UPPSALA UNIVERSITET

IMMUNOGENETICS IN CLL IN THE NGS ERA
Rotterdam, The Netherlands, November 24th 2017

Basic principles of IG sequence analysis: *Immunogenetic analysis: in vitro*

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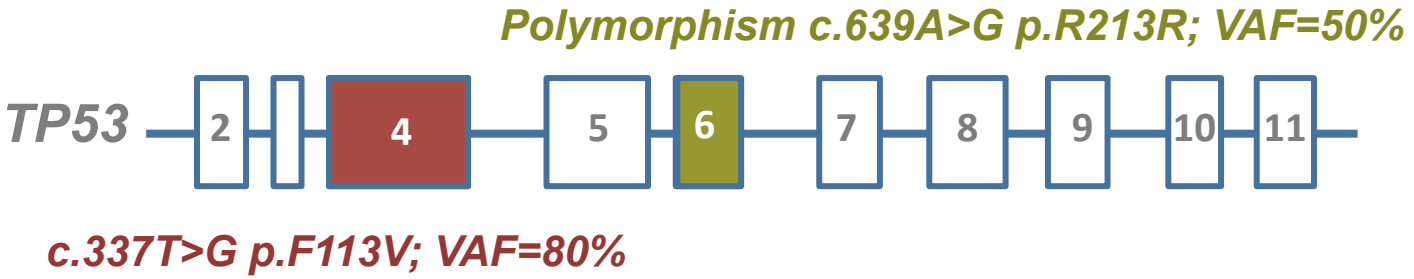
Different nature of IG genes:
IG genes = a unique set of genes

Non-IG genes



Structure:

Single gene – one ➡ many exons

Variations:

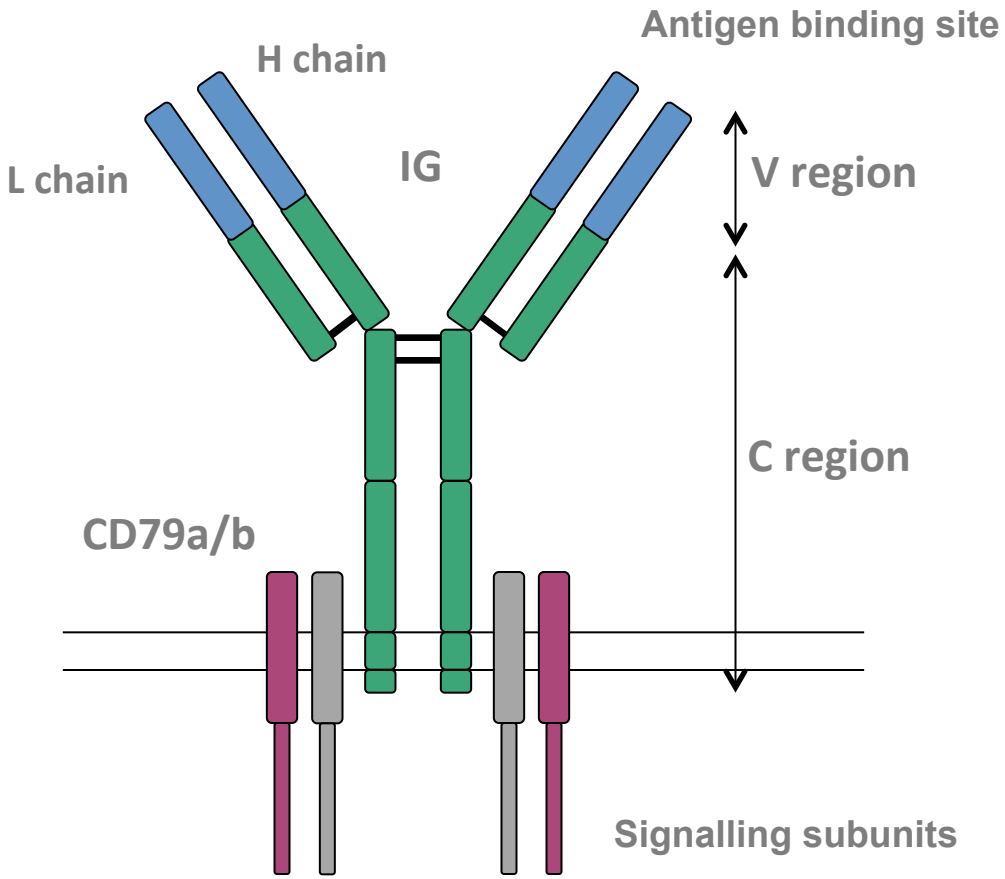
- **SNP**
- **Pathogenic mutations**

Origin of variants:

- **Inherited**
- **Acquired**

Different nature of IG genes:

IG genes = a unique set of genes

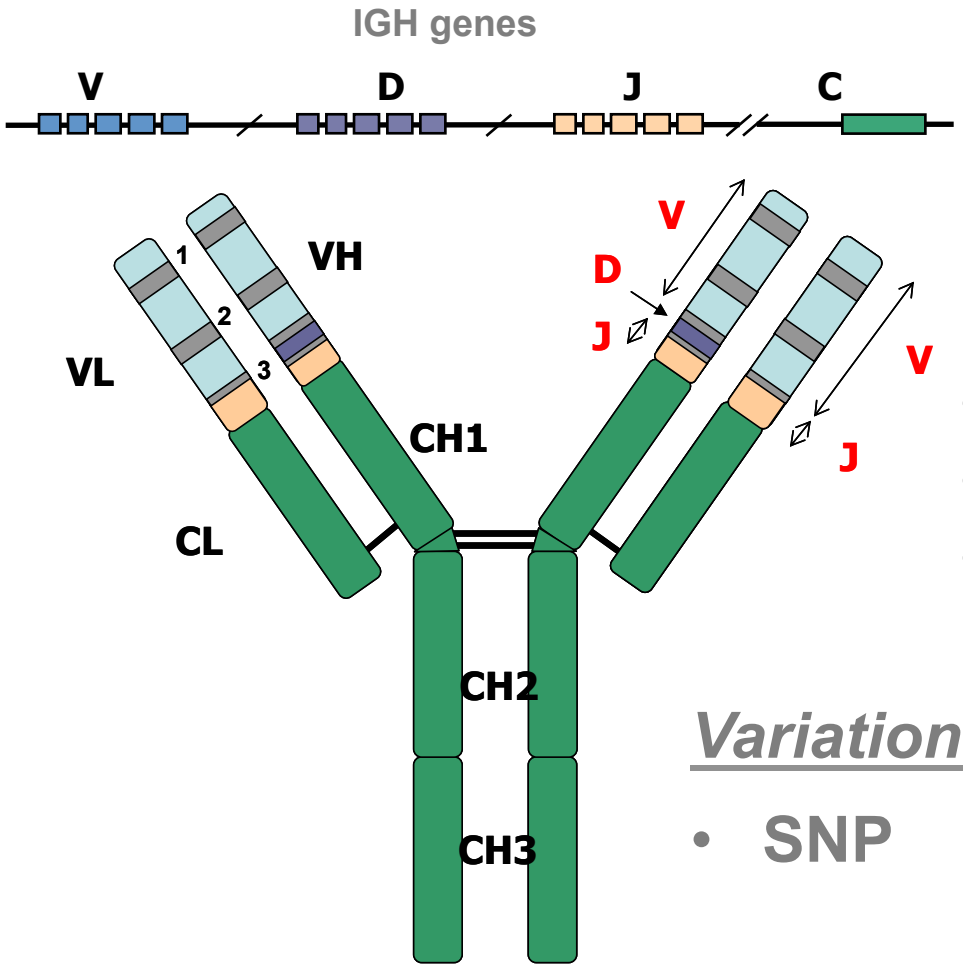


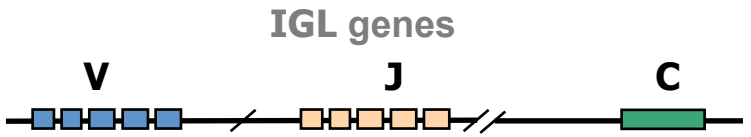
B cell receptor (BcR)

Different nature of IG genes:

IG genes = a unique set of genes

IG genes





Structure

- Rearranged IG gene
- Loss of nucleotide
- Non-templated nucleotides

Variations

- SNP

Generation of diversity

- Acquired

Generation of diversity:

IG genes = a unique set of genes

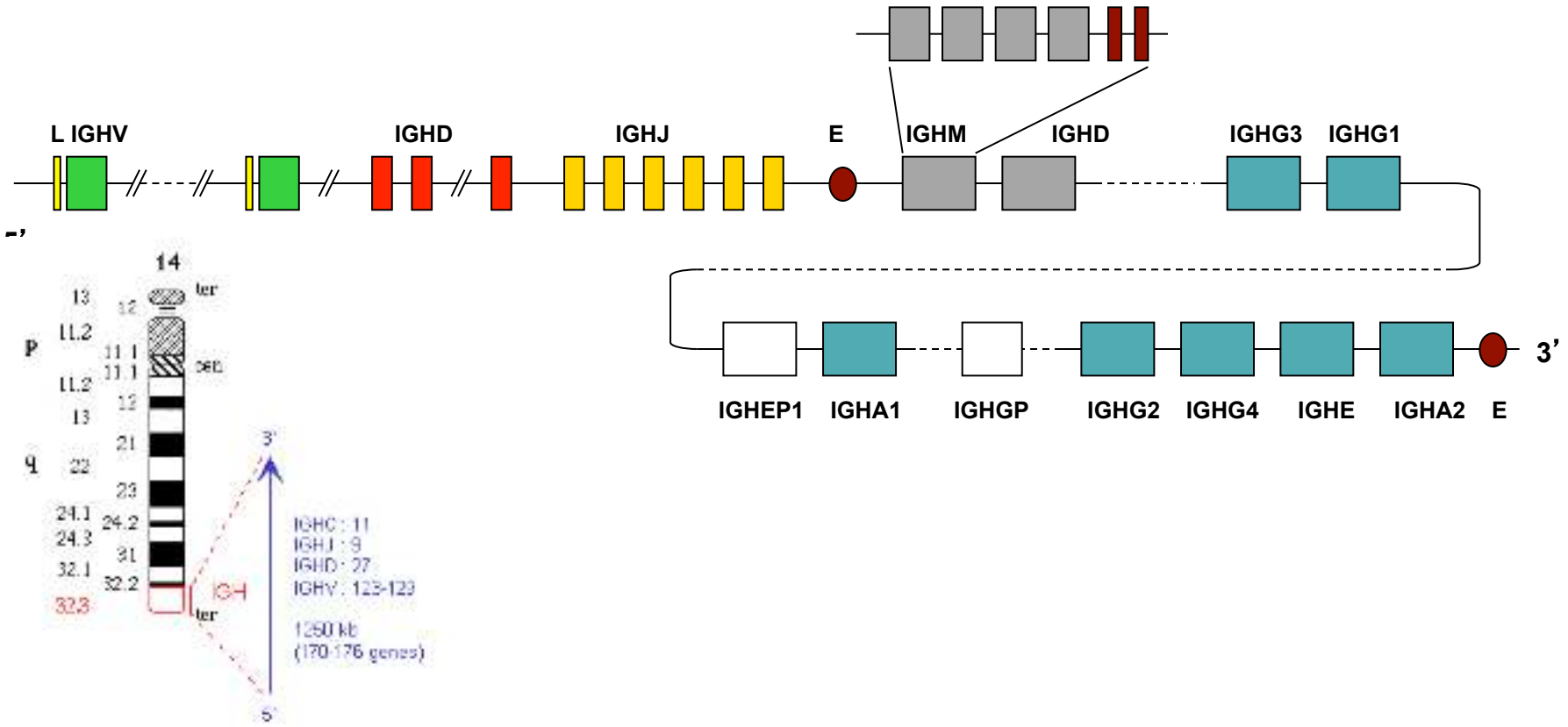
1. ***Combinatorial diversity***
   - *V region encoded by 2 or 3 genes*
   - *Reservoir of multiple IG V, D, J genes*
   - *Random assembly*
2. ***Junctional diversity***
   - *Imprecise joining at the CDR3*
3. ***Combinatorial diversity***
   - *Pairing of heavy and light chain IG genes*

Central
(bone marrow)

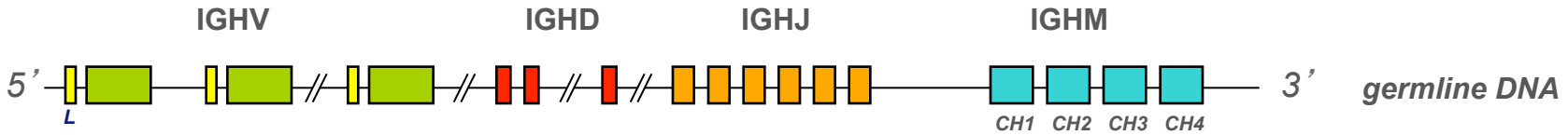
4. ***Maturation diversity***
   - *Somatic hypermutations (SHM)*

Peripheral
(2º lymphoid organs)

Germline organization of the IGH locus

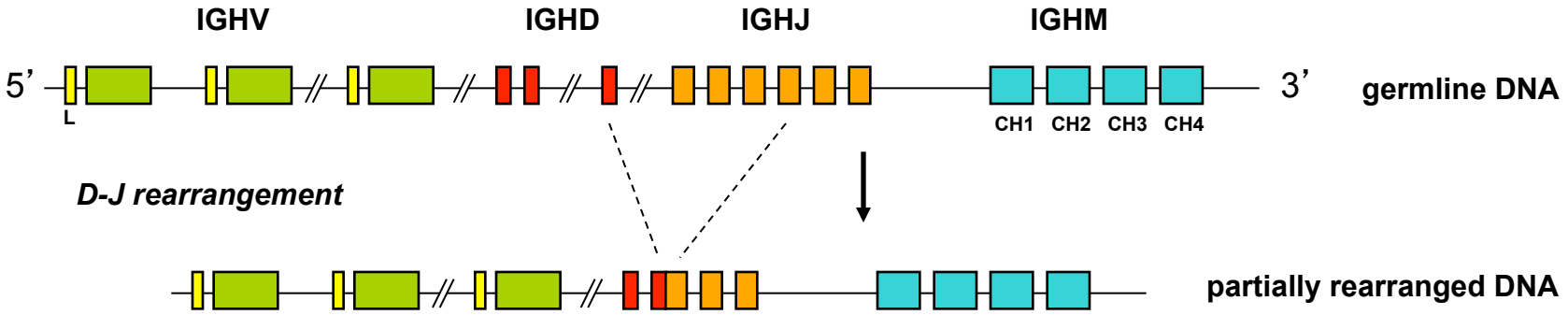


	V	V subgroups	D	J	C
F	55	7	23	6	9
ORF	7		4		
P	46			3	2
Total	108	7	27	9	11



IG V(D)J recombination

IG genes = a unique set of genes



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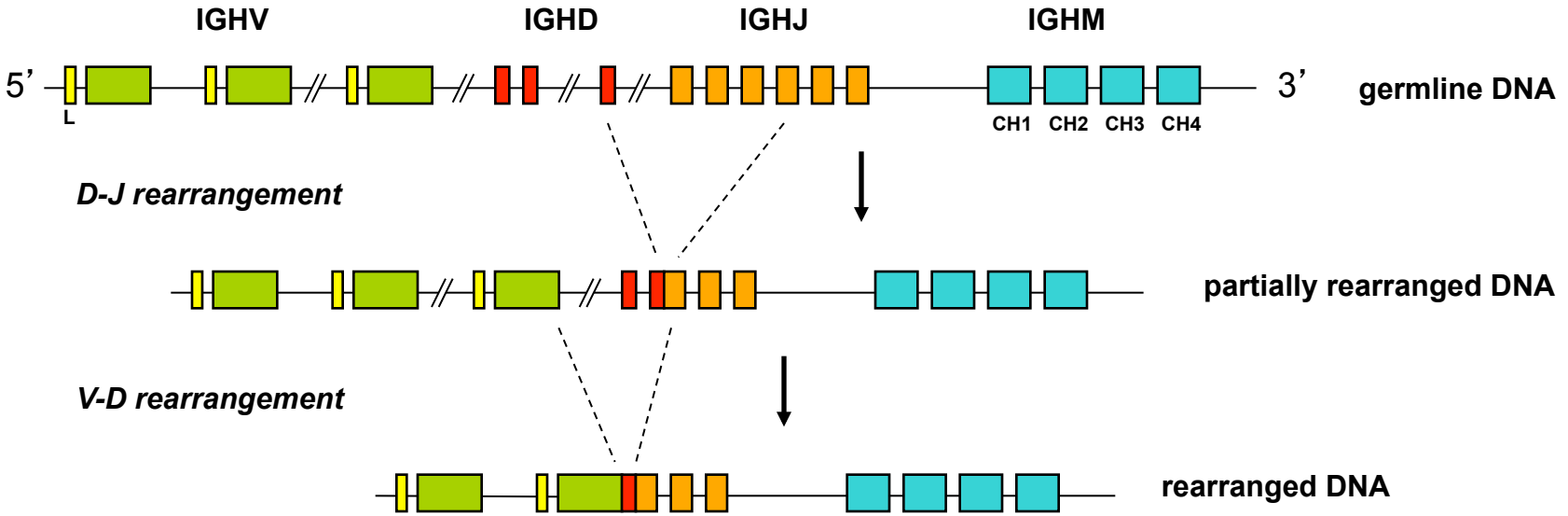

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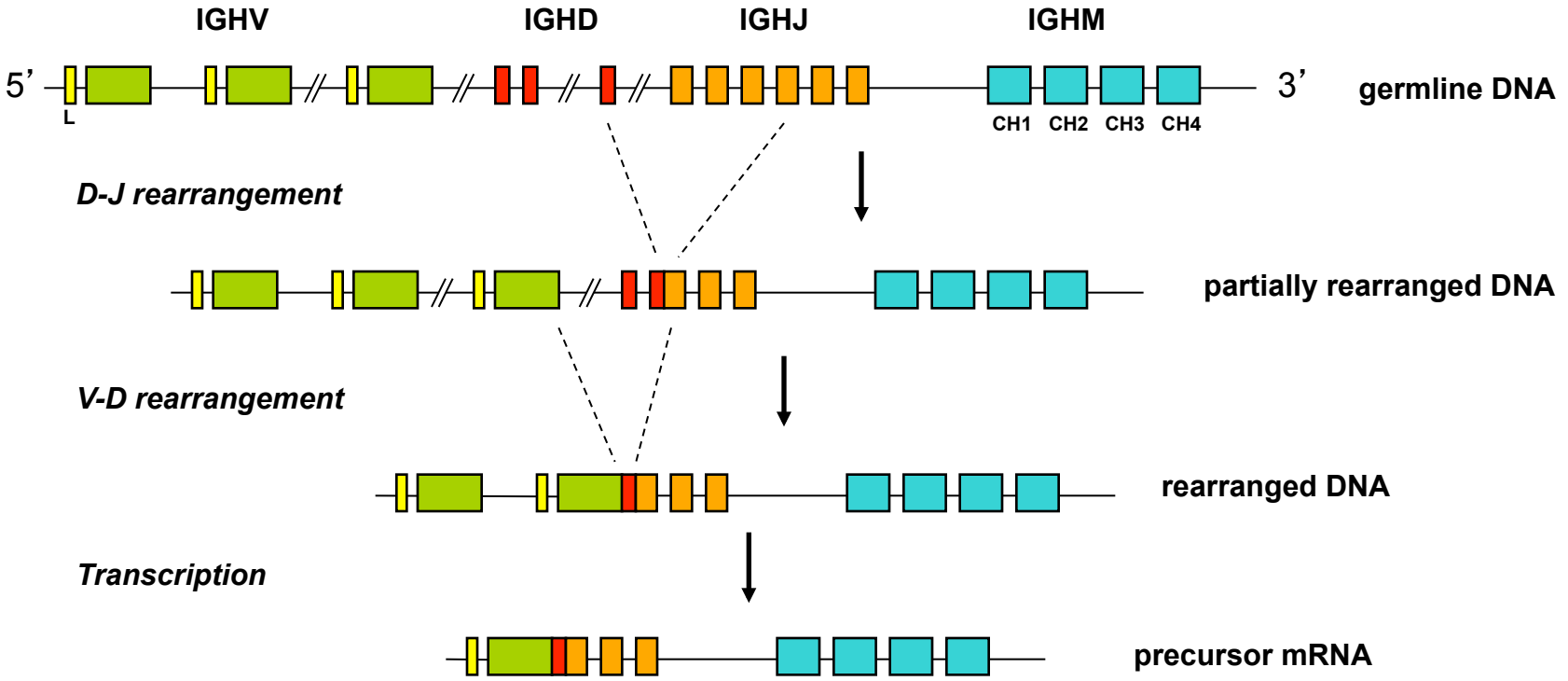

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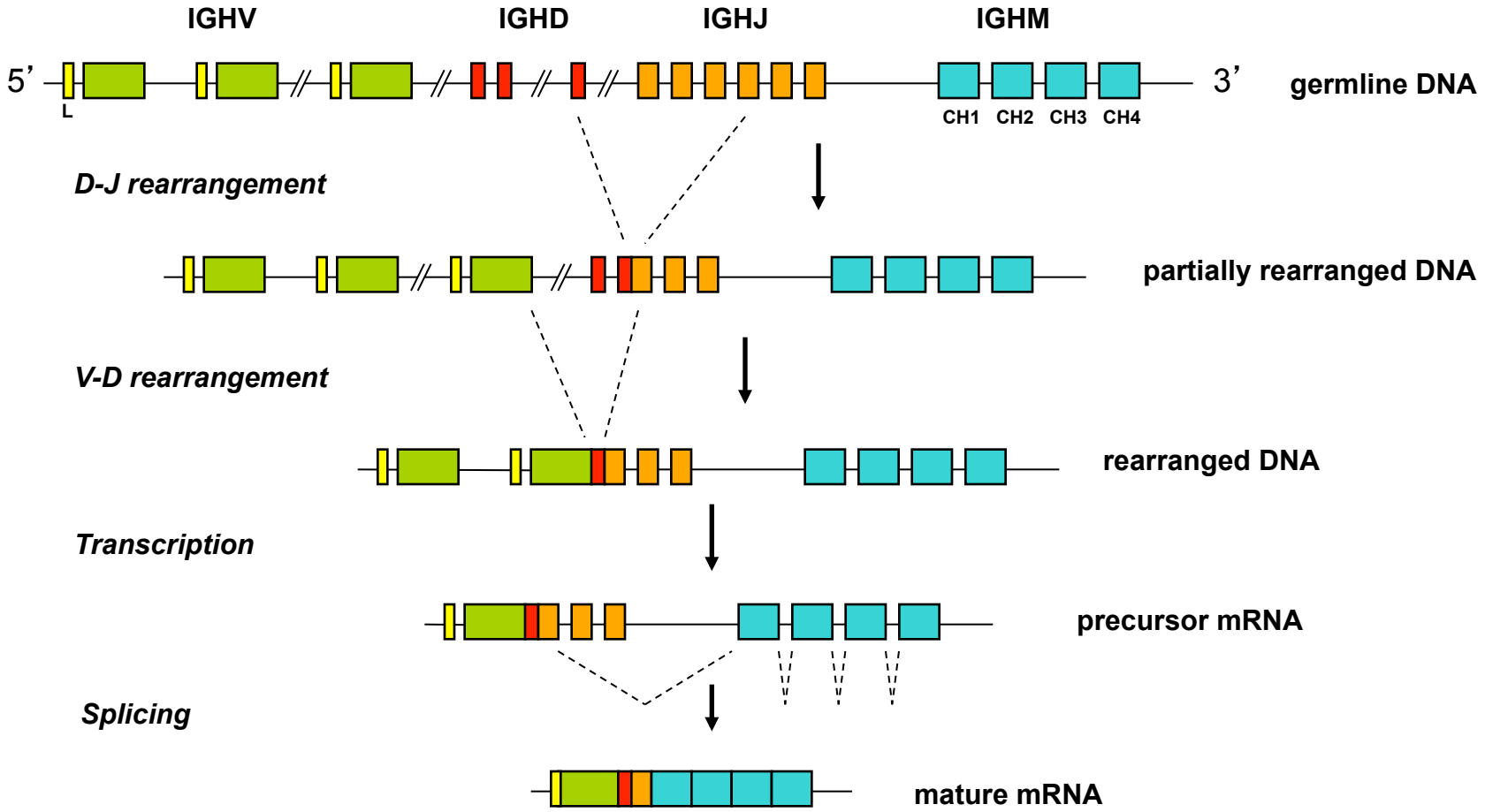

IG V(D)J recombination

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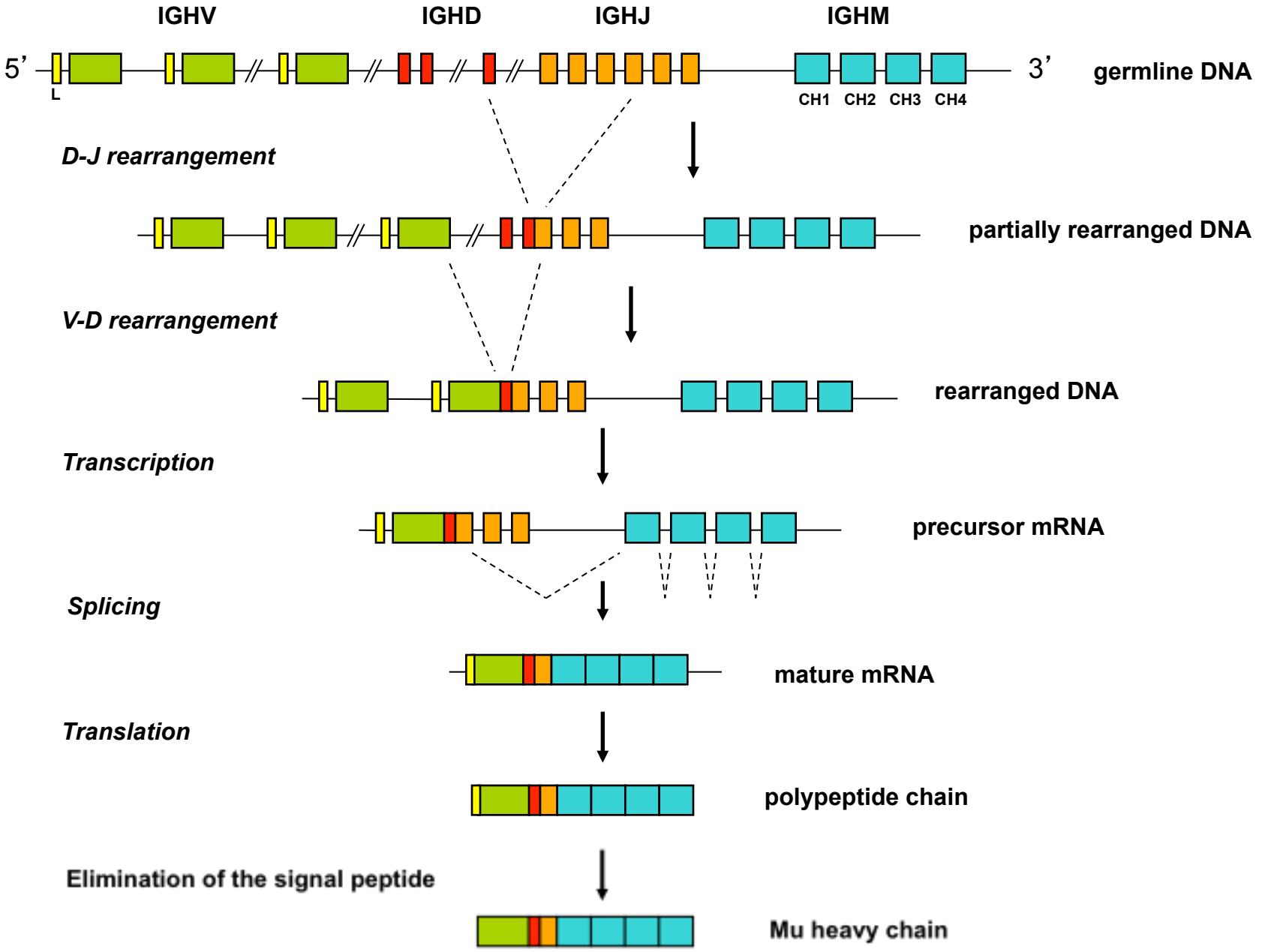
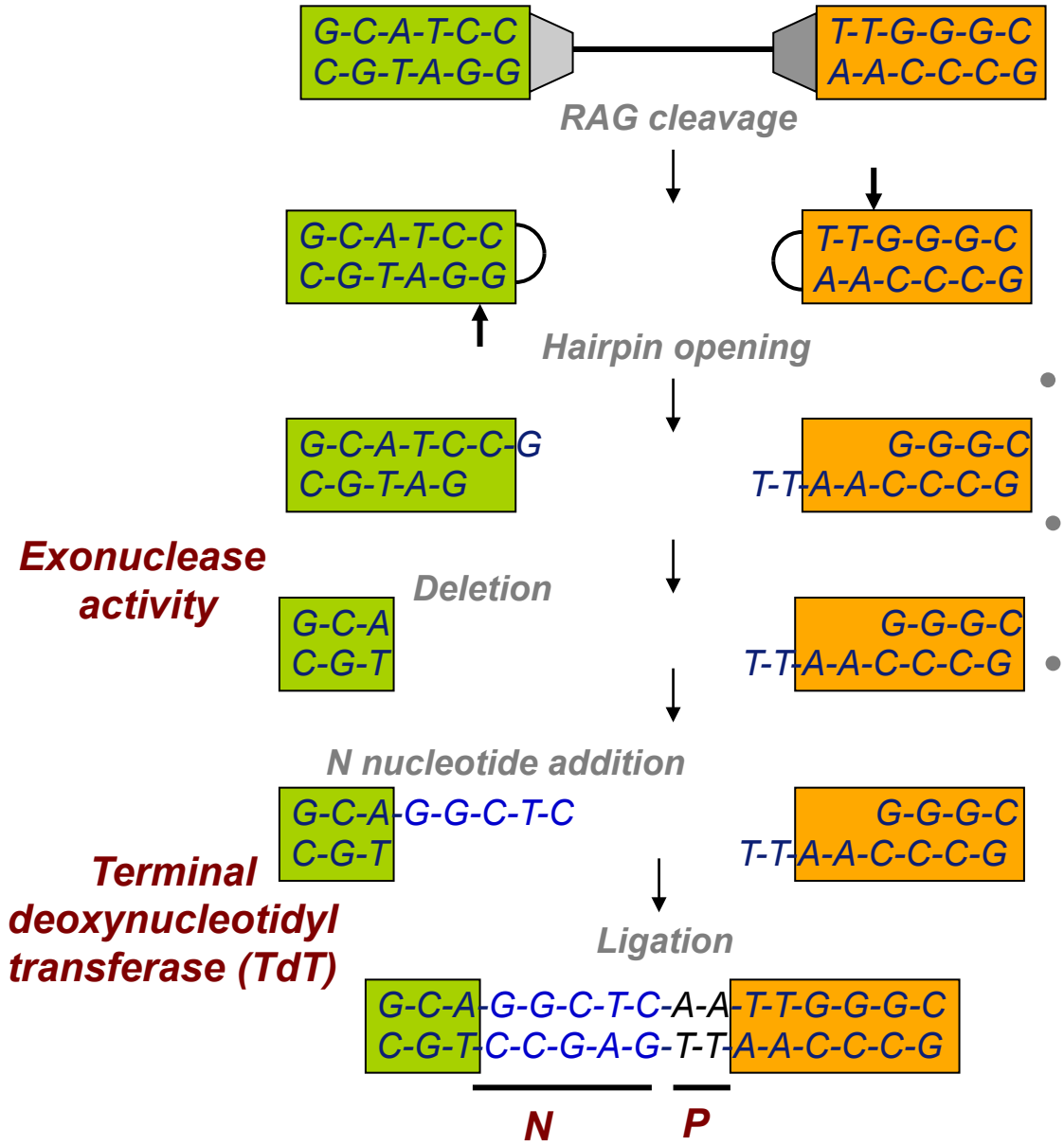

IGHV
IGHD
IGHJ
IGHM
5'
3'
L
CH1
CH2
CH3
CH4
germline DNA
D-J rearrangement
partially rearranged DNA
V-D rearrangement
rearranged DNA
Transcription
precursor mRNA
Splicing
mature mRNA
Translation
polypeptide chain
Elimination of the signal peptide
Mu heavy chain

Junctional diversity:
created by processing of the coding ends

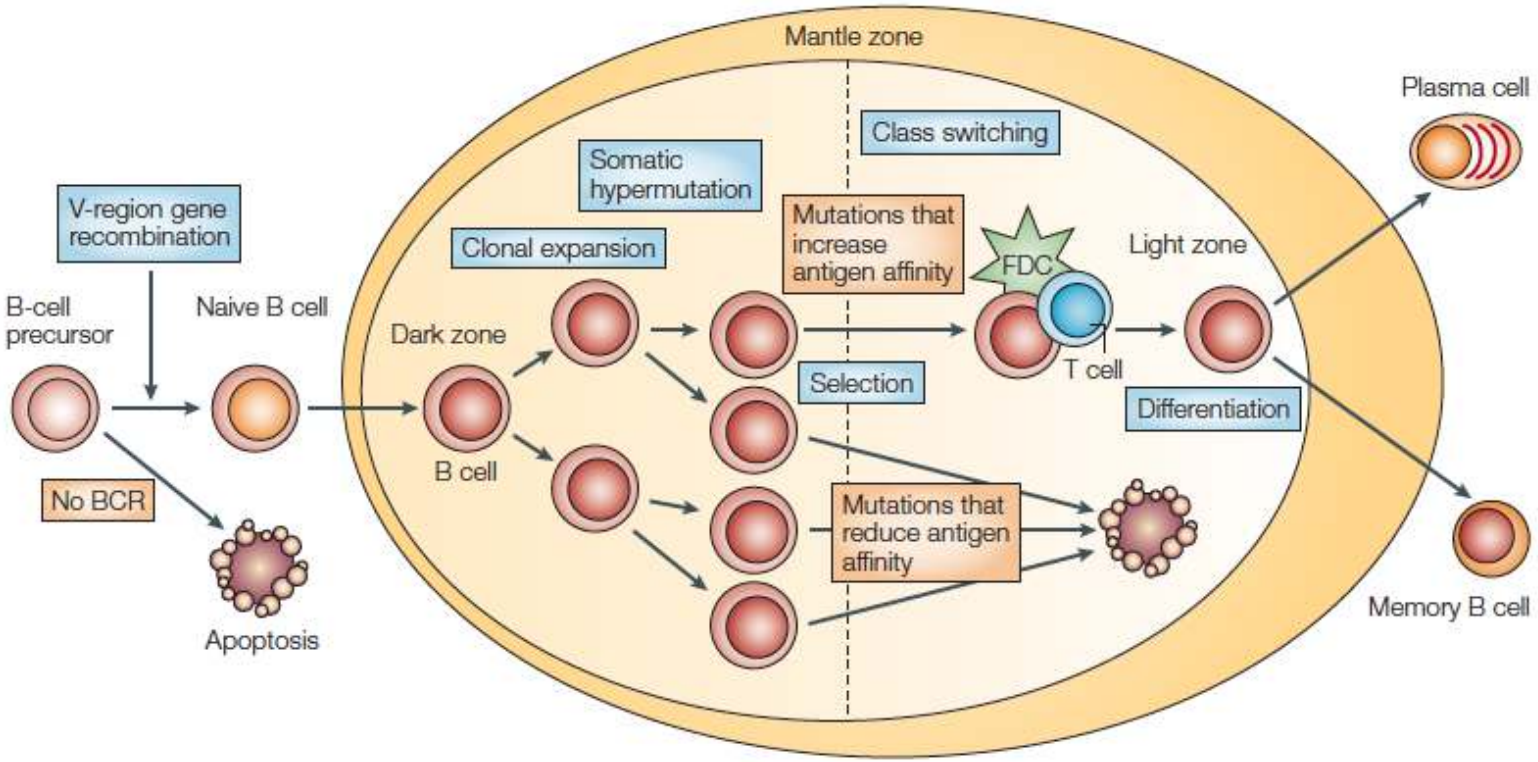


Modification due to:

- **Nucleotide trimming**
- **Palindromic nucleotides**
- **nontemplated nucleotides**

From the bone marrow to the periphery

Antigen exposure further shapes the repertoire



Kuppers, Nat Rev Cancer, 2005

Diversification in the mature B cell repertoire

Several modes of modification

1. ***Somatic hypermutation (SHM)***
   - *V region*
     - ***Mostly single base substitutions***
     - ***Exceptionally high mutational frequency: 10^{-3} to 10^{-4}/base/cell division***
     - ***Localized : start near the 5' end of the V-D-J-EXON and extend for 1-2 kb***
     - ***Preferred target hotspot motifs***
     - ***Transitions more frequent than transversions***
     - ***Silent (S) or replacement (R) or stop codon***
2. ***Class switch recombination (CSR)***
   - *C region*
3. ***Switch from mIGs to secreted Igs***

Diversification in the mature B cell repertoire

Several modes of modification

Insertions/Deletions

Rare events (3%) Subset #2 (25%)

Productive? If reading frame is maintained

- ***Mostly single base substitutions***

- *Exceptionally high mutational frequency: 10^{-3} to 10^{-4}/base/cell division*
- *Localized : start near the 5' end of the V-D-J-EXON and extend for 1-2 kb*
- *Preferred target hotspot motifs*
- *Transitions more frequent than transversions*
- *Silent (S) or replacement (R) or stop codon*

Considerations for protocol optimization

IGHV mutational analysis - ***optimization at two levels***

- technical protocols - generate a ***reliable* IGH sequence**

  - avoid missing sequences
  - avoid incorrect sequence data

- clinical interpretation - generate a ***reliable* report**

  - correct interpretation/implications

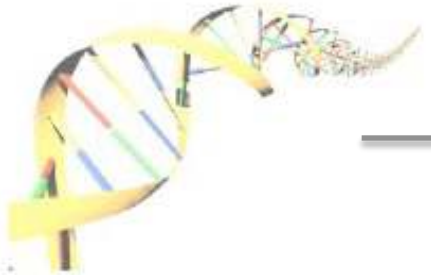
From the patient to an IG sequence:

important parameters

Material:

- Cell source
- Anticoagulant
- Work-up of cells

Type of nucleic acid

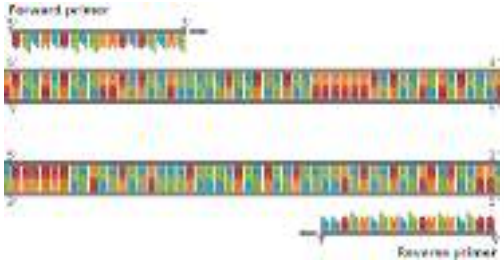


PCR methodology

- PCR protocol
- *Taq* polymerase
- PCR primers

Processing & clonality

Sequencing



IG gene analysis in CLL

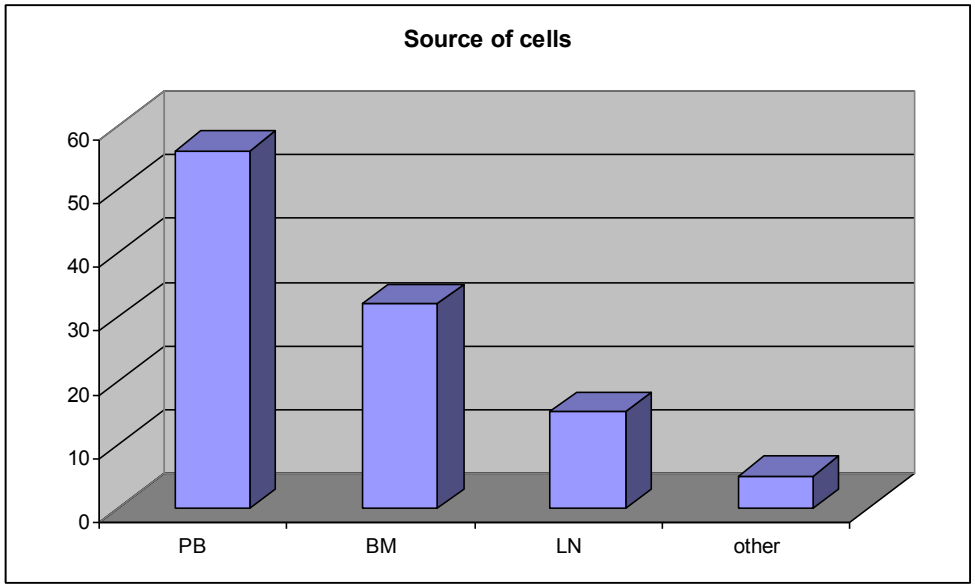
Material: *Cell source*

- **peripheral blood**
- **bone marrow**
- **lymph node**
- **other**

IGHV SHM status

- stable feature irrespective of the leukemic cell source
- stable throughout the disease course

Survey from previous workshop (n=63)

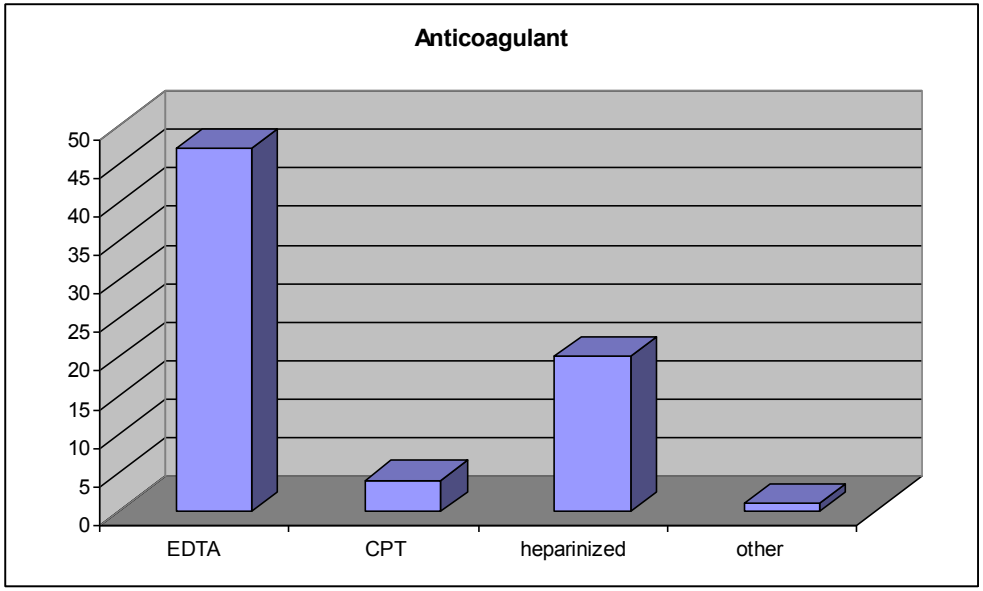


IG gene analysis in CLL

Material: *Anticoagulant*

- **EDTA tubes (Ethylene diamine tetra-acetic acid)**
- **CPT tubes (Citrate/pyridoxal 5'-phosphate/tris)**
- heparinized tubes
- other

Survey from previous workshop (n=63)



IG gene analysis in CLL

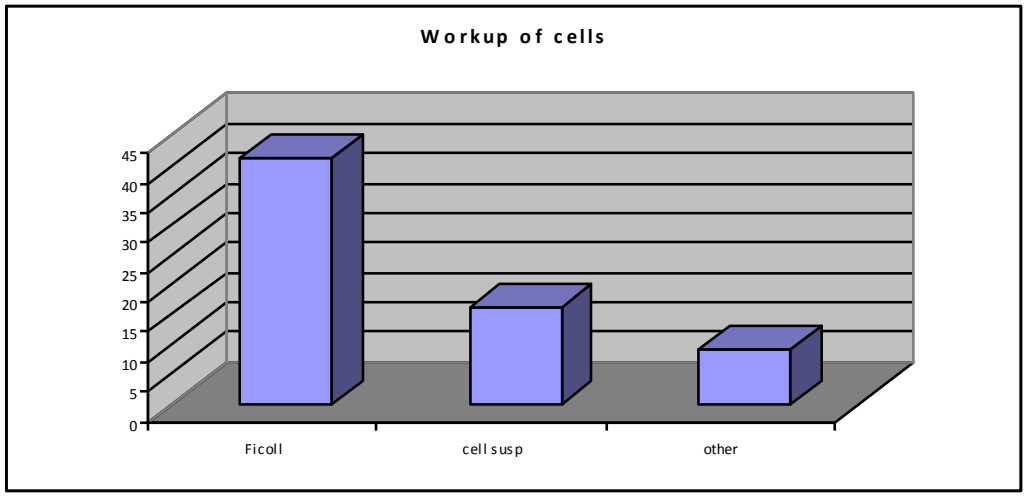
Material: *Work-up of cells*

- **Ficoll gradient (PB / BM)**
- cell suspension of biopsy (e.g. for flow analysis)

other

FFPE (2), whole blood (4), red cell lysis (2), osmotic analysis (1)

Survey from previous workshop (n=63)

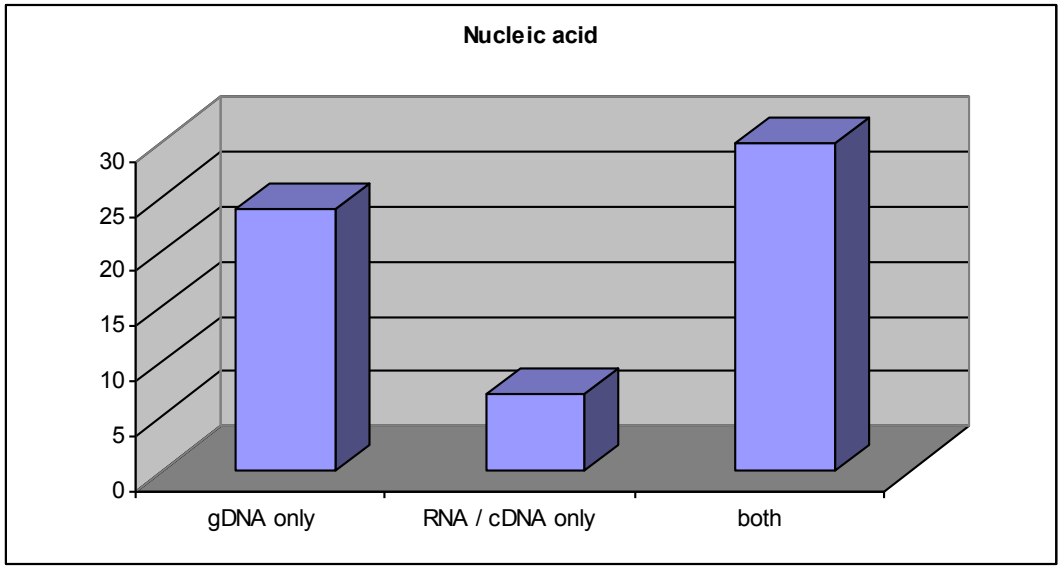


IG gene analysis in CLL

Type of Nucleic acid

- genomic DNA (gDNA)
- RNA / complementary DNA (cDNA)

Survey from previous workshop (n=63)



Quantity of gDNA / RNA

-mostly 100-500 ng gDNA (range 1 ng – 1 ug)

-mostly 1 ug RNA for cDNA reaction (range 400 ng – 2 ug)

IG gene analysis in CLL:

Type of Nucleic acid

Molecule	*advantages*	*disadvantages*
gDNA	- more optimal for long-distance transport - use of archival material	- non-productive rearrangement can also be amplified
RNA/cDNA	- identifies mostly only productive rearrangement - allows isotype identification	- reverse transcription step required

→ no scientific rationale for choosing gDNA or RNA/cDNA

→ advisable to use similar type of nucleic acid in multi-center trials

IG gene analysis in CLL:

Type of Nucleic acid

Table 1 Problematic cases detected in the present multicenter cohort

Category	Number
Unproductive rearrangements	377/4154
cDNA	26/1628
gDNA	351/2526
Single unproductive rearrangements	27/4154
cDNA	7/1628
gDNA	20/2526
Double rearrangements	435/4154
cDNA	61/1628
gDNA	374/2526
Productive+unproductive	350/435
cDNA	19/350
gDNA	331/350
Similar mutational status	326/350
cDNA	18/326
gDNA	308/326
Unmutated productive+mutated unproductive	17/350
cDNA	1/17
gDNA	16/17
Mutated productive+unmutated unproductive	7/350
cDNA	0/7
gDNA	7/7
Double productive	85/435
cDNA	32/85
gDNA	53/85
Similar mutational status	56/85
cDNA	18/56
gDNA	38/56
Discordant mutational status	29/85
cDNA	14/29
gDNA	15/29
Single rearrangement missing 2nd-CYS 104 or J-TRP 118	18/4154
cDNA	9/1628
gDNA	9/2526

Abbreviations: cDNA, complementary DNA; gDNA, genomic DNA.

Unproductive rearrangements are not restricted to gDNA!

9,1% cases carried unproductive rearrangements

RNA/cDNA: 1,6%

gDNA: 13,9%

RNA/cDNA: 0,4%

gDNA: 0,8%

Langerak et al. Leukemia 2011

IG gene analysis in CLL:

Type of Nucleic acid

Table 1 Problematic cases detected in the present multicenter cohort

Category	Number
Unproductive rearrangements	377/4154
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gDNA	331/350
Similar mutational status	326/350
cDNA	18/326
gDNA	308/326
Unmutated productive+mutated unproductive	17/350
cDNA	1/17
gDNA	16/17
Mutated productive+unmutated unproductive	7/350
cDNA	0/7
gDNA	7/7
Double productive	85/435
cDNA	32/85
gDNA	53/85
Similar mutational status	56/85
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Single rearrangement missing 2nd-CYS 104 or J-TRP 118	18/4154
cDNA	9/1628
gDNA	9/2526

Abbreviations: cDNA, complementary DNA; gDNA, genomic DNA.

Double rearrangements are not restricted to gDNA!

RNA/cDNA: 3,8%

gDNA: 14,8%

RNA/cDNA: ≈ 1/3

gDNA: ≈ 2/3

Langerak et al. Leukemia 2011

IG gene analysis in CLL

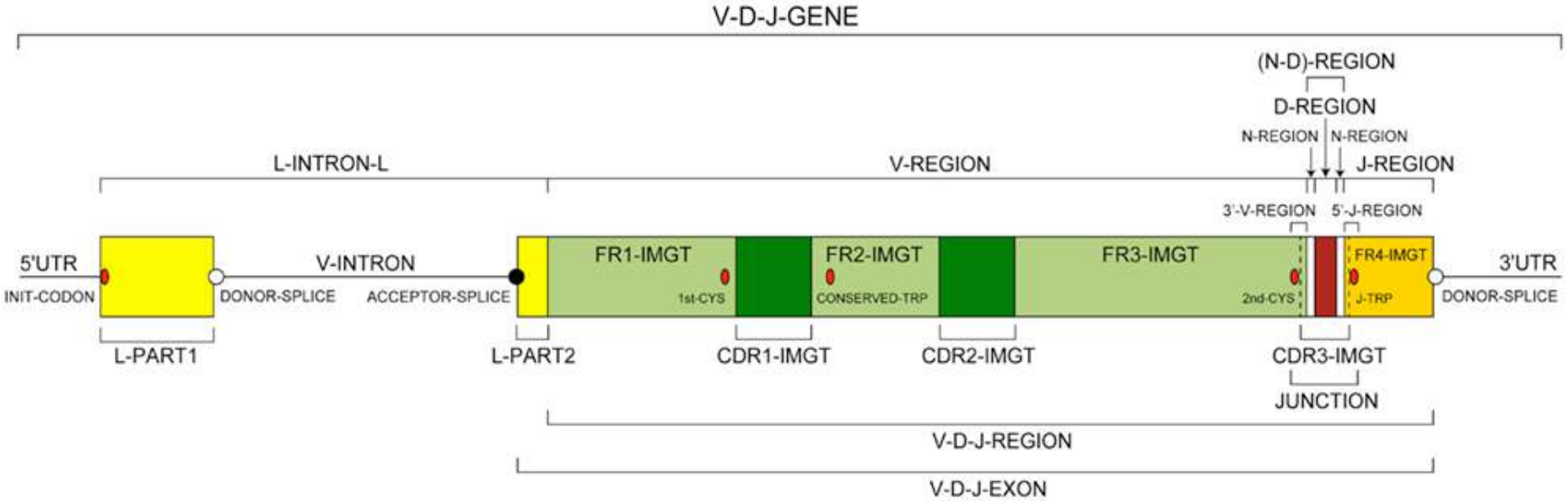
PCR methodology: *PCR primers*

- **IGHV leader primers**
  - LH (family-specific) – CH (*Sahota, Blood 1996*)
  - LH & VH (family-specific) – CH (*Fais, J Clin Invest 1998*)
- **IGHV FR1 primers**
  - FR1 consensus – JH (*Aubin, Leukemia 1995*)
  - FR1 multiplex – JH (BIOMED-2) (*Van Dongen, Leukemia 2003*)
- **IGHV FR2 primers**: short IGHV
- **IGHV FR3 primers**: too short IGHV sequences

- **Downstream primers:** IGHJ or isotype specific IGHC

IG gene analysis in CLL

PCR methodology: ***PCR primers***



IMGT®, http://imgt.cines.fr

IG gene analysis in CLL

PCR methodology: *PCR primers*

Primer sets

- **IGHV leader primers** → **ERIC 2017 UPDATED *RECOMMENDATIONS***
  - LH (family-specific) – CH (*Sahota, Blood 1996*)
  - LH & VH (family-specific) – CH (*Fais, J Clin Invest 1998*)
- **IGHV FR1 primers** → ***only in RARE CIRCUMSTANCES***
  - FR1 consensus – JH (*Aubin, Leukemia 1995*)
  - FR1 multiplex – JH (BIOMED-2) (*Van Dongen, Leukemia 2003*)
- **IGHV FR2 primers** : short IGHV sequences → ***NOT ACCEPTABLE***

  (only recommended upon negative leader / FR1 results, due to SHM)
- **IGHV FR3 primers** : too short IGHV sequences → ***NOT ACCEPTABLE***

IG gene analysis in CLL

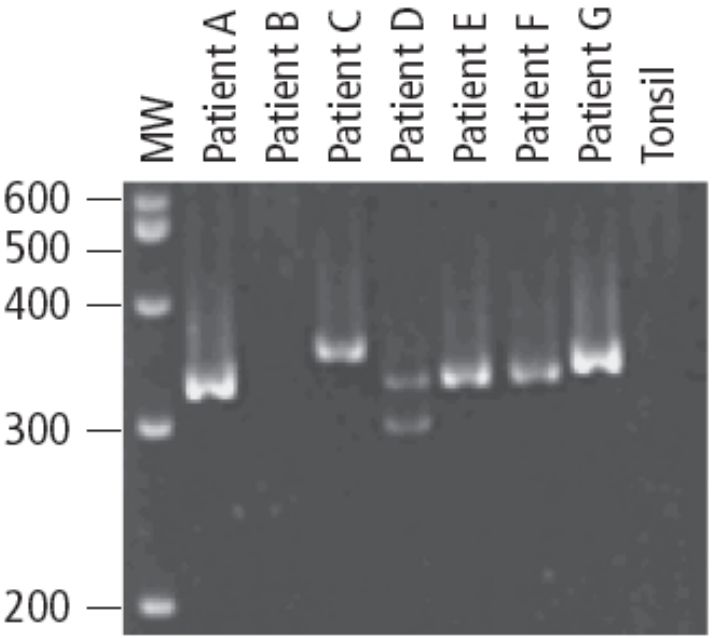
Clonality Testing

Preferred methods for clonality testing

- **heteroduplex analysis**
- **high resolution PAGE**
- **Gene Scan / fragment analysis**
- agarose gel electrophoresis : too low resolution → ***DISCOURAGED***

Strategy	*advantage*	*disadvantage*
HD analysis / PAGE	-unlabeled products allows direct sequencing	-lower detection limit
GS analysis	-higher detection limit -optimal visualization	-labeled products less optimal in sequencing

Heteroduplex analysis in clonality testing:
interpretation and sequencing strategy



Patients A, C, E, F, G

monoallelic

→ direct sequencing

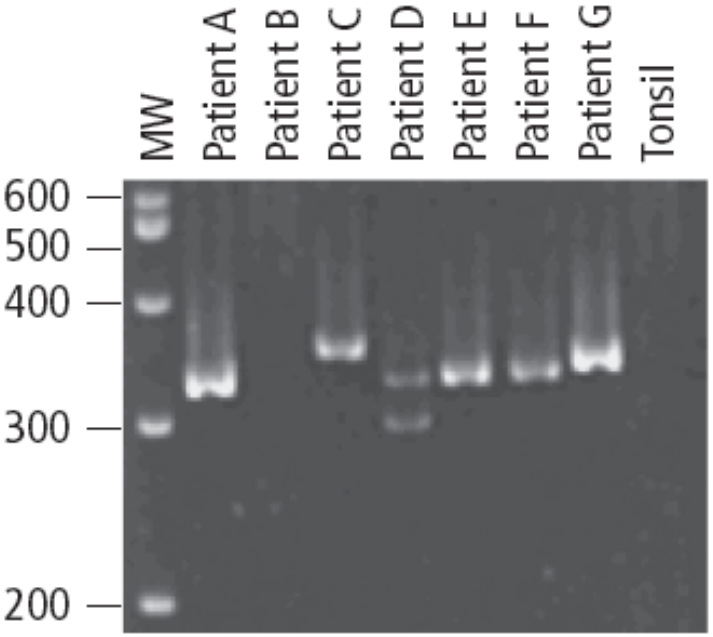
Patient D

bi-allelic

What is the next step ?

- direct sequencing
- single PCR → sequencing
- gel excision → sequencing
- cloning → sequencing

Heteroduplex analysis in clonality testing: *interpretation and sequencing strategy*



Patients A, C, E, F, G

monoallelic

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Patient D

bi-allelic

What is the next step ?

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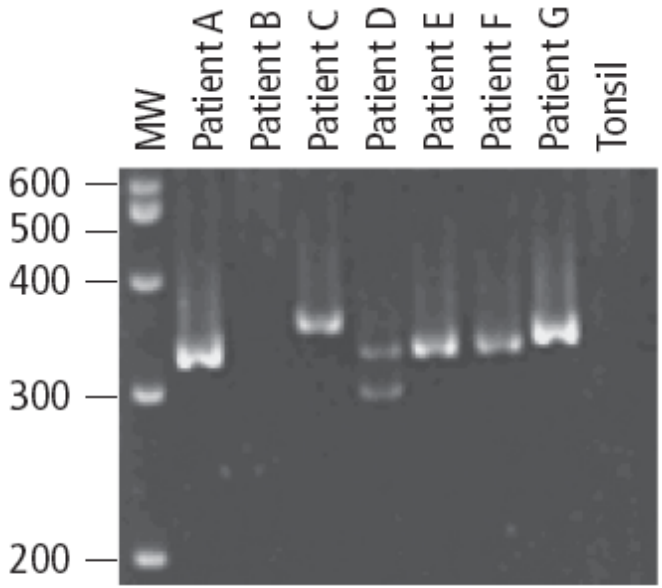
- **gel excision → sequencing**
- cloning → sequencing

Heteroduplex analysis in clonality testing:
interpretation and sequencing strategy



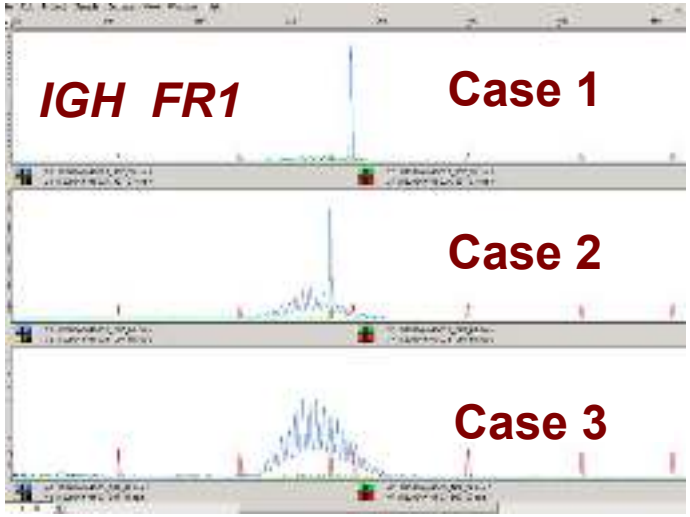
Patient B: What are the possible scenarios?

a. Small clone below detection limit → follow-up sample

b. Clone is present, but not recognized → different primer set required

c. Lymphocytosis not due to CLL clone → check immunophenotype

Genescan analysis in clonality testing:
interpretation and sequencing strategy



Case 1: monoallelic → direct sequencing in principle possible

Case 2: monoallelic + background → 1. direct sequencing ? ; 2. gel excision

Case 3: polyclonal → 1. no clone (no CLL ?); 2. small clone below detection (follow-up sample ?)

IG gene analysis in CLL:

Sequencing strategies

Direct sequencing

- one product from multiplex PCR

→starting from IGHJ / IGHC, then reverse sequence via IGHV family primer

- one product from single PCR

→ sequencing via specific primers from both sides

Sequencing after gel excision + elution

- bi-allelic rearrangement : physical separation of products

Sequencing after subcloning

- final option, e.g. physical separation of bi-allelic rearrangements impossible

IG gene analysis in CLL

ERIC recommendations

Most important and relevant parameters	
• Type of nucleic acid	RNA / cDNA and/or gDNA
• PCR primers	Leader primers
• Clonality analysis	PAGE / HD analysis or GeneScan analysis
• Sequencing	mostly direct (w or w/o gel excision)
	reliable sequence from two strands

→ **Many parameters: no clear scientific rationale for one or the other option**

→ **Some strategies show complementary value**

→ **Clonality testing is an essential phase in the strategy !**

ERIC
european research initiative on CLL

ABOUT US PROJECTS RECOMMENDATIONS DIAGNOSTICS PUBLICATIONS MEETINGS SURVEYS SERVICES

ERIC recommendations

PROGNOSTICATION - IMMUNOGLOBULIN GENE

ERIC recommendations for IGHV gene mutational analysis in CLL (Ghia, Leukemia 2007)

READ

PROGNOSTICATION - IMMUNOGLOBULIN GENE ANALYSIS

ERIC recommendations for IGHV gene mutational analysis in CLL for problematic cases (Langerak, Leukemia 2011)

READ

PROGNOSTICATION - IMMUNOGLOBULIN GENE ANALYSIS

Immunoglobulin gene sequence analysis in chronic lymphocytic leukemia: Updated ERIC recommendations. (Rosenquist, Leukemia 2017)

READ

TP53 ABERRATIONS

ERIC recommendations on TP53 mutation analysis in Chronic Lymphocytic Leukemia (Malcikova, Leukemia 2012)

READ

http://www.ericll.org/eric-recommendations/

ERIC
european research initiative on CLL

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IGHV Gene Analysis Certification

BACKGROUND

CLL cells are endowed with a plethora of communication systems facilitating microenvironmental interactions

READ MORE

AIMS OF THE NETWORK

ERIC aims to promote and/or advance the determination of IGHV gene mutational status in CLL for diagnostic and

READ MORE

STRUCTURE OF THE NETWORK

The IG Network consists of a total of 7 Reference Centres and 7 Certifying Centres.

READ MORE

CERTIFIED CENTRES

ERIC is proud to announce that it currently has 62 certified centres in over 25 countries!

CERTIFICATION ROUNDS

2 certification rounds have been completed. Certification rounds are held just twice a year

PARTICIPATION FORM

Please complete the Participation Form which remains active for the whole year by clicking on read more

http://www.ericll.org/ighv-gene-analysis-certification/

COLLECTION OF PROBLEMATIC CASES AND DATABASE CREATION

'Problematic cases' exist that defy a clear-cut classification and include those showing any of the following features

- Double in-frame rearrangements
- Double in-frame rearrangements with discordant mutation status
- Unmutated in-frame rearrangement coexisting with a mutated out-of-frame rearrangement
- Unmutated heavy chain rearrangement associated with a mutated light chain rearrangement
- Single transcribed out-of-frame rearrangements
- Single transcribed rearrangements carrying stop codons
- Rearrangements carrying any kind of deletions or insertions/duplications
- In-frame rearrangements carrying a mutation either at IMGT codon C-104 (end of HFR3) or at W-118 (start of HFR4)
- Rearrangements without a clearly defined junction

Though these cases are probably known to anyone working in IGHV analysis in CLL, they are limited in frequency, hampering a meaningful analysis at a single-institution level. Therefore, we propose to collect in a dedicated database all problematic cases in order to reach a significant number of them. Everyone in the CLL community is welcome to participate to this joint effort: to submit problematic IGHV sequences and participate to the project please go to the « submission form

COORDINATION

- Lesley Ann Sutton – lesley.sutton@gp.gu.se
- Anastasia Hadzidimitriou – anastasiaxtz@gmail.com

USEFUL LINKS FOR IMMUNOGLOBULIN GENES ANALYSIS

http://www.ericll.org/tools/

ERIC

european research initiative on CLL

the IgCLL group

EuroClonality

a division of ESLHO

EDGE MEDGEN TWINNING

'This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 692298.'