heliX+
User Manual

dynamic
BIOSENSORS

Amine Coupling Kit 2

Coupling of molecules with primary amines to the *ligand strand* - **spin column purification**

Dynamic Biosensors GmbH & Inc.

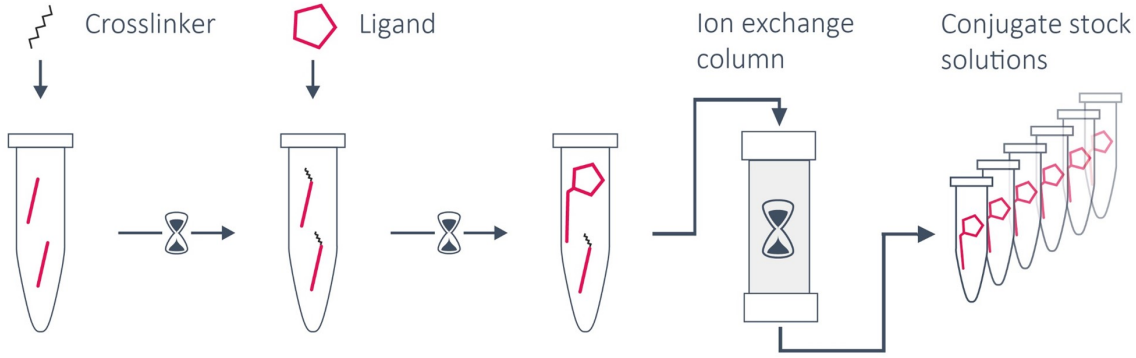
HK-NHS-2 v6.1

Key Features

- Allows for coupling of biomolecules with primary amines (e.g. NH2-terminus, lysines) to the ***Ligand strand*** in a single reaction tube.
- Convenient standard chemistry (NHS chemistry).
- Compatible with **heliX® Adapter Chip**.
- Compatible with **spin column purification** of the ligand-DNA conjugate (> 7 kDa).
- Coupling of multiple ligands can be performed simultaneously.
- Yields > 95 % pure ligand-DNA conjugate.
- Includes reagents for five individual conjugation reactions (approx. 10-50 regenerations each; up to max. 500).
- Compatible with automated standard regeneration process.

Workflow Overview

3-Step Conjugation Workflow



1. DNA Modification	2. Ligand Conjugation	3. Purification	4. Ready-to-use
The ***Ligand strand*** is functionalized with a primary amine reactive NHS.	The biomolecule (ligand) is added to the functionalized ***Ligand strand*** and incubated for at least 1 h.	The ***Ligand strand*** conjugate is purified using the provided anion exchange spin columns. After buffer exchange the conjugates are aliquoted and stored.	The conjugate stock solutions are ready to use in the **heliX®** biosensor.

Timeline: Hands on time < 1 h | Incubation ~ 2 h | Total ~ 3 h

Product Description

Order Number: HK-NHS-2

Table 1. Contents and Storage Information

Material	Cap	Amount	Storage
***Ligand strand* NHS**	Blue	5 x	-20°C
Buffer A [1]	Transparent	1 x 1.8 mL	-20°C
Buffer C [2]	Transparent	5 x 1.8 mL	-20°C
Buffer PE40 [3]	Transparent	5 x 1.5 mL	-20°C
Buffer E48 [3]	Transparent	1.5 mL	-20°C
ddH_2O	Transparent	1.5 mL	-20°C
Crosslinker	Brown	5 x	-20°C
Purification spin column	Red	10 x	2-8°C
2.0 mL reaction tubes for purification spin column		10 x	RT
Anion exchange (AEX) spin column		5 x	RT
Collection tube for AEX spin column		10 x	RT
Centrifugal filter unit (3 kDa MWCO)[4]		5 x	RT
Centrifugation collection tube		10 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 five conjugations of approximately 50-200 µg of biomolecule each.
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 C**. Buffer exchange is recommended prior to the conjugation process.

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. If the pI of the protein is < 6, a low pH kit for conjugation (Order No: HK-NHS-3) is recommended. 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. Dissolve ***Ligand strand NHS*** in **40 µL Buffer A** prior to use, vortex until all solids are completely dissolved and briefly spin down.
2. 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*.
3. Add **10 µL** of the freshly prepared linker solution to one ***Ligand strand*** aliquot. Discard the remaining linker solution from step 2.
4. 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.*

5. In the meantime, 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 C** 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.
6. 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 step 4 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.

II. *Ligand Conjugation*

1. Add approx. **100 µg** (up to a maximum of 200 µg) of the ligand (concentration approx. 0.5 - 50 mg/mL) to the sample from step 6. For optimal conditions use a volume of approx. 50 µL.
   EXAMPLE: Adjust protein concentration to **2 mg/mL** and use **50 µL** for conjugation.

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

2. 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).*

III. *Spin column Purification and Buffer Exchange*

1. Perform a purification reaction using anion exchange spin columns.
   a. To equilibrate the spin column add **400 µL Buffer A** and put the spin column in the provided collection tube. Centrifuge at 2,000 x g for 5 minutes and discard the flow-through.
      Note: To achieve even liquid flow-through the membrane using a fixed-angle rotor, align the printed letter (Q) toward the center of the rotor for all chromatography steps.
   b. Add the complete sample to the spin column, incubate for 1 minute and centrifuge for 2 minutes at 2,000 x g. Discard the flow-through.
   c. Place the column in a new collection tube and add **100 µL of the Elution Buffer E48** to the spin column, incubate for 5 minutes and centrifuge for 2 minutes at 2,000 x g. Repeat the elution step and combine the two flow-throughs.
   d. Add **200 µL of PE40** (or TE40, HE40) buffer to the eluted and combined sample from step c.

IMPORTANT	*If the protein is not stable in PE40 (or TE40, HE40), please check buffer compatibility with the* **switch**SENSE® *compatibility sheet.*

   e. Apply the sample to the centrifugal filter unit and centrifuge at 13,000 x g (up to 14,000 x g) for 10 minutes and discard flow-through. (Please check *Additional information: Buffer Exchange and Concentration with Centrifugal Filter Units*).
2. Add **350 µL of PE40** (or TE40, HE40) buffer and centrifuge at **13,000 x g** for **10 minutes**. Discard the flow-through.
3. Add **350 µL of PE40** (or TE40, HE40) buffer and centrifuge at **13,000 x g** for **15 minutes**. Discard the flow-through.
4. 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.

IV. *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)

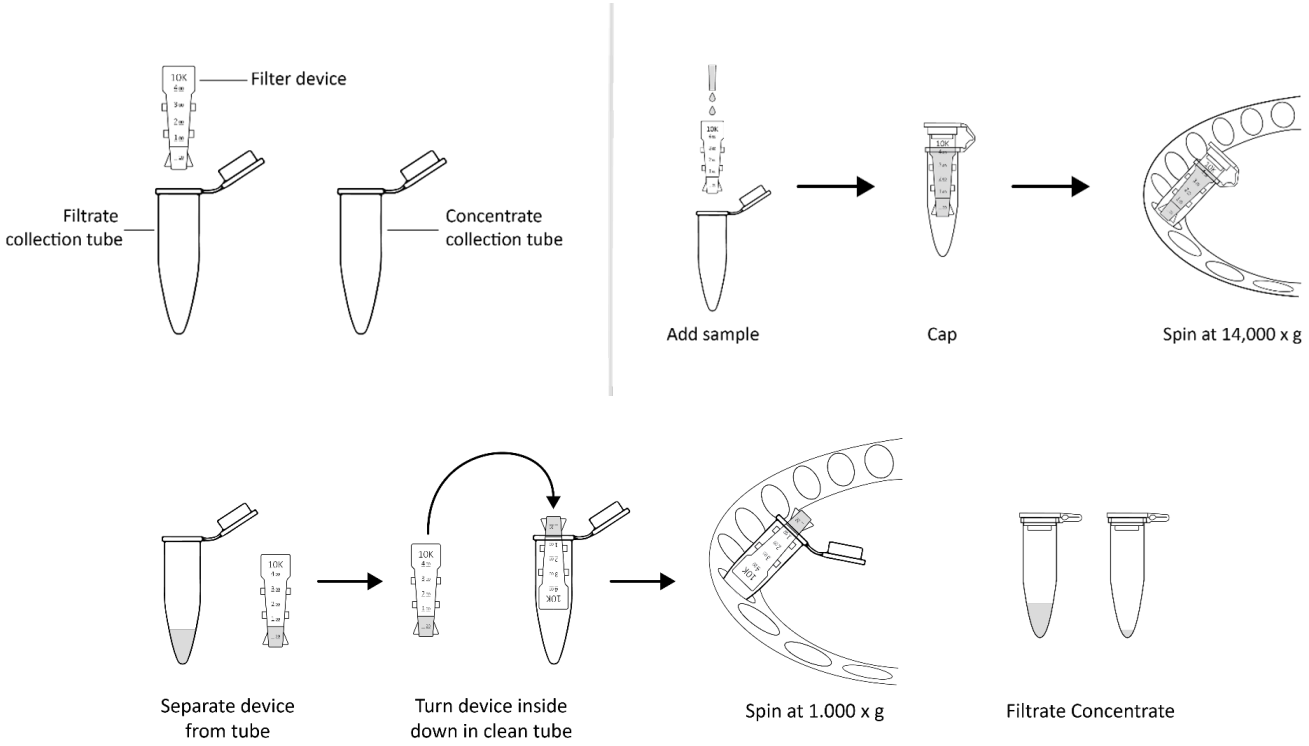
3. For a ready to use solution for a biochip functionalization, please adjust the concentration to **500 nM** (or up to 1 µM) with **PE40** (or TE40, HE40) buffer (including up to 10 % glycerol, if needed) and prepare **20 µL** aliquots.
4. Store between -86 °C and 8 °C, as desired.
   Stability of the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

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
heliX® Amine coupling kit 3 (low pI biomolecules)	5 conjugations	HK-NHS-3
heliX® Thiol coupling kit 1	5 conjugations	HK-MAL-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 [1]	50 mL (yielding 500 mL)	BU-P-150-10
5x Buffer B [5]	50 mL (yielding 250 mL)	BU-P-1000-5
1x Buffer C [2]	12 mL	BU-C-150-1

My Notes

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Instruments and chips are engineered and manufactured in Germany.

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

[**2**] Buffer C: 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 8.0

[**3**] 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

[**4**] 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).

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