

## Supplementary Materials: Comparative miRNA Analysis of Urine Extracellular Vesicles Isolated through Five Different Methods

Felix Royo, Izzuddin Diwan, Michael R Tackett, Patricia Zuñiga, Pilar Sanchez-Mosquera, Ana Loizaga-Iriarte, Aitziber Ugalde-Olano, Isabel Lacasa, Amparo Perez, Miguel Unda, Arkaitz Carracedo and Juan M Falcon-Perez

**Table S1.** Supplemental Table S1. miRNAs assayed by Multiplex miRNA assay of Firefly, and signal intensity for each isolation method (Mean +/- SEM). Last column indicates detection limit of detection for each miRNA. CEN: ultracentrifugation; EXQ: Exoquick-TC; INV: Total Exosome Isolation Solution; LEC: lectin-based purification; NOR: Urine Exosome RNA Isolation Kit.

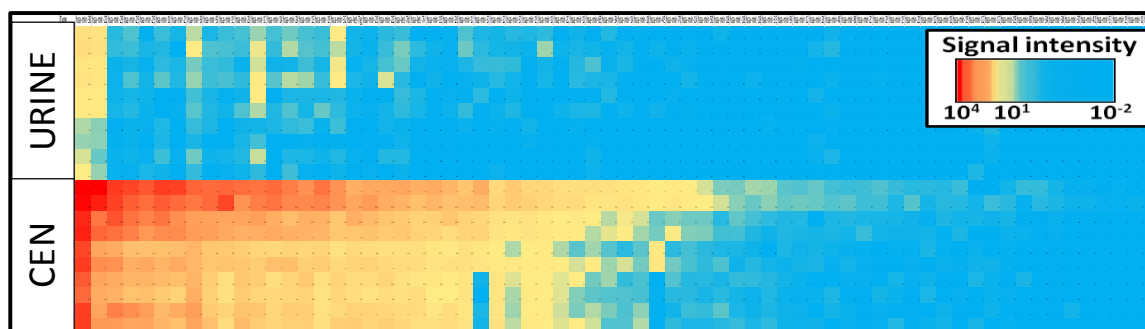
TYPE	CEN	EXQ	INV	LEC	NOR	AVERAGE	LIMIT
hsa-mir-22-3p	3070.82 ± 158.93	1818.82 ± 156.96	4467.62 ± 199.4	515.41 ± 223.51	3943.43 ± 215.65	2763.22	2.48
hsa-mir-30c-5p	1768.45 ± 26.28	593.3 ± 26.01	1567.35 ± 32.12	523.77 ± 39.47	3207.26 ± 38.74	1532.03	0.61
hsa-mir-16-5p	1486.8 ± 42.34	1051.28 ± 51.95	2265.55 ± 57.34	210.53 ± 57.21	2585.24 ± 56.43	1519.88	9.07
hsa-mir-29c-3p	1655.62 ± 294.34	892.9 ± 292.4	2322.51 ± 300.42	355.7 ± 374.25	1870.85 ± 439.05	1419.51	0.87
hsa-mir-29a-3p	1374.69 ± 106.3	786.96 ± 106.29	2059.19 ± 168.19	245.08 ± 241.5	1827.04 ± 234.07	1258.59	0.8
hsa-mir-21-5p	1137.48 ± 311.15	896.77 ± 310.67	2255.3 ± 310.83	178.91 ± 311.1	1652.04 ± 349.34	1224.10	0.51
hsa-mir-320a	1098.31 ± 5.54	629.7 ± 5.53	1662.13 ± 7.4	230.57 ± 7.76	2044.63 ± 7.66	1133.07	0.67
hsa-mir-30a-5p	1019.32 ± 50.34	693.6 ± 50.38	1799.21 ± 50.27	244.18 ± 51.71	1746.44 ± 51.96	1100.55	0.77
hsa-mir-194-5p	937.8 ± 3.26	611.03 ± 3.27	1551.21 ± 3	21.82 ± 4.84	2085.16 ± 4.75	1041.40	0.5
hsa-mir-24-3p	1043.72 ± 14.88	596.78 ± 14.74	1446.12 ± 14.92	131.22 ± 14.81	1888.66 ± 16.75	1021.30	0.7
hsa-mir-29b-3p	1140.33 ± 239.43	548.05 ± 268.01	1377.94 ± 300.25	222.33 ± 326.85	1421.23 ± 343.78	941.97	0.48
hsa-mir-148a-3p	882.22 ± 44.32	685.86 ± 47.45	1573.63 ± 53.18	153.68 ± 61.77	1412.62 ± 68.81	941.60	0.55
hsa-mir-92a-3p	935.63 ± 394.22	669.9 ± 512	1972.25 ± 548.72	287.45 ± 625.47	517.12 ± 663.29	876.47	0.93
hsa-mir-130a-3p	858.18 ± 136.8	464.46 ± 136.32	1332.61 ± 136	184.57 ± 139.11	1335.17 ± 143.91	835.00	0.54
hsa-mir-192-5p	823.2 ± 27.03	463.05 ± 28.24	1199.48 ± 29.29	22.14 ± 28.64	1462.61 ± 27.3	794.10	0.45
hsa-mir-200a-3p	842.04 ± 216.67	461.93 ± 214.09	1213 ± 217.96	207.54 ± 239.75	1001.9 ± 259.84	745.28	0.71
hsa-mir-451a	585.15 ± 5.16	346.81 ± 5.12	1040.78 ± 5.1	18.21 ± 5.1	1144.71 ± 5.15	627.13	3.59
hsa-mir-221-3p	646.87 ± 254.66	331.6 ± 252.42	1168.93 ± 275.69	174.47 ± 306.5	702.78 ± 351.17	604.93	0.74
hsa-mir-17-5p	701.33 ± 116.93	329.25 ± 114.64	802.94 ± 125.29	189.49 ± 125.29	943.38 ± 147.03	593.28	14.83

hsa-mir-223-3p	491.82 ± 3.75	461.65 ± 3.72	766.15 ± 3.7	71.91 ± 3.68	1156.41 ± 3.67	589.59	4.21
hsa-mir-181a-5p	500.89 ± 3.18	258.43 ± 3.15	530.9 ± 3.12	73.23 ± 3.07	939.18 ± 4.31	460.53	0.8
hsa-mir-15a-5p	422.51 ± 36.88	244.35 ± 36.67	773.23 ± 41.82	45.83 ± 45.19	725.56 ± 46.37	442.29	0.55
hsa-mir-378a-3p	476.01 ± 80.34	289.39 ± 80.03	618.79 ± 79.7	26.96 ± 81.35	779.27 ± 82.07	438.08	0.48
hsa-let-7i-5p	445.56 ± 291.65	352.12 ± 292.48	779.72 ± 308.71	76.26 ± 313.46	526.95 ± 321.03	436.12	3.78
hsa-mir-200b-3p	435.09 ± 160.78	265.37 ± 159.48	637.7 ± 165.65	105.52 ± 166.51	669.94 ± 185.97	422.73	0.47
hsa-mir-19a-3p	268.54 ± 10.87	136.1 ± 10.86	409.82 ± 11.12	40.98 ± 11.63	501.02 ± 12.59	271.29	0.55
hsa-mir-375	154.44 ± 3.04	304.91 ± 2.89	459.13 ± 2.56	37.4 ± 2.53	334.31 ± 2.94	258.04	0.53
hsa-let-7g-5p	202.88 ± 268.06	136.15 ± 271.64	304.78 ± 271.06	95.73 ± 270.09	331.28 ± 268.54	214.16	0.75
hsa-mir-222-3p	213.44 ± 215.07	113.55 ± 211.03	324.65 ± 228.01	50.4 ± 294.26	247.69 ± 339.14	189.94	0.8
hsa-let-7d-5p	156.64 ± 4.87	79.11 ± 4.82	173.07 ± 4.83	61.09 ± 4.88	305.14 ± 4.82	155.01	0.6
hsa-mir-185-5p	162.83 ± 25.11	79.95 ± 29.6	207.83 ± 29.97	23.33 ± 30.59	290.11 ± 31.38	152.81	0.48
hsa-mir-34a-5p	138.73 ± 180.5	121.45 ± 178.06	279.19 ± 179.43	15.61 ± 189.58	181.21 ± 236.86	147.24	0.62
hsa-mir-146a-5p	114.99 ± 1.28	90.32 ± 1.28	208.5 ± 1.33	5.39 ± 1.33	295.21 ± 1.39	142.88	0.55
hsa-mir-26b-5p	132.52 ± 4.4	61 ± 4.35	173.8 ± 4.4	39.88 ± 4.64	254.22 ± 4.52	132.28	0.49
hsa-mir-187-3p	141.93 ± 274.84	54.98 ± 271.41	183.78 ± 311.43	53.44 ± 356.5	142.61 ± 413.21	115.35	1.25
hsa-mir-652-3p	100.17 ± 129.04	52.62 ± 127.3	160.76 ± 138.84	24.49 ± 139.11	146.2 ± 137.75	96.85	0.52
hsa-mir-181d-5p	72.57 ± 5.53	46.12 ± 5.51	120.99 ± 5.52	8.38 ± 5.53	143.73 ± 5.75	78.36	0.62
hsa-mir-708-5p	59.88 ± 6.66	33.13 ± 6.69	88.69 ± 6.71	2.87 ± 6.75	133.34 ± 6.78	63.58	0.66
hsa-mir-486-5p	69.19 ± 419.03	50.65 ± 418.55	134.69 ± 440.86	6.3 ± 471.08	51.35 ± 504.06	62.43	3.59
hsa-mir-146b-5p	30.86 ± 1.48	12.96 ± 1.46	48.91 ± 1.38	2.11 ± 1.37	97.28 ± 1.35	38.42	0.56
hsa-mir-503-5p	26.09 ± 0.43	19.98 ± 0.43	46.14 ± 0.69	3.31 ± 0.71	61.38 ± 0.9	31.38	0.43
hsa-mir-33a-5p	16.8 ± 68.03	6.02 ± 67.28	16.01 ± 67.56	3.71 ± 67.22	29.16 ± 71.33	14.34	0.62
hsa-mir-127-3p	18.23 ± 223.52	6.56 ± 232.17	20.4 ± 249.21	1.24 ± 269.65	20.84 ± 269.53	13.45	0.46
hsa-mir-214-3p	26.74 ± 281.19	4.52 ± 281.19	8.78 ± 286.08	0.57 ± 330.28	17.63 ± 326.45	11.65	0.77
hsa-mir-34c-5p	6.47 ± 0.06	11.47 ± 0.06	13.76 ± 0.1	0.34 ± 0.11	25.85 ± 0.11	11.58	0.6
hsa-mir-18a-5p	10.96 ± 36.06	7.54 ± 36.49	16.22 ± 41.71	3.9 ± 42.49	18.51 ± 41.61	11.43	3.07
hsa-mir-125b-5p	10.17 ± 3.14	7.57 ± 3.22	14.92 ± 2.82	1.24 ± 2.69	21.48 ± 2.72	11.08	0.89
hsa-mir-183-5p	10.37 ± 23.54	7.48 ± 23.32	12.1 ± 24.49	3.22 ± 23.96	18.77 ± 25.36	10.39	1.13
hsa-mir-96-5p	10.54 ± 173.75	9.67 ± 172.29	13.68 ± 177.22	1.62 ± 180.75	14.23 ± 185.21	9.95	0.5

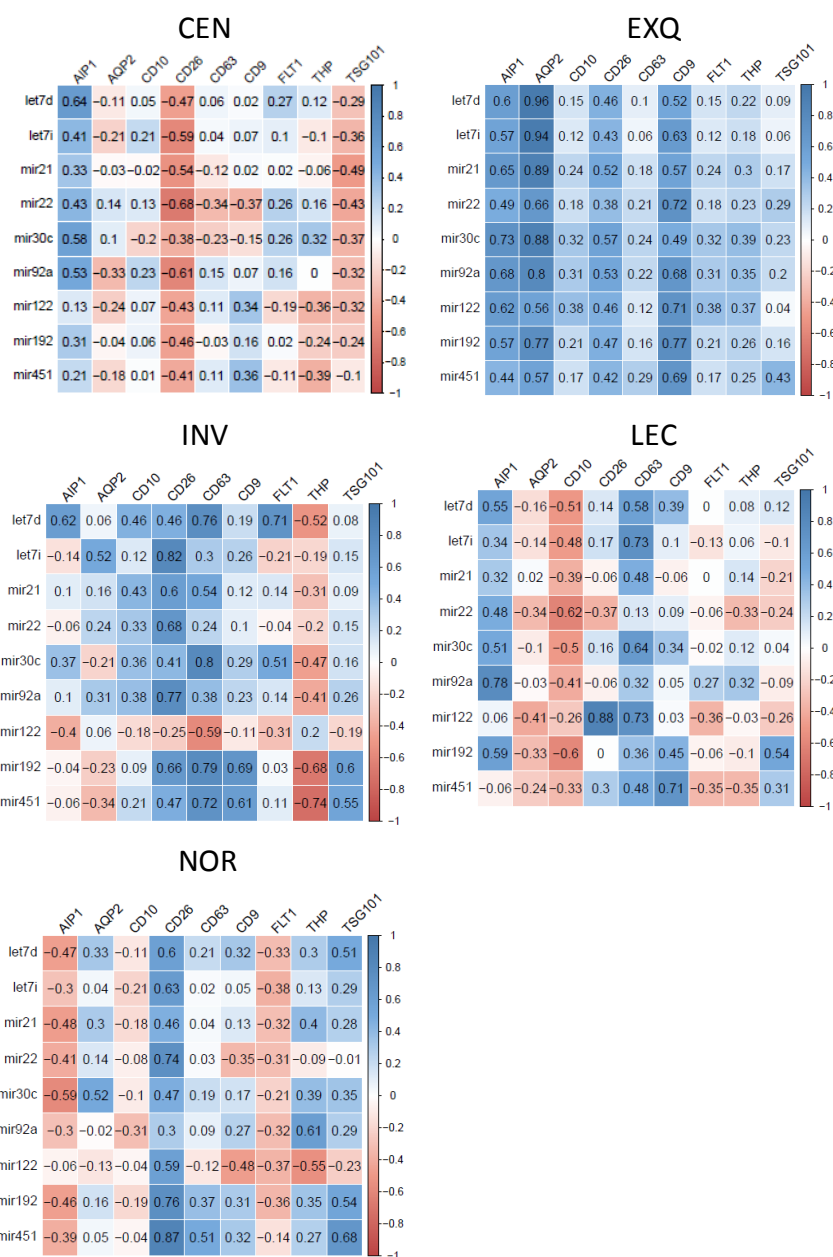
hsa-mir-21-3p	11.5 ± 110.01	4.42 ± 109.93	10.09 ± 112.13	0.65 ± 125.48	17.27 ± 166.82	8.78	0.67
hsa-mir-122-5p	8.03 ± 215.18	10.04 ± 212.38	8.6 ± 253.73	5.7 ± 290.61	9.45 ± 282.3	8.36	2.57
hsa-mir-124-3p	3.23 ± 0.23	9.48 ± 0.23	12.86 ± 0.24	3.88 ± 0.25	10.84 ± 0.25	8.06	12.24
hsa-mir-877-5p	4.22 ± 0.05	5.96 ± 0.05	11.03 ± 0.06	1.47 ± 0.06	17.47 ± 0.08	8.03	1.21
hsa-mir-206	4.57 ± 0.08	8.26 ± 0.08	8.59 ± 0.15	0.61 ± 0.54	16.27 ± 0.64	7.66	7.01
hsa-mir-885-5p	5.96 ± 109.3	3.34 ± 110.35	10.59 ± 116.36	1.63 ± 115.64	8.95 ± 114.39	6.09	0.44
hsa-mir-193a-3p	5.65 ± 2.32	3.94 ± 2.41	6.79 ± 2.35	0.45 ± 2.64	13.24 ± 2.77	6.01	0.52
hsa-mir-296-5p	4.8 ± 1.81	3.91 ± 1.8	9.31 ± 2.14	1.28 ± 2.43	9.88 ± 2.71	5.84	3.88
hsa-mir-155-5p	4.26 ± 1.8	3.97 ± 1.76	3.54 ± 1.79	0.98 ± 1.86	9.92 ± 2.58	4.53	0.67
hsa-mir-101-3p	3.88 ± 160.58	1.68 ± 158.42	10.5 ± 204.41	0.33 ± 235.57	4.96 ± 259.02	4.27	0.42
hsa-mir-34b-5p	3.47 ± 1.3	4.08 ± 1.67	5.41 ± 1.77	0.58 ± 2.42	7.8 ± 2.4	4.27	0.39
hsa-mir-199a-3p	3.69 ± 31.61	1.97 ± 31.9	6.38 ± 32.15	0.76 ± 33.35	6.45 ± 37.6	3.85	1.07
hsa-mir-182-5p	3.27 ± 0.33	1.99 ± 0.43	2.43 ± 0.61	2.25 ± 0.69	8.86 ± 0.77	3.76	0.47
hsa-mir-410-3p	1.71 ± 1.04	0.8 ± 1.04	2.93 ± 1.13	0.14 ± 1.42	4.15 ± 2.01	1.95	0.45
hsa-mir-370-3p	1.07 ± 2.95	2.31 ± 2.93	1.61 ± 2.93	0.14 ± 2.91	2.54 ± 2.91	1.53	0.98
hsa-mir-571	0.69 ± 9.28	0.51 ± 9.24	0.76 ± 9.25	0.64 ± 9.24	1.33 ± 9.31	0.79	0.34
hsa-mir-199a-5p	0.53 ± 293.11	0.31 ± 298.86	1.39 ± 311.93	0.21 ± 314.11	0.63 ± 350.34	0.61	0.54
hsa-mir-337-3p	0.28 ± 2.58	0.26 ± 2.58	0.26 ± 2.58	0.21 ± 2.73	0.53 ± 3.32	0.31	0.59
hsa-mir-183-3p	0.28 ± 3.96	0.16 ± 5.79	0.31 ± 6.09	0.13 ± 5.96	0.52 ± 5.95	0.28	0.8

**Table S2.** Association between the selected miRNAs and cancer were found using the miR2 disease database (<http://www.mir2disease.org/>), and some selected references retrieved by the database are displayed below.

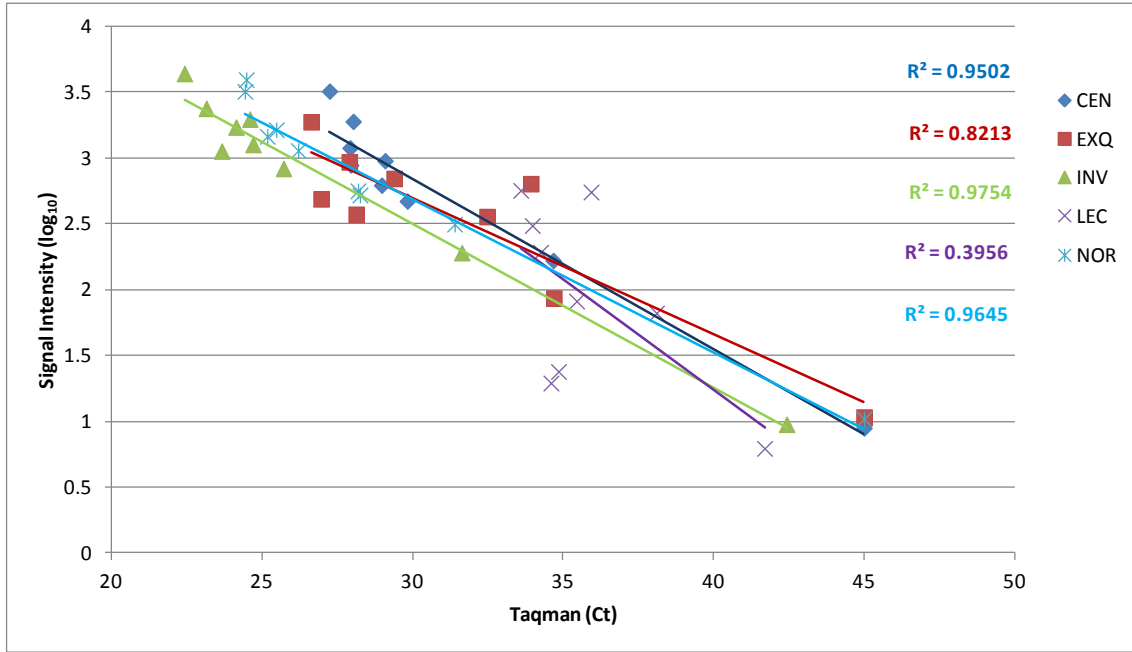
miRNA	Cancer type	Reference
hsa-let-7d-5p	Prostate	Porkka, K.P.; Pfeiffer, M.J.; Waltering, K.K.; Vessella, R.L.; Tammela, T.L.; Visakorpi, T. MicroRNA expression profiling in prostate cancer. <i>Cancer Res.</i> <b>2007</b> , <i>67</i> , 6130–6135. PMID:17616669.
hsa-let-7i	Ovarian	Yang, N.; Kaur, S.; Volinia, S.; Greshock, J.; Lassus, H.; Hasegawa, K.; Liang, S.; Leminen, A.; Deng, S.; Smith, L.; et al. MicroRNA microarray identifies Let-7i as a novel biomarker and therapeutic target in human epithelial ovarian cancer. <i>Cancer Res.</i> <b>2008</b> , <i>68</i> , 10307–10314. PMID:19074899.
hsa-miR-21	Prostate	Prueitt, R.L.; Yi, M.; Hudson, R.S.; Wallace, T.A.; Howe, T.M.; Yfantis, H.G.; Lee, D.H.; Stephens, R.M.; Liu, C.G.; Calin, G.A.; et al. Expression of microRNAs and protein-coding genes associated with perineural invasion in prostate cancer. <i>Prostate</i> <b>2008</b> , <i>68</i> , 1152–1164. PMID:18459106.
hsa-miR-21	Bladder	Dyrskjt, L.; Ostefeld, M.S.; Bramsen, J.B. Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death in vitro. <i>Cancer Res.</i> <b>2009</b> , <i>69</i> , 4851–4160. PMID:19487295.
hsa-miR-22	Prostate	Porkka, K.P.; Pfeiffer, M.J.; Waltering, K.K.; Vessella, R.L.; Tammela, T.L.; Visakorpi, T. MicroRNA expression profiling in prostate cancer. <i>Cancer Res.</i> <b>2007</b> , <i>67</i> , 6130–6135. PMID:17616669.
hsa-miR-30c	Prostate	Volinia, S.; Calin, G.A.; Liu, C.G.; Ambs, S.; Cimmino, A.; Petrocca, F.; Visone, R.; Iorio, M.; Roldo, C.; Ferracin, M.; et al. A microRNA expression signature of human solid tumors defines cancer gene targets. <i>Proc. Natl. Acad. Sci. USA</i> <b>2006</b> , <i>103</i> , 2257–2261. Epub 2006 Feb 3. PMID:16461460.
hsa-miR-30c	Bladder	Wang, G.; Zhang, H.; He, H.; Tong, W.; Wang, B.; Liao, G.; Chen, Z.; Du, C. Up-regulation of microRNA in bladder tumor tissue is not common. <i>Int. Urol. Nephrol.</i> <b>2009</b> . [Epub ahead of print]. PMID:19475496.
hsa-miR-92a	Hepatocellular	Connolly, E.; Melegari, M.; Landgraf, P.; Tchaikovskaya, T.; Tennant, B.C.; Slagle, B.L.; Rogler, L.E.; Zavolan, M.; Tuschl, T.; Rogler, C.E. Elevated expression of the miR-17-92 polycistron and miR-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. <i>Am. J. Pathol.</i> <b>2008</b> , <i>173</i> , 856–864. Epub 2008 Aug 7. PMID:18688024.
hsa-miR-192	colorectal	Braun, C.J.; Zhang, X.; Savelyeva, I.; Wolff, S.; Moll, U.M.; Schepeler, T.; Ørntoft, T.F.; Andersen, C.L.; Dobbstein, M. 53-Responsive micrnas 192 and 215 are capable of inducing cell cycle arrest. <i>Cancer Res.</i> <b>2008</b> , <i>68</i> , 10094–10104. PMID:19074875.
hsa-miR-451	colorectal	Bandres, E.; Bitarte, N.; Arias, F.; Agorreta, J. microRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells. <i>Clin. Cancer Res.</i> <b>2009</b> , <i>15</i> , 2281–2290. Epub 2009 Mar 24. PMID:19318487.



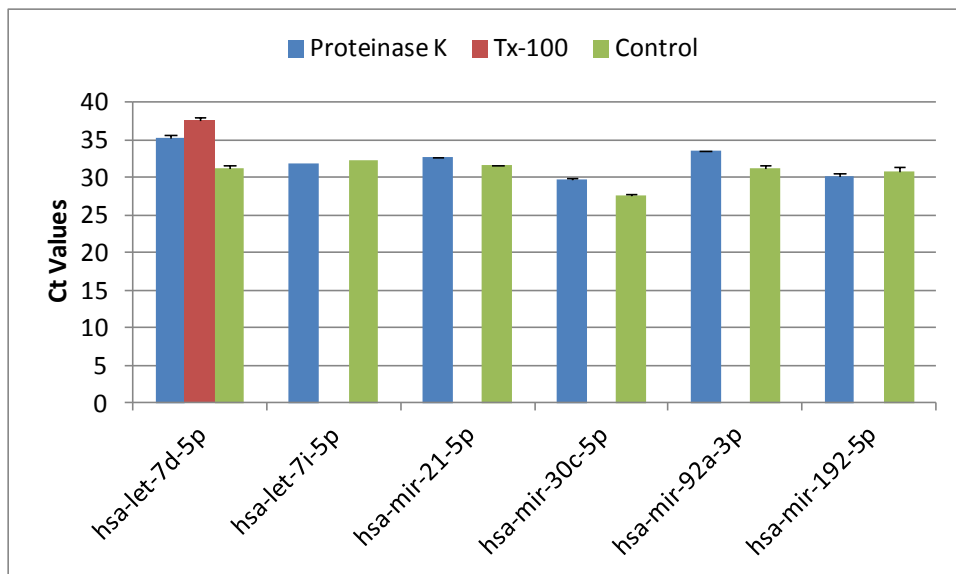
**Figure S1.** Heatmap of signal intensity for Multiplex miRNA assay comparing extracellular vesicles (EVs) isolated from 10 mL of urine using CEN method, with urine (150 µL).



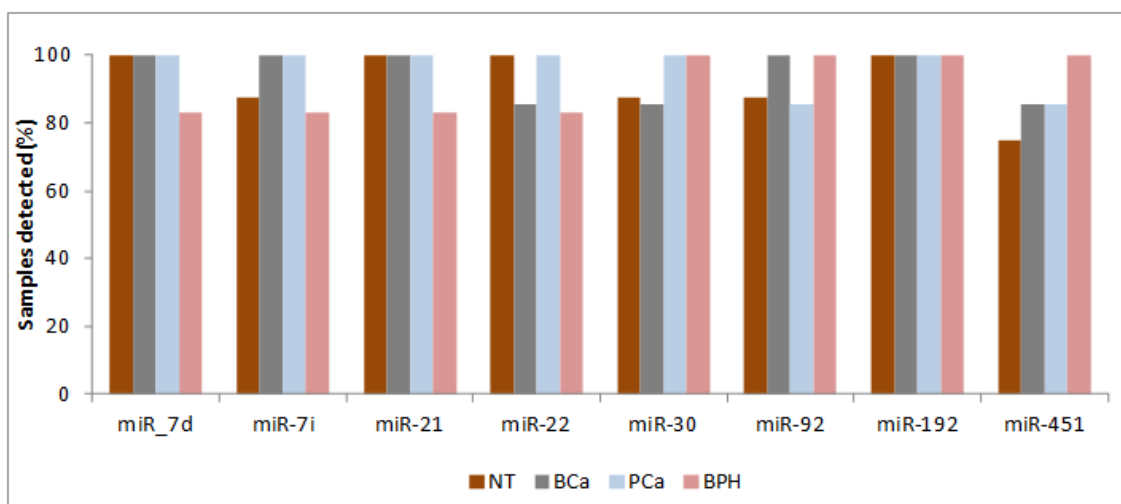
**Figure S2.** Matrix correlations between miRNAs content observed by the multiplex miRNA assay (expressed as signal intensity) and protein content, expressed as densitometry values from Western-blotting for different proteins (data obtained from Royo et al. [13]). Values are correlation coefficients.



**Figure S3.** Correlation analysis between Taqman quantitative PCR (qPCR) and signal intensity from the Multiplex miRNA assay. Each method is treated independently, thus each single point corresponds to the measure of a unique miRNA obtained by a unique method, averaged for all the samples.



**Figure S4.** RNase protection assay. Urine samples were treated with proteinase K or Triton X-100 before incubating with RNase, and uEVs were obtained using NORGEN reagent before proceeding with miRNA qPCR amplification. Note: miRNA amplification was mostly resistant to proteinase-K+RNase treatment, and only when using RNase in the presence of 0.1% Triton X-100 was the amplification abrogated or reduced (hsa-let-7d-5p). Error bars are SD of technical replicates (n = 3).



**Figure S5.** Percentage of detection for each group of patients and miRNA. NT: non-tumor; BCa: bladder cancer; PCa: prostate cancer; BPH: benign prostate hyperplasia.