Review of Minimal Residual Disease Identification Techniques

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Abstract

Minimal Residual Disease (MRD) diagnostic practices are paramount in the assessment of treatment efficacy in patients with neoplasms such as acute lymphoblastic leukemia (ALL) and multiple myeloma (MM)[5]. MRD refers to the number of cancerous cells that remain in a patient after treatment, a number that might be so small that traditional methods cannot detect it. The following review evaluates methods for MRD detection that have been developed over the years, with emphasis on the costs and benefits of older methods (such as multiparameter flow cytometry and PCR) and newer methods (such as high-throughput sequencing). Broad understanding of these techniques will likely lead to newer developments in the field of oncology, thus furthering hope in those with potentially curable blood cancers.

Introduction

With a growing list of drugs that treat blood cancers like ALL and MM, techniques for measuring their efficacy have grown alongside them. While it's evident that methods for measuring MRD have diversified in recent years, how can we properly implement promising techniques at a clinical capacity? The ultimate goal is to achieve levels of remission that deem patients "cancer free," but these levels require techniques that utilize highly sensitive technology. Multiparameter flow cytometry (MFC), considered the traditional MRD detection method, has a tumor load cutoff of 10^{-4}, while more sensitive assays like high-sensitive MFC or high-throughput sequencing (HTS) improve load cutoff by another order of magnitude (at times lower than 10^{-5}). This tumor load cutoff is crucial to a treatment pipeline as it creates a metric for identifying patients who may be at a higher risk of relapsing[1]. It also identifies patients who may need to restart a treatment, or who may need other treatments entirely[2].

A nearly 50-year-old technology, MFC has emerged as a leader in the high-resolution characterization of individual cells. Its capabilities include the identification of cell populations at their antigen expression levels, in addition to identifying and characterizing single plasma cells (PCs) and lymphoid cells via multiple parameters[5]. The technology can also evaluate large quantities of cells in short periods of time and detect both surface and intracellular antigens. The "multiparameter" component of multiparameter flow cytometry refers to the simultaneous evaluation of more than eight cellular markers which allow clonal and normal PCs to be distinguished. However, current MFC PC characterization strategies have the potential of missing cancer stem cells with more immune phenotypes, and likely the greatest pitfall of flow-MRD methods is the lack of standardization in MFC immunophenotyping entirely[4].

In a process called ASO-PCR, the rearrangements of germline V, D, and J segments in the Ig gene complexes of immune cells (B cells and T cells) code for immune cell diversity by creating unique, fingerprint-like regions at V(D)J junction sites[5]. These sites can be identified

and sequenced with standardized technologies in 0.95% of lymphoid malignancies and used for the design of junctional oligonucleotides for a sensitive PCR-based detection of malignant cells at low frequencies[2]. By using these sites as primers, MRD can be analyzed via real-time quantitative PCR (qPCR) and cancerous cells can be detected at frequencies as high as 10^{-5}. However, while the approach yields generally good results, it comes with its own set of challenges. The method is time-consuming and can't easily account for somatic hypermutation (SHM), an Ig molecule maturation process that results in high-affinity antibodies. While this process generally occurs in an immune cell's germinal center, it can also occur in or around junction sites, thus mutating the DNA sequence at PCR primer positions. This phenomenon prevents PCR detection in IgH, IgK, and IgL rearrangements in mature immune cell malignancies, thus creating a disparity in blood cancer analyses and may lead to false negatives[3]. Workarounds have been developed to alleviate some of this (specifically by targeting multiple Ig genes in parallel), but it nevertheless remains difficult to apply the PCR approach to all cases.

The rise of high-throughput sequencing (HTS) has led to entirely new branches of biology and medicine. It introduced an accurate method for analyzing individual antigen receptor genes and has become a powerful tool for detecting immune cell clonal Ig gene rearrangements, specifically in the detection of MRD. By utilizing multiplex primer sets, HTS can detect all rearrangements on a huge sample (frequencies as high as 10^5 cells or more)[1]. Like the PCR MRD method, HTS uses an initial PCR step to anneal primers to Ig gene sequences, though SHM in Ig genes also limits primer annealing. HTS does struggle to identify Ig PCR targets in patients with blood cancers. Consistent MRD quantitation is another pitfall of HTS, as clonal rearrangements are detected between polyclonal Ig rearrangements from healthy immune cells—numbers of which differ greatly from person to person. Potentially the greatest drawback of HTS in the context of MRD is the lack of quality assurance surrounding the technology. Unlike traditional PCR methods which are subject to international quality assurance rounds bi-annually[5], HTS has not yet been standardized. Nevertheless, the utilization of next-generation sequencing (NGS) to detect MRD has achieved commercial success, a hallmark of the core technology at companies like Adaptive Biotechnologies.

clonoSEQ: Striking a Balance

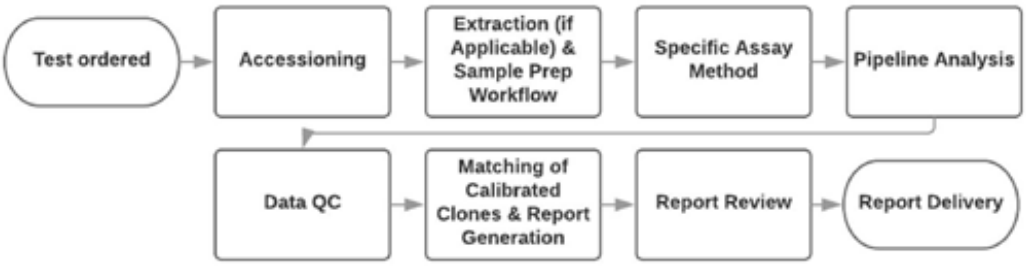

Figure 1: clonoSEQ Assay workflow[2].

Though HTS methods for measuring MRD have yet to experience the kind of global adoption seen with MFC techniques, Adaptive has managed to gain traction in the industry as a harbinger of NGS-based MRD techniques and as a pioneer in the field of immune-driven medicine. The clonoSEQ Assay is an *in vitro* diagnostic that harnesses strengths from both PCR and NGS to detect and quantify Ig gene sequence rearrangements. clonoSEQ specifically targets rearranged IgH (VDJ), IgJH (DJ), IgK, and IgL receptor gene sequences, in addition to translocated BCL1/IgH (J) and BCL2/IgH (J) sequences in DNA from the bone marrow of patients with B cell ALL or MM. The assay includes genomic-region-specific primers for PCR amplification, present as genomic DNA (gDNA). First, gDNA is extracted from a patient's bone marrow, and once it passes quality assessment, rearranged immune receptors are amplified via locus-specific multiplex PCR[6]. Reaction-specific index barcodes are then added to the amplified receptor sequences and sequencing libraries are prepared by pooling barcoded amplified DNA, verified by qPCR. These libraries are then sequenced via NGS (Illumina NextSeq™ 500) and raw sequence data is passed into the bioinformatics pipeline.

QC of the flowcell data is evaluated as the percentage of reads that pass the Illumina quality filter, which must be greater than 70% of reads. Using spike-in PhiX templates, the QC system then calculates error rates. Reads that pass the filter will have a proportion of PhiX reads above 2% and an error rate calculated by the Illumina RTA software of less than 3%. The reads are then demultiplexed using Illumina software and the pipeline undergoes a QC check for evaluating whether unexpected barcodes are observed. If the reads carry a barcode that wasn't specified in the input sample sheet, it will be flagged. The pipeline assigns reads to rearranged receptors after demultiplexing, then clusters reads into clonal receptor sequences. The pipeline performs yet another set of QC checks by validating that sequence data is sufficient and acceptable based on amplification of internal controls and sufficient gDNA.

Additionally, another set of internal controls' presence or absence is used to screen for degradation of residual primers and checks that the detected numbers of PCs and B cells are within a biologically relevant range. Sequences are then compared against a B cell repertoire database and are assigned uniqueness values based largely on locus clonality. This is an important step in estimating an individual patient's limit of detection (LoD) and limit of quantitation (LoQ)[5]. Next, the complete Ig receptor repertoire is assessed again and clonotype sequences are quantified to determine MRD levels in the sample. Once the data are processed, a clonality report is produced that indicates the presence of dominant clones within a malignant lymphocyte clonal population. More specifically, sequence proportions in the sample are assessed and compared to LoD and LoQ values, then the analysis pipeline reports whether ID sequences were detected above the LoQ, above LoD but below LoQ, below LoD, or not detected at all. Once these sequences have been identified in a patient, follow-up samples can be more easily assessed for MRD.

Limitations of clonoSEQ

Though clonoSEQ has proven itself a powerful tool, it's not without its limitations. For example, MRD values obtained with different assays may not be interchangeable, largely due to

differences in assay methods and reagent specificity. The assay may also overestimate MRD frequencies near the LoD, which varies based on the amount of DNA that is tested. Also, like most experiments, positive or false negative results may arise from contamination or human error.

How does clonoSEQ perform compared to MRD?

To assess the clonoSEQ Assay's strength and accuracy, technicians compared the method to MFC, the most common technique for measuring MRD[2]. In a concordance study that evaluated two MM cell lines and two ALL cell lines (selected based on MFC validation), each cell line was tested at five dilutions. Two replicates of each sample were assessed by both the clonoSEQ Assay and by MFC. Concordance of MRD frequency was then plotted (Fig. 2). The blue circles represent MRD concordance, while the orange triangles and red squares denote discordance. These data show that not only does clonoSEQ report MRD at a similar level to MFC, but consistently detects MRD at lower frequencies.

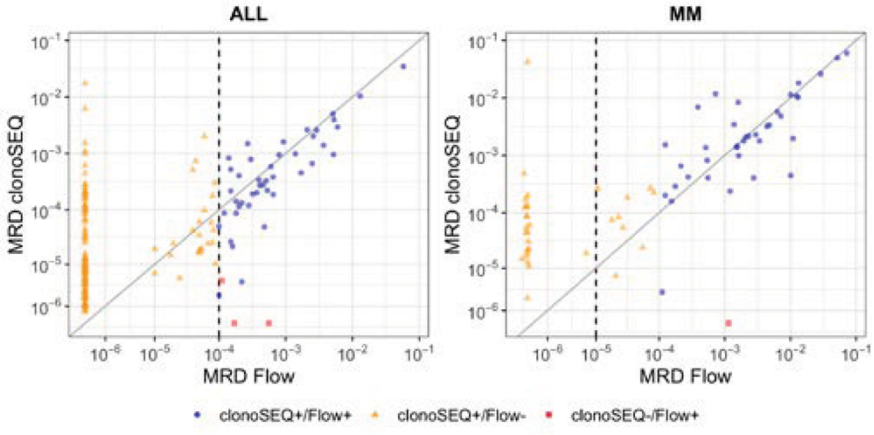

Figure 2: Concordance of clonoSEQ and MFC on samples from ALL and MM patients[2].

The Amplification Bias Problem

Multiplex PCR is a comprehensive method for assessing immune cell diversity, though any variation in annealing kinetics may have a significant effect on primer amplification efficacy. This variation could lead to biased PCR product libraries with amplicon frequencies that are not proportional to the original input template[2], thus leading to potentially undetectable levels of under-amplifying target templates. Alleviating this challenge is imperative for the accurate quantitation of the frequency of specific immune receptor rearrangements, in addition to the necessity of tracking immune repertoires over time.

In one T-ALL experiment, a synthetic analogue of a somatically rearranged immune receptor locus called human TCRG was developed to quantify and correct multiplex PCR amplification bias. However, as immune cell receptor loci are unique like fingerprints, an actual TCRG repertoire is impossible to know. By generating a synthetic repertoire that includes a template for every possible V/J combination, amplification bias can still be identified and corrected. As a sanity check, three mixtures of the TCRG primer mix with modest variation were

run in replicate and showed highly reproducible results. Amplification bias factors derived from the multiplex primer mix allow for the straightforward removal of residual amplification bias bioinformatically. Residual scaling factors are calculated using a pre-to-post-amplification ratio for each template, with each V and J gene segment assigned a mean ratio of its constituent templates. These normalization factors are then used to correct sequencing output[2].

Likely the most compelling data in favor of clonoSEQ's sensitivity is derived from a clinical assay validation study on the TCRG primer mix. To ensure that the multiplex PCR assay is reproducible, the optimized assay was applied to samples from 36 T-ALL patients. Figure 3 displays two panels: both showing MRD detection from MFC (in blue) and PCR (in red) pre- and post-treatment, respectively. It most notably shows that the PCR-based assay can detect MRD in 10 additional patients with greater sensitivity (a clone frequency below 10^{-5}) than MFC. Though this study was performed on T cells, the method is generalizable to other adaptive immune receptor loci and should enable the development of any multiplex PCR system[2].

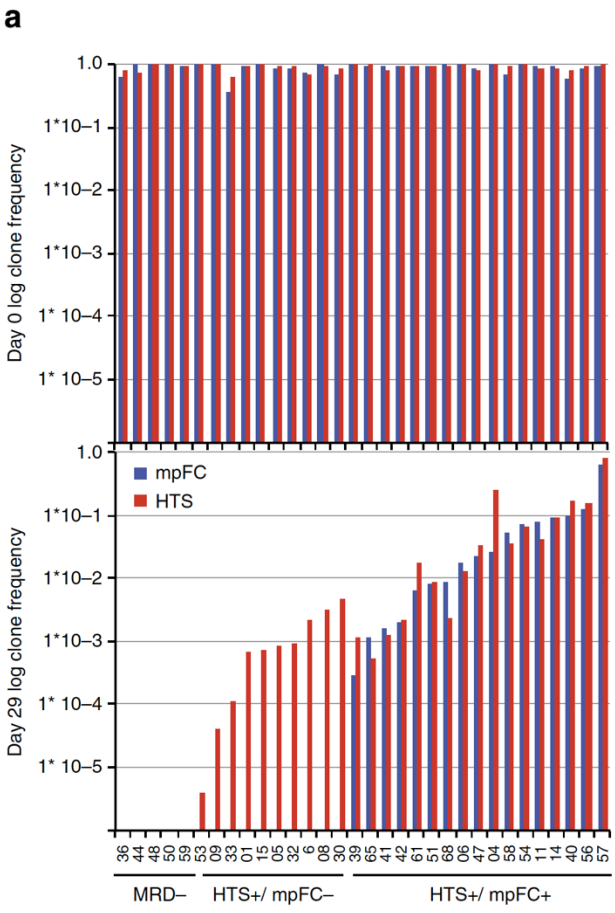

Figure 3: MRD detection in T-ALL with optimized TCRG multiplex PCR assay2.

Conclusion

While newer technologies come with their own set of challenges, they also come with the promise of greater efficiency and sensitivity. The clonoSEQ Assay has shown itself to be a worthy competitor with older methods for measuring MRD (specifically MFC), consistently detecting MRD at lower frequencies than the older technology. In a research environment with increasingly more common MRD studies, we can also expect to see more information that both challenges and reaffirms techniques used in the clonoSEQ Assay, especially in light of Adaptive's recent milestone of being cited in over 500 publications. Though this paper is largely a high-level overview of the core technology at Adaptive, it's worth noting that future papers will focus more closely on the alleviation of amplification bias in the clonoSEQ Assay and the methods for improving upon it, largely via machine learning.

References

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