Supplementary Methods. Candidate biomarker narrowing using scoring system

1. Urine and urinary exosome preparation

For urinary protein extraction, urine was centrifuged for 10 minutes to remove cellular debris. The starting volume was 2.5 mL. After centrifugation at 3,000 ×g for 15 minutes at 4℃, urine supernatants were concentrated with an Amicon Ultra centrifugal filter device (3 kDa MWCO, Millipore) at 14,000 ×g to a volume of ~100 μL. The protein concentration using Bradford assay (Bio-Rad protein assay kit, Bio-Rad, Hercules, CA). For label-free quantification in the discovery phase, 50 μg of urine protein was precipitated by adding a 5-fold volume of ice-cold acetone. For the data-independent acquisition (DIA) in the verification phase, 100 μg of urine protein per individual sample was subjected to digestion. The precipitated proteins were dissolved in sodium dodecyl sulfate (SDS)-containing denaturation buffer (4% SDS, 1 mM TCEP, and 0.1 M Tris-HCl pH 7.4). After heating at 95℃, the denatured proteins were digested using a filter-aided sample preparation (FASP) method [1,2]. Protein digestion was performed with trypsin (enzyme-to-protein ratio of 1:100) at 37℃ overnight.

For urinary exosome analysis, exosomes were isolated using Total Exosome Isolation Reagent (Invitrogen) from 2.5 ml of urine samples after centrifugation at 17,000 ×g for 15 minutes to remove debris. All samples in the discovery and verification phases were prepared according to the manufacturer's instructions. Isolated exosomes were used to extract total exosomal proteins using SDS-containing buffer. After denaturation using sonication and heating at 95℃, protein concentrations were measured using a BCA reducing agent compatible kit (Thermo Fisher Scientific Inc.). In the discovery and verification stages, 25 μg of exosome proteins were precipitated with five volumes of cold acetone at –20°C overnight.

After the precipitated proteins were dissolved in an SDS-containing denaturation buffer, digestion was performed [1,2]. All digested samples were desalted using homemade C18-StagetTips as previously described [1,2]. The desalted peptide samples were dried using a vacuum centrifuge and stored at –80°C until LC-MS/MS analysis.

2. Data analysis of ELISA results

We analyzed the diagnostic performance for bladder cancer detection using the AUROC of each protein. We used logistic regression and machine learning classification algorithms, with or without backward elimination methods, to develop a diagnostic model using a combination of selected proteins. The AUROC of the developed model was compared

with that of urine cytology. The cutoff value was selected by maximizing the true positive rate (sensitivity) and minimizing the false-positive rate. For this reason, we used the Youden index for the threshold. To use the optimized threshold, positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity were calculated for each model. We calculated the AUROC of the combination of urine cytology and each developed model.