Benchmark

BioTechniques

Agarose gel electrophoresis to assess PCR product yield: comparison with spectrophotometry, fluorometry and qPCR

Patrick Wittmeier[1] & Susanne Hummel*,[1]

[1]*Department of Historical Anthropology & Human Ecology, University of Göttingen, Bürgerstraße 50, 37073, Göttingen, Germany; *Author for correspondence: Tel.: +49 551 39 29728; shummel1@gwdg.de*

BioTechniques 72: 155–158 (April 2022) 10.2144/btn-2021-0094

First draft submitted: 24 September 2021; Accepted for publication: 7 March 2022; Published online: 21 March 2022

ABSTRACT

Agarose gel electrophoresis is a relatively easy to use method, commonly applied to evaluate PCR reaction success. Intercalating agents or dyes are used to visualize the amplified fragments. However, it is uncertain to what extent the brightness of bands is informative about the concentration of the amplicons. To more closely examine the suitability of agarose gel electrophoresis to assess PCR product yield, we quantified the brightness of bands on a gel and compared these data with the results from spectrophotometry, fluorometry and qPCR. Evaluation of the results suggests that assessment of the relative quantity of amplicons by band brightness is precise enough even for post-PCR analysis steps requiring PCR product concentrations within a certain range to function properly.

KEYWORDS:

agarose gel electrophoresis ● fluorometry ● PCR product quantification ● quantitative real-time PCR ● spectrophotometry

The assessment of PCR product yield can be necessary when working with DNA, as some post-PCR analysis steps, such as sequencing or fragment length analysis via capillary electrophoresis, require PCR product amounts within a certain range to function properly. Different quantification methods are available to determine the concentration of nucleic acids within a solution, for example spectrophotometry, fluorometry or qPCR [1–3]. Another method to estimate PCR product yield can be agarose gel electrophoresis. This is a relatively inexpensive and easy to use method, originally introduced nearly 50 years ago independently for the separation of topoisomers of mtDNA and the separation of restriction enzyme DNA fragments [4]. Agarose gel electrophoresis is also commonly applied to assess the success of PCR reactions. Nucleic acid fragments are separated by their length while moving through an agarose matrix. By adding a dye or an intercalating agent like ethidium bromide (EtBr), these fragments can be visualized under ultraviolet light [5]. Given that EtBr intercalates in a concentration-dependent manner, it should allow assessment of the number of amplicons in a specific band; furthermore, it should enable comparison of the quantity of PCR products of similar length based on their brightness [6]. Although the predominant opinion on agarose gel electrophoresis is that it is imprecise and therefore not suitable for the assessment of PCR product yield, from our experience a semi-quantitation or at least a rough estimation of PCR product yield, as required for many post-PCR applications, is possible. To more closely evaluate the suitability of agarose gels to assess the amount of PCR products, we quantified the brightness of the bands on a gel and compared it with the respective results of the three different quantification methods mentioned above.

For this study, ten DNA samples were amplified: eight extracted from oral swabs using the EZ1 DNA Tissue Kit and BioRobot EZ1 Advanced according to the manufacturer's instructions (Qiagen, Hilden, Germany) and two control DNA standards (9947A and 9948; Promega, WI, USA). For each sample, two PCR reactions with different amounts of DNA were performed. Fragments of 130 bp in length located close to the *LAC* gene [7] on the q-arm of chromosome 2 were generated. PCR components, parameters and primer sequences are shown in Supplementary Material 1.

For the initial testing of PCR success, a 2.5% agarose gel was prepared by dissolving agarose powder in Tris/Borate/EDTA (TBE) buffer in an Erlenmeyer flask. The flask was covered to prevent evaporation and heated on a magnetic heating stirrer. Heating the agarose gel with a magnetic heating stirrer is slower than, for example, cooking in a microwave oven, and therefore ensures complete dissolving of the agarose powder. After boiling, EtBr was added and mixed thoroughly with the gel prior to casting it into the tray. This way of agarose gel preparation ensures a highly uniform appearance of the set gel, which in our opinion is essential for evaluation of PCR product amounts. After the gel had set, the PCR products were mixed with loading dye and applied to the gel. Electrophoresis was run at 100 V for 45 min. The resulting agarose gel and the detailed description of agarose gel preparation are shown in Supplementary Material 1 & Supplementary Figure 1. Assessment of the brightness of the bands was performed with the image processing open access software ImageJ [8] by generating lane plot profiles, enclosing the peaks and measuring the peak areas. For this assessment, subtraction of the

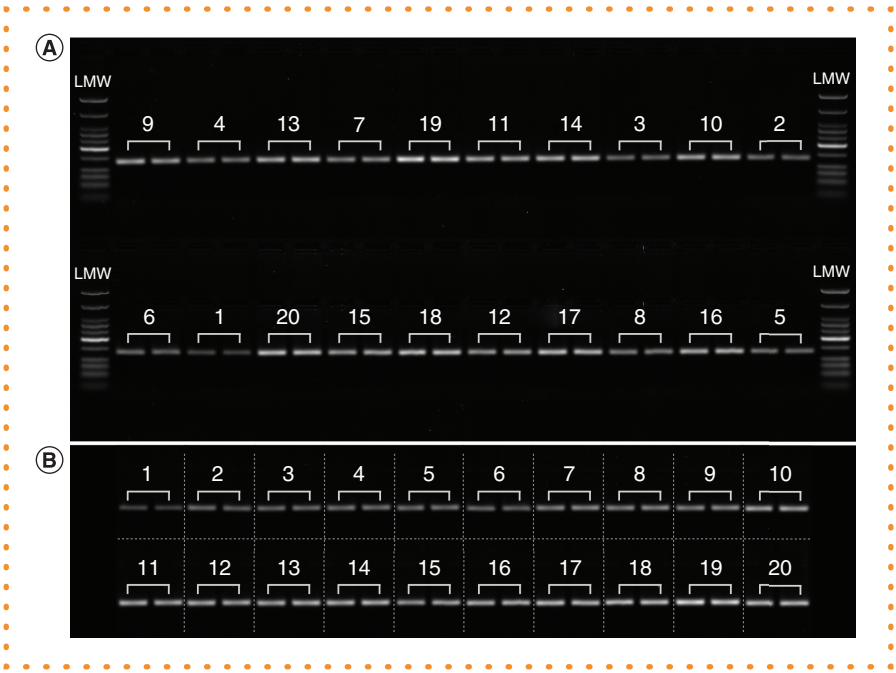

Figure 1. Agarose gels after PCR. Each product was applied twice to assess reproducibility. Samples were arranged according to increasing brightness as determined by ImageJ. **(A)** Original agarose gel with subtracted background. **(B)** Virtual image with bands digitally rearranged by increasing brightness.
LMW: Low-molecular-weight DNA ladder (New England BioLabs, MA, USA).

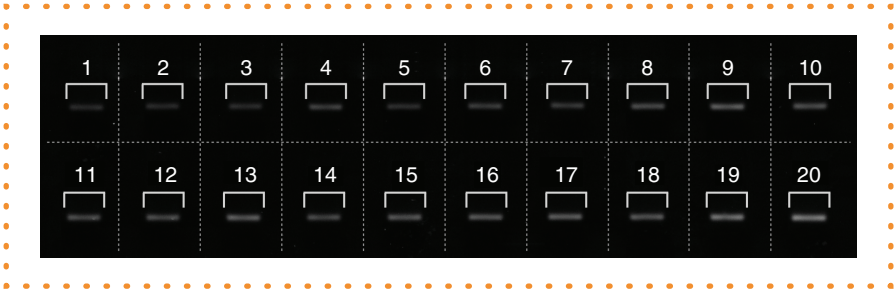

Figure 2. Agarose gel after purification of PCR products. Virtual image with bands digitally rearranged to same order as Figure 1B.

background is recommended, as shown in Figure 1A. Figure 1B shows the bands of the agarose gel virtually rearranged by increasing brightness and thereby represents a relative quantification. A more detailed description of image processing and brightness assessment can be found in Supplementary Material 1.

As spectrophotometry and fluorometry require purified PCR products for analysis, 20 µl of the remaining PCR product was purified using NucleoSEQ® spin columns (Machery-Nagel, Düren, Germany) following the manufacturer's instructions. To account for possible loss of PCR product through the purification process, a second agarose gel electrophoresis was run under the same conditions as before. Figure 2 shows the bands of the gel arranged in the same order as in Figure 1B. The original image is presented in Supplementary Figure 2. For the spectrophotometric measurement, a DS-11 FX+ spectrophotometer/fluorometer (DeNovix, DE, USA) with the dsDNA Absorbance app was used. Each sample was measured five times by applying 1 µl of PCR product onto the lower sample surface. Lower and upper sample surfaces were cleaned with a dry lab wipe between each measurement of the same sample. Prior to measuring the next sample, both sample surfaces were rinsed with dH_2O and a new blank measurement with dH_2O was done. The fluorometric quantification was performed with the DS-11 FX+ and the Fluoro dsDNA app. The DeNovix dsDNA High Sensitivity Assay was used. Two separate assay mixes were prepared for each sample, with 2 and 3 µl PCR product, and each assay mix was measured three times. qPCR was performed with the LightCycler 2.0 system (Roche, Basel, Switzerland) and the QuantiFast® SYBR® Green PCR Kit (Qiagen). A standard curve was generated with PCR product of the control DNA standard 9948, using a 1:2 dilution series ranging from 1:200 to 1:3200. The initial concentration was established from the fluorometric data. For the measurements, dilutions of 1:500 and 1:1000 were prepared for each PCR product. Triplets, each consisting of 2 µl of the dilutions, were analyzed.

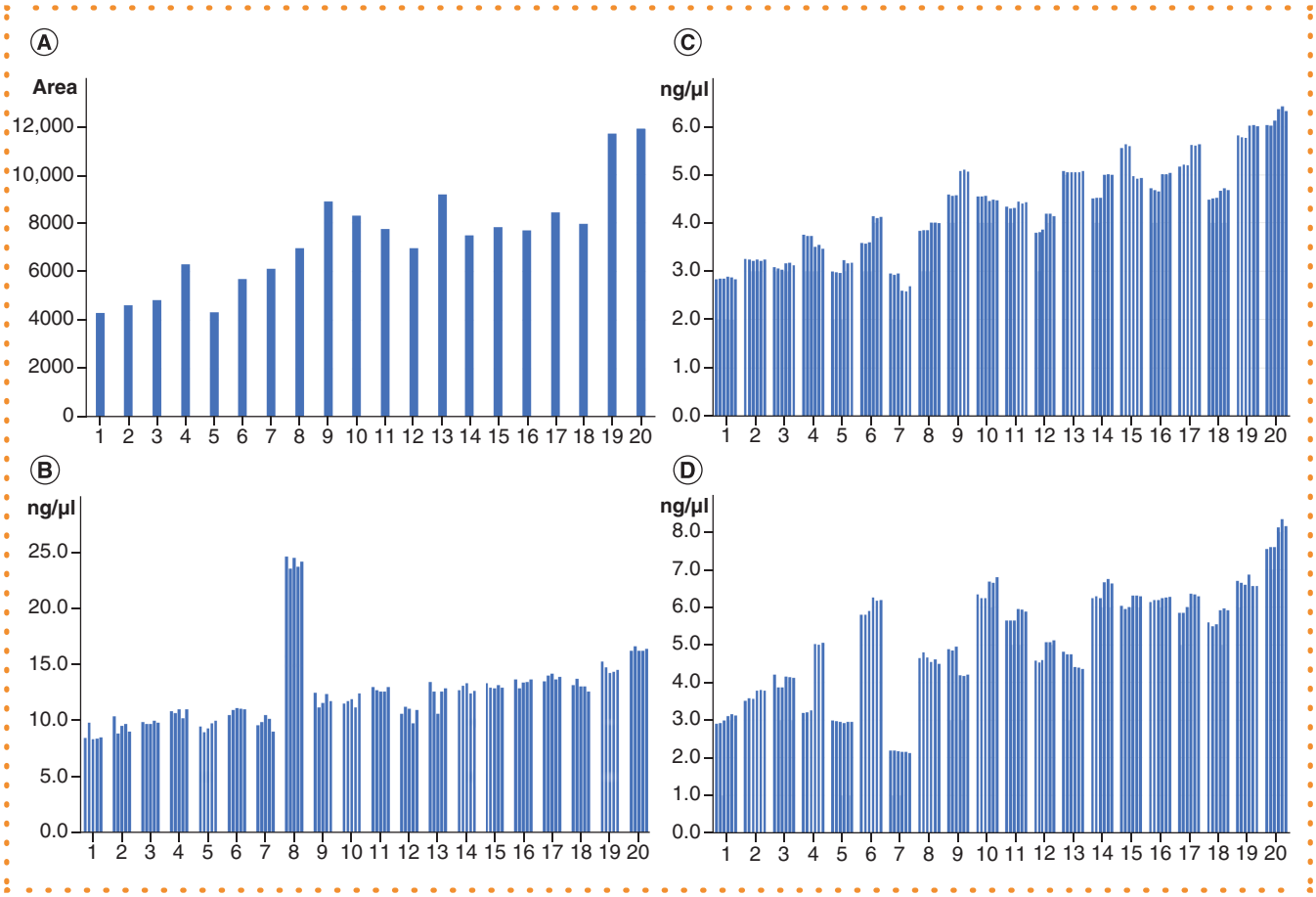

Figure 3. Results of the different quantification methods. Sample arrangement on the x-axis is identical to that in Figure 1B. **(A)** Graphical representation of band brightness based on peak area acquired with ImageJ from agarose gel after purification of PCR products. **(B)** PCR product concentration measured by spectrophotometry. **(C)** PCR product concentration measured by fluorometry. **(D)** PCR product concentration measured by qPCR.

The results of the different quantification methods are presented in Figure 3A–D; for tabular values, see Supplementary Materials 2–5. The general pattern of increasing concentration from left to right can be observed in the results from all quantification methods, although the former order of samples with continuously increasing brightness and respective PCR product concentration is no longer present. These deviations are most likely due to a loss of PCR product of varying degree during the purification procedure, as already indicated through the comparison of the two agarose gels (Figures 1B & 2). The varying PCR product losses are displayed in the results of all methods and are most clearly visible in the results from agarose gel electrophoresis, fluorometry and qPCR. Overall, the determined concentrations by spectrophotometry are the most homogeneous, even if sample 8, which obviously is an outlier and compresses the y-scale, is removed from the diagram. In contrast, the variance between the samples is most pronounced in the results from qPCR.

The comparison of absolute concentrations of amplification products given in ng/µl is possible only for spectrophotometry, fluorometry and qPCR, because the graphical representation of the band brightness acquired by ImageJ is an indirect measure of the concentration. When comparing the absolute PCR product concentrations, the dataset determined by spectrophotometry was the highest overall. One possible explanation is that the applied purification procedure did not fully remove all primers, because spectrophotometry does not distinguish between single-stranded and double-stranded DNA. In contrast, the assay utilized for the fluorometric measurement specifically detects double-stranded DNA. The fact that qPCR and fluorometric data resulted in similar absolute concentrations for the PCR products is caused by the calibration of the standard curve on the basis of fluorometric data (see above). Concerning reproducibility, sample 4 in qPCR is considered to be an outlier, because it reveals a high discrepancy between the two different dilutions. The calculated concentration based on the 1:1000 dilution is approximately 30% higher compared with the 1:500 dilution. This is best explained by possible erroneous pipetting during preparation of the dilutions for sample 4.

To summarize, agarose gel electrophoresis is suitable to compare the product yield of samples following a PCR reaction and thereby allows a relative quantification, provided the amplicons are of similar fragment length and are within the concentration range we describe. This is shown by the comparatively precise ranking of PCR product concentrations that is possible on the basis of an agarose gel. Many post-PCR analysis steps, such as fragment length analysis by capillary electrophoresis or evaluation of PCR parameters, do not require

the determination of absolute PCR product concentrations. Our results show that agarose gel electrophoresis fully complies with these demands. In our opinion, however, this requires careful preparation of agarose gels, focusing on complete dissolving of agarose powder and homogeneous dispersion of EtBr.

Future perspective

Although agarose gel electrophoresis was introduced nearly 50 years ago, it is still a reliable method, commonly applied for the separation of, for example, PCR products. When applying agarose gel electrophoresis to evaluate the success of PCR reactions, the brightness of the bands provides additional information rather than simply indicating the success or failure of the reaction. The evaluation of band brightness can be used to assess the relative quantity of amplicons of similar length. This can be useful for post-PCR applications that require PCR product concentrations within a certain range to function properly, as additional quantification steps would be expendable.

Executive summary

- We examined the suitability of agarose gel electrophoresis to assess PCR product yield by band brightness.
- For comparison, quantification of PCR products by spectrophotometry, fluorometry and qPCR was performed.
- Evaluation of the results suggests that band brightness on ethidium bromide-stained agarose gels indicates PCR product amounts comparatively precisely.
- Band brightness therefore allows a relative quantification of PCR product yield.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2021-0094

Author contributions

P Wittmeier: project design; acquisition, analysis and interpretation of data; writing of the manuscript. S Hummel: project design; interpretation of data; writing of the manuscript.

Acknowledgements

The authors thank the reviewers for their comments and recommendations, which helped to improve the clearity of this article.

Financial & competing interests disclosure

The authors furthermore acknowledge the support by the Open Access Publication Funds of the Göttingen University. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Desjardins RP, Conklin DS. Microvolume quantitation of nucleic acids. *Curr. Protoc. Mol. Biol.* 93(1), 3J (2011).
2. Mardis E, McCombie WR. Library quantification: fluorometric quantitation of double-stranded or single-stranded DNA samples using the Qubit system. *Cold Spring Harb. Protoc.* 2017(6), pdb.prot094730 (2017).
3. Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR. Basic principles of real-time quantitative PCR. *Expert Rev. Mol. Diagn.* 5(2), 209–219 (2005).
4. Borst P. Ethidium DNA agarose gel electrophoresis: how it started. *IUBMB Life* 57(11), 745–747 (2005).

• **Concise review on ethidium bromide agarose gel electrophoresis.**

5. Haines AM, Tobe S, Kobus HJ, Linacre A. Properties of nucleic acid staining dyes used in gel electrophoresis. *Electrophoresis* 36(6), 941–944 (2015).

• **Comparison of the properties of different nucleic acid staining dyes.**

6. Lee PY, Costumbrado J, Hsu CY, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. *J. Vis. Exp.* (62), 3923 (2012).
7. Enattah NS, Sahi T, Savilahti E, Terwilliger JD, Peltonen L, Järvelä I. Identification of a variant associated with adult-type hypolactasia. *Nat. Genet.* 30(2), 233–237 (2002).
8. Schneider C, Rasband W, Eliceiri K. NIH Image to ImageJ: 25years of image analysis. *Nat. Methods* 9(7), 671–675 (2012).

•• **Review on the open-access scientific image analysis tool ImageJ.**