Technical & Report Guidelines

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

Abbreviations: EDTA: ethylenediaminetetraacetic acid; CPT: citrate/pyridoxal 5'-phosphate/Tris; gDNA: genomic DNA; cDNA: complementary DNA; PAGE: polyacrylamide gel electrophoresis.

Item	Recommendations	Remarks
Material		
Anticoagulants	EDTA (or CPT)	
Cells / Tissue	Blood, bone marrow, tissue biopsy	Purification of B cells usually not necessary unless low number of leukemic cells.
Nucleic acid	gDNA or cDNA	cDNA useful when mutations on the IGHJ gene impair amplification.
Production of template for sequencing		
Primers [a]	5': Leader 3': IGHJ or IGHC	5': VH FR1 primers acceptable only if Leader primers fail; VH FR2 and VH FR3 primers not acceptable. 3': IGHJ or IGHC IGHC primers (on cDNA) useful when mutations on IGHJ gene impair amplification.
Amplification	multiplex PCR	Parallel simplex (for each 5' primer) PCR may be useful when more than one rearrangement is present.

Detection of IGH rearrangement	GeneScan or PAGE electrophoresis	Agarose gel electrophoresis strongly discouraged (lack of resolution).
Next generation sequencing	not necessary	Except in rare circumstances when all other approaches fail (alternative sets of primers, utilization of both gDNA and cDNA, processing of a new sample).
Sequencing		
Methodology	direct, both strands	Sequencing of both strands is mandatory for the generation of a single, high quality IGH gene rearrangement sequence.
Sequence alignment	IMGT/V-QUEST (https://www.imgt.org/IMGT_vquest/input)	Adjustable parameter: (i) search for insertions/deletions.
IGHV identity (%)	automatic or adjusted	Adjusted: use option "search for insertions/deletions" when low % identity.
Assignment to stereotyped subsets #2 and #8	IMGT/V-QUEST (https://www.imgt.org/IMGT_vquest/input) ARResT/AssignSubsets (http://bat.infspire.org/arrest/assignsubsets/)	IMGT/V-QUEST: Advanced functionalities, Clinical application: search for CLL subsets #2 and #8.

[a] Leader primers are the only recommended option. That said, in rare cases when the application of a multiplex PCR with leader primers is unsuccessful VH FR1 primers can be used. The result should only be used to facilitate the application of a new round of PCR using IGHV subgroup-specific leader primers. Only if the result is suboptimal again, the report can be based on the VH FR1 PCR but it should be clearly stated that the use of VH FR1 primers might underestimate the total number of IGHV somatic hypermutations since a part of the VH domain is missing.

2. Reporting IGHV gene somatic hypermutation status in CLL.

Recommendations for the assessment of the somatic hypermutation status of the IGHV gene in clonotypic IGHV-IGHD-IGHJ gene rearrangements for standard (A) and difficult (B) cases in CLL.

CLL chronic lymphocytic leukemia, *IG* immunoglobulin, *M-CLL* mutated CLL, *U-CLL* unmutated CLL.

A. STANDARD CASES	
Item	**Recommendations**
1. Methodology	Report type of: primers, PCR product analysis, sequencing method, bioinformatics tools for SHM status assessment, and stereotypy analysis.
2. IGH gene and allele identification	IGHV, IGHD[b], IGHJ genes and alleles.
3. Functionality	SHM status determined only for productive rearrangements; if the rearrangement is unproductive, mention reasons for that (e.g., IG pseudogene, out-of-frame junction, stop codon, large indel).
4. IGHV gene: % of nucleotide identity to the germline to 2 decimal points as reported by IMGT	Classification: U-CLL ≥ 98%; M-CLL < 98%; borderline CLL when 97-97.99%.
5. Subset identification/BcR IG stereotypy	For subsets with well-established prognostic value (currently, subsets #2 and #8).

[b] In a percentage of cases, IGHD identification may be difficult due to: (i) excessive exonuclease trimming of the IGHD gene; and/or (ii) SHM within the VH CDR3, hindering the assignment to the closest germline IGHD gene and allele.

B. CHALLENGING CASES	
Item	**Recommendations**
1. Single unproductive rearrangement	Repeat the PCR with alternative primer sets and using cDNA. Perform NGS to get more detailed information regarding the clonal architecture. SHM status disclosed as not determined only in case all different approaches fail.
2. Double rearrangements	
2.1 One productive and one non-productive	Same as for standard cases: mutational status defined by the productive rearrangement, irrespective of the SHM status of the unproductive rearrangement.
2.2 Double productive	
2.2.1 Concordant SHM status	Same as for standard cases i.e., consider as M-CLL or U-CLL, according to the SHM status.
2.2.2 Discordant SHM status	Check immunophenotype for the presence of 2 clonal populations. Recommend to the physician that it is safer to consider as U-CLL; close follow-up.
3. Multiple (>2) productive rearrangements	Check immunophenotype for the presence of 2 or more clonal populations. Perform NGS to assess the relative frequency of each clonotype and consider the predominant clonotype, if it is clearly identified (NOTE: specific guidelines are still to be provided/developed here).
4. Missing anchors (C104/W118)	Mutational status assessment is possible if evidence for IG expression on leukemic cells and/or preserved G-X-G motif within the VH FR4.