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UNIVERSAL PRIMERS AND FLUORESCENT PROBE DETECT REFERENCE 18S RRNA GENE IN ALL VERTEBRATES

Detection of target genes via real-time PCR-based test-systems requires simultaneous detection of the internal control gene to avoid false negative results. Endogenous Internal Control genes (reference genes) have an advantage over the Exogenous Internal Controls (IC) as they are part of the cellular chromosomal DNA of an organism. Reference genes however have a disadvantage because they vary for each biological species causing additional development of primers and fluorescent probes for each separate biological species, which requires additional time and funds. In recent years, a significant number of studies are devoted to development of universal sets of primers / probes capable of detecting a reference gene in a wide range of biological objects – a whole class or even a living kingdom. Such universal sets of primers / probes will lead to unification of qPCR-based tests used in diagnostic purposes. This paper presents such a set for 18S rRNA reference gene determination in species included in the Vertebrata subtype. Using the developed set, the 18S rRNA gene was detected during the study in DNA samples of Mammals, Sauropsids, Amphibians, and Bony fishes; it showed 100% specificity. The set was also tested in different multiplex qPCR and kept specificity and sensitivity in tetraplex assay.

Key words: Real-time PCR, 18S rRNA, reference gene, vertebrates, internal control gene.

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Универсалды праймерлер және флуоресцентті зонд барлық омыртқалыларда 18s рРНҚ генін анықтады

Нақты-уақыттағы-ПТР негізіндегі сынақ жүйелері арқылы мақсатты гендерді анықтау жалған теріс нәтижелерді болдырмау үшін ішкі бақылау генін бір мезгілде анықтауды талап етеді. Эндогендік ішкі бақылау гендерінің (референстік гендер) экзогендік ішкі бақылауларға (ІБ) қарағанда артықшылығы бар, өйткені олар ағзаның жасушалық хромосомалық ДНҚ бөлігі болып табылады. Референстік гендердің кемшілігі бар, өйткені олар әр биологиялық түр үшін өзгереді, бұл әрбір жеке биологиялық түр үшін праймерлер мен флуоресцентті зондтардың қосымша дамуын тудырады, бұл қосымша уақыт пен қаражатты қажет етеді. Соңғы жылдары көптеген зерттеулер биологиялық объектілердің кең ауқымында – тұтас класста немесе тіпті тірі патшалықта анықтамалық генді анықтауға қабілетті праймерлердің/зондтардың әмбебап жиынтықтарын әзірлеуге арналған. Праймерлердің/зондтардың мұндай әмбебап жинақтары диагностикалық мақсаттарда қолданылатын нақты-уақыттағы-ПТР негізіндегі сынақтарды біріктіруге әкеледі. Бұл құжат омыртқалылар қосалқы түріне кіретін түрлердегі 18S рРНҚ анықтамалық генін анықтауға арналған осындай жинақты ұсынады. Әзірленген жиынтықты пайдалана отырып, 18S рРНҚ гені зерттеу барысында сүтқоректілердің, савропсидтердің, қосмекенділердің және сүйекті балықтардың ДНҚ үлгілерінде анықталды; ол 100% ерекшелігін көрсетті. Жиын сонымен қатар әртүрлі мультиплексті нақты-уақыттағы-ПТР-де сыналған және тетраплекстік талдауда ерекшелік пен сезімталдық сақталды.

Түйін сөздер: Нақты-уақыттағы-ПТР, 18S рРНҚ, референстік ген, омыртқалылар, ішкі бақылау гені.

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Универсальные праймеры и флуоресцентный зонд, выявляют референтный ген 18S рРНК у всех позвоночных

Детекция генов-мишеней с помощью тест-систем на основе ПЦР-в-Реальном-Времени требует одновременного обнаружения гена внутреннего контроля, чтобы избежать ложноотрицательных результатов. Гены эндогенного внутреннего контроля (референсные гены) имеют преимущество перед экзогенным внутренним контролем (ВК), поскольку они являются частью клеточной хромосомной ДНК организма. Однако референсные гены имеют и недостаток, поскольку они различаются для каждого биологического вида, что приводит к дополнительной разработке праймеров и флуоресцентных зондов для каждого отдельного биологического вида, что требует времени и средств. В последние годы значительное количество исследований посвящено разработке универсальных наборов праймеров/зондов, способных обнаруживать референсный ген в широком круге биологических объектов – целого класса или даже живого царства. Такие универсальные наборы праймеров/зондов ведут к унификации тестов на основе ПЦР-в-Реальном-Времени, используемых в диагностических целях. В данной работе представлен такой набор для определения референсного гена 18S рРНК у видов, входящих в подтип Vertebrata. В ходе исследования с помощью разработанного набора в образцах ДНК млекопитающих, завропсид, амфибий и костных рыб ген 18S рРНК детектировался со 100%-ной специфичностью. Этот набор был также исследован в различных мультиплексных реакциях ПЦР-в-Реальном-Времени – специфичность и чувствительность набор сохранял вплоть до увеличения числа мишеней до четырёх в тесте (тетраплекс).

Ключевые слова: ПЦР-в-Реальном-Времени, 18S рРНК, референсный ген, позвоночные, ген внутреннего контроля.

Introduction

Currently, the vast majority of rapid diagnostic methods for determining viral and bacterial diseases are based on real-time PCR (qPCR). Diagnostic test systems that use real-time PCR for their functioning work with pico- and femtomolar amounts of matrices (both DNA and RNA), which often leads to false-negative results [1], especially in flow diagnostics laboratories.

False-negative results can occur in two cases: in the absence of the actual nucleic acid (NA) in the sample or because of the suppression (inhibition) of the PCR reaction [2]. The first option is possible both due to errors in the isolation of NA, and due to the low quality of the initial sample – there may be a temperature regime violation of the samples upon delivery to the laboratory, or an initially small amount of the original sample material. The second option is most often caused by the presence of inhibitors in the isolated NA, or by the failure of one of the components of the test system (possibly due to violations of its storage and use conditions).

In order to level up the listed reasons for the occurrence of false-negative results, the ability to detect an additional non-target gene is usually added to the test-systems. An additional gene is either the Exogenous Internal Control (IC) gene, which is added to all samples when NA is isolated [3], or the Endogenous Internal Control gene (aka the reference gene), which is contained in the sample by default, since it is part of the genome of the studied organism.

The detection of IC cuts off only the second variant of the qPCR failure, since IC is artificially added to the samples it does not give an answer whether the samples actually contain the studied NA. At the same time, the detection of the reference gene allows not only stating the working status of the test-system, but also shows the presence of the desired NA in the sample. Despite such an advantage of the reference gene over the IC, it is IC that is used in most commercial test-systems, since IC is added to the sample during the experiment and works equally well for samples of any type and from any kind of organisms, including environmental samples [4].

The reference genes of eukaryotic organisms most frequently mentioned in the scientific literature are the following: *18S rRNA*, *28S rRNA*, *TUBA* (α-tubulin),*ACTB*(β-actin),*β2M*(β2-microglobulin), *ALB* (albumin), *RPL32* (ribosomal protein L32), *TBP* (TATA-binding protein), *CYCC* (cyclophyllin C), *EF1A* (elongation factor 1α), *GAPDH*

(glyceraldehyde-3-phosphate dehydrogenase), *HPRT* (hypoxanthine phosphoribosyl transferase), *RPII* (RNA polymerase II) [5]. In each individual case of the target gene determination, its own reference gene is selected with a species-dependent sequence.

There are a number of commercial products designed to identify reference genes in various types of organisms. Thus, "Quiagen" produces a number of products under the general name "QuantiTect Primer Assay (200)", designed to determine the endogenous reference gene in the sample – these products are produced separately for each species. At the same time, "Eurogentec" produces the product "18S rRNA Control kit FAM-TAMRA", which is designed for real-time PCR detection of the human, rat, mouse, and rabbit 18S rRNA gene. In addition to this kit, "Eurogentec" produces a number of control kits that determine other reference genes, but in a smaller number of host species – "23S rRNA Control kit" (*E. coli*), "28S rRNA Control kit" (human and mouse) and ten more kits for human specimens only.

In research community is increasing the number of attempts to create universal sets for determining reference genes. In particular, a primer set was created for SYBR-based qPCR to determine the avian homologue of the hydroxymethylbilane synthase (HMBS) gene, which showed universality for a wide range of different birds' species [6]. There are also data on the development of a set of primers and a fluorescent probe to determine the gene for core subunit 5 of mitochondrial NADH:ubiquinone oxidoreductase (*MT-ND5*) for all species of the Canine family [7]. The most universal set of primers and probe, which determines the 28S rRNA gene in all eukaryotic organisms, was successfully tested on 43 animal species belonging to insects, fish, mammals, and plants [8].

The reference gene for a universal set must meet certain requirements – it must not only be present in all organisms belonging to the target group, but also be present in their genomes in several copies. Such a multicopy cluster organization is characteristic of ribosomal RNA (rRNA) genes in absolutely all living cells [9]. One 45S rDNA cluster can contain several dozen copies of the 18S and 28S rRNA genes; Thus, the average number of copies of an individual ribosomal gene in homothermal animals is about 200 [10], while the average human genome contains from 200 to 600 copies [11].

A number of patents is known regarding the 18S rRNA gene as a reference gene. In particular, the patent [12] specifies a number of reference genes for Chinese spruce, including the 18S rRNA gene. In the patent [13], the 18S rRNA gene is included in the combination of reference genes (along with 28S, GAPDH, HSP90, HSP70, RP49) to determine the stable expression of a number of proteins in the Asian ladybug. In the patent [14], the expression of the 18S rRNA gene (along with the IP-10 and CD3ε genes) is considered as a target for the detection of rejection states in renal transplantation. In the patent [15], the 18S rRNA gene is proposed as an endogenous internal control gene, which expression level is stable before and after hypoxic stimulation of ovarian cancer cells. But at the moment there is not a single study devoted to the development of a universal set of primers and a fluorescent probe for the detection of 18S rRNA.

The aim of the proposed work is to design primers and a TaqMan fluorescent probe that are universal for 18S rRNA gene sequence of all organisms belonging to the *Vertebrata* subtype, which will make it possible to use this universal set of primers / probe as an endogenous internal control for a wide range of diagnostic and research test-systems.

Materials and methods

Bacteria cultivation. *B. cereus* and *B. subtilis* strains were obtained as vegetative cells of cultivation controls "CultiControl" ("Liofilchem", Italy). *E. coli* (DH-5α) and *A. tumefaciense* (EHA-101) strains were present in the laboratory. All bacteria were plated on sterile LB agar medium without antibiotics in a biosafety cabinet (class II). Plates were cultured at 37°C for 16 hours.

Isolation of total bacterial DNA. Isolation of DNA samples was carried out by the standard method of phenol-chloroform extraction according to [16]. The determination of the nucleic acids amount was carried out by measuring ultraviolet absorption on a "NanoDrope 2000" ("Thermo") at a wavelength of 260 nm.

Samples for eukaryotic DNA isolation. Total DNA of eukaryotic organisms was isolated from blood samples of the following animal species: cow (*Bos taurus*), sheep (*Ovis aries*), goat (*Capra hircus*), chicken (*Gallus gallus*), duck (*Anas platyrhynchos*), camel (*Camelus dromedarius*), pig (*Sus scrofa*). DNA samples of the muscle tissue were isolated for: edible frog (*Pelophylax esculentus*), salmon (*Salmo salar*), pollock (*Theragra chalcogramma*). Animal blood samples were obtained in the framework of cooperation from the Almaty branch of

the "National Center for Biotechnology" ("NCB"). Muscle tissue samples were taken from commercial food samples purchased from supermarkets.

Total eukaryotic DNA isolation. DNA samples were isolated using the "M-sorb-OOM" ("Synthol") isolation kit according to the manufacturer's instructions. Muscle tissue samples were homogenized after freezing in liquid nitrogen by grinding in a mortar.

Primers and TaqMan fluorescent probe. The primers and the probe were synthesized in the organic synthesis laboratory of "NCB". The primers and probe for 18S rRNA gene detection had the following sequence: CCGGACACGGACAGGATTG (forward primer), CGCTCCACCAACTAAGAACG (reverse primer), ROX-ACGGCCATGCACCACCACC-BHQ2 (TaqMan probe).

Real-time PCR (qPCR). qPCR was carried out on "QuantStudio 5" and "QuantStudio 6 Pro" instruments ("Applied Bioscienses") in the following mode: 1) 95°C 7 min, 1 cycle; 2) 95°C 10 sec, 59°C 30 sec (data acquisition), 72°C 10 sec – 40 cycles. For real-time PCR, HotStart-Taq polymerase ("SibEnzyme") was used with a standard HS-Taq buffer ("SibEnzyme") additionally containing 0.15 mg BSA ("Thermo"), 0.5 mM dNTP ("SibEnzyme"), 2 mM $MgCl_2$ ("SibEnzyme"). Primers and TaqMan probe were added at 250 nM and 180 nM, respectively. From 10 pg to 10 ng of total DNA was used as a template in the reaction.

Results and discussion

Computer analysis of the 18S rRNA gene sequence

BLAST analysis of the reference 18S rRNA gene was carried out for the presence of minimal polymorphism among vertebrates (*Vertebrata*). For each of the classes of modern chordates, a separate BLAST analysis of a selected region of the 18S rRNA gene was performed (the original sequence belonged to the human 18S rRNA gene). The generalized results of the analysis of the selected region of the 18S rRNA gene in six classes of modern vertebrates are shown in Figure 1.

As can be seen from the results of the BLAST analysis, the selected sequence of 18S rRNA gene shows the greatest polymorphism in the *Cyclostomata* class, however, the primers were selected in such a way that there was only one nucleotide substitution in the forward and reverse primer sequences (along with a non-homologous insertion in some species). The region of the 18S rRNA gene complementary to the fluorescent probe for all classes of vertebrates was the most conservative: there were no polymorphic variants in it. Also, polymorphisms were absent in the sequence complementary to the reverse primer (with the exception of *Cyclostomata* class). As for the forward primer, its sequence had a single nucleotide substitution (SNP – single-nucleotide polymorphism) in most representatives of the classes of cartilaginous and bony fish, amphibians, and reptiles.

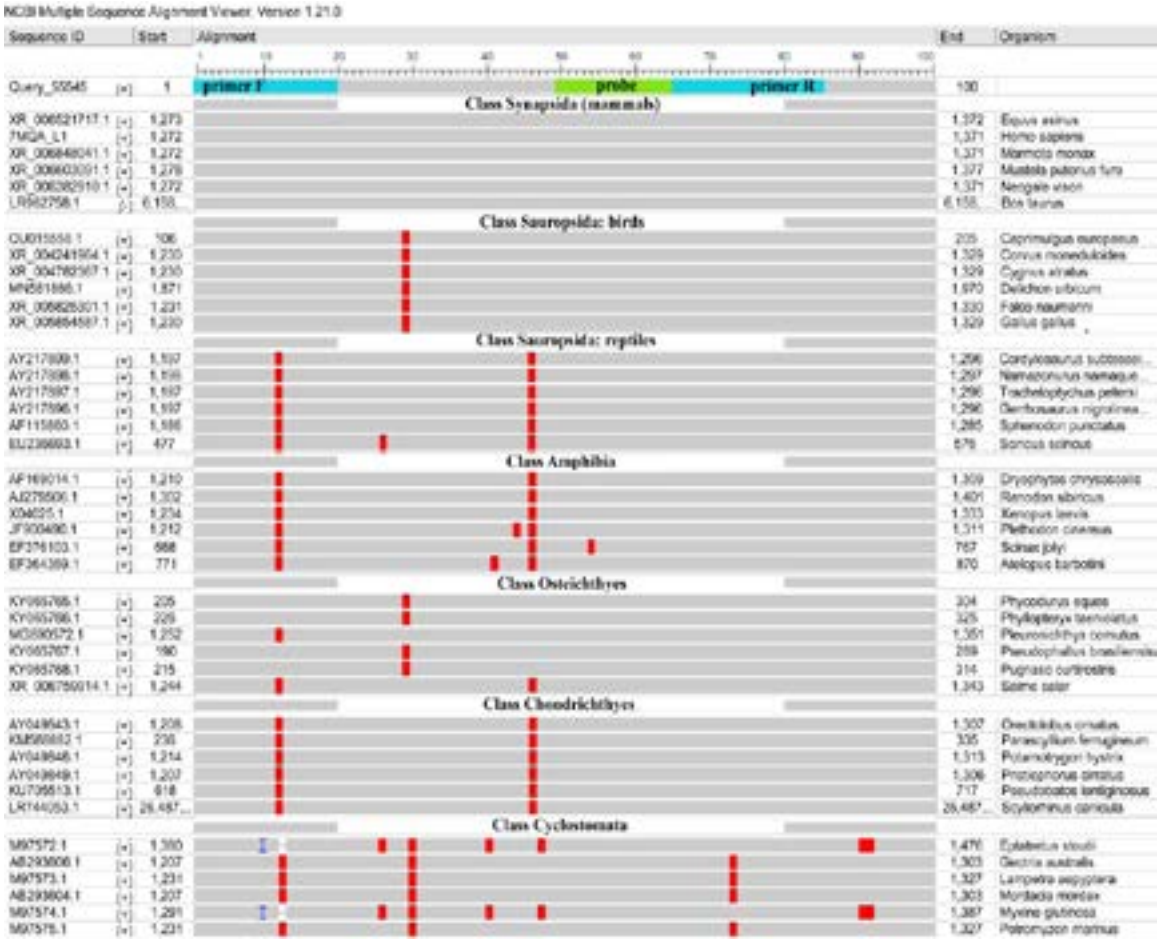
Thus, we determined the region of the 18S rRNA gene sequence with the minimum possible polymorphic variants: SNPs for the mammals and birds (as part of the class *Sauropsida* [17]) were absent; for the cartilaginous and bony fishes, amphibians and reptiles, one SNP was present in the forward primer; for the cyclostomes, one SNP was presented in the forward and reverse primers. BLAST analysis of the sequences of the selected primers and samples among prokaryotic organisms (separately for each class) showed a complete absence of homologous sequences.

All primer sequences were selected ("Vector NTI Suite 11.0") so that the average annealing temperature (Therm.Tm) was from 59°C to 60°C, and for the TaqMan probe it was 61°C. In the "RNAstructure 5.0" program, an analysis of forming duplexes both between primers and between the probe and primers (singly) was made; the results of the analysis are shown in Figure 2. The maximum allowable duplex size was limited to 5 bp, with free energy not higher than -7 kJ.

Specificity and sensitivity determination

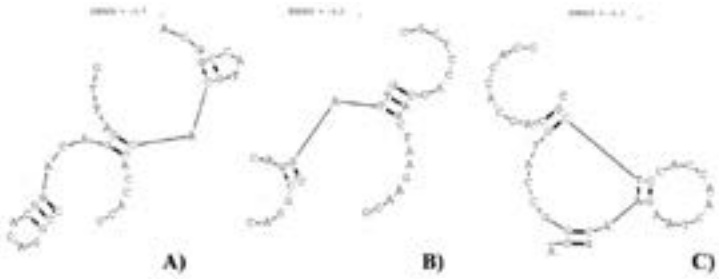
To analyze the specificity of the selected primers and probe, we used DNA samples isolated both from vertebrate animals (cow, sheep, goat, chicken, duck, camel, pig, edible frog, salmon, pollock) and from prokaryotic organisms (*E. coli, B. cereus, B. subtilis, A. tumefaciense*). Graphical results of real-time PCR of all DNA samples from vertebrates (in one repetition) are shown in Figure 3.

Graphical data of real-time PCR for all replicates of prokaryotic DNA samples are presented in Figure 4. Generalized data (the number of repeats n = 10) for the specificity study of the set for the determination of 18S rRNA are presented in Table 1.



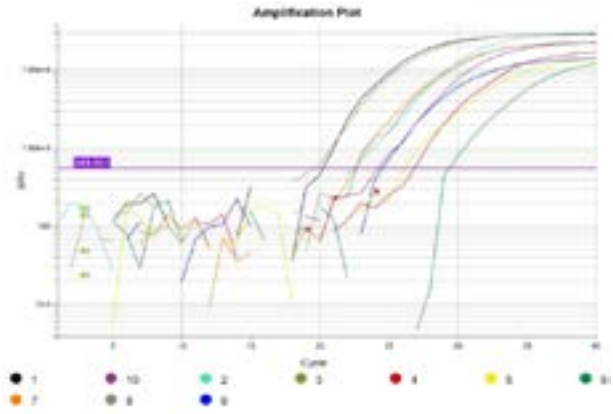
Note: several of the most characteristic representatives are shown for each class. In the upper part of the figure, on the target sequence of 18S rRNA are shown: forward primer (primer F), reverse primer (primer R), and fluorescent probe (probe).

Figure 1 – Results of BLAST analysis of the 18S rRNA gene region for some vertebrate species



Note: forward primer and probe (A), forward and reverse primers (B), reverse primer and probe (C).

Figure 2 – Double-stranded interactions between oligonucleotides designed for 18S rRNA gene detection



Note: Numbers indicate DNA samples of the following animals: 1 – cow, 2 – sheep, 3 – goat, 4 – chicken, 5 – duck, 6 – camel, 7 – pig, 8 – pollock, 9 – edible frog, 10 – salmon.

Figure 3 – qPCR results for the reference 18S rRNA gene on DNA samples from a number of vertebrates.

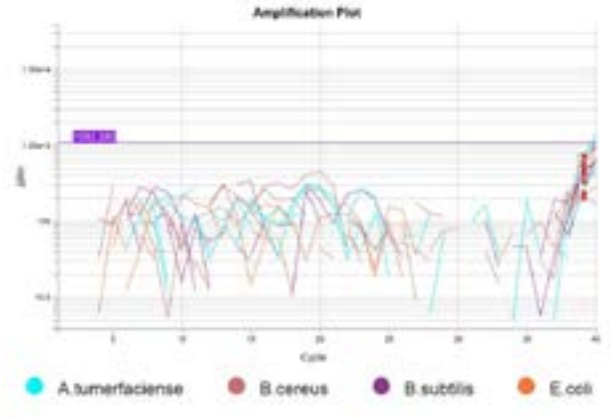


Figure 4 – RT-PCR results for the reference 18S rRNA gene on DNA samples from prokaryotes organisms

Table 1 – The results of qPCR specificity study for the primer and probe set

Species	Ct value, cycle number
cow	20.52 ± 2.28
sheep	26.72 ± 4.46
goat	22.15 ± 3.10
chicken	28.40 ± 2.95
duck	28.65 ± 3.01
camel	30.97 ± 1.33
pig	24.65 ± 1.25
pollock	24.08 ± 1.04
edible frog	25.89 ± 3.66
salmon	26.08 ± 2.64
E. coli	–
B. cereus	–
B. subtilis	–
A. tumefaciense	–

Thus, the specificity of the primers and probe set for the 18S rRNA gene detection in a number of vertebrates turned out to be absolute (the desired sequence was detected in 100% of the studied samples). Moreover, there were no problems with the determination of the 18S rRNA gene for the species from the classes of Bony fish (salmon, pollock) and Amphibians (edible frog), despite the fact that their sequence of the 18S rRNA gene complementary to the forward primer contains SNP (Figure 1). At the same time, in chromosomal DNA samples of both gram-positive (*B. cereus*, *B. subtilis*) and gram-negative (*E. coli*, *A. tumefaciense*) bacteria, the 18S rRNA gene was not detected in 100% of cases either.

To calculate the efficiency of the qPCR reaction and the sensitivity of the developed set of primers / probe, a series of dilutions of domestic pig genomic DNA (*Sus scrofa*) was used, a graphical representation of the results in the form of Ct is shown in Figure 5.

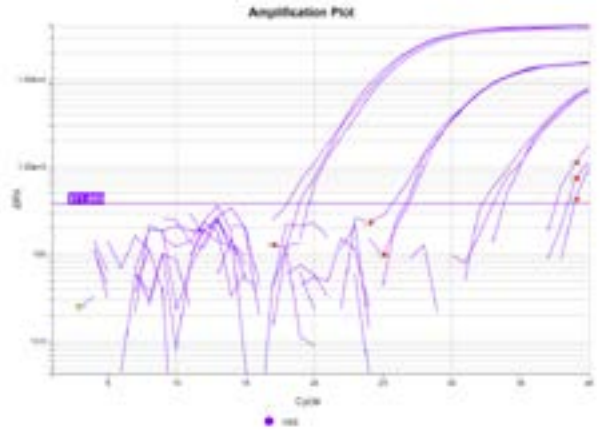


Figure 5 – qPCR results for the 18S rRNA reference gene on a series of dilutions of domestic pig DNA samples

According to [18, 19], the size of the domestic pig genome is about 2.7 Gbp, which made it possible to convert the dilutions of DNA samples into the number of whole genome copies and construct a standard curve shown in Figure 6.

Data analysis was performed in the Q-Gene program according to [20]. The sensitivity of the developed set of primers / probe determined by us was 10 fg of total pig chromosomal DNA. In terms of the genomes number [18], the sensitivity limit was 1.64 genomes. Thus, we have shown that the linear dynamic range (LDR), at which the approximation coefficient R^2 was equal to 0.99, had a length from 1.64 to 1642.5 genome copies per reaction [21]. The efficiency of the qPCR

reaction ranged from 87% to 113% (slope = -3.68) according to [22]. The exact number of copies of the 18S rRNA gene in the pig genome has not yet been established [23]; the number of 45S clusters is known to be from 4 to 6 [24, 25], which, by analogy with the human genome, which has 10 clusters, limits the number of copies of the pig 18S rRNA gene within 300 per genome.

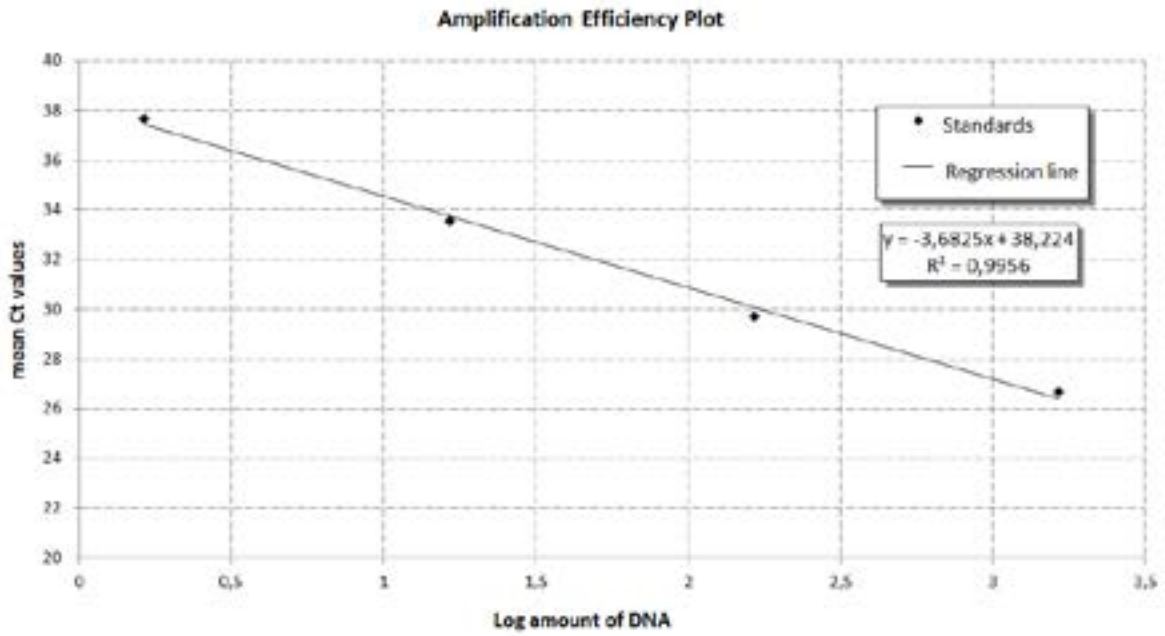


Figure 6 – qPCR data of the series of dilutions of domestic pig chromosomal DNA samples. Q-Gene program.

In order to determine the possibility of using primers and a probe for the 18S gene in a multiplex reaction, we carried out qPCR in parallel with the detection of several porcine genes most often used as reference: β-actin (TaqMan probe labeled with FAM), 23S ribosomal protein (TaqMan probe labeled with JOE), GAPDH (TaqMan probe labeled with Cy5). The obtained results are presented in Table 2, where the first row shows the average threshold cycles for the singleplex reaction, from second to fourth rows show different variations of the duplexes, and the fifth row shows the tetraplex. There were no significant differences between the results for the 18S rRNA gene in different variations of qPCR.

Table 2 – Comparative analysis of qPCR performed in singleplex and multiplexes

qPCR variants	Average values of threshold cycles (n = 5) from fluorescence detection channel			
	ROX (18S)	FAM (β-actin)	JOE (RPS23S)	Cy5 (GAPDH)
1	22.13 ± 0.89	-	-	-
2	22.20 ± 0.91 (0.90)	35.04 ± 0.60	-	-
3	22.14 ± 1.05 (0.99)	-	36.46 ± 0.58	-
4	22.00 ± 1.10 (0.83)	-	-	36.99 ± 0.53
5	21.45 ± 1.35 (0.32)	34.25 ± 0.30	37.16 ± 0.33	36.96 ± 0.44

Note: In brackets is the actual t-test calculated in Microsoft Excel as a two-sample t-test with unequal variances and two-tailed distribution; the number of measurement repetitions was 10 or more (each of the multiplexes was compared with a singleplex). The significance level, indicating that the difference between the samples is significant, was taken as $p \leq 0.05$.

Since the use of multiplex real-time PCR may be associated with the selection of optimal conditions for the operation of all components of the multiplex, we studied the working range of 18S primers / probe depending on the concentration of Mg^{2+} (Figure 7). Previously, we established the optimal concentration of Mg^{2+} equal to 2 mM. The study was conducted on DNA samples from domestic chicken; the minimum established working concentration of Mg^{2+} was 1.2 mM, the maximum was 3 mM. At a concentration of Mg^{2+} below 1.2 mM, the qPCR reaction did not occur; at a concentration above 3 mM, the specificity of the reaction decreased. Thus, to select the reaction conditions in the multiplex, it is possible to vary the concentration of Mg^{2+} within 1.8 mM.

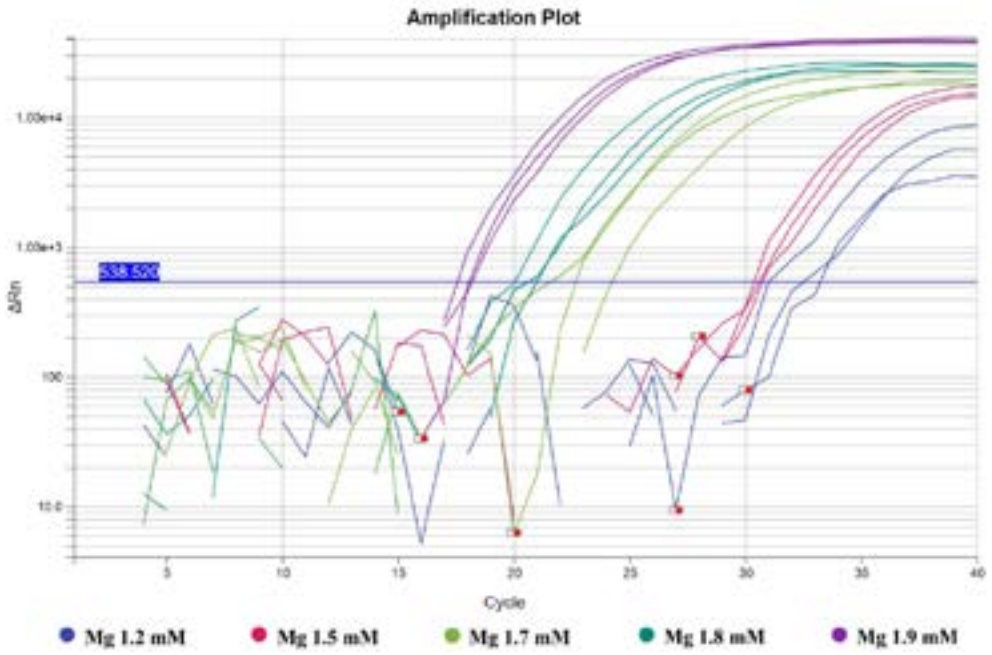


Figure 7 – qPCR results with varying Mg^{2+} concentrations on samples of chicken DNA.

Conclusion

The set of fluorescent probe, forward and reverse primers that we constructed showed 100% specificity for the eukaryotic 18S rRNA gene. DNA samples of cow, sheep, goat, chicken, duck, camel, pig, pollock, edible frog and salmon were examined – the reference gene was detected in all samples. At the same time, the 18S rRNA gene was not detected in the DNA samples of prokaryotic organisms, both gram-negative (*E. coli*, *A. tumefaciense*) and gram-positive (*B. cereus*, *B. subtilis*) bacteria. The versatility of the developed set for the detection of 18S rRNA in vertebrates from the classes of mammals, sauropsids (including birds), amphibians, and bony fishes was confirmed by the conducted qPCRs. The detection limit of the set, measured on the genome of the domestic pig, was 1.6 genomes (about 450 copies). The developed set can be used in multiplex qPCR without reducing sensitivity to the reference 18S rRNA gene, the number of target genes could be increased up to four. We also showed the possibility for varying the reaction conditions of the qPCR by the concentration of Mg^{2+} within 1.8 mM (from 1.2 mM to 3.0 mM) without reducing the specificity of the set.

Conflict of interest

All authors have read and are familiar with the content of the article and have no conflict of interest.

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