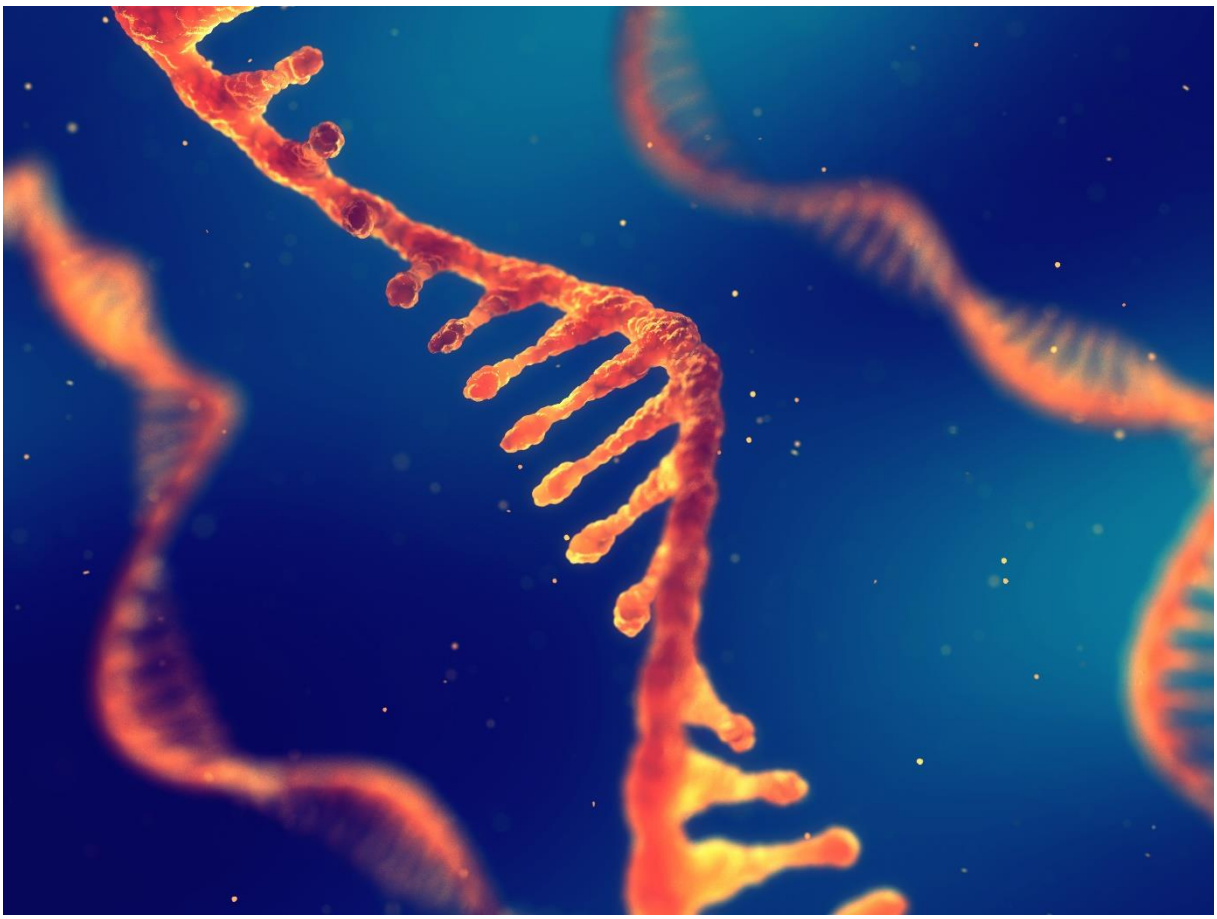
3’ RNA End Labeling Kit 647

- BCK-RNAAA-647 -

User Manual



Version 0.2

Ordering Information:

(for detailed kit content of the kits see Table 1)

3’ RNA End Labeling Kit:

Product Number	Product Name	Detection Filter
BCK-RNAAA-647	**3’ RNA End Labeling Kit 647**	Red (Cy5 filter)

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When describing a procedure for publication using this product, please refer to it as *baseclick 3’ RNA End Labeling Kit*.

3' RNA End Labeling Kit –
For attaching functions to the 3'-end of RNA

The *3' RNA End Labeling Kit* contains reagents to perform 10 enzymatic reactions (25 µL each) and 12 independent labeling reactions (including purification).

Introduction and product description:

Baseclick's *3' RNA End Labeling Kit* has been developed for *in vitro* labeling of mRNA molecules. The kit introduces a single azide at the 3'-end of the mRNA. This azide can be reacted with alkyne-derivates of fluorescent dyes, haptenes and other labels in a highly selective fashion under benign click reaction conditions. Fluorescently labeled mRNA can be used to detect localization of the mRNA inside of cells after transfection.

1. Materials and Storage Conditions

Table 1: Contents of the *3' RNA End Labeling Kit* and storage conditions.

Vial lid	Quantity	Component	Storage
red	30 µL	Cyanine 5-Alkyne (in BCK-RNAAA-647)	–20 °C
blue	15 µL	20x Azide Labeling Mix	–20 °C
orange	60 µL	5x RNA Labeling Buffer	–20 °C
violet	15 µL	Poly(A) Polymerase 600 U/µL	–20 °C
yellow	35 µL	10x Activator² (RNase-free)	–20 °C
green	12x	Reactor 25 (RNase-free)	RT
colorless	14 mL	Binding Buffer	RT
colorless	6 mL	Wash Buffer (concentrated)*	RT
colorless	2.5 mL	Elution Buffer	RT
colorless	24x	BaseClean Columns + Collection Tube	RT
colorless	10 mL	mRNA Transfection Buffer	4-8 °C
colorless	100 µL	mRNA Transfection Reagent	4-8 °C

* upon first usage add 25 mL ethanol (96-100 %) to Wash Buffer

2. Required Material and Equipment not included in this Kit

- RNase free water (e.g. DEPC-treated water)
- Microcentrifuge tubes (RNase free)
- Thermomixer, thermocycler or water bath
- Ethanol absolute
- Polyacrylamide or agarose gel electrophoresis
- Nanophotometer or a possibility to quantify oligonucleotide amounts
- Cell culture lab and fluorescence microscope with Cy5 filter

3. Workflow

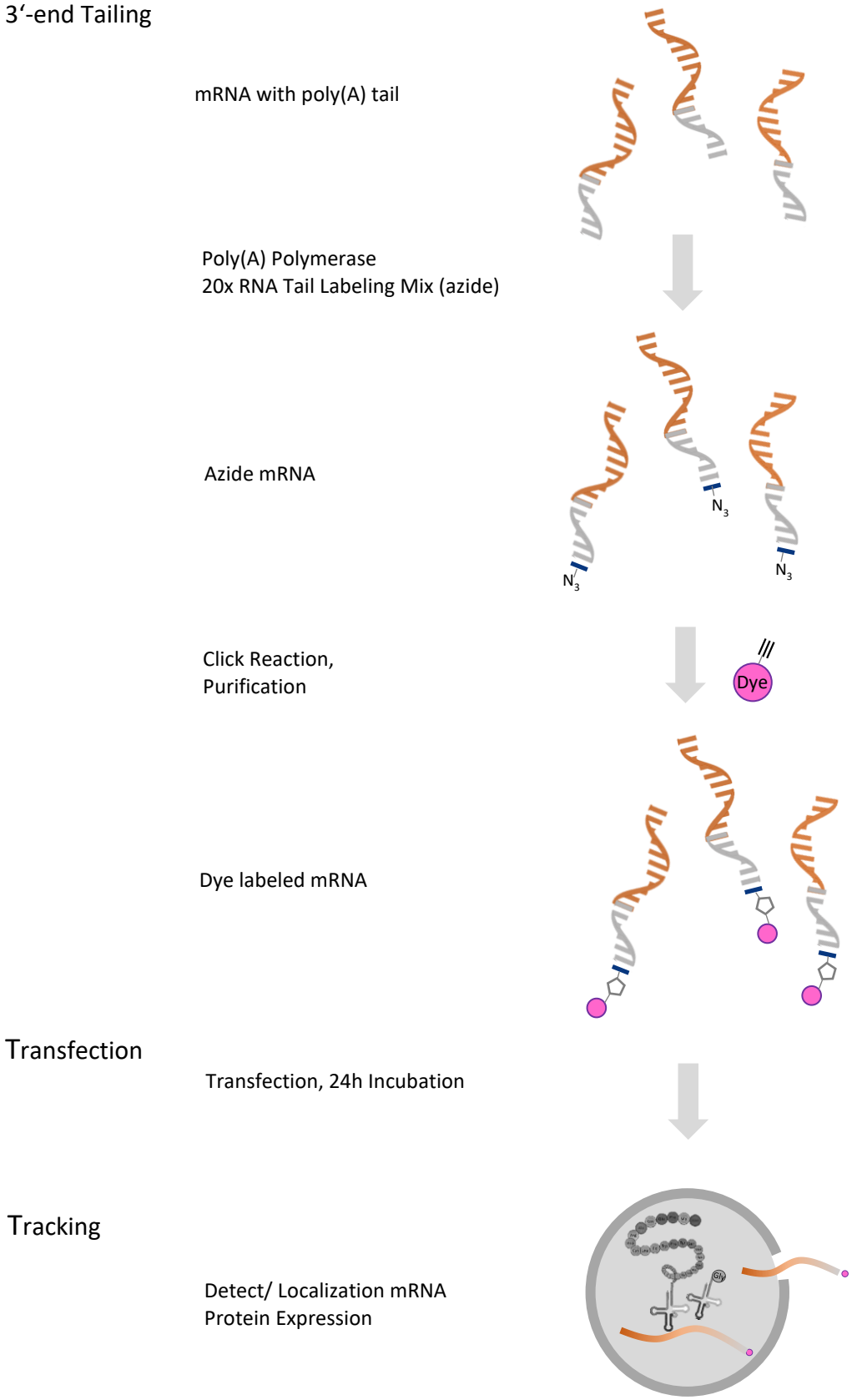

Figure 1: General workflow of the 3’ RNA End Labeling Kit

4. Azide Labeling

In general, 5-10 µg of mRNA can be labeled using the following protocol. It is important that the mRNA preparation does not contain nucleotides as these might interfere with the azide labeling.

- Thaw the 20x RNA Azide Labeling Mix on ice and spin down briefly (blue lid)
- Thaw the 5xRNA Labeling Buffer at room temperature and spin down briefly (orange lid).
- Keep the Poly(A) Polymerase in an enzyme cooler (–20 °C) or on ice (violet lid).
- Prepare the reaction mixture at room temperature:

Table 2: Reagents and amounts for azide labeling.

Vial lid	Amount	Component
Not provided with the kit	5-10 µg	mRNA (280-580 ng/µL)
blue	1.25 µL	20x Azide Labeling Mix
orange	5 µL	5x RNA Labeling Buffer
violet	1 µL	Poly(A) polymerase 600 U/µL
Not provided with the kit	to 25 µL total volume	RNase free water

- Mix by pipetting, spin down briefly and incubate for 30 minutes at 37 °C.
- Inactivate the enzyme at 65 °C for 10 minutes.

5. RNA Purification

After azide labeling, RNA purification is necessary to remove excess azide and the enzyme.

Important Notes:
Buffers provided in this purification system contain irritants.
Wear gloves and lab coat when handling these buffers.
Upon first usage add the required volume of ethanol (24 mL, 96-100% (v/v)) to the Wash Buffer bottle to allow its proper function.
All centrifugation steps are done at 10,000 x g.

Table 3. Reagents and amounts for RNA purification.

Vial lid	Amount for 25 µL mRNA mixture	Component
colorless	125 µL	Binding Buffer
colorless	600 µL	Wash Buffer*
colorless	30 µL	Elution Buffer
colorless	1	BaseClean Columns + Collection Tube
Not provided with the kit	2	RNase free tubes

*add 24 mL ethanol (96-100%) to Wash Buffer (concentrated) when first opened

- Transfer the mixture of the reaction and add 5 volumes of Binding Buffer to a 1.5 mL microcentrifuge tube (not provided) then mix properly.
- Place a BaseClean Column to a Collection Tube and transfer the sample mixture to the BaseClean Column.
- Centrifuge for 1 min, then discard the flow-through.
- Add 600 µL of Wash Buffer (ethanol added) to the BaseClean Column. Centrifuge for 1 min then discard the flow-through.
- Centrifuge for additional 3 min to dry the BaseClean Column. This step will avoid the residual liquid to inhibit subsequent reactions.
- Place the BaseClean Column into a new 1.5 mL microcentrifuge tube (not provided)
- Add e.g. 30 µL of Elution Buffer* to the membrane center of BaseClean Column. Incubate at room temperature for 2 min. (**Note**: Elution volumes down to 10 µL can be used to obtain more concentrated samples)
- Centrifuge for 1 min to elute the RNA. (**Note**: the average eluate volume is 9 µL from 10 µL Elution Buffer volume)

*heating the elution buffer to 70°C can increase the recovery of the mRNA

At this point the mRNA can be stored below –70 °C for long-term storage.

Phenol/chloroform extraction protocol

An alternative purification to the provided column-based kits is the phenol/chloroform extraction protocol, which can provide high-quality RNA. This should be done in a fume hood or well-ventilated space.

- Add 10% volume of 3 M sodium acetate solution to the RNA solution and mix
- Add an equal volume of a 1:1 phenol/chloroform mixture and extract, keep the aqueous phase (upper layer).
- Add an equal volume chloroform and extract, keep the aqueous phase. Repeat the chloroform extraction and collect the aqueous phase in a fresh vial.
- Precipitate the DNA by adding two volumes of ethanol (absolute) and incubate at -20 °C for at least 30 min.
- Centrifuge the sample at maximum speed for 5 min to collect the precipitate.
- Remove the supernatant and rinse the pellet with cold ethanol (70%, 500 µL).
- Centrifuge again the sample at maximum speed for 5 min to collect the precipitate.
- Resuspend the RNA in 10-20 µL water (RNase free).

6. Click Labeling of the mRNA

Table 4. Reagents and amounts for click labeling of the mRNA.

Vial lid	Quantity	Component
red	2.5 µL	Cyanine 5-Alkyne (in BCK-RNAAA-647)
yellow	2.5 µL	10x Activator² (RNase-free)
green	1x	Reactor 25 (RNase-free)

- Add 2.5 µL 10x Activator² (yellow lid) to the Reactor 25 (green lid)
- Add 2.5 µL of dye alkyne (red lid) to the vial.
- Add 20 µL of purified azide labeled mRNA to the vial and mix thoroughly; pipette up and down. Briefly spin down to place the components at the bottom of the vial.
- Close the vial and incubate the mixture at 45 °C, 600 rpm for 20 minutes in a thermomixer. Alternatively, a water bath and slight agitation can be used (shake the vial from time to time).
- After the reaction transfer the supernatant with the clicked mRNA to a new vial (without pellets).
- Purify the clicked mRNA to remove excess of dye alkyne (see section 5, p. 6) and determine the amount using a nanophotometer.

At this point the mRNA can be stored below –70 °C for long-term storage

7. mRNA Transfection and Detection

The mRNA amount required for the experiment depends on the well size used for cell culture and the number of transfections. The following conditions have been determined for a well of a 24-well plate. For other culture formats, please refer to Table 5.

- For optimal mRNA transfection conditions, we recommend using cells which are 60 to 80% confluent at the time of transfection. Typically, for experiments in 24-well plates, 50 000 adherent cells or 100 000 suspension cells are seeded per well in 0.5 mL of cell growth medium 24 h prior to transfection.
- On the day of transfection, dilute 0.5 µg of mRNA into 50 µL Transfection Buffer (colorless lid). Mix by vortexing 10 seconds, spin down briefly.
- Add 1 µL Transfection Reagent (colorless lid), mix by vortexing 10 seconds, spin down briefly.
- Incubate for 15 minutes at RT.
- Add the total volume of transfection mix per well dropwise onto the cells in growth medium (containing serum or not) and/or additives, and distribute evenly.
- Gently rock the plate back and forth and from side to side.
- Perform analysis after 12-48 h incubation (24 h recommended).

Table 5. mRNA transfection guidelines per well according to the cell culture vessel.

Culture vessels	mRNA Transfection Buffer (µL) (colorless lid)	mRNA (µg)	mRNA Transfection Reagent (µL) (colorless lid)
96-well	12.5	0.1 ± 0.05	0.25 ± 0.05
24-well	50	0.5 ± 0.1	1 ± 0.2
12-well	100	1 ± 0.2	2 ± 0.4
6-well	200	2 ± 0.5	4 ± 0.8

Notes:
The provided mRNA Transfection Buffer should be used for successful transfection with mRNA Transfection Reagent.
Prepare a master mix of minimum 50 µL to allow accurate pipetting and homogenous preparation of the complexes.
Performing media change 4 h post-transfection may improve cell viability.
Transfection should be performed in a RNase-free working-environment and mRNA should be diluted and aliquoted in RNase-free water.

8. Detection

For detection of the mRNA, a fluorescence microscope is required which is equipped with a Cy5 filter (BCK-RNAAA-647, excitation 604-644 nm, emission 672-712 nm).

Table 6: Emission and excitation maxima of the available dyes.

Product number	Dye	Exxitation (nm)	Emission (nm)	Filter
BCK-RNAAA-647	Cy5 alkyne	647	663	Cy5

9. Exemplary Results

These are exemplary results from using the components and protocols of the kit for terminal labeling of an eGFP mRNA with a Cy5 dye.

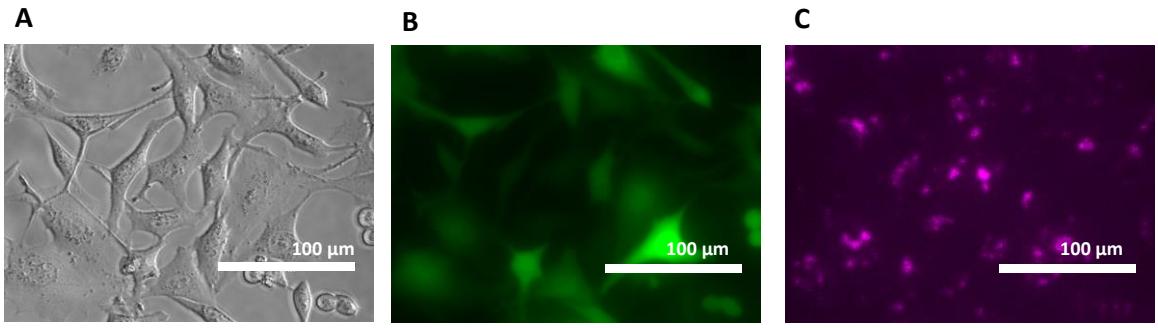

Figure 2. Images of HeLa cells, which have been transfected with Cy5 alkyne labeled mRNA coding for enhanced green fluorescent protein (eGFP) 24 hours prior imaging. Cells were monitored in bright field (A) and fluorescence mode (B-C). Fluorescence of the eGFP was detected using an FITC filter (B), fluorescence of the labeled mRNA was detected using a Cy5 filter (C).

10.Trouble Shooting

No labeled RNA:

Some standard commercial buffer components can decrease the click reaction efficiency or even impair reaction process. For example, TE buffer contains ethylenediaminetetraacetic acid (EDTA), which can chelate Cu^{II} ions and decrease the reaction rate. Make sure not to use TE buffer for elution of the purified mRNA before click labeling. Thiol groups from reducing agents like β-mercaptoethanol or dithiothreitol (DTT) can stop the click reaction.

Low purification yields:

Problems	Possible reason	Solution
Low or no recovery of RNA	More than 100 µL of RNA transcript product applied	If the solution is more than 100 µL, separate it into multiple tubes
	Elution of RNA is not efficient	Make sure that the pH of Elution Buffer or bidistilled water is between 7.0 – 8.0
		Make sure that the elution solution has been completely absorbed by the BaseClean Column membrane before centrifugation
	The size of RNA is larger than 5 Kb	Preheat the elution solution to 60°C before use
Poor performance in the downstream applications	Salt residue remains in eluted RNA	Wash the BaseClean Column twice with Wash Buffer
	Ethanol residue remains in eluted RNA	Do discard the flow-through after washing with Wash Buffer and centrifuge for an additional 3 min

Low mRNA transfection efficiency:

Problem	Solution
Low mRNA transfection efficiency	Optimize the volume of mRNA Transfection Reagent and the amount of mRNA added per well
	Increase the volume of mRNA Transfection Reagent first; if insufficient, increase the amount of mRNA
	The volume of mRNA Transfection Reagent may range between 1.6 – 2.4 µL per µg of mRNA depending on the transfected cell line
	Replace medium containing serum with serum-free medium during transfection
	Replace medium after 4h of transfection.
	Ensure that all reagents are RNase free
	Ensure the medium is permissive to the transfection

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