DIVERSA

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DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES

Enhancing intracellular delivery of a broad range of proteins

USER PROTOCOL – **#DIV031F1**

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ABOUT THE NANOPARTICLES

OVERVIEW

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

DIVERSA PROTEIN DELIVERY NANOPARTICLES, 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 DIVERSA NANOPARTICLES without comprising its structure and activity. Azido (N3- group)-modified proteins will covalently react with the group exposed on the surface of the DIVESA NANOPARTICLES by following easy and mild conditions.

DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES uses strongly labelled fluorescent nanoemulsions that are easily internalized by live cells and 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 DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES.
- 1x Ultracentrifugation filter 0.5 mL- 10 kDa.
- 2x Colector microtubes.
- 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%.
- PBS 2 mM at pH 7.4.
- DMSO.
- Protein/s of interest.

CONSIDERATIONS BEFORE STARTING

- The proteins need to be functionalized with the DIV-LINKER for an efficient association to DIVERSA FLUOGREEN NANOPARTICLES (follow the protocol below).
- The following protocol is optimized for the preparation of 1.5 mL of DIVERSA FLUOGRENN PROTEIN DELIVERY NANOPARTICLES (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 DIVERSA FLUOGREEN NANOPARTICLES within 60 days.
- DIVERSA FLUOGREEN NANOPARTICLES is stable 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/DIVERSA FLUOGRENN PROTEIN DELIVERY NANOPARTICLES.
- Do NOT freeze DIVERSA/DIVERSA FLUOGRENN PROTEIN DELIVERY NANOPARTICLES.
- Do NOT heat up DIVERSA/DIVERSA FLUOGRENN PROTEIN DELIVERY NANOPARTICLES.
- Do NOT centrifuge or vortex DIVERSA/DIVERSA FLUOGRENN PROTEIN DELIVERY NANOPARTICLES.

DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES

PROTOCOL

PROTEIN MODIFICATION STEP:

Note: pH 7.2 - 8 is recommended for protein modification.

1. Add 40 µL of DMSO to the **DIV-LINKER** microtube. Vortex gently and then, spin-down.
2. Select the volume of **DIV-LINKER** microtube based on the MW and the amount of your protein as we recommend in Table 1 (Recommendations of Use and Technical Notes). Then, dilute the **DIV-LINKER** solution up to 250 µL in PBS 2 mM at pH 7.4 or the buffer suggested in Table 2 (Recommendations of Use and Technical Notes).

   Note: Avoid buffers containing b-mercaptoethanol, ammonium salts and primary amines.
3. Add the protein of interest to the **DIV-LINKER** microtube and adjust the final volume to 350 µL with PBS 2 mM at pH 7.4 or the buffer suggested in Table 2 (Recommendations of Use and Technical Notes). Vortex gently and then, spin-down.
4. Incubate the **PROTEIN-LINKER** for 6 h at room temperature (RT) and then, incubate overnight at 4 ºC.

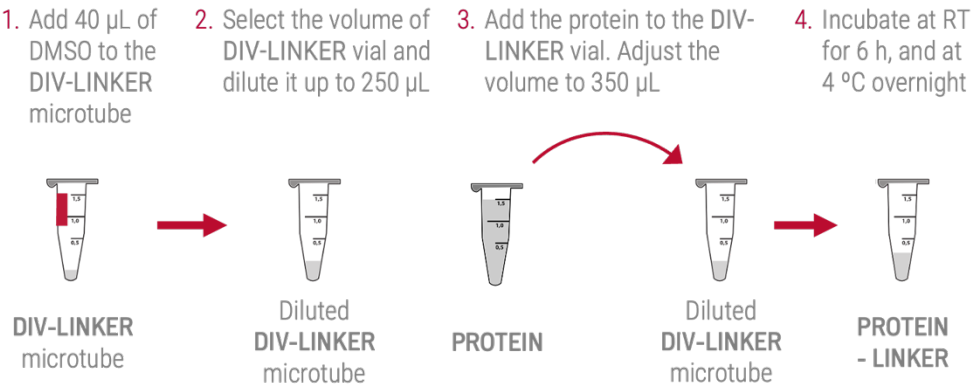

Figure 1. PROTEIN MODIFICATION STEP protocol.

PROTEIN PURIFICATION STEP:

5. Equilibrate the Amicon Ultracentrifugation filter 0.5 mL- 10 kDa with PBS 2 mM at pH 7.4 or the buffer used in the previous protocol. Centrifuge at 14,000 RCF for 5 min at 4 ºC. Discarding the flow-through and the filter is ready to use.
6. After incubation time, transfer the PROTEIN-LINKER to the Amicon Ultracentrifugation filter 0.5 mL- 10 kDa, and top up the volume to 500 µL with PBS 2 mM at pH 7.4 or the buffer suggested in Table 2 (Recommendations of Use and Technical Notes).
7. Centrifuge at 14,000 RCF for 5 min at 4 ºC.

   Note: The volume should drop to ~ 300 µL.
8. Discard the flow-through. Wash the PROTEIN-LINKER four times with PBS 2 mM at pH 7.4 or the buffer suggested in Table 2 (Recommendations of Use and Technical Notes) as performed in the previous step. Centrifuge at 14,000 RCF for 5 min at 4 ºC.

   Note: the centrifugation time of the last wash depends on the desired volume you want to concentrate your protein.

   Note: we recommend concentrate the PROTEIN-LINKER at 2.5 mg/mL.
9. Invert the filter containing the PROTEIN-LINKER into a clean collector microtube, and centrifuge at 1,000 RCF for 2 min at 4 ºC. A 100% of N3-MODIFIED PROTEIN recovery is guaranteed by this method.

The N3-MODIFIED PROTEIN is now ready-to-use. Alternatively, keep it at -20 ºC and use it in the following 3 months.

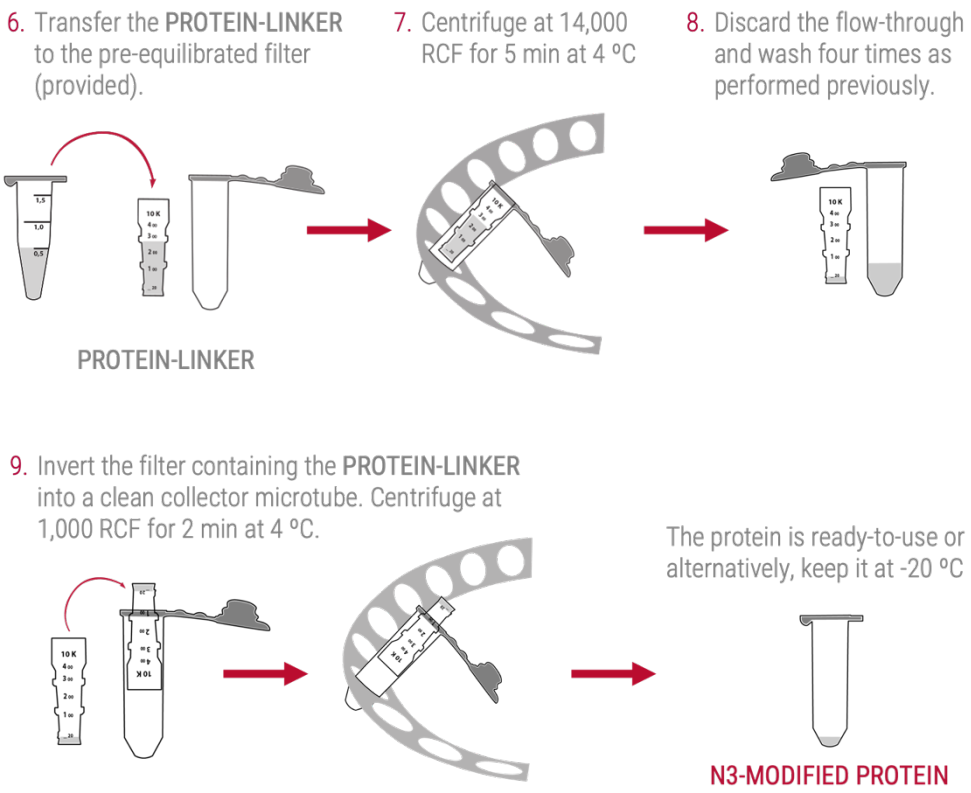

Figure 2. PROTEIN PURIFICATION STEP protocol.

DIVERSA FORMULATION STEP:

10. Reconstitute the DIV031F1 vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids trying to recover all of them form the wall vial and keep the suspension in the vial.
11. Add 900 µL of ultrapure water into the DIVTECH vial.
12. Transfer the whole volume from DIV031F1 vial to the DIVTECH vial, using a micropipette and the 1 mL micropipette tip provided.

IMPORTANT: Before adding the lipids from DIV03F1 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 NANOPARTICLES is now ready for the association of the protein. Alternatively, keep it at 4 °C and use it in the following 60 days.

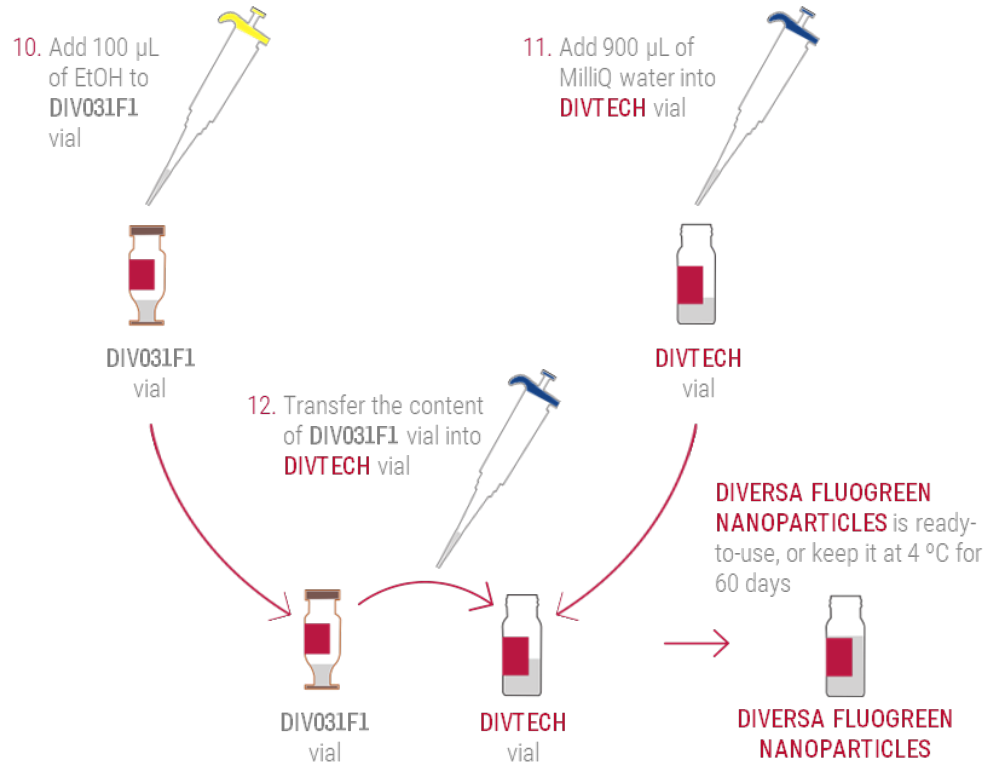

Figure 3. DIVERSA FLUOGREEN FORMULATION STEP protocol.

DIVERSA FLUOGREEN PROTEIN ASSOCIATION STEP:

13. See the following Table to prepare DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES depending on the required quantity of your protein to obtain a maximum yield of association of 6.5 µM:

DIVERSA FLUOGREEN NANOPARTICLES	N3-PROTEIN solution	µg of β-GAL
1 mL	500 µL	1755 µg
500 µL	250 µL	877.5 µg
200 µL	100 µL	351 µg
100 µL	50 µL	175.5 µg
50 µL	25 µL	87.75 µg

Note: Follow this equation to calculate the required quantity of your protein with the recommended concentration at 6.5 µM.

$$\text{Protein (µg)} = 6.5\ \mu\text{M x } \mathbf{V\ (N3 - PROTEIN\ solution)} \text{ x Mw (kDa) x } 10^{-3}$$

Note: For example, to associate 50 µL of N3-modified β-Gal (540 kDa) with 100 µL of DIVERSA FLUOGREEN NANOPARTICLES to obtain a maximum yield of protein associated formulation (6.5 µM), you should add 175.5 µg of N3-modified β-Gal.

$$\text{N3} - \beta - \text{Gal} = 6.5\ \mu\text{M x } \mathbf{50\ \mu L} \text{ x } 540\ \text{kDa x } 10^{-3} = 175.5\ \mu\text{g}$$

14. Add the N3-MODIFIED PROTEIN into DIVERSA FLUOGREEN NANOPARTICLES. Pipette up and down gently for 30 seconds, avoiding any spillage.

15. Incubate the DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES for 4 h at RT and then, incubate overnight at 4 ºC.

The DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES is now ready-to-use. Alternatively, keep it at 4 ºC and use it in the following 30 days.

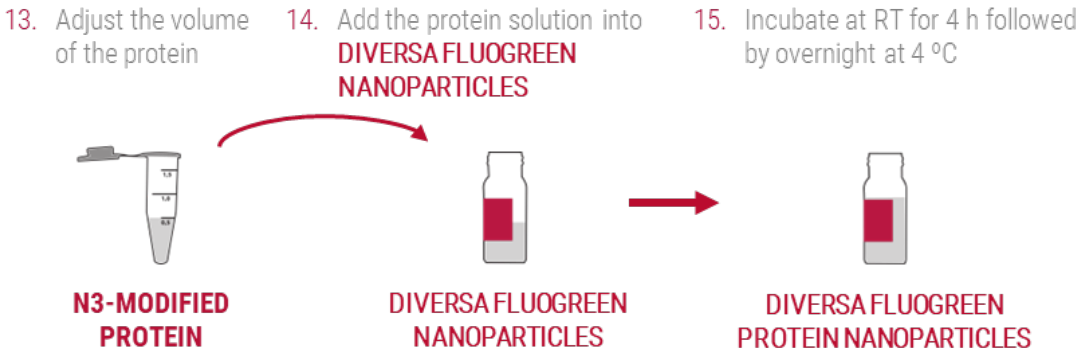

Figure 1. DIVERSA FLUOGREEN PROTEIN FORMULATION STEP protocol.

EXAMPLE OF β-GAL ASSOCIATION PROTOCOL

1. Add 40 µL of DMSO to the **DIV-LINKER** microtube. Vortex gently and then, spin-down.
2. Following the Table 1, to associate 175 µg of β-Gal (540 kDa), take 5 µL of the **DIV-LINKER** microtube and dilute up to 250 µL in dH_2O.
3. Add 35 µL of β-Gal solution (stock concentration at 5 mg/mL) to the **DIV-LINKER** microtube and adjust the final volume to 350 µL with dH_2O. Vortex gently and then, spin-down.
4. Incubate the **PROTEIN-LINKER** for 6 h at RT and then, overnight at 4 ºC.
5. After incubation time, transfer the **PROTEIN-LINKER** to the pre-equilibrated filter and add dH_2O to up 500 µL. Centrifuge at 14,000 RCF for 5 min at 4 ºC.
6. Discard the flow-through and wash the **PROTEIN-LINKER** four times with dH_2O centrifuging at 14,000 RCF for 5 min at 4 ºC.
7. Invert the filter into a clean collector microtube and centrifuge at 1,000 RCF for 2 min at 4 ºC to collect the 100% of the β-Gal-N3.
8. Reconstitute the **DIV031F1** vial with 100 µL of EtOH. Pipette up and down gently for mixing the lipids.
9. Add 900 µL of ultrapure water into the **DIVTECH** vial.
10. Add the content of **DIV031F1** to the **DIVTECH** vial.
11. Transfer the whole volume from **DIV031F1** vial to the **DIVTECH** vial, using a micropipette and the 1 mL micropipette tip provided. The **DIVERSA FLUOGREEN NANOPARTICLES** is now ready for the association of the protein.
12. To associate 175 µg of β-Gal-N3 (540 kDa) and following the previous Table: add 50 µL of the β-Gal-N3 into 100 µL of the **DIVERSA FLUOGREEN NANOPARTICLES**. Pipette up and down gently for 30 seconds, avoiding any spillage.
13. Incubate the **DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES** for 4 h at RT followed by overnight at 4 ºC.

The **DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES** is ready-to-use or alternatively, keep it at 4 ºC and use it in the following 30 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: the cell density should be determined for each cell type and well plate.

2. Prepare the DIVERSA FLUOGREEN PROTEIN 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 PROTEIN DELIVERY NANOPARTICLES to a final volume of 1 mL.

   Note: The final concentration of the DIVERSA FLUOGREEN PROTEIN 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 PROTEIN DELIVERY NANOPARTICLES and, carefully, wash the cells twice with PBS 1X buffer and remove it.

   The DIVERSA FLUOGREEN PROTEIN 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 595 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 PROTEIN DELIVERY NANOPARTICLES performance. Optimize one parameter at a time.

The following parameters can be optimized:

- Amount of DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES: Start fixing the concentration and amount of your protein to be delivered, and then you may vary the quantity of the DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES used.

- Amount of protein to be delivered: you may need to vary the amount of your protein to be delivered. Depending on the sensitivity of your assay, a greater amount of protein and DIVERSA FLUOGREEN PROTEIN DELIVERY NANOPARTICLES may be required.

- Concentration of the protein solution: We recommend protein concentration at 6.5 µM. At lower concentrations, we recommend concentrating your protein using ultracentrifugation filters 0.5 mL- 10 kDa. Use the corresponding buffer in Table 2 (Recommendations of Use and Technical Notes).
  If your protein is in powder, we recommend dissolve it at final concentration of 6.5 µM, using the corresponding buffer in Table 2 (Recommendations of Use and Technical Notes).

- Buffer used in **PROTEIN PURIFICATION STEP**: you can try different buffers as suggested in Table 2 (Recommendations of Use and Technical Notes). For some biomolecules, the buffer used may be critical, for example PBS buffer works well with β-Galactosidase, but not the Tris 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 volume of the **DIV-LINKER** solution based on the required amount and M_W of the protein of interest.

M_W PROTEIN	PROTEIN	DIV-LINKER	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 buffer for the **PROTEIN MODIFICATION STEP** and **PROTEIN PURIFICATION STEP** for *in vivo* experiments.

BUFFER	CONCENTRATION
Physiological saline	0.9% NaCl
Glucose solution for infusion	5-10% w/v
Sodium DL-Lactate solution	60% w/w

Table 3. Suggested buffer solutions for buffer exchange after DIVERSA FORMULATION.

BUFFER SOLUTION	CONCENTRATION
Ultrapure water	N/A
PBS	2-50 mM
NaCl	150 mM
HEPES	10-25 mM
MES	10-50 mM
Tris-HCl	0.1-1M

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
What is the role of the modification of the protein?	The modification of the proteins facilitates their association to DIVERSA FLUOGREEN NANOPARTICLES by following easy and mild procedures that do not compromise its structure and activity. DIVERSA 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 DIVERSA FLUOGREEN PROTEIN NANOPARTICLES 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 final volume of DIVERSA/DIVERSA FLUOGREEN PROTEIN NANOPARTICLES 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.