Development and Application of Calibration Device for Fluorescence Quantitative PCR Analyzer

Songlin Yu *,[a], Yun Tian [b], Zhe Wang [c], Qiangguang Li [d], and Xiaodan Wang [e]

Tianjin Institute of Metrological Supervision and Testing, Nankai district, Tianjin municipality 300192, China

[a] yulinever@tju.edu.cn, [b] tianyun0515@163.com, [c] wangdazhe@sina.com, [d] sansan8608@163.com, [e] wangxd625@163.com

Abstract. This paper proposes a PWM-based light intensity modulation method, and develops a calibration device for fluorescence quantitative polymerase chain reaction (PCR) analyzer. To verify the performance of this calibration device, a temperature calibration system composed of isothermal block, standard platinum resistance thermometer, and thermostatic bath was employed in experimental measurements. Results showed that the absolute value of the indication errors did not exceed 0.05°C, and the uniformities did not exceed 0.06°C. Additionally, the linearity of LED emitting intensity versus relative intensity verified using a luminance meter showed a good fitting coefficient $R^2 \geq 0.9999$. Ultimately, this calibration device was used to calibrate the temperature and optical physical parameters of the fluorescence quantitative PCR analyzer, comprehensively evaluating its technical specifications.

Keywords: Fluorescence quantitative PCR analyzer; Calibration; Temperature; Optical physical parameters

1. Introduction

Fluorescence quantitative PCR analyzers play a vital role in medical clinical diagnosis, fundamental scientific research, pathology studies, in vitro diagnostic reagent production and development, quarantine and disease control, especially as one of the most widely used and accurate devices for COVID-19 nucleic acid testing [1-5]. The real-time fluorescence quantitative PCR-based nucleic acid detection method serves as the gold standard for COVID-19 screening. The temperature parameters of the fluorescence quantitative PCR analyzer directly impact PCR success, while the fluorescence measuring system significantly influences the quantitative results. Hence, the calibration of both the temperature and fluorescence measurement systems of the fluorescence quantitative PCR analyzers is crucial.

Due to the frequent use of fluorescence quantitative PCR analyzer, errors may arise in their temperature control and optical measurement systems, affecting experimental outcomes. Thus, there is an urgent need for the calibration of their temperature and optical parameters simultaneously [6-8]. Currently, domestic calibration institutions mainly approach the calibration of fluorescence quantitative PCR analyzers in two ways: one focuses solely on the thermal field calibration, unable to assess the optical section comprehensively. The other employs temperature sensors and standard materials/reagents to separately calibrate temperature parameters and the optical measurement system [9-11], striving for a full analysis of the metrological performance. However, this calibration method separates the temperature and optical parameters measurements, unable to explain their combined influence on the final quantitative result, the magnitude of errors, or their sources. Moreover, standard materials lack traceability to internationally recognized metrological standards.

This paper proposes a PWM-based light intensity modulation method, and develops a calibration device for fluorescence quantitative PCR analyzers. This calibration device working principle involves simulating the PCR process with micro-LED light sources, emitting light intensity according to set threshold cycles, received by the optical measurement system of the fluorescence

quantitative PCR analyzer to form melting curves, which were then analyzed based on actual temperature data. This device realizes unified calibration of temperature and optical measurement parameters of fluorescence quantitative PCR analyzers, particularly analyzing how temperature performance affects optical measurement errors and their sources, leading to comprehensive assessment of metrological performance indicators. Compared to calibration using standard materials, it simplifies operation, storage, reduces reagent consumption, minimizes potential human errors in reagent preparation, and enhances the reliability of the fluorescence quantitative PCR analyzer's metrology.

2. Experiment System and Method

2.1 Calibration Device for Fluorescence Quantitative PCR Analyzer

The calibration device for fluorescence quantitative PCR analyzer is composed of a control module, temperature sensors, and micro-LED light sources, as shown in figure 1. The control module includes power supply, low dropout (LDO) circuit, resistor distribution networks, analog-to-digital converter (ADC) circuit, microcontroller unit (MCU), PWM control chips, USB serial communication port, and PC. The resistor distribution network divides the voltage signals representing temperature measurements from the temperature sensor array, and ADC convert these divided signals into digital ones, which were then transmitted to the computer for real-time display and processing through USB. Wires between each temperature sensor and ADC are of equal length to enhance temperature measurement consistency. The PC, based on target temperature cycles and actual temperature data from the sensors, issues commands to the micro-LED light sources, with MCU controlling PWM chips to emit light intensity as per instructions. The intensity is received by the fluorescence quantitative PCR analyzer, forming melting curves, and mathematical operations yield measurement results.

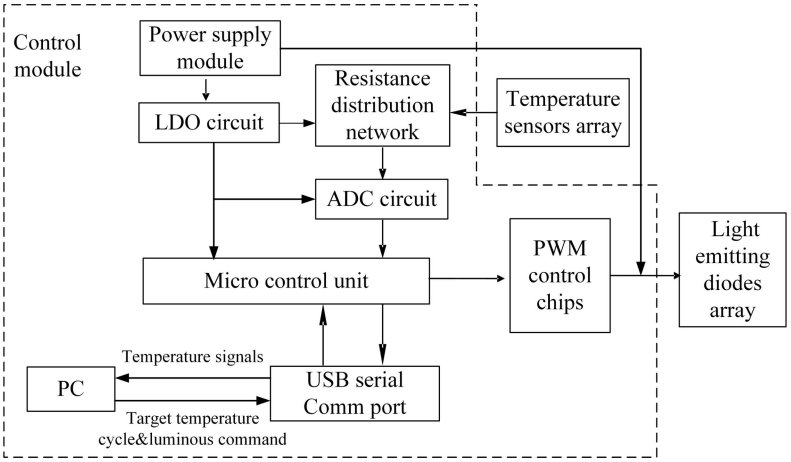

Fig. 1. Schematic diagram of calibration device for fluorescent quantitative PCR analyzer

2.2 PWM based LED Modulation Control

Pulse width modulation (PWM) method was adopted to modulate the emitting light intensity of LED through adjusting the power switch, varying the duty cycle frequency, controlling the illumination time ratio in this paper. Compared with the constant current regulation, the PWM method don't alter the circuit constants like supply current or voltage, make it more efficient compared to constant current regulation [12-14]. PWM-controlled LED sources maintain spectral stability without shift or color temperature variation, offering better linearity. The study utilizes LP5018RSMR chip to construct a 15-channel LEDs intensity control circuit, as depicted in figure 2, powered by 3.3V, enabling simultaneous control of 15 LED channels.

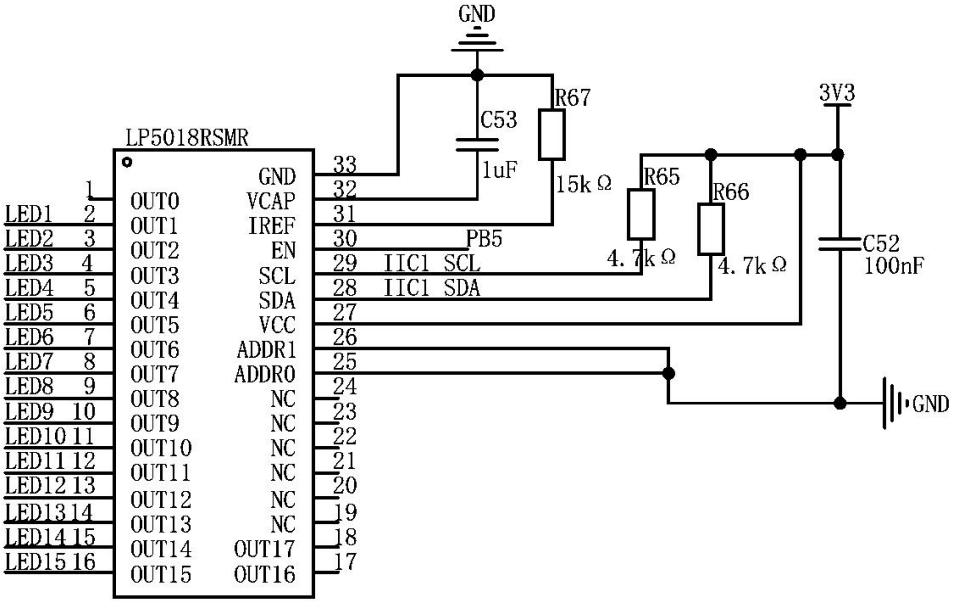

Fig. 2 LED luminescence intensity control circuit based on PWM

3. Results and Discussions

3.1 Calibration Device Indication Error and Uniformity

To validate the accuracy and reliability of this developed calibration device, a series of experiments were conducted using a standardized system composed of isothermal blocks, platinum resistance thermometers, and a thermostat bath.

The test results at calibration points revealed that the absolute value of the indication errors did not exceed 0.05°C and uniformity not exceed 0.06°C, as depicted in figure 3. Due to article space constraints, the raw data were not presented. This rigorous validation underscores the high precision and consistency achieved by the developed calibration device, confirming its capability to accurately measure temperatures within specified tolerances and ensuring reliable PCR performance.

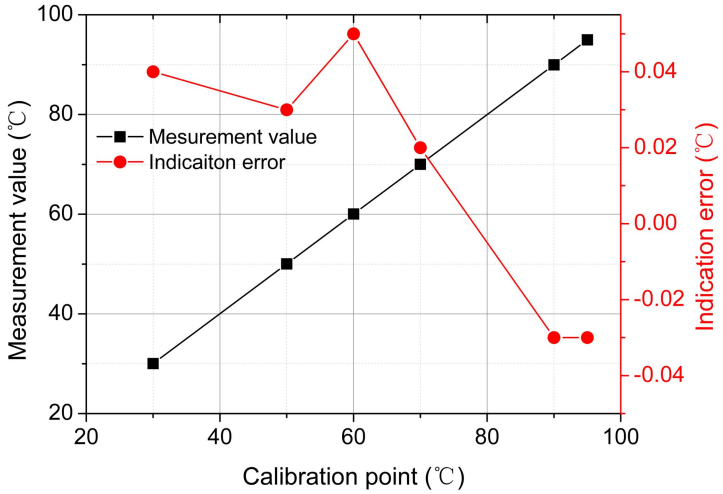

Fig. 3 Curve of measurement value and indication error at calibration point

3.2 Testing of Optical Intensity Output Simulation

In this study, a micro-LED light source serves as a optical simulator, emulating the fluorescent intensity variations occurring in a PCR process. The LED's emission intensity simulates the fluorescent release during gene amplification, directly influences the effectiveness of the optical

amplification simulation. The linearity of the optical simulator's light emission, hence, is critical to the accuracy of simulation.

To assess the linearity of the LED emitting light intensity, a standard luminance meter was employed. The relative intensities of the LEDs were sequentially set to 10%, 20%, 50%, 60%, 80%, and10%, with the brightness values recorded for each intensity level. The resulting data points are depicted in figure 4 (Only one LED data was given due to the length of this paper), revealing a linearity correlation coefficient $R^2 \geq 0.9999$.

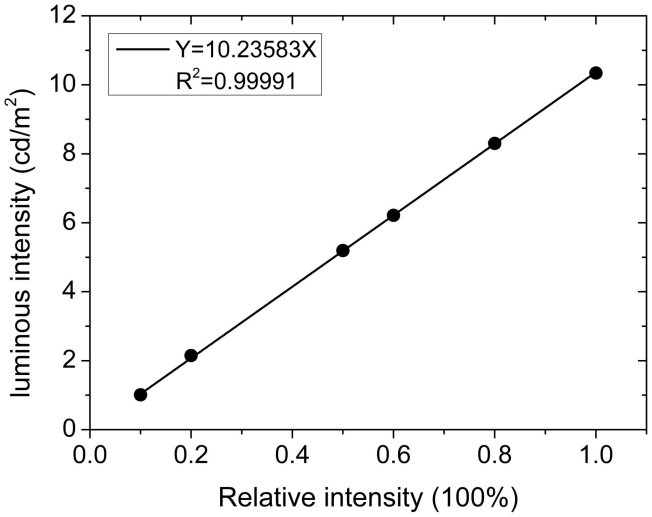

Fig.4 Curve of relative intensity versus luminous intensity of LED

3.3 Temperature Parameters Calibration

The key temperature parameters for a fluorescence quantitative PCR analyzer encompass temperature indication error, uniformity, and average ramp-up/ramp-down rates. Employing the developed equipment, a fluorescence quantitative PCR (Applied Biosysems, Q5) was calibrated, with temperature profiles illustrated in figure 5. From segment 1 to 4, the middle part represents the calibration results indicate the technical parameters. Derived from these measurements, the temperature indication error at 30°C, 50°C, 70°C, 90°C, and 95°C were -0.16°C, -0.13°C, -0.08°C, -0.01°C, -0.08°C, and -0.09°C, respectively, with uniformities of 0.71°C, 0.51°C, 0.64°C, 1.00°C, 1.00°C, and 0.90°C. The average heating and cooling rates were 1.64°C/s and 1.88 °C/s, respectively.

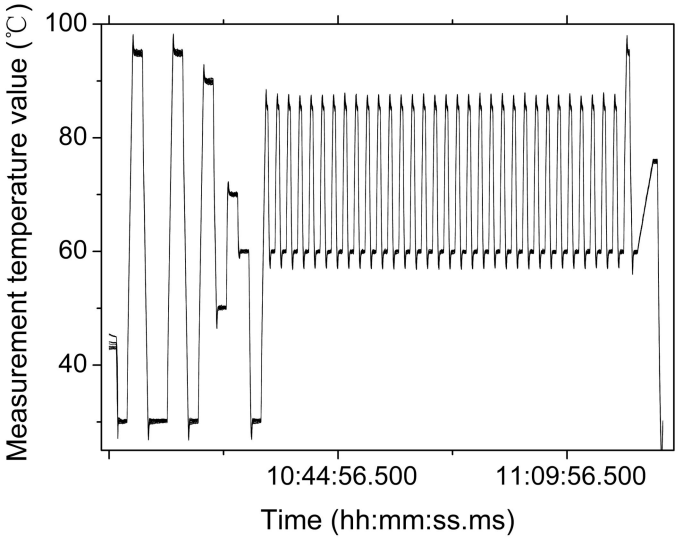

Fig. 5 Temperature measurement curve of fluorescent quantitative PCR analyzer

3.4 Physical Parameters Calibration

The optical physical parameters of a fluorescence quantitative PCR analyzer primarily consist of threshold cycle indication error, uniformity, and precision. In this study, the optical simulator's threshold was set at cycle 24 corresponding to the optical intensity, thus defining the standard value.

The calibration results of a fluorescence quantitative PCR (Applied Biosystems, Q5) was depicted in figure 6. The index growth aligns with threshold cycle, allowing for the calculation of threshold cycle values, revealing threshold cycle indication error, uniformity, and precision of threshold cycle as -0.34, 1.23 and 8.0%, respectively.

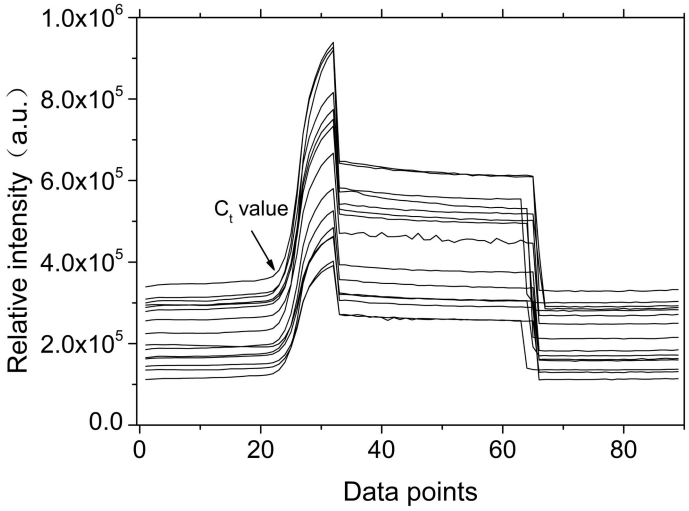

Fig. 6 Curve of relative intensity measured by fluorescent Quantitative PCR Analyzer

4. Summary

To satisfy the full parameter calibration requirements of fluorescence quantitative PCR analyzer, we propose an optical simulator-based calibration method for quantitative PCR and develop a dedicated calibration device, incorporating PWM for multi-channel LED sources. This calibration device is user-friendly, operationally efficient, reducing the need for standard materials and reagents associated with conventional calibration methods, minimizing potential errors in reagent preparation, thus enhancing comprehensive evaluation of PCR performance metrics.

To validate the calibration device performance, a verification system composed of isothermal block, standard platinum resistance thermometer, and thermostatic bath was employed to confirm the indication errors and uniformites of this device. Experimental outcomes indicated that he absolute value of the indication errors did not exceed 0.05°C, and the uniformities did not exceed 0.06°C. Furthermore, linearity correlation coefficients of relative intensity versus luminous intensity of LED were confirmed $R^2 \geq 0.9999$. Ultimately, this calibration device was used to assess temperature parameters performance, mainly including, temperature indication error, uniformity, average heating and cooling rate, and optical physical parameters performance, such as threshold cycle indication error, uniformity and precision. The results demonstrate that this calibration device was capable of fully evaluating all the performance parameters of the fluorescence quantitative PCR analyzer.

Acknowledgements

This work is supported by the Science and Technology Program of the State Administration for Market Regulation (No. 2021MK012).

References

[1] WangYanhu, Sang Xiaoyu, Saeed El-Ashram et al. Establishment of a method for detecting Trichinella spiralis in ovine muscle tissues using real-time fluorescence quantitative PCR. Experimental Parasitology, 2023, 246:108457.

[2] Liu Mengyu, Tian Cuihong, Chen Yequn et al. Effectiveness of a standardized quality control management procedure for COVID-19 RT-PCR testing: a large-scale diagnostic accuracy study in China. Diagnostic Microbiology and Infectious Disease, 2024, 109(2):116287.

[3] Sibra RM Shihab, Bushran N. Iqbal, Shiyamalee Arunasalam. Quality of the sample-based RNA determines the real-time RT-PCR results in the laboratory diagnosis of COVID-19. Diagnostic Microbiology and Infectious Disease, 2024, 108(1):116076.

[4] Fumino Nito, Hitoshi Oya, Takayuki Matsuura et al. Advancing broad bean true mosaic virus detection using conventional RT-PCR and real-time RT-PCR with novel primer set design. Journal of Virological Methods, 2024, 327:114946.

[5] Manoucher Teymouri, Samaneh Mollazadeh, Hamed Mortazavi et al. Recent advances and challenges of RT-PCR tests for the diagnosis of COVID-19. Pathology Research and Practice, 2021, 221(153443):1-8.

[6] Yang Xu, Tingting Ren, Ling jian Zen et al. Studies on optical calibration of real-time fluorescent quantitative polymerase chain reaction (RTFQ-PCR) analyzer. Proceedings of SPIE, 2023, 12343:123430E1-123430E16.

[7] Kangning Wang, Yangyang Jiang, Guo Yu et al. An Optimized Thermal Feedback Methodology for Accurate Temperature Control and High Amplification Efficiency during Fluorescent qPCR. Bioengineering (basel), 2022, 9(6):237.

[8] Hey Reoun An, Gediminas Mainelis, Lori White. Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. 2006, 40(40):7924-7939.

[9] Yunhua Gao, Chun Li, LiQing Wu et al. Device for correcting temperature of PCR analyzer, comprises base and top cover, where top cover comprises base and can seal covered with base, face pointing to top of base is provided with fixing device and temperature sensor probe. 2019, CN209446193(U).

[10] Shangsu Lu, Jinxiong She, Yuanshun Zou et al. Polymerase chain reaction (PCR) temperature calibration system has PCR device, temperature-sensing element, data collect card circuit, and personal computer device that are connected in order. 2014, CN203855583(U).

[11] Tengjie Hu, Yuhong He, Qiumei Zhou. Real-time fluorescent quantitative PCR instrument calibration kit comprises PCR premix, PCR enzyme, standard and primer dry plate, and standard is mixture of two or four of cDNAG1, cDNAG2, cDNAG3, and cDNAG4. 2021, CN113215225(A)-CN113215225(B).

[12] Su Zhao, Weidong Wang, Zhiyi He. A Constant Current LED Driver with Disordered PWM Control An out-of-order PWM controlled LED constant current driver chip. 2013, 30(9):137-140.

[13] Bin Zhao Juncheng Xiao, Quansheng Liu et al. A novel compensation pixel circuit for high bits of AM mini/micro-LED based on PWM method. Energy Reports, 2021, 7(1):343-348.

[14] Linas Svilainis. LED PWM dimming linearity investigation. Display, 2008, 29(3):243-249.