DIVERSA

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DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES

Tracking intracellular delivery of small molecules

USER PROTOCOL - **#DIV010F1**

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ABOUT THE NANOPARTICLES

OVERVIEW

DIVERSA DELIVERY NANOPARTICLES is a biocompatible, biodegradable, and cell-friendly technology for enhancing intracellular delivery of hydrophobic or hydrophilic drugs, paving the way towards clinical translation.

DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES uses strongly labelled fluorescent nanometric emulsions that are easily internalized by live cells that can be visualized by a wide variety of platforms (flow cytometry, microplate assays, fluorescence, and confocal microscopy) in less than 2 h at Ex/Em = 495/503 nm.

DIVERSA FLUOGREEN SMALL MOLECULE 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.

COMPONENTS

- 1x DIV010F1 vials for reconstitution.
- 1x DIVTECH vials for preparation of DIVERSA FLUOGREEN-DRUG NANOPARTICLES.
- 2x Tips for 1 mL micropipette.

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, the 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%.
- Drug of interest.

CONSIDERATIONS BEFORE STARTING

- The following protocol is optimized for the preparation of 1 mL of DIVERSA FLUOGREEN-DRUG NANOPARTICLES (starting from one DIV010F1 vial for reconstitution).
- The drug loading should be optimized regarding the volume used to reconstitute the ethanolic phase of the DIV010F1 vial. For specific recommendations, contact DIVERSA.
- DIVERSA cannot guarantee the optimal characteristics of the final formulation if modifications in the protocol are introduced.
- It is recommended to use DIVERSA FLUOGREEN-DRUG NANOPARTICLES within 24-48 h, considering the stability of the drug.
- DIVERSA FLUOGREEN-DRUG NANOPARTICLES is stable for 6 h in cell culture media at 37 ºC: DMEM and RPMI (with/without FBS).
- Do NOT use any buffer solution containing Triton X-100, SDS or Tween-20 for the preparation or manipulation of DIVERSA FLUOGREEN-DRUG NANOPARTICLES.
- Do NOT freeze DIVERSA FLUOGREEN-DRUG NANOPARTICLES.
- Do NOT heat up DIVERSA FLUOGREEN-DRUG NANOPARTICLES formulation at temperatures higher than 90 ºC for more than 2 h.
- Do NOT centrifuge or vortex DIVERSA FLUOGREEN-DRUG NANOPARTICLES.

DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES

PROTOCOL FOR **HYDROPHOBIC** DRUGS

1. Reconstitute the **DIV010F1** vial with 50 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.

   Note: This volume depends on the solubility of your drug. However, the minimum volume to reconstitute the lipids that we recommend is 20 µL.

2. Add the drug dissolved in one of the solvents suggested in Table 1 (Recommendations of Use and Technical Notes) to **DIV010F1** vial, considering the maximum volume for each solvent.
3. Adjust the volume of the mixture with EtOH up to a final volume of 100 µL.
4. Add 900 µL of ultrapure water into the **DIVTECH** vial or, alternatively, a buffer solution suggested in Table 2 (Recommendations of Use and Technical Notes).
5. Transfer the whole volume from the **DIV010F1** vial containing the drug to the **DIVTECH** vial using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids and drug mixture from **DIV010F1** 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-DRUG NANOPARTICLES** now ready-to-use for *in vitro* and *in vivo* experiments. Alternatively, keep it at 4 ºC and use it in the following 24-48 h.

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.

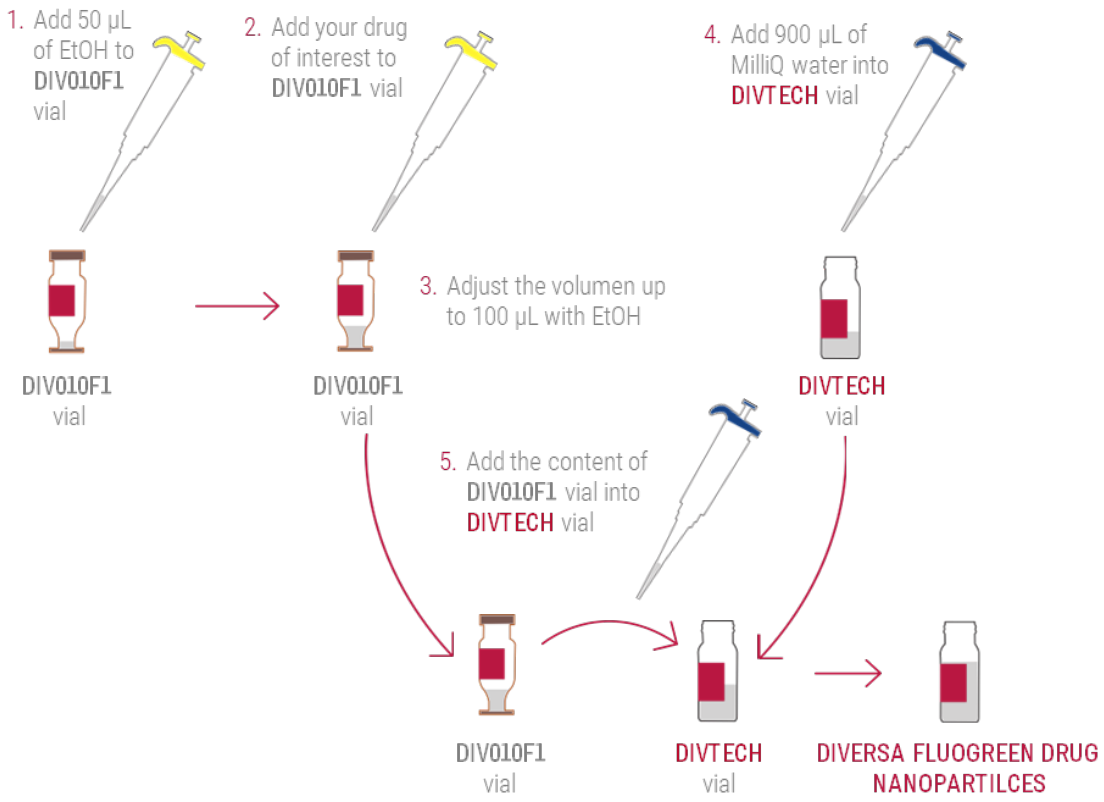

Figure 1. DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES protocol for hydrophobic

PROTOCOL FOR **HYDROPHILIC** DRUGS

1. Reconstitute the DIV010F1 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. Dissolved the drug in 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 2 (Recommendations of Use and Technical Notes).
3. Transfer the whole volume from the DIV010F1 vial the DIVTECH vial containing the drug using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids from DIV010F1 vial to the DIVTECH vial containing the drug, 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-DRUG NANOPARTICLES now ready-to-use for *in vitro* and *in vivo* experiments. Alternatively, keep it at 4 ºC and use it in the following 24-48 h.

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.

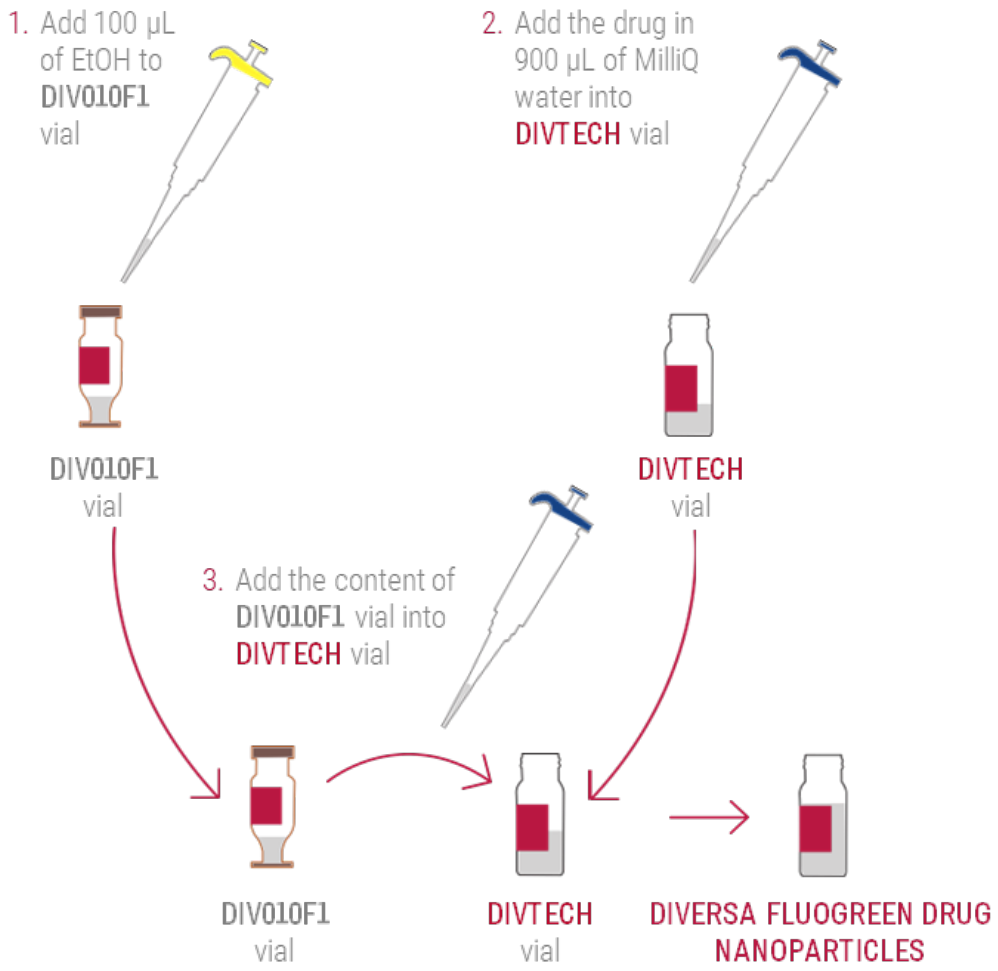

Figure 2. DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES protocol for hydrophilic

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PROTOCOL OPTIMIZATION

PROTOCOL FOR DRUG LOADING OPTIMIZATION

If you do not know the optimal drug loading of your molecule, it is highly recommended to test different drug loadings to get the best DIVERSA NANOPARTICLES performance.

1. Reconstitute the DIV010F1 vial with 50 µL of EtOH for hydrophobic drugs or 100 µL of EtOH for hydrophilic drugs. 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.

   Note: In the case of hydrophobic drugs, the minimum volume to reconstitute the lipids that we recommend is 20 µL.

2. Prepare smaller aliquots from 10 µL or higher as suggested in Table 4 (Recommendations of Use and Technical Notes) depending on the drug loadings you would like to test.

   Example: The total mass of lipids in the DIV010F1 vial is 5.51 mg. In the case of hydrophobic drugs, if you want to test different drug loadings such as 1%, 5%, 10%, 15% and 20% (w/w), you could prepare 5 aliquots of 10 µL, which contain 1.1 mg of total lipid mass. For example, the drug loading of 10% (w/w) will be 110.2 µg of drug, and so on.

   Note: Aliquots can be directly used for drug loading or kept at -20 ºC up to 60 days.

3. From now on, follow the protocol depending on the nature of your drug:
   - For hydrophobic drugs: add the drug dissolved in one of the solvents suggested in Table 1 (Recommendations of Use and Technical Notes) to DIV010F1 vial, considering the maximum volume for each solvent. Then, adjust the volume of the mixture with EtOH up to a final volume of 100 µL. Finally, add 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 2 (Recommendations of Use and Technical Notes).
   - For hydrophilic drugs: dissolved the drug in 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 2 (Recommendations of Use and Technical Notes).

4. Transfer the whole volume from the DIV010F1 vial to the DIVTECH vial using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids from DIV010F1 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-DRUG NANOPARTICLES now ready-to-use for *in vitro* and *in vivo* experiments. Alternatively, keep it at 4 ºC and use it in the following 24-48 h.

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.

EXAMPLE OF ALAMAR BLUE ASSAY CYTOTOXICITY PROTOCOL

1. Seed the recommended number of the cells in a black 96-well plate in 100 µL of complete medium the day before of the cytotoxicity assay.
2. The following day, dilute (if needed) the concentrations of DIVERSA FLUOGREEN-DRUG NANOPARTICLES to be tested (6-8 concentrations are recommended for a dose-response curve) to a final volume of 25 µL.
3. Add this volume to the cells in the 100 µL of complete medium.

   Note$_1$: 4-6 replicates per concentration are recommended.
   Note$_2$: The final concentration of the DIVERSA FLUOGREEN-DRUG NANOPARTICLES in the well represents a 1/5 dilution.
   Note$_3$: if you know the IC50 of your small molecule, you could select 3 higher doses and 3 lower doses. For instance, if the IC50 of the drug is 9 µM, the lower doses could be 0,3 µM, 1 µM and 3 µM and the higher doses could be 27 µM, 81 µM and 143 µM.

4. Add the proper controls:
   - As a negative control (0% cell death): add the same concentration of DIVERSA FLUOGREEN NANOPARTICLES without the drug at the higher dose tested in a final volume of 25 µL.

     Note: you could also use untreated cells, adding the same volume of the media in which the DIVERSA FLUOGREEN-DRUG NANOPARTICLES is resuspended, e.g.: 25 µl/well of ultrapure water or PBS 1X buffer.
   - As a positive control (100% cell death): add Triton 1% (v/v) dissolved in PBS.
   - For the blank (background data): medium and reagent will be placed in one of the empty columns.
5. The incubation times may vary depending on the experiment, from 2 to 72 h.
6. Dilute the Alamar Blue reagent at 10% (v/v) in free-red phenol cell culture medium supplemented with 10% (v/v) of Fetal Bovine Serum.

   Note: We recommend use free red-phenol cell culture medium to avoid signal interference.
7. After incubation time: remove the cell culture medium with the DIVERSA FLUOGREEN-DRUG NANOPARTICLES. Carefully, wash the cells with PBS 1X buffer and remove it.

   Note$_1$: we recommend wash the cells with PBS 1X buffer containing calcium and magnesium ions to avoid maximum detachment of living cells.
8. Add 100 µl of the Alamar Blue reagent at 10% (v/v) per well (Product Ref.: DAL1025, Invitrogen) and incubate for 3-4 h at 37 ºC and protect it from light.

   Note$_2$: We recommend use a multichannel pipette to minimize the variability of the experiment.

9. After incubation time: measure the fluorescence signal using a microplate reader and the following parameters: wavelength of excitation at 570 nm, wavelength of emission at 585 nm.
10. For analysis, background values are subtracted. The cytotoxicity is determined as the ratio between the measured fluorescence values of treated and untreated cells.

EXAMPLE OF UPTAKE ASSAY PROTOCOL

1. Seed the recommended number of the cells in a 24-well plate the day before of the uptake experiment.

   Note: the cell density should be determined for each cell type and well plate.

2. Add 460 µL of fresh cell cultured medium supplemented with 10% (v/v) of FBS and, if necessary, 1 % (v/v) of antibiotics.

3. Add the DIVERSA FLUOGREEN-DRUG NANOPARTICLES to a final volume of 500 µL.

   Note: The final concentration of the DIVERSA FLUOGREEN-DRUG NANOPARTICLES is calculated considering the final volume of 500 µL.

4. 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.

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

    $Note_1$: we recommend wash the cells with PBS 1X buffer containing calcium and magnesium ions to avoid maximum detachment of living cells.

12. For now, you could proceed with the appropriate assay for your desired readout: therapeutic effect of your compound, viability, phenotypic changes in the cells, FACS analysis, fluorescent/confocal microscopy, or a plate reader.

    The DIVERSA FLUOGREEN-DRUG NANOPARTICLES is efficiently internalized inside the cells.

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RECOMMENDATIONS OF USE & TECHNICAL NOTES

Table 1. This table shows the maximum volumes of solvents in which the drugs can be dissolved and incorporated to the DIV010F1 vial.

SOLVENT*	VOLUME
EtOH	Up to 80 µL
DMSO	≤ 25 µL
MeOH	≤ 25 µL
ACN	≤ 25 µL
Acetone	≤ 25 µL
Chloroform	≤ 10 µL

Solvent of preference: EtOH.

Note: kept the drug concentration in the preparation (1 mL) lower than 0.75 mg/mL, irrespective of the solvent.

Table 2. 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 3. 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

Table 4. Recommended volumes to reconstitute the ethanolic phase of the DIV010F1 vial and the aqueous phase of the DIVTECH vial when hydrophobic drug loading optimization is required.

DIV010F1 vial reconstitution volume	Hydrophobic drug solvent volume	DIVTECH vial	Total volume of formulation	Micropipette for formulation
5 µL	5 µL	90 µL	100 µL	2-20 µL
10 µL	10 µL	180 µL	200 µL	20-200 µL
25 µL	25 µL	450 µL	500 µL	

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What is the concentration of the fluorophore in DIV010F1?	The concentration of the fluorophore is 4 µg/mL in the final DIVERSA FLUOGREEN NANOPARTICLES.
How do I concentrate the formulation?	If necessary, the 1 mL of DIVERSA FLUOGREEN -DRUG NANOPARTICLES can be concentrated by using a SpeedVac or Rotavap in mild conditions (avoid overpassing 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 -DRUG NANOPARTICLES can be filtered using 0.22 µm filters of PES membrane.
What should I do if my drug is not soluble in EtOH?	You can use any solvent listed in Table 1. You can also sonicate DIV010F1 vial containing the drug prior addition to the DIVTECH vial.
Can I use buffers other than milliQ water?	Yes, please check Table 2 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-DRUG NANOPARTICLES with 990 µl of MilliQ water.
Can I use DIVTECH for *in vivo* studies?	Yes, DIVERSA FLUOGREEN-DRUG NANOPARTICLES can be used *in vivo*. For specific recommendations and a customized and optimized prototype, contact DIVERSA.

ONLINE RESOURCES

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