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Abstracts

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Introduction: Extracelluar vesicles are released by various cell types, particularly tumour cells, and may be potential targets for body fluid-based cancer diagnosis. However, studies of extracellular vesicles from body fluid have been relatively undermined by time consuming process of isolating extracellular vesicles and lack of effective purification strategies. In this study, we isolated extracellular vesicles by a new method on specific time intervals and detected tumour-derived extracellular vesicles from 2 body fluids, blood and saliva. *Methods*: Six-week old C57BL6 mice had been grown after subcutaneous injection of $10^6 \times$B16BL6 melanoma cells into their bodies. Blood and saliva were collected on specific time intervals from each subject. Extracellular vesicles were isolated from the fluids by ultracentrifugation or aqueous two phase system, a new method which uses a mixed solution of polyethylene glycol and dextran. Isolated melanoma exosome was identified by western blots, and expression of tumour-specific marker was compared. *Results*: In the case of ultracentrifugation, tumour-specific marker was not detected from the great part of body fluid samples because of low isolation efficiency. On the other hand, aqueous two phase system efficiently isolated extracellular vesicles, thus tumour-specific maker was detected with high probability. Especially, aqueous two phase system showed about 10 times higher cancer detection chances than ultracentrifugation when extracellular vesicles was isolated from the body fluids in 8-week old melanoma injected mice. *Summary/conclusion*: To date, it was impossible to use extracellular vesicles for practical cancer diagnosis system because of small amount of extracellular vesicles. We overcame the restriction by using aqueous two phase system. High isolation efficiency of aqueous two phase system gave a new direction for practical cancer diagnosis system.

P-III-11

Comparison of different extraction methods for exosome and exoRNA from cell culture supernatants and body fluid

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Introduction: Exosomes are cell-derived vesicles presenting in many biological fluids. RNAs contained in exosomes could serve as a novel platform for diagnosis. To obtain reliable results, efficient and reproducible methods to isolated exoRNA are mandatory requirements. *Methods*: In our study, nanoparticle tracking analysis was used to measure and characterize exosomes from cell culture supernatants and body fluid isolated by ultracentrifugation, ExoQuick and Total Exosome Isolation Reagent. The quantity and quality of the exoRNA isolated by Trizol-LS, SeraMir ExoRNA Amplification Kit and HiPure Liquid RNA Kit were determined by Bioanalyzer 2100, Nanodrop and Qubit. *Results*: Both ExoQuick (6.94E+10 particles/ml serum) and Exosome Isolation Reagent (4.56E+10) had higher extraction efficiency than ultracentrifugation (1.23E+09). Particles isolated by ExoQuick were more homogeneous distributed than other methods. Total Exosome Isolation Reagent and SeraMir had highest extraction yield of exoRNA (Qubit result: 273 ng/10 ml supernatants) than other combinations of exosome and exoRNA methods. Though ExoRNA concentrations of 500 ul serum were below the detection limits of Quibit (20 ng/ml), Bioanalyzer 2100 result showed that HiPure Liquid RNA Kit had higher extraction efficiency (112.5 ng/500 μl serum) than SeraMir (88.56 ng) and Trizol-Ls (37.38 ng). Supernatants exosome isolated by ultracentrifugation had better RNA size distributions than kits, which may have cellular 18sRNA contamination. However, no contamination was found in exoRNA from serum isolated by kits. *Summary/conclusion*: For cell culture supernatants, 2 new nanomaterial exosome Isolation kits have higher extraction quantity than ultracentrifugation, but exoRNA extracted by kits have lower stability and quality. With respect to serum, the convenient and efficient exosome and exoRNA kits maybe best choice for exosome research.

P-III-12

Scalable isolation and purification of a therapeutic stem cell line exosome product by tangential flow filtration

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Introduction: The ability to produce a commercially valuable therapeutic product from stem cell-derived exosomes demands a standardized stem cell producer line appropriately sourced and isolated, manufactured at scale under GMP and grown in serum-free conditions. ReNeuron's proprietary CTX neural stem cell line fulfils all of these requirements and is a highly efficient producer of therapeutically active exosomes. Current manufacturing scale produces in excess of 25 l of GMP produced conditioned medium from which exosomes can be harvested. However, the use of conventional methods used to isolate and purify exosomes is restricted due to limitations in scalability. We have therefore developed a completely scalable process based on tangential flow filtration (TFF) which enables the isolation and purification of exosomes from large scale production of conditioned medium. *Methods*: A TFF system, comprising of an initial 0.1 μm filter step to separate extracellular vesicles on the basis of size followed by a 300 kDa filter step to remove contaminating protein, was used to isolate and purify an exosome population. Shear stress was maintained at a level of 3,000 s^{-1} or less in order to minimize exosome damage. The concentration and size of the exosome population were measured using nanoparticle tracking analysis, and the protein, DNA and RNA concentrations were quantified. An in vitro model of wound healing and a xenograph model of glioblastoma were used to assess the efficacy of TFF sourced exosomes. *Results*: A yield in excess of 3.0×10^{13} particles per litre of starting material has been achieved with an exosome purity of up to 3.6×10^9 particles/μg protein and an average modal size of 98 nm. TFF isolated exosomes maintained efficacy in both in vitro and in vivo models of disease. *Summary/conclusion*: A scalable TFF based process has been developed to isolate and purify an exosome product as part of the GMP manufacture of a standardized stem cell line with demonstrated patient safety. The resultant exosome product shows efficacy as a therapeutic product in a number of disease models, both in vitro and in vivo.

P-III-13

Comparative analysis of physical-chemical precipitation methods of circulating exosome isolation from human biofluids

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Introduction: Exosome isolation from complex biofluids, such as plasma/serum or urine, is the critical step for downstream applications in diagnostic and therapeutic areas. Differential ultracentrifugation is still the most appreciated method for obtaining an enriched exosome pellet, although is time consuming, requires capital equipment and large sample handling. Numerous commercially reagents have been produced in order to separate exosomes via chemical precipitation. This technique allows a fast and easy microvesicle isolation and is especially useful when small volumes of samples are available. *Methods*: Ultracentrifugation and commercial reagents were used to precipitate exosomes from different small volumes of human biofluids (plasma, serum, urine). The exosome isolation efficiency of chemical reagents was compared with the ultracentrifugation yield, and isolated exosomes were tested for common protein or nucleic acid exosome markers with various techniques (WB, ELISA, qRT-PCR). *Results*: Chemical precipitation revealed consistent advantages compared to ultracentrifugation in terms of time and exosome yield from small volumes of samples. Main disadvantage has been reported in exosome pellet solubilization, very hard for some reagent tested. Remarkable, a chemical reagent showed high efficiency in isolating

exosomes from only 100 μl of plasma/serum, easy pellet solubilization and isolated exosomes were suitable for all downstream performed analyses. *Summary/conclusion*: In this study, we compared the efficiency of different commercial reagents in isolating exosomes from small volumes of complex human biofluids (plasma, serum, urine). Exosome chemical precipitation is an efficient method for isolating exosomes from small volumes of samples, when pellet solubilization is an easy step. This method is really useful as a source of exosome biomarkers for proteomic and trascriptomic with potential development to diagnostic and therapeutic area.

P-III-14

Newly designed size exclusion chromatography columns for isolation and purification of extracellular vesicles in clinical samples

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P-III-15

Exosomes isolation by differential centrifugation: theoretical analysis and the experiment

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Introduction: Differential centrifugation though recognized as "gold standard" method of exosomes isolation may give dissimilar and improper results. It is rather common practice to apply the same centrifugation protocols for different types of rotors, which leads to significant differences in yield and purity of exosome preparations. In present work, we perform the detailed theoretical consideration of the sedimentation process in the 2 types of rotors – swinging bucket (SW) and fixed-angle (FA) and apply the theory to the sedimentation behaviour of extracellular vesicles (EV). *Methods*: HT29 cell culture supernatant was centrifuged at 500 g for 5 minutes, at 2,000 g for 10 minutes, at 10,000 g for 30 minutes and at 100,000 g for 70 minutes. Vesicles sizes and concentrations were measured by NTA. *Results*: General equation, describing the velocity of a particle under centrifugal acceleration was adapted to the form convenient for calculation of EV sedimentation profile. The proportion of pelleted vesicles of a given size and the "cut-off" size of completely sedimented vesicles were presented as dependent on centrifugation force and duration and sedimentation path length for both SW and FA rotors. The theoretical analysis shows that the application of common centrifugation protocol without the account of rotor's sedimentation pathlength may be misleading. The usage of K-factors for adjustment of the centrifugation duration at a change of rotor is reasonable in case of SW rotors, but for FA rotors K-factor makes no sense. Experimentally obtained NTA particle size distributions rather well coincide with theoretically predicted vesicles size distributions. *Summary/conclusion*: We demonstrate for a number of commonly used rotors how the proper centrifugation conditions can be selected using rather simple theoretical estimates of "cut-off" sizes of vesicles. To make easy the adjustment of centrifugation protocol for any rotors in use we present a specially designed web-calculator.

P-III-16

Effective and gentle isolation of extracellular vesicles in human and bovine milk without ultracentrifugation

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Introduction: Milk has over millions of years evolved to provide offspring with crucial developmental components adapted for survival under extreme aspects of early life. A multitude of health promoting milk components supports infant development. Extracellular vesicles in milk represent most likely an additional health promoting component though with a less understood diverse and complex nature. In understanding the molecular- and bio-functional diversity of extracellular vesicles in milk, and their physiological function, gentle isolation procedures and well isolated vesicle fractions are crucial. We have identified and validated a novel and effective milk-EV isolation procedure applicable on both human and bovine milk with focus on purity, and gentle treatment, therefore steps including ultracentrifugation and sedimentation/resuspension are omitted. *Methods*: Untreated fresh human or bovine milk is centrifuged to remove milk fat globules. The resulting skim milk is subjected to centrifugation at 30,000 × g. Resulting supernatant is subjected to size exclusion chromatography to remove remaining casein and whey proteins from the vesicles. The isolated EV fraction, as well as control fractions, are investigated by; SDS-PAGE for primary protein components, EV markers by western blotting and mass spectrometry, phospholipid- and neutral lipid profiles by thin layer chromatography, particle size by nanoparticle tracking analysis and total RNA profiles using a Bioanalyzer. *Results*: Using this purification method extracellular vesicles in milk can be gently and effectively isolated from all major milk proteins without the use of ultracentrifugation. Isolated vesicle fractions are highly enriched in lactadherin, CD63, CD9, MUC1 and Hsc70, compared to control fractions. Isolated milk-EV fractions contain moreover a phospholipid composition similar to the plasma membrane and do not contain triglycerides as opposed to milk fat globules. Particles in the vesicle fraction show a mean diameter of 200 nm. Finally, vesicle fractions are also associated with various RNAs and low amounts of ribosomal RNA. *Summary/conclusion*: The described method enables a successful and gentle isolation of extracellular vesicles from both human and bovine milk without the use of potential detrimental methods. The obtained vesicle fraction shows several extracellular vesicle characteristics and represents a good starting material for further analysis of the bio-diversity and – function of milk vesicles.

P-III-17

From basic research to clinical setting: adapting methods for EV enrichment and analysis

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Introduction: Exosomes are small (30–100 nm) vesicles secreted by all cell types in culture and found in most body fluids such as urine. Depending on the disease and state of progression, the number of exosomes may vary significantly. Urine represents an easy and accessible exosome source for downstream analysis. The main purpose of this study was to establish a workflow for exosome enrichment and characterization applicable to a clinical lab setting. *Methods*: A platform for pre-enrichment and analysis of cell culture exosomes has previously been developed in collaboration with Dr. Oksvold (University of Oslo) and Dr. Vlassov (ThermoFisher, Austin, Texas). This platform has here been optimized for direct capture and analysis of exosomes from cell culture media and clinical urine sample including flow cytometry, western blotting, qRT-PCR. *Results*: Here we demonstrate parameters important for efficient capture kinetics of