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A Practical Guide To Chimerism Analysis: Review of The Literature and Testing Practices Worldwide

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Abstract

Background and Purpose: Currently there are no widely accepted guidelines for chimerism analysis testing in hematopoietic cell transplantation (HCT) patients. The objective of this review is to provide a practical guide to address key aspects of performing and utilizing chimerism testing results. In developing this guide, we conducted a survey of testing practices among laboratories that are accredited for performing engraftment monitoring/chimerism analysis by either the American Society for Histocompatibility & Immunogenetics (ASHI) and/or the European Federation of Immunogenetics (EFI). We interpreted the survey results in the light of pertinent literature as well as the experience in the laboratories of the authors.

Recent developments: In recent years there has been significant advances in high throughput molecular methods such as next generation sequencing (NGS) as well as growing access to these technologies in histocompatibility and immunogenetics laboratories. These methods have the potential to improve the performance of chimerism testing in terms of sensitivity, availability of informative genetic markers that distinguish donors from recipients as well as cost.

Summary:

The results of the survey revealed a great deal of heterogeneity in chimerism testing practices among participating laboratories. The most consistent response indicated monitoring of engraftment within the first 30 days. These responses are reflective of published literature. Additional clinical indications included early detection of impending relapse as well as identification of cases of HLA-loss relapse.

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Dr. Askar has received compensation as a member of the scientific advisory board for CareDx, Inc. Dr. Blouin, Dr. Ye, and Ms. Williams declare no potential competing interests.

Keywords

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1. Introduction

Allogeneic hematopoietic cell transplantation (HCT) is considered to be the only curative intervention for a variety of malignant and non-malignant diseases. More than one million HCT have been performed worldwide during the past six decades [1]. This number is steadily rising and survival rates are improving. Despite its durable curative potential and improved safety, failure of engraftment, graft versus-host disease (GVHD) and disease relapse remain as significant causes of treatment failure. Chimerism analysis is commonly performed to diagnose or identify these conditions [2–5]. Following allogeneic HCT, complete chimerism (CC) implies that only donor hematopoietic cells are detected [6]. Mixed chimerism (MC) is defined by identifying both donor and recipient hemopoietic cell. Therefore, monitoring engraftment kinetics by accurate quantitative analysis of chimerism is critical in detecting impending failure of engraftment and relapse after HCT. Analysis of lineage-specific chimerism has also been reported to improve the sensitivity of the assay [7–9]. Timely detection of MC could provide useful information to guide specific therapeutic interventions, such as tapering of immunosuppression, donor lymphocyte infusion (DLI) or re-transplantation.

Chimerism analysis is based on the detection and quantification of specific genetic differences (i.e., polymorphic markers) that distinguish donor cells from recipient cells (i.e., informative polymorphic markers). It essentially quantifies the proportion of hematopoietic cells of donor origin relative to those of recipient origin in peripheral blood, bone morrow and potentially other tissues. Observing trends in these proportions over time is utilized to monitor and track the donor engraftment status. Various methods have been used for monitoring the chimerism status, all following the same basic principle of distinguishing differences in informative polymorphic genetic markers. Earlier non-molecular methods included red cell phenotyping and cytogenetics-based methods such as fluorescent in situ hybridization (FISH) of sex chromosomes [10–12]. The counterpart molecular methods include restriction fragment length polymorphism (RFLP), variable number of tandem repeats (VNTR) and short tandem repeat (STR) testing by polymerase chain reaction (PCR) followed by fragment analysis [13–15]. With reported sensitivity of 1–5%, STR has widely replaced less-sensitive approaches to become the most commonly used method over the past two decades. Recent years have witnessed the emergence of much more precise methods of chimerism analysis including real-time quantitative PCR (qPCR), digital droplet PCR (ddPCR) and next generation sequencing (NGS). With sensitivity limits of decimals of one percent, these ultra-sensitive methods have been reported to be advantageous in monitoring engraftment kinetics and early relapse detection [16–18]. Despite the appreciable sensitivity advantage of these methods, their clinical utility remains to be established. From a clinical implementation perspective, the optimal methodological approach needs to be informative, sensitive, quantitatively accurate, reproducible and cost effective.

This review highlights the technical considerations for performing chimerism testing using common and emerging molecular assays. We also present the results of a survey on chimerism testing practices in laboratories accredited for performing chimerism testing by either the American Society for Histocompatibility & Immunogenetics (ASHI) and/or the European Federation of Immunogenetics (EFI). We discuss the survey results in the context of published literature as a step towards establishing evidence-based practice guidelines for chimerism testing after HCT. For a number of the critical aspects of performing chimerism analysis where there was not enough published literature, the authors included local experiences in their respective laboratories.

2. Methods

Study Design

In April 2019, a web-based survey was distributed by email to laboratories accredited by ASHI (n= 45) and EFI (n= 51). The survey (Supplemental Table S1) was developed, and data was collected using the web-based application Microsoft Forms (Microsoft Corporation, Redmond, WA). The results were exported and analyzed in Microsoft Excel (Microsoft Corporation, Redmond, WA). The survey recipients were provided a cover email invitation stating that by responding to the survey they agreed to participate in this study. The study was approved by Baylor Scott & White Health Research Institute Institutional Review Board.

The survey included questions related to trends in chimerism testing after HCT. In addition, the survey included questions regarding test utilization, clinical utility and basic characteristics of the transplant programs served by participating laboratories.

3. Results

3.1 Participant Demographics

Fifty-four ASHI (n= 28, 62%)/EFI (n=26, 51%) laboratories accredited for performing chimerism testing/engraftment monitoring responded to the survey. The demographic questions characterized the respondent programs based on geography and type of laboratory. Our participant group represented a broad spectrum of laboratories including a wide geographic distribution. Almost half (46%, n=25) of respondent laboratories were in North America, one of which was from Mexico. The remaining North American respondents represented laboratories in the United States (44%, n=24). Laboratorians from Europe (n=22) comprised 41% of survey respondents, with the most responses received from Italy (n=7, 13% of total responses) and France (n=4, 7% of total responses). The remaining 6 responses (11%) represented laboratories from South America (Argentina, n=1); and the Middle East (Israel n=1, Kuwait n=1, Qatar n=1, Saudi Arabia n=1 and Turkey n=1). There were no responses from Asia, Africa, or Australia.

The majority of respondents described their laboratory as academic/university hospital-based (n=26, 48%). Reference/private labs comprised 17% of participants. The remaining respondents identified their labs as hospital-based (9%), government-based (9%), blood center-based (4%) and organ procurement organization-based (2%). Of note, 19% of survey

responders did not answer this question. The survey included questions directed at the characteristics of the transplant program that the laboratory supports such as program size and types of HCT performed. There were insufficient responses to these questions to conduct a meaningful analysis (only one laboratory responded).

3.2 Technical Considerations

3.2.1 Testing Method—The majority of responding laboratories (87%, n=47) reported using STR for chimerism detection, with some reporting use of multiple methodologies. Higher sensitivity methods reported included qPCR (24%, n=13), NGS (7%, n=4) and ddPCR (2%, n=1) were also reported. One laboratory reported using VNTR. While the majority of respondents used commercially available reagents and analysis software, several reported using in-house developed reagents (n=8) and analysis programs (n=6).

3.2.2 Proficiency Testing: Forty-two laboratories (77%) reported participating in external proficiency testing (PT) programs. Reported providers of PT included ASHI (n=14), National External Quality Assessment Service (NEQAS, n= 8), College of American Pathologists (CAP, n= 7), Istituto Superiore di Sanità (ISS, n=3), EFI (n= 2), the Spanish and Portuguese-Speaking Working Group of the International Society for Forensic Genetics (GHEP-ISFG, n=2), Instand (n=1) and Italian National EPT IN Immunogenetics (n=1).

3.3 Testing Utilization

3.3.1 Chimerism Testing Timepoints—Although there were variations in timepoints at which samples were taken for chimerism testing, there were some commonalities, most notably within the first year post-HCT. The majority of respondents indicated that they tested chimerism samples at 1 month (80%), 3 months (70%) 6 months (61%) and 1 year (67%). Five laboratories (9%) reported testing samples collected earlier than post-HCT day 30. Fifty percent of laboratories also reported testing chimerism at 4 months. Regarding chimerism testing after the first-year post HCT, one respondent specified yearly testing, while another respondent reported yearly testing for 3 years followed by biennial testing. In free text comments, some specified that, as the performing laboratory, they do not determine the timepoints for testing (n=5), or that the timepoints sampled vary by clinician or transplant program (n=4).

3.3.2 Lineage-specific Chimerism Testing—The majority of labs (74%) offer chimerism testing on cell subsets whereas 26% of respondents (n=14) do not. The majority of respondents (68%, n=37) perform chimerism testing on T cell populations (selecting for CD3). Most (52%, n=28) also test myeloid/granulocyte populations (selecting for CD33, CD66b, or CD15). Additional populations tested by the respondents include B cell population (28%, n=15), NK cells (22%, n=12), and CD34+ cells (18%, n=10), CD14+ cells (9%, n=5), and CD71, which is a marker of early erythroid precursors (n=1).

3.3.3 Sample Source/Type—Almost all respondents indicated that peripheral blood (PB) (99%, n=53) and bone marrow (BM) (92%, n=50) are the primary sources used for chimerism testing. PB was the most frequently submitted sample type for the majority of labs (89%, n=47). Among the laboratories that perform chimerism testing on BM, 13%

reported that it was their most frequent specimen type (n=7). Additional specimen sources reported include tissue biopsy (n=9).

3.4 Clinical Utility

All survey respondents reported engraftment monitoring as a primary indication for chimerism testing. The majority also reported using chimerism testing in the diagnosis of relapse (80%, n=43) and in immunotherapy planning, such as DLI (59%, n=32). Additional indications included GvHD (n=3 responses), planning for second transplant (n=1 response) and infectious disease impacting engraftment (n=1 response).

4. DISCUSSION AND REVIEW OF THE LITERATURE

The ultimate goal of HCT is to completely or partially replace host hematopoiesis with that of a donor origin, thereby inducing a state of chimerism. As a part of monitoring effectiveness of HCT, chimerism analysis is performed in the post-transplant period to determine the origin of hematopoiesis. In 2001, the International Bone Marrow Transplant Registry (IBMTR, currently the Center for International Blood and Marrow Transplant Research or CIBMTR) and the National Marrow Donor Program (NMDP) sponsored a workshop to discuss the clinical utility of chimerism analysis after HCT and make recommendations regarding laboratory methods, specimen types, and frequency of analysis [19]. A variety of terms are used in clinical and scientific communication and are defined in table (1) [6]. The detection of MC is dependent on the sensitivity of the testing methodology used (see below).

4.1 Characteristics of participants:

Our survey of practices among ASHI/EFI accredited histocompatibility laboratories from different parts of the world indicated shared themes as well as notable heterogeneity in such practices among these laboratories. Overall, there was a relatively high response rate of 56% of those who were invited to participate. Not surprisingly, representation of laboratories was more heavily weighted towards North America and Europe with less participation from South America and the Middle East. There was no representation from other regions of Asia, Africa or Australia. This representation is consistent with distribution of laboratories accredited by ASHI and/or EFI. These laboratories also represent the geographic regions of the world where a significant proportion of HCT procedures are performed. The majority of responses were received from laboratories with academic affiliation, reflecting the level of complexity and interdisciplinary services typically available in academic institutions. However, some government and private hospitals were also represented. We have not received an adequate number of responses indicating transplant program characteristics or practices. The insufficient responses regarding clinical practices of transplant programs including types of transplants performed as well as indications for performing chimerism testing may suggest inadequate involvement of laboratories in clinical consultation and guiding clinical practices.

4.2 Technical Considerations

4.2.1 *Sample volume* and *Viability*—Peripheral blood or bone marrow specimens are used for chimerism monitoring. A minimum of 1–2 mL of whole blood or 0.5–1 mL of bone marrow is collected in EDTA or ACD tubes and stored at 4–8°C. DNA can be extracted within 7 days of collection when cell subpopulation enrichment is not needed. When subset enrichment is required, a minimum of 2–4 mL of whole blood is required per cell subset. Specimens for enrichment should be processed within 48 hours to maintain the integrity of surface cell markers utilized for cell separation and assessment of fraction purity.

4.2.2 Cell enrichment methods—Positive selection of cell lineages allows faster recovery due to relative ease and efficiency. Cell purity is higher in a positive selection method, but the increase in purity may reduce the overall yield. Negative selection may be advantageous when there is a disproportionately larger number of unwanted cells compared to the number of enrichment target cells present in the specimen [20]. Another advantage of negative selection is the removal of unwanted populations without the binding of a secondary antibody to the target populations, leaving them unaltered for downstream applications.

Having adequate staff is essential for optimal performance of the chimerism assay. As soon as samples arrive in the laboratory for testing, there must be properly trained staff available to perform the cell lineage isolations. If the laboratory does not perform the chimerism testing routinely, or perform this test in batches, there should at least be one individual assigned to the task of performing the initial cell lineage isolation. Once that is done, the isolated cells can be stored at 4°C and nucleic acid extraction performed at a later time.

Several considerations can guide the choice of the method for the enrichment of specific cell lineages for performing chimerism analysis. The two commonly used approaches are separation with immunomagnetic particles and fluorescence-activated cell sorting (FACS) using flow cytometry. Both enrichment techniques were recently reported to offer excellent recovery and purity of CD34+ cells for used in clinical applications [21]. A comparison between the two approaches is presented in Table 2. In addition, the following considerations are also relevant: beads can be purchased in any quantity, but keeping track of reagents in terms of initial quality control and expiration dates for each lot can be a logistical challenge, particularly if not all subsets are performed routinely. For cell-sorters, substantial manipulation is required before starting the sorting process on the instrument. Sorting directly from whole blood, particularly of low frequency populations such as CD34+ cells is not feasible.

4.2.3 Purity of Lineage-Specific Cell Fractions—The majority of labs reported performing chimerism analysis on cell subsets. Notably, the level of purity achieved by different methods can vary from sample to sample and patient to patient. Yield and purity are dependent on total cell count and the frequency of the target cell population [22–24]. In addition, the rate of recovery varies among different cell populations [25]. CD3+ T-cell purity was reported to range from 1–98%, with only 36% of routine clinical samples achieving >90% purity [23]. Willasch *et al.* found that the purity of CD3+ and CD19+

subsets were dependent on sufficient absolute cell counts in the starting blood sample as these populations may be of low frequency following HCT. Particularly in the setting of a T-cell-depleted HCT, the authors suggested that a concurrent complete blood count with a differential was essential to determine achievable purity [24].

There are different approaches to purity assessment and determination of the minimum purity requirements. Some approaches include establishing or verifying achievable purity using samples from healthy donors. However, this will not account for the variation seen in post-HCT patients and may not reliably reflect the purity attained in samples from these patients. The UKNEQAS for Leucocyte Immunophenotyping Chimerism Working Group recommended purity assessment by flow cytometry [10]. Importantly, the recommendation specifically indicated that purity assessment should be performed for all cell lineages and reported with the chimerism analysis results. However, this is often not the case. According to a NEQAS survey almost half of labs that reported performing chimerism testing did not routinely assess the purity of the cell fractions [23]. It is noteworthy that ASHI, EFI and CAP standards require reporting established cell purity [26, 27] but do not specify a minimum level of purity required.

As the purpose of chimerism analysis is to quantify the contribution of donor versus recipient hematopoiesis, the purity of the sample will greatly impact results. In other words, if the enriched fraction is not sufficiently pure, the chimerism analysis will not reflect the true origin of that particular cell population.

Published studies rarely report the approach to purity assessments or minimum acceptable purity threshold. This makes the comparison of findings and conclusions across studies challenging. Acceptable minimum purity levels have not been defined; and studies specifically addressing this in the era of high-sensitivity assays have not been published. In the absence of these data, Hanson et al, suggested that for CD3+ lineage 90% is an achievable minimum purity level[22]. However, other lower frequency populations, such as CD34+ and NK cells, were not assessed. Experiences in our laboratories found that a minimum purity of 80% is more achievable for these cell populations. With the availability of high-sensitivity molecular assays that can detect chimerism well below 1%, the impact of cell lineage purity on test results reported to clinicians may be magnified. This highlights the need for studies to define and standardize minimum purity requirements. Guidelines for purity assessment techniques and minimum purity requirements will provide much needed quality assurance and standardization of chimerism analysis.

4.3 Molecular Methods

In its earlier iterations, chimerism analysis was performed using cytogenetic strategies such as fluorescent in situ hybridization (FISH) and immunology method such as red cell phenotyping. However, these techniques have their own limitations. They can be laborious or time-consuming and of low informativity or low sensitivity. Additionally, their applicability may be restricted to sex-mismatched transplant.

A comparison among different molecular and non-molecular techniques is summarized in Table 3.

4.3.1 STR, VNTR and RFLP—With the advances in molecular techniques and genome sequencing, scientists have identified three types of genomic polymorphisms, namely single nucleotide polymorphisms (SNPs), insertion/deletions (InDels) and microsatellites (VNRT and STR). Most polymorphisms are found in the noncoding regions of the genome [28]. The first method of DNA analysis to take advantage of sequence polymorphisms was restriction fragment length polymorphism (RFLP), where genomic DNA is digested with restriction endonucleases [29, 30]. However, RFLP analysis was time consuming, labor intensive, of low sensitivity and required large amount of high integrity DNA input.

The breakthrough in monitoring chimerism status came when polymerase chain reaction (PCR) techniques were developed [31]. Molecular analysis of microsatellite markers (VNTR or STR) by conventional PCR followed by fragment analysis became the dominant method for chimerism testing [32–37]. VNTRs were among the first genetic markers used to quantitate chimerism analysis. The limited availability of VNTR markers for distinguishing donor/recipient pairs renders the technique non-informative in a number of transplant pairs. In contrast, the STR method provides a solution for this challenge because of the higher degree of polymorphism and relatively shorter fragment length. These characteristics render STR more suited to detect chimerism accurately [33–37]. As a method, STR analysis is rapid, reliable, accurate and reproducible. An additional advantage of this method is the low amount of DNA input required (1–5 ng), which is important when analyzing chimerism in lineages of low frequency. By virtue of being the most commonly used method at present, there are multiple commercially available kits and analysis software available. However, STR testing is not without limitations. It has a limited sensitivity of 1% to 5% with a low accuracy (high coefficient of variation) at low chimerism percentages. In addition, analysis challenges include allelic imbalance (which is differential amplification efficiency of alleles, or amplification imbalances due to preferential amplification of the smallest alleles), stutter peak (which is a PCR-generated artifact and is a result of strand slippage during DNA synthesis of PCR, showing up primarily one repeat before), non-template adenine addition and allele dropouts. It’s also important to note that the amount of input DNA needs to be kept to a minimum to avoid PCR competition and plateau biases.

4.3.2 qPCR—Recent studies have showed that quantitative real-time PCR (qPCR) using TaqMan technology can be used for transplant monitoring and has much high sensitivity than STR [36, 38–41]. qPCR is a direct quantitative method evaluating the cycle threshold (Ct), which is inversely proportional to the original amount of target DNA [42]. The percentage of recipient or donor hematopoietic genomic DNA is obtained by the calculation of differences in threshold cycles (ΔΔCt), where the post-HSCT hematopoietic DNA Ct is compared with the pre-transplant recipient or donor DNA Ct, and the data is normalized with a reference gene Ct. In 2002, Alizadeh et al. first demonstrated that qPCR of 19 SNPs improved sensitivity, detecting 0.1% recipient DNA when 100 ng of DNA used in whole blood samples [36]. Later, a modified qPCR approach using more robust InDel polymorphisms was published [38–41]. Commercial kits are available with 10 – 50 InDel markers and primer/probes. The longer genomic sequence of InDels allowed improved discrimination and reduced assay complexity compared to SNPs, which identify smaller differences among different alleles. The sensitivity of InDel-based qPCR chimerism analysis

has been reported in the range of 0.1–0.01% depending on the selected markers and quantity of DNA input (ranging from 25 ng to 300 ng) [36, 38–41]. Kim et al reported that the limit of detection was 0.11% and 0.024% when used 25 ng and 250 ng of input DNA was used respectively [40].

Nonetheless, the highly sensitive qPCR based chimerism analysis method is limited by a number of technical challenges. The major disadvantage of qPCR is limited accuracy and less precision in quantifying mixed chimerism when the smaller percentage contributor (donor or recipient) DNA level is high (>30–50%). This is explained by the principle of qPCR, which has a variability of threshold cycle value (Ct) up to one cycle and can result in an error up to 0.5 or 2 times the true value [36]. When a percent of the smaller proportion contributor (recipient or donor) allele is high, the inaccuracy of qPCR could be more pronounced [40]. The variability of the quantification is highly influenced by the efficiency of amplification reactions. Calibration curves and multiple replicates are required due to limited technical precision. In addition, quantitative results have to be normalized against an endogenous gene amplified in separate PCR reactions, which also can contribute to the variability in quantification. Although sensitivity is increased with increased DNA input, it leads to a substantial rate of false-positive results [41]. Pre-transplant DNA needs to be included in every qPCR assay. The availability of pre-transplant DNA might be challenging with a limited amount of DNA. This challenge is even more pressing when handling low DNA yield that is a result of disease-associated cytopenia or cell enrichment for the purposes of lineage-specific chimerism.

4.3.3 ddPCR: Digital droplet PCR (ddPCR) is a third-generation PCR technology that complements traditional end-point PCR and real-time PCR. It was developed to overcome limitations of conventional amplification techniques. With ddPCR, samples are partitioned into multiple nanoliter droplets so that each droplet becomes a separate sample with 0, 1 or more copies of the target DNA molecule [43]. The droplets are thermally cycled to end-point and each droplet is counted as positive or negative for specific polymorphism (e.g., InDel). Then Poisson statistics are applied to quantitate the total number of amplifiable targets, from which the absolute number of starting copies can be accurately determined. Therefore, this novel technology offers quantification of the targets at the end-point detection of amplification product in absolute quantities rather than a ratio among different reactions. The efficiency of amplification and the susceptibility to PCR inhibitors is much less of a concern. Consequently, calibration curves, efficiency controls and multiple replicates are not necessary in ddPCR as in qPCR.

Recently, several groups have studied the analytical performance of chimerism analysis by ddPCR, which provides sensitivity values equivalent to qPCR, but with greater accuracy and less variability across the full dynamic range of detection (from 0.05% to 100%) [38, 44–47]. Recently, CE-IVD marked commercial ddPCR kits have become available in the market [48].

Although sensitivity is improved with increasing the DNA input in a similar fashion to qPCR, care must be taken to not generate too many positive droplets such that Poisson statistics can no longer be applied, leading to inaccurate estimations of copy number.

4.3.4 Next generation sequencing (NGS): NGS provides qualitative and quantitative data. The accuracy of quantitative data depends on the depth of sequence data collected for each sample and on the quality of the starting target DNA. For samples with lower abundance targets, many more sequence reads are required to achieve accurate quantification. For chimerism analysis, donor and recipient informative SNP or InDel reads are measured by NGS, and are used to calculate average quantities within a 95% confidence interval of donor and recipient DNA in the post-transplant samples.

Currently, a number of studies have applied NGS chimerism panel using SNP and InDel markers and have shown high reproducibility/precision through a full range of donor-recipient chimerism. They also show a sensitivity of 0.1–1%, which is higher than that of STR-PCR analysis but lower than qPCR and ddPCR [49–51]. Today, several commercially available kits offer an NGS method for chimerism as a complete workflow solution for laboratories, combining reliable testing processes with purpose-designed analytical software.

The technical improvement of NGS platforms has overcome a number of limitations of predecessor methods. High throughput design allows simultaneous detection of hundreds of SNP and InDel markers at a low cost per target [52]. Furthermore, NGS chimerism assays perform reliably with same sensitivity even using significantly lower DNA input amounts (2–5 ng) than those required by qPCR and ddPCR. Doing so enables the monitoring of clinical samples with low DNA yield while still performing cell lineage chimerism, particularly on lower frequency populations such as CD34+ cells. Since NGS instrumentation is becoming more popular in clinical laboratories performing HLA typing for HCT recipients and donors, the novel NGS-based chimerism assay can replace both STR-based and real-time PCR based assays through improved diagnostic performance.

4.4 Test Utilization Considerations

4.4.1 Time points: Routine chimerism testing is performed not only to evaluate success of the transplant and monitor engraftment, but also to diagnose relapse. The most consistent survey response indicated routine chimerism testing at post-transplant day 30 followed by testing at 3- and 12-months post-transplant. This is in line with previously published recommendations from a workshop at the 2001 Tandem Meetings [19]. These consensus-based recommendations also suggest routine monitoring (every 2–4 weeks) until stable engraftment (especially in the context of a nonmyeloablative conditioning regimen or a manipulated graft) as the kinetics and early patterns of chimerism may predict graft loss or relapse. Therefore, routine monitoring may allow for a timely therapeutic intervention.

Since the publication of these recommendations twenty years ago, several groups have investigated the utility of routine monitoring to allow for pre-emptive immunotherapy at the time of MC detection, with conflicting findings. More recent studies have demonstrated that achieving CC at day 30 post-HCT is associated with lower rates of relapse and longer relapse free survival but not overall survival than recipients with late CC [53]. When multiple timepoints were examined (day +30, at 3-, 9-. 12-months), only day 30+ CC was associated with lower rates of relapse [54].

The median time from detection of MC to relapse was reported as 1 month (range: 1–12 months) in relapse cases occurring over a range of 1 to 31 months, supporting the clinical utility well beyond 1-year post-HCT [55].

An important factor in reconciling the findings of these seemingly conflicting studies is the sensitivity of the chimerism testing method used. Many of the studies that implied a lack of utility of routine monitoring used STR or less sensitive methods [56–59]. This strategy would miss patients with low level MC, and misclassify them as CC. In contrast, studies that utilized more sensitive methods, such as qPCR, suggest that early serial testing may be useful. In a prospective study by Qin *et al.*, where qPCR was used for chimerism testing, patients who experienced MC>1% and received pre-emptive immunotherapy had lower rates of relapse than those who did not receive pre-emptive treatment [55].

Taken together, the strongest evidence indicates the clinical relevance of testing at one-month post-transplant, particularly in non-myeloablative conditioning regimens and with manipulated stem cell products as well as when suspecting failure of engraftment or impending relapse.

4.4.2 Lineage-Specific vs Unfractionated—Most laboratories surveyed offer chimerism analysis on lineage-specific cell subsets, in addition to unfractionated samples. T cell and myeloid lineages were the subsets most frequently analyzed by survey participants with fewer laboratories performing chimerism analysis on B cell, NK cell lineages and CD34+ cells. This is consistent with recommendations that subset analysis be used for monitoring, particularly in nonmyeloablative conditioning transplants [19].

Documentation of both myeloid and lymphoid engraftment is particularly relevant in the setting of non-myeloablative conditioning regimens. Several studies have reported detectable lineage-specific MC post-HCT in association with clinical outcomes [4, 37, 60–66]. However, results of lineage-specific chimerism analysis should be interpreted taking into account the dynamic nature of engraftment and rates of immune reconstitution among different cell lineages [25].

In addition to differentiating the origin of specific cell lineages in the recovering hematopoiesis, subset analysis is also suggested to have higher sensitivity compared to chimerism analysis of unfractionated whole blood (WB) [19]. Mixed chimerism of a low frequency cell population such as T cells following T cell depleted/reduced HCT could be below the limit of detection in unfractionated samples but would be detected in T cell-enriched populations.

T cell lineage was the most commonly subset tested by chimerism analysis in our survey. Many studies, including both myeloablative and nonmyeloablative conditioning regimens, have reported detection of MC in the T cell subsets to be associated with increased risk of disease relapse [37, 60, 63, 67]. In contrast, others have found no association of T cell MC and clinical outcomes [68–71]. These conflicting observations may be explained in part by differences in cutoffs (% donor chimerism) for outcomes analysis. Additional challenges in reconciling these studies arise from the fact that critical methodologic details, such as the

validated chimerism analysis test sensitivity and purity of cell enrichments, are often not reported.

The clinical utility of subset chimerism analysis is dependent on achieving sufficient purity of subset-enriched specimens to provide accurate and reliable results. Ideally, technical guidelines should address acceptable purity levels for routine clinical results reporting [10].

4.4.3 *Specimen Source:* Bone Marrow, Peripheral Blood vs. Plasma *(cfDNA)*

—The majority of labs reported peripheral blood (PB) as the main specimen source for post-HCT chimerism analysis whereas bone marrow (BM) was reported as an additional sample type. Previous guidelines have recommended the use of PB over BM for chimerism, especially in the setting of nonmyeloablative transplants, wherein distinguishing myeloid and lymphoid mixed/split chimerism is relevant [72–75]. T cells in PB represent a higher % of total leukocytes than in BM, rendering mixed T cell chimerism detection in PB more readily achievable [19]. Some studies have directly compared the clinical utility of PB to BM for chimerism analysis [76, 77]. A small retrospective study reported a high correlation of chimerism results between the two specimen sources at multiple time points post-transplantation, suggesting that PB chimerism analysis provides equivalent information with less invasive testing [76].

In deciding on the optimal source (bone marrow vs peripheral blood) for early and actionable testing, one must consider that a high sensitivity method may detect chimerism kinetics in the BM that are not necessarily indicative of relapse or graft failure. Also, it has been proposed that the higher recipient DNA in BM samples may reflect marrow stromal cells and not recipient hematopoiesis [78, 79]. In a prospective follow up of patients that showed CC by STR, qPCR revealed that 59% had 0.1–1% recipient DNA in BM while only 7% presented 0.1–1% recipient DNA in PB [80]. Of the latter group, only one of 4 patients relapsed. This suggests that the detection of recipient DNA in PB is much less frequent than in BM but may perform better as a predictor of relapse. In a prospective study by Gambacorta *et al*, 20 post-HCT patients with high-risk and very-high risk AML were monitored by serial chimerism analysis of both BM and PB by qPCR [81]. Comparing BM and PB samples by qPCR showed moderate correlation with significantly higher recipient DNA in BM compared to PB.

Increasing MC detected in PB had an increased sensitivity and specificity in prediction of relapse compared to BM, although not statistically significant. The lower specificity and higher % recipient DNA in BM samples (regardless of relapse), suggest that PB is the superior sample source for relapse prediction, owing to higher specificity and ease of access (compared to invasive bone marrow sampling) for patients at a high risk of relapse. Currently, there is not strong evidence to support using BM compared to PB as a sample source. Additional studies that prospectively compare PB and BM using highly sensitive methods are needed to characterize changes in MC representing biologic variations versus those that portend imminent relapse or graft failure.

Cell-free DNA (cfDNA) consists mainly of small sized (80–200 base-pairs) double-stranded DNA fragments resulting from apoptosis, necrosis, immune-mediated cell damage, or

release of nuclear DNA into the circulation predominantly from hematopoietic cells [82–84]. Technological advances in interrogating cfDNA isolated from plasma and other body fluids has dominated the development of noninvasive prenatal testing (NIPT) and liquid biopsy of malignancies [85, 86]. Emerging applications of cfDNA testing include organ transplantation, autoimmune diseases, cardiovascular diseases, sepsis, stroke and many other disorders [87–93]. The clinical utility of cfDNA as a specimen source for performing chimerism analysis has also been reported in a limited number of studies. Aljurf and colleagues reported that cfDNA chimerism was comparable to that in polymorphonuclear (PMN) cellular chimerism in nonneoplastic disorders [94]. They also reported that in leukemia patients, MC was detected in cfDNA at a higher rate than in cellular specimens in patients with relapse (i.e., more sensitive) but was also detected in patients with no evidence of relapse (i.e., false positive). Another study reported a decrease in the observable percentage of recipient cfDNA in patients who showed improvement in response to treatment of GvHD whereas in patients without improvement, stable or increasing levels of recipient cfDNA were detected [95]. Although it is biologically plausible that the recipient cfDNA could be derived by cell disruption in GvHD target organs or by killing the leukemic cells in GVL, the findings of these studies have not been reproduced in other studies and therefore, the clinical utility of cfDNA chimerism remains to be established.

4.4.4 Practical Approach to Method Sensitivity—

The level of sensitivity required depends on the indication for testing: documentation of engraftment does not require a methodology that can detect microchimerism. If the test is being performed for monitoring in patients at high risk for relapse with the intention to guide immunotherapeutic interventions, the method should be capable of detecting microchimerism (see Table 3) at a sensitivity that allows for timely interventions in the patient population. This method should also have high accuracy and precision to monitor chimerism trends over time.

4.5 Chimerism Analysis with Multiple Donors

When patients are transplanted with multiple donors, chimerism analysis methods must discriminate genetic differences across multiple genomes (i.e., the host and more than one donor). This can be encountered in several transplant scenarios such as double umbilical cord bloods (UCB), combined UCB with HLA-haploidentical family donors, re-transplant or HCT combined with allogeneic immune effector therapy. The key potential challenges are insufficient informative markers and decreasing sensitivity.

The STR method, in addition to being the most widely used for quantitative chimerism analysis, is also well suited for testing multiple donors because of its high degree of polymorphism and consequently high informativity. A single short tandem repeat locus has between 8 and 40 different alleles [96]. The STR loci with a higher number of alleles, are more frequently used in chimerism analysis as they are more informative. Typically, 8–15 STR loci are adequate for determining chimerism in specimens with more than two genomic contributors with a high probability of discrimination. In one of the commercial kits, it was estimated that 15 STR loci showed a combined Power of Discrimination (PD) of 1–1.3729×10^{-17} and a combined Matching Probability (pM) of 1.3729×10^{-17} among Japanese [97]. This essentially means that there would be no other individuals with the same

types across all 15 STR loci in the world, except among monozygotic multiples or the same clone. Therefore, the informativity of STR and the ability to discriminate differences among three unrelated or related individuals (double donors) and even among four unrelated or related individuals (triple donors) is quite adequate. However, the sensitivity of this method is relatively low for single donor (detection level of a minor genotype is between 1% and 5%) and is even less accurate when evaluating specimens with more than one donor, mainly because of PCR competition biases [36].

The selection criterion for an informative marker is not entirely based on maximum informativity and allele frequency, but also on the ability to identify even small amounts of DNA in the mixture. Recent studies have demonstrated the advantages of biallelic markers (SNP and InDel) as being stable, unique and suitable for sensitive quantitative methods for qPCR, ddPCR and NGS chimerism analysis [49–52, 98]. However, these genetic markers are still of limited use due to the complexity of assay design and the limited informativity of SNP markers. A biallelic SNP marker has only three possible genotypes compared to an STR locus with 8 – 40 alleles [96]. However, the abundance of SNP and InDel markers throughout the genome, combined with the multiplex technologies allowing simultaneous testing of hundreds of these markers, affords the high level of informativity needed for multiple donors. Validation data from our laboratories indicated that biallelic markers (SNP and InDel) can distinguish more than two genomes when a large number of markers (>200) spanning all chromosomes are used for chimerism analysis by NGS. Although several qPCR or ddPCR panels with maximum of 50 SNP and InDel markers for chimerism analysis are commercially available, they have limited informativity in specimens with multiple donors. Therefore, the NGS assay offers the high informativity as well as the high sensitivity and accuracy when determining chimerism in specimens of recipients of multiple donors [98].

4.6 Detection of HLA Loss in post-HCT Relapse

Timely detection of relapse that allows for early intervention remains a challenge in the post-HCT period. The recently described HLA-loss relapse was initially identified in post-haploidentical HCT. In this type of relapse the patient-specific HLA alleles (i.e. the GvH mismatched haplotype) become no longer detected on the leukemic cells by HLA genotyping [99]. Further studies demonstrated an underlying copy-neutral loss of heterozygosity of the short arm of chromosome 6 (6p) encompassing the HLA region. This finding is hypothesized to occur through substitution for the “lost” haplotype with a corresponding region from the homologous chromosome, a phenomenon known as acquired partial uniparental disomy. Furthermore, this phenomenon was demonstrated to lead to loss of allorecognition of the malignancy by donor T cells. Since the recipient mismatched HLA haplotype is a target for donor T cell mediated graft versus leukemia (GVL), loss of these HLA targets is a tumor escape mechanism to evade the GvL selective pressure [100]. HLA loss relapse has been identified post-HCT using other donor sources, including 8/8 HLA matched unrelated donors [101–104]. This is possible because these 8/8 matched HCT donor (at HLA-A, -B, -C, -DRB1) may still have mismatches at other HLA loci, commonly DPB1 and possibly DQB1, which may serve as a target of alloreactivity [105].

Detection of HLA-loss in the setting of relapse can have implications for therapeutic management decisions. Relapsed leukemic cells that no longer expresses mismatched HLA-targets may be resistant to the intended GvL benefit of DLI but still carry the same risk of GvHD. Likewise, this may influence donor selection for re-transplant to prefer donors mismatched for the remaining recipient haplotype, which is the remaining target for GvL [106–108]. In terms of testing methodology, it is important to emphasize that this mutation is copy-neutral and therefore undetectable by cytogenetics techniques traditionally used in chimerism. Furthermore, polymorphic markers typically used in chimerism analysis are outside of the MHC. Therefore, routine chimerism analysis will not distinguish these leukemic variants from recipient cells. A diagnostic test to identify the loss of HLA antigens requires polymorphic markers within (i.e., HLA markers) and outside (i.e., non-HLA markers) of the MHC region encoding HLA. The original discovery was made through parallel HLA typing and chimerism STR analysis. Currently, there is one commercially available qPCR test that combines biallelic chimerism markers as well as HLA allele-specific qPCR [101]. The qPCR chimerism analysis based on indel/SNPs differentiates recipient from donor as a reference, while the HLA allele-specific reactions identify allele loss of heterozygosity in recipient. A case of HLA-loss relapse leukemia will be positive for recipient-specific non-HLA markers, but negative for recipient-specific HLA markers. The targeted HLA alleles in the panel include frequent HLA allele groups at the HLA-A, C, DPB1 loci, as these are the loci most often mismatched in both haploidentical and unrelated donor HCTs [103, 104, 109, 110]. In an evaluation of performance of this assay, Ahci *et al* reported high accuracy and reproducibility with a sensitivity of 0.16%. In one study, the panel contained informative markers in at least 70% and 66% of donor-recipient pairs (with HLA mismatches in the GvH direction) in a large series of 454 haplo-HCT and 113 URD-HCT, respectively [101]. Other methodologies, such as NGS have the potential to address known limitations of qPCR by offering higher precision as well as a higher informativity by covering significantly higher number of HLA alleles [102].

In a recent survey of HCT clinicians, the majority indicated that although HLA loss relapse testing is not yet available in their current practice, they are interested in using this test clinically when becomes available (*manuscript in submission)*. The limited availability of commercial kits to test for HLA loss relapse, the relative unfamiliarity with this new test as well as the lack of guidance for utility of this testing modality remain as significant barriers in incorporating this test in routine clinical practice.

4.7 Clinical Utility

The majority of HCT practices utilize chimerism analysis to monitor donor hemopoietic engraftment in the initial 30 days after HCT particularly in myeloablative (MA) conditioning regimens [6, 17, 19]. Beyond that, there is no generally accepted consensus regarding other aspects of the clinical utility of chimerism analysis. Several studies have reported that MA conditioning leads to consistent engraftment [111, 112]. There is also a growing interest in utilizing chimerism analysis in predicting impending relapse and potentially guiding immunotherapy management decisions, such as the administration of donor lymphocyte infusion (DLI) and tapering of immunosuppression in an effort to elicit or enhance the graft versus leukemia (GVL) effect [113–115]. In addition to its role in early detection of classical

relapse, chimerism analysis can also distinguish the clinical entity of HLA loss relapse and guide clinical decision management as discussed earlier [102, 108, 115].

5.0 Limitations

Although we had relatively high rate of participation compared to similar types of surveys, the voluntary nature of participation may have biased the results. In addition, the geographic representation of participants, primarily from North America and Europe may not adequately represent practices in other regions of the world. Nevertheless, there was still reasonable representation from different parts of the world and practice types among laboratories accredited by ASHI and EFI. In addition, we believe that the distribution of responses is in line with the proportions of HCT taking place globally. As this survey invited responses from ASHI- and EFI-accredited labs, practices in laboratories accredited by other accrediting agencies such as CAP were not considered. Furthermore, the is a relative paucity of literature that critically analyzes technical attributes of performing chimerism testing in clinical laboratories. This limitation is even more pronounced in regards to the impact of these technical attributes to the clinical utility of chimerism testing in HCT. We attempted to supplement the findings from the literature with experiences from the laboratories of the authors to address critical issues in performing and interpreting the results of these assays. Lastly, an outstanding gap in the literature analyzing the association between chimerism testing and failure of engraftment is accounting for the presence of donor specific HLA antibodies (DSA) which is increasingly being recognized as a risk factor for primary failure of engraftment [116].

6.0 Concluding remarks

Chimerism analysis is a powerful monitoring and potentially diagnostic tool that might be underutilized in current HCT clinical practice worldwide. The survey of the laboratories revealed two concerning themes. In addition to the remarkable heterogeneity in chimerism testing practices, there is a suggestion of inadequate involvement of the laboratories in clinical consultation to guide utilization as well as interpreting chimerism testing results. This inadequate laboratory involvement in clinical management maybe a contributing factor to the observed heterogeneity in testing practices. There are reports of significant correlation between MC and different clinical outcomes, however, there remains a number of unanswered critical questions, such as the level of MC that is actionable and measured at what time point and on which cell lineage. A contributing factor in answering these questions is the clinical context in which chimerism analysis is performed. Detailed discussion of these clinical considerations is beyond the scope of this review with focus on technical aspects of performing the test. These open questions represent a call to action to engage clinical and laboratory stakeholders to design clinical studies to address these questions as well as encourage clinical consultation to maximize the utility of these tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Post-Transplant Chimerism Terminology

Term	Definition
Complete Chimerism (CC)	Only donor DNA is detected; 100% donor
Mixed Chimerism (MC)	Both donor and recipient DNA are detected
Stable	Both donor and recipient DNA detected; % recipient DNA not changing significantly compared to previously tested timepoint/sample
Increasing	% recipient DNA is increasing compared to previously tested timepoint/sample
Decreasing	% recipient DNA is decreasing compared to previously tested timepoint/sample
Split Chimerism	Complete chimerism in one or more cell subsets with mixed chimerism or 100% recipient in other cell subsets
Microchimerism	Less than 1% recipient DNA detected
Autologous Recovery	Only recipient DNA is detected; 0% donor

Table 2

Comparison of Cell Enrichment Methods

	Cell Sorter	Magnetic Beads
Initial Start-up Cost	High (capital equipment)	Low (reagents)
Technologist Hands-on Time	Low (less manipulation)	High (multiple steps)
Total Isolation Time	High (can be time consuming without some initial clean up)	Low (technologists can process more samples faster)
Purity and Yield	High	High

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Table 3.

Summary of Methodologies for Assessing Chimerism Status and Comparison of Characteristics

Molecular Technique	Genetic Markers	DNA input, ng	Sensitivity, %	Precision	Precision, %CV, for molecular methods		Advantages	Limitations	Informativity	Applicability in Multiple Donors	Reference
					at 1% of chimerism	at 5% of chimerism					
RFLP	cutting sites for restriction enzymes	large amount of DNA needed	5–10%	High	n/a	n/a	Evaluates all nucleated cells, highly informative and needs small sample	Time consuming, labor intensive	High	No	30–31
VNTR-PCR	5–10 VNTR markers (10–100 bp)	100–250 ng	1–5%	High	n/a	n/a	High informativity, Fast and requires low amounts of DNA	Moderate sensitivity, Lower amplification efficiency due to large size of PCR product	High	Yes	32–33
STR-PCR and STR-PCR in subpopulation	12–24 STR markers (2–6 bp)	1–5 ng	1–5%	High	n/a	<20%	High informativity, Fast and requires low amounts of DNA	Moderate sensitivity	High	Yes	34–39, 40, 55
Real-time quantitative PCR (qPCR)	10–50 SNP (1 bp) or InDel (2–12 bp) markers	20–300 ng per well	0.01–0.1%	Moderate, less precise at higher percentage of donor cells	<20%	<20%	Highly sensitivity, Rapid, robust and quantitative	Less accuracy; high amount of DNA amount required	Moderate	Limited by availability of informative markers	40–44
Digital droplet PCR (ddPCR)	10–30 InDel (2–12 bp) markers	20–100 ng per well	0.01–0.1%	High	<10%	<5%	Highly sensitivity and high accuracy	high amount of DNA amount required; low throughput	Moderate	Limited by availability of informative markers	21, 45–49
Next Generation Sequencing (NGS)	24–210 SNP (1 bp) or InDel (2–12 bp) markers	5–50 ng	0.01–1%	High	<10%	<5%	Highly sensitivity and high accuracy; high-throughput	Expensive and labor-intensive	Moderate-High, depend on number of genetic markers used	Yes if higher number of genetic markers used (such as >200)	21, 50–54

RFLP=restriction fragment length polymorphism; FISH=fluorescent *in situ* hybridization; VNTR=Variable Number Tandem Repeats; STR=short tandem repeat markers; InDels=short insertions and deletions; SNP=single-nucleotide polymorphism; n/a=not applicable