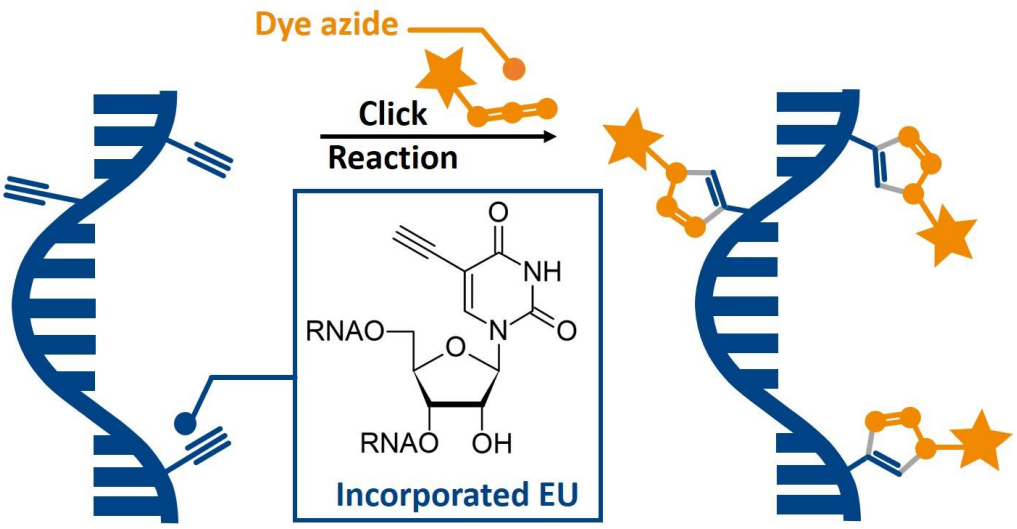
baseclick
DNA & RNA EXPERTS

User Manual

RNA Labeling Kit



Version 3.1

Ordering information

(for detailed kit content see **Table 1**)

RNA Labeling Kits:

Product number	Used Tag Azides
BCK-RNA488-10	6-FAM Azide (Fluorescein analogue)
BCK-RNA555-10	5-TAMRA-PEG3-Azide (Cy3 analogue)
BCK-RNA594-10	5/6-Sulforhodamine 101-PEG3-Azide (Texas-Red analogue)
BCK-RNA647-10	Eterneon-Red 645 Azide (Cy5 Azide analogue)

For References, FAQs and ordering please see online or contact us:

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RNA Labeling Kit

The RNA Labeling Kit contains reagents to perform 10 transcription reactions (50 µL each) and 12 independent labeling reactions.

Introduction and product description:

Baseclick's RNA Labeling Kit has been developed for the production of alkyne-labeled RNA targets by *in vitro* transcription from bacteriophage T7 RNA polymerase promoters. Since multiple rounds of transcription can happen on a single DNA template, many RNA transcripts of the template DNA are produced. The kit utilizes 5-ethynyl-UTP (5-EUTP) to label the RNA with alkynes. The resulting RNA contains alkyne modified uracils, which react with azido-derivates of fluorescent dyes, haptenes and other labels in a highly selective fashion under benign click reaction conditions. The label incorporation can be tuned by the amount of marker azide used in the click reaction.

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Please read the material safety data sheets (MSDS) provided for each product/component.

Literature Citation: When describing a procedure for publication using this product, please refer to it as *baseclick RNA Labeling Kit*.

1. Materials Provided with the Kit and Storage Conditions

Table 1: Contents of the kits and storage conditions

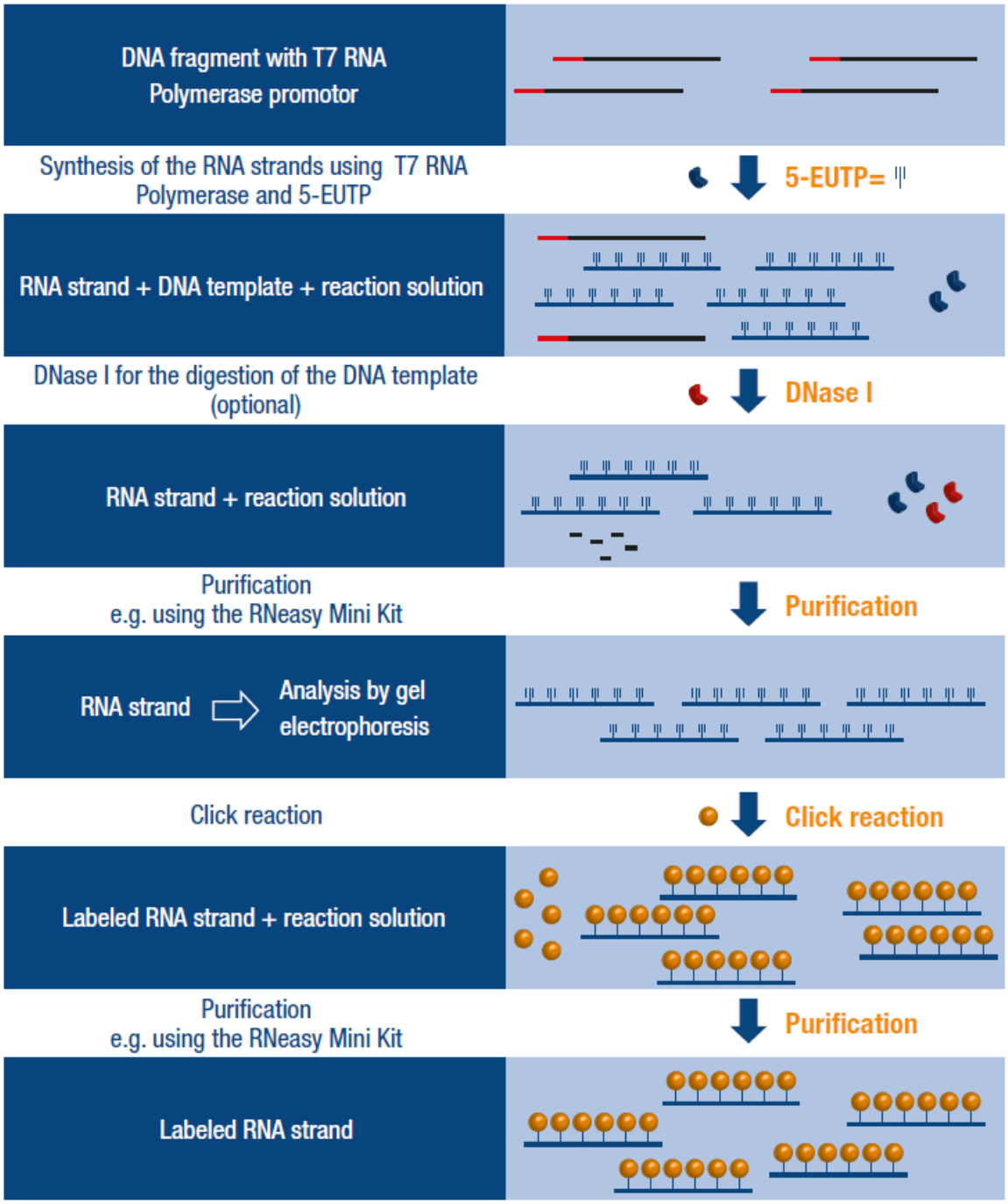
Color code	Name	Amount	Storage
Component A blue	5-Ethynyl-UTP (5-EUTP, 100 mM solution)	12 µL	**Box** - 20 °C Dark
Component B Red	- 6-FAM Azide (BCK-RNA488-10) - 5-TAMRA-PEG3-Azide (BCK-RNA555-10) - 5/6-Sulforhodamine-101-PEG3-Azide (BCK-RNA594-10) - Eterneon-Red 645 Azide (BCK-RNA647-10)	130 µL	
Component C	UTP (100 mM solution)	12 µL	
Component D	ATP (100 mM solution)	12 µL	
Component E	CTP (100 mM solution)	12 µL	
Component F	GTP (100 mM solution)	12 µL	
Component G purple	T7 RNA Polymerase 20 U/µL	310 U	
Component H orange	5x Transcription buffer	110 µL	
Component I yellow	Activator (RNase-free)	110 µL	
Component J green	Reactor-S (RNase-free)	20x	**Bag** RT, *Do not freeze*

This kit is stable up to 1 year after receipt, when stored as directed.

2. Required Material and Equipment not included in this Kit

- Template DNA containing T7-promotor
- DEPC-treated water (RNase free water)
- DNase I (optional)
- Reaction tubes (size depends on the volume of reaction cocktail needed) DNA purification kit (e.g. Qiaquick PCR Purification Kit, optional)
- RNA purification kit (e.g. Qiagen RNeasy Kit from Qiagen) or materials / equipment for self-made purification (for RNA purification RNase free)
- Thermocycler or water bath
- Ribo Lock RNase Inhibitor
- 0,5 M EDTA pH 8.0 (RNase free)

3. Workflow


DNA fragment with T7 RNA Polymerase promotor
Synthesis of the RNA strands using T7 RNA Polymerase and 5-EUTP
5-EUTP=
RNA strand + DNA template + reaction solution
DNase I for the digestion of the DNA template (optional)
DNase I
RNA strand + reaction solution
Purification e.g. using the RNeasy Mini Kit
Purification
RNA strand
Analysis by gel electrophoresis
Click reaction
Click reaction
Labeled RNA strand + reaction solution
Purification e.g. using the RNeasy Mini Kit
Purification
Labeled RNA strand

4. Preparation of Template DNA with T7 Promotor

(Reagents and equipment are not provided in this kit)

Make sure that the template DNA purity / quality is high. The template DNA should be cleaned up using phenol/chloroform extraction followed by an ethanol precipitation. Alternatively, it is also possible to use a purification kit, e.g. Qiaquick PCR Purification Kit. In both cases dilute the purified DNA in DEPC-treated H_2O (RNase free).

4.1. Protocol for Ethanol Precipitation (Example only)

- Add 100 µL of 0.3 M sodium acetate (or 10 µL of 3 M sodium acetate if the starting volume is very high) and mix well.
- Add 1 mL 100% ethanol absolute and mix well. Incubate for at least 20 min at – 20 °C. A longer incubation, e.g. overnight, increases the precipitation yield.
- Centrifuge for 20 min at 2 - 5 °C and 15.300 x g.
- Remove the supernatant and wash with 1 mL 70% Ethanol. Incubate for at least 20 min at – 20 °C.
- Centrifuge for 20 min at 2 - 5 °C and 15.300 x g.
  Optional: Wash a second time with 100% ethanol. Incubation at – 20 °C is not necessary.
- Dry the pellet at room temperature and dilute it in DEPC-treated H_2O (RNase free).

5. *In Vitro* Transcription with T7 RNA Polymerase

5.1. Preparation of the NTP-Mix

The unmodified NTPs (UTP, ATP, CTP, GTP) and the modified NTP (5'-Ethynyl-UTP, 5-EUTP) are provided separately in the kit. It is possible to use unmodified NTPs as a control (see step **5.1.2**). In the modified NTP-mix (prepared in step **5.1.1**) native UTP is completely substituted by 5'-Ethynyl-UTP. For some experiments, it might be more convenient to substitute UTP only partially (see step **5.1.3**).

5.1.1. Preparation of a modified NTP-Mix with 100% substituted UTP (10 µL final volume):

Table 2: Mix the following components in a fresh RNase free vial

Kit Component	Component Name	Volume	C final
Not included	DEPC-treated H_2O (RNase free)	6 µL	-
Component D	ATP	1 µL	10 mM
Component E	CTP	1 µL	10 mM
Component F	GTP	1 µL	10 mM
Component A	5-EUTP	1 µL	10 mM

This mix is ready-to-use for RNA transcription.

5.1.2. *(Optional)* Preparation of an unmodified NTP-Mix as a control (10 µL final volume):

Table 3: Mix the following components in a fresh RNase free vial

Kit Component	Component Name	Volume	C final
Not included	DEPC-treated H_2O (RNase free)	6 µL	-
Component C	UTP	1 µL	10 mM
Component D	ATP	1 µL	10 mM
Component E	CTP	1 µL	10 mM
Component F	GTP	1 µL	10 mM

This mix is ready-to-use for RNA transcription.

5.1.3. *(Alternative)* Preparation of a modified NTP-Mix with variable percentage of 5-EUTP (20 µL final volume). Example for 50% 5-EUTP:

Table 4: Mix the following components in a fresh RNase free vial

Kit Component	Component Name	Volume	C final
Not included	DEPC-treated H_2O (RNase free)	12 µL	-
Component A	5-EUTP	1 µL	5 mM
Component C	UTP	1 µL	5 mM
Component D	ATP	2 µL	10 mM
Component E	CTP	2 µL	10 mM
Component F	GTP	2 µL	10 mM

This mix is ready-to-use for RNA transcription.

5.2. Transcription Setup

Table 5: Reagents and amounts for transcription

Reagent	Vial	Amount	C final
5x Transcription buffer	Component G	10 µL	1X
NTP mix (prepared in 5.1)	-	10 µL	2 mM each NTP
Template DNA (1 µg)	-	depends on concentration	20 ng/µL
Ribo Lock RNase Inhibitor (40 U/µL)*	-	1.25 µL	50 U; 1 U/µL
T7 RNA Polymerase (20 U/µL)*	Component F	1.5 µL	30 U; 0.6 U/µL
DEPC -treated H_2O (RNase free)	-	fill up to 50 µL	-

*keep on ice while using

5.2.1. Prepare the reaction-mix at room temperature and mix well.

5.2.2. Incubate for 2 hours at 37 °C

Optional: Add 2 µL (2 U) DNAse I (RNase free) after incubation to remove template DNA. Mix well and incubate for 15 min at 37 °C.

5.2.3. To stop the RNA Transcription, add 2 µL 0.5 M EDTA pH 8.0 (RNase free) and incubate 10 min at 65 °C.

5.3. RNA Purification

After the RNA Transcription, RNA purification is necessary to remove free 5-EUTP and ETDA. We recommend using a RNA Purification Kit (e.g. Qiagen RNeasy Mini Kit). Use only RNase free equipment and reagents. Dilute the RNA after purification in DEPC-treated H_2O (RNase free).

6. Click Protocol for RNA Labeling

6.1. Calculation of the Label (Dye) Amount

Calculate the total amount of RNA n_{RNA} (nmol) present in your sample:

$$n_{RNA}\ (\text{nmol}) = \frac{\beta\ \left[\frac{\text{ng}}{\mu\text{L}}\right] \times\ \text{V}\ [\mu\text{L}]}{\text{MW}\ \left[\frac{\text{g}}{\text{mol}}\right]}$$

- β (ng/µL): RNA concentration of your sample
- MW (g/mol): molecular weight of your RNA
- V (µL) = volume of your sample

Calculate the total amount of alkyne modifications $n_{alkynes}$ (nmol) in your RNA. This amount corresponds to the amount of uridine in your DNA, if you used a NTP-mix containing 100% 5'-EUTP instead of UTP.

$$n_{alkynes}\ [\text{nmol}]\ =\ \text{number of uridine}\ \times\ n_{RNA}\ [nmol]$$

Calculate the amount of azide n_{azide} (nmol) for labeling the alkyne-modified RNA. Labeling rates depend on the amount of dye azide applied. Normally 1 - 30 equivalents of azide per alkyne group are used, resulting in labeling rates of up to 20%.

$$n_{azide}\ [\text{nmol}]\ =\ n_{alkynes}\ [\text{nmol}]\ \times\ \text{k}$$

- $n_{alkynes}$ (nmol) = calculated above
- k = equivalents of azide (normally 1 – 30)

Calculate the volume of label azide you need for your sample.

$$V_{dye\ azide\ 10\ mM} = \frac{n_{azide}\ [\text{nmol}]}{10\ \frac{\text{nmol}}{\mu\text{L}}}$$

6.2. Click Reaction

6.2.1. Shortly centrifuge the solid catalyst Reactor S (**Component J**) to place all catalyst on the bottom of the vial.

6.2.2. Add 5 µL of the activator (**Component I**). (Be aware that the catalyst is solid and will not be dissolved during the click reaction!)

6.2.3. Add the RNA solution to the solid catalyst and the activator. Use a RNA solution with a concentration of around 100 ng/µL or higher. Overall volume of the reaction mix should be preferably around 20 - 50 µL.

6.2.4. Add the calculated amount of azide solution (**Component H**). For calculation of the Label amount, see calculations outlined in section **6.1**. Mix well and spin down.

Attention: A large excess of dye azide in the reaction can lead to a highly labeled RNA that might be lost during spin column purification! This highly labeled RNA can also lose the typical features of unlabeled RNA like water solubility and hybridization properties!

6.2.5. Incubate for 60 min at 45 °C under gently shaking (around 600 rpm). Make sure that the Reactor S is within the reaction mixture during the whole reaction time.

6.2.6. Transfer the supernatant (without the solid catalyst) containing the reacted RNA into a new vial.

6.2.7. Purify the clicked RNA. We recommend using a RNA Purification Kit (Qiagen RNeasy Kit). Alternatively, it is also possible to purify the RNA by ethanol precipitation (see step **4.1**). Use RNase free reagents and equipment.

7. Calculation of the RNA Labeling Rate

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

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

Table 6: Excitation, molar extinction coefficient and correction factor of fluorescent dyes

Product number	Dye	Excitation (nm)	Extinction coefficient ($cm^{-1} M^{-1}$)	Correction factor
BCK-RNA488-10	6-FAM Azide	496	83.000	0.35
BCK-RNA555-10	5-TAMRA-PEG3-Azide	546	91.000	0.32
BCK-RNA594-10	5/6-Sulforhodamine 101-PEG3-Azide	584	116.000	0.23
BCK-RNA647-10	Eterneon-Red 645 Azide	643	250.000	0.08

7.2. 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:

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

7.3. Calculate an incorporation rate. The dye to base ratio is given by:

$$\text{dye/base} = \frac{A_{dye} \times \varepsilon_{base}}{A_{base} \times \varepsilon_{dye}}$$

$$\text{ØRNA}_{single\ stranded}\ (GC = 50\%): \varepsilon_{base} \approx 12030\ cm^{-1}M^{-1}$$

Table 7: Molar extinction coefficient ε_{Base} is dependent on base composition (single stranded) and should be considered for the sample actually used.

kind of bases	ε per base [$cm^{-1} M^{-1}$]
AMP	15.400
CMP	9.000
GMP	13.700
UMP	10.000

8. Troubleshooting

8.1. If no labeled RNA could be recovered, the labeling rate might have been too high and labeled RNA was lost during spin column purification (dyed spin column). In that case use less amounts of dye for click labeling.

8.2. RNA fragments can have folded structures, which hinder accessibility of the functional groups that are needed for the click reaction. By adding some DMSO (5 - 10% (v/v) final) during click reaction these folded structures are destabilized and improved reaction progress can be observed.

8.3. Some (dye) labels show low solubility in the final reaction mixture, when used at higher concentrations and amounts, especially when high labeling rates are desired. This can impair reaction progress and decrease the yield. Try to use additional or alternative co-solvents like methanol or tetrahydrofuran if possible.

8.4. In some cases, using high labeling rates for the dye conjugation to the RNA alkyne modified fragments can lead to changed properties of the product (e.g. altered solubility). In that case, please decrease the labeling rate.