Supporting Information

A highly sensitive targeted mass spectrometric assay for quantification of AGR2 protein in human urine and serum

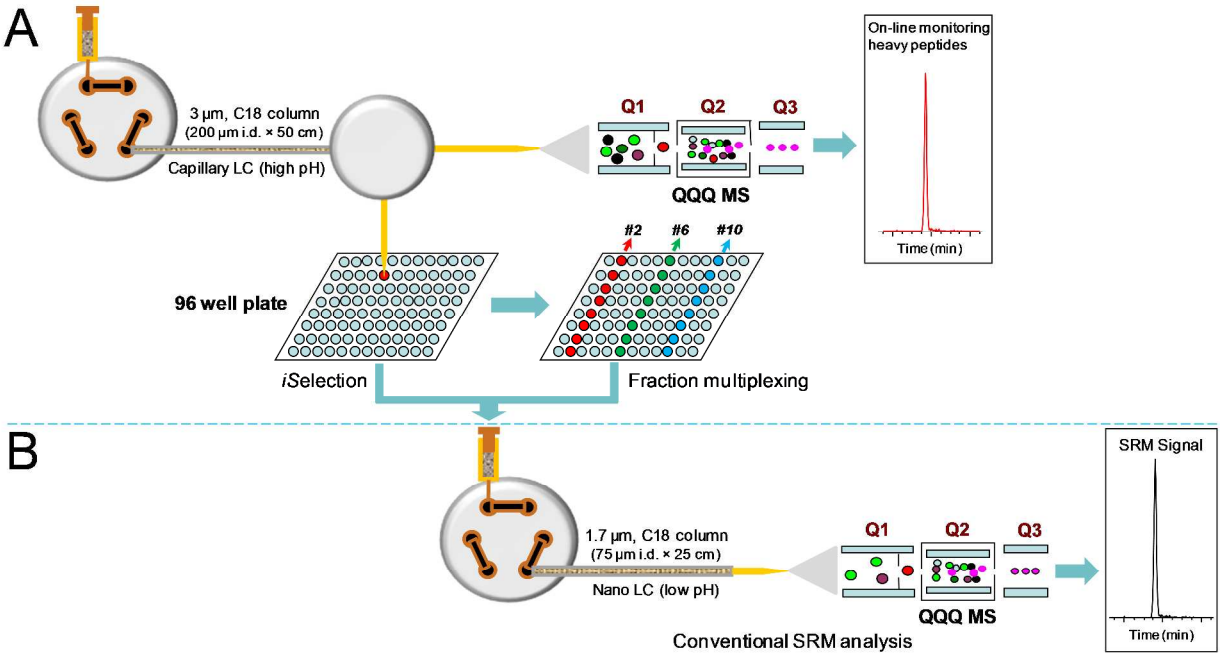
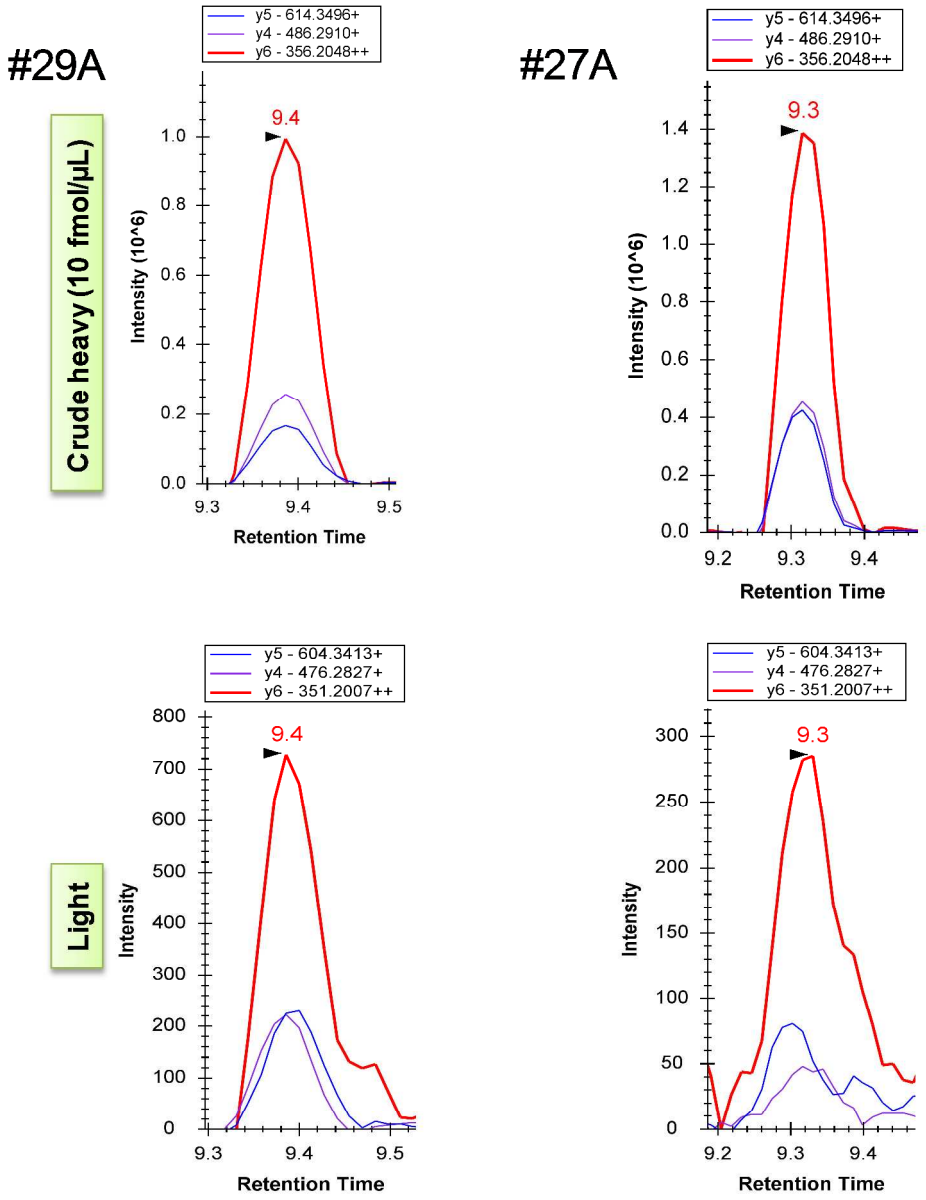


Figure S1. Schematic diagram of the PRISM-SRM workflow. (**A**) PRISM workflow. ~20 µg peptide sample spiked with internal standard (IS) heavy peptides was injected and separated by a high resolution reversed-phase cLC system using high pH mobile phases. The eluent from the cLC column at a flow rate of 3.3 µL/min was split into two flowing streams via a Tee union (the split ratio of flow rates is 1:10): a small fraction (9%) of the column eluent went to a triple quadrupole mass spectrometer for on-line SRM monitoring of IS peptides; a large fraction (91%) of the column eluent was automatically collected every minute into a 96-well plate during a ~100-min LC run. The specific target peptide fractions were either selected based on the same elution times of IS being monitored by the on-line SRM (*i*Selection) or multiplexed. For example, 96 fractions collected along with the first dimensional LC separation can be multiplexed into 12 fractions by combining 8 fractions from the first dimensional LC separation into one fraction for downstream LC-SRM analyses, such as pooling fractions 2, 14, 26, 38, 50, 62, 74, and 86 (marked in red color) into one sample (#2) for the second dimensional LC-SRM. (**B**) Conventional LC-SRM workflow. Following *i*Selection, a target peptide fraction was either directly subjected to nanoLC-SRM with 4 µL sample per injection (~ 45 ng peptides on nanoLC column) or multiplexed with other target fractions with a final volume of 20 µL prior to nanoLC-SRM analysis.


#29A
#27A
Crude heavy (10 fmol/µL)
Light
y5 - 614.3496+
y4 - 486.2910+
y6 - 356.2048++
y5 - 604.3413+
y4 - 476.2827+
y6 - 351.2007++
Intensity (10^6)
Intensity
Retention Time
9.4
9.3

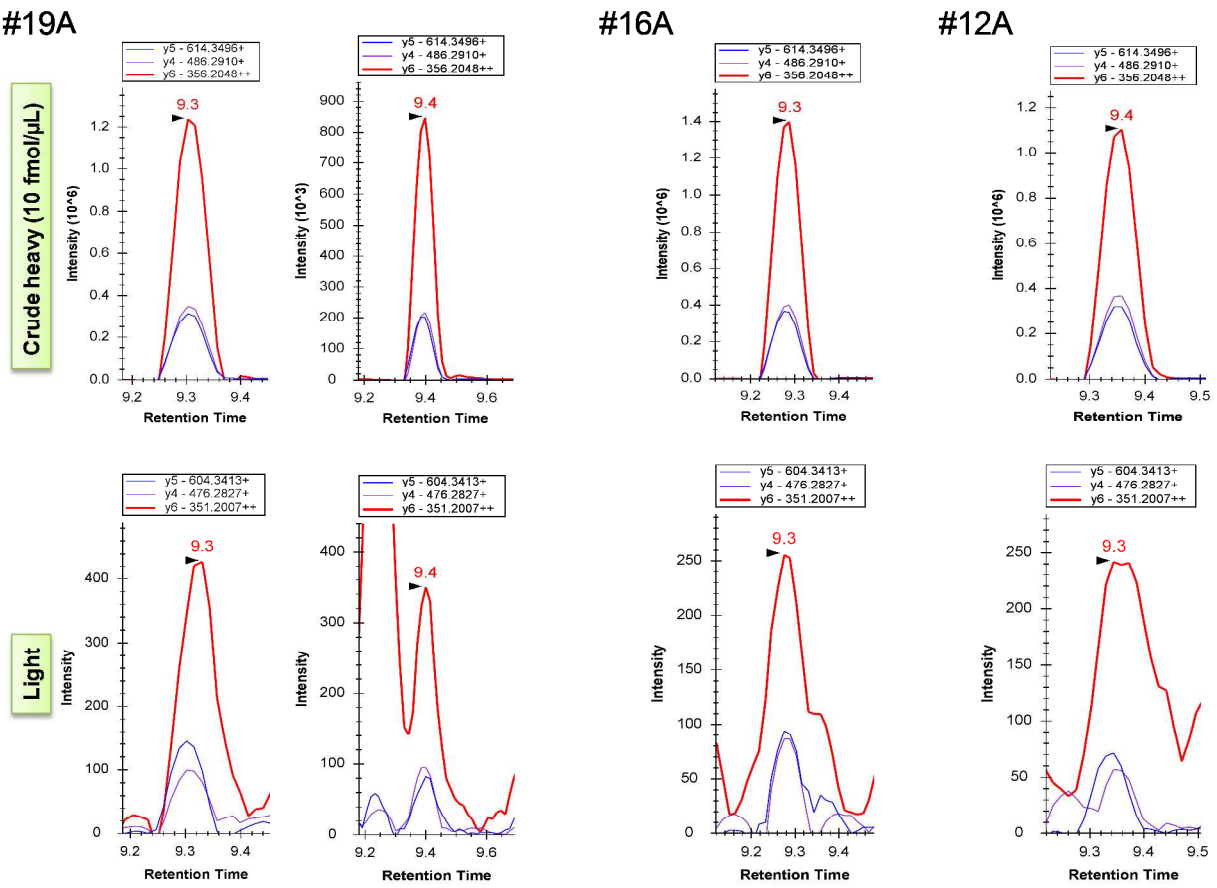


Figure S2. Extracted ion chromatograms of transitions monitored for LPQTLSR derived from AGR2 protein in 5 prostate cancer patient sera.

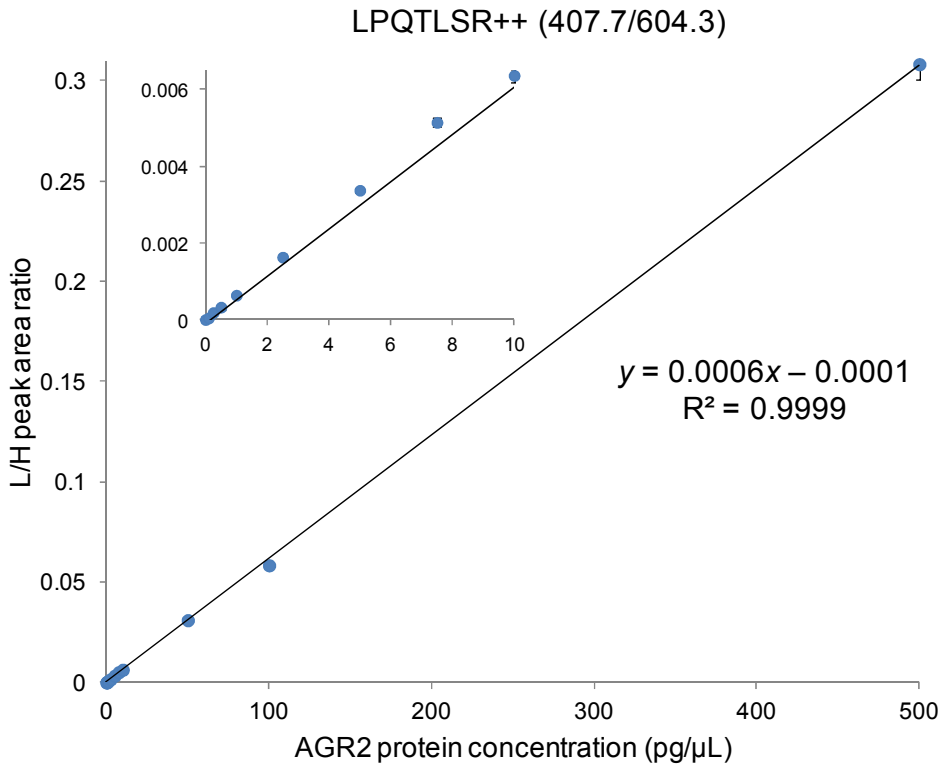


Figure S3. Calibration curve for quantifying AGR2 protein using the transition of 407.7/604.3 from LPQTLSR. Inset plots show details of the low concentration range.

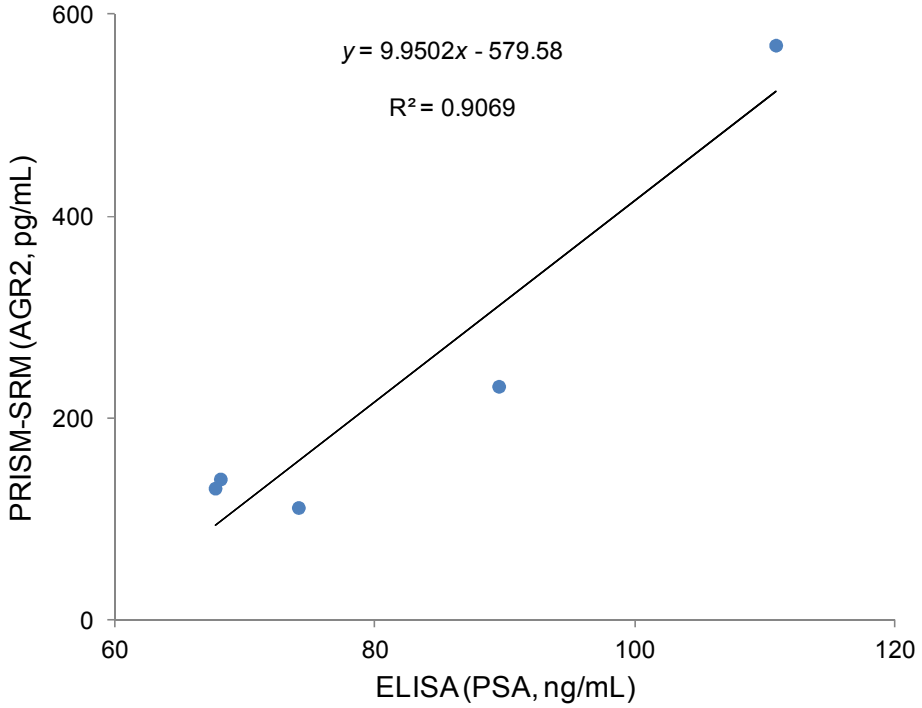


Figure S4. Correlation plot between the AGR2 protein concentrations based on the PRISM-SRM measurements and the PSA protein concentrations determined by ELISA measurements.

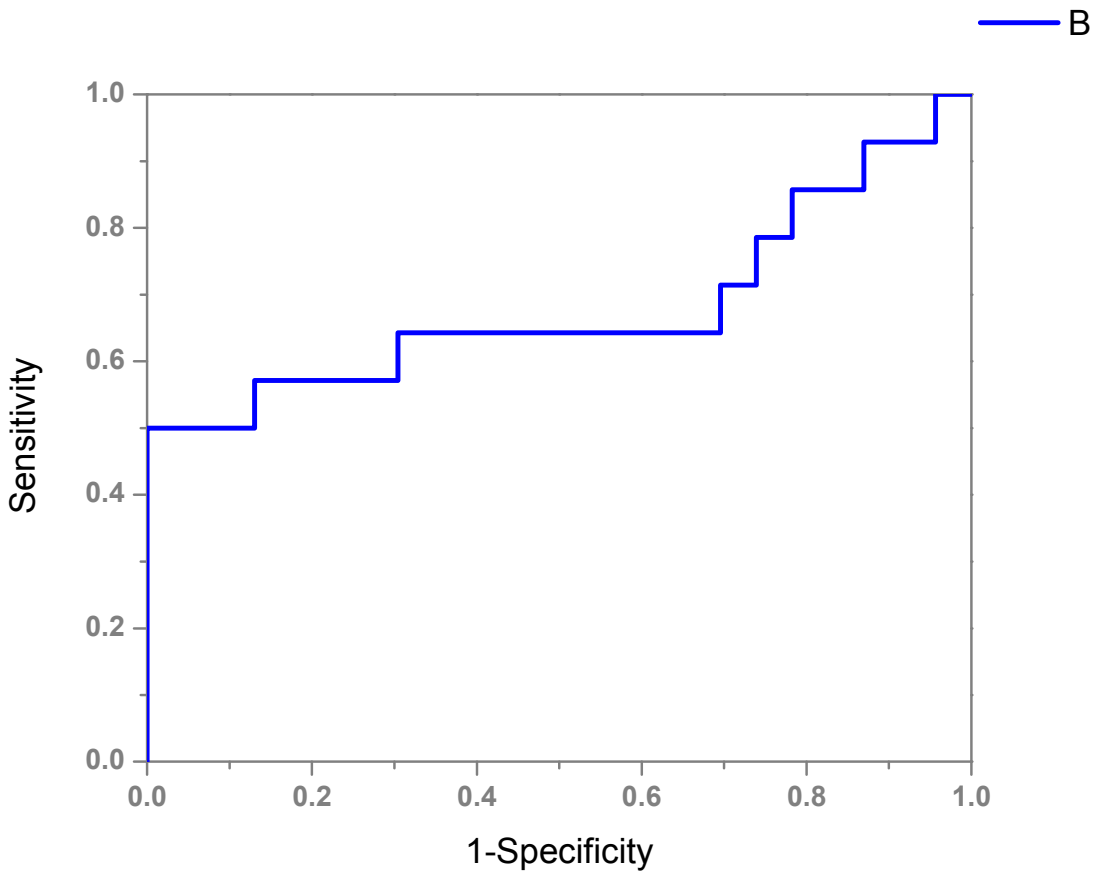


Figure S5. Preliminary ROC curve based on AGR2/PSA ratio values from an initial sets of 37 patients.

Table S1. LC-SRM measurements by spiking tryptic digest of recombinant AGR2 protein into diluted prostate patient urine (20 ng/µL) with the crude heavy peptides included at 100 fmol/µL for generating calibration curves. The transition used is 407.7/351.2 from LPQTLSR.

AGR2 concentration (pg/µL)	0	0.1	0.25	0.5	1	2.5	5	7.5	10	50	100	500
L/H	n/a	6.32E-5	1.68E-4	3.24E-4	6.23E-4	1.45E-3	3.09E-3	4.59E-3	5.81E-3	2.82E-2	5.31E-2	2.94E-1
Standard derivation	n/a	3.88E-6	3.27E-5	3.52E-5	4.53E-5	4.04E-5	3.06E-5	5.70E-5	4.63E-5	2.10E-4	9.60E-4	7.79E-3
CV (%)	n/a	6.1	19.5	10.9	7.3	2.8	1.0	1.2	0.8	0.7	1.8	2.7

Table S2. Summary of all measurements with their corresponding targeted methods.

Regular LC-SRM	PRISM-SRM	IgY14-PRISM-SRM	ELISA
AGR2 calibration curves using diluted urine Endogenous AGR2 in patient urine	Prostate urine	Prostate serum	AGR2 in urine (UW) PSA in serum (JHU)

Table S3. Evaluation of reproducibility of the entire PRISM-SRM (including trypsin digestion and SPE cleanup) starting with five sample aliquots from the same prostate urine specimens.

Sample Name	Repro	407.7>-351.2, 412.7468>-356.2048					407.7427>-476.2827, 412.7468>-486.2910				
		Light	Heavy	L/H	AVERAGE	CV (%)	Light	Heavy	L/H	AVERAGE	CV (%)
1	A	15392	24930706	0.000617	0.0006236	0.9	4013	6793537	0.000591	0.0006346	7.1
	B	15977	25538794	0.000626			4792	7043489	0.00068		
	C	15145	24129245	0.000628			4255	6723093	0.000633		
2	A	16885	24940626	0.000677	0.0007003	6.7	4761	6841364	0.000696	0.0006816	8.6
	B	22101	33003079	0.00067			5543	8978393	0.000617		
	C	15709	20824742	0.000754			4215	5762551	0.000731		
3	A	14597	23610304	0.000618	0.0006465	9.0	4534	6727459	0.000674	0.0006647	6.5
	B	17428	24427595	0.000713			4663	6634333	0.000703		
	C	14689	24163363	0.000608			4318	6993599	0.000617		
4	A	17321	23939689	0.000724	0.0007323	1.8	5167	6848605	0.000754	0.0007197	8.5
	B	14859	19870756	0.000748			4020	5319276	0.000756		
	C	14630	20162437	0.000726			3651	5626398	0.000649		
5	A	11144	15673243	0.000711	0.0007036	7.4	3231	4341533	0.000744	0.0007076	6.5
	B	12108	16109669	0.000752			2979	4538781	0.000656		
	C	10709	16524513	0.000648			3320	4596572	0.000722		
AVERAGE					0.0006813	5.2				0.0006817	7.4
CV (%)					6.6					5.0	

Table S4. Summary of PRISM-SRM measurements of AGR2 in 5 prostate cancer patient serum specimens. The L/H peak area ratio from the transition of 407.7/604.3 and the corresponding calibration curve from the same transition were used to determine the serum AGR2 protein concentrations because the highest abundance transition of 407.7/351.2 showed matrix interference.

Sample ID[a]	L/H[b]	CV	AGR2 concentration (PRISM-SRM, pg/mL)	Total PSA concentration[c] (ELISA, ng/mL)
12	0.000224	9.3	130.9	67.7
16	0.000223	18.9	139.9	68.1
19	0.000429	1.4	231.7	89.5
27	0.000172	7.7	111.7	74.1
29	0.000875	14.7	569.4	110.8

[a] prostate cancer patient sera.
[b] Average L/H for 2 SRM measurements.
[c] Total PSA concentration previously measured by ELISA assay.

Table S5. Summary of ELISA measurements in prostate cancer patient urine specimens.

Samples ID	Vol before concentration (ml)	Volume after concentration (ml)	Protein concentration in μg/μl after concentration	Volume (μl/well) used in ELISA	Amount of protein used (μg/well) for ELISA	OD405	Estimated amount of AGR2 (pg) detected per well in ELISA	Estimated amount of AGR2 (pg) per 100 μg concentrated urine protein	Estimated amount of AGR2 (pg) per ml urine
P06-011	50	1.1	1.28	200	256	0.701	53.6	21	5.9
P07-031	33	1.2	2.4	200	480	3.356	1000	208	181
P07-047	35	1.8	1.3	200	260	0.175	6	2.3	1.5
P07-018	35	0.8	2.7	200	540	2.804	422	78	48
P08-015	50	0.9	1.5	200	300	1.756	187.5	62.5	16.9
P08-032	50	0.9	0.7	200	140	0.28	14	10	1.26

Table S6. The SRM signal ratio of urinary AGR2/PSA protein from SRM measurements in 37 prostate cancer patient urine specimens (crude internal standards for AGR2 and pure internal standards for PSA were spiked at 10 fmol/μL and 5 fmol/μL, respectively).

	Prostate cancer patient urine ID[a]	L/H_{AGR2}[b]	L/H_{PSA}[c]	$(L/H_{AGR2})/(L/H_{PSA})$
Non-cancer	P07022BN	0.001716	1.243	0.00138
	P07036BN	0.003751	18.011	0.00021
	P08018BN	0.000770	0.955	0.00086
	P08022BN	0.002895	1.007	0.00288
	P08036BN	0.001974	3.857	0.00051
	P09040AN	0.007801	0.590	0.01323
	SAP12_003BN	0.00410	1.236	0.003316
	SAP12_008BN	0.00735	1.009	0.007281
	SAP12_014BN	0.00320	0.779	0.004106
	SAP12_016BN	0.00435	0.812	0.005357
	SAP12_017BN	0.00610	1.112	0.005488
	SAP12_026BN	0.02220	1.763	0.012593
	SAP12_028BN	0.00570	3.507	0.001625
	SAP12_030BN	0.00295	1.095	0.002693
	SAP12_033BN	0.00315	0.280	0.011259
	SAP12_034BN	0.00500	1.237	0.004043
	SAP12_035BN	0.01040	2.125	0.004894
	SAP12_039BN	0.01230	1.397	0.008803
	SAP12_050BN	0.01790	28.825	0.000621
	SAP12_052BN	0.00690	2.129	0.003241
	SAP12_071BN	0.00790	2.277	0.003469
	SAP12_072BN	0.00315	3.857	0.000817
	SAP12_077BN	0.00630	1.471	0.004284
Cancer	P06003Pre	0.000373	0.249	0.00149
	P06011Pre	0.003331	0.330	0.01009
	P06017Pre	0.003392	0.169	0.02006
	P07016Pre	0.005759	0.002	3.41433
	P07018Pre	0.012275	0.893	0.01495
	P07019Pre	0.000858	0.003	0.28319
	P07029Pre	0.000107	0.046	0.00232
	P07031Pre	0.026813	0.196	0.13680
	P07040Pre	0.003952	0.261	0.01403
	P07047Pre	0.000734	0.145	0.00481
	P08006rPre	0.001333	3.393	0.00044
	P08015Pre	0.002327	3.240	0.00065
	P08028Pre	0.002061	0.087	0.02370
	P08032Pre	0.000863	0.681	0.00139

[a] Clinical prostate cancer patient urine samples.
[b] Average L/H for 3 SRM measurements. L/H peak area ratio from LPQTLSR was used.
[c] Average L/H for 2 SRM measurements. L/H peak area ratio from IVGGWEC$_{cam}$EK was used.

Supplemental Methods

To accurately determine AGR2 concentration from PRISM-SRM measurements, the digestion efficiency and protein recovery in the SPE cleanup were presumably the same between native AGR2 protein in human serum or urine and standard AGR2 protein for generating calibration curves.

1. Measured urinary AGR2 protein concentration calculation

Based on the L/H ratio of measured AGR2 protein in prostate cancer patient urine, the AGR2 protein concentrations were determined by using standard AGR2 protein calibration curves. Considering the large variation of urinary protein concentration, the original AGR2 protein concentrations in prostate cancer patient urine were then expressed as pg/100 total urine proteins.

$C_{urinary\ AGR2\ protein\ concentration}$ (pg/100 μg total urinary protein) = $C_{AGR2\ protein\ concentration\ determined\ by\ calibration\ curve}$ (pg/μL) × V(μL/100 μg urinary proteins)

V(μL/100 μg urinary proteins) = 100 μg/(0.5 μg/μL) per 100 μg urinary protein = 200 (μL/100 μg urinary protein)

Thus, $C_{urinary\ AGR2\ protein\ concentration}$ (pg/100 μg total urinary protein) = $C_{AGR2\ protein\ concentration\ determined\ by\ calibration\ curve}$ (pg/μL) × 200 (μL/100 μg urinary protein)

2. Measured serum AGR2 protein concentration calculation

To accurately determine the serum AGR2 concentration from PRISM-SRM measurements, the digestion efficiency and protein recovery in the SPE cleanup were presumably the same between native AGR2 protein in human serum and standard AGR2 protein for generating calibration curves. Based on the L/H ratio of measured AGR2 protein in prostate cancer patient sera, the AGR2 protein concentrations at the digested peptide level were determined by using standard AGR2 protein calibration curves. The original AGR2 protein concentrations in prostate cancer patient sera were the AGR2 protein concentrations at the digested peptide level times the dilution factor assuming AGR2 protein recovery was 100% during IgY14 immunoaffinity depletion.

$C_{original\ AGR2\ protein\ concentration} = C_{AGR2\ protein\ concentration\ at\ peptide\ level} \times$ dilution factor

Dilution factor = $V_{peptide\ mixture\ concentration\ at\ 0.5\mu g/\mu L}/V_{original\ serum}$

$V_{original\ serum}$: total volume of starting human serum for IgY14 depletion.

$V_{peptide\ mixture\ concentration\ at\ 0.5\mu g/\mu L}$: total volume of peptide mixtures (from digestion of the IgY14 flow-through) at a concentration of 0.5 μg/μL for PRISM fractionation.

3. Coefficient of variation (CV) calculation

Precision was determined by CV (standard deviation divided by the mean) and expressed as a percentage. For each patient sample, standard deviation and CV calculation were based on three injection replicates of LC-SRM measurements. For the five sample aliquots the PRISM-SRM processing precision was evaluated systematically: five processing replicates involving trypsin digestion, SPE cleanup, and PRISM-SRM measurements. The mean value of the five processing

replicates was obtained from five average L/H area ratio values, and each average L/H value was based on three technical replicates per processing replicate.