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Article

A practical approach for quantitative polymerase chain reaction, the gold standard in microbiological diagnosis

Tímea Mosolygó [1,2,†], Krisztián Laczi [3,†], Gabriella Spengler [1,2,*] and Katalin Burián [1,2]

[1] Department of Medical Microbiology, Albert Szent-Györgyi Health Center and Faculty of Medicine, University of Szeged, Hungary; office.omi@med.u-szeged.hu
[2] MTA-SZTE Microbiology and Health Education Research Group
[3] Department of Biotechnology, University of Szeged, Szeged, Hungary
* Correspondence: spengler.gabriella@med.u-szeged.hu; Tel.: +36-62-545-115
† These authors have contributed equally

Abstract: From gene expression studies to identifying microbes quantitative polymerase chain reaction (qPCR) is widely used in research and medical diagnostics. In transmittable diseases like the Ebola outbreak in West Africa (2014-2016), or the present SARS-CoV2 pandemic qPCR plays a key role in the detection of infected patients. Although the technique itself is decades old with reliable approaches (eg. TaqMan essay) in the diagnosis of pathogens many people showed distrust in it during the SARS-CoV2 outbreak. This came mainly from not understanding or misunderstanding the principles of qPCR. This situation motivated us to design a simple laboratory practical class, in which students have opportunities to understand the underlying principles of qPCR and its advantages in microbiological diagnosis. Moreover, during the exercise, students can develop skills such as handling experimental assays, and the ability to solve problems, discuss their observations. Finally, this activity brings them closer to the clinical practice and they can see the impact of the science on real life. The class is addressed to undergraduate students of biological sciences.

Keywords: laboratory practical class; undergraduate teaching; qPCR

1. Introduction

The Polymerase Chain Reaction (PCR) was invented by Kary Mullis in 1983 and was first used by the team of Cetus Corporation [1]. Since then, PCR technology has undergone a huge development and it has become one of the most valuable and reliable methods used in diagnostics and bioscience. From the original end-point PCR two distinct technologies have emerged for the quantification of nucleic acid concentration. The quantitative PCR (qPCR), which is also known as real-time PCR, and the digital PCR (dPCR). All three technologies are based on the amplification of DNA with thermostable DNA dependent DNA polymerase under 20-40 heat cycles. Each cycle starts with the denaturing of the DNA followed by the annealing of the oligonucleotide primers and finally the elongation of the new strand. The theoretical product number at the end is the initial number of DNA molecules × 2^n where n is the number of cycles. The main difference between the three methods is the way the product is detected. In a traditional end-point PCR the product can be detected with gel electrophoresis and the amount of DNA is determined semi-quantitatively based on the intensity of fluorescence in the gel, therefore it is not suitable for quantification. In contrast, the qPCR can follow the concentration changes in real-time by registering the level of fluorescence after every cycle. This allows the quantitation of genes, transcripts (cDNA) and microbes as well. The dPCR on the other hand kept the end-point detection but it breaks down the reaction into hundreds or even thousands of micro reactions on microwell chips or in droplets. Every well/droplet contains exactly one or zero DNA molecules. After amplification, the positive wells/droplets are counted based on a fluorescent signal, thus the original copy

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number of the sample can be determined without outer standards [2]. Although dPCR has been found superior in precision and efficiency compared to qPCR and even the price/sample is lower for certain platforms dPCR is more time consuming and labour-intensive, the dynamic range is lower than in qPCR and there is an issue with false positives in low input concentration and non-template samples [3]. Therefore qPCR is still preferred in clinical diagnostics and became the gold standard of microbiological detection and quantification [4].

The qPCR operates with fluorescent dyes. As the product number increases cycle by cycle the emitted fluorescent light becomes stronger. The increasing fluorescence can be described with a sigmoid curve. When the signal reaches the lower detection limit of the instrument, the fluorescence starts to increase exponentially. At the upper detection limit, the curve goes into saturation. Where the curve crosses the threshold line the threshold cycle or quantification cycle (C_t or C_q) is defined. The threshold line is set by three rules: the threshold should be 1) above the background noise, 2) on the log phase undisturbed by the plateau, 3) at a point where all amplification curves are parallel. The C_t value is proportional to the initial template concentration [5].

There are two different types of qPCR the intercalating dye-based and probe-based. The probe-based qPCR approach uses target-specific oligonucleotide probes labelled with a fluorescent dye and a quencher molecule. This method is highly specific for the light is emitted only when the probe can hybridize to the target sequence between the primers. Therefore probe-based qPCR is considered a gold standard in microbial diagnostics [6].

The intercalating fluorescent dyes such as SYBR® Green, SYTO dyes, EvaGreen® etc. emit light when bound to the double-stranded DNA and illuminated with UV light [7]. Although this technique is cost-effective compared to the probe-based qPCR the design of the oligonucleotide primers should be carried out carefully. False products like primer dimers may generate bias in quantification. Melting point analysis can indicate the artefact's presence in the samples and the reaction or oligonucleotides can be optimized accordingly. A computational method to correct qPCR results with the help of melting curves has recently been proposed [8].

The outbreak of SARS-CoV-2 sped up the development of new systems using qPCR, e.g. in May of 2020, there were 81 kits and systems approved by the US FDA. Several low-cost intercalating dye-based methods for SARS-CoV2 diagnosis were published to overcome the financial struggle and elevate the throughput of virus diagnostics [9,10]. In the case of SARS-CoV-2 PCR assays targeting ORF1a, ORF1b, S and N genes can detect less, than 10 genome equivalents [11].

In an attempt to facilitate students' understanding of the basic principles of qPCR, we designed a simple exercise for the evaluation of DNA concentration in biological samples. For its cost-effectiveness, we chose the fluorescent dye-based qPCR method to perform this practical class. Although in the diagnosis of SARS-CoV-2 infection reverse transcription-coupled qPCR (RT-qPCR) is used, since this virus possesses RNA genome, in our designed protocol DNA was used as templates, due to the instability of RNA molecules. The goals of this laboratory practical class are for high school students to learn how to perform qPCR and to represent the results obtained in graphs.

2. Materials and Methods

2.1. Biological material

Escherichia coli K-12 (ATCC 10798) was cultivated in Lysogeny Broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 37 °C shaken at 160 rpm until OD_{600}=1 (~$8x10^8$ cells/ml). The cells were collected by centrifugation (10000 x g, 10 minutes) and concentrated 1.25 times in normal saline solution (9 g/l NaCl) resulting in a cell concentration of ~10^9 cells/ml.

2.2. Isolation of DNA

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DNA from 1 ml of the resuspended culture was extracted with Qiagen DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.3. Serial dilution

An eight-step serial dilution with a scale of tens was performed on the purified DNA. The most concentrated sample corresponds to 10^9 cells/ml while the most diluted corresponds to 10^2 cells/ml.

2.4. Quantitative polymerase chain reaction

The concentration of the *E. coli* in each dilution was analysed via qPCR. For the detection of the *uidA* gene, the following primers were used: 5′-CAACGAACTGAACTGGCAG-3′ and 5′-CATTACGCTGCGATGGAT-3′ [12,13]. All primers were synthesized by Integrated DNA Technologies Inc. (Montreal, Quebec, Canada). The qPCR was conducted with the extracted and diluted DNA, primers (10 pmol/µl) and SYBR® Green JumpStart™ Taq ReadyMix (Merck KGaA, Darmstadt, Germany) in a total volume of 20 µl, with a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Thermal cycling was initiated with a denaturation step of 10 min at 95 °C, followed by 40 cycles each of 5 sec at 95 °C, 20 sec at 60 °C and 25 sec at 72 °C. Cycle threshold (C_t) values were determined by automated threshold with Bio-Rad CFX Maestro Software version 2.2.

2.5. Exercise design

The first step in this laboratory session was to divide the students into 4 groups, where each group consisted of 3-4 students. Each group got 1 ml of *E. coli* suspension (10^9 cells/ml), then the students carried out four exercises. First, they extracted DNA from the original suspension of *E. coli*. In the second step, they serially diluted the purified DNA, then they performed the qPCR. In the last step, the students involving the instructor presented and discussed their results obtained from the qPCR graph.

2.6. Safety considerations

At the beginning of the laboratory session, students were briefly informed about the safety rules associated with working with biological samples, in order to avoid accidental contamination. Besides, students are informed about laboratory waste disposal and introduced to the location of the nearest fire extinguisher and first-aid kit. Disposable gloves and a laboratory coat must be worn in the laboratory.

3. Results

The exercises designed were assigned to 4 groups of students. The workflow of the laboratory procedure is summarised in Figure 1. Students in each group extracted the DNA from the original *E. coli* suspension, which corresponds to 10^9 bacterial cells. Then they serially diluted the purified DNA and carried out the qPCR experiments using each dilution. The groups used the primers targeting the *uidA* gene (coding for beta-glucuronidase) and amplify a 121 bps long part of the gene.

Figure 2 shows the C_t values obtained by all groups and the calibration curve calculated from the results. Groups 2 and 4 did not have amplification in the lowest DNA concentration sample. Group 1 had a higher C_t value in the first and third reactions and an abnormal amplification curve. These 4 samples were disclosed from further analysis and are not indicated in Fig. 2. The mean C_t value of undiluted DNA samples (10^9) was 7.08±0.52, while the lowest concentrated samples (10^2) reached the threshold with 31.07±0.06 C_t. On average 3.39 cycles were between the neighbouring dilutions in the same series. This value is close to the theoretical 3.32 cycles difference between the elements of a 10 times dilution series. There was a slight difference between the samples of the groups for the C_t value of the same dilution varies with an average of 0.87 cycles. This deviation between the parallels might be caused by the pipetting error of distinct

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students. The correlation between DNA concentration and cycle number was strong (R^2=0.993) and all parallels fit the trendline well. Groups 3 and 4 measured slightly lower C_t values for the same DNA concentration compared to groups 1 and 2 due to pipetting errors. Although all the data was appropriate building the calibration curve with the equation y= -3.420x+38.079 (Figure 2B). At the end of the laboratory exercise, the students discussed their results and interpreted the calibration curve. To improve their understanding of the main point of the experiment students had to estimate the number of bacterial cells of unknown samples with the aid of the equation of the line.

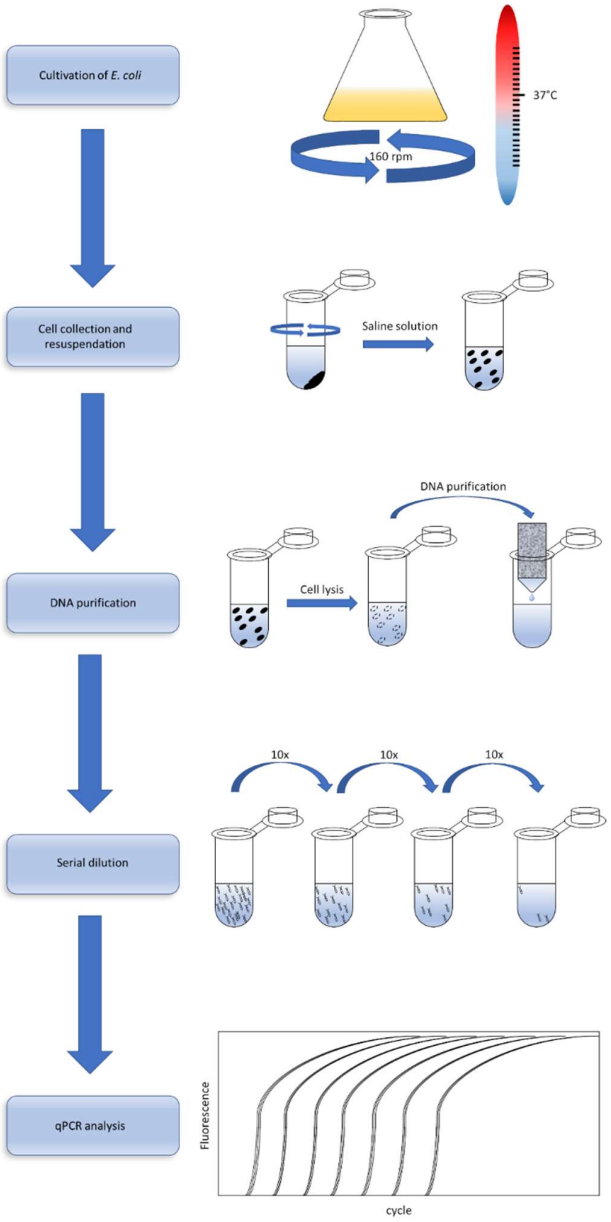

Figure 1. Workflow of the laboratory exercises.

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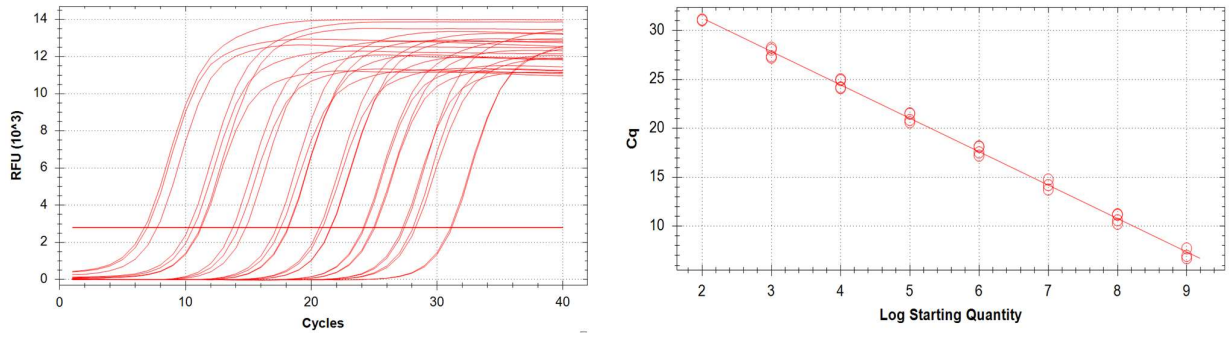

(**a**) (**b**)

Figure 2. Results of the qPCR experiment. The sigmoid curves of the amplification are shown on panel (**a**). RFU: relative fluorescence units The horizontal red line represents the threshold. The calibration curve obtained from the dilution series is shown on panel (**b**). R^2=0.993 and Log Starting quantity equals the theoretical cell number of the samples.

4. Discussion

Emerging and reemerging infections are global public health concerns. Accurate laboratory testing of the causative agent is essential for early discovery, isolation and treatment, in order to cut off the transmission route. The outbreak of SARS-CoV-2 draws tremendous attention to the importance of clinical microbiology and the different molecular and serological methods, such as rapid antigen tests, PCR and evaluation of the serum antibody levels. Viral RNA can be detected in the upper and lower respiratory tract, stool, blood and urine of SARS-CoV-2 infected patients. Due to its sensitivity and specificity of qPCR is the preferred and most widely used method for detecting the presence of viral nucleic acid in these samples [14]. Collective understanding of qPCR's basic principles is essential to increase trust in clinical diagnostics and pull out the venom of sceptic voices who spread disinformation out of profit or gullibility.

Unfortunately, the introduction of different molecular methods to undergraduate students in biology class is hampered by the lack of equipment and the cost of the reagents. Moreover, the extremely rapid development of science led to the fact that relatively few biology teachers have practical experience of DNA techniques during their training.

This situation motivated us to design a simple laboratory practical class, in which students have opportunities to understand the underlying principles of qPCR and its advantages in microbiological diagnosis. Through this activity, students can perform DNA extraction from *E. coli* and carry out qPCR amplification, which are routine diagnostical tools in clinical microbiology. Moreover, during the exercise, students can develop skills such as handling experimental assays, and the ability to solve problems, discuss their observations. Finally, these exercises provide not only insight into the laboratory work, but also connect theory to practice and stimulate interest and enjoyment of science.

At the end of the class, the student should be able to conclude that qPCR can be used for the detection of nucleic acid in clinical samples, and the C_t value negatively correlates with the number of the given microorganism. The designed experiment can be performed over one laboratory class of 4 h or it can be divided into 3 sessions: 1) isolation of the DNA, 2) dilution and PCR assay, 3) interpretation of the results.

The presented protocol was successfully implemented in a microbiological laboratory course held for undergraduate students. The obtained results were appropriate building the calibration curve, only 4 samples were disclosed, due to abnormal amplification.

This practical class can be extended to introduce additional molecular diagnostical methods, such as isolation of RNA, multiplex qPCR or RT-qPCR, where there is a reverse transcription step before the qPCR. Moreover, melting point analysis, which is im-

portant in the specificity of the intercalating dye-based assay also can be discussed during the class.

5. Conclusions

The laboratory exercises presented in this study promotes active learning about the impact of molecular methods and provides students with an opportunity to develop practical skills in the field of laboratory work. Finally, this activity brings the undergraduate students closer to the clinical practice and they can see the importance of science.

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References

1. Saiki, R.; Gelfand, D.; Stoffel, S.; Scharf, S.; Higuchi, R.; Horn, G.; Mullis, K.; Erlich, H. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. *Science* **1988**, *239*, 487–491, doi:10.1126/science.2448875.
2. Dong, L.; Meng, Y.; Sui, Z.; Wang, J.; Wu, L.; Fu, B. Comparison of Four Digital PCR Platforms for Accurate Quantification of DNA Copy Number of a Certified Plasmid DNA Reference Material. *Sci Rep* **2015**, *5*, 13174, doi:10.1038/srep13174.
3. Bosman, K.J.; Nijhuis, M.; van Ham, P.M.; Wensing, A.M.J.; Vervisch, K.; Vandekerckhove, L.; De Spiegelaere, W. Comparison of Digital PCR Platforms and Semi-Nested QPCR as a Tool to Determine the Size of the HIV Reservoir. *Sci Rep* **2015**, *5*, 13811, doi:10.1038/srep13811.
4. Kralik, P.; Ricchi, M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front. Microbiol.* **2017**, *8*, doi:10.3389/fmicb.2017.00108.
5. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **2009**, *55*, 611–622, doi:10.1373/clinchem.2008.112797.
6. Navarro, E.; Serrano-Heras, G.; Castaño, M.J.; Solera, J. Real-Time PCR Detection Chemistry. *Clin Chim Acta* **2015**, *439*, 231–250, doi:10.1016/j.cca.2014.10.017.
7. Gudnason, H.; Dufva, M.; Bang, D.D.; Wolff, A. Comparison of Multiple DNA Dyes for Real-Time PCR: Effects of Dye Concentration and Sequence Composition on DNA Amplification and Melting Temperature. *Nucleic Acids Research* **2007**, *35*, e127, doi:10.1093/nar/gkm671.
8. Ruijter, J.M.; Ruiz-Villalba, A.; van den Hoff, A.J.J.; Gunst, Q.D.; Wittwer, C.T.; van den Hoff, M.J.B. Removal of Artifact Bias from QPCR Results Using DNA Melting Curve Analysis. *The FASEB Journal* **2019**, *33*, 14542–14555, doi:10.1096/fj.201901604R.
9. Zhu, H.; Zhang, H.; Xu, Y.; Laššáková, S.; Korabečná, M.; Neužil, P. PCR Past, Present and Future. *Biotechniques* 10.2144/btn-2020–0057, doi:10.2144/btn-2020-0057.
10. Marinowic, D.R.; Zanirati, G.; Rodrigues, F.V.F.; Grahl, M.V.C.; Alcará, A.M.; Machado, D.C.; Da Costa, J.C. A New SYBR Green Real-Time PCR to Detect SARS-CoV-2. *Sci Rep* **2021**, *11*, 2224, doi:10.1038/s41598-021-81245-0.
11. Chu, D.K.W.; Pan, Y.; Cheng, S.M.S.; Hui, K.P.Y.; Krishnan, P.; Liu, Y.; Ng, D.Y.M.; Wan, C.K.C.; Yang, P.; Wang, Q.; et al. Molecular Diagnosis of a Novel Coronavirus (2019-NCoV) Causing an Outbreak of Pneumonia. *Clinical Chemistry* **2020**, *66*, 549–555, doi:10.1093/clinchem/hvaa029.
12. Abberton, C.L.; Bereschenko, L.; van der Wielen, P.W.J.J.; Smith, C.J. Survival, Biofilm Formation, and Growth Potential of Environmental and Enteric Escherichia Coli Strains in Drinking Water Microcosms. *Appl Environ Microbiol* **2016**, *82*, 5320–5331, doi:10.1128/AEM.01569-16.

13. Machado-Moreira, B.; Monteiro, S.; Santos, R.; Martinez-Murcia, A.; Rajkovic, A.; Smigic, N.; Richards, K.G.; Abram, F.; Burgess, C.M. Impact of Beef Extract Used for Sample Concentration on the Detection of Escherichia Coli DNA in Water Samples via QPCR. *Journal of Microbiological Methods* **2020**, *168*, 105786, doi:10.1016/j.mimet.2019.105786.
14. Zhang, W.; Du, R.-H.; Li, B.; Zheng, X.-S.; Yang, X.-L.; Hu, B.; Wang, Y.-Y.; Xiao, G.-F.; Yan, B.; Shi, Z.-L.; et al. Molecular and Serological Investigation of 2019-NCoV Infected Patients: Implication of Multiple Shedding Routes. *Emerging Microbes & Infections* **2020**, *9*, 386–389, doi:10.1080/22221751.2020.1729071.