Labeling Manual for Andromeda X His-Tag® Labeling Kit

Catalogue Number: AN-030002

For site-specific, purification-free labeling of His-tagged proteins with RED-tris-NTA 2nd Generation dye
For use in Andromeda X

Content and Storage

Andromeda X His-Tag Labeling Kit is shipped at room temperature.

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]**
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 up to 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.

Important Information Before Starting

The protocol describes our best labeling practice for the Andromeda X.

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) or secreted protein targets can be used.

1. Perform lysis of cells expressing His-tagged protein using any method of your choice, e.g. sonication, homogenization, chemical or enzymatic lysis. *For secreted protein targets proceed to the next step.* 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.

2. Centrifuge cell lysate to remove cell debris and protein aggregates for 10 minutes at 20,000 x g and 4 °C. Transfer the supernatant to a clean tube before proceeding to the labeling step (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 100 nM dye solution by mixing 2 µL of dye (2.5 µM) and 48 µL PBS-T. Store remaining dye solution.
4. Predilute lysate containing the His-tagged protein (sample prepared in Step A) by mixing 10 µL of the clear supernatant and 40 µL PBS-T.
5. Add 5 µL of the pre-diluted dye (100 nM) to 50 µL of prediluted lysate prepared in the previous step (clear supernatant). 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. Centrifuge the sample for 10 minutes at 20,000 x g and 4 °C.
8. The protein is now labeled and ready for the experiment.

STEP C
Thermal Unfolding Assay

Load the sample into capillaries (triplicates are recommended). Excitation power will be adjusted automatically while the heating ramp can be adjusted manually. The recommended starting heating rate is 7 °C/min which will result in a full measurement in less than 11 minutes.

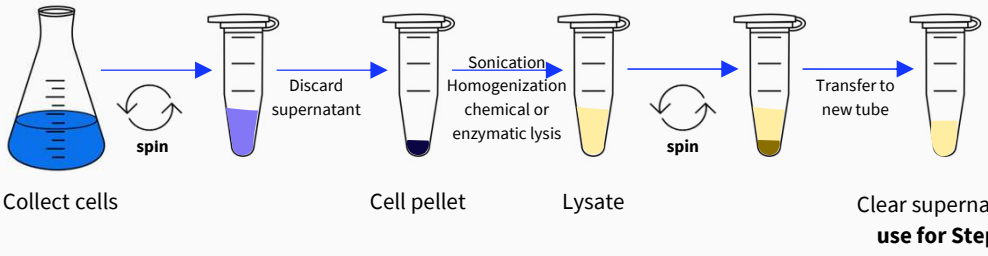
NOTE: We recommned including a negative control (untransfected lysate/cells with empty vector) that can help identify possible unspecific binding.

Quick sheet

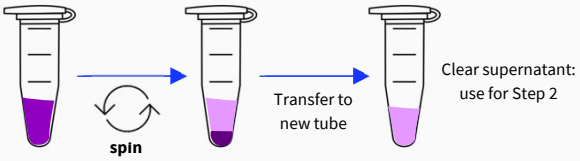
Labeling procedure for His-tagged® targets in Andromeda X

1. Collect & Lyse

Intracellular Target



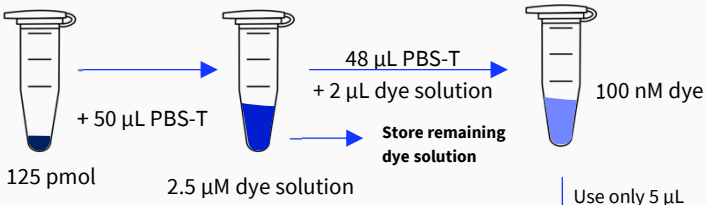
Secreted Target



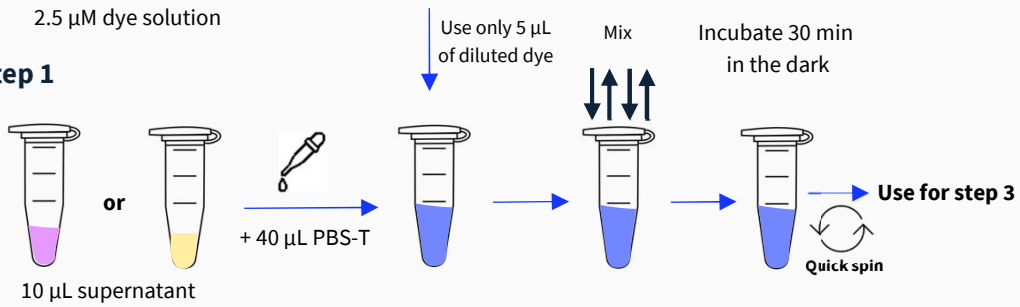
Useful Tips:
- Spin: at 20k x g for at least 10 min
- Use only clear supernatant for step 2. Re-spin if necessary.

2. Target Labeling

a. Prepare Tris-NTA 2nd Gen Dye



b. Label supernatant from Step 1



Useful Tips:
- Use interaction buffer of choice or Phosphate-buffered Saline + 0.05 % Tween™ (PBS-T)
- Depending on the concentration of the His-tagged target, a higher or lower pre-dilution of lysate is advisable. For very low expression, undiluted supernatant may be used

3. Measure

a. Load the sample into capillaries (triplicates are recommended)

b. The recommended starting heating rate is 7 °C/min. This result in a full measurement in less than 11 minutes. The heating rate can be adjusted manually if needed.

Useful Tip:
We recommend including a negative control (untransfected lysate/cells with empty vector)

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 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
	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
	Affinity of the ligand is too low to remain bound during thermal ramp	Increase ligand concentration, if applicable test different ligand

Contact

TECHNICAL SUPPORT

Please get in touch with us for specific questions concerning the product performance. Find all kinds of supporting material or submit a support case via our Support Center **support.nanotempertech.com**

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