Cat# AN-030002

Andromeda His-Tag Labeling Kit

For site-specific, purification-free labeling of His-tagged proteins with RED-tris-NTA 2nd Generation dye
For use in Andromeda, sufficient for 5,000 single-point measurements

Content and Storage

Andromeda His-Tag Labeling Kit is shipped at room temperature.
Each kit contains material sufficient for 5,000 single-point measurements.

2* 125 pmol RED-tris-NTA 2nd Generation 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
- ddH_2O

Protein Labeling Procedure

The Andromeda His-Tag Labeling Kit provides convenient means for the site-specific, purification-free labeling of His-tagged proteins with our fluorescent RED-tris-NTA 2nd Generation dye. This kit can be used for the labeling of any protein carrying a polyhistidine-tag (His-Tag) and contains material sufficient for 5,000 single-point Andromeda 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 nearly 100 % binding of 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 Andromeda and Andromeda Plex instruments.

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 at a pH below 7, these conditions are not advised.

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+}, Ni^{2+}, Zn^{2+}	preloaded protein only**
Polyhistidine-tagged species	none
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+}, Ni^{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
Lysate Preparation

Lysates prepared from any organism (bacteria, yeast, insect or mammalian cells) can be used.

Perform lysis of cells expressing His-tagged protein using any method of your choice, e.g. sonication, homogenization, chemical or enzymatic lysis. Choose a lysis buffer that is compatible with protein labeling, e.g. PBS or HEPES buffer with a pH > 7.0 and a salt concentration of at least 150 mM. Please refer to Table 1 for a list of incompatible substances.

NOTE: Make sure to perform a centrifugation step to remove cell debris and protein aggregates (e.g. 10 minutes at 20,000 x g and 4 °C) before subjecting your samples to protein labeling (Step B).

NOTE: Make sure that your clarified cell extract does not contain any genomic DNA as this might interfere with protein labeling. Disrupt genomic DNA before protein labeling, e.g. by sonication or enzymatic treatment.

STEP B
Protein Labeling

The following protocol describes the labeling procedure for one experiment, with replacement of scientists' interaction buffer of choice for Phosphate-buffered Saline + 0.05 % Tween 20 (PBS-T) if choosing to use an alternate buffer system. Volumes can be scaled up- or down when needed. We recommend sample preparation in PCR tubes or in 96- or 384-well multi-well plates with non-binding surface.

1. Add 8.0 mL ddH2O to the vial containing 5 x PBS-T to obtain 1 x PBS-T.
2. Suspend the content of one vial of RED-tris-NTA 2nd Generation dye (125 pmol) in 50 µL of PBS-T to obtain a 2.5 µM dye solution.
3. Prepare a 25 nM dye solution by mixing 2 µL of dye (2.5 µM) and 198 µL PBS-T.
4. Predilute the lysate sample containing the His-tagged protein by mixing 10 µL of lysate and 90 µL PBS-T.
5. Mix 48 µL of prediluted lysate sample containing the His-tagged protein with 2 µL of dye (25 nM). Mix well by carefully pipetting the sample up and down 4-5 times (do not vortex!).
6. Incubate for 30 minutes at room temperature in the dark.
7. Mix all samples again by carefully pipetting each sample up and down 4-5 times.
8. The protein is now labeled and ready to be subjected to the thermal unfolding assay. The amount of each sample (50 µL) is sufficient to run four technical replicates.

STEP C
Thermal Unfolding Assay

Load the sample into capillaries and measure the samples in the RED channel. Recommended settings are 60 % Excitation Power. At the final dye concentration of 1 nM, the expected fluorescence intensity at 60 % Excitation Power is around 10,000 counts on Andromeda and Andromeda Plex.

FAQ

1. **Can I perform thermal shift assays in crude mixtures with Andromeda?**

   Yes, thermal shift assays to assess binding competency of the His-tagged protein target can be performed even in crude mixtures with Andromeda. Make sure to add ligand at a concentration that is sufficient to saturate the target protein, and to always include proper negative controls (e.g. lysate + ligand solvent) and avoid any buffer mismatches between samples.

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

   Yes, cofactors 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.

3. **The cells expressing my protein of interest were lysed in a buffer which is not compatible with the RED-tris-NTA 2nd Generation dye. What are the alternatives?**

   In this case a predilution of the lysate samples with a compatible buffer (e.g. PBS-T) is recommended. Please refer to Table 1 for a list of incompatible substances and their maximum allowed concentrations, and predilute the lysate samples accordingly. Alternatively, lyse your cells in a buffer that is compatible with the RED-tris-NTA 2^{nd} Generation dye.

4. **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 stock solutions in 5-10 µL aliquots at -20 °C.

5. **How can I be sure that I am really seeing my His-tagged target protein in the thermal unfolding assay?**

   Labeling His-tagged protein with RED-tris-NTA 2nd Generation dye is possible even in crude mixtures due to the dye's high affinity and specificity for His-tagged proteins. However, in complex environments like cell lysate, the use of appropriate controls is always advisable to control for unspecific interactions. It is recommended to always prepare and run a negative control sample alongside, e.g. cell lysates prepared from untransformed cells or cells harboring an empty plasmid backbone. If applicable, the purified His-tagged protein in buffer is an ideal positive control sample that can be run alongside.

Troubleshooting

Observation	Possible Reasons	Remedy
No unfolding detectable, or unfolding signal barely distinguishable from noise	His-tagged protein is not or very poorly expressed	Skip predilution of lysate sample (Step B3.)
	His-tagged protein is unfolded	Optimize protein expression conditions
	Suboptimal buffer conditions for protein labeling	Review assay buffer for incompatibilities with His-tag labeling (pH, EDTA, metal ions, etc.)
	His-tag is not well accessible	Perform control experiment with purified protein and RED-tris-NTA dye in lysis buffer
No thermal shift observable upon ligand addition	Target protein is binding-incompetent	Perform control experiment with purified protein and ligand in lysis buffer
	Ligand is being scavenged by other proteins in the sample	Increase ligand concentration

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.

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