proFIRE
User Manual

dynamic
BIOSENSORS

Amine Coupling Kit 3 for Proteins (> 5 kDa)

functionalization of DBCO-DNA via amines (-NH_2)

Dynamic Biosensors GmbH & Inc.

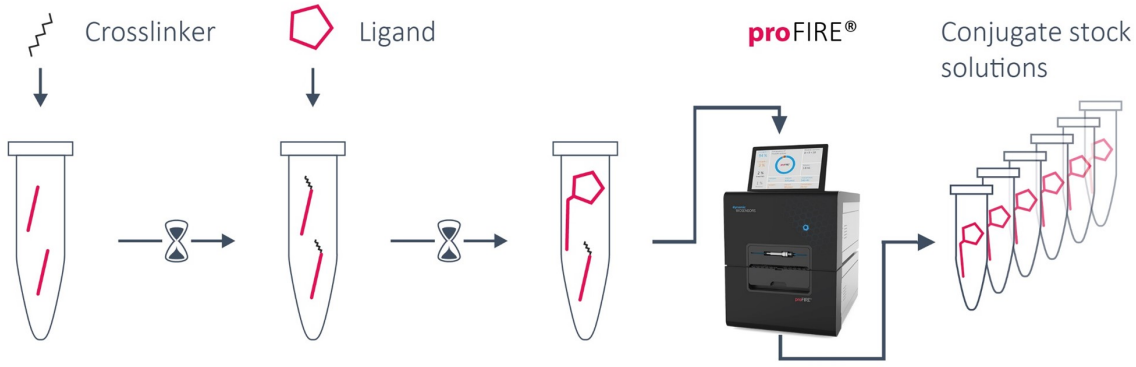
PF-NH2-3 v3.1

Key Features

- Allows for coupling of biomolecules with primary amines (e.g. NH_2-terminus, lysines) to DBCO-modified DNA in a reaction tube.
- Oligos are not included in the kit.
- Convenient standard chemistry.
- Suitable for proteins and peptides (MW > 5 kDa).
- Suitable for any DNA sequence and length up to 150 bases.
- Yields >95 % pure protein-DNA conjugate with controlled quality of your product.
- Coupling of multiple proteins can be performed simultaneously.

Workflow Overview

3-Step Conjugation Workflow



1. DNA Modification	2. Protein Conjugation	3. Purification	4. Ready-to-use
The DNA is activated with amine reactive groups.	After incubation the excess linker is removed by spin column. The protein/peptide is added to the functionalized DNA and incubated for at least **1 h**.	The protein-DNA conjugate is purified using the **pro**FIRE® system. After buffer exchange the conjugates are aliquoted and stored.	The fractions with protein-DNA conjugate are ready for further processing.

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

Product Description

Order Number: PF-NH2-3

Table 1. Contents and Storage Information

Material	Cap	Amount	Storage
Conjugation Buffer	Transparent	5 x 1.8 mL	-20°C
Dilution Buffer	Transparent	1.8 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
Centrifugal filter unit (3 kDa MWCO)[1]		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
DNA	We recommend to use 3 - 4 nmol DNA (modified with DBCO, HPLC grade) for one reaction
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 protein-DNA conjugate's concentration

All necessary solutions and buffers are included in the kit.

Important Notes

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

b. 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.

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

d. To ensure the highest reaction yields, the **ligand should be dissolved in Conjugation Buffer**. Buffer exchange is recommended prior to the conjugation process.

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

f. 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**.

g. If the pI of the protein is < 6, a lower pH buffer is necessary. For more information, please email **support@dynamic-biosensors.com**.

3-Step Conjugation of a Biomolecule to DNA 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. *DNA Modification*

1. Dissolve the DNA in **40 µL Dilution Buffer** 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 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 DNA aliquot. Discard the remaining linker solution from step 3.
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 mantime, 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 Conjugation Buffer** 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. *Protein Conjugation*

1. Add approx. **100 µg** (up to a maximum of 200 µg) of the protein (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. **pro**FIRE® *Purification*

Please refer to the **pro**FIRE® User Manual.

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, fill the missing volume with **Conjugation Buffer**.
   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 contain pure conjugate.
   An example chromatogram is shown in *Additional Information section:* **pro**FIRE® *purification of a protein-DNA conjugate*.
3. Remove the recommended fractions from the fraction collector.

IV. *Buffer Exchange*

1. Add **500 µL** of the first **pro**FIRE® fraction containing the protein-DNA 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 buffer of choice for buffer exchange and centrifuge at **13,000 x g** for **10 minutes**. Discard the flow-through.
4. Add **350 µL** of buffer of choice for buffer exchange and centrifuge at **13,000 x g** for **15 minutes**. Discard the flow-through.
5. To recover the protein-DNA 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.

V. *Aliquots and Storage*

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

$$c_l[M] = \frac{A_{260nm}}{\varepsilon_{260} \cdot d}$$

with
c_l: Concentration of the ligand strand
A_{260nm}: Absorbance at 260 nm
ε_{260}: Extinction coefficient of the DNA at 260 nm
d: Light path length (typically 1 cm; please check your UV-Vis Spectrophotometer's user manual)

3. Store between -86 °C and 8 °C, as desired.
   Stability of the solution is related to the stability of the protein.

Additional Information

proFIRE® purification of a protein-DNA conjugate

1. We recommend using the **pro**FIRE® system equipped with an ion exchange column, Buffer A [2] and Buffer B [3], which have same composition, but different salt concentration, allowing peaks separation.
   In *Figure 1* a typical **pro**FIRE® chromatogram of a protein-DNA 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.
2. After purification, collect the protein-DNA 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.

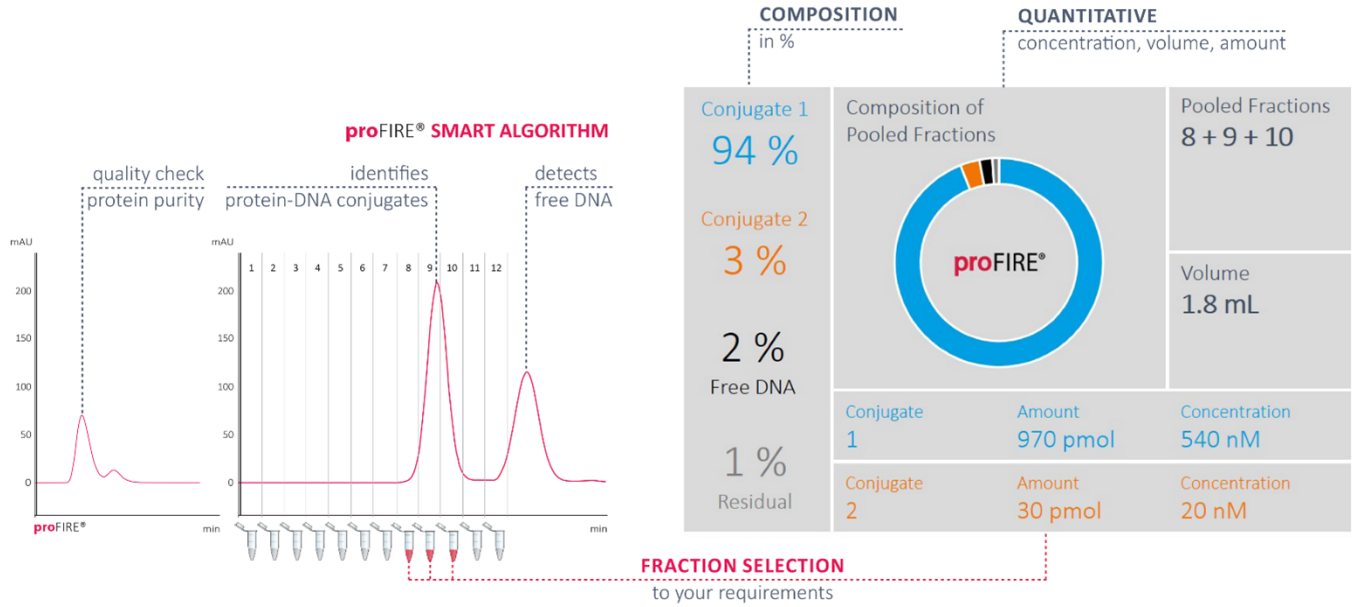
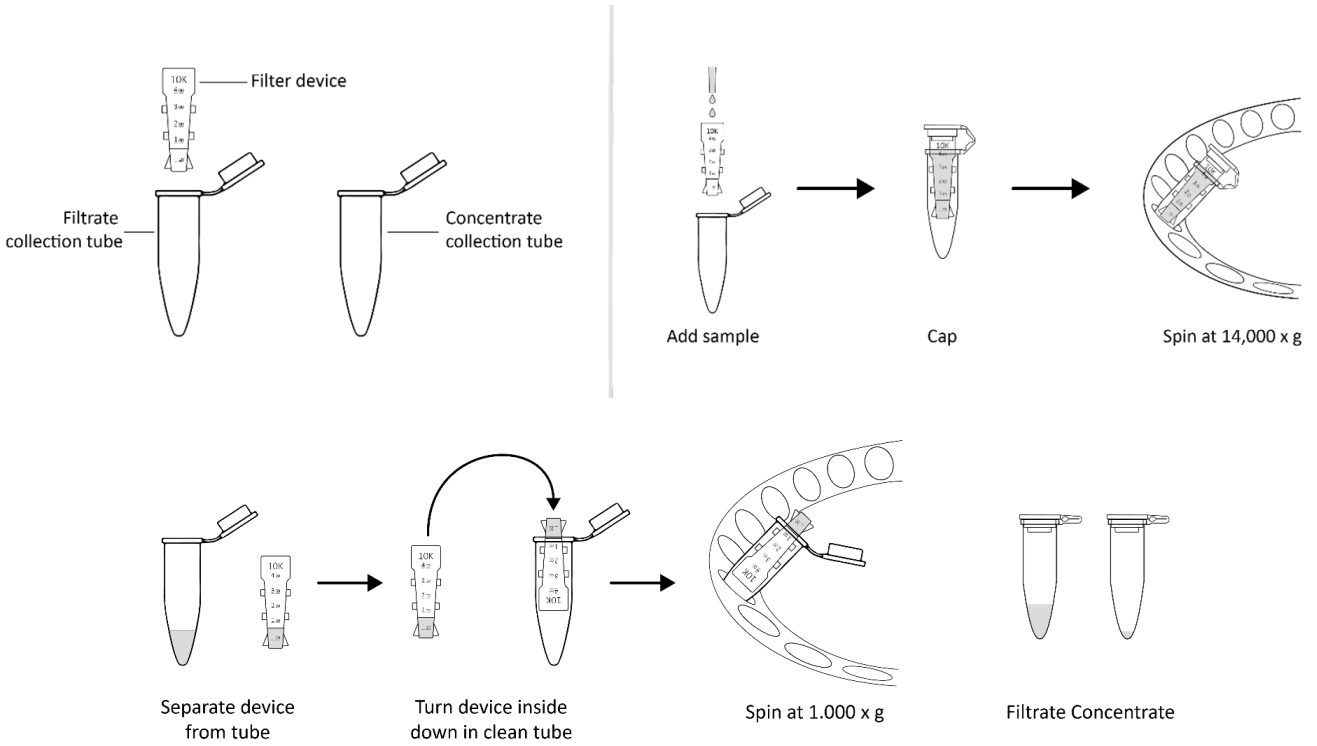


Figure 1. **pro**FIRE® **chromatogram of a ligand strand conjugate purification**. *Used buffers: Buffer A [2]; Buffer B [3]. Column: DBS-chromatographic column. Flow: 1 mL/min. Used program: DNA length* **48**, *Type* **1**.

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 additives. Please note that other additives, which are not listed here may successfully be used for conjugation.

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

Salt concentration

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

When performing conjugation of **strongly charged proteins**, 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
proFIRE® Amine Coupling Kit 1 for Proteins (> 5 kDa);	5 conjugations	PF-NH2-1
proFIRE® Antibody Oligo Conjugation Kit;	3 conjugations	PF-AB-1
proFIRE® Thiol Coupling Kit 1 for proteins (>5 kDa);	5 conjugations	PF-SH-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 [2]	50 mL (yielding 500 mL)	PF-BU-A-10
5x Buffer B [3]	50 mL (yielding 250 mL)	PF-BU-B-5
Conjugation Buffer	12 mL	PF-BU-C-1

My Notes

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Instruments and chips are engineered and manufactured in Germany.

[**1**] 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).

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

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