

DIVTECH FLUOGREEN PROTEIN KIT

Tracking intracellular delivery of a **broad range** of proteins

USER PROTOCOL – #DIV031F1

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ABOUT THE KIT

OVERVIEW

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

DIVTECH FLUOGREEN PROTEIN KIT, based on lipids carrying a reactive group, is suitable for click chemistry. A modification in your protein/s of interest is needed for the association to the **DIVTECH** formulation but it does not compromise its structure and activity. Azido (N₃- group)-modified proteins will covalently react with the group exposed on the surface of the **DIVTECH** formulation by following easy and mild conditions.

DIVTECH FLUOGREEN PROTEIN KIT uses strongly labelled fluorescent nanoemulsions that are easily internalized by live cells that 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.

COMPONENTS

- 1x **DIV-LINKER**.
- 1x **DIV031F1** vial for reconstitution.
- 1x **DIVTECH** vial for preparation of **DIVTECH FLUOGREEN PROTEIN** formulation.
- 1x Purification device.
- 2x Tips for 1 mL micropipette.

STORAGE

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

Shipping temperature may differ from storage temperature. This does not alter the performance of the product.

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EQUIPMENT AND MATERIALS REQUIRED BUT NOT SUPPLIED

- 1 mL micropipette.
- MilliQ water or any other recommended buffer.
- Ethanol 96%.
- DMSO.
- Protein/s of interest.

CONSIDERATIONS BEFORE STARTING

- The proteins need to be functionalized with a linker (via N3-PEG8-NHS ester) for an efficient association to **DIVTECH FLUOGREEN** formulation (follow the protocol below).
- The following protocol is optimized for the preparation of 1 mL of **DIVTECH FLUOGREEN** formulation (starting from one **DIV031F1** 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 **DIVTECH FLUOGREEN** formulation within 60 days.
- **DIVTECH FLUOGREEN** formulation is stable for 24 h in cell culture media at 37 °C: DMEM, RPMI (with/without FBS).
- Do NOT use any buffer solution containing Triton-X, SDS or Tween 20 for the preparation or manipulation of **DIVTECH FLUOGREEN** / **DIVTECH FLUOGREEN-PROTEIN** formulation
- Do NOT freeze **DIVTECH FLUOGREEN** / **DIVTECH FLUOGREEN-PROTEIN** formulation.
- Do NOT heat up **DIVTECH FLUOGREEN** at temperatures higher than 90 °C for more than 2 h.
- Do NOT centrifuge or vortex **DIVTECH FLUOGREEN** / **DIVTECH FLUOGREEN-PROTEIN** formulation.

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DIVTECH FLUOGREEN PROTEIN KIT PROTOCOL

PROTEIN MODIFICATION STEP:

1. Add 40 μL of DMSO to the DIV-LINKER. Vortex gently and spin-down.
2. Select the volume of the linker solution based on the MW and desired quantity (μg) of your protein (see [Table 1](#) below). Take this amount of linker solution in a microcentrifuge tube and dilute up to 250 μL in dH_2O or the buffer of your selection (avoiding buffers containing β -mercaptoethanol, ammonium salts and primary amines).
3. Add the protein of interest into the microcentrifuge tube with the linker and adjust the final volume to 350 μL . Vortex gently.
4. Incubate the mixture at room temperature for 4-6 h or alternatively, overnight at 4 $^{\circ}\text{C}$.

Note: For the protein modification step a pH 7.2-8 is recommended.

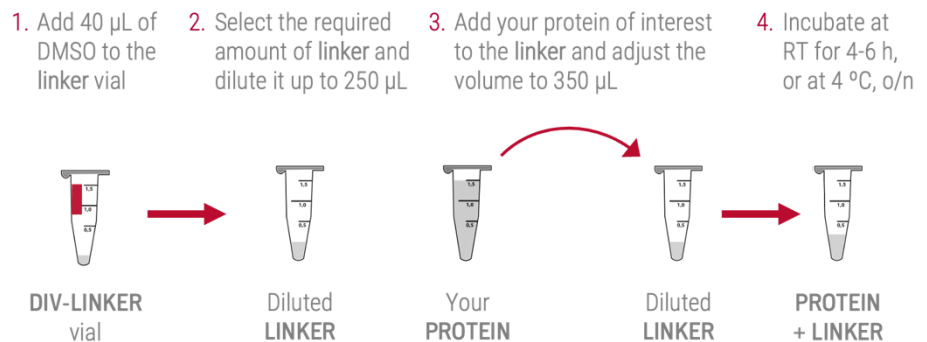


Figure 1. Protein modification step protocol.

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PROTEIN PURIFICATION STEP:

5. Dilute the modified protein to 500 μL with dH_2O and transfer the solution to the provided ready-to-use filters.
6. Centrifuge at 14,000 $\times g$ for 5 min, at room temperature (40° fixed angle rotor).
7. Discard the flow-through and repeat the steps 5-7 two more times.
8. Invert the concentrator with the modified protein (30-50 μL) into a clean microcentrifuge tube. Centrifuge at 1000 $\times g$ for 2 min to collect the concentrated N3-modified protein.
9. The modified protein is ready to be used in the next step or alternatively, keep it at -20 °C.

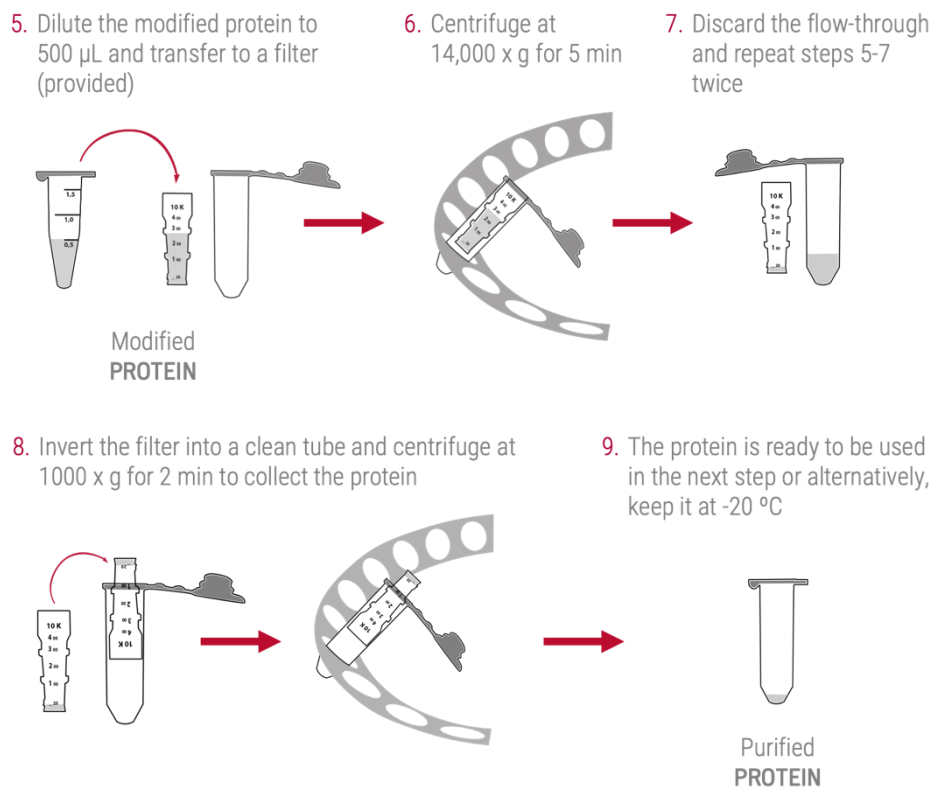


Figure 2. Protein purification step protocol.

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FORMULATION STEP:

10. Reconstitute the **DIV031F1** vial with 100 μL of EtOH and gently pipette up and down for mixing.
11. Add 900 μL of ultrapure water (milliQ) into the **DIVTECH** vial or, alternatively, a buffer solution suggested in **Table 2** (Recommendations of Use and Technical Notes).
12. Add the content of **DIV031F1** to the **DIVTECH** vial, using a 1 mL micropipette and the provided narrow 1 mL micropipette tip, in order to have more air volume for mixing in a fast and vigorous way.
13. The **DIVTECH FLUOGREEN** formulation is now ready for the association of the protein. Alternatively, keep it at 4 $^{\circ}\text{C}$ and use it in the following 60 days.

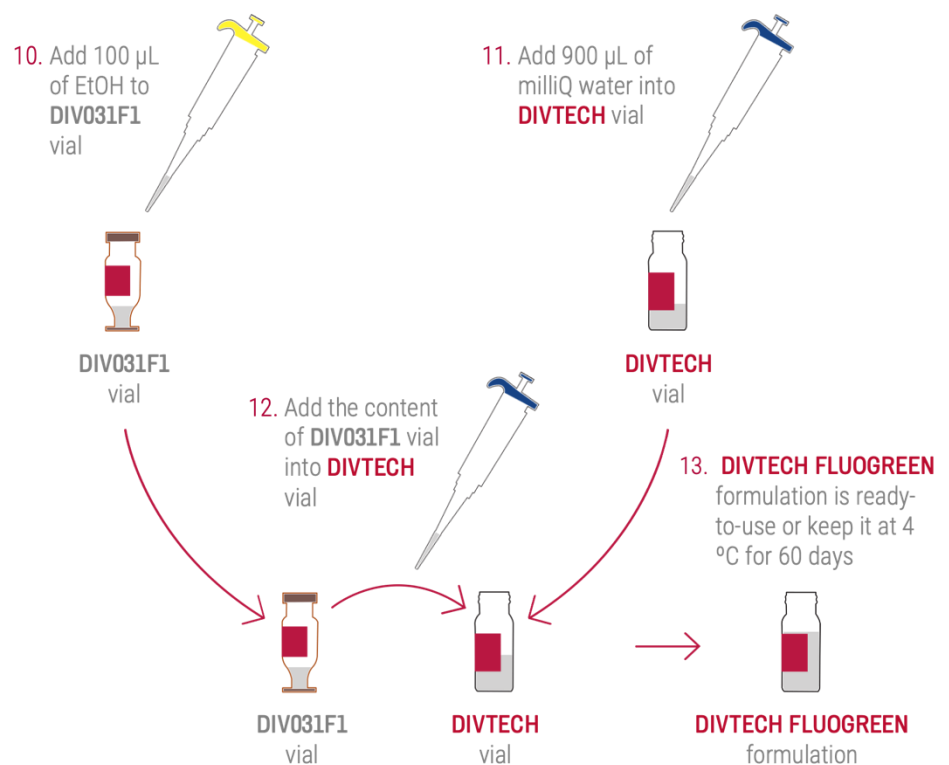


Figure 3. **DIVTECH FLUOGREEN** formulation step protocol

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DIVTECH FLUOGREEN-PROTEIN FORMULATION STEP:

14. Considering the amount of protein that you want to associate (see the note below), adjust the final volume of the modified protein following the recommendations in [Table 3](#).

Note: Follow this equation to calculate the required quantity of your protein:

$$\text{Protein_weight } [\mu\text{g}] = \mu\text{Molar } [\mu\text{mol/L}] \times \text{volume } [\mu\text{L}] \times \text{protein_molar_weight } [\text{Da}] \times 10^{-6}$$

[Conversion factor].

For example, to associate 50 μL of N3-modified BSA (molar weight 69300 Dalton) with 100 μL of DIVTECH formulation and obtain maximum yield of protein associated formulation (5.4 μM), one should add $5.4 \mu\text{M} \times 50 \mu\text{L} \times 69300 \text{ Da} \times 10^{-6} = 18,7 \mu\text{g}$ of N3-modified BSA protein.

15. Add the modified protein solution gently and dropwise into the **DIVTECH FLUOGREEN** formulation. Recommended volumes are provided in [Table 3](#).
16. Incubate the mixture at room temperature for 4-6 h followed by overnight incubation at 4 °C.

The **DIVTECH FLUOGREEN-PROTEIN** formulation is now ready-to-use ([Table 4](#)). Alternatively, keep it at 4 °C and use it in the following 30 days.

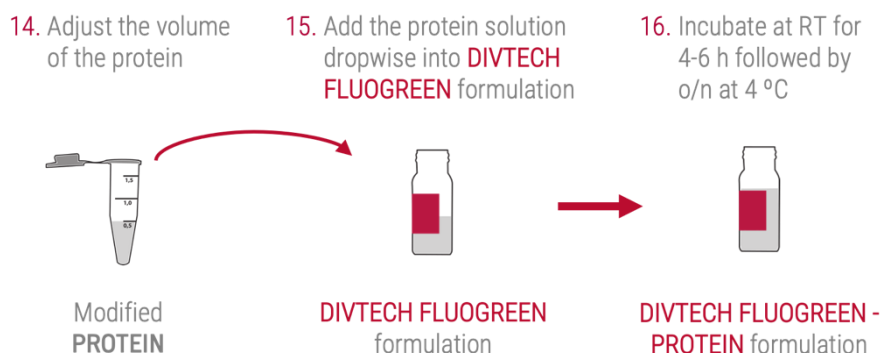


Figure 4. **DIVTECH FLUOGREEN-PROTEIN KIT** protocol.

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EXAMPLE OF β -GAL ASSOCIATION PROTOCOL

1. Add 40 μ L of DMSO to the **DIV-LINKER**. Vortex gently and spin-down.
2. Take 5 μ L of the linker solution (for association of 175 μ g of β -Gal, MW = 540 kDa) and dilute up to 250 μ L in dH₂O.
3. Add 35 μ L of the β -Gal solution (5 mg/ml) to the tube with the linker and adjust the final volume to 350 μ L. Vortex gently.
4. Incubate the mixture 6 h at room temperature and then, overnight at 4 °C.
5. The next day dilute the modified β -Gal-N3 to 500 μ L with dH₂O and transfer the solution to the provided ready-to-use filters.
6. Centrifuge at 14,000 x g for 5 min at room temperature (40° fixed angle rotor).
7. Discard the flow-through and repeat the steps 5-7 two more times.
8. Invert the concentrator with the modified β -Gal-N3 (50 μ L) into a clean microcentrifuge tube. Centrifuge at 1000 x g for 2 min to collect the concentrated β -Gal-N3.
9. Reconstitute the **DIV031F1** vial with 100 μ L of EtOH and gently pipette up and down for mixing.
10. Add 900 μ L of ultrapure water (milliQ) into the **DIVTECH** vial.
11. Add the content of **DIV031F1** to the **DIVTECH** vial.
12. Add 50 μ L of the β -Gal-N3 gently and dropwise into 100 μ L of the **DIVTECH** formulation.
13. Incubate the mixture at room temperature for 4-6 h followed by overnight incubation at 4 °C.
14. The **DIVTECH-PROTEIN** formulation is now ready-to-use.

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RECOMMENDATIONS OF USE AND TECHNICAL NOTES

Table 1. Suggested volume of the linker solution based on the required amount and MW of your protein of interest.

MW PROTEIN	PROTEIN	DIV-LINKER	H ₂ O/BUFFER
> 250 kDa	> 50 µg	5 µL	Up to 350 µL
	20-50 µg	2 µL	
	1-20 µg	1 µL	
15-250 kDa	> 50 µg	40 µL	Up to 350 µL
	20-50 µg	15 µL	
	1-20 µg	5 µL	
< 15 kDa	> 50 µg	40 µL	Up to 350 µL
	20-50 µg	20 µL	
	1-20 µg	10 µL	

Table 2. Suggested aqueous solutions for **DIVTECH** vial.

AQUEOUS SOLUTION	CONCENTRATION
Ultrapure water	N/A
PBS	2-50 mM
NaCl	150 mM
HEPES	10-25 mM

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Table 3. Suggested volumes for **DIVTECH** formulation.

DIVTECH formulation	N3-PROTEIN solution
1 mL*	500 µL
500 µL	250 µL
200 µL	100 µL
100 µL	50 µL

* It is recommended a mild stirring in a shaker (250-300 rpm) during the addition of the protein

Note: The recommended concentration of your protein of interest is up to 6.5 µM in one of the suggested aqueous solutions in Table 2.

Table 4. Recommended volumes for cell culture.

Cell culture vessel	Volume of DIVTECH	Volume of medium	Final volume/well
100 cm	200 µL	4,8 mL	5 mL
6-well	40 µL	960 µL	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

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FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What is the concentration of the fluorophore in DIV021F1 ?	The concentration of the fluorophore is 4 µg/mL in the final DIVTECH FLUOGREEN formulation.
What is the role of the modification of the protein?	The modification of the proteins facilitates their association to DIVTECH formulation by following easy and mild procedures that do not compromise its structure and activity. DIVTECH does not include cationic components.
Can the protein modification alter its biological function?	Usually, the proposed modifications do not alter the native protein biological function. Please contact DIVERSA for advice depending on your specific molecule. DIVERSA can provide alternative procedures.
Are there additional modifications that can be suitable to my protein?	Please contact DIVERSA for advice depending on your specific molecule.
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 DIVTECH FLUOGREEN-PROTEIN formulation with 180 µL of milliQ water.
What if I need to work with higher protein 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.
How do I concentrate the formulation?	If necessary, the 1 mL of DIVTECH FLUOGREEN formulation can be concentrated by using a SpeedVac or Rotavap in mild conditions (avoid surpassing 35 °C or drying out 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.

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