User Guide

CD9 TRIFic™ Exosome Assay

Europium Time-Resolved

Immunofluorescence assay

for detection of exosome antigens

Human CD9 Cat No EX101

Protocol Version 1.4

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Storage

Store Technical Positive Control at -80°C immediately upon receipt. Store all other components at 4°C. The kit has a shelf life of at least three months from receipt.

Product Components

- Streptavidin-coated 96-well Plate — 1 plate
- Technical Positive Control, 25 µg (100 µg/ml) in PBS — 1 vial
- Eu-labeled CD9 mAb; 2.75 µg (in 40 µl TSA buffer, 0.1% BSA) — 1 vial
- Biotinylated CD9 mAb; 22 µg (in 22 µl PBS 7.4 pH, 15 mM NaN_3) — 1 vial

  Note: The CD9 antibody used in this kit is human specific.
- Assay Buffer, 22 ml — 1 bottle
- Europium Fluorescence Intensifier (EFI Solution), 11 ml — 1 bottle
- 25x Wash Buffer, 20 ml — 1 bottle
- User Guide — 1 copy

Equipment and materials required but not supplied with these reagents

- Time-resolved fluorescence microplate reader
- Automatic plate washer
- Plate shaker
- Pipettes for dispensing reagents
- Multichannel micropipette reservoir
- Distilled / MilliQ water
- Phosphate Buffered Saline (PBS)

TRIFic™ Exosome Assay Protocol

Introduction and Assay Principle

In the TRIFic™ exosome assay, the same antibody is used for binding of target to the assay plate and for detection. The assay consists of a monoclonal antibody (labeled with biotin) bound to a streptavidin coated plate which captures protein present on the surface of exosomes (Figure 1). Subsequently, an identical monoclonal antibody (labeled with Europium) is used for detection. Because the capture and detection antibody are identical, they require two linked copies of the same epitope for a signal to be detected. Exosomes provide an ideal structure to link CD9 molecules and allow detection of CD9 in this assay. Exosomes typically have multiple copies of CD9 facing towards the attachment surface and additional CD9 molecules available for detection. Any non specific binding of capture and detection antibodies is unlikely to generate a signal. Using a Europium fluorophore (see box) provides high levels of sensitivity for the assay, which is able to detect small changes in the abundance of the target CD9 protein even within unpurified complex biological samples, such as blood plasma and cerebral spinal fluid.

Fluorophores are chemical substances that emit light following excitation by light or other electromagnetic radiation. The emission of light from a fluorophore is maximal immediately following excitation and decays over a period of time. Time resolved fluorimetry uses fluorophores which have long decay periods. For such fluorophores, measurement of emitted light can be performed when the excitation light is no longer present, thus increasing sensitivity.

Europium is a fluorophore which produces an extended emission decay and has a wide Stokes shift with maximal excitation at 340 nm and peak emission at 615 nm. TRIFic™ assays are Time Resolved Immunofluorescence assays which utilize Europium and have been developed to measure the abundance of CD9 protein specifically associated with exosomes in biological fluids including urine, saliva, cell culture medium, cerebral spinal fluid and blood plasma.

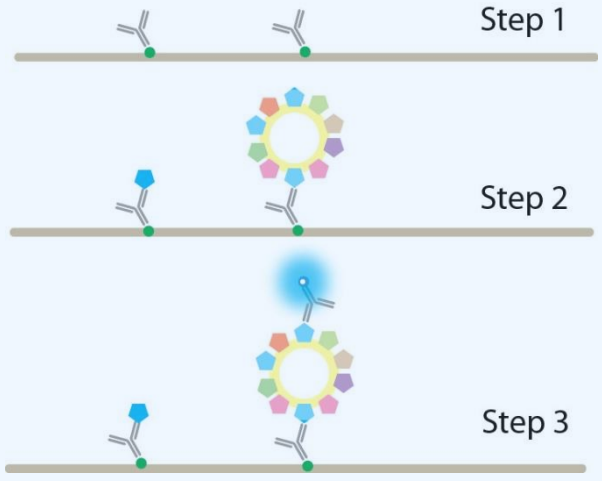


Figure 1. *Schematic for TRIFic™ exosome assay.*

Step 1, Biotinylated antibody is bound to streptavidin coated assay plates.

Step 2, Biological samples are added. Exosomes and any free antigen are captured by the antibody.

Step 3, Europium labeled antibody is added and binds specifically to exosome antigen. The epitopes of bound monomers are already occupied and not detected. Samples are read in a time-resolved fluorescence plate reader.

General Note

The Technical Positive Control provided with the kit can be used to verify that adequate technical and handling procedures have been conducted and a signal can be detected at the end of the protocol. The provided Technical Positive Control cannot be used as a quantification curve for directly quantifying exosomes. Extracellular vesicles of different origin have different CD9 prevalence on their surface.

Suggestion

An internal quantification curve can be obtained for each population of exosomes studied by correlating CD9 TRIFic™ signal intensity with NTA measurements at different concentrations of purified exosomes.

Reagent Preparation

Preparation of the Wash Buffer

Dilute the wash buffer concentrate 25x in MilliQ water (20 ml concentrate in 500 ml MilliQ water). The diluted solution may then be stored at room temperature.

Preparation of the Biotin CD9 in Assay Buffer

[Dilutions should be made on the day the assay is performed]. Briefly centrifuge the vial containing the antibody to gather all the liquid at the base of the vial. Prepare a ~2 ng/µl working solution of biotinylated Ab, by diluting 22 µl (22 µg) in 11 ml of assay buffer.

Preparation of the Europium CD9 in Assay Buffer

[Dilutions should be made on the day the assay is performed]. Briefly centrifuge the vial containing the antibody to gather all the liquid at the base of the vial. Prepare a ~0.25 ng/µl working solution of Eu-labeled CD9 mAb, by diluting 40 µl (2.75 µg) in 11 ml of assay buffer.

Technical Positive Control Preparation

1. 25 µg of exosomes have been purified from cell culture and their total protein concentration determined by Bradford assay. The 25 µg of exosomes are provided in 250 µl PBS, at a concentration of **100 µg/ml**.
2. Prepare 7 microcentrifuge tubes, each with 125 µl of PBS.
3. Prepare dilutions as shown in the table below. Ensure that samples are properly mixed in each tube.

Table 1. Positive control preparation.

Technical Positive Control #	Exosomes	Diluted in PBS (µl)	Exosome concentration (125 µl solution)
1	25 µg (in 250 µl PBS)	250	100 µg/ml
2	125 µl of Tube #1	125	50 µg/ml
3	125 µl of Tube #2	125	25 µg/ml
4	125 µl of Tube #3	125	12.5 µg/ml
5	125 µl of Tube #4	125	6.3 µg/ml
6	125 µl of Tube #5	125	3.1 µg/ml
7	125 µl of Tube #6	125	1.6 µg/ml
8	125 µl of Tube #7	125	0.8 µg/ml

Procedure

Coat the wells with CD9 capture antibody

1. Add 100 µl of the freshly prepared dilute solution (2 ng/µl) of biotin-CD9 antibody (prepared as described above) to each well.
2. Incubate the plate for 1 hour at room temperature on a plate shaker at 750 RPM.
3. Wash the plate using an automatic plate washer. Wash each well three times using 250 µl wash buffer for each cycle.
4. Remove the remaining wash buffer.

Add the sample

5. Clear cells and cellular debris from test samples by centrifuging at 3,000 x g for 20 minutes.
6. Transfer 100 µl of the test sample supernatant to each well. Use 100 µl of PBS instead of sample in order to generate a blank reading.
7. Incubate the plate for 1 hour at room temperature on the plate shaker at 750 RPM.
8. Wash the plate using an automatic plate washer. Wash each well three times using 250 µl wash buffer each time.
9. Remove the remaining wash buffer.

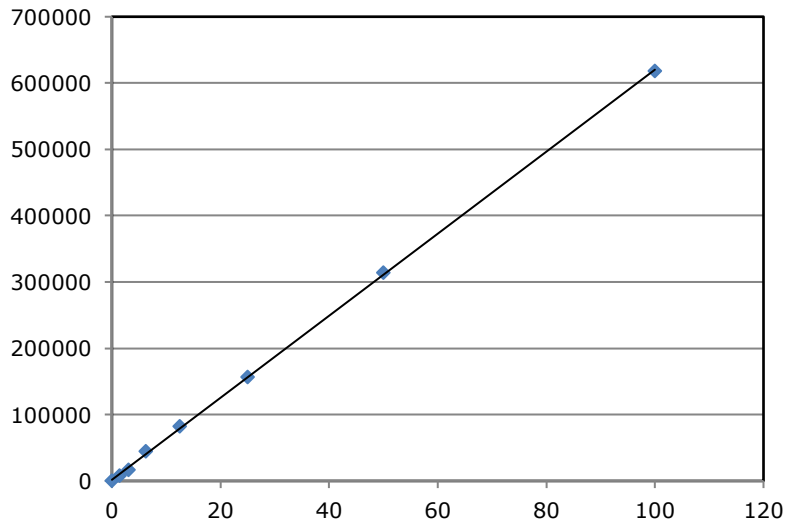
Add the Europium-labeled CD9 detection antibody

10. Add 100 µl per well of the freshly prepared Eu-labeled CD9 antibody dilution (prepared as described above).
11. Incubate the plate for 1 hour at room temperature on the plate shaker at 750 RPM.
12. Wash the plate using an automatic plate washer. Wash each well three times using 250 µl wash buffer each time.
13. Remove the remaining wash buffer.

Signal enhancement and reading

14. Add 100 µl of EFI solution to each well.
15. Incubate the plate for 15 minutes at room temperature on the plate shaker at 750 RPM.
16. Measure fluorescence on a time-resolved fluorescence microplate reader using an excitation wavelength of 340 nm and a measurement wavelength of 615 nm. Measurements should be performed in triplicate. Before taking the readings, make sure that the plate reader is set to read the fluorescence at the bottom of the plate, the integration time is set at 400 µs and the lag time is set at 200 µs.

Technical Positive Control Example



Conc. (µg/ml)	TRF Units
0.75	673
1.5	7520
3.1	16569
6.25	44931
12.5	81920
25	156973
50	314019
100	617999

Figure 2. Example readings obtained using the TRIFic™ exosome assay with the provided technical control sample (exosomes purified from cell culture). Note the linearity of response over a wide range of concentrations.

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