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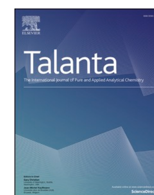

Transferable, easy-to-use and room-temperature-storable PCR mixes for microfluidic molecular diagnostics

Jiasu Xu [a,1], Jin Wang [a,b,1], Xiaosong Su [c], Guofu Qiu [d], Qiurong Zhong [e], Tingdong Li [a], Dongxu Zhang [a], Shiyin Zhang [a,*], Shuizhen He [e,**], Shengxiang Ge [a], Jun Zhang [a], Ningshao Xia [a,b]

[a] State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Public Health, Xiamen University, Xiamen, 361102, China
[b] School of Life Sciences, Xiamen University, Xiamen, 361102, China
[c] Xiang'an Hospital of Xiamen University, Xiamen, 361102, China
[d] Xiamen Innovax Biotech CO., LTD., Xiamen, 361022, China
[e] Haicang Hospital of Xiamen, Xiamen, 361026, China

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ABSTRACT

As the outbreak of coronavirus disease 2019 (COVID-19), on-site molecular diagnosis is becoming increasingly important. In this study, a freeze-drying method was introduced for PCR reagents to meet the requirements of microfluidic molecular diagnosis. Using this method, PCR components were pre-mixed and freeze-dried as a bead, which could be transferred into microfluidic chips easily. As this bead only required reconstitution in water, operational steps of PCR were simplified, pipetting errors and errors associated with improper handling of wet reagents could also be reduced. In addition, 19 PCR mixes for different targets (including both RNA and DNA) detection were stable when stored at room temperature (18–25 °C) for 1–2 years and may be stored longer as activity monitoring remains ongoing. To shorten the stability testing time, accelerated stability testing at higher temperatures was proposed. The evaluation periods of the freeze-dried PCR mixes were shortened to less than one month when stored at 56 °C and 80 °C. When attempts were further tried to predict the shelf lives for freeze-dried PCR mixes, our findings challenged the classic view of the Q_{10} method as a prediction model for freeze-dried PCR mixes and confirmed for the first time that this prediction was influenced by different factors at varying degrees. These studies and findings are important for the development of molecular diagnosis at both central laboratories and resource-limited areas.

1. Introduction

As the outbreak of coronavirus disease 2019 (COVID-19) [1,2], on-site molecular diagnosis is becoming increasingly important [3]. Recent advances in point-of-care (POC) testing, especially microfluidic technology, make it possible to develop rapid, simple, cost-effective and portable molecular diagnostic tools on site [4].

However, traditional PCR reagents typically made for central laboratories are not applicable for microfluidic molecular diagnosis, unless a freeze-drying method is introduced. Firstly, traditional PCR reagents can't be stored at room temperature (RT) as the water molecules they contained drive many destabilization pathways [5,6]. Freeze-drying allows for the preservation of activity in qPCR reagents over the long-term storage at RT because this process removes most of the water molecules [7]. Thus, microfluidic chip contained the freeze-dried reagents can be stored everywhere irrespective of local preservation conditions. Secondly, liquid-form PCR reagents are cumbersome and complicated to prepare, whereas freeze-dried reagents are convenient to use because they only require reconstitution in water. With reduced operating steps, the operational complexity, preparation time, pipetting errors [8] and errors associated with improper handling of wet reagents, requirements for the operating environment and personnel quality can

* Corresponding author.
** Corresponding author.
E-mail addresses: zhangshiyin@xmu.edu.cn (S. Zhang), szhe@xmu.edu.cn (S. He).
[1] Joint first authors.

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all be reduced. Thirdly, instead of loading each component of PCR reagent to microfluidic chip separately, one can transfer all these components into the chip easily if they are freeze-dried as beads (Fig. 1Aii). This can also simplify the design of microfluidic chip as only one chamber is needed to store all the components for PCR. Last but not least, microfluidic molecular diagnostics is typically used to manipulate small volume of liquids, including small volume of samples, which results in reduced detection sensitivity. Freeze-dried PCR mixes can make up for it if they are reconstituted with sample instead of water (Fig. 1B).

Several publications on freeze-dried PCR mixes have been reported during the past 20 years (Table S1) [9–21]. In previous studies, electrophoresis has frequently been used to evaluate the activities of freeze-dried PCR reagents [9,10,12]. However, the nucleic acids detected by electrophoresis represent the final products of the PCR, which are all the same when PCR reaches the plateau phase. Under this circumstance, the band intensities of PCR reagents with different levels of residual activity will appear the same in the electrophoresis assessment (Fig. 1Civ). Quantitative real-time PCR (qPCR) is able to distinguish differences in reagent activity because it can monitor changes in nucleic acids throughout the PCR process. Using this method, the quantification cycle (Cq) values of degraded reagents would be larger than those with 100 % activity (Fig. 1Ciii). However, most freeze-dried PCR reagents

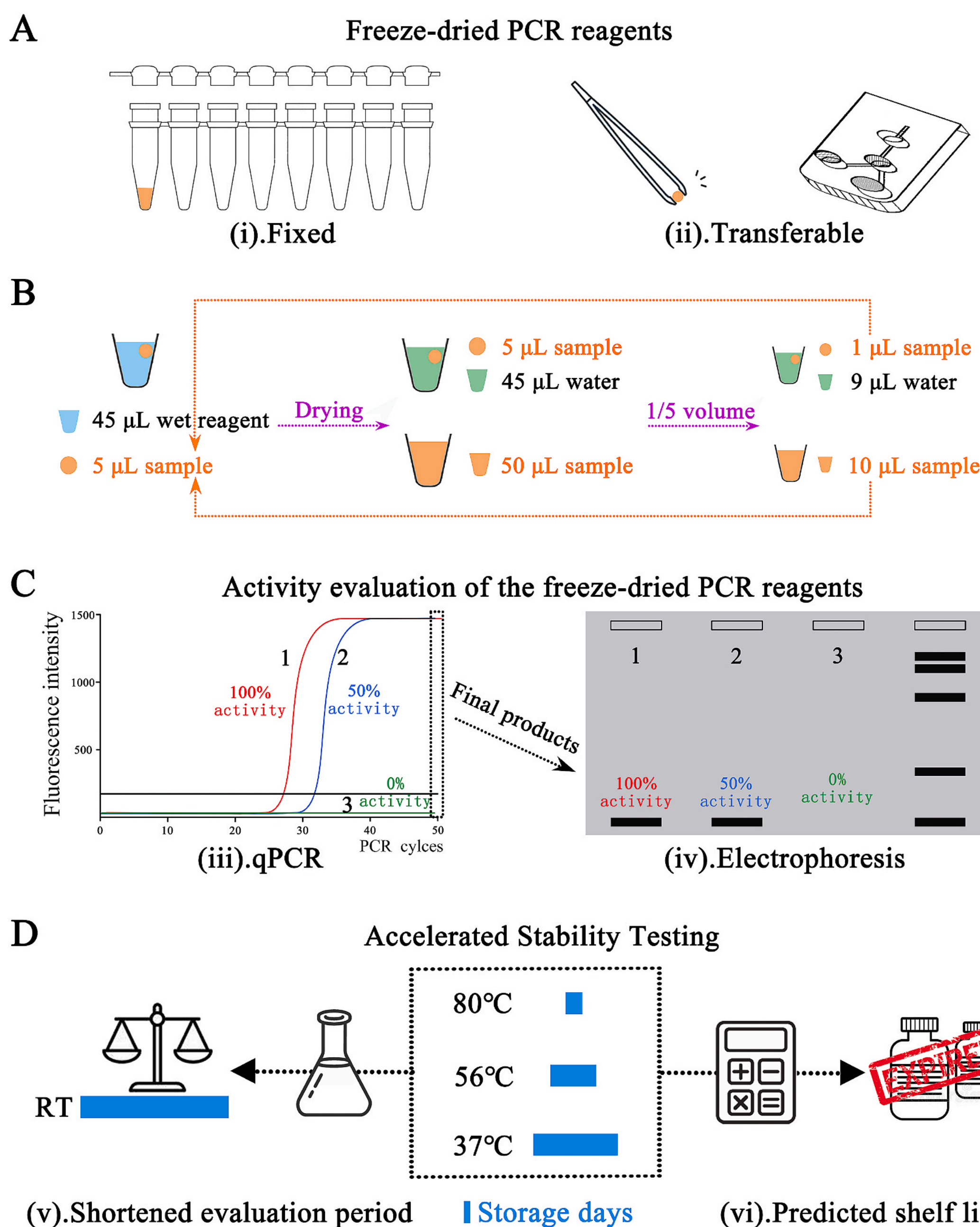

Fig. 1. Schematic diagrams of the freeze-dried PCR mixes and related methods. (A) Freeze-dried PCR mixes. (i) The PCR reagents are freeze-dried in PCR tube strips and cannot be removed after freeze-drying. (ii) The PCR mixes are freeze-dried as a bead and can be transferred by tweezers after freeze-drying. (B) The detection sensitivity of microfluidic reagent is reduced by the smaller reaction volume, which can be made up by reconstituting the freeze-dried PCR mixes with more sample. (C) Residual activity evaluation of the freeze-dried PCR reagents. (iii) Activity of the reagents evaluated by qPCR. In this method, fluorescence was used to report the dynamic changes in nucleic acids during the PCR. (iv) Activity of the reagents evaluated by electrophoresis. In this method, only the final products were measured after PCR. (D) Accelerated stability testing can be used to shorten the evaluation period (v) and predict the shelf lives (vi) of freeze-dried PCR mixes. Higher temperatures are typically associated with shorter storage periods.

detected by qPCR have demonstrated poor stability during long-term storage at RT [11,14,16], especially those designed for RNA targets detection [15,21], which contain the thermally unstable reverse transcriptase [22,23]. And what's worse, currently, most PCR reagents are freeze-dried in PCR tube strips and cannot be transferred (Fig. 1Ai), which poses a greater challenge to current microfluidic diagnosis technology.

Even if the freeze-drying method meets all of the requirements detailed above, the stability testing process over long periods under normal conditions for each production batch is time-consuming, labor-intensive, and not cost-effective. Generally speaking, biological reagents age faster when stored at higher temperatures. Several relevant publications have attempted to use elevated temperatures in accelerated stability testing to shorten the evaluation period for freeze-dried PCR reagents (Fig. 1Dv and Table S1) [10,14,21]. However, the temperatures they selected were incomplete, and the observation periods they reported were relatively narrow due to the poor freeze-drying methods used for their PCR reagents.

Also, even if an accelerated stability testing can be used to shorten the evaluation period, a real-time stability testing at RT remains necessary to establish the correlation between storage periods at higher temperatures and RT, which can still be time-consuming. To get rid of the real-time stability testing at RT, researches have attempted to translate the accelerated stability testing data into a predicted shelf life at RT using mathematical models (Fig. 1Dvi and Table S1) [17,18]. However, in fact, no mathematical method was specially designed and developed to predict the shelf life of freeze-dried PCR reagents, and their accuracy has not been verified up to now.

In this study, we have presented a freeze-drying method to generate transferable, easy-to-use and RT-storable PCR mixes that are suitable for microfluidic molecular diagnosis. Besides, we have introduced accelerated stability testing to shorten the evaluation period of these freeze-dried PCR mixes. In addition, mathematical models were also employed to predict the shelf life of the freeze-dried mixes, with further verification of their accuracy and potential influence factors. The results of this study would foster the development of molecular diagnoses in both central laboratories and resource-limited areas.

2. Materials and methods

2.1. Specimens

Enterovirus 71 (EV71), coxsackievirus A16 (CA16), human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis B virus (HBV), *Escherichia coli* BL21 (*E. coli*), and the human hepatoma cells (HuH-7) were supplied by the National Institute of Diagnostics and Vaccine Development in Infectious Diseases (Xiamen, China). Before use, viruses were inactivated using appropriate methods for each virus.

2.2. Nucleic acid extraction

Nucleic acids were extracted using Viral DNA/RNA Purification Kit, Bacteria DNA Purification Kit, or Tissue/Cell DNA Purification Kit with the DOF-9648 purification system (GenMagBio, China), according to the manufacturer's protocol. The extracted nucleic acids were stored in a 1.5-mL sample tube and maintained at −80 °C before PCR.

2.3. PCR assay

The 40-μL reactions and thermal cycling were the same as the ones described in our previous article [21], except for the sequence specific primers and probes (Table S2).

2.4. Freezing step

All PCR components were added to a 15 mL centrifuge tube, supplemented with trehalose [10 % final concentration (w/v), Sigma-Aldrich], mannitol [2.5 % final concentration (w/v), Sigma-Aldrich] and polyethylene glycol 20,000 [PEG20000, 1.5 % final concentration (w/v), Sigma-Aldrich].

The mixes were aliquoted into liquid nitrogen with a pipette (Thermo Fisher Scientific) to generate spherical reagents (Fig. 2A). After solidification, the beads were transferred to a metallic tray and stored at −20 °C for 1 h to anneal. The tray containing above beads was then transferred to a freeze dryer (Advantage 2.0, VITRIS) whose shelf was pre-cooled to −40 °C.

2.5. Freeze-drying process

After loading the samples, the shelf temperature of the freeze dryer was maintained at −40 °C for 720 min, followed by 25 °C for 180 min. The chamber pressure of the freeze dryer was maintained at 100 mTorr throughout the freeze-drying process. Once freeze-drying was completed, the dried mix was packaged into an aluminum foil bag using a vacuum packaging machine (DZ-400, Shanghai Hongde Packaging Machinery Co. Ltd, China). The entire freeze-drying process was performed in an environment with a humidity of less than 2 %.

2.6. Karl-Fisher titration

The residual moisture content of the freeze-dried reagents was detected using Karl-Fisher titration, as described in detail in our previous article [21].

2.7. Real-time and accelerated stability testing

The packed freeze-dried PCR mixes were stored at RT (18–25 °C), 37 °C, 56 °C, and 80 °C for a long time. During the long-term preservation, activity detections were performed at multiple time points by reconstituting the mixes to their original volumes with nuclease-free water, followed by qPCR.

High, middle, and low concentrations of samples were employed in this study, whose Cq values were approximately 30, 33, and 36 when detected by qPCR, respectively. Differences between the Cq values of the freeze-dried reagents and freshly-prepared wet reagents (ΔCq) were used to evaluate changes in activity. The activities of the freeze-dried mixes were considered to be acceptable ($\Delta Cq < 1$), altered ($1 \leq \Delta Cq < 10$), or lost ($\Delta Cq \geq 10$) according to their corresponding ΔCq values.

3. Results and discussion

3.1. Transferable and easy-to-use freeze-dried PCR mixes

In this study, a freeze-drying method was established to produce microfluidic applicable PCR reagents using pipettes and liquid nitrogen (Fig. 2A). All components in PCR reagents were mixed together and freeze-dried as a bead, which could be transferred to the microfluidic chip designed for nucleic acid testing in our previous article [24] (Fig. 2B). Besides, it's convenient to use this pre-mixed freeze-dried reagent as the only operation is to reconstitute it with water. And the reconstitution process could be done within 5 s (Fig. 2C).

Besides application in microfluidic molecular diagnostics, this freeze-dried PCR reagent can also bring convenience for laboratory testing. With convenient operating steps, the preparation of liquid-form qPCR reagents is no longer complicated, cumbersome or time-consuming. The pipetting errors [8], errors associated with improper handling of wet reagents, requirements for the operating environment and personnel quality can all be reduced.

In all, the characteristics of the freeze-dried PCR mixes described above are suitable for both microfluidic application and laboratory testing. However, cleanliness of the freeze-dried PCR beads may be influenced when they are transferred by tweezers (Fig. 1B). Our future

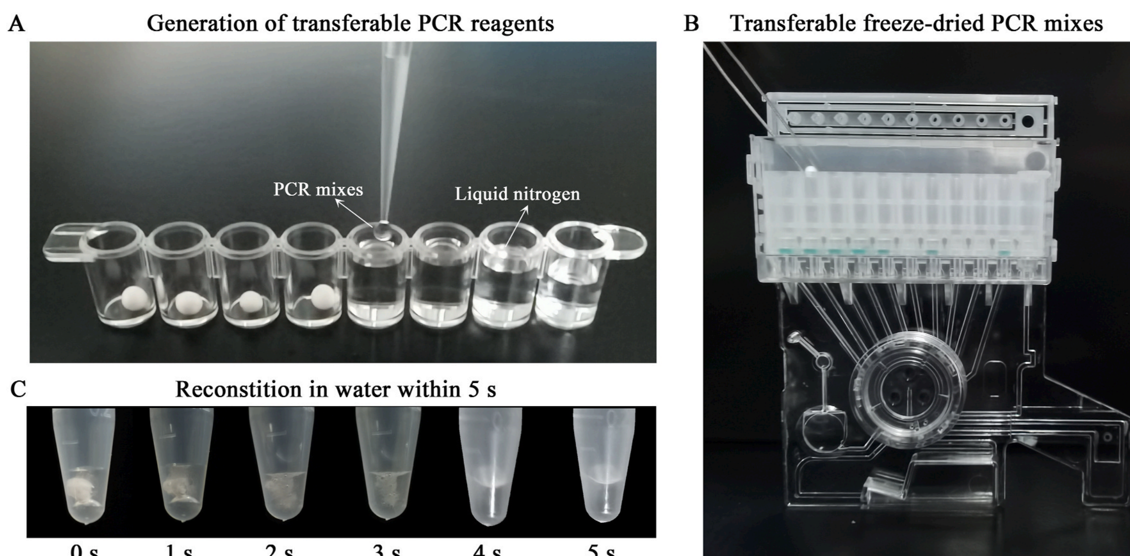

Fig. 2. Characteristics of the freeze-dried PCR mixes suitable for lab and microfluidic application. (A) Generation of spherical PCR reagents by a pipette and liquid nitrogen. (B) The spherical freeze-dried PCR reagents could be transferred to the microfluidic chip designed for nucleic acid testing. (C) The freeze-dried reagents are convenient to use because they only require reconstitution in water, and the reconstitution process can be done in less than 5 s.

researches will focus on developing a specialized method to transfer these beads automatically, through which reagents could be dropped from a clean and sealed bead carrier to the microfluidic chips in turn (Fig. S1).

3.2. Stability of the freeze-dried PCR mixes when stored at RT for 1–2 years

The freeze-dried PCR mixes were then stored at RT for a long time to verify the stability during long-term storage. 19 PCR mixes for different target detection purposes were able to be stored at RT (18–25 °C) for 1–2 years, thus far (Fig. 3), including the reagents for RNA target detection: EV71-1 (569 days, Fig. 3A), EV71-2 (432 days, Fig. 3B), CA16 (312 days, Fig. 3C), HIV-1 (358 days, Fig. 3D), HIV-2 (503 days, Fig. 3E), GAPDH-1 (321 days, Fig. 3F), and GAPDH-2 (358 days, Fig. 3G); and the reagents for DNA target detection: CMV-1 (617 days, Fig. 3H), CMV-2 (362 days, Fig. 3I), CMV-3 (495 days, Fig. 3J), HBV-1 (426 days, Fig. 3K), HBV-2 (426 days, Fig. 3R), *E. coli*-1 (512 days, Fig. 3M), *E. coli*-2 (483 days, Fig. 3N), *E. coli*-3 (483 days, Fig. 3O), *E. coli*-4 (483 days, Fig. 3P), *E. coli*-5 (483 days, Fig. 3Q), ACTB-1 (464 days, Fig. 3R), and ACTB-2 (464 days, Fig. 3S). All tested reagents were able to maintain consistent performance compared with freshly-prepared wet reagents at RT for at least 1–2 years, and their corresponding real-time stability tests at RT remain ongoing.

In above 19 PCR mixes, except for the sequence specific primers and probes (Table S2), the other components, including 10 × PCR Buffer, dNTPs, DNA polymerase, and reverse transcriptase (for RNA target detection only) were all the same. The consistent stability of all the 19 freeze-dried PCR reagents demonstrated that different primers and probes have little influence on the reagent stability and the freeze-drying method described in our study is universal. This conclusion is of great importance: when researchers are going to freeze-dry a new PCR reagent, the only operation required is to provide relevant primers and probes, instead of spending plenty of time optimizing the PCR components, freeze-drying process, lyophilized additive and so on. In an emergency situation, such as the outbreak of coronavirus disease 2019 (COVID-19), this method could allow for pathogen-associated RT-storable PCR reagents to be immediately synthesized, freeze-dried, and distributed.

In the following experiments, reagent EV71-1 was selected as a representative of the reagents stored for RNA target detection because the storage period for this mix is currently the longest (569 days, Fig. 3A). Similarly, reagent CMV-1 was used as a representative mix for DNA target detection due to having the longest storage period (617 days, Fig. 3H).

3.3. Selection of temperatures for accelerated stability testing

Before the accelerated stability testing was employed to shorten the stability testing period, appropriate temperatures were chosen. Because the packaging consumables could not withstand temperatures above 200 °C, temperatures of 60, 80, 100, 120, 140, 160, 180, and 200 °C were chosen to determine the highest temperature that could be used for testing.

When the freeze-dried PCR bead (Fig. 4Ai) was stored at 100 °C or higher, it shrank rapidly (Fig. 4Aii). Then, the bead gradually turned from white to yellow (Fig. 4Aiii) and finally brown (Fig. 4Aiv). These changes occurred faster at higher storage temperatures (Fig. 4B), and led to loss of reagent activity. By contrast, no obvious changes in appearance or activity were observed when the bead was stored at 60 °C or 80 °C for more than 12 h. Thus, 80 °C was chosen as the highest temperature.

To obtain comprehensive evaluation results, 37 °C and 56 °C were also examined, in addition to 80 °C and RT. Therefore, the temperature gradient selected for accelerated stability testing was RT, 37 °C, 56 °C, and 80 °C.

3.4. Shortened evaluation period for the freeze-dried PCR mix

Accelerated stability testing was further performed following storage at the selected temperatures (37 °C, 56 °C, and 80 °C) to verify the feasibility of shortening the evaluation period. The freeze-dried PCR mixes for RNA target detection could be stored at 37 °C, 56 °C, and 80 °C for 99 days, 6 days, and 1 day, respectively (Fig. 5A and Fig. S2). The mixes for DNA target detection could be stored at 37 °C, 56 °C, and 80 °C for 617 days, 26 days, and 21 days, respectively (Fig. 5B and Fig. S2). These results indicated that the evaluation process for freeze-dried PCR mixes could be accelerated using higher temperatures: for the reagents used for RNA detection, 99 days at 37 °C, 6 days at 56 °C or 1 day at 80 °C would be equivalent to 567 days at RT. Similarly, 26 days at 56 °C or 21 days at 80 °C of reagents for DNA detection would be equivalent to 617 days at RT or even 37 °C.

In addition, the higher the temperature was employed, the shorter

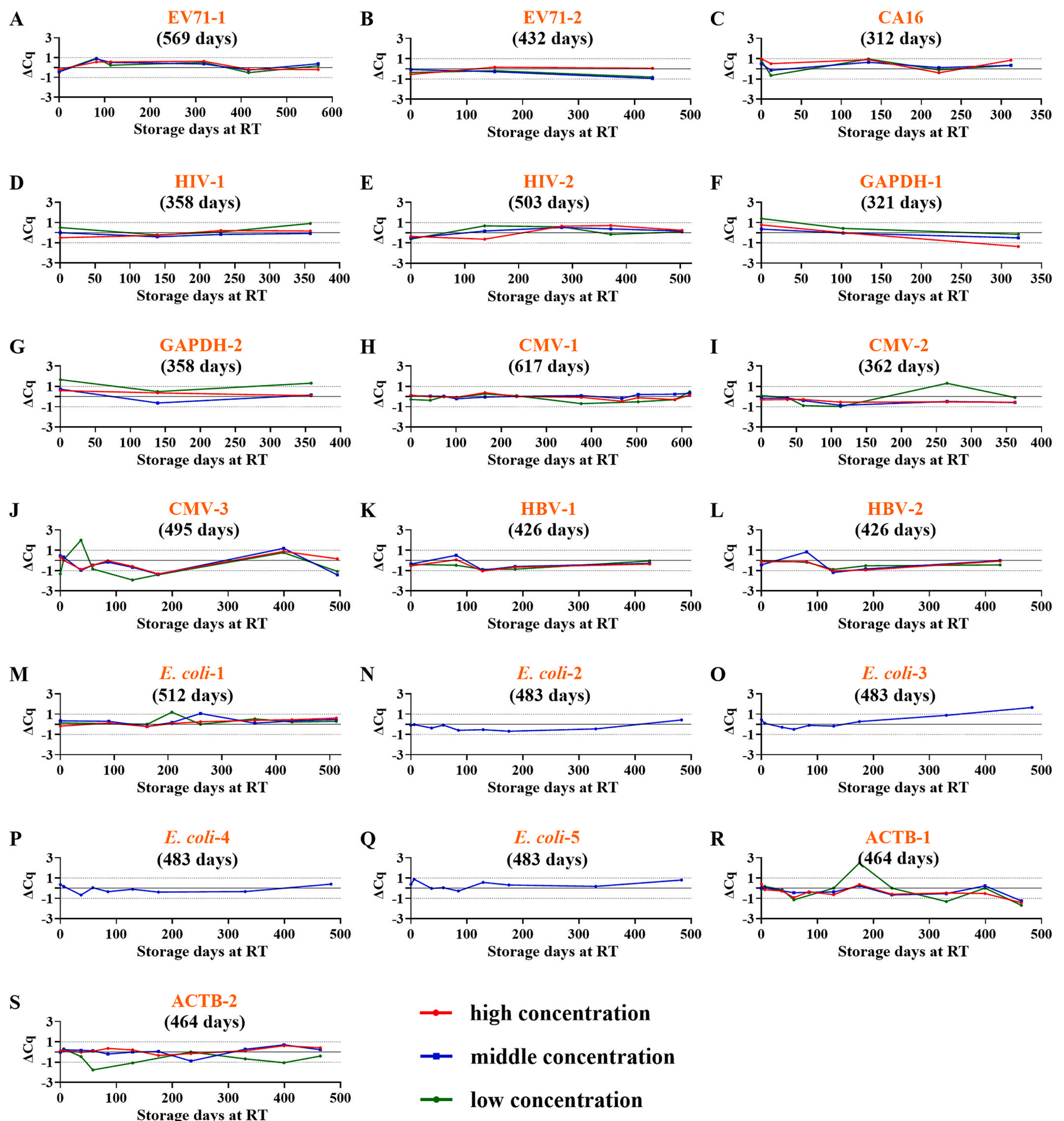

Fig. 3. Activity changes for freeze-dried PCR mixes used for different target detection purposes over the tested storage period at RT. (A) EV71-1; (B) EV71-2; (C) CA16; (D) HIV-1; (E) HIV-2; (F) GAPDH-1; (G) GAPDH-2; (H) CMV-1; (I) CMV-2; (J) CMV-3; (K) HBV-1; (L) HBV-2; (M) *E. coli*-1; (N) *E. coli*-2; (O) *E. coli*-3; (P) *E. coli*-4; (Q) *E. coli*-5; (R) ACTB-1; (S) ACTB-2. Reagents GAPDH-1 and GAPDH-2 were designed to detect the mRNA transcribed from the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in the HuH-7 cell line. Reagents ACTB-1 and ACTB-2 were designed to detect the β-actin (ACTB) gene in the HuH-7 cell line. *: activity of the lyophilized reagent is preserved and requires further observation.

the evaluation process lasted (Fig. 5A–B). When 37 °C was used for accelerated stability testing, the freeze-dried PCR mixes were relatively stable. The evaluation period of reagent for RNA target detection could only be reduced to 99 days, and the one of reagent for DNA target detection was even longer than 617 days. This indicated that 37 °C in accelerated stability testing was not adequate to shorten the evaluation period. On the contrary, the evaluation periods of the freeze-dried PCR mixes could all be shortened to less than one month when stored at 56 °C and 80 °C.

To our knowledge, few studies use 56 °C to perform accelerated stability testing for freeze-dried PCR mixes, and this represents the first study to use 80 °C to perform the same test. However, reagents are more prone to exhibit instability when stored at higher temperatures (Fig. S2). Under the circumstances, subtle variations among different reagents

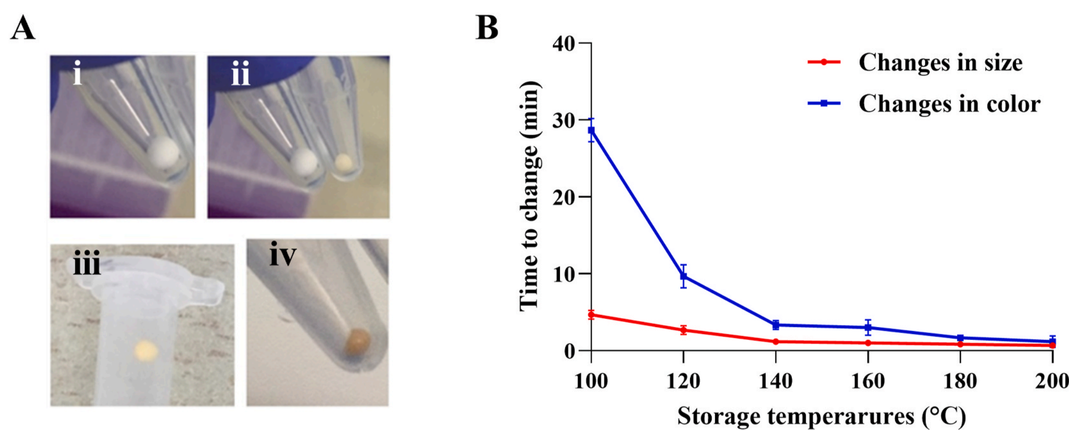

Fig. 4. Selection of appropriate temperatures for accelerated stability testing. (A) Appearance changes over time in freeze-dried beads when stored at 100 °C or higher. (i) The initial freeze-dried bead. (ii) The bead became small. (iii) The bead turned yellow. (iv) The bead became brown. (B) Time when appearances of the freeze-dried beads were changed at different storage temperatures. N = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

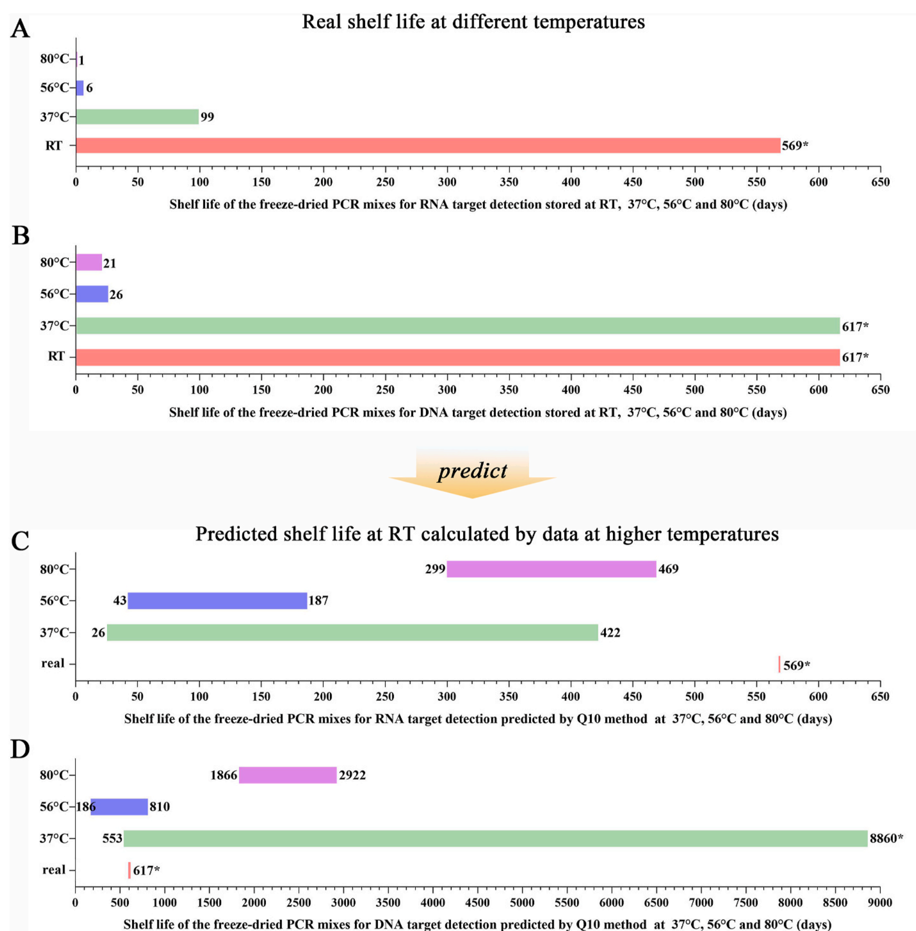

Fig. 5. Shelf lives of freeze-dried PCR mixes. (A and B) Real-world shelf lives of the PCR mixes for RNA (A) or DNA (B) target detection when stored at RT, 37 °C, 56 °C, and 80 °C. In A and B, only the last day when the reagents still remained active was chosen to match between different conditions. For results with multiple time points, please refer to Fig. 3A and H (stored at RT), and Fig. S2 (stored at 37 °C, 56 °C, and 80 °C). (C and D) Predicted shelf lives for PCR mixes for RNA (C) or DNA (D) target detection as calculated by the Q_{10} method. "real" on the Y-axis represents the actual shelf life of the reagents stored at RT. "37 °C," "56 °C," and "80 °C" on the Y-axis represent the predicted shelf lives calculated by the Q_{10} method based on the accelerated stability testing data at the corresponding temperature.

maybe indistinguishable as inconsistent results might be caused by above instabilities. Low temperatures have the opposite effect. To obtain more reliable and comprehensive results, an ideal strategy would include the evaluation of various temperatures for accelerated stability testing.

3.5. Predicted shelf lives of freeze-dried PCR mixes using the Q10 method

To get rid of performing real-time stability testing at RT, attempts have been made to predict the shelf lives of freeze-dried reagents directly, using mathematical models. However, in fact, no mathematical method was specially designed and developed to predict the shelf life of freeze-dried PCR reagents, and their accuracy has not been verified up to now. In this section, we would verify the feasibility and accuracy of the

most widely used mathematical model, the Q_{10} method [17,18] in attempts to determine the shelf lives of PCR reagents.

In the Q_{10} method, the relationship between the shelf life of a biological material at RT and that at higher temperatures is calculated as follows [25]:

$$Shelf\ life = S_x \times \left(1 + Q_{10}^{\frac{T_X - T_{RT}}{10}}\right)$$

where $T_{RT} = 25$ °C; $Tx = X$°C (That is, $T_{37} = 37$ °C, $T_{56} = 56$ °C, $T_{80} = 80$ °C); S_x = the accelerated shelf life at higher temperatures (T_X); Q_{10} = the temperature coefficient. For most biological reagents, Q_{10} ranges from 1.8 to 3 [17,18,26], and a lower (higher) Q_{10} is associated with a shorter (longer) estimated shelf life. Thus, $Q_{10} = 1.8$ and $Q_{10} = 3.0$ were employed together to predict the shelf life ranges of the freeze-dried PCR mixes used in our study.

For the freeze-dried PCR reagents intended for RNA target detection, the shelf lives were predicted to be 299–469 days, 43–187 days, and 26–422 days, based on the shelf lives when stored at 37 °C, 56 °C, and 80 °C, respectively (Fig. 5C). For the freeze-dried PCR reagents intended

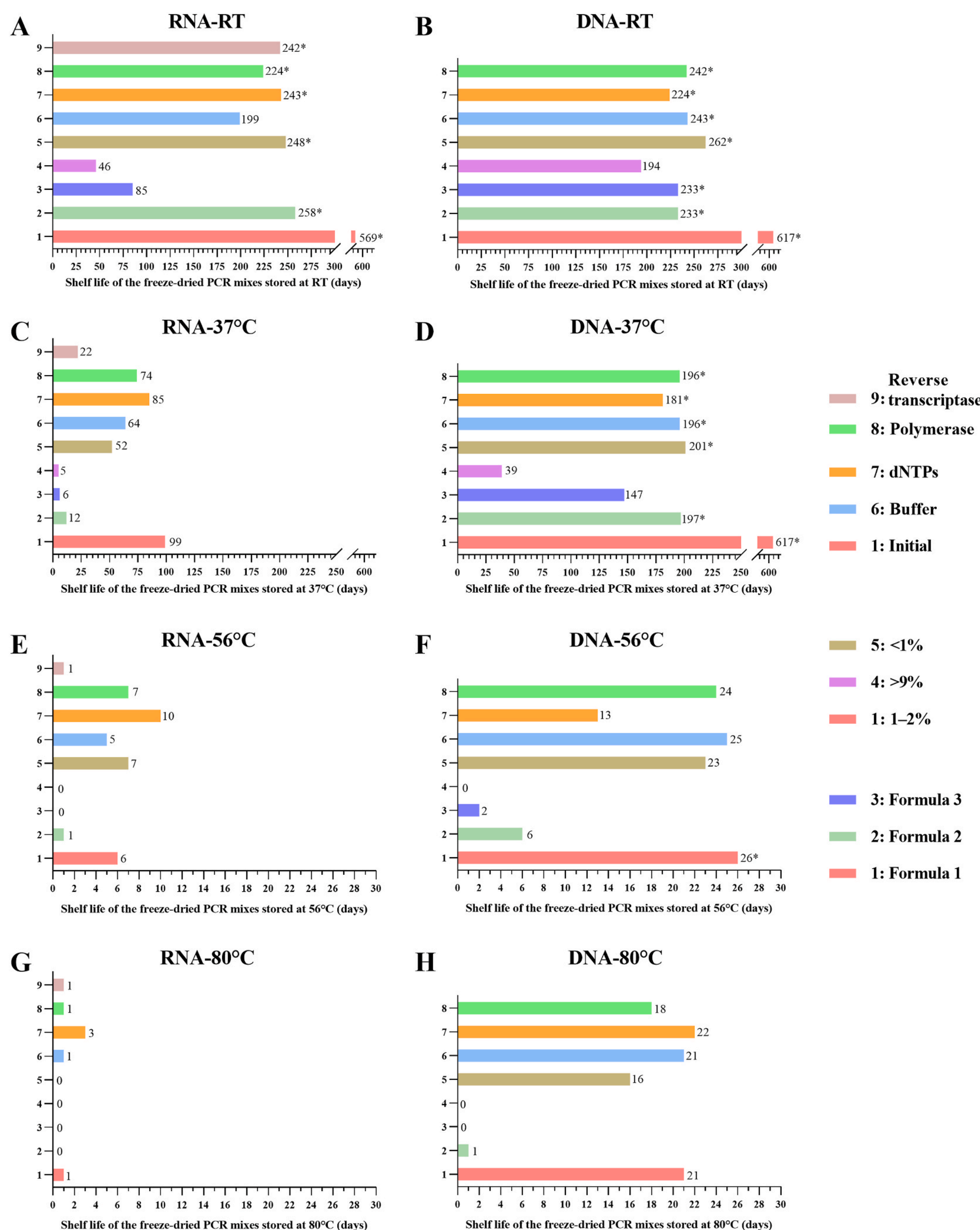

Fig. 6. Storage days of various freeze-dried PCR mixes stored at RT, 37 °C, 56 °C, and 80 °C. (A and B) Reagents for RNA (A) or DNA (B) target detection stored at RT. (C and D) Reagents for RNA (C) or DNA (D) target detection stored at 37 °C. (E and F) Reagents for RNA (E) or DNA (F) target detection stored at 56 °C. (G and H) Reagents for RNA (G) or DNA (H) target detection stored at 80 °C. In each panel, Row 1, Row 2, and Row 3 represent reagents generated using Formula 1, Formula 2, and Formula 3, respectively; Row 1, Row 4, and Row 5 represent PCR mixes with >9 %, 1–2%, and <1 % of residual moisture contents, respectively; Row 1, Row 6, Row 7, Row 8, and Row 9 represent PCR reagents containing different components (the initial reagents replaced by none, buffer, dNTPs, polymerase and reverse transcriptase, respectively). *: the activity of the lyophilized reagent is preserved and requires further observation. For more detailed information about results with multiple time points, please refer to Fig. S3 in the supplementary file.

for DNA target detection, the shelf lives were predicted to be 1866–2922 days, 186–810 days, and 553–8860 days, respectively, based on the shelf lives when stored at 37 °C, 56 °C, and 80 °C (Fig. 5D). Thus, this method was convenient and time-saving to predict the shelf lives of freeze-dried PCR mixes without performing real-time stability tests at RT.

However, some of the shelf-life ranges predicted using the Q_{10} method did not match the real-time stability testing results. For example, the predicted shelf lives for freeze-dried RNA reagents based on the shelf lives at different temperatures were all shorter than the actual RT shelf life (Fig. 5C). In addition, the predicted ranges were so wide and general that they did not provide sufficiently precise information. For example, the shelf life for RNA reagents was predicted in the range of 553–8860 days based on the outcome at 80 °C; however, whether any reagent remained active could not be determined after storage for 2000 days (Fig. 5D). Therefore, this method appears to have limited utility for the accurate prediction of the shelf life of freeze-dried PCR reagents.

To our knowledge, this is the first study focused on verifying the feasibility of using the Q_{10} method to predict the shelf life of freeze-dried PCR reagents. Although this method was time-saving, it was limited in actual usage because the predictions (summarized in Table S3) were wide and inaccurate, which may be due to the potential influences of various factors on the complex prediction process.

3.6. Factors that influence the prediction of shelf life

To explore factors that might affect the predicted shelf life of freeze-dried PCR mixes, various freeze-drying parameters were introduced, including different combinations of lyophilized additives [Formula 1 (the initial combination), Formula 2, and Formula 3 (Table S4),]; residual moisture contents of PCR mixes [>9 %, 1–2% (the initial residual moisture content), and <1 % (these moisture contents were obtained by freeze-drying the PCR mixes at 25 °C for 0 min, 180 min and 300 min) (Table S5),]; and PCR components [Initial (the initial PCR reagents), Buffer (the buffer in the initial PCR reagents was replaced by another one, similarly hereinafter), dNTPs, Polymerase, and Reverse transcriptase (Table S6)]. PCR regents freeze-dried with above various parameters were then stored at different temperatures and detected with middle concentration of samples at multiple time points.

The corresponding results (Fig. 6 and Fig. S3) showed that these freeze-dried regents had inconsistent shelf lives in most cases, either stored at RT or higher temperatures. Overall, lyophilized additives and residual moisture contents had greater impacts on the stability of freeze-dried PCR reagents compared to PCR components. Besides, there were also differences within the same class of parameters. For example, the stability of the freeze-dried PCR reagents containing different residual moisture contents, from high to low, in most cases, were 1–2%, <1 % and >9 %.

When attempts were further tried to relate the storage periods of these various factors at different temperatures, one conclusion could be identified was that reagents with more stability at RT showed longer shelf lives at higher temperatures in most cases. However, there were no obvious mathematical relations between the elevated temperatures and shortened storage days. What's worse, comparisons of shelf lives at different temperatures may come to the opposite conclusion. For DNA target detection, the reagent replaced by another kind of dNTPs had longer storage days than the reagents replaced by another kind of buffer or polymerase when stored at 56 °C, whereas the latter had longer shelf lives when stored at 80 °C.

These results indicated that different freeze-drying parameters would affect the shelf lives of freeze-dried reagents to varying degree, which were reagent specific and unpredictable. In addition, as the mathematical formula such as the Q_{10} method was unable to differentiate the various influencing factors, the individual characteristics of all the freeze-dried PCR reagents cannot be conveyed through a simple mathematical calculation process.

4. Conclusion

This study presented a transferable, easy-to-use and RT-storable PCR mix to meet the requirements of microfluidic molecular diagnosis. The manufacture method is universal as 19 PCR mixes for different DNA and RNA targets detection could be stored at RT for 1–2 years. This is of great importance when researchers are going to freeze-dry a new PCR reagent, as the only operation required is to provide relevant primers and probes. Accelerated stability testing at higher temperatures was also proposed to shorten the evaluation periods to less than 1 month. We have discussed the pros and cons of different temperatures used in accelerated stability testing for freeze-dried PCR mixes. When attempts were further tried to predict the shelf lives for freeze-dried PCR mixes, our findings challenged the classic view of the Q_{10} method as a prediction model for freeze-dried PCR mixes and confirmed for the first time that this prediction was influenced by different factors (lyophilized additives, residual moisture contents, and PCR reagents) at varying degrees. These studies and findings are important to promote burgeoning microfluidic molecular diagnoses in the future.

Credit author statement

Jiasu Xu: Methodology, Formal analysis, Writing – original draft. Jin Wang: Conceptualization, Writing – original draft. Xiaosong Su: Data curation. Guofu Qiu: Resources. Qiurong Zhong: Formal analysis. Tingdong Li: Writing – review & editing. Dongxu Zhang: Writing – review & editing. Shiyin Zhang: Conceptualization, Validation, Writing – review & editing. Shuizhen He: Validation, Writing – review & editing. Shengxiang Ge: Supervision. Jun Zhang: Supervision. Ningshao Xia: Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122797.

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