Data Sheet

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Jena Bioscience

Azide RNA T7 Transcription Kit

Azide functionalization of RNA by T7 RNA Polymerase-mediated *in vitro* Transcription

Cat. No.	Size
PP-501	10 reactions à 40 µl

For *in vitro* use only
Quality guaranteed for 12 months
Store all components at -20°C. Avoid freeze and thaw cycles.

Kit contents

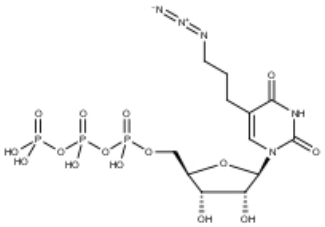
T7 RNA Polymerase (red cap)
5 µl, 200 units/µl
T7 Reaction Buffer (green cap)
100 µl, 5x conc.
A/G/CTP Mix (white cap)
150 µl, 10 mM each ATP, GTP, CTP
UTP solution (white cap)
50 µl, 10 mM
5-Azido-UTP-solution (blue cap)
20 µl, 10 mM (aqueous solution, pH 8.3)
T7 Control Template (purple cap)
10 µl, 200 ng/µl (1423 bp including T7 promoter sequence)
RNase Inhibitor (yellow cap)
12 µl 40 units/µl RNase inhibitor in storage buffer with 50% glycerol (v/v)
RNase free water (white cap)
1 ml, pH 7.5

To be provided by user

Primer and Template
RNA purification tools
DMSO
RNAse free DNAse (optional)
RNAse free PBS, pH 7.6
DBCO-modified detection molecule (Fluorescent Dye or Biotin); for an overview on available DBCO-products refer to: www.jenabioscience.com/click

1. Description

The Azide RNA T7 Transcription Kit enables the efficient preparation of Azide-labeled RNA probes (up to 1400 bp tested) by enzymatic incorporation of a novel Azide-modified UTP analog (Fig. 1) via T7 RNA polymerase–mediated *in vitro* transcription (Fig. 2).



Molecular Weight:
MW = 567 g/mol
Extinction coefficient:
ε_{max} = 8.8 l mmol^{-1} cm^{-1}

Figure 1 Structure and spectroscopic properties of 5-Azido-UTP.

Once purified, the Azide-RNA can be labeled in a second step by a straightforward Copper-free Click Chemistry procedure with **DBCO-containing detection molecules** (e.g. fluorescent dyes or biotin, **not included**) thereby forming a stable, covalent triazole bond (Fig. 2)

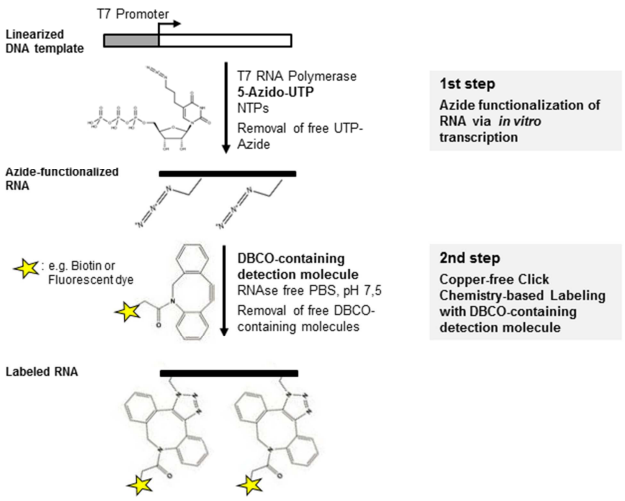


Figure 2 Workflow of 5-Azido-UTP–based RNA modification followed by Copper-free Click Chemistry-based labelling.

Applications:

Azide-labeled RNA probes are suitable for subsequent labelling with DBCO-containing molecules (Copper-free Click Chemistry) or Alkyne-containing molecules (Cu(I)-catalyzed Click Chemistry)[1,2].

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2. Important Notes (Read before starting!)

2.1 Avoiding RNAse Contamination

Although a potent RNase Inhibitor is included during the reaction procedure, creating an RNAse-free work environment and maintaining RNAse-free solutions is critical for performing successful *in vitro* transcription reactions. Therefore, we recommend

a) to perform the all reactions in sterile, RNAse-free tubes using sterile pipette tips.

b) to wear gloves when handling samples containing RNA.

c) to keep all components tightly sealed both during storage and the reaction procedure.

2.2 Requirements for Template DNA

a) Type of template: Any DNA (e.g. linearized plasmid DNA, PCR products) containing a double-stranded T7 promoter region upstream of the target sequence can be used as template for T7 RNA polymerase.

Minimum promoter sequence for efficient transcription:

5'-TAATACGACTCACTATA**GGGAGA**...-3'

↳ Start of transcription

b) Quality of template: The DNA template quality directly influences the yield and quality of the transcription reaction. Generally, a DNA preparation is of sufficient quality if it is RNAse-free and fully digestible with restriction enzymes. If necessary, DNA quality might be improved when treated as follows:

1) Add Proteinase K and SDS with a final concentration of 100-200 µg/µl and 0.5% , respectively and incubate for 30-60 minutes at 37°C.
2) Extract the DNA template by adding an equal volume of a 1:1 mixture of TE-saturated phenol/chloroform
3) Precipitate with Ethanol
4) Remove the supernatant and wash the pellet with 70% Ethanol
5) Resuspend the pellet in RNAse-free 10 mM Tris-HCL pH 7.5, 1mM EDTA

3. *In vitro* Transcription reaction with 5-Azido-UTP

3.1 General Remarks

a) The standard reaction procedure contains 0.4 pmol (200 ng) of linearized 1423 bp-DNA control template. The optimal template amount may need to be individually adjusted.

b) Using the linearized 1423 bp-DNA control template, an optimal balance between reaction efficiency (30%) and labelling efficiency (2-3 dyes per 100 bp, determined with #CLK-A140) is achieved with 25% 5-Azido-UTP substitution. Since reaction and labelling efficiency are strongly template dependent (quality, size, sequence), we strongly recommend to determine the optimal 5-Azido-UTP substitution rate for every template (between 25 - 50%). An 5-Azido-UTP substitution > 50% leads to poor yields. The kit contains sufficient reagents for 10 labeling reactions à 40 µl with 50% 5-Azido-UTP substitution.

d) A control reaction with 0% 5-Azido-UTP should always be performed in parallel.

3.2 General protocol

(Linearized control template & 50% 5-Azido-UTP-substitution)

a) Place the T7 RNA Polymerase on ice and thaw all remaining components at room temperature (RT).

b) Add all components at RT to a nuclease-free microtube (sterile pipette tips) in the following order:

Component	stock conc.	final conc.	1 assay
RNAse-free water, pH 7.5	-	-	X µl
Linearized template DNA with T7 promoter	200 ng/µl (control template)	0.4 pmol (200 ng)	1 µl
T7 Reaction Buffer	5x	1x	8 µl
A/G/CTP Mix	10 mM each	1 mM each	12 µl
UTP	10 mM	0.5 mM	2 µl
5-Azido-UTPsolution(50%)	10 mM	0.5 mM	2 µl
RNase Inhibitor	40 units/µl	1 unit/µl	1 µl
T7 RNA Polymerase	200 units/µl	2.5 units/µl	0.5 µl
Total reaction volume			40 µl

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c) Mix thoroughly by vortexing, spin briefly to collect all drops and **incubate in the dark** at 37°C for 1 h.

3.3 DNAse treatment (optional)

Depending on your down-stream application, removal of the template DNA might be required.

a) Add 1 U/µl RNase-free DNase to the reaction mixture and incubate for 15 min at 37°C to remove template DNA.

b) Add 1 µl 0.5 M EDTA to stop the reaction.

4. Removal of free 5-Azido-UTP

The removal of free 5-Azido-UTP and other nucleotides is necessary for accurate measurements of the Azide-RNA concentration and the subsequent labeling reaction.

We recommend using a gel filtration approach that combines efficient removal of labeled nucleotides and high probe recovery rates. Other RNA purification methods such as LiCl precipitation may work, but have not been tested.
(E.g. Nucleotide/Dye Removal Kit, Cat.-No. PP-216 according to protocol, exception: 1 column / 40 µl reaction)

Please note: The purification by gel filtration does not result in buffer exchange. While nucleotides are efficiently removed, other reaction mixture components e.g. initial target DNA or enzymes are still present. They do not affect the labeling procedure but the absorption measurement at 260 nm. Therefore, prepare a sample of gel filtration purified reaction buffer (incl. template DNA, A/G/CTP-Mix, RNase Inhibitor and T7 RNA Polymerase) in parallel for subsequent blanc correction and correct determination of the Azide-RNA concentration (6.1).

The reagents for Step 5 – 8 are not provided with this kit.

5. Labeling with DBCO-containing detection molecule (Copper-free)

Prior to the labeling reaction, the required amount of DBCO-containing detection molecule for a given amount of Azide-RNA needs to be determined. The labeling efficiency can be influenced by DBCO-containing-molecule/Azide-RNA ratio that has to be adjusted for every new Azide-RNA template. We recommend starting with a 10-fold molar excess of DBCO-containing molecule.

5.1. Calculation of molar amounts of Azide-RNA and DBCO-containing molecule

a) Determine the Azide-RNA concentration $\mathbf{c_{RNA}}$ [ng/µl] by absorbance measurement at 260 nm.

Please note: *Blanc correction with probe buffer solution is required for gel filtration purified Azide-RNA (refer to 5.).*

b) Calculate the total molar amount of Azide- RNA template n_{RNA} [nmol] using the following equations:

$$MW_{RNA}\ (g/mol) = 340\ g/mol \times bp$$

bp = number of basepairs of your RNA template
340 g/mol = average mass of RNA base

$$\mathbf{n_{RNA}}\ [nmol] = (\ c_{RNA}\ [ng/µl] \times V_{RNA}\ [µl])/\ MW_{RNA}\ [g/mol]$$

$\mathbf{V_{RNA}}$ [µl] = total volume of Azide-RNA sample

c) 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:

$$\mathbf{n_{Azide}}\ [nmol] = n_{RNA}\ [nmol] \times bp\ /4$$

d) Calculate the total amount of DBCO-containing molecule n_{DBCO} [nmol] for the labeling reaction (start with 10-fold molar dye excess (k = 10):

$$\mathbf{n_{DBCO}}\ [nmol] = n_{Azide}\ [nmol] \times k$$

k= desired excess of DBCO-containing molecule e.g. 10

e) Calculate the required volume of DBCO-containing molecule solution (10 mM)

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

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***Example**: 25 µl of a 1423 bp Azide-RNA fragment (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 dye access (k=10), 11 nmol of DBCO-containing molecule are required. This results in 25 µl of Azid-RNA fragment (V_{RNA}) and 1.1 µl of a 10 mM DBCO-containing molecule solution for the Labeling reaction.*

5.2. Set-up of Copper-free Click Chemistry-based Labeling Reaction

a) Add the calculated volumes of Azide-RNA (V_{RNA}) and DBCO-containing molecule solution (V_{DBCO}) to a RNAse-free tube and adjust to a total volume of 30-40 µl with RNAse-free 1x PBS, pH 7.5 (black cap). The total reaction volume should be kept as low as possible to enhance the labeling reaction.

b) Mix thoroughly by vortexing, spin briefly to collect all drops and place the vial in a thermomixer. **Gently shake** at 37°C for 1 h **in the dark**.

6. Removal of unbound excess DBCO-containing molecule

Several downstream applications do not require probe purification however, for accurate measurements of the RNA Labeling efficiency free DBCO-containing molecules need to be removed from the reaction mixture.

We recommend using a gel filtration approach (e.g. Nucleotide/Dye Removal Kit, Cat.-No. PP-216). Please refer to 5. for further information.

7. Calculation of the Degree of Labeling (DOL) (for fluorescent dye labeled RNA only)

The efficiency of RNA labeling can be estimated by calculating the ratio of incorporated fluorophores to the number of bases in the RNA fragment (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.

***Please note:** Blanc correction with probe buffer solution (see 5) is required for gel-filtration purified labeled-RNA.*

7.1. Measurement of the RNA-Dye conjugate absorbance

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

7.2. Correction of the A_{260} reading:

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

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

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

$$\text{dye/base ratio} = (A_{dye} \times \varepsilon_{base}) / (A_{base} \times \varepsilon_{dye})$$

RNA: $\varepsilon_{base} = 8250\ cm^{-1}\ M^{-1}$

***Please note:** The path length (d = 1 cm) has been canceled out of the equation since the calculation is a ratio.*

7.4. Calculation of the degree of labelling (DOL)

The degree of labelling (DOL) indicates the number of dyes per 100 bases.

DOL = 100 x dye/base ratio

Example: *A dye/base ratio of 0.02 corresponds to a DOL of 2 that corresponds to 2 dyes per 100 bases of a RNA fragment. Therefore, a 1400 bp RNA fragment contains 28 dye molecules.*

8. Labeling with Alkyne-containing detection molecule (Cu(I)- catalyzed)

Please refer to reference [1] and [2].

Selected References

[1] Rao *et al.* (2012) Enzymatic incorporation of an azide-modified analog into oligoribonucleotides for post-transcriptional chemical functionalization. *Nature Protocols.* **7(6)**:1097

[2] Rao *et al.* (2012) Posttranscriptional chemical functionalization of azide - modified oligoribo-nucleotides by bioorthogonal click and Staudinger reactions. *Chem. Comm.* **48**:498.