Detection of *MYD88* L265P Mutation by Real-Time Allele-Specific Oligonucleotide Polymerase Chain Reaction

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Abstract: *MYD88* L265P mutation has been reported in ~90% of Waldenström's Macroglobulinemia (WM) patients and immunoglobulin M (IgM) monoclonal gammopathies of uncertain significance (MGUS), as well as in some cases of lymphoma and chronic lymphocytic leukemia. The present study aimed to develop a real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) to detect the *MYD88* L265P mutation. We first evaluated the reproducibility and sensitivity of the technique with a diluting experiment of a previously known positive sample. Then, we evaluated the applicability of the methodology by analyzing 30 selected patients (10 asymptomatic WM, 10 symptomatic WM, and 10 IgM MGUS) as well as 10 healthy donors. The quantitative ASO-PCR assay could detect the *MYD88* L265P mutation at a dilution of 0.25%, showing an inverse correlation between the tumor cell percentage and the cycle threshold (CT) value, thus allowing for tumor burden quantitation. In addition, mutated cases were distinguished from the unmutated by >10 cycles of difference between CTs. To sum up, ASO-RQ-PCR is an inexpensive, robust, and optimized method for the detection of *MYD88* L265P mutation, which could be considered as a useful molecular tool during the diagnostic work-up of B-cell lymphoproliferative disorders.

Key Words: Waldenström's Macroglobulinemia, *MYD88*, ASO-PCR, diagnosis

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Waldenström's Macroglobulinemia (WM) is a rare lymphoproliferative disorder (LPD) characterized primarily by monoclonal immunoglobulin M (IgM) hypersecretion associated with bone marrow infiltration by lymphoplasmacytic lymphoma.[1] Although the oncogenic basis of WM has not been defined yet, Next-Generation Sequencing techniques have identified disease-associated mutations that could be responsible for the final phenotype and clinical behavior.[2–6] Among such mutations, the c.794 T > C change, that results in an aminoacid substitution from leucine to proline at position L265P in the *MYD88* gene (38182641 in chromosome 3p22.2),[7] has been reported to be present in approximately 90% of WM patients.[7–11]

Once the *MYD88* L265P mutation was described with high throughput sequencing techniques, it was extensively tested in several hematological malignancies and, apart from WM, it was described to be present at variable frequencies in non-IgM–secreting lymphoplasmacytic lymphoma,[7,9] diffuse large B-cell lymphoma (DLBCL) of the activated B-cell type,[4,12–16] DLBCL of leg-type,[17] primary central nervous system lymphoma,[18,19] mucosa-associated lymphoid tissue lymphoma,[8,12] marginal zone lymphoma,[7–10,20,21] IgM monoclonal gammopathy of uncertain significance (IgM-MGUS),[7,9–11,22] and chronic lymphocytic leukemia.[5,9,11] In addition, it has also been studied, although not found, in multiple myeloma,[7,9,11,23] IgG and IgA MGUS,[9,11] hairy cell leukemia,[9] acute leukemia,[23] ocular mucosa-associated lymphoid tissue lymphoma,[24] and amyloidosis.[9]

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Altogether, these data illustrate that the identification of the presence/absence of the *MYD88* L265P mutation can be very useful during the diagnostic work-up of B-cell LPDs. In addition, several reports have suggested that the use of a sensitive technique could be of great help during the follow-up monitoring of the patients,[10,11] even for a better definition of response criteria of WM.[25] Accordingly, the assessment of the *MYD88* L2645P mutation has been carried out with different strategies, such as PCR amplification and Sanger sequencing,[16–22] high-resolution melting analysis,[15] digestion with restriction enzymes,[8] and allele-specific PCR (AS-PCR).[9–11] However, all of them have some disadvantages mainly concerning the sensitivity and specificity, ability to quantitate, simplicity, cost-effectiveness, laboriousness, or applicability. Therefore, we aimed to develop a cheap, rapid, and reliable tool for the detection of *MYD88* L265P mutation, considering that it could be potentially useful for the diagnosis and management of WM and related lymphoid neoplasms.[10,11]

MATERIALS AND METHODS

Samples

We analyzed bone marrow samples from 30 patients with asymptomatic WM (n = 10), symptomatic WM (n = 10), or IgM-MGUS (n = 10) and peripheral blood samples from 10 healthy donors. Diagnoses had been made according to the latest World Health Organization classification of tumors of hematopoietic and lymphoid tissues.[26] Median percentages of clonal cells evaluated by flow cytometry were 1.73% (0.5 to 5) in MGUS and 8.0% (1.7 to 59.1) in WM. All cases evaluated here had been analyzed in our previous study,[9] using the commercial assay "qBiomarker Somatic Mutation Assay for MYD88_85940" (SABiosciences, Qiagene Co., Hilden, Germany). The patient samples selected for the present study were known to be positive for the *MYD88* L265P mutation, whereas the control samples, obtained from healthy donors, were known to be negative. DNA extraction was automatically carried out with the MagNA Pure System (Roche Diagnostics, Manheim, Germany).

Immunophenotypic Analysis and Clonal Cell Quantification

Immunophenotypic evaluation was carried out using conventional methods, previously described by our group,[27,28] and following the general recommendations of the EuroFlow group for the evaluation of Hematological Malignancies.[29–31] These cases were immunophenotyped using 4-color combinations including up to 20 different

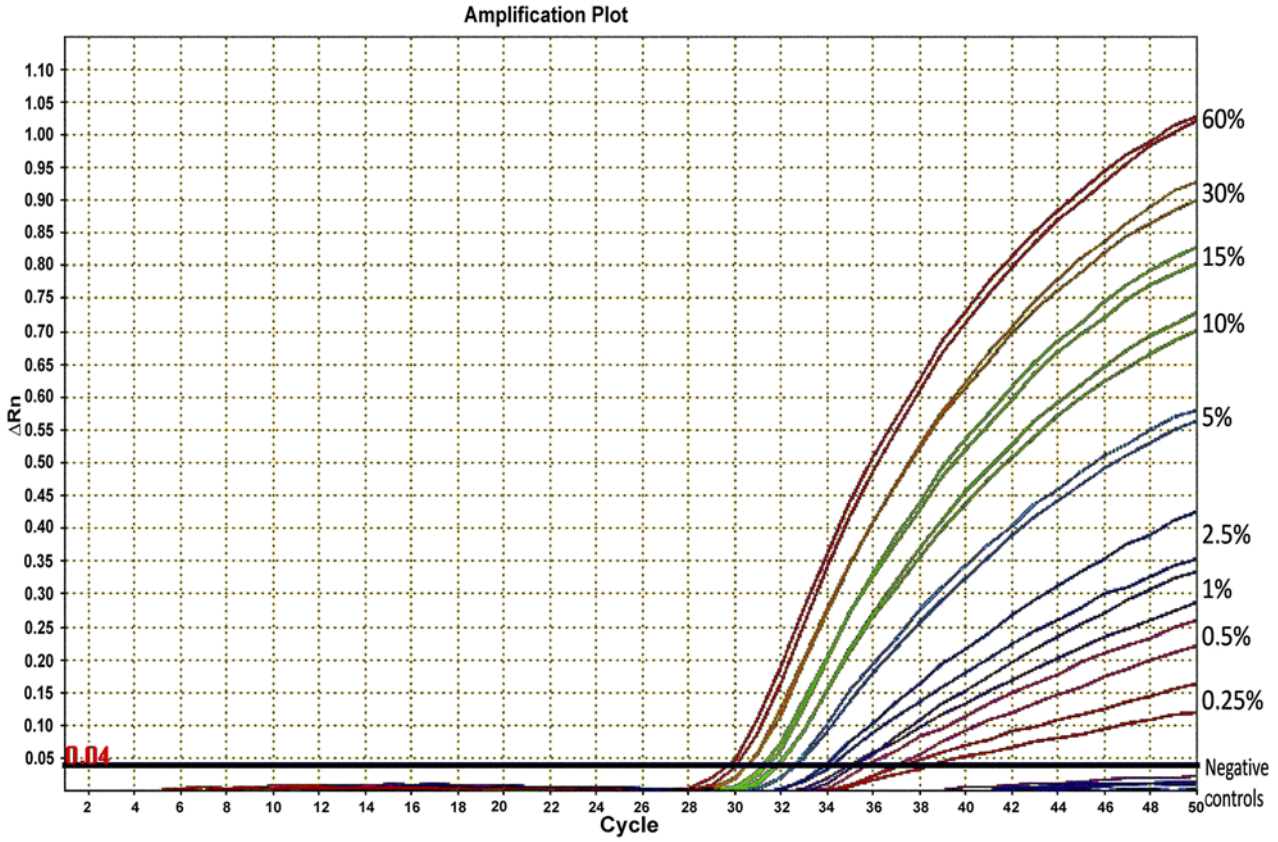

FIGURE 1. Sensitivity plot for quantitative allele-specific polymerase chain reaction. Every serial dilution (60%, 30%, 15%, 10%, 5%, 2.5%, 1%, 0.5%, and 0.25%) was made in duplicate. As it can be observed in the amplification plot, the *MYD88* L265P allele can be detectable to a dilution of 0.25%. Negative controls (also in duplicate) have cycle threshold values >50.

antibodies in addition to surface IgM (sIgM) and cytoplasmatic Ig λ and κ (cyIgλ and cyIgκ). The percentage of clonal cells was determined in with the tube including the sIgM/CD25/CD19/CD38 MoAb. Data acquisition was performed in a FACSCalibur flow cytometer (Becton Dickinson Biosciences—BDB, San Jose, CA) using the FACSDiva software (version 6.1; BDB), and a 2-step acquisition procedure for total and $CD19^+$-only events. Data analysis was performed using the Infinicyt software (Cytognos, Salamanca, Spain).

PCR Design

The real-time allele-specific oligonucleotide PCR (ASO-RQ-PCR) here developed was based on the use of 2 reverse primers differing in the last nucleotide (at 3′ position) so that they are specific either of the wild-type or the mutated allele. To prevent the amplification of the non-matching primer (increasing specificity), an additional nucleotide mismatch ($C > G$) was introduced next to the mutated base. ASO primers were designed using the Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) and were as follows: 5′-CCTTGTACTTGATGGGGAT CA-3′ (wild-type) and 5′-CCTTGTACTTGATGGG GATGG-3′ (mutated). The common forward primer was 5′-ACTTAGATGGGGGATGGCTG-3′, for a final 142 bp PCR product. In addition, a specific TaqMan probe was designed for the PCR assay with the Primer Express Software v3.0.1 (Applied Biosystems, Foster City, CA), that yielded the following 6FAM dye—TAMRA labeled probe: 5′-TTGAAGACTGGGCTTGTCCCACC-3′.

Real-Time ASO-PCR Development

Each experiment required 2 different PCR reactions: one for the detection of the mutation (with the mutated reverse primer) and the other one as a control of the DNA quality (using the wild-type reverse primer). Each reaction was carried out in a final volume of 20 μL, containing 300 nM of each primer (forward and reverse wild-type or mutated), 200 nM of the probe, 1× of the TaqMan Universal PCR Master Mix (Applied Biosystems), and 20 ng of genomic DNA.

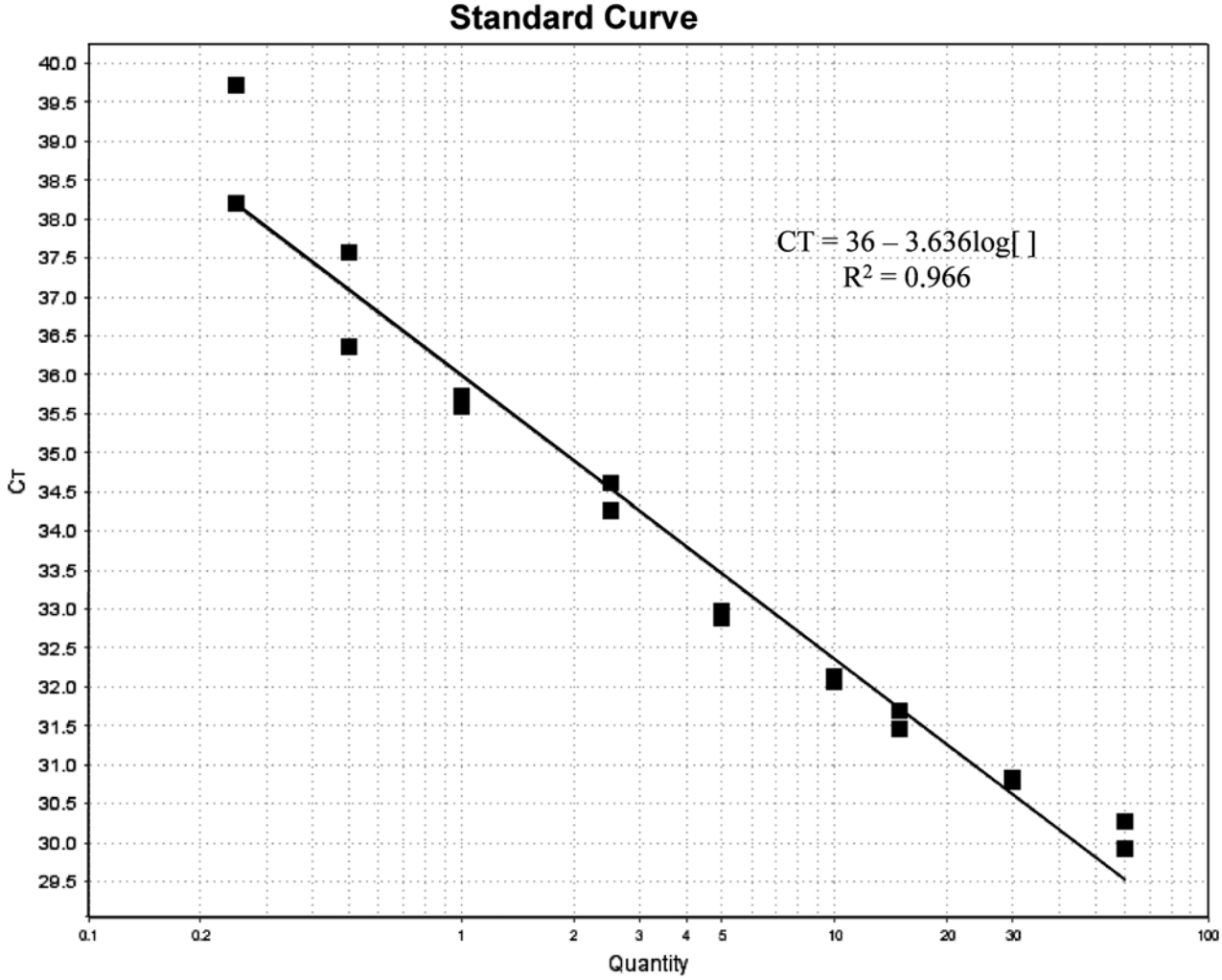

FIGURE 2. Standard curve for AS-RQ-PCR: correlation coefficient, 0.966; slope value = −3.636. CT indicates cycle threshold.

Experiments were performed in a StepOnePlus Real-Time PCR System (Applied Biosystems) and consisted of an initial denaturation step of 10 minutes at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Data were analyzed with the StepOne Software v2.1 (Applied Biosystems). The cycle in which fluorescent emission reaches 10-fold the basal emission is known as the cycle threshold (CT), a value that is proportional to the copy number of the target gene.

RESULTS

Sensitivity Assay

Firstly, we carried out a dilution study to establish the detection limit of our technique. We analyzed the DNA of a patient with the heterozygous mutation (previously found by Sanger sequencing) diluted in wild-type DNA to different concentrations: 60%, 30%, 15%, 10%, 5%, 2.5%, 1%, 0.5%, and 0.25%. All dilutions were tested in duplicate, including 2 controls lacking the mutation. This experiment allowed us to generate the standard curve (SC) for future quantifications.

As Figure 1 shows, ASO-RQ-PCR could detect the *MYD88* L265P mutation at a dilution of 0.25% with >10 cycles of difference from the wild-type DNA background. There was an inverse correlation between the tumor cell percentage and the CT value, which allowed good tumor burden estimation. The correlation coefficient of the SC was 0.966 with a slope value of −3.636 (Fig. 2).

Evaluation of *MYD88* L265P Mutation

A mutation analysis was carried out in the 40 samples selected for this study. All results were in accordance with those already obtained with the commercial assay. The CT value was always >50 in healthy donors, and between 29.6 and 38.1 in MW and 33.4 and 38.4 in MGUS (what means that there were >11 cycle differences between samples with and without the mutation) (Fig. 3).

A dot plot with the percentage of clonal cells evaluated by multiparametric flow cytometry versus the percentage provided by the SC was done to confirm that both estimations correlated well (Fig. 4). Accordingly, the corresponding estimates of both methodologies provided a Pearson correlation coefficient, R, of 0.917 ($P = 1.0 \times 10^{-12}$, Fig. 4).

DISCUSSION

In this study, we have developed and validated a simple inexpensive tool for the detection of the *MYD88*

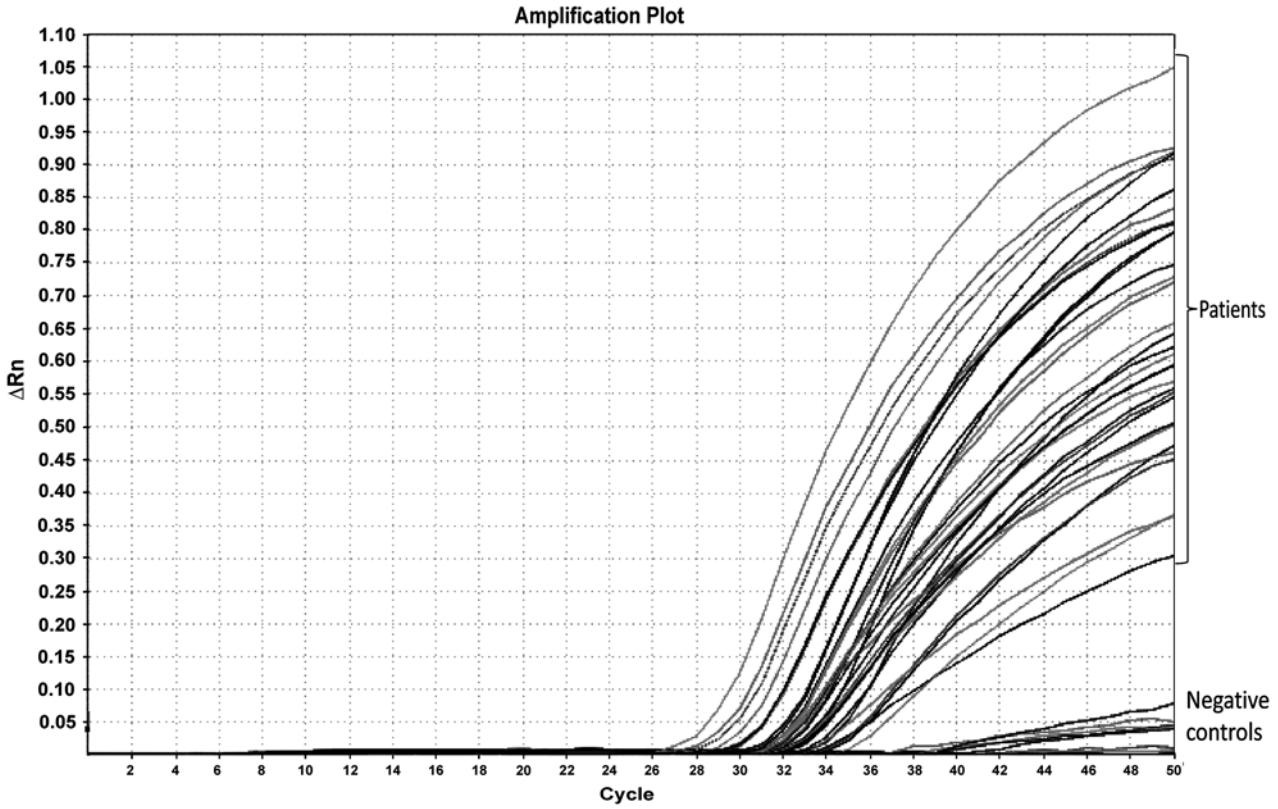

FIGURE 3. Determination of the *MYD88* L265P mutation in 10 asymptomatic MW, 10 symptomatic MW, 10 IgM-MGUS, and 10 healthy donors, by AS-RQ-PCR. Positive cases (in the upper part of the figure) can be distinguished from negatives (at the bottom), whose cycle threshold value is >50.

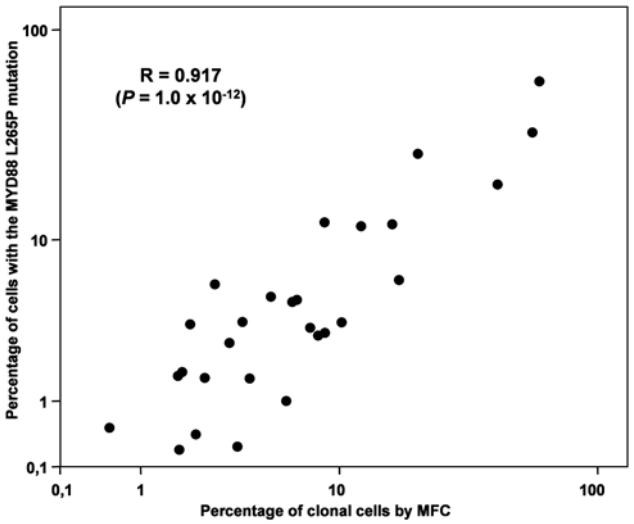

FIGURE 4. Correlation between the percentage of clonal cells evaluated by multiparametric flow cytometry (MFC) versus the percentage provided by the standard curve (SC): Pearson correlation coefficient, $R = 0.917$ ($P = 1.0 \times 10^{-12}$).

L265P mutation which may have a potential use in diagnostic discrimination or as a prognostic marker.

For this purpose, we have used a standard methodology for primers and probes design that has demonstrated to produce robust and reproducible PCR experiments in extensively used minimal residual disease studies.[32] However, we have also introduced some modifications in the mutant-specific amplification primer design that are thought to increase the specificity of the PCR reaction, such as the addition of mismatches close to the 3′ end.[33,34] This allowed us to produce a PCR reaction in which the negative result was always 10 CT (or more) higher than the highest positive result. This has to be combined with the high sensitivity that is offered by the use of TaqMan probes in quantitative PCR systems. In our case, PCR experiment rendered a sensitivity close to 10^{-3}, which means that 1 mutated cell can be detected within 1000 wild-type cells.

There are other methods to analyze the *MYD88* L265P mutation, although ASO-RQ-PCR has some advantages.[8–11,15–22] Sanger sequencing has demonstrated the presence of the mutation in several series,[12,17–19,22] but it has a relatively high cost and low sensitivity, requiring at least 10% to 40% of mutated cells (mean 25%).[15] This sensitivity can be improved. Wang et al[15] demonstrated that PCR and high-resolution melting analysis can achieve a sensitivity of 5%, similar to the achieved by Gachard et al,[8] with the use of PCR followed by enzymatic digestion restriction enzymes. However, this sensitivity is still insufficient for certain entities characterized by low tumor burden, even $<1\%$, such as IgM-MGUS,[28] which is characterized by high prevalence of the mutation ($>50\%$).[9–11,22] Finally, some reports have used AS-PCR followed by agarose gel electrophoresis, which increases sensitivity to 10^{-2}.[10,11] Although post-PCR handling has some disadvantages in terms of laboriousness and risk of cross-contamination, this detection limit approaches the optimal requirements for diagnostic and follow-up purposes. However, there is another disadvantage, as this methodology could lead to false-negative results, especially in the context of MGUS evaluation or monitoring of residual disease after treatment in WM symptomatic patients. The group from Boston has proposed a variation of this PCR by using SYBR Green and a Real-Time PCR equipment, improving their detection limit up to approximately 10^{-3},[11] which is adequate for diagnostic purposes and probably for most monitoring studies. However, this sensitivity gain is hampered by a relatively loss of specificity, as the highest CT value of the positive cases is only 3 cycles below the CT value of the wild-type cases. This raises the concern of possible false-positive results, especially when we require high sensitive values (ie, posttreatment evaluations). Our strategy, with the use of an additional base mismatch at the very 3′ end of the primer, allows to obtain very high CT values for wild-type samples (beyond 50—absolute lack of amplification, or at least cycle >10 cycles respect the mutated samples). This makes our system very robust in terms of specificity, without any loss of sensitivity.

The main limitation of the assay here suggested would be the sensitivity, as 10^{-3} is below to the usual sensitivity reported for ASO-PCR assays.[32] However, we have to remind that the discrimination capacity relies on a single-base change, so any trial to improve the sensitivity could result in a reduction of the specificity. In addition, another commonly recognized disadvantage of our approach is the use of TaqMan probes, which could result in a cost increase respect to other experiments. For instance, SYBR Green assays would be less expensive, keeping the suitability for quantitative mutation detection. Nevertheless, the TaqMan assay adds advantages in terms of specificity[35] with a negligible cost increase for optimized PCR assay in a routine laboratory (<1 €/experiment).

In conclusion, we hereby present an inexpensive, robust and optimized ASO-RQ-PCR assay for the detection of *MYD88* L265P mutation, which could be considered as a useful molecular tool for the evaluation of B-cell LPD.

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