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# Genetically Modified Coelenterazine-Dependent Luciferases as Reporters for *In Vitro* Assay

**Ludmila A. Frank*[a,b], Eugenia E. Bashmakova[a,b], Vasilisa V. Krasitskaya[a] and Alexander N. Kudryavtsev[a]**
[a] *Institute of Biophysics SB RAS*
*Federal Research Center*
*"Krasnoyarsk Science Center SB RAS"*
*50/50 Akademgorodok, Krasnoyarsk, 660036, Russia*
[b] *Siberian Federal University*
*79 Svobodny, Krasnoyarsk, 660041, Russia*

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*Among the most important tasks of modern biotechnology is the development of new analytical techniques suitable for highly sensitive and specific detection of diagnostically important targets rapidly expanding their range with the progress in biomedical science. In this view, particular attention is paid to elaboration of methods based on photosensitive technologies (fluorescent, chemiluminescent, etc.). The studies on bioluminescent systems of various luminous organisms are being carried out in the laboratory of photobiology of the Institute of Biophysics SB RAS for more than 30 years. The genes of many light-emitting proteins, photoproteins and luciferases, were cloned. The recombinant protein analogues were obtained, their spatial structures were determined and the mechanisms of functioning were proposed. With site-directed mutagenesis applied, the luciferase variants with unique beneficial properties were created. Highly sensitive analytical systems for diagnosing a number of biologically active compounds – hormones, oncogenes, infectious agents, etc. were developed on the basis of bioluminescent reactions of these proteins. The paper presents the recent years' results of these studies. These are the method of simultaneous detection of two targets in one sample on the basis of color variants of $Ca^{2+}$-regulated photoprotein obelin and the method for rapid and sensitive detection of tick-borne encephalitis virus based on the unique biospecific fusion protein of Renilla luciferase. The research results testify that the proposed analytical systems are competitive and applicable in modern medical laboratory.*

*Keywords: bioluminescence, $Ca^{2+}$-regulated photoprotein obelin, luciferase, immunoassay, genotyping, hybrid protein.*

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* Corresponding author E-mail address: lfrank@yandex.ru

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# Генетически модифицированные целентеразин-зависимые люциферазы как репортеры для анализа *in vitro*

**Л.А. Франк[а,б], Е.Е. Башмакова[а,б], В.В. Красицкая[а], А.Н. Кудрявцев[а]**
*[а]Институт биофизики СО РАН*
*Федеральный исследовательский центр*
*«Красноярский научный центр СО РАН»*
*Россия, 660036, Красноярск, Академгородок, 50/50*
*[б]Сибирский федеральный университет*
*Россия, 660041, Красноярск, пр. Свободный, 79*

*Одна из важнейших задач современной биотехнологии – создание новых методов анализа, пригодных для высокочувствительного и специфичного выявления диагностически важных мишеней, спектр которых стремительно расширяется с развитием биомедицинской науки. При этом особое внимание уделяется разработке методов, основанных на фотосенсорных технологиях (флуоресцентных, хемилюминесцентных и пр.). В лаборатории фотобиологии Института биофизики СО РАН более 30 лет исследуются биолюминесцентные системы различных светящихся организмов. Клонированы гены многих светоизлучающих белков – фотопротеинов и люцифераз, получены их рекомбинантные аналоги, установлены пространственные структуры и предложены механизмы их функционирования. Сайт-направленным мутагенезом созданы варианты люцифераз с уникальными полезными свойствами. На основе биолюминесцентной реакции этих белков разработаны высокочувствительные аналитические системы для диагностики ряда биологически активных соединений – гормонов, онкогенов, инфекционных агентов и т.д. В настоящей работе представлены результаты этих исследований последних лет. Среди них – способ одновременного выявления двух мишеней в одном образце на основе цветных вариантов* $Ca^{2+}$*-регулируемого фотопротеина обелина, а также способ быстрого и чувствительного выявления вируса клещевого энцефалита на основе уникального биоспецифического гибридного белка люциферазы Renilla. Результаты исследований свидетельствуют о конкурентоспособности предлагаемых аналитических систем и перспективности их применения в условиях современной медицинской лаборатории.*

*Ключевые слова: биолюминесценция,* $Ca^{2+}$*-регулируемый фотопротеин обелин, люцифераза, иммуноанализ, генотипирование, гибридный белок.*

## Introduction

Luciferases are the enzymes that catalyze oxidation of a substrate (luciferin) by molecular oxygen. The reaction product is an oxidized or, very often, decarboxydized substrate derivative in its excited state which relaxes to the ground state with the emission of energy as a visible light. A pair luciferase-luciferin is a key part of bioluminescent systems, which essentially differ depending on the origin of an organism: bacteria, fireflies, coelenterates, etc. Nowadays, the most known luminous organisms are ocean inhabitants: jellyfishes, hydroids, anthozoans, ctenophores, copepods, squids, shrimps et al. (Shimomura, 2006). Bioluminescent systems of the above-mentioned organisms contain luciferases of different types but the one and the same luciferin molecule – coelenterazine (CTZ). The studies on coelenterazine-dependent bioluminescent systems from hydroid *Obelia longissima*, jellyfishes *Obelia geniculata* and *Clytia gregaria*, soft coral *Renilla muelleri*, copepod *Metridia longa* and other luminous organisms are being carried out in the laboratory of photobiology of the Institute of Biophysics SB RAS for more than 25 years. Apart from fundamental research, considerable efforts have been made with regard to analytical applications of those *in vitro* (Frank et al., 1997, 2004a, 2007). The important moment here is that a high quantum yield of the reactions catalyzed by luciferases and a high signal-to-noise ratio provide high sensitivity of the assays based on these enzymes as reporters.

Application of luciferases in bioassay requires thermostability, definite spectral and kinetic bioluminescence characteristics, stability to chemical modifications etc., i.e. the properties, often extrinsic for luciferases of wild type. With genetic engineering applied, several variants of the enzymes of unique practicable properties were obtained. The paper presents analytical techniques based on the novel luciferase variants and demonstrates their practical potential.

## Coelenterazine-dependent luciferases and photoproteins

Coelenterazine-dependent luciferases are relatively small (16-36 kDa) single-chain polypeptides. There are two types of bioluminescent systems: 1) luciferases catalyzing CTZ decarboxylation in accord with classical enzyme-substrate kinetics and 2) photoproteins, which bioluminescent reaction proceeds in two steps (Fig. 1). Firstly, apophotoprotein catalyzes CTZ oxidation by $O_2$ producing hydroperoxide derivative, which is tightly but non-covalently immobilized into hydrophobic cavity of the protein. This complex is named “photoprotein” and its particular property is high stability. Polypeptide chain of the photoprotein contains three $Ca^{2+}$ – binding sites of EF-hand type. Nothing but binding of $Ca^{2+}$ causes fast decomposition of

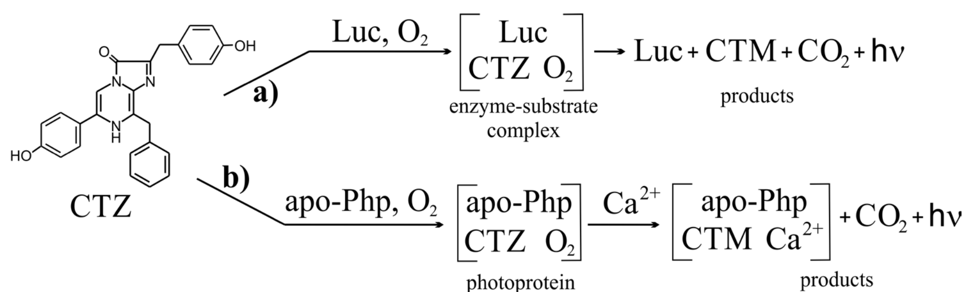


Fig. 1. Two types of coelenterazine-dependent bioluminescent systems. CTZ – coelenterazine; Luc – luciferase; CTM – coelenteramide; apo-Php – apophotoprotein

a photoprotein yielding a quantum of light, $CO_2$, and coelenteramide, bound with apoprotein. So, as opposed to luciferase, the bioluminescence of photoprotein does not depend on oxygen and occurs as a single flash. As the photoprotein directly participates in the reaction, the quantity of light emission is always proportional to its content (at saturated $Ca^{2+}$ concentration).

## Color variants of $Ca^{2+}$-regulated photoprotein obelin and dual assay on their base

A gene encoding apo-obelin was cloned and expressed in *E. coli* cells. Apo-obelin is easily "charged" by chemically synthesized coelenterazine under reducing and $Ca^{2+}$-free conditions. So a practically unlimited source of highly pure recombinant obelin becomes available (Illarionov et al., 2000). Obelin was shown to be stable to chemical modifications and its conjugates with biospecific molecules – immunoglobulins, haptens, oligonucleotides, etc., were obtained and successfully applied as labels in binding assay (Frank et al., 2004b). A spatial structure of the protein was solved and published in 2000 (Liu et al., 2000). Figure 2 presents an active site of the protein, with the amino acids immobilizing peroxycoelenterazine by the net of hydrogen bonds. Bioluminescence spectrum of obelin of wild type (OL-WT) is broad with a maximum at 485 nm and a shoulder at 380 nm. It means that the reaction product – coelenteramide in the excited state forms several structures of different energy (Shimomura, 2006). The replacement of amino acids in CTZ-binding cavity affecting its polarity and hydrogen-bond net may result in the formation of the prevailing structure that changes the bioluminescence spectrum of the mutant. For example, the single exchange W92→F resulted in addition of a new band with $\lambda_{max}$ = 390 nm with the intensity similar to that of the band at 485 nm. Besides, OL-W92F displays the faster bioluminescence kinetics as compared to OL-WT. With site-directed mutagenesis applied we obtained a large group of OL mutant variants with altered spectra and kinetics of bioluminescence. In search of obelin variants suitable for analytical applications we found two stable and active mutants: OL-W92,H22E, emitting fast ($k_d$ = 0.6 $s^{-1}$) blue-shifted signal ($\lambda_{max}$ = 387 nm) and OL-Y138F with slow ($k_d$ = 6.1 $s^{-1}$) red-shifted signal ($\lambda_{max}$ = 498 nm) (Frank et al., 2008; Krasitskaya et al., 2013a). Considerable differences in bioluminescence signals characteristics make it possible to separate them using wide band optic filters and temporal resolution (Fig. 3). The approach was successfully applied to simultaneously detect two gonadotrope hormones – luteinizing (hLH) and follicle stimulating (hFSH) or total and immunoglobulin-bound (macro) prolactins (Kudryavtsev et al., 2012) in clinical sera samples. The approach proved to be useful for large-scale research, providing a fast and sensitive two-target assay that lacks shortcomings of separate determination, to say nothing about cost and labor savings. Of special interest is the method developed for single-nucleotide polymorphisms (SNP) identification based on primer extension reaction (PEXT) with the following dual bioluminescent solid-phase microassay (Krasitskaya et al., 2013a). It includes four steps (Fig. 4):

1) Synthesis of DNA fragments, flanking polymorphic site (templates) by polymerase chain reaction.

2) PEXT reaction using two primers, having a 3'-terminal nucleotide, complementary to either normal (N) or mutant (M) allele. One primer (usually N) is labeled with 6-carboxyfluorescein (FAM), the other one – with oligoadenilate $(dA)_{27}$. In case of complete complementarity of the template and primer, the latter is elongated by DNA polymerase. The reaction mixture contains biotinylated deoxiuridine triphosphate

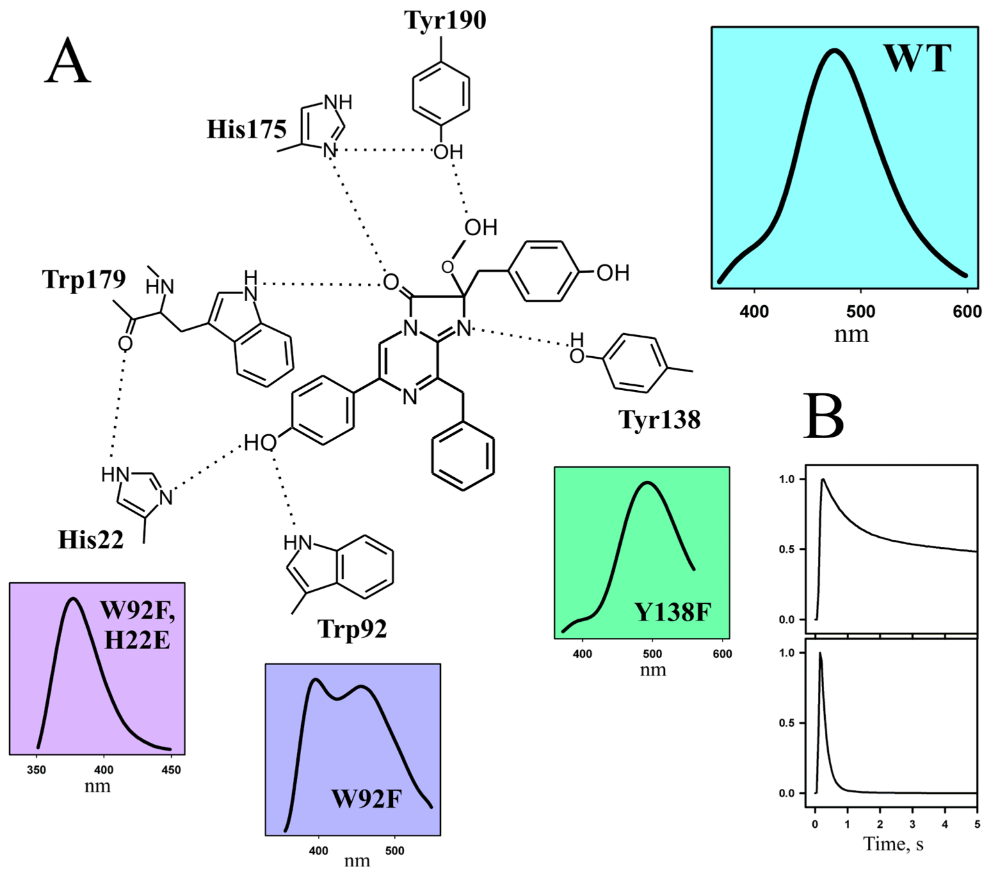


Fig. 2. A. Two-dimensional plot of obelin binding site. Insets: bioluminescence spectra of obelins of wild type (WT) and with denoted mutations. B. Bioluminescence kinetics of OL-Y138F (upper plot) and OL-W92,H22E (lower plot)

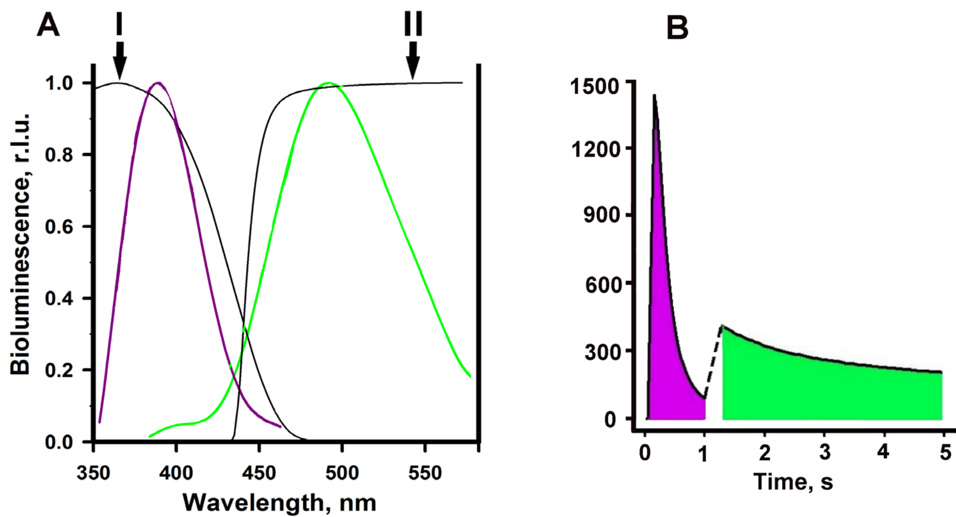


Fig. 3 A. Bioluminescence spectra of W92F,H22E and Y138F (violet and green lines, respectively), black lines show transparences of optic filters VB6 (I) and YB16 (II). B. Bioluminescence of obelin mutants mixture transmitted through filter I (fast violet signal) and filter II (slow green signal); dashed line, time for filter replacement; r.l.u. – relative light units

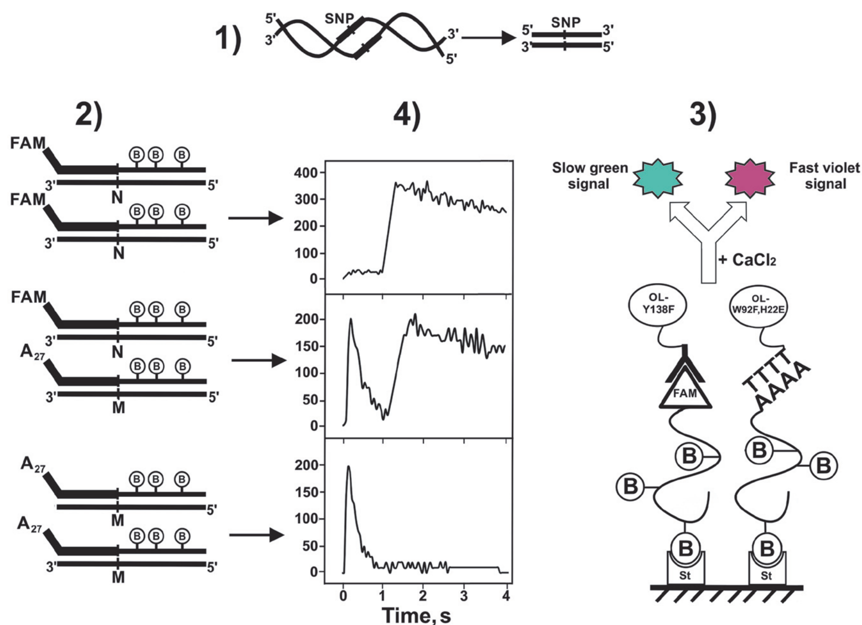


Fig. 4. Simultaneous detection of two gene allelic variants at single-nucleotide polymorphism genotyping. See explanation in the text

(B-dUTP) instead of thymidine triphosphate (TTP) providing inclusion of biotin residues into the resulting extension products.

3) Bioluminescent solid-phase microassay of the PEXT reaction products. Biotinylated amplicons are immobilized on streptavidin-activated surface of the opaque microtiter plate. The conjugates OL-Y138-anti-FAM IgG and OL-W92F, H22E-$(dT)_{28}$ are adhered on the surface in accord with the affinity and their bioluminescent signals are measured as described above.

4) The genotype of each sample is defined by the ratio green-to-violet signal (discrimination factor, D).

The method was applied to identify 4 SNPs associated with risk factors of hemostasis disorders (Bashmakova et al., 2015a) and 3 SNPs in gene of melanocortin-1 receptor (MC1R), associated with melanoma and non-melanoma skin cancer risk (Bashmakova et al., 2015b). Table 1 presents the results of studies of DNAs isolated from clinical samples. The genotype variants differed in D value by almost an order of magnitude or more suggesting by this a reliable genotyping of the assay. All the results were in complete agreement with those, obtained by using conventional RT-PCR techniques or Sanger sequences. At that the developed assay is essentially simpler and cheaper than the above mentioned technologies (Krasitskaya et al., 2013b). The developed technique can be easily applied in many other cases of SNP detection by using the corresponding primer sequences for steps 1 and 2 (Fig. 4).

## Thermostable variant of luciferase soft coral *Renilla muelleri* and assay on its base

The luciferase of soft coral *Renilla muelleri* (RmLuc) is a single-chain relatively small (36 kDa) polypeptide, catalyzing CTZ oxidation with the emission of blue light ($\lambda_{max}$ = 478 nm). The luciferase gene was cloned and the recombinant variant of the protein was

Table 1. Discriminatory factors (D) for the investigated polymorphisms

| Gene, mutation | Genotype variant | D |
|---|---|---|
| FII, **20210 G>A** | Normal homozygous **(GG)**<br>Heterozygous **(GA)** | 31.4±4.2 (n=25)<br>3.4±0.6 (n=5) |
| FV, R506Q, **16916 G>A** | Normal homozygous **(GG)**<br>Heterozygous **(GA)** | 18.7±4.3 (n=69)<br>1.7±0.5 (n=30) |
| FVII R353Q **10976 G>A** | Normal homozygous **(GG)**<br>Heterozygous **(GA)**<br>Mutant homozygous **(AA)** | 12.4±3.8 (n=20)<br>0.9±0.1 (n=11)<br>0.06±0.03 (n=4) |
| MTHFR, **677 C>T** | Normal homozygous **(CC)**<br>Heterozygous **(CT)**<br>Mutant homozygous **(TT)** | 2.73±1.4 (n=47)<br>0.47±0.13 (n=50)<br>0.06±0.022 (n=16) |
| MC1R, R151C **451 C>T** | Normal homozygous **(CC)**<br>Heterozygous **(CT)**<br>Mutant homozygous **(TT)** | 24.9±9.5 (n=316)<br>2.95±1.04 (n=51)<br>0.16±0.033 (n=3) |
| MC1R, R160W **478 C>T** | Normal homozygous **(CC)**<br>Heterozygous **(CT)**<br>Mutant homozygous **(TT)** | 23.15±9.22 (n=299)<br>2.09±0.93 (n=0.93)<br>0.2±0.093 (n=4) |
| MC1R, D294H **880 G>C** | Normal homozygous **(GG)**<br>Heterozygous **(GC)** | 20.9±8.6 (n=386)<br>3.95 (n=2) |

obtained and investigated (Titushin et al., 2008). To obtain a thermostable luciferase variant Rm7, the amino acids substitutions A55T, C124A, C130A, A143M, M185V, M253L, and S287L were sequentially introduced into the luciferase coding sequence (Stepanyuk et al., 2010). Rm7 displays essentially higher resistance to inactivation at 37 °C and almost a 1.5-fold improvement in light output in comparison with the native RmLuc (Fig. 5).

All luciferases are unstable to chemical modifications. Chemical synthesis of conjugates with biospecific molecule as a probe for detecting the corresponding target causes a loss of luciferase bioluminescent activity due to non-specificity of chemical reagents. To develop a bioluminescent probe based on Rm7 it was genetically fused with polypeptides, possessing affinity to the corresponding targets. Several hybrid proteins of the kind were developed in cooperation with the colleagues from the Institute of Chemical Biology and Fundamental Medicine SB RAS (Novosibirsk). Several unique single-chain antibodies (scAb) to human tumor necrosis factor alpha (αTNF) were produced by phage display technology (Vikhrova et al., 2011). The scAbs with the highest affinity were used for construction of hybrids scAb-Rm7. The proteins were expressed in *E. coli*, purified from periplasm and applied as labels in model αTNF solid-phase microassay (patent RF, 2014). The obtained results evidence the use of hybrid protein to provide αTNF detection in concentration of several ng/mL (Fig. 6).

The same approach was applied for the development of bioluminescent immunoassay of tick-borne encephalitis virus (TBEV) in ticks. TBEV is the causative agent of one of the most severe human neuroinfections. Thousands of cases of tick-borne encephalitis occur every year, with over half of them being recorded in Russia. TBEV is transmitted from the saliva of infected ticks within minutes after tick bite, but on the average only 5-10 % of ticks are TBEV-transmitting agents. Rapid and reliable virus detection can significantly decrease the

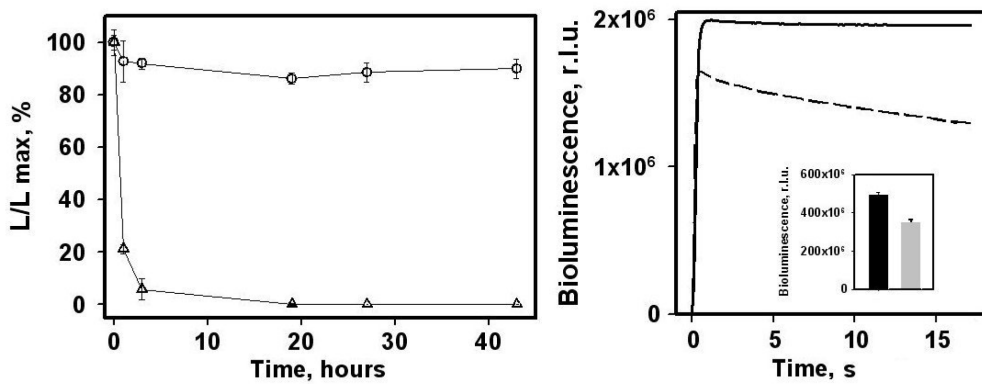


Fig. 5. Left panel: Thermostability of Rm7 (○) and RmLuc (Δ) at 37°C. L/Lmax, the residual bioluminescent capacity expressed as a percentage of initial activity. Right panel: Bioluminescent signal kinetics for Rm7 (solid line) and RmLuc (dashed line). Inset: Integrated bioluminescent signals (integration time 25 s) for Rm7 (black bar) and RmLuc (gray bar)

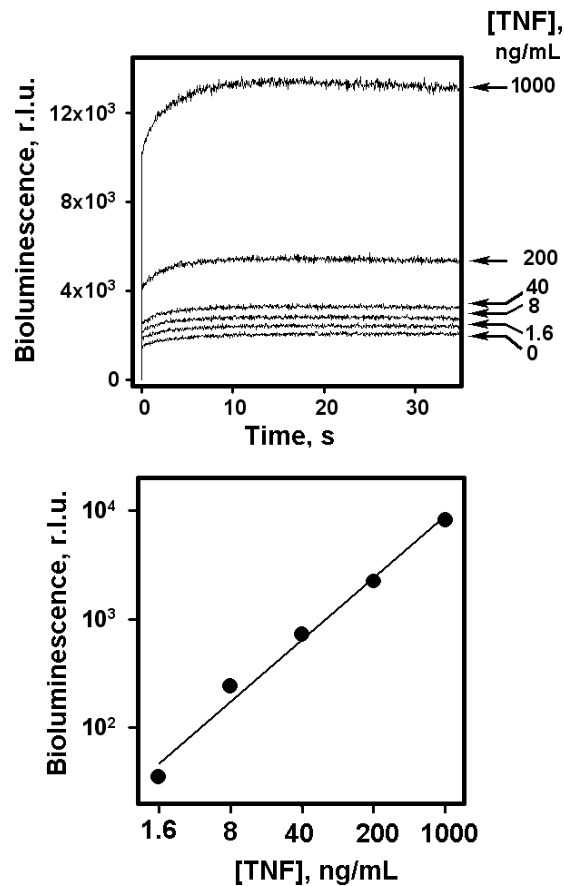


Fig. 6. Model solid-phase bioluminescent microassay of αTNF based on hybrid protein scAb-Rm7. Upper plot: Bioluminescent signals generated from the wells, activated with different quantities of αTNF. Lower plot: Integrated bioluminescent signals (over 40 sec) versus αTNF concentration

risks of unreasonable immunoprophylaxis (administered in Russia) or start the treatment in time. In order to develop a bioluminescent probe to TBEV, the luciferase Rm7 was genetically fused with a single-chain antibody sc14D5a at the C-terminus (Burakova et al., 2015). This hybrid contained variable domains of murine monoclonal antibody with a high affinity to envelope glycoprotein E, luciferase Rm7 connected through a flexible $(GGS)_4$ linker, and C-terminus poly-His-tag to simplify the protein purification (Fig. 7*A*). Eukaryotic proteins produced in bacteria do not carry post-translational modifications; they are often misfolded, and lack specific biological activity. This is a major problem the production of novel hybrid proteins faces. In our case, the proper conformation of single-chain mini-antibody domain is conditioned by the S-S bond formation. Due to the enhanced disulfide bond formation in cytoplasm, the *E. coli* Rosetta-gami strain was selected as the expression host for production of the target hybrid protein. The target hybrid 14D5a-Rm7 was expressed as a soluble protein with a proper folding: both domains revealed their adherent properties – affinity to the target antigen-protein E and bioluminescent activity (Fig. 7*B*). Model experiments with laboratory cultivated ticks (*Dermacentor marginatus*, laboratory strain 20-12, Omsk, Russia) were carried out to demonstrate analytical potential of the obtained hybrid protein. Extracts of five TBEV-infected ticks (virus strain 13120, Siberian subtype) were analyzed by bioluminescent immunoassay, colorimetric immunoassay using Enzyme Immunoassay kit D1154 (Vector-Best, Russia), and RT-qPCR method using Real Best RNA TBEV kit (Vector-Best, Russia). Three non-infected ticks were used as a negative control.

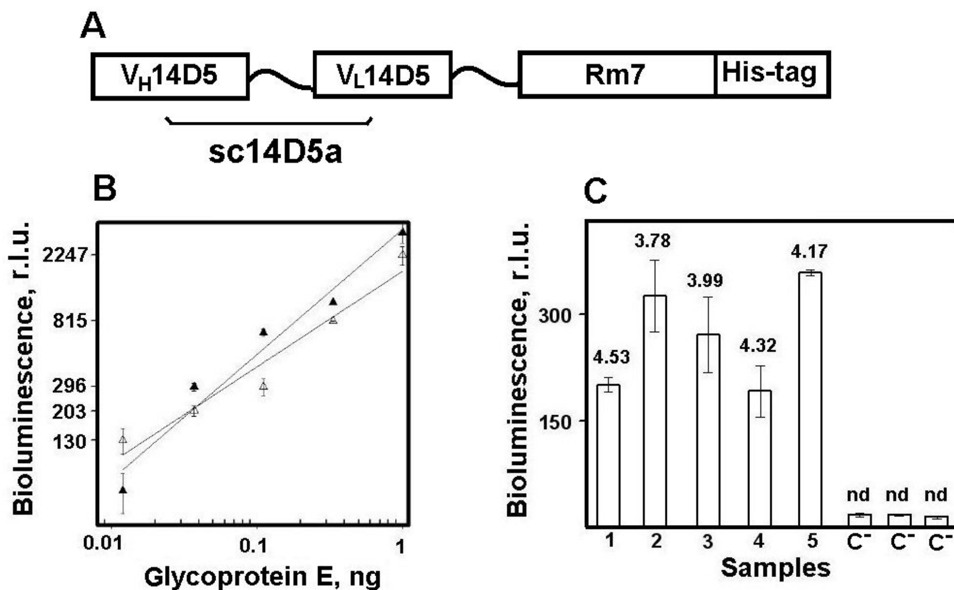


Fig. 7. A. Hybrid protein with 3 domains connected with flexible linkers. B. Solid-phase assay of the protein E using hybrid 14D5a-Rm7 as a bioluminescent probe. (Δ) – freshly prepared hybrid sample, (▲) – after two months of storage in frozen stage. C. Immunoassay of extracts of TBEV-infected and non-infected ($C^-$) ticks. Numbers above the columns show virus load measured as the log of TBEV RNA copies in the corresponding samples, detected by RT-qPCR technique. r.l.u. – bioluminescent signal integrated over 10 s in relative light units; nd – no positive signal detected

Table 2. Results of TBEV bioluminescent detection in native ticks

| D, Sample$^-$/ C$^-$ | C$^+$/C$^-$ | D, Sample$^+$/C$^-$ |
|---|---|---|
| 1.41±0.25, n=135 | 24.3±3.6, n=8 | 4.86±2.4, n=11 |

Colorimetric immunoassay did not detect the virus in both infected and control ticks. All the experiments involving bioluminescent immunoassay and RT-qPCR assay revealed the virus in all five infected ticks and showed a negative result in control samples (Fig. 7*C*). According to the RT-qPCR data, the developed bioluminescent immunoassay allowed the detection of almost $10^4$–$10^5$ virus particles or about 0.1 pg virions. At the same time, bioluminescent immunoassay is much simpler, cheaper, and faster – the bioluminescent signal is integrated during only 10 s.

The assay was applied to detect TBEV in natural ticks. Negative (C$^-$) and positive (C$^+$) samples from the same Vector-Best colorimetric kit were used as controls. The ratio of the sample bioluminescence to bioluminescence of negative control (sample/C$^-$), i.e. discrimination factor D, was proposed as a criterion of TBEV case. The obtained D value of TBEV-positive samples exceeds that of TBEV-negative samples almost 4-fold (Table 2).

## Conclusion

Knowledge of the structure of $Ca^{2+}$-regulated photoprotein obelin and its mechanism of functioning provided a directed search for this protein's variants possessing the improved bioluminescent properties. The color obelins were obtained with site-directed mutagenesis and the method to simultaneously detect two analytes in a single well, based on spectral and time resolutions of bioluminescent signals was developed. Its applicability and advantages were demonstrated at simultaneous detection of several dual targets including two gene allelic variants at SNP identification.

A sequential replacement of 7 amino acids in the luciferase of soft coral *Renilla muelleri* allowed the obtaining of a thermostable variant of the protein that served as a basis for creation of hybrid proteins. Additionally to bioluminescence activity, the genetically fused proteins revealed the affinity to respective targets.

Of particular interest is a hybrid designed for high sensitivity detection of tick-borne encephalitis virus. Sensitivity of the developed analysis is close to that of the RT-qPCR assay, but the assay is much easier and faster to perform.

The research results testify that the proposed analytical systems are competitive and applicable in modern medical laboratory.

## Acknowledgments

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