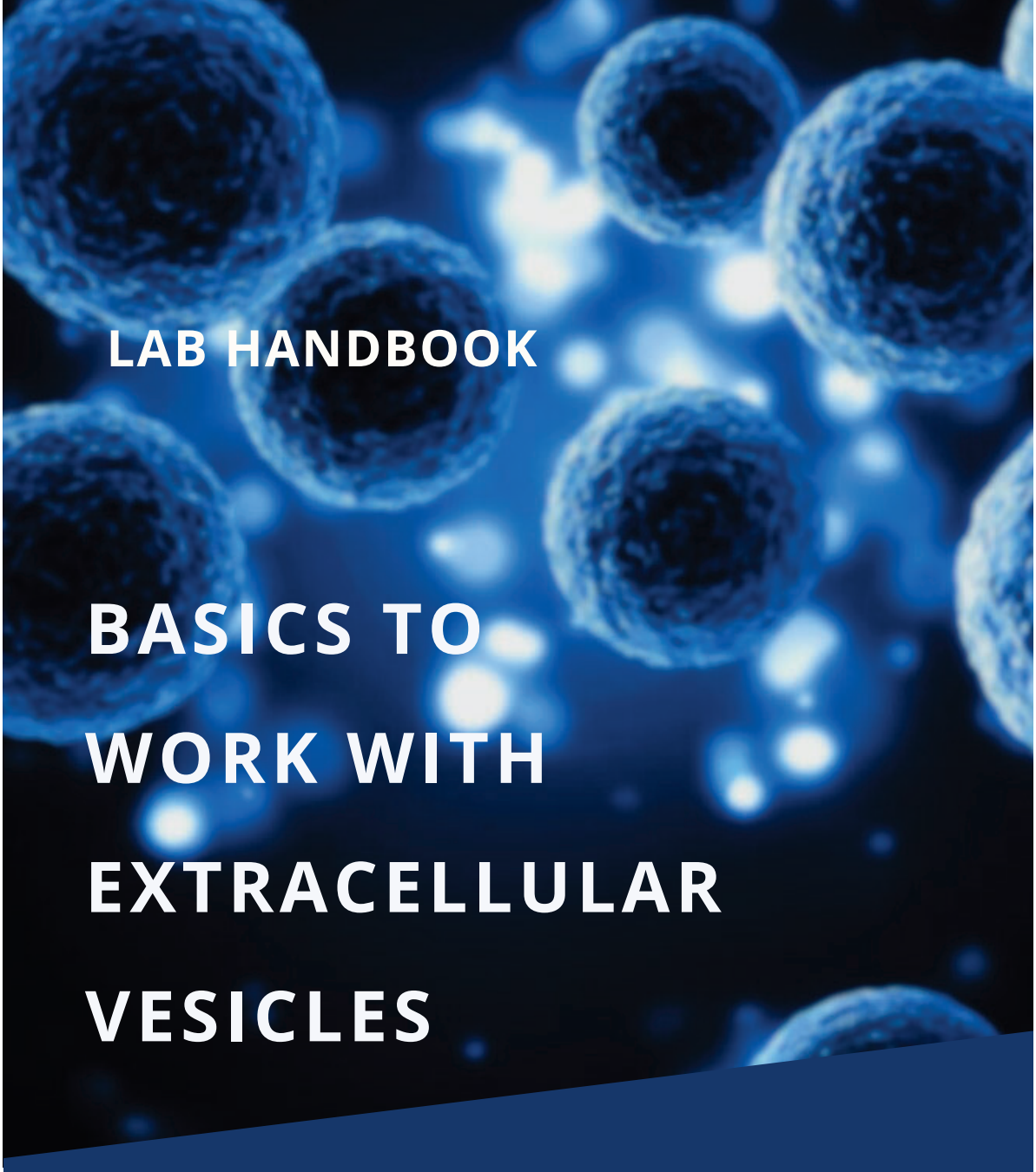

LAB HANDBOOK

BASICS TO WORK WITH EXTRACELLULAR VESICLES

OH-BOOST and MOVE Research School

2nd - 5th of October, 2025

Tartu, Estonia

OH-BOOST

Estonian University of Life Sciences

TARTU ÜLIKOOL UNIVERSITAS TARTUENSIS 1632

HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

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Eesti tuleviku heaks

MOVE
Mobility for Vesicles Research in Europe

BSEVS

Estonian University of Life Sciences

LAB HANDBOOK: BASICS TO WORK WITH EXTRACELLULAR VESICLES

2nd-5th of October 2025

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About OH-Boost & MOVE Joint Research School 2025

The OH-Boost & MOVE Joint Research School 2025 is a collaborative initiative dedicated to uniting leading expertise in extracellular vesicle (EV) research and equipping the next generation of scientists with the knowledge and skills needed to excel in this rapidly evolving field.

This program is co-organised by the OH-Boost project (funded by the European Union's Horizon 2020 Research and Innovation Programme and Horizon Europe Coordination and Support Actions, Grant Agreement No. 101079349) in partnership with the 3rd MOVE Symposium 2025, a dynamic consortium uniting more than 20 European National EV Societies (NEVS) to advance Mobility for Vesicle Research in Europe, with additional organisational support from the Baltic Society of Extracellular Vesicles.

Throughout this immersive training school, participants will gain hands-on experience in the essentials of extracellular vesicle (EV) isolation, characterization, and data analysis. Experienced instructors from the EV field will guide participants through both practical work and conceptual learning. The curriculum combines a wide spectrum of lectures and practical sessions. Core topics include theoretical foundations, best-practice methodologies, troubleshooting strategies, and rigorous quality standards in line with the International Society for Extracellular Vesicles (ISEV). Participants will also be introduced to state-of-the-art approaches such as scalable EV production, size exclusion chromatography, tangential flow filtration, nanoparticle tracking analysis, advanced microscopy techniques for EVs, and EV RNA extraction and characterization, EV glycoprofiling and flow cytometry. This research school is especially tailored for researchers eager to build a strong foundation in extracellular vesicle studies and to take their first steps into this dynamic and rapidly evolving field.

ACKNOWLEDGEMENTS

We acknowledge the support of our sponsors for the OH-Boost and MOVE Research School 2025

Message from the ERA Chair of Comparative Medicine

We are thrilled to welcome you to the interactive training program titled "Basics to Work with Extracellular Vesicles." This program is co-organised by the OH-Boost project in partnership with the 3rd MOVE Symposium 2025. Notably, this marks the 8th research school organised by the Fazeli Lab, with a special focus on developing essential skills and knowledge for working with extracellular vesicles (EVs).

In recent years, interest in EVs has surged across multiple scientific fields, particularly in cancer, infectious diseases, and inflammation. These tiny particles have emerged as central mediators of cellular communication, making them promising targets for biomarker discovery and therapeutic innovation. What was once the domain of human medicine has now expanded to veterinary sciences, plant biology, and microbiology — a clear sign of how rapidly this field is growing.

Since 2023, the OH-Boost project (funded by the European Union's Horizon 2020 Research and Innovation Programme and Horizon Europe Coordination and Support Actions, Grant Agreement No. 101079349) brought Fazeli lab additional expertise and innovation in the field of EVs, guided by the One Health perspective, which emphasizes the interconnections between human, animal, and environmental health. Complementing this, the MOVE initiative, a dynamic consortium formed to unite more than 20 European National EV Societies to advance Mobility for Vesicle Research in Europe, joins hands with OH-Boost to bring together the latest advancements in EV research across Europe. Together, this broad and multidisciplinary collaboration makes the 2025 research school a distinctive hub for interdisciplinary learning.

Through OH-Boost and MOVE, we continue to foster collaboration across diverse research areas, from EV purification to characterization and functional studies. Our work has contributed to advancing science and medicine through peer-reviewed publications and international collaborations, further reinforced by support from networks such as MOVE, which brings together regional experts in the field.

The primary aim of this training school is to introduce participants to the fundamentals of working with EVs. Because of their small size and complex biology, EVs require specialized techniques that may be new even to experienced biomedical researchers. This program provides the foundation needed to develop those skills, nurturing a new generation of researchers ready to contribute to this dynamic and rapidly evolving field.

The continued success of this training school is made possible by the dedication of the Fazeli Lab in Tartu, together with international collaborators and researchers who generously share their expertise. We are deeply grateful to all involved and to you for being part of this year's EV Research School.

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

Organizing Committee

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Seyed Omid Reza Mousavi
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Programme

Methods of EV Isolation- 2nd of October 2025		
9.00-9.20	**Welcoming and Introduction**	*Alireza Fazeli*
9.20-10.05	**Lecture 1** A guide to working with extracellular vesicles: minimal experimental and reporting requirements	*Subhashini Muhandiram*
10.05-10.25	*Tea & Coffee break*	
10.25-11.10	**Lecture 2** Methods for EV purification	*Kasun Godakumara*
11.10-12.10	**Student 3-minute presentations (Part 1)**	
12.10-13:10	*Lunch*	
13.10-14:10	**Workshop 1** Introduction to tangential flow filtration (TFF)	*Omidreza Mousavi and Evgeniy Brik*
14.10-15.10	**Lecture 3** Advanced microscopy to work with EVs	*Sergei Kopanchuk*
15.10-15.30	*Tea & Coffee break*	
15.30-17:00	**Workshop 2** Size exclusion chromatography using manually packed chromatography column	*Omidreza Mousavi and Saumya Gunasekara*
EV Validation Methods- 3rd of October 2025		
9.00-9.40	**Lecture 4** Introduction to ZetaView® nano particle tracking analyser (NTA)	*Bertrand Damart*
9.40-10.20	**Student 3-minute presentations (Part 2)**	
10.20- 10.40	*Tea & Coffee break*	

	Workshop 3	*Bertrand Damart and Deep Bhattacharya*
10.40-11.10	EV size and concentration measurements with NTA	
11.10-12.20	**Workshop 4 (Part 1)** RNA extraction from EVs	*Subhashini Muhandiram*
12.20 –13.20	*Lunch*	
13.20-14.20	**Workshop 4 (Part 2)** RNA quality of integrity analysis	*Subhashini Muhandiram and Omidreza Mousavi*
14.20-15.20	**Workshop 5** Fluoroscence NTA	*Bertrand Damart, Chanaka Premathilaka and Deep Bhattacharya*
15.20-15.40	*Tea & Coffee break*	
15.40-16.45	**Workshop 6** NTA data analysis	*Deep Bhatacharya and Evgeniy Brik*
16.45-17.50	**Lecture 5** CytoFLEX nano: From Calibration to Real-World EV Sample Analysis Beckman Coulter Life Sciences (virtual)	*Dr. Valery Savitski*
EV validation methods and further applications- 4th of October 2025		
9.00-10.00	**Lecture 6** Transmission electron microscopy (TEM)	*Aneta Andronowska*
10.00-10.30	**Lecture 7** Glycoprofiling EVs using solid-phase lectin-based assay	*Maja Kosanovic*
10.30-11.00	*Tea & Coffee break*	

11.00-11.45	**Lecture 8** Simulating EV Analysis Using Imaging Flow Cytometry	*Agnieszka Mostek-Majewska*
11.45-12.20	**Workshop 7** Simulating EV Analysis Using Imaging Flow Cytometry	*Agnieszka Mostek-Majewska*
12.20-13.20	*Lunch*	
13.20-15.00	**Workshop 8 (Part 1)** Glycoprofiling EVs using solid-phase lectin-based assay	*Maja Kosanovic*
15.00-15.20	*Tea & Coffee break*	
15.20-16.20	**Lecture 9** 2-D, 3-D and 4-D Cell Culture. Recapitulation of the in vivo micro-environment with "Histocentric" Hollow fiber Bioreactors.	*John J.S. Cadwell*
16.20-17.20	**Workshop 8 (Part 2)** Glycoprofiling EVs using solid-phase lectin-based assay	*Maja Kosanovic*
17:20-17.45	Final student evaluation	*Evaluation Committee*
Networking Day - 5th of October 2025		
10:30-12:30	**Morning Activity**	
13:00-14:00	**Lunch**	

Lecturers and Instructors

Subhashini Muhandiram

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

Subhashini Muhandiram holds a Bachelor's degree in Medical Laboratory Science and a Master of Philosophy in Molecular and Cancer Biology from the University of Peradeniya, Sri Lanka. During her M.Phil. studies, she developed a low-cost molecular diagnostic assay for early screening of human papillomavirus (HPV) infection, with a focus on less- and non-invasive sampling methods.

She recently completed her PhD at the Estonian University of Life Sciences under the supervision of Prof. Alireza Fazeli. Her doctoral research focused on elucidating the molecular mechanisms of extracellular vesicle-mediated signal transduction during embryo implantation, aiming to uncover novel insights into embryo–endometrial communication. Over the years, she has cultivated expertise in extracellular vesicles, proteomics, biomarker discovery for diagnostic applications, cell biology, and reproductive physiology.

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 career 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.

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 following his research interest focusing on embryo-maternal communication through extracellular vesicles, particularly under stress conditions.

Evgeniy Brik

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

Evgeniy Brik has earned both BSc and MSc degrees in University of Tartu, Estonia. In his work on MSc thesis Evgeniy focused on creating 3D human skin model and its use to investigate potential ways to accelerate skin regeneration. Soon after getting his MSc degree Evgeniy worked from September 2019 to September 2021 in University of Tartu investigating 3D structure of *D. melanogaster* pupal wing in fine details (https://doi.org/10.1016/j.ydbio.2021.09.006). From October 2021 to April 2022, he worked in Fritz Lipmann Institute, Jena, Germany on research of ageing processes in short-living small fish *N. furzeri*. For a short period of time in the end of 2022 Evgeniy worked in Department of Laboratory Genetics in Tartu University Hospital. Starting from September 2024, Evgeniy is a junior researcher and PhD student in Prof. Alireza Fazeli lab in Estonian University of Life Sciences. Here, Evgeniy is investigating microalgae- and cow milk-derived extracellular vesicles (EVs) for their potential application in accelerating human skin regeneration after UV-irradiation caused damages, mechanical wounding and possible alleviating burden of immunological disorders such as atopic dermatitis and psoriasis. EVs have two major advantages: first, they possess significantly lower risk of tumorigenicity due to lack of proliferation, and second, significantly lower risk of allergenic reactions because of EVs' natural origins. These properties of EVs make them promising tool for potential therapeutic approaches.

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.

Saumya Gunasekara

PhD student at Institute of Animal Reproduction and Food Research, Polish Academy of Sciences Olsztyn

Saumya Gunasekara began her academic career with a B.Sc. (Hons) in Agricultural Technology and Management, specializing in Animal Science and Fisheries, from the Faculty of Agriculture, University of Peradeniya. She then pursued postgraduate studies in developmental genetics and artificial intelligence at Chungnam National University, South Korea, earning an M.Sc. in Bio-AI Convergence. During her studies, she developed expertise in both in vivo and in vitro disease modelling. Her master's research focused on the functional validation of rare genetic variants associated with autism spectrum disorders and the identification of underlying molecular mechanisms using the zebrafish model.

She subsequently joined the Institute of Animal Reproduction and Food Research in Olsztyn as a Ph.D. student under the supervision of Prof. Aneta Andronowska and Prof. Alireza Fazeli. Her current research focuses on extracellular vesicles (EVs) secreted by follicular cells and their regulatory influence on sperm function and fertilization. More broadly, her work aims to elucidate how the female reproductive environment naturally modulates male gamete performance, bridging critical gaps in the understanding of inter-gamete communication.

Bertrand DAMART

Sales manager Europe, Particle Metrix GmbH

With a solid background in Biochemistry and Microbiology, Bertrand Damart brings a unique scientific perspective to his role as Sales Manager for Europe & UK (Ex – DACH) at Particle Metrix GmbH. He has a distinguished career in sales, spanning over 25 years, and has dedicated the last five years to the ZetaView®, a leading instrument in nanoparticle tracking analysis. After successfully establishing the ZetaView® in the French market, Bertrand's role was expanded to his current European-wide position, a testament to his expertise and the trust he has built within the scientific community.

Deep Bhattacharya

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

Deep is a graduate of SRM University, Chennai, India. He completed his B.Tech in Biotechnology in 2021 with a focus on Cancer Genetics, Molecular Biology, and Immunology. He furthered his education by obtaining a Master's in Biotechnology from Lund University in 2023, specializing in Synthetic Biology, Probiotics, and Bioprocessing. During his Master's studies, Deep demonstrated notable expertise by securing a gold medal at iGEM, the World Synthetic Biology Competition. His project involved developing a probiotic for bees to address the impact of glyphosate on the declining bee population. Additionally, his master's thesis concentrated on the production and characterization of spider silk protein (Cyllindroform Spidroin), showcasing proficiency in diverse biotechnological applications. Currently, Deep Bhattacharya is a Juniour research fellow at the Estonian University of Life Sciences under the Combivet ERA Chair, working under the guidance of Dr. Suranga Kodithuwakku and Dr. Alireza Fazeli. His research focuses on investigating the role of extracellular vesicles in anti-inflammatory and immunomodulatory diseases.

Chanaka Premathilaka

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

Chanaka Premathilaka began his scientific career at the University of Peradeniya, Sri Lanka. He obtained his bachelor's degree in Molecular Biology and Biotechnology with a minor in Chemistry. Afterward, 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 and Animal Sciences to pursue his Master of Philosophy degree in pathobiology, which was based on assessing and developing a biological intelligence framework for certain vector-borne infectious diseases that recently have become an epidemic in Sri Lanka. With the onset of COVID-19 epidemics around the world in 2020, he took part in research and diagnosis of pandemics. He joined the COMBIVET ERA Chair team in September 2021 as a Ph.D. student specializing in pathophysiology and clinical medicine. The key focus of his work is the development of extracellular vesicles-based tools for understanding host-pathogen interactions in infectious diseases and their utility in disease diagnosis and monitoring.

Dr. Valery Savitski

Senior Field Application Specialist, Beckman Coulter, Germany

Dr. Valery Savitski is a Senior Field Application Specialist at Beckman Coulter, based in Germany. With a background in chemistry and a PhD in biophysics, Valery brings over 20 years of experience in flow cytometry – spanning academic research and technical support. Valery has worked extensively across the Europe and other regions, helping researchers and clinicians optimize their cytometry workflows through training, demonstrations, and hands-on guidance. He is passionate about translating complex scientific concepts into practical applications and enjoys working with diverse teams across disciplines and geographies.

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

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. She is 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

Agnieszka Mostek-Majewska

Researcher, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland

Agnieszka Mostek-Majewska is a researcher at the Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences. Specializing in biochemistry and proteomics, her work is dedicated to improving cattle breeding. She specially investigates post-translational protein modifications during bull sperm capacitation, a critical process for fertilization. Her vital research, published in journals like "Cell Communication and Signaling," aims to enhance the quality of cryopreserved semen for artificial insemination. In recognition of her significant contributions to animal reproduction, she was awarded a scholarship for "outstanding young scientists" by the Minister of Education and Science.

Maja Kosanović

Senior Research Associate at Institute for the application of nuclear energy, University Of Belgrade, Belgrade, Serbia

Maja Kosanović is a Senior Research Associate at the Institute for the Application of Nuclear Energy (INEP), University of Belgrade. A leading expert in the field of extracellular vesicles (EVs), her research explores their fundamental role in cell-to-cell communication and their therapeutic potential. Her research work is particularly focused on the immunomodulatory properties of EVs from the parasite Trichinella spiralis, aiming to develop novel therapies for autoimmune diseases and allergies. She led the "EVTRIS" project, funded by Serbia's Innovation Fund, to develop an antiallergic therapy based on this research.

As the founder and president of the Serbian Society for Extracellular Vesicles (SrbEVs) and a key member of international collaborations like the European COST action BM1202, she is a pivotal figure in the field. In addition to her research, Dr. Kosanović lectures in doctoral programs at the University of Belgrade, mentoring future scientists. Her early-career excellence was recognized with prestigious awards for her doctoral and graduate theses.

John J.S. Cadwell

President/CEO, FiberCell Systems Inc. Frederick, Md. USA

John J.S. Cadwell was born and raised in Chicagoland, Illinois, USA. He attended undergraduate school at Luther College with a year term at the University of Nottingham. He received a master's degree in pharmacology from the University with a year term at the National Institute for Medical Research in Mill Hill, UK. He founded FiberCell Systems in the year 2,000 and for 25 years has contributed articles and publications while giving seminars around the world on the benefits of 3-D perfusion artificial capillary hollow fiber bioreactors and high-density cell culture in histocentric bioreactors. FiberCell holds three primary patents on the scale-up of hollow fiber systems and has over 500 peer reviewed articles citing their technology, including CDM HD. He lives in Frederick, Maryland with his wife, Pwint Cadwell (who is senior managing director at FiberCell) and his teenage stepdaughter, Wendy.

WORKSHOP 1

Tangential Flow Filtration (TFF)

Date and Time: 2nd October 2025, 13.10-14.10

Instructors: Omidreza Mousavi and Evgeniy Brik

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.

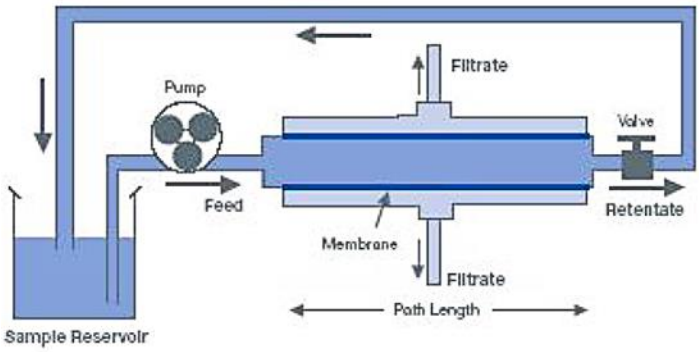

Figure 3: Diagram for the flow of fluids/sample solution in a simple TFF device (Adopted from Pall systems)

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: 2nd October 2025, 15.30-17.00

Instructors: Omidreza Mousavi and Saumya Gunasekara

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.

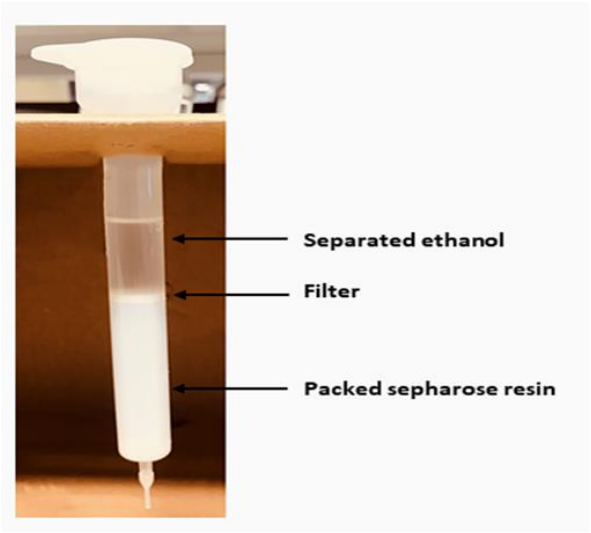

Figure 4: Benchtop column with sepharose resin

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: 3rd October 2025, 10.40-11.10

Instructors: Bertrand Damart and Deep Bhattacharya

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 (Part 1)

RNA cargo extraction from EVs

Date and Time: 3rd October 2025, 11.10-12.20

Instructors: Subhashini Muhandiram

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 4 (Part 2)

Analysis of EV RNA quality and integrity

Date and Time: 3rd October 2025, 13.20-14.20

Instructors: Subhashini Muhandiram and Omidreza Mousavi

Location: SIME General Laboratory, Ravila 14b, Tartu

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WORKSHOP 5

Fluorescence NTA

Date and Time: 3rd October 2025, 14.20-15.20

Instructors: Bertrand Damart, Chanaka Premathilaka and Deep Bhattacharya

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 6

NTA data analysis

Date and Time: 3rd October 2025, 15.40-16.45

Instructor: Deep Bhattacharya and Evgeniy Brik

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

Simulating EV Analysis Using Imaging Flow Cytometry

Date and Time: 4th October 2025, 11.45-12.20

Instructors: Agnieszka Mostek-Majewska

Location: SIME General Laboratory, Ravila 14b, Tartu

Description: This workshop is designed for participants who wish to deepen their understanding of extracellular vesicle (EV) analysis using imaging flow cytometry. Since participants will not have access to the cytometer itself, the workshop has been structured around simulated data interpretation and protocol design exercises. It combines theoretical content with hands-on problem-solving and critical thinking.

The aim is to simulate real laboratory decision-making and data analysis by engaging participants in interpreting imaging data, evaluating staining strategies, and planning their own EV experiments. This format supports both content retention and the development of research-relevant analytical skills.

Learning Objectives:

- Describe the key steps involved in EV isolation, staining, and imaging flow cytometry-based analysis.
- Recognize the importance of specific membrane and marker-based criteria for EV identification.
- Correctly interpret fluorescence scatter plots and imaging data from simulated flow cytometry experiments.
- Understand and evaluate experimental controls, including single-color compensation and detergent-based negative controls.
- Design a simplified EV staining and analysis protocol suited for hypothesis-driven research.

Required materials:

•Laptop or personal computers for analysis

Notes –

WORKSHOP 8

Glyco-profiling EVs using solid-phase lectin-based assay

Date and Time: 4th October 2025, 13.20-15.00 (Part 1) and 16.20-17.45 (Part 2)

Instructor: Maja Kosanovic

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

Description: Use of lectin-based solid-phase assay to analyze glycosilation of EVs

Background:

Glycosylation is a critical biochemical process in which sugar molecules, or glycans, are enzymatically attached to proteins, lipids, or other molecules. In proteins this post-translational modification is essential for protein folding, stability, and function, while also playing a key role in physiological and pathophysiological processes including recognition, immune responses and signaling, making it a potentially useful target for diagnostics and therapeutics.

The glycans on EV surface influence EV biogenesis, stability, and targeting specificity, aiding to precise delivery of cargo to recipient cells. These glycan structures facilitate EV recognition and uptake by target cells through specific receptor interactions. Moreover, altered glycosylation patterns on EVs are implicated in diverse pathologies making them promising biomarkers and therapeutic targets.

Lectins are proteins with high specificity for binding distinct glycan structures, so they can be used as powerful tools for analyzing glycosylation. Lectin-based techniques, such as solid-phase lectin-based assays enable rapid detection of specific glycan motifs on glyco-conjugates. This method, although providing less detailed structural information

compared to mass spectrometry, is cost-effective, relatively simple and specific, making it ideal for fast glyco-profiling of EVs.

Reading materials:

Kosanović M, Janković M. Isolation of urinary extracellular vesicles from Tamm- Horsfall protein-depleted urine and their application in the development of a lectin-exosome-binding assay. Biotechniques. 2014 Sep 1;57(3):143-9. doi: 10.2144/000114208. PMID: 25209049.

Required materials:

1. Microtiter plates
2. Samples
3. Biotinylated lectins
4. BSA for blocking
5. Avidin-biotinilated enzyme system
6. 3,3′,5,5′-Tetramethylbenzidine (TMB)
7. Sulfuric acid
8. Plate reader for measuring absorbance (optical density, OD) at ʎ=450 nm

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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.

GE. (2014). Cross Flow Filtration Method Handbook. Retrieved June 3, 2018, from https://www.bioprocessonline.com/doc/cross-flow-filtration-method-handbook-0001

Heath, N., Grant, L., De Oliveira, T. M., Rowlinson, R., Osteikoetxea, X., Dekker, N., & Overman, R. (2018). Rapid isolation and enrichment of extracellular vesicle preparations using anion exchange chromatography. Scientific Reports, 8(1). https://doi.org/10.1038/s41598-018-24163-y

Kumar, A. M. (2013, July 22). Tangential Flow Filtration: Membrane Types and Process Parameters. Retrieved June 3, 2018.

Kuriyel, R., & Zydney, A. L. (2000). Sterile Filtration and Virus Filtration. In M. A. Desai (Ed.), Downstream Processing of Proteins (Vol. 9, pp. 185–194). Totowa, NJ: Humana Press.

Schwartz, L., & Seeley, K. (2002). Introduction to Tangential Flow Filtration for Laboratory and Process Development Applications Pall Life Sciences Ann Arbor112.RetrievedJune3, 2018.

TRIZOL Reagent technical insert (Invitrogen).

van Reis, R., & Zydney, A. (2001). Membrane separations in biotechnology. Current Opinion in Biotechnology, 12(2), 208–211. https://doi.org/10.1016/S0958-1669(00)00201-9

Veiksina, S., Kopanchuk, S., Mazina, O., Link, R., Lille, A., & Rinken, A. (2015). Homogeneous Fluorescence Anisotropy-Based Assay for Characterization of Ligand Binding Dynamics to GPCRs in Budded Baculoviruses: The Case of Cy3B-NDP-α-MSH Binding to MC4 Receptors.

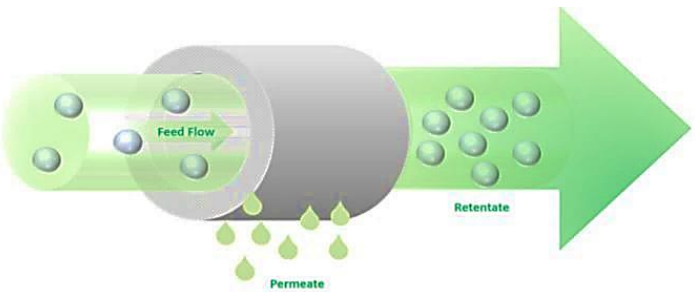
In D. M. F. Prazeres & S. A. M. Martins (Eds.), G Protein-Coupled Receptor Screening Assays (Vol. 1272, pp. 37–50). New York, NY: Springer New York. https://doi.org/10.1007/978-1-4939-2336-6_3

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

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.

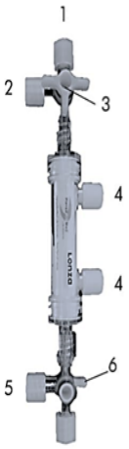


Fig 1

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).

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

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

Fig 2

- 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.

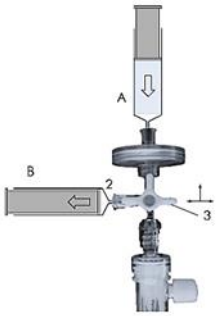
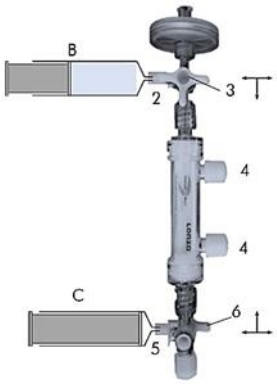


Fig 3

- 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.

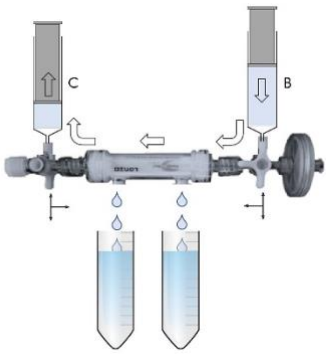


Fig 4

3. Recovery of concentrated EV-s

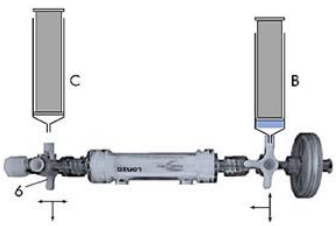


Fig 5

- 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.

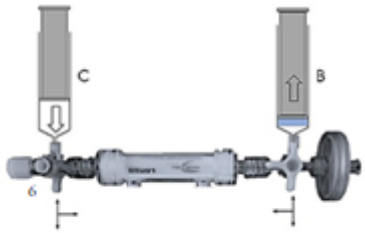


Fig 6

- 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)

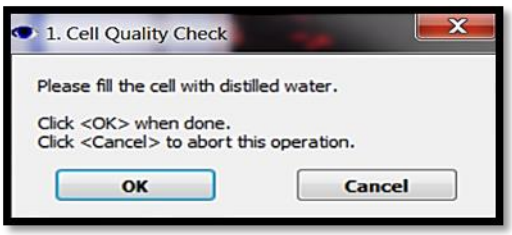


Figure 2.

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).

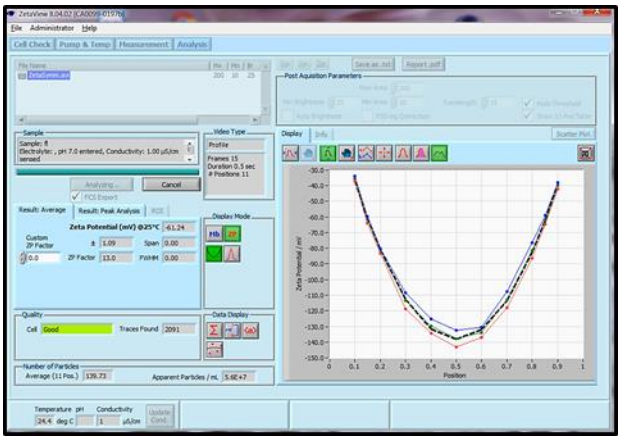


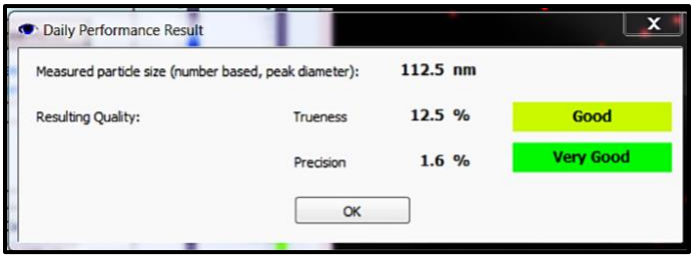
Figure 4.

- **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)

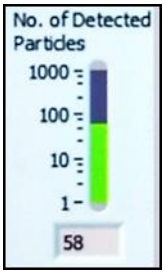


Figure 6.

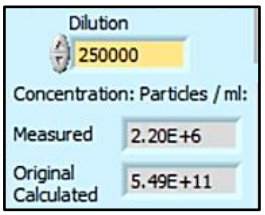


Figure 7.

• 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

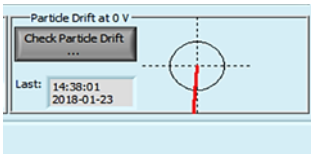


Figure 8.

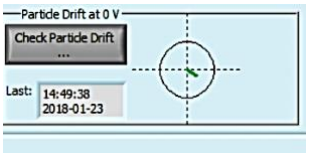


Figure 9.

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.

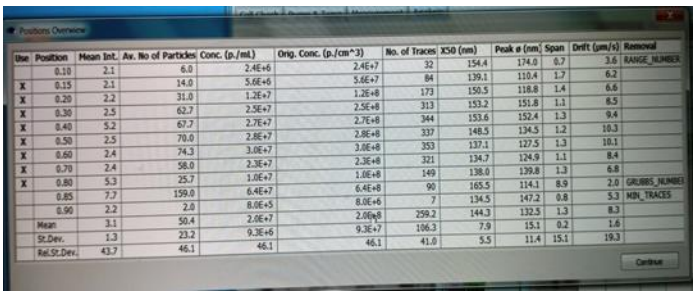
• Go to the 'Measurements' and click 'run video acquisition'. Select the appropriate folder where the measurement data should be stored and label the file.

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 σ (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	8.5	
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	2.0E+8	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	

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)
	o (white) RNA 6000 Pico Conditioning Solution
Syringe Kit	● (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

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they are driven through it electrophoretically. Agilent RNA kits are designed for use with the Agilent 2100 Bioanalyzer instrument only.

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	Buffer compati-bility	50 nM Tris or 50 mM NaCl	50 nM Tris or 50 mM NaCl

individ
ual box)

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:

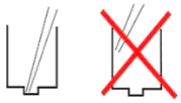
a) Release the lever of the clip and slide it up to the top position.

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).

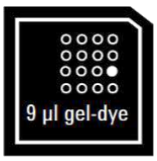
Preparing the Gel

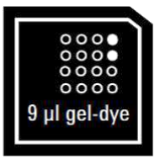
1.Pipette 550 µL of RNA gel matrix (red ●) into a spin filter.
2.Centrifuge at 1500g ± 20 % for 10 min at room temperature.
3.Aliquot 65 µL filtered gel into 0.5 mL RNase-free microcentrifuge tubes. Use filtered gel within 4 weeks. Store at 4 °C.

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



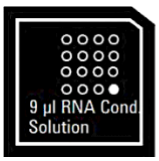


1.Put a new RNA chip on the chip priming station.
2.Pipette 9 µL of gel-dye mix in the well marked

3. Make sure that the plunger is positioned at 1 mL and then close the chip priming station.

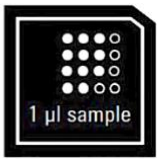
4.Press plunger until it is held by the clip.
5.Wait for exactly 30 s then release clip.
6.Wait for 5 s. Slowly pull back plunger to 1 mL position.
7.Open the chip priming station and pipette 9 µL of gel-dye mix in the wells marked G.
8.Discard the remaining gel-dye mix.

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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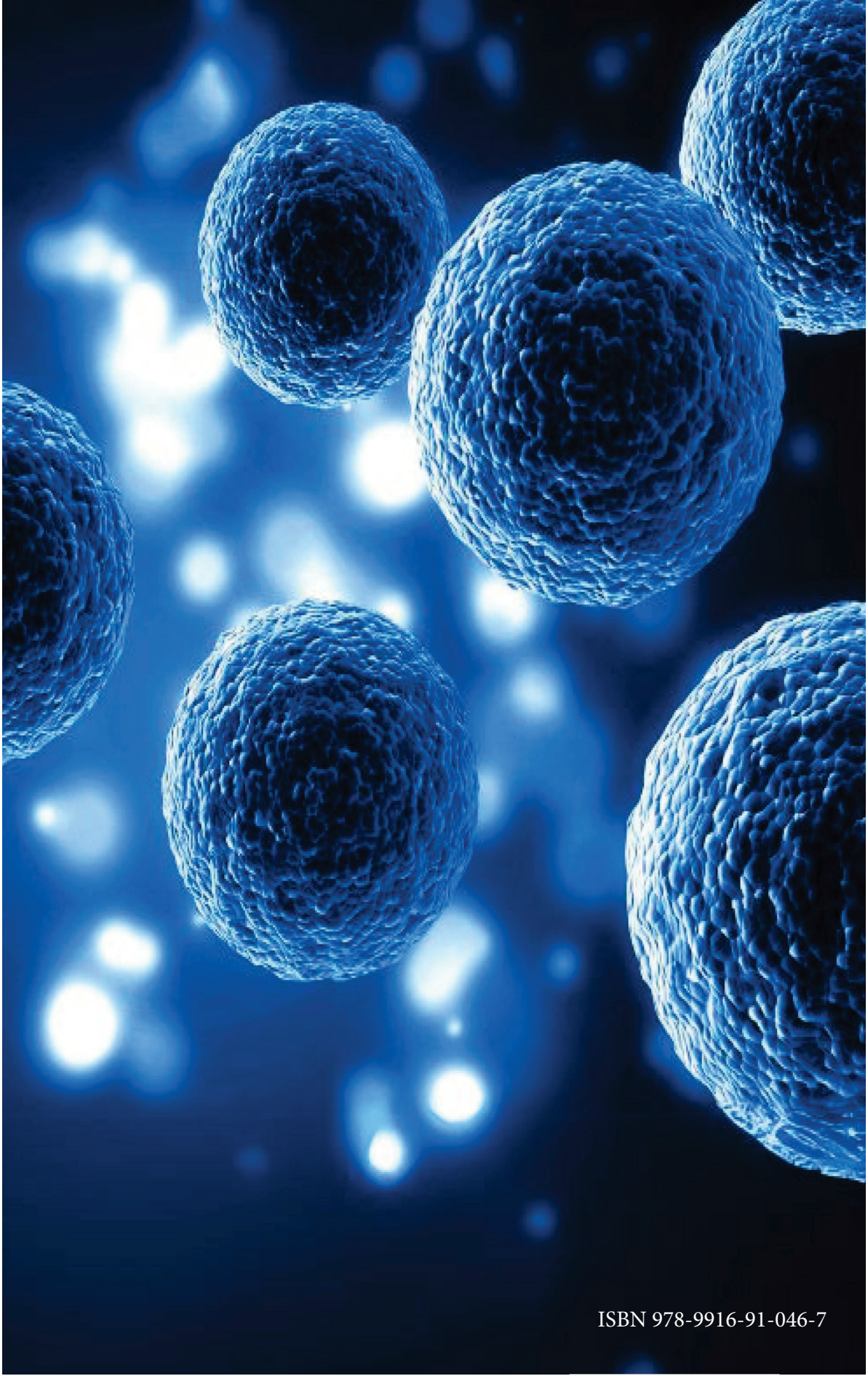
Notes –



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