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Assay Development and Optimization for Isolation of DNA and miRNA from human Saliva and Plasma Exosomes

verfasst von / submitted by

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Abstract

Biomarkers are biological markers and defined as characteristical features that can be objectively measured and evaluated as indicators for normal biological processes, pathogenic processes or pharmacological responses. In short, a biomarker is an indicator of change, which fluctuates of time and biological influences. In this thesis, the focus was put on extracellular vesicles, so-called exosomes, which contain a wide range of biological molecules, especially DNA and numerous types of RNAs, which can serve as potential biomarkers. Extracellular vesicles can be found in different types of body fluids, e.g. plasma and saliva. Within the research project "Epitype 2", saliva and plasma exosomes from type 2 diabetes, prediabetes and gestational diabetes patients will be analyzed to come up with potential epigenetic biomarkers for early disease diagnosis. To provide a solid basis for these planned analysis, the aim of this thesis was to establish protocols for saliva and plasma to isolate DNA and microRNA (miRNA) from their exosomes.

A series of different exosome isolation methods, as well as DNA and RNA isolation kits, were tested in relation to their nucleic acid yield. Six commercial exosomes isolation methods were compared to eachother. Although ultracentrifugation is known as the gold standard for exosome isolation, the final choice fell on the Exiqon miRCURY Exosome serum/plasma kit, which uses simple precipitation for isolation and yielded by far the highest amounts of exosomal DNA and microRNA. For the nucleic acid isolation, seven different kits were tested and analyzed before a decision has been made.

In the end, with some hurdles, it was possible to establish salivary as well as plasma protocols for exosome isolation and subsequent nucleic acid isolation, which build a sound basis for future systemic disease biomarker discovery & diagnostics from saliva as well as plasma.

Abstract

Zusammenfassung

Biomarker sind biologische Marker und sind definiert als charakteristische Eigenschaften, welche objektiv gemessen and ausgewertet werden können. Diese können als Indikatoren für normale biologische Prozesse, pathologische Prozesse oder pharmakologische Antworten dienen. Einfacher gesagt, Biomarker sind Indikatoren, welche auf Veränderungen reagieren und sie können auch Rückschlüsse über biologische Einflüsse geben. In dieser Thesis wurde der Fokus auf extrazelluläre Vesikel, so genannte Exosomen, gelegt. Exosomen beinhalten eine weite Bandbreite an biologischen Molekülen, zum Beispiel DNA und zahlreiche Arten von RNAs, welche als potentielle Biomarker dienen können. Extrazelluläre Vesikel können in den verschiedensten Körperflüssigkeiten, zum Beispiel Plasma und Speichel, vorkommen. Im Rahmen des Forschungprojektes „Epitype 2“ werden Plasma- und Speichelproben von Diabetes-, Prädiabetes- und Schwangerschaftsdiabetes-Patienten analysiert um epigenetische Biomarker für eine frühe Krankheitsdiagnostik zu definieren. Zu diesem Zweck wurde im Rahmen der gegenständlichen Arbeit ein Protokoll erarbeitet, um Exosomen und deren DNA und microRNA (miRNA) aus Plasma und Speichel zu isolieren.

Eine Serie von verschiedenen Exosomen-Isolierungskits, sowie auch DNA - und RNA Isolierungskits wurden in Bezug auf deren Ausbeute an Nukleinsäuren getestet. Sechs kommerzielle Exosomen-Isolierungskits wurden miteinander verglichen. Obwohl Ultrazentrifugation zum Gold Standard in Sachen Exosomen Isolierung zählt, fiel unsere Wahl auf den Exiqon miRCURY Exosome serum/plasma Kit, welcher auf einfacher Präzipitation beruht und die beste Ausbeute an Nukleinsäuren zeigte. Für die Nukleinsäure-Isolierung wurden sieben Kits analysiert und verglichen bevor eine Entscheidung getroffen wurde.

Am Ende, nach einigen Hürden, war es schlussendlich möglich ein Protokoll zu etablieren um Exosomen aus Speichel, sowie aus Plasma zu isolieren und anschließend ausreichende Mengen an DNA und RNA parallel aus der gleichen Ausgangsprobe zu gewinnen. Dieses Protokoll bildet nunmehr eine solide Basis für die Entdeckung von neuen Biomarkern, sowie auch generell für die Biomarker-Diagnostik.

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List of Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
miRNA	Micro ribonucleic acid
NIH	National Institute for Health
SNP	Small nucleotide polymorphism
EDTA	Ethylenediaminetetraacetic acid
MVB	Multivesicular bodies
ILV	Intraluminal vesicles
ESCRT	Endosomal sorting complex required for transport
ER	Endoplasmatic reticulum
ÖRK	Österreichisches Rotes Kreuz
AIT	Austrian Institute of Technology
SOP	Standard operating procedure
Cf	Cell-free
U	Unit
PBS	Phosphate buffered saline
CSF	Cerebrospinal fluid
PED	Polyethersulfone
ds	Double-stranded
ss	Single-stranded
$MgCl_2$	Magnesium chloride
E. coli	Escherichia coli
-OH	Hydroxide
PCR	Polymerase chain reaction
ddH_2O	Double-distilled water
pg	Picogram
mg	Milligram
µl	Microliter
ml	Milliliter
nm	Nanometer
qPCR	quantitative polymerase chain reaction
mM	Millimolar
NTP	Nucleoside triphosphate
RT	Reverse transcription
cDNA	Complementary DNA
LED	Light emitting diode
CCD	Charge-coupled device
nt	Nucleotide

1. Introduction

1.1. Biomarkers

The term “biomarker” is a short term for biological markers and all types of biomarkers have been used in the past by epidemiologists, physicians, and scientists to study diseases (Mayeux, 2004). First described in 1980 by Isaakson, the use of biomarkers was proposed to use urinary nitrogen for an independent measurement of protein intake, which is still one of the most common biomarkers (Hedrick et al., 2012). However, nowadays the research area biomarkers is very large and therefore, biomarkers are defined and classified differently. Biomarkers are nowadays defined as characteristical features that can be objectively measured and evaluated as indicators for normal biological processes, pathogenic processes or pharmacological responses (Puntmann, 2009). These features can include everything from pulse and blood pressure, even basic chemistries to more complex laboratory tests of blood and other tissues, which allows being measured reproducibly (Strimbu & Tavel, 2001). According to the National Institute for Health (NIH) three key definitions were elaborated: (1) biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention”, (2) clinical endpoint defines “a characteristic or variable that reflects how a patient feels, functions or survives”, and (3) a surrogate endpoint reflects “a biomarker intended to substitute for a clinical endpoint with the potential for predicting the clinical benefit or harm on the basis of epidemiological, therapeutic, pathophysiological, or other scientific evidence” (Biomarker Definition Working Group, 2001; Frank & Hargreaves, 2003).

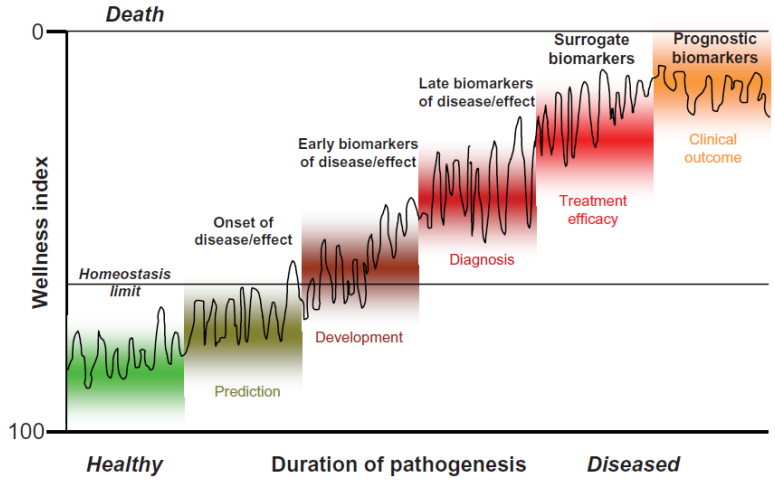


Fig.1 Different types of biomarkers; Classification system for biomarker discrimination (Mueller, 2012).

In simple words, a biomarker is an indicator of change, which fluctuates of time and biological influences (Mueller, 2012). A general classification system was established to discriminate biomarkers: type 0 biomarkers measure the natural history of a disease, which will establish clinical indicators over time. Type 1 biomarkers indicate an intervention effect of drugs and type 2 biomarkers are surrogate endpoint biomarkers (see Fig.1).

In this thesis, the focus was put on extracellular vesicles, so-called exosomes, which were used as type 0 biomarkers. Exosomes contain a wide range of biological molecules, especially DNA and numerous types of RNAs, which can serve as potential biomarkers.

1.1.1. Circulating Biomarkers

The first report of circulating biomarkers was made in 1948 by Mandel and Métais who found free nucleic acids in plasma samples. They detected free RNA and DNA in plasma of healthy as well as non-healthy patients (Mandel & Métais P., 1948). Circulating biomarkers are molecules, e. g. DNA fragments or other smaller molecules like RNAs that can be found cell-free in different human body fluids. Circulating biomarkers comprise nucleic acids, extracellular vesicles, proteins and metabolites from healthy and abnormal cells (Rapisuwon et al., 2016). Circulating nucleic acids, e. g. miRNAs can be incorporated into microparticles such as exosomes, in which they are protected from endonucleases (Valadi et al., 2007). Extracellular vesicles are a promising source for biomarkers because they are easy to identify and can be isolated based on their characteristics. They can act as vectors by transporting and protect biological molecules, such as different types of RNAs, DNA, and proteins (Mueller, 2012).

Circulating miRNAs provide advantages, such as the smaller size, stability and easy detection. However, the fact that miRNAs are limited cannot be ignored and the quantification is difficult (Zhang et al., 2018). MicroRNAs are evolutionary conserved small non-coding RNAs that are involved in RNA silencing as well as post-transcriptional regulation. In addition to that, it could be shown that some microRNAs play a role in proliferation, cell differentiation and apoptosis (Xi et al., 2017). Since the expression levels of RNAs are highly dynamic, the RNA profile can shed a light inside the state of biological systems. DNA can be genetically altered by so-called small nucleotide polymorphisms (SNPs), where only one nucleotide changes occur. Unfortunately, SNPs only explain a small fraction of risk of disease. However, DNA can also be chemically modified. Epigenetic modifications, such as DNA methylation can influence the regulation of gene expression and pathogenesis of human diseases. Epigenetic modifications can also be causal for disease development (Ahsan et al., 2017).

1.1.2. Biomarkers in Plasma

Because blood is a body fluid which communicates directly with nearly all organs and tissues, it is considered and believed to be a promising source for potential biomarkers. Along these lines, most clinical routine laboratory tests are performed from blood, respectively plasma or serum. After blood collection, plasma and serum are obtained by two different biochemical processes. When blood is coagulated, serum can emerge by clotting of fibrin and other related coagulation factors. During centrifugation, these proteins can be separated from serum. Proteins and metabolites are released from platelets into the serum. On the other hand, plasma is obtained by adding an anticoagulant, e.g. EDTA or heparin before blood collection, by which cells can be separated following centrifugation. Yu et al postulated in 2011 that plasma as source for diagnostic biomarkers is better in terms of reproducibility and has higher sensitivity when compared to serum (Yu et al., 2011).

Mitchell et al. demonstrated in 2008 by using a mouse prostate cancer xenografts model that plasma is a great source for microRNAs as blood-based biomarkers. Furthermore, they have shown that circulating miRNAs are stable and detectable in plasma from healthy and prostate cancer patients and that five of six candidate miRNAs displayed increased levels in cancer patients (Mitchell et al., 2008). Especially an increased level of circulating DNA and RNA detected in plasma of cancer patients led to the conclusion that circulating nucleic acids can serve as a non-invasive and sensitive method for diagnostics (Mitchell et al., 2008)

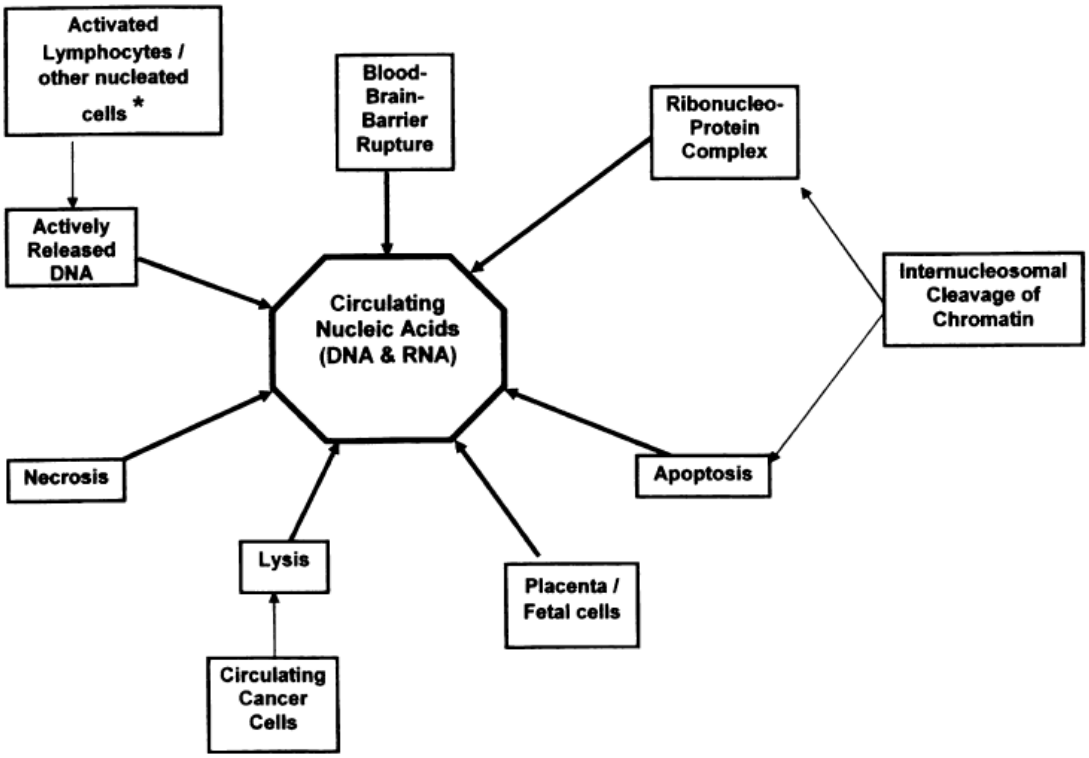


Fig.2 Pathways by which circulating nucleic acids are released into plasma; Different ways of how DNA and RNA can be released into blood-plasma (Swarup & Rajeswari, 2007).

In figure 2, different ways of how DNA and RNA can enter the blood system are illustrated schematically. Apoptosis is the major pathway in healthy patients by which nucleic acids are released into the blood system. Cancer cells can undergo lysis and their DNA and RNA content will be released. Furthermore, it is known that fetal nucleic acids are circulating in maternal plasma, which is nowadays used for prenatal testing. In addition to this examples, other pathways can also lead to freely circulating DNA and RNA in blood, e.g. necrosis, active release of DNA by activated cells or ribonucleoprotein complexes (Swarup & Rajeswari, 2007). However, extracellular vesicles can also be found in plasma samples which lead to the assumption that DNA and RNA are packed into vesicles and therefore protected from nucleases. Fernando et al postulated in 2017 that more than 93% of cell-free DNA is located in plasma exosomes, which makes extracellular vesicles an ideal source for diagnostic biomarkers (Fernando et al., 2017).

1.1.3. Biomarkers in Saliva

Saliva is a promising source of indicators for local, infectious and systemic disorders and serves as a mediator of the body's physiological conditions (Yoshizawa et al., 2013). Saliva in humans is a very heterogeneous body fluid and composed of 99,5% water, 0,3% proteins and 0,2% inorganic substances. The body fluid is clear and slightly acidic with a pH of 6.0 to 7.0 (Humphrey & Williamson, 2001). On average, the salivation ranges from 0.3 to 0.7 ml saliva per minute and per day 1.0 to 1.5 liters can be spilled (Yoshizawa et al., 2013). Three major and a few minor glands located in and around the mouth and throat are producing saliva. However, the three major saliva-producing glands can produce more than 90% of total saliva (Yoshizawa et al., 2013).

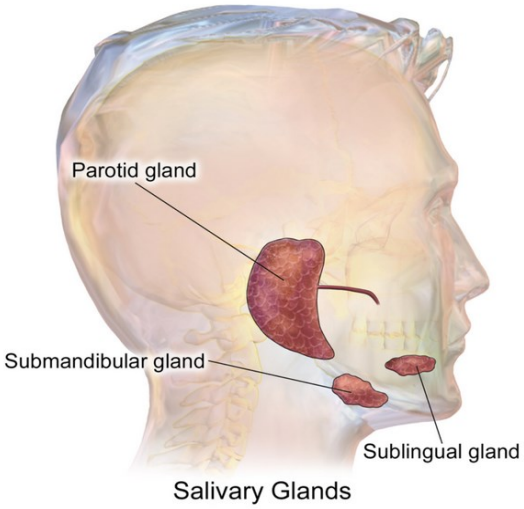


Fig.3 Salivary Glands; The three major glands are parotid, submandibular and sublingual gland. They are located in and around the mouth and throat and produce up to 1 to 1,5 liters saliva every day. More than 90% of total saliva are produced by these 3 major glands (Blausen Gallery, 2014)

Before researchers have postulated that blood-derived molecules can enter the saliva producing tissue, whole saliva was mostly used for diagnosis of local diseases. However, nowadays there is no doubt that circulating biomarkers can be secreted by salivary glands (Kaufman & Lamster, 2002). This led to the conclusion, that oral fluids contain molecular information reflecting the current state of health in an individual. For many years, blood was the main source of measurable biomarkers, but saliva also contains a wide range of biomolecules. The collection of blood is often more expensive, problematic and invasive. When comparing saliva with blood, numerous advantages appear. First, the collection of saliva is very easy and can be done by anybody. The procedure is non-invasive, which means it is painless and can reduce the discomfort of people. Saliva samples are much easier to handle and the shipping and storing requires no specific conditions (Yoshizawa et al., 2013). Furthermore, saliva is basically an unlimited sample matrix. All of the relevant molecule classes, such as DNA, mRNA, miRNA, lipids, and proteins, can be

found in saliva, which can be used as potential biomarkers. Putting all of these advantages together, saliva as sample matrix and novel source for biomarkers has great potential to replace blood in certain disease and application scenarios, e.g. when it comes to molecular test in vulnerable person groups such as very old people and children.

1.2. Exosomes

In 1983 Pan and Johnstone discovered that small vesicles were released from mature blood reticulocytes into the extracellular space (Harding et al., 2013; Pan & Johnstone, 1983). However, history of exosomes began much earlier when plasma was centrifuged at different speeds and high-speed centrifugation for 150 minutes at 31 000xg showed that the clotting time of the supernatant was extended. Additionally, when "the clotting factor of which the plasma is deprived" was added to plasma, the clotting time was shorter. This indicated that cell-free plasma contained a subcellular factor which promotes clotting of blood (Randolph & Service, 1946). In 1967 it was shown by electron microscopy that these subcellular factions consist of small vesicles derived from platelets, called "platelet dust" (Wolf, 1967). Finally, the term "exosomes" was invented a couple of years later by Rose Johnstone when vesicles from sheep reticulocytes were isolated (Johnstone et al., 1987). First, it was thought that these extracellular vesicles were important for externalization of specific plasma membrane proteins (Johnstone et al., 1989). Later, it was discovered that this pathway of protein sorting was highly selective and the importance of exosomes in protein transport could be shown (van der Pol et al., 2012).

Although almost any vesicle from a cell carries cell type-specific membrane and cytosolic components, exosomes are well characterized by certain features. The most important criteria for classification of extracellular vesicles are size, morphology, density and their composition. Exosomes are present in every biological fluid, fractions of body fluids and cultured media of cell culture (van der Pol et al., 2012). The diameter of exosomes ranges from 40 to 100 nm and after negative staining, their morphology is characteristically cup-shaped or round when exermined by transmission and cryo-electron microscopy. The density of exosomes ranges between 1,13 and 1,19 g/ml when floating on sucrose gradient (Simons & Raposo, 2009). Interestingly, the orientation of membrane proteins of exosomes is the same than in cells. Some proteins are enriched in exosomes and therefore can be defined as exosomal marker proteins, including CD 9, CD63, CD81, and CD82 (Simons & Raposo, 2009; van der Pol et al., 2012).

Two different pathways for exosome formation are known: (1) the classical pathway, which is the best studied pathway of exosome formation, and (2) the direct pathway, in which exosomes are released directly from the cell membrane.

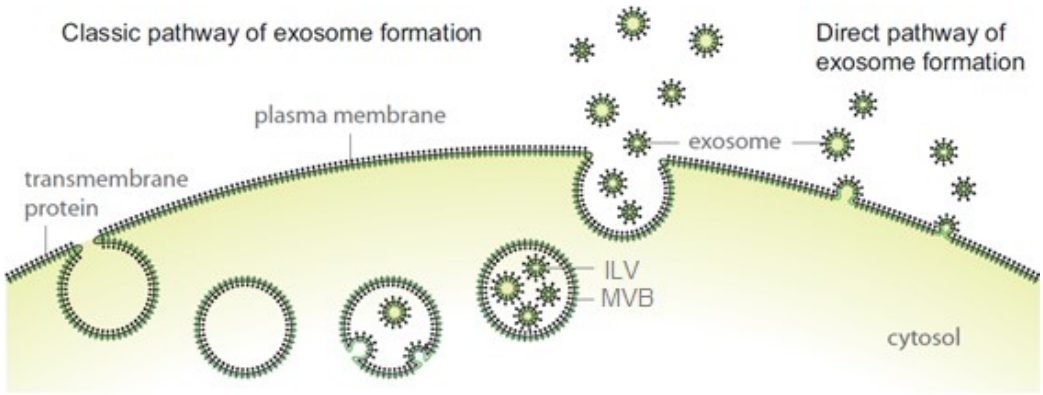


Fig.4 Pathways of Exosome Formation; In the classical pathway (left side) the exosomes are formed within MVBs (multivesivular bodies), which then fuse with the plasma membrane, resulting in the release of exosomes. On the right side, the direct pathway releases exosomes directly from the plasma membrane. (van der Pol et al., 2012).

The release of exosomes via the classical pathway begins with inward budding of membranous vesicles of endosomes. Intraluminal vesicles (ILVs) are formed in multivesicular bodies (MVBs), then the MVBs are transported to the membrane and fusion of MVBs with the plasma membrane results in release of exosomes. The ESCRT (endosomal sorting complex required for transport) machinery is the main force in this process and consists of four protein complexes: ESCRT-0, -I, -II, and –III. The ESCRT-0 together with –I and –II recognize ubiquitinated proteins in the endosomal membrane. ESCRT-III plays a crucial role in membrane budding and scission of ILVs. However, there is still no evidence that ESCRT components are necessary for the formation of ILVs (Hessvik & Llorente, 2018; Rashed et al., 2017; Record et al., 2009). Besides the classical pathway of exosome biogenesis, there is another pathway, called the “direct pathway”. It was shown, that T cells and erythroleukemia cell lines can release exosomes from their plasma membrane directly, or upon expression of HIV Nef or Gag, as well as after cross-linking of surface receptors (Booth et al., 2006; Fang et al., 2007; Lenassi et al., 2010; van der Pol et al., 2012). Interestingly, the formed exosomes from the direct pathway are indistinguishable from vesicles by the classical pathway in terms of their exosome marker and also by their diameter and density (van der Pol et al., 2012).

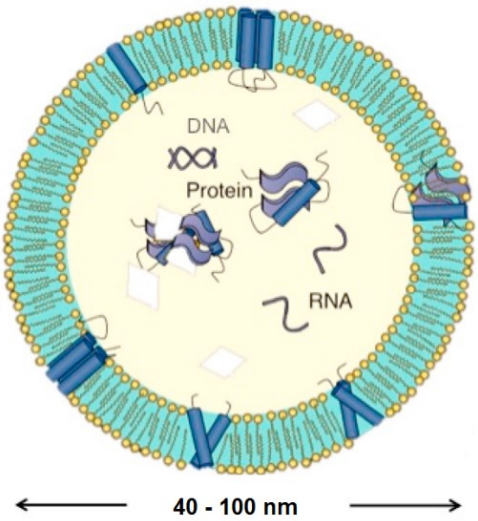


Fig. 5 Schematic illustration of exosomes and their content; The size of exosomes ranges from 40 to 100 nm and they are surrounded by a bilayer membrane, like cells. The molecular composition of exosomes varies and depends on the cell where they originate from. It has been found that exosomes harbor not only proteins, but also single-stranded and double-stranded DNA as well as different types of RNA (e. g. tRNA, mRNA, miRNA). These characteristics are very promising and lead to the presumption that exosomes may play a role in cell-to-cell communication (Li, Zhang, & Knez, 2017).

The molecular composition of exosomes depends on their host cell and contains molecular signatures or footprints of the diseased or healthy cell from which they are descended (Mathivanan et al., 2012). Exosomes were found to harbor bioactive molecules such as proteins, lipids, DNA and different types of RNA, such as miRNAs, mRNAs, and non-coding RNAs as well as other molecules. Studies in 2001 revealed that exosomes contain a particular subset of proteins which have the origin of endosomes, the plasma membrane or the cytosol. Only a very few proteins are from the nucleus, mitochondria, endoplasmatic reticulum (ER) and the Golgi apparatus (Boussac & Ricciardi-castagnoli, 2001). Additionally, exosomes include proteins from their parental cell. However, the most interesting feature of exosomes is, that RNA containing vesicles may play a role in cellular communication. RNAs, especially mRNAs were found out to be functional in exosomes and could be transferred to target cells and later on can be translated into proteins (Beach et al., 2014). MicroRNAs in exosomes can repress or activate gene expression in distant cells and therefore influence their gene expression pattern (Geis-asteggiante et al., 2017). Furthermore, other RNAs were also identified in exosomes, e. g. transfer RNA (tRNA), long noncoding RNA (lncRNA). Recent studies have discovered that exosomes even contain single-stranded DNA in form of cDNA and genomic DNA, as well as larger fragments of double-stranded DNA (Balaj et al., 2011; Kahlert et al., 2014; Thakur et al., 2014). Although the physiological function of exosomes in human body fluids is still a matter of debate, all of these findings together supported the assumption that exosomes may represent an ideal tool for biomarker discovery, especially when miRNA content is taken into consideration.

1.3. Aim of the Thesis

The thesis was done within an ongoing project "Epitype 2" which tries to investigate modifications in DNA-methylation as well as changes in miRNAs in exosomes obtained from saliva and blood of type 2 diabetes patients as novel and gender-optimized biomarkers that allow early and non-invasive type 2 diabetes diagnosis.

Hyperglycemia is the basis on which type 2 diabetes mellitus is diagnosed. The number of people suffering from type 2 diabetes is increasing worldwide. Different factors like genetic predisposition or behavioral and environmental risk factors can contribute to the illness (Tuomilheto et al., 2001). The degree of hyperglycemia is measured by the glycated hemoglobin level, which is a measure of mean blood glucose level or the plasma glucose level. Hyperglycemia results in tissue resistance to insulin, which has a glucose-lowering effect and can lead to B-cell dysfunction (Buchanan, 2001). Type 2 diabetes patients have a higher risk of having cardiovascular disease, premature death, blindness and kidney failures (Metastases et al., 2010). Many longitudinal studies have found out that insulin resistance is often present many years before hyperglycemia is even detectable. In pregnancy, the progressive insulin resistance is severe in the third trimester, which can be compared to the resistance in non-pregnant women with type 2 diabetes (Richard N Bergman, 1989). Therefore, an early diagnosis is very important to avoid type 2 diabetes mellitus.

For the project "Epitype 2", saliva and plasma samples are collected from type 2 diabetes, prediabetes and gestational diabetes patients and their DNA methylation profile, as well as their microRNAs in salivary and plasma exosomes, will be analyzed. Before this study is performed, a protocol had to be established to isolate extracellular vesicles and their DNA as well as their microRNA content from human saliva and plasma. The challenge was to develop a protocol to isolate as many nucleic acids as possible from exosomes because their limited amount represents a significant hurdle. Given the fact that the composition of saliva and plasma showed many differences in terms of behavior when isolating exosomes and with respect to the abundance of DNA and microRNA, these differences needed to be recognized and solved within this thesis. In the end, we were successful in establishing protocols for saliva and plasma which were suited to isolate exosomes and subsequently nucleic acids and served as a sound basis for future systemic disease biomarker discovery and early diagnostics for type 2 diabetes patients.

2. Material and Methods

2.1. Sample Collection

2.1.1. Plasma samples

The blood samples used along protocol optimization were provided by the „Österreichisches Rotes Kreuz“ (ÖRK), which is a private aid organization in Austria. The ÖRK collected blood in EDTA containing tubes (VACUETTE® RÖHRCHEN 9 ml K2E K2EDTA, Greiner Bio-One GmbH, Austria), centrifuged samples at 2000 xg for 10 minutes and prepared aliquots of 1,8 ml which were stored in cryo tubes (2,0ml Graduated Skirted Tube, STARLAB, Germany) at -80°C until use.

2.1.2. Saliva samples

Saliva samples were collected from different volunteers at AIT (Austrian Institute of Technology). The saliva samples were prepared according to following standard operating procedure (SOP). One hour before and while spitting the probands were not allowed to eat, drink or smoke and 10 minutes before spitting the mouth was rinsed with water. Saliva was collected in 50 ml tubes (Falcon™, Fisher Scientific GmbH, Austria). The amount of collected saliva ranged from 5 ml to 20 ml depending on the following experiments. After spitting the samples were stored at 4°C until further processing. The preparation of cell-free (cf) saliva was started by centrifugation at 3000 xg for 20 minutes at 4°C. Right after centrifugation supernatant (cf saliva) was transferred into a new 50 ml tube (Falcon™, Fisher Scientific GmbH, Austria) and aliquots of 2 ml were stored at -20°C. The salivary cell pellet was resuspended with remaining saliva solution and aliquoted in 2 ml tubes (Safe-Lock Tubes, Eppendorf, Germany) and stored at -20°C as well.

2.2 Exosome Isolation Kits

2.2.1. Exiqon

2.2.1.1. miRCURY™ Exosome Isolation Kit – Serum and plasma

This kit provides an exosome isolation method from serum and plasma in which the extracellular vesicles are isolated by precipitation. While the kit was originally developed and distributed by the company Exiqon it is meanwhile offered by Qiagen, which acquired Exiqon. The isolation was performed according to the manufacturer’s instruction. The sample volume could be upscaled to a total volume of 1,5 ml plasma or saliva. When using plasma as a matrix for some experiments thrombin (500U/ml) was added to activate the coagulation cascade, followed by a short centrifugation step for 5

minutes at 10 000 xg. The supernatant was transferred to a new tube and 200 µl precipitation buffer A was added to 0,5 ml sample. The sample mix was then incubated for at least 2 hours, preferably overnight, at 4°C, followed by centrifugation for 5 minutes at 500 xg for plasma samples and 30 minutes at 1500 xg for saliva samples. The resulting pellet was resuspended in 1x PBS (pH 7,4) and filled up to a total volume of 300 µl.

2.2.1.2. miRCURY™ Exosome Isolation Kit – Cells, urine and CSF

The miRCURY™ Exosome Isolation Kit for cells, urine and CSF is designed for processing cell-conditioned media, urine and CSF (cerebrospinal fluid) samples and was also used to isolate exosomes from plasma and saliva. A total volume of 500 µl sample was used to perform the isolation according to the manufacturer's protocol. 200 µl Precipitation buffer B was added to 0,5 ml sample and incubated for at least 2 hours, preferably overnight, at 4°C. Afterwards, the sample mixture was centrifuged for 30 minutes at 10 000 xg at room temperature and the pellet was resuspended in 1x PBS (pH 7,4) to get a total volume of 300 µl.

2.2.2. HansaBioMed Life Sciences

The company HansaBioMed Life Sciences has developed and optimized an effective tool for isolating exosomes in an efficient and fast way. Latex immunobeads are used for generic and specific exosome immunocapture from saliva samples. The immunobeads are covalently coupled with antibodies, which are specific against exosome surface antigens. The isolation was performed according to the manufacturer's protocol. The sample preparation included three centrifugation steps to eliminate cellular debris: (1) 10 minutes at 300 xg, (2) 20 minutes at 1200 xg and (3) 30 minutes at 10 000 xg. 10 µl of pre-coupled beads were added to 500 µl saliva sample and incubated overnight at 4°C in a rotator. Two bead washing steps were then performed with 1 ml 1x PBS (pH 7,4) each, whereby in each step the supernatant was removed by centrifugation at 5000 xg for 10 minutes. The exosome elution finally started with adding 10 µl of exosome elution buffer, followed by vortexing and incubation at room temperature for 5 minutes. 40 µl 1x PBS was then added and the mixture was centrifuged for 10 minutes at 5000 xg. The supernatant was

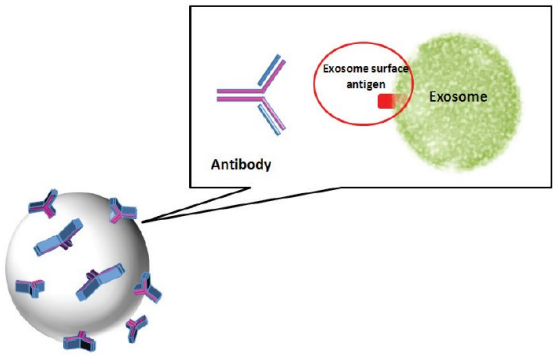


Fig.6 Schematic illustration of latex immunobeads (HansaBioMed); Latex Immunobeads are coupled with antibodies against surface antigens on exosomes (HansaBioMed Life Sciences).

transferred into a clean tube and stored on ice. The elution steps were repeated and the two supernatant fractions were then combined. The end-volume was adjusted to 300 µl with 1x PBS (pH 7,4).

2.2.3. Cell Guidance Systems

The Exo-spin™Kit from Cell Guidance Systems combines size-exclusion and chromatography to isolate exosomes from biological fluids including blood plasma/sera, cell culture media, urine and saliva. To remove cells and cell debris the saliva samples were centrifuged at 300 xg for 10 minutes. The supernatant was centrifuged again at 16000 xg for 30 minutes to remove any remaining cell debris. ½ volume of Exo-spin™ Buffer was added to precipitate exosomes followed by an incubation at 4°C for at least 1 hour, preferably overnight. The sample mixture was then centrifuged for 1 hour at 16 000 xg and the resulting pellet was resuspended in 100 µl 1x PBS (pH 7,4). For exosome purification, the Exo-spin™ column was prepared prior application of the sample. The buffer on top of the column was discarded by using a small pipette. To equilibrate the column, 200 µl 1x PBS (pH 7,4) was added and spun down for 10 seconds at 50 xg. Afterwards, the exosome containing pellet was applied to the column, followed by a centrifugation step at 50 xg for 60 seconds. To eluate the exosomes from the column, 300 µl 1x PBS (pH 7,4) was added to the column and centrifuged again for 60 seconds at 50 xg.

2.2.4. iZON Science

IZON Science provides with qEVoriginal Size Exclusion Column an exosome isolation method from biological samples based on Size Exclusion Chromatography (SEC). In general, the isolation of exosomes was performed according to the protocol. First, the column was equilibrated by rinsing the column with >10 ml 1x PBS (pH 7,4). 500 µl saliva sample was applied to the top. Fractions of 0,5 ml were collected, whereas the first six fractions do not contain any exosomes. The later fractions 7, 8 and 9 did contain extracellular vesicles. These three fractions were evaporated on centrifugal evaporator Univapo 150H (Uniequip). Then the exosomes were resuspended in 100 µl 1x PBS (pH 7,4) and the volumes were put together to get to a total volume of 300 µl.

2.2.5. Pierce™ Protein Concentrators

The company Thermo Scientific™ offers a disposable ultrafiltration centrifugal device that is normally used for concentrating, desalting and buffer exchanging of biological samples. It is available in four distinct molecular-weight cutoffs, whereas in the case of exosome preparation a molecular-weight cutoff of 100K was used. The

concentrators contain a vertical polyethersulfone (PES) membrane, which allows isolation of exosomes by size-exclusion. 0, 5 ml saliva sample was applied to the filter and was then centrifuged for 30 minutes at 15 000 xg. After two washing steps with 300 µl 1x PBS (pH 7,4), the exosomes were recovered in 300 µl 1x PBS (pH 7,4) by resuspension using a pipette.

2.3. EDTA Removal by Centrifugal Devices

To remove EDTA, which is a potential enzyme inhibitor due to its chelating properties, various centrifugal devices were tested on plasma samples.

2.3.1. Amicon® Ultra Centrifugal Filters

Amicon® Ultra-0,5 Centrifugal Filter Devices for volumes up to 500 µl with a molecular-weight cutoff of 100K provide fast ultrafiltration and easy recovery from dilute and complex sample matrices. Exosomes prepared from plasma were loaded onto the filter device and centrifuged at 14 000 xg for 30 minutes. The exosomes were then washed two times with 300 µl 1x PBS (pH 7,4) by resuspending, followed by centrifugation. To recover the exosomes, the Amicon® Ultra filter devices were placed upside down in a clean tube and spun for 2 minutes at 1000 xg. The recovered solution was filled up to 300 µl with 1x PBS (pH 7,4) for further use.

2.3.2. Pierce™ Protein Concentrators

As already mentioned, the Pierce™ Protein Concentrators are typically used for desalting. The centrifugal devices with a molecular-weight cutoff of 100K were used in our case to get rid of EDTA from plasma samples. The removal of EDTA was performed according to the manufacturer's protocol applying the exosome suspension to the concentrator device followed by centrifugation at 15 000 xg for 30 minutes. After two washing steps with 1x PBS (pH 7,4) the exosomes were recovered and the volume was adjusted to 300 µl with 1x PBS (pH 7,4).

2.3.3. Pall Life Sciences

Nanosep® centrifugal devices 100K provide rapid and convenient concentration and removal of salts of biological samples. The membrane consists of polypropylene that provides reduced non-specific adsorption and high recoveries. The exosomes were centrifuged up to 14 000 xg. Biomolecules larger than 100K were retained in the reservoir. EDTA and other low molecular-weight molecules are smaller than 100K and can pass through the membrane. After two washing steps with 1x PBS (pH 7,4), the exosomes were recovered with a pipette and the volume was filled up to 300 µl with 1x PBS (pH 7,4).

2.4. Enzymatic Treatments

2.4.1. DNase I

Deoxyribonuclease I belongs to the family of endonucleases and can digest single- and double-stranded DNA to oligodeoxyribonucleotides, whereby it hydrolizes phosphodiester bonds preferentially at phosphodiester linkages which produce mono- and oligodeoxyribonucleotides with 5'-phosphate and 3'OH groups. In presence of Mg^{2+} DNase I cleaves dsDNA independently in a statistically random fashion. The addition of EDTA to DNase I can inactivate the enzyme by chelating ions, that are required for the digestion. In order to create perfect conditions for digestion, a 10x Digestion Buffer (with $MgCl_2$) needs to be added to the reaction. In this study, 8 U DNase I was used to digest DNA potentially attached to the surface of exosomes. DNase I from Thermo Scientific is a recombinant enzyme that was isolated from *E. coli*, therefore it's optimal incubation temperature was at 37°C for 1 hour. DNase I treatment of exosomes ensure that only DNA within the exosomes is isolated in the following steps.

2.4.2. RNase A

RNase A is an endoribonuclease that selectively degrades single-stranded RNA 3' next to pyrimidine residues (cytosine, uracil), which results in cyclic nucleotide monophosphates to 5'- OH and 2'-, 3'-cyclic monophosphate. RNase A is used to remove single- and double-stranded RNA as well as RNA strands in RNA-DNA hybrids. In this thesis, 0,6 ng RNase A has been used to cleave unwanted nucleic acids that are attached to the exosomes. RNase A from Thermo Scientific is active under a wide range of reaction conditions and it works best at 37°C. For inhibition, a mammalian ribonuclease inhibitor, e.g. Thermo Scientific RiboLock RNase Inhibitor was used for some of the experiments.

2.4.3. Proteinase K

Proteinase K is a serine protease which has a broad substrate specificity. It hydrolyzes a broad range of peptides, therefore it will degrade many proteins in their native conformation. Proteinase K is used in molecular biology to digest proteins from DNA and RNA preparations. In this study, proteinase K from Zymo Research was used to digest unwanted proteins from exosomes and nucleic acids. The source of the enzyme was *Engyodontium album* and it's enzymatic optimum is reached at a pH-value of 4,0 till 12,0 and at a temperature between 25°C and 65°C (Zymo Research, n.d.). 20 mg lyophilized proteinase K was reconstituted by adding 1040 µl proteinase K storage buffer and was stored at -20°C until further use. In order to create perfect conditions for

proteinase K, PK digestion buffer (Zymo Research) was mixed together with the enzyme. Additionally, DNA/RNA Shield™ (2 times concentrated) from Zymo Research was added to the mixture. The properties of the DNA/RNA Shield™ are to inactivate infectious agents, such as viruses and bacteria, and to preserve the genetic integrity and expression profiles of samples (Zymo Research, n.d.). In this study, DNA/RNA Shield™ (2 times concentrated) was used to ensure nucleic acid stability after lysis of exosomes. To prepare 1x solution of DNA/RNA Shield™ (2 times concentrated), an equal amount of the 2x concentrate was mixed with resuspended exosomes. 68 µl PK digestion Buffer and 0,68 mg reconstituted proteinase K was added to the mixture. Subsequently, the solution was incubated at 55°C for 30 minutes. After incubation, the sample was centrifuged at maximum speed for two minutes and the aqueous supernatant was transferred into a clean tube for further nucleic acid isolation.

2.5. DNA Isolation Kits

2.5.1. Roche Applied Science

The High Pure PCR Template Preparation kit from Roche Applied Science purifies nucleic acids from whole blood, cultured cells and tissue. In our case, the kit was used for DNA isolation from exosomes. The isolation was performed according to the manufacturer's manual with some modifications. A sample volume of 300 µl was mixed with 300 µl binding buffer. 0,68 mg Proteinase K was added for degradation of proteins followed by mixing immediately. The mixture was incubated for at least 10 minutes at 70°C to ensure the optimal activity for the enzyme. After adding 250 µl isopropanol, the sample mix was applied twice to a filter tube. 500 µl of the special Inhibitor Removal Buffer was added, which increases the sensitivity and reproducibility. The wash-and-spin steps were performed according to the protocol. The DNA was finally eluted in 100 µl of 70°C prewarmed elution buffer after an incubation of 15 minutes at 45°C.

2.6. RNA Isolation Kits

2.6.1. Exiqon

2.6.1.1. miRCURY™ RNA Isolation – Biofluids

Exiqon's miRCURY™ RNA Isolation Kit – Biofluids provides a method for isolation and purification of RNA from serum, plasma and other biofluids. The isolation is based on spin column chromatography using a proprietary resin as a separation matrix where small RNAs are separated from other cellular components such as proteins. The isolation was performed according to the manufacturer's instructions by using 300 µl sample that was mixed with 90 µl lysis solution BF and incubated for 10 minutes at room

temperature. 30 µl protein precipitation BF was added and the mixture was centrifuged for 3 minutes at 11 000 xg. The supernatant was mixed with 400 µl isopropanol. The sample mix was loaded into the microRNA mini spin column BF, followed by incubation of 2 minutes at room temperature. After some washing-steps with wash solutions 1 and 2 BF, the RNA was eluted in 100 µl ddH_2O.

2.6.1.2. miRCURY™ RNA Isolation – Cell&Plant

The RNA isolation kit for cells and plants is used to isolate and purify total RNA from cultured animal cells, small tissue sample, blood, bacteria, yeast, fungi, bacteria and plants. The purification was performed according to the manufacturer's protocol. The exosomes were lysed with 350 µl lysis solution and 200 µl ethanol 100% was added to the mixture and then the sample mix was loaded onto the column. The RNA was washed three times with 400 µl of the included Wash Solution and the RNA was eluted in 100 µl ddH_2O.

2.6.2. Qiagen

The miRNeasy Serum/Plasma kit is designed for purification of cell-free total RNA, including miRNA from plasma and serum. The isolation is achieved by a combination of phenol/guanidine-based lysis and silica-membrane-based purification of RNA. The following procedure was done as described in the manufacturer's protocol. The sample volume was 500 µl and 2500 µl QIAzol lysis reagent was added to the sample followed by a short incubation of 5 minutes at room temperature. Chloroform was added of an equal volume to starting sample (500 µl), which resulted in the separation into aqueous and organic phase. The sample was centrifuged for 15 minutes at 12 000 xg at 4°C. The upper, aqueous phase was extracted, and 1,5 volume of 100% ethanol (2250 µl) was added to provide appropriate binding conditions for all RNA molecules. The sample was then applied to the RNeasy MinElute spin column. The column was washed several times and the RNA was eluted in a small volume of 100 µl ddH2O.

2.6.3. Zymo Research

Zymo Research provides the Quick-RNA MiniPrep for isolation of total and small/miRNAs from cells and soft tissue. In principle, the isolation was performed according to the manufacturer's protocol. 1 ml RNA lysis buffer was added to 500 µl sample, followed by a short centrifugation for 1 minute at 10 000 xg. The supernatant was applied to the spin-away filter and centrifuged again for 1 minute at 10 000 xg 1 volume of 100% ethanol was mixed with the flow-through and added into the Zymo-Spin™ Columns. After the wash-and-spin steps, the RNA was eluted in 100 µl ddH_2O.

2.7. DNA/RNA Isolation Kits

2.7.1. Zymo Research

The simultaneous isolation of DNA and RNA from small amounts of cells and tissue is possible with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. The isolations of nucleic acids were performed according to manufacturer's protocol. 900 µl DNA/RNA Lysis Buffer was added to 500 µl sample volume. The sample mix was transferred onto Zymo-Spin™ IIIC Column and centrifuged for 1 minute at 12 000 xg. To the flow-through, which contained RNA, 1 or 1,5 volume 100% ethanol was added and was then applied into Zymo-Spin™ IIC Column. Followed by centrifugation at 12 000 xg for 1 minute. The columns were washed several times with DNA/RNA Prep Buffer and DNA/RNA Wash Buffer. The DNA was eluted in 100 µl ddH_2O including an incubation of 5 minutes at room temperature. Whereas the RNA also was eluted in 100 µl ddH_2O, but was only incubated for 1 minute before elution.

2.7.2. Qiagen

AllPrep® DNA/RNA/miRNA Universal from Qiagen enables simultaneous purification of DNA and total RNA from cells and all types of tissue. 500 µl sample was first lysed with 300 µl denaturing guanidine isothiocyanate-containing buffer. The sample mix was then applied to AllPrep DNA Mini spin column and centrifuged at full speed for 30 seconds. This column was washed and pure to elute DNA in 100 µl ddH_2O. The flow-through from the AllPrep DNA Mini spin column was digested by 80 µl proteinase K in the presence of 750 µl 100% ethanol. The mixture was transferred to the RNeasy Mini spin column and was centrifuged at maximum speed for 15 seconds. DNA digestion with 10 µl DNase I in 70 µl Buffer RDD ensures high-yields of RNA. After incubation for 15 minutes at room temperature (RT), 500 µl Buffer FRN was added to the column and centrifuged for 15 seconds at maximum speed. Washing with Buffer RPE was followed by adding 500 µl 100% ethanol onto the spin column and centrifuged for 2 minutes. Purified RNA was eluted in 100 µl ddH_2O.

2.8. Final Protocol for Isolation of DNA and miRNA from human Saliva and Plasma Exosomes

The final protocol for isolation of DNA and miRNA from human saliva and plasma exosomes is described in this chapter. For the isolation of exosomes miRCURY Exosome serum/plasma kit from Exiqon (now Qiagen) was used for saliva and plasma. For the subsequent nucleic acid isolation ZR-Duet™ DNA/RNA MiniPrep from Zymo Research was applied to the plasma- and saliva-derived exosome pellets. Additional material for purification of our sample matrix was needed as desribed underneath.

1,5 ml plasma was previously processed with 15 µl thrombin (500 U/ml) to activate the coagulation cascade. Samples were spun for 5 minutes at 10 000 xg and the supernatant was transferred into a new tube. Then, plasma, as well as saliva exosomes, were isolated from 1,5 ml starting volume with miRCURY Exosome serum/plasma kit from Exiqon by adding 600 µl precipitation buffer A. The solution was mixed and incubated overnight at 4°C. Plasma and saliva samples had to be treated differently. While plasma samples were centrifuged at 500 xg for 5 minutes, saliva samples had to be centrifuged at 1 500 xg for 30 minutes. The supernatant was discarded and the resulting exosome pellet was resuspended in 300 µl 1x PBS (pH=7,4). The next step was the removal of EDTA from plasma samples by simple size-exclusion. For this purpose, Amicon® Ultra-0,5 Centrifugal Filter Devices with a molecular-weight cutoff of 100K were needed to get rid of EDTA from plasma samples. Exosomes from plasma were loaded onto the filter device and centrifuged at 14 000 xg for 30 minutes. The exosomes were then washed two times with 1x PBS (pH=7,4) by resuspending, followed by centrifugation. To recover the exosomes, the Amicon® Ultra filter devices were placed upside down in a clean tube and spun for 2 minutes at 1000 xg. The recovered solution was filled up to 300 µl with 1x PBS (pH=7,4) for further use. Then, plasma and saliva exosomes were treated with DNase I (8 µl, together with 10x Mg^{2+} buffer) and RNase A (1:500 dilution, 3 µl), followed by an incubation for 1 hour at 37°C. After digestion, nucleic acids were protected with DNA/RNA Shield™ (2 times concentrated) from Zymo Research. Then, 34 µl proteinase K (20 mg/ml) together with 68 µl PK digestion buffer (both from Zymo Research) were added to the mixture and samples were incubated for 30 minutes at 55°C. After incubation, the samples were centrifuged at maximum speed for 2 minutes. The aqueous supernatant was transferred into an RNase-free tube. DNA and RNA were isolated simultaneously with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research according to the manufacturer's protocol (see also section 2.7.1.).

2.9. Nucleic Acid Concentration Measurements

2.9.1. PicoGreen DNA Concentration Measurements

DNA concentration measurements were performed with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) according to the manufacturer's protocol. Before the experimental samples could be measured the linear detection range of the standard curve was determined by a concentration range of experimental standard DNA ranging from 0,025 pg/µl to 10,000 pg/µl. The assay mix for Lambda DNA standard was prepared by a 1:2 dilution of 5 µl sample with PicoGreen working solution. After an incubation time of 2 minutes, the fluorescence level was ready to be measured at 525 nm. First, 1 µl blank (water or elution buffer) was put on the optical measurement surface to calibrate the system. For the samples to be measured, different dilutions were prepared ranging from 1:2 to 1:20, depending on the DNA content in the samples. Between each measurement, the surface was cleaned with a lint-free lab wipe. The DNA concentration was analyzed on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific).

2.9.2. RiboGreen RNA Concentration Measurements

Invitrogen offers the Quant-iT™ RiboGreen® RNA Assay Kit to measure RNA in solution. The concentrations of experimental RNA to be used for the standard curve ranged from 0,025 pg/µl to 10,000 pg/µl and were prepared with ribosomal RNA. The assay mix for ribosomal RNA standard was prepared by a 1:2 dilution with RiboGreen working solution. Subsequently, the samples were protected from light and incubated for 2 minutes at room temperature. The Fluorescence level of 1,0 µl at 525 nm was measured on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific). The dilution for the samples to be measured were adjusted correspondingly to the amount of RNA.

2.10. Volume Reduction of DNA/RNA Eluate after Nucleic Acid Extraction

2.10.1. SpeedVac

Centrifugal vacuum concentrator can remove solvents from samples. The centrifugal evaporator Univapo 150H (Uniequip) offers rapid and efficient concentration and drying of small-volume DNA/RNA samples. By vaporizing the elution fluid, in which the sample was dissolved, DNA and RNA samples were concentrated until the requested volume was reached.

2.10.2. Christ Freeze-Drying

Freeze-drying is a method for gentle drying or preservation of thermally sensitive materials. Rotational vacuum concentrator was used to remove aqueous media from samples. Above a process pressure of 0,120 mbar drying occurs directly from a frozen state (-40°C), bypassing the liquid phase. With Alpha 2-4 LSCplus (Martin Christ Gefriertrocknungsanlagen GmbH) frozen DNA and RNA samples were concentrated by removing aqueous solution.

2.10.3. Zymo Research DNA and RNA Concentrator Columns

Zymo Research offers different purification kits that provide purification and concentration of DNA and RNA samples in less than two minutes. For DNA samples DNA Clean & Concentrator-5 was used to concentrate samples for further use. 5 volumes of DNA Binding Buffer were added according to the manufacturer's protocol and the mixture was transferred to the supplied Zymo-Spin™ Column. After centrifugation for 30 seconds and two washing-steps with 200 µl DNA Wash Buffer, DNA was eluted with 10 µl DNA Elution Buffer. For RNA samples, RNA Clean & Concentrator™-5 was needed to concentrate. 2 volumes of RNA Binding Buffer and an equal volume of 100% ethanol was added to the sample and the mixture was bound to the Zymo-Spin™ IC Column. After several washing steps with RNA Prep Buffer and RNA Wash Buffer, RNA was eluted from the column with 10 µl DNase/RNase-Free Water.

2.11. Real-Time Quantitative PCR for DNA/miRNA Quantification

Real-time PCR is a powerful and rapid technique for measuring and quantifying genes or also gene transcripts. The procedure is based on a thermal cycler, which uses a laser to scan a beam of light. Each reaction includes a dye-containing probe that emits fluorescent light when illuminated by a laser. The light emitted by these dyes correlates to the amount of PCR product amplified. The light is captured by a detector, which provides a readout on the amount of fluorescence produced after each PCR cycle. EvaGreen® Dye (Biotum, USA) binds double-stranded DNA. The higher the amount of nucleic acids, the higher the amount of fluorescent light emitted (Klug et. al, 2012). In this thesis, real-time PCR was used to quantify the yields of nucleic acids. A master mix was prepared, sample was added and then the reaction mix was transferred to a 384-well PCR plate and subsequently cycled on the LightCycler® 480 (Roche) using the program as described in Table 2. All of the used solutions and chemicals are listed in Table 1.

Table 1: Chemicals for qPCR

Chemicals	Company
10x Taq Buffer with 15 mM $MgCl_2$	Qiagen, Germany
dNTPs set, 100mM	Thermo Fisher Scientific, USA
EvaGreen 20x	Biotium, USA
HotStar Taq Polymerase [5000U/µl]	Qiagen, Germany

Table 2: Master mix and program of HotStar Taq Polymerase chemistry for DNA-qPCR

master mix	[µl]	program			
ddH_2O	7,12	Activation	95°C	15 min	
10x Taq Buffer with 15 mM $MgCl_2$	1,20	Denaturation	95°C	40 sec	
dNTPs [2mM]	0,96	Annealing	65°C	40 sec	45cycles
EvaGreen 20x	0,168	Elongation	72°C	1 min 20 sec	
Forward Primer [10mM]	0,24	Final Elongation	72°C	7 min	
Reverse Primer [10mM]	0,24				
HotStar Taq Polymerase [5000U/µl]	0,072				
DNA	2,00				
Volume	12,00				

For mircoRNA real-time PCR cDNA was first generated via reverse transcription (see chapter 2.10.1.). The annealing temperature for primers were set to 60°C (table 3). For some of the experiments, HotStar Taq Plus [5U/µl] was used for amplification, whereas the activation temperature was shorter and only 5 minutes for activation were needed.

Table 3: Master mix and program of HotStar Taq Polymerase chemistry for miRNA-qPCR

master mix	[µl]	program			
ddH_2O	7,12	Activation	95°C	15 min	
10x Taq Buffer with 15 mM $MgCl_2$	1,20	Denaturation	95°C	40 sec	
dNTPs [2mM]	0,96	Annealing	60°C	40 sec	45 cycles
EvaGreen 20x	0,168	Elongation	72°C	1 min 20 sec	
Primer Uni Reverse [100mM]	0,24	Final Elongation	72°C	7 min	
Forward Primer [10mM]	0,24				
HotStar Taq Polymerase [5000U/µl]	0,072				
cDNA	2,00				
Volume	12,00				

2.11.1. TaqMan Reverse Transcription (Thermo Fisher Scientific)

With TaqMan® Reverse Transcription Reagents Applied Biosystems by Thermo Fisher Scientific provides a kit with all the necessary components to perform reverse transcription of RNA to cDNA. Reverse transcriptase is used to generate cDNA molecules. RT-PCR can be used to evaluate different gene expression levels, because the amount of cDNA is based on the relative amount of RNA molecules in the sample. In all experiments the TaqMan RT chemistry was used. A master mix was prepared as shown in Table 4. Stem-loop RT primers were used to amplify cDNA from RNA, which are better in efficiency and specificity when compared to conventional ones. Stem-loop RT

primer pool (20nM) was prepared before TaqMan Reverse Transcription master mix was prepared.

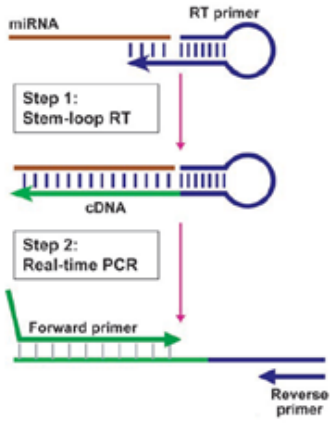


Fig.7 Schematic illustration of TaqMan miRNA assay; First, stem-loop RT primers bind to 3' end of miRNA molecules and reverse transcriptase amplifies cDNA. The next step is the quantification of the RT product using conventional real-time PCR (Chen et al., 2005)

After RNA was added, the sample was incubated in the T3000 Thermocycler (Biometra, Germany). The amplified cDNA was diluted 1:3 before further used in the PCR mastermix.

Table 4: Master mix and program of TaqMan Reverse Transcription

master mix	**[µl]**	**program**			
ddH_2O	5,66	Activation	16°C	30 min	
10x Buffer	1,50	Denaturation	20°C	30 sec	
dNTPs [100mM]	0,15	Annealing	42°C	30 sec	60 cycles
RNase Inhibitor [20U/µl]	0,19	Elongation	50°C	1 sec	
Reverse Transcriptase	1,00	Final Elongation	82°C	5 min	
RT-loop Primer [20nM]	1,50				
RNA	5,00				
Volume	15,00				

2.12. Fragment Analyzer

The Fragment Analyzer™ (Agilent, former Advanced Analytical) system is based on capillary electrophoresis (CE), in which automated and high throughput separation and quantification of nucleic acids is possible. The capillaries contain a separation gel matrix containing a fluorescent intercalating dye, which is automatically primed into the capillaries prior to each run. To separate double-stranded DNA and/or RNA, an electric field is applied through a bore (50µm) fused silica capillary array filled with various conductive gel matrices. By applying a high voltage to the capillary array, the negatively charged DNA/RNA fragments migrate differentially through the gel matrix towards the positive electrode as a function of length or size. At the far end of the capillary, detection of nucleic acids is achieved by an intercalating dye in the separation gel matrix, which fluorescence, when bound to double-stranded DNA or RNA. A high-intensity light emitting diode (LED) excitation light source is focused across the capillary array

detection window and imaged onto a sensitive, two-dimensional charge-coupled device (CCD) detector. The fragment size is measured by the time required to pass through the detection window and the relative emission signal provides the nucleic acid concentration (AATI, 2011).

2.12.1. Advanced Analytical – High Sensitivity RNA Analysis Kit (15nt)

With the High Sensitivity RNA Analysis Kit (15 nt) from Advanced Analytical it is possible to assess the integrity of total RNA and messenger RNA (mRNA) samples with a sizing range from 200 nt – 6,000 nt. The High Sensitivity RNA Analysis Kit (DNF-472) provides accurate quantification, measuring sample concentrations in line with a standard fluorescent method. In this thesis, the kit was used to analyze miRNA from isolated exosomes and was performed according to manufacturer's instructions. In short, an intercalating dye was added to the RNA separation gel and the 5x Capillary Conditioning Solution was diluted to 1x. Then, 1 ml/well of 1x Inlet Buffer was added in one row of a deep-well plate. In another row, 1 ml/well capillary storage solution needed to be replaced every 2-4 week. 0,25x TE Rinse Buffer was placed in one row of a 384 well-plate. The initial RNA latter was diluted to a working concentration of 2 ng/µl. 2 µl RNA sample was denaturized for 2 minutes at 70°C and immediately stored on ice to cool down. Then, the sample was mixed with 18 µl diluent marker and placed in the sample plate. To Unused wells 20 µl ddH_2O was added. Each plate was placed in the associated drawer and the correct positions were selected in the software. Afterwards, the separation was started.

2.12.2. Advanced Analytical – High Sensitivity NGS Fragment Analysis Kit

Advanced Analytical provides with the High Sensitivity NGS Fragment Analysis Kit (DNF-474) a method that efficiently and accurately determines typically the average size of a sequencing library and its concentration. The separation and detection of the fragments is based on the same principle as described above. With this kit it is in general possible to determine the size of DNA fragments ranging from 1 to 6000 bp. The procedure is very similar to the protocol described in chapter 2.11.1. Advanced Analytical – High Sensitivity RNA Analysis Kit (15nt). An intercalating dye was added to genomic DNA separation gel. 5x capillary condition solution was diluted to 1x and 1x fresh Inlet Buffer was added into a deep-well plate and capillary Storage Solution was placed in the deep-well plate as well. Furthermore, 200 µl 0,25x TE Rinse Buffer was added in a 384-well plate. 2 µl sample was mixed with 22 µl diluent marker and unused wells were filled

with 24 µl ddH_2O. Each plate was put in the correct drawer and the right trays were selected. After that, the separation of DNA fragments was started.

3. Results and Discussion

3.1. Nucleic Acid Isolation Kit Test

Exosomes are known to contain a limited amount of DNA and RNA, therefore it was necessary to test different isolation kits to achieve the largest yield possible. Two exosome isolation kits were tested: (1) miRCURY™ Exosome Isolation Kit for Serum and plasma and (2) miRCURY™ Exosome Isolation Kit for Cells, urine and CSF. For nucleic acid isolation, four RNA isolation kits were compared: (1) miRCURY RNA Isolation – Biofluids, (2) miRCURY RNA Isolation kit – Cell&Plant, (3) Qiagen miRNeasy Serum/Plasma and (4) Zymo Research Quick-RNA MiniPrep kit. For DNA isolation, the High Pure PCR Template Preparation kit from Roche Applied Science was used and compared to two DNA/RNA isolation kits: (1) ZR-Duet™ DNA/RNA MiniPrep from Zymo Research and (2) AllPrep® DNA/RNA/miRNA Universal from Qiagen.

Saliva and plasma from healthy humans were collected and 2 different pools for each matrix were prepared, whereas 500µl sample volume was used per isolation. First, exosomes were isolated from both pools with (1) miRCURY™ Exosome Isolation Kit for Serum and plasma and subsequently, RNA and DNA was isolated with the different kits. Nucleic acids were also isolated from cell-free plasma. Exosomes from saliva pools were isolated with (1) miRCURY™ Exosome Isolation Kit for Serum and plasma and (2) miRCURY™ Exosome Isolation Kit for Cells, urine and CSF. For cell-free saliva, none of the exosome isolation kits were used and nucleic acids were isolated straight away. For evaluation of the different isolation kits, quantitative real-time PCR was performed for DNA. In the case of RNA, reverse transcription with TaqMan® Reverse Transcription Reagents Applied Biosystems (Thermo Fischer) was performed first, followed by quantitative real-time PCR. Five specific primers targeting human genes (SNRPN, H19, TBP, TJP2, GATA4) were used to identify the best isolation protocol for DNA. For quantification, a DNA dilution series (64 ng – 0, 0039 ng) was included. To evaluate RNA yield, four different primers targeting specific microRNAs (miR-16, miR-200, miR-21-5p, miR-30c-5p) were used. To quantify the results, a RNA dilution series, ranging from

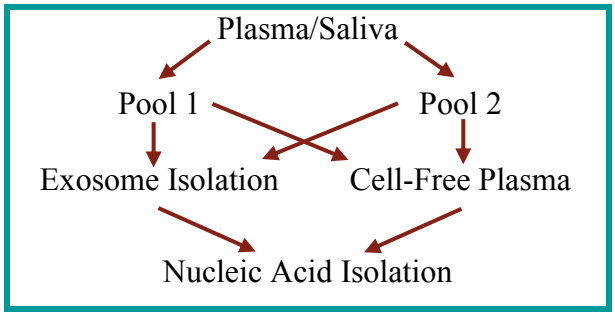


Fig. 8 Schematic illustration of the workflow; Pools of 500 µl were prepared and exosomes were or were not isolated. Subsequently, RNA and/or DNA were isolated with different kits.

100 ng to 0, 00038 ng, was added. The resulting Ct-value tells how many cycles it took to detect a signal from the samples. Therefore, a low Ct-value indicates a higher amount of nucleic acids, because the amplification of target-genes starts earlier. In Fig.9 the results for DNA isolation are illustrated. The isolation of DNA with Qiagen's AllPrep® DNA/RNA/miRNA Universal kit (red bars) was not possible for plasma samples. However, isolation with AllPrep® DNA/RNA/miRNA Universal was successful in saliva samples, but the Ct-value was higher in comparison to the other isolation kits. ZR-Duet™ DNA/RNA MiniPrep from Zymo Research (turquoise bars) achieved the lowest ct-value indicating the highest DNA-yield. The company Roche with its High Pure PCR Template Preparation kit, which only isolates DNA, could achieve high amounts of DNA as well. When comparing the two different miRCURY™ Exosome Isolation kits from Exiqon for salivary exosome isolation, no significant difference in exosome yield can be noticed.

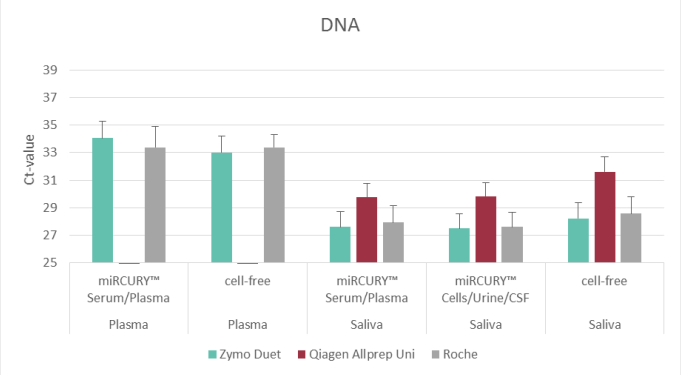


Fig.9 Evaluation of different DNA isolation kits with qPCR; bars are reflecting amount of DNA as Ct-values

To evaluate the best isolation kit for RNA, RT-qPCR was performed to quantify the yields of nucleic acids, which is illustrated in Figure 10. First of all, it is recognizable that both DNA/RNA isolation kits (ZR-Duet™ DNA/RNA MiniPrep from Zymo Research and AllPrep® DNA/RNA/miRNA Universal from Qiagen) in general have worked worse when compared to the RNA isolation kits only. The Quick-RNA MiniPrep kit from Zymo Research (represented by green bars) worked the worst, which can be seen by Ct-values above 36 indicating low amounts of RNA. The best RNA isolation kit was miRCURY RNA Isolation kit for cell and plant (represented by blue bars), because the Ct-values were actually the lowest in almost all cases. A difference can be noticed when comparing the exosome isolation kits.

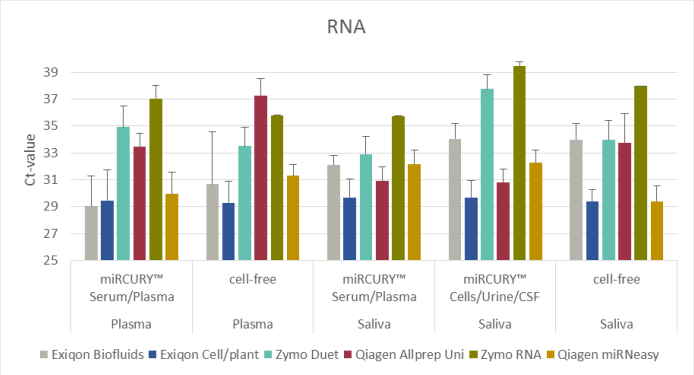


Fig.10 Evaluation of different RNA isolation kits with RT-qPCR; bars are reflecting amount of RNA as Ct-values

The miRCURY™ Exosome Isolation kit for serum and plasma has worked better in saliva than the miRCURY™ Exosome Isolation kit for cells, urine and CSF, since the ct-values are lower in the case of miRCURY™ Exosome Isolation kit for serum and plasma. In addition to quantitative real-time PCR, concentrations were measured on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific) by using a fluorescence dye. First, a blank (water or elution buffer without nucleic acid) was pipetted on the optical measurement surface to calibrate the system. Then 1µl sample was applied and measured. Between each measurement, the surface was cleaned with a lint-free lab wipe. First, a standard curve was measured and a linear equation was set up, from which the sample concentration was calculated. DNA concentrations were measured with Quant-iT™ PicoGreen® dsDNA Assay Kit from Invitrogen.

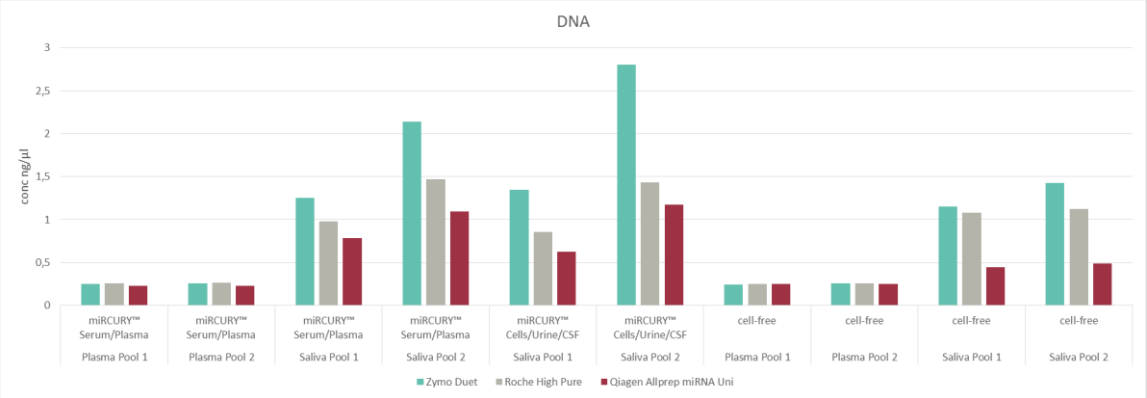


Fig.11 Evaluation of different DNA isolation kits with Quant-iT™ PicoGreen® dsDNA Assay Kit; bars are reflecting concentration of DNA in ng/µl.

Interestingly, both pools have shown the same isolation pattern when isolating only cell-free DNA as well as DNA isolated from exosomes, which is illustrated in figure 11. The highest amount of DNA was achieved by ZR-Duet™ DNA/RNA MiniPrep from Zymo Research (illustrated by turquoise bars) in all cases. With the AllPrep® DNA/RNA/miRNA Universal from Qiagen (red bars) it was not possible to isolate as much DNA as with the ZR-Duet™ DNA/RNA MiniPrep and the lowest amount of DNA was isolated. The High Pure PCR Template Preparation kit from Roche (reflected by grey bars), which only isolates DNA, was in the midrange between ZR-Duet™ DNA/RNA MiniPrep and AllPrep® DNA/RNA/miRNA Universal. It was not possible to isolate as much DNA from plasma compared to saliva. This is true for exosomal DNA as well as for cell-free DNA.

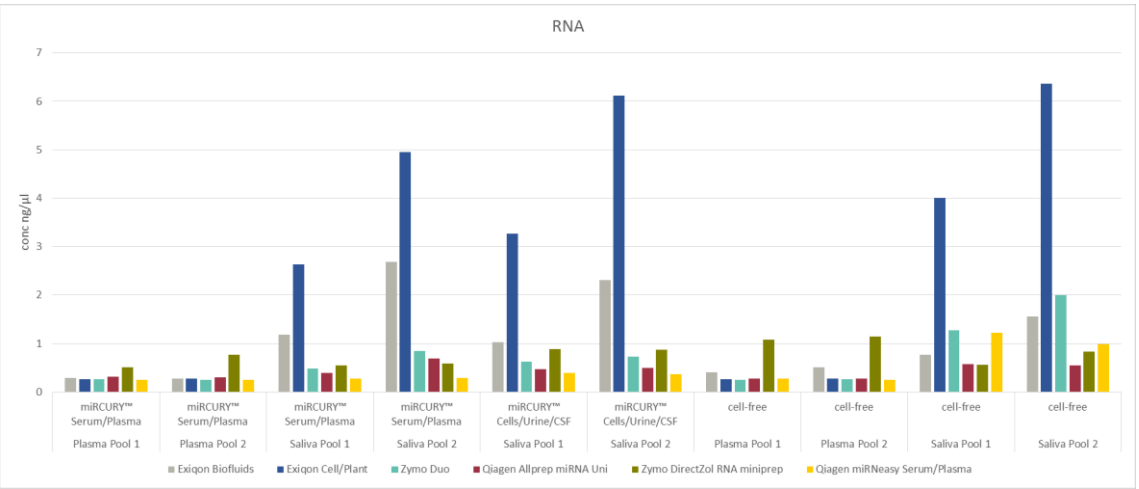


Fig.12 Evaluation of different RNA isolation kits with Quant-iT™ RiboGreen® RNA Assay Kit; bars are reflecting concentration of RNA in ng/µl.

RNA concentrations were measured with Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen. Interestingly, the highest amount of RNA was achieved when isolating with miRCURY RNA Isolation kit for cells and plants (blue bars), which works best for saliva. With the Quick-RNA MiniPrep kit (green bars) from Zymo Research more RNA could be isolated from plasma when compared to the other kits. The difference between the two exosome isolation kits is not recognizable and can be neglected.

To summarize all data from this experiment, the limited amount of sample volume needs to be considered. With regard to the small sample volume available, an isolation kit, which can isolate DNA and RNA from one sample, is preferred. Since only two DNA/RNA isolation kits (ZR-Duet™ DNA/RNA MiniPrep from Zymo Research and AllPrep® DNA/RNA/miRNA Universal from Qiagen) were tested, a decision between them must be made. We figured out, that ZR-Duet™ DNA/RNA MiniPrep from Zymo Research works best for our purpose, because in the case of DNA isolation it worked even better than the High Pure PCR Template Preparation kit from Roche, which isolates DNA only. When working with DNA/RNA isolation kits, loss of RNA is unavoidable. This is illustrated in figure 10 and figure 12, where combined isolation kits work worse than RNA isolation kits only. Although less RNA can be isolated, the decision was taken favoring ZR-Duet™ DNA/RNA MiniPrep from Zymo Research since sample volume is limited and isolating DNA and RNA is possible from one sample. All further experiments were performed with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research to isolate DNA and RNA, if not mentioned before.

3.2. Enzyme Pre-Tests

To avoid co-isolation of nucleic acids that are attached on the outside of exosomes, isolated exosomes were treated with DNase I and RNase A. For this purpose, pre-tests were performed to ensure the right amount of applied enzyme.

3.2.1. Pre-Test DNase I

This experiment was previously performed by Ulrike Kegler. The outcome of this experiment was, that 400 units of DNase I is sufficient to digest the amount of DNA attached on the surface of exosomes. 400 units DNase I, which is represented in 8 µl of DNase I (50U/µl; Thermo Scientific), and 10x reaction buffer for DNase I were added to the resuspended exosome pellet followed by an incubation at an optimal temperature at 37°C for 1 hour. No inactivation of DNase I was needed.

3.2.2. Pre-Tests RNase A

RNase A, DNase and protease-free (10 mg/mL), from Thermo Scientific was used for this experiment. This test was performed in 1x PBS buffer (pH=7,4) and human reference RNA (133 ng/µl) was used to figure out the right amount of RNase A. For inhibition of RNase A, RiboLock RNase Inhibitor (40 U/µL) from Thermo Scientific was added to the mixture.

First, it was calculated, that a low amount of RNase A is sufficient to digest RNA attached on the surface of exosomes. Therefore, a 1:500 dilution with 1x PBS was prepared by adding 2µl RNase A (10 mg/mL) to 998µl 1x PBS. This dilution was always prepared fresh before every experiment. First, the right amount of inhibitor needed to be known.

Table 5: Pre-Test for RNase A with Inhibitor RiboLock; scheme of experimental set-up

	1	2	3	4	5	6	7	8	9
RNA spike	133 ng	133 ng	133 ng	133 ng	-	-	-	-	133 ng
RNase A, 1:500 [µl]	3	3	3	3	3	3	3	3	-
Inkubation	37 C°, 1h								
RiboLock	60 U	80 U	100 U	120 U	60 U	80 U	100 U	120 U	-
Incubation	37 C°, 1h								
RNA spike	-	-	-	-	133 ng	133 ng	133 ng	133 ng	-
Volume (1x PBS) [µl]	300	300	300	300	300	300	300	300	300

Nine different combinations of RNA, RNAse A and inhibitor were tested. The experimental volume was 300µl 1x PBS (pH=7,4). First, in 5 samples 1µl human reference RNA (133 ng/µl) was added to the solution. Then RNase A was added and the mixture was incubated for 1 hour at 37°C. Afterwards, the inhibitor was added in different amounts, which is illustrated in table 5. After an incubation at 37°C for 1 hour, selected samples were spiked-in with RNA (133 ng/µl). RNA was then isolated with miRCURY™

RNA Isolation – Biofluids from Exiqon. To evaluate this experiment, reverse transcription (TaqMan® Reverse Transcription Reagents) quantitative real-time PCR was performed with selected miRNAs (miR-16, miR-21-5p, miR-30c-5p).

Table 6: Inhibitor Pre-Test Results; Exemplary shows RT-qPCR Ct-values for miR-21-5p without spike-in (sample 1-4) and with spike-in (sample 5-8). Positive control (sample 9) only with spike-in RNA.

sample #	1	2	3	4	5	6	7	8	9
Ct-value (miR-21-5p)	40	40	0	40	32,52	33,45	33,44	31,28	32,25

Table 6 exemplary displays RT-qPCR Ct-values of miR-21-5p for each tested combination. A Ct-value of 40 can be set equal to 0, because it means that the amplification starts at cycle 40 or later. Considering that, for sample 1, 2, 3 and 4 a Ct-value of 0 (or 40) means, that no RNA was detectable. Therefore, the digestion of spiked RNA with RNase A, followed by inhibition with RiboLock, worked. Sample 5 to 8 shows whether the inhibition worked or not. A Ct-value of around 32 represents 133 ng RNA (table 6, sample 9). RNA was spiked-in after the inhibition of RNase A with RiboLock. Therefore the Ct-value of sample 5, 6, 7 and 8 represents the spike-in RNA and shows that the inhibition worked in all cases. With these results we decided to use 120 units of RiboLock for the following experiments.

The next step was to figure out the right amount of RNase A to digest nucleic acids attached to the outside of exosomes. For this question, different amounts of spike-in RNA was added to 300µl 1x PBS (pH=7,4).

Table 7: Pre-Test for RNase A; scheme of experimental set-up

	1	2	3	4	5	6	7
RNA spike	133 ng	532 ng	931 ng	1330 ng	1729 ng	2128 ng	133 ng
RNase A, 1:500 [µl]	3	3	3	3	3	3	-
Incubation	37 C°, 1h						
RiboLock	120 U	120 U	120 U	120 U	120 U	120 U	-
Incubation	37 C°, 1h						
RNA spike	-	-	-	-	-	-	-
Volume (1x PBS) [µl]	300	300	300	300	300	300	300

In table 7 the experimental set-up is illustrated. Different amounts of human reference RNA was spiked-in (ranging from 133 ng up to 2128 ng). For the positive control (table 7, sample 7) only 133 ng RNA was spiked-in and no enzyme was added. After adding RNase A (1:500 dilution), the sample mixture was incubated for 1 hour at 37 °C. To inactivate the enzyme, 120U inhibitor were added and the incubation step was repeated. RNA was isolated with miRCURY™ RNA Isolation – Biofluids from Exiqon and to evaluate this experiment, reverse transcription (TaqMan® Reverse Transcription Reagents) quantitative real-time PCR was performed with selected miRNAs (miR-16, miR-21-5p, miR-30c-5p).

Table 8: Pre-Test for RNase A; Results exemplary shows RT-qPCR Ct-values for miR-21-5p testing different amounts of RNA ranging from 133 ng up to 2128 ng (amount increases from sample 1 to sample 6); Positive control (sample 7) only with 133 ng spike-in RNA.

sample #	1	2	3	4	5	6	7
Ct-value (miR-21-5p)	0	0	40	0	0	40	33,31

In tabe 8 the results are illustrated, wheras a Ct-value of 40 can be set equal to 0. The outcome of this experiment shows, that the amount of RNase A used is enough to digest 2128 ng of human reference RNA. The Ct-value of sample 7 reflects the amount of miR-21-5p in 133 ng of human reference RNA (table 8). Since the quantity of RNA is thought to be low on the outside of exosomes, we decided, that 0,6 ng RNase A (3µl of a 1:500 dilution) is sufficient to digest unwanted RNA attached on the surface of exosomes. Therefore, for all the following experiments digestion with 3µl RNase A (10mg/ml) of a 1:500 dilution was carried out.

After that, inhibition of RNase A with lysis buffer was tested. To simplify the RNase A reaction, the inhibitor RiboLock (Thermo Scientific) was replaced by simple lysis buffer from miRCURY™ RNA Isolation – Biofluids (Exiqon). Lysis buffer was added according to the manufacturer's protocol.

	1	2	3	4	5
RNA spike	133 ng	-	133 ng	133 ng	133 ng
RNase A, 1:500 [µl]	3	3	3	3	-
Incubation	37 C°, 1h				
Lysis Buffer [µl]	90	90	-	-	-
RiboLock	-	-	3	-	-
Incubation	37 C°, 1h				
RNA spike	133 ng	133 ng	-	-	-
Volume (1x PBS) [µl]	210	210	300	300	300

Table 9: Inhibition with lysis buffer; Experimental set-up to test inhibition with lysis buffer. The inhibitor RiboLock (Thermo Scientific) was replaced by lysis buffer.

As mentioned in table 9, 90µl lysis buffer from miRCURY™ RNA Isolation – Biofluids (Exiqon) was added to get a total volume of 300µl. For spike-in, 133 ng human reference RNA was added to some samples. RNA was then isolated with miRCURY™ RNA Isolation – Biofluids (Exiqon) and reverse transcription followed with quantitative real time PCR was performed.

sample #	1	2	3	4	5
Ct-value (miR-21-5p)	32,9	33,53	40	0	35,93

Table 10: Inhibition with lysis buffer; Results exemplary shows RT-qPCR Ct-values for miR-21-5p. A Ct-value of 40 can be set equal to 0, which represents no amplified RNA.

Sample 1 and 2 could be seen as duplicates, because after digestion, no RNA will be left. Then, RNA was spiked in again, therefore the Ct-values were positive with values at ~33. Sample 5 had a little bit less RNA, but was still positive and accepted as a positive control. To conclude from these results, 3 µl of a 1:500 dilution RNase A (10mg/ml), which is equal to 0,6 ng RNase A, is sufficient to digest RNA attached on the surface of exosomes.

Furthermore, RNase A can be inhibited by simply adding lysis buffer to the solution instead of RNase inhibitor.

3.2.3. Combination of RNase A and DNase I

To simplify the digestion, combining DNase I and RNase A digestion was tested. It was important, that DNase I is not digesting any RNase A and vice versa. For this experiment, different combinations of DNase I and RNase A were tested on plasma exosomes as well as salivary exosomes.

Table 11: Combination of RNase A and DNase I, Experimental set-up to test combination of RNase A and DNase I; The tested matrices were plasma and saliva. Exosomes were isolated with miRCURY™ Exosome Isolation Kit – Serum and plasma (Exiqon). To purify exosomes, Amicon® Ultra Centrifugal Filters 100 K were used. Then, digestion was performed with RNase A and DNase I. Samples were performed as technical duplicates (1-4 and 5-8).

	1	2	3	4	5	6	7	8
Exosome Isolation	yes	yes	yes	yes	yes	yes	yes	yes
1x PBS	300	300	300	300	300	300	300	300
Amicon 100K	yes	no	yes	yes	yes	no	no	yes
Dnase I [µl]	8	8	-	-	8	8	-	-
10x Mg Buffer	yes	yes	-	-	yes	yes	-	-
RNase A, 1:500 [µl]	3	3	-	-	3	3	-	-
Incubation	37°C, 1h							

The experimental set-up can be seen in table 11. Plasma and saliva were collected and exosomes were isolated with miRCURY™ Exosome Isolation Kit – Serum and plasma (Exiqon) by following the manufacturer's protocol. The resulting exosome pellet was resuspended in 300µl 1x PBS (pH=7,4). For purification of exosomes and to remove EDTA from plasma exosomes, Amicon® Ultra Centrifugal Filters 100K were used. Afterwards, digestion was started by adding 10x Mg-buffer and DNase I. In addition, RNase A was added to the mixture, followed by incubation at 37°C for 1 hour. After that, DNA and RNA were isolated simultaneously with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research according to manufacturer's protocol. To evaluate the results, qPCR and RT-qPCR were performed.

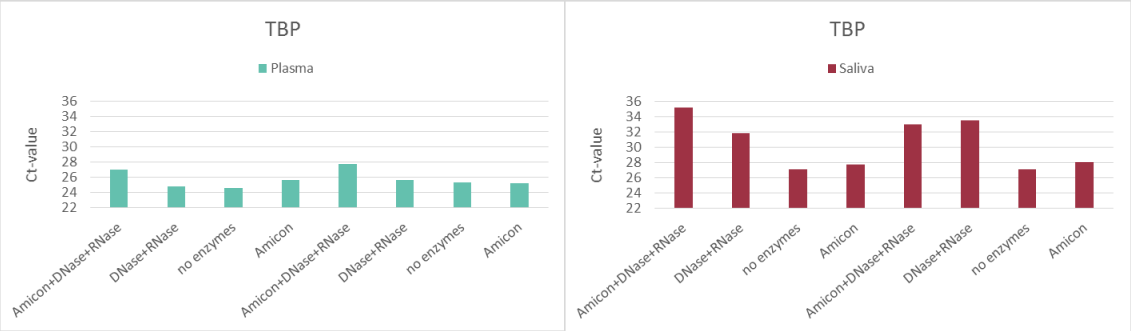


Fig.13 Evaluation of combination of DNase I and RNase A; bars exemplary reflects qPCR results of plasma (turquoise) and saliva (red); samples were performed as duplicates (bars 1-4 and 5-8).

Figure 13 represents qPCR results for plasma (left; turquoise) and saliva (right; red). Primers for TATA-box binding protein (TBP) was used exemplary to illustrate the results. The usage of centrifugal devices, such as Amicon® Ultra Centrifugal Filters 100K is further discussed in chapter 3.3. Centrifugal Devices Test. In short, centrifugal devices are necessary to get rid of EDTA, which was already included in blood collecting tubes. EDTA inhibits DNase I, therefore it has to be eliminated. The conclusion to this is, DNase I without the usage of centrifugal devices, such as Amicon® Ultra Centrifugal Filters 100K, does not digest any DNA. Sample 2 (sample 6) on the left side of figure 13 represents an inactive enzyme, whereas sample 1 (sample 5) in the same figure illustrates an active enzyme which cleaves DNA on the outside of exosomes. RNase A does not affect the amount of DNA, which can be taken from figure 13 (left side; turquoise bars) when comparing 2 and 3 (6 and 7). The use of only Amicon® Ultra Centrifugal Filters 100K does affect the DNA content negatively by losing some DNA on the outside of exosomes (sample 4/8 versus 3/7). For salivary exosomes (figure 13; right side, red bars) centrifugal devices are not necessary, because no EDTA is present in saliva samples. In addition to that, with centrifugal devices the loss of DNA is bigger than expected, but this issue is discussed later in chapter 3.3. Centrifugal Devices Test. Comparing the samples 1/2 with 3 (6 respectively 5/6 with 7), DNase I does digest nucleic acids attached on the surface of exosomes.

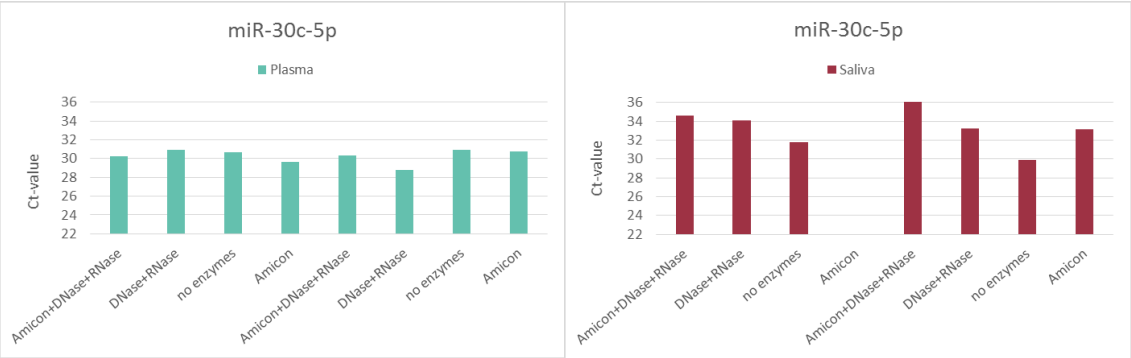


Fig.14 Evaluation of combination of DNase I and RNase A; bars exemplary reflects RT-qPCR results of plasma (turquoise) and saliva (red) for miR-30c-5p; samples were performed as technical duplicates (bars 1-4 and 5-8).

To evaluate the RNA results for plasma and saliva, RT-qPCR was performed with 3 different primers. Figure 14 exemplary shows miR-30c-5p results for plasma (left; turquoise) and saliva (right; red). Barplots 1-4 and 5-8 reflect technical duplicates. Interestingly, when using RNase A for digestion (e.g. sample 6 versus 7), no conclusive results could be obtained as sometimes more miRNA can be detected. Also the duplicates differ in their miRNA amount detected. This can be due to not good technical work, or due to sample preparation. This phenomenon can also be seen in the following experiments and the cause could not be determined.

Nevertheless, these results taken together show quite well that coincubation of DNase I and RNase A does not affect each other and is possible for our purpose. Therefore all of the following experiments, of not mentioned otherwise, were performed with DNase I and RNase A in one reaction mixture.

3.3. Centrifugal Devices Test

In this experiment, three different centrifugal devices were tested for their efficiency to get rid of EDTA in plasma samples. EDTA (ethylenediamine tetraacetic acid) is an anticoagulant and is used to prevent clotting of blood samples. To achieve this EDTA is prefilled into collection tubes. By its characteristic feature to chelate calcium and several other metal ions, EDTA removes calcium and thereby irreversibly prevents clotting within the collection tube (Banfi, Salvano, & Lippi, 2007). Removal of other metal ions, such as magnesium, can inhibit enzymes, because these ions are indispensable for enzymatic reactions. The problem we specifically faced was, that DNase I, the enzyme we used to digest DNA attached on the surface of exosomes, needs magnesium or other divalent metal ions, such as manganese, for its functionality. As the molar mass of EDTA is 292.24 g·mol−1, which is quite small compared to exosomes, it is possible to remove

EDTA with simple size-exclusion. Therefore, three different centrifugal devices with a molecular-weight cutoff of 100K were tested for EDTA removal: (1) Amicon® Ultra-0, 5 Centrifugal Filter Devices 100K, (2) Pierce™ Protein Concentrators 100K and (3) Nanosep® centrifugal devices 100K. Exosomes were isolated with miRCURY Exosome Serum/Plasma Kit (Exiqon) from 1,5 ml plasma. The resulting exosome pellet was resuspended in 300 µl 1x PBS (pH=7, 4). Then size-exclusion was applied to get rid of EDTA before DNase I digestion was performed. Triplicates were performed for each type of centrifugal device. The centrifugation steps were followed according to the manufacturer's protocols, followed by two washing steps with 1x PBS (pH=7, 4). The volume was filled up to 300 µl and digestion with 8 µl DNase I (additionally with 30 µl 10X Mg buffer) and 3 µl RNase A (1:500 dilution) was started. After an incubation at 37°C for 1 hour, DNA and RNA were isolated simultaneously with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. The elution of nucleic acids was performed with 100 µl ddH_20. To evaluate the results, concentrations of isolated nucleic acids were measured with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) and Quant-iT™ RiboGreen® RNA Assay Kit to measure RNA (also from Invitrogen). For Nanosep® centrifugal devices 100K from Pall Life Sciences DNA was not measured with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen), but Ct-values were calculated with quantitative real-time PCR.

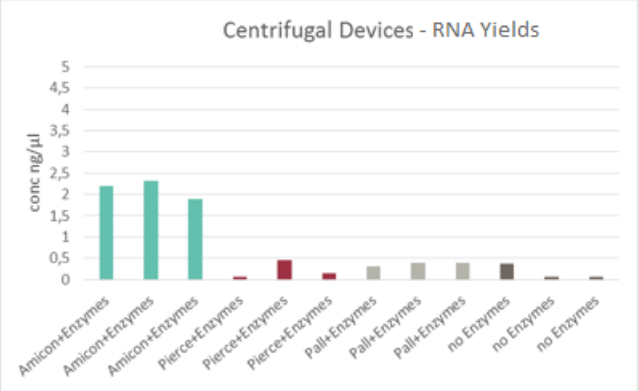


Fig.15 Evaluation of different Centrifugal Devices for EDTA removal; RNA Yields; bars are reflecting RNA concentrations measured with Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen. Three different centrifugal devices were tested: Amicon® Centrifugal Filter Devices, Pierce™ Protein Concentrators, Nanosep® centrifugal devices (all 100K).

Results in figure 15 are showing different amounts of RNA. With the use of Amicon® Ultra-0, 5 Centrifugal Filter Devices 100K a RNA concentration ranging from 1, 89 up to 2, 31 ng/µl could be achieved. When isolating with Pierce™ Protein Concentrators 100K (illustrated as red bars in figure 15), a RNA concentration of 0, 06-0, 45 ng/µl could be reached. Bars in light grey (figure 15) are reflecting the RNA content of exosomes in combination with Nanosep® centrifugal devices 100K from Pall Life Sciences and they range from 0, 31 to 0, 38 ng/µl. Illustrated in dark grey bars are the amounts of RNA from exosome isolation without any enzymes. These concentrations are very low and not as expected. Actually, one would expect a larger amount of RNA when no treatment with RNase A and DNase I was done on exosomes. An explanation could be that here the RNA isolation did not work properly due to the high amount of proteins. When using centrifugal devices, a lot of proteins will also go through the 100K membrane pores and are thereby removed before the RNA isolation. It can be clearly seen on figure 15, that exosome isolation and subsequently RNA isolation works best, when Amicon® Ultra-0, 5 Centrifugal Filter Devices 100K is used for EDTA removal before digestion with enzymes, because the highest amounts of RNA could be obtained (turquoise bars) with this combination.

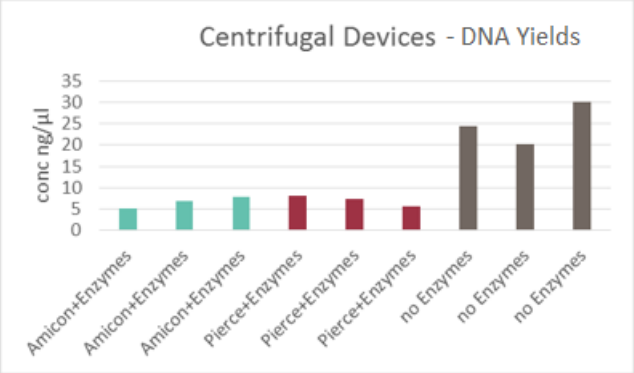


Fig.16 Evaluation of different Centrifugal Devices for EDTA removal; DNA Yields; bars are reflecting DNA concentrations measured with Quant-iT™ PicoGreen® dsDNA Assay Kit from Invitrogen. Two different centrifugal devices were tested: Amicon® Centrifugal Filter Devices and Pierce™ Protein Concentrators (both 100K).

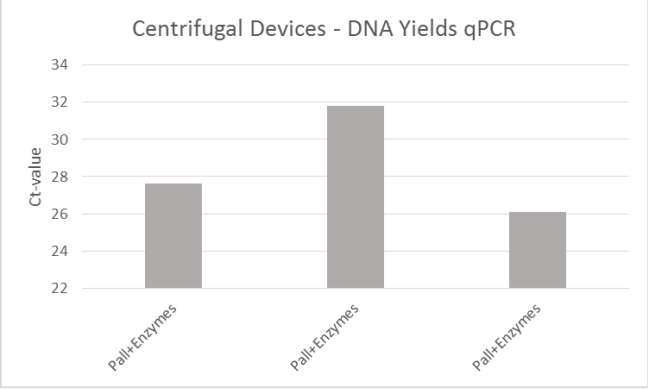


Fig.17 Evaluation of Nanosep® centrifugal device 100K; This figure exemplary shows qPCR Ct-values for the TBP gene as a measure for achievable DNA yields when using Nanosep® centrifugal devices with a molecular-weight cutoff of 100K to get rid of EDTA in plasma samples.

DNA concentrations were measured with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen), but only two types of centrifugal devices were measured on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific). Results are illustrated in figure 16 in different colored bars. Turquoise bars are representing DNA concentrations ranging from 5, 05 to 7, 85 ng/µl with the use of Amicon® Centrifugal Filter Devices 100K. Isolation in combination with Pierce™ Protein Concentrators with a molecular-weight cutoff of 100K (shown as red bars) has given a DNA-yield of 5, 71 to 8, 07 ng/µl. In comparison the use of both centrifugal devices resulted in about the same amount of DNA. For a positive control, no enzymatic digestion was performed (dark grey bars). Here, the DNA concentrations ranged from 20, 27 up to 30, 00 ng/µl, including nucleic acids within the exosomes and also attached on the surface of exosomes.

For Nanosep® centrifugal devices with a molecular-weight cutoff of 100K only quantitative real-time PCR was performed to judge on achievable DNA yields. We decided to do so since RNA concentrations measurements (figure 15) with Quant-iT™ RiboGreen® RNA Assay Kit from Invitrogen gave hardly detectable RNA concentrations fo the Nanosep® centrifugal devices. What can be taken from qPCR values in figure 17 is that DNA isolation with Nanosep® centrifugal devices causes big fluctuations within the triplicates in addition to the overall small DNA yields.
To summarize all of the results, Amicon® Ultra-0, 5 Centrifugal Filter Devices for volumes up to 500 µl with a molecular-weight cutoff of 100K guarantee a fast and easy removal of EDTA from plasma exosomes. Not only the highest concentrations of RNA were achieved, but also in terms of DNA concentrations it was possible to isolate a reasonable amount of nucleic acids.

3.4. Exosome Isolation Kit Test

In this experiment, six different commercially available kits for exosome isolation were compared for their use with saliva. This included different strategies for exosome isolation from human saliva with the subsequent isolation of DNA and microRNA to identify the best isolation protocol.

Table 12: Different strategies for exosome isolation

Exosome Isolation Kits	Preparation Principle
Exiqon miRCURY Exosome serum/plasma kit	Precipitation
iZON qEVoriginal	Size-exclusion chromatography
Cellguidance ExoSpin columns	Size-exclusion chromatography
Hansa Biomed Immunobeads for Overall Exosome capture	Immuno-based
Pierce Protein Concentrator 100K	Size-exclusion
Ultracentrifugation	Centrifugation and Density

The exosome isolation kits to be tested were: (1) Exiqon miRCURY Exosome serum/plasma kit, (2) iZON qEVoriginal, (3) Cellguidance ExoSpin columns, (4) Hansa Biomed Immunobeads for Overall Exosome capture, (5) Pierce Protein Concentrator 100K and (6) ultracentrifugation. The ultracentrifugation, which uses the density of exosomes to isolate them, was performed by the AKH Wien, Allgemeines Krankenhaus der Stadt Wien, because AIT did not have this an ultacentrifugation in-house. Two of the exosome isolation kits which we used in our kit comparison are based on size-exclusion chromatography (iZON qEVoriginal and Cellguidance ExoSpin columns). This is a chromatographic method in which molecules, in this case exosomes, are separated by their size. Exiqon miRCURY Exosome serum/plasma kit uses simple precipitation to isolate exosomes. Another preparation principle is used by Hansa Biomed Immunobeads and it is based on simple latex immunobeads. These beads had been covalently coupled with antibodies against exosome surface antigens before thereby allowing exosome capture from human saliva. Pierce Protein Concentrator with a molecular-weight cutoff of 100K allows the isolation of exosomes by simple size-exclusion separation.

First, saliva was collected from healthy probands according to the SOP described in the Materials & Methods section. Thereof, three pools of cell-free saliva (CFS) were prepared. Triplicates of 1 ml CFS were used as starting volume to compare six different exosome isolation kits (Table 12). Prepared exosomes were once treated with DNase I and RNase A to get rid of nucleic acids attached to the outside of exosomes or left

untreated before DNA and RNA was simultaneously isolated via the ZR-Duet DNA/RNA MiniPrep Kit from Zymo Research.

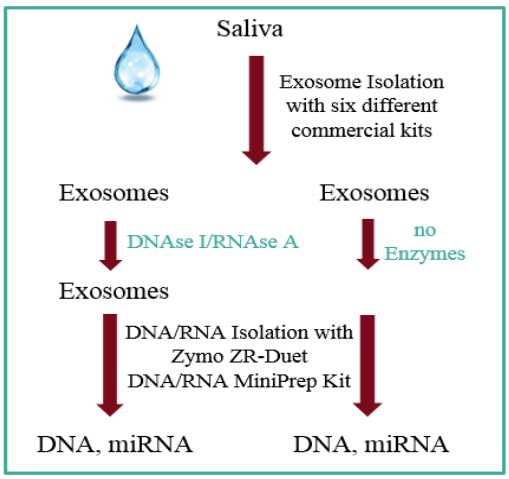


Fig.18 Schematic illustration of workflow for exosome isolation kit test; Saliva was collected from healthy probands and cell free saliva was prepared according to the SOP. Exosomes were isolated with six different isolation kits that are based on different preparation principles (table 12). Then, exosomes were or left untreated with DNase I and RNase A to digest nucleic acids attached on the outside of exosomes. Subsequently, DNA and miRNA were simultaneously isolated with ZR-Duet DNA/RNA MiniPrep Kit from Zymo Research.

The exosome isolations were performed according to the manufacturers protocols. If any changes have been made, then they can be seen from chapter "Material and Methods", within the section "Exosome Isolation". The volume in which the exosomes were dissolved was adjusted to 300 µl with 1x PBS (pH=7, 4). Digestion with the enzymes was carried out and was based on the basis of previous experiments. The elution volume for DNA and miRNA isolation was 100 µl with ddH_2O. Afterwards, the nucleic acids were concentrated 5, 5 times with the centrifugal evaporator Univapo 150H (Uniequip). 55 µl elution volume was reduced under high pressure and heat, so called vaporizing. Then the nucleic acids were dissolved in 10 µl ddH_2O under constant shaking for 1 hour.

To assess the performance of the exosome kits with respect to exosomal DNA/RNA yield and co-isolated non-exosomal nucleic acids, quantitative real-time PCRs for 6 different genes and 6 different microRNAs were performed on exosomes treated with DNAse I and RNase A and exosomes left untreated.

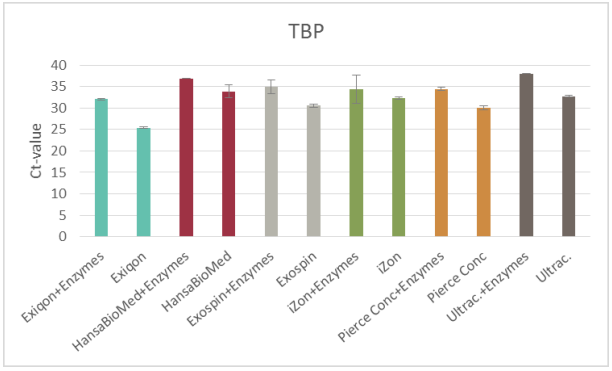


Fig.19 Results Exosome Isolation Kit Test, TBP; Exemplary shows qPCR Ct-values for the TBP gene for every tested kit as a measure for achievable DNA yields and the extent of co-isolated non-exosomal DNA (the latter indicated by an evident difference in the heights of identical colored bars). By concentrating an improvement in Ct-value of about 2 could be achieved and samples became measurable.

In figure 19 qPCR results for the TBP gene are illustrated for every tested kit. Triplicates were performed and the average was calculated for treated exosomes with enzymes and untreated exosomes. A smaller Ct-value means a larger amount of DNA. First, it was interesting, that although other kits, such as Hansa Biomed Immunobeads (red bars) with its covalently coupled antibodies against exosome surface antigens or ultracentrifugation where exosomes were isolated according to their density, were supposed to prepare purer exosomes, a difference between enzyme digested and undigested samples could be even observed here. Meaning that DNA is attached to the surface, regardless of the isolation method. Another interesting observation has been, that exosome isolation with Pierce Protein Concentrator 100K (orange bars) achieved solid results, which could keep up with the other isolation kits. Exiqon miRCURY Exosome serum/plasma kit (turquoise bars) uses simple precipitation to isolate exosomes and was identified to be the best isolation kit, because the highest yield of DNA was achieved with it.

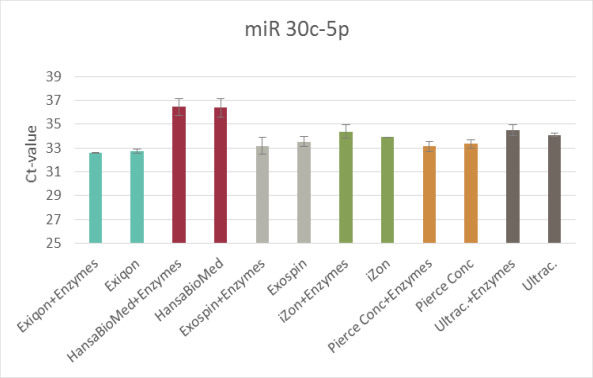


Fig.20 Results Exosome Isolation Kit Test, miR 30c-5p; exemplary shows RT-qPCR Ct-values for the miR 30c-5p gene for every tested kit as a measure for achievable RNA yields and the extent of co-isolated non-exosomal RNA (the latter indicated by a potential difference in the heights of identical colored bars). By concentrating an improvement in Ct-value of about 2 could be achieved and samples became measurable.

The results for microRNA were also evaluated by reverse transcription quantitative real-time PCR. Figure 20 exemplary shows Ct-values for miR 30c-5p gene for every tested kit. Technical triplicates were measured and the average was calculated and illustrated in figure 20. The first bar of every kit represents exosomes treated with DNase I and RNase A and the second bar always indicates untreated exosomes. Interestingly, a difference between the two could not be determined. That would suggest, that only a low amount of RNA is attached to the outside of exosomes. Nevertheless, there are differences in the amount of RNA obtained by the various exosome preparation kits. The lowest Ct-values for Exiqon miRCURY Exosome serum/plasma kit (turquoise bars) reflects the highest amount of RNA that could be isolated via this kit. However, Hansa Biomed Immunobeads (red bars), which represents due to its surface-molecule targeted approach, a very specific method was found to result in the highest Ct-values in this experiment and therefore ended up in the lowest exosome, respectively miRNA yield.

In summary, these quantitative real-time PCR results indicate Exiqon miRCURY Exosome serum/plasma kit (turquoise bars) to be the best kit to isolate salivary exosomes. Moreover, by digesting exosomes with DNase I and RNase A prior to nucleic acid isolation it could be demonstrated that significant amounts of DNA were attached to the surface of exosomes, whereas RNA is only found within the exosomes.

3.5. Volume Reduction Test

Since the concentration of DNA and RNA within exosomes is very low in human plasma and saliva, and the elution volume of our DNA and RNA isolation procedure was with 100 µl rather high for the foreseen downstream procedures, three different methods to reduce sample volume and to concentrate DNA and RNA were tested. (1) Univapo 150H (Uniequip) is a centrifugal vacuum concentrator that can remove liquid from samples. The machine uses heat under vacuum to evaporate ddH_2O from our samples. Freeze-drying is another method that gently dries thermally sensitive materials. With (2) RVC 2-33 CDplus (Martin Christ Gefriertrocknungsanlagen GmbH) DNA and RNA samples were concentrated by removing the aqueous solution. The third method was based on silica membrane columns provided by the company Zymo Research. (3i) DNA Clean & Concentrator-5 was used to concentrate DNA samples for further use. For RNA samples, (3ii) RNA Clean & Concentrator™-5 was applied for concentration. All of the methods were performed according to the manufacturer's protocol. In the first part of this experiment, only (1) Univapo 150H (Uniequip) and (2) RVC 2-33 CDplus were tested and compared to each other. 10 DNA stocks with 50 ng DNA (isolated from human blood samples) per 55 µl were prepared, whereas four samples each were concentrated. Two samples were not concentrated and served as control. Two of these 4 samples were totally concentrated to make sure that the entire liquid disappears. The other two samples were not completely concentrated so that a small volume remains. Then the volume was filled up to 10 µl with ddH_2O and the samples were shaken at room temperature for 1 hour so that the nucleic acid completely dissolves. The same procedure was done with 50 ng RNA in 55 µl ddH_2O and afterwards, the volume was also filled up to 10 µl ddH_2O. To evaluate the results, quantitative real-time PCR was performed for DNA and for RNA reverse transcription was performed before qPCR.

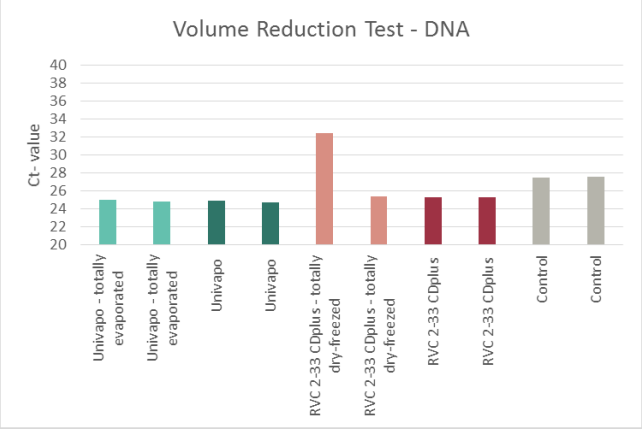


Fig.21 Results Volume Reduction Test, TBP; exemplary shows qPCR Ct-values for the TBP gene for Univapo 150H (turquois bars) and RVC 2-33 CDplus (red bars) indicating achievable DNA concentrations. By concentrating an improvement in Ct-value of about 2,5 could be achieved and samples became measurable. Control samples (grey bars) are not concentrated and represent 50 ng DNA in 55 µl.

To illustrate DNA results, the TBP gene was used in figure 21 to depict the Ct-values. In turquoise bars, the Ct-values are shown for concentrating with Univapo 150H (Uniequip). Whereas dark turquoise indicates concentration where volume was not totally evaporated, light turquoise bars represent total evaporation of ddH_2O. The same applies to the red bars. Light red bars show totally dry-freezed samples and dark red bars represents samples, where ddH_2O was not totally dry-freezed. The first light red bar can be neglected, because when pressure was applied, the sample was flown out from the tube. Firstly, from figure 21 can be seen that completely concentration to a volume of 0 µl does not have an impact on the quantity of DNA. This applies to both volume reduction methods. A Ct-value difference of around 2,5 could be achieved with both of the methods, when comparing the controls (grey bars) to the concentrated samples (turquoise and red bars) and corresponds well to the concentration factor of 5,5 fold.

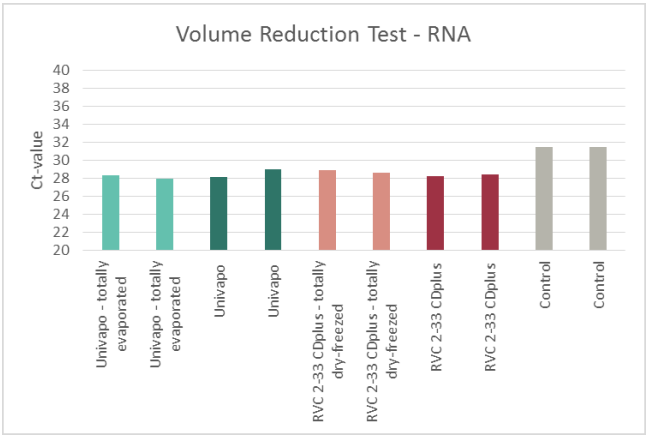


Fig.22 Results Volume Reduction Test, miR 30c-5p; exemplary shows RT-qPCR Ct-values for the miR 30c-5p for Univapo 150H (turquois bars) and RVC 2-33 CDplus (red bars) indicating achievable RNA concentrations. By concentrating an improvement in Ct-value of about 2, 5 could be achieved and samples became measurable. Control samples (grey bars) are not concentrated and represent 50 ng RNA in 55 µl.

The RNA results in figure 22 show the same pattern than the DNA results in figure 21 above. In grey bars, non-concentrated controls are shown and the concentrated samples are illustrated by turquois bars (Univapo 150H (Uniequip)) and red bars (RVC 2-33 CDplus). Again, no difference can be recognized between total evaporation/dry-freezing and not. An improvement of the Ct-values could be achieved with both of the methods indicating that all of the concentration approaches were successful.

Finally the third method, concentration with columns from Zymo Research, was tested. The experimental set-up was a little bit different. 1,5 ml start volume of plasma and saliva was used to isolate exosomes. After that, EDTA was removed from plasma samples by centrifugal devices (Amicon® Ultra-0, 5 Centrifugal Filter Devices 100K) and exosomes were resuspended in 300 µl 1x PBS (pH=7, 4). Next, exosomes were treated with DNase I and RNase A (described in chapter X) and DNA and RNA were simultaneously isolated

with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. For evaluation qPCR was performed for 6 different genes and 6 different microRNAs.

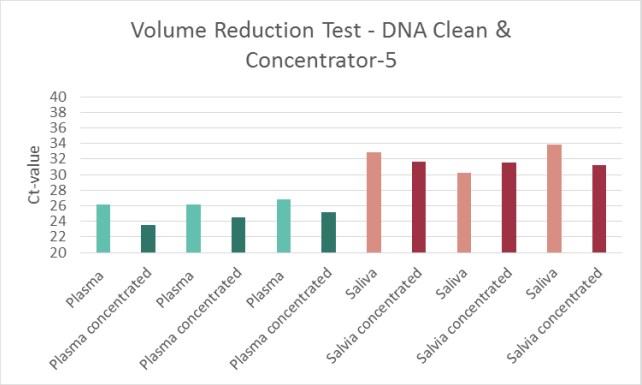
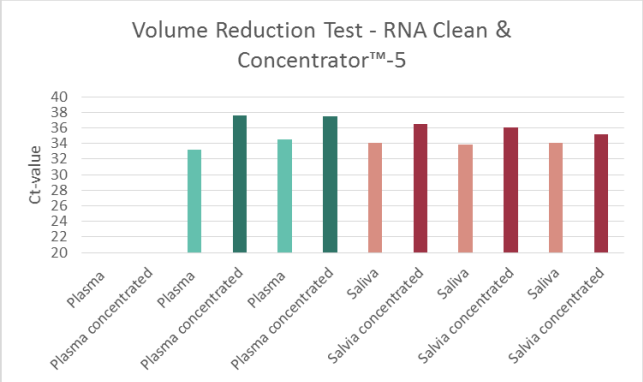


Fig.23 Results Volume Reduction with Columns, TBP gene; exemplary shows qPCR Ct-values for TBP gene for volume reduction test with DNA Clean & Concentrator-5 from Zymo Research. By concentrating an improvement in Ct-value of about 2, 5 could be achieved in plasma (turquoise bars) and saliva (red bars).

Technical triplicates of plasma and saliva samples were performed. For the concentration, 55 µl of isolated DNA was loaded on the column and the isolation was performed according to the protocol. Lastly, the DNA was eluted in 10 µl ddH_2O, whereby a concentration of 5, 5 could be achieved. The darker color (turquoise and red) indicate concentrated samples and a decrease in the Ct-value of about 2, 5 can be observed to non-concentrated samples, which corresponds to the original concentration factor.

Fig.24 Results Volume Reduction with Columns, miR 30c-5p; exemplary shows RT-qPCR Ct-values for miR 30c-5p for volume reduction test with RNA Clean & Concentrator-5 from Zymo Research. By concentrating an improvement in Ct-value of about 2, 5 should be achieved in plasma (turquoise bars) and saliva (red bars), which was not the case.



For microRNA RNA Clean & Concentrator™-5 were used to perform concentration. RT-qPCR results are illustrated in figure 24, whereas plasma samples are shown in turquoise bars and saliva samples in red bars. Interestingly, an increase of Ct-values can be recognized (dark colored bars), which led to the conclusion that concentration of microRNAs is not possible with RNA Clean & Concentrator™-5 kit from Zymo Research. An explanation could be that miRNAs are too small and are not kept in the column and therefore will go through the column and be lost. This explanation is supported by RiboGreen RNA Concentration Measurements with Quant-iT™ RiboGreen® RNA Assay Kit (see Table 13 and explanation underneath).

Table 13: Volume Reduction Test, RiboGreen RNA Concentration Measurements with Quant-iT™ RiboGreen® RNA Assay Kit; Concentration of plasma and saliva samples were measured on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific).

Sample	Non-concentrated ng/µl	5,5 concentrated ng/µl
Plasma	0,06	0,21
Plasma	1,45	9,16
Plasma	1,86	25,25
Saliva	0,22	1,04
Saliva	0,25	1,07
Saliva	0,21	0,96

Table 13 shows how the concentrations in plasma as well as in saliva have increased after concentration. The values are given in ng/µl. For saliva, it was possible to achieve a concentration of 5, 5 when comparing non-concentrated and concentrated measurements. For the three plasma samples concentration with RNA Clean & Concentrator-5 from Zymo Research was also possible, but the values are higher than 5, 5 when comparing them to non-concentrated samples. To summarize the results from (3i) DNA Clean & Concentrator-5 and (3ii) RNA Clean & Concentrator™-5, DNA concentration was possible with columns from Zymo Research for both plasma and saliva. In terms of RNA concentration could also be achieved, but it was impossible with microRNA. MicroRNAs are usually between 21 to 23 nucleotides long and therefore very small. So an explanation could be, that miRNAs were too small and went through the columns and got lost. Therefore this method is not ideal for concentrating miRNAs.

However, when using DNA and RNA from exosomes from plasma and saliva samples, concentration with (1) Univapo 150H (Uniequip) and (2) RVC 2-33 CDplus (Martin Christ Gefriertrocknungsanlagen GmbH) was also problematic for miRNA concentration as results underneath demonstrate.

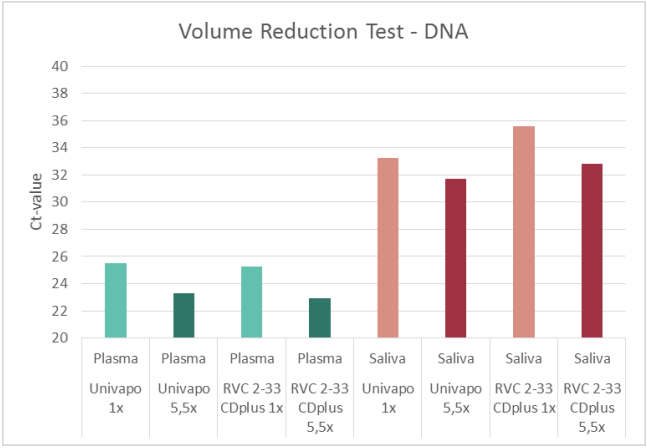


Fig.25 Results Volume Reduction with Univapo and RVC 2-33 CDplus, TBP gene; exemplary shows qPCR Ct-values for TBP gene for volume reduction with Univapo and RVC 2-33 CDplus. Plasma and saliva samples were concentrated 5, 5 times with both methods. 55µl of DNA were concentrated and then DNA was dissolved in 10 µl ddH_2O.

From figure 25 it can be seen that 5, 5 concentration of DNA was achieved with both methods with plasma and saliva samples. Turquoise bars reflect DNA from plasma samples and a Ct-value difference of around 2, 5 could be shown when comparing 1x and 5,5x samples. It was the same for saliva DNA (red bars) where a difference in Ct-values could be detected between 1x and 5, 5x samples.

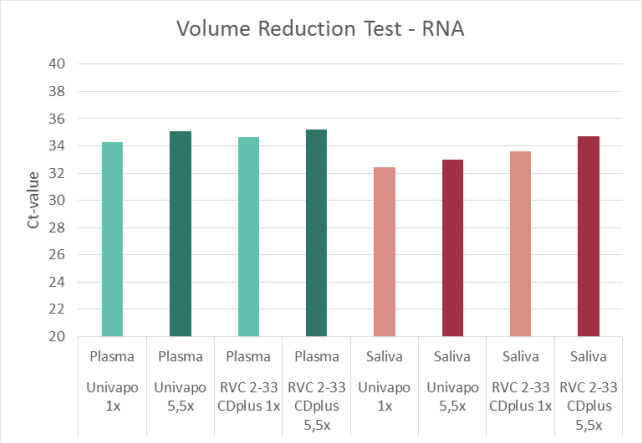


Fig.26 Results Volume Reduction with Univapo and RVC 2-33 CDplus, miR 30c-5p; exemplary shows RT-qPCR Ct-values for miR 30c-5p for volume reduction with Univapo and RVC 2-33 CDplus. Plasma and saliva samples were concentrated 5,5 times with both methods. 55µl of RNA were concentrated and then RNA was dissolved in 10 µl ddH_2O.

Interestingly, the concentration of plasma and saliva RNA was not possible, which can be taken from figure 26. After concentration, the Ct-values were not lower as expected, the values were higher. Higher Ct-values means less RNA and in this case it means, that RNA has been lost during the concentration step with Univapo and RVC 2-33 CDplus. An explanation could be that miRNAs are too small and evaporate with ddH_2O and therefore get lost during this process. Another more likely explanation might be that some inhibitory substance is increased by the concentration process and results in a less efficient PCR amplification of miRNAs. Unfortunately, this problem could not be solved during my practical work at the AIT – Austrian Institute of Technology.

3.6. Proteinase K

Plasma naturally contains a high amount of proteins and the more plasma was used to isolate, the more proteins were there to interrupt with the isolation of DNA and RNA. When using 0,5 ml as starting volume for plasma, a problem with RNA isolation was not recognized. Unfortunately, the volume needed to be increased because of the low amount of exosomes and therefore low amounts of DNA and RNA in plasma and saliva samples. A bad side effect was, that RNA isolation was not possible with 1 ml or even with 1, 5 ml plasma. Therefore, we assumed that the high amount of proteins present in plasma samples was the reason for the given problem. In addition to that, RNAs are often associated with proteins and they rarely occur freely in a cell, which might be even more problematic. Interestingly, this problem only occurred in plasma samples at a volume higher than 1 ml. To solve this problem, proteins were digested with proteinase K from Zymo Research. Proteinase K hydrolyzes a broad range of peptides, therefore it degrades many proteins in their native conformation. In this experiment, 20 mg proteinase K was used to digest unwanted proteins from exosomes and nucleic acids. The lyophilized proteinase K was reconstituted by adding 1040 µl proteinase K storage buffer and was stored at -20°C until further use.

	1	2	3	4
V [ml]	1,5	1,5	1,5	1,5
Exosome Isolation	yes	yes	yes	yes
Centrifugal Device 100K	yes	yes	yes	yes
DNase [µl]	-	8	-	8
Mg Buffer [µl]	30	30	30	30
RNase A, 1:500 [µl]	-	3	-	3
Incubation	37 °C, 1h			
Proteinase K [µl]	12	12	-	-
DNA/RNA Isolation	yes	yes	yes	yes

Table 14: Schematic illustration of Proteinase K Test; Plasma; Exosomes from 1,5 ml plasma were isolated and EDTA was washed away with centrifugal devices 100K. Nucleic acids were digested in some samples and proteinase K was used to digest proteins. Finally, DNA and RNA were isolated simultaneously.

In table 14, a schematic illustration of the experimental set-up for testing proteinase K on plasma exosomes, is shown. The starting volume was 1, 5 ml plasma and exsomes were isolated with miRCURY Exosome Serum/Plasma Kit from Exiqon. In this experiment, thrombin was added before precipitation of exosomes. For that, 15µl thrombin (500U/ml) was added to activate the coagulation cascade, followed by a short centrifugation step for 5 minutes at 10 000 xg. The supernatant was transferred to a new tube and precipitation buffer A was added, followed by further isolation of exosomes. Then, Amicon® Ultra-0, 5 Centrifugal Filter Devices with a molecular-weight cutoff of 100K were used to get rid of EDTA. Digestion was performed with DNase I (and Mg^{2+} Buffer) and RNase A followed by an incubation at 37°C for 1 hour. After that, 12 µl reconstituted proteinase K

was added to the sample mixture and then incubated for 30 minutes at room temperature. The proteins should be digested by proteinase K and the isolation problem should be solved. Finally, DNA and RNA were simultaneously isolated with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research and eluted in 100 µl ddH_2O.

Table 15: Schematic illustration of Proteinase K Test; Saliva; Exosomes from 1, 5 ml saliva were isolated and nucleic acids were digested in some samples and proteinase K was used to digest proteins. Finally, DNA and RNA were isolated simultaneously.

	1	2	3	4
V [ml]	1,5	1,5	1,5	1,5
Exosome Isolation	yes	yes	yes	yes
DNase [µl]	-	8	-	8
Mg Buffer [µl]	30	30	30	30
RNase A, 1:500 [µl]	-	3	-	3
Incubation	37 °C, 1h			
Proteinase K [µl]	8	8	-	-
DNA/RNA Isolation	yes	yes	yes	yes

For saliva, the same exerimental set-up was used (see table 15 above), except the centrifugal device step. Since there was no EDTA in saliva samples, this step could be omitted. Nucleic acids were also isolated with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research and the eluate volume was 100 µl ddH_2O. To evaluate the results, quantitative real-time PCR with 6 different genes for DNA and 6 microRNAs for RNA.

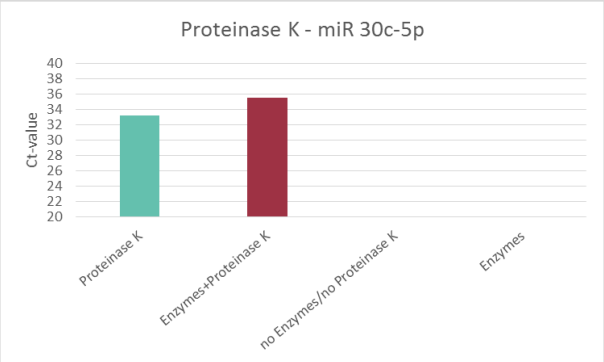


Fig.27: Results Proteinase K Test; Plasma; This figure exemplary shows reverse transcription quantitative real-time PCR results from microRNA 30c-5p. Different combinations with proteinase K, DNase I and RNase A were tested. Enzymes = DNase I, RNase A Finally, DNA and RNA were isolated simultaneously.

Figure 27 illustrates the problem with isolation of miRNA when using a higher volume of plasma. Only in combination with proteinase K, which cleaves all native proteins, it was possible to isolate RNA. This was a big step forward, but still some tests needed to be done on upscaling the starting volume of sample to be used for DNA/RNA isolation from exosomes.

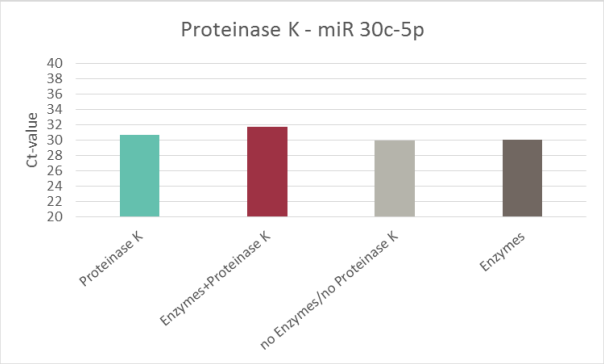


Fig.28: Results Proteinase K Test; Saliva; This figure exemplary represents reverse transcription quantitative real-time PCR results from microRNA 30c-5p. Different combinations with proteinase K, DNase I and RNase A were tested. Enzymes = DNase I, RNase A Finally, DNA and RNA were isolated simultaneously.

For salivary exosomes, the problem regarding the protein content was never an issue. However, the protocol needs to be the same in the sense of comparibility of saliva and plasma research results lateron. Therefore, the proteinase K test was also performed on salivary exosomes. Here, it was possible to isolate miRNA without treatment with proteinase K, but a change in the RNA content could be recognized when treating them with proteinase K. In figure 28, the Ct-values from RT-qPCR are illustrated. The red bar indicates miRNA content when exosomes were treated with DNase I/RNase A in combination with proteinase K. The Ct-value of 31, 75 is slightly higher than the value in samples 4 (dark grey bar), with a Ct-value of 30, 05. A difference of 1, 7 indicates that the amount of miRNA in sample 2 (red bar) is almost four times less the amount than in sample 4 (dark grey bar, only DNaseI/RNase A). This led to the conclusion, that proteinase K not only digested proteins around the exosomes, but also RNA or even miRNA. This result puts the miRNA-results (figure 27) in another picture, which lose credibility. The question was: how trustworthy are the results from figure 27 for miRNA? A new approach was tested with the so called DNA/RNA Shield™ from Zymo Research.

3.6.1. Proteinase K with Shield Buffer (Zymo Research)

Zymo Research offers with its shield buffer a solution, which inactivates infectious agents, such as viruses and bacteria, and preserves the genetic integrity and expression profiles of samples (Zymo Research, n.d.). DNA/RNA Shield™ (2 times concentrated) was used to ensure nucleic acid stability after lysis of exosomes. To prepare 1x solution of DNA/RNA Shield™ (2 times concentrated), an equal amount of the 2x concentrate was mixed with resuspended exosomes. Furthermore, PK digestion buffer was added for proteinase K to create perfect conditions for the enzyme.

Table 16: Schematic illustration of Proteinase K Test with Shield Buffer (2x); Plasma; Exosomes from 1, 5 ml plasma were isolated followed by removing EDTA with centrifugal devices 100K. Nucleic acids were digested and Shield Buffer (2x) was added to protect DNA and RNA from digestion with proteinase K in combination with PK digestion buffer. For RNA isolation, different amount of Ethanol (1x or 1,5x) were added. DNA and RNA were isolated with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. All 4 approaches were prepared in triplicates.

	1	2	3	4
V [ml]	1,5	1,5	1,5	1,5
Exosome Isolation	yes	yes	yes	yes
1x PBS [µl]	300	300	300	300
Centrifugal Devices 100K	yes	yes	yes	yes
DNase I [µl]	8	8	8	8
Mg Buffer [µl]	30	30	30	30
RNase, 1:500 [µl]	3	3	3	3
Incubation	37°C, 1h			
New V [µl]	340	340	340	340
Shield Buffer (2x) [µl]	340	340	340	340
New V [µl]	680	680	680	680
PK Digestion Buffer [µl]	68	68	68	68
Proteinase K (20mg/ml) [µl]	-	-	34	34
Incubation	55°C, 30 min			
DNA/RNA Isolation	yes	yes	yes	yes
Ethanol	1,5x	1x	1x	1,5x

In this experiment, exosomes were isolated from 1, 5 ml plasma and resuspended in 300 µl 1x PBS (pH=7, 4). Centrifugal devices with a molecular-weight cutoff of 100K were needed to get rid of EDTA from plasma samples. Digestion was performed with DNase I and RNase A to get rid of nucleic acids that were attached to the outside of exosomes. After incubation, the new volume reached was 340 µl. Therefore, 340 µl DNA/RNA Shield™ (2 times concentrated) was added to the mixture to protect nucleic acids from further digestion with proteinase K. To create optimal conditions for proteinase K, PK digestion buffer was added. The mixture was incubated for 30 minutes at 55°C and after incubation, the sample was centrifuged at maximum speed for two minutes and the aqueous supernatant was transferred into a clean tube for further nucleic acid isolation with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. For RNA isolation, different amounts of ethanol (100%) were added to the resulting flow-through. DNA and RNA were eluted in 100 µl ddH_2O. To assess the performance of the experiment with shield buffer and proteinase K with respect to exosomal DNA/RNA yield, qPCRs for 6 different genes and 6 different microRNAs were performed.

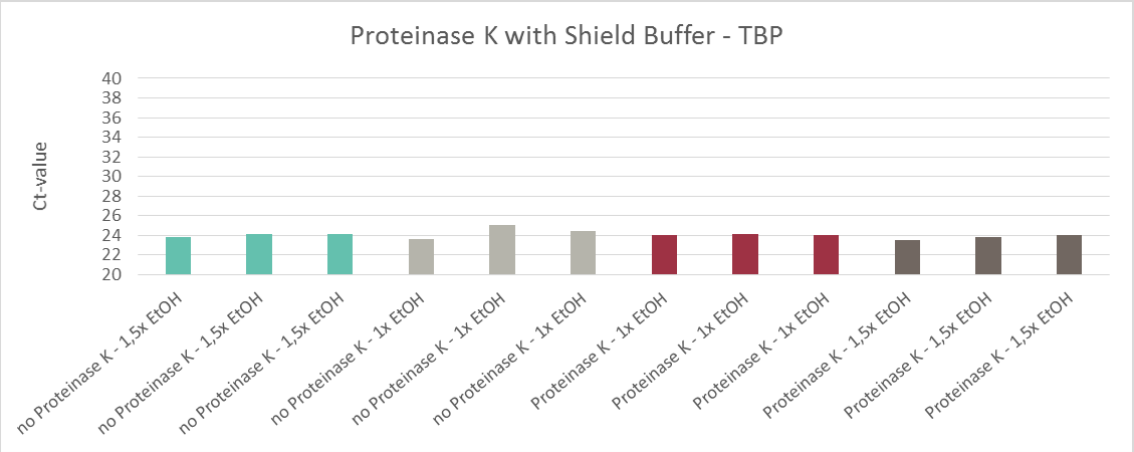


Fig.29: Results of Proteinase K Test with Shield Buffer (2x); TBP; This figure exemplary shows qPCR Ct-values for the TBP gene for different combinations with Shield Buffer (2x) and Proteinase K as a measure for achievable DNA yields from plasma exosomes.

In figure 29 the results for DNA are illustrated, whereas primers for the TBP-gene were used to exemplary display different Ct-values. In the figure, the description for different ethanol amounts can be neglected, because it only becomes important for the RNA. What is obvious at first glance is that the Ct-values are all stable and were around 24. This led to the conclusion, that proteinase K did not digest any DNA and that DNA was protected by DNA/RNA Shield™ (2 times concentrated) from Zymo Research. Another effect that could be observed was the quite low Ct-value that was achieved with this experimental set-up, suggesting high amounts of DNA.

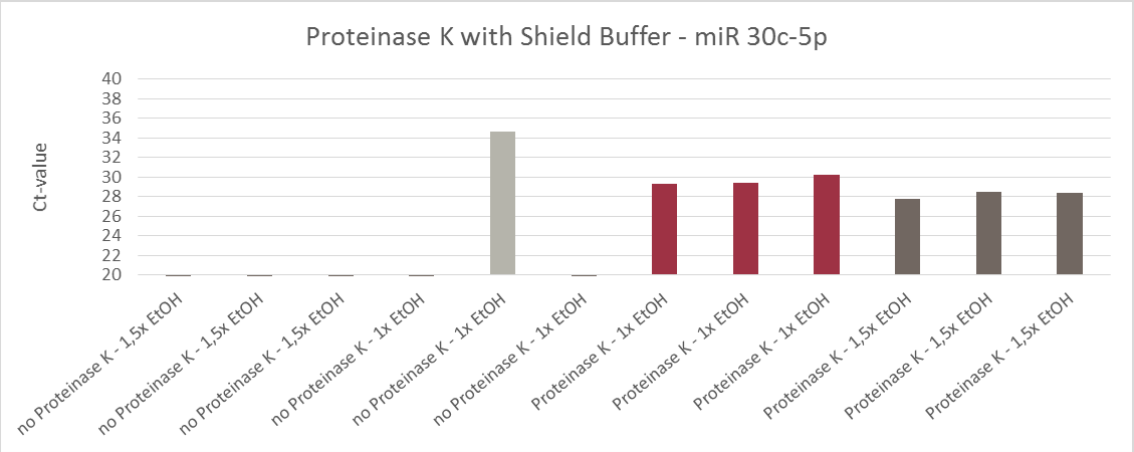


Fig.30: Results of Proteinase K Test with Shield Buffer (2x); miR 30c-5p; This figure exemplary shows RT-qPCR Ct-values for miR 30c-5p for different combinations with Shield Buffer (2x) and Proteinase K as a measure for achievable microRNA yields from plasma exosomes. In addition, different amounts of ethanol were used to isolate RNA, indicated by different colored bars.

In figure 30, the results for microRNA are illustrated in a bar graph, whereas different colored bars indicate different combinations of proteinase K and shield buffer (both from Zymo Research). At first sight it becomes visible that isolation of RNA without proteinase K is not possible, assuming that proteins disrupt RNA isolation at some point. In addition to that, differences in practical work could be observed with respect to the centrifugation step. Interestingly, the filter of the samples that were not treated with proteinase K was blocked what was due to the high amount of proteins. Another interesting thing was, that the amount of ethanol did play a crucial role. It could be find out, that different to manufacturer's protocol from Zymo Research that more ethanol leads to higher miRNA yield. Since the Ct-values are lower with 1,5x ethanol (27, 76 – 28, 48) than with 1x ethanol (29, 31 – 30, 21) to isolate RNA, it can be concluded that isolating RNA with 1,5x ethanol leads to higher yield of RNA.

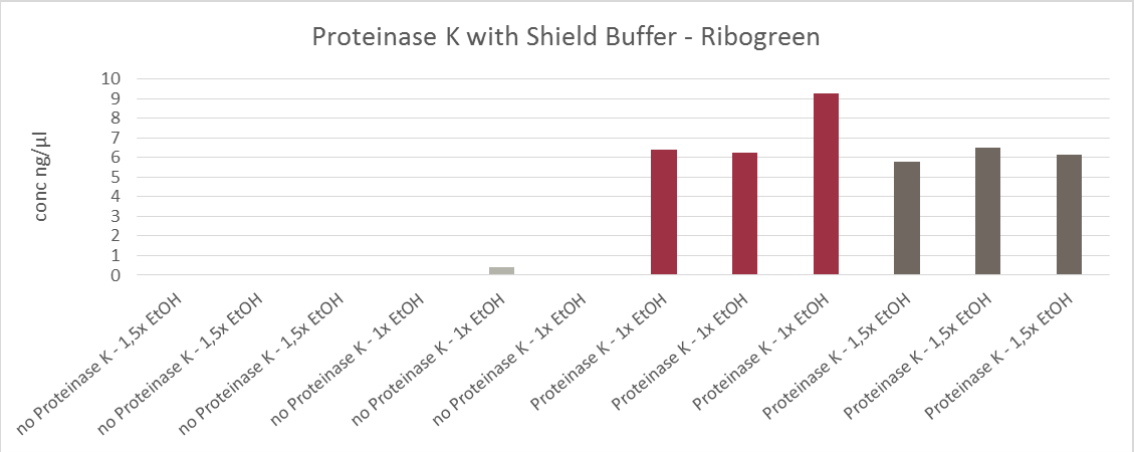


Fig.31: Results of Proteinase K Test with Shield Buffer (2x); Ribogreen; RNA concentrations were measured with Quant-iT™ RiboGreen® RNA Assay Kit on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific). Different combinations with Shield Buffer (2x) and Proteinase K were tested and represented as different colored bars. In addition, different amounts of ethanol for RNA isolation were tested to isolate RNA, indicated by different colored bars.

To underline RT-qPCR results, RNA concentrations were measured with Quant-iT™ RiboGreen® RNA Assay Kit on NanoDrop™ 3300 Fluorospectrometer (Thermo Scientific). Again, no RNA could be measured when no treatment with proteinase K was performed. Fortunately, high concentrations of RNA could be measured in samples that were treated with proteinase K. Interestingly, the overall amount of RNA in general could not be increased when using 1,5x ethanol (100%). A better yield could be achieved on microRNA with 1,5x ethanol. The conclusion from this experiment is, that treatment of exosomes with proteinase K, together with PK digestion buffer and DNA/RNA Shield™

(2 times concentrated) from Zymo Research, is necessary for isolation of RNA. Since microRNAs are the molecules of interest, 1,5x ethanol is sufficient to achieved higher yields of miRNA.

3.7. Upscaling

As already mentioned previously, exosomes are limited in all kind of body fluids. Therefore a higher starting volume of plasma and saliva had to be planned before. Because exosomes are limited, DNA and RNA, especially microRNA, can also found only in a small amount within the exosomes. One major part planned for the project Epitype-2 is smallRNA sequencing, which requires library preparation from miRNA to be done before. The typical starting concentration for library preparation is around 100 ng RNA, therefore the starting sample volume had to be increased. In previous experiments, isolation of nucleic acids from exosomes from 1, 5 ml plasma and saliva was possible and RNA concentrations of 50 ng for plasma and 60 ng for saliva were achieved. Therefore in this experiment upscaling of sample starting volume was performed with 1,5, 3 and 4,5 ml of plasma and saliva. In addition to this, the impact of proteinase K in combination with DNA/RNA Shield Buffer (2x concentrated) was tested on salivary exosomes. Since the maximal volume for exosome isolation is 1,5 ml, for 3 ml and 4,5 ml the exosome isolations were performed separately in 1,5 ml steps and then the exosomes were combined on one column for DNA and RNA isolation. In table X, the experimental set-up for upscaling is illustrated for plasma and saliva. Saliva was collected from healthy participants according to the SOP and a pool was mixed, from which aliquots of 1,5 ml were prepared. For plasma, 1,5 ml aliquots were also prepared from a previously mixed pool.

Table 17: Schematic illustration of Upscaling Test; Plasma and Saliva; Exosomes from 1,5 ml plasma were isolated and EDTA was removed from plasma exosomes with centrifugal devices 100K. For salivary exosomes, no centrifugal device was necessary and therefore this step was omitted. Nucleic acids were digested in some samples and proteinase K was used to digest proteins. Finally, DNA and RNA were isolated simultaneously, whereas for 3 ml and 4, 5 ml two respectively three samples were combined on one column.

	1		2		3			4	5
V [ml]	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5	1,5
Exosome Isolation	yes								
Centrifugal Device 100K	yes								
DNase [µl]	8	8	8	8	8	8	8	8	-
Mg Buffer [µl]	30	30	30	30	30	30	30	30	30
RNase A, 1:500 [µl]	3	3	3	3	3	3	3	3	-
Incubation	37°C, 1h								
New V [µl]	340	340	340	340	340	340	340	340	330
Shield Buffer (2x) [µl]	340	340	340	340	340	340	340	340	330
New V [µl]	680	680	680	680	680	680	680	680	660
PK Digestion Buffer [µl]	68	68	68	68	68	68	68	68	66
Proteinase K (20mg/ml) [µl]	34	34	34	34	34	34	34	34	33
Incubation	55°C 30, min								
DNA/RNA Isolation	yes								

Exosomes were isolated with miRCURY Exosome Serum/Plasma Kit from Exiqon according to the protocol. The isolated exosomes were then resuspended in 300 µl 1x PBS (pH=7, 4) and EDTA was removed from plasma exosomes with centrifugal devices

(Amicon® Ultra-0, 5 Centrifugal Filter Device) with a molecular-weight cutoff of 100K. Salivary exosomes did not contain any EDTA, therefore no centrifugal device was necessary. Afterwards, nucleic acids were digested while incubation for 1 hour at 37°C. After that, an equal amount of DNA/RNA Shield™ (2 times concentrated) was mixed with exosomes. PK digestion Buffer and reconstituted proteinase K was added to the mixture and the solution was incubated at 55°C for 30 minutes. After incubation, the sample was centrifuged at maximum speed for two minutes and the aqueous supernatant was transferred into a clean tube for further nucleic acid isolation with ZR-Duet™ DNA/RNA MiniPrep from Zymo Research. To isolate RNA, 1,5x ethanol (100%) were added to the flow-through. DNA and RNA were eluted in 100 µl H_2O. To evaluate the results, quantitative real-time PCR for 6 genes and 6 specific microRNAs was performed. A Ct-value difference of 1, when upscaling from 1,5 ml to 3 ml, respectively 0,5, when increasing the starting volume from 3 ml to 4,5 ml was expected.

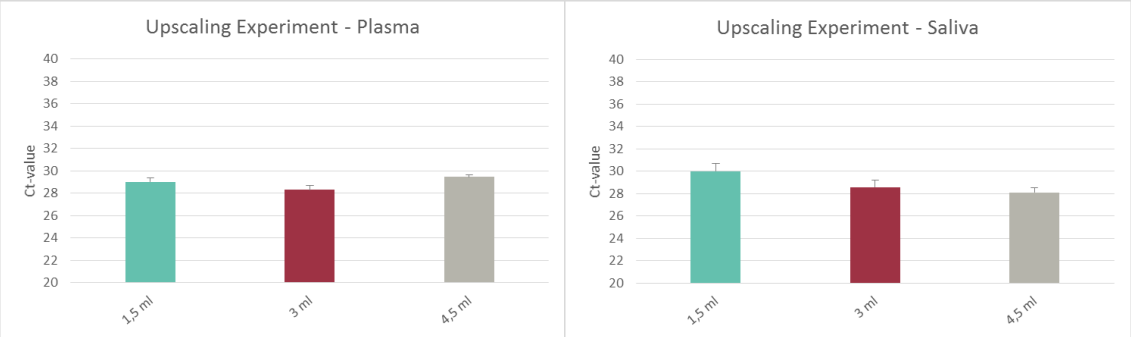


Fig.32: Results of Upscaling Test; Plasma and Saliva; This figure exemplary represents results from reverse transcription quantitative real-time PCR for microRNAs. Upscaling from 1,5 ml until 4,5 ml was tested on plasma and saliva.

In figure 32 the results from reverse transcription quantitative real-time PCR for microRNAs are illustrated. On the left side, results for plasma exosomes are shown. The first bar (turquoise color) represents Ct-values when miRNAs were isolated from exosomes from 1, 5 ml plasma. When comparing the first bar with the second bar (red color), which represents Ct-values for miRNAs from exosomes when isolating from 3 ml plasma, an improvement could be achieved. The Ct-values differ around 0, 7-0, 8, which indicate higher amounts of miRNAs in 3 ml plasma than 1, 5 ml plasma. However, when comparing miRNA yield from 3 ml with 4, 5 ml plasma, an increase of Ct-value could be observed. Therefore, upscaling to 4, 5 ml plasma was not possible. This led to the conclusion, that upscaling to 3 ml plasma was successful, indicated by a Ct-value difference of around 1. For saliva (figure 32, right side), constant improvement of Ct-value could be achieved from 29,98 (1,5 ml) to 28,58 (3 ml), until to 28, 1 (4,5 ml). These

differences in Ct-value indicate more RNA when isolating from 4,5 ml than from 1,5 ml. Therefore, upscaling was successful in saliva, even up to 4,5 ml saliva.

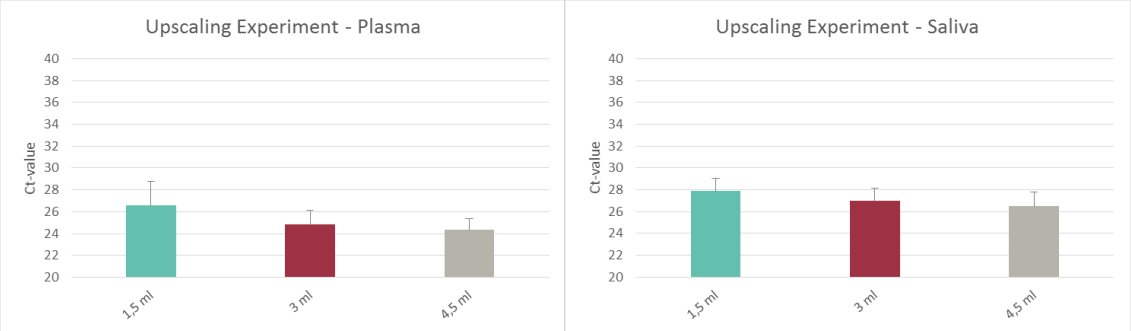


Fig.33: Results of Upscaling Test; Plasma and Saliva; This figure exemplary represents results from quantitative real-time PCR for 3 genes (H19, TBP, JUB). Upscaling from 1,5 ml until 4,5 ml was tested on plasma and saliva.

For DNA, upscaling could also be achieved for plasma and saliva samples. In figure 33, the qPCR results are displayed in a bar graph, whereas on the left side plasma samples are depicted and on the right side saliva samples are illustrated. Upscaling from 1,5 ml to 3 ml plasma was successful (1,5 ml: 26,56; 3 ml: 24,83), indicating by Ct-value differences of >1. Upscaling of DNA yield from plasma samples from 3 ml to 4,5 ml could also be achieved (3 ml: 24,83; 4,5 ml: 24,32). For saliva samples, upscaling was successful as well, which is recognized by a decrease of Ct-values (1,5 ml: 27,87; 3 ml: 26,96; 4,5 ml: 26,48). To summarize these results, it could be observed, that upscaling from 1,5 ml to 4,5 ml plasma or saliva, was successful and an increase of DNA yield, represented by increasing Ct-values of 1 respectively 0,5, could be observed. However, upscaling and the resulting higher amounts of RNA was not possible until 4,5 ml. Nevertheless, higher RNA yield could be observed, which can be taken from the decrease of Ct-value of 1 when comparing values from samples from 1,5 ml to 3,5 ml. After all, this was a huge improvement and allows exosomes isolation and subsequently DNA and RNA isolation from 3 ml plasma and saliva. Another important point of this experiment was the impact of DNA/RNA Shield™ (2 times concentrated) in combination with PK digestion Buffer and proteinase K (both from Zymo Research) on salivary exosomes. It could be found out that these solutions did not have any influence on the quantity of the exosomes. Therefore, this protocol can be used on saliva samples as well.

To assess what the quality of the isolated exosomes was and also what the content of the exosomes looked like, capillary electrophoresis was performed on Fragment Analyzer™.

3.8. Fragment Analyzer™

Capillary electrophoresis (Fragment Analyzer™) was used to test the quality and size of salivary and plasma exosomal DNA/RNA in dependence whether samples were treated with DNase I and RNase A or not. With this method, separation and quantification of double-stranded nucleic acids (DNA/RNA) was possible. The principle behind it is that negatively charged DNA/RNA fragments migrate differentially through the gel matrix towards the positive electrode as a function of length or size, whereas detection of DNA/RNA is achieved by an intercalating dye in the separation gel matrix, which emits fluorescence when bound to double-stranded DNA or RNA. Both, DNA and RNA from exosomes from plasma and saliva were tested with two different kits: (1) Sensitivity NGS Fragment Analysis Kit for DNA and (2) High Sensitivity RNA Analysis Kit (15nt) for RNA. Both kits are provided by the company Advanced Analytical to analyze nucleic acids. The aim of this experiment has been to illustrate the content of exosomes and also to show the difference between digested and undigested exosomes.

Samples from 3.7. Upscaling experiment were tested on the Fragment Analyzer™. For (1) Sensitivity NGS Fragment Analysis Kit for DNA, 2 µl sample dilution was mixed with 22 µl diluent marker in sample plate. Since the concentrations of DNA samples are higher than the detection limit of the Sensitivity NGS Fragment Analysis Kit, different dilution were prepared. For plasma DNA, dilutions of 1:5 were chosen and for saliva samples, 1:3 dilutions were prepared. The separation of DNA fragments was started and the resulting data was analyzed with the Fragments Analyzer Specific ProSize Data Analysis Software.

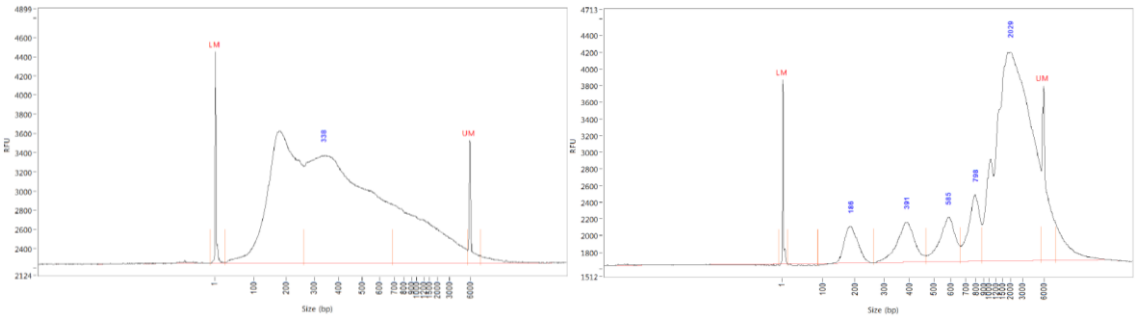


Fig.34: Fragment Analyzer™ Results for Plasma Exosomes digested and undigested; DNA; Plasma exosomes were digested (left) or left untreated (right) and DNA was isolated. Fragment Analyzer™ results for DNA are shown for enzymatic treated exosomes isolated from 3 ml plasma (left) and untreated exosomes isolated from 1,5 ml plasma.

In figure 34 DNA content of treated (with DNase I and RNase A) and untreated plasma exosomes are shown. On the left side, only exosomal DNA is illustrated, whereas on the right side, exosomal DNA and DNA that is attached on the surface of exosomes are shown. At first glance it can be noticed, that a shift from longer DNA (right picture) to smaller or fragmented DNA happened. This indicates, that longer DNA is attached on the outside of exosomes and smaller fragmented DNA is located within plasma exosomes. The appearance of longer DNA attached on the surface of exosomes, would lead to the assumption that it is genomic DNA. Interestingly, the DNA content of salivary exosomes looks a bit different. Again, after digestion with DNase I and RNase A longer DNA seems to be digested, which indicates again that longer DNA is attached on the outside of exosomes.

Still, some longer DNA is left in exosomes, but the proportions are different. Another interesting aspect is, that exosomes from different body fluids differ in their DNA content, considering fragment length.

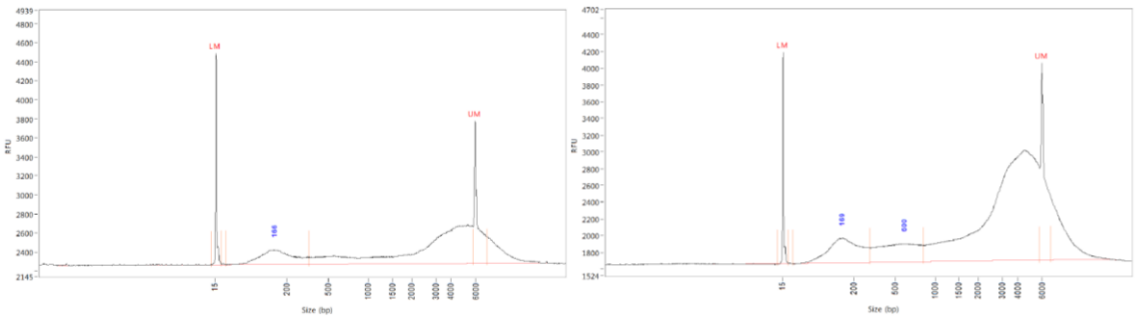


Fig.35: Fragment Analyzer™ Results for Saliva Exosomes digested and undigested; DNA; Salivary exosomes were digested (left) or left untreated (right) and DNA was isolated. Fragment Analyzer™ results for DNA are shown for enzymatic treated exosomes isolated from 3 ml saliva (left) and untreated exosomes isolated from 1, 5 ml saliva.

For RNA samples, no dilutions had to be prepared and 2 µl samples were denaturated and mixed with 18 µl diluent marker. Separation of RNA was started and the resulting data was analyzed with ProSize Data Analysis Software.

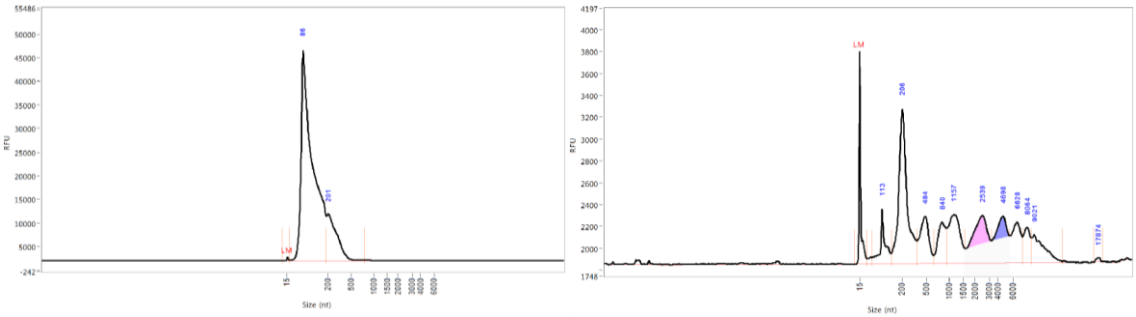


Fig.36: Fragment Analyzer™ Results for Plasma Exosomes digested and undigested; RNA; Plasma exosomes were digested (left) or left untreated (right) and RNA was isolated. Fragment Analyzer™ results for RNA are shown for enzymatic treated exosomes isolated from 3 ml plasma (left) and untreated exosomes isolated from 1,5 ml plasma.

In figure 36 results from (2) High Sensitivity RNA Analysis Kit (15nt) for RNA isolated from plasma exosomes are shown. As expected, only small RNAs were isolated after digestion with RNase A (figure 36, left side). When comparing the right picture (no digestion) to the left one (digestion with RNase A), a shift from longer RNAs attached to the surface of exosomes to small RNAs within the exosomes can be noticed. The content of exosomes is largely made up of small RNAs, respectively microRNAs. Therefore a high peak can be seen at 21-23 nucleotides on the left picture, figure 36.

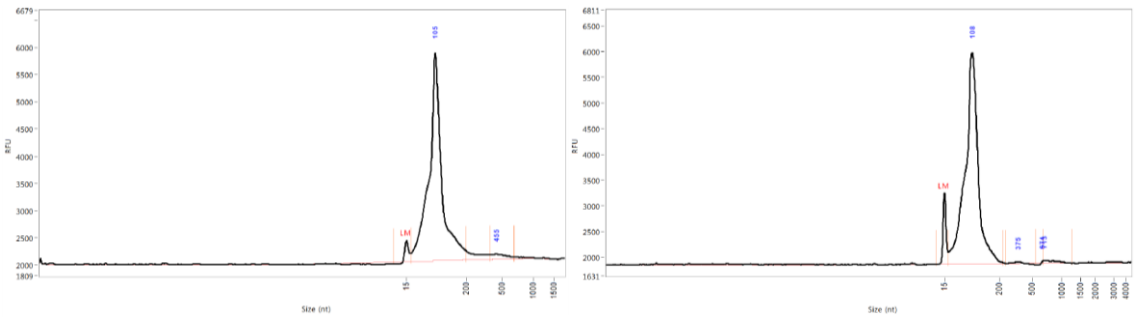


Fig.37: Fragment Analyzer™ Results for Saliva Exosomes digested and undigested; RNA; Salivary exosomes were digested (left) or left untreated (right) and RNA was isolated. Fragment Analyzer™ results for RNA are shown for enzymatic treated exosomes isolated from 3 ml saliva (left) and untreated exosomes isolated from 1, 5 ml saliva.

Interestingly, the difference between non-digested and digested exosomes of salivary exosomes is barely noticeable. Figure 37 demonstrates that salivary exosomes basically only contain small RNA whose size and quantity is not influenced by prior RNase treatment. A high peak at around 20 nucleotides in figure 37, left side, suggest, that only microRNA appears to be present in salivary exosomes. When comparing these results

from salivary exosomes to plasma exosomes, the size of RNA and therefore the content seems to be the same, but a difference on the surrounded RNA attached to the outside of exosomes is noticeable.

To summarize these results from (1) Sensitivity NGS Fragment Analysis Kit for DNA and (2) High Sensitivity RNA Analysis Kit (15nt) for RNA, the DNA content indeed differs between plasma and salivary exosomes. When comparing the RNA content, it seems that there are no differences in terms of size. Salivary exosomes appear to contain only small DNA and RNA. In contrast to RNA, a significant amount of higher molecular weight DNA seems to be attached to the outside surface of exosomes. It seems that plasma exosomes also contain only small fragmented DNA and RNA. Unlike salivary exosomes, plasma exosomes show a high amount of RNAs attached to the surface. However, after digestion with RNase A, only small RNAs were left within the exosomes. These results also show, that isolation of exosomes from plasma and saliva in combination with subsequent digestion with DNase I and RNase A was successful.

4. Summary and Conclusion

In previous experiments, which preceeded this master thesis, 9 different nucleic acid isolation kits were tested and evaluated with quantitative real-time PCR. The selection of kits contained DNA and RNA isolation kits, but also DNA/RNA isolation kits to isolate both nucleic acids simultaneously. From this study the conclusion was drawn, that a combination kit to isolate DNA and RNA at once is the preferred method, because sample volume is only used once. When testing various isolation kits on both cf saliva and plasma as well as thereof derived exosomes, the choice finally fell on ZR-Duet™ DNA/RNA MiniPrep from Zymo Research since it isolates both nucleic acids simultaneously and gave reasonable nucleic acid yields. In the following experiment, several enzyme pre-tests were performed to find out the best conditions for digesting DNA and RNA potentially attached to the surface of exosomes. Therefore, DNase I and RNase A were used to digest unwanted nucleic acids. Another problem was that plasma samples contained EDTA, which inactivates enzymes, such as DNase I, by chelating calcium and other metal ions (e.g. magnesium). To get rid of EDTA three different centrifugal devices were tested for its use in plasma exosomes by simple size exclusion. The outcome of this experiment was, that Amicon® Ultra-0, 5 Centrifugal Filter Devices with a molecular-weight cutoff of 100K works best. EDTA will flow through the membrane and previously isolated exosomes remain on the filter membrane and can be easily recovered. The resulting exosomes are free from EDTA and DNase I treatment can be performed in plasma samples. In addition to that, 6 different exosome isolation kits with different isolation principles were tested and the resulting DNA and RNA were evaluated by quantitative real-time PCR. Interestingly, it turned out that with every tested method, nucleic acids attached to the surface of exosomes were co-isolated. The effect of co-isolated DNA was bigger than with RNA. Because the lowest Ct-values for DNA as well as RNA were achieved with miRCURY Exosome serum/plasma kit from Exiqon we decided to this kit for any further exosome isolation from plasma and saliva In another experiment, 3 different methods to reduce the volume of DNA and RNA samples were tested and evaluated with qPCR. Unfortunately, with none of the tested methods it was possible to reduce volume respectively concentrate samples when they derived from DNase/RNase digested exosomes. This problem still needs to be solved before the next steps can be made (e.g. sequencing). Another problem we had to solve was a high amount of proteins when using higher sample starting volume. This summed up in two

experiments, where a protocol with DNA/RNA Shield™ (2 times concentrated) and proteinase K to digest proteins could be established and upscaling up to 3 ml plasma and saliva was possible. With 3 ml starting volume, enough DNA and RNA could be isolated from exosomes. Results from Fragment Analyzer™ gave an insight into the content of exosomes, which basically consisted of smaller fragmented DNA and microRNAs.

With all these experiments and their results, we have successfully established salivary as well as plasma protocols for exosome isolation and subsequent nucleic acid isolation, which build a sound basis for future systemic disease biomarker discovery & diagnostics from saliva as well as plasma. The whole protocol, which could be established at the end of this thesis, is described in chapter Materials & Methods, 2.8. Final Protocol for Isolation of DNA and miRNA from human Saliva and Plasma Exosomes.

Since the protocol to isolate DNA and RNA from plasma and salivary exosomes is basically established, one of the next steps is for sure to solve the problem for volume reduction. Before samples can be used for small RNA sequencing and therefore compared to each other, the volume needs to be reduced or the sample volume needs to be increased to get a higher yield of DNA and RNA per volume. Another experiment would be to investigate DNA methylation profiles within exosomes by using DNA methylation microarrays. Ideally, specific biomarkers (e.g. miRNAs) and/or DNA methylation changes can be found in exosomes and hopefully, an overlap between plasma and salivary exosomes can be detected. Once newly defined biomarkers can be validated in independent patient samples, early, non-invasive diagnosis of type 2 diabetes could become possible in near future.

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6. Appendix

Primer sequences

— DNA primers:

Primer name	Oligo name	Sequence (5‘-3‘)
H19	H19_fw	TGG GCC GCA GTG CCT CGT GGG
	H19_rev	GGG CGA AGC GGC CAC GGG AG
SNRPN	SNRPN_fw	GTC CCC CAT CCG CCC CCA ACT G
	SNRPN_rev	CCC ACT GCG GTT ACC CCG CAT GCT C
JUB	JUB_fw	GGC ATT GCT CTG CCC ATA GAT GCC TTT G
	JUB_rev	GGA ATC CCT GGT TTT GAC CTG GGG GA
GATA4	GATA4_fw	GCC GGG GTC GCG GAC TGC CA
	GATA4_rev	CGC GCT GCC CCA GGG ATT CCA
TBP	TBP_fw	GGC CCG CGG CTC TGT GCG
	TBP_rev	GTG TCG GAT CCG CAG GCG CAG
TJP	TJP_fw	TGT GCC GCG CGG TTG GGA GG
	TJP_rev	CAG CTT CCT ACG GCG CAT CGG GGA

— RNA primers:

Primer name	Oligo name	Sequence (5‘-3‘)
miR16	miR16_fw	CGC GCT AGC AGC ACG TAA AT
miR21	miR21_fw	GCC CGC TAG CTT ATC AGA CTG ATG
miR30c-5p	miR30c-5p_fw	AGC CGC CTG TAA ACA TCC TAC ACT
miR-205	miR-205_fw	CGC TCC TTC ATT CCA CCG G
miR-26b	miR-26b_fw	CGC CGC TTC AAG TAA TTC AGG AT
miR-92	miR-92_fw	GCCTGT ATT GCA CTT GTC CCC
UNI-rev	UNI_rev	GTG CAG GGT CCG AGG T

Results

Nucleic Acid Isolation Kit

— DNA Results

Mean Ct-values were calculated from 5 different primers: H19, SNRPN, TJP2, GATA4, TBP

(Fig.9)

Isolation Kit		**Zymo Duet**		**Qiagen Allprep**		**Roche**	
Sample	**Matrix**	**Ct-value**	**SD**	**Ct-value**	**SD**	**Ct-value**	**SD**
miRCURY Serum/Plasma	Plasma	34,05	1,24	33,38	1,50	0,00	0,00
cell-free	Plasma	33,00	1,21	33,39	0,91	0,00	0,00
miRCURY Serum/Plasma	Saliva	27,59	1,11	27,93	1,22	29,77	0,97
miRCURY Cells/Urine/CSF	Saliva	27,50	1,08	27,61	1,04	29,80	0,99
cell-free	Saliva	28,23	1,14	28,56	1,24	31,60	1,08

(Fig.11)

Isolation Kit		Zymo Duet	Qiagen Allprep	Roche
Sample	**Matrix**	**conc ng/µl**	**conc ng/µl**	**conc ng/µl**
miRCURY Serum/Plasma	Plasma P1	0,25	0,23	0,26
miRCURY Serum/Plasma	Plasma P2	0,25	0,23	0,26
miRCURY Serum/Plasma	Saliva P1	1,25	0,78	0,98
miRCURY Serum/Plasma	Saliva P2	2,14	1,09	1,47
miRCURY Cells/Urine/CSF	Saliva P1	1,35	0,62	0,85
miRCURY Cells/Urine/CSF	Saliva P2	2,80	1,17	1,43
cell-free	Plasma P1	0,24	0,25	0,25
cell-free	Plasma P2	0,25	0,25	0,26
cell-free	Saliva P1	1,15	0,44	1,08
cell-free	Saliva P2	1,43	0,49	1,12

— RNA Results

Mean Ct-values were calculated from different primers: mirR16, miR200c, miR21-5p, miR30c-5p

(Fig.10)

Isolation Kit		Exiqon Biofluids		Exiqon Cell/Plant		Zymo Duet	
Sample	**Matrix**	**Ct-value**	**SD**	**Ct-value**	**SD**	**Ct-value**	**SD**
miRCURY Serum/Plasma	Plasma	29,03	2,26	29,47	2,29	34,92	1,60
cell-free	Plasma	30,69	3,89	29,25	1,65	33,50	1,42
miRCURY Serum/Plasma	Saliva	32,12	0,68	29,68	1,40	32,91	1,30
miRCURY Cells/Urine/CSF	Saliva	34,05	1,13	29,69	1,25	37,74	1,05
cell-free	Saliva	34,00	1,18	29,40	0,88	33,96	1,47

Isolation Kit		Qiagen Allprep		Zymo RNA		Qiagen miRNeasy	
Sample	**Matrix**	**Ct-value**	**SD**	**Ct-value**	**SD**	**Ct-value**	**SD**
miRCURY Serum/Plasma	Plasma	33,45	1,00	37,03	0,96	29,95	1,60
cell-free	Plasma	37,28	1,25	35,79	0,00	31,30	0,81
miRCURY Serum/Plasma	Saliva	30,90	1,06	35,74	0,00	32,16	1,06
miRCURY Cells/Urine/CSF	Saliva	30,79	1,00	39,46	0,29	32,25	0,95
cell-free	Saliva	33,73	2,17	37,99	0,00	29,42	1,14

(Fig.12)

Isolation Kit		Exiqon Biofluids	Exiqon Cell/Plant	Zymo Duet
Sample	**Matrix**	**conc ng/µl**	**conc ng/µl**	**conc ng/µl**
miRCURY Serum/Plasma	Plasma P1	0,28	0,27	0,26
miRCURY Serum/Plasma	Plasma P2	0,28	0,28	0,26
miRCURY Serum/Plasma	Saliva P1	1,19	2,64	0,48
miRCURY Serum/Plasma	Saliva P2	2,69	4,96	0,84
miRCURY Cells/Urine/CSF	Saliva P1	1,03	3,26	0,62
miRCURY Cells/Urine/CSF	Saliva P2	2,31	6,11	0,73
cell-free	Plasma P1	0,41	0,27	0,25
cell-free	Plasma P2	0,51	0,27	0,26
cell-free	Saliva P1	0,77	4,01	1,27
cell-free	Saliva P2	1,56	6,36	1,99

Isolation Kit		Qiagen Allprep	Zymo DirectZol	Qiagen
Sample	**Matrix**	**conc ng/µl**	**conc ng/µl**	**conc ng/µl**
miRCURY Serum/Plasma	Plasma P1	0,31	0,51	0,25
miRCURY Serum/Plasma	Plasma P2	0,30	0,77	0,25
miRCURY Serum/Plasma	Saliva P1	0,39	0,55	0,27
miRCURY Serum/Plasma	Saliva P2	0,68	0,58	0,28
miRCURY Cells/Urine/CSF	Saliva P1	0,47	0,88	0,39
miRCURY Cells/Urine/CSF	Saliva P2	0,50	0,87	0,36
cell-free	Plasma P1	0,28	1,07	0,28
cell-free	Plasma P2	0,27	1,14	0,25
cell-free	Saliva P1	0,57	0,57	1,22
cell-free	Saliva P2	0,55	0,83	0,99

Combination of RNase A and DNase I

— DNA Results

(Fig.13)

Primer		**TBP**
Sample	**Matrix**	**Ct-value**
Amicon+DNase+RNase	Plasma	26,96
DNase+RNase	Plasma	24,77
no enzymes	Plasma	24,56
Amicon	Plasma	25,68
Amicon+DNase+RNase	Plasma	27,74
DNase+RNase	Plasma	25,63
no enzymes	Plasma	25,32
Amicon	Plasma	25,19
Amicon+DNase+RNase	Saliva	35,18
DNase+RNase	Saliva	31,8
no enzymes	Saliva	27,13
Amicon	Saliva	27,76
Amicon+DNase+RNase	Saliva	33,01
DNase+RNase	Saliva	33,53
no enzymes	Saliva	27,08
Amicon	Saliva	28,06

— RNA Results

(Fig.14)

		miR 30c-5p
Sample	**Matrix**	**Ct-value**
Amicon+DNase+RNase	Plasma	30,28
DNase+RNase	Plasma	30,9
no enzymes	Plasma	30,66
Amicon	Plasma	29,62
Amicon+DNase+RNase	Plasma	30,31
DNase+RNase	Plasma	28,79
no enzymes	Plasma	30,92
Amicon	Plasma	30,74
Amicon+DNase+RNase	Saliva	34,59
DNase+RNase	Saliva	34,07
no enzymes	Saliva	31,79
Amicon	Saliva	0
Amicon+DNase+RNase	Saliva	38,7
DNase+RNase	Saliva	33,23
no enzymes	Saliva	29,86
Amicon	Saliva	33,13

Centrifugal Devices Test

— DNA Results

(Fig.16)

Sample	Matrix	conc ng/µl
Amicon+Enzymes	Plasma	**5,05**
Amicon+Enzymes	Plasma	**6,99**
Amicon+Enzymes	Plasma	**7,85**
Pierce+Enzymes	Plasma	**8,07**
Pierce+Enzymes	Plasma	**7,34**
Pierce+Enzymes	Plasma	**5,71**
no Enzymes	Plasma	**24,37**
no Enzymes	Plasma	**20,27**
no Enzymes	Plasma	**30,00**

(Fig.17)

Primer		TBP
Sample	**Matrix**	**Ct-value**
Pall+Enzymes	Plasma	27,62
Pall+Enzymes	Plasma	31,81
Pall+Enzymes	Plasma	26,11

— RNA Results

(Fig.15)

Sample	Matrix	conc ng/µl
Amicon+Enzymes	Plasma	**2,19**
Amicon+Enzymes	Plasma	**2,31**
Amicon+Enzymes	Plasma	**1,89**
Pierce+Enzymes	Plasma	**0,06**
Pierce+Enzymes	Plasma	**0,45**
Pierce+Enzymes	Plasma	**0,14**
Pall+Enzymes	Plasma	**0,31**
Pall+Enzymes	Plasma	**0,38**
Pall+Enzymes	Plasma	**0,38**
no Enzymes	Plasma	**0,37**
no Enzymes	Plasma	**0,07**
no Enzymes	Plasma	**0,06**

Exosome Isolation Kit Test

— DNA Results

(Fig.19)

Primer		**TBP**	
Sample	**Matrix**	**Ct-value**	**SD**
Exiqon+Enzymes	Saliva	32,07	0,20
Exiqon	Saliva	25,42	0,16
HansaBioMed+Enzymes	Saliva	36,84	0,00
HansaBioMed	Saliva	33,90	1,48
Exospin+Enzymes	Saliva	34,92	1,62
Exospin	Saliva	30,60	0,38
iZon+Enzymes	Saliva	34,33	3,29
iZon	Saliva	32,32	0,25
Pierce Conc+Enzymes	Saliva	34,44	0,38
Pierce Conc	Saliva	30,00	0,50
Ultrac.+Enzymes	Saliva	38,02	0,04
Ultrac.	Saliva	32,73	0,20

— RNA Results

(Fig.20)

Primer		**miR 30c-5p**	
Sample	**Matrix**	**Ct-value**	**SD**
Exiqon+Enzymes	Saliva	32,58	0,07
Exiqon	Saliva	32,71	0,16
HansaBioMed+Enzymes	Saliva	36,44	0,71
HansaBioMed	Saliva	36,37	0,78
Exospin+Enzymes	Saliva	33,16	0,71
Exospin	Saliva	33,53	0,45
iZon+Enzymes	Saliva	34,35	0,57
iZon	Saliva	33,90	0,02
Pierce Conc+Enzymes	Saliva	33,12	0,41
Pierce Conc	Saliva	33,33	0,37
Ultrac.+Enzymes	Saliva	34,48	0,48
Ultrac.	Saliva	34,08	0,15

Volume Reduction Test

— DNA Results

(Fig.21)

Primer		**TBP**
Sample	**Matrix**	**Ct-value**
Univapo - totally evaporated	ddH_2O	25,04
Univapo - totally evaporated	ddH_2O	24,78
Univapo	ddH_2O	24,88
Univapo	ddH_2O	24,73
RVC 2-33 CDplus - totally dry-freezed	ddH_2O	32,47
RVC 2-33 CDplus - totally dry-freezed	ddH_2O	25,41
RVC 2-33 CDplus	ddH_2O	25,24
RVC 2-33 CDplus	ddH_2O	25,33
Control	ddH_2O	27,46
Control	ddH_2O	27,54

(Fig.23)

Primer		**TBP**
Sample	**Matrix**	**Ct-value**
Plasma	Plasma	26,13
Plasma concentrated	Plasma	23,52
Plasma	Plasma	26,18
Plasma concentrated	Plasma	24,55
Plasma	Plasma	26,85
Plasma concentrated	Plasma	25,15
Saliva	Saliva	32,82
Salvia concentrated	Saliva	31,67
Saliva	Saliva	30,25
Salvia concentrated	Saliva	31,57
Saliva	Saliva	33,9
Salvia concentrated	Saliva	31,17

(Fig.25)

Primer		**TBP**
Sample	**Matrix**	**Ct-value**
SpeedVac	Plasma	25,48
SpeedVac 5,5x	Plasma	23,28
Freeze-Dry	Plasma	25,29
Freeze-Dry 5,5x	Plasma	22,92
SpeedVac	Saliva	33,24
SpeedVac 5,5x	Saliva	31,7
Freeze-Dry	Saliva	35,59
Freeze-Dry 5,5x	Saliva	32,84

— RNA Results

(Fig.22)

Primer		**miR 30c-5p**
Sample	**Matrix**	**Ct-value**
Univapo - totally evaporated	ddH_2O	28,31
Univapo - totally evaporated	ddH_2O	27,97
Univapo	ddH_2O	28,2
Univapo	ddH_2O	29
RVC 2-33 CDplus - totally dry-freezed	ddH_2O	28,89
RVC 2-33 CDplus - totally dry-freezed	ddH_2O	28,66
RVC 2-33 CDplus	ddH_2O	28,27
RVC 2-33 CDplus	ddH_2O	28,49
Control	ddH_2O	31,48
Control	ddH_2O	31,48

(Fig.24)

Primer		**miR 30c-5p**
Sample	**Matrix**	**Ct-value**
Plasma	Plasma	0
Plasma concentrated	Plasma	0
Plasma	Plasma	33,2
Plasma concentrated	Plasma	37,65
Plasma	Plasma	34,51
Plasma concentrated	Plasma	37,45
Saliva	Saliva	34,11
Salvia concentrated	Saliva	36,52
Saliva	Saliva	33,87
Salvia concentrated	Saliva	36,03
Saliva	Saliva	34,04
Salvia concentrated	Saliva	35,23

(Fig.26)

Primer		**miR 30c-5p**
Sample	**Matrix**	**Ct-value**
SpeedVac	Plasma	34,28
SpeedVac 5,5x	Plasma	35,07
Freeze-Dry	Plasma	34,65
Freeze-Dry 5,5x	Plasma	35,23
SpeedVac	Saliva	32,46
SpeedVac 5,5x	Saliva	32,99
Freeze-Dry	Saliva	33,59
Freeze-Dry 5,5x	Saliva	34,71

Proteinase K

— RNA Results

(Fig.27, 28)

Primer		**miR 30c-5p**
Sample	**Matrix**	**Ct-value**
Proteinase K	Plasma	33,13
Enzymes+Proteinase K	Plasma	35,48
no Enzymes/no Proteinase K	Plasma	0
Enzymes	Plasma	0
Proteinase K	Saliva	30,7
Enzymes+Proteinase K	Saliva	31,75
no Enzymes/no Proteinase K	Saliva	29,95
Enzymes	Saliva	30,05

Proteinase K with Shield Buffer (Zymo Research)

— DNA Results

(Fig.29)

Primer		**TBP**
Sample	**Matrix**	**Ct-value**
no Proteinase K - 1,5x EtOH	Plasma	23,81
no Proteinase K - 1,5x EtOH	Plasma	24,14
no Proteinase K - 1,5x EtOH	Plasma	24,08
no Proteinase K - 1x EtOH	Plasma	23,58
no Proteinase K - 1x EtOH	Plasma	25,09
no Proteinase K - 1x EtOH	Plasma	24,42
Proteinase K - 1x EtOH	Plasma	23,98
Proteinase K - 1x EtOH	Plasma	24,08
Proteinase K - 1x EtOH	Plasma	24,02
Proteinase K - 1,5x EtOH	Plasma	23,48
Proteinase K - 1,5x EtOH	Plasma	23,77
Proteinase K - 1,5x EtOH	Plasma	24,04

— RNA Results

(Fig.30)

Primer		**miR 30c-5p**
Sample	**Matrix**	**Ct-value**
no Proteinase K - 1,5x EtOH	Plasma	0
no Proteinase K - 1,5x EtOH	Plasma	0
no Proteinase K - 1,5x EtOH	Plasma	0
no Proteinase K - 1x EtOH	Plasma	0
no Proteinase K - 1x EtOH	Plasma	34,62
no Proteinase K - 1x EtOH	Plasma	0
Proteinase K - 1x EtOH	Plasma	29,31
Proteinase K - 1x EtOH	Plasma	29,36
Proteinase K - 1x EtOH	Plasma	30,21
Proteinase K - 1,5x EtOH	Plasma	27,76
Proteinase K - 1,5x EtOH	Plasma	28,48
Proteinase K - 1,5x EtOH	Plasma	28,35

(Fig.31)

Sample	**Matrix**	**conc ng/µl**
no Proteinase K - 1,5x EtOH	Plasma	0,04858
no Proteinase K - 1,5x EtOH	Plasma	0,06065
no Proteinase K - 1,5x EtOH	Plasma	0,05625
no Proteinase K - 1x EtOH	Plasma	0,05434
no Proteinase K - 1x EtOH	Plasma	0,39666
no Proteinase K - 1x EtOH	Plasma	0,04965
Proteinase K - 1x EtOH	Plasma	6,40523
Proteinase K - 1x EtOH	Plasma	6,24899
Proteinase K - 1x EtOH	Plasma	9,28074
Proteinase K - 1,5x EtOH	Plasma	5,80333
Proteinase K - 1,5x EtOH	Plasma	6,50529
Proteinase K - 1,5x EtOH	Plasma	6,13544

Upscaling

— DNA Results

Mean Ct-values were calculated from 3 different primers: H19, TBP, JUB

(Fig.33)

Primer		**Mean**	
Sample	**Matrix**	**Cp-value**	**SD**
1,5 ml	Plasma	26,56	2,23
3 ml	Plasma	24,83	1,32
4,5 ml	Plasma	24,32	1,05
1,5 ml	Saliva	27,87	1,15
3 ml	Saliva	26,96	1,18
4,5 ml	Saliva	26,48	1,28

— RNA Results

Mean Ct-values were calculated from 3 different primers: miR16, miR21, miR 30c-5p

(Fig.32)

Primer		**Mean**	
Sample	**Matrix**	**Cp-value**	**SD**
1,5 ml	Plasma	29,00	0,37
3 ml	Plasma	28,29	0,37
4,5 ml	Plasma	29,44	0,20
1,5 ml	Saliva	29,98	0,68
3 ml	Saliva	28,58	0,59
4,5 ml	Saliva	28,10	0,40