LABELING KIT

ULS™

ULS™ aRNA Fluorescent Labeling Kit

For 25 dual labeling reactions

Product code
EA-002/EA-006/EA-008

For laboratory use only
Research purposes only

KREATECH
BIOTECHNOLOGY

Instruction manual

KREATECH's ULS™ aRNA Fluorescent Labeling Kit

This kit is intended for RESEARCH USE ONLY. IT IS NOT INTENDED FOR DIAGNOSTIC APPLICATIONS and/or COMMERCIAL PURPOSES.

Important

Open the kit immediately and store all components as instructed on page 7

- Read the entire Instruction manual before starting your experiment.
- Do not mix reagents from different kits.
- During the preparation of reagents and throughout the entire procedure please observe the safety regulations issued for laboratories concerning handling of samples.
- Dispose of reagents according to relevant local regulations. Take appropriate safety precautions such as wearing a lab coat, gloves and eye protection.

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A. Assay Materials

I. Components and Storage

Component	Amount	Storage
Cyanine5/Cy5/DY647-ULS™	25 reactions	4°C
Cyanine3/Cy3/DY547-ULS™	25 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	50 pcs	4°C
KREA*block*™	1.5 mL	–20°C*

** KREAblock is shipped at 4°C, store at –20°C upon delivery.*

II. Reagents and Buffers not Included in Kit

Fragmentation reagents (Ambion Cat # 8740)

B. General Information

I. Background

Gene expression profiling using DNA-microarrays has been growing rapidly over the last decade. When working with material from fine needle biopsy, laser capture micro-dissection material and in fact with patient material in general, the amount of target material is limited. This has necessitated the need to carry out target amplification, generally by linear amplification based on a protocol first described by van Gelder and Eberwine[1]. This procedure is based on the reverse transcription from mRNA into double stranded cDNA using an Oligo(dT) primer containing a T7 RNA polymerase promoter sequence. Linear amplification is achieved during the subsequent IVT reaction using T7 polymerase with cDNA as a functional template.

The ULS-aRNA-Fluorescent-Labeling-Kit has been designed to enable the generation of unmodified amplified aRNA using natural nucleotides, which can then be labeled with ULS dyes, thereby providing maximum flexibility for the researcher.

II. Principle of ULS Labeling

The proprietary ULS technology is based on the stable binding properties of a platinum complex to biomolecules. The ULS molecule consists of a platinum complex, a detectable molecule and a leaving group which is displaced upon reaction with the target. This ULS molecule forms a co-ordinative bond, firmly coupling the ULS to the target. ULS labels DNA, and RNA by binding to the N7 position of guanine. In proteins, ULS binds to nitrogen and sulphur containing side chains of methionine, cysteine and histidine (see figure inside back cover). ULS is available coupled to a variety of labels and haptens, including fluorochromes and biotin.
ULS thereby enables one-step non-enzymatic labeling of nucleic acids to be achieved within 15-30 minutes. ULS labeling can be performed with or without enzymatic amplification, prior too labeling.

III. ULS aRNA Fluorescent Labeling Process

The procedure of expression analysis with the ULS aRNA-Fluorescent-Labeling-Kit is as follows: (illustration see figure 2)

1. aRNA is generated from isolated total RNA via linear amplification using natural unmodified nucleotides
2. aRNA is non-enzymatically labeled with ULS reagents (15-30 min)
3. The labeled aRNA is purified with the KREA*pure* column
4. The labeled aRNA is fragmented
5. The fragmented and labeled aRNA is hybridized to a microarray in the presence of KREA*block* (optional)
6. A laser detection system is used to scan and report the relative quantity of the two dyes at any given spot on the microarray
7. Finally, computer-imaging software processes the differential scanning data to analyze the complete array

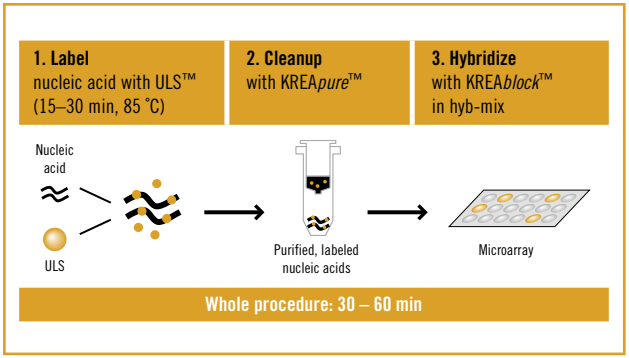


Figure 2: A 30-60 minute protocol for DNA microarray applications

IV. Schematic Overview of the ULS aRNA Fluorescent Labeling Process

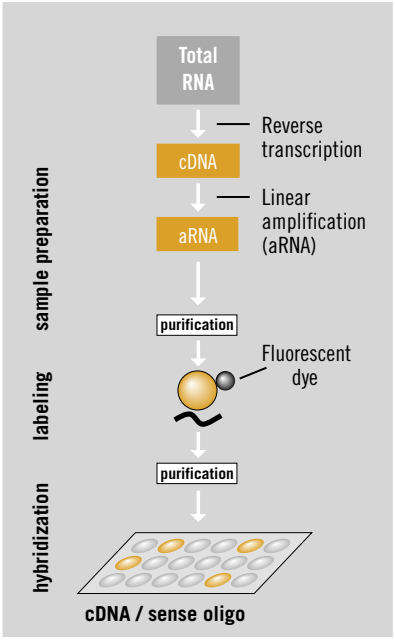


Figure 3. Schematic Overview of ULS aRNA Fluorescent Labeling Process

C Protocol

I. Total RNA Isolation

A wide variety of RNA isolation techniques are available, e.g using Trizol (Invitrogen) extraction followed by RNeasy column purification (QIAGEN). Irrespective of which isolation procedure is used the RNA material should be free from DNA and other contaminants. Assessment of the purity and yield of your RNA should be carried out by

a. Running your RNA on a 1% agarose gel. The integrity of the total RNA is determined by observing the ribosomal bands
b. Determining the OD_{260}. For all RNAs $OD_{260/280}$ should be >1.9 and $OD_{260/230}$ should be >2.1

II. Linear Amplification

ULS technology allows labeling of aRNA generated from a variety of commercially available kits, e.g. Message amp aRNA amplification kit (Ambion), Mega script T7 kit (Ambion).

Important! With the ULS protocol, amplification is carried out prior to labeling. Enzymatic reactions should be carried out using only unmodified nucleotides. This results in better yields, longer fragments and a more stable amplified RNA sample.

Furthermore, aRNA samples need to be clean of divalent cations (e.g. Mg^{2+}) salt and other (wash) buffer components which could disturb the labeling efficiency.
Be aware that some components in silica based purification systems may inhibit the ULS reaction. A final wash step using 80% ethanol (PA) before elution prevents this. Alternatives: alcohol precipitation of eluted material or cleanup using an EDTA, sephadex combination.

III. aRNA Labeling

a. ULS Labeling Procedure

Briefly spin all required reagents to collect contents of tubes

1. Take 2 µg of aRNA ensure final concentration in labeling reaction is above 50 ng/µL. In general suboptimal modification degrees of the labeled material are achieved if final concentration of the aRNA in the labeling mixture is below 50 ng/µL)
2. Add 2 µL of Cyanine5/Cy5/DY647-ULS or Cyanine3/Cy3/DY547-ULS per 2 µg aRNA
3. Add 1/10 volume of 10 x Labeling solution
4. Adjust with RNase-free water to final volume and mix by pipetting (see example set-up below)
5. Label sample by incubation for 15 min at 85°C (if using Cyanine ULS incubate for 30 minutes at 85°C)
6. Place samples on ice, spin down to collect contents of tube before proceeding with purification using the KREA*pure* columns

Example of Cyanine/Cy/DY-ULS labeling of 2 µg aRNA

	Cyanine3/Cy3/ DY547-ULS	Cyanine5/Cy5/ DY647-ULS
aRNA (2 µg) + RNAse free water	16.0 µL	16.0 µL
Cyanine/Cy/DY -ULS	2.0 µL	2.0 µL
10 x labeling solution	2.0 µL	2.0 µL
Total volume	20.0 µL	20.0 µL

b. Dye Removal using KREA*pure* Columns

Removal of free ULS label using KREA*pure* columns

(20800g is equivalent to 14,000 rpm on eppendorf 5417C)

1. Resuspend column material by vortexing
2. Loosen cap ¼ turn and snap off the bottom closure
3. Place the column in a 2 mL collection tube
4. Pre-spin the column for 1 minute at 20800 g
5. Discard flow through and re-use collection tube
6. Wash the column with 300 µL RNase free water
7. Discard collection tube and flow-through
8. Put column in a new (RNase free) 1.5 mL micro centrifuge tube
9. Add ULS-labeled aRNA on to column bed
10. Spin column for 1 minute at 20800 g
11. Flow through is purified labeled aRNA

At this point the degree of labeling (DOL) can be measured (see page 17)

c. aRNA Fragmentation

(Below describes the protocol using the fragmentation reagents from Ambion #8740)

1. Pool Cyanine5/Cy5/DY647-ULS and Cyanine3/Cy3/DY547-ULS labeled samples
2. Transfer the mixture to a microfuge tube and add 1/10 volume of 10x fragmentation buffer (Ambion) to decrease the fragment size to 60-200 bases. (e.g. 4 µL in final volume of 40 µL)
3. Incubate at 70°C for 15 minutes
4. Spin the vial briefly and add 1µL of stop solution Ambion), mix by pipetting (the labeled aRNA can form aggregates which dissolve by pipetting) and place on ice until further use

IV. Preparation of Labeled Material for Hybridization

This kit supplies a KREA*block* solution which can help to reduce background on your array. We recommend that it is used in combination with the Cyanine dyes. When using the Cy-dyes or the DY-dyes if background is an issue then we also suggest you use KREA*block* in your hybridization mixture.

Use of KREA*block*

1. KREA*block* should be added to ¼ final volume of the hybridization mixture (e.g. 25 µL of KREA*block* in a 100 µL hybridization volume)
2. Hybridize and wash slides according to own protocol (we recommend that the KREA*block* solution be used to provide the moisture in the hybridization chamber)

D. Trouble Shooting

I. Total RNA and aRNA Preparation and Analysis

Problem	Possible Reasons and Suggestions
OD_{260} not within parameters	Cause: Impure RNA Remedy: Repeat RNA clean-up kit using commercial kit or precipitate RNA and dissolve again

II. ULS™ Labeling

Problem	Possible Reasons and Suggestions
Degree of labeling too low	Cause: There may be salt present which disturbs labeling Remedy: Clean up aRNA and ensure final 80% ethanol wash step is used with silica based columns (see C II) Cause: Incorrect ratio of labeling reagent to aRNA Remedy: Ensure use of instructed amount of ULS per µg of aRNA Cause: Concentration of the labeling reaction was under 50 ng/µL Remedy: Ensure concentration of the labeling reaction is above 50 ng/µL
High labeling density	Cause: Incorrect ratio of labeling reagent to aRNA Remedy: Ensure use of instructed amount of ULS per µg of aRNA

III. Array Hybridization and Detection

Problem	Possible Reasons and Suggestions
Background on the slide	Cause: Too much sample added to microarray Remedy: Reduce sample amount Cause: Insufficient blocking Remedy: Add more or alternative blockers to pre-hybridization or hybridization buffer Cause: Partial drying of hybridization buffer during hybridization due to insufficient amount of moisture in hybridization vessel Remedy: Ensure sufficient moisture is added to hybridization chamber and vessel is sealed tightly

E. Appendix

I. Determination of RNA Quality

- Measure A_{260} and A_{280} nm using a spectrophotometer and calculate $OD_{260/280}$. For good quality RNA this value should be between 1.9 and 2.1

II. Determination of the Degree of Labeling (DOL)

- Measure A_{260} and A_{550} for determining the DOL of Cyanine3/Cy3/DY547-ULS labeled aRNA
- Measure A_{260} and A_{650} for determining the DOL of Cyanine5/Cy5/DY647-ULS labeled aRNA

$$\text{ng / µL} = \frac{A_{260} \text{ *dilution factor* } 40}{\text{cuvet length (in cm)}}$$

$$\text{pmol / µL} = \frac{A_{\text{dye at max}} \text{* dilution factor}}{\text{cuvet length } * \varepsilon_{\text{dye}} * 10^{-6}}$$

εdye Cyanine3/Cy3/DY547 Reagent = 150.000

εdye Cyanine5/Cy5/DY647 Reagent = 250.000

Degree of labeling (amount of dyes per 100 nucleotides)

$$\text{Labeling \%} = \frac{340 * \text{pmol}_{\text{dye}}}{\text{ng}_{\text{ nucleic acid}} * 1000 \text{ x } 100\%}$$

F. References

Van Gelder RN et al. (1990), Proc Natl Acad Sci USA 87: 1663-1667

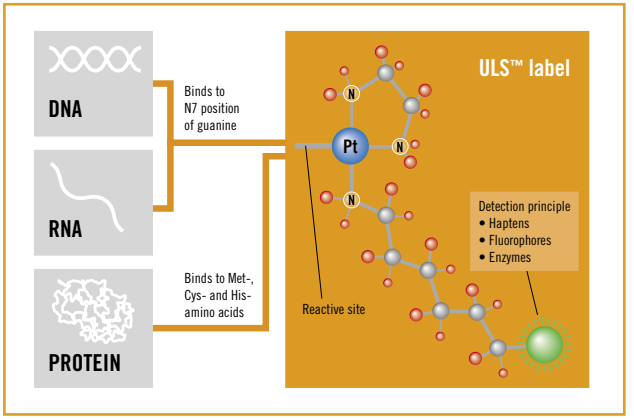
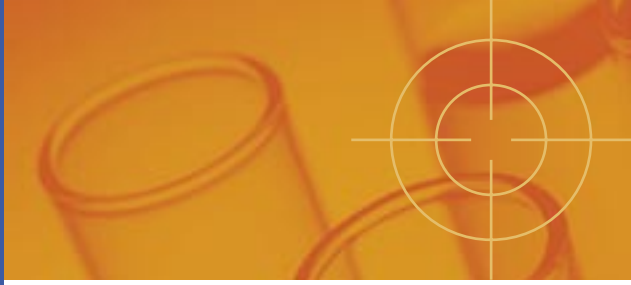


Figure. ULS™ labels RNA by forming a coordinative bond on the N7 position of guanine.



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