DIVERSA

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DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES

Enhancing intracellular delivery of small molecules

USER PROTOCOL - **#DIV010**

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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 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.

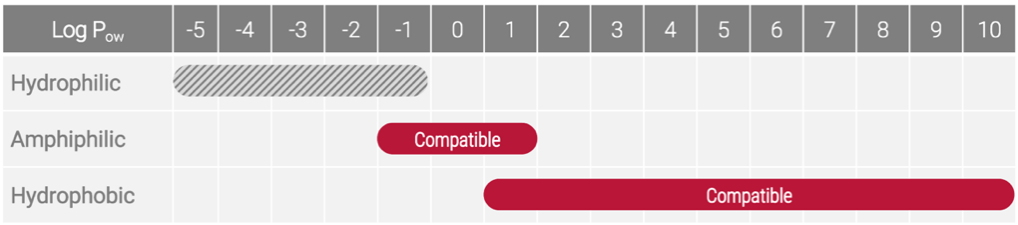

Figure 1. Small molecule Log P_{ow} compatibility with DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES for hydrophobic and amphiphilic drugs.

COMPONENTS

- 4x DIV010 vials for reconstitution.
- 4x DIVTECH vials for preparation of DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES.
- 8x 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.
- 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 SMALL MOLECULE DELIVERY NANOPARTICLES, starting from one DIV010 vial. The box contains four DIV010 vials, allowing for four separate preparations.
- DIVERSA cannot guarantee the optimal formulation performance if any modifications are made to the protocol.
- It is recommended to use DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES within 24-48 hours of preparation for optimal performance.
- DIVERSA 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 SMALL MOLECULE DELIVERY NANOPARTICLES.
- Once formulated, do NOT freeze DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES.
- Do NOT heat over 90 ºC DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES.

DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES

PROTOCOL

1. Add 20 µL of EtOH into the **DIV010** vial. Gently pipette up and down.
2. Add the **dissolved drug** to the previous **DIV010** vial.

   Note$_1$: The maximum volume allowed for the different solvents containing the dissolved drug is indicated in Table 1.

   Note$_2$: The volume of dissolved drug, and therefore the total amount of drug for loading, may need to be adjusted. Please refer to Drug Loading Optimization Protocol for guidance.
3. Adjust the volume of the **DIV010** 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 **DIV010** vial to the **DIVTECH** vial using a 1 mL micropipette and the provided tip.

   Note: Before adding the volume from the **DIV010** 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.

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

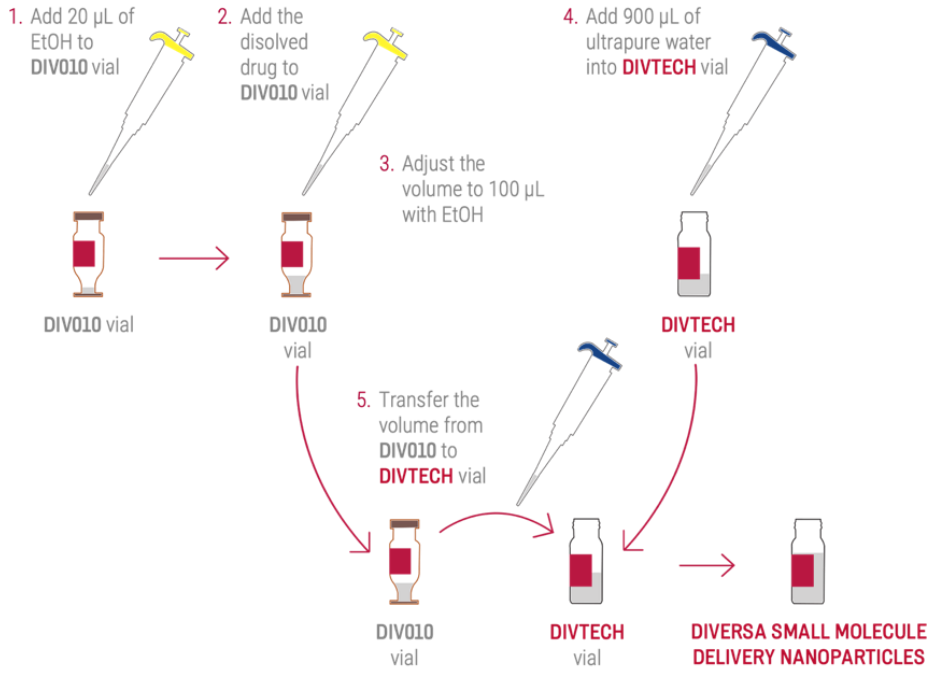

Figure 2. **DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES formulation** protocol.

DRUG LOADING OPTIMIZATION

PROTOCOL

Determine the optimal loading capacity in a reduced formulation size of 200 µL:

1. Add 55 µL of EtOH into the **DIV010** vial. Gently pipette up and down.
2. Divide the content of the **DIV010** vial into aliquots of 10 µL using 0.6 mL microtubes.
3. Add different concentrations of **dissolved drug** to the 0.6 mL microtube aliquots to test various drug loadings.

   Note$_1$: The maximum volume allowed for different solvents containing the dissolved drug is indicated in Table 2.

   Note$_2$: Drug loading is calculated as a percentage of the total lipid mass in each preparation. Each aliquot contains 1 mg of total lipid mass. **Example**: A drug loading of 10% (w/w) would correspond to 100 µg of drug.

   Note$_3$: We recommend an initial assay with 1%, 2.5%, and 5% drug loading. If the formulations are acceptable (please refer to Table 3 for guidance), a subsequent set of experiments can involve testing 10%, 15%, and 20% drug loading.
4. Adjust the volume of the 0.6 mL microtube containing the dissolved drug with EtOH to reach a final volume of 20 µL.
5. Add 180 µL ultrapure water to an empty 0.6 mL microtube.
6. Transfer the 20 µL volume of lipids and dissolved drug to the microtube containing 180 µL of aqueous solution using a 20-200 µL micropipette.

   Note: Before adding the volume containing the lipids and the drug to the 180 µL, 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.

With each drug loading tested, an evaluation must be conducted based on the acceptance criteria outlined in Table 3 to select the optimal loading for the formulation. Loadings that meet these criteria can be used in experiments or scaled up to prepare a larger formulation of 1 mL, following the previous 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 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 SMALL MOLECULE DELIVERY NANOPARTICLES can be concentrated as indicated in the FAQs. DIVERSA 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 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.

TABLES & TECHNICAL NOTES

Table 1. Maximum volumes of solvents suitable for dissolving the drugs that can be incorporated into the DIV010 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. Maximum volumes of solvents suitable for dissolving the drugs that can be incorporated into the 10 µL aliquots of DIV010 for the Drug Loading Optimization Protocol.

SOLVENT	VOLUME
EtOH (preferred)	Up to 20 µL
DMSO	≤ 5 µL
MeOH	≤ 5 µL
ACN	≤ 5 µL
Acetone	≤ 5 µL
Chloroform	≤ 2 µL

Table 3. 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.

Table 4. 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.

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%

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
How do I concentrate the formulation?	DIVERSA 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 SMALL MOLECULE DELIVERY NANOPARTICLES can be filtered using 0.22 µm PES membrane filters if needed.
What should I do if my drug is not soluble in EtOH?	You can use any solvent listed in **Table 1** and **Table 2**. You can also sonicate DIV010 vial containing the drug prior addition to the DIVTECH vial.
How can I determinate the amount of drug that can be loaded?	Optimization steps are outlined in the protocol, please, refer to it and check. If you have specific questions, please, contact DIVERSA because we also offer characterization services, customized nanoparticles and co-development services under request.
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 SMALL MOLECULE DELIVERY NANOPARTICLES with 900 µl of ultrapure water.
Can I use DIVERSA SMALL MOLECULE DELIVERY NANOPARTICLES for *in vivo* studies?	DIVERSA SMALL MOLECULE DELIVERY 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.

Click here to watch the video on preparing DIVERSA SMALL MOLECULE 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.