Monitoring relapse in post-transplant acute myeloid leukemia requires integrated assessment, with flow cytometry showing the highest efficacy

Disease monitoring after allogeneic hematopoietic stem cell transplant (allo-SCT) in patients with acute myeloid leukemia (AML) is critical in guiding clinical management and prognostication.[1] Various diagnostic modalities are currently employed to assess disease status post allo-SCT, including bone marrow morphologic evaluation, multiparameter flow cytometry (MFC) immunophenotyping for minimal residual disease (MRD), polymerase chain reaction (PCR), next-generation sequencing (NGS) analysis, conventional karyotyping, fluorescence *in situ* hybridization (FISH), and chimerism analysis.[1-5] Each provides valuable information but differs in detection sensitivity. Here, we sought to assess the diagnostic performance of these assays in detecting relapse in AML patients following allo-SCT.

Briefly, MFC was performed using our previously characterized two-tube, 12-color panel designed to evaluate MRD.[6] NGS analysis was performed using an 81-gene panel covering genes frequently mutated in myeloid neoplasms.[7] Semi-quantitative PCR to detect *FLT3* mutations and quantitative reverse transcription (qRT)-PCR analysis to detect *RUNX1::RUNX1T1* and *CBFB::MYH11* fusion transcripts were described previously.[8,9] For the *NPM1*-targeted PCR-NGS MRD assay detecting the exon 12 hotspot insertion mutations, molecular barcode-tagged primers were utilized to perform PCR amplification, followed by bidirectional paired-end NGS of the PCR products. Conventional karyotyping and FISH analysis was conducted as described previously.[10] Assessment of stem cell engraftment (chimerism) was performed from sorted T lymphocytes and myeloid cells.[6] The criteria for defining disease relapse and the sensitivities of each assay are provided in *Online Supplementary Table S1*. This study was approved by the Institutional Review Board of our institution.

We reviewed 220 allo-SCT procedures performed in AML patients with detailed clinicopathologic follow-up information and screened them for disease relapse. A total of 48 patients experienced relapse, including seven who underwent two allo-HSCT procedures and relapsed after both. This yielded 55 relapse cases that were included for further analysis. The 48 patients included 31 (64.6%) men and 17 (35.4%) women with a median age of 53 years (range, 2-76) at the time of relapse. The median time to relapse following allo-HSCT was 146 days (range, 32-2,809 days). First, we focused on the specific time point at which disease relapse was detected and summarized the performance of various assays in detecting relapse (Figure 1). Among these 55 cases, disease relapse was detected by MFC-MRD in 52 cases, NGS in 32 cases, cytogenetic studies in 28 cases, chimerism analysis in 19 cases, PCR in 11 cases, and morphology (≥5% blasts) in 24 cases. In detail, MFC-MRD was performed in 54 cases at the time of relapse, of which 52 (96.3%) (cases #1-52, Figure 1) showed aberrant myeloblast populations consistent with relapse. The median percentage of aberrant blasts detected by MFC-MRD was 2.05% of total nucleated cells (range, 0.01-83.5%). Of the two cases (#53 and #54) missed by MFC-MRD, relapse was detected by RT-PCR at a disease level of 0.01% in case #53 (AML with *RUNX1::RUNX1T1*) and by targeted NGS-based *NPM1*-MRD testing at a disease level of <0.005% in case #54 (AML with *NPM1* mutation). MFC-MRD was not performed in case #55 at the time of relapse, and the disease relapse was detected by cytogenetics and morphology (Figure 1). NGS was performed in 45 cases at the time of relapse, and among them, 32 (71.1%) showed mutations consistent with relapsed disease (Figure 1). Only 12 (21.8%) cases harbored PCR-detectable genetic abnormalities: *FLT3*-internal tandem duplications (ITD) (N=5; #3, #21, #23, #43, and #47)

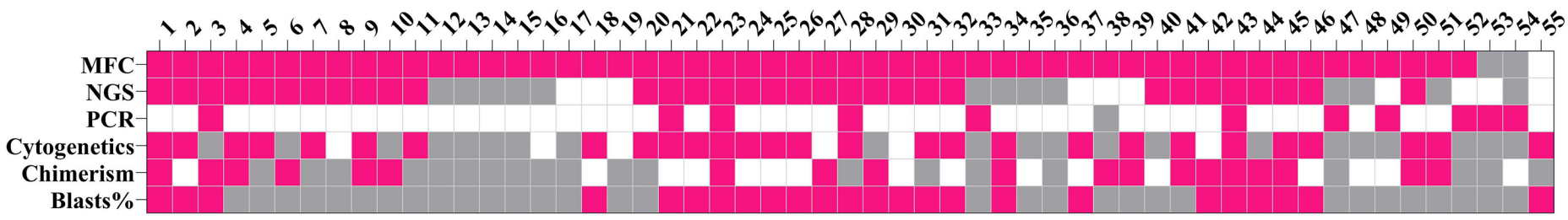


Figure 1. The result of various diagnostic tests at the time of disease relapse. In the 'Blasts%' column, red indicates a blast count ≥5%, while grey represents <5%. In all other columns, red denotes a positive result, grey indicates a negative result, and white indicates that the test was not performed. The criteria for defining disease relapse and the sensitivity of each assay are provided in *Online Supplementary Table S1*. In this study, we separated the highly sensitive polymerase chain reaction next-generation sequencing (PCR-NGS)-based *NPM1* minimal residual disease assay from the regular NGS assay and categorized it under PCR due to its high sensitivity. MFC: multiparameter flow cytometry.

and *FLT3*-tyrosine kinase domain (*TKD*) (N=3; #28, #33, and #49) by semiquantitative PCR, *NPM1* (N=1; #54) by targeted PCR-NGS MRD assay, and *CBFB::MYH11* (N=2; #38 and #52) and *RUNX1::RUNX1T1* (N=1; #53) by qPCR. Among those 12 cases, 11 (91.7%) were positive. Cytogenetic analysis was performed in 49 cases, with 28 (57.1%) positive for cytogenetic abnormalities consistent with disease relapse. Chimerism data were available in 39 patients, with 19 (48.7%) demonstrating recipient-derived cells, consistent with disease relapse. All 55 patients were assessed by morphologic examination, and 24 (43.6%) had morphologic evidence of relapse with ≥5% blasts. Considering all diagnostic studies together, disease relapse was detected exclusively by MFC-MRD in 10 cases (#12-17, #19, #35, #36, and #48), accounting for 18.5% (10/54) of total relapsed cases evaluated by MFC-MRD (Figure 1). The relapse in these ten cases was later confirmed by other diagnostic methods after a median of 65 days (range, 8-274 days) (Figure 2). A representative case of early relapse detected solely by MFC-MRD is illustrated in Figure 3.

Next, we evaluated the status of the aforementioned diagnostic assays during follow-up. The median follow-up was 5.5 months (range, 0.4-90 months). Here, we focused on cases that either had negative results or were not tested by the specific assay at the time of relapse (Figure 2). For MFC-MRD, among the two cases (#53, #54) that were negative at relapse, case #53 became positive after 93 days, while case #54 remained negative after 83 days. Case #55 was not tested at relapse but was positive 18 days later. For NGS, 13 cases were negative, and ten were not tested at relapse. Of the 13 negative cases, ten became positive after a median of 59 days (range, 8-272 days). Of the ten not tested at relapse, seven were subsequently retested, and six became positive. Excluding three cases without follow-up NGS, four of 52 (7.7%) remained negative throughout follow-up. Three of these had mutations at diagnosis

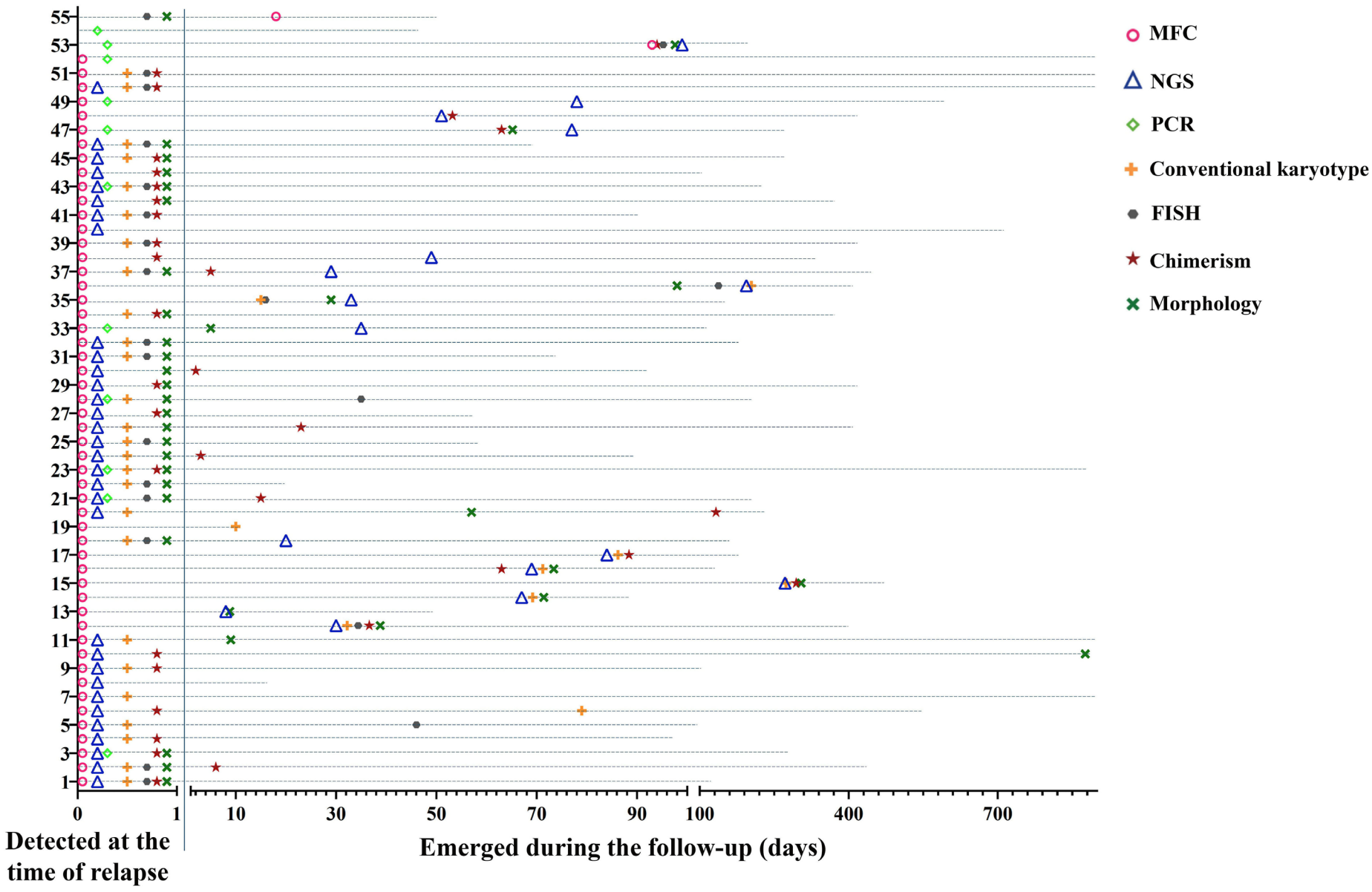
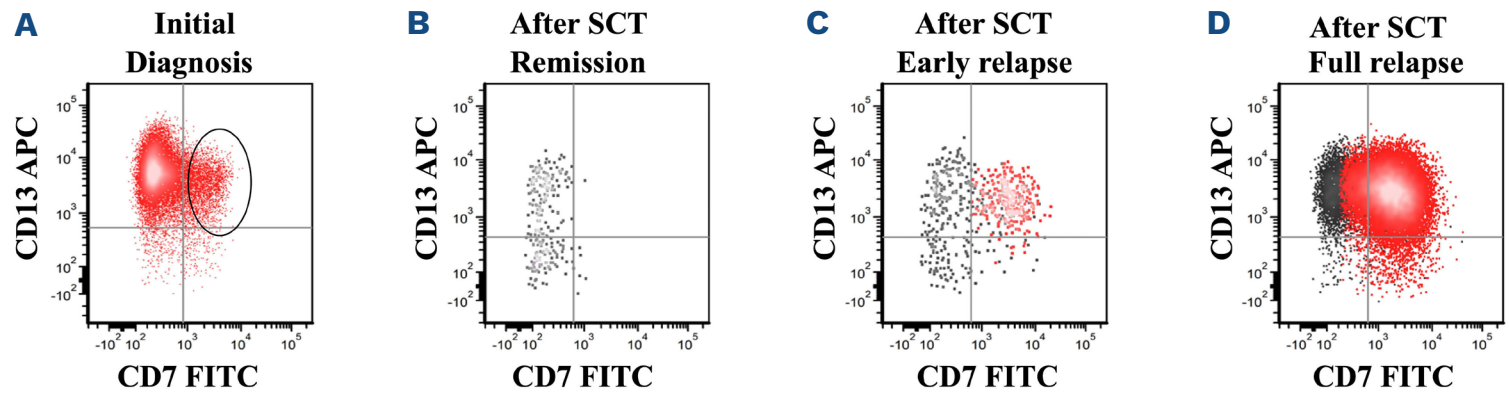


Figure 2. The result of various diagnostic tests during the follow-up after disease relapse. X axis: days after allogeneic stem cell transplantation (SCT). Y axis: case #. Of note, only odd-numbered cases are labeled due to space limitations. Each row represents an individual patient, and the length of the horizontal line corresponds to the duration of follow-up after the first detected relapse. In a few cases (#11, #23, #50, #51, and #52), the follow-up duration exceeded 1,000 days, not fully illustrated in the figure. Notably, an abnormal result from any single assay routinely triggers additional evaluation using other assays if they have not already been performed. Once early disease relapse is established, patients are closely monitored with regular follow-up or initiate treatment as clinically indicated. MFC: multiparameter flow cytometry; NGS: next-generation sequencing; PCR: polymerase chain reaction; FISH: fluorescence *in situ* hybridization.



Flow Cytometry	47% aberrant blasts	0.1% normal blasts	0.15% aberrant blasts	15.4% abnormal blasts
NGS (*JAK2* and *NRAS*)	Positive	Negative	Negative	Positive
Karyotype	46,XY,del(16)(q21q24)[11] /46,XY[9]	Normal	Normal	46,XY,+1,dic(1;14)(p13;q32)del(16)(q21q24)[13]/46,XY,+1,dic(1;2)(p13;q37),del(16)(q21q24)[1]/46,XY[6]
Chimerism	NA	100% donor	100% donor	99% donor
Morphology	51% blasts	2% blasts	2% blasts	22% blasts

Figure 3. Dynamic monitoring of leukemia cell burden using multiple ancillary tests in a patient with acute myeloid leukemia before and after allogeneic stem cell transplantation. (A) This corresponds to case #12 in Figures 1 and 2. At initial acute myeloid leukemia (AML) diagnosis, flow cytometry identified 47% aberrant blasts. Next-generation sequencing (NGS) revealed mutations in *JAK2* and *NRAS*, and conventional karyotyping showed del(16). Morphologic evaluation showed 51% blasts. (B) One month post-transplant, all assays showed normal results. (C) Three months post-transplant, flow cytometry detected a small aberrant blast population (red), representing 0.15% of total cells. This population corresponded to a minor subset of blasts (circled in A) present at initial diagnosis, consistent with early relapse. In addition to the aberrant expression of CD7, myeloblasts also showed multiple other immunophenotypic abnormalities, including decreased CD38 expression and increased expression of CD34, CD54, and CD123 (*data not shown*). The results from all other assays remained normal. (D) Four months post-transplant, the patient developed a full relapse, with all assays showing abnormal findings. In addition to del(16), conventional karyotyping revealed additional abnormalities, indicating clonal evolution. Note: on flow cytometry plots, red indicates aberrant blasts, and black indicates normal myeloid precursors. NA: not applicable.

but were consistently negative after relapse; one had no diagnostic mutation with AML driven by a balanced translocation, precluding NGS monitoring. For PCR analysis, among the 12 cases with genetic targets available for PCR-based monitoring, one case (#38) was PCR-negative at the time of relapse. This case was an AML with a *CBFB::MYH11* fusion at initial diagnosis. At relapse, aberrant myeloblasts (0.94% of total nucleated cells) were detected by flow cytometry and demonstrated an immunophenotypic shift compared with the original leukemic blasts. *CBFB* rearrangement was not detected at relapse or in five subsequent follow-up specimens. Instead, a *GATA2* mutation (variant allele frequency [VAF] 33%) was identified (Figure 2), which was not present at the time of initial diagnosis. These findings illustrate clonal evolution in AML from a *CBFB* rearrangement to a *GATA2* mutation. For cytogenetics, 21 cases were negative and six were not tested at relapse. During follow-up, eight of the 21 negative cases became positive after a median of 82 days (range, 15-274 days), and two of six untested cases were positive. Overall, 17 cases (30.9%) remained negative throughout follow-up. Of these, nine had a normal karyotype and eight had an abnormal karyotype at initial diagnosis. For chimerism, 20 cases were negative, and 16 were not tested at relapse. During follow-up, seven of 20 negative cases became positive after a median of 84 days (range, 30-274). Of the 16 cases not evaluated at the time of relapse, ten had chimerism testing during follow-up, and seven were positive for the detection of recipient-derived cells. Excluding six cases without follow-up testing, 16 of 49 (32.7%) remained negative throughout, mostly associated with low blast counts. For morphology, 31 cases showed no increase in blasts at relapse; 12 later developed ≥5% blasts after a median of 68 days (range, 5-876 days). Thus, 19 of 55 (34.5%) showed no morphologic relapse during the entire follow-up period.

Finally, we explored treatment and outcome. Treatment regimens after disease relapse varied considerably among patients (*Online Supplementary Table S2*). Two patients (#8, #19) did not receive any treatment due to death shortly after relapse. One (#52) was managed with tapering of immunosuppressive therapy only, and the remaining patients received various interventions, including chemotherapy, targeted therapy, and 14 with a second allo-SCT. During follow-up (median 5.5 months; range, 0.4-90 months), 31

patients died. We compared the overall survival of patients whose initial relapse was only detected by MFC-MRD to the remaining patients, and there was no statistically significant difference between these two groups (median: 11.6 *vs*. 9.4 months; *P*=0.28; *Online Supplementary Figure S1A*). This underscores the clinical relevance of MFC-detected relapse and highlights its value as a sensitive and meaningful tool for post-transplant disease monitoring. We also compared the OS of relapses detected by morphology (blasts ≥5%, N=24) *versus* those by ancillary tests only (blasts <5%, N=31), the difference was not statistically significant (median, 7.1 *vs*. 13.4 months; *P*=0.18) (*Online Supplementary Figure S1B*). This finding suggests that, in the post-transplant setting, patient outcome is primarily influenced by the detection of leukemic cells, and the leukemic burden at the time of detection plays a less important role.

In summary, our study demonstrated that the MFC-MRD assay outperformed other techniques by providing reliable and earliest identification of relapse post-allo-SCT. At relapse (Figure 1), MFC-MRD demonstrated the highest positivity of 96.3%, followed by PCR assays at 91.7%, NGS at 71.1%, cytogenetics at 57.1%, chimerism analysis at 48.7%, and morphologic evaluation at 43.6%. Notably, 18.5% of relapses were first detected by MFC-MRD while other methods remained negative, underscoring its overall superior sensitivity in detecting early disease recurrence. In every such case, AML relapse was subsequently confirmed by other diagnostic modalities during follow-up (after a median interval of 65 days), affirming the clinical validity of MFC-MRD. These findings underscore the preeminent role of MFC in early relapse surveillance, enabling detection of relapsing disease well before overt relapse in a significant subset of cases. MFC has gradually become an integral tool in routine clinical practice for detecting relapsed disease in AML.[2,3,6,11] Multiple studies have demonstrated that post-SCT AML MRD detection by MFC carries significant prognostic value.[3,12-14]

Molecular MRD assays, especially qPCR methods, often provide higher analytical sensitivity than MFC-MRD. In our cohort, two cases (#53, 54) with negative MFC-MRD results were found to have disease relapse by molecular MRD testing at levels of 0.01% and <0.005%, respectively. These results align with the validated sensitivity of our MFC assay (~0.1-0.01%) and illustrate that highly sensitive molecular target-specific assays can detect relapse at levels below the threshold of MFC.[6] However, the applicability of molecular MRD testing is inherently restricted to AML subsets with established molecular targets such as *RUNX1::RUNX1T1*, *CBFB::MYH11* rearrangements, and *NPM1* mutations,[1,2] which accounted for only 21.8% cases in our cohort. Moreover, molecular MRD testing is incapable of detecting relapse in cases where clonal evolution leads to the loss of the original molecular targets (such as #38 in our cohort). Conventional NGS, cytogenetic, and chimerism studies generally lack the sensitivity required for early MRD detection.[1,5] Additionally, some AML cases do not harbor cytogenetic abnormalities or somatic mutations that can be reliably used for relapse monitoring. Collectively, the limitations of these assays underscore the indispensable role of MFC, which can be applied broadly across all cases, irrespective of morphologic features, immunophenotypic profiles, mutational status, or cytogenetic abnormalities. It is a more universally applicable approach to post-transplant relapse surveillance. Another advantage of MFC-MRD is its rapid turnaround time (*Online Supplementary Table S1*). Among all monitoring methodologies evaluated in this study, MFC-MRD has the shortest turnaround time.

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Contributions

WJW collected the data, prepared the figures and tables, and drafted the manuscript. WW and SAW designed the research, interpreted the data, and contributed to writing and revising the manuscript. HF, SH, QW, SL, GA, and LJM analyzed the data and revised the manuscript. CZ and PW assisted with statistical analyses. All authors approved the final version of the manuscript.

Data-sharing statement

The data supporting the findings of this study are available within the article and its *Online Supplementary Appendix*. For original data, please contact the corresponding author.

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