The implementation of the Oncomine® Leader-J assay for IGHV somatic hypermutation analysis in CLL

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EAHP

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NEXT GENERATION DIAGNOSTICS

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Overview

- Introduction
- Somatic Hypermutation Status and analysis
- Stereotypy
- Leader vs FR1 primers
- ERIC guidelines
- Assay comparison
- Results
- Discussion & Conclusion

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Introduction

- CLL low-grade B-cell lymphoma WHO classification
- No changes in the 2022 edition
- Numerous prognostic and predicative factors

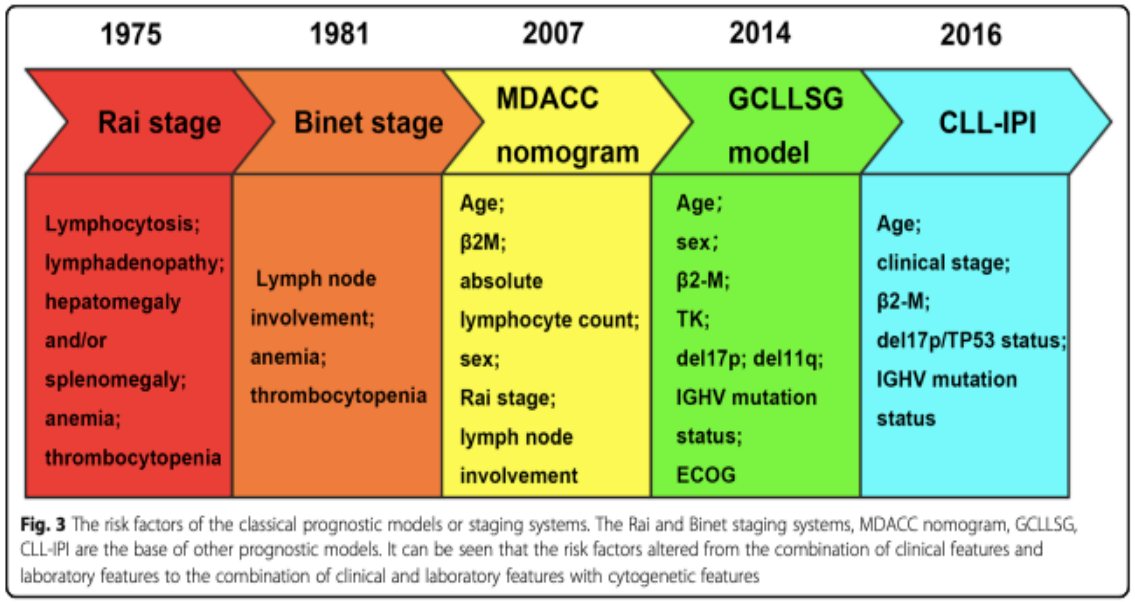

Fig. 3 The risk factors of the classical prognostic models or staging systems. The Rai and Binet staging systems, MDACC nomogram, GCLLSG, CLL-IPI are the base of other prognostic models. It can be seen that the risk factors altered from the combination of clinical features and laboratory features to the combination of clinical and laboratory features with cytogenetic features

Yun et al. Biomarker Research 2020;8:40-47

Prognostic factor	Points
Del17p on FISH or *TP53* mutation	4
Unmutated *IGHV* genes	2
Serum β2 microglobulin >3.5 mg/L	2
Rai stage I–IV	1
Age >65 years	1

Cumulative CLL-IPI score	Risk category	5-year TFS[a]
0–1	Low risk	78%
2–3	Intermediate risk	54%
4–6	High risk	32%
7–10	Very high risk	0%

FISH fluorescence in situ hybridization, *IGHV* immunoglobulin heavy chain gene, *TFS* treatment-free survival
[a]For the Mayo validation cohort

International CLL-IPI Working Group. Lancet Oncol 2016;17(6):779-790

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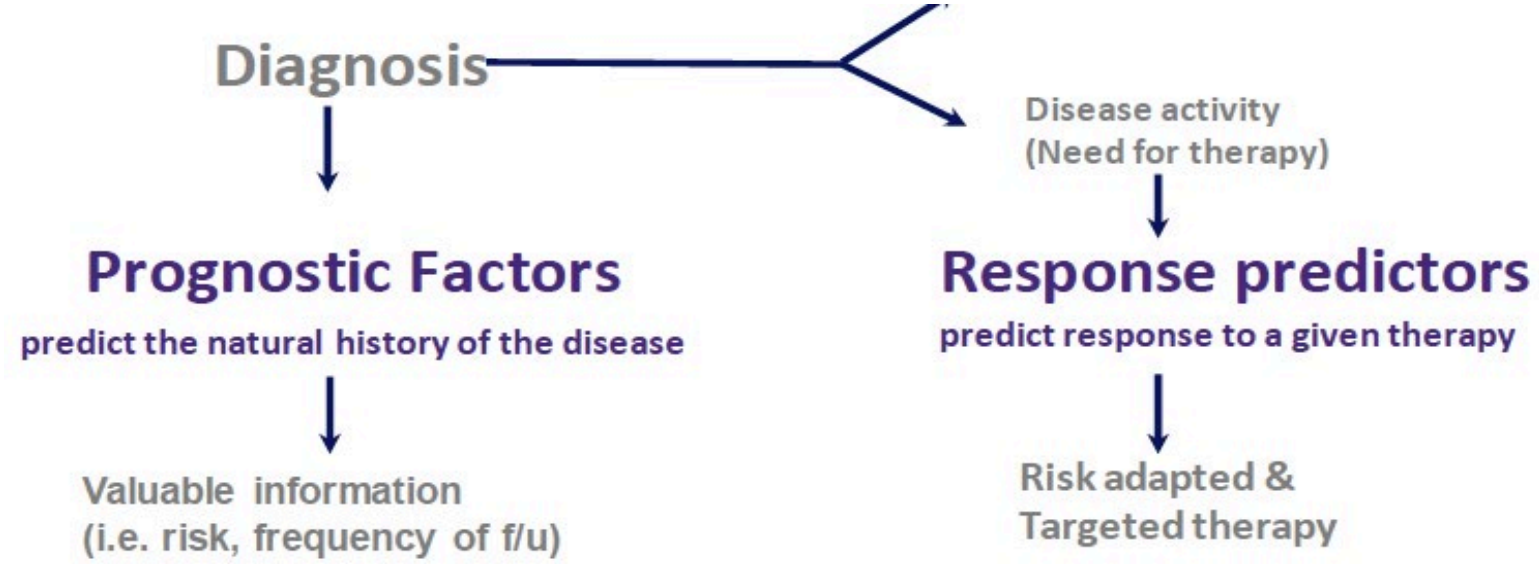
Introduction



IGHV

Prognostic Biomarkers	Predictive Biomarkers
Progression_ Richters	Ibrutinib
Death	FCR- unmutated worse PFS
Toxicity	Treatment tailoring
Frequency of follow up	
Patient counseling	
TTFT and worse OS	

C. Moreno, E Montserrat Blood Rev.2008;22:211-219

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Somatic Hypermutation (SHM) Status

- SHM status mostly reported on dominant clone
- Defined as significant deviation of the variable region (V) of the IGH gene; >2% (mutated) from the closest germline IGHV reference seq
- Typically occurs in context of follicle center reaction
- Involves introduction of point mutations into DNA, with hotspots for mutation being at CDRs coding for areas of maximal Ag contacts
- If B-cells undergo clonal proliferation
- Each cell in clone contains identical IGHV sequence

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SHM analysis

- Stability of intraconal IGHV sequence in CLL
- Some CLL clones have low level ongoing SHM, not enough to hamper SHM analysis
- May be IGHV sequence heterogeneity due to evolution of sub-clones

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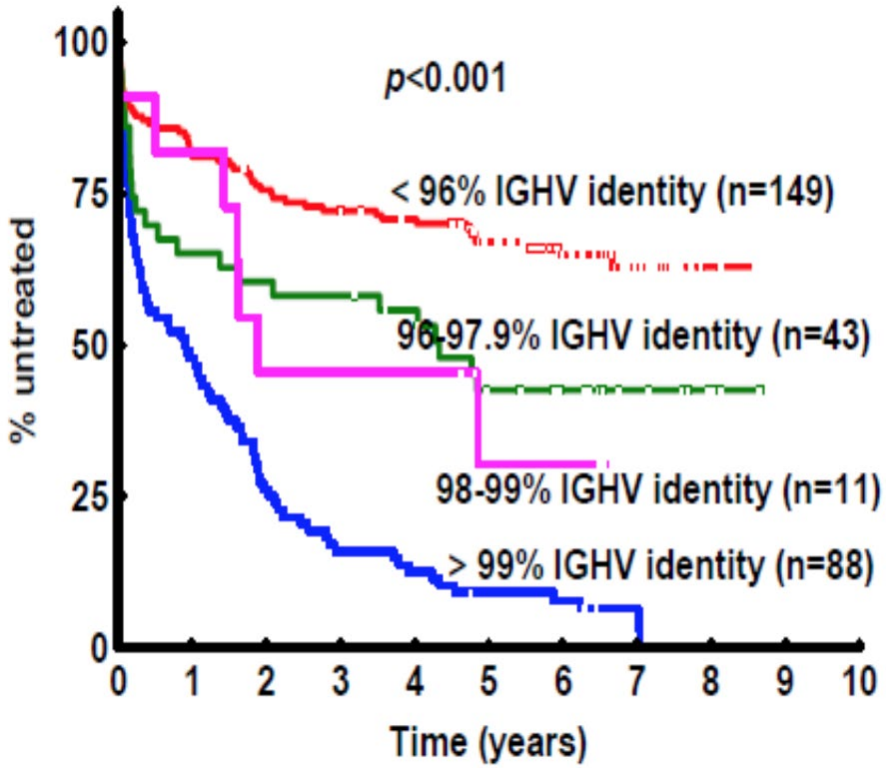
SHM – Borderline cases

- IGHV germline identity between 97-98%
- Not intermediate prognosis
- Mix of cases with aggressive and indolent disease
- TTFT similar to M-CLL except stereotypy subset #2 and #169
- Use of germline % as continuous variable is associated with PFS and OS
- But also an enrichment of cases with #169 and other IGLV3-21 with R110 mutation
- NB: close follow up



Precision medicine in CLL: What is the role of immunoglobulin Gene Analysis: IGHV workshop Paris 4th- 5th July 2019. Diagnostic workshop 4 Jul Stamatopoulos presentation

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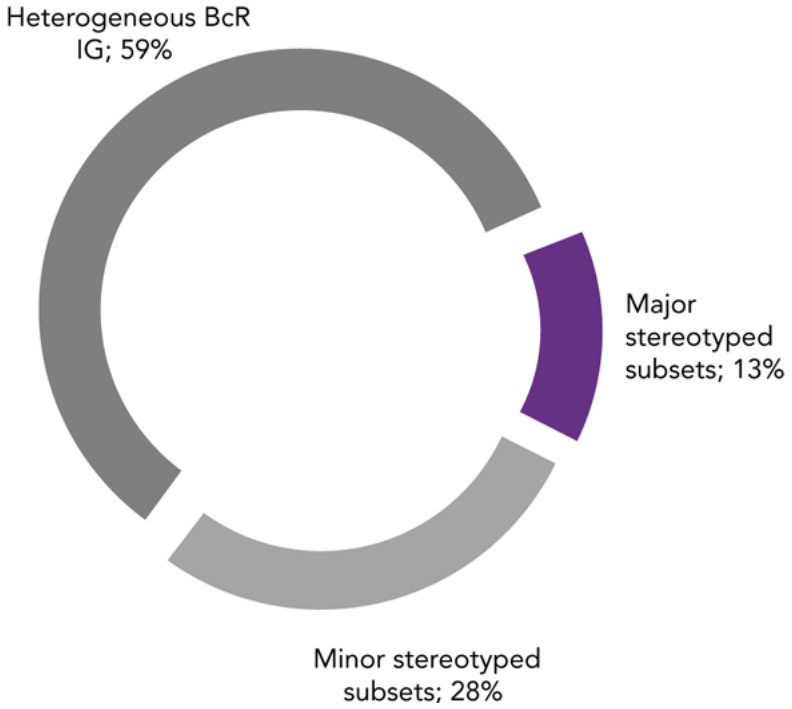
Stereotypy in CLL

- Subcategorization with specific subsets of CLL based on constrained features of the IGHV CDR3
- Proportion of unrelated CLL patients express highly homologous BCRs
- Subsets prognostic significance
- May be independent of SHM status
- The SHM and stereotypy predictive

41% of all CLL can be assigned to subsets with stereotyped B cell receptor (BcR).

29 major subsets were identified corresponding to the 13% of the cohort.



Agathangelidis et al, Blood 2021

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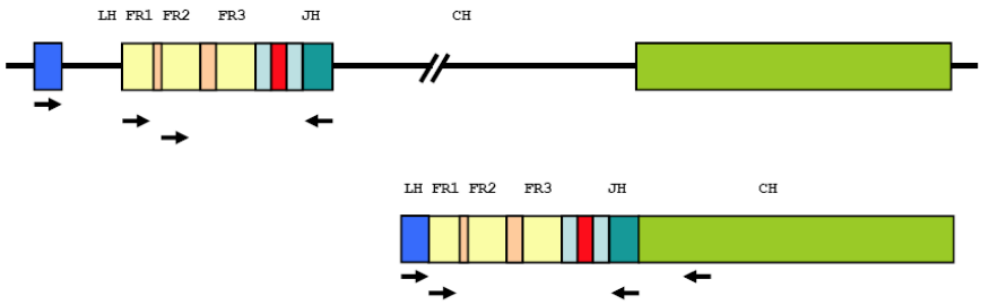
Leader J vs FR1 Primers

	Advantages	Disadvantages
Leader	Accurate, based on whole IGHV gene	Slightly lower detection rate
FR1	Slightly higher detection rate	Estimation of the SHM level
	Widely used in clonality testing	

- Comprehensive approach would include both strategies



- FR1 primers used: complete IGHV region not assessed
- A smaller denominator of nucleotide bases is seen and may result in an overestimation of the mutation percentage

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ERIC guidelines

OPEN

Leukemia (2017) **31**, 1477–1481
www.nature.com/leu

EDITORIAL

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

Leukemia (2017) **31**, 1477–1481; doi:10.1038/leu.2017.125

ERIC
european research initiative on CLL

Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: the 2022 update of the recommendations by ERIC, the European Research Initiative on CLL

Andreas Agathangelidis[1,2], Anastasia Chatzidimitriou[1,3], Thomas Chatzikonstantinou[1,4], Cristina Tresoldi[5], Zadie Davis[6], Véronique Giudicelli[7], Sofia Kossida[7], Chrysoula Belessi[8], Richard Rosenquist[3,9], Paolo Ghia[10✉], Anton W. Langerak[11], Frédéric Davi[12], Kostas Stamatopoulos[1,3] and on behalf of ERIC, the European Research Initiative on CLL

NGS:

More detailed view BcR IG repertoires
Amplification biases and quantification issues
Lack of multicenter validated protocols
Revealed existence minor sub-clones due to intra-clonal diversification or distinct clones

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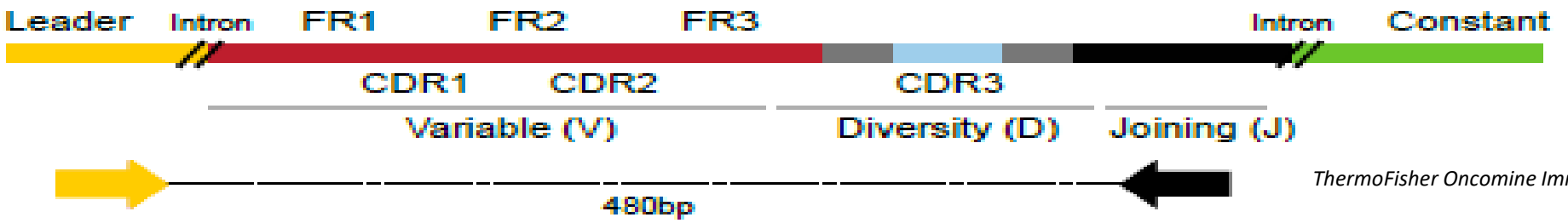
Oncomine® IGHV Leader J Assay



ThermoFisher Oncomine Immune Repertoire User Guide

- Compare LymphoTrack® Dx IGH FR1 assay to ThermoFisher Oncomine® IGHV Leader-J primer assay
- Assessed concordance for SHM status, V-gene usage and mutation frequency rate
- Compared the assignment of stereotypy
- Assessed robustness of the assay in a diagnostic setting

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Assay comparison

- Both assays were run on the Ion S5 XL platform
- Total of 33 samples on both assays for direct comparison
- Different sample types included (PB, BM, sorted)
- Samples multiplexed with an Ampliseq TP53 assay
- Stereotypy and confirmation of software findings assessed online ARResT tool
- Interpretation algorithm developed

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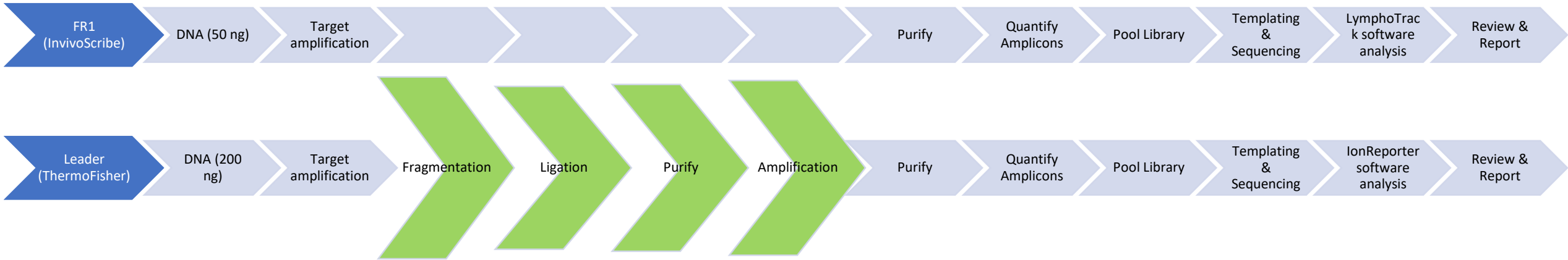
Workflow FR1 vs Leader-J assays



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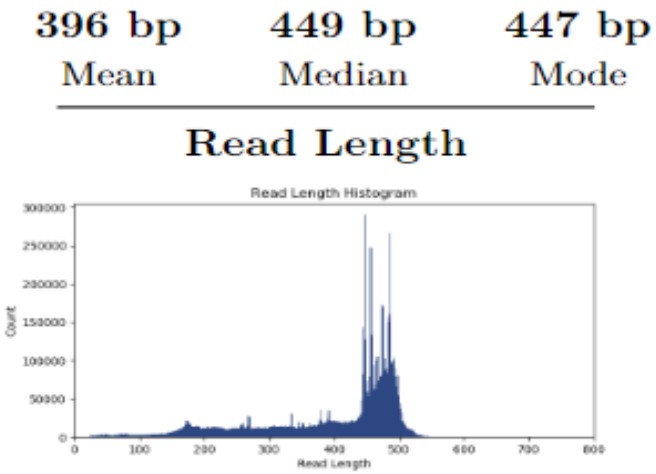
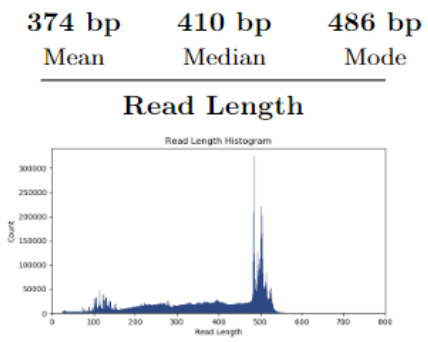
Quality metrics assessment for different runs

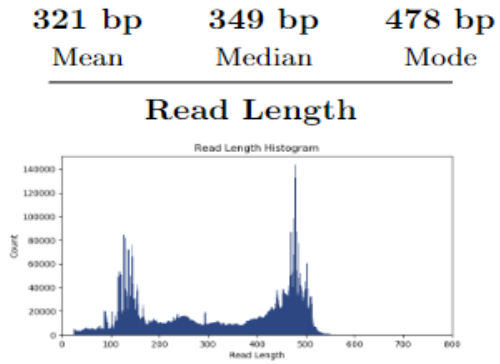
				%					Read length (bp)		
Run ID	Multiplex	Leader_TP53 #	Total Reads	ISP Loading	Usable Reads	Clonal	Low quality	Final library	Mean	Median	Mode
1	N	7	11356977	89	34	57	39	59	397	453	461
2	N	10	11061089	80	37	64	42	57	396	449	447
3	Y	10_3	11094794	85	37	65	43	56	357	399	453
4	N	9	9605651	88	29	65	55	29	377	434	457
5	Y	8_8	8916081	87	28	67	58	41	321	349	478
6	N	8	9198027	90	27	60	54	45	377	444	467
7	N	5	6869376	83	22	62	62	36	391	449	484

- Median read length multiplex =374 vs. Median read length standalone =446



Vs.





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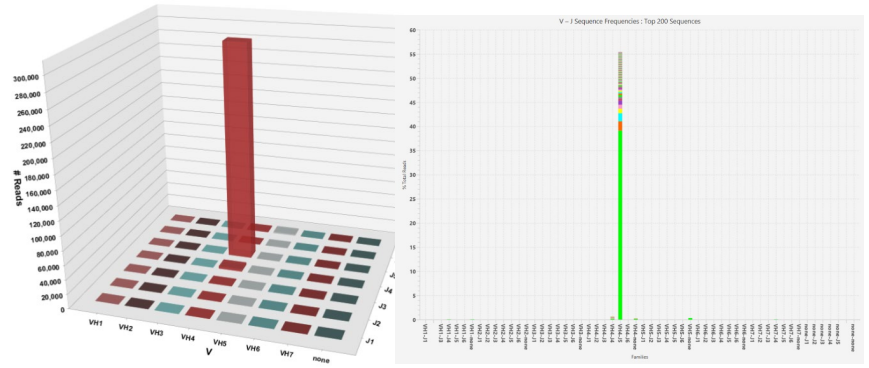
Software analysis

LymphoTrack® FR1

V-J Usage: Top 200 Sequences



Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop codon (Y/N)	V-coverage	CDR3 Seq
1	CACTGTTTCTGGG	279	32538 6	IGHV4-59_01	IGHJ5_02	59.60	59.60	14.04	Y	Y	99.56	GCGAGAGGACCA
2	GTTCTGGATACAG	130	1777	IGHV5-51_04	none	0.33	59.92	0.00	n/a	N	58.04	not found
3	CACTGTTTCTGGG	282	707	IGHV4-59_01	IGHJ4_02	0.13	60.05	14.04	Y	Y	99.56	GCGAGAGGACCA
4	CACTGTTTCTGGG	282	402	IGHV4-59_07	IGHJ5_01	0.07	60.13	14.16	N	N	97.79	not found
5	CTTCTGGATACAC	130	371	IGHV1-2_04	none	0.07	60.19	0.00	n/a	N	58.04	not found

Oncomine® Leader J

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Lineage ID	Functiona	Productiv	Unproduc	Variable	Joining	CDR3 AA	CDR3 NT	Diversity	Plus Coun	Minus Co	Variable M	Total Cour	Frequency
2	1	Productiv	0.824583		IGHV4-59	IGHJ4	ARGPDITG	GCGAGAG	IGHD1-7	63362	314928	0.116	378290	0.658239
3	2	Unproductive		0.962194	IGHV4-59	IGHJ4	CERTRYHV	GCGAGAG	IGHD1-7	103399	8153	0.119	111552	0.194105
4	3	Productiv	0.008974		IGHV5-51	IGHJ4	ARHRYYDS	GCGAGAC	IGHD3-22	1204	2913	0	4117	0.007164
5	4	Productiv	0.007666		IGHV5-51	IGHJ4	ARHAEYSS	GCGAGAC	IGHD6-6	902	2615	0	3517	0.00612
6	5	Productiv	0.005992		IGHV2-5	IGHJ4	AHRMGIA	GCACACA	IGHD6-13	1650	1099	0	2749	0.004783
7	6	Productiv	0.005648		IGHV7-4-1	IGHJ4	ARDYAPE	GCGAGAG	IGHD3-16	1722	869	0	2591	0.004508

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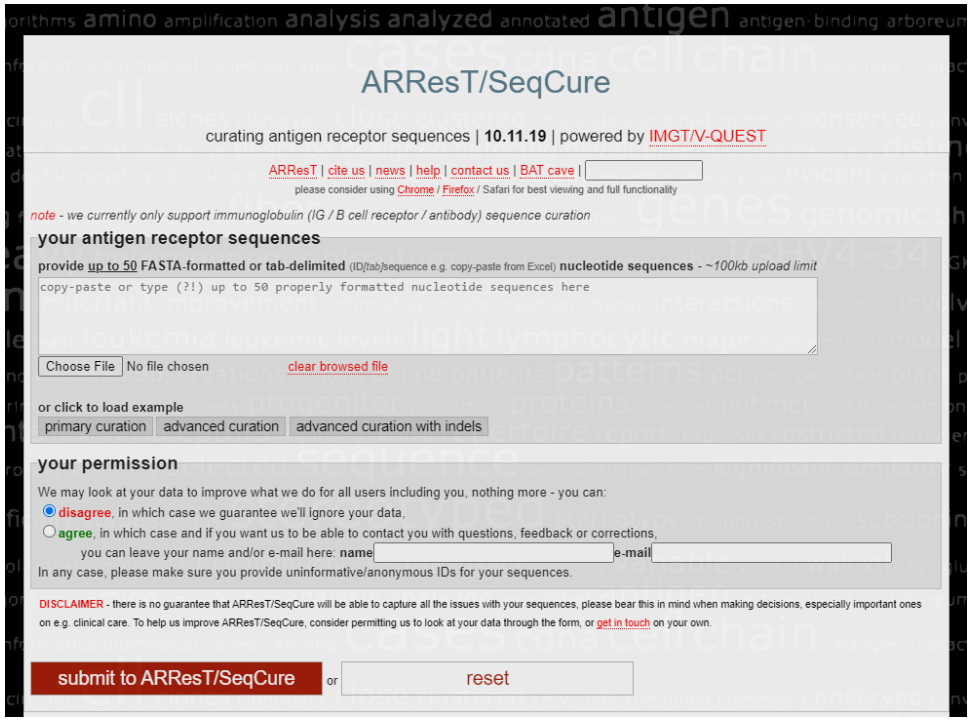
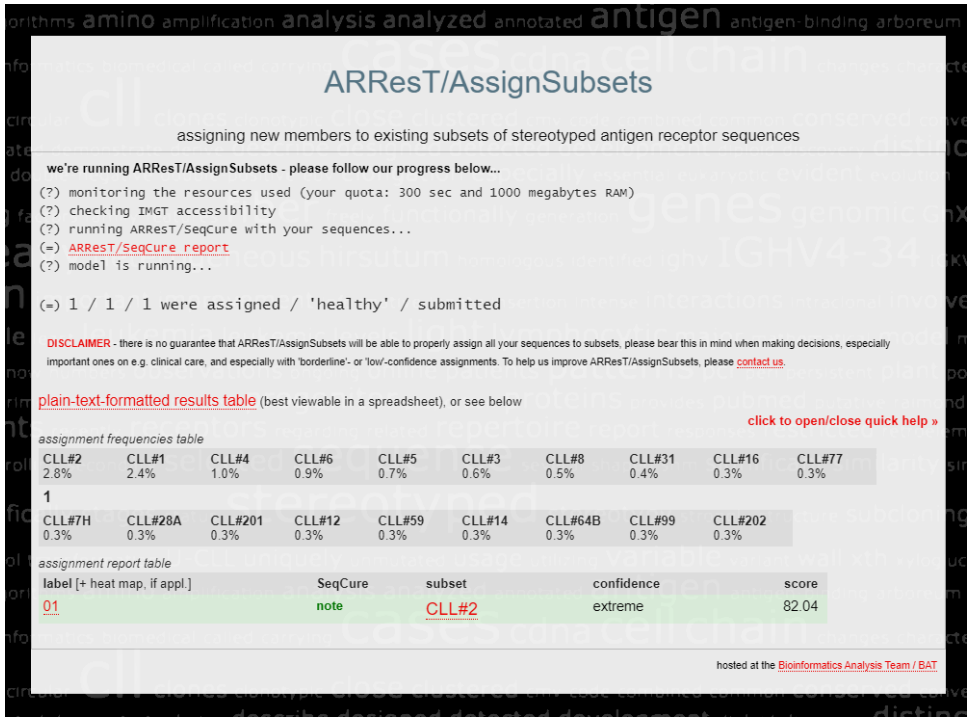
Analysis of V-D-J gene rearrangements and Stereotypy

ARResT tool



ARResT/AssignSubsets tool



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		FR1 result				Leader primer			
Sample no.	Sample type	Status	Family	Mutation Freq	Subset	Status	Family	Mutation Freq	Subset
1	PB	Inconclusive	NA	NA	NA	No clonal rearrangement	NA	NA	NA
2	BMA	No clonal rearrangement	NA	NA	NA	Mutated	IGHV4-34	3.8%	NA
3	PB	Mutated	V4-59	14.04%	CLL#77	Mutated	IGHV4-59	11.6%	CLL#77
4	BMA slide	Mutated, borderline	V1-46	2.65%	Unassigned	Mutated, borderline	IGHV1-46	2.0%	Unassigned
5	Control DNA	Mutated	V4-59	12.28%	Unassigned	No clonal rearrangement	NA	NA	NA
6	Control DNA	Unmutated	V1-46	0%	Unassigned	Unmutated	IGHV1-46	0%	Unassigned
7	Control DNA	No clonal rearrangement	NA	NA	NA	No clonal rearrangement	NA	NA	NA
8	PB	Mutated	V3-33	8.81%	Unassigned	No clonal rearrangement	NA	NA	NA
9	BMA	Mutated	V4-34	3.95%	Unassigned	Mutated	V4-34	4.1%	Unassigned
10	BMA	Mutated	V4-34	6%	CLL#16	Mutated	V4-34	5.2%	CLL#16
11	PB	Mutated	V2-5	7.17%	Unassigned	Mutated	V2-5	8.6%	Unassigned
12	BMA sorted	Unmutated	V5-51	0%	Unassigned	Unmutated	V5-51	0%	Unassigned
13	BMA	Mutated	V3-7	8.15%	Unassigned	Mutated	V3-7	7.1%	Unassigned
14	PB sorted	Unmutated	V1-2	0%	Unassigned	Unmutated	V1-2	0%	Unassigned
15	PB	Mutated, borderline	V4-34	2.19%	Unassigned	Mutated, borderline	V4-34	2.4%	Unassigned
16	PB	Mutated, borderline	V1-69	2.21%	Unassigned	Unmutated	V1-69	1.7%	Unassigned
17	PB sorted	Mutated	V3-15	11.16%	Unassigned	Mutated	V3-15	9.3%	Unassigned
18	BMA sorted	Mutated	V3-33	9,25%	Unassigned	No clonal rearrangement	NA	NA	NA
19	BMA	Mutated	V4-34	8%	Unassigned	Mutated	V4-34	7.6%	Unassigned
20	PB	Mutated	V2-5	7.69%	Unassigned	Mutated	V2-5	6.7%	Unassigned
21	PB	Unmutated	V2-70	0%	Unassigned	Unmutated	V2-70	0%	Unassigned
22	BMA	Unmutated	V3-21	1.76%	CLL#2	Unmutated	V3-21	1.4%	CLL#2
23	BMA	Unmutated	V7-4	0%	CLL#99	Unmutated	V7-4-1	0%	CLL#99
24	PB	Unmutated	V1-3	0%	CLL#1	Unmutated	V1-3	0.3%	CLL#1
25	PB	Mutated, borderline	V3-33	2.20%	Unassigned	Unmutated	V3-33	1.7%	Unassigned
26	BM	Mutated, borderline	V3-23	2.20%	Unassigned	No clonal rearrangement	NA	NA	NA
27	PB	No clonal rearrangement	NA	NA	NA	Unmutated	V4-34	0.0%	Unassigned
28	PB	Inconclusive	NA	NA	NA	No clonal rearrangement	NA	NA	NA
29	DNA	No clonal rearrangement	NA	NA	NA	No clonal rearrangement	NA	NA	NA
30	DNA	Inconclusive	NA	NA	NA	Mutated	V4-31	9%	Unassigned
31	PB sorted	No clonal rearrangement	NA	NA	NA	No clonal rearrangement	NA	NA	NA
32	BM	Inconclusive	NA	NA	NA	Unmutated	V1-69	0%	Unassigned
33	PB	No clonal rearrangement	NA	NA	NA	Mutated	V3-30	5.4%	Unassigned

Results

Total samples run Leader – J assay to date 52 samples

With 33 results for direct comparison to FR1 assay

Sample type:

Sample type	Number
PB	13
BMA	10
PB sorted	3
BMA sorted	2
DNA	5

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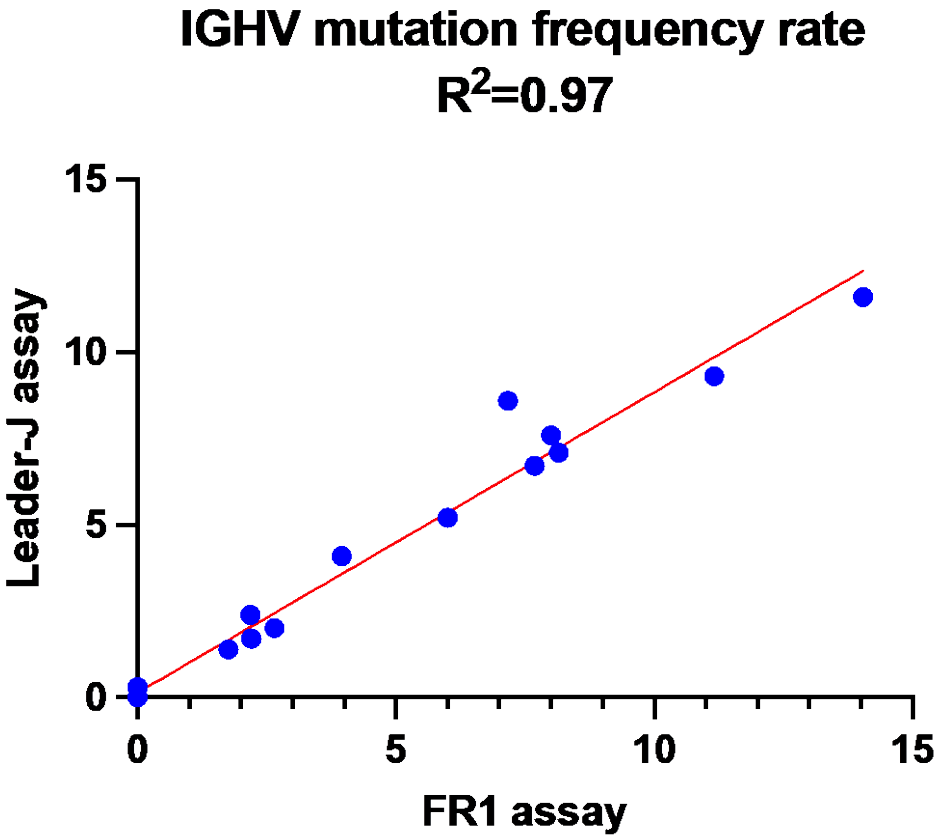
Results



- Mutational frequency rate excellent concordance with R^2 =0.97
- V-gene usage with 100% concordance (n=19)
- SHM status concordant in 89.5% of cases (n=19)
- 2 x discrepant = borderline mutated vs. unmutated
- Stereotypy concordance was 100% (n=19)
- 5 cases with a defined stereotypy – only 26% of cases
- All patient samples reported with a result for this FR1 cohort or Leader-J alone (n=37) 13.5%

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Discussion- Borderline cases

		FR1 result				Leader primer			
Sample no.	Sample type	Status	Family	Mutation Fre	Subset	Status	Family	Mutation Fre	Subset
4	BMA slide	Mutated, borderline	V1-46	2.65%	Unassigned	Mutated, borderline	IGHV1-46	2.0%	Unassigned
15	PB	Mutated, borderline	V4-34	2.19%	Unassigned	Mutated, borderline	V4-34	2.4%	Unassigned
16	PB	Mutated, borderline	V1-69	2.21%	Unassigned	Unmutated	V1-69	1.7%	Unassigned
25	PB	Mutated, borderline	V3-33	2.20%	Unassigned	Unmutated	V3-33	1.7%	Unassigned
26	BM	Mutated, borderline	V3-23	2.20%	Unassigned	No clonal rearrangement			

5x Borderline mutated cases:

- 2 cases remained unchanged
- 2 cases were unmutated on the leader primer – confirmed with sanger
- 1 case with no clonal rearrangement on Leader. Clear clone on FR1

- NB for clinical diagnostics to make the correct call.

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Discussion- Inconclusive

- 4 inconclusive cases with the FR1 primers- All with a single unproductive clone (<0.1%)

		FR1 result				Leader primer			
Sample no.	Sample type	Status	Family	Mutation Freq	Subset	Status	Family	Mutation Freq	Subset
1	PB	Inconclusive	NA	NA	NA	No clonal rearrangement	NA	NA	NA
28	PB	Inconclusive	NA	NA		No clonal rearrangement	Poly		
30	DNA	Inconclusive	NA	NA		Mutated	V4-31	9%	Unassigned
32	BM	Inconclusive	NA	NA		Unmutated	V1-69	0%	Unassigned

- Troubleshooting:
  - Further investigation of the productive rearrangement on the other allele of the IGH locus
  - NGS sequencing errors and/or amplification bias
  - Repeat/ Different primers/ New sample

Sample no	WCC	Lymph	Clinical info
1	6.43	3.55	Already post treatment
28	4.84	1.5	Post treatment with normal flow
30	50.0	39.2	CLL confirmed on flow
32	60.22	43.59	CLL confirmed on flow

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Discussion- No clonal rearrangement

		FR1 result	Leader primer	Further assessment		
Sample type	Sample type	Status	Status	WCC	Lymph	Clinical info
1	PB	Inconclusive	No clonal rearrangement	6.43	3.55	Previous therapy
2	BMA	No clonal rearrangement	Mutated	20.79	9.12	Clonality on flow
5	Control DNA	Mutated	No clonal rearrangement	NA	NA	Primers not binding to control
7	Control DNA	No clonal rearrangement	No clonal rearrangement	NA	NA	Negative DNA control
8	PB	Mutated	No clonal rearrangement	36.09	32.12	Confirmed on Sanger seq
18	BMA sorted	Mutated	No clonal rearrangement	20.5	12.65	Clonality on flow
26	BM	Mutated, borderline	No clonal rearrangement	17.88	10.91	Clonality on flow
27	PB	No clonal rearrangement	Unmutated	70.14	53.94	Just above threshold (low % clone)
28	PB	Inconclusive	No clonal rearrangement	4.84	1.5	Previous therapy
29	DNA	No clonal rearrangement	No clonal rearrangement	6.8	0.96	Previous therapy
31	PB sorted	No clonal rearrangement	No clonal rearrangement	10.48	6.42	Confirmed on flow
33	PB	No clonal rearrangement	Mutated	59.35	54.95	Confirmed on flow

- Higher failure rate with leader primers known
- Having both assays available is preferred or second method

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Discussion- Challenging cases

Double rearrangement (10.5%):

- Productive and unproductive (8.4%)
- NO CLINICAL or biological relevance of unproductive cases
- SHM status assessed only on productive rearrangement

- Discordant (<0.1%) : check flow/report both/ final report as U-CLL

- Multiple >2
  - Check flow
  - Consider predominant clonotype if clearly defined

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Conclusions

- The Leader-J assay showed excellent concordance for variable mutation rate, SHM status and stereotypy in those that were directly comparable.
- FR1 primers used in diagnostic labs but not recommended, with leader primers crucial, esp. in borderline mutated cases as per ERIC guidelines.
- Cut-off of 98% for SHM is arbitrary in terms of clinical outcome with improved prognosis as the IGHV identity becomes increasingly different from the germline. SHM status remains important for motivation of therapy *e.g.* Ibrutinib in unmutated cases.
- A slightly higher failure rate was seen when using the leader assay. Consider using FR1 assay as second line in these cases.
- This Leader-J assay performed well with an excellent correlation to our current assay.
- Easy to use and robust assay which provides accurate results across different sample types and allows multiplexing with improved TATs.

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References

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