Supplementary Data 3 for IJMS-766952

Our centrifugation protocol comprises 4 pre-preparation steps:

1. at 400 x g (5 min, 4ºC)
2. 4,000 x g (20 min, 4ºC)
3. 7,000 x g (20 min, 4ºC)

to remove remaining cells and cellular debris
and one final centrifugation I for ectosome sedimentation at 18,000 x g (20 min, 4ºC). This protocol has been designed and optimized after theoretical considerations, based on the model proposed by Livshits et al. (2015) in their paper [1].
During centrifugation at angular velocity ω, three forces act on a mass particle m:

1. Centrifugal force: $F = m\omega^2 R$
2. Dynamic friction (Stokes force) $T = -6\pi\eta r_c v$
3. Buoyancy force: $W = V\rho_{solv} a$

The condition of force balance $F + W + T = 0$ leads to the following equation (Svedberg equation):

$$v = \frac{dR}{dt} = g_{eff}\frac{d^2}{18\eta}(\rho - \rho_{solv}); \qquad g_{eff} = \omega^2 R \tag{1}$$

where:

m	molecular mass	V	molecule volume
ω	angular velocity	ρ_{solv}	solvent density
R	distance from the axis of rotation	a	centrifugal acceleration
η	lepkość dynamiczna medium	d_S	the Stokes diameter (dynamic dimeter),
r_c	molecule radius (sphere)		
v	molecule sedimentation velocity	ρ	protein density

Equation (1) can be written in the following form:

$$\frac{dR}{dt} = \lambda R;\ \lambda = \frac{\omega^2 d^2}{18\eta}\ (\rho - \rho_{solv}) \tag{2}$$

Then its solution is as follows

$$R(t) = R(0)e^{\lambda t} \tag{3}$$

Based on the above equation and geometric relationships for the fixed angle A27-8x50 rotor Livshits et al. (2015) determined the “efficiency” parameter of the centrifugation process

Pelleted(d) i.e. a fraction of particles of a given diameter *d* which after time *t* travels the distance L_{sed} [1]. We can call this fraction as an enriched fraction

$$Pelleted(d) = \frac{2}{\pi}\left(arcsin\frac{vt}{L_{sed}} + \frac{vt}{L_{sed}}\sqrt{1-\left(\frac{vt}{L_{sed}}\right)^2} \right) \quad (4)$$

Calculator: http://vesicles.niifhm.ru/index.php?do=1

Suppl. Table 1. Centrifugation parameters for the Sorvall LYNX centrifuge (Thermo Scientific) equipped with the fixed angle A27-8x50 rotor (Thermo Scientific)

	A27-8x50
R_{min} [mm]	33
R_{max} [mm]	107
θ [°]	34
tube dimension Ø [mm]	29
ρ [g/cm^3]	1.1 ÷ 1.3
ρ_{solv} [g/cm^3]	1.0
η [cP]	1.0 ÷ 1.5
RCF	18 000 g
T [min]	20, 90

R_{min} minimal rotor radius
R_{max} maximal rotor radius
θ rotor angle
ρ_{solv} solvent density
RCF relative centrifugation force [g]
η dynamic solvent viscosity
T centrifugation time

According to this calculations the enrichment of pellet with soluble/secretory proteins in their average molecular mass around 60kDa (e.g. albumin 63 kDa) is about 0 to 1%, depending on assumed ρ protein density (1.22 to 1.43 g/cm3) [2,3]. If the duration of the centrifugation process is extended to 90 min (more 4 times) protein enrichment will achieve 3% (Suppl Table 2). Cut off d_s is a molecule (protein) dimension to sediment 100% of protein molecules in those centrifugation conditions.

Supplementary Table 2. Calculated protein enrichment in different centrifugation time for typical human plasma proteins

ρ [g/cm3]	Cut off d_s	Albumin enrichment [%]	Fibrinogen enrichment [%]
Centrifugation time 20 min			
1,22 [2]	142	0	3
1,35 [4]	113	0	5
1,43 [3]	102	1	6
Centrifugation time 90 min			
1,22 [2]	67	1	13
1,35 [4]	53	2	21
1,43 [3]	48	3	25

d_S the Stokes diameter (dynamic dimeter), calculated for albumin (7.1 nm) and fibrinogen (21.5 nm) [4]
ρ protein density

1 Livshits M.A., Khomyakova E., Evtushenko E.G., et al. Isolation of exosomes by differential centrifugation: Theoretical analysis of a commonly used protocol [published correction appears in Sci. Rep. 2016; 6:21447. Livshits, Mikhail A. [corrected to Livshits, Mikhail A]]. Sci. Rep. 2015; 5:17319. Published 2015 Nov 30. doi:10.1038/srep17319

2 Andersson K.M., Hovmoller S. The protein content in crystals and packing coefficients in different space groups. Acta Cryst. 2000; D56, 789-790. doi.org/10.1107/S0907444900005163

3 Quillin M.L., Matthews B.W. Accurate calculation of the density of proteins. Acta Cryst. 2000; D56, 791-794. doi.org/10.1107/S090744490000679X

4 Erickson H.P. Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy. Biological Procedures Online, 2009; 11, 32-51. doi:10.1007/s12575-009-9008-x