

Cat# MO-L008

Monolith His-Tag Labeling Kit RED-tris-NTA

Kit for labeling of His-tagged proteins with the RED fluorescent dye NT-647
For use in MST, 500 single-point MST measurements

CONTENT AND STORAGE

Monolith NT™ His-Tag Labeling Kit is shipped at room temperature.
Each kit contains material sufficient for 500 single-point MST measurements.

- 1* 250 pmol RED-tris-NTA dye [store at -20 °C]
- 1* 2 mL 5 x PBS-T (for 10 mL 1 x PBS with 0.05 % Tween 20) [store at -20 °C]
- 1* 1200 pmol His6 Control Peptide [store at -20 °C]

Expiry date: see kit cover

ADDITIONAL MATERIAL REQUIRED

- Variable speed benchtop microcentrifuge
 - 1.5 mL microcentrifuge collection tubes
 - 0.2 mL PCR tubes
 - ddH₂O.
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PROTEIN LABELING PROCEDURE

The Monolith His-tag Labeling Kit RED-tris-NTA provides convenient means for the site-specific, purification-free labeling of small amounts of His-tagged proteins with our NT647 fluorescent dye. This kit can be used for the labeling of any protein or peptide carrying a polyhistidine-tag (His-tag) and contains material sufficient for labeling for 500 single-point MST measurements. Labeling can be completed in 30 min, no removal of excess dye is required. The RED-tris-NTA dye can bind efficiently to His-tags which contain at least six histidines with a K_d in the in the single digit nM range. By following this protocol, optimized for nearly 100 % binding of dye to His-tagged proteins eliminates an additional purification step and ensures the best results. The RED-tris-NTA dye shows fluorescence excitation and emission maxima at approximately 650 nm and 670 nm, respectively.

IMPORTANT INFORMATION BEFORE STARTING

The protocol describes our best labeling practice for the Monolith NT.115, if you are using a Monolith NT.115^{Pico} please refer to FAQ 8 (page 7) for additional information.

His-tags are common protein tags which are routinely used for affinity purification. The His-tag labeling strategy is highly specific, requires only nM concentrations of His-tagged proteins 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. Concentration limits for some buffer components which might interfere with the labeling reaction are listed in Table 1. We recommend using phosphate-buffered saline (PBS) or alternatively HEPES buffer and a pH in the range of 7-8 for the labeling reaction. As the affinity between the dye and the His-tag decreases significantly in Tris buffers and at a pH below 7, these conditions are 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 A). This kit also provides a positive control interaction system with a His6 peptide to evaluate potential interference of buffer components or ligands not listed in Table 1 (please see FAQ 3 for further details).

Table 1: List of common buffer components and their maximum allowed concentration

<i>Component</i>	<i>Maximum allowed concentration</i>
Histidine	1 mM
Imidazole	1 mM
EDTA, EGTA, other chelating agents	0.05 mM
TCEP*	0.5 mM
DTT	5 mM
β -mercaptoethanol	1 mM
GSH	10 mM
GTP, GDP	1 mM
AMP, ADP, ATP	5 mM
Glycerol	10 %
Co ²⁺ , Cu ²⁺ , Ni ²⁺ , Zn ²⁺	preloaded protein only**
Polyhistidine-tagged ligand	none
Tris	not recommended
pH <7	not recommended
SDS	not recommended

* NanoTemper Technologies recommends avoiding the use of TCEP with the red dyes in general.

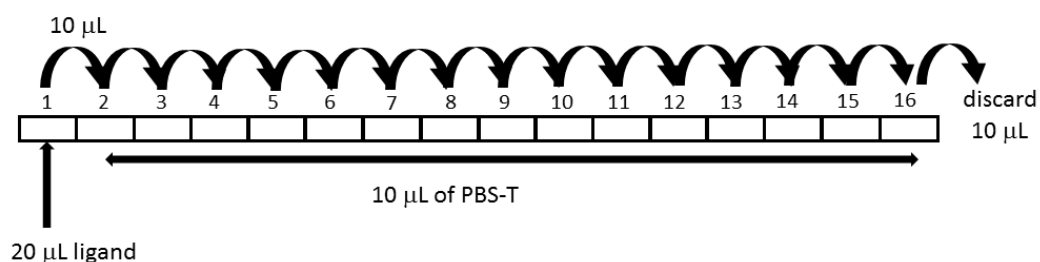
** Co²⁺, Cu²⁺, Ni²⁺, Zn²⁺ ions compete for the binding with RED-tris-NTA dye. Because of that reason only low nanomolar concentrations of listed ions are tolerated.

STEP A
AFFINITY OF DYE TO
TARGET PROTEIN

What is the affinity and efficiency of RED-tris-NTA dye to the His-tagged protein of interest?

To determine the affinity of the His-labeling dye for the His-tagged protein of interest or to determine the labeling efficiency of the protein in the final experimental interaction buffer, the following experimental procedure is recommended, with replacement of scientists' interaction buffer of choice for PBS-T if choosing to use an alternate buffer system:

1. Add 8.0 mL ddH₂O to the vials containing 5 x PBS-T to obtain 1 x PBS-T.
2. Suspend the dye in 50 μ L of PBS-T to obtain 5 μ M dye solution
3. Prepare 200 μ L of 50 nM solution of the RED-tris-NTA dye in PBS-T by mixing 2 μ L of dye (5 μ M) and 198 μ L PBS-T.
4. Prepare 30 μ L of 4 μ M His-tagged protein in PBS-T.
5. Transfer 10 μ L of PBS-T into wells/PCR-tubes **2-16**.
6. Transfer 20 μ L of 4 μ M His-tagged protein solution into the first well/PCR-tube.
7. Transfer 10 μ L of the ligand from well/PCR-tube **1** to well/PCR-tube **2** with a pipette and mix by pipetting up-and-down multiple times. Transfer 10 μ L to well **3** and mix. Repeat the procedure for wells **4-16**. Discard the extra 10 μ L from well 16.



8. Add 10 μ L of 50 nM RED-tris-NTA dye solution to each well (1-16) and mix by pipetting.
9. Incubate for 30 min at room temperature.
10. Load the capillaries and measure the samples at 40 % LED/excitation power and medium MST power (40 % in NT.Control).
11. The K_d can be determined in MO.Control or MO.Affinity Analysis using the K_d fit.

If the affinity of the RED-tris-NTA dye to the His-tagged protein of interest is stronger than 10 nM ($K_d \leq 10$ nM), please continue with Step B protein labeling section I.

If the affinity of the RED-tris-NTA dye to the His-tagged protein of interest is weaker than 10 nM ($K_d > 10$ nM), please continue with Step B protein labeling section II.

If the affinity between RED-tris-NTA dye and your protein of interest is too low ($K_d > 50$ nM) we recommend adjusting your assay buffer to improve the affinity or switching to a covalent labeling strategy for lysine (Cat. # MO-L011) or cysteine (Cat. # MO-L014) residues.

STEP B PROTEIN LABELING

I) Affinity of His-labeling dye to your protein of interest equals or is stronger than 10 nM ($K_d \leq 10$ nM). The following protocol describes the labeling procedure for one experiment with 16 capillaries, with replacement of scientists' interaction buffer of choice for PBS-T if choosing to use an alternate buffer system. If Step A of the protocol was performed proceed directly to 3. Volumes can be scaled up- or down when needed.

1. Add 8.0 mL ddH₂O to the vials containing 5 x PBS-T to obtain 1 x PBS-T.
2. Suspend the dye in 50 μ L of PBS-T to obtain 5 μ M dye solution
3. Prepare a 100 nM dye solution by mixing 2 μ L of dye (5 μ M) and 98 μ L PBS-T.
4. Adjust the protein concentration to 200 nM in a volume of 100 μ L.
5. Mix 90 μ L of protein (200 nM) with 90 μ L of dye (100 nM).
6. Incubate for 30 minutes at room temperature.
7. Centrifuge the sample for 10 min at 4 °C and 15 000 g and transfer the supernatant to a fresh tube.
8. The protein is labeled and ready for the binding assay.

II) Affinity of RED-tris-NTA dye to your protein of interest is weaker than 10 nM ($K_d > 10$ nM). The following protocol describes the labeling procedure for one experiment with 16 capillaries, with replacement of scientists' interaction buffer of choice for PBS-T if choosing to use an alternate buffer system. If Step A of the protocol was performed proceed directly to 3. Volumes can be scaled up- or down when needed.

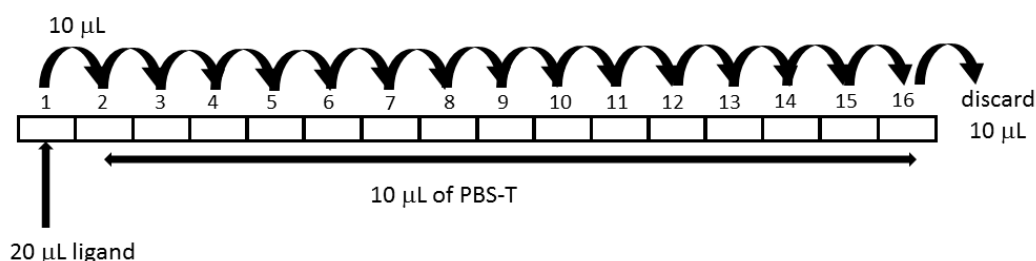
1. Add 8.0 mL ddH₂O to the vials containing 5 x PBS-T to obtain 1 x PBS-T.
2. Suspend the dye in 50 μ L of PBS-T to obtain 5 μ M dye solution
3. Prepare a 100 nM dye solution by mixing 2 μ L of dye (5 μ M) and 98 μ L PBS-T.
4. Adjust the protein concentration to 20 times of the K_d measured in Step A in a volume of 100 μ L (e.g. prepare 100 μ L of 800 nM protein for a K_d of 40 nM between dye and protein. The final protein concentration in the assay is 1/4 of this value = 200 nM)
5. Mix 100 μ L of protein with 100 μ L of dye (100 nM).
6. Incubate for 30 minutes at room temperature.
7. Centrifuge the sample for 10 min at 4 °C and 15 000 g and transfer the supernatant to a fresh tube.
8. The protein is labeled and ready for the binding assay.

Please note: For high affinity interactions the usage of a higher protein concentration can influence your K_d determination (if the protein concentration in the assay is above the K_d of your interaction you can only determine an EC₅₀). If the affinity between RED-tris-NTA dye and your protein of interest is too low ($K_d > 50$ nM) we recommend adjusting your assay buffer to improve the affinity or switching to a covalent labeling strategy for lysine (Cat. # MO-L011) or cysteine (Cat. # MO-L014) residues.

STEP C BINDING ASSAY

We recommend preparation of serial dilutions in PCR tubes or in 384-well multi-well plates with non-binding surface, with replacement of scientists' interaction buffer of choice for PBS-T if choosing to use an alternate buffer system.

1. Prepare 25 μL of the ligand at 2 x concentration (e.g. for a final concentration of 500 nM, prepare ligand at a concentration of 1000 nM). Make sure to avoid buffer mismatches within your titration series.
2. Add 10 μL of PBS-T into the wells/PCR-tubes **2-16**.
3. Transfer 20 μL of the ligand into well/PCR-tube **1**.
4. Transfer 10 μL of the ligand from well/PCR-tube **1** to well/PCR-tube **2** with a pipette and mix by pipetting up-and-down multiple times. Transfer 10 μL to well/PCR-tube **3** and mix. Repeat the procedure for well/PCR-tube **4-16**. Discard the extra 10 μL from well/PCR-tube **16**.



5. Add 10 μL of labeled protein to each well (1-16) and mix by pipetting. The final target protein concentration is 50 nM (or higher, compare Step B II). This concentration should be used for the calculation of the K_d value.
6. Load the capillaries and measure the samples. Recommended settings are 40 % LED/excitation power and medium MST power (40 % MST power in NT.Control). At the final dye concentration of 25 nM the expected fluorescence intensity at 40 % LED/excitation power is around 300 counts on a Monolith NT.115.

FAQ

1. *Even at high MST power settings the signal-to-noise ratio is too poor to allow data analysis. How can I improve the ratio?*

Free dye in the solution might impair the signal-to-noise ratio. In case the concentration of protein prior to labeling has been overestimated, excess dye is present. We recommend to re-check the concentration of your protein or to increase the ratio between the protein and the dye to, e.g. 5:1.

2. *May I dilute labeled protein further?*

Yes, labeled protein can be further diluted. The minimum concentration of the RED-tris-NTA dye we recommend for MST measurements on the Monolith NT.115 is 10 nM. With an LED/excitation power of 100 %, fluorescence intensities of around 250 counts are typically achieved with a negligible bleaching rate. Due to unique chemical properties, the RED-tris-NTA dye is highly resistant to photobleaching.

For high affinity measurements, please see FAQ 8.

3. *With increasing concentrations of the ligand, I noticed ligand-induced fluorescence changes. Does my ligand interfere with the His-labeling or dye?*

To determine if the ligand interferes with the His-labeling procedure, control experiments with the included Control Peptide are recommended:

1. Suspend the lyophilized Control Peptide in 120 μ L of PBS-T to obtain a concentration of 10 μ M.
2. Mix 2 μ L of 10 μ M Control Peptide with 98 μ L of PBS-T.
3. Prepare a 100 nM working dye solution by mixing 2 μ L of dye (5 μ M) and 98 μ L of PBS-T.
4. Mix 90 μ L of peptide (200 nM) with 90 μ L of dye (100 nM).
5. Incubate for 30 minutes at room temperature.
6. Prepare 20 μ L of the 2 x highest ligand concentration used in your assay.
7. Transfer 10 μ L of PBS-T into the wells/PCR-tubes **2-16**.
8. Transfer 20 μ L of the ligand solution into the well/PCR-tube **1**.
9. Transfer 10 μ L of the ligand from well/PCR-tube **1** to well/PCR-tube **2** with a pipette and mix by pipetting up-and-down multiple times. Transfer 10 μ L to well/PCR-tube **3** and mix. Repeat the procedure for well/PCR-tube **4-16**. Discard the extra 10 μ L from well **16**.
10. Add 10 μ L labeled Control Peptide to each well (1-16) and mix by pipetting.
11. Load the capillaries and measure the samples at 40 % LED/excitation power and medium MST power of (40 % in NT.Control).
12. If a binding curve or a ligand-dependent fluorescence change is detected, the ligand likely interferes with the labeling. We recommend switching to a covalent the labeling strategy for lysine (Cat. # MO-L011) or cysteine (Cat. # MO-L014) residues.

4. *My protein requires a divalent cation or co-factor for proper 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 Table 1 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. *I would like to investigate protein-protein interactions. Both proteins have His-tags. How should I proceed?*

The labeling of the protein with the RED-tris-NTA dye is reversible. Although the off-rate is very slow, the dye can “jump” from one His-tagged protein to another. Therefore, we recommend using one binding partner without His-tag. Typically, His-tags can be removed enzymatically from the protein during protein purification if a selective protease recognition sequence (e.g., TEV) was incorporated next to the His-tag.

6. *The protein of interest is stored in a buffer which is not compatible with the RED-tris-NTA dye. What are the alternatives?*

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

7. *Can I store the RED-tris-NTA dye and Control Peptide solution?*

Yes, the solutions may be stored for about 8 weeks. We recommend freezing the solutions in 5-10 μL aliquots at $-20\text{ }^{\circ}\text{C}$.

8. *Can I use RED-tris-NTA labeled proteins at low nM or high pM concentrations, for example when using a Monolith NT.115^{Pico} instrument or when analyzing high affinity interactions (K_d in the pM or low nM range)?*

It depends on the affinity of the dye to the His-tagged protein of interest. Therefore, it is recommended to test this (see Step A) before performing the binding assay, and to use the same labeling conditions as stated in this manual (see Step B) before diluting the labeled target for the binding assay. The interaction of the dye with the His-tagged protein is expected to have a single digit nM affinity. In this case, target concentrations in the same range can be used for binding assays, with the LED/excitation power adjusted accordingly. However, keep in mind that the dye-protein interaction is reversible and may dissociate over time at low concentrations. When using a Monolith NT.115^{Pico}, we recommend final protein and dye concentrations of 50 nM and 10 nM respectively and an LED/excitation power setting of 10 %, which should yield a fluorescence intensity of around 12 000 counts.

9. *Can I use RED-tris-NTA dye to label unpurified His-tagged protein in cell lysates?*

Yes, labeling unpurified His-tagged protein with RED-tris-NTA dye 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 (Step A). 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.

SAFETY INFORMATION

DYE



Hazard statements

H315	Causes skin irritation.
H319	Causes serious eye irritation.
H335	May cause respiratory irritation.

Precautionary statements

P235	Keep cool.
P260	Do not breathe dust/ fume/ gas/ mist/ vapours/ spray.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P304+P312	IF INHALED: Call a POISON CENTER or doctor/ physician if you feel unwell.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

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

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