helix^cyto
User Manual

dynamic BIOSENSORS

Labeling Kit Red dye 2

Labeling of **Analytes** containing a primary amine by a red fluorescent dye

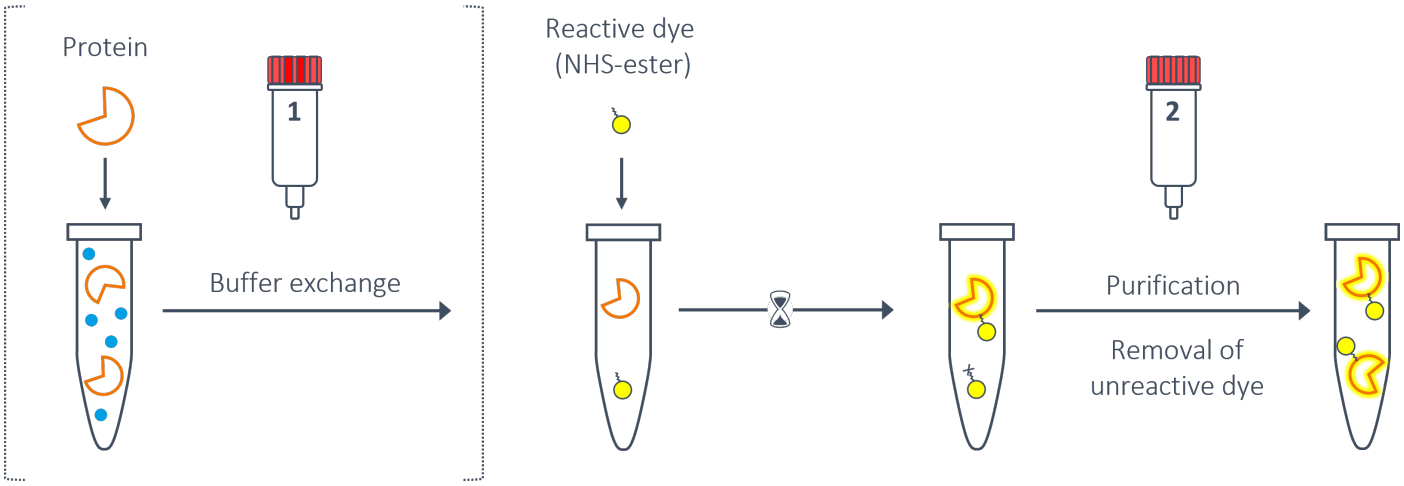
Dynamic Biosensors GmbH & Inc.

CY-LK-R2-1 v1.2

Key Features

- Labeling of biomolecules > 7 kDa by a small red fluorescent dye in a fast and easy reaction
- Convenient standard chemistry (NHS ester) targeting primary amines (e.g. NH_2-terminus, lysines)
- Includes reagents for five individual labeling reactions (25 - 200 µg protein each)
- Labeling of multiple samples can be performed simultaneously
- Degree of labeling (DOL) per molecule can be adjusted
- Compatible with the red channel of all **heliX**® instruments
- Total time needed is less than 45 minutes

Overview 3-Step Labeling Workflow



1. Buffer exchange	2. Dye addition and incubation	3. Purification
The protein of interest is transferred into labeling buffer using provided buffer exchange spin columns.	The fluorescent dye is added to the protein of interest at the desired molar ratio and incubated for 10 minutes.	Free dye molecules are removed and the buffer is exchanged to the desired storage buffer using provided dye removal spin columns.

Timeline: Hands on time < 30 min | Incubation ~ 10 min | Total < 45 min

Product Description

Order Number: **CY-LK-R2-1**

Table 1. Contents and Storage Information

Material	Cap	Amount	Storage	Comment
Red dye (NHS ester, reconstituted to 2 mM stock in anhydrous DMSO)	Blue	5 x 10 µL	-20 °C to -80°C	protect from water, air and light, avoid repeated freeze-thaw cycles
Labeling buffer pH 8.3 (15.7 mM carbonate-bicarbonate, 126 mM NaCl, 2.5 mM KCl, 9.2 mM phosphate)	Transparent	5 x 1 mL	-20 °C	
Storage buffer DPBS (140 mM NaCl, 2.7 mM KCl, 10 mM phosphate)	Transparent	5 x 1 mL	-20 °C	
Buffer exchange spin column (“1”)	Red	5 x	2-8 °C	
Dye removal spin column (“2”)	Red	5 x	2-8 °C	
2.0 mL collection tubes for spin columns		10 x	RT	
1.5 mL protein low-bind collection tubes		10 x	RT	

The kit contains reagents for 5 labeling reactions.

For research use only.

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

Products are shipped at ambient temperature (RT) and should be unpacked upon arrival and stored according to the recommendations in **Table 1**.

The resin slurry of the buffer exchange spin column contains 0.02 % sodium azide and the resin slurry of the dye removal spin column contains 0.05 % sodium azide.

Additional Materials Required

Table 2. Additional Materials

Material	Comment
Benchtop microcentrifuge	Required speed settings 1,500 x g and 1,000 g
UV-Vis spectroscopy (e.g. NanoDrop™)	For concentration and DOL determination of the conjugate

All other necessary solutions, buffers and tubes are included in the kit.

Important Notes

- **DO NOT use buffers containing primary amines** (i.e. Tris, Glycine) during the labeling process.
- Low concentration of sodium azide (< 3 mM) is compatible with the labeling reaction.
- Avoid prolonged exposure of the reactive dye to aqueous solutions, air and light. Upon arrival, **freeze** the **provided dye aliquots** and store at ≤ -20°C until use. Avoid repeated freeze-thaw cycles.
  If necessary, dilute dye directly before use in labeling buffer and do not store leftover diluted dye.
- Before you begin, briefly centrifuge all chemicals to ensure that all material is located at the bottom of the tubes.

3-Step Fluorescent labeling of protein analyte

Please read the entire protocol before starting and **perform all steps without interruption**. *This protocol can be used to label* **25 to 200 µg of protein** *(concentration between 0.25 to 7 mg/ml) per labeling reaction. Sample volumes should be between* **30 to 120 µl**, *independent of the amount of protein used.*

TIP | *This protocol can be performed easily for multiple labeling reactions simultaneously.*

I. Buffer exchange

1. Equilibrate one **Buffer exchange spin column** ("1") per labeling reaction **with Labeling buffer**:
   a. Remove the column bottom seal and loosen cap (do not remove).
   b. Place the column in a 2.0 mL collection tube.
   c. Centrifuge at **1,500 x g** for **1 min** at room temperature to remove the storage solution.
   d. Add 400 µL of Labeling buffer (pH 8.3) to the column.
   e. Centrifuge at **1,500 x g** for **1 min** at room temperature and discard flow-through.
   f. Repeat steps d & e. The Buffer exchange spin column should now be in a dry state.
2. Sample loading:
   a. Transfer the column to a new 1.5 mL protein low-bind collection tube.
   b. Remove the cap and **carefully apply the protein sample to the top of the resin bed**. Do not disturb the resin bed.
   c. Centrifuge at **1,500 x g** for **2 min** to **collect the sample (flow-through)**. Discard the spin column after use.

II. Fluorescent labeling

3. Thaw dye aliquot (2 mM stock concentration in anhydrous DMSO) directly before first use.
4. **Add 1 - 10 µl of the 2 mM dye stock solution to the protein sample** at the desired molar ratio and mix immediately by carefully pipetting up and down. Use the following table as guide for the dye input or see example calculations in Appendix for more details.

	Fab	IgG
Input protein	100 µg ≡ 2 nmol	100 µg ≡ 0.667 nmol
4-fold molar excess of dye	8 nmol	2.7 nmol
Input dye stock [2 mM]	4 µl	1.33 µl

To obtain an average dye-to-protein ratio (= degree of labeling, DOL) of 1 - 2, we recommend a 4-fold molar excess of ***fresh*** *reactive dye.*

IMPORTANT | *Adjustment of the molar excess of dye may be necessary to achieve the desired DOL for individual protein analytes. Optional: Dilute the 2 mM dye stock directly before use with Labeling buffer.*

5. **Incubate** the reaction mixture **for 10 min at room temperature**. Protect from light.

IMPORTANT	*Longer incubation times can lead to reduced protein recovery, whereas shorter incubation times can decrease the labeling efficiency.*

III. Buffer exchange and removal of unbound dye

6. In the meantime, equilibrate one **Dye removal spin column** ("2") per labeling reaction **with Storage buffer (e.g. PBS)**:
   a. Remove the column bottom seal and loosen cap (do not remove).
   b. Place the column in a 2.0 mL collection tube.
   c. Centrifuge at **1,000 x g** for **2 min** at room temperature to remove the storage solution.
   d. Add **400 µL of Storage buffer (DPBS)** to the column.
   e. Centrifuge at **1,000 x g** for **2 min** at room temperature and discard flow-through.
7. Sample loading:
   a. Transfer the Dye removal spin column to a new 1.5 mL protein low-bind collection tube.
   b. Remove the cap and **carefully apply the labeled protein sample to the top of the resin bed**. Do not disturb the resin bed.
   c. Centrifuge at **1,000 x g** for **2 min** to **collect the sample (flow-through)**. *Unreacted dye is retained in the column and stains the resin blue. Discard the Dye removal spin column after use*.

IV. Aliquots and Storage

8. Determine the protein concentration and dye-to-protein ratio (= degree of labeling, DOL), as described in the following section.
9. Aliquot and store between 8 °C and -80 °C. *Labeled proteins should generally be stored under the same conditions as unlabeled proteins.*

IMPORTANT	*Protect labeled proteins from light.*

Appendix

Determination of the dye-to-protein ratio (degree of labeling, DOL)

The average degree of labeling can be calculated from the molar concentrations of protein and dye. Those concentrations can be determined using a UV-Vis spectroscope (e.g. NanoDrop™).

1. Measure the absorbance of the labeled protein sample at 643 nm ($= A_\lambda$) on a NanoDrop in Protein&Labels mode [1]. Determine the concentration of the dye (c_{dye}) by inserting your measured value into this formula:

$$c_{dye}[M] = \frac{A_\lambda}{150,000}$$

2. Measure the absorbance of the labeled protein sample at 280 nm ($= A_p$) on a NanoDrop [1]. Determine the concentration of the protein ($= c_p$) by inserting your measured values from points 1. and 2. into this formula:

$$c_p[M] = \frac{\left\{ A_{280nm} - A_\lambda \cdot 0.04 \right\}}{\varepsilon_{280}}$$

ε_{280} is the extinction coefficient of the protein at 280 nm. Calculate for your protein sequence individually or approximate with typical values:

- ε_{280} of a typical IgG molecule (~150 kDa): 210,000 $M^{-1}cm^{-1}$)
- ε_{280} of a typical Fab molecule (~50 kDa): 74,000 $M^{-1}cm^{-1}$)
- ε_{280} of a typical Nanobody (~15 kDa): 27,000 $M^{-1}cm^{-1}$)

3. The degree of labeling (DOL) is calculated by the formula:

$$DOL = \frac{c_{dye}}{c_p}$$

Example Calculation of dye amount

I. Example calculations for IgG antibodies

1. Labeling of **100 µg** of **IgG** antibody (MW = 150 kDa), which corresponds to **667 pmol**. Optimum analyte volume for this reaction is 50 µl, which corresponds to an initial analyte concentration of 2.0 mg/ml. However, sample volumes between 30 µl and 120 µl can be used.
2. After buffer exchange to Labeling buffer, the fluorescent dye is added directly to the antibody. In order to achieve a DOL of 1 - 2, a **4-fold molar excess of dye** should be added. This corresponds to **2.67 nmol** of dye, which corresponds to **1.33 µl of the 2 mM stock** solution. Alternatively, for more accurate pipetting of the small volume, the dye can be diluted 1:10 with Labeling buffer directly before use and then 13.3 µl of the 0.2 mM solution are used for the labeling reaction.
3. The final, purified labeled analyte should optimally exhibit a concentration > 2 µM and DOL between 1 and 2.

II. Example calculations for Fab fragments

1. Labeling of **100 µg** of **Fab fragment** (MW = 50 kDa), which corresponds to **2 nmol**. Optimum analyte volume for this reaction is 50 µl, which corresponds to an initial analyte concentration of 2.0 mg/ml. However, sample volumes between 30 µl and 120 µl can be used.
2. After buffer exchange to Labeling buffer, the fluorescent dye is added directly to the antibody. In order to achieve a DOL of 1 - 2, a **4-fold molar excess of dye** should be added. This corresponds to **8 nmol** of dye, which corresponds to **4 µl of the 2 mM stock** solution. If required to enable sufficient accuracy of pipetting small volumes, the dye can be diluted 1:10 with Labeling buffer directly before use.
3. The final, purified labeled analyte should optimally exhibit a concentration > 6 µM and DOL between 1 and 2.

Troubleshooting

General considerations: A single protein molecule can be labeled with one or more dye molecules. A higher ratio of dye to protein can increase the signal strength, but also increases the chance of protein aggregation and modification of the binding site. Labeling procedures should generally aim for the introduction of one to two dye molecules per protein molecule. Analytes with low binding affinity might have to be used at higher concentrations during **switch**SENSE® and **Real-Time Interaction Cytometry** (**RT-IC**) measurements, leading to high signal. In this case, labeling procedures should aim for lower DOL of 0.5 - 1.

Table 3. Troubleshooting

Encountered problem	Possible explanation	Improvement
Low yield (concentration) of labeled analyte after purification	Low concentration of analyte solution already before the labeling reaction	Concentration of protein analyte prior to labeling using centrifugal filter units (see Useful order numbers section).
Low DOL after purification	Low concentration of analyte solution before the labeling reaction may also decrease the efficiency of the labeling reaction and promote hydrolysis of dye NHS ester in aqueous solution	Concentration of protein analyte prior to labeling using centrifugal filter units (see Useful order numbers section).
	Dye concentration too low	Increase molar excess of dye up to 10-fold.
	Reaction time too short	Increase reaction time up to 1 h at RT.
	Sample contains impurities, contaminants or carriers (e.g. BSA)	Improve and verify analyte purity using established method of choice. If analyte purity can not be improved, increase molar excess of dye up to 10-fold.
Labeled analyte is getting unstable (unfolding or aggregate formation observed)	DOL too high	Decrease molar excess of dye to 1-fold, or decrease reaction time to 5 min, or decrease reaction temperature to 4 °C.
	Analyte is pH sensitive	Perform labeling reaction in Storage buffer (PBS) or a suitable buffer of choice (pH ≥ 7.2). This might decrease the efficiency of the labeling reaction and the resulting analyte DOL. The molar excess of dye can be increased up to 10-fold or the reaction time can be increased up to 1 h to counter this effect.
	Analyte is temperature sensitive	Perform labeling reaction at 4 °C. This might decrease the efficiency of the labeling reaction and the resulting analyte DOL. The molar excess of dye can be increased up to 10-fold or the reaction time can be increased up to 16 h to counter this effect.

Useful Order Numbers

Table 4. Order Numbers

Product name	Order Number
10 x DPBS	BU-DP-10-1
10 x RT-IC Running Buffer 1	BU-RB-10-1
Centrifugal filter unit (3 kDa MWCO), 5 pcs.	CF-003-5
Centrifugal filter unit (10 kDa MWCO), 5 pcs.	CF-010-5

Contact

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Instruments and chips are engineered and manufactured in Germany.

[**1**] Make sure that the **path length** of measurement is **1 cm** when using above formulas. If it was 1 mm, correct absorbance values by 10x factor. If another UV-Vis spectroscope (other than NanoDrop™) is used, please refer to the general Lambert-Beer-Law for the determination of concentrations.