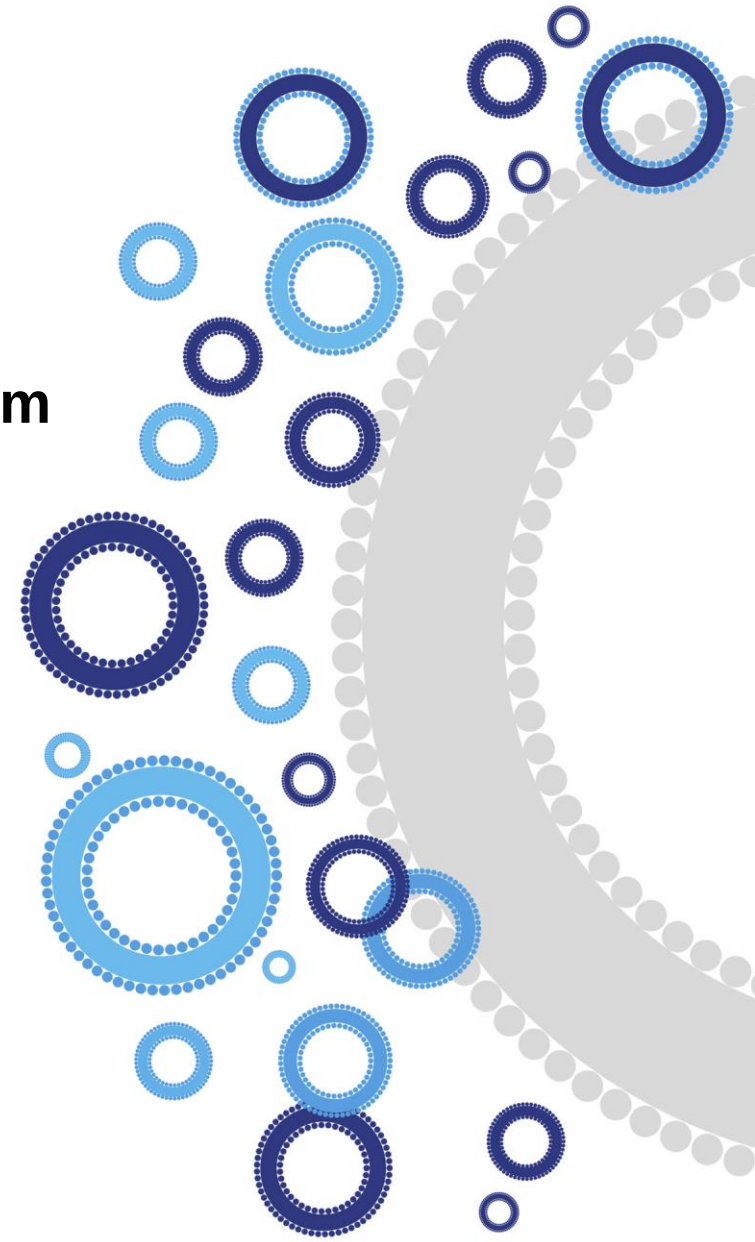
User Guide

CD9 TRIFic™

Exosome Detection System

Europium time-resolved immuno-fluorescence assay for detection of exosome antigens

Cat EX101



Protocol Version 2.4

Contents

CD9 TRIFic™ Exosome Detection System

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

- 1 x Streptavidin-coated 96-well plate (8-well strip format)
- 1 x Technical positive control (100 µg/ml in PBS), 25 µg.

  N.B. The technical positive control is shipped on dry ice in a separate box.
- 1 x Eu-labeled CD9 mAb (in 40 µl TSA buffer, 0.1% BSA), 2.75 µg
- 1 x Biotinylated CD9 mAb (in 22 µl PBS 7.4 pH, 15 mM NaN_3), 22 µg

  The CD9 antibody used in the kit is human specific.
- 1 x Assay buffer, 22 ml
- 1 x Europium fluorescence intensifier (EFI Solution), 11 ml
- 1 x 25x wash buffer, 20 ml
- 1 x User Guide

Equipment and materials required but not supplied with this kit

- Time-resolved fluorescence microplate reader
- Automatic plate washer
- Plate shaker
- Pipettes for dispensing reagents
- Multichannel micropipette reservoir
- Distilled / Milli-Q water
- Phosphate buffered saline (PBS)

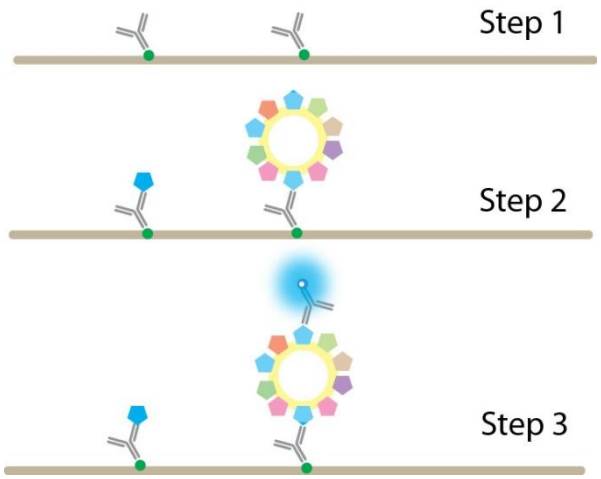
Product information

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 below) 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.



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.

Figure 1. Protocol overview

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 to generate a quantification curve for directly quantifying exosomes. Extracellular vesicles of different origin have different CD9 prevalence on their surface.

If absolute quantification is required, an internal quantification curve can be obtained for each population of exosomes studied by correlating CD9 TRIFic™ signal intensity with Nanoparticle Tracking Analysis (NTA) measurements at different concentrations of purified exosomes.

Reagent preparation

- **Preparation of the wash buffer**: Dilute the wash buffer concentrate 25x in Milli-Q water (20 ml concentrate in 500 ml Milli-Q 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 8 serial dilutions as shown in the table below. Ensure that samples are properly mixed in each tube.
   We recommend beginning with a concentration of 100 µg/ml, as this will ensure that the most diluted sample is within the limit of detection of the assay.

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

See example assay reading on page 9.

Protocol

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. Carefully aspirate 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. Carefully aspirate the remaining wash buffer.

Signal enhancement and reading

14. Add 100 µl of EFI solution to each well.

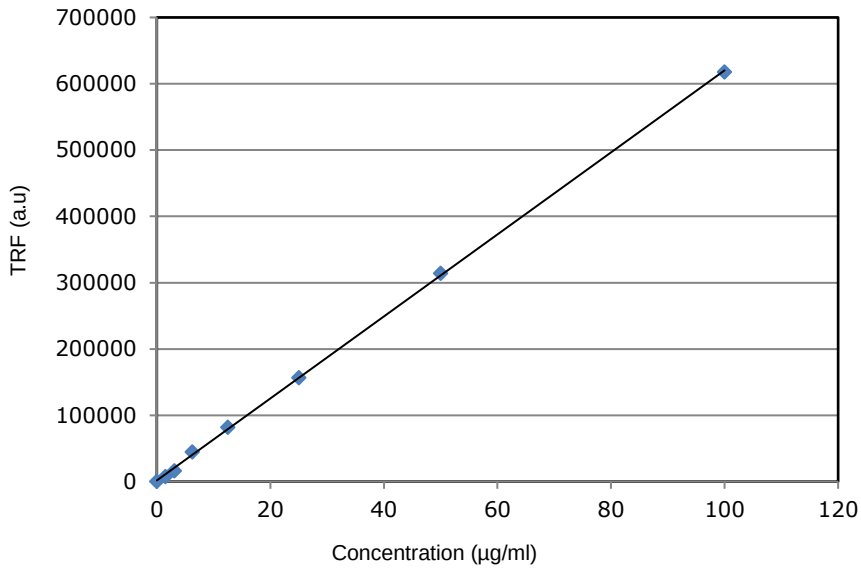
15. Incubate the plate for 15 minutes at room temperature on the plate shaker at 750 RPM.

16. Measure fluorescence on a time-resolved fluorescence microplate reader as shown on the table below. **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.**

Table 2. Instrument settings

Optic settings	**Time resolved fluorescence (TRF)**	
	Filters	Excitation: 340 nm Emission: 615 nm
General settings	Number of flashes	200 with flash lamp
	Settling time	0.3 s
	Integration start/lag time	200 µs
	Integration time	400 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.

Related products

Related products	Product description	Product code
Exosome purification	**Exo-spin™ purification kit**	EX01, EX02, EX03, EX04, EX05
Exosome tracking	**ExoFLARE™ tracking assay**	EX301, EX302, EX303, EX304, EX305, EX306
Nanoparticle Tracking Analysis (NTA) size profiling service	**ZetaView NTA Particle Analysis Service**	ZV-1 and ZV-12

Exo-spin™ purification kit

The Exo-spin™ technology combines precipitation and size exclusion chromatography techniques, making it a superior method for exosome separation and concentration, allowing for high specificity and high recovery of exosomes. Exo-spin™ is available in 5 different configurations represented with catalogue codes EX01, EX02, EX03, EX04, and EX05; specifically designed and optimized for different sample types and downstream applications.

ExoFLARE™ tracking assay

ExoFLARE™ utilizes a combination of a FLARE (FLuorescence Activating Response Element) protein tag together with a pro-fluorophore dye. Neither the protein nor dye exhibit fluorescence in isolation. However, when the protein binds to the dye, it causes a change in structure which results in fluorescence. The dye and protein form an unstable bond with a continuous turnover of the dye, resulting in sustained fluorescence without the levels of photo-bleaching associated with fluorescent proteins (i.e. GFP). This enables ExoFLARE™ to be monitored for extensive periods to allow tracking of dye movement.

NTA size profiling service

Exosome characterization service for analysis of particle size and particle concentration using the ZetaView instrument from Particle Metrix.

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