Markus Hobisch, BSc

Design of a Vector for the Co-expression of Three Enzymes for Enhanced Transaminase Activity

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Abstract

Across many industries chiral amines are important building blocks for chemical synthesis. Using ω-transaminases for chiral synthesis from pro-chiral ketones can be an efficient way for their production, especially when alanine can be utilized as amine donor. However, a major hindrance in this case is the unfavorable equilibrium which leans toward the substrate side. A common way to shift the equilibrium is to remove the by-product pyruvate via lactate dehydrogenase (LDH) and glucose dehydrogenase (LDH), which results in a three enzyme cascade. This thesis explores a way to express those three enzymes polycistronically from one plasmid in *E. coli*. For this purpose 18 co-expression plasmids were constructed. For varying expression levels different gene orders were used in combination with various ribosome binding sites. The resulting cell lysates were tested for their LDH and GDH activity and the enzymes' expression levels examined by densitometric analysis. Additionally, a potential high throughput method for transaminase activity screening was tested: the phenol red assay. It relies on the acids produced by LDH and GDH, lactate and gluconic acid, which is visualized in the color shift of phenol red. Unfortunately, in practice the high background from the cell lysate rendered the assay useless. In the analysis of the co-expression plasmids several candidates with high specific activities were identified, showing up to 5.5 U/mg GDH and 2.6 U/mg LDH activity. Those plasmids can be further used for convenient one pot production of an enzyme mix for enhanced transamination.

Zusammenfassung

Chirale Amine sind wichtige Bausteine für chemische Synthesen in vielen Industriezweigen. Eine effiziente Methode sie zu produzieren ist die chirale Synthese mit ω-Transaminasen, ausgehend von prochiralen Ketonen und mit Alanin als Aminodonor. Ein wesentlicher Nachteil dieser Methode ist jedoch das für die Bildung der gewünschten Amine ungünstige Reaktionsgleichgewicht. Ein geläufiger Weg dies zu umgehen ist das Entfernen des Nebenprodukts Pyruvat mittels Laktatdehydrogenase (LDH) und Glucosedehydrogenase (GDH) in einer dreistufigen Enzymkaskade. Diese Masterarbeit beschäftigt sich mit einer Möglichkeit diese drei Enzyme polycistronisch von einem Plasmid ausgehend in *E. coli* zu produzieren. Für diesen Zweck wurden 18 Coexpressionsplasmide produziert. Um die Expressionsniveaus der Enzyme zu variieren wurden die Gene in unterschiedlichen Reihenfolgen platziert und mit verschiedenen Ribosome Binding Sites versehen. Die daraus resultierenden Zelllysate wurden auf ihre LDH- und GDH-Aktivität untersucht und die Expressionslevels der Enzyme densitometrisch festgestellt. Außerdem wurde ein potentielles Hochdurchsatz-Screening für ω-Transaminasen getestet: der Phenolrot-Assay. Es basiert auf der Freisetzung von Laktat und Glukonsäure durch LDH und GDH, die mit dem Indikator Phenolrot visualisiert wird. Leider erwies sich der Background der Zelllysate als zu große Störung für den Einsatz dieses Assays für diesen Zweck. Die Analyse der Coexpressionsplasmide hingegen lieferte einige Kandidaten mit beachtlichen spezifischen Enzymaktivitäten mit bis zu 5,5 U/mg für GDH und 2.6 U/mg für LDH. Diese Plasmide können in Zukunft zur einfachen One-Pot-Produktion eines potenten Enzymgemisches für diverse Anwendungen von ω -Transaminasen dienen.

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1 Introduction

Chiral amines are important building blocks for the synthesis of a wide range of agrochemicals, fine chemicals and pharmaceutically active compounds. Their stereo chemical nature often defines their characteristics; it can have an impact on their pharmacology, pharmacokinetics, toxicology and the way they are metabolized [1]. D-amphetamine, for example, is 10 times as potent as its L-enantiomer for the induction of locomotor activity in rats [2]. Adderal®, an important drug for treatment of ADHD, containins a 1:3 enantiomeric mixture of L-amphetamine:D-amphetamine [3]. (R)-4-phenylbutan-2-amine is used as a precursor for the synthesis of R,R-labetalol [4], which shows anti-hypertensive effects and can provide relief for patients suffering from high blood pressure [5]. Another example is (S)-2-amino-1-methoxypropane which is used for the production of metolachlor, a herbicide [6]. These examples show that there is a definite necessity to sustainably and stereoselectively produce chiral amines. This was also defined as a key research area in green chemistry [7].

One enzyme sub group that has drawn a lot of attention are the ω-transaminases. They catalyse the transfer of an amino group from an amine donor to a carbonyl group on an amino acceptor. One or both of the two are not an α-keto or α-amino acid. These ω-transaminases require pyridoxal-5'-phosphate (PLP) as a coenzyme. PLP has a shuttle function for electrons and ammonia between amino acceptor and amine donor [8]. Before the reaction, PLP's aldehyde group forms a Schiff base with the residual amino group of a lysine in the enzyme's active site (internal aldimine). When the amino group of the amine donor is pointed towards the aldimine a transamination reaction is facilitated between aldimine and amine donor. This results in the formation of a Schiff base between the amine donor and PLP (external aldimine). A 1,3-prototropic shift then converts the external aldimine to a ketamine. The active site lysine now acts as a base and catalyses the hydrolysis of the ketamine to a ketone, which exits the active site. The resulting pyridoxamine phosphate (PMP) transfers the amino group to a newly bound ketone (amine acceptor). This produces a regenerated PLP forming the internal aldimine and a newly formed amine product [9].

Two biotechnological concepts for the production of enantio-enriched amines employ ω-transaminases: the kinetic resolution of racemic amines and the asymmetric synthesis from prochiral ketone substrates. A notable fact is that if the same ω-transaminase is employed for the two methods, both configurations of amines are accessible. An (S)-selective ω-transaminase can be used to obtain amines in (R)-configuration from kinetic resolution and (S)-amines from asymmetric synthesis. The transamination reaction is employed in both directions, respectively [8].

A simple approach to kinetic resolution of chiral amines is to employ pyruvate as an amine acceptor. This conveniently leads to a thermodynamic equilibrium favouring the formation of the products which are amino acid, ketone and the optically enriched amine. Since this only requires the activity of the ω-transaminase, it has the advantage of employing a single enzyme [8]. However, there are also downsides to this method such as inhibitory effects caused by pyruvate and the produced ketone [10]. Moreover, the co-product alanine can cause problems in product recovery. One method to prevent enzyme inhibition by ketones is the removal of the latter. This has been applied for the inhibitory co-product 2-butanone which is formed during the kinetic resolution of racemic 2-butylamine. As 2-butanone is volatile, its resolution was performed at reduced pressure of 0.200 bar. The removal of less volatile co-products such as α-tetralone or acetophenone can be facilitated by the use of a hydrophobic membrane contactor. Another possibility is the removal of the ketone co-product by employing an alcohol dehydrogenase (ADH) which catalyzes its reduction to the corresponding alcohol. However, there also has to be an enzyme recycling the cofactor NAD(P)H which is depleted by the ADH reaction. The second method against inhibition by ketones relies on the recycling of pyruvate. While there are several approaches to this principle, the simplest method utilizes an amino acid oxidase in addition to the ω-transaminase. This setup only requires catalytic amounts of pyruvate because the amino acid oxidase continuously recycles alanine to pyruvate using molecular oxygen [8].

The other concept to employ ω-transaminases is the asymmetric synthesis of α-chiral primary amines. It can be divided into two groups where one uses an excess of amine donor to overcome the often unfavorable thermodynamic equilibrium, while the other relies on the removal of inhibitory co-products. The simplest method is the use of amine donors in high excess as it only requires the transaminase and no other enzyme. Even though there

were some positive results reported using this method [11, 12], they largely rely on optically pure amines as amine donors. Otherwise the system would have to be more complex and employ a kinetic resolution step for the amine donor. For the removal of the inhibitory co-products there are three major principles. The first one uses 2-propylamine as the amine donor. In this setting, the formed co-product is acetone, which has a lower boiling point than all other components. Thus, it can easily be removed under reduced pressure, which drives the reaction to completion. This method is used for the production of the precursor for the herbicide metolachlor mentioned above. The second co-product removal strategy employs the decarboxylation of pyruvate and an excess of the amine donor alanine. In addition to the ω-transaminase, a decarboxylase is needed to produce acetaldehyde and CO_2 from pyruvate which shifts the reaction equilibrium to the product side. However, this leads to the undesired amination of acetaldehyde which consumes alanine and yields amino ethane. The third way is to remove the co-product pyruvate with a lactate dehydrogenase (LDH). The LDH produces lactate and requires NAD(P)H as a cofactor, which can be regenerated either by a formate dehydrogenase or by a glucose dehydrogenase (GDH). Therefore, this system requires an ω-transaminase, an LDH and either an FDH or GDH, combined with NAD(P)H and an excess of the amine donor alanine. As reducing agent either formate or glucose are needed [8].

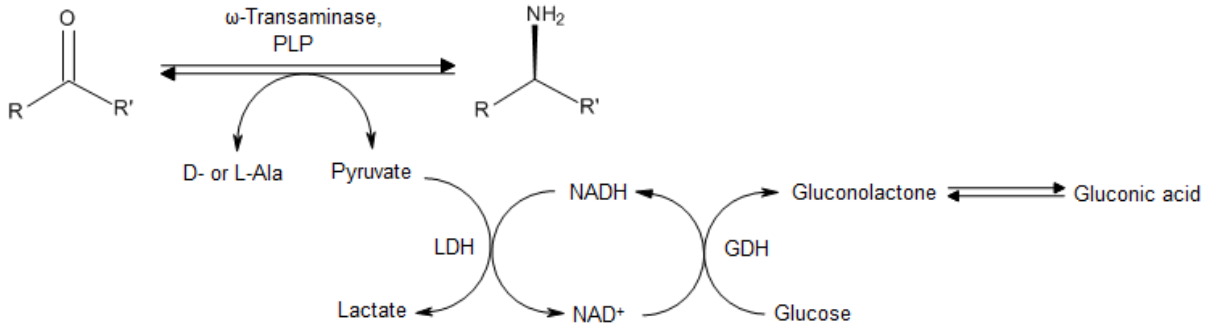

Figure 1: Reaction mechanism of the system employing -transaminase, lactate dehydrogenase (LDH) and glucose dehydrogenase (GDH)

While the system with ω-transaminase, LDH and GDH (**figure 1**) or FDH was already successfully applied [4, 13, 14], it requires the purchase or the separate production of three different enzymes. One way to simplify this process is to express all three enzymes in a single *E. coli* strain. This would reduce the time, cost and reactor volume needed to produce the enzymes required for this amination method for asymmetric synthesis. After looking into the possibilities for the expression of three enzymes from a single plasmid, we settled on a

polycistronic expression system that produces a single mRNA, serving as a template for the translation of all three enzymes. While our industry partner InnoSyn provided us with the ω-transaminase Ate from *Apergillus terreus* and an LDH, we found two possible candidates for the NADH regeneration: a GDH from *Bacillus subtilis* (GDHs) and one from *Bacillus megaterium* (GDHm). Both were tested and the one most suited for the task at hand was incorporated into the system. Important issues for such a co-expression construct are the expression levels of the enzymes. To get a wide variation in this regard, different ribosome binding sites were implemented for LDH and GDH. Additionally, the order of the genes was varied and a total of 18 different plasmids produced and characterized.

Analysis of the conversion rates produced by ω-transaminases is usually done by HPLC, which is quite laborious and time consuming. One method that promises high throughput analysis of transaminase activity should be tested with the cell free extracts produced from our plasmids: the phenol red assay [15]. It relies on the production of lactate and gluconic acid by the LDH and GDH respectively, which lowers the pH of the solution. This pH change is visualized by the indicator phenol red and enables the quantification of the transaminase activity. Since this method was only shown to work with purified enzymes [15], it had to be investigated if and how it can be applied when cell free extract is used instead.

2 Material and Methods

2.1 Strains and Plasmids

Table 1: The strains and plasmids used in this work including relevant features, sources and the BT number of the institute's strain collection.

	Relevant Features	Source	BT Nr.
Strains			
E. coli Top10 F'	F´{lacI$_q$ Tn10 (Tet$_R$)} mcrA Δ(mrr-hsdRMS-mcrBC) Φ80 lacZ ΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL endA1 nupG	Laboratory collection	1482
Plasmids			
pMS470Δ837_eV	AmpR, Ptac, ColE1 origin of replication, *traC* gene	Laboratory collection	-
pMS470Δ837_LDH	Amp^R, *Ptac,* ColE1 origin of replication, *ldh* gene from Innosyn	Laboratory collection	8029
pMS470Δ837_GDHm	Amp^R, Ptac, ColE1 origin of replication, *gdh4* from *B. megaterium*	This work	8027
pMS470Δ837_GDHs	Amp^R, Ptac, ColE1 origin of replication, *gdh* from *B. Subtilis*	This work	8028
pD871_Ate-TA_T274S	Km^R, PrhaBAD, pBR origin of replication, engineered Ate transaminase from *Aspergillus terreus*	Supplied by Innosyn	-
pD871_Ate-TA_T274S_LDH_v1_GDHm_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8031
pD871_Ate-TA_T274S_LDH_v2_GDHm_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8032
pD871_Ate-TA_T274S_LDH_v3_GDHm_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8033
pD871_Ate-TA_T274S_GDHm_v3_LDH_v1	As pD871_Ate-TA_T274S, with LDH and GDHm	This work	8034
pD871_Ate-TA_T274S_GDHm_v3_LDH_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8035
pD871_Ate-TA_T274S_GDHm_v3_LDH_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS	This work	8036

	variation		
pD871_Ate-TA_T274S_LDH_v1_GDHm_v1	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8037
pD871_Ate-TA_T274S_LDH_v1_GDHm_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8038
pD871_Ate-TA_T274S_LDH_v2_GDHm_v1	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8039
pD871_Ate-TA_T274S_LDH_v2_GDHm_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8040
pD871_Ate-TA_T274S_LDH_v3_GDHm_v1	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8041
pD871_Ate-TA_T274S_LDH_v3_GDHm_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8042
pD871_Ate-TA_T274S_GDHm_v1_LDH_v1	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8043
pD871_Ate-TA_T274S_GDHm_v2_LDH_v1	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8044
pD871_Ate-TA_T274S_GDHm_v1_LDH_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8045
pD871_Ate-TA_T274S_GDHm_v2_LDH_v2	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8046
pD871_Ate-TA_T274S_GDHm_v1_LDH_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8047
pD871_Ate-TA_T274S_GDHm_v2_LDH_v3	As pD871_Ate-TA_T274S, with LDH and GDHm, RBS variation	This work	8048

2.2 General Methods

2.2.1 Restriction digestion

Restriction digestions were carried out with Thermo Fisher Scientific FastDigest (FD) enzymes. All reactions were set up as followed and incubated for 1h at 37 °C.

40 µl FD Green Buffer
1 µg DNA
1 µl Enzyme 1
1 µl Enzyme 2
x µl ddH2O
40 µl total

2.2.2 Agarose Gel Electrophoresis

For the separation of DNA samples, agarose gel electrophoresis was used with gels containing 1 % agarose and TAE buffer. The gels were run between 90 V and 120 V. If not stated otherwise, the DNA standard used was the GeneRuler 1 kb DNA Ladder by Thermo Fisher. The loading dye used was the corresponding 6x LD by Thermo Fisher. For a gel of 200 ml, 5 µl of ethidium bromide (10 mg/ml) were added for DNA staining.

2.2.3 Agarose Gel Extraction

DNA extraction from preparative agarose gels was performed using the GeneJET Gel Extraction Kit according to the manual, except for the DNA elution which was done using ddH2O.

2.2.4 Sequencing

For sequencing the samples were sent to LGC genomics or GATC. The primers used are shown below (table 2) and were ordered from Integrated DNA Technologies, Inc.

Table 2: List of the primers used for sequencing

Name	Sequence	Plasmid
pJET_fwd	CGACTCACTATAGGGAGAGCGGC	pJET1.2
pJET_rev	AAGAACATCGATTTTCCATGGCAG	pJET1.2
KST_fwd	CGGATAACAATTTCACAC	pMS470Δ837
KST_rev	TATCAGGCTGAAAATCTTCTCTCATCC	pMS470Δ837
Ate_fwd	ATCGGTCCGATCACCAAG	All co-expression plasmids
GDHm_fwd	TAACATGTCCAGCGTTCACG	All co-expression plasmids
LDHe_fwd	TTGGATTCTGGGTGAGCATG	All co-expression plasmids

2.2.5 Measurement of Protein Concentration

Protein concentrations were measured using the Biorad Bradford Protein Assay. The dye was diluted 1:5 with dH2O. The standards contained 0.05, 0.125, 0.25, 0.375 and 0.5 mg/ml BSA fraction V. Triplicates of the standards and the samples (10 µl, samples in 1:10 and 1:50 dilution in 50 mM KPi buffer at pH 7.5) were pipetted into 96 well plates and incubated with 200 µl of the dye for 5 minutes. 50 mM KPi buffer served as the blank. Afterwards, the absorption was measured at 595 nm. The linear equation obtained from the standards was used to calculate the samples' protein concentration.

2.2.6 SDS-PAGE

SDS-PAGEs were run with 4-12% Bis-Tris NuPAGE Gels from Thermo Fisher in MES buffer. The samples were mixed with 4 x SDS loading dye and heated for 10 minutes at 90 °C. Invitrogen's Powerease 500 drove the electrophoresis at the following limits: 200 V, 120 mA, 25.0 W. Afterwards, the gels were dyed for 20 min in Coomassie Brilliant Blue and destained in deionized water until the bands were clearly visible. The protein standard used was the PageRuler™ Prestained Protein Ladder from Thermo Fisher.

2.2.7 Measurement of DNA Concentration

DNA concentrations were measured using a NanoDrop 1000.

2.2.8 Plasmid Isolation

For plasmid isolation, Thermo Fisher's GeneJET Plasmid Miniprep Kit was used according to the protocol, except for the elution of the DNA which was done with ddH2O. The cell pellets were obtained by centrifuging 4 ml of liquid overnight cultures (5 to 10 ml, with appropriate antibiotic, grown at 37 °C) at 7000 rcf for 1 min.

2.2.9 Transformation by Electroporation

For the transformation of electrocompetent *E. coli* cells, a Biorad MicroPulser at the Ec2 program was used. 40 µl of electrocompetent cells were mixed with 1 µl of DNA, incubated for five minutes and transferred to a 0.2 cm electroporation cuvette. Cells were always kept on ice. After transformation, 1 ml of LB media was added, the cells transferred to a fresh 1.5 ml tube and shaken at 37 °C for 30 min or one hour for ampicillin or kanamycin resistance respectively. Of this cell suspension, 50 µl of a 1:100 dilution were plated onto LB agar plates containing the appropriate antibiotic (100 µg/ml Amp, 40 µg/ml Kan).

2.2.10 Production of Electrocompetent Cells

Electrocompetent *E. coli* TOP10 F' were produced according to a protocol frequently used in our institute. On the first day two ONCs of *E. coli* TOP10 F' and one sterile control were set up in 100 ml baffled flasks with 20 ml LB medium. 1.5 ml reaction tubes, six sterile 500 ml centrifuge tubes, 1 L of 10% sterile glycerol and 2 L sterile water were set to cool at 4 °C. On the next day the ONCs were used to inoculate the main cultures (six times 500ml LB medium in 2 L baffled flasks), were inoculated to an OD_{600} of 0.1 and harvested at an OD600 between 0.6 and 0.9. From here on the cells have to be kept on ice. The flasks were then kept on ice and at 4 °C for 1.25 h. The cooled content of the flasks was then transferred to six centrifuge tubes and centrifuged at 3000 rcf and 4 °C for 10 min. 2 ml 1M sterile Hepes buffer ph 7.4 were added to the cooled water. The resulting buffer was used to resuspend the cell pellets and the contents combined to two centrifuge tubes which were filled up to ¼ of their volume. They were then centrifuged for 10 min at 4000 rcf and 4 °C. The resulting cell pellets were resuspended 1/6 of the tube volume and subsequently filled up to half their volume with 10% glycerol. The cells were then centrifuged for 20 min at 4500 rcf and 4 °C. The resulting pellets were resuspended in 10% glycerol, combined to one tube, which was then

filled up to half its volume with 10% glycerol and centrifuged for 15 min at 5000 rcf at 4 °C. Finally, the cell pellet was resuspended in 6 ml 10% glycerol, aliquoted at 90 µl into the reaction tubes and stored at -70 °C.

2.3 Cloning

The lactate dehydrogenase (LDH) gene was supplied by Innosyn. It was already in use in our laboratory as pMS470Δ837_LDH. It was verified by sequencing.

Both, GDHm [16] and GDHs [17] genes were ordered as GeneArt Genestrings in their original, non codon-optimized form. One recognition sequence for NdeI (CATATG) was added to the 5' end of the coding region, conveniently overlapping with the start codon ATG and one was added for HindIII (AAGCTT) to the 3' end, overlapping with the stop codon TAA.

40 ng of each of those DNA fragments were ligated with the pJET1.2/blunt vector according to the manufacturer's protocol. The ligation mixture was desalted by drop dialysis using a 0.025 µm Millipore membrane (code: VSWP) on ultrapure water for 10 minutes. 2 µl of the mixture were used to transform *E. coli* TOP10 F' cells which were then plated on lysogeny broth (LB) agar containing 100 µg/ml ampicillin. Integration of the Genestrings was checked by colony PCR (cPCR). Seven reactions were set up, three for GDHs and GDHm respectively and one without any template as a negative control.

Master mixture for 8 reactions of 20 µl:
16 µl 10x DreamTaq buffer
3.2 µl dNTPs (10 mM)
4.8 µl pJET_rev (10 µm)
4.8 µl pJET_fwd (10 µM)
0.8 µl DreamTaq DNA Polymerase (5 U/µl)
130.4 µl ddH2O

The product of the cPCR was then analyzed on an agarose gel. The pipet tip used to introduce cell material into the cPCR was also used to start an overnight culture (ONC) for subsequent plasmid isolation. The plasmids of the ONCs corresponding to the correct bands were isolated and sent for sequencing. One correct plasmid for each GDH and the pMS470Δ837_eV were digested with HindIII and NdeI. pMS470Δ837_eV was treated with 1 µl thermosensitive alkaline phosphatase (FastAP, 1 U/µl) for dephosphorylation. The

restriction digests were then separated in a preparative agarose gel, the bands cut out and the DNA extracted. The plasmid backbone of pMS470Δ837 was then ligated with the fragments of GDHs and GDHm respectively. The obtained clones were verified with a cPCR and plasmid DNA of the correct constructs sent for sequencing.

2.4 Cultivation and Gene Expression

Small scale expression

To check for their expression and activity, at first all three enzymes were separately produced in 20 ml LB Amp media in 100 ml baffled flasks. The cultures were inoculated to an OD of 0.1 and grown at 37 °C at 120 rpm. At an OD_{600} of 0.6 to 0.9, protein expression was induced with 0.1 mM Isopropyl-β-D-thiogalactopyranosid (IPTG). The expression then took place at a reduced temperature of 25 °C for 20 h. After cell harvest, the pellet was resuspended in 1.5 ml 50 mM potassium phosphate (KPi) buffer pH 7.5. The cells were then disrupted by sonication (Bronson Sonifier 250, microtip, output control: 3, duty cycle: 40 %, 4x15 seconds on ice) in a 15 ml tube. The disrupted cell suspension was then centrifuged at 3220 rcf for 15 min at 4 °C. The supernatant was used for further analysis.

Large scale expression

Also GDHm, GDHs and LDH were produced in larger scale in 500 ml TB media with appropriate antibiotics in 2 L baffled shaking flasks under the same conditions as in the small scale expression. The cells were harvested by centrifugation at 5000 rcf for 15 minutes. For cell disruption, the cell pellet was weighed and 10 ml 50 mM KPi buffer pH 7.5 were added for resuspension for each gram of cells. The cell suspension was then disrupted by sonication (Bronson Sonifier 250, output control 7.5, duty cycle 70%, for 7 minutes on ice) and the resulting suspension centrifuged for 1 h at 50000 rcf at 4 °C. The supernatant was filtered through a sterile 0.45 µm syringe filter and used for further analysis.

As a negative control for all expressions based on pMS470Δ837 the plasmid pMS470Δ837_eV was used. It carries the *traC* gene coding for a protein necessary for the assembly of extended F pili from mature F-pilin subunits.

2.5 Characterization

The total protein content of the cell free extracts (CFEs) was determined by the Bradford protein assay and the protein expression examined by SDS-PAGE.

2.5.1 GDH Assay

To measure the activity of GDHm and GDHs, an assay based on the increase in concentration of NADH was used. This increase is caused by the reaction of glucose and NAD^+ which is catalyzed by GDH and produces gluconolactone and NADH. The assay was done in a volume of 1 ml in semi-micro cuvettes containing 100 mM KPi pH 7.5, 333 mM D-glucose and 0.67 mM NAD^+. A cuvette with KPi buffer, glucose and NAD^+ was used to blank the photometer. The samples were measured every 15 seconds at 340 nm and 30 °C for 10 minutes. To accurately follow the concentration of NADH, the millimolar extinction coefficient ε was determined experimentally. For this purpose eight concentrations of NADH ranging from 0.05 mM to 0.3 mM were measured with the photometer used for the enzyme assays and a mean ε was calculated using the Beer-Lambert law, where A is the absorption, ε the millimolar extinction coefficient and d the length of the solution the light passes through:

$$A = c \times \varepsilon \times d$$

The activity was then calculated with the following formula:

$$activity\ in\ U/mg = \frac{\Delta abs \times dilution\ factor}{\varepsilon \times 0.005\ ml \times c(protein)}$$

2.5.2 LDH Assay

The assay to measure the activity of the LDH was based on the decrease of NADH over time, caused by the reaction of pyruvate and NADH to lactate and NAD^+. The test was run in a volume of 1 ml in semi-micro cuvettes. The test solution consisted of 100 mM KPi at pH 7.5, 0.24 mM NADH, 2.3 mM pyruvate. A cuvette with KPi buffer, pyruvate and CFE served as a blank. The samples were measured every 15 seconds at 340 nm and 30 °C for 10 minutes. The photometer used for both assays was the Agilent Technology Cary Series UV/Vis Spectrophotometer. The activity was calculated with the following equation:

$$activity\ in\ U/mg = \frac{-\Delta abs \times dilution\ factor}{\varepsilon \times 0.005\ ml \times c(protein)}$$

2.5.3 Heat stability test for GDHs and GDHm

Seven 1:100 dilutions of both CFEs were prepared with 50 mM KPi buffer. Those samples were incubated at 45 °C and one sample each was removed after 0, 2, 5, 10 and 25 minutes and placed on ice. Afterwards their GDH activity was measured.

2.6 Vector Design

The basis for the co-expression construct was the plasmid pD871_*Ate*-TA_T274S (figure 2) supplied by InnoSyn. It carries an engineered version of the omega transaminase *Ate* from *Aspergillus terreus* where the threonine at position 274 is exchanged with a serine. The plasmid harbored a kanamycin resistance gene. It also codes for ROP, an RNA binding protein that keeps plasmid copy numbers low by reducing plasmid replication [18]. The expression of Ate is controlled by the rhaBAD promoter. It promotes expression in the presence of L-rhamnose. The rhaBAD promoter is also affected by catabolite repression. cAMP-CRP is crucial for its activation, which means that high glucose concentrations repress this promoter [19]. The Ate gene is flanked by the restriction sites NdeI and NotI and followed by the phage terminator T7 that facilitates efficient transcription termination.

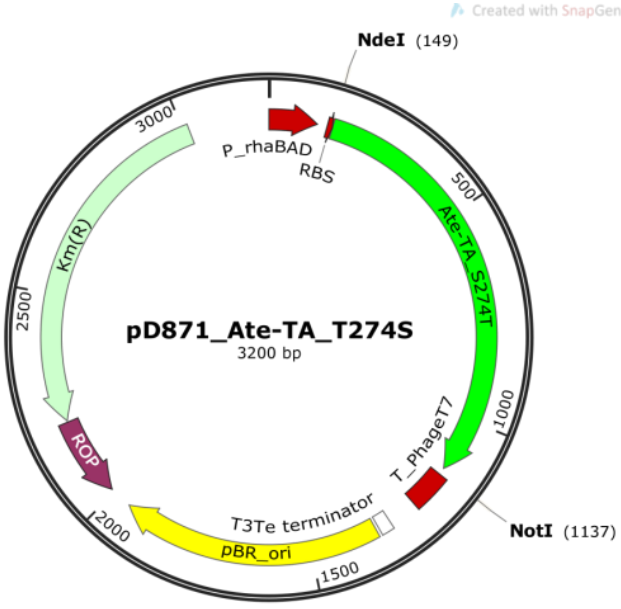

Figure 2: Plasmid map of pD871_Ate-TA_T274S.

For reasons of simplicity, we chose a polycistronic expression, so the rhaBAD promoter controls the transcription of all three coding sequences and one mRNA coding for Ate, LDH and GDHm is produced. To vary their expression levels, two measures were taken. The first was to use different arrangements of the genes: Ate_LDH_GDHm and Ate_GDHm_LDH. Ate was left in the first position since it is the first enzyme in the cascade and I assumed that this would give the best expression for it. The second measure was to vary the ribosome binding sites (RBS) of GDHm and LDH (table 3). For the GDH I chose the native RBS of *gdh* from *Bacillus subtilis* and two native RBS from *E. coli* from the genes *ftsI* and *cpn10*. *ftsI* codes for a peptidoglycan transpeptidase, while *cpn10* codes for a chaperonin. For the LDH I used one RBS simply containing the perfectly conserved Shine-Dalgarno sequence AGGAGG and again two ribosome binding sites native to *E. coli*, namely from *hslU*, a protease and from *gyrB,* a gyrase subunit. These RBSs were chosen based on their suspected expression strength. For example the chaperonin *cpn10* is probably stronger expressed than the peptidoglycan transpeptidase *ftsI*. Those variations make a total of 18 different co-expression constructs.

2.7 Construction of the Co-expression Plasmids

The cloning strategy for the construction of those 18 plasmids was based on the Gibson assembly. With this technique, six variations of the plasmid were created, numbered G1 to G6. They consisted of the two different gene arrangements of GDHm and LDH and the RBS variation for the LDH. For this purpose, I designed 14 primers (table 3) to add the appropriate overhangs for the Gibson assembly. Additionally, I placed restriction sites between each element to introduce a possibility for easy modification of the constructs later on (figure 3 and figure 4). The annealing temperatures (T_a) for each primer pair were calculated with the NEB tm calculator (http://tmcalculator.neb.com) and lowered by one more degree. The PCRs for the necessary modification of the fragments were performed as follows:

1 µl template (pMS470Δ837_GDHm 0.2 ng/µl or pMS470Δ837_LDH 0.3 ng/µl)
10 µl 5x Q5 buffer
1 µl dNTPs (10 mM)
2.5 µl fwd primer (10 µM)
2.5 µl rev primer (10 µM)
0.5 µl Q5-DNA polymerase (2 U/µl)
32.5 µl ddH2O
50 µl total

Temperature profile: 98 °C for 30 s as initial denaturation, 98 °C for 10 s, x °C for 20 s, 72 °C 10 s for 25 cycles, 72 °C 2 min as final extension. The annealing temperature x is listed in table 3.

Due to the length constraints of 60 bp for the primers and the GC rich template, the melting temperature of 2G_fwd was only 51 °C. Thus, all PCRs with 2G_fwd consisted of 5 cycles with a T_a of 49 °C followed by 20 cycles with a T_a of 56 °C.

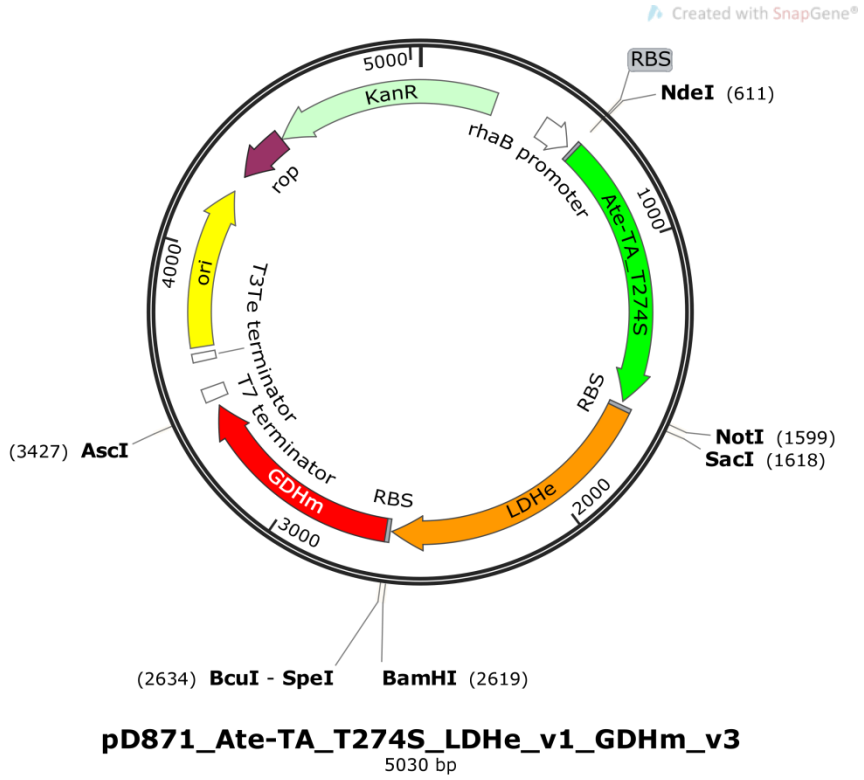

Figure 3: Plasmid map of G1, pD871_Ate-TA_T274S_LDHe_v1_GDHm_v3

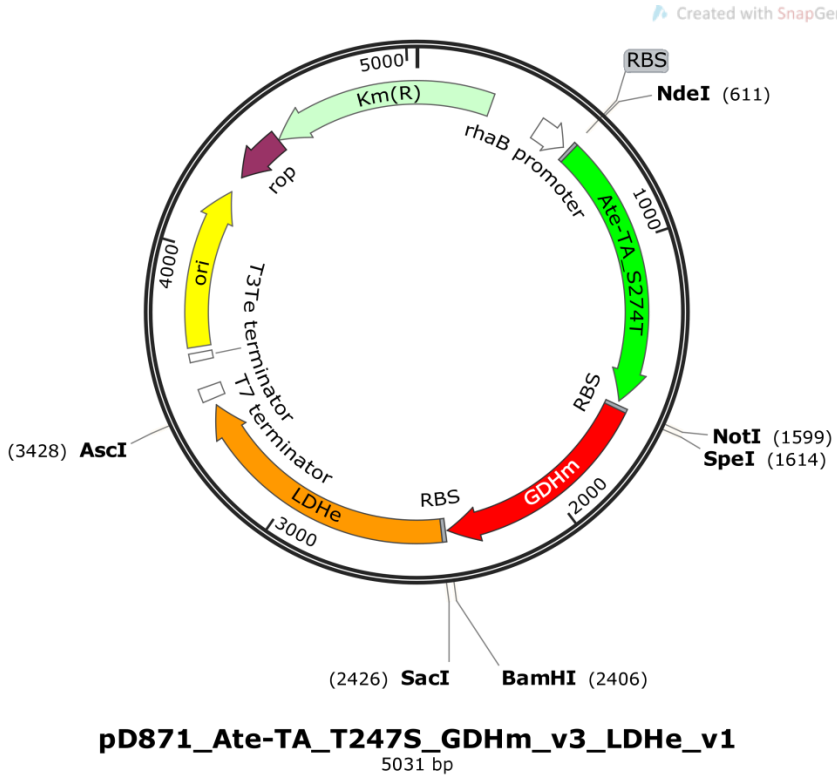

Figure 4: Plasmid map of G4, pD871_Ate-TA_T274S_GDHm_v3_LDHe_v1

Ate_LDH_GDH						
Primer	**Template**	**Sequence**	**bp**	**Tm**	**Ta**	**RBS**
1L1_fwd	LDH	aacgtaattaaggttagagcggccgcaggaggaagagctcATGGCTGCATTGAAAGAC	58	60 °C	56 °C	Synthetic
1L2_fwd	LDH	aacgtaattaaggttagagcggccgcgtaaggagagctcATGGCTGCATTGAAAGAC	57	60 °C		From *hslU*
1L3_fwd	LDH	aacgtaattaaggttagagcggccgcgagcgagaagagctcATGGCTGCATTGAAAGAC	59	60 °C		From *gyrB*
1L_rev	LDH	taactctccgggatccTTAGAACTGCAACTCTTTC	35	56 °C		-
1G_fwd	GDHm	agttctaaggatcccggagagttactagtATGTATACAGATTTAAAAGATAAAGTAGTTG	60	59 °C	59 °C	From *cpn10*
1G_rev	GDHm	agttattgctcagcggtggcggcgcgccTTAGCCTCTTCCTGCTTG	46	61 °C		-
Ate_GDH_LDH						
Primer	**Template**	**Sequence**	**bp**	**Tm**	**Ta**	**RBS**
2G_fwd	GDHm	gtaattaaggttagagcggccgcggagagttactagtATGTATACAGATTTAAAAGATAA	60	51 °C	49 °C and 56 °C*	From *cpn10*
2G1_rev	GDHm	ttcctcctgtggatccTTAGCCTCTTCCTGCTTG	34	61 °C		-
2G2_rev	GDHm	tccttacgctggatccTTAGCCTCTTCCTGCTTG	34	61 °C		-
2G3_rev	GDHm	ttctcgctcaggatccTTAGCCTCTTCCTGCTTG	34	61 °C		-
2L1_fwd	LDH	aagaggctaaggatccacaggaggaagagctcATGGCTGCATTGAAAGAC	50	60 °C	56 °C	Synthetic
2L2_fwd	LDH	aagaggctaaggatccagcgtaaggagagctcATGGCTGCATTGAAAGAC	50	60 °C		From *hslU*
2L3_fwd	LDH	aagaggctaaggatcctgagcgagaagagctcATGGCTGCATTGAAAGAC	50	60 °C		From *gyrB*
2L_rev	LDH	agttattgctcagcggtggcggcgcgccTTAGAACTGCAACTCTTTC	47	56 °C		-

Table 3: Primer list for the construction of the co-expression plasmids. Blue indicates annealing with the template. Green underlining indicates overlap for Gibson assembly. Red indicates the ribosome binding site. bp: number of base pairs. Tm: melting temperature of the annealing region. Ta: Annealing temperature used for the PCR. *: those PCRs consisted of 5 cycles with a Ta of 49 °C followed by 20 cycles with a Ta of 56 °C.

The PCR product was purified by electrophoresis and extracted from the gel slices.

pD871_Ate-TA_T274S was linearized with NotI and also purified in an electrophoresis. NotI cuts nine bases downstream of Ate's stop codon. Since NotI is important for the exchange of amino transaminases later on, it had to be regenerated in the Gibson assembly. For that purpose, the missing part of its recognition sequence was included in all the fragments fused to the 3' end of the linearized plasmid.

2.7.1 Gibson Assembly

All the enzymes necessary for the Gibson assembly (ligase, polymerase and exonuclease) were contained in a master mixture of 15 µl aliquots kindly provided by Kerstin Steiner of ACIB.

73.8 ng of linearized pD871_Ate-TA_T274S were mixed with 25 ng of LDH fragment and 20 ng of GDH fragment equaling 38.4 fmol for each fragment (table 4). The mix was brought to a volume of 5 µl with ddH2O, added to 15 µl of Gibson assembly master mixture and incubated at 50 °C for 60 minutes. 2 µl of the assembly were used to transform 80 µl of electrocompetent *E. coli* TOP10 F'. After 60 minutes of regeneration in 1 ml LB medium, 50 µl and the spun down rest of the cell suspension were plated on LB-Kan agar (40 µg/ml kanamycin). Three colonies of each variant were used to inoculate an ONC for plasmid isolation.

Table 4: Fragment combinations used for the Gibson assembly and the resulting names of the plasmids. The fragments are named after their unique PCR primers. The construct number was the one used in the lab.

LDH fragment	GDH fragment	Plasmid name	Construct number
1L1	1G	pD871_Ate-TA_T274S_LDH_v1_GDHm_v3	G1
1L2	1G	pD871_Ate-TA_T274S_LDH_v2_GDHm_v3	G2
1L3	1G	pD871_Ate-TA_T274S_LDH_v3_GDHm_v3	G3
2L1	2G1	pD871_Ate-TA_T274S_GDHm_v3_LDH_v1	G4
2L2	2G2	pD871_Ate-TA_T274S_GDHm_v3_LDH_v2	G5
2L3	2G3	pD871_Ate-TA_T274S_GDHm_v3_LDH_v3	G6

The correct assembly of the fragments was checked by restriction digest of the plasmids using AscI and BamHI:

2 µl 10x FD Green buffer
5 µl plasmid DNA
0.5 µl FD AscI
0.5 µl FD BamHI
12 µl ddH2O
20 µl total

One correct clone for each construct was then sent for sequencing.

2.7.2 RBS variation for GDHm

The variation of the ribosome binding sites for GDHm was done by restriction cloning. For this purpose, I designed primers adding the various ribosome binding sites and the necessary restriction sites for integration into the previously constructed plasmids (table 5).

Table 5: Primers used for RBS variation of GDHm with the melting temperature of their respective annealing region and the source of their RBS

Primer	Sequence	Tm	RBS
1Gv1_fwd	aaaaGGATCCGGAGGAGGactagtATG TATACAGATTTAAAAGATAAAGTAGTTG	59 °C	From *gdh*
1Gv2_fwd	aaaaGGATCCGATAAACGactagtATG TATACAGATTTAAAAGATAAAGTAGTTG	59 °C	From *ftsI*
1Gv_rev	aaaGGCGCGCCTTAGCCTCTTCCTGCTTG	61 °C	-
2Gv1_fwd	aaaaGCGGCCGCGGAGGAGGactagtATG TATACAGATTTAAAAGATAAAGTAGTTG	59 °C	From *gdh*
2Gv2_fwd	aaaaGCGGCCGCGATAAACGactagtATG TATACAGATTTAAAAGATAAAGTAGTTG	59 °C	From *ftsI*
2Gv_rev	aaaaGGATCCTTAGCCTCTTCCTGCTTG	61 °C	-

The PCR was carried out as explained in section 2.7.1 with a Ta of 59 °C. The template was pMS470Δ837_GDHm. The product was purified using Thermo Fisher's GeneJET PCR Purification Kit according to the manufacturer's manual. After that, the PCR products and the finished plasmids 1-6 were cut with the respective restriction enzymes (table 6) and purified with an agarose gel electrophoresis. The bands were cut out and the DNA was extracted. The fragments were then joined by ligation, resulting in two new variations (v1 and v2) for each of the constructs G1 through G6. The ligation mixture was desalted with the PCR Purification Kit and used for transformation of *E. coli* TOP10 F'. ONCs were inoculated

and plasmid isolations performed for one clone of each of the 12 constructs. The resulting DNA was sent for sequencing. The completion of the RBS variation for GDHm yielded 18 different co-expression vectors.

Table 6: Restriction enzymes, plasmids and fragments used for RBS variation of GDHm, the resulting plasmid names and the short construct numbers.

Restriction enzymes	Plasmids	Fragments	Plasmid name	Construct number
BamHI AscI	G1-G3	1Gv1	pD871_Ate-TA_T274S_LDH_v1_GDHm_v1	G1v1
			pD871_Ate-TA_T274S_LDH_v2_GDHm_v1	G2v1
			pD871_Ate-TA_T274S_LDH_v3_GDHm_v1	G3v1
		1Gv2	pD871_Ate-TA_T274S_LDH_v1_GDHm_v2	G1v2
			pD871_Ate-TA_T274S_LDH_v2_GDHm_v2	G2v2
			pD871_Ate-TA_T274S_LDH_v3_GDHm_v2	G3v2
NotI BamHI	G4-G6	2Gv1	pD871_Ate-TA_T274S_GDHm_v1_LDH_v1	G4v1
			pD871_Ate-TA_T274S_GDHm_v1_LDH_v2	G5v1
			pD871_Ate-TA_T274S_GDHm_v1_LDH_v3	G6v1
		2Gv2	pD871_Ate-TA_T274S_GDHm_v2_LDH_v1	G4v2
			pD871_Ate-TA_T274S_GDHm_v2_LDH_v2	G5v2
			pD871_Ate-TA_T274S_GDHm_v2_LDH_v3	G6v2

2.8 Characterization of the co-expression constructs

The first step was to find suitable expression conditions for the constructs. Since 20 h of expression at 25 °C has proven ideal for the production of amine transaminases (paper ATA) I took this as a starting point and varied the L-rhamnose concentration used for induction. *E. coli* TOP10 carrying construct 1 were cultivated and induced with 0.02, 0.1 and 0.2 % L-rhamnose. Cultivation in 500 ml of TB medium, cell harvest and disruption were done as described in section 2.4 with the addition of 0.1 mM PLP to the buffer used for resuspension of the cell pellet. Additionally, the pellet of the 50,000 rcf centrifugation step was

resuspended in half the volume used for the resuspension of the cell pellet. Bradford protein assay and SDS-PAGE were performed for both fractions. The bands were analyzed with the software Gene Tools 4.3.8 by Syngene. The data for the bands' height in the resulting chromatogram was used for calculation of the bands' portions related to the total protein. The soluble fraction was then further characterized by GDH and LDH assays as described in the sections 2.5.1 and 2.5.2 with the exception that the samples were measured for only 5 minutes.

2.9 Phenol Red Assay

The phenol red assay was reported to be a reliable high throughput screening method for transaminases [15]. The assay was done in 96 well plates containing 80 µl reaction solution and 20 µl CFE. The reaction solution consisted of a 10 mM KPi buffer at pH 7.5, 5 % v/v methanol, 0.036 g/L phenol red, 1 g/L NADH, 9 g/L Glucose (50 mM), 2.4 g/L (20 mM) acetophenone, 45 g/L D-alanine and 0.5 g/L PLP. Additionally, controls containing water or pyruvate (20 mM) instead of acetophenone were measured. The samples used were G1, Ate, eV, LDH+GDHm (diluted to 5 U/mg for GDH and LDH each, at 10 mg/ml protein concentration) and the four dialyzed G1 preparations. The plate was incubated for two hours at 30 °C in a FLUOstar Omega plate reader. Absorbance was measured every 30 seconds at 560 nm and the plate was shaken for one second before each measurement cycle.

2.9.1 Dialysis

Different dialysis methods were applied in an effort to reduce the background in the phenol red assay caused by alternative ketone substrates in the cell lysate (

table 7). To remove RNA and DNA from the sample, RNase and DNase in different concentrations (table 7) were added before the dialysis. Additionally the temperature for dialysis was varied and the different buffers applied to find a balance between RNase and DNase activity, dialysis efficiency and preservation of the target enzymes. All buffers were used at pH 7.5. The dialysis tube was a ZelluTrans/Roth membrane with MWCO of 6000-8000 and 32 mm flat width. After dialysis, the lysate was transferred to a reaction tube and centrifuged for 5 minutes at 16100 rcf and 4 °C to remove denatured protein. Before and after dialysis, the RNA and DNA concentration in the samples were determined by Nanodrop. The protein concentration was measured again after dialysis.

Table 7: The four methods used for dialysis. *: the buffer was changed every 1.5 h while at room temperature.

		Method D1	**Method D2**	**Method D3**	**Method D4**
	Buffer	50 mM KPi	10 mM TRIS HCl	50 mM KPi	50 mM KPi
	Buffer V	800 ml*	2000 ml	2000 ml	2000 ml
	Lysate V	2 ml	1.5 ml	1.5 ml	1.5 ml
	MgCl2	2 mM	2.5 mM	2.5 mM	2.5 mM
	CaCl2	-	0.5 mM	0.5 mM	0.5 mM
	DNase (U/ml)	50	200	200	200
	RNase (U/ml)	50	500	500	500
Time at	**30 °C**	-	0.5 h	0.33 h	0.33 h
	RT	4.5 h	20 h	2 h	20 h
	8 °C	15.5 h	-	18 h	-

3 Results

3.1 Cloning

The gel analysis of the cPCR performed on pJET1.2_GDHm and pJET1.2_GDHs (figure 5) showed two distinct bands for each gene at a size of about 970 bp. This matches the expected size of the GDH genes amplified in the cPCR and indicates correct ligation.

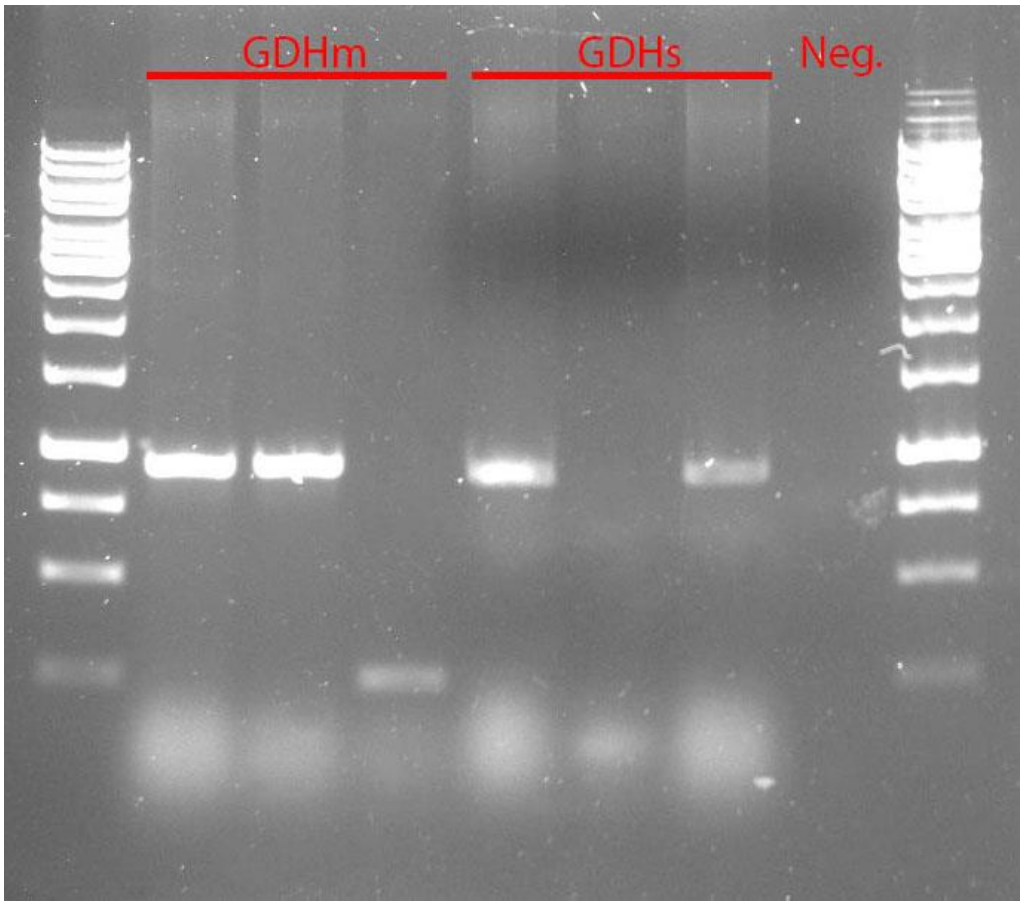

Figure 5: Gel analysis of the cPCR products of pJET1.2_GDHm, pJET1.2_GDHs and the control without template

Sequencing of those four correct ligations showed that the first GDHm clone and the third GDHs clone carried no mutations in the respective genes. They were used for further cloning steps. The preparative agarose gel of the restriction digestion of the two plasmids showed two clear bands for each digested plasmid (figure 6). The bands of interest were at 800 bp for GDHM and GDHs and at 4000 bp for the pMS470Δ837 backbone.

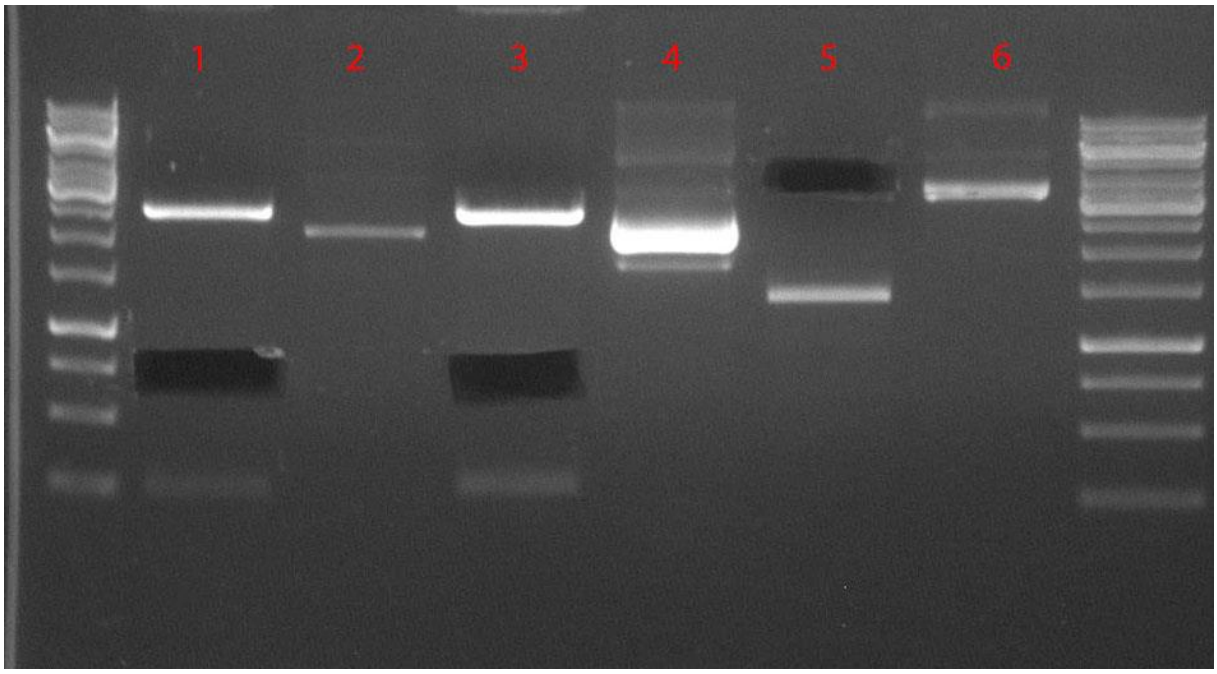

Figure 6: Gel analysis of the restriction digest with NdeI and HindIII. 1: pJET1.2_GDHm cut, 2: pJET1.2_GDHm uncut, 3: pJET1.2_GDHs cut, 4: pJET1.2_GDHs uncut, 5: pMS470Δ837_eV cut, 6: pMS470Δ837_eV uncut

The correct ligation of pMS470Δ837 with GDHm or GDHs was also checked by cPCR and agarose gel electrophoresis (figure 7). The bands visible at around 960 bp indicate correct ligation for all the checked plasmids compared to the control sample of pMS470Δ837_eV showing a band at around 1500 bp.

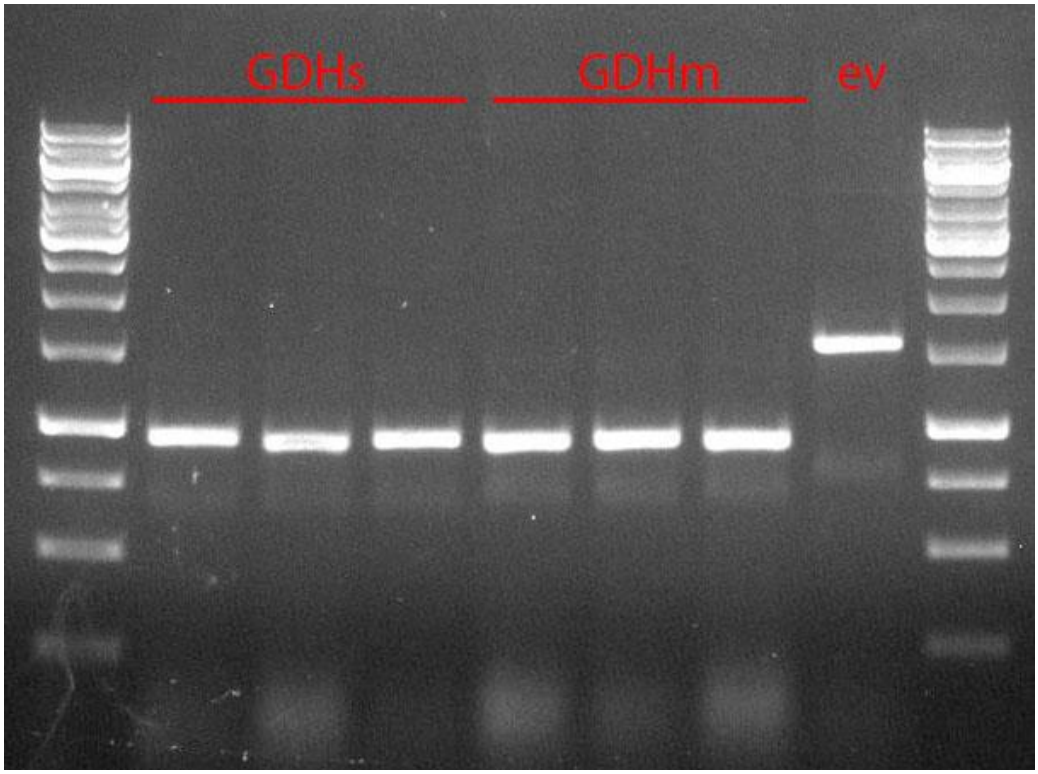

Figure 7: Agarose gel analysis of the cPCR of pMS470Δ837_GDHs, pMS470Δ837_GDHm and pMS470Δ837_eV

3.2 Characterization

3.2.1 Enzymes from Small Scale Production

Bradford protein assay

The Bradford protein assay (figure 8, table 8) for the CFE from the small scale cultivation resulted in protein concentrations between 4 and 5 mg/ml for GDHs and GDHm, whereas the empty vector control eV only contained around 2.5 mg/ml for LDH.

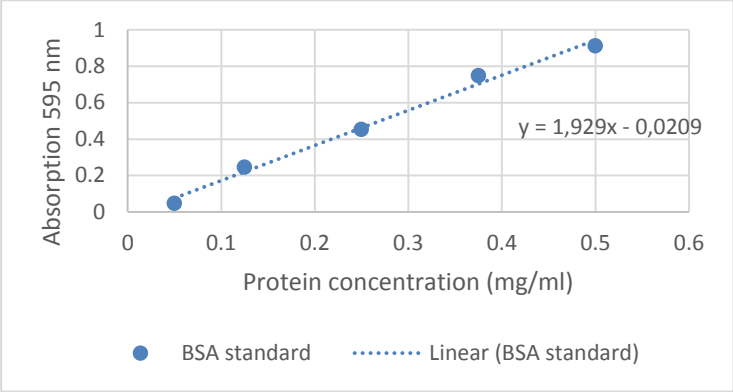

Figure 8: Standard measurements for the Bradford protein assay and the resulting linear equation used for protein concentration calculation

Table 8: Bradford protein assay for cleared lysates of GDHs, GDHm, LDH and the empty vector control (eV) produced on small scale. Absorption values shown are the means of the tree measurements and were subtracted with the blank measurement.

Sample	Dilution	Absorption	Concentration [mg/ml]	Mean Concentration [mg/ml]
GDHs1	1:10	0.893	4.1	3.9
	1:50	0.219	3.8	
GDHs2	1:10	0.866	3.9	4.9
	1:50	0.305	5.9	
GDHm1	1:10	1.047	4.8	5.1
	1:50	0.281	5.3	
GDHm2	1:10	1.000	4.6	4.8
	1:50	0.270	5.0	
LDH1	1:10	0.561	2.7	2.8
	1:50	0.129	2.9	
LDH2	1:10	0.564	2.8	2.7
	1:50	0.120	2.7	
eV1	1:10	0.684	3.7	4.1
	1:50	0.155	4.6	
eV2	1:10	0.684	3.7	4.1
	1:50	0.157	4.6	

SDS-PAGE

The SDS-PAGE analysis for those samples which showed clearly visible bands (figure 9). Those for GDHs and GDHm were found at about 33 kDa while their actual size is 28.2 kDa and 28.1 kDa respectively. This change in migration is probably due to the amino acid composition of the protein. The bands for the LDH appear just below the 40 kDa standard band at around 38 kDa while the enzyme's actual size is 36.6 kDa. The protein expressed via the control plasmid pMS470Δ837_eV appears at around 49 kDa while its actual size is 45 kDa. The intensity of the bands clearly match the results of the Bradford protein assay. We see high expression for both GDH and eV while the LDH was expressed in lower quantities.

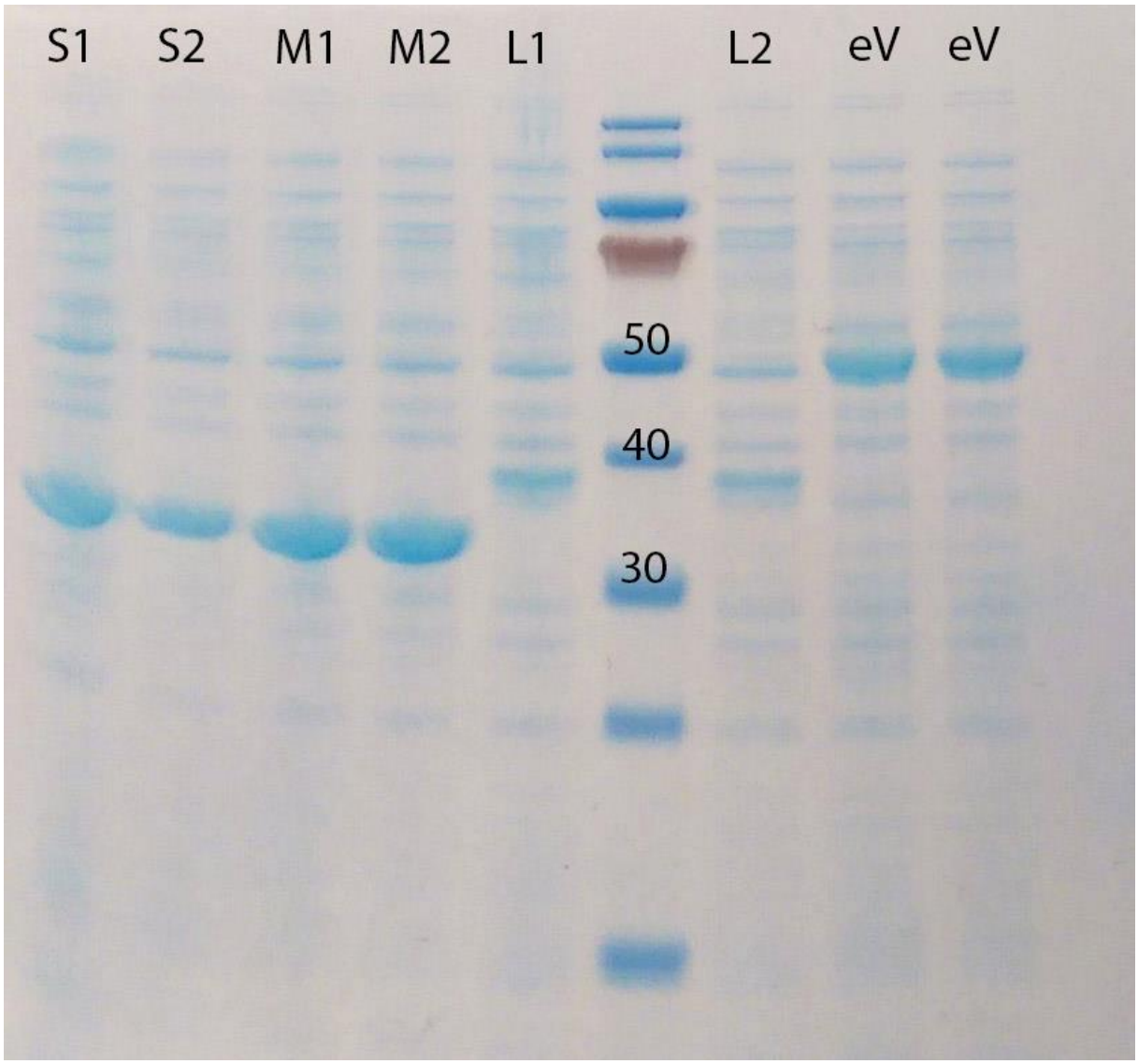

Figure 9: SDS-PAGE analysis of GDHm, GDHs, LDH and eV produced on small scale. The numbers on the standard denote the size of the respective bands in kDa.

Determination of the millimolar extinction coefficient of NADH

The determination of the millimolar extinction coefficient yielded a mean ε of 4.68 L mM^{-1} cm^{-1} (table 9).

Table 9: Photometric determination of the millimolar extinction coefficient of NADH at 340 nm

Concentration [mM]	Absorption at 340 nm	ε [L mM^{-1} cm^{-1}]
0.05	0.238	4.76
0.075	0.357	4.75
0.1	0.482	4.82
0.125	0.591	4.73
0.15	0.710	4.73
0.2	0.941	4.70
0.25	1.119	4.48
0.3	1.338	4.46
	Mean value	**4.68**

GDH assay

The enzyme activity assay of GDHm and GDHs (figure 10) performed with thawed CFE showed a clear increase in the absorption at 340 nm showing conversion of NAD+ to NADH. This translates to activities of 106 U/mg for GDHm1, 109 U/mg for GDHm2, 27 U/mg for GDHs1 and 22 U/mg for GDHs2. This gives a mean activity of 107 U/mg for GDHm and 21 U/mg for GDHs. The control sample eV shows no increase in NADH concentration, ruling out the possibility of native *E. coli* enzymes producing a background for this assay. All CFEs were diluted 1:100.

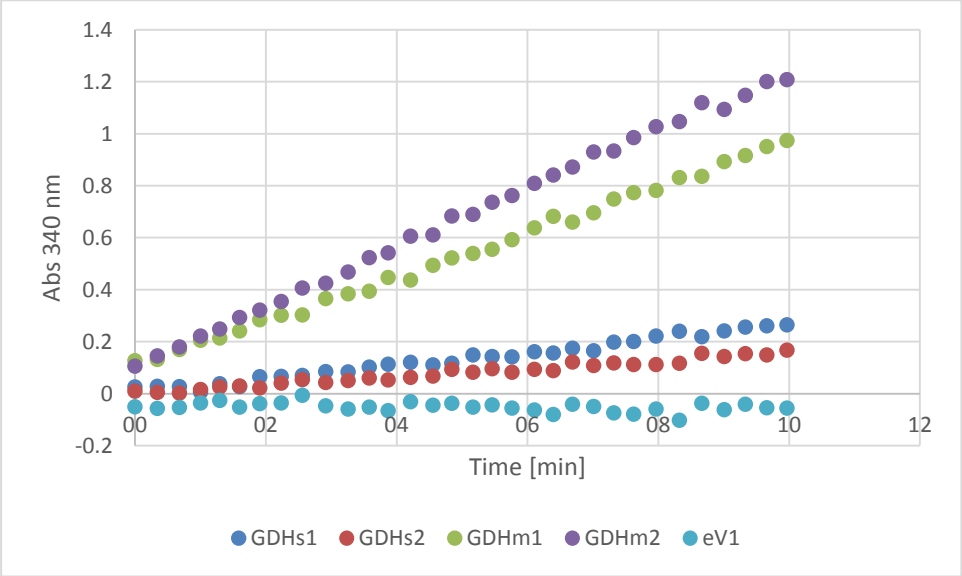

Figure 10: GDH assay for the duplicates of GDHm, GDHs and one sample of eV

LDH Assay

The LDH activity assay (figure 11) also yielded good results. A steady decrease of absorption at 340 nm could be observed after the addition of the LDH CFE, while the addition of eV CFE did not show this effect. Those slopes translate to an activity of 10 U/mg for LDH1 and 13 U/mg for LDH2. All CFEs were diluted 1:10.

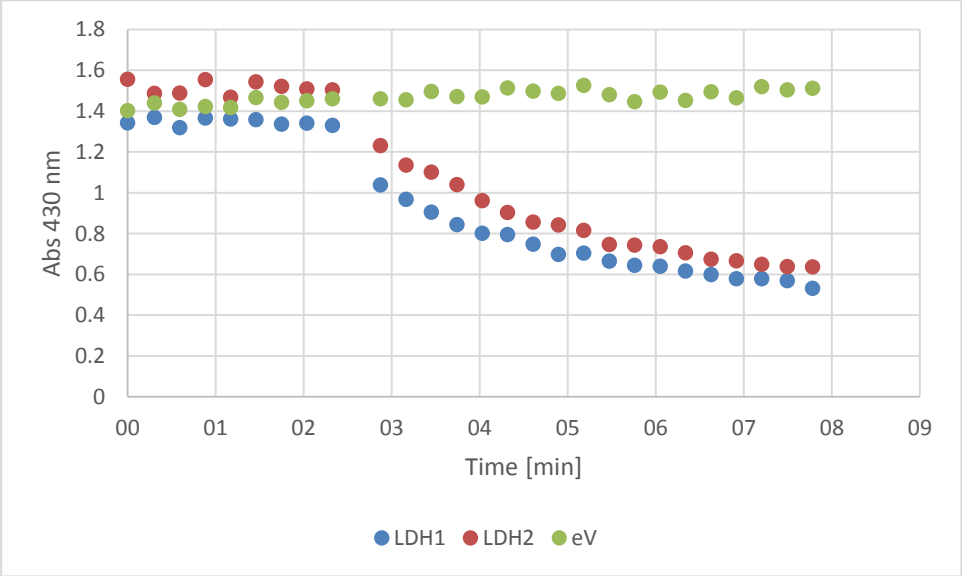

Figure 11: LDH assay for the duplicates of LDH and one sample of eV

Heat Stability Test of GDHm and GDHs

The heat stability test of GDHs (figure 12) and GDHm (figure 13) yielded quite clear results. While GDHm still showed an activity of 20 U/mg after 2 minutes at 45 °C, GDHs showed none at all after the same time. After five minutes also GDHm was not able anymore to produce a detectable increase in absorption at 340 nm. The absorption dipping below 0 at some points is due to the combination of the blank and measurement inaccuracies.

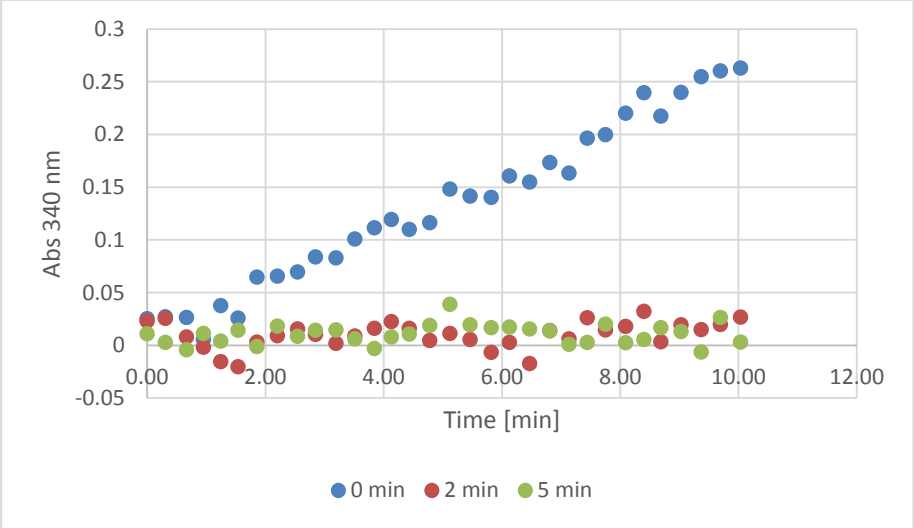

Figure 12: GDHs stability test. The samples were kept on 45 °C for 0, 2 and 5 minutes

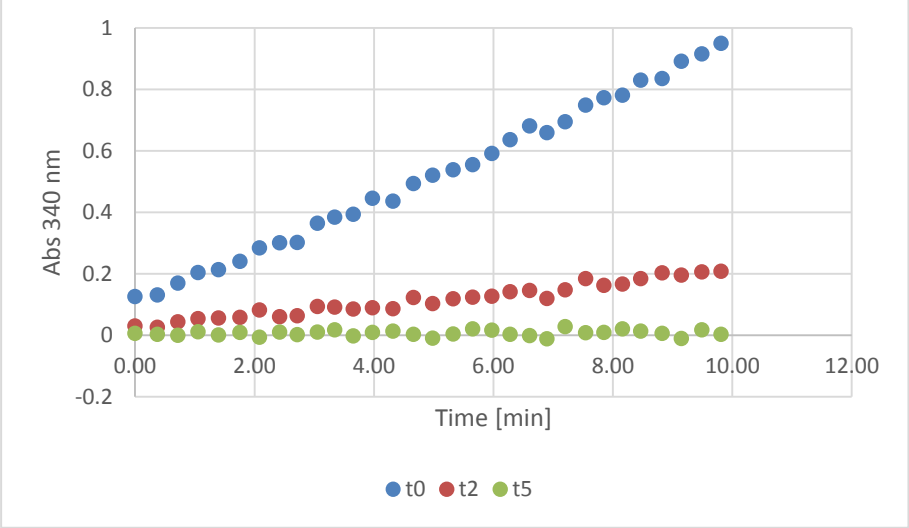

Figure 13: GDHm stability test. The samples were kept on 45 °C for 0, 2 and 5 minutes

3.2.2 Enzymes from Large Scale Production

Bradford Protein Assay

For the cultures grown in 500 ml TB medium, the Bradford protein assay (figure 14, figure 15,) resulted in total protein concentrations of around 12 mg/ml for GDHs, 14 mg/ml for GDHm and around 8 mg/ml for the LDH. The 1:10 dilution for every sample were out of the range of the assay so the 1:50 dilutions were used for the calculation. Even though the protein content obviously is different between the large and small scale process, the concentration for GDHs is still a little lower than for GDHm and the one from LDH is considerably lower than both GDH.

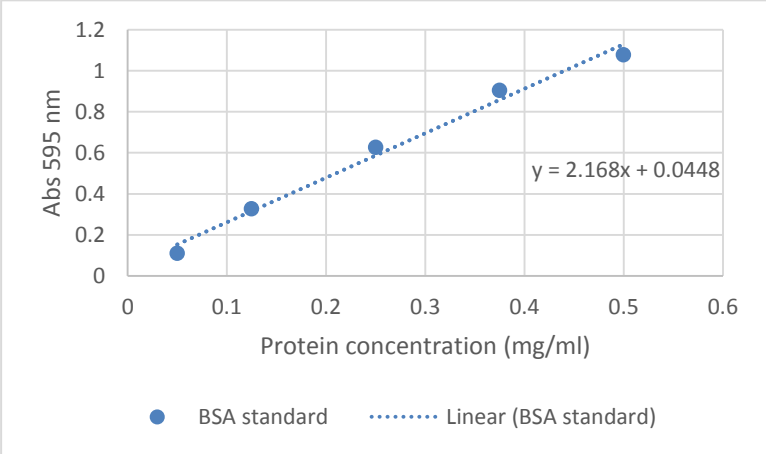

Figure 14: Standard measurements for the Bradford protein assay and the resulting linear equation used for protein concentration calculations of the samples GDHs1, GDHm1 and LDH1

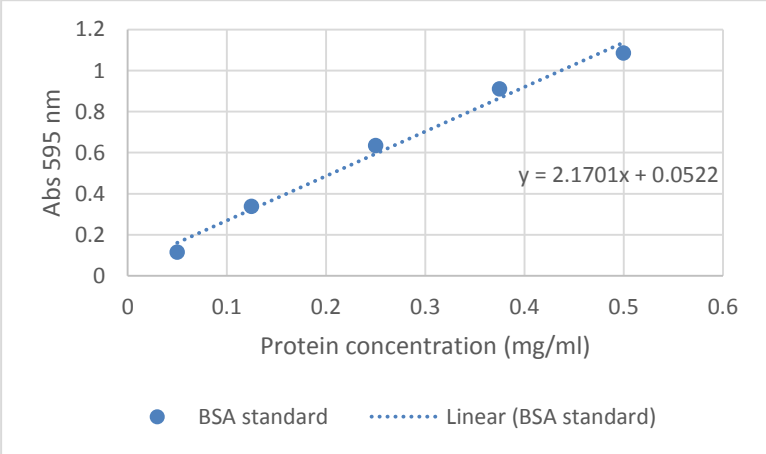

Figure 15: Standard measurements for the Bradford protein assay and the resulting linear equation used for protein concentration calculations of the samples GDHs2, GDHm2 and LDH2

Table 10: Bradford protein assay for CFEs of GDHs, GDHm and LDH produced on large scale. Absorption values shown are the means of the tree measurements and were subtracted with the blank measurement.

Sample	Dilution	Absorption	Concentration [mg/ml]
GDHs1	1:50	0.553	12.1
GDHs2	1:50	0.571	12.0
GDHm1	1:50	0.628	14.0
GDHm2	1:50	0.643	13.6
LDH1	1:50	0.414	8.5
LDH2	1:50	0.390	7.8

GDH Assay

The GDH assay yielded even bigger differences between GDHm and GDHs with calculated activities of 165 U/mg and 35 U/mg respectively resulting from a slope of 0.286 for GDHm and 0.058 for GDHs (figure 16).

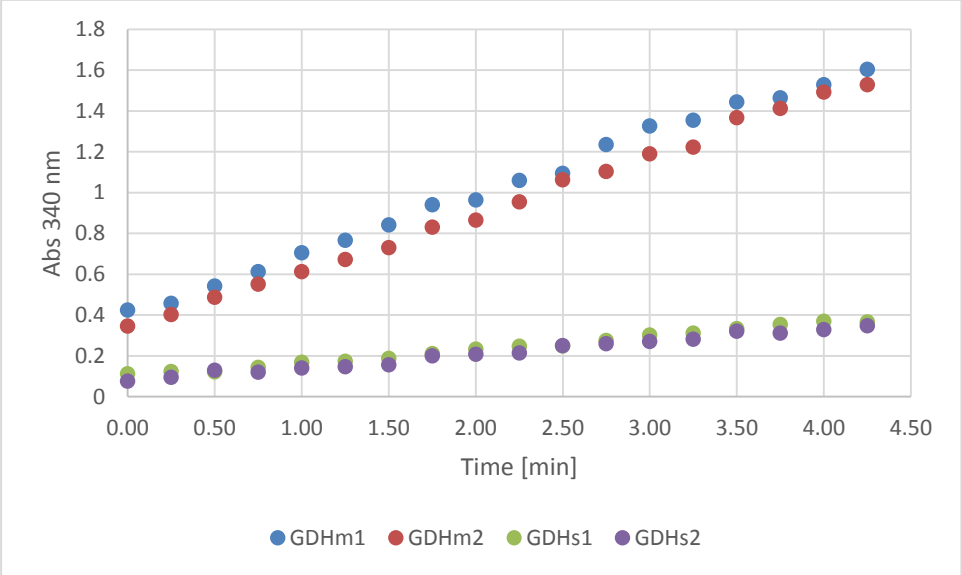

Figure 16: Photometric GDH activity assay for the duplicates of GDHm and GDHs measured at 340 nm

LDH Assay

For the LDH, the activity assay gave a mean slope of -0.126 (figure 17) which translates to an activity of 14 U/mg.

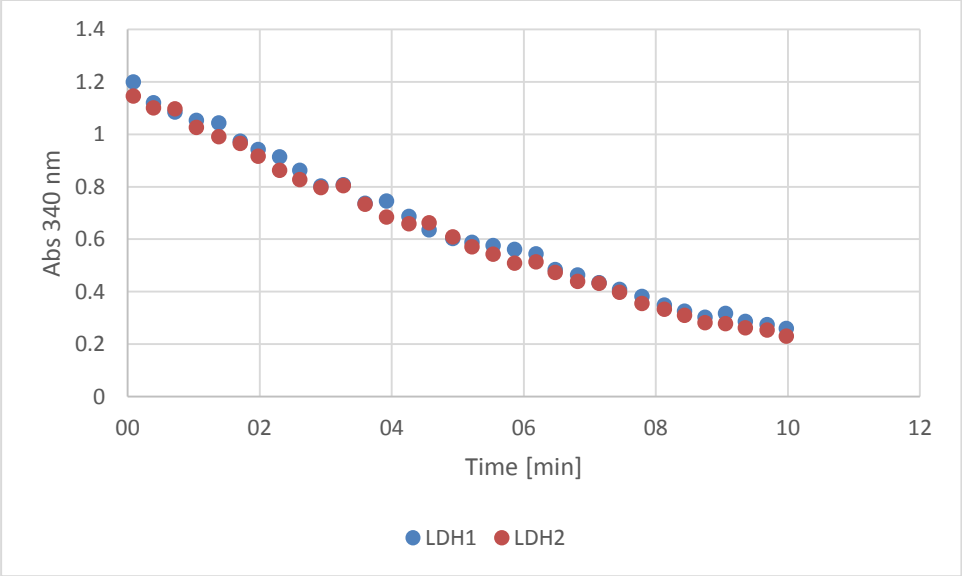

Figure 17: Photometric LDH activity assay at 340 nm for the duplicates of the LDH

SDS-PAGE

The SDS-PAGE analysis of the samples showed a similar presence of GDHm and GDHs in the soluble fraction while the bands for the LDH were far less intense (figure 18).

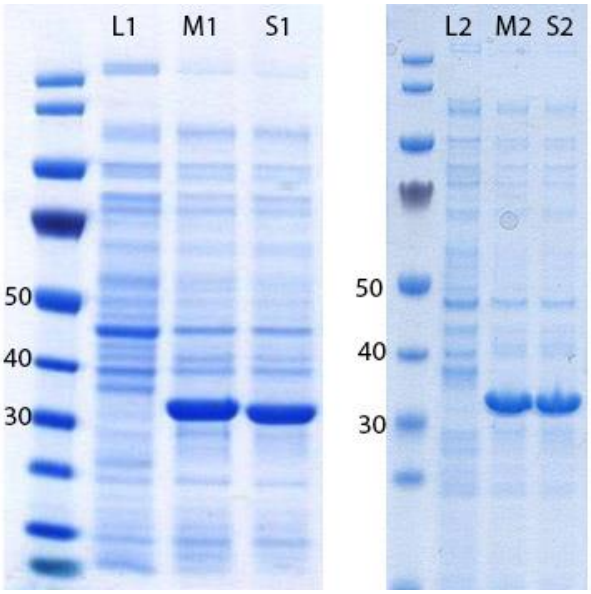

Figure 18: SDS-PAGE analysis of the duplicates for GDHm, GDHs and LDH. The numbers next to the standard denote the corresponding band's size in kDa

Densitometric Analysis

The densitometric analysis (table 11) delivered the portion of the three enzymes in their respective cleared lysate: 8 % for the LDH, 34 % for GDHm and 36 % for GDHs.

Table 11: Densitometric analysis of percentile portion of LDH, GDHm and GDHs of the total soluble protein

	LDH	GDHm	GDHs
% of total protein	8	34	36

3.3 Construction of the Co-expression Plasmids

The plasmid pD871_Ate-TA_T274S linearized with NotI was separated in an agarose gel (figure 19). The bands appeared at around 3200 bp, as expected. However, the uncut plasmid also shows a strong band at approximately the same length. This could be problematic if the digest with NotI was of poor efficiency and left a lot of uncut plasmid in the sample.

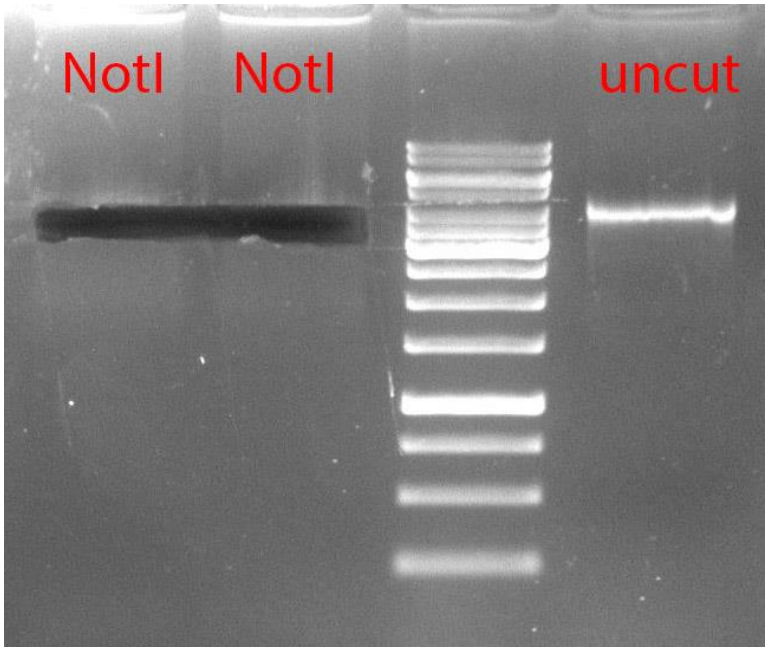

Figure 19: Preparative agarose gel of pD871_Ate-TA_T274S linearization by NotI including a control of the uncut plasmid

The preparative gel of the PCR for the production of the ten Gibson fragments showed quite clear bands at the expected sizes: ~1050 bp for all LDH fragments, ~850 bp for all GDH Gibson fragments and ~820 bp for the GDH fragments for RBS variation. Unfortunately, the

gel with the cut out bands was structurally not sound enough for the transfer into the UV chamber to be photographed.

After the Gibson assembly reaction was done, the resulting mix was used for transformation of *E. coli*. Several ONCs were inoculated with the resulting colonies for each construct and plasmid isolation was performed. The results of the control cuts of those plasmids with AscI and BamHI show correct assembly for all constructs (figure 20). AscI and BamHI excise the last of the three genes in the construct. This is GDHm for the constructs G1 to G3 and the LDH for G4 to G6. As expected, we find one band at ~ 805 bp for G1, G2 and G3 and one at ~ 1022 bp for G4, G5 and G6. We also see the plasmid backbone at ~ 3204 bp for all the correct constructs. The negative control, pD871_Ate-TA_T274S does not contain the restriction sites in question and therefore does not show such bands. The correct assembly of all 18 checked plasmids also confirms that a vast majority of the original plasmid was actually linearized. One plasmid for each of the constructs was sent for sequencing and none of them carried any unwanted mutation.

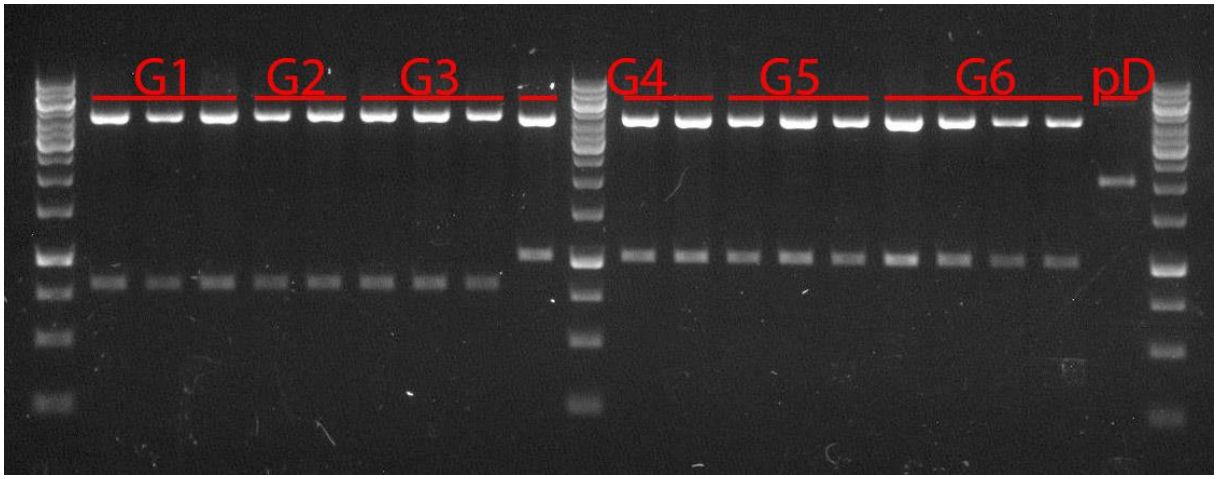

Figure 20: Agarose gel analysis of the constructed plasmids digested with AscI and BamHI. pD: original plasmid pD871_Ate-TA_T274S

Because the gel extraction caused substantial loss of DNA, I performed another PCR for the RBS variation of GDHm. The control gel yielded well defined bands at around 820 bp (figure 21).

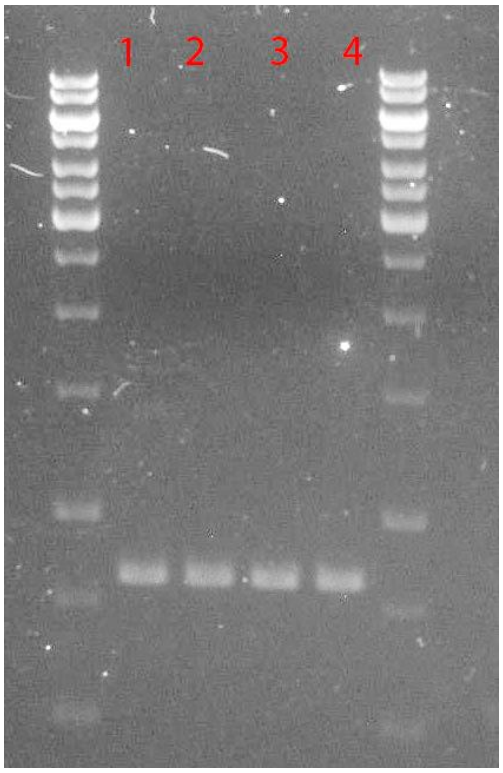

Figure 21: Control gel of PCR for GDHm RBS variation. 1: G1v1, 2: G1v2, 3: G2v1, 4: G2v2

The restriction digest of the six previously constructed plasmids showed the expected bands at 800 bp for GDHm and just above 4000 bp for the backbone (figure 22).

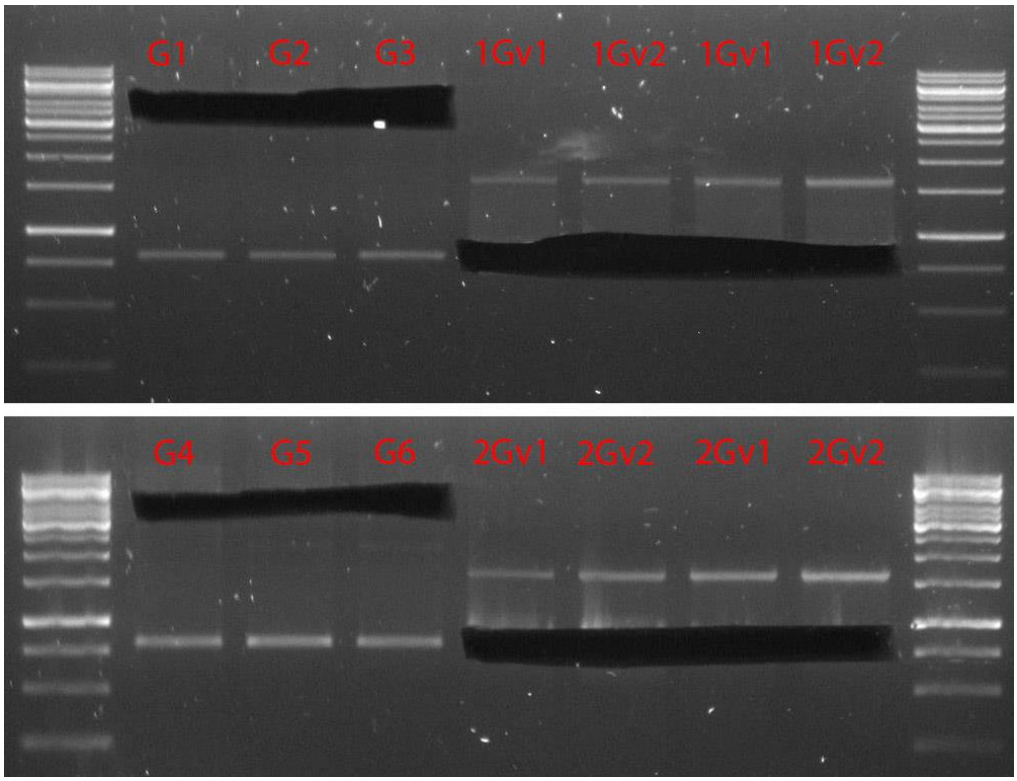

Figure 22: Preparative agarose gel for the RBS variation of GDHm with previously constructed plasmids and PCR products. DNA in upper gel cut with NotI and BamHI, in lower gel with BamHI and AscI

3.4 Characterization of the Co-expression Constructs

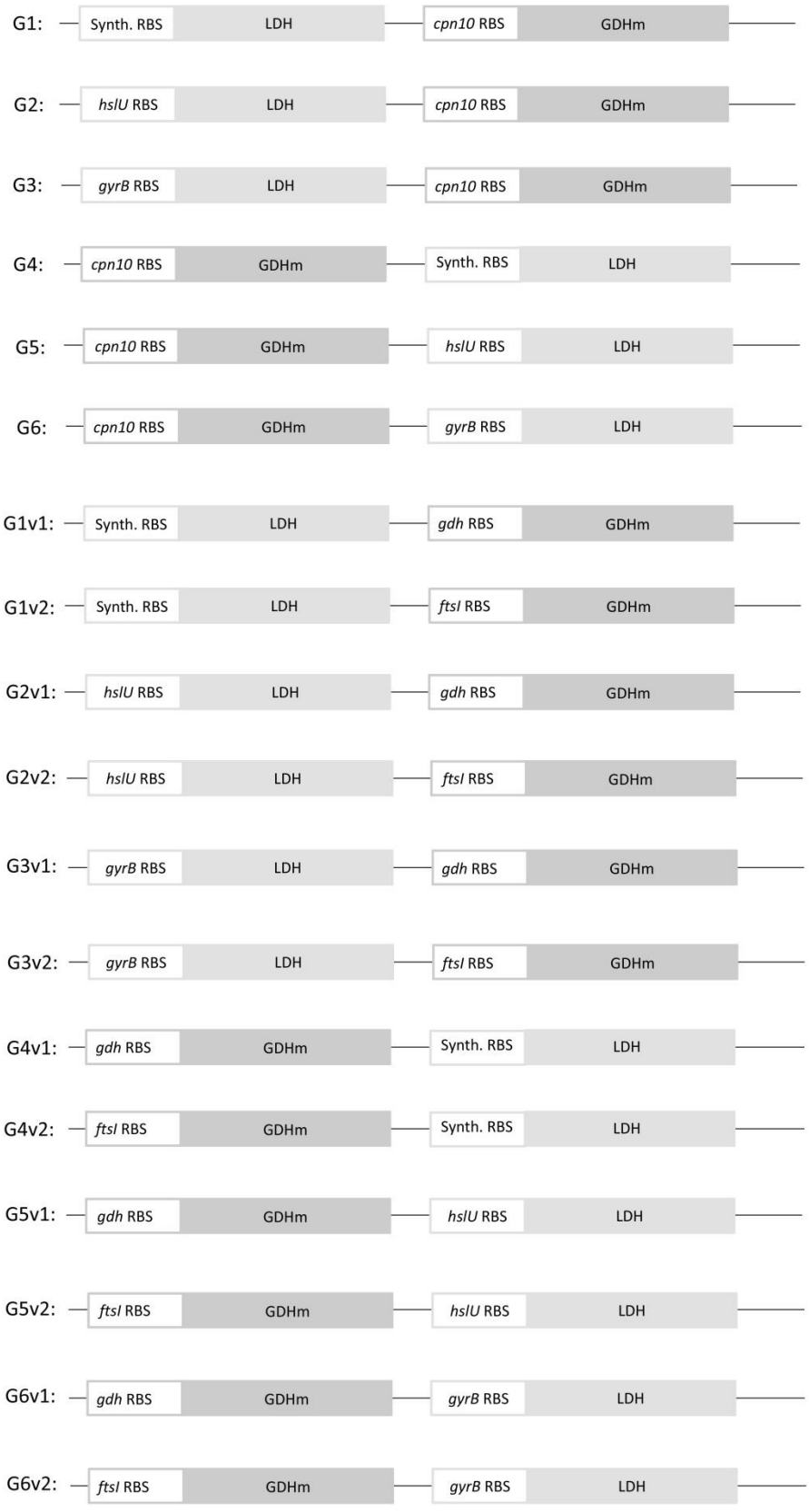

Figure 23: Overview of the co-expression constructs. Gene order and the used ribosome binding sites (RBS) are shown. Upstream of them all Ate is located (not shown).

The first step to characterize the co-expression plasmids (**figure 23**) was to find a suitable L-rhamnose concentration for inducing protein expression. The total protein concentration of the cell free extract was 11.9 mg/ml for 0.02 %, and 12.1 mg/ml for both 0.1% and 0.2% L-rhamnose. The insoluble fraction after centrifugation had a concentration of 0.5 mg/ml for all three concentrations. All these numbers do not show any notable difference in protein production.

Densitometric analysis of the SDS-PAGE (figure 24) gave more valuable results. As Ate and LDH are very similar in size (36.2 kDa and 36.6 kDa respectively) they are visible as a single band. The portion of Ate/LDH in the soluble fraction was found to increase with the L-rhamnose concentration (table 12). It made up 7 %, 12 % and 14 % for 0.02 %, 0.1 % and 0.2 % L-rhamnose respectively. The amount of insoluble Ate/LDH increased at a similar rate from 3 %, through 5 % up to 6 %. Thus, 0.2 % L-rhamnose was used for induction, since the amount of enzyme in the soluble fraction was the largest while also no excessive amounts of insoluble protein were produced.

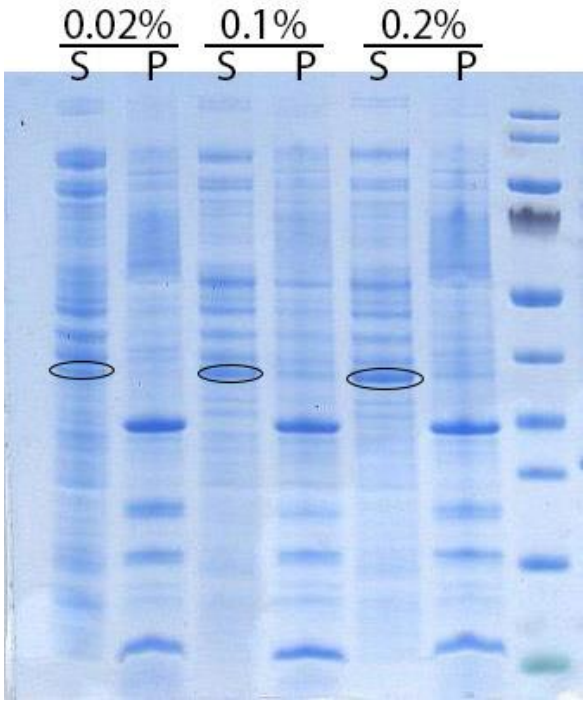

Figure 24: SDS-PAGE of soluble (S) and insoluble (P) fractions of the different L-rhamnose concentrations (0.02 %, 0.1 %, 0.2 %) used for induction. The marked bands show Ate/LDH.

Table 12: Results of the densitometric analysis determining the portion of the Ate/LDH band of the total protein in the soluble (S) and insoluble (P) fraction.

	0.02 % S	**0.02 % P**	**0.1 % S**	**0.1 % P**	**0.2 % S**	**0.2 % P**
Portion Ate/LDH in %	7	3	12	5	14	6

3.4.1 Protein Concentration

The CFEs of the 18 variations showed a range of total protein content in the soluble fraction from 8.1 mg/ml for G3.2 up to 16.9 mg/ ml for G2v1.2 (table 13). Most duplicates of the same constructs show similar values, only G3 and G2v1 show a larger deviation of around 3 mg/ml. This is most probably due to problems in cell disruption in one of the duplicates. The protein concentrations of the resuspended pellet fractions were between 1.6 mg/ml and 3.2 mg/ml.

Table 13: Calculated protein concentrations for the soluble and the resuspended pellet fraction of the duplicates of the co-expression constructs

Construct	Protein concentration [mg/ml]		Construct	Protein concentration [mg/ml]	
	Soluble	**Insoluble**		**Soluble**	**Insoluble**
G1.1	10.9	2.1	**G2v2.1**	11.5	2.2
G1.2	10.3	1.8	**G2v2.2**	11.3	2.3
G2.1	9.9	2.8	**G3v1.1**	11.9	2.4
G2.2	9.7	2.5	**G3v1.2**	10.7	2.1
G3.1	10.7	2.3	**G3v2.1**	10.8	2.2
G3.2	8.1	2.4	**G3v2.2**	10.3	2.1
G4.1	8.6	1.8	**G4v1.1**	9.2	2.3
G4.2	9.0	1.7	**G4v1.2**	10.4	1.9
G5.1	9.6	1.7	**G4v2.1**	11.4	2.1
G5.2	9.4	1.6	**G4v2.2**	11.2	2.0
G6.1	8.8	1.9	**G5v1.1**	10.1	1.9
G6.2	8.8	2.2	**G5v1.2**	9.0	2.0
G1v1.1	14.5	3.2	**G5v2.1**	11.2	2.2
G1v1.2	13.7	2.4	**G5v2.2**	10.7	2.1
G1v2.1	13.6	3.0	**G6v1.1**	11.1	2.3
G1v2.2	13.0	2.5	**G6v1.2**	11.1	2.0
G2v1.1	13.8	3.2	**G6v2.1**	11.0	2.1
G2v1.2	16.9	2.9	**G6v2.2**	9.7	1.7

3.4.2 SDS-PAGE

Looking at the results of the SDS-PAGE (figure 25 to figure 30), an obvious difference can be found in the band of GDHm (the lower marked band in figure 25). It is best seen when looking at G3v1.2 and G3v2.1 (figure 25). We see a clearly visible band in G3v1 but not in G3v2. The only difference between the two plasmids is the RBS for GDHm and it results in a much lower expression of the enzyme in the v2 construct. That v1 and the standard RBS give more expression than v2 is also true for all other constructs. The insoluble fractions (P) of

the samples show a clear band for Ate/LDH at 38 kDa which indicates that some of the produced enzymes are not properly folded, coagulate and get removed during centrifugation. The samples of the insoluble fraction of G3v1.1 and G3v2.1 (figure 25) resulted in too much of a smear to be properly analyzed, so a lower amount of them was applied to an SDS gel once more (figure 28).

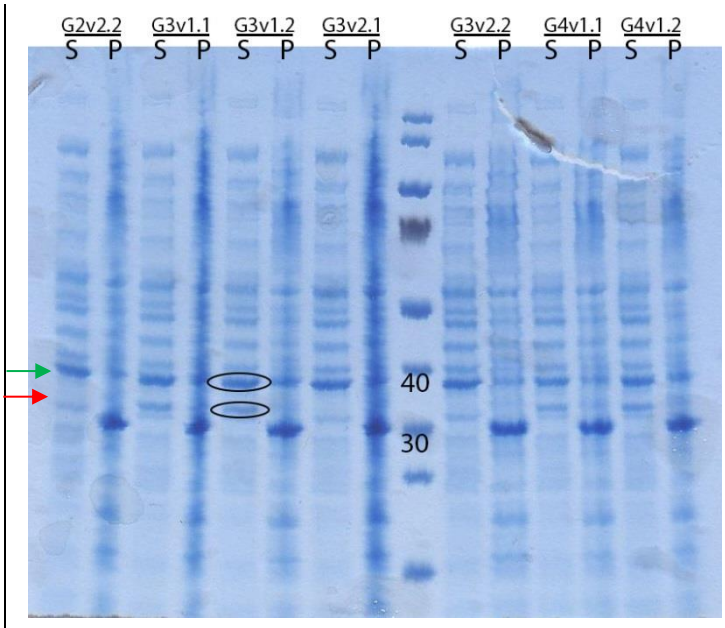

Figure 25: SDS-PAGE of the samples G2v2.2 to G4v1.2. S=soluble fraction, P=pellet after centrifugation. The upper marked band is Ate/LDH and the lower is GDHm. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

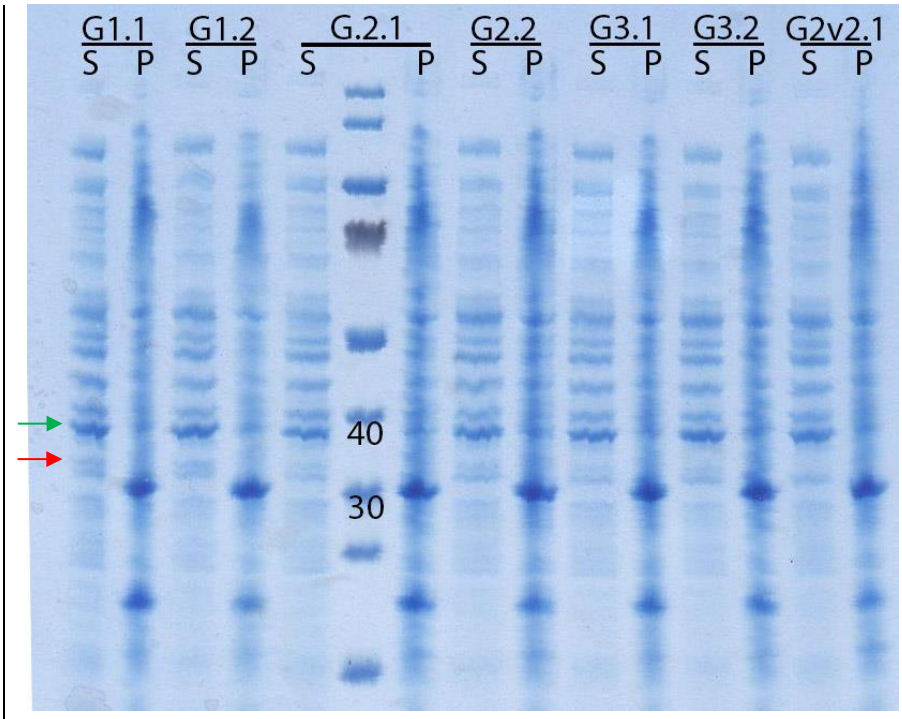

Figure 26: SDS-PAGE of the samples G1.1 to G3.2 and G2v2.1 . S=soluble fraction, P=pellet after centrifugation. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

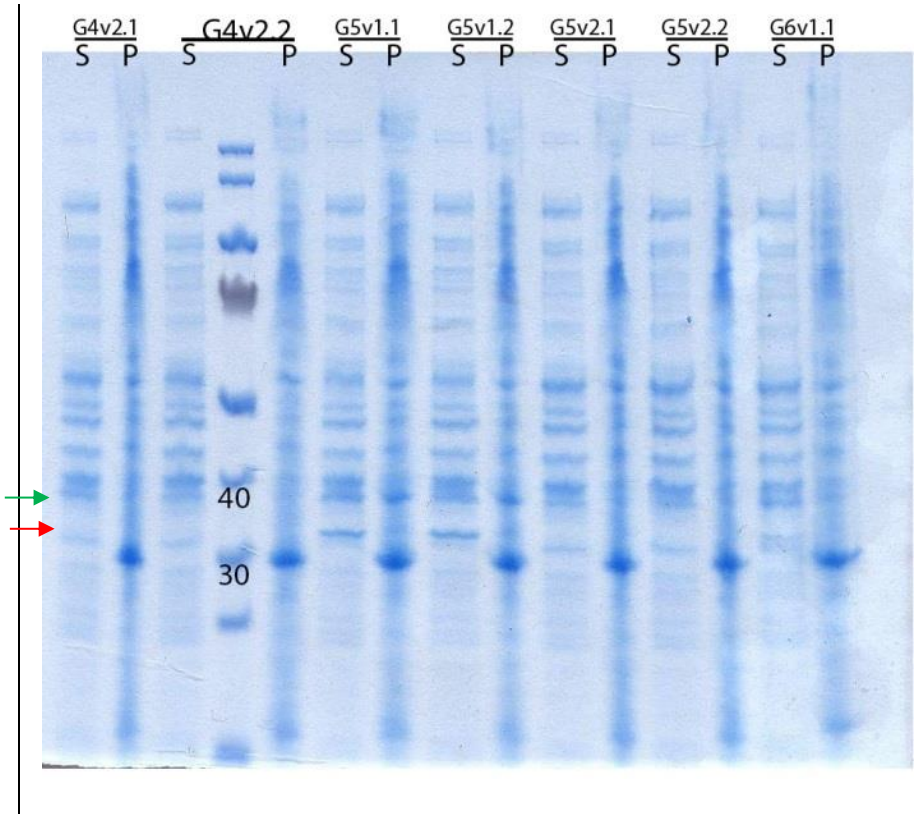

Figure 27: SDS-PAGE of the samples G4v2.1 to G6v1.1. S=soluble fraction, P=pellet after centrifugation. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

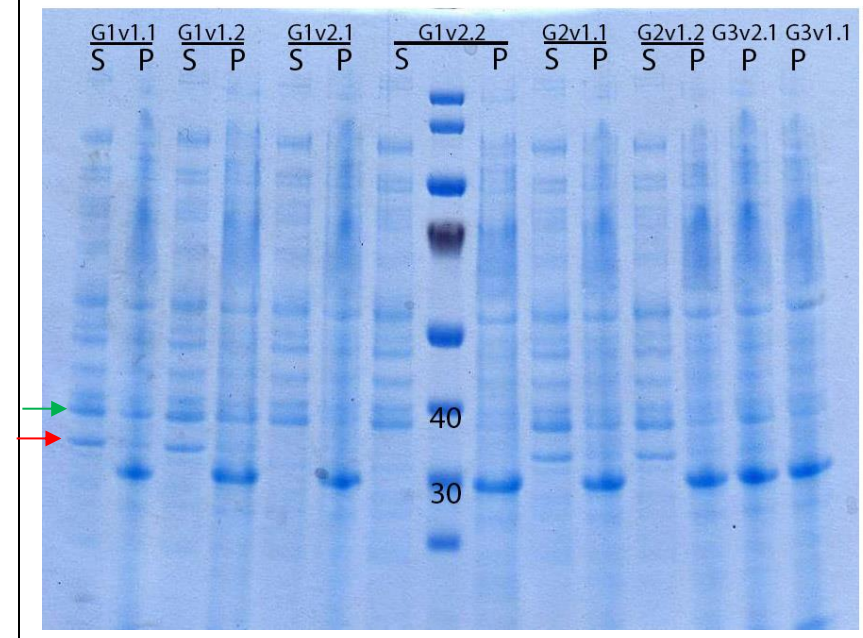

Figure 28: SDS-PAGE of the samples G1v1.1 to G3v1.1. S=soluble fraction, P=pellet after centrifugation. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

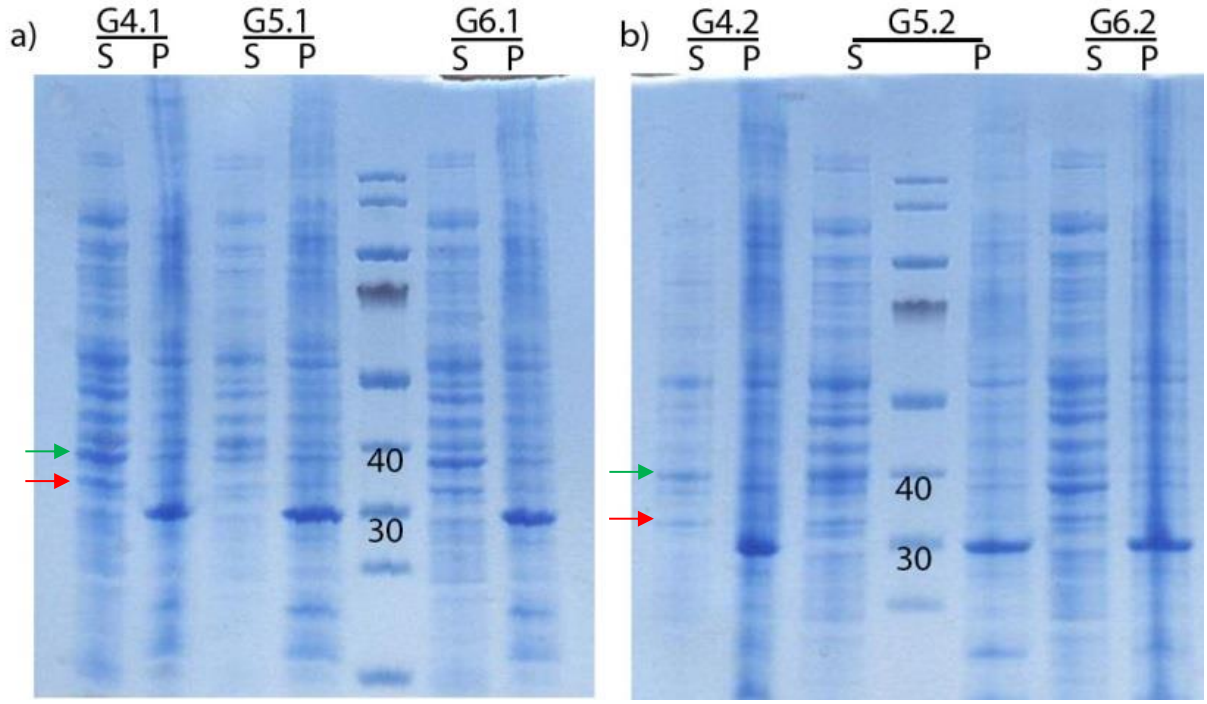

Figure 29: SDS-PAGE of the samples G4.1 to G6.1 (a) and G4.2 to G6.2 (b). S=soluble fraction, P=pellet after centrifugation. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

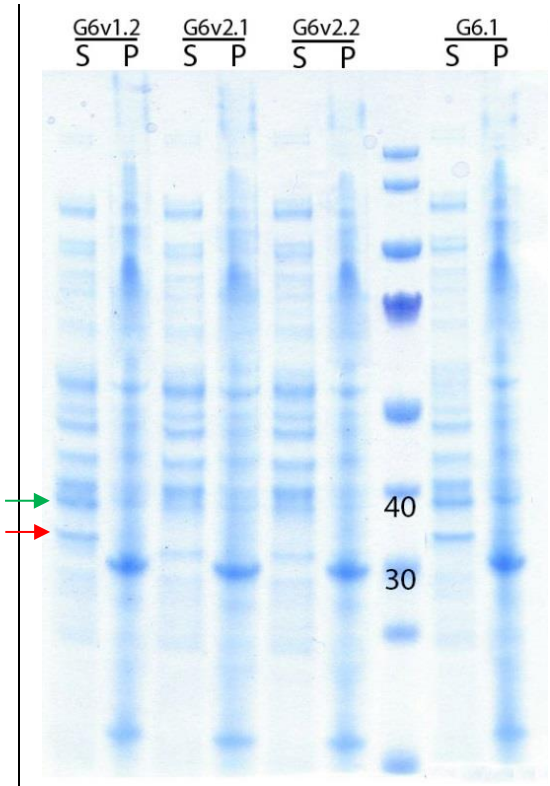

Figure 30: SDS-PAGE of the samples G6v1.2 to G6v2.2 and G6.1. S=soluble fraction, P=pellet after centrifugation. The arrows indicate Ate/Ldh (Green) and Gdh (red), respectively. The numbers on the standard denote the size of the bands in kDa

3.4.3 Densitometric analysis

The densitometric analysis of the SDS-PAGE bands for Ate/LDH and GDHm (figure 31 to figure 33) show interesting results. For the soluble fractions of G1, G2 and G3 there is a strong Ate/LDH band making up between 15 % and 18 % of the total protein on the gel while GDHm only accounts for 3 % to 6 %. The insoluble pellet fraction for G1, G2 and G3 contain 7 % to 10 % of Ate/LDH while no band for GDHm was detected. For G4, G5 and G6 the results are very different. The portion of soluble Ate and LDH are much lower with 9 % for G4, 8 % for G5 and 11 % for G6. The soluble GDHm reaches 11 % for G4 and 7 % for both G5 and G6. Here, we also see GDHm in the insoluble fraction where it makes up 2 % for G4 and 4 % for both G5 and G6 while Ate/LDH account for 6 % for G4 and 7 % for G5 and G6 (all figure 29).

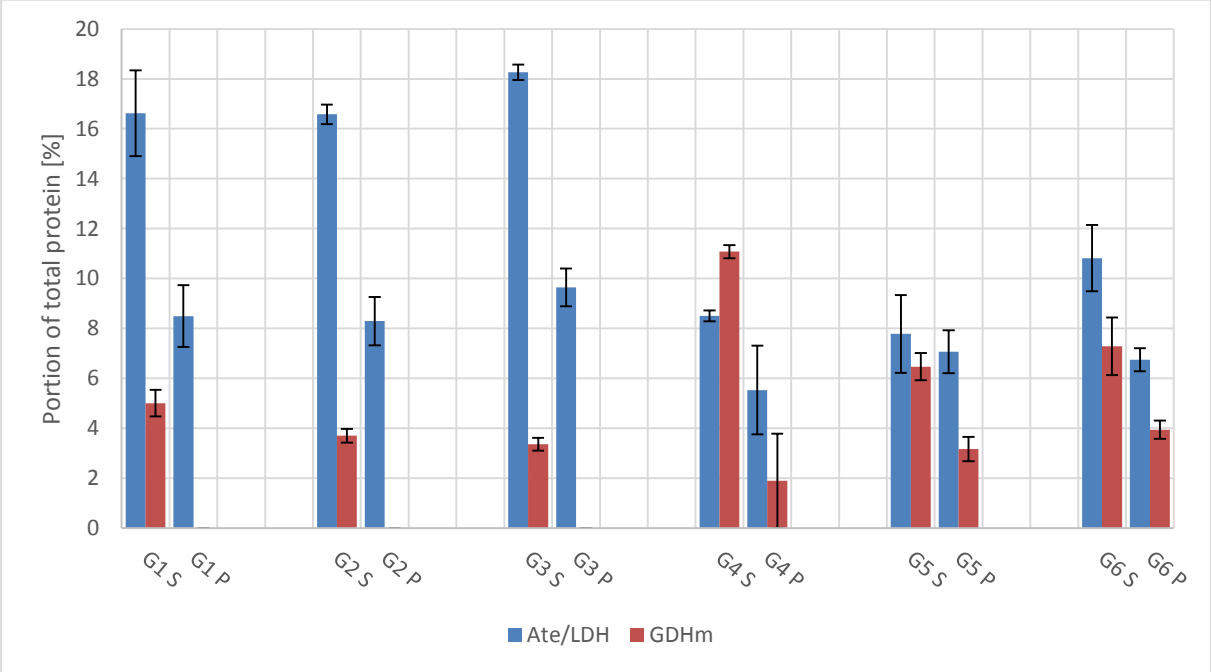

Figure 31: Results of the densitometric analysis of the SDS-PAGE for G1, G2, G3, G4, G5 and G6. S: soluble fraction, P: insoluble pellet fraction. Error bars show the deviation of the duplicates from the mean.

For G1v1, there are 13 % of the soluble protein consisting of Ate/LDH and GDHm each, while the insoluble fraction contains 13 % Ate/LDH and 5 % GDHm. In G1v2, Ate/LDH makes up 14 % of the soluble and 6 % of the insoluble fraction while no band for GDHm was detected. There are high levels of soluble Ate/LDH for G2v2, G3v1 and G3v2 ranging between 17 % and 20 % while we find between 8 % and 13 % in the insoluble fraction. In G3v1 we see 12 % soluble and 4 % insoluble GDHm. This is also true for all other v2 constructs. G2v1 shows 14 % Ate/LDH and 11 % GDHm in the soluble fraction while we find 9 % and 3 % respectively in the insoluble fraction (all figure 30).

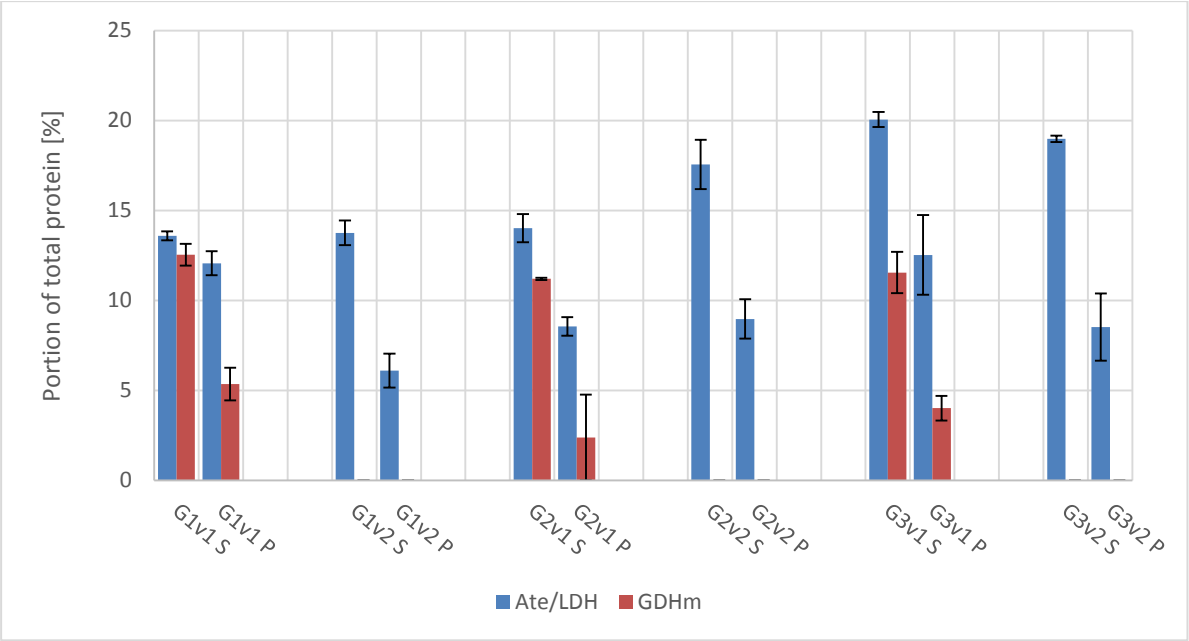

Figure 32: Results of the densitometric analysis of the SDS-PAGE for G1v1, G1v2, G2v1, G2v2, G3v1 and G3v2. S: soluble fraction, P: insoluble pellet fraction. Error bars show the deviation of the duplicates from the mean.

In G4v1, 14 % Ate/LDH and 9 % GDHm were detected in the soluble fraction while the insoluble fraction contained 12 % and 3 % respectively. For G4v2, 9 % of the soluble protein and 9 % of the insoluble protein were found to be Ate/LDH. G5v2 contained 12 % soluble and 14 % insoluble Ate/LDH while 14 % of the soluble protein and 5 % of the insoluble protein was GDHm. G5v2 showed a rather low expression of Ate/LDH which made up only 7 % of the soluble and 4 % of the insoluble protein. For G6v1, there are 12 % Ate/LDH in the soluble and 7 % in the insoluble fraction. GDHm makes up 8 % of its soluble protein and was not detected in the insoluble fraction. In G6v2, Ate/LDH was found to make up 8 % of the soluble fraction. In the insoluble fraction of G6v2 the only large deviation between duplicates in this analysis occurred as indicated by the error bars (all figure 33).

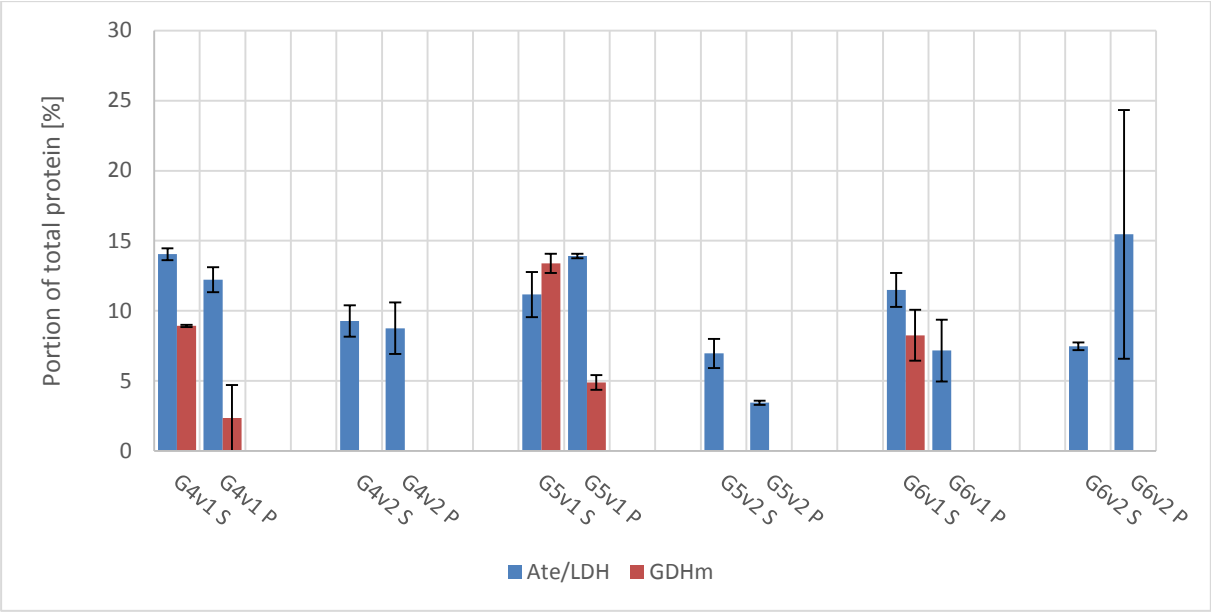

Figure 33: Results of the densitometric analysis of the SDS-PAGE G4v1, G4v2, G5v1, G5v2, G6v1 and G6v2. S: soluble fraction, P: insoluble pellet fraction. Error bars show the deviation of the duplicates from the mean.

3.4.4 GDH Assay

The results of the GDH assay confirm the results from the SDS-PAGE and the densitometric analysis thereof. We generally see a stronger increase in absorption at 340 nm for the six original constructs and their v1 variations than for the v2 constructs (figure 37 to figure 42). These slopes translate to an activity of up to 6.0 U/mg and 4.9 U/mg for the duplicates of G1v1. G4, G5 and G5v1 showed an activity just below 3 U/mg (table 14). No v2 construct showed any notable activity. The duplicates generally gave similar results with the exception of G6v1. G6v1.1 had an activity of 0.5 U/mg while G6v1.2 had a much stronger activity of 4.5 U/mg.

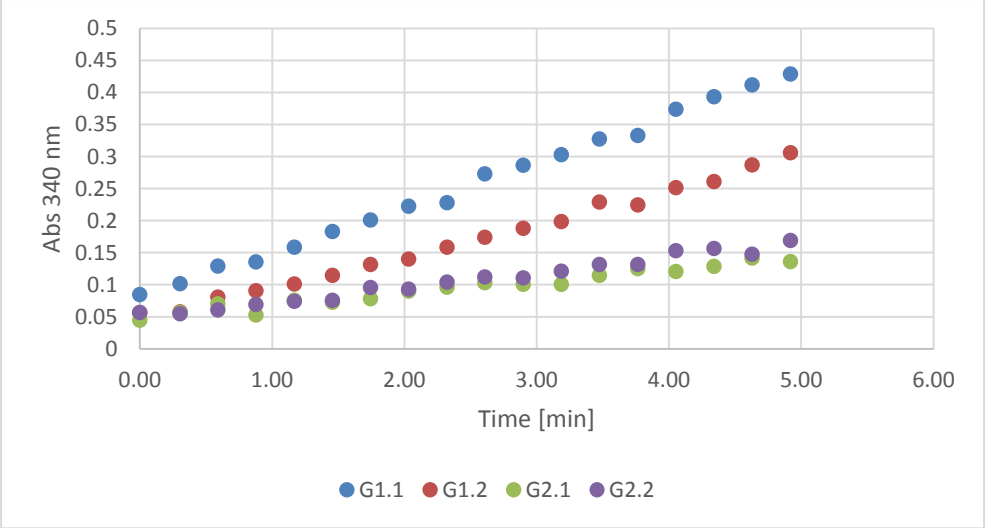

Figure 34: Photometric GDH assay for the duplicates of G1 and G2

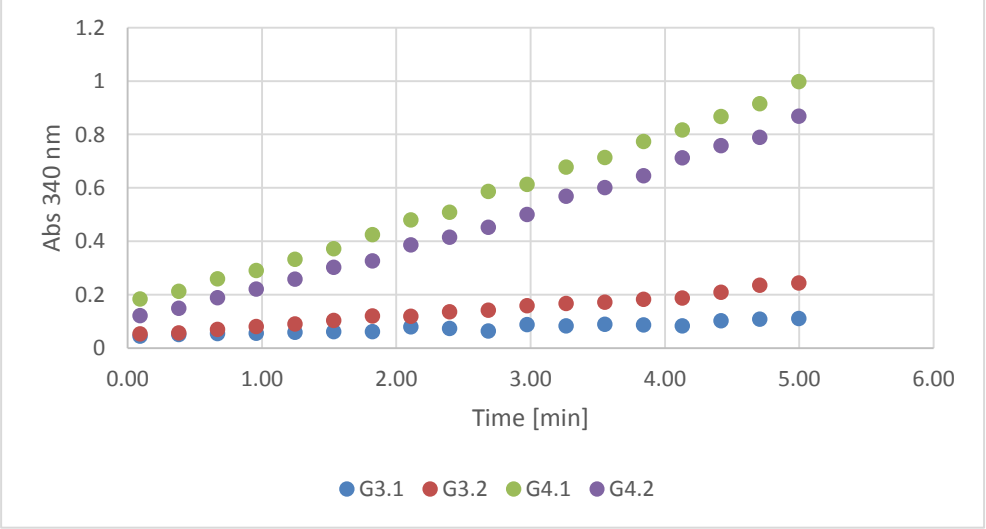

Figure 35: Photometric GDH assay for the duplicates of G3 and G4

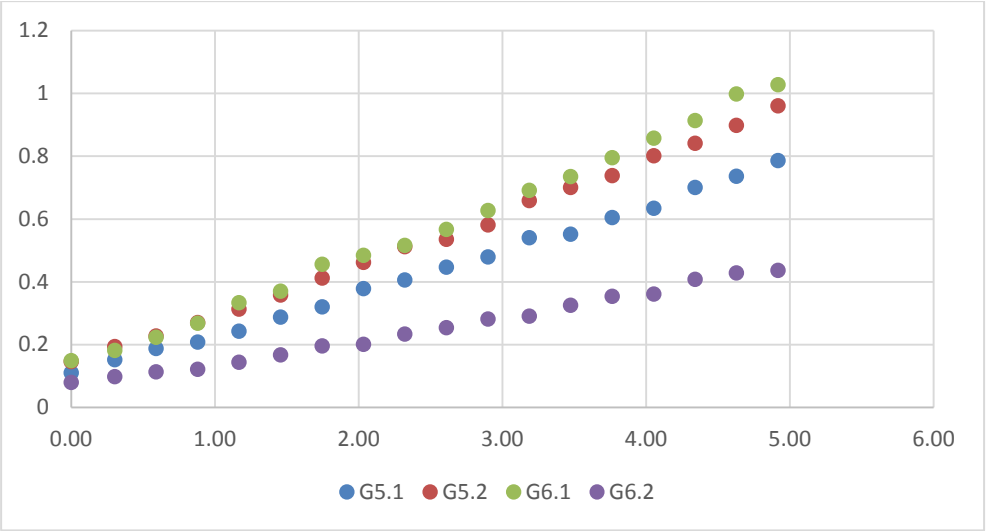

Figure 36: Photometric GDH assay for the duplicates of G5 and G6

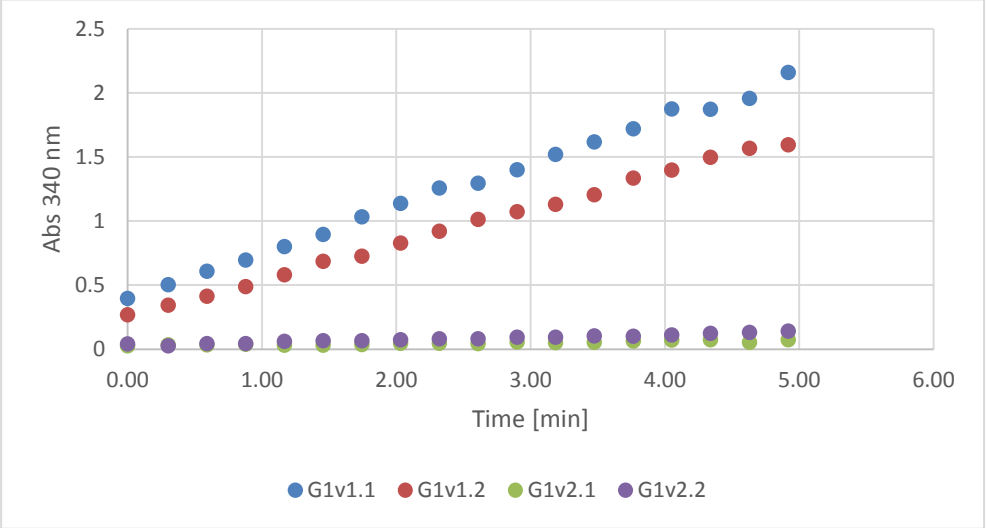

Figure 37: Photometric GDH assay for the duplicates of G1v1 and G1v2

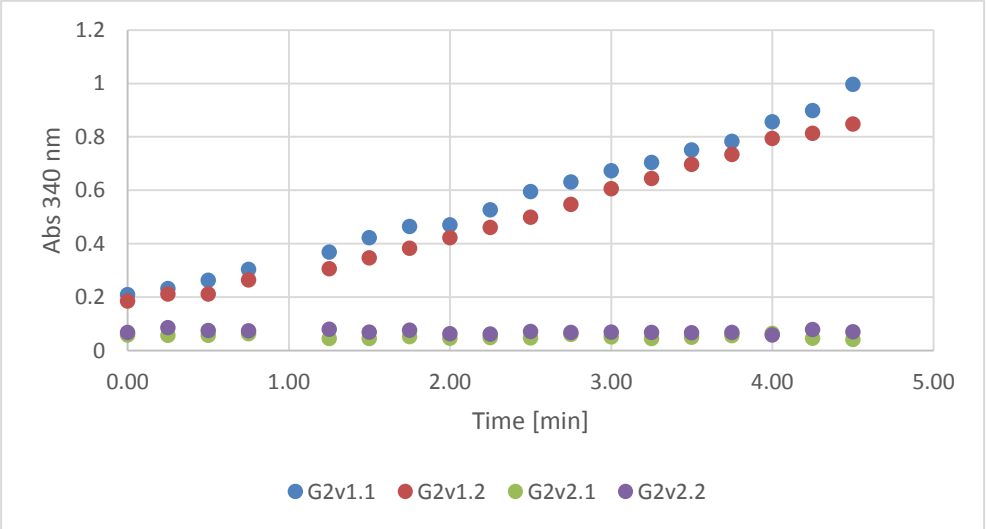

Figure 38: Photometric GDH assay for the duplicates of G2v1 and G2v2

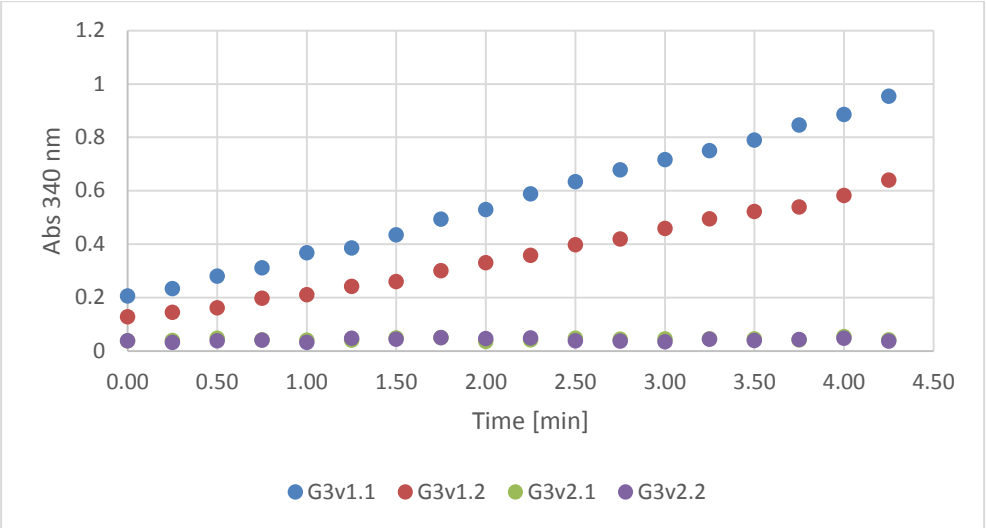

Figure 39: Photometric GDH assay for the duplicates of G3v1 and G3v2

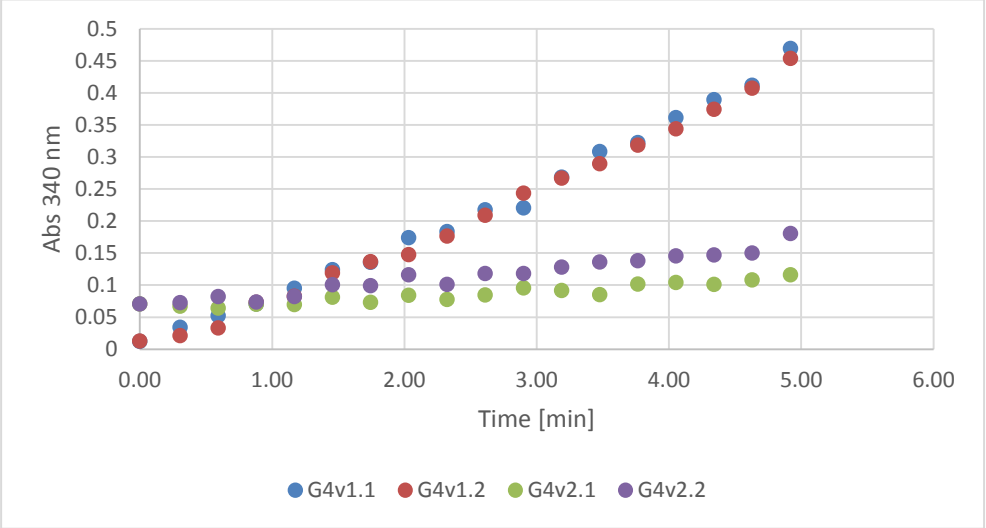

Figure 40: Photometric GDH assay for the duplicates of G4v1 and G4v2

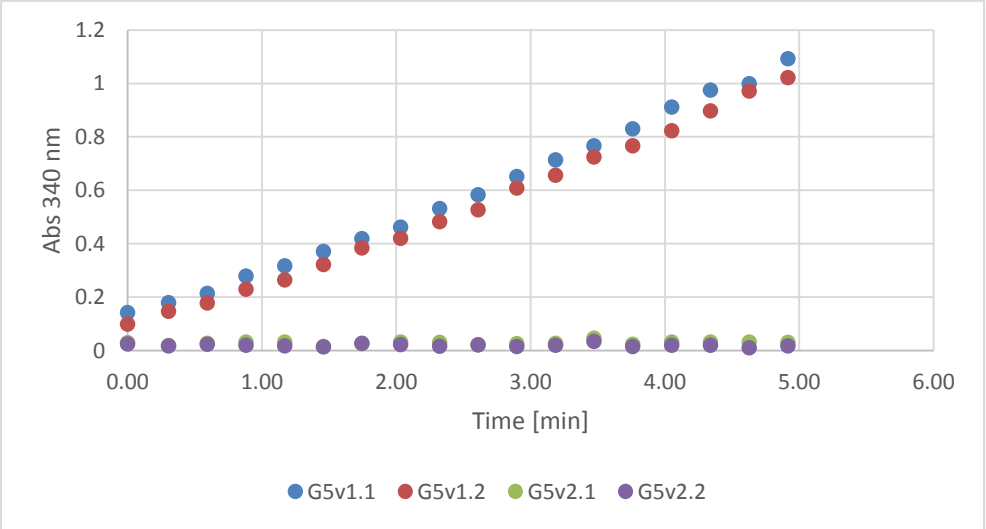

Figure 41: Photometric GDH assay for the duplicates of G5v1 and G5v2

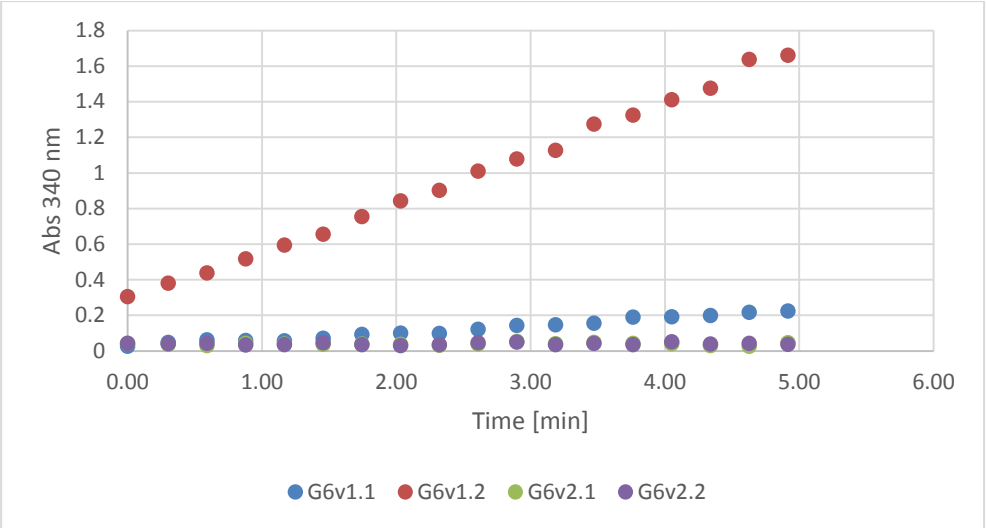

Figure 42: Photometric GDH assay for the duplicates of G6v1 and G6v2

Table 14: Slopes of the absorption changes at 340 nm and the calculated GDH activities for the duplicates of the co-expression constructs

Construct	Slope	GDH activity [U/mg]	Construct	Slope	GDH Activity [U/mg]
G1.1	0.0668	1.1	**G2v2.1**	-0.0030	0.0
G1.2	0.0445	0.8	**G2v2.2**	-0.0038	0.0
G2.1	0.0169	0.3	**G3v1.1**	0.1616	2.8
G2.2	0.0211	0.4	**G3v1.2**	0.0972	1.7
G3.1	0.0124	0.2	**G3v2.1**	0.0020	0.0
G3.2	0.0378	0.6	**G3v2.2**	0.0046	0.0
G4.1	0.1652	2.8	**G4v1.1**	0.0773	1.3
G4.2	0.1512	2.6	**G4v1.2**	0.0734	1.3
G5.1	0.1362	2.3	**G4v2.1**	0.0084	0.0
G5.2	0.1645	2.8	**G4v2.2**	0.0181	0.1
G6.1	0.1686	2.9	**G5v1.1**	0.1676	2.9
G6.2	0.0694	1.2	**G5v1.2**	0.1646	2.8
G1v1.1	0.3537	6.0	**G5v2.1**	0.0002	0.0
G1v1.2	0.2844	4.9	**G5v2.2**	-0.0013	0.0
G1v2.1	0.0080	0.0	**G6v1.1**	0.0300	0.5
G1v2.2	0.0209	0.1	**G6v1.2**	0.2614	4.5
G2v1.1	0.1475	2.5	**G6v2.1**	0.0017	0.0
G2v1.2	0.1130	1.9	**G6v2.2**	0.0012	0.0

If we compare the densitometric data for the GDHm bands of the soluble fractions with the GDH activity (table 15), we see a clear correlation between the two. The two samples with the highest percentile portion of GDHm in their total protein, also show the highest GDH activity. However, the two samples G1v1 and G5v1 both show a portion of 13 %. While G1v1

has an activity of 5.5 U/mg G5v1 only has 2.9 U/mg. The v2 samples that showed no GDHm bands at all, also show no activity.

Table 15: Comparison of the densitometric data regarding GDHm expression and GDHm activity. The values shown are the mean values of the duplicates

Construct	% GDHm	GDH activity [U/mg]	Construct	% GDHm	GDH activity [U/mg]
G1	5	1.0	**G2v2**	0	0.0
G2	4	0.4	**G3v1**	12	2.3
G3	3	0.4	**G3v2**	0	0.0
G4	11	2.7	**G4v1**	9	1.3
G5	6	2.6	**G4v2**	0	0.1
G6	7	2.1	**G5v1**	13	2.9
G1v1	13	5.5	**G5v2**	0	0.0
G1v2	0	0.1	**G6v1**	8	2.5
G2v1	11	2.2	**G6v2**	0	0.0

Another interesting analysis is to look at the impact the different ribosome binding sites and the position of the GDHm coding sequence had on the amount of soluble and insoluble GDHm produced and on the resulting average activity (table 16). The RBS v3 worked better if GDHm was in the middle position (G4, G5 and G6), producing an average of 2.4 U/mg, making up 8 % of the soluble and 3 % of the insoluble fraction. In the rear position (G1, G2 and G3) it only produced an activity of 0.6 U/mg, 4 % of the soluble and 0 % of the insoluble fraction. The RBS variant v1 delivered an activity of 2.2 U/mg in the middle position with 10 % of the soluble and 2 % of the insoluble protein fraction. In the rear position it produced an even stronger activity of 3.3 U/mg, 12 % of the soluble and 4 % of the insoluble protein. The v2 variant apparently did not work at all. It gave no activity and no detectable bands in either position.

Table 16: The different ribosome binding sites and their positions on the tricistronic mRNA compared to the portion GDHm takes up in the soluble and insoluble fraction and the mean GDH activity produced

RBS and its position	% of soluble fraction	% of insoluble fraction	GDH activity [U/mg]
v1, middle	10	2	2.2
v1, rear	12	4	3.3
v2, middle	0	0	0.0
v2, rear	0	0	0.0
v3, middle	8	3	2.4
v3, rear	4	0	0.6

3.4.5 LDH Assay

Since I was not able to discriminate the LDH from the Ate in the SDS gels, the LDH assay was important to find out whether the LDH was actually expressed in cells harboring the co-expression plasmids. While there was practically no decrease in absorption at 340 nm for G3, G3v1, G3v2, G5v2 and G6v2, the samples G4, G4v1, G4v2 and G5v1 caused a more rapid decrease, indicating stronger LDH activity. The other nine samples showed a moderate decrease (figure 43 to figure 51). The activities were calculated (table 17) from the slope of the absorption change. They ranged from 0 U/mg of total protein for samples like G5v2 to 3.0 U/mg for G4v1. Generally spoken, there was a low deviation between the duplicates. The only exception is G6 where G6.1 showed an activity of 2.1 U/mg and G6.2 only 0.2 U/mg.

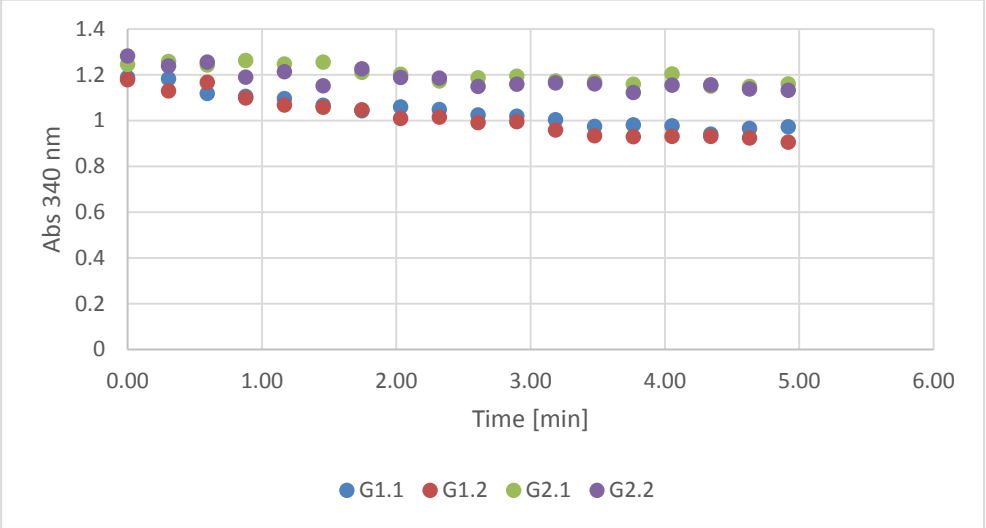

Figure 43: Photometric LDH assay for the duplicates of G1 and G2

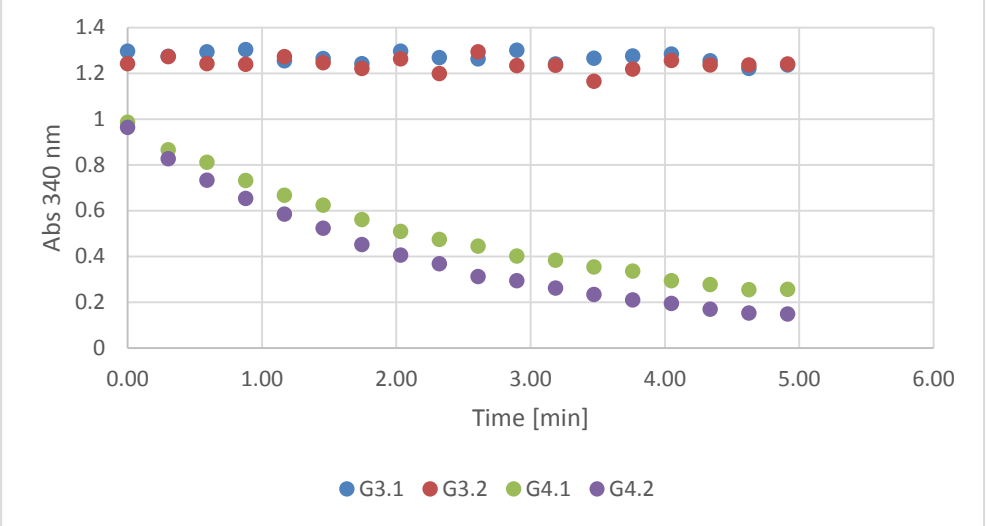

Figure 44: Photometric LDH assay for the duplicates of G3 and G4

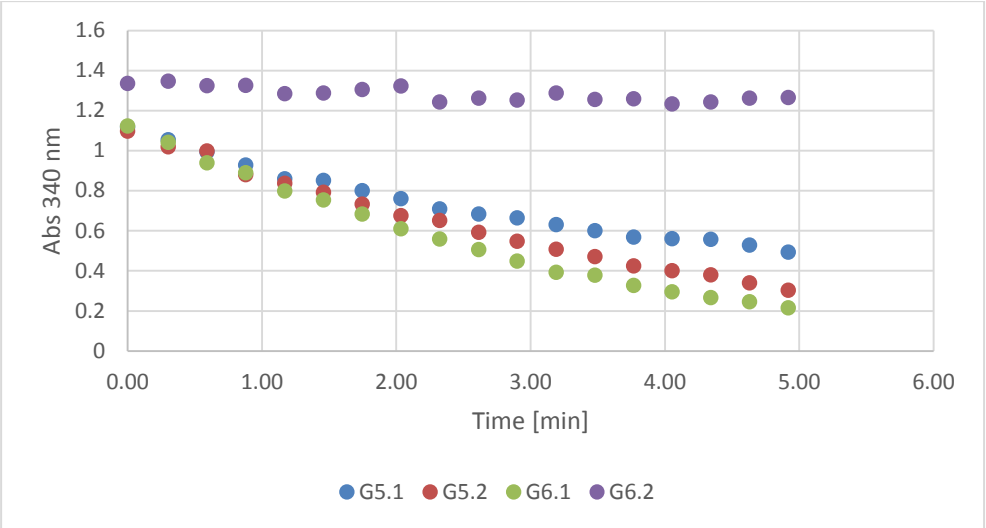

Figure 45: Photometric LDH assay for the duplicates of G5 and G6

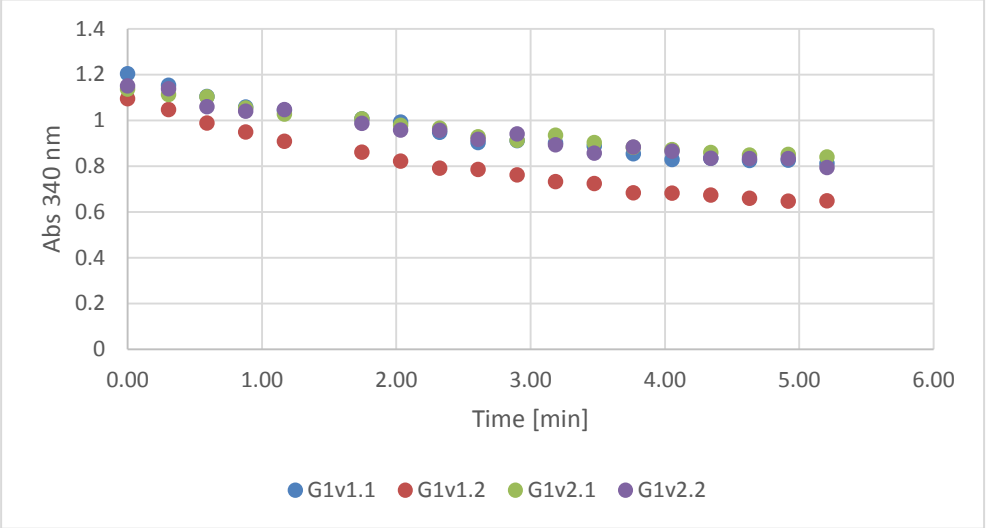

Figure 46: Photometric LDH assay for the duplicates of G1v1 and G1v2

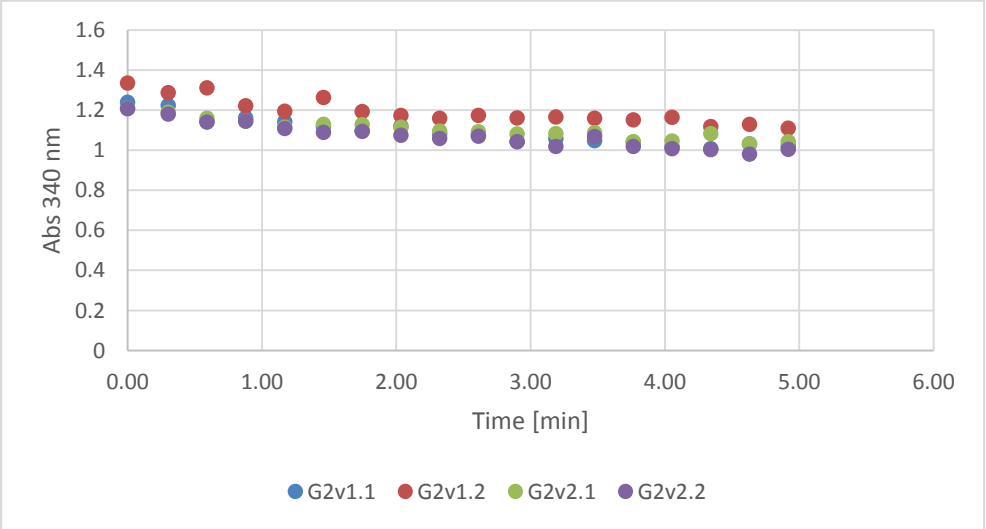

Figure 47: Photometric LDH assay for the duplicates of G2v1 and G2v2

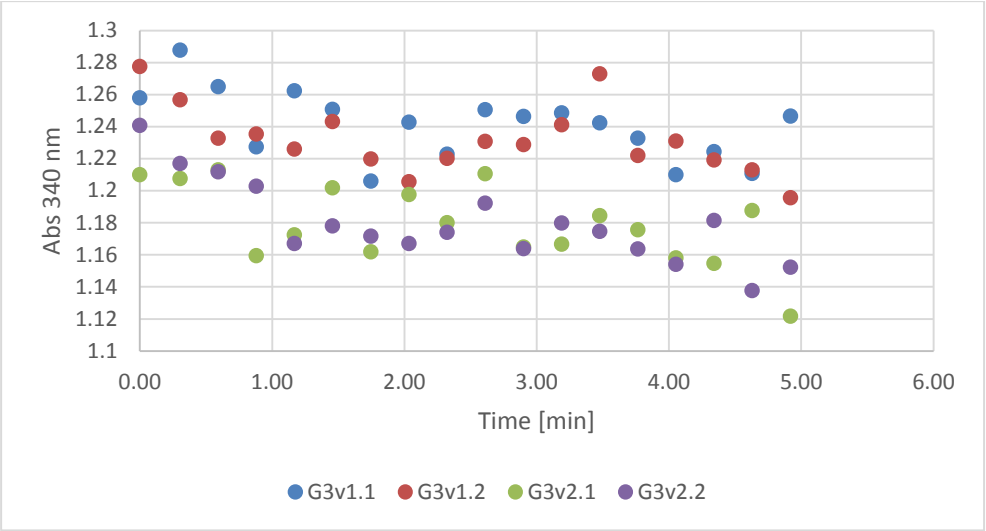

Figure 48: Photometric LDH assay for the duplicates of G3v1 and G3v2

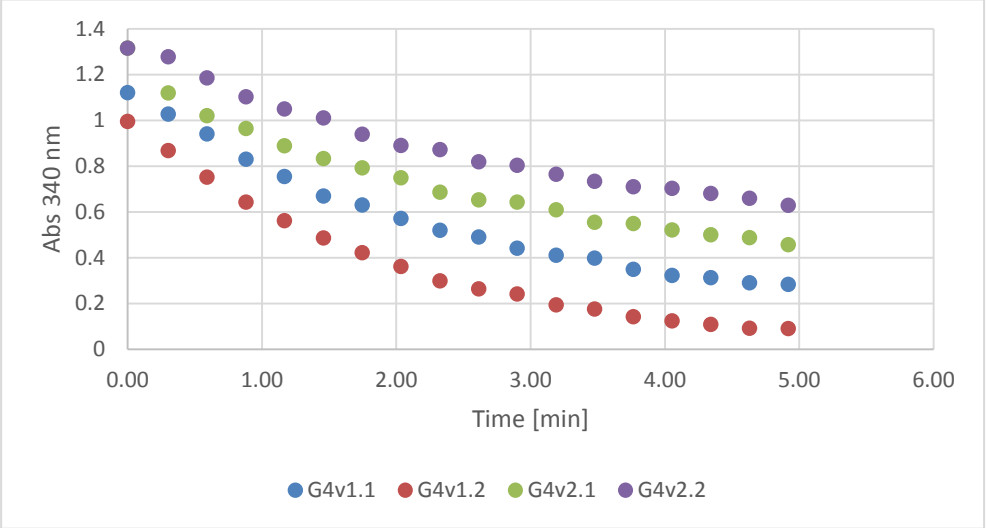

Figure 49: Photometric LDH assay for the duplicates of G4v1 and G4v2

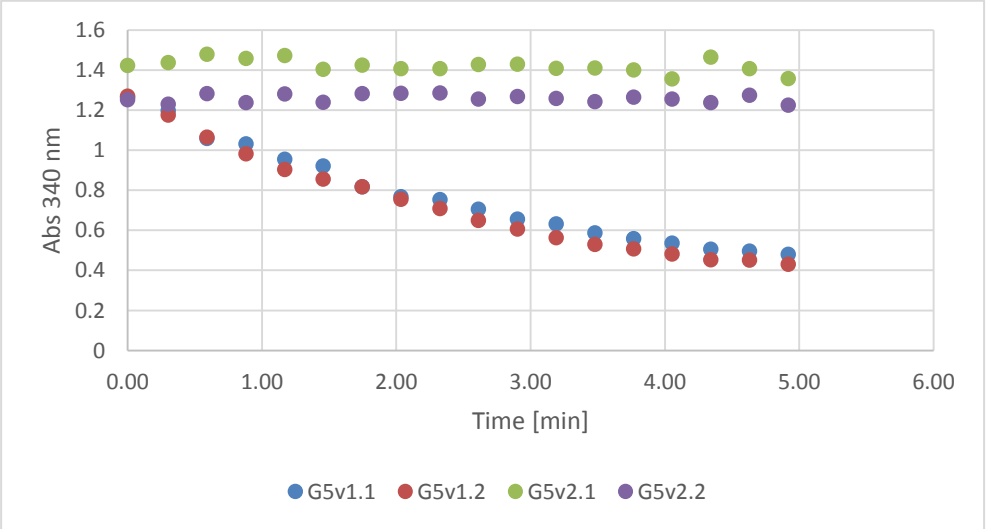

Figure 50: Photometric LDH assay for the duplicates of G5v1 and G5v2

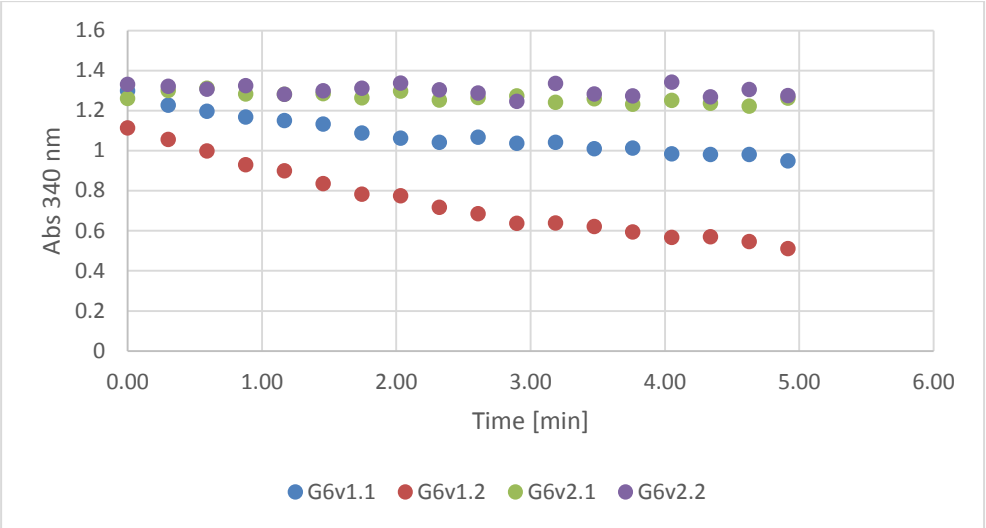

Figure 51: Photometric LDH assay for the duplicates of G6v1 and G6v2

Table 17: Slopes of the absorption changes at 340 nm and the calculated LDH activity for the duplicates of the co-expression constructs

Construct	Slope	LDH activity [U/mg]	Construct	Slope	LDH activity [U/mg]
G1.1	-0.0870	0.7	**G2v2.1**	-0.0729	0.6
G1.2	-0.0841	0.7	**G2v2.2**	-0.0451	0.4
G2.1	-0.0244	0.2	**G3v1.1**	-0.0214	0.2
G2.2	-0.0411	0.4	**G3v1.2**	-0.0275	0.2
G3.1	-0.0124	0.1	**G3v2.1**	-0.0138	0.1
G3.2	-0.0021	0.0	**G3v2.2**	-0.0362	0.3
G4.1	-0.2263	1.9	**G4v1.1**	-0.3190	2.7
G4.2	-0.2667	2.3	**G4v1.2**	-0.3749	3.2
G5.1	-0.1752	1.5	**G4v2.1**	-0.3470	3.0
G5.2	-0.2073	1.8	**G4v2.2**	-0.2420	2.1
G6.1	-0.2478	2.1	**G5v1.1**	-0.2437	2.1
G6.2	-0.0189	0.2	**G5v1.2**	-0.2918	2.5
G1v1.1	-0.1009	0.9	**G5v2.1**	-0.0009	0.0
G1v1.2	-0.1306	1.1	**G5v2.2**	0.0042	0.0
G1v2.1	-0.0786	0.7	**G6v1.1**	-0.0603	0.5
G1v2.2	-0.0928	0.8	**G6v1.2**	-0.1176	1.0
G2v1.1	-0.1016	0.9	**G6v2.1**	-0.0118	0.1
G2v1.2	-0.0644	0.6	**G6v2.2**	-0.0068	0.1

Comparing the LDH activity with the percentile portion of the Ate/LDH band (table 18) there is not such a clear correlation as with GDHm. For example, in G3 the band makes up 18 % of the soluble protein, but shows only 0.1 U/mg while G4 shows 8 % but a relatively high activity of 2.1 U/mg.

Table 18: Comparison of the densitometric data regarding LDH and Ate expression and LDH activity. The values shown are the mean values of the duplicates

Construct	% Ate/LDH	LDH activity [U/mg]	Construct	% Ate/LDH	LDH activity [U/mg]
G1	17	0.7	**G2v2**	18	0.5
G2	17	0.3	**G3v1**	20	0.2
G3	18	0.1	**G3v2**	19	0.2
G4	8	2.1	**G4v1**	14	3.0
G5	8	1.7	**G4v2**	9	2.6
G6	11	1.2	**G5v1**	11	2.3
G1v1	14	1.0	**G5v2**	7	0.0
G1v2	14	0.8	**G6v1**	11	0.8
G2v1	14	0.8	**G6v2**	7	0.1

For the LDH, I also compared the portion of the respective bands in the soluble and insoluble protein fraction and the average activity, depending on the RBS and the position of the LDH coding sequence (table 19). The synthetic RBS with the LDH coding sequence in the middle (G1, G1v1 and G1v2) led to an activity of 0.8 U/mg with 15 % Ate/LDH in the soluble and 9 % in the insoluble fraction. In the rear position (G4, G4v1 and G4v2), an activity of 2.5 U/mg was obtained. 11 % of the soluble and 9 % of the insoluble fraction were accounted for Ate/LDH. The RBS from *hslU* in the middle position (G2, G2v1 and G2v2) delivered an activity of 0.5 U/mg, 16 % of the soluble and 9 % of the insoluble fraction. In the rear position (G5, G5v1, G5v2) it produced 1.3 U/mg, 9 % in the soluble and 8 % in the insoluble fraction. The *gyrB* RBS in the middle position (G3, G3v1 and G3v2) yielded 0.2 U/mg, 19 % of the soluble and 10 % of the insoluble fraction. In the rear position (G6, G6v1 and G6v2) it produced 0.7 U/mg and 10 % of each protein fraction.

Table 19: The different RBS and their positions on the tricistronic mRNA compared to the portion Ate/LDH take up in the soluble and insoluble fraction and the mean GDH activity produced

RBS and its position	% of soluble fraction	% of insoluble fraction	LDH activity [U/mg]
Synthetic, middle	15	9	0.8
Synthetic, rear	11	9	2.5
From *hslU*, middle	16	9	0.5
From *hslU*, rear	9	8	1.3
From *gyrB*, middle	19	10	0.2
From *gyrB*, rear	10	10	0.7

To get a better overview, the results of the two enzyme assays are compared side by side (

table **20**). We find several different configurations of enzyme activities. G5v1 is an example for high balanced activity with 2.3 U/mg LDH and 2.9 U/mg GDH activity. Intermediate balanced activity can be provided with G1 as it showed 0.7 U/mg LDH and 1.0 U/mg GDH activity. A low balanced activity was achieved with G2 as it yielded 0.3 U/mg and 0.4 U/mg for LDH and GDH activity respectively. There are also unbalanced systems like G4v1 that had a higher LDH activity with 3.0 U/mg and only 1.3 U/mg GDH activity or G1v1 with 1.0 U/mg LDH and 5.5 U/mg GDH activity.

Table 20: Overview of LDH and GDH activities for the co-expression constructs

Construct	LDH activity (U/mg)	GDH activity (U/mg)	Construct	LDH activity (U/mg)	GDH activity (U/mg)
G1	0.7	1.0	**G2v2**	0.5	0.0
G2	0.3	0.4	**G3v1**	0.2	2.3
G3	0.1	0.4	**G3v2**	0.2	0.0
G4	2.1	2.7	**G4v1**	3.0	1.3
G5	1.7	2.6	**G4v2**	2.6	0.1
G6	1.2	2.1	**G5v1**	2.3	2.9
G1v1	1.0	5.5	**G5v2**	0.0	0.0
G1v2	0.8	0.1	**G6v1**	0.8	2.5
G2v1	0.8	2.2	**G6v2**	0.1	0.0

3.5 Phenol Red Assay

The first attempt to see if the phenol red assay was suitable for the detection of esterase activity with this given setup gave mixed results (figure 52). While the signal of the *E. coli* background control eV remains relatively stable, indicating no transaminase activity and therefore no acid production, the transaminase expressing clone shows a slight decline in absorption at 560 nm over time. The decrease for G1 however is much stronger. Surprisingly, the samples without added acetophenone (x –S) show nearly the same signal change as the ones with added substrate. However, the signal increases slightly in the first few minutes, but then decreases even stronger than for the samples with acetophenone.

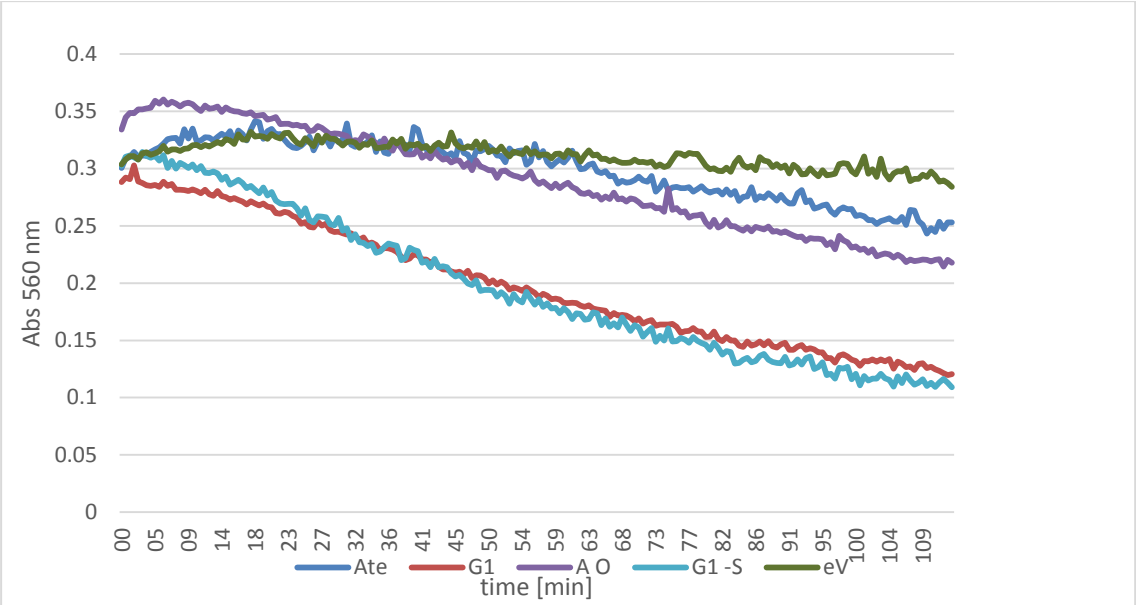

Figure 52: Photometric measurements at 560 nm of the phenol red assay for A, G1 and eV without dialysis. A: CFE of Ate produced from pD871_Ate-TA_T274S, eV: CFE from pMS470Δ837_eV, -S: sample without the substrate acetophenone

3.5.1 Dialysis

The G1 samples treated with the various dialysis methods were measured in regard of protein, RNA and DNA concentration (table 21). D1 was the method that led to the least precipitation of proteins, 8.2 mg/ml were left after dialysis. D2 only left 4.7 mg/ml protein, D3 6.0 mg/ml and D4 6.4 mg/ml. The most efficient RNA degradation and removal were found in D2 and D4 where no measurable amount was left. In contrast to that, there were still 132 µg/µl RNA in D1 and 372 µg/µl in D3. DNA degradation and removal was only complete in D2, with D4 as the second best with 247 µg/µl, followed by D3 with 478 µg/µl and D1 with 539 µg/µl. In all samples there was insoluble protein visible after centrifugation.

Table 21: Results of the concentration measurements of protein, RNA and DNA for untreated G1 as well as for G1 treated with dialysis methods D1, D2, D3 and D4

	G1	D1	D2	D3	D4
Protein [mg/ml]	10.9	8.2	4.7	6.0	6.4
RNA [µg/µl]	2857	132	0	372	0
DNA [µg/µl]	3556	539	0	478	247

While the results above look quite promising, it yet had to be tested if they actually had an impact on the phenol red assay. Every dialysis sample was measured in three setups: acetophenone as substrate (AcPh), no substrate (-S) and pyruvate (Pyr) as substrate (figure 53 to figure 58). One striking result is the absorption increase occurring after 30 to 60 minutes in the all samples supplied with pyruvate, except Ate (figure 54). The results of the four dialysis methods are rather disappointing. They show no visible improvement in background reactions: the samples without substrate exhibit a stronger signal change than the ones with acetophenone. One exception for this statement is the sample treated with dialysis method D2 (figure 56), as it shows no signal change when incubated without substrate, but also only a very minor decrease for the sample with acetophenone.

Another interesting piece of information can be gathered from the assay with the mix of LDH and GDH (figure 59): The sample without substrate shows a stronger absorption decrease than the one with acetophenone.

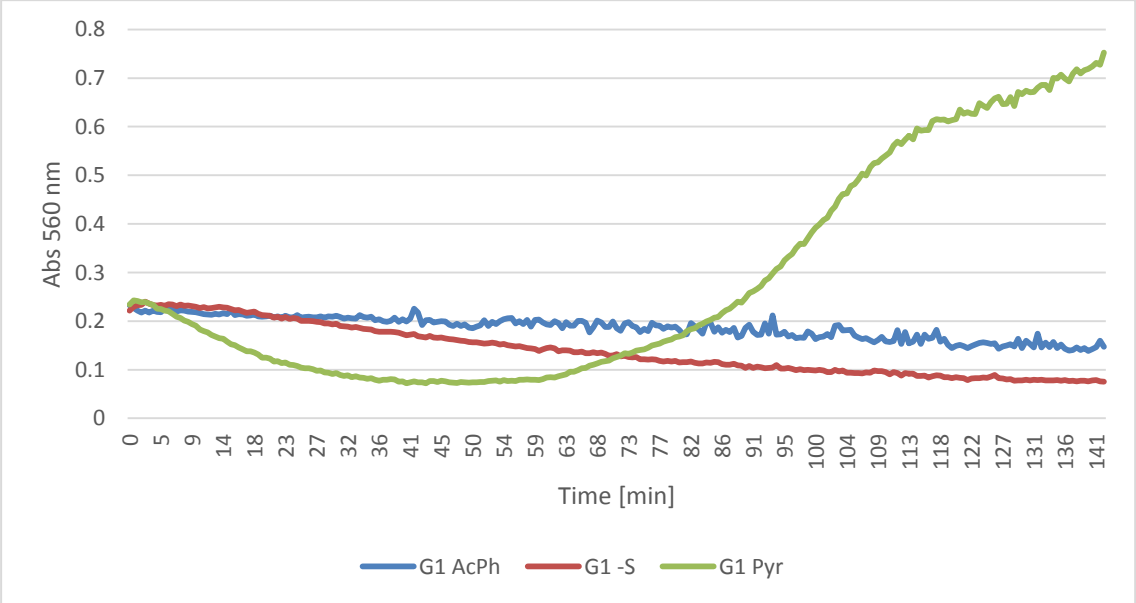

Figure 53: Phenol red assay of G1 with equal molar amounts of acetophenone (AcPh) or pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

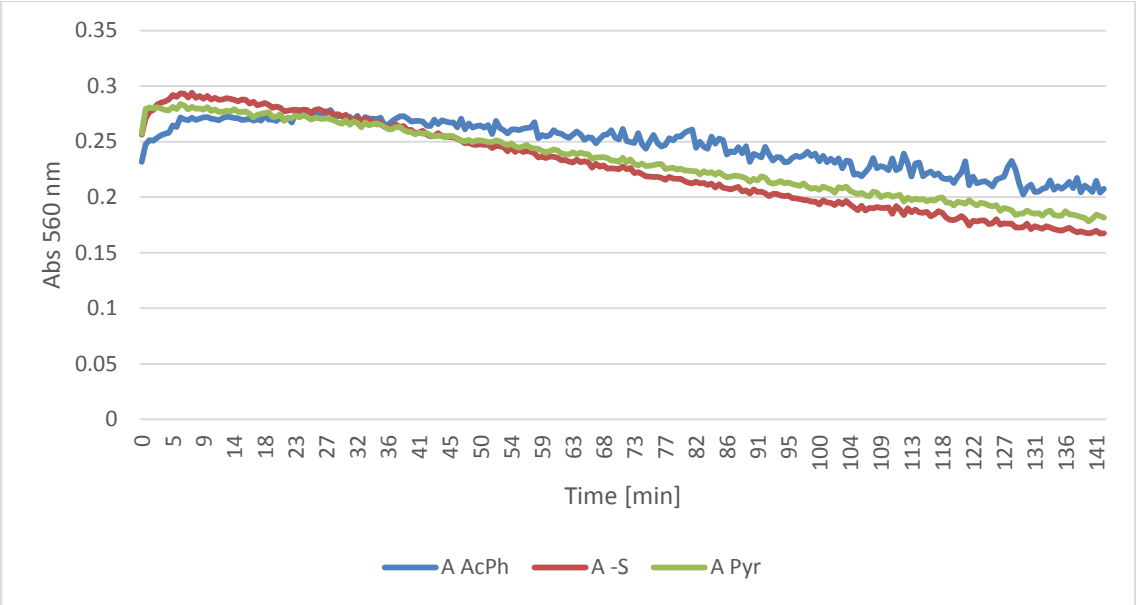

Figure 54: Phenol red assay of Ate with equal molar amounts of acetophenone (AcPh) or pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

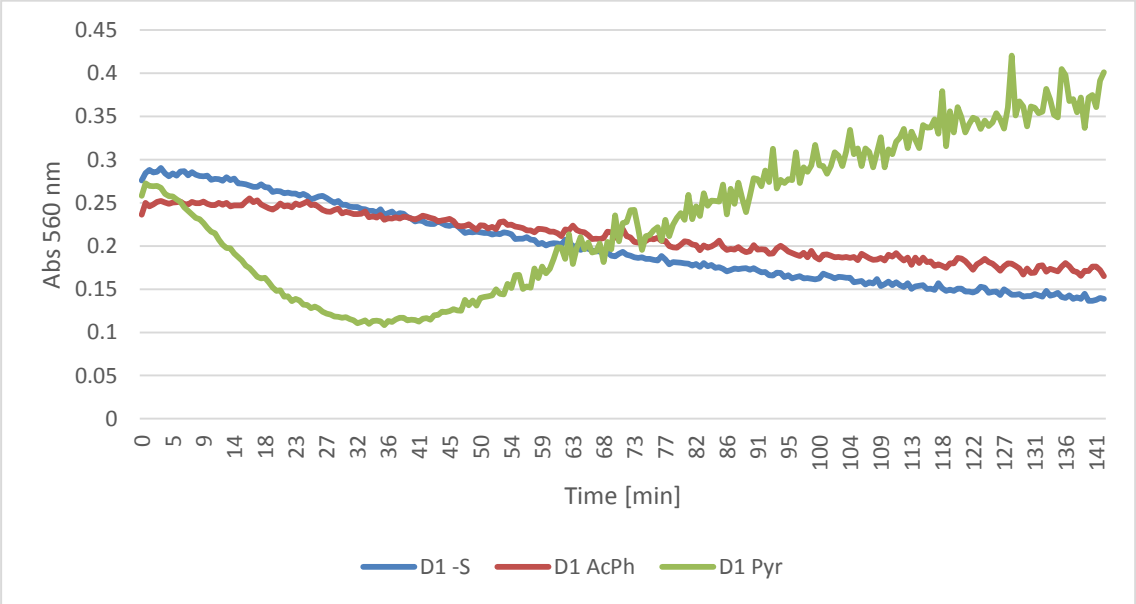

Figure 55 Phenol red assay of G1 treated with dialysis method D1 with equal molar amounts of acetophenone (AcPh) and pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

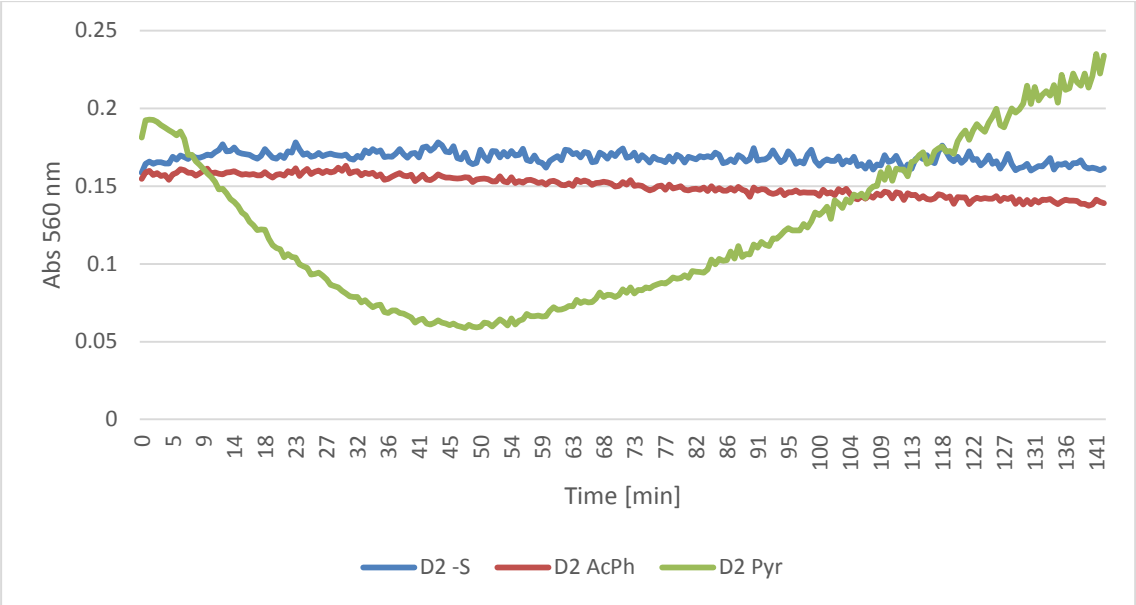

Figure 56 Phenol red assay of G1 treated with dialysis method D2 with equal molar amounts of acetophenone (AcPh) and pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

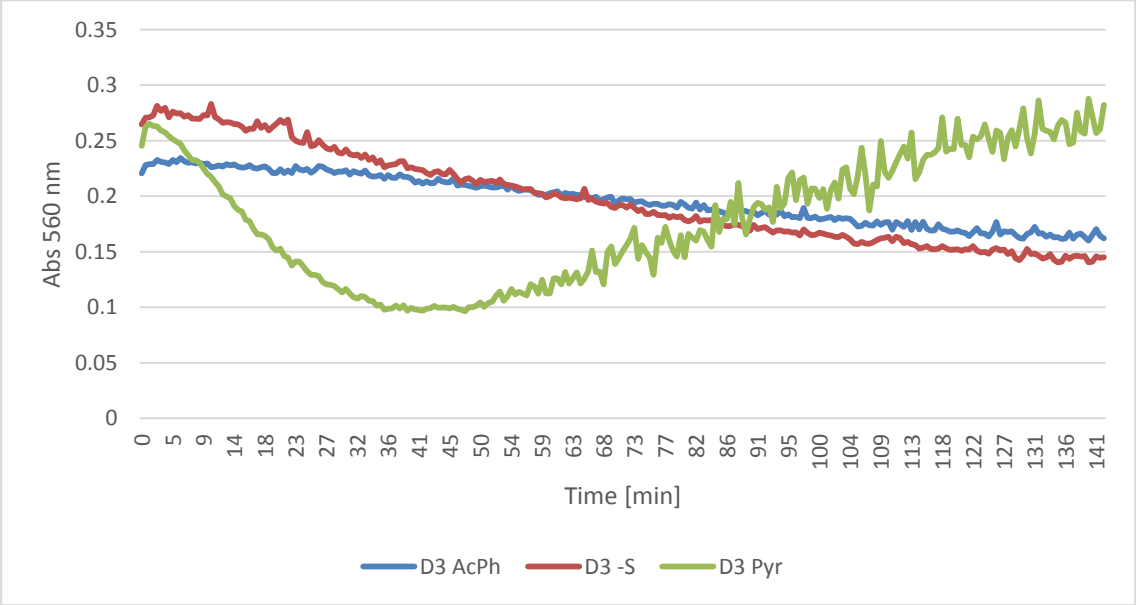

Figure 57: Phenol red assay of G1 treated with dialysis method D3 with equal molar amounts of acetophenone (AcPh) and pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

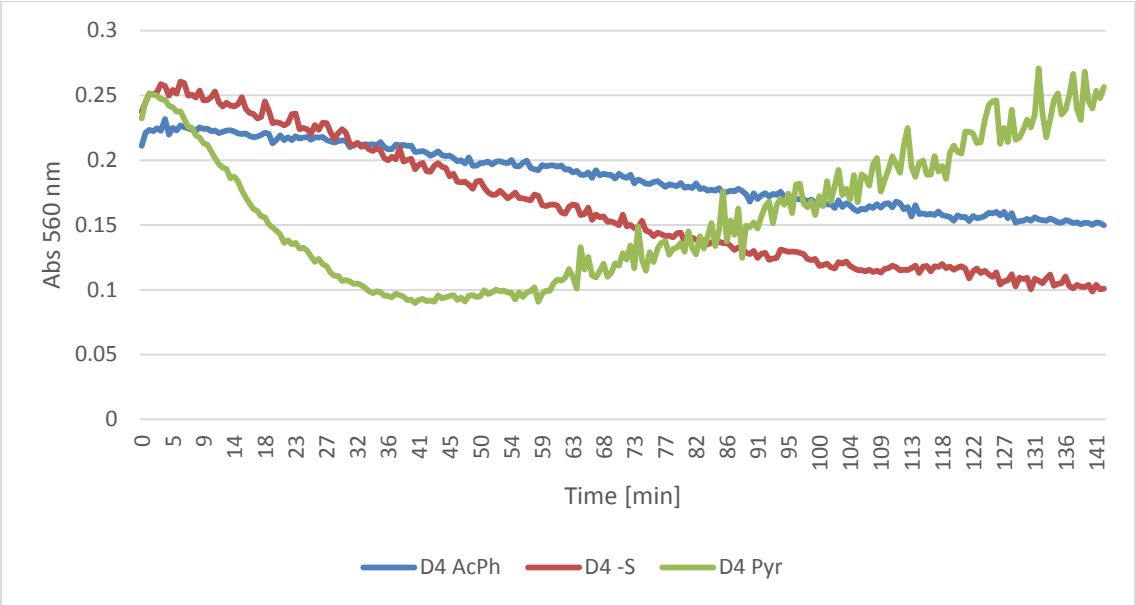

Figure 58: Phenol red assay of G1 treated with dialysis method D4 with equimolar amounts of acetophenone (AcPh) and pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

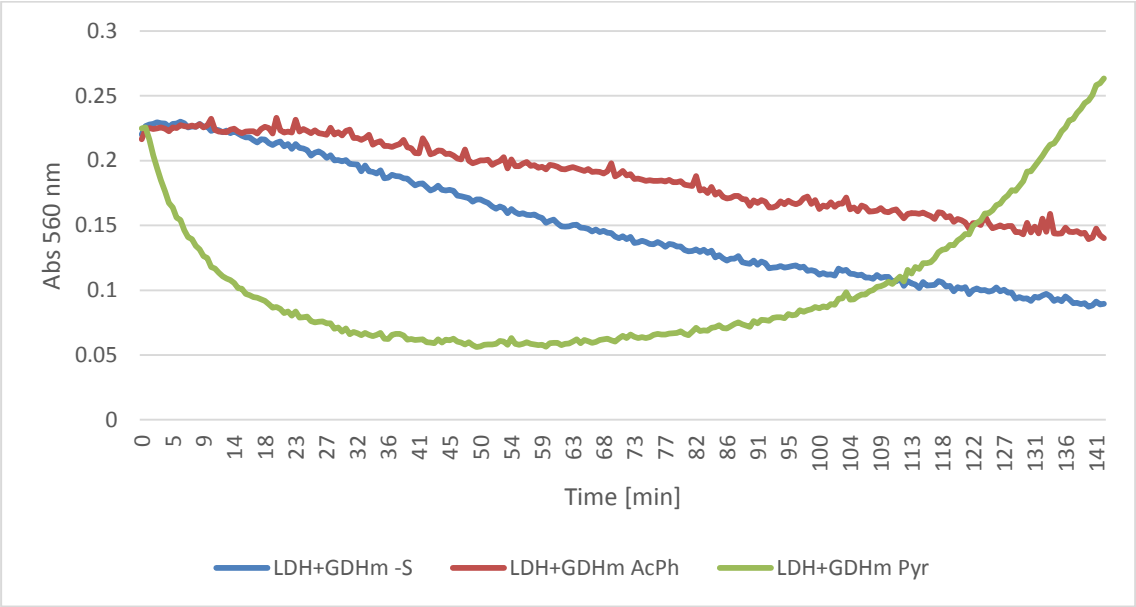

Figure 59: Phenol red assay of a mix of LDH and GDHm with equimolar amounts of acetophenone (AcPh) and pyruvate (Pyr) as substrate and without addition of substrate (-S). The mean of three measurements is shown.

4 Discussion

4.1 Cloning and characterization of the two GDH variants

The cloning steps for GDHm and GDHs worked quite flawlessly. All restriction and ligation steps worked on the first try. Also the cPCR proved to be a very robust method to detect correct ligations, of course only if they result in a size change of the amplified DNA region. For example it was of great use to check if the integration of GDHm and GDHs into pMS470Δ837 is correct, because this resulted in a size change of approximately 400 bp which was visualized in the agarose gel.

In the small scale experiments, GDHm showed a much higher activity than GDHs. However, the small volume of the cell free extract did only allow centrifugations in the table top apparatus which was limited to 3220 rcf. At this speed usually still a lot of not totally correctly folded enzymes remain in the supernatant which influences the specific activity measured in the GDH and LDH assays. To really determine which GDH showed higher specific activity the large scale expression was very useful. The disrupted cells could be centrifuged at 50,000 rcf. Also the resulting SDS-PAGE was analyzed densitometrically to determine whether GDHm is better expressed than GDHs. The similar portions of approximately 30% of the total soluble protein for both enzymes showed that GDHm is definitely more efficient in catalyzing the reaction from glucose and NAD+ to gluconolactone and NADH.

The heat stability test could have been repeated with a lower temperature where maybe a more gradual decline in activity could be shown, but also in those apparently harsh conditions the result was quite clear: GDHm is less prone to heat inactivation than GDHs.

To determine the activity of LDH and GDH as accurate as possible, the determination of the millimolar extinction coefficient ε for NADH was absolutely viable. The value commonly found in literature is 6.22 ± 0.2 L mM^{-1} cm^{-1} while the one I determined was 4.68 L mM^{-1} cm^{-1}. This deviation is due to the fact that molar absorption depends on several factors such as temperature, substance purity and calibration of the photometer [20]. So it is very useful to determine the molar absorption coefficient of the analyte at the temperature, with the photometer and from the production batch that are actually used for the measurements.

This change in the value of ε results in quite a drastic increase regarding the calculated enzyme activity. For example, the GDHm produced on large scale had an activity of 125 U/mg using the millimolar extinction coefficient found in literature, while the actual ε brought the activity to 165 U/mg which is a difference of 32%.

4.2 Construction of the Co-expression Plasmids

The first potential problem I encountered in this step of the project was the linearization of pD871_Ate-TA_T274S. Here, the bands of the linearized and the uncut plasmid appeared at very similar heights. That means if there were some uncut plasmids left they would have been extracted together with the linearized ones, but since no unwanted original plasmid was detected in the following steps, we can assume that this restriction digest was pretty much complete.

Another possible issue was the low melting temperature of the primer 2G_fwd, as only a primer over 60 bp could have provided a higher one, but this would have created substantial extra costs. However, the strategy to run 5 cycles with a T_a of 49 °C and then 20 more rounds with a T_a of 56 °C worked very well. As a more sophisticated approach to this problem, a gradient PCR could have been used.

One issue I find looking back at my work is the lack of control gels for some of the preparative agarose gels. In an attempt to utilize as much of the DNA produced in the PCRs I settled for this way. Even though you also see where the bands were cut out on the pictures of the preparative gels, it would look a lot neater with separate control gels and I probably could have speared one or two microliters of the PCR for that purpose.

4.3 Characterization of the Co-expression Constructs

Cultivation and protein expression worked very well with the 0.2 % L-rhamnose induction. While it has to be noted that this is possibly not the ideal concentration, it resulted in sufficient expression for this work. It also has to be noted that the strain I used was far from ideal for this plasmid since it does metabolize L-rhamnose. In the actual production process,

a strain that does not metabolize L-rhamnose would be used. This guarantees a stable concentration thereof and results in a more constant expression level.

4.3.1 SDS-PAGE

The SDS-PAGEs of the co-expression strains were of mixed quality. One issue was the application of an appropriate amount of the insoluble fractions of protein to the gel. Their bands often showed a smear which can be attributed to overloading of the gel. One explanation could be that the protein concentrations determined by Bradford assay were inaccurate. Here this was not a major problem and I simply ran the samples once more with less protein which resulted in clearer bands. An alternative reason for this smear could be the physical properties of the insoluble protein samples. They sometimes appeared to not be totally solubilized, showed a slightly gel-like consistency and sometimes even tended to stick to the pipet tip after injection into the gel slots.

Another issue with the SDS-PAGE was that Ate (36.2 kDa) and LDH (36.6 kDa) are of such similar size that their bands could not be distinguished on the gel. A possible solution for this would be to experiment with different combinations of buffers and gels, as this can influence the running properties of proteins and therefore may result in separated bands for the two enzymes.

4.3.2 GDH Assay and GDHm RBS Variation

The GDH assay for the co-expression constructs worked very well. Even though it could have been possible that the also present LDH would counter the GDH catalyzed NADH formation in the beginning of the reaction, while utilizing substrate it has available in the diluted cell lysate, but this could not be observed. The increase in absorption was very swift and without delay for the samples that exhibited notable GDH activity.

A positive result is that the modulation of GDHm expression was successful over the different co-expression constructs. The RBS variant v1, the one with the strongest expression, was derived from *gdh* of *B. subtilis* with the sequence GGAGGAGG. Surprisingly, it delivered more activity, more soluble and more insoluble GDHm when the GDHm coding sequence was the last of the three on the polycistronic mRNA. Usually, such a result is not expected due to the fact that, in bacteria, the translation of the first cistron begins before

the others have even been transcribed [21]. So the coding sequences closest to the promoter should be the strongest translated ones, which was also reported several times before [22, 23]. Another relevant factor for translation is mRNA degradation. Regarding its effect on polycistronic mRNA I found conflicting information. One explanation is that mRNA degradation begins on its 3' end which automatically leads to less translated protein the closer the corresponding cistron is located to the 3' end [24]. Another view of this matter is that mRNA degradation is a two-step process, starting with fragmentation of the mRNA beginning at the 5' end, followed by degradation of the fragments starting from their 3' end [21].

The RBS from *E. coli's cpn10* with the sequence CGGAGAGTT also delivered good GDH activities. This one however meets my expectations and shows higher expression levels if the GDHm coding sequence is in the second position of the polycistronic mRNA. As mentioned above there was no activity and no soluble or insoluble enzyme detected for the v2 RBS variant. It came from *E. coli's ftsI* and has the sequence GATAAACG. Looking also at the other two ribosome binding sites, one possible explanation could be their degree of similarity to the consensus of the Shine Dalgarno sequence AGGAGG which interacts with the 16S rRNA of the small ribosome subunit which is crucial for transcription initiation [21]. The RBS of *gdh* contains the whole consensus sequence and delivers the highest expression, the RBS of *cpn10* matches GGAG while you cannot find a proper match in the RBS of *ftsI.* While this apparently works in its natural setting, it may simply not offer strong enough ribosome binding to yield any substantial expression under the far from ideal conditions in a cell expressing three different heterologous proteins.

4.3.3 LDH Assay and LDH RBS Variation

The LDH assay also worked quite well for the co-expression constructs. As in the GDH assay, I could not observe any interference from the other enzyme, in this case GDHm. The decrease in absorption was very clear for the samples that showed notable activity.

The modulation of LDH expression was also successful. As with GDHm, the RBS containing the whole preserved Shine Dalgarno sequence led to the highest activity. The second highest was produced by the RBS from *hslU* with the sequence GTAAGGA, sharing an AGG with the consensus sequence. The lowest activity was achieved by the RBS from *gyrB* with the

sequence AGCGAGAA, where we can make out a match in GAG. Regarding the organization of the genes and its influence on the expression we see some very interesting results. For every RBS, the LDH activity was always higher when LDH was on the last position of the tricistronic mRNA. As described in the previous section, this does not meet my expectations at all.

As already mentioned above, the densitometric data from the SDS-PAGE does not match the measured LDH activity. This may be due to the fact that the analyzed band also contains the transaminase Ate. Since the band's intensity does not directly correlate to the measured LDH activity, this may indicate that the expression levels of Ate vary strongly between the constructs. Looking at the constructs, there is no direct genetic reason for this. Ate is always in the first position and behind the same RBS. So this variation is probably caused by the translation of LDH and GDHm. If they are strongly translated a lot of the cell's resources go into this process and reduce Ate production. However, if LDH and GDHm are controlled by weaker ribosome binding sites their translation is reduced, which lowers the metabolic load [25] and allows more resources to be funneled towards Ate production. To completely reveal this relation, a lot more research would still be needed.

Considering the equimolar nature of the catalyzed reaction, as we always need 1 mol glucose to regenerate 1 mol NADH that was used to reduce 1 mol of pyruvate to lactate. So theoretically we should aim for equal levels of GDH and LDH activity. This ensures efficient removal of the by-product pyruvate and continuous regeneration of the cofactor NADH during the reaction, while during the gene expression no cellular resources are wasted on the production of enzyme that is not necessarily needed. Considering this, I can find several candidates in my collection of co-expression constructs that provide different levels of activity. Some with balanced high activity like G5v1, some with balanced intermediate activity like G1 and some with balanced low activity like G2. Other constructs showed higher LDH activity like G4v1 and some higher GDH activity like G1v1. Those could also be useful if for certain applications the activity of one of the enzymes is somehow impaired by the reaction conditions. Also these results may vary when Ate is replaced with another transaminase, that is expressed better or worse. So since only the v2 constructs proved to be non-functional due to a lack of GDHm expression, this leaves us with a range of 12 co-

expression plasmids that can be used for future one pot production of transaminase enzyme cascades.

4.4 Phenol Red Assay

While the signal of the eV control remained relatively stable, the signal of the Ate sample declined over time of the measurement. This decline is probably due to native lactate and glucose dehydrogenases producing lactate from the pyruvate released by the transaminase and gluconic acid from the glucose present in the sample. As expected, we see a much stronger decrease in absorption for G1, where GDH and LDH are overexpressed and produce way more acid from pyruvate, the by-product of the transaminase reaction with acetophenone as the substrate. However, this reaction is apparently not the only source of substrate converted to acids lowering the pH as the samples without added substrate also show a decrease in absorption at 560 nm. As this is true for G1 as well as for LDH+GDHm, we must assume that there are alternative substrates for Ate as well as for GDHm and LDH in the cell free extract. Possible candidates for LDH and GDHm are pyruvate and glucose, while among others, the keto groups of nucleic acids provide possible targets for unspecific transaminase reactions. Also the discrepancy between the measurements with and without acetophenone may indicate some kind of inhibitory effect on one or more enzymes.

4.4.1 Dialysis

The measurements of protein, RNA and DNA concentrations for the dialyzed samples looked quite promising. We saw a substantial removal of RNA and DNA from the samples and even complete removal in D2. However, we also see a correlation between RNA and DNA removal and loss of protein during the dialysis. Mostly, this can be attributed to precipitation on the dialysis membrane where insoluble protein was visible after dialysis and centrifugation. The degradation and removal of DNA and RNA seemed to have worked best in D2. Unfortunately, this method also led to the greatest loss in soluble protein.

The only dialysis method that showed an improvement in reducing the unspecific signal change in the assay was D2. However this apparently came at the cost that no functional transaminase was left behind, since neither D2 –S nor D2 AcPh show any notable absorption

decrease at all. The biggest difference between D2 and the other methods was that it was done in 10 mM Tris HCl buffer. The D2 with added pyruvate however showed a strong decrease in absorption followed by the same increase as in other samples with added pyruvate. This implies that LDH and GDHm are still functional and thus are less susceptible to denaturation during dialysis.

The setup with pyruvate as substrate serves as an approach to investigate the potential of the reactions catalyzed by LDH and GDHm. The decrease of absorption is the strongest in the samples containing additional pyruvate for all preparations of G1. This fact suggests that the initial transaminase reaction is much slower than the following reactions. If LDH and GDH were the limiting factor in the cascade, there would not have been such a strong difference between the two setups. Even the moderate activity of G1 (LDH: 0.7 U/mg, GDH: 1.1 U/mg) seems to be more than enough.

Looking at the results of the phenol red assay another thing immediately strikes the eye. For all samples, except Ate, provided with pyruvate as substrate the absorption decreased rather rapidly only to increase again after 30 to 60 minutes. Since this absorption change is not caused by the phenol red [26], it is most probably due to precipitates that formed during incubation and were visible afterwards. Possibly they were caused by the low pH occurring after substantial acid formation by LDH and GDHm. The reason why Ate (figure 54) does not show this absorption increase is because it does not contain overexpressed GDH and LDH like the others.

Another interesting piece of information can be gathered from the assay with the mix of LDH and GDH (figure 59). The sample without substrate shows a stronger absorption decrease than the one with acetophenone. This means that the inhibitory effect mentioned above apparently affects LDH and/or GDHm.

To really utilize the potential possibilities of the phenol red assay to quantify transaminase activity it is important to find an efficient dialysis method. A setup that provides conditions for sufficient degradation and removal of DNA and RNA but does not lead to denaturation of Ate, LDH and GDHm has to be found. Also to get numeric information on the activity visualized by the assay the establishing of a standard curve from lactate and gluconic acid is necessary. This is an effort I did not make here, because it would not have been of much use, with an assay including such a massive background of unspecific reactions.

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6 Supplementary Information

Bradford assays for the Co-expression constructs

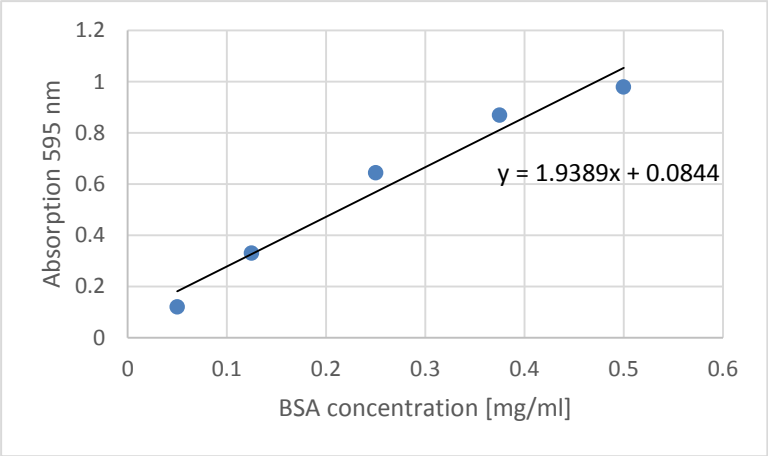

Figure 60: BSA standard measurement used for the duplicates of G1, G2 and G3, linear regression and the resulting equation used for calculation of the protein concentration

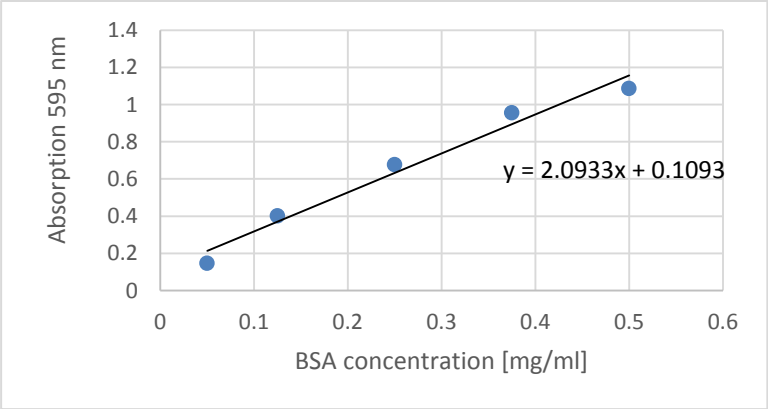

Figure 61: BSA standard measurement used for the duplicates of G4, G5 and G6, linear regression and the resulting equation used for calculation of the protein concentration

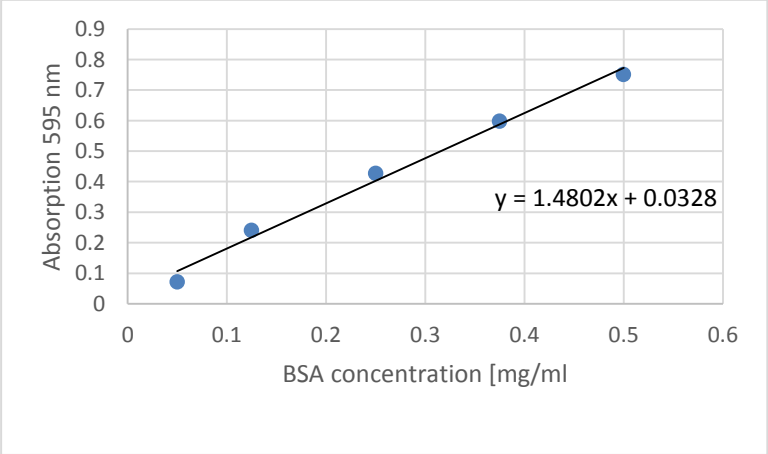

Figure 62: BSA standard measurement used for the duplicates of G1v1, G1v2 and G2v1, linear regression and the resulting equation used for calculation of the protein concentration

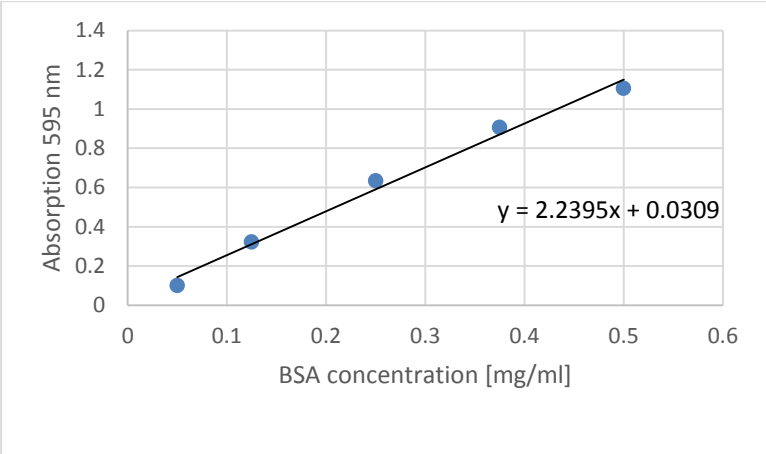

Figure 63: BSA standard measurement used for the duplicates of G2v2, G3v1, G3v2 and G3.2, linear regression and the resulting equation used for calculation of the protein concentration

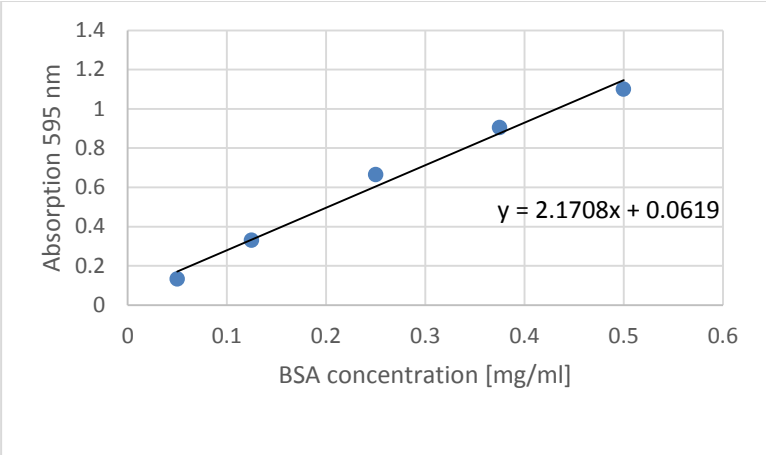

Figure 64: BSA standard measurement used for the duplicates of G4v2, G5v1 and G5v2, linear regression and the resulting equation used for calculation of the protein concentration

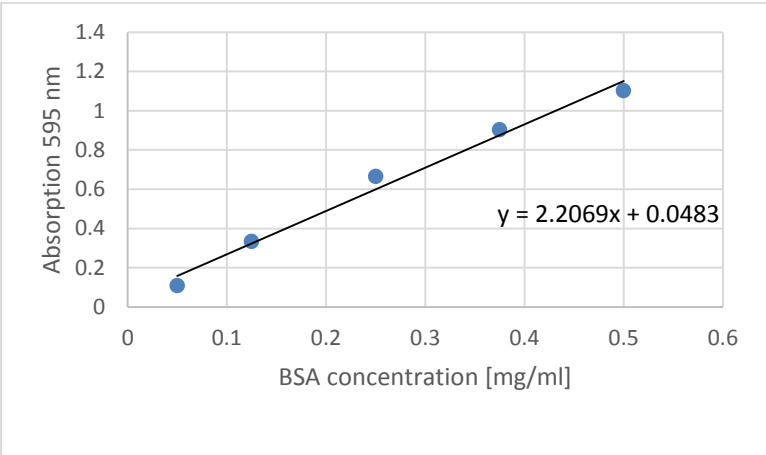

Figure 65: BSA standard measurement used for the duplicates of G5v2, G6v1 and G6v2, linear regression and the resulting equation used for calculation of the protein concentration

Table 22: Photometric measurements at 595 nm for the Bradford assay of the co-expression constructs. S: suluble fraction, 1:50 dilution. P: insoluble fraction, 1:10 dilution

G1.1 S	**G1.1 P**	**G1.2 S**	**G1.2 P**	**G2.1 S**	**G2.1 P**
0.897	0.911	0.906	0.974	0.858	0.99
0.917	0.956	0.889	1.064		0.934
0.946	0.877	0.897	1.071	0.89	1.025
G2.2 S	**G2.2 P**	**G3.1 S**	**G3.1 P**	**G3.2 S**	**G3.2 P**
0.927	0.94	0.902	0.973	0.976	0.973
0.882	0.967	0.721	0.944	1.062	0.944
0.931	0.945	0.668	0.949	0.988	0.949
G4.1 S	**G4.1 P**	**G4.2 S**	**G4.2 P**	**G5.1 S**	**G5.2 P**
0.886	0.868	0.899	0.845	0.908	0.875
0.882	0.897	0.925	0.906	0.925	0.906
0.925	0.952	0.914	0.921	0.984	0.906
G6.1 S	**G6.1 P**	**G6.2 S**	**G6.2 P**	**G1v1.1 S**	**G1v1.1 P**
0.929	0.88	0.9	0.898	0.944	1.005
0.921	0.861	0.915	0.963	0.983	1.061
0.943	0.885	0.895	0.934	1.016	1.002
G1v1.2 S	**G1v1.2 P**	**G1v2.1 S**	**G1v2.1 P**	**G1v2.2 S**	**G1v2.2 P**
0.952	0.887	0.936	0.962	0.889	0.933
0.97	0.88	0.979	1.027	0.941	0.922
0.951	0.949	0.95	0.99	0.976	0.931
G2v1.1 S	**G2v1.1 P**	**G2v1.2 S**	**G2v1.2 P**	**G2v2.1 S**	**G2v2.1 P**
0.95	0.99	1.031	1	1.039	0.996
1.002	1.006	1.028	0.952	1.004	0.99
0.929	1.073	1.098	0.992	1.05	1.005
G2v2.2 S	**G2v2.2 P**	**G3v1.1 S**	**G3v1.1 P**	**G3v1.2 S**	**G3v1.2 P**
1	1.009	1.032	1.035	0.954	0.986
1.011	1.038	1.014	1.045	1.006	0.992
1.045	1.038	1.092	1.054	1.026	0.984
G3v2.1 S	**G3v2.1 P**	**G3v2.2 S**	**G3v2.2 P**	**G4v1.1 S**	**G4v1.1 P**
0.974	0.99	0.963	1.016	0.92	1.318
1.019	1.013	0.966	0.983	0.97	0.889
0.999	1.05	1.002	0.979	0.931	0.928
G4v2.1 S	**G4v2.1 P**	**G4v2.2 S**	**G4v2.2 P**	**G5v1.1 S**	**G5v1.2 P**
0.98	0.953	0.982	1.01	1.018	0.956
0.975	0.973	1.006	1.006		0.998
1.024	0.956	1.13	0.991	1.038	0.987

G5v2.1 S	G5v2.1 P	G5v2.2 S	G5v2.2 P	G6v1.1 S	G6v1.1 P
1.014	1.014	1.008	0.995	1.031	1.009
1.023	1.092	1.02	1.034	1.047	1.084
1.089	1.003	1.035	0.999	1.04	1.071
G6v1.2 S	**G6v1.2 P**	**G6v2.1 S**	**G6v2.1 P**	**G6v2.2 S**	**G6v2.2 P**
1.004	0.987	1.045	1.017	0.989	0.906
1.05	0.938	1.014	1.01	0.955	0.899
1.063	1.02	1.048	0.994	0.989	0.942

Nucleotide sequences:

Sequence of GDHs:

ATGTATCCGGATTTAAAAGGAAAAGTCGTCGCTATTACAGGAGCTGCTTCAGGGCTCGGAAAGGCGATGGCCATTCGCTTCGGCAAGGAGCAGGC
AAAAGTGGTTATCAACTATTATAGTAATAAACAAGATCCGAACGAGGTAAAAGAAGAGGTCATCAAGGCGGGCGGTGAAGCTGTTGTCGTCCAA
GGAGATGTCACGAAAGAGGAAGATGTAAAAAATATCGTGCAAACGGCAATTAAGGAGTTCGGCACACTCGATATTATGATTAATAATGCCGGTCT
TGAAAATCCTGTGCCATCTCACGAAATGCCGCTCAAGGATTGGGATAAAGTCATCGGCACGAACTTAACGGGTGCCTTTTTAGGAAGCCGTGAAGC
GATTAAATATTTCGTAGAAAACGATATCAAGGGAAATGTCATTAACATGTCCAGTGTGCACGAAGTGATTCCTTGGCCGTTATTTGTCCACTATGCG
GCAAGTAAAGGCGGGATAAAGCTGATGACAGAAACATTAGCGTTGGAATACGCGCCGAAGGGCATTCGCGTCAATAATATTGGGCCAGGTGCGA
TCAACACGCCAATCAATGCTGAAAAATTCGCTGACCCTAAACAGAAAGCTGATGTAGAAAGCATGATTCCAATGGGATATATCGGCGAACCGGAG
GAGATCGCCGCAGTAGCAGCCTGGCTTGCTTCGAAGGAAGCCAGCTACGTCACAGGCATCACGTTATTCGCGGACGGCGGTATGACACAATATCC
TTCATTCCAGGCAGGCCGCGGTTAA

Sequence of GDHm:

ATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGC
AAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAA
GGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTT
GAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCA
ATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGC
AAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGA
ACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAA
GTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTT
TCCAAGCAGGAAGAGGCTAA

Sequence of the Co-expression Backbone:

GCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGGTAACG
AATTCAAGCTTGATATCATTCAGGACGAGCCTCAGACTCCAGCGTAACTGGACTGCAATCAACTCACTGGCTCACCTTCACGGGTGGGCCTTTCTTC
GGTAGAAAATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTT
GCCGGATCAAGAGCTACCAACTCTTTTTCCGAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGC
CACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCG
GGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTA
CACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTC
GGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCATCGATT
TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCAGAAAGGCCCACCCGAAGGTGAGCCAGGTGATTACATTTGGGC

CCTCATCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCTGCGGTAAAGCTCATCAGCGTGGTCGTGAAGCGATTCACAGATGTCTGCC
TGTTCATCCGCGTCCAGCTCGTTGAGTTTCTCCAGAAGCGTTAATGTCTGGCTTCTGATAAAGCGGGCCATGTTAAGGGCGGTTTTTTCCTGTTTGG
TCATTTAGAAAAACTCATCGAGCATCAAGTGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAA
GGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTC
CCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGACT
TGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCACCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGC
GATCGCCGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGA
TATTCTTCTAATACCTGGAATGCTGTTTTCCCTGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGA
AGAGGCATAAATTCCGTCAGCCAGTTTAGCCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCG
CATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGA
ATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCTT
ATTTCATTATGGTGAAAGTTGGAACCTCTTACGTGCCGATCAAGTCAAAAGCCTCCGGTCGGAGGCTTTTGACTTTCTGCTATGGAGGTCAGGTAT
GATTTAAATGGTCAGTATTGAGCGATATCTAGAGAATTCGTCCACCACAATTCAGCAAATTGTGAACATCATCACGTTCATCTTTCCCTGGTTGCCA
ATGGCCCATTTTCCTGTCAGTAACGAGAAGGTCGCGAATTCAGGCGCTTTTTAGACTGGTCGTAATGAAATTCTTTTTAAGAAGGAGATATACATAT
GGCGTCTATGGACAAAGTATTCGCTGGTTACGCTGCACGTCAGGCTATCCTGGAATCCACTGAAACTACCAACCCGTTCGCTAAAGGTATCGCATG
GGTTGAAGGTGAACTGGTACCGCTGGCTGAAGCGCGTATCCCGCTGCTGGATCAGGGCTTCATGCACTCTGACCTGACTTACGACGTTCCGTCTGT
ATGGGACGGTCGTTTCTTCCGTCTGGATGACCACATCACTCGTCTGGAAGCGTCCTGCACCAAACTGCGTCTGCGTCTGCCGCTGCCGCGCGACCA
GGTTAAGCAGATCCTGGTTGAAATGGTTGCTAAATCTGGTATCCGTGACGCATTCGTTGAGCTGATCGTTACTCGCGGTCTGAAAGGCGTTCGTGG
TACTCGTCCGGAAGATATCGTTAACAACCTGTACATGTTCGTTCAGCCGTACGTATGGGTAATGGAACCGGATATGCAGCGCGTTGGTGGTTCTGC
TGTTGTTGCGCGTACCGTTCGTCGCGTTCCGCCAGGTGCAATCGACCCGACCGTTAAAAACCTGCAGTGGGGCGACCTGGTTCGTGGTATGTTCGA
AGCAGCTGACCGCGGTGCAACTTACCCGTTCCTGACTGACGGTGACGCACACCTGACTGAAGGTTCTGGCTTCAACATCGTTCTGGTTAAAGACGG
CGTACTGTACACTCCGGACCGCGGTGTTCTGCAGGGCGTAACTCGTAAGTCTGTTATCAACGCTGCTGAAGCGTTCGGTATCGAAGTTCGCGTTGA
GTTCGTTCCGGTTGAACTGGCTTACCGCTGCGACGAAATCTTCATGTGTAGTACTGCAGGTGGTATCATGCCAATCACCACTCTGGACGGTATGCC
GGTTAACGGTGGTCAGATCGGTCCGATCACCAAGAAAATCTGGGACGGTTACTGGGCGATGCACTACGACGCTGCTTACTCCTTCGAAATCGACTA
CAACGAACGTAATTAAGGTTAGAGC

Sequence of the insert for G1 (pD871_Ate-TA_T274S_LDH_v1_GDHm_v3):

GGCCGCAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGT
GGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGAT
AAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAA
CTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCAT
CATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGT
TTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTT
GCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCC
GGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTAT
ACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGG
CCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCG
AAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCCGGAGAGTTACTAGTATGTA
TACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAG
TTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGAC
GTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAAC
CCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAAT
ATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAA
AGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACAC
CAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCA
GCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAG
CAGGAAGAGGCTAAGGCGCGCC

Sequence of the insert for G2 (pD871_Ate-TA_T274S_LDH_v2_GDHm_v3):

GGCCGcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGG
GCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACT
GAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGA
AGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCC
GAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCG
AAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCAC
GGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGC
TGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCAC
CTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGT
ATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAA
GAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCCGGAGAGTTACTAGTATGTATACAGA
TTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTGTTAT
TAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGTAACA
AAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCCAGTT
CCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATATTTCG
TTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAGGCG
GTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCAATT
AACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGCAGT
TGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCAGGA
AGAGGCTAAGGCGCGCC

Sequence of the insert for G3 (pD871_Ate-TA_T274S_LDH_v3_GDHm_v3):

GGCCGcgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGT
GGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAA
CTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTC
GAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCAT
CCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTT
CCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGC
CACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGG
AGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATAC
CACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCC
TGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAA
GAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCCGGAGAGTTACTAGTATGTATAC
AGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTG
TTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGT
AACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCC
AGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATAT
TTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAG
GCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCA
ATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGC
AGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCA
GGAAGAGGCTAAGGCGCGCC

Sequence of the insert for G4 (pD871_Ate-TA_T247S_GDHm_v3_LDH_v1):

GGCCGCGGAGAGTTACTAGTATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTT
CGTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCG
GACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTT
ATGATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCAT
TCTTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCC
ATTATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAA
CATTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTA
CATCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGG
TATGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCACAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAAC
TTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGA
CCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCC
CTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGT
TTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCC
GGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCG
CTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCG
GCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGT
GGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAA
AATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAG
AACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAG
AGTTGCAGTTCTAAGGCGCGCC

Sequence of the insert for G5 (pD871_Ate-TA_T247S_GDHm_v3_LDH_v2):

GGCCGCGGAGAGTTACTAGTATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTT
CGTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCG
GACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTT
ATGATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCAT
TCTTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCC
ATTATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAA
CATTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTA
CATCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGG
TATGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCagcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGC
TGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCT
GGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTA
AGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTG
AACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGT
TGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTAC
CTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCAT
GAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTG
GATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCT
GCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACG
GTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTT
GCAGTTCTAAGGCGCGCC

Sequence of the insert for G6 (pD871_Ate-TA_T247S_GDHm_v3_LDH_v3):

GGCCGCGGAGAGTTACTAGTATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTT
CGTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCG

GACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTT
ATGATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCAT
TCTTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCC
ATTATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAA
CATTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTA
CATCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGG
TATGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCtgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGC
TGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCT
GGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTA
AGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTG
AACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGT
TGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTAC
CTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCAT
GAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTG
GATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCT
GCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACG
GTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTT
GCAGTTCTAAGGCGCGCC

Sequence of the insert for G1v1 (pD871_Ate-TA_T274S_LDH_v1_GDHm_v1):

GGCCGCAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGT
GGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGAT
AAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAA
CTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCAT
CATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGT
TTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTT
GCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCC
GGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTAT
ACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGG
CCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCG
AAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGGAGGAGGactagtATGTATA
CAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTT
GTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACG
TAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACC
CAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATA
TTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAA
GGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACC
AATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAG
CAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGC
AGGAAGAGGCTAAGGCGCGCC

Sequence of the insert for G1v2 (pD871_Ate-TA_T274S_LDH_v1_GDHm_v2)

GGCCGCAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGT
GGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGAT
AAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAA
CTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCAT
CATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGT
TTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTT

GCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCC
GGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTAT
ACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGG
CCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCG
AAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGATAAACGactagtATGTATAC
AGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTG
TTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGT
AACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCC
AGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATAT
TTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAG
GCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCA
ATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGC
AGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCA
GGAAGAGGCTAAGGCGCGCC

Sequence of the insert for G2v1 (pD871_Ate-TA_T274S_LDH_v2_GDHm_v1):

GGCCGcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGG
GCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACT
GAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGA
AGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCC
GAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCG
AAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCAC
GGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGC
TGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCAC
CTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGT
ATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAA
GAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGGAGGAGGactagtATGTATACAGATT
TAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTA
ACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGTAACAAA
AGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCCAGTTCC
TTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATATTTCGTT
GAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAGGCGGT
ATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCAATTAA
CGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGCAGTTG
CAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCAGGAAG
AGGCTAAGGCGCGCC

Sequence of the insert for G2v2 (pD871_Ate-TA_T274S_LDH_v2_GDHm_v2):

GGCCGcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGG
GCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACT
GAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGA
AGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCC
GAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCG
AAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCAC
GGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGC
TGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCAC
CTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGT
ATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAA

GAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGATAAACGactagtATGTATACAGATT
TAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTA
ACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGTAACAAA
AGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCCAGTTCC
TTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATATTTCGTT
GAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAGGCGGT
ATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCAATTAA
CGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGCAGTTG
CAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCAGGAAG
AGGCTAAGGCGCGCC

Sequence of the insert for G3v1 (pD871_Ate-TA_T274S_LDH_v3_GDHm_v1):

GGCCGcgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGT
GGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAA
CTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTC
GAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCAT
CCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTT
CCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGC
CACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGG
AGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATAC
CACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCC
TGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAA
GAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGGAGGAGGactagtATGTATACA
GATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTGT
TATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGTA
ACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCCA
GTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATATT
TCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAGG
CGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCAA
TTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGCA
GTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCAG
GAAGAGGCTAAGGCGCGCC

Sequence of the insert for G3v2 (pD871_Ate-TA_T274S_LDH_v3_GDHm_v2):

GGCCGcgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGT
GGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAA
CTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTC
GAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCAT
CCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTT
CCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGC
CACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGG
AGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATAC
CACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCC
TGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAA
GAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAGTTCTAAGGATCCGATAAACGactagtATGTATACAG
ATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCGTTTCGGTCAAGAAGAAGCAAAAGTTGTTA
TTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGACAAGCAATCATCGTTCAAGGCGACGTAAC
AAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATGATTAATAACGCTGGTGTTGAAAACCCAGT

TCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTTAGGAAGCCGTGAAGCAATTAAATATTTC
GTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATTATTTGTTCACTACGCAGCAAGTAAAGGC
GGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACATTGGACCAGGTGCGATGAACACACCAAT
TAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACATCGGTAAGCCAGAAGAAGTAGCAGCAG
TTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTATGACGAAATACCCTTCTTTCCAAGCAGG
AAGAGGCTAAGGCGCGCC

Sequence of the insert for G4v1 (pD871_Ate-TA_T247S_GDHm_v1_LDH_v1):

GGCCGCGGAGGAGGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTC
GTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGG
ACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTAT
GATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTC
TTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCAT
TATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACA
TTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACA
TCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTA
TGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCACAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAACTT
GCTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGAC
CTGGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCC
TAAGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTT
TGAACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCG
GTTGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCT
ACCTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGC
ATGAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGG
TGGATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAAT
CTGCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAAC
GGTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGT
TGCAGTTCTAAGGCGCGCC

Sequence of the insert for G4v2 (pD871_Ate-TA_T247S_GDHm_v2_LDH_v1):

GGCCGCGATAAACGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCG
TTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGA
CAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATG
ATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTT
AGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATT
ATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACAT
TGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACAT
CGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTAT
GACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCACAGGAGGAAGAGCTCATGGCTGCATTGAAAGACCAACTGATCCACAACTTG
CTGAAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCT
GGCGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTA
AGATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTG
AACCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGT
TGATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTAC
CTGATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCAT
GAACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTG
GATAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCT
GCGCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACG

GTATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTT
GCAGTTCTAAGGCGCGCC

Sequence of the insert for G5v1 (pD871_Ate-TA_T247S_GDHm_v1_LDH_v2):

GGCCGCGGAGGAGGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTC
GTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGG
ACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTAT
GATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTC
TTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCAT
TATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACA
TTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACA
TCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTA
TGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCagcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTG
AAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGG
CGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAG
ATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAA
CCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTG
ATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCT
GATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGA
ACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGA
TAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGC
GCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGT
ATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGC
AGTTCTAAGGCGCGCC

Sequence of the insert for G5v2 (pD871_Ate-TA_T247S_GDHm_v2_LDH_v2):

GGCCGCGATAAACGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCG
TTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGA
CAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATG
ATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTT
AGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATT
ATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACAT
TGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACAT
CGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTAT
GACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCagcgtaaggagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGA
AAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGC
GGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGA
TTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAAC
CTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGA
TATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTG
ATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAA
CGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGAT
AGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCG
CCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTAT
TAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAG
TTCTAAGGCGCGCC

Sequence of the insert for G6v1 (pD871_Ate-TA_T247S_GDHm_v1_LDH_v3):

GGCCGCGGAGGAGGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTC
GTTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGG
ACAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTAT
GATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTC
TTAGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCAT
TATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACA
TTGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACA
TCGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTA
TGACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCtgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTG
AAAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGG
CGGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAG
ATTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAA
CCTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTG
ATATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCT
GATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGA
ACGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGA
TAGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGC
GCCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGT
ATTAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGC
AGTTCTAAGGCGCGCC

Sequence of the insert for G6v2 (pD871_Ate-TA_T247S_GDHm_v2_LDH_v3):

GGCCGCGATAAACGactagtATGTATACAGATTTAAAAGATAAAGTAGTTGTAATTACAGGTGGATCAACAGGTTTAGGACGTGCAATGGCTGTTCG
TTTCGGTCAAGAAGAAGCAAAAGTTGTTATTAACTATTACAACAATGAAGAAGAAGCATTAGATGCGAAAAAAGAAGTAGAAGAAGCAGGCGGA
CAAGCAATCATCGTTCAAGGCGACGTAACAAAAGAAGAAGACGTTGTAAACCTTGTTCAAACAGCTATTAAAGAATTCGGAACATTAGACGTTATG
ATTAATAACGCTGGTGTTGAAAACCCAGTTCCTTCTCATGAGCTATCTTTAGACAACTGGAACAAAGTTATTGATACAAACTTAACAGGTGCATTCTT
AGGAAGCCGTGAAGCAATTAAATATTTCGTTGAAAATGACATTAAAGGAAACGTTATTAACATGTCCAGCGTTCACGAAATGATTCCTTGGCCATT
ATTTGTTCACTACGCAGCAAGTAAAGGCGGTATGAAACTAATGACGGAAACATTGGCTCTTGAATATGCGCCAAAAGGTATCCGAGTAAATAACAT
TGGACCAGGTGCGATGAACACACCAATTAACGCTGAAAAATTCGCTGATCCTGTACAACGTGCAGACGTAGAAAGCATGATTCCAATGGGTTACAT
CGGTAAGCCAGAAGAAGTAGCAGCAGTTGCAGCATTCTTAGCATCATCACAAGCAAGCTATGTAACAGGTATTACATTATTTGCTGATGGTGGTAT
GACGAAATACCCTTCTTTCCAAGCAGGAAGAGGCTAAGGATCCtgagcgagaagagctcATGGCTGCATTGAAAGACCAACTGATCCACAACTTGCTGA
AAGAAGAACACGTACCACAAAACAAGATCACCGTGGTGGGCGTTGGTGCGGTTGGCATGGCCTGTGCGATTAGCATCCTGATGAAGGACCTGGC
GGACGAGCTGGCACTGGTTGACGTGATGGAAGATAAACTGAAGGGTGAAATGATGGATCTGCAACATGGTAGCCTGTTTCTGCGCACCCCTAAGA
TTGTTAGCGGCAAGGATTACAGCGTCACCGCGAACTCGAAGCTGGTCATTATCACTGCGGGTGCTCGTCAGCAAGAGGGCGAGTCTCGTTTGAAC
CTGGTCCAACGTAACGTCAATATCTTCAAGTTCATCATCCCGAATGTGGTCAAGTATTCTCCGCACTGTAAATTGCTGGTGGTTTCCAATCCGGTTGA
TATCCTGACGTACGTTGCCTGGAAGATTAGCGGTTTTCCGAAAAATCGTGTGATTGGTTCCGGTTGCAATCTGGACAGCGCCCGTTTCCGCTACCTG
ATGGGTGAACGCCTGGGCGTGCATGCTTTGTCTTGCCACGGTTGGATTCTGGGTGAGCATGGTGACAGCAGCGTTCCGGTCTGGAGCGGCATGAA
CGTTGCGGGTGTTAGCCTGAAAACGCTGCACCCGGAGCTGGGTACCGATGCCGACAAAGAGCAGTGGAAACAGGTCCACAAACAGGTGGTGGAT
AGCGCGTACGAGGTTATCAAGCTGAAAGGTTATACCACCTGGGCGATTGGCCTGAGCGTCGCAGACTTGGCGGAGAGCATCATGAAAAATCTGCG
CCGTGTCCATCCGATCTCCACGATGCTGAAGGGCCTGTATGGCATTAAAGAGGATGTGTTTCTGAGCGTGCCGTGCGTCCTGGGTCAGAACGGTAT
TAGCGATGTTGTGAAGGTAACGCTGACCAGCGAAGAAGAAGCACACCTGAAGAAAAGCGCAGACACCCTGTGGGGCATTCAGAAAGAGTTGCAG
TTCTAAGGCGCGCC