

A new, non-specific thermolabile nuclease active at low temperature, in wide pH range and high concentration in salts

The subject matter of the invention is a new, thermolabile, non-specific PPR nuclease or its enzymatically active fragment, or a sequence that shares at least 40% of its identity, active at low temperature, in wide pH range and high concentrations of salts (e.g. NaCl, KCl, MgCl₂, MgSO₄, (NH₄)₂SO₄). The subject matter of the invention is also a gene-encoding PPR nuclease or its enzymatically active fragment; a particle of nucleic acid-encoding PPR nuclease or its enzymatically active fragment; expression plasmid including the sequence of the PPR-encoding gene; a recombinant strain of *Escherichia coli* JM109(DE3) pD454-PPR-AmpR and a strain of *Escherichia coli* ArcticExpress (DE3) pD454-PPR-AmpR; the method of PPR nuclease protein production, application of the PPR nuclease in processes of purification of recombinant proteins of significantly lower DNA content as well as for decontamination of reagents and mixtures for PCR, qPCR, RT-PCR, RT-qPCR and NGS in order to obtain higher sensitivity and specificity of relevant genetic analyses; application of the PPR nuclease in the processes of virus vector purification (particularly lentiviruses [LV], adenoviruses [AV, AAV] and retroviruses [RV] used in modern gene and cell therapies (chimeric antigen receptor [CAR] T-cell immunotherapy); application of the PPR nuclease in exosome purification processes for therapeutic or diagnostic purposes; application of the PPR nuclease in processes of recombinant protein purification, particularly enzymes, antibodies, vaccination antigens, products used in cell therapies, and other therapeutic proteins.

Currently, the most popular non-specific nuclease is Benzonase[®] (Merck, USA), whose optimal temperature of activity is 37°C, and the main disadvantages include no possibility of effective inactivation by high temperature and limited tolerance to increased concentration of salts. Similar parameters are exhibited by Benzonase[®]-related products of generic character, such as e.g. Denarase, produced in another host - *Bacillus sp.* (c-LEcta, Germany). Another example of an enzyme of similar characteristics is Cyanase[™] nuclease, derived from another microorganism (RiboSolutions, USA). However, the main assumption of inventors is to obtain a non-

specific nuclease derived from psychro- and halophile microorganisms, retaining significant activity below 20°C and even in refrigerated conditions (4-8°C), maintaining optimal activities in a wide spectrum of salt concentrations and pH, and characterized by a lower temperature of irreversible enzyme inactivation. Currently, on world markets there are only two non-specific nucleases exhibiting significant activity at lower temperatures. These are Cryonase™ (Takara, Japan), derived from a psychotropic organism and HL-SAN (ArcticZymes, Norway). However, compared to the subject invention, these enzymes are characterised by a lower tolerance to high concentrations of salts, weaker activity at low temperatures (<20°C) and a narrower pH tolerance range (residual activity at pH < 7.0).

DNA contamination, commonly occurring in protein products produced in microorganisms, poses a significant problem during industrial manufacturing of recombinant proteins and enzymes, especially for diagnostic, therapeutic and scientific purposes.

Enzymes of a significantly lower nucleic acid contamination (so called "DNA-free") are ideal for precise diagnostics based on amplification and/or DNA ligation (among others: PCR, qPCR, RT-qPCR, NGS, RCA, LAMP), where the highest sensitivity, specificity and the lack of ambiguous or false positive results are required. Even traces of foreign DNA may lead to obtaining artefacts in the above-mentioned, supersensitive techniques. The problem of DNA contamination escalates when detected DNA is in a low quantity. The signal from contaminated DNA may interfere with low-copy DNA detection, being the subject of the measurement, significantly affecting the sensitivity and reliability of the test.

Commercial suppliers of enzymes and reagents (among others DNA polymerases, PCR master mixes, reagents for NGS) confirm the significance of nucleic acid contamination, and offer DNA-free products, which differ from conventional reagents in terms of their production technology and quality control. However, the level of contamination of these products is very often far from expectations as it strongly relies on the sensitivity of the DNA contamination detection method (according to literature findings, the majority of companies offer DNA-free polymerases, containing from 10 to 1000 copies of the DNA genome per 1 U of enzyme).

The production of therapeutic proteins and active substances for medicinal products, due to high quality standards, also requires the removal of process contaminants, particularly related to DNA (host and exogenous). The amount of the remaining DNA must usually be limited to 100 pg per drug dosage (e.g. in the case of therapeutic antibodies) and for some vaccines to 10 ng per drug dosage. These values are determined by the guidelines of the World Health Organization (WHO), as well as the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

An ideal tool for the purification of contaminating nucleic acids seems to be the application of an appropriate non-specific and universal nuclease, characterized by high activity at low temperatures (4-22°C), wide range of pH (6.0-10.0), and a high concentration of salts and other additives commonly used in purification processes (*downstream processing*) which could be inactivated at a temperature that is safe for enzymes and biopharmaceuticals that are being purified (proteins, enzymes, antibodies, antigens, virus vectors for gene therapy etc.).

Particularly in terms of effective purification from nucleic acids of virus vectors (among other lentiviruses (LV), adenoviruses (AV, AAV), retroviruses (RV)), used in modern gene and cell therapies (such as CAR-T therapies), it is highly desirable to apply purification conditions at high salt concentrations (250-1000 mM NaCl) and pH within 6.0-8.0 (Kramberger et al., Hum Vaccin Immunother. 2015;11(4):1010-21. doi: 10.1080/21645515.2015.1009817), i.e. the optimal conditions for PPR nuclease action. Such conditions significantly facilitate the digestion of nucleic acids of the host cells composing chromatin, as well as change the viscosity of solutions containing virus vectors or proteins facilitating their purification. Additionally, they are indispensable for effective binding of purified virus vectors or proteins to the stationary phase. This is of key importance for increased efficiency of the production processes and significant reduction in production costs.

In recent years interest regarding enzymes from psychrophilic microorganisms (i.e. ones adapted to living at low temperatures) has been growing. The great significance of these enzymes is related to their high activity at low temperatures (generating production savings) and thermal liability, thanks to which their effective,

fast and selective inactivation is possible (after purification process) by a slight temperature increase (that does not damage the product which is under enzymatic treatment).

The subject-matter, a thermolabile, non-specific nuclease, may be applied for the production of enzymes that are free from nucleic acids (e.g. DNA-free polymerases, reverse transcriptases, or ligases). These are very expensive and not widely available enzymes, which are often desirable for specialist technologies of molecular biology and *in vitro* diagnostics. The PPR nuclease, the subject matter of our invention, may also be used by pharmaceutical and cosmetics companies for the purification of products of natural origin from nucleic acids. For this purpose the pharmaceutical market currently uses mesophilic Benzonase[®] (Merck), which is characterized by low tolerance to monovalent and bivalent salts in the reaction environment.

An international publication, WO2006095769, has described a polypeptide of endonuclease activity derived from *Shewanella sp.*, psychrophile microorganisms which exhibit high activity at low temperatures. It may remove any nucleic acid present in protein solution as well as lower viscosity of protein extract. However, its inactivation poses certain difficulties as, in accordance with literature reports (Saramiento et al., Front Bioeng Biotechnol. 2015; 3: 148.), it requires incubation at 70°C for 30 minutes (at such a high temperature many recombinant proteins may denature).

Moreover, an international publication, WO2013/121228, presents a non-specific endonuclease and its enzymatically active fragment, available under the HL-SAN trade name. This invention regards endonucleases, which are inactivated by mild temperature conditions, exhibiting thermolabile properties. This invention also includes the removal of contaminating polynucleotides from biologicals by the application of this endonuclease. This invention also concerns the prevention of false positive results in the amplification reaction of nucleic acid by the application of endonuclease, in particular, in the amplification reaction by the PCR method.

The aim of this invention was to obtain a thermolabile, non-specific nuclease of better properties maintaining high activity at temperatures below 20°C, in particular in refrigerated conditions (4-8°C), in high concentrations of salts and possibly wide pH

range. Moreover, PPR nuclease is compatible with the majority of buffers and additives used in bioprocess. This enzyme, hydrolyzing nucleic acid, may be a highly valuable tool used for the production of recombinant proteins with a lower content of nucleic acids, enzymes, antibodies, vaccination antigens, exosomes, virus vectors for gene or cell therapies, the preparation of products used in cell therapies, and the purification of other therapeutic proteins from DNA and RNA contaminations (e.g. enzymes for molecular biology and precision *in vitro* diagnostics, proteins and virus vectors for the biopharmaceutical industry, and biological components for the veterinary and cosmetics industries).

The subject matter of the invention is a PPR nuclease or its enzymatically active fragment, where the nuclease sequence is SEQ.2 or the sequence that shares at least 40% of its identity.

The PPR nuclease or its enzymatically active fragment is irreversibly inactivated after incubation for 15 minutes at 52°C in the presence of 1-5 mM DTT, and it is possible to lower the inactivation temperature by longer incubation with DTT.

The PPR nuclease or its enzymatically active fragment is generally active in concentrations of the following salts: NaCl: 0-1400 mM, MgCl₂ 5-200 mM, urea: 0-6000 mM, ammonium sulphate: 0-200 mM, imidazole: 0-400 mM.

The gene encoding PPR nuclease or its enzymatically active fragment with a sequence is presented as SEQ.1.

The particle of nucleic acid encoding the PPR nuclease or its enzymatically active fragment as defined in Claims 1-3 or encoding the protein containing the above-mentioned PPR nuclease or its enzymatically active fragment.

Expression plasmid, pD454-PPR-AmpR, containing the sequence of the PPR-encoding gene in accordance with Claim 4. In addition, the plasmid includes: a T7 phage promoter or another one active in the *E. coli* expression system. The plasmid has the SEQ.4 sequence.

Recombinant strains of *Escherichia coli* JM109(DE3) pD454-PPR-AmpR or *Escherichia coli* ArcticExpress (DE3) pD454-PPR-AmpR are transformed with the above-described plasmid.

The manner of PPR nuclease protein production, where recombinant strains of *Escherichia coli* JM109(DE3) pD454-PPR-AmpR or *Escherichia coli* ArcticExpress

(DE3) pD454-PPR-AmpR are cultured on medium and later induction of the PPR nuclease gene expression is performed by adding IPTG; the protein is isolated and purified.

The manner of isolation and purification of the PPR nuclease, or its enzymatically active fragment as defined above, which involves expression of the previously described nuclease or its fragment in a relevant host cell and consequently separation of the nuclease from the host cell and/or medium where the cells were cultured.

PPR nuclease application in the processes of the purification of recombinant proteins of significantly lower DNA content and for decontamination of reagents and reaction mixtures for PCR, qPCR, RT-PCR, RT-qPCR, RCA, LAMP and NGS in order to obtain increased sensitivity and specificity of the relevant genetic analyses.

Application of the PPR nuclease in the processes of virus vector purification (particularly lentiviruses [LV], adenoviruses [AV, AAV] and retroviruses [RV]), used in modern gene and cell therapies (e.g. chimeric antigen receptor [CAR] T-cell immunotherapy).

Application of the PPR nuclease in processes to purify exosomes used for therapeutic and diagnostic purposes.

Application of the PPR nuclease in the processes of recombinant protein purification, particularly of enzymes, antibodies, vaccination antigens, products used in cell therapies and other therapeutic proteins.

Application of the PPR nuclease in the pharmaceutical, veterinary and cosmetics industries.

Application of the PPR nuclease in the pharmaceutical and biotechnology industries to remove DNA contamination in culture media for mammalian fermentation and microbiological processes.

The terminology used above in Description and Patent Claims has the following meaning:

Nuclease - this term refers to the enzyme that hydrolyzes a phosphodiester bond in the polynucleotide chain of nucleic acids (DNA or RNA).

Non-specific nuclease - enzyme hydrolyzing all types of nucleic acids (including ssDNA, dsDNA, circular DNA, ssRNA, dsRNA).

Psychrophilic organism - organism that lives at low temperatures (below 20°C).

Psychrotropic organism - organism tolerating low temperatures (may live at low temperatures but it is not necessary).

Halophilic organism - organism tolerating high concentration of salts, that lives in saline waters or soils.

PPR nuclease - non-specific nuclease that is the subject matter of the invention of SEQ ID sequence no. 2.

Description of figures and sequences:

Fig. 1 - showing the scheme of the pD454-PPR expression plasmid.

Fig. 2 - showing pH influence on PPR nucleolytic activity depending on NaCl salt concentration. Measurement using modified Kunitz's test, conditions: 20 mM MgCl₂, temperature of 22°C.

Fig. 3 - showing temperature and high salts (500 mM NaCl + 100 mM MgCl₂) influence on PPR nucleolytic activity. Measurement using modified Kunitz's test.

Fig. 4 - showing influence of Mg²⁺ ions on PPR nucleolytic activity in selected pH and temperature conditions. Maximum PPR activity is obtained at 50-150 mM concentration at 37°C in a pH 8.0 buffer. At ambient temperature (22°C) in a pH 6.5 buffer optimal activity is obtained at significantly lower Mg²⁺ ion concentrations (20-50 mM).

Fig. 5 - showing PPR inactivation in various temperature conditions in the presence of 5 mM DTT. Complete PPR inactivation is obtained at 52°C.

Fig. 6 - showing the comparison of nucleolytic activity values of PPR, HL-SAN and Benzonase[®] nucleases in buffers of various NaCl concentrations (0, 250, 500 mM), in pH 7.0, 8.0, 9.0, respectively. The remaining reaction conditions are as follows: temperature 22°C, 50 mM Tris, 20 mM MgCl₂ (5 mM MgCl₂ used for Benzonase[®]). The greatest competitive advantages PPR shows in high NaCl concentrations (250-500 mM), commonly used in the processes of purification of recombinant proteins and virus vectors. The advantage of PPR over the HL-SAN nuclease, whose characteristics are the most similar, increases in correlation with decreasing pH (7.0-8.0).

Fig. 7 - showing the comparison of values of the PPR, HL-SAN and Benzonase[®] nucleases' nucleolytic activities in DMEM medium, commonly used for mammalian *in vitro* cell culture, in which recombinant proteins, virus vectors and other biological therapeutics are produced.

Fig. 8 - showing the comparison of values of the PPR, HL-SAN and Benzonase[®] nucleases' nucleolytic activities in buffers with similar physiological salt concentration (PBS and TBS) with the addition of 500 mM NaCl, commonly used in the methodology of recombinant protein purification.

Fig. 9 - showing detection of contaminating host DNA (*E. coli*) using qPCR methods in samples of UDGase (UDG) and purified UDGase using the PPR nuclease (UDG+PPR).

Fig. 10 – showing the removal of genomic DNA contamination from post-culture medium of CHO cells producing cetuximab and bevacizumab monoclonal antibodies.

SEQ.1 – showing the PPR nuclease nucleotide sequence.

SEQ.2 – showing the amino acid sequence of the PPR nuclease protein.

SEQ.3A – showing the amino acid sequence of the PelB signal peptide.

SEQ.3B – showing His6-tag, allowing for purification.

SEQ.4 – showing the sequence of the recombinant pD454-PPR-AmpR expression plasmid.

The invention is illustrated by the following examples of its performance, without any limitation of its application.

Example 1

Obtaining the expression plasmid pD454-PPR-AmpR.

To obtain the expression plasmid pD454-PPR-AmpR, purified by ethanol precipitation, the DNA fragment of the SEQ.1 pattern is digested by the SapI restriction enzyme, and following that it is ligated with DNA of the pD454-SR plasmid vector (ATUM, Newark, CA 94560, USA), digested by the same restriction enzyme.

The ligation mixture transforms the competent TOP10F *Escherichia coli* cells that are placed onto Petri dishes with LA medium (1% peptone; 0.5% yeast extract; 1% NaCl; 1.5% agar) containing ampicillin 100 µg/ml. As a result of plasmid DNA isolation, the expression plasmid of the pD454-PPR-AmpR and SEQ.4 sequence is obtained from the developed bacterial colonies. The map of the pD454-PPR-AmpR plasmid is shown in Fig. 1.

Example 2

Obtaining recombinant strains of *E. coli* JM109(DE3) pD454-PPR-AmpR or *E. coli* ArcticExpress (DE3) pD454-PPR-AmpR.

To obtain recombinant strains of *E. coli* JM109(DE3) pD454-PPR-AmpR or *E. coli* ArcticExpress (DE3) pD454-PPR-AmpR transformations of *E. coli* JM109(DE3) or *E. coli* ArcticExpress (DE3) cells are performed with circular DNA of pD454-PPR-AmpR expression plasmid obtained as described in Example 1 (SEQ.4). Bacteria cells are placed onto LB medium (1% peptone; 0.5% yeast extract; 1% NaCl) containing ampicillin 100 µg/ml and then the obtained colonies of recombinant strains of *E. coli* JM109(DE3) pD454-PPR-AmpR or *E. coli* ArcticExpress (DE3) pD454-PPR-AmpR are used for biosynthesis of the PPR nuclease.

Example 3

Obtaining the PPR nuclease using the cells of recombinant strains of *E. coli* JM109(DE3) pD454-PPR-AmpR or *E. coli* ArcticExpress (DE3) pD454-PPR-AmpR.

The recombinant strains of *E. coli* JM109(DE3) pD454-PPR-AmpR or *E. coli* ArcticExpress (DE3) pD454-PPR-AmpR obtained in accordance with Example 2 are cultured in LB medium (1% peptone; 0.5% yeast extract; 1% NaCl) containing ampicillin 50 µg/ml at 37°C for 16-18 h. Following that, the overnight culture is used to inoculate a medium of identical contents, at a 1:50 ratio. The culture is continued at 30°C until OD₆₀₀ (optical density) = 0.4-0.5 is obtained, and then induction of PPR nuclease gene expression is performed by adding IPTG to a final concentration of 0.2 mM. The culture is maintained at 18°C for 20-22 h and then bacteria cells are

separated from the medium by centrifugation. Cell sedimentation suspended in the buffer contains 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, 5 mM MgCl₂; at least 5 ml of buffer solution per 1 g of cell sedimentation.

Following that, the suspension of cells undergoes disintegration using ultrasounds, performing 3 cycles of sonication; energy intensity 100 J/ml of suspension. The obtained cell lysate is centrifuged at 16000 RCF in order to remove insoluble proteins and cell fragments, and then it is filtrated through a 0.2 µm membrane. The PPR nuclease protein is separated from the remaining bacterial proteins applying the immobilized metal affinity chromatography method (IMAC) using the stationary phase with immobilized bivalent ions of nickel. The PPR nuclease bound to the stationary phase is then washed out by the buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 300 mM imidazole, 5 mM MgCl₂. The fraction containing the PPR nuclease undergoes dialysis with a buffer solution containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM MgCl₂; at least 100 ml per 1 ml enzyme, for at least 18 hours in refrigerated conditions. The obtained enzyme formula is mixed 1:1 with glycerol and frozen at -20°C. Measurement of protein concentration is performed using the spectrophotometric method, at 280 nm wavelength.

Example 4

Examination of the enzymatic properties of the PPR nuclease recombinant protein.

Specific nucleolytic activity regarding nucleic acids necessary to define the optimal conditions of enzyme activity and inactivation was determined for the obtained recombinant PPR nuclease in accordance with Example 3.

In order to determine PPR nuclease nucleolytic activity serial dilutions of the enzyme are incubated in a reaction buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM MgCl₂ and 1 µg pUC19 plasmid DNA of 20 µl volume for 10 min. The reaction is stopped by adding 5 µl 25 mM DTT solution to a final concentration of 5 mM and the sample is heated to 55°C for 10 min. As a control, 1 µg of pUC19 plasmid DNA is incubated without the addition of nuclease. Subsequently, the samples are loaded onto a 1% agarose gel and DNA separation is performed in gel at 130 voltage for 40 min. The remaining, nondegraded DNA in gel is stained with ethidium bromide and documented by taking a photography of the gel. 1 U activity is determined as the enzyme quantity essential for total degradation of 1 µg of pUC19 plasmid DNA in 10 min. at 37°C.

In order to determine PPR nuclease specific activity serial dilutions of the enzyme are incubated in a reaction buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM MgCl_2 and 100 μg of herring sperm genomic DNA of 300 μl volume at 37°C for 30 min. The reaction is stopped by adding 300 μl 4% solution of perchloric acid to 2% final concentration and sample incubation on ice for 60 min. As a control, 100 μg DNA is incubated without the addition of nuclease. Afterwards, the samples are centrifuged for 10 minutes until separation of the precipitate of non-degraded DNA. The content of free nucleotides and oligonucleotide fragments smaller than 10 bp is determined in supernatant by measuring absorbance at 260 nm wavelength. 1 U nuclease activity is determined as enzyme quantity causing increase of absorbance in the studied sample at 260 nm wavelength of 1.0 within 30 minutes of reaction at 37°C.

Example 4A

Determination of optimal pH for activity of nucleolytic PPR nuclease.

In order to determine optimal pH for enzyme nucleolytic activity reaction is conducted as described in Example 4. The solutions of pH 6.0-10.0 with 1 U enzyme in reaction mixtures containing 0, 250, 500 mM NaCl, respectively. PPR nuclease exhibits highest nucleolytic activity in a buffer of pH 8.0 at 500 mM NaCl concentration, as shown in Fig. 2.

Example 4B

Determination of optimal temperature for PPR nuclease nucleolytic activity.

The determination of the optimal temperature for enzyme nucleolytic activity reaction is performed as described in Example 4 at various temperatures from 6°C to 45°C with 1 U enzyme in the reaction mixture containing 50 mM Tris, pH 8.0 and variable concentrations of MgCl_2 (5 mM and 100 mM). The PPR nuclease maintains nucleolytic activity in the whole range of the tested temperatures (Fig. 3). However, the highest activity is exhibited at 37°C and with high concentration of NaCl (500 mM) and MgCl_2 (100 mM). It needs to be highlighted that in these conditions 100% of standard activity may be obtained in refrigerated conditions at 6°C, by applying the relevant salt concentration: NaCl (500 mM) and MgCl_2 (100 mM).

Example 4C**Determination of optimal concentration of Mg^{2+} ions for PPR nuclease nucleolytic activity.**

To determine the effects of Mg^{2+} ion concentration on PPR nucleolytic activity reaction is performed as described in Example 4 in solutions of varied content of Mg^{2+} ions; 5 mM to 200 mM with 1 U enzyme in the reaction mixture. In a slightly alkaline environment (pH 8.0) the PPR nuclease shows the highest nucleolytic activity in the buffer containing Mg^{2+} ions at 150 mM concentration (optimum 50-150 mM), as shown in Fig. 4. In a low-pH environment (pH 6.5) PPR shows the highest nucleolytic activity in the buffer containing Mg^{2+} ions at 20 mM concentration (optimum 20-50 mM). In all observed ranges of $MgCl_2$ (5-200 mM) PPR concentrations show high specific activity.

Example 4D**Confirmation of potential inhibitor effects on PPR enzymatic activity.**

To determine the scope of enzyme tolerance to popular ion components used in the preparation of recombinant proteins, present in the reaction buffer, a nucleolytic reaction is performed as described in Example 4 in solutions of various amounts of individual potential inhibitors: NaCl, urea, ammonium sulphate, imidazole. The PPR nuclease retains high nucleolytic activity in the presence of increased concentrations of tested substances, as shown in Table 1.

Table 1. Effects of inhibitors on PPR nucleolytic activity

Substance	Non-inhibiting concentration
NaCl/KCl	0-1400 mM
Urea	0-6000 mM
Ammonium sulphate	0-200 mM
Imidazole	0-400 mM

Example 4E**Thermal inactivation of PPR enzymatic activity**

To determine the parameters of PPR nuclease inactivation series of 0.2 ml test probes for PCR are prepared containing 100 U PPR in a 50 μ l reaction buffer containing 5 mM DTT, as described in Example 4A, excluding pUC10 plasmid DNA. Afterwards, the probes are incubated for 15 minutes at relevant temperatures and then for 5 minutes on ice. As a control, 100 U PPR nucleases in the same buffer are kept on ice. Following that, nucleolytic reaction is performed using a 5 μ l PPR solution from the previous stage, after inactivation described in Example 4A. As a control, only plasmid DNA in the reaction buffer (negative control) and plasmid DNA with 100 U PPR kept on ice (positive control) are incubated in the same conditions as reaction trials. The degree of plasmid DNA degradation in the trials is analyzed on an agarose gel. As shown in Figure 5, PPR is completely inactivated in the presence of DDT at 52°C or higher.

Example 4F**Determination of nucleolytic activity of PPR, HL-SAN, Benzonase[®] nucleases in buffers of varied concentration of salts and pH.**

Determination of nucleolytic activity of PPR, HL-SAN and Benzonase[®] nucleases is performed at various temperatures; 6°C, 22°C and 37°C, with varied addition of NaCl to the final concentration; 0, 250, 500 mM, in pH 7.0, 8.0, 9.0, respectively in the presence of 50 mM Tris, 20 mM MgCl₂ (5 mM MgCl₂ for Benzonase[®]). As shown in Figure 6, the PPR nuclease exhibits the highest nucleolytic activity of all tested nucleases in the buffers with increased content of salt (250 and 500 mM NaCl) in pH 7.0, 8.0 and 9.0 (only HL-SAN shows slightly higher activity in 500 mM NaCl and pH 9.0). The Benzonase[®] nuclease practically does not function in any conditions of increased salt concentration (250, 500 mM NaCl), irrespective of pH value. In conditions of lower pH (7.0, 8.0) and ambient temperature (22°C) the PPR nuclease is significantly more active compared with HL-SAN and Benzonase[®]. A similar correlation was observed for 6°C and 37°C (data not shown).

Example 4G**Determination of nucleolytic activity of PPR, HL-SAN, Benzonase® nucleases in DMEM medium.**

Determination of the nucleolytic activity of PPR, HL-SAN and Benzonase® nucleases are performed as described in Example 4 at various temperatures: 6°C, 22°C and 37°C but instead of a reaction buffer DMEM medium (commonly used in cultures of mammalian cells, e.g. CHO, HEK, for the production of recombinant proteins and virus vectors) is used. As shown in Figure 7, the PPR nuclease, added directly to the DMEM medium at all tested temperatures shows the highest nucleolytic activity of all tested enzymes. At 37°C PPR is six times more active than the Benzonase® nuclease. In refrigerated conditions (6°C) and ambient temperature (22°C) PPR is approx. three times more active than the other tested nucleases, i.e. HL-SAN and Benzonase® (Figure 7). The activity for every enzyme in conditions recommended by the manufacturer was assumed as 100% activity.

Example 4H**Determination of nucleolytic activity of PPR, HL-SAN, Benzonase® nucleases in PBS and TBS buffers.**

Determination of the nucleolytic activity of PPR, HL-SAN and Benzonase® nucleases is performed as described in Example 4 at 6°C, 22°C and 37°C but instead of a reaction buffer the following buffers are used: PBS (Phosphate Buffered Saline) pH 7.4 (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄; 2.7 mM KCl; 137 mM NaCl) and TBS (Tris Buffered Saline) (50 mM Tris-Cl, pH 7.6; 150 mM NaCl), commonly used in processes of recombinant protein purification. Moreover, the activity of nucleases in TBS with the addition of 500 mM NaCl was compared. As presented in Figure 8, the PPR nuclease in all tested buffers, i.e. PBS, TBS and TBS with high content of salt (500 mM NaCl) and at all tested temperatures shows the highest nucleolytic activity of all tested enzymes. The activity for every enzyme in conditions recommended by the manufacturer was assumed as 100% activity (Figure 8).

Example 5**Application of the recombinant PPR nuclease.****Example 5A****Application of the PPR nuclease in the production of recombinant UDCase of lower host DNA content.**

Following Example 3, the obtained PPR nuclease is used in the process of purification of other recombinant enzymes commonly used in scientific studies and molecular diagnostics, particularly in polymerases, ligases and UDases containing significantly lower host DNA contaminants. The standard protocol of UDase purification of *E. coli* bacteria has been modified so that PPR nuclease is added to the prepared bacteria lysate containing overproduced UDase in the following manner: 40 U per 1 ml of lysate and subsequent incubation at 20-25°C for 1 hour using a magnetic mixer set at 200 rotations/min. Consequently, the lysate is processed in accordance with standard procedures for UDase. Content measurement of host DNA contamination is performed using the qPCR method with 16S bacteria-specific primers. UDase purified using the additional step with the PPR nuclease contains 100 times fewer host DNA contaminants in comparison with an enzyme purified without the PPR nuclease, which is shown in Figure 9. The UDase enzyme purified in such a manner used in scientific studies or molecular diagnostics increases the sensitivity of the method and significantly lowers the risk of potential false positive results.

Example 5B**Application of the PPR nuclease for the removal of DNA contamination in the process of monoclonal antibody purification of mammalian cells.**

The PPR nuclease enzyme obtained as described in Example 3 is used for the removal of DNA contaminants in the process of purification of recombinant monoclonal antibodies isolated from Chinese hamster ovary (CHO) cells. Mammalian cells are cultured on a relevant medium for 5 days. Afterwards, the cells are separated by centrifugation and a supernatant is used to purify antibodies by standard chromatographic methods. In the medium, apart from antibodies and medium

components, a large amount of genomic DNA derived from host cells degraded during culture is present. Performance of the preliminary stage of incubation of the post-culture medium with the PPR nuclease significantly reduces the content of DNA contamination in the medium, and thus the efficiency of antibodies binding to the stationary phase is increased. The stage of post-culture medium incubation with the addition of the PPR nuclease to 50 U/ml of medium for 60 minutes at 20-22°C was introduced into the standard process of the purification of antibodies. After this time, from 1 ml of medium, treated and untreated with the PPR nuclease, DNA is isolated using the genomic DNA isolation kit. The total obtained DNA is loaded onto a 1% agarose gel with the addition of ethidium bromide to visualize nucleic acids. Then, the medium undergoes the standard procedure of purification of antibodies. As a control, a post-culture medium is incubated in the same conditions without the addition of the PPR nuclease. As shown in Figure 10, the addition of the PPR nuclease significantly decreases the contamination of genomic DNA in the post-culture medium, accumulated during the growth of cells secreting cetuximab and bevacizumab antibodies outside into the medium.

Patent claims:

1. The PPR nuclease or its enzymatically active fragment, where the nuclease sequence is SEQ.2 or the sequence that shares at least 40% of its identity.
2. The PPR nuclease or its enzymatically active fragment in accordance with Claim 1, characterized in that it is irreversibly inactivated after incubation for 15 minutes at 52°C in the presence of 1-5 mM DTT with the possibility to lower the temperature by prolonged incubation with DTT.
3. The PPR nuclease or its enzymatically active fragment in accordance with Claim 1, characterized in that it is principally active in concentrations of the following salts: NaCl: 0-1400 mM, MgCl₂ 5-200 mM, urea: 0-6000 mM, ammonium sulfate : 0-200 mM, imidazole: 0-400 mM.
4. The gene-encoding PPR nuclease or its enzymatically active fragment with the sequence has been presented as SEQ.1.
5. The particle of nucleic acid encoding the PPR nuclease or its enzymatically active fragment as defined in Claims 1-3 or protein-encoding containing the above-mentioned PPR nuclease or its enzymatically active fragment.
6. The expression plasmid pD454-PPR-AmpR, containing the sequence of the PPR-encoding gene in accordance with Claim 4, which additionally includes: the T7 phage promoter or another one active in the *E. coli* expression system; the plasmid has the SEQ.4 sequence.
7. The recombinant *Escherichia coli* strains of JM109(DE3) pD454-PPR-AmpR and *Escherichia coli* ArcticExpress (DE3) pD454-PPR-AmpR transformed by the plasmid in accordance with Claim 6.
8. The manner of PPR nuclease protein production, characterized in that recombinant strains of *Escherichia coli* JM109(DE3) pD454-PPR-AmpR or *Escherichia coli* ArcticExpress (DE3) pD454-PPR-AmpR are cultured on a medium, and later the induction of the PPR nuclease gene expression is performed by adding IPTG, and the protein is isolated and purified.
9. The manner of isolation and purification of the PPR nuclease, or its enzymatically active fragment as defined in any Claim 1-8, characterized in that the manner involves expression of the previously described nuclease or its

fragment in a relevant host cell, and consequently the separation of the nuclease from the host cell and/or medium where the cells were cultured.

10. Application of the PPR nuclease in the processes of purification of recombinant proteins of significantly lower DNA content, and for decontamination of reagents and reaction mixtures for PCR, qPCR, RT-PCR, RT-qPCR, RCA, LAMP and NGS in order to obtain increased sensitivity and specificity of the relevant genetic analyses.
11. Application of the PPR nuclease in the processes of virus vector purification (particularly lentiviruses [LV], adenoviruses [AV, AAV] and retroviruses [RV]), used in modern gene and cell therapies (e.g. chimeric antigen receptor [CAR] T-cell immunotherapy).
12. Application of the PPR nuclease in the processes to purify exosomes used for therapeutic and diagnostic purposes.
13. Application of the PPR nuclease in the processes of recombinant protein purification, particularly of enzymes, antibodies, vaccination antigens, products used in cell therapies and other therapeutic proteins.
14. Application of the PPR nuclease in the pharmaceutical, veterinary and cosmetics industries.
15. Application of the PPR nuclease in the pharmaceutical and biotechnology industries to remove DNA contamination in culture media for mammalian fermentation and microbiological processes.