

**EuroKUP COST Meeting
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Losehill Hall, Derbyshire**

Tamm-Horsfall Protein and Urinary Exosome Isolation

Patricia Fernández-Llama^{1, 2}, Sookkasem Khositseth¹, Patricia A. Gonzales¹, Robert A. Star³, Trairak Pisitkun¹, and Mark A. Knepper¹

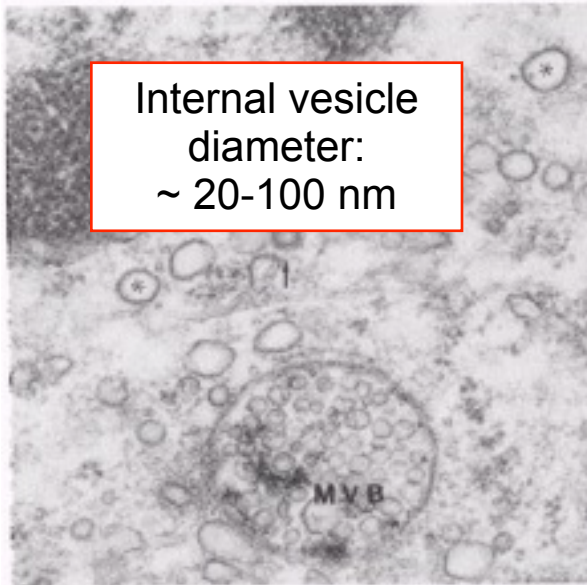
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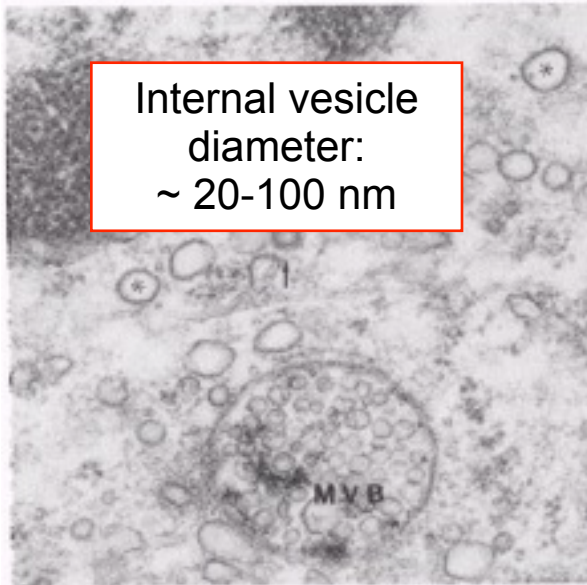
Introduction: Urinary Exosomes

- Exosomes are small (20-100 nm) membrane vesicles that are released from the multivesicular body lumen into the extracellular environment (including plasma and urine) upon fusion of the multivesicular bodies with the plasma membrane
- Urinary exosomes contain proteins that are characteristic of every renal tubule epithelial cell type, as well as podocytes and transitional epithelia from the urinary collecting system



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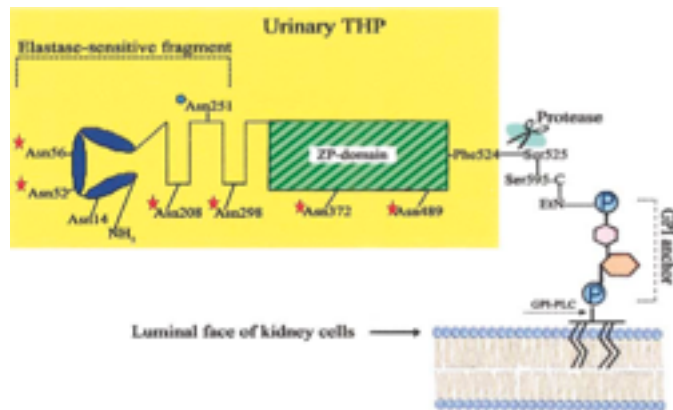


Urinary exosomes provide a suitable starting material for **biomarker discovery** relevant to a variety of diseases



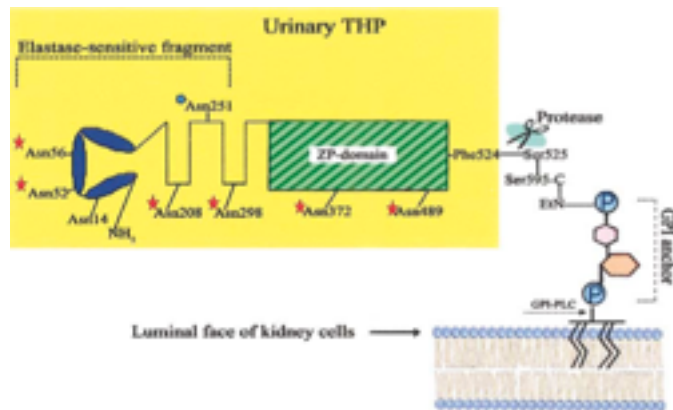
Introduction: Tamm-Horfall protein (THP)

- Glycosylphosphatidylinositol (**GPI**)-linked protein synthesized in the thick ascending limb of the loop of Henle. Is targeted to the plasma membrane and is secreted into the urine via an extracellular proteolytic cleavage
- THP is found in the urine as a high-molecular-weight polymer assembled into filaments or matrices
- THP has a **zona pellucida** (ZP) domain of approximately 260 amino acids including 8-10 conserved cysteine residues. The ZP domain presumably functions as a polymerization module



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THP is the urine's most abundant protein



Introduction: Urinary Exosomes

- Current protocols for urinary exosome isolation involved a two-step differential centrifugation process
- Due to their low density, exosomes are expected to remain in the 17,000 Xg supernatant and to **sediment** only when the sample is spun at **200,000 Xg**
- The large **polymeric THP network** has the potential to trap exosomes in the 17,000 Xg pellet preventing efficient and reproducible isolation in the subsequent 200,000 Xg spin



1. Investigate the effect of THP polymerization on the isolation of urinary exosomes
2. Improve the reproducibility and consistency of the urinary exosome isolation technique

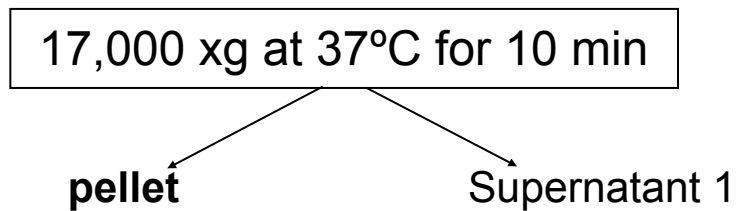


Material and Methods

1. Urine collection from healthy volunteers

- First morning void urine or 12h collection (exosome counting experiment)
- In sterile container with a protease inhibitor mixture

2. Isolation of exosomes by ultracentrifugation



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17,000 xg at 37°C for 10 min

pellet

Supernatant 1

Incubation **w** or **wo**
DTT (200 mg/ml) at
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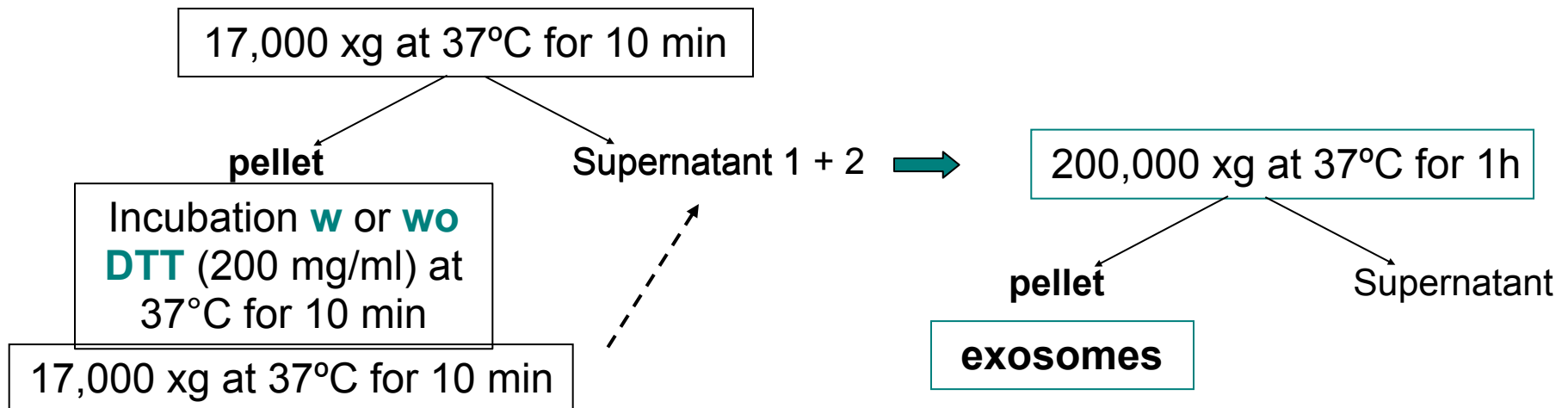


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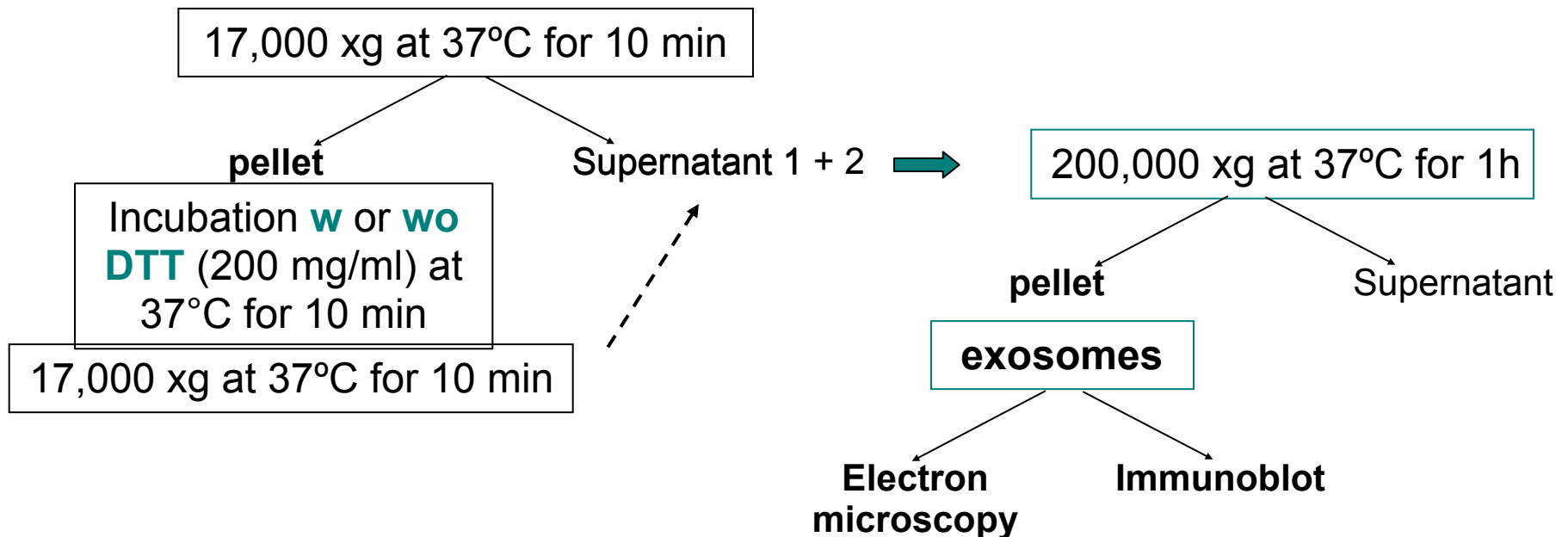


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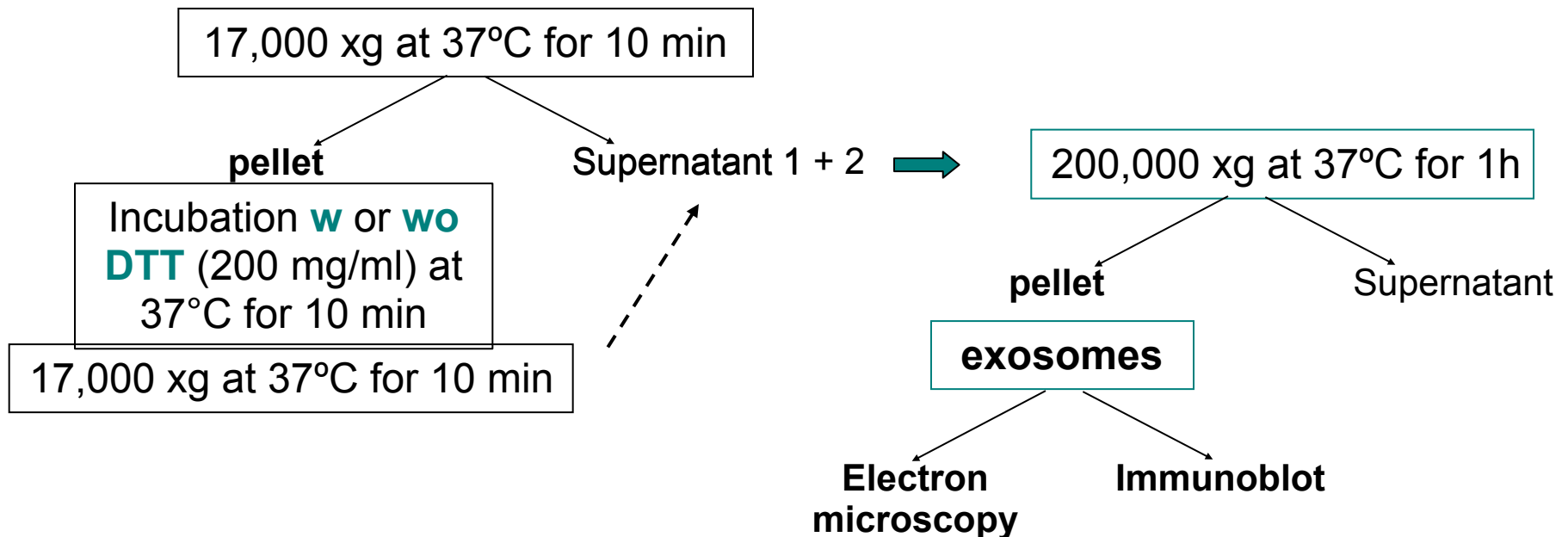


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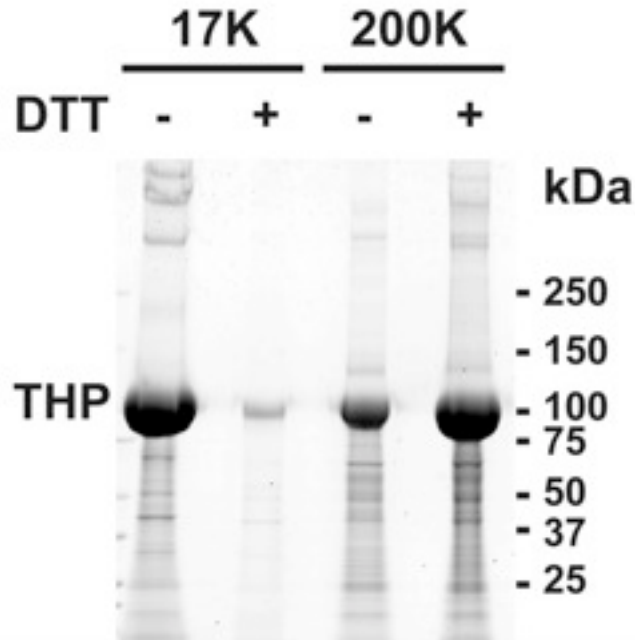
2. Isolation of exosomes by ultracentrifugation



3. Stored conditions: room temperature, 4°C and -80°C



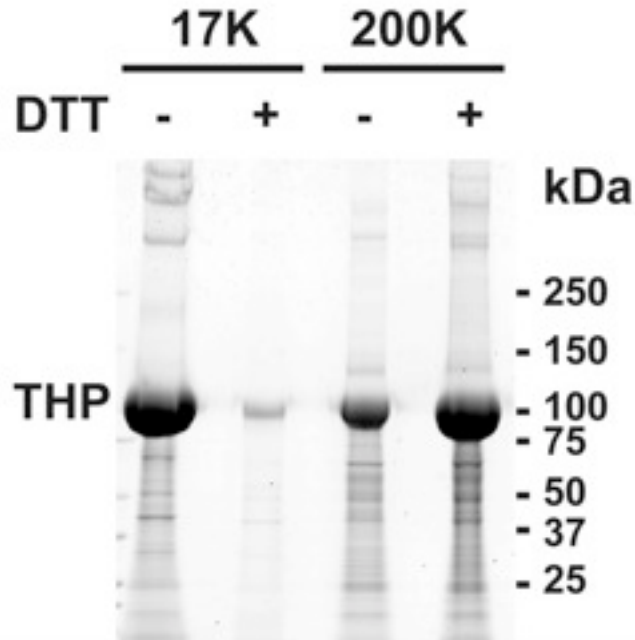
Results: THP sedimentation during centrifugation



Coomassie-stained gel of the 17,000 Xg and 200,000 Xg pellets from normal human urine with and without addition of DTT at the time of the 17,000 Xg centrifugation.



Results: THP sedimentation during centrifugation



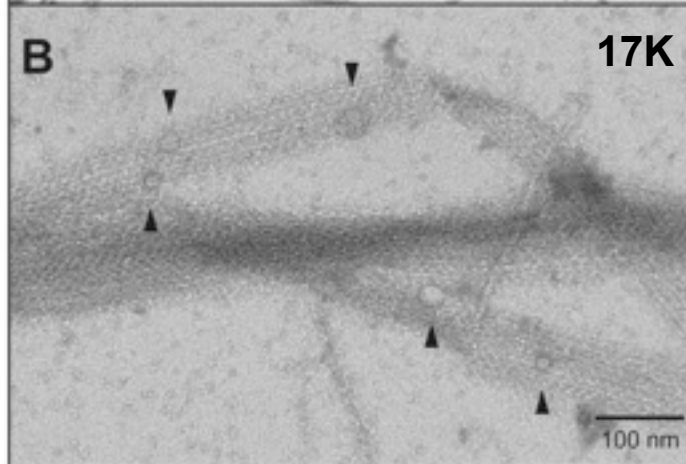
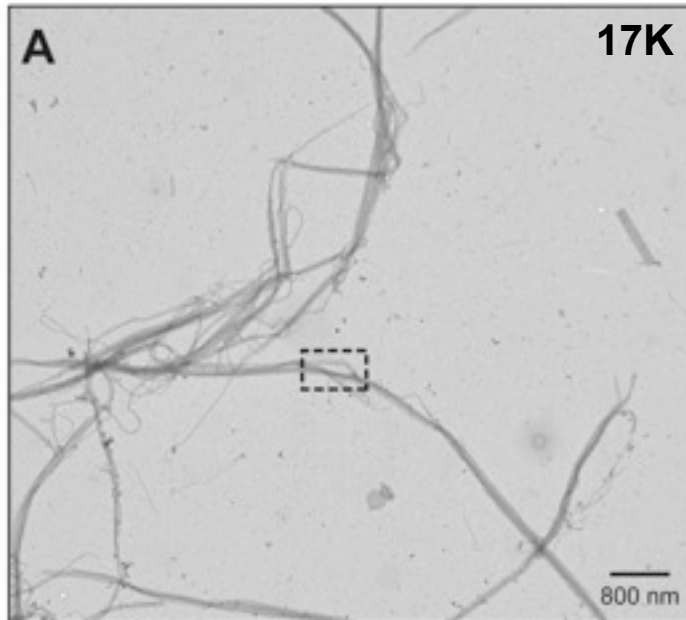
Coomassie-stained gel of the 17,000 Xg and 200,000 Xg pellets from normal human urine with and without addition of DTT at the time of the 17,000 Xg centrifugation.

A substantial amount of THP is present in the 17,000 Xg pellet without reduction and most of it moved to the 200,000 Xg fraction when the sample is reduced

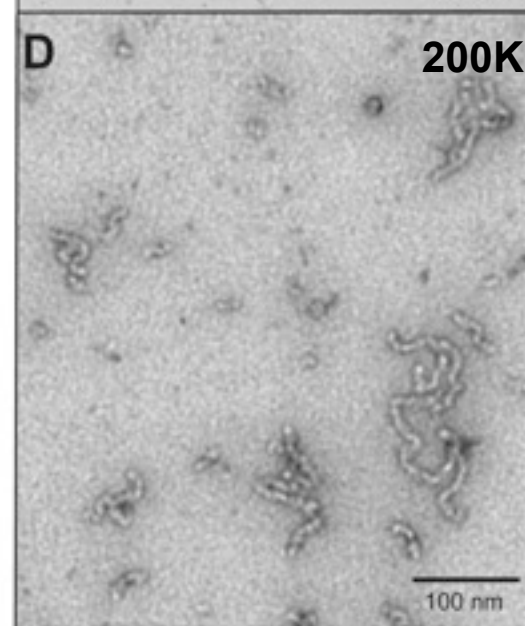
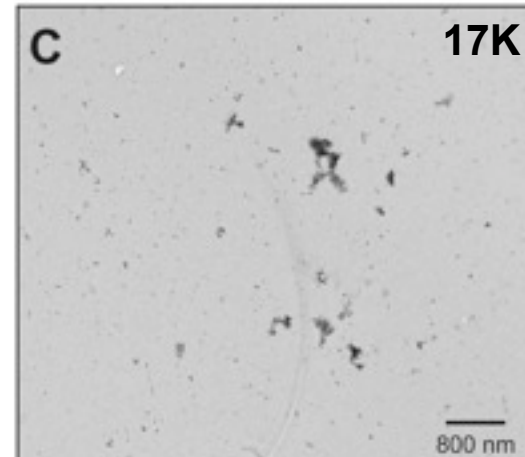


Results: electron microscope (EM) images

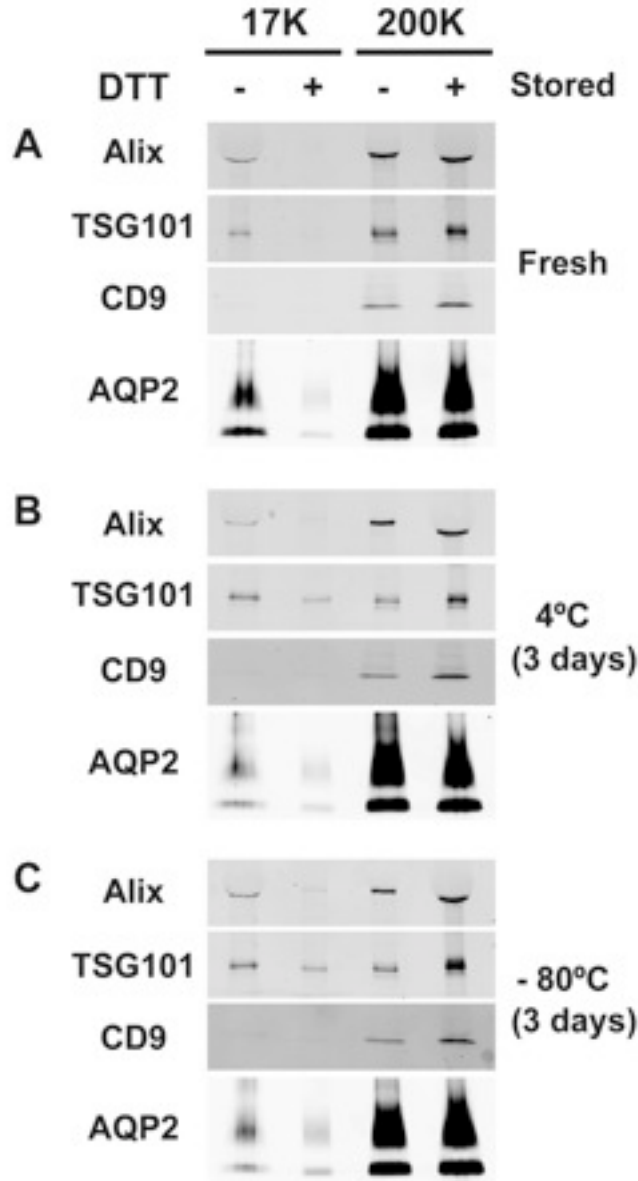
Without DTT



With DTT



Results: exosomal proteins and stored conditions

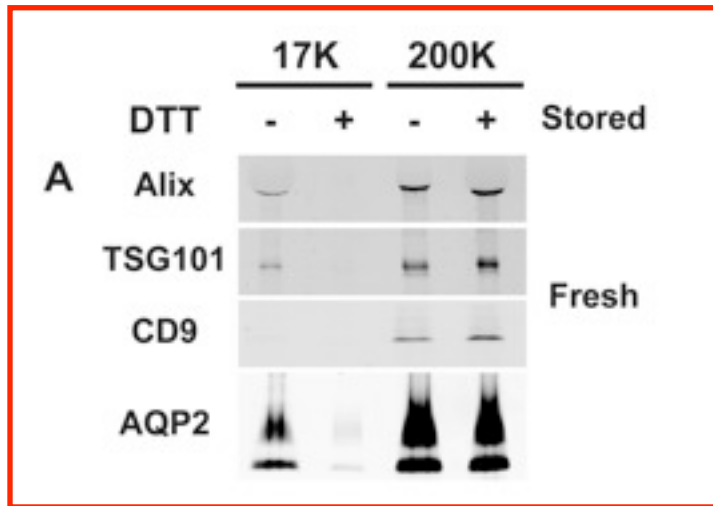


- Three aliquots from pooled urine from three volunteers were processed: one immediately (named fresh), one after stored 3 days at 4°C and the other after stored 3 days at -80°C

- Gel loading of urinary exosome was based on the same percentage of the pellet's final volume

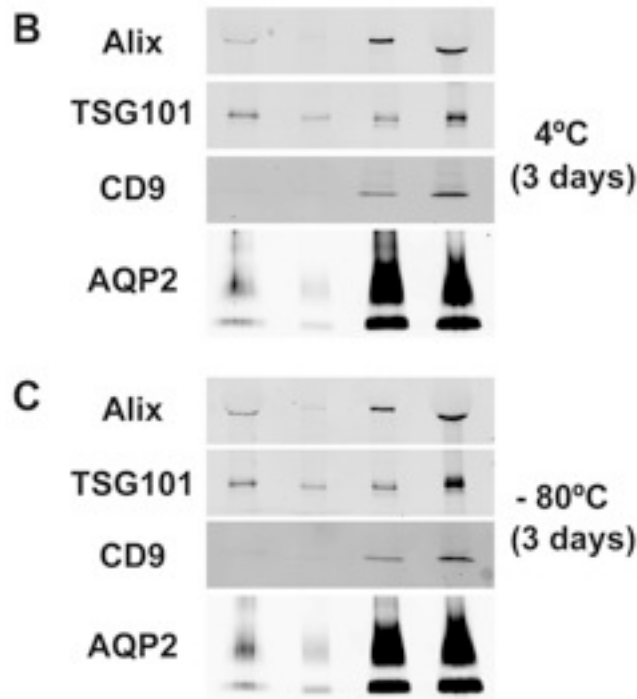


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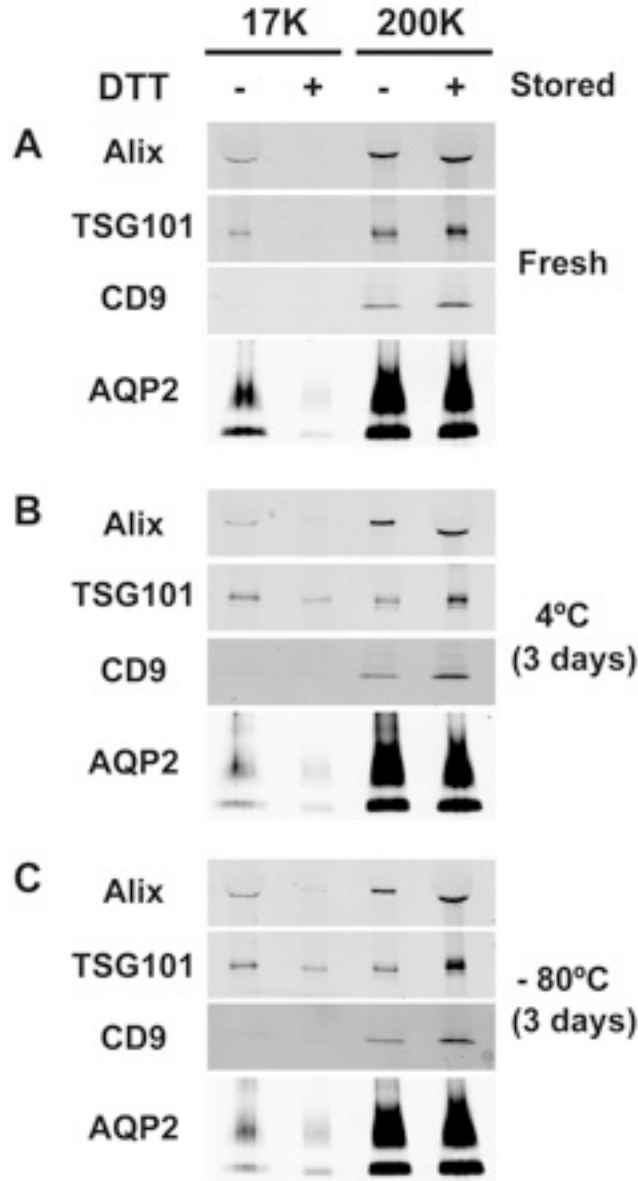


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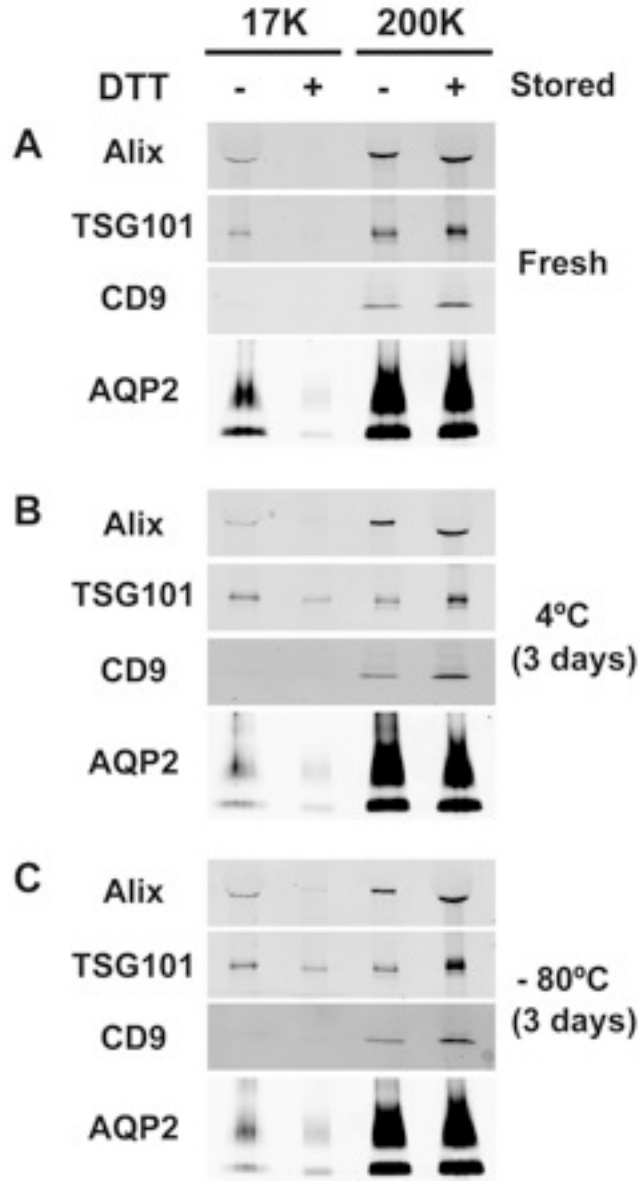


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Significant entrapment of exosomes by the unreduced THP polymeric network

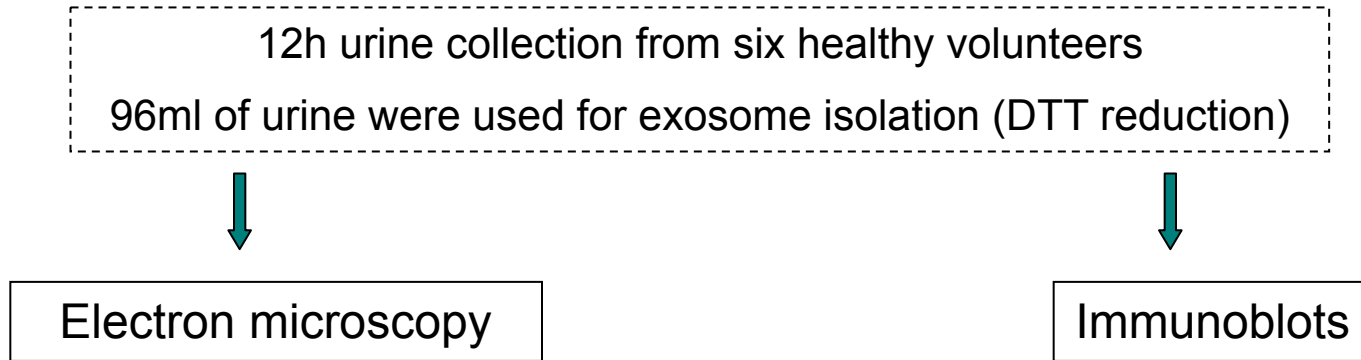


Results: exosome-like particle counting by electron microscopy

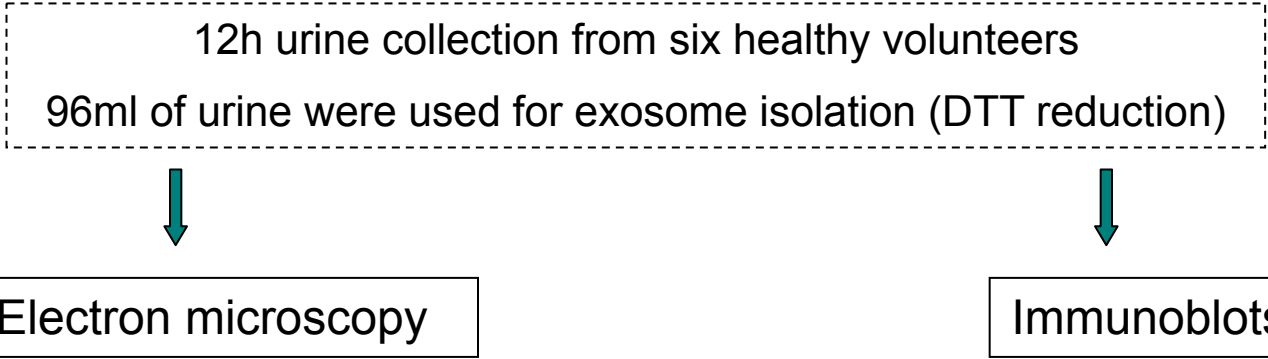
12h urine collection from six healthy volunteers
96ml of urine were used for exosome isolation (DTT reduction)



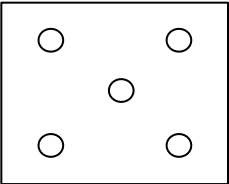
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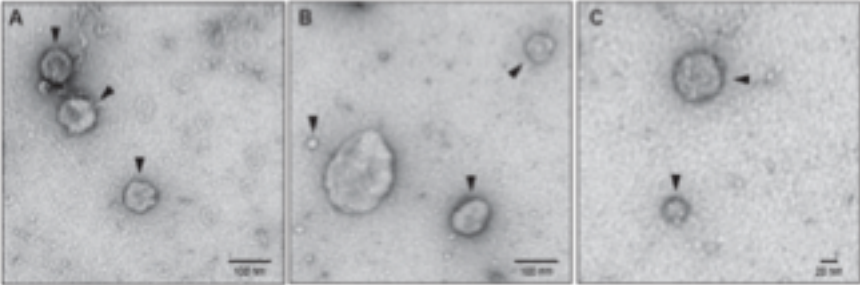
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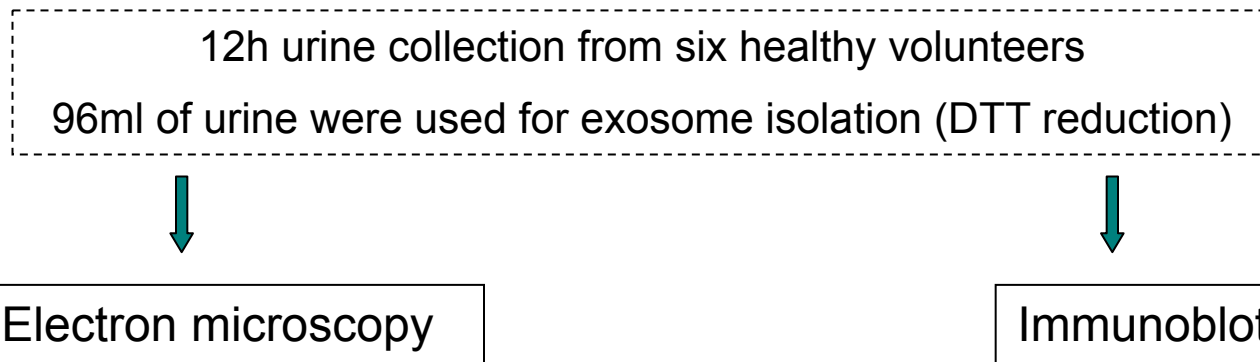
• The same fraction of the pellet was placed on EM grids



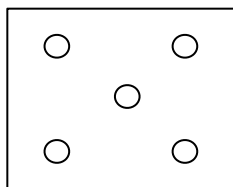
• Exosome-like vesicles were counted from 20 electron micrographic fields at 20,000X magnification from each subject



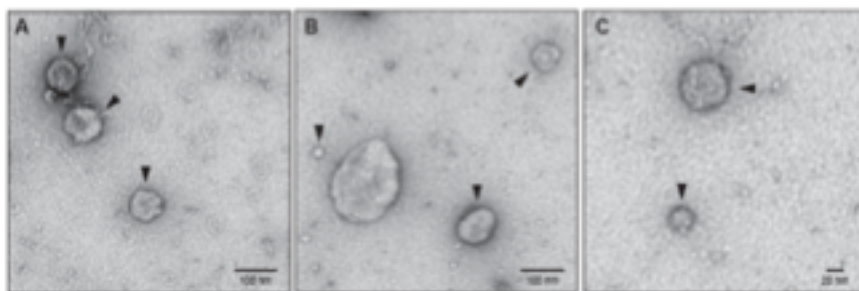
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- Obtained an estimation of the number of exosome-like vesicles in each sample
- Calculated the “minimal” rate of excretion **4440±1639** [SD] particles per minute



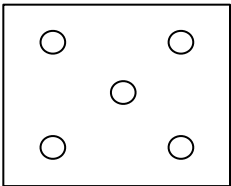
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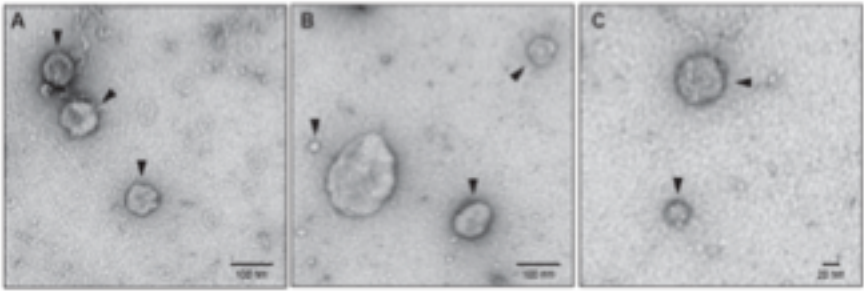
Electron microscopy

Immunoblots

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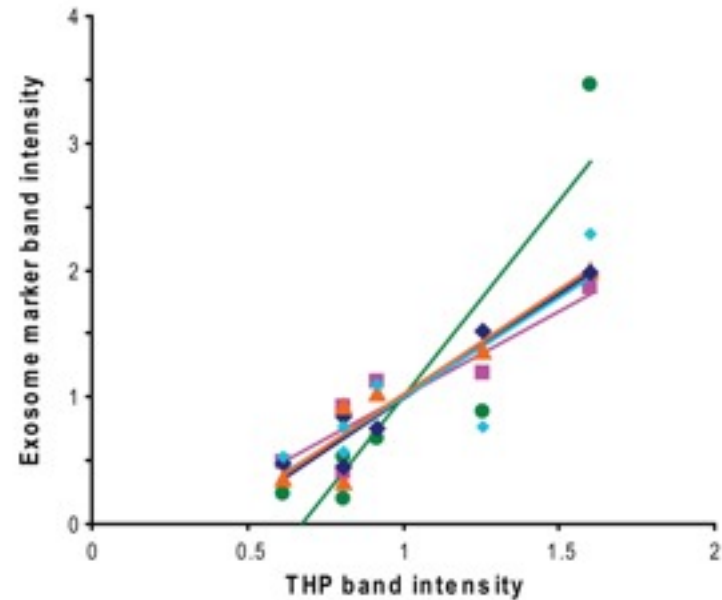
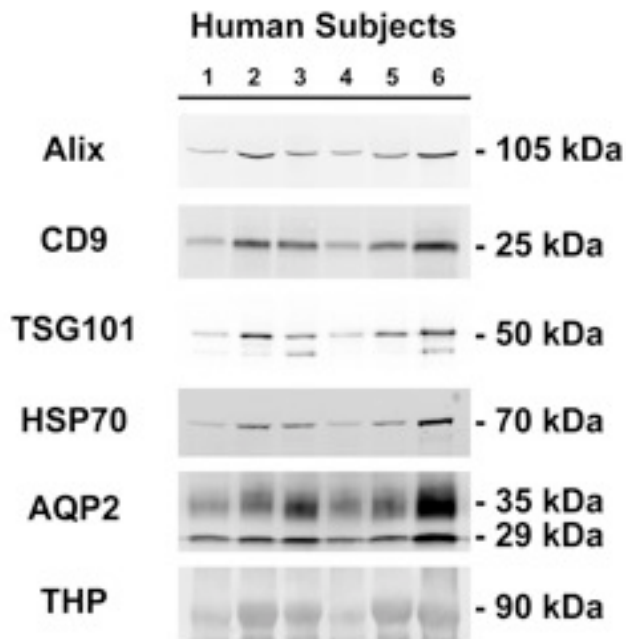


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Results: relation between exosome-like particle counts and exosomal marker proteins

Gel were loaded based on an equal number of exosome-like particles

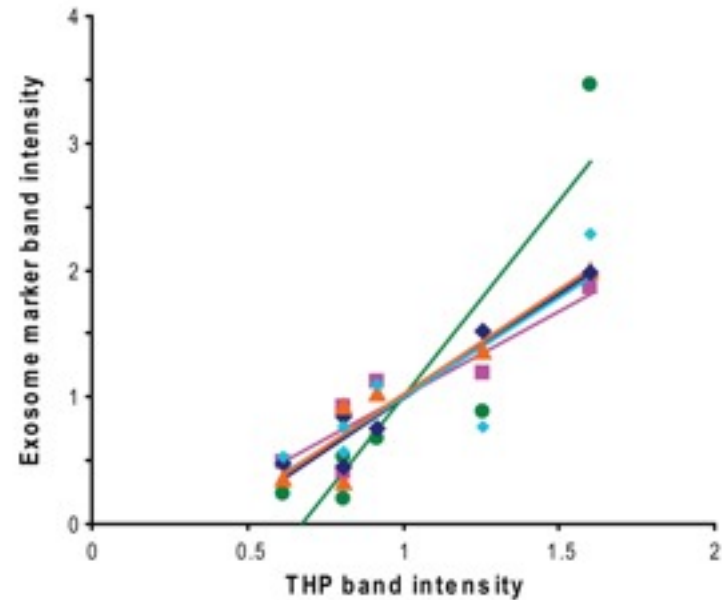
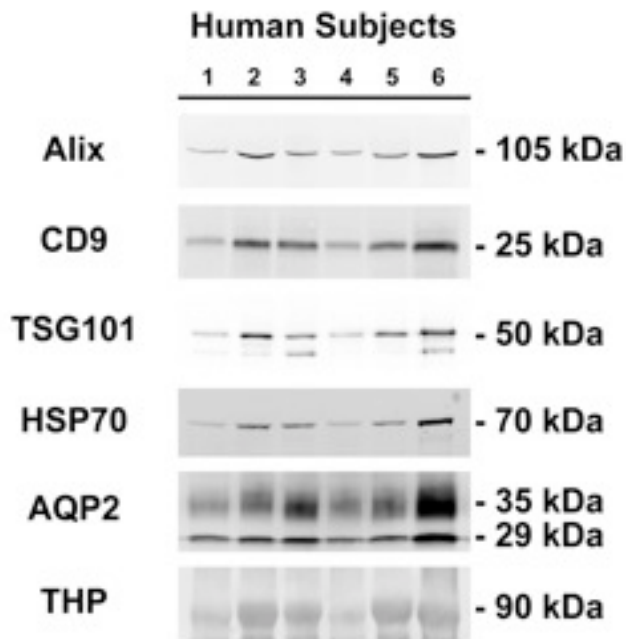


Markers	<i>r</i>	<i>p</i> -value
◆ Alix	0.99	0.002
■ CD9	0.96	0.010
▲ TSG101	0.98	0.003
● HSP70	0.90	0.040
◆ AQP2	0.84	0.075



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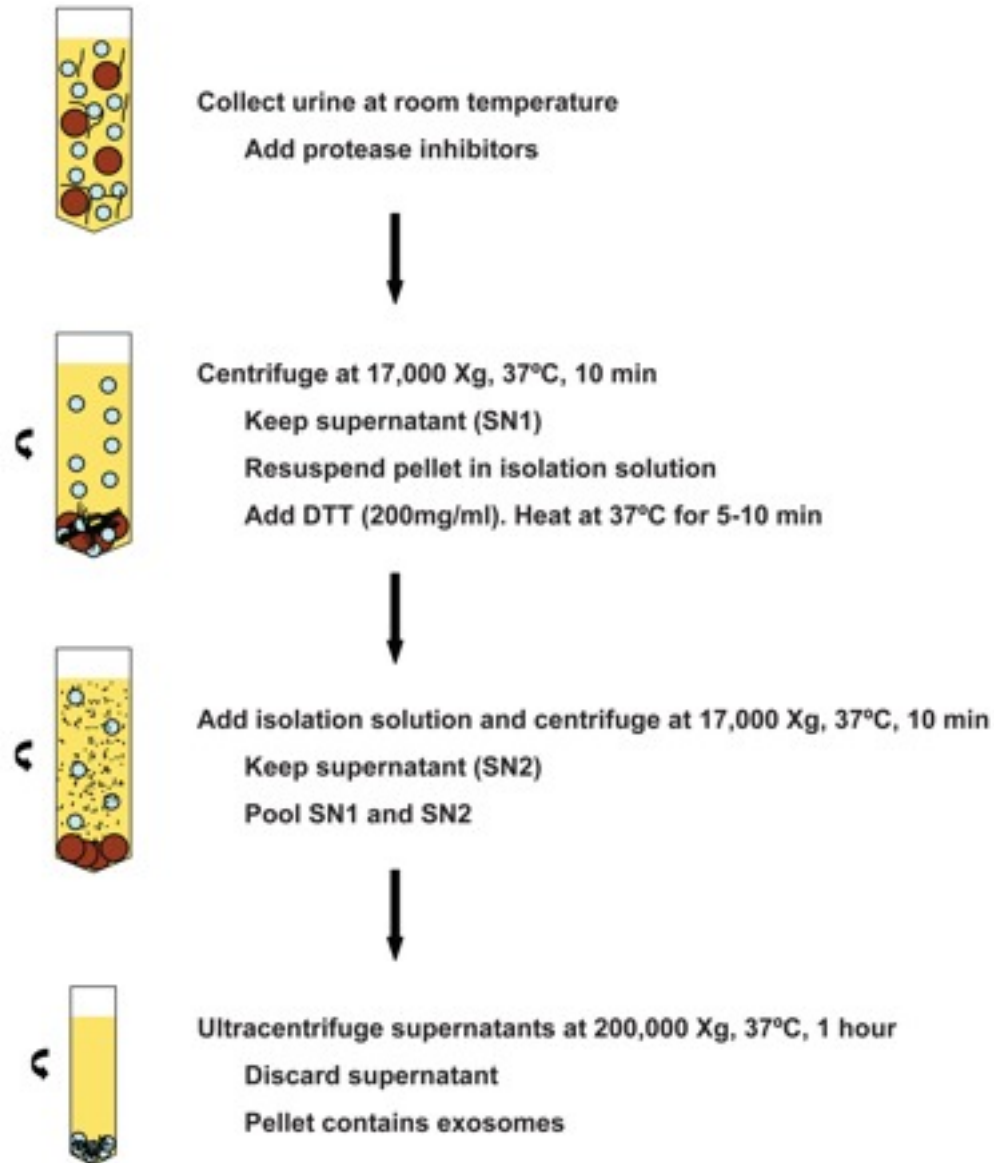
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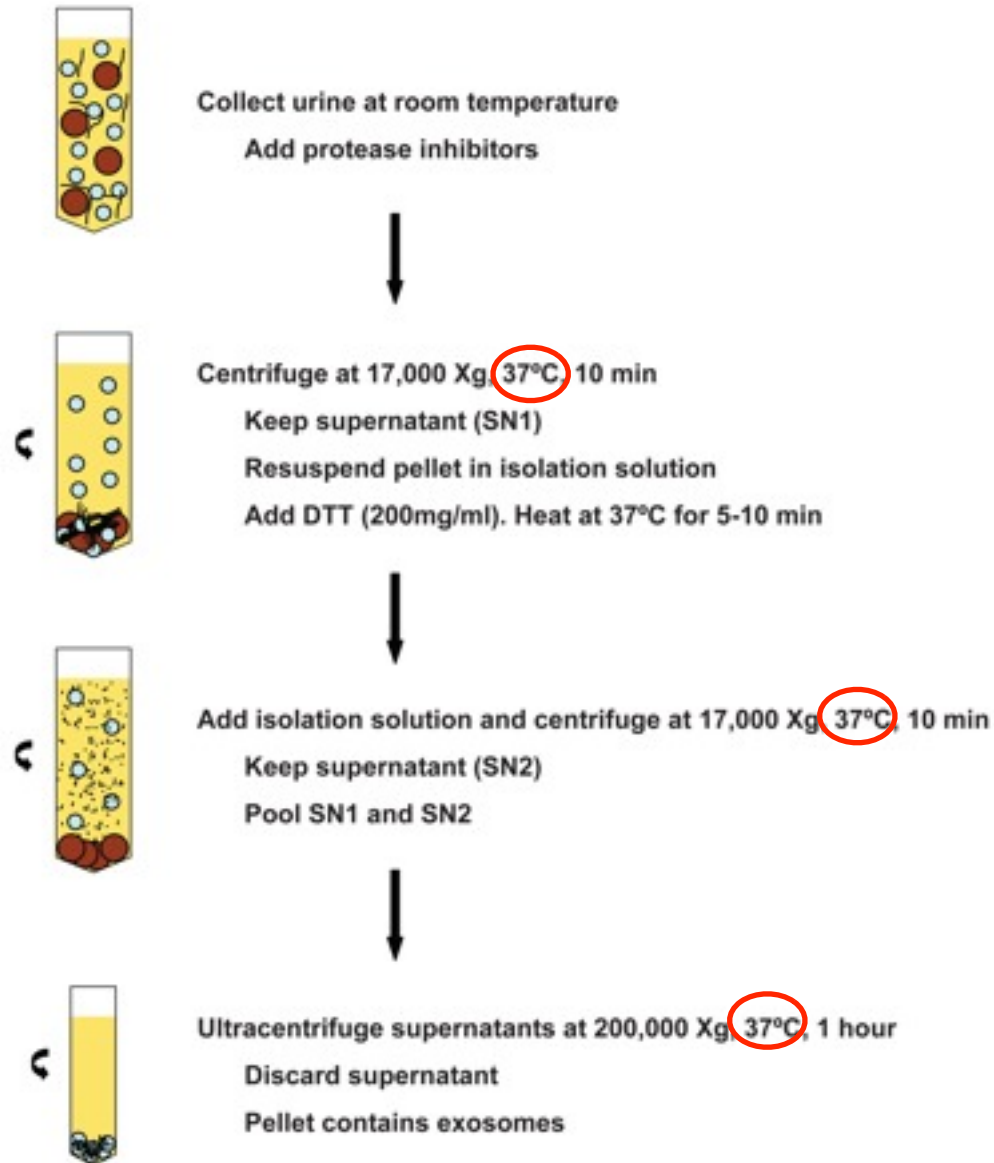
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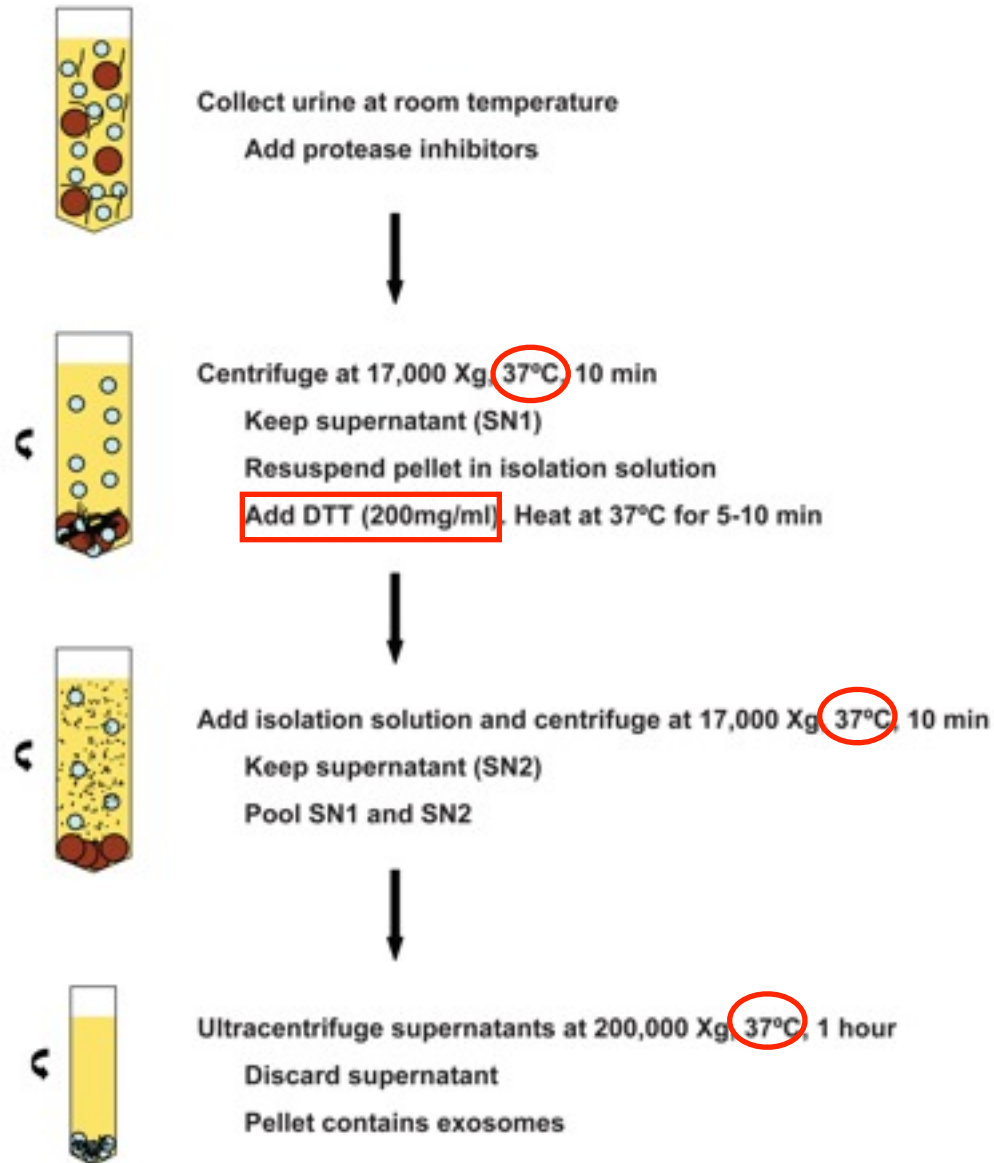
New protocol for urinary exosomes isolation



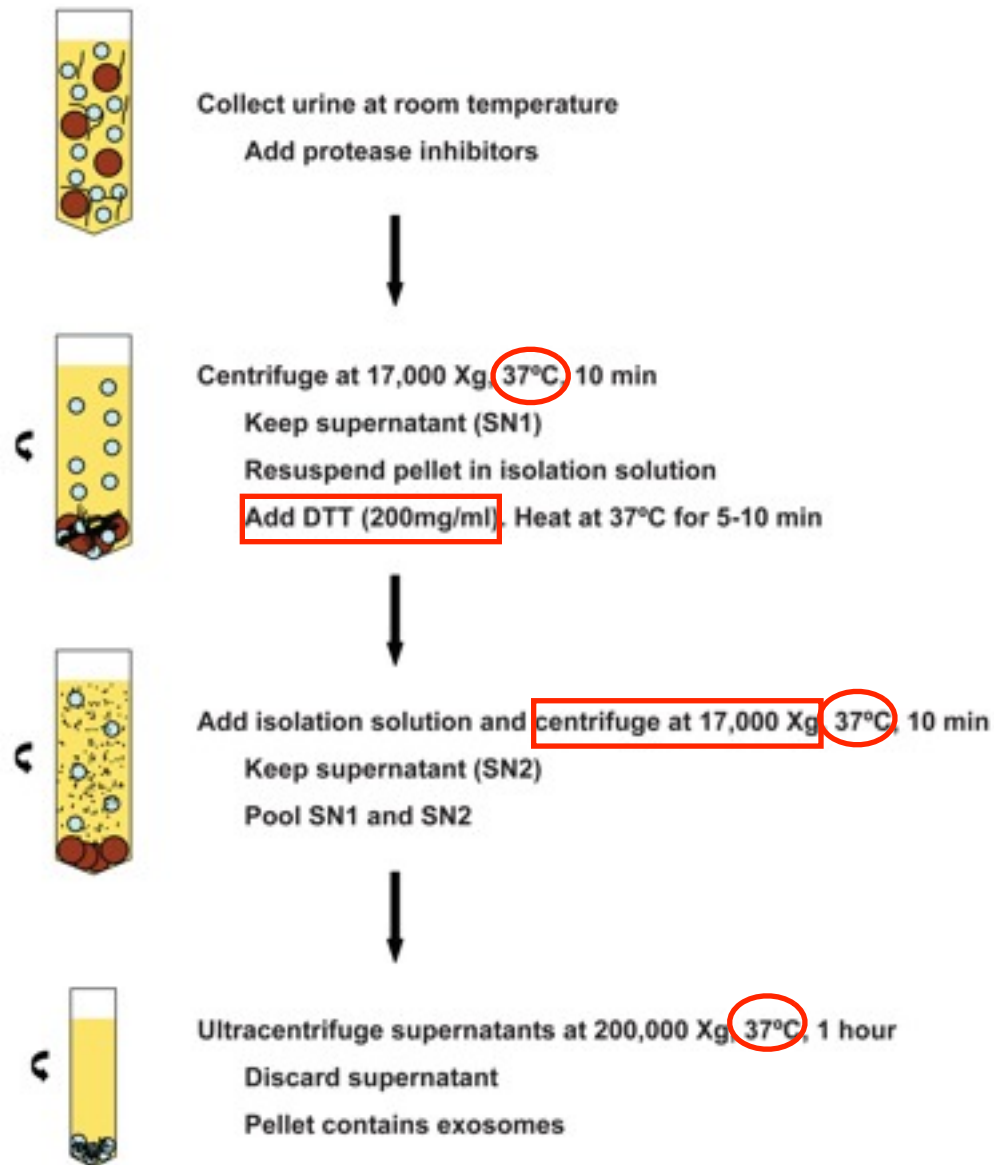
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Conclusions

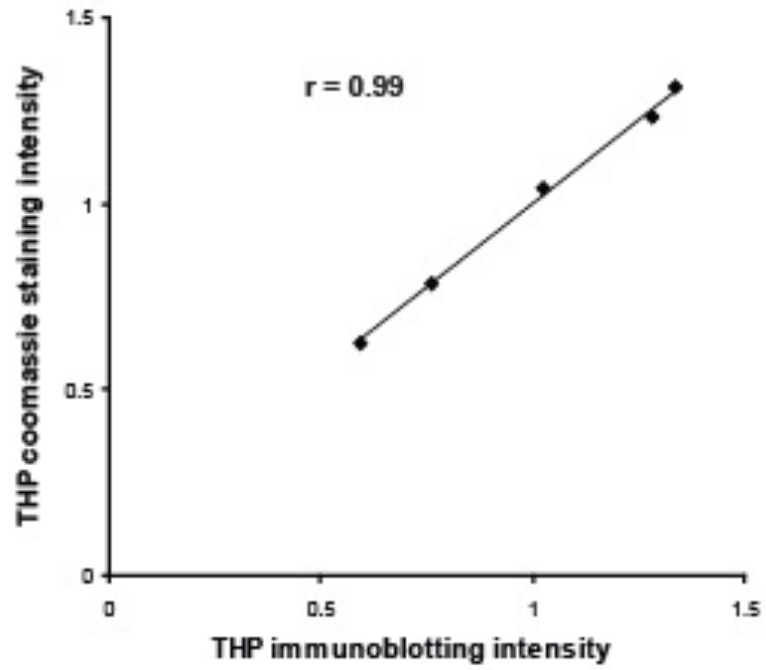
- Urinary exosomes are entrapped by polymeric THP in the 17,000 xg spin
- Chemical reduction of disulfide bonds with DTT, that depolymerized THP, eliminated the entrapment of exosomes allowing an improve in the efficiency and reproducibility of the urinary exosome isolation by differential centrifugation
- The amount of THP in urinary exosomes is representative of the amount of exosomal markers therefore it can potentially be used to normalized samples from spot urine





Acknowledgments





Proteomic Analysis of Urinary Exosomes

1. Separation of urinary vesicles from urine of human volunteers by differential centrifugation (200,000 Xg pellet from 17,000 Xg supernatant).
2. Solubilize in detergent (SDS) and separation by molecular weight by 1D SDS-PAGE.
3. Slice gel into 35 blocks.
4. Trypsinize and extract peptides from each block.
5. Analysis by LC-MS/MS.
6. Identify original proteins by database comparison.

