

REVIEW

EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations

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PCR-based immunoglobulin (Ig)/T-cell receptor (TCR) clonality testing in suspected lymphoproliferations has largely been standardized and has consequently become technically feasible in a routine diagnostic setting. Standardization of the pre-analytical and post-analytical phases is now essential to prevent misinterpretation and incorrect conclusions derived from clonality data. As clonality testing is not a quantitative assay, but rather concerns recognition of molecular patterns, guidelines for reliable interpretation and reporting are mandatory. Here, the EuroClonality (BIOMED-2) consortium summarizes important pre- and post-analytical aspects of clonality testing, provides guidelines for interpretation of clonality testing results, and presents a uniform way to report the results of the Ig/TCR assays. Starting from an immunobiological concept, two levels to report Ig/TCR profiles are discerned: the technical description of individual (multiplex) PCR reactions and the overall molecular conclusion for B and T cells. Collectively, the EuroClonality (BIOMED-2) guidelines and consensus reporting system should help to improve the general performance level of clonality assessment and interpretation, which will directly impact on routine clinical management (standardized best-practice) in patients with suspected lymphoproliferations.

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INTRODUCTION

The diagnosis of lymphoid malignancies is greatly supported and facilitated by clonality testing. Depending on the experience of the hematopathologists and the type of histopathological requests, 5–15% of cases could benefit from molecular clonality diagnostics.^{1,2} Of the many different markers that can be used for clonality testing in suspected lymphoproliferations, immunoglobulin (Ig) and T-cell receptor (TCR) antigen receptor gene rearrangements stand out as the most widely applied targets. These Ig and TCR rearrangements are formed from the earliest stages of B-cell and T-cell development onwards.^{3,4} Random coupling between one of many *V*, (*D*) and *J* genes results in the formation of a unique V(D)J exon that encodes the actual

antigen-binding moiety of the Ig or TCR chain. Owing to the huge diversity in Ig/TCR rearrangements, the diversity of different Ig or TCR molecules is estimated to be in the order of 10¹². As a consequence each lymphocyte has a unique antigen receptor molecule on its membrane and the chance that two different lymphocytes coincidentally bear the same receptor is almost negligible. Hence, identical rearrangements are not derived from multiple independently generated cells, but rather reflect the clonal nature of the involved cell population. Evaluation of the homogeneous vs heterogeneous nature of the rearrangements is thus at the basis of clonality testing.

In the last two decades, PCR-based analysis of Ig/TCR rearrangements has gradually replaced Southern blot analysis as

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gold standard method for clonality testing.^{1,5–14} However, the earliest PCR strategies suffered from false negativity (lack of recognition of all possible rearrangements) and false positivity (inability to accurately distinguish monoclonal from polyclonal PCR products). False negativity was at least in part also caused by the fact that most laboratories only used TCR gamma (TCRG) and complete IG heavy chain (IGH) V–J gene rearrangements as PCR targets for reasons of limited primer usage and relatively simple gene structure. These drawbacks prompted the design of completely novel assays for Ig/TCR rearrangement detection in the European BIOMED-2 network (now called EuroClonality consortium).¹⁵ This effort has resulted in standardized multiplex PCR assays for nearly all Ig/TCR targets, which collectively show an unprecedentedly high rate of detection in the most common B- and T-cell malignancies.^{2,16–18} This high detection rate was not only achieved by optimized primer design, but also by inclusion of extra Ig/TCR targets (IG kappa, IGK and TCR beta, TCRB as well as incomplete IGH D–J and TCRB D–J rearrangements).¹⁵ Meanwhile, the BIOMED-2/EuroClonality PCR protocols have been extensively validated in studies of many groups outside the consortium.^{19–22} As a result these multiplex assays have now become the world standard for PCR-based Ig/TCR clonality testing.

Owing to the successful development of the BIOMED-2/EuroClonality multiplex PCR protocols, the analytical phase of clonality testing has thus largely been standardized. Because of this standardization, Ig/TCR clonality testing has now become technically feasible in a routine diagnostic setting. This is reinforced by the availability of commercial kits to run these assays (InVivoScribe, San Diego, CA, USA). An important consequence of the technical standardization and commercialization is that clonality assays can easily be performed in routine laboratories, even in (smaller) laboratories that only occasionally receive clonality testing requests, and thus have limited experience. However, background knowledge and ample experience are more than ever required for Ig/TCR target choice and accurate interpretation of the PCR results.²³ In an attempt to make interpretation less subjective, interpretation algorithms have been introduced, especially in the United States.^{24–26} These algorithms take into account peak heights and peak ratios to define 'truly clonal' rearrangements. Although clear clones readily fulfill such criteria, the cutoff values used in these algorithms create a false sense of accuracy and might even lead to false-positive or false-negative interpretation. The main problem is that multiplex clonality PCRs, which use primers of different efficiencies, are not quantitative, but merely qualitative assays. Thus, clonality testing much more concerns recognition of molecular patterns, for which accurate interpretation and reporting guidelines are mandatory. Hence, standardization of the pre-analytical and post-analytical phases is urgently needed to prevent misinterpretation and incorrect conclusions of the clonality data obtained.

For this reason standardization of interpretation and quality control are major aims of the EuroClonality consortium, next to education and further innovation in molecular hemato-oncology (see: <http://www.euroclonality.org>). While setting up an external quality assessment (EQA) scheme for Ig/TCR clonality testing, the need for guidelines on how to interpret and report Ig/TCR clonality data has become even more apparent, given the lack of objective criteria to evaluate Ig/TCR data. During recent years, the EuroClonality group has therefore been working on standardization of pre- and post-analytical aspects, including the development of clear guidelines for analysis, interpretation and reporting of the EuroClonality (BIOMED-2) Ig/TCR assays.

Here, we discuss important pre- and post-analytical aspects of clonality testing, provide guidelines for interpretation of clonality results and present a uniform way to report the results of Ig/TCR assays. Collectively, these aspects should help to improve the general performance level of clonality assessment and

interpretation, which will directly impact on routine clinical management (standardized best-practice) in patients with suspected lymphoproliferations.

PRE-ANALYTICAL PHASE

In the pre-analytical phase several aspects are of utmost importance for optimal clonality testing results. These include the 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.

Material type and sample preparation

Particularly relevant for final interpretation is key information on the suspected cell population. This information should come from either (histo)morphology/immunohistochemistry and/or flow cytometric immunophenotyping, and typically concerns the suspected cell lineage and the tumor size, as well as the background of non-suspicious (normal or reactive) lymphoid cells.

It is obvious that the performance of the multiplex Ig/TCR clonality assays is highest with fresh or frozen cell material, as these PCR assays have been validated and standardized for that type of material. However, the use of formalin-fixed paraffin-embedded (FFPE) tissues has also proven to be a realistic option,²⁷ provided that the DNA is not too degraded. Although most nucleic acid isolation procedures (traditional methods and/or kits) generally result in a good yield and purity, it might be important to further purify the DNA isolated from FFPE blocks to remove PCR inhibitors. Because of the inhibitory effect, it is advised to test FFPE DNA at different concentrations (at least two) in the Ig/TCR multiplex PCRs. In this respect, FFPE DNA integrity should be checked upfront to adapt the concentration used in the Ig/TCR assays. In contrast, for fresh-frozen tissues it is sufficient to check DNA integrity afterwards, only when unexpected negative results are obtained in the Ig/TCR tests. DNA integrity can be checked via one of many available PCRs that target a single gene. Though these control tests might have their value, the BIOMED-2 control gene test, being a multiplex assay of differently sized amplicons, is preferred as it probably best reflects the multiplex conditions under which the actual Ig/TCR clonality tests are run¹⁵ and provides a good view of the product sizes that can be expected to be amplified properly. Given the amplicon size of most Ig/TCR targets, amplification of fragments of 300 nucleotides (nt) in the control PCR predicts reliable Ig/TCR testing results. Nevertheless, even when amplicons of maximally 200 nt are obtained, smaller Ig/TCR amplicons might still be evaluated reliably (Table 1).²⁸

Selection of targets

Once the DNA quality is checked and approved, the next important aspect of the pre-analytical phase concerns the selection of the Ig/TCR targets to be evaluated. Target selection typically depends on the amount of available DNA, the DNA integrity and naturally the clinical question, including the type of suspected cell population. If the DNA amount and integrity are not limiting factors, target selection is solely determined by the clinical question.

In the EuroClonality network, an algorithm for target selection has been proposed that depends on the suspected cell population (Figure 1).² Thus, in case of suspected B-cell clonality, generally the three different IGH FR targets are chosen, in parallel to or followed by the IGK targets.¹⁷ Although the consecutive use of IGH and IGK PCRs might be the more cost-efficient approach,²¹ a parallel approach is more time-efficient for both the clinician and the patient. Even though the combination of IGH V–J and IGK targets should be sufficient in the vast majority of cases (>95%), evaluation of the IGH D–J and IGL targets might occasionally be

Table 1. Ig/TCR multiplex PCR: preferred method of analysis, expected size ranges and nonspecific bands^a

Multiplex PCR	Preferred method of analysis	Size range (nt)	Nonspecific bands (nt)
IGH V _H -J _H	GS and HD both suitable	Tube A: 310–360 Tube B: 250–295 Tube C: 100–170	Tube A: ~ 85 Tube B: ~ 228 ^b Tube C: ~ 211 ^b
IGH D _H -J _H	HD slightly preferred over GS (Amplicon variation hampers GS)	Tube D: 110–290 (D _H 1/2/4/5/6-J _H) 390–420 (D _H 3-J _H) Tube E: 100–130	Tube D: ~ 350 ^c Tube E: 211 ^d
IGK	GS and HD have complementary value (Small CDR3 + amplicon variation hamper GS)	Tube A: 120–160 (V _K 1f/6/V _K 7-J _K) 190–210 (V _K 3f-J _K) 260–300 (V _K 2f/V _K 4/V _K 5-J _K) Tube B: 210–250 V _K 1f/6/V _K 7-Kde 270–300 (V _K 3f/intron-Kde) 350–390 (V _K 2f/V _K 4/V _K 5-Kde)	Tube A: ~ 217 ^b Tube B: ~ 404 ^b
IGL	HD slightly preferred over GS (Small CDR3 hampers GS)	Tube A: 140–165	Tube A:—
TCRB	GS and HD both suitable	Tube A: 240–285 Tube B: 240–285 Tube C: 170–210 (Dβ2) 285–325 (Dβ1)	Tube A: ~ 213 ^{b,e} , ~ 273 ^b Tube B: ~ 93, ~ 126, ~ 221 ^{b,e} Tube C: ~ 128, ~ 337 ^{b,e}
TCRG	GS and HD both suitable	Tube A: 145–255 Tube B: 80–220	Tube A:— Tube B:—
TCRD	HD slightly preferred over GS (Low template amount + amplicon variation hamper GS)	Tube A: 120–280	Tube A: ~ 90, ~ 123

Abbreviations: GS, GeneScan; HD, heteroduplex; Ig, immunoglobulin; IGH, IG heavy chain; IGK, IG kappa; IGL, IG lambda; nt, nucleotide; TCR, T-cell receptor; TCRB, TCR beta; TCRD, TCR delta; TCRG, TCR gamma. ^aUpdate of table 25 of earlier BIOMED-2/EuroClonality report. ^bParticularly seen in samples with low numbers of contaminating lymphoid cells. ^cNonspecific 350-bp band is the result of cross-annealing of the D_H2 primer to a sequence upstream of J_H4. In GeneScanning this nonspecific band does not comigrate with D–J products. ^dThe 211-bp PCR product represents product from germline D_H7–J_H1 region; when PCR amplification is very efficient, also longer PCR products might be obtained based on primer annealing to downstream J_H genes; for example, 419 bp (D_H7–J_H2), 1031 bp (D_H7–J_H3), and so on. ^eDetection of nonspecific band depends on quality of primers (batch-dependent).

helpful as second-line approach.¹⁷ This should be applied to cases with strong suspicion of B-cell clonality that is not confirmed by IGH V–J and IGK testing. Analogous to B cells, suspected T-cell clonality can best be addressed by evaluating two TCR targets (TCRB and TCRG), either in parallel or consecutively. Traditionally, TCRB is the gold standard target, but our own results in the EuroClonality network show that TCRB is at least equally informative as first-line target.¹⁶ Importantly, TCR delta (TCRD) (generally together with TCRG) should only be used as a target for well-defined clinical requests, that is, suspected TCRγδ T-cell proliferations or immature (lymphoblastic) T-cell proliferations.^{2,16} Usage of TCRD in other situations merely creates difficulties due to the fact that most TCRD rearrangements are removed in TCRαβ lineage T cells upon rearrangement of the TCR alpha (TCRA) locus; thus, the paucity of TCRD templates might easily give rise to preferential amplification and pseudoclonality. Moreover, even authentic clonal TCRD rearrangements might not be associated to a malignant lymphoid proliferation.²⁹

Priority in selection of targets for cases with low DNA quality or low DNA amount

In case of FPPE specimens with low-quality DNA, some targets (IGH FR3, IGK V–J and TCRG) might still be optimal because of their smaller amplicon size, whereas other targets with larger amplicons (IGH FR1, IGH FR2, IGK Kde and TCRB) could be less reliable. Nevertheless, amplification of the targets with larger amplicons might still be possible in some cases and should not be discouraged upfront. For example, despite a size range of 210–400 nt for IGK Kde amplicons, IGK Kde PCR can be very valuable, even in samples of suboptimal DNA quality.^{20–28}

The priority of targets might not solely depend on amplicon sizes. In case of suspected T-cell proliferations, some targets (TCRG V–J tube B and TCRB D–J) are notoriously difficult when evaluated in isolation. Moreover, also the complementarity of the multiplex PCRs will influence selection of targets that should be assessed in duplicate. Thus, both TCRB V–J tubes and both TCRG V–J tubes

should preferably be analyzed in parallel. Hence, for suspected T-cell proliferations with limited DNA amount and low DNA quality, the recommended multiplex PCRs would be at least TCRG-tube A and TCRB tube A preferably with TCRB tube B. For suspected B-cell proliferations, target choice is based on amplicon sizes but also on the type of suspected lymphoma. For (post-) germinal center lymphomas, IGH D–J and IGK Kde are ideal PCR targets as these are not prone to somatic mutations. However, it should be stressed that for all cases with limited DNA integrity and DNA amount, duplicate assessment of a single PCR tube is highly preferred over testing multiple PCR targets.

Finally, for cases with a low percentage of suspected B or T cells, reproducibility of the profiles is essential. A low number of lymphocytes in, for example, skin or intestinal lesions can easily result in overinterpretation of coincidental dominant peaks. To prevent misinterpretation, assessment of the targets in duplicate as well as adjustment of the amount of DNA by increasing the DNA concentration, and hence the number of cells per PCR, are strongly recommended.

POST-ANALYTICAL PHASE: PITFALLS AND SOLUTIONS

Accurate interpretation and reporting of clonality testing results heavily depends on detailed knowledge on the structure of Ig/TCR genes, their rearrangement patterns and awareness of all nonspecific amplifications and cross-lineage rearrangements. Many of these issues have been addressed extensively over the years in multiple publications on behalf of the EuroClonality group.^{23,30,31} Recently, even a whole issue of the *Journal of Hematopathology* was devoted to multiple aspects of clonality testing.^{27,28,32–37} Owing to their impact on interpretation and reporting, the most relevant technical and immunobiological pitfalls are briefly discussed here (see also Table 2).

Technical pitfalls

If no PCR products (that is, neither clonal nor polyclonal products) are obtained in the multiplex PCR, it is worth checking the underlying reason, as this will have direct consequences for

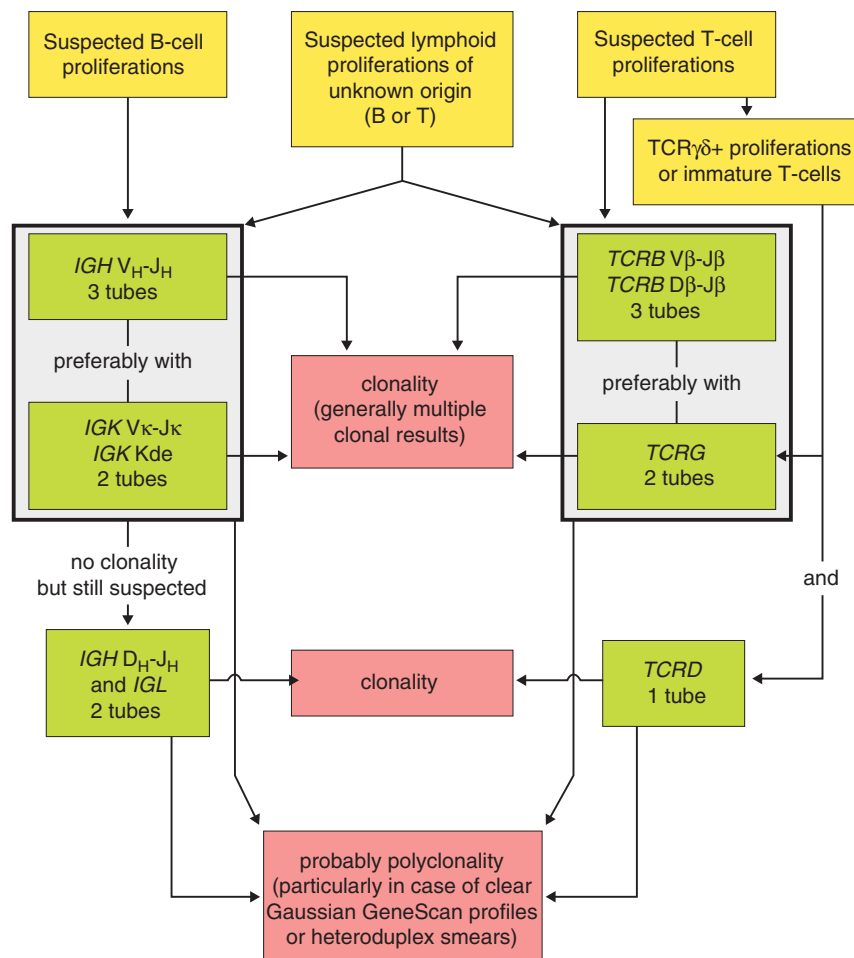


Figure 1. Strategy for PCR-based clonality diagnostics in suspected lymphoproliferations with an inconclusive diagnosis or with unusual histology, immunophenotype or clinical presentation, using the EuroClonality/BIOMED-2 multiplex PCR protocols. In case of a suspected B-cell proliferation, IGH V_H-J_H multiplex PCR analysis is best performed first. As a second step, IGK PCR analysis (V_κ-J_κ and Kde rearrangements) can be chosen. Preferably, these two steps are combined to avoid delay in the diagnostic process. Finally, IGH D_H1-6-J_H PCR analysis (potentially combined with IGL analyses) can be reserved for remaining suspected cases, in which the preceding PCR assays have failed to detect mono-clonality and have not shown clear signs of polyclonality either. For suspected T-cell proliferations, TCRB multiplex PCR is generally slightly more informative than TCRG PCR, but the order of analysis of these two loci can be changed as they provide complementary information; preferably both targets should be used in parallel. Only in case of suspected TCRγδ⁺ T-cell proliferations and immature T-cell proliferations (suspicion of lymphoblastic malignancies), combined TCRG and TCRD PCR analysis is preferred. In case of suspected lymphoproliferations of unknown origin, both Ig and TCR genes should be used as PCR targets. It should be noted that in such cases the clonal Ig/TCR results cannot be used straightforwardly for B-/T-lineage assignment. A full-proof diagnosis of polyclonality remains difficult, but a high probability of polyclonality is supported by clear Gaussian GS curves or HD smears in the absence of clonal results.

interpretation and alternative strategies. Lack of Ig/TCR (mono-clonal and polyclonal) products because of poor DNA quality of the FFPE tissue can be confirmed by checking DNA quality in a control gene multiplex PCR (see above). Furthermore, B- and T-cell numbers can be rechecked using histological slides or flow cytometry data to understand whether the lack of detectable PCR products is caused by lack of B or T cells in the sample. If so, an alternative specimen should be analyzed. For B-cell clonality it is important to verify whether the patient has received CD20 antibody therapy. Finally, extensive somatic mutation in Ig genes might prevent optimal annealing of Ig primers and thus block efficient amplification of the clonal Ig rearrangement. In such cases, alternative Ig targets that are less prone to somatic hypermutation (IGH D-J, IGK Kde rearrangements) should be evaluated (concept of complementarity of targets).^{15,17,38} False-negative results can also be caused by the presence of t(11;14) and t(14;18) aberrations in mature B-cell proliferations. As these aberrant IGH rearrangements are not amplified in the IGH multiplex PCRs, an IGH V-J PCR failure could be expected from

such alleles. Thus, information concerning complementary laboratory tests (for example, fluorescence *in situ* hybridization analysis) should be taken into account when interpreting clonality data.

Based on primer positions and the expected extent of nt insertion at the junctions, amplicon size ranges were previously established for all Ig/TCR multiplex PCR reactions (Table 1). However, as these size ranges represent the approximate 5–95% intervals, amplicons that are a few nts shorter or longer may still represent true rearrangements. Even clearly undersized or oversized amplicons may be considered as true rearrangements in the absence of evidence of nonspecific products in other samples,^{36,39,40} especially in IGH and IGK targets that harbor deletions and insertions through the somatic hypermutation process.⁴¹ Also, amplification from a downstream J gene might create extended PCR amplicons, which could occur as an additional PCR product in the same or the another V-J multiplex reaction (in case of TCRB, IGH and IGK) or as the sole PCR product (in case of IGH and IGK).^{36,42} In the latter situation, this is mostly due to disrupted primer annealing to the J gene of the V(D)J junction owing to the

Table 2. Technical and biological pitfalls in Ig/TCR clonality testing

Phenomenon	Pitfall	Potential solution
Lack of clonal signal and lack of polyclonal Gaussian curve	1. Poor DNA quality 2. Few T/B cells 3. Clonal signal not detected due to SHM in malignant cells	1. Check DNA quality in control PCR 2. Check T-/B-cell content by histology or flow cytometry 3. Evaluate other framework or Ig target
Bands/peaks outside size range	1. CDR3 regions/junctions outside 5–95% size interval 2. Nonspecific product	1. True rearrangement product; in case of doubt, sequence for confirmation 2. Check Table 1 for sizes of nonspecific products
Undersized bands/peaks	For example, internal deletion in <i>VH/Vκ/Vλ</i> gene (SHM related)	Potential rearrangement product; sequence for confirmation
Oversized bands/peaks	For example, extended amplification from downstream <i>J</i> gene (for example, due to SHM in rearranged <i>JH</i> gene)	Potential rearrangement product; sequence for confirmation
Multiple clonal signals	Bi-allelic rearrangements Biclonality	Consider the number of potential rearrangements per allele/locus ^a and judge whether this fits with clonality (with bi-allelic rearrangements) or biclonality
Selective amplification and pseudoclonality, due to low level of specific template	Few T/B cells in sample	Repeat multiple PCRs (same tissue, second independent DNA isolation and/or related tissue) → compare patterns for consistency
Oligo-/monoclonality in histologically reactive lesion	Exaggerated immune response with dominant specificity (for example, large germinal centers)	1. Repeat multiple PCRs (same tissue, second independent DNA isolation and/or related tissue) → compare patterns for consistency between samples and multiple targets 2. Re-evaluate histopathology
Oligoclonal T-/B-cell repertoire in peripheral blood of elderly individuals, immunodeficient patients or transplant patients	Incomplete immune system, for example, in case of immunosenescence or reduced/suppressed lymphocyte production	Repeat multiple PCRs (same tissue, second independent DNA isolation and/or related tissue) → compare patterns for consistency and compare with primary process (in case of staging)

Abbreviations: CDR, complementarity-determining region; HD, heteroduplex; Ig, immunoglobulin; IGK, IG kappa; SHM, somatic hypermutation; TCR, T-cell receptor; TCRB, TCR beta. ^aIn TCRB and IGK loci multiple rearrangements can be detectable per allele, which influences the number of peaks/bands that is compatible with a single clone.³⁵ Complex patterns may be seen after HD analysis.

Table 3. Typical expected Ig/TCR profiles under different immunobiological conditions

Immunobiological condition	Examples	Expected profile in PCR reaction
No lymphocytes	Non-hematopoietic tissue	No peaks/bands (w/o background) ^a
Paucity of lymphocytes	Small infiltrate, small sample (for example, skin)	(Minor) peaks/bands, not reproducible
(Immune)activation with dominant clones	Dominant immune response (for example, infection, autoimmunity)	(Multiple) peaks/bands, reproducible
Reactive lymphocytes	Broad immune response	(Irregular) Gaussian curve/smear
Monoclonality (mono-/bi-allelic)	Leukemia, lymphoma, (clone of unknown significance) ^b	One or two peaks/bands ^c
Monoclonality + polyclonal background	Idem, (small) clone between normal/reactive lymphocytes ^b	One or two peaks/bands ^c + Gaussian curve/smear
Monoclonality (somatically mutated)	Idem, (post-)follicular B-cell process	No peaks/bands ^d (or Gaussian curve/smear from remaining normal lymphocytes) ^a

Abbreviations: Ig, immunoglobulin; IGK, IG kappa; TCR, T-cell receptor; TCRB, TCR beta. ^aNonspecific peak(s)/band(s) might be observed. ^bClone of unknown significance is mostly seen under conditions in which there is some residual background of polyclonal cells. ^cNumber of peaks/bands is dependent upon competition in PCR reaction; for IGK and TCRB loci up to four clonal products may be compatible with one clone. ^dRepresents false-negative result.

somatic mutations. Furthermore, in some cases multiple peaks/bands are observed that result from the same IGK V–J rearrangement, which is explained by some cross-annealing of V family-specific primers to genes of other V families and the fact that these V primers are not clustered.¹⁵

Finally, a general technical pitfall of multiplex PCR reactions is that they run a higher risk of nonspecific amplification than monoplex PCR. This especially holds for those samples in which competition for true Ig/TCR templates is limited due to low numbers of B or T cells. Though several of these nonspecific amplicons have been described before, recently some novel nonspecific products have been found. Therefore, we have now updated the original information of the 2003 publication in Table 1.

Biological pitfalls

Beside the technical issues, also multiple biological pitfalls can pose difficulties in interpretation of clonality testing results. These pitfalls not only concern immunobiological aspects, but also locus-intrinsic aspects.

Translating the number of apparently clonal peaks into the number of clones is not always straightforward. Most lymphocytes undergo rearrangements on both alleles of a particular Ig or TCR locus. Hence, two clonal peaks are more likely to reflect bi-allelic rearrangement patterns than biclonality.⁴³ Nonetheless, true biclonality can occur in up to 5% of mature B-cell lymphoproliferative disorders, and in these cases careful interpretation of IGH/IGK rearrangement patterns must be performed in conjunction with morphology, immunophenotyping

Table 4. The EuroClonality uniform system for technical description

Type of profile per tube (in duplicate)	Technical description per tube	Optional: more detailed technical description ^a
No peaks/bands (but: poor DNA quality)	No (specific) product, poor DNA quality	
No peaks/bands (w/o background)	No (specific) product	Nonspecific product(s) (... nt)
One or two reproducible clonal peaks/bands ^b	Clonal (... nt)	Weak clonal (... nt) Clonal (... nt) + polyclonal background (Gaussian curve/smear)
One or two non-reproducible (clear) peaks/bands ^b	Pseudoclonal	
Multiple ($n \geq 3$) non-reproducible peaks/bands ^b	Pseudoclonal	
Multiple ($n \geq 3$) reproducible peaks/bands ^b	Multiple products ($n = \dots$) (... nt)	
Gaussian curve/smear ^c (with or without minor reproducible peaks/bands ^b)	Polyclonal (not clonal ^c)	Irregular polyclonal (not clonal ^c)
Pattern that cannot be categorized as one of the above	Not evaluable ^d	

Abbreviations: HD, heteroduplex; nt, nucleotide. ^aExamples of more detailed technical description options that can be chosen by the user. ^bIn HD analysis the number of bands does not necessarily reflect the number of different PCR products, as additional HDs can be formed between products. ^cIn HD analysis a polyclonal smear may not always be smooth or clear, despite specific product in gel; hence this is scored as 'not clonal'. ^dIn <5% of PCR results the description per tube cannot be made.

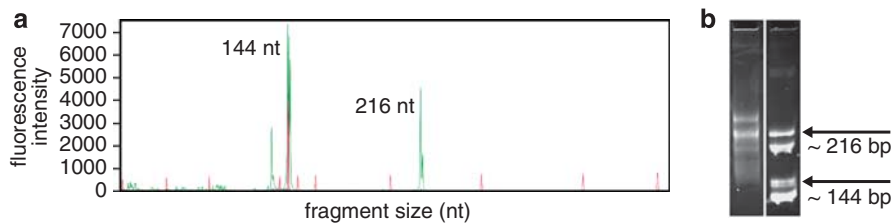


Figure 2. Example highlighting the difficulty to correlate the number of bands in HD analysis to the number of rearrangements. Using TCRG multiplex PCR tube A, two clonal peaks are observed (biallelic rearrangements) in GS analysis (a). In contrast, in HD analysis (b) four bands are seen, two representing the homoduplexes of ~144 and ~216 bp, and the other two representing clonal HDs between the two clonal rearrangements (indicated by arrows).

and/or immunohistochemistry.^{43,44} Furthermore, because of the specific configuration of some Ig/TCR loci (especially IGK and TCRB), multiple rearrangements can be present on the same allele. Consequently, this will have an impact on defining the number of clones, as the presence of up to four different IGK or TCRB rearrangements is still compatible with a single clonal cell population (explained in detail in Langerak *et al.*³⁵).

Additional immunobiological aspects to be considered when interpreting Ig/TCR clonality data, have to do with the number of lymphocytes, in the sample and with the immune reactivity under inflammatory or ageing conditions. The presence of only few B or T cells in the sample (for example, in skin tissues) might cause preferential amplification, leading to the false impression of monoclonality (pseudoclonality). This stresses the importance of evaluating duplicate PCR reactions to establish reproducibility of clonality patterns and products. Under certain conditions (infection and inflammation) multiple clonal peaks might be present as a result of an exaggerated immune response. These immune response-related clonal expansions of lymphocyte populations should not be misinterpreted as signs of malignancy.^{18,45} Detection of an oligoclonal T-cell repertoire in the blood of elderly individuals and immunodeficient patients or transplant patients should be considered as potential sign of an incomplete immune system due to ageing of the system (immunosenescence) and reduced or suppressed T-cell production, respectively.¹ The same might apply to the B-cell

repertoire in an ageing immune system, though possibly to a lesser extent.

EXTERNAL QUALITY ASSESSMENT

To ensure correct performance and accurate interpretation of results in laboratories performing Ig/TCR clonality assays, a robust EQA scheme is an essential instrument. The initial challenge was therefore to standardize EQA for clonality analysis among EuroClonality laboratories and to develop guidelines for analysis, interpretation and reporting of clonality testing results.

In the first five EQA rounds that were organized in the EuroClonality network, the performance of BIOMED-2-based Ig/TCR clonality assessment was tested using DNA samples from different hematological and histopathological cases.⁴⁶ As readout systems both GeneScan (GS) fragment analysis and polyacrylamide heteroduplex (HD) analysis were used.¹⁵ Cases in the various EQA rounds were selected by the individual EQA round organizers to include both classical lymphoproliferations as well as more difficult cases. Each center processed the diagnostic DNA according to their routine PCR workflow dependent on the suspected diagnosis. For several EQA rounds, these DNA samples were accompanied by data files of GS analysis profiles and photographic images of HD gels, which were evaluated by the participants. These electronic data files and images allowed a more accurate assessment of the reproducibility of interpretation

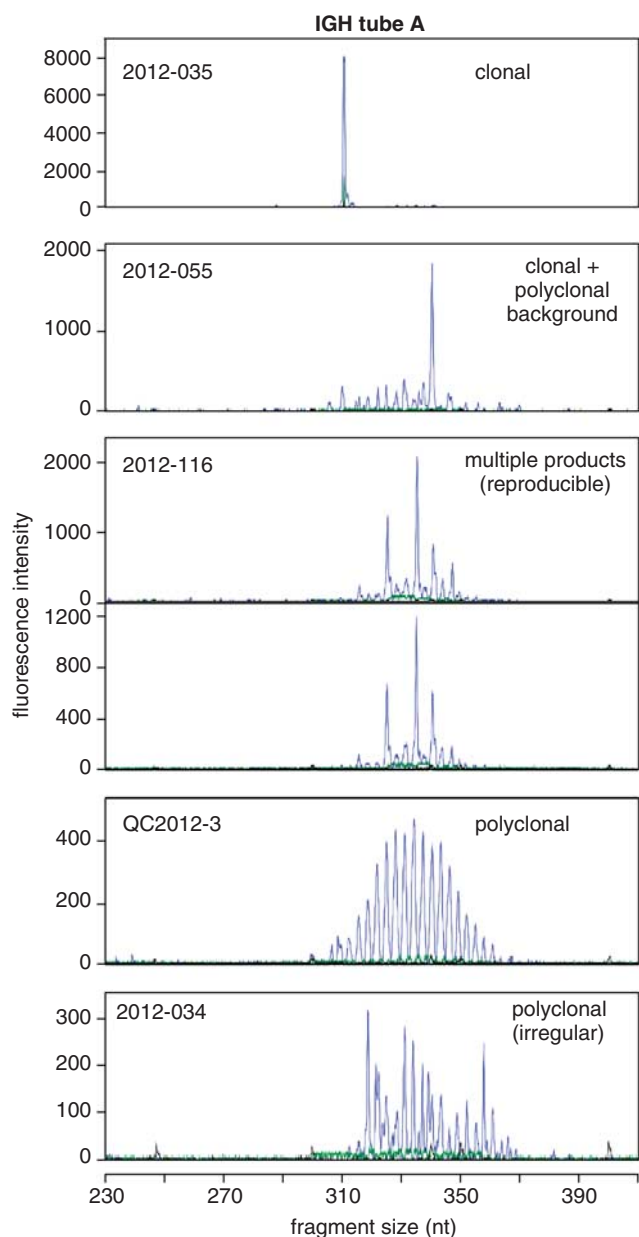


Figure 3. Examples of technical descriptions of different IGH GS profiles. Representative examples of profiles are shown for IGH multiplex tube A. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

and reporting of the data, as in these electronic EQA rounds variation in the analytical phase was eliminated.⁴⁶

EUROCLONALITY UNIFORM DESCRIPTION AND REPORTING OF IGH/TCR DATA

The post-analytical phase of diagnostic PCR-based clonality testing comprises three different levels, (1) technical description per PCR; (2) overall molecular interpretation of clonality testing data (separate for Ig and TCR); and (3) integration of the clonality testing results with morphological, immunophenotypical and clinical data. Therefore, from the first EQA round onwards, a process of developing a standardized technical description of

individual multiplex tubes (level 1) was started. In the later EQA rounds the uniform technical description was further refined to a standardized reporting system. In parallel, reporting of the overall molecular interpretation of Ig and TCR clonality data (level 2) was also standardized. As clinical interpretation is dependent upon morphological, immunophenotypical and/or clinical data, standardization of level 3 was considered to be beyond the scope of EuroClonality. Finally, the revised standardized description system was evaluated by each EuroClonality center using 50 consecutive routine diagnostic cases that were submitted for Ig/TCR clonality testing.

Immunobiological concept as starting point

To create a conceptual basis for the proposed EuroClonality uniform description and reporting of clonality data, we first defined the typical Ig/TCR profiles that could be expected in particular immunobiological conditions (Table 3). Notably, reproducibility of the profiles is an important aspect of this conceptual basis. We therefore strongly advocate the use of duplicates (independent PCR amplifications from the same DNA isolate, or from a second DNA isolate), such that results from the duplicate analyses can be taken into account. In our view and experience having the results from duplicate experiments is pivotal for accurate interpretation of the molecular profiles.

In DNA samples from a specimen without lymphocytes, no rearranged Ig/TCR genes are to be expected. This implies that in such samples no specific Ig/TCR amplicons can be formed, however, it should be realized that nonspecific PCR products of the multiplex assays might become more easily amplified in such samples (Table 1). When few lymphocytes are present in the specimen (so called paucicellular specimens), for example, in a case of a small lymphocyte infiltrate in a skin sample, selective amplification might occur. This is due to a nonrandom distribution of DNA template in the aliquot that is used in the PCR reaction. The resulting Ig/TCR profile will show several peaks or bands that are not reproducible in duplicates. In specimens with numerous reactive lymphocytes an Ig/TCR profile with a more or less complete Gaussian distribution is to be expected, whereas a dominant immune response might create (multiple) peaks/bands that are reproducible in duplicates. Finally, the presence of a monoclonal population of lymphocytes, as in a lymphoid malignancies or as a clone of unknown significance, is predicted to give rise to one or two clear (reproducible) peaks or bands (monoallelic and mostly biallelic rearrangements, respectively), possibly in combination with a polyclonal Gaussian profile of the non-clonal reactive lymphocytes. It should be noted that for some targets (IGH, TCRB and TCRG) the presence of clear monoclonal peaks/bands in one multiplex PCR reaction might result in lack of specific Ig/TCR amplicons in the complementary multiplex PCR targeting the same locus, owing to the lack of remaining reactive lymphocytes. An exceptional situation could be expected in the case of B-cell clones that show a heavy somatic mutation load, in which case the monoclonal product would remain undetected resulting in a Gaussian Ig profile (when reactive cells are present in the background) or a profile without Ig peaks/bands (in case of a high tumor load without reactive cells).

Technical description of individual multiplex PCR reactions

Based on the expected profiles presented in Table 3, we defined a list of technical descriptions for the expected profiles (level 1 of post-analytical interpretation). These technical descriptions are primarily meant to standardize reporting and interpretation within and between laboratories. Nevertheless, these descriptions sometimes appear on the final report to the clinician or pathologist. At first we started with an extensive list of technical descriptions taking into account all kind of subtleties, but gradually this list shortened, as it appeared to be too difficult to find consensus on

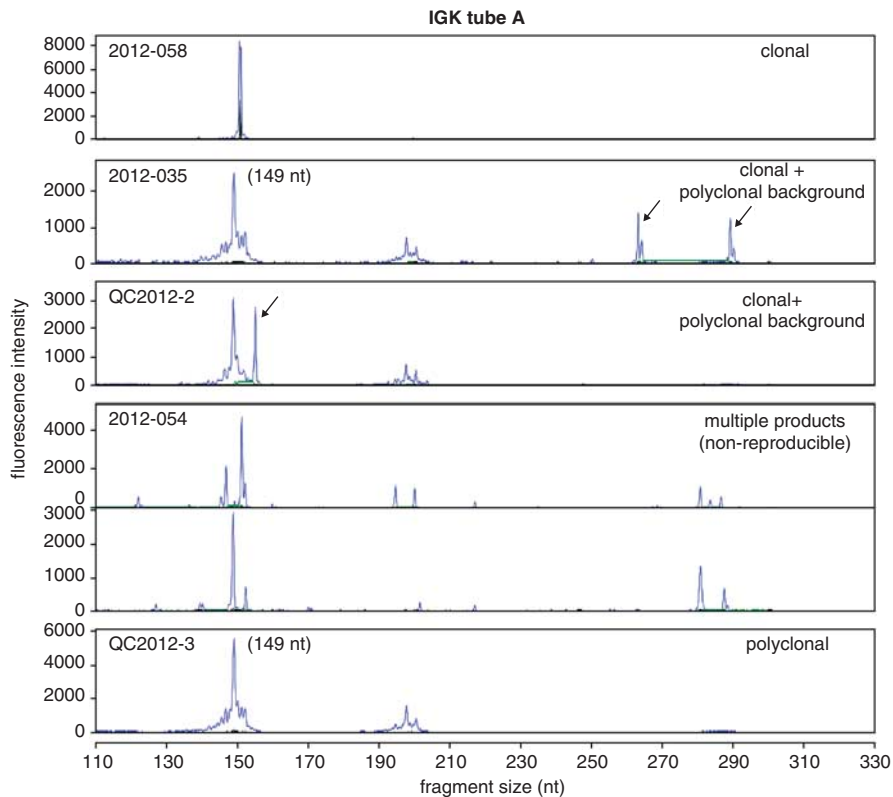


Figure 4. Examples of technical descriptions of different IGK GS profiles. Representative examples of profiles are shown for IGK multiplex tube A. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

distinguishing between several of the more subtle terms. After four EQA rounds, we therefore agreed to make a more limited series of consensus technical descriptions that should clearly distinguish between the main profiles of the multiplex PCRs (Table 4). To describe additional aspects of the profiles, we agreed to have the option of a more detailed description in addition to the main description category. The uniform system for technical descriptions was intended for both GS fragment analysis and gel-based HD analysis, however, it appeared that some of the options are clearly applicable to GS analysis but are not very suitable for describing HD results (Table 4).

When applying these descriptions in daily practice, profiles without peaks/bands will be referred to as 'no (specific) product', with the possibility to indicate that DNA quality appeared to be poor. Optionally, nonspecific PCR products and their amplicon sizes are mentioned. Profiles with one or two clear peaks or bands are called 'clonal', with the option to indicate that the intensities are weak or that a polyclonal background is seen. Even cases that show a relatively weak clonal peak/band in a polyclonal background can be truly clonal, as long as the pattern is reproducible and preferably seen in multiple targets. When one to two peaks or bands are identified that are clearly non-reproducible, this is referred to as 'pseudoclonal'; implicit in this description is that clonal signals are seen, but that they differ in size between the duplicates. Profiles with multiple (defined as three or more) peaks or bands are indicated with the description 'multiple products'. Such profiles can be non-reproducible or reproducible; the former option is very close to 'pseudoclonal', whereas the latter reflects multiple consistent clones probably due to a dominant immune response. It should be noted that in HD analysis the number of bands is not necessarily equal to the number of rearrangements

due to the presence of both homoduplex band(s) as well as HD band(s) formed between the different rearrangements (Figure 2).

The Gaussian profile in GS analysis and the smear in HD analysis are described as 'polyclonal', although the HD smear may not always be clearly visible in which case this profile is best referred to as 'not clonal'. If the Gaussian distribution is not perfectly shaped (especially in GS analysis), the option 'irregular polyclonal' can be used to more accurately describe a profile that is largely polyclonal with some minor peaks. Finally, any profile that cannot be categorized as one of these categories should be referred to as 'not evaluable'. One example might be those profiles in which the signal intensity is in the range of background noise.

To illustrate the main technical descriptions of the various Ig/TCR profiles in GS analysis, we have selected several routine diagnostic cases that showed representative profiles for one or more of the Ig/TCR loci (Figures 3–8).

Molecular conclusion on Ig/TCR gene rearrangement pattern

Based upon the results of the individual PCR targets and using the knowledge of the Ig/TCR loci, the molecular conclusion on the Ig/TCR rearrangement pattern is defined.

Analogous to the list of technical descriptions, we also started with a long list of overall molecular interpretations and conclusions of clonality testing results (level 2 of post-analytical interpretation). These molecular interpretations are based on the technical descriptions of all available Ig/TCR profiles, dealing with B and T cells separately. It should be noted that cross-lineage rearrangements may occur occasionally,^{16,17} and with high frequency in immature lymphoid malignancies.⁴⁷ After careful evaluation in several EQA rounds a consensus system consisting of

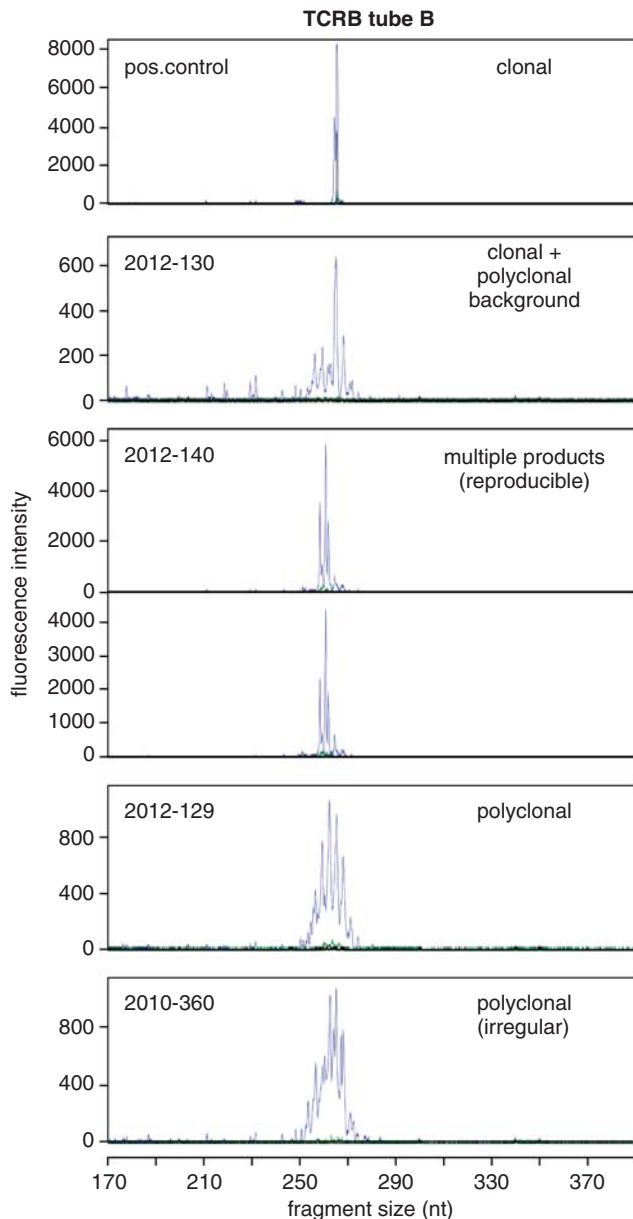


Figure 5. Examples of technical descriptions of different TCRB (V–J) GS profiles. Representative examples of profiles are shown for TCRB multiplex tube B. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

relatively few categories emerged (Table 5). Once again, the more detailed conclusions are optional.

In case of poor DNA quality or if profiles in the Ig or TCR targets are not evaluable, the overall conclusion is 'not evaluable'. When no specific products are seen in any of the Ig or TCR targets, the overall molecular conclusion is 'no rearrangements in Ig/TCR targets detected'. Pseudo-clonal profiles and profiles with multiple non-reproducible products are grouped under the conclusion 'no clonality detected, suggestive of low template amount'. The other three main categories of molecular conclusions are as follows: 'clonality detected', 'oligoclonality/multiple clones detected' and 'polyclonality detected (or no clonality detected in case of weak or faint smears in HD analysis)'. Each of these main conclusions comes with one or more optional conclusions that contain more

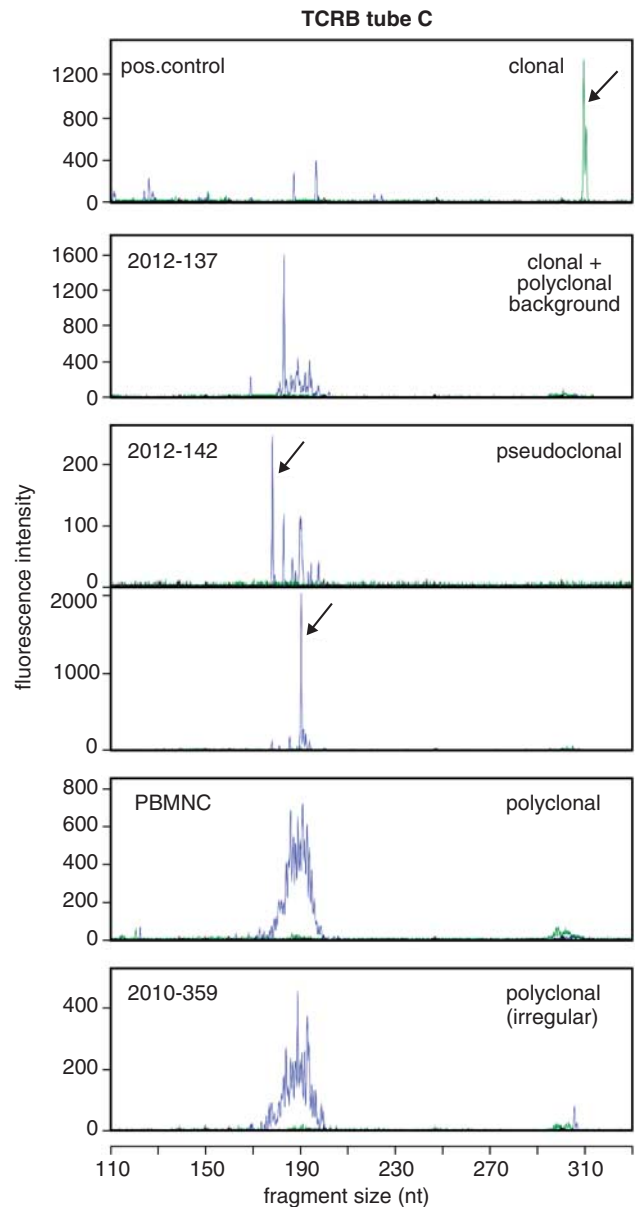


Figure 6. Examples of technical descriptions of different TCRB (D–J) GS profiles. Representative examples of profiles are shown for TCRB multiplex tube C. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

details with respect to biallelic products, minor clonal products or an oligo/polyclonal background. Importantly, the molecular conclusion of 'clonality detected' can be made, even if not all profiles show a clonal pattern.

Validation of the EuroClonality uniform description and reporting system

We aimed to develop a description and reporting system that should be applicable to >95% of diagnostic cases. To validate the uniform system presented here, each of the EuroClonality laboratories scored 50 consecutive cases that were submitted for routine Ig/TCR clonality testing in their institutes. In this way, the system was evaluated on a total of >1150 cases, representing >750 B-cell clonality requests and >620 T-cell clonality requests.

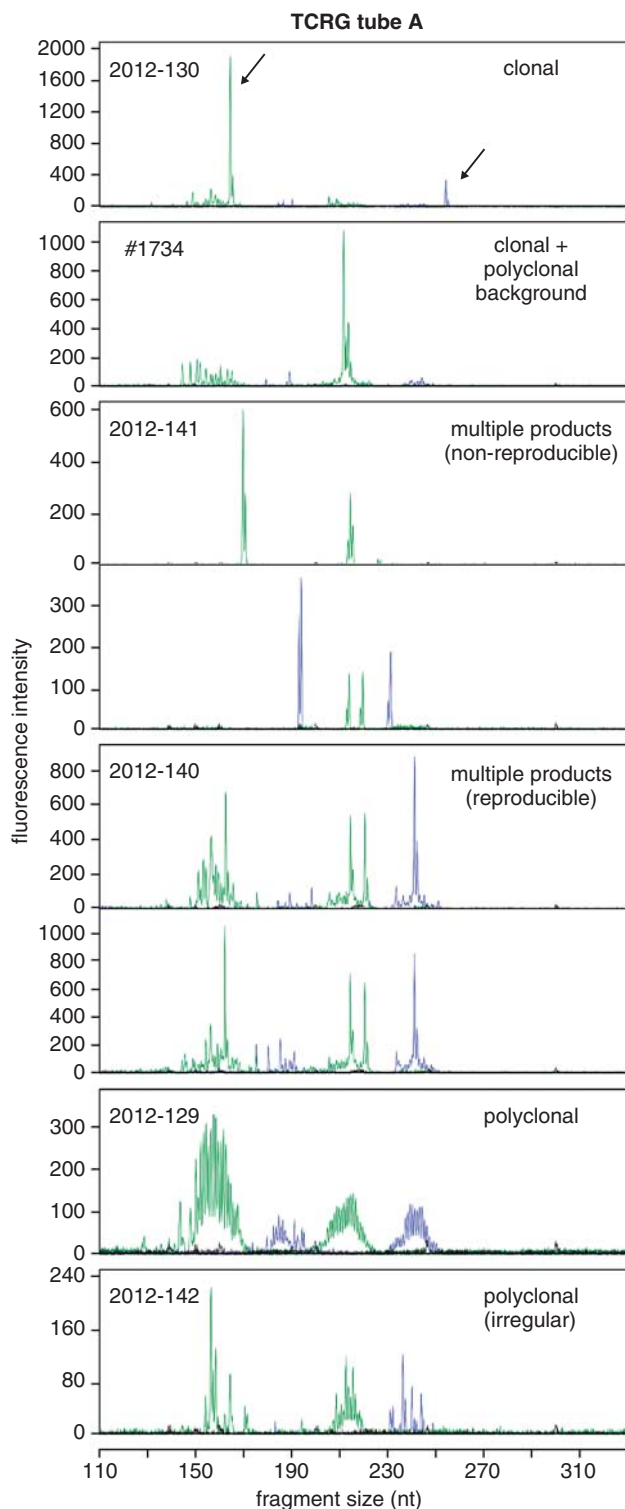


Figure 7. Examples of technical descriptions of different TCRG GS profiles. Representative examples of profiles are shown for TCRG multiplex tube A. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

Overall, the description and reporting system was difficult to apply in only 36/1150 (~3.1%) cases. Thus, applicability was above the 95% target threshold, implying that the system works

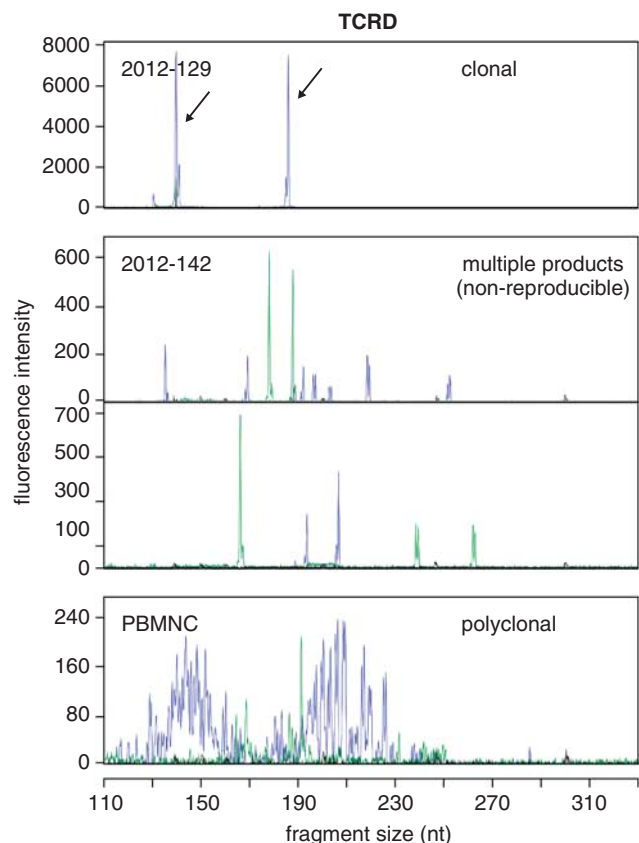


Figure 8. Examples of technical descriptions of different TCRD GS profiles. Representative examples of profiles are shown for TCRD multiplex tube. All assays have been performed in duplicate, but owing to space constraints duplicates are only shown for some technical descriptions in which the reproducibility of the pattern is crucial for proper choice of the term.

well for routine clonality diagnostics in multiple centers that have harmonized techniques and interpretation. Most of the difficulties appeared to be centered around low-intensity clonal signals resulting in a description of 'clonality' or 'polyclonality' with the additional remark that a minor clone might be present. This is one of the well-known gray areas in interpretation that will be hard to completely standardize between centers.

CONTINUING MEDICAL EDUCATION THROUGH WORKSHOPS

Standardization and quality control is critically important for optimizing clinical diagnostics for patient management. The other important prerequisite for increasing quality of diagnostics is continuous education, not only for new laboratories but also for experienced users. Education is consequently another major aim of the EuroClonality consortium. For this reason, annual educational workshops on clonality testing are organized for the diagnostic community. These 3-day workshops are especially meant for laboratories with some experience with the multiplex PCR assays for Ig/TCR gene rearrangements that bring their problematic cases for discussion. In addition, the workshop is also relevant for those laboratories that have only just started to perform PCR-based clonality testing in acquiring a broad understanding of the technical and biological pitfalls. Apart from lectures on basic aspects on gene rearrangements and immunobiological and technical pitfalls of clonality testing, the main purpose of these workshops is to have interactive sessions between participants (pathologists or hematologists plus molecular biologists) and faculty. In the various sessions of the

Table 5. The EuroClonality uniform system for molecular conclusion

<i>Overall technical description for all Ig or TCR targets</i>	<i>Molecular interpretation/conclusion</i>	<i>Optional: more detailed molecular interpretation^a</i>
No (specific) product, poor DNA quality No (specific) product	Not evaluable, due to poor DNA quality No rearrangement in Ig/TCR targets detected	
Clonal (... nt) ^f	Clonality detected	Clonality detected (biallelic products) Clonality detected (biclinality) Clonality detected (minor clonal product) Clonality detected (isolated rearrangement) clonality detected (with caution, plus advice for follow-up analysis/new sample) Clonality detected in addition to background of B/T cells
Pseudoclonal (one or more non-reproducible products)	No clonality detected, suggestive of low template amount	
Multiple reproducible products ($n \geq 3$) ^b	Oligoclonality/multiple clones detected	Dominant clone in oligo/polyclonal background
Polyclonal (not clonal ^c)	Polyclonality detected (no clonality detected ^c)	Polyclonality detected plus minor clone of unknown significance ^d
Not evaluable	Not evaluable ^e	

Abbreviations: HD, heteroduplex; Ig, immunoglobulin; IGK, IG kappa; nt, nucleotide; TCR, T-cell receptor; TCRB, TCR beta. ^aExamples of more detailed molecular interpretation options that can be chosen by the user. ^bFor IGK and TCRB loci up to four clonal products may be compatible with one clone. ^cIn HD analysis a polyclonal smear may not always be clearly detectable, despite specific product in agarose gel; hence this is scored as 'not clonal'. ^dFor those cases in which minor reproducible peaks/bands are detected in the polyclonal background. ^eIn <5% of cases the molecular interpretation cannot be made. ^fClonal peaks/bands are not necessarily seen for every Ig/TCR target analyzed to reach the molecular conclusion 'clonality detected'.

workshop, difficult cases with respect to histopathology and molecular clonality data are presented by the participants themselves, and interpretations are discussed. These multidisciplinary meetings perfectly illustrate the idea of an integrated approach that is advocated to optimize interpretation of clonality data. More details on the workshops can be found at the EuroClonality website (<http://www.euroclonality.org/workshop.html>).⁴⁸

FREQUENTLY ASKED QUESTIONS (FAQS) AND REVIEW OF DIFFICULT CASES

A FAQs section is also present on the EuroClonality website. This FAQs section contains a list of the most frequent issues from the past years, ranging from 'detection rate of clonality tests' to 'software used in clonality analysis'. The FAQs have been answered by an expert team of molecular biologists routinely involved in clonality testing in a pathological or immuno-hematological setting. For questions that are not addressed in the FAQ section, EuroClonality offers an online support service.⁴⁸ To this end, a Review Board of experts is available to respond to queries on difficult cases that pose trouble in interpretation. Scientists who wish to use this service can upload their data files and query via the EuroClonality website (<http://www.euroclonality.org/Support.php>). Subsequently, teams of experts from the areas of hematopathology and hematology-immunology will provide advice on interpretation, reporting and further testing within 10 working days. Many requests have been submitted to and addressed by the Review Board, which clearly illustrates the role that such an online service has in addition to publications, guidelines and expert opinion papers.

CONCLUSION

Following earlier standardization of the analytical phase of Ig/TCR clonality testing, the EuroClonality consortium now developed a uniform reporting system for describing results and conclusions of Ig/TCR clonality assays. At the outset several criteria were defined for the reporting system: (i) it should address two levels, that is, (a)

technical description of individual (multiplex) PCR results and (b) overall molecular conclusion for B and T cells; (ii) it should be useful for reporting both HD gel profiles and GS profiles; and (iii) the system should be applicable to at least 95% of routine cases. After validation in multiple EQA rounds and consequent finetuning, we now have a consensus reporting system that fulfils all these criteria. Clearly, two different levels are concerned with the reporting system. To use the system in reporting both HD and GS analysis results, we have defined a successful approach, although it should be stressed that some of the options are more difficult to apply to gels and will therefore be less commonly chosen for HD-based results. This especially holds true for describing the number of bands in HD analysis as there is a less direct relation between the number of bands and the number of different rearrangements, due to the presence of additional HDs between the different rearrangements and due to single-strand DNA molecules. Finally, we reached the predefined aim to design reporting guidelines that are applicable in >95% of routine diagnostic cases, with only 3% of difficult cases in a large cohort of >1150 routine cases that we studied within the EuroClonality consortium. The uniform reporting system will also become available in other languages via the website (<http://www.euroclonality.org>). It can now be further validated in other EQA schemes and may eventually be used to judge performance of individual laboratories in this type of diagnostics.

CONFLICT OF INTEREST

The EuroClonality (BIOMED-2) consortium is an independent scientific consortium that aims at innovation, standardization and education in the field of diagnostic clonality studies. All acquired knowledge and experience are shared with the scientific and diagnostic community during lectures and educational workshops. The relevant intellectual property has been protected by the patent 'Nucleic acid amplification primers for PCR-based clonality studies' (PCT/NL2003/000690), which is collectively owned by the EuroClonality (BIOMED-2) consortium and licensed to InVivoScribe. The revenues of the patent are exclusively used for EuroClonality (BIOMED-2) consortium activities, such as for covering (in part) the costs of the consortium meetings, the EuroClonality Educational Workshops and the costs of collective experiments.

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