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DIVERSA mRNA DELIVERY NANOPARTICLES

DIVERSA lipid nanoparticles for promoting effective intracellular transfection of mRNA.

USER PROTOCOL – **#DIV053**

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ABOUT THE NANOPARTICLES

OVERVIEW

DIVERSA's nanoparticles are a biocompatible, biodegradable, and cell-friendly technology designed to enhance the intracellular delivery of nucleic acids, paving the way for clinical translation.

DIVERSA's nanoparticles demonstrate exceptional efficacy, ensuring safe mRNA delivery while maintaining cell integrity, standing out from viral and cationic-based transfection methods. They can accommodate a wide range of mRNA sizes (900 – 4500 nt), expanding research possibilities. For different size requirements, additional information is available in our FAQs.

DIVERSA's 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.

COMPONENTS

- 4x **DIV053** vial for reconstitution.
- 4x **DIVTECH** vial for preparation of DIVERSA's nanoparticles.
- 4x sterile, non-toxic, pyrogenic-free polypropylene 1 mL syringes.
- 4x 21G ½ sterile needles (0.8 x 38 mm).

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, the mRNA-loaded nanoparticles should be storage at 2-8 ºC.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1.5 mL sterile RNase-free microtubes
- 0.6 mL sterile RNase-free microtubes
- 2 mL sterile RNase-free microtubes
- 15 mL sterile RNase-free tube
- Amicon Ultra Centrifugal Filter
- 0.22 µm filter of PES membrane
- RNase contamination remover (e.g. RNaseZAP or RNase AWAY Surface Decontaminant)
- RNase-free water (Molecular Grade)
- Dulbecco's phosphate-buffered saline 1X (DPBS) (without calcium and magnesium)
- Ethanol (EtOH) 96%
- mRNA of interest
- Citrate buffer 10 mM at pH3 (10 mL):
  - Weight 2.8 mg of Sodium Citrate Tribasic Dihydrate (CAS No: 6132-04-3) and 17.4 mg of Citric Acid Monohydrate (CAS No: 5949-29-1).
  - Dissolve both components in 8 mL of RNase-free water (Molecular Grade).
  - Measure the pH and, if necessary, adjust to pH 3. Clean the sensor to avoid contamination by RNAses.
  - Adjust the final volume to 10 mL with RNAse-free water.
  - Store at 2-8 ºC in an RNAse-free container.

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CONSIDERATIONS BEFORE STARTING

- The following protocol is optimized for the formulation of 5 µg of mRNA, starting from one **DIV053** vial. A protocol for the formulation of 1 µg of mRNA is also provided. The box contains four **DIV053** vials, allowing four separate nanoparticle preparations.
- DIVERSA cannot guarantee optimal formulation performance if any modifications are made to the protocol.
- It is recommended to use DIVERSA's nanoparticles within 48 hours of preparation for optimal performance.
- Transfection with DIVERSA's nanoparticles is stable in supplemented cell culture media for at least 24 h at 37 ºC.
- Do not use any buffer solution containing Triton-X, SDS, or Tween-20 for the preparation of DIVERSA's nanoparticles.
- Once formulated, do NOT freeze DIVERSA's nanoparticles.
- Do NOT heat over 90ºC DIVERSA's nanoparticles.

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DIVERSA mRNA DELIVERY NANOPARTICLES

PROTOCOL for the encapsulation of 5 µg of mRNA

Click here to view the video on the preparation of **DIVERSA mRNA DELIVERY NANOPARTICLES**.

Clean the workspace and micropipettes before starting with 70% EtOH, followed by RNase contamination remover solution.

1. Add 300 µL of EtOH into the **DIV053** by inserting the needle through the septum using the provided syringe and needle. Then vortex the vial.

   Note$_1$: DO NOT remove the metal cap from the vial to avoid spilling.

   Note$_2$: You will need the same syringe and needle for step 4. Do not discard them.

2. Add 895 µL of 10 mM Citrate Buffer (pH 3) and 5 µL of mRNA to the **DIVTECH** vial.

   Note$_1$: Prepare just a prior injection and avoid leaving the mRNA citrate phase at RT.

   Note$_2$: The specified volumes are based on an mRNA stock concentration of 1 mg/mL. Please adjust accordingly for different stock concentrations.

3. Remove the metal cap from the **DIV053**. Take the volume from the **DIV053** vial reusing the same syringe and needle from step 1. Before injection, ensure an approximately 0.3 mL air gap in the syringe. Then, inject the volume into the **DIVTECH** vial containing the mRNA with a sudden, vigorous downward motion, resulting in a final volume of 1.2 mL of **DIVERSA**'s nanoparticles.

4. Leave the **DIVTECH** vial open for 35 min at room temperature (RT), protected from light.

5. Transfer the 1.2 mL of **DIVERSA**'s nanoparticles from the **DIVTECH** vial to a 15 mL sterile RNase-free tube containing 6 mL of DPBS 1X at 4 ºC.

   Subsequently, either proceed with the CONCENTRATION PROTOCOL or store the diluted formulation at 4 ºC for later use.

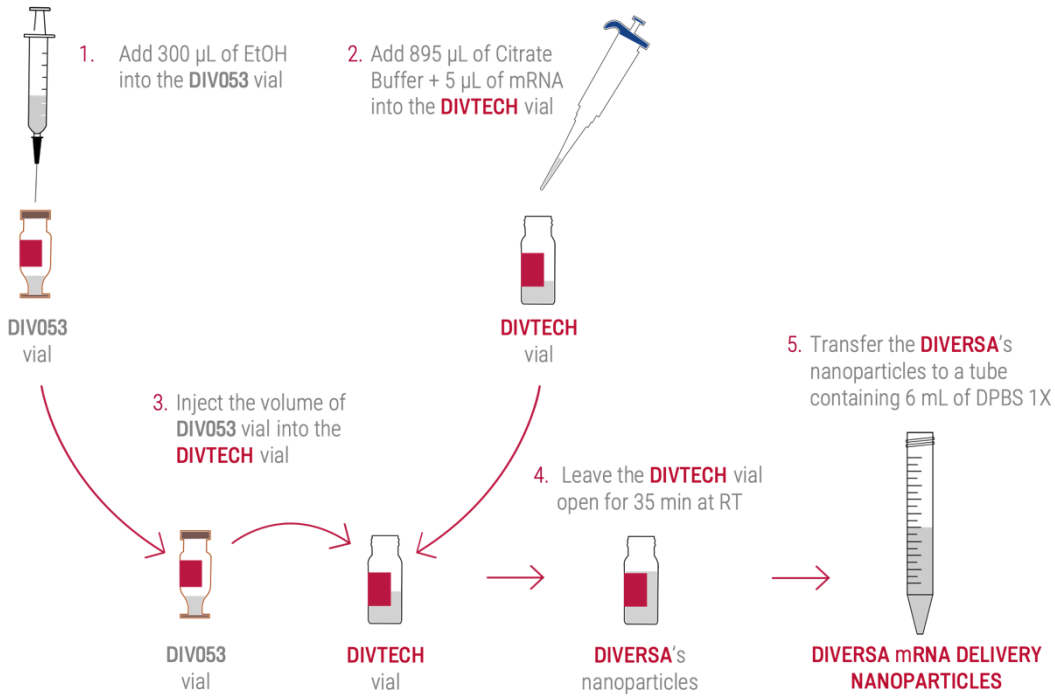

Figure 1. DIVERSA mRNA DELIVERY NANOPARTICLES. Formulation protocol.

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PROTOCOL for the encapsulation of 1 µg of mRNA

Please note that the variability in the formulation increases when working with smaller mRNA quantities. Ensure careful handling to maintain consistency in nanoparticle formation and performance.

1. Inject 310 µL of EtOH into the **DIV053** by inserting the needle through the septum using the provided syringe and needle. Then vortex the vial.

   Note: DO NOT remove the metal cap from the vial to avoid spilling.

2. Remove the metal cap from the **DIV053**. Divide the volume of **DIV053** vial into 5 aliquots of 60 µL in 0.6 mL sterile RNase-free microtubes.

   Note: Unused **DIV053** aliquots can be stored at 4ºC for up to 2 weeks. Ensure the microtubes are tightly closed to prevent evaporation of ethanol.

3. Add 175 µL of 10 mM Citrate Buffer (pH 3) and 5 µL of mRNA in 0.6 mL sterile RNase-free microtubes.

   $Note_1$: Prepare just a prior injection and avoid leaving the mRNA citrate phase at RT.

   $Note_2$: The specified volumes are based on an mRNA stock concentration of 0.2 mg/mL. Please adjust accordingly for different stock concentrations.

4. Using a 200 µL micropipette, add 60 µL from the previously reconstituted **DIV053** vial into the 0.6 mL sterile RNase-free microtube containing the citrate buffer solution and the mRNA, resulting in a final volume of 240 µL of **DIVERSA**'s nanoparticles.

   Note: Before adding the volume from the **DIV053** vial into the microtube, set the micropipette at the maximum volume and add the solution with a sudden, vigorous downward motion. Pipette up and down several times with confidence to ensure proper mixing and nanoparticle formation.

5. Leave the microtube containing the **DIVERSA**'s nanoparticles open for 35 min at room temperature (RT), protected from light.

6. Transfer the 240 µL of **DIVERSA**'s nanoparticles to a 2 mL sterile RNase-free microtube containing 1.760 mL of DPBS 1X at 4 ºC.

   Subsequently, either proceed with the CONCENTRATION PROTOCOL or store the diluted formulation at 4 ºC for later use.

CONCENTRATION PROTOCOL

Select the most appropriate Amicon Ultra Centrifugal Filter.

Note: A molecular weight cut-off (MWCO) between 10 kDa and 100 kDa is preferred. The total volume of the diluted formulation will depend on the selected formulation protocol.

- **PROTOCOL** for the encapsulation of 5 µg of mRNA. If possible, select a filter with a capacity greater than 7.2 mL. If the capacity is lower, the diluted formulation (from step 5, FORMULATION PROTOCOL) can be added in multiple steps. In such cases, the formulation must always be kept at 4ºC during the waiting period.

- **PROTOCOL** for the encapsulation of 1 µg of mRNA. If possible, select a filter with a capacity greater than 2 mL. If the capacity is lower, the diluted formulation (from step 6, FORMULATION PROTOCOL) can be added in multiple steps. In such cases, the formulation must always be kept at 4ºC during the waiting period.

1. Equilibrate the membrane of the Amicon Ultra Centrifugal Filter by adding 1X DPBS at 4 ºC, ensuring the membrane is fully covered. Centrifuge at 2,500 RCF at 4 ºC (5-10 min) and discard the flow-through.
2. Add the diluted formulation and centrifuge at 2,500 RCF at 4 ºC.

   Note$_1$: Please check Table 1 for recommendations on final working concentrations.

   Note$_2$: The centrifugation time may vary depending on the molecular weight (MW) of the mRNA and the characteristics of the Amicon Ultra Centrifugal Filter, so adjust the centrifugation time accordingly.

   As a reference, DIVERSA's nanoparticles loaded with an mRNA of 1922 nt using an Amicon Ultra Centrifugal Filter (15 mL-30 kDa) and centrifuged for 35 min resulted in approximately 200 µL.

   Note$_3$: Ensure that the DIVERSA's nanoparticles remain in suspension, and DO NOT allow them to dry completely.

3. Discard the flow-through and collect the DIVERSA's nanoparticles from the upper part of the Amicon Ultra Centrifugal Filter.
4. Transfer the DIVERSA's nanoparticles to a 0.6 mL sterile RNase-free microtube and store at 4 ºC until use.

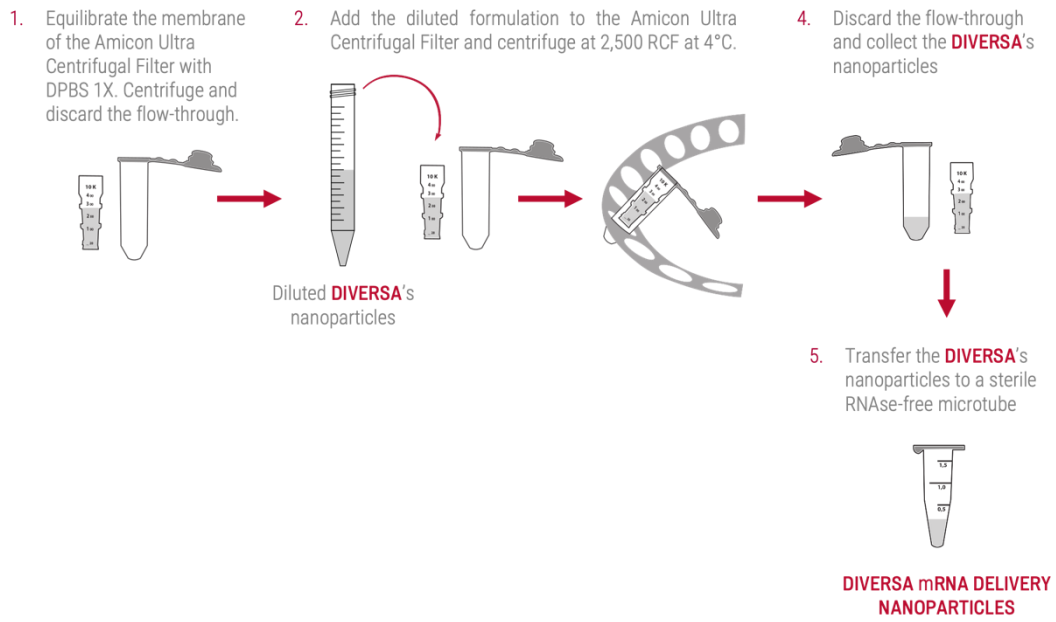

Figure 2. DIVERSA mRNA DELIVERY NANOPARTICLES. Concentration protocol

TRANSFECTION ASSAY

EXAMPLE PROTOCOL

1. Seed the recommended number of the cells in a 96-well plate with 100 µL of supplemented medium the day before the transfection assay.

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

2. Prepare the DIVERSA's nanoparticles for 5 µg of mRNA according to the provided FORMULATION PROTOCOL. Concentrate it up to 250 µL for a final mRNA concentration of 20 µg/mL.

3. Add the DIVERSA's nanoparticles at the desired transfection concentration in, at least, triplicate (e.g., 5 µL of nanoparticles in a final volume of 100 µL, achieving a final concentration in the well of 1 µg/mL of mRNA encapsulated in DIVERSA's nanoparticles).

   Note: This concentration can be modified depending on the type of mRNA and the specific cells of interest, but a minimum amount of 1 µg/mL of mRNA is recommended for the first set of experiments.

4. The read out can be performed after different incubation times depending on the mRNA of interest.

   For example:

   - HEK293 cells transfected with 1 µg/mL FLuc mRNA encapsulated in DIVERSA's nanoparticles can be analyzed 24 h post-transfection with the ONE-Glo™ Luciferase Assay (Promega (Ref.: E6120).
   - HEK293 cells transfected with 1 µg/mL GFP mRNA encapsulated in DIVERSA's nanoparticles can be analyzed 24 h post-transfection by Flow Cytometry Analysis, fluorescence or confocal microscopy.

TABLES AND TECHNICAL NOTES

Table 1. Recommended volume of DIVERSA's formulation to transfect 100 ng of mRNA in 100 µL using a 96-well plate.

Final volume of DIVERSA's nanoparticles	500 µL	250 µL	100 µL
Amount of mRNA in DIVERSA's nanoparticles		5 µg	
mRNA concentration in DIVERSA's nanoparticles	10 ng/µL	20 ng/µL	50 ng/µL
Volume of mRNA loaded DIVERSA's nanoparticles to transfect 100 ng	**10 µL**	**5 µL**	**2 µL**

Table 2. Recommended volumes for cell culture transfection to achieve a final mRNA concentration in the well of 1 µg/mL, starting from a mRNA concentration in DIVERSA's nanoparticles of 20 µg/mL.

Cell culture vessel	Amount of mRNA/well	Volume of DIVERSA's nanoparticles*	Volume of medium/ well*	Total volume/well*
100 cm	5000 ng	250 µL	4,75 mL	5 mL
6-well	1000 ng	50 µL	950 µL	1 mL
12-well	500 ng	25 µL	475 µL	500 µL
24-well	250 ng	12.5 µL	237.5 µL	250 µL
96-well	100 ng	5 µL	95 µL	100 µL

* These volumes are recommended for an incubation time of 4 hours. If DIVERSA's nanoparticles are incubated for longer than 4 hours, it is recommended to double the volumes of both the nanoparticles and the medium.

Table 3. Example of cells successfully transfected using DIVERSA's formulation.

Immortalized Cells	Embryonic kidney cells (HEK293) Epithelial breast cancer cells (MDA-MB-231) Epithelial lung cancer cells (A549) Human monocytes (THP-1) Mouse macrophages (RAW264) Mouse fibroblasts (NIH/3T3) Mouse cardiomyocytes (HL-1) Human cardiomyocytes (AC10) Human fibroblasts (HFF-1)
Primary cells	Human primary monocytes-derived macrophages Cortical neurons Human primary monocytes-derived macrophages Cortical neurons
Organoids	From brain cells

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FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
Can I use mRNA to encode any protein?	Yes, DIVERSA's nanoparticles can be loaded with mRNAs encoding for any protein of interest.
DIVERSA's nanoparticles can be used for other types of nucleic acids?	No, DIVERSA can provide customized formulations for other types of nucleic acid.
What is the maximum amount of mRNA to encapsulate in DIVERSA's nanoparticles?	The maximum amount of mRNA to encapsulate is 5 µg per **DIV053** vial. However, higher amounts can be achieved as a customized formulation. Contact DIVERSA.
What is the amount of the fluorophore in **DIV053?**	The fluorophore amount is 5 µg per vial.
Can I filter the formulation?	Yes, if necessary, DIVERSA's nanoparticles can be filtered using small 0.22 µm PES membrane filters.
How can I measure the size of the final formulation?	Diameter size can be measured by an equipment as Dynamic Light Scattering (DLS) analysis.
Can I use DIVERSA's nanoparticles for *in vivo* studies?	DIVERSA's nanoparticles can be used *in vivo*. Contact DIVERSA for specific recommendations for *in vivo* experiments.

ONLINE RESOURCES

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

CHANGELOG

Version	Date	Change Description
1.0	1 APR 2024	Initial release of the protocol.
2.0	8 NOV 2024	Clarified protocol steps for preparing DIVERSA's nanoparticles; added recommendations.