

# Site-specific terminal and internal labeling of RNA by poly(A) polymerase tailing and copper-catalyzed or copper-free strain-promoted click chemistry

Marie-Luise Winz, Ayan Samanta, Dirk Benzinger and Andres Jäschke\*

Institute of Pharmacy and Molecular Biotechnology, Heidelberg University, Im Neuenheimer Feld 364, Heidelberg 69120, Germany

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## ABSTRACT

The modification of RNA with fluorophores, affinity tags and reactive moieties is of enormous utility for studying RNA localization, structure and dynamics as well as diverse biological phenomena involving RNA as an interacting partner. Here we report a labeling approach in which the RNA of interest—of either synthetic or biological origin—is modified at its 3'-end by a poly(A) polymerase with an azido-derivatized nucleotide. The azide is later on conjugated via copper-catalyzed or strain-promoted azide-alkyne click reaction. Under optimized conditions, a single modified nucleotide of choice (A, C, G, U) containing an azide at the 2'-position can be incorporated site-specifically. We have identified ligases that tolerate the presence of a 2'-azido group at the ligation site. This azide is subsequently reacted with a fluorophore alkyne. With this stepwise approach, we are able to achieve site-specific, internal backbone-labeling of *de novo* synthesized RNA molecules.

## INTRODUCTION

In the post-genomic era, RNA has become one of the most interesting objects of investigation in diverse fields of research. Chemical RNA modification is often indispensable, not only for visualization (1), studies of structure and dynamics (2,3), purification (4), immobilization (5), or cross-linking of RNA to its biomolecular interaction partners (6,7), but also for modulation of its properties (8).

Certain modifications can be incorporated during chemical oligonucleotide synthesis (9,10), limited to shorter sequences that can be connected by enzymatic

ligation to obtain longer ones. During *in vitro* RNA synthesis (e.g. by T7 RNA polymerase—T7 RNAP), it is possible to incorporate 5'-terminal modifications (11,12) and internal modifications, which are site-specific only when non-natural base pairs are used (13). Evidently, co-synthetic methods are unusable for RNA isolated from living organisms, where post-synthetic modification can be a valuable alternative. For natural and *in vitro* synthesized RNA, the 5'-terminus is often blocked by mono- and triphosphates, or cap structures. Thus, the 3'-terminus can be labeled more easily. Besides the chemical modification of the 3'-terminal vicinal diol (14,15), various enzymatic methods employing ligases (16–18) or nucleotidyl transferases, like terminal deoxynucleotidyl transferase (TdT) (19) or poly(A) polymerase (PAP) (20) have been reported. For post-synthetic, internal labeling of RNA, DNAzymes (21), twin ribozymes (22) or hybridization-based functionality transfer reactions (23) can be used, all of which rely on chemically synthesized, modified oligonucleotides. Although the conversion of terminal modifications to internal ones by ligases or nucleotidyl transferases has been reported (7), this approach is limited to the introduction of small modifications. Bulky modifications, such as fluorophores have not been introduced using these methods, presumably due to the severe sterical hindrance imposed by the modification. On the one hand, this sterical hindrance hampers the enzymatic introduction of the modified nucleotide, e.g. PAP is efficient only with certain modified NTPs (20). On the other hand, it also inhibits further enzymatic manipulations involving the modified end. One solution to circumvent both problems is to employ a modified nucleotide that does not contain the label itself, but rather provides a small reactive handle for a subsequent chemical reaction, in which the desired label can be attached to the RNA of interest. Here, the copper-catalyzed click reaction, or copper-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) (24,25) is of particular interest. In this reaction, which has been used to label DNA

\*To whom correspondence should be addressed. Tel: +49 6221 54 4851, Fax: +49 6221 54 6430; Email: jaeschke@uni-hd.de

since 2003 (26–28), and more recently also to label RNA (6,29–33), the reactive groups (terminal alkynes and azides) are relatively small. Thus, they cause only minor structural changes in nucleic acids and their building blocks, so that enzymes are more likely to accept them as substrates. Indeed, for CuAAC labeling, enzymatic incorporation of alkyne-modified nucleotides into DNA *in vitro* (34–37), and of pyrimidines bearing an ethynyl modification at position C-5 into nucleic acids *in vivo* (29,38,39) are possible. Post-synthetic approaches, using methyltransferases to transfer alkyne-modified adenosine to DNA (40), or an extended alkynyl moiety to tRNA (30) have equally been reported. However, it can be advantageous to incorporate the azide instead of its alkyne counterpart, since azides have also been shown to participate in other, metal-free, click reactions, such as the strain promoted azide–alkyne cycloaddition (SPAAC) (41). While the introduction of alkyne groups into DNA (42–45) and RNA (31) has been achieved by standard phosphoramidite chemistry, the azido-group is chemically unstable under phosphoramidite coupling conditions (46). This has so far prevented the extensive exploitation of azides for both, DNA and RNA labeling. In contrast, enzymes have been successfully applied for providing nucleic acids with azides for further chemical conversion (32,47,48). For RNA, the incorporation of N<sub>3</sub>-modified nucleotides by a mutated T7 RNAP has been reported before the advent of CuAAC (48). In a recent study, an N<sub>3</sub>-modified initiator nucleotide has been incorporated at the 5'-terminus of RNA by T7 RNAP, for further conversion by CuAAC (32). In the same study, 3'-N<sub>3</sub>-2',3'-ddATP was added to the 3'-terminus of RNA by *Escherichia coli* PAP, followed by CuAAC, which does, however, not permit further enzymatic 3'-modifications due to the missing 3'-hydroxyl group.

Here, we set out to expand the scope of RNA click labeling. For 3'-terminal RNA labeling, we tested four different nucleotidyl transferases [yeast and *E. coli* PAP, Cid 1 poly(U) polymerase (PUP) (49) and TdT] for their ability to incorporate a set of modified NTPs bearing azides at different positions. We developed a toolbox for reliable labeling of RNA with fluorophores and other relevant functional tags via copper-catalyzed (CuAAC) or metal-free strain-promoted (SPAAC) click chemistry for all four possible nucleotides (A, C, G and U), at a wide range of concentrations. Moreover, after introduction of nucleotides modified at position C-8 or C-2', the resulting 3'-termini contain free hydroxyl groups. Those

RNAs could be subjected to enzymatic manipulations involving their modified 3' termini, namely, addition of poly(A)-tails, 3'-adapter ligation and splinted ligation to other RNA sequences. Doing so, we converted the terminal azide modifications into internal ones, for which we optimized CuAAC reaction conditions, to achieve site-specific, internal RNA labeling of *de novo* synthesized RNA. This protocol does not demand either *de novo* synthesis of modified entities or characterization and manipulation of new enzymes, and involves only commercially available reagents and enzymes, making it more accessible to future users.

## MATERIALS AND METHODS

### Materials

RNA1 and all DNAs (Table 1) were purchased from IBA. RNA2 and RNA3 were kindly provided by Prof. Mark Helm (Institute of Pharmacy and Biochemistry, University of Mainz). RNA4 was purchased from Dharmacon. Modified NTPs were purchased either from BIOLOG, IBA or tebu-bio (Supplementary Table S1). Yeast PAP was purchased from USB. *Escherichia coli* PAP, Cid1 PUP and RNA ligase 2, truncated (RNL2, tr.) were purchased from New England Biolabs. T4 polynucleotide kinase (PNK), TdT, T4 DNA ligase (DNL), T4 RNA ligase (RNL1) and DNase I were purchased from Fermentas. T4 RNA ligase 2 (RNL2) was prepared in-house. Alexa Fluor 647 alkyne, Alexa Fluor 488 alkyne and biotin-alkyne were purchased from Invitrogen. Aza-dibenzocyclooctyne (DIBAC) Fluor 488 (sold as Dibenzylcyclooctyne-Fluor 488) was purchased from Jena Bioscience. Tris-(3-hydroxypropyl)triazolylmethylamine (THPTA) was synthesized according to the published procedure (50). *Escherichia coli* cells of strain JM 109 were purchased from Promega.

### Preparation of 5'-radioactively labeled oligonucleotides

For 5'-labeling, RNA and [ $\gamma$ -<sup>32</sup>P]-ATP (10  $\mu$ Ci/ $\mu$ l, 3000 Ci/mmol; Hartmann Analytic), (end concentration: both 1  $\mu$ M) were incubated with PNK (1 U/ $\mu$ l) in 1 $\times$  T4 PNK buffer A (50 mM Tris-HCl (pH 7.6 at 25°C), 10 mM MgCl<sub>2</sub>, 5 mM DTT, 100  $\mu$ M spermidine) for 60 min at 37°C. Gel-purified oligonucleotides were recovered by precipitation with ethanol (RNA1) or isopropanol (RNA2, RNA3). To image radioactive bands, we exposed storage

**Table 1.** Sequences of oligonucleotides

Name	Sequence	Features
RNA1	5'-GUGACCGCGGAUCGACUUCACCGCGCAGUG-3'	10 nt loop, 5 nt dangling ends
RNA2	5'-GCAAGCUGACCCUGAAGUUCAU-3'	siRNA (sense)
RNA3	5'-GAACUUCAGGGUCAGCUUGCCG-3'	siRNA (antisense)
RNA4	5'-pGCCCCGACGAUGCUUCAGCAACCAGUGUAAUGGCG-3'	Ligation fragment <sup>a</sup>
DNA1	5'-CGCCATTACTGGTTGCTGAAGCATCGTCGGGCCACTGCGCGGTGAGT CGATCCGCGGTCCAC-3'	DNA splint <sup>a</sup>
DNA2	3'-Ap-5'-5'-pCTGTAGGCACCATCAAT-3'-block	Adapter: 3'-block, 5' adenylated
DNA3	5'-CGCCATTACTGGTTGCTGAAGCATCGTCGGGTGAAGTCGATCCGCGGTCCAC-3'	Helper DNA, assisting CuAAC

<sup>a</sup>For splinted ligation of RNA1 (+G) to RNA4.

phosphor screens (GE Healthcare) to the gels and scanned them with a Typhoon 9400 scanner (GE Healthcare). If not stated otherwise, whenever quantifications are given, radioactive bands were background-corrected (to the object average of a region in the gel not containing any radioactive bands), and quantified using ImageQuant software (Molecular Dynamics; version 5.2).

### Screening and optimization of enzymatic incorporation of azide-containing nucleotides to the 3' terminus of RNA oligonucleotides

To enable visualization of RNA after analysis by denaturing sequencing PAGE (seqPAGE), non-labeled RNA was generally doped with the respective 5'-radiolabeled RNA (final concentrations: 1–20 nM). For initial tests, 5 pmol of RNA1 was incubated with the respective N<sub>3</sub>-NTP (500 μM) and the respective nucleotidyl transferase in its recommended buffer at 37°C for 60 min (both PAPs and Cid1 PUP) or 90 min (TdT). Amounts of nucleotidyl transferase and buffer used were: 600 U of yeast PAP in 1× USB yeast poly(A) polymerase buffer [20 mM Tris-HCl (pH 7.0 at 25°C), 0.6 mM MnCl<sub>2</sub>, 0.02 mM EDTA, 100 μg/ml acetylated BSA, 10% glycerol] in a final volume of 25 μl; 5 U of *E. coli* PAP in 1× NEB *E. coli* poly(A) polymerase buffer [50 mM Tris-HCl (pH 7.9 at 25°C), 250 mM NaCl, 10 mM MgCl<sub>2</sub>] in a final volume of 20 μl; 2 U of Cid1 PUP in 1× NEBuffer 2 [10 mM Tris-HCl (pH 7.9 at 25°C), 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT] in a final volume of 25 μl; 20 U of TdT in 1× Fermentas terminal deoxynucleotidyl transferase buffer [25 mM Tris (pH 7.2 at 25°C), 200 mM potassium cacodylate, 0.01% (v/v) Triton X-100, 1 mM CoCl<sub>2</sub>] in a final volume of 20 μl. Reaction mixtures were optionally stored at –20°C until analysis by 15 or 18% seqPAGE. For unit definitions, see Supplementary Methods.

For N<sub>3</sub>-NTPs modified at positions C-2' and C-8, in combination with yeast and *E. coli* PAP, RNA concentrations and incubation times were varied, as indicated. Buffer conditions were maintained, if not stated otherwise. For the 2'-N<sub>3</sub>-NTPs (except for the very initial study), the PAP was heat-inactivated by incubation at 65°C for 10 min (yeast) or 20 min (*E. coli*). For 2'-N<sub>3</sub>-2'-dATP, in combination with yeast PAP, lower NTP concentrations (50 μM) were also employed, whereas with *E. coli* PAP, double enzyme concentration (0.5 U/μl) and addition of MnCl<sub>2</sub> (2.5 mM) were tested at 500 μM NTP concentration.

### Modification of RNA with single 2'-N<sub>3</sub>-nucleotides for CuAAC

The exact reaction conditions used for single nucleotide incorporation at 4 μM or 5 μM RNA concentration, employing yeast PAP or *E. coli* PAP, respectively, are summarized in Table 2.

### CuAAC with RNA after PAP reaction with N<sub>3</sub>-modified NTPs

PAP reaction mixtures were purified either by ethanol precipitation (RNA1), isopropanol precipitation (RNA2,

**Table 2.** Conditions that lead to single nucleotide addition

Modified NTP	PAP from	Reaction time
2'-N <sub>3</sub> -2'-dATP	<i>Escherichia coli</i> (0.5 U/μl)	16 h (–MnCl <sub>2</sub> )
	<i>Escherichia coli</i> (0.25 U/μl)	20 min (+MnCl <sub>2</sub> ) <sup>a</sup>
2'-N <sub>3</sub> -2'-dCTP	Yeast	20 min
2'-N <sub>3</sub> -2'-dGTP	Yeast	2 h
2'-N <sub>3</sub> -2'-dUTP	Yeast	5 min
3'-N <sub>3</sub> -2',3'-ddATP	Yeast	Not optimized
3'-N <sub>3</sub> -2',3'-ddTTP	Yeast	Not optimized
8-N <sub>3</sub> -ATP	Not possible	–

General conditions: 5 μM RNA, 0.25 or 0.5 U/μl PAP (*E. coli*) or 4 μM RNA, 24 U/μl PAP (yeast), commercial buffers (additives as indicated), 500 μM NTP, 37°C. Samples were heat inactivated by incubation at 65°C for 10 min (yeast PAP) or 20 min (*E. coli* PAP).

<sup>a</sup>Preferred conditions (2.5 mM MnCl<sub>2</sub>).

RNA3), by size exclusion chromatography employing MicroSpin™ G-25 columns (GE Healthcare) or by QIAquick Nucleotide Removal Kit (Qiagen).

The CuAAC was generally performed with an RNA concentration of 50 nM, 1 μM or 10 μM in 50 mM phosphate buffer (pH 7 at 25°C), 100 μM CuSO<sub>4</sub>, 500 μM THPTA, 1 mM sodium ascorbate [for *in situ* generation of Cu(I)] in a final volume of 10–100 μl, and varying concentrations of either Alexa Fluor 488 alkyne, Alexa Fluor 647 alkyne or biotin alkyne. Reactions were performed for 2 h at 37°C. At RNA concentrations above 10 μM (e.g. 50 μM), the concentrations of CuSO<sub>4</sub>, THPTA and sodium ascorbate were increased 5-fold.

If not stated otherwise, reaction mixtures were purified by phenol extraction (only for Alexa Fluor alkynes) in presence of EDTA (10 mM) and Na-acetate (0.3–0.6 M, pH 5.5), followed by ethanol or isopropanol precipitation, and then analyzed by 12 or 15% seqPAGE. Bands were visualized by Storage Phosphor Screen scan, as well as fluorescence scan on the Typhoon 9400 (Alexa Fluor 488: ex: 488 nm, em: 520 nm BP 40; Alexa Fluor 647: ex: 633 nm, em: 670 nm BP 30). To compare the different scans, gels were registered using the ImageJ plugin Turboreg (51). For monitoring biotinylation by streptavidin gel shift, the oligonucleotides were incubated at room temperature with a streptavidin solution (Thermo Scientific) of at least 5-fold molar excess for 5 min prior to gel loading.

### Fluorescent labeling of N<sub>3</sub>-modified RNA by SPAAC

RNA 1 (doped with radioactively labeled RNA 1) was labeled with a single N<sub>3</sub>-A, -C, -G and -U, using the reaction conditions described in Table 2 and was purified by ethanol precipitation. N<sub>3</sub>-modified RNA (1 μM) was reacted with DIBAC Fluor 488 (50 μM) in 50 mM phosphate buffer (pH 7 at 25°C) for 2 h at 37°C. The reacted RNA was purified by ethanol precipitation in the presence of 0.3 M Na-acetate and analyzed by 15% seqPAGE. Bands were visualized by Storage Phosphor Screen scan, as well as fluorescence scan on the Typhoon 9400 (fluorescence scan: ex: 488 nm, em: 520 BP 40). To compare the different scans, gels were registered, as described above.



### Liquid chromatography–mass spectrometry analysis of PAP and CuAAC reaction products

Liquid chromatography–mass spectrometry (LC–MS) experiments were carried out using a microTOFQII ESI mass spectrometer (Bruker) connected to a 1200 Series HPLC system (Agilent), equipped with a Phenomenex kinetex C18 2.6  $\mu\text{m}$  column (2.1  $\times$  100 mm). The solvent system used for elution consisted of 100 mM hexafluoroisopropanol/8.6 mM triethylamine (pH 8.3) as solvent A and methanol (LC–MS grade) as solvent B. The column was pre-equilibrated with 95% solvent A and 5% solvent B for 10 min. Precipitated oligonucleotides were re-dissolved in water, injected onto the column and were eluted with a gradient starting from 5% solvent B to 35% over 30 min. Absorption signals at 254 nm (oligonucleotide absorption) and at 492 nm (Alexa Fluor 488 maximal absorption) were recorded with a multi wavelength detector. For internal calibration (enhanced quadratic mode) of high-resolution mass spectra, we used ESI Tune Mix (Fluka). Data analysis was carried out using the DataAnalysis software (version 4.0 SP 4) for analysis of mass spectra that were deconvoluted using the maximum entropy deconvolution, and Hyphenation Star PP software (version 3.2.44.0) for analysis of LC chromatograms (both Bruker Daltonik). Molecular masses refer to the  $m/z$  values given by the DataAnalysis software.

### Fluorescent labeling of a natural RNA isolate

Total RNA was isolated from *E. coli* JM 109 using the GenElute Total RNA Purification Kit (Sigma Aldrich). In all, 28  $\mu\text{g}$  of total RNA ( $\sim$ 50 pmol) were modified in a reaction containing 2'-N<sub>3</sub>-2'-dUTP (500  $\mu\text{M}$ ), yeast PAP (600 U) in 1 $\times$  USB yeast poly(A) polymerase buffer (25  $\mu\text{l}$ ) for 30 min at 37°C. The enzyme was removed by phenol extraction and the RNA recovered by ethanol precipitation in the presence of Na-acetate.

To perform CuAAC, the modified total RNA ( $\sim$ 1  $\mu\text{M}$ ) was incubated with Alexa Fluor 647 alkyne (500  $\mu\text{M}$ ), CuSO<sub>4</sub> (100  $\mu\text{M}$ ), THPTA (500  $\mu\text{M}$ ), Na-ascorbate (1 mM) in phosphate buffer (50 mM, pH 7) at 25°C for 30 min. RNA was purified immediately after the reaction by phenol extraction in the presence of EDTA (10 mM) and Na-acetate (0.6 M, pH 5.5) followed by ethanol precipitation. About 5 pmol of non-reacted, PAP-reacted and clicked RNA were analyzed by 8% denaturing PAGE and first scanned for Alexa Fluor 647 signals. Then, RNA was stained unspecifically with SYBR Gold (Invitrogen) and scanned again (ex: 532 nm, em: 526 nm SP). Gel images were registered as described above.

### Polyadenylation of N<sub>3</sub>-modified RNA

Polyadenylation of N<sub>3</sub>-nucleotide-containing, purified RNA by yeast PAP or *E. coli* PAP was performed by adding 2.5 pmol of ethanol-precipitated, 2'-N<sub>3</sub>-2'-dNTP-reacted RNA, 2  $\mu\text{l}$  of unpurified N<sub>3</sub>-NTP containing yeast PAP mix or 1.6  $\mu\text{l}$  of N<sub>3</sub>-NTP containing *E. coli* PAP mix into a PAP reaction as described above, containing ATP (500  $\mu\text{M}$ ) instead of the modified NTP

and fresh PAP. All reaction mixtures were analyzed by 12 or 15% seqPAGE.

Shifted bands or smears, representing polyadenylated RNA were quantified against the different bands representing non-polyadenylated sequences, differentiating between non-modified and N<sub>3</sub>-nucleotide-bearing RNA, whenever different bands were distinguishable.

### Adapter ligation of N<sub>3</sub>-modified RNA

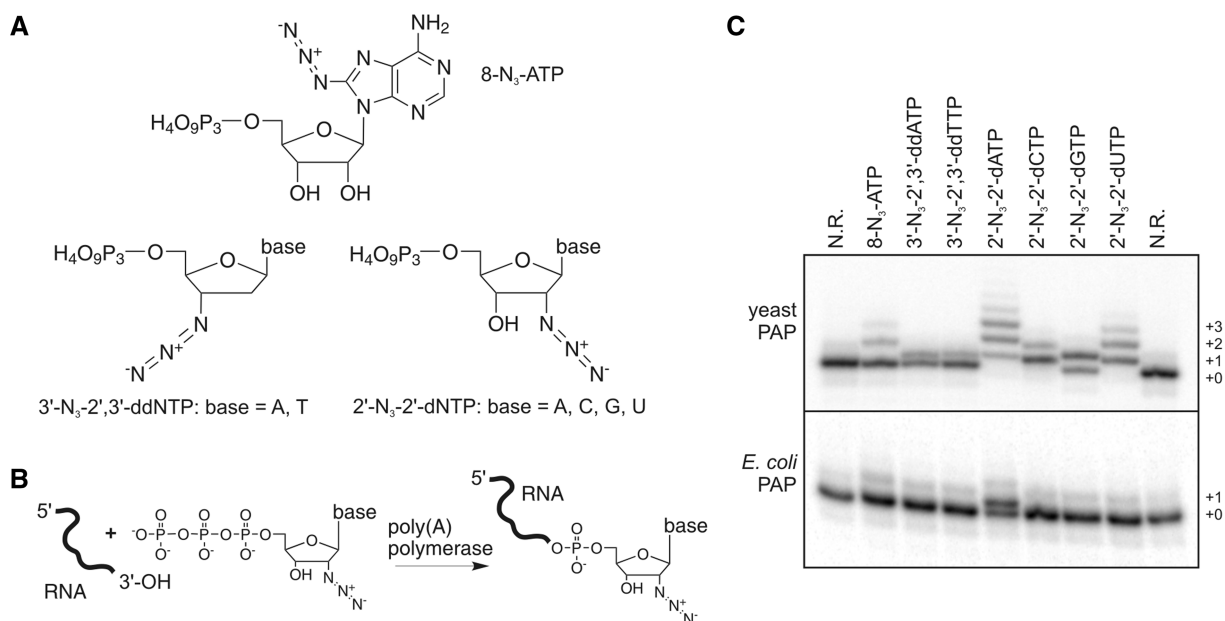
The 3'-adapter ligation was performed using an adenylated adapter (DNA2) prepared according to a published protocol (52). In all, 2  $\mu\text{l}$  of unpurified N<sub>3</sub>-NTP containing yeast PAP or 1.6  $\mu\text{l}$  of N<sub>3</sub>-NTP containing *E. coli* PAP mix were added to the ligation mixture, consisting of adapter (7.5–10  $\mu\text{M}$ ), RNL1 (1 U/ $\mu\text{l}$ ) and RNL2, tr. (20 U/ $\mu\text{l}$ ) in ligation buffer (50 mM Tris–HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, final volume: 10  $\mu\text{l}$ ). Ligation mixtures were incubated at 4°C overnight and were analyzed by 12 or 15% seqPAGE. Bands containing non-ligated RNA or ligated RNA (shifted bands) were quantified. The ligation yield (consumption of non-ligated material) was determined ratiometrically, differentiating between non-N<sub>3</sub>-modified and N<sub>3</sub>-nucleotide-bearing RNA, whenever different bands were distinguishable.

### Splinted ligation of N<sub>3</sub>-modified RNA

To convert the 3'-terminal modification into an internal one, splinted ligation was carried out, employing non-purified, 2'-N<sub>3</sub>-2'-dGTP-reacted RNA1 (0.5  $\mu\text{M}$ ), RNA4 (2.5  $\mu\text{M}$ ), DNA1 splint DNA (2.25  $\mu\text{M}$ ), and DNL (1.5 U/ $\mu\text{l}$ ) or RNL2 (500 nmol/ $\mu\text{l}$ ) in 1 $\times$  ligation buffer [50 mM Tris–HCl (pH 7.4 at 25°C), 10 mM MgCl<sub>2</sub>, 200  $\mu\text{M}$  ATP and 5 mM DTT; final volume: 20  $\mu\text{l}$ ]. For those samples subjected to CuAAC, the DTT concentration in the ligase buffer was decreased to 0.5 mM. The mixture was denatured for 30 s at 90°C and then cooled to room temperature for 15 min prior to addition of the enzyme. The reaction mixture was incubated either at 37°C for 1 h or at 16°C overnight. The reaction was stopped by heating to 80°C for 10 min. The DNA splint was removed by the addition of DNase I (0.5 U/ $\mu\text{l}$ ) and incubated at 37°C for 15 to 30 min, followed by 10 min of heat inactivation at 80°C or phenol extraction (for samples subjected to click reaction). The reaction mixture was purified by ethanol precipitation. Reactions were analyzed by 15% seqPAGE. Shifted bands, representing ligated RNA were quantified against the bands representing non-ligated sequences.

### CuAAC with internally N<sub>3</sub>-modified RNA

The CuAAC with Alexa Fluor 647 alkyne or Alexa Fluor 488 alkyne was carried out with 250 nM ligated RNA, 2 mM Alexa Fluor alkyne, 500  $\mu\text{M}$  CuSO<sub>4</sub>, 2.5 mM THPTA and 5 mM Na-ascorbate. In a different approach, the ligated RNA was first annealed to a partially reverse complementary DNA (DNA3, in 40-fold excess over ligation product) that forces the modified position into a 9-nt bulge-loop, and carried out as described above. RNA was then purified by phenol extraction in the presence of EDTA (10 mM) and Na-acetate



**Figure 1.** Incorporation of different  $N_3$ -modified NTPs by different PAPs. **(A)** Overview of the modified NTPs employed in this study. **(B)** Schematic representation of the addition of a  $2'$ - $N_3$ -modified nucleotide to the  $3'$ -terminus of an RNA sequence by PAP. **(C)** Addition of various  $N_3$ -modified nucleotides by yeast and *E. coli* PAP to the  $3'$ -terminus of RNA1. N.R.: no reaction control. Analysis by 18% seqPAGE. Radioactive bands are shown. Bandshifts indicate the incorporation of one or more nucleotides.

(300–600 mM, pH 5.5) followed by ethanol precipitation. Then, DNA2 was digested with DNase I in DNase I buffer with  $MgCl_2$  [10 mM Tris-HCl (pH 7.5 at 25°C), 2.5 mM  $MgCl_2$ , 0.1 mM  $CaCl_2$ ] for 15 min, and the enzyme was heat-inactivated for 10 min at 65°C. Reactions were analyzed by 15% seqPAGE. Shifted bands, representing clicked RNA, were quantified against the bands representing non-clicked sequences.

## RESULTS

### Screening of nucleotidyl transferases and modified nucleotides

To achieve both, base- and backbone modifications, we employed NTPs bearing the azide modification at position C-2', C-3' or C-8 (see Figure 1A for an overview). We determined which of the four nucleotidyl transferases (yeast and *E. coli* PAP, Cid 1 PUP and TdT) are best suited to efficiently transfer the different  $N_3$ -modified nucleotides to the  $3'$ -termini of RNA (shown schematically in Figure 1B for modification of RNA with  $2'$ -modified nucleotides and PAP). The results are summarized in Table 3.

For TdT, the efficiency of  $N_3$ -nucleotide addition was very low (Supplementary Figure S1A), which was expected since TdT usually modifies DNA (53) and is known to have a very low efficiency on RNA (54).

Cid 1 PUP accepted  $2'$ - $N_3$ - $2'$ -dNTPs with moderate yield, but no significant incorporation of NTPs modified at positions C-3' or C-8 could be detected (Supplementary Figure S1B and S1C). Additionally, this enzyme produced heterogeneous tails in most cases.

**Table 3.** Results of the first screening of nucleotidyl transferases for incorporation of  $N_3$ -nucleotides

Modified NTP	No. of residues added			
	Yeast PAP	<i>Escherichia coli</i> PAP	Cid 1 PUP	TdT
8- $N_3$ -ATP	1–2	–	0–2 <sup>a</sup>	–
$2'$ - $N_3$ - $2'$ -dATP	Multiple	1(–2) <sup>b</sup>	0–mult. <sup>a</sup>	–
$2'$ - $N_3$ - $2'$ -dCTP	1–2	–	0–mult. <sup>a</sup>	–
$2'$ - $N_3$ - $2'$ -dGTP	1–2	–	0–mult. <sup>a</sup>	0–1 <sup>a</sup>
$2'$ - $N_3$ - $2'$ -dUTP	1–2	–	0–mult. <sup>a</sup>	–
$3'$ - $N_3$ - $2',3'$ -ddATP	0–1	–	–	ND
$3'$ - $N_3$ - $2',3'$ -ddTTP	0–1	–	–	ND

Generally, reactions were carried out with commercial buffers, 500  $\mu$ M NTP, at 37°C for 60 min (TdT: 90 min). Enzyme-specific conditions were the following: 0.2  $\mu$ M RNA, 24 U/ $\mu$ l yeast PAP; 0.2  $\mu$ M RNA, 0.08 U/ $\mu$ l Cid1 PUP; 0.25  $\mu$ M RNA, 0.25 U/ $\mu$ l *E. coli* PAP, 0.25  $\mu$ M RNA, 1 U/ $\mu$ l TdT. No heat inactivation.

<sup>a</sup>Mostly 0.

<sup>b</sup>Only at long incorporation times or with  $MnCl_2$ .

Yeast PAP exhibited the highest incorporation activity for all modified NTPs (Figure 1C). In accordance with the published data (55), one to two C-8 modified nucleotides were incorporated. We made similar observations for NTPs modified at positions C-2', except for  $2'$ - $N_3$ - $2'$ -dATP, where we observed the addition of multiple nucleotides. For NTPs modified at position C-3', the addition of a single nucleotide is possible with limited efficiency.

With *E. coli* PAP, only the incorporation of  $2'$ - $N_3$ - $2'$ -dATP proceeded with a good efficiency, leading to the addition of one nucleotide (Figure 1C).

Taking together these results, we focused on yeast and *E. coli* PAP for more detailed investigations.

### Yeast PAP reaction with the base-modified 8-N<sub>3</sub>-ATP

For the base-modified ATP analog 8-N<sub>3</sub>-ATP, yeast PAP added either one or two nucleotides to the RNA, after which further nucleotide additions did not occur, irrespective of RNA concentration and reaction time. The reaction was more efficient at an RNA concentration of 0.2 μM than at higher concentrations (0.8 μM and 4 μM), and the fraction of modified RNA sequences increased at longer reaction times (120 min) (Supplementary Figure S2). Using 8-N<sub>3</sub>-ATP, it is thus possible to modify RNA sequences with one to two moieties that can react in further CuAAC so that the length of the RNA is only slightly altered. The extent of modified sequences can, to a certain degree, be controlled by varying the RNA concentration and reaction time.

### Optimization of single incorporation for sugar-modified nucleotides

For some applications, it is crucial that only a single modification is added to each oligonucleotide. While only a single nucleotide can be incorporated in case of 3'-modified (and therefore 3'-blocked) NTPs, for 2'-modified NTPs that carry a free 3'-OH, multiple incorporation is, in principle, possible. However, having observed the relatively short tails generated by yeast PAP with 2'-N<sub>3</sub>-2'-dCTP, -GTP and -UTP (Figure 1C), we attempted to control the reaction, so as to specifically achieve the addition of a single modified nucleotide.

The optimal reaction conditions for single nucleotide addition at 4 (yeast PAP) or 5 μM (*E. coli* PAP—only for 2'-N<sub>3</sub>-2'-dATP) RNA concentration were determined by screening over reaction times between 2 and 240 min for RNA1, RNA2 and RNA3 and are given in Table 2.

For 2'-N<sub>3</sub>-2'-dGTP, we observed the highest amount of single nucleotide addition after 2 h of reaction time. Longer incubations led to an increase of multiple nucleotide addition and thereby reduction of products containing a single modified nucleotide. Similarly, we determined the optimal reaction times to be 5 min for 2'-N<sub>3</sub>-2'-dUTP, and 20 min for 2'-N<sub>3</sub>-2'-dCTP (Supplementary Figure S3).

Since the use of yeast PAP with 2'-N<sub>3</sub>-2'-dATP led to the addition of multiple moieties, even at low NTP concentration and short reaction time (Supplementary Figure S4A), *E. coli* PAP seemed to be more suitable to achieve single nucleotide addition in this particular case. To facilitate the reaction, we added 2.5 mM MnCl<sub>2</sub> to the reaction buffer (already containing 10 mM MgCl<sub>2</sub>). In the absence of MnCl<sub>2</sub>, acceptable yields were only achieved after 16 h of incubation at 37°C, using double amounts of enzyme (Supplementary Figure S4B), which led to substantial RNA degradation and severe fluctuations in reaction yield. In contrast, MnCl<sub>2</sub> at a concentration of 2.5 mM has been reported to increase the efficiency of *E. coli* PAP by ~5-fold (56). Indeed, we observed a considerable acceleration of 2'-N<sub>3</sub>-A-incorporation, so that generally reaction yields of >50% were obtained for the product containing a single modified nucleotide, after as little as

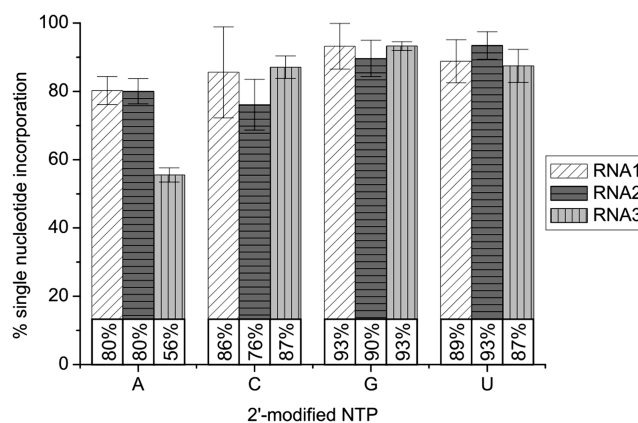
20 min reaction time (Supplementary Figure S5). Comparing initial rates of the first and second nucleotide incorporation, we found that the second incorporation proceeds with only 5–10% of the initial rate of the first incorporation, so that single nucleotide incorporation is favored after short reaction times.

### Incorporation efficiencies under optimized conditions

Figure 2 shows a triplicate determination of single nucleotide incorporation efficiencies under the optimized conditions (Table 2) for each of the three test sequences. In 11 out of the 12 combinations, single-addition product yields ≥75% were achieved. Altogether, we were able to identify conditions under which single nucleotide incorporation is prevalent for each of the 2'-modified NTPs, requiring relatively short reaction times and preserving the integrity of the RNA sequences.

### Dynamic range of PAP reaction

Depending on the application, the amount of RNA to be modified may vary considerably. While certain RNA samples from natural sources can be scarce, highly abundant natural RNAs or chemically synthesized sequences might need to be modified in bigger amounts and therefore in higher concentrations, demanding our labeling strategy to be equally efficient under very high as well as low RNA concentrations. Thus, to investigate the robustness of our approach, we sought to find out the dynamic range of the yeast PAP reaction where we varied the reaction time employing any one of the four 2'-N<sub>3</sub>-modified NTPs and RNA1 at 0.2 and 4 μM concentration (for an example, see Supplementary Figure S6), or RNA2 at 4 to 80 μM concentration in a reaction with 2'-N<sub>3</sub>-2'-dCTP and -UTP (Supplementary Figure S7). The overall reaction yield was generally lower at 0.2 μM than at 4 μM concentration. However, the reaction kinetics



**Figure 2.** Determination of single nucleotide incorporation efficiencies for all 2'-N<sub>3</sub>-2'-dNTPs with three different RNA sequences under conditions optimized for 4 or 5 μM (only 2'-N<sub>3</sub>-2'-dATP) RNA. RNA1, 2, and 3 were subjected to PAP reactions under optimized conditions (in triplicate, except RNA3 + 2'-N<sub>3</sub>-2'-dGTP—duplicate). Analysis by 15% seqPAGE. All radioactive bands were quantified with ImageQuant software and the percentages of singly modified product were calculated for all samples. Of these, average values are shown. Error bars indicate 1 SD.



were similar. At elevated RNA concentrations, we found that the relative rate of the reaction was decreased. Nonetheless, efficient addition of a single nucleotide was possible at prolonged reaction times (maximally 2 h for 80  $\mu$ M RNA with 2'-N<sub>3</sub>-2'-dCTP).

To further confirm the successful incorporation of a single modified nucleotide, we performed an LC-MS analysis of the purified product of a reaction containing 80  $\mu$ M RNA2 and 2'-N<sub>3</sub>-2'-dCTP (Supplementary Figure S8), incubated for 2 h. In this analysis, we determined a reaction yield of  $\sim$ 80%. The identity of the PAP reaction product could be confirmed according to the molecular mass.

### CuAAC at the 3'-terminus

To convert the azide modification to various functional tags, we employed the CuAAC using biotin-alkyne, Alexa Fluor 488 alkyne or Alexa Fluor 647 alkyne, together with a water-soluble Cu-stabilizing ligand, THPTA (50). A Cu-stabilizing ligand is necessary to protect the biomolecule, i.e. RNA from degradation by free copper ions (57) and oxidation products of ascorbate (50). RNA modified with any one of the four 2'-N<sub>3</sub>-2'-dNTPs (Figure 3A) or with 8-N<sub>3</sub>-ATP (Supplementary Figure S9) could be efficiently conjugated with various functional moieties: fluorophores (Figure 3A) or biotin (Supplementary Figure S10). Purification of the

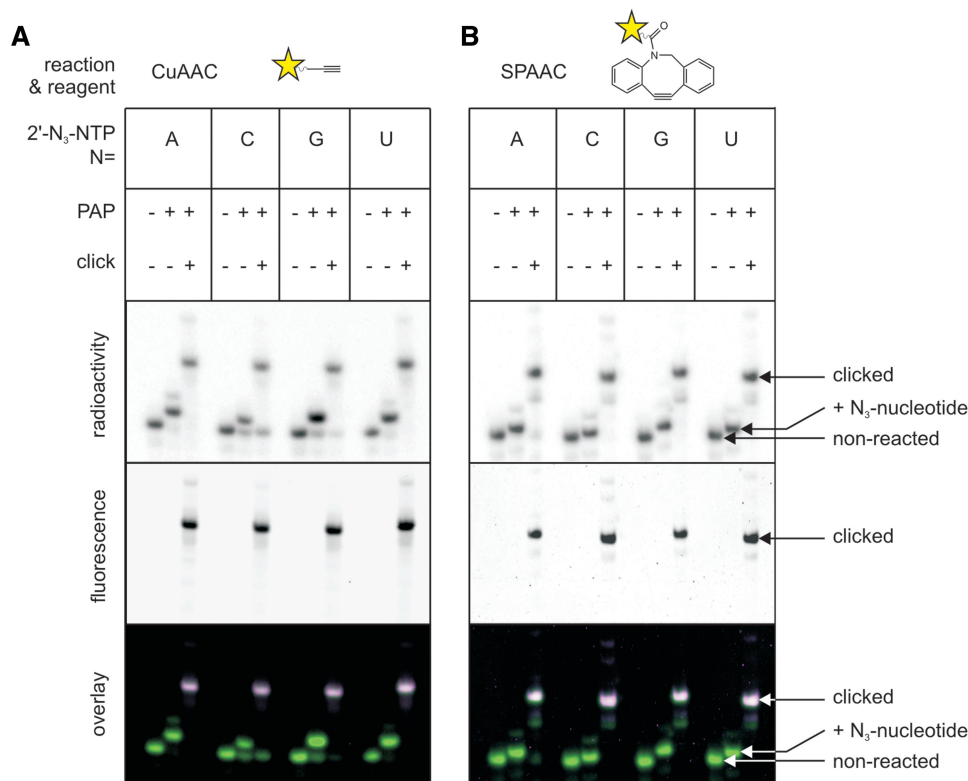
RNA from the PAP reactions by size exclusion chromatography, ethanol or isopropanol precipitation proved to be efficient enough in removing excess azido-NTPs prior to CuAAC. Under carefully optimized conditions, we achieved high yields (from  $>$ 80% up to quantitative) at RNA concentrations ranging from 50 nM to 50  $\mu$ M. Yields and concentrations of the respective azide-bearing RNA, alkyne, as well as Cu(II), THPTA and ascorbate, are summarized in Table 4. In all cases, it was sufficient to incubate the reactions for 2 h at 37°C, which lead to excellent reaction yields without causing significant RNA degradation.

An LC-MS analysis was performed for RNA2 and RNA3, reacted with 2'-N<sub>3</sub>-2'-dCTP at an RNA concentration of 20  $\mu$ M and conjugated with Alexa Fluor 488 alkyne at an RNA concentration of 10  $\mu$ M. Here, the reaction products with the respective expected molecular

**Table 4.** Yields and corresponding reaction conditions of CuAAC at different N<sub>3</sub>-RNA concentrations

[N <sub>3</sub> -RNA]	[alkyne]	[Cu(II)]	[THPTA]	[Asc.]	Yield
50 nM	50 $\mu$ M	100 $\mu$ M	500 $\mu$ M	1 mM	$>$ 80%
1–10 $\mu$ M	500 $\mu$ M	100 $\mu$ M	500 $\mu$ M	1 mM	Quantitative
50 $\mu$ M	2 mM	500 $\mu$ M	2.5 mM	5 mM	Quantitative

General conditions: 50 mM phosphate buffer, 37°C, 2 h.



**Figure 3.** Fluorescent labeling of RNA by CuAAC or SPAAC. RNA1, modified with each of the four 2'-N<sub>3</sub>-2'-dNTPs under optimized conditions and further conjugated with fluorescent dyes. Analysis by 15% seqPAGE. Radioactivity scan (upper panel), fluorescence scan (middle panel) and an overlay of both (green: radioactivity; magenta: fluorescence; white: both; lower panel) are given. (A) Conjugation with Alexa Fluor 647 alkyne by CuAAC. (B) Conjugation with DIBAC Fluor 488 by SPAAC.

masses were present in > 94% of overall RNA content, according to the UV absorption at 260 nm (Supplementary Figure S11 and S12).

### Fluorescent labeling of RNA by SPAAC

To prove the generality of our approach, which can be extended to copper-free techniques, we performed an exemplary SPAAC reaction (Figure 3B), employing a heterocyclic aza-dibenzocyclooctyne (DIBAC) (58) previously shown to exhibit one of the highest reaction rates among the known cyclooctyne compounds (59). Similar to the case of CuAAC, RNA modified with any one of the four 2'-N<sub>3</sub>-modified nucleotides reacted quantitatively with the fluorescent DIBAC, providing thus a second possibility of high yielding RNA modification.

### Detection limit for fluorescently labeled RNA

Figure 4 shows the determination of the detection limit for RNA that was fluorescently labeled, using our strategy, employing CuAAC. After incorporation of either one of the four 2'-N<sub>3</sub>-nucleotides (A, C, G or U) and conjugation with Alexa Fluor 647 alkyne, RNA amounts of as little as 100 amol still resulted in clearly visible bands on a 12% denaturing sequencing gel. Even lower amounts like 25 or 50 amol were still distinguishable as blurry spots. Thus, this labeling approach permits an excellent sensitivity.

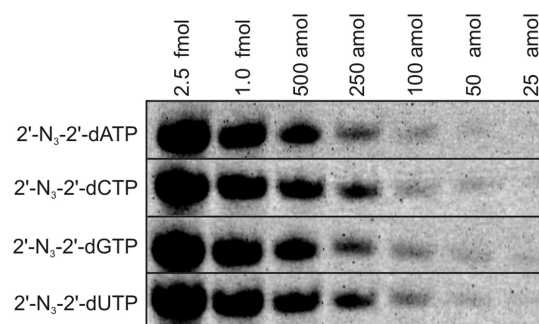
### Fluorescent labeling of an RNA isolate from *E. coli*

To test the robustness of our labeling approach and its applicability to RNA from biological sources, we isolated total RNA from *E. coli*, reacted this isolate with 2'-N<sub>3</sub>-2'-dUTP and yeast PAP. We employed a 30-min incubation time to assure that every sequence carries at least one modified nucleotide, and performed the CuAAC with Alexa Fluor 647 alkyne afterwards. To prevent degradation of the total RNA sample, both, reaction time and temperature were decreased (30 min incubation at 25°C). In the PAGE analysis (Figure 5), the appearance of fluorescent bands that perfectly overlay with the bands visible after non-specific RNA staining and the absence of degradation products indicate the applicability of this labeling approach toward long natural RNA.

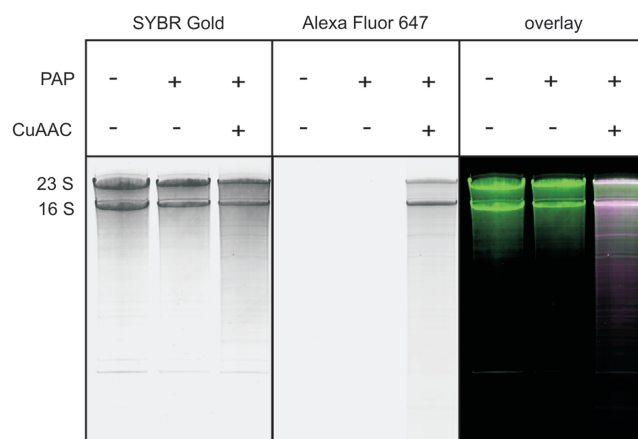
### Enzymatic reactions involving the azido-modified 3'-termini of PAP reaction products

Since the azido-modifications at the base or the sugar moieties of the nucleotides employed in this study only constitute minor changes with respect to their natural analogs, we assumed that they would not interfere with certain enzymatic downstream reactions. To test this assumption, we investigated the feasibility of (i) further tailing these RNAs with yeast and *E. coli* PAP employing the enzyme's preferred substrate ATP; (ii) carrying out an adapter ligation to add a short DNA strand; and (iii) a splinted ligation in order to add a second RNA strand to the modified 3'-terminus.

The results of polyadenylation and adapter ligation are summarized in Table 5. While yeast PAP could not efficiently polyadenylate most of the templates containing a



**Figure 4.** Detection limit for RNA1 modified with each of the four 2'-N<sub>3</sub>-2'-dNTPs and conjugated with Alexa Fluor 647 alkyne. Samples (same as in Figure 3A) were diluted and analyzed by 12% seqPAGE in amounts from 25 amol to 2.5 fmol. Fluorescence scan (Typhoon 9400, pixel size: 100 μm, PMT: 800 V, high sensitivity) is shown.



**Figure 5.** Fluorescent labeling of a natural RNA sample. A total RNA isolate from *E. coli* was reacted with 2'-N<sub>3</sub>-2'-dUTP and yeast PAP, and further subjected to CuAAC with Alexa Fluor 647 alkyne. Analysis by 8% denaturing PAGE. SYBR Gold scan (left panel), Alexa Fluor 647 scan (middle panel) and overlay (green: radioactivity; magenta: fluorescence; white: both; right panel) are given. The two main bands represent 23S and 16S ribosomal RNA.

2'-N<sub>3</sub>-modified nucleotide at their 3'-termini, the polyadenylation reaction proceeded quantitatively when carried out with *E. coli* PAP (Figure 6A). For RNA containing 8-N<sub>3</sub>-A at the 3'-terminus (Figure 6B), near-quantitative polyadenylation by yeast PAP was observed for those sequences that contained a single 8-N<sub>3</sub>-A moiety. Interestingly, the polyadenylation reaction was inhibited when two 8-N<sub>3</sub>-A moieties were present.

We also investigated the possibility of ligating pre-adenylated DNA adapters to the modified 3'-termini, employing the ligases RNL1 and RNL2, tr (Figure 6B, for reaction yields see Table 5). The RNA strands containing one 8-N<sub>3</sub>-A were accepted almost as well as the unmodified RNA, whereas there was a strong bias against sequences with two 8-N<sub>3</sub>-A modifications. For 2'-N<sub>3</sub>-nucleotide-containing sequences, such a preference was not observed (Supplementary Figure S14). In addition to



the desired RNA-adapter ligation product, we observed some concatenated (running higher) and circularized RNAs (running lower) in the  $^{32}\text{P}$  scans of the gels. Both of these are due to the presence of a monophosphate at the 5'-terminus of the radiolabeled RNAs ( $\leq 2\%$ ), as observed by others [e.g. (60)]. Non-labeled RNA sequences do not contain a 5'-monophosphate and can thus not form these adducts, which amount to  $<0.35\%$  of the total RNA in any of the samples.

Encouraged by these results, we investigated the possibility of joining a second RNA strand to RNA carrying a 2'- $\text{N}_3$ -nucleotide at the 3'-terminus, employing splinted

**Table 5.** Enzymatic manipulation of 3'-termini of RNA previously modified in PAP reactions

3'-terminal modified nt	Polyadenylation <sup>a</sup> by		Adapter ligation
	Yeast PAP	<i>Escherichia coli</i> PAP	
None	+ (~93%)	+ (>95%)	+ (>95%) <sup>c</sup>
2'- $\text{N}_3$ -2'-dA	+ (~65%)	+ (>95%)	+ (~75%) <sup>c</sup>
2'- $\text{N}_3$ -2'-dC	- (<5%) <sup>b</sup>	+ (>95%)	+ (~90%) <sup>c</sup>
2'- $\text{N}_3$ -2'-dG	- (<5%)	+ (>95%)	+ (~85%) <sup>c</sup>
2'- $\text{N}_3$ -2'-dU	- (<5%)	+ (>95%)	+ (~80%) <sup>c</sup>
3'- $\text{N}_3$ -2',3'-ddA	Not possible	Not possible	Not possible
3'- $\text{N}_3$ -2',3'-ddT	Not possible	Not possible	Not possible
(1×) 8- $\text{N}_3$ -A	+ (~84–89%)	ND	+ (~50–80%) <sup>c</sup>
(2×) 8- $\text{N}_3$ -A	- (<5%)	ND	(+) (~8–40%) <sup>c</sup>

<sup>a</sup>In presence of 500  $\mu\text{M}$  ATP, 1 h, 37°C.

<sup>b</sup>A higher fraction of polyadenylated sequences was attributed to remaining, non-modified RNA.

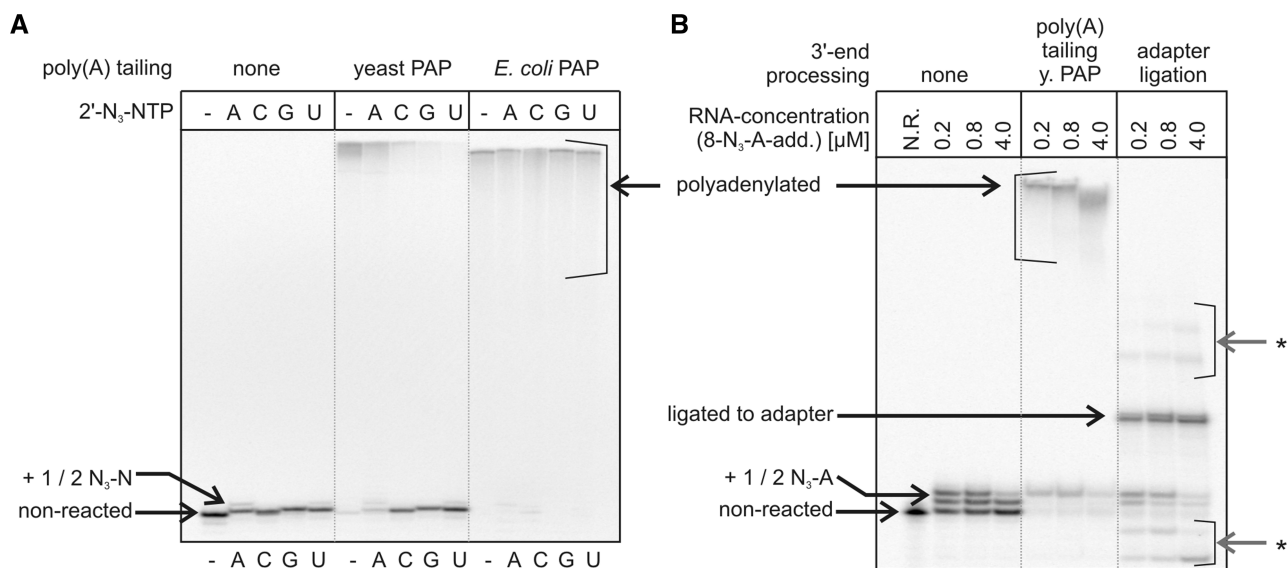
<sup>c</sup>Consumption of starting material (RNA).

ligation. In this method, the use of a splint oligonucleotide enables the correct ligation of the sequences of choice in high yield (61). In our case, this would convert the 3'-terminal  $\text{N}_3$ -modification into an internal modification within a defined RNA sequence and considerably extend the scope of the approach.

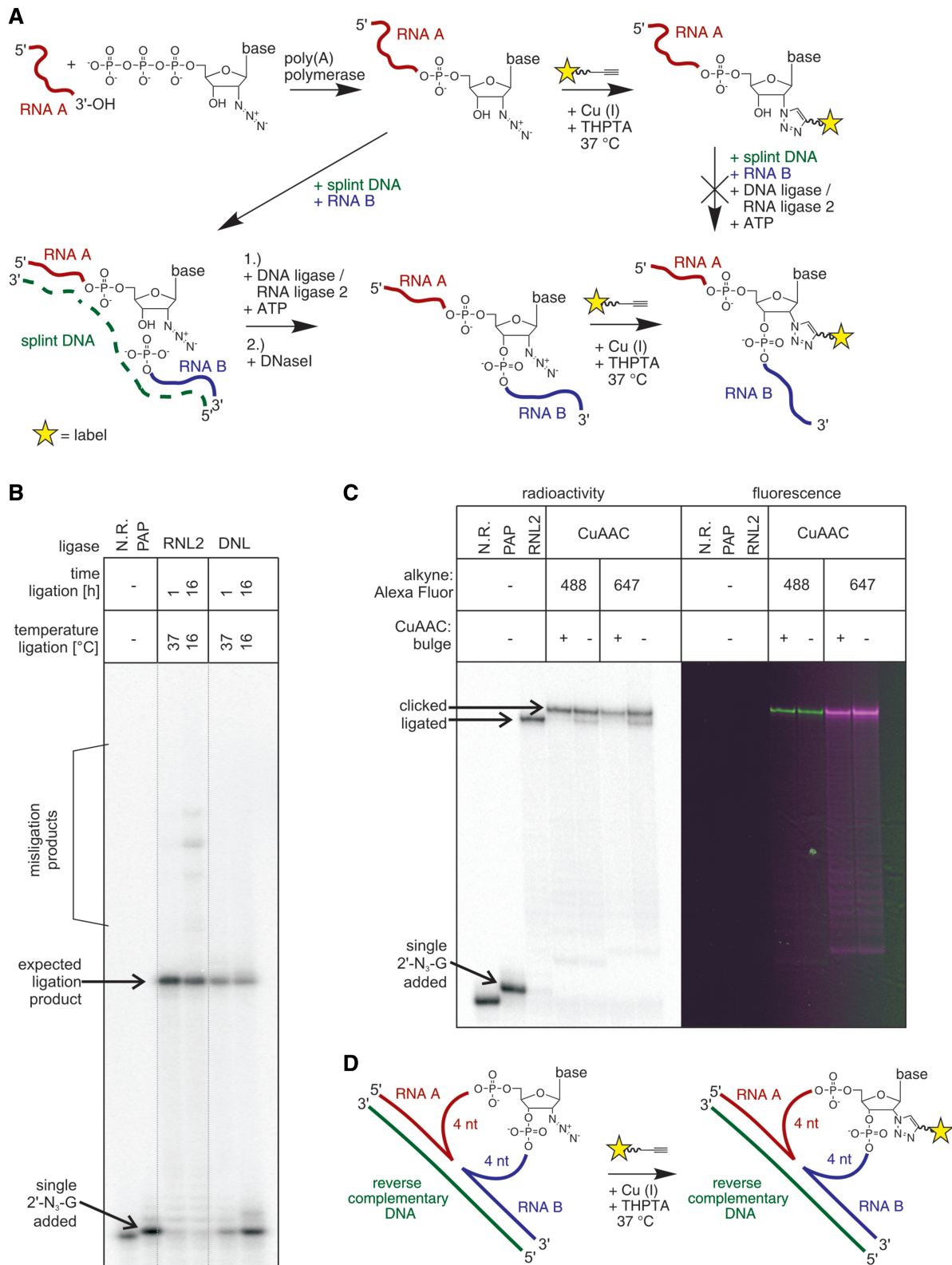
An RNA oligonucleotide modified with 2'- $\text{N}_3$ -2'-dGTP was subjected to splinted ligation (as shown schematically in Figure 7A), demonstrating the feasibility of the approach. Both, DNL and RNL2 accept the modified 3'-terminus as substrate (Figure 7B), with RNL2 showing better efficiencies than DNL. For both enzymes, an incubation for 1 h at 37°C produced significantly higher yields than 16 h at 16°C. With a 5-fold excess of the second ligation fragment and incubation times of 1 h at 37°C, ~50% ligation efficiency could be achieved with DNL, while the ligation with RNL2 had an excellent efficiency of ~80% of desired product. In contrast to this, as expected, ligation was not possible any longer when a bulkier modification like a fluorophore was attached by CuAAC prior to the ligation (Supplementary Figure S15). In this case, the modification at the 2'-position of the 3'-terminal nucleotide is much bulkier than just an azide and inhibits the ligase.

### CuAAC of internally azido-modified RNA

After ligation, the former terminal modification has been converted to an internal one. As a side effect of this, the accessibility of the azide for both, alkyne reactants and copper-catalyst is reduced during CuAAC. Depending on whether or not the modified position is situated in



**Figure 6.** Enzymatic manipulation of  $\text{N}_3$ -modified 3'-termini. (A) Polyadenylation of RNA1 modified with each of the four 2'- $\text{N}_3$ -nucleotides by yeast PAP and *E. coli* PAP. RNA1, reacted with each of the four 2'- $\text{N}_3$ -NTPs under optimized conditions, and further reacted either with yeast PAP or with *E. coli* PAP in the presence of ATP. Analysis by 12% seqPAGE. (B) RNA1 modified with 8- $\text{N}_3$ -ATP at the indicated concentrations, and further subjected to polyadenylation with yeast PAP or adapter ligation. N.R.: no reaction control. Analysis by 15% seqPAGE. Asterisk indicates bands that appear as artifacts, due to the presence of a monophosphate at the 5'-terminus of the radiolabeled RNA, which was doped into the reaction for visualization. Lower running bands represent circularized RNA, while higher running bands represent products of RNA-RNA ligation (with or without adapter added). Both radioactive scans shown here have been scaled. A non-scaled representation is given in Supplementary Figure S13.



**Figure 7.** Creating an internal fluorescent label. **(A)** General reaction scheme for creation of terminal (upper right corner) or internal modifications (lower right corner). An internal modification can be created by first adding an N<sub>3</sub>-modified nucleotide to the 3'-terminus of the RNA sequence, connecting this RNA to a second RNA sequence via splinted ligation, and subjecting the product, with an internal N<sub>3</sub>-modification to CuAAC. **(B)** Splinted ligation of RNA1 and RNA4, employing different ligases (RNL2 and DNL) under different reaction conditions (time and temperature). Analysis by 15% seqPAGE. **(C)** Addition of 2'-N<sub>3</sub>-guanosine to RNA1, followed by splinted ligation to RNA4 (using RNL2), and CuAAC with Alexa Fluor 488/647 alkyne, with or without the use of a helper DNA that forces the modified position into a 9-nt bulge loop. Analysis by 15% seqPAGE. Radioactivity scan (left panel), and an overlay of Alexa Fluor 488 scan (green, middle two lanes) and Alexa Fluor 647 scan (magenta, right two lanes) are shown. **(D)** Formation of 9 nt bulge loop to assist CuAAC. N.R.: no reaction control.

a base-paired region or in a more flexible loop, the cycloaddition can be hampered, which has also been observed for click reactions of 5-ethynyl-deoxyuridine in double-stranded DNA (36). Indeed, in our test system, where the modified nucleotide is situated in a partially base paired structure, we observed a decrease in cycloaddition efficiency under standard conditions. In order to improve the labeling yields, we successfully applied forced conditions involving 5-fold higher Cu concentrations and  $\geq 10$ -fold higher alkyne concentrations, which led to efficiencies in the range of 75–85%. A further increase in labeling efficiency could be achieved by hybridizing a DNA strand with partial reverse complementarity to the ligated sequence that forced 4 nt both, up- and downstream of the modification site into a non-paired bulge loop structure (Figure 7C and D). Here, we again observed near-quantitative reaction yields (>92%) as observed before with 3'-terminal modifications.

## DISCUSSION

In our modular RNA labeling approach, we employ enzymes (PAPs) to incorporate a convertible nucleotide at the 3'-terminus of the RNA, in order to provide a reactive handle (an azide) which is further converted to a modification of interest in a bio-orthogonal click reaction, namely CuAAC or SPAAC. The azide is not stable under the standard conditions of solid-phase chemical oligonucleotide synthesis (46), and can therefore not be incorporated easily with these methods, making enzymatic reactions an attractive alternative, which has the additional advantage of not being limited to short sequences.

As we have shown, yeast PAP can readily add one (or sometimes more) azido-modified nucleotide(s) (modified at C-8 or C-2') to the 3'-terminus of RNA but can, in most cases, not easily extend this reaction product, even with the preferred PAP substrate ATP. In a reaction mixture containing the above-mentioned modified NTPs, this leads to the dominance of RNA containing a single (or sometimes two) modified nucleotide(s).

Similarly, *E. coli* PAP can well accept 2'-N<sub>3</sub>-2'-dATP as an NTP-substrate, and can also easily extend RNA substrates containing a 2'-N<sub>3</sub>-modification at their 3'-termini with standard ATP. However, when both, the NTP and the 3'-terminus of the RNA-substrate are 2'-N<sub>3</sub>-modified, the *E. coli* PAP reaction is considerably slowed down (by 10- to 20-fold), which again makes it possible to achieve a prevalence of singly modified RNA sequences.

Unlike most other RNA 3'-labeling approaches, we provide a method that is capable of introducing each of the four (A, C, G, U) nucleotides in a chemically modified form. We took advantage of kinetic control of the enzymatic reaction to ensure single label incorporation. This enables the 3'-terminal modification of sequences from any origin (synthetic or natural), causing only minimal mutation, and thereby preserving function.

Both, the PAP reactions, and the CuAAC can be adjusted to a wide range of RNA concentrations (at least 200 nM to 80  $\mu$ M for PAP, at least 50 nM to 50  $\mu$ M for CuAAC), thereby making our labeling strategy applicable for very little as well as large amounts of RNA. Even at the lowest RNA concentration (50 nM), CuAAC reaction yields of  $\geq 80\%$  could be achieved under optimized conditions. For such low concentrations of nucleic acids carrying single reactive handles, no previous reports exist for CuAAC modification. At higher concentrations, we routinely observed quantitative reaction yields.

Since both reactions proceed with high yield and without significant degradation, the overall efficiency of our two-step functionalization strategy is very high. As a consequence, an outstanding sensitivity can be achieved upon fluorescent labeling, enabling the detection of RNA amounts <100 amol, which exceeds the only comparable report of RNA detection sensitivity after CuAAC labeling (32) by more than three orders of magnitude.

Another major advantage of our labeling protocol arises from the kinetic control over the PAP reactions that we employ in order to achieve the incorporation of a single label, instead of using a 3'-protected, and thus blocked nucleotide (lacking a 3'-hydroxyl function), as in the case of the recently reported RNA labeling using *E. coli* PAP and 3'-N<sub>3</sub>-2',3'-ddATP (32). RNA that is end-modified with a 3'-blocked nucleotide cannot be subjected to further enzymatic manipulations of that modified 3'-end. Contrary to this, in our experimental setup, the modified 3'-terminus bears a free 3'-hydroxyl group, which is available for further enzymatic downstream processing, such as polyadenylation (with non-modified ATP) by *E. coli* PAP or ligation employing a number of different ligases. Thereby, the terminal azido-modification can be converted into an internal modification, which is still able to undergo CuAAC. To achieve near-quantitative labeling, also with the sterically challenging internal backbone modifications, we had to further optimize the CuAAC conditions. In order to circumvent the problems of sterical clashes in base-paired regions, we introduced a simple but efficient strategy. Using a helper oligonucleotide that forces the modified nucleotide into an available position, we were able to recover full reactivity.

Noteworthy, due to the possibility of attaching the nucleotide of choice (A, C, G or U) prior to ligation, we are capable of targeting any internal position within a *de novo* synthesized RNA, without mutating its sequence. This should help preserving the integrity of functional RNAs that can be studied after labeling.

To even further broaden the scope of azide incorporation into oligonucleotides, other click reactions can be employed to convert azides, which is a subject of ongoing research in our laboratory. The copper-free reactions include the Staudinger ligation, which has already been successfully applied to RNA (62) and the SPAAC (41), which has also been applied to nucleic acids (63–66). For the latter, we exemplarily show the fluorescent labeling of N<sub>3</sub>-modified RNA with a DIBAC-fluorophore. Similar to our observations with CuAAC, the reaction proceeded with excellent efficiency. Copper-free reactions are more compatible with living cells than the CuAAC and



may therefore allow for new *in vivo* applications. In our decision in favor of incorporating azides instead of alkynes, this was a crucial factor.

In conclusion, we provide a simple method for chemoenzymatic RNA functionalization, which does not require any special skills or lab equipment for chemical synthesis. Since all components are commercially available, the technique should be readily available to prospective users from different disciplines. Our method can be applied to many different RNA substrates, at variable concentrations, to create either terminal or internal modifications with an ever-growing number of possible labels. Owing to the modular nature of the approach, a set of only four different modified NTPs is sufficient to target RNA for conjugation with a multitude of different labels. Therefore, comparatively little optimization is needed, while high yields can be expected even at concentrations that are relatively high for standard enzymatic reactions. The only requirement for a sequence to be modified is that it is a substrate of the respective PAP. Due to the popularity of copper-catalyzed, as well as metal-free click reactions, a growing number of possible labels are commercially available. Therefore, the technique is open to a broad range of applications, e.g. *in vitro* studies of RNA structure and dynamics, RNA imaging, as well as the modulation of RNA properties through chemical modification.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR online: Supplementary Figures 1–15, Supplementary Table 1 and Supplementary Methods.

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