



HighYield T7 AF647 RNA Labeling Kit (CTP-based)

Preparation of randomly AF647-modified RNA probes by *in vitro* transcription with CTP-PEG5-AF647

Cat. No.	Amount
RNT-401-AF647	20 reactions x 20 µl

For general laboratory use.

Shipping: shipped on gel packs

Storage Conditions: store at -20 °C

Additional Storage Conditions: avoid freeze/thaw cycles

Shelf Life: 12 months

Spectroscopic Properties: λ_{exc} 648 nm, λ_{em} 671 nm, ϵ 270.0 L mmol⁻¹ cm⁻¹ (Tris-HCl pH 7.5)

Description:

HighYield T7 AF647 RNA Labeling Kit (CTP-based) is designed to produce randomly AF647 modified RNA probes via *in vitro* transcription. Such probes are ideally suited for *in situ* hybridization and Northern Blot experiments. AF647 (also known as Alexa Fluor® 647) is a hydrophilic dye with increased photostability compared to Fluorescein.

CTP-PEG5-AF647 is efficiently incorporated into RNA as substitute for its natural counterpart CTP using an optimized reaction buffer and T7 RNA Labeling Polymerase Mix. 35 % CTP-PEG5-AF647 substitution typically results in an optimal balance between reaction and labeling efficiency. Individual optimization of CTP-PEG5-AF647/CTP ratio however, can easily be achieved with the single nucleotide format. The resulting AF647-modified RNA probe can subsequently be detected by fluorescence spectroscopy.

The kit contains sufficient reagents for 20 labeling reactions of 20 µl each (35 % CTP-PEG5-AF647 substitution, 2.5 mM ATP, GTP, UTP, 0.2 mM CTP, 0.1 mM CTP-PEG5-AF647).

A 20 µl reaction yields about **5-7 µg RNA after 30 min incubation (1 µg T7 control template, 1.4 kb RNA transcript)**. Yields may however vary depending on the template (promotor design, sequence length, secondary structure formation).

Content:

HighYield T7 RNA Labeling Polymerase Mix

2x 40 µl, incl. RNase inhibitor and 50 % glycerol (v/v)

HighYield T7 Reaction Buffer

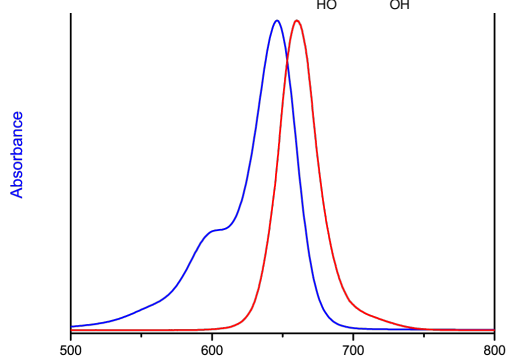
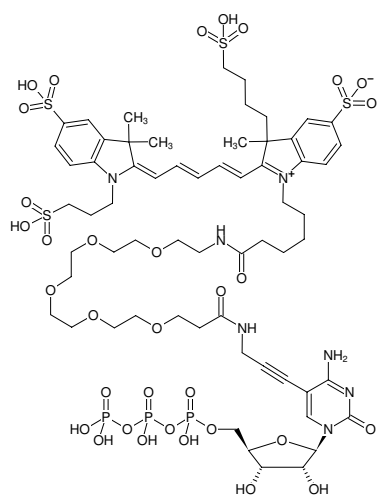
1x 200 µl (10x), HEPES-based

ATP - Solution

1x 100 µl (100 mM)

GTP - Solution

1x 100 µl (100 mM)



Excitation and Emission spectrum of AF647



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CTP - Solution

1x 100 µl (100 mM)

UTP - Solution

1x 100 µl (100 mM)

CTP-PEG5-AF647

1x 10 µl (5 mM)

T7 G-initiating control template (1.4 kbp)

1x 10 µl (200 ng/µl), 1.4 kbp PCR fragment plus T7 class III phi6.5 promoter resulting in approx. 1400 nt RNA transcript

PCR-grade water

1x 1.2 ml

DTT

1x 150 µl (100 mM)

To be provided by user

T7 Promotor-containing DNA template
RNA purification tools
RNase-free DNase I (optional)

1. Important Notes (Read before starting)

1.1 Prevention of RNase contamination

Although a potent RNase Inhibitor is included, creating a RNase-free work environment and maintaining RNase-free solutions is critical for performing successful *in vitro* transcription reactions. We therefore recommend

- to perform all reactions in sterile, RNase-free tubes using sterile pipette tips.
- to wear gloves when handling samples containing RNA.
- to keep all components tightly sealed both during storage and reaction procedure.

1.2 Template requirements

- **Template type:** Linearized plasmid DNA or PCR products containing a double-stranded T7 class II phi2.5 or class III phi6.5 promoter region upstream of the target sequence. Transcription initiation from T7 class III promoter is generally more efficient than initiation from T7 class II promoter. Minimum T7 promoter sequences:

T7 class III phi6.5 promoter
5'-TAATACGACTCACTATAGNN...-3'

Bold: First base incorporated into RNA, NN: ideally CG

or

T7 class II phi2.5 promotor

5'-TAATACGACTCACTATTAGNN...-3'

Bold: First base incorporated into RNA, NN: ideally CG

- **Template quality:** DNA template quality directly influences yield and quality of transcription reaction. Linearized plasmid DNA needs to be fully digested and to be free of contaminating RNase, protein and salts. We recommend selecting restriction enzymes that generate blunt ends or 5'-overhangs and purification by phenol/chloroform extraction. A PCR mixture can be used directly however, better yields will usually be obtained with purified PCR products (e.g. via silica-membrane based purification columns).

2. Preparation of working solutions

2.1 Preparation of 10 mM ATP/GTP/UTP working solution

- Thaw 100 mM ATP, 100 mM GTP and 100 mM UTP solutions on ice, vortex and spin-down briefly.
- Prepare a 1:10 dilution with PCR-grade water to achieve a final concentration of 10 mM (e.g. 5 µl 100 mM ATP + 5 µl 100 mM GTP + 5 µl 100 mM UTP + 35 µl PCR-grade water).
- 10 mM ATP/GTP/UTP working solution can be stored at -20°C. Avoid freeze/thaw cycles.

2.2 Preparation of 10 mM CTP working solution

- Thaw 100 mM CTP solution on ice, vortex and spin-down briefly.
- Prepare a 1:10 dilution with PCR-grade water to achieve a final concentration of 10 mM (e.g. 5 µl 100 mM CTP + 45 µl PCR-grade water).
- 10 mM CTP working solution can be stored at -20 °C. Avoid freeze/thaw cycles.

3. In vitro Transcription protocol

The protocol is optimized for 0.5 µg - 1 µg DNA template.

An optimal balance between reaction and labeling efficiency is typically achieved with 35% CTP-PEG5-AF647 substitution following the standard protocol below however, individual optimization might improve results for individual applications (e.g. variation of CTP-PEG5-AF647/CTP ratio between 30 - 50 %).

- Place HighYield T7 RNA Labeling Polymerase Mix on ice.
- Thaw all remaining components at room temperature (RT), mix by vortexing and spin down briefly.
- Assemble all components at RT to a nuclease-free microtube (sterile pipette tips) in the following order:
- Mix PCR-grade water, HighYield T7 Reaction Buffer and DTT by vortexing and spin down briefly.



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- Add nucleotide solutions and template DNA, vortex and spin down briefly.
- Add HighYield T7 RNA Labeling Polymerase Mix vortex and spin down briefly.
- Incubate for 30 min at 37 °C in the dark (e.g. PCR cycler). Depending on the RNA probe individual optimization may increase product yield (2h – 4h at 37 °C).

RNA concentration can be determined by absorbance measurement at 260 nm (A_{260}) according to the Law-of-Lambert-Beer ($A_{260} = 1$ correspond to 40 $\mu\text{g/ml}$ ssRNA).

Incorporation rate of fluorophore

The efficiency of RNA labeling can be estimated by calculating the ratio of incorporated fluorophores to the number of bases (dye / base).

[Please note: Blanc correction with probe buffer solution is required.]

Component	Volume	Final concentration
PCR-grade water	X μl	
HighYield T7 Reaction Buffer (10x)	2 μl	1x
100 mM DTT	2 μl	
10 mM ATP/GTP/UTP working solution (s. 2.1)	5 μl	2.5 mM
10 mM CTP working solution (s. 2.2)	0.4 μl	0.2 mM
5 mM CTP-PEG5-AF647	0.4 μl	0.1 mM
Template DNA	X μl	0.5 - 1 μg
HighYield T7 RNA Labeling Polymerase Mix	2 μl	
Total volume	20 μl	

1. Measurement of the nucleic acid-dye conjugate absorbance:

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

2. Correction of A_{260} reading:

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

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

Correction Factor for AF647: $CF_{260} = 0.05$

3. Calculation of dye to base ratio by the law of Lambert-Beer ($A = c \times \epsilon \times d$):

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

Extinction coefficients:

$$\text{AF647: } \epsilon_{dye} = 270,000 \text{ cm}^{-1} \text{ M}^{-1}$$

$$\text{ssRNA: } \epsilon_{base} = 12,030 \text{ cm}^{-1} \text{ M}^{-1} \text{ (average, 50\% GC)}$$

3. Calculation of the degree of labeling (DOL)

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

$$\text{DOL} = 100 \times \text{dye/base ratio}$$

Example: A dye/base ratio of 0.02 corresponds to a DOL of 2 that corresponds to 2 dyes per 100 bases.

Related Products:

5-Propargylamino-CTP-PEG5-AF647, #NU-831-PEG5-AF647

Please note: Reagents for the following steps are not provided within this kit.

DNA template removal (optional)

Depending on the down-stream application, removal of template DNA might be required. We recommend a salt-resistant, high efficiency DNAase such as Turbo™ DNAse (ThermoFisher). Follow the manufacturer instructions.

RNA purification

Purification of RNA is required for certain applications such as measurement of AF647-labelled RNA probe concentration. Spin column purification will remove proteins, salts and unincorporated nucleotides. Please follow the manufacturer instructions and ensure that the columns match with product size and possess a sufficient binding capacity (e.g. RNA Clean & Concentrator™ columns (Zymo Research) or Monarch® RNA Cleanup kit (NEB)). Other RNA purification methods such as LiCl precipitation may work but have not been tested.

Total RNA quantitation