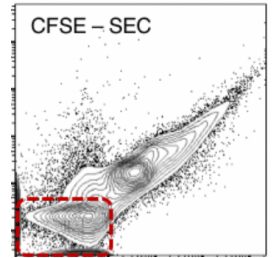


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## Bulk EV staining with CFDA-SE (with NAP-5 purification)

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Aizea Morales Kastresana<sup>1</sup>, Bryce Killingsworth<sup>1</sup>, Jennifer Jones<sup>1</sup>

<sup>1</sup>Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

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Jennifer Jones

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**Protocol status:** Working

**We use this protocol and it's working**

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**Protocol Integer ID:** 35361



**Keywords:** flow cytometry, EVs, CFSE, bulk staining,

## Disclaimer

This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

## Abstract

A protocol for bulk CFSE staining of EVs.

## Guidelines

- When possible CFDA-SE dye and CFSE-stained EVs should be protected from significant light exposure
- Fresh aliquots of CFDA-SE should be prepared from solid CFDA-SE dye every few months to avoid using dye with diminished dying ability due to hydrolysis (however in our lab older aliquots of dye have been used without reduced staining capacity)
- See this link for useful information about handling CFSE and using it for labeling cells for flow cytometry: <https://med.virginia.edu/flow-cytometry-facility/wp-content/uploads/sites/170/2015/10/Protocol-for-CFSE-Labeling.pdf>



## Materials

### MATERIALS

⊗ DPBS Invitrogen - Thermo Fisher Catalog #14190

⊗ DMSO, Anhydrous Thermo Fisher Catalog #D12345

⊗ Vybrant<sup>®</sup> CFDA SE Cell Tracer Kit Thermo Fisher Catalog #V12883

⊗ illustra NAP-5 Columns GE Healthcare Catalog #17085301






#### For preparing CFDA-SE aliquots:

- 1 vial of solid dye from Vybrant CFDA-SE Cell Tracer Kit
- Anhydrous DMSO
- Small volume tubes for aliquoting CFDA-SE (0.5 mL or PCR tubes)
- Large volume tube (5 mL snap tube or 15 mL conical) for diluting CFDA-SE








#### For staining EVs with CFDA-SE:

- 1 aliquot of CFDA-SE
- 1 Nap-5 column
- 1 500 mL bottle of DPBS
- 1 ring stand to hold Nap-5 column
- Eppendorf 0.5 mL and 1.5 mL LoBind tubes
- Preparation of EVs to stain
- Foil or other cover to protect stained EVs from light
- 37 °C water bath or heat block

## Preparing CFDA-SE dye aliquots








- 1 Determine whether or not you need to prepare new aliquots of CFDA-SE dye.
- 2 Let a new vial of solid CFSE-DA dye warm to RT (should take less than 10 minutes.)
- 3 Per the Vybrant™ CFDA SE Cell Tracer Kit instructions, dissolve the solids in the vial in  90  $\mu\text{L}$  anhydrous DMSO (based on the quantity of dye in each vial this creates a  10 millimolar (mM) solution.)
- 4 Dilute the solution to  4500  $\mu\text{L}$  total volume with the same anhydrous DMSO, creating a  200 micromolar ( $\mu\text{M}$ ) solution.
- 5 Aliquot volumes of choice into tubes and freeze at   $-20\text{ }^{\circ}\text{C}$  , under desiccating conditions to prevent dye hydrolysis during storage.

## Dying EVs with CFDA-SE

- 6 Warm one  200 micromolar ( $\mu\text{M}$ ) aliquot of CFDA-SE in your hand, or in a   $37\text{ }^{\circ}\text{C}$  water bath until just thawed.
- 7 Dilute the CFDA-SE with DPBS to a concentration of  40 micromolar ( $\mu\text{M}$ ) , pipetting then vortexing to ensure the DMSO in the aliquot fully mixes with the DPBS.
- 8 Prepare a mixture of EVs in DPBS, diluting the mixture to  50  $\mu\text{L}$  in a 0.5 mL low protein binding tube.
- 9 Pipette  50  $\mu\text{L}$  of  40 micromolar ( $\mu\text{M}$ ) CFDA-SE into the tube with the EV mixture and vortex to mix
- 10 Cover with foil and float onto a   $37\text{ }^{\circ}\text{C}$  water bath for 2 hours, inverting and flicking the tube halfway through the incubation



## Removing free dye with Nap-5 size exclusion column

- 11 Remove top and bottom caps from column and equilibrate with ~  10 mL of DPBS. Ensure that the column is flushed continuously and does not dry
- 12 Mark 2 Eppendorf LoBind 1.5 mL tubes at the 0.5 mL line on the side of the tube with a pen
- 13 Vortex and quickly spin down stained EV mixture
- 14 The moment after the last drop of buffer elutes from the Nap-5 column, load the entire  100  $\mu$ L volume of EVs on top of the gel bed of the column and immediately begin collecting eluate in the first marked 1.5 mL tube.
- 15 The moment after the loaded EV mixture has been absorbed into the Nap-5 gel, gently fill the buffer reservoir at the top of the column to the top, using ~  3 mL of DPBS
- 16 After  0.5 mL of eluate has filled the first 1.5 mL tube, start collecting eluate in the second 1.5 mL tube
- 17 Upon elution of  0.5 mL into the second 1.5 mL tube, the Nap-5 column can be filled with DPBS and flushed
- 18 The first 1.5 mL tube is considered Fractions 1 and 2 and should consist of void DPBS and the second 1.5 mL tube is considered Fraction 3 and 4 and should consist of eluted EVs
- 19 The column should be flushed with ~  10 mL of DPBS then loaded with  3 mL of filtered 20% Ethanol for storage