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A novel method for the isolation of extracellular vesicles and RNA from urine

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Abstract

The discovery of urinary extracellular biomarkers has been impeded by the lack of efficient methods for the isolation of extracellular vesicles (EVs: exosomes and microvesicles) and extracellular nucleic acid (RNA and DNA) from urine. Ultracentrifugation (UC), considered the gold standard for vesicle isolation from many biofluids, is efficacious but laborious, and, like most commercially available methods, is unable to isolate enough material from small volumes for protein or RNA-based biomarker discovery. We have developed a novel precipitation method for the isolation of EVs and nucleic acids from urine. The method, which is now commercially available, takes less than 30 min and does not require polyethylene glycol. Transmission electron microscopy and Nanosight particle analysis confirm that the method isolates intact vesicles with a similar size, shape, and number to UC. Immunoblot analysis of preparations made from a variety of urine samples demonstrates that the method isolates multiple vesicle protein markers more efficiently than other commercial kits, especially from more diluted samples. Bioanalyzer, quantitative reverse transcription polymerase chain reaction, and array analysis show that the method is extremely efficient at the isolation of extracellular miRNAs. The Ymir Genomics EV and Extracellular RNA Isolation Kits offer an efficient and rapid alternative to UC and other commercial kits.

Keywords

Extracellular vesicles, exosomes, urine biomarkers, microRNA

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Introduction

A goal for the diagnostics field is the isolation of disease biomarkers from easily acquired biofluids such as blood, urine, and saliva. Such biomarkers make it possible to replace invasive tissue biopsies with the so-called "liquid biopsies." For blood, there has been much success in this endeavor. Numerous studies have used circulating tumor cells, circulating DNA, extracellular vesicles (EVs), and miRNA isolated from blood as biomarkers for many diseases.[1] Progress in the discovery of urinary extracellular biomarkers was relatively slow. One reason is the lack of efficient methods for the isolation of EVs, proteins, and nucleic acids from urine. Ultracentrifugation (UC), considered the gold standard for vesicle isolation from many biofluids, is efficacious but laborious. Given that any biomarker should be readily and robustly isolatable by clinical laboratories that typically do not have an ultracentrifuge, this technique is not ideal. To fill this void, several companies have introduced EV isolation kits that do not require UC.[2–4] These kits use binding columns or precipitation reagents such as polyethylene glycol to isolate EVs from a variety of biofluids.

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Ymir Genomics has developed a novel, proprietary, and rapid precipitation method that does not require polyethylene glycol.[5] Here, we compare the method with UC and three other commercial precipitation methods for their ability to isolate EV protein markers and extracellular RNA.

Materials and methods

Urine sample isolation

Urine samples were collected from healthy volunteers (members of Ymir Genomics' staff and family). All samples were first-void and clean-catch collections. Samples remained at room temperature (RT) and were processed within 2 h, where applicable creatinine and protein levels were assessed.

EV isolation

EVs were isolated from urine samples using Ymir Genomics' EV Isolation Kit (Ymir Genomics, Cambridge, Massachusetts, USA), miRCURY Exosome Isolation Kit (Exiqon, Woburn, Massachusetts, USA), ExoQuick-TC (SBI, Mountain View, California, USA), and Total Exosome Isolation Reagent (Life Technologies, Carlsbad, California, USA) following manufacturers' directions. EVs were also isolated by UC as follows: urine sample was sequentially centrifuged for 10 min at 2000 $\times$ g and at 17,000 $\times$ g to remove cells and cellular debris, and then the resulting supernatant was centrifuged at 200,000 $\times$ g for 60 min at 24°C to sediment exosomes.

Immunoblot analysis

EV samples were combined with SDS sample buffer (Boston Bioproducts, Ashland, Massachusetts, USA), separated by denaturing electrophoresis on precasting 4–12% Novex or 4–12% Bolt gels (Life Technologies, Carlsbad, California, USA), and then transferred onto a nitrocellulose membrane (Life Technologies) by applying 20 V for 7 min at RT. The blots were blocked with 5% milk in TBST (tris buffered saline-tween 20; Boston Bioproducts, Ashland, Massachusetts, USA) for 1 h at RT, and incubated with rabbit anti-CD9 (1:1000; Cell Signaling Technologies, Danvers, Massachusetts, USA), rabbit anti-Rab5 (1:300; Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-CD63 (1:200, Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-aquaporin-2 (1:1000; Abcam, Cambridge, Massachusetts, USA), and rabbit anti-Hsp70 (1:1000; Abcam) antibodies in blocking buffer overnight at 4°C. After incubation, the blots were washed with TBST and incubated for 1 h at RT with goat anti-rabbit HRP antibody (1:2000; Cell Signaling Technologies) in blocking buffer. Western Bright ECL (BioExpress, Kaysville, Utah, USA) was used to develop the blots. The equivalent of 3 ml of urine EVs was loaded per lane. In Figure 3(b), the immunoblot band intensity for AQ2, CD9, and Rab5 is represented in a heat map (Excel), with red being the most intense and blue being the least intense.

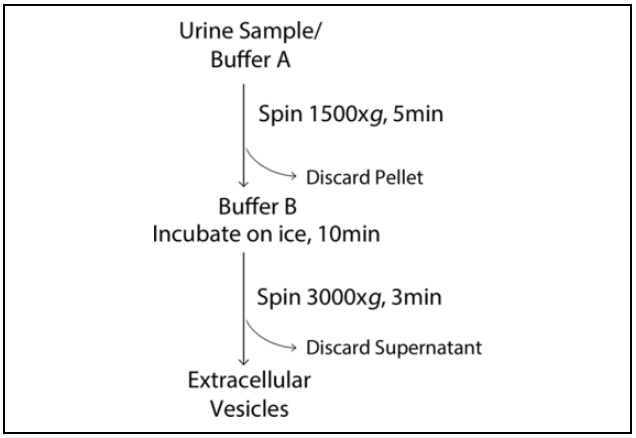

Figure 1. Outline of Ymir Genomics' EV Isolation Kit protocol. EV: extracellular vesicle.

Nanoparticle tracking analysis

EVs, diluted in PBS, were analyzed by nanoparticle tracking using the NanoSight NS300 system (Malvern Instruments, Malvern, UK) equipped with 405 nm laser. Videos were collected and analyzed using the NTA software (version 3.0 0060).

miRNA isolation and profile

A 36 ml urine sample from a single volunteer was used. The sample was split into two equal halves and EVs were isolated by the Ymir EV kit or differential UC. miRNA was isolated from urinary EVs with mirVANA Paris Kit (Life Technologies). miRNA quality and concentration were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) and Small RNA Kit (Agilent Technologies, Santa Clara, California, USA). The miRNA profile was examined with an Abcam Circulating miRNA Oncology Fixed Panel version 1.0 (Abcam, Cambridge, Massachusetts, USA) and analyzed with Abcam Analysis Workbench.

Quantitative reverse transcription polymerase chain reaction

Complementary DNA (cDNA) was synthesized from urinary exosomal RNA using the TaqMan MicroRNA RT Kit (Life Technologies) according to the manufacturer's instructions. Quantitative PCR for mature miRNAs was performed using TaqMan miRNA Gene Assays (Life Technologies) and Gene Expression Master Mix (Life Technologies). Primers for hsa-mir-10b, hsa-mir-223, and hsa-mir-200c were obtained from Life Technologies.

Transmission electron microscopy. Cryogenic electron microscopy was performed at the University of Pennsylvania, School of Medicine's Electron Microscopy Resource

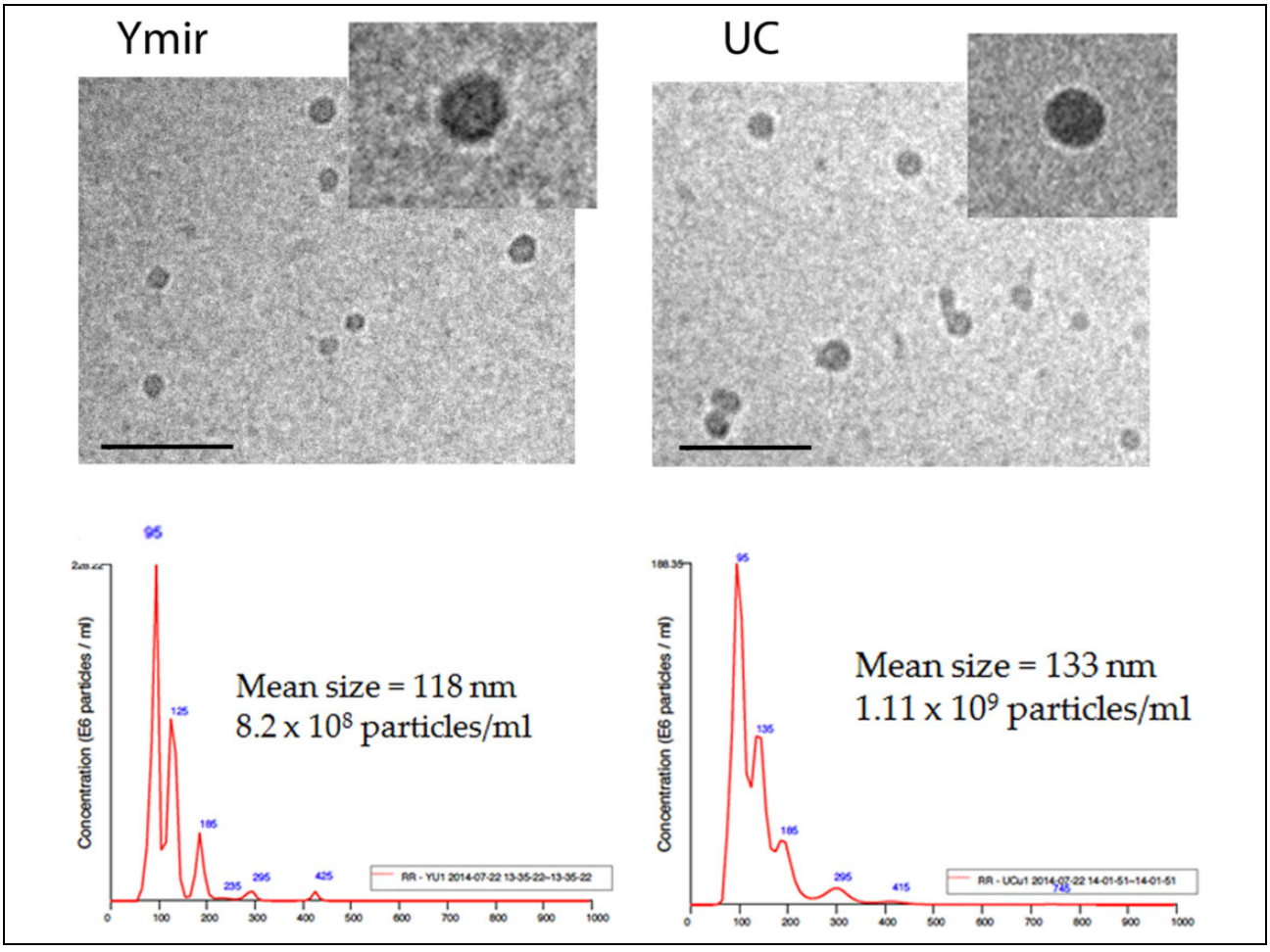

Figure 2. A comparison of urine extracellular vesicle size, number, and shape between UC and the Ymir Genomics' EV Isolation Kit. Top: Urine vesicle size and shape as measured by TEM. Top left (Ymir): Electron micrograph of urine vesicles isolated with the Ymir Genomics' Extracellular Vesicle Kit. Top right (UC): Electron micrograph of urine vesicles isolated by differential UC. Bar represents 1 μm. Bottom: Nanoparticle tracking analysis using a Nanosight device of urine vesicles isolated by the Ymir Genomics' Extracellular Vesicle Kit (Ymir) and differential UC. UC: ultracentrifugation; EV: extracellular vesicle; TEM: transmission electron microscopy.

Laboratory. Briefly, 3–5 μl of EV preparations from a single-pooled sample (three individuals contributing 1 ml each) were adsorbed to a holey carbon transmission electron microscopic (TEM) grid, blotted with filter paper, and plunged into liquid ethane cooled to −180°C. Samples were held at cryogenic temperature in a Gatan 626 (Gatan Inc, Pleasanton, California, USA) holder while observed at 200 keV on an FEI Tecnai F20 microscope (Gatan Inc, Pleasanton, California, USA). Images were digitally collected on a 2K×2K Gatan ORIUS camera at the indicated magnification for each image (Gatan Inc, Pleasanton, California, USA).

Cell survival assay. EVs were isolated from urine by UC and Ymir method. Relative concentration of EVs was determined by the method of Bradford (BioRad, Hercules, California, USA). HeLa cells in complete media (Dulbecco's modified Eagle medium (DMEM), Gibco, Carlsbad, California, USA; 5% fetal bovine serum (FBS), Gibco, Carlsbad, California, USA) were treated with 10 μg of exosomes, phosphate buffered saline (PBS) (Gibco, Carlsbad, California, USA; negative control), or pH9.0 alkaline buffer (positive control), and then incubated at 37°C with 5% carbon dioxide for 18 h. Cell survival determined by alamar blue (BioRad, Hercules, California, USA) assay was carried out following the manufacturers' instructions. Each treatment was performed in triplicate.

Results

The Ymir Genomics EV isolation method has a simple and rapid protocol (Figure 1).To determine its efficacy, a healthy volunteers sample was divided into two and processed by the Ymir Genomics protocol and by a standard UC protocol.[6] The resulting preparations were analyzed by TEM and Nanosight particle analysis.[7] The size, shape, and number of particles observed in each preparation were similar (Figure 2).

To determine whether the protocol isolates vesicle protein markers, multiple first-void urine samples of varying

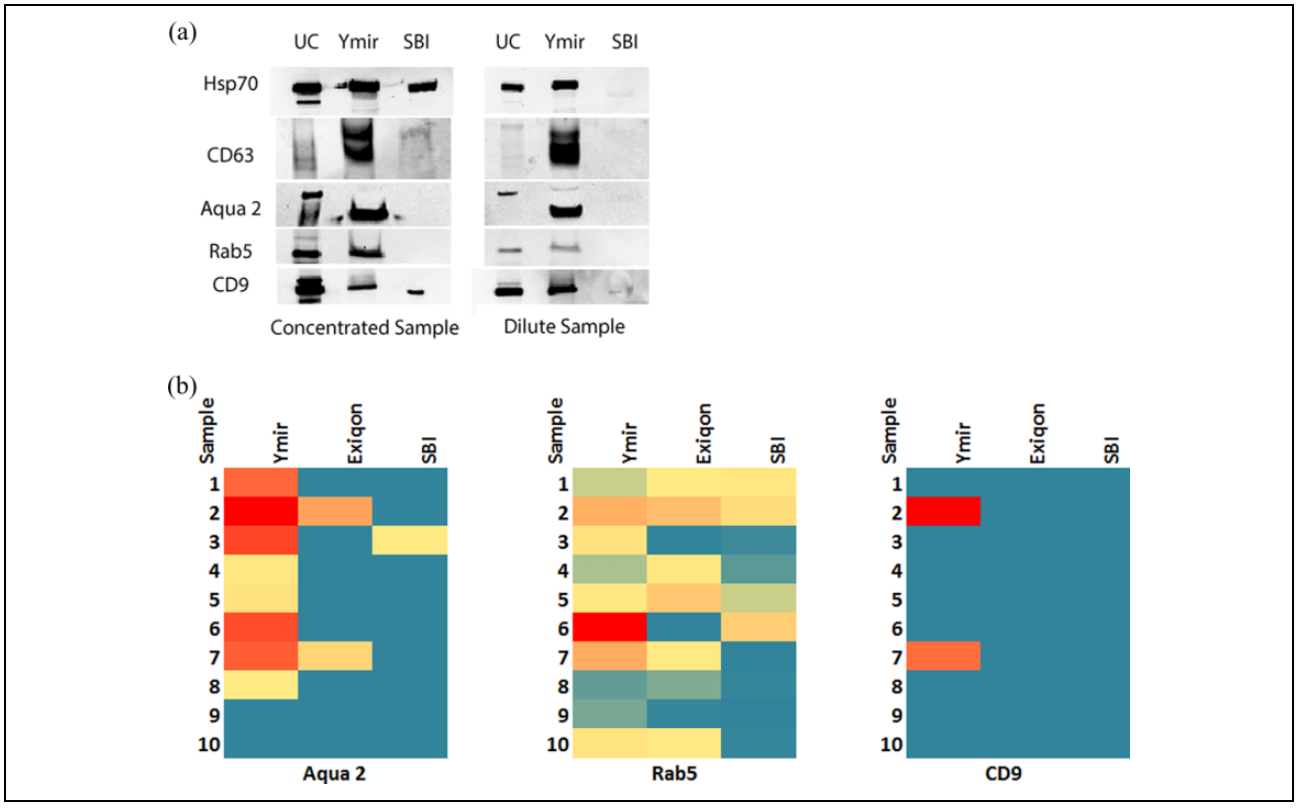

Figure 3. A comparison of yield for EV protein markers. (a) A composite immunoblot using antibodies specific for five published markers for urine EVs is shown. Two 9 ml urine samples, namely, one displaying a relatively high protein concentration (above 0.1 mg/ml as judged by Bradford assay) and another displaying a relatively low protein concentration (below 0.05 mg/ml as judged by Bradford assay) were split into three equal parts and processed via differential UC, the SBI Exosome Isolation Kit, and the Ymir Genomics' EV Isolation Kit. These data are representative of three similar experiments with different samples. (b) A heat map representing the yield of three published markers is shown for urine EVs from 10 separate urine samples processed by the Ymir Genomics' method, Exiqon's miRCURY Exosome Isolation Kit, and the SBI Exosome Isolation Kit. Red/orange denotes strong signal, yellow denotes weak signal, and blue denotes little or no signal. EV: extracellular vesicle; UC: ultracentrifugation.

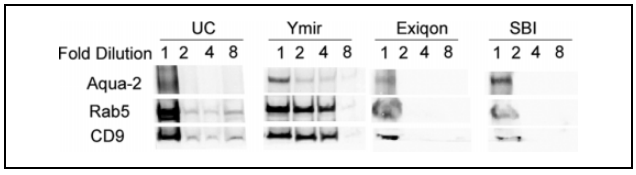

Figure 4. A comparison of EV protein markers over the course of multiple dilutions. A single relatively high concentration sample (above 0.1 mg/ml as judged by Bradford assay) was sequentially diluted with PBS. Each diluted sample was split into four equal parts and subjected to the indicated EV isolation method followed by immunoblotting with three markers for urine EVs. EV: extracellular vesicle.

protein concentrations from different donors were divided into 5 × 1 ml samples and subjected to EV isolation via standard UC and four commercial kits from Life Technologies, Exiqon, SBI, and Ymir Genomics. The preparations were subjected to immunoblot analysis using antibodies specific for the known vesicle marker CD63, Hsp70,[8] exosomal/endosomal markers such as Rab5 and CD9,[9] and a urine-specific vesicle marker Aquaporin 2.[6] As Figure 3(a) shows for the more concentrated sample, all the methods isolate detectable amounts of Hsp70, CD63, Rab5, and Aquaporin 2, although the Ymir Genomics method isolates significantly more of the Aquaporin 2 marker. However, for the more diluted sample, only the Ymir Genomics method isolates all three of these markers. A repeat of this experiment with 10 different samples with a range of protein concentrations yielded similar results (Figure 3(b); Supplemental Figure S1), suggesting the Ymir Genomics method is the best method for a variety of samples. To probe the concentration lower limit of this method, we took a separate sample and diluted it 2×, 4×, and 8× with PBS and then applied various vesicle isolation methods followed by immunoblot for Aq2, CD9, and Rab5. Once again, the Ymir Genomics' kit is the only method that isolates all three of these vesicle markers from the diluted sample (Figure 4). Strikingly, this method efficiently isolates all three markers even from the same sample diluted 4× while neither of the other commercial kits function at the 2× dilution. Even UC does not efficiently isolate these markers at the 4× dilution.

Ymir Genomics also makes a urine extracellular RNA extraction kit that is based on the same precipitation

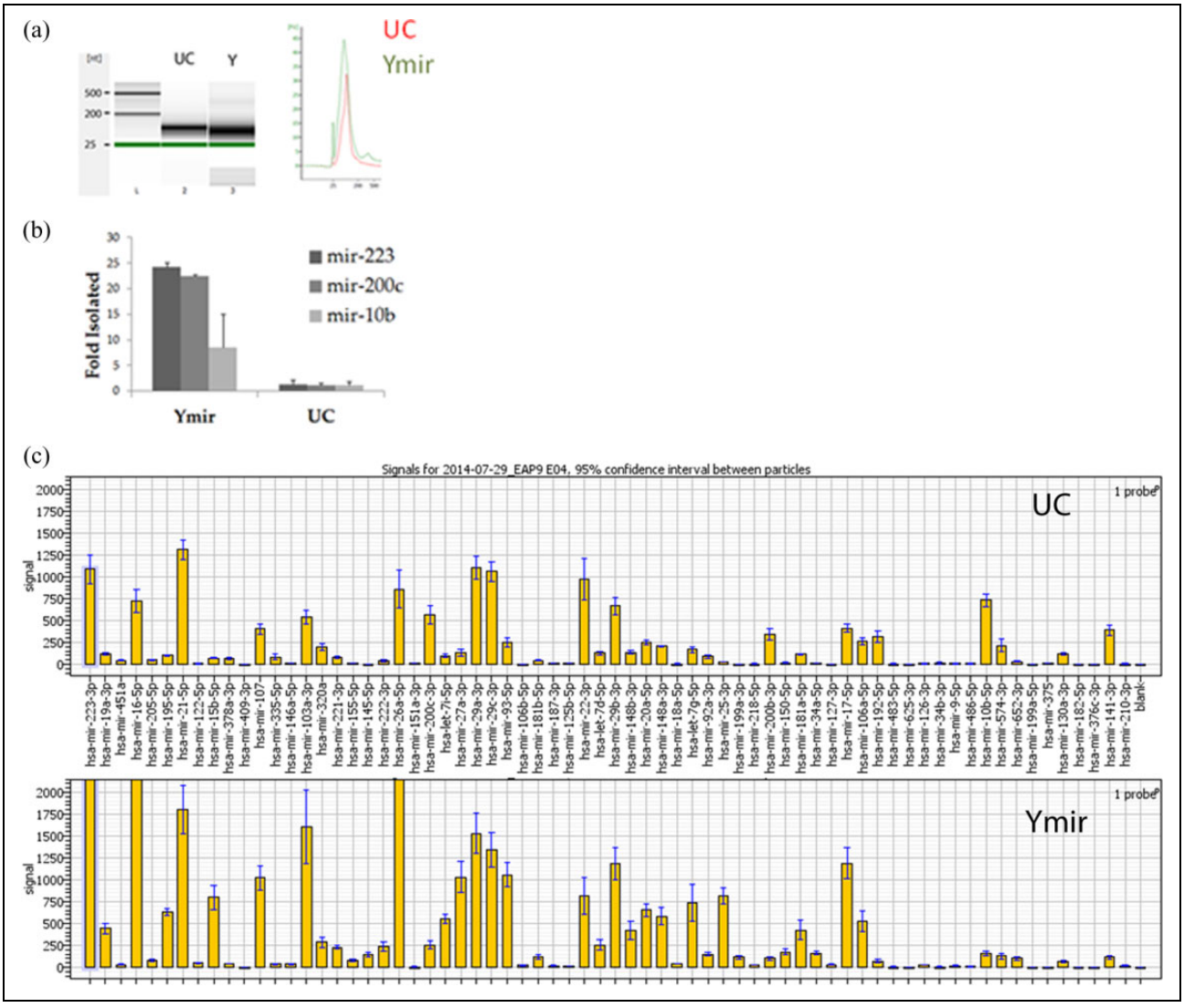

Figure 5. A comparison of RNA isolated from urine EV preparations. A single urine sample was divided into two equal portions and processed by differential UC and the Ymir EV Isolation Kit (Y or Ymir). (a**)** Bioanalyzer analysis of both preparations. (b) RT-PCR analysis of miRNAs such as 223, 200c, and 10b. (c) Firefly miRNA array analysis of miRNAs found in urine EV preparation using differential UC and Ymir EV Isolation Kit (Ymir). Un-normalized arbitrary units describe relative amounts of each of the 69 miRNAs. Note: The values for mir-16 (AU = 4621.9 ± 401.9), mir-26a (2887.0 ± 388.8), and mir-223 (7769.6 ± 633.0) were cutoff for the Ymir Genomics' preparation for aesthetic reasons. EV: extracellular vesicle; UC: ultracentrifugation; RT-PCR: reverse transcription polymerase chain reaction.

reagent. Bioanalyzer analysis of RNA produced from a single sample by this protocol or UC followed by RNeasy RNA purification shows a similar yield and a similar size profile (Figure 5(a)). Interestingly, when quantitative reverse transcription PCR analysis was used to look for three miRNAs known to be in the extracellular fraction of urine, then the yield was significantly higher using the Ymir Genomics' kit compared to UC (Figure 5(b)). To expand on this result, similar preparations were applied to the Abcam Oncology microRNA array that simultaneously assesses the relative amounts of 69 microRNAs implicated cancer.[10] Figure 5(c) shows the raw data from that array. In general, both methods agree as to which miRNAs are present in the sample. However, the Ymir Genomics preparation consistently yields a stronger signal for a majority of the miRNAs detected (Figure 5(c)). In all, 27 of the detected miRNAs show a significantly stronger signal from the Ymir Genomics preparation compared to 3 having a stronger signal from UC and 5 being within error bars. These results strongly suggest that the Ymir Genomics preparation is more efficient at isolating extracellular miRNAs compared to UC. Given that the Ymir Genomics' method uses a novel small molecule to induce precipitation, we wanted to make sure that the Ymir Genomics' preparations would not be toxic to cells. To test this, we isolated EVs from a single-pooled urine sample using the Ymir method and differential UC and then added 10 µg of each preparation to HeLa cells in standard media for 18 h

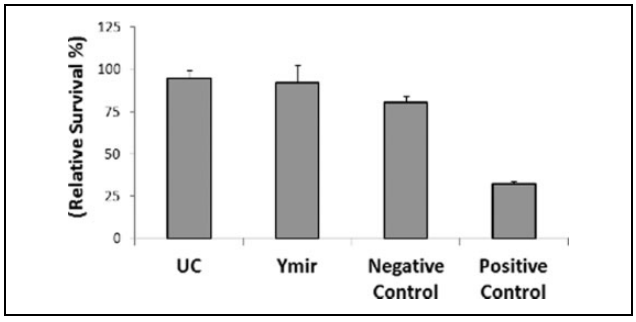

Figure 6. Ymir Genomics' EV preparations are not toxic to cells. A single-pooled urine sample was divided into two equal portions and processed by differential UC and the Ymir EV Isolation Kit (Ymir). A Bradford protein assay determined yield. Ten micrograms of each preparation were applied to HeLa cells. After 18 h, the cell survival was determined by alamar blue assay. EV: extracellular vesicle; UC: ultracentrifugation.

followed by bromodeoxyuridine viability assay. Eighteen hours is more than a typical time frame and 10 μg is a typical amount for EV delivery studies.[11] Figure 6 shows that the EV preparation from Ymir Genomics does not adversely affect these cells compared to UC and the negative control PBS while the positive control basic wash does.

Discussion

Despite its easy, noninvasive collection, relative sterility, and richness in a variety of biomolecules, currently, only about 3% of discovery research for extracellular biofluid biomarkers is performed in urine.[12] One reason for this is that the concentration of biomarkers is significantly less in urine when compared to blood. For instance, EVs are approximately 1000× more concentrated in blood than in urine.[13] This makes the isolation of EVs more challenging, particularly when using precipitation methods. Urine is also a more variable medium than blood in terms of pH and osmolality. Unlike blood which is almost always pH 7, urine varies daily from pH 5 to pH 8. Depending on what an individual eats and drinks and alcohol or drugs taken, the osmolality, protein, and creatinine levels can also vary widely. These challenges lead to significant sample-to-sample variability in the efficiency of extracellular biomolecule isolation. Unlike other commercial kits, the Ymir Genomics' Urine EV and RNA Isolation Kits use a sample treatment buffer to ameliorate initial sample pH differences. Furthermore, the method uses a novel precipitation reagent that is less dependent on concentration (as judged by protein; Figure 5). Interestingly, consistent with a recent report[14] that looked at density, we see that UC is not as effective for less concentrated samples. Only the Ymir Genomics method worked on diluted samples. The method is the most rapid of the methods attempted (30 min for the EV preparation) and does not require special equipment. Furthermore, EV preparations from the method are not toxic to cells (Figure 6). Thus, the method should make biomarker isolation from urine more accessible to research and clinical laboratories.

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Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: The authors are all employees of Ymir Genomics LLC and benefit from the sale of the Ymir Genomics Urine EV Isolation Kit.

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Supplemental material

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