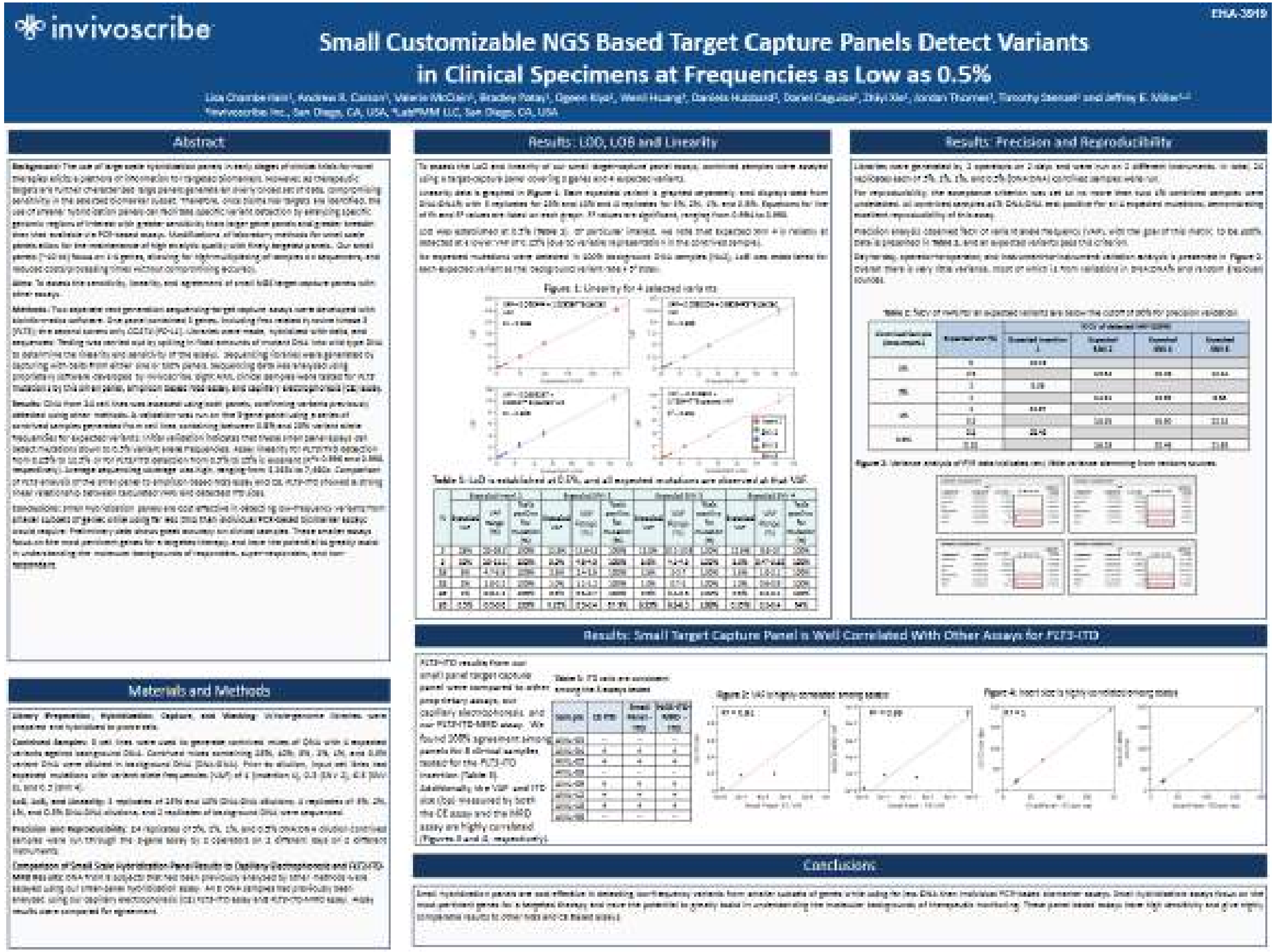
Results: Combined Sensitivity and Specificity

Results: Clinical Samples

Conclusions

EHA 2017

https://invivoscribe.com/Posters/2017/EHA/05172017_LMC_poster_EHA_small-panel-flt-3-across-panels_final.pdf



AMP 2017

https://invivoscribe.com/Posters/2017/AMP/Invivoscribe_AMP2017_PosterTT45_TRB_2Combo.pdf

TT45

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

Prosha Shah[1], Ying Huang[1], Kasey Hutt[1], Jeff Pangan Ibarra[1], Gilgar Vigil[1], Roshanak Bob[1], Mark D. Ewalt[1], James L. Zehnder[2], Tim Stenzel[3], and Jeffrey E. Miller[1]

[1]Invivoscribe Technologies, Inc., San Diego, CA USA; [2]Institute for Pathodiagnostik Berlin, Germany; [3]University of Colorado, Denver, CO USA; [4]Department of Pathology, Stanford University Medical Center, Stanford, CA USA

Background

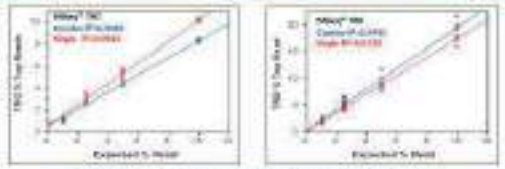
Material and Methods

- The LymphoTrack® TRG and TRB Assays for the MiSeq® were cGMP manufactured, QC tested, and analyzed using LymphoTrack® bioinformatics software developed under full design control consistent with 21CFR(1400) compliant regulatory systems.
- Limit of detection (LoD), linearity, precision and reproducibility (R/R) were tested using clonal control DNA diluted in wild-type peripheral (normal) DNA for TRB assay.
- DNA from 68 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^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} sensitivity with an internal control spiked in to reactions at approximately 1,000 cell equivalence.
- Libraries were prepared with amplicons generated by PCR using proprietary multiplex master mixes with the consensus primers targeting all TRG and TRB V, D, and J exon fa'milies, synthesized with MiSeq® specific adapters and 24 index sequences optimized for the MiSeq platform.
- Libraries were either sequenced for TRB and TRG individually or for TRB + TRG combined.
- LymphoTrack® Software - MiSeq® was used to analyze FASTQ data generated from the MiSeq®.
- Where 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®.

Conclusion

- The LymphoTrack® MiSeq® TRB assay demonstrated excellent linearity (R^2>0.99), sensitivity (as low as 2.5%), and reproducibility (<3%CV) testing serial dilutions of control cell line DNA.
- Concordance between the LymphoTrack® TRB - MiSeq® and IdentiClone® TRB assays was 86.4%.
- Concordance between the LymphoTrack® TRG - MiSeq® and IdentiClone® TRG assays was 96.3%.
- Concordance between the combined TRB+TRG LymphoTrack® MiSeq® and IdentiClone® assays was 84.3%.
- Excellent linearity (R^2>0.90) for combined cell line, reproducibility (<20% CV) for control cell line, and clonal detection for MRD at 10^{-5} sensitivity was demonstrated for LymphoTrack® MiSeq® TRG and TRB assays.

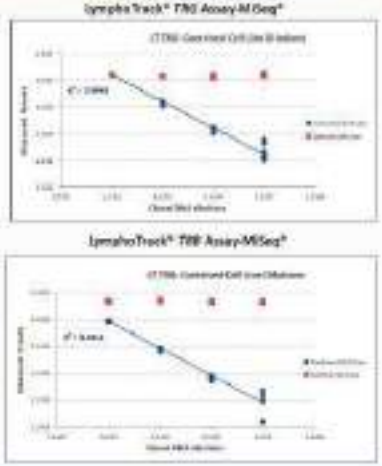
Results: TRG / TRB LoD, LoB and Linearity



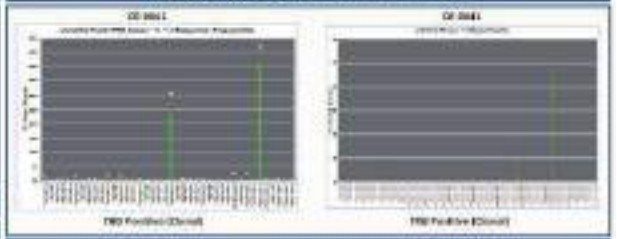
Results: TRG/TRB Precision and Reproducibility

Clonal Control Dilutions (%)	TRG MiSeq®			TRB MiSeq®		
	Size (bp)	Mean % Clonal	CV%	Size (bp)	Mean % Clonal	CV%
10	141	9.66	10.4	298	11.83	16.7
5	147	4.90	12.0	298	5.35	18.4
2.5	147	2.39	11.7	298	5.05	18.6
1	147	0.94	13.8	298	2.84	28.0
0	varies	0.61	38.8	varies	0.17	68.8

Results: TRG/TRB MRD Detection

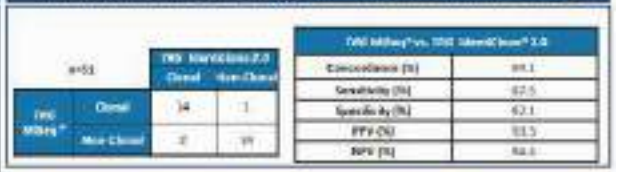


Results: TRG/TRB Positive Clinical Sample

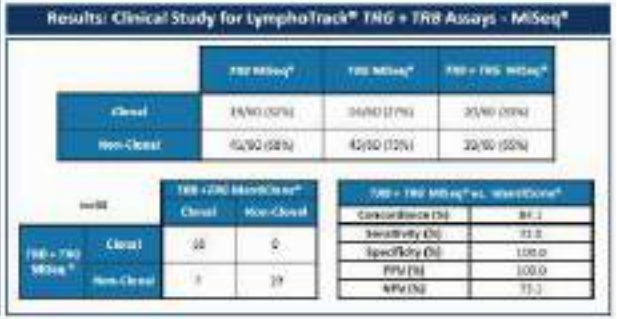


Results: Clinical Study between TRB MiSeq® and IdentiClone® Assays

Results: Clinical Study Comparing TRG MiSeq® and IdentiClone® Assays



Results: Clinical Study for LymphoTrack® TRG + TRB Assays - MiSeq®



AMP 2017

https://invivoscribe.com/Posters/2017/AMP/Invivoscribe_AMP2017_PosterH58.pdf

H58

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

Lisa Chamberlain[1], Valerie McClain[1], Andrew R. Carson[1], Angelo Arias[1], Ogeen Kiva[1], Wenli Huang[1], Christopher Chandler[2], Selena Zheng[1], Daniela Hubbard[1], Daniel Caguioa[1], Zhiyi Xie[1], Jordan Thomes[1], Bradley Patay[1], Timothy Stenzel[1], and Jeffrey E. Miller[1,2] - [1]Invivoscribe, San Diego, CA USA, [2]LabPMM, LLC, San Diego, CA USA

Introduction

The uniform detection of clinically relevant mutations is critical to drug and diagnostics development. Technologies such as next-generation sequencing (NGS) have improved sensitivity of detection, but no clear 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 sensitivity and scale, utilizing technologies ranging from capillary electrophoresis (CE) to next-generation sequencing. For example, Invivoscribe's proprietary NGS large panel-based assays (such as Myleme® and MyAML®) and NGS medium panel-based assays (MyMRD®) can be useful in early investigational stages of drugs and treatments, while highly targeted amplicon based assays (FLT3-ITD MRD) can more specifically home in on clinically relevant biomarkers in later stages of development. Here we demonstrate the ability to reliably detect clinically actionable NPM1, FLT3-ITD, and FLT3-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 small- to 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 50+ AML samples were run on the CE and NGS-amplicon assays for FLT3 variants. In this study, small-scale NGS panels are defined as a few genes, and medium-scaled panels are 20+ genes. The DNA input for the assays ranged from 50-700 ng of high-quality genomic DNA. In NGS-Amplicon assays, regions containing variants were amplified by PCR. Up to 24 samples were purified, pooled, and sequenced. Target capture libraries were generated for NGS panel assays and sequenced. The Illumina MiSeq® platform was used for all NGS assays in this study. Sequencing data was analyzed using proprietary Invivoscribe MyInformatics® software, which produced variant read frequencies (VRF) defined as variant reads divided by total reads.

Table Legend	
CE	Capillary Electrophoresis
SR	Signal Ratio
VRF	Variant Read Frequency
+	Positive detection
-	Not detected
	Sample not tested

Results: Small Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS assays to identify a four base pair insertion in NPM1. 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 NPM1 insertions in five samples.

Table 1. Variant: NPM1

Sample Name	Dilution	CE Data	NGS-Amplicon		Medium-Scale NGS Panel	
		Call	Variant	VRF(%)	Variant	VRF(%)
AML-01	1	-	-	N/A	-	N/A
AML-04	1	+	[illegible]	[illegible]	[illegible]	[illegible]
	1/10		[illegible]	[illegible]	[illegible]	[illegible]
	1/100				[illegible]	[illegible]
AML-05	1		[illegible]	[illegible]	[illegible]	[illegible]
	1/10				[illegible]	[illegible]
AML-08	1	-	-	N/A	-	N/A
AML-09	1		[illegible]	[illegible]	[illegible]	[illegible]
	1/10		[illegible]	[illegible]	[illegible]	[illegible]
	1/100		[illegible]	[illegible]	[illegible]	[illegible]
AML-12	1	+	[illegible]	[illegible]	[illegible]	[illegible]
	1/10				[illegible]	[illegible]
	1/100				[illegible]	[illegible]
AML-18	1		[illegible]	[illegible]	[illegible]	[illegible]
	1/10				[illegible]	[illegible]
	1/100				[illegible]	[illegible]
AML-50	1	-	-	N/A	-	N/A

Results: Large Insertion Detection

We compared performance of CE, amplicon-based, and panel-based NGS methodologies to identify internal tandem duplications (ITDs) in FLT3. Of the eight clinical samples examined, five were positive for the FLT3-ITD; all three assays detected FLT3-ITD at similar frequencies (Table 2). Following dilution of the clinical samples, low frequency mutations are no longer observable in the less sensitive CE assay. However, using the more sensitive NGS assays, variants are still detected at frequencies of 10^{-3} and 10^{-4}.

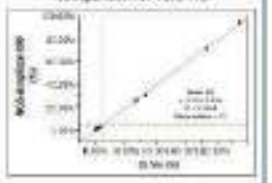
Table 2. Variant: FLT3-ITD

Sample Name	Dilution	CE Data				NGS-Amplicon		NGS-Panel			
		Call	Size	SR	Var (%)	Size	VRF (%)	Small-Scale		Medium-Scale	
								Size	VRF (%)	Size	VRF (%)
AML-01	1	-	N/A	N/A	N/A	-	N/A	-	N/A	-	N/A
AML-04	1	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/10					[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/100									[illegible]	[illegible]
AML-05	1	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AML-08	1	-	-	N/A	-	-	N/A	-	N/A	-	N/A
AML-09	1		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/10		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/100									[illegible]	[illegible]
AML-12	1	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/10									[illegible]	[illegible]
	1/100									[illegible]	[illegible]
AML-18	1		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	1/10									[illegible]	[illegible]
	1/100									[illegible]	[illegible]
AML-50	1	-	N/A	N/A	N/A	-	N/A	-	N/A	-	N/A

Measured allele frequencies are highly similar between the assays, with R^2 values of linear fit ranging from 0.77 to 0.98 (Figure 1). The lowest R^2 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^2 value of 0.998 (Figure 2).

Figure 1. Concordance between methods (R^2)

Figure 2. NGS-Amplicon by CE comparison for FLT3-ITD



Results: Single Nucleotide Variant Detection

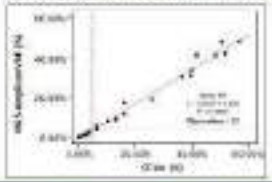
We compared performance across the three assays of a single nucleotide variant in the tyrosine kinase domain of FLT3 (FLT3-TKD). Two clinical samples were positive for the mutation (as determined by CE) and were detected by all three assays (Table 3). As with the other variants, NGS panel assays detected lower frequency variants that were not detected by the CE assay at frequencies of 10^{-3} and 10^{-4}.

Table 3. Variant: FLT3-TKD

Sample Name	Dilution	CE Data	NGS-Amplicon		NGS-Panel			
		Call	Variant	VRF (%)	Small-Scale		Medium-Scale	
					Variant	VRF (%)	Variant	VRF (%)
AML-01	1	-	-	N/A	-	N/A	-	N/A
AML-04	1	-	-	N/A	-	N/A	-	N/A
	1/10				-	N/A	-	N/A
	1/100							N/A
AML-05	1	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
AML-08	1	-	-	N/A	-	N/A	-	N/A
AML-09	1	-	-	N/A	-	N/A	-	N/A
	1/10				-	N/A	-	N/A
	1/100				-	N/A	-	N/A
AML-12	1	-	-	N/A	-	N/A	-	N/A
	1/10							N/A
	1/100	+	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
			[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
							[illegible]	[illegible]
							[illegible]	[illegible]
AML-50	1	-	-	N/A	-	N/A	-	N/A

Additionally, 33 clinical AML samples with varying TKD variant allelic frequencies (2%-58%) as detected by the CE-based CDx assay were tested using the NGS-amplicon based FLT3-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 FLT3-TKD



Conclusions

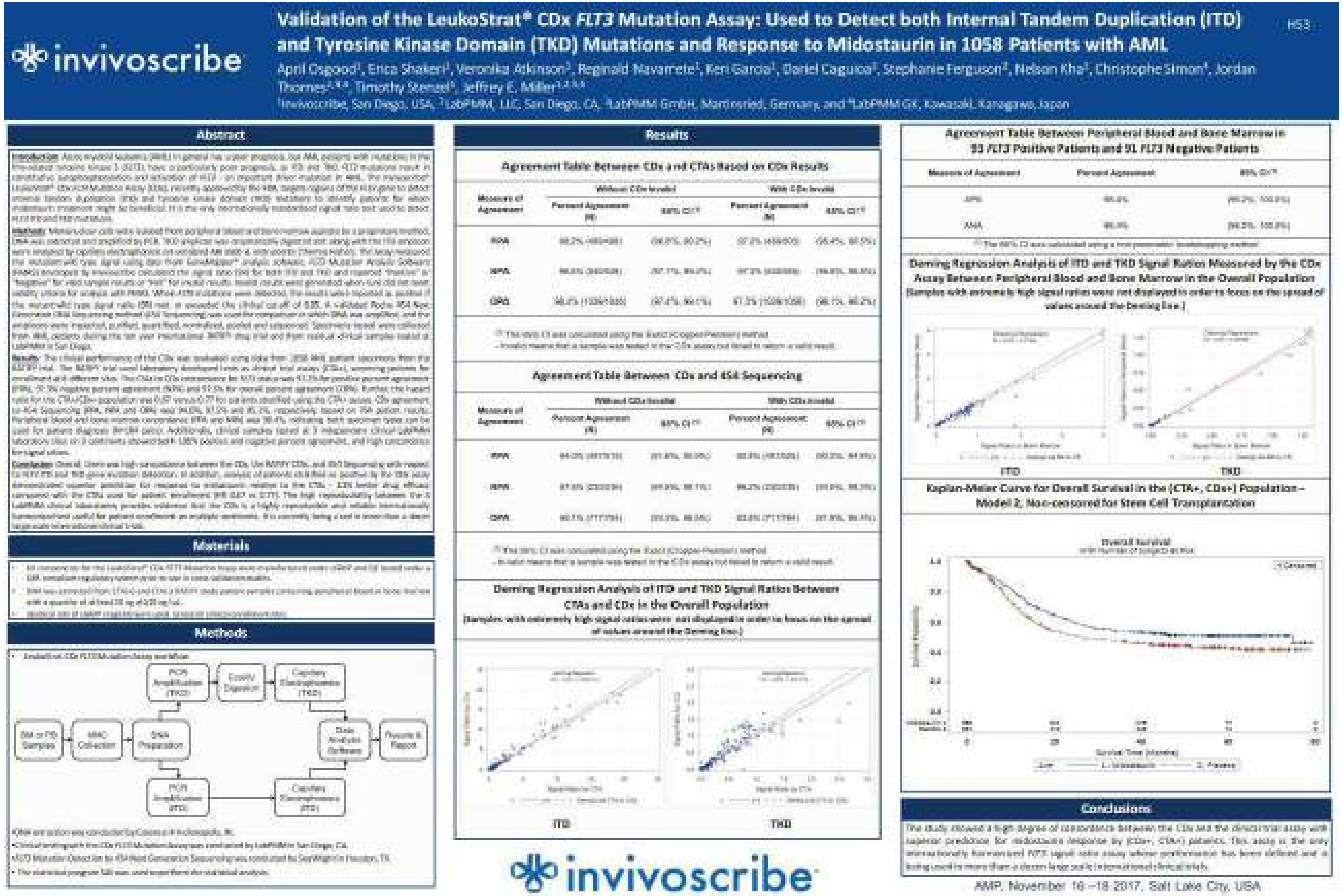
The ever-changing landscape of drug and molecular diagnostics development requires the diligent application of both cost-effective and scale-appropriate assays. Invivoscribe® assays offer excellent detection and frequency concordance of clinically actionable variants in this set of AML clinical samples, 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 improvement of patient outcomes and personalized medicine.

invivoscribe

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

AMP 2017

https://invivoscribe.com/Posters/2017/AMP/Invivoscribe_AMP2017_PosterH53.pdf



invivoscribe®
Improving Lives with Precision Diagnostics®

AMP 2017

https://invivoscribe.com/Posters/2017/AMP/Invivoscribe_AMP2017_PosterH45.pdf

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

H45

Ying Huang[1], Kasey Hutt[1], Jeff Panganiban[1], Austin Jacobsen[1], Fresha Shah[1], Edgar Vigil[1], Selena Zheng[1], Zhiyi Xie[1], Roshanak Bob[2], Tim Stenzel[1], and Jeffrey E. Miller[1]
[1]Invivoscribe, San Diego, USA; [2]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® assays with accompanying bioinformatics using the MiSeq® and PGM NGS platforms, as well IdentiClone® assays on the ThermoFisher® 3500 capillary platform.

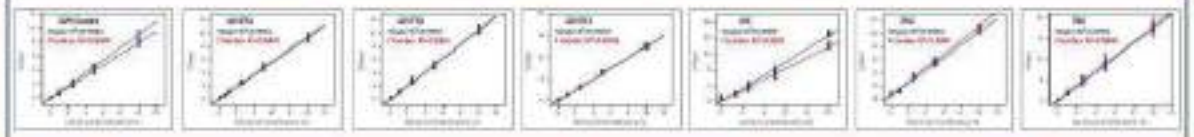
Materials and Methods

- LymphoTrack® NGS-based Assays for the MiSeq® (24 indices) and Ion PGM™ (12 indices), and IdentiClone® CE-based Assays 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 (P/R) of the LymphoTrack® Assays – MiSeq® and PGM™ were validated using clonal control DNA diluted in wild-type polyclonal (tonsil) DNA. Only data from MiSeq® 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® Assays and IdentiClone 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® Software – MiSeq®, or 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®.

Conclusions

This study demonstrated that the comprehensive NGS LymphoTrack® 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: LoD, Linearity, Precision and Reproducibility for LymphoTrack® Assays – MiSeq®



Clonal Control DNA mass (ng)	N	IGH/TCRγ Leader			IGH FR1			IGH FR2			IGH FR3			IGK			TRG			TRB		
		Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%
10	32	490	7.37	12.6	295	5.08	17.5	243	15.74	8.9	334	12.99	6.2	322	7.11	22.8	147	8.65	30.4	338	12.65	16.7
5	48	490	3.73	14.2	295	2.58	19.4	243	7.72	8.2	334	7.08	8.4	322	3.86	21.9	147	4.59	32.0	338	6.36	18.4
2.5	48	490	2.10	12.9	295	1.28	14.3	243	3.83	8.2	334	3.58	8.0	322	1.81	21.1	147	2.39	31.7	338	5.25	19.6
1	48	490	0.70	12.9	295	0.50	19.8	243	1.52	12.5	334	1.43	16.1	n/a	n/a	n/a	147	0.42	33.8	338	2.06	28.0
0	16	varies	0.04	28.2	varies	0.01	34.8	varies	0.06	26.5	334	0.07	28.6	varies	0.01	35.3	varies	0.02	40.3	varies	0.17	46.8

Results: Clinical Study between LymphoTrack® – MiSeq®, LymphoTrack® – Ion PGM™, and IdentiClone® Assays

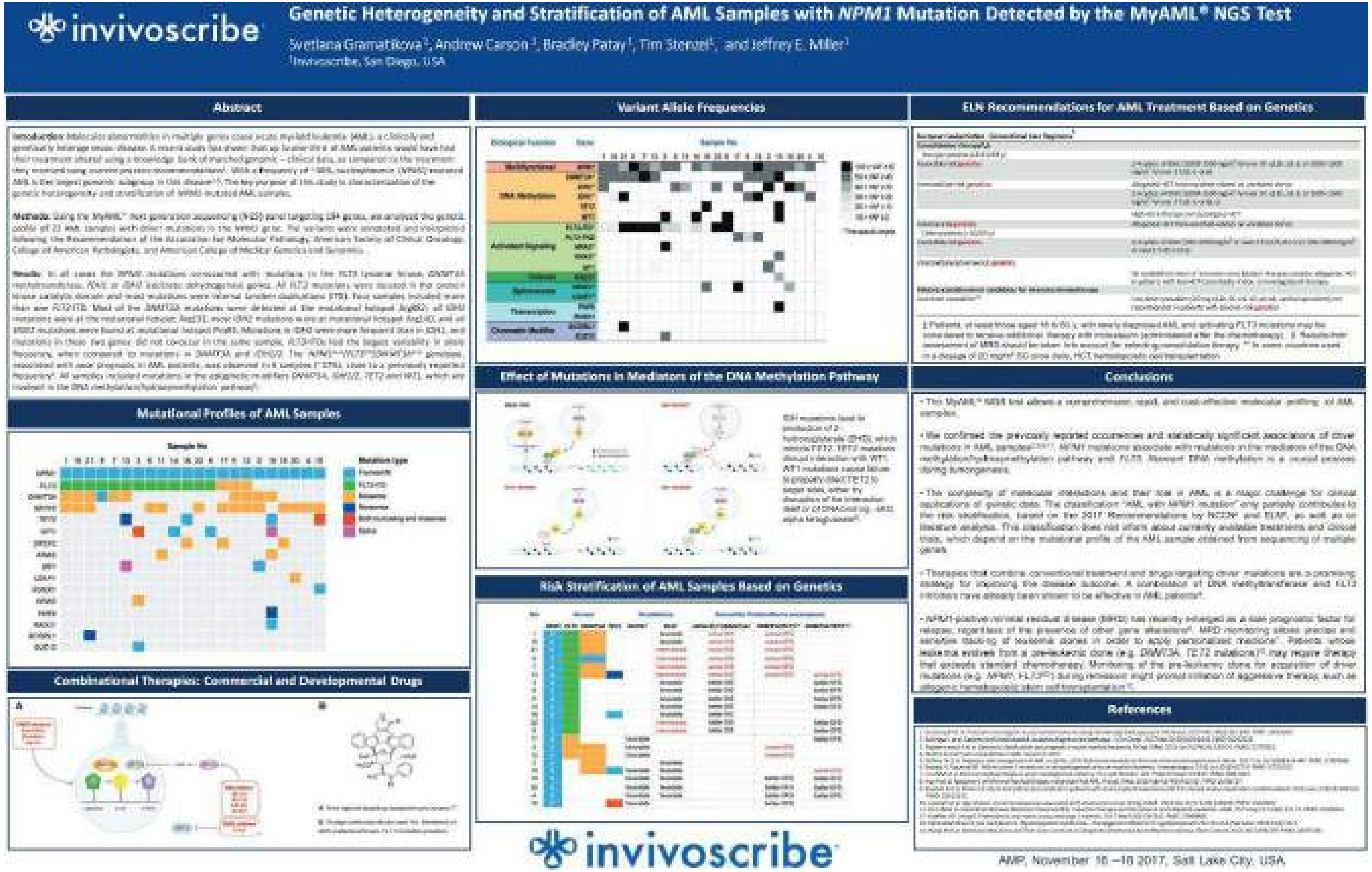
	LymphoTrack® Assays – MiSeq®				LymphoTrack® Assays – PGM™		IdentiClone® Assays			
	IGH FR1/2/3	IGK	TRG	TRB	IGH FR1/2/3	IGK	IGH Tube A/B/C	IGK Tube A/B	TRG 2.0	TRB Tube A/B/C
Clonal (%)	25/59 (42%)	20/59 (34%)	16/60 (27%)	15/60 (25%)	15/40 (38%)	15/55 (27%)	16/59 (28%)	22/59 (37%)	16/58 (27%)	24/60 (40%)
Non-Clonal (%)	32/59 (54%)	35/59 (59%)	41/60 (72%)	41/60 (68%)	22/40 (55%)	37/55 (64%)	28/59 (34%)	35/59 (59%)	35/58 (59%)	28/60 (33%)

Assay	No. of Samples that Met Criteria for Both Methods	No. of Samples with Indicated Result				Concordance (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		*CE+/NGS+	CE+/NGS-	CE-/NGS+	CE-/NGS-					
MiSeq IGH FR1/2/3	44	24	1	1	18	95.5	96.0	94.7	96.0	94.7
MiSeq IGK	55	18	1	0	35	98.2	95.0	100	100	97.2
MiSeq TRG	53	14	2	1	34	94.1	87.5	97.1	93.3	94.4
MiSeq TRB	44	18	6	0	20	86.4	75.0	100	100	76.9
PGM IGH FR1/2/3	41	22	1	0	18	97.6	95.7	100	100	94.7
PGM IGK	43	15	0	0	34	100	100	100	100	100

*CE, IdentiClone® Assays, capillary electrophoresis; NGS, LymphoTrack® Assays – MiSeq®; PPV, positive predictive value; NPV, negative predictive value.

AMP 2017

https://invivoscribe.com/Posters/2017/AMP/Invivoscribe_AMP2017_PosterH19.pdf



AMP Europe 2018

https://invivoscribe.com/Posters/2018/AMP/AMPEuro_LT_S5_final_Final.pdf

EHA 2018

https://invivoscribe.com/Posters/2018/EHA/MyMRD%20cfDNA%20EHA2018_Final%20MD.pdf

P5994

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

Zhiyi Xie, Lisa Chamberlain*, Andrew Carson, Valerie McClain, Ogwen Kiya, Bradley Patay, Martin Blankfard, Timothy Stenzel, Jeffrey Miller
Invivoscribe, Inc., San Diego, United States

Background

Methods

Results: cfDNA Preparation

Results: LOD and Linearity

Results: Variant Detection of SeraCare samples

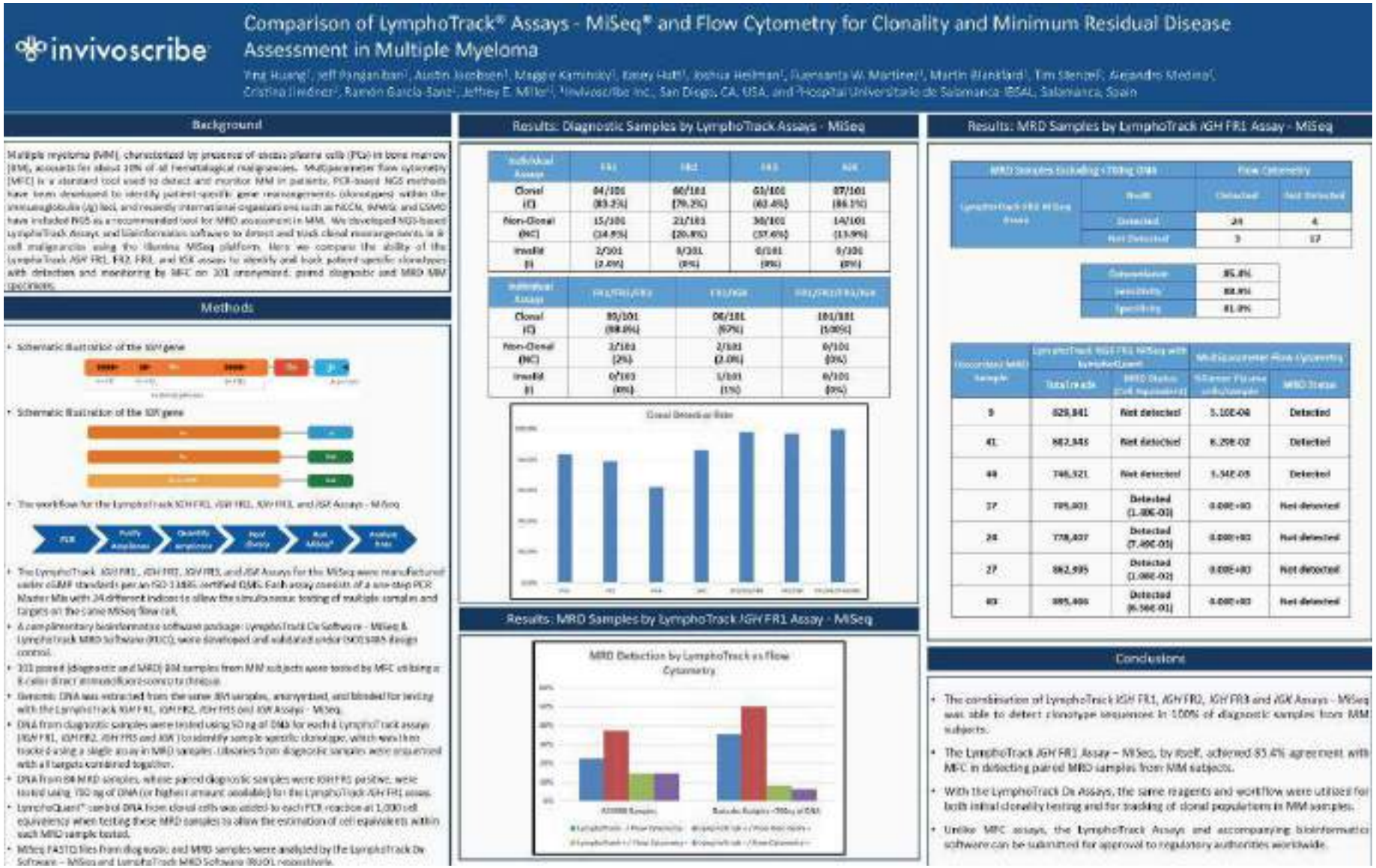
Results: Linearity of SeraCare Samples

Results: Depth of Coverage of Synthetic Plasma Samples

Conclusions

EHA 2018

https://invivoscribe.com/Posters/2018/EHA/EHA2018_LymphoTrack_MRD_MM_FINAL.pdf



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

Ying Huang[1], Jeff Panganiban[1], Austin Jacobsen[1], Maggie Kaminsky[1], Kasey Hall[1], Joshua Heilman[1], Fuensanta V. Martinez[1], Martin Blankfard[1], Tim Stenzel[1], Alexandra Medina[1], Cristina Fiedler[2], Ramón García-Sanz[2], Jeffrey E. Miller[1], [1]Invivoscribe Inc., San Diego, CA, USA; and [2]Hospital Universitario de Salamanca-IBSAL, Salamanca, Spain

Background

Multiple myeloma (MM), characterized by presence of clonal plasma cells (PCs) 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 (clonotypes) within the immunoglobulin (Ig) loci, and recently international organizations such as NCCN, EMMRG, and ESMO have included NGS as a 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 FR1, FR2, FR3, and IGK assays to identify and track patient-specific clonotypes with detection and monitoring by MFC on 101 anonymized, paired diagnostic and MRD MM specimens.

Methods

- Schematic illustration of the IGH gene

- Schematic illustration of the IGK gene

- The workflow for the LymphoTrack IGH FR1, IGH FR2, IGH FR3, and IGK Assays - MiSeq

- The LymphoTrack IGH FR1, IGH FR2, IGH FR3, and IGK Assays for the MiSeq were manufactured under cGMP standards per an ISO 13485 certified QMS. 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 Dx Software - MiSeq & LymphoTrack MRD Software (RUO), were developed and validated under IVD/GMP design control.
- 101 paired (diagnostic and MRD) BM samples from MM subjects were tested by MFC utilizing a 8-color direct immunofluorescence technique.
- Genomic DNA was extracted from the same BM samples, anonymized, and blinded for testing with the LymphoTrack IGH FR1, IGH FR2, IGH FR3 and IGK Assays - MiSeq.
- DNA from diagnostic samples were tested using 50 ng of DNA for each LymphoTrack assays (IGH FR1, IGH FR2, IGH FR3 and IGK) to identify sample-specific clonotype, which was then tracked using a single assay in MRD samples. Libraries from diagnostic samples were sequenced with all targets combined together.
- DNA from 88 MRD samples, whose paired diagnostic samples were IGH FR1 positive, were tested using 150 ng of DNA (or highest amount available) for the LymphoTrack IGH FR1 assay.
- 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 equivalents within each MRD sample tested.
- MiSeq FASTQ files from diagnostic and MRD samples were analyzed by the LymphoTrack Dx Software – MiSeq and LymphoTrack MRD Software (RUO), respectively.

Results: Diagnostic Samples by LymphoTrack Assays - MiSeq

Individual Assays	IGH FR1	IGH FR2	IGH FR3	IGK
Clonal (C)	84/101 (83.2%)	80/101 (79.2%)	83/101 (82.4%)	87/101 (86.1%)
Non-Clonal (NC)	15/101 (14.9%)	21/101 (20.8%)	16/101 (15.8%)	14/101 (13.9%)
Invalid (I)	2/101 (2.0%)	0/101 (0%)	2/101 (2%)	0/101 (0%)

Individual Assays	IGH FR1/FR2/FR3	IGH FR1/FR2	IGH FR1/FR2/FR3/IGK
Clonal (C)	99/101 (98.0%)	98/101 (97%)	101/101 (100%)
Non-Clonal (NC)	2/101 (2%)	2/101 (2.0%)	0/101 (0%)
Invalid (I)	0/101 (0%)	1/101 (1%)	0/101 (0%)

Results: MRD Samples by LymphoTrack IGH FR1 Assay - MiSeq

Results: MRD Samples by LymphoTrack IGH FR1 Assay - MiSeq

MRD Samples Including >700ng DNA	Flow Cytometry	
LymphoTrack IGH FR1 MiSeq Assay	Detected	Not Detected
Detected	39	4
Not Detected	3	17

Concordance	85.8%
Sensitivity	88.6%
Specificity	81.0%

Discordant MRD Samples	LymphoTrack IGH FR1 MiSeq with LymphoQuant		Multiparameter Flow Cytometry	
	Total reads	MRD Status (Cell Equivalent)	%Cancer Plasma cells/sample	MRD Status
9	828,841	Not detected	5.10E-04	Detected
41	982,945	Not detected	6.29E-02	Detected
48	746,321	Not detected	5.54E-05	Detected
37	789,801	Detected (1.30E-03)	0.00E+00	Not detected
24	778,837	Detected (7.49E-05)	0.00E+00	Not detected
27	862,935	Detected (1.06E-02)	0.00E+00	Not detected
83	885,486	Detected (6.56E-01)	0.00E+00	Not detected

Conclusions

- The combination of LymphoTrack IGH FR1, IGH FR2, IGH FR3 and IGK Assays - MiSeq was able to detect clonotype sequences in 100% of diagnostic samples from MM subjects.
- The LymphoTrack IGH FR1 Assay – MiSeq, by itself, achieved 85.4% agreement with MFC in detecting paired MRD samples from MM subjects.
- With the LymphoTrack Dx 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.

AMP 2018

https://invivoscribe.com/Posters/2018/AMP/AMP2018_PosterH045_v8.pdf

H045

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

Ying Huang[1], Austin Jacobsen[1], Jeff Panganiban[1], Edgar Vigil[1], Kasey Hutt [1], Joshua Heilman[1], Martin Blankfard[1], Even H Rustad[2], Malin Hultcrantz[2], Ola Landgren[2] and Jaffrey E. Miller[1]

[1]Invivoscribe, Inc., San Diego, USA, [2]Memorial Sloan Kettering Cancer Center, New York, USA

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 193 anonymized/blinded MM specimens using LymphoTrack Assays. These assays identify B-cell populations by targeting the IGHV Leader, IGH FR1, FR2, FR3, and IGK. Thirty (30) follow-up samples were also tested to demonstrate MRD utility.

Materials and Methods

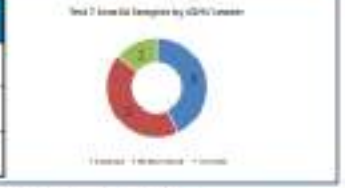
- Five (5) LymphoTrack® Assays (IGHV Leader, IGH FR1, IGH FR2, IGH FR3 and IGK) for the MiSeq®, each with available with 24 indices, were designed, developed, and manufactured per cGMP, then QC tested and validated under a QSR-compliant regulatory system.
- 50 ng of genomic DNA (only ~7,700 cell equivalents) from 193 MM bone marrow (BM) baseline specimens were procured, anonymized and blinded prior to testing with the four LymphoTrack Assays (IGH FR1, FR2, FR3 and IGK) using the MiSeq® 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 5th LymphoTrack Assay, IGHV Leader.
- The most prominant 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® internal control (IC; 100 cell equivalents) was added to each PCR reaction in follow up specimens.
- LymphoTrack® Software (MiSeq®) 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 (58ng ~= 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 procedures 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 diagnosis 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 N=193	IGH FR1	IGH FR2	IGH FR3	IGK	Overall (IGH FR1/FR2/FR3 + IGK)
C (Clonal)	88/193 (45.6%)	81/193 (42.0%)	90/193 (46.6%)	106/193 (54.9%)	152/193 (78.8%)
NC (Non-clonal)	83/193 (43.0%)	109/193 (56.5%)	101/193 (52.3%)	76/193 (40.4%)	34/193 (17.6%)
I (Invalid)	22/193 (11.4%)	3/193 (1.6%)	2/193 (1.0%)	9/193 (4.7%)	7/193 (3.6%)



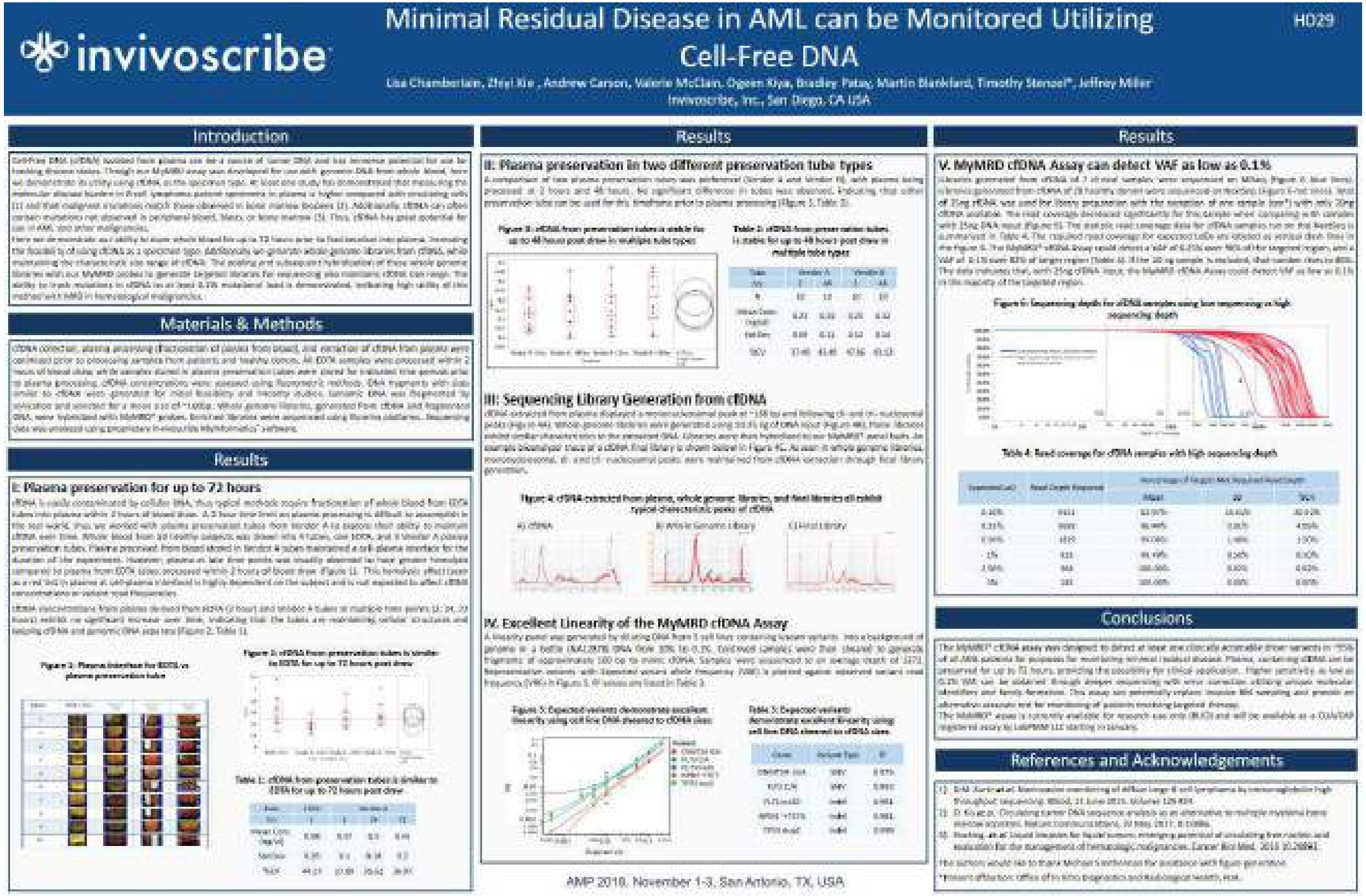
Results: Using LymphoTrack® Assays - MiSeq® to Track Sequences in Follow up MM Samples

Follow-up Sample	Assay	MRD Results	% clonal cells in sample	LymphoQuant Frequency
Sample1_TP1	FR2	Detected	0.166	1.66E-01
Sample1_TP2	FR2	Detected	1.876	2.81E-01
Sample1_TP3	FR2	Detected	1.628	2.98E-01
Sample1_TP4	FR2	Detected	0.001	2.16E-02
Sample2_TP1	FR2	Detected	0.004	1.22E-01
Sample2_TP2	FR2	Detected	0.003	1.16E-01
Sample3_TP1	IGK	Detected	0.002	2.83E-01
Sample3_TP2	IGK	Detected	0.001	2.71E-01
Sample4_TP1	FR3	Not Detected	0.000	7.32E-03
Sample5_TP1	IGK	Detected	1.717	1.17E-01
Sample5_TP2	IGK	Detected	0.093	1.44E-01
Sample6_TP1	IGK	Detected	0.001	2.15E-01
Sample6_TP2	IGK	Detected	0.003	7.35E-03
Sample6_TP3	IGK	Detected	0.004	9.65E-02
Sample6_TP4	IGK	Detected	0.019	1.05E-01
Sample6_TP5	IGK	Detected	0.002	1.25E-01
Sample7_TP1	FR3	Detected	2.773	3.33E-01
Sample7_TP2	FR3	Detected	0.168	1.36E-01
Sample8_TP1	FR3	Detected	0.011	4.14E-03
Sample8_TP2	FR3	Detected	0.044	9.97E-03
Sample8_TP3	FR3	Detected	0.070	1.77E-01
Sample8_TP4	FR3	Detected	0.882	1.55E-01
Sample8_TP5	FR3	Detected	0.081	2.38E-02
Sample8_TP6	FR3	Detected	38.228	7.08E-01
Sample9_TP1	FR2	Detected	1.190	1.19E-01
Sample9_TP2	FR2	Detected	0.089	2.07E-01
Sample10_TP1	FR3	Not Detected	0.000	2.83E-01
Sample10_TP2	FR3	Detected	0.003	1.91E-01
Sample11_TP1	FR1	Not Detected	0.000	8.33E-01
Sample12_TP1	FR3	Not Detected	0.000	2.46E-01
Overall detecting samples: 28/30 (87%)				

invivoscribe

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

https://invivoscribe.com/Posters/2018/AMP/AMP2018%20H029%20v10_ASH.pdf

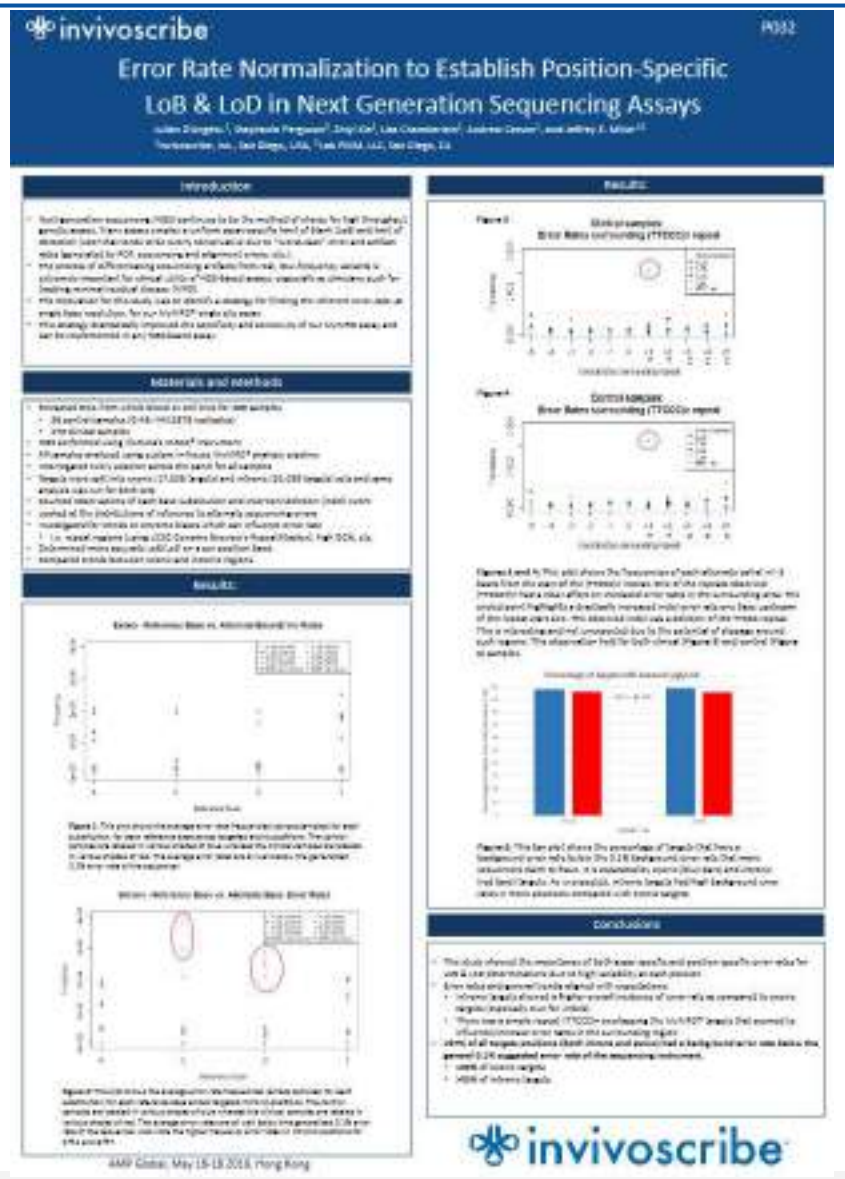


invivoscribe

Improving Lives with Precision Diagnostics

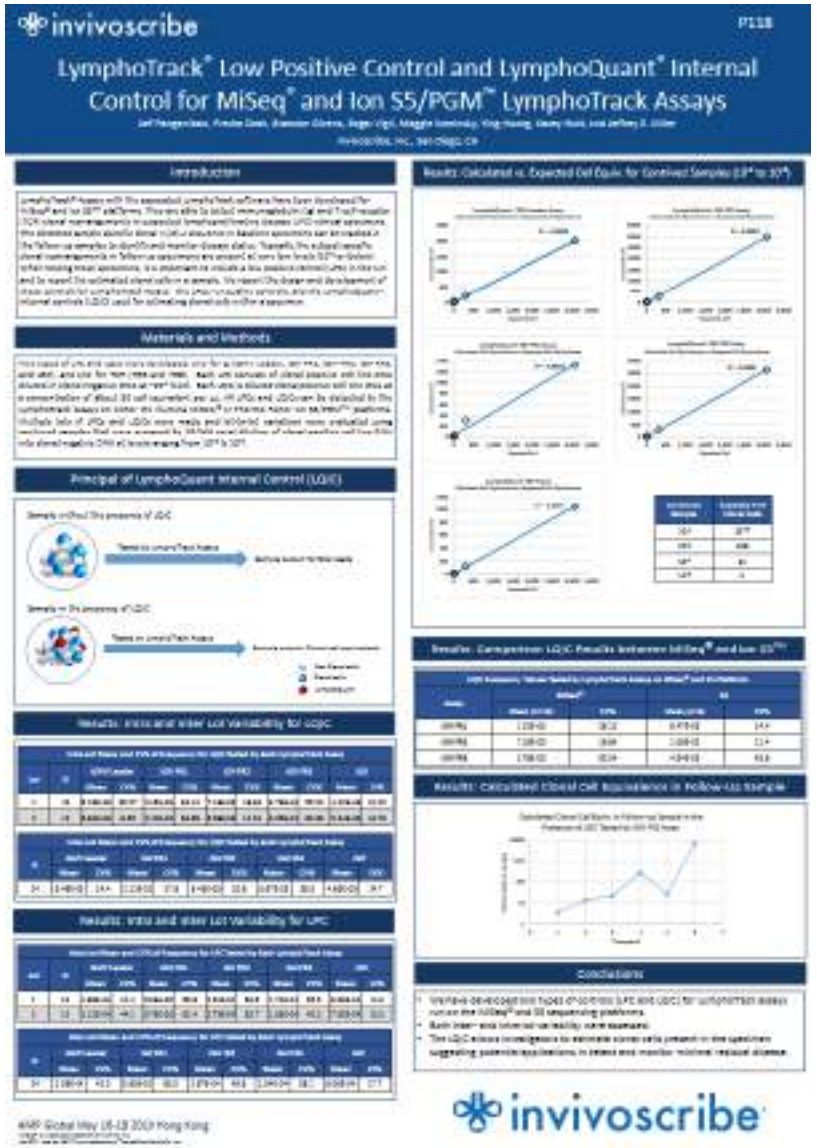
AMP Global 2019

https://invivoscribe.com/Posters/2019/AMP/FINAL_Poster_Presentation_ErrorRateNormalization.pdf



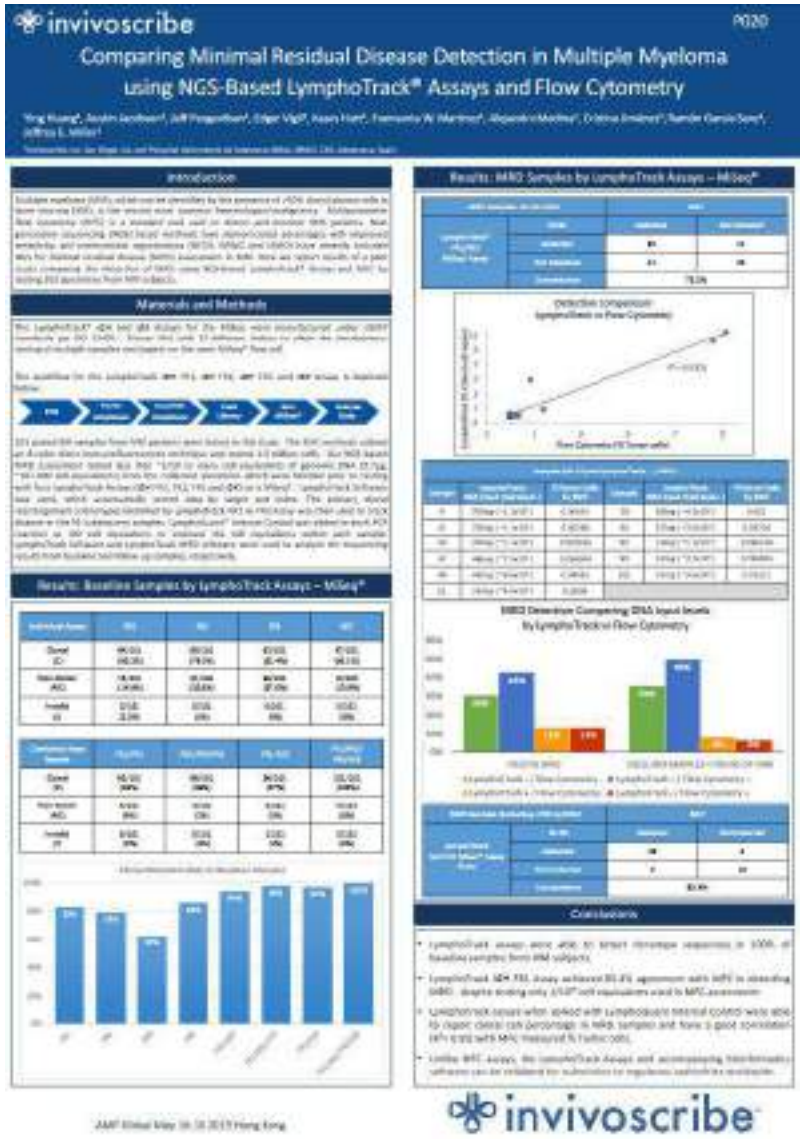
AMP Global 2019

https://invivoscribe.com/Posters/2019/AMP/AMPGlobal2019_poster_LQIC_LPC_Final.pdf



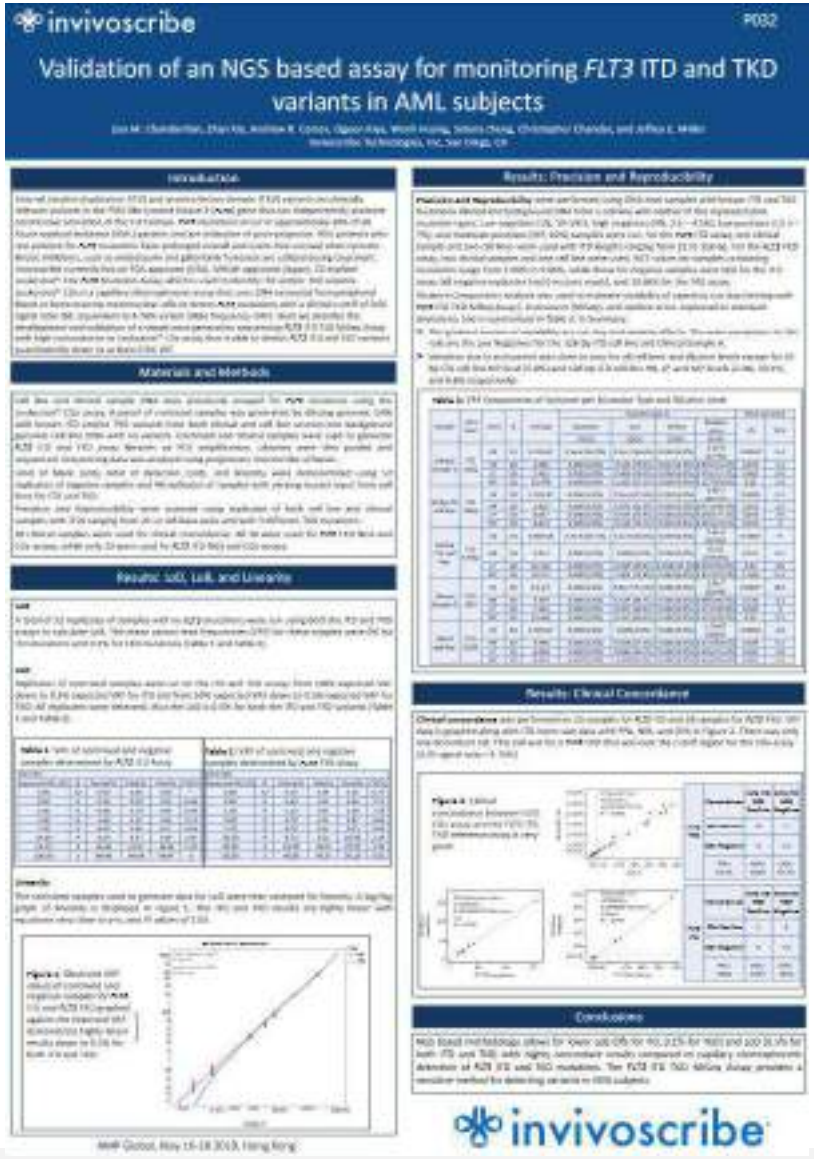
AMP Global 2019

https://invivoscribe.com/Posters/2019/AMP/AMPG2019_LymphoTrack_MRD_MM_FINAL.pdf



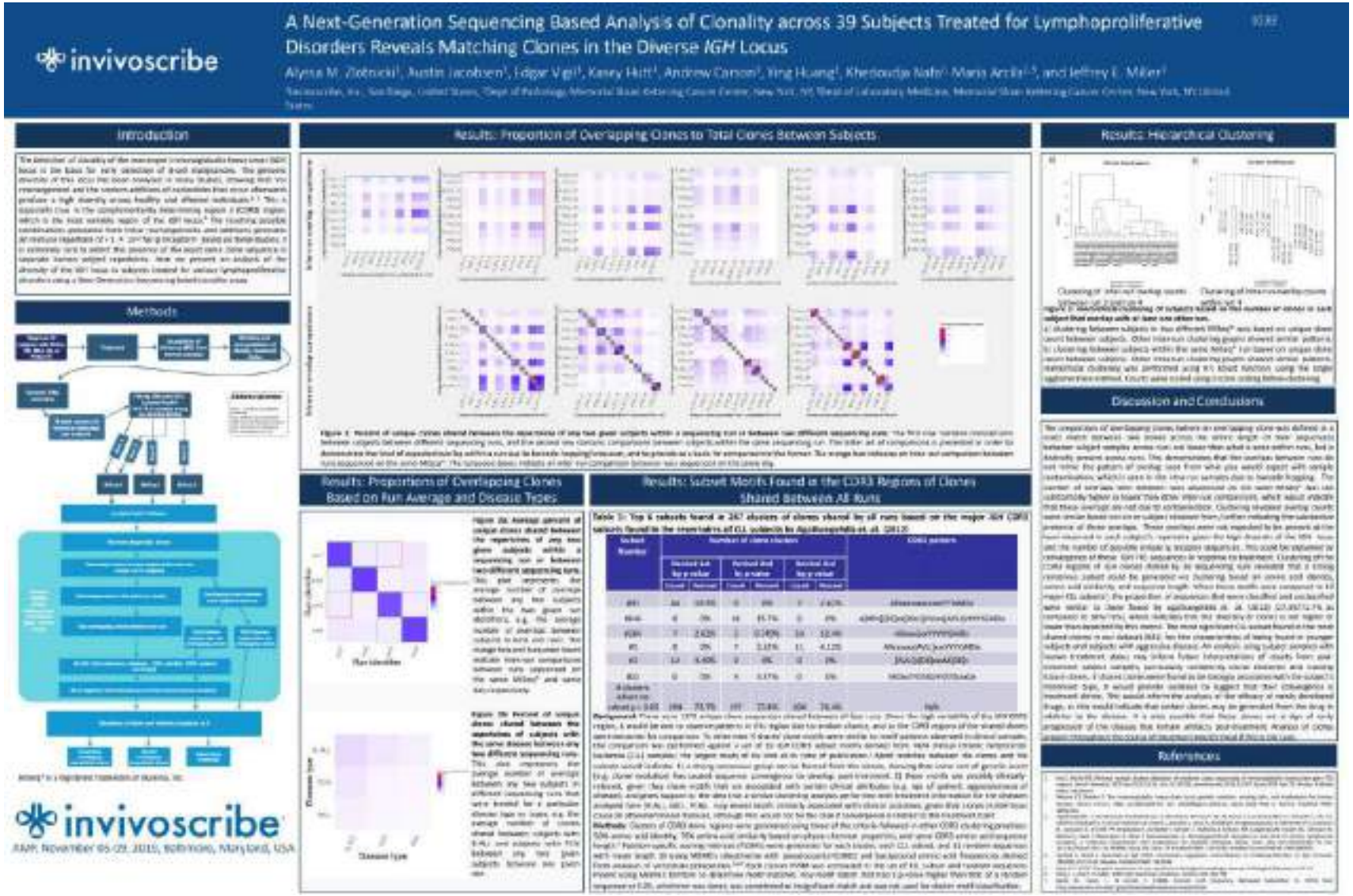
AMP Global 2019

https://invivoscribe.com/Posters/2019/AMP/086%20poster%20AMPGlobal2019_final_V3.pdf



AMP 2019

https://invivoscribe.com/Posters/2019/AMP/AMP_2019_poster_AMZ_final.pdf



AMP 2019

https://invivoscribe.com/Posters/2019/AMP/AMP2019_Poster_TT054.pdf

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
Invivoscribe, Inc., San Diego, USA

Introduction

Materials and Methods

Results

Discussion and Conclusions

AMP 2019

https://invivoscribe.com/Posters/2019/AMP/AMP2019_TT057_FINAL.PDF

Evaluation of An Alternative Fragmentation Method in High Throughput NGS Sample Testing of Minimal Residual Disease in Hematological Malignancies

TT057

Angelo Arias, Lisa Chamberlain, Julian D'Angelo, Ogeen Kiya, Andrew Carson, Zhiyi Xie, Jeffrey Miller
Invivoscribe, Inc. San Diego, CA USA

Introduction

Invivoscribe® collaborates with leading pharmaceutical companies testing large batches of samples using target capture-based next generation sequencing (NGS) assays such as MyMRD® and MyAML® to identify clinically actionable variants. The Invivoscribe MyMRD assay is a multi-gene targeted NGS panel that covers 23 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 for 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-handling time and dramatically increase throughput. We compared enzymatic fragmentation (using the KAPA™ HyperPlus kit) to our standard mechanical shearing protocol (KAPA™ HyperPrep kit) in the context of the MyMRD assay. In this presentation, the two methods will be referred to as MyMRD-MS (Mechanical Shearing, KAPA™ HyperPrep), and MyMRD-EF (Enzymatic Fragmentation, KAPA™ HyperPlus).

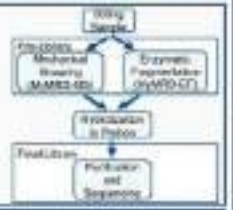
We present similar performance between enzymatic fragmentation and mechanical shearing in both wet lab metrics (yield, average size) and dry bio-metrics (assay reportable variant, or SNV/Indel limit of detection or LoD). Notably, the assay present limitations in the detection of structural variants (SVs). as MyMRD-EF was less sensitive to the detection of SVs than MyMRD-MS.

Materials & Methods

Genomic DNA was enzymatically fragmented using the KAPA™ HyperPlus kit (Roche) or mechanically fragmented using a Covaris® M220 Focused Ultrasonicator. Fragmented DNA was then ligated to Illumina adapters to generate NGS libraries ("Pre-Libraries") which were quality checked by LabChip® (PerkinElmer®) or BioAnalyzer (Agilent). These libraries were checked for minimum criteria of percent purity and average size. Yield was obtained using Quant-it™ (ThermoFisher™) for DNA quantitation.

These Pre-Libraries are then hybridized to oligonucleotide probes that target the 23 genes in the MyMRD assay.

Finally, these hybridized "Final Libraries" are purified and prepared for sequencing using Illumina platforms (MiSeq®). Sequencing data was analyzed using proprietary bioinformatics (MyInformation® software). SNV, indel, and SV calls made from the sequencing data were analyzed.



Results

I: Optimization of fragmentation time

To validate enzymatic fragmentation as robust DNA fragmentation method, we first sought to establish a suitable fragmentation method that would produce usable fragments for the MyMRD assay. To compare the two methods, we used Pre-Library data from a previous study in which we validated the MyMRD-MS method. The top panel in Figure 1 illustrates titration of fragmentation time from 5 minutes to 15 minutes. We used average size as the primary output. As expected, longer fragmentation times produced shorter fragments, with the 10' incubation fragments meeting the 500bp average size threshold for the MyMRD assay. 8 minutes was selected as the optimal fragmentation time to reproducibly obtain fragments >500bp. We also analyzed yield and percent purity at the MyMRD Pre-Library stage. Percent purity was defined as the amount of useful product in the 300-700bp range. A minimum of 150ng Pre-Library yield and purity of >30% is required for the MyMRD assay. All four fragmentation time points (5', 8', 10', and 15') produced sufficient yield and purity for the assay (Figure 1).

Figure 1: Enzymatically fragmented Pre-Libraries meet MyMRD criteria for average size, yield, and % purity

II: Comparison of mechanically and enzymatically fragmented libraries

Since fragmentation only involves the Pre-Library stage of library construction, we set out to establish whether enzymatically fragmented DNA (using KAPA™ HyperPlus) was equivalent to mechanically fragmented DNA (using KAPA™ HyperPrep). Using a 500ng DNA input (standard for MyMRD), we compared the average size, yield, and % purity as measured by Quabird BioAnalyzer / LabChip between both methods. Figure 2 displays performance between historical data from mechanical shearing (N=234) and our entire dataset obtained using enzymatic fragmentation (N=105). These data demonstrate the equivalence at the Pre-Library stage between MyMRD-EF and MyMRD-MS.

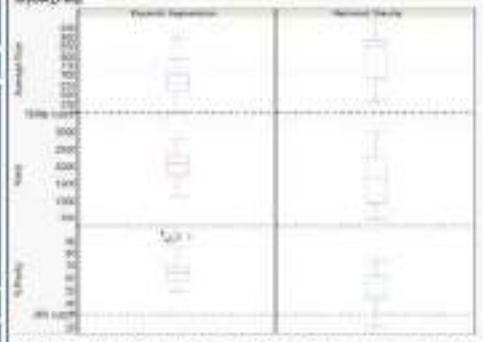


Figure 2: Enzymatically fragmented Pre-Libraries are similar to mechanically sheared Pre-Libraries with respect to average size, yield, and % purity.

III: Expected and Observed Read Frequency display excellent linearity

A series of reactional linearity samples that were previously run on the MyMRD-MS assay were re-run using the MyMRD-EF workflow to demonstrate equivalence in linearity using the alternate library prep kit. We compared expected variant VAFs to observed VAFs and noted excellent linearity between expected and observed read frequency (R^2=0.985). These data demonstrate equivalent linearity between MyMRD-EF and MyMRD-MS.

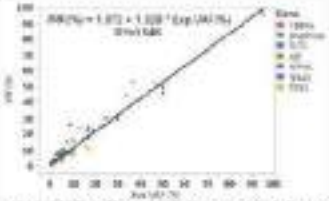


Figure 3: Expected variant VAFs vs Observed VAFs from enzymatically fragmented samples.

Results

IV. Limit of Detection: SNVs, Indels, SVs

Gene	Variant	Type	Expected VAF	Observed VAF
FLT3	ITD	Indel	0.10%	0.10%
		SNV	0.17%	0.46%
		SNV	10%	10.73%
		SNV	0.46%	0.34%
		SNV	2.86%	2.64%
		Indel	10%	10.39%
		Indel	1.00%	1.40%
		Indel	1.43%	2.07%
		Indel	0.00%	0.00%
KRAS		Indel	0.28%	0.28%
		Indel	0.76%	0.55%
		Indel	0.24%	0.46%
NRAS		SNV	1.03%	0.64%
		Indel	0.48%	0.16%
		SNV	0.00%	0.17%
IDH1/2		Indel	3.80%	4.40%
DNMT3A		SNV	0.42%	0.35%
		SNV	2.23%	2.36%
ASXL1		SNV	0.19%	0.00%
		SNV	1.73%	0.35%
		SNV	1.00%	0.04%
CEBPA		Indel	0.48%	0.62%
		SNV	2.45%	2.04%

Table 1: Indel and SNV detection using the MyMRD-EF workflow. MyMRD-MS and MyMRD-EF are equivalent in Indel and SNV detection.

Previous studies using the MyMRD-MS method revealed the limit of detection (LoD) for indels and single nucleotide variants (SNVs) to be 0.34% and 0.5%, respectively. These studies defined LoD as the lowest VAF for which the variant is detected in all replicates (4/4 replicates). To confirm LoD using enzymatic fragmentation, 4 replicates of contrived samples from the MyMRD-MS study were re-run using the MyMRD-EF workflow. Detection of variants in the MyMRD panel with varying expected VAFs for indels and SNVs are listed in Table 1 below. All expected indels and SNVs were detected down to 0.10% and 0.17%, respectively. These data establish equivalency between MyMRD-MS and MyMRD-EF in indel and SNV detection.

Gene	Variant	Detected	Expected VAF
		3 of 4	0.10%
		3 of 4	0.60%
		3 of 4	0.91%
		2 of 4	2.80%
		4 of 4	0.26%
		4 of 4	1.80%
		4 of 4	10.40%
		4 of 4	7.70%

Table 2: SV detection using the MyMRD-EF workflow. MyMRD-EF failed to detect SVs previously detected by MyMRD-MS.

Pre-structural variants (SVs), the MyMRD-MS LoD was found to be 1.6%. Using the MyMRD-EF workflow, we were able to detect all (4 of 4) RUNX1/CBFA2T1 translocations at the 1.0% level as expected. However, only 2 of 4 RUNX1/CBFA2T1 translocations were detected at 0.50% expected VAF. Detection failure was traced to low coverage resulting from inversion artifacts detected in the sequencing data. These inversions were not present in mechanically fragmented samples and varied in size, position, and frequency, indicating these artifacts are exclusive to enzymatic fragmentation. These data suggest MyMRD-EF is not compatible with structural variant calling.

Conclusions

With the notable exception of inversion artifacts, enzymatic fragmentation using the KAPA® HyperPlus kit with the MyMRD 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 50% in enzymatically fragmented samples that were detected using MyMRD with mechanical shearing. Incorporating enzymatic fragmentation to current and future assays could provide a cost-effective way to process large batches of samples and enable automated construction of NGS libraries; however, mechanical shearing is still required for specimens requiring structural variant detection at low prevalence.

EHA 2020

https://invivoscribe.com/Posters/2020/EHA-2020-KHUTT_v4.pdf

EUROPEAN HEMATOLOGY ASSOCIATION

TRG Clonality Interrogation by CE and NGS: Bridging the Gap Between Classical and Leading Edge Technologies

invivoscribe
Improving Lives with Precision Diagnostics

K. HUTT[1], A. ARIAS[1], E. FINNEGAN[1], I. PANGAMBAN[1], D. VISSERS[2], N. KHAT[1], I. DANNER[2], O. MEDINA[1], D. CAGUIOA[1], J. LASSITER[2], Y. HUANG[2]
1 Invivoscribe, Inc., 10222 Barnes Canyon Road, Building 1, San Diego, CA 92121-2711 USA
2 LabPMM LLC, 10222 Barnes Canyon Road, Building 1, San Diego, CA 92121-2711 USA

INTRODUCTION

TRG clonality is a powerful tool to assist in diagnosing numerous 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, elucidating 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 suspect T-cell lymphoproliferative diseases routinely tested using TRG assays were tested using both the LymphoTrack® TRG Dx assay (in development) on the Illumina® MiSeqDx® 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 sample) was used as the metric for calling positives and negatives. For CE plots, a standard Relative-Peak-Ratio (RPR) calculation was used as a metric for calling positives and negatives.

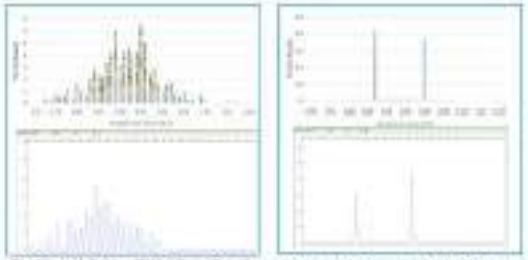
The results from the NGS-based assay were converted, *in silico*, to NGS-E plots, by taking the sizes of the sequenced amplicons and plotting them on a histogram. On CE-based assays, electropherograms are the standard output. The two different groups were then compared (NGS-E vs CE). The NGS-E plots 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).

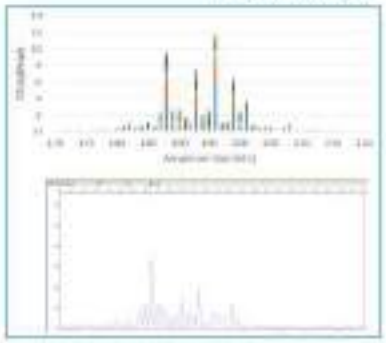
$$H(X) = -\sum_{i=1}^{n} P(x_i) \log_b P(x_i)$$

To ensure fair comparisons between samples and size columns that had different numbers of participating amplicons, each size column was initialized with 65 participating amplicons (representing the highest number of participating amplicons across all samples/sizes), and each initialized amplicon was given a minimum %Total Reads value of 0.0001525. 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 65 participants contributes 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

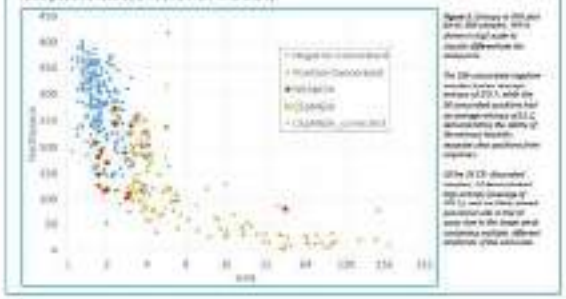
385 clinical samples were run on both NGS and CE assays. Examples of concordant results between these two assays are shown in *Figure 1* and *2* for negative and positive TRG clonality, respectively. An example of a discordant result between NGS and CE is shown in *Figure 4*. As seen in this example, 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 shows that this sample should be negative for TRG clonality.





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 (86.0%, average entropy 279.7, 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 (10.9%) were discordant. Of the discordant samples, 28 were NGS+ (red dots, *Figure 5*), while 28 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 28 were highly complex peaks (average entropy of 202.3, yellow dots with blue squares, *Figure 5*) that were correctly distinguished in the NGS data as being polyclonal. These 14 represent missed 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 diversity. 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%).



CONCLUSIONS

A significant hurdle 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 times 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 labs can use to become more accustomed to NGS data, as they address potential concerns about converting to NGS-based testing.

CONTACT INFORMATION

Dr. Kasey Hutt
khutt@invivoscribe.com
1.858.224.0800