Protocol

Jena Bioscience

Copper-free Click labeling of Azide-modified RNA

To be used with RNA probe synthesized with HighYield T7 Azide RNA Labeling Kit (#RNT-101-AZ)

1. Removal of free 5-Azido-C_3-UTP

Removal of free 5-Azido-C_3-UTP and unmodified nucleotides is required for accurate measurement of Azide-RNA probe concentration and subsequent copper free CLICK labeling reaction. We recommend spin column purification such as gel filtration or silica membrane-based approaches. In some cases (e.g after labeling with a bulky fluorescent dye), gel filtration leads to higher RNA probe recovery rates. Other RNA purification methods such as LiCl precipitation may work but have not been tested.

2. Copper-free CLICK labeling reaction

CLICK labeling efficiency depends on the molar ratio of DBCO-functionalized detection reagent to Azide-RNA probe. We recommend a 10-fold molar excess of DBCO-containing detection reagent as a starting point however, individual optimization might be required.

2.1 Calculation of molar amount of Azide-RNA probe and DBCO-functionalized detection

- Determine the Azide-RNA probe concentration **c_{RNA}** [ng/µl] by absorbance measurement at 260 nm.
- Calculate the total molar amount of Azide- RNA probe n_{RNA} [nmol] using the following equations:

  MW_{RNA} (g/mol) = 340 g/mol x bp

  bp = number of basepairs of your RNA template

  340 g/mol = average mass of RNA base

  n_{RNA} [nmol] = (c_{RNA} [ng/µl] x V_{RNA} [µl])/ MW_{RNA} [g/mol]

  V_{RNA} [µl] = total volume of Azide-RNA sample
- Calculate the total amount of possible Azide modifications n_{Azide} [nmol] in the sample assuming an equal distribution of all four bases and 100% substitution efficiency:

  n_{Azide} [nmol] = n_{RNA} [nmol] x bp /4
- Calculate the total molar amount of DBCO-containing detection reagent n_{DBCO} [nmol] (start with 10-fold molar excess (k = 10):

  n_{DBCO} [nmol] = n_{Azide} [nmol] x k

  k= desired molar excess of DBCO-containing detection molecule e.g. 10
- Calculate the required volume of DBCO-containing detection reagent solution (10 mM)

  V_{DBCO} [µl] = n_{Dye} [nmol] / c_{Dye} [mM] , c_{Dye} = 10 mM

***Example**: 25 µl of a 1423 bp Azide-RNA probe (c_{RNA} = 60 ng/µl) correspond to 0.0031 nmol of RNA (n_{RNA}) and contain 1.1 nmol of Azide groups (n_{Azide}). If labeled with a 10-fold molar access (k=10), 11 nmol of DBCO-containing detection reagent is required. This corresponds to 25 µl of Azide-RNA probe (V_{RNA}) and 1.1 µl of 10 mM DBCO-containing detection reagent (V_{DBCO}).*

2.2 Set-up of copper-free CLICK labeling reaction

- Add calculated volumes of Azide-RNA probe (V_{RNA}) and DBCO-containing detection reagent (V_{DBCO}) to a sterile, RNAse-free tube.
- Adjust to a total volume of 30-40 µl with RNAse-free 1x PBS e.g. pH 7.6 or 100 mM phosphate buffer pH 8. Ensure a final pH of the reaction mixture of 7 – 8.5 and keep total reaction volume as low as possible.
- Mix thoroughly by vortexing, spin down briefly and incubate for 1h at 37°C **in the dark**. Prolonged incubation at reduced temperature (e.g. 6h at 26°C) may increase labeling efficiency in some cases.
- Remove unreacted DBCO-containing detection reagents for an accurate measurement of RNA probe labeling degree (see 1. & 3.).

3. Degree of Labeling (DOL) calculation

(for fluorescent RNA probes only)

RNA probe labeling efficiency can be estimated by calculating the ratio of incorporated fluorophores to the number of bases (dye / base). Multiplication of this ratio with 100 leads to the Degree of Labeling (DOL) that indicates the number of dyes per 100 bp of RNA probe.

6.1. Measurement of RNA-Dye conjugate absorbance

Measure absorbance of the purified labeled RNA probe at 260 nm (A_{260}) and at the excitation maximum (λ_{Ex}) of attached fluorescent dye (A_{dye}).

6.2. Correction of A_{260} reading:

To obtain an accurate absorbance measurement for the nucleic acid, the contribution of the dye at 260 nm has to be corrected. Use the following equation:

$$A_{base} = A_{260} - (A_{dye} \times CF_{260})$$

6.3. Calculation of dye to base ratio by the law of Lambert-Beer (A = c x ε x d)

dye/base ratio = (A_{dye} x ε_{base}) / (A_{base} x ε_{dye})

RNA: ε_{base} = 8250 cm^{-1} M^{-1}