DIVERSA

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DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES

DIVERSA lipid nanoparticles for promoting effective intracellular transfection of mRNA.

USER PROTOCOL – **#DIV053F1**

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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 FLUOGREEN mRNA DELIVERY NANOPARTICLES uses strongly labelled fluorescent lipid nanoparticles that are easily internalized by live cells and can be visualized by a wide variety of fluorescent platforms (flow cytometry, microplate assays, fluorescence, and confocal microscopy) in less than two hours at Ex/Em = 495/503 nm.

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

- 4x DIV053F1 vial for reconstitution.
- 4x DIVTECH vial for preparation of DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
- 4x sterile polypropylene, non-toxic, pyrogenic-free 1 mL syringes.
- 4x 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.
- Amicon Ultra-4 centrifugal filter 30 kDa or
- Amicon Ultra-0.5 centrifugal filter 100 kDa
- 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 FLUOGREEN mRNA DELIVERY NANOPARTICLES for 5 µg of mRNA.

  Note: An increased mRNA loading above 5 µg per formulation can be achieved. However, the lipid composition of the **DIV053F1** 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 FLUOGREEN mRNA DELIVERY NANOPARTICLES within 24 hours to obtain maximum expression.
- The transfection of DIVERSA FLUOGREEN 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 FLUOGREEN mRNA DELIVERY NANOPARTICLES.
- Do NOT freeze DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
- Do NOT heat DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.

DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES

PROTOCOL FOR LOADING 5 µg RNA PER FORMULATION

PRIOR TO THE 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 a 0.22 µm filter of PES membrane.
6. Store it at 4 ºC 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 one of DIV053F1 vial with 300 µL of EtOH with the help of the syringe provided. Vortex to dissolve and mix the lipids and keep the suspension inside the vial.

   Note: DO NOT remove the metal cap from the vial.

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

   $Note_1$: We recommend using mRNA stock concentration at 1 mg/mL to increase the reproducibility.

   $Note_2$: Do not leave the mRNA into Citrate Buffer for more than 5 min.

9. Inject the whole volume from the DIV053F1 vial to DIVTECH vial using the syringe and the needle provided.

   Note: Before adding the lipids from DIV053F1 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.

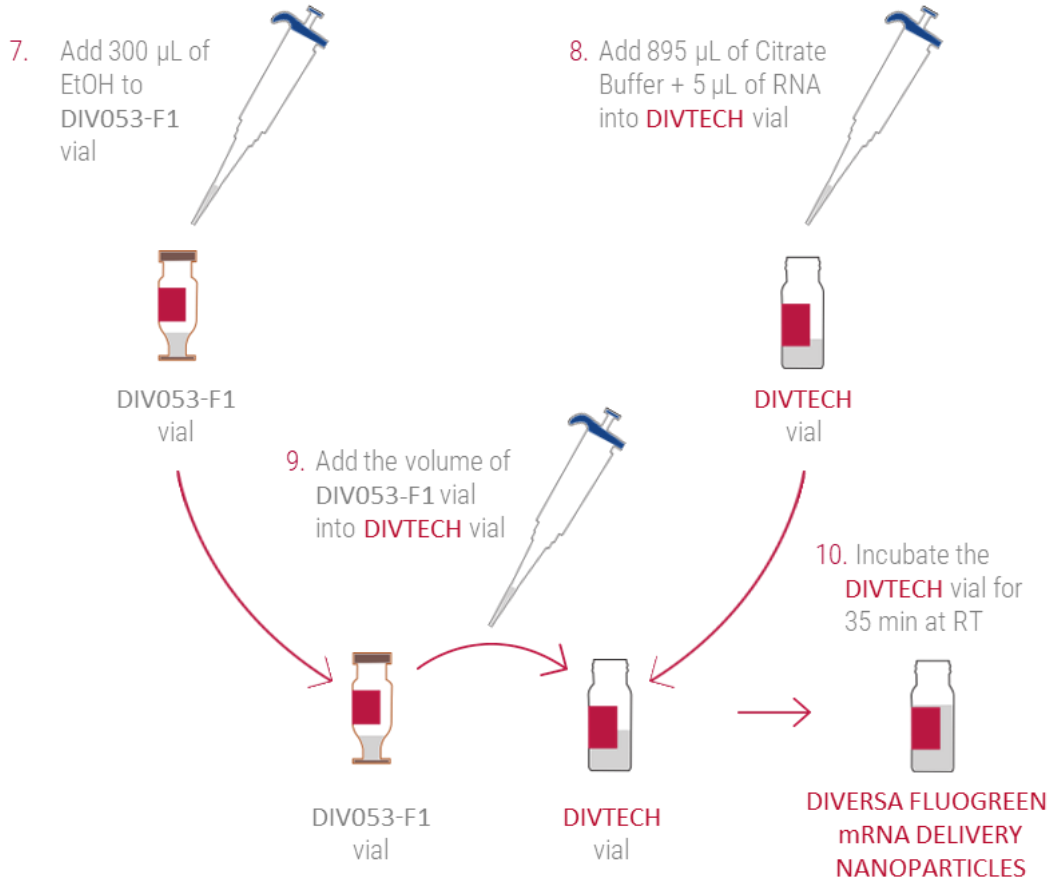

Figure 1. DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES protocol.

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.

Note: the porosity of the filter membrane depends on the molecular weight of the RNA.

11. Before using the 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 ºC.
12. After incubation, transfer the DIVERSA FLUOGREEN 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 ºC.

    Note: The remaining volume in the filter should be approximately 1 mL. If higher, please increase the centrifugation time.
14. Discard the flow-through. For a second washing step, add 3-4 mL of DPBS 1X to the filter containing the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
15. Centrifuge at 2,500 RCF for 7 min.
16. From now on, the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES can be concentrated at 4ºC depending on the desired working RNA concentration. The final volume should be concentrated to approximately 250 µL recommended in Table 1 (Recommendations of Use and Technical Notes).

    Note$_1$: Please adjust the centrifugation time to control de volume of the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.

    Note$_2$: The formulation can be concentrated to a maximum of 100 µL. However, please be sure not to let them completely dry.

    Note$_3$: As a reference, for final volume of 100 µL, centrifugation time may need to exceed 10 minutes.
17. Discard the flow-through. Collect the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES at pH 7 into RNase-free standard microtubes, ensuring they are protected from light.

The DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES is now ready-to-use. Alternatively, keep at 4 ºC and use it in the following 24 hours.

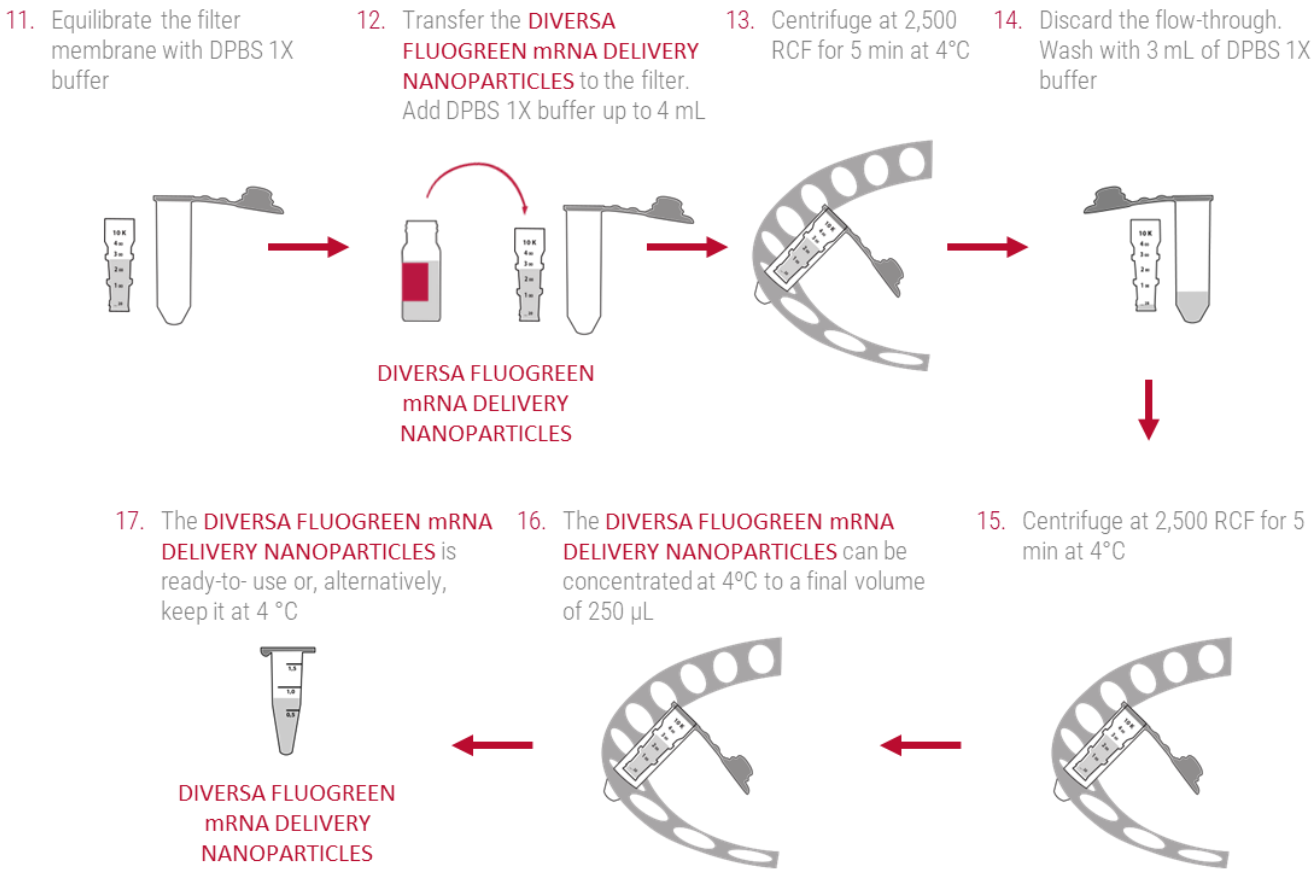

Figure 2. DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES Buffer Exchange

EXAMPLE OF TRANSFECTION PROTOCOL

1. Seed 20,000 HEK293 cells/well in a white 96-well plate in 100 µL of supplemented medium the day before the transfection assay.

   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.

2. Prepare the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES following the provided protocol.

3. Add the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES at the desired transfection concentration, at least, in triplicate (i.e.: 5 µL of nanoparticles in a final volume of 100 µL for a final concentration of 1 µg/mL mRNA-encapsulated in DIVERSA's NANOPARTICLES).

   Note: this concentration can be modified depending on the type of mRNA and the specific cells of interest.

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

   Note: For example:

   4.1. HEK293 cells transfected with 1 µg/mL FLuc mRNA-encapsulated in DIVERSA's NANOPARTICLES were analyzed at 24 h post-transfection adding.

   4.2. 25 µL of ONE-Glo™ Luciferase Assay (Promega (Ref.: E6120)

   4.3. HEK293 cells transfected with 1 µg/mL GFP mRNA-encapsulated in DIVERSA's NANOPARTICLES were analyzed at 24 h post-transfection by FACS.

EXAMPLE OF UPTAKE PROTOCOL

1. Seed 1x10^6 HEK293 cells/well in a transparent 6-well plate in 1 mL of supplemented medium the day before the uptake assay.

   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.

2. Prepare the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES following the provided protocol.

3. Add the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES at the desired uptake concentration (i.e.: 50 µL of nanoparticles in a final volume of 1 mL for a final concentration of 1 µg/mL mRNA-encapsulated in DIVERSA's NANOPARTICLES).

   Note: this concentration can be modified depending on the type of mRNA and the specific cells of interest.

4. Incubate the cells for 2-4 hours at 37 ºC.

   Note: Depending on de type of readout assay performed, incubation times may influence delivery efficiency.

5. After incubation time, remove the cell culture medium with the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES and, carefully, wash the cells twice with PBS 1X buffer and remove it.

   The DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES is efficiently internalized inside the cells.

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

6. Adjust the concentration for the analysis using the flow cytometer and the following parameters: wavelength of excitation at 495 nm, wavelength of emission at 503 nm.

   Note: we recommended analysis on the same day of the experiment. However, for extended storage (> 16h), we recommended resuspend the cells in 4% (*v/v*) of paraformaldehyde to prevent cell deterioration.

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Mini-**DIVERSA** FLUOGREEN mRNA DELIVERY NANOPARTICLES

PROTOCOL FOR LOW RNA LOADING

FORMULATION STEP (pH 3):

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.

1. Reconstitute one of **DIV053F1** vial with 310 µL of EtOH with the help of the syringe provided. Vortex to dissolve and mix the lipids and keep the suspension in the vial.

   Note: DO NOT remove the metal cap from the vial.

2. Now, remove the metal cap from the vial.
3. Divide the volume of **DIV053F1** vial in 5 aliquots of 60 µL in brown 0.6 mL RNAse-free standard microtubes.

   Note: The unused **DIV053F1** aliquots can be stored at 4ºC for up to 2 months.

4. In brown 0.6 mL RNAse-free standard microtubes, add 175 µL of 10 mM Citrate Buffer at pH 3 and 5 µL of mRNA.

   $Note_1$: In this step, we recommend using **mRNA stock concentration at 0.2 mg/mL** to increase the reproducibility.

   $Note_2$: Do not leave the mRNA into Citrate Buffer for more than 5 min.

   $Note_3$: These 5 aliquots contain 1 µg of mRNA.

5. Add the 60 µL of the lipids to the RNAse-free standard microtube containing the mRNA in 180 µL using the 200 µL micropipette. Pipette up-down several times with confidence.

   Note: Before adding the 60 µL, set the micropipette at the maximum volume and add the lipids in a vigorous way, placing the tip without touching the buffer. Please, do it with confidence.

6. Leave the brown 0.6 mL RNase-free standard microtubes open and incubate for 35 min at room temperature (RT), protected from light. This will allow for the formation of the nanoparticles.

BUFFER EXCHANGE (pH 7):

For this purpose, an ultrafiltration filter 0.5 mL- 100 kDa is needed.

Note: the porosity of the filter membrane depends on the molecular weight of the RNA.

7. Before using the ultrafiltration filter, equilibrate the membrane with DPBS 1X by adding 0.5 mL of DPBS 1X, and centrifuge at 2,500 RCF for 5 min at 4 ºC.
8. After incubation, transfer the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES to the ultrafiltration filter 0.5 mL-100 kDa, and top up the volume to 0.5 mL with DPBS 1X.
9. Centrifuge at 2,500 RCF for 5 min at 4 ºC.

   Note: The remaining volume in the filter should be approximately 100 µL. If higher, please increase the centrifugation time.
10. Discard the flow-through. For a second-washing step, add 400 µL of DPBS 1X to the filter containing the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
11. Centrifuge at 2,500 RCF for 7 min at 4 ºC.

    Note: The remaining volume in the filter should be approximately 230 µL. If higher, please increase the centrifugation time.
12. Discard the flow-through. For a third-washing step, add 270 µL of DPBS 1X to the filter containing the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
13. Centrifuge at 2,500 RCF for 5 min at 4 ºC.
14. Discard the flow-through. For a four-washing step, repeat steps 12 and 13 steps one more time.
15. From now on, the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES can be concentrated at 4ºC depending on the desired working RNA concentration. The final volume should be concentrated to approximately 250 µL recommended in Table 1 (Recommendations of Use and Technical Notes).

    Note$_1$: Please adjust the centrifugation time to control the volume of the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.
    Note$_2$: The formulation can be concentrated to a maximum of 100 µL. However, please be sure not to let them completely dry.

    Note$_3$: As a reference, for final volume of 100 µL, centrifugation time may need to exceed 10 minutes.
16. Discard the flow-through. Collect the Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES at pH 7 into RNase-free sterile standard microtubes, ensuring they are protected from light.

The Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES is now ready-to-use. Alternatively, keep at 4 ºC and use it in the following 24 hours.

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OPTIMIZATION GUIDELINES

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

The following parameters can be optimized:

- Amount of RNA to be formulated: the recommended amount of RNA to be formulated is 5 µg. A protocol to formulate smaller amounts (Mini-DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES for 1 µg of RNA) has also been developed. However, if you need to formulate higher amounts of RNA, the content of the lipid DIV053F1 vial must be adjusted. This can provide as CUSTOMIZED DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES. Please, contact DIVERSA for further assistance.

- 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 volume of the DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES to transfect 100 ng of mRNA in 100 µL using a 96-well plate.

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

Note: Recommended concentration of DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES of 20 ng/µL.

Table 2. Recommended volume of DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES to transfect a final concentration of 1 µg/mL mRNA starting from a concentration of 20 µg/mL.

Cell culture vessel	RNA/well	Volume of **DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES***	Volume of medium	Final 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

Table 3. Example of cells transfected using DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES.

Cells	HEK293 MDA.MB-231 A549 THP-1 HI-1 Dendritic cells
Primary cells	Human primary monocytes-derived macrophages Cortical neurons Human primary monocytes-derived macrophages Cortical neurons Human fibroblasts (HFF-1) Human cardiomyocytes (AC10) Mouse fibroblasts (NIH/3T3) Mouse cardiomyocytes (HL-1)
Organoids	From brain cells

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
Can I use RNA encoding any protein?	Yes, DIVERSA FLUOGREEN 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 another type of nucleic acid instead of RNA?	Yes, DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES can be loaded with any polynucleotide such as pDNA or siRNA. For specific customized prototype, contact DIVERSA.
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 FLUOGREEN mRNA DELIVERY NANOPARTICLES?	The concentration DIVERSA FLUOGREEN 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 FLUOGREEN mRNA DELIVERY NANOPARTICLES can be concentrated in the buffer exchange step up to desired up to 100 µL.
Can I filter the formulation?	Yes, if necessary, DIVERSA FLUOGREEN mRNA DELIVERY NANOPARTICLES can be filtered using small 0.22 µm filters of PES membrane.

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 FLUOGREEN mRNA DELIVERY NANOPARTICLES for *in vivo* studies?	Yes, DIVERSA FLUOGREEN 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.