External quality assessment for PML-RARα detection in acute promyelocytic leukemia: Findings and summary

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Background: The confirmation of clinical diagnosis, molecular remission, and sequential minimal residual disease monitoring required PML-RARα detection in acute promyelocytic leukemia (APL). The current status of PML-RARα detection in various laboratories remains unknown.

Methods: In 2018, external quality assessment (EQA) for PML-RARα detection was carried out in China. Three EQA sample panels for PML-RARα isoform L/S/V were prepared by different mock leukocyte samples. The performances of PML-RARα detection, including admission screening, and qualitative and quantitative detection by real-time quantitative reverse transcription PCR (RT-qPCR), were assessed based on APL simulated clinical case.

Results: The mock leukocyte samples met the requirements of a clinically qualified sample for PML-RARα EQA panel. Among the laboratories, 13/50 (26.0%) were “competent,” 21/50 (42%) classified as “acceptable,” and 16/50 (32.0%) classified as “improvable.” One (1/50, 2.0%) laboratory reported one screening mistake. Twenty-six (26/50, 52.0%) laboratories reported 29 false-positive and 19 false-negative results. Twenty-three (23/50, 46.0%) laboratories reported 42 quantitative incorrect results.

Conclusion: Significant differences were not found in PML-RARα detection performance among laboratories that used different extraction methods. The performances of qualitative and quantitative RT-qPCR detection were worse accurate for PML-RARα isoform V. Quantitative variation was higher for low-level samples. Further continuous external assessment and education are needed in the management of PML-RARα detection.

Keywords
acute promyelocytic leukemia, external quality assessment, PML-RARα, real-time quantitative reverse transcription PCR
Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) with characteristic biological and clinical features, comprising approximately 10% of de novo AML cases in younger adults. APL is present of a specific t(15;17) chromosomal translocation in the leukemic blast, which involves the promyelocyte (PML) gene on chromosome 15 to the retinoic acid receptor-alpha (RARA) gene on chromosome 17. According to different breakpoints in PML and RARA, there are three isoforms of PML-RARA fusion gene (FG): long (L, 55%), variant (V, 5%), and short (S, 45%).

PML-RARA FG is present in almost all APL cases and is a biomarker for APL diagnosis, disease burden, minimal residual disease (MRD) monitoring, and molecular remission. Detection methods for t(15;17) or PML-RARA FG include conventional chromosome analysis, fluorescence in situ hybridization, and polymerase chain reaction (PCR). Compared with common reverse transcription PCR (RT-PCR), real-time quantitative reverse transcription PCR (RT-qPCR) for PML-RARA has higher precision and reliability, and is routinely used, especially in molecular hematology laboratories.

Clinical detection of the PML-RARA fusion gene is important in APL development. APL can be diagnosed in patients with abnormal hematopoiesis and characteristic cytogenetic abnormalities with t(15;17), regardless of the percentage of marrow blasts. PML-RARA FG transcript level can reflect the abnormal leukemia blasts load, quantitatively document disease burden, and confirm molecular remission. The goal of consolidation therapy for APL is a durable molecular remission, defined as undetectable PML-RARA FG.

Rigorous sequential MRD monitoring by RT-qPCR coupled with pre-emptive therapy can help reduce clinical relapse rates in APL patients.

External quality assessment (EQA) programs of common RT-PCR for PML-RARA FG test were first performed nearly 20 years ago. These programs used RNA, cDNA, or plasmid as EQA samples, and examined the heterogeneous sensitivities of PML-RARA FG RT-PCR detection. In 2003, the Europe Against Cancer (EAC) program established RT-qPCR standardization and quality control analysis for the PML-RARA FG transcript and recommended the ratio of FG copy number to control genes (GC) copy number (FG\text{CN}/GC\text{CN}) as the PML-RARA FG transcript level. The MRD value is a ratio between the FG transcript level in follow-up ([FG\text{CN}/GC\text{CN}]\text{FUP}) and diagnostic samples ([FG\text{CN}/GC\text{CN}]\text{DIAG}). These studies promoted the improvement of the PCR detection sensitivity and accuracy for PML-RARA FG, especially the EAC-sanctioned RT-qPCR. However, there existed some limitations. For example, some defects, total RNA, cDNA, recombinant plasmid, and NB4 cells were not suitable as EQA samples. Little is known about the evaluation of PML-RARA isoform V detection. These EQA programs only assessed the accuracy of the RT-PCR or RT-qPCR methodology, but did not analyze MRD monitoring results for PML-RARA based on APL clinical information. The EQA scoring criteria for BCR-ABL1 are unsuitable for PML-RARA, because only the accuracy of quantitative RT-qPCR detection was analyzed, with no admission screening and qualitative test.

We made MS2 armored RNAs for PML-RARA FG transcript, CG transcript, and 23s rRNA. Armored RNAs are stable, nuclease-resistant, and precisely quantifiable synthetic RNAs. They were already used as BCR-ABL1 and control gene standards. The EQA panel of PML-RARA isoform L/S/V with simulated APL clinical information was designed. We prepared mock leukocyte samples as EQA samples by mixing different amounts of the aforementioned armored RNAs, which can simulate total RNA yields extracted from BM by adding a large amount of 23s rRNA armored RNA. The PML-RARA detection was assessed, including RNA extraction, admission screening, and qualitative and quantitative RT-qPCR test.

2 | MATERIALS AND METHODS

2.1 | Design of APL simulated case

According to the NCCN Clinical Practice Guidelines in Oncology Acute Myeloid Leukemia (version 3.2017) and Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet, we designed APL simulated clinical case for isoforms L/S/V (see Appendix S1).

2.2 | Preparation and evaluation of mock leukocyte samples for EQA panel

Total RNA extracted from BM was divided into three components, including PML-RARA FG transcript RNA, CG transcript RNA, and other non-target RNA. We used MS2 virus-like particle packaging technology to make mock leukocytes samples, which consisted armored RNAs of PML-RARA FG L/V/S, chimeric CGs (AR-CG), and 23s rRNA (AR-23s). Firstly, the recombinant plasmids, pACYC-MS2-PML-RARA L/V/S, pACYC-MS2-CGs, and pACYC-MS2-23s rRNA, were constructed separately. Then, five armored RNAs were expressed and purified as previously described. The armored RNAs were identified by transmission electron microscopy, enzymatic digestion test, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and RT-PCR.

We constructed PML-RARA FG EQA panel which consisted of limited positive and negative samples with different FG\text{CN}/GC\text{CN} ratio and MRD value (Table 1). The positive mock leukocyte samples were obtained by mixing AR-FG L/V/S and AR-CG at different concentrations after adding 30 μL AR-23s. Negative mock leukocyte samples were prepared from specified concentrations of AR-CG after adding 30 μL AR-23s. All mock leukocyte samples were freeze-dried and stored at −20°C.

The EQA panel was evaluated using a routine detection process. Total RNA was extracted by TRIzol reagent and spin column, quantified using NanoDrop 2000c (Thermo Fisher). Using the one-step or two-step RT-qPCR method, qualitative and quantitative detection of PML-RARA FG and CG was performed by the manufacturer’s instructions on ABI 7500 Instrument (Applied Biosystems).
2.3 | Organization of the EQA

Before sample processing, the EQA samples should be centrifuged at 12 000 r/min for 1 min and did not need the reconstitution and the lysis of red blood cells. Total RNA extraction was performed by using routine operating procedure of individual laboratory. Participating laboratories first performed screening tests for the admission sample (A1711, B1721, and C1731) based on APL simulated clinical case; then, RT-qPCR was carried out, and the FG\_CN/CG\_CN ratio and MRD value were calculated. EQA panel A or B set was randomly assigned to the participants beside EQA panel C delivery to all laboratories. Each participant was asked to report the results on the data sheet within 2 weeks.

2.4 | Laboratory performance scoring

Accurate detection of the PML-RARα FG was prerequisite for APL diagnosis and MRD monitoring. Any result distinct from the established value was considered as "incorrect result" which will affect evaluation of treatment effect for APL MRD. Any error in RT-qPCR is multiplicative, rather than additive, data distributions from RT-qPCR-based EQA testing program produce a lognormal distribution, that is an asymmetric distribution of results with a strong positive skew. The log reduction was calculated by using the admission sample in each EQA panel as the baseline. The reduction in PML-RARα levels from this baseline value was then calculated for each correct qualitative positive EQA sample and reported as a log reduction.

| EQA panel | Sample No. | Isoform classification | FG\_CN/CG\_CN ratio | MRD value | Log reduction | No. of correct/Total no. tested (%) |
|-----------|------------|------------------------|---------------------|-----------|---------------|------------------------------------|
| A         | A1711      | L                      | 10.00%              | 1         | 0             | 25/25 (100)                        |
| A         | A1712      | L                      | 2.00%               | 0.2       | 0.6990        | 22/25 (88)                         |
| A         | A1713      | Negative               | 0                   | Negative  | Negative      | 22/25 (88)                         |
| A         | A1714      | Negative               | 0                   | Negative  | Negative      | 24/25 (96)                         |
| A         | A1715      | L                      | 0.02%               | 0.002     | 2.6990        | 19/25 (76)                         |
| B         | B1721      | S                      | 10.00%              | 1         | 0             | 25/25 (100)                        |
| B         | B1722      | S                      | 2.00%               | 0.2       | 0.6990        | 17/25 (68)                         |
| B         | B1723      | Negative               | 0                   | Negative  | Negative      | 20/25 (80)                         |
| B         | B1724      | Negative               | 0                   | Negative  | Negative      | 20/25 (80)                         |
| B         | B1725      | S                      | 0.02%               | 0.002     | 2.6990        | 22/25 (88)                         |
| C         | C1731      | V                      | 130.00%             | 1         | 0             | 49/50 (98)                         |
| C         | C1732      | Negative               | 0                   | Negative  | Negative      | 42/50 (84)                         |
| C         | C1733      | V                      | 0.02%               | 0.00015   | 3.8239        | 35/50 (70)                         |
| C         | C1734      | Negative               | 0                   | Negative  | Negative      | 43/50 (86)                         |
| C         | C1735      | V                      | 0.21%               | 0.0015    | 2.8240        | 36/50 (72)                         |
| C         | C1736      | V                      | 10.00%              | 0.077     | 1.1135        | 36/50 (72)                         |

The EQA scores based on qualitative and quantitative results were classified as "competent" (100% satisfied results), "acceptable" (<2 incorrect results), or "improvable" (more than 2 incorrect results).

2.5 | Statistical analyses

All data were analyzed using SPSS version 16.0. PML-RARα detection sensitivity, specificity, accuracy, and variation distribution between different samples or groups were compared using t test or one-way ANOVA or Fisher chi-square test. P values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Quality assessment of armored RNAs

Armored RNAs were constructed and expressed successfully by validation of sequencing and a series of experiments (Figure 1), and by TEM to detect the diameters of the armored RNAs of MS2 VLPs (about 30 nm; Figure 1A) and SDS-PAGE to clear molecular weight of proteins (about 14 KD; Figure 1B). Digesting with RNase A and DNase I for 1 hour at 37°C, only one single band between 1 kb and 2 kb was visible using 1% agarose gel electrophoresis (Figure 1C). RT-PCR was performed respectively to confirm encapsulation of the five target sequences (Figure 1D), followed by sequencing. To verify their stability and availability of the armored RNAs for the EQA study before panel distribution, stability analyses were performed and approved that armored RNAs were stable.
FIGURE 1 Evaluation of armored RNAs. A, Identification of five armored RNAs by transmission electron microscopy. The diameter of armored RNAs was approximately 30 nm. Number 1, 23s rRNA armored RNA (AR-23s); Number 2, chimeric CGs armored RNA (AR-CG); Number 3, PML-RARα FG L armored RNA (AR-FG L); Number 4, PML-RARα FG S armored RNA (AR-FG S); and Number 5, PML-RARα FG V armored RNA (AR-FG V). B, After purification by gel exclusion chromatography, freshly prepared armored RNAs were loaded onto an SDS-polyacrylamide gel and subjected to electrophoresis in tricine buffer. Proteins were visualized by staining the gel with Coomassie brilliant blue. Lane M, PageRuler Prestained Protein Ladder; Lane 1, AR-23s; Lane 2, AR-CG; Lane 3, AR-FG L; Lane 4, AR-FG S; Lane 5, AR-FG V; Lane 6, negative control (blank); and Lane 7, positive control (MS2). C, Identification of five armored RNA by agarose gel electrophoresis after enzymatic digestion test. Freshly prepared armored RNAs were incubated with RNase A and DNase I at 37°C for 1 h and subsequently analyzed on a 1% agarose gel, producing bands between 1 kb and 2 kb. Lane M, molecular weight marker; Lane 1, AR-23s without incubation with RNase A and DNase I; Lane 2, AR-23s incubated with RNase A and DNase I; Lane 3, AR-CG without incubation with RNase A and DNase I; Lane 4, AR-CG incubated with RNase A and DNase I; Lane 5, AR-FG L without incubation with RNase A and DNase I; Lane 6, AR-FG L incubated with RNase A and DNase I; Lane 7, AR-FG S without incubation with RNase A and DNase I; Lane 8, AR-FG S incubated with RNase A and DNase I; Lane 9, AR-FG V without incubation with RNase A and DNase I; and Lane 10, AR-FG V incubated with RNase A and DNase I. D, Ethidium bromide-stained 1% agarose gel of RT-PCR amplification products of RNA extracted from armored RNAs. Lane M, two kinds of molecular weight marker; Lane 1, RT-PCR products of RNA extracted from AR-FG L; Lane 2, RT-PCR products of RNA extracted from AR-FG S; Lane 3, RT-PCR products of RNA extracted from AR-FG V; Lane 4, RT-PCR products of RNA extracted from AR-CG; and Lane 5, RT-PCR products of RNA extracted from AR-23s.
for at least 2 weeks at 37°C, room temperature, 4°C, and −20°C (data not shown).

3.2 | Evaluation of mock leukocyte samples

Total RNA yields extracted by TRIzol reagent ranged from 15.2 to 24.5 μg/sample and spin column from 9.3 to 14.1 μg/sample, respectively. The quantitative validation results of the positive samples are slightly different between one-step and two-step method which is in agreement with expected values (See Appendix S2).

3.3 | Panel distribution and response

Fifty laboratories submitted their detection results and experimental data. TRIzol reagent was widely used by 46/50 (92%) participants, the spin column method was used by 3/50 (6%) laboratories, and only one laboratory used the magnetic bead method. The 37/50 (74%) laboratories used PML-RARa fusion gene RT-qPCR kit (YUANQI BIO Co., Ltd. Shanghai, China) for one-step method, and 12/50 (24%) laboratories used two-step in-house RT-qPCR. One laboratory (2%) used PML-RARa fusion gene RT-qPCR kit (SYBio Co., Ltd. Shanghai, China) for two-step method (Table 2).

3.4 | Performance of laboratories

The mock leukocyte samples had good adaptability to various RNA extraction methods. We did not find significant differences in RNA extraction performance among laboratories that used different extraction methods (P = 0.79; Figure 2A). RNA yields extracted by TRIzol reagent between EQA samples in panel C were consistent (P = 0.99; Figure 2B). All 50 laboratories used ABL1 as the control gene. Excluding 6 results from one laboratory, other laboratories had control gene ABL1 CN >10^4 and the median of CG CN ranged from 1.14 × 10^4 to 4.57 × 10^7 (Figure 2C). The different RNA extraction methods had no effect on PML-RARa detection accuracy and no significant difference (P = 0.40; Figure 2E).

Among the laboratories, 13/50 (26.0%) laboratories were “competent,” 21/50 (42%) classified as “acceptable,” and 16/50 (32.0%) classified as “improvable.” The performances of the different RT-qPCR assays used for the qualitative and quantitative tests indicated overall accuracy, sensitivity, and specificity were 91.1%, 94.0%, and 86.0%, respectively; the accuracy of in-house methods was better than commercial kits, and EQA panel C for isoform V detection was worse than that of EQA panels A and B (Tables 1 and 3).

| TABLE 2 | Qualitative and quantitative performance of different assays for each EQA panel |
|---------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| EQA panel | Assay | YUANQI Qualitative | SY Qualitative | In-house Qualitative | YUANQI Quantitative | SY Quantitative | In-house Quantitative |
| | No. of data sets | | | | | | |
| A | A1711 | 19/19 (100) | NT | 6/6 (100) | 6/6 (100) |
| | A1712 | 19/19 (100) | 16/19 (84.2) | NT | 6/6 (100) | 6/6 (100) |
| | A1713 | 16/19 (84.2) | NT | 6/6 (100) | / |
| | A1714 | 18/19 (94.7) | NT | 6/6 (100) | / |
| | A1715 | 18/19 (94.7) | 13/18 (72.2) | NT | 6/6 (100) | 6/6 (100) |
| B | B1721 | 18/18 (100) | 18/18 (100) | NT | 5/6 (83.3) | / |
| | B1722 | 17/18 (94.4) | 11/17 (64.7) | NT | 5/6 (83.3) | / |
| | B1723 | 14/18 (77.8) | NT | 5/6 (83.3) | / |
| | B1724 | 14/18 (77.8) | NT | 5/6 (83.3) | / |
| | B1725 | 18/18 (100) | 17/18 (94.4) | NT | 5/6 (83.3) | 5/5 (100) |
| C | C1731 | 36/37 (97.3) | 37/37 (100) | NT | 12/12 (100) | 12/12 (100) |
| | C1732 | 33/37 (89.2) | NT | 8/12 (66.7) | / |
| | C1733 | 30/37 (81.1) | 25/29 (86.2) | 0/1 (0) | 11/12 (91.7) |
| | C1734 | 33/37 (89.2) | NT | 9/12 (75.0) | / |
| | C1735 | 32/37 (84.5) | 26/32 (81.3) | 0/1 (0) | 10/12 (83.3) |
| | C1736 | 36/37 (97.3) | 25/36 (69.4) | 1/1 (100) | 10/12 (83.3) |
| Total | | 371/407 (91.2) | 207/243 (85.2) | 9/11 (81.8) | 72/79 (91.2) | 78/83 (94.0) |

In-house, in-house-developed RT-qPCR assay; NT, not tested; SY, PML-RARa fusion gene RT-qPCR kit (Shanghai SYBio Co., Ltd.); YUANQI, PML-RARa fusion gene RT-qPCR kit (Shanghai YUANQI BIO Co., Ltd.).
Concerning admission screening test, 49 (49/50, 98.0%) participating laboratories were excellently proficient. One laboratory made PML-RARα FG isoform V identification mistake. Forty laboratories (40/50, 80.0%) identified isoform L/S/V, while the remaining 10 (10/50, 20.0%) did not.

Of 550 qualitative results received, 48 (8.7%) were incorrect, including 18 false-negative (FN) results and 30 false-positive (FP) results. In 18 FN results, case set C accounted for 15 incorrect results, with the remaining 2 ones from B, and one from A. Of 30 FP results, case sets A/B/C occupied 4, 10, and 16 incorrect results, respectively (Table 3). Six laboratories that did not apply isoform-classified reagents reported 19 incorrect qualitative results, 12 FN results, and 7 FP results; 16 isoform-classified laboratories had 29 incorrect results, 6 FN, and 23 FP (Table 3).

### TABLE 3 Qualitative incorrect results of different reagents and EQA panels

| Incorrect results | Reagent type          | No. of qualitative incorrect results for EQA panel (No. of laboratory) |
|-------------------|-----------------------|-----------------------------------------------------------------------|
|                   |                       | A          | B          | C          | Total       |
| False-negative    | Isoform classified    | 1 (1)      | 1 (1)      | 4 (3)      | 6 (4)       |
|                   | Not classified        | 0 (0)      | 1 (1)      | 11 (5)     | 12 (6)      |
| False-positive    | Isoform classified    | 4 (3)      | 8 (4)      | 11 (11)    | 23 (15)     |
|                   | Not classified        | 0 (0)      | 2 (1)      | 5 (4)      | 7 (4)       |
| Total             |                       | 5 (4)      | 12 (7)     | 31 (21)    | 48 (26)     |

**FIGURE 2** Detection performance of mock leukocyte samples for EQA panels. A, RNA yields extracted by different methods. Error bars represent standard deviation. B, RNA yields extracted by TRIzol between EQA samples. C, Control gene copy number of each mock leukocyte sample. D, Differences in the slope of RT-qPCR standard curve between different laboratory groups. 1, correct detection group; 2, quantitative incorrect group; 3, only qualitative incorrect group. Error bars represent standard deviation. **P < 0.001 vs 2. E, Effect of different RNA extraction methods for PML-RARα detection accuracy.
In quantitative RT-qPCR test, 42 incorrect results were reported by 23 participating laboratories. The mean, median, standard deviation (SD), and coefficient of variation (CV) of log reduction for PML-RARα quantitative results are summarized (Table 4). The CV of case set C was greater than the value of case sets A and B. The CV value increased at a higher PML-RARα level in each sample set (Table 4). Case sets A/B/C contributed 8, 8, and 26 incorrect results, separately. The range was from −2.19 to −4.15 for the slope and 0.96 to 1.00 for the R² value. According to RT-qPCR quantitative results, we divided the participating laboratories into 3 groups, including correct detection group, quantitative incorrect group, and only qualitative incorrect group. Using the difference in slope as an index, the inconsistencies in amplification efficiency of PML-RARα FG and CG in the quantitative incorrect group were statistically significantly greater than those in the other two groups (Figure 2D).

| Log reduction | EQA panel A | EQA panel B | EQA panel C |
|---------------|-------------|-------------|-------------|
|               | A1712       | A1715       | B1722       | B1725       | C1733       | C1735       | C1736       |
| Mean          | 0.6376      | 2.6109      | 0.7012      | 2.7032      | 3.5326      | 2.5189      | 1.2524      |
| SD            | 0.0629      | 0.1577      | 0.0648      | 0.2184      | 0.4545      | 0.3255      | 0.1813      |
| CV (%)        | 9.87        | 6.04        | 9.24        | 8.08        | 12.87       | 12.92       | 14.48       |
| Median        | 0.6675      | 2.6244      | 0.7007      | 2.7241      | 3.5066      | 2.5149      | 1.2182      |
| Minimum       | 0.1260      | 2.1543      | 0.2273      | 2.2450      | 2.2040      | 1.6345      | −0.6487     |
| Maximum       | 1.1837      | 3.7677      | 1.1222      | 3.4919      | 4.2936      | 3.8516      | 2.2852      |
| Range         | 1.0577      | 1.6134      | 0.8949      | 1.2469      | 2.0896      | 2.2171      | 2.9339      |
| No.           | 25          | 24          | 24          | 24          | 42          | 44          | 49          |

CV, coefficient of variation; SD, standard deviation.

**4 | DISCUSSION**

We successfully designed mock leukocyte samples as the EQA panel for qualitative and quantitative RT-qPCR detection performance. Thirty-seven of the laboratories reported incorrect qualitative or quantitative results of PML-RARα detection. The detection performance of the laboratories using in-house methods for PML-RARα was significantly better than those using commercial reagents. Among three sample sets, the detecting ability to rare isoform V was worse than L or S. In the same sample set, the detection accuracy of PML-RARα low-level samples was lower than the high-level samples which prompt these participants needed to improve RT-qPCR test reliability.

The mock leukocyte samples met the requirements of a clinically qualified sample for PML-RARα EQA panel. By mixing different armored RNAs of PML-RARα FG, CG, and 23s rRNA, we simulated the composition and RNA yield of total RNA of BM-nucleated leukocytes to prepare mock leukocyte samples. The qualified samples were judged by copy number (CN) of control gene, ABL1 is >10⁴, or location of Ct value is in the range of 21.9–29.3. The copy number of control gene in each EQA sample tested by laboratories complied with the above requirements. RNA yields extracted by TRIzol reagent between EQA samples were consistent with the range of RNA yields/sample reported. The RNA yields did not seem to affect PML-RARα FG RT-qPCR detection, because laboratories achieved accurate detection results (Figure 2E, Table 2). This conclusion was consistent with previous findings.

The degree of agreement with the established value reached 91.3% (502/550) in PML-RARα qualitative RT-qPCR detection for all EQA samples. This qualitative accuracy rate was slightly higher than previously reported. Except for accidental specimen loading errors, laboratory aerosol and instrument contamination may be the main cause of false-positive results, especially for ultra-low PML-RARα copies. By analyzing the reported RT-qPCR false-positive results, we found that laboratories using in-house method had more false-positive results than those using commercial reagents. The in-house method needs to open the lid between cDNA synthesis step and PCR amplification step, and there is an increased chance of residual contamination. In addition, the intensive distribution of EQA-positive samples, larger load volumes, and repeated detection of specific samples may have more opportunities for cDNA synthesis and PCR contamination.

We found that commercial reagents had lower sensitivity than in-house method. Five laboratories using commercial unclassified reagents reported 11 false-negative results for medium-level and low-level isofom V samples. This may be due to the low detection sensitivity of the PML-RARα unclassified reagents for rare isofom V, especially low-level sample. These laboratories were obliged to improve program documentation to accommodate PML-RARα rare isofom V detection. Only one laboratory reported ABL1 CG <10⁴ copies, and the FN results were due to incorrect preservation of RNA of the laboratory leading to RNA degradation.

There was a great deal of PML-RARα FG quantitative variation between not only reagents but also case sets (Table 4). We observed that commercial reagents reported more quantitative improper results than in-house method, especially for PML-RARα isoform V (Table 2). The possible reasons for that are as follows. The inconsistency of the RT-qPCR detection efficiency between each sample
and every EQA case sets accompanies a evident divergence. The variation of RT-qPCR quantitative detection for commercial reagents (YUANQI) was greater than in-house methods, especially the high PML-RARα level (Table 4). The quantitative variation had a lot to do with the intrinsic procedure, for example, the determination of standard curve. The higher difference in slope of PML-RARα FG and CG in the quantitative incorrect group was in charge of quantitative wrong (Figure 2D). In addition, unequal amplification efficiency between the plasmid calibration standard and the RNA template will bring about an potential augment in quantitative detecting inaccuracy; thus, standard curve should satisfy both slope range (from -3.2 to -3.6) and $R^2 > 0.980$ like BCR-ABL1. Laboratories should optimize and validate the RT-qPCR procedures to achieve consistent quantitative detection capacity of different isoforms.

Mock leukocyte samples successfully can be used to assess PML-RARα detection. Significant differences were not found in PML-RARα detection performance among laboratories that used different extraction methods. The performances of qualitative and quantitative RT-qPCR tests were worse in the PML-RARα detection process, especially for PML-RARα FG rare isoform V. Quantitative variation was higher for fusion gene low-level samples. To improve PML-RARα FG detection, laboratories should conduct internal quality control and anti-contamination, optimize RT-qPCR methodology, and regularly maintain and calibrate PCR instrument to ensure the accuracy of qualitative and quantitative detection. Our study highlights the need for further continuous external assessments and education in the management of APL PML-RARα detection process.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS’ CONTRIBUTIONS

Qisheng Wu designed the research study, performed the research, analyzed the data, and wrote the manuscript; Jinming Li reviewed and critically revised the manuscript, and approved the submitted and final versions; Rui Zhang reviewed and critically revised the manuscript; Yu Fu, Jiawei Zhang, and Kun Chen performed the research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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