Manual

Spectral Shift Optimized Protein Labeling Kit

For His-Tag

Size L

Cat# NT-L128

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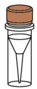
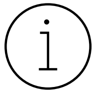
1. About the kit

This kit provides convenient means for the site-specific, purification-free labeling of small amounts of His-tagged proteins with our Spectral Shift-optimized fluorescent dye. It can be used for the labeling of any protein or peptide carrying a polyhistidine-tag (His-tag). Labeling can be completed in a few minutes and no removal of excess dye is required. The Spectral Shift-optimized dye binds efficiently to His-tags which contain at least six histidines with a K_d in the single digit nanomolar range.

The kit contains material sufficient for 4,000 data points.

	Included in this kit	Quantity	Storage
	**Spectral Shift-Optimized Dye, For His-Tag (800 pmol)** Carries a tris-NTA group with high affinity for His-tagged proteins.	1	-20 °C



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.

IMPORTANT INFORMATION BEFORE STARTING

His-tags are common protein tags which are routinely used for affinity purification. The His-tag labeling strategy is highly specific, requires only nanomolar concentrations of the His-tagged protein and no dye removal step. Labeling can be carried out even with unpurified samples, in cell lysate or other complex bioliquids. Moreover, His-tag labeling is robust towards a variety of common storage and assay buffer components.

The table below lists the concentration limits for certain buffer components that may interfere with the labeling reaction. For optimal results, we recommend using phosphate-buffered saline (PBS) or alternatively HEPES buffer, maintaining the pH between 7 and 8, supplemented with 0.005% Tween 20. Since the dye's affinity for the His-tag decreases substantially at pH values below 7, operating under those conditions is not advised.

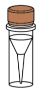
To ensure a high labeling efficiency, we recommend to initially determine the affinity between the dye and the His-tagged protein of interest (Step 2). This kit does not include a positive control; however, any His-tagged protein can be used for the purpose of evaluating potential interference of buffer components or ligands not listed in the table.

Component	Maximum allowed concentration
Histidine	1 mM
Imidazole	1 mM
EDTA, EGTA, other chelating agents	0.05 mM
DTT	5 mM
TCEP	0.5 mM
β-mercaptoethanol	1 mM
GSH	10 mM
Glycerol	10 %
Tween 20, Tween 80, Triton X-100, other detergents	Use concentration **below CMC**, e.g. 0.005% Tween 20
Polyhistidine-tagged ligand	None
Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}	Not recommended
Tris	Not recommended
pH < 7	Not recommended
SDS	Not recommended

2. Affinity between dye and His-tagged protein

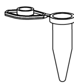
For optimal results in the ligand binding assay, it is essential that the dye is fully bound to the His-tagged protein. Typically, the affinity between the dye and the His-tag falls within the low nanomolar range, though this can vary depending on the accessibility of the tag. To ensure accurate results, it is recommended to determine the binding affinity in a preliminary experiment.

For the assay, we suggest using a PBS buffer supplemented with **0.005% Tween-20** (PBS-T) as the interaction buffer. However, if you prefer to use a different buffer system, you can substitute it for PBS-T in the following protocol. Volumes can be scaled up- or down when needed.



The dye vial contains 400 µL of dye dissolved in PBS-T at a concentration of **2 µM**.

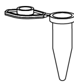
Briefly spin down the content of the vial to collect the dye solution at the vial bottom.



Take a PCR tube and mix 2 µL of dye (2 µM) with 198 µL of PBS-T.

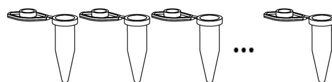
This yields 200 µL of a **20 nM** dye solution.

Mix carefully by pipetting up and down several times.



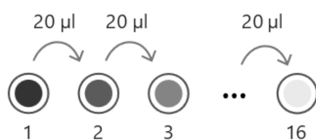
Take a 0.5 mL microcentrifuge tube and mix 160 µL of the 20 nM dye solution with 160 µL of PBS-T.

This yields 320 µL of **10 nM** dye solution.



Prepare a rack with 16 PCR tubes. Transfer 20 µL from the **10 nM** dye solution into tubes **2** to **16**.

Then, mix 20 µL of **20 nM** dye with 20 µL of **1 µM** of your His-tagged protein of interest in tube **1**.



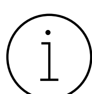
Prepare a serial dilution by transferring **20 µL** from tube to tube. Mix carefully by pipetting up and down.

This will result in a dilution series with a starting protein concentration of **500 nM** and a constant dye concentration of **10 nM**.

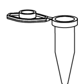
Incubate for 10 minutes at room temperature before proceeding with the affinity measurement between dye and protein.

3. Protein labeling

The following protocol describes the procedure for preparing **200 µL** of labeled His-tagged protein of interest, sufficient for one experiment with 16 data points (**20 µL** final volume), with replacement of your interaction buffer of choice for PBS-T if choosing to use an alternate buffer system. Volumes can be scaled up- or down when needed.



If the affinity between dye and target protein determined in **Step 2** is very low (K_d **> 50 nM**) we recommend adjusting your assay buffer to improve the affinity or switching to a covalent labeling strategy for lysine or cysteine residues.



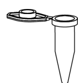
For His-tagged proteins with high affinity to the dye (K_d **≤ 5 nM**):
Adjust the protein concentration to **100 nM** in 100 µL of PBS-T.

For His-tagged proteins with lower affinity (Kd **> 5 nM**):
Adjust the protein concentration to **20-times the K_d** value in 100 µL of PBS-T.

(Example: For a K_d of 20 nM, prepare 400 nM protein.)

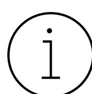
The dye vial contains 400 µL of dye dissolved in PBS-T at a concentration of **2 µM**.

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



Add 2 µL of dye (2 µM) to the 100 µL of protein, then add 98 µL of PBS-T and mix carefully by pipetting up and down several times.

This yields 200 µL of **2X** labeled protein (20 nM dye).

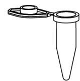


As an optional step, we recommend centrifuging the sample at the typical maximum speed of a benchtop centrifuge (around 20,000 × g) for 10 min. After centrifugation, carefully transfer the supernatant (top layer) to a fresh PCR tube, avoiding any potential pellet.

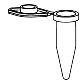
The protein is ready for the binding assay, you can continue with Step 4.

4. Ligand binding assay

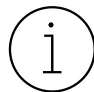
We recommend preparing the serial dilution of your ligand of interest in PCR tubes with non-binding surface. If you are using a different buffer system, replace PBS-T with your chosen interaction buffer. Adjust the volumes as needed to fit the scale of your assay.



Prepare 25 µL of your ligand of interest at a **2X** concentration in PBS-T.

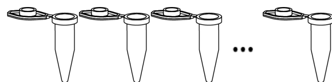


Take a 0.5 mL microcentrifuge tube and mix 160 µL of **2X** labeled protein (prepared in Step 3) with 160 µL of PBS-T.



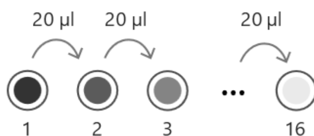
If the ligand requires a solvent like DMSO, use PBS-T containing the same concentration of DMSO to ensure that there are no buffer inconsistencies throughout the dilution series.

This yields 320 µL of **1X** labeled protein.



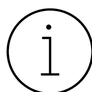
Prepare a rack with 16 PCR tubes. Transfer 20 µL of the **1X** labeled protein solution into tubes **2** to **16**.

Then, mix 20 µL of your ligand at **2X** concentration with 20 µL of **2X** labeled protein in tube **1**.



Prepare a serial dilution by transferring 20 µL from tube to tube. Mix carefully by pipetting up and down.

Incubate the dilution series for at least **10 minutes** at room temperature before proceeding with the affinity measurement between protein and ligand.



If you labeled 100 µL of **100 nM** of His-tagged protein in Step 3, the final target concentration in the dilution series is **25 nM**.

Note that for ligands binding with very high (e.g. pM) affinity to your protein, only EC_{50} values can be determined.

5. Frequently asked questions (FAQ)

5.1 How can I determine if my ligand interferes with His-tag labeling of my protein?

If your ligand is a protein, ensure that it does not also contain a His-tag. If both your target protein and the ligand have His-tags, the dye may transfer from the labeled target protein to the ligand at high ligand concentrations. This can result in a signal change that mimics a typical binding dose-response, potentially leading to misinterpretation of the data.

Additionally, some small molecules can interfere with the labeling process by interacting with the dye. To rule out such interferences, perform a control experiment by measuring a dilution series of the ligand against dye alone, in the absence of His-tagged protein.

5.2 How can I improve the signal-to-noise ratio in my ligand binding assay?

Poor signal-to-noise ratio can be caused by the presence of excess free dye in the solution. This issue can arise if the affinity between dye and protein has not been characterized (Step 2) and the protein concentration was overestimated before labeling, resulting in an insufficient amount of protein to bind all the dye molecules. We recommend to re-check the concentration of your His-tagged protein or to increase the ratio between protein and the dye to ensure that all dye is bound.

5.3 My protein requires a divalent cation or co-factor for function. May I add it to PBS-T buffer during labeling?

Yes, co-factors required for the protein function can be added directly to the PBS-T buffer. Please check the table in the first section of this manual for limitations. Divalent ions like Ca^{2+} cannot be added to PBS as this will result in precipitation. Alternatively, HEPES buffered saline can be used as labeling and assay buffer.

5.4 My protein is stored in a buffer that is not compatible with the dye. What are the alternatives?

In this case, a buffer exchange is recommended. You can use a buffer exchange column of your choice.

5.5 Can I also label unpurified His-tagged protein in cell lysates?

Yes, labeling unpurified His-tagged protein is possible if the dye retains strong affinity towards the His-tagged protein in cell lysate. This should be tested prior to performing the binding assay (see Step 2). Strong detergents often present in lysis buffers should be avoided (e.g. SDS) since they tend to disrupt binding interactions. It is recommended to use mechanical force (e.g. Dounce homogenizer) and PBS buffer for cell homogenization. In complex environments like cell lysate, the use of appropriate controls is always advisable to control for unspecific interactions.

Need help? Visit the NanoTemper Support Center at **https://support.nanotempertech.com.**

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7. Notes

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