Edificio Emprendia, Campus Sur
15782 Santiago de Compostela
(A Coruña)
www.diversatechnologies.com
info@diversatechnologies.com

DIVERSA mRNA DELIVERY NANOPARTICLES

DIVERSA lipid nanoemulsions for promoting effective intracellular transfection of mRNA.

USER PROTOCOL – **#DIV053**

Edificio Emprendia, Campus Sur
15782 Santiago de Compostela
(A Coruña)

www.diversatechnologies.com
info@diversatechnologies.com

ABOUT THE NANOPARTICLES

OVERVIEW

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

DIVERSA mRNA DELIVERY NANOPARTICLES uses lipidic nanosystems for simple and efficient delivery of mRNA in a broad range of mammalian cells, even in difficult-to-transfect cells.

DIVERSA's nanoparticles do not require specialized tools. It minimizes material loss, ensuring maximum research value. Furthermore, it can accommodate a broad range of mRNA sizes, which broadens research possibilities.

Our nanoparticles show cases of exceptional efficacy, rendering results comparable to LNPs, approved for clinical applications. It's also bio-inspired, ensuring safe mRNA delivery and prioritizing cell integrity over virus and cationic transfection nanoparticles.

The formulation is easily internalized by live cells allowing efficient release of mRNA and expression of protein/s of interest that can be easily identified and visualized. These nanoparticles were selected based on high transfection efficiency, low cytotoxicity, and great stability of the formulation.

COMPONENTS

- 1x DIV053 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVERSA mRNA DELIVERY NANOPARTICLES.
- 1x sterile polypropylene, non-toxic, pyrogenic-free 1 mL syringes.
- 1x 21G ½ sterile needles (0.8 x 38 mm).

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, store them at 4 ºC up to 24h.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- Sterile standard microtubes.
- RNAse Zapp.
- Sodium Citrate Tribasic Dihydrate (CAS No: 6132-04-3).
- Citric Acid Monohydrate (CAS No: 5949-29-1).
- RNAse-free water (Molecular Grade).
- Dulbecco's phosphate-buffered saline 1X (DPBS) (no calcium, no magnesium).
- Ethanol (EtOH) 96%.
- mRNA of interest.

  Note: Recommended mRNA stock concentration at 1 mg/mL

CONSIDERATIONS BEFORE STARTING

- The following protocol is optimized for the preparation of DIVERSA mRNA DELIVERY NANOPARTICLES for 5 µg of mRNA.

  Note: An increased mRNA loading can be achieved. However, the lipid concentration of DIV001 vial must be adapted. For a customized prototype, contact DIVERSA.

- DIVERSA cannot guarantee the optimal characteristics of the final formulation if modifications in the protocol are introduced.
- It is recommended to use the DIVERSA mRNA DELIVERY NANOPARTICLES within 24 hours to obtain maximum expression.
- The transfection of DIVERSA mRNA DELIVERY NANOPARTICLES can be performed in supplemented and non-supplemented media.
- Do NOT use any buffer solution containing Triton-X, SDS or Tween-20 for the preparation or manipulation of DIVERSA mRNA DELIVERY NANOPARTICLES.
- Do NOT freeze DIVERSA mRNA DELIVERY NANOPARTICLES.
- Do NOT heat up DIVERSA mRNA DELIVERY NANOPARTICLES.

DIVERSA mRNA DELIVERY NANOPARTICLES

PROTOCOL

PRIOR TO FORMULATION STEP:

Prepare 10 mL of 10 mM Citrate Buffer at pH 3:

1. Weight out 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).
2. Solubilize both components in 8 mL of RNAse-free water (Molecular Grade).
3. Measure the pH and, if necessary, adjust pH to 3 using NaOH or HCl.
4. Adjust the volume to 10 mL.
5. Sterile filter the whole buffer using 0.22 µm filter of PES membrane.
6. Store it at 4 °C in a sterile container.

FORMULATION STEP (pH 3):

Note: Click here to see the video of the FORMULATION STEP.

Note: Prepare the working space by cleaning up with 70% EtOH, followed by RNAse Zapp spray. Use RNAse Zapp to clean out the micropipettes. Ideally, work in a nuclease free hood.

7. Reconstitute the DIV053 vial with 300 µL of EtOH. Pipette up and down gently to efficiently dissolve the lipids trying to recover all of them from the wall vial. Keep the suspension in the vial.

   Note: Remove completely the metal cap from the vial with the help of a crimping tool.

8. Add 895 µL of 10 mM Citrate Buffer at pH 3 and 5 µL of mRNA in the DIVTECH vial.

   Note: We recommend using mRNA stock concentration at 1 mg/mL to increase the reproducibility.

   Note: Do not leave the mRNA into Citrate Buffer for more than 5 min.

9. Inject the whole volume from the DIV053 vial to DIVTECH vial using the syringe and the needle provided.

   Note: Before adding the lipids from DIV053 vial to DIVTECH vial, leave an air gap of 0.300 µL in the syringe and inject the lipids in a vigorous way. Please, do it with confidence.

10. Leave the DIVTECH vial open and incubate for 35 min at room temperature (RT), protected from light. This will allow for the formation of the particles.

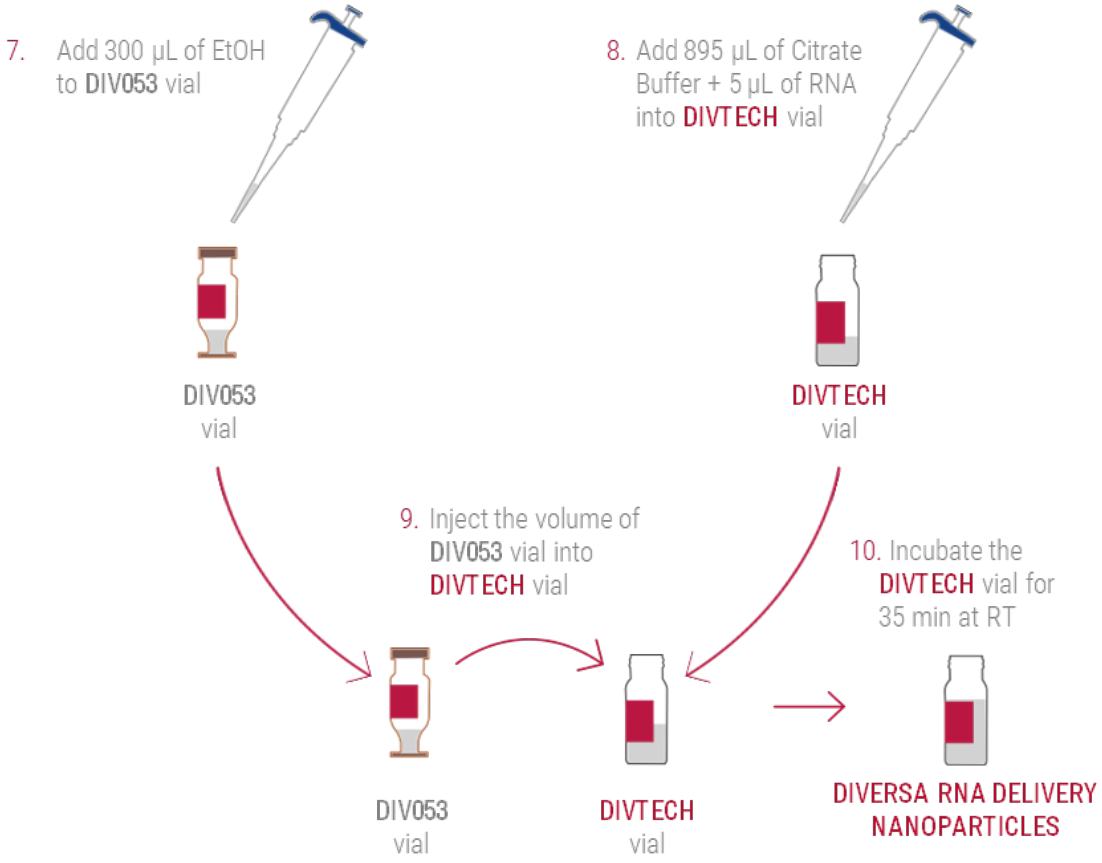

Figure 1. DIVERSA mRNA DELIVERY NANOPARTICLES protocol.

BUFFER EXCHANGE (pH 7):

The buffer exchange aims to obtain formulations at pH 7, suitable for *in vitro* and *in vivo* studies. For this purpose, an ultrafiltration filter 4mL- 30 kDa is needed.

11. Prior to the use of ultrafiltration filter, equilibrate the membrane with DPBS 1X by adding 4 mL of DPBS 1X, and centrifuge at 2,500 RCF for 5 min at 4 °C.
12. After the incubation time, transfer the DIVERSA mRNA DELIVERY NANOPARTICLES to the ultrafiltration filter 4 mL- 30 kDa, and top up the volume to 4 mL with DPBS 1X.
13. Centrifuge at 2,500 RCF for 5 min at 4 °C.

    Note: The volume should drop to ~1 mL.
14. Discard the flow-through. Wash the DIVERSA mRNA DELIVERY NANOPARTICLES again with DPBS 1X as performed in the previous step. Centrifuge at 2,500 RCF for 7-11 min, depending on the desired volume, at 4°C. The final volume should be around 250 µL recommended in **Table 1** (Recommendations of Use and Technical Notes).

    Note: if needed, the formulation can be concentrated up to 100 µL.

    Note: Increased mRNA concentrations can also be achieved by increasing the RNA loading above 5 µg per formulation. To do so, the lipid composition of DIV053 vial must be adapted. For a customized prototype, contact DIVERSA.
15. Discard the flow-through. Collect the DIVERSA mRNA DELIVERY NANOPARTICLES at pH 7 into sterile standard microtubes protected from light.

The DIVERSA mRNA DELIVERY NANOPARTICLES is now ready-to-use for *in vitro* or *in vivo* experiments. Alternatively, keep at 4 °C and use it in the following 24 hours.

Note: the concentration of DIVERSA mRNA DELIVERY NANOPARTICLES depends on the required concentrations for *in vitro* or *in vivo* studies.

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.

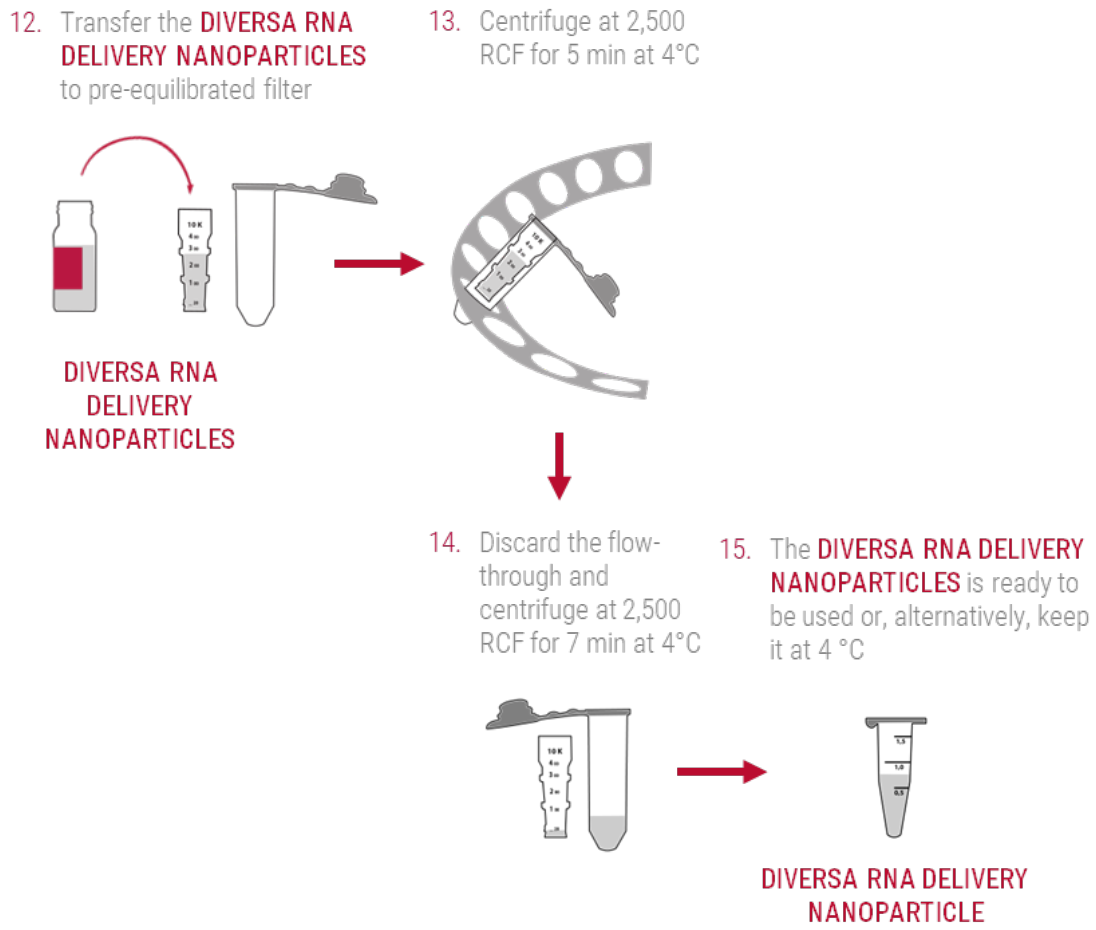

Figure 2. DIVERSA mRNA DELIVERY NANOPARTICLES Buffer Exchange protocol.

EXAMPLE OF TRANSFECTION PROTOCOL

1. Seed 10,000 HEK293 cells/well in a white 96-well plate in 100 µL of supplemented medium the day before the transfection assay.
2. Prepare the DIVERSA mRNA DELIVERY NANOPARTICLES following the provided protocol.
3. Add the DIVERSA mRNA DELIVERY NANOPARTICLES at the desired transfection concentration, at least, in triplicate (i.e.: 10 µL of nanoparticles in a final volume of 100 µL for 1 µg/mL mLuc and for mGFP).

   Note: this concentration can be modified depending on the formulation concentration achieved.
4. The read out can be performed upon different incubation times depending on the RNA of interest (i.e.: 24 h for RNA encoding Luciferase adding 25 µL of ONE-Glo™ Luciferase Assay (Promega (Ref.: E6120) and for RNA encoding GFP by FACS).

PROTOCOL OPTIMIZATION

PROTOCOL FOR RNA LOADING OPTIMIZATION

Edificio Emprendia, Campus Sur
15782 Santiago de Compostela
(A Coruña)

www.diversatechnologies.com
info@diversatechnologies.com

OPTIMIZATION GUIDELINES

It is **highly recommended** to **optimize your conditions** to get the best DIVERSA mRNA DELIVERY NANOPARTICLES performance.

The following parameters can be optimized:

- **Amount of RNA to be delivered**: the recommended amount of RNA to be delivered is 5 µg.
  However, if you need to deliver higher amounts of RNA, the lipid proportion of DIV053 vial must be adapted. For customized prototypes, contact DIVERSA.

- **Cell type and density**: you may need to optimize cell numbers. Delivery efficacy may be sensitive to the confluency of the cells in culture.

- **Incubation times for *in vitro* assays**: you may vary incubation times, depending on the type of functional assay performed, shorter or longer incubation time may influence delivery efficiency.

RECOMMENDATIONS OF USE AND TECHNICAL NOTES

Table 1. Recommended final concentration of the DIVERSA mRNA DELIVERY NANOPARTICLES.

Final volume of DIVERSA mRNA DELIVERY NANOPARTICLE	mRNA in DIVERSA mRNA DELIVERY NANOPARTICLE	Final concentration	Volume of DIVERSA mRNA DELIVERY NANOPARTICLE to transfect*
500 µL	5000 ng	10 ng/µL	10 µL
250 µL		20 ng/µL	5 µL
100 µL		50 ng/µL	2 µL

*Note: Recommended volume to transfect 100 ng of mRNA in 100 µL using a 96-well plate.

Table 2. Recommended volumes for cell culture*.

Cell culture vessel	RNA/well	Volume of DIVERSA RNA NANOPARTICLE REAGENT*	Volume of medium	Final volume/well
100 cm	5000 ng	500 µL	4,5 mL	5 mL
6-well	1000 ng	100 µL	900 µL	1 mL
12-well	500 ng	50 µL	450 µL	500 µL
24-well	250 ng	25 µL	225 µL	250 µL
96-well	100 ng	10 µL	90 µL	100 µL

*Note: To get a final concentration of 1 µg/mL RNA per well.

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
Can I use RNA encoding any protein?	Yes, DIVERSA MRNA DELIVERY NANOPARTICLES can be loaded with any RNA encoding for your protein of interest. We recommend using this RNA at 1mg/mL.
What is the maximum amount of RNA to encapsulate?	You can encapsulate higher amounts. However, the lipid proportion of DIV001 vial must be adapted. For customized prototypes, contact DIVERSA.
Can I use a mix of RNA and DNA?	Yes, if necessary, DIVERSA NANOPARTICLE REAGENT can mix 5 µg RNA (stock: 1 mg/mL) with 5 µg DNA (stock: 1 mg/mL) in a final volume of 900 µL of 10 mM Citrate Buffer pH 3.
Can I use RNA at different concentrations?	It is recommended to use the RNA at 1 mg/mL. If the concentration of your RNA is lower, you could concentrate it using an Amicon or by SpeedVac.
What should I do if I cannot concentrate my RNA at 1 mg/mL?	Add the volume of your RNA corresponding to encapsulate 5 µg into 900 µL of the Citrate Buffer.
What is the concentration of the DIVERSA RNA NANOPARTICLE REAGENT?	The concentration DIVERSA MRNA DELIVERY NANOPARTICLES depends on the final volume obtained after the buffer exchange step, over 200-250 µL.
How do I concentrate the formulation	If necessary, the DIVERSA MRNA DELIVERY NANOPARTICLES can be concentrated in the buffer exchange step up to desired up to 100uL.
Can I filter the formulation?	Yes, if necessary, DIVERSA MRNA DELIVERY NANOPARTICLES can be filtered using small 0.22 µm filters of PES membrane. I

Edificio Emprendia, Campus Sur
15782 Santiago de Compostela
(A Coruña)

www.diversatechnologies.com
info@diversatechnologies.com

QUESTION	ANSWER
How can I measure the size of the final formulation?	Diameter size can be measured by Dynamic Light Scattering (DLS) analysis upon 10x dilution in DPBS.
Can I use DIVERSA MRNA DELIVERY NANOPARTICLES for *in vivo* studies?	Yes, DIVERSA MRNA 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 see the video of the FORMULATION STEP.