DIVERSA

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DIVTECH PROTEIN KIT

Enhancing intracellular delivery of a broad range of proteins

USER PROTOCOL – **#DIV031**

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ABOUT THE KIT

OVERVIEW

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

DIVTECH 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.

COMPONENTS

- 1x DIV-LINKER.
- 1x DIV031 vial for reconstitution.
- 1x DIVTECH vial for preparation of DIVTECH formulation.
- 1x Purification device.
- 2x Tips for 1 mL micropipette.

STORAGE

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

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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 funcionalized with a linker (N3-PEG8-NHS ester) for an efficient association to DIVTECH formulation (follow the protocol below).
- The following protocol is optimized for the preparation of 1 mL of DIVTECH-PROTEIN formulation (starting from one DIV031 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 formulation within 60 days.
- DIVTECH formulation is stable for 24 h in cell culture media at 37 ºC: 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 / DIVTECH-PROTEIN formulation.
- Do NOT freeze DIVTECH / DIVTECH-PROTEIN formulation.
- Do NOT heat up DIVTECH at temperatures higher than 90 ºC for more than 2 h.
- Do NOT centrifuge or vortex DIVTECH / DIVTECH-PROTEIN formulation.

DIVTECH 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 solutions 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 ºC.

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

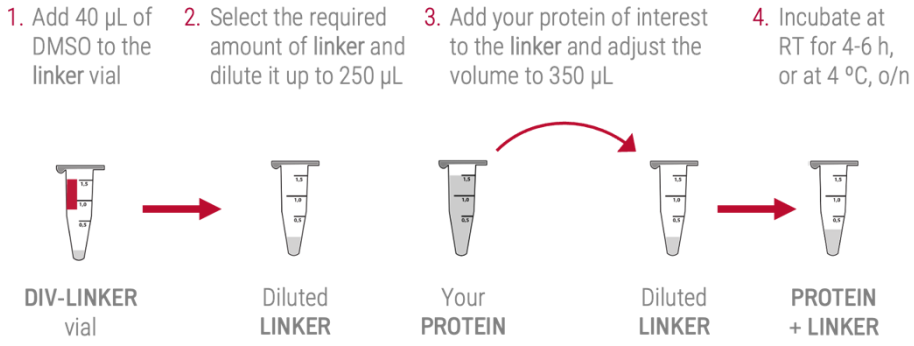

Figure 1. Protein modification step protocol.

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 x g for 5 min, at room temperature (40º 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 x 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 ºC.

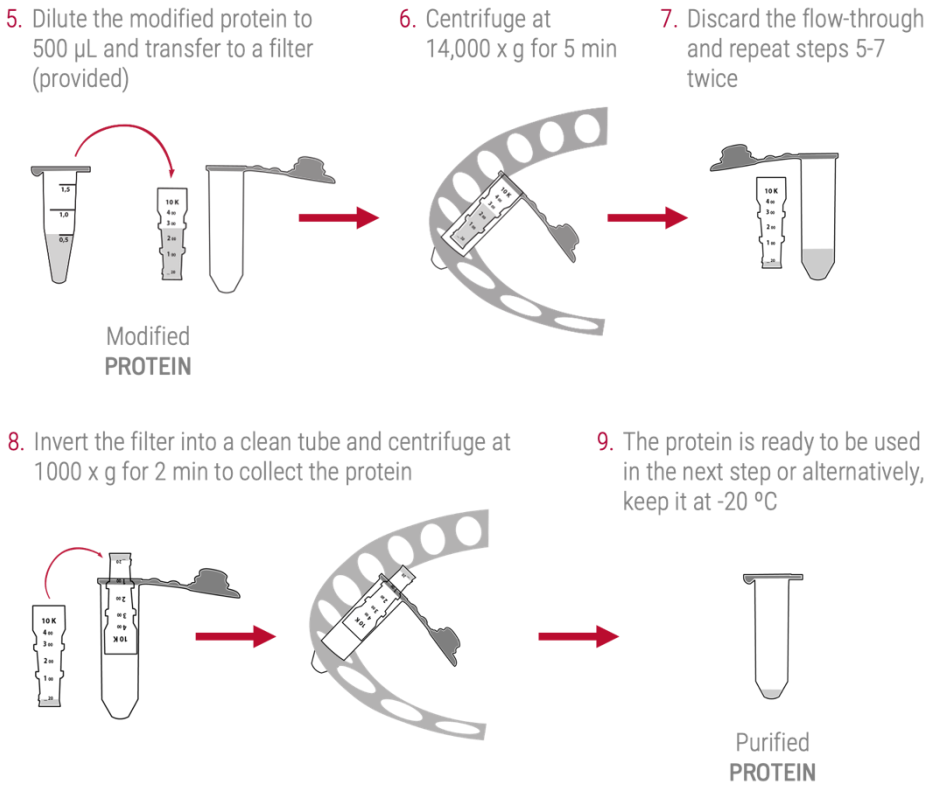

Figure 2. Protein purification step protocol.

FORMULATION STEP:

10. Reconstitute the **DIV031** 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 **DIV031** 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** formulation is now ready for the association of the protein. Alternatively, keep it at 4 °C and use it in the following 60 days.

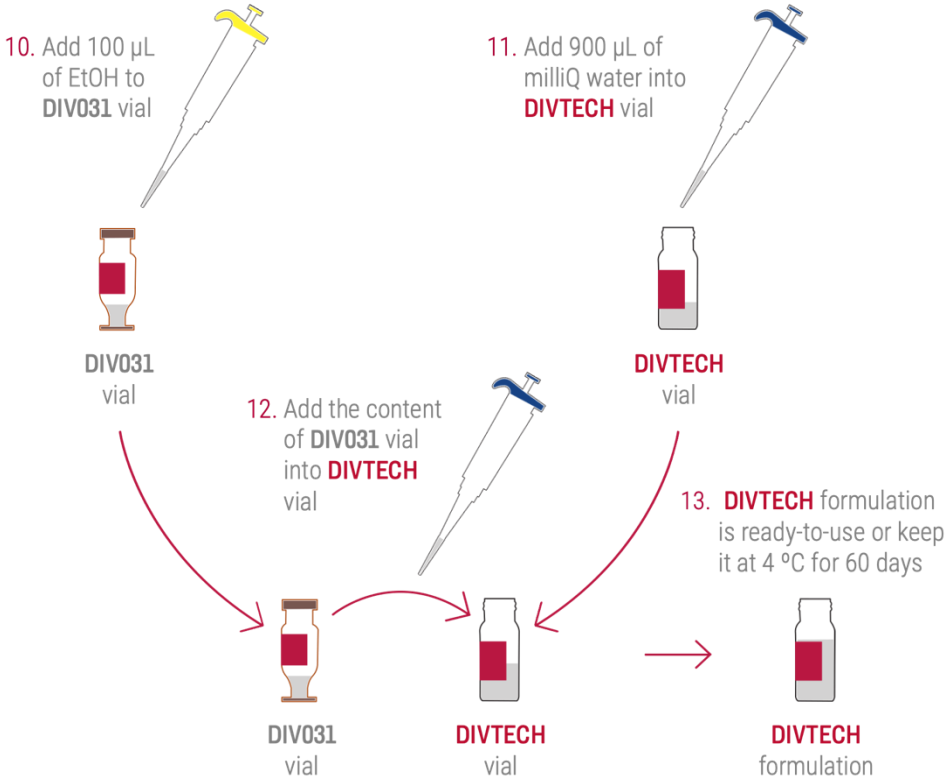

Figure 3. **DIVTECH** formulation step protocol

DIVTECH-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:

    Protein_weight [µg] = µMolar [µmol/L] x volume [µL] x protein_molar_weight [Da] x 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 µM x 50 µL x 69300 Da x 10^{-6} = 18,7 µg of N3-modified BSA protein.

15. Add the modified protein solution gently and dropwise into the DIVTECH 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 ºC.

    The DIVTECH-PROTEIN formulation is now ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 30 days.

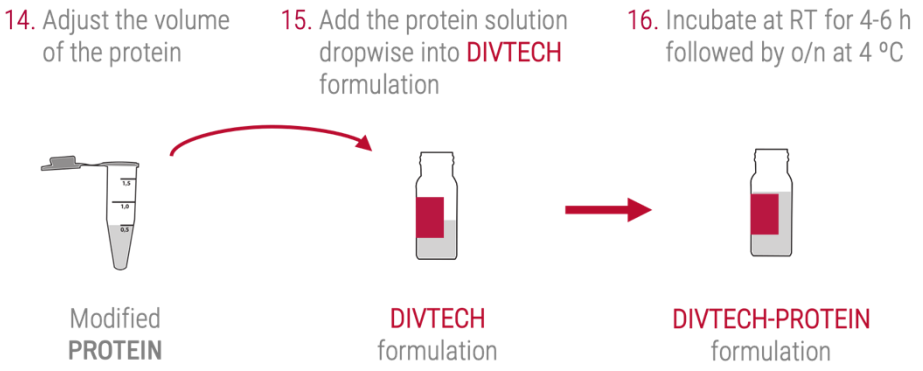

Figure 4. DIVTECH-PROTEIN KIT protocol.

EXAMPLE OF β-GAL ASSOCIATION PROTOCOL

1. Add 40 µL of DMSO to the **DIV-LINKER**. Vortex gently and spin-down.
2. Take 5 µL of the linker solution (for association of 175 µg of β-Gal, MW = 540 kDa) and dilute up to 250 µL in dH_2O.
3. Add 35 µL of the β-Gal solution (5 mg/ml) to the tube with the linker and adjust the final volume to 350 µL. Vortex gently.
4. Incubate the mixture 6 h at room temperature and then, overnight at 4 ºC.
5. The next day dilute the modified protein β-Gal-N3 to 500 µL with dH_2O and transfer the solution to the provided ready-to-use filters.
6. Centrifuge at 14,000 x g for 5 min at room temperature (40º 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 β-Gal-N3 (50 µL) into a clean microcentrifuge tube. Centrifuge at 1000 x g for 2 min to collect the concentrated β-Gal-N3.
9. Reconstitute the **DIV031** vial with 100 µL of EtOH and gently pipette up and down for mixing.
10. Add 900 µL of ultrapure water (milliQ) into the **DIVTECH** vial.
11. Add the content of **DIV031** to the **DIVTECH** vial.
12. Add 50 µL of the modified β-Gal-N3 gently and dropwise into 100 µL of the **DIVTECH** formulation.
13. Incubate the mixture at room temperature for 4-6 h followed by overnight incubation at 4 ºC.
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_2O/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

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.

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
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-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 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.