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 are a biocompatible, biodegradable, and cell-friendly technology designed to enhance the intracellular delivery of small molecules, paving the way for clinical translation.

DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES are optimized for hydrophobic and amphiphilic small molecules (Fig. 1), providing an efficient method for drug delivery with higher loading capacity than conventional liposomes. These nanoparticles are easily internalized by cells and can penetrate more complex structures, such as 3D cell cultures and organoids. Additionally, they can be adapted to various routes of administration for evaluation in animal models, maximizing targeted biodistribution and enhancing their therapeutic effect. Contact DIVERSA for specific recommendations for *in vivo* experiments.

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 formulation can be used as a positive control for cell internalization and for testing the efficiency of associated molecules in specific cell lines of interest.

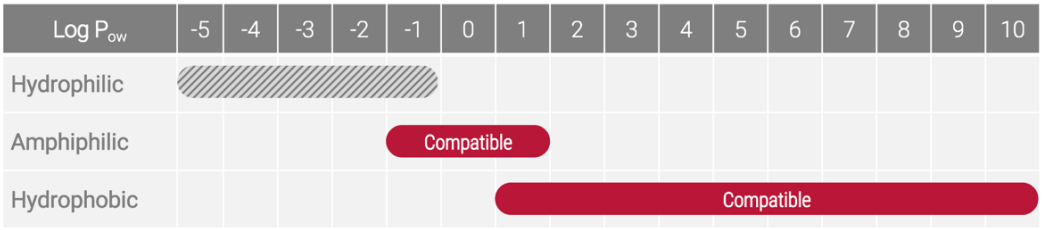

Figure 1. Small molecule Log P_{ow} compatibility with DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES for hydrophobic and amphiphilic drugs.

COMPONENTS

- 1x DIV010F1 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.
- 2x Tips for 1 mL micropipette.

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, the drug-loaded preparation should be stored at 2-8 ºC for up to 48 hours.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1 mL micropipette.
- Amber 0.6 mL microtubes.
- Ultrapure water.
- Ethanol (EtOH) 96%.
- Drug of interest.

CONSIDERATIONS BEFORE STARTING

- The following protocol is optimized for the preparation of 1 mL of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.
- DIVERSA cannot guarantee the optimal formulation performance if any modifications are made to the protocol.
- You may need to determine first the optimal loading capacity for your small molecule by testing different concentrations. We recommend using the DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES, which includes four vials to test different loadings before proceeding with DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES. Alternatively, you can empirically select a loading based on previous successful results with our nanoparticles (see Table 3).
- It is recommended to use DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES within 24-48 hours of preparation for optimal performance.
- DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES are stable in cell culture media under the following tested conditions: for at least 24 h at 37 ºC in DMEM and RPMI, supplemented with 10% (v/v) of FBS and 1% (v/v) of penicillin/streptomycin.
- Do NOT use any buffer solution containing Triton X-100, SDS or Tween-20 for the preparation and manipulation of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.
- Once formulated, do NOT freeze DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.
- Do NOT heat over 90ºC DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.

DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES

PROTOCOL

1. Add 20 µL of EtOH into the DIV010F1 vial. Gently pipette up and down.
2. Add the dissolved drug to the previous DIV010F1 vial.

   Note: The maximum volume allowed for the different solvents containing the dissolved drug is indicated in Table 1.
3. Adjust the volume of the DIV010F1 vial with EtOH to reach a final volume of 100 µL.
4. Add 900 µL of ultrapure water to the DIVTECH vial.
5. Transfer the entire volume from the DIV010F1 vial to the DIVTECH vial using a 1 mL micropipette and the provided tip.

   $Note_1$: Before adding the volume from the DIV010F1 vial into the DIVTECH vial, set the micropipette at the maximum volume and add the solution with a sudden, vigorous downward motion. Pipette up and down for 5-10 times with confidence.

   $Note_2$: It's important to evaluate the selected drug loading based on the acceptance criteria provided in Table 3. This will help ensure the chosen loading is appropriate for your application.

The DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES are now ready to use or can be stored at 2-8 ºC for 24-48 hours.

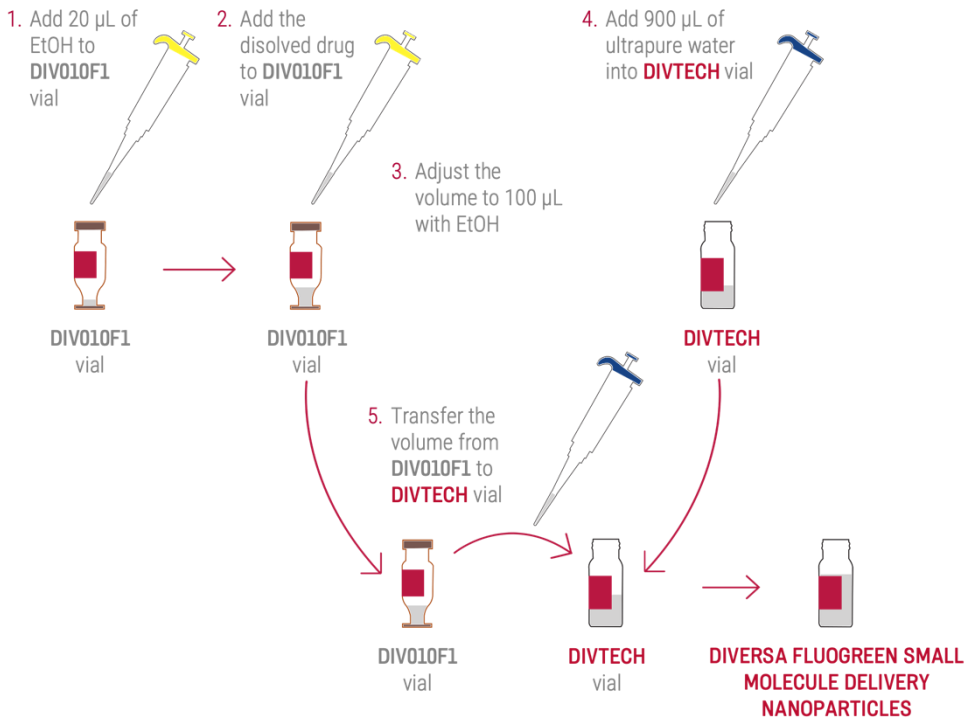

Figure 1. DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES protocol.

CITOTOXICITY ASSAY

EXAMPLE PROTOCOL

1. Seed the recommended number of cells in 96-well plate with 100 µL of complete medium the day before conducting the cytotoxicity assay.

   Note$_1$: Optimizations should be performed depending on the cell type and the length of the experiment.

   Note$_2$: Cell culture medium supplemented with 10% (v/v) of FBS and 1% (v/v) of Penicillin-Streptomycin is recommended.

   Note$_3$: When using Alamar Blue, we recommend using black plates.

2. Select the concentrations of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES to be tested and prepare the corresponding working volumes.

   Note: We recommend using 6-8 different concentrations for a dose-response curve, with 4-6 replicates for each concentration.

   Important: to set-up a range of concentrations, DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES can be concentrated as indicated in the FAQs. DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES can also be diluted, preferably in ultrapure water.

3. On the following day, add a fixed volume of the different concentrations of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES (e.g., 25 µL) to the cells previously seeded in 100 µL of complete medium. There is no need to replace the medium prior to the addition of the nanoparticles.

   Note: Add the same fixed volume of ultrapure water to the control cells to ensure similar dilution of the cell culture medium.

   Recommended controls:

   - Add 1% (v/v) Triton X-100 as a positive control (100% cell death).
   - For the blank, place the medium in one of the empty columns (no seeded cells) to obtain background.
   - Formulations prepared following the formulation protocol but without the drug (blank formulations) can also be added under the same conditions for control purposes.

4. The incubation times may vary depending on the experiment.
5. Various cell viability assays, such as Alamar Blue and MTT, can be performed according to the provider's protocol to assess cell viability.

CELL INTERNALIZATION ASSAY

EXAMPLE PROTOCOL

1. Seed the recommended number of the cells in a 24-well plate, chamber, or similar device, the day before the experiment.

   Note$_1$: Optimizations should be performed depending on the cell type and the length of the experiment.

   Note$_2$: Cell culture medium supplemented with 10% (v/v) of FBS and 1% (v/v) of Penicillin-Streptomycin, is recommended.

2. The day of the experiment, remove the medium for the wells, add 225 µL of fresh culture medium and 25 µL of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES.

   Note$_1$: The volume of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES should be adjusted based on your specific drug and loading used. Consider testing different volumes.

   Note$_2$: If different formats are used, adjust the volumes accordingly (see suggestions in Table 4.

3. Incubate the cells at 37 ºC in a CO_2 incubator under standard conditions for at least 2-4 hours.

   Note: Depending on the type of readout assay performed, shorter or longer incubation time may influence delivery efficiency.

4. After incubation, remove the medium, wash the cells twice with DPBS 1X and proceed with the appropriate assay for your desired readout (FACS analysis, fluorescent/confocal microscopy, or a plate reader).

   Note: We recommended washing the cells with DPBS 1X buffer containing calcium and magnesium ions to avoid maximum detachment of living cells.

5. Adjust the equipment settings to visualize DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES, setting the excitation wavelength to 495 nm and the emission wavelength to 503 nm.

TABLES & TECHNICAL NOTES

Table 1. Maximum volumes of solvents suitable for dissolving the drugs that can be incorporated into the DIV010F1 vial.

SOLVENT	VOLUME
EtOH (preferred)	Up to 80 µL
DMSO	≤ 25 µL
MeOH	≤ 25 µL
ACN	≤ 25 µL
Acetone	≤ 25 µL
Chloroform	≤ 10 µL

Table 2. Parameters for selecting the optimal drug loading of the Formulation*.

VISUAL PARAMETER	OUTCOME
The suspension is uniform with no visible aggregates	Acceptable
The suspension appears slightly opalescent or milky, typical of nanoparticle formulations	Acceptable
The formulation is homogeneous with no visible precipitation	Acceptable
The formulation becomes transparent	Not acceptable
Two distinct phases can be observed	Not acceptable
Precipitates or clumps appear	Not acceptable

* For deeper characterization using specialized equipment and methodologies, please contact DIVERSA.

Drug	MW (g/mol)	Log P_{ow}	Mass (mg)	% Loading (w/w)	Molar (mM)	% EE
Resveratrol	228.20	3.1	0.03	0.5%	0.13	>90%
Disulfiram	296.54	3.9	0.75	15%	2.53	>90%
Curcumin	368.40	3.2	0.03	0.5%	0.08	>98%
Galunisertib	369.42	2.4	0.75	15%	2.03	>90%
Simvastatin	418.56	4.68	0.4	7%	1	>99%
Methotrexate	454.44	-1.85	0.05	2%	0.04	>99%
Doramapimod	527.66	5.7	0.75	15%	1.42	>99%
Oleuropein	540.51	-0.4	0.75	15%	1.39	>99%
Doxorubicin	543.52	1.3	1	20%	1.84	>90%
Etoposide	588.56	0.6	0.05	1%	0.09	>80%
Paclitaxel	853.91	2.5	0.05	1%	0.06	>80%
Rose Bengal	973.67	8.5	1	20%	1.03	>95%

Table 3. Examples of different small molecules with different molecular weights (MW) and Log P_{ow} values successfully encapsulated in DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES. This table can guide the selection of potential drug loading concentrations to optimize the formulation of your small molecule.

Table 4. Recommended volumes for cell culture for an incubation time of 2-4 hours.

Cell culture vessel	Volume of DIVERSA NANOPARTICLES	Volume of medium	Final volume/well
100 cm	500 µL	4,5 mL	5 mL
6-well	100 µL	900 µL	1 mL
12-well	50 µL	450 µL	500 µL
24-well	25 µL	225 µL	250 µL
96-well	10 µL	90 µL	100 µL

*If DIVERSA FLUOGREEN NANOPARTICLES are incubated for more than 4 hours, it is recommended to double the volumes of the nanoparticles and medium.

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 SMALL MOLECULE DELIVERY NANOPARTICLES.
Is the green fluorochrome the only available option?	Currently, we just offer the green fluorochrome, but DIVERSA can provide custom formulations with alternative dyes such as Cy5, Cy7.5, and others.
Is the fluorochrome pH-sensitive?	No, it is stable across a wide pH range.
Does the fluorochrome affect the biological activity?	No, it does not. The fluorochrome is covalently linked to the lipids and does not interfere with the biological activity of the cells.
How do I concentrate the formulation?	DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES can be concentrated using an Amicon Ultra Centrifugal Filter. Samples can be concentrated up to 4-fold their original volume (i.e., to a final volume 250 µL).
Can I filter the formulation?	Yes, DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES can be filtered using 0.22 µm PES membrane filter if needed.
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.
How can I measure the size of the final formulation?	Measure particle size using Dynamic Light Scattering (DLS) by adding to the cuvette 100 µl of DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES with 900 µl of ultrapure water.
Can I use DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES for *in vivo* studies?	DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES can be used for cell or organ analysis by flow cytometry or confocal microscopy, and we offer customized solutions to adjust fluorophore concentration as needed. For whole-body imaging, DIVERSA provide customized reagents labeled with fluorophores such as Cy7.5, tailored to your experimental settings.

ONLINE RESOURCES

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

Click here to watch the video on preparing **DIVERSA FLUOGREEN SMALL MOLECULE DELIVERY NANOPARTICLES**.

CHANGELOG

Version	Date	Change Description
1.0	1 APR 2022	Initial release of the protocol.
2.0	1 NOV 2024	Updated loading instructions; clarified visual parameters for nanoparticle formulation evaluation; improved readability and flow of the protocol for ease of use.