MicroRNA in biofluids – Robust biomarkers for disease

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Introduction

Circulating or extracellular microRNAs in biofluids is an emerging class of biomarkers with the great advantage of being minimally invasive in diagnostic use. These microRNAs originate from various circulating cells or from tissue cells including cells shed from organs and cancers (dying cells & apoptotic bodies). microRNAs have been shown to be stabilized and protected from RNase degradation by inclusion in various protein complexes and membranous particles such as exosomes or microvesicles.

Exosomes are nano-sized extracellular vesicles of endocytic origin participating in cell-to-cell communication. Their proposed role as intercellular hormone like messenger together with their stability as carrier of proteins and RNA makes them ideal in the search for biomarkers for a variety of biological questions. Exosomes are released by many cell types and found in many biofluids including serum, plasma, CSF and urine.

Challenges of detecting microRNA in Biofluids:
- Low abundance of the target microRNAs
- Presence of PCR inhibitors
- Samples being susceptible to many pre-analytical variables
- Small size of microRNAs, corresponding to a standard primer
- High sequence similarity especially among closely related family members
- Varying GC content

Exiqon Solutions:
- LNA™ in PCR primers, allowing design of two short, highly specific primers for the target microRNA
- Synthetic RNA spike-ins for monitoring purification efficiency and co-purification of inhibitors/Rnases
- Optimized RNA isolation kit for biofluids that ensures high PCR performance and reproducibility
- Use carrier RNA (e.g. MS2) during purification
- Optimized Exosome Isolation Kit for improved detection of microRNA in biofluids

miRCURY™ Exosome Isolation Kit enables microRNA detection in low abundant microRNA samples

Exiqon has developed two easy-to-use kits for isolation of exosomes that can improve detection of microRNA from subcompartments from various biofluids and for enrichment of depleted samples:

- miRCURY™ Exosome Kit – Cells, urine and CSF
- miRCURY™ Exosome Kit – Serum and plasma

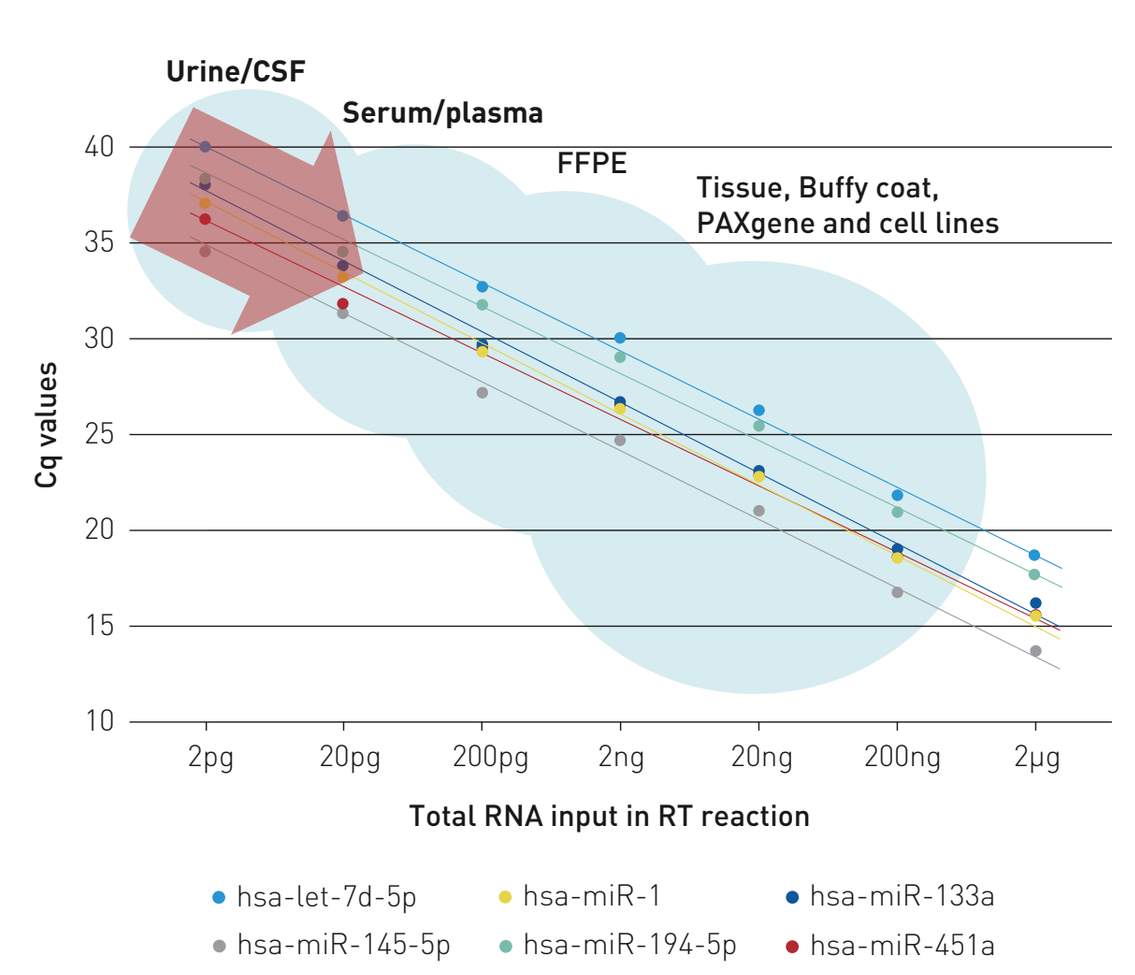

Figure 1. Typical microRNA expression levels in different sample types which are all accurately detected within the linear range of Exiqon's LNA™-enhanced microRNA PCR platform. Red arrow indicate possible improvement of microRNA detection from biofluid samples of low microRNA expression levels when applying miRCURY™ Exosome Isolation Kit. Data from the amplification of 6 microRNAs in serial dilutions of human reference RNA are shown. All microRNA assays exhibit linear read-out with correlation coefficients R2 > 0,99.

Highly efficient isolation of exsomes from urine and serum using the miRCURY™ Exosome Isolation Kit

Nanosight data prove that the miRCURY™ Exosome Isolation Kit precipitates microvesicles of the correct size to include exosomes. As the majority of microRNAs are retained in the enriched exosomes (pellet), the miRCURY™ Exosome Isolation Kit for microRNA isolation from various biofluids is an ideal platform for diagnostic biomarker discovery.

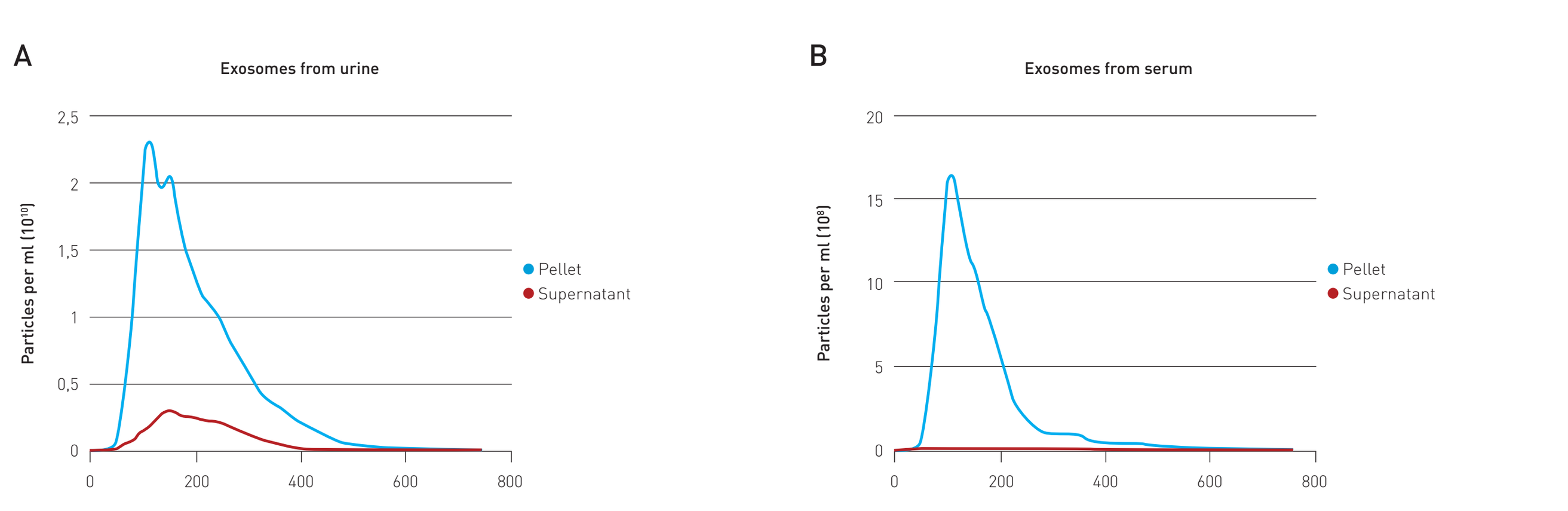

Figure 2. Nanosight data comparing enriched exosomes (pellet) with supernatant from A) urine and B) serum. Nanoparticle Tracking Analysis (NTA) allows visualization and analysis of particles in liquids from 10-2000nm. The particles contained in the sample are visualized by light scatter when illuminated by laser light and captured by a digital camera. The motion of each particle is tracked by the NTA software. The size calculation occurs after determination of the rate of Brownian motion of each individual particle.

microRNA profiles from serum/plasma derived exosomes

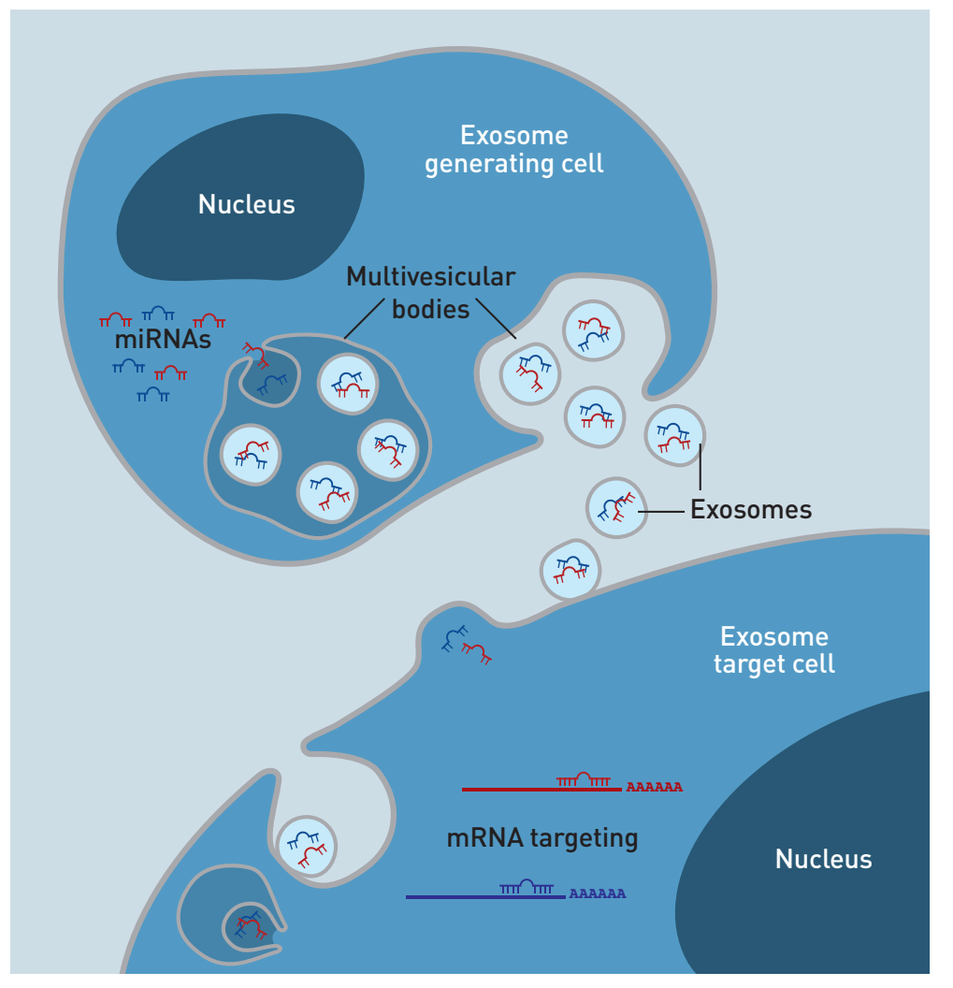

Figure 3. Exosomes transfer microRNAs between cells

- Exosomes are secreted into the blood stream by a wide range of mammalian cell types under normal and pathological conditions
- When released by neoplastic cells exosomes potentially carry arrays of oncogenic molecules including proteins and microRNAs
- The unique signature of exosomal microRNAs may reveal the cell of origin and the condition of those cells and hold potential as biomarkers for early detection of malignancy
- Exosomal microRNA as the starting point for early biomarker studies can reduce the risk of false negative results when the study involve detection of low abundance microRNAs

Improved detection of microRNA in low abundant plasma samples

The Exosome Isolation Kit greatly improves detection of microRNAs from plasma. By moving the detection to a higher signal-to-noise level more robust differentiation between healthy and diseased patients is possible thereby enabling more accurate biomarker studies.

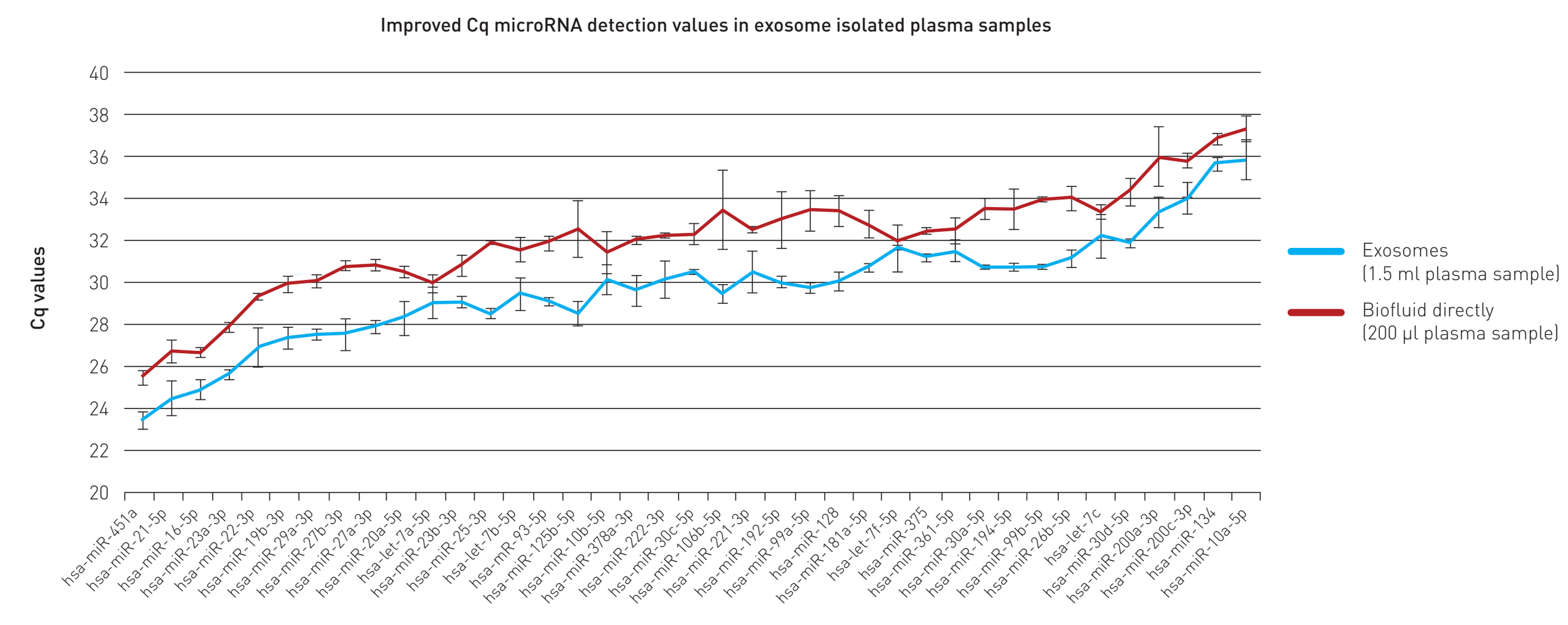

Figure 4. Average Cq values of for four biological replicates from 1.5 ml normal plasma using miRCURY™ Exosome Isolation Kit – Serum and plasma and direct extraction from 0.2 ml normal plasma using the miRCURY™ Biofluid isolation kit only. Samples are profiled on miRCURY LNA™ Universal RT microRNA PCR, human miRNome panel , v3.

Majority of microRNA detected in whole plasma and serum are contained in the exosome pellets

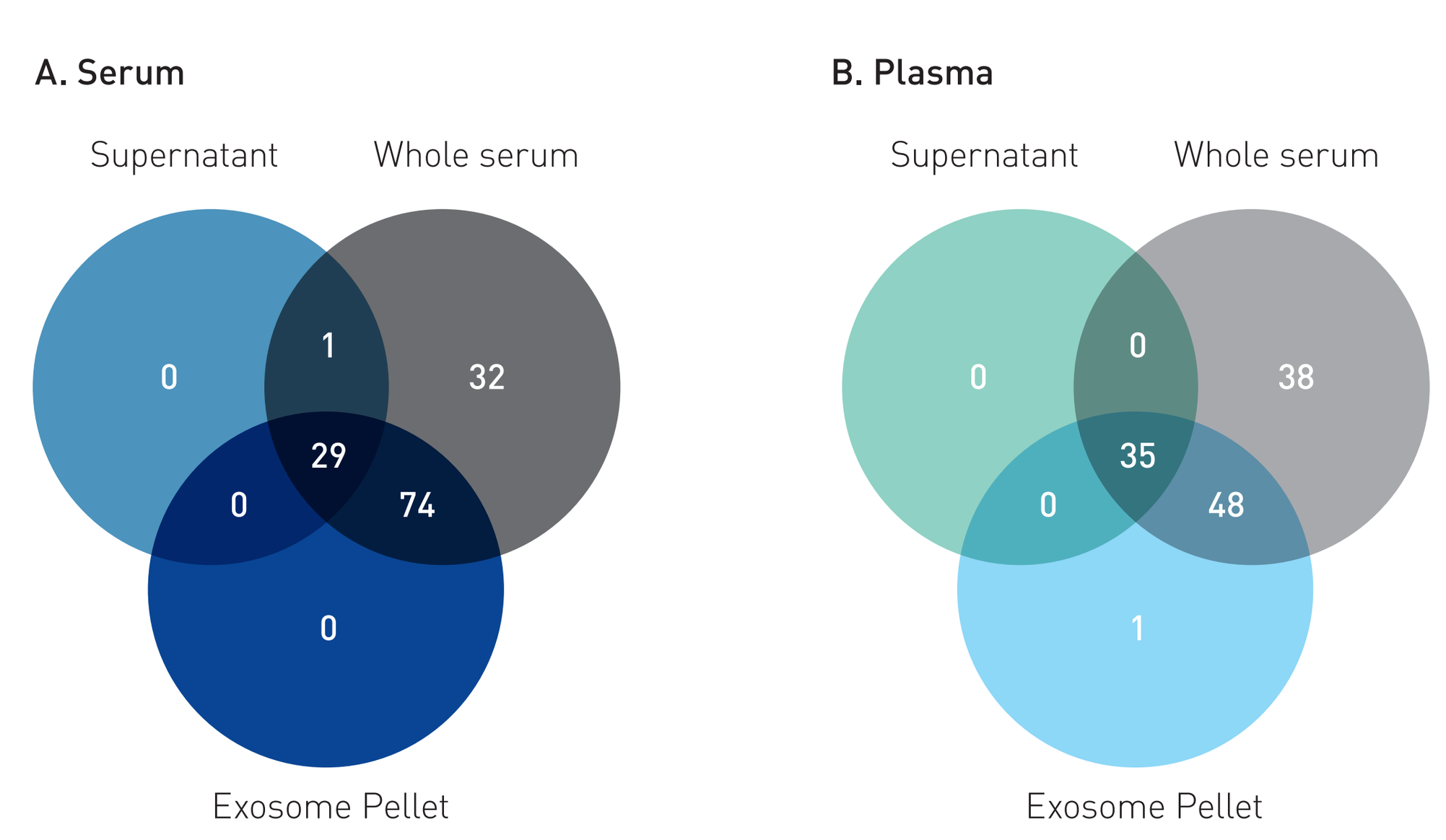

Figure 5. Venn diagrams of the microRNAs found in different sub-fractions versus the corresponding whole biofluid. All samples were isolated and profiled in duplicate. **A)** Whole serum pool and the exosome pellets. **B)** Whole plasma pool and the exosome pellets.

Increased microRNA detection and call rate in cell free urine samples by increasing sample input volumes

Cell-free microRNAs in urine samples may be an accurate biomarker for Urologic cancers and toxicology, but their low abundances makes them difficult to detect robustly. The miRCURY™ Exosome Isolation Kit greatly improves detection, by increasing the possible sample input volumes (up to 10 ml), yet avoiding the issue with culmination of PCR inhibitors enabling the use of cell free microRNA from urine as biomarkers in diagnostics.

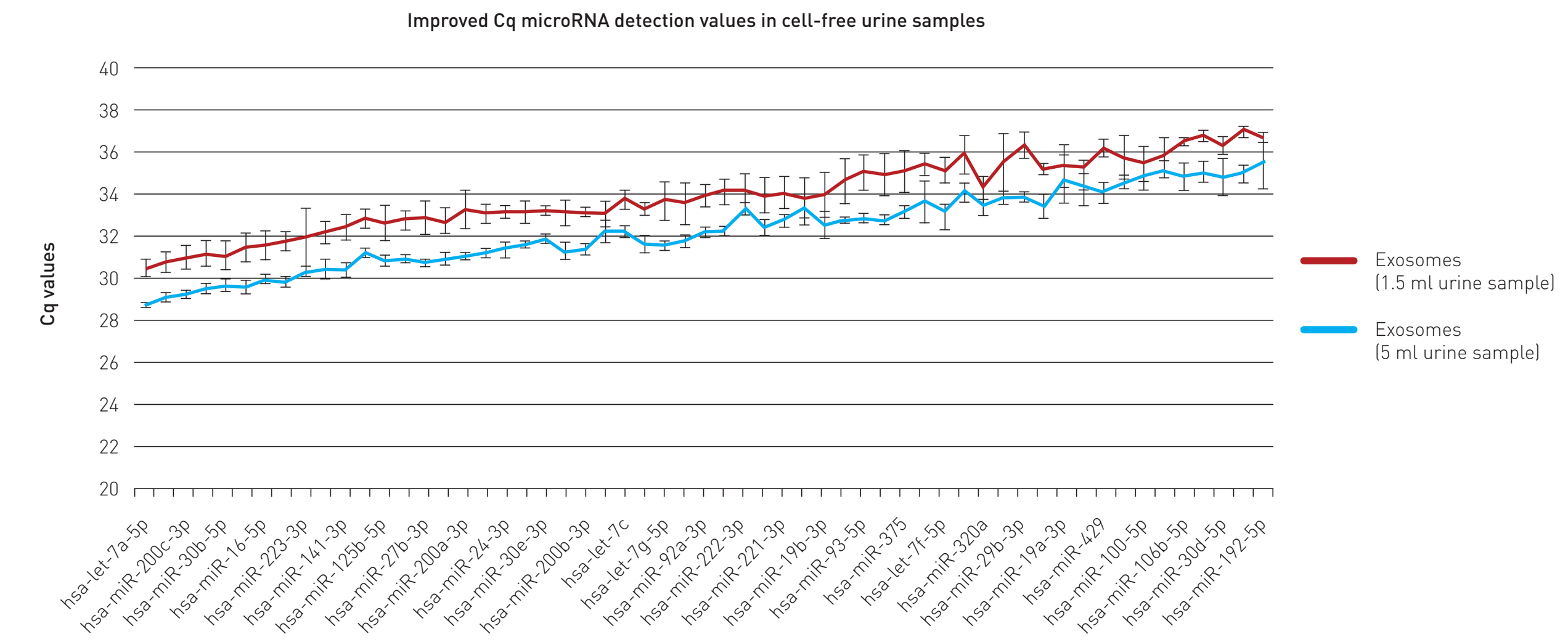

Figure 6. Average Cq values of for four biological replicates from 1.5 ml and 5 ml normal urine supernatant using miRCURY™ Exosome Isolation Kit – Cells, urine and CSF. Samples are profiled on miRCURY LNA™ Universal RT microRNA PCR, human miRNome panel , v3.

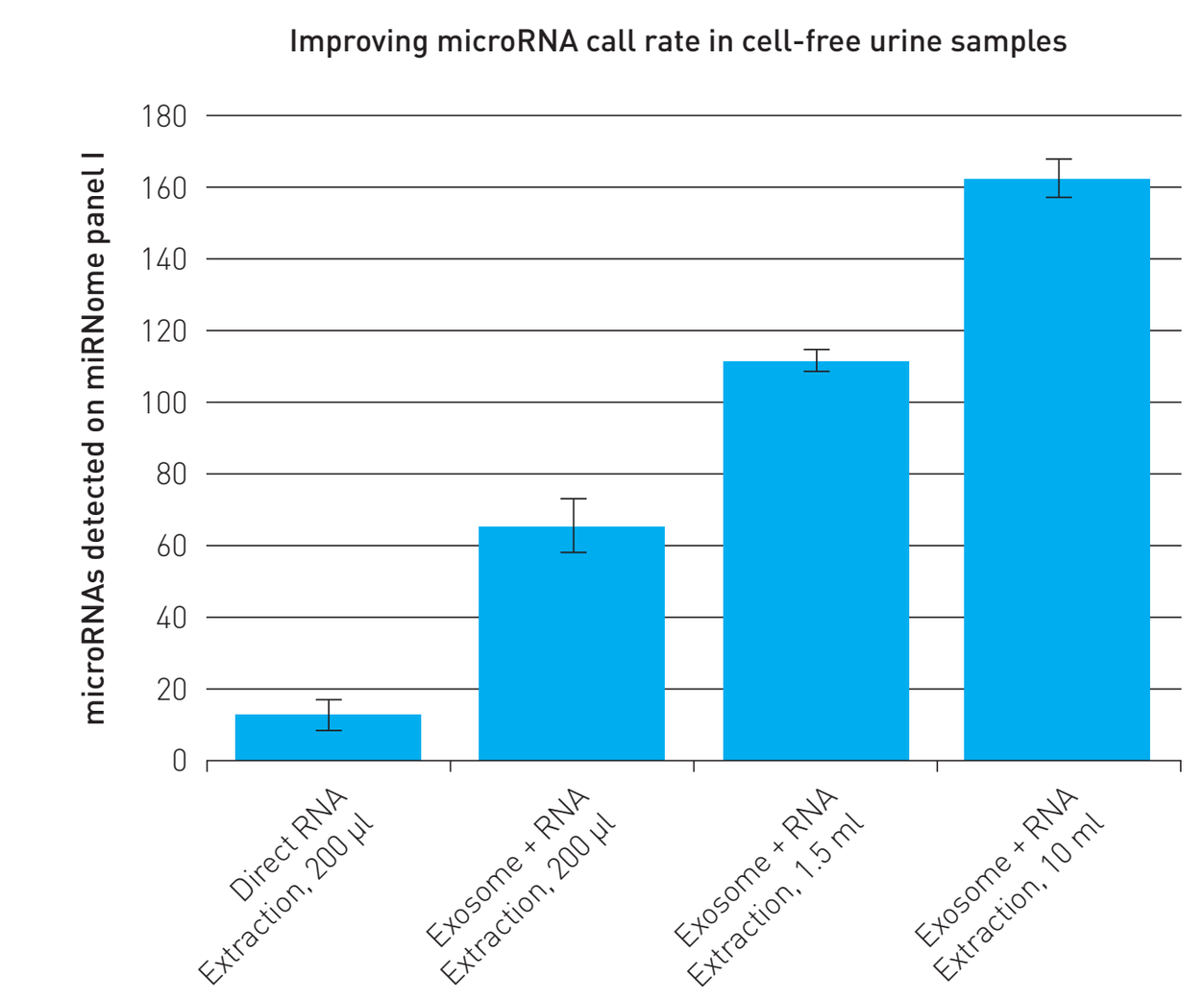

Figure 7. miRCURY™ Exosome Isolation Kit improves microRNA call rate in urine. microRNAs from cell-free urine extracted using miRCURY™ RNA Isolation Kit – Cell & Plant only, or extracted in combination with the miRCURY™ Exosome Isolation Kit– exosomes from 200 µL, 1.5 mL and 10 mL urine, respectively. Samples are profiled on miRCURY LNA™ Universal RT microRNA PCR, human miRNome panel , v3.

microRNA detection in prostate cancer

Prostate cancer is one of the most common cancers in the Western World. Most newly diagnosed cancers are classified as low to medium risk, and the vast majority of low risk cancers will never progress. However, due to lack of adequate diagnostics to identify those cancers most likely to progress, a significant proportion of early stage patients are over-treated, resulting in unnecessary side-effects (impotence, incontinence) with very limited benefit. There is thus an unmet need for better diagnostics to identify early stage cancers with poor prognosis.

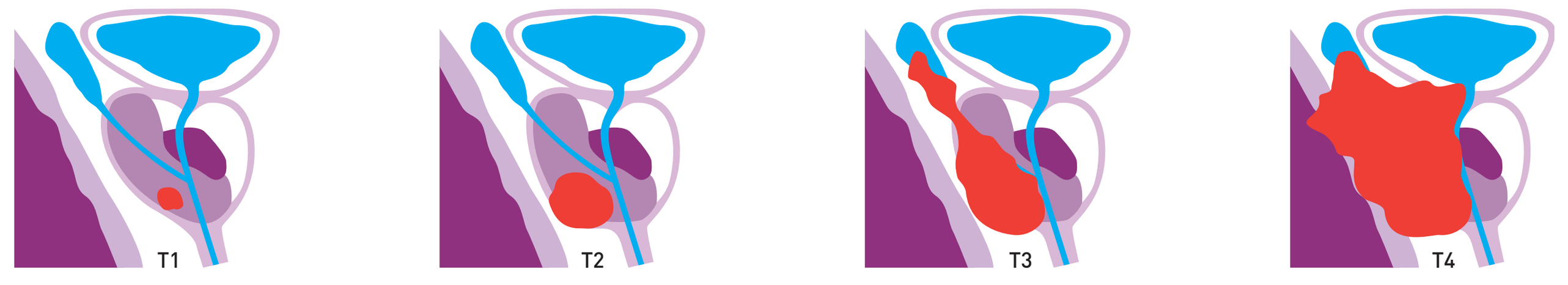

Figure 8. Prostate cancer stages.

Differential urine microRNA levels in early and late stages of prostate cancer

We obtained a set of urine samples collected after digital prostatic massage from a set of controls, early stage prostate cancer, and late stage prostate cancer patients. These were profiled for the expression of 742 human microRNAs by qPCR. The dataset was normalized using a global mean approach, employing the microRNAs expressed in all samples. Using the set of microRNAs expressed in all samples, we were able to separate late stage cancers from early stage/benign samples by PCA (Figure 6A). Statistical analysis of the dataset generated a list of microRNAs that were differentially expressed between early and late stage samples. Using a set of just two microRNAs, we could separate late stage cancers from early stage/benign samples with a ROC AUC of 0.93 (Figure 6B).

Potential applications:
- Staging of prostate cancer in urine samples - will require validation in an independent sample set
- Prognosis of early stage cancers- will require validation in an independent set of early stage cancers with known outcome

A: PCA plot of prostate cancer samples based on microRNA levels in urine

B: ROC curve for early/benign vs late stage prostate cancer based on two microRNAs, AUC=0.85

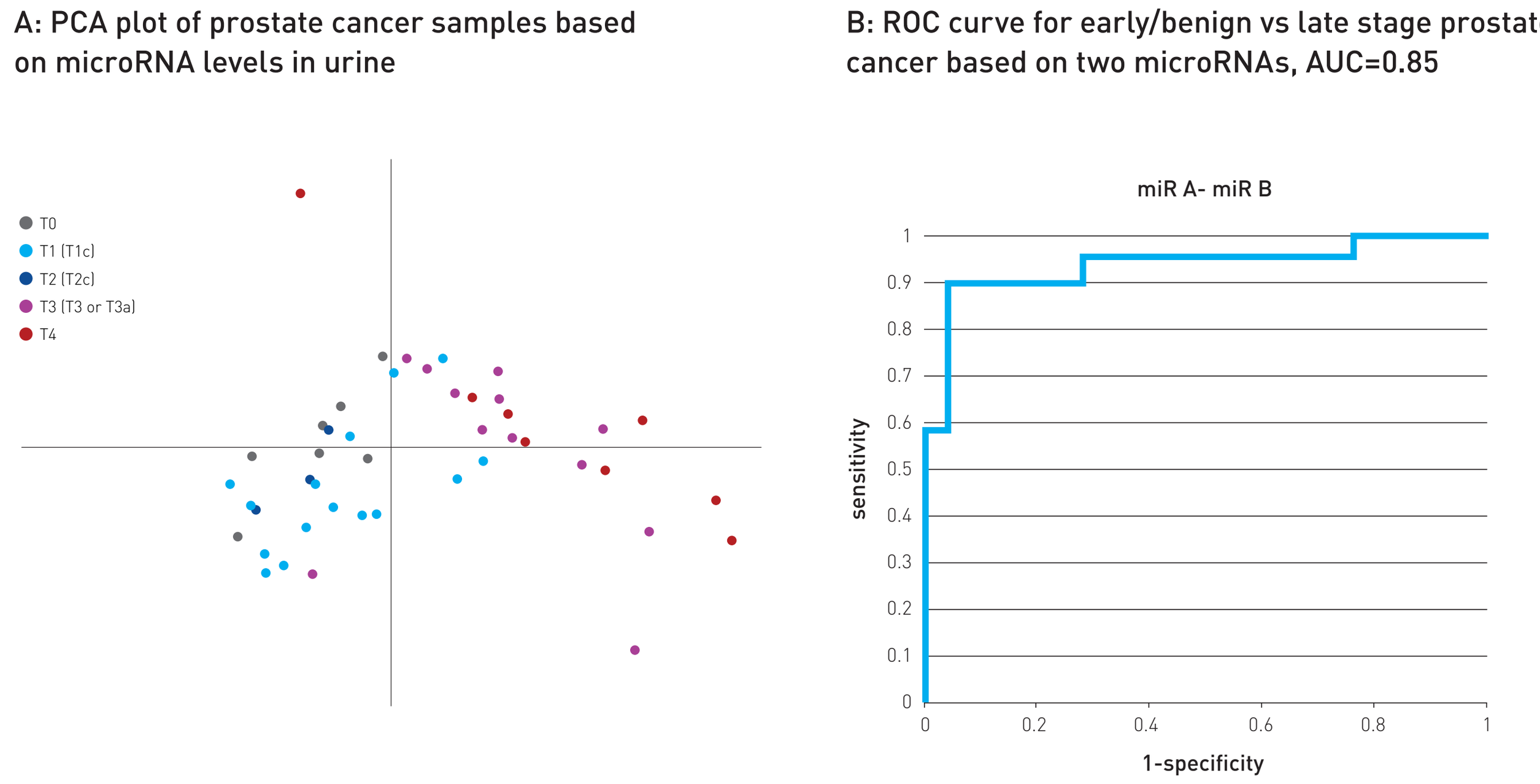

Figure 9. Prostate cancer stages can be separated by microRNA levels in urine. A) PCA plot of urine samples separated by microRNA expression. Samples are colored by stage, with late stage (T3 and T4) in shades of red, early stage (T1 and T2) in shades of blue, and benign samples in gray. **B)** ROC analysis of the urine dataset using just two microRNAs. The curve was generated from raw PCR values.

Concluding remarks

The above data was obtained from prostata massaged urine samples. This proccedure is applied to enrich the urine for prostata sheeded cells. It will be interesting to verify if the same promissing results can be found from standard urine samples applying the miRCURY™ Exosome Isolation Kit.

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