Research Article

A New Approach for Accurate Identification of Allele State Based on Real-Time PCR for Biomedical Tasks

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Abstract. Genotyping of single nucleotide polymorphisms (SNPs) is an important task in medicine, veterinary medicine and biology. Precise differentiation of SNPs can be challenging. Methods based onTaqman can lead to false positive results due to non-specific annealing of the probe. The aim of this research was to develop a new approach for the accurate differentiation of SNPs based on real-time PCR with Taqmanprobes and their rivals.The rivals competed with the Taqmanprobes for annealing to the site. The rivals blocked the nonspecific allele so that the Taqmanprobe could not anneal to it. Thus,the Taqmanprobe only detected specific alleles.This approach madeit possible to fine-tune the diagnostic system by selecting the ratio of Taqmanprobes and rivals (in non-equimolar amounts too).The new approach was tested on several diagonally significant SNPs in veterinary medicine.Using Taqman probes and rival probes showed a significantly greater specificity and efficiency in the determination of both homozygotes and heterozygotes than when conventional systems based only on Taqmanwere used.

Keywords: SNP, allele identification, real-time PCR, fluorescent dye

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1. Introduction.

Genotyping of a single nucleotide polymorphism (SNP) is an urgent and common task for genetic mapping of complex traits and, in particular, for the diagnosis of hereditary diseases [1,2,3,4]. The most popular methods for solving this type of problem are methods based on the polymerase chain reaction due to the simplicity of the analysis and relatively low price.A fairly large number of suchlike techniques have been developed [6], for example, allele-specific PCR [3, 8], oligonucleotide ligation [8], and others. But all of them have a common significant drawback –they are multistage, which negatively affects the capacity and cost of such systems [9, 10] and increases the risk of contamination due to the necessity to work directly with PCR products.One-step

detection systems based on RT-PCR have also been developed, such as the reaction with two allele-specific TaqMan-probes annealed at one site [11], as well as the reaction with ETL-primers [9].However, this method also has certain restrictions.In particular, it is not always possible to select the appropriate ETL-primers [9], which does not allow to detect many SNPs.The reaction with allele-specific TaqMan-probes also has its drawbacks. First, it lacks specificity, leading to erroneous interpretation of the results. [12].One of the options for solving this problem is to add complex modifications to the probe - MGB or LNA nucleotides - which increase the efficiency of allele discrimination. However, this will increase the cost of such systems lot.The additionof LNA nucleotides in large quantities leads to a shortening of the probe, which can increase the number of alternative nonspecific binding sites in genomic DNA.Unequal optical characteristics of various dyes and possible overlapping of their fluorescence spectra complicate the analysis of the results [13].

We have developed an alternative method for genotyping with a high specificity, at least comparable to that when using MGB probes or LNA nucleotides. This method doesn't require the addition of expensive modifications and can be fine-tuned.The reaction is carried out in two separate volumes, each of which contains the components of the reaction mix, primers, a probe with a FAM dye, complementary to one of the alleles, and a "probe-rival", complementary to the other allele.The main condition for the work of the proposed system is the ability to fine-tune the specificity of the reaction by changing the concentration ratios of the "probe-rival" and the probe, which is impossible in "ordinary" multiplex reactions, where the probes are equimolar.Moreover, another advantage is that diagnostic systems for both alleles are supplied with the same dye."Probe-rivals" are standard oligonucleotides with 3'-end blocked (for example,with an amino group). Despite a large number of oligonucleotides, the use of "probe-rivals" is profitable, since the cost of even one LNA nucleotide in a probe exceeds the cost of one"probe-rival".

The above technique has been tested on a number of diagnostic systems, including the systems for the diagnosis of the c.693 + 304G>A mutation in the PKLR gene of a domestic cat(*FelisSilvestrisCatus*). [14,15,16]

2. Material and Methods

The sequences of the designed primers, probes and probe-rivas for the diagnostic system for the mutation in the PKLR gene of the domestic cat were developed manually

Table 1: primers, probes, ”probe-rivals” used in the diagnostic system for the PKLR gene.

PrimerF	GACACGGGTTCCTGATTTCCT
Primer R	CCTGCAGGCATGGGAAGAG
Probemut	FAM- CCCGCTCCACAACTCTGCCCCC-BHQ1
Probe-rivalwt	CCGCTCCACGACTCTGCCCCC-NH2
Probewt	FAM- CCGCTCCACGACTCTGCCCCC –BHQ1
Probe-rival mut	CCCGCTCCACAACTCTGCCCCC-NH2

Table 2: probes for the diagnostic system without “probe-rivals”.

Probewt	Probemut
ROX- CCGCTCCACGACTCTGCCCCC - BHQ2	FAM- CCCGCTCCACAACTCTGCCCCC-BHQ1

using the Vector NTI 10.0.1 software (Thermo Fisher Scientific, USA). They are presented in the Table 1.

The RT-PCR reactionmix withtheprobe-rivals is divided into two volumes, each of which contains the following oligonucleotides:

1. Forward primer (F)
2. Reverse primer (R)
3. Probe complementary to one of the alleles (wt – wild-type, mut – mutant allele)
4. Oligonucleotide – “probe-rival”, complementary to another allele.

For comparison, we have developed a diagnostic system for detecting mutations in the PKLR gene in the classical version (without probe-rivals), working in the multiplex mode. The reaction mix included two probes for detecting the wild-type and mutant alleles (Table 2) with different dyes. Other components of the reaction mix are the same as in the first case.

Preparation of positive controls for the wild-type and mutant test system.

For the test systems checking and tuning, as well as for their further use, control DNA samples that imitate both homozygotes and the heterozygous carrier of the mutationare required.

For this, we created two genetic constructions - plasmids containing a wild-type and a mutant variant of the PKLR gene fragment.A positive control for the wild-type test system was obtained by amplifying a fragment of the PKLR gene of a healthy cat and cloning it into the pJet1.2 vector (Fig. 1.).The mutant variant was made by adding a substitution into the “wild-type” genetic construction using PCR (Fig. 2.). The heterozygous carrier

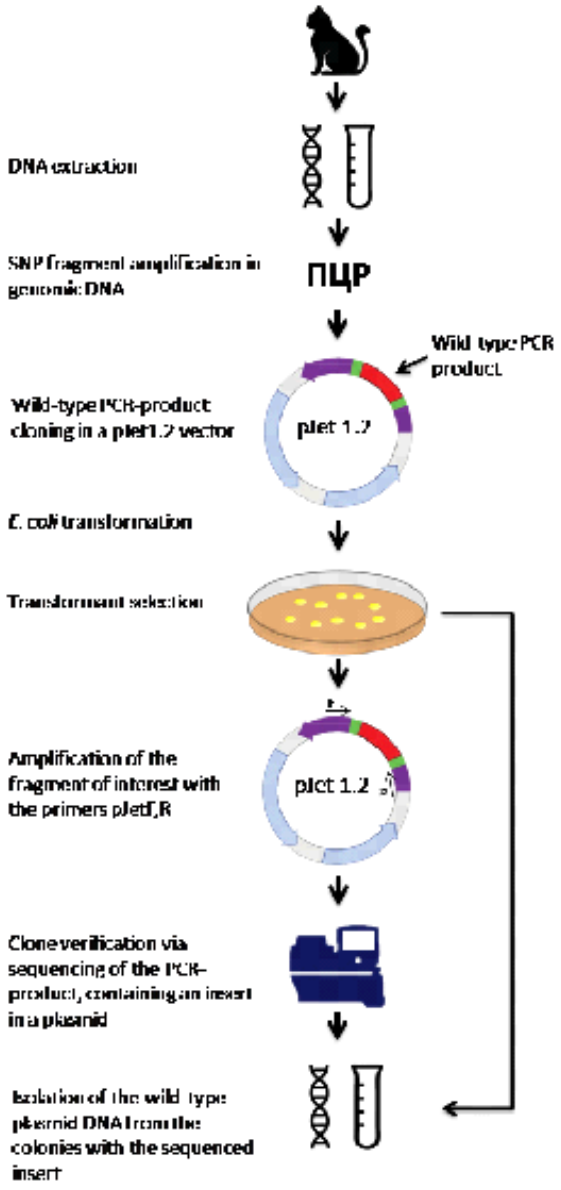

Figure 1: Experiment scheme: wild-type control obtaining.

imitation was made by equimolar mixing of both types of plasmids after measuring their concentration by agarose gel electrophoresis.

Figure 2: **Total DNA extraction of acat** wascarried out from buccal epithelium using a method adapted for animals with cetyl-trimethyl-ammonium bromide (CTAB) [17].

Amplification of a fragment of genome DNA, containing SNP. We used primers for the diagnostic system itself, indicated in the Table 1. AmplificationwascarriedoutonTercik thermal cycler (DNA-Technology, Russia). Theprogramfortheamplification: 94°C – 200s, then 40 cycles: 94°C– 18s, 60°C– 40s, 72°C– 40s, final elongation- 72°C 5 min.

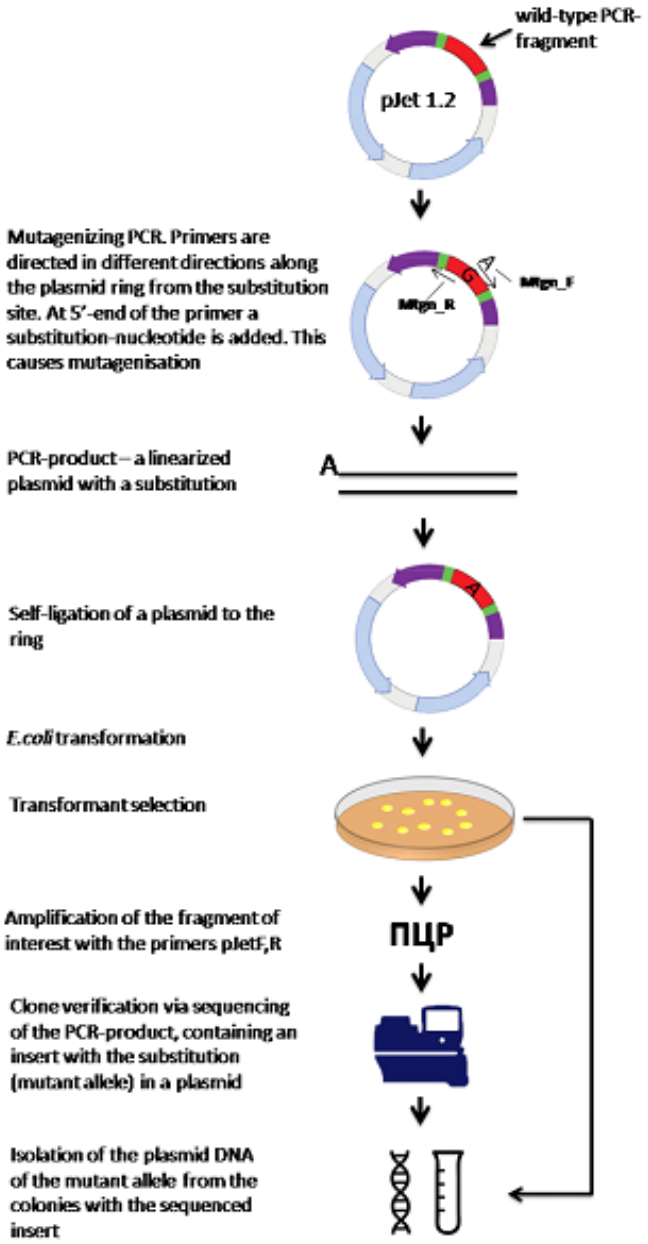

Figure 2: Experiment scheme: mutant control obtaining.

We used *Taq*-polymerase, *Taq*-buffer, $MgCl_2$, and dNTPmanufactured by LTD "Beagle", Russia.

Amplicon was cloned into the pJET1.2 vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA). Plasmid was used to transform cells of *E. coli* strain DH5α.Colonies were verified by PCR with primers flanking the insert (primers pJetF and pJetR, supplied with the kit). Theprogramfortheamplification: 94°C – 5 min, then 35 cycles: 94°C- 15s, 58°C-30s, 72°C-30s., final elongation - 72°C 5 min. PCR products of the

Table 3: Mutagenizing primers.

Forward primer	PO4-AACTCTGCCCCCGGCTCGCCCC
Reverse primer	PO4-GTGGAGCGGGGGCACGGGGC

target length were sequenced.Colonies containing the target sequence were cultured in liquid LB medium [18] to produce plasmid DNA.

Plasmid DNA isolation was performed by alkaline lysis[19].

The addition of a substitution into the wild-type plasmid (Fig. 2) was performed to obtain an artificial fragment of the mutant allele.ForthisweappliedaPCRmethod. The sequences of the designed mutagenizing primers are shown in the Table 3. The reaction was carried out with the fragment LongAmp® TaqDNAPolymerase (NewEnglandBiolabs, USA) in the thermal cycler Tercik (DNA-Technology, Russia).

A substitution is added to the 5'-end of the "forward" primer.The product of the PCR reaction is a full-length linearized plasmid (Fig. 2) with a substitution at the end, which was then self-ligated into a ring using T4 ligase from the PCR Cloning Kit and cloned into *E. coli* DH5α bacteria.The selection of transformants was carried out by sequencing the PCR products obtained with the primers flanking the insert (pJetF and pJetR).

The diagnostic system was tested in a RT-PCR thermal cyclerANK-16 (Institute for Analytical Instrumentation, Russia).Theprogramofamplification: 94°C – 200s, then 40 cycles: 94°C– 18s, 60°C– 40s, 72°C– 40s, final elongation- 72°C 5 min.

Results and discussion

We have tested the PKLR diagnostic system in the classical version (multiplex PCR with detection of the wild-type and mutant alleles in separate optical channels).It was also used to select the optimal annealing temperature, which turnedout to be 60 °Cfor a given combination of primers / probes and device.

One can see that the specificity of the diagnostic system based on the classical multiplex without additional modifications is insufficient.The nonspecific reaction of the mutant probe on the wild-type matrix is well expressed, the probe for the wild-type allele does not distinguish even one homozygote from another.

The use of "probe-rivals" and separate detection of alleles in two different reaction volumes makes it possible to achieve the best balance between the specificity and efficiency of the system and fine-tune the specificity by changing the concentration ratio of the probe and the "probe-rival".The PKLR diagnostic system was tested in a range of "probe-rivals"-to-probe ratios from 1: 1 to 5: 1. The results are presented in Tables 4 and 5(PKLR wt diagnostic system and PKLR mut diagnostic system, respectively). For a

Table 4: Relationship of the efficiency of the reaction for detecting a wild-type allele and the ratio of the concentrations of the ”probe-rival” and the probe. The efficiency is calculated by the software of the ANK-16.

Diagnostic system	Matrix	1:1	2:1	3:1	4:1	5:1
PKLR_wt	wt	11,4	16,28	19,47	14,19	19,2
	heterozygote	12	7,66	7,76	6	7,46
	mut	6,5	2,86	1,7	0	0
	ΔEff	5,5	4,8	6	6	7,46

Table 5: Relationship of the efficiency of the reaction for detecting a mutant allele and the ratio of the concentrations of the ”probe-rival” and the probe. The efficiency is calculated by the software of the ANK-16.

Diagnostic system	Matrix	1:1	2:1	3:1	4:1	5:1
PKLR_mut	wt	4,87	7,35	4,2	4,2	3,3
	heterozygote	13,6	14,35	16	12	13,38
	mut	20,04	14,88	18,93	12,24	15,92
	ΔEff	8,73	7	11,8	7,8	10,08

quantitative assessment, the ΔEff parameter was used. It shows the difference in the efficiency of a specific reaction on a heterozygous matrix (which is always lower than the reaction on a specific homozygous matrix) and a non-specific reaction.

Based on the data presented (Tables 4, 5), one can notice a significant difference in the effectiveness of specific and non-specific reactions for all diagnostic systems. The optimal probe: probe-rival ratio for the wild-type and mutant diagnostic systems was different (5: 1 and 3: 1, respectively).

Note that for the wild-type (Table 4) system, wehave achieved a total absence of a nonspecific reaction, which is completely blocked when the concentration ratio of the probe and the probe-rival is 4:1 or more. In these cases, it is possible to move from quantitative analysis to qualitative. Note that in the ”classical” version of detection using multiplex PCR, it is impossible to achieve such fine tuning, since a change in the concentration ratio of probes, which increases the specificity of reaction of one of them, automatically reduces the specificity of reaction of the second, and the only degree of freedom is a change in the annealing temperature of the probes by changing their length, which is clearly not enough.Attempts to increase specificity by raising the annealing temperature in the amplification program to an extremely high (but still allowing the reaction) leads to a decrease in the efficiency of allele discrimination due to a very strong drop in the efficiency of the reaction on a heterozygous matrix.

We checked the reproducibility of the obtained resultsof the diagnostic system for the detection of the PKLR wild-type allele (Table 6).Based on the data obtained, one can see that the ranges of efficiency values for nonspecific reaction and reaction on

TABLE 6: The reproducibility of the obtained results of the diagnostic system for the detection of the PKLR wild-type allele (5: 1).

Experiment №	wt/mut	mut/mut
1	5,6	0
2	7,74	0
3	7,75	0
4	6,77	1,42
5	5,96	0
6	6,61	0
7	5,11	0
Average efficiency	6,5	0,2
Minimum ΔEff (between the worst result in a heterozygote and the best in a non-specific reaction)	3,69	

a heterozygous matrix do not overlap with each other.The minimum ΔEff (between the worst result in a heterozygote and the best in a nonspecific reaction) was 3.69, which allows accurate genotyping of the presented samples.Comparing the difference in efficiency between specific and nonspecific reactions in our system, and in the system, which uses MGB probes in multiplex mode [1], one can see that in the second case, the nonspecific reaction is not effectively inhibited. In particular, its significant growth is observed on the mutant allele. This can lead to erroneous detection of this variant as a heterozygote.

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