DIVERSA

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DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES

Tracking intracellular and extracellular delivery of a broad range of proteins

USER PROTOCOL – **#DIV031F1**

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ABOUT THE NANOPARTICLES

OVERVIEW

DIVERSA PROTEIN DELIVERY NANOPARTICLES are a biocompatible, biodegradable, and cell-friendly technology designed to enhance the intracellular and extracellular delivery of proteins, paving the way for clinical translation.

DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES associate proteins very efficiently, irrespective of their molecular weight and isoelectric point. A tag in the protein(s) of interest is required for efficient association with DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES without compromising protein structure or activity.

DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES enable efficient intracellular delivery of proteins, ensuring their release within the cell after uptake, while also supporting extracellular applications.

DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES uses strongly labelled fluorescent nanometric emulsions that are easily internalized by live cells that can be visualized by a wide variety of platforms (flow cytometry, microplate assays, fluorescence, and confocal microscopy) in less than 2 hours at Ex/Em = 495/503 nm.

DIVERSA FLUOGREEN formulation can be used as a positive control for cell internalization and for testing the efficiency of associated proteins in specific cell lines of interest.

COMPONENTS

- 1x DIV-LINKER.
- 1x DIV031F1 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES
- 1x Amicon Ultra Centrifugal Filter 0.5 mL- 10 kDa.
- 2x Collector microtubes.
- 2x Tips for 1 mL micropipette.

STORAGE

Before formulating, store the vials at -20 ºC. Once formulated, the preparation should be stored at 2-8 ºC for up to 60 days without the protein, or up to 2 days with the associated protein.

EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1 mL micropipette.
- 0.6 mL microtubes.
- 1.5 mL microtubes.
- Ultrapure water.
- Ethanol (EtOH) 96%.
- PBS 2 mM at pH 7.4 (PBS).
- DMSO.
- Protein/s of interest.

CONSIDERATIONS BEFORE STARTING

- The proteins must be tagged with the **DIV-LINKER** for efficient association with **DIVERSA FLUOGREEN** nanoparticles.
- The following protocol is optimized for the preparation of 1.5 mL of **DIVERSA FLUOGREEN PROTEIN** nanoparticles.
- **DIVERSA** cannot guarantee the optimal formulation performance if any modifications are made to the protocol.
- It is recommended to use **DIVERSA FLUOGREEN** nanoparticles (prior association of the protein) within 60 days of preparation for optimal performance.
- After protein association, it is recommended to use **DIVERSA FLUOGREEN PROTEIN** nanoparticles within 48 hours of preparation for optimal performance.
- **DIVERSA FLUOGREEN PROTEIN** nanoparticles are stable in cell culture media under the following tested conditions: for at least 24 h at 37 ºC in DMEM and RPMI, supplemented with 10% (v/v) of FBS and 1% (v/v) of penicillin/streptomycin.
- Do NOT use any buffer containing Triton-X, SDS or Tween-20 for the preparation of **DIVERSA FLUOGREEN PROTEIN** nanoparticles.
- Once formulated, do NOT freeze **DIVERSA FLUOGREEN PROTEIN** nanoparticles.
- Do NOT heat over 90ºC **DIVERSA FLUOGREEN PROTEIN** nanoparticles.

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DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES PROTOCOL

PROTEIN TAGGING STEP

Click here to view the video on the preparation of DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES.

Please check the **EQUATIONS** found in the Tables and Technical Notes section before starting.

Note that this step requires an overnight incubation period.

1. Add 40 µL of DMSO to the DIV-LINKER vial. Gently pipette up and down until fully dissolved.
2. Calculate the required volume of **DIV-LINKER** to tag the **Desired Protein Amount** for association with DIVERSA FLUOGREEN nanoparticles, based on your experimental needs (refer to EQUATION 1 and EQUATION 2 in Tables and Technical Notes). Transfer the calculated volume of **DIV-LINKER** into a microtube.

   Note: The remaining **DIV-LINKER** can be used to tag more of the same protein or other different proteins. Store the leftover **DIV-LINKER** at -20 ºC and use it within 3 months.
3. Add the protein to the microtube containing the **DIV-LINKER** and adjust the final volume to 500 µL with PBS. Vortex gently.

   Note$_1$: If your protein is in powder form, we recommend dissolving it in PBS 2 mM at pH 7.4.

   Note$_2$: DO NOT use buffers containing β-mercaptoethanol, ammonium salts, or primary amines.
4. Incubate the **PROTEIN** with the **DIV-LINKER** for 6 hours at room temperature (RT), followed by overnight incubation at 2-8 ºC. After this step, the protein is successfully tagged and referred to as **PROTEIN-TAG**.

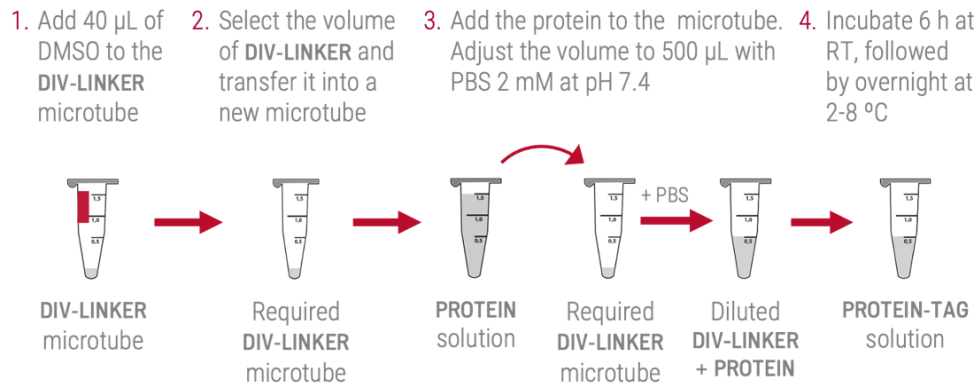

Figure 1. DIVERSA PROTEIN DELIVERY NANOPARTICLES PROTOCOL: PROTEIN TAGGING STEP.

PROTEIN PURIFICATION STEP

1. Equilibrate the membrane of the provided Amicon Ultra Centrifugal Filter (0.5 mL-10 kDa) by adding PBS, ensuring it is fully covered. Centrifuge at 14,000 RCF at 4 ºC (5-10 min). Discard the flow-through.
2. Transfer the 500 µL of **PROTEIN-TAG** to the filter and centrifuge at 14,000 RCF for 5 min at 4 ºC. Discard the flow-through.
3. Wash the **PROTEIN-TAG** four times with 500 µL of cold PBS, following the same centrifugation settings.
4. After the final wash, adjust the volume of **PROTEIN-TAG** for association with DIVERSA FLUOGREEN nanoparticles (refer to EQUATION 3 and EQUATION 4 of Tables and Technical Notes for volume calculations):
    a) If concentration is required: Perform an additional centrifugation cycle to achieve the desired volume. Invert the filter into a clean collector microtube and centrifuge at 1,000 RCF for 2 min at 4 ºC to collect the **PROTEIN-TAG**.
    b) If dilution is required: Invert the filter into a clean microtube, centrifuge at 1,000 RCF for 2 min at 4 ºC, and dilute the **PROTEIN-TAG** with PBS to the required volume.

The **PROTEIN-TAG** is now ready to use in the Protein Association Step or can be stored at -20 ºC for up to 1 month.

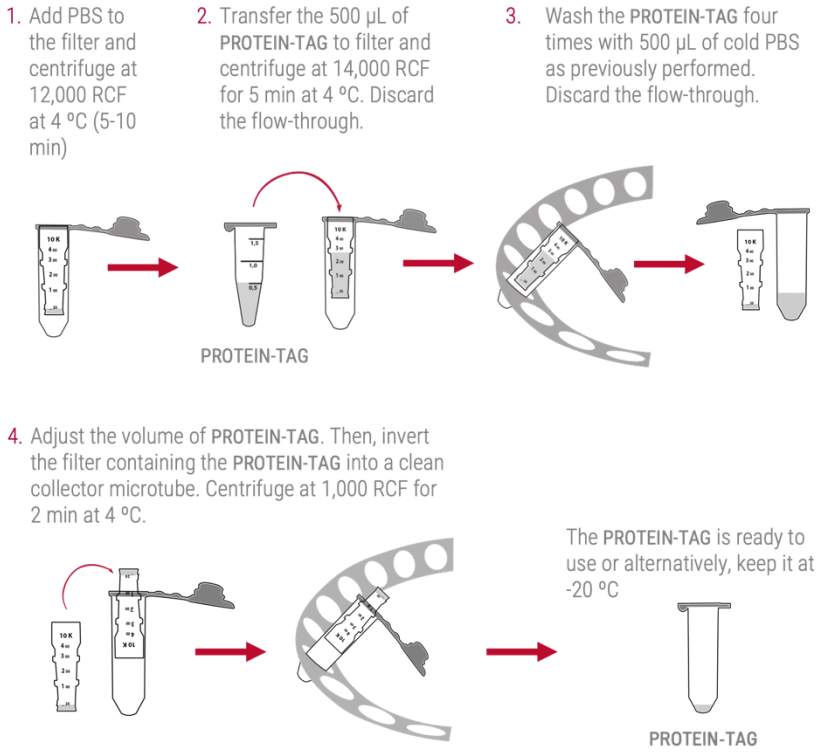

Figure 2. DIVERSA DELIVERY NANOPARTICLES PROTOCOL: PROTEIN PURIFICATION STEP.

FORMULATION STEP

1. Add 100 µL of EtOH in the **DIV031F1** vial. Gently pipette up and down.
2. Add 900 µL of ultrapure water into the **DIVTECH** vial.
3. Transfer the entire volume from **DIV031F1** vial to the **DIVTECH** vial using a 1 mL micropipette and the provided tip.

   Note: Before adding the volume from **DIV031F1** vial to the **DIVTECH** vial, set the micropipette at the maximum volume, and add the solution with a sudden, vigorous downward motion. Pipette up and down for 5-10 times with confidence.

The **DIVERSA FLUOGREEN** nanoparticles are now ready to use or can be stored at 2-8 °C for up to 60 days. They can be used in the Protein Association Step either all at once for the formulation of a single protein or split into fractions for different timepoints or proteins, depending on the experimental needs.

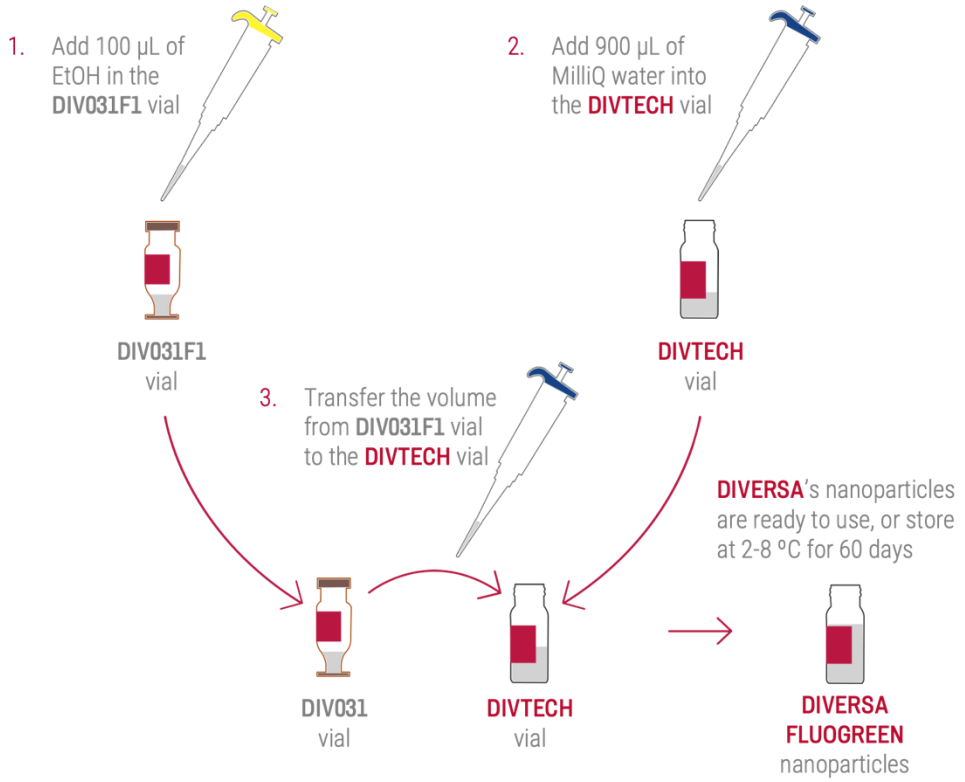

Figure 3. DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES: FORMULATION STEP.

PROTEIN ASSOCIATION STEP

Note: This step requires an overnight incubation period.

1. Transfer the calculated volume of **DIVERSA FLUOGREEN** nanoparticles (refer to EQUATION 3 of Tables and Technical Notes) to a microtube.
2. Add the required volume of the **PROTEIN-TAG** (refer to EQUATION 4 of Tables and Technical Notes) to the microtube in a sudden vigorous movement. Pipette up and down for 5-10 times with confidence.
3. Incubate the **DIVERSA FLUOGREEN PROTEIN** nanoparticles at room temperature (RT) for 4 h, followed by an overnight incubation at 2-8 ºC.

The **DIVERSA FLUOGREEN PROTEIN** nanoparticles are now ready to use or can be stored at 2-8 ºC for up to 2 days.

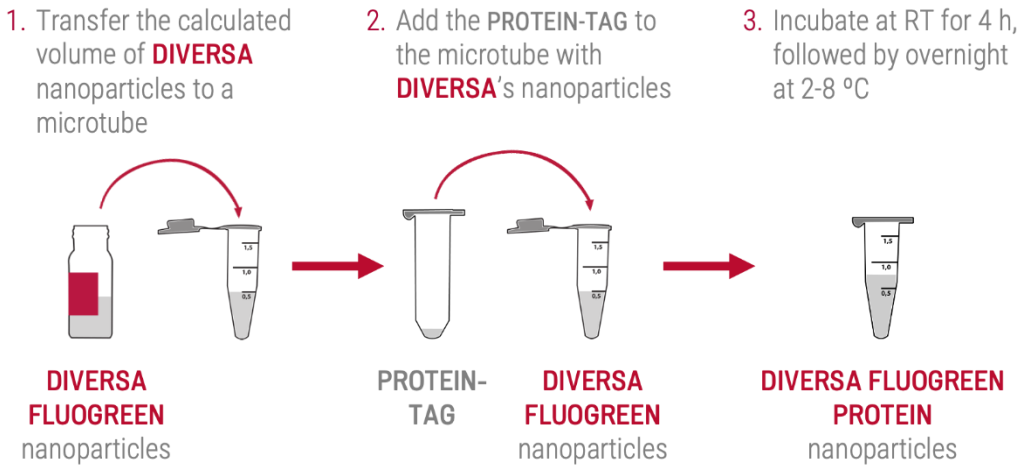

Figure 4. DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES: PROTEIN ASSOCIATION STEP

X-GAL STAINING FOR β-GAL DELIVERY

EXAMPLE PROTOCOL

Note that the DIVERSA FLUOGREEN PROTEIN formulation should be ready for the experiment. Please review the protocols prior to starting. The PROTEIN TAGGING and the PROTEIN ASSOCIATION protocols require an overnight incubation period.

1. Seed the recommended number of cells in a 24-well plate with 500 µL of complete medium the day before the experiment. Cells should be 70-80% confluent on the day of the experiment.

   Note$_1$: for *in vitro* experiments, the adherent cells must be between 70-80% confluent on the day of the experiment. However, optimizations should be performed depending on the cell type and the length of the experiment.

2. Start with the PROTEIN TAGGING & PURIFICATION STEP.
3. In this protocol, the amount of β-Gal protein (540 kDa) to associate with DIVERSA FLUOGREEN nanoparticles is set to 190 µg.
4. The calculation of the needed volume of DIV-LINKER for 190 µg of β-Gal protein (540 kDa) according to EQUATION 1 and EQUATION 2 of Tables and Technical Notes is 4 µL.

$$\text{Max Protein (µg)} = 3.54 \times 540 = 1912 \text{ µg}$$

$$\text{Volume of DIV-LINKER needed} = \frac{40 \times 190}{1912} = 4 \text{ µL}$$

5. Transfer 4 µL of DIV-LINKER into a microtube and add 190 µg of β-Gal protein to tag (38 µL of a stock solution at 5 mg/mL). Adjust the final volume to 500 µL with 458 µL of PBS. Vortex gently.
6. Now finish the PROTEIN TAGGING STEP and follow the steps 1-3 of the PROTEIN PURIFICATION STEP.

   Note: After the final wash, a volume of 86 µL of β-Gal-TAG is obtained.

7. The calculated volume of DIVERSA FLUOGREEN nanoparticles to associate the 190 µg of β-Gal-TAG, according to EQUATION 3 of Tables and Technical Notes, is 100 µL.

$$\text{Volume of DIVERSA nanoparticles (mL)} = \frac{190}{1912} \times 1 \text{ mL} = 0{,}1 \text{ mL} = 100 \text{ µL}$$

8. The calculated final volume of **β-Gal-TAG** to associate with 100 µL of **DIVERSA FLUOGREEN** nanoparticles, according to EQUATION 4 of Tables and Technical Notes, is 50 µL.

$$\text{Volume of } \beta\text{–GAL–TAG } (\mu L) = \frac{100\ \mu L}{2} = 50\ \mu L$$

9. Concentrate the **β-Gal-TAG** solution from 86 µL to 50 µL with an additional centrifugation cycle. Then, invert the filter into a clean collector microtube and centrifuge at 1,000 RCF for 2 min at 4 ºC to collect the 50 µL of **β-Gal-TAG**. The **β-Gal-TAG** is now ready to use in the DIVERSA PROTEIN ASSOCIATION STEP or can be stored at -20 ºC for up to 1 month.
10. Formulate **DIVERSA FLUOGREEN** nanoparticles following the steps 1-3 of the FORMULATION STEP.
11. Associate 50 µL of **β-Gal-TAG** with 100 µL of **DIVERSA FLUOGREEN** nanoparticles following the steps 1-3 of the PROTEIN ASSOCIATION STEP. The **DIVERSA FLUOGREEN β-Gal DELIVERY NANOPARTICLES** are ready to use or can be stored at 2-8 ºC for to up 2 days.
12. The day of the experiment, add 1.6 µL of **DIVERSA FLUOGREEN β-Gal** nanoparticles (2 µg of β-Gal) per well in a final volume of 250 µL of complete medium (supplemented with 10% (v/v) of FBS and 1% (v/v) of penicillin/streptomycin).

    Note: The volume of **DIVERSA FLUOGREEN** nanoparticles should be adjusted based on your specific protein and experiment. Consider testing different volumes.
13. Incubate the cells at 37 ºC in a CO_2 incubator under standard conditions for at least 2-4 hours.

    Note: Depending on the type of readout assay performed, shorter or longer incubation time may influence delivery efficiency.
14. After incubation, remove the medium, wash the cells twice with DPBS 1X for 5 minutes.
15. Fix the cells with 2% (v/v) PFA; 0.2% (v/v) Glutaraldehyde in DPBS 1X for 15 minutes at RT.
16. Remove the fixing solution, wash the cells twice with DPBS for 5 minutes.
17. Add X-GAL staining solution containing 1 mg/ml X-gal (just enough to the cover cells) and incubate overnight at 37 ºC in darkness.
18. Remove the staining solution, wash the cells twice with distilled water.
19. Add 1X DPBS and check the cells to determine whether cells are turning blue. For long term storage, wrap in parafilm and store at 2-8°C.

CELL INTERNALIZATION ASSAY

EXAMPLE PROTOCOL

1. Seed the recommended number of the cells in a 24-well plate, chamber, or similar device, the day before the experiment.

   Note$_1$: Optimizations should be performed depending on the cell type and the length of the experiment.

   Note$_2$: Cell culture medium supplemented with 10% (v/v) of FBS and 1% (v/v) of Penicillin-Streptomycin, is recommended.

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

3. Remove the medium for the wells, add 225 µL of fresh culture medium and 25 µL of DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES

   Note$_1$: The volume of DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES should be adjusted based on your specific protein and loading used. Consider testing different volumes.

4. Incubate the cells at 37 ºC in a CO_2 incubator under standard conditions for 2-4 hours.

   Note: Depending on the type of readout assay performed, shorter or longer incubation time may influence delivery efficiency.

5. After incubation time, remove the medium, wash the cells twice with DPBS 1X and proceed with the appropriate assay for your desired readout (FACS analysis, fluorescent/confocal microscopy, or a plate reader).

   Note: We recommend washing the cells with DPBS 1X buffer containing calcium and magnesium ions to avoid maximum detachment of living cells.

   Adjust the equipment settings to visualize DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES, setting the excitation wavelength to 495 nm and the emission wavelength to 503 nm.

TABLES AND TECHNICAL NOTES

DIV-LINKER VOLUME CALCULATOR

EQUATION 1 calculates the maximum amount of protein that can be tagged with the provided 40 µL of DIV-LINKER, considering the protein's molecular weight (MW).

$$\text{Max Protein (µg)} = 3.54 \times \text{MW of your protein (kDa)}$$

Example: For a protein with a molecular weight of 35,000 g/mol (35 kDa), the maximum amount of protein you can tag with the 40 µL of DIV-LINKER is:

$$\text{Max Protein (µg)} = 3.54 \times 35 = 124 \text{ µg}$$

EQUATION 2 calculates the proportionally smaller volume of linker needed if you wish to tag a smaller amount of protein, based on your experimental needs and the intended amount of protein to associate with the nanoparticles.

$$\text{Volume of LINKER needed (µL)} = \frac{\text{40 µL of LINKER} \times \text{Desired Protein Amount (µg)}}{\text{Max Protein (µg)}}$$

Example: If you only need 50 µg of your protein (35 kDa) for your experiments, the volume of DIV-LINKER required is 16.13 µL.

$$\text{Volume of LINKER needed} = \frac{40 \times 50}{124} = 16.13\ \mu L$$

NANOPARTICLES VOLUME CALCULATOR

EQUATION 3 calculates the volume of DIVERSA FLUOGREEN nanoparticles needed to associate the Desired Protein Amount.

$$\text{Volume of DIVERSA nanoparticles (mL)} = \frac{\text{Desired Protein Amount (µg)}}{\text{Max protein (µg)}} \times 1\ mL$$

Note: The minimum volume of DIVERSA FLUOGREEN nanoparticles is 30 µL. If EQUATION 3 yields a smaller volume, increase the amount of Desired Protein (µg).

Example: For associating 50 µg of a protein of 35 kDa, the volume of DIVERSA FLUOGREEN nanoparticles required will be:

$$\text{Volume of DIVERSA nanoparticles (mL)} = \frac{50}{124} \times 1 = 0.403 \text{ mL} = 403 \text{ µL}$$

PROTEIN-TAG VOLUME CALCULATOR

EQUATION 4 calculates the final volume of PROTEIN (PROTEIN-TAG) needed for association with the volume of DIVERSA FLUOGREEN nanoparticles.

$$\text{Volume of PROTEIN–TAG } (\mu L) = \frac{\text{Volume of DIVERSA's nanoparticles } (\mu L)}{2}$$

Note: The minimum volume of PROTEIN for association is 15 µL. If EQUATION 4 yields a smaller volume, increase the amount of Desired Protein (µg) in EQUATION 3.

Example: For 403 µL of DIVERSA FLUOGREEN nanoparticles the volume of PROTEIN-TAG required will be:

$$\text{Volume of PROTEIN } (\mu L) = \frac{403}{2} = 201{,}5\ \mu L$$

TABLE OF EXAMPLES

Table 1. Examples of required volumes of DIV-LINKER, DIVERSA FLUOGREEN nanoparticles, and PROTEIN-TAG calculated using the provided equations for varying amounts of proteins with different molecular weights (MW).

MW PROTEIN	Amount of PROTEIN[1]	Volume of DIV-LINKER[2]	Volume of DIVERSA FLUOGREEN NANOPARTICLES[3]	Volume of PROTEIN-TAG[4]
500 kDa	1771 µg	40 µL	1 mL	500 µL
	886 µg	20 µL	500 µL	250 µL
	531 µg	12 µL	300 µL	150 µL
	89 µg	2 µL	50 µL	25 µL
250 kDa	886 µg	40 µL	1 mL	500 µL
	443 µg	20 µL	500 µL	250 µL
	266 µg	12 µL	300 µL	150 µL
	44 µg	2 µL	50 µL	25 µL
100 kDa	354 µg	40 µL	1 mL	500 µL
	177 µg	20 µL	500 µL	250 µL
	106 µg	12 µL	300 µL	150 µL
	18 µg	2 µL	50 µL	25 µL
50 kDa	177 µg	40 µL	1 mL	500 µL
	89 µg	20 µL	500 µL	250 µL
	53 µg	12 µL	300 µL	150 µL
	9 µg	2 µL	50 µL	25 µL
15 kDa	53 µg	40 µL	1 mL	500 µL
	27 µg	20 µL	500 µL	250 µL
	16 µg	12 µL	300 µL	150 µL
	3 µg	2 µL	50 µL	25 µL

[1] To be defined by end-users according to the experimental needs.
[2] Calculate according to EQUATION 1 and EQUATION 2.
[3] Calculate according to EQUATION 3.
[4] Calculate according to EQUATION 4.

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FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What is the amount of the fluorophore in **DIV031F1**?	The fluorophore amount is 4 µg.
Is the fluorochrome pH sensitive?	No, it is stable across a wide pH range.
Does the fluorochrome affect the biological activity?	No, it does not. The fluorochrome is covalently linked to the lipids and does not interfere with the biological activity of the cells.
What is the purpose of adding a tag to the protein?	The tag in the protein facilitates its association to DIVERSA's nanoparticles by following easy and mild procedures that do not compromise its structure and activity.
Can I filter the formulation?	Yes, DIVERSA FLUOGREEN PROTEIN nanoparticles can be filtered using 0.22 µm PES membrane filters if needed.
How can I measure the size of the final formulation?	Diameter size can be measured using an equipment as Dynamic Light Scattering (DLS) analysis.
Can DIVERSA FLUOGREEN nanoparticles be used to increase the intracellular concentration of a specific protein replacing gene transfection experiments?	DIVERSA FLUOGREEN nanoparticles can indeed lead to an increased in the intracellular content of the associated protein in much simple and easy way in relation to conventional transfection protocols. The reagents can be added when the cell culture media is replaced, or at specific time points. Please, contact DIVERSA's team for advice.
Can I use DIVERSA FLUOGREEN nanoparticles for in vivo studies?	DIVERSA FLUOGREEN nanoparticles can be used for cell or organ analysis by flow cytometry or confocal microscopy, and we offer customized solutions to adjust fluorophore concentration as needed. For whole-body imaging, DIVERSA provide customized reagents labeled with fluorophores such as Cy7.5, tailored to your experimental settings.

ONLINE RESOURCES

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

Click here to watch the video of the DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES.

CHANGELOG

Version	Date	Change Description
1.0	1 MAR 2022	Initial release of the protocol.
2.0	20 NOV 2024	Updated equations and examples for better clarity of volume calculations; improve readability.