heliX+
User Manual

dynamic
BIOSENSORS

Amine Coupling Kit 5

His-mediated coupling of his-tagged molecules to the *ligand strand* - **proFIRE purification**

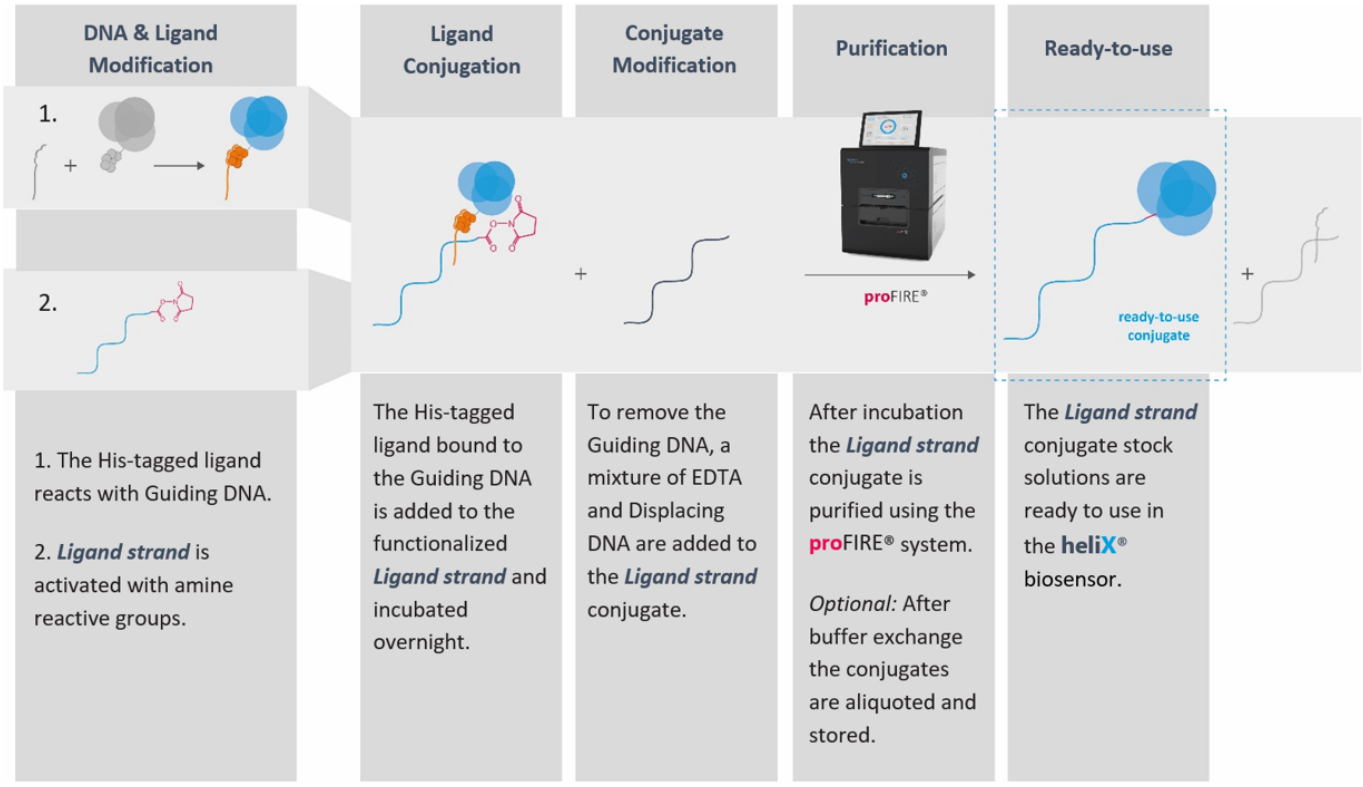
Dynamic Biosensors GmbH & Inc.
HK-NHS-5 v8.1

Key Features

- Allows **site-specific** covalent coupling of **his-tagged biomolecules** with primary amines (e.g. NH2-terminus, lysines) to the ***Ligand strand*** in a single reaction tube.
- **Oriented coupling** in the proximity of the His-tag.
- Convenient standard chemistry (NHS chemistry).
- Compatible with **heliX® Adapter Chip**.
- Compatible with **proFIRE® purification** for pure ligand-DNA conjugates (> 5 kDa).
- Coupling of multiple ligands can be performed simultaneously.
- Yields > 95 % pure ligand-DNA conjugate with user determined quality of final product.
- Includes reagents for five individual conjugation reactions (approx. 10-30 regenerations each; up to max. 300).
- Compatible with automated standard regeneration process.

Workflow Overview

3-Step Conjugation Workflow



Timeline: Hands on time < 1 h | Incubation ~ 12-16 h

Product Description

Order Number: HK-NHS-5

Table 1. Contents and Storage Information

Material	Cap	Amount	Storage
LS His NHS	Blue	3 x	-20°C
Buffer H	Transparent	3 x 1.8 mL	-20°C
Guiding DNA LS	Orange	3 x 36 µL	-20°C
Displacing DNA LS	Purple	3 x 12 µL	-20°C
Loading Solution (500 µM)	Transparent	50 µL	-20°C
EDTA Solution (500 mM)	Transparent	100 µL	-20°C
Buffer PE40 [1]	Transparent	5 x 1.5 mL	-20°C
ddH_2O	Transparent	1.5 mL	-20°C
Crosslinker	Brown	3 x	-20°C
Purification spin column	Red	6 x	2-8°C
2.0 mL reaction tubes for purification spin column		6 x	RT
Centrifugal filter unit (3 kDa MWCO)[2]		3 x	RT
Centrifugation collection tube		6 x	RT

For research use only.

This product has a limited shelf life, please see expiry date on label.

IMPORTANT

Products may be shipped at different temperatures, but storage should adhere to the guidelines outlined in the Table.
The kit contains reagents sufficient for three conjugations of approximately 0.7 nmol of biomolecule each. The Loading solution contains 500 µM $NiCl_2$. The resin slurry in the purification spin column contains 0.02 % sodium azide.

Additional Materials Required

Table 2. Additional Materials

Material	Comments
Benchtop microcentrifuge	Required speed range of between 1,000 x g to 13,000 x g
Vortex	
1.5 mL reaction tubes	
UV-Vis Spectrophotometer (e.g. Nanodrop)	For determination of the ***Ligand strand*** conjugate's concentration

All necessary solutions and buffers are included in the kit.

Important Notes

a. The lyophilized ***Ligand strand*** may not always be found at the bottom of the tube; it could remain on the tube wall or in the tube cap. Please always check for the presence of the lyophilized ***Ligand strand***, identifiable by its clear pellet appearance (you may need to remove the tube label to see it). If it is not at the bottom, please centrifuge the tube at high speed for a couple of minutes before dissolving the DNA in buffer. Alternatively, place the tip of your pipette near the DNA pellet and dispense the buffer directly onto it; the DNA will quickly dissolve.

b. The crosslinker will be linked to the primary amine groups ($-NH_2$) of the ligand. Primary amines exist at the N-terminus of each polypeptide chain and in the side-chain of lysine amino acid residues.

c. Avoid using any buffers containing primary amines (i.e. Tris, Glycine) during the conjugation process (Please check *Compatibility Sheet* section).

d. Up to 1 mM of Dithiothreitol (DTT) can be used during the conjugation process. Avoid using 2-Mercaptoethanol or any other thiol-based reducing agents during the conjugation process. If a reducing agent is necessary, TCEP is recommended up to 1 mM.

e. **Avoid using partially purified protein samples or protein samples containing carriers** (e.g. BSA).

f. To ensure the highest reaction yields, the **ligand should be dissolved in Buffer H**. Buffer exchange is recommended prior to the conjugation process. **The ligand solution must not contain EDTA**.

g. Before starting, briefly centrifuge all tubes with blue, brown and transparent caps to ensure that all material is at the bottom of the tubes.

h. For molecules with a molecular weight around or lower than 5 kDa, extra caution is required during the purification process. Small molecules and some peptides may not be properly purified using the provided chromatographic column. For more information please email **support@dynamic-biosensors.com**.

3-Step Conjugation of a Biomolecule to a *Ligand strand* in a Reaction Tube

Please read the entire protocol before starting and **perform all steps without interruption**.

TIP	*This protocol can be performed simultaneously for multiple coupling reactions.* *Avoid using partially purified protein samples or protein samples containing carriers (e.g., BSA).*

Before starting allow the crosslinker to reach room temperature before use.

I. *Nanolever Modification*

1. Equilibrate **two** purification spin columns (red cap) for one coupling reaction:
   a. Remove the column's bottom seal and loosen cap (do not remove cap).
   b. Place the column in a 2.0 mL reaction tube.
   c. Centrifuge at **1,500 × g** for **1 minute** to remove the storage solution.
   d. Add **400 µL of Buffer H** to the column's resin bed. Centrifuge at **1,500 × g** for **1 minute** to remove buffer.
   e. Repeat step d and discard the resulting buffer from the reaction tube. The purification spin column should now be in a dry state.
2. Dissolve ***LS His NHS*** in **40 µL Buffer H** prior to use, vortex until all solids are completely dissolved and briefly spin down.
3. Dissolve the **crosslinker** (brown cap) by adding **100 µL ddH_2O**, vortex until all solids are completely dissolved and briefly spin down. **IMPORTANT**: *Always use fresh compound*.
4. Add **5 µL** of the freshly prepared linker solution to one ***LS His NHS*** aliquot. Discard the remaining linker solution from step 2.
5. Vortex the reactants for 10 sec, spin down and incubate for **20 minutes** at room temperature.

IMPORTANT	*Do not exceed incubation time or the reaction yield will decrease.*

II. *Ligand Modification*

1. Prepare approx. **0.7 nmol** of the **His-tagged ligand**. For optimal conditions dissolve / dilute ligand in Buffer H and use a volume of approx. 30 - 40 µL. **EXAMPLE**: Use **35 µL** of a **20 µM** protein solution to get a final concentration of 0.7 nmol.

IMPORTANT	*Ensure the storage buffer of the ligand does not contain any primary amines, e.g. Tris buffers, glycine (please check Important Notes).*

2. Add **36 µL Guiding DNA LS** and **10 µL Loading solution** to the His-tagged ligand. Mix the reaction by pipetting up and down and let it react at room temperature for **15 minutes**.

III. *Ligand Conjugation*

1. Sample loading
   a. Place the columns from step 5 in new 1.5 mL reaction tubes.
   b. Remove the cap of spin column number 1 and apply the sample from *Ligand modification* step to the top of the resin bed.
   c. Centrifuge at **1,500 × g** for **2 minutes** to collect the sample (flow-through). Discard the Purification spin column after use.
   d. Remove the cap of spin column number 2 and apply the sample from step c to the resin bed.
   e. Centrifuge at **1,500 × g** for **2 minutes** to collect the sample (flow-through). Discard the Purification spin column after use.
2. Add the ligand mix from the *ligand modification* step to this sample. Mix the reaction by pipetting up and down and let it react at room temperature for **at least 1 hour**.

IMPORTANT | *Do not vortex. If necessary, the reaction can be carried out at 4 °C with a longer reaction time (e.g. overnight).*

IV. *Removing of Guiding DNA LS*

1. Add **12 µL of Displacing DNA LS** and **20 µL of EDTA solution** to your conjugate. Mix the reaction by pipetting up and down and let it react at room temperature for **2 hours**.

V. **pro**FIRE® *Purification*

1. Perform a purification using the appropriate **pro**FIRE® workflow (please refer to the **pro**FIRE® User Manual). Please make sure that the sample volume is **160 µL**.
   a. If the volume is less than 160 µL, make up the missing volume with **Buffer A**.
   b. If the volume exceeds 160 µL, please perform additional 160 µL runs until all the sample is consumed.
2. Use the Data Viewer software of the **pro**FIRE® to identify which fractions contains pure conjugate. An example chromatogram is shown in *Additional Information section:* **pro**FIRE® *purification of a* ***Ligand strand*** *conjugate*.
3. Remove the recommended fractions from the fraction collector.

TIP | *Do not keep the* ***Ligand strand*** *conjugate for prolonged time in the* **pro**FIRE® *running buffer. Proceed immediately with the buffer exchange.*

VI. *Buffer Exchange*

1. Add **500 µL** of the first **pro**FIRE® fraction containing the ***Ligand strand*** conjugate to the centrifugal filter unit. Centrifuge at **13,000 x g** (up to 14,000 x g) for **10 minutes** and discard flow-through.
2. Add the remaining fractions to the same filter unit and repeat the centrifugation step in order to collect all samples in one tube. (Please check *Additional information: Buffer Exchange and Concentration with Centrifugal Filter Units*).
3. Add **350 µL of PE40** (or TE40, HE40) buffer and centrifuge at **13,000 x g** for **10 minutes**. Discard the flow-through.

IMPORTANT | *If the protein is not stable in PE40 (or TE40, HE40), please check buffer compatibility with*

the **switch**SENSE® *compatibility sheet.*

4. Add **350 µL of PE40** (or TE40, HE40) buffer and centrifuge at **13,000 x g** for **15 minutes**. Discard the flow-through.
5. To recover the ***Ligand strand*** conjugate, place the centrifugal filter unit upside down in a new centrifugal collection tube (provided in the kit).
   Spin at **1,000 x g** for **2 minutes** to transfer the sample to the tube.

VII. *Aliquots and Storage*

1. Measure the absorbance of the ***Ligand strand*** conjugate at 260 nm (= A_{260nm}) on a UV-Vis Spectrophotometer (e.g. Nanodrop).
2. Determine the concentration of the ***Ligand strand*** conjugate (c_l) by inserting (A_{260nm}) into the following equation:

$$c_l[M] = \frac{A_{260nm}}{490,000 \frac{L}{mol\ cm} \cdot d}$$

   where *d* is the path length (usually equal to 1 cm; however, please check the UV-Vis Spectrophotometer user manual)

3. For a ready to use solution for a biochip functionalization, please adjust the concentration to **500 nM** (or up to 1 µM) with **PE40** (or TE40, HE40) buffer (including up to 10 % glycerol, if needed) and prepare **20 µL** aliquots.
4. Store between -86 °C and 8 °C, as desired.
   Stability of the solution is related to the stability of the ligand molecule.

IMPORTANT | *Before a* **switch**SENSE® *interaction measurement, please add the appropriate adapter strand to the conjugate solution.*

Additional Information

proFIRE® purification of a *Ligand strand* conjugate

1. To ensure best results from a measurement, no free ***Ligand strand*** should be present on the chip. Therefore, crude ***Ligand strand*** conjugates must be purified by ion exchange chromatography prior to measurement. This quality control step gives you additional useful information about your sample purity.
2. We recommend using the **pro**FIRE® system equipped with an ion exchange column, Buffer A [3] and Buffer B [4], which have same composition, but different salt concentration, allowing the peak separation.
   In *Figure 1* a typical **pro**FIRE® chromatogram of a ***Ligand strand*** conjugate purification is depicted, where the peak of the protein-DNA conjugate is separated from the free protein (left) and the free DNA (right).
   IMPORTANT: The **pro**FIRE® system owns a tailored software for automatic recognition and quantitation of DNA conjugates.
3. After purification, collect the ***Ligand strand*** conjugate fractions (*Figure 1*: fractions 8-10), concentrate the conjugate, and exchange buffer with your buffer of choice using a Centrifugal filter unit, as described in section II.

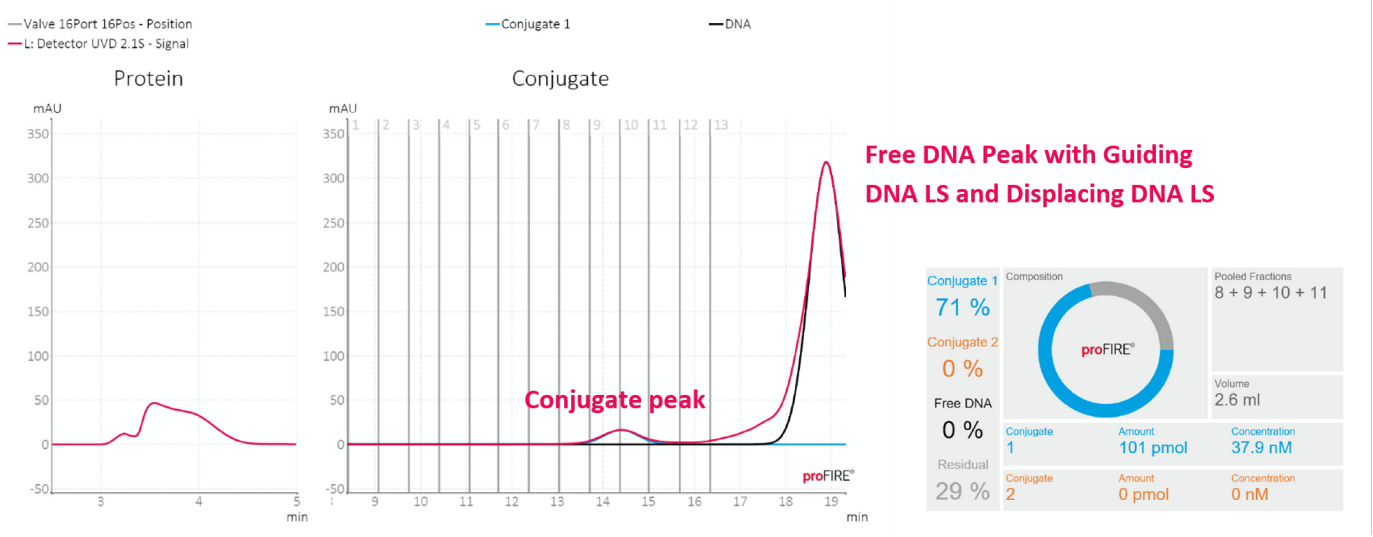
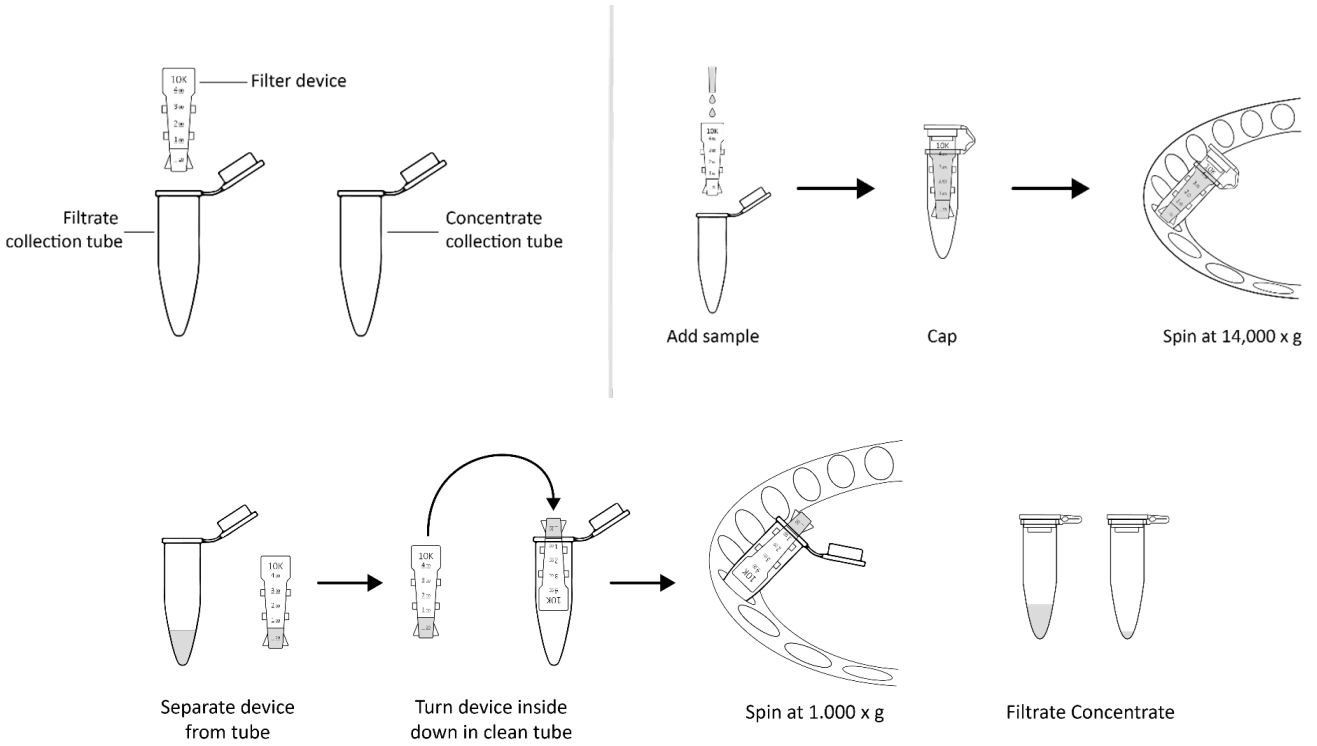


Figure 1. **proFIRE® chromatogram of a ligand strand conjugate purification**. *Used buffers: Buffer A [3]; Buffer B [4]. Column: DBS-chromatographic column. Flow: 1 mL/min. Used program: DNA length* **48**, *Type* **1**.

Buffer Exchange and Concentration with Centrifugal Filter Units

1. Take one centrifugal filter unit, add the appropriate volume of buffer in the filter device, and cap it.
2. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
3. Spin the device at 13,000 x g (or 14,000 x g) for the given time.
4. Remove the flow through and repeat steps 1-3.
5. Remove the assembled device from the centrifuge and separate the filter device from the microcentrifuge tube.
6. To recover the conjugate, place the filter device upside down in a clean centrifugal tube, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 x g to transfer the sample from the device to the tube.



Compatibility Sheet

Buffer additives

The conjugation of ligands with all available coupling kits can be performed with many different additives. The following list shows all tested ones, but please note that others not listed here may also be successfully used.

Additive	Up to	Suitability Amine Coupling	Suitability Thiol Coupling
EDTA	1 mM	●○○	●○○
DTT*	1 mM	●○○	○○●
TCEP	1 mM	●○○	●○○
Tris**	1 mM	○○●	●○○
DMSO	2 %	●○○	●○○
ATP	0.5 mM	●○○	●○○
$MgCl_2$	2.5 mM	●○○	●○○
Glycine**	—	○○●	○●○
Mannitol	8 %	●○○	●○○
Glycerol	10 %	●○○	●○○
Trehalose	8 %	●○○	●○○
Histidin**	30 mM	○○●	○●○
Acetonitrile***	50 %	●○○	●○○
Trifluoroacetic acid	0.1 %	●○○	●○○

** thiol-based reducing agents*
*** contains primary amines*
**** caution, may harm the ligand*

pH/pI

The pH value for the conjugation buffer may range from pH 5.0 to pH 8.0, depending on the ligand characteristics. When performing a conjugation of proteins with a **pI of < 6**, please note that using a buffer with lower pH may result in a better yield of conjugate.

Buffer	pH	Order No	Composition
Phosphate-Citrate Buffer	pH 5	-	50 mM buffer salt, 150 mM NaCl
Buffer M	pH 6.5	BU-M-150-1	50 mM MES, 150 mM NaCl
Buffer A	pH 7.2	BU-P-150-10	50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl
Buffer C	pH 8.0	BU-C-150-1	50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl

Salt concentration

For standard conjugations, 50 mM buffer salt and 150 mM NaCl (monovalent salt) are used.

When performing conjugation of **strongly charged ligands**, make sure that the concentration of NaCl is sufficiently high (**up to 400 mM NaCl is recommended**). Otherwise, precipitation of DNA may occur.
The shielding effect of monovalent sodium cations leads to DNA stabilization through neutralization of the negative charge on the sugar phosphate backbone.

Useful Order Numbers

Table 3. Order Numbers

Product Name	Amount	Order No
heliX® Amine coupling kit 1 (**pro**FIRE® purification)	5 conjugations	HK-NHS-1
Centrifugal filter unit (3 kDa MWCO)	5 pcs.	CF-003-5
Centrifugal filter unit (10 kDa MWCO)	5 pcs.	CF-010-5
10x Buffer A [3]	50 mL (yielding 500 mL)	BU-P-150-10
5x Buffer B [4]	50 mL (yielding 250 mL)	BU-P-1000-5

My Notes

Contact

Dynamic Biosensors GmbH
Perchtinger Str. 8/10
81379 Munich
Germany

Dynamic Biosensors, Inc.
300 Trade Center, Suite 1400
Woburn, MA 01801
USA

Order Information **order@dynamic-biosensors.com**

Technical Support **support@dynamic-biosensors.com**

www.dynamic-biosensors.com

Instruments and chips are engineered and manufactured in Germany.

[**1**] Buffer PE40: 10 mM Na_2HPO_4/NaH_2PO_4, 40 mM NaCl, pH 7.4, 0.05 % Tween, 50 µM EDTA, 50 µM EGTA

[**2**] For conjugation of proteins with a molecular weight higher than 20 kDa: Centrifugal filter units with a MWCO of 10 kDa can be ordered for a faster concentration process (Order No: CF-010-5).

[**3**] Buffer A: 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.2

[**4**] Buffer B: 50 mM Na_2HPO_4/NaH_2PO_4, 1 M NaCl, pH 7.2