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LIFE SCIENCES PAPER

Expressing Human Lactate Dehydrogenase A (LDHA) in *E. coil* through Molecular Cloning

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Certain DNA can be genetically modified and inserted into organisms, such as *E. coli*, and then relevant proteins can be expressed in large amounts. After transforming *E. coli* by inserting human LDHA gene into the cell, protein qualitative and functional assays (including immunoblot assay and activity assay) confirmed that human M type lactate dehydrogenase monomer (M-ldh) encoded by human LDHA was expressed. *E. coli* is able to use its own transcription and translation machinery to produce proteins from foreign DNA. Expressing proteins encoded by other species' genes should increase the number of phenotypes, and, as a consequence, increase the probability of gene-scale therapy through molecular genetic engineering.

LDHA, expression, recombinant

INTRODUCTION

Lactate dehydrogenase (LDH) has an important role in energy production in organisms and has been actively researched [1; p. 1]. LDH should be purified with a sufficient amount before it can be studied and analyzed. However, it is difficult to obtain human tissue samples, and it is difficult to properly extract the proteins or enzymes of interest from those samples. Therefore, to obtain purified active LDH, the human LDHA gene was inserted into *E. coli* to transform the cell, and human LDH was produced through the transcriptional and translational machinery of the *Escherichia coli* (*E. coli*). As a result, active human LDH was produced from the heterogeneous cell. In summary, *E. coli* is now used in experiments by biologists because of its versatility at expressing inter-species genes.

METHODS

Human LDHA cDNA – The complementary DNA (cDNA) of human LDHA (Fig. 1; National Center for Biotechnology Information (NCBI) accession No. CR541714) that is carried on pDNR-LIB vector was purchased from Thermo Scientific (MHS6278-202840043). The cells containing the plasmid were streaked on a LB-Chloramphenicol plate (LB-Chl, 25 ug/ml) and the plate was incubated at 37˚C for 18 hours.

Escherichia coli expression vector – pTrc99A (Fig. 2) owes its usefulness as an expression vector to its own strong promoter called *trc promoter* [2; p. 214]. To subclone human LDHA gene into pTrc99A, pSaI14 (Plasmid 8614), which comprises pTrc99A backbone and ribonuclease inhibitor gene as an insert between the restriction sites of *Nco*I and *Hind*III, was purchased (Addgene, USA).

Transformation – pSaI14 was transformed into DH5α competent cells. 25 ul of the cells were incubated with about 100 ng of vector on ice for 10 minutes. Then cells were allowed to be exposed to a heat shock at 42˚C for 1 minute. Subsequently, cells were transferred from heat bath to ice for 2 minutes. 1 ml of Luria Broth (LB) liquid media was introduced and thereby cells were recovered in the media at 37˚C for 1 hour. 200 ul of cells was plated on LB-Ampicillin (100 ug/ml) plate Then, the plates were incubated at 37˚C for 18 hours.

The isolation of plasmid pDNR-LIB – A single colony from LB-Chl plate was selected and grown in 3 ml of the liquid media at 37˚C for 18 hours. The cells were centrifuged at 3000 *g*, 4˚C for 10 minutes. The plasmid containing human LDHA cDNA was prepared

by using Accuprep® Nano-Plus Mini Extraction Kit according to the manufacturer's protocol (Bioneer, South Korea).

Polymerase Chain Reaction (PCR) to amplify Human LDHA cDNA – The cDNA of human LDHA gene was amplified by PCR. 200 ng of pDNR-LIB was used as a template. The sequence of forward primer containing a *Nco*I restriction site (boldface) is 5' TCC **ACC ATG G**CA ACT CTA AAG GAT CAG 3'. Reverse primer containing a *Hind*III restriction site is 5' CAG A**AA GCT T**TA AAA TTG CAG CTC C 3'. 5U of *Taq* DNA polymerase (TAKARA, Japan) was used in the total reaction volume of 50 ul. PCR was performed under the following conditions: 95˚C for 5 minute for initial denaturation, 95˚C for 30 seconds for denaturation, 58˚C for 30 seconds for primer annealing, 72˚C for 1 minute for elongation, and 72˚C for 10 minutes for final elongation. Cycling steps were repeated 30 times.

Restriction enzyme digestion – 5 ug of PCR amplified human LDHA gene with *Nco*I and *Hind*III sites and 5 ug of pSaI14 were double digested by 20U of *Nco*I and 30U *of Hind*III in a tube at 37˚C for 1 hour.

Agarose gel electrophoresis followed by gel extraction — Double cut fragments of human LDHA gene (insert) and pTrc99A backbone (vector) from pSaI14 were seperated by gel electrophoresis, and the resulting bands were extracted from the gel by HiYield™ Gel/PCR DNA fragments Extraction Kit (RBC bioscience, South Korea).

Ligation – Extracted insert fragment and vector fragment were incubated with T4 DNA ligase (Invitrogen, USA) at 37˚C for 1 hour. The ligated DNA was introduced into DH5α cells as described earlier.

Positive clone screening – Four individual colonies on the plate were selected and transferred into 3 ml of LB-Amp liquid media, respectively and allowed to grow for 18 hours at 37˚C. The plasmids were isolated from each colony, and then confirmed whether human LDHA gene had been well inserted into pTrc99A by restriction enzyme digestion.

The preparation of total protein lysate from E. coil – The positive clone (LDHA cells) was grown in 200 ml of LB-Amp liquid media at 37˚C for 18 hours. Then, the cell pellet was collected by centrifugation at 12,000 *g*, 4˚C for 10 minutes. The cells were re-suspended with 10 ml of lysis buffer. The cell walls were broken by 60 cycles of sonication. Each cycle had a sonic pulse for 2 seconds and a rest interval for 4 seconds. The sonicated pellet underwent centrifugation again at 12,000 *g*, 4˚C for 10 minutes. Ultimately, supernatant was saved into a new tube and the pellet was discarded. The protein concentration of the total cell lysate was measured by Bradford assay (Biosesang, Korea) according to the manufacturer's instructions.

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis – 30 ug of total cell lysate from control cells and LDHA cells were separated respectively according to the difference in molecular weight by gel electrophoresis at 200 V until the loading dye reached the bottom of the gel. To analyze the gel through two different techniques, the same gel was made concurrently, and thus two duplicated SDS gels were made.

Coomassie blue gel staining – The SDS gel submerged into the staining reagent for 15 minutes, and then into a destaining reagent (AcOH) for 15 minutes, twice. Further destaining and conservation of the gel proceeded with the gel submerging into ddH_2O for a few days until background staining or non-band staining is sufficiently removed.

Western blot – The proteins on the gel were transferred onto nitrocellulose membrane. Ponceau staining reagent was used to cover the surface of the membrane to confirm whether SDS gel was successfully transferred onto the membrane. After Ponceau reagent was wiped out from the membrane, its surface was blocked by 5% skim milk in PBS-T, or PBS with 0.05 % of Tween 20, at 4˚C for 1 hour to inhibit non-specific binding of antibodies. The membrane was incubated in 0.1% solution of mouse monoclonal anti- human M-ldh antibody in PBS-T for 2 hours, followed by 0.03% solution of goat anti-mouse IgG antibody with horseradish peroxidase (HRP) conjugated in PBS-T for 1 hour. The surface was washed out with PBS-T right after

each immnuostaining. WEST-ZOL® Plus western blot detection system was employed to develop the membrane.

Enzyme activity assay – 25 ug and 100 ug of endogenous protein and recombinant protein were introduced into the mixture of 600 ul of lactate stock, 400 ul of 6 mM NAD+ stock, and 200 ul of bicarbonate stock to observe absorbance change that is ascribed to the formation of NADH, and hence the reactivity of LDH that catalyzes the conversion of lactate and NAD+ into pyruvate and NADH. The absorbance change was obtained at 340 nm.

RESULTS

Polymerase Chain Reaction – The human LDHA insert was amplified by PCR (Fig. 3). The expected size was 999 bp and the sequence is shown in Fig. 1. Amplified insert present band was located around 1000 bp as expected. The amplified insert contained the *Nco*I and *Hind*III restriction sites.

The confirmation of positive clone through the restriction enzyme digestion of expression vector – Positive clones were confirmed by the restriction enzyme digestion of the plasmid containing pTrc99A backbone and human LDHA insert followed by gel electrophoresis. An uncut circular plasmid band migrated slower than expected size (Fig. 4; Lane 2). *Nco*I or *Hind*III-cut linearized plasmid bands, respectively, are shown near the expected size of 5122 bp, whose expression map is shown at Figure 3, an aggregate of the backbone and the insert (Fig. 4; Lane 3 and 4). Two distinct bands, which each have a length of 4.0kb (Fig. 4; lane 5, red-arrow) and 1.0kb (lane 5, blue-arrow) that are in accordance with pTrc99A backbone and human LDHA gene respectively, double-cut by both of *Nco*I and *Hind*III, are shown. A band noticed above pTrc99A backbone (lane 5, black-arrow) is thought to be a single cut plasmid fragment by either restriction enzyme, i.e. an incomplete digestion. Thus, the black-arrowed band migrated similarly as the bands shown at lane 3 and 4.

Protein qualitative analysis – The two duplicated SDS gels underwent coomassie blue staining and immunostaining, respectively. Dye molecules in coomassie blue R-250 staining reagent (BIO-RAD, USA) adhere to protein molecules and SDS gel [3; p. 372], and thereby highlight invisible protein molecules inside the SDS gel in blue color. The expression of M type of Lactate dehydrogenase monomer (M-ldh), which is encoded by human LDHA gene, was confirmed by coomassie blue gel staining (Fig. 5A). A thicker band compared to a counterpart at the same position of lane 1 is indicated by a blue arrow at lane 2, which is considered the band containing overexpressed recombinant M-ldh. The molecular size of M-ldh is estimated to be around 36.5kD, which is in accordance with the expected size of M-ldh composed of 332 amino acids, whose sequence is shown at Fig. 6. The counterpart is thought to contain many kinds of proteins whose sizes are about 36.5kD and perhaps includes endogenous LDH from the *E. coli*. Furthermore, whether the band with the blue arrow contains overexpressed human M-ldh was more precisely confirmed by immunostaining with the duplicated gel. Chemi-luminescent (CL) signal from the action of horseradish peroxidase (HRP) was detected. HRP was conjugated with specific antibody against a primary antibody against human M-ldh. In the developing step, the substrate of HRP was added, reacted with HRP, and release CL signal. Therefore, the signal was detected where the presence of M-ldh presents immune reactivity. As a result, no band was detected at Fig. 5B; lane 1, which suggests that there was no immune-reactive band toward the antibody against M-ldh. Meanwhile, a prominent signal was located at the same position with the blue-arrowed band at Fig. 5; lane 2. Thus, human M-ldh was successfully overexpressed in *E. coli*.

Protein functional analysis – After the overexpression of M-ldh confirmed, it was identified whether human LDH is enzymatically functional. Only after four M-ldh monomers compose a M4 tetramer form does lactate dehydrogenase becomes active. An active

M4 tetramer catalyzes the conversion of lactate and NAD+ to pyruvate and NADH. Changes in absorbence according to the production of NADH, at 340 nm where NADH shows maximum absorptivity [4; p. 151], was measured in lactate-NAD stock mixed with protein lysate. An enzymatic activity of proteins in a lysate from control cells was hardly shown (Fig. 7; graph A). On the contrary, the activity of proteins in a lysate from LDHA cells present Michaelis-Menten kinetics (Fig. 7; graph B and C). Recombinant M-ldh tetramer shows the higher initial activity when the more enzyme was introduced: The initial activity, ΔA/minute, is 0.336/minute for 25 ug of the protein lysate, and 1.242/minute for 100 ug.

DISCUSSION

An uncut expression vector plasmid in circular form was located higher than single-cut linearized plasmid (Fig. 2; lane 2-4) because DNA in circular form pass more slowly through an agarose gel than the DNA in a linear form even though they are the same in length. On that account, a turbid smear emerging above the outstanding band at lane 3 is thought to be an intact circular plasmid resulting from incomplete digestion of *Nco*I. This suggests that 4U of restriction enzyme per 1 ug of DNA may be insufficient, considering that 20U of NcoI and 30U of HindIII were used for 5 ug of DNA. Another incomplete digestion occurred at lane 5 (black-arrow), which ought better to be ascribed to the short of *Nco*I enzyme.

An enzymatic activity of recombinant M-ldh tetramer was measured and obtained (Fig. 7). As the concentration of the enzyme increased from 25 ug per reaction stock 1200 ul to 100 ug, the initial activity increased accordingly. The activity graph follows a Michaelis-Menten kinetics equation, with the activity becoming saturated as enzymatic reaction proceed, which are shared by many typical enzymes.

A band that has been thought to contain proteins within the size of about 36.5kD or the analogous with M-ldh in size was detected, and hence the enzymatic activity or the presence of M-ldh has been expected to be shown through Western blotting and activity assay though they would be less than recombinant. Far from the anticipation, however, it is regarded that *E. coli* lacks its own LDH. First of all, immunoblot analysis did not show any trace of M-ldh (Fig. 5B; lane 1), which means that the antibody against M-ldh does not present cross-reactivity between human M-ldh and the endogenous LDH of *E. coli*. Secondly, sizewise analogous proteins, shown at Fig. 5A; lane 1, does not show any functionality (Fig. 7). In other words, this is the same as saying that *E. coli* is able to produce exogenous proteins that could not be made by the *E. coli* itself.

Ultimately, this suggests that *E. coli* or other organisms having plasmids containing strong promoter, for example, mammals [5; p. 5322], are able to produce exogenously when a gene from another species, including human, is inserted into them. To confirm this probability, there are three experiments that should be conducted: 1) inserting human genes into other species or bacterium such as *Zymomonas mobilis*, instead of *E. coli*, 2) inserting non-human gene such as chicken genes into *E. coli*, and 3) inserting non-human genes into other species; namely, inserting genes into heterogeneous species. *E. coli* is already under the edifice of modern genetic engineering and biotechnology.

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FIGURE LEGENDS

FIGURE 1. **Human LDHA complementary DNA sequence.**

Total 999 nucleotides with stop codon reproduced from www.ncbi.nih.gov/nuccore/CR541714.1

FIGURE 2. **Cloning plasmid (Human LDHA inserted pTrc99A) expression map.**

The sequence of pTrc99A reproduced from http://www.addgene.org/vector-database/4402/

FIGURE 3. Polymerase Chain Reaction of Human LDHA gene.

PCR-amplified human LDHA cDNA fragments separated by electrophoresis on 1.2% ararose gel. Forward primer: 5' TCC ACC ATG GCA ACT CTA AAG GAT CAG 3'. Reverse primer: 5' CAG AAA GCT TTA AAA TTG CAG CTC C 3'.

FIGURE 4. The confirmation of positive clone through the restriction enzyme digestion of expression vector.

Lane 1: 1kb plus DNA ladder (Thermo scienfic, USA). Lane 2: no restriction enzyme added. Lane 3: *Nco*I. Lane 4: *Hind*III. Lane 5: Both.

FIGURE 5. The confirmation of the expression of human LDHA.

A: Coomassie blue gel staining. Lane 1: Total protein lysate from control cells. Lane 2: Total protein lysate from LDHA cells. Overexpressed recombinant human M-ldh is indicated by a blue arrow. B: Immunoblot. Lane 1: Total protein lysate from control cells; no band has been detected. Lane 2: Total protein lysate from LDHA cells. Primary antibody: mouse monoclonal anti- Human LDHA antibody (Santa Cruz, USA). Second antibody: goat anti- mouse IgG antibody with horseradish peroxidase (HRP) conjugated.

FIGURE 6. Human LDHA amino acid sequence.

Total 332 amino acids reproduced from www.ncbi.nih.gov/nuccore/CR541714.1

FIGURE 7. Enzyme activity assay.

Time[unit: sec] versus absorbance at 340nm[A]. Absorbance change according to the production of NADH at 340nm was measured by a spectrometer in lactate-NAD stock mixed with protein lysate. Reaction has occurred in lactate stock 600ul, 6mM NAD+ stock 400ul and bicarbonate stock 200ul with protein lysates mixed.

FIGURE 8. Human LDHA 3D structure.

Each M-ldh subunit is colored by blue, green, brown and pink. NAD binding sites are highlighted yellow (A). Lactate binding sites are highlighted yellow (B). Rendered by Cn3D (NCBI, USA). PDB ID: 4AJP, which is available on http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=98243. Information on active sites reproduced from http://www.ncbi.nlm.nih.gov/protein/CAG46515.1

Figure 1

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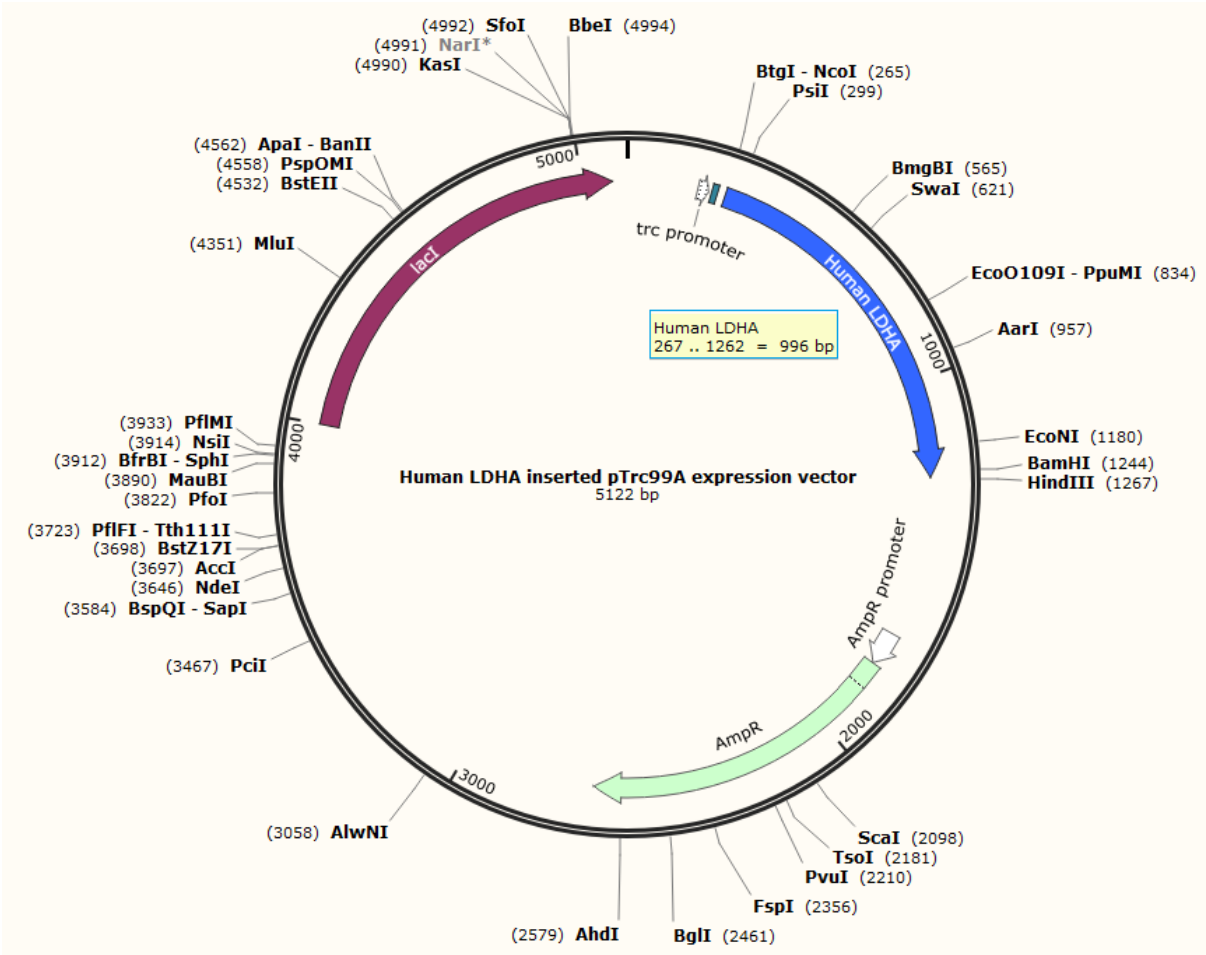
Figure 2



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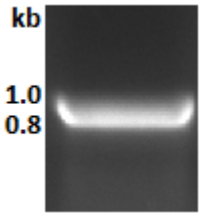

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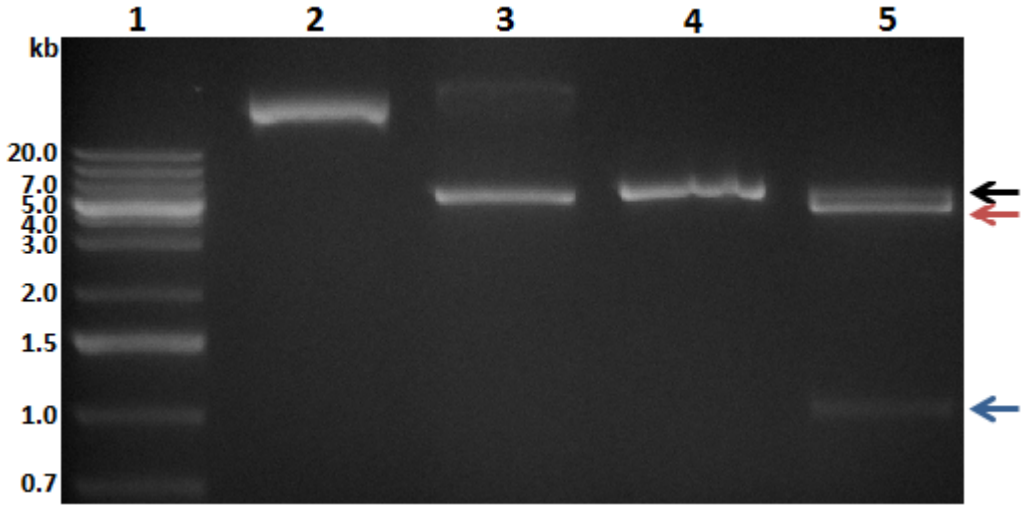

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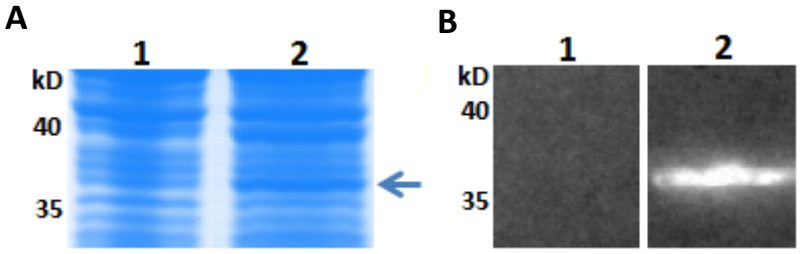

Figure 6

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Figure 7

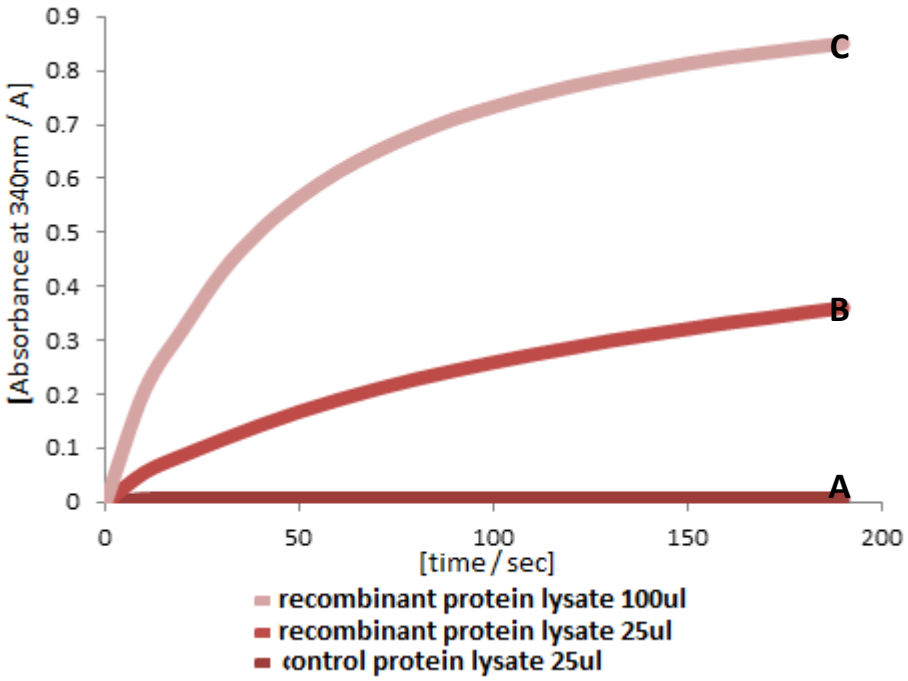

Figure 8