Biomolecular Diagnostics, Laboratory

Scientific Report

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Abstract

In the scope of this report eight basic techniques performed in molecular biology are presented, namely the use of the BCA protein assay, the triglyceride assay, pH and glucose measurement, western blot, plasmidic DNA isolation, enzyme cutting and the DNA gel electrophoresis. These techniques are to either isolate, quantify or detect a biomolecule of interest which may have further implications in early detection of known diseases or show the validity of an implemented treatment. The results and their validity are analysed and discussed according to established standard values.

1 Introduction

Molecular diagnostics has become an indispensable field of research, applying molecular biology to medical testing. For this reason many applications require a basic understanding of the most standard procedures. Therefore, a series of eight diagnostic measurements were performed describing the necessary materials and methods, as well as a discussion of the results.

Proteins are large biomolecules and necessary for many processes within an organism. They consist of long chains of amino acid residues which give them their form and function. A simple and robust method to quantify the total amount of protein within a sample is the bicinchoninic acid (BCA) protein assay. It is based on the fact that the peptide bonds in proteins reduce divalent copper ions to monovalent ones proportionally. The amount of Cu^{+} ions can be made visible by adding BCA, which forms purple coloured complexes with the copper. The amount of protein can be determined by measuring the absorption of the sample solution with the usage of a reference ladder

For a specific analysis of proteins of interest a western blot is a common procedure that is performed. Through steps that include sample preparation, gel electrophoresis, staining, blocking and primary and secondary antibody incubation, specific proteins of interest can be detected and extracted from a given sample.

Carbohydrates are an important energy source that is required by all cells and organs of the body. Simple carbohydrates are composed of sugars such as fructose and glucose and irregularities in the metabolism of these sugars can indicate a variety of diseases such as diabetes, pancreatitis, and different types of cancers. Therefore, it is important to understand if the body regulates glucose levels after carbohydrate intake through the use of glucose measuring devices such as glucometers. These sensors apply a series of redox reaction to convert glucose to electrons whose resulting current can be measured by the device. Standard values are taken after fasting (80-100 $\frac{mg}{dl}$), after eating (170-200$\frac{mg}{dl}$), and a few hours after eating (120-140 $\frac{mg}{dl}$). Deviations from these values may indicate signs of impaired glucose metabolism or a marker for diabetes.

Lipids are an important macromolecule in the human body responsible for energy storage, insulation, and cellular communication. Fats, a subgroup of lipids, also known as triglycerides are an important measure of human heart health and can diagnose

hyperlipidemias such as diabetes mellitus and endocrine disorders . Therefore, they are used as a standard diagnostic measure in routine checkups as part of the lipid panel. Triglycerides are found in the plasma portion of the blood and consist of a glycerol head attached by an ester linkage to three chains of fatty acids. The normal range for triglycerides is less than 150 mg/dL whereas values from 150-199 mg/dL are considered borderline high and above 199 mg/dL the triglyceride level can be considered either high or very high.

Nucleic acids are the building blocks of all organisms and can broken down into two major groups; DNA and RNA. Specifically, bacteria have two types of DNA known as chromosomal DNA and plasmidic DNA. Plasmidic DNA has been used in recent years for cloning, transferring and manipulating genes of interest. Steps which are necessary to correctly inject or extract specific genes can be broken down into plasmidic DNA isolation from bacterial cells where the plasmidic DNA is separated from other intercellular components and chromosomal DNA, enzyme cutting of plasmidic DNA by restriction enzymes to manipulate genes at certain locations in the plasmid and gel electrophoresis as a quality control to see if the restriction enzymes are working as anticipated and if the potential gene has been implanted correctly.

A simple but highly diagnostic test is the pH measurement. The pH of a certain solution is a measure of the concentration of hydrogen ions in a solution. When a solution has a high number of hydrogen ions it is considered acidic and has a pH value below 7, whereas when it has a high number of hydroxide ions it has a pH value larger than 7 and is considered basic. Since the organs of the body can operate at certain pH levels, abnormal pH levels that can be detected in bodily fluids such as urine and blood can show early signs of diseases such as urinary tract infections, renal failures, chronic obstructive pulmonary disease and diarrhea.

2 Materials and methods

2.1 Protein isolation and quantification assay with BCA Protein Assay Kit

To isolate the proteins from 58 mg mice liver, a 2 ml mixture of radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor cocktail (PIC) was added in a ratio of 1:20000 to the sample in an Eppendorf reaction tube (1,5 ml). The RIPA was necessary to break the cells and extracellular matrix up while the PIC protects the proteins. After adding 15 ceramic beads, the tube was placed two times for 20 seconds at 6500 rpm in the homogenizer (Precellys 24, BERTIN). Then the probe was on ice for 5 minutes before putting it in the centrifuge (5415R, EPPENDORF) at 4 °C and 10 000 rpm. Due to the force all solid parts were at the bottom of the tube while the liquid phase was on top. The top liquid with the proteins was pipetted into a new reaction tube.

To evaluate the protein weight, a reference ladder was necessary. Therefore five Eppendorf reaction tubes were prepared. In the first tube 100 µl of a protein stock solution were pipetted. This stock solution consisted out of 2 $\frac{\mu g}{\mu l}$ bovine serum albumin in 0.9%-NaCl. The other four tubes were filled with 50 µl of 0.9%-NaCl solution. From the 100 µl stock, 50 µl were transferred to second tube. After homogenizing (MS2 Minishaker,IKA), another 50 µl were transferred from the second to the third tube. This procedure was repeated again reaching the fourth tube. In the fifth one no proteins were added so that the reference ladder could be corrected by the blank. With this a concentration cascade was created, from the first to the last tube as $2, 1, 0.5, 0.25$ and $0\ \frac{\mu g}{\mu l}$.

To evaluate the sample with the absorbance spectrophotometer (xMark, BIORAD) a 96-well plate was prepared. 10 µl of the reference ladder were pipetted to the well plate and diluted with additional 2 µl 0.9%-NaCl solution. For the sample, 2 µl of the probe were transferred to the plate and diluted with 10 µl saline solution. The reason for the different volumes was the otherwise too high protein weight of the sample. To get more reliable data, this assay was executed in the form of a triplicate. To quantify the amount of proteins, 200 µl of BCA were added to all of the prepared wells. The BCA used was the Pierce BCE Assay Kit from the company THERMO FISCHER SCIENTIFIC and reagent A:B was mixed in a ratio of 49:1.

The well plate was then incubated (G2545A, SHEL LAB) for 30 minutes at 37 °C to let the substances react. The absorbance of the, to the protein proportional, BCA complexes was then measured at a wavelength of 562 nm with the absorbance spectrophotometer.

2.2 Measurement of the blood glucose level

The glucose was measured three times for each subject. The measurements were conducted in the morning after fasting for more than 8 hours, 10-15 minutes after eating lunch and then 2-3 hours after having eaten lunch. The hands were disinfected with an ethanol solution and a finger was chosen from which to draw the blood. Usually the middle or ring finger was chosen for drawing the blood.

A test stripe was placed into the glucometer (CALLA Light, WELLION) and the glucometer was ready to use when a symbol appeared that resembled a drop of blood. Then a lancet was placed into the lancing device and the safety cover was removed. Once activating, the tip of the finger was pierced with the help of the lancing device. The blood droplet was placed onto the tip of the test stripe and after a few seconds the glucometer showed the glucose concentration in $\frac{mg}{dl}$. The lancet and the test stripe were then discarded into a closable, puncture-proof container to prevent cross contamination and a risk for injury.

2.3 Triglyceride assay with blood plasma

To isolate the blood plasma a the blood sample 5 µl of 0.5 M EDTA solution were first placed into an Eppendorf tube. This served to prevent coagulation of the blood through removal of the calcium ions. The blood from two test subjects was drawn with the help of a lancet. The tip of a finger was first thoroughly cleaned with warm water and then pierced. Approximately 50 µl of blood was then drawn using a pipette and placed into the same Eppendorf tube as the EDTA solution. The blood was then mixed well and placed on ice.

In order to separate the blood plasma from the hematocrit the blood sample was centrifuged (5415R, EPPENDORF) at 3500 rpm for 10 minutes. The clear supernatant was then pipetted carefully into a new reaction tube and placed on ice. The old tube was discarded since it did not contain any blood plasma.

To quantify the triglyceride concentration the standard curve method was used. Six Eppendorf reaction tubes were labeled 1-6 for the different dilutions needed for the standard curve. In the first tube 10 µl of the 4 mM glycerine solution was placed. In the

sixth tube a blank was created with 10 µl of the 0.9%-NaCl. For tubes 2-5 a cascade was created with mixtures of the glycerine solution and NaCl. After each tube was completed the contents were homogenized (MS2 Minishaker, IKA). In the second tube, 10 µl of the 4 mM glycerine solution and 10 µl of the 0.9%-NaCl solution were pipetted. In the third tube, 10 µl of the solution from the second tube was placed along with 10 µl of the 0.9%-NaCl solution. This procedure was repeated for the fourth and fifth tubes pipetting 10 µl of the solution from the previous tube and adding 10 µl of the 0.9%-NaCl solution.

In order to measure the results with an absorbance spectrophotometer (xMark, BIO-RAD) a 96-well plate was prepared. Each of the six dilutions for the standard curve along with both blood triglyceride samples were performed in triplicate in order to ensure that the final data was accurate and outliers could be discarded. 2 µl of each of the dilutions and both of the blood triglyceride samples was pipetted into the wells of the 96-well plate. To each of these 24 wells, 200 µl of the triglyceride reagent was added.

Then the plate was incubated for 10 minutes at a temperature of 37 °C to let the reagent react with the samples. This reaction consists of four steps by which the triglycerides are first enzymatically hydrolysed by lipase to free fatty acids and glycerol. This glycerol is then phosphorylated by ATP with glycerol kinase to create glycerol-3-phosphate and ADP. This glycerol-3-phosphate is then further oxidized by dihydroxyacetone phosphate by glycerolphosphate oxidase to create hydrogen peroxide. The final step is where this hydrogen peroxide reacts with 4-aminoantipyrine and 4,5-dichloro-2-hydroxybenzene sulfanate to produce a red colored dye.

The plate with the red colored dye in the wells was then read at a wavelength of 500 nm with the absorbance spectrophotometer. The absorbance measured was proportional to the concentration of triglycerides present in the sample. The standard curve was calculated using the measured extinction on the y-axis and the mg/ml of the sample on the x-axis. The glycerine concentration from each of the blood plasma samples was then calculated by using the standard curve generated.

2.4 pH value of a test sample

Three samples were prepared in order to be able to measure them using a pH sensor. The three samples were a heavily concentrated grapefruit tea, LDS buffer, and a human saliva sampled. Before conducting the measuring the pH meter needed to be calibrated with calibration solutions consisting of the pH values 4.01, 7.00 and 10.00. The pH meter (peqMeter 1.14) could be calibrated by simply pressing the `CAL` button and choosing the

fitting calibration solutions and pressing CAL once again. The calibration solutions that were not used were then unchecked on the screen.

The electrode on the tip of the pH sensor was then removed from the storage buffer and rinsed properly with distilled water. The electrode was placed into the first calibration solution and Calibrate button on the display of the pH meter was chosen and CAL was pressed. The voltage measured was -18 mV and the pH was set to 7.00. For the two subsequent calibration solutions the same steps were followed and the electrode was cleaned with the distilled water after each calibration. The resulting voltage measured was 157 mV and -167 mV for the 4.01 and 10.00 pH respectively. Once the calibration was complete the samples could be measured. The used samples were a concentrated grapefruit tea, the LDS buffer from other experiments and human saliva. Between each trial the electrode was cleaned with the distilled water. The electrode was then placed into each of the samples and the pH value was read from the display. Once the measurements were completed the electrode was placed back into the storage buffer after cleansing.

2.5 Western Blot

Using the liver protein extracted in the protein isolation experiment a western blot was performed to separate the beta-actin. 3.15 $\frac{\mu g}{\mu l}$ was the total concentration of protein that was determined and extracted. Four different amounts of protein were used and their values were 70 μg, 60 μg, 30 μg, and 10 μg. Each well was to receive 30 μl of a prepared solution for each of the four protein amounts. Therefore, each solution consisted of 5 μl of LDS sample buffer to make sure that all proteins have a equal negative charge so that when performing the gel electrophoresis they would be separated purely by size and not by charge and 1 μl 0.5 M DTE to break the disulfide bonds and aid in the heating to break up higher order protein structures. The desired protein weight was then divided by the concentration to get the volume of protein solution needed. This was, together with the LDS buffer and DTE, subtracted from the wanted 30 μl volume to get the amount of distilled water needed. Each sample was then prepared and centrifuged (Sprout mini, BIOZYM). The samples were then incubated (Thermomixer Compact, EPPENDORF) at 70 °C to denature the protein and reveal only the primary structure so that separation during the gel electrophoresis depended purely on the size of the sample.

The next phase was preparing and conducting the gel electrophoresis. First, 30 ml of the 20x MOPS running buffer were diluted in a measuring cylinder by adding 600 ml of distilled water. The polyacrylamide gel (Invitrogen 4-12% Bis-Tris) was then taken out of the packaging, rinsed with water and the comb and white stripe were removed. This gel contained a stacking section where the proteins could quickly pass through and then a resulting section where they would be separated based on their size with the smallest

proteins going the quickest through the pores of the gel. The gel with the label facing outward was then placed into the gel chamber (XCell SureLock). The space between the gel and the temporary plastic gel holder was filled with the MOPS running buffer and 500 μl of the antioxidant solution was added to the running buffer. The remaining buffer was then filled into the chamber and the slots of the gel were rinsed via pipetting the buffer solution up and down.

First, the protein standard (SeeBlue Plus2 Pre-Stained Protein Standard) was pipetted into the first well and the samples were pipetted into the subsequent wells starting from the 10 μg, 30 μg, 60 μg, and finally 70 μg protein amounts. In order to prevent proteins from sliding to the voltage applied to the empty wells, 10 μl of LDS sample buffer was added to the empty slots. The gel chamber was then closed and the gel electrophoresis (Bio Rad PowerPac Basic) was run for 1 hour at 175 volts.

Once the gel electrophoresis was completed, the gel packaging was opened and the gel was placed together with the membrane into a transfer chamber. Via a sandwiching method the membrane was placed compactly alongside the membrane with the sandwich layers being the sponge, white filter, membrane, gel, two white filters and another sponge. To make sure that there were no encapsulated air bubbles, the sandwich was pressed flat from one side to the other. Using the stirring plate (MR 3001 K, HEIDOLPH) and an electrophoresis power supply (E835, CONSORT) the transfer was run for 1.5 hours with 500 mA to transfer the proteins from the gel to the high-affinity membrane. Once the proteins were transferred a visual inspection was completed by PonceauS staining the membrane to make the transferred proteins visible.

The membrane was then blocked for one hour with 5% BSA solution to make sure that the membrane would lose the protein affinity at the locations where no protein was present. This prevented the primary and secondary antibodies from binding to the normally high-affinity membrane. After blocking, the primary antibody was incubated with the membrane overnight. To prevent any unbounded primary antibodies from staying on the membrane it was washed three times for 10 minutes each with TBST buffer and then incubated with the secondary antibody for two hours. The membrane was then washed again in the same cycle with TBST buffer to remove any unbound antibodies and incubated with an ECL reagent (New England Biolabs) to make the protein bands visible under the UV detector (G:BOX Chemi XX6, SYNGENE).

2.6 Plasmidic DNA isolation from bacterial cells

An *E.coli* cell culture with spliced multiple cloning site (MCS) was grown beforehand on a fitting growth medium. To make sure that no other cells were grown, an antibiotic

solution was added. 1.5 ml of the harvested culture were then centrifuged (5415D, EPPENDORF) for 5 minutes at 5000 rpm. The supernatant was discarded. The left over transparent pallet was soluted in 50 μl of P1 buffer to destroy the cell membrane. For the buffer 50 mM Tris-HCl (pH 8.0), 10mM EDTA and 100 $\frac{\mu g}{\mu l}$ of RNase A were used. 300 μl of TENS and 100 μl of 3 M sodium acetate solution (pH 5.2) were then added to the sample, each step followed by inverting it for mixing. This was necessary to get rid of all unwanted biomolecules. The TENS solution had been prepared beforehand with 50 mM NaOH, 5 mM HCl and 0.5% SDS. The probe was then centrifuged for 10 minutes at 5000 rpm. After that, the supernatant with the dissolved plasmids was pipetted into another tube. To concentrate the solution, first 1 ml of ice cooled 100%-ethanol was added and then stored overnight at -20°C in the freezer (comfort nofrost, LIEBHERR) for incubation. The following day, the solution was centrifuged again for 10 minutes at 12000 rpm. The supernatant was then removed and the pellet washed with 0.5 ml of 70%-ethanol by inverting it carefully. After removing the abundant ethanol, the tube with the pellet was placed in a holder with open lid for about 10 minutes to let all of the alcohol evaporate. Lastly, the pellet of plasmidic DNA was then soluted in 70 μl distilled water to create a known volume. To make sure that the DNA was distributed homogeneous, the tube was homogenized for several seconds. To determine the exact concentration, 1 μl of the sample was placed in the spectrophotometer (DS-11+, DENOVIX) and measured at 260 nm. Before the first measurement a blank containing pure water was used for calibration. To check for the purity of the probe, the $^{260}/_{280}$ ratio was recorded. The entire process was done for two samples.

2.7 Enzyme cutting of plasmidic DNA

The isolated plasmidic DNA samples from 2.6 were reused in this task. Due to quality problems one of the prepared samples had to be discarded. Therefore, both of the probes for the enzyme cutting come from the same sample with the concentration 1290.8 $\frac{ng}{\mu l}$. The vector used for cutting was the pcDNA^{TM}4/HisMax C with 5527 base pairs.

To 4 μg DNA sample, which are around 3 μl of the solution, 2 μl of each enzyme and 2 μl buffer were added. With another 11 μl of water, each of the two probes had a total volume of 20 μl. For homogenization, the tube was tilted slightly and rotated. For pipetting the enzymes, filtered tips were used. The DNA sample volume was calculated by dividing the desired weight with the measured concentration from before. The cutting enzymes chosen were the combination BglII/XbaI and BglII/EcoRV with the NEBuffer 3.1 for both. The incubation was done in a 37°C bath for about 1 hour. Both samples were stored on ice until evaluation using DNA gel electrophoresis.

2.8 Gel electrophoresis with DNA

As with the western blot, DNA strands can travel different distances in the electrophoresis gel depending on their length which is given in base pairs. This means that this method can be used to separate DNA sample by their size.

Therefore, the first step was to prepare the gel. For 70 ml of 1% agarose gel 0.7 g of agarose (Universal-Agarose peqGOLD, VWR) and 70 ml of the 1x TAE buffer were added into an Erlenmeyer flask. The mixture was then heated in a kitchen microwave at full power until no crystals were visible anymore, meaning that everything was in the solution. After cooling the solution down to around 30°C, 6 μl of DNA dye (Atlas Clear Sight, BIOATLAS) were added. The lower temperature was needed to not destroy the dye which was necessary so that the gel would be visible in the detection step. After that, the agarose solution was poured into the casting chamber. Before putting the chamber into the fridge (LKUv 1610-24, LIEBHERR) all air bubbles in the gel, on the walls and on the slot comb were removed carefully. When the gel became solid, it was placed in the gel chamber and the comb was removed. The chamber was then flooded with 1x TAE buffer until the gel was fully covered.
As for the samples, 5 samples in total were prepared for the gel electrophoresis. Two of them were the cut vector pieces from the previous enzyme cutting step (BglII/XbaI and BglII/EcoRV). The third was the uncut vector which was used for the enzyme cutting. The fourth sample was a plasmid which was isolated using a different protocol which had a higher success rate compared to the one presented. The last probe was the EcoRI/BamHI cut version of the fourth sample. All samples with cut vectors were heated for a short time to stop the enzyme activity. 5 μl 6x Loading dye was added to each sample to a respective volume of 25 μl. The dye consisted of bromophenol blue which inhibits the enzyme activity and glycerin which makes the dye heavier than the TAE buffer and therefore sinks to the bottom of the well.
The probes were then loaded into gel together with the 1 kilobase DNA reference ladder from New England Biolabs. The two empty slots of the 8 slot gel were filled with 10 μl of the 6x loading dye.
The electrophoresis was supplied (MP-300V, CLEAVER SCIENTIFIC) with a voltage of 110 volt for 45 minutes. After that, the gel was removed from the chamber and placed on a plastic plate inside of the fluorescence imaging detector (G:BOX Chemi XX6, SYNGENE).

3 Results

3.1 Protein isolation and quantification assay

Figure 3.1 shows the well plate and the colouring of the solutions with different protein concentration. The columns 1-5 are the reference ladder with 1 having a total protein weight of 10 µg and 5 being the blank. The columns 6 and 7 are from the tissue sample and therefore have the same concentration.

Table 3.1 shows the absorption of the different wells as well as the total protein weight of the references in μg.

Table 3.1: Absorption of the well plate measured for each triplicate (row) and their average. The total protein weight of the reference ladder is shown for the corresponding columns.

	Reference					Sample	
Protein weight	10	5	2.5	1.25	0	-	-
Unit	μg						
Absorption	1	2	3	4	5	6	7
A	1.26	0.727	0.416	0.254	0.092	1.026	0.92
B	1.285	0.714	0.44	0.264	0.169	0.811	0.899
C	1.273	0.731	0.44	0.271	0.093	0.842	0.834
Average	1.273	0.724	0.432	0.263	0.093	0.827	0.884

The plot in figure 3.2 shows the five points from the reference and their corresponding linear fit. The plot has been corrected by the blank. The data points shown are the average from the triplicates. An exception are the values from B5 and A6 which were omitted since they seem to be outliers. The values of the tissue sample are plotted as crosses.

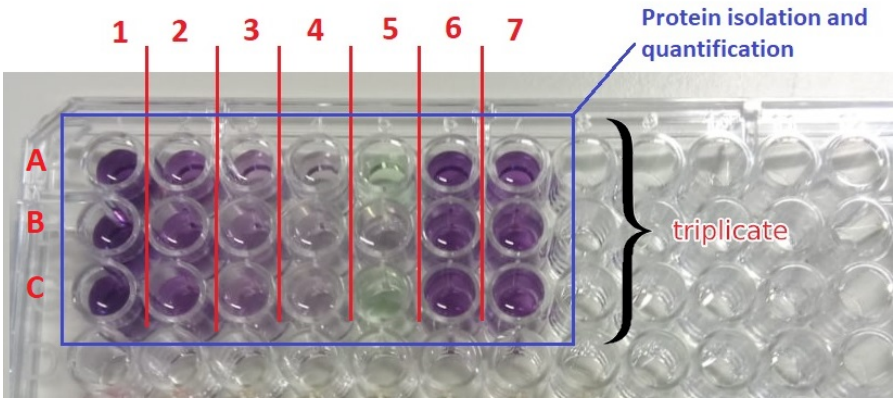

Figure 3.1: 96-well plate with 1-5 as the reference ladder and 6-7 as the sample.

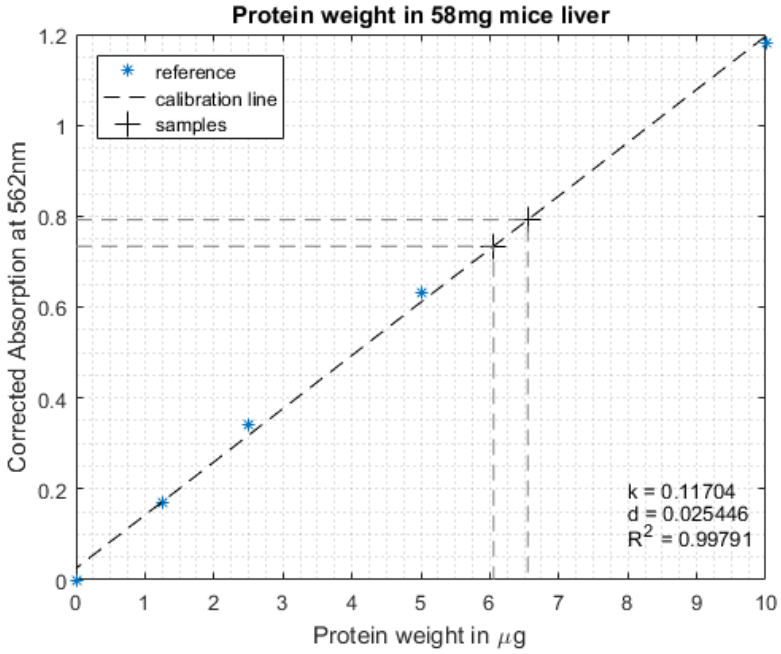

Figure 3.2: Plot of the absorption as a function of the protein weight. The black dotted line is the linear fit of the reference measurements. The tissue sample values are shown as black crosses.

The actual weight of protein in the sample was calculated using the values k = 0.11704 μg^{-1} and d = 0.025446 and equation 3.1.

$$\text{Protein\_weight} = \frac{\text{corrected\_absorption} - d}{k} \tag{3.1}$$

Table 3.2: Calculated protein weight of the tissue samples. The average absorption as in table 3.1 was corrected by the blank.

Sample column	corrected absorption	Protein weight
6	0.7340	6.054 μg
7	0.7918	6.548 μg

3.2 Measurement of the blood glucose level

Figure 3.3 shows how the glucose level in $^{mg}/_{dL}$ changed over time for two participants. The plots display the time before (green) and after (red) eating, as well as the measurement points. Both participants had a different diet. The first, which is labeled as 'Bernhard', had a banana together with two 0.3 l glasses of juice. This diet can be considered as sugary with a low fat and protein content. The second, labeled as 'Omar' had a burrito bowl with chicken, vegetable and rice . This can be considered as a low sugar, low fat, and high protein diet.

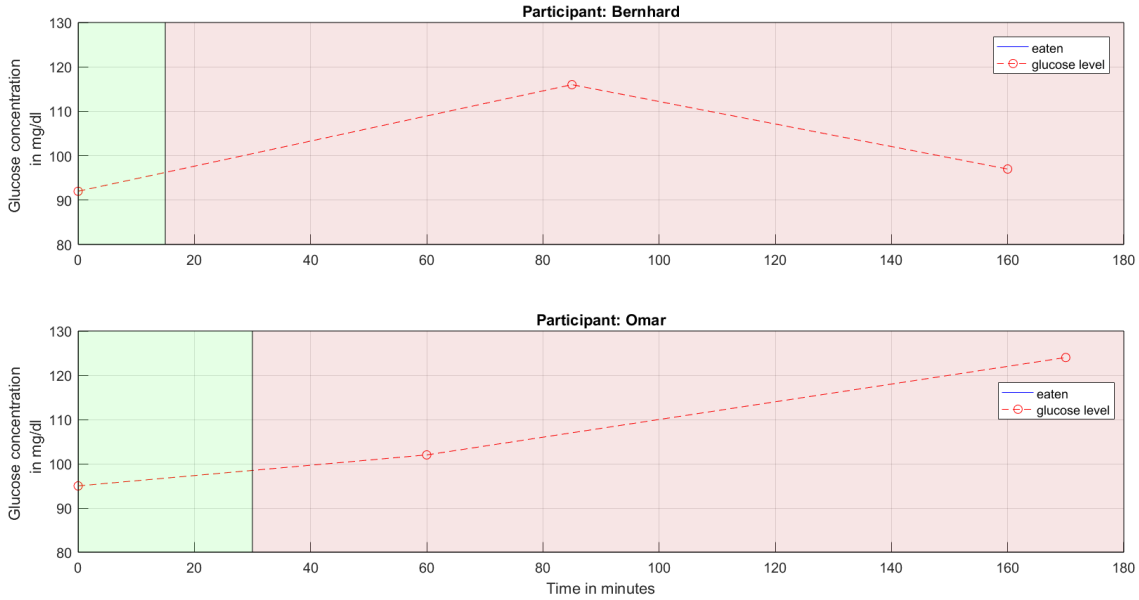

Figure 3.3: Glucose measurement over time.

3.3 Triglyceride assay with blood plasma

Figure 3.4 shows the well plate and the the different colored solutions with varying glycerine concentrations. The first six columns represent the reference ladder with column one having 4 mM glycerine and column six having 0 mM glycerine. The columns 7 and 8 are the blood samples for the subjects 'Bernhard' and 'Omar' respectively.

Table 3.3 shows the absorption of the different wells with each column being performed in triplicate and the glycerine concentrations of the reference ladder.

Table 3.3: Absorption of the well plate measured for each triplicate (row) and their average. The total glycerine concentration of the reference ladder is shown for the corresponding columns.

	Reference						Sample	
Glycerine conc.	4	2	1	0.5	0.25	0	-	-
Unit	mM							
Absorption	1	2	3	4	5	6	7	8
E	0.569	0.348	0.233	0.183	0.155	0.140	0.234	0.201
F	0.642	0.346	0.233	0.183	0.157	0.140	0.241	0.209
G	0.586	0.345	0.234	0.179	0.157	0.132	0.242	0.206
Average	0.599	0.346	0.233	0.182	0.156	0.137	0.239	0.205

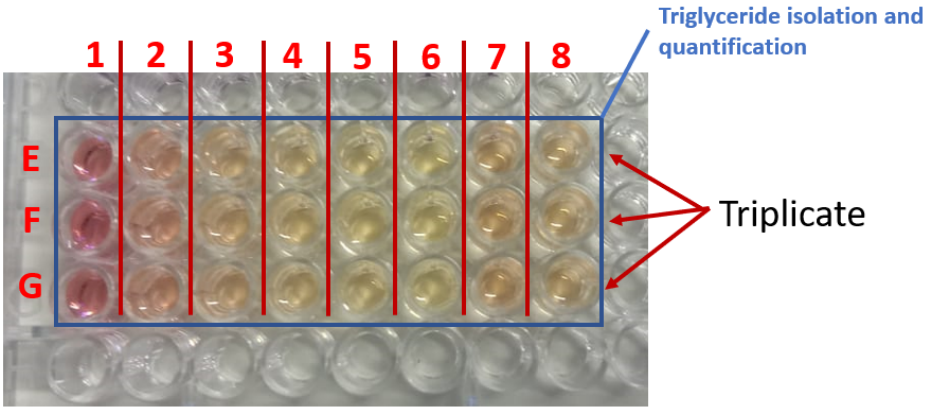

Figure 3.4: 96-well plate with 1-6 as the reference ladder and 7-8 as the sample.

The plot in figure 3.5 shows the six points from the reference and their corresponding linear fit. The plot has been adjusted to account for the blank and the data points shown are based on the average of the triplicate samples. The values for the blood plasma samples were then plotted as crosses depending on the subject's blood plasma. The values for extracted glycerine concentration based on the corrected absorption can be seen in Table 3.4.

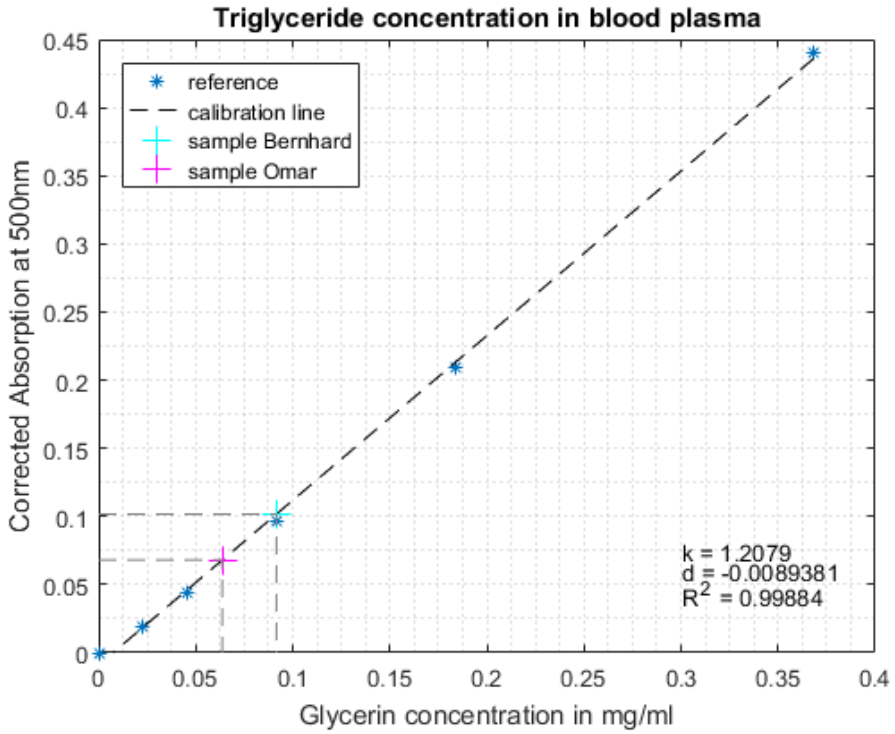

Figure 3.5: Plot of the absorption as a function of the glycerine concentration. The black dotted line is the linear fit of the reference measurements. The glycerine sample values are shown as light blue and purple crosses.

Table 3.4: Calculated glycerine concentration of each of the blood plasma samples. The average absorption as in table 3.3 was corrected by the blank.

Sample column (Subject)	Corrected Absorption	Glycerine Concentration	Triglyceride Concentration
7 (Bernhard)	0.1017	0.0921 mg/ml	0.901 mg/ml
8 (Omar)	0.0680	0.0637 mg/ml	0.623 mg/ml

The calculation from glycerine to triglyceride concentration was done by comparing their molecular weight. One mole of glycerine is stated as 9.2 g while one mole of triglycerides weighs around 90 g [1][2]. The exact weight for the triglycerides is unknown since it depends on the fatty acids attached. Since the concentration of glycerine measured is equal to the concentration of triglyceride in the blood, a conversion factor can be used which is calculated from similarities. Equation 3.2 shows the formula that was used for the computation of the concentrations.

$$\text{triglyceride} = \text{glycerine} \cdot \left(\frac{90}{9.2}\right) \tag{3.2}$$

3.4 pH value of a test sample

Table 3.5 shows the measured pH values for three different samples of interest.

Table 3.5: Measured pH values of various test samples.

Test sample	**pH value**
Concentrated grapefruit tea	2.91
LDS buffer	4.89
Human saliva	7.29

3.5 Western Blot

Figure 3.6 shows the results of the gel electrophoresis from the protein samples analyzed in the western blot. The first column is the colored version of the second column which was taken in the UV imaging machine. This colored reference ladder could be more accurately analyzed and the bands correctly grouped based on the two additional known colored bands present at 198 kDA and 17 kDa. The columns labeled 1-4 had different amounts of the same beta-actin protein inputted into the gel starting from 10 μg, 30 μg, 60 μg, and 70 μg. This corresponds to the intensity of the bands which can be seen to increase as the amount of protein used also increased. The isolated protein beta-actin was supposed to be located at 42 kDa, however from the gel in figure 3.6 it can be seen that for each column the value is between 49 kDa and 62 kDa.

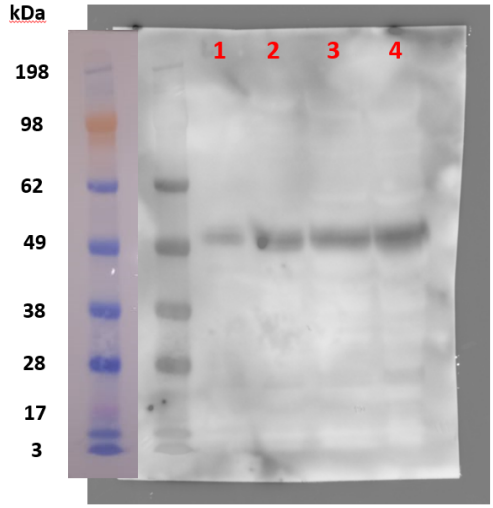

Figure 3.6: Visualization of the protein bands with varying amounts of protein sample.

3.6 Plasmidic DNA isolation from bacterial cells

The concentration measurements of the plasmidic DNA solution were performed three times per sample. Table 3.6 shows the measured concentrations of the isolated plasmidic DNA solutions and the $^{260}/_{280}$ ratio.

Table 3.6: Plasmidic DNA concentration of the isolated solutions and $^{260}/_{280}$ ratios

Nr.	Preparator	Concentration	$^{260}/_{280}$ ratio	Average Concentration	Average $^{260}/_{280}$ ratio
1	Bernhard	255.73 ng/μl	1.48	261.49 ng/μl	1.49
		245.46 ng/μl	1.50		
		283.27 ng/μl	1.48		
2	Omar	1292.00 ng/μl	2.00	1290.80 ng/μl	2.02
		1257.55 ng/μl	2.03		
		1322.84 ng/μl	2.03		

3.7 Gel electrophoresis of the enzyme cut plasmid DNA

Table 3.7 is a summary of the samples used. The numbers on the top row refer to those from figure 3.7. Sample 2 and 3 were provided from the laboratory and were prepared using an isolation method with higher success rate, which is not in the scope of this report. The numbers in the brackets are the base number where each enzyme cuts in a pcDNA^{TM}4/HisMax C vector. The total number of base pairs of this plasmid is 5257.

Table 3.7: Summary of the samples used for the gel electrophoresis

	Samples					
	1	2	3	4	5	6
Description	reference ladder	uncut vector	cut vector	uncut vector	cut vector	cut vector
Isolation Protocol	-	not described		as described		
Enzyme 1 (base pair)	-	-	EcoRI (1194)	-	BglII (12)	BglII (12)
Enzyme 2 (base pair)	-	-	BamHI (1179)	-	XbaI (1233)	EcoRV (1206)
Buffer	-	-	NEBuffer 3.1	-	NEBuffer 3.1	NEBuffer 3.1

Figure 3.7 shows an image of the the gel from the electrophoresis inside the UV-light chamber. The small green line on the left border of the image and the reference ladder indicates the 3 kilobase line of the reference. The red box shows barely visible bands of two samples. The blue line is connecting their position straight to the ladder and ends at the position of the 4 kilobase reference. The black structure in the background is the separated loading dye.

The gel from figure 3.8 was imaged by another laboratory group. The red boxes and blue lines are used as before. The orange box is used to visualize a different sample between the other two. The blue lines indicate DNA lengths of 1-2 kilo and 4-5 base pairs.

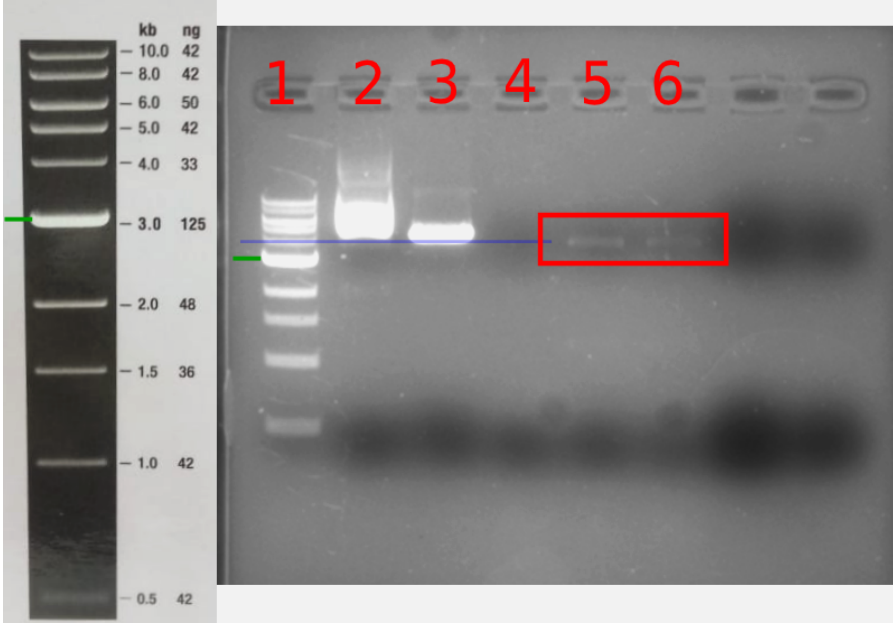

Figure 3.7: Gel after the electrophoresis of the DNA samples. On the left is the reference ladder information.

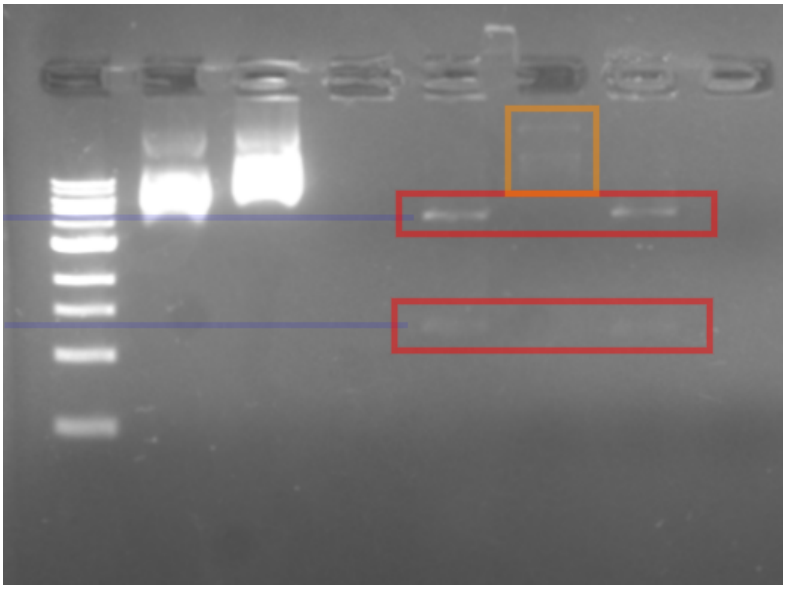

Figure 3.8: Gel after the electrophoresis of DNA samples, done by another group.

4 Discussion

The **protein isolation and quantification assay** showed that 58 mg of mice liver has around 6.3 µg of protein. Taking into account that just 2 µl of the probe were used a total concentration of 3.15 $\frac{\mu g}{\mu l}$ was determined. The difference of 0.5 µg of the sample columns 6 and 7 meets a relative difference of approximately 8%. When looking at the corresponding columns in table 3.1 a comparatively large variance can be observed. An explanation for this could be that the resting time of the probe, before pipetting it into the well plate, led to an inhomogeneous protein distribution within the reaction tube.

The **measurement of the blood glucose level** showed varying curves depending on which subject was measured and what was eaten. For Bernhard a common curve can be seen which indicates that after fasting the blood sugar level is low since no glucose had been digested for the past eight hours. However, after eating the blood sugar levels rose and then tapered off approximately 2 hours after eating. On the other hand, Omar who had a less sugary diet showed an abnormal curve that was marked with a system that adapted to food intake slower than expected. The glucose measurement approximately two hours after eating was 124 $\frac{mg}{dl}$ and much larger than the value measured shortly after eating 102 $\frac{mg}{dl}$. The fasting values were in the normal glucose range for both subjects, however, for both subjects the values directly after eating show lower than normal values. For future measurements it would be advised to take more measurements so that the glucose metabolism can more accurately and precisely be measured and conclusive statements can be reached.

The results of the **triglyceride assay** suggest that both subjects, Bernhard and Omar, have healthy amounts of triglycerides. The values obtained for each subject were 90.1 $\frac{mg}{dl}$ and 62.3 $\frac{mg}{dl}$ for Bernhard and Omar respectively. These values are within the normal range for triglycerides which is below 150 $\frac{mg}{dl}$ whereas values above this range indicate may indicate an elevated risk for cardiovascular diseases [3].

The **pH value of a test sample** showed interesting results for very different sample types. The most surprising was the the strong acidic solution of the grapefruit tee. Since it contains pieces of fruits which are known as sour, it was not unexpected that the sample tended to be more acidic, but not to this extent. After investigation of the exact ingredients the result makes more sense due to four types of acidifiers being used. Also, the high concentration had a major impact on the low pH. The LDS buffer originally had a pH of

5.2 but the results showed the this value changed quite a bit. Not surprising was the last sample which was expected to have a neutral behaviour. This value is rather close to the pH of the blood with values usually between 7.35 and 7.45 which also makes sense.

The **western blot** aimed at separating the protein beta-actin from mice liver samples. This specific type of protein was inspected due to its high levels in all types of tissues and their importance in cell motility, structure and integrity [4]. The expected locations of the beta-actin bands on the gel after gel electrophoresis had been performed were at 42 kDa, however, from the results it can be seen that the bands are between 49 kDa and 62 kDa based on the reference ladder [5]. One reason that might indicate different than expected values can be due to the reference ladder being run in a different running buffer than is suggested by the manufacturer. According to the standard protocol provided in the laboratory for SeeBlue Plus2 Pre-Stained protein standard it is meant to be run with MES SDS running buffer but for this experiment MOPS running buffer was used. This might result in the reference ladder running the bands slower or faster than normal which would then not allow for comparison between the protein bands of interest and the reference ladder. There might have been other potential reasons for the protein bands not being at the expected locations compared to the standard which may be due to mistakes with the primary or secondary antibodies, incorrect binding, or potential higher order structures and binding of the protein.

The results from the **plasmidic DNA isolation** display very different values. While the three measurement points of one sample do not deviate too much from each other, the difference between sample 1 and 2 is huge. The same applies to the $^{260}/_{280}$ ratios. In general, a ratio of 1.8 means that the sample is highly pure and consists mainly of DNA. Values at 2 are a indicator for a lot of RNA in the probe since RNA has a higher absorbance at 260 nm and a value below 1.8 is a sign for protein contamination.
This means that sample 1 did just have a much lower concentration as expected, it also contained too much protein. A reason for the low ratio could be the low concentration because less DNA means a lower absorption at 260 nm and therefore shifts the balance down. The reason for the low concentration could be either due to the amount of *E. Coli* used, which could not be quantified exactly or due to a pipetting error. The concentration of the second sample lies in the suspected range. The high $^{260}/_{280}$ ratio leads to the conclusion that a lot of of RNA is present.
The overall result suggests that the first sample should be discarded and not used for the enzyme splitting.

The **plasmidic DNA isolation** and **DNA gel electrophoresis** led to very weak bands. Regarding figure 3.7 you can see that the reference ladder and the two samples with the not described isolation method have bright and broad bands meaning that they had a very high DNA concentration. Sample 3 is, compared to sample 2, thinner and less smeared. This makes sense, since the first sample is the uncut vector. Uncut plasmids are

twisted in their structure often leading to more or less bulky formations which can travel different far in the gel. This means that sample 3 was cut successfully. The enzymes used for this probe cut very close to each other leading to extremely different strand sizes. The longer one can be seen bright in the gel in nearly the same height as the uncut vector at around 5 kilo bases. This is because they have nearly the same number of base pairs wich should be around 5200. The smaller piece should consist of just 15 bases and has probably traveled through the entire length of the gel and is therefore not visible.
Sample 4 cannot be identified in the image. The reason is probably the low initial intensity combined with the smearing behaviour of uncut vectors. Sample 5 and 6 show hardly visible lines at 4 kilo bases. This fits perfectly with the expectation because the samples should have strands at 4000 and 1200 bases due to the enzymes used. Thus, the shorter pieces should be in the range of 1 to 1.5 kilo bases but are not visible in the image.
In figure 3.8 the second line can be seen, which lies in the predicted range. The base number of the bands in the upper red box is a bit above 4 kilo bases as in the other gel. In addition, also the smearing of the uncut vector is visible, as it is marked with the orange box. But since the intensity is not high enough, the image does not allow for a quantification of the bases.

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