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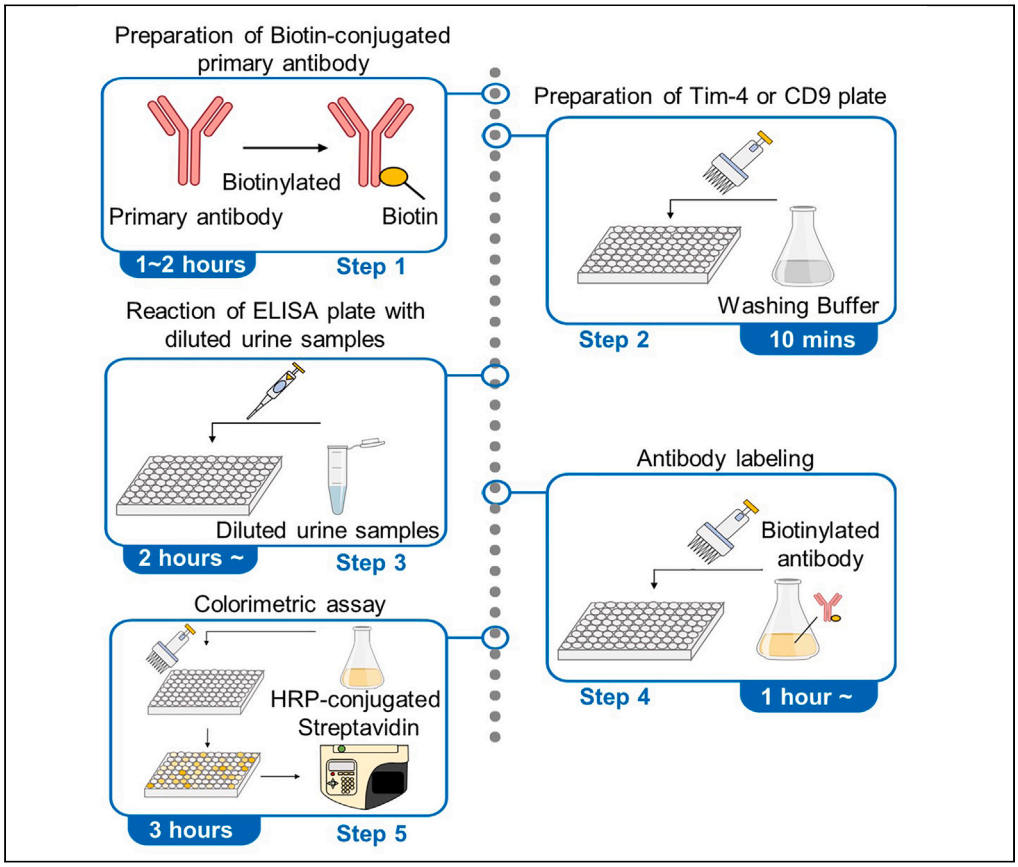
Protocol

Enzyme-linked immunosorbent assay to detect surface marker proteins of extracellular vesicles purified from human urine



The molecular profile of extracellular vesicles released in urine reflects the pathophysiological processes occurring within originating cells located in diverse nephron segments. Here, we present an enzyme-linked immunosorbent assay for quantitative membrane protein detection in extracellular vesicles in human urine samples. We describe steps for preparing urine samples, biotinylated antibodies, and microtiter plates to purify extracellular vesicles and detect membrane-bound biomarkers. The specificity of signals and the limited variability by freeze-thaw cycles or cryopreservation have been verified.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for membrane protein detection in EV in clinical urine samples by ELISA

Steps for the preparation of urine samples, antibodies, and microtiter plates

Applicable to investigating the molecular characterization of urinary EV

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Enzyme-linked immunosorbent assay to detect surface marker proteins of extracellular vesicles purified from human urine

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SUMMARY

The molecular profile of extracellular vesicles released in urine reflects the pathophysiological processes occurring within originating cells located in diverse nephron segments. Here, we present an enzyme-linked immunosorbent assay for quantitative membrane protein detection in extracellular vesicles in human urine samples. We describe steps for preparing urine samples, biotinylated antibodies, and microtiter plates to purify extracellular vesicles and detect membrane-bound biomarkers. The specificity of signals and the limited variability by freeze-thaw cycles or cryopreservation have been verified.

For complete details on the use and execution of this protocol, please refer to Takizawa et al. (2022).[1]

BEFORE YOU BEGIN

Background

Urinary extracellular vesicles (uEVs) are a source of potentially useful biomarkers because they reflect physiological and pathological conditions in kidney and urinary tract.[2,3] This protocol describes an enzyme-linked immunosorbent assay for quantitative membrane protein detection in extracellular vesicles in human urine samples.

Institutional permissions

This study was approved by the ethics committee of the University of Tokyo.

Preparation of urine

Timing: 30 min

1. Collect urine in a sterile container with a screw cap. If possible, collect a mid-stream sample of random catch or first-morning urine.
2. Insert the centrifugation tube into a benchtop centrifuge with a bucket rotor and spin at 500 g for 5 min. Where necessary, use counterbalance.
3. Using an electronic pipettor and serological pipette, collect the supernatant and transfer it to a fresh, sterile 1.5 mL tube.
4. If immediately preparing the sample after collection is not possible, refrigerate the supernatant at −80°C.

Note: If urine left over from clinical testing is to be used, it can be frozen and stored at this stage.

5. (After thawing) Centrifuge at 1,200 × *g* for 5 min at 4°C to remove cell debris and urine salts.
6. Collect the supernatant in a new sterile 1.5 mL tube using an electronic pipettor and serological pipette.

Dilution of urine samples and standard curve preparation for quantitative measurement

Timing: 20 min

7. Dilute the urine samples two or more times with Sample Reaction Buffer.

※ Since the appropriate dilution rate of the sample varies greatly depending on the concentration of extracellular vesicles in the sample and the amount of surface protein marker to be detected, it is recommended to serially dilute the sample and preliminarily examine the appropriate dilution rate.

8. Dilute a reference urine at an appropriate concentration with Reaction Buffer. Prepare seven steps of 2-fold serial dilution.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-MUC1 (1:333)	Santa Cruz Biotechnology	sc-7313, RRID:AB_626983
Mouse monoclonal anti-CD9 (clone 12A12) (1:10000)	Shionogi & Co	SHI-EXO-M01
Mouse monoclonal anti-CD63 (clone 3-13) Biotinylated (Included in Exosome ELISA kits) (1:100)	Wako Pure Chemical Industries	019-27713
Critical commercial assays		
Biotin labeling kit-NH_2	Dojindo Molecular Technologies	LK-03
PS Capture Exosome ELISA Kit (Streptavidin HRP)	Wako Pure Chemical Industries	298-80601
PS Capture Exosome ELISA Kit (Anti Mouse IgG POD)	Wako Pure Chemical Industries	297-79201
CD9 Capture Exosome ELISA Kit (Streptavidin HRP)	Wako Pure Chemical Industries	296-83701
Other		
iMark™ Microplate Absorbance Reader	Bio-Rad Laboratories	168-1130J5
Multiskan Skyhigh	Thermo Scientific	A5119500C
Wellwash	Thermo Scientific	5165000
MS 3 control (Microplate shaker)	IKA	0020016017

MATERIALS AND EQUIPMENT

- Keep urine samples and kit reagents away from skin and mucous membranes.
- When allowing the plate to incubate in each step, always label a plate seal to protect the wells from drying, contamination with foreign matters, uneven temperature, and evaporation of the dispensed reagents.
- The environment in the lab can have an impact on ELISA. The temperature in the laboratory should be maintained at 20°C–25°C. Also, avoid measurement in an environment with wind current (including air conditioner wind) and low humidity.
- By changing tips between adding samples, standards, and reagents, you can prevent cross-contamination of your samples or reagents.

STEP-BY-STEP METHOD DETAILS

Preparation of biotin-conjugated primary antibody

Timing: 1–2 h

Here we describe how to conjugate primary antibody using the Biotin labeling kit-NH_2, but biotinylation using other products (e.g., Biotin Protein Labeling Kit (Roche), Biotin-XX Microscale Protein Labeling Kit (Molecular Probes), Biotinylation Kit/Biotin Conjugation Kit (Fast, Type A) - Lightning-Link® (abcam)) is also acceptable.

Alternatives: If you are using an already biotinylated primary antibody, these processes are unnecessary.

Alternatives: If you use HRP-conjugated secondary antibody as a detection antibody (such as the one in PS Capture Exosome ELISA Kit (Anti Mouse IgG POD), these processes are unnecessary.

1. Add 100 μL WS Buffer and antibody containing 50–200 μg protein to a Filtration Tube. Pipettes to mix and centrifuge at 8,000 × *g* for 10 min.
2. Add 10 μL DMSO to NH_2-Reactive Biotin, and dissolve with pipetting. Proceed to the next step immediately.
3. Add 100 μL reaction buffer to the filtration tube, followed by 8 μL of NH_2-Reactive Biotin solution, and mix with pipets.
4. Incubate the tube at 37°C for 10 min.
5. Wash the filtration tube three times with WS buffer.
   a. Add 100 μL WS buffer to the filtration tube, and centrifuge for 10 min at 8,000 × *g*. Discard the filtrate. After centrifugation, if the solution is still on the membrane, spin at 8,000 × *g* for an additional 5 min.
   b. Add 200 μL WS buffer to the filtration tube and centrifuge it at 8,000 × *g* for 10 min. Discard the filtrate. Repeat this step (b) one more time.
6. To recover the conjugate, pipette around 10 times with 200 μL of WS buffer added to the filter tube. Transfer the solution to a microtube.

Preparation of Washing Buffer

Timing: 5 min

⚠ CRITICAL: When using PS Capture Exosome ELISA Kit (Tim4-coated plate), addition of Exosome Binding Enhancer to Washing Buffer is essential for extracellular vesicles to bind with a plate.

7. Bring Washing Buffer (10×) to room temperature before opening and dispense the necessary amount.
   a. [PS Capture Exosome ELISA Kit] Dilute 10 times with purified water. Exosome Binding Enhancer (100×) should be added to the diluted solution and thoroughly mixed.

   Note: As components have a tendency to deposit, use Washing Buffer (1×) no later than 8 h after the addition of Exosome Binding Enhancer (100×).

   b. [CD9 Capture Exosome ELISA Kit] Dilute 10 times with purified water.

Preparation of Tim-4 or CD9 plate

Timing: 10 min

8. Wash each well of a 96-well plate 3 times with 300–350 μL of Washing Buffer (1×). The plate should be inverted and carefully blotted with clean paper towels.

Reaction of ELISA plate with diluted urine samples

Timing: 2 h ~

9. Fill each well with 100 μL of sample dilution, standard dilution, and Reaction Buffer as a blank (Figure 1). Put a plate seal over the plate. Incubate for 2 h at 20°C–25°C room temperature.

Note: It is advised to shake the microplate during incubation at a speed of 300–500 rpm because the standing condition could result in low detection sensitivity and significant well-to-well variance. Overnight incubation may be necessary depending on the reactivity of the primary antibodies.

10. Dispose of the solution and wash three times with 300–350 μL of Washing Buffer (1×). The plate should be turned over and carefully blotted with clean paper towels.

Antibody labeling

Timing: 1h ~

11. Fill each well with 100 μL of biotinylated antibody diluent. As a negative control, add 100 μL of Reaction Buffer to a well instead of antibody diluent. Cover with a plate seal.
12. Incubate for 1 h at room temperature (20°C–25°C) with shaking at 300–500 rpm using a microplate shaker.

Note: The dilution and appropriate incubation time vary greatly depending on the primary antibody. If appropriate sensitivity is not obtained, increase concentration, or extend the incubation time. Overnight incubation may be applicable.

13. Repeat the wash as in step 5.

Colorimetric assay

Timing: 3 h

14. Add 100 μL of HRP-conjugated Streptavidin (1×) to each well. Put a plate seal over it.

Alternatives: If you use HRP-conjugated secondary antibody as a detection antibody (such as the one in PS Capture Exosome ELISA Kit (Anti Mouse IgG POD)), dispense 100 μL of the secondary detection antibody reaction solution in each well.

15. Incubate for 2 h at room temperature (20°C–25°C) with shaking at 300–500 rpm using a microplate shaker.

Note: We usually proceed immediately to the next step, but overnight incubation may be applicable.

Alternatives: If you use HRP-conjugated secondary antibody as a detection antibody, incubate for 1 h.

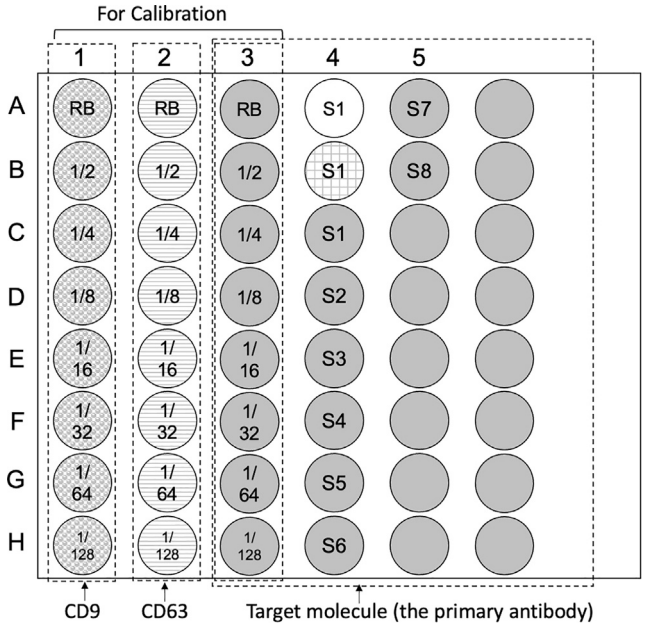

Figure 1. An example of how to arrange urine samples in ELISA plate

Columns 1 and 2 contain dilution standards for CD9 or CD63 antibodies, and column 3 contains standards for the primary antibody to be examined. Reaction Buffer (RB) instead of urine is placed for background measurements. Columns 4 and 5 contain the actual samples to be assayed, including negative control without primary antibody (A-4). Control with the antibody absorbed with antigens can be placed to confirm specificity (B-4) (See also Figure 3).

16. Discard the solution in the well and wash 5 times with 300–350 μL of Washing Buffer (1×). The plate should be turned over and carefully blotted with fresh paper towels.
17. Pour each well with 100 μL of TMB Solution that has been warmed to room temperature. Use a microplate shaker to stir for approximately 1 min. Put a new plate seal on top.
18. Incubate for 30 min at 20°C–25°C room temperature.
19. Pour each well with 100 μL of Stop Solution that has been warmed to room temperature.
20. Using a microplate shaker, stir for approximately 5 s. Measure the absorbance immediately using a microplate reader with wavelengths of 450 nm and 620 nm (600–650 nm).

Note: Deposits may be seen on a plate's surface.

EXPECTED OUTCOMES

Using the present protocol, the exosome markers (CD9 and CD63) can be detected in all the tested samples. By testing two-fold serial dilutions of urine samples, linearly reduced reactivity with urine dilution is confirmed (Figure 2). We have applied this ELISA to detect several marker proteins including MUC1.[1]

The specificity of signals can be checked by using antibody pretreated with antigen. The MUC1 antibody absorption nearly eliminates the signals (Figure 3).

Using urine samples from healthy subjects, it was determined how many freeze-thaw cycles and whether or not protease inhibitors were present affected the expression levels in uEVs. The values of CD9 and MUC1 were unaffected by the number of freeze-thaw cycles up to three (Figure 4) or by the lack of protease inhibitors (Figure 5).

The stability of vesicles in urine cryopreserved at −80°C for a month did not interfere with the detection of CD9 (Figure 6), indicating that uEVs are stable in frozen urine

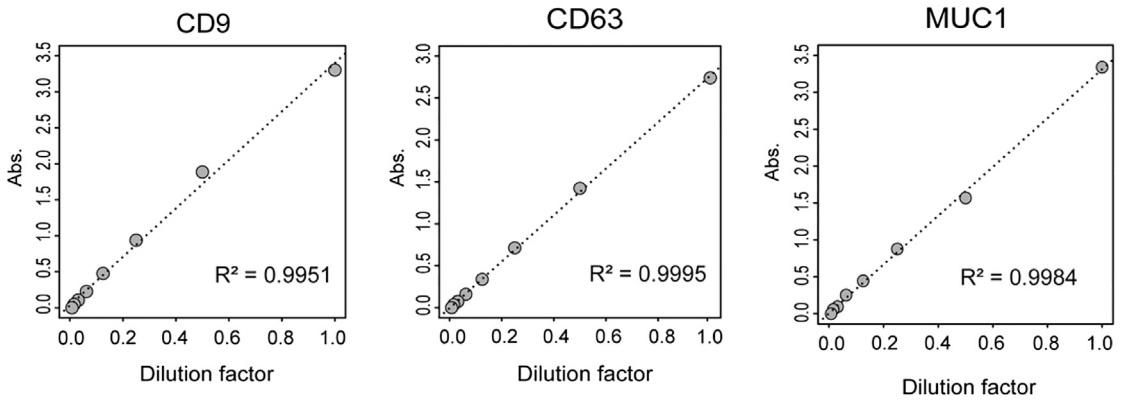

Figure 2. Standard curve of exosome markers (CD9 and CD63) and MUC1

The figure is obtained by plotting the absorbance at 450 nm (y-axis) against the standard concentration values (x-axis). R2 is the correlation coefficient for the standard curve.

QUANTIFICATION AND STATISTICAL ANALYSIS

Use the calculated value at 450 nm obtained by subtraction the absorbance values at 620 nm (600–650 nm) in the following calculations (e.g., A1 = A1(450 nm)-A1(620 nm)).

1. Subtract the absorbance readings of the blank (C1, for example, in Figure 1) from the readings of the reference standard's dilution series (C2–8, for example, in Figure 1) to determine the value.
2. Plot the absorbance readings (Y-axis) against standard concentration values (X-axis) to create a standard curve.

   ⚠ CRITICAL: For each experiment run, prepare a new standard curve.

3. Subtract the absorbance readings of the diluted samples from the blank measurements to determine the value.

   ⚠ CRITICAL: If the absorbance value of the diluted sample exceeds the absorbance range of the standard curve, re-dilute the sample to the proper concentration and perform the measurement.

4. Calculate the concentration of the unknown sample using the standard curve, and then multiply the result by the dilution factor.

LIMITATIONS

- The mechanism of the Tim4-based purification system is that the extracellular IgV-like domain of Tim4 binds phosphatidylserine on the surfaces of EVs.[4] Because this binding is Ca^{2+}-dependent,[4] urine must be diluted at least twice with Sample Reaction Buffer attached to the kit. When sensitivity is so weak that undiluted urine must be used, addition of 1/100 volume of Exosome Binding Enhancer (100×) is required.
- Because phosphatidylserine also exists on the surface of apoptotic cells, Tim4 bind to apoptotic cells or apoptotic bodies. Therefore, the results could be impacted by the presence of cell debris or apoptotic cells.[5] We confirmed that apoptotic bodies or autophagic extracellular vesicles were eliminated from Tim4-purified uEVs by not detecting their markers (Annexin V or LC3B-PE, p62)[6] in any of the urine samples.[1]

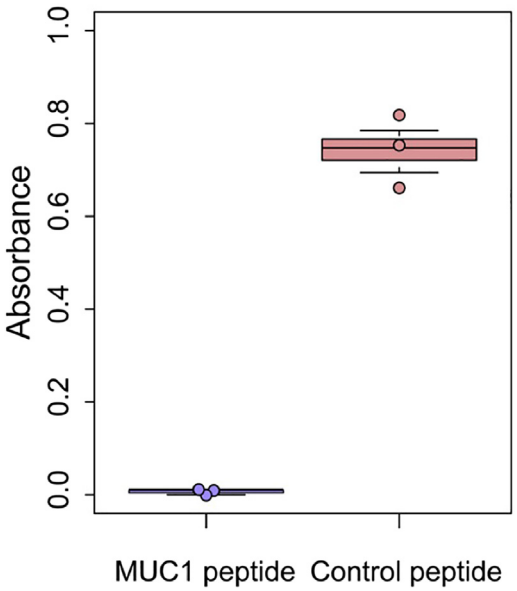

Figure 3. Comparing the pretreatment of MUC1 antibody with the antigen MUC1 peptide or a control peptide

- ELISA plates with antibody to exosome markers can purify EVs from urine. Because the majority of uEVs do not express CD9 or CD63,[7] the properties of the uEVs adsorbed on the plate may differ depending on the type of plate used, such as the cells from which the uEVs are derived. Therefore, it is possible that the relative values obtained by CD9- or CD63-plates may differ from Tim4-plates.
- Since there is no absolute reference sample, this protocol detects relative quantitation within samples. If the sample is spread across multiple plates, the same reference can be placed on multiple plates for relative evaluation.
- This protocol does not include the process of making cytosolic antigen accessible by the antibody. Therefore, the primary antibody should recognize the target molecule's extracellular (extravesicular) region.

TROUBLESHOOTING

Problem 1

No color development (step 20).

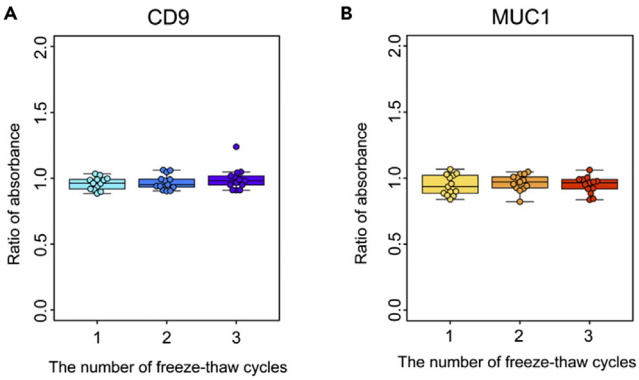

Figure 4. Effect of freeze-thaw cycles

Change in expression levels of CD9 (A) and MUC1 (B) by a number of freeze-thaw cycles. The ratios of expression levels of one, two, three freeze-thaw cycle(s) to that of no freeze-thaw are shown.

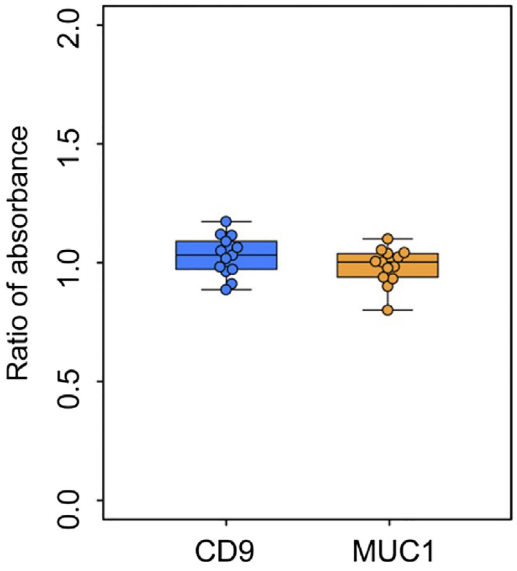

Figure 5. Change in expression levels of CD9 and MUC1 with and without protease inhibitor (PI) immediately after urine collection

Samples were prepared with or without PI and subjected to two freeze-thawing cycles. The ratios of the expression levels of samples without PI to those with PI are shown.

Reason 1: Omission of key reagents.

Reason 2: Primary antibody does not bind to the antigen in ELISA.

Reason 3: Improper conjugation of primary antibody.

Potential solution

Solution 1: Check that all reagents have been added in the correct order.

Solution 2: Use a different antibody.

Solution 3: Confirm the reaction with your own biotinylated CD9 antibody.

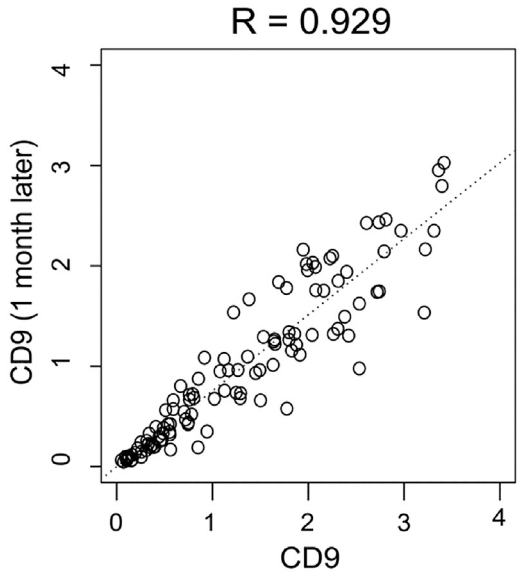

Figure 6. The correlation of CD9 measured by ELISA at one-month intervals is shown

Correlation coefficients are expressed as Pearson's R.

Problem 2
The signal is too high (step 20).

Reason 1: Sensitivity of the antibody is too high.

Reason 2: Concentration of the molecule of interest un uEVs is too high.

Potential solution
Solution 1: Ab dilutions showing a saturated signal should be avoided. The preparation and testing of several primary antibody dilutions is necessary (e.g., 1:250, 1:500, 1:1000, 1:2000, and 1:3000). It is best to select an Ab dilution that exhibits a linear reactivity with successively diluted standard samples.

Solution 2: Increase the dilution factor of urine.

Problem 3
High OD values of blank (step 20).

Reason 1: The plates weren't washed thoroughly enough.

Reason 2: Contamination of urine.

Reason 3: The signals are not specific to the antigen.

Potential solution
Solution 1: Carefully adhere to the wash regimen.

Solution 2: During the addition of the negative and positive controls, switch micropipette tips.

Solution 3: Change the primary antibody.

Problem 4
Poor reproducibility of test.

Reason 1: Dispensing errors.

Reason 2: Improper washing.

Reason 3: The signals are not specific to the antigen.

Potential solution
Solution 1: Calibrate micropipettes. Verify other dispensing equipment.

Solution 2: If an automatic plate washer is employed, make sure the Wash Buffer is flowing uniformly via all the ports and manifolds. Clean the ports if there are any obstructions.

Solution 3: Check that the signals can be eliminated by preincubating the primary antibody with the antigen (Figure 3).

Problem 5
Irregular color development (step 20).

Reason 1: Improper washing of wells.

Reason 2: Dryness of plate due to improper coverage with a plate seal.

Reason 3: Standing condition during incubation.

Potential solution

Solution 1: Utilize an automated plate washer, if one is available.

Solution 2: Cover properly during the reaction period.

Solution 3: Incubate with shaking at using a microplate shaker or speed up the shaker.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yutaka Harita (haritay-ped@h.u-tokyo.ac.jp)

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper does not report original code.
- The lead contact, upon request, will share data reported in this paper.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.K., Y.H; ELISA optimization, T.K., N.T.; supervision, Y.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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