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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Development of a Multiplex PCR Test System for the Determination of a Transgene Based on the pBC1 Plasmid and Its Derivatives for the Expression of Recombinant Proteins in *Mus musculus* Milk

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Abstract—A multiplex PCR test system for identification of the regulatory sequences of genetic constructs for transformation (promotor, insulator, and terminator) in the *Mus musculus* genome and for transgenic animal selection by genotyping with horizontal agarose gel electrophoresis detection was developed. The proposed system was validated by genotyping mouse strains producing human lactoferrin, heat shock protein HSP 70, firefly luciferase, and lysozyme, which were obtained by microinjections of linearized DNA into murine zygote pronucleus with random transgene integration into the genome using the pBC1 plasmid for expression of the gene of interest in milk of transformed animals (milk expression vector kit).

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The technology of creating genetically modified animals producing recombinant proteins in milk reaches a level of industrial use. The first pharmaceutical products based on milk of such animals have been created. When expressed in milk, the protein of interest can be posttranslationally modified in animal cells, and it is economically advantageous to isolate it from milk in preparative amounts [1]. The construction of the vector for transformation involves the use of functional sequences for efficient vector operation, including the *cis*-regulatory elements of animal origin (e.g., *Capra hircus* promoter and *Gallus gallus* insulator).

A conventional vector for creating animals producing recombinant protein in milk is the pBC1 Milk Expression Vector Kit (Invitrogen, United States), based on the cryptic plasmid *Bacillus subtilis* [2]. It was used to create constructs for expression of recombinant proteins in milk of different animal species, both large farm animals (goat [3, 4], cow [5], and pig [6]) and laboratory animals (mice [7–10]).

The basic method used to determine the presence of insertion of a transgene in the genome of a producer animal is PCR. Although the PCR technology has been developed sufficiently well, the key step in the use of this method is the creation and validation of a test system for obtaining representative results [11]. Unfortunately, the manufacturer did not propose a universal test system for determining genetically modified animals derived on the basis of the pBC1 vector, and creating a construct each time requires selection of primers, conditions for PCR, and its validation. The difficulty in designing a stable test system is associated with the high probability of a random multicopy insertion of the construct in this transgenesis method, with the risk of fragmentation of the constructs at microinjection as well as a tandem multicopy insertion in the forward or reverse coordination into the animal genome. Moreover, all these variants of transformational events may occur at the late stages of embryo development; as a result, the transgene will be present only in a small number of primary transgenic mouse cells. All this increases the probability of false-negative results, particularly in respect of the primary transgenic animals. In addition, it is necessary to ensure a high specificity of primers due to an increased homology between the regulatory elements of the genomes of different species, so that the resulting test system would effectively cut off the false-positive results.

To test a large number of strains of mice producing various human proteins that are maintained at the Core Facility of the Institute of Gene Biology, Russian Academy of Sciences, we have developed a universal multiplex test system for transgene determination based on the pBC1 vector and its derivatives, which

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Table 1. Structure of designed primers

To *Carpa hircus* β-casein promoter ("b-cas")	
Forward primer	AACAAATCCCCACTATCTAGAGAATAAGAT
Reverse primer	CTTAAGCTATAATGGAGAAAGTAACAAGCT
To *Gallus gallus* β-globulin insulator ("b-glob")	
Forward primer	TTTAGGCTGAAAGAGAGATTTAGAATGACA
Reverse primer	TCTTTGTCCTTCTATCCTATCTTCTTATCC
To *mus musculus* IGHC-2A-γ ("IGG")	
Forward primer	GATTCCCTCTTGGAAACTTCTAACTATGAC
Reverse primer	CTTTATTTATACAAGGGAAGCATGGAGATG

makes it possible to detect the *Capra hircus* β-casein promoter and *Gallus gallus* β-globin insulator in the transformation constructs that remain unchanged when inserted properly into the mouse genome. These elements are crucial for efficient transgene expression at random insertion into the genome, and their presence is not less important than the presence of the coding sequence, for which it is still necessary to develop a test system de novo. In this paper, we describe this development stage by stage.

Selection of primer sequences. Primers were selected taking into account the ultimate pattern of electrophoretic distribution of PCR products in gel to obtain amplicons 426 bp long for the β-casein promoter and 443 bp long for the β-globin insulator.

To cut off the false-negative test results, we selected primers to amplify the gene region encoding the constant γ-chain of mouse immunoglobulin A 1174 bp long (Gene ID: 380793) [12].

To simulate electrophoregrams, we used the Geneious 11 software tool for smart electrophoresis [13]. To select primers with desired parameters, we used the basic local alignment search in the NCBI Primer BLAST software [14].

Optimization of reaction conditions. In the standard reaction conditions, 1 μM solution of primers for the standard reaction volume of 20 μL is used. For the multiplex PCR, it is recommended to use primers for a multiplex system in the concentration range 2–10 μM [15]. An oligonucleotide concentration of 5 μM in a total reaction volume of 20 μL was used as a starting point for selecting primer concentration in the system.

Polymerase added by the manufacturer in the Isogene lyophilized PCR premix kit greatly reduces the temperature of the solution (in the range 2–2.5°C). Thermocycler detects the temperature at the site of the contact with the microtube wall rather than in solution. Therefore, with allowance for this fact, the actual reaction temperature specified in the thermocycler programmer should be greater than the calculated one (56°C).

Course of reaction. The reaction is performed under the following conditions: primary denaturation at 96°C for 5 min (40 cycles); secondary denaturation at 95°C for 40 s; annealing at 60°C for 40 s, at 72°C for 40 s, and at 72°C for 10 min.

The structure of the developed primers is shown in Table 1. The primers were synthesized by Evrogen (Russia). The initial reaction conditions we found unsatisfactory, because the reaction products (amplicons) showed a very high yield in the gel and, consequently, a low resolution of β-cas and β-glob signals. To increase the polymerase functioning efficiency, we performed an experiment with sequential increase in the magnesium chloride concentration to determine the optimal concentration of Mg^{2+} (Fig. 1). To achieve the optimum signal resolution in the agarose gel, we performed reactions with $MgCl_2$ concentrations of 1.6, 2, 2.5, and 3.5 μM. According to the electrophoretogram, the most effective concentration was 3.5 μM.

After testing the reaction conditions using the B-cas and B-glob primers pairs, we supplemented the system with the IgG-encoding oligonucleotides. As a result, we obtained positive genotyping results with the formation of all three amplicons (Fig. 2).

The presented system of the multiplex PCR assay for detecting the regulatory elements from *Capra hircus* and *Gallus gallus* genomes that are exogenous with respect to *Mus musculus* made it possible to reliably detect the transgene.

The system developed by us can be optimized in terms of the amplicon length for the producer animal genome by selecting to another conserved sequence or by reducing the amplicon length to 800 bp.

In addition, this system can be adapted to work with model animals of various species (mice, goats, rabbits, etc.), for which it is necessary to use the sequences of primer pairs with a high affinity for the animal genome for a positive control of the reaction and to refuse from the detection of the promoter region in the analysis of transgenic goats.

Genotyping the primary transgenic mice showed a high sensitivity of this system and the reproducibility

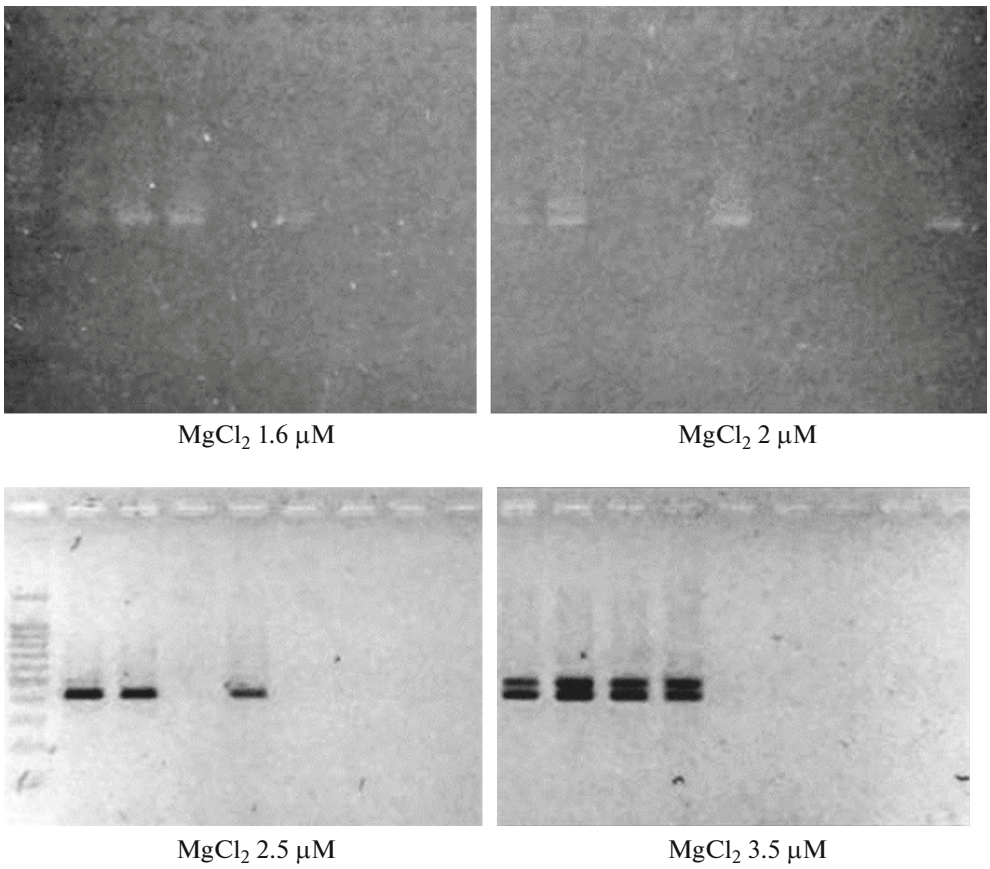

Fig. 1. Electrophoregram of multiplex PCR products (Hsp 70 with primers "b-cas" and "b-glob" in 2% agarose gel (TAE buffer)) at different magnesium chloride concentrations.

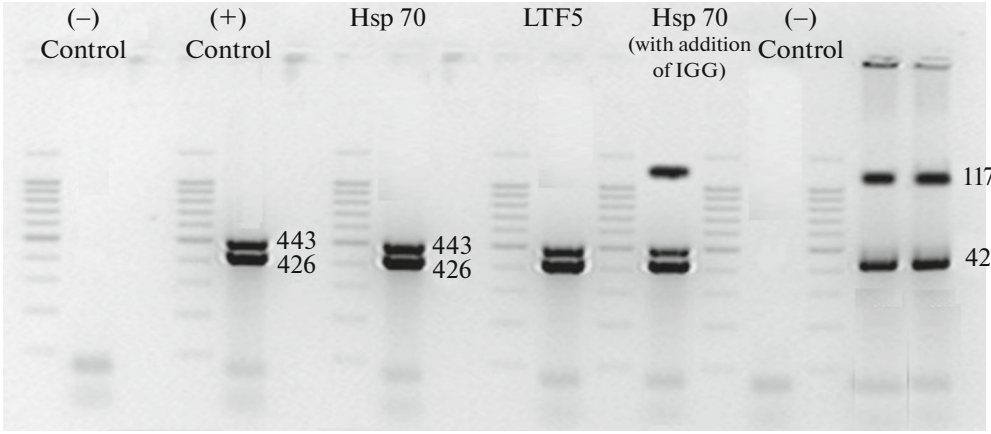

Fig. 2. Electrophoregram showing the distribution of multiplex PCR products in 2% agarose gel (TAE buffer). Primers "b-cas," "b-glob," and "IGG." Designations: LTF5—lactoferrin, Hsp 70—heat shock protein 70.

of results. In the study of a sample including more than 2000 mice of different strains, the repeatability of results was over 98.5%. In the analysis of the genome of the primary transgenic mice (200 F1 descendants), we detected the insertion of the construct with a high mosaicism. Only 7% of the tested animals carried the recombinant cassette in the genome.

The system presented in this paper can provide certification of animals used in preclinical trials in order to improve the quality of research and to make results

of these experiments more consistent with the high standards of medical and pharmaceutical industry.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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