DIVERSA

Edificio Emprendia, Campus Sur
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(A Coruña)

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DIVERSA FLUOGREEN DELIVERY NANOPARTICLES

Tracking fluorescent DIVERSA for an effective cell internalization

USER PROTOCOL – **#DIV000F1**

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ABOUT THE NANOPARTICLES

OVERVIEW

DIVERSA is a biocompatible, biodegradable, and cell-friendly technology for enhancing intracellular delivery of small molecules and biomolecules, paving the way towards clinical translation.

DIVERSA FLUOGREEN DELIVERY NANOPARTICLES uses strongly labelled fluorescent nanometric emulsions that are easily internalized by live cells. They can be visualized by a wide variety of platforms (flow cytometry, microplate assays, fluorescence, and confocal microscopy) in less than two hours at Ex/Em = 495/503 nm,

DIVERSA FLUOGREEN DELIVERY NANOPARTICLES can be used as a positive control for cell internalization before testing the efficiency of associated molecules in specific cell lines of interest.

DIVERSA FLUOGREEN DELIVERY NANOPARTICLES can also be used as a positive control to normalize the values obtained with other types of delivery systems, as well as with exosomes.

COMPONENTS

- 1x DIV000F1 vial for reconstitution.
- 1x DIVTECH vial for preparation of each DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.
- 2x Tips for 1 mL micropipette.

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, storage is recommended at 4 ºC.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1 mL micropipette.
- Sterile standard microtubes.
- MilliQ water or any other recommended buffer.
- Ethanol (EtOH) 96%.

CONSIDERATIONS BEFORE STARTING

- The following protocol is optimized for the preparation of 1 mL of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES (starting from one DIV000F1 vial for reconstitution).
- DIVERSA cannot guarantee the optimal characteristics of the final formulation if modifications in the protocol are introduced.
- It is recommended to use DIVERSA FLUOGREEN DELIVERY NANOPARTICLES within the following 60 days.
- DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is stable for 24 h in cell culture media at 37 ºC: DMEM and RPMI (with/without FBS).
- Do NOT use any buffer solution containing Triton-X, SDS or Tween-20 for preparation or manipulation of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.
- Do NOT freeze DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.
- Do NOT heat up DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.
- Do NOT centrifuge or vortex DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.

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DIVERSA FLUOGREEN DELIVERY NANOPARTICLES

PROTOCOL

1. Reconstitute the DIV000F1 vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids trying to recover all of them from the vial wall and keep the suspension in the vial.
2. Add 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 1 (Recommendations of Use and Technical Notes).
3. Transfer the whole volume from the DIV000F1 vial to the DIVTECH vial using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids from DIV000F1 vial to the DIVTECH vial, set the micropipette at the maximum volume to have dead air volume in the tip for mixing in a faster and vigorous way. Then, place the 1 mL micropipette tip into the buffer solution of DIVTECH vial, and pipette up and down for 30 seconds, avoiding any spillage.

The DIVERSA FLUOGREEN DELIVERY NANOPARTICLES now is ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 60 days.

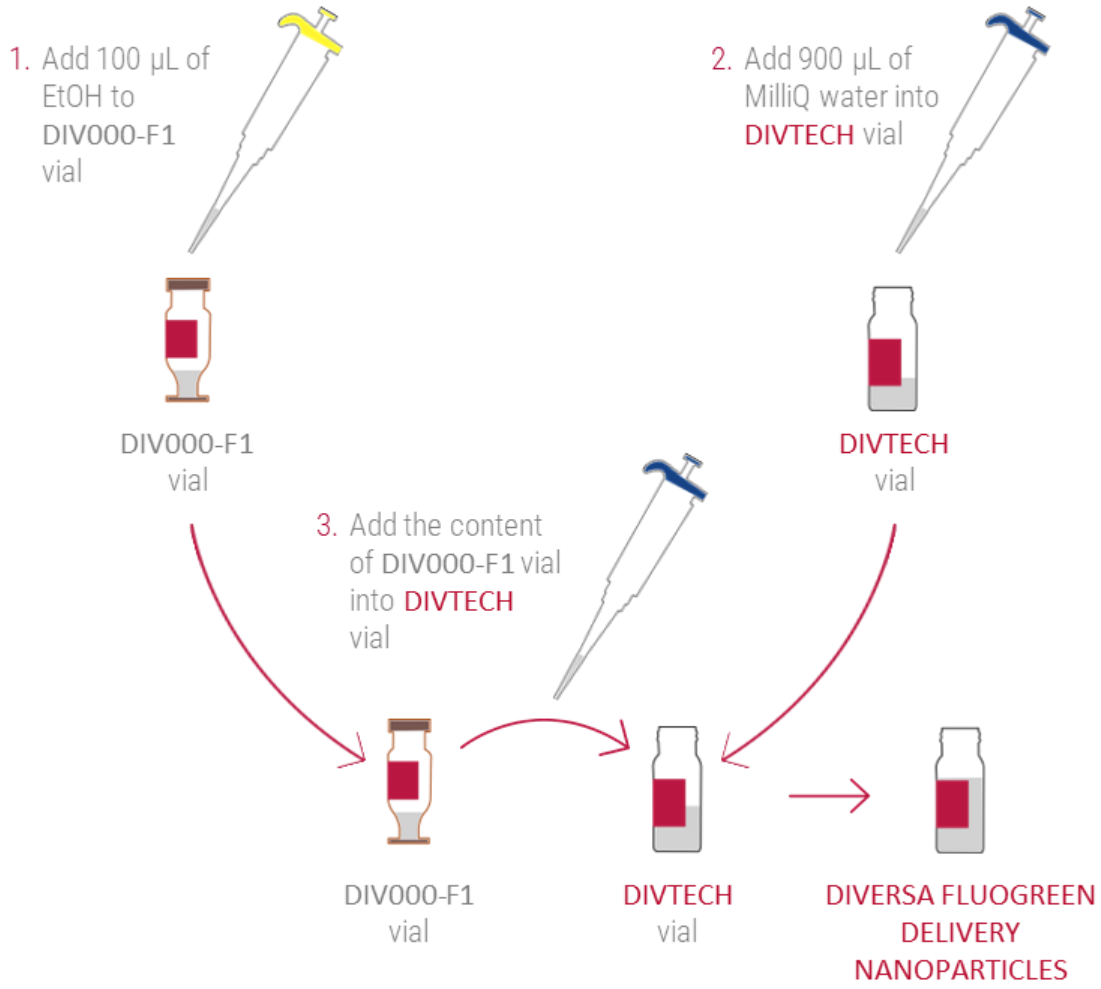

Figure 1. DIVERSA FLUOGREEN DELIVERY NANOPARTICLES protocol.

EXAMPLE OF UPTAKE ASSAY PROTOCOL

1. Seed the recommended number of the cells in a 6-well plate the day before of the uptake experiment for FACS analysis.

   Note: for *in vitro* experiments, the adherent cells must be between 70-80% of confluency on the day of the experiment. However, optimizations should be required depending on the cell type.

2. Reconstitute the DIV000F1 vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids trying to recover all of them from the vial wall and keep the suspension in the vial.
3. Add 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 1 (Recommendations of Use and Technical Notes).
4. Transfer the whole volume from the DIV000F1 vial to the DIVTECH vial using a micropipette and the 1 mL micropipette tip provided.

   IMPORTANT: Before adding the lipids from DIV000F1 vial to the DIVTECH vial, set the micropipette at the maximum volume to have dead air volume in the tip for mixing in a faster and vigorous way. Then, place the 1 mL micropipette tip into the buffer solution of DIVTECH vial, and pipette up and down for 30 seconds, avoiding any spillage.

5. Add 960 µL of fresh cell cultured medium supplemented with 10% (v/v) of FBS and, if necessary, 1 % (v/v) of antibiotics.
6. Add the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES to a final volume of 1 mL.

   Note: The final concentration of the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is calculated considering the final volume of 1 mL.

7. Incubate the cells for 2-4 hours at 37 ºC.

   Note: Depending on the type of readout assay performed, incubation times may influence delivery efficiency.

7. After incubation time: remove the cell culture medium with the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES and, carefully, wash the cells twice with PBS 1X buffer and remove it.

   The DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is efficiently internalized inside the cells.

   Note: we recommend washing the cells with PBS 1X buffer containing calcium and magnesium ions to avoid maximum detachment of living cells.

8. Adjust the concentration for the analysis using the flow cytometer and the following parameters: wavelength of excitation at 495 nm, wavelength of emission at 503 nm.

   Note: we recommend analysis on the same day of the experiment. However, for extended storage (> 16 h), we recommend resuspend the cells in 4% (v/v) of paraformaldehyde to prevent cell deterioration.

RECOMMENDATIONS OF USE & TECHNICAL NOTES

Table 1. Suggested buffer for DIVTECH vial.

BUFFER SOLUTION	CONCENTRATION
Ultrapure water	N/A
PBS	2-50 mM
NaCl	150 mM
HEPES	10-25 mM
DPBS	1X

Table 2. Recommended volumes for cell culture.

Cell culture vessel	Volume of **DIVTECH**	Volume of medium	Final volume/well
100 cm	200 µL	4,8 mL	5 mL
6-well	40 µL	960 mL	1 mL
12-well	20 µL	996 µL	500 µL
24-well	10 µL	240 µL	250 µL
96-well	4 µL	96 µL	100 µL

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What is the concentration of the fluorophore in DIV000F1?	The concentration of the fluorophore is 4 µg/mL in the final DIVERSA FLUOGREEN DELIVERY NANOPARTICLES.
Is the green fluorochrome the only available fluorochrome so far?	Currently, we offer the green fluorochrome. However, you can contact DIVERSA for a customized formulation, where we can incorporate alternative fluorochromes such as Cy5, Cy7.5, and other fluorescent dyes.
Is the fluorochrome pH sensitive?	No, it is not. It can be used at a wide range of pH.
How stable is the signal from the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES?	The green signal of the green fluorochrome is stable for up to 1 year at -20 ºC, according to the manufacturer. Prior to formulation, DIVERSA FLUOGREEN DELIVERY NANOPARTICLES, should be stored at these conditions to preserve the stability. Upon formulation, you can use it for up to 7 days if it is stored at 4 ºC and protected from light. Regarding stability upon addition to cell cultures, we have tracked it in live cells for up to 8 days.
Does the fluorescence of the formulation come from molecules that have any biological effect?	No, it does not. The fluorescence arises from molecules that are covalently linked to the lipids, but have no intrinsic effect. The fluorescence does not interfere with the efficacy of the formulation or the activity of the associated drug/ biomolecule.
How do I concentrate the formulation?	If necessary, the 1 mL of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES can be concentrated by using a SpeedVac or Rotavap in mild conditions (avoid surpassing 35 °C or drying out the samples). Samples can be concentrated up to 4-fold its original volume (i.e., to a final volume 250 µL).
Can I filter the formulation?	Yes, if necessary, DIVERSA FLUOGREEN DELIVERY NANOPARTICLES can be filtered using 0.22 µm filters of PES membrane.
Can I use buffers other than MilliQ water?	Yes, please check Table 1 for other recommended buffers.

How can I measure the size of the final formulation?	Diameter size can be measured by Dynamic Light Scattering (DLS) analysis adding to the cuvette 10 µL of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES with 990 µL of MilliQ water.
Can I use DIVERSA FLUOGREEN DELIVERY NANOPARTICLES for *in vivo* studies?	No, for *in vivo* tracking using whole-body imaging systems, it is necessary to use other fluorophores like Cy7.5. DIVERSA can offer this customized reagent. Other available reagents are labeled formulations with Cy5. For specific recommendations and a customized and optimized prototype, contact DIVERSA. If the purpose is to extract organs/cells and perform analysis by flow cytometry or confocal microscopy, then the answer is yes.

ONLINE RESOURCES

Visit our website www.diversatechnologies.com for further information.

Click here to watch the video of the FORMULATION STEP.