Chapter 6

Quality Control for IG/TR Marker Identification and MRD Analysis

Eva Fronkova, Michael Svaton, and Jan Trka

Abstract

Selection of the proper target is crucial for clinically relevant monitoring of minimal residual disease (MRD) in patients with acute lymphoblastic leukemia using the quantitation of clonal-specific immunoreceptor (immunoglobulin/T cell receptor) gene rearrangements. Consequently, correct interpretation of the results of the entire analysis is of utmost importance. Here we present an overview of the quality control measures that need to be implemented into the process of marker identification, selection, and subsequent quantitation of the MRD level.

Key words Minimal residual disease, Acute lymphoblastic leukemia, Quality control, Next-generation sequencing, PCR

1 Introduction

Minimal residual disease (MRD) monitoring became the standard tool for acute lymphoblastic leukemia (ALL) patient risk stratification. Development of the methodology, as started by the leading pediatric international consortia, has led to the wide acceptance of this approach by both pediatric and adult hematologists alike. Among all potentially available strategies for MRD follow-up analysis, detection and subsequent quantitation of immunoreceptor gene (immunoglobulin/T cell receptor; IG/TR) rearrangements have become the gold standard. IG/TR-based MRD monitoring is currently not only used in frontline treatment of ALL patients but also for the prediction of outcome after relapse of ALL and for follow-up analysis of patients before and after hematopoietic stem cell transplantation (SCT).

As really crucial treatment decisions are being made based on the results of MRD measurement, the accuracy of the method is critical. At particular time-points of treatment, both potential false-negative and false-positive results may have serious consequences.

Anton W. Langerak (ed.), *Immunogenetics: Methods and Protocols*, Methods in Molecular Biology, vol. 2453, https://doi.org/10.1007/978-1-0716-2115-8_6, © The Author(s) 2022

Therefore, quality controls must be an integral part of this approach throughout all the critical procedures of MRD marker identification, selection, and follow-up analysis.

Here, we summarize the critical steps in marker identification and MRD analysis together with the description of related quality control measures.

2 IG/TR Marker Identification

2.1 PCR-Based Marker Identification

A classical approach of clonal marker identification includes PCR amplification, clonality assessment, and Sanger sequencing of PCR products. The strategy for choosing IG/TR markers for amplification differs based on the type of malignancy. In CLIP laboratories, we prefer to use separate singleplex PCR reactions for ALL (25 for B-ALL, 20 for T-ALL), as described by the BIOMED-1 consortium [1, 2], with frozen premixes including primer pairs and polymerase for each rearrangement, complemented by T cell receptor beta (TRB) detection via three multiplex PCR reactions, as described by the BIOMED-2 consortium [3].

2.1.1 Control Samples

Cell line or patient samples with respective rearrangements are used as positive control, and water is used as negative control to check for possible contamination. Using 20–25 single reactions, it is not possible to add positive and negative controls to each mix. One positive control and one negative control are used for each marker screening, with positive control changing (rotating) for each screening round to control all the PCR premixes.

2.1.2 Distinguishing Between Monoclonal and Polyclonal PCR Products

In case of positive amplification, it is necessary to distinguish monoclonal PCR products from oligo/polyclonal ones. This was previously done using heteroduplex analysis on polyacrylamide gels [3]. Currently, GeneScan analysis or technologies of automated electrophoresis (Agilent Bioanalyzer or similar) are preferred due to significantly reduced hands-on time. We use Agilent Bioanalyzer on-a-chip electrophoresis for clonality detection, because it does not require fluorescently labeled primers as in GeneScan, while providing a similar degree of size distinction. Moreover, PCR products can be directly used for further analysis. The TRB multiplex interpretation is difficult due to possible unspecific bands. Therefore, polyclonal control samples consisting of a mix of at least ten healthy donor “buffy coat” samples should be used for each TRB multiplex tube, together with positive controls and water, to discern nonspecific bands. The monoclonal products are then sequenced and clone-specific primers are designed (see below).

2.2 NGS-Based Marker Identification

Alternatively—and currently more frequently—the methods used for the screening of IG/TR gene rearrangements as clonal markers in ALL are routinely based on next-generation sequencing (NGS), providing a rapid and full overview of the rearrangements present in the sample. These methods, usually based on amplicon sequencing for particular markers (IG/TR rearrangements), rely on multiplex PCR with a large number of specific primers, and thus a reliable and standardized quality control is needed in routine practice to obtain reliable results. When focusing on noncommercial and thus freely available solutions, EuroClonality-NGS assays and approaches that were developed to standardize routine diagnostic practice for both the wet lab and bioinformatic parts of marker identification are optimal [4].

2.2.1 Quality Control of the Library Preparation

To ensure that all possible IG/TR gene rearrangements that are present in the diagnostic DNA sample can be detected, a routine control of the PCR primer mixes should regularly be performed using a polyclonal quality control sample (PC-QC). A mixture of polyclonal DNA samples isolated from the PBMCs obtained from multiple healthy donors is easily accessible in routine laboratory practice and provides a diverse repertoire of IG/TR gene rearrangements. NGS library preparation from the PC-QC is required each time a new working dilution of the primer mix is prepared to test the correct performance of all primers and should be periodically repeated to assess stable primer mix composition over longer periods of time.

Standard quality control (QC) of the NGS library is required for each sequencing run and consists of gel electrophoresis of the final products to assess a good specific amplification of the library at the expected amplicon length and quantitation of the purified specific products.

For the purpose of assessing correct PCR amplification during each NGS library preparation, a central in-tube quality/quantitation control (cIT-QC) is used and added to the PCR reaction to undergo the whole process in parallel with the diagnostic sample. The cIT-QC consists of selected human B and T cell lines with defined IG/TR rearrangements [5] and serves as a positive control for all the IG/TR gene loci, including the ones that were not rearranged in the patient's malignant cells and would otherwise lack specific rearrangements. Reads from the cIT-QC are used during the bioinformatic analysis to confirm correct NGS library preparation and aid with the normalization of all the other reads to cell counts.

2.2.2 Data Analysis

A large number of specifically developed software tools exist for the analysis of IG/TR gene rearrangements, with the ARResT/Interrogate [6] and Vidjil [7] applications being developed in collaboration with the EuroClonality-NGS working group to be well suited

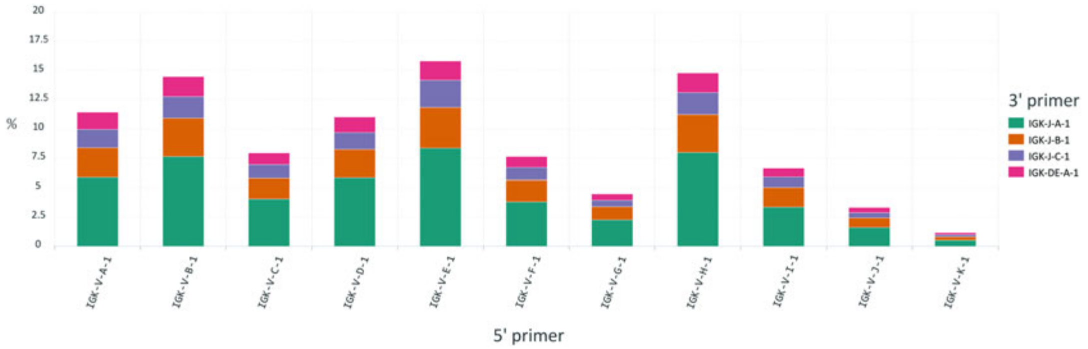

Fig. 1 Primer usage in a mixed polyclonal sample. Individual primers from the EuroClonality-NGS IGK-VJ-Kde primer mix are shown with 5′ primers on the x axis and 3′ primers in different colors. The y axis shows the relative abundance of reads identified with the respective primer sequence in the NGS library

for MRD marker identification including the automatic quality control of the libraries prepared according to the EuroClonality-NGS working group protocols. An essential prerequisite for the analysis is sufficient sequencing coverage of the NGS libraries with good base quality for reliable identification of all IG/TR rearrangements present in the DNA sample, including the cIT-QC. This is taken into consideration during the bioinformatic analysis with these tools.

Using correct primer annotation, the usage of specific 5′ and 3′ primers can be examined in each sample to assess their individual performance. An example of such analysis of IGK-VJ-Kde primer usage in a polyclonal sample is shown (Fig. 1). Although influenced by the gene usage in a healthy polyclonal repertoire, it is a reliable indicator of any errors that may have occurred during the primer mix preparation. Primer mix performance should be checked regularly using a PC-QC.

Reads corresponding to the cIT-QC are identified during the bioinformatic analysis and serve as an amplification control for each individual library. An automatic QC determines that all expected rearrangements of the cIT-QC are present in the respective libraries and a quantitation factor is calculated based on the DNA input of the cIT-QC as well as the patient's sample. A potential failure to detect some of the cIT-QC rearrangements may occur in a situation with a low coverage of the NGS library and a high infiltration of blasts in the patient's sample with monoclonal rearrangement. In such cases usually only some of the cIT-QC rearrangements are not covered and the MRD marker can still be clearly identified. In samples with limited polyclonal IG/TR background, the cIT-QC makes up a large proportion of reads.

2.3 Choosing Markers for MRD and Optimization of the Clonal-Specific RQ-PCR Systems

There have been many debates on the subject of (preferential) selection of the most specific and stable markers. However, in the real-life situation, prioritization of markers is not really an issue; for the sake of time of routine diagnostic throughput, usually all available (mono)clonal markers identified from Sanger sequencing are used for clonal-specific primer design and subsequent RQ-PCR optimization. Sequential testing of potential markers and primers is not preferred as the total time spent on the entire selection-optimization process must fit in the diagnostic window for MRD monitoring. Markers are therefore mostly selected based on their real, rather than predicted performance during the optimization process.

However, with the advent of NGS-based marker identification, more information is available on every marker. First, the real abundance of the clonal marker in the analyzed DNA sample can be estimated based on the cIT-QC and the background, and second, and perhaps most importantly, its specificity can be confirmed against a large dataset of IG/TR rearrangements from other patients and polyclonal samples. Detailed description of this is well beyond the scope of this chapter.

Ultimately, the real performance of the selected clonal marker-primer in RQ-PCR is the criterion for its use in MRD monitoring.

The EuroMRD (former ESG-MRD-ALL) consortium has established strict criteria for defining sensitivity and specificity of RQ-PCR systems [8].

Reaching adequate sensitivity and specificity based on EuroMRD criteria represents a QC of a well-designed RQ-PCR system per se. Similar rearrangements in normal B and T cells are the source of possible false-positivity, and background amplification is unavoidable in some markers. The extent of nonspecific amplification (NSA) depends on the involved genes and the number of inserted and deleted nucleotides in the junction. It has been estimated that NSA occurs in 35% of IGH markers and in more than 90% of TCRG markers [9]. IGK markers are also highly prone to NSA. IGK-KDE rearrangements are recommended as first-choice markers due to their stability, but based on our NGS data, the presence of highly similar rearrangements with resulting NSA is extremely high in polyclonal controls (unpublished data).

Therefore, it is mandatory to use adequate polyclonal controls. At least six wells of polyclonal DNA (preferentially from at least 10 healthy donors PB samples) should be used together with MRD samples in the RQ-PCR assay. Usually, 2–3 specific primers are tested for each monoclonal rearrangement, and two independent markers with the lowest NSA and sufficient sensitivity are selected and further optimized if needed. To reduce NSA, it is possible to slightly correct RQ-PCR conditions, i.e., to increase the annealing temperature by 2–4 °C or to titrate the primer concentration, usually by decreasing the clonal marker-specific primer concentration.

3 Interpretation of RQ-PCR MRD Analysis Results

The EuroMRD consortium has also defined and published guidelines for the correct interpretation of RQ-PCR MRD monitoring results. These criteria not only reflect the potential biological issues of the approach but also the clinical relevance of the result.

Consequently, the criteria for MRD positivity were defined more strictly for situations, where possible false-positivity would lead to unjustified treatment intensification. This is typically the situation of an emerging molecular relapse, most commonly during regular follow-ups after stem cell transplantation (SCT). In the opposite situation, i.e., when treatment reduction would be the outcome of false-negative MRD result, the criteria are intentionally stricter toward negativity [8].

In summary, sample is considered to be MRD positive in the context of therapy reduction (e.g., risk group stratification into lower risk group) if:

- The CT value of at least one of the three replicates is ≥1.0 CT lower than the lowest CT of background

  and.
- The CT value of at least one of the three replicates is within 4.0 CT from the highest CT value of the previously defined "sensitivity."

A sample is considered to be MRD positive in the context of therapy intensification (e.g., therapeutic intervention after SCT) if:

- The CT value of at least one of the three replicates is ≥3.0 CT lower than the lowest CT of background

  and.
- The CT value of at least one of the three replicates is within 4.0 CT from the highest CT value of the previously defined "sensitivity."

3.1 Identification of False-Positive and False-Negative Results

In an intra-laboratory setting, a newly emerged low MRD positivity remains a diagnostic challenge. Before the era of NGS methods, the extent of false-positivity was assessed only indirectly. Van der Velden et al. retested the low-positive samples in different timepoints of ALL using MRD assays designed for different (irrelevant) markers and concluded that the NSA differs between timepoints and markers and is mostly present in IGH markers with background amplification in PB (buffy coats) in post-maintenance treatment phases. Their study concluded that the background for IGH markers was lowest at the end of induction treatment (day 33) and that EuroMRD criteria sufficiently excluded most of the false-positives [10].

Our group focused on MRD positivity during the post-SCT period. Starting 140 days post-SCT, we frequently observed positive results fulfilling EuroMRD criteria for therapy intensification in patients who turned negative in the following examinations. Using indirect methods, we showed that the positives were nonspecific and their occurrence correlated with intense B cell regeneration, which is usually very intense post-SCT [11]. With the development of NGS-based MRD methods, we expanded the previous cohort and reanalyzed post-SCT RQ-PCR-positive samples by NGS. A vast majority of RQ-PCR positive samples in patients who subsequently did not progress into hematological relapse were negative using NGS. NGS sequences of amplified physiological rearrangements were highly similar to ASO primer sequences, suggesting that RQ-PCR amplification was not specific [12].

Based on these data, we decided to recheck every MRD result post-SCT that was concluded by RQ-PCR to be "positive, nonquantifiable." The size of the nonspecific RQ-PCR products is usually different from the expected size of the amplified marker. Therefore, it is helpful to keep RQ-PCR products and check their size using the Agilent Bioanalyzer together with products of the standard curve dilution (usually 10–1 and 10–4) as size standard and with buffy coats that previously showed positive signals. Based on our experience, up to 30–40% of low-positive (nonquantifiable) RQ-PCR results can be identified as false-positive, because the length of the "clonal-specific" product differs from its original size and overlaps with buffy coat amplification (unpublished data). In the remaining cases, the sizes of all products including buffy coat are in the same size range and thus cannot be distinguished. With IG/TR NGS available, it is possible to reevaluate the remaining positive RQ-PCR result via NGS. However, to ensure that NGS has the same (or better) sensitivity as RQ-PCR, it is crucial to test the sensitivity of NGS using the diluted sample (e.g., 10–4), preferentially in a separate NGS run to avoid sample cross-contamination.

4 Conclusion

MRD monitoring using an IG/TR-based quantitation method is an elegant and clinically relevant approach. However, as several steps are prone to technical and interpretational errors, adequate quality control measures must be included throughout the process. Some of the basic and more advanced tips have been listed in this chapter. On top of these, intra-laboratory procedures and interlaboratory measures can be introduced as well.

Acknowledgments

This work was supported by the Ministry of Health of the Czech Republic, grant NV20-03-00284.

References

1. Pongers-Willemse M, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P et al (1999) Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets report of the BIOMED-1 CONCERTED ACTION: investigation of minimal residual disease in acute leukemia. Leukemia 13:110–118. https://doi.org/10.1038/sj.leu.2401245
2. Szczepański T, Pongers-Willemse MJ, Langerak AW, Harts WA, Wijkhuijs AJ, van Wering ER et al (1999) Ig heavy chain gene rearrangements in T-cell acute lymphoblastic leukemia exhibit predominant DH6-19 and DH7-27 gene usage, can result in complete V-D-J rearrangements, and are rare in T-cell receptor alpha beta lineage. Blood 93:4079–4085
3. Van Dongen JJM, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL et al (2003) Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 17:2257–2317. https://doi.org/10.1038/sj.leu.2403202
4. Brüggemann M, Kotrová M, Knecht H, Bartram J, Boudjogrha M, Bystry V et al (2019) Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation study. Leukemia 33:2241–2253. https://doi.org/10.1038/s41375-019-0496-7
5. Knecht H, Reigl T, Kotrová M, Appelt F, Stewart P, Bystry V et al (2019) Quality control and quantification in IG/TR next-generation sequencing marker identification: protocols and bioinformatic functionalities by EuroClonality-NGS. Leukemia 33:2254–2265. https://doi.org/10.1038/s41375-019-0499-4
6. Bystry V, Reigl T, Krejci A, Demko M, Hanakova B, Grioni A et al (2017) ARResT/interrogate: an interactive immunoprofiler for IG/TR NGS data. Bioinformatics 33:435–437. https://doi.org/10.1093/bioinformatics/btw634
7. Giraud M, Salson M, Duez M, Villenet C, Quief S, Caillault A et al (2014) Fast multiclonal clusterization of V(D)J recombinations from high-throughput sequencing. BMC Genomics 15:409. https://doi.org/10.1186/1471-2164-15-409
8. Van der Velden VHJ, Cazzaniga G, Schrauder A, Hancock J, Bader P, Panzer-Grumayer ER et al (2007) Analysis of minimal residual disease by Ig/TCR gene rearrangements: guidelines for interpretation of real-time quantitative PCR data. Leukemia 21:604–611. https://doi.org/10.1038/sj.leu.2404586
9. Van der Velden VHJ, Hochhaus A, Cazzaniga G, Zczepanski T, Gabert J, van Dongen JJ (2003) Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17:1013–1034. https://doi.org/10.1038/sj.leu.2402922
10. Van der Velden VHJ, Wijkhuijs JM, van Dongen JJM (2008) Non-specific amplification of patient-specific Ig/TCR gene rearrangements depends on the time point during therapy: implications for minimal residual disease monitoring. Leukemia 22:641–644. https://doi.org/10.1038/sj.leu.2404925
11. Fronkova E, Muzikova K, Mejstrikova E, Kovac M, Formankova R, Sedlacek P et al (2008) B-cell reconstitution after allogeneic SCT impairs minimal residual disease monitoring in children with ALL. Bone Marrow Transplant 42:187–196. https://doi.org/10.1038/bmt.2008.122

12. Kotrova M, Van der Velden VHJ, van Dongen JJM, Formankova R, Sedlacek P, Brüggemann M et al (2017) Next-generation sequencing indicates false-positive MRD results and better predicts prognosis after SCT in patients with childhood ALL. Bone Marrow Transplant 52(7):962–968. https://doi.org/10.1038/bmt.2017.16

Open Access This chapter is licensed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license and indicate if changes were made.

The images or other third party material in this chapter are included in the chapter's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the chapter's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.