PROTEIN LABELING KIT

ULS™

Platinum*Link* Protein Labeling Kit

For 4 Labeling Reactions

(100 µg per labeling)

Product code

PLK-007, BIO-ULS
PLK-008, DNP-ULS
PLK-009, FLU-ULS
PLK-010, RHO-ULS

For Research Use Only

Not for diagnostic purposes

KREATECH
BIOTECHNOLOGY

Instruction manual

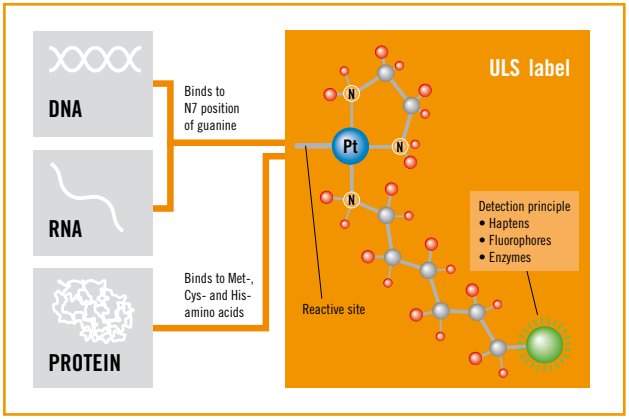

Figure 1. ULS™, the Universal Linkage System that labels your DNA, RNA and proteins.

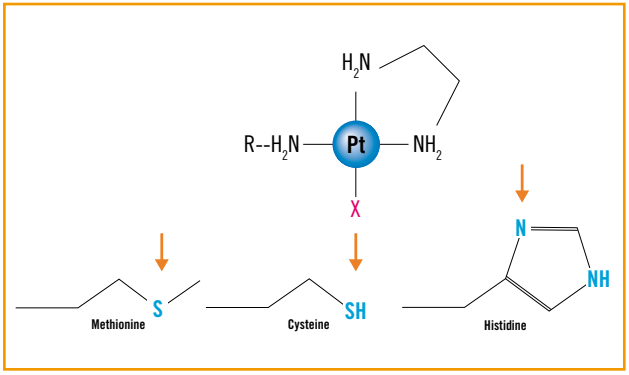

Figure 2. ULS™, labels proteins by forming a coordinative bond on:
- *Sulphur atoms of Methionine and Cysteine*
- *Nitrogen atom in Histidine (pH>4)*

KREATECH's Platinum*Link* Protein Labeling Kit

For labeling of proteins and antibodies with biotin, dinitrophenol, fluorescein or rhodamine

This kit is intended for RESEARCH USE ONLY. IT IS NOT INTENDED FOR DIAGNOSTIC APPLICATIONS and/or COMMERCIAL PURPOSES.

Important

Open the kit immediately and store all components at 4 °C
- Read the entire instruction manual before starting your experiment.
- Do not mix reagents from different kits.
- During the preparation of reagents and throughout the entire procedure please observe the safety regulations issued for laboratories concerning handling of samples.
- Dispose reagents according to relevant local regulations. Take appropriate safety precautions such as wearing a lab coat, gloves and eye protection

Limited Product Warranty – Patent Disclaimer

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Technical Services

We highly value your opinion on our products. If you have any questions, requests or suggestions concerning this product or any other KREATECH products, please contact our Technical Services at techservices@kreatech.com

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A. Assay Materials

I. Components and Storage

All components should be stored at 4ºC upon arrival.

Component	Description of function	Amount
10 x PL buffer	10 x Protein Labeling buffer	40 µl
Label: BIO-ULS, **or** DNP-ULS, **or** FLU-ULS, **or** RHO-ULS	Labeling reagent	20 µl
ULS-Trap columns	Column for removal of non-reacted ULS	4
Collection tubes	Use in combination with ULS-Trap columns	8
Manual		1

II. Materials Needed but not Included in Kit

- 1.5 ml reaction vials (*e.g.* Eppendorf)
- Table top microcentrifuge (*e.g.* Eppendorf)
- 37 ºC incubator or waterbath
- Purified water (*e.g.* MilliQ)
- Protein of interest

III. Optional Materials not Included in Kit

- Vortex
- Spectrophotometer
- Materials for buffer-exchange (*e.g.* protein desalting columns and corresponding buffers).
- Assay for determination of Biotin-concentration (e.g HABA assay Pierce # 28010)
- Assay for determination of protein concentration (e.g BCA assay Pierce #23225 and/or Rc Dc Protein Assay Bio-Rad # 500-0120)

B. General Information

I. Introduction and Product Description

KREATECH's Platinum*Link* Kits provide a convenient way to conjugate biotin (BIO), dinitrophenol (DNP), fluorescein (FLU) or rhodamine (RHO) to antibodies or other proteins. The kits contain all components necessary to perform four separate labeling reactions including subsequent removal of non-reacted label using ULS-Trap columns. The standard protocol in this manual is optimized for the labeling of 100 µg antibody at a final concentration of 1 mg/ml during labeling. Other proteins like recombinant proteins or Fab'-fragments can be labeled as well. In general, following this standard protocol a labeling degree of 2-4 is obtained.

For labeling of proteins, a labeling technology is needed that is robust and efficient. The Universal Linkage System (ULS™) is robust, stable in aqueous solutions, and flexible in use. It is compatible with commonly used buffers, salts and detergents and with high incubation temperatures. The proprietary ULS technology is based on the stable binding properties of a platinum complex to biomolecules (see figure 1). In proteins, ULS binds to sulphur and nitrogen containing side chains of **METHIONINE**, **CYSTEINE** and **HISTIDINE** residues (see figure 2). Since these residues are less likely to be involved in protein-protein interactions than lysine (which is targeted by

isothiocyanate or N-hydroxysuccinimide (NHS) labeling), there is a lower risk of interference with target epitope recognition.

ULS labeling is compatible with commonly used biochemical buffers including Tris and glycine, with detergents like Triton X-100, and with many salts. Incompatible substances during the labeling reaction include thioaldehydes, thioketones (*e.g.* thiourea), thioethers, and thiols (for instance dithiothreitol, ß-mercaptoethanol), and strongly reducing agents like tris-(2-carboxyethyl) phosphine (TCEP) and tributylphosphine (TBP). After labeling, these compounds may be added to the solution as long as prolonged exposure is avoided.

After labeling of proteins with fluorescent dyes or haptens it is important to remove free label, since this can interfere with downstream applications. Therefore, the Platinum*Link* kits include ULS-Trap columns to remove the non-reacted ULS label. This also enables a more accurate determination of labeling densities. In addition to the highly efficient removal of free label, ULS-Trap columns give a very high recovery of labeled proteins. Because free label removal is based on affinity purification instead of size exclusion there is no molecular size cut-off resulting in the preservation of small proteins and peptides in the purified labeled protein solution.

II. Procedural Overview and General Considerations

The procedure of protein labeling using the Platinum*Link* Protein Labeling Kit is as described below. The flow scheme in figure 3 gives an overview of the protein labeling procedure. The entire procedure takes less than half-an-hour hands-on time to complete.

Note: The Platinum*Link* Kits have been developed for labeling of single purified proteins and antibodies to be used for different downstream applications. For labeling of complex protein mixtures, such as serum or cell lysate samples, KREATECH has developed other kits. Information on these kits is available at www.kreatech.com

In general, the protocol consists of the following steps:

0. Optional: sample preparation
1. ULS labeling of proteins
2. Removal of free label using ULS-Trap columns

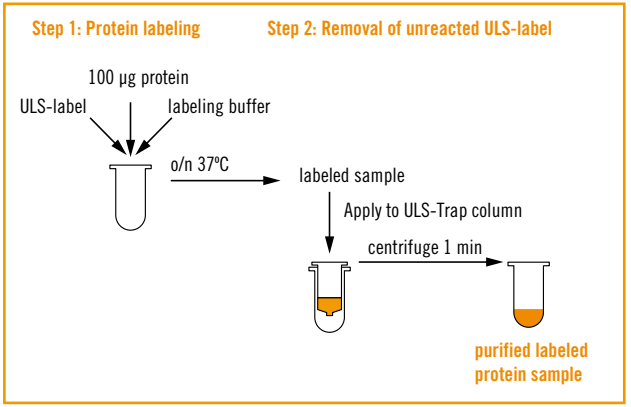

Figure 3. Schematic overview of ULS protein labeling process.

0. OPTIONAL: sample preparation

The ratio of proteins to ULS-label during incubation is important for successful labeling. In case the protein concentration of the protein stock is unknown, it is important to determine the protein concentration prior to labeling. We recommend using the BCA protein assay. We strongly discourage the use of carrier proteins during labeling, since all proteins present in the reaction mixture will be labeled. In case a carrier protein is present in your protein stock and can not be removed, its concentration has to be included in the protein concentration.

In general, purification of your protein stock solution prior to labeling is not necessary, since ULS labeling is compatible with most biochemical buffers including tris(hydroxymethyl) aminomethane, glycine, ≤ 15% glycerol, detergents including Triton X-100, and with many salts, including ≤ 40 mM EDTA and sodium azide at a maximum concentration of 0.02% *during* labeling. Incompatible substances *during* the labeling reaction include thioaldehydes, thioketones, thioethers, and thiols (for instance, dithiothreitol, ß-mercaptoethanol and thiourea), and strongly reducing agents like tris-(2-carboxyethyl) phosphine (TCEP) and tributylphosphine (TBP). In case any incompatible substances are present in your protein stock solution, we recommend buffer exchange with saline or Tris.

1. ULS Labeling of Proteins

Since ULS-labels are very stable, BIO-ULS, DNP-ULS, FLU-ULS and RHO-ULS are provided as aqueous stock solutions. This makes time consuming weighing of precious labeling agents obsolete.

The labeling protocol described below has been optimized for the labeling of 100 µg of protein in a 100 µl labeling volume.

For example, 10 µl of a 10 mg/ml stock solution is added to the 100 µl labeling volume (or 20 µl of a 5 mg/ml stock, 50 µl of a 2 mg/ml stock, etc). If the concentration of your protein stock solution is lower than 1.18 mg/ml, addition of 100 µg protein to a 100 µl end volume will not be feasible. In that case, consider concentrating your protein stock before labeling. Alternatively, add as much protein as possible. When the protein concentration in the labeling mixture is ≤ 0.5 mg/ml, the amount of label has to be adjusted according to Table 1 in order to prevent over-labeling.

Protein concentration in labeling mixture	Amount of ULS-label needed
1 mg/ml	5 µl
0.5 mg/ml	3 µl
0.1 mg/ml	1 µl

Table 1: Amount of ULS-label needed when using lower protein concentrations in the labeling mixture.

The labeling time has been optimized to overnight (16 hours) at 37°C. If preferred, one may decrease the labeling time by performing the labeling reaction at 50°C for four hours instead.

With the protocol described in this manual we have obtained very good results without using protease inhibitors.

However, if protease inhibitors are required during labeling, Roche Complete™ has been found to be compatible with ULS-labeling.

2. Removal of Free Label using ULS-Trap Columns

Removal of free label after protein labeling is essential, since non-reacted labels might interfere in the downstream application. To this end, many researchers first inactivate the excess of label with a soluble target and use gel filtration to remove the inactivated free label. This method removes 95-98% of free label, but small proteins and peptides are removed as well.

KREATECH has developed a free label removal spin column that specifically binds non-reacted ULS. Therefore, de-activation of free label prior to column purification is not needed. Non-reacted ULS is very efficiently (>99.5%) removed and as the ULS-Trap column is not based on size exclusion, even small proteins and peptides are recovered. The method is rapid and purification is performed in less than 5 minutes. The rate of protein loss using ULS-Trap has been determined for several individual proteins and protein mixtures at a concentration of 100 µg protein in 100 µl labeling buffer. On average, protein recovery turned out to be >85%. In case you notice severe protein loss over the column, a carrier protein might be added to the reaction mixture immediately before loading on the ULS-Trap column. (Note: do not add a carrier protein *before or during* the labeling reaction!). As described above, ULS-Trap columns are not based on size exclusion. As a consequence, buffer exchange does not take place. In case your downstream application will be strongly influenced by buffer composition, buffer exchange after removal of free label should be considered.

C. Protocol

Please read the entire protocol before starting

- Before use, shortly centrifuge all vials to collect fluids at the bottom of the vial.
- BIO-ULS, DNP-ULS and RHO-ULS come as aqueous solutions. FLU-ULS comes as a suspension; prior to use thoroughly resuspend the FLU-ULS by pipetting.

OPTIONAL: Sample preparation

- Generally, purification of protein stocks prior to ULS-labeling is not necessary. However, in case your protein stock solution contains incompatible substances as described on page 10, buffer exchange is recommended.
- In case the protein concentration of your protein stock is not known, determine protein concentration using for instance a BCA Protein Assay Kit (*e.g.* Pierce # 23225). Follow the manufacturer's instructions.

I. ULS labeling of proteins

- Prepare one 1.5 ml Eppendorf vial for each labeling reaction (vials not provided).
- Add the reagents in the order as is shown in Table 2.

NOTE: if protein stock concentration is lower than 0.5 mg/ml, the amount of label has to be adjusted according to Table 1 (page 11).

Table 2. protein labeling with ULS labels

	volumes
Protein of interest	100 µg (*e.g.* 10 µl of 10 mg/ml stock)
Purified water	Adjust volume to 85 µl
10x PL buffer	10 µl
BIO-ULS **or** DNP-ULS **or** FLU-ULS **or** RHO-ULS	5 µl
Total volume	**100 µl**

- Thoroughly mix the samples by pipetting and incubate overnight (16 hrs) at 37°C in a water bath or incubator.
- Optional: the samples may be incubated at 50 °C for four hours to reduce labeling time.

II. Free label removal using ULS-Trap columns

Preparing the ULS-Trap column

Prepare one ULS-Trap column for each 100 µl labeling reaction:

- Invert the column several times to resuspend the content
- Snap off the bottom of the column and **remove the cap**
- Place the column in a 2 ml polypropylene sample collection tube
- Spin column dry: 1 minute at maximum speed (~20,000 g) in a table top microcentrifuge
- Discard the 2 ml collection tube together with its content
- Place the column in a new 2 ml polypropylene sample collection tube

Removal of free label

- Load 100 µl labeled sample onto the center of a ULS-Trap Column.
- Centrifuge column with sample for 1 minute at maximum speed (~20,000 g) in a table top microcentrifuge; the flow through contains the labeled protein, non-reacted ULS is retained on the column.
- Transfer your purified, labeled protein to a storage vial (not provided).
- If desired, desalt the solution and/or add preservative (*e.g.* EDTA, glycerol, carrier protein)

III. Determination of the degree of labeling

There may be occasions that one wishes to check the degree of labeling of the proteins. This is done by measuring the absorbance of DNP, FLU or RHO and calculating the amount of ULS molecules using the molar extinction coefficient. For BIO this is not possible, but instead, biotin might be determined using the HABA/avidin method (Pierce, Cat no # 28010). Essential to both methods is that after the labeling reaction the excess of free label is removed.

Determination of label to protein ratio for DNP, FLU or RHO labeled proteins:

- Label the protein as described
- Remove non-reacted ULS using ULS-Trap column
- Perform a Protein assay on a fraction of the labeled and purified material. In contrast to the protein determination prior to labeling, we recommend using Bio-Rad's Rc Dc protein assay (Bio-Rad # 500-0120), since the BCA assay is not compatible with proteins purified over ULS-Trap columns. Moreover, with Bio-Rad's assay no correction factors for absorbance of the label is needed since the

protein concentration is measured at 750 nm.

- Measure absorbance in a spectrophotometer at the wavelength indicated in Table 3.
- Calculate the label / protein ratio using the following formula:

$$\text{label / protein} = \frac{A^* MW}{\varepsilon * c * l}$$

with A: absorbance at indicated wavelength
ε: molar extinction coefficient as given in Table 3
l: cuvette path length (cm)
c: protein concentration in mg/ml
MW: molecular weight of the protein in g/mol.

Label	Absorbance maxium (nm)	Emission maximum (nm)	Extinction coefficient (l mol^{-1} cm^{-1})	Solvent
DNP-ULS	363	N.A.	18,000	MeOH
FLU-ULS	495	517	83,000	pH 9
RHO-ULS	550	573	91,000	MeOH

Table 3: Spectral characteristics of labels

Determination of Biotin-ULS to Protein ratio:

- Label the protein as described
- Remove excess BIO-ULS™ using ULS-Trap column
- Perform a Protein assay on a fraction of the labeled and purified material. In contrast to the protein determination before labeling, we recommend to use BioRad's *Rc Dc* protein assay, since the BCA assay is not compatible with proteins purified over ULS-Trap columns.
- Determine the molar ratio of biotin incorporated into a protein using the HABA-Avidin method. The HABA dye (2-hydroxyazobenzen-4'-carboxylic acid) binds to avidin to produce a yellow-orange colored complex which absorbs at 500 nm. Free biotin will displace the HABA dye and cause the absorbance to decrease. A standard curve can be established using free biotin to estimate the number of moles of biotin incorporated after biotinylation of a protein. We recommend to use HABA from Pierce (catalogue #28010) following the manufacturer's instructions.

D. Trouble Shooting

Problem	Possible Cause	Suggestions
Weak signal in downstream application	Under-labeling	• Check L/P ratios. Increase degree of labeling by increasing labeling time and/or temperature, or by decreasing protein concentration during labeling. • Increase concentration of labeled protein in downstream application. • Make sure the used protein stock buffer is ULS-compatible. If not: change buffer.
	Over-labeling	• Check L/P ratios. Decrease degree of labeling by decreasing labeling time and/or temperature, or by decreasing the amount of ULS-label used. • Decrease concentration of labeled protein in downstream application.

Problem	Possible Cause	Suggestions
Low protein concentration after free label removal	Protein loss over ULS-Trap column	• Add a carrier protein immediately before applying to the ULS-Trap column (not during labeling). • Consider an alternative non-reacted label removal method: - gel filtration (using e.g. Pierce's protein desalting columns or Bio-Rad's BIOspin columns) - dialysis.
	Protein precipitation after labeling	• see Problem: Protein precipitation after labeling.
Protein precipitation after labeling	Protein degradation	• Use freshly prepared samples. • Add (more) protease inhibitors.
	Over-labeling	• Check L/P ratios. Decrease degree of labeling by decreasing labeling time and/or temperature or by decreasing the amount of ULS-label used.

E. References

Van Gijlswijk RP, Talman EG, Peekel I, Bloem J, van Velzen MA, Heetebrij RJ, Tanke HJ (2002). Use of horseradish peroxidase- and fluorescein-modified cisplatin derivatives for simultaneous labeling of nucleic acids and proteins. *Clin Chem*; 48(8),1352-9.

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