LABELING KIT

ULS™

ULS™ cDNA synthesis and labeling kit

DSK-001 with Cy3 and Cy5
DSK-002 with Cy5
DSK-003 with Cy3
DSK-004 with Biotin
DSK-005 with Biotin + Streptavidin Phycoerytrin

For 20 reactions

Product code
Product code DSK-001/DSK-002/DSK-003/DSK-004/DSK-005

For laboratory use only
Research purposes only

KREATECH
BIOTECHNOLOGY

Instruction manual

KREATECH's ULS™ cDNA Synthesis and 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 5.

- 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.
- MSDS on request or available from our website: www.kreatech.com

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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 – Important

The ULS cDNA Synthesis and Labeling kit contains 20 reactions to generate cDNA from total RNA and label this cDNA with ULS.

Component	Amount	Storage
DSK-001 Cy3/Cy5-ULS	10 Cy3 and 10 Cy5 reactions	2-6º C
DSK-002 Cy5-ULS	20 reactions	2-6º C
DSK-003 Cy3-ULS	20 reactions	2-6º C
DSK-004 Biotin-ULS	20 reactions	2-6º C
DSK-005 Biotin-ULS and SA-PE	20 reactions	2-6º C
DNA purification columns	20 pcs	2-6º C
DNA binding buffer	20 mL	2-6º C
Collection tubes	20 pcs	2-6º C
10 x Labeling buffer	100 µL	2-6º C
KREA*pure* columns	20 pcs	2-6º C
0.5M NaOH	400 µL	2-6º C
1M HCL	200 µL	2-6º C

Component	Amount	Storage
5x M-MLV reaction buffer	400 µL	-20ºC
Oligo dT (0.5 µg/µl)	40 µL	-20ºC
dNTP mix (2.5 mM each)	400 µL	-20ºC
M-MLV reverse transcriptase (RT) 5,000 units	50 µL	-20ºC
KREA*block*	1.5 mL	-20ºC

II. Reagents and buffers not included in kit

100% ethanol (for preparation of fresh 80% ethanol)

B. General Information

I. Background

The ULS cDNA Synthesis and Labeling Kit generates labeled cDNA for use in microarray hybridization experiments. First unmodified cDNA is made via a reverse transcription from mRNA into cDNA using an Oligo(dT) primer. Unlike other methods, there are no unnatural modified nucleotides used in the reverse transcription reaction. This unmodified cDNA is then labeled in a one step 30 min. reaction with ULS labeling reagent followed by removal of non reacted ULS with the KREA*pure* purification column.

II. Principle of ULS Labeling

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 stable coordinative bond, firmly coupling the ULS to the target. ULS labels DNA, and RNA by binding to the N7 position of guanine (see figure inside back cover). ULS is available coupled to a variety of labels and haptens, including fluorochromes and biotin. ULS enables one-step non-enzymatic labeling of nucleic acids to be achieved within 30 minutes.

III. ULS cDNA Synthesis and Labeling Process

The procedure of expression analysis with the ULS cDNA Synthesis and Labeling-Kit is as follows:

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

IV. Schematic Overview of the ULS cDNA Synthesis and Labeling Process

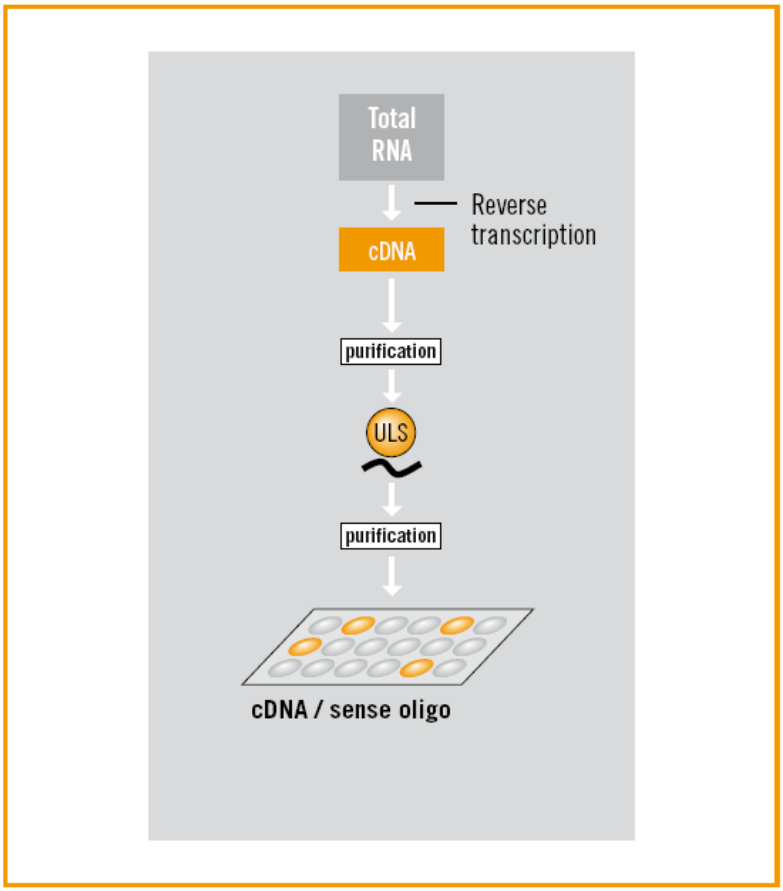

Fig.1 Schematic Overview of the ULS cDNA Synthesis and Labeling Process

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. cDNA Synthesis

This single strand cDNA synthesis kit is designed to generate first strand unmodified cDNA using total RNA as a template. The synthesized cDNA is then labeled using the ULS labeling technology.

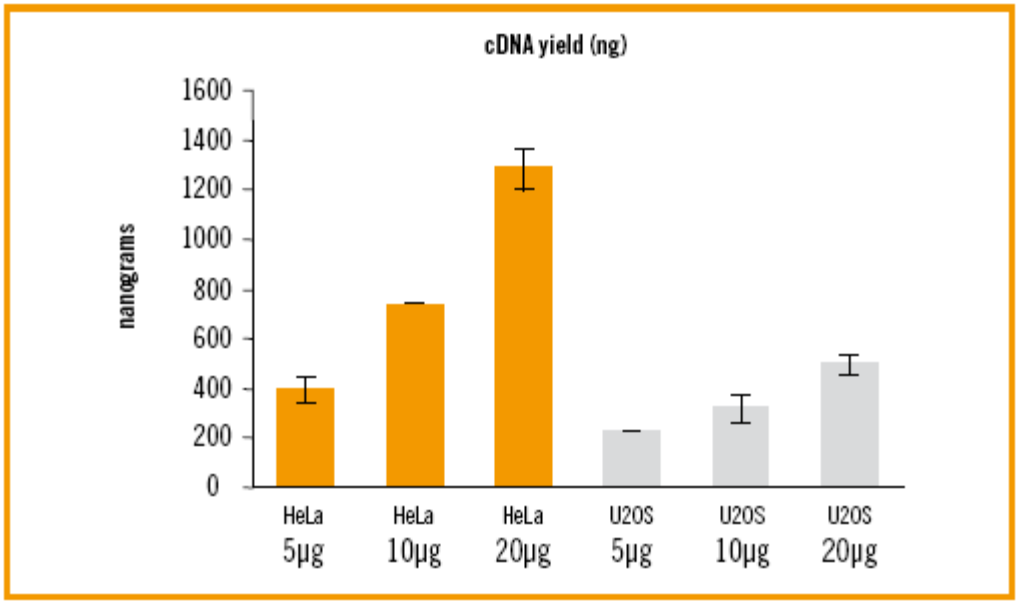

Fig.2 cDNA yields in nanograms.
The amount of RNA starting material needed is dependent on the source material (shown here: cell lines Hela and U2OS).

a. First Strand cDNA Synthesis

IMPORTANT

The minimum amount of starting RNA material needed to produce sufficient cDNA (min 250 ng) is dependent on the gene activity in the source material. As a rule of thumb, the cDNA yield is about 5%. In general, 5-10 µg of total RNA will yield sufficient cDNA for one array hybridization.

Protocol for first strand cDNA from total RNA (we recommend starting with 10 µg total RNA)

1. Add the following to a 1.5 mL tube

Component	Volume
Total RNA	10µg
Oligo dT(0.5 µg/µL provided)	2 µL
Water	fill to 25 µL
Total reaction volume	25 µL

2. Heat the reaction mixture to 70ºC for 5 minutes and then place it on ice.
3. Add the following reagents to the tube:

Component	Volume
5 x M-MLV RT reaction buffer	20 µL
5 x dNTP mix (2.5 mM each)	20 µL
Water	32.5 µL
M-MLV RT 200 unit/µL	2.5 µL

4. Mix and incubate the tube at 42°C for 50 minutes and then shift it to 70°C for 10 minutes. Place on ice.
5. Add 20 µL of the Hydrolysis solution (0.5M NaOH), and incubate 30 mins at 65°C
6. Add 10 µL of Neutralization solution (1M HCl) and place on ice.
7. Store any unused first strand cDNA synthesis reaction mixture at -20°C. First strand cDNA can be stored at -20°C for up to three months.

b. cDNA Purification

The DNA purification columns employ a single-buffer system that allows for efficient DNA adsorption onto the matrix of the column. The DNA is washed then eluted with a small volume of water.

Reagent Preparation

- Prepare fresh 80% ethanol from 100% ethanol.

1. In a 1.5 mL tube, add 500 µL of DNA Binding Buffer to the 130 µL of cDNA sample. Mix briefly by vortexing.
   Note: The sample capacity of the column is 800 µL.
2. Transfer mixture to a cDNA purification column in a collection Tube.
3. Centrifuge at ≥10,000 rpm for 30 seconds.
4. Discard the flow-through. Re-use collection tube
5. Add 200 µL of freshly prepared 80% ethanol to the column.
6. Centrifuge at ≥10,000 rpm for 30 seconds.

7. Repeat step 5 and 6.
8. Add 6-10 µL water directly to the column matrix and let stand for 1 minute.
9. Transfer the column to a 1.5 mL tube and centrifuge at ≥10,000 rpm for 30 seconds to elute the DNA.
10. Ultra-pure DNA in water is now ready for labeling.
    Note: Elution of DNA from the column is dependent on pH. Water used should have a pH >5.0.

III. cDNA Labeling

IMPORTANT

1. The final concentration of cDNA in this labeling reaction should be 50 ng/ µL or higher. A final cDNA concentration as low as 25 ng/ µL can be used, but a slightly lower cDNA labeling density will be obtained.
2. For optimal labeling density, the ratio of cDNA:ULS-dye should always be 1 µg cDNA: 1 µL ULS-dye. The labeling reaction can be scaled up or down (see example below). When doing this it is important to note that regardless of the amount of cDNA you label that you keep this ratio of ULS reagent per µg cDNA constant i.e. If you double the amount of cDNA target you need to double the amount of ULS reagent.
   Note: In general suboptimal modification degrees of the labeled material are achieved if final concentration of the aRNA in the labeling mixture is below 25 ng/µL)

a. ULS labeling reaction

Spin all required reagents to collect contents of tubes

1. Take 0.25 – 5 µg cDNA
2. Add 1 µL of Cy5-ULS, Cy3-ULS or BIO-ULS per 1 µg of cDNA.
3. Add 1/10 volume of 10 x labeling solution e.g. 1 µL of labeling solution in a 10 µL reaction volume
4. Adjust with DNase-free water to final volume and mix by pipetting (see example set-up below).
5. Label sample by incubation for 30 minutes at 85°C.
6. **Note**: for low volumes (10 µL), a PCR machine with hot lid is advised. Larger volumes can be incubated in a water bath.
7. Place samples on ice, spin down to collect contents of tube before proceeding with purification using the KREA*pure* column

Example of ULS labeling of cDNA

	0.25 µg	1 µg	5 µg
cDNA + DNase free water	8.75 µL	17 µL	13 µL
ULS reagent	0.25 µL	1 µL	5 µL
10 x Labeling solution	1 µL	2 µL	2 µL
Total Volume	10 µL	20 µL	20 µL

The important factors to consider when setting up the labeling experiments are:

1. To ensure the concentration of nucleic acid in the labeling reaction is > 25 ng/µL. i.e. if labeling 1 µg in 20µL as in the example set up the nucleic acid concentration = 50 ng/µL.
2. The KREA*pure* column capacity is 50 µL. If the labeling reaction volume exceeds this then concentration will be necessary before purification over the KREA*pure* column.

b. Dye Removal Using KREApure Columns

Removal of free ULS label using KREApure columns (20800 x g is equivalent to 14,000 rpm on eppendorf 5417C)

1. Resuspend column material by vortexing
2. Loosen cap 1/4 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. Add 300 µL DNase free water to the column
7. Spin column for 1 minute at 20800 x g
8. Discard collection tube and flow-through
9. Put column in a new (DNase free) 1.5 mL micro centrifuge tube
10. Add ULS-labeled cDNA on to column bed - **careful not to pipette to the sides of the column but directly onto the column material**
11. Spin column for 1 minute at 20800 x g
12. Flow through is purified labeled cDNA
13. Determine the degree of labeling (DoL) as follows: measure absorbance at 260 nm and 550 nm (for Cy3-ULS) or 650 nm (for Cy5-ULS) using a Nanodrop or other spectrophotometer. Calculate the DoL value as explained in the box on the next page or use the interactive calculator on our web site (www.kreatech.com)

NOTE: The DoL value should preferably be 2-3% (indicating an average of 2-3 Cy-ULS molecules per 100 nt). However a DoL value of between 1.0-3.6% is acceptable. DoL values lower than 1.0% may not produce enough signal, whereas DoL values higher than 3.6% might cause either high background levels or quenching of signal. In these cases please refer to the trouble shooting section or contact us at techservices@kreatech.com.

c. Determination of the Degree of Labeling

Measure A_{260} and A_{550} for determining the DoL of Cy3-ULS labeled cDNA
Measure A_{260} and A_{650} for determining the DoL of Cy5-ULS labeled cDNA

Degree of Labeling (DoL) calculation

$$X = \text{ng (nucleic acid) / µl} = \frac{(OD260 - (ODmax * \text{corr. factor})) * \text{dilution factor} * 40}{\text{cuvette path length (cm)}}$$

$$Y = \text{pmol (dye) / µl} = \frac{OD_{dye} * \text{dilution factor}}{\text{cuvette path length (cm)} * \varepsilon \text{ dye} * 1 \times 10^{-6}}$$

$$\textbf{DoL} = \frac{340 * Y}{X * 1000} * 100\%$$

ε dye Cy3-ULS = 150,000 $mol^{-1} * L * cm^{-1}$
ε dye Cy5-ULS = 250,000 $mol^{-1} * L * cm^{-1}$
Correction factor for Cy3 = 0.08
Correction factor for Cy5 = 0.05

Please check out our website: www.kreatech.com for an easy-to-use DoL calculator.

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 this is used in initial experiments, although on some microarray platforms it may not be necessary.

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 cDNA Preparation and Analysis

Problem	Possible Reasons and Suggestions
OD260 not within parameters	**Cause:** Impure RNA **Remedy:** Repeat RNA clean-up using a commercial kit or precipitate RNA and dissolve again

II. Labeling, Array Hybridization and Detection

Problem	Possible Reasons and Suggestions
Low Signals	Inefficient labeling of cDNA **Cause:** There may be salt present which disturbs labeling **Remedy:** Clean up cDNA and ensure final 80% ethanol wash step is used with purification columns (see C IIb) **Cause:** Incorrect ratio of labeling reagent to cDNA **Remedy:** Ensure use of instructed amount of ULS per µg of cDNA **Remedy:** Ensure concentration of the labeling reaction is above or equal to 25 ng/µL

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 Microarray **Cause:** Too high concentration of detection Antibody **Remedy:** Dilute detection antibody

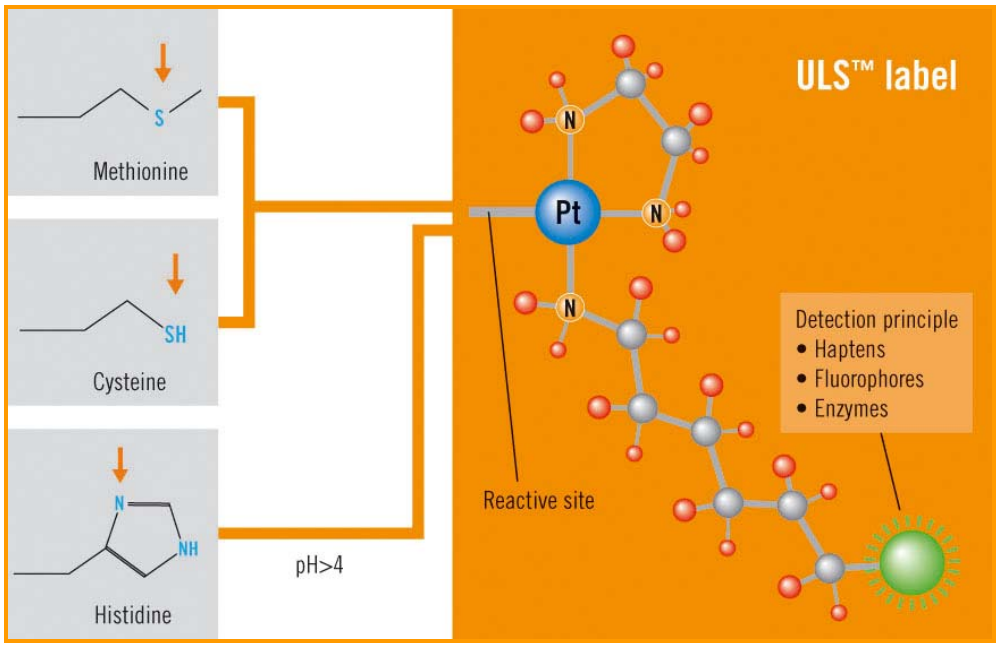

Fig.3 ULS, the Universal Linkage System that labels you DNA, RNA and proteins

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