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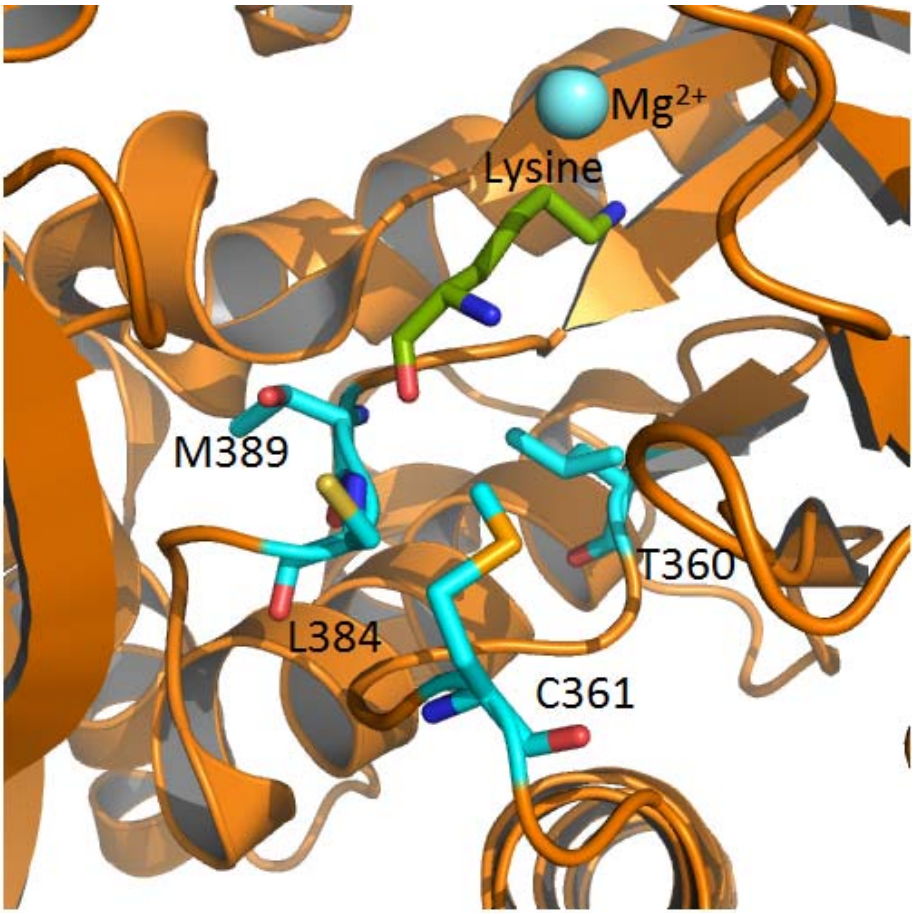

Engineering of 3-methylaspartate ammonia-lyases for bio-based adipic acid production

The deamination of lysine

Master's thesis in Biology and Biological Engineering

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Gothenburg, Sweden 2017

Foreword

I would like to thank all the people that have made it possible for me to do my master thesis at Chalmers tekniska högskola. The assistance and the numerous conversations that enriched my experience in Sweden. The guidance and help that I received for the exchange and my master thesis.

Firstly, I would like to thank Dr. Veronica Saez Jiminez for the guidance during my stay, the help on the thesis work, the open ear for all my questions and the interesting conversation over the project. The guidance to aim for a more critical view towards the result and the thesis work. Finally, to help me grow in the lab environment and to lay confidence in me during the lab experiments.

Secondly, I would like to thank Dr. Valeria Mapeli and Dr. Lisbeth Olsson for the opportunity of doing my master thesis in the 'Biology and Biological Engineering' department with specifically the adipic acid project group.

Thirdly, I would like to thank the IndBio staff/researchers for helping me with any question I asked and guiding me to find the right answer. The happy and enlightened atmosphere in the lab during the experiments and the Monday meetings. The aid with finding an apartment in Göteborg for my stay and the continuous support.

Finally, I would like to thank my fellow master students; Adam Larsson, Louis Samuelsson, Amanda Ristinmaa, Zélie Jeannot and Vasilis Diamadopoulos for the warm and open welcome. The happy conversations, the critical thoughts on my concerns and the collective listening ear that made all the stressful moments melt away.

I may have forgotten some persons during my round of thanks and therefor my sincere apologies. But it is thanks to all the major help and the inspiring words of everyone that this master thesis has come to a successful end.

My sincere thanks to all,

Robin Van Havere

Abstract

The current traditional adipic acid production is a highly polluting process. Researchers have been studying bio-based pathways to produce adipic acid in an environmentally sustainable way. One of these bioprocesses could involve the use of a metabolic pathway to produce adipic acid from lysine. The pathway first enzymatic reaction is the deamination of lysine into 6-aminohex-2-enoic acid. No enzyme has been discovered yet to catalyse this reaction.

The study of an enzyme able to do this conversion was the main aim of this thesis. Several ammonia lyases were considered as interesting enzymes to study since these enzymes are able to do a deamination on aminoacids, though different than than lysine. The enzyme studied in this research project is **3-methylaspartate ammonia lyase (MAL)** which catalyses the deamination reaction on 3-methylaspartic acid. The 3-methylaspartic acid has a comparable structure to lysine and makes the MAL enzyme an appropriate candidate to study the conversion. However, the MAL enzyme did not show any activity towards lysine. Preliminary results obtained from computational structural biology experiments of MAL in the presence of lysine suggested some mutations that could be done on the enzyme to gain activity on lysine. Therefore, the suggested mutations were implemented in the MAL enzyme, aiming at increasing the size of the binding pocket of MAL to give lysine more space to fit in the active site and make the reaction more feasible. The mutations were successfully introduced in the MAL gene with a site-directed **mutagenic PCR** protocol. The mutated MAL genes were then **expressed** in *E.coli* BL21 (DE3) and the protein purified. The **protein purification** was done with two methods that used the same basic principle: an **Immobilized Metal-ion Affinity Chromatography**. After the purification, the activity of the mutated enzymes was monitored by various **activity assays**. The assays provided the **kinetic constants** of the conversion of the natural substrate, 3-methyl aspartate, by the mutated enzymes and their plausible activity on lysine by detecting the theoretical conversion products**: 6-aminohex-2-enoic acid** and **ammonia**.

No activity on lysine was detected, but still valuable information was retrieved from the activity assays. Some of the mutated enzymes like L384A, C361A and C361S showed a tremendous impairment in the activity, which led me to conclude that those residues that were altered are important for the enzyme catalysis.

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Abbreviations

UV light	Ultra violet light
E.coli	*Escherichia coli*
6-AHEA	6-aminohex-2-enoic acid
3MA	3-methylaspartic acid
MAL	3-methylaspartate ammonia lyase
kDa	Kilodalton
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
kb	Kilobases
kbp	Kilobase pares
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
DTT	Dithiothreitol
6X His-Tag	6 times histidine tag
Vmax	Maximum velocity
Km	Michaelis constant
Kcat	Turnover number
Kcat/Km	Specificity constant

1. Introduction

In this master thesis, a description is given on how the research and experiments were done to facilitate the enzymatic conversion of lysine into 6-aminohex-2-enoic acid, the first step of a metabolic pathway under study for the bio-based production of adipic acid. The project is valuable because adipic acid is still a product that is derived from fossil resources. The use of fossil resources is commonly accompanied with pollution for the environment and the world needs more biologically made products to be sustainable and more durable for future generations.

2. Background

2.1. From the traditional fossil-based economy to the future bio-based economy

The production of fuels and chemicals is still mostly derived from fossil resources and uses traditional refineries to produce these products. The traditional production makes many by-products like carbon dioxide, nitrous oxide, etc., which contribute to the emission of greenhouse gasses and the effect of global warming. The need for more sustainable processes is growing every day, because of the detrimental effects on the environment of the traditional refineries and the depletion of the fossil resources. One alternative could be the use of biotechnological processes to obtain the products in a bio-based way. This implies that the production of certain products is done by microorganisms or enzymes and that the feedstocks are not fossil-based, but from renewable sources. The new way of production often uses the term 'Biorefinery'. One definition of biorefinery is: 'a refinery that uses biomass and converts this to valuable products'(Berntsson et al. 2012). The biomass can come from different resources like: paper, agriculture, forestry, algae, etc., and can be converted into products as fuels, chemicals, electricity or even just heat (Figure 1). Known biomass sources that are mainly used nowadays are: starch, straw, wood and algae. These bio-based processes usually have no environmentally damaging by-products. Biological production has been known for centuries. It already exists from the middle ages, for example for the production of beer and bread, which are produced by yeast fermentations. (IEA Bioenergy 2011; Berntsson et al. 2012).

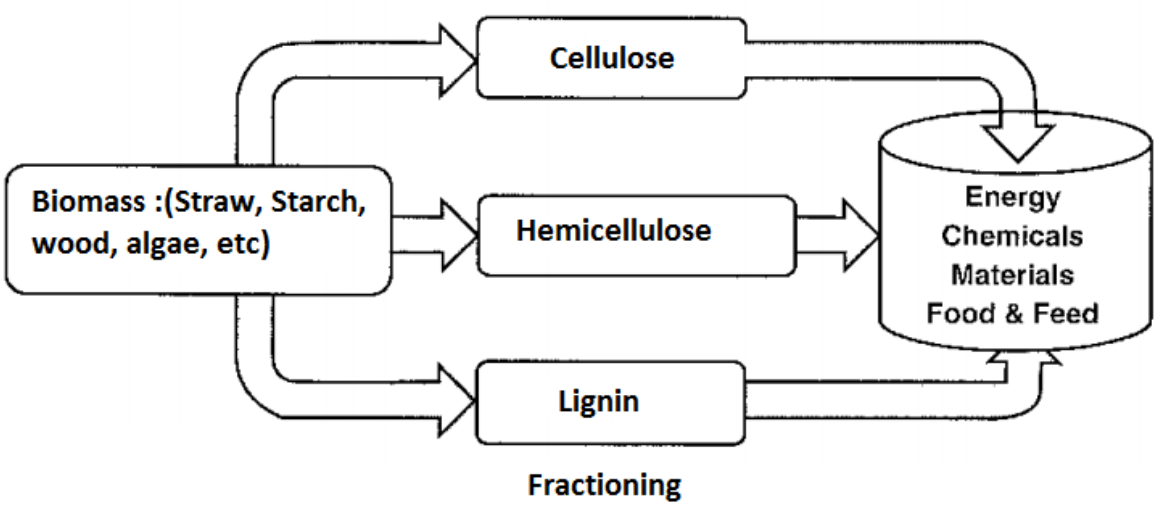

Figure 1: Diagram of a bio refinery process which uses biomass as feedstock and different production pathways for the obtaining of different fuels and chemicals.

2.2. Adipic acid

Adipic acid (

Figure 2) is a very widely used chemical and is mainly used for the production of the plastic polymer Nylon 6-6 polyamide. Adipic acid is also an additive in the food industry, pharmaceutical industry and in an array of industrial coatings to improve the flexibility and resilience. It is estimated that every year around 2,85 million tons of adipic acid are produced. This makes adipic acid the most important synthetic dicarboxylic acid annually produced for commercial purposes (Deng et al. 2016).

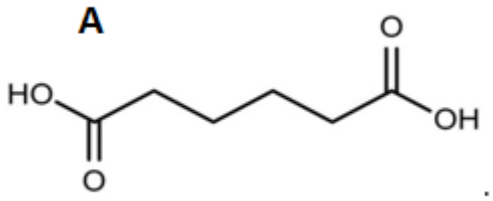

Figure 2: Adipic acid chemical structure

2.3. Current adipic acid production

The traditional production of adipic acid is derived from fossil resources and uses a chemical process combined with high pressure and temperature. The main process to produce adipic acid uses benzene as feedstock (Figure 3.A) (Noack et al. 2011). The benzene is hydrogenated under high pressure to form cyclohexane. The cyclohexane then is oxidized to a mixture of cyclohexanol and cyclohexanone, also called KA oil. The KA oil mixture will react with nitric acid and air to form adipic acid. To make the production process 'greener' and avoid the use of nitric acid and the generation of N_2O emissions (deleterious for the environment), two other process schemes have been drafted. The first one uses cyclohexane and ozone with UV light to produce adipic acid (Figure 3B) (Hwang and Sagadevan 2014). The second one starts with cyclohexane and hydrogen peroxide. The hydrogen peroxide molecules are used to oxidize cyclohexane in successive steps to produce adipic acid (Figure 4) (Sato et al. 1998).

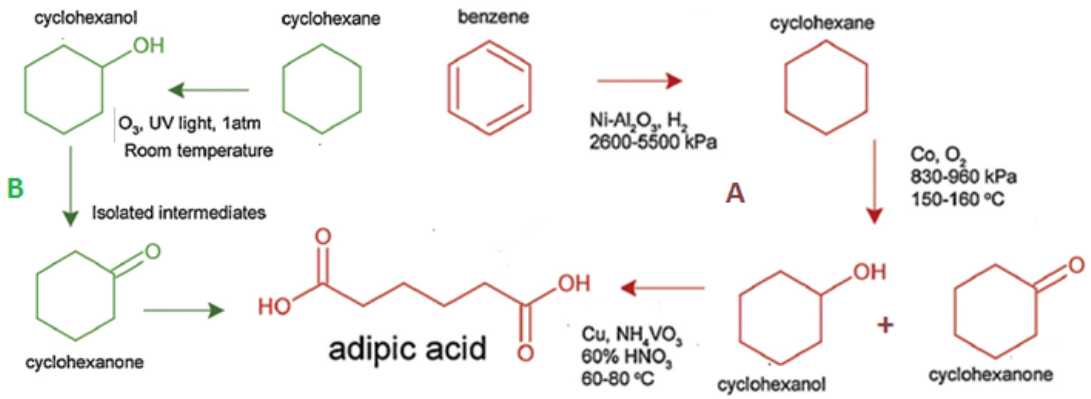

Figure 3: A, The traditional production of adipic acid through the conversion of benzene with high pressure, temperature and nitric acid; B, production of adipic acid from the oxidation of cyclohexane by ozone and UV light (Deng et al. 2016).

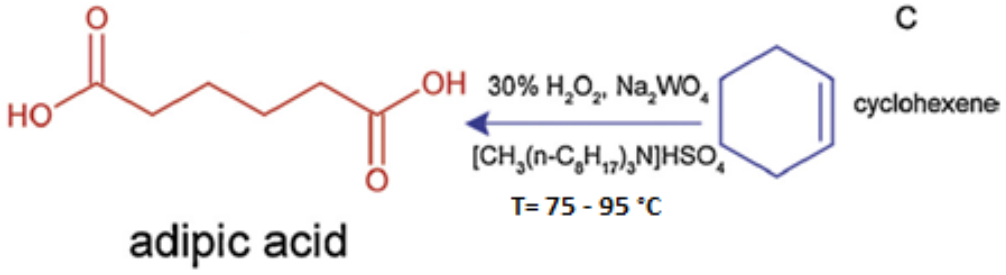

Figure 4: Process of producing adipic acid by the oxidation of cyclohexene with hydrogen peroxide (Deng et al. 2016)

Still the chemical production methods are not environmental friendly: petrochemicals are used as reagents and the usage of these reagents is still detrimental for the environment. The traditional production process is the most common production process, because it is the only one that is economically feasible and efficient enough. Still the current process has a few disadvantages: an overall low product yield (4-11 %), the nitric acid used corrodes the reaction vessels, the production of N_2O gas, CO_2 gas and other hazardous compounds, the use of the toxic and carcinogenic compound benzene and a high-energy consumption. It is estimated that around 0,3 kg N_2O / kg adipic acid is released in the environment. The N_YO_X gasses emitted are approximately 10 % of the world-wide emission in one year. (Polen et al. 2013; Deng et al. 2016)

2.4. Bio-based adipic acid production

In order to reduce the traditional processes in favour of more environmentally friendly ones, new bio-based production pathways for adipic acid production are being researched. Some of the processes lead to the biological production of precursors that can be chemically converted into adipic acid and other processes lead to direct production of adipic acid. In the following paragraph, I will describe several production processes that have been researched and tested until this moment.

2.4.1. Biosynthesis of adipic acid precursors

In the first place, the production methods that will be reviewed are the ones that lead to the indirect bio-based production of adipic acid. The study of production processes that are partially bio-based and use less chemical steps is a positive set up for the future. In these processes, precursors are produced through fermentation and converted to adipic acid with a final chemical step. At the moment, there are two main precursors researched for adipic acid production: cis, cis-muconic acid and D-glucaric acid.

2.4.1.1. Cis, cis-muconic acid

There are two indirect pathways to produce cis, cis-muconic acid (Figure 5) that were engineered into host organisms for uncomplicated production (Deng et al. 2016).

The first pathway (Figure 5.A) that will be discussed uses D-glucose as starting substrate that is converted to 3-dehydroshikimic acid through the microorganism's native shikimate pathway. This is then converted with a 3-dehydroshikimic acid dehydrogenase (aroZ gene) to protocatechuic acid. The protocatechuic acid is then converted with the protocatechuate decarboxylase (aroY gene) that removes a carboxylic group of the molecule and produces catechol. The last step that renders cis, cis-muconic acid involves breaking open the aromatic circle. The catechol 1,2-dioxygenase (CatA, CatA2) catalyze this reaction and cuts the double bound between the two hydroxyl carbons to form cis, cis-muconic acid. The discussed pathway has been implemented in *Escherichia coli*. Three genes have been introduced in the DNA of *E.coli*: the aroY-, the aroZ- gene and the CatA/CatA2-gene. The theoretical yield of the conversion of glucose into cis, cis muconic acid is 43 % (mol/mol), but the practical lab tests showed a titer of 38,6 g/l with a 0,22 mol cis, cis-muconic acid/mol glucose conversion. The low efficiency of the process can be due to the low acidity resistance of *E. coli*. Tests with *Saccharomyces cerevisiae* have also been tried but the titer obtained was 141 mg/L (Draths and Frost 1994; Deng et al. 2016)

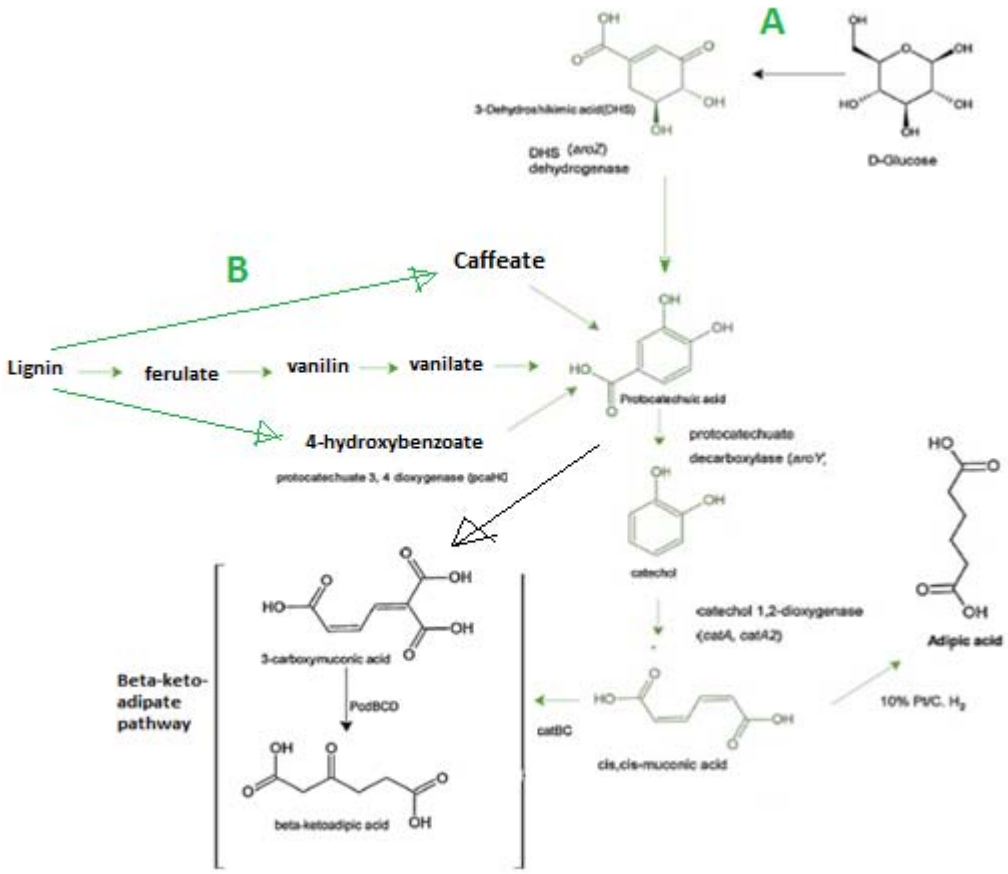

Figure 5: A, The fermentation process of D-glucose to cis, cis-muconic acid through different enzymatic steps. B, Lignin aromatic derivatives conversion to cis-cis muconic acid through different enzymatic steps in the native pathway of P. putida (Deng et al. 2016)

The second pathway (Figure 5.B) uses lignin derivatives as feedstock. Lignin is an important renewable source of aromatic molecules in nature, and as happens with D-glucose, is not an important food source. The lignin can be broken down by either enzymatic hydrolysis or a chemical solvent mixtures. The derivates that are produced have been used in the drafted pathway to produce protocatechuic acid through the β-ketoadipate pathway from *Pseudomonas putida* KT2440. The problem addressed was that the pathway finally converts the protocatechuic acid to β-ketoadipate and not cis, cis-muconic acid. A solution was found which blocks the further conversion of protocatechuic acid by protocatechuate 3,4 dioxygenase by excluding the pcatHG gene that produces this enzyme. Now it can be converted to catechol with a protocatechuate decarboxylase (AroY gene) and the carboxylic group can be removed. This will again be converted to cis, cis-muconic acid by cleaving the double bound between the two hydroxyl carbon bound groups with catechol 1,2-deoxygenase (CatA, CatA2). The engineered microorganism that is used for the pathway is *Pseudomonas putida* KT2440-jD1. This organism was isolated after a mutagen had been used to treat *Pseudomonas putida* KT2440 (Van Duuren et al. 2011). The genes that were incorporated are the AroY-, the CatA/CatA2-gene. The yield for this process is not discussed, but the concerns of this pathway is the low conversion rate of lignin to the derivates at the moment. The depolymerization is a key part of getting higher yields with this particular pathway (McFall et al. 1998; Polen et al. 2013; Deng et al. 2016).

2.4.1.2. D-glucaric acid

The biological pathway (Figure 6) to produce D-glucaric acid from D-glucose has been researched as a different indirect pathway for adipic acid production. The process starts with the conversion of D-glucose to Glucose-6-phosphate by phosphoenolpyruvate-dependent phosphotransferase system (PTS). Then the phosphate group is transferred to a different location on the molecule by myo-inositol-1-phosphate synthase (ino1) to produce myo-inositol-1-phosphate. The phosphate group is replaced with a hydroxyl group by a phosphatase (SuhB) to convert it to myo-inositol. This is converted to D-glucuronic acid by transferring two hydroxyl group to a carboxyl group and an ester binding by a myo-inositol oxygenase (miox). Subsequently, the cyclic bound is cleaved at the ester bound by urinate dehydrogenase (udh) to form D-glucaric acid. The described pathway has been engineered in *Escherichia coli* in which four different genes needed to be introduced. The genes are the ino1-, the SuhB-, the miox- and udg-gene. The efficiency of the production of D-glucaric is impeded by the instability of the recombinant MIOX enzyme and its activity correlation with the concentration of myo-inositol. (Moon et al. 2009; Deng et al. 2016).

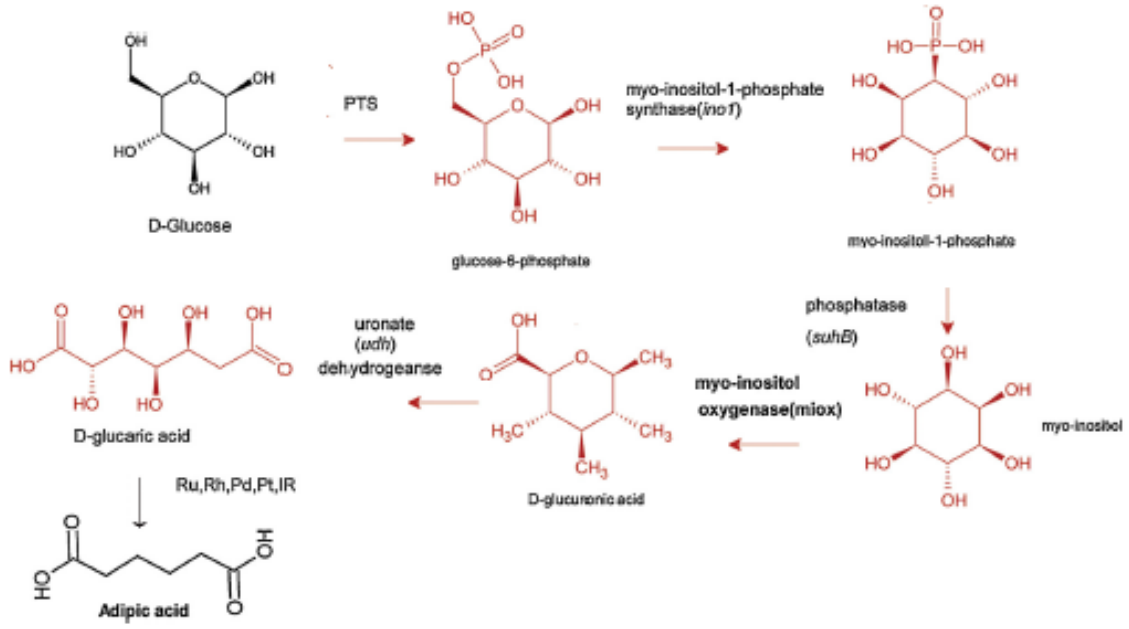

Figure 6: The fermentation process of D-Glucose to D-glucaric acid through different enzymatic steps and chemical conversion to adipic acid (Deng et al. 2016)

2.4.2. Direct bio-based conversion to adipic acid

The production of adipic acid precursors are a good step in the right direction since adipic acid is produced with less chemical steps. However, complete bio-based processes that will use microbial fermentation to produce adipic acid have been researched. Several metabolic pathways have been drafted for bio-based production of adipic acid. The most important ones will be reviewed in this paragraph.

The first bio-based pathway (Figure 7.A) used the substrate D-glucose. In this case, the reverse of the adipate degradation pathway has been used to convert D-glucose to adipic acid. The pathway is similar to the fatty acids oxidation and thermodynamically feasible. It starts with D-glucose that is converted to acetyl-CoA and succinyl-CoA by glycolysis and the citric acid cycle. These are converted to 3-oxoadipyl-CoA by β-ketothioase. The next step is converting a ketone to a hydroxyl group by a 3-hydroxylacyl-CoA dehydrogenase to form 3-hydroxyadipyl-CoA. Then a dehydratation is done by 3-hydroxyadipyl-CoA dehydrogenase and a double bound is formed. This double bound is reduced again by 5-carboxy-2-pentenoyl-CoA reductase to adipyl-CoA. The last step will replace the S-CoA group by a hydroxyl group to form adipic acid. The metabolic pathway has been engineered in *E.coli*. The yield for this production pathway is very low, around 600 µg/l. Some factors affecting the yield are the accumulation of the intermediates that are inhibiting the cell growth and expression of the enzymes (Yu et al. 2014; Deng et al. 2016).

The second pathway (Figure 7.B) uses long carbon chains like n-alkanes or fatty acids as substrate. This pathway is build up around the ω-oxidation pathway. The specific substrate proposed is coconut oil which undergoes different enzymatic steps to form adipic acid. It begins with the conversion of coconut oil to malonyl-CoA and acetyl-CoA. Then these are transformed to hexanoic acid by hexanoate synthase (HexS). The hexanoic acid is then treated with Cytochrome P_{450} reductase (CPR) and P_{450} monooxygenase (CYP_{450}) to change a methyl group in a hydroxyl group and obtain 6-hydroxycaproic acid. The next step forms 6-oxohexanoic acid and can be done through two different conversions. To change the hydroxyl group in an aldehyde group, a Fatty alcohol dehydrogenase (ADH) or a Fatty alcohol oxidase (FAO) can be used. The last step is to convert to adipic acid by Fatty aldehyde dehydrogenase (Ald DH). This pathway has been implemented in *Candida tropicalis* (Probst 2002). This strategy rendered a yield of 4 g/l of adipic acid after 6 days cultivating with coconut oil. After this test, Picataggio et al, developed the fermentation process to gain a 50 g/l yield in 120 h (Probst 2002; Deng et al. 2016).

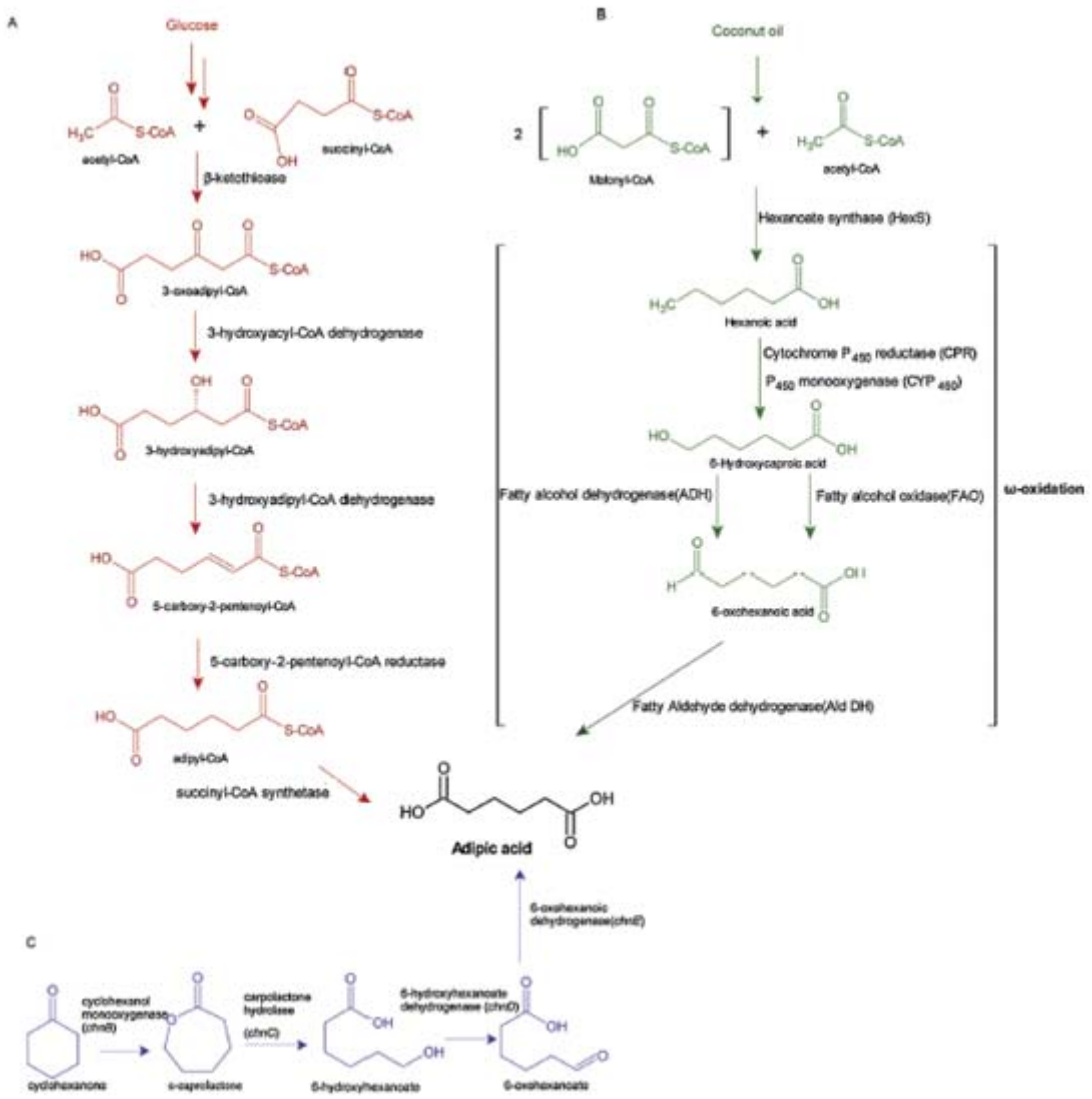

Figure 7: Direct bio-based adipic acid production. A: from glucose by using the reverse adipate degradation pathway. B: from long chained carbon substrate through β-or ω-oxidation. C: from cyclohexanone by an array of different enzymes (Deng et al. 2016)

The third pathway (Figure 7.C) uses cyclohexanone as feedstock. Here four enzymes convert cyclohexanone to adipic acid. The cyclohexanone is oxidized by a cyclohexanol monooxygenase (chnB) to introduce an ester bound in the cyclohexanone molecule and form ε-caprolactone. The ε-caprolactone is then converted to 6-hydroxyhexanoate by a caprolactone hydrolase which cuts the cyclo-part and adds hydroxyl groups at each end. Next a 6-hydroxyhexanoate dehydrogenase (chnD) transforms a hydroxyl group into an aldehyde group. Then the 6-oxohexanoic dehydrogenase (chnE) rearranges the structure and changes the aldehyde group to a carboxylic group to produce adipic acid. The enzymes that compose the metabolic pathway were found in *Arthrobacter* species and *Rhodococcus* species isolated from wastewater bioreactors. The genes were expressed in E.coli and during the tests a trace amount of adipic acid was detected (Brzostowicz et al. 2003; Deng et al. 2016).

The fourth pathway (Figure 8) that will be discussed uses lysine as a substrate to produce adipic acid. Lysine can be produced through fermentation and this production process is being researched because lysine is an important food additive for animal feed (Tosaka et al. 1983). The pathway describes four steps of the theoretical metabolic conversion to produce adipic acid. The first step involves the deamination of lysine to 6-aminohex-2-enoic acid (6-AHEA) by an ammonia lyase. In the second step, an oxidoreductase takes place on the double bound of the molecule to produce 6-aminocaproic acid. The third conversion involves a deamination at the end of the molecule and introduce an aldehyde group by a transaminase. The last step will convert adipic acid semialdehyde in adipic acid by an oxidoreductase called 6-oxohexonoate dehydrogenase. Here an aldehyde is change to a carboxylic group. The entire pathway has been reviewed and described with what kind of enzymes are needed. But only for the fourth step an enzyme has been found that can do the conversion. The rest of the enzymes are not found yet, nevertheless research is being done at this moment on these conversions (Burgard et al 2015).

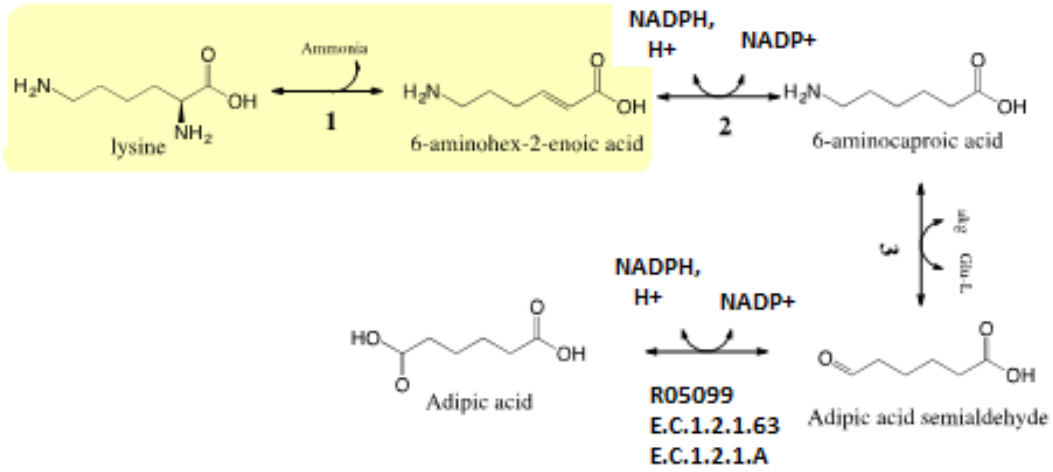

Figure 8: Bio-based production of adipic acid with as substrate Lysine (Burgard et al 2015).

In this master thesis, we will focus on this particular pathway. More precisely on the first reaction of the pathway: the lysine conversion to 6-AHEA. The principle behind this conversion is the deamination of the lysine at the α-carbon (Figure 9). This deamination reaction would be typically done by an ammonia lyase, but there is not a described ammonia lyase able to catalyze this reaction for Lysine.

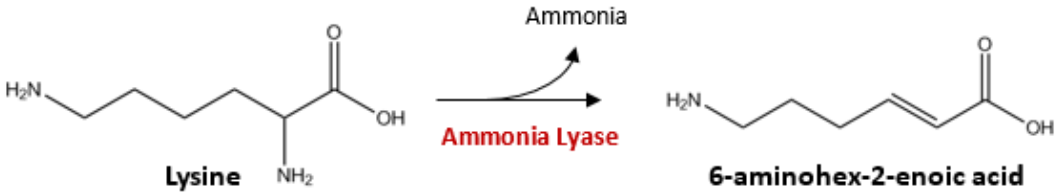

Figure 9:The theoretical conversion of lysine by an ammonia lyase

2.5. MAL (3-methylaspartate ammonia lyase)

The family of the carbon-nitrogen lyases (EC 4.3.1) consists of a range of different enzymes like histidine ammonia lyase, aspartate ammonia lyase and 3-methylaspartate ammonia lyase that mainly catalyse the same reaction with different substrates: the α, β-deamination of the alpha carbon bound ammonia group and make a double bound between the alpha and beta carbon of the molecule (Figure 10). The 3-methylaspartate ammonia lyase (MAL) is one of the most studied ammonia-lyases. The crystal structure, physiological function, kinetic constants and the catalytic residues for conversion have been determined (Asano et al. 2004). MAL was first discovered in a cell-free extract of *Clostridium tetanomorphum* in 1959 by Barker et al. MAL is found in a range of different organisms such as bacteria (*Clostridium sp.*, *Pseudomonas sp.*, *Citrobacter sp.*). But out of all these microorganisms, the MAL of *Citrobacter amalonaticus* (Ca MAL) is best described and characterized in literature (Botting et al. 1988; Levy et al. 2002; Asano et al. 2004).

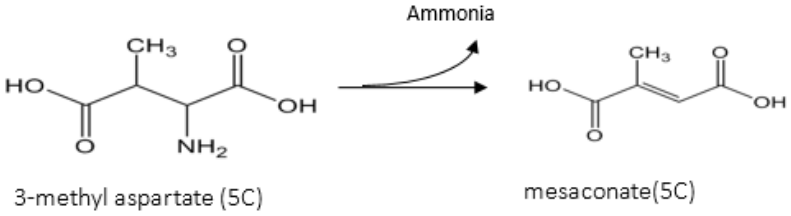

Figure 10: The conversion of 3-methylasapartate to mesaconate and ammonia, reaction catalysed by 3-methyl aspartate ammonia lyase

The crystallization and the structural analysis of *Citrobacter amalonaticus* MAL gave valuable information of the characteristics and catalytic mechanism. The enzyme is a homodimer with a molecular weight of ~ 80 kDa (Figure 11**Error! Reference source not found.** B1 and B2). The analysis showed the binding pocket where the substrate L-threo-3-methyl aspartate binds for conversion to mesaconate. Also, a Mg $^{2+}$-ion was detected, which is essential for the activity. It further determined that the amino acids highlighted in Figure 11 B3 were important for the activity of the enzyme. An essential amino acid for the activity is lysine 331, which will substract a hydrogen from the beta-carbon of the substrate. This will lead to a reorganization of the electrons in the substrate. This reconfirmation is stabilized by the Mg^{2+}-ions (Asano et al. 2004; Raj et al. 2009; De Villiers et al. 2012).

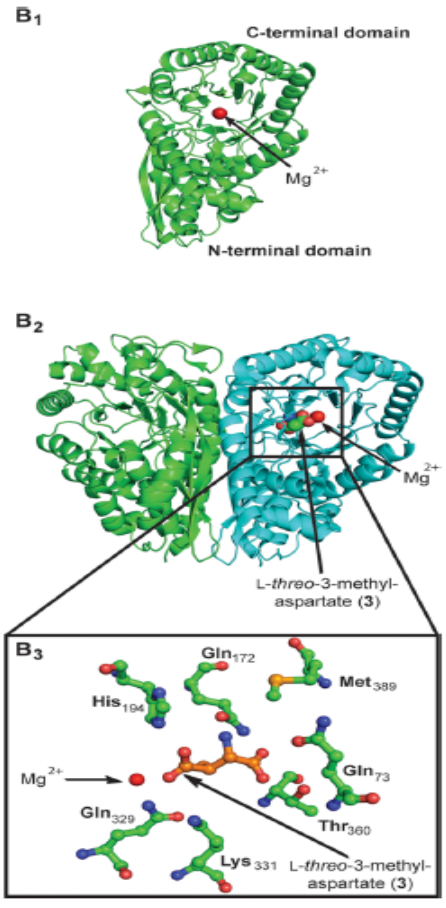

Figure 11: B1, MAL monomer; B2, MAL enzyme with substrate in binding pocket; B3, enlargement of binding pocket with substrate (De Villiers et al. 2012).

3. Aim of the master thesis

The general aim of the project wherein this thesis fits in is the production of adipic acid in a bio-based process using a metabolic pathway from the substrate lysine. The first reaction in this pathway involves the deamination of lysine to produce 6-AHEA (Figure 9). There is not yet a described enzyme that is able to catalyze this conversion. So, the main and specific aim of the project is the search and/or engineering of an enzyme able to convert L-lysine into 6-AHEA.

The enzymatic activity necessary to catalyze the deamination of lysine is defined as ammonia lyase (EC 4.3.1). There are ammonia lyases able to catalyse the deamination reaction on various substrates like histidine, phenylalanine, aspartic acid and 3-methylaspartate. One of these enzymes is 3-methylaspartate ammonia lyase (MAL), whose substrate is 3-methyl aspartate. Since 3-methyl aspartate has a similar structure than lysine (Figure 12), MAL could be a suitable candidate to be engineered for the activity on lysine.

The enzyme studied in this master thesis was the MAL from *Citrobacter amalonaticus*. The enzyme was chosen because it has been thoroughly studied in past research: the crystal structure is available and the catalytic mechanism, cofactors and amino acids important for activity of the enzyme have been researched (Asano et al. 2004; Raj et al. 2009; De Villiers et al. 2012).

The main differences between 3-methylaspartic acid and lysine are the length of the carbon chain and the presence of a carboxyl group instead of an amino group (Figure 12). These differences represent a challenge since the substrate specificity of MAL must be changed.

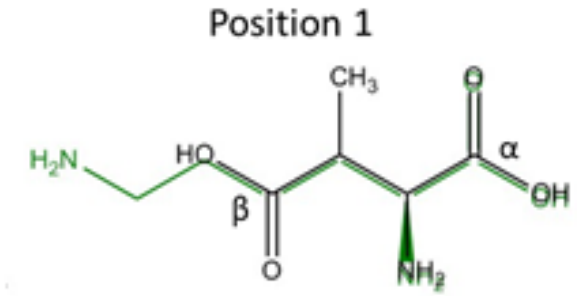

Figure 12: Overlay of L-lysine (green) with 3-methyl aspartate (black).

Structural biology and protein engineering tools were combined in the project to achieve the aim of changing the substrate specificity from 3-methyl aspartate to lysine. Previous computational studies were done in collaboration with the Computational Biology Laboratory in Copenhagen in order to understand how lysine could fit in the binding pocket of the enzyme. Preliminary results obtained with the program MutateX (https://github.com/ELELAB/mutatex) lead to the suggestion of mutating some residues. The mutation of these residues should help to accommodate lysine in the catalytic pocket as well as led to a stable enzyme-substrate complex. Considering the preliminary results from the computational studies as well as the information available in the literature, the following mutations that look scientific relevant were studied: T360A, T360S, C361S, C361A, L384A and M389A.

These mutations were introduced in the MAL gene by site directed mutagenesis. A polymerase chain reaction (PCR) was done to alter the amino acid sequence and mutate the enzyme. After the mutagenesis, expression and purification of the different enzyme variants, different activity assays were done to determine if the enzymes were still active towards the natural substrate and calculate the kinetic constants. Finally, the activity towards lysine or β-lysine was measured (Figure 13). The various activity assay will be explained and discussed further in the thesis.

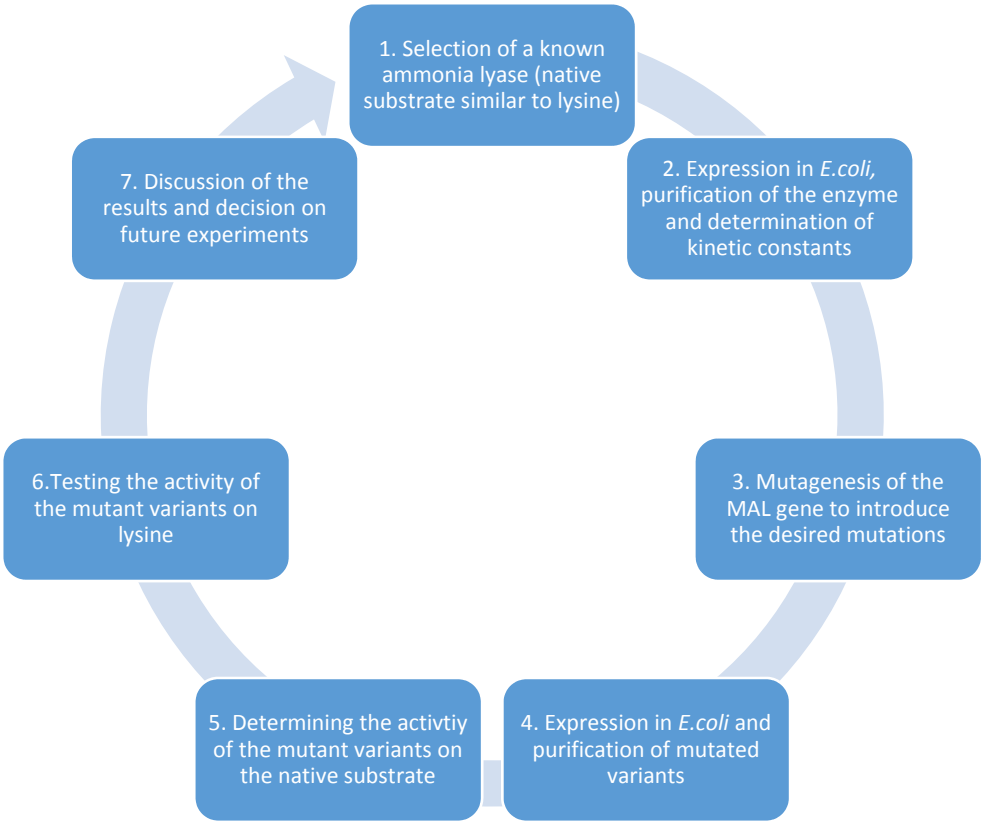

Figure 13: Flow-chart of the strategy followed in the present master thesis

The major issues or scientific questions that were investigated in the thesis are:

i. Can MAL be expressed and purified?
ii. Is MAL active, if yes? What are the kinetic constants of MAL?
iii. Are the site directed mutagenesis experiments successful?
iv. Can the mutant enzymes be expressed and purified?
v. Are the MAL mutants still active towards the native substrate?
vi. If active, what are the kinetic constants of the mutated enzymes?
vii. Have the native MAL or the mutant MAL activity towards lysine?
viii. Are the purification, quantification and activity assays applicable? Do they need to be optimized?

4. Methods and materials

This chapter of the master thesis will describe the chosen techniques and materials. They will be explained and discussed to the fullest of their use in the master thesis. The first part will revolve around the mutagenesis protocol used to produce the mutated proteins and their expression and purification. The second part will explain the methods used for measuring the activity towards the different substrates tested.

4.1. Plasmid construction and bacterial strains

Before discussing all the methods that have been used to obtain the aim of the master thesis, the plasmid construct used for protein expression and mutagenesis protocols is presented. The plasmid construct was build up out of the pET28b vector (Figure 14) and the *Citrobacter amalonaticus* MAL gene sequence (Appendix. A). The MAL sequence was cloned in the vector with the help of restriction enzymes NcoI and XhoI (Figure 15). A remarkable fact is that there is no stop codon at the end of the MAL gene sequence that was cloned in the vector. This has a definitive reason, after the XhoI restriction site, a poly histidine sequence and a stop codon are found. This will allow that a 6X Histidine-Tag is pasted after the MAL enzyme when it was expressed. This way, the expressed proteins can be purified through an immobilized metal-ion affinity chromatography (see section 4.8.2). On the other hand, the plasmid has a T7-promotor for gene expression, a lac operon and a gene that confers kanamycin resistance which will help with selection procedures.

The plasmid was transformed in *Escherichia coli*. Two different *E.coli* strains were used: *E.coli* DH5α, which was used for plasmid storage and propagation, and E.coli BL21 (DE3), was used for protein expression.

pET-28a(+) sequence landmarks	
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.

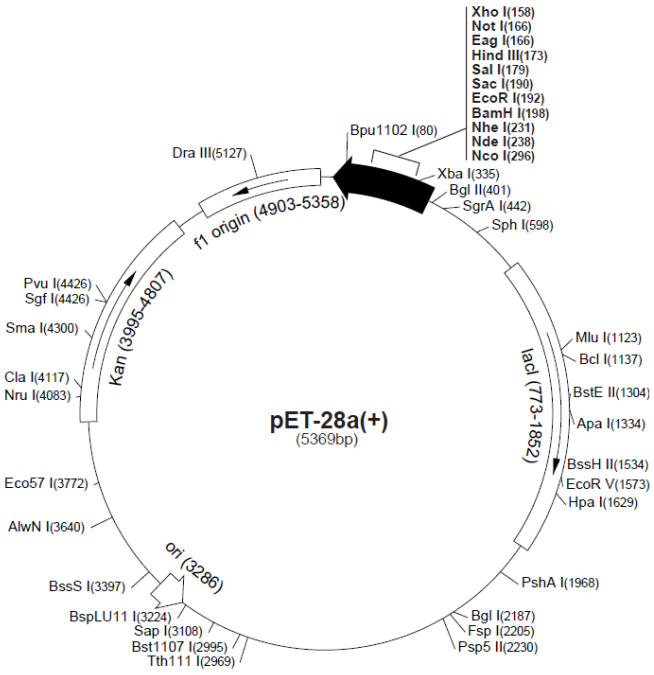

Figure 14: The pET28b vector used for bacterial expression. Size: 5368 bp. It contains the T7-promotor and -terminator regions, a kanamycin resistance gene, 6X Histidine Tag that can be set up at N-terminal tail of the enzyme (possibility for C-Terminal). Sequence numbering done by pBR322 convention (Tabka et al. 2012).

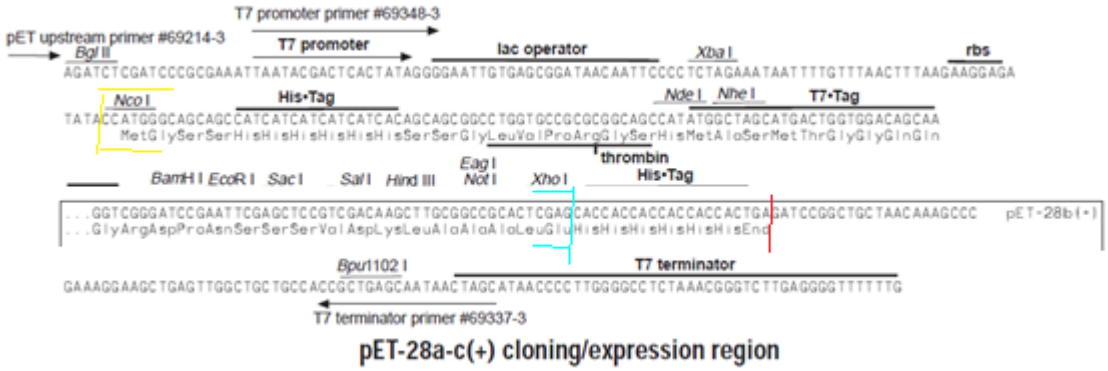

Figure 15: The pET28b plasmid cloning/expression region and the restriction sites that are used to introduce the MAL gene sequence into the pET28b plasmid. The part between the yellow (NcoI) and teal (XhoI) line is digested and replaced with the MAL gene sequence. The red line shows where the stop codon of the protein sequence is (Tabka et al. 2012).

4.2. Site-directed Mutagenesis

A site directed mutagenesis method was carried out on the pET28b-MAL plasmid construct in order to introduce the mutations suggested by the computational experiments in the MAL sequence. The mutagenesis was done by designing two primers containing a mutation to be inserted in the gene sequence and carrying out a mutagenic PCR. After the PCR, the newly formed plasmids were transformed in *E.coli.*

4.2.1. Primer design

The primers that were used in the mutagenic PCR where specially designed to have an alteration in their sequence compared with the MAL gene sequence in a way that an amino acid is changed by other in the enzyme. To design the primers the following guidelines were used (QuikChange Site-Directed mutagenesis kit, Strategene):

- Both the forward and reverse complementary primer need to have the desired mutation.
- Primers should be between 25 and 45 bases long with a melting temperature (Tm) of ≥78 °C.
- $T_m = 81{,}5 + 0{,}41.(\%GC) - \left({}^{675}/_{N}\right) - \%mismatch$
  - N = length in bases of the primer
  - %GC = amount of GC bp / N
  - % mismatch = amount of nucleotide changed / N
- The mutation should be in the middle of the primer, circa 10-15 bases around the mutation
- A minimum of 40 % GC should be acquired in the primer

All the primers were designed followed these rules (Table 1). They were synthetized by Eurofins genomics (Ebersberg, Germany). The primers were diluted with milli-Q water to a concentration of 2 µg/µl and stored at – 20 °C. 50 ng/µl dilutions were made for their use in the mutagenic PCR reactions.

Table 1: Primers used in the site directed mutagenesis protocol. The highlighted parts show the triplet that codify for the amino acid that was changed. The underlined and bold letters are those that replaced the original nucleotides. (Appendix A) * The name is constructed like: Citrobacter amalonaticus, Forward or Reverse Complementary, Original Amino Acid, place in sequence, New Amino Acid.

Primer	Name*	sequence	N	% GC	Tm (°C)
C361A (forward)	Ca_F_C361A*	5'- GCC.TAC.CAG.GGC.GGT.ACC.**GCT**.AAC.GAA.ACC.GAA.ATC.AGC.GCC -3'	42	26	86
C361A (Reverse)	Ca_Rc_C361A	3'- GGC.GCT.GAT.TTC.GGT.TTC.GTT.**AGC**.GGT.ACC.GCC.CTG.GTA.GGC -5'			
C361S (forward)	Ca_F_C361S	5'-GCC.TAC.CAG.GGC.GGT.ACC.**TCT**.AAC.GAA.ACC.GAA.ATC.AGC.GCC-3'	42	25	88
C361S (reverse)	Ca_Rc_C361S	3'-GGC GCT GAT TTC GGT TTC GTT **AGA** GGT ACC GCC CTG GTA GGC -5'			
M389A (forward)	Ca_F_M389A	5'- CTG.ATC.AAG.CCG.GGC.**GC**G.GGC.TTC.GAT.GAA.GGT.CTG -3'	36	23	83
M389A (reverse)	Ca_Rc_M389A	3'- CAG.ACC.TTC.ATC.GAA.GCC.C**GC**.GCC.CGG.CTT.GAT.CAG -5'			
T360A (forward)	Ca_F_T360A	5'- GCC.TAC.CAG.GGC.GGT.**GCC**.TGT.AAC.GAA.ACC.GAA.ATC.AGC.GCC -3'	42	26	89
T360A (reverse)	Ca_Rc_T360A	3'-GGC.GCT.GAT.TTC.GGT.TTC.GTT.ACA.GG**C**.ACC.GCC.CTG.GTA.GGC-5'			
T360S (forward)	Ca_F_T360S	5'- GCC.TAC.CAG.GGC.GGT.**TCC**.TGT.AAC.GAA.ACC.GAA.ATC.AGC.GCC -3'	42	25	88
T360S (reverse)	Ca_Rc_T360S	3'- GGC.GCT.GAT.TTC.GGT.TTC.GTT.ACA.GG**A**.ACC.GCC.CTG.GTA.GGC -5'			
L384A (forward)	Ca_F_L384A	5'- CGT CCG.ATG.CGT.ATG.**GCG**.ATC.AAG.CCG.GGC -3'	30	20	80
L384A (reverse)	Ca_Rc_L384A	3'- GCC.CGG.CTT.GAT.**CGC**.CAT.ACG.CAT.CGG ACG -5'			

4.2.2. Polymerase Chain Reaction

To introduce the mutations in the plasmid construct pET28b-MAL, a Polymerase Chain Reaction (PCR) was done. The principle of a PCR is the replication of DNA. This process (Figure 16: Global overview of a mutagenic PCR (https://www.researchgate.net/figure/256334332_fig1_Schematic-diagram-of-the-PCR-based-site-directed-mutagenesis-procedure-used-to-produce)Figure 16) is composed of different steps: (I), the denaturation of the template DNA from ds DNA to ss DNA; (II), the annealing of the primers to the ss DNA template and (III), the extension step in which the polymerase replicates the complementarity DNA strand. This process is repeated several times to amplify the amount of newly formed ds DNA strands. A mutagenic PCR has one difference, the primers have some different nucleotides than the parental DNA, these nucleotides are the wanted alteration that needed to be implemented.

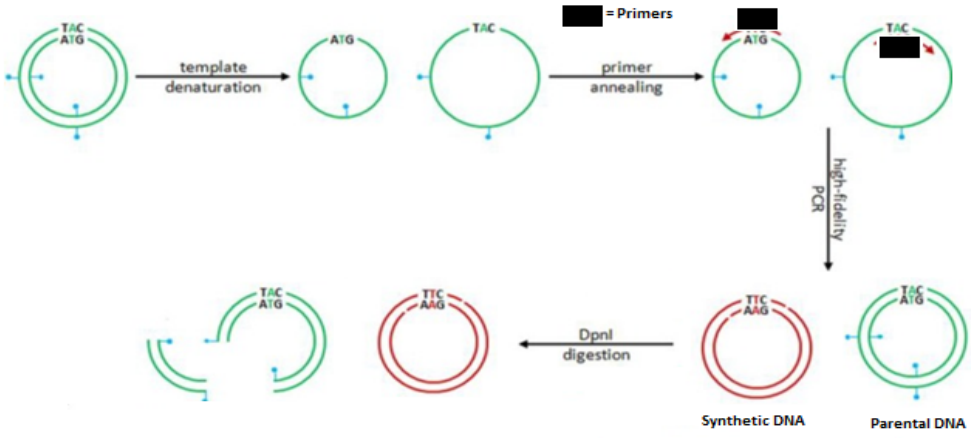

Figure 16: Global overview of a mutagenic PCR (https://www.researchgate.net/figure/256334332_fig1_Schematic-diagram-of-the-PCR-based-site-directed-mutagenesis-procedure-used-to-produce)

The site directed mutagenesis was done with a mutagenic PCR protocol to alter the nucleotides with the designed synthetic primers. All the PCR reactions were done with the Eppendorf 5331 Mastercycler gradient, unless it is specifically mentioned it was not. The PCR reaction mixture (Table 2) used 10 ng of template DNA, 125 ng of both forward and reverse complementary primers, 250 µM of each dNTPs, 2,5 U of Phusion DNA polymerase (Thermo Scientific) and the Phusion HF buffer. The PCR was build up as followed: (I) hot start denaturation of the plasmid DNA (98 °C, 1 min); (II) eighteen cycles that start with a second denaturation (98 °C,20 s) step, followed by an annealing step (52-68 °C, 30-50 s) in which the synthetic primers are bound to the denatured plasmid DNA, followed by the elongation step (72 °C, 6 min) in which the Phusion HF DNA polymerase is activated to extend the DNA chain; and (III) an extra elongation step (72 °C, 10 min). Then the PCR mixture was cooled down to 4 °C.

Now that the newly mutated plasmid was obtained, the parental non-mutated plasmid needed to be destroyed. This was done with the restriction enzyme DpnI (Figure 16), which digests DNA that is methylated, specifically at the N6-methyladenine site. This enzyme will not cut the newly formed plasmids because it was produced synthetically and it is not methylated. Methylation of DNA happens in the cell by an enzyme called DNA methyltransferase, so only the parental DNA produced in *E.coli* will be restricted. When the enzyme was added (1 µl), it was incubated for 1 h at 37 °C.

Table 2: PCR reaction mixture

Solutions used	Amount
Milli-Q water	31,25 µl
HF buffer 5X	10 µl
Plasmid DNA stock (10 ng/µl)	2 µl
Forward primer stock (50 ng/µl)	2,5 µl
Reverse primer stock (50 ng/µl)	2,5 µl
Phusion HF polymerase	0,5 µl (2,5 U)
dNTP's stock (10 mM)	1,25 µl

4.3. Agarose gel electrophoresis

To assess that the PCR was successful and that the plasmids were amplified, a gel electrophoresis was done on the Mupid-EXu Submarine electrophoresis system. The method is based on size exclusion. The agarose matrix pores will allow that smaller DNA fragments are less withheld than larger DNA fragments, while a positive charged anode pulls the negatively charged DNA fragments through the matrix. The pores will allow the smaller DNA fragments to migrate faster through the matrix and the different DNA fragments were separated. The Generuler 1kb DNA ladder (Thermo Scientific) was run together with the samples. A ladder is a mix which has different sized DNA fragments of known size that were used as a reference for the used samples. The range of this ladder is 250 bp to 10 kbp (Appendix B). When the matrix is prepared GelRed™nucleic aicd stain solution (1X) is added. The GelRed dye helped with the detection of the different DNA bands in the matrix. When GelRed bound to the DNA in the agarose matrix It fluoresced and this could be measured with the Biorad GelDoc ™ EZ imager which provided a graphical picture which showed if the plasmids were formed during the PCR (Figure 17).

The protocol was executed as followed: (I) an 0,8 % agarose gel was made with GelRed ™ nucleic acid stain solution (X1, Biotium Inc), (II) the samples (2 µl of the PCR product) were mixed with loading dye (4 µl, Thermo Scientific) and loaded in the wells of the 0,8 % agarose gel, (III) a ladder (5 µl, Thermo Scientific) was run as a reference, (IV) an electric filed is applied to the gel, 100 volts for 30 min. The gel was imaged by the Biorad GelDoc ™ EZ imager (Figure 17).

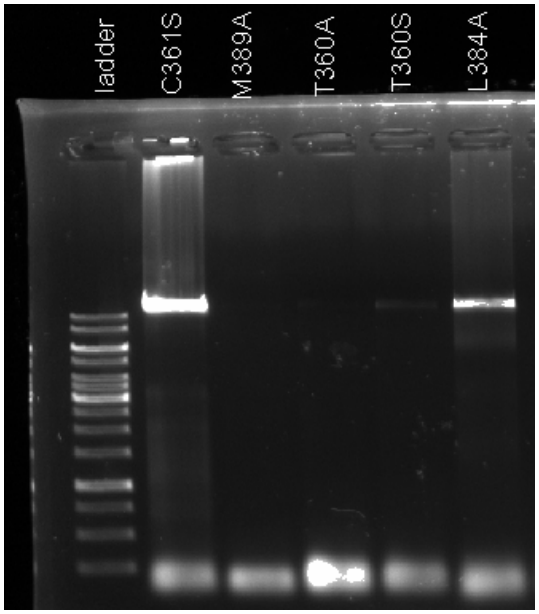

Figure 17: Example of an 0,8% agarose electrophoresis gel with plasmid samples (PCR products).

4.4. Transformation of *E. coli*

The transformation of the newly formed plasmid was done into two different *E.coli* strains. The first is the transformation to *E.coli* DH5α which was used for the storage and propagation of the plasmid. The second is the transformation to *E.coli* BL21 (DE3) which was used for protein expression. The plasmid will be transferred in the competent cell with a heat shock protocol. This protocol is based on the observation that certain microorganism can ingest foreign DNA from their environment. This heat shock protocol used a sudden raise of temperature to form pores in the plasma membrane of the host cells which let the plasmid DNA migrate inside. After the heat shock the cells are put on ice to close the pores and restore the plasma membrane. The protocol was executed as followed: (I) the plasmid (the PCR product of 10 µl or ~100 µg/ml sample) was added to 100 µl of competent cells and incubated on ice for 20 min, (II) the cells are heated at 42 °C for 1,5 min; (III) the cells are cooled down on ice for 2 min, (IV) 800 µl of LB media is added and the mixture is incubated for 1 h at 37 °C and (V) cells are centrifuged and plated on a LB with kanamycin agar (Appendix C).

4.5. Plasmid propagation and purification

In case that the transformation was successful, *E.coli* DH5α colonies grew on the plate. Some of the colonies were selected to grow in Lysogenic Broth (LB) media with kanamycin to get enough biomass of bacterial culture to isolate the plasmid DNA. The protocol was as followed: in a 50 ml falcon, 5 ml of LB media, 5 µl of kanamycin (50 ng/µl) and one ticked colony of the plate was added. The culture was grown overnight at 37 °C.

The Gene Jet Plasmid MiniPrep Kit (Thermo Scientific) was used to extract the plasmid from the grown cultures. The protocol was as followed: (I) The bacteria cultures were spun down at 4000 rpm at 4 °C for 5 min in the Beckman Coulter Allegra 25R centrifuge, (II) the supernatant was discarded and the pellet was resuspended in 250 µl of the kit resuspension buffer and transferred to a 1,5 ml Eppendorf tube, (III) 250 µl of lysis solution was added to break open the cells, (IV) 350 µl of neutralization solution was added to stop the lysis, (V) the tube was centrifuged for 5 min at 14000 rpm in the Eppendorf 5417R centrifuge, (VI) the supernatant containing the plasmid is transferred to the Gene Jet spin column (no cell debris should be taken) and centrifuged for 1 min at 13000 rpm to bind the plasmid DNA to the membrane, (VII) the flow-through is discarded and 500 µl of washing buffer is added to the column and centrifuged 1 min at 13000 rpm, (VIII) 50 µl of the kit elution buffer is added and the column incubated for 2 min at room temperature, (XI) to eluted the plasmid DNA the column is centrifuged for 2 min at 13000 rpm. To determine that the isolation of plasmid DNA was successful, an 0,8% agarose electrophoresis gel was run and spectrophotometric measurement with the Nanodrop spectrophotometer was done.

4.6. Nanodrop spectrophotometer for DNA and protein quantification

The NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used to quantify the concentrations of the plasmids and expressed proteins. The general idea is that with a spectrophotometrically measurements the concentration can be calculated with the help of the law of Lambert-Beer (Figure 18). The formula explains that a nucleic acid concentration can be calculated if the absorbance measurement is done (at 260 nm for nucleic acids) when the path length and the extinction coefficient is known. The absorbance of the measurements should be between 0,25 – 2 A to be a representable result. The protocol for plasmid quantification was done as followed: (I) choosing the nucleic acid protocol (which reads the absorbance at 260 nm) for ds DNA, (II) setting up the blank with the buffer in which the plasmid DNA is stored, (III) pipetting an aliquot (1 µl) of the sample on the measuring lens and measure the spectrum from 220 – 340 nm, (IV) drying the lens with a dust-free wipe and check the measurements obtained. Then the nucleic acid concentration was calculated with Law of Lambert-Beer (Figure 18).

$$c = \frac{(A * \varepsilon)}{b}$$

Figure 18: The law of Lambert-Beer (c = nucleic acid concentration in ng/µl, A = absorbance in UA at 260 nm, ε= extinction coefficient in ng.cm/µl and b = path length in cm).

The protein protocol is a little different and was as followed: (I) choosing the method for measurement of proteins A280 and fill the required parameters (molecular weight of the MAL enzyme, 90924 g/moles, and extinction coefficient, 74720 l/moles), (II) setting up the blank with milli-Q water, (III) pipetting an aliquot (1 µl) of the sample on the measuring lens, measuring the absorbance at 280 nm, (IV) drying the lens with a dust-free wipe and check the results. The Law of Beer (Figure 19) was used to calculate the protein concentration.

$$C = \left(\frac{A}{(\varepsilon * b)}\right) * MW$$

Figure 19: The law of Beer (c = protein concentration in g/l or µg/µl, A = absorbance in A, ε= extinction coefficient in l/moles.cm, b = path length in cm, MW = proteins molecular weight in g/moles).

4.7. Plasmid sequencing

The purified plasmid was send off for sequencing by Eurofins Genomics (Ebersberg, Germany). The sample was sequenced with a T7-promotor and T7-terminator primer since the plasmid construct pET28-MAL contain the T7 promoter and T7 terminator regions. When the sequencing results were available, a sequence alignment with the program Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was done. The native MAL sequence was used as the reference to check if the desired mutations were introduced in the gene and no other mutations occurred.

4.8. Protein expression and purification

4.8.1. Protein expression

If the results of the plasmid sequencing were positive and only the wanted mutation was implemented in the MAL gene, the transformation to the host cell *E.coli* BL21 (DE3) was done for protein expression. The transformation to *E.coli* BL21 (DE3) was done with the same protocol as for the *E.coli* DH5α transformation. First, a preculture was grown to gain a sustainable amount of microorganism before starting the protein expression. From the transformed *E.coli* BL21 (DE3) plate four colonies were selected and added to a 50-ml Falcon tube with 5 ml of LB media with kanamycin (50 ng/ml). The preculture was incubated 6-8 h at 37 °C.

The protein expression was achieved by growing the cells in LB/kanamycin media supplemented with the auto-induction solution. The auto-induction media ensured that the protein expression by *E.coli* BL21 (DE3) happened. It contained glucose, glycerol, lactose and needed salts (Appendix D). The expression of the protein is based on the lactose operon (*lac* operon). When the cultures are inoculated the microorganisms prefer to consume glucose first, the moment the glucose is depleted, the lactose is consumed and will induce the *lac* operon to stop repressing the promotor for protein expression (Figure 20).

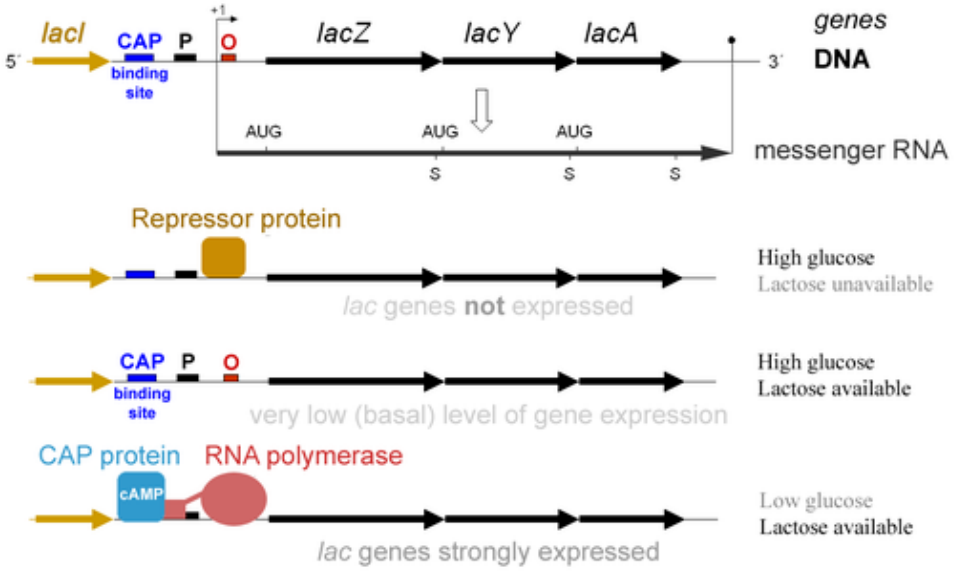

Figure 20: Scheme of how the *Lac* Operon works with different mixture of glucose and lactose.

The cultures were grown overnight at 30 °C and at 200 rpm in the KS 501 Digital IKA®-WERKE shake incubator. After that the cultures had grown, the microorganisms were separated from the LB media through centrifugation. The cultures were centrifuged at 4000 rpm, 4 °C for 5 min in the Beckman Coulter Allegra 25R centrifuge. The pellet was frozen at – 20 °C to help the lysis of the cells.

The next step involved the sonication of the cells which breaks the cell walls and frees the expressed proteins. The Branson Digital Sonifier Models 250 and 450 with an 1/8 inch tip adequate for volumes between 1-10 ml was used to break open the cells and gain the lysate. The protocol was as followed: (I) Around 10 ml of lysis buffer (50 mM phosphate buffer pH 8, 300 mM NaCl and 10 mM imidazole) was added to the pellet, (II) the pellet was dissolved in the buffer, (III) the sonication was done at 30 % amplitude for intervals of 1 min for 5 times, (IV) the lysate was centrifuged and the supernatant filtered with a 0,45 µm cellulose filter. This gained filtrate containing the expressed protein was used in the purification methods.

4.8.2. SDS-PAGE

Before the purification protocol was carried out, the presence of the expressed protein in the lysate was checked through a Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The test was done with the Biorad PowerPac ™ basic. The method uses sodium dodecyl sulphate that is a detergent and which is mixed with the protein samples. The mixture is heated and the proteins will be denatured by the Sodium Dodecyl Sulphate molecule that coated the denatured proteins and turned them is linear negatively charged polypeptide chains. The added DTT disrupted the disulphide bonds and boosted the denaturation of the protein. The protein mix is then loaded on a polyacrylamide gel and an electrical field is set. The polypeptide chains will migrate towards the anode and are separated by their molecular weight. The 3-metylaspartate ammonia lyase monomer has a molecular weight of circa 45 kDa.

The protocol was as followed: (I) Check the protein sample if it is clear or if precipitation is present, (II) the protein sample (23 µl) was mixed with 4X Sample Buffer (7,5 µl), (III) the samples were denatured in a heating block at 95 °C for 10 min, (IV) the samples were centrifuged for 5min at 12000rpm in order to pellet any cell debris, (V) the samples (5, 7, 15 µl) and the Precision Plus Protein™ Unstained Standards ladder (5 µl with a range of 10 to 250 kDa, Appendix E) were loaded onto the gel, (VI) the gel was run at 300 volts for 30-40 min, (VII) the gel was imaged by the Biorad GelDoc ^{TM} EZ imager.

4.8.3. Protein purification

The purification of the protein was done with two different methods. They were tried and tested to investigate which is the most effective and efficient way to purify the protein. Both methods are based on an immobilized metal-ion affinity chromatography (IMAC). The principle of the immobilized metal-ion affinity chromatography is based on the fact that the protein to be purified is expressed with a 6X His-Tag in the C-terminal tail when produced by the *E.coli* BL21 (DE3). The Histidine-tag has a high selective affinity for Ni^{2+} that is immobilized in the column matrix. Consequently, the proteins containing a His-Tag are selectively bound to the matrix of the column when loaded. A step with washing buffer (50 mM phosphate pH8, 20 mM imidazole, 300 mM NaCl) ensures that the rest of unwanted proteins that are non-specifically bounded the column are washed off. The protein is eluted from the column with an elution buffer (50 mM phosphate pH8, 500 mM imidazole, 300 mM NaCl). This buffer contains a high concentration of imidazole. Imidazole has a similar molecular structure than histidine so, it competes against the MAL protein and the column released the MAL that is eluted.

4.8.4. Ni-NTA Spin Column protein purification

The first purification method used a Ni-NTA spin column from Qiagen. The spin columns bound the wanted proteins that have a Histidine-Tag with a maximum capacity of 300 µg/ml, and let the other unwanted proteins and intracellular material flow-through the column. The protocol suggested by the manufacturer was optimized for maximum recovery of the MAL enzymes. Instead of washing with only 20 mM imidazole, a second washing step with higher concentration of imidazole in the buffer (200 mM) was added to clear the nonspecific bounded proteins and have a higher purity of the MAL protein. The protocol was done using the Eppendorf 5417R centrifuge and was as followed: I) The spin column was loaded with 600 µl of lysis buffer (50 mM phosphate buffer pH 8, 300 mM NaCl and 10 mM imidazole) and centrifuged at 2900 rpm for 2 min to calibrate the column, II) the filtered lysate was loaded and centrifuged at 1600 rpm for 5 min, III) the previous step was repeated until all the lysate was centrifuged, IV) 600 µl of washing buffer (50 mM phosphate pH8, 30 mM imidazole, 300 mM NaCl) was loaded and centrifuged at 2900 rpm for 2 min, V) previous step was repeated, VI) 600 µl of the second washing buffer (50 mM phosphate pH8, 200 mM imidazole, 300 mM NaCl) was loaded and centrifuged at 2900 rpm for 2 min, VII) 300 µl of elution buffer (50 mM phosphate pH8, 500 mM imidazole, 300 mM NaCl) was loaded and centrifuged at 2900 rpm for 2 min to release the protein of the column.

4.8.5. ÄKTA purifier protein purification

The second purification method used a Histrap™ Excel column in an ÄKTA purifier system. The Histrap™ Excel column is packed with a Ni^{2+} sepharose matrix that bounded the Histidine-tagged proteins. The protocol was as followed: (I) all the used solutions were vacuum filtered so, no solids or air bubbles could get stuck in the lines of the ÄKTA purifier, (II) the ÄKTA system was washed with milli-Q water for 15 ml, (III) the column was inserted, (IV) the column was cleaned with milli-Q water for 10 ml in order to remove the ethanol solution (20%) in which the column is stored, (V) a blank run was carried out in order to remove any lingering proteins or contaminants in the column, (VI) the protein was loaded onto the column and washed with buffer A (50 mM phosphate pH8, 20 mM imidazole, 300 mM NaCl), (VI) a gradient (Figure 21) that started with 100 % of buffer A (50 mM phosphate pH8, 20 mM imidazole, 300 mM NaCl) and gradually switched to 100 % of Buffer B (50mM phosphate pH8, 500 mM imidazole, 300mM NaCl) was set up to elute the protein and collect the different fractions, (VII) the elution of proteins was followed through the monitoring of the absorbance at 280nm.

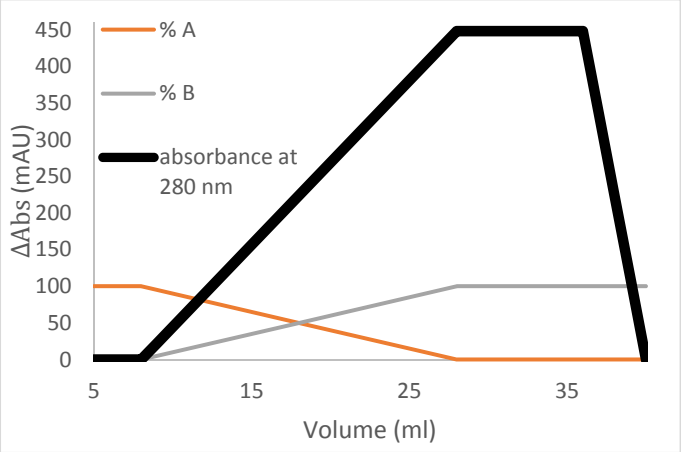

Figure 21: Blank run of the ÄKTA with the gradient of % A and % B.

4.8.6. Protein concentration

The purified protein samples were checked in an SDS-PAGE to verify if the protein was pure enough and not lost in the purification process. The Amicon Ultra 15 centrifugal filters were used for concentrating the proteins and exchanging the buffer. This step was done with the intention of removing the imidazole and salts from the previous buffers and store the enzymes in a suitable buffer. The Amicon Ultra 15 has a membrane which withheld all molecules that have a higher molecular weight than 10 kDa. The protocol was executed in the Beckman Coulter Allegra 25R at 3900 rpm and at 4°C as followed: (I) loading the column with milli-Q water and centrifuge for 5 min in order to wash the membrane, (II) discarding the liquid that went through the membrane, loading the sample containing the purified protein and centrifuging for 15-20 min, (III) repeating until all the protein sample has been loaded, (IV) rinsing the column with 9 ml of storing buffer (50 mM phosphate buffer, 2 mM MgCl2 and 0,1 mM KCl) and centrifuging for 20 min or until less than 2 ml is left, (V) repeat the previous step, (VI) pipette carefully the retained liquid out of the falcon in a 1,5-ml tube. The final mixture was quantified in the Nano drop spectrophotometer define the protein concentration and then the mixture was frozen in the – 80 °C freezer.

4.9. Activity assays

4.9.1. Measurement of the activity of MAL enzyme on 3-methyl aspartate, L-Lysine and β-Lysine

Once the protein was purified and concentrated, activity assays were carried out in order to determine the activity and the kinetic constants of the native and mutated MAL enzymes on the 3-methylaspartate, and the new substrates L-lysine and β-lysine. In one of the assays, the focus lay on the detection of the product 6-aminohex-2-enoic acid and other put the focus on detecting the product ammonia. These should be the two products of the theoretical conversion of Lysine with the MAL enzymes (Figure 22). All the activity assays were spectrophotometrically measured with the FluoStar Omega plate reader. The absorbance was measured at specific wavelengths to determine if an increment or decrease in absorbance can be detected in the mixture during the reactions. The reactions were done in triplicates to calculate the standard deviation for the determined results. The received absorbance measurements were used together with the law of Lambert-Beer (Figure 18) to calculated the different kinetic activities.

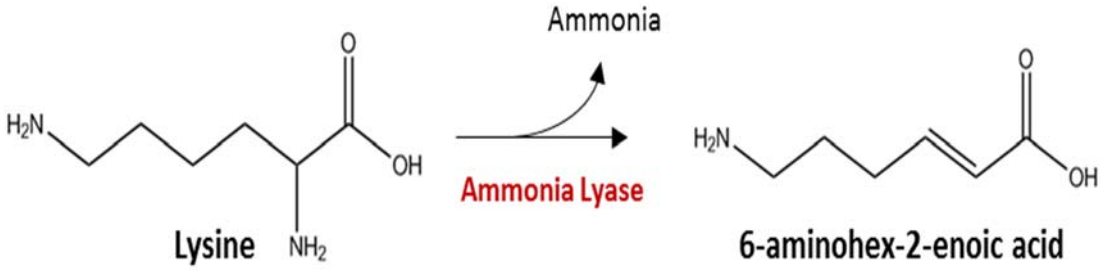

Figure 22:The theoretical conversion of lysine by an ammonia lyase enzyme

4.9.2. Activity assay on 3 methyl-aspartate: detection of mesaconate

The activity of the MAL enzyme and mutant variants on the natural substrate 3-methylaspartate was measured spectrophotometrically. This assay was used to determine the kinetic constants of the native enzyme and if the kinetic constants of the variants were changed due to the mutations that were introduced. The assay measured the production of mesaconate at 240 nm. The mesaconate has a higher absorbance at 240 nm than 3-methylaspartate, so when it is formed an increment in the absorbance should be detected. The assay was done at 30 °C with the following conditions: 500 mM Tris-HCl pH 9.2, 20 mM M_gCl_2, 1 mM KCl, paired with different concentration of 3-methyl aspartate (0,5-20 mM) and enzyme concentration (5-10 µg/ml) (Botting et al. 1988) (Appendix F).

4.9.3. Activity on lysine: detection of 6-aminohex-2-eonic acid

The assay was based on the theoretical knowledge that when lysine is converted by the MAL enzyme, 6-aminohex-2-eonic acid (6-AHEA) is formed. The absorbance of 6-AHEA is higher than that of lysine. This implies that the moment that the MAL enzyme converts lysine, an increase in the absorbance should be measured. The absorbance was measured at 230 and 240 nm. The initial absorbance of the reaction mixture at 230 nm was higher than at 240 nm due to the higher absorbance of lysine at the former wavelength. Because of this, the 240 nm measurement was used in the calculations. The conditions of the assay were the same as the 3-methylaspartate assay. The assay was done at 30 °C with the following conditions:500 mM Tris-HCl pH 9.2, 20 mM M_gCl_2, 1 mM KCl). paired with different concentrations of L-lysine or β-lysine (10-30 mM) and enzyme concentrations (5 - 10 µg/ml) (Botting et al. 1988).

4.9.4. Activity on lysine: detection of ammonium

The last assay determined spectrophotometrically the amount of ammonium produced during the conversion of lysine or 3-methyl aspartate. The Ammonia Assay Kit (Sigma-Aldrich) was used for the determination of ammonia in the samples. The fundamentals of this test (Figure 23) is that the ammonium reacts with α-ketoglutaric acid and reduces nicotinamide adenine dinucleotide phosphate (NADPH) when L-glutamate dehydrogenase is present. The oxidation of the NADPH will give a decrease in absorbance at 340 nm. This is directly proportional with the amount of ammonium in the mixture. Ammonium concentrations between 0,2-15 µg/ml can be measured with this kit (Appendix G).

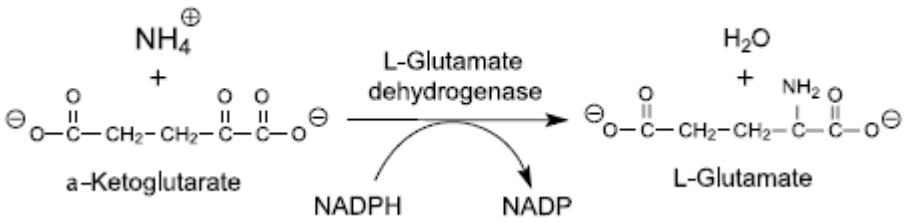

Figure 23: The coupled reaction used for the detection of ammonia.

5. Results

5.1. Mutagenesis of the MAL gene.

The mutagenic PCR as explained in the methods and materials section of the thesis (4.2. mutagenesis) had a similar build up for the generation of all the mutations (Table 3). Still, for all the different reactions that were done, the right annealing temperature for the primers needed to be optimized. If the primers do not bind to the denatured plasmid DNA, the DNA will not be replicated and no mutated plasmids will be formed.

Table 3: Overview of the mutagenic PCR protocol. Z and Y are the parameters that were optimized in the different PCR

Cycles	Process	Temperature (°C)	Time
X1	Denaturation I	98	1 min
X18	Denaturation II	98	20 sec
	Annealing	Z	Y sec
	Elongation I	72	6 min
X1	Elongation II	72	10 min
X1	Cooling down	4 to 10	30 min
X1	DpnI digestion	37	60 min

A first mutagenic PCR was done as a test to assure that the protocol and the concentration of primers, polymerase, dNTP mix and plasmid used, were adequate for the replication of the plasmid DNA. The experiment was done with an annealing temperature of 68 °C for 30 seconds using the forward and reverse primers for the mutation of Cysteine 361 to Alanine (C361A). The mutagenic PCR (Figure 24 A) was done in duplicates to procure more amplified plasmid in case the yield of the reaction was low.

The gel electrophoresis after the first experiment (Figure 24 A) displayed two intense bands which indicates that the plasmid had been amplified. This meant that the protocol and the concentration of the different components of the PCR mixture were appropriate. The band of the plasmid mixtures was around 10 kbp, which is higher than the expected 7 kbp. This could be due to a different DNA conformation that makes the plasmid migrate slower than normal. In the next experiment, mutagenic PCR to obtain the remaining plasmids with the desired mutations were carried out with an annealing temperature of 72 °C for 30 seconds (Figure 24 B**Error! Reference source not found.**).

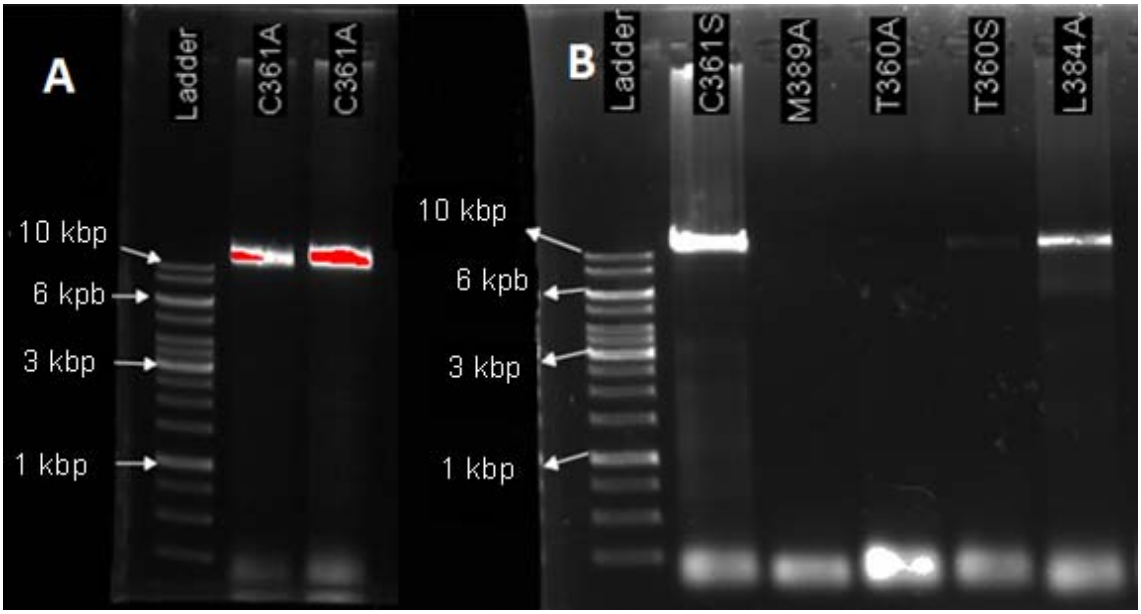

Figure 24: Gel electrophoresis of the PCR products obtained in the first mutagenic PCR with the intended mutation of C361A (A) and B: Gel electrophoresis of the mutagenic PCR with the remaining mutations (C361S, M389A, T360A, T360S and L384A).

The second experiment revealed (Figure 24 B) that the bands for C361S and L384A are clearly visible on the gel which meant that the amplification of the plasmid was successful. The PCR of M389A, T360A and T360S display no or very faint bands, which indicated the amplification was not executed optimally. After that these mutagenic PCR failed, the primers were checked through a spectrophotometric measurement with the NanoDrop to make sure that the concentration of the primers were adequate (~ 50 ng/µl). The PCR to introduce the mutations M389A, T360A and T360S in the MAL gene were done again in the following experiment (Figure 25 A) were the PCR were optimized by changing the annealing temperature to 64 °C and the annealing time to 50 seconds.

The gel (Figure 25 A) reveals that the plasmids that should contain the mutations M389A and T360A were amplified in a high quantity. The measurement of the bands on the gel were saturated, this means the maximum amount to be measured with the detection method was exceeded. So, lowering the annealing temperature to 64 °C for 50 seconds was a good strategy for obtaining amplification with these two mutations. However, the amplification of the plasmid with the T360S mutation was not successful. The annealing temperature for this reaction was already set to 72 and 64 °C. So, to determine the optimal annealing temperature, the next mutagenic PCR (Figure 25 B) was run applying range of various annealing temperatures from 58 °C to 70 °C for 40 seconds. The PCR was executed in the Biorad MyCycler™ Thermal cycler. This thermal cycler can produce different annealing temperatures at the same time in a multi-annealing temperature experiment.

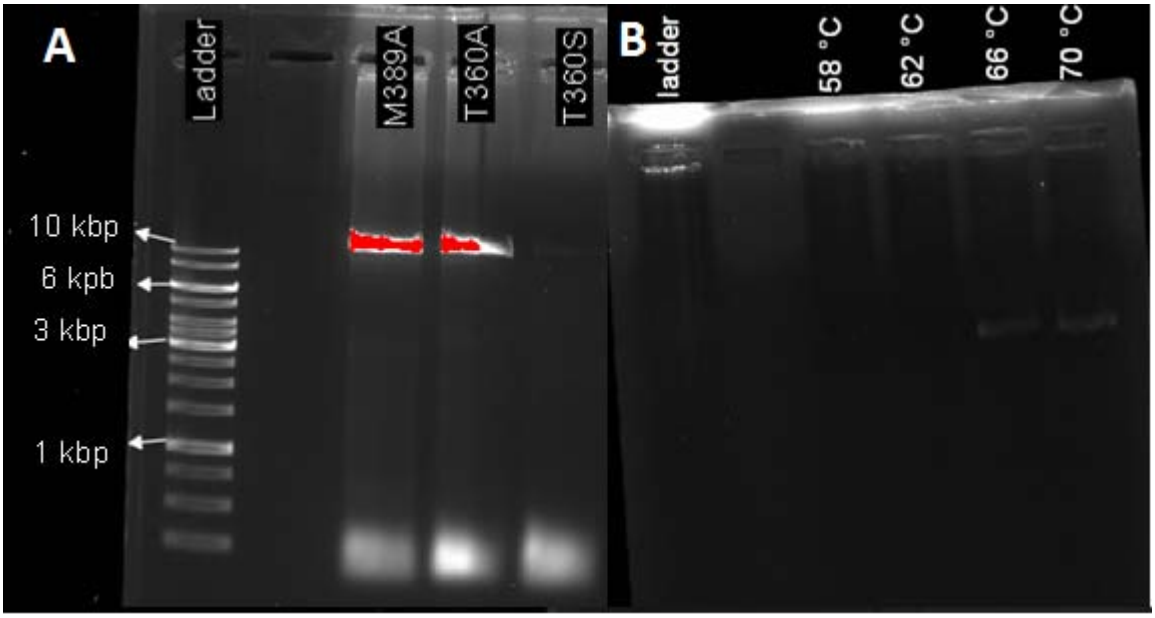

Figure 25: A: Gel electrophoresis of the mutagenic PCR for the mutations M389A, T360A and T360S. B: Gel electrophoresis of the mutagenic PCR with the T360S mutation with various annealing temperatures (58, 62, 66 and 70 °C).

The gel made for checking the multi-annealing mutagenic PCR (Figure 25 B) showed faint bands for the reactions in which annealing temperatures of 66 and 70 °C were used. The new ladder did not run properly during the gel electrophoresis experiment. The reactions which used lower temperatures did not render bands. It can be concluded that these annealing temperatures were not optimum and that the optimal annealing temperature for this reaction should be around 70 °C. The various annealing temperature that were used reveal that this factor is not the cause for the low amplification.

It was decided to prepare new stock solutions: new primer solutions were made to 50 ng/µl, new milli-Q water was sterilized and a new plasmid stock was used. Figure 24 B displayed a faint band of the T360S mutation with the annealing conditions of 72 °C for 30 seconds. The conditions were repeated, but done in quadruplets to get enough plasmid material (Figure 26 A). The gel that checked this mutagenic PCR showed that with these conditions the expected faint bands were present. The three successful PCR mixtures were put together and used for the transformation protocol.

Due to the low yield obtained in the amplification of the plasmid with the T360S mutation, a last experiment was tried in order to optimize the conditions of the PCR. The following PCR used the mutations T360S to gain more plasmid material and the mutation C361S was used as a positive control. The optimal annealing condition for C361S is 72 °C for 30 seconds. This was changed to optimize it for the T360S mutations by prolonging the time to 40 seconds. The gel of the PCR products (Figure 26 B) displayed that the amplifications were successful for the C361S samples and not for the T360S samples. So, the problem for the failure of the T360S mutation probably relates with the primers or the annealing temperature. But, because time management, the samples were anyway used for the transformation into *E. coli* DH5α.

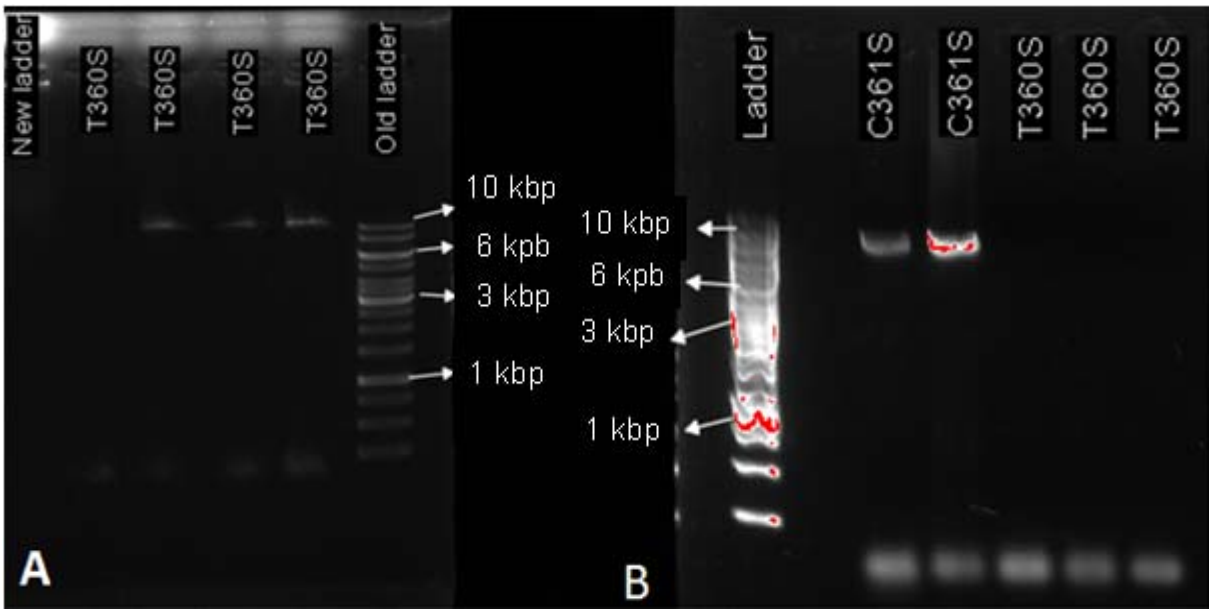

Figure 26:A: Gel electrophoresis of the mutagenic PCR with quadruplets of the T360S mutation. B: Gel electrophoresis of the mutagenic PCR with triplets of the T360S mutation and the mutation C361S is run as a reference.

The transformations to *E.coli* DH5α were done with all the mutated plasmids that were obtained with the mutagenic PCR experiments. In the case of the plasmid with the T360S mutation, all the different acquired PCR mixtures were combined into one and used for the transformation. This transformation was successful even if the quantity of plasmid was low. All the transformation plates showed signs of growth (Figure 27).

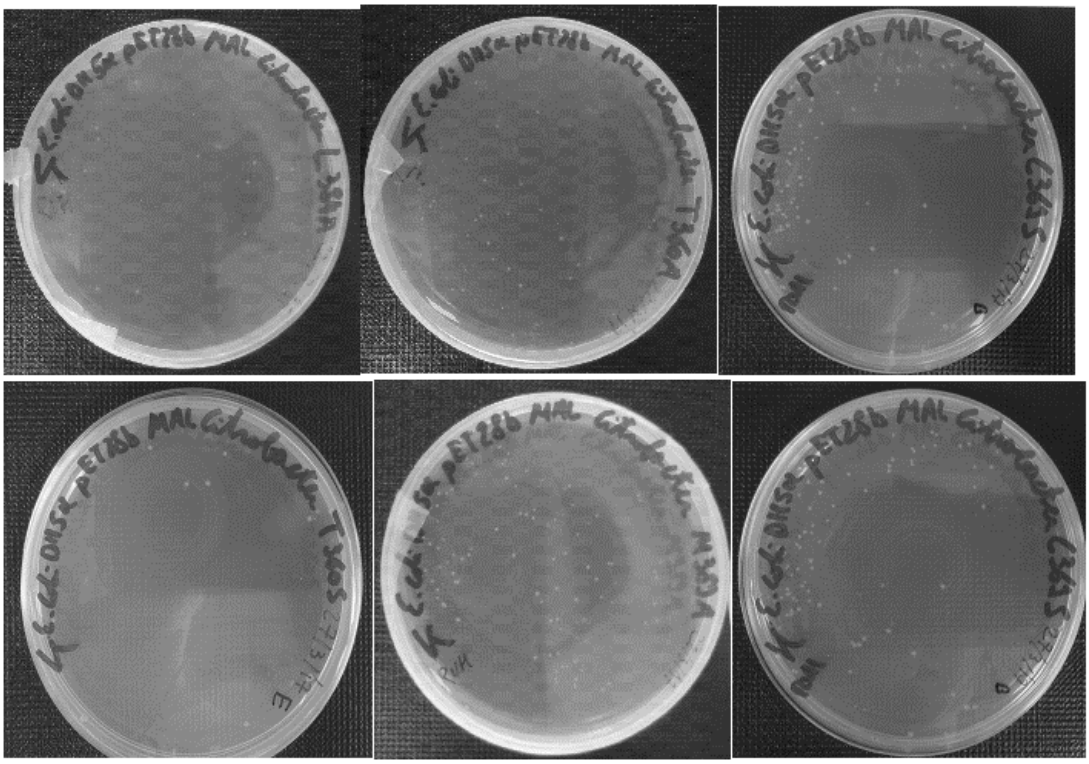

Figure 27: Picture of the transformed *E.coli* DH5α plates in which isolated colonies can be seen. Left top to right down: *E.coli* transformed with the plasmids construct pET28-MAL containing the following mutations, L384A, T360A, C361S, T360S, M389A and C361A.

From the transformed *E.coli* DH5α plates isolated colonies were selected and grown in LB media supplemented with kanamycin to increase the amount of biomass before the plasmid purification. The Gene Jet Plasmid Miniprep Kit (Thermo Scientific) was used to extract the plasmid from the grown cultures and a gel electrophoresis experiment was done to determine if the plasmids were still present after the purification (Figure 28).

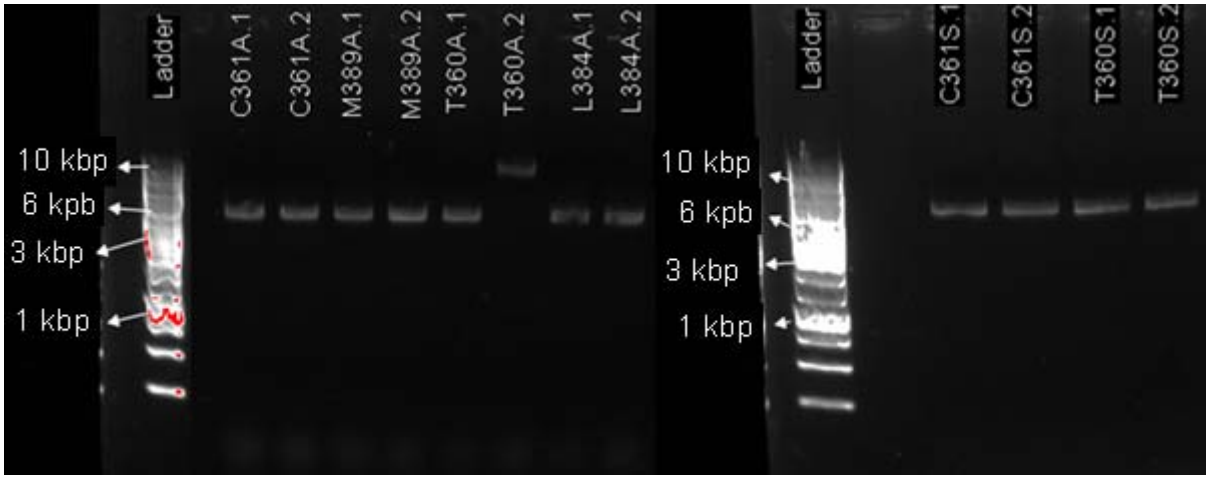

Figure 28: Gel electrophoresis of the purified plasmid samples after the Gene Jet Miniprep. The 1 or 2 behind the mutation indicates which colony was selected of the *E.coli* DH5α plate.

The gel made to check the plasmid purification displayed that all the plasmids were present in the samples. All the bands of the samples were around 7 kbp of length, which is the expected size. Only the sample corresponding to the plasmid with the T360A mutation isolated from the colony 2 displayed a higher size (Figure 28, T360A.2 sample). It showed a size of around 10 kbp, this could indicate that there are extra nucleotides inserted in the sequence of the mutated plasmid or a different topology of the plasmid. The samples were prepared for sequencing (Eurofins Geonomics). After the sequencing results were available, they were aligned using the Clustal mega program. This created an alignment with the mutated MAL sequences and the original MAL sequence to display if the desired mutation was introduced in the gene and if other unwanted mutations happened.

The alignments revealed that the mutations C361A, C361S, T360A, T360S and L384A were successfully implemented (Figure 29). But the sequence alignment of the plasmid from some of the colonies had unwanted mutations. These samples were discarded and only the plasmid with the desired mutations and no others were used for the transformation into *E.coli* BL21 (DE3).

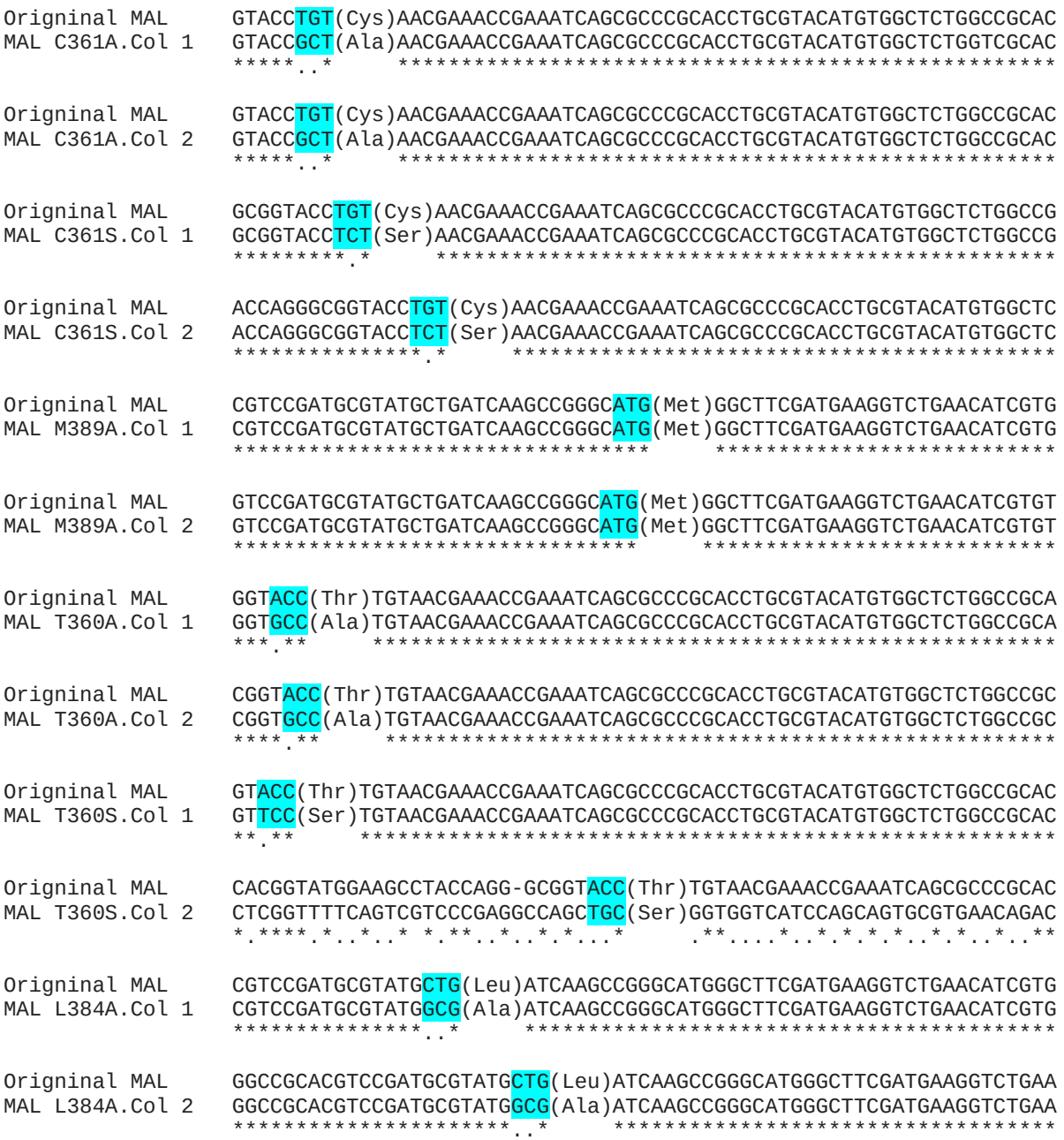

Figure 29: Detail of the alignments of the sequencing results of the mutated MAL sequences with the original MAL sequence in which the introduced mutations are shown. Teal = the codon of the wanted mutation. The name is build up as followed: MAL, original amino acid, location amino acid, substitution amino acid, colony 1 or 2. (The full sequence alignment is found in Appendix H).

The M389A mutation entailed to change the methionine codon ATG to the alanine codon GCG. The mutation however, was not implemented in the plasmids obtained from both colonies. In this case, the PCR for introducing the M389A mutation was carried out again. The mutagenic PCR was executed with the same conditions, with an annealing temperature of 64 °C for 30 seconds (Figure 30 A). The results of this mutagenic PCR were contradictory. The test was done in duplicates and only one sample did the amplification. So, another PCR was done and new primer stocks were made for better amplification because the acquired amount of plasmid was probably too low to do a transformation. The annealing temperature was again 64 °C, but the annealing time was prolonged to 45 seconds to make sure that the primers had enough time to bind to the denatured plasmid DNA. The test was done in quadruplets to assure that enough plasmid was procured (Figure 30 B). The results were the same as the previous test and in total not enough plasmid was amplified. For the next PCR, a multi-annealing PCR was done to see if 64 °C was really the optimal annealing temperature. The various annealing temperatures were 55, 60 and 64 °C and each temperature was done in triplicates in the Biorad MyCycler™ Thermal cycler (Figure 30 C). The results of this PCR were not that conclusive. All the annealing temperatures did not create the plasmid in a great quantity. The amplification was low, but with all the different samples of M389A, the transformation could be done. All the replicated PCR mixtures were put together and were desalted with the Gene Jet PCR purification kit (Thermo Scientific). The desalting was done because the salts originated from the used HF buffer can affect the transformation negatively. The concentrated sample was then transformed to *E.coli* DH5α (Figure 31).

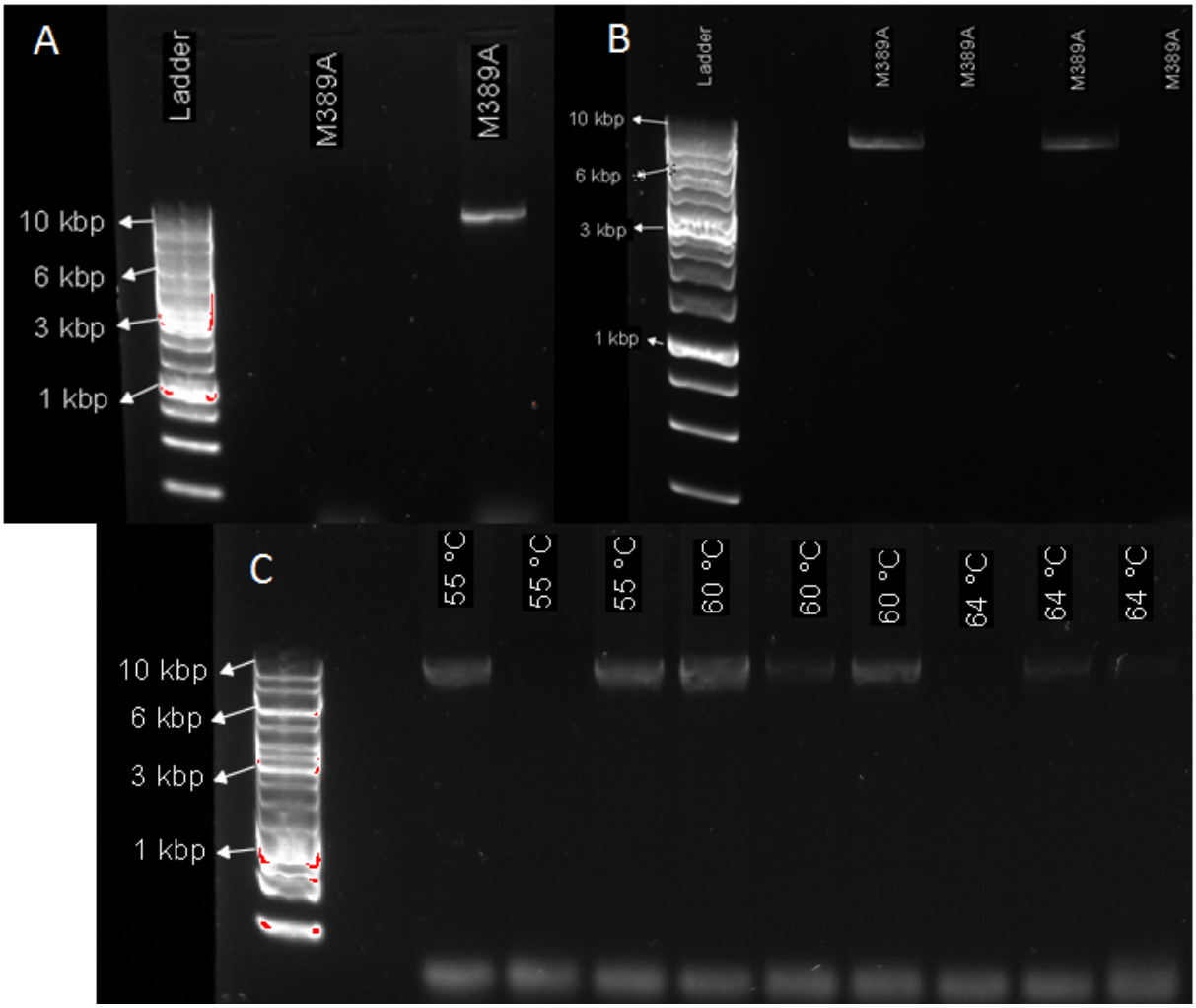

Figure 30: The gel electrophoresis experiment for the different PCR test do to acquire the M389A mutation.

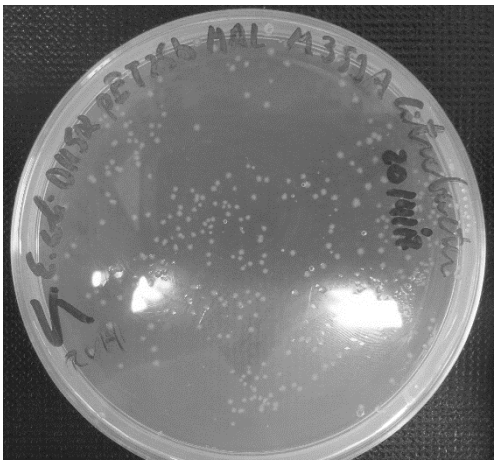

Figure 31: LB agar plate supplemented with kanamycin with the transformed *E.coli* DH5α colonies containing the plasmid with the mutation M389A in the MAL gene.

The plasmid purification of a newly formed *E.coli* DH5α colony was executed and confirmed in an agarose gel where a band of 7 kbp was visible. Samples were send off for sequencing (Eurofins Genomics). The alignment of the obtained sequencing results revealed that the mutation was implemented in the plasmid DNA and no unwanted mutation occurred (Figure 32).

```
Orginal MAL     GTCCGATGCGTATGCTGATCAAGCCGGGCATG(Met)GGCTTCGATGAAGGTCTGAACATCGTGT
MAL M389A.Col 3 GTCCGATGCGTATGCTGATCAAGCCGGGCGCG(Ala)GGCTTCGATGAAGGTCTGAACATCGTGT
                ****************************  *     ****************************
```

Figure 32: The sequencing results of the plamid construct pET28-MAL containing the M389A mutation (Full alignment in Appendix H).

All the mutated plasmids were sequenced and the alignment revealed only the wanted mutation were implement. So, the plasmids were transformed to *E.coli* BL21 (DE3), the *E.coli* strain used for protein expression (Figure 33).

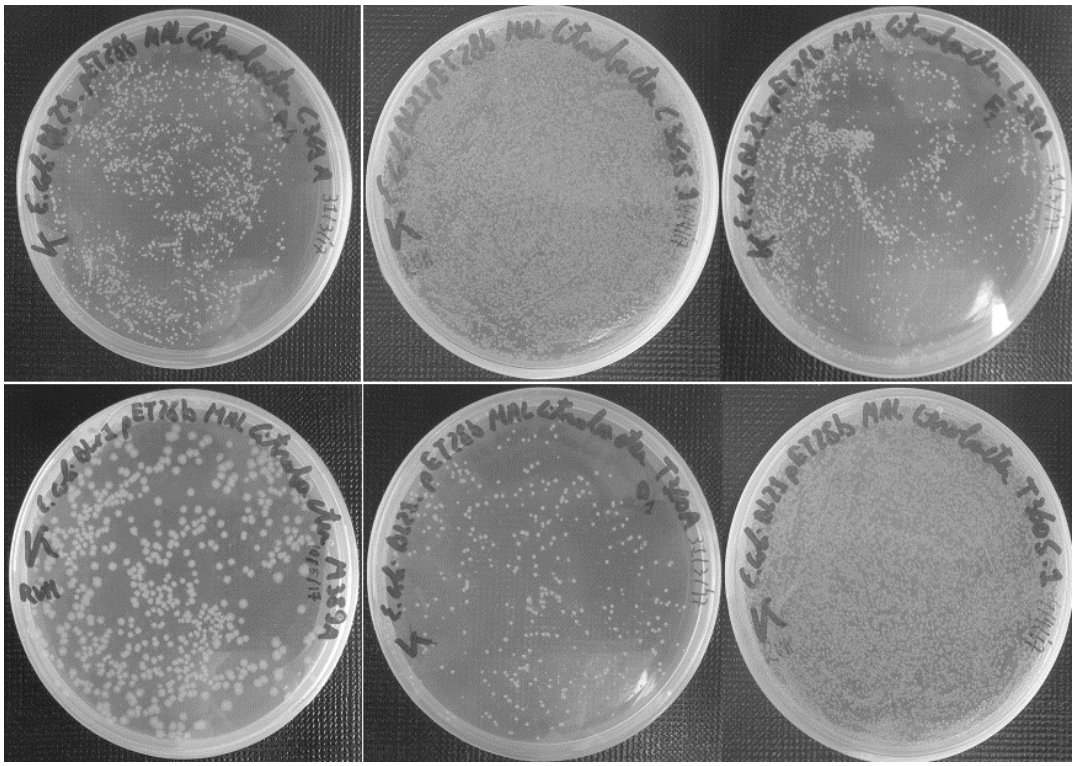

Figure 33: Transformed *E.coli* BL21 (DE3) plates. Left top to right down: *E.coli* transformed with the plasmids construct pET28-MAL containing the following mutations C361A, C361S, L384A, M389A, T360A and T360S.

5.2. Protein expression

The protein was expressed in *E.coli* BL21 (DE3) growing in LB media enriched with kanamycin and auto-induction media as explained in section 4.8.1. When the cultures had grown and the protein had been expressed, the protein was released out of the microorganism through sonication and centrifuged to separate the cell debris from the lysate. The presence of the expressed protein was checked in both the insoluble fraction (cell pellet) and the soluble fraction (lysate). If the protein was present in the cell pellet, it was an indication that it was expressed as an inclusion body. The presence of all the MAL enzyme and the MAL mutant variants in the lysate (intense band of ~ 45kDa) verified that the protein was expressed in a soluble way which is a good indication that the different proteins are well folded and active (Figure 34). The filtered lysate was then used in the protein purification.

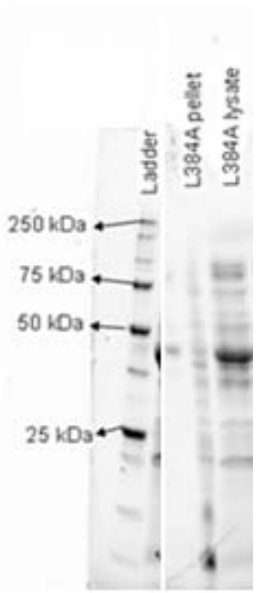

Figure 34: SDS-PAGE to inspect if the MAL L384A mutant variant was expressed. When the expression of the rest of the variants was checked similar results were obtained.

5.3. Protein purification

As explained, two purification methods were executed during the master thesis. The first method involved the use of the ÄKTA purifier system and the second method the Ni-NTA spin columns. To determine if the proteins were purified, SDS-PAGE gels were run to display the results.

5.3.1. ÄKTA purifier system

The ÄKTA purifier system has a built-in detector which monitors the absorbance of the stream at 280 nm. The spectrophotometer allows to detect when the protein samples are being eluted and in which fractions. But it does not verify if a certain chromatographic peak is the wanted protein, it only detects a protein.

The chromatograms obtained during the purification processes of the different variants (Figure 35) are composed of a black line that symbolizes the absorbance at 280 nm (mAU) that is measured and two grey lines that are the outer boundaries for the fractions that were collected with the eluted proteins. If we study the blank run (in which just buffer is present, not protein sample is loaded) (Figure 35 F), it shows a linear increment in the absorbance that correlates with the increasing imidazole concentration in the buffer. A comparison of the chromatograms of the proteins that were purified against the blank run displayed an extra peak (between 7-21 ml of the volume of the run) detected at the middle of the slope of the black line. The peak corresponded to a protein that was detected with the spectrophotometer. The height of this peak correlated with the quantity of protein eluted. After the protein was eluted, an SDS-PAGE was done on the selected fractions that showed proteins. The SDS-PAGE checked the size and the purity of the protein. The results from the SDS-PAGE and activity assays confirmed the identity of the MAL variants.

All the MAL variants were purified and detected during the ÄKTA run (Figure 35). The SDS-PAGE gels (Figure 36 and Figure 37) showed that all the variants had the right size of ~45 kDa because the bands were around the 50 kDa marker of the ladder and the purity was of a high degree because no contaminations were observed. However, not every purification was exactly the same.

The MAL variant C361A and C361S showed a similar visible peak in the ÄKTA run (Figure 35 A and B) and clear bands on the gels were detected (Figure 36). The detection of the MAL variant T360A and L384A with the ÄKTA was not very clear. There was only a small hump noticeable on the graph (Figure 35 C and E). This also translated in fainter bands that were displayed on the SDS-PAGE gel (Figure 37). The MAL variant T360S detection with the ÄTKA was very clear (Figure 35 D). The peak was the highest of all the proteins and together with the saturations on the SDS-PAGE gel (Figure 37), it indicated that the protein concentration was higher than the other proteins.

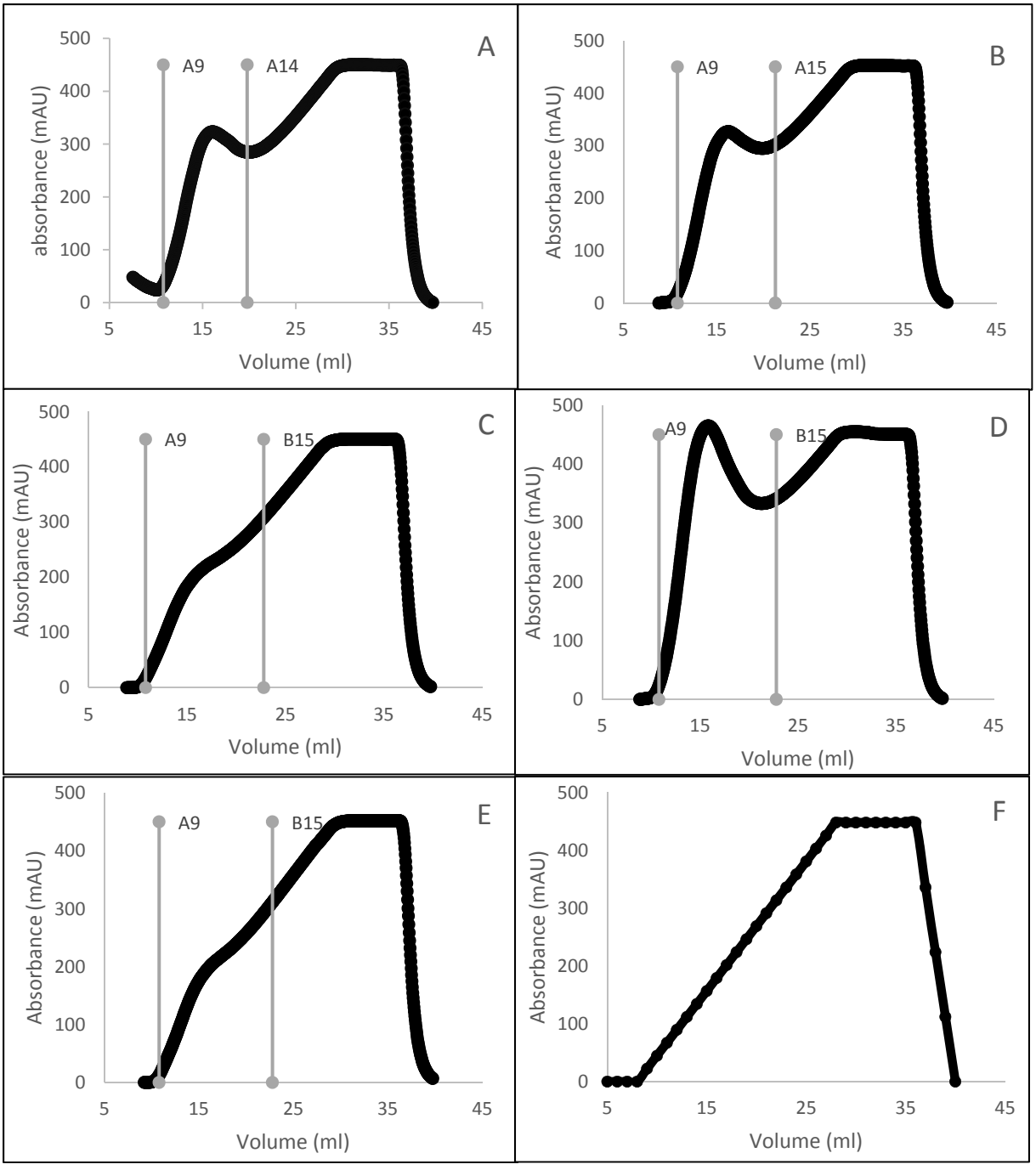

Figure 35: The chromatograms of the purification of the MALs variants through IMAC. A: MAL C361A, B: MAL C361S, C: MAL T360A, D: MAL T360S, E: MAL L384A and F: blank run. The space between the vertical grey lines represent the protein fractions that were selected.

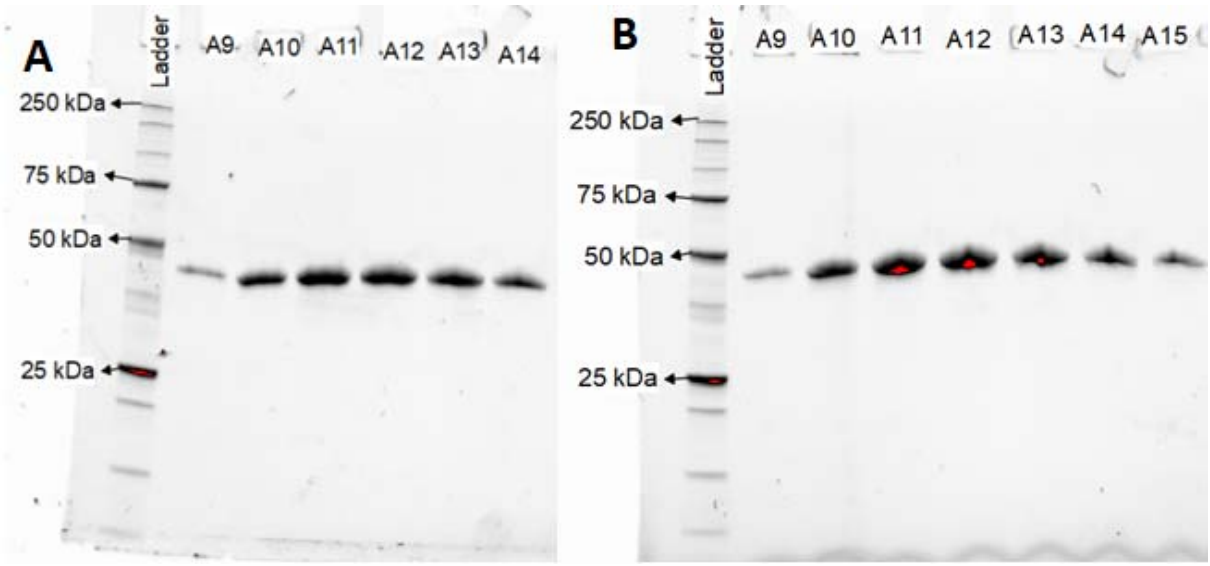

Figure 36: A: SDS-PAGE of fractions A9-A14 from the ÄKTA purification of MAL variant C361A. B: SDS-PAGE of fractions A9-A15 from the ÄKTA purification of MAL variant C361S

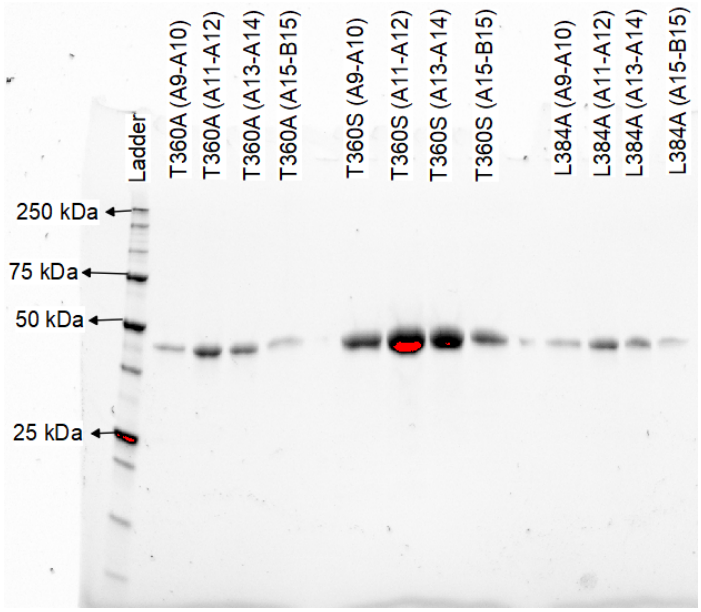

Figure 37: SDS-PAGE of the different samples obtained from the IMAC experiment for the protein purification of MAL variants: T360A, T360S and L384A. The different labels A9 to B15 represent the different fractions that were checked.

5.3.2. Ni-NTA spin columns

The purification of the different variants was also done with the Ni-NTA spin columns (Qiagen) as an alternative way of purification. The aim was to compare the performace of both methods of putification. After the purification, an SDS-PAGE was done to check if the proteins were pure. The SDS-PAGE was build up with various samples from the purification process. The first sample was the pellet which corresponded with the insoluble fraction of the culture were the protein was expressed. The second was the lysate that corresponded with the soluble fraction of the culture. The third was the flow through that did not bind to the column when loading the lysate. The next was the various washing steps and the last was the elution step to acquire the protein.

During the spin column tests, different concentrations of imidazole were used in the washing buffer to optimize the retrieving of a purer protein sample that had less contaminations and a higher yield.

The first experiment used the spin columns and a washing buffer of 20 mM imidazole (Figure 38 A). The figure shows that the matrix in the column did not bind all the expressed protein that is in the lysate since the flow through lanes showed an intense band around 45kDa that corresponds to the protein with the His-Tag. Probably the matrix in the column was saturated. The wash step with 20 mM imidazole did not do a great elimination of unwanted proteins. The lanes corresponding to the washing steps displays no or a few faint bands, which indicates that the 20 mM imidazole concentration is to low and there are still impurities present in the protein lanes (Figure 38 B).

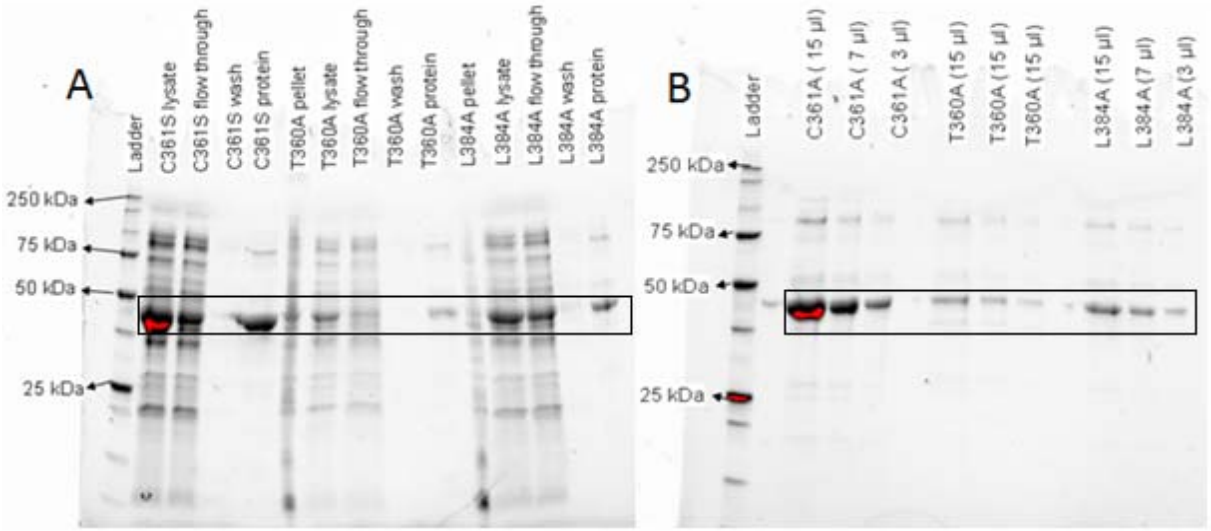

Figure 38: A: SDS-PAGE of the different fractions obtained during the purification with the spin columns using washing buffer of 20 mM imidazole. B: SDS-PAGE of the purified proteins (in different loading volumes) obtained. The black box is the desired protein that should be purified.

The next round of experiments raised the imidazole concentration of the washing buffer to 30 mM (Figure 39 A). The result of the SDS-PAGE gel was similar than in the previous purification experiment. The protein of the lysate did not bind completely to the column (as can be seen in the flow through lines) and the washing step did not remove all impurities of the sample. But the washing lane now indicates faint bands. So, the increase in the imidazole concentration did help to remove certain impurities and get a purer protein. In the following experiment, a higher concentration of imidazole in the washing buffer (40 mM) was used (Figure 39 B). The protein lanes looked even purer than the previous experiment, but there was still some contamination. Especially, a band of around 100 kDa can be observed in the gel. The proteins with lower molecular weight were washed from the matrix column by the 40 mM imidazole washing buffer.

In the last experiment that was done with the spin columns, an array of buffers with different concentration of imidazole was used to display if the protein could be purified to a greater extend (Figure 40). The purification was better with higher imidazole concentrations. The difference between the various washing steps was clearly visible, each higher concentration of imidazole removed more impurities away. But this also involved that with higher imidazole concentrations a part of the target protein was eluted before the elution step. This will mean that a choice needsto be made: a higher amount of protein or a very pure sample.

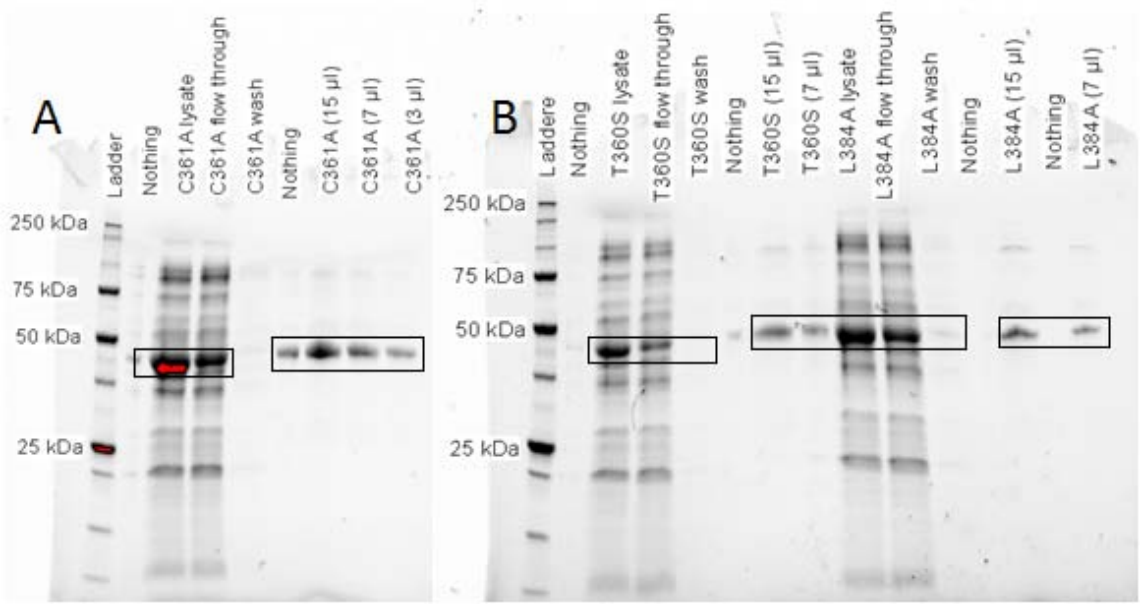

Figure 39: SDS-PAGE of the different fractions obtained during the purification with the spin columns using washing buffer with A, 30 mM imidazole and B, 40mM of imidazole. The black box is the desired protein that should be purified.

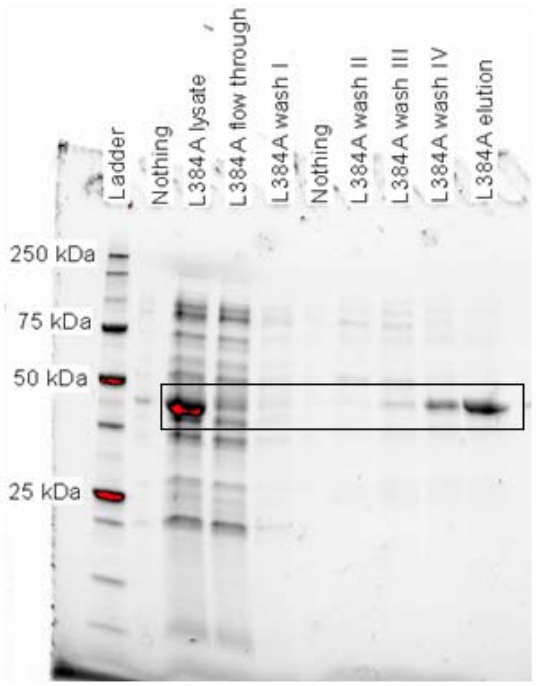

Figure 40: SDS- PAGE of the different fractions obtained during the purification with the spin columns using using array of washing buffers with different concentration of imidazole: I) 20 mM, II) 100 mM, III) 200 mM, IV) 300 mM. The black box is the desired protein that should be purified.

5.4. Measurement of the activity of the mutated and native MAL enzymes on 3-methylaspartic acid, L-lysine and β-lysine

5.4.1. Comparison of activity of the various enzymes on 3-methyl aspartate

The first test to evaluate the activity of the enzymes was an activity assay on 3-methylaspartic acid. The test determined how and if the native and the mutated enzymes were active using 3-methyalaspartic acid (3MA)as a substrate. Upon MAL activity, 3-methylaspartic acid is converted to mesaconate, that is thenmonitored as increase of absorbance at 240 nm. The catalytic constants Km and Kcat were calculated for the native MAL and the different mutant variants.

After the experiments, the gained sprectrophotometrical measurements expressed in absorbance were used to calculate the activity of the enzyme (observed constants, k_{obs}, expressed in s^{-1}) for each 3MA concentration that was used. The increment of absorbance per minute was first converted to U/l using the extinction coefficient (74720 l/mol). This was divided by the amount of enzyme used in the experiment gained the activity of the enzyme expressed in U/mg. Finally, using the molecular weight (90924 g/moll), the activity was expressed in s^{-1}. The different s^{-1} of each 3MA concentration then can be fitted with the concentrations of 3MA using the Michaelis-Menten equation that will render the constants kcat (1/s) and Km (mM) (Figure 41).

The graph of the kinetics of the different enzymes shows that all the mutations had a detrimental effect on the activity. The kinetic constants for the native enzyme: Km equal to 0,7606 mM and Kcat equal to 0,0251 s-1 are higher than does of the mutated enzymes (Table 4). The enzyme MAL T360S has the least decrease in activity and is still comparable to the native enzyme. The enzymes C361S and C361A both have their activity impaired, the Kcat value is around 3 times lower than the native enzyme. The last mutation L384A has its activity impaired with a major amount. The Kcat is 140 times lower than the native enzyme and the activity of the enzyme is almost completely reduced to zero. The Kcat/Km or the specificity constant of the native enzyme is 2 mM^{-1}. s^{-1}. The analysis of the data in Table 4 displays that all the mutated enzymes except T360S have a reduced specificity constant. Which means that the enzyme will need more substrate to have the same conversion rate as the native enzyme. Only T360S has a higher Kcat/Km which could indicate that the activity is similar or higher than the native enzyme.

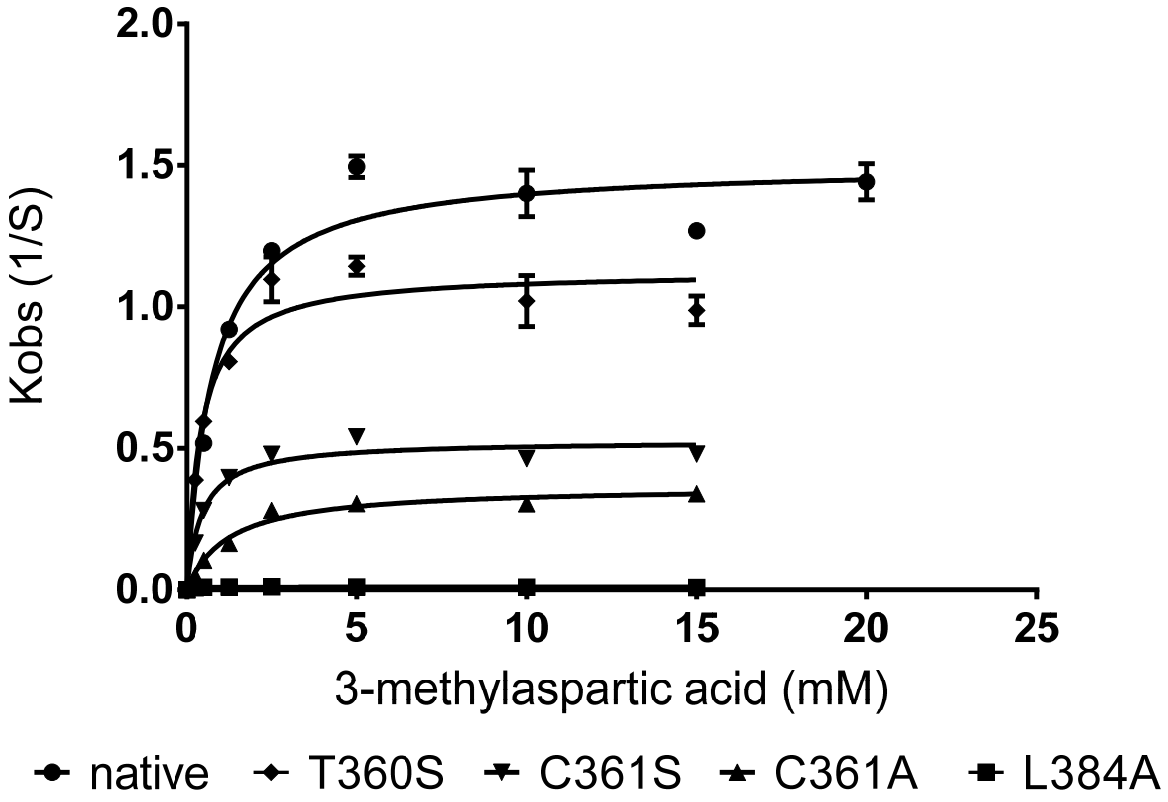

Figure 41: Graph of the kobs (s^{-1}) in function of the substrate concentration with standard deviation bars. (If the standard deviation bar is not visible, it will be smaller than the marker).

Table 4: The kinetic constants from the mutated enzymes compared against the native enzyme.

Mutation done	Kcat (S^{-1})		Km (mM)		Kcat/Km ($mM^{-1}. S^{-1}$)
	Value	St. Error	Value	St. Error	
Native	1,505	0,072	0,761	0,191	2,0
C361A	0,367	0,018	1,231	0,234	0,3
C361S	0,527	0,023	0,436	0,096	1,2
T360S	1,126	0,057	0,419	0,107	2,7
L384A	0,011	0,0005	0,114	0,034	0,1

5.4.2. Activity assessment on lysine: detection of 6-aminohex-2-enoic acid (6-AHEA)

The determination of the activity of the enzymes towards lysine was done spectrophotometrically monitoring the increase of absorbance and 240 nm due to 6-aminohex-2-enoic acid formation. Two substrates of lysine were considered in this thesis: L-lysine and β-lysine. β-lysine could be more suitable for the activity because the amino group is in the same location that 3-methylaspartic acid. A set of reactions were drafted to test if the enzymes were active towards the new substrates using a reaction with the native substrate 3-methylaspartic acid as a positive control. Negative controls were tested to ensure if there was an increase in absorbance not due to the enzyme activity.

In the graphs (Figure 42) the different reactions are visible. The first reaction is called “L-lysine + MAL enzyme”, this reaction contained 30 mM of L-Lysine together with 10 µg/ml enzyme. The negative control for this reaction was “L-lysine”, which was done in the same conditions that the previous one but without enzyme. A similar approach was used for the reactions “β-Lysine + Mal enzyme” and the “β-Lysine” reaction, instead now the L-Lysine is replaced with β-Lysine. The “no substrate” reaction is another negative control which contained 10 µg/ml MAL enzyme and no substrate, and the” 3-Methylaspartic acid + MAL” enzyme was build up out of 15 mM 3MA and 10 µg/ml MAL enzyme. It was the positive control.

Figure 42 displays the results of the activity assays with the different MAL variants. A first notice is that all the enzymes still have activity towards 3-methylaspartic acid as proven in the previous tests, even if they are impaired. But there was not an increment over time that could be appointed to the conversion of lysine to 6-AHEA. So, none of the enzymes gained an activity towards lysine that could be detected. The negative controls sometimes do reveal an increment over time, this was surprising and could be attributed to a background reaction that is taking place.

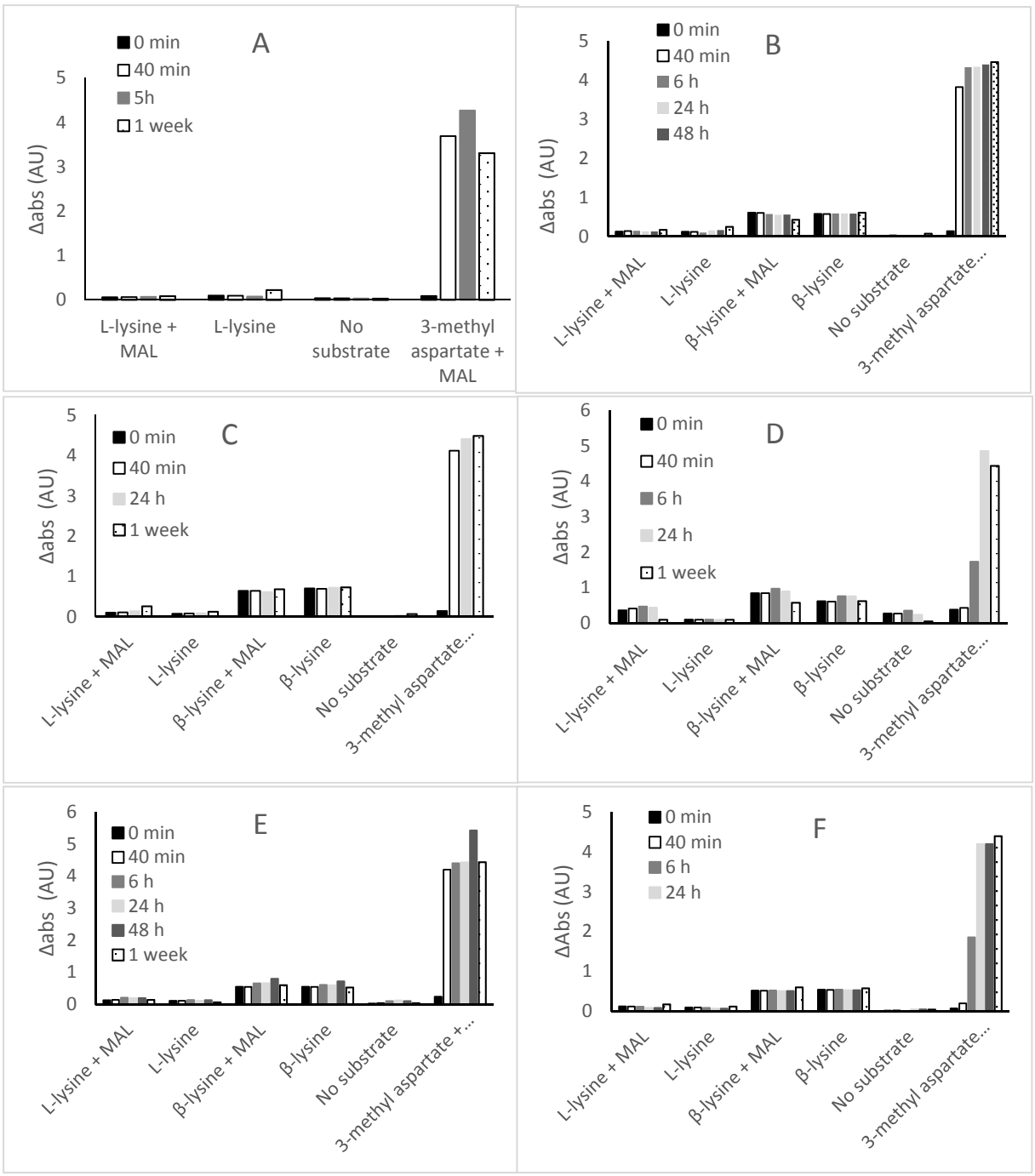

Figure 42: The graphs for the detection of activity on lysine over a set amount of time for the native and the mutated enzymes. A: native, B: C361A, C: C361S, D: T360A, E: T360S and F: L384A.

5.4.3. Activity assessment on lysine: Detection of ammonium

The second activity assay revolving around the detection of activity on lysine was done using the ammonia assay kit (Thermo Scientific). The fundamentals of the assay is based on the knowledge that when lysine is converted by the MAL enzyme ammonia is produced together with 6-AHEA. The ammonia produced can be detected in small amount in the ammonia assay kit (from 0,2 to 15 µg/ml). The experiments carried out used the same reactions tested in the previous section, but now the concentration of the substrates and enzyme were higher.

For all the reactions that were used in this assay, the based conditions were: 500 mM Tris-HCl, pH 9,2; 20 mM $MgCl_2$ and 1 mM KCl. The reactions I or II had 100 mM of L-lysine or β-Lysine as substrate and the positive control had a 3-methylaspartic acid concentration of 15 mM. The negative controls for lysine had the same concentration as the reaction. A third negative control was used to detect if the substrate on its own would give any reading. The different experiments had a few variations like; the used enzyme and the used enzyme concentration (Table 5, Table 6, Table 7, Table 8 and

Table 9).

The results of this activity assay are not conclusive. The major problem with the results is that the measurement for the same reactions differ to vastly from each other (Table 5). This can be seen in certain standard deviations that are too high, to be acceptable. Also, a lot of measurement that are done reveal to be negative or errors which cannot be used for the assessment of the activity (Table 6). The negative controls with lysine often show a higher ammonia quantity then the actual reaction I and II do (Table 7). This is not plausible because no reaction should occur and no ammonia should be present. As last the measurements of the negative controls with only enzyme and no substrate fluctuate extremely. The measurements go from 2 till 11 µg/ml ammonia, when again no ammonia should be formed (Table 5 and

Table 9).

Table 5: Comparison of the amount of ammonia produced during 3h, 24h, 4 days and 1 week of activity with the C361A enzyme measured with the Ammonia Assay Kit. "- "= error. (The enzyme concentration = 100 µg/ml):

samples	µg NH3/ml 3 h	µg NH3/ml 24 h	µg NH3/ml 4 days	µg NH3/ml 7 days
reaction I (L-Lysine + MAL)	3.7 ± 0.4	1.6 ± 1.7	2.6 ± 0.5	1.7 ± 0.9
reaction II (β-lysine + MAL)	4.1 ± 2	5.4 ± 1.2	5.8 ± 0.2	5.5 ± 2.3
negative control (L-lysine)	7.1 ± 0.6	8.9 ± 3	2.9 ± 1	8.2 ± 1.1
negative control (β-lysine)	5.6 ± 1.8	6.8 ± 0.2	5.4 ± 0.1	15 ± 5
positive control (3MA + MAL)	35.9 ± 2.5	32.5 ± 0.1	32.4 ± 0.2	33.5 ± 0.3
Negative control (MAL)	"-"	12.0	3.2	8.3

Table 6: Comparison of the amount of ammonia produced during 3h, 24h, 4 days and 1 week of activity with the C361S enzyme measured with the Ammonia Assay Kit. "- "= error. (The enzyme concentration = 100 µg/ml):

samples	µg NH3/ml 3 h	µg NH3/ml 24 h	µg NH3/ml 4 days	µg NH3/ml 7 days
reaction I (L-Lysine + MAL)	"-"	2.3 ± 2	"-"	5.7 ± 1.2
reaction II (β-lysine + MAL)	4.1 ± 1.6	7.6 ± 3.7	6.8 ± 1.3	8.4 ± 1.6
negative control (L-lysine)	7.1 ± 0.6	8.9 ± 3	2.9 ± 1	8.2 ± 1.1
negative control (β-lysine)	5.6 ± 1.8	6.8 ± 0.2	5.4 ± 0.1	15 ± 5
positive control (3MA + MAL)	36.9 ± 1.2	34.3 ± 0.9	34.0 ± 0,4	34.0 ± 1,9
Negative control (MAL)	7.2	7.0	10.2	10.4

Table 7: Comparison of the amount of ammonia produced during 3h, 24h, 4 days and 1 week of activity with the T360A enzyme measured with the Ammonia Assay Kit. "- "= error. (The enzyme concentration = 50 µg/ml):

samples	µg NH_3/ml 3 h	µg NH_3/ml 24 h	µg NH_3/ml 4 days	µg NH_3/ml 7 days
reaction I (L-Lysine + MAL)	4.9 ± 2	7.5 ± 0.6	7 ± 1.4	6.4 ± 1.9
reaction II (β-lysine + MAL)	9.7 ± 2.3	6.4 ± 1.5	9.6 ± 0.23	9 ± 1
negative control (L-lysine)	7.1 ± 0.6	8.9 ± 3	2.9 ± 1	8.2 ± 1.1
negative control (β-lysine)	5.6 ± 1.8	6.8 ± 0.2	5.4 ± 0.1	15 ± 5
positive control (3MA + MAL)	28.7 ± 0.9	35.1 ± 1.0	35.0 ± 0.6	37.7 ± 0.7
Negative control (MAL)	7.9	13.1	10.8	11.8

Table 8: Comparison of the amount of ammonia produced during 3h, 24h, 4 days and 1 week of activity with the T360S enzyme measured with the Ammonia Assay Kit. "- "= error. (The enzyme concentration = 100 µg/ml):

samples	µg NH3/ml 3 h	µg NH3/ml 24 h	µg NH3/ml 4 days	µg NH3/ml 7 days
reaction I (L-Lysine + MAL)	4.3 ± 0.9	4.3 ± 2	3.6 ± 1	3.2 ± 0.7
reaction II (β-lysine + MAL)	5 ± 0.75	5.1 ± 0.3	8 ± 1.4	9.8 ± 0.9
negative control (L-lysine)	7.1 ± 0.6	8.9 ± 3	2.9 ± 1	8.2 ± 1.1
negative control (β-lysine)	5.6 ± 1.8	6.8 ± 0.2	5.4 ± 0.1	15 ± 5
positive control (3MA + MAL)	36.1 ± 0.4	35.4 ± 0.2	31.0 ± 1.8	33.4 ± 0.2
Negative control (MAL)	8.9	8.0	10.8	9.5

Table 9: Comparison of the amount of ammonia produced during 3h, 24h, 4 days and 1 week of activity with the L384A enzyme measured with the Ammonia Assay Kit. "- "= error. (The enzyme concentration = 50 µg/ml):

samples	µg NH_3/ml 3 h	µg NH_3/ml 24 h	µg NH_3/ml 4 days	µg NH_3/ml 7 days
reaction I (L-Lysine + MAL)	4.7 ± 0.7	5.3 ± 0.6	6.5 ± 0.5	6.3 ± 0.7
reaction II (β-lysine + MAL)	5.1 ± 1.4	6.7 ± 0.4	7.7 ± 1.4	9.1 ± 0.8
negative control (L-lysine)	7.1 ± 0.6	8.9 ± 3	2.9 ± 1	8.2 ± 1.1
negative control (β-lysine)	5.6 ± 1.8	6.8 ± 0.2	5.4 ± 0.1	15 ± 5
positive control (3MA + MAL)	36 ± 0.1	34.1 ± 0.7	34.9 ± 1.5	35.5 ± 0.4
Negative control (MAL)	5.8	6.7	10.8	6.5

6. Discussion

In this master thesis, the ability of the MAL from *C. amalonaticus* and some mutant variants to catalyse the deamination of L-lysine or β-lysine was investigated through activity assays. First the native enzyme was expressed and purified. Then its activity towards lysine was measured. Because no activity was detected, various mutant variants were produced which had a single amino acid substitutions around the binding pocket.

The selection of the mutations to be introduced in the MAL gene was done based on the preliminary results of computational experiments and on data present in literature (Levy et al. 2002; De Villiers et al. 2012). The selected mutations were all near the binding pocket. The mutations introduced are meant to create more space to fit lysine in the binding pocket while still receiving an active and stable enzyme. The increase in space was needed because lysine has two more carbon molecules compared to 3-methylaspartic acid. This lead to a site directed mutagenesis were certain bulky amino acids were substituted with smaller amino acids. The increased space in the binding pocket could have facilitated an activity towards lysine, without losing the activity on the 3-methylaspartic acid. The selected mutations were: T360A, T360S, C361A, C361A, M389A and L384A.

The activity assays on 3-methylaspartic acid (3MA) with the various enzymes gained notable results. The native kinetic constants (Km = 0,76 mM and Kcat = 1,505 s^{-1}) were compared to the results of the native enzyme in the literature (Km =1 mM. and Kcat = 233 s^{-1}) (Kato, Y. Asona 1995). The Kcat from the article is circa 155 times bigger, but the Km is similar. This means the rate of conversion for our native enzyme is much lower, but the affinity to the substrate is similar. This major difference could come from the differences in the assay conditions (e.g. the temperature used in the assay) or could be due to the presence of the His-Tag in the enzyme. The results from the mutated variants indicated that the activity towards 3MA was lower than our native enzyme. The variant T360S displayed similar kinetic constants as the native enzyme. This means that the substitution of the threonine by a serine does not affect the activity of the enzyme to a great extent. If we look at the chemical structure of threonine and serine it displays that the side chains are very similar. A carboxylic group is converted to a hydroxyl group. The serine could still make a hydrogen bound with the substrate like threonine did (Kato, Y. Asona 1995). The variants C361A and C361S have similar impaired kinetic constants when compared to the native enzyme, but still have an activity towards the substrate. However, the L384A variant had its kinetic constants impaired extremely. The leucine that is substituted for the alanine lead to a decrease in Kcat of circa 140 times and a decrease of Km of circa 7 times. This means that the leucine 384 has a significant role in the enzyme catalysis. It is described that the leucine stabilizes the methyl group of the substrate through Van Der Waals packing interactions (Kato, Y. Asona 1995).

After the kinetic constants towards 3MA were determined, the activity of the mutants on lysine was measured. The activity assays that should monitor the formation of 6-AHEA displayed that there was no activity towards lysine that could be detected. The mutation that were done did increase the binding pocket but did not result in any deamination activity towards lysine. This result is maybe expected, because lysine is still a quite different substrate then 3-methylaspartic acid. Lysine has two more carbons and instead of the carboxylic group at the end of the chain there is an amino group. Also, lysine does not have a methyl group like 3MA. Probably, more than one mutation needs to be executed to obtain activity with the enzyme on lysine. Perhaps a good strategy could be to find an intermediate structure between 3-methylaspartic acid and lysine as a starting point for further experiments.

The aim of the thesis is not achieved, but valuable information is gathered for further experiments. Other strategies can be tried to gain activity on the lysine or an intermediate between lysine and 3MA. This could involve an error prone PCR to randomly mutated an ammonia lyase and check the activity on lysine. This should not be confided to the 3-methylaspartate ammonia lyase, but could be expanded to histidine ammonia lyase and aspartate ammonia lyase.

The purification of the wanted enzyme was done with an immobilized metal-ion affinity chromatography that will bind the His-Tag that is added to the wanted protein and which be eluted by adding high concentrations of imidazole. Two different methods were used that have their individual pros and cons. This will be discussed in the following paragraphs.

The first method involved the use of the ÄKTA purifier system. This method had certain advantages and disadvantages. The main disadvantage was that the purification of one protein takes 3 to 4 hours. The great amount of time is due to the continuous preservation of the ÄKTA system and the used column. Every time the ÄKTA is run the machine needs to be cleaned with water. The column needed to be inserted and be cleaned with water. The flow rate for the ÄKTA system is 1ml/min and cannot be higher due to maintaining an adequate pressure level in the system and not damaging the used purification column or the ÄKTA system. Then a calibration of the column was done and a blank run of the purifying protocol was done to ensure no protein contamination was present. This was done because the column was used for all the different enzymes that were produced. After the purification, the column and the ÄKTA was again cleaned and then stored in 20 % ethanol. Apart from this negative point, the purification results with the ÄKTA were outstanding. All the proteins were purified with no extra contamination to be seen on the SDS-PAGE gels. Even if it takes longer, the purification results are worth the occupation of time.

The protein purification with the Ni-NTA spin columns was the second method that was tried. The columns were easy to use and the protein purification went very fast. In one hour, a protein could be purified. All the different purifications could be done at the same time, which gave the columns a clear advantage against the ÄKTA system. But the main disadvantage of the columns was the low yield and the persistent contamination that was displayed on the SDS-PAGE gels. The contamination can be removed till a very low quantity compared to the amount of wanted protein but not fully and this purification method would also remove a part of the wanted protein which explains the lower yield. Secondly, the maximum amount of protein that could be bound on the column was only 300 µg. The spin columns are a suitable alternative if an ÄKTA purifying system is not available. It still purifies the protein in a high enough quantity to be useful for the thesis.

The optimization of the activity assays had been tried in function of the optimal conditions of the MAL enzyme. During the master thesis, different reaction conditions were tested. In this paragraph, they will be quickly discussed. The pH of the reaction was one of the parameters optimized. The pH optimum for the activity of MAL is 9,7(Kato, Y. Asona 1995; Asano et al. 2004). This pH optimum couldn't be achieved with the Tris-HCl buffer so, a different buffer was needed. The search of the buffer lead to the Glycine-NaOH buffer. But this buffer wasn't suitable because it couldn't be used for the experiments involving the use of the ammonium assay kit. For the measurements using the kit, the pH of the reaction should be between 7 and 8. However, it was not possible to lower the pH of the glycine-NaOH to these values due to the low buffering capacity of the buffer in this range. As an alternative, Tris-HCl buffer was used instead at a pH of 9. The second optimization involved the decrease of the concentration of the Tris-HCl buffer used in the sprectrophotometrical measurements from 500 mM to 100 mM. This was done to lower the background interference that Tris-HCl gave and maybe give a clearer detection of the possible activity on lysine. But when the reaction mixtures with 30 mM β-lysine were made the pH was not 9 anymore but 8,3. β-lysine had a buffering capacity that lowered the pH of the reaction mixture. This would mean that the measurement was done at a different condition than L-lysine and 3MA. So, it was finally decided to increase the amount of Tris-HCl in the reaction mixture to 500 mM. The last adjustment was that the activity assays would be done at 45 °C. The 45°C for the activity assay was contemplated because it is the optimal temperature for the activity of the enzyme following certain articles (Kato, Y. Asona 1995; Asano et al. 2004). This was tested and the enzyme lost his activity after two days. Because the increment that could happen would be very low, long term measurements were needed. So, the high temperature was scrapped and replaced with 30 °C which had less of an activity lowering capability.

The conditions of the activity assay in which the 3MA activity was measured were good. There were no interferences due to the used reaction mixture and solutions. The used pH and temperature were suitable for the activity of the enzyme.

The activity assay on lysine was not yet established in the laboratory. So, two different methods were tried in order to measure the activity on lysine by the various MAL enzymes. The performance and the appropriateness are discussed in the next paragraphs.

The first lysine detection protocol that was used detected the 6-aminohex-2-enoic acid (6-AHEA) that could be produced. The increase in absorbance for 6-AHEA could be detected. If there was any activity towards lysine it would have shown with the detection of the plate reader. The reaction conditions of the samples did not interfere with the measurements. This detection assay was suitable and could be used to detect the activity on lysine in the future. A second lysine detection protocol was used the detect the ammonia that could be formed. This was done by a commercial ammonia assay kit. The kit was a good try to detect the formation of ammonium in the reaction samples. But the results were to differentiated and chaotic to determine if something did happen. The results of the negatives and the actual reaction samples were too close to each other, also with some tests the negatives indicated to have a higher amount of ammonia than the actual reaction samples and standard of the kit. This should be impossible. There for the test was a valid try, but is not an effective way to detect the ammonia that is formed from the theoretical reaction with lysine. The future detection of lysine can be done with the 6-AHEA detection method. But the ammonium assay kit can't be used effectively. Another method that could be used to monitor the activity on lysine is an Ion chromatography.

The mutagenesis of the different mutations was done with a mutagenic PCR. There was only one difference between the different mutagenic PCR. This was the annealing conditions of the various primers. The optimal annealing conditions for the different mutations were sought out to have a successful result. For the mutations C361A, C361S, T360A and L384A an optimal annealing temperature was found and the results were positively replicated in more than one experiment. Only the annealing conditions of M389A and T360S were not that successful. The M389A mutation showed a good PCR an annealing temperature of 64 °C for 50 seconds. But the second experiment didn't gain the same result. Only 50 % of the PCR samples were successful. The rate of success could be contributed to the annealing of the primers to the denatured plasmid DNA. If the annealing is not working well, the replication of the DNA will not be done. The T360S mutation also did not have satisfactory results. Every annealing condition that was tried gained only faint bands in the gel electrophoresis which displayed that the mutagenic PCR was not executed completely to the optimal conditions. The only change in annealing conditions that was not tried is the extension of the annealing time. This could have provided more time for the primers to bind and a better replication of the plasmid DNA.

The transformation of the plasmid DNA to the two *E. coli* host cells was done by a heat shock protocol. The plasmid was inserted to the competent cells. So, the heat shock protocol was successful every time it was used in the master thesis. The plasmids were introduced and growth was seen on the spread-out petri dishes. The effectiveness of the method is high and easy to do. So, this was a positive note for the thesis work. The transformations could have been done by an electroporation protocol, but due to the effectiveness of the chosen heat shock transformation, this other method was not used. The heat shock protocol is easy to do and the results were outstanding.

7. Conclusions

The general aim of the project is to find an enzyme that can catalyze the deamination reaction of lysine. This reaction is part of a metabolic pathway for the bio-based production of adipic acid from the substrate lysine. The enzyme that was used in this thesis work was the 3-metylaspartate ammonia lyase (MAL), an enzyme that catalyzes the deamination of 3-methyl aspartic acid, a substrate that has a similar structure than lysine. The strategy followed to gain activity on lysine was to procure more space in the binding pocket of the MAL enzyme through changing some of the amino acid that were known to be around the substrate. Computational experiments were carried out to define the amino acid mutations that needed to happen and still gain an active enzyme.

From the experiments that were done during the thesis following conclusion could be deduced:

- The native MAL enzyme from *C. amalonaticus* did not show any activity on lysine.
- The mutagenic PCR successfully implemented all the various mutation, although no real conclusions on the optimal annealing temperatures and times were concluded.
- The two methods tried to purify the protein have certain advantages and disadvantages. The comparison of the methods displayed that the ÄKTA purifier system was more capable of purifying the protein in a higher concentration, quantity and with less contaminations then the Qaigen Ni-NTA spin columns.
- The activity assay on 3-methylaspartic acid results display that all the mutations involved a decrease of the activity of the enzyme to different extents. This reveals that the mutated residues do have an important part in the activity of the MAL enzyme
    - The C361A and C361S mutations were cysteine 361 was substituted for alanine or serine had a similar impairment of the enzymes kinetic activity which indicates that the replace of cysteine by the two smaller amino acid did affect the catalytic conversion of the enzyme.
    - The T360S mutation of the MAL enzyme had a similar kinetic activity then the native enzyme. This reveals that the exchange of Threonine 360 for serine did not affect the activity of the MAL enzyme and is a decent mutation to create more place in the biding pocket.
    - The T360A mutation activity toward 3-methylaspartic acid was reduced with a tremendous amount.
    - The L384A mutation of the MAL enzyme were leucine 384 was exchanged with alanine had an immense impairment of the enzymes activity. This indicates that the leucine 384 is important for the catalysis of the MAL enzyme.
- The assay for the detection of 6-aminohex-2-enoinc acid was confirmed to be an effective protocol after certain optimizations were done.
- The commercial ammonia assay kit was not suitable for detecting the ammonia mainly because a background measurement that was noticed in the negative controls and the test is time consuming.
- The strategy followed to gain activity on lysine through the production of various mutants was not successful since the mutant variants which should have a bigger binding pocket did not show any activity towards lysine. Although the obtained results give useful information in the understanding of how the enzyme works.

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9. Appendix

A. Gene sequence of MAL *Citrobacter amalonaticus* (UniProt O66145)

CC.ATG.GCA.AAA.ATT.AAA.CAG.GCG.CTG.TTC.ACC.GCT.GGC.TAC.TCC.TCT.TTC.TAT.TTT.GAT.G
AC.CAG.CAG.GCG.ATC.AAA.AAC.GGC.GCA.GGT.CAT.GAC.GGT.TTT.ATT.TAT.ACC.GGC.GAT.CC
G.GTC.ACC.CCG.GGC.TTT.ACT.TCT.GTG.CGC.CAG.GCC.GGC.GAG.TGC.GTT.TCC.GTA.CAG.CTG.
ATT.CTG.GAA.AAC.GGT.GCG.GTG.GCG.GTG.GGT.GAT.TGC.GCC.GCG.GTG.CAG.TAC.TCC.GGT.G
CC.GGT.GGT.CGC.GAT.CCG.CTG.TTC.CTG.GCT.GAA.CAT.TTT.ATT.CCG.TTC.CTG.AAC.GAC.CAC.
ATT.AAA.CCG.CTG.CTG.GAA.GGT.CGC.GAC.GTG.GAT.GCG.TTC.CTG.CCG.AAC.GCC.CGT.TTC.TT
C.GAC.AAA.CTG.CGT.ATC.GAC.GGT.AAC.CTG.CTG.CAT.ACC.GCC.GTT.CGC.TAC.GGT.CTG.TCT.C
AG.GCA.CTG.CTG.GAT.GCC.ACC.GCG.CTG.GCC.TCG.GGC.CGC.CTG.AAA.ACC.GAA.GTG.GTG.TG
T.GAT.GAA.TGG.CAA.CTG.CCG.TGC.GTA.CCG.GAA.GCC.ATT.CCG.CTG.TTT.GGT.CAG.AGC.GGC.
GAC.GAT.CGC.TAC.ATC.GCC.GTC.GAC.AAG.ATG.ATC.CTG.AAA.GGT.GTT.GAC.GTC.CTG.CCG.CA
T.GCG.CTG.ATC.AAC.AAC.GTG.GAA.GAG.AAG.CTG.GGT.TTC.AAA.GGC.GAA.AAA.CTG.CGT.GA
G.TAC.GTG.CGC.TGG.CTG.TCC.GAC.CGT.ATT.CTG.AGC.CTG.CGC.AGC.AGC.CCG.CGC.TAC.CAT.
CCG.ACC.CTG.CAT.ATC.GAT.GTG.TAT.GGC.ACC.ATC.GGT.CTG.ATC.TTC.GAT.ATG.GAC.CCG.GT
A.CGC.TGC.GCC.GAG.TAC.ATC.GCC.AGC.CTG.GAA.AAA.GAG.GCT.CAG.GGT.CTG.CCG.CTG.TAC.
ATT.GAA.GGC.CCG.GTT.GAT.GCA.GGC.AAC.AAG.CCG.GAT.CAG.ATC.CGC.ATG.CTG.ACC.GCC.A
TC.ACC.AAA.GAG.CTG.ACC.CGC.CTG.GGT.TCC.GGC.GTG.AAA.ATT.GTC.GCA.GAC.GAA.TGG.TG
T.AAC.ACC.TAT.CAG.GAC.ATT.GTG.GAC.TTC.ACC.GAT.GCC.GGT.AGC.TGC.CAC.ATG.GTG.CAG.
ATC.AAA.ACC.CCG.GAT.CTG.GGT.GGC.ATT.CAC.AAC.ATC.GTT.GAC.GCG.GTG.CTG.TAC.TGC.AA
C.AAA.CAC.GGT.ATG.GAA.GCC.*TAC.CAG.GGC.GGT.****ACC***.
TGT.*AAC.GAA.ACC.GAA.ATC*.AGC.GCC.CGC.ACC.TGC.GTA.CAT.GTG.GCT.CTG.GCC.GCA.CGT.CC
G.ATG.CGT.ATG.**CTG**.ATC.AAG.CCG.GGC.**ATG**.GGC.TTC.GAT.GAA.GGT.CTG.AAC.ATC.GTG.TTT.
AAC.GAA.ATG.AAC.CGC.ACC.ATC.GCG.CTG.CTG.CAG.ACT.AAG.GAT.CTG.GTG.CCG.CGC.GGC.A
GC.CTC.GAG

- NcoI restriction site + extra Ala (because of the last G in NcoI site. initial codon (ATG) in red.
- No stop codon (we are using the one on the plasmid after the HisTag).
- Thrombin site
- XhoI restriction site
- No NdeI, XhoI or NcoI restriction sites, no thrombin recognition site (LVPRGS).
- Mutation locations:
    - T360A and T360S
    - C361A and C361S
    - L384A
    - M389A

B. Generuler 1kb ladder range chart

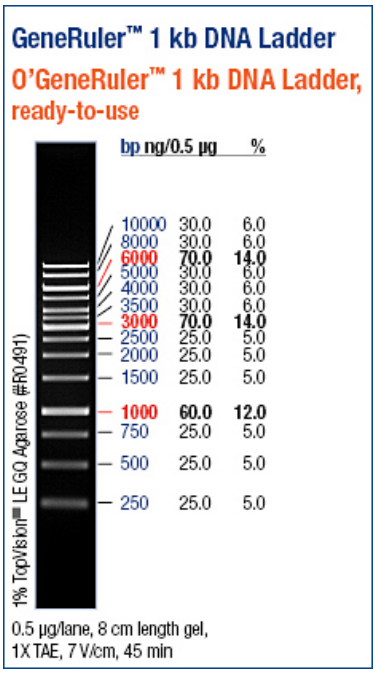

Figure 43: Generuler 1kb DNA ladder chart

C. Transformation protocol

1. The experiment starts with the rotavapor where the excess of liquid is vaporized till dry or 10 -15 µl volume.
2. If dry add 10 µl of sterile milli-Q H_2O (sterile fume hood).
3. Take out the competent cells from the - 80-degree freezer and put them directly on ice.
4. Pipette the plasmid sample with the competent cells and rest for 20-30 min on ice (sterile fume hood)
5. Then a heat shock is done at 42 °C for 1:30 min. Then put the samples back on ice for 1-2 min.
6. Add 800 µl of LB to the sample (sterile fume hood). Incubate the sample now for 45 min - 1 h at 37 °C in a thermomixer.
7. After the incubation, the samples will be centrifuged for 3' at 12000 rpm. 800 µl of LB is removed, there will be around 100 µl above the pellet.
8. Invert this and mix it with the pellet and plate them on an LB-agar with kanamycin (sterile fume hood). Place the petri dishes in the incubator at 37 °C overnight

D. Cultures preparation for protein expression

Main culture build up:

- In a 500-ml flask add:
  - 93 ml LB
  - 2 ml preculture
  - 300 µl Kanamycin
  - 2 ml auto induction solution 1
  - 5 ml auto induction solution 2
  - 100 µl auto induction solution 3
  - Incubate for circa 16-18 h at 30 °C

All the solutions described in the following tables are autoclaved before they are used in either the precultures or main expression cultures. This is done to ensure that no extra bacterial contaminations can occur during the experiments.

LB-broth

Peptone	8 g
Yeast extract	4 g
Sodium Chloride	8 g
Milli-Q water	800 ml

Auto-induction solution I

Glucose	2,5 g
Glycerol	25 g
Lactose	10 g
Milli-Q water	100 ml

Auto-induction II

Potassium dihydrogen phosphate	6,8g
Sodium dihydrogen phosphate	7,1 g
Milli-Q water	100 ml

Auto-induction III

Magnesium sulphate heptahydrate	9,86 g
Milli-Q water	20 ml

Kanamycin stock solution

Kanamycin	25 mg
Milli-Q water	500 µl

E. Precision Plus Protein™ Unstained Standards ladder

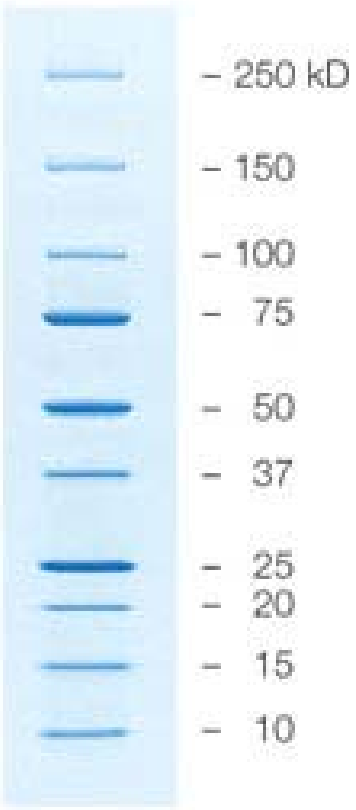

Figure 44: Precision Plus Protein™ Unstained Standards ladder with a range from 10 to 250 kDa

F. Activity assay on 3-methylaspartic acid protocol

Reaction buffer mix:

- 500 mM Tris-HCl buffer: out of 1000 mM stock take 1875 µl for end volume of 3750 µl
- 20 mM MgCl2: out of 100 mM stock take 750 µl for end volume of 3750 µl
- 1 mM KCL: out of 100 mM stock take 37,5 µl for end volume of 3750 µl
- 10 µg/ml enzyme add X µl (depends of concentration of the enzyme) for 3750 µl end volume
    - substrate preparations (because the substrate we have is 50 % (2S,3S)-3-methyl aspartic acid and 50 % (2S,3R)-3-methyl aspartic acid)

wanted concentration	Dilution factor only 50%	normale beginning concentration	dilution by adding buffer and substrate together	end concentration needed for 30 µl inoculation
0,5 mM	X2	1 mM	X5	5 mM
1,25 mM	X2	2,5 mM	X5	12,5 mM
2,5 mM	X2	5 mM	X5	25 mM
5 mM	X2	10 mM	X5	50 mM
10 mM	X2	20 mM	X5	100 mM
15 mM	X2	30 mM	X5	150 mM
20 mM	X2	40 mM	X5	200 mM

Blank solution:

- 500 mM Tris-HCl, take 500 µl of 1 M stock for 1000 µl
- 20 mM MgCl2, take 200 µl of 100 mM stock for 1000 µl
- 1 mM KCl, take 10 µl of 100 mM stock for 1000 µl
- Add 290 µl milli-Q water

Experiment protocol:

1. Place the reaction buffer mix in the thermomixer for 30 ' at 30 °C
2. Fill all the wells with 30 µl of the right substrate
3. Put the plate reader on and set the Temperature on 30 °C
4. Using a multi-dispenser pipette fill the reaction wells with 120 µl of reaction buffer mix.
5. Start the run (60 cycles, 240 nm & 230 nm for 40 sec cycle time, total experiment time > 30 min)

G. Ammonia assay with the ammonia assay kit

Reaction buffer mix:

- 500 mM Tris-HCl: Take 450 µl of 1M Tris-HCl
- 20 mM MgCl2: Take 180 µl of 100 mM MgCl2
- 1 mM KCl: Take 9 µl of 100 mM KCl
- Enzyme adding:
    - MAL variant C361A (stable with 100 µg/ml): 26,7 µl
    - MAL variant C361s (stable with 100 µg/ml): 27 µl
    - MAL variant T360A (unstable with 100 µg/ml, use 50 µg/ml): 54,7 µl
    - MAL variant T360S (stable with 100 µg/ml): 18,5 µl
    - MAL variant L384A (unstable with 100 µg/ml, use 50 µg/ml): 50,8 µl
    - Add the right amount of milli-Q water to get an end volume of 720 µl

Buffer mix (no enzyme):

- 500 mM Tris-HCl: Take 300 µl of 1M Tris-HCl
- 20 mM MgCl2: Take 120 µl of 100 mM MgCl2
- 1 mM KCl: Take 6 µl of 100 mM KCl
- Then add 54 µl of mQ H2O to get an end volume of 480 µl

Test scheme will be as followed:

- L = L-lysine, L^- = L-lysine negative (no enzyme), B= β-Lysine, B^-= β-Lysine negative (no enzyme), E = no substrate, 3 = 3MA, S = standard, B = water & RB= reagent blank

MAL A	MAL B	MAL D	MAL E	MAL F	negative	positive
L	L	L	L	L	L^-	S
L	L	L	L	L	L^-	S
L	L	L	L	L	L^-	B
B	B	B	B	B	B^-	B
B	B	B	B	B	B^-	
E	E	E	E	E	RB	
3	3	3	3	3	RB	
3	3	3	3	3	RB	

- The test will be carried out with 20 µl of reaction sample with 100 µl of AAK reagent
- Remark: only the Standard is a 10 µl sample otherwise it would be too high to measure
- The measurements are done at 3 h, 24h, 4 days and 1 week

Experiment protocol:

1. Make reaction buffer mix and put at 30 °C to let the reactions take place.
2. Take 20 µl of test sample and adjust the pH to 7 from 9,2
    i. L and L-: add 3,6 µl of 1M HCl
    ii. B, B- and 3: add 2 µl of 1M HCl
    iii. E: add 2,8 µl of 1M HCl
3. Then transfer the sample to the micro plate and add 100 µl of AAK reagent with a multi dispenser pipette
4. Mix well. Let it react for 5 min at 30°C and measure at 340 nm.
5. Add the 1 µl of enzyme in all the wells except the reagent blank and the blank. Mix and let it react again for 5 min at 30 °C
6. measure again at 340 nm

H. Alignment of all the sequenced plasmids.

To understand all the results, all the colours that are used in the alignment results will be explained first. The teal markings in the location of the codon of the desired mutations. The yellow markings are the extra mutation that occurred outside of the desired mutation. The pink markings are the reference for the forward and reverse primers to compare if the extra mutation can be ignored and filtered away or if they are not able to be disposed of.

MAL C361A.col 1

Forward

```
origninal         -------------------------------------CCATGGCAAAAATTAAACAGGCG
MAL C361A.Col 1   TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGCG
                                                       **********************

origninal         CTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGC
MAL C361A.Col 1   CTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGC
                  ************************************************************

origninal         GCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTG
MAL C361A.Col 1   GCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTG
                  ************************************************************

origninal         CGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTG
MAL C361A.Col 1   CGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTG
                  ************************************************************

origninal         GGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCT
MAL C361A.Col 1   GGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCT
                  ************************************************************

origninal         GAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTG
MAL C361A.Col 1   GAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTG
                  ************************************************************

origninal         GATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTG
MAL C361A.Col 1   GATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTG
                  ************************************************************

origninal         CATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCG
MAL C361A.Col 1   CATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCG
                  ************************************************************
```

```
origninal         GGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCC
MAL C361A.Col 1   GGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCC
                  ************************************************************

origninal         ATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTG
MAL C361A.Col 1   ATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTG
                  ************************************************************

origninal         AAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTC
MAL C361A.Col 1   AAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTC
                  ************************************************************

origninal         AAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGC
MAL C361A.Col 1   AAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGC
                  ************************************************************

origninal         AGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATC
MAL C361A.Col 1   AGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATC
                  ************************************************************

origninal         TTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGA-GGCTCA
MAL C361A.Col 1   TTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAAAAGGCTCA
                  ************************************************** * ******

origninal         GGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCG
MAL C361A.Col 1   GGGTCTGCCGCTGTACATTGAAGGCCC--------------------------------
                  ********************* * **

origninal         CATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGC
MAL C361A.Col 1   ------------------------------------------------------------

origninal         AGACGAATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCA
MAL C361A.Col 1   ------------------------------------------------------------

origninal         CATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCT
MAL C361A.Col 1   ------------------------------------------------------------

origninal         GTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAAT
MAL C361A.Col 1   ------------------------------------------------------------

origninal         CAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAA
MAL C361A.Col 1   ------------------------------------------------------------

origninal         GCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCAT
MAL C361A.Col 1   ------------------------------------------------------------

origninal         CGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL C361A.Col 1   ---------------------------------------------
```

Reverse

```
origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL C361A.Col 1   ------------------------------------------------------------

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL C361A.Col 1   ------------------------------------------------------------

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL C361A.Col 1   ------------------------------------------------------------

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL C361A.Col 1   ------------------------------------------------------------

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL C361A.Col 1   ------------------------------------------------------------

origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL C361A.Col 1   ------------------------------------------------------------
```

```
origninal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL C361A.Col 1   ------------------------------------------------------------

origninal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL C361A.Col 1   ------------------------------------------------------------

origninal         AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL C361A.Col 1   ------------------------------------------------------GCTACA
                                                                     ******

origninal         TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL C361A.Col 1   TCGCCGTCGACAAGATGATCCTGAAAGGTGTGACGTCTTGCCCGCATGCGCTGATCAACA
                  ******************************          ******************

origninal         ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL C361A.Col 1   ACGTGGAAGAGAAGCTGGGT-TCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
                  ******************* ****************************************

origninal         CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL C361A.Col 1   CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
                  ************************************************************

origninal         TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL C361A.Col 1   TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
                  ************************************************************

origninal         CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
MAL C361A.Col 1   CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
                  ************************************************************

origninal         GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
MAL C361A.Col 1   GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
                  ************************************************************

origninal         GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
MAL C361A.Col 1   GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
                  ************************************************************

origninal         TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
MAL C361A.Col 1   TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
                  ************************************************************

origninal         ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
MAL C361A.Col 1   ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
                  ************************************************************

origninal         GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
MAL C361A.Col 1   GTACCGCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGTCGCAC
                  *****  ********************************************* *****

origninal         GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
MAL C361A.Col 1   GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
                  ************************************************************

origninal         TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
MAL C361A.Col 1   TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
                  ************************************************************

origninal         GCCTCGAG-------------------
MAL C361A.Col 1   GCCTCGAGCACCACCACCACCACCAATG
                  ********
```

MAL C361A.col 2

Forward

```
origninal         ------------------------------------------------------------
MAL C361A.Col 2   CGTTTGGGGGAACCCCTTCCCCTCTAGATTAATCTTGTTTAACTTTAAGACTGAGATATA

origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL C361A.Col 2   CCATGTCTAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
                  ***** * ************************************************

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL C361A.Col 2   ACCAGCAGGTCATCAAAAACGGCGCAGGTCATGACGGCTTTATTTATACCGGCGATCCGG
                  *********  ************************* ********************

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL C361A.Col 2   TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACTTCTGATTC
                  ************************************************  *******

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL C361A.Col 2   TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
                  ************************************************************

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL C361A.Col 2   GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
                  ************************************************************

origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL C361A.Col 2   CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
                  ************************************************************

origninal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL C361A.Col 2   TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
                  ************************************************************

origninal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL C361A.Col 2   TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATTGG
                  ************************************************************ *

origninal         AAC-TGCCGTGCGTACCG-GAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTA
MAL C361A.Col 2   CAACTGCCGTGCGTACCGGAAGCCCTTTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTA
                   *  **************  *  ** ***********************************

origninal         CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAA
MAL C361A.Col 2   CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATTCA
                  ************************************************************  *

origninal         CAACGTGGA--AGAGAAGCTGGGTTTCAAAGGC-GAAAAACTGCGTGAGTACGTGCGCTG
MAL C361A.Col 2   ACAACGTGGTATGAGAAGCTGGGTTTCAAAGGTTAAAAACTGCTTTGAGTACGTGCGCTG
                    *    *    ********************   ****      ***************

origninal         GCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATAT
MAL C361A.Col 2   GCTGTTCGACCGTATTCTGAGCCTGCGCAGCAGCTCCGCGCTACCATCGATCCTGCATAT
                  ***** ***************************** *       *   *** *********

origninal         CGATG-TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTAC--GCTGCGCCGA
MAL C361A.Col 2   CGATTGTGTATGGCACATCGGTCCTGATCTTCGATATGGACCCTGGAAACGCTGCGTCGA
                  ****  **********    *  ******************** * *   ****** ***

origninal         GTACATCGCCAGCCTGGAAAAAGAGGCTCA---GGGTCTGCCGC-TGTACATTGAAGGCC
MAL C361A.Col 2   GTACATCGCATGCCTGAAAAAAATGAGTGCTCAGGGTCTGCCGCCTGTACATTGAAATGC
                  *********  ***** *****  *  *     *********** ***********   *

origninal         CGG---TTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAG
MAL C361A.Col 2   CCCGCTTGGATGCAGGTCAACCAGCCGGATCGATCCGCAAGCGTACGCCATCTCTAAGTG
                  *     * ********  *     * *    ******** **  **  *       *  *
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origninal         AGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATC
MAL C361A.Col 2   ATCTG-ATCCGCTGGATTCGGGCTGGTAAGTTGTGTCCGAGAACTGAC------------
                  * ***   *  **** *** ***  * ** ****  * **  * **

origninal         AGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         ATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         AAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         GTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TGGTGCCGCGCGGCAGCCTCGAG
MAL C361A.Col 2   -----------------------
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Reverse

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origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL C361A.Col 2   -------------------------------------------------------------

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL C361A.Col 2   -------------------------------------------------------------

origninal         AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL C361A.Col 2   -------------------------------------------------------------

origninal         TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL C361A.Col 2   -------------------------------------------------------------

origninal         ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL C361A.Col 2   -----------------------------------------------------TGGGCTGT
                                                                        ******

origninal         CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL C361A.Col 2   CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
                  ************************************************************

origninal         TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL C361A.Col 2   TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGATGCGCCGAGTACATCG
                  ****************************************** ****************
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origninal          CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
MAL C361A.Col 2    CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
                   ************************************************************

origninal          GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
MAL C361A.Col 2    GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
                   ************************************************************

origninal          GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
MAL C361A.Col 2    GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
                   ************************************************************

origninal          TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
MAL C361A.Col 2    TCACCGATGCCGGTAGCTGCCTCATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
                   ********************* ************************************

origninal          ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
MAL C361A.Col 2    ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
                   ************************************************************

origninal          GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
MAL C361A.Col 2    GTACCGCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
                   *****  ***************************************************

origninal          GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
MAL C361A.Col 2    GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
                   ************************************************************

origninal          TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
MAL C361A.Col 2    TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
                   ************************************************************

origninal          GCCTCGAG----------------------------------------------------
MAL C361A.Col 2    GCCTCGAGCACCACCACCACCACCACTGAGATCCGGATGCTAACAAAGCCAAAAAAGGAA
                   ********

origninal          -------------
MAL C361A.Col 2    AATGAAGGTCCCT
```

MAL C361S.col 1

Forward

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origninal          ------------------------------------CCATGGCAAAAATTAAACAGGCG
MAL C361S.Col 1    AGGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGCG
                                                       ***********************

origninal          CTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGC
MAL C361S.Col 1    CTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGC
                   ************************************************************

origninal          GCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTG
MAL C361S.Col 1    GCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTG
                   ************************************************************

origninal          CGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTG
MAL C361S.Col 1    CGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTG
                   ************************************************************

origninal          GGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCT
MAL C361S.Col 1    GGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCT
                   ************************************************************
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origninal          GAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTG
MAL C361S.Col 1    GAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTG
                   ************************************************************

origninal          GATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTG
MAL C361S.Col 1    GATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTG
                   ************************************************************

origninal          CATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCG
MAL C361S.Col 1    CATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCG
                   ************************************************************

origninal          GGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCC
MAL C361S.Col 1    GGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCC
                   ************************************************************

origninal          ATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTG
MAL C361S.Col 1    ATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTG
                   ************************************************************

origninal          AAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTC
MAL C361S.Col 1    AAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTC
                   ************************************************************

origninal          AAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGC
MAL C361S.Col 1    AAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGC
                   ************************************************************

origninal          AGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATC
MAL C361S.Col 1    AGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATC
                   ************************************************************

origninal          TTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAG
MAL C361S.Col 1    TTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAG
                   ************************************************************

origninal          GGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGC
MAL C361S.Col 1    GGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGC
                   ************************************************************

origninal          ATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCA
MAL C361S.Col 1    ATGCTGACCGCCATCACCAAAGA-----------------------------------
                   ***********************

origninal          GACGAATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCAC
MAL C361S.Col 1    ------------------------------------------------------------

origninal          ATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTG
MAL C361S.Col 1    ------------------------------------------------------------

origninal          TACTGCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATC
MAL C361S.Col 1    ------------------------------------------------------------

origninal          AGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAG
MAL C361S.Col 1    ------------------------------------------------------------

origninal          CCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATC
MAL C361S.Col 1    ------------------------------------------------------------

origninal          GCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL C361S.Col 1    ---------------------------------------------
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Reverse

```
origninal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL C361S.Col 1    ------------------------------------------------------------

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL C361S.Col 1   ------------------------------------------------------------

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL C361S.Col 1   ------------------------------------------------------------

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL C361S.Col 1   ------------------------------------------------------------

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL C361S.Col 1   ------------------------------------------------------------

origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL C361S.Col 1   ------------------------------------------------------------

origninal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL C361S.Col 1   ------------------------------------------------------------

origninal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL C361S.Col 1   ------------------------------------------------------------

origninal         AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGA-GCGGCGACGATCGCTAC
MAL C361S.Col 1   ----------------------ATTCCGCTGTTTGGTCAGAGCGGGCGACGATCGCTAC
                                        ******************   ***************

origninal         ATCGCCGTCGACAAG--ATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCA
MAL C361S.Col 1   ATCGCCGTCGACAAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCA
                  **************      *   **********************************

origninal         ACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGC
MAL C361S.Col 1   ACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGC
                  ************************************************************

origninal         TGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCG
MAL C361S.Col 1   TGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCG
                  ************************************************************

origninal         ATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACA
MAL C361S.Col 1   ATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACA
                  ************************************************************

origninal         TCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATG
MAL C361S.Col 1   TCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATG
                  ************************************************************

origninal         CAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCC
MAL C361S.Col 1   CAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCC
                  ************************************************************

origninal         TGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGG
MAL C361S.Col 1   TGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGG
                  ************************************************************

origninal         ACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCA
MAL C361S.Col 1   ACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCA
                  ************************************************************

origninal         TTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGG
MAL C361S.Col 1   TTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGG
                  ************************************************************
```

```
orginal          GCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCG
MAL C361S.Col 1  GCGGTACCTCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCG
                 ********* **************************************************

orginal          CACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCG
MAL C361S.Col 1  CACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCG
                 ************************************************************

orginal          TGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCG
MAL C361S.Col 1  TGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCG
                 ************************************************************

orginal          GCAGCCTCGAG--------------------------
MAL C361S.Col 1  GCAGCCTCGAGCACCACCACCACCACTGAGATCC
                 ***********
```

MAL C361S.col 2

Forward

```
orginal          ------------------------------------------------------------
MAL C361S.Col 2  AAATATAATGTTAACTAAAATTTCCCCTCTATCATAATTTTGTTTAACTTTTAGAAGGAG

orginal          -----CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTT
MAL C361S.Col 2  ATATACCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTT
                      ******************************************************

orginal          TGATGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGA
MAL C361S.Col 2  TGATGACCATTAGGCGATCAAAAACGGCGCAGGTCCTGACGGTTTTATTTATACCGGCGA
                 *********  ************************ ************************

orginal          TCCGGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCT
MAL C361S.Col 2  TCCGGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCT
                 ************************************************************

orginal          GATTCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGC
MAL C361S.Col 2  GATTCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGC
                 ************************************************************

orginal          CGGTGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACAT
MAL C361S.Col 2  CGGTGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACAT
                 ************************************************************

orginal          TAAACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGA
MAL C361S.Col 2  TAAACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGA
                 ************************************************************

orginal          CAAACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGC
MAL C361S.Col 2  CAAACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGC
                 ************************************************************

orginal          ACTGCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGA
MAL C361S.Col 2  ACTGCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGA
                 ************************************************************

orginal          ATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCG
MAL C361S.Col 2  ATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCG
                 ************************************************************

orginal          CTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGAT
MAL C361S.Col 2  CTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGAT
                 ************************************************************
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orginal          CAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTG
MAL C361S.Col 2  CAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTG
                 ************************************************************

orginal          GCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATAT
MAL C361S.Col 2  GCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATAT
                 ************************************************************

orginal          CGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTA
MAL C361S.Col 2  CGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTA
                 ************************************************************

orginal          CATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGA
MAL C361S.Col 2  CATCGCCAGCCTGGAAAAAGAGGCTCAGGTTCTGCCGCTGTACATTTGAAAGCCCGGGTT
                 **************************** ***************   *   ** *

orginal          TGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCG-CCATCACCAAAGAGCTGACCC
MAL C361S.Col 2  GATGCAGCAACAGCCGGATCCGATCCGCAGGCTGAACCGTCATCACCCAAGAGCTGACCC
                       *  ********* ******** ***** *   ******* ************

orginal          GCCTGGGT-TCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATT
MAL C361S.Col 2  GGCCTTGGTTCCGGCCGTGAAATGGTTCGCCAGAACCAGGTGGGTGTTACCACCCTATT
                 * *   *  * * *   *  ** **   *    ***    **      ** **    ***

orginal          GTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGT
MAL C361S.Col 2  CA------------------------------------------------------------

orginal          GGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTAC
MAL C361S.Col 2  ------------------------------------------------------------

orginal          CAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTG
MAL C361S.Col 2  ------------------------------------------------------------

orginal          GCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAAC
MAL C361S.Col 2  ------------------------------------------------------------

orginal          ATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCG
MAL C361S.Col 2  ------------------------------------------------------------

orginal          CGCGGCAGCCTCGAG
MAL C361S.Col 2  ---------------
```

Reverse

```
orginal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL C361S.Col 2  ------------------------------------------------------------

orginal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL C361S.Col 2  ------------------------------------------------------------

orginal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL C361S.Col 2  ------------------------------------------------------------

orginal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL C361S.Col 2  ------------------------------------------------------------

orginal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL C361S.Col 2  ------------------------------------------------GCCAGAAACGAC
                                                                 **** *    **

orginal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL C361S.Col 2  CGATTCTCGGAAC-----------------------AAAACTTGGCGTATCGGAC----
                 ** * ** * *                       **     *  * *  ***
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orignal          TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL C361S.Col 2  --CGTAACCATGGTGGCAATACCGGCGGTCCGCTACAGGTCCGTCTTCAAGGACAACTGC
                   **** * * ***  *             *  * *    * ****         *****

orignal          TGGAT---GCCACCGCGCTGGCCTCGGGCCGC----CTGAAAACCGAAGTGGTGTGTGAT
MAL C361S.Col 2  TAGAATGGCCCCCGCCGCTGACTCGGAGCCGCTGGAAAAACCGAAGTGGTTGTGTGGATG
                 * **     ** *  ***** *   * *****       *     *  ** *****

orignal          GAATGGCAACTGCCGTGCGT-ACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGA
MAL C361S.Col 2  AAATGGCAACTGCCGTGCGTACCGGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGA
                  ******************  * ************************************

orignal          TCGCTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCT
MAL C361S.Col 2  TCGCTACATCGCCGTCGACAAGATGATC-TGAAAGGTGTTGACGTCCTGCCGCATGCGCT
                 **************************** *******************************

orignal          GATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCG-AAAAACTGCGTGAGTACGTGC
MAL C361S.Col 2  GATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAAACTGCGTGAGTACGTGC
                 ************************************ *********************

orignal          GCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGC
MAL C361S.Col 2  GCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGC
                 ************************************************************

orignal          ATATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCG
MAL C361S.Col 2  ATATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCG
                 ************************************************************

orignal          AGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGG
MAL C361S.Col 2  AGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGG
                 ************************************************************

orignal          TTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGA
MAL C361S.Col 2  TTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGA
                 ************************************************************

orignal          CCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACA
MAL C361S.Col 2  CCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACA
                 ************************************************************

orignal          TTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGG
MAL C361S.Col 2  TTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGG
                 ************************************************************

orignal          GTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCT
MAL C361S.Col 2  GTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCT
                 ************************************************************

orignal          ACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTC
MAL C361S.Col 2  ACCAGGGCGGTACCTCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTC
                 *************** ********************************************

orignal          TGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGA
MAL C361S.Col 2  TGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGA
                 ************************************************************

orignal          ACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGC
MAL C361S.Col 2  ACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGC
                 ************************************************************

orignal          CGCGCGGCAGCCTCGAG-----------------------------------------
MAL C361S.Col 2  CGCGCGGCAGCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCC
                 *****************

orignal          ----------------------------
MAL C361S.Col 2  GAAAAGAAAGCTGGCTGGCCTTAATGGA
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MAL M389A.col 1

Forward

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origninal         ------------------------------CCATGGCAAAAATTAAACAGGCGCTGTT
MAL M389A.Col 1   ATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGCGCTGTT
                                                ****************************

origninal         CACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGG
MAL M389A.Col 1   CACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGG
                  ************************************************************

origninal         TCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCA
MAL M389A.Col 1   TCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCA
                  ************************************************************

origninal         GGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGA
MAL M389A.Col 1   GGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGA
                  ************************************************************

origninal         TTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACA
MAL M389A.Col 1   TTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACA
                  ************************************************************

origninal         TTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGC
MAL M389A.Col 1   TTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGC
                  ************************************************************

origninal         GTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATAC
MAL M389A.Col 1   GTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATAC
                  ************************************************************

origninal         CGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCG
MAL M389A.Col 1   CGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCG
                  ************************************************************

origninal         CCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCC
MAL M389A.Col 1   CCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCC
                  ************************************************************

origninal         GCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGG
MAL M389A.Col 1   GCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGG
                  ************************************************************

origninal         TGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGG
MAL M389A.Col 1   TGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGG
                  ************************************************************

origninal         CGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAG
MAL M389A.Col 1   CGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAG
                  ************************************************************

origninal         CCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGA
MAL M389A.Col 1   CCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGA
                  ************************************************************

origninal         TATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCT
MAL M389A.Col 1   TATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCT
                  ************************************************************

origninal         GCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCT
MAL M389A.Col 1   GCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGA--------------
                  *******************************************
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orginal         GACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGA
MAL M389A.Col 1 ------------------------------------------------------------

orginal         ATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGT
MAL M389A.Col 1 ------------------------------------------------------------

orginal         GCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTG
MAL M389A.Col 1 ------------------------------------------------------------

orginal         CAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         CCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGG
MAL M389A.Col 1 ------------------------------------------------------------

orginal         CATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCT
MAL M389A.Col 1 ------------------------------------------------------------

orginal         GCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL M389A.Col 1 -----------------------------------------
```

Reverse

```
orginal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL M389A.Col 1 ------------------------------------------------------------

orginal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL M389A.Col 1 ------------------------------------------------------------

orginal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL M389A.Col 1 ------------------------------------------------------------

orginal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL M389A.Col 1 ------------------------------------------------------------

orginal         AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL M389A.Col 1 --------------GAAAGCCAATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
                             *  * ************************************

orginal         TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCC-TGCCGCATGCGCTGATCAAC
MAL M389A.Col 1 TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTTGCCGCATGCGCTGATCAAC
                ************************************ ******************

orginal         AACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTG
MAL M389A.Col 1 AACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTG
                ************************************************************

orginal         TCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGAT
MAL M389A.Col 1 TCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGAT
                ************************************************************

orginal         GTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATC
MAL M389A.Col 1 GTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATC
                ************************************************************
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origninal          GCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCA
MAL M389A.Col 1    GCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCA
                   ************************************************************

origninal          GGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTG
MAL M389A.Col 1    GGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTG
                   ************************************************************

origninal          GGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGAC
MAL M389A.Col 1    GGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGAC
                   ************************************************************

origninal          TTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATT
MAL M389A.Col 1    TTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATT
                   ************************************************************

origninal          CACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGC
MAL M389A.Col 1    CACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGC
                   ************************************************************

origninal          GGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCA
MAL M389A.Col 1    GGTACCGCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCA
                   ******  ***************************************************

origninal          CGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTG
MAL M389A.Col 1    CGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTG
                   ************************************************************

origninal          TTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGC
MAL M389A.Col 1    TTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGC
                   ************************************************************

origninal          AGCCTCGAG-------------------------------------------
MAL M389A.Col 1    AGCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCC
                   *********
```

MAL M389A.col 2

Forward

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origninal          --------------------CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTA
MAL M389A.Col 2    AACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTA
                                       ************************************

origninal          CTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTT
MAL M389A.Col 2    CTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTT
                   ************************************************************

origninal          TATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTG
MAL M389A.Col 2    TATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTG
                   ************************************************************

origninal          CGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGT
MAL M389A.Col 2    CGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGT
                   ************************************************************

origninal          GCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTT
MAL M389A.Col 2    GCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTT
                   ************************************************************

origninal          CCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAA
MAL M389A.Col 2    CCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAA
                   ************************************************************
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origninal         CGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTA
MAL M389A.Col 2   CGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTA
                  ************************************************************

origninal         CGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGA
MAL M389A.Col 2   CGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGA
                  ************************************************************

origninal         AGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCA
MAL M389A.Col 2   AGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCA
                  ************************************************************

origninal         GAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCT
MAL M389A.Col 2   GAGCGGCGACGATCGCTACATCGC----------------------------------
                  ************************

origninal         GCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCG
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCA
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGT
MAL M389A.Col 2   ------------------------------------------------------------

origninal         ACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACAT
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCAC
MAL M389A.Col 2   ------------------------------------------------------------

origninal         CAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACAC
MAL M389A.Col 2   ------------------------------------------------------------

origninal         CTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAAC
MAL M389A.Col 2   ------------------------------------------------------------

origninal         CCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGG
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGT
MAL M389A.Col 2   ------------------------------------------------------------

origninal         ACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGA
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAA
MAL M389A.Col 2   ------------------------------------------------------------

origninal         GGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL M389A.Col 2   ----------------------------
```

Reverse

```
origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL M389A.Col 2   ------------------------------------------------------------

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL M389A.Col 2   ------------------------------------------------------------

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL M389A.Col 2   ------------------------------------------------------------
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orginal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL M389A.Col 2  ------------------------------------------------------------

orginal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL M389A.Col 2  ------------------------------------------------------------

orginal          TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL M389A.Col 2  ------------------------------------------------------------

orginal          TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL M389A.Col 2  ------------------------------------------------------------

orginal          AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL M389A.Col 2  ------------------------------------------------------------

orginal          TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL M389A.Col 2  ------------------------------------------------------------

orginal          ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL M389A.Col 2  ------------------------------------------------------------

orginal          CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL M389A.Col 2  ----------------------------------TACCATCCGACCGTGCATATCGATG
                                                   ************ ************

orginal          TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL M389A.Col 2  TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
                 ************************************************************

orginal          CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
MAL M389A.Col 2  CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
                 ************************************************************

orginal          GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
MAL M389A.Col 2  GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
                 ************************************************************

orginal          GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
MAL M389A.Col 2  GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
                 ************************************************************

orginal          TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
MAL M389A.Col 2  TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
                 ************************************************************

orginal          ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
MAL M389A.Col 2  ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
                 ************************************************************

orginal          GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
MAL M389A.Col 2  GTACCGCTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
                 *****  ***************************************************

orginal          GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
MAL M389A.Col 2  GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
                 ************************************************************

orginal          TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
MAL M389A.Col 2  TTAACGAAATGAACCGCACCATCGCGCTGCTG--------------------------
                 *********************************

orginal          GCCTCGAG
MAL M389A.Col 2  --------
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MAL M389 col 3

Forward

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origninal         -------------------------------------CCATGGCAAAAATTAAACAGGC
MAL M389A.Col 3   CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGC
                                                       **********************

origninal         GCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGG
MAL M389A.Col 3   GCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGG
                  ************************************************************

origninal         CGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGT
MAL M389A.Col 3   CGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGT
                  ************************************************************

origninal         GCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGT
MAL M389A.Col 3   GCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGT
                  ************************************************************

origninal         GGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGC
MAL M389A.Col 3   GGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGC
                  ************************************************************

origninal         TGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGT
MAL M389A.Col 3   TGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGT
                  ************************************************************

origninal         GGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCT
MAL M389A.Col 3   GGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCT
                  ************************************************************

origninal         GCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTC
MAL M389A.Col 3   GCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTC
                  ************************************************************

origninal         GGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGC
MAL M389A.Col 3   GGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGC
                  ************************************************************

origninal         CATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCT
MAL M389A.Col 3   CATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCT
                  ************************************************************

origninal         GAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTT
MAL M389A.Col 3   GAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTT
                  ************************************************************

origninal         CAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCG
MAL M389A.Col 3   CAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCG
                  ************************************************************

origninal         CAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGAT
MAL M389A.Col 3   CAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGAT
                  ************************************************************

origninal         CTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCA
MAL M389A.Col 3   CTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCA
                  ************************************************************

origninal         GGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCG
MAL M389A.Col 3   GGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCG
                  ************************************************************
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origninal          CATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGC
MAL M389A.Col 3    CATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGC
                   ************************************************************

origninal          AGACGAATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCA
MAL M389A.Col 3    AGACGAATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCA
                   ************************************************************

origninal          CATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCT
MAL M389A.Col 3    CATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCT
                   ************************************************************

origninal          GTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAAT
MAL M389A.Col 3    GTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAAT
                   ************************************************************

origninal          CAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAA
MAL M389A.Col 3    CAGCGCCCGCACCTGCGTACAT-------------------------------------
                   **********************

origninal          GCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCAT
MAL M389A.Col 3    ------------------------------------------------------------

origninal          CGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL M389A.Col 3    ---------------------------------------------
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Reverse

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origninal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL M389A.Col 3    ------------------------------------------------------------

origninal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL M389A.Col 3    ------------------------------------------------------------

origninal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL M389A.Col 3    ------------------------------------------------------------

origninal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL M389A.Col 3    ------------------------------------------------------------

origninal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL M389A.Col 3    ----------------------------------------------GACCACATTAAAC
                                                                  ************

origninal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL M389A.Col 3    CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
                   ************************************************************

origninal          TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL M389A.Col 3    TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
                   ************************************************************

origninal          TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL M389A.Col 3    TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
                   ************************************************************

origninal          AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL M389A.Col 3    AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
                   ************************************************************

origninal          TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL M389A.Col 3    TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
                   ************************************************************
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```
origninal          ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL M389A.Col 3    ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
                   ************************************************************

origninal          CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL M389A.Col 3    CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
                   ************************************************************

origninal          TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL M389A.Col 3    TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
                   ************************************************************

origninal          CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
MAL M389A.Col 3    CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
                   ************************************************************

origninal          GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
MAL M389A.Col 3    GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
                   ************************************************************

origninal          GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
MAL M389A.Col 3    GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
                   ************************************************************

origninal          TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
MAL M389A.Col 3    TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
                   ************************************************************

origninal          ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
MAL M389A.Col 3    ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
                   ************************************************************

origninal          GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
MAL M389A.Col 3    GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
                   ************************************************************

origninal          GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
MAL M389A.Col 3    GTCCGATGCGTATGCTGATCAAGCCGGGCGCGGGCTTCGATGAAGGTCTGAACATCGTGT
                   ****************************  ****************************

origninal          TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
MAL M389A.Col 3    TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
                   ************************************************************

origninal          GCCTCGAG-----------------------------------------------
MAL M389A.Col 3    GCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAA
                   ********
```

MAL T360A.col 1

Forward

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origninal          ------------------------------CCATGGCAAAAATTAAACAGGCGCTGTT
MAL T360A.Col 1    ATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAGGCGCTGTT
                                                  ****************************

origninal          CACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGG
MAL T360A.Col 1    CACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAACGGCGCAGG
                   ************************************************************

origninal          TCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCA
MAL T360A.Col 1    TCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCTGTGCGCCA
                   ************************************************************
```

```
orginal          GGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGA
MAL T360A.Col 1  GGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCGGTGGGTGA
                 ************************************************************

orginal          TTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACA
MAL T360A.Col 1  TTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTGGCTGAACA
                 ************************************************************

orginal          TTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGC
MAL T360A.Col 1  TTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGACGTGGATGC
                 ************************************************************

orginal          GTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATAC
MAL T360A.Col 1  GTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATAC
                 ************************************************************

orginal          CGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCG
MAL T360A.Col 1  CGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCG
                 ************************************************************

orginal          CCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCC
MAL T360A.Col 1  CCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCC
                 ************************************************************

orginal          GCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGG
MAL T360A.Col 1  GCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGG
                 ************************************************************

orginal          TGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGG
MAL T360A.Col 1  TGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGG
                 ************************************************************

orginal          CGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAG
MAL T360A.Col 1  CGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAG
                 ************************************************************


orginal          CCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGA
MAL T360A.Col 1  CCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGA
                 ************************************************************

orginal          TATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCT
MAL T360A.Col 1  TATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCT
                 ************************************************************

orginal          GCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCT
MAL T360A.Col 1  GCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCT
                 ************************************************************

orginal          GACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGA
MAL T360A.Col 1  GACCGCCATCACCAAAGA----------------------------------------
                 ******************

orginal          ATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGT
MAL T360A.Col 1  ------------------------------------------------------------

orginal          GCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTG
MAL T360A.Col 1  ------------------------------------------------------------

orginal          CAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGC
MAL T360A.Col 1  ------------------------------------------------------------

orginal          CCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGG
MAL T360A.Col 1  ------------------------------------------------------------

orginal          CATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCT
MAL T360A.Col 1  ------------------------------------------------------------
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origninal         GCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL T360A.Col 1   -------------------------------------
```

Reverse

```
origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL T360A.Col 1   ------------------------------------------------------------

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL T360A.Col 1   ------------------------------------------------------------

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL T360A.Col 1   ------------------------------------------------------------

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL T360A.Col 1   ------------------------------------------------------------

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL T360A.Col 1   ------------------------------------------------------------

origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL T360A.Col 1   ------------------------------------------------------------

origninal         TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL T360A.Col 1   ------------------------------------------------------------

origninal         TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL T360A.Col 1   ------------------------------------------------------------

origninal         AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL T360A.Col 1   ----------------------------GCTGTTTGGTCAGAGCGGCGACGATCGCTACA
                                              ********************************

origninal         TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL T360A.Col 1   TCGCCGTCGACAAGATGATCTGAAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
                  ********************  ************************************

origninal         ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL T360A.Col 1   ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
                  ************************************************************

origninal         CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL T360A.Col 1   CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
                  ************************************************************

origninal         TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL T360A.Col 1   TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
                  ************************************************************

origninal         CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAG-GCCCGGTTGATGCA
MAL T360A.Col 1   CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAAGGCCCGGTTGATGCA
                  ******************************************  **************

origninal         GGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTG
MAL T360A.Col 1   GGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTG
                  ************************************************************

origninal         GGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGAC
MAL T360A.Col 1   GGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGAC
                  ************************************************************

origninal         TTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATT
MAL T360A.Col 1   TTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATT
                  ************************************************************
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origninal          CACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGC
MAL T360A.Col 1    CACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGC
                   ************************************************************

origninal          GGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCA
MAL T360A.Col 1    GGTGCCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCA
                   *** ********************************************************

origninal          CGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTG
MAL T360A.Col 1    CGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTG
                   ************************************************************

origninal          TTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGC
MAL T360A.Col 1    TTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGC
                   ************************************************************

origninal          AGCCTCGAG------------------------------
MAL T360A.Col 1    AGCCTCGAGCACCACCACCACCACCAATGAGATCCGGCTG
                   *********
```

MAL T360A.col 2

Forward

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origninal          ------------------------------------------------------------
MAL T360A.Col 2    GGAGAACCGGTTAATTTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA

origninal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL T360A.Col 2    CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
                   ************************************************************

origninal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL T360A.Col 2    ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
                   ************************************************************

origninal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL T360A.Col 2    TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
                   ************************************************************

origninal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL T360A.Col 2    TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
                   ************************************************************

origninal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL T360A.Col 2    GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
                   ************************************************************

origninal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL T360A.Col 2    CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
                   ************************************************************

origninal          TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL T360A.Col 2    TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
                   ************************************************************


origninal          TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL T360A.Col 2    TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
                   ************************************************************

origninal          AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL T360A.Col 2    AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
                   ************************************************************
```

```
orginal          TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL T360A.Col 2  TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
                 ************************************************************

orginal          ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL T360A.Col 2  ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
                 ************************************************************

orginal          CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL T360A.Col 2  CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCCAACCCTGCATATCCAA
                 ****************************************** * **

orginal          TGTATGGCAC----CATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTAC
MAL T360A.Col 2  TGTGTATGGGCACCATCGGGCCTGATCTTCCAAATGGGACCCGGGACCCCTGCCGCCAAG
                 *** *                ** ********* * ****  ** *    *   ****   *

orginal          A---TCGCCAG--CCTGGAAAA--AGAGGCTCAGGGTCTG---CCGCTGTACATTGAAGG
MAL T360A.Col 2  AACATCCCCCAGCCCTGGAAAAATGAAGGCTCAGGGGCCTGCCCGCTTGTACATTTGAAG
                 *   ** **    *********    ********** *     *   ********  * *

orginal          CCC---GGTTGATGCAGGCA----ACAAGCCGGATCAGATCCG--CATGCTGACC--GCC
MAL T360A.Col 2  GGCCCGGGTTGATGCCTGGCAACAAAGCCCGGAATCAGAATCCCGTATGCTTGACCCGCC
                   *   *********  *       *    * * ******  *    *****   *  ***

orginal          ATCACC---AAAGAGCTGAC-CCGCCTGGG-TTCCG--GCGTG-AAAATTGTCGCAGACG
MAL T360A.Col 2  ATCCACCAAAAGAAGCTGAACCCGCTTGGGGTTCCCGGGCGTGAAAAATTGGCCCAAACG
                 ***  *   **  ******  **** **** ****   ***** ******* * ** ***

orginal          AATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGG
MAL T360A.Col 2  AATGGGTGAAAACTCCTTATCC--------AGG--GAAATTTGGGGGAACTTTCACACGA
                 *****   ** **   * *             *        *   ** * **  **** *

orginal          TGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACT
MAL T360A.Col 2  TGGCCCGTTAA-----------------------G----CCTGGCCCAATGGGGTG--
                 **       **                              ** * *  **  **

orginal          GCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCG
MAL T360A.Col 2  -CCAAATACAAAAACCCCCGGTAAATCAGGGGGGGTTATTTCCAAAAATCTCCTGTGTAA
                  * * * ***          *   * ***** **    * *    *** *

orginal          CCCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGG
MAL T360A.Col 2  CCCGGGGGGTCTTGGACTTGGCAAACAAAAAA---CCGGGG--GTTTTTGGGAAACCC--
                 ****       * *  *  ****          *   ***  *    *  **   ** **

orginal          GCATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGC
MAL T360A.Col 2  --CTTACCCACGGG--GGGGGGGGGCTTTTGGTAAAAGAAAA----------------
                     *   *  **     **   *    *  ** * ** ****

orginal          TGCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL T360A.Col 2  ----------------------------------------
```

Reverse

```
orginal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL T360A.Col 2  ------------------------------------------------------------

orginal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL T360A.Col 2  ------------------------------------------------------------

orginal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL T360A.Col 2  -----CCAGGTTTTAC-----------------------CGGATCGTCCCCGCTTTTTT
                       ** ** *****                     **  **    * ***  **

orginal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTA--CTCCGGTGCCGG
MAL T360A.Col 2  TGCCAGCGGGGGGGTTCCTAAAGTTTTGAAACGTCGGGGCGGGGGATGCCCCGGCCAATT
                 **  *   ** * * *       *  *     ** ** ** *  * *  * ****
```

```
orginal          TGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAA
MAL T360A.Col 2  CCGTCCGGGTCGG-TCGCGTTCAGGGGACATTTTTC----GTTCTGAAGGC--CCATTAA
                    ***  * ** * *     *    * *******       * ***** *    ******

orginal          ACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAA
MAL T360A.Col 2  CCG--TGTTGAA--GTCCGGA---CGGGATGCTTCCTCCGAACCCCGTTTTT---GGCAA
                  *   ** ** *  ***  **    *    * * *  ****** **  ***    * ***

orginal          ACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACT
MAL T360A.Col 2  -ATGGGTTT-GACGGTACCTGT---GCATACGCCGTTCGTTCGGTTGTTTCAGCACTGCT
                    ** ** * ******* *           ********** *  * * * **       **

orginal          GCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATG
MAL T360A.Col 2  GGTGC-------ACGCGCTGCCTCGGGCCGCCTGAAA-CCGAAGT--GGTGTTGATGAAG
                 * **                ****************** *******   ****      * *

orginal          GCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTA
MAL T360A.Col 2  GCAACTGCCGTGCGTACG--GAAGCCATTCCGCTGTTGGTCAGAGCGGCGACGATCGCTA
                 *****************    *  *  * *     *************************

orginal          CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAA
MAL T360A.Col 2  CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAA
                 ************************************************************

orginal          CAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCT
MAL T360A.Col 2  CAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCT
                 ************************************************************

orginal          GTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGA
MAL T360A.Col 2  GTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGA
                 ************************************************************

orginal          TGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACAT
MAL T360A.Col 2  TGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACAT
                 ************************************************************

orginal          CGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGC
MAL T360A.Col 2  CGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGC
                 ************************************************************

orginal          AGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCT
MAL T360A.Col 2  AGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCT
                 ************************************************************

orginal          GGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGA
MAL T360A.Col 2  GGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGA
                 ************************************************************

orginal          CTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCAT
MAL T360A.Col 2  CTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCAT
                 ************************************************************

orginal          TCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGG
MAL T360A.Col 2  TCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGG
                 ************************************************************

orginal          CGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGC
MAL T360A.Col 2  CGGTGCCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGC
                 **** ********************************************************

orginal          ACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGT
MAL T360A.Col 2  ACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGT
                 ************************************************************

orginal          GTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGG
MAL T360A.Col 2  GTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGG
                 ************************************************************
```

```
origninal         CAGCCTCGAG--------------------------------------------------
MAL T360A.Col 2   CAGCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAAGA
                  **********

origninal        -------------
MAL T360A.Col 2   AAAGTGATGCTCT
```

MAL T360S.col 1

Forward

```
origninal         ----------------------------------------CCATGGCAAAAATTAAACAG
MAL T360S.Col 1   CTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGCAAAAATTAAACAG
                                                          ********************

origninal         GCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAAC
MAL T360S.Col 1   GCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGACCAGCAGGCGATCAAAAAC
                  ************************************************************

origninal         GGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCT
MAL T360S.Col 1   GGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTCACCCCGGGCTTTACTTCT
                  ************************************************************

origninal         GTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCG
MAL T360S.Col 1   GTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTGGAAAACGGTGCGGTGGCG
                  ************************************************************

origninal         GTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTG
MAL T360S.Col 1   GTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGTCGCGATCCGCTGTTCCTG
                  ************************************************************

origninal         GCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGAC
MAL T360S.Col 1   GCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCGCTGCTGGAAGGTCGCGAC
                  ************************************************************

origninal         GTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTG
MAL T360S.Col 1   GTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTG
                  ************************************************************

origninal         CTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCC
MAL T360S.Col 1   CTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCC
                  ************************************************************

origninal         TCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAA
MAL T360S.Col 1   TCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAA
                  ************************************************************

origninal         GCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATC
MAL T360S.Col 1   GCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATC
                  ************************************************************

origninal         CTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGT
MAL T360S.Col 1   CTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGT
                  ************************************************************

origninal         TTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTG
MAL T360S.Col 1   TTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTG
                  ************************************************************

origninal         CGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTG
MAL T360S.Col 1   CGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTG
                  ************************************************************

origninal         ATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCT
MAL T360S.Col 1   ATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCT
                  ************************************************************
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orginal          CAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATC
MAL T360S.Col 1  CAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATC
                 ************************************************************

orginal          CGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTC
MAL T360S.Col 1  CGCAT-------------------------------------------------------
                 *****

orginal          GCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          CACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTG
MAL T360S.Col 1  ------------------------------------------------------------

orginal          CTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAA
MAL T360S.Col 1  ------------------------------------------------------------

orginal          ATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          AAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          ATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL T360S.Col 1  -------------------------------------------------
```

Reverse

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orginal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL T360S.Col 1  ------------------------------------------------------------

orginal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL T360S.Col 1  ------------------------------------------------------------

orginal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL T360S.Col 1  ------------------------------------------------------------

orginal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL T360S.Col 1  ------------------------------------------------------------

orginal          TGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGC
MAL T360S.Col 1  ------------------------------------------TGTTTCAGGCACTGC
                                                            *** ***********

orginal          TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
MAL T360S.Col 1  TGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGC
                 ************************************************************

orginal          AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
MAL T360S.Col 1  AACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACA
                 ************************************************************

orginal          TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
MAL T360S.Col    TCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACA
                 ************************************************************

orginal          ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
MAL T360S.Col 1  ACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGT
                 ************************************************************
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origninal          CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
MAL T360S.Col 1    CCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATG
                   ************************************************************

origninal          TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
MAL T360S.Col 1    TGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTACATCG
                   ************************************************************

origninal          CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
MAL T360S.Col 1    CCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGCAG
                   ************************************************************

origninal          GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
MAL T360S.Col 1    GCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGCCTGG
                   ************************************************************

origninal          GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
MAL T360S.Col 1    GTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTGGACT
                   ************************************************************

origninal          TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
MAL T360S.Col 1    TCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGCATTC
                   ************************************************************

origninal          ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
MAL T360S.Col 1    ACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAGGGCG
                   ************************************************************

origninal          GTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
MAL T360S.Col 1    GTTCCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCCGCAC
                   ** ********************************************************

origninal          GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
MAL T360S.Col 1    GTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATCGTGT
                   ************************************************************

origninal          TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
MAL T360S.Col 1    TTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGCGGCA
                   ************************************************************

origninal          GCCTCGAG----------------------------------------
MAL T360S.Col 1    GCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGC
                   ********
```

MAL T360S.col 2

Forward

```
origninal          ------------------------------------------------------------CC
MAL T360S.Col 2    GAGGGCGAAACATTTTCCCTTCTATAATAATTTTGTTTAACTTTAAGAAGGAGATATACC
                                                                              **

origninal          ATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGAC
MAL T360S.Col 2    ATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATGAC
                   ************************************************************

origninal          CAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTC
MAL T360S.Col 2    CAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGGTC
                   ************************************************************

origninal          ACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTG
MAL T360S.Col 2    ACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTCTG
                   ************************************************************
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orginal          GAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGT
MAL T360S.Col 2  GAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTGGT
                 ************************************************************

orginal          CGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCG
MAL T360S.Col 2  CGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAACCG
                 ************************************************************

orginal          CTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTG
MAL T360S.Col 2  CTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAACTG
                 ************************************************************

orginal          CGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTG
MAL T360S.Col 2  CGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACTGCTG
                 ************************************************************

orginal          GATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAA
MAL T360S.Col 2  GATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATGGCAA
                 ************************************************************

orginal          CTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATC
MAL T360S.Col 2  CTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTACATC
                 ************************************************************

orginal          GCCGTCGACAAGATGAT-CCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAA
MAL T360S.Col 2  GCCGTCGACAAGATGATTCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAACAA
                 ***************** ****************************************

orginal          CGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTC
MAL T360S.Col 2  CGTGGAAGAGAAGCTGGGTTTCACAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTC
                 ********************** ************************************

orginal          CGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATCGATGT
MAL T360S.Col 2  CGACCGTATTCTGAGCCTGCGCACCAGCCCGCGCTACCATCCGACCCTGCATATCGATGT
                 ********************** ************************************

orginal          GTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCC-GAGTACATCG
MAL T360S.Col 2  GTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTTGCGCCGAGTACATCG
                 ******************************************    * **********

orginal          CCAGCCTGGAA--AAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGATGC
MAL T360S.Col 2  CCAGCCTGTAATAAAGAAGGCTCATGTTCTGCCGCTGTACATTGAAAGGTCCCGGGTTGA
                 ******** **  **  ******* * ****************** *  *    * **

orginal          AGGC----AACAAGCCGGATCAGATCCGCATGCTGACC-----GCCATCACCAAAGA-GC
MAL T360S.Col 2  ATGCAGGCTACAAGCTGGATCAGAATTCCCGCATGCCTGAACCGCCATCACCAAAGAAGC
                 * **     ****** ********    *    ** *      ************** **

orginal          TGACCCGCCT-GGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAG
MAL T360S.Col 2  TGACTCGTCTTGGGATTCCTGGCGGAATTGGTCGCAGAACGAATGGGGGTTAACCACCTC
                 **** ** ** *** * *   * * ** * ********   *  * *    * * * *

orginal          GACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGAT
MAL T360S.Col 2  TATACTCAG----------------------------------------------------
                   * * *  *

orginal          CTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAA
MAL T360S.Col 2  ------------------------------------------------------------

orginal          GCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTG
MAL T360S.Col 2  ------------------------------------------------------------

orginal          GCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGT
MAL T360S.Col 2  ------------------------------------------------------------

orginal          CTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTG
MAL T360S.Col 2  ------------------------------------------------------------
```

```
origninal         GTGCCGCGCGGCAGCCTCGAG
MAL T360S.Col 2   ---------------------
```

Reverse

```
origninal         CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL T360S.Col 2   ------------------------TGCCCTA------CTCTATCTTTTCTGGCTTTTGTT
                                          **  *        **    ** **     ***  *

origninal         ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL T360S.Col 2   ACCAGCCGGATCTCAGTGGTGGT-------------------------------------
                  ****** **   ***     **

origninal         TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL T360S.Col 2   ------------------------------------------------------GGTGG
                                                                       * *

origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL T360S.Col 2   T------------------GGTGCTCGAGGCTGCCGCGCGGCACCAGAT------CCTTA
                  *                  ****   **    ******  ***   *         *  *

origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL T360S.Col 2   GTC---------TGCAGCAGCGCGATGGTGCGGTTCATTTCGTTAAACACGATGTTCAGA
                  ***         **   * *   **   *    ***  *** * **  ** *  ** *

origninal         CGCTGCTGGAAGGTCGCGACGTGGATG---------CGTTCCTGCCGAACG---------
MAL T360S.Col 2   CCTTCATCGAAGCCCATGCCCGGCTTGATCAGCATACGCATCGGACGTGCGGCCAGAGCC
                  *  *  * ****  *  * *  *  **          **   * * **  **

origninal         ---------CCCGTTTCTTCGACAAACTGCGTATCGACGGTAACCTGCTGCATACCGCCG
MAL T360S.Col 2   ACATGTACGCAGGTGCGGGCGCTGATTTCGGTTCGTTA-CAGGAACCGCCCTGGTAGGC
                         *  **     **   *  *  ** ***     *     *  * *

origninal         TTCGCTACGGTCTGTCTCAGGCACTGCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGA
MAL T360S.Col 2   TTCCATACCGTGTTT--GTTGCAGT-----ACAGCACCGCGT------------------
                  ***  *** ** * *     *** *     *   *******

origninal         AAACCGAAGTGGTGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTG-
MAL T360S.Col 2   -CAACGATGTTG-------TGAATGCCAC------------------CCAGATCCGGGGT
                    * *** ** *       ****** **                    *   ****  *

origninal         TTTGGTCAG---AGCGGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGGT
MAL T360S.Col 2   TTTGATCTGCACCATGTGGCAGCTACCGGCATCGGTGAAGTCCACAATGTCCTGATAGGT
                  **** ** *       *  *  * *  *  *****  *  * * * *   ****** ****

origninal         GTTGACGTCCTGCCGCATGCGCTGATCAACAACGTGGAAGAGAAGCTG-----GGTTTCA
MAL T360S.Col 2   GTTACACCATTCG--TCTGCGACAATTTTCACGCCGGAACCCAGGCGGGTCAGCTCTTTG
                  ***        *      ****   **   **    ****   * ** *        **

origninal         AAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCA
MAL T360S.Col 2   GTGATGGCGGTCAGCATGCGGATCTGATCCGGCTTGTTGCCTGCAT--------------
                    *  *     * ** ** * *  **  * ****    * * * **

origninal         GCAGCCCGCGCTACCATCCGACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCT
MAL T360S.Col 2   -CAACCGGG-----------CCTTCAATGTACAGCGG---CAGACCCTGAGCCTCTTTT
                   ** ** *            ** *  ** *  *   *      *** *  * **  * *

origninal         TCGATATGGACCCGGTACGCTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGG
MAL T360S.Col 2   TCCAGGCTGGCGATGTACTCGGCGCAGCGTACCGGGTCCATATCGAAGATCAGACCCGAT
                  ** *    * *   **** * **** * ****   * *    * *** *  ** * *

origninal         ----GTCTGCCGCTGTACA------------TTGAAGGCCCGGTTGATGCAG--------
MAL T360S.Col 2   GGTGCCATACACATCGATATGCAGGGTCGGATGGTAGCGCGGGCTGCTGCGCAGCTCAGA
                         * *   *  * *             * * **  * ** ** ***
```

```
orginal          ------GCAACAAGCCGGATCAGATCCGCATG---CTGACCGCCATCACCAAAGAGCTGA
MAL T360S.Col 2  ATACGGTCGGACAGCCAGCGCACGTACTCACGCAGTTTTCGCCTTTGAAACCCAG----
                       *    **** *  **  * * ** *    *   **** *    *   **

orginal          CCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGA-CGAATGGTGTAACACCTATCAGGAC
MAL T360S.Col 2  ----CTTCTCTTCCACGTTGTTGATCAGCGCATGCGGCAGGACGTCACACCCTTTCAGAT
                     * *   ****    **   **   ****    * * *  ** *****  *   **

orginal          ATTGTGGACTTCACCGATGCCGGTAGCTGCCACATG--------------GTGCAGATC
MAL T360S.Col 2  CATCTTGTCGACGGCGATGTAGCGATCGTCGCCGCTCTGACCAAACAGCGAATGGCCTTC
                   * * * *  *  *****  *  * *  *  *                    **    **

orginal          AAAACCCCGGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAA
MAL T360S.Col 2  CGGGTACGCACGCCAGTTGCCATTCATCACACACC----------------------A
                       *     *  * ** ******  ***                        *

orginal          CACGGTATGGAAGCCTACCAGG-GCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCAC
MAL T360S.Col 2  CTCGGTTTTCAGTCGTCCCGAGGCCAGCTGCGGTGGTCATCCAGCAGTGCGTGAACAGAC
                 * **** *  *  * * **  *  * *   * **    *  * * * *  * *  *  **

orginal          CTGCGTACATGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGG
MAL T360S.Col 2  CCGTACGGACCGCGGTATGCACCAGTTACCGGTCGATACGCAGTGG-------------
                 * *     *    * * **  *      *** *   ** ** *

orginal          CTTCGATGAAGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCA
MAL T360S.Col 2  -----------CCAAGAAACCGGGCGTTTCGGACAGGTAACGCATTCCACAGGTTC----
                           ***    *   **   ** * * * ****       * * *

orginal          GACTAAGGATCTGGTGCCGCGCGGCAGCCTCGAG
MAL T360S.Col 2  ---------------------------------
```

MAL L384A.col 1

Forward

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orginal          --CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGA
MAL L384A.Col 1  TACCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGA
                   ************************************************************

orginal          TGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCC
MAL L384A.Col 1  TGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCC
                 ************************************************************

orginal          GGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGAT
MAL L384A.Col 1  GGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGAT
                 ************************************************************

orginal          TCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGG
MAL L384A.Col 1  TCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGG
                 ************************************************************

orginal          TGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAA
MAL L384A.Col 1  TGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAA
                 ************************************************************

orginal          ACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAA
MAL L384A.Col 1  ACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAA
                 ************************************************************

orginal          ACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACT
MAL L384A.Col 1  ACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACT
                 ************************************************************
orginal          GCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATG
MAL L384A.Col 1  GCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATG
                 ************************************************************
```

```
orginal          GCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGAT-CGCT
MAL L384A.Col 1  GCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCCGCT
                 ********************************************************* ****

orginal          ACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCA
MAL L384A.Col 1  ACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCA
                 ************************************************************

orginal          ACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGA-AAAACTGCGTGAGTACGTGCGCTGG
MAL L384A.Col 1  ACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAAACTGCGTGAGTACGTGCGCTGG
                 ********************************** ************************

orginal          CTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATC
MAL L384A.Col 1  CTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATC
                 ************************************************************

orginal          GATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTAC
MAL L384A.Col 1  GATGTGTATGGCACCATCGGTCTGATCTTCGATATGGATCCCGGTACGCTGCGCGAGTAC
                 ************************************ ** *          *******

orginal          ATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGTTGAT
MAL L384A.Col 1  ATCGTCAGCCTGGAAAAAGAGGCTCAAGGGTCTGCCGCTGTACATTGAAGGCCGGTTGAT
                 **** ********************** **     *          *  * * *********

orginal          GCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGACCCGC
MAL L384A.Col 1  GCAGCCACTAGCCGGATCAGATTCGGCATGCTTGACGGCA-ATCACCAGAGCTGACCTGC
                 **** **  *  * *       ** *******   ** **   *   ********** **

orginal          CTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACATTGTG
MAL L384A.Col 1  TTGG-ATTCGGCCTGAAAATTGTCGCAGACTGAACTGGGTGGTA----------------
                   ***  * **** *****************  *    *

orginal          GACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGGTGGC
MAL L384A.Col 1  ------------------------------------------------------------

orginal          ATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTACCAG
MAL L384A.Col 1  ------------------------------------------------------------

orginal          GGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCTGGCC
MAL L384A.Col 1  ------------------------------------------------------------

orginal          GCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAACATC
MAL L384A.Col 1  ------------------------------------------------------------

orginal          GTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCCGCGC
MAL L384A.Col 1  ------------------------------------------------------------

orginal          GGCAGCCTCGAG
MAL L384A.Col 1  ------------
```

Reverse

```
orginal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL L384A.COL 1  ------------------------------------------------------------

orginal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL L384A.COL 1  ------------------------------------------------------------

orginal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL L384A.COL 1  ------------------------------------------------------------
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```
origninal         TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL L384A.COL 1   ------------------------------------------------------------


origninal         GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL L384A.COL 1   ------------------------------------------------------------


origninal         CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAAAC
MAL L384A.COL 1   -----CAGCGAGCCGATGGGAGTGGCGTTTCCTTGCCGAACGACCGGTTCTCG-ACCAAC
                       * *  **     *      *  *  * * ********* *** ****   ** ***


origninal         TGCGTATCG-ACGG-TAACCTG----------CTGCATACCGCCGTTCGCTACGGTCTGT
MAL L384A.COL 1   TGCGTATTCGACGGGTAAACTGCTGACATAACGTCCGTTTCGCTAACCGGTCCTGTCTCA
                  *******   **** *** ***          * * *  ***    ** * * ****


origninal         CTCAGGCACTGCTGGATGCCACCGCG-CTGGCCT-CGGGCC-GCCTGAAAACCGAAGTGG
MAL L384A.COL 1   GGGCACTACTTGG-ATTGCCACCGCGCTTGGCCTCGGGCCCGCCTGAAAACTGAAGTGGG
                        ***      *********  ******  ** **  *   ***    *   **


origninal         TGTGTGATGAATGGCAACTGCCGTGCGTACCGG-AAGCCATTCCGCTGTTTGGTCAGAGC
MAL L384A.COL 1   TGTGTGATGAATGGCAACTGCCGTGCGTACCGGAAGGCCATTCCGCTGTTTGGTCAGAGC
                  ********************************* * ************************


origninal         GGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCG
MAL L384A.COL 1   GGCGACGATCGCTACATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCG
                  ************************************************************


origninal         CATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAG
MAL L384A.COL 1   CATGCGCTGATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAG
                  ************************************************************


origninal         TACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATC-C
MAL L384A.COL 1   TACGTGCGCTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCC
                  ************************************************************ *


origninal         GACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACG
MAL L384A.COL 1   GACCCTGCATATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACG
                  ************************************************************


origninal         CTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGA
MAL L384A.COL 1   CTGCGCCGAGTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGA
                  ************************************************************


origninal         AGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAA
MAL L384A.COL 1   AGGCCCGGTTGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAA
                  ************************************************************


origninal         AGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTA
MAL L384A.COL 1   AGAGCTGACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTA
                  ************************************************************


origninal         TCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCC
MAL L384A.COL 1   TCAGGACATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCC
                  ************************************************************


origninal         GGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTAT
MAL L384A.COL 1   GGATCTGGGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTAT
                  ************************************************************


origninal         GGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACA
MAL L384A.COL 1   GGAAGCCTACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACA
                  ************************************************************


origninal         TGTGGCTCTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGA
MAL L384A.COL 1   TGTGGCTCTGGCCGCACGTCCGATGCGTATGGCGATCAAGCCGGGCATGGGCTTCGATGA
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origninal          AGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGA
MAL L384A.COL 1    AGGTCTGAACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGA
                   ************************************************************

origninal          TCTGGTGCCGCGCGGCAGCCTCGAG
MAL L384A.COL 1    TCTGGTGCCGCGCGGCA--------
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MAL L384A.col 2

Forward

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origninal          ------------------------------------------------------------
MAL L384A.Col 2    TGGGACTTTTATCTTCCCCTCTCTTTTTATTAATTTTTGTTTAACTTTAAGAAGGAGATA

origninal          --CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGA
MAL L384A.Col 2    TACCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGA
                     ************************************************************

origninal          TGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCC
MAL L384A.Col 2    TGACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCC
                   ************************************************************

origninal          GGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGAT
MAL L384A.Col 2    GGTCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGAT
                   ************************************************************

origninal          TCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGG
MAL L384A.Col 2    TCTGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGG
                   ************************************************************

origninal          TGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAA
MAL L384A.Col 2    TGGTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAA
                   ************************************************************

origninal          ACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAA
MAL L384A.Col 2    ACCGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCTTCGACAA
                   ************************************************************

origninal          ACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACGGTCTGTCTCAGGCACT
MAL L384A.Col 2    ACTGCGTATCGACGGTAACCTGCTGCATACCGCCGTTCGCTACTGTCTGTCTCAGGCACT
                   ****************************************** ****************

origninal          GCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATG
MAL L384A.Col 2    GCTGGATGCCACCGCGCTGGCCTCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATGAATG
                   ************************************************************

origninal          GCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTA
MAL L384A.Col 2    GCAACTGCCGTGCGTACCGGAAGCCTTTCCGCTGTTTGGTCAGAGCGGCGACGATCGCTA
                   ************************* **********************************

origninal          CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCTGATCAA
MAL L384A.Col 2    CATCGCCGTCGACAAGATGATCCTGAAAGGTGTTGACTTCCTGCCGCATGCGCTGATCAA
                   ************************************ **********************

origninal          CAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCT
MAL L384A.Col 2    CAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCGCTGGCT
                   ************************************************************
origninal          GTCCGACCGTATTCTGAGCCTG-CGCAGCAGCCCGCGCTACCATCCGACCCTGCATA-TC
MAL L384A.Col 2    GTCCGACCGTATTCTGAGGCTTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCATATTC
                   ****************** **  ********************************** **

origninal          GATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGAGTAC
MAL L384A.Col 2    GATGTGTATGGCACCATCGGTCTGATCTCGATATGGACCCGGTACGGCTGCGCCGAGTAC
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orginal          ATCGCCAGCCTGGAAAAAGAG-----GCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGG
MAL L384A.Col 2  ATCGTCCAGCCTGCAAAACATGTAGGCTCAAGGTTCTGCCGCTGTACATTGAGGTCCCGG
                 **** *   *  * **** *          *** ****************** * *****

orginal          TTGATGCAGGCAACAAGCCGGATCA-GATCCGCATGCTGACCGCCATCACCAAAGAGCTG
MAL L384A.Col 2  TGA-TGCAGCCACAAGCCGGGATCCGAATCCGCATGCTGACGC-CATCAC-TATGAGCTG
                 *   ***** **  *  * *****   **************   ******  * ******

orginal          ACCCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGAC
MAL L384A.Col 2  ACCCGCATGGATATGCGT--AAACTGGTTCCAAGACGACGTGGGGTA------------
                 ****** *** *    *    **     **  ******   * *  *

orginal          ATTGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          GGTGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCC
MAL L384A.Col 2  ------------------------------------------------------------

orginal          TACCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCT
MAL L384A.Col 2  ------------------------------------------------------------

orginal          CTGGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          AACATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          CCGCGCGGCAGCCTCGAG
MAL L384A.Col 2  ------------------
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Reverse

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orginal          CCATGGCAAAAATTAAACAGGCGCTGTTCACCGCTGGCTACTCCTCTTTCTATTTTGATG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          ACCAGCAGGCGATCAAAAACGGCGCAGGTCATGACGGTTTTATTTATACCGGCGATCCGG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          TCACCCCGGGCTTTACTTCTGTGCGCCAGGCCGGCGAGTGCGTTTCCGTACAGCTGATTC
MAL L384A.Col 2  ------------------------------------------------------------

orginal          TGGAAAACGGTGCGGTGGCGGTGGGTGATTGCGCCGCGGTGCAGTACTCCGGTGCCGGTG
MAL L384A.Col 2  ------------------------------------------------------------

orginal          GTCGCGATCCGCTGTTCCTGGCTGAACATTTTATTCCGTTCCTGAACGACCACATTAAAC
MAL L384A.Col 2  ------------------------------------------------------------

orginal          CGCTGCTGGAAGGTCGCGACGTGGATGCGTTCCTGCCGAACGCCCGTTTCT--TCGACAA
MAL L384A.Col 2  ----------------CGTGAAAATTGCGTTCCTGCCGGAACGCCCGATTTCTGACAGCT
                               **       ************* *   **   * *     *

orginal          ACTGCGTATCGACGGTAACCTGCTGCATACCGCCG-TTCGCTACGGTCTGTCTCAGGCAC
MAL L384A.Col 2  GCGATATCGAAACGGTAACTGGCTGCATACAGACGGTTCGGTAACGGTCGTATTCAGCAC
                  *    *    ********  ********* * ** **** **  *   ** *   ****

orginal          TGCTGG--ATGCCACCGCGCTGGCC-TCGGGCCGCCTGAAAACCGAAGTGGTGTGTGATG
MAL L384A.Col 2  TGCTTGAATGACCACCGCGATGACCTCCGGACGCCTGAAAAACCGAAGTGGTGTGTGATG
                 **** *     ******** ** **  *** *  *   ********************
orginal          AATGGCAACTGCCGTGCGTACCGGAAGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATC
MAL L384A.Col 2  AATGGCAACTGCCGTGCGTACCGG-AGCCATTCCGCTGTTTGGTCAGAGCGGCGACGATC
                 ********************** ***********************************

orginal          GCTACATCGCCGTCGACAAGATG--ATCCTGAAAGGTGTTGACGTCCTGCCGCATGCGCT
MAL L384A.Col 2  GCTACATCGCCGTCGAACAAGATGATCCTGAAAAGGTGATGACGTCCTGCCGCATGCGCT
                 ***************  *       *   ******* ********************
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origninal         GATCAACAACGTGGAAGAGAAGCTGGGTTTCAAAGGCGAAAAACTGCGTGAGTACGTGCG
MAL L384A.Col 2   GATCAACAACGTGGAAGAGAAGCTGGGT-TCAAAGGCGAAAAACTGCGTGAGTACGTGCG
                  **************************** ********************************

origninal         CTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCA
MAL L384A.Col 2   CTGGCTGTCCGACCGTATTCTGAGCCTGCGCAGCAGCCCGCGCTACCATCCGACCCTGCA
                  ************************************************************

origninal         TATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGA
MAL L384A.Col 2   TATCGATGTGTATGGCACCATCGGTCTGATCTTCGATATGGACCCGGTACGCTGCGCCGA
                  ************************************************************

origninal         GTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGT
MAL L384A.Col 2   GTACATCGCCAGCCTGGAAAAAGAGGCTCAGGGTCTGCCGCTGTACATTGAAGGCCCGGT
                  ************************************************************

origninal         TGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGAC
MAL L384A.Col 2   TGATGCAGGCAACAAGCCGGATCAGATCCGCATGCTGACCGCCATCACCAAAGAGCTGAC
                  ************************************************************

origninal         CCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACAT
MAL L384A.Col 2   CCGCCTGGGTTCCGGCGTGAAAATTGTCGCAGACGAATGGTGTAACACCTATCAGGACAT
                  ************************************************************

origninal         TGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGG
MAL L384A.Col 2   TGTGGACTTCACCGATGCCGGTAGCTGCCACATGGTGCAGATCAAAACCCCGGATCTGGG
                  ************************************************************

origninal         TGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTA
MAL L384A.Col 2   TGGCATTCACAACATCGTTGACGCGGTGCTGTACTGCAACAAACACGGTATGGAAGCCTA
                  ************************************************************

origninal         CCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCT
MAL L384A.Col 2   CCAGGGCGGTACCTGTAACGAAACCGAAATCAGCGCCCGCACCTGCGTACATGTGGCTCT
                  ************************************************************

origninal         GGCCGCACGTCCGATGCGTATGCTGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAA
MAL L384A.Col 2   GGCCGCACGTCCGATGCGTATGGCGATCAAGCCGGGCATGGGCTTCGATGAAGGTCTGAA
                  ********************  ************************************

origninal         CATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCC
MAL L384A.Col 2   CATCGTGTTTAACGAAATGAACCGCACCATCGCGCTGCTGCAGACTAAGGATCTGGTGCC
                  ************************************************************

origninal         GCGCGGCAGCCTCGAG----------------------------------------------
MAL L384A.Col 2   GCGCGGCAGCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCTCG
                  ****************

origninal         ------------------------
MAL L384A.Col 2   AAAAGAAAACTAAGCTTGTATTTC
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