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Next-generation sequencing for measurable residual disease detection in acute myeloid leukaemia

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Summary

Acute myeloid leukaemia (AML) is a blood cancer characterized by acquired genetic mutations. There is great interest in accurately establishing measurable residual disease (MRD) burden in AML patients in remission after treatment but at risk of relapse. However, inter- and intrapatient genetic diversity means that, unlike in the chronic myeloid and acute promyelocytic leukaemias, no single genetic abnormality is pathognomonic for all cases of AML MRD. Next-generation sequencing offers the opportunity to test broadly and deeply for potential genetic evidence of residual AML, and while not currently accepted for such use clinically, is likely to be increasingly used for AML MRD testing in the future.

Keywords: minimal residual disease, acute myeloid leukaemia, genetics, measurable residual disease, mutation detection, myeloid leukaemia.

The definition of complete remission (CR) in acute myeloid leukaemia (AML), established over 60 years ago as a bone marrow myeloblast count of < 5% determined by cytomorphology (Bisel, 1956), has recently been refined by the inclusion of measurable residual disease (MRD) negativity in clinical response criteria (Dohner *et al.*, 2017). The strong correlation between MRD test result status and risk of subsequent relapse in AML patients has driven efforts to develop techniques that can detect residual leukaemic cells with higher sensitivity (Buckley *et al.*, 2017; Hourigan *et al.*, 2017; Schuurhuis *et al.*, 2018). Currently, the two most prominent techniques for sensitive AML MRD detection, multiparameter flow cytometry (MPFC) and quantitative real-time PCR (qPCR), have notable limitations (Voskova *et al.*, 2007; Grimwade & Freeman, 2014; Hokland *et al.*, 2015; Hourigan *et al.*, 2017).

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Next-generation sequencing (NGS) has considerable potential as a method for AML MRD detection. NGS operates via the simultaneous sequencing of millions of DNA fragments, enabling the survey of many loci in a single experiment and the potential detection of trackable variants in over 90% of AML patients (Papaemmanuil *et al.*, 2016). Furthermore, NGS in some cases has a sensitivity comparable to qPCR (10^{-5} or better) (Levis *et al.*, 2018) but with the ability to detect variants in multiple genes using a single assay without the need to design and validate multiple mutation-specific assays (Hokland & Ommen, 2011). Additionally, NGS allows for the multiplexing of patient samples, providing superior throughput, with potential for quick translation into the clinic and the requirement to establish only a single workflow.

Despite being a promising technique for AML MRD detection, there is currently little consensus on how to address the limitations of NGS for this use case. Here, we outline the current state of NGS-based MRD detection in AML, and offer our thoughts on target selection, technical considerations in the laboratory, bioinformatic approaches and suggested minimal reporting standards. It is our hope this information may support best practices and continued progress in the use of NGS for AML MRD in the future.

Current state of NGS MRD detection in AML

Unlike qPCR and MPFC, NGS-based detection of AML MRD has, appropriately, been used for research rather than clinical use. Recent studies utilizing NGS for the detection of MRD in AML vary greatly in their design and technical aspects. Cohorts studied have included AML patients undergoing allogeneic haematopoietic cell transplantation (alloHCT) (Getta *et al.*, 2017; Kim *et al.*, 2018; Thol *et al.*, 2018; Zhou *et al.*, 2018; Press *et al.*, 2019), receiving standard induction chemotherapy (Klco *et al.*, 2015; Gaksch *et al.*, 2018; Jongen-Lavrencic *et al.*, 2018; Morita *et al.*, 2018; Onecha *et al.*, 2018; Rothenberg-Thurley *et al.*, 2018; Thol *et al.*, 2018; Wong *et al.*, 2018), receiving novel therapies in clinical trials (Levis *et al.*, 2018), or having only specific mutations (Thol *et al.*, 2012; Kohlmann *et al.*, 2014; Salipante *et al.*, 2014; Levis *et al.*, 2018; Patkar *et al.*, 2018; Zhou *et al.*, 2018;

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Patel *et al.*, 2019). Also, since most studies to date have had access to diagnostic samples, *de novo* leukaemia-associated mutation discovery using remission samples alone remains an important unmet challenge. Few studies have utilized error-corrected sequencing techniques, the value of which will be discussed in more detail in later sections. There is also substantial variation in the sample type sequenced, input amount of nucleic acid, and library preparation technique and bioinformatics approaches employed. Consequently, the reported limit of detection (LOD) in these studies at specific genomic positions ranges anywhere from 0·005% (Onecha *et al.*, 2018; Thol *et al.*, 2018) to 1·4% (Cheng *et al.*, 2014; Getta *et al.*, 2017) for single nucleotide variants (SNVs) and 0·001% (Onecha *et al.*, 2018; Zhou *et al.*, 2018) to 0·9% (Cheng *et al.*, 2014; Getta *et al.*, 2017) for small insertions or deletions (indels), with many studies not reporting a LOD (Klco *et al.*, 2015; Jongen-Lavrencic *et al.*, 2018; Kim *et al.*, 2018; Morita *et al.*, 2018; Rothenberg-Thurley *et al.*, 2018). Furthermore, the technical details of the NGS MRD experiment employed are often not explicitly reported. Despite this wide heterogeneity in the literature to date there is widespread enthusiasm, based largely on the explosion of knowledge of the genetic aetiology of AML over the past ten years (Ley *et al.*, 2010; Walter *et al.*, 2012; Ley *et al.*, 2013; Papaemmanuil *et al.*, 2016; Tyner *et al.*, 2018), that NGS represents the future of AML residual disease detection.

Target selection

In 2018, the European Leukemia Network AML Measurable Residual Disease Expert Committee published consensus guidelines for MRD testing in AML including the use of flow cytometry and also qPCR for molecular MRD detection against well-validated targets (Schuurhuis *et al.*, 2018). Notably absent from these recommendations was guidance on the clinical use of NGS for AML MRD detection. The genetic complexity of AML, both between patients and within a single patient, offers opportunities as well as challenges for target selection in AML MRD. Clonal evolution, preleukaemic founder mutations, and germline predisposition mutations all represent how this complexity may manifest into challenges for informative target selection.

It is known that the clonal composition of a patient's leukaemia can vary over time, as selection pressures from treatment, sampling bias or differing proliferation rates can reduce or expand subclones of differing genomic profiles (Farrar *et al.*, 2016). Consequently, a mutation detected at diagnosis may only be present in a subclone that responds to treatment, rendering it undetectable at CR, while another treatment-resistant clone subsequently expands, resulting in relapse. Conversely, a subclone not detected at diagnosis may expand and be responsible for relapse. This was recently demonstrated directly using a multigene panel to assess relapse samples for patients who tested MRD-negative, revealing loss of the original MRD marker and emergence of previously undetected or untracked markers (Thol *et al.*, 2018). Therefore, mutations commonly lost or gained at relapse [e.g., *IDH1/2*, *KRAS*, *NRAS*, *MLL-PTD*, *FLT3*-ITD (internal tandem duplication), and *FLT3*-TKD (tyrosine kinase domain)] may be useful if positive in a CR patient but might need to be supplemented with testing for more stable mutations to define a patient as MRD-negative (Schuurhuis *et al.*, 2018). Perhaps due to the financial burden of MRD-level sequencing, tracking of only mutations detected at diagnosis has been the typical approach for retrospective academic studies. As costs decline over time the use of a broad panel including not just those mutations present at diagnosis but also potential 'escape' mutations may provide greater sensitivity for detecting an impending relapse in a clinical setting particularly when targeting the MRD marker therapeutically (e.g. *FLT3*-ITD, *IDH* mutations). Draft guidance in 2018 from the US regulatory agency (Food & Drug Administration, 2018) however proposed that '*analysis of the risk of false-positive and false-negative results for each marker individually and for the panel as a whole*' should be reported when testing for AML MRD with multiple markers, suggesting the current published literature is insufficient to meet the evidentiary requirements required to adopt such an approach in clinical trials in the US.

Concurrently, not all variants detected by sequencing using broad panels are specific to leukaemic cells. Age-related clonal haematopoiesis (ARCH), also known as clonal haematopoiesis of indeterminant potential (CHIP), is a recently reported age-related phenomenon in which haematopoietic cells acquire mutations that are also commonly found in AML in the absence of haematologic disease (Steensma *et al.*, 2015; Young *et al.*, 2016; Shlush, 2018). This has important implications in AML MRD, since some of the most common mutations seen in age- related clonal haematopoiesis (ARCH)/CHIP (e.g., *DNMT3A*, *ASXL1*, and *TET2*) are also often early founder mutations in AML clonal architecture and therefore may persist in ancestral clones and are, when used in isolation, not predictive of relapse (Jongen-Lavrencic *et al.*, 2018; Thol *et al.*, 2018). These mutations may be most useful if negative, particularly in the post-alloHCT setting from a donor who does not have such mutations. Persistence from diagnosis to remission of detectable mutations in *IDH1* or *IDH2* (sometimes misclassified as ARCH/CHIP mutations) can however be predictive of relapse (Debarri *et al.*, 2015; Thol *et al.*, 2018; Ok *et al.*, 2019).

Germline mutations in several genes have been implicated in increasing the risk of AML development (e.g., *CEBPA*, *GATA2*, *RUNX1*, *ANKRD26*, and *DDX41*) (Porter, 2016). Tracking a mutation that is germline and therefore not exclusive to leukaemic cells in an attempt to monitor MRD is not recommended, again except potentially in the post-alloHCT setting.

To overcome these factors, several considerations should be used for MRD target selection. First, in cases where a previously well-validated AML MRD target (e.g. mutated *NPM1*) (Patkar *et al.*, 2018; Zhou *et al.*, 2018; Patel *et al.*, 2019) is not appropriate for monitoring of a specific patient,

broad gene panels should be considered to ensure adequate coverage of targets present at initial diagnosis but also those potentially responsible for relapse. As sequencing costs continue to decrease, this method will become increasingly feasible although regulatory challenges remain. Second, whether a mutation is associated with ARCH/CHIP or is present in the germline should be determined if possible. For patients in cytomorphological remission, mutations detected with a variant allele frequency (VAF) of> 5% might suggest the presence of non-leukaemia-specific mutations, while VAFs of around 50% or 100% might suggest germline origin, particularly if remaining stable on repeat testing in different clinical scenarios (e.g. diagnosis and remission) (Fig 1). Where possible, germline variants should be confirmed by sequencing genomic material from an appropriate non-haematological source (e.g. skin biopsies or expanded fibroblasts) to be formally excluded from analysis. The context of testing (e.g., diagnostic, relapse, remission at count recovery, germline, blood vs. bone marrow, pre- or post-alloHCT) is important when evaluating the significance of detection or non-detection of targets.

While DNA sequencing has been the primary focus of MRD detection in AML, it is limited in its ability to detect gene fusions commonly found in AML patients (e.g., *PML-RARA*, *CBFB-MYH11*, and *RUNX1-RUNX1T1*). Although qPCR has been widely employed for fusion-based MRD detection (Schuurhuis *et al.*, 2018), targeted RNA-seq has been demonstrated to achieved a comparable LOD while also having high multiplexing capacity and the ability to identify novel fusion breakpoints or insertion sequences that vary between patients (Dillon *et al.*, 2018). In the future, both DNA and RNA sequencing will ideally be used in tandem for full coverage of both fusion and non-fusion mutations for AML MRD detection.

Laboratory considerations

Not all NGS assays capable of detecting mutations in an AML sample at diagnosis are also appropriate for use in MRD measurement, and details of technical aspects are of great importance in determining the reliability of the results of such testing. Detection of MRD by NGS is contingent upon the ability to confidently distinguish variants due to true patient leukaemic biology from those due to background error generated during library preparation and sequencing. Although different sequencing methods have different error rates, sequencing-by-synthesis, the chemistry used by Illumina, has an average error rate of about 0·1% (Glenn, 2011; Fox *et al.*, 2014). Additionally, PCR error introduced during library preparation increases this background error rate to about 1% (Glenn, 2011; Fox *et al.*, 2014). Thus, the goal when considering the myriad of variables involved in NGS is to minimize background error by implementing technical considerations that allow true variants to be called with statistical significance.

One method for distinguishing background error is via the addition of unique molecular indices (UMIs), also known as molecular barcodes (MBCs), to the experimental design (Fig 2A) (Young *et al.*, 2015; Waalkes *et al.*, 2017). This involves tagging each individual DNA molecule with a unique short sequence of nucleotides, typically 6- to 12-nt in length, prior to PCR amplification (Roloff *et al.*, 2017). Since each target molecule has its own unique random barcode attached prior to amplification, the resulting PCR progeny (read family) can be identified by aligning amplicons with the same unique molecular barcode. True mutations in the target molecule can be distinguished from PCR/sequencing errors, since true variants should be present in each member of the read family, while PCR/sequencing errors will be intermittently dispersed (Roloff *et al.*, 2017). This 'consensus clustering' using UMI read families allows for the removal of PCR and sequencing errors (lowering the observed background error rate) and provides an accurate quantification of each original molecule. Duplex sequencing takes this principle further, where each double-stranded DNA fragment is tagged with unique duplex UMI barcodes, allowing for the identification of PCR/sequencing errors that are strand-specific, thus potentially providing additional power to lower background error rates and improving test sensitivity (Schmitt *et al.*, 2012; Salk *et al.*, 2019).

An often overlooked variable in many NGS MRD studies is the quantity of sample DNA used for library preparation.

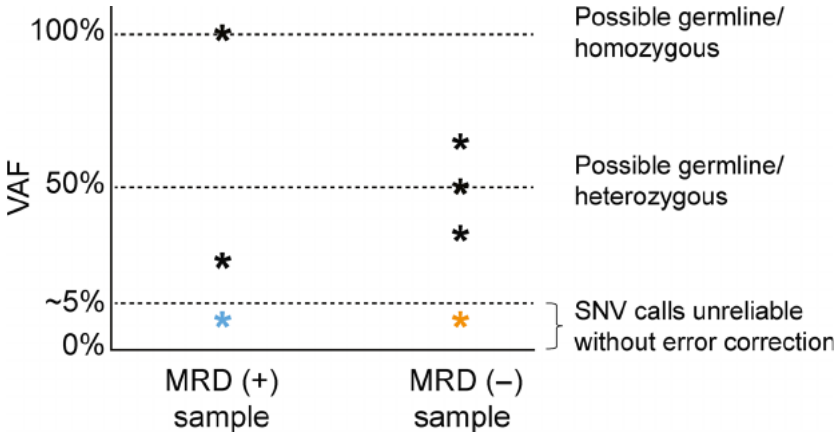

Fig 1. Important considerations for variants detected at given allele frequencies. Not all deleterious variants detected in a sample are informative or associated with a patient's leukaemia. For example, variant allele frequencies (VAFs) around 50% and 100% could be of heterozygous or homozygous germline origin. Variants below 5% VAF fall within the error rate of next-generation sequencing (NGS), making them unreliable to call, especially in a variant discovery setting, without the use of error-correction techniques (true positive:-green, false positive:red). SNV, single nucleotide variant.

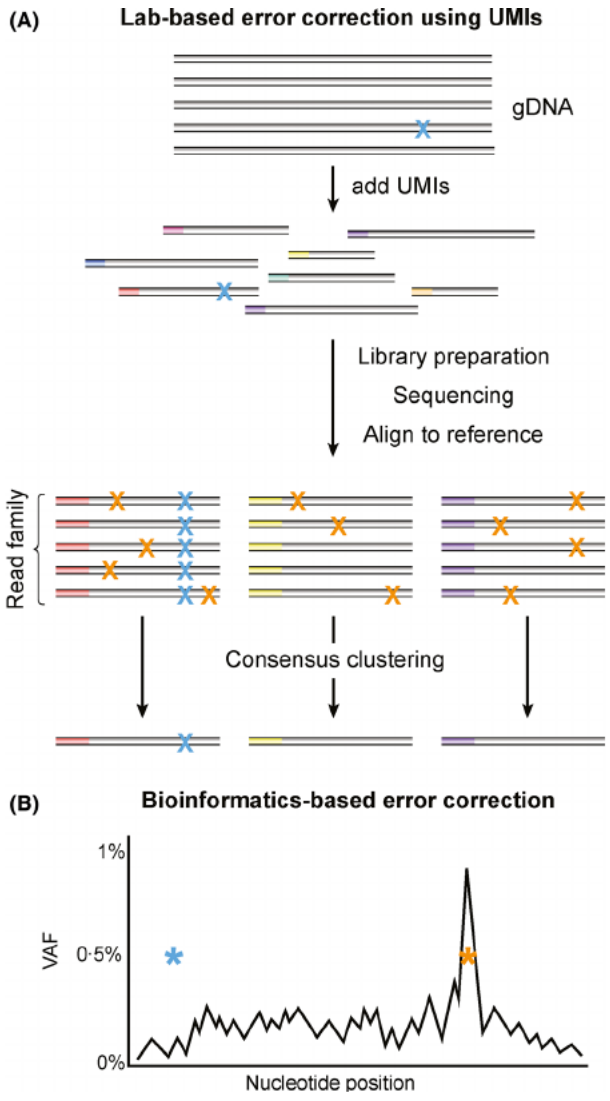

Fig 2. Overview of common laboratory and bioinformatics-based next-generation sequencing (NGS) error-correction methods. (A) The use of unique molecular indices (UMIs) during library preparation can be employed to reduce background error in NGS. UMIs are added to DNA molecules prior to amplification and are maintained throughout library preparation. Consensus clustering of UMI read families allows for the identification of errors introduced during library preparation and sequencing and the discrimination of true positive (green) from false-positive variants (red). (B) Bioinformatic approaches employing position-specific background error models improve variant detection sensitivity by determining the probability of detecting any observed variant relative to the background error rate. For example, given two variants with the same variant allele frequency (VAF) detected at different nucleotide positions, one variant (green) is significantly above the position-specific error rate (black line) and can be confidently called while the other variant (red) falls below the position-specific error rate and cannot be confidently called.

DNA input ultimately is a key determinant of the potential LOD of any assay. For example, if the goal is to detect one variant allele in 10,000 normal alleles (0·01% VAF) and the mass of the haploid human genome is approximately 3.3 pg, a theoretical minimum input of 33 ng DNA is required. However, library preparation is not 100% efficient and DNA is always lost during this process (Aigrain *et al.*, 2016). Additionally, non-uniform gene coverage and the need for multiple variant observations to distinguish true variants from background noise must be accounted for. With the efficiency of reaching a desired coverage differing between library preparation methods, sequencing chemistries, and specific target gene regions, increased DNA input and sequencing coverage is required to achieve any particular LOD (Sims *et al.*, 2014) and should be determined experimentally. In practice, most high-quality studies of AML MRD with NGS have used at least 200 ng of genomic DNA input, although some technologies will require considerably more.

The platform used for sequencing is another important technical variable. While the Illumina platform is frequently found in facilities performing DNA sequencing, the Ion Torrent platform is also commonly employed. Many other sequencing modalities are available or are in development. Each platform and sequencing instrument differs in cost, throughput, error profile, and potential read length. Thus, it is important to consider the strengths and weaknesses of each platform and sequencer. For example, errors generated by the Illumina platform are typically in the form of substitutions while the Ion Torrent platform has a reportedly higher error rate in homopolymer regions resulting in insertions/deletions (Goodwin *et al.*, 2016). This is notable in the context of AML MRD detection as it may result in variability in error rates for different genes tested. In addition to the brand of sequencer and type of chemistry used, the sequencer model and even the specific individual instrument may have varying error rates and sequence biases.

Higher-capacity systems are now available capable of sequencing up to hundreds of patients in a single run. This opportunity also presents challenges. Illumina flow cells with patterned nanowells, which allow for increased data output at a lower cost, exhibit higher rates of sample index hopping compared to non-patterned flow cells (Illumina, 2018). Index hopping is a recently reported phenomenon when multiplexing samples, due to misassignment of sample indices during multiplex sequencing (Sinha *et al.*, 2017; Costello *et al.*, 2018; Illumina, 2018) (Table I). With rates as high as 6% depending on the sequencer and library preparation method (Costello *et al.*, 2018), the phenomenon of index hopping poses a particular problem in the context of MRD in AML, which requires the detection of variants at or below the rate of index hopping. In addition to modifications of the sequencing platform used, index hopping can be mitigated through the use of non-redundant dual indices for sample multiplexing (as opposed to redundant dual indexing, or single indexing) (Bartram *et al.*, 2016; Costello *et al.*, 2018). It may also be prudent practice to avoid running diagnostic samples (with high mutation burden) on the same sequencing run as remission samples.

Finally, it is important to remember that increasing sequencing depth or DNA input does not necessarily increase

Table I. Current limitations of NGS for AML MRD and potential solutions.

Limitations of NGS for AML MRD	Potential solutions
Index hopping	Unique dual sample indices for multiplexing Avoid diagnostic and remission samples on same run
Unknown significance of detected mutations	Larger clinically annotated data sets, ideally from randomized trials Confirmation of potential germline, CHIP/ARCH variants
Marker instability/clonality	Broad sequencing at sequential timepoints
Single-cell sequencing	
Sequencing cost	Sample multiplexing and use of newer, lower cost-per-base sequencing platforms Exclusion of proven non-informative markers Significantly cheaper than interventions (*e.g.* alloHCT)
Intrinsic error/false-positive rate	Error-correction approaches essential for VAF < 5% Orthogonal validation of NGS results (*e.g.* ddPCR)
Discordance with flow cytometry	Studies with paired NGS and flow cytometry Single-cell sequencing with immunophenotype
Reproducibility/lack of uniform reporting standards	Require sequencing data sharing in public databases Establish consensus minimal reporting standard
Unknown significance of mutation level, functional consequence or co-occurrence	NGS data sharing (large clinically annotated data sets) Novel analytical approaches (e.g. machine learning)

CHIP, clonal haematopoiesis of indeterminant potential; ARCH, age-related clonal haematopoiesis; alloHCT, allogenic haematopoietic cell transplantation; VAF, variant allele frequency; ddPCR, digital droplet PCR.

the specificity of the assay (Malmberg *et al.*, 2018; Onecha *et al.*, 2018). The intrinsic error rate of the platform used will typically generate some false-positive data. Careful selection of the experimental design (including sample source and input), library preparation methodology (including the use of pre- and post-PCR workspaces, UMIs and potentially high-fidelity polymerases) and finally the choice of sequencing instrument and chemistry can help mitigate some sources of laboratory-induced error, making the bioinformatic challenge of calling only true AML MRD variants present at low VAF easier if not easy.

Bioinformatic approaches

Despite being a crucial step in NGS MRD detection, the bioinformatics pipelines used to call variants and detect MRD above background noise are not emphasized in the literature. Currently, there is no consensus on which algorithms are most robust for AML MRD determination, as published studies typically utilize pipelines developed, modified or adopted locally. If NGS is to be translated into the clinic for MRD detection, efforts should be made to benchmark, optimize and harmonize pipelines to develop standardized analysis guidelines.

The first major consideration is if MRD is to be defined as the persistent detection of mutations in a remission sample that were already known to be present in the same patient at the time of AML diagnosis. This approach reduces the bioinformatic complexity considerably, as well as potentially reducing the laboratory cost if sequencing at remission is personalized by sequencing only the specific regions known to be mutated in that patient at diagnosis. In contrast, *de novo* discovery of mutations at very low VAF, without a prior estimate of the likelihood that the region is mutated, is significantly more challenging and requires considerably more sequencing in the laboratory, and consequently more computational resources and bioinformatic analyst time. For the reasons given earlier regarding clonal evolution, however, and for cases where no diagnostic mutational data are available, it may nevertheless be preferable to be able to accurately call new variants in a remission sample.

There is currently insufficient evidence to suggest one particular algorithm should be used for variant calling in AML remission samples. Mutations seen in AML are varied, including SNVs, indels, chromosomal translocations, and large copy number variations (Papaemmanuil *et al.*, 2016). Many variant-calling algorithms exist, each with differing sensitivities and specificities based on the variant type being analysed and the sequencing data collected. Some have more stringent criteria than others, employing strict cut-offs to minimize false-positive calls, but that run the risk of filtering out true low-VAF somatic events (Krøigård *et al.*, 2016). Also, while the level of agreement between general-purpose variant callers (e.g., MuTect, Samtools, VarScan, Strelka) increases as sequencing depth increases, the performance of some callers is particularly dependent on sequencing depth (Krøigård *et al.*, 2016). Thus, instead of employing general-purpose variant callers that have highly variable performance, algorithms designed to detect specific types of variants may perhaps be employed, especially for those variants that are challenging to call. For example, one particular area of difficulty has been in calling *FLT3*-ITD mutations, chiefly long ITDs using short-read sequencing (Hang Au *et al.*, 2016). When a relatively short ITD is located at the centre of a sequencing read, the adjacent sequences can be used for alignment, and common variant callers such as VarScan, GATK, and Samtools can identify the inserted nucleotides. However, if an ITD is close to the end of an amplicon or is longer than the sequencing read, the reference sequence may be too short for alignment or not present, causing the read to be discarded (Hang Au *et al.*, 2016). This results in low sequencing depth at these regions, making MRD-level variant

calling challenging. Given this, callers specific for *FLT3*-ITDs have been developed (Hang Au *et al.*, 2016; Blatte *et al.*, 2019). In the near future, the use of long-read sequencing techniques which generate reads spanning the entire ITD, such as Pacific Biosciences Single Molecule, Real-Time (SMRT) sequencing or Oxford Nanopore MinION technology, might help overcome this obstacle (Gurdasani *et al.*, 2018).

Additionally, since the fundamental challenge of NGS-based MRD methods is distinguishing true mutations from background error, establishing the probability of producing false-positive reads is crucial. While some studies have used a single representative gene (Rothenberg-Thurley *et al.*, 2018) or the average error rate of their platform (Morita *et al.*, 2018) to establish background error, different genotypic landscapes have different overall error rates (Nakamura *et al.*, 2011). To achieve the lowest and most accurate LOD in an NGS experiment, the frequency of variant alleles should be compared to a position-specific background error (Malmberg *et al.*, 2018) (Fig 2B). Methods of doing so include, but are not limited to, calculating average VAFs over a range of positions around each mutation for each patient (Thol *et al.*, 2018), calculating site-specific average VAFs in other AML patients within the cohort who did not present a particular mutation at diagnosis (Jongen-Lavrencic *et al.*, 2018), and using a cohort of individuals without AML to determine average site-specific error rates for genes of interest (Malmberg *et al.*, 2018). Tracking of insertions such as *FLT3*-ITD or mutated *NPM1* may however be a particularly powerful methodology when using the Illumina platform. Given the typical pattern of false-positive errors using this technology is substitutions rather than indels, it is possible, thanks to low background error rates, to achieve highly sensitive and specific tracking of these mutations (Levis *et al.*, 2018; Patkar *et al.*, 2018), both of which are not typically seen in healthy individuals.

Finally, it should be noted that bioinformatics, like nucleic acid sequencing, is a rapidly developing field with new advancements frequently reported. While scientifically this is exciting it does pose a challenge for benchmarking and harmonization across studies. Dissemination not just of processed final results, but also uploading of raw sequencing data into public databases will be essential to allow future comparisons between studies and bioinformatic approaches.

Ultimately, future work comparing different locked pipelines on multiple AML MRD data sets, ideally with orthogonal validation of true variants, should be performed with hopes of moving towards more standardized, potentially clinically applicable, NGS MRD bioinformatic benchmarks with known performance characteristics.

Conclusions

Currently, there are no uniform reporting standards for MRD NGS data, complicating the comparison of different studies. Details such as the number of reads per UMI needed to call a variant and how many distinct UMI families per variant are required to make a positive MRD call are sparse, despite their meaningful implications. Additionally, the genomic equivalents input and total number of reads per sample sequenced are often unreported, despite their importance in contributing to the LOD of an MRD test. Details including sample source, clinical stage, controls, replicates, and sequencing platforms together with detailed description of bioinformatic approaches and uploading of sequences into public databases would allow for comparison between studies and facilitate faster progress. Good recent examples of such guidelines for the use of NGS technology in cancer diagnostics already exist and have relevance to this issue (Jennings *et al.*, 2017; Roy *et al.*, 2018) but do not directly address the specific 'use case' of AML MRD. While consensus guidelines for the use of NGS for AML MRD in particular are likely to follow, we suggest here some minimal details required to interpret a publication reporting NGS-based AML MRD data (Box 1).

There are a number of additional 'known unknowns' not covered in this review. First, the relationship between AML MRD measured by flow cytometry and that measured by NGS is not absolute, with discordance noted in both directions (Jongen-Lavrencic *et al.*, 2018). Also, the significance of detecting more than one mutation by NGS in the same sample is unclear, but it is conceivable that this has additional prognostic significance. Notably, single-cell sequencing may offer a way to answer both of these questions. The VAF of a mutated sequence detected may have differing predictive significance for different subsequent clinical interventions, and

Box. Some basic reportable details for NGS AML MRD publications

- Patient demographic and AML diagnostic information
- Clinical timepoint (count recovery?, stage of treatment)
- Type of sample (blood *versus* marrow)
- Nucleic acid type (DNA/RNA), quality, quantity (genomic equivalents)
- Experimental design (e.g. controls, replicates)
- Library preparation method (amplicon *versus* hybrid capture *versus* other)
- Library panel content (specific regions of interest)
- Error-correction approaches used
- Sequencer used (company, model)
- Depth of coverage (including unique read families and duplication rate)
- Bioinformatics pipeline (quality scores, filters, error correction)
- Threshold for positive MRD call
- Test performance characteristics (e.g. lower limit of detection).

the functional consequence of a mutation within a gene almost certainly has importance that is not currently modelled in AML MRD studies by NGS. Ultimately the goal will be not just to determine the *quantity* of residual disease, but also the *quality* in terms of genetic characteristics and therapeutic susceptibilities (Tyner *et al.*, 2018).

Ultimately, with all of the promise that NGS offers for the detection of residual disease in AML, fully realizing its potential requires an understanding of both disease biology, clinical evolution and the technical aspects of NGS assay design and data analysis. To achieve maximum sensitivity and specificity using NGS, the considerations outlined here may help optimize study design. Like all developing technologies, NGS for the detection of MRD in AML has limitations. Our hope is that by highlighting challenges and suggesting some potential solutions here, we will not only allow better understanding of these issues but also motivate the invention of NGS more perfectly aligned with the goal of accurately detecting residual disease before evident relapse in patients with AML.

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Conflicts of Interest

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