Large Volume His-Tag Labeling Kit RED-tris-NTA 2nd Generation

For Dianthus, Monolith NT.115 Series and NT.Automated Instruments with a RED Detector

Cat # NT-L118

Content And Storage

Large Volume His-Tag Labeling Kit RED-tris-NTA 2nd Generation is shipped at room temperature.
Each kit contains material sufficient for 3840 single-point TRIC/MST measurements.

4* 250 pmol RED-tris-NTA 2nd Generation dye **[store at -20 °C]**
2* 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
- 384 microwell plates or 0.2 mL PCR tubes
- Microwell plate sealing foil
- Buffer of choice (PBS-T recommended)

Protein Labeling Procedure

The Large Volume His-Tag Labeling Kit RED-tris-NTA 2nd Generation provides convenient means for the site-specific, purification-free labeling of small amounts of His-tagged proteins with our 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 3840 single-point TRIC/MST measurements. Labeling can be completed in 30 min, no removal of excess dye is required. The RED-tris-NTA 2nd Generation dye can bind efficiently to His-tags which contain at least six histidines with a K_d in the single digit nM range. By following this protocol, optimized for binding of RED-tris-NTA dye to His-tagged proteins eliminates an additional purification step and ensures the best results. RED-tris-NTA 2nd Generation 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 Dianthus NT.23 and Monolith NT.115, if you are using a Dianthus NT.23Pico or Monolith NT.115^{Pico} please refer to FAQ 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) supplemented with 0.005 % Tween 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

Component	*Maximum allowed concentration*
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+}, 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.

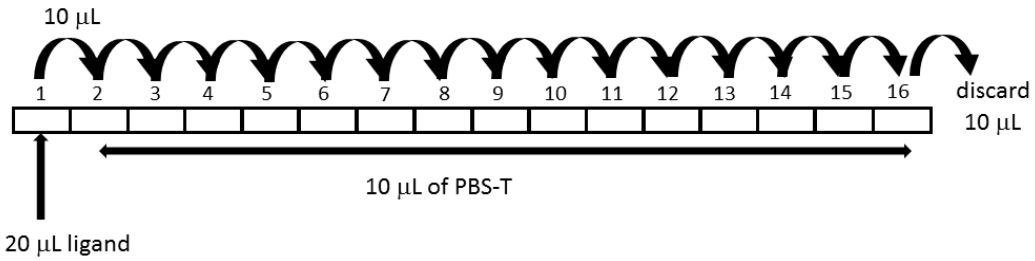
** Co^{2+}, Cu^{2+}, Zn^{2+} ions compete for the binding with RED-tris-NTA 2nd Generation dye. Because of that reason only low nanomolar concentrations of listed ions are tolerated.

STEP A: Affinity of Dye to Target Protein

What is the affinity and efficiency of the RED-tris-NTA 2nd Generation 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. PBS-T (0.005 % Tween) buffer can be replaced by a different assay buffer if required by the user.

1. Suspend one vial of dye in 100 µL of PBS-T to obtain a 2.5 µM dye solution.
2. Prepare 200 µL of 25 nM solution of the RED-tris-NTA 2nd Generation dye in PBS-T by mixing 2 µL of dye (2.5 µM) and 198 µL PBS-T.
3. Prepare 30 µL of 4 µM His-tagged protein in PBS-T.
4. Transfer 10 µL of PBS-T into wells/PCR-tubes **2-16**.
5. Transfer 20 µl of 4 µM His-tagged protein solution into the first well/PCR-tube.
6. 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 wells **4-16**. Discard the extra 10 µL from well/PCR-tube 16.



7. Add 10 µl of 25 nM RED-tris-NTA 2nd Generation dye solution to each well/PCR-tube (1-16) and mix by pipetting. If you are using a Dianthus, spin the microwell plate and then seal the plate with sealing foil.
8. Incubate for 30 min at room temperature.
9. Measure your samples in the Dianthus using auto-excitation or load the capillaries and measure the samples at 60 % LED/excitation power and medium MST power in the Monolith.
10. The K_d can be determined in DI.Control/MO.Control or DI.Screening Analysis/MO.Affinity Analysis using the K_d fit.

If the affinity of the RED-tris-NTA 2nd Generation 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 2nd Generation 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 2nd Generation 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. # NT-L111) or cysteine (Cat. # NT-L114) 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 ≤ 10 nM). The following protocol describes the labeling procedure for one experiment with 16 capillaries.* PBS-T (0.005 % Tween) buffer can be replaced by a different assay buffer if required by the user*. If Step A of the protocol was performed proceed directly to 2. Volumes can be scaled up- or down when needed.*

1. Suspend the dye in 100 µL of PBS-T to obtain 2.5 µM dye solution.
2. Prepare a 50 nM dye solution by mixing 2 µL of dye (2.5 µM) and 98 µL PBS-T.
3. Adjust the protein concentration to 200 nM in a volume of 100 µL.
4. Mix 90 µL of protein (200 nM) with 90 µL of dye (50 nM).
5. Incubate for 30 minutes at room temperature.
6. Centrifuge the sample for 10 min at 4 °C and 15 000 g and transfer the supernatant to a fresh tube.
7. The protein is labeled and ready for the binding assay.

II) *Affinity of RED-tris-NTA 2nd Generation 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 wells/capillaries.* PBS-T (0.005 % Tween) buffer can be replaced by a different assay buffer if required by the user. *Volumes can be scaled up- or down when needed.*

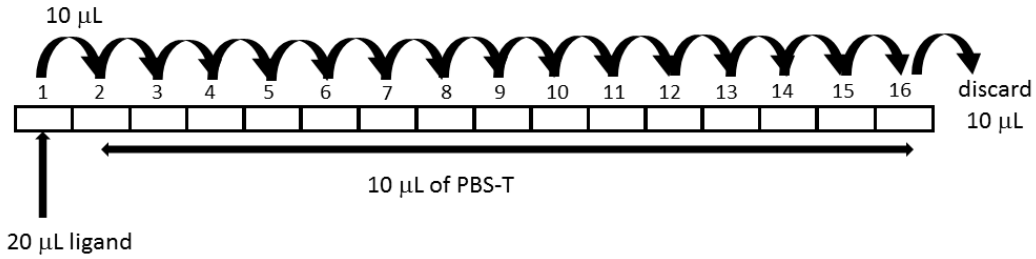
1. Suspend the dye in 100 µL of PBS-T to obtain 2.5 µM dye solution.
2. Prepare a 50 nM dye solution by mixing 2 µL of dye (2.5 µM) and 98 µL PBS-T.
3. Adjust the protein concentration to 20 times of the K_d measured in Step A in a volume of 100 µL (e.g. prepare 100 µL of 800 nM protein for a K_d of 40 nM between dye and protein. The final protein concentration in the assay will be 1/4 of this value = 200 nM)
4. Mix 90 µL of protein with 90 µL of dye (50 nM).
5. Incubate for 30 minutes at room temperature.
6. Centrifuge the sample for 10 min at 4 °C and 15 000 g and transfer the supernatant to a fresh tube.
7. 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 2nd Generation 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. # NT-L111) or cysteine (Cat. # NT-L114) 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. PBS-T (0.005 % Tween) buffer can be replaced by a different assay buffer if required by the user.

1. Prepare 25 µL of the ligand at 2 x concentration in PBS-T or your assay buffer of choice (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/PCR-tube (1-16) and mix by pipetting. The final target protein concentration in the assay is 50 nM (or higher, compare Step B II). This concentration should be used for the calculation of the K_d value. If you are using a Dianthus, spin the microwell plate and then seal the plate with sealing foil.
6. Measure your samples in the Dianthus using auto-excitation or load the capillaries and measure the samples at 60 % LED/excitation power and medium MST power in the Monolith. At the final dye concentration of 12.5 nM the expected fluorescence intensity at 60 % LED is around 250 counts on a Monolith NT.115.

FAQ

1. *The signal-to-noise ratio of my experiment 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 may be 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. *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 µL of PBS-T to obtain a concentration of 10 µM.
   2. Mix 2 µL of 10 µM Control Peptide with 98 µL of PBS-T.
   3. Prepare a 100 nM working dye solution by mixing 4 µL of dye (2.5 µM) and 96 µL of PBS-T.
   4. Mix 90 µL of peptide (200 nM) with 90 µL of dye (100 nM).
   5. Incubate for 30 minutes at room temperature.
   6. Prepare 25 µL of the 2 x highest ligand concentration used in your assay.
   7. Transfer 10 µL of PBS-T into the wells/PCR-tubes **2-16**.
   8. Transfer 20 µl of the ligand solution into the well/PCR-tube **1**.
   9. 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 **16**.
   10. Add 10 µl labeled Control Peptide to each well/PCR-tube (1-16) and mix by pipetting. If you are using a Dianthus, spin the microwell plate and then seal the plate with sealing foil.
   11. Measure your samples in the Dianthus using auto-excitation or load the capillaries and measure the samples at 60 % LED/excitation power and medium MST power in the Monolith.
   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 labeling strategy for lysine (Cat. # NT-L111) or cysteine (Cat. # NT-L114) residues.

3. *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.

4. *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 2nd Generation 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.

5. *The protein of interest is stored in a buffer which is not compatible with the RED-tris-NTA 2nd Generation dye. What are the alternatives?*

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

6. *Can I store the RED-tris-NTA 2nd Generation 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 °C.

7. *Can I use RED-tris-NTA 2nd Generation labeled proteins at low nM or high pM concentrations, for example 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, page 4) before performing the binding assay, and to use the same labeling conditions as stated in this manual (see Step B, page 5) 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 Dianthus NT.23 Pico turn off the Pico sensitivity for the measurement. Using a Monolith NT.115^{Pico}, we recommend an LED/excitation power setting of 10 %, which should yield a fluorescence intensity of around 12 000 counts.

8. *Can I use RED-tris-NTA 2nd Generation dye to label unpurified His-tagged protein in cell lysates?*

   Yes, labeling unpurified His-tagged protein with RED-tris-NTA 2nd Generation 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, page 4). 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

P264	Wash hands thoroughly after handling.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.
P302+P352	IF ON SKIN: Wash with plenty of water.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P337+P313	If eye irritation persists: Get medical advice/attention.

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

Contact

TECHNICAL SUPPORT

Please get in touch with us for specific questions concerning the product performance.

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