NGS-Based Clonality Testing

Assessing Clonality Status, Somatic Hypermutation and Monitoring Minimum Residual Disease (MRD)

Maria Arcila, M.D.
Memorial Sloan Kettering Cancer Center

Educational Goals

- Review principles of clonality testing
- Discuss the role of Next generation sequencing in assessing clonality and somatic hypermutation
- Describe the role of NGS in disease monitoring and minimal residual disease assessment
- Discuss bioinformatics software and data analysis

Additional educational goals for this lecture

- Understand:
  - Why and when should next generation sequencing be considered.
  - Benefits and potential pitfalls.

Clonality Testing in Diagnosing and Monitoring Lymphoid Malignancies

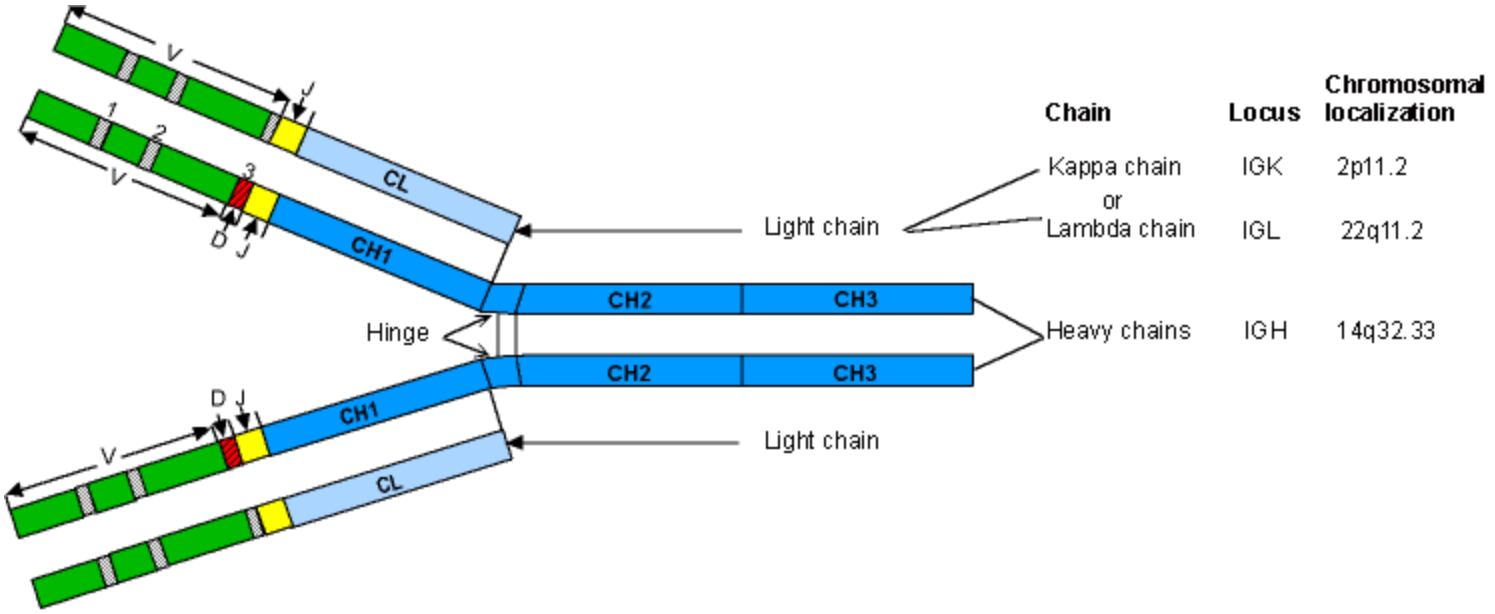
- Clonality testing greatly facilitates the diagnosis of lymphoid malignancies
  - IG and TCR gene rearrangements are the most widely applied targets
  - PCR-based analysis of Ig/TCR rearrangements - Gold standard method in the last 2 decades
- Use for monitoring of residual disease: more limited due to intrinsic relatively low sensitivity of routine standardized assays

Principles of clonality assessment

- Rearrangement of antigen receptor genes occur during lymphoid proliferation (physiologic and pathologic)
  - Products are unique in length and sequence in each cell.
- Establishing the unique length or sequence allows discrimination of monoclonality and polyclonality
  - Polyclonal – generally considered benign
  - Monoclonal - generally considered neoplastic. One product over-represented

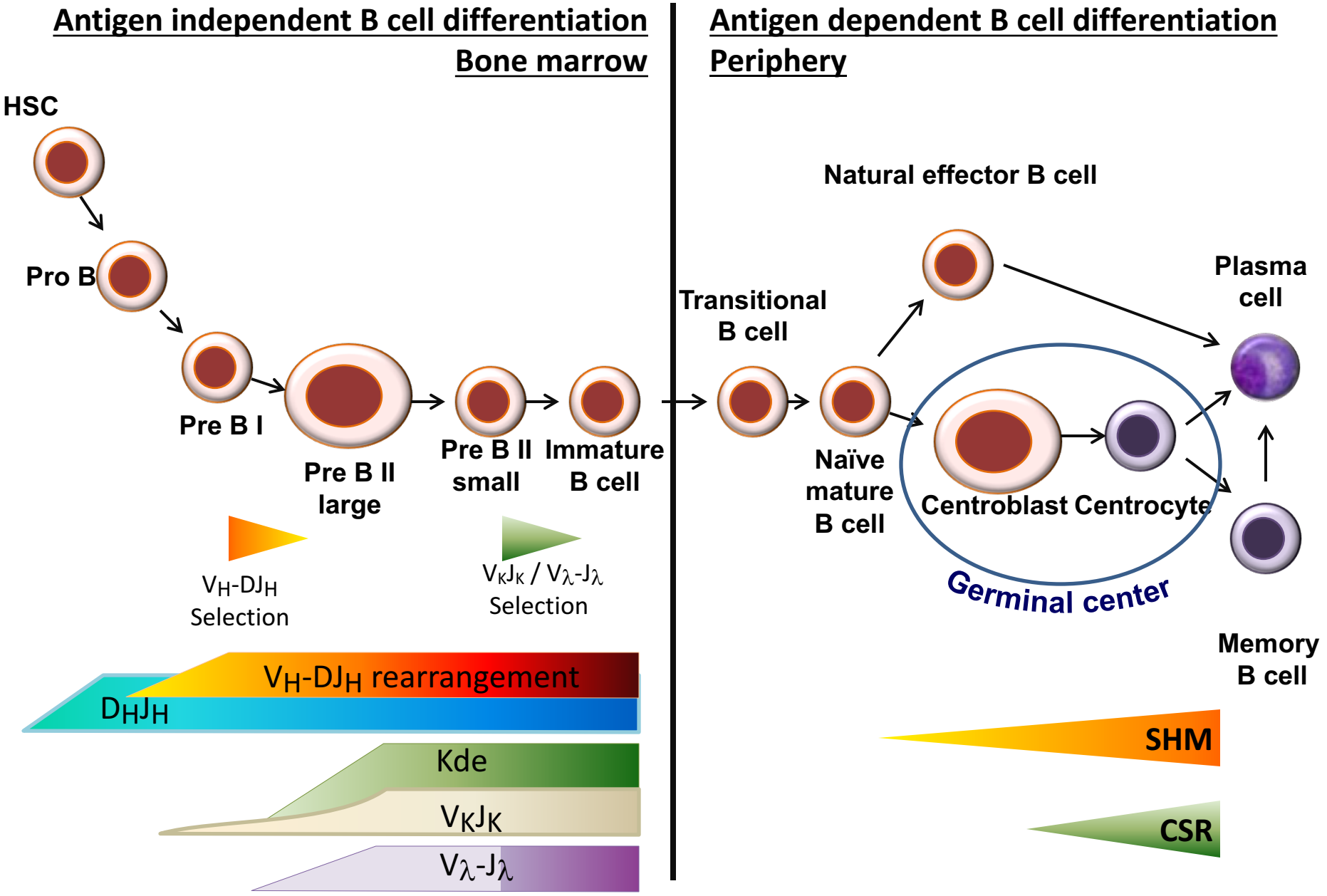
Review of B cell differentiation

- B cell development occurs through several stages
- Each stage represents a change in the genome content at the antibody loci.

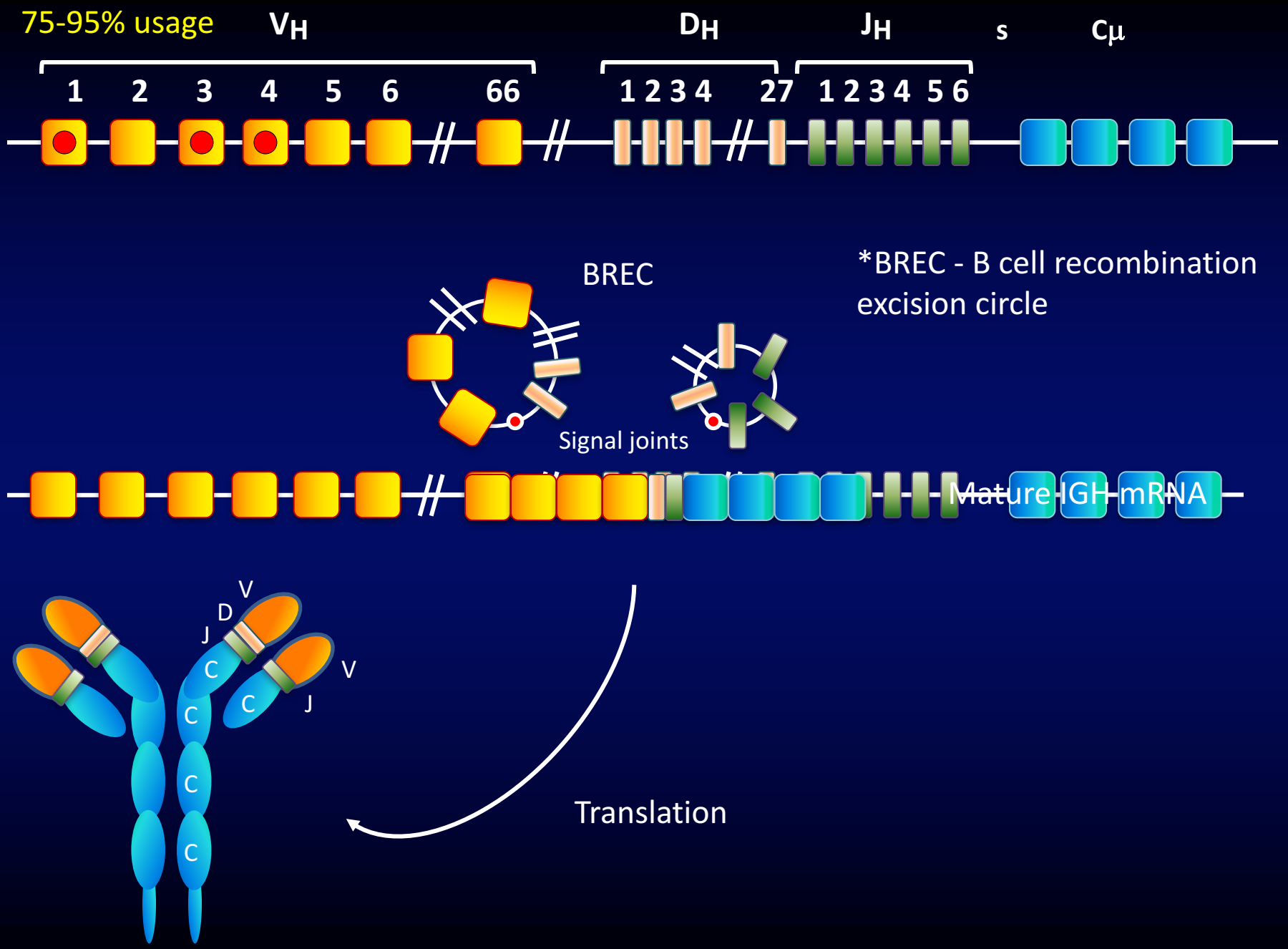


Schematic representation of an immunoglobulin molecule

Molecular processes in precursor and peripheral B-cells



VH - 46-52 functional , ~30 non-functional

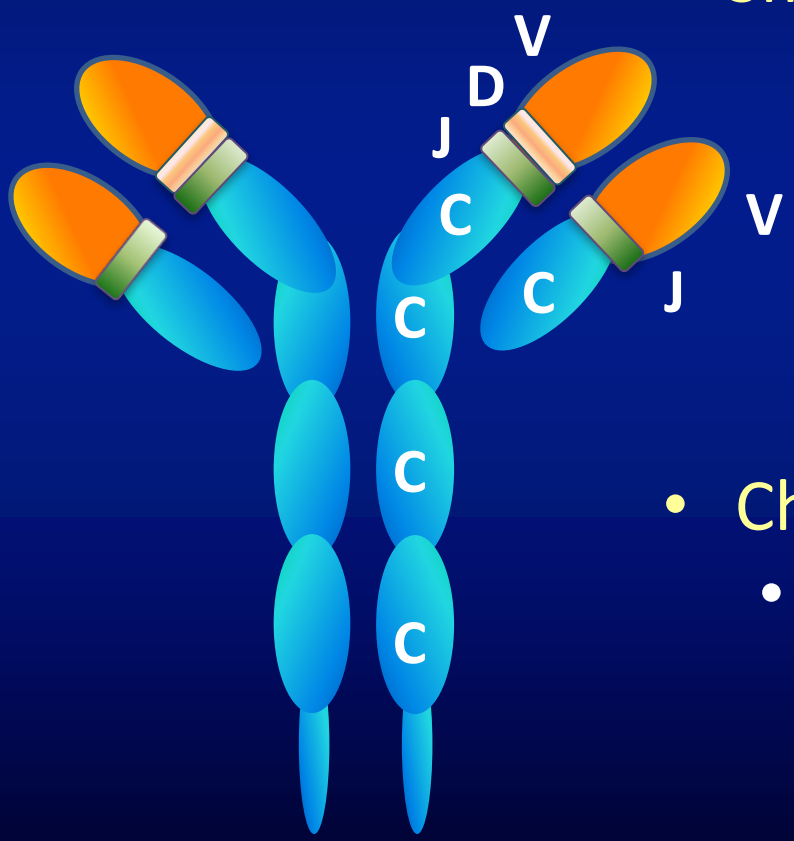


Resulting Diversity in IgH and TCR molecules

	Ig			TCR			
	H	κ	λ	α	β	γ	δ
Gene segments							
V - variable	~44	~43	~38	~46	~47	6	6
D – diversity	27	-	-		2	-	3
J – joining	6	5	4	53	13	5	4
Combination Diversity	>2X10^6			>2X10^6		>5000	
Junctional Diversity	++	+/-	+/-	+	++	++	++++
Total Diversity	>10^{12}			>10^{12}		>10^{12}	

Data Source - JJM van Dongen, Dept. of Immunology, Erasmus MC, Rotterdam

Further modifications during B cell maturation



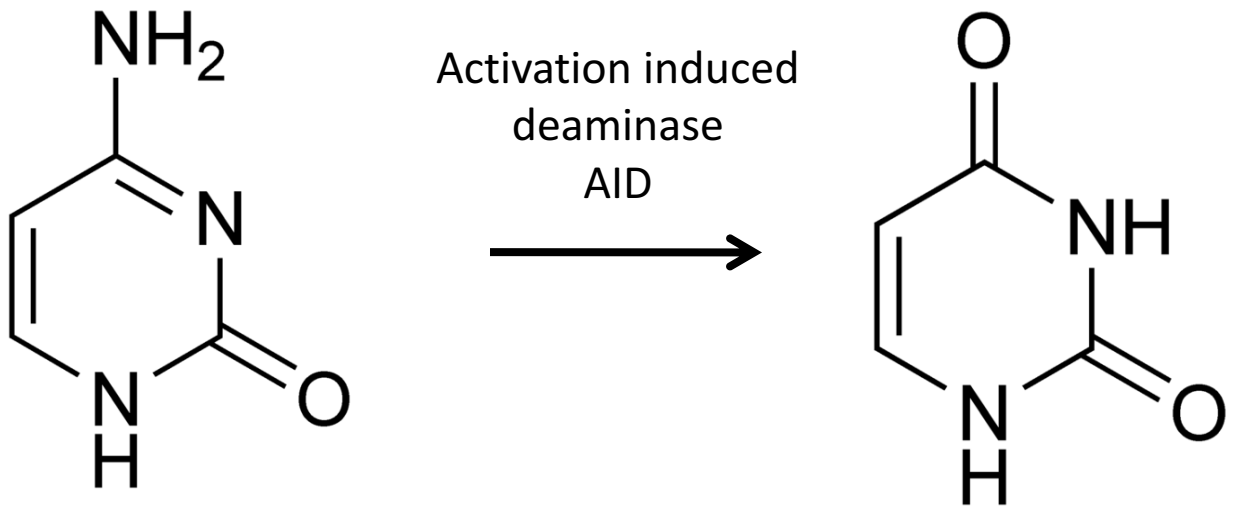
Small changes in variable regions:

- Somatic hypermutation (SHM)
  – higher affinity

- Changes of constant domains
  - Class switch recombination (CSR)
    - Change effector function

SOMATIC HYPERMUTATION

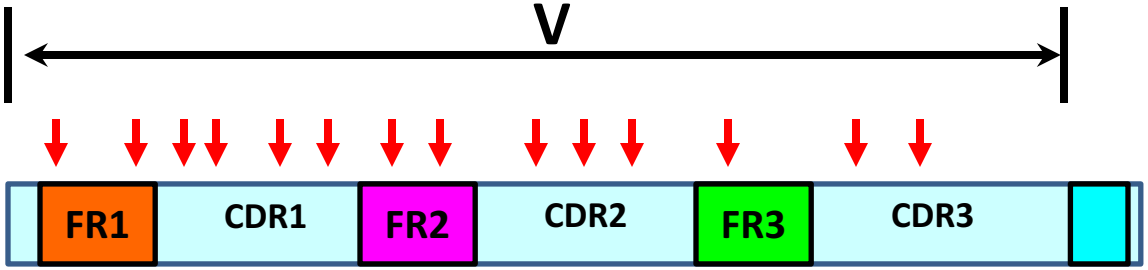
cytosine: guanine pairs mutated to a uracil:guanine mismatch



cytosine

uracil

- Generally repaired by high-fidelity DNA mismatch repair enzymes – remove uracil
- Error-prone DNA polymerases are recruited to fill in the gaps and create mutations
- Rapidly-proliferating population of B cells - production of thousands of B cells, possessing slightly different receptors and varying specificity for the antigen, from which the B cell with highest affinities for the antigen can be selected.



Alignments

```
                                        <FR1-><-------CDR1-IMGT------><--------------------FR2-IMGT---------------------><-------C
                                         A  S  A  F  T  F  S  D  Y  Y  M  N  W  I  R  Q  A  P  G  K  G  L  E  W  V  S  Y  I  S  S
                    Query_1       1     GCCTCTGCATTCACCTTCAGTGACTACTACATGAACTGGATCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATACATCAGTAGT  90
V  96.9% (219/226)  IGHV3-11*01   70     .......G.............................G.....................................................T......  159
                                         A  S  G  F  T  F  S  D  Y  Y  M  S  W  I  R  Q  A  P  G  K  G  L  E  W  V  S  Y  I  S  S
V  96.5% (218/226)  IGHV3-11*04   70     .......G.............................G.....................................................T......  159
V  94.2% (213/226)  IGHV3-11*05   70     .......G.............................G.....................................................T......  159

                                        DR2-IMGT------><-----------------------------------------------------FR3-IMGT--------------
                                         S  G  D  T  I  Y  Y  A  D  S  V  K  G  R  F  T  M  S  R  D  N  A  K  N  S  L  Y  L  Q  M
                    Query_1       91    AGTGGTGATACCATATACTATGCAGACTCTGTGAAGGGCCGATTCACCATGTCCAGGGACAACGCCAAGAACTCACTGTATCTGCAAATG  180
V  96.9% (219/226)  IGHV3-11*01   160    ......AG............C.............................C.....................................  249
                                         S  G  S  T  I  Y  Y  A  D  S  V  K  G  R  F  T  I  S  R  D  N  A  K  N  S  L  Y  L  Q  M
V  96.5% (218/226)  IGHV3-11*04   160    ......AG............C.............................C.....................................  249
V  94.2% (213/226)  IGHV3-11*05   160    ...A..AG.TA..C.A....C.............................C.....A.................................  249

                                        ------------------------------------><-------------CDR3-IMGT------------>
                                         N  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R  G  R  A  G  T  G  D  F  D  Y  W  G  Q  G  T
                    Query_1       181   AACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGAGGGCGGGCTGGAACCGGGGACTTTGACTACTGGGGCCAGGGAACC  270
V  96.9% (219/226)  IGHV3-11*01   250    ..........................................-----------------------------------------  295
                                         N  S  L  R  A  E  D  T  A  V  Y  Y  C  A  R
V  96.5% (218/226)  IGHV3-11*04   250    ........................T..................-----------------------------------------  295
V  94.2% (213/226)  IGHV3-11*05   250    ..........................................-----------------------------------------  295
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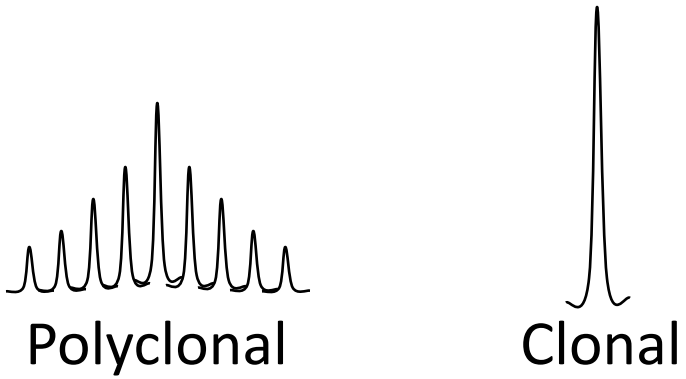
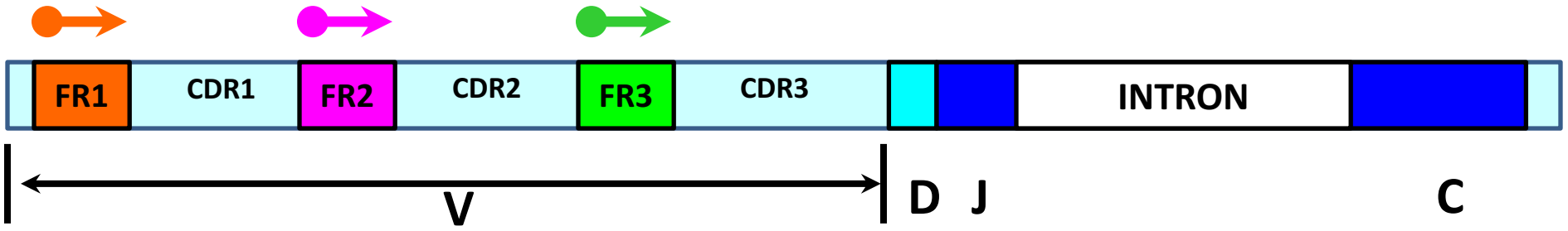
PCR-based analysis of Ig/TCR rearrangements: Gold standard method in the last 2 decades

Analytical phase extensively validated and largely standardized

- Multiplex PCR assays initially designed by BIOMED2 network (Euroclonality consortium)
- Further optimized by Invivoscribe
- High rate of detection in the most common B- and T-cell malignancies



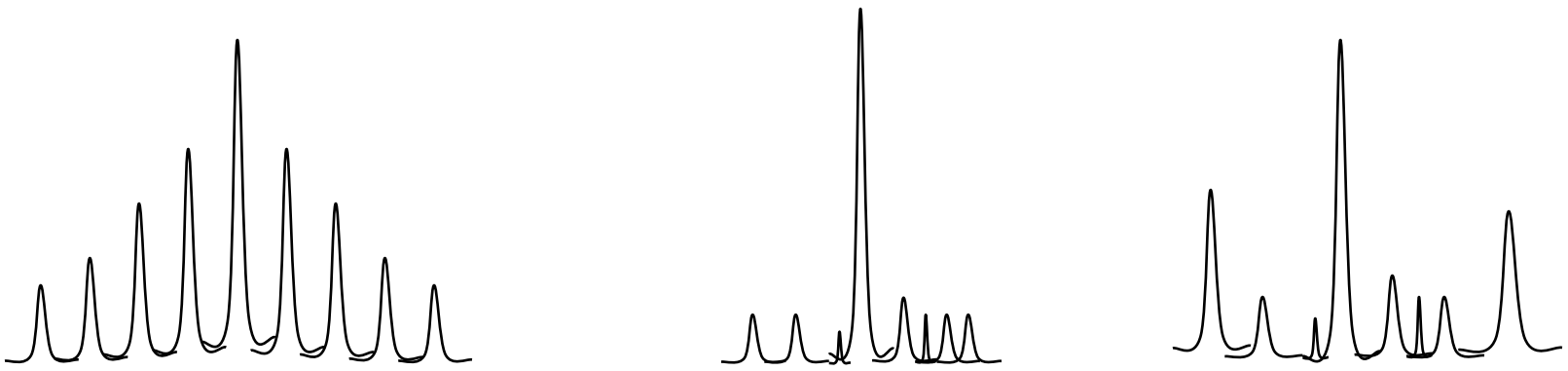


IgH Gene - Chromosome 14

FR Framework – highly conserved regions

CDR Complementarity determining regions – preferred sequences for SHM

Assessment of clonality



Polyclonal Clonal ??

- Background knowledge and ample experience required for accurate interpretation
- Interpretation algorithms exist - Take into account peak heights and peak ratios to define ‘truly clonal’ rearrangements.
- Cutoff values used in algorithms create a false sense of accuracy and might even lead to false- positive or false-negative interpretation.

The Pros and Cons

Pros

- Simple process, robust, rapid analysis
- Low DNA input requirements
- Relatively inexpensive
- Successfully exploits the size and overall composition of rearrangement fragments - sensititvity ~5-10%
- Available and easily instituted in to most laboratories

Cons

- Separates PCR products by the length of rearrangement not by unique sequence
- May be subjective in its interpretation
- Relatively low sensitivity
- Unsuitable for minimal residual disease assessment

Clonality assessment by NGS methods

Marked advantages over length–based analysis

- Allow identification of the full range of clonal populations
- Determine specific DNA sequence of clonal rearrangements
- Detect clonal events hidden in a polyclonal distribution
- Track residual disease – low level and MRD
- For B cell processes - Examine Somatic Hypermutation (SHM) as a prognostic marker

Beyond the clone detection

- May identify both stable and dynamic aspects of the immune repertoire that differ under normal and disease conditions
- Provide a high-resolution picture of the spectrum of immunity found in lymphoid malignancies.
- Define initial behaviors of clonal tumor populations, suppression or re-emergence of these populations following treatment

NGS Based Clonality Testing

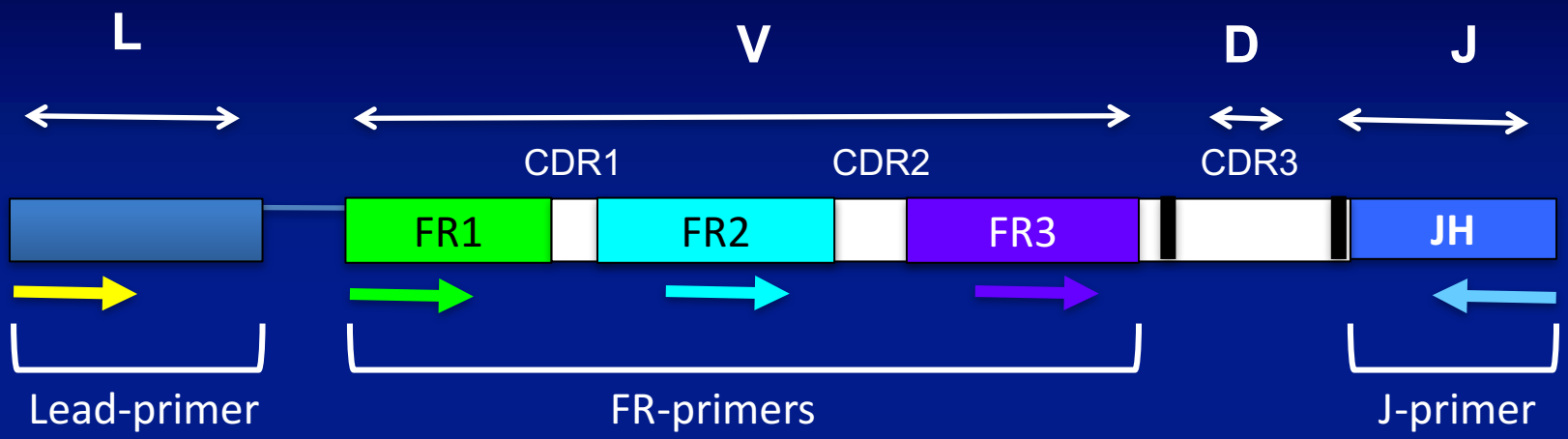
- Utilize capture based approaches – in-house developed or commercially available

- Invivoscribe – Lymphotrack assays
  - Commercially available kits to enable assessment of *IGH, IGK, TRG, TRB*
  - Use with the leading Next-Generation Sequencing (NGS) platforms
    - Optimized multiplex PCR master mixes
    - Primers with platform specific adapters
    - Specimen tracking Seq ID tags for single step workflow.
    - Comprehensive bioinformatics package
      1. DNA sequence
      2. clonal prevalence
      3. V-J family identity for each gene rearrangement.

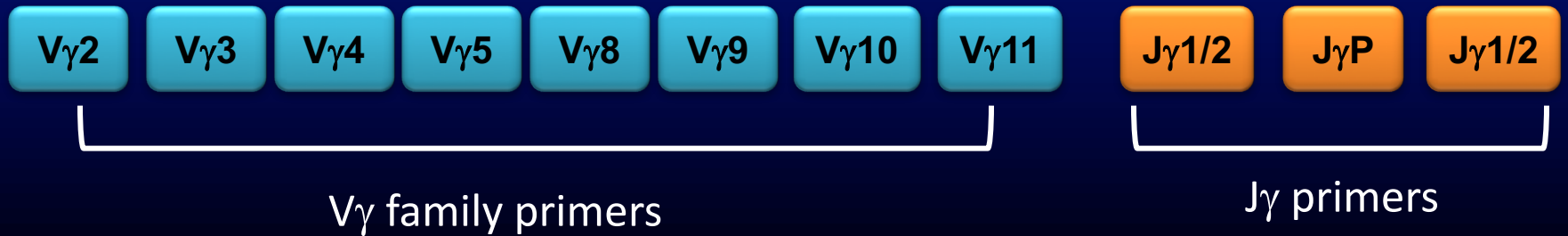
Illumina MiSeq

Ion Torrent PGM

IGH GENE



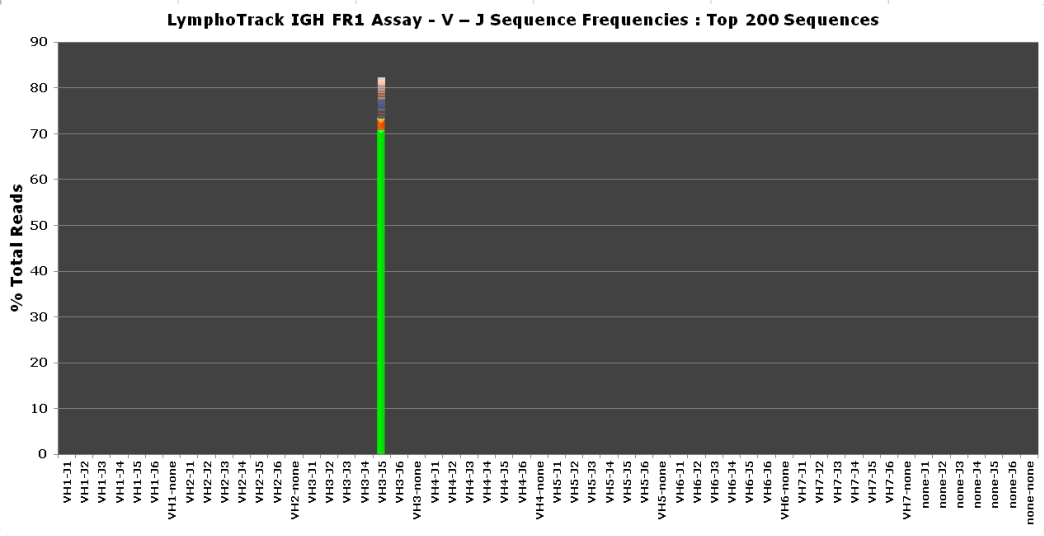
TRG GENE

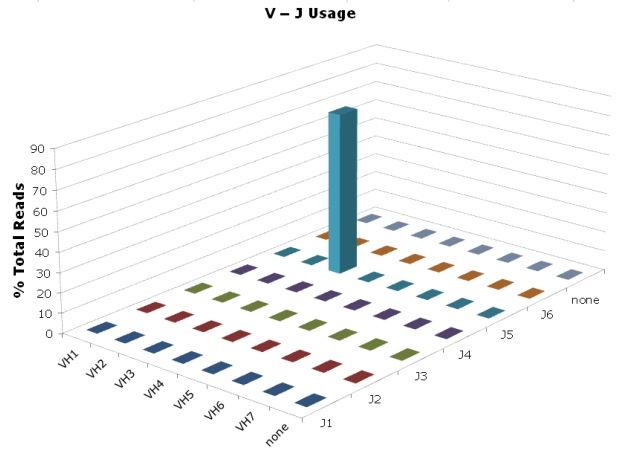


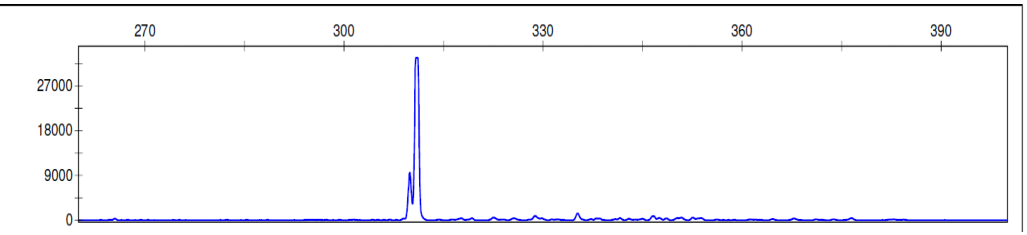
Establishing the diagnostic clone - Software output

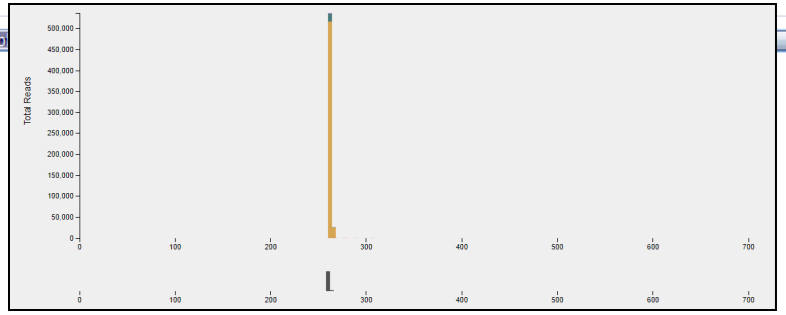
Total count	662,765										
Rank	**Sequence**	**Length**	**Merge count**	**V-gene**	**J-gene**	**% total read**	**Cumulative %**	**ate to partial**	**frame (**	**top codon**	**V-coverage**
1	GCCTCTGGATTCA	260	544830	IGHV3-21_02	IGHJ5_02	82.21	82.21	7.93	Y	Y	99.12
2	GCCTCTGGATTCA	260	15907	IGHV3-21_02	IGHJ5_02	2.40	84.61	8.81	Y	Y	99.12
3	TTCAGCCTCTGGA	264	744	IGHV3-21_02	IGHJ5_02	0.11	84.72	7.93	Y	Y	100.00
4	GCCTCTGGATTCA	305	256	IGHV3-11_04	IGHJ6_02	0.04	84.76	0.00	Y	Y	99.56
5	GCCTCTGGATTCA	275	191	IGHV3-11_04	IGHJ4_02	0.03	84.79	0.00	Y	Y	100.00
6	GCCTCTGGATTCA	260	184	IGHV3-15_02	none	0.03	84.81	3.00	n/a	N	99.57
7	GCCTCTAAATTCA	295	175	IGHV3-30_01	IGHJ6_02	0.03	84.84	14.54	N	N	98.24
8	GCCTCTGGATTCA	255	175	IGHV3-9_01	IGHJ6_02	0.03	84.87	0.00	n/a	N	94.32

GCCTCTGGATTCACTTTCAGTAGCTATAACATGAACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCATATATTAGTGGTAGAAGTGATTACATATA
CTACGCAGACTCAGTGAAGGGCCGATTCACCGTCTCCCGAGACAACGCCAAGAATTCGCTGTTTCTGCAAATGGACAGCCTGAGAGTCGACGACACGGCTGTTTAT
TACTGTACGAGAAGTCGTTTTTCCGACCTCTGGGGCCAGGGAACCCT





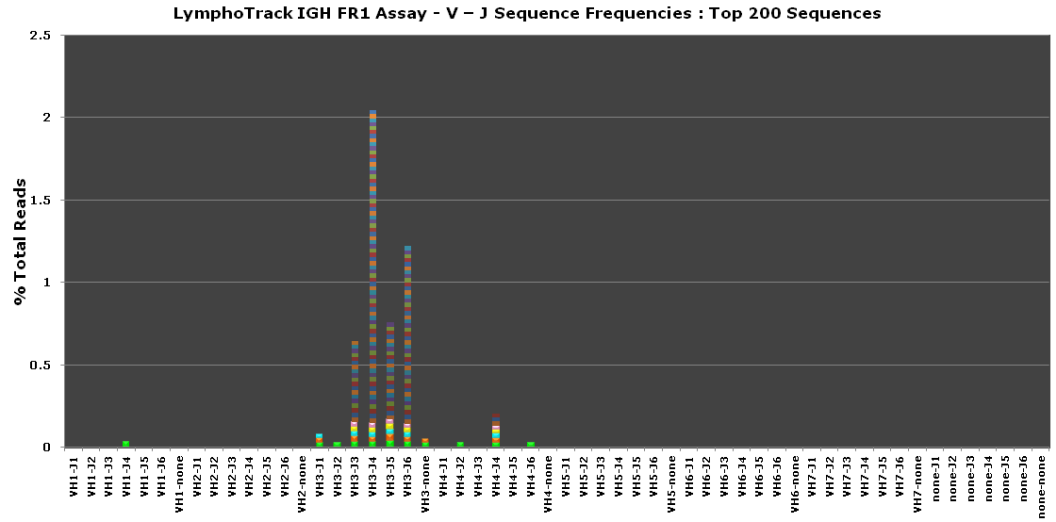


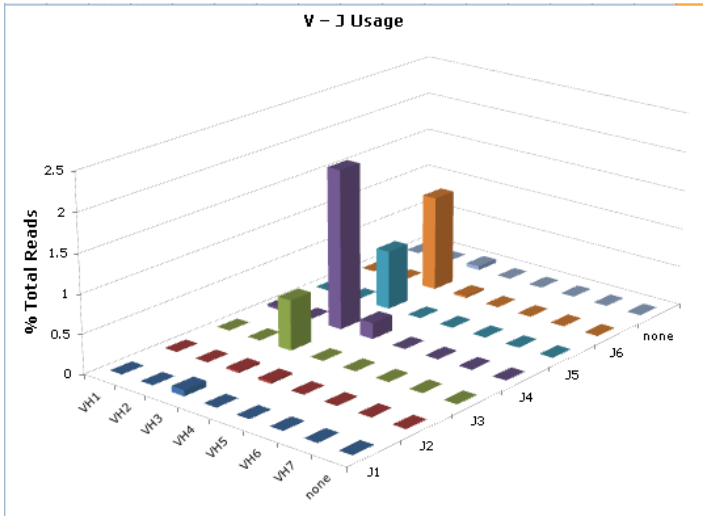


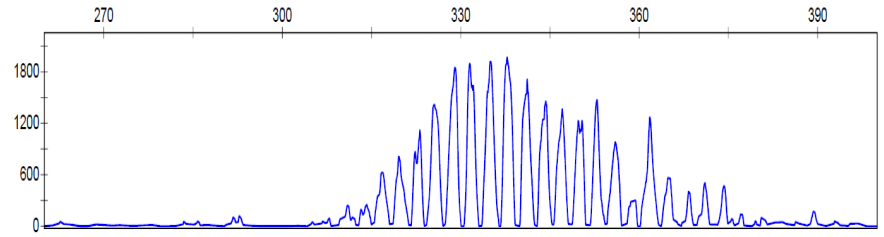
Polyclonal - Software output

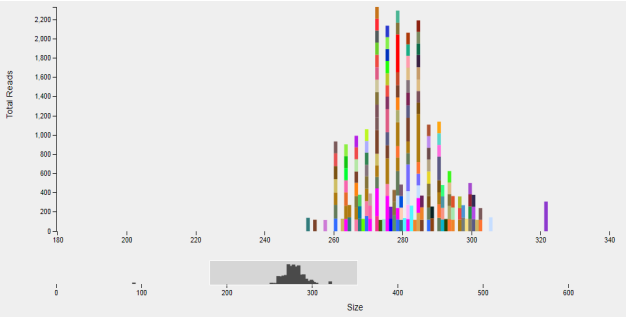
Total count	529,354

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
1	GCTTCTGGATTCA	284	210	IGHV3-49_05	IGHJ5_02	0.04	0.04	2.58	Y	Y	99.57
2	GCCTCTGGATTCA	209	200	IGHV3-13_01	IGHJ5_02	0.04	0.08	1.79	n/a	N	60.27
3	GCCTCTGGATTCA	269	174	IGHV3-7_01	IGHJ3_02	0.03	0.11	0.00	Y	Y	96.92
4	GCCTCTGGATTCA	272	168	IGHV3-9_01	IGHJ3_02	0.03	0.14	0.00	Y	Y	99.13
5	GCGTCTGGATTCA	272	167	IGHV3-33_01	IGHJ4_02	0.03	0.17	0.00	Y	Y	100.00
6	CTTCTCAATACTC(	277	164	IGHV1-2_02	IGHJ4_02	0.03	0.20	6.19	Y	Y	100.00
7	GCGTCTGGATTCA	284	163	IGHV3-33_01	IGHJ4_02	0.03	0.24	0.00	Y	Y	99.56
8	GCCTCTGGATTCA	269	162	IGHV3-48_01	IGHJ5_02	0.03	0.27	5.29	Y	Y	99.56
9	GCCTCTGGATTCA	204	161	IGHV3-66_04	IGHJ5_02	0.03	0.30	0.89	N	N	34.82
10	GCCTCTGGATTCA	281	159	IGHV3-13_01	IGHJ3_02	0.03	0.33	0.45	Y	Y	99.11

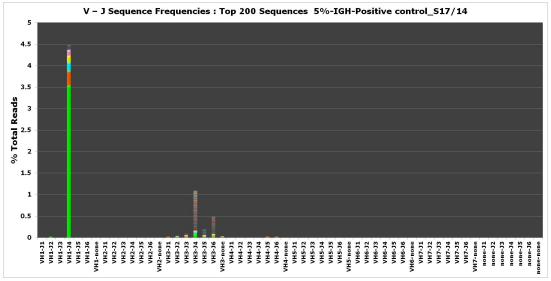








Assay sensitivity for Clonality Assessment



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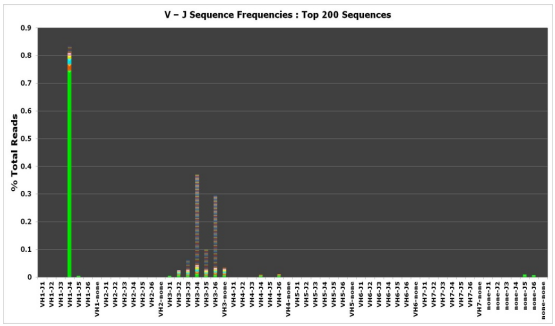

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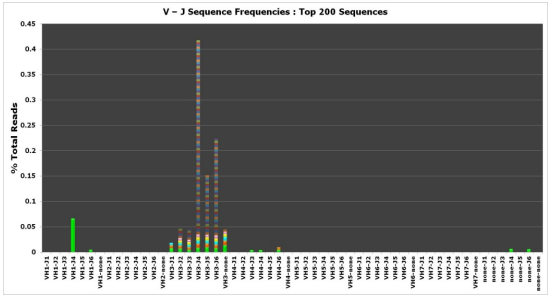

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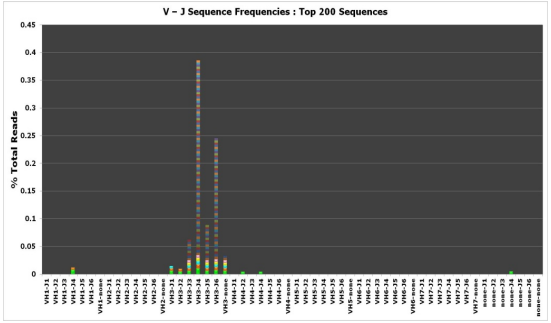

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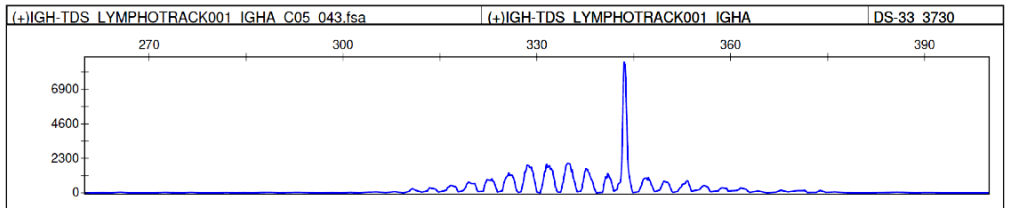
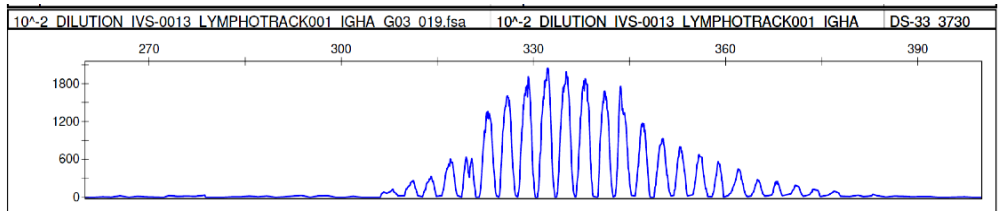
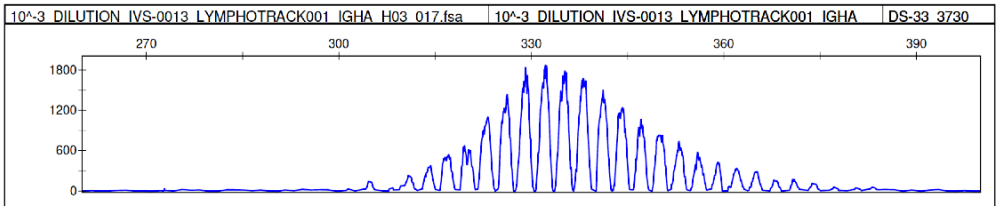
IGH Sensitivity Study







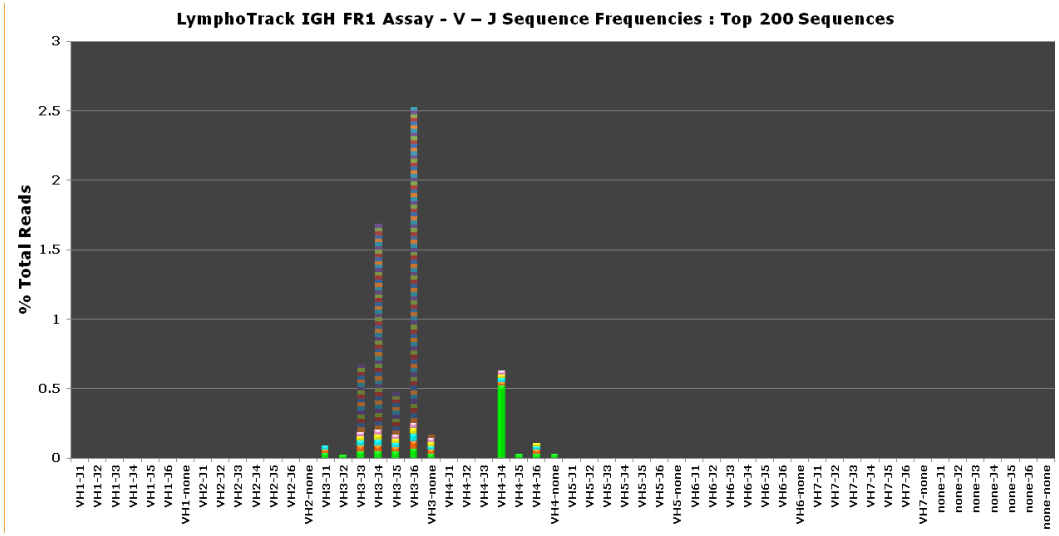
Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads
1	CATCTGGATACAC	295	5028	IGHV1-46_03	IGHJ4_02	0.76
2	CTTCTGGAGGCAC	289	131	IGHV1-69_13	IGHJ6_02	0.02
3	GCCTCTGGATTCA	293	74	IGHV3-33_01	IGHJ4_02	0.01
4	GCCTCTGGATTCA	117	57	IGHV3-13_04	IGHJ3_02	0.01
5	GCCTCTGGATTCA	147	53	IGHV3-9_01	IGHJ4_02	0.01
6	GCCGGACTCTGT(	121	50	IGHV3-9_02	none	0.01
7	GCCTCTGGATTCA	257	48	IGHV3-72_01	IGHJ6_03	0.01
8	GCCTCTGGATTCA	275	46	IGHV3-9_01	IGHJ4_02	0.01
9	GCCTCTGGATTCA	296	43	IGHV3-30-3_01	IGHJ6_03	0.01
10	GCCTCTGAATTCA	150	43	IGHV3-11_05	IGHJ4_02	0.01

Example of inter and intra assay reproducibility

Reproducibility - Inter assay			
SAMPLE ID	**DS1%**	**DS2%**	**Total reads**
IGH1062	27.94	24.70	**328,229**
IGH1062	26.50	26.00	**400,856**
IGH1062	24.80	24.04	**813,738**

Reproducibility - Intra assay			
SAMPLE ID	**DS1%**	**DS2%**	**Total reads**
IGH1062-1	26.36	26.02	**252,465**
IGH1062-2	27.19	26.31	**276,897**
IGH1062-3	27.68	25.79	**290,364**

Interpretation of clonality in low tumor samples



Total count	499,814

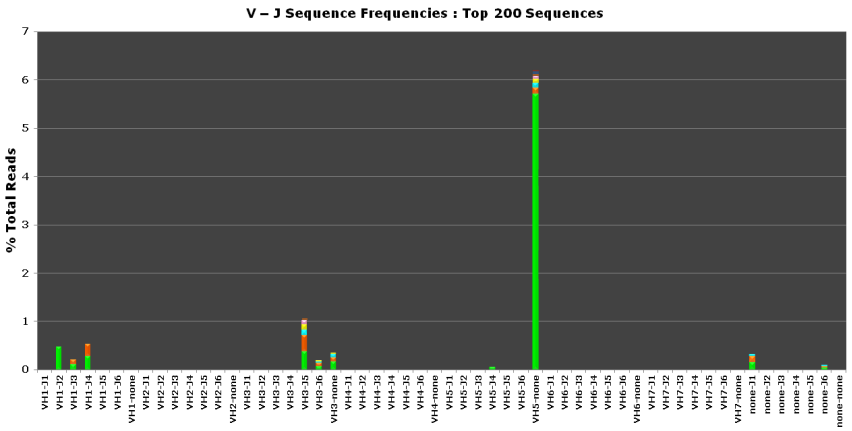
Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop codon (Y/N)	V-coverage
1	CATTGTCTCTGGT	273	2706	IGHV4-59_01	IGHJ4_02	0.54	0.54	10.53	Y	Y	98.25
2	GCCTCTGGATTCA	254	339	IGHV3-21_02	IGHJ6_04	0.07	0.61	0.00	Y	Y	99.56
3	GCCTCTGGATTCA	308	267	IGHV3-30_18	IGHJ6_03	0.05	0.66	0.00	Y	Y	100.00
4	GCCTCTGGATTCA	305	256	IGHV3-23_04	IGHJ6_03	0.05	0.71	0.00	Y	Y	99.56
5	GCCTCTGGATTCA	284	251	IGHV3-7_03	IGHJ4_02	0.05	0.76	0.00	Y	Y	99.56
6	GCCTCTGGATTCA	272	248	IGHV3-23_04	IGHJ3_02	0.05	0.81	0.00	Y	Y	99.12
7	GCCTCTGGATTCA	276	237	IGHV3-35_01	IGHJ5_02	0.05	0.86	3.96	n/a	N	95.15
8	GCCTCTGGATTCA	272	208	IGHV3-13_01	IGHJ6_03	0.04	0.90	0.45	Y	Y	100.00
9	GCCTCTGGATTCA	290	207	IGHV3-74_01	IGHJ3_02	0.04	0.94	0.00	Y	Y	100.00
10	GCCTCTGGATTCA	287	206	IGHV3-30_18	IGHJ4_02	0.04	0.99	0.00	Y	Y	99.12

Interpretation rules for primary diagnosis

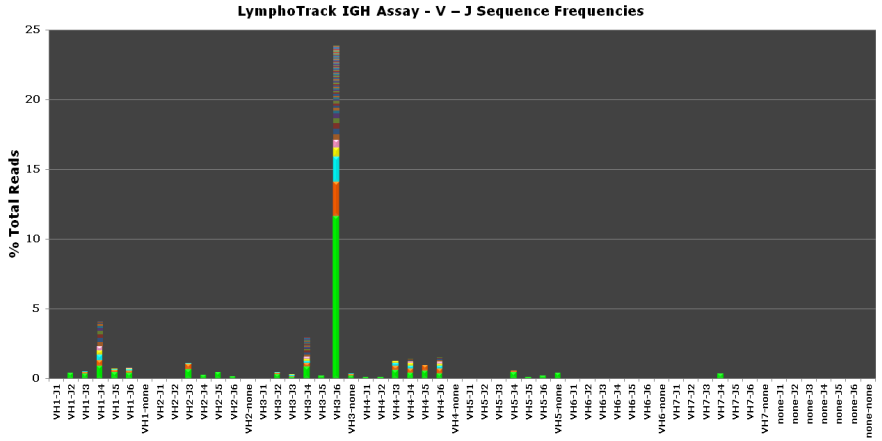
Total reads for sample is ≥50,000	One or two reads ≥ 2.5% and ≥ 5X the % reads for the 4th most frequent unique sequence	Evidence of clonality detected
	Top read is ≥ 2.5%, but the top read is < 5X the % reads for the 4th most frequent unique sequence.	No evidence of clonality detected
	Three or more reads ≥ 2.5%	No evidence of clonality detected[1]
	All reads < 2.5%	No evidence of clonality detected

Recommendations

- Extremely important to standardize DNA input and protocols.
- Set very stringent criteria for clonality calling for the diagnostic sample
- Beware of post treatment samples with very low level disease for initial clonality assessment
- Beware of low template samples – may require duplicate testing
- Interpretation of clonality must be made in the context of the disease and other ancillary testing



Pt. with follicular lymphoma
Post treatment BM sample – submitted for initial characterization of clone. No morphologic disease



Retrospective sequencing of FFPE tissue from the diagnostic lymph node.

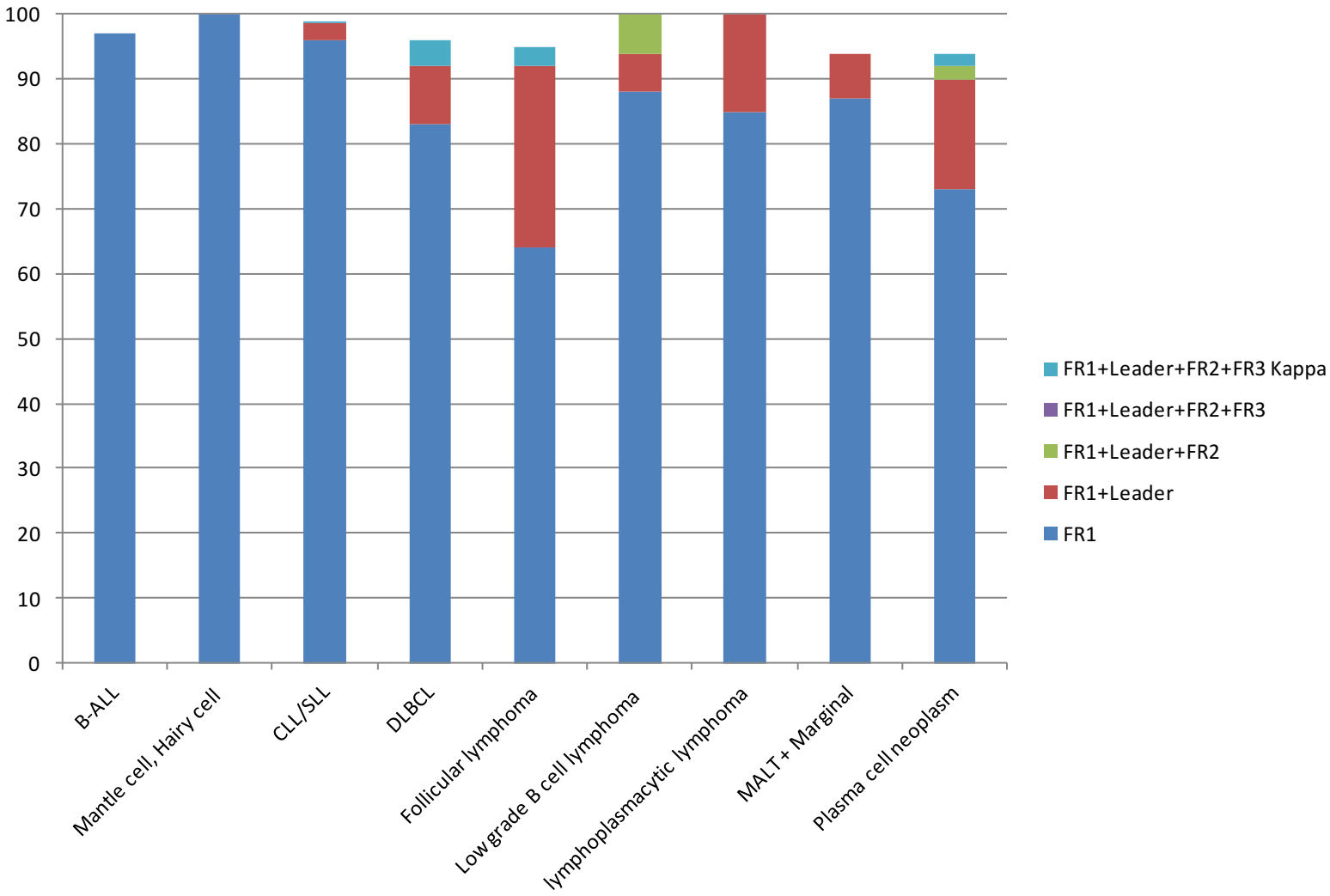
FROM:

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

P A S Evans, Ch Pott, P J T A Groenen, G Salles, F Davi, F Berger, J F Garcia, J H J M van Krieken, S Pals, Ph Kluin, E Schuuring, M Spaargaren, E Boone, D González, B Martinez, R Villuendas, P Gameiro, T C Diss, K Mills, G J Morgan, G I Carter, B J Milner, D Pearson, M Hummel, W Jung, M Ott, D Canioni, K Beldjord, C Bastard, M H Delfau-Larue, J J M van Dongen, T J Molina and J Cabeçadas

	IGH (three V_H–J_H tubes: FR1, -2 and -3)[a]				*IGK (two tubes: V_κ–J_κ and Kde)*				*IGH (V_H–J_H) + IGK*			
	Total	***1***	***2***	***>2***	***Total***	***1***	***2***	***>2***	***Total***	***1***	***2***	***⩾3***
MCL (*n*=54)	100%	0%	0%	100%	100%	0%	27%	73%	100%	0%	0%	100%
	54/54	0/54	0/54	54/54	54/54	0/54	15/54	39/54	54/54	0/54	0/54	54/54
B-CLL/SLL (*n*=56)	100%	2%	4%	94%	100%	0%	43%	57%	100%	0%	0%	100%
	56/56	1/56	2/56	53/56	56/56	0/56	24/56	32/56	56/56	0/56	0/56	56/56
FL (*n*=109)	84%	10%	28%	47%	84%	32%	32%	20%	100%	9%	18%	73%
	92/109	11/109	30/109	51/109	92/109	35/109	35/109	22/109	109/109	10/109	20/109	79/109
MZL (*n*=41)	87%	10%	17%	60%	83%	39%	20%	24%	97%	12%	5%	80%
	36/41	4/41	7/41	25/41	34/41	16/41	8/41	10/41	40/41b	5/41	2/41	33/41
DLBCL (*n*=109)	79%	17%	22%	39%	80%	38%	34%	8%	96%	18%	14%	64%
	86/109	19/109	24/109	43/109	87/109	41/109	37/109	9/109	105/109b	20/109	15/109	70/109
TOTAL (*n*=369)	88%	9%	17%	62%	88%	25%	32%	30%	98%	9%	10%	79%
	324/369	34/369	63/369	227/369	323/369	92/369	119/369	112/369	363/369	34/369	37/369	292/369

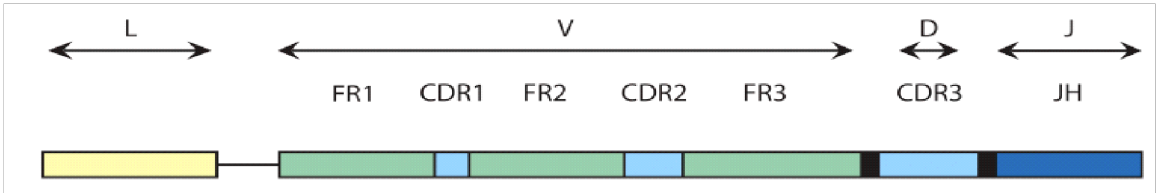
Comparison of NGS and CE (n=500)



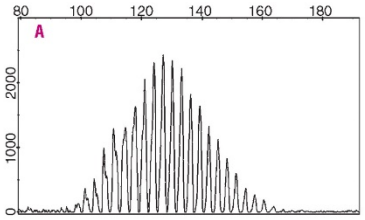
Somatic Hypermutation Assessment

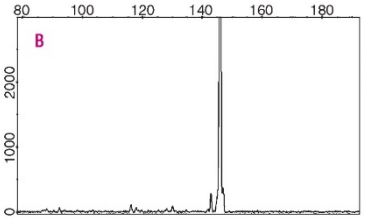
- Greatly facilitated by NGS
- Demonstrates higher sensitivity than traditional methods
- Multidimensional clinically relevant data captured with single assay
  - Simultaneous identification of unique clonal IGHV sequences (both productive and unproductive), and concurrent determination of SHM status
  - Easily characterize multiple clones when present

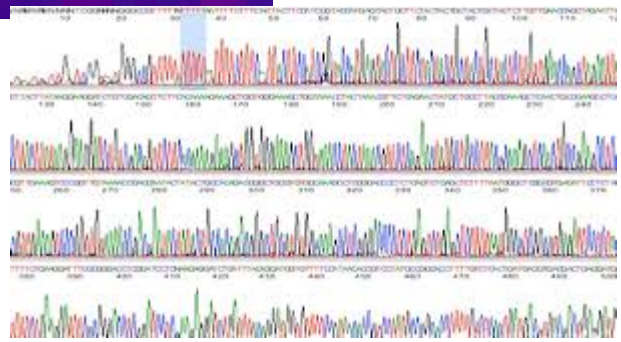
Traditional clonality& SHM testing



- Primers by BIOMED-2/ EuroClonality/ Invivoscribe
- PCR amplification
- Visualization of products by gel or capillary electrophoresis







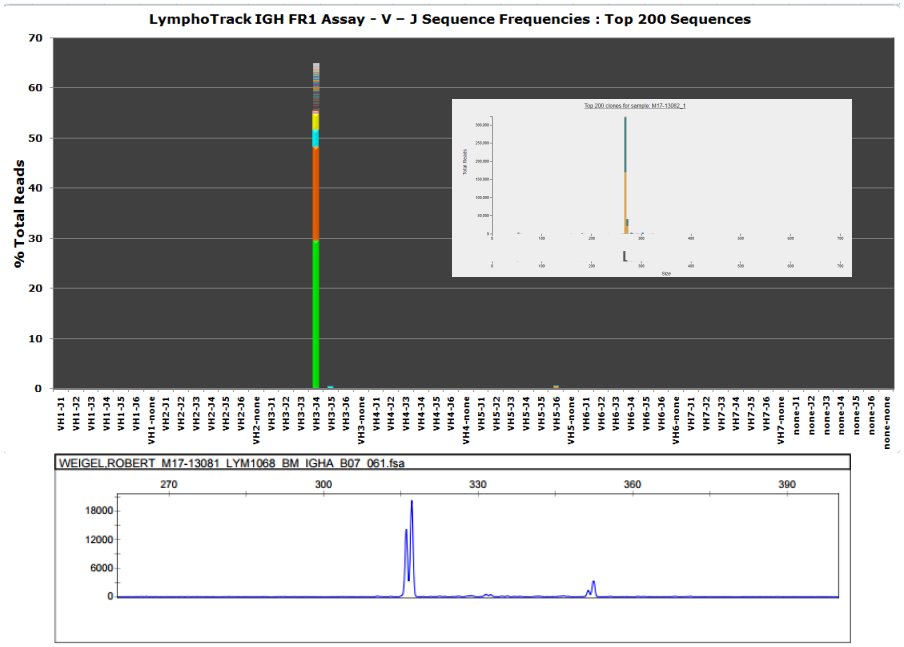
SHM
- Genomic or cDNA as template
- Cloning into plasmid
- Alignment
  - SHM: >2% discordance from germline

Slide provided by Dr. Petrova-Druss

Somatic hypermutation by NGS methods

Total count	480,438

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
1	GCCTCCGGATTCA	266	176876	IGHV3-74_02	IGHJ4_02	36.82	36.82	11.11	Y	Y	100.00
2	GCCTCTGGATTCA	266	124972	IGHV3-23_04	IGHJ4_02	26.01	62.83	1.76	Y	Y	99.12
3	GCCTCTGGATTCA	266	15372	IGHV3-23_04	IGHJ4_02	3.20	66.03	3.96	Y	Y	99.12
4	GCCTCTGGATTCA	266	5827	IGHV3-23_04	IGHJ4_02	1.21	67.24	1.76	Y	Y	99.12
5	GCCTCTGGATTCA	278	2364	IGHV3-23_04	IGHJ4_02	0.49	67.73	6.61	Y	Y	99.12
6	GTTTTGGATATAA	301	1342	IGHV5-51_01	IGHJ6_02	0.28	68.01	9.29	Y	Y	100.00
7	GTTCTGGATATAA	301	883	IGHV5-51_01	IGHJ6_02	0.18	68.20	8.85	Y	Y	100.00
8	GCCTCTGGATTCA	266	856	IGHV3-23_04	IGHJ4_02	0.18	68.37	2.20	Y	Y	99.12
9	GCCTCTGGATTCA	266	737	IGHV3-23_04	IGHJ4_02	0.15	68.53	3.52	Y	Y	99.12
10	GCCTCTGGATTTA	290	642	IGHV3-21_02	IGHJ6_02	0.13	68.66	4.41	Y	Y	100.00



Sequence Alignment

```
CLUSTAL O(1.2.0) multiple sequence alignment

1       GCCTCCGGATTCACCTTCAGTGACTACTGGATGCACTGGGTCCGCCAAGTTCCAGGGAAG
2       GCCTCTGGATTCACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAG
        ***** ********** ** ..***    ***..***********.* **********

1       GGGCTGACGTGGCTTTCAGTTATCAATACTGACGGGAAAACCACAAACTACGCGGACTCC
2       GGGCTGGAATGGGTCTCAGCTATTAGTGATAGTGGTGGTAGCACATACTACGCAAACTCC
        ******...*** * **** *** *.*..*.. ** ..:* ****:*******..*****

1       GTGAAGGGCCGATTCACCGTCTCCAGAGACAACGCCAAGAACACACTCTATGTGCAAATG
2       GTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTCTCTGCAAATG
        **********.******.*************  **********.** *.* ********

1       GACAGTCTGAGAGCCGAGGACACGGCTGCGTATTTCTGTGGAAGAAGTTTGTGGAATAGC
2       AACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAACTTATACTGGAACC
        .**** ******************* * .****:***** .*.** **: :  ..:* *

1       CTTGACCACTGGGGCCAGGGAGCCCT
2       CTTGACTCCTGGGGCCAGGGAACCCT
        ****** .*************.****
```

Accuracy of SHM in CLL specimens

- SHM status was evaluated by conventional criteria
  - Mutation rate >2% compared to germline IGHV sequence
- Comparison to reference lab
  - 50 specimens
- 100% concordant
  - Excellent inter- and intra-assay reproducibility and precision
  - Identical mutation rates on multiple repeats

FR1

Length	Raw count	V-gene	J-gene	% total reads	Mutation rate (%)
297	123101	IGHV4-59_01	IGHJ2_01	60.54	4.82

Leader

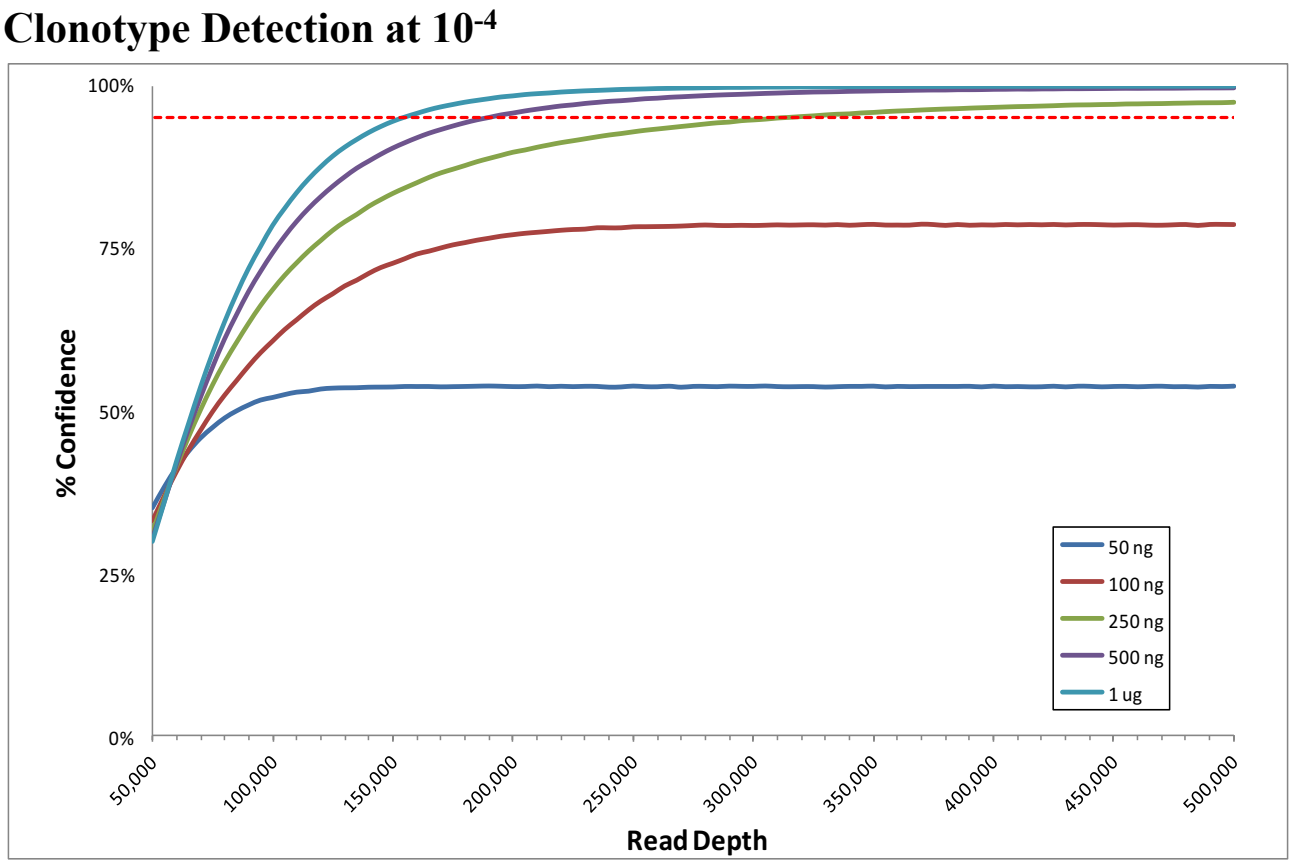
Length	Raw count	V-gene	J-gene	% total reads	Mutation rate (%)
479	86115	IGHV4-59_01	IGHJ2_01	24.31	4.1

Disease Monitoring

- Monitoring of low level and minimal residual disease is one of the main advantages of NGS
- Following initial characterization of the disease associated clone, it is possible to track the clone in subsequent samples at levels beyond those allowed by flow cytometry
- DNA input is critical for MRD testing

Relationship between the amount of DNA and number of total reads required for detecting a clonotype with 95% confidence

- **Theoretical yield:**
  - 6.5pg of DNA per cell; test 700 ng ~=100,000 cell equivalents
  - Often detect positives 10^{-6}



Figures provided by Invivoscribe

95% PROBABILITY OF DETECTING 5 READS OF THE TARGET SEQUENCE				
SENSITIVITY	DNA PER REPLICATE	# REPLICATES	READ DEPTH PER REPLICATE	# OF DIFFERENT SAMPLES FOR CLONOTYPE TRACKING PER RUN
$1x10^{-4}$	200 ng*	1 replicate of 200 ng	700,000	22 samples per run plus 2 controls on 24 Index Run
$1x10^{-5}$	700 ng**	5 replicates of 700ng each	700,000	4 samples per run plus 2 controls on 24 Index Run or
	2 µg***	2 replicates of 2 µg each	1,400,000	5 samples per run plus 2 controls on 12 Index Run

Note: A replicate is an independent PCR reaction with input DNA from the same subject.

* Assuming 20ng/µl of DNA (achievable without secondary DNA concentrating step).

** Assuming 70ng/µl of DNA (achievable only with secondary DNA concentrating step).

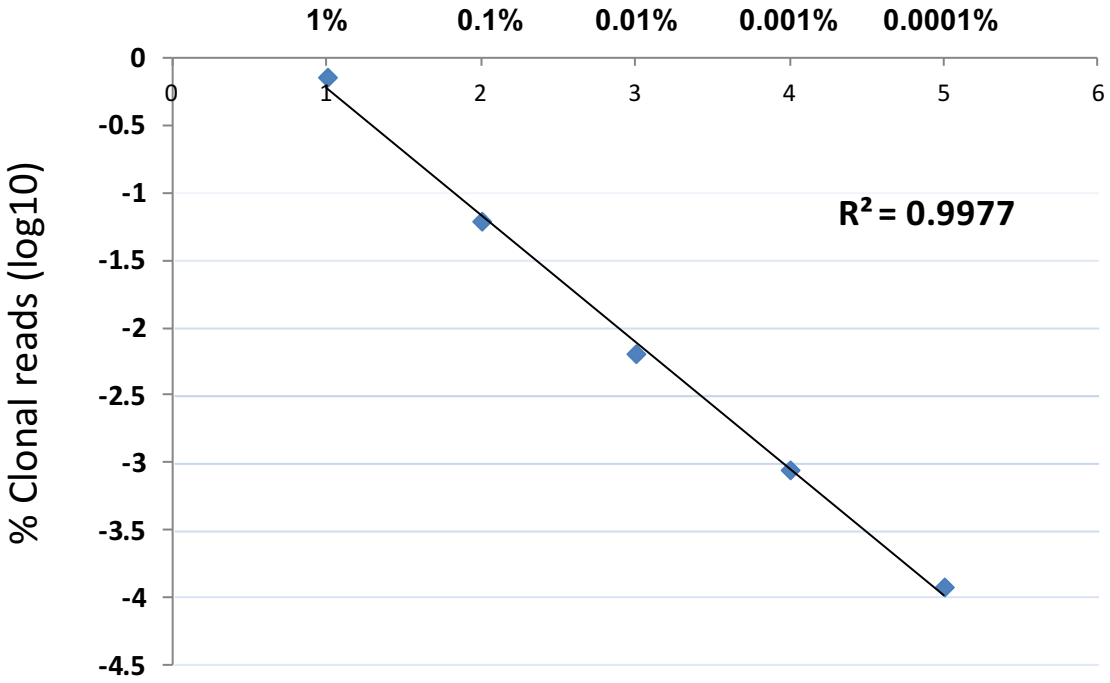
*** Assuming 200ng/µl of DNA (achievable only with secondary DNA concentrating step).

$1x10^{-6}$ PROVIDED FOR INFORMATIONAL PURPOSES ONLY				
$1x10^{-6}$	2 µg***	18 replicate of 2 µg each	2,100,000	1 sample over 3 runs on 8 Index Run

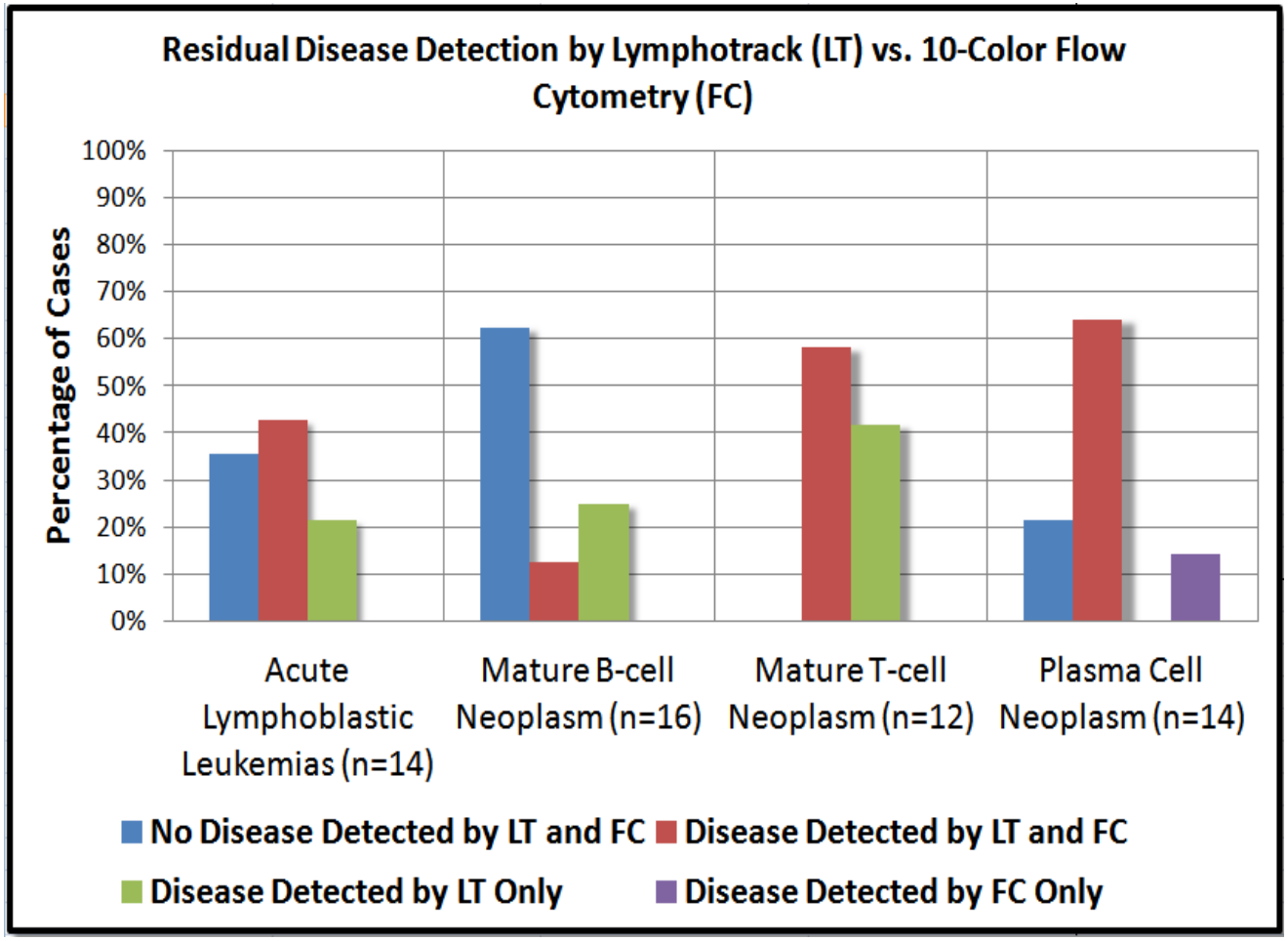
Note: Detection at $1x10^{-6}$ in any of the above experimental set-ups is possible, including $1x10^{-4}$. However, to achieve 95% confidence that a sample is truly negative at $1x10^{-6}$ sensitivity, testing requires the above experimental set-up.

IGH dilution study
(cell line - 2ug total input)

Dilution (IVS-0013 into IVS 0000) Undiluted clone 72%	Raw Count (target sequence)				Total read count per run				Total reads (All runs)		% clonal read
	1	2	3	4	1	2	3	4	Target sequence	Total reads	
1% (1/100)	5152	5898	5073	5299	498,330	565,180	731,526	694614	21,422	2,489,650	**0.86044**
0.1% (1/1000)	381	447	502	425	396,417	470,979	690,803	800424	1,755	2,358,623	**0.07441**
0.01% (1/10,000)	64	27	34	66	637,782	422,769	760,124	787451	191	2,608,126	**0.00732**
0.001% (1/100,000)	17	4	13	2	431,755	356,569	797,245	891913	36	2,477,482	**0.00145**
0.0001% (1/1,000,000)	0	3	1	1	425,060	387,118	826,528	837627	5	2,476,333	**0.00020**



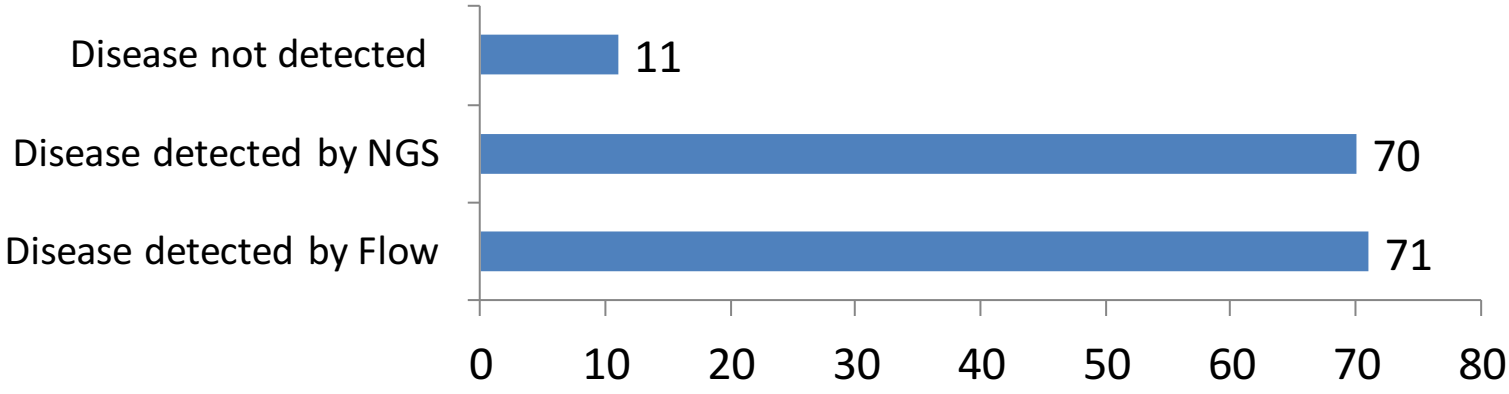
Comparison of NGS and Flow cytometry



Provided by Dr. Caleb Ho

AMP 2016 DOI:10.1016/S1525-1578(16)30178-7

Comparison of NGS and CE – Plasma cell neoplasms (n=83 samples, 71 patients)



3 discrepant cases

2 cases: detected by flow not NGS: 0.0005%, 0.00095%

1 case detected by NGS and not flow: 0.02% of the total rearranged IGH reads

Examples of the benefits of NGS testing

- Detection of diagnostic clonal sequence at high sensitivity
- Provide a high-resolution picture of the spectrum of immunity found in lymphoid malignancies.
- Define behaviors of clonal tumor populations, suppression or re-emergence of these populations following treatment
- May identify both stable and dynamic aspects of the immune repertoire

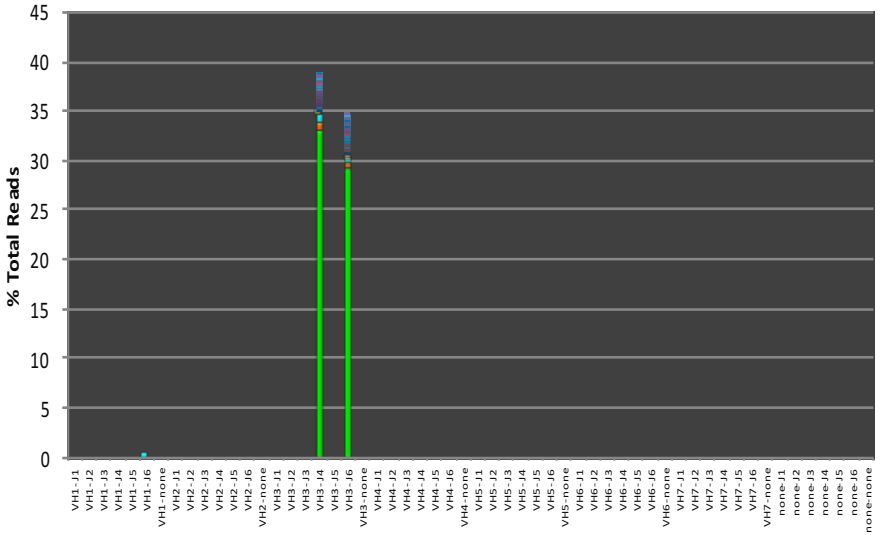
Case 1 - 2 yo male BM B-ALL – diagnostic sample (ETV6-RUNX1 fusion +)

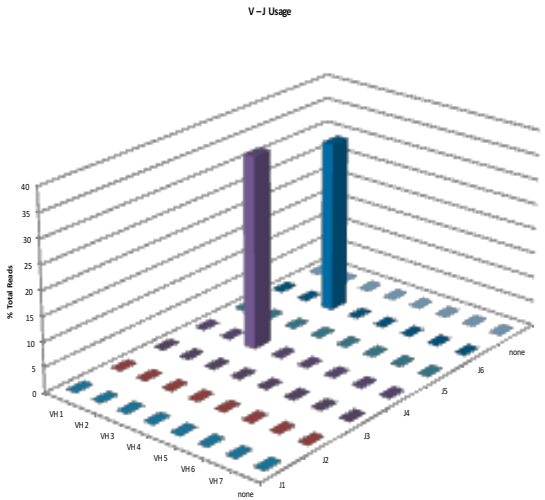
Total count	766,700

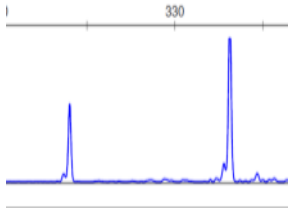
Rank	Sequence	Length	Merge count	V-gene	J-gene	total r	umulative	to pa	rame	op codo	/-covera
1	GCCTCTGGATTCA	261	316398	IGHV3-7_02	IGHJ4_02	41.27	41.27	0.00	n/a	N	94.22
2	GCCTCTGGATTCA	288	282740	IGHV3-7_01	IGHJ6_02	36.88	78.15	0.00	N	N	99.56
3	GCCTCTGGATTCA	303	1278	IGHV3-9_01	IGHJ6_02	0.17	78.31	0.00	N	N	99.56
4	CTTCTGGATACAC	295	508	IGHV1-8_01	IGHJ6_02	0.07	78.38	0.00	Y	Y	99.56
5	CTTCTGGATACAC	288	430	IGHV1-8_01	IGHJ6_02	0.06	78.43	0.00	N	N	98.67
6	GCCTCTGGATTCA	286	280	IGHV3-11_01	IGHJ6_02	0.04	78.47	0.00	N	N	96.92
7	GCCTCTGGATTCA	287	262	IGHV3-23_04	IGHJ6_02	0.03	78.50	0.00	Y	Y	96.48
8	CTTCTGGATACAC	295	246	IGHV1-8_01	IGHJ6_02	0.03	78.54	0.00	Y	Y	98.67

GCCTCTGGATTCACCTTTAGTAGCTATTGGATGAGCTGGGTCCGCCAGGCTCCAGGGAAAGGGCTGGAGTGGGTGGCCAACATAAAGCAAGATGGAAGTGAGAAATACTATGTGGACTCTGTG
AAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTACCTTAAAGGGGGTGGTGACTGCTAAGGGCTACTG
GGGCCAGGGAACCCT

GCCTCTGGATTCACCTTTAGTAGCTATTGGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTGGCCAACATAAAGCAAGATGGAAGTGAGAAATACTATGTGGACTCTGT
GAAGGGCCGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGAGGGCGAAGACTATGATAGTT
CCTCTTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC



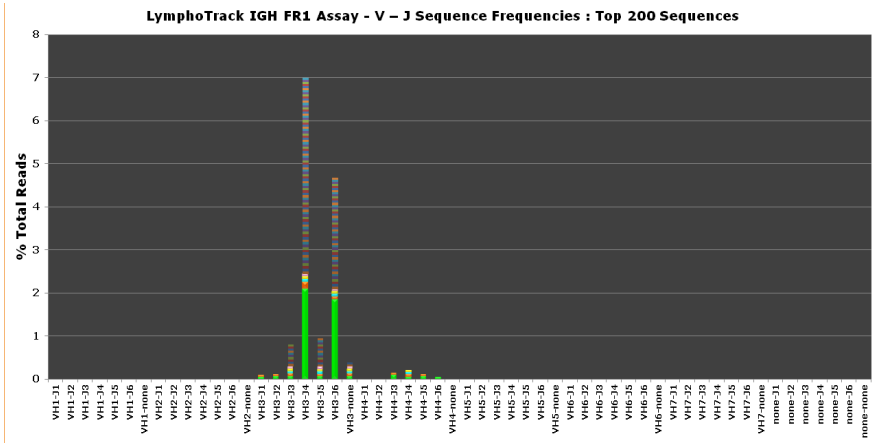


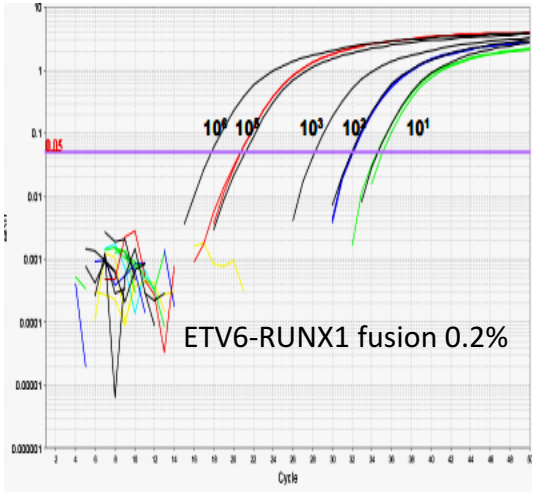


2 yo male BM B-ALL – Monitoring sample

- Morphology – negative
- Flow – negative
- FISH – negative
- Clonal sequence 2.5% of rearranged IGH reads (35,896 reads / 907,564)

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads
1	GCCTCTGGATTCA	261	8882	IGHV3-7_02	IGHJ4_02	2.34
2	GCCTCTGGATTCA	288	7139	IGHV3-7_01	IGHJ6_02	1.88
3	CACTGTCTCTGGT	119	347	IGHV4-39_07	IGHJ3_02	0.09
4	CACTGTCTCTGGA	270	256	IGHV4-59_08	IGHJ5_02	0.07
5	GCCTCAAGATTCT	268	255	IGHV3-33_06	IGHJ4_02	0.07
6	GCCTCTTAATTCA	253	253	IGHV3-7_02	IGHJ6_02	0.07
7	GCCTCTGGATTCA	265	252	IGHV3-30_18	IGHJ4_02	0.07
8	GCGTCTGGATTCA	259	250	IGHV3-33_06	IGHJ4_02	0.07
9	GCCTCTGGATTCA	257	249	IGHV3-30-3_01	none	0.07
10	GCCTCTCGATTCA	239	248	IGHV3-7_02	IGHJ4_02	0.07





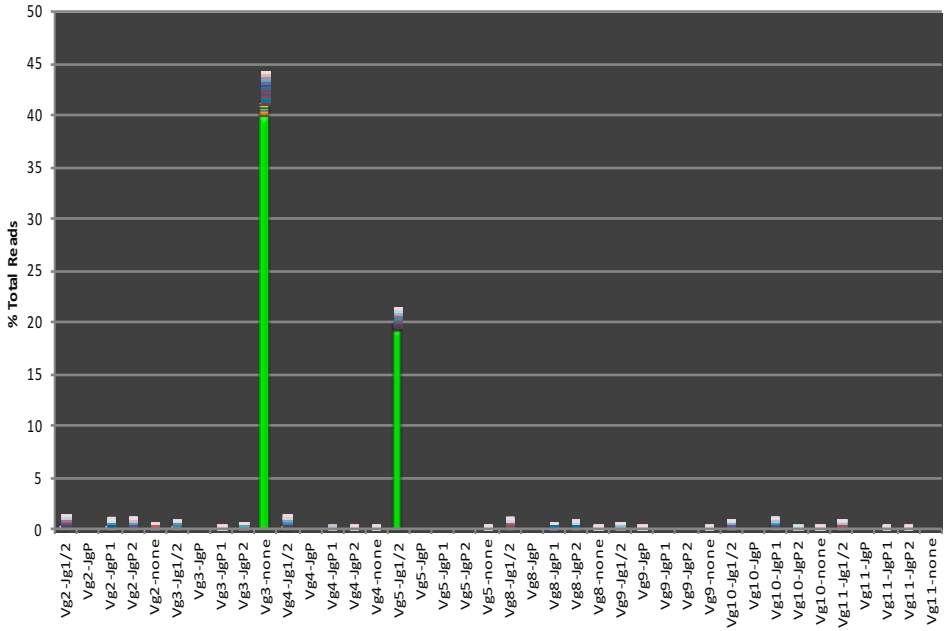
Case 2 - 52 year old male with B-ALL – diagnostic sample

Total count **788,733**

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %
1	AGAATCAGTAGA	126	348022	Vg3	none	44.1241840	44.1241840
2	GGACTCAGTCC	151	163509	Vg5	Jg1/2	20.7305894	64.8547734
3	GGAATCAGCCC	143	739	Vg4	Jg1/2	0.0936946	64.9484680
4	GGAGTCAGTCC	157	648	Vg2	Jg1/2	0.0821571	65.0306251
5	GAAGACTAAGA	133	601	Vg11	Jg1/2	0.0761982	65.1068232

AGAATCAGTAGAGGAAAGTATTTTACTTATGCAAGCATGAGGAGGAGCTGGAAATTGATATTGCAAAATCTAATTGAAAATGATTCTGGATCTATTACTGTGCCGCGT
AGGGGCGCTTTGGCAGTG

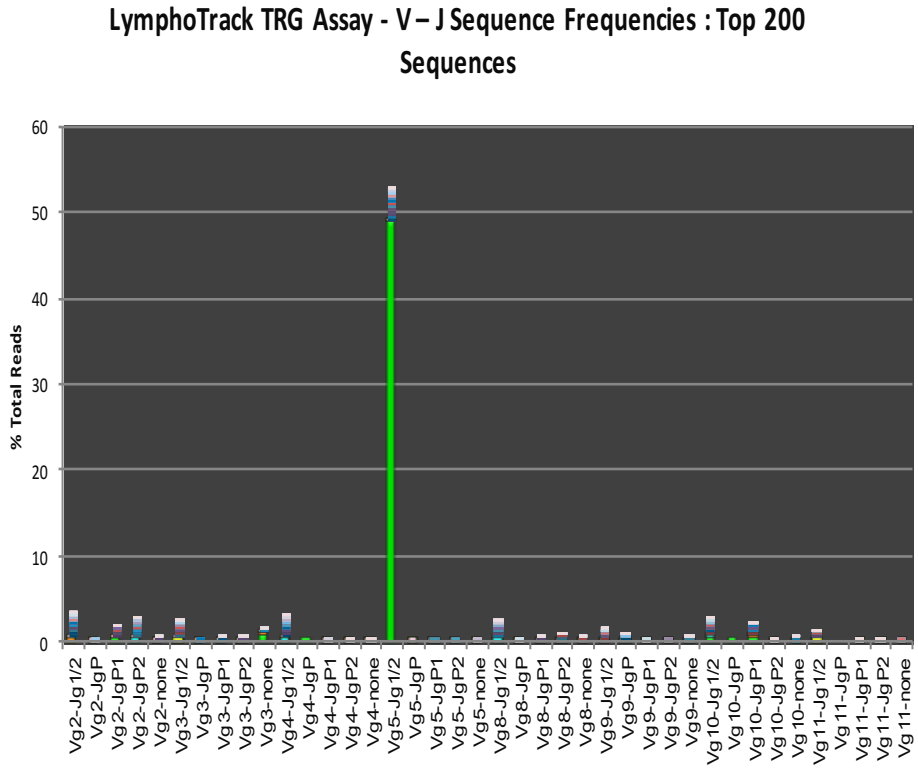
GGACTCAGTCCAGGAAAGTATTATACTCATACACCCAGGAGGTGGAGCTGGATATTGATACTACGAAATCTAATTGAAAATGATTCTGGGGTCTATTACTGTGCCACC
TGGGACAGGCCTCGGGATTATTATAAGAAACTCTTTGGCAGTG



52 year old male with B-ALL – relapse 1 yr

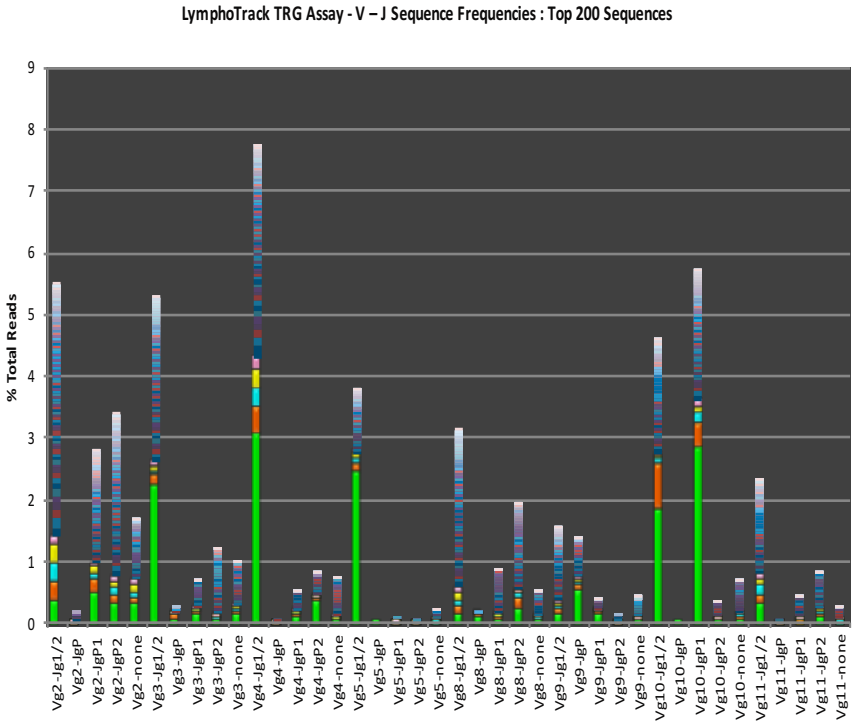
Total count **1,282,820**

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads
1	GGACTCAGTCCAGGAAAGT	151	665920	Vg5	Jg1/2	51.9106344
2	AGAATCAGTAGAGGAAAGT	126	11361	Vg3	none	0.8856270
3	TGGGTAAGACAAGCAACAA	151	4804	Vg10	JgP1	0.3744875
4	TGGGTAAGACAAGCAACAA	156	4204	Vg10	Jg1/2	0.3277155
5	GGAGTCAGTCCAGGGAAG	153	3917	Vg2	JgP1	0.3053429
6	TGGGTAAGACAAGCAACAA	147	3148	Vg10	JgP1	0.2453969

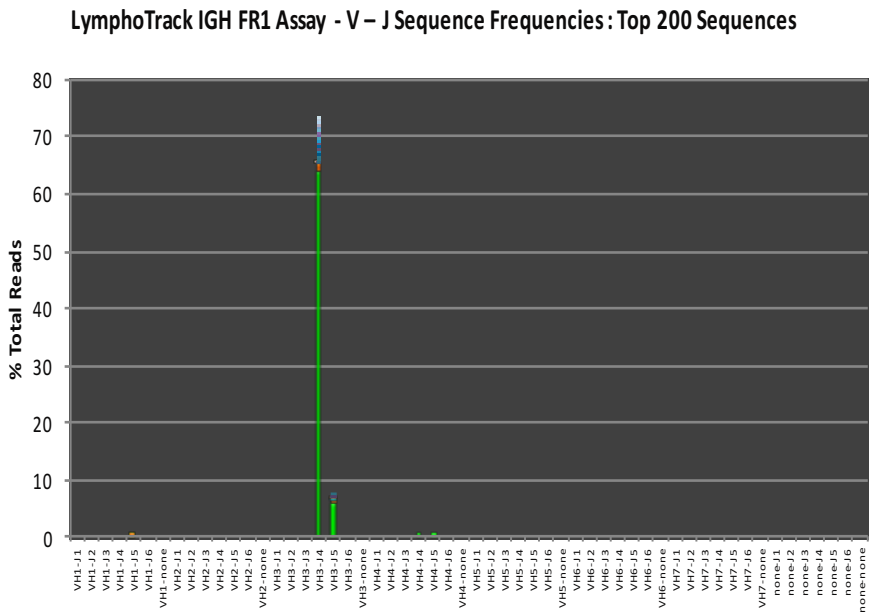


Pre-transplant sample

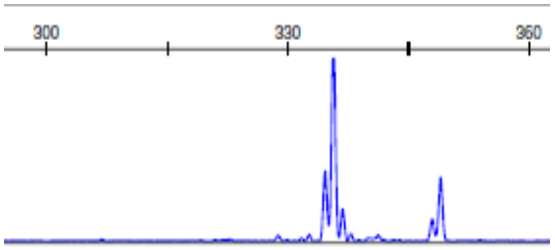
Total count		824,287	1,005,938				
Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
1	GGAATCAGCCCAGG	143	24955	Vg4	Jg1/2	3.0274649	3.0274649
2	TGGGTAAGACAAGC	151	23292	Vg10	JgP1	2.8257148	5.8531798
3	GGACTCAGTCCAGG	151	20087	Vg5	Jg1/2	2.4368939	8.2900737
4	GGACTCAGTCCAGG	156	18111	Vg3	Jg1/2	2.1971716	10.4872453
5	TGGGTAAGACAAGC	156	14966	Vg10	Jg1/2	1.8156298	12.3028751

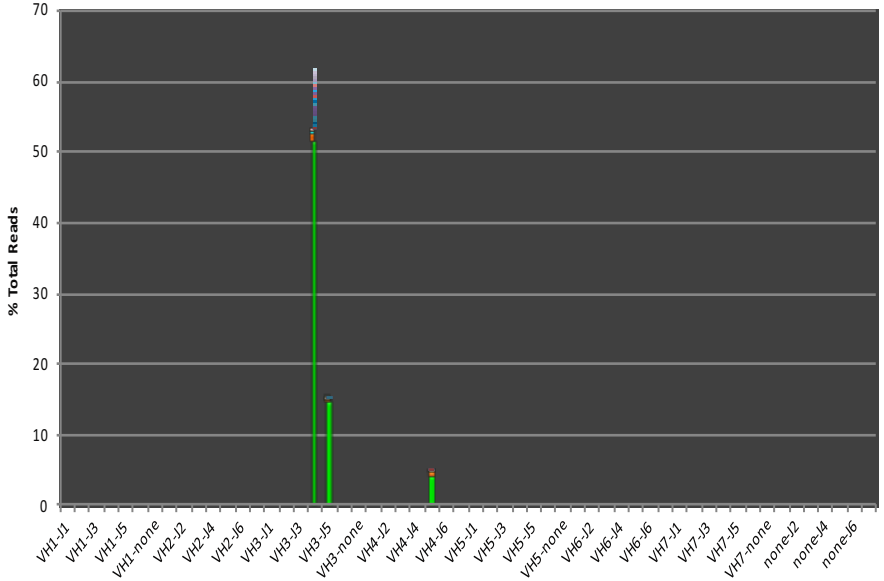


Case 3 - Monitoring of B-ALL

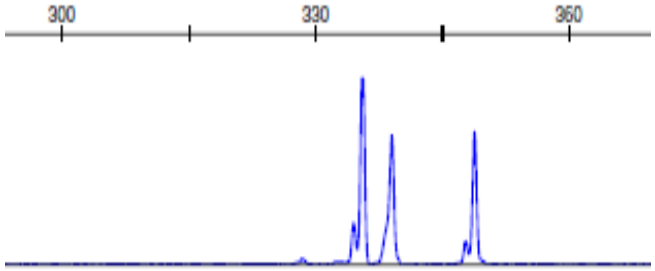


Diagnosis





Relapse



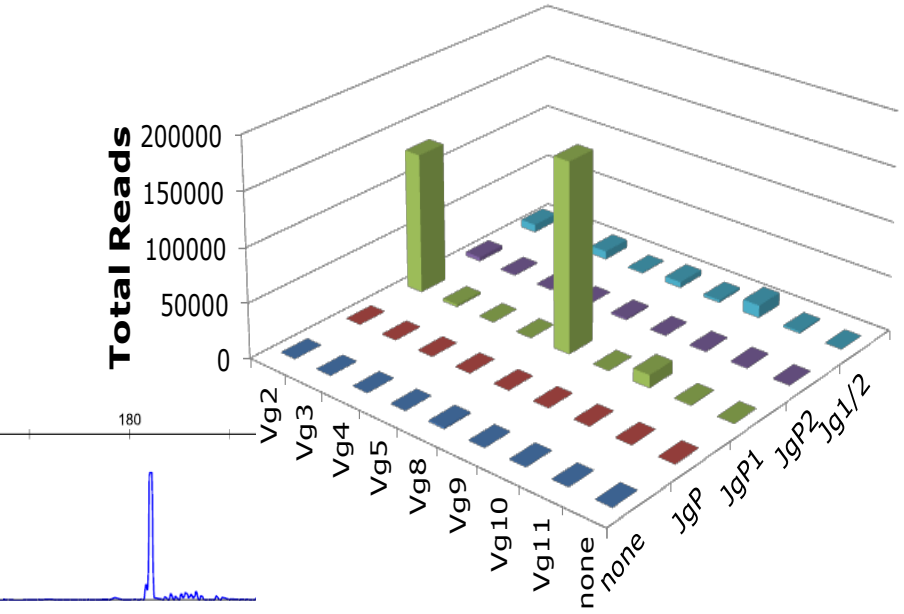
Case 4 - 49yo male with right parotid lesion
Peripheral T-cell Lymphoma NOS

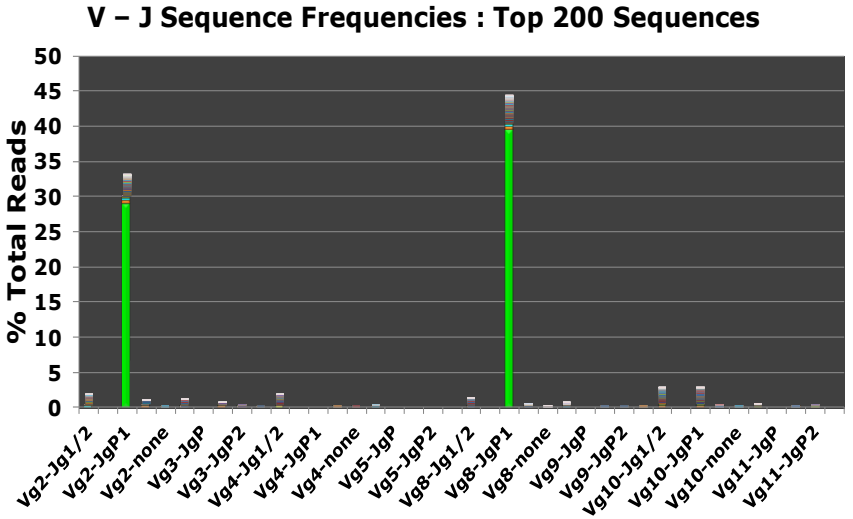
Top 2 clones of same size but different usage

3	Total count	369,093						
4								
5	Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
6	1	GGAATCAGTCGAC	111	144722	Vg8	JgP1	39.2101720	39.2101720
7	2	GGAGTCAGTCCAC	111	106312	Vg2	JgP1	28.8035807	68.0137526
8	3	GGAATCAGTCGAC	110	2099	Vg8	JgP1	0.5686914	68.5824440
9	4	GGAGTCAGTCCAC	110	1584	Vg2	JgP1	0.4291601	69.0116041
10	5	GGATTCAGTCCAG	111	1403	Vg2	JgP1	0.3801210	69.3917251
11	6	GAATCAGTCGAGA	110	1168	Vg8	JgP1	0.3164514	69.7081765
12	7	GAGTCAGTCCAG	110	829	Vg2	JgP1	0.2246046	69.9327812
13	8	TGGGTAAGACAAC	122	697	Vg10	JgP1	0.1888413	70.1216225
14	9	GGACTCAGTCCAC	133	656	Vg3	JgP1	0.1777330	70.2993554
15	10	GGAATCAGTCGAC	112	586	Vg8	JgP1	0.1587676	70.4581230

GGAATCAGTCGAGAAAAGTATCATACTTATGCAAGCACAGGGAAGAGCCTTAAATTTATACTGGAAAATCNAATTGAACGTGACTCTGGGGTCTATTACTGTGCCACCTACC
ACTGGTTGGTTCAAGATATTTGCTG

GGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCACAAGGAACAACTTGAGATTGATACTGCAAAATCNAATTGAAAATGACTCTGGGGTCTATTACTGTGCCACCTGGG
ACGGGTTGGTTCAAGATATTTGCTG

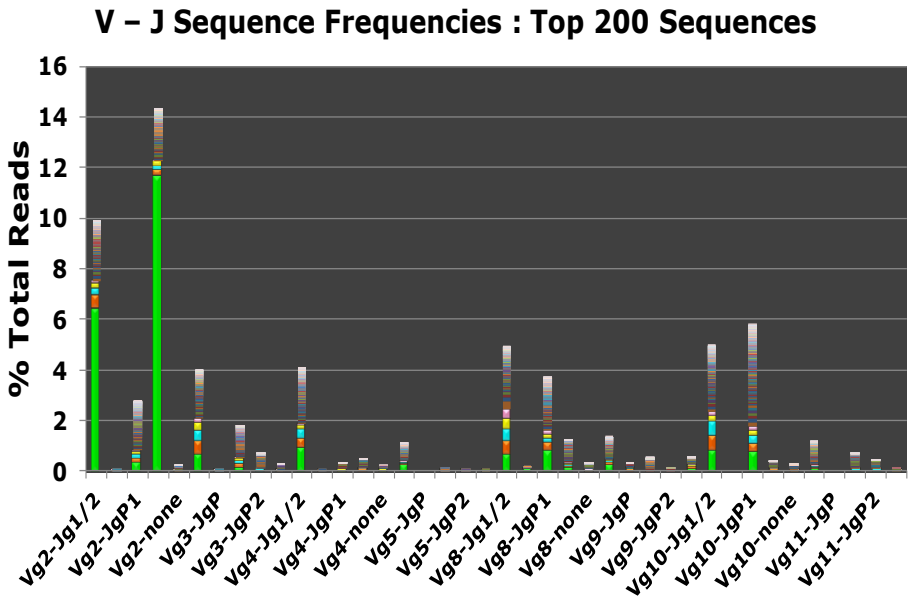


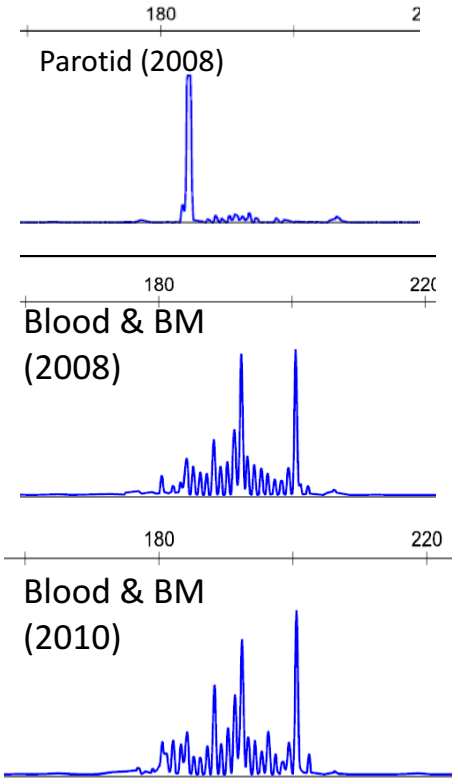


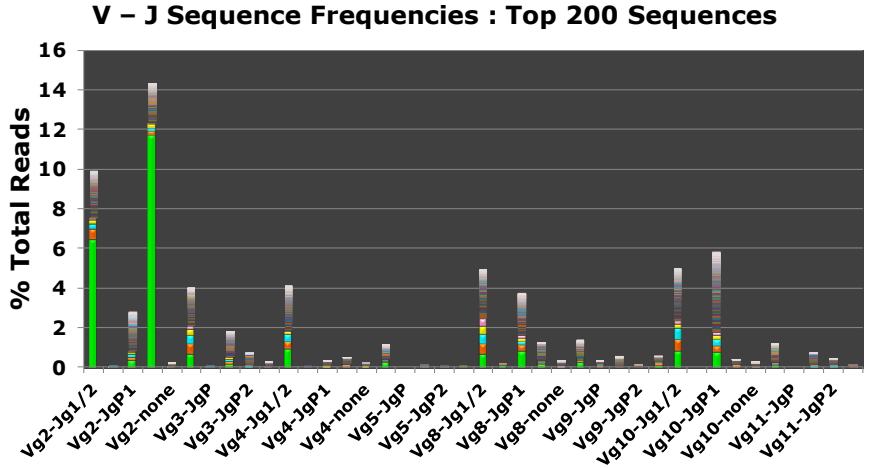
Blood and bone marrow 2008

3	Total count	398,357						
4								
5	Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
6	1	GGAGTCAGTCCAC	128	46313	Vg2	JgP2	11.6260038	11.6260038
7	2	GGAGTCAGTCCAC	146	25559	Vg2	Jg1/2	6.4161041	18.0421080
8	3	GGAATCAGCCCAC	134	3744	Vg4	Jg1/2	0.9398605	18.9819684
9	4	TGGGTAAGACAAC	139	3211	Vg10	Jg1/2	0.8060609	19.7880293
10	5	GGAATCAGTCGAC	120	3167	Vg8	JgP1	0.7950155	20.5830449
11	6	TGGGTAAGACAAC	129	3147	Vg10	JgP1	0.7899949	21.3730398
12	7	GGAATCAGTCGAC	138	2648	Vg8	Jg1/2	0.6647304	22.0377701
13	8	AGAATCAGTAGAC	146	2600	Vg3	Jg1/2	0.6526809	22.6904510
14	9	TGGGTAAGACAAC	146	2358	Vg10	Jg1/2	0.5919314	23.2823824

GGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCACAAGGAACAACTTGAGATTGATACTGCAAAATCTAATTGAAAATGA
CTCTGGGGTCTATTACTGTGCCACCTGGGACGGGCCCTGGGGAGTAGTGATTGGATCAAGACGTTTGCAA





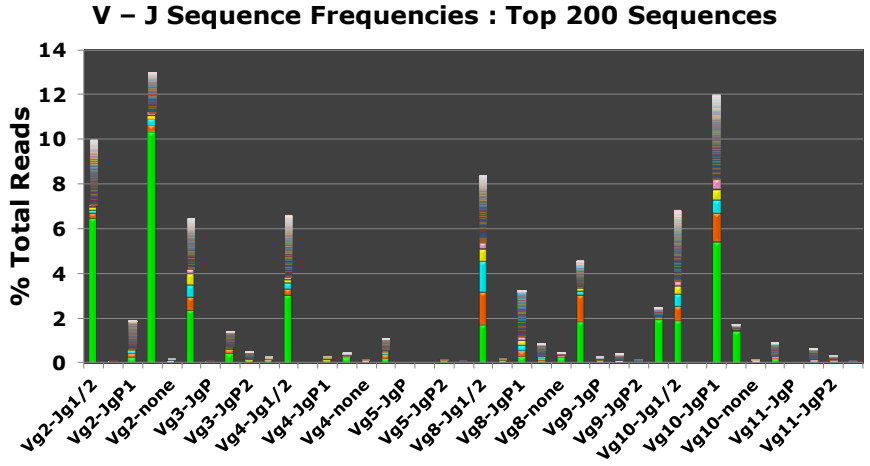


BM 2010

GGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCACAAGGAACAACTTG
AGATTGATACTGCAAAATCTAATTGAAAATGACTCTGGGGTCTATTACTGTGC
CACCTGGGACGGGCCCTGGGGAGTAGTGATTGGATCAAGACGTTTGCAA

3	Total count	361,609
4		

	Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
6	1	GGAGTCAGTCCAC	128	37161	Vg2	JgP2	10.2765694	10.2765694
7	2	GGAGTCAGTCCAC	146	23241	Vg2	Jg1/2	6.4271077	16.7036772
8	3	TGGGTAAGACAAC	124	19456	Vg10	JgP1	5.3803971	22.0840742
9	4	GGAATCAGCCCAC	134	10850	Vg4	Jg1/2	3.0004784	25.0845527
10	5	AGAATCAGTAGAC	146	8406	Vg3	Jg1/2	2.3246103	27.4091629
11	6	CGGCATTCCGTCA	145	6926	Vg9	none	1.9153284	29.3244914
12	7	TGGGTAAGACAAC	143	6784	Vg10	Jg1/2	1.8760595	31.2005509
13	8	CGGCATTCCGTCA	142	6572	Vg9	Jg1/2	1.8174326	33.0179835
14	9	GGAATCAGTCGAC	138	6001	Vg8	Jg1/2	1.6595273	34.6775108
15	10	GGAATCAGTCGAC	142	5355	Vg8	Jg1/2	1.4808813	36.1583921

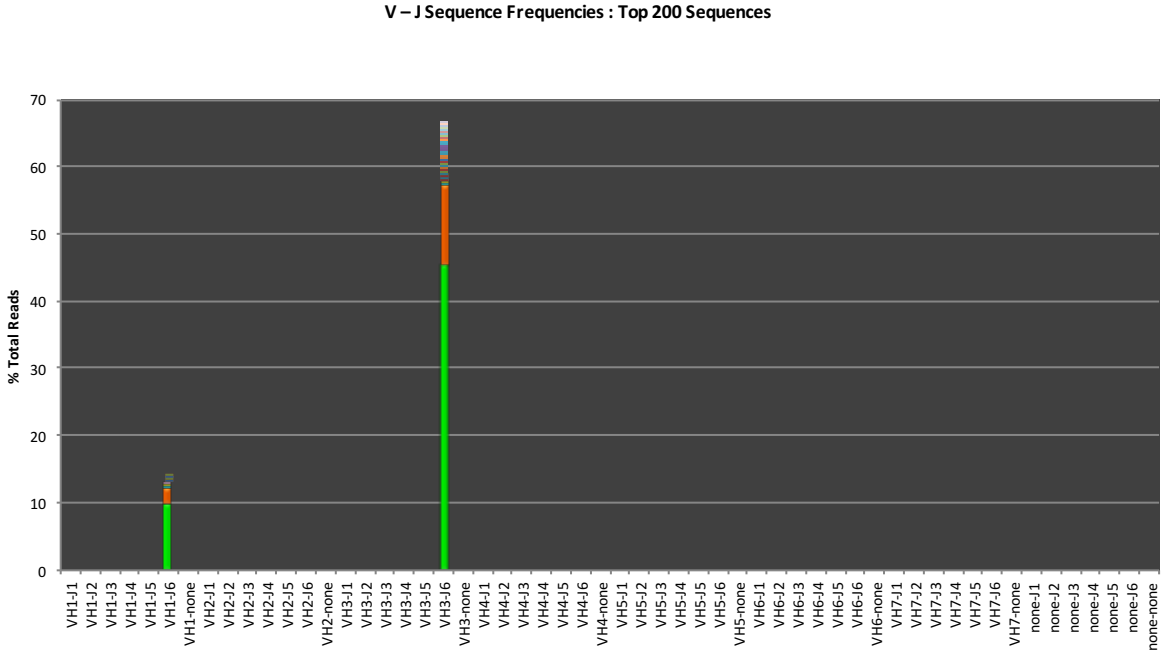


GGAGTCAGTCCAGGGAAGTATTATACTTACGCAAGCACAAGGAACAACTTG
AGATTGATACTGCAAAATCTAATTGAAAATGACTCTGGGGTCTATTACTGTGC
CACCTGGGACGGGCCCTGGGGAGTAGTGATTGGATCAAGACGTTTGCAA

BM 2013

Case # 5 - 54yo male with thrombocytopenia and neutropenia

Total count	**656,700**						
Rank	Sequence	Length	Raw count	V-gene	J-gene	% total reads	Cumulative %
1	GCCTCTGGATTCAC	309	295220	IGHV3-30_18	IGHJ6_02	44.9550784	44.9550784
2	GCCTCTGGATTCAC	287	78430	IGHV3-30_03	IGHJ6_03	11.9430486	56.8981270
3	CTTCTGGAGGCACC	295	63830	IGHV1-69_13	IGHJ6_03	9.7198112	66.6179382
4	CTTCTGGAGGCACC	301	15749	IGHV1-69_13	IGHJ6_02	2.3982031	69.0161413
5	CGCCGTCTCTGGTC	279	2483	IGHV4-4_02	IGHJ5_02	0.3781026	69.3942439
6	GCCTCTGGATTCAC	309	2423	IGHV3-30_18	IGHJ6_02	0.3689660	69.7632100
7	GCCTCTGGATTCAC	309	2227	IGHV3-30_18	IGHJ6_02	0.3391198	70.1023298
8	CTTCTGGAGGCACC	292	2163	IGHV1-69_13	IGHJ6_03	0.3293741	70.4317040
9	CTTCTGGAGGCACC	295	1733	IGHV1-69_13	IGHJ6_03	0.2638952	70.6955992
10	CTTCTGGAGGCACC	295	1528	IGHV1-69_13	IGHJ6_03	0.2326785	70.9282778



Flow cytometry:
3 abnormal populations
- Hairy cell leukemia (lambda)
-CLL (lambda)
-CD5+ B cell lymphoma (kappa)

When should you consider assessing clonality by NGS

- Important questions:
  - Intended use:
    - If only diagnostic and rapid TAT required – PCR+CE
    - For diagnostic cases with relatively low tumor content to resolve clonal vs not clonal
    - Use NGS if intended use is for further monitoring of the patient.
    - Decisions of use for staging and monitoring are institution dependent and vary depending on the team and current guidelines for minimal residual disease monitoring
  - For monitoring use –
    - Consider sensitivity and feasibility to meet expected TAT required by the clinical team
    - Are other ancillary methods available that can provide same level of disease monitoring – qPCR, Flow
    - Consider the disease process
      - NGS preferable for T cell malignancies, B-ALL, and some low grade B cell lymphomas.

Potential Pitfalls

- New technology – not readily available in all labs
- Analytical phase still without the extensive validation and standardization compared to CE assays
- As for any other assay –
  - Pre-and post-analytical phases highly variable
    - Clinical context, selection of representative material, preservation and sample handling, isolation of nucleic acid (yield, purity and integrity) and selection of Ig/TCR rearrangements as PCR targets
    - Accurate interpretation heavily depends on individual experience, detailed knowledge on both the technology and disease process.

Conclusions

- Clonality testing provides distinct advantages
- Efficiently detects IgH and *TRG gene* rearrangements using a streamlined approach
- Results are highly reproducible and provide a more objective way to determine clonality
- Easy assessment of somatic hypermutation
- Enables the use of clonality testing for monitoring of disease
- Defines behaviors of clonal tumor populations, suppression or re-emergence following treatment
- Compared to flow cytometry, NGS provides higher detection of residual disease in mature B and T cell neoplasms as well as B-ALL. Similar capabilities as flow for plasma cell neoplasms
- Further studies needed to define clinically significant levels of MRD, establishment of guidelines based on longitudinal follow up and patient outcomes

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