DIVERSA

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DIVERSA ANIONIC PEPTIDE DELIVERY NANOPARTICLES

Enhancing intracellular delivery of anionic peptides

USER PROTOCOL – **#DIV042**

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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 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 DIV042 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVERSA 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 NANOPARTICLES (starting from one DIV042 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 NANOPARTICLES within 60 days.
- DIVERSA NANOPARTICLES is compatible with supplemented cell culture media 37 ºC: DMEM, RPMI.
- Do NOT use any buffer solution containing Triton-X, SDS or Tween-20 for the preparation or manipulation of DIVERSA/DIVERSA-PEPTIDE NANOPARTICLES.
- Do NOT freeze DIVERSA/DIVERSA-PEPTIDE NANOPARTICLES.
- Do NOT heat up DIVERSA/DIVERSA-PEPTIDE NANOPARTICLES.
- Do NOT centrifuge or vortex DIVERSA/DIVERSA-PEPTIDE NANOPARTICLES.

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DIVERSA ANIONIC PEPTIDE DELIVERY NANOPARTICLES

PROTOCOL

1. Reconstitute the DIV042 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 vial to the DIVTECH vial using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids from DIV042 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 NANOPARTICLES is now ready for the association of the anionic peptide. Alternatively, keep it at 4 ºC and use it in the following 60 days.

4. Add the DIVERSA 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-PEPTIDE NANOPARTICLES is now ready-to-use for *in vitro* and *in vivo* experiments. Alternatively, keep it at 4 ºC and use it in the following 2 days, or depending on the stability of your cationic peptide.

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).

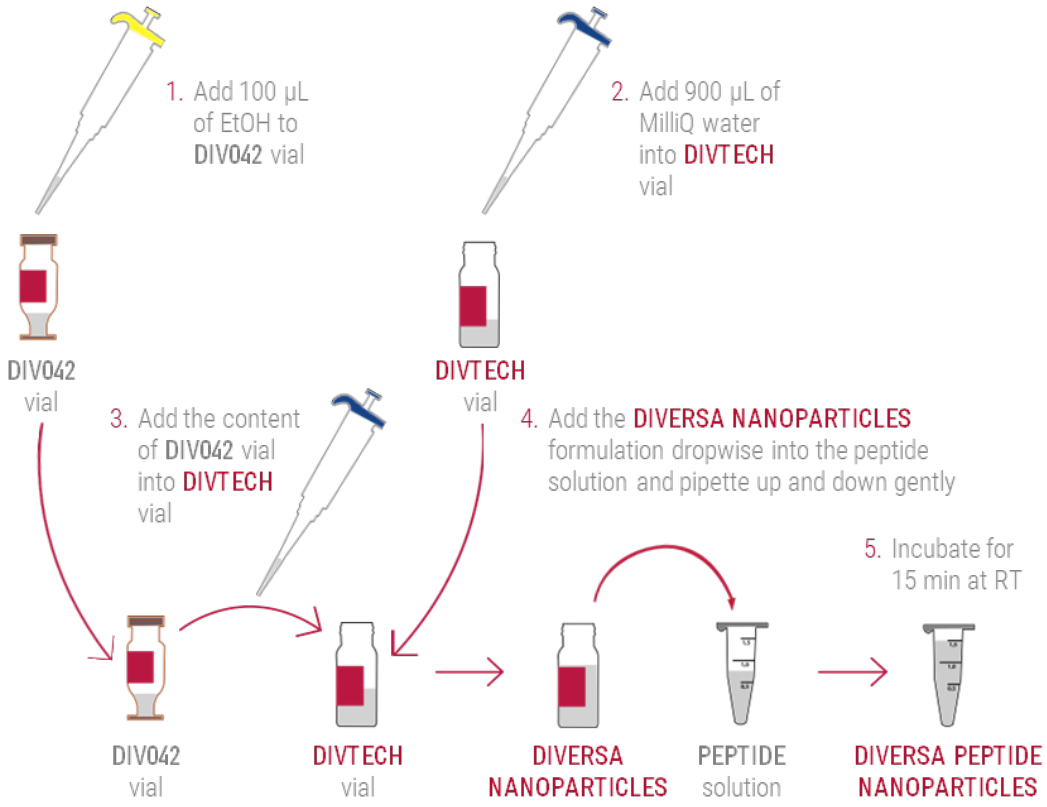

Figure 1. DIVERSA ANIONIC PEPTIDE DELIVERY NANOPARTICLES protocol.

EXAMPLE OF *TITYUS STIGMURUS* ANIONIC PEPTIDE (Tanp) ASSOCIATION PROTOCOL

1. Reconstitute the DIV042 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 to the DIVTECH vial using a micropipette and the provide narrow 1 mL micropipette tip.

IMPORTANT: Before adding the lipids from DIV042 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 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 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-PEPTIDE NANOPARTICLES is now ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 2 days.

OPTIMIZATION GUIDELINES

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

The following parameters can be optimized:

- Amount of DIVERSA 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 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 ANIONIC PEPTIDE DELIVERY NANOPARTICLES may be required. For higher amounts of peptide, you can scale up the volume of DIVERSA 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.

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RECOMMENDATIONS OF USE AND TECHNICAL NOTES

Table 1. Suggested buffer solution 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-PEPTIDE 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 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, 2-3x10^5 cells must be seeded per well. Note: the cell density should be optimized for each cell model.

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
Can I filter the formulation?	Yes, if necessary, DIVERSA 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 10 µL of DIVERSA-PEPTIDE NANOPARTICLES with 990 µL of MilliQ water.
Can I use DIVERSA-PEPTIDE NANOPARTICLES for research *in vivo* studies?	Yes, DIVERSA-PEPTIDE NANOPARTICLES can be used for research *in vivo* studies. For specific recommendations and a customized and optimized prototype, contact DIVERSA.
What if I need to work with higher 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 peptide.
How do I concentrate the formulation?	If necessary, the 1 mL of DIVERSA-PEPTIDE 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.