DIVERSA

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

www.diversatechnologies.com
info@diversatechnologies.com

# **DIVERSA** FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES

Tracking intracellular delivery of anionic peptides

## USER PROTOCOL – **#DIV042-F1**

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

www.diversatechnologies.com
info@diversatechnologies.com

# ABOUT THE NANOPARTICLES

## OVERVIEW

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

DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES, based on cationic lipids, is suitable for an efficient association of your anionic peptides (pH > pI) mainly due to electrostatic interactions.

## COMPONENTS

- 1x DIV042F1 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.
- 2x Tips for 1 mL micropipette.

## STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, storage is recommended at 4 ºC.

## EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1 mL micropipette.
- Sterile standard microtubes
- MilliQ water or any other recommended buffer.
- Ethanol (EtOH) 96%.
- Anionic peptide of interest.

## CONSIDERATIONS BEFORE STARTING

- The following protocol is directed for anionic peptides where the isoelectric point (pI) must be higher than the pH of the buffer.
- The following protocol is optimized for the preparation of 1 mL of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES (starting from one DIV042-F1 vial for reconstitution).
- DIVERSA cannot guarantee the optimal characteristics of the final formulation if modifications in the protocol are introduced.
- It is recommended to use DIVERSA FLUOGREEN DELIVERY NANOPARTICLES within 60 days.
- DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is compatible with supplemented cell culture media at 37 ºC: DMEM, RPMI.
- Do NOT use any buffer solution containing Triton-X, SDS or Tween-20 for the preparation or manipulation of DIVERSA FLUOGREEN/DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.
- Do NOT freeze DIVERSA FLUOGREEN/DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.
- Do NOT heat up DIVERSA FLUOGREEN/DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.
- Do NOT centrifuge or vortex DIVERSA FLUOGREEN/DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.

# **DIVERSA** FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES

## PROTOCOL

1. Reconstitute the DIV042-F1 vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids trying to recover all of them from the wall of the vial and keep the suspension in the vial.
2. Add 900 µL of ultrapure water into the DIVTECH vial or, alternatively, a buffer solution suggested in Table 1 (Recommendations of Use and Technical Notes).
3. Transfer the whole volume from DIV042-F1 vial to the DIVTECH vial using a micropipette and the 1 mL micropipette tip provided.

   IMPORTANT: Before adding the lipids from DIV042-F1 vial to DIVTECH vial, set the micropipette at the maximum volume to have dead air volume in the tip for mixing in a faster and vigorous way. Then, place the 1 mL micropipette tip into the buffer solution of DIVTECH vial, and pipette up and down for 30 seconds, avoiding any spillage.

   The DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is now ready for the association of the anionic peptide/s. Alternatively, keep it at 4 ºC and use it in the following 60 days.

4. Add the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES gently and dropwise into the anionic peptide solution and pipette up and down gently. Recommended volumes are provided in Table 2.

   Note: we recommend using peptide stock concentration at 4 mg/mL to increase the reproducibility.

5. Incubate the mixture for 15 min at room temperature (RT). Agitation is not required.

   The DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES is now ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 2 days.

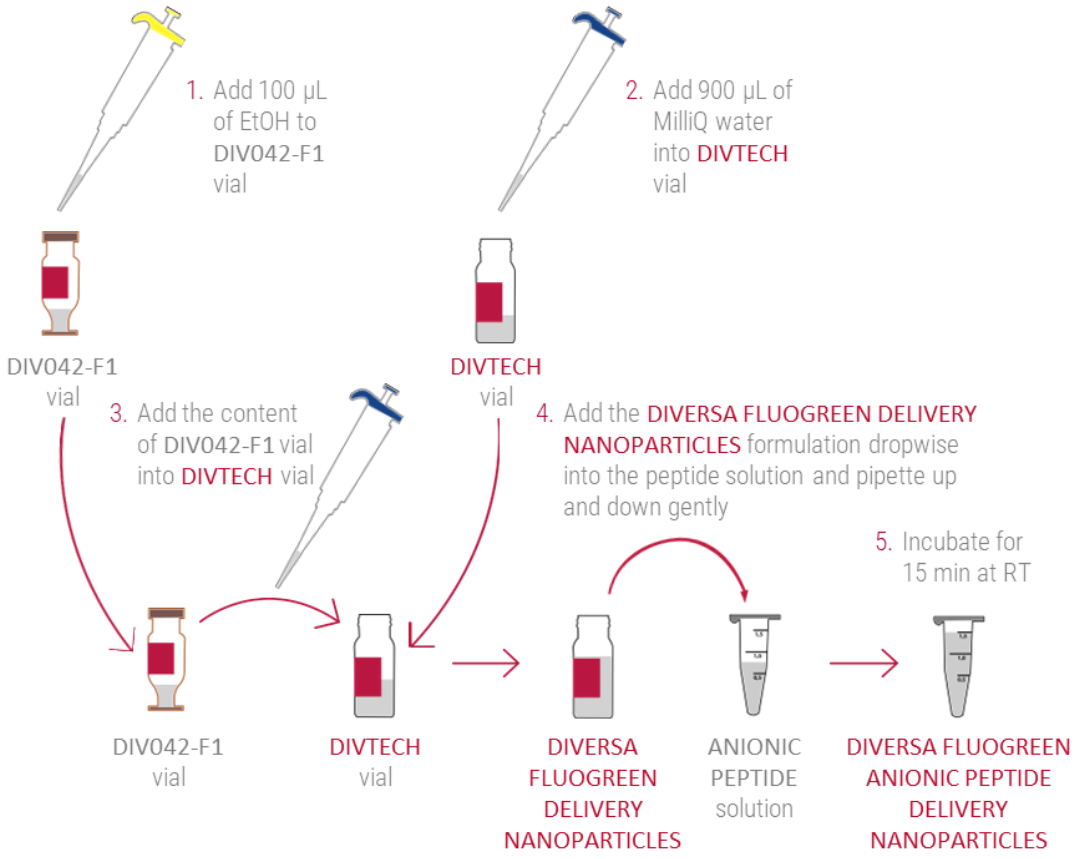


**Figure 1.** DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES protocol.

# EXAMPLE OF *TITYUS STIGMURUS* ANIONIC PEPTIDE (TanP) ASSOCIATION PROTOCOL

1. Reconstitute the DIV042-F1 vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids trying to recover all of them from the vial wall and keep the suspension in the vial.
2. Add 900 µL of ultrapure water into the DIVTECH vial.
3. Add the content of DIV042-F1 to the DIVTECH vial using a micropipette and the provide narrow 1 mL micropipette tip.

IMPORTANT: Before adding the lipids from DIV042-F1 vial to DIVTECH vial, set the micropipette at the maximum volume to have dead air volume in the tip for mixing in a faster and vigorous way. Then, place the 1 mL micropipette tip into the buffer solution of DIVTECH vial, and pipette up and down for 30 seconds, avoiding any spillage.

The DIVERSA FLUOGREEN DELIVERY NANOPARTICLES is now ready for the association of the TanP peptide. Alternatively, keep it at 4 ºC and use it in the following 60 days.

4. Add 20 µL of the DIVERSA FLUOGREEN DELIVERY NANOPARTICLES dropwise into 5 µL of TanP solution and pipette up and down gently.

   Note: we recommend using peptide stock concentration at 4 mg/mL to increase the reproducibility.

5. Incubate the mixture for 15 min at RT.

The DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES is now ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 2 days.

# EXAMPLE OF UPTAKE ASSAY PROTOCOL

1. Seed the recommended number of the cells in a 6-well plate the day before of the uptake experiment for FACS analysis.

   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 (see Table 3 in Recommendations of Use and Technical Notes).

2. Prepare the DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES following the provided protocol.

3. Add 960 µL of fresh cell cultured medium supplemented with 10% (v/v) of FBS and, if necessary, 1 % (v/v) of antibiotics.

4. Add the DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES to a final volume of 1 mL.

   Note: The final concentration of the DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES is calculated considering the final volume of 1 mL.

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

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

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

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

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

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

# OPTIMIZATION GUIDELINES

It is highly recommended to optimize your conditions to get the best DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES performance. Optimize one parameter at a time.

The following parameters can be optimized:

- Amount of DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES: Start fixing the concentration and amount of your anionic peptide to be delivered, and then you may vary the quantity of the DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.

- Amount of anionic peptide to be delivered: you may need to vary the amount of your anionic peptide to be delivered as we recommended in Table 2 (Recommendations of Use and Technical Notes). Depending on the sensitivity of your assay, a greater amount of peptide and DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES may be required. For higher amounts of peptide, you can scale up the volume of DIVERSA DELIVERY NANOPARTICLES according to the amount of peptide.

- Concentration of the peptide solution: We recommend peptide concentration at 4 mg/mL. At lower concentrations, we recommend concentrating your peptide using ultracentrifugation filters 0.5 mL- 10 kDa. If your protein is in powder, we recommend dissolve it at final concentration of 4 mg/mL in its corresponding buffer.

- 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. Suggested aqueous solutions for DIVTECH vial.

| BUFFER SOLUTION | CONCENTRATION |
|---|---|
| Ultrapure water | N/A |
| NaCl | 150 mM |
| HEPES | 10-25 mM |
| DPBS | 1X |

Table 2. Suggested volumes for DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES.

| **DIVERSA NANOPARTICLES** | **PEPTIDE** solution | Amount of **PEPTIDE*** |
|---|---|---|
| 50 µL | 5 µL | 20-50 µg |
| 20 µL | 5 µL | 10-20 µg |
| 10 µL | 5 µL | 1-10 µg |

*For higher amounts of peptide, you can scale up the volume of DIVERSA FLUOGREEN DELIVERY NANOPARTICLES according to the amount of peptide (e.g., 200 µL of formulation for up to 200 µg of peptide, ideally in 5 µL, however, this peptide solution volume can be increased for higher quantities).

Table 3. Recommended volumes for cell culture.

| Cell culture vessel | Volume of **DIVERSA** | Volume of medium | Final volume/well |
|---|---|---|---|
| 100 cm | 200 µL | 4,8 mL | 5 mL |
| 6-well | 40 µL | 960 mL | 1 mL |
| 12-well | 20 µL | 996 µL | 500 µL |
| 24-well | 10 µL | 240 µL | 250 µL |
| 96-well | 4 µL | 96 µL | 100 µL |

*In 6-well plates, 2-3x$10^5$ cells must be seeded per well. Note: the cell density should be optimized for each cell model.

# FREQUENTLY ASKED QUESTIONS

| QUESTION | ANSWER |
|---|---|
| What is the concentration of the fluorophore in DIV042-F1? | The concentration of the fluorophore is 4 µg/mL in the final DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES. |
| Is the green fluorochrome the only available fluorochrome so far? | Currently, we offer the green fluorochrome. However, you can contact DIVERSA for a customized formulation where we can incorporate alternative fluorochromes: Cy5, Cy7.5, and other fluorescent dyes. |
| Is the fluorochrome pH sensitive? | No, it is not. It can be used a wide range of pH |
| How stable is the signal from the DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES? | The green signal of the green fluorochrome is stable for up to 1 year at -20 ºC, according to the manufacturer. Prior to formulation, DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES, should be stored at these conditions to preserve the stability. Upon formulation, you can use it for up to 7 days if it is stored at 4 ºC and protected from light. Regarding stability upon addition to cell cultures, we have tracked it in live cells for up to 8 days. |
| Does the fluorescence of the formulation come from molecules that have any biological effect? | No, it does not. The fluorescence arises from molecules that are covalently linked to the lipids, but have no intrinsic effect. The fluorescence does not interfere with the efficacy of the formulation or the activity of the associated drug/biomolecule. |
| Can I filter the formulation? | Yes, if necessary, DIVERSA FLUOGREEN ANIONIC NANOPARTICLE REAGENT can be filtered using 0.22 µm filters of PES membrane. |
| How can I measure the size of the final formulation? | Diameter size can be measured by Dynamic Light Scattering (DLS) analysis adding to the cuvette 20 µL of DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES with 180 µL of MilliQ water. |
| Can I use DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES for research *in vivo* studies? | No, for *in vivo* using whole-body imaging systems, it is necessary to use other fluorophores. DIVERSA can offer a customized reagent labeled formulations with Cy5. |

| | |
|---|---|
| | If the purpose is to extract organs/cells and perform analysis by flow cytometry or confocal microscopy, then the answer is yes. |
| What if I need to work with higher protein/peptide concentrations than the ones provided in Table 2? | You can concentrate the formulation (see next question), or alternatively, contact DIVERSA for advice depending on your specific protein/peptide. |
| How do I concentrate the formulation? | If necessary, the 1 mL of DIVERSA FLUOGREEN ANIONIC PEPTIDE DELIVERY NANOPARTICLES can be concentrated by using a SpeedVac or Rotavap in mild conditions (avoid surpassing 35 °C or drying the samples). Samples can be concentrated up to 4-fold its original volume (i.e., to a final volume 250 µL). |

# ONLINE RESOURCES

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