8 Standardization of Molecular Monitoring for Chronic Myeloid Leukemia: 2021 Update

Matthew Salmon, Helen E. White, Nicholas C. P. Cross, and Andreas Hochhaus

8.1 Background

Studies to explore the possibility of molecular monitoring of chronic myeloid leukemia (CML) patients by reverse transcriptase polymerase chain reaction (RT-PCR) were initiated more than 30 years ago, when the principal clinical challenge was to develop a methodology to detect early relapse after bone marrow transplantation (BMT). The first studies were qualitative, using two-step, nested RT-PCR and standard agarose gel electrophoresis to determine whether *BCR-ABL1* mRNA was detectable or undetectable in patient samples, with a control for adequate cDNA quality being provided by single step amplification of a housekeeping control gene. Standardization at this time focused mainly on the need to eliminate false-positive results arising from contamination of amplification reactions with previously amplified products [1].

Although some of these early studies were able to identify groups of patients that were more or less likely to relapse, the predictive value for individual cases was very limited [2–6], and thus there was a need to develop quantitative RT-PCR approaches that might be able to give an indication of the level of disease in specimens that tested positive for *BCR-ABL1,* and also the kinetics of any changes in the size of the malignant clone over time. Initial quantitative procedures were based on the use of competitive PCR, which relies on the addition of known numbers of molecules of a competitor plasmid to a series of amplification reactions, with the number of *BCR-ABL1* targets in the sample being estimated by determining the point at which the competitor and *BCR-ABL1* amplicons are of equivalent fluorescent intensity on an agarose gel. Using competitive PCR it was shown that rising *BCR-ABL1* levels on sequential analysis predicted relapse after BMT and provided prognostically useful information for patients in complete cytogenetic remission (CCyR) on interferon alpha [7–12]. Competitive PCR was thus effective but extremely labor intensive and was only performed on a research basis in a small number of transplant centers. The development and subsequent commercialization of reverse-transcription real-time quantitative PCR (RT-qPCR) in the late 1990s [13] along with the introduction of highly effective targeted therapy for CML provided the means and the need for widespread adoption of molecular monitoring. However, there was no standard approach as to how the assay should be performed and different methodologies

M. Salmon · H. E. White · N. C. P. Cross (✉)
Faculty of Medicine, University of Southampton, Southampton, UK

Wessex Regional Genetics Laboratory, Salisbury, UK
e-mail: ncpc@soton.ac.uk

A. Hochhaus
Abteilung Hämatologie/Onkologie, Klinik für Innere Medizin II, Universitätsklinikum Jena, Jena, Germany

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proliferated, resulting in results that were difficult or impossible to compare between centers.

8.2 Measurement of Residual Disease in the Laboratory

To understand the problem of standardization it is first necessary to understand how the test is performed. Anticoagulated peripheral blood or bone marrow samples are received in the testing laboratory, ideally within 24 hours of collection. Generally peripheral blood is preferred as this is less invasive, and results are comparable to bone marrow provided that total leukocytes are recovered by lysis of red cells [14]; in contrast to the analysis of residual disease in acute leukemia, mononuclear cells isolated by density gradients such as Lymphoprep® or Ficoll should not be used for CML. Leucocytes are lysed in a chaotropic agent that inactivates pervasive RNA-degrading enzymes, and RNA is extracted and reverse transcribed to cDNA, typically using random hexamer primers (Fig. 8.1). Differences in the amount of RNA extracted, the integrity of that RNA, and the efficiency with which it is reverse transcribed may vary widely between samples, even in established laboratories. This means that the sensitivity with which *BCR-ABL1* can be detected or excluded is also highly variable. It is generally agreed that the best way to take this variation into account is to relate the number of copies of *BCR-ABL1* to those of an housekeeping reference gene, which serves as an internal control for both the quantity and quality of the cDNA for each sample [15].

Two measurements are made by RT-qPCR for all samples: an estimate of the number of *BCR-ABL1* transcripts and an estimate of the number of transcripts of the housekeeping reference gene. Different laboratories use various processes to derive these estimates; for example, some measure *BCR-ABL1* and the reference gene singly, in duplicate or in triplicate from an identical cDNA specimen; others make a single measurement from independent cDNA preparations. In addition, different criteria are used to define whether a result is considered detectable or undetectable based on replicate results and technical parameters.

Results for specimens that are positive for *BCR-ABL1* are expressed as the ratio of *BCR-ABL1* transcript numbers divided by the number of reference gene transcripts in the same volume of cDNA. For samples that test negative for *BCR-ABL1*, the number of reference gene transcripts

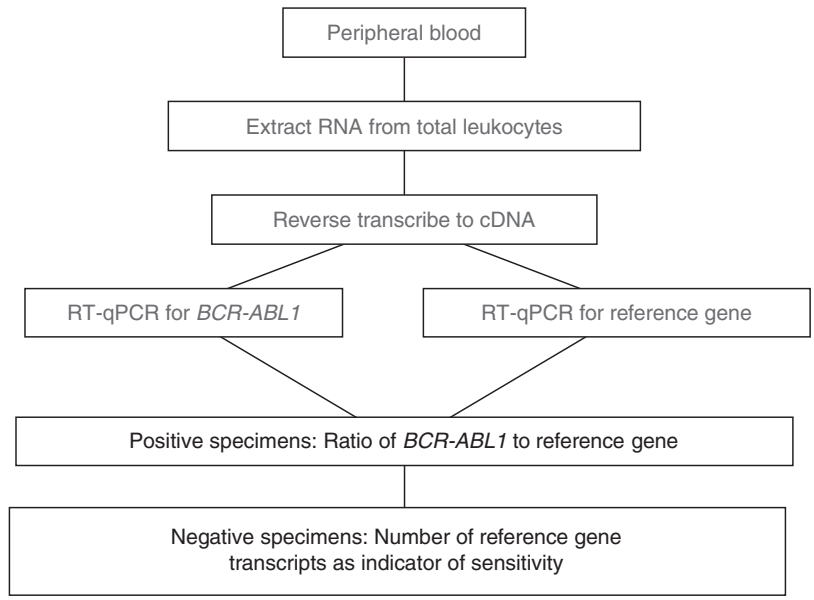

Fig. 8.1 Schematic outline of *BCR-ABL1* RT-qPCR analysis. Positive specimens mean those in which *BCR-ABL1* mRNA is detected; negative specimens are those in which *BCR-ABL1* mRNA is not detected

gives an indication of the sensitivity with which residual disease can be excluded for that particular specimen. It is very important that testing laboratories monitor closely the variability of their assay and reject runs that are considered as outliers, for example, by regularly measuring high and low standards [16, 17]. Establishment of RT-qPCR requires extensive validation, for example, using the methodology described by the Molecular Oncology Resource Committee of the College of American Pathologists [18].

8.3 Choice of Reference Gene

One of the critical variables between centers has been the choice of reference gene. An ideal reference gene would be expressed uniformly in different cell types regardless of their proliferative status, unaffected by therapeutic regimens, invariant between individuals, and expressed at a level similar to *BCR-ABL1*. Unfortunately such a perfect reference gene does not appear to exist and instead several alternatives have emerged. The most widely used is *ABL1;* this is partly a historical accident as the plasmid constructs that were used for competitive PCR quantification could also be used to quantify normal *ABL1* expression [7]. Subsequently, however, the Europe Against Cancer (EAC) group undertook an extensive analysis of candidate reference genes and concluded that *ABL1,* beta-2-microglobulin (*B2M*), and beta-glucuronidase (*GUSB*) were suitable for normalization of RT-qPCR results [15]. *BCR* is also widely used as an internal control for CML, based on the rationale that both normal *BCR* and *BCR-ABL1* are driven by the same promoter, and thus they are likely to be transcribed at similar rates in different cell types [16]. The great majority of testing laboratories worldwide use *ABL1* as an internal reference but *BCR* or *GUSB* is also used in many centers. The use of other reference genes is not recommended. This means that there are at least three distinct units of measurement in widespread use for the estimation of residual disease in CML: *BCR-ABL1*/total *ABL1* (i.e., *ABL1* + *BCR-ABL1*), *BCR-ABL1*/*BCR*, and *BCR-ABL1*/*GUSB*.

Although the use of different reference genes used to be the principal reason for limited comparability of results between centers, there are other important factors that are particularly relevant for laboratory-developed tests. Laboratories using the same reference gene may use different probe/primer combinations, partly as a result of concerns about infringements of intellectual property rights. In addition, laboratories may differ in their approach to the setting of user-defined parameters such as the threshold, what constitutes an acceptable result in terms of slope of the standard curve, minimum number of points to construct a standard curve, what cycle threshold (C_t) value is accepted as a positive result, and the reproducibility between duplicate or triplicate replicates. Finally, in order to achieve sensitive detection of residual disease, it is essential to analyze a sufficiently large sample. Clearly it is impossible to achieve a sensitivity of 1 in 10^5 if only the equivalent of 10^4 cells or fewer are analyzed. Some of these issues have been addressed by the EAC and consensus guidelines published [19, 20]; in addition many commercially available kits provide detailed guidance for RT-qPCR set up and analysis.

8.4 The International Scale for *BCR-ABL1* Measurement

The International Randomized Study of Interferon and STI571 (IRIS) study demonstrated the dramatic superiority of imatinib over interferon-based regimens. In this trial, RT-qPCR analysis was centralized in three centers (Adelaide, London, and Seattle) that used different laboratory procedures and two different control genes [21]. Large, differences in median *BCR-ABL1* values at specific timepoints between the three centers were noted, which prompted the need for an urgent alignment of their respective results. In the absence of any independent reference or calibration materials, an essentially arbitrary decision was made that each center would measure the level of disease in a common set of 30 pretreatment CML patient samples using *BCR* as a control gene, and that results would be normalized to

this standardized baseline. Reanalysis of the data showed improved comparability of results between the three laboratories and the standardized baseline was used to normalize all subsequent trial results [21]. Thus, major molecular response (MMR), for example, was defined as a three log reduction from the IRIS-standardized baseline and not a three log reduction from pretreatment material for each individual case.

The scale used in the IRIS trial subsequently formed the basis of the international scale (IS) for *BCR-ABL1* measurement, which was proposed as a means to enable laboratories to continue to use their own methods but produce results that are more comparable between centers [22]. Although the samples used to define the IRIS standardized baseline were very limited in quantity and therefore quickly exhausted, excellent traceability was provided by the detailed internal quality control data accrued by the Adelaide laboratory [16], thus enabling the IS to be linked directly to measurements made for IRIS. The IS expresses detectable disease as a percentage, with 100% BCR-ABL^{IS} defined as the IRIS standardized baseline and 0.1% BCR-ABL^{IS} corresponding to MMR (also known as MR^3). A level of 1% BCR-ABL corresponds roughly to the limit at which Ph-positive metaphases can be detected by standard cytogenetics, and thus levels of disease <1% are consistent with complete cytogenetic remission (Fig. 8.2) [23].

The initial focus of the IS was on detectable residual disease and in particular whether a patient had or had not achieved defined milestones, for example, 10% or 0.1% BCR-ABL^{IS}. Second-generation TKIs produce faster and deeper responses compared to imatinib and the need arose for robust, standardized, and workable definitions of DMR [24]. Such definitions are particularly important for the selection of patients who may achieve treatment-free remission (TFR).

Definitions were proposed [24] and accepted by the European LeukaemiaNet (ELN) in their 2013 recommendations for the management of CML patients [25]. These definitions have been elaborated by the European Treatment and Outcome Study (EUTOS) group to enable testing laboratories to score DMR in a comparable fashion [26] and remained unchanged in the 2020 update to the ELN recommendations [27]. The definitions are:

- MR^4 (≥4-log reduction from IRIS baseline) = either (i) detectable disease ≤0.01% BCR-ABL^{IS} or (ii) undetectable disease in cDNA with 10,000–31,999 *ABL1* transcripts or 24,000–76,999 *GUSB* transcripts*.

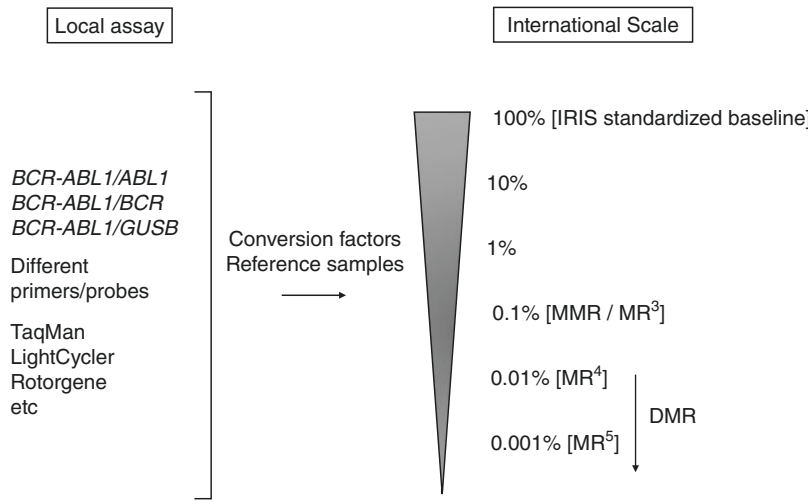

Fig. 8.2 The International Scale for *BCR-ABL1* RT-qPCR measurement. Centers continue to use their established assays for *BCR-ABL1* and convert results to the international scale (IS) using CFs or calibrated reference reagents. MMR = major molecular response; MR^3, MR^4, and MR^5 are 3, 4, and 5 log reductions, respectively, from the IRIS standardized baseline. DMR = deep molecular response is MR^4 or lower

- $MR^{4.5}$ (≥4.5-log reduction from IRIS baseline) = either (i) detectable disease ≤0.0032% $BCR\text{-}ABL^{IS}$ or (ii) undetectable disease in cDNA with 32,000–99,999 *ABL1* transcripts or 77,000–239,999 *GUSB* transcripts*.
- MR^{5} (≥5-log reduction from IRIS baseline) = either (i) detectable disease ≤0.001% $BCR\text{-}ABL^{IS}$ or (ii) undetectable disease in cDNA with ≥100,000 *ABL1* transcripts ≥240,000 *GUSB* transcripts*.

*Numbers of *ABL1* or *GUSB* transcripts in the same volume of cDNA used to test for *BCR-ABL1*. Equivalent numbers of *BCR* reference gene transcripts for each level of MR have not been defined.

In addition, essential quality criteria with respect to reference gene transcripts numbers must be met and are given in Table 8.1. The definitions depend critically on the ability of testing laboratories to measure absolute numbers of reference gene transcripts in a comparable manner (see below).

Although the terms "complete molecular response" or "complete molecular remission" have been used in the past, it is difficult to define these terms in any meaningful way. Instead, definitions of deep response need to be qualified with the level of sensitivity achieved for that sample, particularly for specimens where *BCR-ABL1* is not detected which should be referred to as "molecularly undetectable leukemia" and specifying the number of reference gene transcripts and/or the level of response [27].

Table 8.1 Summary of reference gene numbers required for scoring deep molecular response

	MR^{4}	$MR^{4.5}$	MR^{5}
Minimum sum of reference gene transcripts[a]	10,000 *ABL1* 24,000 *GUSB*	32,000 *ABL1* 77,000 *GUSB*	100,000 *ABL1* 240,000 *GUSB*
$BCR\text{-}ABL^{IS}$ level for positive samples[b]	≤0.01%	≤0.0032%	≤0.001%

[a]Irrespective of whether *BCR-ABL1* is detected or not. Numbers of reference gene transcripts in the same volume of cDNA that is tested for *BCR-ABL1*. The minimum number in any individual replicate should be 10,000 *ABL1* or 24,000 *GUSB*

[b]Provided that the minimum reference gene copy numbers in the row above are fulfilled

8.5 Implementing the International Scale

Although the concept of the IS is very attractive, international implementation has proven to be challenging. Initially, the only mechanism for laboratories to adopt the IS was to establish a laboratory-specific conversion factor (CF) using a process initiated by the Adelaide laboratory [28]. For a testing laboratory to establish a CF, a series of samples (typically 20–30) are exchanged with a reference laboratory that span at least three logs of detectable disease but do not exceed an IS value of 10%. Samples are analyzed by both centers over a period of 2–3 months to take into account common intralaboratory variables, e.g., different operators and different batches of reagents. The results for the reference and test laboratories (using the IS and local units, respectively) are compared and the CF for the testing laboratory derived by a straightforward mathematical calculation. To validate the CF, a further set of samples are exchanged which are again analyzed in a similar manner, i.e., in both centers over a period of time. If the converted values for the test laboratory show a bias of within ±1.2-fold compared to the reference laboratory, then the CF is considered validated and suitable for conversion of the test laboratory results to the IS. Of 38 test laboratories which undertook this process (using 19 different methods and 5 different control genes), 22 (58%) successfully established validated CFs, testifying to the success of the process [28]. The reason that the validation process failed in the remaining test laboratories is unclear, but presumably indicates that their assays are nonlinear or unstable over time.

Since it is impossible for a single reference laboratory to standardize all other testing laboratories in the world, the concept of regional or national reference laboratories has been developed, for example, in Europe through EUTOS. Following derivation of a CF with Adelaide, the laboratory in Mannheim has performed sample exchanges and derived further CFs with more than 50 testing centers that can then serve in turn as reference centers for their countries or regions [29]. Although this process worked well, at least for laboratories with stable assays, it is arguably intrinsically flawed as any errors will be propagated along the line. Furthermore there are other obvious issues, for example, (a) derivation of CFs is time consuming and expensive; (b) due to the requirement to involve an established reference laboratory, the process is only open to a limited number of testing laboratories at any given time; (c) many centers struggle to accrue sufficient numbers of suitable samples; (d) it is unclear how often CFs need to be revalidated; (e) it is unclear what happens to the 50% of laboratories who fail to achieve the defined performance criteria; (f) it is unclear what constitutes a stable or unstable CF and how testing laboratories should accommodate CFs that change over time.

8.6 Development of Reference Reagents and Calibrated Kits

While the development of CFs was a major step forward and provided an important proof of principle, it is obvious that this approach is not sustainable in the long term. Ideally, any testing laboratory should be able to access reference standards or use a kit that enables them to convert patient results directly to the IS. The development of standards and kits initially required the development of a process by which these tools could be calibrated to the IS. An important milestone in this process was the establishment in 2010 of the First World Health Organization International Genetic Reference Panel for quantitation of *BCR-ABL1* mRNA [30]. The reference panel comprises four different dilution levels of freeze-dried preparations of K562 cells diluted in HL60 cells that were assigned fixed % *BCR-ABL1*/reference gene values on the IS following an international calibration process. Due to the scale of molecular monitoring, it was not physically possible to manufacture and validate a sufficiently large quantity of reference material to satisfy worldwide demand, and thus the principal function of these primary reagents was limited to the calibration of secondary reference reagents. These secondary reference reagents may be manufactured and calibrated by companies, reference laboratories, or other agencies and made available to testing laboratories either on a commercial basis or as part of specific national or regional standardization initiatives (Fig. 8.3).

An international evaluation of a panel of such secondary reference material demonstrated these reagents can be used to derive laboratory-specific CFs for a wide range of *BCR-ABL1* testing protocols while mitigating some of the logistical challenges of the sample exchange method [31]. This study also highlighted that many local RT-qPCR assays showed signs of poor optimization, and that individual laboratories need to robustly determine the optimal conditions for their protocols, a process for which a panel of calibrated reference material is ideally suited. Local secondary panels have also been produced to harmonize molecular monitoring results in Latin America [32] and China [33]. Recently, the AcroMetrix™ *BCR-ABL* Panel (ThermoFisher) has become the first commercially available set of secondary reference reagents in the form of lyophilized cellular material. These will hopefully enable laboratories to undertake analytical validation and performance monitoring of *BCR-ABL1* assays from RNA extraction through to generation of results of the IS as well as enabling on-demand derivation of CFs.

As indicated above, standardization of DMR requires testing laboratories to be able to estimate absolute numbers of reference gene transcripts in a comparable manner as an indication of the quality of the sample. Determination of the number of *BCR-ABL1* and reference gene transcripts is typically performed by using an external plasmid calibrator; however, different calibrators

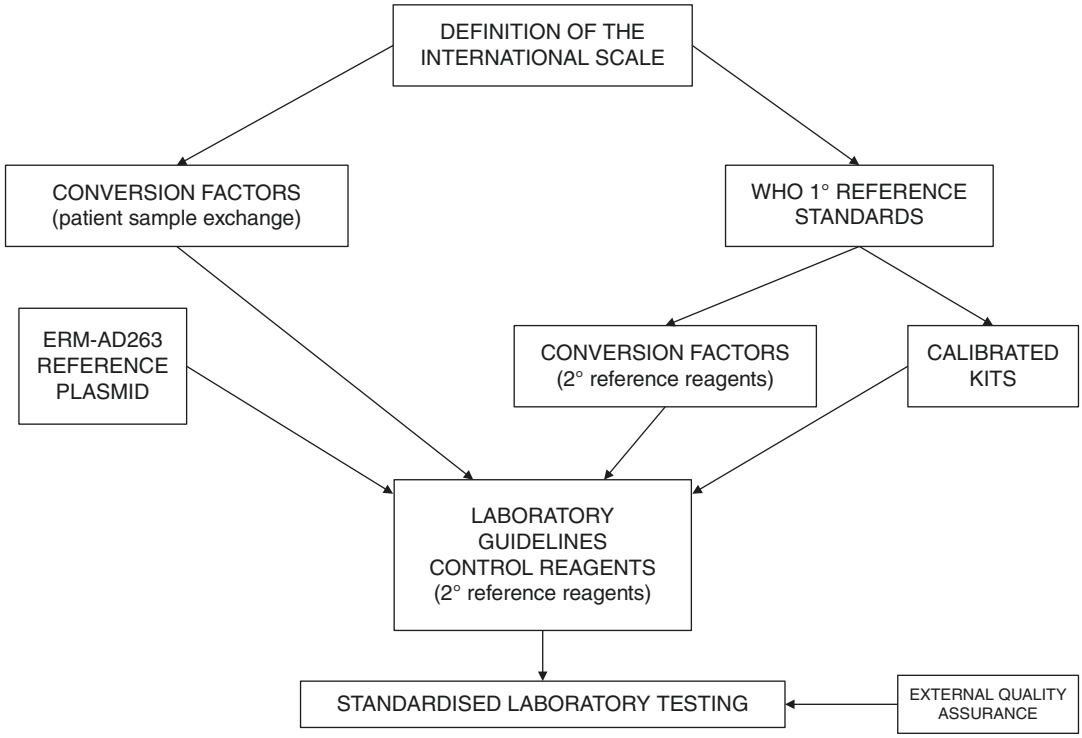

Fig. 8.3 Components of the standardization process for molecular monitoring of CML

(developed in house or commercially available) are in use worldwide and until 2015, no common reference material existed to which they could be aligned. In response to this, an internationally accepted certified reference plasmid, ERM-AD623, was developed that includes *BCR-ABL1* and the three most commonly used reference genes (*ABL1*, *BCR*, and *GUSB*) [34]. The direct or indirect use of this plasmid helps to improve the accuracy of results prior to conversion (Fig. 8.4) as well as the accuracy of reference gene copy number estimates for samples where *BCR-ABL1* is not detected.

A number of different kits, systems, and secondary reagents are available that enable testing laboratories to derive patient results on the IS [35–37]. Comparative data from a large EQA scheme involving laboratories using a diversity of methods showed that, in general, the performance of each IS conversion method in use was acceptable, but significant systematic intermethod differences were apparent [37]. It is not clear which of these methods provides the "correct" result.

8.7 What Is Achievable by Standardization?

The combination of CFs, calibrated reagents, and ERM-AD623 should help testing laboratories to generate more standardized results. Indeed, widespread adoption of the IS seems to have reduced the interlab variability [37]. However, as noted above, some lack of agreement between different laboratories using diverse methodologies and control genes remains, particularly at lower levels of *BCR-ABL1* [37]. Whether this remaining disagreement is acceptable depends on the effect it has on clinical interpretation. When evaluating the performance characteristics of a method, two factors should be considered: trueness (i.e., the degree of closeness of mean measured quantity value and the true quantity value) and the precision (i.e., the degree to which repeated measurements under unchanged conditions show the same results). The trueness of a method can be estimated comparing the average value obtained from several replicate

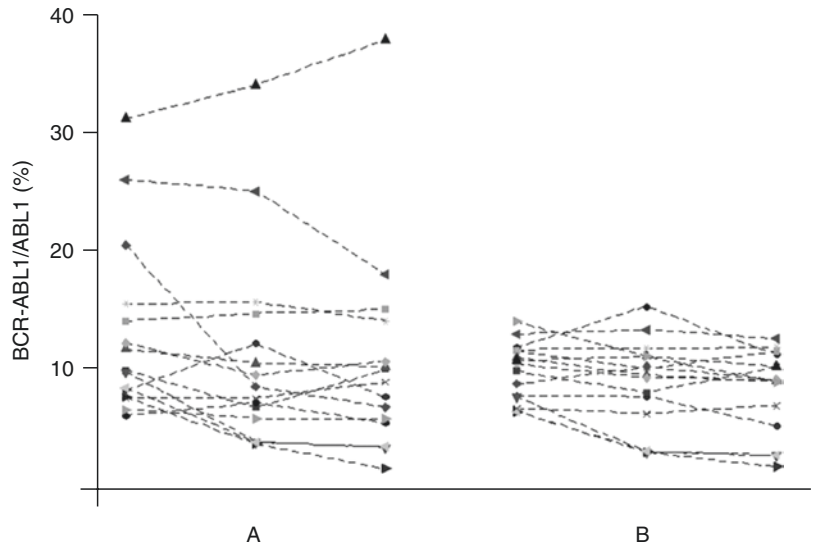

Fig. 8.4 Identical samples were measured in triplicate in 14 different laboratories using either local plasmid calibrators (**a**) or a common plasmid calibrator ERM-AD623 (**b**). It is apparent that the distribution of results is tighter when a common calibrator is used

measurements on a reference material with an established IS value. The precision of a method can be estimated from the 95% limit of agreement of all the individual measurement results obtained for the reference material. Existing experience with the set-up and validation of CFs has shown that an average difference within ±1.2-fold of the established value and 95% limits of agreement within ±five fold of the established value were achieved by the best performing methods [28]. This led to an MMR concordance rate of 91%, a level of agreement which probably represents the maximum that can be achieved using current RT-qPCR technology. However this figure of 91% critically depends on the set of samples that are used and would be expected to be substantially lower if the sample set was restricted to samples that were close to MMR. It is important, therefore, to consider intrinsic assay variation when assessing the response of a patient against specific milestones such as those recommended by the ELN [25]. In addition, at very low levels of disease, variation between replicates is inevitably greater than that seen at higher levels due to the fact that small numbers of molecules are being sampled. This should be taken into account when interpreting changes in levels of disease on sequential analysis, for example, a four-fold increase from 0.002% IS to 0.008% IS might be considered as a prompt to perform repeat analysis at the next scheduled visit, whereas an increase from 0.07% to 0.28% would be considered sufficient for rapid repeat analysis and possibly mutation testing [38].

Standardization of molecular monitoring is an ongoing process and critically requires testing laboratories to implement robust internal quality control to monitor assay drift and reproducibility [16]. In principle, if a laboratory can demonstrate assay stability over time then they only need to derive a CF once, although a new CF will have to be derived either internally or externally if processes or equipment are changed [28]. Laboratories using calibrated kits need to validate or verify that the kit is working correctly in their hands and that all preanalytical steps are optimized.

Of note, approximately 1–2% of CML patients harbor atypical *BCR-ABL1* mRNA fusion transcripts that cannot be monitored by standard *BCR-ABL1* RT-qPCR tests. It is important to recognize these fusions early in the disease course to avoid false-negative MRD assessments, and they can be monitored using bespoke RT-qPCR tests. However, such results cannot be expressed on the IS, and thus the common molecular milestones and triggers for treatment discontinuation are difficult to apply.

8.8 Standardization of Deep Molecular Response

Currently, a major focus of investigation in CML is the concept of TFR; around half of patients who have a prolonged and sustained DMR remain in sustained remission after stopping treatment. A general requirement to consider discontinuing treatment is DMR (MR^4 or better) for at least 2 years, and ideally at least 2 years in $MR^{4.5}$ or 3 years in MR^4 [27, 39, 40]. Standardization of molecular monitoring at this level of deep response is, therefore, particularly important, not only to meet the recommended criteria for attempting TFR, but also to detect patients who relapse as early as possible, as DMR is usually reachieved upon prompt resumption of treatment [41].

A program of QC rounds within the EUTOS consortium has been addressing the standardization of DMR in laboratories throughout Europe by repeated auditing of routine local results as well as distribution and testing of evaluation samples, including an IS-calibrated secondary reference panel. An in-depth analysis of this data is ongoing, but encouragingly, almost all participants could reliably detect *BCR-ABL1* at $MR^{4.5}$. This work has also enabled the monitoring of laboratory CFs over time, which for most labs were generally stable.

Given the increasing technical sensitivity required, a better understanding of the limits of a given assay's performance is crucial, and establishing or verifying the limit of detection (LoD), limit of quantitation (LoQ), and limit of blank (LoB) of an assay is important [42]. The LoD/LoQ values are dependent on the background signal (the LoB), which in an ideal *BCR-ABL1* assay is zero (i.e., there is a $\leq 5\%$ probability of a false-positive result from a true-negative sample). However, an analysis by the EUTOS group found that some testing laboratories had LoBs above acceptable levels, thus potentially compromising their ability to accurately report DMR [43].

On the other hand, an assay with a poorly optimized LoD may not be able to detect very low levels of *BCR-ABL1* transcripts, potentially leading to overestimation of the depth of response, and/or the generation of false-negative results. For example, a hypothetical laboratory with a CF of 0.8 may test a sample in duplicate, detecting a total of 34,500 *ABL1* copies. If the LoD was well optimized, it may also detect 2 copies of *BCR-ABL1* in one replicate and 1 copy of *BCR-ABL1* in the second replicate. As per the guidelines for scoring DMR [26], the result for this sample would be: (sum *BCR-ABL1* = 6)/(sum *ABL1* = 34,500) × 0.8 × 100 = 0.014% = MMR. However, if the laboratory had a poorly optimized LoD, then it may not detect *BCR-ABL1* in either replicate of the sample, leading to: undetectable *BCR-ABL1* in 34,500 *ABL1* = $MR^{4.5}$. In the latter case, the inability to reliably detect very low levels of *BCR-ABL1* results in a false-negative result and misclassification of the molecular response from MMR to $MR^{4.5}$.

8.9 Effects of *BCR-ABL1* Transcript Type

An emerging issue is the fact that multiple studies have described an inferior molecular response to treatment for patients carrying the e13a2 *BCR-ABL1* transcript, compared to those with e14a2 [44–46]. There is some evidence that this difference may be at least partially explained by an amplification bias toward the e13a2 transcript when using the EAC RT-qPCR assay [47, 48] which, if confirmed, would necessitate careful evaluation of patient transcript type and would be an additional factor to consider in the standardization of molecular monitoring. Indeed, our preliminary analysis shows that accounting for the amplification efficiency of each transcript may reduce differences in relative amplification of *BCR-ABL1* and the reference gene. Furthermore, an individualized approach to molecular monitoring (i.e., measuring the reduction of *BCR-ABL1* relative to a baseline of the patient's *BCR-ABL1* level at diagnosis or start of treatment) also appears to negate the differential response to treatment [49].

8.10 Droplet Digital PCR

Droplet digital PCR (ddPCR) has been proposed as a solution to some of the challenges faced by RT-qPCR. The main advantage of ddPCR is that measurement of target copy number does not rely on an exogenous calibration curve, making inter-laboratory comparison of results potentially more straightforward. Several studies have now shown that ddPCR produces comparable results to RT-qPCR, and that it may improve the precision of measurement [50–52]. ddPCR also brings improvements in sensitivity (largely through the ability to more effectively test multiple replicates), which might allow for a more granular stratification of patients at or below MR^4, potentially identifying patients that may be at greater risk of relapse after stopping treatment [53, 54]. Additionally, a CE-IVD marked ddPCR assay for monitoring *BCR-ABL1* on the IS is now commercially available (QxDX BCR-ABL %IS Kit, BioRad), which seems to show improved sensitivity and precision compared to RT-qPCR [51], although a reduction in the variability of results may be a natural consequence of comparing a single ddPCR method against a diverse set of RT-qPCR protocols. Further work on developing a standardized ddPCR approach will likely be required before its widespread adoption in routine *BCR-ABL1* monitoring, and this technique remains cost-prohibitive for many.

8.11 Other Approaches

There has been considerable interest in the fact that *BCR-ABL1* mRNA levels vary between CML patients at diagnosis and that measurement of reductions in disease levels from pretreatment levels of individual patients may provide additional prognostic information [55, 56]. Hanfstein et al. determined the level of *BCR-ABL1* transcripts for each patient at diagnosis and compared this to the level after 3 months of treatment, using *GUSB* as the reference gene, and found a median 1.4-log reduction in *BCR-ABL1*. Those patients achieving a 0.46-log reduction of *BCR-ABL1* transcripts at 3 months had significantly better overall and progression-free survival, compared with patients not achieving a 0.46-log reduction [55]. In a slightly different approach (and using *BCR* as the reference gene), Branford et al. noted that despite some patients failing to reach the milestone of 10% $BCR\text{-}ABL1^{IS}$ after 3 months of treatment, there exists a subgroup of these patients that go on to achieve a good response to treatment. The authors calculated the time taken for *BCR-ABL1* levels to reach that of half the diagnostic level for this subgroup and found that patients whose *BCR-ABL1* had reduced by at least half within 76 days had significantly better outcomes than those whose halving time was >76 days, identifying an additional risk factor for patients that fail to reach the 10% milestone at 3 months [56]. Several further studies have shown the halving time to be prognostic of response when using *ABL1* as a control gene [57, 58] and with the use of second-generation TKIs [59]. However, it should be noted that this is an as yet completely unstandardized metric and is not included in current guidelines for routine monitoring of CML.

Alternative approaches such as amplification of patient-specific genomic DNA *BCR-ABL1* fusions might provide greater insights into the dynamics of the malignant clone [60–62]. Recent work has shown levels of genomic *BCR-ABL1* relative to the diagnostic sample after 3 months of treatment may be predictive of optimal response [63]. There also appears to be good agreement between the reduction of levels of *BCR-ABL1* gDNA and mRNA relative to the pretreatment baseline, and, interestingly, the presence of gDNA in mRNA negative samples may be predictive of a loss of DMR during TFR attempts [64], pointing to the presence of a population of CML stem cells that are not actively expressing *BCR-ABL1* mRNA.

The cartridge-based GeneXpert system (Cepheid) offers a more automated approach to *BCR-ABL1* monitoring. The system is RT-qPCR based but does not require the use of a standard curve. Instead, each production-lot of reagents are supplied precalibrated, allowing the delta-Ct between *ABL1* and *BCR-ABL1* to be measured and then used to calculate the ratio of *BCR-ABL1*:*ABL1*

[65]. The latest generation of this assay (Xpert *BCR-ABL* Ultra) is calibrated with secondary reference material aligned to the WHO *BCR-ABL1* genetic reference panel, allowing results to be reported directly on the IS and is sensitive enough to allow monitoring of DMR [66].

The application of single-cell sequencing to CML is also beginning to be investigated. Initial studies have demonstrated the heterogeneity of CML stem cells and revealed distinct subpopulations that persist through TKI treatment [67, 68]. The single-cell approach has been used to enhance molecular monitoring in acute myeloid leukemia [69] and may have the potential to provide similar benefits in CML.

Currently it is not clear if the measurement of *BCR-ABL1* mRNA levels pretreatment and/or using DNA-based approaches are really going to become routine practice, but both will require further standardization since the IS breaks down above levels of 10% when different reference genes are used [6], and it is unclear how to relate DNA-based results to the IS. It seems likely that RT-qPCR on the IS will continue to be the method of choice for monitoring CML patients in most centers for the foreseeable future although digital PCR could have a major impact if it was cheaper.

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