BASICS TO WORK WITH EXTRACELLULAR VESICLES

LAB HAND BOOK

Research School
5-7 th of September, 2023
Tartu, Estonia

COMBIVET · BSEVS · Eesti Maaülikool Estonian University of Life Sciences · Tartu Ülikool Universitas Tartuensis 1632 · H-BOOST · Helsingin yliopisto Helsingfors universitet University of Helsinki · Universitas Studiorum Mutinensis · Euroopa Liit Euroopa Regionaalarengu Fond · Eesti tuleviku heaks

Estonian University of Life Sciences

LAB HANDBOOK: BASICS TO WORK WITH EXTRACELLULAR VESICLES

5th-7th of September 2023

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About COMBIVET & OH-Boost Joint Research school 2023

This collaborative research school is a dynamic partnership between the Combivet ERA Chair of Comparative Medicine at the Institute of Veterinary Medicine and Animal Sciences of the Estonian University of Life Sciences, and the OH-Boost project. It is a collective effort involving four esteemed institutions, Estonian University of Life Sciences (EMU), Tartu University (UT), University of Helsinki (UH), and University of Milan (UniMi). The research school is funded by the European Union's Horizon 2020 research and innovation program, under grant agreement No 857418, and Horizon Coordination and Support Actions under grant agreement № 101079349 respectively.

Throughout this exclusive research school, students will engage in hands-on training, mastering the essentials of extracellular vesicle (EV) isolation, characterization, and associated data analysis. Participants will be guided throughout the program by experienced instructors in the EV field.

The curriculum includes a diverse collection of lectures, covering vital theoretical foundations, optimal methodologies, practical tips, troubleshooting strategies, and steadfast adherence to quality benchmarks set by the International Society for Extracellular Vesicles (ISEV). Furthermore, the program explores cutting-edge advancements, including scalable EV production and crucial techniques such as size exclusion chromatography, tangential flow filtration, nanoparticle tracking analysis, flow cytometry for EVs, and EV RNA extraction and characterization.

This research school is designed to meet the needs of researchers focused on extracellular vesicles (EVs). Especially for those looking to build solid foundational knowledge to begin their exploration of the EV research field.

ACKNOWLEDGEMENTS

We acknowledge the support of our sponsors for the COMBIVET and OH-Boost joint conference 2023

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Message from the COMBIVET ERA chair

We are delighted to extend a warm welcome to the hands-on training school titled "Basics to Work with Extracellular Vesicles." This event is a collaborative effort between the COMBIVET ERA Chair of Comparative Medicine at the Institute of Veterinary Medicine and Animal Sciences of the Estonian University of Life Sciences, the Faculty of Biomedicine and Biotechnology at the University of Tartu, and the One Health Boost (OH-Boost) project . Notably, this marks the 6th research school organized by the Fazeli lab specifically centered around "Basics to Work with Extracellular Vesicles."

Recent times have witnessed a surge of interest in Extracellular Vesicles (EVs) across various scientific communities, particularly in fields dealing with cancer, infectious diseases, and inflammation. These tiny particles have emerged as vital mediators of cellular interactions, rendering them highly appealing targets for biomarker discovery and therapeutic applications. What was once primarily a subject of interest within Human medicine has now expanded to captivate researchers in veterinary medicine, as well as those studying plants and microorganisms. This remarkable field of science is truly gaining traction.

Under the guidance of the COMBIVET ERA Chair of Comparative Medicine at the Institute of Veterinary Medicine and Animal Sciences of the Estonian University of Life Sciences, we've fostered collaboration among diverse researchers exploring EVs. Our work spans EV purification, characterization, and the functionality of EVs. We have communicated our insights in respected journal publications with applications in science and medicine. The Baltic Society of Extracellular Vesicles strengthens our partnership with regional EV research experts.

Starting this year, the OH-Boost project joins us, adding expertise and innovation to our journey of advancing EV understanding and applications. This aligns with the One Health perspective, emphasizing human, animal, and environmental health interconnections—an essential research domain.

The primary objective of the ongoing research school is to introduce participants to the fundamentals of working with EVs. Given their minuscule size and intricate characteristics, studying EVs requires specific skills that may be novel even to experienced biomedical researchers. Our mission through this training school is to impart these crucial skills, nurturing a new generation of researchers poised to contribute to this exciting realm of science.

This training school owes its existence to the dedication of the Fazeli lab members in Tartu, who played a key role in organizing the event, alongside our global collaborators and researchers who generously shared their expertise throughout the research school. We extend our gratitude to all involved, including you, for your participation in the EV research school.

Alireza Fazeli
Professor
ERA Chair of Comparative Medicine in the Institute of Veterinary Medicine and Animal Sciences
Estonian University of Life Sciences
Tartu, Estonia

Organizing Committee

Event Planing and logistics

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Suranga Kodithuwakku
Subhashini Muhandiram
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Programme

Methods of Extracellular Vesicle (EVs) Isolation- 5th of September 2023		
9.00-9.20	**Welcoming and Introduction**	Alireza Fazeli
9.20-10.05	**Lecture 1** A guide to working with EVs: minimal experimental and reporting requirements	Kasun Godakumara
10.05-10.25	*Tea & Coffee break*	
10.25-11.10	**Lecture 2** Methods for EV purification	Qurat Ul Ain Reshi
11.10-12.10	Students 3-minute presentations	
12.10-13:10	*Lunch*	
13.10-15.10	**Workshop 1** Introduction to tangential flow filtration (TFF)	Getnet Midekessa Gayandi Ekanayake
15.10-15.30	*Tea & Coffee break*	
15.30-17.00	**Workshop 2** Size exclusion chromatography using manually packed chromatography column	Qurat Ul Ain Reshi Madhusha Prasadani Signe Parts
EV characterization and validation methods- 6th of September 2023		
9.00-9.40	**Lecture 3** Introduction to zeta view nano particle tracking analyser	Sascha Raschke
9.40-10.20	**Lecture 4** Flow cytometric analysis of EVs	Olavi Reinsalu
10.20- 10.40	*Tea & Coffee break*	
10.40-11.10	Student 3-minute presentations	
11.10-12.20	**Workshop 3** EV size and concentration measurements with NTA	Getnet Midekessa Chanaka Premathilaka Omid Mousavi
12.20 –13.20	*Lunch*	

13.20-14.20	**Workshop 4** Fluorescence NTA	Getnet Midekessa Chanaka Prematilaka Omid Mousavi
14.20-15.20	**Workshop 5** EV zeta-potential measurements with NTA	Getnet Midekessa Chanaka Prematilaka Omid Mousavi
15.20-15.40	*Tea & Coffee break*	
15.40-17.10	**Workshop 6** NTA data analysis	Johanna Piibor
17.10-17.50	**Workshop 7** Flow cytometric analysis of EVs	Olavi Reinsalu Dulmini N. Sapugahawatte
19.00 onwards	*Research School Dinner*	
EV validation methods and further applications- 7th of September 2023		
9.00-9.40	**Lecture 5** Transmission electron microscopy (TEM)	Aneta Andronowska
9.40-10.20	**Lecture 6** Advanced fluorescent microscopy methods to work with EVs	Sergei Kopantšuk
10.20-10.40	*Tea & Coffee break*	
10.40 – 11.40	**Workshop 8** Advanced fluorescent microscopy methods to work with EVs	Sergei Kopantšuk Caius Miller
11.40-12.20	**Lecture 7** Lipidomics as a tool in EV research	Minna Holopainen
12.20-13.20	*Lunch*	
13.20-15.00	**Workshop 9** EV RNA cargo analysis and RNA extraction from EVs	Kasun Godakumara Subhashini Muhandiram
15.00-15.20	*Tea & Coffee break*	
15.20-17.00	**Workshop 10** Analysis of EV RNA quality and integrity	Kasun Godakumara Subhashini Muhandiram
17.00-17.30	Final Student Evaluation	Evaluation committee

Lecturers and Instructors

Kasun Godakumara

Research Fellow, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Kasun Godakumara is a research fellow attached to the Combivet research group at the Estonian University of Life Sciences. His earlier research carrier was focused on intercellular communication in hard tissues. He earned his bachelor's in medical laboratory science and Master of Philosophy in Biotechnology from the University of Peradeniya in 2013 and 2016 respectively. After being employed as a laboratory analyst in the chemical pathology sector, he joined the University of Tartu as a doctoral student in 2018. He completed his PhD in Medicine in the year 2022. His studies are focused on extracellular vesicle-based embryo maternal communication.

Qurat Ul Ain Reshi

Specialist, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

I pursued my B.Sc in Zoology and M.Sc in Clinical biochemistry from the University of Kashmir. Afterwards, as an masters intern I worked on a project related to DNA replication machinery of Plasmodium falciparum. in the Special Centre of Molecular Medicine (SCMM), Jawarharlal Nehru University, New Delhi, India.

In 2018, I joined to Department of Pathophysiology, University of Tartu and started my PhD studies under the supervision of Prof. Alireza Fazeli. In Tartu, I am working in a developmental biology lab where I aim to understand the role of extracellular vesicles (EVs) in interactions between spermatozoa and oviductal epithelial cells in cattle. In addition, I am also working as a specialist at the Estonian University of life sciences with Prof. Ülle Jaakma where we aim to decipher the differences in seminal plasma-derived EVs of bulls with different fertility indices.

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Getnet Midekessa

Research Fellow, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Estonia

Getnet Midekessa received his BSc in Applied Chemistry from Hawassa University, Ethiopia. Following graduation, he worked in both the food and pharmaceutical industries in Ethiopia. Later he completed his master's studies in protein science and biotechnology at the University of Oulu, Finland. During his master's studies and post-graduation, he worked on projects involving protein characterization and extracellular vesicles research at the Faculty of Biochemistry and Molecular Medicine at the University of Oulu.

Getnet started his doctoral study in March 2018 at the Institute of Biomedicine and Translational Medicine, University of Tartu under the supervision of Prof. Fazeli. His research interests focus on the detection of surface extracellular vesicles using different biosensing technologies. As part of his research, he is also developing a tool to investigate the colloidal stability of extracellular vesicles for future drug delivery systems.

Chanaka Premathilaka

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Chanaka Premathilaka began his scientific career atthe University of Peradeniya, Sri Lanka. He obtained his bachelor's degree in molecular biology with a minor in Chemistry. Afterwards, he completed his MSc in Experimental Biotechnology at the Faculty of Medicine and Postgraduate Institute of Science focused on Bone Physiology where he examined biochemical changes of bone microarchitecture in an osteoporotic mouse model treated with some traditional cures for osteoporosis. He later enrolled in the Faculty of Veterinary Medicine to pursue his Master of Philosophy degree in Pathobiology, which was based on Assessing and Developing a Biological Intelligent Framework for Vector-Borne Infectious Diseases in Sri Lanka. With the onset of COVID-19 epidemics around the world in 2020, he took part in the research and diagnosis of the pandemic outbreak in Sri Lanka. He joined the COMBIVET ERA Chair team in September 2021 as a PhD student specializing in the pathophysiology of infectious diseases.

Seyed Omid Reza Mousavi

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Seyed Omid Reza Mousavi did his Master's degree thesis on cellular and molecular interactions of spermatozoa with fallopian tube epithelial cells, and completed his Master's degree in Genetics in 2019 from the Medical Sciences branch of Azad University in Iran. Currently, he is a PhD student at the Institute of Veterinary Medicine and Animal Sciences at the Estonian University of Life Sciences. As a member of Prof Fazeli's research group, he is working on the toll-like receptor-mediated functions of extracellular vesicles in embryo-maternal interactions.

Gayandi Ekanayake

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Gayandi Ekanayake began her scientific career at the University of Kelaniya in Sri Lanka, where she graduated in 2018 with a BSc. (Honors) in Botany (Plant Sciences) and in 2020 with an MSc. in Crop Protection and Plant Biotechnology. She participated in two India-Sri Lanka joint research projects while pursuing her master's degree, including "High throughput genotyping to expedite the genetic characterization and dissection of important agronomic traits of tea" and "Genetic analysis of plant pigments of tea (*Camellia Sinensis* L.)".

Under the direction of Professors Alireza Fazeli and Rajeev Bhat, Gayandi enrolled as a doctoral student at the Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences in 2021 for a combined PhD study between the ERA Chairs of Combivet and Valortech. She also works at the Estonian University of Life Sciences as a junior research fellow. Her PhD work focuses on the purification and characterisation of extracellular vesicles (EVs) derived from plant waste materials and their byproducts

Madhusha Prasadani

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Madhusha Prasadani completed her BSc degree (Agriculture Technology and Management, majoring in Animal Science) in the year 2019 from the Faculty of Agriculture, University of Peradeniya, Sri Lanka. She continued her master's (MSc) degree in Biotechnology at the Postgraduate Institute of Agriculture, University of Peradeniya and completed it in 2021. She has worked as a graduate research assistant in the fields of environmental studies and oral cancer studies. She joined the COMBIVET ERA Chair team in March 2022 as a PhD student in Veterinary Medicine and Animal Science at the Estonian University of Life Sciences. She is currently working on identifying the potential of milk extracellular vesicles as a biomarker in the discovery of insulin resistance in dairy cattle and humans under the supervision of Prof. Alireza Fazeli, Prof. Hanno Jaakson and Prof. Vallo Volke.

Johanna Piibor

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Johanna Piibor graduated from Veterinary Medicine at the Estonian University of Life Sciences (MVD). During her studies she developed an interest in reproductive biology and began her transcendence from large animals to mammalian cells. In 2020, she started doing her PhD in molecular studies of subclinical endometritis. Her work aims to characterize molecular changes in the uterine tissue of cattle with subclinical endometritis and its potential use in diagnostics.

Sascha Raschke

Application and Scientific support, Particle Metrix GmbH, Germany

Sascha Raschke studied biology at the University of Düsseldorf, Germany. After graduating in molecular biology and oncology, he did his PhD in radiation biology. After a postdoc position in a radiobiological research project in Essen, he worked in scientific project management, setting up and implementing an interdisciplinary project with a medical and a physical research institute. In 2015 he joined Particle Metrix and was responsible for the scientific and technical support of the ZetaView® instrument. In 2020 he took over the Applications division and has since become increasingly involved in scientific exchange and cooperations as well as application development. He is also responsible for the production of video tutorials, writing and updating operating instructions and taking care of NTA-related inquiries.

Olavi Reinsalu

Research Fellow in Biomedical Technology, Faculty of Science and Technology, University of Tartu, Tartu, Estonia

Olavi Reinsalu received his diplomas for his bachelor's and master's degrees in the curriculum of gene technology at the University of Tartu in 2012 and 2015, respectively. For the research of his master's thesis he studied virus-like particles carrying melanoma antigens. After graduation he worked for three years as a researcher at the University of Tartu and the Competence Center of Health Technologies developing a bovine fluorescence in situ hybridization technique as part of a R&D project for agricultural enterprises. Starting in the fall of 2018 he became a doctoral student at theUniversity of Tartu studying in the curriculum of technology and engineering specializing in biomedical technology and later defended his thesis in 2022. His PhD study is a continuation of his research from master's and is also about extracellular vesicles and virus-like particles carrying melanoma antigens. He received his PhD in 2022. Now he is working as a post-doc studying synthetic vesicles (also known as liposomes) as drug delivery vehicles.

Aneta Andronowska

Professor of reproductive endocrinology at Department of Hormonal Action Mechanisms, Institute of Animal Reproduction and Food Research of Polish Academy of Sciences, Olsztyn, Poland

Prof. Aneta Andronowska is the professor of reproductive endocrinology at the Department of Hormonal Action Mechanisms, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Olsztyn, Poland. Professor Aneta Andronowska, a specialist in immuno-endocrine and molecular mechanisms involved in: embryo-uterine interactions during the maternal recognition of pregnancy and implantation; estrus synchronization and its effect on the oviduct, as well as the influence of the immune system on corpus luteum function. In the current studies her team is involved in the study of the role of chemokines in peri-implantation period in porcine uterus and ovary. She is also involved in the creation of a new laboratory for cell and tissue imaging on light and electron microscopy levels. For many years she is also the Head of PhD studies at the IAR&FR PAS, Olsztyn, Poland

Sergei Kopanchuk

Research Fellow, Institute of Chemistry, University of Tartu, Tartu, Estonia

Over the past 20 years at the Universities of Tartu and Uppsala, Sergei Kopanchuk has gained expertise in studying the molecular mechanisms of the functioning of various G protein-coupled receptors, focusing on the dynamics of system behavior. He is involved in the development of various types of assays for drug screening and biosensors. In the last decade, he has built and configured microscopes with different fluorescence imaging modes (confocal, wide-field and TIRF/HILO) for various measurement techniques such as FLIM in time and frequency domain, TGL, FCS and single molecule/particle detection/tracking. In addition, he has enhanced these optical setups with super-resolution options, STED and localization microscopy.

Caius Miller

PhD student, Institute of Chemistry, University of Tartu, Tartu, Estonia

Caius Miller completed his bachelors and masters in University of Tartu on the subject of materials science. I worked in the Laboratory of Laser Spectroscopy with fluorescent nanodiamonds. Afterwards I moved onto my Phd where the idea has been to combine my previous work with fluorescent nanoparticles and use it in a bioorganic environment. To achieve this I have been working in the Laboratory of Bio-organic Chemistry in Tartu University using fluorescence to detect single EVs through EV specific tetraspanin markers.

Minna Holopainen

Postdoctoral researcher, University of Helsinki Lipidomics Unit (HiLIPID), Finland

Minna Holopainen received her Doctoral degree in Physiology and Neuroscience from the University of Helsinki in 2020. Her research topic was related to the immunomodulation of mesenchymal stromal cells, their extracellular vesicles and their (metabo)lipidomics. She conducted the research at Finnish Red Cross Blood Service and also started working at Helsinki University Lipidomics Unit (HiLIPID) in 2018. Currently, she is a postdoctoral researcher at HiLIPID and runs the lipidomics core facility services and method development.

Signe Parts

PhD Student, Faculty of Biomedicine, University of Tartu, Tartu, Estonia

Signe Parts began her studies at Tartu University, Estonia with BSc studies in Gene Technology where she had a great opportunity to collaborate with AS Icosagen in cell line development and gene editing with the CRISPR system to create a more efficient selection system for super-producer cells. In 2020 she continued her studies in the Biomedicine curriculum. Her Master's thesis focused on diabetic patients' urinary extracellular vesicles isolation methods. In September 2022, she started her PhD studies as a junior researcher in Prof. Fazeli's research group under the supervision of Prof. Vallo Volke and Prof. Alireza Fazeli. During her PhD studies, Signe will continue her work with diabetic patients and extracellular vesicles. In addition to her research, she enjoys popularizing science in Estonia. She is working as a lecturer at the University of Tartu Youth Academy where she is teaching biology workshops.

Subhashini Muhandiram

PhD Student, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Subhashini Muhandiram gained her bachelor's degree in Medical Laboratory Science from the University of Peradeniya, Sri Lanka. She has later completed Master of Philosophy degree in Molecular and cancer biology. During this time she has worked on establishing a low-cost molecular diagnostic assay for early screening of Human papillomavirus infection (HPV) among females on emphasising less and non-invasive sampling methods. Over the years, she has developed an interest in biomarker screening for diagnostic assays, cell biology and reproductive physiology.

She is currently a doctoral student attached to the ERA chair of Combivet at the Estonian University of Life Sciences under the supervison of Prof. Alireza Fazeli. Her PhD study is focused on elucidating the molecular mechanism of extracellular vesicle-mediated signal transduction during embryo implantation.

Dulmini Nanayakkara

Research Fellow, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, Estonia

Dulmini received her bachelor's degree with first class and Distinction from Mangalore University, India majoring in Microbiology in the year 2013. Then, she completed her master's degree in Medical Microbiology at the University of Peradeniya, Sri Lanka in 2016. Later, she completed her PhD in the year 2022 at the Department of Microbiology, Faculty of Medicine, the Chinese University of Hong Kong, Hong Kong SAR in Microbiology. Her doctoral research focus was "Characterization of Streptococcus agalactiae from humans and animals' and it involved the investigation of antimicrobial resistance, detection of possible zoonotic traits of GBS and application of novel MALDI-TOF-MS based typing method in collaboration with Swiss Tropical and Public Health Institute (Swiss TPH), Switzerland apart from genomics. After the doctoral training, she worked as a postdoctoral research fellow at the Chinese University of Hong Kong and dealt with several research works related to antimicrobial resistance, one-health and microbiome. She is currently a research fellow at EMU and working on antimicrobial properties of plant and animal-derived extracellular vesicles. Dulmini won President's Award for Scientific Research in 2019 and National Research Council (NRC) Merit Awards for Scientific Research in 2021 for her previous research work.

WORKSHOP 1

Tangential Flow Filtration (TFF)

Date and Time: 5th of Septemeber 2023, 13.10-15.10

Instructors: Getnet Midekessa, Gayandi Ekanayake

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Students will get hands-on experience in using the TFF method to purify EVs from cell-conditioned media. More details related to the workshop can be found in **Appendix 1.**

Demonstration: JAr cell-conditioned media will be used to demonstrate EV purification by Tangential Flow Filtration (TFF).

Principles of the TFF (PALL Life Sciences) system

Background information on membranes and membrane filtration

Membranes are an essential element in most biotechnological processes. These membranes used to filter biological relevant biomolecules from contaminants based on the size of pores on the membrane. In other words, filtration methods employed membrane chemistry that depend on the size and charge difference separations with high-throughput and high-resolution needs. Advances in membrane filtration technology involve high-resolution applications, such as separation of proteins from virus-like particles, protein purification using Tangential Flow Filtration (TFF) and updated membrane chromatography (van Reis & Zydney, 2001).

Classifications of membrane filtration: Normal flow (dead end) filtration and tangential flow (cross-flow) filtration are the two types of filtration modes that can use either microfiltration or ultrafiltration membranes. L ikewise, TFF can be classified into microfiltration and ultrafiltration based on the nature of membrane porosity, i.e. the size of the pores in the filter

membrane determines what passes through and what's retains in the system (Schwartz & Seeley, 2002). As the name tells, the membrane pore size for microfiltration is in micron level and it ranges from 1-10um, whereas ultrafiltration membranes have a pore size range of 0.001-0.1um (Kumar, 2013). In normal flow filtration, a fluid/feed stream is applied perpendicular to the membrane surface and larger particles in the feed streams pile up at the membrane surface, gradually blocking the membrane due to the formation of filter cake /gel. Formation of gel onto the membrane surface caused the membrane to foul and the flow of liquid through the membrane lowered. This membrane filtration method results in lower separation efficiency for the product we want to filter. In most of the cases, normal flow filtration is used in sterile filtration of bacteria and small microorganisms, particles from feedstock solutions and medium exchange (Kuriyel & Zydney, 2000).

With TFF (see Figure 3 below), the feed stream flows through the feed channel and move across the membrane i.e. tangentially to the membrane as well as through the membrane. The cross-flow movement of the feed stream averts the accumulation of molecules at the surface of the membrane that can cause membrane fouling. Retained product /sample of interest is circulated to sample holder and will continue to loop through the membrane for the given process time. The simple example to understand the principle of TFF is to consider how to separate sand from pebbles using a siever (sifting screen). In this scenario, the sand (i.e. permeate) will pass through the sifter pores when sifter is shaking and this results in pebbles (i.e. retentate) to remain on the top of the membrane being concentrated.

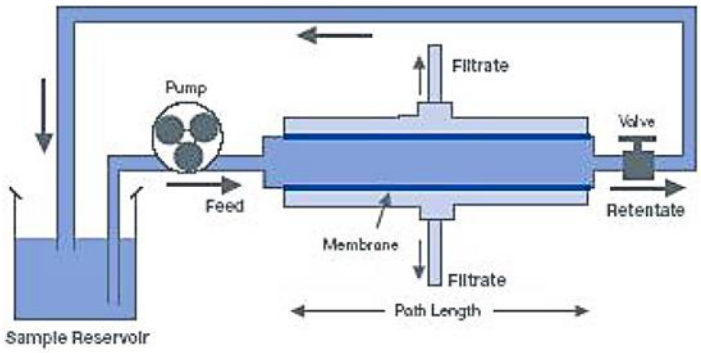

Figure 3: Diagram for the flow of fluids/sample solution in a simple TFF device (Adopted from Pall systems)

Both transmembrane pressure and cross-flow velocity are important features in TFF devices, as the pressure is needed to drive the flow of fluid through the membrane, along with permeable molecules. Whereas, the cross-flow velocity is the rate determining step of the flow through the feed channel and along the membrane. This velocity adds the force to disrupt molecules that can cause membrane fouling (GE, 2014).

Applications of TFF: Primarily TFF is designed for product concentration (of biomolecules) as well as diafiltration (i.e. removal of salts and buffer exchange roles) in research, biopharmaceutical, and medical industries. In addition, TFF can be applied in cell harvesting and product fractionation (GE, 2014).

Some clinical studies introduced TFF to isolate EVs from conditioned cell culture media. Upon comparison with EVs isolated with anion exchange chromatography, TFF purified EVs were contaminated with proteins and lipid droplets. This might suggest that the need for additional EV isolation step to be introduced for removal of contaminants (Heath et al., 2018).

Protocol for TFF: The TFF-Easy (HansaBioMed) system employs polysulfone membranes with a 20 nm pores to concentrate and remove small proteins and biomolecules from conditioned media and biological

sources. The EV concentration and purification involving TFF-Easy include the following steps:

1. Tangential flow filtration (TFF) is performed following sequential centrifugation of the conditioned medium prior to the purification of EVs by TFF. TFF-Easy filtration-based devices were used to concentrate the EVs (HansaBioMed, Tallinn, Estonia).

2. After washing the TFF-Easy filtration device three times with Milli-Q water, the conditioned medium was loaded into a pre-connected syringe-1 via the sample injection nozzle.

3. Following the loading of the conditioned medium into pre-connected syringe-1, the conditioned medium was pushed into pre-connected syringe-2 by closing a valve. Following the opening of the permeate nozzles on the device, the concentration process of the conditioned medium began.

4. By pushing syringes 1 and 2 alternatively up and downwards, the conditioned medium was retained and concentrated to around 1.6 to 1.9 mL.

5.The collected concentrated conditioned media was transferred to a sterile tube. The collected conditioned media was further concentrated to 500 µL using a 10 kDa cut-off concentrator before being subjected to the SEC column. A detailed description of TFF-easy can be found in Appendix 1.

Notes –

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WORKSHOP 2

Size exclusion chromatography using manually packed chromatography column

Date and Time: 5th of September 2023, 15.30-17.00

Instructors: Qurat Ul Ain Reshi, Madhusha Prasadani, Signe Parts

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Purification of Extracellular Vesicles derived from bovine follicular fluid by Size-exclusion chromatography (bench-top column)

Reading materials:

Reshi Q.U.A., Hasan M.M., Dissanayake K., Fazeli A. (2021) Isolation of Extracellular Vesicles (EVs) Using Benchtop Size Exclusion Chromatography (SEC) Columns. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_14

Background

Size-exclusion chromatography (SEC), also known as molecular sieve chromatography, is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers.

For separation of different molecules in SEC, we use sepharose resin, which is a porous matrix of spherical particles that lack reactivity and has adsorptive properties. After sample has been applied, molecules larger than the pores are unable to diffuse into the beads, so they elute first. Molecules that range in size between the very big and very small can penetrate the pores to varying degrees based on their size.

Benchtop columns also know as gravity flow columns (Figure 4), is one kind of size-exclusion chromatography column, which is packed with Sepharose. Sepharose is a trade name for a cross-linked, beaded-form of agarose, a polysaccharide polymer material extracted from seaweed.

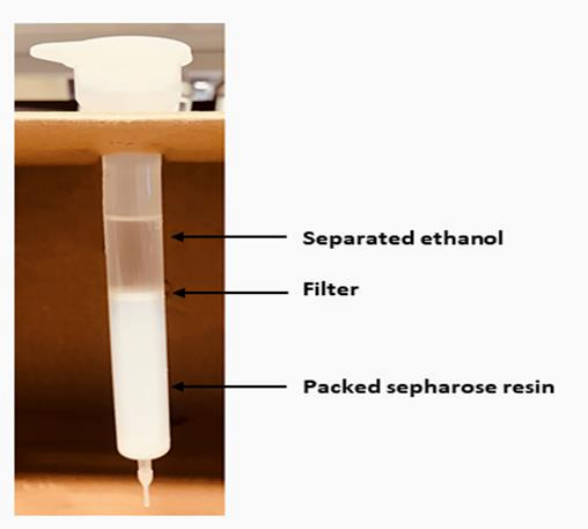

Figure 4: Benchtop column with sepharose resin

Required materials:

1. Benchtop column (Econo-pac® Disposable chromatography column).

2. Sepharose (Sepharose™ 4 fast flow)

3. Amicon Ultra-15 Centrifugal filters 10kDa (Millipore)

A. Sample Preparation:

Despite the differences in sample types, all the samples need to be pre-processed before using in the benchtop SEC columns. The larger particles and cell debris are required to be removed from the samples using differential centrifugation. Centrifugation speed could vary, depending on the samples and must be optimized accordingly. The centrifugation speeds mentioned below were used for bovine follicular fluid samples.

1. First, centrifuge the sample at 300g for 10 min at 4˚C to remove different and larger cell types.

2. Then, collect the supernatant and centrifuge again for 10 min at 2000xg at 4˚C in a new centrifuge tube.

3. Now, centrifuge the collected supernatant at 10,000xg for 30 min at 4˚C to remove the cell debris and apoptotic bodies. Transfer the supernatant to a new tube.

4. Now, using a centrifugal filter unit (10 kDa) concentrate the sample up to 500 µl by centrifuging at 3000xg for 20 minutes at 4˚C and keep it on ice. The samples are ready for isolation of EVs using benchtop columns.

B. Packing a benchtop column with Sepharose:

1. Take out one benchtop column with a closed end.

2. Shake the Sepharose container well to mix ethanol with the beads.

3. Fill the column with 15 ml of Sepharose**.**

4. Mount the column vertically and let the column to stand for 2 hours or more until the Sepharose beads settle down and separate from the 20% ethanol, as shown in figure 4.

5. Put the filter carefully on the top of the Sepharose layer using forceps with blunt ends without disrupting the layer of Sepharose and press the filter down with the forceps.

C. Prewash of the column before use:

1. Take out a benchtop column prepared by the protocol mentioned.

2. Break the lower tip of the column and let the ethanol flow freely through the column.

3. When the level of ethanol is closer to the level of the filter, prepare yourself to add 15 ml MQ directly into the column. After the ethanol passes down the filter immediately add 15 ml of MQ, followed by 15 ml of PBS**.**

D. Purification of the EVs in the sample:

1**.** Start with labelling your 1.5 ml microcentrifuge tubes and place them on ice until you start collecting the fractions.

2. Take the pre-processed sample (max 500 µl).

3. Add the sample on top of the filter inside the column.

4. Allow the sample to pass through the filter and add 10 ml of PBS directly into the column.

5. Immediately start the fraction collection (max 500 µl). In order to determine EV fractions from your sample, collect 20 fractions in 1.5 ml microcentrifuge tubes each with 500 µl of the eluted sample.

6. Place the collected fractions on ice.

E. Washing the benchtop column for re-use:

1. After collecting all the fractions, let the PBS in the column drain.
2. Then add 15 ml of Milli Q to wash the column.
3. Once Milli Q ran through the column, add 15 ml 0.1 M NaOH.
4. Then wash the column twice with 15 ml of Milli Q.
5.Wash the column again with 15 ml of PBS.
6.Now, load the new sample and isolate EVs as mentioned previously.

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WORKSHOP 3

EV size and concentration measurements with NTA

Date and Time: 6th of September 2023, 11.10-12.20

Instructor: Getnet Midekessa, Chanaka Premathilaka and Omid Mousavi

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Demonstration of the operation of the Zetaview. Participants will be allowed to operate the machine and measure samples (EVs isolated from JAr cells). More details related to the workshop can be found in **Appendix 2.**

Reading materials:

Dissanayake K., Midekessa G., Lättekivi F., Fazeli A. (2021) Measurement of the Size and Concentration and Zeta Potential of Extracellular Vesicles Using Nanoparticle Tracking Analyzer. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_1

Required materials:

- 100 nm polystyrene beads standard solution 110-0020
- Dulbecco`s Phosphate Buffered Saline – 500 ml × 6
- Double deionized water (Milli-Q)

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WORKSHOP 4

Fluoroscence NTA

Date and Time: 6th of September 2023, 13.20-14.20

Instructor: Getnet Midekessa, Chanaka Premathilaka and Omid Mousavi

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Demonstration of the measurement of fluorescently labelled EVs using the Zeta view. Participants will be allowed to operate the machine and measure samples (EVs isolated from JAr cells)

Reading materials:

Midekessa, G.; Godakumara, K.; Dissanayake, K.; Hasan, M.M.; Reshi, Q.U.A.; Rinken, T.; Fazeli, A. Characterization of Extracellular Vesicles Labelled with a Lipophilic Dye Using Fluorescence Nanoparticle Tracking Analysis. Membranes 2021, 11, 779. https://doi.org/10.3390/membranes11100779

Required materials:

- YG-PS-100nm fluorescent alignment suspension beads
- Dulbecco`s Phosphate Buffered Saline – 500 ml × 6
- Double deionized water (Milli-Q)
- Membrane dye – CellMask™ Green

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WORKSHOP 5

EV zeta-potential measurements with NTA

Date and Time: 6th of September 2023, 14.20 -15.20

Instructor: Getnet Midekessa, Chanaka Premathilaka and Omid Mousavi

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Demonstration of the operation of the Zetaview. Participants will be allowed to operate the machine and measure the zeta potential of samples (EVs isolated from JAr cells)

Reading materials:

Dissanayake K., Midekessa G., Lättekivi F., Fazeli A. (2021) Measurement of the Size and Concentration and Zeta Potential of Extracellular Vesicles Using Nanoparticle Tracking Analyzer. In: Brevini T.A., Fazeli A., Turksen K. (eds) Next Generation Culture Platforms for Reliable In Vitro Models. Methods in Molecular Biology, vol 2273. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1246-0_15

Required materials:

- 100 nm polystyrene beads standard solution 110-0020
- Dulbecco`s Phosphate Buffered Saline – 500 ml × 6
- Double deionized water (Milli-Q)

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WORKSHOP 6

NTA data analysis

Date and Time: 6th of September 2023, 15.40-17.10

Instructor: Johanna Piibor

Location: SIME Seminar Room (2nd floor), Ravila 14b, Tartu

Description: Looking at the raw data produced by Zetaview. Get acquainted with the different output formats, what the raw data can tell us, and different data analysis approaches

Lecture: what kind of data do we obtain from ZetaView NTA and what we can do with it.

- Understands what different type of files are obtained from ZetaView
- Knows where to find information about
- Particle size and ZP distribution
- ZP peak data
- Average concentration and particle size
- Knows different strategies for visualizing ZetaView NTA data and applicable statistical tests

Practical session: how to perform simple analysis in Excel.

- Following independently a short tutorial knows how to visualise provided data

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WORKSHOP 7

Flow cytometric analysis of EVs

Date and Time: 6th of September 2023, 17.10-17.50

Instructor: Olavi Reinsalu, Dulmini N. Sapugahawatte

Location: SIME Seminar Room (2nd floor), Ravila 14b, Tartu

Notes –

WORKSHOP 8

Advanced fluorascent microscopy methods to work with EVs

Date and Time: 7th of September 2023, 10.40-11.40

Instructor: Sergei Kopantšuk, Caius Miller

Location: SIME Seminar Room (2nd floor), Ravila 14b, Tartu

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WORKSHOP 9

EV RNA cargo analysis and RNA extraction from EVs

Date and Time: 7th of September 2023, 13.20-15.00

Instructors: Kasun Godakumara and Subhashini Muhandiranm

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: Extraction of RNA from EV is an essential step in EV function and characterization studies. It is somewhat challenging because of the low amount of RNA contained in EVs. This workshop will introduce a simple method to isolate an appreciable amount of RNA using phenol-chloroform separation. More details related to the workshop can be found in **Appendix 3.**

Required material

1. TriZol reagent
2. Chloroform
3. Glycogen
4. Isopropanol
5. Absolute Ethanol
6. RNase-free water
7. Bioanalyzer chips and reagents (RNA 6000 Pico kit)

RNA extraction protocol

HOMOGENIZATION:

1.Add 0.7 ml of TriZol reagent to the concentrated EV sample (preferably > 1x 10^{11} EV per ml).

2.Homogenize by pipetting.

3.Incubate the homogenized sample for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes.

PHASE SEPARATION:

4. Add 0.2 ml of chloroform. Cap sample tubes securely. Mix samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at no more than 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer the upper aqueous phase carefully without disturbing the interphase into the fresh tube. Measure the volume of the aqueous phase (The volume of the aqueous phase is about 60% of the volume of TRIZOL Reagent used for homogenization).

RNA PRECIPITATION:

5. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 ml of isopropyl alcohol. Add 15 µg of glycogen to the solution. Incubate samples at RT for 10 minutes and centrifuge at 18,000 x g speed for 30 min at RT. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

RNA WASH:

6. Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol. Mix the samples by vortexing and centrifuge at 18,000 x g for 1 minute at 4 °C. Repeat the above washing procedure twice. Remove all leftover ethanol.

RE-DISSOLVING RNA:

7. Air-dry or vacuum dry RNA pellet for 5-10 minutes. Do not dry the RNA pellet by centrifuge under a vacuum. Dissolve RNA in 20 µl of DEPC-

treated water by passing the solution a few times through a pipette tip. Heat the RNA solution for 10 min at 60°C.

SPECTROPHOTOMETRIC ANALYSIS:

8. Use the Qubit to measure the concentration of RNA (standard protocol attached).

9. Use the Bioanalyzer to measure RNA quality (standard protocol attached). Qubit 2.0 High sensitivity RNA assay.

1. Set up the required number of 0.5-mL tubes for standards and samples. The Qubit® RNA HS Assay requires 2 standards.

2. Label the tube lids.

3. Prepare the Qubit® working solution by diluting the Qubit® RNA HS Reagent 1:200 in Qubit® RNA HS Buffer. Use a clean plastic tube each time you prepare a Qubit® working solution. Do not mix the working solution in a glass container.

4. Add 190 µL of Qubit® working solution to each of the tubes used for standards.

5. Add 10 µL of each Qubit® standard to the appropriate tube, then mix by vortexing for 2–3 seconds. Be careful not to create bubbles.

6. Add 197 µL of Qubit® working solution to individual assay tubes for each sample.

7. Add 2 µL of Spike-in solution to each sample assay tube.

8. Add 1 µL sample to the assay tube.

9. Mix by vortexing for 2-3 seconds. The final volume in each tube should be 200 µL.

10. Allow all tubes to incubate at room temperature for 2 minutes.

11. On the Home screen of the Qubit® 2.0 Fluorometer, press RNA, then select RNA: High Sensitivity as the assay type. The "Standards" screen is displayed.

12. On the Standards screen, press Yes to read the standards.

13. Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read. When the reading is complete (~3 seconds), remove Standard #1.

14. Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read. When the reading is complete, remove Standard #2.

15. When the calibration is complete, the instrument displays the Sample screen.

16. Insert a sample tube into the sample chamber, close the lid, then press Read. When the reading is complete (~3 seconds), remove the sample tube.

WORKSHOP 10

Analysis of EV RNA quality and integrity

Date and Time: 7th of September 2023, 15.20-17.00

Instructors: Kasun Godakumara and Subhashini Muhandiranm

Location: SIME General Laboratory, Ravila 14b, Tartu

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References

Chomczynski P, Mackey K. (1995) Short technical report. Modification of the TRIZOL reagent procedure for isolation of RNA from Polysaccharide- and proteoglycan-rich sources. Biotechniques 19(6): 942-5.

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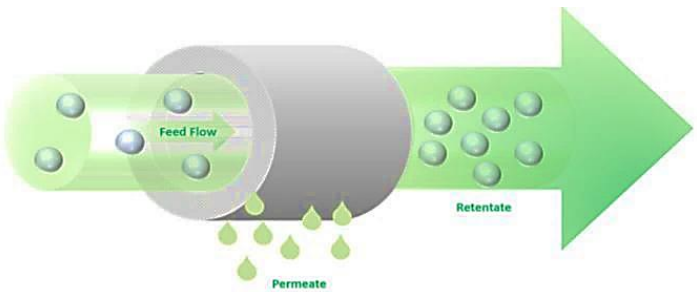
In D. M. F. Prazeres & S. A. M. Martins (Eds.), G Protein-Coupled Receptor Screening Assays (Vol. 1272, pp. 37–50). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-2336-6_3

APPENDIX 1

TFF-Easy: Tangential flow filtration for Extracellular Vesicle concentration

About TFF-Easy

TFF-Easy is a filter cartridge containing polysulfone hollow fibers (5 nm pores), which allows the concentration and the removal of small proteins and molecules from diluted matrices (cell-conditioned media, urine, etc.), prior to the EV purification. Water and small molecules (< 100 kDa) pass through the hollow fibre pores, whereas EVs are concentrated in the retentate. EVs can be easily recovered with a syringe from the filter cartridge.



Specifications: Sterility: The TFF-Easy is provided sterile. Once used the filter can be sterilized by Beta irradiation. Do not autoclave the TFF-Easy.

Storage: Store the device at room temperature.

EV concentration procedure

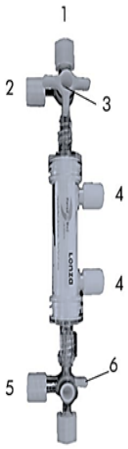


Fig 1

1	Sample injection nozzle
2	Tangential flow filtration nozzle
3	Flow valve 1
4	Permeate nozzles
5	Tangential flow filtration nozzle
6	Flow valve 2

Sample	Volume range	Reusable
Cell media	5 - 1000 ml	20-30 times
Urine *	5- 1000 ml	10-20 times
Plasma/serum	5-20 ml	5-10 times

* TFF-Easy can be used for concentrating all diluted body fluids (urine, CSF, bronchoalveolar lavage etc).

1. Sample injection by filtration (optional).

- Remove the screw from the sample injection nozzle (position 1).

- Insert a syringe filter in the nozzle. The device is compatible with multiple filter typology. The dimension of the filter pores can be chosen by the user according to the necessity.

In order to keep all the EV subtypes it is recommended to use filter pores larger than 0.22 μm.

Fig 2

- Pick up the sample with syringe A and insert it on the top of the filter.

- Insert a clean empty syringe in the Tangential flow filtration nozzle (position 2).

- Rotate the valve 3 to the position indicated by arrows in Figure 3.

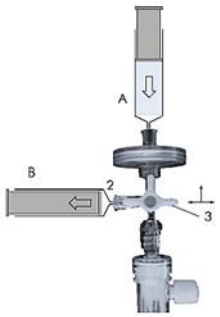
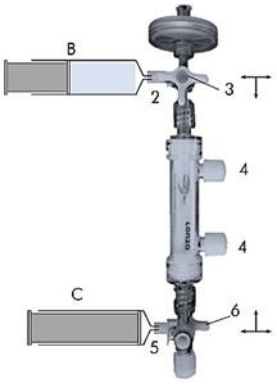


Fig 3

- Inject the sample into the filter by pushing the piston of syringe A. The sample passes through the filter, filling the syringe B.

2. Tangential Flow Filtration



- Rotate the valve 3 to the position indicated in Figure 4.

- Open the permeate nozzles, removing the screws.

- Insert a new clean syringe (C) in position 5.

- Set 2 permeate collection tubes under the nozzles in position 4, as indicated in Figure 4.

- Start the concentration process by pushing the syringes B and C alternatively upwards and downwards.

- Continue the concentration process until the desired volume is obtained.

- The permeate starts to flow to the collection tubes, while the retentate in the syringes contains the concentrated EVs.

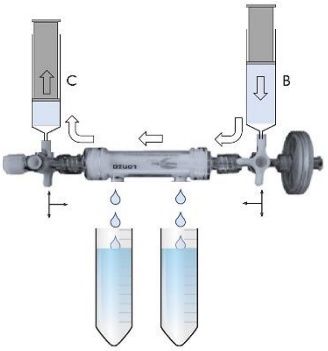


Fig 4

3. Recovery of concentrated EV-s

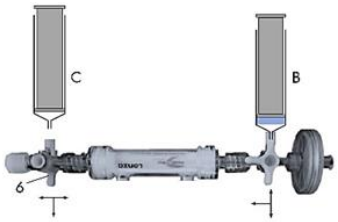


Fig 5

- In order to collect the concentrated EVs, push the concentrated products into syringe B.

- Rotate the valve 6 in the position indicate in figure 5 and disconnect the syringe C.

- Load air into syringe C.

- Rotate valve 6 to the position indicated in Figure 6.

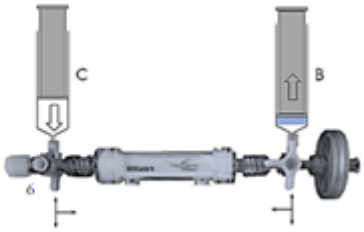


Fig 6

- Insert the syringe C and inject air into the device and pull up the piston of syringe B in order to collect all the residual volume of the concentrated sample.

- Rotate the valve 3 to the position indicated by arrows and disconnect the syringe B.

- Collect the concentrated samples in a clean tube.

Fast dialysis procedure

TFF-Easy can be used for EV dialysis and buffer exchange.

- Inject the buffer (buffer 1) containing EVs into the device as indicated in Figure 4.

- Push the syringes B and C alternatively upwards and downwards until all buffer has passed to the permeate collection tubes.

- Load the syringe B with the new buffer (buffer 2) and inject it into the device. Repeat the operation indicated in the point above at least 5 times.

- Concentrate the EVs in buffer 2 until the desired volume.

Example

Dialysis progress	Conductivity (µS/cm)	Particle concentration (particle number/ml)
EVs in buffer 1 (PBS 1X) 5 ml	15000	$5.8x10^{11}$
1- Removal of buffer 1 by TFF	15000	
2- Injection of buffer 2 (NaCl 100 mM) in TFF cartridge		
3- Removal of buffer 2 and buffer 1 residues	4100	
4- Injection of buffer 2		
5- Removal of buffer 2	624	
6- Injection of buffer 2		
7- Concentration of buffer 2 up to 5 ml	621	$4.9x10^{11}$

Washing procedure

Once the concentration process is ended the filter cartridge has to be washed with aboundant MilliQ water. If the cartridge is used for concentrating complex fluids it is recommended to wash as described to point 1, Easy fouling.

Procedure for MilliQ water washing

- Use 2 clean syringes and load one with MilliQ water. - Connect the syringe containing water to the position 2 and the empty one to the position 5

- Rotate the valves 3 and 6 in order to open the device (see Figure 6)

- Inject the water in the device and continue the washing step by pushing alternatively the two syringes upwards and downwards until all the water is passed to the permeate collection tubes.

- Repeat the operation. It is recommended to wash with at least 3 volumes of water.

- Let the device dry at room temperature. If after washing the fibres look colored or if the filter is clogged proceed as indicated:

1- Easy fouling

A- Wash the filter with warm MilliQ water (40-50°C), carefully, applying low pressure with syringes.

B- Wash the filter with a solution of NaOH 0.5 N, then wash with at least 3 volumes of MilliQ water.

2- Moderate fouling

A- Prewash the filter with warm MilliQ water, as indicated above.

B- Wash the filter with a solution of NaOH 1 N, then with at least 3 volumes of MilliQ water. If the fouling persists, use a warm solution of NaOH 1N (35-40°C), then wash the filter with 3 volumes of MilliQ water.

3- Hard fouling

Prewash the filter with warm MilliQ water as indicated above. Wash the filter with a water solution of NaClO (0.05 %), then wash with at least 5 volumes of MilliQ water.

Optional: a wash with Ethanol 96% (5 - 10 ml) can be performed for the removal of potential pyrogenic agents. Wash finally with at least 3 volumes of MilliQ water.

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APPENDIX 2

ZetaView®- Operational Steps

Preparing the machine for experiments

• Switch on the machine.

• Click the ZetaView icon on the desktop of the computer connected to the machine. This software has 4 platforms i.e. Cell Check, Pump and Temp, Measurement, and Analysis (figure 1).

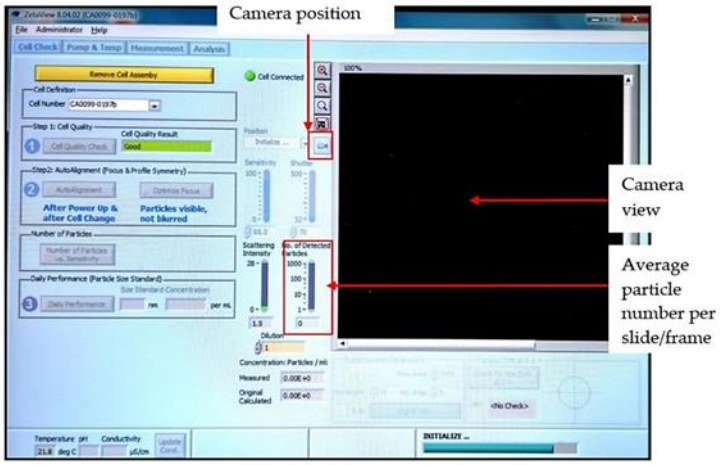


Figure 1.

• Run pump 1, which is connected to the distilled water bottle, or flush the cell manually using 5ml of distilled water.

Note – Pump 1 is connected to the distilled water bottle and Pump 2 is connected to the PBS bottle. The flow rate can be adjusted.

The next 3 steps (steps 1,2 and 3) evaluate the 'cell' of the machine (500µl chamber into which the samples are loaded for measurements) for its suitability for measuring samples and calibrate the system using particles of known sizes (100nm bead suspension). The software takes you through these steps automatically.

• Run pump 1, which is connected to the distilled water bottle, or flush the cell manually using 5ml of distilled water.

Note – Pump 1 is connected to the distilled water bottle and pump 2 is connected to the PBS bottle. The flow rate can be adjusted.

Next 3 steps (Step 1,2 and 3) evaluate the 'cell' of the machine (500µl chamber into which the samples are loaded for measurements) for its suitability for measuring samples and calibrate the system using particles of known sizes (100nm bead suspension). The software takes you through these steps automatically.

• **Step 1: Cell quality check**

The machine requests to fill the cell with distilled water to check the quality of the cell for measurements (figure 2)

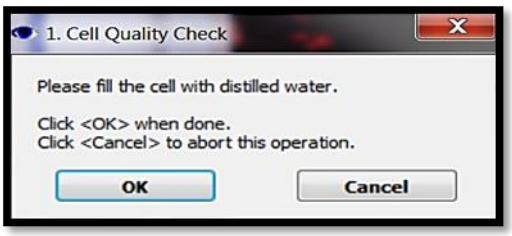


Figure 2.

Inject a volume not less than 500 µl to the cell through the injection port. The surplus volume will be sent to the waste bin. By injecting a larger volume the air in the cell are sent out of the cell. Remember, during all the downstream measurements the cell should be air-free as they may interfere with the measurements. After injecting distilled water, click OK. Let the machine to analyze the cell quality.

If the cell quality is good, the cell quality results appear as 'Good'.

If the Cell quality is good, the following message box appears (figure 3). Move to the step 2.

Figure 3.

- **Step 2: Auto-alignment**

If the cell quality is good, the next step is to align the cell with particles of known diameter. Inject 102 nm bead alignment suspension to the cell (dilution 1:250000), in order to calibrate the machine. Click OK. Let the machine to perform the alignment and autofocus.

If the auto-alignment is good, a message box appears requesting to load the cell with the sample

Note – The 1:250 000 diluted bead suspension should not be older than 1 week. If not prepare it using the stock standard (1:1000 dilution). The stock standard should not be older than 1 month. If it is older than 1 month, prepare a new stock standard from the original suspension.

At this stage, daily performance may be carried out before injecting the sample.

- **Step 3: Daily performance**

While the bead suspension is still in place, click the daily performance tab. It performs 5 measurements and depending on the selected display mode (histogram or parabola), it generates 5 graphs which ideally tends to overlap each other (figure 4).

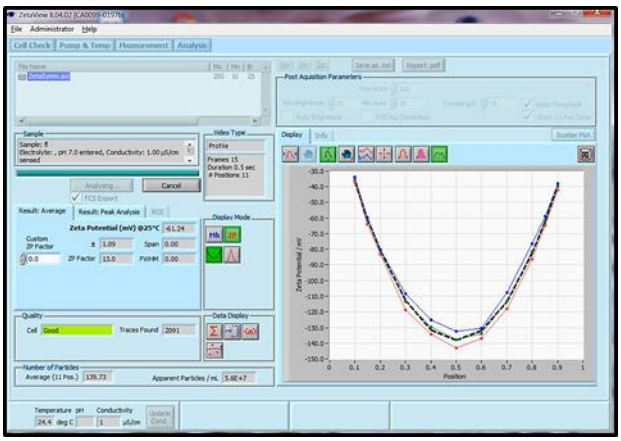


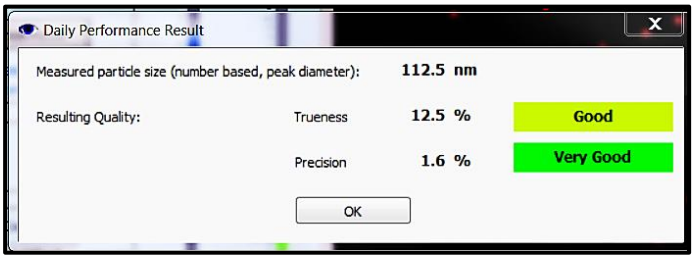
Figure 4.

- **Step 4**

When the daily performance is completed, result box appears stating the `trueness' and `precision' of the measurement of bead suspension. The result should be good or very good to proceed (figure 5).

Clean the cell by injecting Distilled water or PBS before injecting the samples. Cleaning process should be done before measuring each sample.

Figure 5.



Measuring the size profile and concentration

- Prepare the samples to be measured beforehand and keep at room temperature at least 30 minutes until the sample temperature reached room temperature.

- Fill the chamber with the sample

• For accurate measurements by the machine, in average, 100-150 particles per frame should exist. This is indicated by the indicator as green (figure 6). If the average number of particles per frame is too low the indicator goes orange and if it is too high the indicator foes red. Avoid both extremes and dilute the sample for measurements based on the general particle density of the sample. Enter the correct dilution factor of the sample(figure 7)

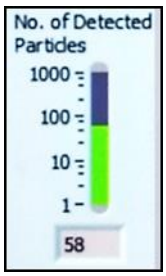


Figure 6.

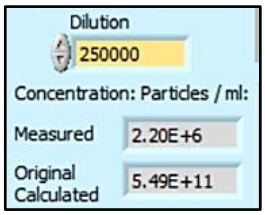


Figure 7.

• Check particle drift by clicking on the ´check particle drift button'. (the drift indicator start as a red line (figure 8) and will be changed to green once the particles in the cell reach the Brownian motion (figure 9). Click the ´check particle drift button' and proceed to the measurements

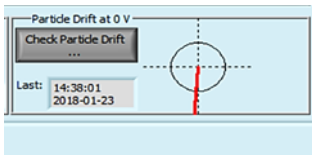


Figure 8.

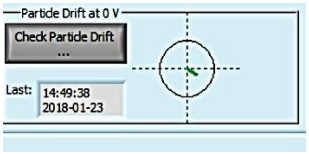


Figure 9.

Note - the measurements should be taken when the particles are at Brownian motion. Therefore, once the sample is injected into the cell it takes a few minutes for the red indicator to become green.

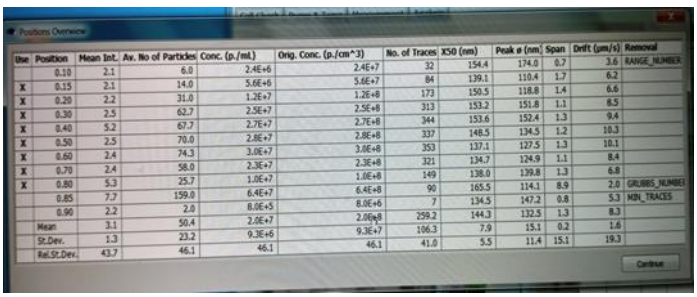
• Go to the 'Measurements' and click 'run video acquisition'. Select the appropriate folder where the measurement data should be stored and label the file.

Select the appropriate parameters.

- Experiment: Size profiling
- Positions: 11
- Cycles:- 3,
- High
- Frame Rate: 30 fps
- sensitivity: 85
- shutter: 70

• Click `OK`

• After measuring the size profile, the machine gives a report mentioning the measurements at the 11 frames (figure 10). Furthermore, the machine may have deselected certain frames from calculation due to some reasons (minimum traces, particle drift etc).



Use	Position	Mean Int.	Av. No of Particles	Conc. (p./ml.)	Orig. Conc. (p./cm^3)	No. of Traces	X50 (nm)	Peak ø (nm)	Span	Drift (µm/s)	Removal
	0.10	2.1	6.0	2.4E+6	2.4E+7	32	154.4	174.0	0.7	3.6	RANGE_NUMBER
X	0.15	2.1	14.0	5.6E+6	5.6E+7	84	139.1	110.4	1.7	6.2	
X	0.20	2.2	31.0	1.2E+7	1.2E+8	173	150.5	118.8	1.4	6.6	
X	0.30	2.5	62.7	2.5E+7	2.5E+8	313	153.2	151.8	1.1	6.5	
X	0.40	5.2	67.7	2.7E+7	2.7E+8	344	153.6	152.4	1.3	9.4	
X	0.50	2.5	70.0	2.8E+7	2.8E+8	337	148.5	134.5	1.2	10.3	
X	0.60	2.4	74.3	3.0E+7	3.0E+8	353	137.1	127.5	1.3	10.1	
X	0.70	2.4	58.0	2.3E+7	2.3E+8	321	134.7	124.9	1.1	8.4	
X	0.80	5.3	25.7	1.0E+7	1.0E+8	149	138.0	139.8	1.3	6.8	
	0.85	7.7	159.0	6.4E+7	6.4E+8	90	165.5	114.1	8.9	2.0	GRUBBS_NUMBER
	0.90	2.2	2.0	8.0E+5	8.0E+6	7	134.5	147.2	0.8	5.3	MIN_TRACES
	Mean	3.1	50.4	2.0E+7	2.06e8	259.2	144.3	132.5	1.3	8.3	
	St.Dev.	1.3	23.2	9.3E+6	9.3E+7	106.3	7.9	15.1	0.2	1.6	
	Rel.St.Dev.	43.7	46.1	46.1	46.1	41.0	5.5	11.4	15.1	19.3	

Continue

Figure 10.

Click ´continue` .

• The machine provides the final report for the size measurements in different formats which are stored in the selected folder.

1. **Measuring the Zeta potential**

• Click on the Zeta potential as the experiment. The storage folder and file name will be automatically selected corresponding to the size measurement.

• Select the following parameters and click OK

- Experiment – zeta potential
- Positions: 2
- Pulsed,
- Frame Rate 30
- Sensitivity: 85
- Shutter: 70

• Click `OK` and let the machine to measure the zeta potential.

1. **Finishing the measurements**

• When all the samples are analyzed, wash the chamber with distilled water (you may use the pump 1) and then dry the cell by injecting air into the cell.

• Close the software and turn off the machine.

• Clean the workstation properly

APPENDIX 3

Agilent RNA 6000 Pico Kit - Quick Start Guide

The complete RNA 6000 Pico Guide can be found in the online help of the 2100 Expert software.

Agilent RNA 6000 Pico Kit (reorder-no 5067-1513)	
Agilent RNA 6000 Pico Chips	Agilent RNA 6000 Pico Reagents (reorder-no 5067-1514) & Supplies
25 RNA Pico Chips	● (blue) RNA 6000 Pico Dye Concentrate[1]
3 Electrode Cleaners	● (green) RNA 6000 Pico Marker (4 vials)
Syringe Kit	o (white) RNA 6000 Pico Conditioning Solution ● (red) RNA 6000 Pico Gel Matrix (2 vials)
1 Syringe	● (yellow) RNA 6000 Pico Ladder (reorder-no 5067-1535) (1 vial, 10x concentrate)
	4 Spin Filters (reorder-no 5185-5990)
Tubes for Gel-Dye Mix	
30 Safe-Lock Eppendorf Tubes PCR clean (DNase/RNase free) for gel-dye mix	

Assay Principles

Agilent RNA kits contain chips and reagents designed for analysis of RNA fragments. Each RNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. Agilent RNA kits are designed for use with the Agilent 2100 Bioanalyzer instrument only.

[1] This product is provided under a license by Life Technologies Corporation to Agilent Technologies. The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product only as described in accompanying product literature. The sale of this product is expressly conditioned on the buyer not using the product or its components (1) in manufacturing; (2) to provide a service, information, or data to an unaffiliated third party for payment; (3) for therapeutic, diagnostic or prophylactic purposes; (4) to resell, sell or otherwise transfer this product or its components to any third party, or use for any use other than use in the subfields of research and development, quality control, forensics, environmental analysis, biodefense or food safety testing. For information on purchasing a license to this product for purposes other than described above contact Life Technologies Corporation, Cell Analysis Business Unit, Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354." **Research use only,** Not for use in Diagnostic Procedures

Applications and Kits

Agilent RNA kits are designed for the analysis of total RNA (eukaryotic, prokaryotic, and plant) and mRNA samples. Agilent RNA kits: RNA 6000 Nano Kit (reorder-no 5067-1511), RNA 6000 Pico Kit (reorder-no 5067-1513), and Small RNA Kit (reorder-no 5067-1548)

Storage Conditions

• Freeze unopened RNA ladder at -20 °C. Prepared ladder aliquots need to be stored at -70 °C. Keep all other reagents and reagent mixes refrigerated at 4 °C when not in use to avoid poor results caused by reagent decomposition.

• Protect dye and dye mixtures from light. Remove light covers only when pipetting. Dye decomposes when exposed to light.

Equipment Supplied with the Agilent 2100 Bioanalyzer System

• Chip priming station (reorder-no 5065-4401)

• IKA vortex mixer

Additional Material Required (Not Supplied)

•RNaseZAP® recommended for electrode decontamination only (Ambion, Inc. cat. no. 9780)

• RNase-free water recommended for routine electrode cleaning

• Pipettes (10 µL and 1000 µL) with compatible tips (RNase-free, no filter tips, no autoclaved tips)

• 0.5 mL and 1.5 mL microcentrifuge tubes (RNase-free Eppendorf Safe-lock PCR clean or Eppendorf DNA LoBind microcentrifuge tubes are highly recommended)

• Microcentrifuge (> 13000g)

• Heating block or water bath for ladder/sample preparation

• Mandatory: Dedicated bayonet electrode cartridge (reorder-no 5065-4413) for RNA 6000 Pico and Small RNA assays.

Sample Preparation

• Prepare RNA samples in deionized water. For estimation of RNA concentration, the total RNA concentration in the sample must be between 200 – 5000pg/µL. The mRNA concentration must be between 500 – 5000pg/µL.If concentration of a sample is above this range, dilute with RNase-free water.

Specifications

Physical Specifications		**Analytical Specifications**		
Type	**Specifi-cation**	**Specifi-cation**	**Total RNA Assay**	**mRNA Assay**
Analysis run time	30 min	Qualitative range	50-5000 pg/µL (in water) (Signal/ Noise > 3)	250 – 5000 pg/µL (in water) (Signal/ Noise > 3)
Number of Samples	11 samples / chip	Quantitation reproduci-bility	20 % CV	20 % CV
Sample volume	1 µL	Quantitation accuracy	30 % CV (for ladder as sample)	30 % CV (for ladder as sample)
Kit stability	4 months (Storage tempera ture see individ ual box)	Buffer compati-bility	50 nM Tris or 50 mM NaCl	50 nM Tris or 50 mM NaCl

Due to the high sensitivity of the assay, different ions and higher salt concentrations might influence the performance of the assay. It is recommended to analyze the samples in water, as this will yield the highest sensitivity.

Setting up the Chip Priming Station

1.Replace the syringe:

a) Unscrew the old syringe from the lid of the chip priming station.

b) Release the old syringe from the clip. Discard the old syringe.

c) Remove the plastic cap of the new syringe and insert it into the clip.

d) Slide it into the hole of the luer lock adapter and screw it tightly to the chip priming station.

2. Adjust the base plate:

a) Open the chip priming station by pulling the latch.

b) Using a screwdriver, open the screw at the underside of the base plate.

c) Lift the base plate and insert it again in position C. Retighten the screw.

3. Adjust the syringe clip:

a) Release the lever of the clip and slide it up to the top position.

Essential Measurement Practices

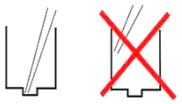
• Handle and store all reagents according to the instructions on the label of the individual box.

• Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.

• Allow all reagents to equilibrate to room temperature for 30 min before use. Thaw samples on ice.

• Protect dye and dye mixtures from light. Remove light covers only when pipetting. The dye decomposes when exposed to light and this reduces the signal intensity.

• Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.



• Always wear gloves when handling RNA and use RNase-free tips, microcentrifuge tubes and water.

• It is recommended to heat denature all RNA samples and RNA ladder before use for 2 min and 70 °C (once) and keep them on ice.

• Do not touch the Agilent 2100 Bioanalyzer instrument during analysis and never place it on vibrating surface.

• Always vortex the dye concentrate for 10 s before preparing the gel-dye mix and spin down afterwards.

• Use a new syringe and electrode cleaners with each new kit.

• Use loaded chips within 5 min after preparation. Reagents might evaporate, leading to poor results.

• To prevent contamination (e.g. RNase), it is strongly recommended to use a dedicated electrode cartridge for RNA assays. For running the RNA 6000 Pico assay, the 16 pin bayonet cartridge is mandatory.

Agilent RNA 6000 Pico Assay Protocol

WARNING

Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

➔ Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples.

➔ Handle solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

Preparing the RNA Ladder

1.Spin the ladder down and pipette in an RNase-free vial.
2.Heat denature the ladder for 2 min at 70 °C.
3. Immediately cool the vial on ice.
4.Add 90 µL of RNase-free water and mix thoroughly.
5.Prepare aliquots in recommended 0.5 mL RNase-free vials with the required amount for typical daily use.
6.Store aliquots at -70 °C. After initial heat denaturation, the frozen aliquots should not require repeated heat denaturation.
7. Before use, thaw ladder aliquots on ice (avoid extensive warming).

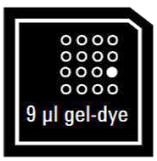
Preparing the Gel

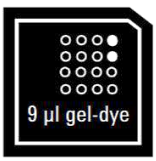
1.Pipette 550 µL of RNA gel matrix (red ●) into a spin filter.
2.Centrifuge at 1500g ± 20 % for 10 min at room temperature.
3.Aliquot 65 µL filtered gel into 0.5 mL RNase-free microcentrifuge tubes. Use filtered gel within 4 weeks. Store at 4 °C.

Preparing the Gel-Dye Mix

1.Allow the RNA dye concentrate (blue ●) to equilibrate to room temperature for 30 min.
1.Vortex RNA dye concentrate (blue ●) for 10 s, spin down and add 1 µL of dye into a 65 µL aliquot of filtered gel.
2.Vortex solution well. Spin tube at 13000g for 10 min at room temperature. Use prepared gel-dye mix within one day.

Loading the Gel-Dye Mix

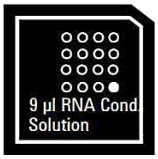




1.Put a new RNA chip on the chip priming station.
2.Pipette 9 µL of gel-dye mix in the well marked G
3. Make sure that the plunger is positioned at 1 mL and then close the chip priming station.
4.Press plunger until it is held by the clip.

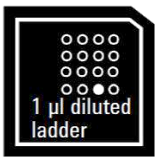
5.Wait for exactly 30 s then release clip.
6.Wait for 5 s. Slowly pull back plunger to 1 mL position.
7.Open the chip priming station and pipette 9 µL of gel-dye mix in the wells marked G .
8.Discard the remaining gel-dye mix.

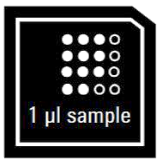
Loading the Conditioning Solution and Marker



1.Pipette 9 µL of the RNA conditioning solution (white o) into the well marked CS.
2.Pipette 5 µL of RNA marker (green ●) in all 11 sample wells and in the well marked.

Loading the Diluted Ladder and Samples





1.Pipette 1 µL of the heat denatured and aliquoted ladder in the well marked .
2.Pipette 1 µL of sample in each of the 11 sample wells. Pipette 1 µL of RNA Marker (green ●) in each unused sample well.
3.Put the chip horizontally in the IKA vortexer and vortex for 1 min at 2400rpm.
4.Run the chip in the Agilent 2100 Bioanalyzer instrument within 5 min.

Technical Support

Please visit our support web page http://www.agilent.com/genomics/contactus to find information on your local Contact Center.

Further Information

Visit the 2100 Bioanalyzer site at http://www.agilent.com/genomics/bioanalyzer. You can find useful information, support and current developments about the products and the technology.

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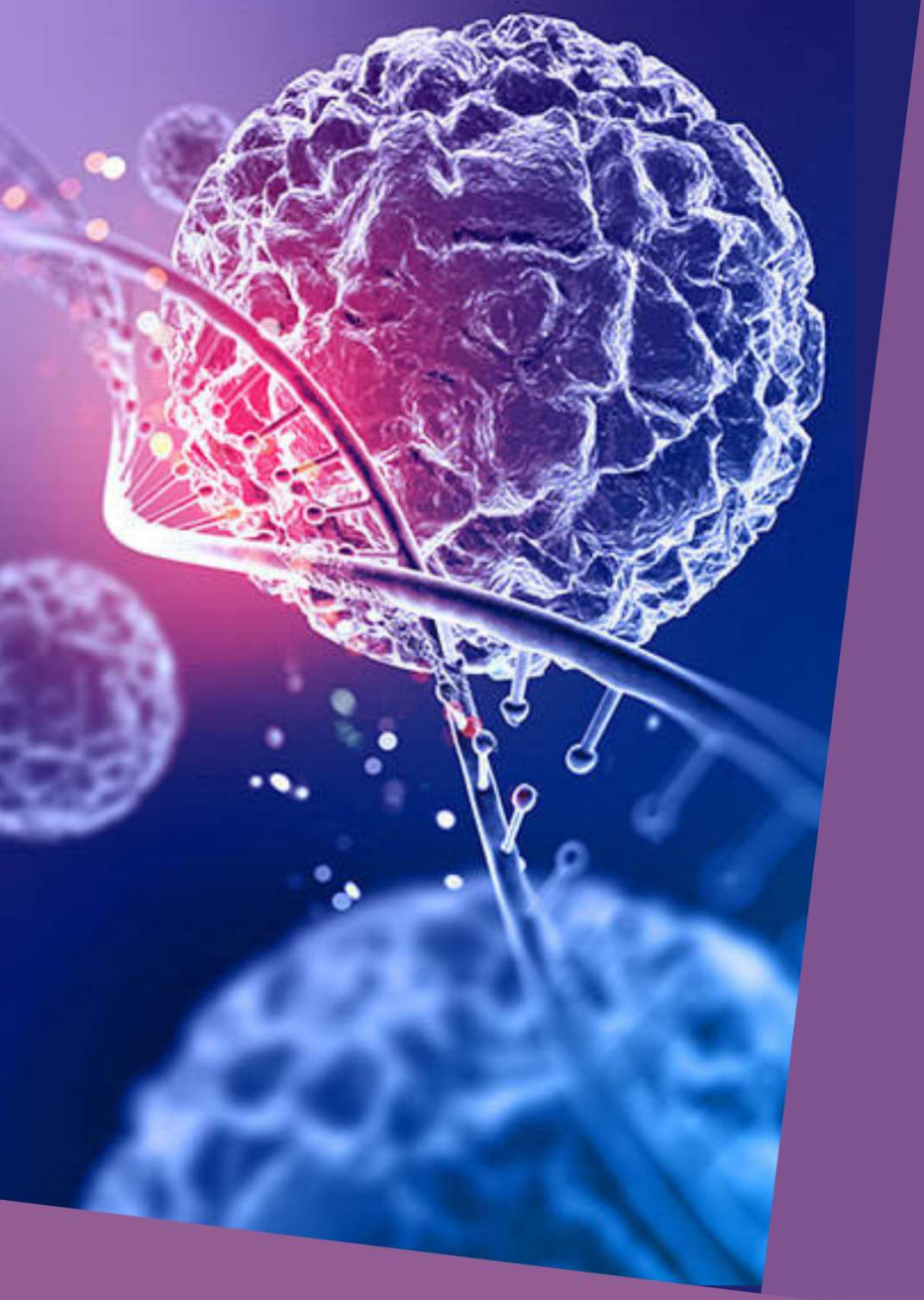

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