LABELING KIT

ULS™

ULS™ Labeling Kit For CombiMatrix arrays

with Cy3 and Cy5 ULS
with Cy5 ULS
with Cy3 ULS
with Biotin ULS
with Biotin ULS and SA-PE

For 25 dual labeling reactions - EA-024
For 25 single color labeling reactions - EA-025/ EA-026/EA-027/EA-028

Product code
EA-024/EA025/EA-026/EA-027/EA-028

For laboratory use only
Research purposes only

KREATECH
BIOTECHNOLOGY

Instruction manual

KREATECH's ULS™ Fluorescent Labeling Kit for CombiMatrix 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.

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A. Assay Materials

I. Components and Storage

For product code EA-024

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*™	1.5 mL	–20°C*

For product code EA-025 or EA-026

Component	Amount	Storage
Cy5-ULS (EA-025) **or** Cy3-ULS (EA-026)	25 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	25 pcs	4°C
KREA*block*™	1.5 mL	–20°C*

For product code EA-027 or EA-028

Component	Amount	Storage
Biotin ULS	25 reactions	4°C
10 x Labeling Buffer	100 µL	4°C
KREA*pure*™ columns	25 pcs	4°C
KREA*block*™	1.5 mL	–20°C*
EA-028-Streptavidin Phycoerythrin (1mg/mL)	300µL	4°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

The ULS labeling kit for CombiMatrix arrays can be used to label either amplified or unamplified RNA or DNA. Since ULS labeling is a chemical labeling enzymatic steps are carried out efficiently with natural unmodified nucleotides instead of bulky modified nucleotides. Generating unmodified target also allows more flexibility. The unmodified RNA or DNA can be used in a variety of ways e.g. it can be labeled with either fluorescent or biotin labels, some can be used for a microarray experiment and the rest for an RT-PCR validation.

This kit has been formatted for convenient use with CombiMatrix arrays.

I. Background

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

Product Code	Name	Reactions
	Amplification and Labeling Kits	
GEA-020	RNA ampULSe – amplification and labeling kit with Cy3 and Cy5 ULS for CombiMatrix arrays	20
GEA-022	RNA ampULSe – amplification and labeling kit with Cy5 ULS for CombiMatrix arrays	20
GEA-024	RNA ampULSe – amplification and labeling kit with Cy3 ULS for CombiMatrix arrays	20
GEA-026	RNA ampULSe – amplification and labeling kit with Biotin ULS for CombiMatrix arrays	20
GEA-028	RNA ampULSe – amplification and labeling kit with Biotin ULS and Streptavidin Phycoerythrin (SA-PE) for CombiMatrix arrays	20

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 customarray microarrays from CombiMatrix.

II. ULS Labeling Process (Figure 2)

1. Non-enzymatic labeling of RNA or DNA with ULS reagents (15-30 min)
2. Purification of the labeled RNA or DNA with the KREA*pure* column
3. RNA fragmentation of the labeled (RNA targets only)
4. Hybridization labeled DNA or RNA to a microarray in the presence of KREA*block* (optional)
5. Scanning of the microarray
6. Analysis of the data using appropriate software

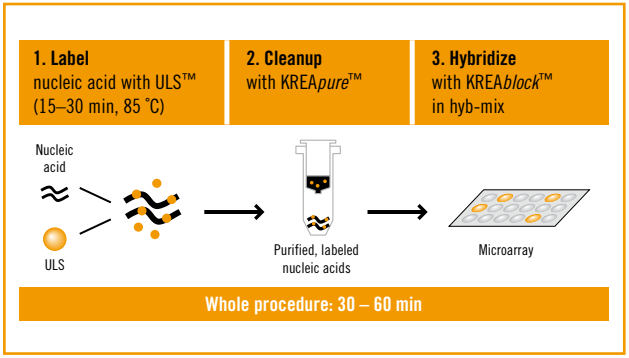

Figure 2: A 30-60 minute protocol for DNA microarray applications

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. Enzymatic steps

There are a number of reason for introducing an enzymatic step.

1. The probes on the microarray are sense orientation and therefore it is necessary to convert the sense mRNA into antisense target.
2. There is a limited amount of starting material and an amplification step is needed.

ULS technology allows labeling of RNA or DNA generated from a variety of commercially available kits e.g. RNA ampULSe; RNA amplification and labeling kit (Kreatech).

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

Important, samples before labeling 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. Labeling

a. ULS Labeling Procedure

CombiMatrix recommends hybridizing with 1-5ug for their Customarray™ 12K microarray and 1-1.5ug for their Customarray™ 2x4K. Each labeling reaction can label up to 5ug of RNA or DNA. it is recommend that 5ug is labeled per reaction and that after checking the labeling density (in the case of the fluorescently labeling samples), the amount needed is taken out and hybridized.

Briefly spin all required reagents to collect contents of tubes.

1. Take 1-5 µg of RNA or DNA 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 RNA or DNA in the labeling mixture is below 50 ng/µL)
2. Add 1 µL of Cy5-ULS, Cy3-ULS or BIO-ULS per 1 µg RNA or DNA (Always keep the ratio of µg of RNA/DNA 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 set-up 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/BIO-ULS labeling of 5 µg RNA or DNA

	Cy3-ULS	Cy5-ULS	BIO-ULS
aRNA (5 µg) + RNAse free water	13 µL	13 µL	13 µL
ULS -dye	5 µL	5 µL	5 µL
10 x labeling solution	2 µL	2 µL	2 µL
Total volume	20 µL	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 sample 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 sample

At this point the degree of labeling (DOL) can be measured when using fluorescent dyes (see page 18)

c. aRNA Fragmentation

When working with RNA samples it is necessary to fragment. (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 or DNA 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 RNA or DNA 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 RNA or DNA 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 RNA or DNA Remedy: Ensure use of instructed amount of ULS per µg of RNA or DNA

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

$$\text{ng / µL} = \frac{A_{260} \text{ *dilution factor* 40 (RNA) 50 dsDNA or 33 ssDNA}}{\text{cuvet length (in cm)}}$$

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

$\varepsilon_{\text{dye Cy3 Reagent}} = 150{,}000$

$\varepsilon_{\text{dye Cy5 Reagent}} = 250{,}000$

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

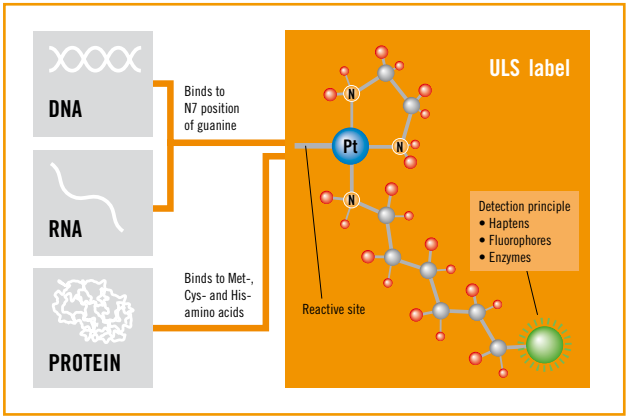

Figure. ULS, the Universal Linkage System that labels your DNA, RNA and proteins.

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