

## CAS13A: PURIFICATION AND USE FOR DETECTION OF VIRAL RNA

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The use of CRISPR-Cas systems in genome editing has recently become one of the major research areas. Meanwhile, CAS proteins can be employed to develop novel techniques for molecular diagnostics. Traditional approaches to the identification of microorganisms have a few drawbacks: they are time-consuming (microbiological methods), insufficiently sensitive (immunoassays), expensive or labor-intensive (PCR, sequencing). The aim of this work was to obtain a functionally active Cas13a protein that could be used as a diagnostic tool and study its behavior under different conditions and at various target concentrations. We constructed an expression vector with the *cas13a* gene of *Leptotrichia wadei* under the control of T7 promoter. We obtained a functionally active Cas13a RNase with pre-programmed activity, guide RNA, and a fragment of influenza B RNA sequence serving as a target. The functional activity of Cas13 RNase was assessed by fluorescence in the reaction mix containing guide RNA, target RNA, and a molecular RNA beacon. The obtained protein Cas13a was able to specifically recognize the target and did not exhibit any non-specific RNase activity. This study can become a basis for developing a novel, rapid, specific and sensitive method for pathogen detection.

**Keywords:** PCR, diagnostics, infectious diseases, CRISPR-Cas system, Cas13a

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**Received:** 31.05.2018 **Accepted:** 07.06.2018

**DOI:** 10.24075/brsmu.2018.021

## CAS13A: ПОЛУЧЕНИЕ И ИСПОЛЬЗОВАНИЕ ДЛЯ ОПРЕДЕЛЕНИЯ ВИРУСНОЙ РНК

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Использование CRISPR-Cas систем для редактирования геномов организмов в последнее время стало одним из магистральных научных направлений. Между тем белки системы CAS можно применять для разработки методов молекулярной диагностики. Традиционные подходы к идентификации микроорганизмов имеют ряд недостатков: они времязатратны (культуральные методы диагностики), недостаточно чувствительны (иммунологические методы), имеют высокую себестоимость и методически сложны (ПЦР, секвенирование). Целью работы было получение функционально активного препарата белка Cas13a и изучение его поведения в различных условиях, в том числе при изменении концентрации мишени, для дальнейшего использования в диагностических целях. Была создана генетическая экспрессионная конструкция, имеющая на 5'-конце T7-промотор и ген *cas13a* бактерии *Leptotrichia wadei*. Получены препараты функционально активной программируемой РНКазы белка Cas13a, направляющей РНК, а также РНК вируса гриппа Б (РНК-мишень). Функциональную активность РНКазы белка Cas13a определяли по появлению флуоресцентного сигнала в реакционной смеси, содержащей направляющую РНК, РНК-мишень, молекулярный РНК-маячок. Показано, что полученный препарат белка Cas13a способен специфически выявлять мишень на примере фрагментов РНК вируса гриппа Б и не обладает неспецифическими видами РНКазной активности. Данное исследование может стать основой для создания нового быстрого специфического и чувствительного метода идентификации микроорганизмов.

**Ключевые слова:** ПЦР, диагностика, инфекционные заболевания, CRISPR-Cas система, Cas13a

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**Статья получена:** 31.05.2018 **Статья принята к печати:** 07.06.2018

**DOI:** 10.24075/vrgmu.2018.021

Mankind has been fighting infectious diseases for many decades now. A serious threat is posed by those infections that cause outbreaks or epidemics [1]. Because a successful treatment outcome depends in the first place on the accuracy of the diagnosis, a search for novel diagnostic approaches continues. Unlike classical microbiological methods of

pathogen identification based on the use of differential culture media, polymerase chain reaction (PCR) ensures rapid detection of microorganisms regardless of the specifics of their life cycle. Evolution of sequencing techniques and free access to public databases containing sequencing data encourage a more active use of PCR [2].

Still, there is a need for novel molecular diagnostic techniques. There are a few obstacles preventing a wider application of PCR, including high equipment costs and a lack of laboratory facilities and qualified personnel. Among the proposed alternatives to PCR are mobile biosensors based on a combination of physical and biological approaches [3–5] and techniques that do not rely on complex equipment [6, 7]. We believe that the most promising technique that provides high specificity and sensitivity for single molecule detection is Specific High-Sensitivity Enzymatic Reporter Unlocking, or SHERLOCK [8, 9].

SHERLOCK combines isothermal amplification of total nucleic acids and Cas13a activity, allowing detection of both DNA and RNA molecules. Isothermal amplification ensures accumulation of target molecules, while Cas13a acts as a sensor capable of accurate target recognition, including single nucleotide polymorphisms [9].

Cas13a nuclease activity is initiated when CRISPR guide RNA (crRNA) binds to Cas13a entailing significant conformational changes in the protein structure aimed to form a channel for the binding of a target RNA [10]. When Cas13a “meets” target RNA, a guide crRNA/target RNA duplex is formed in a positively charged NUC lobe channel. The target RNA serves as an activator: duplex formation catalyzes the movement of catalytic domains towards each other, followed by the formation of an RNA cleavage site. The use of RNA probes enables a visual representation of fluorescence signal accumulation as Cas13 exerts its activity.

The aim of this work was to obtain a functionally active Cas13a protein and study its behavior under different conditions, including varying concentrations of a target molecule represented by an RNA fragment of the influenza B virus.

## METHODS

### Synthesis of the recombinant protein LwCas13a

To obtain the recombinant protein Cas13a of *Leptotrichia wadei* (LwCas13a), a codon-optimized gene synthesized *de novo* by Evrogen, Russia, was incorporated into the gene expression vector pET42b(+) under the control of *lacT7* promoter. Gene expression was induced in the cells of *Escherichia coli* BL21(DE3) pLysS, driven by isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The resulting Cas13a protein was tagged with octo-histidine on its C-terminus. The frozen bacterial cells were resuspended in the lysing buffer (20 mM Tris HCl, pH of 8.8, 500 mM NaCl, 5 mM  $\beta$ -mercaptoethanol) and lysed by exposure to cyclic pulsed ultrasound. The lysate was centrifuged for clarification at 15,000 g for 20 min; the supernatant was then used for

the affinity chromatography on automated medium-pressure system NGC Discover™ 10 (Bio-Rad, USA) with the 20 ml HisPrep FF 16/10 column (GE, Germany) pre-charged with Ni<sup>2+</sup> ions. To remove non-specifically bound impurities, Triton X-100 was added to the buffer solutions at a final concentration of 0.1%. The protein was eluted using a linear imidazole gradient (the final concentration was 0.5M). After chromatography, Cas13a-containing fractions were combined and dialyzed against a storage buffer (20 mM Tris HCl, pH of 8.0, 200 mM NaCl, 0.1 mM EDTA). Protein concentrations were measured spectrophotometrically at 280 nm wavelength using Implen NanoPhotometer (IMPLEN, Germany). Concentrations were calculated accounting for the extinction coefficient [11, 12].

### Acquisition of target and guide RNAs

Guide and target RNAs were obtained through PCR followed by the transcription of PCR products using the qPCRmix-HS SYBR kit (Evrogen, Russia) according to the manufacturer's protocol. Guide RNA was obtained using artificially synthesized oligonucleotide primers with self-complementary regions. To get a target RNA molecule, we amplified a plasmid fragment carrying a sequence of the influenza B virus and a sequence of the MS2 phage. *In vitro* transcription of the amplicons was aided by the MEGAscript® T7 Kit (Thermo Fisher Scientific, USA).

### Testing LwCas13a for non-specific nuclease activities

To test LwCas13a for non-specific nuclease activities, we measured fluorescence. Briefly, the fluorescence signal is emitted when the reporter RNA molecule RNaseAlert v2 Substrate (Thermo Fisher Scientific, USA) is cleaved. The reporter RNA molecule is an oligonucleotide beacon with a fluorescent dye sitting on its 5'-end and a quencher on its 3'-end. When the molecule is cleaved, the dye is separated from the quencher emitting light in the green spectrum at 520 nm wavelength. The final LwCas13a concentration of 450 nM was incubated at 37 °C for 2 hours in the reaction mix containing a nuclease buffer and the reporter RNA (40 mM Tris HCl, pH of 7.3, 60 mM NaCl, 6 mM MgCl<sub>2</sub>, 125 nM RNaseAlert v2 Substrate); fluorescence was measured in real time on the QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA). RNase A (Thermo fisher scientific, USA) was used for positive control; pure reporter RNA, for negative.

### Testing LwCas13a endonuclease activity

The reaction mix for testing LwCas13a endonuclease activity consisted of a nuclease buffer (40 mM Tris HCl, pH of 7.3,

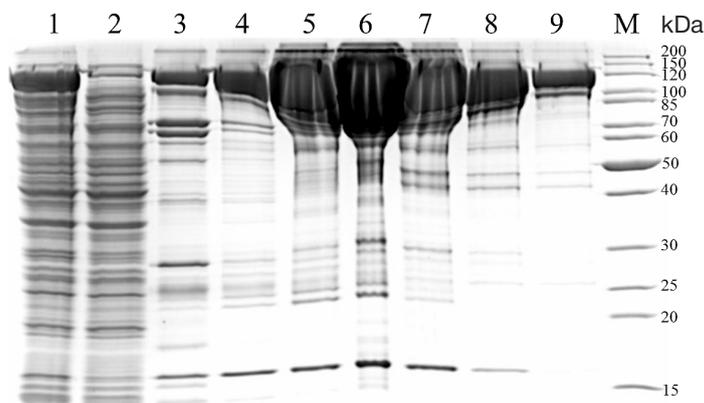


Fig. 1. SDS-PAGE of recombinant LwCas13a. Fraction analysis after IMAC: 1 — total protein; 2 — flow through; 3–9 — eluted fractions; M — molecular weight markers

60 mM NaCl, 6 mM MgCl<sub>2</sub>), 450 mM LwCas13a, 22.5 nM crRNA, 125 mM RNaseAlert v2 Substrate, 2 µl RiboLock RNase Inhibitor, 100 ng RNA of a tobacco mosaic virus (for the background), and different concentrations of target RNA. Fluorescence was measured in real time for 2 hours at 37 °C using the QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA).

## RESULTS

### Purification of the recombinant LwCas13a

The recombinant protein LwCas13a was obtained using affinity chromatography. Chromatography products were analyzed by denaturing electrophoresis, which revealed that induced *E. coli* cells had produced a water-soluble protein with a molecular weight comparable with the predicted LwCas13a weight (139.8 kDa) (Fig. 1).

### Optimization of reporter RNA (RNaseAlert) concentrations in the reaction mix

To achieve optimal fluorescence intensity, we conducted a series of model tests using RNase A (Fig. 2) and selected the

substrate concentration of 125 nM for further experiments. At this particular value the maximal dynamic range of 100, 000 arbitrary fluorescence units was provided.

### Testing LwCas13a for non-specific RNase activities

The obtained LwCas13a protein was tested for non-specific RNase activities by 2-h incubation with the fluorescent RNaseAlert v2 Substrate in the absence of crRNA and target RNA. The fluorescence signal was not changed during incubation. For positive control, we incubated the fluorescent substrate with RNase A; for negative control, the reporter RNA was incubated without any additives (Fig. 3). We found that our method yielded the LwCas13a protein that did not exhibit any non-specific RNase activity, which allowed us to proceed to the study of characteristics of its pre-programmed RNase activity.

### Testing method sensitivity using the influenza B virus

Our LwCas13a-based detection method was tested for sensitivity in a series of model tests, for which we employed a fragment of influenza B viral RNA sequence, which served as a target. The lower sensitivity threshold observed was 10<sup>7</sup> molecules of viral RNA (Fig. 4 and fig. 5).

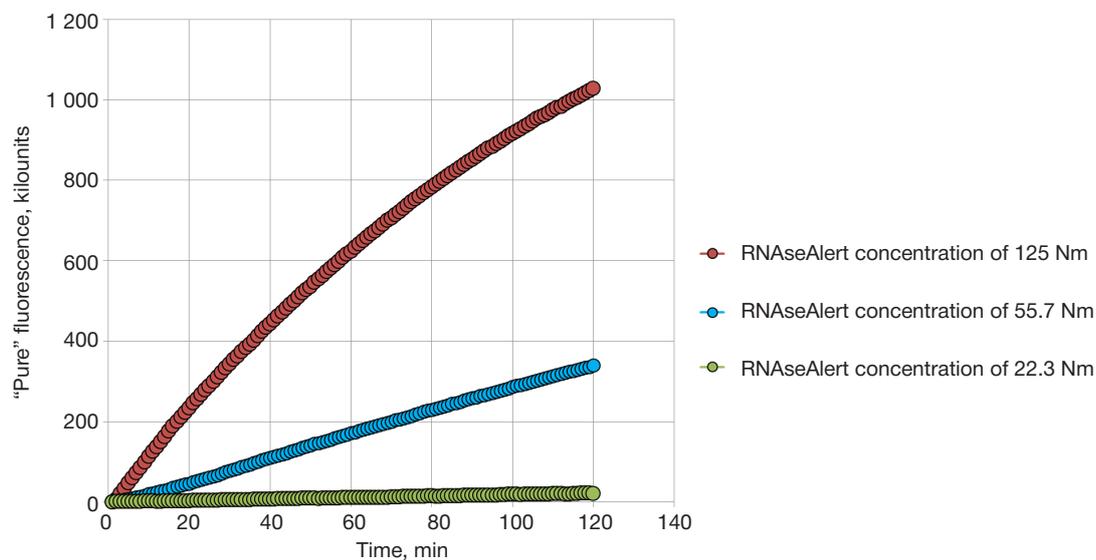


Fig. 2. Fluorescence kinetics during incubation of different RNaseAlert

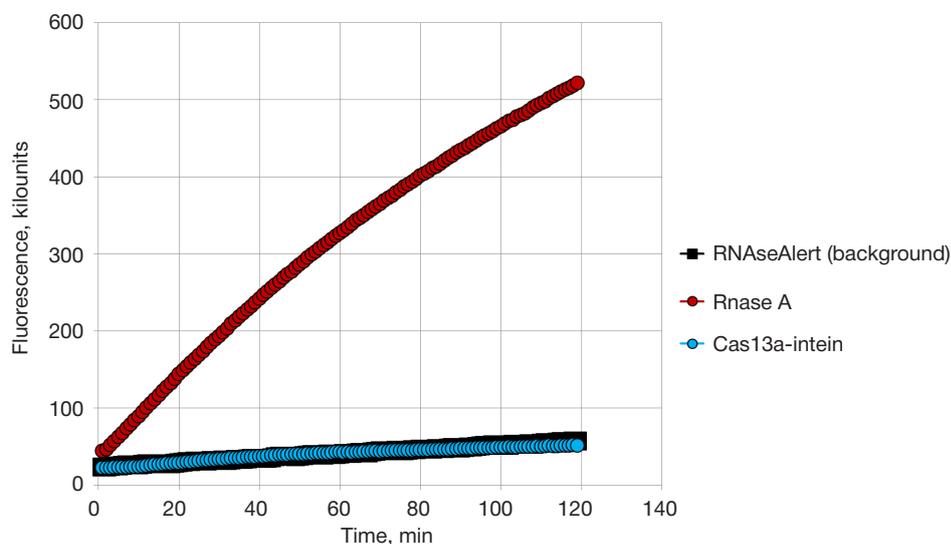


Fig. 3. Fluorescence kinetics during the study of non-specific RNase activities of LwCas13a

## DISCUSSION

LwCas13a-based pathogen detection opens up new diagnostic horizons. In 2017 Cas13 was adapted for the use in a platform called SHERLOCK [7, 9]. The latter combines Cas13a-based detection of RNA targets pre-amplified by recombinase polymerase amplification (RPA) and T7-transcription. All reactions take place in the same reaction mix. Using this approach, the researchers were able to design a diagnostic platform for Zika detection. Its attomolar sensitivity and specificity proved to be no less inferior to those of quantitative PCR (qRT-PCR) and droplet digital PCR. The researchers studied Cas13a orthologs to obtain a stable and reliable fluorescent signal emitted when Cas13a started to exert its RNase activity. They found that Cas13a of *Leptotrichia wadei* was capable of detecting up to 50 pM of target RNA [7]. So, this enzyme served as a basis for the SHERLOCK platform. Although the sensitivity demonstrated by Cas13-based detection was high, the researchers decided to investigate a possibility of combining it

with different types of isothermal amplification and established that RPA combined with transcription and the effect of Cas13a RNase activity could improve the sensitivity of the method even more. SHERLOCK is able to discriminate between target RNAs that differ in only one nucleotide and are present in the solution at very low concentrations; the platform can also be used as a portable tool [7, 9].

In our research work we have synthesized a codon-optimized variant of LwCas13a. Unlike the previously described protein [7, 9], our protein had a different sequence changed for more effective expression in *E. Coli* cells. The protein yield was up to 10 mg from 1L of culture (Fig. 1). We have also elaborated a method for Cas13a purification and tested it to discover that programmed RNase does not exhibit any non-specific RNase activity. We ran a few *in vitro* tests to evaluate the specificity of Cas13 RNase activity against the fragments of viral RNA. Without target pre-amplification by previously described RPA and T7-transcription [7, 9], the sensitivity of our technique was  $10^7$  molecules per reaction.

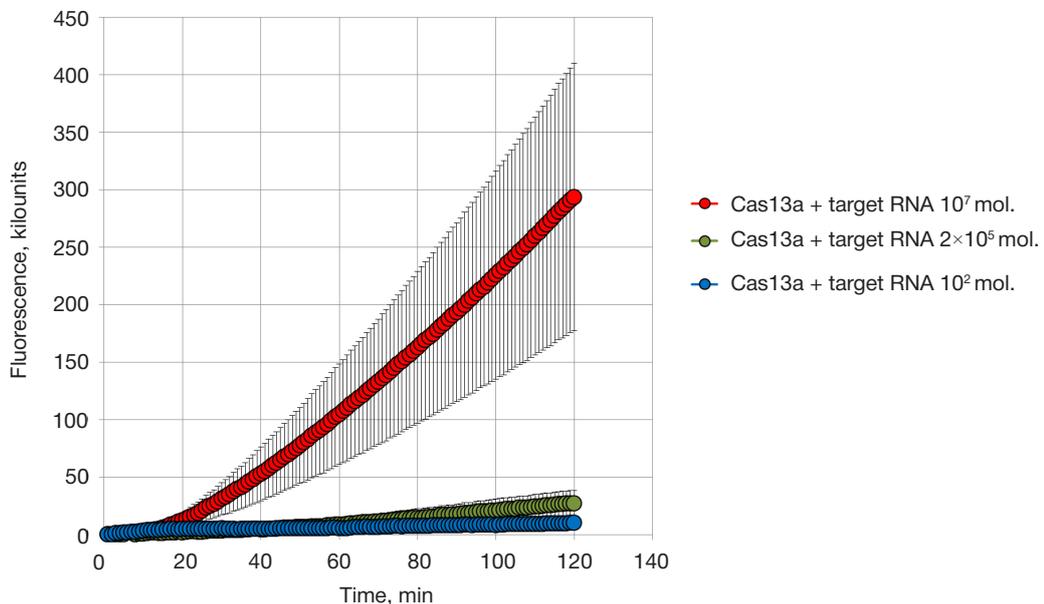


Fig. 4. Fluorescence kinetics at different concentrations of target RNA

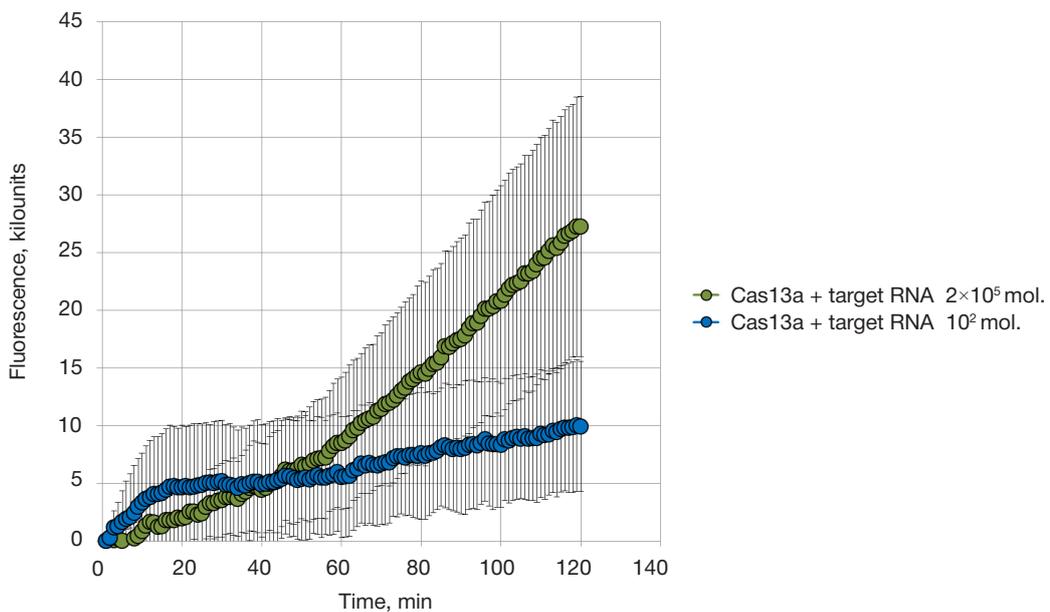


Fig. 5. Fluorescence kinetics at small concentrations of target RNA of the influenza B virus

## CONCLUSIONS

In the course of this work we synthesized a codon-optimized variant of *Leptotrichia wadei* Cas13a that exhibits specific RNase activity in *E. coli* cells. The obtained protein is deprived of non-specific nuclease activities and can specifically detect the target, which in our case was a fragment of influenza RNA.

Further research will be aimed at perfecting the technique, improving its sensitivity, studying programmed RNase specificity, and increasing multiplexity. Besides, we will attempt to create a Cas13a-based diagnostic system for portable use. Field diagnostic tools can be of great assistance in monitoring the agents of infection in their natural reservoirs, preventing the invasion of pathogens into the human population [13–15].

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