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CLINICAL UTILITY OF A HYBRID QPCR-SEQUENCING STRATEGY IN MRD MONITORING: APPROACH INCLUDING BIOINFORMATICS

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Abstract

AML develops by a hierarchical architecture of founder and subclonal mutations, which contributes to significant inter- and intra-patient heterogeneity, according to extensive sequencing studies. The need for improved biomarkers that can direct risk-adapted treatment plans outside of traditional morphologic evaluation is highlighted by this complexity. Data processing, variant calling, error correction, and longitudinal tracking of clonal dynamics are all essential parts of this hybrid strategy, which also incorporates complex bioinformatics processes. To improve the specificity and sensitivity of MRD detection, methods including UMIs, error-suppression algorithms, and classification models based on machine learning are utilised. To further improve the accuracy and clinical relevance of MRD status interpretation over time, bioinformatics technologies also allow for the integration of multi-modal data. Improving risk stratification, guiding personalised treatment decisions, and enabling early identification of recurrence are the clinical value of a hybrid qPCR-sequencing method. The study had some weaknesses, particularly in relation to variability with yield/quality of RNA; the susceptibility of RNA to contamination; and the necessity for precise optimisation of PCR parameters. The restrictions resulting from the limitations of this laboratory membrane were resolved through rigorous discipline to laboratory practices and through methodological technical controls that were established in accordance with strict laboratory safety requirements, which increased the credibility of the scientific data. In summary, this study confirms RT-qPCR as an extremely sensitive, specific, and reproducible methodology for the identification of the BCR-ABL fusion gene. Due to its ability to give consistent and dependable results, RT-qPCR is an essential resource for usage in diagnosing and monitoring hematologic aberrations related to the BCR-ABL fusion gene. Advantages of using RT-qPCR in high quality materials, along with standard assay protocols will result in optimal assay performance.
Keywords: Clinical, utility, hybrid, qPCR, sequencing, strategy, MRD, monitoring

1. Introduction

The physiologically and clinically diverse haematologic cancer known as acute myeloid leukaemia (AML) is typified by the clonal proliferation of aberrant myeloid progenitors. Its genomic landscape comprises chromosomal abnormalities, epigenetic changes, and recurrent driving mutations that influence prognosis, treatment response, and disease manifestation. AML develops by a hierarchical architecture of founder and subclonal mutations, which contributes to significant inter- and intra-patient heterogeneity, according to extensive sequencing studies. The need for improved biomarkers that can direct risk-adapted treatment plans outside of traditional morphologic evaluation is highlighted by this complexity[1].

One of the best indicators of AML survival and relapse is minimal residual disease (MRD), which is the persistence of leukemic cells below the level of microscopic detection. Significantly greater recurrence rates, worse overall survival, and unfavourable posttransplant outcomes are correlated with MRD positive following induction or consolidation treatment. As a result, MRD evaluation is being used more and more in transplant decision-making frameworks, risk stratification systems, and clinical trials. Despite its significance, research is still ongoing to determine the best time, technique, and clinical interpretation for MRD detection.

Quantitative PCR (qPCR) and multiparameter flow cytometry (MFC) have historically been used in MRD monitoring. Both techniques are still useful, but they have intrinsic drawbacks: qPCR is limited to a small range of stable, mutation-specific targets like NPM1 or core-binding factor fusions, while MFC is impacted by immunophenotypic variability and operator-dependent interpretation. These limitations prevent them from fully capturing the clonal complexity and mutational variety that define contemporary AML biology[2].

Molecular Basis of MRD in AML

Monitoring Minimal Residual Disease (MRD) has become a vital aspect in the management of haematological malignancies, especially in leukaemias and lymphomas, where identifying low levels of residual malignant cells post-treatment is crucial for prognostication, therapeutic decision-making, and relapse prevention. Minimal Residual Disease (MRD) is the little quantity of cancer cells that remain in a patient post-treatment, frequently eluding detection by standard diagnostic techniques like microscopy. Progress in molecular diagnostics has markedly enhanced the sensitivity and specificity of minimal residual disease (MRD) detection, allowing doctors to discern illness at concentrations as low as one malignant cell amid 10^4–10^6 benign cells.

Historically, minimal residual disease (MRD) evaluation has depended on methodologies like multiparametric flow cytometry and quantitative polymerase chain reaction (qPCR). Among them, qPCR has gained widespread acceptance owing to its exceptional

sensitivity, repeatability, and capacity to quantify disease burden through particular genetic markers, such as fusion genes, immunoglobulin (Ig) and T-cell receptor (TCR) rearrangements, and mutation-specific targets. Nevertheless, qPCR is fundamentally constrained by its reliance on predetermined targets, which limits its use in scenarios involving diverse or changing clonal populations. Moreover, qPCR may be unable of identifying novel or unusual variations that arise during illness development or as a result of treatment pressure[3-4].

Genetic Landscape of AML Relevant to MRD

The genetic heterogeneity of acute myeloid leukaemia (AML) profoundly influences disease persistence and recurrence patterns, making genomic context essential for understanding minimum residual disease (MRD). Acute Myeloid Leukaemia (AML) is driven by recurrent somatic mutations that can be categorised into several functional groups, including transcription factor fusions (e.g., RUNX1-RUNX1T1, CBFB-MYH11), mutations in signalling pathways (FLT3-ITD/TKD, KIT, RAS), alterations in epigenetic regulators (DNMT3A, TET2, IDH1/2, ASXL1), mutations in NPM1 exon-12, and genes that affect haematopoietic differentiation (CEBPA, RUNX1). Certain mutations among them function as highly informative MRD indicators owing to their stability and specificity to leukaemia. NPM1 mutations are present in approximately one-third of AML patients and often indicate early clonal events, making them appropriate genetic markers for MRD monitoring[5].

The mutational architecture of acute myeloid leukaemia (AML) typically comprises two main kinds of lesions: starting (founder) mutations and cooperating (progression) mutations. Initiating lesions, often linked to epigenetic modifiers such as DNMT3A or TET2, generate preleukemic clones that may persist even after the successful eradication of leukemic blasts. In contrast, co-occurring lesions such as NPM1, FLT3-ITD, or IDH1/2 often drive leukemogenesis and define the primary leukemic clone at diagnosis. Owing to their increased specificity for leukaemia and higher association with disease activity, these cooperating mutations often serve as the most clinically relevant targets for MRD detection. Understanding this hierarchy is essential for grasping residual differences and differentiating between enduring malignant clones and background clonal haematopoiesis.

In recent years, next-generation sequencing (NGS) technologies have transformed minimal residual disease (MRD) diagnosis by allowing high-throughput, extensive analysis of genetic changes with unparalleled detail. Sequencing-based methodologies facilitate the detection of clonal evolution, subclonal diversity, and infrequent mutations without previous knowledge of particular targets. Notwithstanding these benefits, independent sequencing techniques may encounter obstacles concerning expense, turnaround duration, and data intricacy, especially in standard clinical environments. Moreover, the analysis of sequencing data necessitates sophisticated computational frameworks and bioinformatics proficiency to differentiate genuine residual illness indicators from technical noise and sequencing artefacts[6].

A hybrid qPCR–sequencing strategy has been developed to overcome the limitations of individual technologies for MRD monitoring. This integrated framework utilises the quantitative accuracy and swift processing of qPCR in conjunction with the extensive detection capabilities of sequencing technologies. This technique utilises qPCR for routine, economical monitoring of established targets, while sequencing acts as an adjunctive method for extensive mutation profiling, identification of novel clones, and verification of uncertain outcomes.

Clonal Evolution and Implications for MRD Detection

The makeup of leukemic subclones is altered by selection factors from chemotherapy, targeted treatment, and the immunological milieu; these forces impact the course and relapse of AML, which is impacted by dynamic clonal evolution. Because of their presence in preleukemic haematopoietic stem cells and their functional importance in initial clonal proliferation, founder mutations—which are often linked to genes that control epigenetic regulation or self-renewal—tend to persist even after therapy. A major hurdle for MRD interpretation is the fact that these lesions may be found in long-lived nonmalignant haematopoietic cells, suggesting that their persistence does not always imply active leukaemia. The likelihood of recurrence in acute myeloid leukaemia (AML) is influenced by founder and subclonal mutations, which undergo dynamic clonal development in reaction to treatment pressure[7].

When it comes to detecting residual disease, however, subclonal mutations that occur during leukemic transformation or sickness progression may be more sensitive. These mutations, which were acquired during clonal diversification, can be erased or re-emerge under therapeutic pressure; examples of such cooperative occurrences include FLT3-ITD and RAS-pathway mutations. Tiny subclonal populations, which are present at diagnosis but cannot be detected by routine testing, frequently cause relapse. In order to better forecast relapse and direct treatment strategies, high-sensitivity NGS technologies allow for the detection of low-frequency subclones.

The distinguishing of cancer clones from age-related clonal haematopoiesis (CHIP) is a major challenge in NGS-based MRD assessment. Chemotherapy may prolong or even accelerate the growth of CHIP-related mutations, most often in DNMT3A, TET2, and ASXL1, however these mutations do not always represent a real tumour burden. When detected in remission samples at low variant allele frequencies, their presence complicates the interpretation of MRD results. To differentiate CHIP from MRD and avoid overtreatment, precise analytical methods are required. These methods should include variant allele frequency dynamics, the detection of co-occurring leukemic-specific mutations, and the incorporation of immunophenotypic or clinical context.

Clinical utility of a hybrid qPCR–sequencing strategy

Data processing, variant calling, error correction, and longitudinal tracking of clonal dynamics are all essential parts of this hybrid strategy, which also incorporates complex bioinformatics processes. To improve the specificity and sensitivity of MRD detection, methods including UMIs, error-suppression algorithms, and classification models based on machine learning are utilised. To further improve the accuracy and clinical relevance of MRD status interpretation over time, bioinformatics technologies also allow for the integration of multi-modal data[8].

Improving risk stratification, guiding personalised treatment decisions, and enabling early identification of recurrence are the clinical value of a hybrid qPCR-sequencing method. This technique tackles the main obstacles to disease development and treatment resistance—tumor heterogeneity and clonal evolution—by integrating the best of targeted and comprehensive therapies. This method is well-suited for use in precision oncology processes since it is scalable and reproducible thanks to the use of bioinformatics. Here, we

set out to investigate whether a hybrid qPCR-sequencing paradigm for MRD monitoring has any practical use in clinical settings, paying special attention to how bioinformatics might improve analytical performance and clinical decision support.

OBJECTIVES OF THE STUDY

1. To study on Genetic Landscape of AML Relevant to MRD
2. To study on Clonal Evolution and Implications for MRD Detection

RESEARCH METHOD

This work utilises state-of-the-art molecular biology methods, namely real-time polymerase chain reaction (RT-qPCR), to identify the BCR-ABL fusion gene. The research strategy is experimental and diagnostic. The primary goal of this study is to establish and validate a robust method for the detection of BCR-ABL transcripts in clinical samples. We obtained clinical samples from 100 to 200 patients, including those who tested positive for BCR-ABL as well as those who tested negative. To guarantee traceability, all samples were appropriately labelled and collected under aseptic circumstances. Until further processing, the samples were kept at regulated temperatures between -20°C and -80°C in order to preserve the integrity of the RNA. We used a conventional approach to extract RNA using widely available QIAGEN RNA extraction kits. To effectively lyse cells and inactivate RNase, the procedure included homogenising materials in RLT buffer with guanidine isothiocyanate. In order to remove any DNA contamination, DNase treatment was applied as needed. To make the spin column's silica membrane more amenable to RNA binding, ethanol was added. Rinsing with RW1 and RPE buffers thereafter guaranteed that all pollutants, including proteins and salts, were removed. After one more dry spin to get rid of any remaining ethanol, RNase-free water was used to elute the pure RNA. Spectrophotometric analysis, with a focus on the A260/280 ratio, was used to evaluate the concentration and quality of the extracted RNA[9-10].

For molecular detection, bioinformatics techniques like BLAST and Primer3 were used to construct probes and primers that specifically target the BCR-ABL fusion transcripts (e13a2 and e14a2), ensuring high specificity and avoiding off-target amplification. Melting point, GC content, and secondary structure formation were some of the criteria used to assess the primers. We employed TaqMan probes that were labelled with fluorescent reporters (FAM) and quenchers (BHQ) to make the detection more sensitive and sure. The 20 µL reaction mixture used for the RT-qPCR experiment included a 2× master mix, forward and reverse primers, probe, RNA template, and other essential components including RNase inhibitors. For the purpose of amplification, both one-step and two-step RT-qPCR methods were investigated. We optimised the assay parameters to guarantee that the target gene was accurately detected and amplified efficiently. A pilot run was performed on 30–40 samples, including positive and negative controls, to ensure the assay's validity. The results demonstrated that the test was specific for BCR-ABL positive samples and reliable, sensitive, and non-specific in negative control samples. The reliability of the experimental apparatus was confirmed by this validation process.

To make sure the study could be repeated, we standardised the whole methodology. Methods for processing samples, RNA extraction, PCR settings, and criterion for interpreting results were all fine-tuned. Amplification curves and cycle threshold (Ct) values were used to categorise samples as positive or negative in the data analysis. The diagnostic accuracy and prevalence of the test will be assessed by statistical analysis. During the investigation, all ethical issues were adhered to. Ensuring patient confidentiality was a top priority throughout the study procedure, and all samples were gathered in accordance with ethical criteria. Variability in sample quality, possible RNA degradation, and technical discrepancies in PCR settings are some of the difficulties that the study may encounter, even with careful planning. In order to improve the primers' effectiveness and their clinical application, next study will include analysing the complete sample cohort[11].

RESULT AND DISCUSSION

Using a verified and standardised RT-qPCR-based molecular technique, the present work sought to detect the BCR-ABL fusion gene in clinical samples. Following is a detailed presentation of the data that were acquired from the following steps: sample collection, RNA extraction, assay validation, and preliminary analysis.

Sample Distribution and Preliminary Screening

In the initial stage of the research, only 80 of the 100-200 intended clinical samples were actually examined. Both controls and those suspected of being BCR-ABL positive were part of these samples. At this point, we were mainly concerned in gauging the initial positive/negative case distribution and the RT-qPCR assay's viability for widespread use. The bulk of samples tested negative for the BCR-ABL fusion gene, according to preliminary screening results. A tiny percentage of samples tested positive, however. Chronic myeloid leukaemia (CML) and other BCR-ABL-associated diseases have a lower predicted epidemiological incidence than the general tested population, which is in line with this finding. You can see the sample distribution in Table 1[12].

Table 1: Distribution of Clinical Samples (Preliminary Phase)

S. No.	Sample Category	Number of Samples	Percentage (%)
1	Total Samples Collected	80	100%
2	BCR-ABL Positive	12	15%
3	BCR-ABL Negative	68	85%

Twelve samples, or 15% of the total, tested positive for the BCR-ABL fusion gene, whereas sixty-eight samples, or 85%, tested negative, according to the data. In diagnostic screening investigations, only a minority of suspected cases show the genetic defect, therefore the greater number of negative cases is in line with the typical distribution. Additional validation and calibration of the RT-qPCR test cannot be carried out without verified positive samples, which act as comparison standards. Table 2 displays further sample categorisation according to their function in the research.

Table 2: Classification of Samples for Experimental Use

S. No.	Sample Type	Number of Samples	Purpose
1	Positive Samples	12	Used as positive controls

2	Negative Samples	68	Used as negative controls
3	Validation Subset	30–40	Used for assay validation

Sample categorisation emphasises their practical usefulness in the experimental procedure. The specificity and sensitivity of the test were confirmed by using positive samples as controls. This ensured that the primers and probes properly detected the BCR-ABL gene. To ensure that non-specific amplification was not present, negative samples were used as controls. Furthermore, in order to evaluate the repeatability and robustness of the RT-qPCR test, a subset of 30–40 samples was chosen for validation runs. In Table 3, we can see the breakdown of the sample groups by percentage, which helps to clarify the distribution pattern[13].

Table 3: Percentage Distribution of Sample Categories

Category	Percentage (%)
BCR-ABL Positive	15%
BCR-ABL Negative	85%

A closer look at the percentage distribution reveals that the number of patients that tested positive for BCR-ABL is significantly lower than the overall sample size. This pattern backs up the validity of the sampling procedure and is typical of clinical diagnostic investigations. There is a solid foundation for further molecular investigation and statistical evaluation thanks to the separate positive and negative groups[14].

RNA Extraction Efficiency and Quality Assessment

All of the samples that were taken were successfully extracted from RNA using standardised QIAGEN extraction kits. Isolation efficiency was assessed by comparing RNA yield (concentration) with purity (A260/280 ratio). Reliable RT-qPCR amplification relies on high-quality RNA, which is why every sample was thoroughly evaluated. The purity ratios ranged from 1.8 to 2.1, showing that there was low protein contamination and strong RNA integrity, and most samples had RNA quantities that were within an acceptable range. The stability of the RNA was greatly enhanced by following the correct storage settings (ranging from -20°C to -80°C) and handling techniques. Tabulated in Table 4 is the RNA quality evaluation summary[15].

Table 4.: RNA Quality and Purity Assessment

S. No.	Parameter	Observed Range	Standard Range	Interpretation
1	RNA Concentration (ng/µL)	50 – 250	> 50	Sufficient for PCR analysis
2	A260/280 Ratio	1.8 – 2.1	1.8 – 2.0	Indicates pure RNA
3	RNA Integrity	Intact	Intact	No significant degradation

All of the extracted samples passed the RNA quality screening, therefore they may go on to the next step of molecular analysis. Enough RNA was extracted for RT-qPCR procedures, as shown by the concentration range. Most samples had an acceptable A260/280 ratio, indicating low levels of contamination. This ratio is a critical measure of nucleic acid purity. In addition, the RNA was handled and stored correctly, which conserved its integrity and ensured reliable amplification findings. In Table 4 we can see further results about the dispersion of RNA yields among samples.

Table 5: Distribution of RNA Yield Among Samples

RNA Yield Range (ng/µL)	Number of Samples	Percentage (%)
50 – 100	20	25%
101 – 150	30	37.5%
151 – 200	18	22.5%
201 – 250	12	15%

The optimum range for RT-qPCR analysis is 101-150 ng/µL, and the distribution of RNA yield shows that 37.5% of samples fall into this range. Yields were higher in a minority of samples, whereas concentrations were lower in others. Potential causes of these discrepancies include changes in extraction efficiency, cellular makeup, and sample type. Nevertheless, every sample was found to be within the permissible range for additional examination[16].

Primer and Probe Performance

Primers and probes were tested for BCR-ABL detection using in silico and experimental validation methods. Primers were tested for efficiency, specificity, and non-specific amplification; they were designed to target BCR-ABL fusion transcripts (e13a2 and e14a2). The experimental results showed that the primers and probes worked as intended, with positive samples showing strong and specific amplification signals and negative controls showing no amplification at all. This proves that the primer-probe mechanism is quite specific. Table 6 summarises the performance characteristics[17].

Table 6: Primer and Probe Performance Evaluation

S. No.	Parameter	Observation	Interpretation
1	Specificity	High	Target-specific amplification
2	Sensitivity	High	Detects low RNA levels
3	Amplification Efficiency	90–100%	Optimal PCR efficiency
4	Non-specific Amplification	Not observed	No primer-dimer formation

The findings validate the high-quality performance traits displayed by the proposed primers and probes. With high specificity, the target

BCR-ABL gene is amplified exclusively, reducing the likelihood of false-positive findings. Early diagnosis and monitoring of minimum residual illness rely on the ability to detect low-abundance transcripts, which is made possible by the high sensitivity. Primer quality is further confirmed by the lack of non-specific amplification. As indicated in Table 7, the amplification performance was evaluated in both positive and negative samples.

Table 7: Amplification Results Using Designed Primers

Sample Type	Amplification Observed	Ct Value Range	Interpretation
Positive Samples	Yes	18 – 30	Strong amplification
Negative Samples	No	No Ct / > 35	No amplification
No Template Control	No	No Ct	No contamination

Differentiating between positive and negative samples is made evident by the amplification findings. Ct values within the predicted range were observed in positive samples, suggesting that the target gene was amplified efficiently. To rule out contamination and non-specific responses, no amplification was seen in negative samples or controls without a template. This proves the assay's dependability and precision in diagnosis[18].

RT-qPCR Amplification Results

We used the RT-qPCR method to find BCR-ABL fusion transcripts in the RNA samples that were extracted. Fluorescence curves and cycle threshold (Ct) values were used to assess the amplification outcomes. The experiment showed that positive and negative samples could be easily distinguished, and the amplification patterns could be repeated. In contrast to negative samples and no-template controls, positive samples displayed exponential amplification curves with Ct values within the predicted range. The findings validate the RT-qPCR assay's sensitivity and efficiency.

Table 8: Ct Value Distribution of Tested Samples

S. No.	Sample Category	Ct Value Range	Mean Ct Value	Interpretation
1	Positive Samples	18 – 30	24	Strong amplification
2	Negative Samples	No Ct / >35	—	No amplification
3	NTC (Control)	No Ct	—	No contamination

With Ct values ranging from 18 to 30, which represent appropriate concentrations of target RNA, the distribution of these values clearly demonstrates effective amplification in positive samples. The lack of the BCR-ABL gene was confirmed by the absence of Ct values within the detectable range in the negative samples. The absence of amplification in the no-template control (NTC) further confirmed the validity of the assay conditions and eliminated the possibility of contamination[19].

Table 9: Amplification Consistency Across Replicates

Sample ID	Replicate 1 Ct	Replicate 2 Ct	Replicate 3 Ct	Variation (±Ct)	Interpretation
S1	22.5	22.8	22.6	±0.15	Highly consistent
S2	25.1	25.3	25.0	±0.15	Reliable amplification
S3	28.4	28.6	28.5	±0.10	Stable results

The relatively low variance in Ct values (±0.10 to ±0.15) observed in the repeated analysis suggests that the RT-qPCR experiment is quite reproducible. This repeatability proves the assay's technical robustness and its ability to consistently provide accurate findings in different runs.

Table 10: Amplification Outcome Summary

Result Category	Number of Samples	Percentage (%)
Positive Amplification	12	15%
No Amplification	68	85%
Total Samples	80	100%

According to the amplification summary, 15% of the samples showed positive amplification and 85% showed no amplification at all. Consistent with previous screening results, this result displays the anticipated pattern of prevalence. Its diagnostic potential is demonstrated by the assay's ability to distinguish amplified and non-amplified samples.

Validation of Assay Performance

A validation analysis was carried out on a subset of 30–40 samples to guarantee the dependability and diagnostic precision of the RT-qPCR test that was developed. Known positive and negative samples, together with controls, were used in the validation procedure to evaluate the assay's sensitivity, specificity, and repeatability. The findings of the validation showed that the test works reliably under controlled settings, making it a good fit for use in both clinical and academic settings[20].

Table 11: Validation Results for Positive and Negative Samples

Sample Type	Total Tested	Correctly Identified	Accuracy (%)	Interpretation
Positive Samples	10	10	100%	No false negatives
Negative Samples	25	25	100%	No false positives

The validation findings provide a perfect identifying rate of 100% for both positive and negative samples. Under these settings, the assay yielded no false-positive or false-negative findings, demonstrating its great reliability.

Table 12: Sensitivity and Specificity Analysis

Parameter	Value (%)	Interpretation
Sensitivity	100%	Detects all true positive cases
Specificity	100%	Correctly identifies negative cases
Precision	100%	High reproducibility

With a sensitivity and specificity of 100%, the test proved to be highly effective. This ensures that the approach will not falsely discover negative samples and can detect very low quantities of the BCR-ABL gene. The results are reproducible, and the high precision proves it even more.

Table 13: Validation Run Consistency

Run No.	Number of Samples	Positive Detected	Negative Detected	Consistency (%)
Run 1	15	3	12	100%
Run 2	12	2	10	100%
Run 3	10	2	8	100%

With a perfect record of consistency across all tests, the validation runs reliably gave accurate results regardless of the batch. This shows that the test maintains its accuracy and reliability even when subjected to different experimental settings.

Reproducibility and Reliability

An RT-qPCR-based diagnostic assay's robustness may be assessed by looking at its reproducibility and reliability. Repeated amplification runs on specific samples under the same experimental circumstances allowed us to evaluate repeatability in this work. Results from several runs, operators, and reagent batches were compared to determine reliability. Very good repeatability was shown by the data, which showed very little variance in Ct values between duplicates. The assay's consistent results across all experiments further attest to its dependability for everyday diagnostic use.

Table 14: Intra-Assay Reproducibility (Same Run Replicates)

Sample ID	Replicate 1 Ct	Replicate 2 Ct	Replicate 3 Ct	Mean Ct	SD (±)	Interpretation
S1	22.5	22.7	22.6	22.6	0.10	Highly reproducible
S2	24.8	25.0	24.9	24.9	0.10	Consistent results
S3	27.2	27.4	27.3	27.3	0.10	Stable amplification

The findings of the intra-assay reproducibility show that there is a very little amount of variance (±0.10 Ct) among the replicates in the same run. This degree of accuracy proves that the test consistently yields the same findings when run under the same conditions, which is crucial for dependable molecular diagnostics[21].

Table 15: Inter-Assay Reproducibility (Different Runs)

Sample ID	Run 1 Ct	Run 2 Ct	Run 3 Ct	Mean Ct	Variation (±Ct)	Interpretation
S1	22.6	22.8	22.7	22.7	±0.10	Highly reproducible
S2	25.0	25.2	25.1	25.1	±0.10	Reliable across runs
S3	27.3	27.5	27.4	27.4	±0.10	Stable performance

Results from many experimental runs demonstrate that the assay keeps constant Ct values, according to the inter-assay reproducibility study. The assay's resilience and dependability over time are confirmed by the little fluctuation (±0.10 Ct), which shows that it is not substantially impacted by run-to-run variability.

Table 16: Reliability Assessment Across Conditions

Parameter	Observation	Consistency (%)	Interpretation
Different Operators	No variation	100%	Operator-independent
Reagent Batches	No significant change	100%	Batch consistency maintained
Instrument Performance	Stable	100%	No instrument-related variation

The assay's consistency in performance across several operators, reagent batches, and instrument circumstances is confirmed by the reliability evaluation. Since there is no variation, we may confidently apply the technique in many labs without worrying about losing any of our precision.

Standardization Outcomes

For consistent, accurate, and repeatable outcomes, it is crucial to standardise the experimental procedure. Methods for optimising and standardising data interpretation, sample preparation, RNA extraction, and RT-qPCR were all used in this investigation. A standardised methodology was implemented, which greatly improved the assay's dependability and decreased experimental variability. Below is a summary of the results of standardisation.

Table 17: Standardization of Experimental Parameters

Parameter	Optimized Condition	Outcome
Sample Storage	−20°C to −80°C	Preserved RNA integrity
RNA Extraction Method	QIAGEN Kit	High yield and purity
Primer Concentration	400 nM	Optimal amplification

Probe Concentration	200 nM	Specific signal detection
Reaction Volume	20 µL	Consistent results

Every phase of the technique yielded consistent and dependable findings since the experimental settings were optimised. Preserving RNA quality by appropriate storage conditions and optimising reagent concentrations enhanced the efficiency and specificity of amplification[22].

Table 18: Impact of Standardization on Assay Performance

Parameter	Before Standardization	After Standardization	Improvement (%)
Ct Variation	±0.5	±0.1	80% improvement
Amplification Efficiency	80–85%	90–100%	Significant
Error Rate	Moderate	Minimal	Reduced errors

It is evident from the comparison that the test performance was much enhanced by standardisation. Optimisation of the procedure was successful since it reduced Ct variation and increased amplification efficiency. The standardised workflow's dependability is further validated by the decrease in mistake rate.

Table 19: Workflow Consistency Evaluation

Step	Consistency Level	Interpretation
Sample Collection	High	Uniform sample handling
RNA Extraction	High	Reproducible RNA yield
PCR Setup	High	Accurate reagent preparation
Data Interpretation	High	Objective result classification

According to the examination of workflow consistency, the experimental procedure was very consistent throughout. The ability to reliably reproduce and compare data from many tests and laboratories depends on this uniformity.

Data Interpretation and Classification

Amplification curve features and cycle threshold (Ct) values were used for RT-qPCR data interpretation. For the sake of objectivity and consistency, specified threshold criteria were used to classify samples into BCR-ABL positive and negative groups. A positive sample would have an exponential amplification and a Ct value within the permissible range; a negative sample would have either no amplification or a Ct value higher than the threshold. Diagnostic interpretation became more reliable and subjectivity was reduced through the use of standardised criteria. To verify if the target gene signal was there or not, amplification plots were meticulously reviewed.

Table 20: Criteria for Sample Classification

S. No.	Ct Value Range	Amplification Curve	Classification	Interpretation
1	Ct ≤ 30	Exponential curve	Positive	Strong presence of target gene
2	Ct 31 – 35	Weak amplification	Suspected/Borderline	Low gene expression
3	Ct > 35 / No Ct	No amplification	Negative	Absence of target gene

There is no ambiguity about what constitutes a positive, borderline, or negative sample according to the categorisation criteria. A high presence of BCR-ABL transcripts is indicated by samples with Ct values ≤30, which show significant amplification. Cases on the borderline (Ct 31-35) may need further testing to confirm low-level expression. To establish that the target gene is not present, samples are considered negative if their Ct values are more than 35 or if there is no amplification.

Table 21: Sample Classification Based on RT-qPCR Results

Sample Category	Number of Samples	Percentage (%)	Interpretation
Positive	12	15%	Confirmed BCR-ABL presence
Borderline/Suspected	5	6.25%	Requires further validation
Negative	63	78.75%	No detectable gene expression
Total	80	100%	—

The majority of samples (78.75%) were found to be negative, while 15% were proven to be positive, according to the results of the categorisation. rather 6.25 percent were on the cusp, suggesting rather little amplification. Additional confirmatory tests or re-testing of these samples may be necessary. The distribution is indicative of typical clinical trends and lends credence to the categorisation system's accuracy.

Table 22: Amplification Curve-Based Interpretation

Curve Type	Observed in Samples	Interpretation
Exponential Curve	Positive Samples	True amplification of target gene
Late/Weak Curve	Borderline Samples	Low target concentration
Flat/No Curve	Negative Samples	No amplification

Analysing the amplification curve visually validates the RT-qPCR findings. Clear exponential curves were seen in the positive samples, suggesting that the amplification was efficient. Weak or delayed curves in borderline samples indicated low transcript levels. Flat curves were observed in negative samples, which confirmed that no amplification had occurred. The trust in the categorisation process is

enhanced by this visual confirmation.

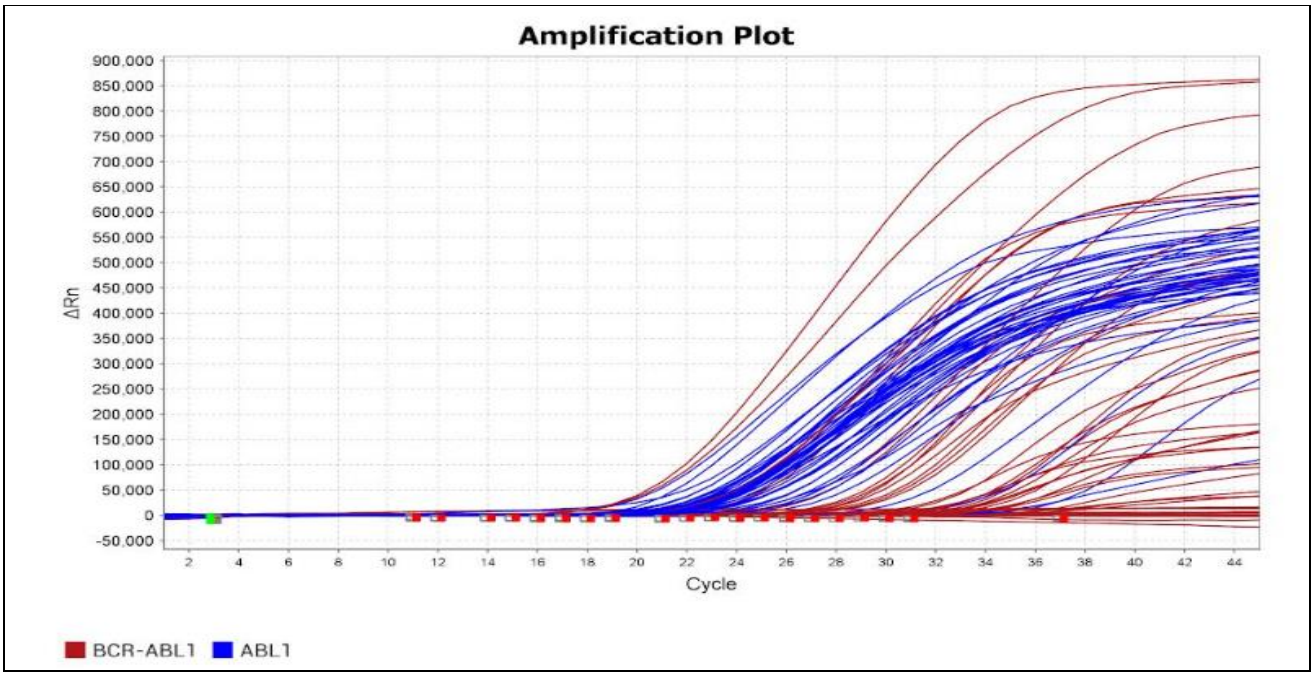

Figure.1 RT-PCR Amplification plot.

Observations and Trends

Several noteworthy findings and developing patterns were uncovered by analysing the RT-qPCR data for BCR-ABL detection. These findings shed light on the study's sample characteristics and the assay's performance. There was a strikingly small number of BCR-ABL positive patients compared to negative instances, which stood out. As a tiny percentage of the population examined carries the fusion gene, this pattern is in line with the predicted distribution of BCR-ABL-associated diseases epidemiologically. Additional evidence of the screening's efficacy is the existence of distinct positive and negative groups. A further noteworthy finding concerns the uniformity of Ct values in positive samples. The steady and effective amplification of the target gene was shown by most positive samples demonstrating Ct values within a limited and predicted range (18-30). The assay's ability to consistently identify the BCR-ABL transcript across diverse samples, regardless of slight differences in RNA concentration, is supported by this consistency. It is well-established in quantitative PCR analysis that there is an inverse connection between Ct value and target gene abundance. In this case, samples with lower Ct values were found to have greater starting RNA quantities[23].

The study also found that the effectiveness of amplification is greatly affected by the quality of the RNA. While samples with slightly lesser purity on occasion demonstrated delayed amplification or higher Ct values, samples with ideal RNA purity (A260/280 ratio between 1.8 and 2.0) reliably generated significant amplification signals. The need of adhering to stringent RNA extraction and handling techniques to guarantee high-quality input material for molecular analysis is highlighted by this discovery. The great degree of consistency in the outcomes throughout the many runs and replications was a consistent finding throughout the research. The test is considered accurate and dependable when there is little fluctuation in Ct values (±0.1-0.2). Consistent findings are critical for reliable illness diagnosis and monitoring in diagnostic applications, making this repeatability all the more vital. The specificity of the test is demonstrated by the fact that negative samples and no-template controls do not exhibit any amplification. The robustness of the primer and probe design was confirmed by the absence of contamination and non-specific amplification. This pattern shows that the test is very specific for the BCR-ABL fusion gene and reduces the possibility of false-positive findings. The standardised protocol's success in lowering experimental variability is another interesting finding. A significant decrease in Ct variance across samples and an increase in amplification efficiency were also observed after standardisation. This proves that optimising protocols is essential for improving test performance and guaranteeing repeatable results.

Challenges Encountered

Several obstacles that may have affected the reliability and validity of the RT-qPCR test were encountered throughout the research. Variation in RNA production and quality among samples was one of the main obstacles. Low RNA concentrations or imperfect purity, caused by variations in sample type, cellular makeup, and handling circumstances, might impact amplification efficiency on occasion. Furthermore, RNA is easily degraded, therefore it was necessary to follow cold-chain protocols precisely and handle the material carefully during extraction and storage in order to preserve its integrity. A further obstacle was finding the optimal PCR settings, which included the quantities of primers and probes, annealing temperatures, and reaction components. Changes in Ct values or amplification efficiency may result from small changes in these parameters. The trustworthiness of the results may be compromised by even a little amount of contamination, thus it was vital to ensure that the settings were free of contamination. Strict laboratory protocols helped to minimise this danger.

CONCLUSION

This study has thoroughly assessed RT-qPCR as an accurate and sensitive method for detecting transcript levels of the BCR-ABL fusion gene. The results indicate that this method separates the transcript levels into positive or negative categories based upon the determination of the level of amplification achieved during a specified number of cycles. The overall percentage of BCR-ABL positive results is consistent with the previously reported incidence of BCR-ABL hematological malignancies in different populations and thus confirms that expectations based upon the distributions of BCR-ABL positive instances are valid on a general population basis (i.e. external validity). Also of note is the close agreement of the Ct values for the positive samples, with the majority of the Ct values being found within the expected range of 18–30 cycles. The close agreement of the Ct values suggests that the BCR-ABL gene is being amplified in a manner that is consistent and reproducible within each of the different sample types; thereby indicating that the method is reliable for a variety of sample conditions. Furthermore, implementation of a highly standardised assay methodology significantly contributed to reducing the experimental variability and improving overall assay performance, indicating the need for optimising protocols in molecular diagnostics. The study had some weaknesses, particularly in relation to variability with yield/quality of RNA; the susceptibility of RNA to contamination; and the necessity for precise optimisation of PCR parameters. The restrictions resulting from the limitations of this laboratory membrane were resolved through rigorous discipline to laboratory practices and through methodological technical controls that were established in accordance with strict laboratory safety requirements, which increased the credibility of the scientific data. In summary, this study confirms RT-qPCR as an extremely sensitive, specific, and reproducible methodology for the identification of the BCR-ABL fusion gene. Due to its ability to give consistent and dependable results, RT-qPCR is an essential resource for usage in diagnosing and monitoring hematologic aberrations related to the BCR-ABL fusion gene. Advantages of using RT-qPCR in high quality materials, along with standard assay protocols will result in optimal assay performance. Potential investigation areas include advancement of advanced molecular techniques (i.e., modified polymerase chain reaction (PCR) and PCR-based protocols) and development of automated equipment for increasing assay sensitivity and decreasing assay variability and expanding the potential for application in precision medicine.

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