LABELING KIT

ULS™

ULS™ Fluorescent Labeling Kit for Agilent arrays

with Cy3 and Cy5 - ULS
with Cy5 - ULS
with Cy3 - ULS

For 25 dual labeling reactions - EA-021
For 25 single labeling reactions - EA-022/EA-023

Product code
EA-021/EA-022/EA-023

For laboratory use only
Research purposes only

KREATECH
BIOTECHNOLOGY

Instruction manual

KREATECH's ULS™ Fluorescent Labeling Kit for Agilent arrays

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.

Limited Product Warranty – Patent Disclaimer

This warranty limits our liability to replacement of this product. No other warranties of any kind expressed or implied, including without limitation, implied warranties or fitness for a particular purpose, are provided by KREATECH. KREATECH shall have no liability for any direct, indirect, consequential, or incidental damages arising out of use, or the inability to use this product.

Ordering Information and Technical Services

KREATECH Biotechnology B.V.

Visiting address
Vlierweg 20
1032 LG Amsterdam
The Netherlands

Postal address
P.O. Box 37078
1030 AB Amsterdam
The Netherlands

Phone: +31 20 691 9181
Fax : +31 20 696 3531
E-mail: info@kreatech.com
www.kreatech.com

Technical Services
techservices@kreatech.com

Table of contents

Limited Product Warranty and Patent Disclaimer 3
Ordering Information and Technical Services 4

A. Assay Materials 7
I. Components and Storage 7
II. Reagents and Buffers not Included in Kit 7

B. General Information 8
I. Background 8
II. Principle of ULS Labeling Procedure 8
III. ULS Fluorescent Labeling Process 10
IV. Schematic Overview of ULS Fluorescent Labeling Process 11

C. Protocol 12
I. Total RNA Isolation 12
II. Linear Amplification of aRNA 12
III. aRNA Labeling 13
a. ULS Labeling Procedure 13
b. Dye Removal Using KREA*pure* Columns 14
c. aRNA Fragmentation 15
IV. Preparation of Labeled Material for Hybridization 15

D. Trouble Shooting 16
I. Total RNA and aRNA Preparation and Analysis 16
II. ULS Labeling 16
III. Array Hybridization and Detection 17

E. Appendix 17
I. Determination of RNA Quality 17
II. Determination of Degree of Labeling (DOL) 17

F. References 18

A. Assay Materials

I. Components and Storage

For product code EA-021

Component	Amount	Storage
Cy5-ULS	25 reactions	4°C
Cy3-ULS	25 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	50 pcs	4°C
KREA*block*™	3 mL	–20°C*

For product code EA-022 or EA-023

Component	Amount	Storage
Cy5-ULS (EA-022) or Cy3-ULS (EA-023)	25 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	25 pcs	4°C
KREA*block*™	3 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 with patient material in general, the amount of target material is limited. This has made it necessary 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 Fluorescent Labeling Kit for Agilent arrays 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.

This kit has been formatted to be convenient for use on Agilent's gene expression microarrays

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.

In addition to ULS Labeling Kits for Agilent microarrays, KREATECH Biotechnology also provides kits to amplify your RNA before labeling: the RNA ampULSe: Amplification and Labeling Kits for Agilent microarrays. These kits employ Ambion's widely used MessageAmp™II aRNA amplification reagents followed by labeling with KREATECH's proprietary ULS technology. There are three available formats for use with Agilent microarrays:

Product Code	Name	Reactions
	Amplification and Labeling Kits	
GEA-014	RNA ampULSe – amplification and labeling kit with Cy3 and Cy5 ULS for Agilent gene expression microarrays	20
GEA-016	RNA ampULSe – amplification and labeling kit with Cy5 ULS for Agilent gene expression microarrays	20
GEA-018	RNA ampULSe – amplification and labeling kit with Cy3 ULS for Agilent gene expression microarrays	20

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 to labeling. ULS labeling of RNA and DNA is compatible with all DNA microarray platforms. This set of kits are specially formatted to be convenient for use with the Agilent arrays.

III. ULS aRNA Fluorescent Labeling Process (Figure 2)

1. Generation of aRNA from isolated total RNA via linear amplification using natural unmodified nucleotides
2. Non-enzymatic labeling of aRNA with ULS reagents (15-30 min)
3. Purification of the labeled aRNA with the KREA*pure* column
4. Fragmentation of the labeled aRNA
5. Hybridization of the fragmented and labeled aRNA to a microarray in the presence of KREA*block* (optional)
6. Scanning of the microarray
7. Analysis of the data using appropriate software

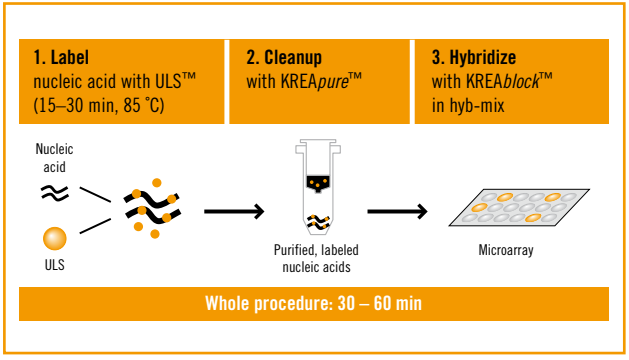

Figure 2: A 30-60 minute protocol for DNA microarray applications

IV. Schematic Overview of the ULS aRNA Fluorescent Labeling Process

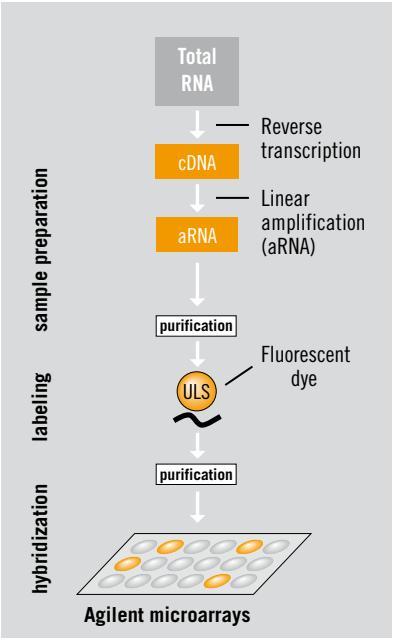

Figure 3. Schematic Overview of ULS aRNA Fluorescent Labeling Process

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+}), salts 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

Depending on the Agilent microarray being use the amount needed for hybridization may vary between 0.25 µg and 1 µg. This kit is set up to allow labeling of up to 2 µg per labeling reaction. We recommend that where possible 1-2 µg is labeled per reaction. This allows for controls such as labeling density measurements to be carried out. Afterwards the desired amount can be taken for hybridization.
Briefly spin all required reagents to collect contents of tubes

1. Take 1- 2 µg of aRNA ensure final concentration in labeling reaction is above 50 ng/µL. Optimal modification degrees of the labeled material are not achieved if final concentration of the aRNA in the labeling mixture is below 50 ng/µL)
2. Add 1 µL of Cy5-ULS or Cy3-ULS per 1 µg aRNA (Always keep the ratio of µg of aRNA to µL ULS 1:1 when increasing or decreasing the amount to be labeled).
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 on page 14)
5. Label sample by incubation for 15 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 Cy labeling of 2 µg aRNA

	Cy3-ULS	Cy5-ULS
aRNA (2 µg) + RNAse free water	16 µL	16 µL
Cy-ULS	2 µL	2 µL
10 x labeling solution	2 µL	2 µL
Total volume	**20 µL**	**20 µL**

b. Dye Removal using KREA*pure* Columns

Removal of free ULS label using KREA*pure* columns

(20800 x g 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 x g
5. Discard flow through and re-use collection tube
6. Wash the column with 300 µL RNase free water
7 Spin column for 1 minute 20800 x g
8. Discard collection tube and flow-through
9. Put column in a new (RNase free) 1.5 mL micro centrifuge tube
10. Add ULS-labeled aRNA on to column bed - careful not to pipette on the sides of the column but directly on the column material
11. Spin column for 1 minute at 20800 x g
12. 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. If carrying out a dual color hybridization both colors can be pooled for fragmentation
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. If background is an issue we 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
Degree of labeling too high	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 Cy3-ULS labeled aRNA
- Measure A_{260} and A_{650} for determining the DOL of Cy5-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 Cy3 Reagent = 150,000

εdye Cy5 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

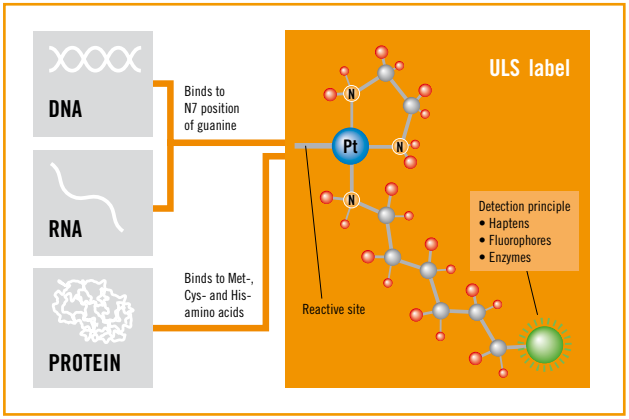

Figure. ULS, the Universal Linkage System that labels your DNA, RNA and proteins.

KREATECH Biotechnology B.V.
Vlierweg 20
1032 LG, Amsterdam
The Netherlands
Tel. +31 20 691 9181
Fax +31 20 696 3531
E-mail info@kreatech.com
www.kreatech.com

KREATECH
BIOTECHNOLOGY

Published August 2006