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Original Article

A degenerate base primer correction significantly improves qPCR performance for adenovirus detection: a case study of the modified Jothikumar kit

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Abstract

Background: Primer–template mismatches are an important yet frequently underestimated cause of reduced sensitivity in qPCR assays. Even a single mismatch, particularly near the 3′ terminus, can impair polymerase extension, delay amplification, and increase the limit of detection. The widely used Jothikumar adenovirus qPCR assay is known to perform suboptimally for human adenovirus type 3 (HAdV3) due to mismatches within the binding region of the forward primer.
Materials and methods: In this study, we assessed how correcting a degenerate base mismatch affects qPCR performance for HAdV3. Using SYBR Green–based qPCR and ten-fold serial dilutions of quantified viral DNA, we compared amplification efficiency, Cq values, linearity, and analytical sensitivity between the original primer and a modified version designed to restore effective complementarity to HAdV3 targets.
Results: The original primer showed poor amplification efficiency, a shallow standard-curve slope, and a substantially elevated detection threshold. In contrast, the corrected primer achieved near-optimal efficiency, excellent linearity, improved reproducibility, and at least a ten-fold lower limit of detection. Across matched dilution points, the modified primer amplified approximately twenty cycles earlier than the unmodified version, demonstrating a profound impact on assay sensitivity.
Conclusion: These results show that even minimal optimization of primer sequence can markedly improve diagnostic performance. Routine in silico evaluation and periodic reassessment of primer–template compatibility should therefore be standard practice, especially for genetically variable viral targets such as adenoviruses.

Keywords: AdV3; adenovirus detection; myocarditis; SYBR Green; primer design; primer–template mismatch; qPCR; qPCR sensitivity; viral diagnostics

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1. Introduction

Primers are short DNA fragments that initiate the polymerase chain reaction by annealing to the template strand. For efficient amplification, primers must bind their target sequence with high fidelity. Any mismatch between the primer and the template decreases the stability of this interaction, reducing the likelihood of successful polymerase binding. This reduced binding efficiency can manifest in PCR as delayed amplification, visible as an increase in the Cq value, thereby lowering the sensitivity of the assay [1]. Notably, Stadhouders et al. (2010) demonstrated that a single primer-template mismatch could increase the Cq value by up to ≥7 cycles [2]. More recently, Huang et al. (2024), analyzing 111 different mismatches, showed that the type of mismatch, its position, and the properties of the polymerase (e.g., proofreading activity) critically determine amplification

performance-particularly 3′ mismatches of the G→C, C→A, T→G, and T→A types, which significantly impair PCR efficiency [3]. Such mismatches therefore directly reduce viral detection performance. This effect has also been documented in SARS-CoV-2 diagnostics, where primer–template mismatches markedly decreased RT-qPCR sensitivity [4].

The Jothikumar assay is a high-sensitivity TaqMan qPCR system designed for the detection of multiple adenovirus serotypes (A–F). Under optimal conditions, it can detect very low numbers of viral DNA copies, for example, 5 copies for human adenovirus 40 (HAdV40) and 8 copies for HAdV41. However, detection of HAdV3 is notably less sensitive, requiring ≥350 copies for reliable amplification. This reduced sensitivity is attributed to multiple primer–template mismatches, highlighting how sequence mismatches directly limit assay performance [5].

Given that primer–template mismatches can drastically impair qPCR sensitivity and efficiency, as exemplified by the reduced detection of certain adenovirus serotypes with the Jothikumar assay, mismatches represent a major limitation of such diagnostic systems. Even a single nucleotide mismatch can markedly decrease reaction efficiency, yet this problem has not been systematically analyzed in the context of adenoviruses. Therefore, the aim of this study was to evaluate how a single-position degenerate-base correction of the primer sequence influences the performance of a widely used adenovirus qPCR assay. To achieve this, we compared the performance of the original and corrected primer sets in terms of slope, R^2, amplification efficiency, and limit of detection (LoD). This study demonstrates that even minor primer sequence optimization can yield substantial improvements in qPCR performance, with direct implications for adenovirus diagnostics.

2. Materials and Methods

2.1 Viral template and nucleic acid extraction

Human adenovirus type 3 (HAdV3; ATCC VR-3™) was used as the reference template. Viral DNA was extracted using the NucleoSpin Dx kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Template copy number was quantified by droplet digital PCR (ddPCR), yielding a stock concentration of 3.49×10^6 copies/µl.

2.2 Primer design and modification

The primer sequences described by Jothikumar et al. were evaluated by BLASTn analysis against adenovirus sequences to identify mismatches [6][7]. A single-position primer modification was introduced by replacing one nucleotide with a degenerate base (S = G/C) to accommodate known sequence variability in HAdV-3. Both the original and modified primer sequences are provided in Figure 1.

	Forward																						
Primer	G	G	A	C	G	C	C	T	C	G	G	A	G	T	A	C	C	T	G	A	G		
KX384949.1 (ATCC VR-1572™)	G	G	A	C	G	C	T	T	C	G	G	A	G	T	A	C	C	T	G	A	G		
AY599834.1 (ATCC VR-3™)	G	G	A	T	G	C	T	T	C	G	G	A	G	T	A	C	C	T	C	A	G		
New primer	G	G	A	C	G	C	C	T	C	G	G	A	G	T	A	C	C	T	S	A	G		
	Reverse																						
Primer	T	T	G	T	T	C	A	A	A	T	C	T	T	T	G	G	G	G	T	G	N	C	A
KX384949.1 (ATCC VR-1572™)	T	T	G	T	T	T	A	A	A	T	C	C	T	T	G	G	G	G	T	G	C	C	A
AY599834.1 (ATCC VR-3™)	T	T	G	T	T	T	A	A	A	T	C	T	T	T	G	G	G	G	T	G	N	C	A

Figure 1. The original and modified primer sequences

2.3 Primers properties

GC% and estimated Tm were calculated using the Wallace rule (Tm ≈ 2°C × (A+T) + 4°C × (G+C)); for degenerate bases we assumed S = G/C and N = 0.5 GC. For publication-quality thermodynamic Tm calculations, consider reporting values from Primer3/IDT with specified ionic conditions. Basic primer properties are listed in Table 1.

Table 1. Basic primers properties

Primer	Length (nt)	GC (%)	Tm (°C)
Default Forward Primer	21	67	60
New Forward Primer	21	67	60
Reverse Primer	23	39-43	52-53

2.4 PCR conditions

QPCR assays were performed using the PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) in 10 µl reaction volumes, containing 500 nM of each primer and 2 µl of template DNA. Reactions were carried out in duplicate using a CFX Opus Dx Real-Time PCR System (Bio-Rad).

PCR amplification was performed under the following conditions: initial denaturation at 95 °C, followed by 59 cycles of 95 °C for 15 s, 57 °C for 16 s, and 72 °C for 60 s with fluorescence acquisition at the extension step. A melt-curve analysis was included at the end of the run, ranging from 65 to 95 °C in 0.5 °C increments.

2.5 Standard curve and limit of detection

Ten-fold serial dilutions of the HAdV3 template (10^0–10^{-4}) were prepared, corresponding to 6.98×10^6, 6.98×10^5, 6.98×10^4, 6.98×10^3, and 6.98×10^2 copies per reaction.

2.6 Data analysis

Amplification efficiency (E) was calculated from the slope of the standard curve using the formula:

$$E = 10^{(-1/\text{slope})} - 1$$

The coefficient of determination (R^2) was derived from linear regression analysis of Cq versus $\log_{10}$ (copy number). All data represent the mean values of duplicate reactions.

Amplification specificity was assessed by melt-curve analysis (60–95°C, 0.3°C increments). Only reactions showing a single, primer-set–specific melt peak and no amplification in the negative control (NTC) were considered positive.

3. Results

3.1. Performance of the modified primer set (MOD_AdV3)

qPCR assays performed with the modified forward primer (MOD_AdV3) demonstrated robust amplification across all five dilution points (10^0–10^{-4}). The standard curve exhibited a slope of −3.526 with an R^2 of 0.9998, corresponding to an amplification efficiency of 92.1% (95% CI: 89.3–95.2%). Within the tested dilution range, amplification was observed down to ~7×10^2 copies per reaction (10^{-4} dilution), with mean Cq values of approximately 35.3. These results indicate that the single-position degenerate-base correction yielded highly efficient and reproducible amplification over a dynamic range spanning at least four orders of magnitude.

3.2. Performance of the original primer set (STD_AdV3)

In contrast, the original Jothikumar primer set (STD_AdV3) exhibited suboptimal performance. Amplification was observed only across four dilution points (10^0–10^{-3}), with failure of detection at 10^{-4}. The resulting standard curve showed a slope of −5.358 and an R^2 of 0.960, corresponding to a low and unstable efficiency of 53.7% (95% CI: 30–209%). The limit of detection was approximately 7×10^3 copies per reaction (10^{-3} dilution), with amplification observed in only one of two replicates (mean Cq ≈ 54.3).

3.3. Comparative analysis

Direct comparison between the primer sets revealed a striking improvement in assay performance following the single-position degenerate-base correction. Across shared dilution points, the modified primer reduced Cq values by an average of −20.31 cycles relative to the original primer. Moreover, within the tested dilution range, the apparent limit of detection improved by at least one order of magnitude (≥10×). Together, these results demonstrate that correcting a single mismatch in the primer sequence dramatically enhances qPCR sensitivity, efficiency, and reproducibility for adenovirus detection. The exact Cq values for individual

dilutions are included in Table 2. A comparison of Cq values depending on the dilutions between the original and modified primers is shown in Figure 2. Standard curves for both the original and modified primers are in Figure 3. For direct comparison, standard curves for both primer sets were constructed using the shared dilution range (10^0–10^{-3}); the 10^{-4} dilution was therefore excluded from regression analysis, although amplification was observed for MOD_AdV3 at this dilution.

Table 2. ΔCq at matched dilutions (MOD – STD)

Dilution	MOD mean Cq	STD mean Cq	ΔCq
0,001	31,97	54,33	-22,36
0,01	28,34	51,28	-22,94
0,1	24,82	42,66	-17,84
1,0	21,25	39,34	-18,09

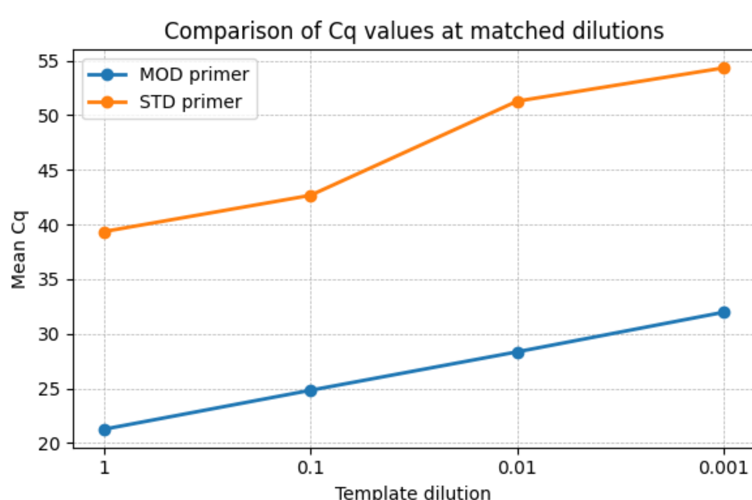

Figure 2. Comparison of Cq values at matched dilutions for original (STD) and modified (MOD) primers. Mean Cq values (n=2) are plotted for four serial dilutions (10^{-3}–10^0). The modified primer consistently amplified ~20 cycles earlier than the original Jothikumar primer across all tested dilutions, highlighting the substantial improvement in sensitivity and efficiency following single-position degenerate-base correction.

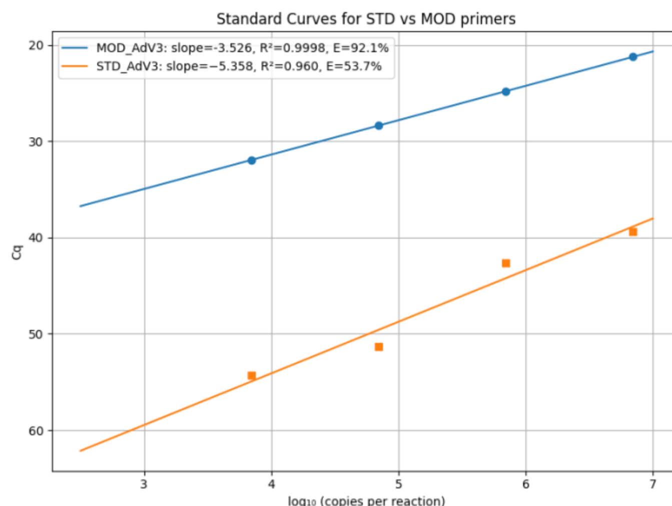

Figure 3. Standard curves for adenovirus detection using original (STD) and modified (MOD) primer sets. Linear regression analysis of quantification cycle (Cq) values plotted against $\log_{10}$ of input template copies. The modified primer (MOD, blue) showed markedly improved performance compared with the original primer (STD, orange), with a steeper slope, higher coefficient of determination (R^2), and amplification efficiency (~92%) close to the theoretical optimum. In contrast, the STD primer displayed a shallow slope, poor efficiency (~54%), and reduced reproducibility, consistent with impaired binding due to primer–template mismatch. Error bars indicate replicate variation where applicable

3.4. In silico analysis

In silico analysis (Table 3.) revealed that the forward primer reported by Jothikumar (STD_AdV3) aligns well with multiple HAdV types; however, in reference sequences of HAdV-3 (KX384949.1, AY599834.1), mismatches are observed: a single mismatch at position 7 (KX384949.1) and two mismatches, including one at the 3′ end (AY599834.1). The presence of a 3′-terminal mismatch is a critical limitation and may severely impair PCR efficiency. The modified primer (MOD_AdV3), incorporating a degenerate base S (G/C), corrects the critical 3′ mismatch in AY599834.1. This modification is consistent with experimental data showing markedly improved amplification efficiency (E increase from ~54% to ~92%) and a ΔCq shift of approximately −20 cycles. Importantly, this modification does not represent a fixed single-nucleotide substitution but rather the introduction of controlled degeneracy at a single primer position.

Primer	Target accession	Target type/serotype	Match length (nt)	Number of mismatches	Mismatch position (from 5′)	Critical location (3′)	Comments
STD_AdV3	OR753125.1	HAdV-1	21	1	7	No	Potential decrease in stability
STD_AdV3	AY599834.1	HAdV-3	21	2	4, 21	Yes	High risk of loss of amplification
MOD_AdV3 (S=G/C)	KX384949.1	HAdV-3	21	1	7	No	Mismatch maintained but not critical
MOD_AdV3 (S=C)	AY599834.1	HAdV-3	21	1	4	No	Critical mismatch at 3′ eliminated

Table 3. In silico BLASTn analysis of primer sequences.

3.5. MIQE checklist

Minimum information for publication of quantitative real-time PCR experiments (MIQE) checklist is available as Supplementary S1.

3.6. Amplification specificity

Melt-curve analysis confirmed a single specific amplicon for both primer sets, with no detectable primer-dimer formation, even at late amplification cycles (Supplementary Figure S1).

4. Discussion

As previously mentioned, proper primer–template annealing at the 3′ end is essential for DNA polymerase activity. Even a single-nucleotide mismatch can markedly reduce elongation efficiency. Interestingly, this phenomenon has been deliberately exploited in allele-specific PCR, where a mismatch at the 3′ terminus is used to discriminate allelic variants [8]. In contrast, in our study a single mismatch reduced amplification efficiency from 92% to 54% and caused a shift in Cq of approximately 20 cycles. These findings clearly illustrate how even one mismatch, particularly near the 3′ terminus, can critically impair primer performance.

BLAST analysis demonstrated that the newly designed primer aligns well with multiple adenovirus types and shows no significant cross-reactivity with the human genome or unrelated viruses such as HSV, CMV, or enteroviruses. This broad compatibility reduces the risk of false-positive amplifications and increases the overall reliability of detection. In silico validation should therefore be considered a crucial step in primer design.

Importantly, we found that the original primer set failed to consistently detect the target virus. The observed mismatch decreased amplification efficiency and increased the limit of detection by approximately one order of magnitude. In a diagnostic setting, this effect could lead to false-negative results, particularly in samples with low viral loads. Our findings highlight that commonly used “classical” primers may underperform for certain adenovirus subtypes, thereby posing a risk of false-negative outcomes.

Furthermore, adenoviruses are recognized causes of viral myocarditis, which in some cases may progress to heart failure. The European Society of Cardiology (ESC) myocarditis position statement recommends identifying the underlying etiological agent whenever feasible, as it may guide both prognosis and management. Therefore, reliable detection of adenoviruses is essential for accurate diagnosis and appropriate clinical decision-making. A primer–template mismatch that increases the detection threshold by an order of magnitude, as demonstrated in our study, could thus have direct clinical consequences by contributing to false-negative results in patients with low viral loads [9–11].

This study has several limitations. First, only a single adenovirus strain (ATCC VR-3™) was tested, which does not allow direct extrapolation to all viral types. Second, amplification was performed with a SYBR Green chemistry, which is more prone to non-specific products compared with hydrolysis probe–based assays (e.g., TaqMan). Third, the limit of detection was assessed based on serial dilutions tested in duplicate and should therefore be interpreted as an apparent LoD within the tested dilution range rather than a probabilistic LoD. Replicate-based estimation of detection probability at low template concentrations was not performed and should be addressed in future validation studies. Finally, we did not validate the assay across different thermocyclers, reagent kits, or clinical samples, which limits the generalizability of our conclusions.

The relatively high number of amplification cycles (59) and the late Cq values observed for the original primer set warrant careful interpretation. The extended cycling protocol was intentionally selected to allow direct comparison between primer sets under identical conditions and to explore the performance limits of the assay at low template concentrations. In SYBR Green–based assays, late-cycle amplification can raise concerns regarding non-specific products or primer-dimer formation; however, in the present study, amplification specificity was assessed by melt-curve analysis. For reactions considered positive, a single, well-defined melt peak at the expected melting temperature was consistently observed, whereas no amplification or only non-specific melt profiles were detected in no-template controls. Importantly, late Cq values obtained with the original primer set were accompanied by poor amplification efficiency and reduced linearity of the standard curve, indicating inefficient priming rather than true analytical sensitivity. In contrast, the modified primer set yielded markedly earlier Cq values, near-optimal efficiency, and robust linearity, supporting the conclusion that the observed improvements reflect genuine gains in assay performance rather than artifacts of extended cycling.

Based on these findings, we recommend routine in silico evaluation of primer sets, particularly for use in diagnostic viral panels. For regions with high sequence variability, the introduction of degeneracy (e.g., N, R, Y bases) may help broaden coverage without compromising efficiency. Future studies should include validation of clinical specimens and with probe-based chemistries to further improve specificity and robustness.

Our study emphasizes that routine BLAST verification of primer-template compatibility should be a standard step in diagnostic assay design, as even a single mismatch may compromise detection sensitivity by an order of magnitude.

4.1. Practical implications

The findings of this study highlight that even a single primer–template mismatch can critically affect qPCR performance, reducing amplification efficiency, delaying Cq values by several cycles, and substantially increasing the limit of detection. In practical diagnostic settings, such impairments translate directly into a higher risk of false-negative results, especially in samples with low viral loads or partially degraded nucleic acids. The corrected primer evaluated here restores near-optimal amplification efficiency and lowers the detection threshold by at least one order of magnitude, demonstrating that minor sequence adjustments can have major diagnostic consequences. These results emphasize the importance of regular in silico verification of primer sequences, particularly for assays targeting genetically diverse viruses such as adenoviruses. Routine reassessment of primer–template compatibility - especially before large-scale clinical screening-may significantly improve diagnostic reliability and reduce the likelihood of overlooked infections.

5. Conclusion

This study demonstrates that even a single primer–template mismatch can markedly compromise qPCR performance, leading to reduced efficiency, delayed Cq values, and an increased risk of false-negative results. Introducing a single-position degenerate-base modification restored efficient and reproducible amplification and substantially improved analytical sensitivity. These findings highlight the critical importance of routine in silico primer–template evaluation and targeted primer optimization, even at the single-nucleotide level, to ensure reliable molecular diagnostics. Given the clinical relevance of adenovirus detection, particularly in the context of viral myocarditis, such optimization represents a simple yet impactful strategy to enhance diagnostic robustness.

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References

1. Wu, J. H., Hong, P. Y., & Liu, W. T. (2009). Quantitative effects of position and type of single mismatch on single base primer extension. *Journal of microbiological methods*, *77*(3), 267–275. https://doi.org/10.1016/j.mimet.2009.03.001
2. Stadhouders, R., Pas, S. D., Anber, J., Voermans, J., Mes, T. H., & Schutten, M. (2010). The effect of primer-template mismatches on the detection and quantification of nucleic acids using the 5' nuclease assay. *The Journal of molecular diagnostics: JMD*, *12*(1), 109–117. https://doi.org/10.2353/jmoldx.2010.090035
3. Huang, K., Zhang, J., Li, J., Qiu, H., Wei, L., Yang, Y., & Wang, C. (2024). Exploring the Impact of Primer-Template Mismatches on PCR Performance of DNA Polymerases Varying in Proofreading Activity. *Genes*, *15*(2), 215. https://doi.org/10.3390/genes15020215
4. Zimmermann, F., Urban, M., Krüger, C., Walter, M., Wölfel, R., & Zwirglmaier, K. (2022). In vitro evaluation of the effect of mutations in primer binding sites on detection of SARS-CoV-2 by RT-qPCR. *Journal of virological methods*, *299*, 114352. https://doi.org/10.1016/j.jviromet.2021.114352
5. Jothikumar, N., Cromeans, T. L., Hill, V. R., Lu, X., Sobsey, M. D., & Erdman, D. D. (2005). Quantitative real-time PCR assays for detection of human adenoviruses and identification of serotypes 40 and 41. *Applied and environmental microbiology*, *71*(6), 3131–3136. https://doi.org/10.1128/AEM.71.6.3131-3136.2005
6. Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797
7. Huggett JF, et al. *The digital MIQE guidelines update: dMIQE2020.* Nat Protoc, 2020.
8. Tóth, T., Csaba, Á., Bokor, A., & Ács, N. (2023). Variable fragment length allele-specific polymerase chain reaction (VFLASP), a method for simple and reliable genotyping. *Molecular and cellular probes*, *69*, 101910. https://doi.org/10.1016/j.mcp.2023.101910

9. Jeanette Schulz-Menger, Valentino Collini, Jan Gröschel, Yehuda Adler, Antonio Brucato, Vanessa Christian, Vanessa M Ferreira, Estelle Gandjbakhch, Bettina Heidecker, Mathieu Kerneis, Allan L Klein, Karin Klingel, George Lazaros, Roberto Lorusso, Elena G Nesukay, Kazem Rahimi, Arsen D Ristić, Marcin Rucinski, Leyla Elif Sade, Hannah Schaubroeck, Anne Grete Semb, Gianfranco Sinagra, Jens Jakob Thune, Massimo Imazio, the ESC Scientific Document Group , 2025 ESC Guidelines for the management of myocarditis and pericarditis: Developed by the task force for the management of myocarditis and pericarditis of the European Society of Cardiology (ESC)
Endorsed by the Association for European Paediatric and Congenital Cardiology (AEPC) and the European Association for Cardio-Thoracic Surgery (EACTS), *European Heart Journal*, Volume 46, Issue 40, 21 October 2025, Pages 3952–4041, https://doi.org/10.1093/eurheartj/ehaf192
10. Alida L. P. Caforio, Sabine Pankuweit, Eloisa Arbustini, Cristina Basso, Juan Gimeno-Blanes, Stephan B. Felix, Michael Fu, Tiina Heliö, Stephane Heymans, Roland Jahns, Karin Klingel, Ales Linhart, Bernhard Maisch, William McKenna, Jens Mogensen, Yigal M. Pinto, Arsen Ristic, Heinz-Peter Schultheiss, Hubert Seggewiss, Luigi Tavazzi, Gaetano Thiene, Ali Yilmaz, Philippe Charron, Perry M. Elliott, Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases, *European Heart Journal*, Volume 34, Issue 33, 1 September 2013, Pages 2636–2648, https://doi.org/10.1093/eurheartj/eht210
11. Theresa A McDonagh, Marco Metra, Marianna Adamo, Roy S Gardner, Andreas Baumbach, Michael Böhm, Haran Burri, Javed Butler, Jelena Čelutkienė, Ovidiu Chioncel, John G F Cleland, Andrew J S Coats, Maria G Crespo-Leiro, Dimitrios Farmakis, Martine Gilard, Stephane Heymans, Arno W Hoes, Tiny Jaarsma, Ewa A Jankowska, Mitja Lainscak, Carolyn S P Lam, Alexander R Lyon, John J V McMurray, Alexandre Mebazaa, Richard Mindham, Claudio Muneretto, Massimo Francesco Piepoli, Susanna Price, Giuseppe M C Rosano, Frank Ruschitzka, Anne Kathrine Skibelund, ESC Scientific Document Group , 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) With the special contribution of the Heart Failure Association (HFA) of the ESC, *European Heart Journal*, Volume 42, Issue 36, 21 September 2021, Pages 3599–3726, https://doi.org/10.1093/eurheartj/ehab368