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# AN ERIC PRACTICAL GUIDE FOR IMMUNOGLOBULIN GENE SEQUENCE ANALYSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA: FROM PATIENT MATERIAL TO SEQUENCE INTERPRETATION

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**TITLE:**

# Immunoglobulin Gene Sequence Analysis In Chronic Lymphocytic Leukemia: From Patient Material To Sequence Interpretation

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**KEYWORDS**

Chronic lymphocytic leukemia (CLL), somatic hypermutation (SHM), immunoglobulin, immunoglobulin heavy variable (IGHV), complementarity determining region 3 (CDR3), International ImMunoGeneTics information system (IMGT)

**SUMMARY**

Herein, we present a protocol that details the technical aspects and essential requirements to ensure robust IG gene sequence analysis in patients with chronic lymphocytic leukemia (CLL), based on the accumulated experience of the European Research initiative on CLL (ERIC).


## ABSTRACT

During B cell maturation, the complex process of immunoglobulin (IG) gene V(D)J recombination coupled with somatic hypermutation (SHM) gives rise to a unique DNA sequence within each individual B cell. Since B cell malignancies result from the clonal expansion of a single cell, IG genes represent a unique molecular signature common to all the malignant cells within an individual patient; thus, IG gene rearrangements can be used as clonal markers. In addition to serving as an important clonal identifier, the IG gene sequence can act as a 'molecular timeline' since it is associated with specific developmental stages and hence reflects the history of the B cell involved in the neoplastic transformation. Moreover, for certain malignancies, in particular chronic lymphocytic leukemia (CLL), the IG gene sequence holds prognostic and potentially predictive capabilities. That said, extrapolating meaningful conclusions from IG gene sequence analysis would be impossible if robust methods and tools were not available to aid in their analysis. This article, drawing on the vast experience of the European Research Initiative on CLL (ERIC), details the technical aspects and essential requirements necessary to ensure reliable and reproducible IG gene sequence analysis in CLL, a test that is now recommended for all CLL patients prior to treatment. More specifically, the various analytical stages are described ranging from the identification of the clonotypic IG gene rearrangement and the determination of the nucleotide sequence to the accurate clinical interpretation of the IG gene sequence data.


## INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common form of leukemia in adults in western countries, is characterized by the clonal expansion of mature neoplastic B cells[1]. From a clinical perspective, the disease course is extremely variable with some patients experiencing an aggressive disease, requiring treatment very early after diagnosis, and often relapsing or being refractory to therapy. This is in stark contrast to a substantial proportion of patients (~30%) who present with an indolent disease, never require treatment, and have a life expectancy similar to healthy age-matched individuals[2]. This clinical heterogeneity is reflected in the diversity of the molecular abnormalities found in CLL patients which ultimately drive the pathogenesis and progression of this disease[3].

Establishing an accurate CLL diagnosis is usually straightforward, however, the aforementioned clinical heterogeneity may hinder the effective management of CLL patients and underscores the need for prognostic and predictive markers that could assist in treatment decision-making[2]. Numerous studies have attempted to refine the prognostication of CLL, culminating in an abundance of novel clinical and biological markers being proposed[4]. The mutational status of the clonotypic immunoglobulin heavy variable (IGHV) gene is one of the most robust prognostic markers in CLL, largely due to the fact that (i) it remains stable over time and as the disease progresses and (ii) it is independent of other clinical and biological parameters[5]. That

notwithstanding, very few, if any markers, including IGHV mutational status, are currently applied in clinical routine at the time of diagnosis.

Initial reports on the clinical utility of IG gene sequencing in CLL date as far back as 1999, when 2 independent studies reported that patients with no or a minimal somatic hypermutation (SHM) load (unmutated CLL, U-CLL) have a worse prognosis than patients carrying a higher SHM burden (mutated CLL, M-CLL)[6,7]. More specifically, the U-CLL group consisted of cases harboring clonotypic IGHV genes with few or no SHM and hence a high percent identity to the closest germline IGHV gene (≥98%), whereas M-CLL consisted of cases with a higher mutational load (percent identity to the closest germline IGHV gene <98%). Since these early studies, it has been continually demonstrated that U-CLL cases display a shorter time-to-first treatment (TTFT) and overall survival (OS) compared to M-CLL.

In hindsight, these seminal studies highlighted the pivotal role of the B cell receptor (BcR) IG in CLL pathobiology, thus paving the way for extensive research into CLL-microenvironmental interactions which, in turn, led to a more comprehensive appreciation of the biological heterogeneity of this disease[8,9]. More recent studies have provided additional support for the importance of immunogenetic analyses in CLL by revealing that individual cases may cluster in subsets due to sharing (quasi)identical BcR IG gene sequences, a phenomenon termed BcR IG stereotypy[10-13]. Accumulating evidence supports the notion that patients assigned to the same stereotyped subset harbor similar clinicobiological properties that clearly separate them from other CLL patients within the same SHM category but with different IG gene sequences; it has indeed been reported that the categorization of CLL patients based on BcR IG stereotypy further refines the bulk segregation of CLL patients into U-CLL or M-CLL groups[14-20].

From a clinical perspective, it is noteworthy that the SHM status of the clonotypic IGHV gene seems to correlate with a specific response to chemoimmunotherapy. In particular, M-CLL patients treated with a combination of fludarabine/cyclophosphamide/rituximab (FCR), the gold standard treatment for medically fit CLL patients lacking *TP53* gene defects, exhibited significantly longer progression-free survival (PFS) and OS compared to U-CLL patients who received the same treatment[21-23]. Therefore, identification of the SHM status has become important in particular for assisting in the therapeutic management of CLL patients *i.e.,* a predictive marker, rather than for assessing the clinical course of the disease *i.e.,* a prognostic marker. In fact, according to the recent update of the NCI-sponsored guidelines from the International Workshop on CLL, the SHM status of the rearranged IGHV genes should be determined in all CLL cases prior to treatment initiation in both general practice and clinical trials[24]. Moreover, owing to the recent approval of novel treatment agents, and the growing number of drugs in trials, the SHM status of the IG molecule may play an even greater role in the therapeutic management of patients with CLL in the near future[5].

This report details all aspects of IG gene sequence analysis, beginning with choosing the appropriate material and culminating with the clinical interpretation of the sequencing results. In-depth assessment of these steps is important in diagnostic routine for the production of reliable results and to ensure accurate patient stratification within clinical trials. Protocols are

provided to aid in the harmonization of IG gene sequence analysis, thus ensuring that results are comparable amongst different laboratories.

**PROTOCOL**

The research protocol was approved by the Ethics Committee of the San Raffaele Scientific Institute, Milan, Italy.

## 1. Material Selection

### 1.1. Choice of starting material

Note: In most cases of CLL the tumor cell count is high at diagnosis (>80-90%). Thus, cell sorting or enrichment of the leukemic cells is usually not necessary.

1.1.1. If using Peripheral blood (PB), the most common material of choice for IG gene sequence analysis, use a blood volume between 10-20 mL.

1.1.2. Alternatively, in cases with a low CLL cell burden, use cell sorting of PB samples or material from other tissues, like bone marrow (BM) or lymph nodes (LN).

### 1.2. Sampling time

1.2.1. Sampling time is not crucial given that the IG SHM status is stable overtime; that said, avoid sampling at certain periods, such as immediately after or during therapy, since the low number of CLL cells may complicate the analysis and lead to unreliable results.

### 1.3. Material collection and storage

Note: Collection and storage of starting material depends strongly on the material choice.

1.3.1. For PB or BM samples, use EDTA or CPT (citrate-tris-pyridossalphosphate) collection tubes to prevent inhibition of the PCR amplification process; alternatively, use heparin tubes.

1.3.2. For LN samples, collection options are either cell suspensions, biopsies from fresh frozen tissue or, in rare instances formalin-fixed paraffin-embedded (FFPE) tissues.

## 2. Density Gradient Separation

Note: If PB or BM is the starting material of choice, which is the most frequent scenario, mononuclear cell isolation using density gradient separation is recommended.

2.1. Add 200 µL of EDTA (0.5 M EDTA/ pH 8.0) to a 50 mL sterile collection tube. Add 20 mL of PB and 15 mL of RPMI. Mix by pipetting (2-3 times is sufficient).

2.2. Add 15 mL of density gradient medium to a new 50 mL collection tube.

Note: In case the PB volume is less than 20 mL, the volumes of RPMI (previous step) and density gradient should be modified accordingly.

2.3. Slowly layer in the solution from step 2.1 so that it does not disrupt the gradient. Centrifuge at 800 x g for 20 minutes with the centrifuge brake off.

2.4. Collect the mononuclear cell ring that has formed between the upper plasma/platelet layer and the density gradient medium layer using a sterile Pasteur pipette, then transfer to a sterile 15 mL collection tube.

2.5. Add RPMI to a final volume of 12 mL and mix. Centrifuge at 750 x g for 8 minutes. Discard the supernatant.

2.6. Resuspend the cell pellet using 10 mL of RPMI and repeat step 2.5.

2.7. Dissolve the cell pellet in 1 mL of PBS (DPBS, no calcium, no magnesium). Perform a cell count using a Neubauer plate by mixing 20 µL of sample and 80 µL of 1:10 Türk's solution.

2.8. If downstream processing of the sample is not scheduled for the same day, centrifuge the sample at 750 x g for 8 minutes. Discard the supernatant and store the cell pellet at -80$^{o}$ C.

## 3. Nucleic Acid Extraction

3.1. Decide on a substrate for analysis of the SHM status of IG genes. Genomic DNA (gDNA) is the most popular choice as there is no need for a reverse transcription step; alternatively, the use of RNA/complementary DNA (cDNA) ensures that, at least at the transcriptional level, the productive, functional clonotypic IG rearrangement is the “favored” target.

3.2. Use one of numerous commercially available kits suitable for gDNA or total RNA extraction and the synthesis of cDNA (see **Table of Materials**).

3.3. Assess the integrity of the DNA or RNA using sensitive nucleic acid quantification systems. If using FFPE material for the analysis, assess the quality and quantity of the extracted gDNA/cDNA by PCR amplification of a known housekeeping gene, *e.g.* retinoic acid receptor alpha (*RARα*), beta-actin, *etc.*.

## 4. Amplification and Sequencing of IGHV-IGHD-IGHJ Gene Rearrangements

4.1. **IGH PCR primer selection**

4.1.1. Preferably, perform multiplex PCR with IGHV subgroup specific 5' primers and IGHJ gene specific 3' primers[25,26]. If results are sub-optimal, prepare separate PCR mixes using single IGHV subgroup-specific primers.

4.1.2. Use 5' primers that anneal to either the leader region located immediately upstream of the IG rearrangement or within the coding region (*e.g.* framework 1, FR1) of the IGHV gene.

4.1.2.1. Use leader primers that allow for the amplification of the entire rearranged IGHV-IGHD-IGHJ gene sequence, which in turn will enable the accurate estimation of SHM levels. Thus, IGHV leader primers are recommended by ERIC in their updated guidelines for IG gene sequence analysis[27].

4.1.2.2. In instances where leader primers fail to amplify the clonotypic IG rearrangement, use IGHV FR1 primers. However, framework primers do not amplify the entire clonotypic IG gene rearrangement, which may lead to inaccurate determination of the level of SHM. For this scenario, clearly state in the clinical report that the use of IGHV FR1 primers may overestimate the true percent identity to the closest germline gene.

4.1.3. For the 3' end use consensus primers targeting the IGHJ genes, multiplex sets of IGHJ gene-specific primers or isotype-specific primers, depending on the substrate. Primer sequences are provided in **Table 1**, whereas **Figure 1** depicts the various locations for primer annealing.

4.2. **PCR amplification of IGHV-IGHD-IGHJ gene rearrangements**

4.2.1. Prepare the primer mix using equimolar quantities of each subgroup primer(s) to ensure unbiased amplification.

4.2.1.1. For example, when using leader primers, two different primers are used to amplify rearrangements belonging to the IGHV1 subgroup. Thus, use a quantity of these 2 primers (IGHV1L and IGHV1bL) that is half compared to IGHV4L, which is the only primer for IGHV4 subgroup rearrangements.

4.2.1.2. Apply the opposite approach in the case of the IGHJ1-2 and IGHJ4-5 primers. As these primers target two different IGHJ gene subgroups, double the quantity compared to the IGHJ3 and IGHJ6 primers.

4.2.1.3. Use **Table 1** to determine the exact primer quantities when preparing relevant primer mixes.

4.2.2. Mix PCR reagents as listed in **Table 2.** After combining all the PCR reagents in a PCR tube, add 0.5 µL of Taq polymerase and 100 ng of gDNA or 2 µL of cDNA (cDNA should be prepared using 1 µg of RNA).

261 Note: A Taq enzyme with proofreading activity is not essential as the amplification error rate is
262 extremely low and will therefore not affect the level of SHM.
263
264 4.2.3. Run the thermal PCR protocol detailed in **Table 3**.
265
266 4.3. **Clonality assessment**
267
268 Note: A critical next step involves evaluating the clonality pattern of the PCR product. When
269 assessing clonality in CLL patients, the most common finding is a single, monoclonal peak/band.
270
271 4.3.1. Use methods with high-sensitivity, such as fragment or heteroduplex analysis, for clonality
272 assessment[28,29].
273
274 4.3.2. Fragment analysis
275
276 4.3.2.1. Perform multiplex PCRs using 5'-labeled IGHV leader or FR1 subgroup specific PCR
277 primers; this will yield PCR products that can be separated by capillary electrophoresis, thereby
278 permitting clonality assessment of the IGH PCR products based on their IGHV complementarity
279 determining region 3 (CDR3) lengths.
280
281 4.3.2.2. Use primers, reagents and thermal conditions identical to those previously mentioned
282 (see **Tables 1-3**). Custom 5´- labeled primers are fluorescently labeled oligos with a choice of dyes
283 on the 5' end. Examples of reporter dyes include 6-FAM, HEX, NED or TET. Thus, 2 separate
284 reactions are required based on the use of 3 different dyes per reaction.
285
286 4.3.3. Assess the size of the PCR products on a six percent (6%) polyacrylamide gel (this
287 percentage results in optimal resolution), using 1x TBE as a running buffer. Run at 80 V for 45
288 min.
289
290 Note: It is recommended that PCR products are allowed to migrate on the gel for sufficient time
291 so that monoclonal samples can be separated from the polyclonal background and the DNA size
292 marker can separate adequately. Factors such as the size and thickness of the gel can affect
293 migration so no single setting (voltage and time) can guarantee an optimal result That said,
294 setting the voltage at 80 V for 45 minutes is a good starting point as it minimizes the risk of the
295 sample migrating too quickly and 'running' off the gel.
296
297 4.3.4. Check for PCR products of approximately 500 base pairs when using IGHV leader primers
298 and 350 base pairs when using the IGHV FR1 primer mixes.
299
300 Note: In rare instances, CLL cases have been found to carry double, monoclonal products and in
301 even rarer instances, cases may exhibit an oligoclonal pattern. An intercalating agent such as
302 Ethidium bromide (EtBr) or less hazardous and more sensitive commercially available DNA stains
303 can be used for the visualization of DNA on acrylamide gels using UV excitation or blue-light.
304

305 4.4. **PCR product purification**
306
307 Note: PCR products are purified to remove any polyclonal background originating from normal B
308 cells within the CLL sample as well as primer dimers that may lead to suboptimal sequencing. Use
309 any method that removes unincorporated dNTPs and primers for PCR clean-up, such as an
310 enzymatic treatment, *e.g.,* Sap (shrimp alkaline phosphatase)/Exo (Exonuclease I), or column-
311 based purification.
312
313 4.4.1. Load the total volume of the PCR product onto a 3% low-melting agarose gel.
314
315 4.4.2. Allow the PCR products to run on the gel until they separate from the background.
316
317 4.4.3. Excise the sharp, prominent PCR bands, purify and elute. If two rearrangements are
318 detected, both bands should be excised and sequenced seperately.
319
320 4.4.4. To perform Sap/Exo clean-up, follow the manufacturer's instructions regarding the specific
321 volumes to use; however a typical reaction may contain 1 µL Sap, 0. 5 µL Exo and 2 µL 5x
322 incubation buffer per 5-10 µL PCR reaction. Mix by gently vortexing each sample and incubate on
323 a PCR block at 37 °C for 30 minutes. Inactivate the enzymes by heating to 85 °C for 15 minutes.
324
325 4.4.5. Load a small quantity of the PCR products on a 3% agarose gel to assess the purity of the
326 product.
327
328 **5. Sanger Sequencing**
329
330 Note: Several sequencing strategies exist, however, regardless of the exact approach, direct
331 sequencing of both strands is mandatory to ensure a reliable result.
332
333 5.1. **General sequencing protocols**
334
335 5.1.1. If amplification has been performed using single primers, proceed with sequencing using
336 the appropriate IGHV- and IGHJ- or constant region-specific primers. This approach can also be
337 adopted if multiplexed fluorescently labeled primers have been used and the PCR product was
338 assessed using fragment analysis (sequencing primers should be not labeled).
339
340 5.1.2. If multiplexed primers have been used and clonality was assessed on a gel, then use a
341 downstream primer at the first step of sequencing *e.g.* a consensus IGHJ primer with the
342 sequence being 5'-GTGACCAGGGTNCCTTGGCCCCAG-3'. Alternatively, a CH primer can be used if
343 cDNA was used as substrate. Next, use subgroup-specific IGHV leader or FR1 primers for the
344 second sequencing step.
345
346 5.2. **Example sequencing protocol**
347

348 Note: Several sequencing protocols exist and an example protocol is detailed below.
349
350 5.2.1. Dilute 33 ng of the PCR product in a total volume of 11.5 µL using, *e.g.,* sterile water.
351 Denature the PCR product by heating to 95 °C for 5 minutes. Immediately place on ice for 10
352 minutes to prevent formation of secondary structures.
353
354 5.2.2. Add 8 µL of master mix to each tube along with 0.5 µL (corresponding to 5 pmol) of the
355 primer.
356
357 5.2.3. As a control, prepare a tube containing 8 µL of master mix, 0.5 µL pUC18 control template,
358 2 µL (-)47 sequencing primer and 9.5 µL sterile water. The final total volume in each tube should
359 be 20 µL.
360
361 5.2.4. Use thermal cycling conditions for the sequencing reaction as detailed in **Table 4**.
362
363 5.2.5. Prepare the Stop Solution by mixing 2 µL of sodium acetate 3M (pH 5.2), 2 µL EDTA 100
364 Mm (pH 8.0) and 1 µL of glycogen (concentration 20 mg/mL), then add 5 µL to each sample.
365 Transfer the product to a new microcentrifuge tube.
366
367 5.2.6. Add 60 µL of 100% ethanol (-20 °C) and gently mix. Centrifuge at 14,000 rpm for 15 minutes
368 (4 °C). Discard the supernatant.
369
370 5.2.7. Add 100 µL of 70% ethanol (-20 °C) and gently mix. Centrifuge at 14,000 rpm for 10 minutes
371 (4 °C). Discard the supernatant. Repeat once.
372
373 5.2.8. Dry the pellet for 90 minutes at room temperature. Add 40 µL of sodium dodecyl sulfate
374 (SDS) solution to each sample.
375
376 5.2.9. Transfer the sample to a sequencing plate, cover with strip caps or tape seals, load to the
377 machine and perform Sanger sequencing. The sequencing plate is usually a 96-well, v-bottom,
378 full-skirt PCR plate compatible with the sequencer. The plates may be barcoded depending on
379 whether the sequencing is to be performed in-house, at a commercial company or at an academic
380 sequencing center/platform.
381
382 5.3. After sequencing, 'stitch together' the 2 reads generated from the individual sequencing
383 steps to form a complete IGHV gene rearrangement *i.e.,* the consensus sequence.
384
385 **6. Sequence Analysis**
386
387 Note: The following sections focus on the output obtained from the IMGT/V-QUEST tool[30] when
388 analyzing rearranged IG sequences, and IMGT/JunctionAnalysis[31], both of which are particularly
389 relevant for clinical reporting and research studies. IMGT/V-QUEST is an alignment tool that
390 compares user submitted IG sequences with the IMGT reference directory sets[30]. The tool output
391 includes several sections within which the user can define certain parameters.

6.1. Specify the species, *e.g., Homo sapiens* (human), and receptor type or locus (*e.g.* IGH, IGK or IGL).

6.2. Paste sequences to be analysed, in FASTA format and including headers, into the text area (in batches of up to 50 sequences per run). Alternatively, an option to enter the path to a local file containing the FASTA sequences is provided.

6.3. Choose one of the following 3 display options for the results:

6.3.1. Detailed view: choose this option in order to get the results for each sequence individually.

6.3.2. Synthesis view: choose this option to compile a summary table ordered by IGHV gene and allele name or by sequence input order. This option also provides an alignment of sequences that, in any submitted batch, are assigned to the same IGHV gene and allele.

6.3.3. Excel file: choose this option to provide all output files as spreadsheets incorporated into a single file. All viewing options have a large selection of basic and advanced parameters to choose from, however only the factors most pertinent to CLL IG sequence analysis are discussed below in the 'Representative Results' section.

**7. ARResT/AssignSubsets Tool**

7.1. To investigate BcR IG stereotypy within CLL (additional details provided below in the 'Representative results' section), submit IGHV-IGHD-IGHJ FASTA sequences to the ARResT/AssignSubsets tool[32]. The tool reports the assignment to major CLL stereotyped subsets. The subset-assignment tool together with detailed instructions are available on the ARResT/AssignSubset website[32] and also on the ERIC website under the services tab.

## REPRESENTATIVE RESULTS

Examples provided below illustrate results obtained from IG gene sequencing analysis following the steps detailed above. Although DNA and/or RNA extraction are routine procedures for most clinical/research laboratories, a first critical step involves checking the quality/quantity of gDNA and/or RNA using a spectrophotometer or other suitable technology with high sensitivity. The next crucial step involves PCR amplification of the clonotypic IGHV-IGHD-IGHJ gene rearrangement. As mentioned above, only the use of IGHV leader primers enables amplification of the full length of the rearranged IGHV gene sequence, thus permitting the true SHM load to be determined; therefore, IGHV leader primers are the recommended primer choice (**Figure 2**). In rare instances where IGHV leader primers cannot amplify the clonotypic IG rearrangement, IGHV FR1 primers may be used. As evidenced in **Figure 3**, several PCR products/bands may be observed, especially when gDNA is the starting material; these bands should be excised and sequenced seperately. If both IGHV leader and IGHV FR1 primers fail to yield results, the analysis

435 should be repeated using a new patient sample (when possible). The last checkpoint prior to
436 sequencing is determining the clonality of the sample using fragment analysis (**Figure 4**).
437
438 Sequences obtained should be analyzed using the IMGT/V-QUEST tool[30]. User-selected
439 parameters include species, receptor type or locus, search for insertion, and deletion option, *etc.*
440 and are listed together with the number of sequences analyzed. Each FASTA sequence is
441 displayed and the V-DOMAIN analyzed by IMGT/V-QUEST is highlighted in green. Additionally, if
442 the input sequence was in the opposite orientation (antisense), the program automatically
443 provides the complementary reverse sequence and states this above the FASTA sequence.
444
445 The Result summary table is provided directly below the FASTA sequence, at the top of the
446 'Detailed View' results page, and contains information important for the clinical reporting of IG
447 gene sequence analysis in CLL. Each row of this table is explained below.
448
449 ***Result summary.*** The first row in the results table states the functionality of the input sequence:
450 an IG rearranged sequence can be either productive or unproductive (**Figure 5A & 5B**). An IG
451 gene rearrangement is unproductive if stop codons are present within the V-D-J-REGION (for VH)
452 or within the V-J-REGION (for VL) and/or if the rearrangement is out-of-frame due to
453 insertions/deletions. A third output is provided when the functionality cannot be determined,
454 *i.e.,* if the IGHV-IGHD-IGHJ junction could not be identified; in this instance, the result summary
455 would read as 'No rearrangement found' or 'No junction found'. It is important to note that only
456 productive and, thus, functional rearrangements should be analyzed further. If the sole amplified
457 IG rearrangement is not functional (unproductive or 'no rearrangement found'), the PCR
458 amplification process should be repeated. If the result remains negative a new patient sample
459 should be obtained.
460
461 ***V-GENE and allele.*** The 'V-GENE and allele' row indicates the closest germline IGHV gene and
462 allele with its alignment score and the percent identity. If several gene(s) and alleles give the
463 same high score, all are listed. The percent identity between the submitted sequence and the
464 closest IGHV gene and allele is of particular importance since it determines the SHM status. This
465 value is calculated from the first nucleotide of the V-REGION to the 3' end of the V-REGION
466 excluding the CDR3. If IGHV FR1 primers are used, the sequence corresponding to that of the
467 primer should always be removed to ensure as accurate a result as possible. It should be noted
468 that a low identity percentage (<85%) may result from an insertion or deletion (discussed further
469 below) and, should this be the case a 'Warning' note will appear in the 'Result summary' row to
470 alert the user.
471
472 ***J-GENE and allele.*** As described for the V-gene and allele above, the 'J-GENE and allele' row
473 indicates the closest germline IGHJ gene and allele with its alignment score and the percent
474 identity. However, as the IGHJ gene is rather short and its 5' end may have been trimmed due to
475 exonuclease activity, alternative choices are also searched by the tool, based on the highest
476 number of consecutive identical nucleotides.
477

***D-GENE and allele by IMGT/JunctionAnalysis.*** The 'D-GENE and allele by IMGT/JunctionAnalysis' indicates the closest germline IGHD gene and allele with the D-REGION reading frame identified by the tool in the user sequence. IMGT/JunctionAnalysis[31] provides a detailed and accurate analysis of the junction and has been integrated into the IMGT/V-QUEST tool interface. This tool manages all aspects of difficulty linked to IGHD gene and allele identification *i.e.,* (i) the short length of the D-REGION; (ii) use of 2 or even 3 reading frames; (iii) exonuclease trimming at both ends of the gene; and, (iv) the presence of mutations.

***FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION.*** This row provides the length of the IGHV FRs and CDRs shown within brackets and separated by dots and the amino acid (AA) junction. The AA sequence of the junction illustrates (i) an in- or out-of-frame rearrangement (out-of-frame positions are indicated by the '#' sign), (ii) the presence or absence of stop codons (a stop codon is shown by an asterisk '*'), and (iii) the presence or absence of the anchors of the VH CDR3-IMGT: C (2nd-CYS 104) and W (J-TRP 118).

Somatic hypermutations predominantly consist of single nucleotide changes, however, small insertions and deletions within the V-region can be observed, albeit at a much lower frequency[33]. When using the default IMGT/V-QUEST parameters, insertions and deletions are not automatically detected; however, strong indications that a sequence may carry such alterations, *i.e.,* a low percent identity and/or different IGHV CDR1-IMGT and CDR2-IMGT lengths between the submitted user sequence and the closest germline gene and allele, are detected by the tool and a warning alert appears below the Results summary table (**Figure 5C**).

IMGT provides an option called 'Search for insertions and deletions' in the 'Advanced parameters' section located below the 'Display results' section. In cases where relevant warnings appear, it is advisable to use this functionality. When insertions and deletions are detected, comprehensive details of the finding are provided in the 'Result summary' section including (i) where the insertion or deletion is located *i.e.,* VH FR-IMGT or CDR-IMGT; (ii) the number of inserted/deleted nucleotides; (iii) the sequence (only for insertions); (iv) whether a frameshift has occurred; (v) the V-REGION codon from which the insertion or deletion starts; and (vi) the affected nucleotide position in the submitted sequence (**Figure 5D**). For insertions, the tool removes the insertion(s) from the submitted user sequence and then re-analyzes the sequence using the standard IMGT/V-QUEST parameters. If deletions are detected, the tool adds gaps, represented by dots, to replace the deleted nucleotides before repeating the analysis.

As for every diagnostic or prognostic test performed in clinical laboratories, stringent standards and a high level of reproducibility are of utmost importance. The IMGT/V-QUEST output provides much of the information necessary for reporting the results of IG gene sequence analysis in CLL, *i.e.,* (i) the IGHV, IGHD and IGHJ genes and alleles utilised; (ii) the functionality of the clonotypic IGHV-IGHD-IGHJ gene rearrangement *i.e.,* whether the rearrangement is productive or unproductive; and (iii) the percent identity of the rearranged IGHV gene and allele in comparison to its closest germline IGHV gene and allele. For comprehensive details regarding the clinical reporting of IG genes in CLL, the interpretation of problematic or technically challenging cases

521 and recommendations for the accurate and robust determination of IGHV gene SHM status in
522 CLL, the reader is directed to recently updated ERIC guidelines and reports[27,34].
523
524 Finally, owing to our growing knowledge concerning BcR IG stereotypy in CLL, an additional
525 recommended requirement for the clinical reporting of IG gene rearrangements in CLL relates to
526 the assignment of cases to major stereotyped subsets. It is now recommended to state in the
527 clinical report whether the analyzed productive IGHV gene rearrangement can be assigned to
528 one of the major stereotyped subsets, namely subsets #1, #2, #4 or #8[12,27]. Assignment to major
529 CLL stereotyped subsets should be performed with the use of the ARResT/AssignSubsets tool
530 (**Figure 6**)[32].
531
532 **FIGURE & TABLE LEGENDS**
533
534 **Table 1. Primer sequences and quantities for the PCR amplification of the clonotypic IGHV-**
535 **IGHD-IGHJ gene rearrangement.**
536
537 **Table 2. Reagents for the PCR amplification of the clonotypic IGHV-IGHD-IGHJ gene**
538 **rearrangement.**
539
540 **Table 3. Thermal cycling conditions for the PCR amplification of the clonotypic IGHV-IGHD-IGHJ**
541 **gene rearrangement.**
542
543 **Table 4. Thermal cycling conditions for the IG gene sequencing reaction.**
544
545 **Figure 1. Schematic representation of an IGHV-IGHD-IGHJ gene rearrangement with various**
546 **primer annealing locations indicated.** 5' IGHV primers anneal to the leader sequence that is
547 located upstream of the IGHV coding sequence. 5' IGHV framework (FR) primers anneal to the
548 start of the FR1 region, which is located within the rearranged IGHV gene, whereas the 3' IGHJ
549 primers anneal to the end of the rearranged IGHJ gene. UTR: untranslated region.
550
551 **Figure 2. PCR amplification of the IGHV-IGHD-IGHJ gene rearrangement in 7 patient samples**
552 **using 5' IGHV leader primers.** The eighth lane represents a positive control whereas the negative
553 control for the PCR was loaded into the ninth lane. The expected PCR product is approximately
554 500 base pairs in size when using IGHV leader primers. The asterisk in the last column of the gel
555 indicates the 100 bp DNA ladder.
556
557 **Figure 3. PCR amplification of the IGHV-IGHD-IGHJ gene rearrangement in 13 patient samples**
558 **using IGHV FR1 primers.** The fourteenth lane contains the positive control whilst the negative
559 control was loaded into the fifteenth lane. As evidenced in lane 2, for which DNA was the starting
560 material, several PCR bands are observed, which should be excised and sequenced seperately.
561 Samples in lanes 5, 7, and 13 yielded polyclonal results (evidenced by the smear in the gel) and,
562 thus, the PCR step should be repeated. If a second PCR fails to amplify the rearranged IG gene(s)
563 the analysis should be performed on a new sample (if possible) or using a different primer set.

564 The expected PCR product is approximately 350 base pairs in size when using IGHV FR1 primer
565 mixes. The asterisk in the last column of the gel indicates the 100 bp DNA ladder.
566
567 **Figure 4. Assessment of clonality using genescan analysis.** In genescan analysis, monoclonal PCR
568 products give rise to a dominant peak of fluorescent products of identical size (upper panel),
569 whereas polyclonal PCR products result in a more normal distribution of product sizes (lower
570 panel). The red traces are peaks from a fluorescently-labeled DNA ladder that is loaded in the
571 same capillary injection as the sample being analysed. The size standard fragments are subjected
572 to the same electrophoretic force as the sample and are therefore exposed to the same injection
573 conditions. The uniform spacing of the size standard fragments confirms precise size calling. The
574 blue traces represent the monoclonal (top panel) or polyclonal (bottom panel) nature of the
575 samples.
576
577 **Figure 5. Examples of the result summary tables provided by IMGT/V-QUEST. (A)** Result
578 summary table for a productive IGHV-IGHD-IGHJ gene rearrangement (no stop codon(s) and an
579 in-frame sequence junction). The V-GENE and allele and the J-GENE and allele identified in the
580 user sequence by IMGT/V-QUEST in this case are IGHV4-34*01 and IGHJ6*02 (on the basis of the
581 highest alignment score evaluation). The percent identity is 99.65% due to a single somatic
582 mutation. The D-GENE and allele identified by IMGT/JunctionAnalysis is the IGHD3-3*01 in
583 reading frame 1. The lengths of the 4 framework regions (FRs) as well as the 3 complementarity
584 determining regions (CDRs) are indicated in brackets. The amino acid (AA) sequence of the IGHV-
585 D-J junction is also provided. In this example the junction comprised 22 AAs. **(B)** Result summary
586 table for an IGHV-IGHD-IGHJ gene rearrangement sequence that is unproductive due to an out-
587 of-frame junction. An X for the CDR3-IMGT length indicates that for this sequence the length
588 could not be determined. The '#' signs observed in the AA junction sequence indicate a
589 frameshift. **(C)** Result summary table warning of low V-REGION identity (60.94%) and indicating
590 that the 'insertions and deletions' option in the 'Advanced parameters' section should be used.
591 **(D)** Result summary table for the case with germline low percent identity illustrated in (C) after
592 repeating the sequence analysis using the 'Search for insertions and deletions' option. The
593 correct identity percent is displayed in brackets and is calculated considering the insertion as a
594 single mutational event.
595
596 **Figure 6. Subset assignment report table generated by the ARResT/AssignSubsets tool.** The
597 FASTA IG sequences from 10 CLL patients (A1-A10) were submitted to the ARResT/AssignSubsets
598 tool. Case A4 was assigned to CLL stereotyped subset #2 (patients within this group are known
599 to have a poor prognosis irrespective of SHM load) with high confidence. Seven of the other
600 cases/sequences were not assigned to a major stereotyped subset and hence classed as
601 unassigned. Two of the submitted sequences (A7 and A8) could not be used for subset
602 assignment and were instead classed as skipped/unhealthy due to issues with the sequence, *i.e.,*
603 due to an out-of-frame junction or the presence of stop codons. A comprehensive explanation of
604 the table headings and the tool is available at the ARResT/AssignSubsets website[32].
605
606 **DISCUSSION**

607 The study of IG genes in CLL has been vital not only for providing a better understanding into
608 disease pathobiology but also for improving patient risk stratification. It is therefore paramount
609 that the multi-step procedure of IG gene sequence analysis and subsequent interpretation of the
610 sequence data is performed in a standardized manner. The availability of suitable and sufficient
611 patient material and the timing of sampling should not impact on the ability to perform IG gene
612 mutational analysis since the test can be performed on PB and on any sample with a high CLL
613 tumor cell count. The separation of blood mononuclear cells using gradient separation methods
614 is routinely performed in diagnostic laboratories, and as always, care should be taken when
615 adding the blood/RPMI solution to the tube containing the gradient; this task should be
616 performed in a slow, gentle manner to avoid disturbing the gradient and prevent the loss of cells.
617
618 Following cell separation, nucleic acids are extracted. Both gDNA and cDNA are suitable for SHM
619 analysis of the clonotypic IGH rearrangement, however, there are advantages and disadvantages
620 for the use of either material. The laboratory workflow proceeds quicker when using gDNA since
621 no reverse transcription has to be performed prior to PCR amplification. That said, using gDNA as
622 the substrate for PCR may result in the amplification of both productive and unproductive
623 rearrangements which, in turn, can lead to additional hands-on laboratory work, *i.e.,* purification
624 or gel excision of multiple PCR products and individual sequencing of all rearrangements. When
625 comparing the gDNA and cDNA approaches, for the latter a reverse transcription step must be
626 performed before proceeding with PCR amplification. However, in this instance, for most cases,
627 only productive IG rearrangements will be amplified and subsequently sequenced. Thus, only a
628 single PCR product will be purified and sequenced, while results interpretation is more
629 straightforward.
630
631 The efficiency of amplification largely depends on the quality of the gDNA/cDNA, which should
632 be assessed using a sensitive quantification method, and the primers utilized. The primers used
633 are critical to the entire analytical procedure because the accurate determination of the SHM
634 level can only be achieved by amplifying the entire sequence of the rearranged IGHV gene. A full-
635 length rearrangement can only be assessed if 5' IGHV leader primers are used, thereby justifying
636 the recent recommendations by ERIC for the use of leader primers[27]. The use of alternative
637 primers, leading to the amplification of incomplete IGHV-IGHD-IGHJ gene rearrangements, is not
638 appropriate for IGHV gene mutational analysis, and hence strongly advised against. The sole
639 exception pertains to cases which repeatedly produce negative results when IGHV leader primers
640 are used and analysis of new patient material is either not possible or also does not yield results.
641 If use of a different patient sample and/or material (gDNA/cDNA) and various primers sets still
642 fail to produce a PCR product, possible reasons could be that the tumor cell burden is too low or
643 that lymphocytosis is not due to a CLL clone (in which case the immunophenotype should be re-
644 evaluated). Primers used for the analysis should always be stated in the clinical report.
645
646 As CLL results from the accumulation of monoclonal B cells bearing the same IGHV-IGHD-IGHJ
647 gene rearrangement, for the vast majority of cases clonality assessment will reveal a unique
648 monoclonal peak, *i.e.,* a single clone. On rare occasions, double rearrangements or even
649 oligoclonal patterns may be observed[35]. In such cases, gel electrophoresis is not the preferred
650 technique for clonality assessment and more sensitive methods such as fragment analysis should

be applied. Once clonality has been confirmed, the final step prior to sequencing relates to the purification of the PCR product to ensure that sequences of high quality are obtained. Finally, the IMGT/V-QUEST tool is the resource recommended for IG sequence analysis by ERIC. As the IMGT databases are constantly updated and report results in a consistant and well annotated manner, this tool ensures standardized analysis and facilitates comparative analysis amongst different clinical or academic facilities.

As immunogenetic analysis in CLL has prognostic and, in particular, predictive implications, robust analysis of the IGHV-IGHD-IGHJ gene rearrangement including the assignment to major CLL stereotyped subsets, is no longer only desirable but of key importance. This is particularly evident in this era of novel therapeutics and the potential that IG gene analysis has to guide treatment decisions[5,21-23,36-38], as officially indicated by the recent update of the NCI-sponsored guidelines from the International Workshop on CLL[24]. That said, rigorous standards are warranted, especially as determination of the SHM status of the clonotypic rearranged IGHV gene in CLL patients is not a trivial matter and involves a multi-step process. Importantly, certain experimental steps, most notably the choice of primers, yield superior results when specific options and/or approaches are adhered to, other technical aspects are amenable to some degree of flexibility, such as material selection or the method for assessing clonality, due to the fact that they lead to subtle differences, if any, with regard to the final results.

Over the last decade, ERIC has strived to promote the standardization and harmonization of various methods pertinent to CLL diagnostics and prognostication. This is reflected in the published recommendations and guidelines for immunogenetic analysis[27,34], numerous organized educational workshops and events, the establishment of an IG Network, as well as an expert online forum to discuss and provide guidance on IG gene sequence interpretation in CLL. The overall goal of ERIC through these actions is to promote optimal clinical management and patient care. Despite intense efforts, this task is far from complete, and in fact has become more complex owing to the development of novel high-throughput sequencing aimed at immune profiling. Nevertheless, although challenges persist, intensive efforts for the robust standardization of IG gene analysis in CLL continue and are currently the focus of ongoing activities within ERIC.

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**DISCLOSURES**

The authors have no relevant conflict of interest to disclose.

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Figure 1

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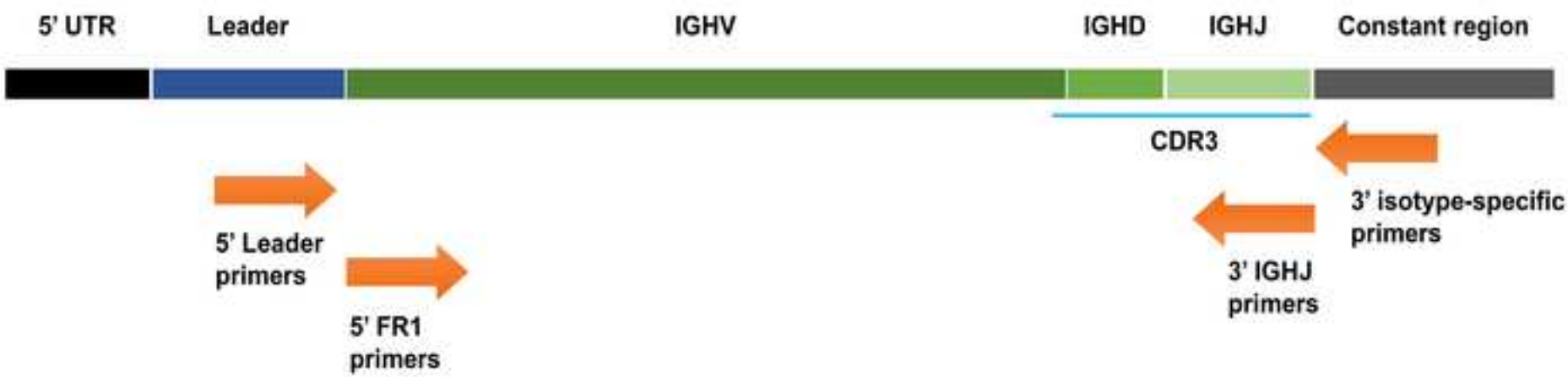

Figure 2

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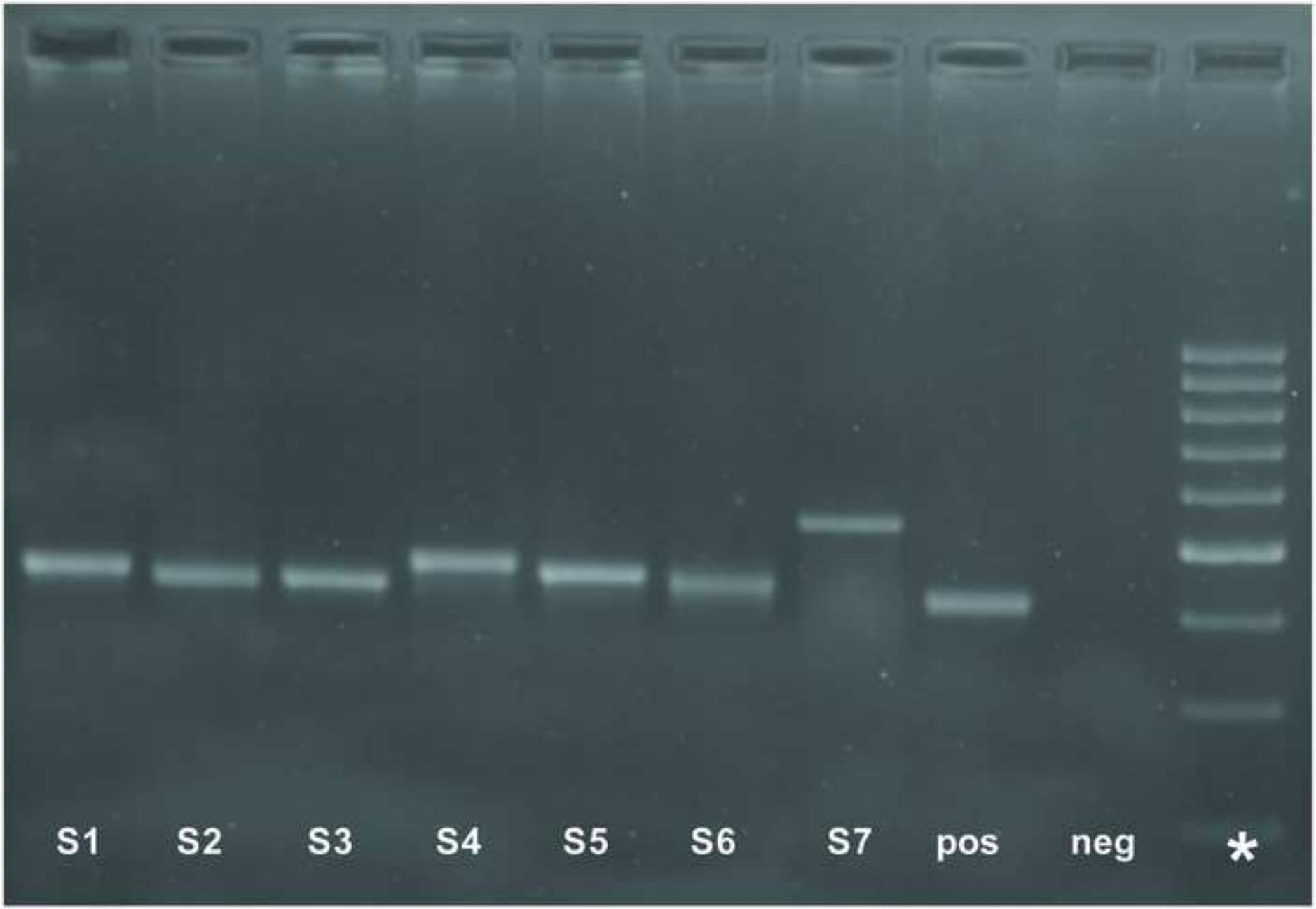


* 100 bp DNA ladder

Figure 3

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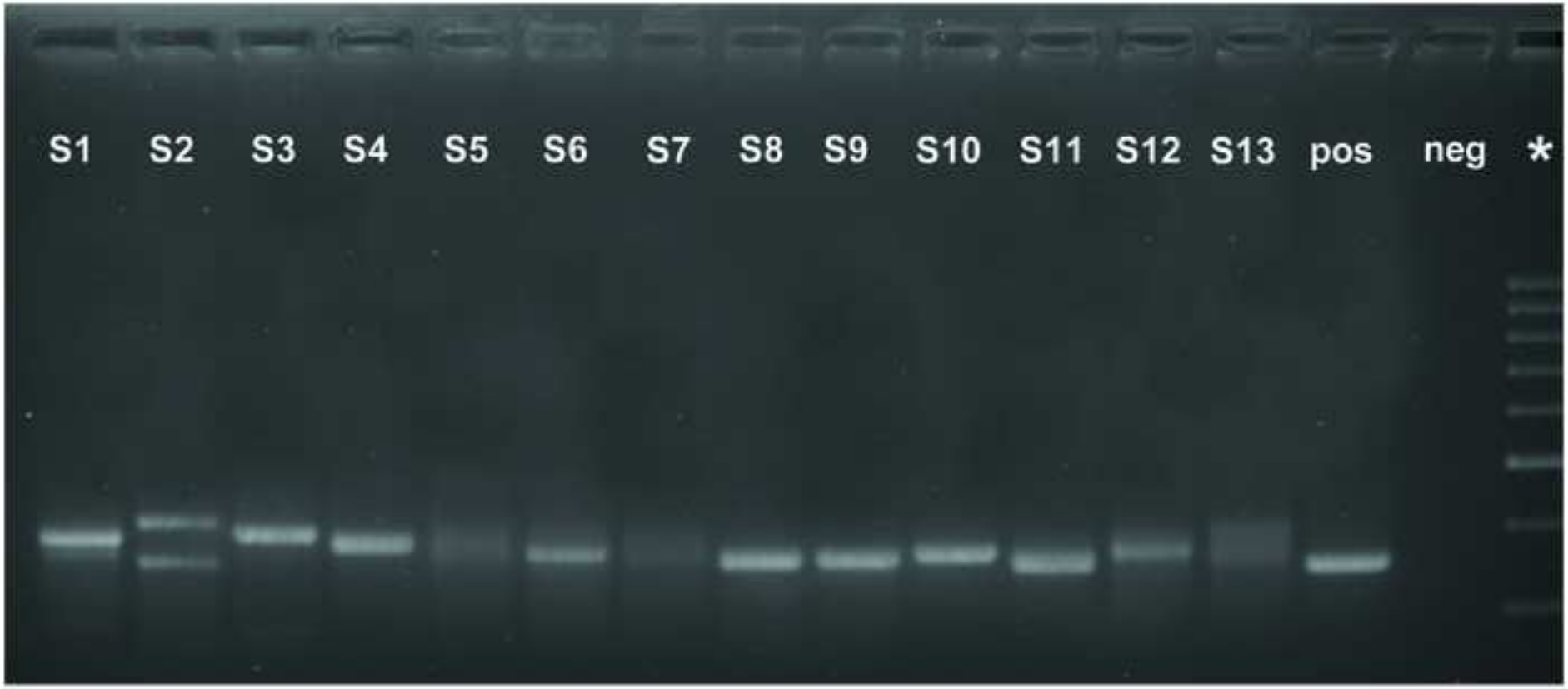


* 100 bp DNA ladder

Figure 4

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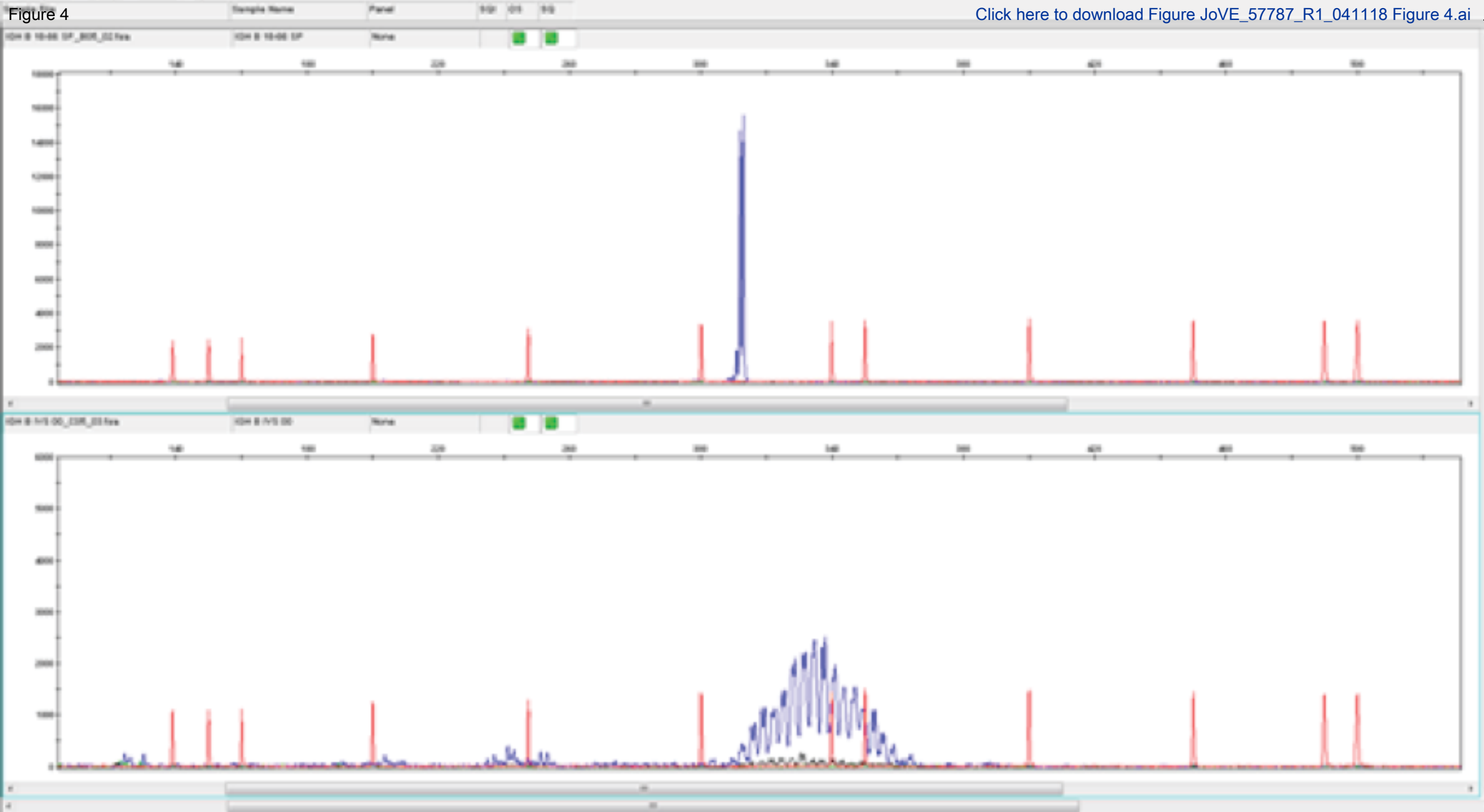

Figure 5

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A

| Result summary: | Productive IGH rearranged sequence: (no stop codon and in-frame junction) | | |
|---|---|---|---|
| V-GENE and allele | Homsap IGHV4-34*01 F | score = 1411 | identity = 99.65% (284/285 nt) |
| J-GENE and allele | Homsap IGHJ6*02 F | score = 291 | identity = 98.33% (59/60 nt) |
| D-GENE and allele by IMGT/JunctionAnalysis | Homsap IGHD3-3*01 F | D-REGION is in reading frame 1 | |
| FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION | [25.17.38.10] | [8.7.22] | CARGLPLLEWLLGPYYYYYGMDVW |

B

| Result summary: | Unproductive IGH rearranged sequence (out-of-frame junction) | | |
|---|---|---|---|
| V-GENE and allele | Homsap IGHV3-64*01 F | score = 1435 | identity = 100.00% (288/288 nt) |
| J-GENE and allele | Homsap IGHJ6*03 F | score = 215 | identity = 90.38% (47/52 nt) |
| D-GENE and allele by IMGT/JunctionAnalysis | Homsap IGHD5-12*01 F | D-REGION is in reading frame 2 | |
| FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION | [25.17.38.7] | [8.8.X] | CASAGSPSMVATR##YYYYYMDVW |

C

| Result summary: | Productive IGH rearranged sequence: (no stop codon and in-frame junction) (a) | | |
|---|---|---|---|
| V-GENE and allele | Homsap IGHV6-1*01 F, or Homsap IGHV6-1*02 F | score = 341 | identity = 60.94% (142/233 nt) |
| J-GENE and allele | Homsap IGHJ4*02 F | score = 159 | identity = 81.25% (39/48 nt) |
| D-GENE and allele by IMGT/JunctionAnalysis | Homsap IGHD2-15*01 F | D-REGION is in reading frame 3 | |
| FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION | [3.17.38.11] | [10.9.16] | CARGVGWEIPEAHGCDYW |

(a) Low V-REGION identity (60.94%): this may indicate potential nucleotide insertion(s) and/or deletion(s). Try 'Search for insertions and deletions' in 'Advanced parameters' at the bottom of the Search page

D

Result summary:

Nucleotide insertions have been detected and automatically removed for this analysis: they are displayed as capital letters in the user submitted sequence above.

| localization in V-REGION | nb of inserted nt | inserted nt | causing frameshift | from V-REGION codon | from nt position in user submitted sequence |
|---|---|---|---|---|---|
| CDR2-IMGT | 3 | AAC | no | 60 | 102 |

IMGT/V-QUEST results after removal of the insertion(s):
Potentially productive IGH rearranged sequence: no stop codon and in-frame junction
(Check also your sequence with BLAST against IMGT/GENE-DB reference sequences to eventually identify out-of-frame pseudogenes)

| | | | |
|---|---|---|---|
| V-GENE and allele | Homsap IGHV6-1*01 F, or Homsap IGHV6-1*02 F | score = 1019 | identity = 93.91% (216/230 nt) [93.48% (215/230 nt)] |
| J-GENE and allele | Homsap IGHJ4*02 F | score = 159 | identity = 81.25% (39/48 nt) |
| D-GENE and allele by IMGT/JunctionAnalysis | Homsap IGHD2-15*01 F | D-REGION is in reading frame 3 | |
| FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION | [2.17.38.11] | [10.9.16] | CARGVGWEIPEAHGCDYW |

Figure 6

Click here to download Figure JoVE_57787_R1_041118 Figure 6.ai

*assignment report table*

| label [+ heat map, if appl.] | SeqCure | subset | confidence | score |
|---|---|---|---|---|
| A4 | OK | CLL#2 | extreme | 82.54 |
| A10 | warning | unassigned | extreme | 13.47 |
| A1 | OK | unassigned | extreme | -Inf |
| A2 | OK | unassigned | extreme | -Inf |
| A3 | OK | unassigned | extreme | -Inf |
| A5 | warning | unassigned | extreme | -Inf |
| A6 | warning | unassigned | extreme | -Inf |
| A9 | warning | unassigned | extreme | -Inf |
| A7 | warning | skipped/unhealthy | | |
| A8 | warning | skipped/unhealthy | | |

Table 1

Click here to download Table Table 1 052918.xlsx

| 5’ IGHV FR1 primers |
|---|
| IGHV1 |
| IGHV2 |
| IGHV3 |
| IGHV4 |
| IGHV5 |
| IGHV6 |
| **5’ IGHV leader primers** |
| IGHV1L |
| IGHV1bL |
| IGHV2aL |
| IGHV2bL |
| IGHV3aL |
| IGHV3bL |
| IGHV4L |
| IGHV5L |
| IGHV6L |
| **3’ IGHJ primers** |
| IGHJ1-2 |
| IGHJ3 |
| IGHJ4-5 |
| IGHJ6 |

| Primer sequence |
| --- |
| CAGGTGCAGCTGGTGCAGTCTGG |
| CAGGTCAACTTAAGGGAGTCTGG |
| GAGGTGCAGCTGGTGGAGTCTGG |
| CAGGTGCAGCTGCAGGAGTCGGG |
| GAGGTGCAGCTGTTGCAGTCTGC |
| CAGGTACAGCTGCAGCAGTCAGG |
| |
| AAATCGATACCACCATGGACTGGACCTGGAGG |
| AAATCGATACCACCATGGACTGGACCTGGAG(C/A) |
| AAATCGATACCACCATGGACACACTTTGCT(A/C)AC |
| AAATCGATACCACCATGGACATACTTTGTTCCAC |
| AAATCGATACCACCACCATGGAGTTTGGGCTGAGC |
| AAATCGATACCACCACCATGGA(A/G)(C/T)T(G/T)(G/T)G(G/A)CT(G/C/T)(A/C/T)GC |
| AAATCGATACCACCATGAAACACCTGTGGTTCTT |
| AAATCGATACCACCATGGGGTCAACCGCCATC |
| AAATCGATACCACCATGTCTGTCTCCTTCCTC |
| |
| TGAGGAGACGGTGACCAGGGTGCC |
| TGAAGAGACGGTGACCATTGTCCC |
| TGAGGAGACGGTGACCAGGGTTCC |
| TGAGGAGACGGTGACCGTGGTCCC |

| Quantity for 60 µL of primer mix |
| --- |
| 10 |
| 10 |
| 10 |
| 10 |
| 10 |
| 10 |
| |
| 5 |
| 5 |
| 5 |
| 5 |
| 5 |
| 5 |
| 10 |
| 10 |
| 10 |
| |
| 20 |
| 10 |
| 20 |
| 10 |

Table 2

Click here to download Table Table 2 052918.xlsx

| Reagent | Volume (µL) |
|---|---|
| RB x 10 buffer | 5 |
| $MgCl_2$ (50 mM) | 3 |
| dNTPs (10 mM) | 2 |
| Primer IGHV leader/FR1 mix (10 µM) | 3 |
| Primer IGHJ mix (10 µM) | 3 |
| $H_2O$ | 31.5 |
| Total volume | 47.5 |

Table 3

Click here to download Table Table 3 052918.xlsx

| Temperature | Duration | Cycle number | Description |
|---|---|---|---|
| 94 °C | 5 minutes | 1 | polymerase activation |
| 94 °C | 1 minute | 39 | denaturation |
| 59 °C | 1 minute | | annealing |
| 72 °C | 1.5 minutes | | extension |
| 72 °C | 10 minutes | 1 | final extension |
| 18 °C | ∞ | 1 | preservation |

Table 4

Click here to download Table Table 4 052918.xlsx

| Temperature | Duration | Cycle number | Description |
|---|---|---|---|
| 94 °C | 5 minutes | 1 | polymerase activation |
| 96 °C | 20 seconds | 30 | denaturation |
| 50 °C | 20 seconds | | annealing |
| 60 °C | 4 minutes | | extension |
| 4 °C | ∞ | 1 | preservation |

Click here to download Table of Materials JoVE_57787_Materials_052918.xlsx

| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| BD Vacutainer Plastic Blood Collection Tubes with K2EDTA: Tube Stopper | ThermoFisher scientific | 02-689-4 |
| BD Vacutainer CPT Mononuclear Cell Preparation Tube - Sodium Heparin | Becton Dickinson | 362753 |
| BD Vacutainer Heparin Blood Collection Tubes FAQ | Daigger Scientific | 366480 |
| UltraPure 0.5 M EDTA pH 8.0 | Invitrogen | 15575038 |
| Centrifuge tubes 15 mL CS500 | Corning | 352096 |
| Centrifuge tubes PP 50 mL CS500 | Corning | 352070 |
| RPMI Medium 1640 (1X) liquid | Invitrogen | 21875-091 |
| Ficoll-Paque PLUS, 6 x 100 mL | STEMCELL Technologies | 17-1440-02 |
| Serological pipettes 5 mL | Sarstedt | 861,253,001 |
| Serological pipettes 10 mL | Sarstedt | 861,254,001 |
| Serological pipettes 25 mL | Sarstedt | 861,685,001 |
| Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X) | Invitrogen | 14190250 |
| Neubauer plate | Marienfeld-Superior | 640010 |
| Tuerk solution | Sigma-Aldrich | 93770 |
| PureLink Genomic DNA Mini Kit | Invitrogen | K1820-01 |
| QIAamp RNA Blood Mini Kit | Qiagen | 52304 |
| AllPrep DNA/RNA Mini Kit | Qiagen | 80204 |
| 5X first-strand buffer | Invitrogen | P/N Y02321 |
| Random Primers, 20 µg | Promega | C1181 |
| RNaseOUT™  Recombinant Ribonuclease Inhibitor | Invitrogen | 10777019 |
| DTT | Invitrogen | P/N Y00147 |
| SuperScript II Reverse Transcriptase | Invitrogen | 18064-014 |
| 10 mM dNTP Mix | Invitrogen | 18427013 |
| PCR Buffer | Invitrogen | O00065 |
| MgCl2 (magnesium chloride) (25 mM) | ThermoFisher scientific | AB0359 |
| Taq DNA Polymerase, recombinant | Invitrogen | 10342-020 |
| Applied Biosystems 5’ Labeled Primers | ThermoFisher scientific | - |
| Agilent High Sensitivity DNA Kit | Agilent Technologies | 5067-4626 |
| UltraPure TBE Buffer, 10X | ThermoFisher scientific | 15581044 |
| UltraPure Ethidium Bromide, 10 mg/mL | ThermoFisher scientific | 15585011 |

| | | |
|---|---|---|
| SYBR Safe DNA Gel Stain | ThermoFisher scientific | S33102 |
| 10X BlueJuice Gel Loading Buffer | Invitrogen | 16500100 |
| DNA Ladder 1000 bp/1 kb ladder BLUE ready-to-use | Geneon | 305-105 |
| UltraPure Agarose | ThermoFisher scientific | 16500500 |
| Agarose low gelling temperature | Sigma-Aldrich | A9414-250G |
| Novex TBE Gels, 10%, 10 well | ThermoFisher scientific | EC6275BOX |
| ExoSAP-IT PCR Product Cleanup Reagent | ThermoFisher scientific | 78201.1.mL |
| QIAquick Gel Extraction Kit (50) | Qiagen | 28704 |
| GenomeLab DTCS Quick Start Kit | Beckman Coulter | 608120 |
| Sodium Acetate Solution (3 M), pH 5.2 | ThermoFisher scientific | R1181 |
| Glycogen, molecular biology grade | ThermoFisher scientific | R0561 |
| Ethanol absolute | EMD Millipore | 1024282500 |
| SafeSeal tube 1.5 mL | Sarstedt | 72,706 |
| Multiply-Pro cup 0.2 mL, PP | Sarstedt | 72,737,002 |
| Centrifuge | | |
| PCR machine | | |
| Bioanalyzer | Agilent Technologies | |

| Comments/Description |
| --- |
| material storage |
| material storage |
| material storage |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| cell processing |
| DNA extraction |
| RNA extraction |
| DNA/RNA extraction |
| cDNA synthesis |
| cDNA synthesis |
| cDNA synthesis |
| cDNA synthesis |
| cDNA synthesis |
| cDNA synthesis, PCR |
| PCR |
| PCR |
| PCR |
| PCR/Clonality assessment |
| PCR product quantification |
| gel electrophoresis |
| gel electrophoresis |

gel electrophoresis
gel electrophoresis
gel electrophoresis
gel electrophoresis
gel electrophoresis
gel electrophoresis
PCR product clean-up
PCR product clean-up
Sanger sequencing
Sanger sequencing
Sanger sequencing
Sanger sequencing
general use
general use
equipment
equipment
equipment

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Author(s): ANDREAS AGATHANGELIDIS, LESLEY-ANN SUTTON, ANASTASIA HADZIDIMITRIOU, ANTON W. LANGERAK, CHRYSOULA BELESSI, FREDERIC DAVI, RICHARD ROSENQUIST, KOSTAS STAMATOPOULOS, PAOLO GHIA

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Article Title: AN ERIC PRACTICAL GUIDE FOR IMMUNOGLOBULIN GENE SEQUENCE ANALYSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA: FROM PATIENT MATERIAL TO SEQUENCE INTERPRETATION

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Rebuttal Letter

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To: Philip Steindel, PhD

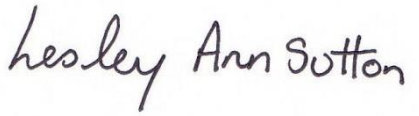
Review Editor, JoVE Publications

Stockholm, 14th May 2018

Dear Dr. Steindel,

We thank you for your favorable appraisal of our manuscript (JoVE57787) entitled "AN ERIC PRACTICAL GUIDE FOR IMMUNOGLOBULIN GENE SEQUENCE ANALYSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA: FROM PATIENT MATERIAL TO SEQUENCE INTERPRETATION". We are resubmitting with the requested formatting modifications including our responses to the editorial comments.

Sincerely,



Lesley Ann Sutton, PhD, Department of Molecular Medicine and Surgery, Clinical genetics, Karolinska Institutet, Stockholm, Sweden

E-mail: lesley-ann.sutton@ki.se

**Editorial comments:**

**1.** I have edited this to Jove format (imperative tense with notes; consolidating some shorter steps and splitting up some longer ones); however, there are still issues with the selections for filming. For the protocol section of the video (what the highlighting is for), we will only be able to film concrete steps as done in the lab (e.g., most of section 2) or use of software with a graphical user interface (GUI; e.g., most of 6). Vague steps like choosing primers or deciding on a substrate will not be able to be filmed (implications of these choices can be discussed in the results section of the video; no highlighting will be necessary in that section of the manuscript, though).

**Reply:** The sections that are to be included in the film are highlighted in yellow in the revised version and, in brief, cover the following process:
Section 1: Material requirements, collection and storage.
Section 2: Ficoll gradient separation
Section 3: Nucleic acid extraction
Section 4: Amplification and sequencing of IGHV-IGHD-IGHJ rearrangements - preparation of the mastermix and the gel, clonal assessment and purification of the PCR product(s)
Section 6: Sequence analysis

2. There are still necessary details missing for the particular protocols you intend to show; see below.

1. 4.3.2.1: How are primers fluorescently labeled (which fluorophores, where on the molecule, etc.)?

**Reply:** The following text 'Custom 5´- labeled primers are fluorescently labeled oligos with a choice of dyes on the 5' end. Examples of reporter dyes include 6-FAM, HEX, NED or TET.' has been added to the appropriate section at lines 282-283 (highlighted in green).

2. 4.3.3: How long to run the gel and what settings?

**Reply:** The following text 'It is recommended that PCR products are allowed to migrate on the gel for sufficient time so that monoclonal samples can be separated from the polyclonal background and the DNA size marker can separate adequately. Factors such as the size and thickness of the gel can affect migration so no single setting (voltage and time) can guarantee an optimal result. That said, setting the voltage at 80V for 45 minutes is a good starting point as it minimizes the risk of the sample migrating too quickly and 'running' off the gel.' has been added to the appropriate section at lines 287-293 (highlighted in green).

3. 4.3.4: How should the gel be imaged?

**Reply:** The following text 'An intercalating agent such as Ethidium bromide (EtBr) or less hazardous and more sensitive commercially available DNA stains can be used for the visualization of DNA on acrylamide gels using UV excitation or blue-light.' has been added to the appropriate section at lines 298-300 (highlighted in green).

4. 4.4.4: This is not the protocol I found for ExoSAP-IT (2 µL of a single reagent per 5 µL product; see https://www.thermofisher.com/order/catalog/product/78201.1.ML); also, 0.05 µL is a very small amount to reliably add.

**Reply:** At our center we follow the guidelines provided and optimized by the genome center where sequencing is performed. Admittedly, 0,05 ul is a very small volume however we prepare a mastermix of the reagents and from this mix dispense to all reactions. Nevertheless, to avoid confusion for the reader we have modified the text (lines 317-320) to read as follows: **'**To perform

Sap/Exo clean-up, follow the manufacturer's instructions regarding the specific volumes to use, however a typical reaction may contain 1 µl Sap, 0. 5 µl Exo and 2 µl 5x incubation buffer per 5-10 µl PCR reaction. Mix by gently vortexing each sample and incubate on a PCR block at 37°C for 30 minutes. Inactivate the enzymes by heating to 85°C for 15 minutes.'

5. 5.2.9 (formerly 5.3.16): What exactly is supposed to be repeated here? The original 5.3.11 and 5.3.12 instruct the user to centrifuge and discard supernatant, but there doesn't seem to be any supernatant to discard at this point (maybe some residual).

**Reply:** The relevant sentences (lines 367-371) have been clarified and read as below:
5.2.7. Add 100 µl of 70% ethanol (-20°C) and gently mix.
5.2.8. Centrifuge at 14,000 rpm for 10 minutes (4°C). Discard the supernatant.
5.2.9. Repeat steps 5.2.7 and 5.2.8.

6. 5.2.11: What is the 'sequencing plate'? How is the sequencing done (e.g., sent off elsewhere; an in-house machine, etc.)?

**Reply:** The relevant section (lines 376-380) has been expanded and now reads as follows: Transfer the sample to a sequencing plate, cover with strip caps or tape seals, load to the machine and perform Sanger sequencing. The sequencing plate is usually a 96-well, v-bottom, full-skirt PCR plate compatible with the sequencer. The plates may be barcoded depending on whether the sequencing is to be performed in-house, at a commercial company or at an academic sequencing center/platform.