Manual

Spectral Shift Optimized Protein Labeling Kit

Cysteine-Reactive

Size L

Cat# NT-L024

NANOTEMPER

Content

1. About the kit

This advanced, Cysteine-reactive kit offers a rapid and efficient method for covalently labeling thiols, particularly cysteine residues. Building upon our previous MALEIMIDE labeling technology, this kit is specifically engineered for optimal performance with spectral shift measurements. The kit is optimized for labeling and purification of proteins with a molecular weight higher than 5 kDa.

The kit contains material sufficient for 4 independent labeling reactions.

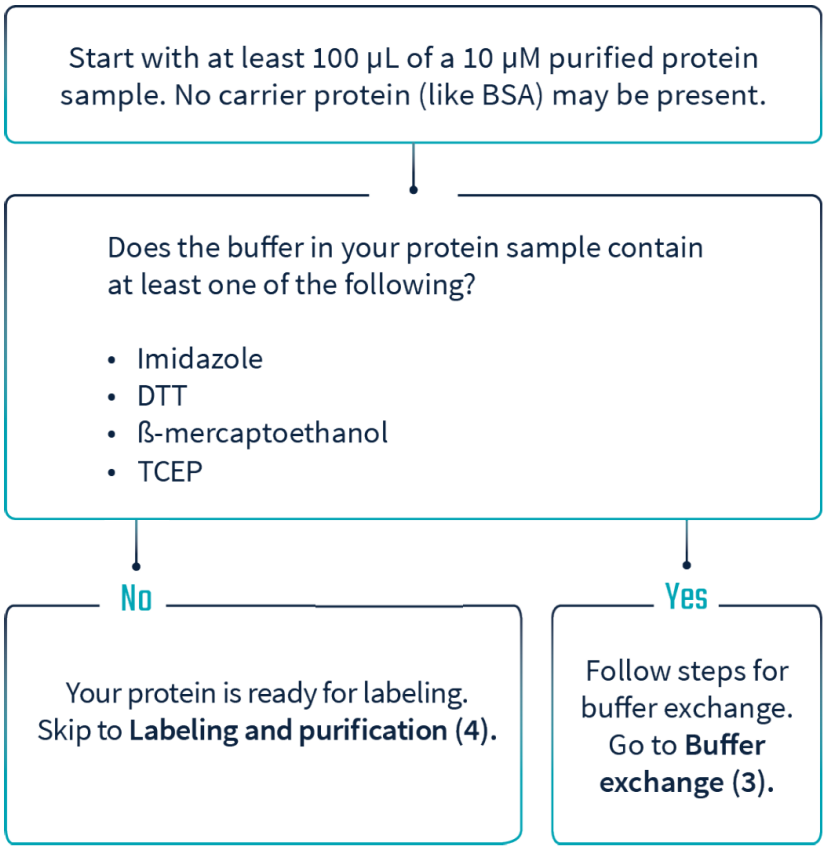
Included in this kit	Quantity	Storage
 **Spectral Shift-Optimized Dye, Cysteine-Reactive (3 nmol)** Carries a reactive MALEIMIDE group that reacts with thiol groups to form a covalent bond.	4	-20 °C
 **Labeling Buffer MALEIMIDE** 17.7 mM NaH_2PO_4, 32.5 mM Na_2HPO_4, 100 mM NaCl, pH 7.0 at room temperature	4	4 °C
 **A-Column** The A-Column provided can be used for the buffer exchange of 40-100 µL of a protein with a molecular weight higher than 5 kDa.	4	4 °C
 **B-Column** The B-Column provided can be used to purify proteins with a molecular weight higher than 5 kDa.	4	4 °C
 Adapter (for 15 mL centrifuge tube)	2	RT

Bring all components to room temperature before use. When stored appropriately, the kit components should be stable for approximately 12 months.

Do not exceed recommended centrifuge time or speed.

2. Plan your labeling

Before you start protein labeling make sure that your sample is prepared in a compatible buffer. The decision tree below is intended to help you to determine if you need to perform an initial **Buffer exchange** or if you can directly start with **Labeling and purification**.



IMPORTANT INFORMATION

To label proteins with the MALEIMIDE-dye, it is essential to ensure that free cysteines are available for reaction. In the absence of a reducing agent, cysteines can form disulfide bonds (cystine), which will prevent labeling. To avoid this, the protein must be reduced before labeling.

To reduce any unwanted disulfide bonds, we recommend adding TCEP to the protein sample at a final concentration of 1 mM and to incubate the mixture for 1 hour at room temperature. After the reduction step, excess TCEP needs to be removed by performing a buffer exchange. This step is critical as TCEP may interfere with the labeling reaction.

Be aware that longer incubation times or higher TCEP concentrations may reduce structural disulfides located within the protein core, which could affect protein stability and function. If stability is a concern, optimize the TCEP concentration (e.g., 0.5 mM) and incubation time to balance effective cysteine reduction while maintaining protein integrity.

3. Buffer exchange

You will have to subject your protein sample to a buffer exchange if it's dissolved in an incompatible buffer. Your buffer cannot contain imidazole, DTT, β-mercaptoethanol, TCEP or other reducing agents and should be within pH 6.9-7.2.

If your protein is in a compatible buffer for labeling, skip this section and go straight to **Labeling and purification**.

Do not exceed recommended centrifuge time and speed.

To convert your protein concentration from mg/mL to µM use the following formula:

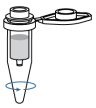
$$\text{Concentration (µM)} = 1{,}000 \times \frac{\text{Concentration (mg/mL)}}{\text{Molecular Weight (kDa)}}$$

Materials required	Quantity
Labeling Buffer MALEIMIDE Reconstitute with 3 mL of ddH_2O right before use. The final pH will be 7.0 at room temperature. You will use only 1 mL in this step. The other 2 mL will be used for labeling your protein.	1
A-Column The A-Column provided can be used for the buffer exchange of 40-100 µL of a protein with a molecular weight higher than 5 kDa.	1
10 µM highly pure (> 90 %) protein sample	100 µL
1.5 mL microcentrifuge tubes	2
Variable speed benchtop microcentrifuge (min. speed 1,500 x g)	1

Buffer exchange protocol

Invert the A-Column 3 times to resuspend slurry.

Twist off bottom and remove cap.



Place **A-Column** in a fresh microcentrifuge tube and centrifuge at 1500 g for 1 minute to remove storage solution.

Discard flow through from microcentrifuge tube and place **A-Column** back in the microcentrifuge tube.

Add 300 µL **Labeling Buffer MALEIMIDE** to equilibrate the **A-Column**. Avoid contacting the inner walls of the column and load the buffer directly in the center of the resin bed.

Centrifuge at 1500 g for 1 minute and discard flow through.

Perform this step three times.

Place A-Column in a fresh microcentrifuge tube.

Pipette 100 µL of your 10 µM protein sample in the center of the column resin. Centrifuge at 1500 g for 2 minutes.

Your protein is in the collected flow through. The used column can be discarded.

4. Labeling and purification

Before you start with the labeling procedure, make sure your protein's concentration is ~10 µM. If it's significantly different, please see **Frequently Asked Questions** for suggestions.

Is your protein too concentrated? We recommend diluting it in the **Labeling Buffer MALEIMIDE** to ensure compatibility with the labeling protocol.

To convert your protein concentration from mg/mL to µM use the following formula:

$$\text{Concentration } (\mu\text{M}) = 1{,}000 \times \frac{\text{Concentration } (\text{mg/mL})}{\text{Molecular Weight } (\text{kDa})}$$

Materials required	Quantity
Spectral Shift-Optimized Dye, Cysteine-Reactive (3 nmol) The dye is dissolved in 5 µL of anhydrous DMSO at a concentration of 600 µM and ready to use.	1
Labeling Buffer MALEIMIDE If you performed the buffer exchange, use the remaining 2 mL of reconstituted labeling buffer for this step. Otherwise, reconstitute one vial with 3 mL of ddH_2O right before use. The final pH will be 7.0 at room temperature.	1
B-Column The B-Column provided can be used to purify proteins with a molecular weight higher than 5 kDa.	1
Adapter (for 15 mL centrifuge tube)	1
10 µM highly pure (> 90 %) protein sample in MALEIMIDE-compatible buffer	90 µL

1.5 mL microcentrifuge tubes	3
15 mL centrifuge tube	1
Assay or equilibration buffer of choice	12 mL
Variable speed benchtop microcentrifuge (min. speed 1,500 x g)	1

Use gloves when handling DMSO and dye. Use dye immediately after mixing with Labeling Buffer MALEIMIDE.

Do not vortex your protein as it might compromise its integrity. Instead, gently pipette up and down to mix.

Do not exceed recommended incubation time.

Final DMSO concentration in the labeling reaction should not exceed 5 %.

Protein labeling protocol

Briefly spin down the content of the dye vial to collect the dye solution at the vial bottom.

Add 5 µL **Labeling Buffer MALEIMIDE** to the vial containing 5 µL of 600 µM dye.

Mix carefully by pipetting up and down several times.

Add 90 µL of your **10 µM protein sample** to the vial.

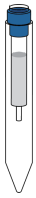
Mix carefully by pipetting up and down several times. This yields 100 µL of dye-protein solution with an approximately 3-fold excess of dye.

Incubate for 30 minutes at room temperature in the dark.

In the meantime: equilibrate your B-Column (~ 20 minutes)

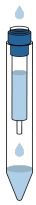
Remove top cap from the **B-Column** and pour off the storage solution.

Then remove the bottom cap. Save both caps and set aside.



Replace the cap from the 15 mL centrifuge tube with the **Adapter**.

Then place the column on top of the adapter and in the tube.



Fill column with assay or equilibration buffer of choice and allow buffer to enter the packed resin bed completely by gravity flow.

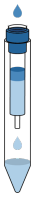
Discard the flow through collected.

Repeat this step 3 more times.

About 8-10 mL of buffer should be used in total for all 4 steps. Discard the last flow through so the tube is ready for the next step.

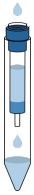
If you finish the equilibration before the 30 minutes labeling incubation is completed, place the caps you set aside back on the column to prevent the resin bed from drying out.

Purify your labeled protein by removing free dye



After the labeling reaction incubation, transfer the 100 µL of dye-protein solution to the B-Column you just equilibrated. Avoid contacting the inner walls of the column and load your sample directly in the center of the resin bed.

Let sample enter the resin bed completely.



Add 550 µL of assay or equilibration buffer of choice and allow buffer to enter the resin bed completely.

Have a fresh microcentrifuge tube ready to collect your labeled protein.

To elute your protein, place the fresh microcentrifuge tube under the column.

Add 450 µL of assay or equilibration buffer onto column and collect the flow through that contains your labeled protein. Avoid contacting the inner walls of the column and load your buffer directly in the center of the resin bed.

The used column can be discarded.

As an optional step, we recommend centrifuging the sample at the typical maximum speed of a benchtop centrifuge (around 20,000 × g) for 10 minutes. Aggregated proteins, if present, will form a blue pellet at the bottom of the tube. Carefully transfer the supernatant (top layer) to a fresh tube without disturbing the pellet.

Most proteins can be stored after labeling for several weeks at -80 °C when aliquoted and flash-frozen in liquid nitrogen. Avoid repeated freeze-thaw cycles.

5. Concentration and degree-of-labeling (DOL)

We recommend measuring the concentration and the degree-of-labeling (DOL) of your labeled protein before you start your binding experiments. The DOL describes how many dye molecules are bound to your protein, e.g. a DOL of 1 refers to a dye:protein ratio of 1:1.

- Measure absorption A_{280} and A_{650}.
- If your protein does not contain tyrosine or tryptophan residues, the absorption A_{205}, which arises primarily from the peptide bond, can be measured instead.
- Depending on the sensitivity of your spectrophotometer and the concentration of your sample, you may be out of detection range.
- Typical path length *d* of a spectrophotometer is 1 cm.
- Correction factor at 280 nm: 0.04
- Correction factor at 205 nm: 0.19
- Molar absorbance of dye: 250,000 $M^{-1}cm^{-1}$

Concentration determination at 280 nm

Provide the extinction coefficient $\varepsilon_{280,\,Protein}$ of your protein.
Measure absorption at 280 nm and 650 nm.
Calculate protein concentration with the following equation:

$$c(M) = \frac{A_{280} - (A_{650} \times 0.04)}{\varepsilon_{280,Protein} \times d}$$

Concentration determination at 205 nm

Provide the molecular weight $MW_{Protein}$ of your protein.
Measure absorption at 205 nm and 650 nm.
Calculate protein concentration with the following equation:

$$c(M) = \frac{A_{205} - (A_{650} \times 0.19)}{31 \times MW_{Protein}(Da)}$$

Calculate degree-of-labeling with the following equation

$$DOL = \frac{A_{650}}{250{,}000\ M^{-1}cm^{-1} \times c(M)}$$

- Typical protein yield results are 50% to 70% after the purification step. Optimal DOL is 0.5-1.
- A DOL greater than 1 should be avoided for most proteins since it can lead to adverse effects on protein function.
- A DOL below 0.5 should be avoided since it can lead to reduced signal-to-noise ratio. For more details see Frequently asked questions (FAQ).
- For the best result from your assay, start with 20 nM of your labeled protein (Nano-RED and Spectral Shift detector) or 5 nM (Pico-RED detector), respectively.
- Always use the latest version of MO.Control Software for optimal assay planning.

6. Frequently asked questions (FAQ)

6.1 Can I use a different buffer from the Labeling Buffer MALEIMIDE?

The provided Labeling Buffer MALEIMIDE is the preferred buffer for the labeling reaction. It's a phosphate buffer with pH 7.0 at room temperature. However, you cannot use buffers that contain imidazole, DTT, β-mercaptoethanol or TCEP. Use of other buffers may decrease labeling efficiency.

6.2 What adjustments should I make if the concentration of the protein sample I want to label is different than the recommended 10 µM?

This table shows the adjustments you have to make to the dye solution if you want to label the protein at other concentrations. We do not recommend to label less than 2 µM of protein.

The dye is provided as a 600 µM solution in DMSO.

Calculate the needed volumes of dye and buffer using the equations below.

Protein concentration (µM)	Dye excess	Dye concentration (µM)
2 – 5	5-fold	10 – 25
6 – 10	3-fold	18 – 30
11 - 20	2-fold	22 - 40

Calculate the volume of dye needed with the following equation:

$$\mathrm{Volume_{Dye}(\mu L)} = \frac{\mathrm{Concentration_{Protein}(\mu M)} \times \mathrm{Dye_{Excess}}}{600\ \mu\mathrm{M}} \times 100\ \mu\mathrm{L}$$

Calculate the volume of Labeling Buffer MALEIMIDE needed with the following equation:

$$\mathrm{Volume_{Buffer}(\mu L)} = 10\ \mu\mathrm{L} - \mathrm{Volume_{Dye}(\mu L)}$$

- Mix Labeling Buffer MALEIMIDE with dye solution as calculated above.
- Add 10 µL of this dye solution (100-400 µM) to 90 µL of your protein stock.
- Mix carefully by pipetting up and down several times.
- Incubate for 30 minutes at room temperature in the dark and continue with
- the standard protocol for **labeling and purification**.

6.3 Can I increase the volume of my labeling reaction?

Yes, you can increase it up to 500 µL, which is the maximum volume the provided B-Column can process. Adjust the volume to 650 µL after the sample has entered the bed by using assay or equilibration buffer.

6.4 The calculated DOL is significantly higher than recommended. What could be the reason?

If your calculated DOL is significantly higher than recommended, your protein is most likely over-labeled. Reduce the ratio of dye to protein.

6.5 The calculated DOL is significantly less than recommended. What could be the reason?

If your calculated DOL is significantly less than recommended, your protein is most likely under-labeled. Here is a list of potential reasons and proposed solutions:

Reason	Explanation	Solution
MALEIMIDE is non-reactive.	MALEIMIDES can remain stable for several hours to days in aqueous buffer at near-neutral pH, but hydrolyze relatively quickly (within hours) at higher pH values. Also, higher temperatures can increase the rate of hydrolysis.	Use the diluted dye solution immediately after preparing it.
The cysteines in the protein are not reduced.	In the absence of a reducing agent, free cysteines can form disulfide bonds, either within the same protein (intramolecular) or between different proteins (intermolecular).	Ensure cysteines are reduced and reactive by incubating with a reducing agent, then remove it with a buffer exchange using the supplied A-column before labeling.
The buffer contains free sulfhydryls.	Free sulfhydryls (like DTT or protein impurities) present in the buffer will react with the labeling dye and decrease the labeling efficiency.	Perform a buffer exchange prior to the labeling using the supplied A-column.
Protein purity is too low.	The presence of carrier proteins like BSA or casein will interfere with the labeling and subsequent binding assays.	Use a protein sample that is appropriately pure (> 90 %).
pH of the labeling reaction is not optimal.	The maleimide reaction is most efficient near neutral pH (pH 6.9 to 7.2).	Perform a buffer exchange prior to the labeling using the supplied A-column.
Protein concentration is too low.	An optimal ratio between protein and dye is required for successful labeling.	Concentrate your protein, e.g. by using spin concentrators (not included in this kit).
	Need help? Visit the NanoTemper Support Center at **https://support.nanotempertech.com**.	

7. Safety information

B-Column

Hazard statements

H317	May cause an allergic reaction.

Precaution statements

P261	Avoid breathing dust/fume/gas/mist/vapors/spray.
P272	Contaminated work clothing should not be allowed out of the workplace.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P302 + P352	IF ON SKIN: Wash with plenty of water.
P333 + P313	If skin irritation or rash occurs: Get medical advice/attention.
P362 + P364	Take off contaminated clothing and wash it before reuse.

For more information, please consult the respective Safety Data Sheets (SDS). SDS are available from NanoTemper Technologies upon request.

8. Purchase notification

NanoTemper grants the buyer the non-transferable right to use the purchased product for research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or its components for commercial purposes.

Limited warranty.

NanoTemper will replace any product that does not meet the specifications. This warranty limits NanoTemper's liability only to the cost of the product when within the expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. NanoTemper assumes no responsibility or liability for any indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive.

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9. Notes

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